THE USE OF EFFECT-BASED METHODS FOR WATER SAFETY PLANNING IN SOUTH AFRICA

Volume 2: Sample processing procedures for appropriate bioassays for the assessment of selected modes of action (MOA) relevant for water safety planning

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WATER RESEARCH COMMISSION

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Report to the
Water Research Commission

by

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This report forms part of a set of two reports. The other report is *The Use of Effect-Based Methods for Water Safety Planning in South Africa*. *Volume 1: Use of bioassays for the assessment of selected modes of action (MOA) relevant for water safety planning*. (WRC Report No.TT 908/1/22)

EXECUTIVE SUMMARY

BACKGROUND

The water industry in South Africa (SA) today is faced with the challenge of ensuring a sustained and safe supply of drinking water. Surface waters can contain a wide range of substances referred to as micropollutants that include industrial compounds, agricultural compounds like pesticides, pharmaceuticals, personal care products and plasticisers/ microplastics. These compounds have the potential to adversely affect the ecosystems in which they are found. By identifying, their sources point (e.g. wastewater treatment plants) or diffuse (e.g. agriculture) within the ecosystem, and attempting to find solutions may help to reduce the presence and bioactivity of the compounds. These compounds are present as mixtures in the aquatic ecosystem and can affect the endocrine system, for example reproduction, cancers, neurodevelopmental effects and obesity. The focus has been mostly on the effects of endocrine disrupting chemicals (EDCs) with estrogenic activity. However, the endocrine system is not limited to estrogenic activity, other hormone-driven signalling systems like androgens, progestogens, glucocorticoids, retinoids and thyroid hormones play a critical role in maintaining processes throughout the different life-stages in humans and animals. Present in the aquatic environment, these chemicals and their degradation products are difficult to assess for risk and impact on human and environmental health. Many of the compounds are found at low doses in the environment, but may have a high potency. It is also important to note that while individual compounds might not elicit effects at these low concentrations, their combined presence may still be important, especially if they have a similar mode of action (MOA).

RATIONALE

The Global Water Research Coalition (GWRC) has embarked on a new study investigating the use Effect-based Monitoring program (EBM) for Water Safety Planning (WSP). For water quality assessment and risk management, carcinogenesis, adverse effects on reproduction and development, effects on xenobiotic metabolism, modulation of hormone systems, DNA reactivity and adaptive stress responses are considered the most relevant toxicological endpoints.

This project will focus on the relevant bioassays for the assessment of selected modes of action (MOA) for treated water safety in SA.

PROJECT AIMS

The aims of the project were:

1. To develop a list of relevant stakeholders (particular reference to SA) and get an overview of ongoing pre-regulatory to fit in with protocols that will be developed for risk and effect- based monitoring plan for WP2 of the GWRC EBM in Water Safety Planning.

2. Based on the GWRC Toolbox to detect endocrine activity, compile a recommended short list of appropriate and available bioassays in SA to develop a toolbox for the assessment of selected endocrine activity endpoints relevant to the South African scenario.

3. Select bioassays based on Mechanisms of Action (MOA) inspired by both Food safety approach & One-Health approach and elaborate innovative and smart combination of effect-based methods (EBM).

4. Provide effect-based trigger values (EBTV) specifically relevant to ecosystem as well as human health targets.

OBJECTIVES

The objectives of this project were to:

1. Compile a list of appropriate bioassays for the assessment of selected MOA and relevant for WSP.
2. Review the Estrogenic activity Toolbox from 2011.
3. Include *in vitro* assays with additional hormone receptor-mediated effects like androgenic and thyroid activity, as well other endpoints based on MOA for example, oxidative stress, and cell toxicity.
4. Include low complexity i*n vivo* bioassays for example the AMES test and FETAX.

METHODOLOGY

A comprehensive literature review was done by the GWRC (May 2020 report) to identify applicable bioassays for EBM. This report was used as a guideline to select the relevant bioassays applicable to the South African scenario. The list of assays was dependent on capacity, infrastructure and which assays are currently available or under development within SA. The report addressing the aims and objectives stated in Section 1.3 is presented in two volumes:

- Volume 1 – covering literature review on the use of effect-based methods for water safety planning in South Africa and factsheets on potential bioassays for assessing different modes of action (MoAs) relevant for water safety planning in South Africa
- Volume 2 – contains a toolbox for sample processing for selected *in vitro* and *in vivo* bioassays **(this report)**

CONCLUSIONS AND RECOMMENDATIONS

Chemical analysis comes with limitations and challenges (capacity, cost and infrastructure, etc.), but also with the vast number of chemicals that may be present in the environment, particularly in SA. The approach of effect-based monitoring using *in vitro* bioassays and well plate-based *in vivo* assays has been recommended for water quality assessment. *In vitro* bioassays can be used to investigate different stages of cellular toxicity pathways, including induction of xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and cytotoxicity. These assays are useful as high-throughput screens which is essential for routine water quality monitoring. These assays can be used to develop a toolbox of EBMs not just estrogenic activity for water quality and safety in SA. One of the aims of developing a toolbox of assays is to allow water quality laboratories to build capacity and use these assays to test water quality from different water sources, from treated water to surface and groundwater on a regular basis. It will also enable water stakeholders to design a suitable fit-for-purpose bioassay test battery for a particular water type or source.

While a battery of three to four bioassays is recommended, there are some situations or constraints at specific locations that may not allow for this. Therefore, even a simple cytotoxicity assay can be considered. At the same time, depending on the type of water being investigated other assay endpoints, for example androgenic activity, thyroid activity and other receptor-mediated endpoints, should also be included in the battery of assays.

Effect-based method use in SA is limited. This may be for a number of reasons, for example, the cost of the bioassays and the infrastructure required such as cost, capacity and policy. The current SANS 241 that is currently under revision should include EBMs and the accompanying EBTVs for drinking water. This stands true for other water quality guidelines and WSPs. It is clear from the literature that SA's water is contaminated with chemicals of emerging concern (CECs), pharmaceutical and personal care products (PPCP) and EDCs. Using EBMs in monitoring programmes is important. Case studies looking at using the toolbox in parallel with chemical analysis will facilitate the development of these tools to assess the health risk of these compounds to humans and animals. The factsheets, together with the updated toolbox, should be used by water stakeholders in order to improve and increase the sustainability of water quality and use in SA.

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1 BACKGROUND

1.1 INTRODUCTION

The water industry in South Africa (SA) today is faced with the challenge of ensuring a sustained and safe supply of drinking water. Surface waters can contain a wide range of substances referred to as micropollutants that include industrial compounds, agricultural compounds like pesticides, pharmaceuticals, personal care products and plasticisers/microplastics. These compounds have the potential to adversely affect the ecosystems in which they are found. By identifying their sources point, (e.g. wastewater treatment plants) or diffuse (e.g. agriculture) within the ecosystem, and attempting to find solutions may help to reduce the presence and bioactivity of the compounds. These compounds are present as mixtures in the aquatic ecosystem and can have effects on the endocrine system for example reproduction, cancers, neurodevelopmental effects and obesity. The focus has been mostly on the effects of endocrine disrupting chemicals (EDCs) with estrogenic activity. However, the endocrine system is not limited to estrogenic activity, other hormone-driven signalling systems like androgens, progestogens, glucocorticoids, retinoid and thyroid hormones play a critical role in maintaining processes throughout the different life-stages in humans and animals. Present in the aquatic environment, these chemicals and their degradation products are difficult to assess for risk and impact on human and environmental health. Many of the compounds are found at low doses in the environment, but may have a high potency. It is also important to note that while individual compounds might not elicit effects at these low concentrations, their combined presence may still be important, especially if they have a similar mode of action (MOA).

The Global Water Research Coalition (GWRC) has embarked on a new study investigating the use of an Effect-based Monitoring program (EBM) for Water Safety Planning (WSP). For water quality assessment and risk management, carcinogenesis, adverse effects on reproduction and development, effects on xenobiotic metabolism, modulation of hormone systems, DNA reactivity and adaptive stress responses are considered the most relevant toxicological endpoints. This report will focus on the relevant bioassays for the assessment of selected modes of action (MOA) for water safety in SA.

1.2 PROJECT AIMS AND OBJECTIVES

The aim of this project was to develop a toolbox of *in vitro* and *in vivo* bioassays with different modes of action for the assessment of water quality for a water safety plan

The objectives of the project:

5. To compile a toolbox of *in vitro* and *in vivo* bioassays with different modes of action for the assessment of water sources which will be available and relevant for application in a water safety plan for SA.
6. To update the methods in the first toolbox “The compilation of a toolbox of bio-assays for detection of estrogenic activity in water” from 2011.

1.3 PROJECT SUMMARY

1.3.1 A historical perspective:

Research on EDCs in South Africa started around 1999 when the WRC held a meeting to discuss the status of EDC research in South Africa. The ground work was laid to develop a strategic plan for research and to

develop the biological and analytical capacity for the identification of these compounds known as EDCs with the help of WRC funding. The GWRC was also interested in developing an international toolbox to determine estrogenic activity that could be implemented in programs and the WRC as a member of the coalition funded the work by a consortium of universities that took part in developing the first toolbox for estrogenic activity using a battery of bioassays. In 2011 a toolbox that suited the South African scenario was developed using assays that were available in South Africa. Later the GWRC toolbox was expanded to include bioassays with other endocrine endpoints (or MOA) that was published in 2014, at this time the thyroid and androgenic bioassays were added to the SA toolbox in 2017.

1.3.2 Current report

A comprehensive literature review was done by the GWRC (May 2020 report) to identify applicable bioassays for EBM. This report was used as a guideline to select the relevant bioassays applicable to the South African scenario. The list of assays was dependent on capacity, infrastructure and which assays are currently available or under development within South Africa. The report addressing the aims and objectives stated in Section 1.3 is presented in two volumes:

- Volume 1 – covering literature review on the use of effect-based methods for water safety planning in South Africa and factsheets on potential bioassays for assessing different modes of action (MoAs) relevant for water safety planning in South Africa (this report)
- Volume 2 – contains a toolbox for sample processing for selected *in vitro* and *in vivo* bioassays.

2 EXTRACTION PROCEDURES FOR AQUEOUS AND SOLID ENVIRONMENTAL SAMPLES

Compiled by: E Archer, C Truter and G Wolfaardt

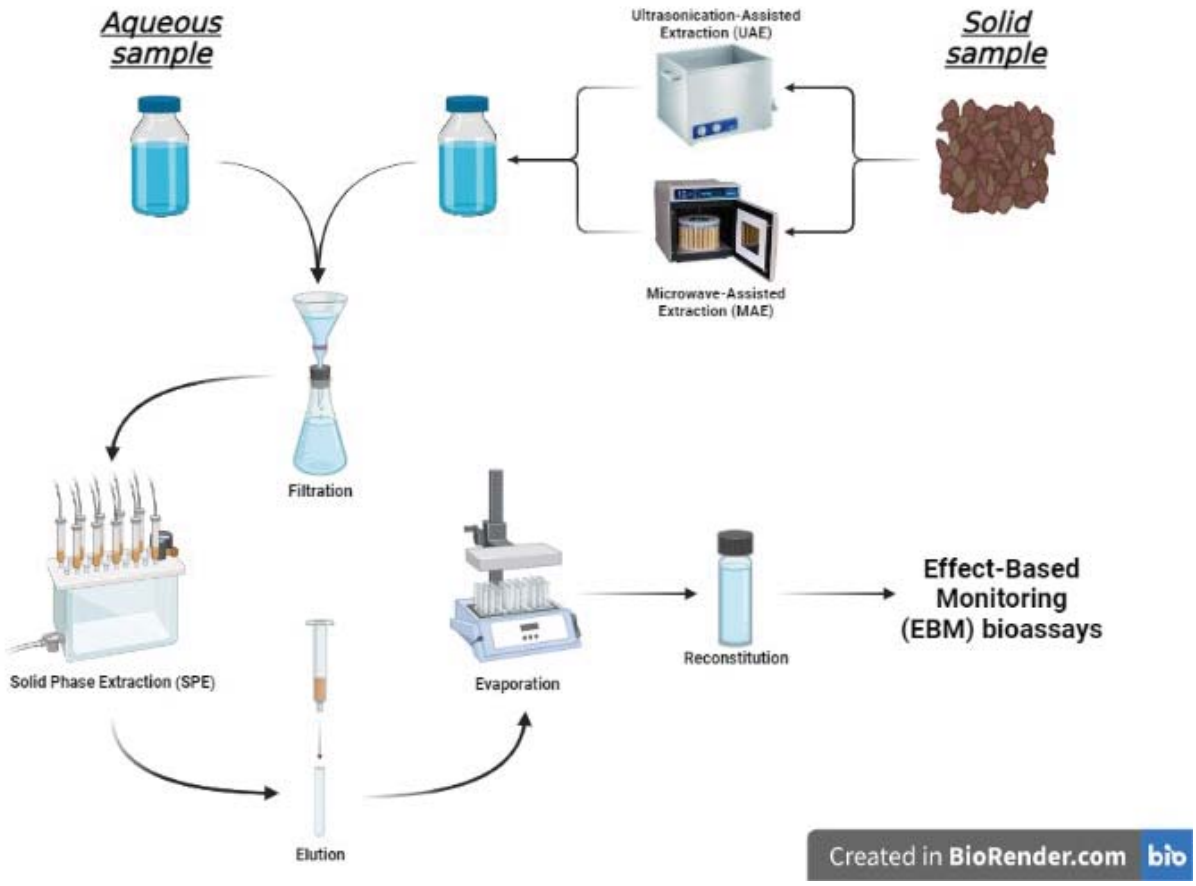

Figure 2-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples

2.1 ACRONYMS & ABBREVIATIONS

ddH_2O	Double Distilled Water
EDC	Endocrine Disrupting Compound
HLB	Hydrophilic-Lipophilic Balanced copolymer
HPLC	High-Performance Liquid Chromatography
KPa	Kilopascal
MAE-SPE	Microwave-Assisted Extraction coupled with Solid Phase Extraction
MeOH	Methanol
SPE	Solid Phase Extraction
UAE-SPE	Ultrasonicated-Assisted Extraction coupled with Solid Phase Extraction

2.2 PRINCIPLE OF THE ASSAY

This protocol describes the extraction of organic pollutants from aqueous samples by solid phase extraction (SPE), and extraction of organic pollutants from solid samples through ultrasonicated-assisted extraction coupled with solid phase extraction (UAE-SPE) or microwave-assisted extraction coupled with solid phase extraction (MAE-SPE). These methodologies are needed to isolate a selected range of pollutants based on their physico-chemical interaction with a polymer sorbent, followed by reconstitution into a solvent, thereby allowing for the sample to be purified and concentrated for its use in downstream analytics (chemical analyses or effect-based monitoring).

2.3 REQUIREMENTS

2.3.1 Staff training (technical skills)

- Good laboratory practice
- Basic training in practical analytical chemistry

2.3.2 Laboratory (test environment)

- General laboratory
- A low traffic area is required, containing a fume hood and nitrogen gas supply.

2.3.3 Apparatus

- Millipore Milli-Q synthesis ultrapure water system or equivalent system to produce double distilled water (ddH2O). The system must be equipped with an EDS filter (Cat. No. EDSPAK001, Microsep) to remove endocrine disrupting compounds (EDCs) from the water.
- Vacuum pump.
- Vacuum manifold, 12 columns (Chromabond® Manifold Cat. No. 730150) or equivalent.
- Glass filtration funnels (500 mL).

- Clamps to connect the filtration funnel and the funnel sieve.
- Glass vacuum filtration flasks, 1-2 L.
- Rubber tubing to connect flask to vacuum pump.
- pH meter
- Pipettes
- 9 Port Reacti-vap evaporator including a heating stirring module and Reacti-vap needles and plugs (Thermo Cat. No. TS-18825 or equivalent).
- Filter forceps (blunt nose), (Millipore, Cat. No. XX6200006 or equivalent).
- Microwave-assisted extractor (MARS, CEM Corporation or similar) containing heating mantles

  or

  Ultrasonication bath with heat and timer function.
- Vortex (Vortex-genie 2, Scientific Industries or similar)
- Centrifuge containing a 50mL falcon tube rotor and specifications to maintain 5000rpm and temperature of 4°C.

2.3.4 Consumables/materials and reagents

Tables 2-1 and 2-2 provide lists of materials and reagents required for the extraction of environmental samples.

Table 2-1: List of consumables/materials required extraction

Name	Cat no	Supplier
Duran® laboratory bottles, with caps (1L)	Z305170	Merck
Amber glass bottles	154515	Chromatography research supplies
50mL falcon tubes	P10487	Whitehead Scientific
Whatman® glass microfiber filters, Grade GF/F	WHA1825047	Merck
Supelclean™ ENVI™-18 SPE Tube	57064	Sigma-Aldrich
or		
Oasis® HLB 6 cc (200mg) Vac Cartridge	186000115	Waters Corporation
Pipette tips (200 µL & 1000 µL)	113-G & P10102	Whitehead Scientific

Table 2-2: List of reagents required extraction

Name	Cat no	Supplier
Hydrochloric acid (ACS reagent, 37%)	320331	Sigma-Aldrich
Methanol 215 SpS (HPLC-grade)	67-56-1	Romil Pure Chemistry
Ethanol absolute SpS (HPLC-grade)	64-17-5	Romil Pure Chemistry
Formic acid (high purity)	AE0A600396	Merck

2.4 METHOD – EXTRACTION OF AQUEOUS MATRICES

NOTE: Throughout the extraction method, ddH_2O refers to double-distilled water that went through the EDS filter to remove EDCs from the water.

NOTE: Inclusion of a field blank, consisting of ultrapure water, should be done for additional quality control of the SPE process. This includes both the filtration and SPE process.

2.4.1 Collection and pre-treatment of samples

1) Collect 2×1 L of aqueous samples such as sewage, surface water, groundwater or tap water in methanol rinsed glass sample bottles. Amber bottles are preferred to prevent chemical breakdown by UV light. Do not rinse out the bottle with water from the source prior to collection.
   a. Preferably use PTFE lined caps such as Schott GL45 PBT type. Alternatively, line the lid on the inside with tin foil to prevent the sample from coming in contact with the plastic lid of the bottle which can be a possible source of EDC contamination.
2) Measure the original pH of the water using the pH strip or bench-top pH meter and make a note.
3) Bring the sample back to the laboratory as soon as possible and store in the dark at 4°C and extract as soon as possible. Minimize contact to direct sunlight during the collection and transport of samples.
4) This step is necessary if you are dealing with raw sewage samples (or similar samples), otherwise proceed to step 8. Raw sewage samples require pre-filtration.

NOTE: If the samples are sewage samples the extraction process should be started within 4-6 hours of collection or if necessary, they may be stored overnight at 4°C. Surface water samples need to be extracted within 48 hours.

5) Raw sewage and very turbid samples require pre-filtration. Assemble the glass filtration unit (see Figure 2-1).
   a. Place 1-2 glass wool filters between the loading reservoir and the sieve funnel.
6) Connect the filtration unit to the vacuum inlet and pass the entire sample through the unit under vacuum. Once the entire sample has been pre-filtered, rinse the reservoir thoroughly with HPLC-grade methanol and ddH_2O and proceed to step 8.
7) Assemble the glass filtration unit as shown in Figure 2-2.
   a. Load a 0.45µm filter (47 mm diameter) onto the funnel/sieve membrane and clamp down the filtration reservoir.
   b. Using a 0.7µm glass fibre filter (GF/F, 47mm diameter) is also sufficient to remove most sediment to prevent clogging of the SPE cartridges during the SPE procedure.
8) Connect the filtration unit to the vacuum inlet. Pass 250-300 mL of the sample at a time through the filtration unit under vacuum.
   a. The filter may need to be replaced if it gets clogged, but this will depend on the sample type. Once the entire sample (1 L) has passed through the filter, transfer the filtrate to a separate sample bottle (clearly labelled) and proceed to solid phase extraction.

b. Remove the tubing connecting the arm of the filtration flask to the vacuum pump and proceed to connect to the vacuum inlet to the SPE vacuum manifold (see Figure 2-1).

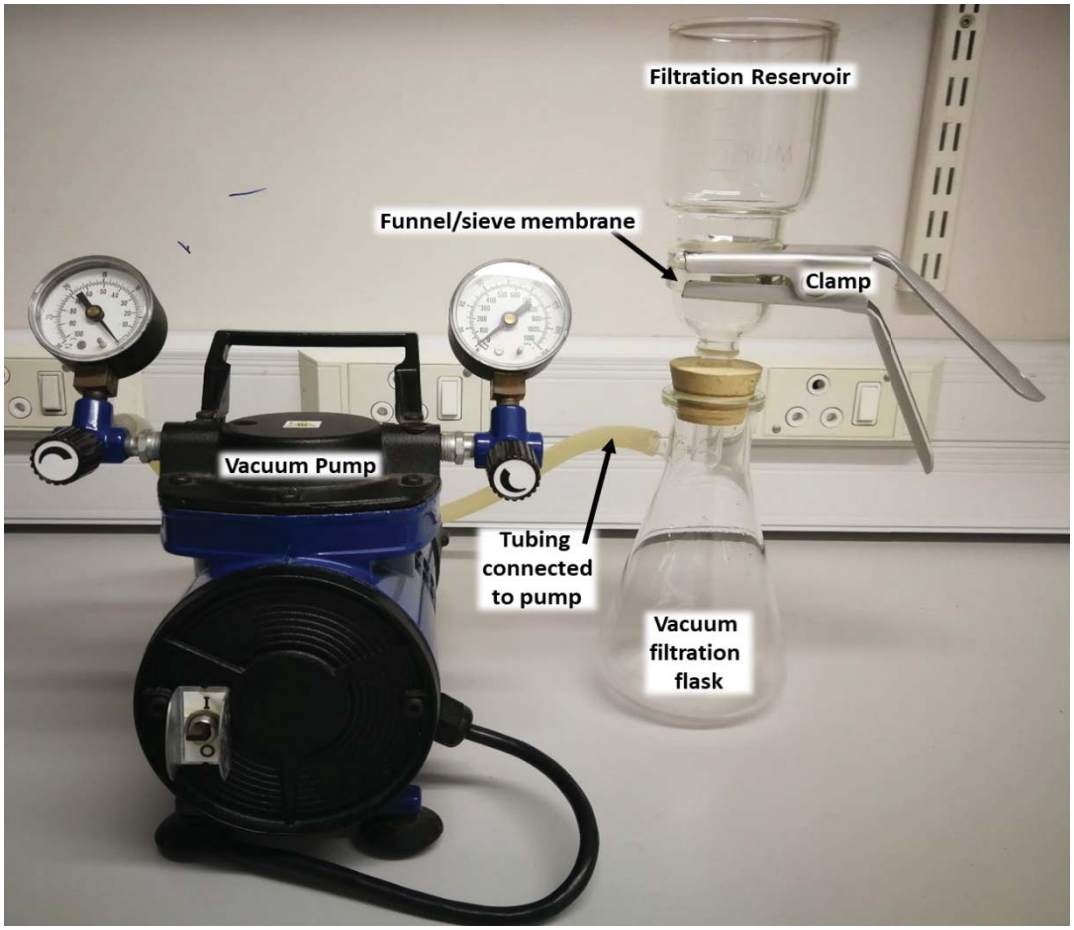

Figure 2-2: Filtration setup for water extraction procedure

2.4.2 Solid phase extraction – pre-conditioning of the cartridge

IMPORTANT NOTE:	The most used sorbents are porous silica particles bonded with C18 or other hydrophobic alkyl groups. Therefore, it is important to first condition the cartridge with a water-miscible organic solvent to solvate the alkyl chains. Then equilibrate the cartridge with water or buffer solution. Do not allow the sorbent bed of the SPE cartridge to run dry during the extraction. This can significantly reduce the retention efficiency of the cartridge and result in low analyte recoveries and poor assay to assay reproducibility.

1) Load the SPE cartridges (6cc, 200mg packing) onto the SPE manifold and open the vacuum valves (see Figure 2-3).
   a. It may occur that one SPE cartridge may get blocked when passing the entire volume of water. If this occurs, another SPE cartridge may be used with the volumes that has passed through each clearly marked, and then combined again during the elution step.
2) Add 5 mL methanol (HPLC grade) to the SPE cartridge reservoir (see Figure 2-3) and allow to pass through by gravity.

3) Just before the methanol reaches the top frit of the SPE cartridge packing material (see Figure 2-3), add 5 mL ddH_2O and allow to pass through by gravity and just before the water reaches the top frit, close the vacuum valve at the bottom of the cartridge.

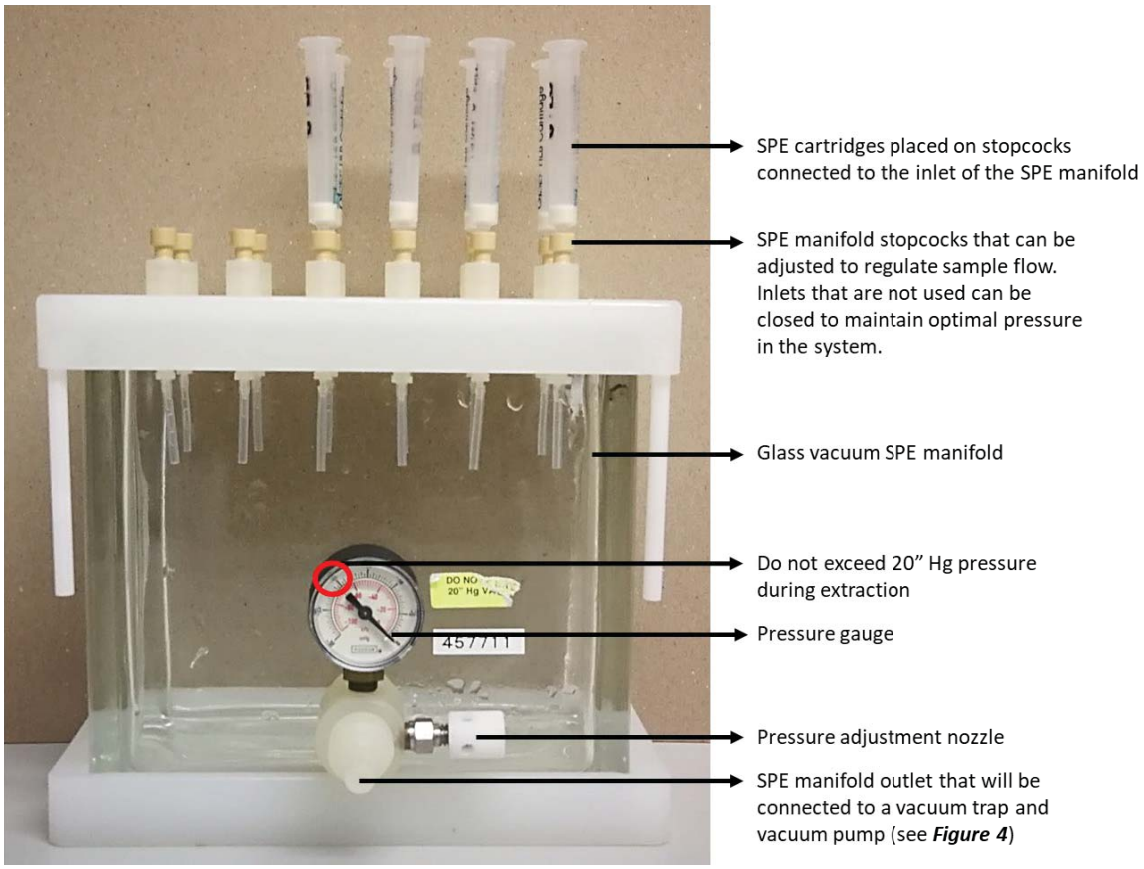

Figure 2-3: An example of a solid phase extraction manifold

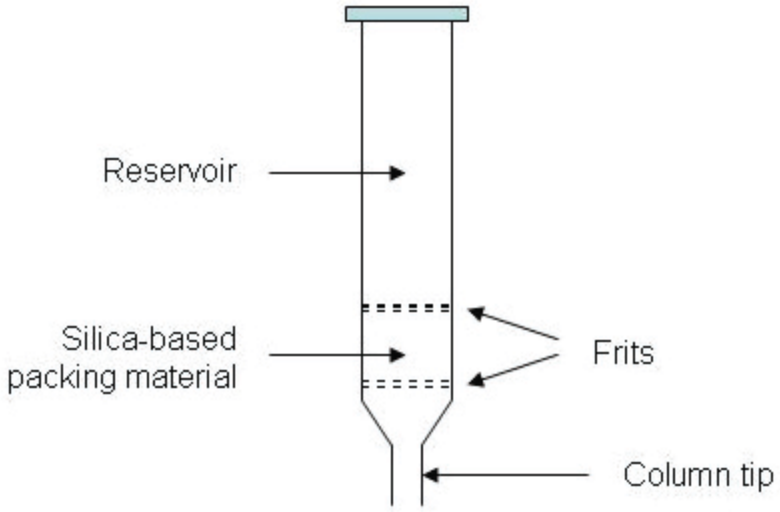

Figure 2-4: A solid phase extraction column/cartridge

2.4.3 Solid phase extraction – main extraction procedure

1) Connect the SPE manifold to the vacuum trap (see Figure 2-3).
2) If you have a large sampler adaptor, connect it to the top of the SPE cartridge (make sure the seal between the adaptor and cartridge is tight and drop the weight at the other end of the tube into the water sample.
3) If you do not have a sample adaptor, you will need to use a sterile 10 mL glass pipette per sample and gently fill the reservoir of the cartridge with sample (± 5 mL).
4) Open the vacuum valve for all samples and gently turn on the vacuum.

NOTE:	If you have a sample adaptor, you need to check that each sample is flowing from the sample bottle to the SPE cartridge. If this is not the case, you need to check that the seal between the adaptor and cartridge is tight. To do this, close the vacuum valve for that sample and tweak the connection until it is sealed tightly.
CAUTION:	DO NOT let the sorbent bed run dry while doing this. If necessary, fill up the reservoir with ddH_2O.

5) Adjust the vacuum strength to achieve a flow rate of approximately 10 mL/min (equal to ± 3 drops/s). As the process continues you might need to increase the vacuum, but do not exceed 70 kPa (20 mmHg).
6) If you do not have a large sample adaptor, gently keep filling the cartridge reservoir with the aqueous sample, preventing the cartridge from running dry.
7) When the vacuum trap is full, close the valves, stop the vacuum, disconnect the trap, empty the contents down the drain and reconnect the trap. Turn the vacuum back on, open the valves and set the flow rate again.
8) After the entire 1 L sample has passed through the column, disconnect the large volume sample if required, leave the cartridge on the manifold (with vacuum) to dry and then close the valves.

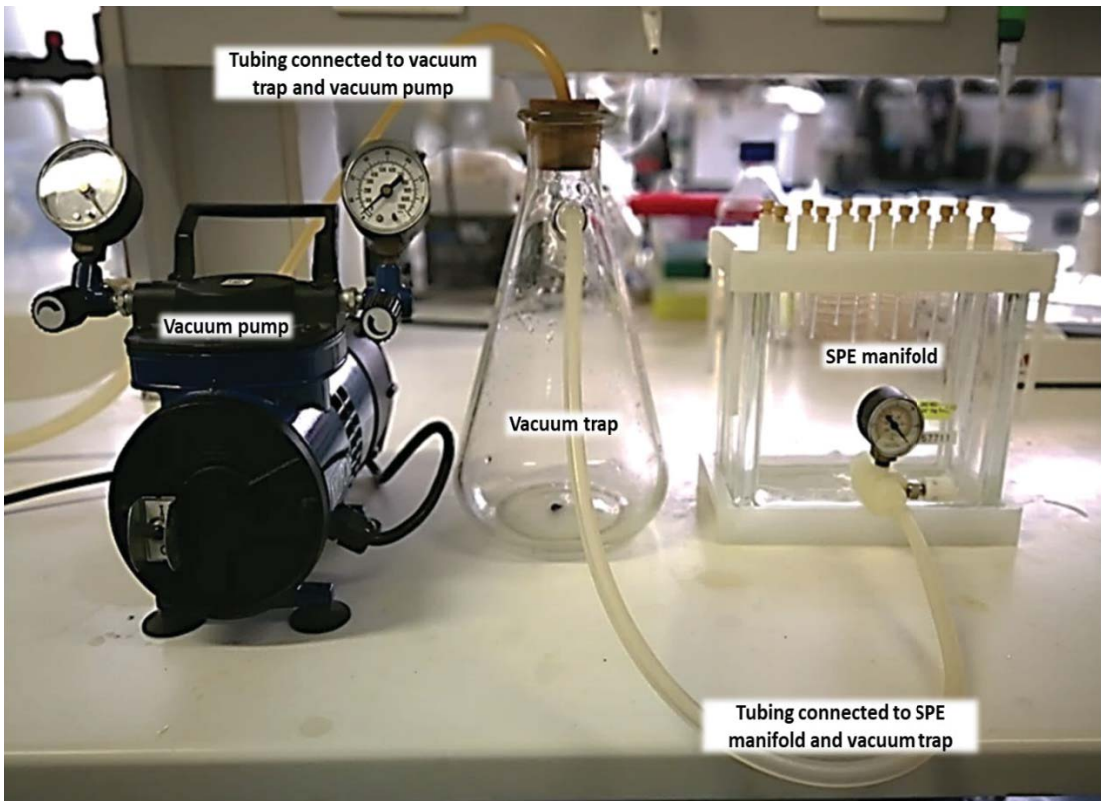

Figure 2-5: An example of the filtration setup for sample extraction

2.4.4 Elution

1) Remove the manifold lid carefully with cartridges still in place and insert a tube rack with a Reacti-vap conical tube or equivalent in the appropriate holes to correspond with the cartridges on the manifold lid.
2) Add 3-5 mL methanol to each cartridge reservoir and allow the solvent to percolate through the sorbent bed.
3) Open the valves and allow eluting with gravity alone into the tubes.
4) Once most of the methanol has eluted, connect to vacuum pump, and gently turn the vacuum on to remove the remaining solvent from the sorbent bed (about 2-3 minutes). The vacuum should be reduced to 5 mmHg, to prevent the methanol from passing through too quickly.
5) Once all the solvent has been eluted, the samples can be carefully removed from the manifold and placed in the Reacti-vap evaporator in a fume hood to be blown down.
6) The SPE manifold can be dismantled and cleaned thoroughly with methanol and ddH_2O.

2.4.5 Evaporation and reconstitution

NOTE: A high degree of volatile analyte loss is expected during the evaporation process. This may negatively affect the experimentation outcome by exhibiting false negative results. For this reason, the addition of a small amount of a high-boiling-point solvent, known as “keepers” is recommended to be added before the sample is being evaporated (Dabrowski, 2006). However, care should be taken to ensure that the “keeper” solvent does not interfere with the bioassay or chemical analysis further down the line.

1) In a fume hood, load the Reacti-vap tubes with the eluent into the Reacti-vap evaporator. Lower the needles of the blow-down unit into the tubes and turn on the nitrogen flow to create a gentle flow on the surface of the samples (not too strong to cause splashes).

2) Lower the needles every 30 min to keep a constant, gentle flow on the surface of the samples. It should take approximately 1-2 hours to blow the sample to dryness.
3) Once completely dry, remove the tubes from the unit. Reconstitute each sample by adding 1 mL ethanol (sample concentrated 1000×) to each tube. Mix the samples thoroughly by vortexing or sonification.
4) Place the eluent into sterile glass amber vials (4 mL volume) and store at -20°C prior to analysis.

2.5 METHOD – EXTRACTION OF SOLID MATRICES

The protocol that follows include two extraction methods for isolation from solid matrices (sediment, soil, sludge, plant material, animal tissue) using either a microwave-assisted extraction coupled with solid phase extraction (MAE-SPE) or an ultrasonication-assisted extraction coupled with solid phase extraction (UAE-SPE).

2.5.1 Collection and pre-treatment of samples

1) Collect the desired sample using PET plastic bottles or 50 mL Falcon tubes.
    a. For sludge/solid particulate matter from water samples, ensure that enough volume is taken to generate a sufficient pellet size after centrifugation.
        i. Normally 2 L for raw wastewater to obtain at least 2 g (dry mass) (duplicates of 1g)
        ii. Normally 8-10 L of surface water, depending on the turbidity of the water matrix.
        iii. For solids (sediment, soil, plants, tissue), ensure that you take enough wet-mass sample to obtain a duplicate of 1g dried mass sample.
2) Centrifuge the water sample/solid sample (5000rpm, 4°C) for 10 minutes to remove excess water.
    a. The supernatant can either be discarded or used for separate SPE of the aqueous matrix.
3) Freeze-dry samples in 50 mL falcon tubes.
    a. Samples should be frozen solid before it is placed in the freeze-dryer.
    b. Freeze the samples on an angle to increase the surface area.
    c. Freeze-dry overnight.
4) Homogenise the freeze-dried samples using a mortar and pestle if necessary.
    a. This will ensure that the sample is thoroughly mixed before a duplicate 1g dried mass is taken for further processing.

5) Continue to either the microwave-assisted extraction coupled with solid phase extraction (MAE-SPE) or ultrasonication-assisted extraction coupled with solid phase extraction (UAE-SPE).

2.5.2 Procedure: Microwave-assisted extraction coupled with solid phase extraction (MAE-SPE)

1) Place the 1g freeze-dried and homogenised samples (in duplicate) in separate MAE cartridges (see Figure 2-6).
2) Add 25 mL of a 50:50 MeOH:H_2O mixture (pH adjusted to 3 using formic acid) to each of the MAE cartridges.

NOTE: Ensure that the cap of the MAE cartridge is securely fastened but avoid over-tightening by making use of the tightening device. A loud 'click' noise will be heard when the cap is securely tightened.

Figure 2-6: Illustration of the microwave-assisted extractor (MAE) and the setup of the MAE vessel for sample processing

3) Place the MAE cartridge inside the heating mantle of the MAE vessel and transfer the MAE vessel to the machine.
4) Program the MAE machine to the following method:
   a. Heat to 110°C for 10 mins.
   b. Maintain at 110°C for 30 mins.
5) Allow samples to cool down to room temperature before opening the lids of the MAE tubes.
   a. Pressure is built up within the MAE tubes during the heating process and can cause harm to the user or loss of the sample if the cap is opened above room temperature.
6) Discard the contents of each MAE tube into labelled 300 mL plastic bottles.
7) Wash each MAE tube with 225 mL ddH_20 and add the contents to each 300 mL glass Schott bottle.
   a. This result in a final sample volume of 250 mL and ensures that the MAE tubes are thoroughly cleaned and that each sample contain less than 5% of MeOH that may cause unnecessary e lution of the sample during the SPE process.

8) Proceed with the Solid Phase Extraction (SPE) procedure (Section 3; solid-phase extraction of aqueous sample matrices).
   a. Pre-conditioning of the cartridge
   b. Extraction procedure
   c. Elution
   d. Evaporation and re-constitution

2.5.3 Procedure: Ultrasonication-assisted extraction coupled with solid phase extraction (UAE-SPE)

1) Place the 1g freeze-dried and homogenised samples (in duplicate) into 50 mL Falcon tubes.
2) Add 10 mL of a 50:50 HPLC-grade methanol:Milli-Q H_20 mixture (pH adjusted to 3 using formic acid) to each dried solid sample/pellet.
   a. Vortex the sample after addition of the 10 mL aqueous mixture and prior to the sonication step
3) Place sample on a floating bed inside a sonication water bath (Figure 2-6) at a water temperature of 40°C and sonicate for 15 minutes.
4) Remove the Falcon tubes from the sonication bath and centrifuge the sample for 10 minutes (5000rpm, 4°C) and decant the supernatant into a new 500mL Schott sample bottle.
   a. KEEP THE CENTRIFUGED PELLET in the original Falcon tube to repeat the sonication procedure.

Figure 2-7: Illustration of an ultrasonication bath for sample processing

5) Repeat steps (2)-(4) twice using the original centrifuged pellet.

a. This results in three sonication cycles in total for each sample to ensure analyte recovery.
b. After the sample pellet has been sonicated three times, the remaining pellet can be discarded.
c. A final volume of 30 mL solvent containing the extracted analytes will thus be generated and taken for further sample clean-up and extraction.

6) Add 270 mL Milli-Q H_20 to the sonicated solvent sample in the new 500mL Schott sample bottle.
   a. This result in a final volume of 300 mL sample (30 mL sample extract and 270 mL ddH_20) that will ensure that the concentration of methanol is below 5% to avoid elution of the analytes during further processing.

7) Proceed with the Solid Phase Extraction (SPE) procedure
   a. Pre-conditioning of the cartridge
   b. Extraction procedure
   c. Elution
   d. Evaporation and re-constitution

2.6 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

2.6.1 Advantages

- The procedure has a high level of selectivity and repeatability compared to other chemical extraction procedures (such as liquid-liquid extraction).
- The SPE procedure allows for improved chemical stability if the sample needs to be stored through freezing (rather than storing the sample itself) if the sample cannot be analysed immediately.
- Reconstitution of the sample into a working solvent (mostly ethanol or methanol) is a requirement for loading samples into assay plates of the effect-based monitoring methods.
- Exclusive extraction of polar and slightly non-polar organic compounds (reduce matrix effects) to be taken forward for effect-based monitoring.
- Provide the ability to pre-concentrate samples for sensitivity experimentations or when EDCs are present in low concentrations that are close to assay detection limits.
- Ability to perform toxicity analysis of the entire environment, or over various sample matrices (aqueous, soil/sludge/suspended solids).

2.6.2 Limitations

- The SPE cartridges are more costly than consumables for other extraction procedures.
- The SPE cartridges may be subject to clogging if the sample matrix is too dirty or not pre-filtered well enough before processing.
- The evaporation process may lead to the loss of volatile organics and non-organic compounds that may also provide endocrine-disrupting responses.
- The procedure for the extraction of the solid matrices is time consuming and may complicate routine sample processing using available staff.

2.6.3 Recommendations

- Use of alternative SPE cartridges that may tailor the research outcome, as some cartridges may be more selective to extract basic- or acidic organic compounds, while others may be more selective for strong acids or strong bases and quaternary amines.
- The use of the UAE-SPE technique has been shown to yield acceptable chemical recovery and the equipment may be more readily available than MAE-SPE. Where possible, and if available, the use of an accelerated solvent extractor is most preferable and include an automated system throughout the extraction process that saves time and potential human error.

2.7 REFERENCES

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2.8 USEFUL CONTACTS

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3 H4IIE-LUC REPORTER GENE ASSAY

Compiled by: A Kruger, R Pieters and S Horn

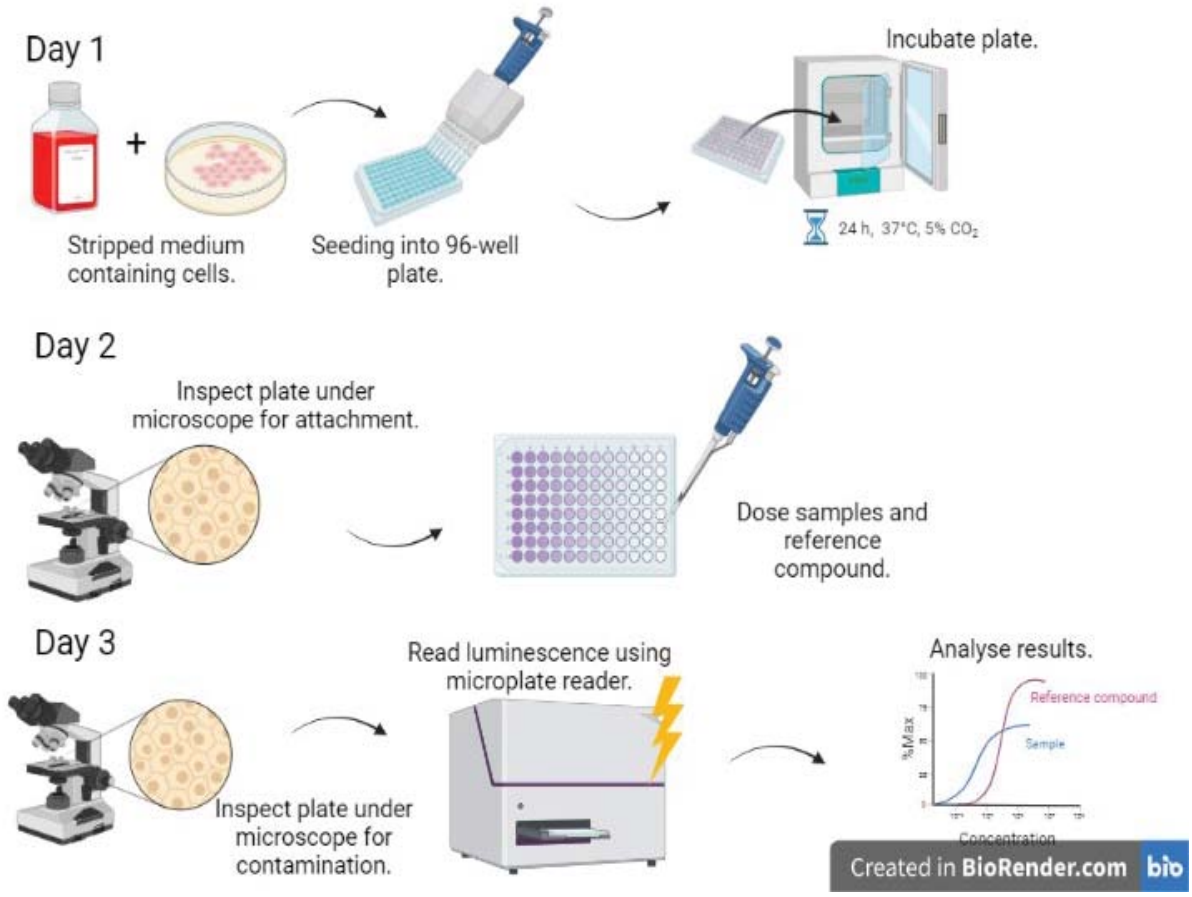

Figure 3-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the H4IIE-LUC reporter gene assay

3.1 ACRONYMS & ABBREVIATIONS

2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASE	Accelerated solvent extraction
ATP	Adenosine triphosphate
BC	Blank control
BEQ	Bio-assay equivalent
C_3H_2O	Isopropanol
$CaCl_2$	Calcium chloride
CALUX	Chemical activated luciferase gene expression
cdt	Charcoal dextran treated
CoA	Coenzyme A
CV	Coefficient of variation
DCM	Dichloromethane
dl-PCB	Dioxin-like polychlorinated biphenyl
DMEM	Dulbecco's modified Eagles' medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DR-CALUX	Dioxin responsive chemical activated luciferase gene expression
DRE	Dioxin-response-element
DTT	Dithiothreitol
EC_{20-80}	Effective concentration range
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FBS	Foetal bovine serum
HCl	Hydrochloric acid
HSP	Heat shock protein
LAR	Luciferase assay reagent
MeOH	Methanol
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
OD	Optimal density

PAH	Polycyclic aromatic hydrocarbon
PCDD/F	Polychlorinated dibenzofuran
RCF	Relative centrifugal force
REP_{20-50}	Relative potency range
RLU	Relative light unit
SPSS	Statistical package for the social sciences
stdev	Standard deviation
VC	Vehicle control

3.2 PRINCIPLE OF THE ASSAY

H4IIE-*luc* is a recombinant cell line derived from rat hepatoma cells and has the aryl hydrocarbon receptor (AhR) (Tillit et al., 1991). Under the transcriptional control of several dioxin-responsive enhancers this cell line has been stably transfected with the luciferase reporter gene. This cell line exhibits AhR-mediated luciferase expression and therefore these cells can measure the induction of cytochrome P450. Light is produced when the AhR is activated. The amount of light produced is directly proportional to the amount of AhRs activated (Aarts, 1993).

The AhR is a ligand-activated transcription factor that regulates gene expression of P450 enzymes (Tian et al., 2015). The P450 enzymes mediate both toxicological and physiological effects when the AhR is activated by exogenous and/or endogenous chemicals. The P450 enzymes have roles in cell differentiation, host defence, and drug metabolism and detoxification. The AhR plays an important role in the development and differentiation of lymphocytes and likely has a role in atherogenesis. Activation of the AhR leads to immunosuppressive effects. The AhR is involved in hepatic fibrogenesis, steatosis, autoimmune hepatitis as well as neurotransmission impairment (Tian et al., 2015).

This assay had been established originally to detect dioxin-like compounds such as polychlorinated dibenzo-para-dioxins, polychlorinated dibenzofurans (PCDD/Fs), dioxin-like polychlorinated biphenyls (dl-PCBs) and a few polycyclic aromatic hydrocarbons (PAHs), just to name a few toxicants (Aarts et al., 1995). All of these elicit a response via the AhR and mediate detrimental health effects such as impairments in reproduction, immune and nervous systems (Tian et al., 2015).

When ligands diffuse into the cytoplasm of a cell and bind to the AhR complex, the complex translocates into the nucleus and binds to the aryl hydrocarbon receptor nuclear translocator (ARNT) forming a heterodimer (Figure 3-2) (Larigot et al., 2018, Nguyen & Bradfield, 2008). The heterodimer recruits coactivators (SRC-1, SRC-2 and SRC-3) and binds to specific DNA sequences referred to as dioxin-response-elements (DRE) (Figure 1). This stimulates the transcription of target genes such as CYP1A1, CYP1A2 and AhRR (Larigot et al., 2018, Tian et al., 2015). The activity expressions of CYP1A1 and CYP1A2 are different in the liver and non-liver tissues and are often tissue-specific (Newman, 2014).

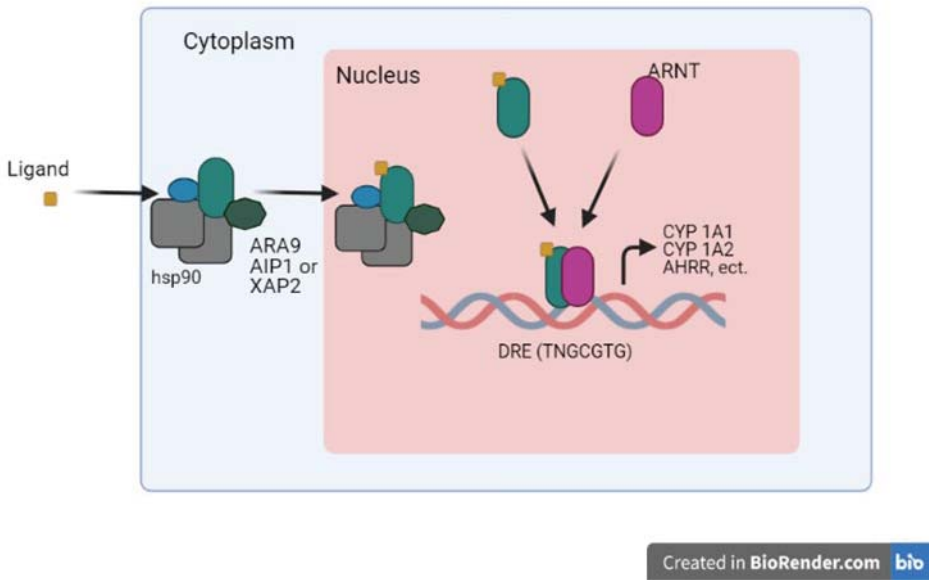

Figure 3-2: Illustration of the AhR mediated response in H4IIE-luc genetically modified cells (adapted from Nguyen & Bradfield, 2008).

Negative regulation of the AhR: after activation of the AhR and the receptor is exported out of the nucleus to the cytoplasm then a) the receptor is degraded and b) activity of the AhR-ARNT complex is reduced (HSP: heat shock protein; ARA9: aryl hydrocarbon receptor-associated protein 9: AIP1: aryl hydrocarbon receptor-interacting protein 1; XAP2: X-associated protein: AhR: aryl hydrocarbon receptor; DRE: dioxin response element; ARNT: AhR nuclear translocator; AHRR; aryl hydrocarbon receptor repressor).

Since there is a wide variety of compounds both harmless and harmful that have been identified as ligands of the AhR, it should be clear for which purpose this assay is required because this will determine with which solvents extracts are prepared. Dioxin-like compounds and their ilk are apolar and should be extracted with apolar solvents such as n-hexane (Osman & Saim, 2013). Also, because these types of pollutants are apolar, they partition to the sediment when transported in natural aquatic systems. It is for this reason that it is advisable to also collect sediment. The hexane extracts contain all apolar compounds from the sediment, including the lesser persistent compounds such as PAHs. In order to target specifically the more potent persistent compounds, the extract should be treated with sulphuric acid (Hilscherova et al., 2001). An acid treatment destroys all non-persistent compounds. When the extract elicits a response from the cells after this treatment, the presence of these harmful compounds has been established.

Water samples extracted for pharmaceuticals and hormones following a protocol with a more polar solvent such as ethanol, may also be tested in this assay, however, because an acid treatment is not included in the extraction protocol, any response by the cells might be due to either harmful or harmless compounds.

Advantages of this bio-assay are that it is relatively rapid, and results are consistent. However, because there is a large variety of naturally occurring ligands that may elicit a response via the AhR, such as flavonols (teas,

red wine, fruits, and vegetables) and flavanones (citrus fruit and beans) (Ashida et al., 2000, Tian et al., 2015), it is important to target ligands that are dioxin-like and also persistent.

3.3 REQUIREMENTS

3.3.1 Acquisition of the cell line

The H4IIE-luc cell line was provided by the University of Saskatchewan (Canada). Commercial cell lines include the DR-CALUX cell line, available from BioDetection Systems in The Netherlands (https://biodetectionsystems.com). Prof. Michael Denison from University of California, Davis (https://etox.ucdavis.edu/denison-michael) gives away similar cell lines (H4L1.1c2). He retired middle 2021, but the cells might still be available from UCDavis. (The H4L1.1c2 is the synonym for H4IIE-luc (Cellosaurus, 2021)).

3.3.2 Permits

These must be obtained from the relevant governmental departments: to import mammalian cells, the Department of Agriculture and Land Reform must be contacted and the Department of Health has to be contacted for importing human cells.

3.3.3 Staff training (technical skills)

- Training in tissue culture techniques.
- Good laboratory practice.

3.3.4 Laboratory (test environment)

- Dedicated to tissue culturing.

3.3.5 Software

- Microsoft Excel® is used to analyse the raw data.
- Software capable of performing descriptive statistics.

3.3.6 Apparatus, consumables/materials and reagents

Tables 3-1 to 3-3 provide lists of the apparatus, consumables/materials and reagents required for the H4IIE-LUC reporter gene assay.

Table 3-1: Lists of the apparatus required for the H4IIE-LUC reporter gene assay

Name	Specifications
Autoclave	
Accelerated Solvent Extractor	Thermo Scientific™ Dionex™ ASE ™ 150 Accelerated Solvent Extractor
Balance	maximum 200 g, d=0.1 mg
Bio-safety hood	Biological safety cabinet class II, protection for product and personnel against any ordinary microbiological agents
Centrifuge	With swing-out rotor
Cell counter	Something similar to Luna-II Automated Cell Counter or the manual haemocytometer
Cryo storage	Dewar containing liquid nitrogen or -150°C liquid nitrogen freezer
Freezer	-20˚C
Freezer	-80˚C
Glass beakers	250 mL, 500 mL, 1 L
Measuring cylinder	1 L
Microplate reader	Specifications: capable of quantifying luminescence and absorbance (560 nm)
Mr Freeze®	Nalgene, 5100-0001
Multi-channel pipette	20-200 µL
Multipipette	12 channel, 10-100 µL volume range
pH meter	
Phase contrast inverted microscope	
Pipette	1-10 µL variable volume
Plate shaker	
Refrigerator	4°C
Repeat pipette	
Schott bottles, glass	250 mL, 500 mL, 1 L
Spatula	
Incubator	Capable of maintaining humidified air and supplementation with 5% CO_2 (i.e. Thermo Electron Corporation Forma Series II)
Vortex mixer	
Water purification system	Producing water quality of 18.2 MΩ.cm

Table 3-2: Lists of the consumables/materials required for the H4IIE-LUC reporter gene assay

Name	Cat no	Supplier
96-well, transparent walled microplates with flat transparent bottom, with lid.	92196	TPP
96-well white-walled microplates with flat transparent bottom with lid.	655098	Greiner Bio-one
Bottle top filters, 0.22 µm, 250 mL	596-520	AEC Amersham SOC
Eppendorf Combitips advanced	Z763039-100EA	Sigma-Aldrich
High-clarity polypropylene conical tube 15 mL	352096	Falcon
High-clarity polypropylene conical tube 50 mL	352070	Falcon
Nitrile gloves (powder-free)		
Pasteur pipettes	F1016S	Labocare
Permanent marker pens		
Pipette tips, sterile, 10 µL	RC-L10	Microsep
Pipette tips, sterile, 200 µL	301 – 02 – 121	Axygen scientific
Pipette tips, sterile, 1000 µL	301 – 01 – 401	Axygen scientific
Sterile serological pipettes (12 mL)	SSP1001	Aquilon
Tin foil		
Tissue culture dishes, 100 x 20 mm	664160	Cellstar, Greiner Bio-one
Tissue paper/Paper towel		

Table 3-3: Lists of reagents required for the H4IIE-LUC reporter gene assay

Name	Cat no	Supplier
2,3,7,8-tetrachlorodibenzo-*p*-dioxin	DRE-GA09011171TO	Industrial Analytical
Adenosine triphosphate	B3003	Melford
Beetle luciferin	E160C	Promega
Calcium chloride	C-2661	Sigma-Aldrich
Coenzyme A	MEL-C70800-0.1	Melford
Dextran treated charcoal	C6241-20G	Sigma-Aldrich
DMEM (low glucose)	D2902-1L	Sigma-Aldrich
DMSO (≥99.7%)	34869-1L	Sigma-Aldrich
DPBS	D5652-10L	Sigma-Aldrich
DTT	D11000-5.0	Melford

Name	Cat no	Supplier
EDTA	318884-500ML	Sigma-Aldrich
Ethanol (Reagent Alcohol, ≥89.0%)	270741-1L	Sigma-Aldrich
FBS	S181G-500	Biowest
Hexane UV (Analytical Grade)	212-4	Honeywell
Hydrochloric acid		
Isopropanol	SAAR5075040LC	Merck
Methanol	230-4	Honeywell
$MgSO_4 \cdot 7H_2O$	10034-99-8	Sigma-Aldrich
$Mg(CO_3)_4 \cdot Mg(OH)_2$	M5671-500G	Sigma-Aldrich
pH buffers (pH 4, pH 7)		
Reporter lysis buffer	ADE3971	Promega
Sodium bicarbonate	S5761-1KG	Sigma-Aldrich
Sodium hydroxide		
Tetrazolium salt thiazolyl blue (MTT)	M5655-1G	Sigma-Aldrich
Tricine	T5816-25G	Sigma-Aldrich
Trypan blue stain, 0.4%	T13001	Logos Biosystems
Trypsin	S181G-500	Biowest

3.4 GENERAL METHODS

3.4.1 Preparation of glassware

Used glassware are washed with soap and water, rinsed with distilled water and allowed to air dry. The mouth of the glassware and the lids are covered with a double layer of foil before it is autoclaved at 121˚C for 15 minutes on a dry cycle.

3.4.2 Dulbecco's modified Eagle’s medium (DMEM)

The medium is prepared in a 1 L measuring cylinder:
Add 1 bottle of DMEM (15.6 g) with low glucose powder for 1 L medium to 900 mL deionised and autoclaved water (18.2 MΩ.cm) in a measuring cylinder. Add a magnetic stirrer bar inside the measuring cylinder on top of a magnetic stirrer to assist in dissolving the powder. Add 3.7 g sodium bicarbonate. When all components are dissolved, add the remaining 100 mL water. Adjust the pH to 7.1 (using NaOH or HCl) and filter the medium into a 1 L sterilised glass bottle using a 0.22 µm bottle top filter. Do this inside the bio-safety hood. Label the

bottle with date, identity of contents and name of the preparer. Store at 4˚C. Unused medium should be discarded after three months.

3.4.3 Maintenance medium

The medium used to maintain the cells is DMEM supplemented with 10% foetal bovine serum (FBS). The required volume of DMEM is added to 0.22 µm bottle top filter together with required volume of FBS and then filtered. This takes place inside the bio-safety hood (i.e. 450 mL DMEM + 50 mL FBS). Label the bottle with date, identity of contents and name of the preparer. Store at 4˚C. Unused supplemented medium should be discarded after 14 days.

3.4.4 DMEM freeze medium (10 mL)

Mix 5 mL FBS, 0.4 mL tissue culture quality dimethyl sulfoxide (DMSO) and 4.6 mL supplemented DMEM. Label and store at -20˚C in 15 mL centrifuge tubes or container of similar quality. It can be stored for three months.

3.4.5 Assay medium

This media is stripped from hormones through supplementing DMEM with 10% stripped FBS (charcoal dextran treated FBS). The hormones are removed from the FBS using dextran coated charcoal. Label the bottle with date, identity of contents and name of the preparer. Store at 4˚C. Discard unused stripped medium after 14 days.

3.4.6 Stripped FBS

Heat the FBS (56ºC for 30 min) and gradually pour into a 50 mL tube containing 0.006 g/mL dextran coated charcoal. Shake for 45 min at 45˚C. Centrifuge the mix for 20 min at 1000 g-force and decant the supernatant into 50 mL tubes containing 0.006 g/mL dextran coated charcoal before shaking the mix again for 45 min at 45˚C. Centrifuge the tubes for 20 min at 1000 rcf and filter the supernatant. Aliquot the filtered stripped FBS into 50 mL tubes. Label the tubes to indicate that it is stripped FBS, add the date and initials (of the person who stripped the FBS). Store at -20˚C.

3.4.7 Dulbecco's phosphate-buffered saline (DPBS) without Mg^{2+} and Ca^{2+} salts

Add 9.64 g DPBS powder into 1 L deionised water (18.2 MΩ.cm) into a 1 L measuring cylinder. Use a magnetic stirrer bar inside the measuring cylinder on a magnetic stirrer to assist in dissolving the powder. Adjust the pH to 7.4. Transfer into a clean 1 L Schott bottle. Autoclave the DPBS before use. Clearly indicate the contents and date on the label on the bottle. Store at 4˚C.

3.4.8 DPBS with Mg^{2+} and Ca^{2+} salts

To prepare 1 L follow the same instructions as above. Add also:

0.1 g $CaCl_2$

0.2465 g $MgSO_4 \cdot 7H_2O$

Make sure the salts dissolve. It can be stored at room temperature and does not need to be autoclaved.

3.4.9 Reference compound 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (TCDD)

10 µg/mL stock is used to prepare the dilution range (0.006, 0.037, 0.22, 1.33, 8 and 48 ng/mL) in hexane. This is prepared in 2 mL amber vials with polytetrafluoroethylene (PTFE) lids. The vials are labelled and stored at -20°C with Parafilm® wrapped around the lids to prevent evaporation of the solvent.

3.4.10 Reporter lysis buffer

For 100 mL: Add 20 mL lysis buffer to 80 mL deionised water (18.2 MΩ.cm).

3.4.11 Luciferase assay reagent (LAR)

To prepare 500 mL LAR, mix the following by weighing all the components and adjust pH to 7.8:

1.792 g	Tricine (20 mM)
0.329 g	$MgSO_4 \cdot 7H_2O$ (2.67 mM)
2.569 g	DTT (33.3 mM)
0.075 g	Beetle luciferin (470 µM)
0.1036 g	CoA (270 µM)
0.146 g	ATP (530 µM)
5 mL	EDTA (0.1 mM)
5.35 mL	$Mg(CO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$ solution (1.07 mM) (see preparation below)

Add deionised water (18.2 MΩ.cm) to prepare 500 mL LAR.

Preparation of 20 mL $Mg(CO_3)_4.Mg(OH)_2 \cdot 5H_2O$ solution: Add 0.967 g $Mg(CO_3)_4.Mg(OH)_2$, into 15 mL deionised water (18.2 MΩ.cm) and slowly add 2 mL concentrated HCl until the solution turns clear. Finally add 3 mL deionised water (18.2 MΩ.cm). Final volume is 20 mL. Aliquot into 50 mL tubes and label appropriately. Label and store at -80°C.

Precautionary note: LAR is light sensitive! Protect storing tubes against UV by covering them with aluminium foil. When thawing the tubes ahead of use, keep them protected from UV radiation.

Store at -80˚C.

3.4.12 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution

0.5 mg/mL MTT powder in stock medium.
One plate requires 6.6 mL MTT solution.
Solution should be prepared in the dark.

3.5 GENERAL CELL CULTURE TECHNIQUES

3.5.1 Aseptic conditions

The tissue culture laboratory is a sterile environment and all the counter tops, as well as the bio-safety hood must be cleaned regularly with 70% ethanol before and after working with the cells. Media changes, passaging of the cells and exposures are done inside the bio-safety hood to prevent microbial contamination (Davis, 2002).

It is important to always wear nitrile gloves that is regularly sprayed with 70% ethanol. Wearing face masks are optional, but advisable because the nutrient media is not usually supplemented with antibiotics and humans may inadvertently transmit microbes to the cells when working with them. Only open glass bottles containing media and tubes containing FBS, trypsin, or antibiotics inside the bio-safety hood. Spray the bottles and tubes with 70% ethanol before placing it inside the bio-safety hood. Spray consumables with 70% ethanol before placing it in the bio-safety hood. Glass bottles should be autoclaved before using it again.

3.5.2 Thawing the frozen cells (start-up cell-line) (Figure 3-2)

1. Frozen H4IIE-*luc* cells (1 mL) are collected from cryopreservation and are placed in a water bath to allow quick thawing (30 sec).
2. Add the 1 mL cells from the cryo-vial into 11 mL supplemented medium (pre-warmed to room temperature, or 37°C) in a 15 mL centrifuge tube.
3. Centrifuge the tube at 125 relative centrifugal force (rcf) for ten minutes (Figure 3-3).
4. Discard the supernatant and resuspend the pellet using 12 mL supplemented medium.
5. Transfer the medium containing the cells into a 100 by 20 mm tissue culture dish (label the culture dish) and place in an incubator under stable conditions (humidified air supplemented with 5% CO_2 and at 37ºC).

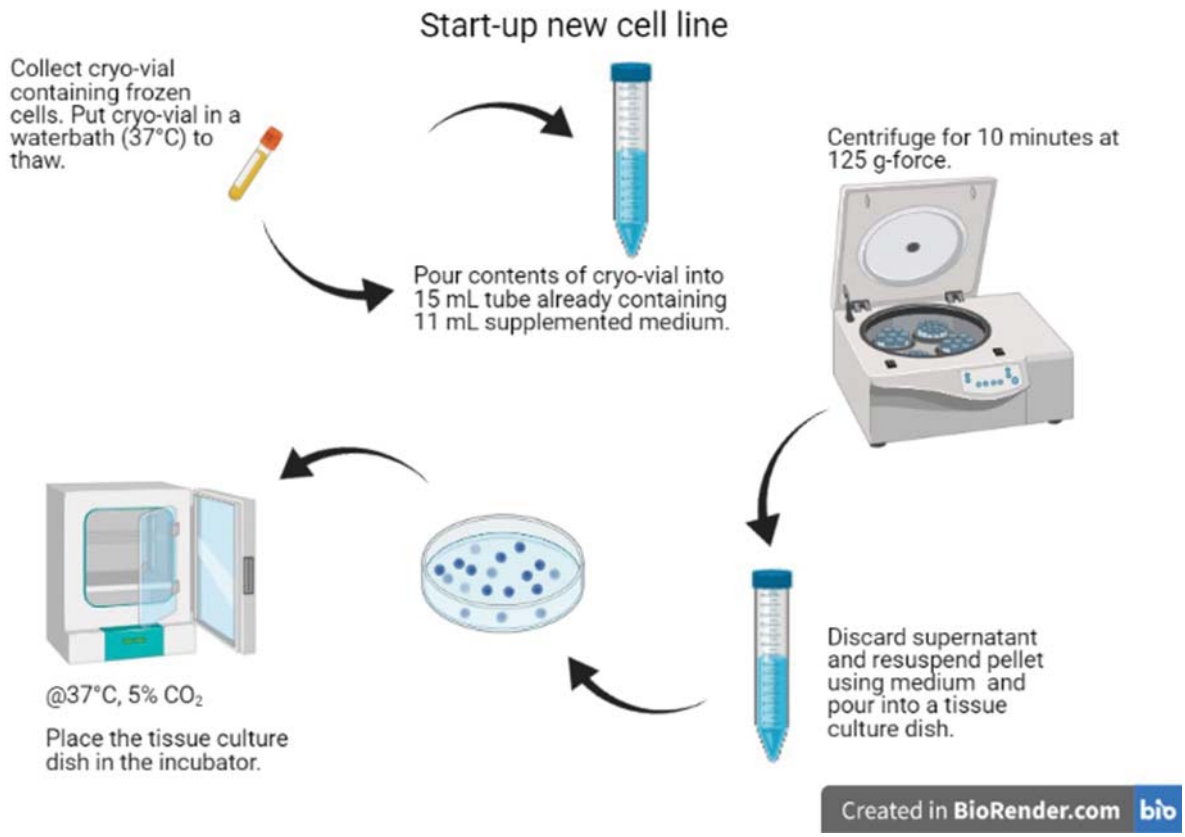

Figure 3-3: Diagram illustrating the start-up process of new H4IIE-*luc* cells from cryostorage.

3.5.3 Subculturing the cells

When the cells reach 100% confluency, they need to be sub cultured.

1. Passaging the cells requires the medium to be removed from the culture dish and cells to be washed with DPBS three times (Figure 3-4).
2. Use a sterile Pasteur pipette to transfer 1.5 mL trypsin into the culture dish and return the culture dish to the incubator for 3 minutes (Whyte et al., 2004). The trypsin loosens the cells from the culture dish.
3. Discard the trypsin and use 12 mL supplemented medium to create a suspension in which the cells are transferred to a new tissue culture dish.
4. Seed each new plate with 3 or 4 mL and add supplemented medium to create the 12 mL working volume of the plates.

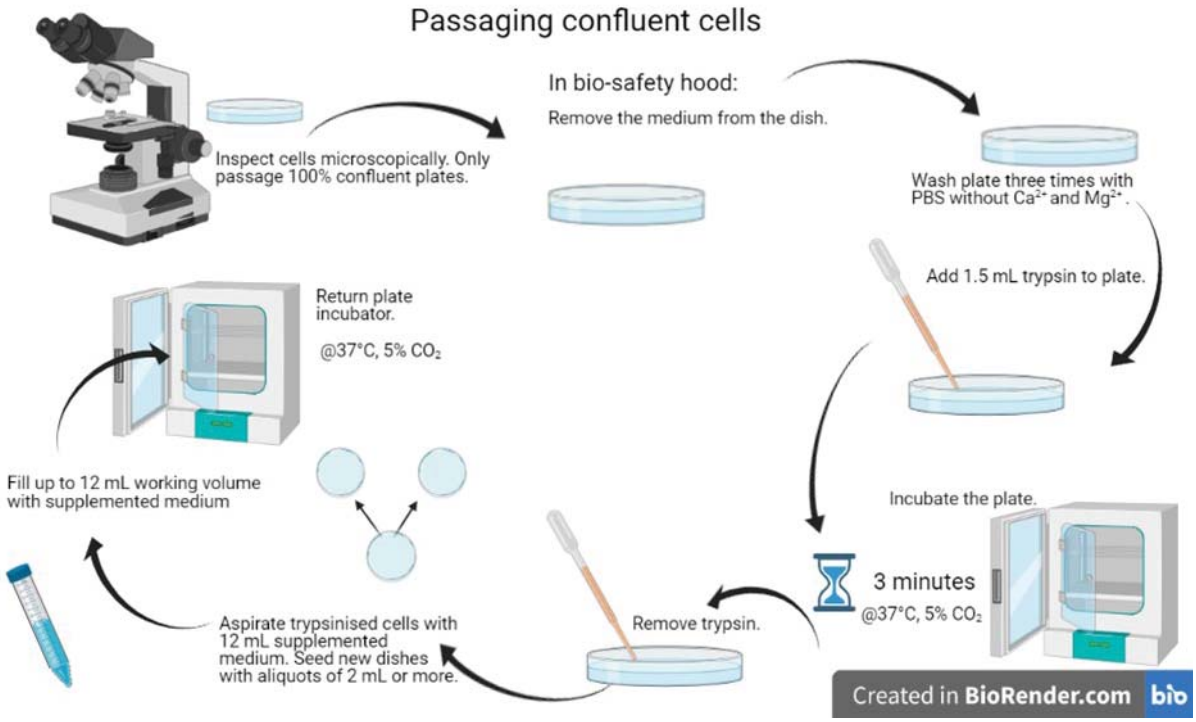

Figure 3-4: Method for passaging cells to new culture dishes.

3.5.4 Freezing stock cultures

1. The first three steps for this method are the same as the steps for subculturing the cells but instead of placing the cells into a culture dish, it is transferred into a 15 mL tube (Figure 3-5).
2. The tube is centrifuged for 10 minutes at 380 rcf.
3. The supernatant is discarded, and the pellet is resuspended using 1 mL freeze medium.
4. The suspension is poured into a cryo-vial and the lid is closed tightly.
5. Place the cryo-vial in the Mr Freeze® container which is filled with isopropanol/propan-2-ol (Note: The isopropanol/propan-2-ol should be replaced after every fifth use).
6. Place the Mr Freeze® container in the -80˚C for the next 24 h before moving the cryo-vial to the dewar containing liquid nitrogen. It is important to label the cryo-vial properly.

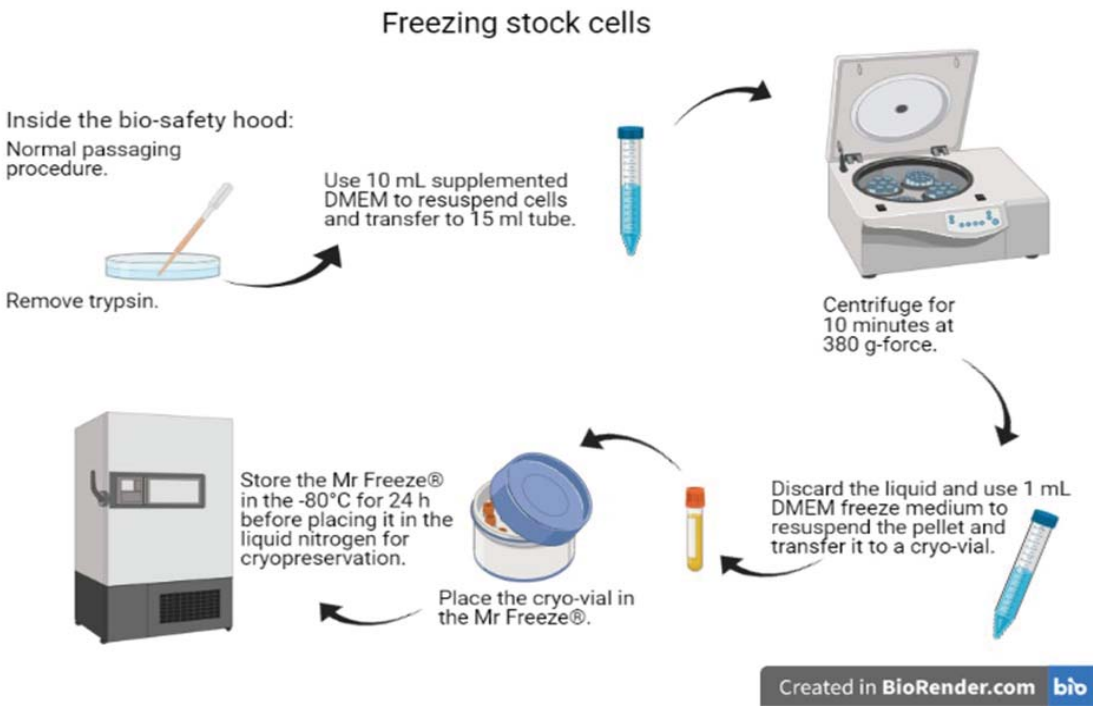

Figure 3-5: Illustration for freezing stock cells in.

3.6 H4IIE-*LUC* REPORTER GENE ASSAY PROCEDURE

Day 1

1. Trypsinise the cells in a confluent cell culture dish and use 12 mL assay medium to create a suspension (Figure 3-6).
2. Pool cells in a 15 mL tube and determine the density of the viable H4IIE-l*uc* cells in suspension with a cell counting apparatus. Adjust the density (80 000 cells/mL) to the required volume needed: 15 mL per 96-well plate.
3. Seed the cells at 80 000 cells/mL into a 96-well white-walled microplate with a flat transparent bottom in aliquots of 250 µL per well.
4. Seed only the inner 60 wells and fill the outer 36 wells with DPBS. This creates a uniform microclimate across all cell bearing wells.
5. Place the plate in incubator for 24 h.

Counting cells with the Luna-II Automated Cell Counter:

1. Add 100 µL pooled cells into a cryo-vial.
2. Place 100 µL trypan blue in the cryo-vial. Mix well.
3. Transfer 12 µL of the trypan blue cell suspension onto the reading slide and place the slide in the Luna.
4. Count the cells with on-board software of the instrument.

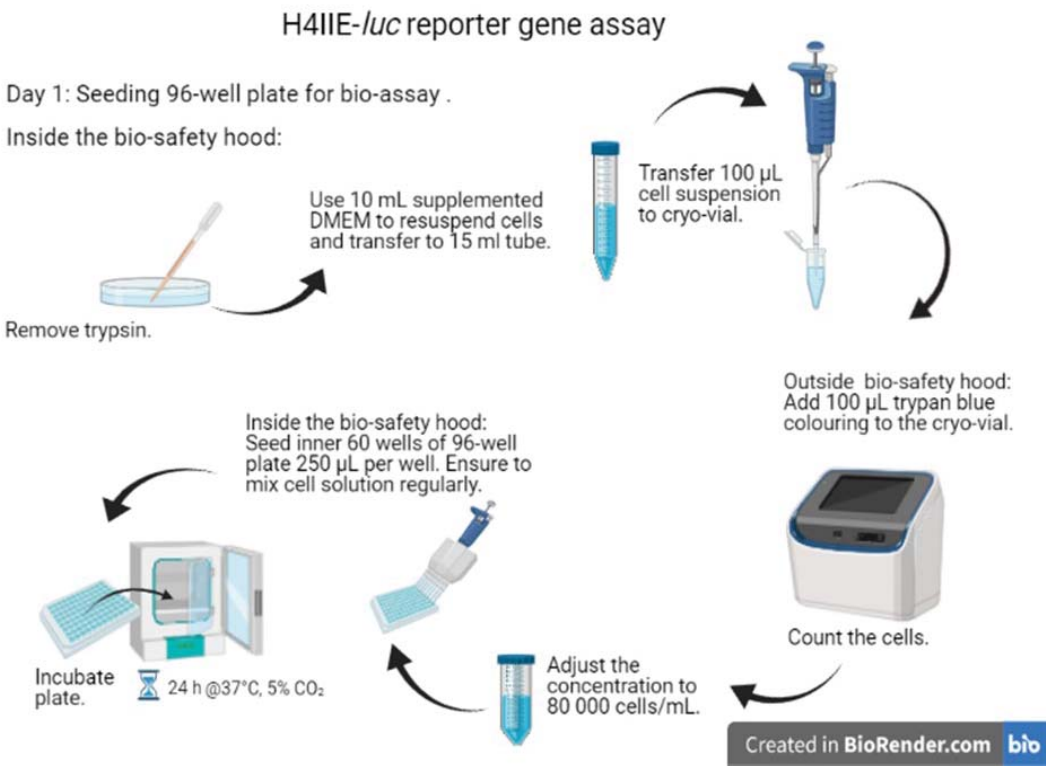

Figure 3-6: Diagram for day one of the reporter gene assay using H4IIE-*luc* assays.

Day 2

1. Vortex the sample and reference compound containing amber screw top vials spray with 70% ethanol to remove microbes and place vials in the bio-safety hood.
2. Inspect the cells with an inverted phase contrast microscope to confirm cells have attached (Figure 3-7).
3. Dose each well with 2.5 µL sample, or medium (blank control; BC) or vehicle control (VC) (three replicates per concentration and six concentrations per sample).
4. Each plate must receive a positive control, which is 2,3,7,8-TCDD
5. See the lay-out in Figure 3-8
6. After dosing the samples and the standard, place the plate in incubator for 72 h.

Use sterile tips for the dosing. The first concentration is the pure extract followed by 2x or 3x serial dilution ranges, depending on the sample.

		Sample 1				Sample 2			TCDD (ng/mL)			
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
PBS							BC		0.48		PBS
PBS							BC		0.08		PBS
PBS							BC		0.0133		PBS
PBS							VC		0.0022		PBS
PBS							VC		0.00037		PBS
PBS							VC		0.000061		PBS
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Figure 3-7: Plate layout. The grey shading indicates all the wells containing PBS. The green and blue shadings illustrate the wells exposed to samples tested. The purple shaded wells receive the positive control (2,3,7,8-TCDD (ng/mL)) (BC: blank control, VC: vehicle control)

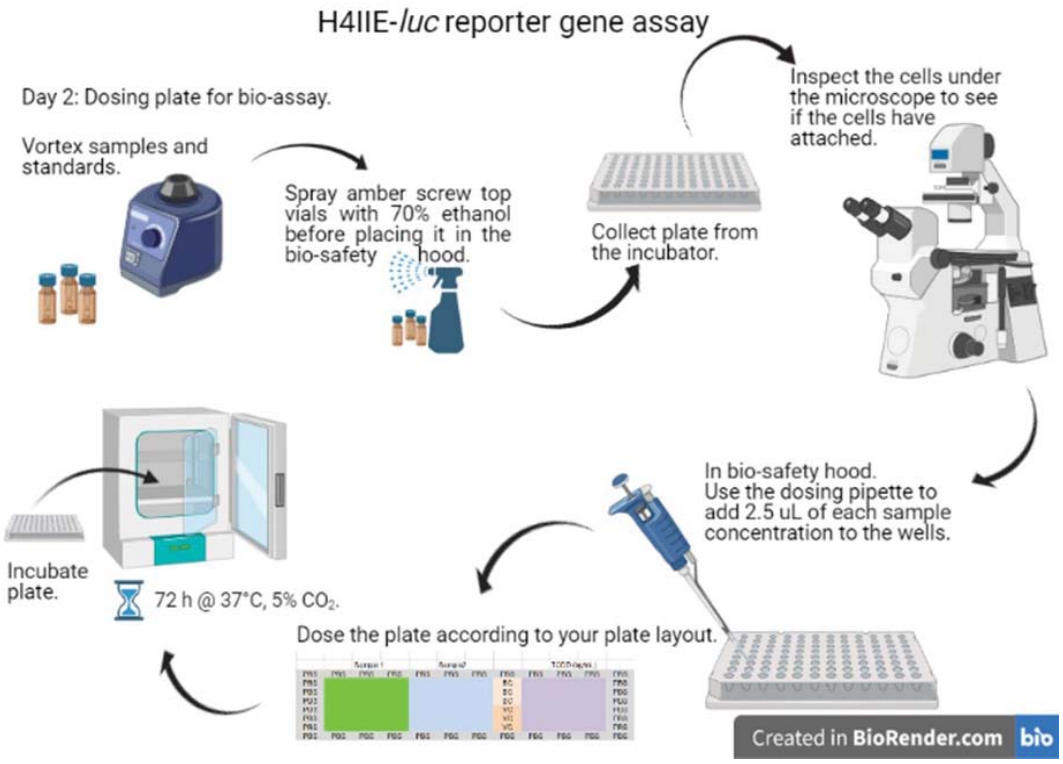

Figure 3-8: Diagram for day 2 of the reporter gene assay using H4IIE-*luc* cells for dosing the plate.

Day 5

Sterilised conditions are not necessary from this point forward.

1. Remove LAR from -80˚C to thaw and switch the lights off (Figure 3-9).
2. Remove the plate from the incubator and inspect visually for any signs of cytotoxicity. Note this on the plate lay-out sheet because this will help interpret the results.
3. Remove the media from the wells by shaking it over a waste tray.

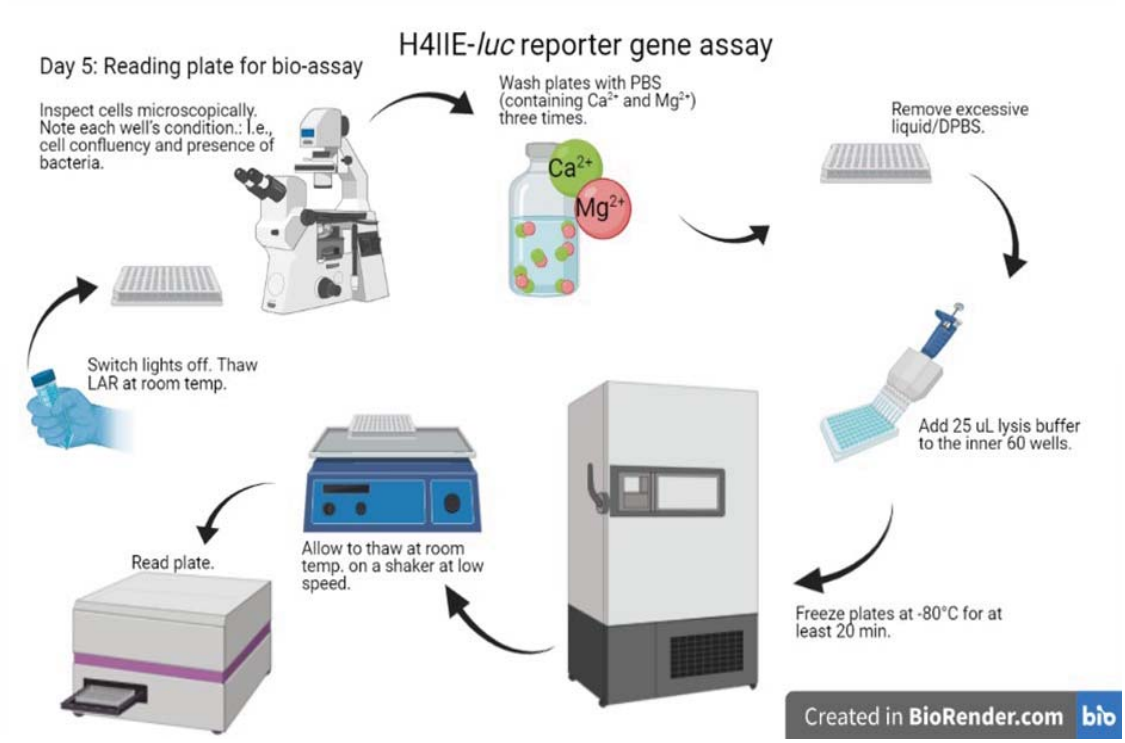

Figure 3-9: Day 5 of the reporter gene assay illustration.

4. Wash the cells three times with DPBS containing Mg^{2+} and Ca^{2+} salts, and pat the plate dry on tissue paper.
5. Add 25 µL reporter lysis buffer to each of the inner 60 wells.
6. Freeze the plate for 20 minutes at -80˚C to ensure lysis of the cells.
7. After at least 20 minutes, remove the plate and thaw on the plate shaker before reading the luminescence. The plates may be stored in the freezer for a maximum period of three months before reading the results. The luciferase activity is recorded/quantified as relative light units (RLU).
8. While waiting for the plate to thaw, it is important to use a clean 96-well plate to prime the microplate reader. Open the installed program on the computer and place the injector in use (i.e. injector 4) in the LAR and prime the lines from the injector to the plate (Select prime under the instrument option).
9. After the plate is primed inspect the plate for evidence of LAR inside wells, this will indicate a successful prime. Now the microplate reader is ready. Set the plate reader to inject 100 µL LAR into each well, briefly shake to mix the contents and read the luminescence immediately before continuing to the next well.
10. After all the plates are read it is important to rinse the injector with deionised H_2O. This is usually done using the same plate as the one used to prime the microplate reader. Insert the plate and on the program select wash under the instrument option. Wash plate twice to ensure the injector and the lines are properly cleaned.

3.7 MTT VIABILITY ASSAY

This colorimetric assay measures the metabolic activity of cells and is based on the principle that active cells can reduce the yellow MTT solution through mitochondrial dehydrogenase to form violet-blue formazan crystals (Mossmann, 1983).

This assay is done simultaneous to the reporter gene assay but in separate plates. This assay is performed to determine the viability of the cells after exposure to environmental samples with unknown compounds. If cytotoxicity is observed in the MTT viability assay, the luminescence assay results should be reconsidered: the absence of light in the luminescence assay may be due to dead or dying cells, and not the lack of ligands. A dilution adjustment is required to determine a safe concentration series at which the cells can be exposed. The luminescence assay should be repeated with the adjusted concentrations series to learn if there are any ligands capable of binding to the AhR.

Day 1

1. Seed the cells in the inner 60 wells as well as the entire column 12.
2. Seed cells in a transparent 96-well microplate.

Day 2

1. Dosing is done the same as for the reporter gene assay. The cells in column 12 are left unexposed – to be treated at the end of the assay (Day 5).

Day 5

1. Prepare the MTT solution in the dark.
2. Remove the plate from the incubator and inspect visually.
3. Remove media from column 12 and kill the cells with 200 µL methanol in each well of this column. Wait five minutes. This provides an absorbance value for dead cells and provide the negative control whereas the absorbance from the VC cells represents the 100% viable cells (positive control).
4. Remove media and methanol from the plate and wash three times with DPBS. Remove the excessive liquid on tissue paper.
5. Add 100 µL MTT solution into the wells and place the plate in the incubator for 30 minutes.
6. Remove plate from incubator and discard the MTT solution from the wells.
7. Add 200 µL DMSO to all cell-containing wells. Leave the plate for another 30 minutes before reading the absorbance with the microplate reader.

3.8 CALCULATIONS AND REPORTING OF RESULTS

3.8.1 Reporter gene assay

1. The raw data can be worked in Microsoft Excel®.
2. Calculate the mean, standard deviation, and coefficient of variation for the triplicate values of each concentration dosed.

3. Express each mean as a percentage of the maximum response elicited by the reference compound (%TCDD-max).
4. Plot the dose-response curve for each sample and the reference compound separately with the %TCDDmax on the y-axis and the logarithm of the concentration (in the case of the reference compound) or the logarithm of the volume dosed (in the case of the sample) on the x-axis.
5. Determine the slope, and intercept for the straight-line section (y = mx + c) of each dose-response curve. Use all the points on the straight-line section of the graph. There should be a minimum of three. If there are not at least three, the curve cannot be used for calculations. If this is the situation for the reference compound, the assay must be repeated.
6. Determine the effective concentration (EC) at 20, 50 and 80% of the sample curves as well as the reference compound curve.
7. Determine the relative effects potency for EC20-50 by dividing the EC20 of the reference compound curve with the EC20 of the sample curve. This is also referred to as the bio-assay equivalent (BEQ). This is done to determine if the slope of the sample curve is parallel to the slope of the reference compound. In the case of parallel slopes, the relative potency range (REP20-50) will be the same value. This will indicate that the compounds in the sample have the same response on the cells as the reference compound, acting as a dilution (higher concentration) of the reference compound (Villeneuve et al., 2000). For nonparallel dose-response relationships, relative potency is a function of dose and the relationship at a single level of response, such as the EC50, is not constant over the entire range of responses for the compounds being compared. Calculating the REP20-50 will return different values indicating that reporting only a REP50, for example, would be misleading (Villeneuve et al, 2000). Therefore, it is best to report all three, REP20-50 but to clearly indicate which responses were achieved by the cells, and which ones are only extrapolated values. Experience showed that REP20 and REP50 are often elicited by environmental samples, but not REP80.
8. Back calculate the REP to take the volume of water or mass of sediment that had been extracted into consideration.
9. These values can be compared with international guideline levels for minimum allowable levels of dioxin-like compounds in the environment.

3.8.2 MTT viability assay results

1. For each triplicate sample dilution, and the VC and MeOH killed cells, calculate the mean, standard deviation, and coefficient of variation (%CV) of the optimal densities (ODs). This can be done on an Excel® spread sheet (Figure 3-10). The CV indicates the quality of the data points: the smaller the variation between them the more valid are they. The %CV should be no more than 20%. If this is the case, one data point may be removed to improve the CV. This same point must be kept out of all future calculation.
2. Subtract the mean OD of the MeOH killed cells from each of the individual readings. This will give you a new set of data.
3. Determine % viability for each data point, including the VC and the MeOH by dividing its new value (i.e. the value after subtracting MeOH OD). Again, calculate mean and standard deviation for every triplicate sample dilution, VC and killed cells. Again, determine mean and standard deviation.

4. Plot the means on a bar graph and add the standard deviation.
5. Statistically significant differences between a sample and the VC should be determined using the Mann-Whitney U test ($p < 0.05$) because of the limited number of data points. This will give an indication whether any of the results is statistically significant compared to the VC.

	Conc				Mean	Stdev	%CV
VC	mg/mL	A	B	C			
Sample 1							
Sample 2							
Sample 3							
Sample 4							
MeOH killed cells							

Figure 3-10: Indication of a template for the raw MTT data (Conc: concentration; Stdev: standard deviation; %CV: coefficient of variation; VC: vehicle control;)

3.9 SEDIMENT EXTRACTION

There is more than one method to extract the sediment (Soxhlet and accelerated solvent extraction (ASE) methods).

The steps of the ASE extraction method (alternatively known as pressurised liquid extraction (PLE)) will be provided below. The model of the instrument described is: Dionex150 (US EPA, 1998).

Note: All equipment and utensils that come into contact with the sample should be pre-rinsed with acetone (three times) followed by hexane (three times). The rinse step removes potential polar (acetone) and apolar contaminants (hexane) that might contaminate the sample.

1. Air dry or freeze dry the sediment samples for several days until completely dry. Samples should be protected from UV radiation to prevent possible degradation of target samples.
2. Grind the sample to a fine powder using for example a mortar and pestle. Grinding increases the available surface from which target compounds can be extracted.
3. Sieve the sample using a copper sieve with a mesh size of 0.5 mm to create a homogenous sample matrix.
4. Take 40 g sediment (note the exact mass) and mix it with an equal volume of anhydrous diatomaceous earth to remove any trace water molecules that might still lurk within the sample. The presence of water will prevent efficient extraction in the presence of organic solvents to be used. The diatomaceous earth should have been previously cleaned by extracting with a 3:1 mixture of dichloromethane (DCM) and hexane.

5. Wash the ASE extraction cell with phosphate-free soap and rinse it with 18.2 MΩ.cm water, afterwards rinse it three times with acetone and hexane.
6. Allow the ASE cell to dry completely before placing the cellulose filter in the outlet end of the cell.
7. Place the dried sediment in the cell and a cellulose filter on top. Close the lid tightly.
8. Use a DCM and hexane mixture (3:1) as the extraction solvent.
9. Extract the sediments using the parameters: 100˚C, 1500 psi (10 min heat and 5 min static), 60% flash volume, nitrogen purge time of 100 seconds and the cycles (McCant et al., 1999).
10. Collect the extract in a collection bottle which was pre-cleaned and allow it to cool.
11. Evaporate the extract to almost dryness (1 mL) using a gentle stream of nitrogen gas (i.e. can use TurboVap II).
12. Transfer the remaining extract to a tube and adjust volume to 10 mL using hexane.
13. Sediment extracts contain elemental sulphur which is cytotoxic to H4IIE-*luc* cells. A copper treatment can be applied to the extract to remove the sulphur from the sediments (US EPA, 1986).
14. Add 0.5 g copper shavings (freshly activated) to 10 mL sample extract in a test tube. (The copper shavings can be activated through using 32% hydrochloric acid, rinse the shavings with acetone and hexane).
15. Swirl the tube to mix the extract and copper shavings and allow it to stand for 10 min.
16. Add copper shavings until they do not turn black anymore.
17. Transfer the sample quantitatively to separation funnels for the acid treatment.
18. Acid treatment: Add 15 mL 98% concentrated sulphuric acid (this will remove all non-dioxin-like compounds) and mix it carefully.
19. Let it stand until the two phases are clearly separated. This takes approximately 1 hour. Carefully tap off the acid phase and discard safely.
20. Repeat steps 18 and 19 until the acid phase stays clear and does not turn black anymore
21. Wash the sample-containing-hexane phase with 5% aqueous sodium chloride and leave it to stand for the phases to separate. Tap off the aqueous phase and discard safely.
22. Wash the sample with 20% aqueous potassium hydroxide (tap the KOH-solution off immediately after separation of the phases, because it can breakdown the compounds of interest).
23. Lastly wash the sample with a second 5% NaCl-solution.
24. Filter the hexane phase through pre-cleaned glass wool which is covered with anhydrous sodium sulphate.
25. Concentrate the crude extract to 0.5 mL using nitrogen gas and adjust the volume to 1 mL with hexane.
26. Store the extract in 2 mL amber vials and store at 4˚C until it is used in a bio-assay. These should be clearly labelled and efforts to prevent undue evaporation of the hexane should be taken do not store the sample too long, and wrap the lids with Parafilm® strips.

3.10 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

3.10.1 Advantages

- Like all bio-assays this assay provide a summarised biological effect mediated through the same mechanisms of action. In this case the mechanism of action is the binding to the AhR.
- The outcome of this assay can be compared to a guideline level based on the toxic equivalent quotient calculated from an instrumental analysis of the sample.

3.10.2 Limitations

- Effect-based trigger values are still in the process of being established.
- There are many AhR ligands in the environment and to distinguish between the more and less potent ones, an acid wash step should form part of the sample preparation steps.
- The cells used by the commercial DR-CALUX method are expensive and annual licensing fees are required. The cells used by research laboratories are available without charge, but not easily accessible without the right contacts.

3.10.3 Recommendations

- There is an international method validation process currently ongoing by the International Organization for Standardization (ISO) to validate this assay. Although the above method is based on preliminary versions of the ISO method, the final ISO method should be established in a monitoring/regulatory laboratory.

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3.12 USEFUL CONTACTS

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4 YEAST (ANTI)ESTROGEN AND (ANTI)ANDROGEN SCREENS

Compiled by: E Archer, C Truter, G Wolfaardt and N Aneck-Hahn

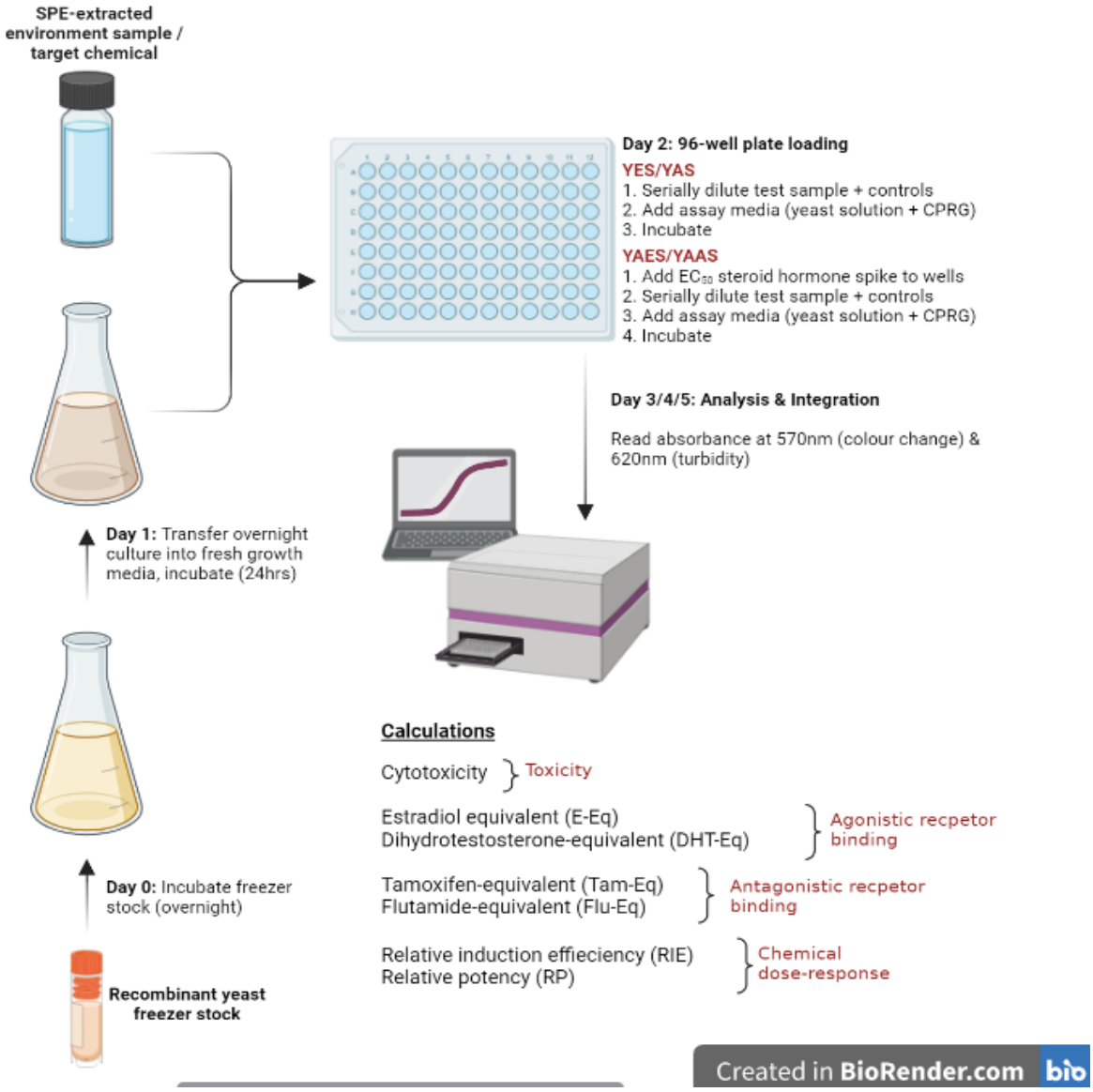


Figure 4-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the yeast (anti)estrogen and (anti)androgen screens

4.1 ACRONYMS & ABBREVIATIONS

ARE	Androgen Response Element
CPRG	Chlorophenol red-b-D-galactopyranoside
DHT	Dihydrotestosterone
E_2	Estradiol
EC_{50}	Effect Concentration At 50%
EDC	Endocrine Disrupting Contaminant
ERE	Estrogen Response Element
ERα/ERβ	Estrogen Receptor Alpha or Beta
FLU	Flutamide
hAR	Human Androgen Receptor
hER	Human Estrogen Receptor
MeOH	Methanol
OD	Optical Density
RIE	Relative Induction Efficiency
RP	Relative Potency
TAM	Tamoxifen
YAAS	Yeast Antianrogen Screen
YAES	Yeast Antiestrogen Screen
YAS	Yeast Androgen Screen
YES	Yeast Estrogen Screen

4.2 PRINCIPLE OF THE ASSAY

4.2.1 Yeast Estrogen Screen (YES) and Yeast Androgen Screen (YAS) assays

Recombinant Saccharomyces cerevisiae yeast strains, PGKhER and PGKhAR, were developed to identify compounds that can interact with the human estrogen receptor (hER) and human androgen receptor (hAR) respectively (Figure 4-2). The PGKhER cell line was transfected with the hERα gene into the main genome, along with an expression plasmid containing an estrogen response element (ERE)-linked lac-Z gene complex that encode for the enzyme β-galactosidase, whilst the PGKhAR cell line was transfected with the hAR gene into the main genome, along with an expression plasmid containing an androgen response element (ARE)-linked lac-Z gene complex that also encode for the enzyme β-galactosidase. The hER or hAR in the contrasting cell lines are expressed (Figure 4-2-1) in a form capable of binding to ERE/AREs within a hybrid promoter on the expression plasmid (Figure 4-2-2). Activation of the receptor by binding of a ligand (steroid hormone or EDC) (Figure 4-2-3) causes expression of the reporter gene Lac-Z (Figure 4-2-4) that promotes the transcription and translation for the enzyme β-galactosidase. This enzyme is secreted into the assay medium (Figure 4-2-5) and result in the hydrolysis of the chromogenic substrate chlorophenol red-b-D-galactopyranoside (CPRG) in the assay medium from a yellow- to a red product (Figure 4-2-6), which can be measured by absorbance (Routledge and Sumpter, 1996; Urbatzka et al., 2007; Jobling et al., 2009).

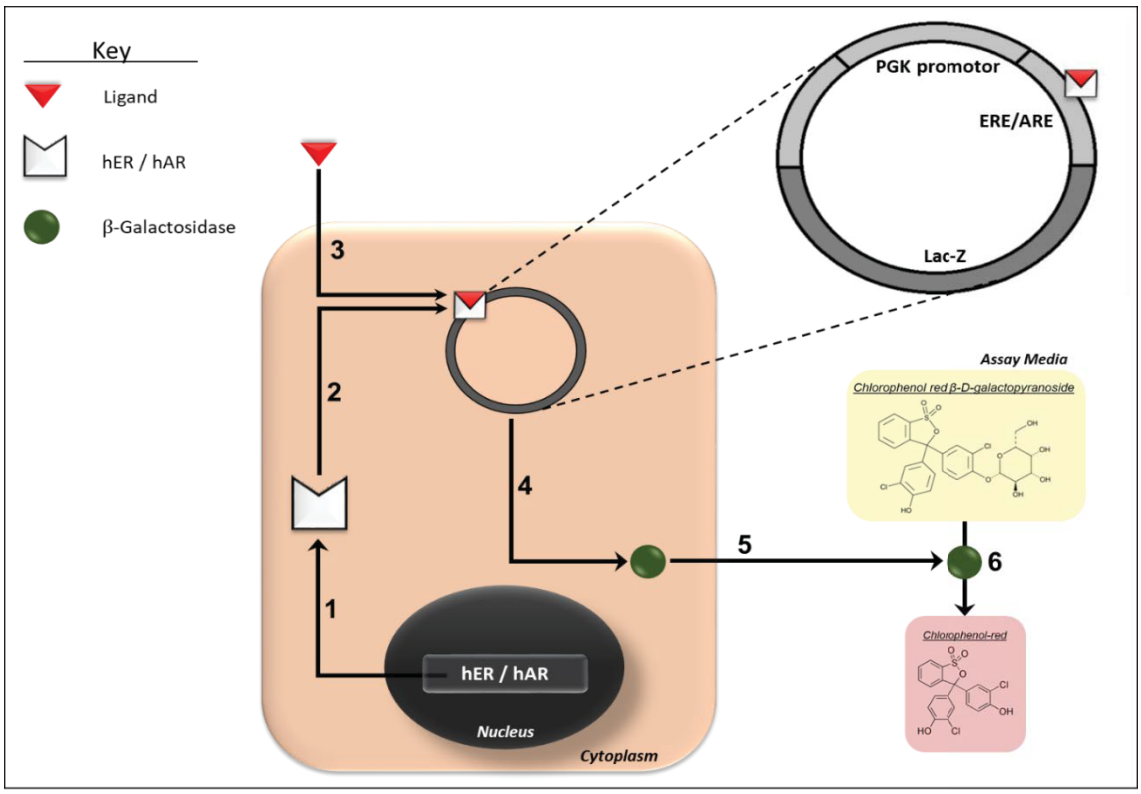


Figure 4-2: Schematic representation of the estrogen/androgen receptor-inducible expression system in the yeast cell lines

The concentration of ligands in the test sample that can initiate steroid hormone receptor-dependent β-galactosidase production, and subsequent CPRG hydrolysis in the assay media, is thus directly correlated to the presence of natural and/or anthropogenic substances that interfere with hERα or hAR binding in a dose-dependent manner.

4.2.2 Modifications of the YES and YAS to investigate both agonistic- or antagonistic steroid hormone receptor binding

Both the YES and YAS assays allows for investigating whether ligands in the test sample will either bind to the respective steroid hormone receptors in either:

(1) an agonistic manner by having similar estrogenic/androgenic effects as a model steroid hormone, or
(2) an antagonistic manner by interfering with normal binding of a steroid hormone in the assay, therefore having anti-estrogenic or anti-androgenic properties.

Investigation for estrogenic/androgenic responses (i.e. steroid hormone agonism), the standard protocol for the YES and/or YAS is followed (Figure 4-3) that include comparing the potency of a test chemical or test sample against a standard curve of a model steroid hormone, namely 17β-estradiol (E_2) for the YES and dihydrotestosterone (DHT) for the YAS. These assays thus allow to either calculate relative potencies (RP)

and relative induction efficiencies (RIE) of individual/mixture test chemicals or E_2-equivalent concentrations (E-EQ) and DHT-equivalent concentrations (DHT-Eq) respectively for environmental test samples.

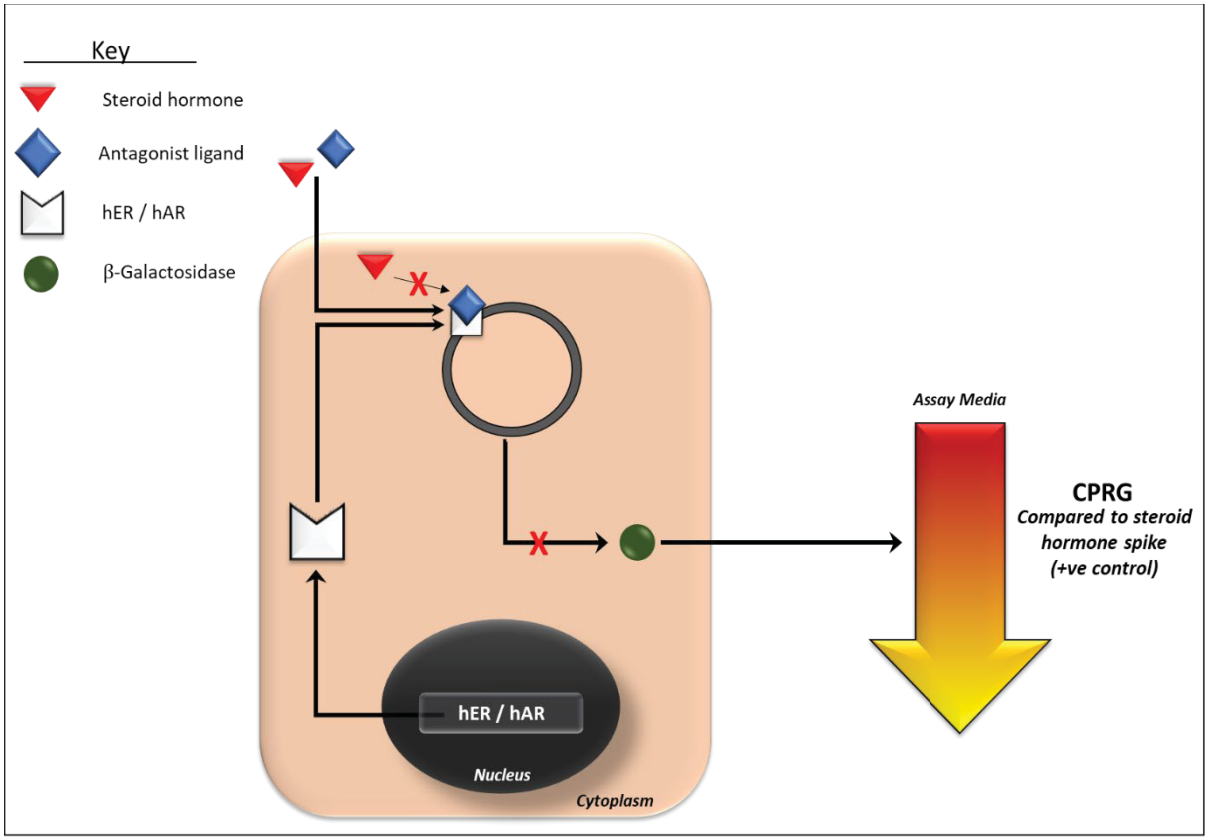


Figure 4-3: Schematic representation of steroid hormone antagonism expression in the yeast cell lines

In contrast, investigating anti-estrogenic and/or anti-androgenic responses (i.e. steroid hormone antagonism), a modified protocol namely a yeast anti-estrogen screen (YAES) and/or yeast anti-androgen screen (YAAS) is followed. In this manner, the ability of any ligand in the test sample to either interfere with the normal binding of a model steroid hormone to the steroid hormone receptor or any downstream pathway that inhibit the production of the enzyme β-galactosidase will be expressed through the inability of CPRG to be hydrolised from a yellow to a red product in the assay media. The most predominant modifications to the YAES and YAAS include and additional spiking step of an EC_{50} concentration of the model steroid hormone in the assay plate wells of each test chemical/environmental test sample and replacing the reference standard curve with a model steroid hormone receptor antagonist, namely tamoxifen (TAM) for the YAES and flutamide (FLU) for the YAAS alongside a spike of an EC_{50} concentration of the model steroid hormone (E_2 and DHT respectively). These assays thus allow to either calculate relative potencies (RP) and relative induction efficiencies (RIE) of individual/mixture test chemicals or tamoxifen-equivalent concentrations (TAM-EQ) and/or flutamide-equivalent concentrations (FLU-Eq) of environmental test samples.

4.3 REQUIREMENTS

4.3.1 Acquisition of the cell line

The yeast cell lines can be obtained from Xenometrix, Switzerland (YES Cat. No. N05-230-E; YAS Cat. No. N05-230-A). The local distributor is ToxSolutions Kits and Services:

E-mail: hesmarie@toxsolutions.co.za

4.3.2 Permits

The Department of Agriculture, Land Reform and Rural Development is responsible for issuing a permit for importing genetically modified organisms:

Postal address: Directorate Genetic Resources, Private Bag X973, Pretoria, 0001
E-mail: GMO@dalrrd.gov.za
Website: https://www.dalrrd.gov.za/Branches/Agricultural-Production-Health-Food-Safety/Genetic-Resources/Biosafety

Ms Refilwe Ngoepe
Tel: 012 3196364
E-mail: RefilweN@daff.gov.za

Ms Bathobile Mahlangu
Tel: 012 319 6364
E-mail: BathobileM@daff.gov.za

4.3.3 Staff training (technical skills)

- Good laboratory practice
  - For cell culture laboratory guidelines refer to Clitherow et al. (2006)
- Training in cell culture techniques
- Software package knowledge (e.g. Excel, Graphpad Prism or similar)

4.3.4 Laboratory (test environment)

- Dedicated cell culture laboratory

o For cell culture laboratory guidelines refer to Wigley (2006)

4.3.5 Software

- Graphpad Prism version 4.0 or higher
- Microsoft Excel

4.3.6 Apparatus

- Assay dedicated glassware:
    - Beakers (25 mL, 50 mL, 100 mL, 200 mL, 500 mL)
    - Schott Bottles, 25 mL, 50 mL, 100 mL, 250 mL, 500 mL and 1 L glass bottles (Cat. No.21801-145/175/245/365/445/545, Merck)
    - Erlenmeyer Flasks (25 mL, 50 mL, 100 mL, 250 mL)
    - Test tubes, 25 mL (for long term stock cultures)
    - Glass syringe (5-10 mL)
- Autoclave
- Centrifuge with swing-out rotor
- Conical centrifuge tubes, glass or plastic, 50 mL (Sigma Cat. No. Z14,575-0 or equivalent)
- Incubator, natural ventilation (Heraeus, B290 or equivalent)
- Millipore Milli-Q synthesis ultrapure water system or equivalent system to produce double distilled water (ddH_2O), fitted with an EDS filter (Cat. No. EDSPAK001, Microsep).
- Multiskan Titertek 96 well plate reader (Titertek Multiskan MCC/340 or equivalent)
- Pipette, 8 or 12 channel, volume 5-50 µL
- Pipette, 8 or 12 channel, volume 30-300 µL
- Pipette, 2-20 µL
- Pipette, 20-200 µL
- Pipette, 200-1000 µL
- Refrigerator/freezer combination
- Shaker (Heidolph Titramax 100 or equivalent)
- Type I or II biological safety cabinet
- Hemocytometer
- Light microscope

4.3.7 Consumables/materials and reagents

Tables 4-1 and 4-2 provide lists of the consumables/materials and reagents required for the yeast (anti)estrogen and (anti)androgen screens.

Table 4-1: List of the consumables/materials required for the yeast (anti)estrogen and (anti)androgen screens

Name	Cat no	Supplier
• Tin foil		
• Autoclave tape		
• Cryovials, sterile, 1.8 mL	377267	AEC-Amersham
• Petri dishes, 90 x 15mm, sterile	PLAS-HP0005	Lasec
• Microtiter plates, clear 96 well flat bottomed (Greiner)	M3061	Sigma-Aldrich

Name	Cat no	Supplier
• Microtiter plates with lid, sterile, clear 96 well flat bottomed (Corning®)	CLS3997	Sigma-Aldrich
• Nitrile gloves	RLAS1GL014	Lasec
• Pipette tips (200 µL)	Z740030	Sigma-Aldrich
• Pipette tips (1000 µL)	Z740095	Sigma-Aldrich
• Reagent reservoirs (Corning® Costar®)	CLS4870	Sigma-Aldrich
• Whatman PURADISC filters, 0.2 µm	6780-2502	Microsep
• Inoculation needle, loop, sterile	13-079A	Thermo Scientific

Table 4-2: List of the reagents required for the yeast (anti)estrogen and (anti)androgen screens

Name	Cat no	Supplier
Potassium dihydrogen phosphate (KH_2PO_4)	P-0662	Sigma
Ammonium sulphate $(NH_4)_2SO_4$)	R 0350/500g	NT Laboratory Supplies
Potassium hydroxide (KOH pellets)	1310-58-3	Sigma
Anhydrous magnesium sulfate ($MgSO_4$)	7487-88-9	Sigma
Ferric sulphate ($Fe_2(SO_4)_3$)	F-1135	Sigma
L-leucine	371213W	BDH
L-histidine	372214E	BDH
Adenine	1.00838	Merck
L-arginine-HCl	1.01543	Merck
L-methionine	371315E	BDH
L-tyrosine	371562R	BDH
L-isoleucine	371236G	BDH
L-lysine-HCl	371293P	BDH
L-phenylalanine	1.07256	Merck
L-glutamic acid	371024T	BDH
L-valine	37160	BDH
L-serine	371465R	BDH
Thiamine	440055N	BDH
Pyroxidine	449865Q	BDH
Pantothenic acid	111 993	Merck
Inositol	380443M	BDH
Biotin	44011 4H	BDH
D-glucose	ART.8337	Merck
L-aspartic acid	370225W	BDH
L-threonine	371505Y	BDH
Copper (II) sulphate, anhydrous ($CuSO_4$)	7758-98-7	Sigma
Chlorophenol red-β-D-galactopyranoside (CPRG)	10884308001	Roche Diagnostics

Name	Cat no	Supplier
17β-Estradiol	50-28-2	Sigma
Tamoxifen	10540-29-1	Sigma
Dihydrotestosterone	521-18-6	Sigma
Flutamide	13311-84-7	Sigma
Ethanol	27,0741	Sigma
Glycerol	G2025	Sigma
Agar	A9915	Sigma
Parafilm	P7793	Sigma

4.4 METHOD – PREPARATION FOR THE BIOASSAY

4.4.1 Media components

Note: When preparing the assay components, ddH_2O that went through an EDS filter (to remove EDCs) should be used.

4.4.2 Minimal Medium (pH 7.1)

Table 4-3: Minimal medium is prepared by dissolving the following media components in ddH_2O

Chemical	Mass (g)	Volume (mL)
KH_2PO_4	13.6	
$(NH_4)_2SO_4$	1.98	
KOH pellets	4.2	
$MgSO_4$	0.2	
$Fe_2(SO_4)_3$ solution (40 mg/50 mL ddH_2O)[a]		1
L-arginine-HCl	0.020	
L-methionine	0.020	
L-phenylalanine	0.025	
L-tyrosine	0.030	
L-isoleucine	0.030	
L-lysine-HCl	0.030	
L-leucine	0.050	
L-histidine	0.050	
Adenine	0.050	
L-glutamic acid	0.100	
L-valine	0.150	
L-serine	0.375	
ddH_2O		1000

[a] Ferric sulphate solution: 40 mg $Fe_2(SO_4)_3$ in 50 mL ddH_20

Aliquot 45mL of the final solution into 200 mL glass Schott bottles and sterilise by autoclaving at 121°C for 20 minutes.

- *Store at room temperature for up to 6 months*.

4.4.3 Vitamin solution

Table 4-4: The vitamin solution is made by dissolving the following in ddH2O

Chemical	Mass (g)	Volume (mL)
Thiamine	0.008	
Pyroxidine	0.008	
Pantothenic acid	0.008	
Inositol	0.040	
Biotin solution[a]		20
ddH_2O		180

[a]Dissolve 2 mg biotin in 100 mL ddH_2O

Sterilise the solution by filtering through a 0.2µm pore size Whatman PURADISC filter into sterile glass bottles (10 mL aliquot per bottle)

- *Store at 4°C for a maximum of 3 months*.

4.4.4 Glucose solution (20% w/v)

Dissolve 20 g D-glucose in 100 mL ddH_2O and sterilise in 20 mL aliquots by autoclaving at 121°C for 20 minutes.

- *Store at 4°C for a maximum of 3 months*.

4.4.5 L-aspartic acid solution (4mg/mL)

Dissolve 400 mg of L-aspartic acid in 100 mL ddH_2O and sterilise in 20 mL aliquots by autoclaving at 121°C for 20 minutes.

- *Store at 4°C for a maximum of 3 months*.

4.4.6 L-threonine solution (24mg/mL)

Dissolve 1200 mg of L-threonine in 50 mL ddH_2O and sterilise in 5 mL aliquots by autoclaving at 121°C for 20 minutes.

- *Store at 4°C for a maximum of 3 months*.

4.4.7 Copper (II) sulphate solution (20 mM)

Dissolve 31.92 mg copper (II) sulphate in 100 mL ddH_2O and filter sterilise through a 0.2 µm pore size Whatman, PURADISC filter into sterile glass bottles.

- *Store at room temperature*.

4.4.8 Chlorophenol red-β-D-galactopyranoside (CPRG)

Prepare a 10 mg/mL stock solution by dissolving 100 mg CPRG in 10 mL ddH_2O and filter sterilise through 0.2 µm pore size Whatman, PURADISC filters into sterile amber or foil-covered glass bottles.

- *Store at 4°C for 3 months*.

4.4.9 Reference standard chemicals (for standard curve)

4.4.9.1 *17β-Estradiol (E_2) stock solution (200 nM)*

A 54.58 g/L stock solution of 17β-estradiol is prepared in HPLC grade ethanol in a sterile glass bottle and stored at -20°C.

4.4.9.2 *Dihydrotestosterone (DHT) stock solution (100 µM)*

A 29.045 mg/L stock solution of dihydrotestosterone is prepared in HPLC grade ethanol in a sterile glass bottle and stored at -20°C.

4.4.9.3 *Hydroxy-tamoxifen (OH-TAM) stock solution (100 µM)*

A 38.75 mg/L stock solution of hydroxy-tamoxifen is prepared in HPLC grade ethanol in a sterile glass bottle and stored at -20°C.

4.4.9.4 *Flutamide (FLU) stock solution (100 µM)*

A 27.62 mg/L stock solution of flutamide is prepared in HPLC grade ethanol in a sterile glass bottle and stored at -20°C.

4.5 METHOD FOR PREPARATION AND STORAGE OF YEAST STOCK CULTURES

4.5.1 Long-term stock cultures

Long-term stock cultures are prepared on minimal medium agar slopes. Minimal medium must be prepared, then add 1g agar per 100 mL medium (1% agar). After autoclaving 90 mL must be cooled to 50°C before adding the following growth medium components:

- 10 mL glucose
- 2.5 mL L-aspartic acid
- 1 mL Vitamin solution
- 0.8 mL L-threonine
- 250 µL Copper (II) sulphate

This must be gently swirled to mix and directly pour 10 mL into sterile glass tubes (universals). The tubes must be left at an angle of approximately 45° for the agar to set. Approximately 2 µL of the original yeast stock culture (stock culture from Prof JP Sumpter's laboratory, UK) is spread over the surface of the slope. The slope must be incubated at 32°C for 3 days, then 1 mL sterile 100% glycerol is drizzled down the slope and the cells are suspended in the medium using a sterile loop or a 1000 µL pipette with a filter tip. Aliquots of the glycerol yeast suspension are then transferred to sterile 1.8 mL cryovials and subsequently stored at -70°C or -80°C for a maximum of 6 months.

4.5.2 Short-term stock cultures (10× concentrated yeast stock)

Day 1

A flask of growth medium must be prepared as described above (see section 4.8). Add 125 µL of the long-term yeast stock to the flask. Incubate at 28°C for approximately 24 hours on a shaker at 150-155 rpm.

Day 2

Prepare two flasks of growth medium as described above. Place 1 mL of the 24-hour yeast culture into each flask. Incubate both flasks at 28°C for 24 hours on a shaker at 150-155 rpm.

Day 3

Transfer the 24-hour culture to two sterile 50 mL Pyrex glass or polypropylene centrifuge tubes. Centrifuge at 4°C for 10 minutes at 2000 × gc.

c) Working out the G-force of the centrifuge

G-force (or g) = (1.118 × 10-5) r s2 = 0.0000118 × r × s2

Where:
s = revolutions per minute (i.e. the speed you spin at)
r = the radius (the distance in centimetres from the centre of the rotor to the bottom of the bucket holding the tubes when the bucket is in the swing-out position)
Moodley et al. (2008)

Decant the supernatant and resuspend the pellet in 5 mL sterile minimal medium with 15% sterile glycerol (by adding 8 mL glycerol to 45 mL minimal medium). This 10× concentrated yeast stock culture must be dispensed into sterile 1.8 mL cryovials as 0.5 mL aliquots. These stock cultures can be stored at -20°C for a maximum of 4 months.

4.6 GROWTH MEDIUM FOR THE ASSAY

Prepare growth medium by combining the following medium components:

- 45 mL Minimal medium
- 5 mL Glucose solution
- 1.25 mL L-Aspartic acid solution
- 0.5 mL Vitamin solution
- 0.4 mL L-Threonine solution
- 125 µL Copper (II) sulphate solution

4.7 PREPARATION FOR SAMPLE ANALYSIS

Note: The assay should be carried out in a type I or II laminar flow air cabinet, to minimise aerosol formation.

Caution: After seeding and during the exposure period (incubation period), the climate disturbances in the incubator should be kept to a minimum (i.e. limited opening and closing of the incubator). All the microtiter plates should be placed separately of each other, not stacked as this can affect the results of the assay.

4.8 ASSAY PROCEDURE – PREPARATION OF YEAST CULTURE AND ASSAY MEDIUM

- **DAY 0 – FREEZER STOCK CULTURING**
    - Prepare a new flask with *GROWTH MEDIUM* (section 4.8).
    - Add 125 µL of the *10X concentrated yeast stock* (section 4.10.2) in the fresh media.
    - Incubate for 24hrs at 28°C on a shaker inside an incubator at 150-155 rpm until turbid.

- **DAY 1 – 1ST PASSAGE**
    - Prepare a new flask with *GROWTH MEDIUM* (section 4.8).
    - Add 2 mL of the overnight-incubated yeast stock into the fresh growth medium.
    - Incubate for 24 hours at 28°C on a shaker inside an incubator at 150-155 rpm until turbid.

- **DAY 2 – PREPARATION OF THE ASSAY MEDIUM (ON THE DAY OF THE BIOASSAY)**
    - Prepare a new flask of *GROWTH MEDIA* (section 4.8)
    - The yeast cell concentration needs to be adjusted to **4x10^{7} cells/50mL** in the bioassay (Sohoni & Sumpter, 1998). Therefore, the yeast concentration in the 24-hr culture needs to be determined using a hemocytometer and light microscope (see procedure at https://www.asbcnet.org/lab/getstarted/Documents/Yeast-4.pdf), thus determining the volume of overnight yeast stock culture that need to be transferred to the fresh growth medium. Alternatively, a volume of 2 mL from the 24-hr yeast culture (with an absorbance at 620 nm of 1.0) can simply be added to the fresh growth medium, granted that the OD_{620} of the 24-hr stock is adjusted to 1.0 using fresh growth medium (Routledge & Sumpter, 1996).
    - Add 0.5 mL of the chlorophenol red-β-D-galactopyranoside (CPRG) solution to the fresh growth medium.
        - This media is now ready to be used immediately for seeding the 96-well assay plates and are FROM HEREON REFERRED TO AS THE ***ASSAY MEDIUM***

4.9 METHOD – THE YEAST ESTROGEN SCREEN (YES) AND YEAST ANDROGEN SCREEN (YAS)

4.9.1 Preparation of the sample dilutions, control and blank – CHEMICAL DOSE-RESPONSE

The protocol that follows is for the determination whether test chemicals (e.g. pesticides, pharmaceuticals, personal care products, industrial chemicals) will have affinity to bind to the hER (YES) and/or hAR (YAS) in reference to a dose-dependent manner similar to a model estrogen hormone (17β-estradiol) or androgen hormone (dihydrotestosterone) respectively.

4.9.2 Serial dilutions of the test chemicals and controls

The dilutions of the test chemicals and reference standard controls must be made in a separate 96 well microtiter plates (***dilution plate***) in the following way.

Note: Each plate must contain at least one row of assay medium and solvent (ethanol/DMSO) that serve as a negative control (blank) and a standard curve for 17β-Estradiol (positive control in the YES) ranging from 1 × 10^{-8} M to 4.8×10^{-12} M (2.274 µg/L to 1.3 ng/L) that can be extended to a concentration of 1.19×10^{-15} M (0.324 pg/L) if needed, or a standard curve for dihydrotestosterone (positive control in the YAS) ranging from 5 × 10^{-6} M to 9.8×10^{-9} M (20.328 µg/L to 1.3 ng/L). Starting concentrations of test chemicals are usually from a 2 g/L master stock that is serially diluted in the ***dilution plate*** and then transferred to the ***assay plate***.

1. Place 100 µL of the solvent (ethanol or DMSO), in wells 2-12 on the plate.
2. Place 200 µL of the test sample master stock/control/blank solvent into the first well.
3. Using an 8 multichannel pipette serially dilute (100 µL) across the plate, changing the tip for each carry over.
4. Transfer 10 µL of the dilution series across to a new sterile 96 well, optically flat bottom microplate with a low evaporation lid (***assay plate***) (see Figure 4-4 below).

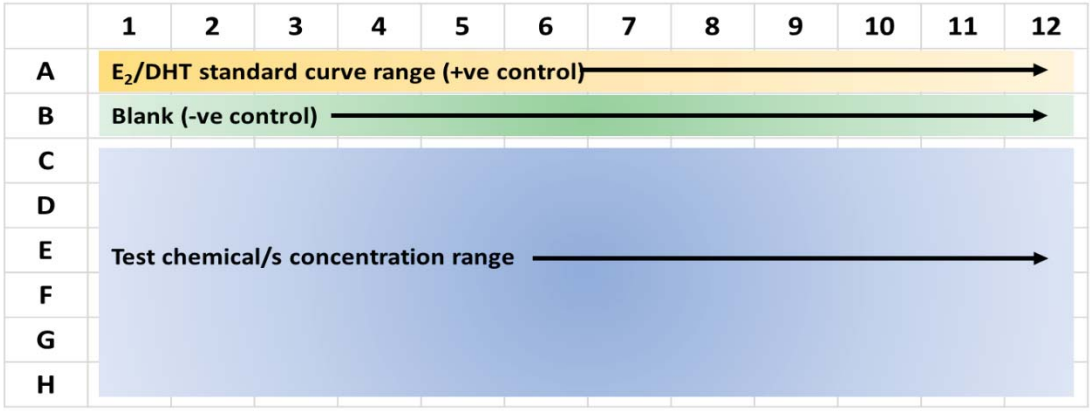
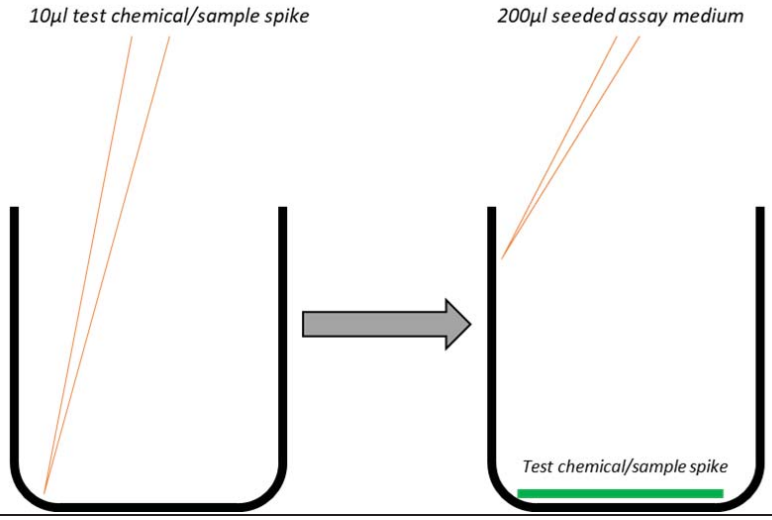


Figure 4-4: Assay plate layout for the yeast estrogen screen (YES) or yeast androgen screen (YAS)

5. Allow to evaporate to dryness on the assay plate.

6. Dispense 200 µL of the seeded assay medium that contains the chromogenic substrate (CPRG) into each sample well using a multichannel pipette.

Note: Use a new set of pipette tips for each transfer of the seeded assay medium into the assay plate. Avoid splashing when transferring the seeded assay medium by discarding the contents on the upper side of the well to avoid contact with the test chemical spike (see figure below).



7. Secure the lid and seal the plates carefully with parafilm.
    a. Place in a naturally ventilated incubator at 32°C for 3 to 5 days.
8. After 3 days incubation, the colour development of the medium must be read on the plate reader (Titertek Multiskan MCC/340) at an optical density (OD) absorbance of 570 nm for colour change (OD_{570}) and 620 nm for turbidity (OD_{620}) of the yeast culture. After reading the plate, it must be returned to the incubator for a further 24 (Day 4) and 48 (Day 5) hours, to obtain data with the best contrast between the positive control (sigmoidal dose-response of the standard curve) and the blank (negative control).
    a. Negative control wells (blank) should appear yellow to light orange in colour due to background expression of β-galactosidase and turbidity of yeast growth.
    b. Positive wells are indicated by a deep red colour accompanied by yeast growth (turbidity), usually in a dose-dependent manner.
    c. Clear wells containing no growth/turbidity indicates lysis of the cells and the colour may vary from yellow to light orange.
9. All experiments must be performed in triplicate.

Caution: Some chemicals may leach across the plate and contaminate adjacent wells (Figure 4-4). By leaving empty rows between samples, this problem can be eliminated.

Figure 4-5: An example of a YES assay plate (3 days incubation) (YAS show similar results)

Row A contains a positive sample, row B a sample with cytotoxicity (clear yellow well in column 1), row C a sample with cytotoxicity and estrogenic activity and row D a sample below the detection limit of the assay. Row F contains the assay blank and row G and H the E2 positive control standard and extended curve.

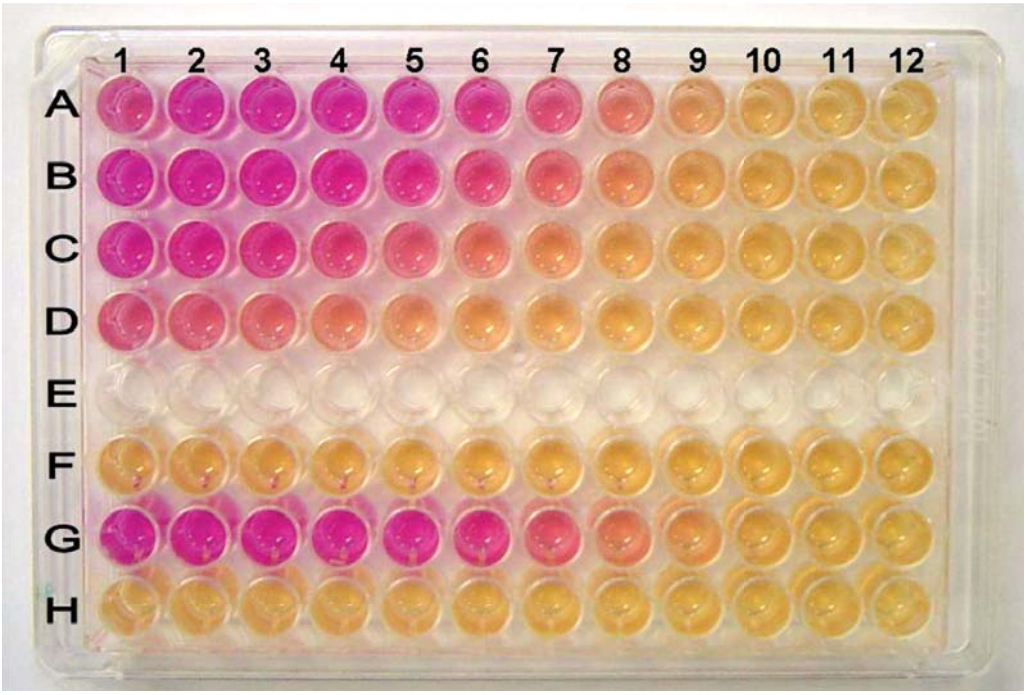


Figure 4-6: An example of leaching in the YES assay plate

Row A contains serial dilutions of a test chemical and row B to D contains only the assay medium (blank). This figure illustrates that the chemical leached across and down the plate into the adjacent wells.

4.9.3 Preparation of the sample dilutions, control and blank – ***ENVIRONMENTAL SAMPLES***

The protocol that follows is for the determination whether pre-extracted environmental samples contain chemicals, or mixtures thereof, that will have affinity to bind to the hER in a dose-dependent manner similar to a model estrogen hormone (17β-Estradiol).

4.9.4 Serial dilutions of pre-extracted environmental samples and controls

Test samples and reference standard controls must be made in a separate 96 well microtiter plate (***dilution plate***) in the following way.

Note: Each plate must contain at least one row of assay medium and solvent (ethanol/DMSO) that serve as a negative control (blank) and a standard curve for 17β-Estradiol (positive control) ranging from 1×10^{-8} M to 4.8×10^{-12} M (2.274 µg/L to 1.3 ng/L) which can be extended to a concentration of 1.19×10^{-15} M (0.324 pg/L) if needed. A 1000x or 500x pre-concentrated environmental sample that has previously been extracted and reconstituted in ethanol or methanol will be serially diluted in the dilution plate and then transferred to the assay plate.

1. Place 100 µL of the solvent (ethanol), in wells 2-12 on the plate.
2. Place 200 µL of the test environmental sample/control/blank solvent into the first well
3. Using an 8 multichannel pipette serially dilute (100 µL) across the plate, changing the tip for each carry over.
4. Transfer 10 µL of the dilution series across to a new sterile 96 well, optically flat bottom microplate with a low evaporation lid (***assay plate***) (see Figure 4-6 for sample layout in the assay plate).

Note: A 20x dilution factor will be applied to the pre-concentrated sample when the assay medium is added in the assay plate. Therefore, a starting concentration of, e.g. 1000x will equate to a 50x concentrated sample in the assay medium. For this reason, it is advised that environmental samples where a low level of pollutants are expected should be concentrated to at least 1000x during SPE so that a concentration range of at least 50x to 1.6x are achieved in the assay plate. On the contrary, environmental samples that are expected to be high in contaminants (e.g. raw wastewater) should include a wider range of serial dilutions (e.g. 50x to 0.78x), as a highly concentrated sample may introduce a high level of cytotoxic elements in the assay well that is not suitable for quantification.

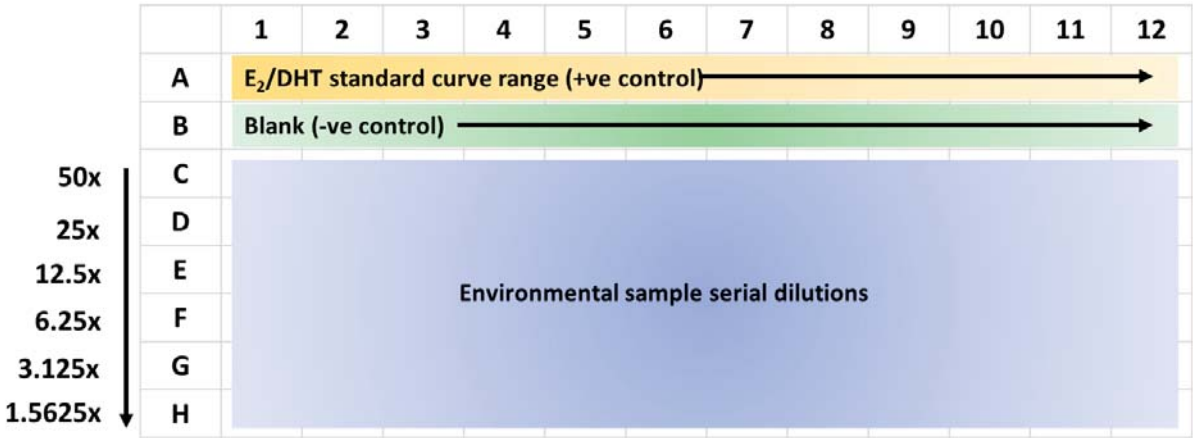
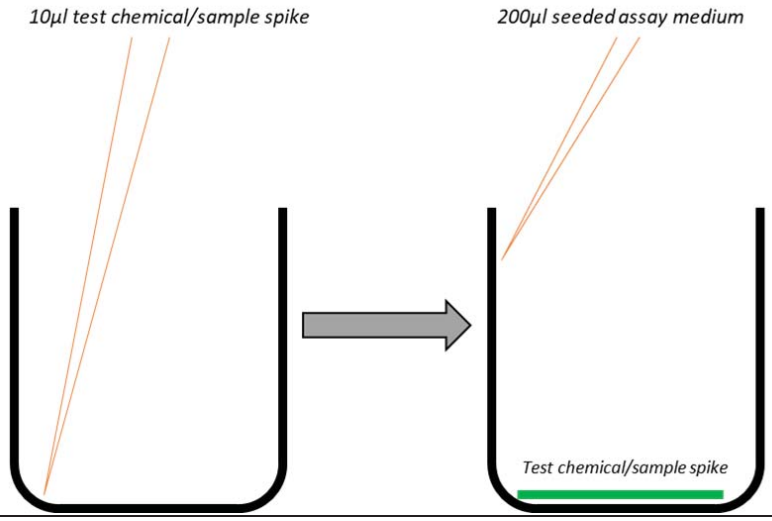


Figure 4-7: Assay plate layout for the yeast estrogen screen (YES) or yeast androgen screen (YAS)

5. Allow to evaporate to dryness on the assay plate.
6. Dispense 200 µL of the seeded assay medium that contains the chromogenic substrate (CPRG) into each sample well using a multichannel pipette.

Note: Use a new set of pipette tips for each transfer of the seeded assay medium into the assay plate. Avoid splashing when transferring the seeded assay medium by discarding the contents on the upper side of the well to avoid contact with the test chemical spike (see figure below).



7. Secure the lid and seal the plates carefully with parafilm.
    a. Place in a naturally ventilated incubator at 32°C for 3 to 5 days.
8. After 3 days incubation, the colour development of the medium must be read on the plate reader (Titertek Multiskan MCC/340) at an optical density (OD) absorbance of 570 nm for colour change (OD_{570}) and 620 nm for turbidity (OD_{620}) of the yeast culture. After reading the plate, it must be returned to the incubator for a further 24 (Day 4) and 48 (Day 5) hours, to obtain data with the best contrast between the positive control (sigmoidal dose-response of the standard curve) and the blank (negative control).
    a. Negative control wells (blank) should appear yellow to light orange in colour due to background expression of β-galactosidase and turbidity of yeast growth.
    b. Positive wells are indicated by a deep red colour accompanied by yeast growth (turbidity), usually in a dose-dependent manner.
    c. Clear wells containing no growth/turbidity indicates lysis of the cells and the colour may vary from yellow to light orange.
9. All experiments must be performed in triplicate.

Caution: Some chemicals may leach across the plate and contaminate adjacent wells (Figure 4-4). By leaving empty rows between samples, this problem can be eliminated.

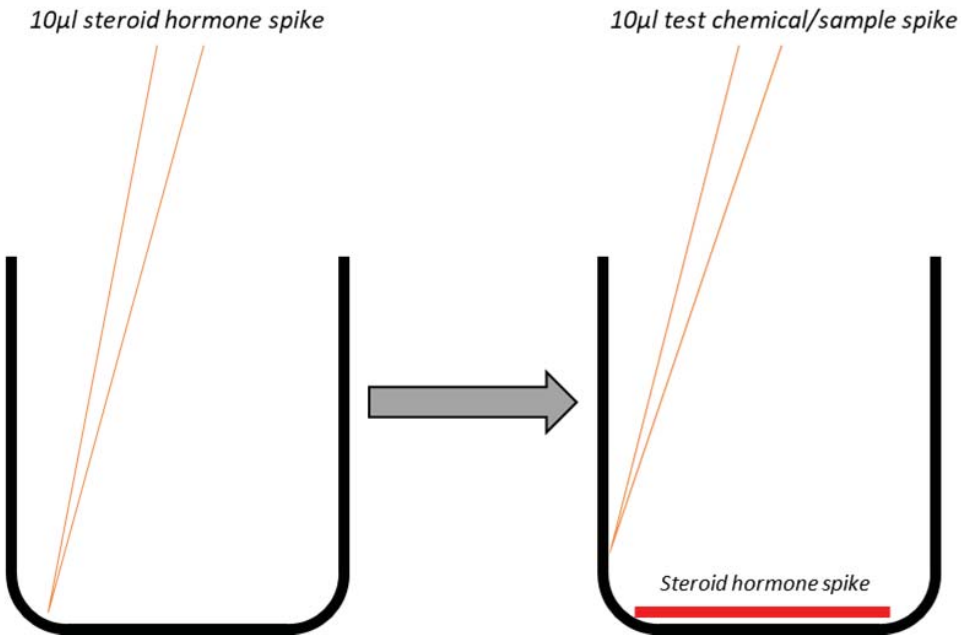
4.10 METHOD – THE YEAST ANTI-ESTROGEN SCREEN (YAES) AND YEAST ANTI-ANDROGEN SCREEN (YAAS)

The YAES and YAAS evaluate the ability of any ligand in the test sample to either interfere with the normal binding of a model steroid hormone to the steroid hormone receptor or any downstream pathway that inhibit the production of the enzyme β-galactosidase, which will be expressed through the inability of CPRG to be hydrolised from a yellow to a red product in the assay media.

The composition of assay media and test sample preparation (serial dilution transfer from a dilution plate to the assay plate) remain the same as outlined for the YES and YAS procedure, with some slight modifications, namely:

(1) Spiking each well in the 96-well assay plate (except for blanks) with a sub-maximal (EC_{50}) concentration of the model steroid hormone
    a. 17β-estradiol (E_2) for the YAES
    b. Dihydrotestosterone (DHT) for the YAAS
(2) Addition of both spike- (sub-maximal steroid hormones) and negative controls (ethanol) in addition to the positive control (tamoxifen for the YAES and flutamide for the YAAS), and
(3) Replacing the reference standard curve with a model steroid hormone receptor antagonist,
    a. Tamoxifen (TAM) for the YAES
    b. Flutamide (FLU) for the YAAS

Caution: As most wells in the 96-well assay plate will first be coated with a sub-maximal concentration of the steroid hormones, followed by the addition of the test chemical/test sample, it is vital to follow a systematic spiking approach to avoid cross-contamination or any unnecessary interference of the insertion of the test chemical/sample spike after the plates have been coated with the steroid hormone spike. The most feasible manner to avoid this is to ensure that the steroid hormone spike is inserted at the bottom of each flat-bottomed well (the same set of pipette tips may be used), followed by the test chemical/test sample addition onto the lower side of the well, using a new set of pipette tips for each transfer (see figure below)



4.10.1 Preparation of the sample dilutions, control and blank – ***chemical dose-response***

The same preparation protocol as for the YES and/or YAS applies to the YAES and/or YAAS with some slight modifications that will be listed below.

The protocol that follows is for the determination whether test chemicals will have affinity to interfere with the binding of a model steroid hormone to the hER or hAR in reference to a model steroid hormone receptor antagonist (i.e. tamoxifen for anti-estrogenicity or flutamide for anti-androgenicity).

Serial dilutions of the test chemicals/samples and controls must be made in a separate 96 well microtiter plate (***dilution plate***) in the following way.

Note: Each plate must contain at least one row of assay medium and solvent (ethanol) that serve as a negative control (blank) and a standard curve for tamoxifen (+ve control in the YAES) ranging from 1.8 mg/L to 14.5 µg/L, or a standard curve for flutamide (+ve control in the YAAS) ranging from 2.3 µg/L to 1.3 ng/L. This means that the starting sample concentration should be 20x higher during the preparation of the standards (37.2 mg/L for tamoxifen and 386.6 µg/L for flutamide) due to the dilution of the standards with the assay media.

1. Place 10 µL of the sub-maximal concentration (EC_{50}) of the model steroid hormone for each assay in each well of the ***assay plate***, except for wells B1-B6 (-ve control wells; Fig. 5.1).
    a. Leave the assay plate in the biological safety cabinet and allow wells to dry completely.
2. Place 100 µL of the solvent (ethanol), in wells 2-12 on the ***dilution plate***.
3. Place 200 µL of the test chemical master stock/control/blank solvent into the first well
4. Using an 8 multichannel pipette serially dilute (100 µL) across the plate, changing the tip for each carry over.
5. Transfer 10 µL of the dilution series across to the ***assay plate*** (see Figure 4-8 for sample layout in the assay plate).

a. Allow to evaporate to dryness in the biological safety cabinet.

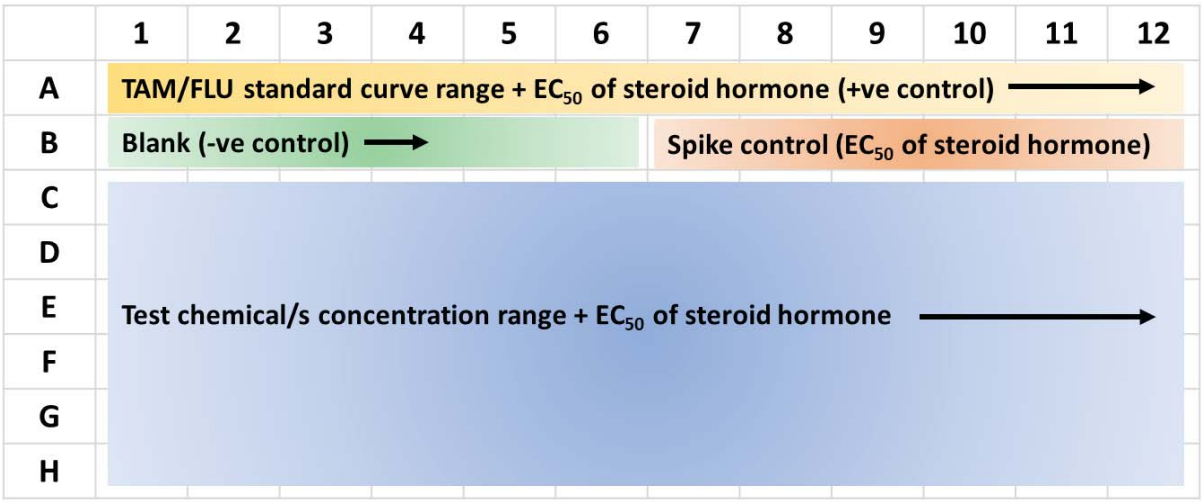
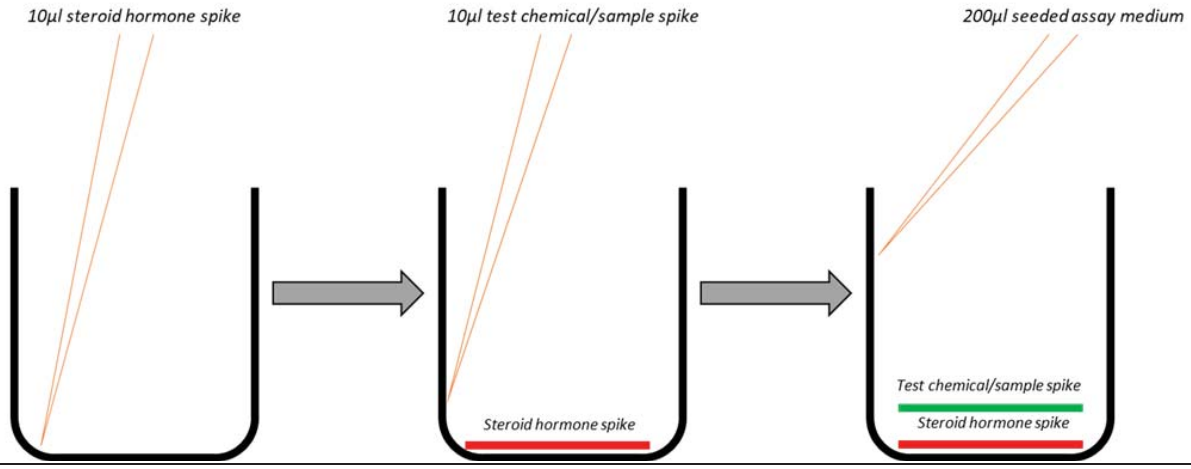


Figure 4-8: Assay plate layout for the yeast anti-estrogen screen (YAES) or yeast anti-androgen screen (YAAS)

6. Dispense 200 µL of the seeded assay medium that contains the chromogenic substrate (CPRG) into each sample well using a multichannel pipette.

Note: Use a new set of pipette tips for each transfer of the seeded assay medium into the assay plate. Avoid splashing when transferring the seeded assay medium by gently discarding the contents on the upper wall of the well (see figure below).



7. Secure the lid and carefully seal the edges of the plate and lid with parafilm.
    a. Keep the assay plate upright whilst sealing with parafilm to avoid cross-contamination of the wells.
    b. Place in a naturally ventilated incubator at 32°C for 3 to 5 days.
8. After 3 days incubation, the colour development of the medium must be read on the plate reader (Titertek Multiskan MCC/340) at an optical density (OD) absorbance of 570 nm for colour change (OD_{570}) and 620 nm for turbidity (OD_{620}) of the yeast culture. After reading the plate, it must be returned to the incubator for a further 24 (Day 4) and 48 (Day 5) hours, to obtain data with the best contrast between the positive control (sigmoidal dose-response of the standard curve) and the blank (negative control).

a. Negative control wells (blank) should appear yellow to light orange in colour due to background expression of β-galactosidase and turbidity of yeast growth.
b. All spike control wells should appear bright/deep red in colour due to sufficient expression of β-galactosidase from the model steroid hormone.
c. Positive wells are indicated by a yellow to light orange colour accompanied by yeast growth (turbidity), usually in a dose-dependent manner because of ER/AR-antagonists that inhibited β-galactosidase expression from the added steroid hormone spike.
d. Clear wells containing no growth/turbidity indicates lysis of the cells and the colour may vary from yellow to light orange.

9. All experiments must be performed in triplicate.

4.10.2 Preparation of the sample dilutions, control and blank – ***environmental samples***

The same preparation protocol as for the YES and/or YAS applies to the YAES and/or YAAS with some slight modifications that will be listed below.

The protocol that follows is for the determination whether pre-extracted environmental samples contain chemicals, or mixtures thereof, that will have affinity to interfere with the binding of a model steroid hormone to the hER or hAR in a dose-dependent manner.

Serial dilutions of the pre-extracted environmental samples and controls must be made in a separate 96 well microtiter plate (***dilution plate***) in the following way.

Note: Each plate must contain at least one row of assay medium and solvent (ethanol) that serve as a negative control (blank) and a standard curve for tamoxifen (+ve control in the YAES) ranging from 1.8 mg/L to 14.5 µg/L, or a standard curve for flutamide (+ve control in the YAAS) ranging from 2.3 µg/L to 1.3 ng/L. This means that the starting sample concentration should be 20x higher during the preparation of the standards (37.2 mg/L for tamoxifen and 386.6 µg/L for flutamide) due to the dilution of the standards with the assay media.

1. Place 10 µL of the sub-maximal concentration (EC_{50}) of the model steroid hormone for each assay in each well of the ***assay plate***, except for wells B1-B6 (-ve control wells).
    a. Leave the assay plate in the biological safety cabinet and allow wells to dry completely.
2. Place 100 µL of the solvent (ethanol), in wells 2-12 on the ***dilution plate***.
3. Place 200 µL of the test environmental sample/control/blank solvent into the first well
4. Using an 8 multichannel pipette serially dilute (100 µL) across the plate, changing the tip for each carry over.
5. Transfer 10 µL of the dilution series across to a new sterile 96 well, optically flat bottom microplate with a low evaporation lid (***assay plate***) (see Figure 4-9 for sample layout in the assay plate).
    a. Allow to evaporate to dryness on the assay plate.

Note: A 20x dilution factor will be applied to the pre-concentrated sample when the assay medium is added in the assay plate. Therefore, a starting concentration of, e.g. 1000x will equate to a 50x concentrated sample in the assay medium. For this reason, it is advised that environmental samples where a low level of pollutants are expected should be concentrated to at least 1000x during SPE so that a concentration range of at least 50x to 1.6x are achieved in the assay plate. Alternatively, environmental samples that are expected to be high in contaminants (e.g. raw wastewater) should include a wider range of serial dilutions (e.g. 50x to 0.78x), as a highly concentrated sample may introduce a high level of cytotoxic elements in the assay well that is not suitable for quantification.

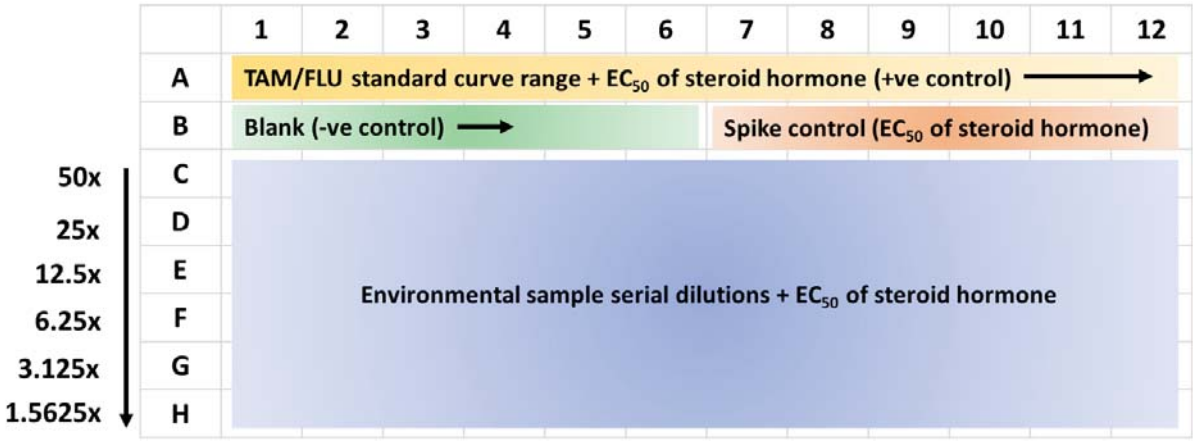


Figure 4-9: Assay plate layout for the yeast anti-estrogen screen (YAES) or yeast anti-androgen screen (YAAS)

6. Dispense 200 µL of the seeded assay medium that contains the chromogenic substrate (CPRG) into each sample well using a multichannel pipette.
7. Secure the lid and seal the plates carefully with parafilm.
    a. Place in a naturally ventilated incubator at 32°C for 3 to 5 days.
8. After 3 days incubation, the colour development of the medium must be read on the plate reader (Titertek Multiskan MCC/340) at an optical density (OD) absorbance of 570 nm for colour change (OD_{570}) and 620 nm for turbidity (OD_{620}) of the yeast culture. After reading the plate, it must be returned to the incubator for a further 24 (Day 4) and 48 (Day 5) hours, to obtain data with the best contrast between the positive control (sigmoidal dose-response of the standard curve) and the blank (negative control).
    a. Negative control wells (blank) should appear yellow to light orange in colour due to background expression of β-galactosidase and turbidity of yeast growth.
    b. Positive wells are indicated by a deep red colour accompanied by yeast growth (turbidity), usually in a dose-dependent manner.
    c. Clear wells containing no growth/turbidity indicates lysis of the cells and the colour may vary from yellow to light orange.
9. All experiments must be performed in triplicate.

4.11 METHOD – CALCULATIONS

4.11.1 Calculating and reporting the relative potency (RP) and relative induction efficiency (RIE) values for ***chemical dose-response***

1. Transfer raw data to Excel (absorbance readings of 570 nm and 620 nm)
2. Determine whether the test samples were cytotoxic by using the following equation Leusch (2008)

Cytotoxicity = average of blank OD_{620} - (standard deviation blank OD_{620} * 3)

Any samples with an OD_{620} below this cytotoxicity threshold should be excluded from further calculations but kept as an estimate of yeast cytotoxicity that is observed in the test samples.

3. Calculate the corrected absorbance to correct for turbidity in each well (Urbatzka et al., 2007):

Corrected absorbance = OD_{570} – (OD_{620} – median blank OD_{620})

4. Determine the detection limit of the assay (Dhooge et al., 2006):

Detection limit = average of blank corrected absorbance + (standard deviation of corrected absorbance * 3)

Any samples that are below this detection limit should be considered as a qualitative result and should be excluded for quantitative determination.

The calculation of Estradiol equivalents (EEq), dihydrotestosterone equivalents (DHT-Eq), tamoxifen equivalents (TAM-eq) or flutamide equivalents (FLU-Eq), depending on which assay was followed, can be calculated for samples with 3 or more points above the detection limit.

5. Log-transform the corrected absorbance values and express values as a percentage of the maximum steroid hormone response from the standard curve in the YES or YAS, or the maximum steroid hormone spike in the YAES or YAAS.

Log Max E_2/DHT = Log test corrected absorbance / Log maximum E_2/DHT corrected absorbance * 100 *or* Log Max E_2/DHT spike = Log test corrected absorbance / Log maximum E_2/DHT spike corrected absorbance * 100

6. Transfer data to Graphpad Prism (version 4 or higher), with E_2/DHT/TAM/FLU concentrations as X-values and log max values as Y-values.
7. Log transform X-values.
8. Fit the test chemical or E_2/DHT/TAM/FLU standard curve (sigmoidal function, variable slope) to obtain the EC_{50} value.

9. Calculate the relative induction efficiency (RIE) using the following formula (Fang et al., 2000):

RIE = Max absorbance chemical/max absorbance E_2/DHT/TAM/FLU × 100

RIE gives an indication of the maximum (anti)estrogenic or (anti)androgenic activity, depending on the type of bioassay used, that could be obtained for a test chemical as a percentage of the positive control. For example, a RIE of 50 means that the maximum estrogenic response of a test chemical is only 50% of the response that could be obtained with the positive control. This means that the test chemical will never reach the maximum activity that could be obtained by the positive control, not even by increasing the concentration of the test chemical.

10. Calculate the relative potency (RP) with the following formula (Fang et al., 2000):

RP = EC_{50} of E_2/DHT/TAM/FLU / EC_{50} of sample * 100

RP gives an indication of the potency of a test chemical compared to the positive control (positive control = 100%), e.g. a RP of 10 means that the test chemical is 10% as potent as the positive control and that a 10 times greater concentration would be needed to obtain the EC_{50} compared to the positive control.

4.11.2 Calculation and reporting of results for ***environmental samples***

11. Transfer raw data to Excel (absorbance readings of 570 nm and 620 nm)
12. Determine whether the test samples were cytotoxic by using the following equation Leusch (2008)

Cytotoxicity = average of blank OD_{620} - (standard deviation blank OD_{620} * 3)

Any samples with an OD_{620} below this cytotoxicity level should be excluded from further calculations but kept as an estimate of yeast cytotoxicity that is observed in the test samples.

13. Calculate the corrected absorbance to correct for turbidity in each well (Urbatzka et al., 2007):

Corrected absorbance = OD_{570} – (OD_{620} – median blank OD_{620})

14. Determine the detection limit of the assay (Dhooge et al., 2006):

Detection limit = average of blank corrected absorbance + (standard deviation of corrected absorbance * 3)

Any samples that are below this detection limit should be considered as a qualitative result and should be excluded for quantitative determination.

The calculation of Estradiol equivalents (EEq), dihydrotestosterone equivalents (DHT-Eq), tamoxifen equivalents (TAM-eq) or flutamide equivalents (FLU-Eq), depending on which assay was followed, can be calculated for samples with 3 or more points above the detection limit.

15. Log-transform the corrected absorbance values and express values as a percentage of the maximum steroid hormone response from the standard curve in the YES or YAS, or the maximum steroid hormone spike in the YAES or YAAS.

Log Max E_2/DHT = Log test corrected absorbance / Log maximum E_2/DHT corrected absorbance * 100 *or* Log Max E_2/DHT spike = Log test corrected absorbance / Log maximum E_2/DHT spike corrected absorbance * 100

16. Transfer data to Graphpad Prism (version 4 or higher), with E_2/DHT/TAM/FLU concentrations as X-values and log 10% max values as Y-values.
17. Log transform X-values.
18. Fit the E_2/DHT/TAM/FLU standard curve (sigmoidal function, variable slope) and determine X-values for all unpaired Y-values.

4.12 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

4.12.1 Advantages

- The yeast cultures are inexpensive to maintain and grow in the lab compared to other mammalian cell-based assays.
- The assay allows for evaluation of cytotoxicity, as well as steroid hormone agonism or antagonism, thus allowing for a range of effect-based outcomes.
- The yeast assays are more robust against cytotoxicity in test samples compared to mammalian cell assays, making it possible to evaluate steroid hormone receptor modulation in highly contaminated environments such as wastewater.

4.12.2 Limitations

- The yeast cells contain only ERα and may therefore not be as sensitive as other bioassays containing both ERα and ERβ.
- The yeast cell wall may impede active and passive transport of test chemicals and environmental samples into the intracellular space, resulting in false negatives compared to its mammalian cell line counterparts.
- Yeast-based assays may require significant sample enrichment (up to 1000 times concentration) in clean water matrices where a low level of contaminants is expected.
- Matrix interference resulting in cytotoxicity may be an issue and results in masking estrogenic activity.
- Variable results occur in this assay when octyl or nonylphenols are present in high concentration in

the sample, due to leaching (creeping) of the compound across the assay plate.

4.12.3 Recommendations

- Mammalian cell reporter gene assays such as the T47D-KBluc or ER-CALUX representing estrogen signalling and the MDA-kb2 or AR-CALUX for androgen signalling can be performed to supplement the yeast screen data. These screens offer higher sensitivity and represent both ERα and ERβ
- The yeast assays can be complimented with analytical chemistry
- For more in-depth investigations to aid in the identification of chemical classes responsible for the observed interference with hER or hAR signalling, the yeast screens can be run in combination with high-performance thin-layer chromatography (HPTLC) (Baetz et al. 2021).

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4.14 USEFUL CONTACTS

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5 THE T47D-KBluc REPORTER GENE ASSAY

Compiled by: MC Van Zijl and NH Aneck-Hahn

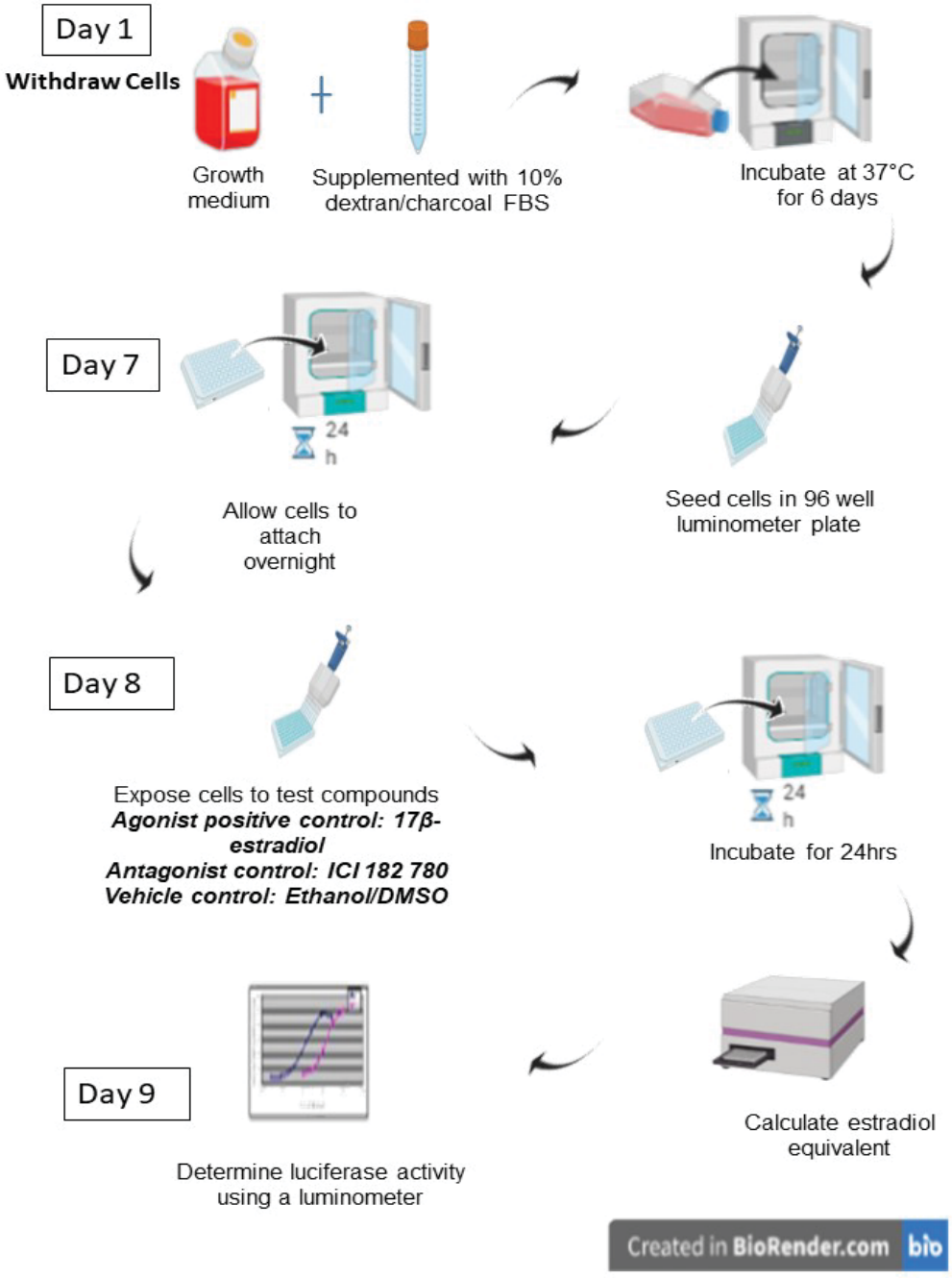

Figure 5-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the T47D-KBluc reporter gene assay

5.1 ACRONYMS & ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
E_2	17 β-Estradiol
EC_{50}	Half maximal effective concentration
EDC	Endocrine disrupting compound
EEq	Estradiol equivalents
ER	Estrogen receptor
ERE	Estrogen-responsive element
FBS	Fetal bovine serum
FI	Fold induction
HBSS	Hanks' balanced salt solution
HPLC	High performance liquid chromatography
PBS	Phosphate buffered saline
RIE	Relative induction efficiency
RLU	Relative light units
RP	Relative potency

5.2 PRINCIPLE OF THE ASSAY

The US EPA developed an estrogen-dependent stable cell line, that can be used to screen chemicals for estrogenic and anti-estrogenic activity. The T47D human breast cancer cells, which contain both endogenous estrogen receptor (ER)-α and -β, were transfected with a triplet estrogen-responsive elements (ERE)-promoter-luciferase reporter gene construct. This provides an in vitro system that can be used to evaluate the ability of chemicals to modulate the activity of estrogen-dependent gene transcription (Wilson et al., 2004).

In principle, compounds enter the cell; estrogen receptor ligands bind to the ER; two ligand-bound receptors dimerize and bind coactivators; then the dimer binds to the ERE on the reporter gene construct and activates the luciferase reporter gene. The presence of the luciferase enzyme can then be assayed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of estrogenic activity of the test chemical. When testing chemicals using the T47D-KBluc cells, an estrogen is defined as a chemical that induced dose dependent luciferase activity, which could be specifically inhibited by the anti-estrogen ICI 182,780. Agonists stimulate luciferase expression and are compared to the vehicle control or to the 17β-estradiol (E_2) control. Anti-estrogens block the E_2-induced luciferase expression, which is compared to the E_2 control (Wilson et al., 2004).

Advantages of this assay are that it is relatively rapid, eliminates the need for transfection and can be conducted in 96 well plates and consistent results are produced.

5.3 REQUIREMENTS

5.3.1 Acquisition of the cell line

The cells can be obtained from LGC Standards South Africa (catalog no ATCC-CRL-2865).

Information and contact details:

Website: www.lgcstandards.com

E-mail: atccrsa@lgcgroup.com

Tel: +27 11 466 4321

5.3.2 Permits

An import permit will also be required from the Department of Health, RSA.

Contact details:
E-mail: importexportpermit@health.gov.za

5.3.3 Staff training (technical skills)

- Training in cell culture techniques
- Software package knowledge (Excel and Graphpad Prism or similar)

5.3.4 Laboratory (test environment)

- Dedicated cell culture laboratory
  For cell culture laboratory guidelines refer to Wigley (2006)

5.3.5 Software

- Graphpad Prism 4, or equivalent
- Microsoft Excel

5.3.6 Apparatus

- Autoclave
- Balance (maximum 110 g, d = 0.1 mg)
- Beakers, glass, 100 mL; 250 mL and 1 L (for preparing reaction buffer and rinsing glassware)
- Bottles, autoclavable, glass, 25 mL; 500 mL and 1 L (Duran Cat. No. 218011453; 218014459 and 218015455 or equivalent)
- Centrifuge with swing-out rotor (buckets must be able to contain 50 mL conical tubes)
- CoolCell alcohol-free cell freezing containers (BioCision Cat. No. BCS-136 or equivalent)
- Freezer (-20°C)
- Freezer (-80°C)

- Fridge (4°C)
- Incubator, humidified 5% CO_2
- Inverted microscope
- Measuring cylinder, 1 L
- Microplate luminometer with two dispensers (LUMIstar Omega, BMG Labtech or equivalent)
- Milli-Q Integral 3 water purification system or equivalent with a point-of-use ultrafiltration cartridge to remove endocrine disrupting compounds (EDCs) from water (Merck Millipore EDS-Pak® polisher, Cat. No. EDSPAK001)
- Oven (for drying glassware)
- pH meter
- Pipette, 12 channel, 10-100 µL variable volume
- Pipette, 12 channel, 30-300 µL variable volume
- Pipette, 1-10 µL variable volume
- Pipette, 20-200 µL variable volume
- Pipette, 200-1000 µL variable volume
- Pipettor for serological pipettes
- Plate warmer/shaker
- Spatulas
- Type II biohazard safety cabinet
- Vortex mixer
- Water bath

5.3.7 Consumables/materials and reagents

Tables 5-1 and 5-2 provide lists of the consumables/materials and reagents required for the T47D-KBluc reporter gene assay

Table 5-1: List of the consumables/materials required for the T47D-KBluc reporter gene assay

Name	Cat no	Supplier
Bottle top filters, 0.22 µm, 500 mL	430513	Corning
Centrifuge tubes, sterile, 50 mL	352070	Corning (Falcon)
Cryovials, sterile, 1.8 mL	377267	Thermo Fisher Scientific (Nunc)
Eppendorf tubes, 2 mL	0030120094	Eppendorf
Hemocytometer		
Liquinox phosphate-free liquid detergent		
Luminometer plates, 96 well, white with clear bottom	3610	Corning
Nitrile gloves		
Permanent marker pens		
Pipette filter tips, sterile, 10 µL	4135	Corning (DeckWorks)
Pipette filter tips, sterile, 1000 µL	4140	Corning (DeckWorks)

Name	Cat no	Supplier
Pipette filter tips, sterile, 200 µL	4138	Corning (DeckWorks)
Pipette tips, sterile, 250 µL	9400263	Thermo Fisher Scientific
Reagent reservoir (100 mL, autoclavable)		
Serological pipettes, sterile, 2 mL	170365	Thermo Fisher Scientific (Nunc)
Serological pipettes, sterile, 5 mL	170366	Thermo Fisher Scientific (Nunc)
Serological pipettes, sterile, 10 mL	170367	Thermo Fisher Scientific (Nunc)
Tin foil		
Tissue culture flasks, sterile, 25 cm^2	430372	Corning
Tissue culture flasks, sterile, 75 cm^2	430641U	Corning

Note: It is recommended to use consumables from the suppliers as stated. Consumables from other suppliers must first be tested as it may affect the outcome of the assay.

Table 5-2: List of the reagents required for the T47D-KBluc reporter gene assay

Name	Cat no	Supplier
Antibiotic/antimycotic solution	15240062	Thermo Fisher Scientific (Gibco)
Adenosine triphosphate (ATP)	A7699	Sigma-Aldrich
Beetle luciferin, potassium salt	E1603	Promega
Bovine serum albumin (BSA) (Fraction V)	A7906	Sigma-Aldrich
Cell culture freezing media	12648010	Thermo Fisher Scientific (Gibco)
D (+)-glucose	108337	Merck Millipore
17 β-estradiol (E_2)	E8875	Sigma-Aldrich
Ethanol, gradient grade for liquid chromatography	111727	Sigma-Aldrich (Supelco)
Fetal bovine serum (FBS)	SH30071.03	HyClone
FBS, charcoal/dextran treated	SH30068.03	HyClone
Glycylglycine	50200	Sigma-Aldrich
Hanks' balanced salt solution (HBSS), 10x	14185045	Thermo Fisher Scientific (Gibco)
HEPES buffer, 1M	15630056	Thermo Fisher Scientific (Gibco)
Hydrochloric acid (HCl), 5N		Preferred supplier
ICI 182 780	1047	Tocris
Magnesium chloride ($MgCl_2$), 1 M	M1028	Sigma-Aldrich
Methanol, gradient grade for liquid chromatography	106007	Sigma-Aldrich (Supelco)
Phosphate buffered saline (PBS), 10x	14080048	Thermo Fisher Scientific (Gibco)
pH buffers (pH 4, pH 7, pH 10)		Preferred supplier
Reporter lysis buffer	E3971	Promega
RPMI 1640 medium with glutamine, without phenol red	R8755	Sigma-Aldrich
Sodium bicarbonate ($NaHCO_3$)	S5761	Sigma-Aldrich
Sodium hydroxide (NaOH), 5 N		Preferred supplier

Name	Cat no	Supplier
Sodium pyruvate, 100 mM	11360039	Thermo Fisher Scientific (Gibco)
Trypsin-EDTA, 10x	15400054	Thermo Fisher Scientific (Gibco)

Note: It is recommended to use reagents from the suppliers as stated. Reagents from other suppliers should first be tested in the assay as it may affect the outcome of the assay.

5.4 METHODS – GENERAL

Nitrile gloves (latex free) must be worn when preparing assay components and when doing the assay. Working with latex gloves may affect the outcome of the assay. All glassware must be prepared by washing in Liquinox phosphate-free liquid detergent, rinsing ten times in tap water, five times in ultrapure EDC free water and twice in HPLC grade methanol consecutively. Dry glassware in oven and cover with foil. If required, sterilize glassware by autoclaving at 121°C for 20 minutes.

Note: It is advisable to have dedicated glassware for the assay.

5.5 PREPARATION OF ASSAY COMPONENTS

5.5.1 RPMI medium

Maintenance medium is prepared by adding the following media components together in a 1 L glass bottle:

1 bottle RPMI 1640 powder for 1 L medium

2.5 g D-glucose

1.5 g $NaHCO_3$

10 mL 1 M HEPES solution

10 mL 100 mM Sodium pyruvate solution

980 mL Ultrapure EDC free water

Adjust the pH to 7.3 (with 5 N, HCl or NaOH) and filter sterilize with a 0.22 µm bottle top filter into a 1 L glass bottle that has been sterilised by autoclaving at 121°C for 20 minutes. Store at 4°C.

Note: Discard unused media after three months.

5.5.2 Maintenance Medium

Maintenance medium consists of RPMI medium supplemented with 10% FBS and 100U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin (add 100 mL fetal bovine serum and 10 mL Gibco antibiotic/antimycotic solution to 900 mL medium). Store medium at 4°C.

5.5.3 Withdrawal Medium

Assay medium consists of RPMI medium supplemented with 10% dextran/charcoal FBS (add 100 mL FBS to 900 mL medium). Store medium at 4°C.

5.5.4 Assay Medium

Assay medium consists of RPMI medium supplemented with 5% dextran/charcoal FBS (add 50 mL FBS to 950 mL medium). Store medium at 4°C.

Note: Do not add any antibiotics to the withdrawal and assay medium.

5.5.5 HBSS

Autoclave 1 L of ultrapure EDC-free water in a glass bottle to sterilize. Working in a biohazard safety cabinet, add 50 mL 10x HBSS to 950 mL autoclaved water in a sterile 1 L glass bottle, using a sterile measuring cylinder. Store HBSS at 4°C.

5.5.6 Trypsin

Divide 10x concentrated trypsin in 5 mL aliquots into sterile centrifuge tubes and store at -20°C. Prepare 1x trypsin by adding 45 mL HBSS to 5 mL 10x trypsin. Work under sterile conditions. Store 1x trypsin at 4°C.

5.5.7 PBS

Add 100 mL 10x PBS to 900 mL ultrapure water. Store at 4°C.

5.5.8 Lysis buffer

Add 1 mL 5x lysis buffer to 4 mL ultrapure water, just prior to adding lysis buffer to cells.

5.5.9 Glycylglycine

Make a 1 M stock solution by adding 6.605 g glycylglycine (MW 132.1) to 50 mL ultrapure water, pH to 7.8 (with 5 N, HCl or NaOH), sterile filter and store in 50 mL centrifuge tube or another suitable sterile container at 4°C.

5.5.10 ATP solution

Make a 0.1 M stock solution by adding 2.755 g ATP (MW 551.1) to 50 mL ultrapure water, pH to 7.8 (with 5 N, HCl or NaOH) and aliquot into 5 mL volumes in a freezable container. Store at -80°C.

5.5.11 BSA

Make a 50 mg/mL stock by adding 10 mL ultrapure water to 500 mg BSA. Aliquot into 1 mL volumes in Eppendorf tubes or another suitable freezable container. Store at -20°C

Note:
- Do not agitate the solution. Add the ultrapure water slowly to the BSA and leave on the bench for a couple of minutes to dissolve. Agitation may cause BSA to stick to the sides of the glass beaker and cause foaming and air pockets, which will increase the time to dissolve.

5.5.12 Reaction buffer

Reaction buffer is prepared by adding the following together:

90 mL ultrapure water

2 mL 1 M Glycylglycine

5 mL 0.1 M ATP

1 mL BSA solution

1.5 mL 1 M $MgCl_2$

pH the solution to 7.8 (with 5 N, HCl or NaOH). Aliquot into 10 mL volumes. Store at 4°C.

Note:
- The pH of the reaction buffer is critical.
- Cloudiness of reaction buffer does not affect results.

5.5.13 Luciferin

Make a 1 mM stock solution by adding 159.21 mg D-luciferin (potassium salt MW 318.41) to 500 mL ultrapure water. Aliquot into 10 mL volumes in freezable containers. Store at -80°C.

Note:
- Luciferin is light sensitive and aliquots should be stored in amber vials or wrapped in foil.
- Luciferin may be refrozen.

5.5.14 E_2 stock solution

Make a stock solution of 10 mM E_2 by adding 27.239 mg E_2 (MW 272.39) to 10 mL HPLC grade ethanol in a new glass bottle, prepared by rinsing twice in HPLC grade methanol. Use an amber bottle or wrap bottle in foil and store at -20°C.

5.5.15 ICI stock solution

Make a stock solution of 1 mM ICI by adding 6.067 mg ICI (MW 606.77) to 10 mL HPLC grade ethanol in a new glass bottle, prepared by rinsing twice in HPLC grade methanol. Use an amber bottle or wrap bottle in foil and store at -20°C.

5.6 GENERAL CELL CULTURE TECHNIQUES

5.6.1 Aseptic conditions

- Always work in a biohazard safety cabinet to reduce the possibility of contaminating the tissue cultures.
- Disinfect the cabinet by wiping down the surface with 70% ethanol before and after working in the cabinet.
- Always wear nitrile gloves when working in the cabinet and decontaminate gloves by spraying with 70% ethanol each time before entering the cabinet.
- All materials and reagents that come into direct contact with cell cultures must be sterile.
- Decontaminate everything with 70% ethanol before entering the cabinet.
- Do not work directly above open bottles, flasks, plates, etc. and don't leave bottles/lids unnecessarily open inside the cabinet.
- Close lids before removing bottles from the cabinet.

Refer to McAteer and Davis (2006) for more comprehensive guidelines.

5.6.1.1.1.1.1 **Note:** Female technologists should always be aware and particularly careful when working with the estrogen sensitive cells as estrogenic contamination may occur due to natural excretion of hormones. This may therefore give a false positive reaction in the cells. Nitrile gloves should always be worn when working with the cells.

5.6.2 Thawing the frozen cells

1. Prior to thawing the cells, place 10 mL maintenance media in a 25 cm^2 tissue culture flask, using aseptic techniques. Place the flask in a CO_2 incubator for at least 15 minutes, in order for the media to reach 37°C and to equilibrate.
2. Thaw the vial containing the frozen cells in a 37°C water bath, with gentle agitation, and without submerging the cap in the water.
3. Decontaminate the vial by spraying the outside surface with 70% ethanol.
4. Transfer the contents of the vial to the cell culture flask, using aseptic techniques. Label the flask with the name of the cell line, the date and passage number.
5. Place the flask back in the CO_2 incubator and allow cells to attach to the surface overnight.
6. Discard the medium and replace with fresh medium the following day. Trypsinize if necessary.

5.6.3 Subculturing cells

Subculture cells when they reach 80-90% confluency.

1. Place media and HBSS in the CO_2 incubator for at least 15 minutes before trypsinizing cells, in order for the media to reach 37°C and its normal pH. All procedures from this point forward should be carried out in a biohazard safety cabinet, using aseptic techniques.
2. Discard culture media from flasks and rinse cells twice with HBSS (5-10 mL for a 75 cm^2 flask or 3-5 mL for a 25 cm^2 flask).

3. Add 3 mL trypsin to a 75 cm^2 flask or 1 mL to a 25 cm^2 flask and return to the incubator for 2 minutes.
4. Remove excess trypsin.
5. Detach cells by gently tapping the flask against the palm of your hand.
6. Add 10 mL medium to a 75 cm^2 flask or 5 mL to a 25 cm^2 flask.
7. Transfer 1/3 or 1/4 to a new culture flask and add maintenance or assay media (20 mL for 75 cm^2 flask or 10 mL for 25 cm^2 flask). Label the new flasks and remember to update the passage number.
8. Return flasks to incubator until cells reach 80-90% confluency.

Note:
- Transfer the required amount of media and HBSS to cell culture flasks before heating in the incubator, to avoid repeated heating/cooling of the media. This will also ensure that in the event of contamination, it is limited to the one flask and not contamination of the whole bottle of medium or HBSS. If the culture flask does not have a vented cap, the cap should be slightly loose, to allow for the exchange of CO_2.
- Media should be changed twice a week.
- When subculturing cells from a 25 cm^2 flask to a 75 cm^2 flask, transfer all of the trypsinized cells from the 25 cm^2 flask to the 75 cm^2 flask and add 15 mL media.

5.6.4 Freezing of stock cultures

1. Grow cells in a 75 cm^2 flask until they reach 80-90% confluency.
2. Place media and HBSS in the CO_2 incubator for at least 15 minutes before trypsinizing cells, in order for the media to reach 37°C and its normal pH. All procedures from this point forward should be carried out in a biohazard safety cabinet, using aseptic techniques.
3. Discard culture media from flasks and rinse cells twice with 5-10 mL HBSS.
4. Add 3 mL trypsin and return to the incubator for 2 minutes.
5. Remove excess trypsin.
6. Detach cells by gently tapping the flask against the palm of your hand and add 10 mL maintenance medium to the flask.
7. Transfer medium containing cells to a 50 mL conical tube and centrifuge at 15°C, 172 x g, for 10 minutes.
8. Remove media using a sterile pipette, taking care not to disturb the pellet.
9. Add 6 mL cell freezing media and pipet gently two to three times to disperse cells.
10. Aliquot 1 mL per cryovial and label the vial with the name of the cell line, date and passage number. Freeze the cells at -80°C using a cryofreezing container [a].

Note:
- New stock cell cultures should be prepared every 6-12 months for cells frozen at -80°C.
- Transfer frozen cells to liquid nitrogen for long-term storage of cells.
- Never use a working flask for making stock cultures. Keep the passage numbers of the frozen stock cultures as low as possible, because the responsiveness of the cells to E_2 may decrease with increasing passage numbers. Keep one vial of the original lot to make new stock cultures and use the last vial of each new batch of stock cultures for the next batch of stock cultures.
- It is advised to store stock cultures in two different locations, to ensure that stocks are not lost in the case of a physical disaster at the primary location.
- It is strongly recommended to check frozen cell cultures for viability before the stock culture is terminated.

a) Freezing cells using the CoolCell alcohol-free cell freezing container

Using the CoolCell alcohol-free cell freezing container ensures that the optimal cooling rate of -1°C/min is achieved when freezing the cells, in order to minimize damage to the cells due to osmotic imbalance and ice crystal formation.

1. Make sure that the chambers and cryovials are dry to avoid tubes sticking upon freezing.
2. The container and core (black ring) should be at room temperature.
3. Place the core at the bottom of the central cavity.
4. Place sample vials containing 1 mL of cell suspension in each well. Each of the 12 wells should contain a filled vial. If the freezing batch is less than 12 vials, empty wells should be filled with filler vials, containing equivalent volumes of freezing media.
5. Fully seat the lid on the CoolCell container.
6. Place the container upright in a -80°C freezer, allowing at least 2.5 cm free space around the container.
7. Freeze for at least four hours before transferring the vials to storage boxes or liquid nitrogen. It is recommended to use dry ice when transferring cryovials to permanent storage to avoid temperature rise and cell damage.

5.7 ASSAY PROCEDURE

Day 1 (e.g. Tuesday)

Grow cells in a 75 cm^2 flask until they reach 80-90% confluency. Trypsinize and split cells 1:3. Add 10 mL withdrawal media to flask (media containing 10% dextran/charcoal treated FBS) and incubate for one week in 5% CO_2 incubator.

Day 4 (e.g. Friday)

Change withdrawal media.

Day 7 (e.g. Monday)

1. Trypsinize cells and add 10mL assay medium (medium containing 5% dextran/charcoal treated FBS).
2. Pool cells in a 50 mL conical tube if cells from more than one flask is used and gently mix cell suspension by inverting the tube.
3. Count the cells using a Hemocytometer [b].
4. Seed cells at 5 x 10^4 cells per well in 96-well luminometer plates (100 µL per well) [c], place plates in the incubator and allow cells to attach overnight. Remember to note the passage number of the cells for each experiment.

b) Counting cells using a Hemocytometer:

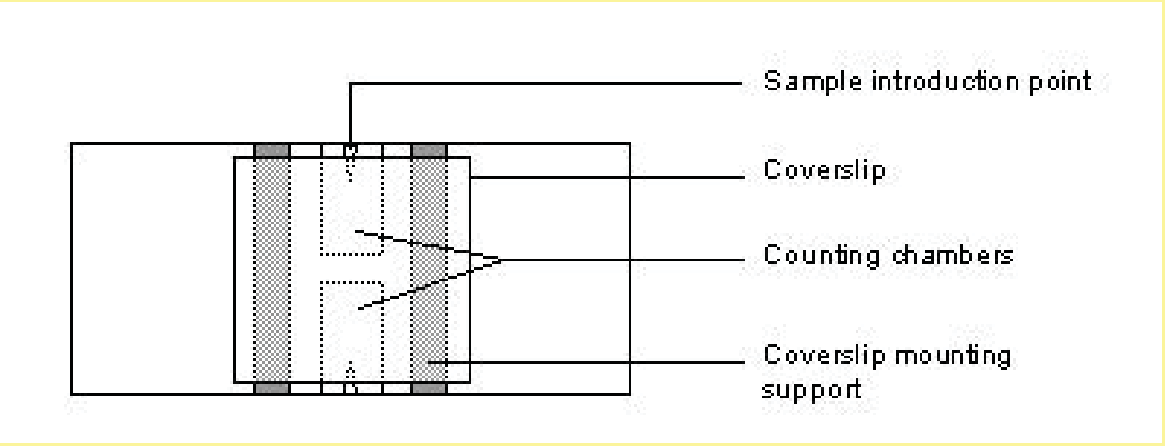

Figure 5-2: Diagram of a hemocytometer

1. Slightly dampen the coverslip mounting support areas with water, place the coverslip squarely on top of hemocytometer and gently, but firmly press the coverslip over the support areas. Appearance of rainbow rings (like oil when it lies on water) indicates that the coverslip has formed a tight seal and ensures the depth of the chamber is 0.1 mm.
2. Load cell suspension (10-12 µL) onto the hemocytometer using a pipette. Place the pipette tip to the groove on one side of the hemocytometer and gently force the fluid out and allow it to be drawn under the coverslip by capillary action. The fluid must cover the entire polished surface of each chamber. Take care not to overload the counting chambers.

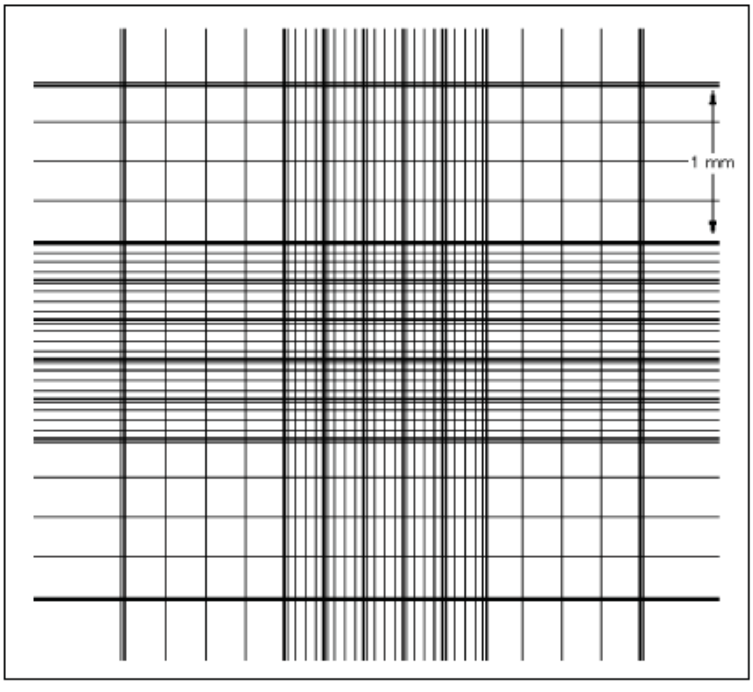

Figure 5-3: Diagram of a counting chamber grid

3. Use the 10x objective of the microscope to focus on the grid lines in the chamber (Figure 2). Count the cells in the four corner 1 mm^2 grid areas (one 1mm^2 grid area consists of 4 x 4 squares surrounded by three grid lines) (Figure 5-3).

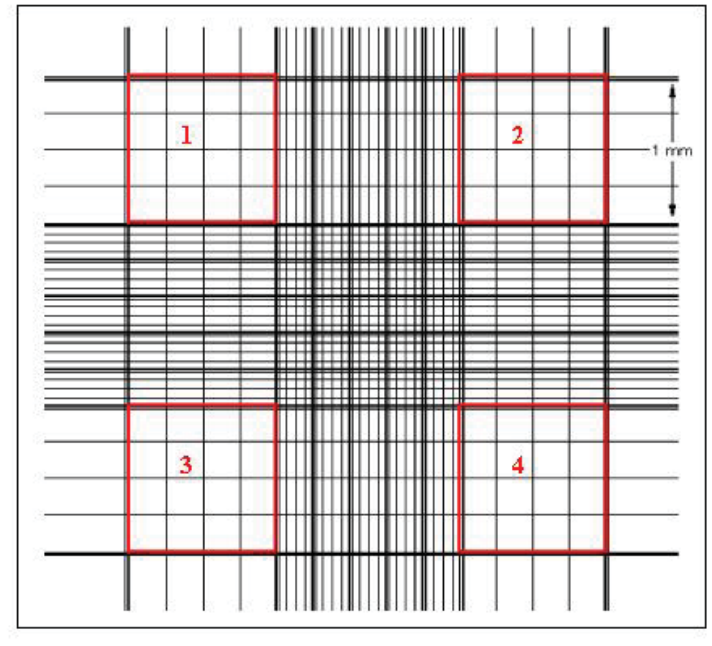

Figure 5-4: An example of the four corner squares on a counting chamber grid

Note:

- In order to prevent cells from being counted twice, the cells touching the upper or left boundaries are counted, but those that touch the bottom or right boundaries are not.
- If more than 10% of cells are clustered together, attempt to disperse the original cell suspension further by pipetting and start again. Clusters containing more than five cells are counted as five.
- If there are too many cells to count realistically, perform a dilution using assay medium. If there are less than 100 cells per 1 mm^2 grid area, additional squares must be counted to improve the accuracy of the count.

4. Determine the cell concentration with the following formula:

   Cells/mL = average count per 1 mm^2 square x 10 000 x dilution factor

   (The depth of 1 mm square is 0.1mm and the volume is therefore 0.0001 mL. The cell count per 1 mm^2 (average of squares 1-4) must therefore be multiplied by 10 000 to get the cells/mL).

   McAteer and Davis (2006) and http://www.nuncbrand.com/en/frame.aspx?ID=1174

c) Seeding cells at 5 x 10^4 cells per well:

- Prepare 11 mL of the final cell dilution per plate.
- Cells must be seeded at 5 x 10^4 cells per well or 5 x 10^4 cells per 100 uL (0.1 mL), which is equal to 500 000 cells/mL and 5 500 000 cells/11 mL.
- Determine the volume of the original cell suspension that will contain 5 500 000 cells by dividing 5 500 000 by the number of cells per mL counted on the hemocytometer. Add assay medium to obtain a final volume of 11 mL and transfer to a sterile reagent reservoir.
- The outer rows of the plates may evaporate and are therefore not used. Fill the outer row of the plate with 200 µL medium or HBSS and fill the rest of the plate with 100 µL cell suspension, using a multichannel pipette.

Day 8 (Tuesday)

Dosing of the plates

1. Prepare 1000x concentrated dosing solutions for controls and test chemicals in HPLC grade ethanol in 2 mL Eppendorf tubes and vortex to mix.
   a) ICI control

   Add 10 µL of the 1 mM ICI stock to 990 µL ethanol (10 µM)

   b) E_2 control and standard curve

   Add 10 µL of the 10 mM E_2 stock to 990 µL ethanol (100 µM)

   Add 10 µL of the 100 µM E_2 stock to 990 µL ethanol (1 µM)

   Add 100 µL of the 1 µM E_2 stock to 900 µL ethanol (100 nM)

   Add 300 µL of the 100 nM E_2 stock to 700 µL ethanol (30 nM)

   Add 100 µL of the 100 nM E_2 stock to 900 µL ethanol (10 nM)

   Add 100 µL of the 30 nM E_2 stock to 900 µL ethanol (3 nM)

   Add 100 µL of the 10 nM E_2 stock to 900 µL ethanol (1 nM)

   Add 100 µL of the 3 nM E_2 stock to 900 µL ethanol (0.3 nM)

   Add 100 µL of the 1 nM E_2 stock to 900 µL ethanol (0.1 nM)

   Add 100 µL of the 0.3 nM E_2 stock to 900 µL ethanol (0.03 nM)

   c) *Samples*

   Make dilutions of the sample in ethanol at 1000x the desired concentration.
2. Dilute 1000x concentrations in assay medium (containing 5% dextran/charcoal treated FBS) to double the desired final concentration to allow for the dilution factor when vehicle control (VC), E_2 or ICI are co-incubated with the samples and controls. Vortex to mix.
   a) Vehicle control (VC)

   Add 20 µL HPLC grade ethanol to 10 mL medium

   b) ICI control

   Add 20 µL of the 10 µM ICI to 10 mL medium (0.02 µM)

   c) E_2 control

   Add 20 µL of the 100 nM E_2 to 10 mL medium (0.2 nM)

   d) E_2 standard curve

   Add 2 µL of the 100 nM E_2 stock to 1 mL medium (0.2 nM/200 pM)

   Add 2 µL of the 30 nM E_2 stock to 1 mL medium (60 pM)

   Add 2 µL of the 10 nM E_2 stock to 1 mL medium (20 pM)

   Add 2 µL of the 3 nM E_2 stock to 1 mL medium (6 pM)

   Add 2 µL of the 1 nM E_2 stock to 1 mL medium (2 pM)

   Add 2 µL of the 0.3 nM E_2 stock to 1 mL medium (0.6 pM)

   Add 2 µL of the 0.1 nM E_2 stock to 1 mL medium (0.2 pM)

   Add 2 µL of the 0.03 nM E_2 stock to 1 mL medium (0.06 pM)

   e) *Samples*

   Add 2 µL of sample concentrations in ethanol to 1 mL medium.
3. Remove the cell culture medium from the 96 well plates containing the cells and add 50 µL of the dosing solutions to the appropriate wells. Add 50 uL medium containing E_2 or ICI to wells that should be co-incubated with the controls. Wells that are not co-incubated with E_2 or ICI must receive an additional 50

µL medium containing the vehicle control. An example of how to dose the plates is illustrated in figure 5-5 and the resulting final concentrations in figure 5-6.

Final concentrations:

a) Vehicle control

0.2% (v/v)

b) ICI control

10 nM

c) E_2 *control*

0.1 nM (100 pM)

d) E_2 *standard curve*

100 pM; 30 pM; 10 pM; 3 pM; 1 pM; 0.3 pM; 0.1 pM

4. Incubate plates for 24h in a 5% CO_2 incubator.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS
B	Medium/ HBSS	100 µL VC	50 µL 200 pM E2 +50 µL VC	50 µL 200 pM E_2 +50 µL VC	50µL 60 pM E_2 +50 µL VC	50µL 20 pM E_2 +50 µL VC	50µL 6 pM E_2 +50 µL VC	50µL 2 pM E_2 +50 µL VC	50µL 0.6 pM E_2 +50 µL VC	50µL 0.2 pM E_2 +50 µL VC	50µL 0.06 pM E_2 +50 µL VC	Medium/ HBSS
C	Medium/ HBSS	100 µL VC	50 µL 200 pM E2 +50 µL VC	20x sample 1 +50 µL VC	6x sample 1 +50 µL VC	2x sample 1 +50 µL VC	0.6x sample 1 +50 µL VC	0.2x sample 1 +50 µL VC	0.06x sample 1 +50 µL VC	0.02x sample 1 +50 µL VC	0.006x sample 1 +50 µL VC	Medium/ HBSS
D	Medium/ HBSS	100 µL VC	50 µL 200 pM E2 +50 µL VC	20x sample 2 +50 µL VC	6x sample 2 +50 µL VC	2x sample 2 +50 µL VC	0.6x sample 2 +50 µL VC	0.2x sample 2 +50 µL VC	0.06x sample 2 +50 µL VC	0.02x sample 2 +50 µL VC	0.006x sample 2 +50 µL VC	Medium/ HBSS
E	Medium/ HBSS	50 µL 0.02 µM ICI +50 µL VC	50 µL 0.02 µM ICI 50 µL 200 pM E2	20x sample 1 50 µL 200 pM E2	6x sample 1 50 µL 200 pM E2	2x sample 1 50 µL 200 pM E2	0.6x sample 1 50 µL 200 pM E2	0.2x sample 1 50 µL 200 pM E2	0.06x sample 1 50 µL 200 pM E2	0.02x sample 1 50 µL 200 pM E2	0.006x sample 1 50 µL 200 pM E2	Medium/ HBSS
F	Medium/ HBSS	50 µL 0.02 µM ICI +50 µL VC	50 µL 0.02 µM ICI 50 µL 200 pM E2	20x sample 2 50 µL 200 pM E2	6x sample 2 50 µL 200 pM E2	2x sample 2 50 µL 200 pM E2	0.6x sample 2 50 µL 200 pM E2	0.2x sample 2 50 µL 200 pM E2	0.06x sample 2 50 µL 200 pM E2	0.02x sample 2 50 µL 200 pM E2	0.006x sample 2 50 µL 200 pM E2	Medium/ HBSS
G	Medium/ HBSS	50 µL 0.02 µM ICI +50 µL VC	50 µL 0.02 µM ICI 50 µL 200 pM E2	20x sample 1 50 µL 0.02 µM ICI	6x sample 1 50 µL 0.02 µM ICI	2x sample 1 50 µL 0.02 µM ICI	0.6x sample 1 50 µL 0.02 µM ICI	20x sample 2 50 µL 0.02 µM ICI	6x sample 2 50 µL 0.02 µM ICI	2x sample 2 50 µL 0.02 µM ICI	0.6x sample 2 50 µL 0.02 µM ICI	Medium/ HBSS
H	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS	Medium/ HBSS

Figure 5-5: An example of the dosing protocol on a plate

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Vehicle control	0.1 nM E_2	0.1 nM E_2	30 pM E_2	10 pM E_2	3 pM E_2	1 pM E_2	0.3 pM E_2	0.1 pM E_2	0.03 pM E_2	
C		Vehicle control	0.1 nM E_2	10x sample 1	3x sample 1	1x sample 1	0.3x sample 1	0.1x sample 1	0.03x sample 1	0.01x sample 1	0.003x sample 1	
D		Vehicle control	0.1 nM E_2	10x sample 2	3x sample 2	1x sample 2	0.3x sample 2	0.1x sample 2	0.03x sample 2	0.01x sample 2	0.003x sample 2	
E		10 nM ICI	10 nM ICI	10x sample 1	3x sample 1	1x sample 1	0.3x sample 1	0.1x sample 1	0.03x sample 1	0.01x sample 1	0.003x sample 1	
			0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	
F		10 nM ICI	10 nM ICI	10x sample 2	3x sample 2	1x sample 2	0.3x sample 2	0.1x sample 2	0.03x sample 2	0.01x sample 2	0.003x sample 2	
			0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	0.1 nM E_2	
G		10 nM ICI	10 nM ICI	10x sample 1	3x sample 1	1x sample 1	0.3x sample 1	10x sample 2	3x sample 2	1x sample 2	0.3x sample 2	
			0.1 nM E_2	10 nM ICI	10 nM ICI	10 nM ICI	10 nM ICI	10 nM ICI	10 nM ICI	10 nM ICI	10 nM ICI	
H												

Figure 5-6: An example of the final concentration in the wells

Note:

- Ethanol (vehicle) concentrations in the final dilutions of controls or test substances may not exceed 0.2%.
- During the extraction procedure, samples are concentrated 1000x, but are diluted 1000x when introduced to the cells in order to comply with the 0.2% vehicle limitation. When higher concentrations are needed to get sufficient points for more accurate estradiol equivalents (EEq) calculations, transfer the required volume of sample to a Eppendorf tube, allow to evaporate and resuspend in medium containing 0.2% vehicle control. For example, to obtain a final concentration equal to 10x concentrated sample, evaporate 20 µl of sample and add 1 mL vehicle control medium.
- Each assay plate should contain the agonist positive control (E_2), negative control (vehicle only), antagonist control (E_2 plus ICI) and background control (vehicle plus ICI).
- Each sample should be tested alone as well as in the presence of 0.1 nM E_2 or ICI.
- Plates should be dosed in triplicate. When a lot of samples needs to be done, a single screening plate containing the samples and controls may be dosed. Only the samples with estrogenic activity are then done in triplicate with the full dose-response curve of E_2.
- Where possible, dosing plates should be kept separate from tissue culture flasks containing cells.

Anti-estrogenic activity

To test for anti-estrogenic activity, samples are co-incubated with 0.1 nM E_2 and the standard curve is ICI, co-incubated with 0.1 nM E_2. The plates must also contain the agonist positive control (E_2), negative control (vehicle only), antagonist control (E_2 plus ICI) and background control (vehicle plus ICI).

ICI standard curve

1. Prepare 1000x concentrated dosing solutions in HPLC grade ethanol in 2 mL Eppendorf tubes and vortex to mix.

   Add 10 µL of the 1 mM ICI stock to 990 µL ethanol (10 µM)
   Add 300 µL of the 10 µM ICI stock to 700 µL ethanol (3 µM)
   Add 100 µL of the 10 µM ICI stock to 900 µL ethanol (1 µM)
   Add 100 µL of the 3 µM ICI stock to 900 µL ethanol (0.3 µM)
   Add 100 µL of the 1 µM ICI stock to 900 µL ethanol (0.1 µM)
   Add 100 µL of the 0.3 µM ICI stock to 900 µL ethanol (0.03 µM)
   Add 100 µL of the 0.1 µM ICI stock to 900 µL ethanol (0.01 µM)
   Add 100 µL of the 0.03 µM ICI stock to 900 µL ethanol (0.003 µM)

2. Dilute 1000x concentrations in assay medium (containing 5% dextran/charcoal treated FBS) to double the desired final concentration to allow for the dilution factor when co-incubating with E_2. Vortex to mix.

   Add 2 µL of the 10 µM ICI to 1 mL medium (0.02 µM/20 nM)
   Add 2 µL of the 3 µM ICI to 1 mL medium (6 nM)
   Add 2 µL of the 1 µM ICI to 1 mL medium (2 nM)
   Add 2 µL of the 0.3 µM ICI to 1 mL medium (0.6 nM)
   Add 2 µL of the 0.1 µM ICI to 1 mL medium (0.2 nM)
   Add 2 µL of the 0.03 µM ICI to 1 mL medium (0.06 nM)
   Add 2 µL of the 0.01 µM ICI to 1 mL medium (0.02 nM)

Add 2 µL of the 0.003 µM ICI to 1 mL medium (0.006 nM)

3. Add 50 µL of the dosing solutions to the appropriate wells containing 0.1 nM E_2.
   Final concentrations of ICI curve:
   10 nM; 3 nM; 1 nM; 0.3 nM; 0.1 nM; 0.03 nM; 0.01 nM; 0.003 nM

Day 9 (Wednesday)

Aseptic conditions are not necessary from this point forward and the following procedures should be carried out outside the biological safety cabinet to avoid contaminating the cabinet.

1. Remove assay plates from incubator and assess plates under the microscope for any signs of cytotoxicity, e.g. condensed cell contents or "weathered" cells.
2. Remove dosing solution by shaking plate gently over a waste tray. (Dispose of the waste according to the applicable safety specifications and guidelines).
3. Wash cells by filling each well with 200 µL PBS, at room temperature, using a multichannel pipette and discard PBS.
4. Use a pipette to remove as much of the PBS as possible from each well, taking care not to disturb the cells.
5. Add 25 µL lysis buffer to each well using a multichannel pipette in order to lyse the cells.
6. Place microplates in a freezer. The lysis buffer is activated by one freeze/thaw cycle and the plates can be stored in a freezer for a couple of days if it is not possible to read the plates immediately.
7. Thaw the lysed cells at 37°C while gently shaking on a plate warmer/shaker.
8. Determine luciferase activity using a luminometer with two dispensers programmed to inject 25 µL reaction buffer, followed by 25 µL 1 mM D-luciferin 5 s later, into each well. Luciferase activity is quantified as relative light units (RLU).

5.8 CALCULATION AND REPORTING OF RESULTS

5.8.1 Environmental samples

Estradiol equivalent (EEq) values are calculated for environmental samples.

1. Transfer raw data to Excel.
2. Convert RLU to fold induction (FI) relative to the vehicle control for samples and positive control (RLU/average of vehicle control).

Note:
- Plates should be discarded if the FI of 0.1 nM E_2 control is less than 3-fold.
- If the fold induction values (FI) for samples incubated together with 0.1 nM E_2 are below the FI values for 0.1 nM E_2 incubated alone, cytotoxicity or anti-estrogenic activity is present.

3. Estradiol equivalent values can be calculated for samples with RLU values above the EC_{10} of the E_2 curve. The EC10 can be calculated by the following formula:

   (max RLU E_2 - min RLU E_2) / 10 + min RLU E_2.

4. Express FI values as a percentage of 0.1 nM E_2.
5. Transfer data to Graphpad Prism, with E_2 concentrations as X-values and RLU values expressed as a percentage of E_2 max values as Y-values.
6. Log transform X-values.
7. Fit the E_2 standard curve (sigmoidal function, variable slope) and determine X-values for all unpaired Y-values.
8. Transform X-values back using $X = 10^X$ to obtain the EEq concentrations.
   EEq values must be corrected for the dilution factor to obtain the EEq value for the original undiluted and unconcentrated sample.

5.8.2 Chemicals

The half maximal effective concentration (EC_{50}), relative potency (RP) and relative induction efficiency (RIE) values are calculated and reported for estrogenic chemicals.

1. Transfer raw data to Excel
2. Convert relative light units (RLU) to fold induction relative to the vehicle control for samples and positive control (RLU/average of vehicle control).

Note: If the fold induction values (FI) for test chemicals incubated together with 0.1 nM E_2 are below the FI values for 0.1 nM E_2 incubated alone, anti-estrogenic activity is present.

3. Express FI values as a percentage of 0.1 nM E_2.
4. Transfer data to Graphpad Prism (version 4), with test chemical or E_2 concentrations as X-values and RLU values expressed as a percentage of E_2 max as Y-values.
5. Log transform X-values.
6. Fit the test chemical or E_2 curve (sigmoidal function, variable slope) to obtain the EC_{50} value.
7. Calculate the relative induction efficiency (RIE) with the following formula:

RIE = Max RLU chemical/max RLU E_2 x 100

Fang et al., 2000

RIE gives an indication of the maximum estrogenic activity that could be obtained with a test chemical as a percentage of the positive control. For example, a RIE of 50 means that the maximum estrogenic response of a test chemical is only 50% of the response that could be obtained with the positive control. This means that the test chemical will never reach the maximum activity that could be obtained by the positive control, not even by increasing the concentration of the test chemical.

8. Calculate the relative potency (RP) with the following formula:

RP = EC_{50} of E_2/EC_{50} of sample x 100

Fang et al., 2000

RP gives an indication of the potency of a test chemical compared to the positive control (positive control = 100%), e.g. a RP of 10 means that the test chemical is 10% as potent as the positive control and that a 10 times greater concentration would be needed to obtain the EC_{50} compared to the positive control.

Caution:
The responsiveness of the cells to E_2 may decrease with increasing passage numbers. Therefore, it is very important to keep note of the passage number of the cells that was used for each experiment and to use a new batch of stock cultures when the E_2 response starts to decrease.

5.9 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

5.9.1 Advantages

- The assay is relatively rapid, eliminates the need for transfection, can be conducted in 96 well plates and consistent results are produced.
- The cell line is freely available from ATCC.

5.9.2 Limitations

- Requires a dedicated cell culture laboratory.
- Requires specialised training in cell cultures.
- Special care needs to be taken in order to prevent estrogenic contamination.
- Matrix interference in the form of cytotoxicity, although less than in the YES assay.

5.9.3 Recommendations

- This assay can be used in place of the ER-CALUX as it is less expensive, but equally sensitive (Leusch, 2008).

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5.11 USEFUL CONTACTS

Technical queries on method and training

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Recommended reading

DAVIS JM editor (2006). *Basic cell culture. 2nd edition*. Oxford University Press, New York.

6 THE MDA-KB2 REPORTER GENE ASSAY

Compiled by: A Kruger, R Pieters and S Horn

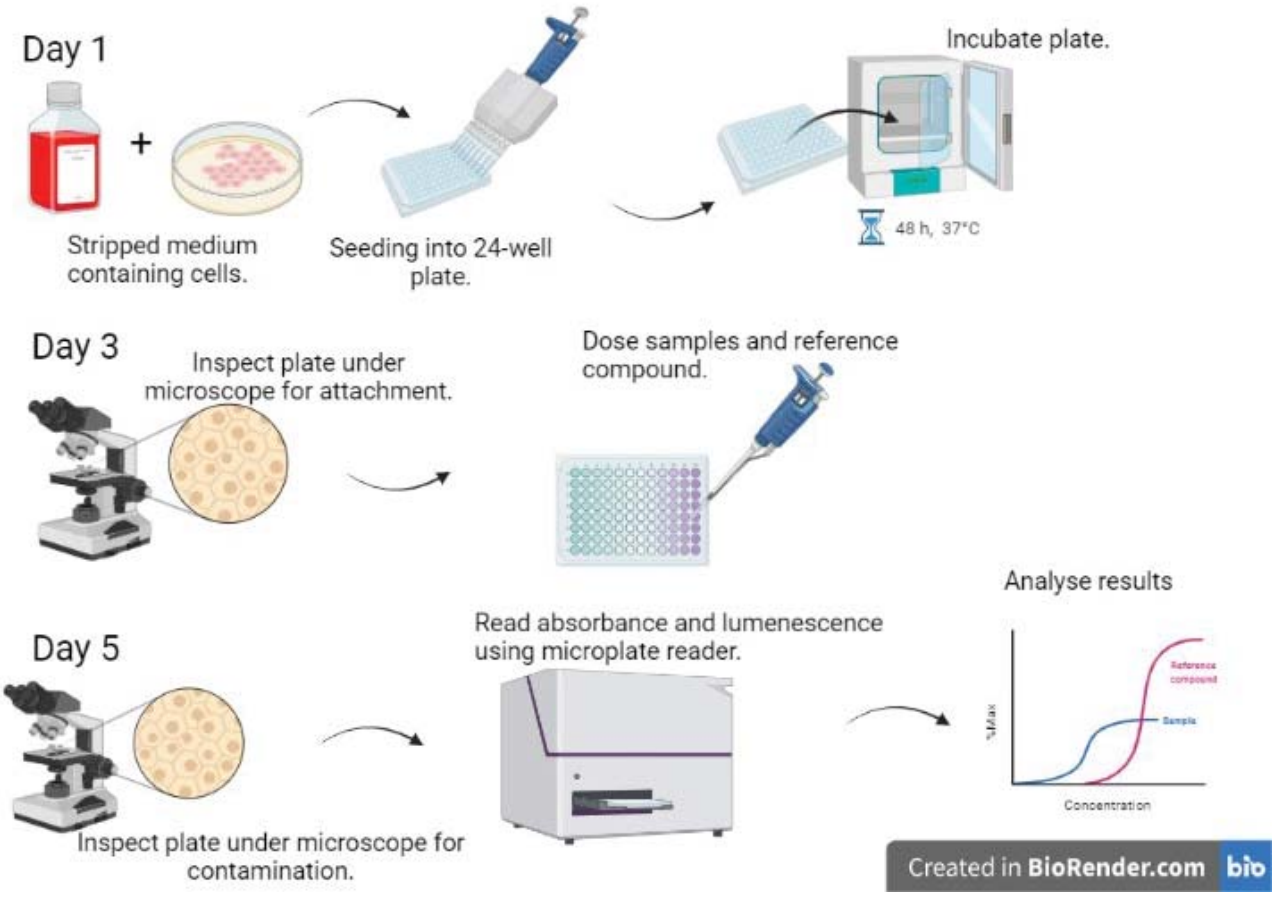

Figure 6-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the MDA-KB2 reporter gene assay

6.1 ACRONYMS & ABBREVIATIONS

AR	Androgen receptor
ATP	Adenosine triphosphate
BC	Blank control
BEQ	Bio-assay equivalent
C_3H_2O	Isopropanol
$CaCl_2$	Calcium chloride
CALUX	Chemical activated luciferase gene expression
cdt	Charcoal dextran treated
CoA	Coenzyme A
CV	Coefficient of variation
DCM	Dichloromethane
dl-PCB	Dioxin-like polychlorinated biphenyl
DMEM	Dulbecco's modified Eagles' medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
EC_{20-80}	Effective concentration range
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FBS	Foetal bovine serum
GR	Glucocorticoid receptor
HCl	Hydrochloric acid
L-15	Leibovitz's L-15
LAR	Luciferase assay reagent
MeOH	Methanol
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
OD	Optical density
RCF	Relative centrifugal force
REP_{20-50}	Relative potency range
RLU	Relative light unit
SPSS	Statistical package for the social sciences
stdev	Standard deviation
VC	Vehicle control

6.2 PRINCIPLE OF THE ASSAY

The parent cell line (MDA-MD-453) was stably transformed into the MDA-kB2 cell line using a luciferase reporter plasmid which is androgen responsive (Wilson et al., 2002). This plasmid is driven by a mouse mammary tumour virus promoter (MMTV). The MDA-kB2 cell line is used to study the activation of glucocorticoid and androgen receptors through hormonal antagonists and agonists (Wilson et al., 2002).

Both the androgen receptor (AR) and the glucocorticoid receptor (GR) are ligand activated transcription factors and members of the steroid receptor superfamily (Cunninghams et al., 2012) and occur in the MDA-kB2 cells and both receptors can act through the MMTV promoter. Androgens play a role in sexual differentiation of the male reproductive tract, accessory reproductive organs, and other tissues during foetal development (Wilson et al, 2002). The glucocorticoid hormone is important in maintaining homeostasis which is stress-related and to regulate physiological functions that is essential for life (Klopčič et al., 2014).

When the assay is performed to test for activation, both the AR and GR are available. In the event of luminescence, the assay has to be repeated with one of the receptors, often the AR, is blocked with a suitable inhibitor. If luminescence occurs again, it means that it was the GR that was activated (Wilson et al., 2002)

6.3 REQUIREMENTS

6.3.1 Acquisition of the cell line

The cell line is commercially available from ATCC (www.atcc.org) under catalogue number CRL-2713™. Other commercial cell lines available are AR CALUX®, AR-anti CALUX® and GR CALUX® from BioDetection Systems in the Netherlands (https://biodetectionsystems.com).

6.3.2 Permits

- All the requirements for obtaining the cells can be found on the ATCC website (www.atcc.org).
- An import permit will also be required from the Department of Health, RSA. Allow a minimum of 8 weeks before expecting the permit.

6.3.3 Staff training (technical skills)

- Training in tissue culture techniques.
- Good laboratory practice.

6.3.4 Laboratory (test environment)

- Dedicated to tissue culturing.

6.3.5 Software

- Microsoft Excel® is used to analyse the raw data.
- Software capable of performing the Mann-Whitney U test.

6.3.6 Apparatus, consumables/materials and reagents

Tables 6-1, 6-2 and 6-3 provide lists of the apparatus, consumables/materials and reagents required for the (anti)androgen and glucocorticoid screens

Table 6-1: List of the apparatus required for the MDA-KB2 reporter gene assay

Name	Specifications
Autoclave	
Balance	maximum 200 g, d=0.1 mg
Bio-safety hood	Biological safety cabinet class II, protection for product and personnel against any ordinary microbiological agents
Centrifuge	With swing-out rotor
Cell counter	Something similar to Luna-II Automated Cell Counter or the manual haemocytometer
Cryo storage	Dewar containing liquid nitrogen or -150°C liquid nitrogen freezer
Freezer	-20˚C
Freezer	-80˚C
Glass beakers	250 mL, 500 mL, 1 L
Measuring cylinder	1 L
Microplate reader	Specifications: capable of quantifying luminescence and absorbance (560 nm)
Mr Freeze®	Nalgene, 5100-0001
Multi-channel pipette	20-200 µL
Multipipette	12 channel, 10-100 µL volume range
pH meter	
Phase contrast inverted microscope	
Pipette	1-10 µL variable volume
Pipette	20-200 µL variable volume
Pipette controller for serological pipettes	
Plate shaker	
Refrigerator	4°C
Repeat pipette	
Schott bottles, glass	250 mL, 500 mL, 1 L
Spatula	
Incubator	capable of maintaining humidified air (i.e. Thermo Electron Corporation Forma Series II)
Vortex mixer	
Water purification system	producing water quality of 18.2 MΩ.cm

Table 6-2: List of the consumables/materials required for the MDA-KB2 reporter gene assay

Name	Cat no	Supplier
96-well, transparent walled microplates with flat transparent bottom, with lid.	92196	TPP
96-well white-walled microplates with flat transparent bottom with lid.	655098	Greiner Bio-one
Bottle top filters, 0.22 µm, 250 mL	596-520	AEC Amersham SOC
Eppendorf Combitips advanced	Z763039 – 100EA	Sigma-Aldrich
High-clarity polypropylene conical tube 15 mL	352096	Falcon
High-clarity polypropylene conical tube 50 mL	352070	Falcon
Nitrile gloves (powder-free)		
Pasteur pipettes	F1016S	Labocare
Permanent marker pens		
Pipette tips, sterile, 10 µL	RC-L10	Microsep
Pipette tips, sterile, 200 µL	301–02–121	Axygen scientific
Pipette tips, sterile, 1000 µL	301–01–401	Axygen scientific
Sterile serological pipettes (12 mL)	SSP1001	Aquilon
Tin foil		
Tissue culture dishes, 100 x 20 mm	664160	Cellstar, Greiner Bio-one
Tissue paper/Paper towel		

Table 6-3: List of the reagents required for the MDA-KB2 reporter gene assay

Name	Cat no	Supplier
Adenosine triphosphate	B3003	Melford
Beetle luciferin	E160C	Promega
Calcium chloride	C-2661	Sigma-Aldrich
Coenzyme A	MEL-C70800-0.1	Melford
Dexamethasone	D4902-100MG	Sigma-Aldrich
Dextran treated charcoal	C6241-20G	Sigma-Aldrich
DMSO (≥99.7%)	34869-1L	Sigma-Aldrich
DPBS	D5652-10L	Sigma-Aldrich
DTT	D11000-5.0	Melford
EDTA	318884-500ML	Sigma-Aldrich

Name	Cat no	Supplier
Ethanol (Reagent Alcohol, ≥89.0%)	270741-1L	Sigma-Aldrich
FBS	S181G-500	Biowest
Flutamide	F9397-1G	Sigma-Aldrich
Hexane UV (Analytical Grade)	212-4	Honeywell
Hydrochloric acid		
Isopropanol	SAAR5075040LC	Merck
Leibovitz's L-15	L4386-50L	Sigma-Aldrich
Methanol	230-4	Honeywell
$MgSO_4 \cdot 7H_2O$	23,039-1	Sigma-Aldrich
$Mg(CO_3)_4 \cdot Mg(OH)_2$	M5671-500G	Sigma-Aldrich
pH buffers (pH 4, pH 7)		
Reporter lysis buffer	ADE3971	Promega
Sodium bicarbonate	S5761-1KG	Sigma-Aldrich
Sodium hydroxide		
Testosterone	86500-1G	Sigma-Aldrich
Tetrazolium salt thiazolyl blue (MTT)	M5655-1G	Sigma-Aldrich
Tricine	T5816-25G	Sigma-Aldrich
Trypan blue stain, 0.4%	T13001	Logos Biosystems
Trypsin	S181G-500	Biowest

6.4 GENERAL METHODS

6.4.1 Preparation of glassware

Used glassware are washed with soap and water and then rinsed with distilled water and allowed to air dry. The mouth of the glassware and the lids are covered with a double layer of foil before it is autoclaved at 121˚C for 15 minutes on a dry cycle.

6.4.2 Leibovitz's L-15 (L-15) medium

The medium is prepared in a 1 L measuring cylinder:
Add 13.8 g Leibovitz's L-15 (L-15) with phenol red powder for 1 L medium to 900 mL deionised and autoclaved water (18.2 MΩ.cm) in a measuring cylinder. Use a magnetic stirrer bar inside the measuring cylinder on a

magnetic stirrer to assist in dissolving the powder. When all components are dissolved, add remaining 100 mL. Adjust the pH to 7.3 (using NaOH or HCl) and filter the medium into a 1 L sterilised glass bottle using a 0.22 µm bottle top filter. Do this inside the bio-safety hood. Label the bottle with date, identity of contents and name of the preparer. Store at 4˚C. Unused medium should be discarded after three months.

6.4.3 Maintenance medium

The medium used to maintain the cells is L-15 supplemented with 10% foetal bovine serum (FBS). The required volume of L-15 is added to 0.22 µm bottle top filter together with required volume of FBS and then filtered. This takes place inside the bio-safety hood. (i.e. 450 mL L-15 + 50 mL FBS). Label the bottle with date, identity of contents and name of the preparer. Store at 4˚C. Unused supplemented medium should be discarded after 14 days.

6.4.4 L-15 Freeze medium (10 mL)

Mix 5 mL FBS, 0.4 mL tissue culture quality dimethyl sulphoxide (DMSO) and 4.6 mL supplemented L-15. Label and store in at -20˚C in 15 mL centrifuge tubes or container of similar quality. It can be stored for three months.

6.4.5 Assay medium

This media is stripped from hormones through supplementing L-15 with 10% stripped FBS (charcoal dextran treated FBS). The hormones are removed from the FBS using dextran coated charcoal. Label the bottle with date, identity of contents and name of the preparer. Store at 4˚C. Discard unused stripped medium after 14 days.

6.4.6 Stripped FBS

Heat the FBS (56ºC for 30 min) and gradually pour in a 50 mL tubes containing 0.006 g/mL dextran coated charcoal, shake for 45 min at 45˚C. Centrifuge the mix for 20 min at 1000 g-force and decant the supernatant into 50 mL tubes containing 0.006 g/mL dextran coated charcoal before shaking the mix again for 45 min at 45˚C. Centrifuge the tubes for 20 min at 1000 g-force and filter the supernatant. Aliquot the filtered stripped FBS into 50 mL tubes. Label the tubes to indicate that it is stripped FBS, add the date and initials (of the person who stripped the FBS). Store at -20˚C.

6.4.7 Dulbecco's phosphate-buffered saline (DPBS) without Mg^{2+} and Ca^{2+} salts

Add 9.64 g DPBS powder into 1 L deionised water (18.2 MΩ.cm) in 1 L measuring cylinder. Use a magnetic stirrer bar inside the measuring cylinder on a magnetic stirrer to assist in dissolving the powder. Adjust the pH to 7.4. Transfer into clean 1 L Schott bottle. Autoclave the DPBS before use. Clearly indicate the contents and date on the label on the bottle. Store at 4˚C.

6.4.8 DPBS with Mg^{2+} and Ca^{2+} salts

To prepare 1 L follow the same instructions as above. Add also:

0.1 g	$CaCl_2$
0.2465 g	$MgSO_4 \cdot 7H_2O$

Make sure the salts dissolve. It can be stored at room temperature and does not need to be autoclaved.

6.4.9 Reference compound testosterone

10 ng/mL stock is used to make the dilutions range (0.8, 4.7, 14.2, 28.3, 56.7 and 85 ng/mL (concentration which cells receive is x100 smaller)) in methanol. This is prepared in 2 mL amber vials with polytetrafluoroethylene (PTFE) lids. The vials are labelled and stored at -20°C with Parafilm® wrapped around the lids to prevent evaporation of the solvent.

6.4.10 Reference compound flutamide

1 mg/mL stock is used to make the dilutions range (2.1, 6.2, 18.5, 55.6, 166.7 and 500 µg/mL (concentration which cells receive is x100 smaller)) in methanol. This is prepared in 2 mL amber vials with polytetrafluoroethylene (PTFE) lids. The vials are labelled and stored at -20°C with Parafilm® wrapped around the lids to prevent evaporation of the solvent.

6.4.11 Reference compound dexamethasone

10 ng/mL stock is used to make the dilutions range (90, 450, 2 250, 11 250, 56 250 and 84 375 ng/mL (concentration which cells receive is x100 smaller)) in methanol. This is prepared in 2 mL amber vials with polytetrafluoroethylene (PTFE) lids. The vials are labelled and stored at -20°C with Parafilm® wrapped around the lids to prevent evaporation of the solvent.

6.4.12 Reporter lysis buffer

For 100 mL: Add 20 mL lysis buffer to 80 mL deionised water (18.2 MΩ.cm).

6.4.13 Luciferase assay reagent (LAR)

To prepare 500 mL LAR, mix the following by weighing all the components and adjust pH to 7.8:

1.792 g	Tricine (20 mM)
0.329 g	$MgSO_4 \cdot 7H_2O$ (2.67 mM)
2.569 g	DTT (33.3 mM)
0.075 g	Beetle luciferin (470 µM)
0.1036 g	CoA (270 µM)
0.146 g	ATP (530 µM)
5 mL	EDTA (0.1 mM)
5.35 mL	$Mg(CO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$ solution (1.07 mM) (see preparation below)

Add deionised water (18.2 MΩ.cm) to prepare 500 mL LAR.
Preparation of 20 mL $Mg(CO_3)4{\cdot}Mg(OH)_2{\cdot}5H_2O$ solution: Add 0.967 g $Mg(CO_3)_4{\cdot}Mg(OH)_2$, into 15 mL deionised water (18.2 MΩ.cm) and slowly add 2 mL concentrated HCl until the solution turns clear. Finally add 3 mL deionised water (18.2 MΩ.cm). Final volume is 20 mL. Label and store at room temperature.

Precautionary note: LAR is light sensitive! Protect storing tubes against UV by covering them with aluminium foil. When thawing the tubes ahead of use, keep them protected from UV radiation.
Aliquot into 50 mL tubes and label appropriately.

Store at -80˚C.

6.4.14 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution

0.5 mg/mL MTT powder in stock medium.
One plate requires 6.6 mL MTT solution.
Solution should be prepared in the dark.

6.5 GENERAL CELL CULTURE TECHNIQUES

6.5.1 Aseptic conditions

The tissue culture laboratory is a sterile environment and all the counter tops, as well as the bio-safety hood must be cleaned regularly with 70% ethanol before and after working with the cells. Media changes, passaging of the cells and exposures are done inside the bio-safety hood to prevent microbial contamination (Davis, 2002).

It is important to always wear nitrile gloves that are regularly sprayed with 70% ethanol. Wearing face masks are optional, but advisable because the nutrient media is not usually supplemented with antibiotics and humans may inadvertently transmit microbes to the cells when working them. Only open glass bottles containing media and tubes containing FBS, trypsin, antibiotics inside the bio-safety hood. Spray the bottles and tubes with 70% ethanol before placing it inside the bio-safety hood. Spray consumables with 70% ethanol before placing it in the bio-safety hood.

Glass bottles should be autoclaved before using it again.

6.5.2 Thawing the frozen cells (start-up cell-line)

1. Frozen MDA-kB2 cells (1 mL) are collected from cryopreservation and are placed in a water bath to allow quick thawing (30 sec).
2. Add the 1 mL cells from the cryo-vial into 11 mL supplemented medium (pre-warmed to room temperature, or 37°C) in a 15 mL centrifuge tube.

3. Centrifuge the tube at 125 relative centrifugal force (rcf) for ten minutes (Figure 6-2).
4. Discard the supernatant and resuspend the pellet using 12 mL supplemented medium.
5. Transfer the medium containing the cells into a 100 by 20 mm tissue culture dish (label the culture dish) and place in an incubator under stable conditions (humidified air at 37ºC with no additional CO_2).

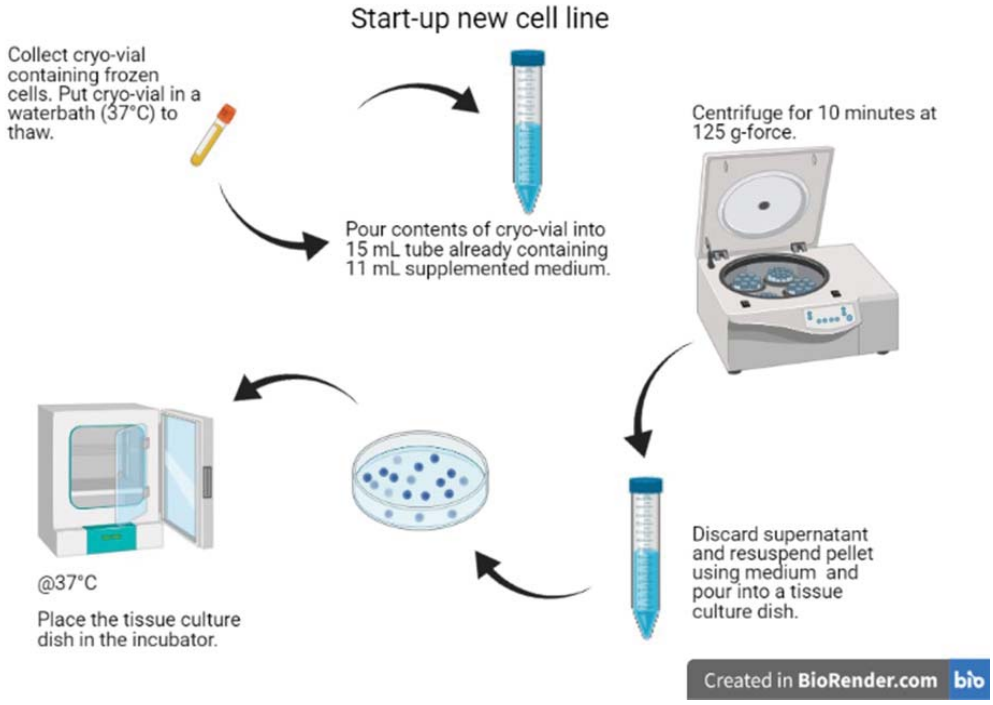

Figure 6-2: Diagram illustrating the start-up process of new MDA-kB2 cells from cryostorage.

6.5.3 Subculturing the cells

When the cells reach 100% confluency, they need to be sub cultured.

1. Passaging the cells requires the medium to be removed from the culture dish and cells to be washed with DPBS three times (Figure 6-3).
2. Use a sterile Pasteur pipette to transfer 1.5 mL trypsin in the culture dish and return the culture dish to the incubator for 3 minutes (Whyte et al., 2004). The trypsin loosens the cells from the culture dish.
3. Discard the trypsin and use 12 mL supplemented medium to create a suspension in which the cells are transferred to a new tissue culture dish.
4. Seed each new plate with 3 or 4 mL and add supplemented medium to create the 12 mL working volume of the plates.

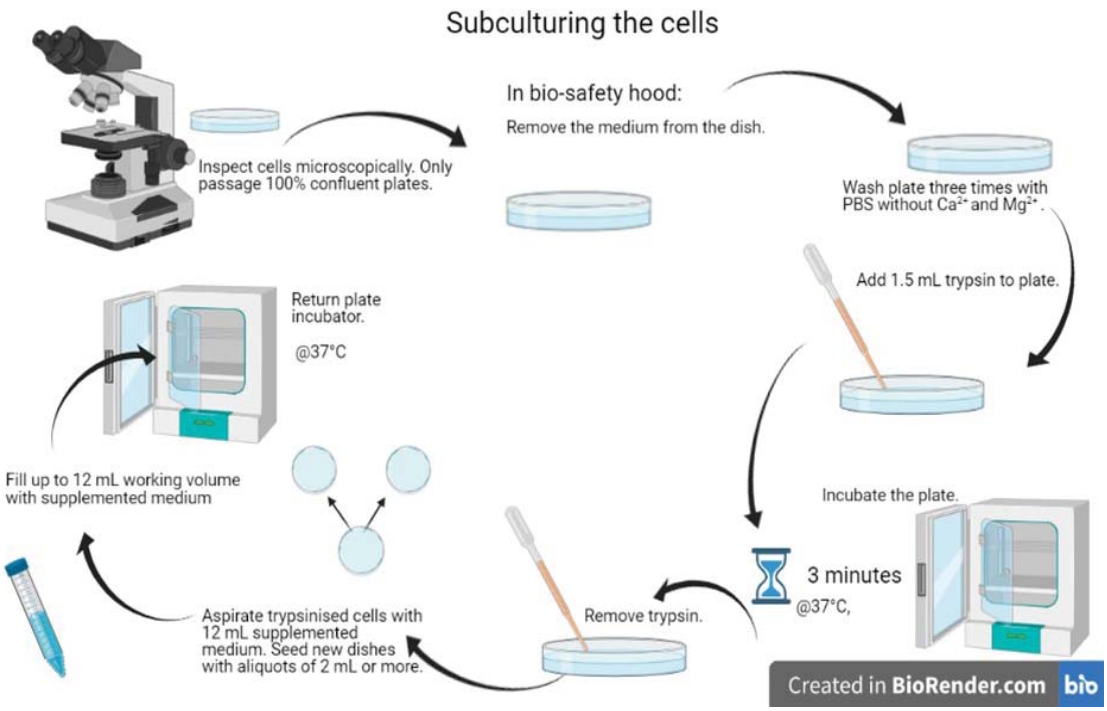

Figure 6-3: Method for passaging cells to new culture dishes.

6.5.4 Freezing stock cultures

1. The first three steps for this method are the same as the steps for subculturing the cells but instead of placing the cells into a culture dish, it is transferred into a 15 mL tube (Figure 6-4).
2. The tube is centrifuged for 10 minutes at 380 rcf.
3. The supernatant is discarded, and the pellet is resuspended using 1 mL freeze medium.
4. The suspension is poured into a cryo-vial and the lid is closed tightly.
5. Place the cryo-vial in the Mr Freeze® container which is filled with isopropanol/propan-2-ol (note the isopropanol/propan-2-ol should be replaced after every fifth use).
6. Place the Mr Freeze® container in the -80˚C for the next 24 h before moving the cryo-vial to the dewar containing liquid nitrogen.

It is important to label the cryo-vial properly.

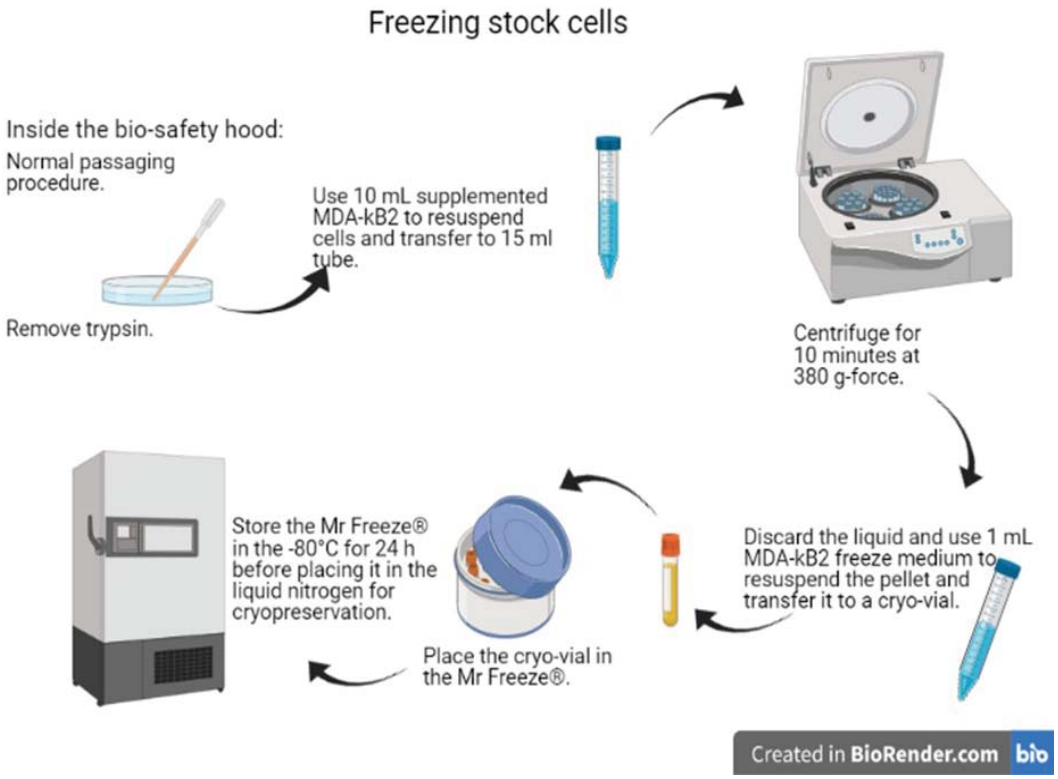

Figure 6-4: Illustration for freezing stock cells

6.6 MDA-KB2 REPORTER GENE ASSAY PROCEDURE

Day 1

1. Trypsinize the cells in a confluent cell culture dish and use 12 mL assay medium to create a suspension (Figure 6-4).
2. Pool cells in a 15 mL tube and determine the density of the viable MDA-kB2 cells in suspension with a cell counting apparatus. Adjust the density (120 000 cells/mL) to the required volume needed: 15 mL per 96-well plate.
3. Seed the cells at 120 000 cells/mL into a 96-well white-walled microplate with a flat transparent bottom in aliquots of 250 µL per well.
4. Seed only the inner 60 wells and fill the outer 36 wells with DPBS. This creates a uniform microclimate across all cell bearing wells.
5. Place the plate in incubator for 48 h.
6. When the assay is run in antagonistic mode, i.e. checking for anti-androgenic effects, add 0.283 ng/mL testosterone to the cell suspension before seeding the plates.
7. When an assay is run to test for glucocorticoid activation, the androgen receptors are blocked through adding 0.556 µg/mL flutamide to the stripped medium before seeding the plate.

Counting cells with the Luna-II Automated Cell Counter:

1. Add 100 µL pooled cells in a cryo-vial.

2. Place 100 µL trypan blue in the cryo-vial. Mix well.
3. Transfer 12 µL of the trypan blue cell suspension onto the reading slide and place the slide in the Luna.
4. Count the cells with on-board software of the instrument

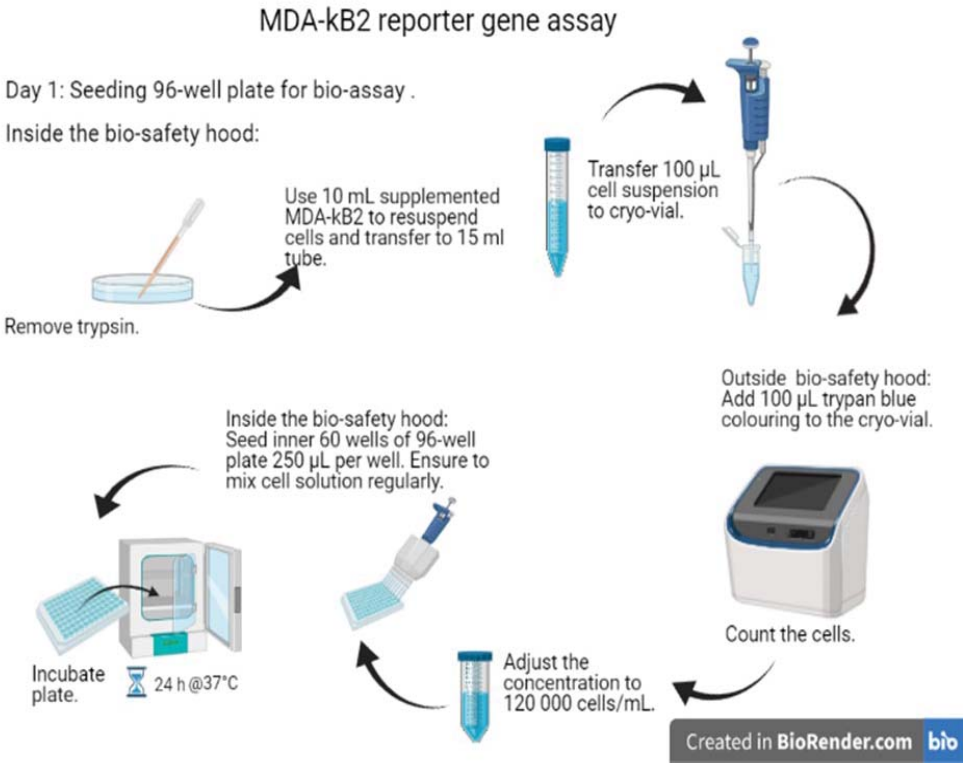

Figure 6-5: Diagram for day one of the reporter gene assay using MDA-kB2 assays.

	Reference compound			Sample				Sample			
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
PBS							BC				PBS
PBS							BC				PBS
PBS							BC				PBS
PBS							VC				PBS
PBS							VC				PBS
PBS							VC				PBS
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Figure 6-6: Example of a typical plate layout.

Depending on the assay performed it will determine which reference compound will be used as well as whether the stripped medium will be spiked or not and with what. Androgen inhibition the stripped medium is spiked with 0.283 ng/mL testosterone and for glucocorticoid activation the 0.556 µg/mL is added to the assay medium.

Day 3

1. Vortex the sample and reference compound containing amber screw top vials spray with 70% ethanol to remove microbes and place vials in the bio-safety hood.
2. Inspect the cells with an inverted phase contrast microscope to confirm cells have attached (Figure 6-6).
3. Dose each well with 2.5 µL sample, or medium (blank control; BC) or vehicle control (VC) (three replicates per concentration and six concentrations per sample).

4. When the activation assay is run for the first time, include the testosterone reference compound to each plate. In the case of luminescence from any of the sample wells, the assay is to be repeated. During day 1 the cell suspension must contain flutamide to block the AR (0.556 ng/mL). The reference compound that should be included then is dexamethasone
6. After dosing the samples and the standard, place the plate in incubator for 48 h.
   Use autoclaved tips for the dosing. The first concentration is the pure extract followed by 2x or 3x serial dilution ranges, depending on the sample.

Day 5

Sterilised conditions are not necessary from this point forward.

1. Remove LAR from -80˚C to thaw and switch the lights off (Figure 6-7).
2. Remove the plate from the incubator and inspect visually for any signs of cytotoxicity. Note this on the plate lay-out sheet because this will help interpret the results.
3. Remove the media from the wells by shaking it over a waste tray.
4. Wash the cells three times with DPBS containing Mg^{2+} and Ca^{2+} salts, and pat the plate dry on tissue paper.
5. Add 25 µL reporter lysis buffer to each of the inner 60 wells.
6. Freeze the plate for 20 minutes at -80˚C to ensure lysis of the cells.
7. After at least 20 minutes, remove the plate and thaw on the plate shaker before reading the luminescence. The plates may be stored in the freezer for a maximum period of three months before reading the results. The luciferase activity is recorded/quantified as relative light units (RLU).

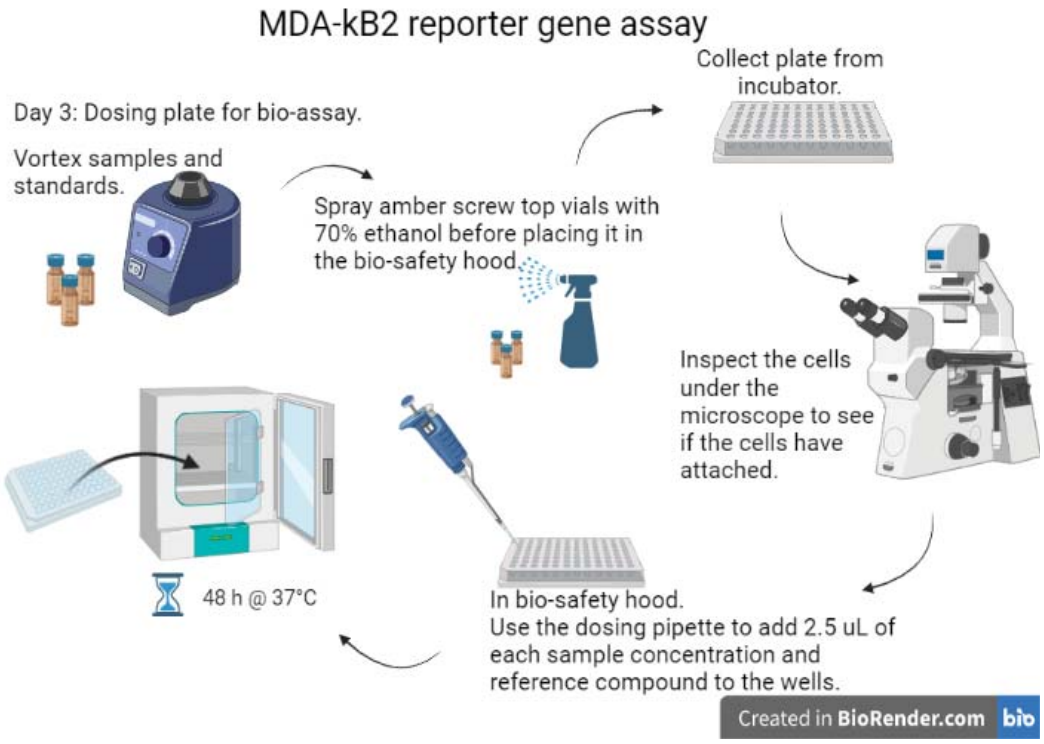

Figure 6-7: Diagram for day 3 of the reporter gene assay using MDA-kB2 cells for dosing the plate

8. While waiting for the plate to thaw, it is important to use a clean 96-well plate to prime the microplate reader. Open the installed program on the computer and place the injector in use (i.e. injector 4) in the LAR and prime the lines from the injector to the plate (Select prime under the instrument option).
9. After the plate is primed inspect the plate for evidence of LAR inside wells, this will indicate a successful prime. Now the microplate reader is ready. Set the plate reader to inject 100 µL LAR into each well, briefly shake to mix the contents and read the luminescence immediately before continuing on to the next well.
10. After all the plates are read it is important to rinse the injector with deionised H_2O. This is usually done using the same plate as the one used to prime the microplate reader. Insert the plate and on the program select wash under the instrument option. Wash plate twice to ensure the injector and the lines are properly cleaned.

6.7 MTT VIABILITY ASSAY

This colorimetric assay measures the metabolic activity of cells and is based on the principle that active cells can reduce the yellow MTT solution through mitochondrial dehydrogenase to form violet-blue formazan crystals (Mossmann, 1983).

This assay is done simultaneous to the reporter gene assay. This assay is performed to ensure that the cells are completely viable at the end of the exposure period and that the unknown environmental sample was not cytotoxic. If cytotoxicity is observed in the MTT viability assay, the luminescence assay results should be reconsidered: the absence of light in the luminescence assay may be due to dead or dying cells, and not the lack of ligands. A dilution adjustment is required to determine a safe concentration series at which the cells can be exposed. The luminescence assay should be repeated with the adjusted concentrations series to learn if there are any ligands capable of binding to the AR or GR.

Day 1

1. Seed the cells in the inner 60 wells as well as the entire column 12.
2. Seed cells in a transparent 96-well microplate.

Day 3

1. Dosing is done exactly the same as for the reporter gene assay. The cells in column 12 are left unexposed – to be treated at the end of the assay (Day 5).

Day 5

1. Prepare the MTT solution in the dark.
2. Remove the plate from the incubator and inspect visually.
3. Remove media from column 12 and kill the cells with 200 µL methanol in each well of this column. Wait five minutes. These provide an absorbance value for dead cells and provide the negative control whereas the absorbance from the VC cells represent the 100% viable cells (positive control).
4. Remove media and methanol from the plate and wash three times with DPBS. Remove the excessive liquid on tissue paper.

5. Add 100 µL MTT solution into the wells and place the plate in the incubator for 30 minutes.
6. Remove plate from incubator and discard the MTT solution from wells.
7. Add 200 µL DMSO to all cell-containing wells. Leave plate for another 30 minutes before reading the absorbance with the microplate reader.

6.8 CALCULATIONS AND REPORTING OF RESULTS

6.8.1 Reporter gene assay

1. The raw data can be worked in Microsoft Excel®.
2. Calculate the mean, standard deviation, and coefficient of variation for the triplicate values of each concentration dosed.
3. Express each mean as a percentage of the maximum response elicited by the reference compound (testosterone, flutamide, dexamethasone).
4. Plot the dose-response curve for each sample and the reference compound separately with the %flutamidemax (%testosteronemax or %dexamethasone) on the y-axis and the logarithm of the concentration (in the case of the reference compound) or the logarithm of the volume dosed (in the case of the sample) on the x-axis.
5. Determine the slope, and intercept for the straight-line section ($y = mx + c$) of each dose-response curve. Use all the points on the straight-line section of the graph. There should be a minimum of three. If there are not at least three, the curve cannot be used for calculations. If this is the situation for the reference compound, the assay must be repeated.
6. Determine the effective concentration (EC) at 20, 50 and 80% of the sample curves as well as the reference compound curve.
7. Determine the relative effects potency for EC20-50 by dividing the EC20 of the reference compound curve with the EC20 of the sample curve. This is also referred to as the bio-assay equivalent (BEQ). This is done to determine if the slope of the sample curve is parallel to the slope of the reference compound. In the case of parallel slopes, the Relative potency range (REP)20-50 will be the same value. This will indicate that the compounds in the sample have the same response on the cells as the reference compound, acting as a dilution (higher concentration) of the reference compound (Villeneuve et al., 2000). For nonparallel dose-response relationships, relative potency is a function of dose and the relationship at a single level of response, such as the EC50, is not constant over the entire range of responses for the compounds being compared. Calculating the REP20-50 will return different values indicating that reporting only a REP50, for example, would be misleading (Villeneuve et al, 2000). Therefore, it is best to report all three, REP20-50 but to clearly indicate which responses were actually achieved by the cells, and which ones are only extrapolated values. Experience showed that REP20 and REP50 are often elicited by environmental samples, but not REP80.
8. Back calculate the REP to take the volume of water or mass of sediment that had been extracted into consideration.
9. These values can be compared with international guideline levels for minimum allowable levels of dioxin-like compounds in the environment.

6.8.2 MTT viability assay results

1. For each triplicate sample dilution, and the VC and MeOH killed cells, calculate the mean, standard deviation, and coefficient of variation (%CV) of the optimal densities (ODs). This can be done on an Excel® spread sheet. The CV indicates the quality of the data points: the smaller the variation between them the more valid are they. The %CV should be no more than 20%. If this is the case, one data point may be removed to improve the CV. This same point must be kept out of all future calculation.
2. Subtract the mean OD of the MeOH killed cells from each of the individual readings. This will give you a new set of data.
3. Determine % viability for each data point, including the VC and the MeOH by dividing its new value (i.e. the value after subtracting MeOH OD). Again, calculate mean and standard deviation for every triplicate sample dilution, VC and killed cells. Again, determine mean and standard deviation.
4. Plot the means on a bar graph and add the standard deviation.
5. Statistically significant differences between a sample and the VC should be determined using the Mann-Whitney U test ($p < 0.05$) because of the limited number of data points. This will give an indication whether any of the results is statistically significant compared to the VC.

	Conc				**Mean**	**Stdev**	**%CV**
VC	**mg/mL**	A	B	C			
Sample 1							
Sample 2							
Sample 3							
Sample 4							
MeOH killed cells							

Figure 6-8: Indication of a template for the raw MTT data

(Conc: concentration; Stdev: standard deviation; %CV: coefficient of variation; VC: vehicle control)

6.9 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

6.9.1 Advantages

- (Anti-)androgenic activity of the total mixture of compounds in an environmental sample can be determined using the bio-assay. If present, GR activation can also be quantified

6.9.2 Limitations

- Effect-based trigger (EBT) values are still in the process of being established. The EBT for water is further developed than for sediment
- The calculations for the antagonistic effect is not yet well established and the reader will do well to invest in literature searches where mention is made of the calculation of the suppression ratio.

6.9.3 Recommendations

- Sample enrichment is usually necessary. This is done by performing an extraction method for the target compounds and concentrating the compounds into a small volume of solvent. Only then the concentration becomes high enough to elicit a response in the cell line.

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6.11 USEFUL CONTACTS

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7 THE GH3.TRE-Luc REPORTER GENE ASSAY

Compiled by: MC Van Zijl and NH Aneck-Hahn

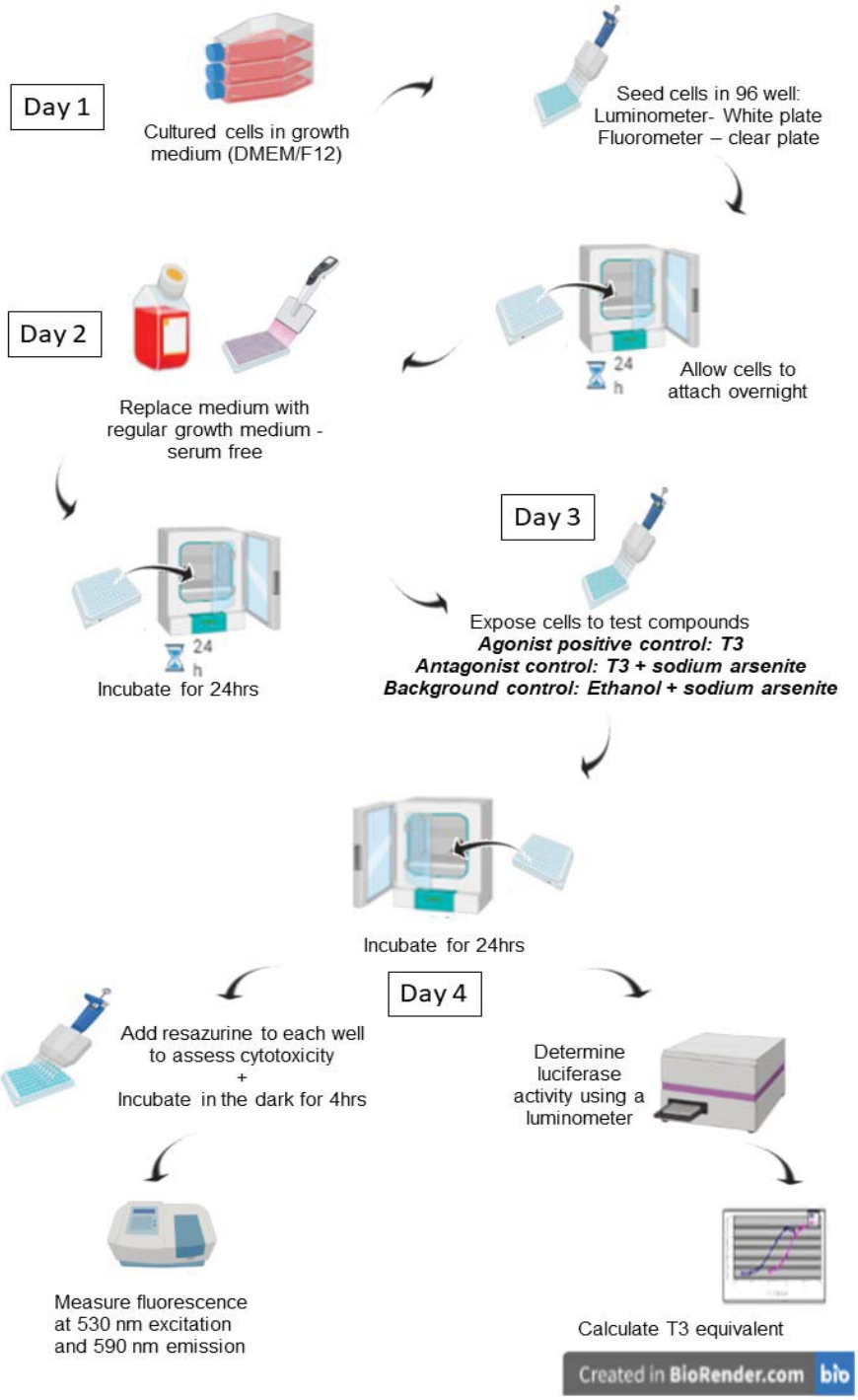

Figure 7-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the GH3.TRE-Luc reporter gene assay

7.1 ACRONYMS & ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
FBS	Fetal bovine serum
FI	Fold induction
HBSS	Hanks' balanced salt solution
HPLC	High performance liquid chromatography
MTA	Material transfer agreement
PBS	Phosphate buffered saline
RFU	Relative fluorescence units
RLU	Relative light units
SA	Sodium arsenite
T3	3,3′,5-Triiodo-L-thyronine
T3Eq	T3 equivalents
VC	Vehicle control

7.2 PRINCIPLE OF THE ASSAY

The GH3.TRE-Luc assay is an in vitro luciferase reporter gene assay that was developed to measure receptor mediated thyroid hormone activity. The rat pituitary tumor GH3 cell line constitutively expresses both thyroid hormone receptor (THR) isoforms and has been modified to contain a luciferase reporter gene (Freitas et al., 2011). The presence of the luciferase enzyme can then be assessed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of thyroid hormone activity of the test chemical. Agonists stimulate luciferase expression and are compared to the vehicle control or to the triiodothyronine (T3) control. The antagonist control is sodium arsenite (SA). Cytotoxicity is assessed using resazurine.

7.3 REQUIREMENTS

7.3.1 Acquisition of the cell line

The cells can be obtained from Wageningen University (The Netherlands) and will require a material transfer agreement (MTA).

7.3.2 Permits

An import permit will also be required from the Department of Health, RSA.

Contact details:
E-mail: importexportpermit@health.gov.za

7.3.3 Staff training (technical skills)

- Training in cell culture techniques
- Software package knowledge (Excel and Graphpad Prism or similar)

7.3.4 Laboratory (test environment)

- Dedicated cell culture laboratory
  For cell culture laboratory guidelines refer to Wigley (2006)

7.3.5 Software

- Graphpad Prism 4, or equivalent
- Microsoft Excel

7.3.6 Apparatus

- Autoclave
- Balance (maximum 110 g, d = 0.1 mg)
- Beakers, glass, 100 mL; 250 mL and 1 L (for preparing stock solutions and rinsing glassware)
- Bottles, autoclavable, glass, 25 mL; 500 mL and 1 L (Duran Cat. No. 218011453; 218014459 and 218015455 or equivalent)
- Centrifuge with swing-out rotor (buckets must be able to contain 50 mL conical tubes)
- CoolCell alcohol-free cell freezing containers (BioCision Cat. No. BCS-136 or equivalent)
- Freezer (-20°C)
- Freezer (-80°C)
- Fridge (4°C)
- Incubator, humidified 5% CO_2
- Inverted microscope
- Microplate luminometer with two dispensers (LUMIstar Omega, BMG Labtech or equivalent)
- Milli-Q Integral 3 water purification system or equivalent with a point-of-use ultrafiltration cartridge to remove endocrine disrupting compounds (EDCs) from water (Merck Millipore EDS-Pak® polisher, Cat. No. EDSPAK001)
- Oven (for drying glassware)
- pH meter
- Pipette, 12 channel, 10-100 µL variable volume
- Pipette, 12 channel, 30-300 µL variable volume
- Pipette, 1-10 µL variable volume
- Pipette, 20-200 µL variable volume
- Pipette, 200-1000 µL variable volume
- Pipettor for serological pipettes
- Plate warmer/shaker

- Spatulas
- Type II biohazard safety cabinet
- Vortex mixer
- Water bath

7.3.7 Consumables/materials and reagents

Tables 7-1 and 7-2 provide lists of the consumables/materials and reagents required for the GH3.TRE-Luc reporter gene assay

Table 7-1: List of the consumables/materials required for the GH3.TRE-Luc reporter gene assay

Name	Cat no	Supplier
Centrifuge tubes, sterile, 50 mL	352070	Corning (Falcon)
Cryovials, sterile, 1.8 mL	377267	Thermo Fisher Scientific (Nunc)
Eppendorf tubes, 2 mL	0030120094	Eppendorf
Hemocytometer		
Liquinox phosphate-free liquid detergent		
Luminometer plates, 96 well, white with clear bottom	3610	Corning
Nitrile gloves		
Permanent marker pens		
Pipette filter tips, sterile, 10 µL	4135	Corning (DeckWorks)
Pipette filter tips, sterile, 1000 µL	4140	Corning (DeckWorks)
Pipette filter tips, sterile, 200 µL	4138	Corning (DeckWorks)
Pipette tips, sterile, 250 µL	9400263	Thermo Fisher Scientific
Reagent reservoir (100 mL, autoclavable)		
Serological pipettes, sterile, 2 mL	170365	Thermo Fisher Scientific (Nunc)
Serological pipettes, sterile, 5 mL	170366	Thermo Fisher Scientific (Nunc)
Serological pipettes, sterile, 10 mL	170367	Thermo Fisher Scientific (Nunc)
Syringes		
Syringe filters, 0.2 µm	SLGP033RS	Merck Millipore
Tin foil		
Tissue culture flasks, sterile, 25 cm^2	430372	Corning
Tissue culture flasks, sterile, 75 cm^2	430641U	Corning

Note: It is recommended to use consumables from the suppliers as stated. Consumables from other suppliers must first be tested as it may affect the outcome of the assay.

Table 7-2: List of the reagents required for the GH3.TRE-Luc reporter gene assay

Name	Cat no	Supplier
Adenosine triphosphate (ATP)	A7699	Sigma-Aldrich
Beetle luciferin, potassium salt	E1603	Promega
Bovine insulin	I6634	Sigma-Aldrich
Bovine serum albumin (BSA) (Fraction V)	A7906	Sigma-Aldrich
Cell culture freezing media	12648010	Thermo Fisher Scientific (Gibco)
DMEM/F12 (1:1) with 15 mM HEPES	31330095	Thermo Fisher Scientific (Gibco)
Ethanolamine	E0135	Sigma-Aldrich
Ethanol, gradient grade for liquid chromatography	111727	Sigma-Aldrich (Supelco)
Fetal bovine serum (FBS)	SH30071.03	HyClone
Glycylglycine	50200	Sigma-Aldrich
Hanks' balanced salt solution (HBSS), 10x	14185045	Thermo Fisher Scientific (Gibco)
Human apotransferrin	T1147	Sigma-Aldrich
Magnesium chloride ($MgCl_2$), 1 M	M1028	Sigma-Aldrich
Methanol, gradient grade for liquid chromatography	106007	Sigma-Aldrich (Supelco)
Phosphate buffered saline (PBS), 10x	14080048	Thermo Fisher Scientific (Gibco)
pH buffers (pH 4, pH 7, pH 10)		Preferred supplier
Reporter lysis buffer	E3971	Promega
Resazurin sodium salt	R7017	Sigma-Aldrich
Sodium arsenite (SA)	S7400	Sigma-Aldrich
Sodium hydroxide (NaOH), 5 N		Preferred supplier
Sodium selenite	S5261	Sigma-Aldrich
3,3′,5-Triiodo-L-thyronine (T3)	T2877	Sigma-Aldrich
Trypsin-EDTA, 10x	15400054	Thermo Fisher Scientific (Gibco)

Note: It is recommended to use reagents from the suppliers as stated. Reagents from other suppliers should first be tested in the assay as it may affect the outcome of the assay.

7.4 GENERAL METHODS

Nitrile gloves (latex free) must be worn when preparing assay components and when doing the assay. All glassware must be prepared by washing in Liquinox phosphate-free liquid detergent, rinsing ten times in tap water, five times in ultrapure EDC free water and twice in high performance liquid chromatography (HPLC) grade methanol consecutively. Dry glassware in oven and cover with foil. If required, sterilize glassware by autoclaving at 121°C for 20 minutes.

7.5 PREPARATION OF ASSAY COMPONENTS

7.5.1 Regular growth medium

Regular growth medium consists of DMEM/F12 (1:1) with 15 mM HEPES supplemented with 10% fetal bovine serum (FBS) (add 50 mL FBS to 450 mL DMEM/F12). Store at 4°C.

7.5.2 PCM-medium components

Prepare 1000x stock concentrations of bovine insulin, ethanolamine, sodium selenite, human apotransferrin, bovine serum albumin and fetuin in ultrapure water, aliquot (100 µL in Eppendorf tubes) and store at -20°C.

7.5.2.1 Bovine insulin

Prepare 10 mg/mL stock concentration of bovine insulin in ultrapure water (add 20 mg bovine insulin to 2 mL ultrapure water).

7.5.2.2 Ethanolamine

Prepare 10 mM stock concentration of Ethanolamine (MW 61.08) in ultrapure water (add 1.2 µL ethanolamine to 2 mL ultrapure water)

7.5.2.3 Sodium selenite

Prepare 10 µg/mL stock concentration of sodium selenite in ultrapure water (add 10 mg sodium selenite to 10 mL ultrapure water for a 1 mg/mL stock, and then transfer 20 µL of the 1 mg/mL stock to 1 980 µL ultrapure water for the final stock of 10 µg/mL).

7.5.2.4 Human apotransferrin

Prepare 10 mg/mL stock concentration of human apotransferrin in ultrapure water (add 20 mg apotransferrin to 2 mL ultrapure water).

7.5.2.5 Bovine serum albumin (BSA)

Prepare 500 mg/mL stock concentration of BSA in ultrapure water (add 1 000 mg BSA to 2 mL ultrapure water).

7.5.3 PCM medium

PCM medium (withdrawal media) should be prepared fresh prior to every experiment. Thaw aliquots of the 1000x stock concentrations of bovine insulin, ethanolamine, sodium selenite, human apotransferrin and bovine serum albumin. Add 100 µL of each component to 100 mL of DMEM/F12 (1:1) with 15 mM HEPES and filter sterilize using a 0.22 µm syringe filter.

7.5.4 HBSS

Autoclave 1 L of ultrapure EDC-free water in a glass bottle to sterilize. Working in a biohazard safety cabinet, add 50 mL 10x HBSS to 950 mL autoclaved water in a sterile 1 L glass bottle, using a sterile measuring cylinder. Store HBSS at 4°C.

7.5.5 Trypsin

Divide 10x concentrated trypsin in 5 mL aliquots into sterile centrifuge tubes and store at -20°C. Prepare 1x trypsin by adding 45 mL HBSS to 5 mL 10x trypsin. Work under sterile conditions. Store 1x trypsin at 4°C.

7.5.6 PBS

Add 100 mL 10x PBS to 900 mL ultrapure water. Store at 4°C.

7.5.7 Lysis buffer

Add 1 mL 5x lysis buffer to 4 mL ultrapure water, just prior to adding lysis buffer to cells.

7.5.8 Glycylglycine

Make a 1 M stock solution by adding 6.605 g glycylglycine (MW 132.1) to 50 mL ultrapure water, pH to 7.8 (with 5 N, HCl or NaOH), sterile filter and store in 50 mL centrifuge tube or another suitable sterile container at 4°C.

7.5.9 ATP solution

Make a 0.1 M stock solution by adding 2.755 g ATP (MW 551.1) to 50 mL ultrapure water, pH to 7.8 (with 5 N, HCl or NaOH) and aliquot into 5 mL volumes in a freezable container. Store at -80°C.

7.5.10 BSA

Make a 50 mg/mL stock by adding 10 mL ultrapure water to 500 mg BSA. Aliquot into 1 mL volumes in Eppendorf tubes or another suitable freezable container. Store at -20°C

Note:

- Do not agitate the solution. Add the ultrapure water slowly to the BSA and leave on the bench for a couple of minutes to dissolve. Agitation may cause BSA to stick to the sides of the glass beaker and cause foaming and air pockets, which will increase the time to dissolve.

7.5.11 Reaction buffer

Reaction buffer is prepared by adding the following together:

90 mL ultrapure water

2 mL 1 M Glycylglycine

5 mL 0.1 M ATP

1 mL BSA solution

1.5 mL 1 M $MgCl_2$

pH the solution to 7.8 (with 5 N, HCl or NaOH). Aliquot into 10 mL volumes. Store at 4°C.

Note:

- The pH of the reaction buffer is critical.
- Cloudiness of reaction buffer does not affect results.

7.5.12 Luciferin

Make a 1 mM stock solution by adding 159.21 mg D-luciferin (potassium salt MW 318.41) to 500 mL ultrapure water. Aliquot into 10 mL volumes in freezable containers. Store at -80°C.

Note:

- Luciferin is light sensitive and aliquots should be stored in amber vials or wrapped in foil.
- Luciferin may be refrozen.

7.5.13 3,3′,5-Triiodo-L-thyronine (T3) stock solution

Make a stock solution of 200 µM T3 by adding 2.604 mg T3 (MW 650.97) to 20 mL HPLC grade ethanol. Use an amber glass bottle or wrap bottle in foil and store at -20°C.

7.5.14 Sodium arsenite (SA) stock solution

Make a stock solution of 20 mM SA by adding 52 mg SA (MW 129.91) to 20 mL HPLC grade ethanol. Use an amber glass bottle or wrap bottle in foil and store at -20°C.

7.5.15 Resazurin

Make a stock solution of 400 µM resazurin by adding 10 mg resazurin (MW 251.17) to 100 mL PBS (pH 7.4). Filter sterilize using a 0.22 µm syringe filter and aliquot 1 mL volumes in Eppendorf tubes. Cover with foil and store at -20°C.

7.6 GENERAL CELL CULTURE TECHNIQUES

7.6.1 Aseptic conditions

- Always work in a biohazard safety cabinet to reduce the possibility of contaminating the tissue cultures.
- Disinfect the cabinet by wiping down the surface with 70% ethanol before and after working in the cabinet.
- Always wear nitrile gloves when working in the cabinet and decontaminate gloves by spraying with 70% ethanol each time before entering the cabinet.
- All materials and reagents that come into direct contact with cell cultures must be sterile.
- Decontaminate everything with 70% ethanol before entering the cabinet.
- Do not work directly above open bottles, flasks, plates, etc. and don't leave bottles/lids unnecessarily open inside the cabinet.
- Close lids before removing bottles from the cabinet.

Refer to McAteer and Davis (2006) for more comprehensive guidelines.

7.6.2 Thawing the frozen cells

1. Prior to thawing the cells, place 10 mL regular growth media in a 25 cm^2 tissue culture flask, using aseptic techniques. Place the flask in a CO_2 incubator for at least 15 minutes, in order for the media to reach 37°C and to equilibrate.
2. Thaw the vial containing the frozen cells in a 37°C water bath, with gentle agitation, and without submerging the cap in the water.
3. Decontaminate the vial by spraying the outside surface with 70% ethanol.
4. Transfer the contents of the vial to the cell culture flask, using aseptic techniques. Label the flask with the name of the cell line, the date and passage number.
5. Place the flask back in the CO_2 incubator and allow cells to attach to the surface overnight.
6. Discard the medium and replace with fresh medium the following day. Trypsinize if necessary.

7.6.3 Subculturing cells

Subculture cells when they reach 80-90% confluency.

1. Place media and HBSS in the CO_2 incubator for at least 15 minutes before trypsinizing cells, in order for the media to reach 37°C and its normal pH. All procedures from this point forward should be carried out in a biohazard safety cabinet, using aseptic techniques.
2. Discard culture media from flasks and rinse cells twice with HBSS (5-10 mL for a 75 cm^2 flask or 3-5 mL for a 25 cm^2 flask).
3. Add 3 mL trypsin to a 75 cm^2 flask or 1 mL to a 25 cm^2 flask for 30 seconds.
4. Remove excess trypsin.
5. Detach cells by gently tapping the flask against the palm of your hand.
6. Add 10 mL medium to a 75 cm^2 flask or 5 mL to a 25 cm^2 flask.

7. Transfer 1/3 or 1/4 to a new culture flask and add maintenance or assay media (20 mL for 75 cm^2 flask or 10 mL for 25 cm^2 flask). Label the new flasks and remember to update the passage number.
8. Return flasks to incubator until cells reach 80-90% confluency.

Note:
- Transfer the required amount of media and HBSS to cell culture flasks before heating in the incubator, to avoid repeated heating/cooling of the media. This will also ensure that in the event of contamination, it is limited to the one flask and not contamination of the whole bottle of medium or HBSS. If the culture flask does not have a vented cap, the cap should be slightly loose, to allow for the exchange of CO_2.
- Media should be changed twice a week.
- When subculturing cells from a 25 cm^2 flask to a 75 cm^2 flask, transfer all of the trypsinized cells from the 25 cm^2 flask to the 75 cm^2 flask and add 15 mL media.

7.6.4 Freezing of stock cultures

1. Grow cells in a 75 cm^2 flask until they reach 80-90% confluency.
2. Place media and HBSS in the CO_2 incubator for at least 15 minutes before trypsinizing cells, in order for the media to reach 37°C and its normal pH. All procedures from this point forward should be carried out in a biohazard safety cabinet, using aseptic techniques.
3. Discard culture media from flasks and rinse cells twice with 5-10 mL HBSS.
4. Add 3 mL trypsin for 30 seconds.
5. Remove excess trypsin.
6. Detach cells by gently tapping the flask against the palm of your hand and add 10 mL maintenance medium to the flask.
7. Transfer medium containing cells to a 50 mL conical tube and centrifuge at 15°C, 172 x g, for 10 minutes.
8. Remove media using a sterile pipette, taking care not to disturb the pellet.
9. Add 6 mL cell freezing media and pipet gently two to three times to disperse cells.
10. Aliquot 1 mL per cryovial and label the vial with the name of the cell line, date and passage number. Freeze the cells at -80°C using a cryofreezing container [a].

Note:
- New stock cell cultures should be prepared every 12 months for cells frozen at -80°C.
- Transfer frozen cells to liquid nitrogen for long-term storage of cells.
- Never use a working flask for making stock cultures. Keep the passage numbers of the frozen stock cultures as low as possible, because the responsiveness of the cells to T3 may decrease with increasing passage numbers. Keep one vial of the original lot to make new stock cultures and use the last vial of each new batch of stock cultures for the next batch of stock cultures.
- It is advised to store stock cultures in two different locations, to ensure that stocks are not lost in the case of a physical disaster at the primary location.
- It is strongly recommended to check frozen cell cultures for viability before the stock culture is terminated.

a) Freezing cells using the CoolCell alcohol-free cell freezing container

Using the CoolCell alcohol-free cell freezing container ensures that the optimal cooling rate of -1°C/min is achieved when freezing the cells, in order to minimize damage to the cells due to osmotic imbalance and ice crystal formation.

8. Make sure that the chambers and cryovials are dry to avoid tubes sticking upon freezing.
9. The container and core (black ring) should be at room temperature.
10. Place the core at the bottom of the central cavity.
11. Place sample vials containing 1 mL of cell suspension in each well. Each of the 12 wells should contain a filled vial. If the freezing batch is less than 12 vials, empty wells should be filled with filler vials, containing equivalent volumes of freezing media.
12. Fully seat the lid on the CoolCell container.
13. Place the container upright in a -80°C freezer, allowing at least 2.5 cm free space around the container.
14. Freeze for at least four hours before transferring the vials to storage boxes or liquid nitrogen. It is recommended to use dry ice when transferring cryovials to permanent storage to avoid temperature rise and cell damage.

7.7 ASSAY PROCEDURE

The assay is performed according to Freitas et al. (2011), with modifications from Mengeling and Furlow (2015) and Nkosi (2020).

Day 1

1. Grow cells in regular growth medium in a 75 cm^2 flask until they reach 80-90% confluency.
2. Trypsinize cells and add 10 mL regular growth medium.
3. Pool cells in a 50 mL conical tube if cells from more than one flask is used and gently mix cell suspension by inverting the tube.
4. Count the cells using a Hemocytometer [b].
5. Seed cells at 5 x 10^4 cells per well in 96-well luminometer plates (100 µL per well) [c], place plates in the incubator and allow cells to attach overnight. Remember to note the passage number of the cells for each experiment.

b) Counting Cells using a Hemocytometer:

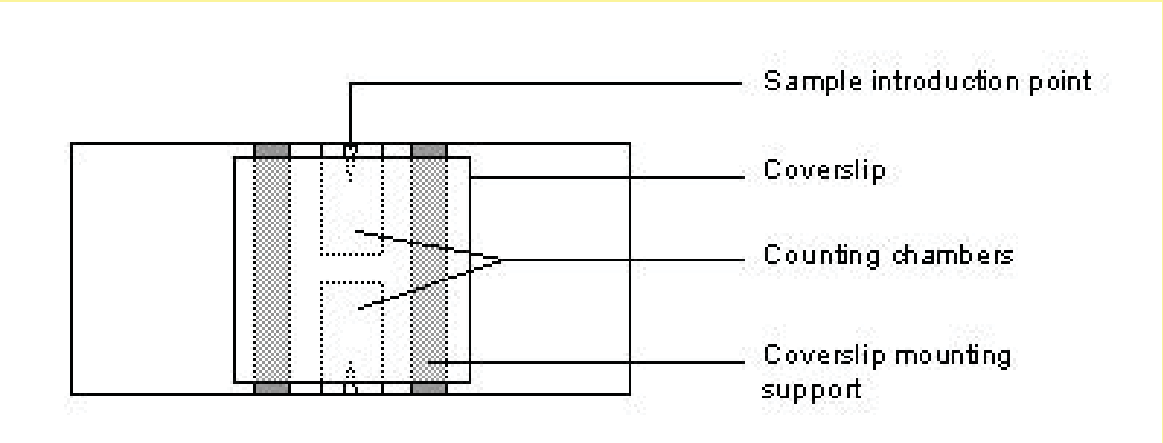

Figure 7-2: Diagram of a Hemocytometer

1. Slightly dampen the coverslip mounting support areas with water, place the coverslip squarely on top of hemocytometer and gently, but firmly press the coverslip over the support areas. Appearance of rainbow rings (like oil when it lies on water) indicates that the coverslip has formed a tight seal and ensures the depth of the chamber is 0.1 mm.
2. Load cell suspension (10-12 µL) onto the hemocytometer using a pipette. Place the pipette tip to the groove on one side of the hemocytometer and gently force the fluid out and allow it to be drawn under the coverslip by capillary action. The fluid must cover the entire polished surface of each chamber. Take care not to overload the counting chambers.

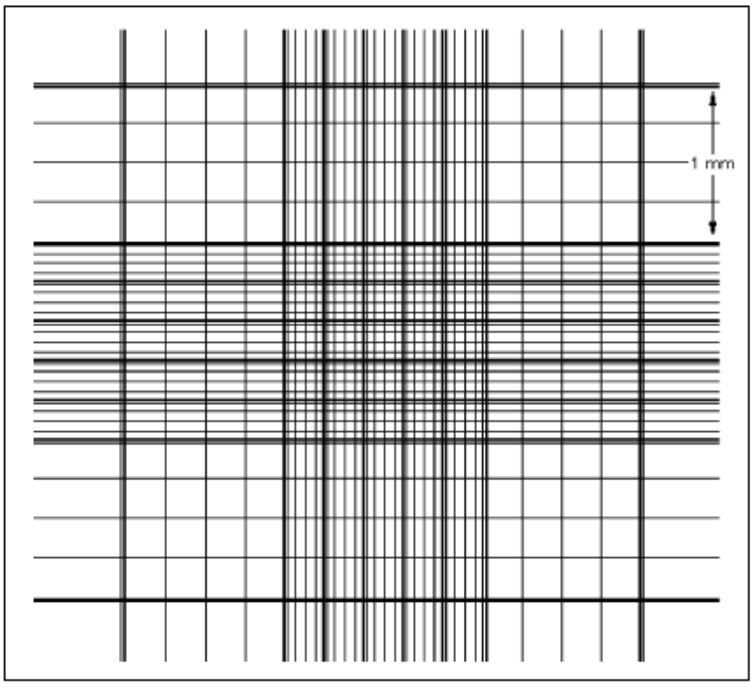

Figure 7-3: Diagram of a counting chamber grid

4. Use the 10x objective of the microscope to focus on the grid lines in the chamber (Figure 2). Count the cells in the four corner 1 mm^2 grid areas (one 1mm^2 grid area consists of 4 x 4 squares surrounded by three grid lines) (Figure 3).

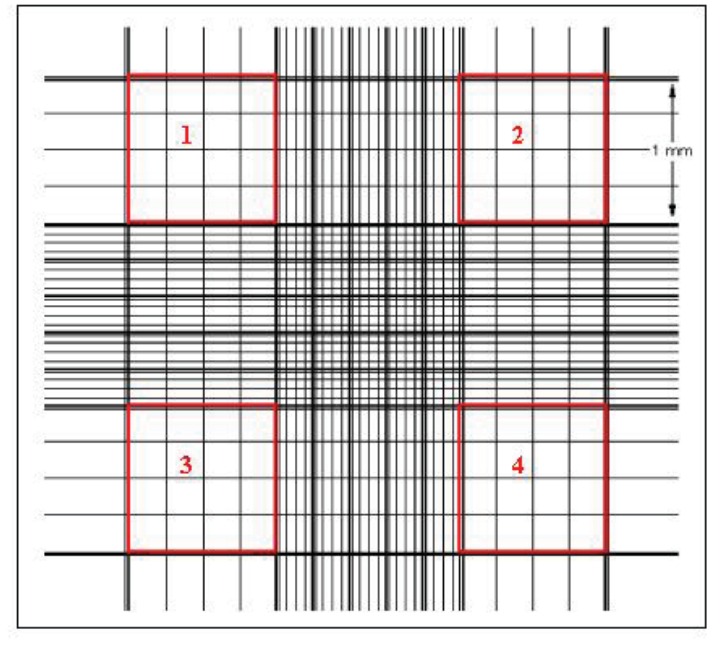

Figure 7-4: An example of the four corner squares on a counting chamber grid

Note:

- In order to prevent cells from being counted twice, the cells touching the upper or left boundaries are counted, but those that touch the bottom or right boundaries are not.
- If more than 10% of cells are clustered together, attempt to disperse the original cell suspension further by pipetting and start again. Clusters containing more than five cells are counted as five.
- If there are too many cells to count realistically, perform a dilution using assay medium. If there are less than 100 cells per 1 mm^2 grid area, additional squares must be counted to improve the accuracy of the count.

4. Determine the cell concentration with the following formula:

   Cells/mL = average count per 1 mm^2 square x 10 000 x dilution factor

   (The depth of 1 mm square is 0.1mm and the volume is therefore 0.0001 mL. The cell count per 1 mm^2 (average of squares 1-4) must therefore be multiplied by 10 000 to get the cells/mL).

   McAteer and Davis (2006) and http://www.nuncbrand.com/en/frame.aspx?ID=1174

c) Seeding cells at 5 x 10^4 cells per well:

- Prepare 11 mL of the final cell dilution per plate.
- Cells must be seeded at 5 x 10^4 cells per well or 5 x 10^4 cells per 100 µL (0.1 mL), which is equal to 500 000 cells/mL and 5 500 000 cells/11 mL.
- Determine the volume of the original cell suspension that will contain 5 500 000 cells by dividing 5 500 000 by the number of cells per mL counted on the hemocytometer. Add assay medium to obtain a final volume of 11 mL and transfer to a sterile reagent reservoir.
- The outer rows of the plates may evaporate and are therefore not used. Fill the outer row of the plate with 200 µL medium or HBSS and fill the rest of the plate with 100 µL cell suspension, using a multichannel pipette.

Day 2

1. Prepare serum free medium (PCM medium, refer to 3.2.3).
2. Remove regular growth medium from plates.
3. Add 100 µL HBSS to each well.

4. Remove HBSS, add 100 µL PCM medium to each well.
5. Return plates to the incubator for 24 hours.

Day 3

Dosing of the plates

1. Prepare 1:10 dilution series for controls and test chemicals in HPLC grade ethanol in 2 mL Eppendorf tubes and vortex to mix.
2. Dilute 1000x concentrations in PCM medium to double the desired final concentration to allow for the dilution factor when vehicle control (VC), T3 or SA are co-incubated with the samples and controls. Vortex to mix.
3. Remove the cell culture medium from the 96 well plates containing the cells and add 100 µL of the dosing solutions to the appropriate wells. Add 100 uL medium containing T3 or SA to wells that should be co-incubated with the controls. Wells that are not co-incubated with T3 or SA must receive an additional 100 µL medium containing the vehicle control. An example of a plate layout is illustrated in figure 7-5.
4. Incubate plates for 24h in a 5% CO_2 incubator.

Note:

- Ethanol (vehicle) concentrations in the final dilutions of controls or test substances may not exceed 0.2%.
- During the extraction procedure, samples are concentrated 1000x, but are diluted 1000x when introduced to the cells in order to comply with the 0.2% vehicle limitation. When higher concentrations are needed to get sufficient points for more accurate T3 equivalents (T3Eq) calculations, transfer the required volume of sample to an Eppendorf tube, allow to evaporate and resuspend in medium containing 0.2% vehicle control. For example, to obtain a final concentration equal to 10x concentrated sample, evaporate 20 µl of sample and add 1 mL vehicle control medium.
- Each assay plate should contain the agonist positive control (T3), negative control (vehicle only), antagonist control (T3 plus SA) and background control (vehicle plus SA).
- Each sample should be tested alone as well as in the presence of 1 nM T3 or 100 nM SA.
- Plates should be dosed in triplicate. When a lot of samples needs to be done, a single screening plate containing the samples and controls may be dosed. Only the samples with activity are then done in triplicate with the full dose-response curve of T3.
- Where possible, dosing plates should be kept separate from tissue culture flasks containing cells.
- Dosing plates should not be stacked on one another to avoid cross-contamination.

Anti-thyroid activity

To test for anti-thyroid activity, samples are co-incubated with 1 nM T3 and the standard curve is SA, co-incubated with 1 nM T3. The plates must also contain the agonist positive control (T3), negative control (vehicle only), antagonist control (T3 plus SA) and background control (vehicle plus SA).

Cytotoxicity

A separate plate is dosed to assess samples for cytotoxicity.

Note:
According to the original protocol by Freitas et al. (2011), the rezazurine assay for cytotoxicity is done on the same plate that is used to measure T3 activity. However, a study by Nkosi (2020) revealed that the addition of resazurin to the wells increased the background activity of the assay resulting in poor fold inductions (FI) for relative light unit (RLU) readings. It is therefore recommended to do the cytotoxicity assay on a separate plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Vehicle control	1 nM T3	100 nM T3	10 nM T3	1 nM T3	0.1 nM T3	0.01 nM T3	0.001 nM T3	0.0001 nM T3	0.00001 nM T3	
C		Vehicle control	1 nM T3	Sample 1	3x sample 1	1x sample 1	0.3x sample 1	0.1x sample 1	0.03x sample 1	0.01x sample 1	0.003x sample 1	
D		Vehicle control	1 nM T3	10x sample 2	3x sample 2	1x sample 2	0.3x sample 2	0.1x sample 2	0.03x sample 2	0.01x sample 2	0.003x sample 2	
E		100 nM SA	100 nM SA	10x sample 1	3x sample 1	1x sample 1	0.3x sample 1	0.1x sample 1	0.03x sample 1	0.01x sample 1	0.003x sample 1	
			1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	
F		100 nM SA	100 nM SA	10x sample 2	3x sample 2	1x sample 2	0.3x sample 2	0.1x sample 2	0.03x sample 2	0.01x sample 2	0.003x sample 2	
			1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	1 nM T3	
G		100 nM SA	100 nM SA	10x sample 1	3x sample 1	1x sample 1	0.3x sample 1	10x sample 2	3x sample 2	1x sample 2	0.3x sample 2	
			1 nM T3	100 nM SA	100 nM SA	100 nM SA	100 nM SA	100 nM SA	100 nM SA	100 nM SA	100 nM SA	
H												

Figure 7-5: An example of a typical plate layout

Day 4

Aseptic conditions are not necessary from this point forward and the following procedures should be carried out outside the biological safety cabinet to avoid contaminating the cabinet.

Resazurin assay (cytotoxicity)

1. Remove assay plates from incubator and add 8 µL resazurin to each well.
2. Incubate in the dark for 4 hours (37°C, 5% CO_2).
3. Measure fluorescence at 530 nm excitation and 590 nm emission. Readings are quantified as relative fluorescence units (RFU).

Luciferase activity (T3 activity)

1. Remove assay plates from incubator and assess plates under the microscope for any signs of contamination or cytotoxicity, e.g. condensed cell contents or “weathered” cells.
2. Remove dosing solution by shaking plate gently over a waste tray. (Dispose of the waste according to the applicable safety specifications and guidelines).
3. Wash cells by filling each well with 200 µL PBS, at room temperature, using a multichannel pipette and discard PBS.
4. Use a pipette to remove as much of the PBS as possible from each well, taking care not to disturb the cells.
5. Add 25 µL lysis buffer to each well using a multichannel pipette in order to lyse the cells.
6. Place microplates in a freezer. The lysis buffer is activated by one freeze/thaw cycle and the plates can be stored in a freezer for a couple of days if it is not possible to read the plates immediately.
7. Thaw the lysed cells at 37°C while gently shaking on a plate warmer/shaker.
8. Determine luciferase activity using a luminometer with two dispensers programmed to inject 25 µL reaction buffer, followed by 25 µL 1 mM D-luciferin 5 s later, into each well. Luciferase activity is quantified as relative light units (RLU).

7.8 CALCULATION AND REPORTING OF RESULTS

T3 equivalent (T3Eq) values are calculated for environmental samples. Cytotoxic concentrations may not be used for calculating T3Eq. Samples are considered cytotoxic if the FLU of the sample is less than the FLU of the vehicle control minus 3x standard deviation.

1. Transfer raw data to Excel.
2. Convert RLU to fold induction (FI) relative to the vehicle control for samples and positive control (RLU/average of vehicle control).

Note:
- Plates should be discarded if the FI of 1 nM T3 control is less than 3-fold.
- If the FI for samples incubated together with 1 nM T3 are below the FI values for 1 nM T3 incubated alone, cytotoxicity or anti-thyroid activity is present.

3. T3 equivalent values can be calculated for samples with RLU values above the EC_{10} of the T3 curve. The EC10 can be calculated by the following formula:

(max RLU T3 - min RLU T3) / 10 + min RLU T3

4. Express FI values as a percentage of 1 nM T3.
5. Transfer data to Graphpad Prism, with T3 concentrations as X-values and RLU values expressed as a percentage of T3 max values as Y-values.
6. Log transform X-values.
7. Fit the T3 standard curve (sigmoidal function, variable slope) and determine X-values for all unpaired Y-values.
8. Transform X-values back using $X = 10^X$ to obtain the T3Eq concentrations.
   T3Eq values must be corrected for the dilution factor to obtain the T3Eq value for the original undiluted and unconcentrated sample.

7.9 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

7.9.1 Advantages

- The assay is relatively rapid, eliminates the need for transfection and can be conducted in 96 well plates.

7.9.2 Limitations

- Requires a dedicated cell culture laboratory.
- Requires specialised training in cell cultures.
- Matrix interference in the form of cytotoxicity.

7.9.3 Recommendations

- This assay can be incorporated in a suite of bioassays measuring different endpoints for effect-based monitoring.

7.10 REFERENCES

FREITAS J, CANO P, CRAIG-VEIT C, GOODSON M, FURLOW JD and MURK AJ (2011). Detection of thyroid hormone receptor disruptors by a novel stable in vitro reporter gene assay. Toxicology in vitro **25** 257-66.

MCATEER JA and DAVIS JM (2006). Basic cell culture technique and the maintenance of cell lines. In: DAVIS JM editor. *Basic cell culture. 2nd edition*. Oxford University Press, New York, p 135-189.

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NKOSI SS (2020). Assessment of thyroid hormone disrupting activity from source water, sediments and drinking water (Dissertation). University of Pretoria, Pretoria.
WIGLEY CB (2006). The cell culture laboratory. In: DAVIS JM editor. *Basic cell culture. 2nd edition*. Oxford University Press, New York, p 1-27.

7.11 USEFUL CONTACTS

Technical queries on method and training

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8 THE AREc32 ASSAY

Compiled by: MC Van Zijl and NH Aneck-Hahn

(According to the National Research Centre for Environmental Toxicology (Entox) Standard Operating Procedure, version 4, 27 March 2012)

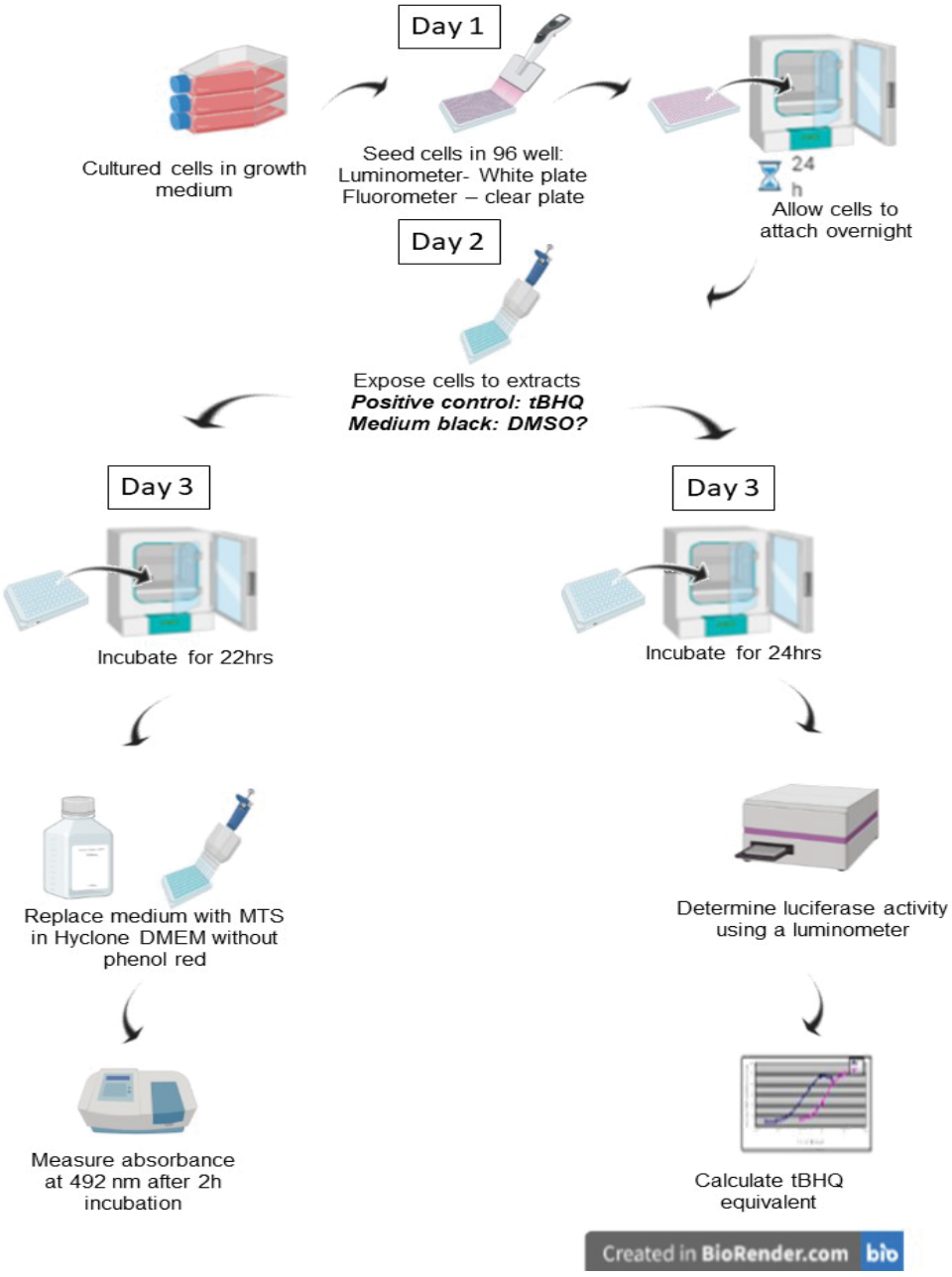

Figure 8-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the AREc32 assay

8.1 ACRONYMS & ABBREVIATIONS

ARE	Antioxidant Response Element
ATP	Adenosine triphosphate
DMSO	Dimethylsulfoxide
ECACC	European Collection of Authenticated Cell Cultures
EDC	Endocrine disrupting compound
Entox	National Research Centre for Environmental Toxicology
FBS	Fetal bovine serum
IR	Induction ratio
HPLC	High performance liquid chromatography
Nrf2	NF-E2-related factor 2
PBS	Phosphate buffered saline
RLU	Relative light units
tBHQ	Tert-Butylhydroquinone
TEQ	Toxic equivalent concentrations

8.2 PRINCIPLE OF THE ASSAY

The mammalian cellular defence mechanism against oxidative stress is primarily mediated at the transcriptional level by Nrf2 (NF-E2-related factor 2), which is responsible for the induction of detoxification and antioxidant genes. Nrf2 activates the transcription of sequences containing the Antioxidant Response Element (ARE), which is a cis-element found in the promoter region of genes encoding proteins that protect the cell from damage by counteracting the harmful effects of reactive oxygen species and environmental carcinogens.

The AREc32 assay is an *in vitro* assay to assess the oxidative stress response and cytotoxicity of chemicals in water samples. The AREc32 cell line was generated by Wang et al. (2006). It is derived from the MCF-7 human breast cancer cell line, with the addition of a luciferase gene construct attached to the ARE. Antioxidant Response Element is activated by Nrf2, which activates the cellular defence mechanism against oxidative stress. In this system, the induction of Nrf2 is proportional to the amount of luciferase produced by the cells, which can be assessed by a bioluminescence assay (Esher et al., 2012). As a control, the cell viability is assessed with the MTS assay. MTS (tetrazolium) is bioreduced by cells into an aqueous, soluble formazan product by dehydrogenase enzymes found in metabolically active cells (Mosman, 1983). When cells die, they rapidly lose the ability to reduce these products due to mitochondrial dysfunction. The absorbance of the formazan product at 490nm is directly proportional to the number of living cells in culture.

8.3 REQUIREMENTS

8.3.1 Acquisition of the cell line

The cells can be obtained from the European Collection of Authenticated Cell Cultures (ECACC), cat no 16071902. Merck is the distributor of these cells in South Africa.

Information and contact details:
Website: www.sigmaaldrich.com/ZA/en
E-mail: rsa@merckgroup.com
Tel: 086 006 3725

The cells can also be obtained from Signosis, Inc., California, USA, cat no SL-0010-NP.
Website: www.signosisinc.com
E-mail: info@signosisinc.com
Tel: 1-408-747-0771

8.3.2 Permits

An import permit will also be required from the Department of Health, RSA.

Contact details:
E-mail: importexportpermit@health.gov.za

8.3.3 Staff training (technical skills)

- Training in cell culture techniques
- Software package knowledge (Excel and GraphPad Prism or similar)

8.3.4 Laboratory (test environment)

- Dedicated cell culture laboratory
  For cell culture laboratory guidelines refer to Wigley (2006)

8.3.5 Software

- Graphpad Prism 4, or equivalent
- Microsoft Excel

8.3.6 Apparatus

- Autoclave
- Balance (maximum 110 g, d = 0.1 mg)
- Beakers, glass, 100 mL and 1 L (for preparing stock solutions and rinsing glassware)

- Bottles, autoclavable, glass, 25 mL; 500 mL and 1 L (Duran Cat. No. 218011453; 218014459 and 218015455 or equivalent)
- CoolCell alcohol-free cell freezing containers (BioCision Cat. No. BCS-136 or equivalent)
- Freezer (-20°C)
- Freezer (-80°C)
- Fridge (4°C)
- Incubator, humidified 5% CO_2
- Inverted microscope
- Measuring cylinder, 1 L
- Microplate luminometer
- Microplate fluorometer
- Milli-Q Integral 3 water purification system or equivalent with a point-of-use ultrafiltration cartridge to remove endocrine disrupting compounds (EDCs) from water (Merck Millipore EDS-Pak® polisher, Cat. No. EDSPAK001)
- Oven (for drying glassware)
- pH meter
- Pipette, 12 channel, 10-100 µL variable volume
- Pipette, 12 channel, 30-300 µL variable volume
- Pipette, 1-10 µL variable volume
- Pipette, 20-200 µL variable volume
- Pipette, 200-1000 µL variable volume
- Pipettor for serological pipettes
- Spatulas
- Type II biohazard safety cabinet
- Vortex mixer
- Water bath

8.3.7 Consumables/materials and reagents

Tables 8-1 and 8-2 provide lists of the consumables/materials and reagents required for the AREc32 assay

Table 8-1: List of the consumables/materials required for the AREc32 assay

Name	Cat no	Supplier
Centrifuge tubes, sterile, 50 mL	352070	Corning (Falcon)
Cryovials, sterile, 1.8 mL	377267	Thermo Fisher Scientific (Nunc)
Eppendorf tubes, 2 mL	0030120094	Eppendorf
Hemocytometer		
Liquinox phosphate-free liquid detergent		
Luminometer plates, 96 well, white with clear bottom	3610	Corning
Nitrile gloves		

Name	Cat no	Supplier
Permanent marker pens		
Pipette filter tips, sterile, 10 µL	4135	Corning (DeckWorks)
Pipette filter tips, sterile, 1000 µL	4140	Corning (DeckWorks)
Pipette filter tips, sterile, 200 µL	4138	Corning (DeckWorks)
Reagent reservoir (100 mL, autoclavable)		
Sealing film, clear, polyester, sterile	AX/PCR-SP-S/S	Axygen
Serological pipettes, sterile, 2 mL	170365	Thermo Fisher Scientific (Nunc)
Serological pipettes, sterile, 5 mL	170366	Thermo Fisher Scientific (Nunc)
Serological pipettes, sterile, 10 mL	170367	Thermo Fisher Scientific (Nunc)
Tin foil		
Tissue culture flasks, sterile, 75 cm^2	430641U	Corning

Note: It is recommended to use consumables from the suppliers as stated. Consumables from other suppliers must first be tested as it may affect the outcome of the assay.

Table 8-2: List of the reagents required for the AREc32 assay

Name	Cat no	Supplier
Adenosine triphosphate (ATP)	A7699	Sigma-Aldrich
CellTiter 96 Aqueous One Solution Assay	G3580	Promega
Coenzyme A	C4282	Sigma-Aldrich
Dimethylsulfoxide (DMSO)	D4540	Sigma-Aldrich
DMEM high/modified (with 4500 mg/L glucose, 110 mg/L sodium pyruvate, no phenol red, no L-glutamine)	SH30604.01	HyClone
DMEM with sodium pyruvate and L-glutamine, high glucose	11995065	Thermo Fisher Scientific (Gibco)
DTT	D9779	Sigma-Aldrich
EDTA	E6758	Sigma-Aldrich
Ethanol, gradient grade for liquid chromatography	111727	Sigma-Aldrich (Supelco)
Fetal bovine serum (FBS)	10099141	Thermo Fisher Scientific (Gibco)
Geneticin	10131027	Thermo Fisher Scientific (Gibco)
Glutamax	35050061	Thermo Fisher Scientific (Gibco)
HEPES buffer, 1M	15630080	Thermo Fisher Scientific (Gibco)
Luciferin (VivoGlo)	P1041	Promega
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	M2773	Sigma-Aldrich
Methanol, gradient grade for liquid chromatography	106007	Sigma-Aldrich
Penicillin-streptomycin	15140122	Thermo Fisher Scientific (Gibco)
Phosphate buffered saline (PBS), without calcium and magnesium	003002	Thermo Fisher Scientific

Name	Cat no	Supplier
Sodium pyruvate, 100 mM	11360070	Thermo Fisher Scientific (Gibco)
Tert-Butylhydroquinone (tBHQ)	112941	Sigma-Aldrich
Tricine	T5816	Sigma-Aldrich
Tris	T1503	Sigma-Aldrich
Trypsin-EDTA, 0.5%	15400054	Thermo Fisher Scientific (Gibco)
Trypsin-EDTA, 0.25%	25200056	Thermo Fisher Scientific (Gibco)

Note: It is recommended to use reagents from the suppliers as stated. Reagents from other suppliers should first be tested in the assay as it may affect the outcome of the assay.

8.4 GENERAL METHOD

This method is according to the National Research Centre for Environmental Toxicology (Entox) Standard Operating Procedure, version 4, 27 March 2012

Nitrile gloves (latex free) must be worn when preparing assay components and when doing the assay. All glassware must be prepared by washing in Liquinox phosphate-free liquid detergent, rinsing ten times in tap water, five times in ultrapure EDC free water and twice in high performance liquid chromatography (HPLC) grade methanol consecutively. Dry glassware in oven and cover with foil. If required, sterilize glassware by autoclaving at 121°C for 20 minutes.

8.5 PREPARATION OF ASSAY COMPONENTS

8.5.1 Cell lysis buffer

Prepare the following stock solutions for the cell lysis buffer:

- Tris (1 M), pH 7.8 - make up 500 mL and store at 4°C.
- DTT (100 mM) - make up as 1 mL aliquots and store at -20°C.
- EDTA (500 mM) - make up 50 mL and store at 4°C.

Prepare 250 mL 5x cell lysis buffer in autoclaved milli-Q water:

125 mM Tris, pH 7.8
5% Triton- X 100
10 mM EDTA
50% glycerol

Store at 4°C

Prepare 1x cell lysis buffer on the day of the experiment using milli-Q water:

25 mM Tris, pH 7.8
1% Triton-X 100

2mM EDTA

2mM DTT

10% glycerol

Note:
Do not add DTT to the 5x buffer. Add the required volume to the 1x buffer made on the day of the experiment.

8.5.2 Luciferin substrate buffer

Prepare the following stock solutions for the luciferin substrate buffer:

- Tricine (1 M), pH 7.8-make up 500 mL and store at 4°C.
- $MgSO_4.7H_20$ (1 M) - make up 50 mL and store at 4°C.
- DTT (1 M) - make up as 2 mL aliquots and store at -20°C.
- EDTA (500 mM) - make up 50 mL and store at 4°C.
- Coenzyme A (100 mM) - make up 1 mL aliquots and store at -20°C.
- ATP (100 mM) - make up 1 mL aliquots and store at -20°C.
- VivoGlo luciferin (100 mM) - make up a single 2mL aliquot and store at -20°C.

Prepare 500 mL 5x cell lysis buffer in autoclaved milli-Q water:

100 mM Tricine, pH 7.8

13.35 mM $MgSO_4.7H_2O$

0.5 mM EDTA

Prepare 1x luciferin substrate buffer on the day of the experiment using milli-Q water:

20 mM Tricine, pH 7.8

2.67 mM $MgSO_4.7H_2O$

0.1 mM EDTA

33.3 mM DTT

261 µM Coenzyme A

530 µM ATP

470 µM Luciferin

Note:
DTT, coenzyme A, ATP and luciferin must only be added to the 1x buffer on the day of the experiment.

8.5.3 Preparation of complete DMEM medium

Add 8 mL geneticin (G418), 50 mL FBS, 5 mL penicillin-streptomycin and 5 mL glutamax into 500 mL DMEM. Homogenise the medium carefully by swirling a few times, avoid bubbles. Store the medium at 4°C. Discard medium after 4 weeks.

Note:
Aliquot geneticin (8 mL), FBS (50 mL), penicillin-streptomycin (5 mL) and glutamax (5 mL) and store at -20°C. Thaw one tube of each in a water bath before preparing the medium.

8.5.4 Preparation of complete white hyclone medium for MTS

Add 5mL each of HEPES buffer (1 M), non-essential amino acids (10 mM), and sodium pyruvate (100 mM) into Hyclone media. Pour 50 mL FCS, 5 mL penicillin-streptomycin, and 5 mL glutamax into Hyclone media. Homogenise the medium carefully by swirling a few times, avoid bubbles. Store the medium at 4°C. Discard medium after 8 weeks.

8.5.5 Preparation of tBHQ for dosing

Make up a 10 mg/mL stock of tBHQ to use as a master stock: weigh out 10 mg of tBHQ powder and dissolve in 100% methanol in the biosafety cabinet. Make aliquots of 1 mg/mL (10 µL of master stock to 90 µL of methanol) into 1.5 mL HPLC vials and label with your name, the concentration, the chemical, and the date. These are single use vials for each experiment, and should not be refrozen. Avoid thawing and freezing the master stock as much as possible.

8.6 GENERAL CELL CULTURE TECHNIQUES

8.6.1 Aseptic conditions

- Always work in a biohazard safety cabinet to reduce the possibility of contaminating the tissue cultures.
- Disinfect the cabinet by wiping down the surface with 70% ethanol before and after working in the cabinet.
- Always wear nitrile gloves when working in the cabinet and decontaminate gloves by spraying with 70% ethanol each time before entering the cabinet.
- All materials and reagents that come into direct contact with cell cultures must be sterile.
- Decontaminate everything with 70% ethanol before entering the cabinet.
- Do not work directly above open bottles, flasks, plates, etc. and don't leave bottles/lids unnecessarily open inside the cabinet.
- Close lids before removing bottles from the cabinet.

Refer to McAteer and Davis (2006) for more comprehensive guidelines.

8.6.2 Thawing the frozen cells

1. Thaw one cryovial of AREc32 cells in a 37°C water bath until only a small sliver of ice is visible. Alternatively, you can rub the tube vigorously in your hands to thaw it.
2. Wipe the outside of the cryovial properly with 70% ethanol and place in cabinet.
3. Pipette 10 mL of complete DMEM into a T75 flask.
4. Resuspend the cells in the cryovial with 1 mL complete DMEM using a 1 mL pipette and sterile tip.
5. Transfer all contents from the cryovial into T75 flask and label the flask with your name, date, cell passage number.
6. Incubate the T75 flask in a 37°C 5% CO_2-incubator.

7. After few hours, note if most of the cells have attached to the bottom of the flask. In that case, do just a media change (in order to remove the DMSO from the previous medium) and incubate the T75 flask at 37°C 5% CO_2 incubator.
8. Observe growth daily and passage when cells reach approximately 80% confluence. This can take from 2-5 days, depending on how vigorously they have been split.

Note:
Cells should be discarded if:

Cells are 100% confluent and many floating cells are visible.

Growth medium looks cloudy.

Presence of bacteria or fungi contamination under microscope.

Cell growth rate has diminished and they are failing to grow to 80% confluency.

The cell passage number is higher than 25.

8.6.3 Subculturing cells

1. Thaw 0.25% pink trypsin from freezer (10 mL aliquots).
2. Warm up 1 bottle of complete DMEM and take your room temperature PBS.
3. Homogenise the medium carefully by swirling a few times, avoid bubbles.
4. Pipette 10 mL complete DMEM into T75 flasks (refer to step 13). Label with name, date, ratio that the cells are being split (e.g. 1:3), and the new cell passage number.
5. Retrieve the culture flask with cells to subculture from CO_2-incubator and place them in the safety cabinet (don't forget to lightly spray ethanol on the bottom of the flasks).
6. Aspirate off the old medium into a waste container using a sterile pipette (10 or 25 mL).
7. Carefully pour or pipette 10 mL PBS into the culture flask and swirl gently.
8. Aspirate off the PBS.
9. Repeat steps 6 and 7.
10. Carefully pipette 1.5 mL trypsin into the culture flask and swirl. Allow cells to detach. This should take approximately 2-3 minutes. If the cells are not detaching in may be because the trypsin is no longer at 37°C, so place the flask(s) in the 37°C incubator for an additional 1-2 minutes.
11. Pipette 8 mL (if doing a 1:3 split) or 7 mL (if doing a 1:8 split) of complete DMEM into the flask and rinse the inside surface of the culture flask several times.
12. Resuspend the cells for approximately 10-20 times until no clumps are visible.
13. Transfer an aliquot of cells into the new culture flasks. (The amount to be transferred depends on the growth rate of the cells and the number of days to be planned for next subculturing).

Guidelines for the number of cells to be subcultured: (for reference only)

Confluence	Days to next subculture	Split ratio
90-100%	3-4	1:8
90-100%	2	1:3
80-90%	4-5	1:8
80-90%	2-3	1:3

Rough experimental guide: For an AREc32 experiment, one 80% confluent flask should be enough to seed out two 96 well plates. Subculture cells when they reach 80-90% confluency.

Note:
- Subculture cells when they reach 80-90% confluency.
- For an AREc32 experiment, one 80% confluent flask should be enough to seed out two 96 well plates.

8.7 ASSAY PROCEDURE

Day 1

Harvest cells

1. Thaw white 0.5% trypsin from freezer.
2. Warm up 1 bottle of complete DMEM.
3. Place sterile PBS in the biosafety cabinet.
4. Homogenise the medium carefully by swirling a few times, avoid bubbles.
5. Retrieve the culture flask with cells from CO_2-incubator and place them in the cabinet.
6. Aspirate off medium into the waste container using a sterile pipette.
7. Rinse cells with 10 mL PBS.
8. Aspirate off PBS.
9. Repeat steps 6 and 7.
10. Pour 1.5 mL trypsin into the culture flask and swirl (tapping the flask may help cell dislodgement).
11. Pipette 10 mL of complete DMEM and dislodge cells from the sides and bottom of the culture flask.
12. Resuspend the cells ~20-30 times by pipetting up and down repeatedly to ensure dispersion of individual cells.
13. Transfer everything from the culture flask into a 50 mL Falcon tube. Resuspend cells further if necessary.

Cell counting

1. Transfer 50 µL of cell suspension into an Eppendorf tube.
2. Clean the hemocytometer and cover slip with 70% ethanol and paper towel.
3. Place 10 µL of cell suspension mixed with Trypan Blue under the cover slip of the hemocytometer at the bevelled edge. Make sure not to get any bubbles trapped under the cover slip.
4. Using the 100x magnification objective on the inverted microscope, each of the counting squares will nearly fill the field of view. Count the outermost 4 squares. A total of at least 200 cells should be counted in all 4 counting squares for the estimate to be accurate.
5. Calculate the average number of cells in one square.
6. Since the volume of each square is 10^{-4} mL, the final concentration of cells in cell suspension per mL = average number of counted cells in one square × 10^4.
7. After counting, rinse the hemocytometer and coverslip with 70% ethanol and wipe dry.

Plating cells in 96-well plate for AREc32 induction

1. Adjust final concentration of cells to 1.2 × 10^5 cells/mL using complete DMEM. Calculate 12 mL of cell suspension per plate (if doing more than two plates, calculate 10 mL per plate and then add 5 mL to account for any error).
2. Calculate the volume of cell solution to use:
   (Cells/mL you need)/(cells/mL you have) x volume you need in mL
   For example: if you want to seed out 6 x 96 well plates: you calculate that you have 185 000 cells per mL, and you want 120 000 cells per mL, you would proceed with the following calculation:
   12 0000/185 000 x 65 = 42.17 mL
   You would then add 42.17 mL of the cell solution to 65 - 42.17 = 22.83 mL of media.
2. Transfer 100 µL of the cell solution into each well of a sterile 96-well white tissue culture microplate using a multichannel pipette. If you are also performing the MTS cell viability assay, you will need an additional and equal number of clear 96 well plates. Proceed with seeding the cells as per above.
3. Incubate the 96-well plate at 37°C for 24 hours.

Day 2

Preparation of sample extracts for dosing

1. Warm complete DMEM media in the water bath.
2. Use the clear 96 well tissue culture plates; each well has a capacity for 350 µL. For the tBHQ or reference compound dosing, use the 96 well “masterblock” plates with 1 mL capacity per well (unless a linear concentration-effect curves is done).
3. Calculate how much of the reference chemical/sample you want to dose in the top well. You will need to add twice the amount you want to finish with as we do a serial dilution by half each time. The sample will be diluted in a total volume of 60 µL of methanol, and then 30 µL is added to the proceeding wells for the serial dilution. To prepare, add the amount of methanol to the top well that will allow a total volume (including the sample) of 60 µL. Then add 30 µL of methanol to the remaining wells.

For example: if 10 µL of sample needs to be added to the top well for the desired concentration, dilute it in 50 µL of methanol (for a total volume of 60 µL). Then add 30 µL of methanol to the following wells. Mix the top well thoroughly and then take 30 µL from the top well and add it to the next well, mixing thoroughly. Then take 30 µL from this well and add it to the next one along, and repeat until serial dilution is complete. For controls, just add 30 µL of methanol to each well and allow it to evaporate at the same time.

4. Put the tissue culture plate aside in the biosafety cabinet and allow methanol to evaporate for around 1 hour, or until the wells are completely dry. Take note of the time it took to evaporate. Then add the desired amount of media. For one luciferase and one MTS plate, add 300 µL of media to each of the 96 wells in the plate and mix well.
5. On each plate, do one series with the reference chemical t-BHQ, for QC/QA and also for the calculation of toxic equivalent concentrations tBHQ-EQ (do this after you have added the media to the samples, so they are all ready to dose at once):
    a. If samples are tested and a serial dilution series is easier to do, use the following dilutions: Add 1 x 10^{-5} M of the tBHQ (reference chemical) to the top wells (600 µl), mix well, then do a serial dilution taking 300 µl from the first well, transferring to the next, mixing well, then transferring 300 µl from this to the next well and repeat until you get to the last well.
    b. If a linear dilution series is possible, then use the following dilutions of tBHQ (reference chemical): add 300 µl of medium in each well and then add the following amounts of the in medium diluted tBHQ stock.

    For example: if the original stock of tBHQ in methanol is 1 mg/mL, then it must be diluted 1:50 with medium directly before dosing. This stock is called “medium stock solution”.

MW	166.22	g/mol
Stock tBHQ in methanol	1	mg/mL
dilute the stock 1:50 with medium directly before dosing		
medium stock	0.02	mg/mL
	1.20E-04	M

Then add 20 to 6 µL of this medium stock to 300 µL of medium as follows:

add x µL of medium stock solution into 300 µL medium	t-BHQ concentration (M)	t-BHQ concentration (mg/L)	methanol content %
20	7.52E-06	1.25	0.13%
18	6.81E-06	1.13	0.11%
16	6.09E-06	1.01	0.10%
14	5.36E-06	0.89	0.09%
12	4.63E-06	0.77	0.08%
10	3.88E-06	0.65	0.06%
7	2.74E-06	0.46	0.05%
5	1.97E-06	0.33	0.03%

The concentrations can be varied but the methanol content should not exceed 0.1%

Tip out the old media from the plates and transfer 100 µl of the media with the reference compounds/samples/controls into the correct wells. (*Pipetting is done at an angle of 45°against the wall of the 96-well plate. Release the dosing solution very slowly to prevent disturbing the cells)*

6. Incubate the plates again in the 37°C incubator for 24 hours.

	1	2	3	4	5	6	7	8	9	10	11	12 tBHQ*
A	S1	S1	S1	S1	S1	S1	S1	S1	C	C	C	$1x10^{-5}$ M
B	S2	S2	S2	S2	S2	S2	S2	S2	C	C	C	$5x10^{-6}$ M
C	S3	S3	S3	S3	S3	S3	S3	S3	C	C	C	$2.5x10^{-6}$ M
D	S4	S4	S4	S4	S4	S4	S4	S4	C	C	C	$1.25x10^{-6}$ M
E	S5	S5	S5	S5	S5	S5	S5	S5	C	C	C	$6.27x10^{-7}$ M
F	S6	S6	S6	S6	S6	S6	S6	S6	C	C	C	$3.13x10^{-7}$ M
G	S7	S7	S7	S7	S7	S7	S7	S7	C	C	C	$1.57x10^{-7}$ M
H	S8	S8	S8	S8	S8	S8	S8	S8	C	C	C	$7.83x10^{-8}$ M

Figure 8-2: Suggested plate layout

S= sample (each colour is a separate sample); C= control. 1 represents the highest dose and 1-8 the serial dilution. *serial dilution, preferred alternative is a linear dilution down from approx. $1x10^{-5}$ M.

Day 3

Measurement of fluorescence activity – MTS assay
(To be done 22 hours after dosing the cells)

1. Warm the Clear Hyclone media in the water bath.
2. Warm an aliquot of MTS in the water bath (you will need 2.4 mL per 96 well plate).
3. After 22 hours incubation, retrieve the **clear** 96-well plate from 37°C incubator.
4. Examine cells microscopically for overt signs of toxicity and record the cell morphology (e.g. round shape, apoptosis, dead cells floating).
5. **Per plate:** Add 12 mL of Hyclone media and 2.4 mL MTS to a 50 mL falcon tube. Mix well. *(MTS is light sensitive so make it fresh each time and avoid contact with light for too long).*
6. Tip media from the clear 96 well plates and add 120 µL of MTS solution to each well, using a multichannel pipette.
7. Transfer the plate to the 37°C incubator for 2 hours.
8. Read fluorescence on a fluorometer [Configuration for Fluostar Optima: position delay 0.5 s, no. of kinetic windows 1, no. of cycles 1, measurement start time 0, no. of flashes 20, cycle time 1, no. of multichromatics 1, excitation filter A-492, emission filter - none, gain 974 (remember to do a gain adjustment before each plate), well scanning none].

Luciferase Activity:

(To be done 24 hours after dosing the cells)

1. After 24 hour incubation, retrieve the **white** 96-well plate from 37°C incubator.
2. Examine cells microscopically for overt signs of toxicity and record the cell morphology (e.g. round shape, apoptosis, dead cells floating).
3. Prepare 1x cell lysis buffer (refer to 2.2.1).
4. Prepare 1x luciferin substrate buffer (refer to 2.2.2) in a falcon tube/glass beaker. Wrap the falcon tube/beaker with aluminium foil to prevent light from interfering with the luciferin substrate. Adjust the pH of the entire solution to between 7.7-7.8 (usually at this pH range the luciferin will emit a bright yellow colour). **Note**: this is a very critical step and if forgotten the assay will fail.
5. Pour sterile PBS into an autoclaved multichannel tray inside the biosafety cabinet. Tip out media from the first 96-well plate and use a multichannel pipette to transfer 100 µL of PBS into each well. Tip out and repeat.
6. Tip out the second PBS washing and attach a white Perkin Elmer backing sticker to the back of the plate (making sure that all the wells are covered by the sticker). Pour the 1x cell lysis buffer into the second multichannel tray. Add 30 µL of this cell lysis buffer to each well using a multichannel pipette.
7. After addition of the cell lysis buffer, wait for exactly 5 minutes, giving time for the cell lysis buffer to lyse all the cells properly. (*To ensure proper lysis, you can also keep the plate on a shaker for 5 minutes.).*
8. After the incubation period is over, pour the prepared 1x luciferase substrate buffer to the third multichannel reagent tray and immediately add 100 µL of this buffer to each of the wells using a multichannel pipette. Cover the plate quickly with an aluminium foil, take it to the luminometer and read the plate. [Configuration for Fluostar Optima: position delay 0.5s, no. of kinetic windows 1, no. of cycles 1, measurement start time 0, cycle time 36, measurement start time 0, measurement interval time 1s, emission filter lens, gain 4000, last req value 90%, positioning delay 0.5s, shaking width 1mm, shaking mode orbital, additional shaking 20s before cycle, reading direction 1, well scanning none, pause before cycle 0, pause duration 0].

8.8 DATA EVALUATION

The data evaluation is according to the Entox SOP, version 4, 27 March 2012.

Interference by cytotoxicity causes a suppression of the induction signal (Figure 8-3) and those concentrations cannot be used for the induction data evaluation.

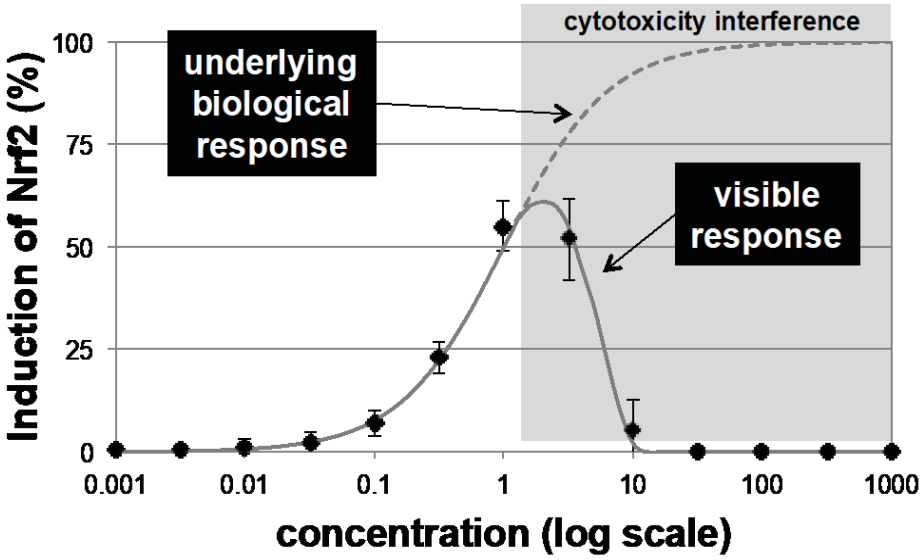

Figure 8-3: Concentration-effect curves. Theoretical graph showing that cytotoxicity can suppress the induction signal (Entox SOP, version 4, 27 March 2012).

8.8.1 Cell viability

The cell viability in each well can be calculated by the absorbance at 492 nm of the sample divided by the average absorbance of the controls (only medium) (equation 1).

$$\text{cell viability} = \frac{OD_{492nm}(sample)}{\frac{\sum_{i=1}^{n} OD_{492nm}(control)}{n}} \quad (1)$$

Then a log-logistic concentration effect curve (equation 2) is fitted using GraphPad Prism software. The maximum is set to 1 (all cells alive) and the minimum is 0 (all cells dead). Adjustable parameters are the slope s and the effect concentration causing 50% reduction of cell viability, EC_{50}. An example is given in Figure 8-4.

$$\text{cell viability} = 1 - \frac{1}{1 + 10^{s \cdot (\log EC_{50} - \log(\text{concentration of tBHQ or REF of sample}))}} \quad (2)$$

Often due to lack of data (as cytotoxicity is not the target endpoint), no full concentration effect curves are obtained. Partial concentration effect curves can be fitted if the slope is fixed to 1. In these cases, the 50% cytotoxicity level is not reached and instead of the EC_{50} an EC_{10}.

$$\log EC_{10} = \log EC_{50} - \frac{1}{s} \log\left(\frac{10}{90}\right) \quad (3)$$

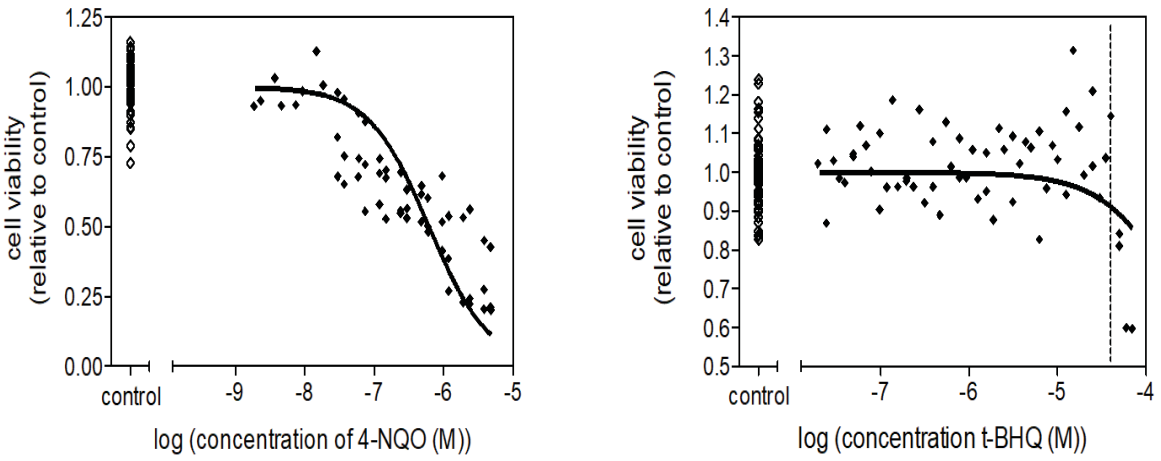

Figure 8-4: Concentration-effect curves for cytotoxicity. Left: full concentration-effect curve for 4-NQO; right: partial concentration-effect curve for t-BHQ (Entox SOP, version 4, 27 March 2012).

Only concentrations that were not cytotoxic should be evaluated for induction. The EC_{10} can be used as a guidance value for the selection of the valid data. The variability of the control is also in the range of 10% (i.e. when calculating the standard deviation of the controls, a value from 0.05 to 0.1 if often obtained).

8.8.2 Induction of Nrf2 (luminescence)

The induction of Nrf2 is proportional to the amount of luciferase produced, which can be assessed by a bioluminescence assay. The relative light units (RLU) are a relative measure of the amount of luciferase. The induction ratio (IR) is defined as the ratio of the RLU of the sample divided by the average RLU of the controls (equation 4).

$$IR = \frac{RLU(sample)}{\frac{\sum_{i=1}^{n} RLU(control)}{n}} \qquad (4)$$

The IR values are then plotted against the concentration (Figure 4, left). Because a clear maximum cannot be reached in most instances, only the low concentrations and effect levels up to an IR of 5 are used to derive a linear concentration-effect curve (Figure 4, right). The assessment endpoint is the concentration that induces an IR of 1.5 ($EC_{IR1.5}$). The $EC_{IR1.5}$ can be derived using the linear regression function in prism, forcing the line through the point x=0, y=1 (control) (Figures 8-5 and 8-6).

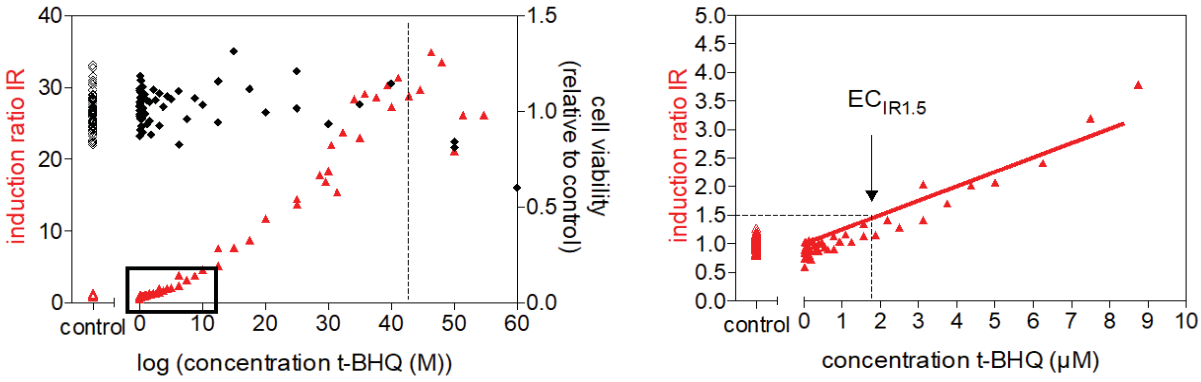

Figure 8-5: Concentration-effect curves for induction. Left: full concentration-effect curve for t-BHQ; right: insert of the linear part of the concentration-effect curve for t-BHQ.

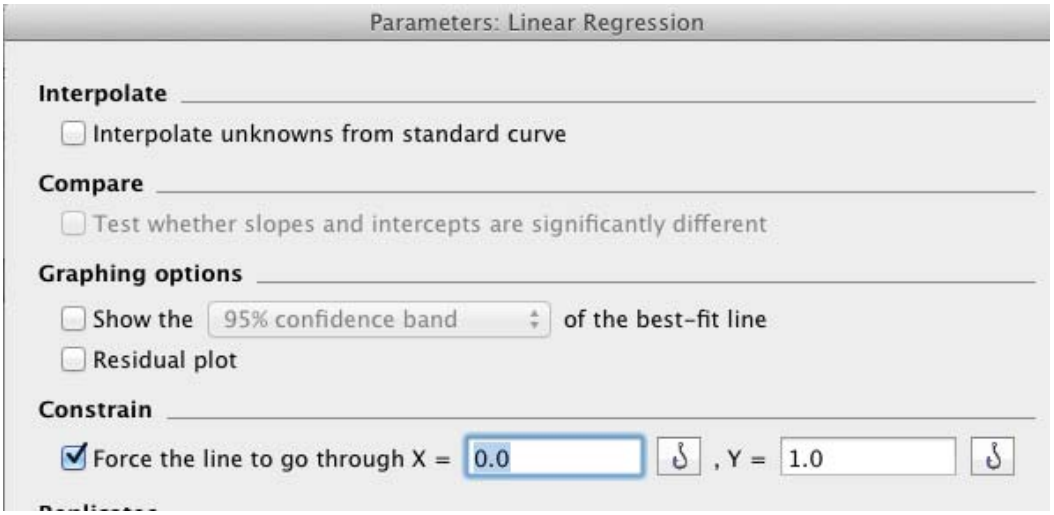

Figure 8-6: Screenshot of GraphPad Prism for linear regression forced through (0/1)

8.8.3 Reporting of results: toxic equivalency concept

Toxic equivalent concentrations (TEQ) are calculated as the ratio of $EC_{IR1.5}$ values of the reference compound to the $EC_{IR1.5}$ of the sample (equation 5).

$$tBHQ\text{-}EQ = \frac{EC_{IR1.5}(\text{reference compound t-BHQ})}{EC_{IR1.5}(\text{sample})} \qquad \left[\frac{\frac{g}{L_{bioassay}}}{\frac{L_{water\ sample}}{L_{bioassay}}} = \frac{g}{L_{water\ sample}}\right] \qquad (5)$$

8.9 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

8.9.1 Advantages

- The assay is relatively rapid, eliminates the need for transfection and can be conducted in 96 well plates.

8.9.2 Limitations

- Requires a dedicated cell culture laboratory.
- Requires specialised training in cell cultures.
- Matrix interference in the form of cytotoxicity.

8.9.3 Recommendations

- It is recommended to include the AREc32 assay, for the induction of oxidative stress and cytotoxicity, to compliment the suite of bioassays measuring other endpoints.

8.10 REFERENCES

ESCHER BI, DUTT M, MAYLIN E, TANG JYM, TOZE S, WOLF CR and LANG M (2012) Water quality assessment using the AREc32 reporter gene assay indicative of the oxidative stress response pathway. Journal of Environmental Monitoring **14** 2877-2885.

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MOSMAN, T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods **65**(1-2) 55-63.

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8.11 USEFUL CONTACTS

Technical queries on method and training

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9 OXIDATIVE STRESS DETERMINATION

Compiled by: S Horn, A Kruger and R Pieters

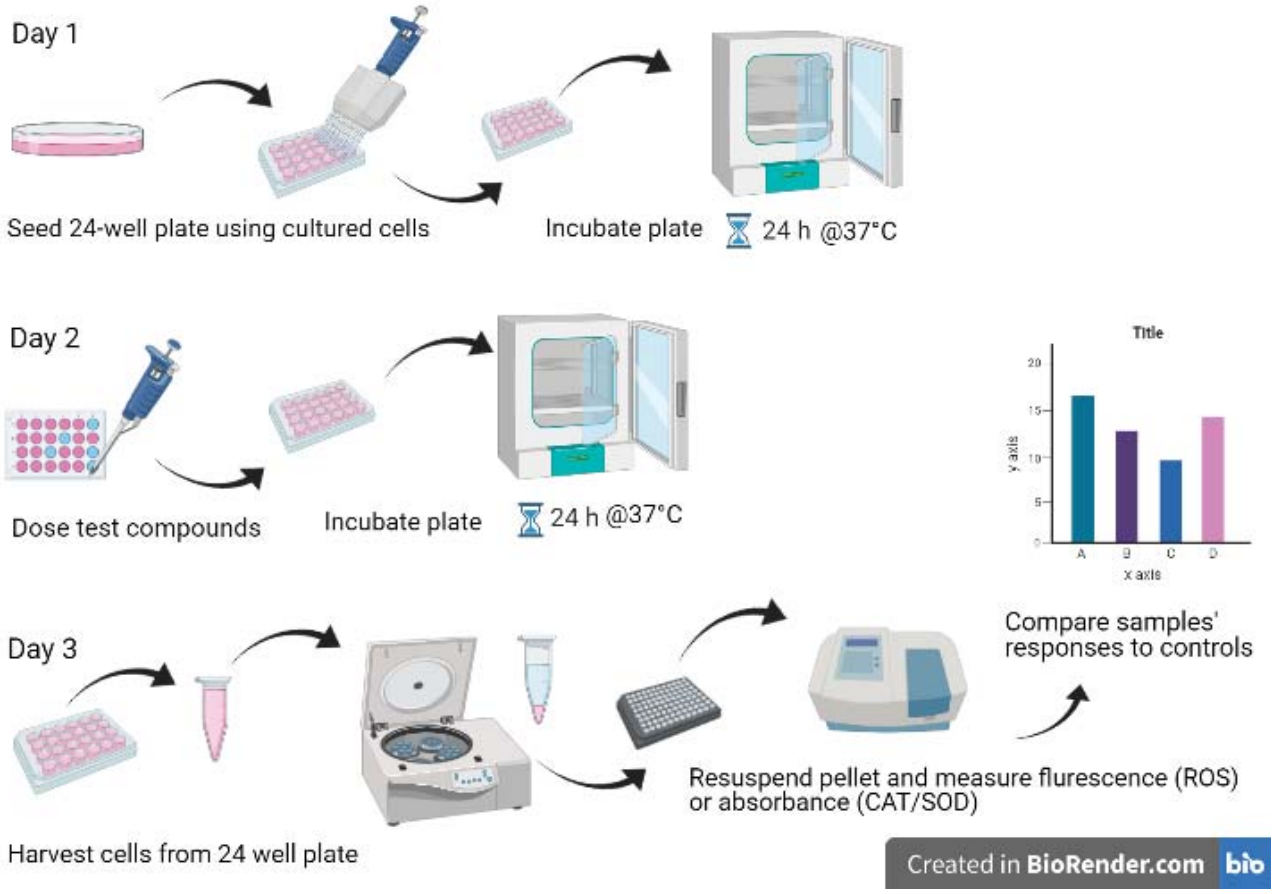

Figure 9-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for oxidative stress determination

9.1 ACRONYMS & ABBREVIATIONS

BSA	bovine serum albumin
CAT	catalase
DCF	2'-7'dichlorofluorescein
DCFH	2'-7'dichlorodihydrofluorescein
DPBS	Dulbecco's phosphate-buffered saline
DTPA	diethylene triamine penta-acetic acid
H_2DCFDA	2'-7'dichlorodihydrofluorescein diacetate
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
KH_2PO_4	monopotassium phosphate
K_2HPO_4	dipotassium phosphate
$KMnO_4$	potassium permanganate
RFU	relative fluorescence unit
ROS	reactive oxygen species

9.2 PRINCIPLE OF THE ASSAY

9.2.1 Reactive Oxygen Species (ROS)

The level of ROS, including hydrogen peroxide (H_2O_2), can be measured in cells using a fluorogenic dye such as 2'-7'dichlorodihydrofluorescein diacetate (H_2DCFDA) (Katerji et al., 2019). The H_2DCFDA diffuses into the cells and is hydrolysed to 2'-7'dichlorodihydrofluorescein (DCFH) where it remains trapped within the cells. If H_2O_2 is produced by the cells as a result of increased oxidative stress, the DCFH will react with the H_2O_2 and generate fluorescent 2'-7'dichlorofluorescein (DCF) (Katerji et al., 2019) which can be measured at excitation and emission wavelengths of 480 nm and 535 nm using a fluorescence plate reader. The relative fluorescence unit (RFU) of ROS produced by the cells can then be calculated as the difference between experimental and blank measurements. The negative control will consist of untreated cells stimulated with 0.03% H_2O_2, one hour before the assay.

9.2.2 Catalase (CAT)

Catalase is the enzyme responsible for the degradation of H_2O_2 to water (H_2O) and oxygen (O_2) (Katerji et al., 2019) and therefore CAT determination is based on the principle of measuring the enzyme-catalysed decomposition of H_2O_2 (Cohen et al., 1970). H_2O_2 is added to the cells and after incubation, H_2SO_4 is used to stop the reaction. The amount of H_2O_2 remaining after catalase action in the cells will be determined by titration with potassium permanganate ($KMnO_4$), a very strong oxidizing reagent. The potassium permanganate which did not react (residual) with the H_2O_2 is measured spectrophotometrically at a

wavelength of 490 nm and the amount remaining is inversely proportional to the activity of the catalase enzyme. This is followed by the addition of excess potassium permanganate ($KMnO_4$) to react with H_2O_2. Catalase activity is determined by measuring absorbance at a wavelength of 490 nm. The response is compared to the control and a change in catalase activity indicates oxidative stress.

9.2.3 Superoxide Dismutase (SOD)

Superoxide dismutase catalyses the partitioning of superoxide radicals (O_2^-) into ordinary O_2 and H_2O_2 and pyrogallol is an organic compound that auto-oxidises rapidly. Superoxide dismutase activity can therefore be quantified in a kinetic reaction using pyrogallol auto-oxidation. The pyrogallol assay for SOD activity investigates the ability of SOD to inhibit the auto-oxidation of pyrogallol into a yellow solution (Katerji et al., 2019), which can be determined by measuring the optical density at 560 nm. A yellow-brown colour indicates autoxidation and thus no SOD activity, while a white colour indicates no autoxidation and SOD activity. The response is compared to the control and a change in catalase activity indicates oxidative stress.

9.3 REQUIREMENTS

9.3.1 Acquisition of the cell line

The methods listed in this section of the toolbox were set up to determine oxidative stress biomarkers by using adherent tissue cultures. Cell lines should be grown in sterile conditions and under each specific cell line's environmental requirements. Commercially available adherent cell lines are available from American Type Culture Collection (ATCC) LGC Standards South Africa (ATCC) or Sigma Aldrich.

9.3.2 Permits

An import permit will be required depending on the nature of the cell line to be used.

9.3.3 Staff training (technical skills)

- Training in tissue culture techniques.
- Good laboratory practice.

9.3.4 Laboratory (test environment)

- Dedicated to tissue culturing.

9.4 Software

- Microsoft Excel® is used to analyse the raw data.
- Statistical software capable of performing univariate statistics.

9.4.1 Apparatus, consumables/materials and reagents

Tables 9-1, 9-2 and 9-3 provide lists of the apparatus, consumables/materials and reagents required for oxidative stress determination.

Table 9-1: Lists of the apparatus required for oxidative stress determination

Name	Cat no
Balance	maximum 200 g, d=0.1 mg
Centrifuge	With swing-out rotor
Microplate reader	Read absorbance at 560 nm
Multi-channel pipette	12 channel, 10-100 µL and 2-200 uL volume range
pH meter	
Research pipettes	1-10 µL; 0-200 µL variable volume
Pipette controller for serological pipettes	
Refrigerator	4°C
Repeat pipette	
Spatula	
Incubator	Able to maintain humidified air and supplemented with 5% CO_2 (e.g. Thermo Electron Corporation Forma Series II)

Table 9-2: Lists of the consumables/materials required for oxidative stress determination

Name	Cat no	Supplier
Cells and all their requirements		
24-well tissue culture treated non-pyrogenic, transparent, flat bottom with lid, sterile	3527 Corning® Costar®	The Scientific Group
High-clarity polypropylene conical tube 15 mL	352096, Falcon	Lasec
High-clarity polypropylene conical tube 50 mL	352070, Falcon	Lasec
Microcentrifuge tubes (2 mL)	CLS430917	Sigma-Aldrich
Nitrile gloves (powder free, short sleeved)		
Permanent marker pens		
Pipette tips, sterile, 10 µL	RC-L10	Microsep
Pipette tips, sterile, 200 µL	301-02-121	The Scientific Group
Pipette tips, sterile, 1000 µL	301-01-401	The Scientific Group

Table 9-3: Lists of the reagents required for oxidative stress determination

Name	Cat no	Supplier
Bovine serum albumin	A9418-10G	Sigma-Aldrich
Bradford's reagent	B6916-500ML	Sigma-Aldrich
DPBS	D5652-10L	Sigma-Aldrich
DTPA	D6518-10G	Sigma-Aldrich
H_2O_2	H1009-100ML	Sigma-Aldrich
H_2DCFDA	D6883-50MG	Sigma-Aldrich
H_2SO_4	258105-2.5L	Sigma-Aldrich
K_2HPO_4	1370101000	Sigma-Aldrich
KH_2PO_4	1370391000	Sigma-Aldrich
$KMnO_4$	223468-25G	Sigma-Aldrich
Pyrogallol	16040-100G-R	Sigma-Aldrich
Tris buffer		
Trypsin	S181G-500	Biowest

9.5 METHOD – REACTIVE OXYGEN SPECIES (ROS) DETERMINATION

9.5.1 Assay procedure

This assay should ideally be conducted in a black 96 well plate, but alternatively, the assay can be done in sterile six, 12- or 24-well plates and transferred to a black 96 well plate (non-sterile) for the last step.

1. One millilitre of cells is seeded into 24 well plates (cells are seeded according to previously established density and growth requirements. These parameters are cell line-specific).
2. After attachment (±24 h), cells are exposed to the test compounds for 24 h in triplicate.
3. After the 24 h exposure time, the media of the three untreated cells (negative control) are removed and these cells are stimulated with 1 mL of 0.03% hydrogen peroxide (H_2O_2) for 45 min.

Assay performed in the dark from here

4. Remove all media and wash the plate three times with 500 µL DPBS.
5. Add 200 µL of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (10 µM prepared in stock medium) to all wells and incubate for 30 min at 37°C in the dark.
6. Remove H_2DCFDA from all the wells and wash cells three times with 500 µ DPBS.
   (If assay was done in a black 96 well plate, add 200 µ DPBS and skip to step 16).
7. Trypsinise cells (150 trypsin) for three minutes.
   (The time is dependent on the cell line)
8. Stop trypsin activity with 500 µL DPBS.
9. Transfer cell suspension to a 2 mL centrifuge tube
   (ensure that tubes are correctly labelled ROS#1-?)

10. Centrifuge tubes at 1 000 x g-force for four minutes.
11. Discard the supernatant.
12. Re-suspend the pellet with 800 µL DPBS.
13. Add 200 µL per well of the cell suspension to each of the black 96 well plates.
14. Measure fluorescence with excitation at 480 nm and emission at 535 nm.

The relative fluorescence units (RFU) of the intracellular ROS are calculated as the difference between the treated and untreated cells (blank control) measurements (Yao et al. 2015 and Wu et al., 2011).

9.5.2 Calculation of results

1. The raw data can be worked in Microsoft Excel®.
2. For each triplicate sample, untreated vehicle control cells and ROS positive control cells (H_2O_2 stimulated), calculate the mean, standard deviation, and coefficient of variation (%CV) of the relative fluorescence units (RFUs). This can be done on an Excel® spreadsheet. The CV indicates the quality of the data points: the smaller the variation between them the more valid are they. The %CV should be no more than 20%. If this is the case, one data point may be removed to improve the CV. This same point must be kept out of all future calculations.
3. Plot the mean RFU values on a bar graph (mean RFU on the y-axis and sample names on the x-axis).
4. Statistically significant differences between the sample and the untreated control cells should be determined using the Mann-Whitney U test ($p < 0.05$) because of the limited number of data points. This will indicate whether any of the results are statistically significant compared to the untreated cells that represent the basal ROS produced by cells naturally.

9.6 CATALASE (CAT) DETERMINATION

9.6.1 Assay procedure

1. Seed cells into 24 well plates.
   (Cells are seeded according to previously established density and growth requirements. These parameters are cell line-specific.).
2. After attachment (±24 h), cells are exposed to the test compounds for 24 h in triplicate.
3. Remove media from the whole plate and wash cells three times with 500 µL DPBS.
4. Trypsinise cells (150 µL trypsin) for three minutes.
5. Stop trypsin activity with 500 µL DPBS.
6. Transfer cell suspension to a 2 mL centrifuge tube
   (Ensure that tubes are correctly labelled CAT#1-?)
7. Centrifuge tubes at 1 000 g for four minutes.
8. Discard the supernatant
9. Re-suspend the cell pellet in 270 µL ice-cold phosphate buffer
   (Phosphate buffer recipe: 0.09 M K_2HPO_4 (base) adjusted to pH 7.4 with 0.09 M KH_2PO_4 (acid)).

10. Lyse cells through sonication at medium intensity for 30 seconds
11. Centrifuge at 10 000 g for 10 min at 4°C.

This supernatant is used for catalase (CAT), SOD and protein content determination.
Supernatant can be frozen for up to a week

12. Add 93 µL H_2O_2 (6 mM) to a 96 well plate
13. Add 10 µL of supernatant to the H_2O_2 in a 96 well plate and incubate for three minutes.
14. Reaction is stopped by adding 19 µL of H_2SO_4 (6 N).
15. Add 130 µL MnO_4 (1.9 mM) to each well and determine the absorbance at 490 nm within 30 to 60 seconds.

All tests were done in triplicate and results were reported as a mean of three readings and expressed as µmol H_2O_2/min/mg protein (Cohen et al., 1970).

9.6.2 Calculation of results

1. The raw data can be worked in Microsoft Excel®.
2. For each triplicate sample and untreated vehicle control cells, calculate the mean, standard deviation, and coefficient of variation (%CV) of the optical density values obtained. This can be done on an Excel® spreadsheet. The CV indicates the quality of the data points: the smaller the variation between them the more valid are they. The %CV should be no more than 20%. If this is the case, one data point may be removed to improve the CV. This same point must be kept out of all future calculations.
3. Subtract the mean OD of the blank (deionised water only) from each of the individual samples' readings.
4. A catalase standard was included and contained: $KMnO_4$ (1.9 mM), phosphate buffer and H_2SO_4 (6 N).
5. Determine the S0 (substrate concentration at time zero) by subtracting the mean absorbance of the deionised water blanks from the spectrophotometric CAT standard).
6. Determine the S3 (substrate concentration at time 3 minutes) by subtracting the mean absorbance of the samples' OD (calculated in nr. 3) from the spectrophotometric CAT standard which is S0.
7. Determine the first-order reaction rate constant, k (enzyme-catalysed decomposition of H_2O_2 by CAT) by using the following equation for each sample:

$$k = log(S0/S3)\ x\ 2.3t$$

where t = the time interval over which the reaction is measured (3 minutes); S_0 = the substrate concentration at time zero (was calculated by subtracting the mean absorbance of the deionised water blanks from the spectrophotometric CAT standard); S_3 = the substrate concentration at time 3 minutes (the mean absorbance of the reaction samples was subtracted from the spectrophotometric CAT standard); 2.3 = the first-order kinetic conversion factor.

8. Express all the responses of samples (treated and untreated) in terms of their corresponding protein concentration.
9 The values were converted to µmol by multiplying with 1 000 and consequently expressed as µmol H_2O_2/min/mg protein (Cohen *et al*., 1970; Mennillo *et al*., 2019).

10. Plot the concentration means on a bar graph (µmol H_2O_2/min/mg protein on the y-axis and sample names on the x-axis).
11. Statistically significant differences between the sample and the untreated control cells should be determined using the Mann-Whitney U test ($p < 0.05$) because of the limited number of data points. This will indicate whether any of the results are statistically significant compared to the untreated cells.

9.7 SUPEROXIDE DISMUTASE (SOD) DETERMINATION

9.7.1 Assay procedure

1. Seed cells into 24 well plates.
   (Cells are seeded according to previously established density and growth requirements. These parameters are cell line-specific.).
2. After attachment (±24 h), cells are exposed to the test compounds for 24 h in triplicate.
3. Remove media from the whole plate and wash cells three times with 500 µL DPBS.
4. Trypsinise cells (150 µL trypsin) for three minutes.
5. Stop trypsin activity with 500 µL DPBS.
6. Transfer cell suspension to a 2 mL centrifuge tube
   (Ensure that tubes are correctly labelled SOD#1-?)
7. Centrifuge tubes at 1 000 g for four minutes.
8. Discard the supernatant
9. Re-suspend the cell pellet in 270 µL ice-cold phosphate buffer
   (Phosphate buffer recipe: 0.09 M K_2HPO_4 (base) adjusted to pH 7.4 with 0.09 M KH_2PO_4 (acid)).
10. Lyse cells through sonication at medium intensity for 30 seconds
11. Centrifuge at 10 000 g for 10 min at 4°C.

This supernatant is used for catalase (CAT), SOD and protein content determination.
Supernatant can be frozen for up to a week

12. Use supernatant as prepared in CAT protocol steps 1-11.
13. A 4 µL cell lysate sample (supernatant) and Tris-buffer-blank are added in triplicate to the wells in a 96 well tissue culture plate in triplicate.
14. Add 245 µL of 1:49 (v/v) diethylene triamine penta-acetic acid (DTPA)/Tris buffer to each well.
    (1 nM diethylenetriaminepentaacetic acid (DTPA) and 50 mM Tris buffer (pH 7.5))
15. The reaction is initiated by adding 4 µL pyrogallol (24 nM in 10 mM HCl) to each well
16. Absorbance is measured at 560 nm every 30 seconds for five minutes (10 readings in total).

The SOD activity is expressed as ng SOD/mg protein (Del Maestro & McDonald, 2018).

9.7.2 Calculation of results

1. The raw data can be worked in Microsoft Excel®.

2. For each triplicate sample and untreated vehicle control cells, calculate the mean, standard deviation, and coefficient of variation (%CV) of the optical density values obtained. This can be done on an Excel® spreadsheet. The CV indicates the quality of the data points: the smaller the variation between them the more valid are they. The %CV should be no more than 20%. If this is the case, one data point may be removed to improve the CV. This same point must be kept out of all future calculations.
3 Determine the reaction gradient of the samples and Tris experimental blanks.
4. Determine the reaction tempo by calculating the change in absorbance per minute (mean optical density for each sample/5 minutes).
5. One unit of SOD is defined as the amount of enzyme necessary to inhibit 50% of the reaction (pyrogallol autoxidation). Determine % inhibition of pyrogallol autoxidation by multiplying by 100.
6. Convert the amount of SOD in units to a SOD concentration in ng/mL (one SOD unit equals 125 ng/mL SOD). The fraction responsible for SOD activity was determined by multiplying with the dilution factor (sample:reagents).
7. Obtained values were normalised against protein content in each cell lysate and expressed as SOD activity in ng/mg protein (Marklund & Marklund 1974; Del Maestro & McDonald, 1989).
8. Plot the concentration means on a bar graph (SOD activity in ng/mg protein on the y-axis and sample names on the x-axis).
9. Statistically significant differences between the sample and the untreated control cells should be determined using the Mann-Whitney U test ($p < 0.05$) because of the limited number of data points. This will indicate whether any of the results are statistically significant compared to the untreated cells.

9.8 BRADFORD'S METHOD FOR PROTEIN CONTENT MEASUREMENT

The protein contents of each sample of each batch were determined using the Bradford (1976) method. This method is based on the binding of Coomassie brilliant blue (active ingredient in Bradford reagent) dye to proteins which can then be quantified using a spectrophotometer. The protein content is determined by the use of a protein standard (bovine serum albumin) calibration curve.

9.8.1 Assay procedure

1. Use supernatant as prepared in CAT (SECTION 9.5.1) and SOD (SECTION 9.6.1) protocol steps 1-11
2. Add 5 µL cell lysate (supernatant) in triplicate to the wells in a 96 well tissue culture plate.
3 Prepare protein standard (bovine serum albumin) calibration curve ranging from 0-2 000 µg/ml.
4. Also add 5 µL of each BSA concentration (calibration curve) as the positive control in triplicate to the wells in a 96 well tissue culture.
5. Measure the absorbance at 590 nm.

9.8.2 Calculation of results

1. The raw data can be worked in Microsoft Excel®.

2. Calculate the mean, standard deviation, and coefficient of variation for the triplicate values of samples and standards.
3. Plot the means of the optical density obtained by the protein standard (bovine serum albumin) on y-axis and the corresponding concentrations used for the protein standard (0-2 000 µg/ml) on the x-axis.
4. Determine the slope, and intercept for the straight-line section (y = mx + c) of the standard.
5. Determine total protein concentrations in the cells exposed to samples by using y = mx + c.

9.9 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

9.9.1 Advantages

- The assays are relatively rapid and can be conducted in 24 well plates and consistent results are produced.

9.9.2 Limitations

- Requires a dedicated cell culture laboratory.
- Some cell lines, e.g. liver tissue cells, rapidly produce catalase in response to oxidative stress. The level of ROS is therefore too low (already converted) to be compared to control cells, and does not accurately reflect the stress response due to ROS.

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9.10 USEFUL CONTACTS

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10 AMES TEST

Compiled by: L Swart and H Pearson

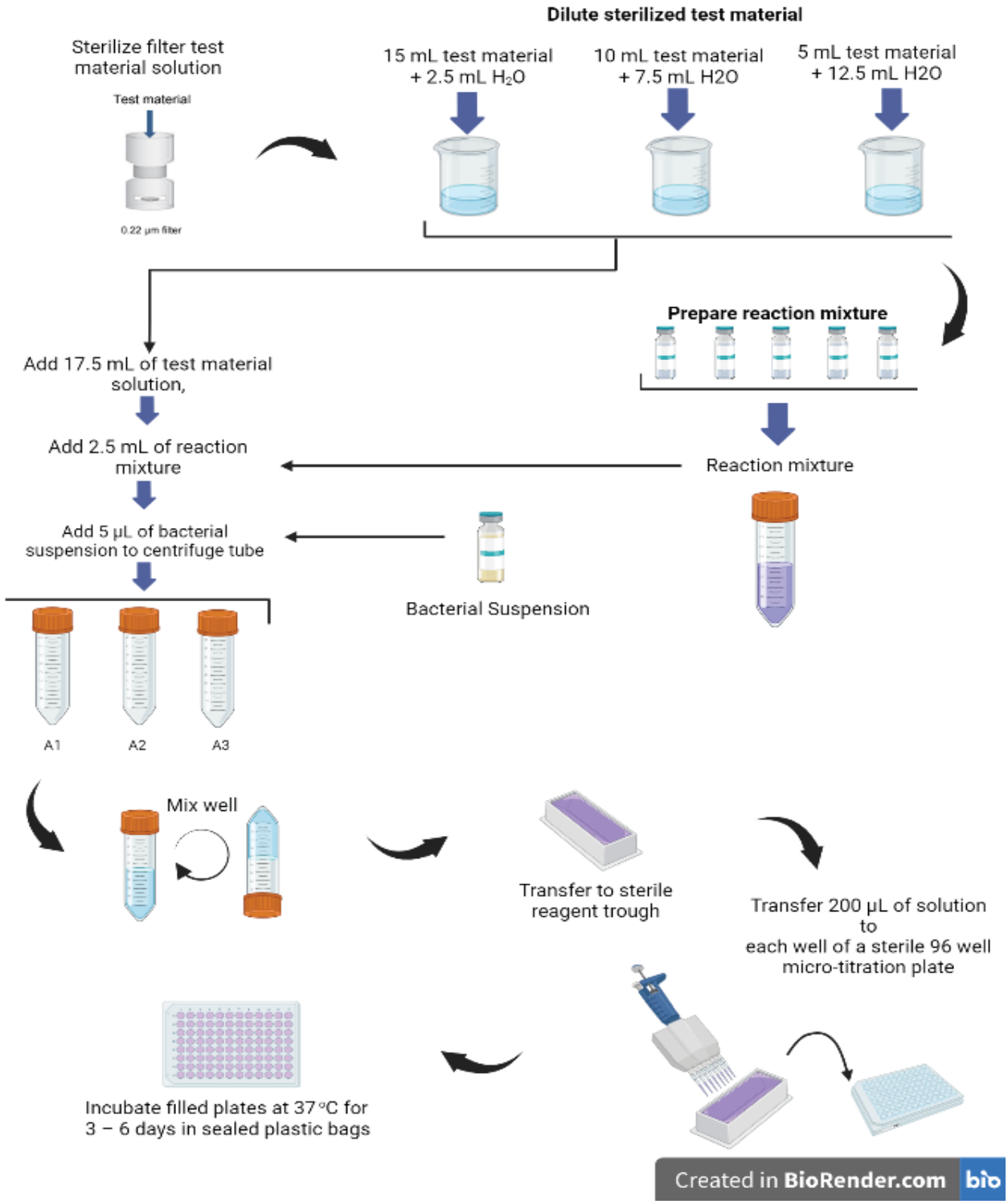

Figure 10-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the AMES test

10.1 ACRONYMS & ABBREVIATIONS

AR	Analytical Grade
$C_{14}H_{11}N$	2-aminoanthracene (2-AA)
$C_{16}H_{18}N_3NaO_4S$	Ampicillin sodium salt
$C_{10}H_{16}N_2O_3S$	D-Biotin
$C_{21}H_{16}Br_2O_5S$	Bromocresol purple, sodium salt
ºC	Degree(s) Celsius
$C_6H_8O_7$ H_2O	Citric acid monohydrate
d	Day(s)
C_2H_6SO	Dimethyl sulfoxide (DMSO)
$C_6H_{12}O_6$	D-Glucose, anhydrous
G-6-P-Na2, $C_6H_{11}Na_2O_9P{\cdot}2H_2O$	D-Glucose-6-phosphate disodium salt hydrate
g	Gram(s)
HCl	Hydrochloric acid
h	Hour(s)
KCl	Potassium chloride
L	Litre(s)
$MgCl_2$ $6H_2O$	Magnesium chloride hexahydrate
$MgSO_4$ $7H_2O$	Magnesium sulphate heptahydrate
mg	Milligram(s)
mg/L	Milligram(s) per Litre
mL	Millilitre(s)
µL	Microliter(s)
µm	Micrometre(s)
µg/L	Microgram(s) per litre
µS /cm	Micro-siemens per centimetre
mol/L	Mol per Litre(s)
nm	Nanometre(s)
±	Plus, or minus
%	Percentage
KCl	Potassium chloride
K_2HPO_4	Di-potassium hydrogen phosphate
RA	Reagent Grade
$NaNH_4HPO_4$ $4H_2O$	Sodium ammonium hydrogen phosphate tetrahydrate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate, anhydrous
Na_2HPO_4	Di-sodium hydrogen phosphate, anhydrous
NaOH	Sodium hydroxide solution
$C_{21}H_{27}N_7NaO_{17}P_3$	β-Nicotinamide adenine dinucleotide phosphate sodium salt (NADP)
$C_8H_6N_4O_5$	Nitrofurantoin (NF)
$C_6H_7N_3O_2$	4-Nitro-o-henylenediamine (4-NOPD)
S9-fraction	Liver homogenate; induced by phenobarbital/β-naphtoflavone
$C_6H_9N_3O_2$	L-Histidine

10.2 PRINCIPLE OF THE ASSAY

This International Standard (ISO/DIS 11350, 2011) specifies a method for the determination of the genotoxic potential of water and wastewater using the bacterial strains *Salmonella typhimurium* TA98 and TA100 in a fluctuation assay. This combination of strains can measure genotoxicity of chemicals that induce point mutations (base pair substitutions and frameshift mutations) in genes coding for enzymes that are involved in the biosynthesis of the amino acid histidine.

Note: For measuring genotoxicity of samples containing DNA crosslinking agents ISO 13829 (2000) should be applied.

The bacteria are exposed under defined conditions to various concentrations of the test sample and incubated for 100 minutes at 37 ± 1°C in 24-well plates. Due to this exposure, genotoxic agents enclosed in the test sample may be able to induce mutations in one or both marker genes of the bacterial strains used (hisG46 for TA100 and hisD3052 for TA98) in correlation to the applied concentrations. Induction of mutations will cause a concentration-related increase in the number of mutant colonies.

After exposure of the bacteria, reversion indicator medium, containing the pH indicator dye bromocresol purple, is added to the wells. Subsequently, the batches are distributed to 384-well plates (48 wells for each parallel) and incubated for 48 hours to 72 hours.

Mutagenic activity of the test sample is determined by counting the number of purple to yellow shifted wells (per 48 wells of each parallel), treated with the undiluted or the diluted test sample, compared to the negative control.

The lowest dilution (1: N) of the test sample which induces no mutagenic effect under all experimental conditions (if any mutagenic effect is induced by the test sample) is the criterion for evaluating the mutagenic potential. Sample dilutions above this (1 : A, A < N) shall induce a mutagenic effect according to the criteria of this standard in at least one strain under at least one activation condition (with or without addition of S9-mix). The respective D_{min}-value is N. If no mutagenic effect is observed under all experimental conditions, this dilution is 1 : 1 and the respective D_{min}-value is 1.

10.3 REQUIREMENTS

10.3.1 Acquisition of test organisms

The procedure described is based on the, commercially obtained, MutaChromoPlate test kit, used to measure for the presence of mutagenic compounds.

10.3.2 STAFF TRAINING (TECHNICAL SKILLS)

To perform the test, the analyst should be proven competent in

- Good Laboratory Practice (GLP)
- Trained in an aquatic ecotoxicology laboratory
- Software packages knowledge (e.g. EXCEL)

10.3.3 LABORATORY (TEST ENVIRONMENT)

- The testing facility must be free of vapours, odours and dust that may be toxic to the test organisms
- Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage
- Do not use equipment made of copper
- Temperature control equipment should be adequate to maintain the recommended environmental conditions as stated in the standard during storing of samples, testing and culture maintenance

10.3.4 SOFTWARE

- EXCEL spreadsheet

10.3.5 APPARATUS

The following apparatus will be required to perform the test:

- Temperature- and time-controlled incubator, 37 ± 1°C
- pH meter
- Analytical balance
- Steam steriliser
- Dry steriliser
- Magnetic stirrer
- Rotary mixer
- Freezer, at least ≤ -18°C and at ≤ -70°C
- Pipettes, 0,1 mL, 0,5 mL, 1 mL, 2 mL, 5 mL, 10 mL
- 25 mL, glassware or plastics
- Storage bottles, 250 mL and 1 000 mL
- Graduated cylinders, 100 mL and 200 mL
- Graduated flasks, 20 mL, 200 mL and 500 mL
- Erlenmeyer flasks, 50 mL, 100 mL and 250 mL
- Inoculating loops
- 8-channel multi-stepper pipette (repeater pipette)
- 8-channel pipettes, 5 µL to 50 µL and 50 µL to 300 µL
- Spectrophotometer

- Microplate photometer for 24-well plates and optionally for 384-well plates, filters: 420 nm ± 15 nm and 595 nm ± 10 nm
- Petri dishes with venting ribs, diameter approximately 94 mm, height approximately 16 mm
- Cryogenic vials, sterile, 1 mL, 10 mL

10.3.6 CONSUMABLES/MATERIALS

Table 10-1: Lists of the consumables/materials

Name	Cat no	Supplier
MutaChromoPlate kit	B5051S9-1SP	Environmental Bio-detection Products Inc.
Storage bottles, 250 mL and 1 000 mL	-	General supplier
Sterile filters, 0,2 µm and 0,45 µm	-	General supplier
Transparent sterile polystyrene 24-well and 384-well plates with flat bottom and lid	-	General supplier
Pipette tips	-	General supplier
Petri dishes	-	General supplier
Cryogenic vials (1 mL & 10 mL)	-	General supplier

10.3.7 Reagents

Use chemicals of recognised analytical grade (AR grade), unless otherwise specified.

Table 10-2: Lists of the reagents to be used

Name	Cat no	Supplier
•Water, grade 1 (ISO 3696, 1987)	-	General supplier
≤ 5 µS/cm sterile filtration (0,2 µm) or autoclaving	-	General supplier
•2-aminoanthracene (2-AA)	CAS: 613-13-8	General supplier
•Ampicillin sodium salt	CAS: 69-52-3	General supplier
D-Biotin	CAS: 58-85-5	General supplier
•Bromocresol purple, sodium salt	CAS: 62625-30-3	General supplier
•Citric acid monohydrate	CAS: 5949-29-1	General supplier
•Dimethyl sulfoxide	CAS: 67-68-5	General supplier
•D-Glucose, anhydrous	CAS: 50-99-7	General supplier
•D-Glucose-6-phosphate disodium salt hydrate,	CAS: 3671-99-6	General supplier
•Hydrochloric acid solution	-	
•Magnesium chloride hexahydrate	CAS: 7791-18-6	General supplier
•Magnesium sulphate heptahydrate	CAS: 10034-99-8	General supplier
•Potassium chloride	CAS: 7447-40-7	General supplier
•Di-potassium hydrogen phosphate	CAS: 7758-11-4	General supplier
•Sodium ammonium hydrogen phosphate tetrahydrate	- CAS: 7583-13-3	General supplier
•Sodium chloride	CAS: 7647-14-5	General supplier
•Sodium dihydrogen phosphate, anhydrous	CAS: 7558-80-7	General supplier
•Di-sodium hydrogen phosphate, anhydrous	CAS: 7558-79-4	General supplier
•Sodium hydroxide solution	-	
•β-Nicotinamide adenine dinucleotide phosphate sodium salt	- CAS: 698999-85-8	General supplier
•Nitrofurantoin	CAS: 67-20-9	General supplier
•4-Nitro-o-henylenediamine	CAS: 99-56-9	General supplier
•Nutrient broth powder	-	

Name	Cat no	Supplier
•S9-fraction (liver homogenate)	-	
•L-Histidine	CAS: 71-00-1	General supplier
Phosphate buffer	-	General supplier

- As far as possible, use" reagent grade" chemicals. If chemicals with different amounts of water are used, calculate the needed amounts accordingly
- If autoclaving is necessary always autoclave for 20 minutes at 121°C ± 2°C. Cover vessels loosely (e.g. with aluminium foil). Never seal air-tight
- Water, grade 1, as defined in ISO 3696 (1987), respectively water with a conductivity of ≤ 5 µS/cm. If sterile water is needed, sterilise by sterile filtration (0,2 µm) or autoclaving

10.4 METHOD

10.4.1 sampling

10.4.2 Sample collection

- Take samples as specified in ISO 5667-1 (2020), ISO 5667-3 (2018), ISO 5667-14 (2014), and ISO 5667-16 (2017)
- Test the samples immediately after sampling. If this is not possible, keep water samples at 0°C to 5°C (< 48 hours) or below -18°C (up to 2 months)
- For multiple testing divide larger samples in advance into appropriate portions, since thawed samples can only be used on the same day
- Samples containing solids should be centrifuged to separate them. In this case, only the supernatant is processed further
- Sterilise all samples using sterile filters (0,45 µm). Homogenise test samples by thoroughly shaking before use
- Adjust the sample to a pH of 7,2 ± 0,2 using either HCl or NaOH solution. Select the acid or alkali concentrations such that the added volumes are as small as possible. Avoid over-titration. Take into account the change of the sample's pH and resulting effects (ISO 5667-16, 2017)

10.4.3 Sample preparation

- Filter sterilize the sample to be tested using a 0.22 µm membrane filter.
- Prepared sample dilutions, in sterile distilled water, in a 50 mL sterile tubes supplied in kits.
- Volume of each tube should be 17.5 mL (combination of samples and sterile distilled water).

10.5 TEST PREPARATION

10.5.1 Preparation of solutions

- **Sodium dihydrogen phosphate buffer**, c(NaH_2PO_4) = 0,2 mol/L. Dissolve 14,39 g NaH_2PO_4 (alternatively 16,55 g $NaH_2PO_4 \cdot H_2O$) in 600 mL of water
- **Disodium hydrogen phosphate buffer**, c(Na_2HPO_4) = 0,2 mol/L. Dissolve 28,39 g Na_2HPO_4 in 1 000 mL of water. Add solution together until a pH value of 7,4 is reached and autoclave. Store at room temperature in the dark. The solution is stable for at least one year
- **D-Biotin solution**. Dissolve 12,2 mg D-Biotin in 100 mL of water by boiling up. After cooling sterilise by filtration (0,2 µm filter). Store 10 mL aliquots at ≤ -18°C in sterile cryogenic vials. Aqueous solutions stored as frozen aliquots are stable for at least one year
- **L-Histidine solution**. Dissolve 50 mg of L-Histidine in 50 mL of water and sterilise by filtration (0,2 µm filter). Store 1,5 mL aliquots at ≤ -18°C in sterile cryogenic vials. Aqueous solutions stored as frozen aliquots are stable for at least one year
- **Glucose-6-phosphate solution**. Dissolve 0,68 g of D-Glucose-6-phosphate in 10 mL of water and sterilise by filtration (0,2 µm). Store aliquots (e.g. 200 µL) at ≤ -18°C in sterile cryogenic vials. Aqueous solutions stored as frozen aliquots are stable for at least one year
- **NADP solution**, c(NADP) = 0,04 mol/L. Dissolve 0,31 g of NADP in 10 mL of water and sterilise by filtration (0,2 µm). Store aliquots (e.g. 700 µL) at ≤ -18°C in sterile cryogenic vials. Aqueous solutions stored as frozen aliquots are stable for at least one year

Note: NADP is provided with different amount of crystal water. The actual molecular weight is specified in the product data sheet. Calculate amount of NADP needed according to the given molecular weight.

- **Potassium chloride solution**. Dissolve 74,56 g of KCl in 1 000 mL of water and autoclave. Store at room temperature. The solution is stable for at least one year
- **$MgCl_2 \cdot 6\ H_2O$ solution**. Dissolve 50,83 g of $MgCl_2 \cdot 6\ H_2O$ in 1 000 mL of water and autoclave the solution. Store at room temperature. The solution is stable for at least one year
- **Bromocresol purple solution**. Dissolve 51 mg of bromocresol purple sodium salt in 30 mL of water. Prepare this solution freshly before addition to the reversion indicator medium
- **Ampicillin solution**. Dissolve 500 mg of ampicillin in 10 mL of water and sterilise by filtration (0,2 µm filter). Store 500 µL aliquots at ≤ -18°C in sterile cryogenic vials. The solution is stable for at least six months
- **Growth medium**. Dissolve 4,7 g of nutrient broth powder and 0,31 g of sodium chloride in 200 mL of water. Adjust pH to 7,5 ± 0,1. Add water to 250 mL and autoclave the solution. Per 1000 mL growth medium the following final concentrations shall result:
  - 7,5 g meat extract
  - 7,5 g peptone
  - 5,0 g sodium chloride

Note Use nutrient broth powder containing 40% meat extract, 40% peptone, and 20% sodium chloride Solutions stored under sterile conditions as frozen aliquots are stable for at least one year.

- **Exposure medium**. Dissolve consecutively the following ingredients in 900 mL water:
  - 0,2 g magnesium sulphate heptahydrate
  - 2,0 g citric acid
  - 10,0 g di-potassium hydrogen phosphate
  - 3,5 g sodium-ammonium hydrogen phosphate tetrahydrate
  - 4,0 g D-glucose

Add water to 1 000 mL, adjust the pH to 7,0 ± 0,2, if necessary, and sterilise by filtration (0,2 µm filter). Store medium at 2°C to 8°C.

Add per 100 mL 0,6 mL of D-biotin solution and 0,1 mL of L-histidine solution under sterile conditions. Prepare only the amount of medium that is used in the next two weeks. Store medium at 2°C to 8°C.

- **Exposure medium concentrate**. Dissolve consecutively the following ingredients in 70 mL water:
  - 0,2 g magnesium sulphate heptahydrate
  - 2,0 g citric acid
  - 10,0 g di-potassium hydrogen phosphate
  - 3,5 g sodium-ammonium hydrogen phosphate tetrahydrate
  - 4,0 g D-glucose

Add water to 93 mL, adjust the pH, if necessary, and sterilise by filtration (0,2 µm filter). Store medium concentrate at 2°C to 8°C.

Add 6 mL of D-biotin solution and 1 mL of L-histidine solution under sterile conditions. Prepare only the amount of medium that is used in the next two weeks. Store medium concentrate at 2°C to 8°C.

- **Reversion indicator medium**

Solution I: Dissolve the following ingredients in 950 mL water in the given order:

- 0,4 g magnesium sulphate heptahydrate
- 4,0 g citric acid
- 20,0 g di-potassium hydrogen phosphate
- 7,0 g sodium ammonium hydrogen phosphate tetrahydrate

Add water to 1 000 mL and add 30,0 mL of bromocresol purple solution. Transfer the solution one half each into two 1 000 mL flasks and autoclave.

Solution II: Dissolve 8,0 g of D-glucose in 800 mL of water. Transfer the solution one half each into two 1000 mL flasks and autoclave.

After cooling to ambient temperature, mix 515 mL of Solution I with 400 mL of Solution II under sterile conditions. Add 20 mL of D-biotin solution under sterile conditions to each flask.

Store the medium at room temperature in the dark. The medium is stable for at least one month.

- **Control solutions**

  Negative controls: For preparation of the negative controls, always use the same solvent as for the samples to be tested. This is usually water when testing water samples and DMSO when testing chemicals.

  Positive controls: In general, dissolve 10 mg of each positive control substance in 10 mL of DMSO. Prepare 50 µL aliquots as stock solutions in sterile cryogenic vials and store them at ≤ -18°C. Under these conditions stock solutions are stable for at least one year. On the day of the test, unfreeze one aliquot.

10.5.2 Strains

Use mutant strains of *Salmonella typhimurium* LT2, which enable detection of point mutations, to determine the mutagenic potential of a test sample. Since point mutations can be subdivided into two classes (frameshift mutations and base pair substitutions), the two tester strains TA98 and TA100 are used. TA98 contains as a marker the frameshift mutation (+2 type) hisD3052, whereas TA100 bears the base pair substitution hisG46.

10.5.3 Overnight culture

- Under sterile conditions, pipette 20 mL of growth medium supplemented with 20 µL of ampicillin solution into a 100-mL Erlenmeyer flask closed permeable to air with caps or aluminium foil and mix by gentle agitation
- Add 20 µL of the respective tester strain (TA98 or TA100) immediately after thawing
- Incubate the culture at 37 ± 1°C for not more than 10 h. A clock timer may be used
- Use a shaking rate of at least 150 rpm
- From inoculation of test bacteria to the beginning of incubation at 37°C ensure that the temperature of the incubation bath remains < 23°C. If necessary, add thermal packs or ice

10.5.4 Preparation of S9-mix

Treatment for enzyme induction and preparation of the S9-fraction are described in ISO/DIS 11350 (2011) If the S9-fraction is purchased commercially, it should also be prepared according to

Prepare the S9-mix freshly on the day of testing. Mix:

- 66 µL KCl solution
- 64 µL MgCl2 ·6H2O solution
- 50 µL glucose 6-phosphate solution
- 200 µL NADP solution
- 1 000 µL phosphate buffer

- 20 µL water (sterile)
- 600 µL S9-fraction

This mixture is sufficient for 2 exposure plates. In the case of more than 2 plates, increase the amount of S9-mix proportionally.

Keep the S9-mix permanently on ice for not more than 1 h and use it only on the same day. Discard remaining S9-mix at the end of this day.

10.6 TEST PROCEDURE

10.6.1 Preparation of tester strains

In the test cultures a cell density of 180FAU[3] for TA98 and 45FAU for TA100 is recommended. Laboratory-specific adaptation of tester strain density may be necessary to achieve the number of negative controls revertant wells as defined in ISO/DIS 11350 (2011).

- In the test culture the tester strain inoculum is diluted 10-fold (see ISO/DIS 11350, 2011 Table 2 and Table 3). Therefore, adjust the cell density in the overnight culture inoculum to 1 800 FAU for TA98 and 450 FAU for TA100
- Calculate the required dilution factor and the volume of 1x exposure medium that must be added to the overnight culture in order to adjust the cell density according to the Equations in ISO/DIS 11350 (2011)
- For the determination of the actual cell density of the overnight culture dilute 100 µL of the overnight culture with 900 µL growth medium, otherwise the FAU will be out of range
- Measure cell densities (OD595) of this dilution of the tester strains TA98 and TA100 immediately before exposure and calculate FAU values of this dilution using a FAU calibration curve (see ISO 7027, 2016)
- Set up the calibration curve using OD_{595}. Calculate the dilution factor according to Equations below:

In the case of strain TA98:

$$d = \frac{OD_{595}[FAU]}{180}$$

Where:
d is the dilution factor.

OD_{595}[FAU] is the FAU value of the 10-fold diluted overnight culture of strain TA98 that is determined as described above.

In the case of strain TA100:

$$d = \frac{OD_{595}[FAU]}{45}$$

d is the dilution factor.

OD_{595}[FAU] is the FAU value of the 10-fold diluted overnight culture of strain TA100 that is determined as described above.

Use the calculated dilution factor to determine the required volume of 1x exposure medium that must be added to the overnight culture in order to adjust the cell density according to the Equation below.

$$Vol_{add} = (Vol_{culture} \cdot d) - Vol_{culture}$$

where

d is the dilution factor according to Equation 1 and 2 above;

Vol_{add} is the volume of 1x exposure medium to be added to the overnight culture in millilitres, mL; $Vol_{culture}$ is the volume of the undiluted overnight culture in millilitres, mL.

10.6.2 Preparation of test culture plate A without S9-mix

Prepare test cultures according to Table 2 in ISO/DIS 11350 (2011), using sterile 24-well plates (plate A). For each culture incubate at least three replicates. Perform under sterile conditions.

- If calculation of cytotoxicity is applied measure initial OD595 (t = 0 minutes) and final OD595 (t = 100 minutes) of plate A using a microplate photometer. It is recommended to use only tester strain TA98 for measurement of growth since cell density of TA100 remains low
- Incubate plate A in the dark at 37 ± 1°C for 100 minutes with shaking (150 rpm)
- Fill 2,5 mL of reversion indicator medium into each well of another 24-well plate (plate B)
- Immediately thereafter, transfer 500 µL of test culture from plate A into plate B by using a multi-stepper pipette and mix thoroughly
- Subsequently, transfer the content of one well of plate B to 48 wells of a 384-well plate (plate C) in 50 µL aliquots using a multi-stepper pipette
- Incubate the 384-well plate in the dark at 37 ± 1°C for 48 hours without shaking. Avoid evaporation-promoting conditions (e.g. ventilation)
- In case of low number of wells with revertant growth in the positive controls, extend the incubation time in 384-well plates to 72 hours
- If calculation of cytotoxicity is not applied, fill plate A with half of the volume of each ingredient
- After 100 minutes of incubation, directly add the reversion indicator medium into plate A and mix thoroughly
- Transfer the cultures to the 384-well plate and incubate

10.6.3 Preparation of test culture plate A with S9-mix

Prepare test cultures according to Table 3 in ISO/DIS 11350 (2011), using sterile 24-well microplates (plate A). For each culture incubate at least three replicates. Perform under sterile conditions.

- If calculation of cytotoxicity is applied measure initial OD595 (t = 0 minutes) and final OD595 (t = 100 minutes) of plate A using a microplate photometer. It is recommended to use only tester strain TA98 for measurement of growth since cell density of TA100 remains low
- Incubate the plates in the dark at 37 ± 1°C for 100 minutes with shaking (150 rpm)
- Fill 2,5 mL reversion indicator medium into each well of another 24-well plate (plate B)
- Immediately thereafter, transfer 500 µL of test culture from plate A into plate B by using a multi-stepper pipette and mix thoroughly
- Then, transfer the content of one well of plate B to 48 wells of a 384-well plate (plate C) in 50 µL aliquots using a multi-stepper pipette
- Incubate the 384-well plate in the dark at 37 ± 1°C for 48 hours without shaking. Avoid evaporation-promoting conditions (e.g. ventilation)
- In case of low number of wells with revertant growth in the positive controls extend the incubation time in 384-well plates to 72 hours
- If calculation of cytotoxicity is not applied, fill plate A with half of the volume of each ingredient
- After 100 minutes of incubation, directly add reversion indicator medium into plate A and mix thoroughly
- Transfer to the 384-well plate and incubate

10.6.4 Scoring of the test results

Score each 384-well plate for the number of positive (yellow) and negative (purple) wells in each 48-well area. Plate scoring may be performed manually or by using a 384-well plate photometer (420 nm ± 10 nm).

10.7 CALCULATIONS OF RESULTS

For calculation of cytotoxicity, use OD_{595} values as measured. Calculate mean OD_{595} values ± SD of sample wells and negative control wells. Calculate cytotoxicity (CT) according to Equation below.

$$CT = 100 - 100\left(\frac{St=100 - St=0}{NCt=100 - NCt=0}\right)$$

where

CT is the cytotoxicity in percent (%)

S is the OD_{595} of sample

NC is the OD_{595} of negative control

t = 0 is the initial value at t = 0 min

t=100 is the final value at t=100min

10.8 TEST VALIDITY

The test is valid if

- the mean value for negative controls is ≥ 0 and ≤ 10 wells with revertant growth per 48-well area at all testing conditions (± S9-mix, tester strains TA98 and TA100)
- the mean value for positive controls is ≥ 25 wells with revertant growth per 48-well area at all testing conditions (± S9-mix, tester strains TA98 and TA100)

If one or both of these criteria are not met, a part of the test (e.g. only one testing condition) or the entire test is invalid.

10.9 INTERPRETATION OF RESULTS

Plates are scored visually. Yellow and partial yellow wells are scored as positive. Purple wells are scored as negative.

Observe the “blank” (sterility assessment) wells. Proceed only if the “blank wells” are sterile (purple). If the “blank wells are turbid or yellow, the assay might be contaminated, or the sample is interfering with the reagents and results will be invalid.

10.10 TEST REPORT

This test report shall contain at least the following information:

- the test method used, together with a reference to this International Standard (ISO/DIS 11350, 2011)
- identity of the test sample (origin and date of sampling, pH value, conductivity)
- negative and positive control substances (chemical name, source, batch number or comparable data, if available)
- storage of sample and preparation of test sample (storage conditions (if not tested directly), adjustment of pH value, centrifugation (including g and time), filtration (including filter material and pore size) and other manipulations)
- tester strains (strain, source, date of arrival in the laboratory, storage conditions, date of stock culture preparation, and date of genotype checking (if this date deviates from stock culture preparation)), obtained OD_{595} of the overnight culture, adjusted OD_{595} of the inoculum)
- metabolizing system (preparation and origin of S9-fraction, protein content, date of preparation, storage conditions)
- testing environment (address of testing laboratory, date of test, method of counting)
- incubation time and test results (individual numbers of wells with revertant growth per treatment induction rate indication of cytotoxicity (if any), statistical evaluation, D_{min} values, other observations (e.g. precipitation, bacterial growth without colour shift)

10.11 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

10.11.1 application

According to ISO/DIS 11350 (2011), this method is applicable to:

- Freshwater
- Wastewater
- Aqueous extracts and leachates
- Eluates of sediments (freshwater)
- Porewater
- Aqueous solutions of single substances or of chemical mixtures
- Drinking water

10.11.2 advantages

The Ames test is an easy and inexpensive bacterial assay for determining the mutagenicity of any chemical. Results are robust, and the Ames test can detect suitable mutants in large populations of bacteria with high sensitivity. It does not require any special equipment or instrumentation.

10.11.3 Limitations

Bacterio-toxic effects of the test sample may lead to a reduction of viable bacteria and to a reduction of wells with revertants due to a repression of revertant growth.

This method includes sterile filtration of water and wastewater prior to the test. Due to this filtration, solid particles are separated from the test sample. Thus, genotoxic substances adsorbed on particles might not be detected.

10.12 RECOMMENDATIONS

The MutaChromoPlate reagents, bacteria and other consumables are supplied ready-to-use in a non-specialised laboratory. Assay endpoints are easy to read colorimetric changes that require no specialised training. These kits are highly sensitive to low mutagen concentrations. It is ideal for quick screening of water samples.

10.13 REFERENCES

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO (International Standards Organisation) 3696 (1987) Water for analytical laboratory use – Specification and test methods ISO/TC 47 Chemistry pp5.
ISO (International Standards Organisation) 13829 (2000) Water quality – Determination of the genotoxicity of water and wastewater using the umu-test ISO/TC 147/SC 5 Biological methods pp18.
ISO (International Standards Organisation) 21427-2 (2006) Water quality – Evaluation of the genotoxicity by measurement of the induction of micronuclei – Part 2: Mixed population method using the cell line V79 ISO/TC 147/SC 5 Biological methods pp20.
ISO/TS (International Standards Organisation /Technical Standard) 20281 (2006) Water quality – Guidance on statistical interpretation of ecotoxicity data ISO/TC 147/SC 5 Biological methods pp252.
ISO/DIS (International Standards Organisation /Draft International Standard) 11350 (2011) Water quality – Determination of the genotoxicity of water and wastewater – *Salmonella*/microsome fluctuation test (Ames fluctuation test) ISO/TC 147/SC 5 Biological methods pp37.
ISO (International Standards Organisation) 5667-14 (2014) Water quality – Sampling – Part 14: Guidance on quality assurance of environmental water sampling and handling ISO/TC 147/SC 6 Sampling (general methods) pp34.
ISO (International Standards Organisation) 7027 (2016) Water quality – Determination of turbidity – Part 1: Quantitative methods ISO/TC 147/SC 2 Physical, chemical and biochemical pp9
ISO (International Standards Organisation) 5667-16 (2017) Water quality – Sampling – Part 16: Guidance on biotesting of samples ISO/TC 147/SC 6 Sampling (general methods) pp24.
ISO (International Standards Organisation) 5667-3 (2018) Water quality – Sampling – Part 3: Preservation and handling of water samples ISO/TC 147/SC 6 Sampling (general methods) pp52.
ISO (International Standards Organisation) 5667-1 (2020) Water quality – Sampling – Part 1: Guidance on the design of sampling programmes and sampling techniques ISO/TC 147/SC 6 Sampling (general methods) pp39.
ISO (International Standards Organisation) 5667-10 (2020) Water quality – Sampling – Part 10: Guidance on sampling of wastewaters ISO/TC 147/SC 6 Sampling (general methods) pp45

10.14 USEFUL CONTACTS

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11 *ALIIVIBRIO FISCHERI* BIOLUMINESCENT TEST

Compiled by: L Swart and H Pearson

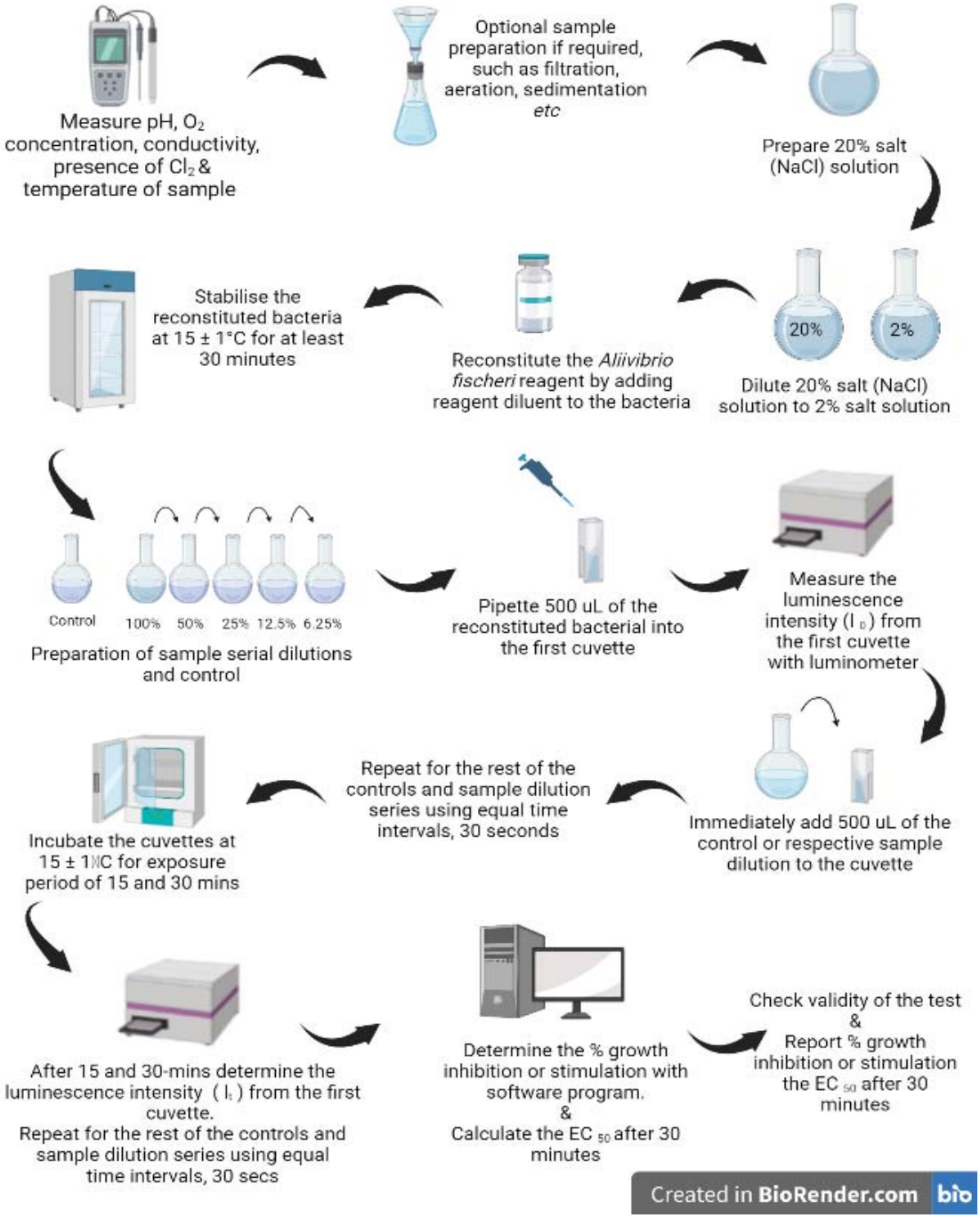

Figure 11-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the ALIIVIBRIO FISCHERI test

11.1 ACRONYMS & ABBREVIATIONS

AR	Analytical Grade
Cr	Chrome
CV	Coefficient of Variation
ºC	Degree(s) Celsius
EC_{20}	Effective concentration at 20%
EC_{50}	Effective concentration at 50%
h	Hour(s)
HCl	Hydrochloric acid
L	Litre(s)
µg/L	Microgram(s) per litre
µL	Microliter(s)
mg/L	Milligram(s) per litre
mL	Millilitre(s)
%	Percentage
±	Plus or minus
$K_2Cr_2O_7$	Potassium dichromate
NaCl	Sodium chloride
Zn	Zinc

11.2 PRINCIPLE OF THE ASSAY

The stimulation or inhibition of the luminescence is determined by combining different dilutions of the test sample with the luminescent bacteria *Aliivibrio fischeri*. The increase or decrease of light intensity is measured after a contact time of 5 (optional), 15 and 30 minutes. The stimulation or inhibitory effect of dilutions is compared to a toxin free control to give the percentage stimulation or inhibition (%). The value is plotted against the dilution factor and the resultant curve is used to calculate the EC_{50} (effective concentration causing 50% inhibition of light output) of the sample.

This procedure is based on SANS 11348-3 (2013) “Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test) – Part 3 for the method using freeze-dried bacteria”.

The procedure described is based on the BioTox™ WaterTox™ EVO commercial kit used to measure the bioluminescence inhibition of the luminescent bacteria, *Aliivibrio fischeri* (formerly *Vibrio fischeri*), with a luminometer.

11.3 REQUIREMENTS

11.3.1 ACQUISITION OF KITS

The procedure described is based on the BioTox™ WaterTox™ EVO commercial kit used to measure the bioluminescence inhibition of the luminescent bacteria, *Aliivibrio fischeri* (formerly *Vibrio fischeri*), with a luminometer.

11.3.1.1 Kit contents

Commercial BioTox™ WaterTox™ EVO kits contain the following:

- 6 vials *Aliivibrio fischeri* reagent containing lyophilised *Aliivibrio fischeri* NRRL B-11177 together with stabilisers
- 6 vails reagent diluent
- NaCl tablet

11.3.1.2 Kit storage

- The BioTox™ kit should be stored at -18 ± 1°C. The best before/expiry date is a minimum of 24 months from date of arrival to you if stored at -18 ± 1°C or lower
- Reagent diluent and salt solutions should be stored in a fridge at 4 ± 2°C, once prepared

11.3.2 STAFF TRAINING (TECHNICAL SKILLS)

To perform the test, the analyst should be deemed competent in:

- Good laboratory practice
- Trained in an aquatic ecotoxicology laboratory
- Software package knowledge (e.g. EXCEL, BioTox™)

11.3.3 LABORATORY (TEST ENVIRONMENT)

- The testing facility must be free of vapours, odours and dust that may be toxic to the test organisms
- Temperature control equipment should be adequate to maintain the recommended environmental conditions as stated in the standard
- Use temperature-controlled equipment, at 15 ± 1°C, for testing and keeping of samples during testing
- Monitor the fridge/freezer and room/chiller block temperatures daily and/or during testing

11.3.4 SOFTWARE

- BioTox™ Software (optional) for calculations
- EXCEL

11.3.5 APPARATUS

The following apparatus will be required to perform the test:

- Luminometer
- Dry/cooling block incubator, set at 15 ± 1°C
- Adjustable transfer pipettes and pipette tips (10-5000 μL)
- pH-meter (accuracy 0,1 pH units)
- Oxygen meter
- Conductivity meter
- Thermometers
- Stopwatch
- Fridge and freezer

11.3.6 CONSUMABLES/MATERIALS

Table 11-1: List of consumables/materials

Name	Cat no	Supplier
BioTox™ WaterTox™ EVO kit	1243-500	**Environmental Bio-detection Products Incorporated**
Cuvettes	-	General supplier
Pipette tips	-	General supplier
50 mL glass beakers	-	General supplier
45 mL measuring cylinders	-	General supplier
450 mL measuring cylinders	-	General supplier

11.3.7 REAGENTS

Use chemicals of recognised analytical grade (AR grade), unless otherwise specified.

Table 11-2: List of reagents

Name	Cat no	Supplier
Solid NaCl (pro analysis grade)	-	General supplier
NaOH 0,1 M and/or 0,01 M	-	General supplier
HCl 0,1 M and/or 0,01 M	-	General supplier
Nitric acid, HNO_3	-	General supplier
Acetone	-	General supplier
Reference stock solution, such as 1 g/L Potassium dichromate, 3,5-dichlorophenol or 2,2 mg/L zinc sulfate heptahydrate	-	General supplier
Water, deionized or of equivalent purity (conductivity <10 µS/cm)	-	General supplier
Chlorine testing kits	-	General supplier

11.4 SAMPLING METHOD

11.4.1 Sample collection

- Collect samples in chemically inert, clean containers as specified in ISO 5667-16 (1998)
- Fill the containers completely and seal them
- Test the samples as soon as possible after collection
- Where necessary, store samples at (5 ± 2°C) in the dark in the containers for no longer than 48 hours
- For periods up to two months, store samples at −18 ± 1°C
- Do not use chemicals to preserve the samples
- Perform the necessary pH-adjustment and salt addition immediately before testing

11.4.2 Sample preparation

Prepare the original sample for the toxicity assay as follow:

- If the pH of the sample is not between 6 and 8.5, adjust the pH to 7.0 ± 0.2 with NaOH or HCl (the volume of the sample should not increase more than 5%)
- Adjust the salinity of the sample to be equivalent to 2% NaCl solution. With freshwater or low salinity samples add either solid NaCl to a final concentration of 2% w/v. Alternatively, use 1:10 dilution, e.g. 1 mL of the 20% salt solution in 10 mL sample, to adjust the salinity
  If the oxygen concentration of the undiluted sample is less than 3 mg/L, oxygenate the sample by aeration or stirring

11.5 TEST PREPARATION

11.5.1 20% and 2% salt (NaCl) solution preparations

- Dissolve the salt (NaCl) tablet in 45 mL distilled water to obtain 20% NaCl solution
- Transfer 20 mL of the 20% salt solution into 180 mL distilled water to obtain 200 mL of a 2% salt solution to be used as sample diluent
- Adjust the pH of the salt solutions to 7.0 ± 0.2, if necessary, by adding either hydrochloric acid or sodium hydroxide

11.5.2 Preparation of test bacteria

- Reconstitute the *Aliivibrio fischeri* reagent by adding the contents of the cooled (4 ± 2°C) reagent diluent to the reagent vial containing the bacteria
- The reconstituted reagent should be equilibrated at 4 ± 2°C for at least 30 minutes
- Then stabilise the reagent at 15 ± 1°C for at least 30 minutes before pipetting it into the cuvettes arranged in the chiller
- The reconstituted reagent must be used within the same day, and it cannot be frozen or used the next day

11.5.3 Preparation of dilution series

While the bacterial reagent is being reconstituted, prepare a sample dilution series according to SANS 11348-3 (2013), as follows:

- Equilibrate all solutions at 15 ± 1°C prior to preparing the sample serial dilution
- Prepare the sample dilution series by using 2% NaCl solution as diluent
- Dilutions are prepared by means of a graduated dilution and combines two geometric series (D = 2, 4, 8, 16, etc., and D = 3, 6, 12, 24, etc.). Suitable dilutions are chosen depending on the expected toxicity of the sample

The dilution series can be prepared from two stock solutions:

- Dilution 1:1, undiluted sample (dilution factor at final assay will be 1:2). 3000 µL undiluted sample
- Dilution 2:3, (dilution factor at final assay will be 1:3). 2000 µL sample and 1000 µL 2% NaCl solution

All the other dilutions can be done from the two stock dilutions by serial dilution using only the dilution factor 1:1 (for example 1.5 mL of previous dilution and 1.5 mL of diluent). The principle of the dilution procedure is shown in the picture below:

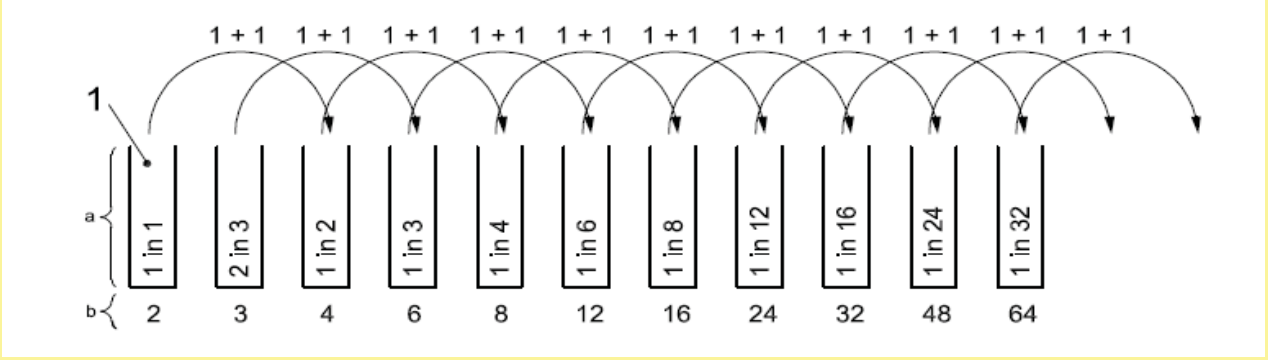

Figure 11-2: Serial dilutions sample preparation.
1 = sample, a = dilution of sample, b = final dilution level D after the addition of the test suspension.

- Mix the dilutions by repeatedly pipetting the solutions
- The length of the dilution series must be determined separately for each sample, but with most samples about 5 -10 dilutions is sufficient for the determination of EC_{50}

11.6 TEST PROCEDURE

- Pipette 500 µL of the reconstituted bacterial suspension into the cuvettes needed for performing the tests
- It is recommended to do duplicates for the control and sample dilutions
- Temperate all samples and dilutions to 15 ± 1°C for at least 15 minutes
- Measure the luminescence intensity (I_0) from the first cuvette containing bacterial suspension

- Immediately add 500 µL of the control or sample dilution to the cuvette. Repeat for all sample dilutions using equal time intervals between each sample

Note: If it is desired to test nearly undiluted water samples, it is possible to add 800 µL of the undiluted water sample to 200 µL of a test suspension. The dilution then is 1:1,25. Prepare similar control sample.

- Incubate sample dilutions at 15 ± 1°C for the chosen contact time (either 5, 15 or 30 minutes)
- After 5-, 15- and 30-minutes contact time determine the luminescence intensity (I_t) from the first sample (cuvette number 1). Repeat for all samples using the same time interval as during the first measurement

Notes: Temperature changes during the measurement may affect the results. Ensure that all reagents have reached the same temperature (15 ± 1°C).

The contact time should be exactly the same for all the samples and the controls. The most used contact times are 5, 15 and 30 minutes.

11.7 CALCULATION OF RESULTS

Calculate the results using computer software, such as a spreadsheet which automatically performs all calculations needed or perform calculations manually.

If the software is not used, the inhibition percentage (INH%) is calculated as follows (in this example the contact time is 15 minutes).

$KF = IC_{15}/IC_0$

And

$INH\% = 100 - 100 \times (IT_{15} / KF \times IT_0)$

Where:

KF	=	Correction factor
IC_{15}	=	Luminescence intensity of control after contact time (15 min) in RLU
IC_0	=	Initial luminescence intensity of control sample in RLU
INH%	=	Percentage inhibition
IT_{15}	=	Luminescence intensity of test sample after contact time (15 min) in RLU
IT_0	=	Initial luminescence intensity of the test sample in RLU

The EC_{50} and EC_{20} values are determined by using standard linear regression analysis. If the range of value pairs cannot be linearized, the EC_{50} value can be determined graphically using a double logarithmic co-ordinate system. The Gamma value (Γ_t = ratio of light lost to the amount of light remaining at time t) is plotted on the y-axis and the concentration (in mg/L, mol/L or % of original sample) on the x-axis.

For further information about the calculation, refer to the ISO Standard 11348-3 (2007) or ISO Standard 21338 (2010).

11.8 TEST VALIDITY

For the test performed to be valid, the three reference substances, solutions not neutralized, should cause 20% to 80% inhibition after 30 minutes contact time at the following concentrations in the final test suspension:

- 18,7 mg/L Cr(VI), equivalent to 52,9 mg/L potassium dichromate
- 3,4 mg/L 3,5-dichlorophenol and
- 2, 2 mg/L Zn(II), equivalent to 9,67 mg/L zinc sulfate heptahydrate
- The correction factor (KF) after 30 minutes incubation should range between 0,6-1,8
- The parallel determinations do not deviate from their mean by more than 3% for the control samples

11.9 TEST REPORT

The test report should contain the following information:

- Identity of the water sample, including sampling, storage time and conditions
- pH and oxygen concentration, in mg/L or % saturation of the original water sample
- Date of test performance
- Sample pre-treatment, if any, e.g. pH after adjustment
- Origin of the bacteria, batch number; date of delivery and expiration date
- Storage temperature of the bacteria
- Expression of the results in accordance with ISO 11348-3 (2007)
- Any deviation from this method and information on all circumstances which might affect the results
- Test results with reference substances for the batch of bacteria and the actual test

11.10 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

11.10.1 APPLICATION

The *Aliivibrio fischeri* bioluminescence test is applicable to the following sample types:

- Wastewater
- Aqueous extracts and leachates
- Freshwater
- Sea and brackish water
- Eluates of sediments
- Porewater and
- Single substances

Soil and sediment samples can also be tested with the kinetic modification of the test (see ISO 21338, 2010). Water quality – Kinetic determination of the inhibitory effects of sediment, other solids, and colored samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test).

11.10.2 ADVANTAGES

- The BioTox™ WaterTox™ EVO commercial kit is used for the determination of toxicity of water-soluble samples. The inhibitory effect of the sample on the light emission of luminescent bacteria, *Aliivibrio fischeri*
- The kit provides a rapid, easy to use method for measuring toxicity of aqueous samples
- The significance of the *Aliivibrio fischeri* bioluminescent test is to help in the assessment of possible risk to similar species in the natural environment, as an aid in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes and identifying sensitive tests species to water pollution
- Toxicity tests, such as the *Aliivibrio fischeri* bioluminescent test, are applied to assess water pollution and are primarily used to screen for toxic substances in the aquatic environment and to some extent to predict the toxic effect of environmental impacts on algae
- A further significance of the test is to comply with the "The Management of Complex Industrial Wastewater Discharges, Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) Approach" (DWAF, 2003).

11.10.3 LIMITATIONS

- Insoluble, slightly soluble, volatile substances or substances which react with the dilution water or the suspension, or alter their state during the test period, may affect the result or impair the reproducibility of the test results
- Losses of luminescence caused by light absorption or light scattering may occur in the case of strongly coloured or turbid waters. This interference can be compensated by a sample treatment for turbidity or, for example, by using a double-chambered absorption correction test tube
- Since oxygen is required for the bioluminescence, samples with a high oxygen demand (and/or a low oxygen concentration) may cause a deficiency of oxygen and be inhibitory
- Readily biodegradable nutrients in the sample may cause a pollutant-independent reduction in bioluminescence
- Samples with a pH outside the range of pH = 6,0 and pH = 8,5 affect the luminescence of the bacteria. An adjustment of the sample is required when the toxic effect of pH is not wanted
- As the test organism *Aliivibrio fischeri* is a marine bacterium, testing salt-water samples with the standard procedure often leads to stimulation effects of bioluminescence, which may mask inhibition effects. Salt concentrations in the initial sample exceeding 30 g/L NaCl, or contents of other compounds giving equal osmolarity may lead, together with the salt spiking required by the test, to hyperosmotic effects. The resulting salt concentration in the test samples should not exceed the osmolarity of a 35 g/L NaCl solution to avoid these effects

11.10.4 RECOMMENDATIONS

This method provides an easy-to-use bioassay for measuring acute toxicity of substances/pollution to bacteria, such as *Aliivibrio fischeri*. The significance of the *Aliivibrio fischeri* bioluminescence test is to assist in the assessment of possible risks to bacteria in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other bacterial species for comparative purposes. The *Aliivibrio fischeri* bioluminescence test should form part of a battery of at least 3 tests representing different trophic levels in the aquatic environment (others include but are not limited to invertebrates, algae, vertebrates and protozoa amongst others). The reason for this is the variation between the sensitivity of the different species to different substances, and therefore using several different trophic levels increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

11.11 REFERENCES

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.

ISO (International Organization for Standardization) 11348-3 (2007) Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) – Part 3: Method using freeze-dried bacteria. ISO/TC 147/SC 5 Biological methods pp21.

ISO (International Organization for Standardization) 21338 (2010) Water quality – Kinetic determination of the inhibitory effects of sediment, other solids and colored samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test). ISO/TC 147/SC 5 Biological methods pp21.

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LAPPALAINEN J, JUVONEN R, NURMI J and KARP M (2001) Automated colour correction method for *Vibrio fischeri* toxicity test. Comparison of standard and kinetic assays. Chemosphere **45** 635-641.

SANS (South African National Standards) 11348-3 (2013) Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test) – Part 3 for the method using freeze-dried bacteria.

11.12 USEFUL CONTACTS

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12 *PSEUDOKIRCHNERIELLA SUBCAPITATA* GROWTH INHIBITION TEST

Compiled by: L Swart and H Pearson

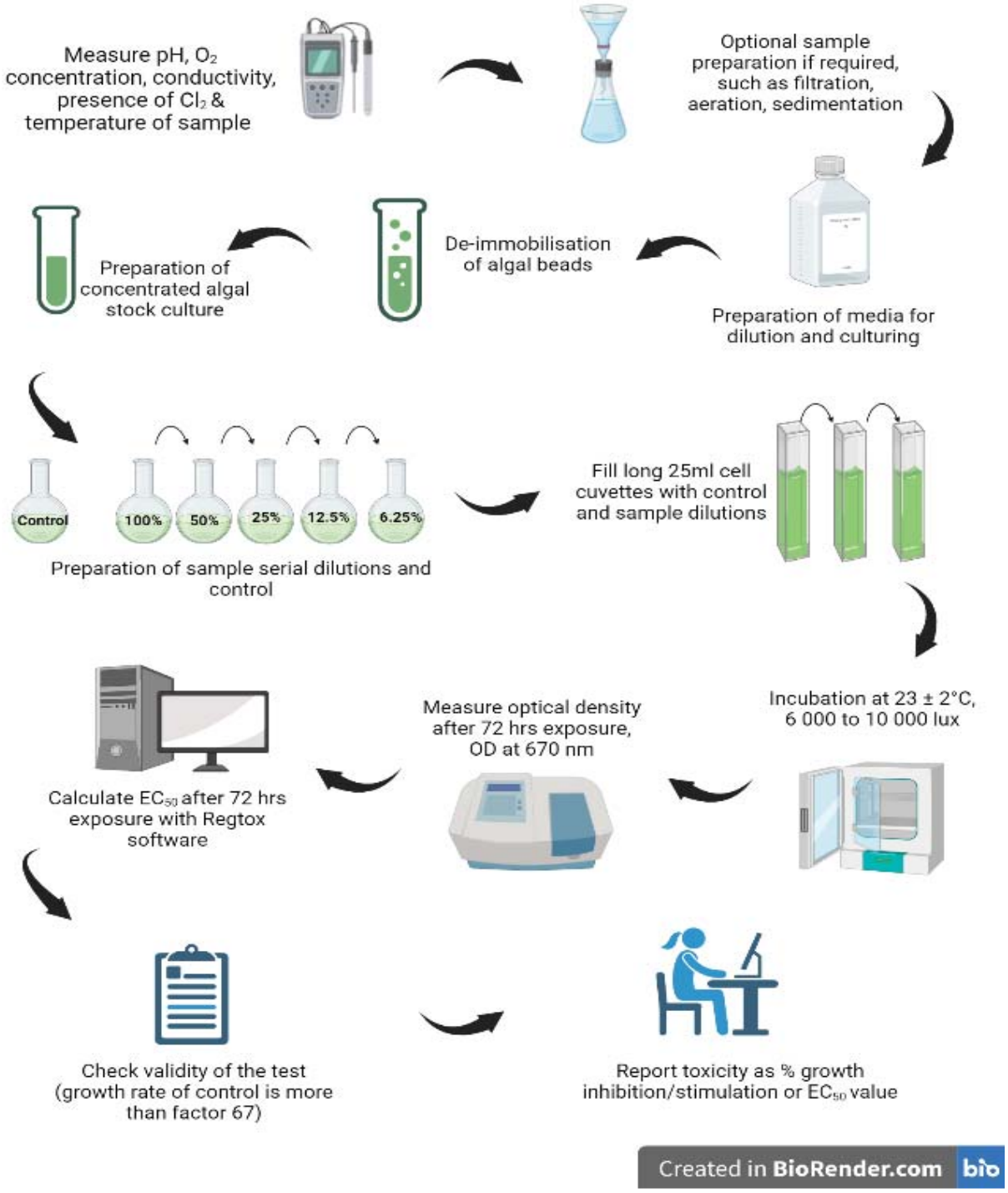

Figure 12-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the PSEUDOKIRCHNERIELLA SUBCAPITATA growth inhibition test

12.1 ACRONYMS & ABBREVIATIONS

NH_4Cl	Ammonium chloride
AR	Analytical Grade
H_3BO_3[1]	Boric acid
$CaCl_2 \cdot 2H_2O$	Calcium dichloride dihydrate
CO_2	Carbon dioxide
Cr	Chrome
$CoCl_2 \cdot 6H_2O$	Cobalt (II) chloride
CV	Coefficient of Variation
$CuCl_2 \cdot 2H_2O$	Coper (II) chloride hexahydrate
ºC	Degree(s) Celsius
EC_{20}	Effective concentration at 20%
EC_{50}	Effective concentration at 50%
E_rC_{10}	EC_{10} value based on growth rate
E_rC_{50}	EC_{50} values based on growth rate
$Na_2EDTA \cdot 2H_2O$	Ethylenediaminetetraacetic acid, Disodium dihydrate
h	Hour(s)
HCl	Hydrochloric acid
$FeCl_3 \cdot 6H_2O$	Iron (III) chloride hexahydrate
K	Kelvin
L	Litre(s)
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
$MgSO_4 \cdot 7H_2O$	Magnesium (II) sulphate heptahydrate
$MnCl_2 \cdot 4H_2O$	Manganese (II) chloride
µg/L	Microgram(s) per litre
µL	Microliter(s)
mg/L	Milligram(s) per litre
mL	Millilitre(s)
KH_2PO_4	Monopotassium phosphate
nm	Nanometre(s)
OD	Optical density
%	Percentage
±	Plus, or minus
$K_2Cr_2O_7$	Potassium dichromate
rpm	Rotations per minute
$NaHCO_3$	Sodium bicarbonate
NaCl	Sodium chloride
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate dihydrate
$ZnCl_2$	Zinc chloride

12.2 PRINCIPLE OF THE ASSAY

Mono-species algal strains are cultured for several generations in a defined medium containing a range of concentrations of the test sample, prepared by mixing appropriate quantities of growth medium, test sample, and an inoculum of exponentially growing algal cells. The test batches are incubated for a period of 72 ± 2 hours during which the cell density in each test solution is measured at least every 24 hours. Growth is determined in terms of optical density (OD) (at 670 nm). The tests are performed in disposable spectrophotometric cells of 10 cm path-length as test vials. Inhibition is measured as a reduction in specific growth rate relative to control cultures grown under identical conditions.

This procedure is based on SANS 8692 (2015). "Water Quality – freshwater algal growth inhibition test with unicellular green algae". This International Standard specifies a method for the determination of the growth inhibition of unicellular green algae by substances and mixtures contained in water or by wastewater. This method is applicable for substances that are easily soluble in water. A rapid algal growth inhibition screening test for wastewater is described in Annex A. An alternative test procedure with algae from algal beads, with direct measurement of algal growth in spectrophotometric cells, is described in Annex B of SANS 8692 (2015).

With modifications to this method, as specified in ISO 14442 (2006) and ISO 5667-16 (1998), the inhibitory effects of poorly soluble organic and inorganic materials, volatile compounds, heavy metals, and wastewater can be tested. Testing of coloured test solutions requires specific modifications as specified in ISO 14442 (2006).

The *Pseudokirchneriella subcapitata* growth inhibition test measures the short-chronic toxicity of effluents, receiving waters, chemicals/products and/or leachates to the green algae *Selenastrum capricornutum* (first named *Raphidocelis subcapitata* and presently named *Pseudokirchneriella subcapitata*).

12.3 REQUIREMENTS

12.3.1 ACQUISITION OF TEST Material AND KITS

The procedure described in this document is based on the ALGALTOXKIT F™ commercial kit used to measure the growth inhibition or stimulation of the algae *Selenastrum capricornutum* (first named *Raphidocelis subcapitata* and presently named *Pseudokirchneriella subcapitata*) with a spectrophotometer. This procedure is based on ISO 8692 (2012) Edition 3 or SANS 8692 (2015) Edition 1. "Water Quality – freshwater algal growth inhibition test with unicellular green algae".

Note: According to ISO 8692 (2012) Edition 3, the algae beads supplied by MicroBioTests Inc. are an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if validity criteria specified in this International Standard are fulfilled.

12.3.1.1 Kit contents

The commercial ALGALTOXKIT F™ contains all the materials, including the test species as immobilized in algal beads, reagents and test containers, to perform the algal growth inhibition test according to the internationally accepted Standard ISO 8692 (2012).

The Algaltoxkit F ™ consists of:

- Two tubes containing small algal beads with *Pseudokirchneriella subcapitata* microalgae immobilized in an inert matrix. The tubes with the storage medium and the algal beads should be kept in the refrigerator in darkness at 5 ± 2°C until use
- One glass bottle containing the special medium to dissolve the matrix in which the microalgae are immobilized. The matrix dissolving medium must be stored in the refrigerator in darkness at 5 ± 2°C until use
- Five glass bottles containing concentrated algal growth media solutions of various chemicals to make up 2 litres of algal culturing medium with deionized water, according to the formula of the standard. The vials should be stored in the refrigerator at 5 ± 2°C in darkness until use
- Two sets of 18 disposable 10 cm path-length long cells with lids, each in a transparent holding tray provided with two plastic strips. The long cells serve as test vials and allow for direct measurement of the OD in the test containers
- Two long cells with lids, one for zero calibration of the spectrophotometer and one cell for scoring of the OD of the concentrated algal suspension
- Specification sheets and graphs

12.3.2 STAFF TRAINING (TECHNICAL SKILLS)

To perform the test, the analyst should be deemed competent in:

- Good laboratory practice
- Trained in an aquatic ecotoxicology laboratory
- Software package knowledge (e.g. EXCEL/ REGTOX)

12.3.3 LABORATORY (TEST ENVIRONMENT)

- The testing facility must be free of vapours, odours and dust that may be toxic to the test organisms
- Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage
- Do not use equipment made of copper
- Temperature control equipment should be adequate to maintain the recommended environmental conditions as stated in the standard during storing of samples, testing and culture maintenance

12.3.4 SOFTWARE

- EXCEL spreadsheet or

- software supplied by the supplier, such as REGTOX for calculations

12.3.5 APPARATUS

The following apparatus will be required to perform the test:

- Spectrophotometer reading at 670 nm
- Incubator with lights or growth chamber
- Lux meter
- Centrifuge set at 3000 rpm
- Vortex shaker
- Adjustable transfer pipettes and pipette tips (10-5000 μL)
- pH-meter (accuracy 0,1 pH units)
- Conductivity meter
- Oxygen meter
- Thermometers
- Stopwatch
- Fridge

12.3.6 CONSUMABLES/MATERIALS

Table 12-1: List of consumables/materials

Name	Cat no	Supplier
ALGALTOXKIT F™	TK41	MicroBioTests Inc.
100 mL volumetric flasks	-	General Supplier
1 L volumetric flasks	-	General Supplier
25 mL measuring cylinders	-	General Supplier
100 mL measuring cylinders	-	General Supplier
250 mL plastic/glass beakers	-	General Supplier

12.3.7 REAGENTS

Use chemicals of recognised analytical grade (AR grade).

Table 12-2: List of reagents

Name	Cat no	Supplier
Sodium hydroxide (NaOH), 1N	-	General Supplier
Hydrochloric acid (HCl), 1N	-	General Supplier
Nitric acid, HNO_3	-	General Supplier
Acetone	-	General Supplier
	-	General Supplier

Reference stock solution, such as 1 g/L Potassium dichromate	-	General Supplier
Water, deionized or of equivalent purity (conductivity <10 µS/cm)	-	General Supplier
Chlorine testing kits	-	General Supplier
NH_4Cl	-	General Supplier
$MgCl_2 \cdot 6H_2O$	-	General Supplier
$CaCl_2 \cdot 2H_2O$	-	General Supplier
$MgSO_4 \cdot 7H_2O$	-	General Supplier
KH_2PO_4	-	General Supplier
$FeCl_3 \cdot 6H_2O$	-	General Supplier
$Na_2EDTA \cdot 2H_2O$	-	General Supplier
H_3BO_3	-	General Supplier
$MnCl_2 \cdot 4H_2O$	-	General Supplier
$ZnCl_2$	-	General Supplier
$CoCl_2 \cdot 6H_2O$	-	General Supplier
$CuCl_2 \cdot 2H_2O$	-	General Supplier
$Na_2MoO_4 \cdot 2H_2O$	-	General Supplier
$NaHCO_3$		

12.4 SAMPLING METHOD

12.4.1 Sample collection

- Collect samples in chemically inert, clean containers as specified in ISO 5667-16 (1998)
- Fill the containers completely to the top of container and seal if possible
- Test the samples as soon as possible after collection as indicated in the standard
- Where necessary, store samples at 5 ± 2°C in the dark in the containers for no longer than 48 hours
- Do not use chemicals to preserve the samples

12.4.2 Sample preparation

Measure the pH, dissolved oxygen, conductivity, and free chlorine of the sample prior to analysis. Usually, the test shall be carried out without adjustment of the pH of the medium after addition of the test sample. However, some substances may exert a toxic effect due to extreme acidity or alkalinity. In order to determine the toxicity of a sample independent of pH, adjust the pH of the aqueous sample or stock solution (before the dilution in series) to that of the culture medium using either 1 mol/L hydrochloric acid (HCl) or 1 mol/L sodium hydroxide solution (NaOH) (see ISO 5667-16, 2018).

Note: Record the appearance of the sample (e.g. colour, turbidity, odour) prior to testing.

12.5 TEST PREPARATION

12.5.1 Algal growth media/nutrient sock solution

Prepare the four nutrient stock solutions in deionised water, according to the compositions given in Table 12-3. These solutions are eventually diluted to achieve the final nutrient concentrations in the test solutions.

Table 12-3: Mass concentrations of nutrients in the test solution.

	Nutrient	Mass concentration in stock solution	Final mass concentration in test solution
Macronutrients	NH_4Cl	1,5 g/L	15 mg/L (N: 3,9 mg/L)
	$MgCl_2 \cdot 6H_2O$	1,2 g/L	12 mg/L (Mg: 2,9 mg/L)
	$CaCl_2 \cdot 2H_2O$	1,8 g/L	18 mg/L (Ca: 4,9 mg/L)
	$MgSO_4 \cdot 7H_2O$	1,5 g/L	15 mg/L (S: 1,95 mg/L)
	KH_2PO_4	0,16 g/L	1,6 mg/L (P: 0,36 mg/L)
Fe-EDTA	$FeCl_3 \cdot 6H_2O$	64 mg/L	64 µg/L (Fe: 13 µg/L)
	$Na_2EDTA \cdot 2H_2O$	100 mg/L	100 µg/L
Trace elements	H_3BO_3[1]	185 mg/L	185 µg/L (B: 32 µg/L)
	$MnCl_2 \cdot 4H_2O$	415 mg/L	415 µg/L (Mn: 115 µg/L)
	$ZnCl_2$	3 mg/L	3 µg/L (Zn: 1,4 µg/L)
	$CoCl_2 \cdot 6H_2O$	1,5 mg/L	1,5 µg/L (Co: 0,37 µg/L)
	$CuCl_2 \cdot 2H_2O$	0,01 mg/L	0,01 µg/L (Cu: 3,7 ng/L)
	$Na_2MoO_4 \cdot 2H_2O$	7 mg/L	7 µg/L (Mo: 2,8 µg/L)
$NaHCO_3$	$NaHCO_3$[1]	50 g/L	50 mg/L (C: 7,14 mg/L)

[1] H_3BO_3 can be dissolved by the addition of 0,1 mol/L NaOH.

12.5.2 Test organisms

The freshwater unicellular green algae, *Pseudokirchneriella subcapitata* ((Korshikov) Hindak (ATCC® 22662TM, CCAP 278/4 or 61.81 SAG) is used as the test organism to perform this bioassay. This algal species is a non-motile, crescent-shaped algae, 40 to 60 µm in size. The organisms are ubiquitous in most freshwaters. The algae do not clump because it is free of complex structures and do not form chains. It is therefore easy to count them. This algae species is a planktonic green alga belonging to the order of *Sphaeropleales* (Chlorophyta, Chlorophyceae) and are usually unicellular in culture.

This species is formerly known as *Scenedesmus subspicatus* (Chodat) also formerly known as *Selenastrum capricornutum* (Prinz). The new name is currently cited by culture collections.

Stock cultures can be maintained in the medium specified in the standard. However, frequent subculturing is necessary (once a week) to prevent failure of growth. The stock culture can be maintained for extended

periods on richer algal media such as those recommended by the culture collection. Alternatively, algae can be stored for several months on agar plates or in alginate beads without losing their viability. The algae can be easily recovered from the agar or liberated from the algal beads when needed to perform the bioassay.

The appearance of the cells and the identity of the test organisms should be confirmed by microscopy.

12.5.3 Demobilisation of algal beads

- Take one of the two tubes containing algal beads and pour out the liquid; take care not to eliminate any of the algal beads during the process
- Open the vial labelled "Matrix dissolving medium" and transfer 5 mL to the tube
- Cap the tube and handshake vigorously. Repeat the shaking for two minutes or until the matrix immobilizing the algae is totally dissolved. The algae should be entirely freed within 5 to 10 minutes. A Vortex shaker may be used to speed up the process
- Centrifuge the tube for 10 minutes at 3000 rpm in a conventional lab centrifuge
- Pour out the supernatant and replace it by 10 mL deionized water. Cap the tube and shake it vigorously to re-suspend the algae homogenously
- Centrifuge the tube again at 3000 rpm for 10 minutes and decant the supernatant
- Resuspend the algae in 10 mL algal culturing medium

12.5.4 Preparation of algal stock

- Pour the algal suspension from the tube into a 25 mL calibrated flask and add algal growth medium to the 25 mL mark. Stopper and shake to homogenize the algal suspension
- Take the two long cells with the labels "Calibration long cell" and "Algal stock cell". Fill the calibration cell with 25 mL algal culturing medium and close the cell with the lid. Put this cell in the spectrophotometer and zero-calibrate the instrument
- Transfer the algal suspension into the “Algal stock cell” and tightly close the cell with the lid. Shake this cell thoroughly to distribute the algal suspension evenly. Put the “Algal stock cell” in the spectrophotometer and read the optical density (OD1) after 10 seconds
- Take the optical density/algal number (OD/N) sheet and look up the number of algae (N1) corresponding with OD1. With N2 equal to 1.10^6 algae/mL, calculate from the N1/N2 ratio the dilution factor needed to reach an optical density equal to OD2, corresponding to an algal density of 1.10^6 cells/mL
- Transfer the algal suspension from the “Algal stock cell” into a 100 mL flask and add the volume of algal culturing medium needed to make up a 1.10^6 cells/mL suspension
- Stopper and shake the flask thoroughly to distribute the algae evenly

12.6 TEST PROCEDURE

12.6.1 Test sample

Test samples may be aqueous (e.g. wastewater) or non-aqueous (e.g. chemical substance or mixture of chemicals) for which the inhibitory effects on the growth of algae shall be determined.

If the test sample is aqueous (e.g. wastewater), pre-treatment (e.g. filtration, neutralization) should be considered depending on the nature of the sample and the purpose of the test. Add nutrient stock solutions, to the sample.

For non-aqueous test samples, preparation of stock solutions is generally necessary. The method for preparation of the stock solutions should be carefully chosen based on the properties of the sample. Stock solutions are usually prepared by dissolving the test sample in growth medium. Modifications are necessary when the test sample does not readily dissolve in the growth medium as specified in ISO 14442 (2006) and ISO 5667-16 (1998).

12.6.2 Preparations of test and control sample

- Prepare the test samples by mixing the appropriate volumes of test sample or test sample stock solutions, growth medium and inoculum in the test vessels. The total volume, concentrations of added growth medium nutrients and cell density shall be the same in all vessels
- Prepare at least three replicate batches for each test sample concentration
- The initial cell density shall be sufficiently low to allow exponential growth in the control culture throughout the test duration without a pH drift of more than 1,5 pH units. Therefore, the initial cell densities shall not exceed 10^4 cells/mL
- Prepare three replicate control samples by adding the appropriate volume of inoculum to growth medium. Measure the pH of a replicate batch at each test concentration and in one control replicate
- If appropriate, prepare a single concentration series of the test sample without algae to serve as background for the cell density determinations

12.6.3 Sample concentration

- Algae should be exposed to concentrations of the test sample in a geometric series with a ratio not exceeding 3,2 (e.g. 1,0 mg/L, 1,8 mg/L, 3,2 mg/L, 5,6 mg/L, and 10 mg/L)
- The concentrations should be chosen to obtain at least one inhibition below and one inhibition above the intended E_rC_X parameter. Additionally, at least two levels of inhibition between 10% and 90% should be included in order to provide data for regression analysis
- A screening/limit test with only one concentration can be conducted to demonstrate absence of toxicity. The number of replicates for this one concentration should be at least three

- A suitable concentration range is best determined by carrying out a preliminary range-finding test covering several orders of magnitude of difference in test concentration. Replication of test concentrations is not a requirement in the preliminary test

12.6.4 Incubation

The test vessels shall be sufficiently covered to avoid airborne contamination and to reduce water evaporation, but they shall not be airtight in order to allow CO_2 to enter the vessels (a small hole is sufficient). Incubate the test vessels at 23 ± 2°C, under continuous white light. The light intensity at the average level of the test media shall be homogeneous within ± 10% and in the range 60 µmoL/(m^2·s) to 120 µmoL/(m^2·s) when measured in the photosynthetically effective wavelength range of 400 nm to 700 nm, using an appropriate receptor.

The light intensity specified can be obtained using four to six fluorescent lamps of the universal white (natural) type, i.e. a rated colour of standard colour 2 (colour temperature of 4 300 K). The optimum distance of the lamps is approximately 0,35 metre from the algal culture medium.

For light-measuring instruments calibrated in lux, an equivalent range of 6 000 lx to 10 000 lx is acceptable for the test.

Note: Continuously shake, stir, or aerate the cultures in order to keep the cells in free suspension and to facilitate CO_2 mass transfer from air to water, and in turn reduce pH drift.

12.6.5 Measurements

- Measure the cell density in each test batch (including the controls) at least every 24 hours.
- Mix the test batches thoroughly before measurement
- The nominal cell density can be used as the initial cell density and no initial cell density measurement is then required. The test shall last for 72 ± 2 hours
- At the end of the test, measure the pH of samples of at least one replicate batch at each test sample concentration and one control replicate

12.7 CALCULATIONS OF RESULTS

12.7.1 Plotting of growth curve

Tabulate the cell density measurements for each test batch according to the concentration of the test sample and the duration of measurement.

Plot a growth curve for each test concentration and control, as a graph of the logarithm of the mean cell density against time. A linear growth curve indicates exponential growth, whereas a levelling off indicates that cultures have entered the stationary phase.

If the control cultures show declining growth rate towards the end of the exposure period, inhibited cultures may tend to catch up with the controls, falsely indicating a decreased growth-inhibiting effect. In this case, perform the calculations of growth rate and growth inhibition based on the last measurement within the exponential growth period in the control cultures.

12.7.2 Expression of results

Denote EC_{10} and EC_{50} values based on growth rate as E_rC_{10} and E_rC_{50}. Also clearly indicate the time span used for the determination, e.g. E_rC_{50} (0 to 72 hours). Report E_rC_{10} and E_rC_{50} values in milligrams per litre or as percentages with the corresponding confidence intervals.

When testing wastewater by means of a graduated dilution, *D*, the test medium with the highest concentration at which an inhibition < 5% is observed is termed the lowest ineffective dilution (LID). This dilution is expressed as the reciprocal of the volume fraction of wastewater in the test medium (e.g. if the wastewater content is one part in four (25% volume fraction), the dilution factor is *D* = 40); see ISO 5667-16 (1998), Annex A.

12.8 TEST VALIDITY

Consider the test valid if the following conditions are met:

- The average growth rate in the control replicates shall be at least 1.4 d^{-1}. This growth rate corresponds to an increase in cell density by a factor 67 in 72 hours
- The variation coefficient of the growth rate in the control replicates shall not exceed 5%
- The pH in the control shall not have increased during the test by more than 1.5 relative to the pH of the growth medium. An increase in pH during the test can have significant influence on the results and therefore a limit of 1,5 units is set. These variations, however, should always be kept as low as achievable, e.g. by performing continuous shaking during the test
- If these criteria are not met, examine experimental techniques and use inoculate from other sources, if necessary.

12.9 INTERPRETATION OF RESULTS

EC_{10} and EC_{50} values are toxicological data derived from a laboratory experiment carried out under defined standard conditions. They give an indication of potential hazard but cannot be used directly to predict effects in the natural environment.

When interpreting EC_{10} and EC_{50}, take into consideration the shape of the growth curves. Certain features of these curves (e.g. delayed onset growth, good initial growth but not sustained) may help to indicate the mode of action of the toxic substance concerned.

12.10 TEST REPORT

The test report shall contain at least the following information:

- Sampling: identity of the water sample, including sampling, storage time and conditions; pH and oxygen concentration, in mg/L or % saturation of the original water sample; all data required for complete identification of the test sample
- Sample pre-treatment, if any, e.g. pH after adjustment
- Test details: start date and duration of the test; method of preparation of sample and test batches; concentrations tested; composition of medium; culturing apparatus and incubation procedure; light intensity and quality; test temperature; pH of test solutions including the controls at start and end of test and method for measuring cell density and, if appropriate and method to correct for background values
- Test organism: origin of the algae such as batch number, date of delivery and expiration; storage temperature of the algae; test organism species; origin; strain number and method of cultivation
- Expression of the results (the percentage growth inhibition or growth stimulation in each sample after 72 hours is reported as the percentage effect or the 72-hour EC_{20} and EC_{50} values. Cell density in each batch at each measuring point. Mean cell density for each test concentration (and control) at each measuring point. Growth curves (logarithm of mean cell density against time) for each test concentration and control. Relationship between the concentration and effect (percentage inhibition values against concentration) in tabular or graphical representation, e.g. percentage of inhibition on probit-scaled ordinate against concentration in logarithmic-scaled abscissa. ErCx values such as ErC_{10} and ErC_{50} with their confidence intervals, including the method of determination or, alternatively, if wastewater is tested by means of a graduated dilution, the LID. Other observed effects such as bleaching of algal cells
- A reference to this International Standard (ISO 8692, 2012) and any deviation from this method and information on all circumstances which might affect the results
- Test results with reference substances for the batch of algae and the actual test

12.11 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

12.11.1 APPLICATION

The *Pseudokirchneriella subcapitata*. growth inhibition test is applicable to the following sample types:

- Wastewater
- Aqueous extracts and leachates
- Freshwater
- Sea and brackish water
- Eluates of sediments
- Porewater and
- Single substances

12.11.2 Advantages

Bioassays, such as the *Pseudokirchneriella subcapitata* growth inhibition test, are applied to assess water pollution and are primarily used to screen for toxic substances in the aquatic environment and to some extent to predict the toxic effect of environmental impacts on algae.

The major advantage of MicroBioTests kits, in comparison to "conventional" bioassays, is that the test organisms are incorporated in the kits in a "resting" or "immobilized" form, from which they can be activated "on demand" prior to performance of the toxicity test. This eliminates the need for continuous recruitment and/or stock culturing of test organisms, and hence the major cost factor. Furthermore, all MicroBioTests kits have been "miniaturized" into low-cost kits which can be performed with conventional laboratory materials and equipment, on little bench space.

The significance of the *Pseudokirchneriella subcapitata*. growth inhibition test is to help in the assessment of possible risk to similar species in the natural environment, as an aid in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes and identifying sensitive tests species to water pollution.

Toxicity tests, such as the *Pseudokirchneriella subcapitata*. growth inhibition test, are applied to assess water pollution and are primarily used to screen for toxic substances in the aquatic environment and to some extent to predict the toxic effect of environmental impacts on algae.

A further significance of the test is to comply with the “The Management of Complex Industrial Wastewater Discharges, Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach” (DWAF, 2003).

12.11.3 LIMITATIONS

Limitations of the test include:

- High salt concentrations in samples can lead to precipitation upon algal medium addition, which can interfere with OD measurements. If a precipitate forms during testing, note this on the data sheet – such results are omitted from the battery of tests during the hazard classification process
- Volatile substances might inhibit growth of algae in other wells, including that of the control. Ensure that controls and other samples are separated from such samples
- Pathogenic and/or predatory organisms in the samples may affect survival
- Coloured natural samples may interfere with OD readings of the algal suspensions, especially when the colour shows absorption at the (670 nm) wavelength which is used to measure algal density. It is important to note that the determination of the toxicity of highly coloured samples to microalgae is automatically biased by interference of the colour with light penetration in the medium containing the algae

12.11.4 RECOMMENDATIONS

This method provides an easy-to-use bioassay for measuring toxicity of substances/pollution to algae, such as *Pseudokirchneriella subcapitata*. The significance of the *Pseudokirchneriella subcapitata* growth inhibition test is to assist in the assessment of possible risks to algal species in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with testing of other algal species for comparative purposes. The *Pseudokirchneriella subcapitata* growth inhibition test should form part of a battery of at least 3 tests representing different trophic levels in the aquatic environment (others include but are not limited to bacteria, invertebrates, algae, vertebrates, and protozoa amongst others). The reason for this is the variation between the sensitivity of the different species to different substances, and therefore using several different trophic levels increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

12.12 REFERENCES

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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12.13 USEFUL CONTACTS

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Algal strains collections

The strains recommended are available in unialgal, non-axenic cultures from the following collections:

- SAG – Sammlung von Algenkulturen Göttingen [Göttingen Algal Culture Collection], Germany, www.epsag.uni-goettingen.de (viewed 2012-01-30);
- ATCC – American Type Culture Collection, USA, www.atcc.org (viewed 2012-01-30);
- CCAP – Culture Collection of Algae and Protozoa, UK, www.ccap.ac.uk (viewed 2012-01-30);
- ALCP – Algothèque du Laboratoire de Cryptogamie, France, www.mnhn.fr (viewed 2012-01-30).

13 *DAPHNIA MAGNA/PULEX* ACUTE TOXICITY TEST (IMMOBILIZATION TEST)

Compiled by: L Swart and H Pearson

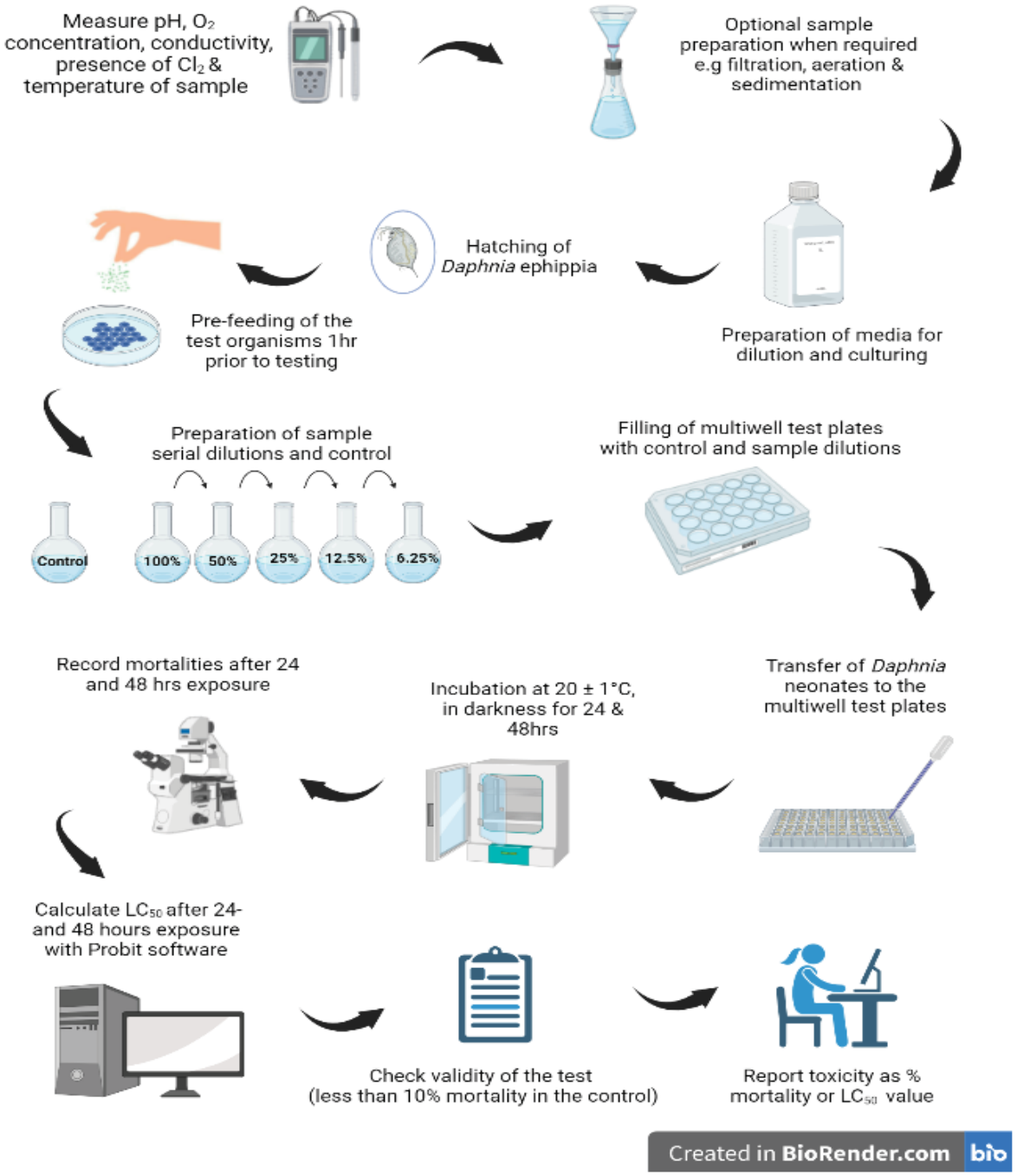

Figure 13-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the DAPHNIA MAGNA/PULEX acute toxicity test (immobilization test)

13.1 ACRONYMS & ABBREVIATIONS

AR	Analytical Grade
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
Cr	Chrome
CV	Coefficient of Variation
d	Day(s)
ºC	Degree(s) Celsius
g	Gram(s)
h	Hour(s)
HCl	Hydrochloric acid
LC_{10}	Lethal concentration at 10%
LC_{50}	Lethal concentration at 50%
L	Litre(s)
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
µg/L	Microgram(s) per litre
mg	Milligram(s)
mg/L	Milligram(s) per litre
mL	Millilitre(s)
HNO_3	Nitric acid
%	Percentage
±	Plus, or minus
KCl	Potassium chloride
$K_2Cr_2O_7$	Potassium dichromate
$NaHCO_3$	Sodium bicarbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide

13.2 PRINCIPLE OF THE ASSAY

The *Daphnia magna /pulex* acute toxicity test (immobilization test) measures the short-term acute toxicity of wastewater discharges and receiving water to the freshwater Cladocera *Daphnia magna or pulex*. In this test, a range of concentrations of substance are tested to investigate the different degrees of toxic effects on the swimming capability of *Daphnia sp.* under otherwise identical test conditions after 48 hours. This International Standard specifies a method for the determination of the acute toxicity to *Daphnia magna* Straus (Cladocera, Crustacea).

This procedure can be based on SANS 6341 (2015). “Water Quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – acute toxicity test”, OECD Guideline 202 (2004). “*Daphnia sp*., Acute Immobilisation Test” as well as other international standards such as US EPA (2002). “Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms Testing of Chemicals”.

13.3 REQUIREMENTS

13.3.1 Acquisition of test materials and kits

Although inhouse cultures can be used as per requirements noted in ISO 6341 (2012), this protocol describes the use of commercially supplied Toxkits. The kits contain all the materials (including the test organisms) necessary to perform simple, rapid, sensitive, and reproducible toxicity tests at low cost. Toxkit tests are particularly suited for routine toxicity testing of chemicals and wastes released in aquatic as well as in terrestrial environments. The DAPHTOXKIT *magna* F^{TM} (*Daphnia magna*) tests can be performed in accordance with testing conditions prescribed by SANS 6341 (2015). "Water Quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) – acute toxicity test".

Note*:* According to ISO 6341: 2012, MicroBioTests Inc. are an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if validity criteria specified in this International Standard are fulfilled.

13.3.1.1 Kit contents

The commercial DAPHTOXKIT *magna* F^{TM} contains all the materials, including the test species, reagents, and test containers, to perform the *Daphnia magna* acute toxicity test according to the internationally accepted Standard ISO 6341 (2012).

The DAPHTOXKIT *magna* F^{TM} consists of:

- Six 1 mL plastic tubes covered by aluminium foil, containing ephippia of *Daphnia magna*, to be stored in a refrigerator at 5°C ± 2°C until use. The number of neonates obtained from each vial suffices for one full toxicity test
- Two sets of four small glass bottles, each containing a concentrated solution of one salt, to make up two times 2 litre Standard Freshwater (ISO medium, formula according to ISO, 2012) with deionized or distilled water, for preparation of the hatching and toxicant dilution medium. Composition:
  vial 1: $NaHCO_3$ (129.5 mg - dissolved in 2 litre = 64.75 mg/L)
  vial 2: $CaCl_2.2H_2O$ (588 mg - dissolved in 2 litre = 294 mg/L)
  vial 3: $MgSO_4.7H_2O$ (246.5 mg - dissolved in 2 litre = 123.25 mg/L)
  vial 4: KCl (11.5 mg - dissolved in 2 litre = 5.75 mg/L)
- Six polystyrene petri dishes of 5 cm diameter, for the hatching of the ephippia
- Six polycarbonate test plates composed of 6 rinsing wells and 24 wells for the toxicant dilutions
- Six 1 mL plastic tubes containing a small amount of *Spirulina* powder for "prefeeding" the test organisms prior to the toxicity test
- Six Parafilm strips for sealing the multi-well plates to minimize evaporation during the incubation period

- Six polyethylene micropipettes for transfer of the test organisms
- A small micro-sieve, for rinsing of the ephippia

13.3.2 STAFF TRAINING (TECHNICAL SKILLS)

To perform the test, the analyst should be proven competent in

- Good Laboratory Practice (GLP)
- Trained in an aquatic ecotoxicology laboratory
- Software packages knowledge (e.g. EXCEL)

13.3.3 LABORATORY (test environment)

- The testing facility must be free of vapours, odours and dust that may be toxic to the test organisms
- The use of controls also allows checking that the test is performed in an atmosphere free from toxic dusts and vapours
- Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage
- The exposure of organisms as specified in this International Standard shall be carried out either in the dark or under a 16 and 8 hours light and dark photoperiod, in a temperature-controlled room or incubator at 20 ± 2°C in the test containers
- Temperature control equipment should be adequate to maintain the recommended environmental conditions as stated in the standard, during storing of samples, testing and culture maintenance
- The ephippia is hatched in an environmentally controlled facility under the following illumination conditions 5400-6600 lux

13.3.4 Software

- EXCEL spreadsheet
- REGTOX
- TSK (Trimmed Spearman Karber)
- Probit

13.3.5 Apparatus

The following apparatus will be required to perform the test:

- Temperature controlled room
- Incubator or growth chamber
- Lightbox
- Adjustable transfer pipettes and pipette tips (10-10 000 μL)
- pH-meter (accuracy 0,1 pH units),
- Oxygen meter

- Conductivity meter
- Thermometers
- Stopwatch
- Fridge

13.3.6 CONSUMABLES/MATERIALS

Table 13-1: List of consumables/materials

Name	Cat no	Supplier
DAPHTOXKIT *magna* F™	TK33	MicroBioTests Inc.
100 mL volumetric flasks	-	General supplier
2 L volumetric flask	-	General supplier
25 mL measuring cylinders	-	General supplier
100 mL measuring cylinders	-	General supplier
50 mL plastic/glass beakers	-	General supplier

13.3.7 Reagents

Use chemicals of recognised analytical grade (AR grade), unless otherwise specified.

Table 13-2: List of reagents

Name	Cat no	Supplier
Sodium hydroxide (NaOH), 1N	-	General supplier
Hydrochloric acid (HCl), 1N	-	General supplier
Nitric acid, HNO_3	-	General supplier
Acetone	-	General supplier
Reference stock solution, such as 1 g/L Potassium dichromate	-	General supplier
Water, deionized or of equivalent purity (conductivity <10 µS/cm)	-	General supplier
Chlorine testing kits	-	General supplier
$CaCl_2 \cdot 2H_2O$	-	General supplier
$MgSO_4 \cdot 7H_2O$	-	General supplier
$NaHCO_3$	-	General supplier
KCl	-	General supplier

13.4 SAMPLING METHOD

13.4.1 Sample collection

- Sampling, transportation, and storage of the samples should be performed as specified in ISO 5667-16 (1998)
- Collect samples in chemically inert, clean containers
- Rinse the bottle with the sample before filling it
- Fill the containers completely to the top of container and seal if possible
- Test the samples as soon as possible after collection as indicated in the standard
- Where necessary, store samples at 5 ± 2°C in the dark
- Do not use chemicals to preserve the samples

13.4.2 Sample preparation

- Carry out the toxicity test as soon as possible, preferably within 12 hours of collection. If this time interval cannot be met, cool the sample to 5 ± 2°C and test the sample within 24 hours. If it is not possible to perform the test within 72 hours, the sample may be frozen as soon as possible after sampling and maintained deep-frozen (below -18°C) for testing within two months of collection (see ISO 5667-16, 1998)
- Immediately test the frozen samples after complete thawing, e.g. in a water bath at a maximum temperature of 30°C. Do not use a microwave for thawing the samples
- At the time of testing, homogenize the sample to be analysed by shaking manually
- High concentrations of suspended inorganic or organic solids in a sample can be harmful to filter-feeding *Daphnia magna*. Compensation for this interference can be made by a sample treatment for turbidity. If necessary, allow to settle for a maximum of 2 hours in a container, and sample, e.g. by drawing off the required quantity of supernatant using a pipette, maintaining the end of the pipette in the centre of the section of the test container and halfway between the surface of the deposited substances and the surface of the liquid. If the raw sample of the decanted supernatant is likely to interfere with the test (due to presence of residual suspended matter, protozoa, microorganisms, etc.), centrifuge, for example, for 10 minutes at 5 000*g* or filter the raw or decanted sample. Test the residual toxicity of the supernatant. The kind of filter to be used should be checked by a test with control medium run through the filters

Note Some filters and apparatus can add measurable toxicity, sometimes because of wetting agents added to the filters. A filter paper can also absorb toxic substances and remove them from the sample filtrate.

- The sample obtained by either of these methods is the sample submitted to testing
- Usually, no aeration of sample or prepared test concentrations is necessary. If, and only if, the dissolved oxygen is < 40% saturation, a pre-aerate of the sample or all test solutions for at most 20

minutes by appropriate methods, e.g. aeration or stirring may be performed. Any supersaturation should be remedied

- Report any pre-aeration of test solutions or sample
- Measure the pH (as specified in ISO 10523, 2008) and the dissolved oxygen concentration (as specified in ISO 5814, 2012) and record these values in the test report
- Tests shall be carried out without pH adjustment of the test sample. The pH of test batches is measured at the beginning and at the end of the test and reported. However, in some cases, the final pH of a test solution may significantly differ from original pH of the test sample due to the concentration range selected and as a result of the buffer capacity of the dilution water or test sample. If toxic effects are observed at concentrations where the pH is not compatible with the survival of the organisms (i.e. outside the pH 6,0 to pH 9,0 range), the test(s) can be repeated with pH adjustment of the test sample

Note: Adjustment of the pH can alter the nature of the sample.

- If the pH is to be adjusted, the recommendation is to adjust to the pH of the dilution water. Choose the concentration of the hydrochloric acid or the sodium hydroxide solutions to restrict the volume fraction added to not more than 5%. If, as a result of pH adjustment, there is an issue with suspended matter, separate the suspended matter from the remaining sample as specified in ISO 5667-16 (1998). Any pH adjustment shall be included in the test report
- Adjust the temperature of the pre-treated sample to the test temperature
- Measure the pH, dissolved oxygen, conductivity, and free chlorine of the sample prior to analysis. Usually, the test shall be carried out without adjustment of the pH of the medium after addition of the test sample. However, some substances may exert a toxic effect due to extreme acidity or alkalinity. In order to determine the toxicity of a sample independent of pH, adjust the pH of the aqueous sample or stock solution (before the dilution in series) to that of the culture medium using either 1 mol/L hydrochloric acid (HCl) or 1 mol/L sodium hydroxide solution (NaOH) (see ISO 5667-16, 1998)
- Note the appearance of the sample (e.g. colour, turbidity, odour) prior to testing

13.5 TEST PREPARATION

13.5.1 Preparation of standard freshwater used for dilution and culturing

- Natural water (surface or groundwater), reconstituted water or dechlorinated tap water are acceptable as culturing and dilution water if *Daphnia magna* survives in it for the duration of the culturing, acclimation, and testing without showing signs of stress. Waters in the range pH 6 to pH 9, with hardness between 140 mg/L and 275 mg/L (as $CaCO_3$) are recommended
- As an example, the preparation of standard freshwater/dilution water meeting the requirements is described below

- Dissolve known quantities of reagents in distilled water. The dilution water prepared shall have a pH of 7,8 ± 0,5, a hardness of (225 ± 50 mg/L expressed as $CaCO_3$), a molar Ca + Mg ratio close to 4 + 1 and a dissolved oxygen concentration above 7 mg/L

Prepare the standard freshwater as follows:

- Dissolve 11,76 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) in distilled water and make up to 1 litre with distilled water
- Dissolve 4,93 g of magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) in distilled water and make up to 1 litre with distilled water
- Dissolve 2,59 g of sodium bicarbonate ($NaHCO_3$) in distilled water and make up to 1 litre with distilled water
- Dissolve 0,23 g of potassium chloride (KCl) in distilled water and make up to 1 litre with distilled water
- Mix 25 mL of each of the four solutions and make up to 1 litre with distilled water
- The dilution water shall be aerated until the dissolved oxygen concentration has reached saturation and the pH has stabilized. The dilution water prepared in this way shall not be further aerated before use
- If necessary, adjust the pH to 7,8 ± 0,5 by adding sodium hydroxide (NaOH) solution or hydrochloric acid (HCl)
- Store the standard freshwater in the fridge at 4 ± 2°C

The Standard Freshwater will be used as hatching medium for the ephippia, as dilution medium for preparation of the toxicant dilution series and for the control samples.

13.5.2 Test organisms

The test organisms used are obtained in the form of dormant eggs (ephippia) of the crustaceans *Daphnia magna* which are used worldwide for toxicity testing. The eggs are protected by a chitinous capsule called ephippium and can be stored for long periods of time without losing their viability. When the ephippia are placed in specific environmental conditions and triggers, the eggs will develop into neonates in about three days which can then be used immediately for the toxicity tests.

The test organisms are neonates of *Daphnia magna* Straus (Cladocera, Crustacea), obtained by a cyclical parthenogenesis under specified breeding conditions (see ISO 6341, 2012, Annex C).

The animals used for the test shall be less than 24 hours old and should not be first brood progeny. The test organisms shall be from a healthy stock, showing no signs of stress such as mortality > 20% in 2 days, presence of males, ephippia, or discoloured animals, and there shall be no delay in the production of the first brood. Isolate gravid females and collect newly released neonates within 24 hours.

If the culture conditions differ significantly from test conditions, it is recommended that one generation be acclimated under the test conditions for about one week to avoid stressing the parent animals and the offspring.

The age of the stock culture and the source (including clone, if possible) of the culture shall be indicated in the test report, since the sensitivity of the test organisms to toxicants can be affected by the source of the culture.

The test organisms may also derive from the hatching of ephippia obtained from laboratory cultures of the crustacean as described in ISO 6341 (2012). The neonates hatched from the ephippia may be used directly as test organisms if they comply with all validity criteria given in this International Standard.

13.5.3 Stock culturing

Loading of stock culture should be 25 to 50 animals per litre. They should be kept in mass stocks.

Note A loading of stock culture like the loading to be employed in the test is recommended. For example, a stock loading of 25 animals per litre would be suitable for test regimes employing replicates of five animals in approximately 200 mL of test solution.

The stock culture should be fed freshly prepared unicellular green algae from laboratory cultures. Food algae should be in the exponential growth phase. Algae cultures can be used as long as they are growing without showing degradation effects. Harvested algae should not be stored at room temperature for a long time, because then degradation processes occur. They can be stored in a refrigerator in the dark or can even be frozen as long as cultures fed with these algae are still healthy and in a good reproduction status. Food should not be overdosed. It is recommended that stock cultures be fed with carbon levels at 0,1 mg per organism day to 0,2 mg per organism day. To avoid the transfer of algae growth medium to stock culture it is recommended that the algae be separated from the algae growth medium and resuspended in culture medium. For stock culture a regular maintenance is necessary. Change the medium at least 2 to 3 times per week. Transfer the adults to a clean vessel with new medium and separate the neonates from the stock. Remove the exuviae, the dead and/or discoloured animals and the feeding residues.

Stock culturing of *Daphnia magna* shall be carried out under a 16 hour light and 8 hours dark photoperiod of diffuse daylight or artificial daylight. The testing atmosphere shall be at 20 ± 2°C and free from vapours or dusts toxic to *Daphnia magna*.

13.5.2 Hatching of ephippia

Hatching (See Figure 13-2) of the ephippia must be initiated 3 to 4 days prior to the start of the toxicity test.

- Mark a 250 mL airline cup with the date, the ISO medium batch number and the ephippia batch number

- Pour the contents of one vial with ephippia into the micro sieve and ensure that all the ephippia is transferred
- Rinse the ephippia thoroughly with tap water to eliminate all traces of the storage medium (± 2 minutes)
- Add 100 mL prepared ISO medium to a 250 mL airline cup and transfer the rinsed ephippia into the cup
- Cover the hatching 250 mL airline cup and incubate for ± 72 hours, at 21 ± 1°C under continuous illumination of light intensity as described under “Light conditions” above

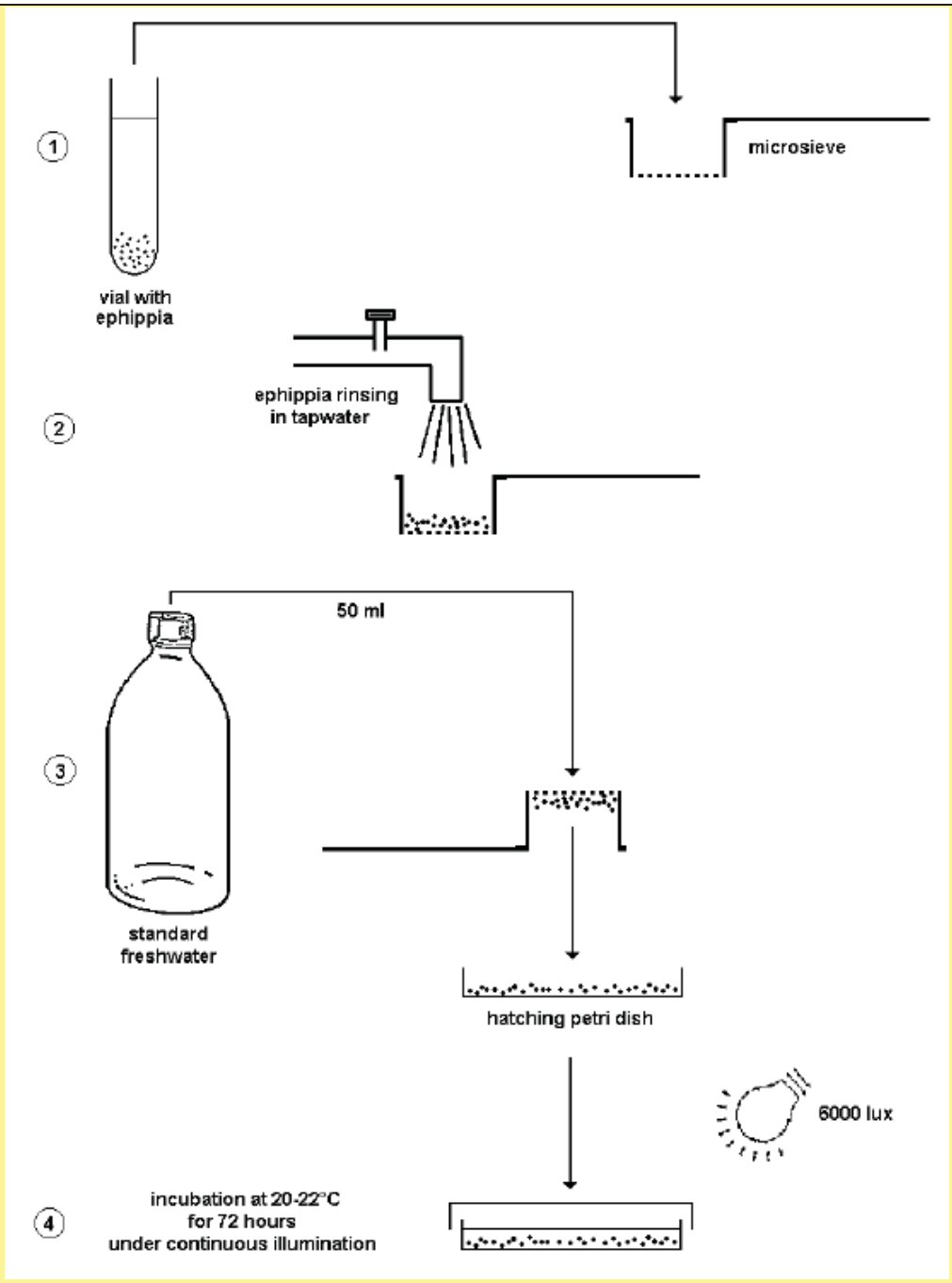

Figure 13-2: Hatching procedure for *Daphnia magna* ephippia.

Important considerations regarding ephippia hatching:

- The embryonic development of *Daphnia magna* eggs takes about three days in optimal conditions
- Under the illumination and temperature conditions indicated, the first neonates may even appear before 72 hours incubation, but the largest hatching will occur between 72 and 80 hours of incubation
- Considering that a minimum of 120 neonates are needed to perform one complete test and that the neonates should not be older than 24 hours at the start of the toxicity test the organisms must be collected at the latest 90 hours after the start of the incubation

13.5.3 Pre-feeding of the test organisms

- Pre-feeding of neonates provides them with an “energetic reserve” and precludes mortality by starvation (which would bias the test results) during the subsequent 48 hour test exposure during which the organisms are not fed
- In order to provide the neonates hatched from the ephippia with food prior to the test, a 2 hour “pre-feeding” is applied with a suspension of *Spirulina* micro-algae
- Fill one vial *Spirulina* powder with ISO medium (± 1 mL)
- Shake the vial thoroughly to homogenize the contents
- Two hours prior to collecting the neonates for the test, pour the *Spirulina* suspension into the hatching 250 mL airline cup and mix the contents gently for even distribution by re-pipetting the medium a few times in the cup. Take note not to suck up the neonates

13.6 TEST PROCEDURE

13.6.1 Test dilutions

- The test should comprise at least five concentrations of the sample to be tested. Select the dilutions within a geometric series with a separation factor which depends on the nature of the sample to be analysed (chemical substances, effluents, waters or extracts) and on the type of assay (range finding or definitive)
- For the range finding test with chemical substances, the separation factor for the serial dilutions is usually 10 (one order of magnitude difference between two successive dilutions)
- For treated or untreated wastewater, freshwater, porewater or extracts, a separation factor of two between dilutions is usually performed (i.e. dilution of the previous dilution by half)
- Dilution series for the definitive test on chemical substances are prepared with a separation factor not exceeding 3,2
- If steep concentration-response curves are expected, it is recommended that a separation factor not exceeding 2,2 be used
- Each dilution is preferably carried out in four replicates with a control also in four replicates
- Substances which are poorly soluble in water may be solubilized or dispersed directly in pure water or dilution water by suitable means using ultrasonic devices or solvents of low toxicity to *Daphnia*

magna. Solvents should be used only when the LC_{50} is greater than the solubility of the test substance. If a solvent is used, the concentration of the solvent in the final test solution shall not exceed 0,1 mL/L, and two control solutions, one with no solvent, the other with the maximum concentration of solvent, shall be included in the test. Consider special requirements concerning test design for chemicals with solvents, e.g. additional solvent-control and statistical evaluations according to ISO/TS 20281 (2006)

13.6.2 Test

- Prepare a dilution series with the test sample and the dilution water
- Combine increasing volumes of the test solution with the dilution water, so as to obtain the desired concentrations for the test and transfer to the test containers
- To obtain a test and solution temperature of 20 ± 2°C, for example, place the containers in a temperature-controlled room
- As soon as this temperature is attained, introduce the *Daphnia magna* into the test containers with the pipette, taking care to add as little hatching medium as possible, and release the crustaceans under the water surface
- At least 20 animals, preferably divided into four groups of five animals each, should be used at each test concentration and for the controls. At least 2 mL of test solution should be provided for each animal (i.e. a volume of 10 mL for five test organisms per test container)
- For each series of tests, prepare a control having a volume of dilution water equal to the volume of the test solutions and introduce the same number of *Daphnia magna* as in the test solutions. If a solvent is used to solubilize or disperse substances, prepare a second control with the dilution water containing the solvent at the maximum concentration used (i.e. not greater than 0,1 m/L)
- Animals shall not be fed during the test and test containers shall be maintained in a temperature-controlled room or chamber at a temperature of 20 ± 2°C. Observations of test organism responses are made at the end of the exposure time
- At the end of the test period of 24 or 48 hours, count the immobile *Daphnia magna* in each container. Those which are not able to swim after gentle agitation of the liquid for 15 seconds shall be considered to be immobilized, even if they can still move their antennae
- Determine the concentration range giving 0% to 100% immobilization and note anomalies (e.g. lethargy, floating on the surface, abnormal rotating, or circling) in the behaviour of the *Daphnia magna*

13.6.3 Preliminary test

This test enables determination of the range of concentrations over which the definitive test is to be carried out. For this purpose, use only a single series of concentrations (generally chosen in geometric progression) of stock solution or sample. Five *Daphnia magna* should be exposed to each test concentration, and no replicates are necessary. Depending on the purpose of the test and the statistical requirements concerning

the test results, other dilution designs with concentrations in a geometric or a logarithmic series can also be appropriate.

13.6.4 Definitive test

- This test determines the percentage of *Daphnia magna* which are immobilized by different concentrations, the 24 hour LC_{50} or 48 hour LC_{50}
- For the calculation of an LC_{50} value, it is desirable that the range of concentration chosen results in at least three percentages of immobilization between 10% and 90%
- For each concentration and each control, use a minimum of 20 *Daphnia magna*, preferably divided into four replicates, with five animals per test container
- Immediately after counting the immobilized *Daphnia magna*, measure the dissolved oxygen concentration (see ISO 5814, 2012) in the test containers with the control batch and with the most concentrated test batch (if necessary, pour the contents of all containers corresponding to this concentration into one container, taking suitable precautions so as not to modify the dissolved oxygen content)
- If the dissolved oxygen concentration in the most concentrated test batch drops below 2 mg/L, the dissolved oxygen concentration shall be measured in the other test batches to check whether they meet the required 2 mg/L minimum concentration. Any test batches with dissolved oxygen concentration below 2 mg/L shall not be considered for the final calculations

13.6.5 Filling the test plate

For filling the test plates (see Figure 13-3 below)

- For a statistically acceptable evaluation of the effects, each test concentration as well as the control must be assayed in four replicates. Each multi-well plate is provided with four test wells for the controls and four test wells for each toxicant concentration
- Additionally, the multi-well test plates are provided on the left side with a column of "rinsing wells". These rinsing wells serve to prevent dilution of the toxicant in the multi-well cups during the transfer of the test organisms from the hatching cup to the test plate

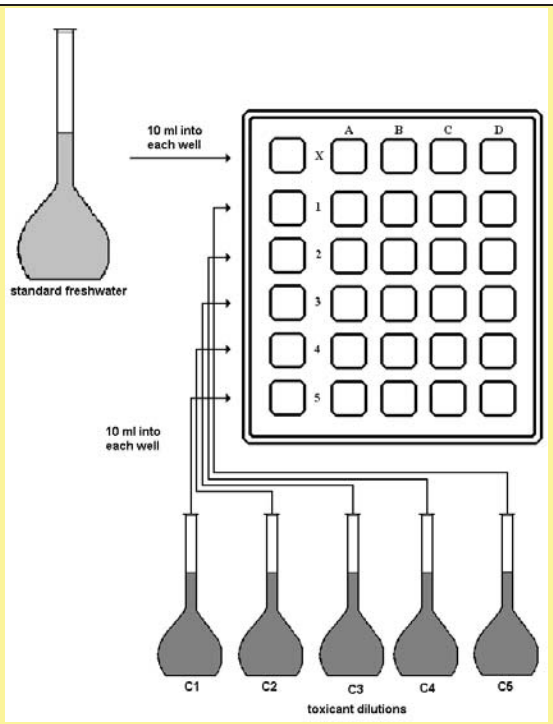

Figure 13-3: Filling of the test plates

- The test wells in each column are labelled A, B, C and D and the rows are labelled X (controls), 1, 2, 3, 4 and 5 for the five toxicant dilutions. All the wells of each row must be filled with one toxicant dilution (or with the dilution medium for the control row)
- Transfer 10 mL dilution water into each well of the control row and 10 mL of the respective toxicant concentrations into each well of the corresponding rows, in the sequence of increasing toxicant concentrations

13.6.6 Transfer of neonates to the test well and incubation

- After filling the test plates the neonates are ready for transfer. The transfer of the neonates (see Figure 13-4) into the test wells is performed with the aid of a micropipette. Place the multi-well plate on the lightbox to enhance vision when transferring neonates
- Transfer 20 neonates (active and swimming) from the hatching airline cup into the rinsing wells of the multi-well plate with the use of a micropipette. Alternatively, the neonates may be transferred into a 5 mL petri dish first and then transferred to a multi-well plate
- Ensure to carry over as little as possible liquid from the Petri dish /Airline cups to the wells during this transfer and rinse the micropipette thoroughly after each transfer to prevent contamination
- Transfer exactly five neonates from each rinsing well into the four wells of each row. Count the neonates as they exit the micropipette to be sure of the transfer of exactly five test organisms per well
- Cover the multi-well plate with a strip of parafilm to prevent evaporation
- Place the multi-well plate in a temperature-controlled environment at 20 ± 1°C, in darkness

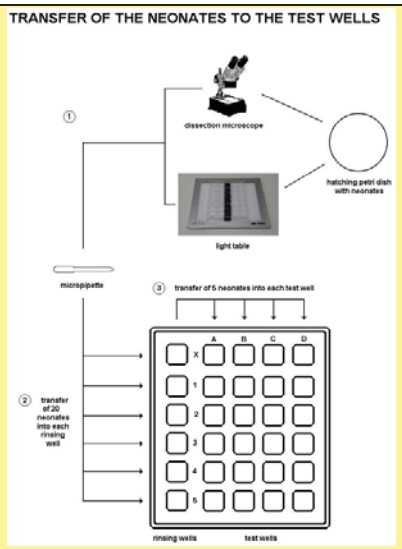

Figure 13-4: Transfer of neonates to the test wells

13.6.7 Floating test organisms

Daphnids are quite susceptible to being trapped at the surface of the medium in the test wells, by the phenomenon of “surface tension”. Once floating, some organisms may not be able to free themselves from the surface and may die as a result. In order to avoid floating organisms which can jeopardise the results, it is of utmost importance during the transfer of the test organisms to put the tip of the micropipette in the solution and not to drop the organism onto the surface of the test solution. If floating during the test drop one or two drops of the test solution on the floating animal.

13.6.8 Scoring of the test results

- Mortalities are recorded after 24 and 48 hours of the incubation period. With the multi-well plate on the lightbox and/or under the dissection microscope, record the number of dead and immobilized neonates, versus that of the actively swimming test organisms in each well

- If any neonates are floating drop one or two drops of test solution on floating organism in order to submerged them

- The neonates who are not able to swim after gentle agitation of the liquid for 15 seconds shall be considered immobilized, even if they can still move their antennae. Death is indicated by a lack of movement after gentle prodding with a needle/pipette tip. Score the number of dead neonates on the relevant data sheets

13.7 CALCULATIONS OF RESULTS

13.7.1 Estimation of the LC_{50}

At the end of the 24 hour or 48 hour test, calculate the percentage immobilization for each concentration in relation to the total number of *Daphnia magna* used. Determine the 24 hour LC_{50} or 48 hour LC_{50} by an appropriate statistical method (Probit analysis, moving average, binomial methods or graphical estimation).

The concentration of the test substance should be measured, as a minimum at the highest and lowest test concentration, at the beginning and end of the test. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within ± 20% of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

If the data are insufficient or calculation of the LC_{50} is not required, quote the minimum concentration corresponding to 100% immobilization and the maximum concentration corresponding to 0% immobilization.

Record the mean percentage immobilization in the control and in each test concentration.

13.7.2 Expression of results

Express the LC_{50}, and the values corresponding to 0% and 100% immobilization:

- as a percentage, in the case of effluents, waters, eluates or extracts or as
- in milligrams per litre, in the case of chemical substances

Determination of the initial concentration (i.e. the concentration present at the beginning of the test) which, in 24 or 48 hours, immobilizes 50% of exposed *Daphnia magna*, under the conditions specified in this International Standard. This concentration, known as the effective initial inhibitory concentration, is designated 24 hour LC_{50} or 48 hour LC_{50}. An indication of the lowest concentration tested which immobilizes all the *Daphnia magna* and the highest concentration tested which does not immobilize any of the *Daphnia magna* is desirable and provides useful information in cases where the LC_{50} cannot be determined.

The test is carried out in one or two stages:

- a preliminary test which determines the range of concentrations to be tested in the definitive toxicity test and gives an approximate value of the 24 hour LC_{50} or 48 hour LC_{50}
- a definitive test, conducted when the approximate value given by the preliminary test is not sufficient, which permits calculation of the 24 hour LC_{50} or 48 hour LC_{50}, and determines concentrations corresponding to 0% and 100% immobilization

If the method specified in this International Standard is used for biotesting of chemical substances, a limit test can be performed at 100 mg/L or at a lower concentration, at which the substance is soluble or is in

stable dispersion under the conditions of the test. If it provides useful information, a limit test may also be performed at concentrations above 100 mg/L as long as the substance is soluble or in stable dispersion.

13.8 TEST VALIDITY

Consider the results as valid if the following conditions are satisfied at the end of the test:

- the percentage immobilization of the controls is less than or equal to 10%
- the 24 hour LC_{50} of the potassium dichromate is within the range 0,6 mg/L to 2,1 mg/L

13.9 INTERPRETATION OF RESULTS

Following the 48 hour check, the data should be entered onto the relevant electronic data sheet where automatically calculations of the total number of dead and immobile neonates for each toxicant concentration will take pace. This must include the mean and the percentage effect.

13.9.1 Screening test calculations

Calculate the percentage mortalities for each sample in relation to the number of test organisms exposed as follows:

Percentage mortality = $100 - [(N_{t0}-N_{tx})/ N_{t0} \times 100]$

N_{t0} Number of test organisms exposed at time zero

N_{tx} Number of dead test organisms after 24 or 48 hours exposure

13.9.2 Definitive test calculations

Calculate the 48 hour LC_{10} and LC_{50} values (toxicity endpoints) by applying an appropriate statistical method (e.g. TSK or Probit). Express toxicity endpoints as a percentage (e.g. LC_{50} of 30%) or sample concentration (e.g. 30 mg/L). In cases where limited mortalities were recorded for the definitive test and a statistical method could not be used to calculate the endpoints (> 50%), these endpoints should be reported as a LC_{10} of 50-100% or as a LC_{50} > 100% or as TU< 1.

13.10 TEST REPORT

This test report shall contain at least the following information:

- the test method used, together with a reference to this International Standard (ISO 6341, 2012)
- all information required for the complete identification of the original sample (before treatment) or of the chemical substance under test
- the methods of preparation of the samples
    - for effluents, waters, eluates and extracts, the method and the storage time of the samples, the pH and the dissolved oxygen concentration of the original sample, if need be, the conditions in which the decantation, filtration or centrifugation of the sample and a possible adjustment of the pH were carried out
    - for chemical substances, the method of preparation of the stock and test solutions
- all biological, chemical, and physical information relative to the test set out in this International Standard, including the origin and the age of the stock culture of the *Daphnia magna* used
- the results of the test in the form of the 24 hour LC_{50} or 48 hour LC_{50}, the method of calculation, and, if possible, the 95% confidence limit; in the case of chemical analysis of the substances, the method used
- the results of the limit test, if conducted
- the minimum tested concentration corresponding to 100% immobilization and the maximum tested concentration corresponding to 0% immobilization in 24 hours or 48 hours
- any abnormal behaviour of the *Daphnia magna* under the test conditions (e.g. lethargy, floating on the surface, abnormal rotating or circling)
- any operating details not specified in this International Standard and incidents which may have affected the results
- the results obtained with the reference chemical as well as the date of the reference test
- data to prove that the validity criteria are met
- name and address of the testing laboratory, the persons carrying out the test, and the person approving the report

13.11 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

13.11.1 Application

According to ISO 6341 (2012) this method is applicable to:

- Chemical substances which are soluble under the conditions of the test, or can be maintained as a stable suspension or dispersion under the conditions of the test
- Industrial or sewage effluents
- Treated or untreated wastewater
- Aqueous extracts and leachates

- Freshwater (surface and groundwater)
- Eluates of freshwater sediment
- Porewater of freshwater sediments

13.11.2 Advantages

The significance of the *Daphnia magna*/*pulex* acute toxicity test is to help in the assessment of possible risk to similar invertebrate species in the natural environment, as an aid in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes (US EPA, 2002). Like rotifers and copepods, Cladocera's such as *Daphnia sp.* are ecologically very important members of freshwater aquatic communities. *Daphnia* are the most used crustacean test species for determination of the effects of xenobiotics on primary consumers in freshwater aquatic ecosystems. Toxicity tests, such as the *Daphnia magna*/*pulex* acute toxicity test, is applied to assess water pollution and are primarily used to screen for toxic substances in the aquatic environment and to some extend to predict the toxic effect of environmental impacts on aquatic invertebrates. A further significance of the test is to comply with the "The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach" (DWAF, 2003).

13.11.3 Limitations

- Dark coloured samples and samples containing high loads of suspended solids may impede the observation of the test organisms
- Samples containing oils and surface tension altering compounds may cause test organisms to float
- Pathogenic and /or predatory organisms in the samples may affect survival
- Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may have an effect on the bioavailability or solubility of certain hazardous substances

13.11.4 RECOMMENDATIONS

This method provides an easy-to-use bioassay for measuring acute toxicity of substances/pollution to invertebrates, such as *Daphnia magna*. The significance of the *Daphnia magna* acute toxicity test is to assist in the assessment of possible risks to invertebrate species in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other invertebrate species for comparative purposes. This test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment (others include but are not limited to invertebrates, algae, vertebrates, and protozoa amongst others). The reason for this is the variation between the sensitivity of the different species to different substances, and therefore using several different trophic levels increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

13.12 REFERENCES

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

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ISO/TS (International Organization for Standardization/Technical Standard) 20281 (2006) Water quality – Guidance on statistical interpretation of ecotoxicity data. ISO/TC 147/SC 5 Biological methods pp252.

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MicroBioTests Inc., Belgium. DAPHTOXKIT F™. Freshwater Toxicity Test with *Daphnia*. Standard Operational Procedure.

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SLABBERT L (2004) Methods for direct estimation of ecological effect potential (DEEEP) WRC Report 1313/01/04. Water Research Commission, Pretoria.

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Protection Agency, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington DC, 20460. Fifth Edition.

13.13 USEFUL CONTACTS

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14 *POECILIA RETICULATA* ACUTE TOXICITY TEST (IMMOBILIZATION TEST)

Compiled by: L Swart and H Pearson

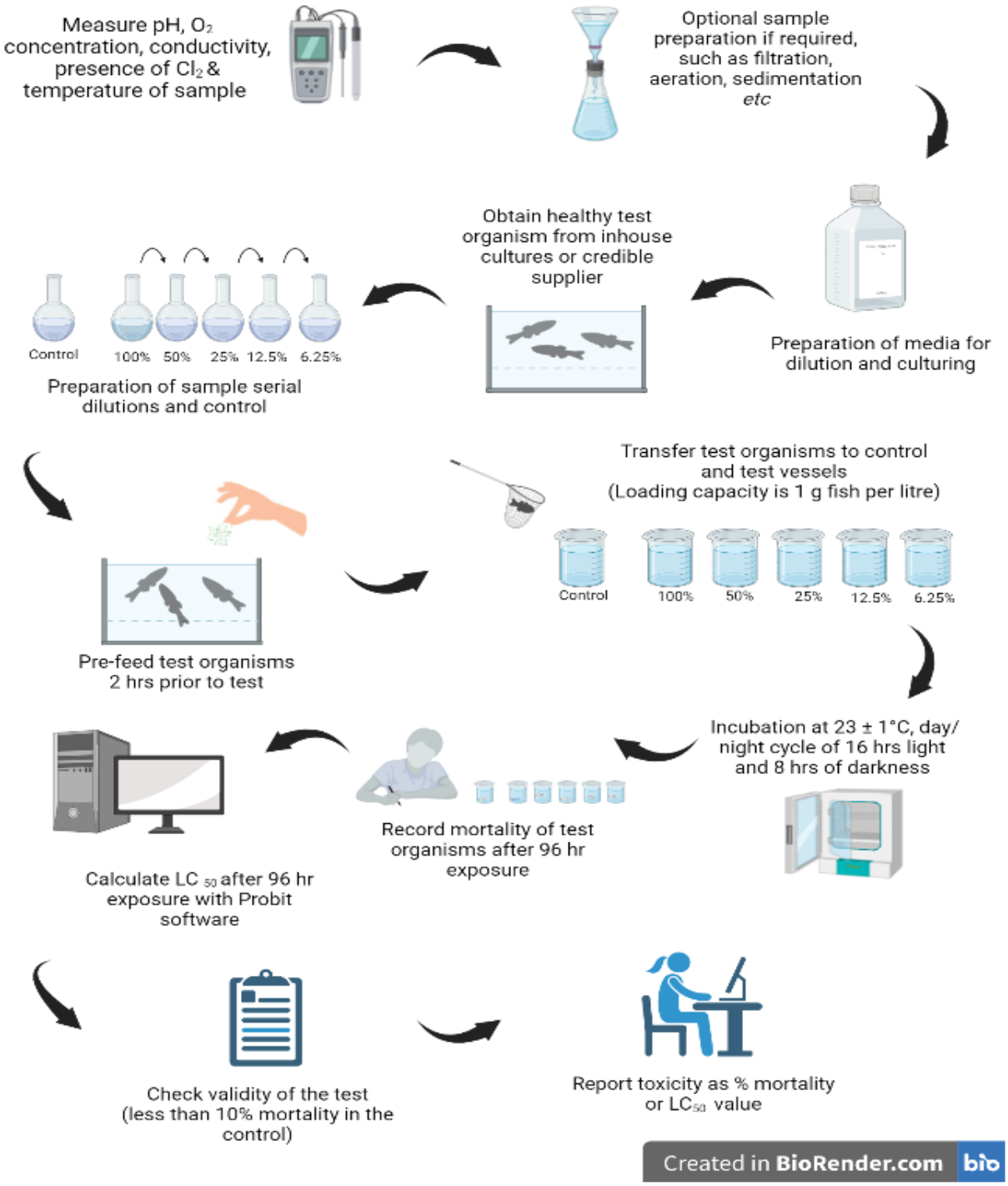

Figure 14-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the POECILIA RETICULATA acute toxicity test (immobilization test)

14.1 ACRONYMS & ABBREVIATIONS

AR	Analytical Grade
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
Cr	Chrome
CV	Coefficient of Variation
d	Day(s)
ºC	Degree(s) Celsius
g	Gram(s)
h	Hour(s)
HCl	Hydrochloric acid
LC_{10}	Lethal effective concentration at 10%
LC_{50}	Lethal effective concentration at 50%
L	Litre(s)
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
µg/L	Microgram(s) per litre
µL	Microliter(s)
mg	Milligram(s)
mg/L	Milligram(s) per litre
mL	Millilitre(s)
HNO_3	Nitric acid
%	Percentage
±	Plus, or minus
KCl	Potassium chloride
$K_2Cr_2O_7$	Potassium dichromate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide

14.2 PRINCIPLE OF THE ASSAY

The fish acute toxicity test measures the short-term acute toxicity of various water samples to specific freshwater fish species, such as *Poecilia reticulata*. Determination, under specified conditions of the concentrations at which a substance is lethal to 50% of a test population of *Poecilia reticulata* after exposure periods of 24 hours, 48 hours, 72 hours and 96 hours to that substance in the ambient water. These median lethal concentrations are designated the 24 hour LC_{50}, 48 hour LC_{50}, 72 hour LC_{50} and 96 hour LC_{50}.

This procedure can be based on SANS 7346-1 (2013) or ISO 7346-1 (1996), “Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]. Part 1: Static method as well as other international standards such as US EPA:

(2002). “Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms Testing of Chemicals”.

14.3 REQUIREMENTS

14.3.1 Acquisition of test organisms

The fish should be in good health and free from any apparent malformation. The fish can be bred and cultivated either in fish farms or in the laboratory, under disease- and parasite-controlled conditions, so that the test fish will be healthy and of known parentage.

If fish are not bred inhouse the test organisms must be obtained from approved suppliers at an age of ± 7 days on receipt. These juveniles are then acclimatized for at least three days (from on-site supplier) and 3 to 5 days for external suppliers, before they are used for testing. The batches should be monitored during this period and a decision made at the discretion of laboratory as to whether a batch can be used for testing or not. Juvenile guppies of this age are generally 7 mm to 15 mm in length and should not be used after the tails are defined (male or female) which occurs after ± 21 days of age as per available literature.

When guppies are delivered, the container should be placed in a guppy holding tank to adapt to temperature for 15 to 30 minutes. If most of the water in the plastic holder or container will fill the tank the guppies can be transferred immediately. However, this water (e.g. wetland) should be diluted with at least a quarter of inhouse water after which daily top ups with this water must be performed to ensure that, e.g. high salt loads (used for treatment of diseases by breeders) are diluted before placing the fish in the fresh testing water. Dates of receipt and use are noted to keep track of ages and no fish older than 21 days may be used for testing.

Mark the holding tank with the delivery date and name of the supplier for traceability purposes. Fish must be held under the following conditions:

- Light: 12 to 16 hours photoperiod daily
- Temperature: appropriate to the species
- Oxygen concentration: at least 90% of air saturation value
- Fish should be fed three times per day and half an hour before the test is started (e.g. last feed with acclimatization period in testing room)

14.3.2 STAFF TRAINING (TECHNICAL SKILLS)

To perform the test, the analyst should be proven competent in

- Good Laboratory Practice (GLP)
- Trained in an aquatic ecotoxicology laboratory
- Software packages knowledge (e.g. EXCEL)

14.3.3 LABORATORY (TEST ENVIRONMENT)

- The preparation and storage of solutions, the holding of fish, and all the manipulations and tests shall be carried out in premises with an atmosphere free from harmful concentrations of airborne contaminants
- Take care to avoid any unwanted disturbance that may change the behaviour of the fish. Carry out all tests under normal laboratory illumination with a daily photoperiod of 12 hours to 16 hours
- The testing facilities must be separate from the holding/culturing facilities
- The use of controls also allows checking that the test is performed in an atmosphere free from toxic dusts and vapours
- Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage
- The exposure of organisms as specified in this International Standard shall be carried out either in the dark or under 16 hours light and 8 hours dark photoperiod, in a temperature-controlled room or incubator at 23 ± 1°C in the test containers
- Temperature control equipment should be adequate to maintain the recommended environmental conditions as stated in the standard, during storing of samples, testing and culture maintenance

14.3.4 SOFTWARE

- EXCEL spreadsheet
- REGTOX for calculations
- TSK (Trimmed Spearman Karber) or
- Probit

14.3.5 APPARATUS

The following apparatus will be required to perform the test:

- Temperature controlled room
- Adjustable transfer pipettes and pipette tips (10-10 000 μL)
- pH-meter (accuracy 0,1 pH units),
- Conductivity meter
- Oxygen meter
- Thermometers
- Stopwatch
- Fridge
- Fish tanks and accessories

14.3.6 CONSUMABLES/MATERIALS

Table 14-1: List of consumables/materials

Name	Cat no	Supplier
Juvenile Guppies	NA	Local supplier
2 L volumetric flask		General supplier
25 mL measuring cylinders		General supplier
100 mL measuring cylinders		General supplier
250 mL measuring cylinders		General supplier
1000 mL measuring cylinders		General supplier
50 mL plastic/glass beakers		General supplier

14.3.7 Reagents

Use chemicals of recognised analytical grade (AR grade), unless otherwise specified.

Table 14-2: List of reagents

Name	Cat no	Supplier
Sodium hydroxide (NaOH), 1N	-	General supplier
Hydrochloric acid (HCl), 1N	-	General supplier
Nitric acid, HNO_3	-	General supplier
Acetone	-	General supplier
Reference stock solution, such as 1 g/L Potassium dichromate	-	General supplier General supplier
Water, deionized or of equivalent purity (conductivity <10 µS/cm)	-	General supplier General supplier
Chlorine testing kits	-	General supplier
$CaCl_2 \cdot 2H_2O$	-	General supplier
$MgSO_4 \cdot 7H_2O$	-	General supplier
$NaHCO_3$	-	General supplier
KCl	-	General supplier

14.4 SAMPLING METHOD

14.4.1 Sample collection

- Sampling, transportation and storage of the samples should be performed as specified in ISO 5667-16 (1998)
- Collect samples in chemically inert and clean containers
- Rinse the bottle with the sample before filling it.
- Fill the containers completely to the top of container and seal if possible
- Test the samples as soon as possible after collection as indicated in the standard
- Where necessary, store samples at $5 \pm 2°C$
- Do not use chemicals to preserve the samples

14.4.2 Sample preparation

- Carry out the toxicity test as soon as possible, preferably within 12 hours of collection. If this time interval cannot be met, cool the sample to 5 ± 2°C and test the sample within 24 hours
- At the time of testing, homogenize the sample to be analysed by shaking manually
- High concentrations of suspended inorganic or organic solids in a sample can be harmful to the test organisms. Compensation for this interference can be made by a sample treatment for turbidity. If necessary, allow to settle for a maximum of 2 hours in a container, and sample, e.g. by drawing off the required quantity of supernatant using a pipette, maintaining the end of the pipette in the centre of the section of the test container and halfway between the surface of the deposited substances and the surface of the liquid. If the raw sample of the decanted supernatant is likely to interfere with the test (due to presence of residual suspended matter, protozoa, microorganisms, etc.), centrifuge, for example, for 10 minutes at 5 000*g* or filter the raw or decanted sample. Test the residual toxicity of the supernatant. The particular kind of filter to be used should be checked by a test with control medium run through the filters
- Usually, no aeration of sample or prepared test concentrations is necessary. If, and only if, the dissolved oxygen is < 40% saturation, a pre-aerate of the sample or all test solutions for at most 20 minutes by appropriate methods, e.g. aeration or stirring may be performed. Any supersaturation should be remedied. Report any pre-aeration of test solutions or sample
- Measure the pH (as specified in ISO 10523, 2008) and the dissolved oxygen concentration (as specified in ISO 5814, 2012) and record these values in the test report. Tests shall be carried out without pH adjustment of the test sample. The pH of test batches is measured at the beginning and at the end of the test and reported. However, in some cases, the final pH of a test solution may significantly differ from original pH of the test sample due to the concentration range selected and as a result of the buffer capacity of the dilution water or test sample. If toxic effects are observed at concentrations where the pH is not compatible with the survival of the organisms (i.e. outside the pH 6,0 to pH 9,0 range), the test(s) can be repeated with pH adjustment of the test sample.

Note: Adjustment of the pH can alter the nature of the sample.

- If the pH is to be adjusted, the recommendation is to adjust to the pH of the dilution water selected. Choose the concentration of the hydrochloric acid or the sodium hydroxide solutions to restrict the volume fraction added to not more than 5%. If, as a result of pH adjustment, there is an issue with suspended matter, separate the suspended matter from the remaining sample as specified in ISO 5667-16 (1998). Any pH adjustment shall be included in the test report
- Adjust the temperature of the pre-treated sample to the test temperature
- Measure the pH, dissolved oxygen, conductivity, and free chlorine of the sample prior to analysis. Usually, the test shall be carried out without adjustment of the pH of the medium after addition of the test sample. However, some substances may exert a toxic effect due to extreme acidity or alkalinity. In order to determine the toxicity of a sample independent of pH, adjust the pH of the aqueous sample or stock solution (before the dilution in series) to that of the culture medium using either 1 mol/L hydrochloric acid (HCl) or 1 mol/L sodium hydroxide solution (NaOH) (see ISO 5667-16, 1998)

Note: the appearance of the sample (e.g. colour, turbidity, and odour) prior to testing.

14.5 TEST PREPARATION

14.5.1 Preparation of standard freshwater used for dilution and culturing

- The freshly prepared standard dilution water shall have a pH of 7,8 + 0,2, and a calcium hardness of approximately 250 mg/L, expressed as calcium carbonate, and shall contain the following concentrations of salts dissolved in distilled or deionized water
    - 294,0 mg/L $CaCl_2.2H_2O$
    - 123,3 mg/L $MgSO_4.7H_2O$
    - 63,0 mg/L $NaHCO_3$
    - 5,5 mg/L KCl
- Aerate the dilution water until the concentration of dissolved oxygen reaches at least 90% of its air saturation value and the pH is constant at 7,8 + 0,2. If necessary, adjust the pH of the solution by adding sodium hydroxide solution or hydrochloric acid. The dilution water thus prepared shall receive no further forced aeration before use in the tests
- If necessary, adjust the pH to 7,8 ± 0,2 by adding sodium hydroxide (NaOH) solution or hydrochloric acid (HCl)
- Store the standard freshwater in the fridge at 4 ± 2°C

The standard freshwater will be used as hatching medium for the test organism, as dilution medium for preparation of the toxicant dilution series and for the control samples

14.5.2 Loading

The volume of the test vessels should be sufficient that a loading rate of 1 g of fish per litre of water should not be exceeded at any time during the test.

14.5.3 Pre-feeding of the test organisms

Pre-feeding of neonates provides them with an “energetic reserve” and precludes mortality by starvation (which would bias the test results) during the subsequent 96 hours test exposure during which the organisms are not fed.

14.6 TEST PROCEDURE

14.6.1 Screening test

- A screening test is performed on water samples with an unknown toxicity. Receiving water toxicity tests generally consist of 100% receiving water and a control
- Preparation room temperature should be at 23 ± 1°C
- Samples should be inverted prior to testing to ensure homogeneity and the re-suspension of matter. At least 200 mL water sample will be required for a screening test
- All tests are done in duplicate with 5 or more test organisms per vessel as follows
- Mark two 250 mL cups as controls and add 100 mL dilution medium in each
- Mark two 250 mL cups with the relevant sample name and add 100 mL of the sample in each
- Gently add 5 test organisms to each cup
- Note the date and time at the start of the test
- After 24, 48, 72 and 96 hours note the mortality in each cup

14.6.2 Definitive test

- The test should comprise at least five concentrations of the sample to be tested. Select the dilutions within a geometric series with a separation factor which depends on the nature of the sample to be analysed (chemical substances, effluents, waters or extracts) and on the type of assay (range finding or definitive)
- For treated or untreated wastewater, freshwater, pore water or extracts, a separation factor of 2 between dilutions is usually performed (i.e. dilution of the previous dilution by half)
- Dilution series for the definitive test on chemical substances are prepared with a separation factor not exceeding 3, 2
- At least 600 mL water sample will be required for a definitive test
- In cases where the objective of the test is to estimate the degree of toxicity of the receiving water, a definitive, multi-concentration test is performed by preparing dilutions of the receiving water
- Mark two 250 mL cups as control and add 100 mL dilution medium to each cup
- Mark two 250 mL cups with the relevant sample name and concentration (100%, 50%, 25% 12%, 6%)
- Prepare the serial dilution of the sample
- Gently add 5 test organisms to each cup
- Note the date and time at the start of the test
- After 24, 48, 72 and 96 hours note the mortality in each cup

14.6.3 Transfer of test organisms

- Prepared sample, control and temperature control samples are now moved to the guppy testing lab, where room temperature will be adjusted to increase water temperature to the desired level (23 ± 1°C). A verified thermometer is placed in the temperature control cup to monitor temperature
- Fry are caught in the guppy holding room and placed in guppy lab for ± 30 minutes in a separate container to adjust to temperature. Check the temperature of the water from which they are transferred and ensure that it doesn't differ more than 2°C from the testing water before transferring fry. Minimum of 10 fry is used per screening test and per definitive dilution concentration (five per 250 mL cup)
- Transfer fry to airline cups with the aid of a micro-sieve net, guppy fry should be handled with utmost care to prevent injury and stress as this will increase mortality rate. Ensure that the least possible liquid is transferred to samples with micro-sieve net to prevent dilution of the sample. Recount fry after transfer to ensure that 10 or more fry per sample is transferred (5 or more per cup). Rinse micro-sieve net when in contact with sample to prevent cross-contamination
- Record guppy batch used. Record the number of guppies used on the laminated register in guppy lab to ensure the guppies are used within expiry date specified
- Make a note on the relevant report form of any change in appearance of the water sample during dilution (e.g. colour change, precipitation, flocculation, and the release of volatile substances)

14.6.4 Scoring of the test results

- Mortalities are recorded after 24, 48, 72 and 96 hours of the incubation period
- Record the number of dead and immobilized fry, versus that of the actively swimming test organisms in each cup
- Dead test organisms are not removed during the test

14.7 CALCULATIONS OF RESULTS

14.7.1 Estimation of the LC_{50}

Where a simple graphical estimation of the LC_{50} is considered adequate, this can be obtained by plotting mortality (expressed as a percentage of test fish in each test vessel) against concentration of test sub-stance. Using axes with linear scales, this will produce a sigmoid relationship from which the LC_{50} can be derived by interpolating the concentration expected to cause 50% mortality.
It is more appropriate to plot the data on graph paper having axes with logarithmic and probability scales. Data plotted in this way should produce a linear relationship from which the LC_{50} can be interpolated.

If insufficient data are available to estimate the LC_{50} at 24 hours and 48 hours and if available, at 72 hours and 96 hours, record the minimum concentration in which 100% mortality occurred and the maximum concentration giving 0% mortality at 24 hours; 48 hours; 72 hours and 96 hours.

14.7.2 Expression of results

Express the LC_{50}, and the values corresponding to 0% and 100% immobilization:

- As a percentage, in the case of effluents, waters, eluates or extracts or as
- In milligrams per litre, in the case of chemical substances

14.8 TEST VALIDITY

The results shall be considered valid if the following requirements are fulfilled:

- the dissolved oxygen concentration in the test solutions during the test was at least 60% air saturation value
- the concentrations of the test substance were not known (or suspected) to have declined significantly throughout the test
- the mortality of the control fish did not exceed 10% or one per tank
- the proportion of control fish showing abnormal behaviour did not exceed 10% or one per tank
- the 24 h, LC_{50} of the reference chemical (e.g. potassium dichromate), if available, for the stock of fish was in reasonable agreement with results obtained previously in the same laboratory

14.9 INTERPRETATION OF RESULTS

Following the 96 hours check, the data should be entered onto the relevant electronic data sheet where calculations of the total number of dead and immobile neonates for each toxicant concentration will automatically take pace. This must include the mean and the percentage effect.

14.9.1 Screening test calculations

Calculate the percentage mortalities for each sample in relation to the number of test organisms exposed as follows:

Percentage mortality = $100 - [(N_{t0}-N_{tx})/ N_{t0} \times 100]$

N_{t0} Number of test organisms exposed at time zero

N_{tx} Number of dead test organisms after 96 hours exposure

14.9.2 Definitive test calculations

Calculate the 48 hour LC_{10} and LC_{50} values (toxicity endpoints) by applying an appropriate statistical method (e.g. TSK or Probit). Express toxicity endpoints as a percentage (e.g. LC_{50} of 30%) or sample concentration (e.g. 30 mg/L). In cases where limited mortalities were recorded for the definitive test and a statistical method could not be used to calculate the endpoints (> 50%), these end-points should be reported as a LC_{10} of 50-100% or as a $LC_{50} > 100\%$ or as $TU < 1$.

14.10 TEST REPORT

The test report shall include the following information:

- a reference to this part of ISO 7346 (1996)

- the chemical identity and any additional available information about the test substance (e.g. water solubility, volatility, octanol/water partition coefficient, degradation rate)
- the method of preparing the dilution water, stock solutions and test solutions
- all chemical, biological and physical data pertaining to the test and not otherwise specified in ISO 7346 (1996), including details of the acclimatization conditions of the test fish, and the mass of fish, in grams per litre
- the data to considered when assessing the validity of the test:
    - concentration of dissolved oxygen
    - mortality observed among control fish
    - proportion of control fish showing abnormal behaviour
    - LC_{50} of the reference substance
- a list showing the nominal concentrations tested (with chemical analytical values, where available), and the total percentage mortalities in each 24 hours, 48 hours, 72 hours, and 96 hours after the start of the test
- the LC_{50} values and confidence limits, if available, at 24 hours, 48 hours, 72 hours, and 96 hours of the substance tested; reference should be made to the method of calculation, and the method of chemical analysis, where applicable
- the slope of the concentration-response curve (and its 95% confidence limit if available)
- a graphical illustration of the concentration- response relationship
- any unusual reactions by the fish under the test conditions and any visible external effects produced by the test substance
- any deviation from the procedure specified in ISO 7346 (1996), and the reason for it

14.11 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

14.11.1 APPLICATION

According to ISO 73416 (1996), this method is applicable to:

- Chemical substances which are soluble under the conditions of the test, or can be maintained as a stable suspension or dispersion under the conditions of the test
- Industrial or sewage effluents
- Treated or untreated wastewater
- Aqueous extracts and leachates
- Freshwater (surface and groundwater)
- Eluates of freshwater sediments
- Porewater of freshwater sediments

14.11.2 advantages

The significance of the *Poecilia reticulata* acute toxicity test is to help in the assessment of possible risk to similar vertebrate species in the natural environment, as an aid in determination of possible water quality

criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes (US EPA, 2002). Toxicity tests, such as the *Poecilia reticulata* acute toxicity test, is applied to assess water pollution and are primarily used to screen for toxic substances in the aquatic environment and to some extend to predict the toxic effect of environmental impacts on aquatic invertebrates. A further significance of the test is to comply with the "The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach" (DWAF, 2003).

The significance of the acute toxicity fish test is to help in the assessment of possible risk to similar species in the natural environment, as an aid in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes (US EPA, 2002).

14.11.3 Limitations

- Dark coloured samples and samples containing high loads of suspended solids may impede the observation of the test organisms
- Pathogenic and/or predatory organisms in the samples may affect survival
- Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may influence the bioavailability or solubility of certain hazardous substances

14.11.4 RECOMMENDATIONS

This method provides an easy-to-use bioassay for measuring acute toxicity of substances/pollution to vertebrates, such as *Poecilia reticulata*. The significance of the *Poecilia reticulata* acute toxicity test is to assist in the assessment of possible risks to fish species in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other invertebrate species for comparative purposes. This test should form part of a battery of at least 3 tests representing different trophic levels in the aquatic environment (others include but are not limited to bacteria, invertebrates, algae, vertebrates, and protozoa amongst others). The reason for this is the variation between the sensitivity of the different species to different substances, and therefore using several different trophic levels increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

14.12 REFERENCES

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.
ISO (International Organization for Standardization) 8402 (1994) Quality Management and Quality Assurance - Vocabulary. ISO/TC 176/SC 1 Concepts and terminology pp39.
ISO (International Organization for Standardization) 7346-1 (1996) Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)] – Part 1: Static method. ISO/TC 147/SC 5 Biological methods pp11.
ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.
ISO (International Organization for Standardization) 10523 (2008) Water quality – Determination of pH. ISO/TC 147/SC 2 Physical, chemical and biochemical pp13.
ISO (International Organization for Standardization) 5814 (2012) Water quality – Determination of dissolved oxygen – Electrochemical probe method. ISO/TC 147/SC 2 Physical, chemical and biochemical pp14.
ISO/TS (International Organization for Standardization/Technical Standard) 20281 (2006) Water quality – Guidance on statistical interpretation of ecotoxicity data. ISO/TC 147/SC 5 Biological methods pp252.
SANS (South African National Standard) 7346-1 (2013) Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyrinidae)] Part 1: Static method.
SLABBERT L (2004) Methods for direct estimation of ecological effect potential (DEEEP) WRC Report 1313/01/04. Water Research Commission, Pretoria.

US EPA (United States Environmental Protection Agency) (2002) Method for measuring the acute toxicity of effluents and receiving water to freshwater and marine organisms. EPA-821-R-02-012. U.S. Environmental Protection Agency, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington DC, 20460. Fifth Edition.

14.13 USEFUL CONTACTS

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15 *SPIRODELA POLYRHIZA* GROWTH INHIBITION TEST

Compiled by: L Swart and H Pearson

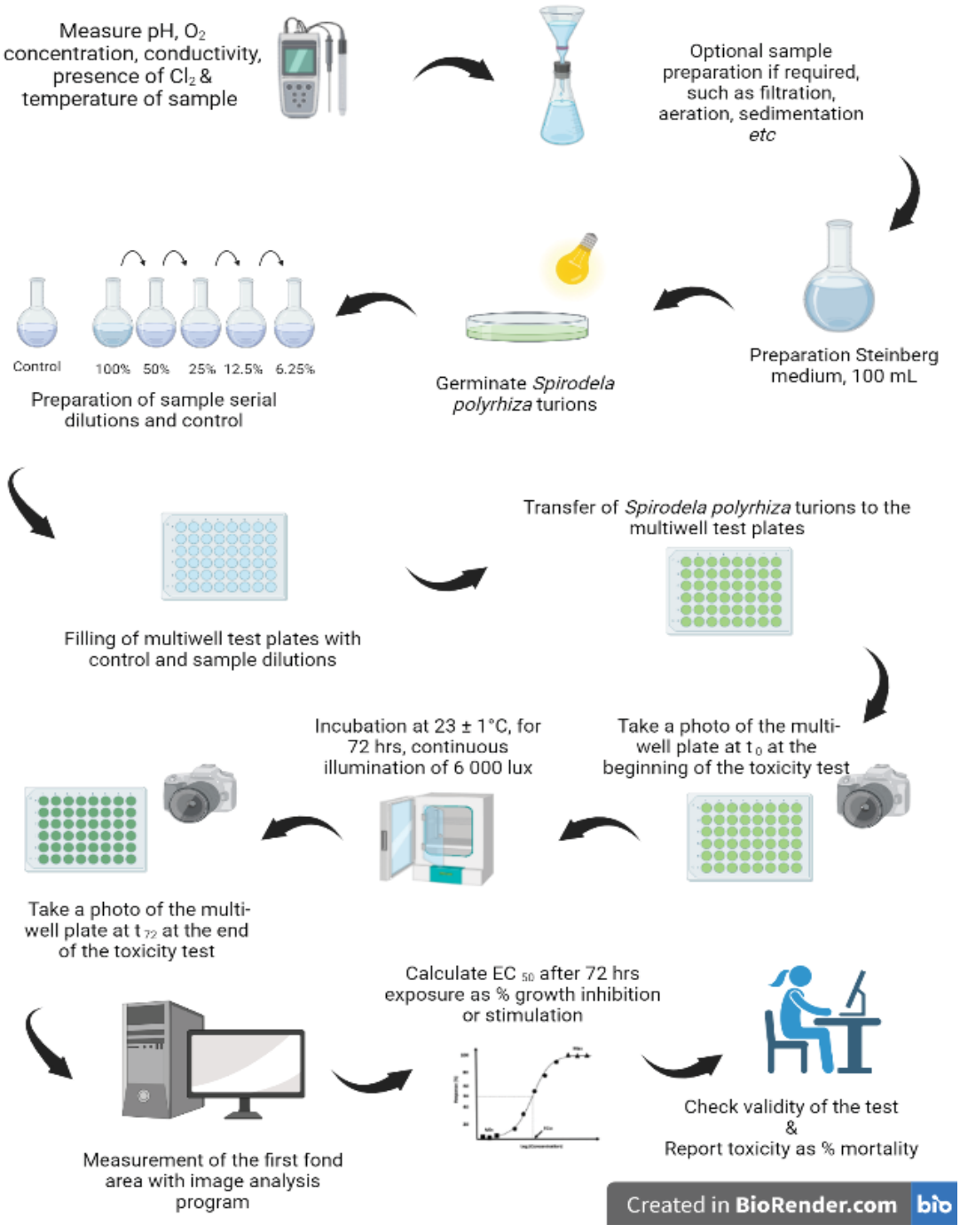

Figure 15-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the SPIRODELA POLYRHIZA growth inhibition test

15.1 ACRONYMS & ABBREVIATIONS

AR	Analytical Grade
H_3BO_3	Boric Acid
$Ca(NO_3)_2 \cdot 4H_2O$	Calcium nitrate tetrahydrate
CV	Coefficient of Variation
d	Day(s)
ºC	Degree(s) Celsius
$C_{10}H_{18}N_2Na_2O_{10}$	EDTA disodium-dihydrate
EC_{20}	Effective concentration at 20%
EC_{50}	Effective concentration at 50%
g	Gram(s)
h	Hour(s)
HCl	Hydrochloric acid
$FeCl_3 \cdot 6H_2O$	Iron trichloride hexahydrate
L	Litre(s)
$MnCl_2 \cdot 4H_2O$	Manganese (II) chloride solution
$MgSO_4 \cdot 7H_2O$	magnesium sulphate heptahydrate
µg/L	Microgram(s) per litre
mg	Milligram(s)
mg/L	Milligram(s) per litre
mL	Millilitre(s)
HNO_3	Nitric acid
%	Percentage
±	Plus, or minus
KCl	Potassium chloride
KNO_3	Potassium Nitrate
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Potassium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate dihydrate
$ZnSO_4 \cdot 7H_2O$	Zinc sulphate heptahydrate

15.2 PRINCIPLE OF THE ASSAY

Vegetative buds (turions) of the duckweed species *Spirodela polyrhiza* stored in test tubes are “germinated” in a Petri dish containing growth medium. The Petri dish is incubated for 3 days at 25°C under continuous illumination of 6 000 lux.

The germinated turions are transferred into the cups of a 48 cups multi-well test plate containing the toxicant dilutions series, and a digital picture of the test plate is then taken and saved on a computer. The test plate is subsequently incubated for 3 days at 25°C with continuous illumination, after which a second digital picture is taken.

The size (the area) of the small “first frond” of the duckweed is measured in the test cups at the start of the toxicity test and after 3 days incubation, with the aid of an image analysis program, such as Image J. The growth of the duckweed is calculated by subtracting the mean initial size at t_0 hours of the first frond from the mean final size at t_{72} hours, in the control and in the different toxicant concentrations. The percentage growth inhibition of the duckweed in the respective toxicant concentrations can then be calculated, followed by the evaluation of the 72 hour EC_{50}.

The method measures the growth inhibition of samples to *Spirodela polyrhiza* and is based on the ISO 20227 (2017) method, titled: “Water quality – Determination of the growth inhibition effects of wastewaters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* – Method using a stock culture independent Microbiotest”.

15.3 REQUIREMENTS

15.3.1 Acquisition of test materials and kits

This protocol describes the use of commercially supplied Toxkits. The kits contain all the materials (including the test organisms) necessary to perform simple, rapid, sensitive, and reproducible toxicity tests at low cost. Toxkit tests are particularly suited for routine toxicity testing of chemicals and wastes released in aquatic as well as in terrestrial environments. The Duckweed TOXKIT F™ tests can be performed in accordance with testing conditions prescribed by ISO 20227 (2017).

Note*:* According to ISO 20227 (2017), MicroBioTests Inc. are an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if validity criteria specified in this International Standard are fulfilled).

15.3.1.1 Kit contents

The commercial Duckweed TOXKIT F™ contains all the materials, including the test species, reagents, and test containers, to perform the *Spirodela polyrhiza* growth inhibition toxicity test according to the internationally accepted Standard ISO 20227 (2017).

The Duckweed TOXKIT F™ consists of:

- Two polystyrene multi-well test plates (9 x 13 cm) with 48 cups (1 mL) which will serve as test containers
- Two polystyrene Petri dishes (9 cm diameter) with cover, to be used for the germination of the turions
- Two polystyrene test tubes (10 mL) each containing *Spirodela polyrhiza* turions in a storage medium
- A small micro-sieve with 100 µm mesh for eliminating the storage medium
- Two small plastic spatulas for easy transfer of the turions
- Three 25 mL glass bottles labelled A, B and C, and two 12 mL glass bottles labelled D and E, for preparation of the Steinberg growth and test dilution medium
- A detailed brochure with all instructions for performance of “range finding” and/or “definitive” assays on pure chemicals or polluted water samples
- An abbreviated version of the Standard Operational Procedure manual
- A Specification Sheet indicating the batch number of the turions, the batch number of the concentrated salt solutions, the expiry date of the Duckweed TOXKIT F™ and the 72 hour EC_{50} for the test with the reference chemical potassium chloride KCl

15.3.2 STAFF TRAINING (TECHNICAL SKILLS)

To perform the test, the analyst should be proven competent in

- Good Laboratory Practice (GLP)
- Trained in an aquatic ecotoxicology laboratory
- Software packages knowledge (e.g. EXCEL)

15.3.3 LABORATORY (test environment)

- The testing facility must be free of vapours, odours and dust that may be toxic to the test organisms
- The use of controls also allows checking that the test is performed in an atmosphere free from toxic dusts and vapours
- Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage
- Temperature control equipment should be adequate to maintain the recommended environmental conditions as stated in the standard, during storing of samples, testing, and culture maintenance

15.3.4 Software

- EXCEL spreadsheet

- REGTOX program

15.3.5 Apparatus

The following apparatus will be required to perform the test:

- Temperature controlled room
- Incubator or growth chamber
- Lux meter
- Digital camera and image analysis system, such as Image J
- Adjustable transfer pipettes and pipette tips (10-10 000 μL)
- pH-meter (accuracy 0.1 pH units),
- Oxygen meter
- Conductivity meter
- Thermometers
- Stopwatch
- Fridge
- Analytical balance

15.3.6 CONSUMABLES/MATERIALS

Table 15-1: List of consumables/materials

Name	Cat no	Supplier
Duckweed TOXKIT F™	TK63	MicroBioTests Inc.
100 mL volumetric flasks	-	General supplier
2 L volumetric flask	-	General supplier
25 mL measuring cylinders	-	General supplier
100 mL measuring cylinders	-	General supplier
50 mL plastic/glass beakers	-	General supplier

15.3.7 reagents

Use chemicals of recognised analytical grade (AR grade), unless otherwise specified.

Table 15-2: List of reagents

Name	Cat no	Supplier
Sodium hydroxide (NaOH), 1N	-	General supplier
Hydrochloric acid (HCl), 1N	-	General supplier
Nitric acid, HNO_3	-	General supplier
Acetone	-	General supplier
Reference stock solution, such as 3,5-dichlorophenol or potassium chloride	-	General supplier
Water, deionized or of equivalent purity (conductivity <10 µS/cm)	-	General supplier
Chlorine testing kits	-	General supplier
KNO_3	-	General supplier
KH_2PO_4	-	General supplier

Name	Cat no	Supplier
K_2HPO_4	-	General supplier
$MgSO_4 \cdot 7H_2O$	-	General supplier
$Ca(NO_3)_2 \cdot 4H_2O$	-	General supplier
H_3BO_3	-	General supplier
$ZnSO_4 \cdot 7H_2O$	-	General supplier
$Na_2MoO_4 \cdot 2H_2O$	-	General supplier
$MnCl2 \cdot 4H_2O$	-	General supplier
$FeCl3 \cdot 6H_2O$	-	General supplier
EDTA disodium-dihydrate	-	General supplier

15.4 SAMPLING METHOD

15.4.1 Sample collection

- Sampling, transportation, and storage of the samples should be performed as specified in ISO 5667-16 (1998)
- Collect samples in chemically inert, clean containers
- Rinse the bottle with the sample before filling it
- Fill the containers completely to the top of container and seal if possible
- Test the samples as soon as possible after collection as indicated in the standard
- Where necessary, store samples at 5 ± 2°C in the dark
- Do not use chemicals to preserve the samples

15.4.2 Sample preparation

- Carry out the toxicity test as soon as possible, preferably within 24 hours of collection. If this time interval cannot be met, cool the sample to 5 ± 2°C and test the sample within 48 hours
- At the time of testing, homogenize the sample to be analysed by shaking/stirring manually
- Measure the pH (as specified in ISO 10523, 2008) and the dissolved oxygen concentration (as specified in ISO 5814, 2012) and record these values in the test report
- Tests shall be carried out without pH adjustment of the test sample. The pH of test batches is measured at the beginning and at the end of the test and reported. However, in some cases, the final pH of a test solution may significantly differ from original pH of the test sample due to the concentration range selected and as a result of the buffer capacity of the dilution water or test sample. If toxic effects are observed at concentrations where the pH is not compatible with the survival of the organisms (i.e. outside the pH 6.0 to pH 9.0 range), the test(s) can be repeated with pH adjustment of the test sample

Note: Adjustment of the pH can alter the nature of the sample.

- If the pH is to be adjusted, the recommendation is to adjust to the pH of the dilution water. Choose the concentration of the hydrochloric acid or the sodium hydroxide solutions to restrict the volume

fraction added to not more than 5%. If, as a result of pH adjustment, there is an issue with suspended matter, separate the suspended matter from the remaining sample as specified in ISO 5667-16 (1998). Any pH adjustment shall be included in the test report

- Adjust the temperature of the pre-treated sample to the test temperature
- Measure the pH, dissolved oxygen, conductivity, and free chlorine of the sample prior to analysis. Usually, the test shall be carried out without adjustment of the pH of the medium after addition of the test sample. However, some substances may exert a toxic effect due to extreme acidity or alkalinity
- In order to determine the toxicity of a sample independent of pH, adjust the pH of the aqueous sample or stock solution (before the dilution in series) to that of the culture medium using either 1 mol/L hydrochloric acid (HCl) or 1 mol/L sodium hydroxide solution (NaOH) (see ISO 5667-16, 1998)
- Note the appearance of the sample (e.g. colour, turbidity, odour) prior to testing

15.5 TEST PREPARATION

15.5.1 Preparation of the stock solution

Prepare the eight stock solutions by adding the prescribed weight of the chemicals to 1 Litre of pure water as specified in Table 15-3 and 4: Macro- and micro-element stock solutions.

Table 15-3: Macro-elements stock solutions.

Macro-elements (50-fold concentrated)		**g/L**
Stock solution 1	KNO_3	17.50
	KH_2PO_4	4.50
	K_2HPO_4	0.63
Stock solution 2	$MgSO_4 \cdot 7H_2O$	5.00
Stock solution 3	$Ca(NO_3)2 \cdot 4H_2O$	14.75

Table 15-4: Micro-elements stock solutions.

Microelements (1 000-fold concentrated)		**mg/L**
Stock solution 4	H_3BO_3	120.00
Stock solution 5	$ZnSO_4 \cdot 7H_2O$	180.00
Stock solution 6	$Na_2MoO_4 \cdot 2H_2O$	44.00
Stock solution 7	$MnCl_2 \cdot 4H_2O$	180.00
Stock solution 8	$FeCl_3 \cdot 6H_2O$ EDTA disodium-dihydrate	760.00 1 500.00

Note: Stock solutions 2 and 3, and 4 to 7 may be pooled (considering the required concentrations).

15.5.2 Preparation of the growth and test dilution medium

The growth medium will be used for the germination of the *Spirodela* turions and as growth medium for the duckweed and at the same time as dilution medium for the toxicants in the toxicity test. The composition of

the growth medium is that of the "*Steinberg medium*" prescribed by ISO for the *Lemna* toxicity tests (ISO 20079, 2005).

Prepare the Steinberg medium as follows (see Figure 15-2: Preparation of duckweed growth and test dilution medium):

- Transfer about 300 mL pure water (deionized or distilled) to a 500 mL volumetric flask
- Uncap vials A, B and C and transfer 10 mL from each bottle in the volumetric flask
- Uncap vials D and E and transfer 0.5 mL from each bottle in the volumetric flask
- Fill the flask up to the 500 mL mark with pure water, stopper the flask and shake thoroughly to homogenize the medium
- Store the prepared Steinberg medium in the refrigerator in darkness until use

Note: This medium has a relatively short shelf life and should be used within two weeks after preparation.

A similar (500 mL) volume of Steinberg medium shall be prepared at the time of performance of the second toxicity test.

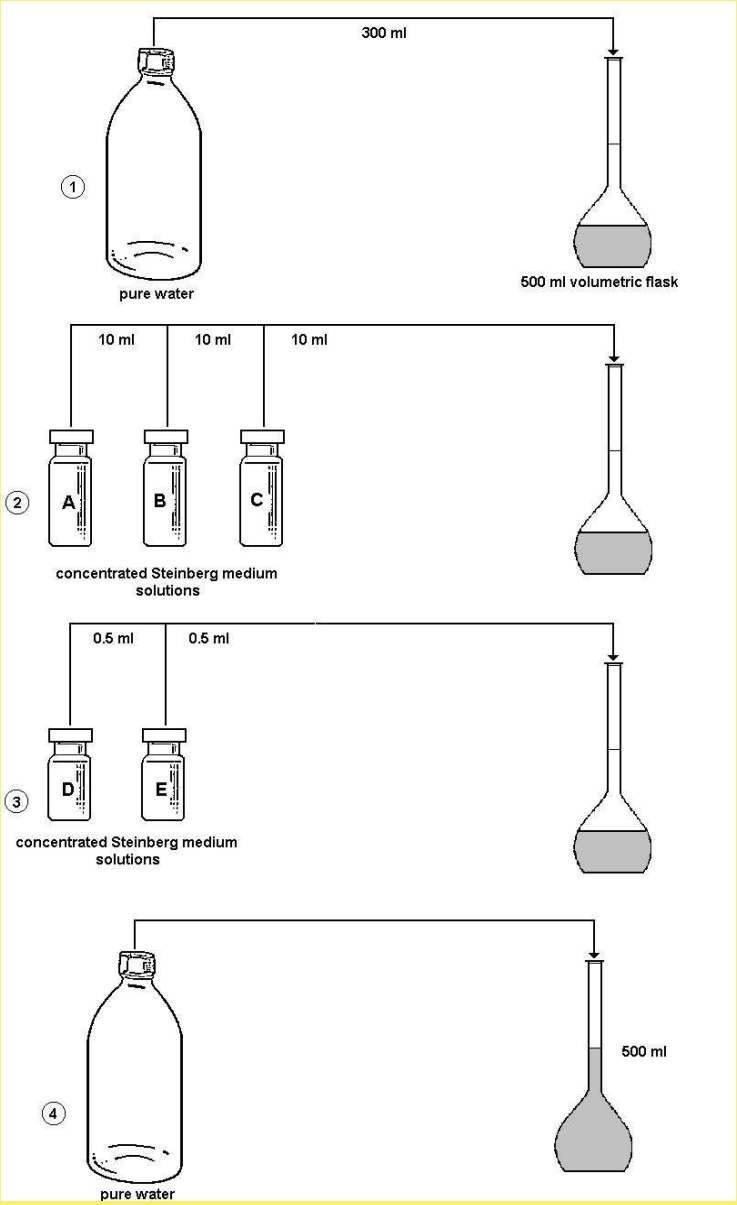

Figure 15-2: Preparation of duckweed growth and test dilution medium.

15.5.3 Test organisms

Duckweed are free-floating higher water plants commonly used in ecotoxicological research for the assessment of the toxicity of wastewaters, natural waters and chemicals (see ISO 20227, 2017).

Duckweed are fast growing plants, many of which have a cosmopolitan distribution, and they are, hence, well suited as primary producers for hazard assessment of pollutants in freshwater environments.

Contrary to terrestrial plants, for which bioassays can be started from the "dormant" life stages (seeds), toxicity tests with duckweed require continuous culturing and maintenance of live stocks, with the inherent biological, technical, and financial costs. A few duckweed species, however, produce dormant vegetative buds (turions) which can be stored for long periods of time, and which can be germinated on demand at the time of performance of the bioassay. One of the duckweeds producing turions is *Spirodela polyrhiza*, and

this species was eventually selected for a simple and practical MicroBioTest Inc. toxkits which is independent of the stock culturing and maintenance of live stocks. *Spirodela polyrhiza* was found to be as sensitive to toxicants as the conventional bioassays with other duckweeds.

15.5.4 Germination of the *Spirodela polyrhiza* turions

The tubes with the turions and the vials with the solutions must be stored in the refrigerator prior to use.

Germinate the turions as follows:

- Take a tube with *Spirodela polyrhiza* turions and shake it slightly to resuspend the turions
- Pour the contents of the tube in the micro-sieve and rinse with pure water to remove the storage medium
- Make sure that all the turions are transferred to the micro-sieve
- Put 10 mL Steinberg medium in one of the 9 cm Petri dishes.
- Turn the micro-sieve upside down and flush all the turions in the Petri dish, by pouring 10 mL Steinberg medium over the surface of the micro-sieve
- Fill the Petri dish further by adding 10 mL Steinberg medium
- Cover the Petri dish with the transparent lid and place it in the incubator
- Incubate the Petri dish for 3 days (72 hours ± 1 hour) at 25°C with continuous "top" illumination (6 000 lux at the surface of the Petri dish)

Note: Both germination of the turions and the growth of the first fronds are "very substantially" dependent on temperature and illumination conditions. It is therefore most important that the prescribed values (25°C and 6000 lux) be respected as closely as possible.

15.6 TEST PROCEDURE

15.6.1 Preparation of the effluent dilution series

For a test on an effluent, a small volume of concentrated Steinberg growth medium must first be added to the effluent prior to making the dilution series.

15.6.2 Addition of concentrated Steinberg growth medium solutions to the effluent

Prepare the effluent dilutions series as follows (see Figure 15-3: Addition of concentrated Steinberg medium to the effluent):

- Transfer about 80 mL effluent in a 100 mL calibrated flask
- Uncap vials A, B and C and transfer 2 mL from each vial in the calibrated flask
- Uncap vials D and E and transfer 100 µL from each vial in the calibrated flask
  Fill the flask up to the 100 mL mark with effluent, stopper the flask and shake thoroughly to homogenize the contents

Note: The addition of 6.2 mL growth medium to 93.8 mL effluent dilutes the effluent sample by about 6%, which means that the highest effluent concentration which will be tested is about 94% of the original effluent.

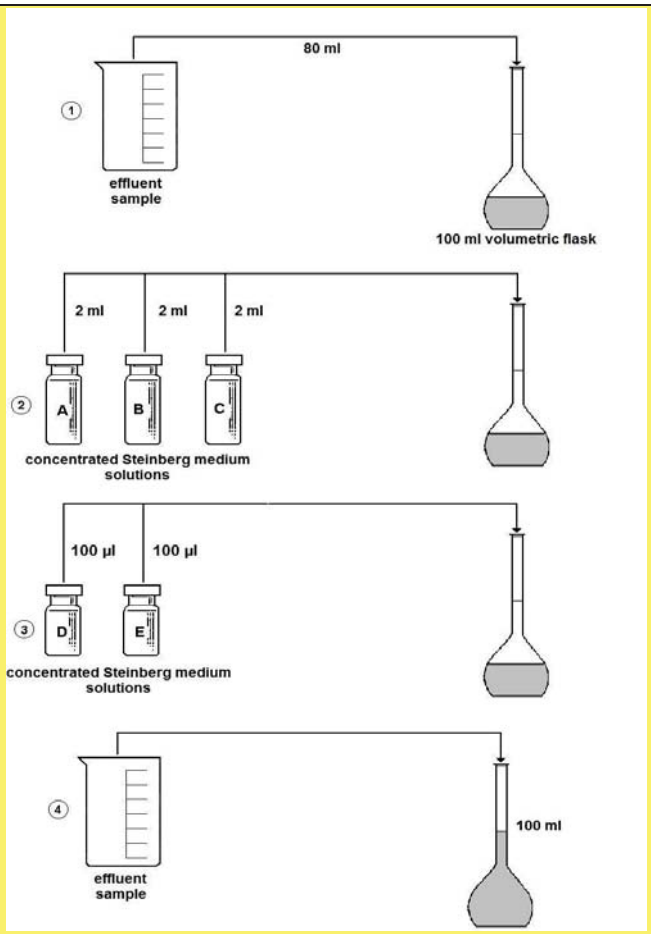

Figure 15-3: Addition of concentrated Steinberg medium to the effluent.

15.6.3 Preparing the 1:1 dilution series from this 94% effluent

To prepare the toxicant dilution (See Figure 15-4):

- Take five 20 mL test tubes and label them C1, C2, C3, C4 and C5
- Add 20 mL effluent (containing growth medium) to test tube C1
- Add 10 mL Steinberg medium (as dilution medium) to the tubes C2, C3, C4 and C5
- Transfer 10 mL effluent from tube C1 to tube C2. Cap and shake the test tube
- Transfer 10 mL test dilution from tube C2 to tube C3. Cap and shake the test tube
- Repeat this procedure for the next dilutions, i.e. 10 mL from tube C3 to tube C4, and 10 mL from tube C4 to tube C5

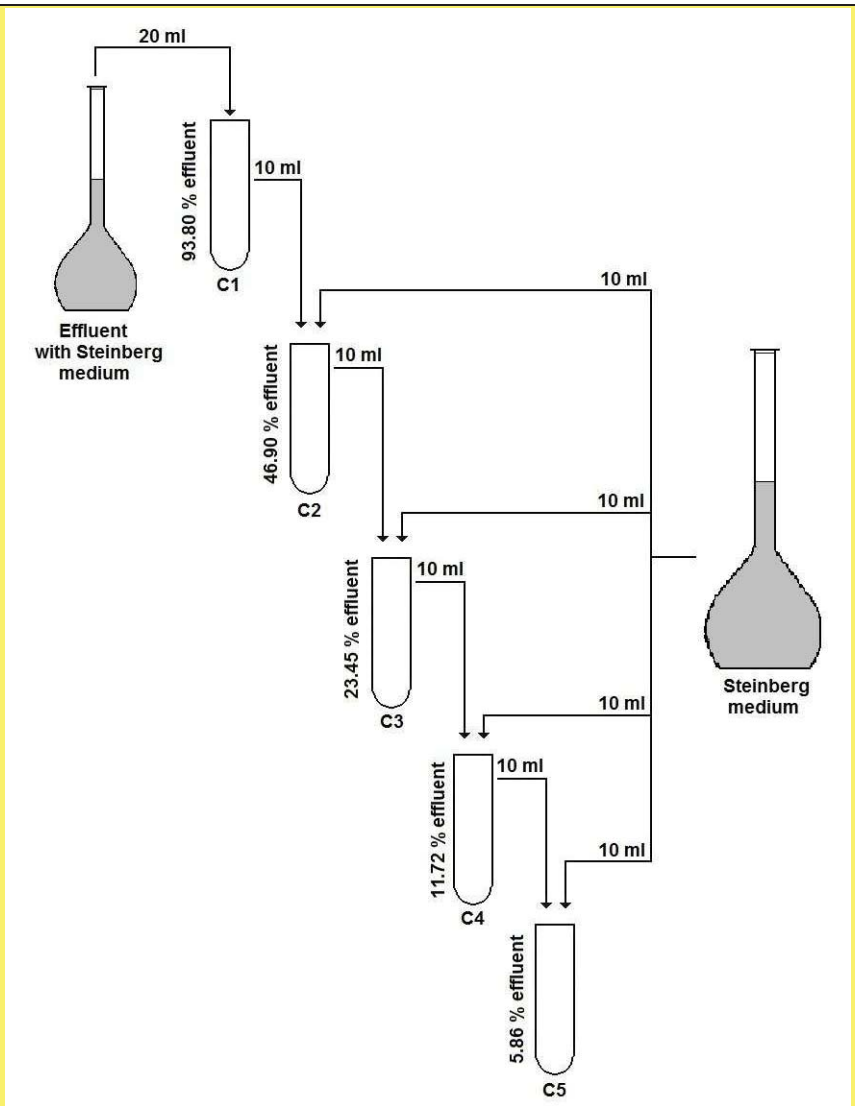

Figure 15-4: Preparation of the toxicant dilution

15.6.4 Definitive test

- The dilution series to be prepared spans the range of the lowest concentration producing 100% effect,
  to the highest one producing less than 10% effect in the range finding test
- A dilution series is prepared, starting with a test concentration of the toxicant which was the lowest
  one giving 100% growth inhibition in the range finding test
- A logarithmic dilution series (e.g. 10 mg/L, 5.6 mg/L, 3.2 mg/L, 1.8 mg/L, 1 mg/L and the negative control) is then prepared in test tubes, with the volumes of growth medium and toxicant

15.6.5 Filling of the test plates with the effluent dilutions

Each dilution is transferred into all the cups of one row in the multi-well plate (See Figure 15-5). The rows are labelled from A to F and the cups from 1 to 8.

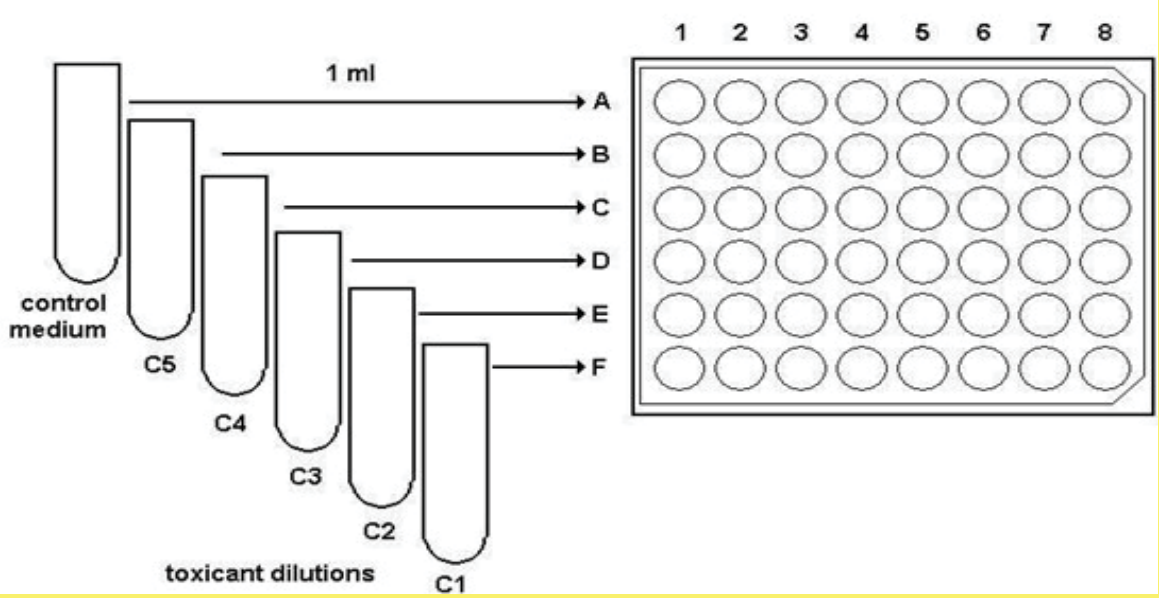

Figure 15-5: Filling of the test plates with the effluent dilutions.

Note: The distribution of the test solutions must always be carried out starting with the control row (row A) on top of the multi-well plate with Steinberg growth medium (dilution medium), followed in sequence by the rows containing increasing toxicant concentrations, up to the highest test concentration in the bottom row (row F).

Fill the test plates as follows (see Figure 15-5: Filling of the test plates with the effluent dilutions):

- Put 1 mL Steinberg medium from the control tube in the 8 cups of row A (the control row)
- Put 1 mL of the tube containing the C5 toxicant concentration in the 8 cups of Row B
- Repeat this procedure with the tubes C4, C3, C2 and C1 for the 8 cups in the rows C, D, E and F respectively

15.6.6 Transfer of the germinated turions to the test cups

Take the Petri dish with the germinated turions out of the incubator and check if the turions have germinated.

Note: Germinated turions can easily be distinguished from those which have not germinated, by the presence of a (small) first frond on one side of the turion and small roots.

With the aid of the spatula, transfer 1 germinated turion into each cup of the Control row (row A). Repeat this operation with the other rows “from the top to the bottom of the multi-well plate”, i.e. starting with the row containing the lowest test concentration (C5 in row B) down to the row with the highest test concentration (C1 in row F).

The transfer of the germinated turions to the cups of the multi-well must be carried out “randomly”, i.e. one has to avoid picking up and transferring first the germinated turions which have “the largest fronds”.

15.6.7 Taking of the photo of the multi-well plate at the start of the toxicity test

- A digital photo (See Figure 15-6) of the multi-well plate containing the germinated turions (with their small first fronds) must be taken at the start of the 3 days toxicity test.
- To take the photo, place the test plate on a horizontal surface and take a photo of the multi-well plate with a digital camera. If possible, put the multi-well plate on a light table to take the photo. This will increase the contrast between the germinated turions and the first small fronds and allow for a better distinction between the turion and the first frond.
- Alternatively, the multi-well plate can be placed on a white background, but by no means the plate should be placed on a “dark” background to take the photo.
- To take the photo, the digital camera should not be held too close to the multi-well plate, since this will lead to a “distortion” of the view of the cups in the columns on the left and right side of the multi-well plate. It is important that the edges of all the lateral wells also have a round (and not an oval) look!
- Transfer the photo of the multi-well plate to a computer file.

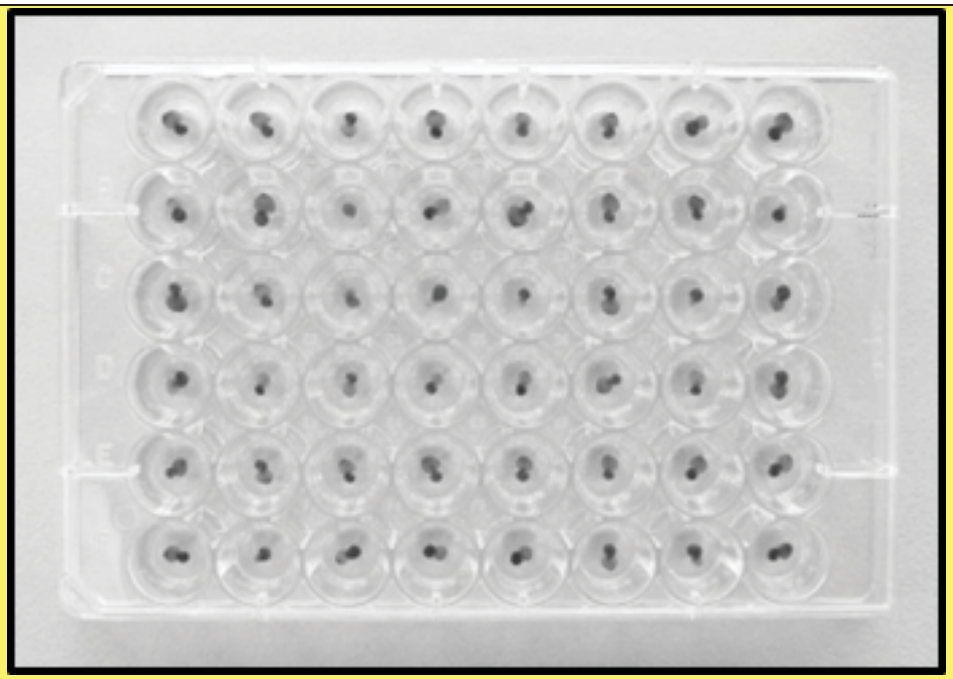

Figure 15-6: Photo of the multi-well plate with the germinated turions and their small first fronds (at t_0 hours)

15.6.8 Incubation of the test plate

Put the cover on the multi-well plate and put the plate in the incubator. Incubate the test plate at 25°C for 3 days (72 ± 1 hour), with a continuous illumination of 6 000 lux (at the top of the multi-well).

Note: Same remark as for the germination conditions - the prescribed 25°C and 6 000 lux illumination must be respected as closely as possible.

15.6.9 Taking of the photo of the multi-well plate at the end of the toxicity test

- A digital photo of the multi-well plate containing the grown fronds must be taken again at the end of the 3 days toxicity test
- It is also advised to again put the plate on a "light table" (or on a white background) to take the photo, for a better distinction between the turions and the first fronds
- To take the photo take the multi-well plate from the incubator and remove the lid
- Take a quick look at the fronds in each cup. If some fronds are not laying totally "horizontally" (and hence don't show their total surface) they must be put in a horizontal position with the aid of the spatula
- Take a photo of the multi-well plate again (see photo) and transfer the photo to a computer file

15.6.10 Measurement of the first fond area

- The measurement of the areas of the first fronds can be made immediately after taking the photo of the multi-well or can be postponed to any appropriate time
- Area measurements of the first fronds must be made in all the test cups, on the (computer stored) photos of the multi-well test plate taken at t_0 hour and at t_{72} hours
- The area measurements are made with the aid of an appropriate image analysis program (e.g. image J, which is an open access image analysis program)
- In some cups, after 3 day incubation, a second frond will have developed from the germinated turion but the area measurements shall be restricted to the surface of the largest frond (the first frond)
- The data of all the area measurements shall be scored on the data report templates for the t_0 hours and the t_{72} hours measurements (see Table 15-5 and Table 15-6)

Table 15-5: Data report template for the area measurements at the start of the toxicity test (t_0 h values).

Replicate	Control	C5	C4	C3	C2	C1
1						
2						
3						
4						
5						
6						
7						
8						

Table 15-6: Data report template for the area measurements at the end of the toxicity test (t_{72} hour values)

Replicate	Control	C5	C4	C3	C2	C1
1						
2						
3						
4						
5						
6						
7						
8						

The area measurements are made with the aid of an appropriate “image analysis” program (such as, e.g. “Image J” which is accessible free of charge on the Internet).

15.7 CALCULATIONS OF RESULTS

The effect parameter measured in this document on the *Spirodela polyrhiza* is the growth inhibition of the test organism. The growth inhibition is calculated as the difference (the decrease) of the size of the first fronds in the cups of the rows with toxicant concentrations versus their size in the cups in the control row after 3 days exposure.

With the data scored in the data report templates (Table 15-5 and 6) calculate for the eight replicates in the control and in the five test concentrations, the mean area of the initial first frond (size at t_0 hours) and of the final first frond (size at t_{72} hours).

Score these values on the data treatment sheet (Table 15-7).

Table 15-7: Data treatment sheet.

	Control	C5	C4	C3	C2	C1
Mean area of initial first frond at t_0 h (I)						
Mean area of final first frond at t_{72} h (F)						
Mean growth of the first frond (F-I)						
Percentage growth inhibition						

Calculate for the control and for the five test concentrations, the mean growth of the first frond (i.e. the difference between the mean t_{72} hours area values and the mean t_0 hour area values).

Calculate the percentage growth inhibition using: A-B/A X 100

Where:

A is the mean growth of the first frond in the control

B is the mean growth of the first frond in the five test concentrations.

The 72 hours EC_{50} (and other EC_x values, if needed) can be calculated from the percentage's growth inhibition with the aid of an appropriate statistical program. It is recommended to use a nonlinear regression of the concentration-response curve with a suitable model.

15.7.1 Estimation of the EC_{50}

A Data Treatment Excel sheet, supplied by MicroBioTests Inc., allows to transfer the data of the "initial first frond areas" and the "final first frond areas" into this Excel sheet which then calculates the growth of the first fronds, the percentage growth inhibition in each test concentration, and the 72 hours EC_{50} with the 95% confidence limits.

15.8 TEST VALIDITY

The "mean growth" of the first fronds in the cups of the control column after 3 days incubation at 25°C and under 6 000 lux illumination (the mean t_{72} hours - t_0 hours area), must be at least 10 mm^2.

15.9 INTERPRETATION OF RESULTS

Following the 3 day incubation, the data should be entered onto the relevant electronic data sheet where automatically calculations of the growth inhibition for each toxicant concentration will take pace. This must include the mean and the percentage effect.

15.10 TEST REPORT

This test report shall contain at least the following information:

- the test method used, together with a reference to this document, i.e. ISO 20227 (2017)
- name of the laboratory performing the test
- date of test performance
- test organism (species, origin)
- designation of test material (type of the sample or name of the chemical tested)
- sample pre-treatment (if any)
- dilutions or concentrations tested
- image analysis program used for the area measurements
- result sheet with the mean initial and final frond areas, and the calculation of the growth inhibition
- statistical data treatment method used for the EC calculations
- comments on the test results, if necessary

15.11 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

15.11.1 Application

According to ISO 20227 (2017) this method for the determination of the inhibition of the growth of the first fronds of *Spirodela polyrhiza* germinated from turions is applicable to substances and mixtures contained in water or wastewater, including treated municipal wastewater and industrial effluents. The test is also applicable to pure chemicals and in particular, plant protection products and pesticides.

15.11.2 Advantages

The *Spirodela polyrhiza* test is very simple and easy to perform:

- the assay does not require culturing or maintenance of live stocks of the test species, and can be performed “anytime, anywhere” by the use of stored turions
- stored turions have a shelf life of several months with a high germination success
- the test requires minimal bench and incubation space, and minimal equipment
- the area measurements of the first fronds do not need to be made immediately and can be postponed to an appropriate timing
- the area measurements by image analysis are very rapid and precise, and take less than 1 hour for a complete test

15.11.3 Limitations

- Dark coloured samples and samples containing high loads of suspended solids may impede the observation of the test organisms
- Pathogenic and /or predatory organisms in the samples may affect survival

- Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may influence the bioavailability or solubility of certain hazardous substances

15.12 RECOMMENDATIONS

This method provides an easy-to-use bioassay for measuring acute toxicity of substances/pollution to invertebrates, such as duckweed. The significance of the duckweed growth test is to assist in the assessment of possible risks to invertebrate species in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other invertebrate species for comparative purposes. This test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment (others include but are not limited to bacteria, invertebrates, algae, vertebrates, and protozoa amongst others). The reason for this is the variation between the sensitivity of the different species to different substances, and therefore using several different trophic levels increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

15.13 REFERENCES

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ASTM (American Society for Testing and Materials) E1415-91 (2012) Standard guide for conducting static toxicity tests with *Lemna gibba* G3. *Annual book of ASTM Standards* Vol. 11.05. Pesticides, antimicrobials and alternative control agents; environmental assessment; hazardous substances and oil spill response pp1727.

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

EC (Environment Canada) (2007) Biological Test Method: Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*. EPS 1/RM/37, Second Edition pp 137.

ISO (International Organization for Standardization) 8402 (1994) Quality Management and Quality Assurance - Vocabulary. ISO/TC 176/SC 1 Concepts and terminology pp39.

ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.

ISO (International Organization for Standardization) 20079 (2005) Water quality – Determination of the toxic effect of water constituents and wastewater on duckweed (*Lemna minor*) – Duckweed growth inhibition test. ISO/TC 147/SC 5 Biological methods pp23.

ISO (International Organization for Standardization) 20227 (2017) Water quality – Determination of the growth inhibition effects of wastewaters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* – Method using a stock culture independent microbiotest. ISO/TC 147/SC 5 Biological methods pp20.
MicroBioTests Inc., Belgium. SPIRODELA DUCKWEED TOXKIT F™. Toxicity Test with *Spirodela polyrhiza*. Standard Operational Procedure.
OECD (Organisation for Economic Co-operation and Development) 221 (2006) *Lemna sp*. Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris pp22. https://doi.org/10.1787/9789264016194-en.
Report on the International Interlaboratory Comparison of the *Spirodela* duckweed microbiotest. In: Publications – Extensive Reports on the website www.microbiotests.be.

15.14 USEFUL CONTACTS

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16 *THAMNOCEPHALUS PLATYURUS* ACUTE TOXICITY TEST (MORTALITY TEST)

Compiled by: L Swart and H Pearson

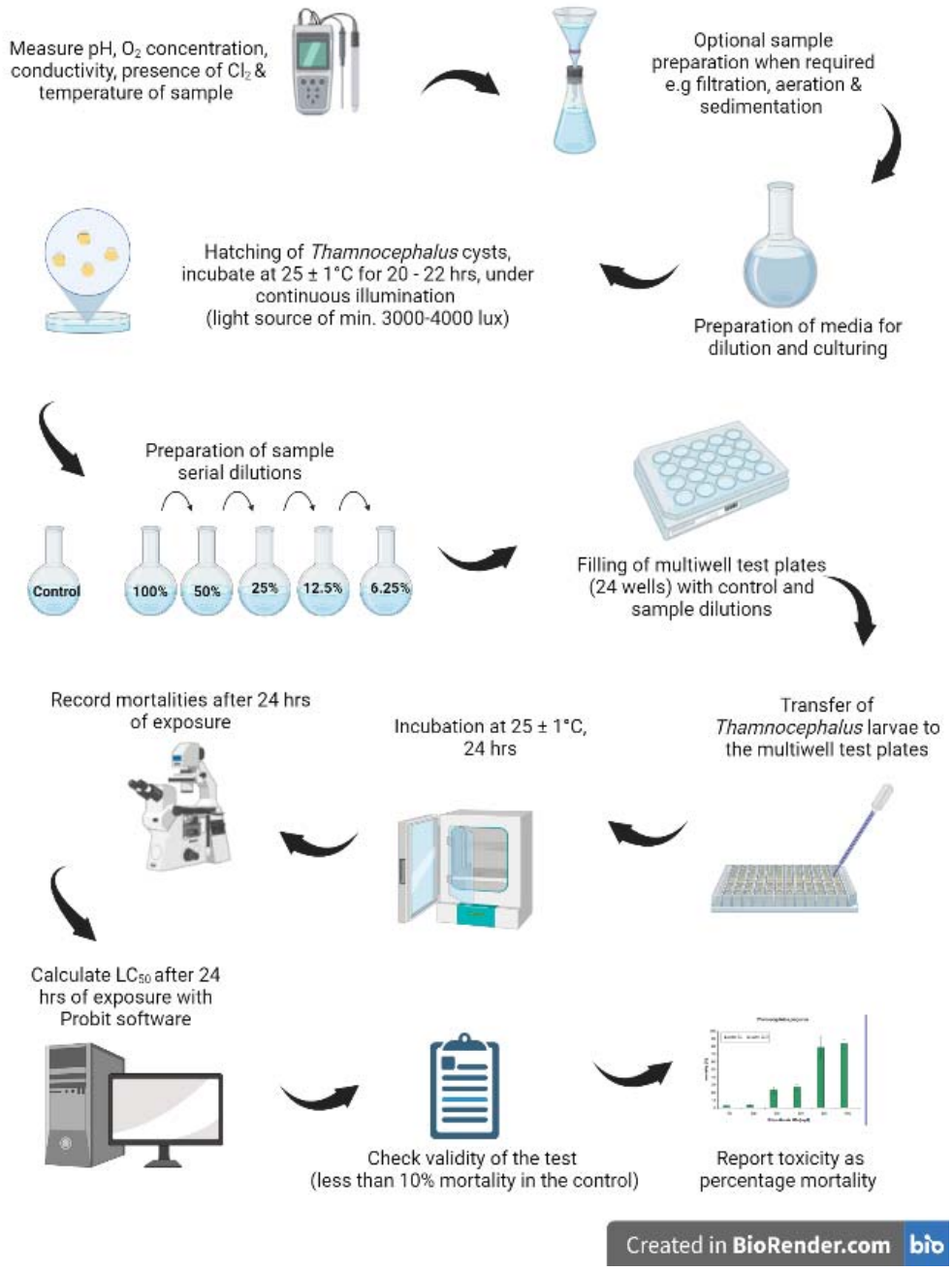

Figure 16-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the THAMNOCEPHALUS PLATYURUS acute toxicity test (mortality test)

16.1 ACRONYMS & ABBREVIATIONS

AR	Analytical Grade
$CaSO_4 \cdot 2H_2O$	Calcium sulphate dihydrate
cm	Centimetre(s)
Cr	Chrome
ºC	Degree(s) Celsius
h	Hour(s)
HCl	Hydrochloric acid
LC_{10}	Lethal concentration at 10%
LC_{50}	Lethal concentration at 50%
L	Litre(s)
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
µL	Microlitre (s)
Mg	Milligram(s)
mg/L	Milligram(s) per litre
mL	Millilitre(s)
HNO_3	Nitric acid
%	Percentage
±	Plus, or minus
KCl	Potassium chloride
$K_2Cr_2O_7$	Potassium dichromate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide

16.2 PRINCIPLE OF THE ASSAY

The *Thamnocephalus platyurus* acute toxicity test (mortality test) measures the short-term acute toxicity of wastewater discharges and receiving water to the freshwater Crustacea *Thamnocephalus platyurus* (Fairy Shrimp). In this test, a range of concentrations of substance are tested to investigate the different degrees of toxic effects on the survival capability of *Thamnocephalus platyurus* under otherwise identical test conditions after 24 hours. The International Standard specifies a method for the determination of the acute toxicity to *Thamnocephalus platyurus* Straus (Cladocera, Crustacea). This procedure can be based on SANS 14380 (2014). “Water Quality – Determination of the acute toxicity *Thamnocephalus platyurus* (Crustacea, Anostraca).

16.3 REQUIREMENTS

16.3.1 Acquisition of test materials and kits

This protocol describes the use of commercially supplied Toxkits. The kits containing all necessary materials, including the test organisms to perform simple, rapid, sensitive and reproducible toxicity tests at low cost. Toxkit are suited for toxicity testing of chemicals and wastes, released in aquatic as well as terrestrial environments. THAMNOTOXKIT F™ (*Thamnocephalus platyurus*) tests can be performed in accordance with testing conditions prescribed by SANS 14380 (2014). "Water Quality – Determination of the acute toxicity of *Thamnocephalus platyurus* (Crustacea, Anostraca)".

Note: According to ISO 14380 (2011), MicroBioTests Inc. are an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if validity criteria specified in this International Standard are fulfilled.

16.3.1.1 Kit contents

The commercial THAMNOTOXKIT F™ contains all the materials, including the test species, reagents, and test containers, to perform the *Thamnocephalus platyurus* acute toxicity test according to the internationally accepted Standard ISO 14380 (2011).

The THAMNOTOXKIT F™ consists of:

- Six 1 mL plastic vials containing cysts of the fairy shrimp *Thamnocephalus platyurus,* which should be stored in a refrigerator at 5 ± 2°C until use. The number of larvae obtained from each vail will exceed the number of test organisms needed for the toxicity test
- Five small glass bottles, each containing a concentrated solution of one salt, to make up one litre Standard Freshwater (moderately hard synthetic water, US EPA formula) with deionized or distilled water for preparation of the hatching and toxicant dilution medium
  Composition:
  Vial 1: $NaHCO_3$ (96 mg - dissolved in 1 L = 96 mg/L)
  Vial 2: $CaSO_4.2H_2O$ (60 mg - dissolved in 1 L = 60 mg/L)
  Vial 3: $CaSO_4.2H_2O$ (60 mg - dissolved in 1 L = 60 mg/L
  Vial 4: $MgSO_4.7H_2O$ (123 mg - dissolved in 1 L = 123 mg/L)
  Vial 5: KCl (4 mg - dissolved in 1 L = 4 mg/L)
- Two polystyrene Petri dishes of 5 cm diameter with cover, for the hatching of the *Thamnocephalus* cysts
- Six polystyrene plates of 9 x 13 cm with 24 wells (3 mL) which will serve as test containers
- Six parafilm strips for sealing the multi-well plate to minimize evaporation during the incubation period
- Six polyethylene micropipettes for transferring the larvae

16.3.2 Staff training (technical skills)

To perform the test, the analyst should be proven competent in

- Good Laboratory Practice (GLP)
- Trained in an aquatic ecotoxicology laboratory
- Software packages knowledge (e.g. EXCEL)

16.3.3 LABORATORY (test environment)

- The testing facility must be free of vapours, odours and dust that may be toxic to the test organisms
- The use of controls also allows checking that the test is performed in an atmosphere free from toxic dusts and vapours
- Take special care to avoid contamination of the water by inorganic or organic substances during preparation and storage
- The exposure of organisms as specified in this International Standard shall be carried out in the dark in a temperature-controlled room or incubator at 25 ± 1°C in the test containers for 24 hours
- Temperature control equipment should be adequate to maintain the recommended environmental conditions as stated in the standard, during storing of samples, testing and culture maintenance
- The ephippia is hatched in an environmentally controlled facility under the following illumination conditions of 3000-4000 lux

16.3.4 software

- EXCEL spreadsheet
- REGTOX
- TSK (Trimmed Spearman Karber)
- Probit

16.3.5 Apparatus

The following apparatus will be required to perform the test:

- Temperature controlled room
- Incubator or growth chamber
- Stereomicroscope
- Lightbox
- Adjustable transfer pipettes and pipette tips (10-10 000 μL)
- pH-meter (accuracy 0,1 pH units)
- Oxygen meter
- Conductivity meter
- Thermometers
- Stopwatch
- Fridge

- Analytical balance

16.3.6 Consumables/materials

Table 16-1: List of consumables/materials

Name	Cat no	Supplier
THAMNOTOXKIT F™	TK31	MicroBioTests Inc.
25 mL volumetric flasks	-	General supplier
100 mL volumetric flasks	-	General supplier
1 L volumetric flask	-	General supplier
25 mL measuring cylinders	-	General supplier
100 mL measuring cylinders	-	General supplier

16.3.7 Reagents

Use chemicals of recognised analytical grade (AR grade), unless otherwise specified.

Table 16-2: List of reagents

Name	Cat no	Supplier
Sodium hydroxide (NaOH), 1N	-	General supplier
Hydrochloric acid (HCl), 1N	-	General supplier
Nitric acid	-	General supplier
Acetone	-	General supplier
Reference stock solution, such as 1 g/L potassium dichromate	-	General supplier
Water, deionized or of equivalent purity (conductivity <10 µS/cm)	-	General supplier
Chlorine testing kits	-	General supplier
$NaHCO_3$	-	General supplier
$CaSO_4.2H_2O$	-	General supplier
$MgSO_4.7H_2O$	-	General supplier
KCl	-	General supplier

16.4 SAMPLING METHOD

16.4.1 Sample collection

- Sampling, transportation, and storage of the samples should be performed as specified in ISO 5667-16 (1998)
- Collect samples in chemically inert, clean containers
- Rinse the bottle with the sample before filling it
- Fill the containers completely to the top of container and seal if possible
- Test the samples as soon as possible after collection as indicated in the standard
- Where necessary, store samples at 5 ± 2°C in the dark
- Do not use chemicals to preserve the samples

16.4.2 Sample preparation

- Carry out the toxicity test as soon as possible, preferably within 24 hours of collection. If this time interval cannot be met, cool the sample to 5 ± 2°C and test the sample within 48 hours (see ISO 5667-16, 1998)
- At the time of testing, homogenize the sample to be analysed by shaking manually
- High concentrations of suspended inorganic or organic solids in a sample can be harmful to filter feeding *Thamnocephalus platyurus*. Compensation for this interference can be made by a sample treatment for turbidity. If necessary, allow to settle for a maximum of 2 hours in a container, and sample, e.g. by drawing off the required quantity of supernatant using a pipette, maintaining the end of the pipette in the centre of the section of the test container and halfway between the surface of the deposited substances and the surface of the liquid. If the raw sample of the decanted supernatant is likely to interfere with the test (due to presence of residual suspended matter, protozoa, microorganisms, etc.), centrifuge, for example, for 10 minutes at 5 000*g* or filter the raw or decanted sample. Test the residual toxicity of the supernatant. The kind of filter to be used should be checked by a test with control medium run through the filters

Note: Some filters and apparatus can add measurable toxicity, sometimes because of wetting agents added to the filters. A filter paper can also absorb toxic substances and remove them from the sample filtrate.

- The sample obtained by either of these methods is the sample submitted to testing
- Usually, no aeration of sample or prepared test concentrations is necessary. If, and only if, the dissolved oxygen is < 40% saturation, a pre-aerate of the sample or all test solutions for at most 20 minutes by appropriate methods, e.g. aeration or stirring may be performed. Any supersaturation should be remedied
- Report any pre-aeration of test solutions or sample
- Measure the pH (as specified in ISO 10523, 2008) and the dissolved oxygen concentration (as specified in ISO 5814, 2012) and record these values in the test report
- Tests shall be carried out without pH adjustment of the test sample. The pH of test batches is measured at the beginning and at the end of the test and reported. However, in some cases, the final pH of a test solution may significantly differ from original pH of the test sample due to the concentration range selected and because of the buffer capacity of the dilution water or test sample. If toxic effects are observed at concentrations where the pH is not compatible with the survival of the organisms (i.e. outside the pH 6,0 to pH 9,0 range), the test(s) can be repeated with pH adjustment of the test sample

Note: Adjustment of the pH can alter the nature of the sample.

- If the pH is to be adjusted, the recommendation is to adjust to the pH of the dilution water. Choose the concentration of the hydrochloric acid or the sodium hydroxide solutions to restrict the volume fraction added to not more than 5%. If, as a result of pH adjustment, there is an issue with suspended

matter, separate the suspended matter from the remaining sample as specified in ISO 5667-16 (1998). Any pH adjustment shall be included in the test report

- Adjust the temperature of the pre-treated sample to the test temperature
- Measure the pH, dissolved oxygen, conductivity, and free chlorine of the sample prior to analysis. Usually, the test shall be carried out without adjustment of the pH of the medium after addition of the test sample. However, some substances may exert a toxic effect due to extreme acidity or alkalinity. In order to determine the toxicity of a sample independent of pH, adjust the pH of the aqueous sample or stock solution (before the dilution in series) to that of the culture medium using either 1 mol/L hydrochloric acid (HCl) or 1 mol/L sodium hydroxide solution (NaOH) (see ISO 5667-16, 1998)
- Note the appearance of the sample (e.g. colour, turbidity, odour) prior to testing

16.5 TEST PREPARATION

16.5.1 Preparation of standard freshwater used for dilution and culturing

- As an example, the preparation of standard freshwater/dilution water meeting the requirements is described below
- Dissolve known quantities of reagents in distilled water. The dilution water prepared shall have a pH of 7,6 ± 0,3, a hardness of 80 mg/L - 100 mg/L expressed as $CaCO_3$

Prepare the standard freshwater as follows:

- Dissolve 60 mg of calcium sulphate dihydrate ($CaSO_4 \cdot 2H_2O$) in distilled water and make up to 1 litre with distilled water
- Dissolve 60 mg of magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) in distilled water and make up to 1 litre with distilled water
- Dissolve 96 mg of sodium bicarbonate ($NaHCO_3$) in distilled water and make up to 1 litre with distilled water
- Dissolve 4mg of potassium chloride (KCl) in distilled water and make up to 1 Litre with distilled water
- Mix 25 mL of each of the four solutions and make up to 1 litre with distilled water
- The dilution water shall be aerated until the dissolved oxygen concentration has reached saturation and the pH has stabilized. The dilution water prepared in this way shall not be further aerated before use
- If necessary, adjust the pH to 7,6 ± 0,3 by adding sodium hydroxide (NaOH) solution or hydrochloric acid (HCl)
- Store the standard freshwater in the fridge at 4 ± 2°C

The standard freshwater will be used as hatching medium for the larvae, as dilution medium for preparation of the toxicant dilution series and for the control samples.

16.5.2 Test organisms

The test organisms used are obtained in the form of dormant cysts of the crustaceans *Thamnocephalus platyurus* which are used worldwide for toxicity testing. The cysts are protected by a strong shell and can be stored for long periods of time without losing their viability. When the cysts are placed in specific environmental conditions and triggers, the cysts will rehydrate and hatch in about 24 hours and can then be used immediately for the toxicity tests.

The test organisms are neonates of *Thamnocephalus platyurus* (Crustacea, Anostraca) cultured under specified laboratory breeding conditions (see SANS 14380, 2014, Annex B) or obtained as dried cyst from a specialized company.

The animals used for the test shall be less than 22 hours old and the test organisms may also derive from the hatching of cysts obtained from laboratory cultures of the crustacean as described in SANS 14380 (2014). The larvae hatched from the cysts may be used directly as test organisms if they comply with all validity criteria given in this International Standard.

16.5.3 Hatching of cysts

Hatching (See Figure 16-2) of the cysts must be initiated 20-22 hours prior to the start of the toxicity test by doing the following.

- Open a tube with cysts and fill it with diluted hatching medium (approximately 1 mL)
- Close the tube with the stopper and shake it at regular intervals during a 30-minute period
- Empty the contents of the vial with pre-hydrated cysts into one of the two small petri dishes (mark the petri dish with the date and time) make sure most of the cysts are transferred by rinsing the tube with diluted standard freshwater
- Add 10 mL diluted standard freshwater medium to the petri dish and swirl gently to distribute the cysts evenly
- Cover the hatching petri dish and incubate at 25 ± 1°C for 20-22 hours, under continuous illumination (light source of min. 3000-4000 lux)

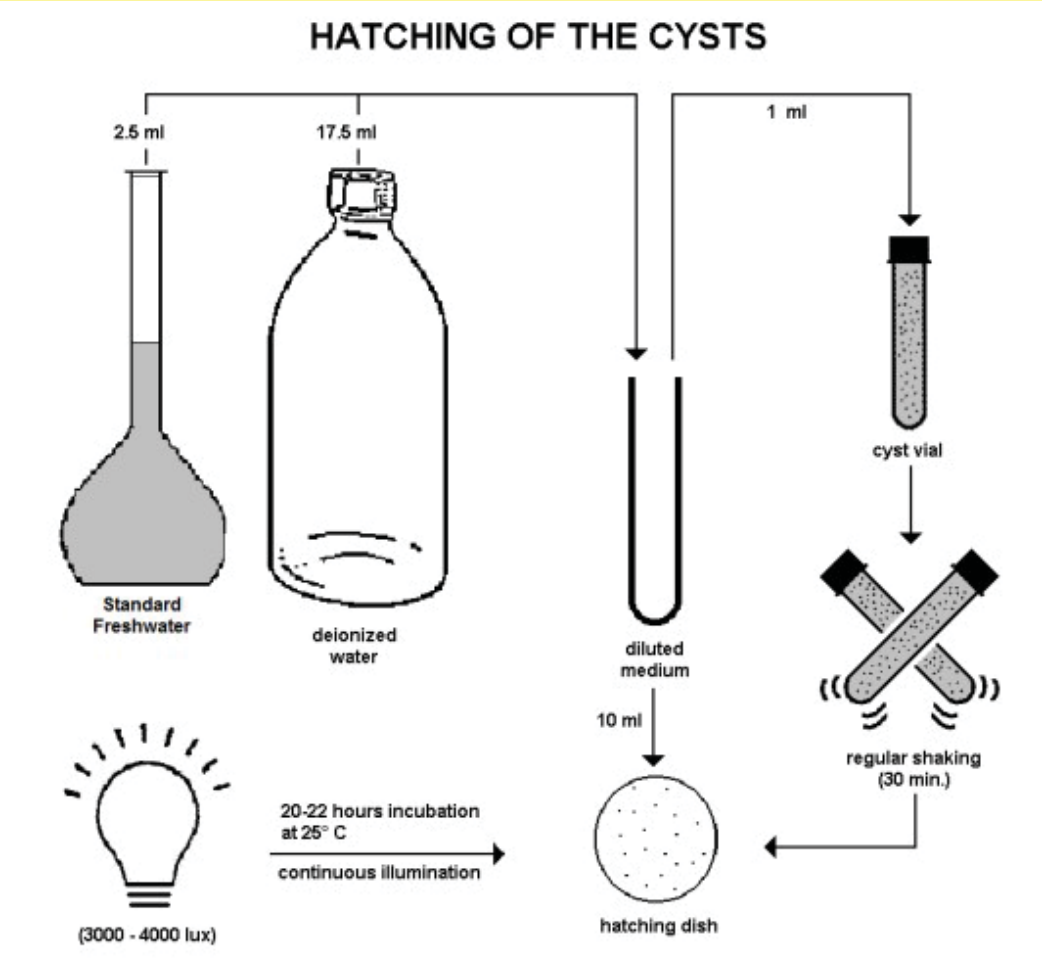

Figure 16-2: Hatching procedure for *Thamnocephalus platyurus* cysts.

16.6 TEST PROCEDURE

16.6.1 Test dilutions

- The test should comprise at least five concentrations of the sample to be tested. Select the dilutions within a geometric series with a separation factor which depends on the nature of the sample to be analysed (chemical substances, effluents, waters or extracts) and on the type of assay (range finding or definitive)
- For the range finding test with chemical substances, the separation factor for the serial dilutions is usually 10 (one order of magnitude difference between two successive dilutions)
- For treated or untreated wastewater, freshwater, porewater or extracts, a separation factor of two between dilutions is usually performed (i.e. dilution of the previous dilution by half)
- Dilution series for the definitive test on chemical substances are prepared with a separation factor not exceeding 3.2
- If steep concentration-response curves are expected, it is recommended that a separation factor not exceeding 2.2 be used
- Each dilution is preferably carried out in three replicates with a control also in three replicates

- Substances which are poorly soluble in water may be solubilized or dispersed directly in pure water or dilution water by suitable means using ultrasonic devices or solvents of low toxicity to *Thamnocephalus platyurus*. Solvents should be used only when the LC_{50} is greater than the solubility of the test substance. If a solvent is used, the concentration of the solvent in the final test solution shall not exceed 0.1 mL/L, and two control solutions, one with no solvent, the other with the maximum concentration of solvent, shall be included in the test. Consider special requirements concerning test design for chemicals with solvents, e.g. additional solvent-control and statistical evaluations according to ISO/TS 20281 (2006)

16.6.2 Test

- Prepare a dilution series with the test sample and the dilution water
- Combine increasing volumes of the test solution with the dilution water, to obtain the desired concentrations for the test and transfer to the test containers
- To obtain a test and solution temperature of 25 ± 1°C, for example, place the containers in a temperature-controlled room or incubator
- As soon as this temperature is attained, introduce the *Thamnocephalus platyurus* into the test containers with the micro-pipette, taking care to add as little hatching medium as possible, and release the crustaceans under the water surface
- At least 30 animals, preferably divided into three groups of ten animals each, should be used at each test concentration and for the controls
- For each series of tests, prepare a control having a volume of dilution water equal to the volume of the test solutions and introduce the same number of *Thamnocephalus platyurus* as in the test solutions. If a solvent is used to solubilize or disperse substances, prepare a second control with the dilution water containing the solvent at the maximum concentration used (i.e. not greater than 0.1 m/L)
- Animals shall not be fed during the test and test containers shall be maintained in a temperature-controlled room or chamber at a temperature of 25 ± 1°C. Observations of test organism responses are made at the end of the exposure time
- At the end of the test period of 24 hours, count the number of dead *Thamnocephalus platyurus* in each container and note down these mortalities. Those which are not able to swim after gentle agitation of the liquid for 10 seconds shall be considered to be dead
- Determine the concentration range giving 0% to 100% mortality and note anomalies (e.g. lethargy, floating on the surface, abnormal rotating, or circling) in the behaviour of the *Thamnocephalus platyurus*

16.6.3 Preliminary test

This test enables determination of the range of concentrations over which the definitive test is to be carried out. For this purpose, use only a single series of concentrations (generally chosen in geometric progression) of stock solution or sample. Ten test organisms should be exposed to each test concentration, and no

replicates are necessary. Depending on the purpose of the test and the statistical requirements concerning the test results, other dilution designs with concentrations in a geometric or a logarithmic series can also be appropriate.

16.6.4 Definitive test

- This test determines the percentage mortality of *Thamnocephalus platyurus* by different concentrations and the 24 hour LC_{50}
- For the calculation of an LC_{50} value, it is desirable that the range of concentration chosen results in at least three percentages of mortality between 10% and 90%
- For each concentration and each control, use a minimum of 30 test organisms, preferably divided into three replicates, with ten animals per test container

16.6.5 Filling the test plate

For filling the test plates (see Figure 2 below)

- Each toxicant dilution must be transferred into all the wells of one column in the multi-well plate
- The wells are labelled from 1 to 6 horizontally and from A to D vertically
- The distribution of the test solutions will always be carried out starting with the control (left, column 1) towards the highest concentration (right, column 6)
- Controls: Add 1 mL standard freshwater to each well of column 1 (wells A1, B1, C1, D1)
- Toxicant dilutions: Shake each test tube thoroughly and transfer 1 mL of test tube 5 to each well of column 2 (wells A2, B2, C2, D2)
- Repeat this procedure (steps 2 and 3) with test tubes 4, 3, 2 and 1 to fill the wells of columns 3, 4, 5 and 6, respectively

16.6.6 Transfer of neonates to the test well and incubation

Transfer of the fairy shrimp larvae (See Figure 2) to the multi-well plate is accomplished in two steps:

- Transfer the larvae (approximately 50) from the petri dish into the rinsing wells of the multi-well plate (D1 to D6)
- Transfer approximately 10 larvae from the rinsing wells to each test wells (rows A, B, C)

Note: The intermediate transfer of the larvae through rinsing wells (row D) minimizes the dilution of the toxicant solutions in the test wells (rows A, B, C).

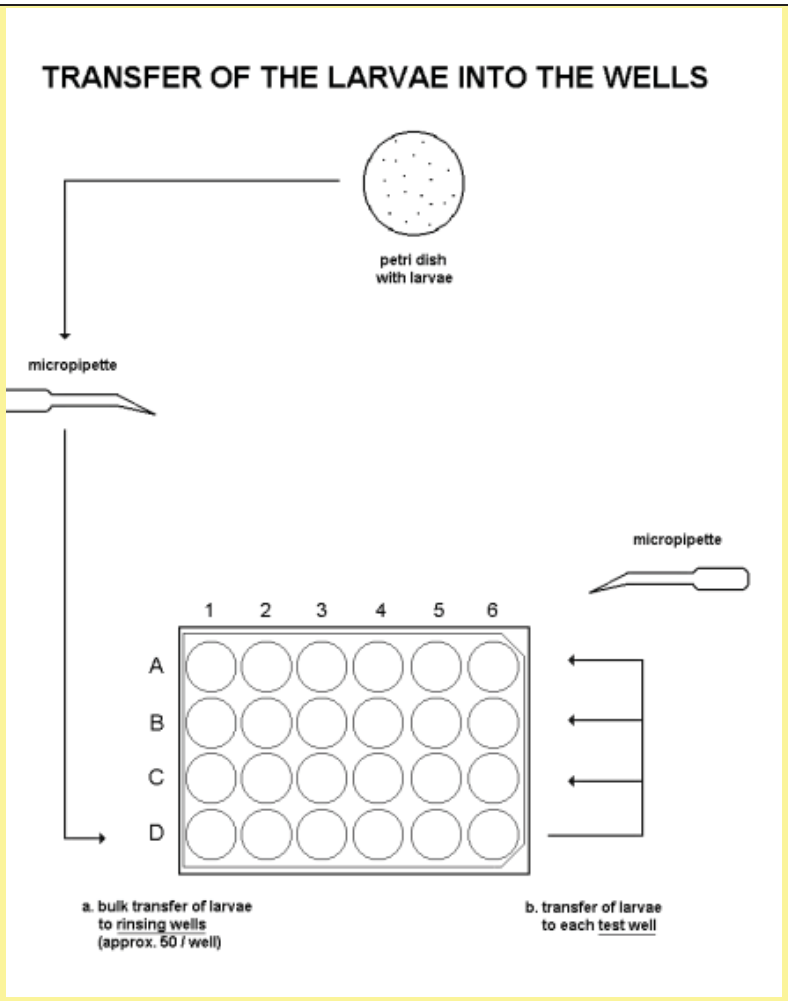

Figure 16-3: Transfer of larvae to the test wells

16.6.7 Scoring of the test results

- Mortalities are recorded after the 24 hours incubation period
- Take the multi-well plate out of the incubator and put it under the dissection microscope
- Check all the wells of row A, B, and C and record the number of dead larvae. The larvae are considered dead if they do not show any movement during 10 seconds of observation
- Record the mortality figures on the results sheet
- Total the number of dead larvae for each concentration and calculate the % mortality

16.7 CALCULATIONS OF RESULTS

16.7.1 Estimation of the LC_{50}

At the end of the 24 hour test, calculate the percentage mortality for each concentration in relation to the total number of *Thamnocephalus platyurus* used. Determine the 24 hour LC_{50} by an appropriate statistical method (REGTOX or Probit analysis, moving average, binomial methods or graphical estimation).

The concentration of the test substance should be measured, as a minimum at the highest and lowest test concentration, at the beginning and end of the test. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within ± 20% of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

If the data are insufficient or calculation of the LC_{50} is not required, quote the minimum concentration corresponding to 100% mortality and the maximum concentration corresponding to 0% mortality. Record the mean percentage mortality in the control and in each test concentration.

16.7.2 Expression of results

Express the LC_{10} and LC_{50}, and the values corresponding to 0% and 100% mortality:

- as a percentage, in the case of effluents, waters, eluates or extracts or as
- in milligrams per litre, in the case of chemical substances

Determination of the initial concentration (i.e. the concentration present at the beginning of the test) which, in 24 hour cause 50% mortality of exposed *Thamnocephalus platyurus*, under the conditions specified in this International Standard. This concentration, known as the effective initial inhibitory concentration, is designated 24 hour LC_{50}. An indication of the lowest concentration tested which cause the mortality of all the *Thamnocephalus platyurus* and the highest concentration tested which does not cause any mortality of the *Thamnocephalus platyurus* is desirable and provides useful information in cases where the LC_{50} cannot be determined.

The test is carried out in one or two stages:

- a preliminary test which determines the range of concentrations to be tested in the definitive toxicity test and gives an approximate value of the 24 hour LC_{50}.
- a definitive test, conducted when the approximate value given by the preliminary test is not sufficient, which permits calculation of the 24 hour LC_{50} and determines concentrations corresponding to 0% and 100% mortality

If the method specified in this International Standard is used for biotesting of chemical substances, a limit test can be performed at 100 mg/L or at a lower concentration, at which the substance is soluble or is in stable dispersion under the conditions of the test. If it provides useful information, a limit test may also be performed at concentrations above 100 mg/L as long as the substance is soluble or in stable dispersion.

16.8 TEST VALIDITY

Consider the results as valid if the following conditions are satisfied at the end of the test:

- the percentage mortality in the controls is less than or equal to 10%
- the 24 hour LC_{50} of the potassium dichromate is within the range 0.052 mg/L to 0.148 mg/L

16.9 INTERPRETATION OF RESULTS

Following the 24 hour check, the data should be entered onto the relevant electronic data sheet where automatically calculations of the total number of dead larvae for each toxicant concentration will take place. This must include the mean and the percentage effect.

16.9.1 Screening test calculations

Calculate the percentage mortalities for each sample in relation to the number of test organisms exposed as follows:

Percentage mortality = $100 - [(N_{t0}-N_{tx})/ N_{t0} \times 100]$

N_{t0} Number of test organisms exposed at time zero

N_{tx} Number of dead test organisms after 24 exposures

16.9.2 Definitive test calculations

Calculate the 24 hour LC_{50} values (toxicity endpoints) by applying an appropriate statistical method (e.g. TSK or Probit). Express toxicity endpoints as a percentage (e.g. LC_{50} of 30%) or sample concentration (e.g. 30 mg/L). In cases where limited mortalities were recorded for the definitive test and a statistical method could not be used to calculate the endpoints (> 50%), these endpoints should be reported as a LC_{50} > 100% or as TU < 1.

16.10 TEST REPORT

This test report shall contain at least the following information:

- the test method used, together with a reference to this International Standard (ISO 14380, 2011)
- all information required for the complete identification of the original sample (before treatment) or of the chemical substance under test
- the methods of preparation of the samples
    - for effluents, waters, eluates and extracts, the method and the storage time of the samples, the pH and the dissolved oxygen concentration of the original sample, if need be, the conditions in which the decantation, filtration or centrifugation of the sample and a possible adjustment of the pH were carried out
    - for chemical substances, the method of preparation of the stock and test solutions
- all biological, chemical, and physical information relative to the test set out in this International Standard, including the origin of the *Thamnocephalus platyurus* used
- the results of the test in the form of the 24 hour LC_{50}, the method of calculation, and, if possible, the 95% confidence limit; in the case of chemical analysis of the substances, the method used

- the results of the limit test, if conducted
- the minimum tested concentration corresponding to 100% mortality and the maximum tested concentration corresponding to 0% mortality in 24 hours
- any abnormal behaviour of the *Thamnocephalus platyurus* under the test conditions (e.g. lethargy, floating on the surface, abnormal rotating or circling)
- any operating details not specified in this International Standard and incidents which may have affected the results
- the results obtained with the reference chemical as well as the date of the reference test
- data to prove that the validity criteria are met
- name and address of the testing laboratory, the persons carrying out the test, and the person approving the report

16.11 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

16.11.1 Application

According to ISO 14380 (2011) this method is applicable to:

- Chemical substances which are soluble under the conditions of the test, or can be maintained as a stable suspension or dispersion under the conditions of the test
- Industrial or sewage effluents
- Treated or untreated wastewater
- Aqueous extracts and leachates
- Freshwater (surface and groundwater)
- Toxins of blue green algae

16.11.2 Advantages

The significance of the *Thamnocephalus platyurus* acute toxicity test is to help in the assessment of possible risk to similar invertebrate species in the natural environment, as an aid in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other species for comparative purposes (US EPA, 2002).

Like rotifers and copepods, Anostraca's such as *Thamnocephalus platyurus* are ecologically very important members of freshwater aquatic communities. Toxicity tests, such as the *Thamnocephalus platyurus* acute toxicity test, is applied to assess water pollution and are primarily used to screen for toxic substances in the aquatic environment and to some extend to predict the toxic effect of environmental impacts on aquatic invertebrates. A further significance of the test is to comply with the "The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach" (DWAF, 2003).

The major advantage of MicroBioTests kits, in comparison to "conventional" bioassays, is that the test organisms are incorporated in the kits in a "resting" or "immobilized" form, from which they can be activated "on demand" prior to performance of the toxicity test. This eliminates the need for continuous recruitment and/or stock culturing of test organisms, and hence the major cost factor. Furthermore, all MicroBioTests kits have been "miniaturized" into low-cost kits which can be performed with conventional laboratory materials and equipment, on little bench space.

16.11.3 Limitations

- Dark coloured samples and samples containing high loads of suspended solids may impede the observation of the test organisms
- Samples containing oils and surface tension altering compounds may cause test organisms to float
- Pathogenic and /or predatory organisms in the samples may affect survival
- Living organisms as used during these tests, also have species specific requirements for culture conditions, which may necessitate adjustment of, e.g. the sample pH or salinity or oxygen concentration and these actions may have an effect on the bioavailability or solubility of certain hazardous substances

16.11.4 RECOMMENDATIONS

This method provides an easy-to-use bioassay for measuring acute toxicity of substances/pollution to invertebrates, such as *Thamnocephalus platyurus*. The significance of the *Thamnocephalus platyurus* acute toxicity test is to assist in the assessment of possible risks to invertebrate species in the natural environment. It also aids in determination of possible water quality criteria for regulatory purposes and for use in correlation with acute testing of other invertebrate species for comparative purposes. This test should form part of a battery of at least three tests representing different trophic levels in the aquatic environment (others include but are not limited to bacteria, invertebrates, algae, vertebrates, and protozoa amongst others). The reason for this is the variation between the sensitivity of the different species to different substances, and therefore using several different trophic levels increases the reliability of the test interpretation. Secondly, no single species can indicate all the substantial endpoints. This is a way to obtain a better view of the effects on ecosystem functioning and on different trophic levels.

16.12 REFERENCES

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

DWAF (Department of Water Affairs and Forestry) (2003) The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effects Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

ISO (International Organization for Standardization) 5667-16 (1998) Water quality – Sampling – Part 16: Guidance on biotesting of samples. ISO/TC 147/SC 6 Sampling (general methods) pp24.
ISO/TS (International Organization for Standardization/Technical Standard) 20281 (2006) Water quality – Guidance on statistical interpretation of ecotoxicity data. ISO/TC 147/SC 5 Biological methods pp252.
ISO (International Organization for Standardization) 10523 (2008) Water quality – Determination of pH. ISO/TC 147/SC 2 Physical, chemical and biochemical pp13.
ISO (International Organization for Standardization) 5814 (2012) Water quality – Determination of dissolved oxygen – Electrochemical probe method. ISO/TC 147/SC 2 Physical, chemical and biochemical pp14.
ISO (International Organization for Standardization) 14380 (2011) "Water Quality – Determination of the acute toxicity *Thamnocephalus platyurus* (Crustacea, Anostraca). ISO/TC 147/SC 5 Biological methods pp21.
MicroBioTests Inc., Belgium. THAMNOTOXKIT F™. Freshwater Crustacean Toxicity Screening Test. Standard Operational Procedure.
SANS (South African National Standards) 14380 (2011) Water quality – Determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca) pp 24.
SLABBERT L (2004) Methods for direct estimation of ecological effect potential (DEEEP) WRC Report 1313/01/04. Water Research Commission, Pretoria.
US EPA (United States Environmental Protection Agency) (2002) Method for measuring the acute toxicity of effluents and receiving water to freshwater and marine organisms. EPA-821-R-02-012. U.S. Environmental Protection Agency, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington DC, 20460. Fifth Edition.

16.13 USEFUL CONTACTS

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17 FROG EMBRYO TERATOGENESIS ASSAY OF XENOPUS (FETAX)

Compiled by: Edward Archer and Christoff Truter

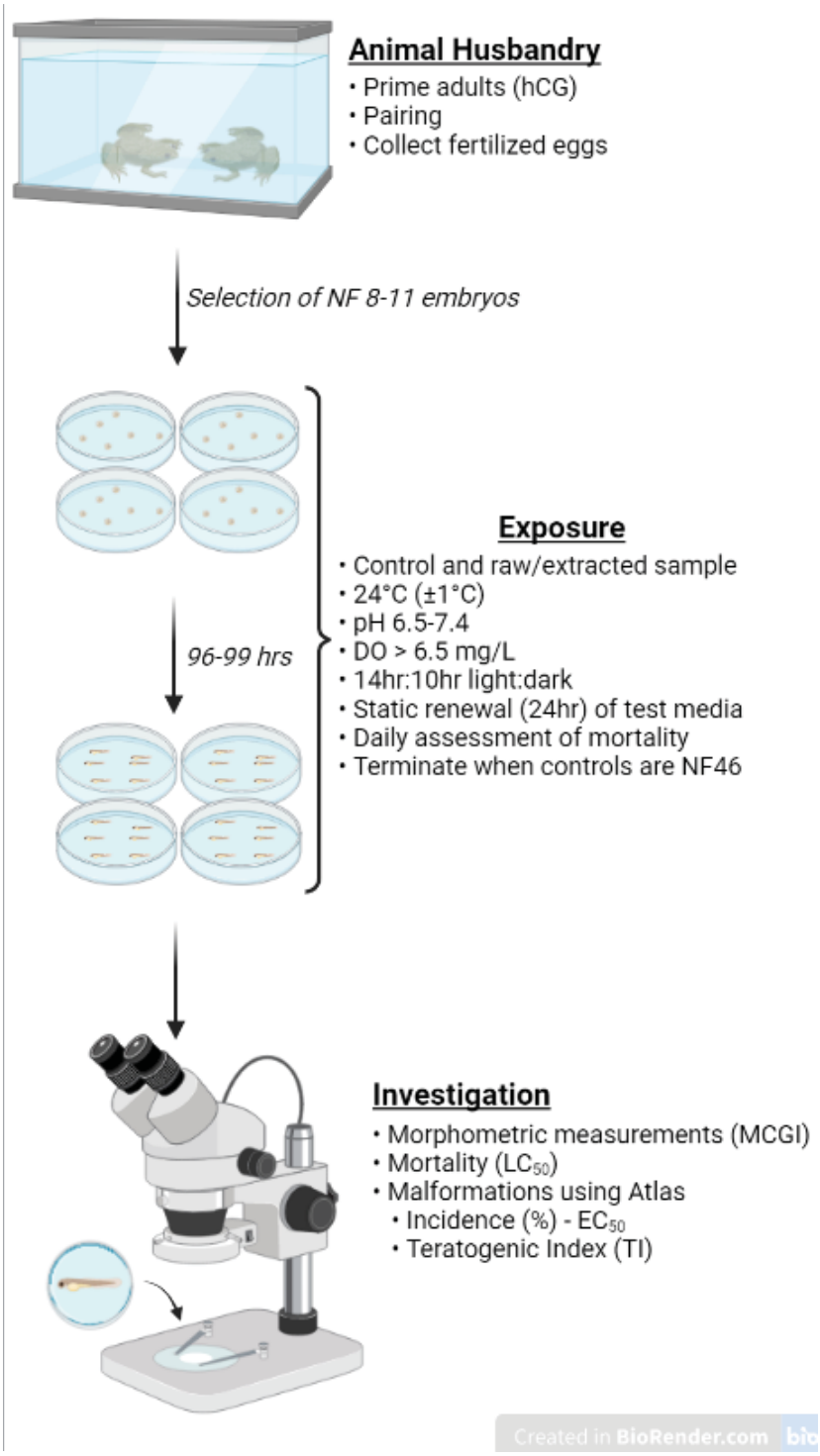

Figure 17-1: Schematic showing a typical procedure for the extraction of aqueous and solid environmental samples for the FROG EMBRYO teratogenesis assay

17.1 ACRONYMS & ABBREVIATIONS

FETAX	Frog Embryo Teratogenesis Assay for Xenopus
hCG	Human Chorionic Gonadotropin
ASTM	American Society of Testing and Materials
EC50	Effect Concentration for 50% of the test subjects
DO	Dissolved Oxygen
OECD	Organisation for Economic Co-operation and Development
NF	Nieuwkoop and Faber
TI	Teratogenic Index
MCGI	Minimum Concentration for Growth Inhibition
IU	International Units
SPE	Solid Phase Extraction

17.2 PRINCIPLE OF THE ASSAY

The Frog Embryo Teratogenesis Assay of Xenopus (FETAX) is an acute, *in vivo* whole-embryo bioassay to determine teratogenesis and developmental toxicity of test chemicals, complex mixtures and/or environmental samples using the African clawed frog (*Xenopus laevis*) as sentinel organism.

Developmental toxicity serves as a sensitive *in vivo* bioassay to show toxicity in test samples at lower concentrations than where toxicity is observed for adult organisms. Embryos of *X. laevis* thus serve as good sentinel organisms due to their direct nature of contact with environmental matrices, their ease of in-house breeding, and relatively quick and well-documented developmental phases (Nieuwkoop and Faber, 1975). Test environmental samples or test chemicals/mixtures can be prepared at varying concentrations and exposed to the embryos at the onset of a specific developmental stage, upon which mortality is continuously monitored and developmental malformations recorded upon completion of the 96-hour exposure period using an established *X. laevis* embryo malformation index (Bantle et al., 1999).

This 96-hour toxicity assay is well described in guidelines from the American Society of Testing and Materials (ASTM, 1998) to serve as an alternative toxicity screen using mammalian organisms to evaluate developmental toxicity *in vivo*. Moreover, the target endpoints that include mortality and the degree of embryonic malformations are valuable to assess the developmental toxicity in test environmental samples such as treated surface- or wastewater and even drinking water quality.

17.3 REQUIREMENTS

17.3.1 PERMITS

Ethical clearance and permits from the research institution need to be obtained prior to the experimentation. The procedure and contact details of obtaining such ethical clearance for experimentation and animal holding permits may vary between institutions and should thus be arranged well ahead of performing the experiments.

17.3.2 STAFF TRAINING (TECHNICAL SKILLS)

- Good laboratory practice
- Training in cell basic light and stereo microscopy
- Experience in aquatic/anuran husbandry
- Thorough knowledge of the ASTM guidelines for FETAX (REF)
- Software package knowledge (for managing datasets, statistics, and microscope imaging)

17.3.3 LABORATORY (TEST ENVIRONMENT)

- Dedicated animal housing facility (wet room or aquarium)
    - Equipped with aeration for the holding tanks and light/dark cycles
- Large incubators or climate rooms
    - Capable of maintaining light/dark cycles and temperatures of 23°C ±1°C
- Microscope and imaging facility

17.3.4 SOFTWARE

- Statistical programme for data visualisation and exposure comparisons
- Microsoft Excel
- Microscope imaging (e.g. Leica LazEz or similar)

17.3.5 APPARATUS

- Stereo Microscope

17.3.6 CONSUMABLES/MATERIALS

- Glass petri dishes with lids (100mm x 15mm)
- Syringes and needles, sterile
- Aquarium net
- Plastic syringes
- Nylon or plastic mesh

17.3.7 REAGENTS

Table 17-1: List of reagents

Name	Cat no	Supplier
Formalin (37%)	252549	Sigma-Aldrich
l-cysteine	52-90-4	Sigma-Aldrich
Sodium chloride (NaCl)	7647-14-5	Sigma-Aldrich
Sodium bicarbonate ($NaHCO_3$)	144-55-8	Sigma-Aldrich
Potasium chloride (KCl)	7447-40-7	Sigma-Aldrich
Calcium chloride ($CaCl_2$)	10035-04-8	Sigma-Aldrich
Calcium sulfate dihydrate ($CaSO_4.2H_2O$)	10101-41-4	Sigma-Aldrich
Magnesium sulphate ($MgSO_4$)	10034-99-8	Sigma-Aldrich
Human Chorionic Gonadotropin (hCG)	9002-61-3	Sigma-Aldrich
Tricaine Methane Sulfonate (MS222)	886-86-2	Sigma-Aldrich
Acetone, Reagent Grade	179124	Sigma-Aldrich

17.4 METHOD – PREPARATION OF FETAX SOLUTIONS AND TEST SAMPLES

Refer to the American Society of Testing and Materials Guidelines for FETAX (ASTM, 1998; section 10) for a detailed description of sample preparation.

17.4.1 FETAX SOLUTION

Prepare the FETAX solution by adding the following reagents to 1 litre of Milli-Q water (ASTM, 1998).

NaCl .....625 mg
$NaHCO_3$ .....96 mg
KCl.....30 mg
$CaCl_2$ .....15 mg
$CaSO_4.2H_2O$ .....60 mg
$MgSO_4$ .....75 mg

17.4.2 L-CYSTEINE SOLUTION

- Prepare a 2% w/v l-cysteine solution in Milli-Q water and adjust the (pH to 8.1) using 1M sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl).

17.4.3 MS222 (Tricaine methane sulfonate) SOLUTION

- Add 200 mg/L MS222 into Milli-Q water and buffer with 0.42-1.05 g/L sodium bicarbonate ($NaHCO_3$).

17.4.4 ENVIRONMENTAL SAMPLES

Test environmental samples should be collected in clean glass bottles (washed with detergent, followed by two ethanol rinses and two Milli-Q water rinses) and transported to the testing facility/laboratory on ice. Same-day processing of aqueous sample extracts or freeze-dried sediment/soil samples should be done to preserve the chemical integrity of the test sample.

17.4.5 AQUEOUS SAMPLES

Environmental aqueous samples may be exposed directly to the test organisms or concentrated using SPE to generate a dilution series of the test water sample using FETAX Solution (ASTM, 1998) as solvent. All samples and sample concentrations should be performed in triplicate.

17.4.6 WHOLE SOIL/SEDIMENT

Soiled or sediment testing can be performed by replacing the petri dishes with glass tubes that can accommodate a Teflon mesh insert that serves as an exposure vessel. Up to 35 g of sediment (dry weight) can be placed in the bottom of the vessel, the Teflon mesh insert added, and filled with 140 mL of FETAX Solution (ASTM, 1998). If the soil will be diluted, it should be verified that the dilution material (such as laboratory reference soil) is non-toxic and as chemically/physically like the test matrix as possible. All samples and sample concentrations should be performed in triplicate.

17.4.7 TEST CHEMICALS AND/OR MIXTURES

All test material should be reagent- or analytical-grade unless a specific test mixture/formulation/commercial product is being tested. In the case of the latter, information of the active ingredients and impurities should be recorded, along with information regarding its biohazardous toxicity, solubility and stability in water, recommended handling procedures and other physico-chemical properties (pH, hardness, alkalinity, conductivity, etc.). Test chemicals can mostly be added directly to the test petri dishes containing the fertilized eggs using freshly made stock solutions on the day of exposure. The preferred FETAX Solution (ASTM, 1998) should be used as solvent for chemical and/or mixture stock solutions where possible. Volatile solvents such as acetone, ethanol, methanol should be avoided as this may interfere with embryonic development. All samples and sample concentrations should be performed in triplicate.

17.4.8 CLEANING OF GLASSWARE

Ensure that all glassware is cleaned as referred to in section 7.4.1. of the ASTM (1998) guideline:

3.7.1. Soak 15 min, and scrub with tissue culture compatible detergent in tap water.

3.7.2. Rinse twice with tap water.
3.7.3. Rinse once with fresh, dilute (10%, v/v) hydrochloric acid to remove scale, metals, and bases.
3.7.4. Rinse twice with tap water.
3.7.5. Rinse once with full strength reagent-grade acetone to remove organic compounds.
3.7.6. Rinse well with hot tap water.
3.7.7. Rinse well with distilled water or FETAX solution.
3.7.8. Heat the glassware in an oven at 350°C for 3 h to drive off any residual acetone.

17.5 METHOD – ANIMAL HUSBANDRY

All animal husbandry, treatment and handling should be performed according to the South African Standard: the care and use of animals for scientific purposes (SANS 10386:200X). An in-house protocol for *X. laevis* handling and husbandry can also be found from the Stellenbosch University Research Ethics Committee (Protocol #: SU-ACUM14-00002). Details of the *X. laevis* husbandry will follow here.

Synthetically induced ovulation on *X. laevis* was done according to Haywood et al. (2004) and the main procedure described below.

- Day 1: Inject 300 IU of human chorionic gonadotropin (hCG) into the dorsal lymph sac of female adult frogs to induce ovulation.
    - The hCG should be injected in 50-100 IU doses.
- Day 3: Inject the same female adult frogs with a second dose of hCG (750 IU) and male adult frogs with 200 IU hCG.
    - The hCG should be injected in 50-100 IU doses.
    - The breeding pairs are ready when the males develop a darkened keratinized skin (nuptial pads) on the forearms, and a red, swollen cloacal labia for the females.
- Pair the adult male and females in breeding tanks filled with de-chlorinated water.
    - The breeding tanks should be fitted with a false bottom that should be 30mm from the bottom to allow fertilized eggs to fall to the bottom without disturbances of the adult *frogs.*

17.6 METHOD – FETAX PROCEDURE

The 1998 ASTM guideline includes detailed information for the FETAX procedure, along with appendices on concentration steps for range-finding tests, microsome isolation reagents, and nicotinamide adenine dinucleotide phosphate (NADPH)-generating system components (ASTM, 1998). Refer to the ASTM guidelines (ASTM, 1998) for a comprehensive guideline on performing the FETAX (https://www.astm.org/e1439-12r19.html; NICEATM, 2000). A brief method summary is described below:

NOTE: The exposure conditions for the FETAX requires exposure of embryos between the mid-blastula to early gastrula developmental stage according to the Nieuwkoop and Faber (1975) atlas of *X. laevis* development classification. This window of embryonic development (NF stage 8-11) falls between **4-8 hours post-fertilisation**. As a result, all the experimental setup (cleaned exposure dishes, test solutions and handling equipment) should be ready once successful breeding and spawning has been observed to ensure that the exposure conditions fall within the criteria of the assay.

17.6.1 preparation and Selection of embryos

- Upon the completion of *X. laevis* husbandry:
    - Collect the fertilised eggs using a soft aquarium net or a plastic pipette.
    - Make sure not to damage the eggs whilst handling.
- Place the harvested eggs in a large petri dish containing a 2% l-cysteine solution (Dawson and Bantle 1987).
    - Refer to section 7.4.1. of the ASTM (1998) guideline for the cleaning of glassware for the experimentation.
    - The l-cysteine solution removes the jelly coat around the egg but does not damage the egg itself.
- Carefully select normal cleaving embryos using a stereo microscope and referral to the Nieuwkoop and Faber (1975) developmental atlas.
    - The selection criteria include embryos in the mid-blastula to early gastrula stage of development (NF stage 8-11; Figure 17-2), which is between 4- and 8-hours post-fertilization.
    - Keep all embryos that qualify under these specifications in a separate clean glass petri dish containing FETAX solution until the desired number of embryos are obtained that falls within the NF stage 8-11 developmental range.

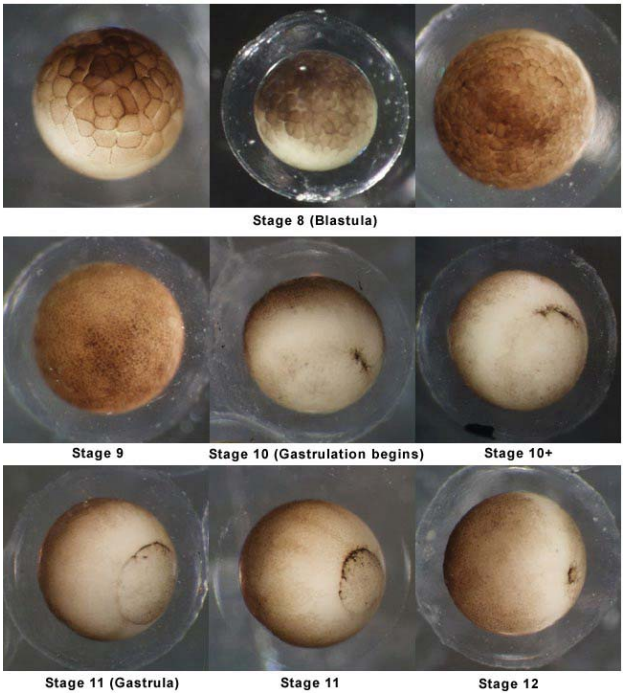

Figure 17-2: Example of the Nieuwkoop and Faber (1975) classification of the late blastula to early gastrula staging for the selection criteria in the FETAX procedure. Image derived from https://www.swarthmore.edu/NatSci/sgilber1/DB_lab/Frog/frog_staging.html.

17.6.2 Exposure conditions and experimental setup

Ensure that all test solutions and exposure samples (in the case of raw water samples) are pre-conditioned to 24°C (±1°C) to avoid unnecessary temperature shock to the embryos during the initial preparation and/or routine changing of exposure solutions.

- Prepare the glass petri dishes (100 mm diameter) for the various exposure conditions (including controls) by adding 40 mL of a FETAX solution to each.
    - Do not add the test chemicals or raw environmental water samples to the exposure groups before all embryos are staged and distributed into the test petri dishes
    - An exception when this cannot be avoided include direct exposure of environmental sample that require a defined percentage of the final water volume.
- Distribute twenty-five (25) haphazardly selected NF stage 8-11 embryos (Figure 17-2) into each of the exposure glass petri dishes.
    - All exposure conditions are done in duplicate, except for the negative controls (blank) that are done in quadruplicate containing only FETAX solution
    - This may result in many petri dishes that need to be prepared, including provision for their incubation space in the temperature-controlled facility (Figure 17-3).

Figure 17-3: Example of a typical FETAX layout of the glass petri dishes in the temperature-controlled room.

- Spike all the test conditions after all the NF stage 8-11 embryos have been added into the petri dishes using a pipette and gently swirl the dish in a figure of eight motion to distribute the spiking sample evenly throughout the dish.
- Place all the exposure embryos in a controlled climate room with the following conditions (ASTM, 1998; OECD 2008; Babalola et al., 2021):
    - Water temperature: 24°C (±1°C)
    - pH: 6.5 to 7.4
    - DO: > 6.5 mg/L
    - Photoperiod: 14hr:10hr light:dark
- Replace the test solution every 24 hours (static renewal) by drawing up the old solution using a glass pipette and immediately replacing with new test solution.
    - This includes the negative controls containing only FETAX solution.

- Ensure that enough additional FETAX solution is incubated at 24°C (±1°C) for the replacement of the test solution each day.
- Monitor the larvae daily for mortalities using either a stereomicroscope or tactile stimuli and remove deceased individuals immediately.

- Upon completion of the 96-hour exposure, the control tadpoles should have reached NF stage 46 with less than xxx% mortality (NICEATM, 2000)
  - Observe the stage of the control larvae inside the petri dishes using a stereoscopic microscope and stereo microscope and referral to the Nieuwkoop and Faber (1975) developmental atlas.
  - The NF stage 46 is characterised by the onset of hind limb development as illustrated in Figure 17-4.

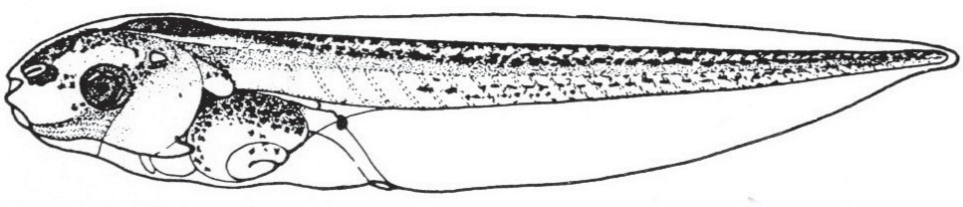

Figure 17-4: Illustration of a Nieuwkoop and Faber (1975) NF stage 46

  - The exposure period may be extended to 99 hours if the desired developmental stage has not been reached yet in the control exposure group. The experiment should subsequently be terminated and repeated if the desired NF stage is not reached after 99 hours, which suggest that undesired factors are limiting normal development of the embryos.
- If the desired NF stage 46 has been successfully reached in the control groups with less than 10% mortality (NICEATM, 2000):
  - Euthanize all larvae using a MS 222 solution (OECD, 2008) and transfer the specimens to a 10% buffered formalin solution.

17.7 ASSESSMENT

17.7.1 Mortality and Growth Inhibition

- Record the daily mortalities in the test solutions
  - For testing of individual chemicals/pollutants, the cumulative mortality data throughout the exposure period is used to calculate the lethal concentration of 5%, 50% and 95% of test subjects (LC_5, LC_{50} and LC_{95} respectively).
- Perform morphometric measurements of each tadpole specimen
  - snout-to-vent length (mm), body length (mm), tail length (mm), and body mass (g)
- If individual chemicals are tested or a range of mixture concentrations, determine the minimum concentration for growth inhibition (MCGI) by comparing the total lengths of individuals from the respective concentrations of the different exposure groups with that of the control group.

- Determine the developmental stage of each tadpole using the classification atlas of Nieuwkoop and Faber (1975).

17.7.2 Malformations and Teratogenic Index

Each specimen should be evaluated for developmental malformations using the atlas of abnormalities as described in Bantle et al. (1999) and elsewhere (Babalola et al., 2021; Fort and Paul, 2002; Hu et al., 2015). Briefly, all observed abnormalities that are listed under the established FETAX index are scored according to the level of severity (see example in Figure 17-5).

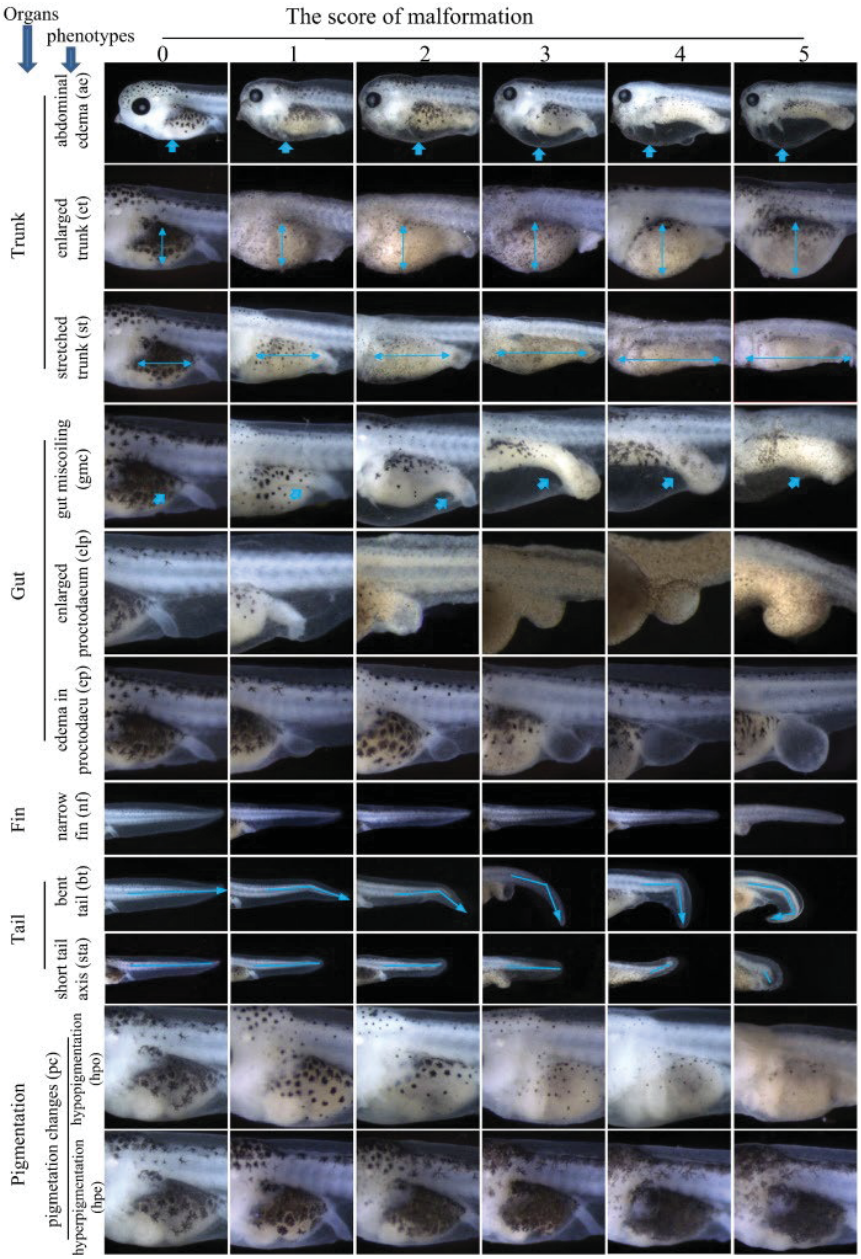

Figure 17-5: A partial example of the FETAX atlas of abnormalities as illustrated in Hu et al. (2015), showing the variation of items that are investigated (Y-axis) and the scoring of the level of severity of the abnormality (X-axis).

- Teratogenicity is quantified as the 50% effective concentration for malformation (EC_{50}), and the malformation incidence (%) using the Atlas of Abnormalities (Bantle et al., 1999).

- The Teratogenic Index (TI) is calculated by calculating the LC_{50}/EC_{50} ratio.

17.8 ADVANTAGES, LIMITATIONS & RECOMMENDATIONS

17.8.1 Advantages

- The assay is relatively rapid for an *in vivo* bioassay (4 days).
- The assay provides a variety of developmental malformation endpoints that can be evaluated through an established malformation index and can be extrapolated to higher vertebrates.
- The use of an ecologically relevant sentinel aquatic organism (*X. laevis* embryos) provides concrete evidence of direct teratogenicity of test material to higher trophic level aquatic organisms.
- The FETAX can be applied on treated effluents, surface/groundwaters, leachates, and solid-phase samples (soils, sediments, and particulate matter), allowing for a high-tier ecological risk characterisation tool.

17.8.2 Limitations

- Ethical clearance from the research institution will be required for the in-house breeding and experimentation.
- Access to animal housing, breeding aquaria and incubation rooms may be less available.
- Breeding success of *X. laevis* breeding pairs may vary between seasons.
- High potential loss of organisms during the bioassay procedure if test conditions are not regularly monitored.
- The FETAX protocol as provided in the ASTM guidelines (ASTM, 1998) has strict ranges of acceptable mortality and malformation outcomes in the control sample subset, which may require for the bioassay to be repeated several times.

17.8.3 Recommendations

- The FETAX can be complemented with additional molecular evaluation for the investigation of other teratogenic- or endocrine-disrupting biomarkers (such as thyroid disruption). This can be achieved by increasing the number of specimens in each experiment, whereby a subset of the test organisms is processed for molecular investigation rather than fixation for morphometric evaluation.
- Additionally, by increasing the number of specimens in each test condition, a subset of surviving specimens can be used for an extended Xenopus metamorphosis assay (XEMA) (OECD, 2008). This is done through a chronic (28-day) exposure of *X. laevis* tadpoles from NF stage 46, which co-incidentally is also the end stage of the FETAX, until NF stage 51. Histopathology of the thyroid hormone, as well as molecular evaluation of markers of the thyroid endocrine system is then evaluated, which thus produce a comprehensive range of effect-based outcomes.

17.9 REFERENCES

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