The Compilation of a Toolbox of Bio-assays for Detection of Estrogenic Activity in Water

Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

INTRODUCTION

The WRC has been funding a structured and well planned Endocrine Disruptor Chemical (EDC) and Toxicant research programme from 2001, consisting of several research projects in different Key Strategic Areas (KSAs). These projects have to be coordinated to make sure that the objectives of the programme are being met. The sampling, biological and analytical procedures used in this programme and in collaboration with the Global Water Research Coalition (GWRC), now need to be taken a step further to the development of user friendly manuals. It is therefore of utmost importance to the success of the programme and benefits to the country that the training manuals will be ready during the third phase of the EDC programme, and the implementation of the National Toxicant Monitoring Programme (NTMP) of DWAF.

After a Workshop at the d'Ouwe Werf in Stellenbosch on 4-5 June 2007, it was decided to divide the manual into different volumes:

Volume 1:	Monitoring and Assessment
Volume 2:	Sampling Guide
Volume 3:	Bio-assay Methods
Volume 4:	Management Options

This Volume (3) will focus on the identification, selection and standardization of biological assays for EDCs, with special reference to estrogenic compounds.

BACKGROUND AND JUSTIFICATION

The presence of estrogenic compounds in drinking waters, source waters and wastewater is of international concern because of potential adverse health effects on wildlife and humans. Chemical analysis in environmental matrices has been problematic due to both the large numbers of compounds with endocrine disrupting capabilities that may be present in the environment and the ultra-low concentrations that have been reported in the literature to cause estrogenic effects. Biological methods are becoming increasingly popular as screening tools because the specific chemical nature of an environmental sample is not always known. As the effects of chemical mixtures cannot always be inferred from their concentrations, bioassays are an important component of examining the presence of and integrating the effects of complex mixtures of endocrine disrupting chemicals. However, no single assay can accurately predict the total estrogenic activity of complex samples to all organisms. Therefore, there is a need to develop a recommendation for a suite of suitable and reliable methods available for conducting this analysis.

Project summary

Substantial effort in recent years has been put into the development of biological methods to assess the estrogenicity of water including drinking water, ground water, surface water and wastewater.

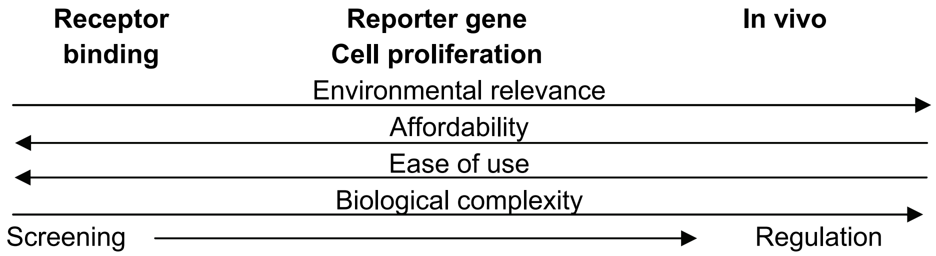
Methods that rely on biological activity are finding increasing utility as screening tools, because the chemical nature of the endocrine disrupting sample may be unknown and difficult to identify, and the biological method may be the best (or only) indicator of biological activity.

Initially the project team investigated the available methods from the literature and examined a suite of existing bioassays suitable for laboratory screening in one or more of the matrices outlined above. Initial method selection was based on parameters such as:

- local and global applicability,
- sensitivity or limits of detection,
- reproducibility,
- robustness,
- ease of use,

- application to more than one matrix,
- appropriate sample preparation and isolation procedures,
- environmental relevance,
- cost,
- repeatability,
- others as determined during the study.

The framework using these criteria is summarized below:



These bioassays should be representative of potential adverse effects that could impact aquatic ecosystems and/or animal health and be applicable for future evaluation of water and wastewater treatment technologies. After completion of the initial evaluation of the bioassay suitability, some selected methods were identified for inclusion in the manual. Ideally this final selection should consist of two *in vitro* and one *in vivo* method. At a Workshop held on 22 September 2008 at the School of Health Systems & Public Health, University of Pretoria, the panel decided to include the following assays:

YES Assay (Routledge & Sumpter)
T47D-Kbluc
E-screen
VTG ELISA

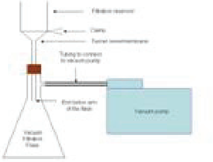
Methods were selected in close consultation with the project reference group. The complete standard operating procedures (SOPs) have been developed for the selected methods. The availability of such tools would allow the regulator / health authorities to make decisions based on sound scientific data when faced with a new water quality problem or the presence of new chemicals with unknown properties and will be able to base decisions on the precautionary principle.

A literature review was written on the identified bio-assays as part of associated project K8/920. This appears on the WRC web site under the Special Publications Number SP 29/11. In addition, an available report of the Global Water Research Coalition (GWRC) is provided under the special Publications Number 30/11. To access these please go to www.wrc.org.za > Knowledge Hub > Special Publications > enter the SP number in the search block. After completion of the initial evaluation of the bioassay suitability, some selected methods were included in the manual, and guidelines for chemical analysis of endocrine disrupter chemicals in water resources have also been added to Volume 3.

EXTRACTION PROCEDURE FOR WATER SAMPLES FOR THE ASSESSMENT OF ESTROGENIC ACTIVITY IN THE RECOMBINANT YEAST ESTROGEN ASSAY AND THE T47D-KBLUC ASSAY

1ℓ sample

pH to 3



↓

Pre-filtrate turbid samples

↓

Solid-phase extraction

5 ml dd H_2O

↓

5 ml MeOH

↓

5 ml dd H_2O

Pre-conditioning

↓

Sample

↓

Dry cartridge

↓

3-5 ml MeOH

Elution

↓

Evaporate

↓

Reconstitute with 1 ml EtOH

↓

Store sample at -20°C

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

dd H_2O	Double distilled water
EDCs	Endocrine disrupting compounds
ec	Endcapped
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
SPE	Solid phase extraction

EXTRACTION PROCEDURE FOR WATER SAMPLES FOR THE ASSESSMENT OF ESTROGENIC ACTIVITY IN THE RECOMBINANT YEAST ESTROGEN ASSAY AND THE T47D-KBLUC ASSAY

Compiled by: NH Aneck-Hahn, MC Van Zijl and C de Jager

This protocol describes the extraction of organics from water samples by solid phase extraction (SPE). These extracts are suitable for the assessment of estrogenic activity and androgenic activity in the bioassays.

Hazards:

- It must be kept in mind that environmental samples may contain hepatitis and other sewage-related and waterborne diseases. In this case, adequate vaccination and proper protective wear, such as gloves and standard laboratory wear is required.
- The correct safety procedures should be followed when working with the relevant solvents (methanol, ethanol), washing reagents and nitrogen gas as well as disposal of solvents and other waste materials.
- Do not exceed the maximum recommended vacuum (70 kPa, 20 mmHg) on the SPE.

Note:

- All glassware for sample storage, filtration and extraction of water samples must be washed in chromic acid and rinsed once in methanol (Cat No. 34860, Riedel-de Haën) and twice in ethanol (Cat.No. 27,0741, Sigma-Aldrich).
- If gloves are worn, it should be nitrile gloves and not latex gloves.

REQUIREMENTS

Staff training (technical skills)

- Good laboratory practice
- Basic training in practical analytical chemistry

Laboratory (test environment)

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

Apparatus

- Millipore Milli-Q synthesis ultrapure water system or equivalent system to produce double distilled water (dd H_2O). 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 column (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
- 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)

Consumables/Materials

- Aluminium/tin foil
- Amber glass bottles 4 mℓ (Chromatography research supplies, Cat. No.154515)
- C18 ec (endcapped), SPE cartridge 6 mℓ/500 mg (Chromabond Macherey-Nagel, Cat. No. 730 014 or Oasis HLB SPE cartridges, 6 cc 500 mg, Waters Corp Cat. No. 186000115)
- Glass bottles, 1 ℓ (for sampling purposes, can also be amber bottles)
- Glass Pasteur pipette with rubber bulb
- Glass wool filters (Macherey-Nagel, Cat. No. 000904 or equivalent)
- pH 0 -14 indicator strips
- Serological pipettes, sterile, 1 mℓ and 10 mℓ (Corning Costar Cat. No. 4012 and 4101, Scientific Group or equivalent)
- Sterile filters, 0.45 µm, 47 mm diameter (MicroSep, Osmonics Cat. No. E04WG047S1 or equivalent)

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.

Reagents

- Concentrated HCl
- Double distilled water
- Methanol – High performance liquid chromatography (HPLC) grade
- Ethanol – HPLC grade

METHOD

Note: Throughout the extraction method, dd H_2O refers to dd H_2O that went through the EDS filter to remove EDCs from the water.

Collection and pre-treatment of samples

1. Collect 2×1 ℓ of aqueous samples such as sewage, surface water, ground water or tap water in methanol rinsed sample bottles.
   Line the lid on the inside with tin foil to prevent the sample from coming into 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 and make a note.
3. Drop the pH of the sample to 3 by adding concentrated HCl drop wise with a glass Pasteur pipette. Check using pH strips.
4. 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.

Note: If the samples are sewage samples the extraction process should be started within 4-6 hours of collection or if absolutely necessary they may be stored overnight at 4°C.

5. 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.
6. Raw sewage and very turbid samples require pre-filtration. Assemble the glass filtration unit (see Figure 1). Place 1-2 glass wool filters between the loading reservoir and the sieve funnel.
7. 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 methanol and dd H_2O and go to step 8.
8. Assemble glass filtration unit (as in Figure 1) and load with 0.45 µm, sterile filters (47 mm diameter).

9. Connect the filtration unit to the vacuum inlet. Pass the sample through the filtration unit under vacuum, 250-300 mℓ at a time. You may need to replace the filter if it gets clogged, but this will depend on the sample. Once the sample (1 ℓ) has been filtered, you can proceed to the solid phase extraction.

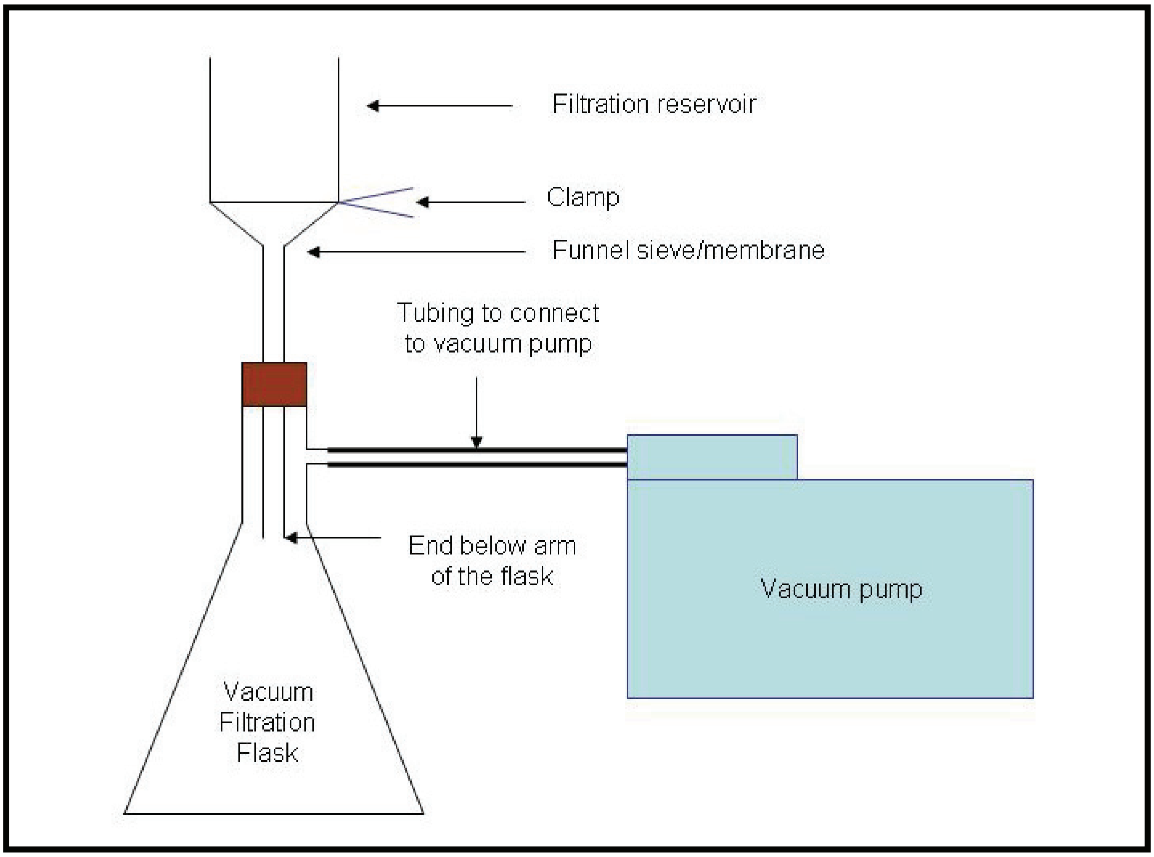


Figure 1: **Filtration setup for water extraction procedure**

Note: Place the membrane for clarifying (removing particles) from the water onto the funnel sieve/membrane and clamp the funnel closed. Place the filtration funnel (with the glass stem of the funnel through a rubber stopper) into the vacuum filtration flask. Make sure the bottom of the stem of the funnel is as deep as possible, preferably below the level of the side arm of the flask. Connect the arm of the vacuum filtration flask to the inlet of the vacuum pump. Switch on the vacuum pump and add the sample into the funnel. Make sure no liquid gets sucked into the vacuum pump. Once the sample has been filtered, remove the pipe from the arm of the filtration flask and connect to the vacuum inlet of the SPE vacuum manifold (see Figure 4).

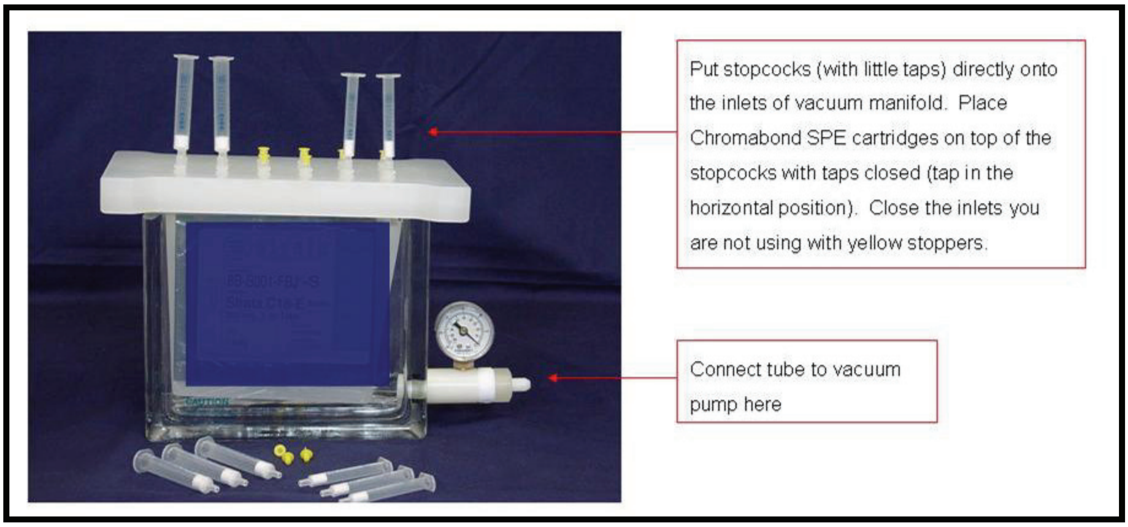
Solid phase extraction – pre-conditioning of the cartridge

Important note: The most commonly used sorbents are porous silica particles bonded with C_{18} 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 onto the SPE manifold and open the vacuum valves (see Figure 2a and b).
2. Add 5 mℓ dd H_2O to the reservoir of each cartridge and allow to pass through by gravity.

3. Just before the water reaches the top frit (see Figure 3) add 5 mℓ methanol (HPLC grade) and allow to pass through by gravity.
4. Just before the methanol reaches the top frit add 5 mℓ dd H_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.

Note: If you have a large sample adaptor you will need to fill the reservoir in step 4 with distilled water.



Put stopcocks (with little taps) directly onto the inlets of vacuum manifold. Place Chromabond SPE cartridges on top of the stopcocks with taps closed (tap in the horizontal position). Close the inlets you are not using with yellow stoppers.

Connect tube to vacuum pump here

Figure 2a: **An example of a solid phase extraction manifold**

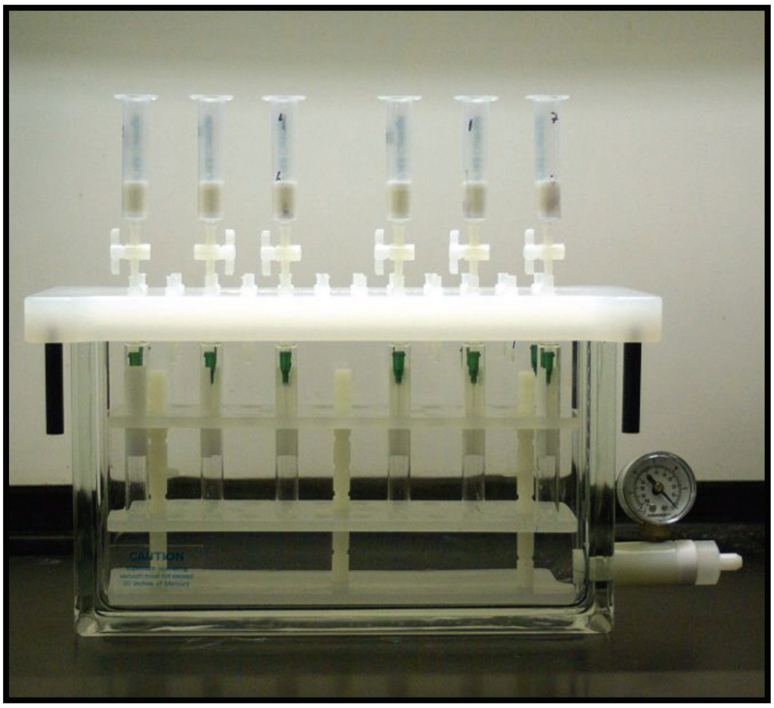


Figure 2b: **An example of a solid phase extraction manifold**

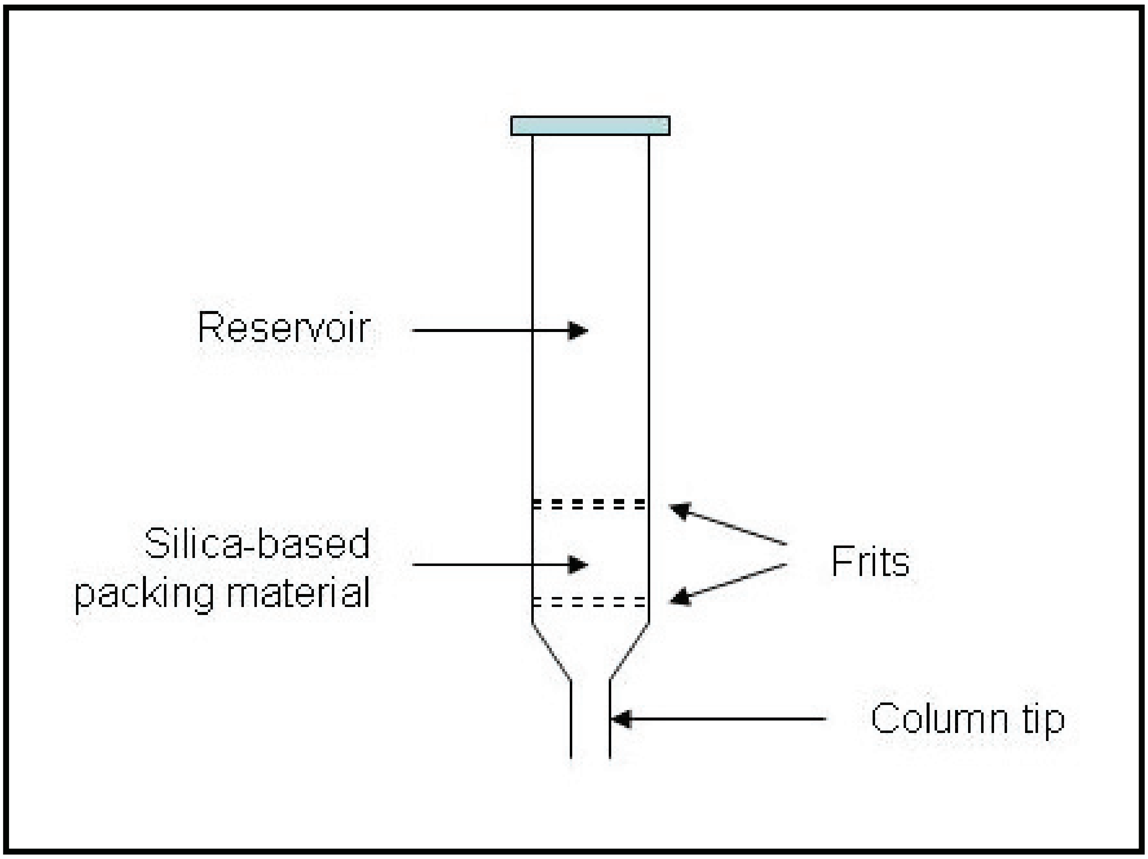


Figure 3: **A solid phase extraction column/cartridge**

Extraction procedure

1. Connect the SPE manifold to the vacuum trap (see Figure 4).
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 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. In order to do this, close the vacuum valve for that sample and tweak the connection until it is sealed tightly.

Caution: NEVER let the sorbent bed run dry while doing this. If necessary fill up the reservoir with dd H_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 don't 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.

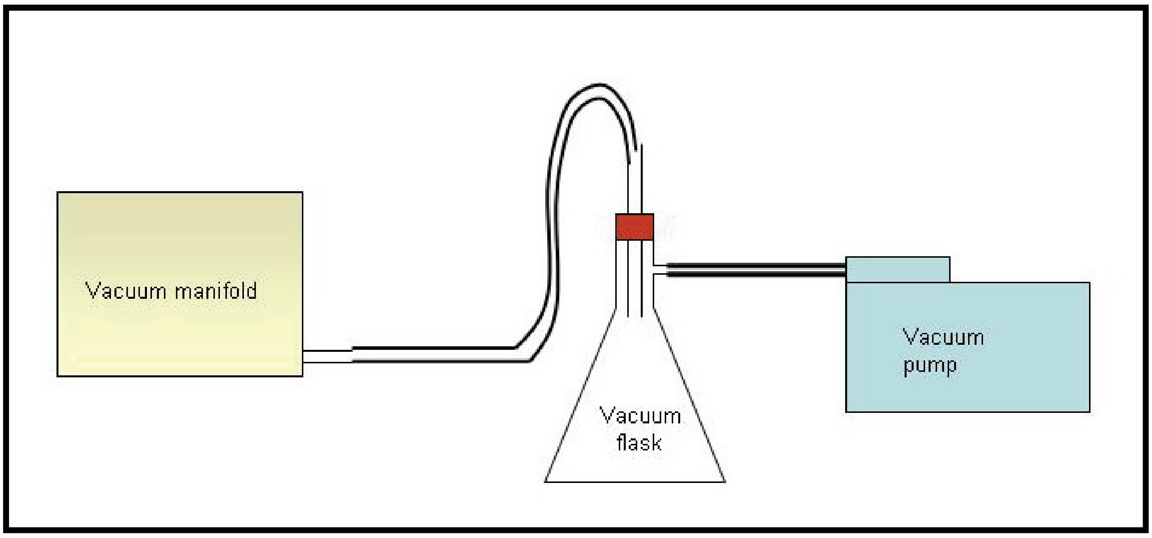


Figure 4: **An example of the filtration setup for sample extraction**

Note: The vacuum manifold should be connected according to Figure 4 so that the waste can collect in the vacuum flask.

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 tube.
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 dd H_2O.

Evaporation and reconstitution

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 sonifying.
4. Place the eluent into sterile glass amber vials (4 mℓ volume) and store at -20°C prior to analysis.

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ANECK-HAHN NH, DE JAGER C, BORNMAN MS and DU TOIT D (2005). Oestrogenic activity using a recombinant yeast screen assay (RCBA) in South African laboratory water sources. *Water SA* **31**: (2) 253-256.

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WATERS (2003). Oasis® Applications Notebook. Agrochemicals, Food, Pharmaceuticals, Forensics, Environmental. Waters Corporation, Milford, MA, USA.

USEFUL CONTACTS

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THE RECOMBINANT YEAST ESTROGEN SCREEN (YES)

Yeast-screen

Detection limit: 0.6-2.7 ng/ℓ

Incubate yeast overnight in rotating waterbath

↓

Day 0
Serially dilute test compounds and controls in 96-well plates and evaporate to dryness
Positive control: 17β-estradiol
Vehicle control: Ethanol/DMSO

↓

Add yeast and CPRG

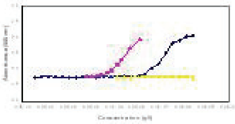
↓

Incubate plates

↓

Day 3,4,5
Read absorbance at 540nm (for colour) and 620nm (for turbidity)

↓



Calculate estradiol equivalents

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

CPRG	Chlorophenol red-β-D-galactopyranoside
dd H_2O	Double distilled water
E_2	17β-Estradiol
EC_{50}	Half maximal effective concentration
EEq	Estradiol equivalents
hER	Human estrogen receptor
ERE	Estrogen response element
RIE	Relative induction efficiency
RP	Relative potency
YES	Recombinant yeast estrogen screen

THE RECOMBINANT YEAST ESTROGEN SCREEN (YES)

Compiled by: NH Aneck-Hahn, MC van Zijl and C de Jager

PRINCIPLE OF THE ASSAY

In this assay a recombinant yeast strain using *Saccharomyces cerevisiea* was developed in the Genetics Department at Glaxo to identify compounds that can interact with the human estrogen receptor (hER). This yeast was transfected with the human ERα gene, and a plasmid containing an estrogen response element (ERE)-linked *lac-Z* gene encoding the enzyme *β*-galactosidase. Active ligands bind to the receptor and induce *β*-galactosidase expression in a dose dependent manner (Routledge and Sumpter, 1996) (Figure 1).

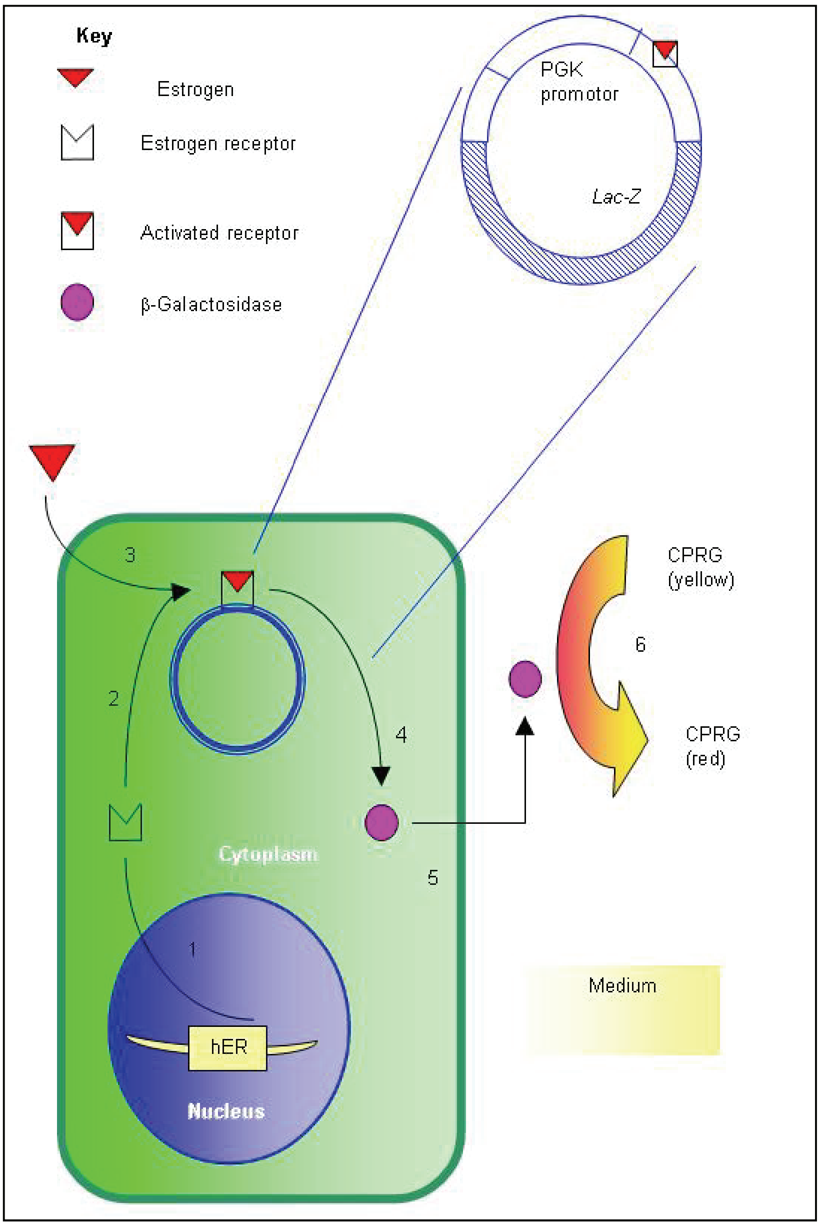


Figure 1: Schematic representation of the estrogen-inducible expression system in the yeast.

The human estrogen receptor gene is integrated into the main genome and is expressed (1) in a form capable of binding to EREs within a hybrid promoter on the expression plasmid (2). Activation of the receptor (3), by binding of ligand, causes expression of the reporter gene *Lac-Z* (4) which produces the enzyme β-galactosidase.

This enzyme is secreted into the medium (5) and metabolises the chromogenic substrate CPRG (normally yellow) into a red product (6), which can be measured by absorbance (Routledge and Sumpter, 1996).

REQUIREMENTS

Acquisition of the yeast cells

The yeast was obtained from Prof JP Sumpter's laboratory, in the Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex in the United Kingdom.
Contact details:
Name: Professor John Sumpter
Job title: Head of Institute for Environment, Brunel University, Uxbridge, UK
E-mail: John.Sumpter@brunel.ac.uk (current at time of print)
Institution: http://www.brunel.ac.uk
Tel: + 01895 274 000 Ext: 66303
Fax: + 01895 269 761

Permits

The Department of Agriculture is responsible for issuing a permit for importing genetically modified organisms. The necessary forms can be accessed from the following website (accessed on 31/03/2009): www.nda.agric.za/docs/geneticresources/geneticresources.htm
Alternatively contact the National office:
City: Pretoria
Tel: 012 319 6253
Fax: 012 319 6329
Physical address: Harvest House, Room 261, 30 Hamilton Street, Arcadia, Pretoria
Postal address: Directorate Genetic Resources, Private Bag X973, Pretoria, 0001
E-mail: MichelleV@nda.agric.za (current at time of print)

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)

Laboratory (test environment)

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

Software

- Graphpad Prism 4, or equivalent
- Microsoft Excel

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 (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 (dd H_2O). The system must be equipped with an EDS filter (Cat. No. EDSPAK001, Microsep) to remove endocrine disrupting compounds (EDCs) from the water
- Multiskan Titertek 96 well plate reader (Titertek Multiskan MCC/340 or equivalent)
- Pipette, 12 channel, volume 5-50 µℓ
- Pipette, 12 channel, volume 30-300 µℓ
- Pipette, 2-20 µℓ
- Pipette, 20-200 µℓ
- Pipette, 200-1000 µℓ
- Pipettor for serological pipettes
- Refrigerator/freezer combination
- Shaker (Heidolph Titramax 100 or equivalent)
- Type II Biohazard safety cabinet
- Water bath with rotator (Grant OLS 200 or equivalent)

Consumables/Materials

- Cotton wool (non-absorbant)
- Cryovials, sterile, 1.8 mℓ (Nunc, Cat. No. 377267, AEC-Amersham or equivalent)
- Microtiter plates with lid, sterile, clear 96 well flat bottomed (Labsystems, Cat. No. 95029780, AEC-Amersham or equivalent)
- Nitrile gloves
- Permanent marker pens
- Pipette filter tips, sterile (200 µℓ) (Thermo Finntip filter 200, Cat. No. 94052300, AEC-Amersham)
- Pipette filter tips, sterile (300 µℓ) (Thermo Finntip filter 300, Cat. No. 94052350, AEC-Amersham)
- Pipette tips, sterile (250 µℓ) (Thermo Finntip 250, Cat. No. 9400263, AEC-Amersham)
- Pipette tips, sterile (300 µℓ) (Thermo Finntip 250, Cat. No. 9401253, AEC-Amersham)
- Pipette tips, sterile (1000 µℓ) (Thermo Finntip 1000, Cat. No. 9401113, AEC-Amersham)
- Reagent reservoirs
- Serological pipettes (1 mℓ, 2 mℓ, 10 mℓ) (Corning Costar Cat. No 4012; 4021; 4101, Scientific Group)
- Tin foil
- Whatman PURADISC filters 0.2 µm pore size (Cat. No. 6780-2502, Microsep)

Reagents

• Potassium dihydrogen phosphate (KH_2PO_4)	(Cat. No. P-0662, Sigma)
• Ammonium sulphate $(NH_4)_2 SO_4$)	(Cat. No. R 0350/500g,NT Laboratory Supplies)
• Potassium hydroxide (KOH pellets)	(Cat. No. 504 44 00, Saarchem)
• Anhydrous magnesium sulfate ($MgSO_4$)	(Cat. No. 291184P, BDH)
• Ferric sulphate ($Fe_2 (SO_4)_3$)	(Cat. No. F-1135, Sigma)
• L-leucine	(Cat. No. 371213W, BDH)
• L-histidine	(Cat. No. 372214E, BDH)
• Adenine	(Cat. No. 1.00838, Merck)
• L-arginine-HCl	(Cat.No. 1.01543, Merck)
• L-methionine	(Cat. No. 371315E, BDH)
• L-tyrosine	(Cat. No. 371562R, BDH)
• L-isoleucine	(Cat. No. 371236G, BDH)
• L-lysine-HCl	(Cat. No. 371293P, BDH)

L-phenylalanine	(Cat. No. 1.07256, Merck)
L-glutamic acid	(Cat. No. 371024T, BDH)
L-valine	(Cat. No. 37160, BDH)
L-serine	(Cat. No. 371465R, BDH)
Thiamine	(Cat. No. 440055N, BDH)
Pyroxidine	(Cat. No. 449865Q, BDH)
Pantothenic acid	(Cat. No. 111 993, Merck)
Inositol	(Cat. No. 380443M, BDH)
Biotin	(Cat. No. 44011 4H, BDH)
D-glucose	(Cat. No. ART.8337, Merck)
L-aspartic acid	(Cat. No. 370225W, BDH)
L-threonine	(Cat. No. 371505Y, BDH)
Copper (II) sulphate, anhydrous ($CuSO_4$)	(Cat. No. 278504G, BDH)
Chlorophenol red-β-D-galactopyranoside (CPRG)	(Cat. No. 10884308001, Roche Diagnostics)
17β-Estradiol	(Cat. No. E8875, Sigma)
Ethanol	(Cat. No. 27,0741, Sigma-Aldrich)
Glycerol	(Cat. No.G2025, Sigma)
Agar	(Cat. No. A9915, Sigma)
Parafilm	(Cat. No. P7793, Sigma)

METHOD

Preparation of assay components

Note: When preparing the assay components, dd H_2O that went through the EDS filter to remove EDCs from the water should be used.

Minimal Medium (pH 7.1)

Minimal medium is prepared by adding the following media components together.

13.6 g	KH_2PO_4
1.98 g	$(NH_4)_2SO_4$
4.2 g	KOH pellets
0.2 g	$MgSO_4$
1 mℓ	$Fe_2(SO_4)_3$ solution (40 mg/50 mℓ water)[a]
50 mg	L-leucine
50 mg	L-histidine
50 mg	Adenine
20 mg	L-arginine-HCl
20 mg	L-methionine
30 mg	L-tyrosine
30 mg	L-isoleucine
30 mg	L-lysine-HCl
25 mg	L-phenylalanine
100 mg	L-glutamic acid
375 mg	L-serine
150 mg	L-valine
1 ℓ	dd H_2O

Aliquot 45 mℓ into 200 mℓ glass Schott bottles, sterilised by autoclaving at 121°C for 20 minutes and stored at room temperature.

a) Ferric sulphate $Fe_2(SO_4)_3$ solution (40 mg/50 mℓ water)

The ferric sulphate solution is made up by dissolving 40 mg $Fe_2(SO_4)_3$ in 50 mℓ dd H_2O.

Vitamin solution

The vitamin solution is made by dissolving the following in dd H_2O.

8 mg	Thiamine
8 mg	Pyroxidine
8 mg	Pantothenic acid
40 mg	Inositol
20 mℓ	Biotin solution (2 mg/100 mℓ water)[b]
180 mℓ	dd H_2O

Sterilise the solution by filtering the solution through 0.2 μm pore size Whatman PURADISC filters into sterile glass bottles (10 mℓ aliquot per bottle) and store at 4°C.

b) Biotin solution (2 mg/100 mℓ water)
The biotin solution is made by dissolving 2 mg biotin in 100 mℓ dd H_2O.

Glucose solution

A 20% weight/volume solution of D-glucose is made by dissolving 20 g glucose in 100 mℓ dd H_2O. Once dissolved the solution must be sterilised in 20 mℓ aliquots by autoclaving at 121°C for 20 minutes. This solution can be stored at 4°C for a maximum of 3 months.

L-aspartic acid solution (4 mg/mℓ)

A stock solution is prepared by dissolving 400 mg of L-aspartic acid in 100 mℓ dd H_2O. Once dissolved the solution must be sterilised in 20 mℓ aliquots at 121°C for 20 minutes and stored at 4°C for 3 months.

L-threonine solution (24 mg/mℓ)

A stock solution is prepared by dissolving 1200 mg of L-threonine in 50 mℓ dd H_2O. Once dissolved the solution must be sterilised in 5 mℓ aliquots by autoclaving at 121°C for 20 minutes. The solution can be stored for a maximum of 3 months at 4°C.

Copper (II) sulphate solution (20 mM)

A 20 mM copper (II) sulphate solution is prepared by dissolving 31.92 mg copper (II) sulphate in 100 mℓ dd H_2O and filter sterilised through a 0.2 μm pore size Whatman, PURADISC filters into sterile glass bottles. The solution can be stored at 4°C for a maximum of 3 months.

Chlorophenol red-β-D-galactopyranoside (CPRG)

Prepare a 10 mg/mℓ stock solution by dissolving 100 mg CPRG in 10 mℓ dd H_2O and filter sterilise through 0.2 μm pore size Whatman, PURADISC filters into sterile amber or foil covered glass bottles and stored at 4°C.

Growth medium for the assay

Prepare growth medium by adding together the following:

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

17β-Estradiol (E_2) stock solution

A 54.58 μg/ℓ stock solution of 17β-Estradiol is prepared in ethanol in a sterile glass bottle and stored at -20°C.

PREPARATION AND STORAGE OF YEAST STOCK CULTURES

Long-term stock cultures

Long-term stock cultures are prepared on minimal medium agar slope. 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. 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.

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

Day 1

A flask of growth medium must be prepared as described above. Add 125 µℓ of the long-term yeast stock to the flask. Incubate at 28°C for approximately 24 hours in a rotating water bath at 150-155 upm.

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 in a rotating water bath (150-155 upm).

Day 3

Transfer the 24-hour culture to two sterile 50 mℓ Pyrex glass centrifuge tubes. Centrifuge at 4°C for 10 minutes at 2000 × g^c. 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 in 0.5 mℓ aliquots. These stock cultures can be stored at -20°C for a maximum of 4 months.

c) Working out the G-force of the centrifuge

G-force (or g) = $(1.118 \times 10^{-5})\ r\ s^2 = 0.0000118 \times r \times s^2$

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)

Preparation for sample analysis

For sample collection and extraction procedures refer to Volume 1 (Monitoring and Assessment) *and Volume 2* (Sampling Guide) respectively *or alternatively* to the section on the extraction procedure (EXTRACTION PROCEDURE FOR WATER SAMPLES FOR THE ASSESSMENT OF ESTROGENIC ACTIVITY, pg 9).

Note: The assay should be carried out in a type 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.

Assay medium

Prepare growth medium as described above. Inoculate with 125 μℓ of the 10× concentrated yeast stock (refer to short-term stock cultures above) and incubate at 28°C in a rotating water bath at 150-155 upm until turbid (24 hours). Prepare a new flask of growth medium and add 0.5 mℓ CPRG, this medium must be inoculated with 0.5 mℓ of the 24-hour yeast culture.

Preparation of the sample dilutions, control and blank

Serial dilutions must be made of the sample extracts and controls, in 96 well microtiter plates in the following way.

1. Place 100 μℓ of the solvent (ethanol or DMSO), in wells 2-12 on the plate.
2. Place 200 μℓ of the sample extract/control/blank 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.
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.
7. Each plate must contain at least one row of assay medium and solvent ethanol (blank) and a standard curve for 17β-Estradiol 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).
8. Seal the plates carefully with parafilm and placed in a naturally ventilated incubator at 32°C for 3 to 5 days.
9. After 3 days incubation, the colour development of the medium must be read on the plate reader (Titertek Multiskan MCC/340) at an absorbance (abs) of 540 nm for colour change and 620 nm for turbidity 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, in order to obtain data with the best contrast between the positive control and the blank.
10. After incubation the control wells appear yellow to light orange in colour, due to background expression of β-galactosidase and turbid due to the growth of the yeast.
11. Positive wells are indicated by a deep red colour accompanied by yeast growth (turbid) usually in a dose dependent manner (Figure 2).
12. Clear wells, containing no growth indicates lysis of the cells and the colour can vary from pink to yellow (no turbidity) (Figure 2).
13. All experiments must be performed in triplicate.

Caution: Some chemicals may leach across the plate and contaminate adjacent wells (Figure 3). By leaving empty rows between samples, this problem can be eliminated.

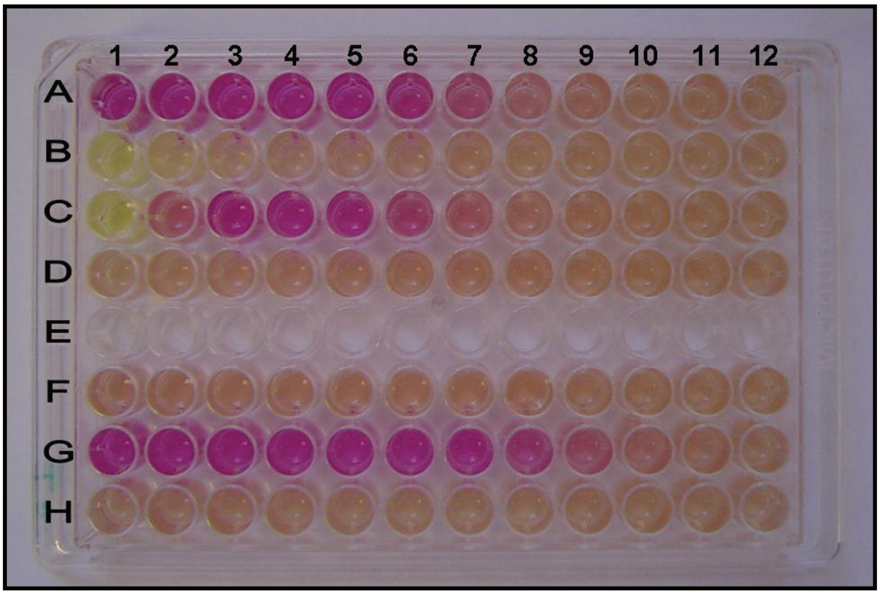


Figure 2: An example of a YES assay plate (3 days incubation)
Row A contains a positive sample, row B a sample with cytotoxicity (clear yellow well in column 1), row C a sample with cytotoxicity and estrogenic activity and row D a sample below the detection limit of the assay. Row F contains the assay blank and row G and H the E_2 positive control standard and extended curve.

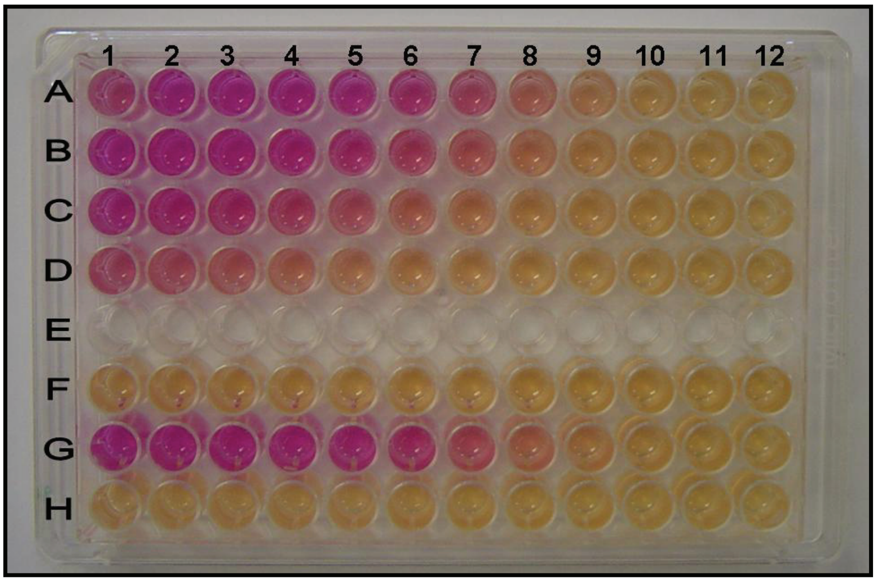


Figure 3: An example of leaching in the YES assay plate
Row A contains serial dilutions of a test chemical and row B to D contains only the assay medium (blank). This figure illustrates that the chemical leached across and down the plate into the adjacent wells.

Calculation and reporting of results for environmental samples

1. Transfer raw data to Excel.
2. The following equation must be applied to correct for turbidity in the well:

Corrected value = test abs (540 nm) – [test abs (620 nm) – median blank abs (620 nm)]
Dhooge et al. (2006)

3. Determine the detection limit of the assay (average of blank plus 3 times the standard deviation) (Dhooge et al., 2006). Estradiol equivalents (EEq) can be calculated for samples with 3 or more points above the detection limit.

Note: Samples are considered to be cytotoxic at concentrations where the 620 nm turbidity reading of the sample is less than the average of the blank (at 620 nm) minus 3 times the standard deviation.

Leusch (2008)

4. Transform the corrected absorbance values to log and express values as a percentage of the maximum E_2 response.
5. Transfer data to Graphpad Prism (version 4), with E_2 concentrations as X-values and log 10% 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.

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.

Calculating and reporting the half maximal effective concentration (EC_{50}), relative potency (RP) and relative induction efficiency (RIE) values for estrogenic chemicals

1. Transfer raw data to Excel.
2. Correct for turbidity in the well:

Corrected value = test abs (540 nm) – [test abs (620 nm) – median blank abs (620 nm)]

Dhooge et al. (2006)

3. Determine the detection limit of the assay (average of blank plus 3 times the standard deviation). EC_{50} values can be calculated for samples with 3 or more points above the detection limit.
4. Transform the corrected absorbance values to log and express values as a percentage of the maximum E_2 response.
5. Transfer data to Graphpad Prism (version 4), with test chemical or E_2 concentrations as X-values and log10% max values as Y-values.
6. Log transform X-values.
7. Fit the test chemical or E_2 curve (sigmoidal function, variable slope) to obtain the EC_{50} value.
8. Calculate the relative induction efficiency (RIE) using the following formula:

RIE = Max absorbance chemical/max absorbance E_2 × 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.

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

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

Fang et al. (2000)

RP gives an indication of the potency of a test chemical compared to the positive control (positive control = 100%), for 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.

LIMITATIONS OF THE ASSAY

- 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.
- Poor predictor of whole organism response.
- 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.

RECOMMENDATIONS

The YES assay is the most widely used yeast-based reporter assay. There is a large amount of data available for individual chemicals. This assay is robust and can be successfully applied as a screen for environmental water and sediment samples. It is suitable for a high volume of samples and is also relatively quick, but does allow for the measurement of slow reacting compounds (Beresford et al., 2000; Gaido et al., 1997; Leusch et al., 2005). It is recommended that it is used in conjunction with an additional estrogenic bioassay, such as the T47D-KBluc reporter gene assay or the E-screen.

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THE T47D-KBluc REPORTER GENE ASSAY

T47D-KBluc assay

Detection limit: 0.4 -1.3 ng/ ℓ

Day 1
Withdraw cells

↓

Day 7
Seed cells in 96 well luminometer plates and allow cells to attach overnight

↓

Day 8
Expose cells to test compounds
Agonist positive control: 17ß-estradiol
Antagonist control: ICI 182 780
Vehicle control: Ethanol/DMSO

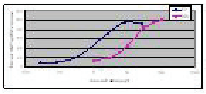
↓

Incubate plates overnight

↓

Day 9
Determine luciferase activity using a luminometer

↓



Calculate estradiol equivalents

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
E_2	17β-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

The T47D-Kbluc REPORTER GENE ASSAY

Compiled by: MC van Zijl, NH Aneck-Hahn and C de Jager

PRINCIPLE OF THE ASSAY

The US EPA developed an estrogen-dependent stable cell line. The T47D human breast cancer cells, which contain both endogenous estrogen receptor (ER)-α and -β, were transfected with an estrogen-responsive element (ERE) 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. The cell line has the potential to be used both for screening chemicals and as an aid in defining mechanism of action of chemicals with estrogenic and anti-estrogenic activity. This is valuable for a first-pass type *in vitro* assay, as a ligand for either receptor could drive the luciferase reporter gene, thereby eliminating the need for a separate assay for ERα and ERβ (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. Agonists stimulate luciferase expression and are compared to the vehicle control (media plus ethanol) 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.

REQUIREMENTS

Acquisition of the cell line

The cells were obtained from the American Type Culture Collection (ATCC, catalog no CRL-2865), Manassas, USA.

Information and contact details:

Website:	www.atcc.org
E-mail:	tech@atcc.org
Tel:	+703 365 2700
Fax:	+703 365 2750

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.

Information and contact details:
City: Pretoria
Tel: 012 312 0816
Fax: 012 323 0093
Physical address: Hallmark Building, 226 Vermeulen Str, Pretoria
Postal address: Private Bag X828, Pretoria, 0001

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)

Laboratory (test environment)

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

Software

- Graphpad Prism 4, or equivalent
- Microsoft Excel

Apparatus

- Autoclave
- Balance (OHAUS Voyager VP114CN (110 g × 0.1 mg) or equivalent)
- Centrifuge with swing-out rotor (buckets must be able to contain 50 mℓ conical tubes)
- Cryofreezing container (Nalgene Cat. No. 1500-0001, AEC-Amersham or equivalent)
- Erlenmeyer Flask, 5 ℓ
- Freezer (-20°C)
- Freezer (-80°C)
- Fumehood
- Glass tubes with lids, 5 mℓ
- Incubator, humidified 5% CO_2
- Inverted microscope
- Measuring cylinder, 1 ℓ
- Microplate luminometer with two dispensers (LUMIstar OPTIMA or equivalent)
- Millipore Milli-Q synthesis ultrapure water system or equivalent with EDS filter to remove endocrine disrupting compounds (EDCs) from water (Cat. No. EDSPAK001, Microsep)
- Oven
- pH meter
- Pipette, 12 channel, 10-100 µℓ volume range
- Pipette, 1-10 µℓ variable volume
- Pipette, 200-1000 µℓ variable volume
- Pipette, 20-200 µℓ variable volume
- Pipettor for serological pipettes
- Plate warmer/shaker (THERMOstar or equivalent)
- Protective gloves for working with acid
- Refrigerator (4°C)
- Safety goggles
- Schott bottles, glass, 25 mℓ; 50 mℓ; 100 mℓ; 500 mℓ and 1 ℓ (Cat. No. 21801-145; -175; -245; -445 and -545, Merck)

- Spatulas
- Type II biohazard safety cabinet
- Vortex mixer
- Water bath with rotator (Grant OLS 200 or equivalent)

Consumables/Materials

- Bottle top filters, 0.22 µm, 500 mℓ (Corning Cat. No. 430513, Scientific Group or equivalent)
- Conical tubes, sterile, 50 mℓ (Corning Cat. No. 430292, Scientific Group or equivalent)
- Eppendorf tubes, 1.5 mℓ (Cat. No. HK0030123425, Merck or equivalent)
- Hemocytometer
- Luminometer plates, 96 well, white with clear bottom (Corning Costar Cat. No. 3610, Scientific Group or equivalent)
- Nitrile gloves
- Pipette tips, sterile, 10 µℓ (Thermo Finntip 10, Cat. No. 9400300, AEC-Amersham or equivalent)
- Pipette tips, sterile, 1000 µℓ (Thermo Finntip 1000, Cat. No. 9401113, AEC-Amersham or equivalent)
- Pipette tips, sterile, 200 µℓ (Thermo Finntip filter 200, Cat. No. 94052300, AEC-Amersham or equivalent)
- Pipette tips, sterile, 250 µℓ (Thermo Finntip 250, Cat. No. 9400263, AEC-Amersham or equivalent)
- Permanent marker pens
- Reagent reservoir (100 mℓ, autoclavable)
- Sterile cryovials (Nunc Cat. No. 377267/100, AEC-Amersham or equivalent)
- Sterile serological pipettes (1 mℓ; 2 mℓ; 10 mℓ; 25 mℓ) (Corning Costar Cat. No 4012; 4021; 4101; 4251, Scientific Group or equivalent)
- Tin foil

- Tissue culture flasks, sterile (25 cm^2; 75 cm^2) (Corning, Cat. No. 430372; 430641, Scientific Group or equivalent)

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.

Reagents

Antibiotic/antimycotic solution	(Gibco Cat. No. 15240-062, Scientific Group)
Adenosine triphosphate (ATP)	(Cat. No. A7699, Sigma-Aldrich)
Beetle luciferin, potassium salt	(Promega Cat. No. E1603, Whitehead Scientific)
Bovine serum albumin (BSA) (Fraction V)	(Cat. No. A7906, Sigma-Aldrich)
Cell culture freezing media	(Gibco Cat. No. 11101-011, Scientific Group)
D (+)-glucose	(Cat. No. 8337, Merck)
17β-Estradiol (E$_2$)	(Cat. No. E8875, Sigma-Aldrich)
Ethanol, high performance liquid chromatography (HPLC) grade	(Cat. No. 34870, Sigma-Aldrich)
Fetal bovine serum (FBS)	(HyClone Cat. No. SH30071.03, Separations)
FBS, charcoal/dextran treated	(HyClone Cat. No. SH30068.03, Separations)
Glycylglycine	(Cat. No. G7278, Sigma-Aldrich)
Hanks' balanced salt solution (HBSS), 10×	(Gibco Cat. No. 14185-045, Scientific Group)
1 M HEPES buffer	(Gibco Cat. No. 15630-056, Scientific Group)
Hydrochloric acid (HCl), 5N	(Preferred supplier for pH purposes)
ICI 182 780	(Cat. No. 1047, Tocris)
Isopropanol (C_3H_8O)	(Cat. No. 34863, Riedel-deHaën)

Magnesium chloride ($MgCl_2$), 1 M solution	(Cat. No. M1028, Sigma-Aldrich)
Phosphate buffered saline (PBS), 10×	(Gibco Cat. No. 14080-048, Scientific Group)
pH buffers (pH 4, pH 7, pH 10)	
Potassium dichromate ($K_2Cr_2O_7$)	(Cat. No. 20,924-4, Sigma-Aldrich)
Reporter lysis buffer	(Promega Cat. No. E3971, Whitehead Scientific)
RPMI 1640 medium (with glutamine, without phenol red)	(Cat. No. R8755, Sigma-Aldrich)
Sodium bicarbonate ($NaHCO_3$)	(Cat. No. S5761, Sigma-Aldrich)
Sodium hydroxide (NaOH), 5 N	(Preferred supplier for pH purposes)
100 mM Sodium pyruvate	(Gibco Cat. No. 11360-039, Scientific Group)
Sulphuric acid (technical grade)	(Cat. No. 30741, Riedel-deHaën)
Trypsin-EDTA (10×)	(Gibco Cat. No. 15400-054, Scientific Group)

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.

METHOD

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.

Preparation of glassware

All glassware must be prepared by washing in chromic acid for 30 minutes, rinsing in tap water, double distilled EDC free water and HPLC grade ethanol 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.

Preparation of assay components

Chromic acid

Prepare chromic acid in a 5 ℓ Erlenmeyer flask by adding

80 g	$K_2Cr_2O_7$
150 mℓ	Double distilled water

Carefully add 2 ℓ concentrated sulphuric acid and store in a dark bottle at room temperature.

Caution:

- The solution gets very hot when adding the sulphuric acid – work carefully.
- Always work in a fume hood and always wear appropriate gloves and safety goggles when working with the acid.

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	Double distilled 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.

Maintenance Medium

Maintenance medium consists of RPMI medium supplemented with 10% FBS and 100 U/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.

Assay 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.

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

HBSS

Autoclave 1 ℓ of EDC-free double distilled water in a glass bottle to sterilize. Working in a biohazard safety cabinet, add 50 mℓ 10× HBSS to 950 mℓ autoclaved water in a sterile 1 ℓ glass bottle, using a sterile measuring cylinder. Store HBSS at 4°C.

Trypsin

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

PBS

Add 100 mℓ 10x PBS to 900 mℓ distilled water. Store at 4°C.

Lysis buffer

Add 1 mℓ 5x lysis buffer to 4 mℓ distilled water, just prior to adding lysis buffer to cells.

Glycylglycine

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

ATP solution

Make a 0.1 M stock solution by adding 2.755 g ATP (MW 551.1) to 50 mℓ distilled 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.

BSA

Make a 10 mℓ stock by adding 500 mg BSA to 10 mℓ distilled water. Aliquot into 1 mℓ volumes in a freezable container. Store at -20°C

Reaction buffer

Reaction buffer is prepared by adding the following together:
90 mℓ distilled 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). Store at 4°C

Note:

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

Luciferin

Make a 1 mM stock solution by adding 159.21 mg D-luciferin (potassium salt MW 318.41) to 500 mℓ distilled 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.

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, prepared glass bottle. Use an amber bottle or wrap bottle in foil and store at -20°C.

ICI stock solution

Make a stock solution of 10 mM ICI by adding 60.677 mg ICI (MW 606.77) to 10 mℓ HPLC grade ethanol in a new, prepared glass bottle. Use an amber bottle or wrap bottle in foil and store at -20°C.

GENERAL CELL CULTURE TECHNIQUES

Aseptic conditions

(refer to McAteer and Davis (2006) for more comprehensive guidelines)

- 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.

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.

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 by gentle agitation in a 37°C water bath, 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.

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 observe cells under an inverted microscope until cell layer is dispersed (approximately 2 minutes).
4. Detach cells by gently tapping the flask against the palm of your hand.
5. Add 10 mℓ medium to a 75 cm^2 flask or 5 mℓ to a 25 cm^2 flask.
6. 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.
7. Return flasks to incubator until cells reach 80-90% confluency.

Note:

- 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.

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 observe cells under an inverted microscope until cell layer is dispersed (approximately 2 minutes).
5. Detach cells by gently tapping the flask against the palm of your hand and add 10 mℓ maintenance medium to the flask.
6. Transfer medium containing cells to a 50 mℓ conical tube and centrifuge at 15°C, 172 × g, for 10 minutes.
7. Remove media using a sterile pipette, taking care not to disturb the pellet.
8. Add 6 mℓ cell freezing media and pipet gently two to three times to disperse cells.
9. 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.

a) Freezing cells using the Nalgene cryofreezing container

Using the Nalgene cryofreezing 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. Remove the vial holder and foam insert from the cryofreezing container.
2. Add 250 mℓ isopropanol to the bottom part of the container.
3. Replace the foam insert and vial holder.
4. Insert vials containing stock cultures and close the lid.
5. Place cryofreezing container in a -80°C freezer for at least 4 hours.
6. Transfer vials to storage boxes or liquid nitrogen.

Note:

- The cryofreezing container can be stored at room temperature when not in use.
- Replace isopropanol after every fifth use.
- Thaw one vial after 48 h to determine whether the cells are viable, i.e. survived the freezing procedure.

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 assay 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 assay media.

Day 7 (e.g. Monday)

1. Prepare medium containing 5% dextran/charcoal treated FBS by mixing equal volumes of RPMI medium and assay medium containing 10% dextran/charcoal treated FBS.
2. Trypsinize cells and add 10 mℓ medium containing 5% dextran/charcoal treated FBS.
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 × 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:

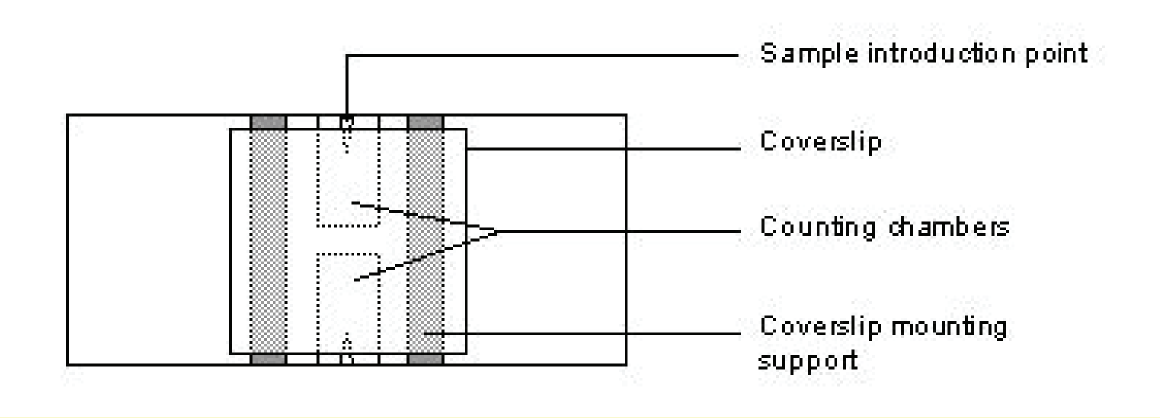


Figure 1: Diagram of a hemocytometer

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.

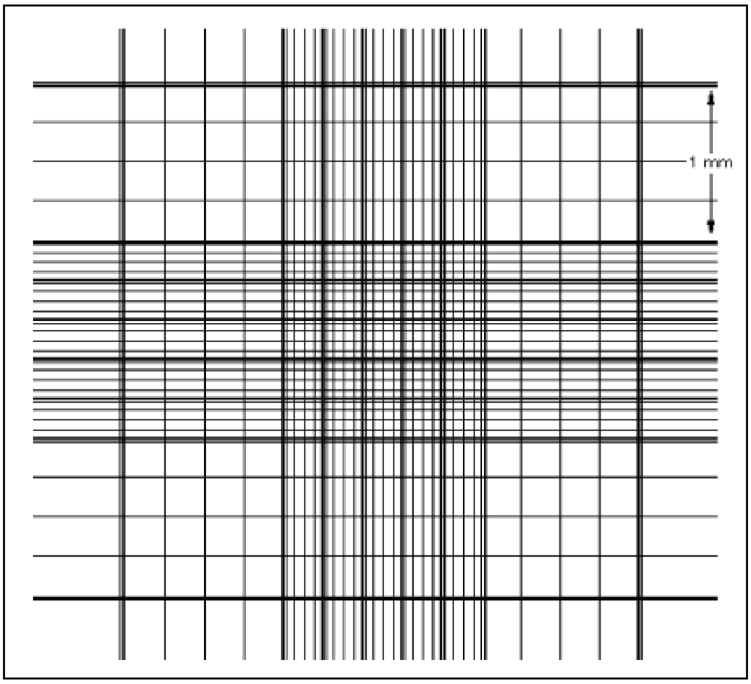


Figure 2a: Diagram of a counting chamber grid

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

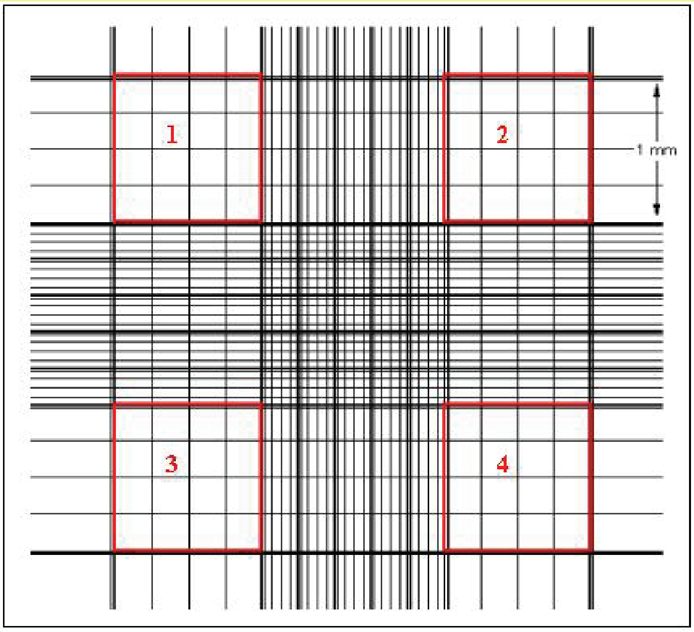


Figure 2b: An example of the four corner squares on a counting chamber grid

Count the cells in the four corner squares (1, 2, 3, 4) 1 mm^2 grid areas (one 1 mm^2 grid area consists of 4 × 4 squares surrounded by three grid lines) indicated in red.

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 × 10 000 × dilution factor
   (The depth of 1 mm square is 0.1 mm 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) <u>Seeding cells at 5 × 10^4 cells per well</u>:

- Prepare 11 mℓ of the final cell dilution per plate.
- Cells must be seeded at 5 × 10^4 cells per well or 5 × 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 amount of cells per mℓ counted on the hemocytometer. Add assay medium to obtain a final volume of 11 mℓ and transfer to a 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 1000× 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 10 mM ICI stock to 1 mℓ ethanol (100 µM)
   b) E_2 control and standard curve
   Add 1 µℓ of the 10 mM E_2 stock to 1 mℓ ethanol (10 µM)
   Add 10 µℓ of the 10 µM E_2 stock to 1 mℓ 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 300 µℓ of the 10 nM E_2 stock to 700 µℓ ethanol (3 nM)
   Add 100 µℓ of the 10 nM E_2 stock to 900 µℓ ethanol (1 nM)
   Add 300 µℓ of the 1 nM E_2 stock to 700 µℓ ethanol (0.3 nM)
   Add 100 µℓ of the 1 nM E_2 stock to 900 µℓ ethanol (0.1 nM)
   c) *Samples*
   Make dilutions of the sample in ethanol at 1000× the desired concentration (For sample collection and extraction procedures refer to Manual 1 and 2 respectively or alternatively to our section on extraction).
2. Dilute 1000× concentrations in medium containing 5% dextran/charcoal treated FBS to double the desired final concentration to allow for the dilution factor when vehicle, E_2 or ICI are co-incubated with the samples and controls. Vortex to mix.
   a) Vehicle control
   Add 20 µℓ HPLC grade ethanol to 10 mℓ medium (VC)
   b) ICI control
   Add 20 µℓ of the 100 µM ICI stock to 10 mℓ medium (0.2 µM)
   c) E_2 control
   Add 20 µℓ of the 100 nM E_2 stock 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)
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)

e) *Samples*

Add 2 µℓ of sample concentrations in ethanol to 1 mℓ medium.

3. Add 50 µℓ of the dosing solutions to the appropriate wells. Add 50 µℓ 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 3a and the resulting final concentrations in Figure 3b.

   Final concentrations:

   a) *Vehicle control*
   0.2% (v/v)

   b) *ICI control*
   0.1 µM

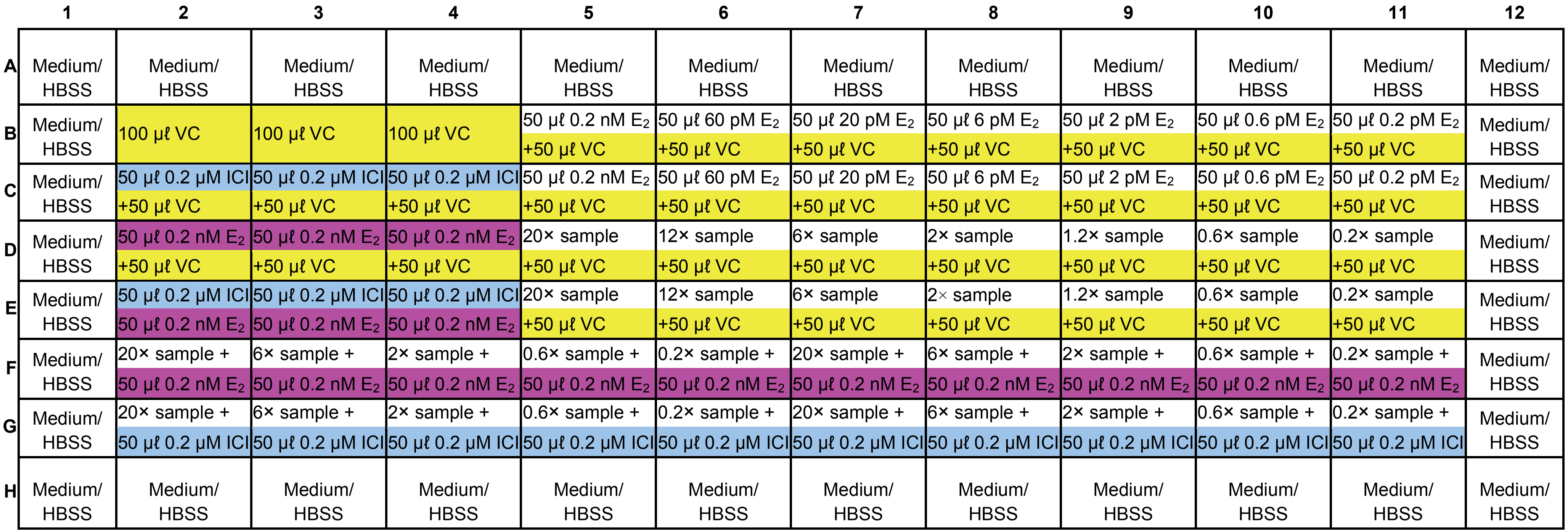
   c) E_2 *control*
   0.1 nM

   d) E_2 *standard curve*
   0.1 nM; 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.

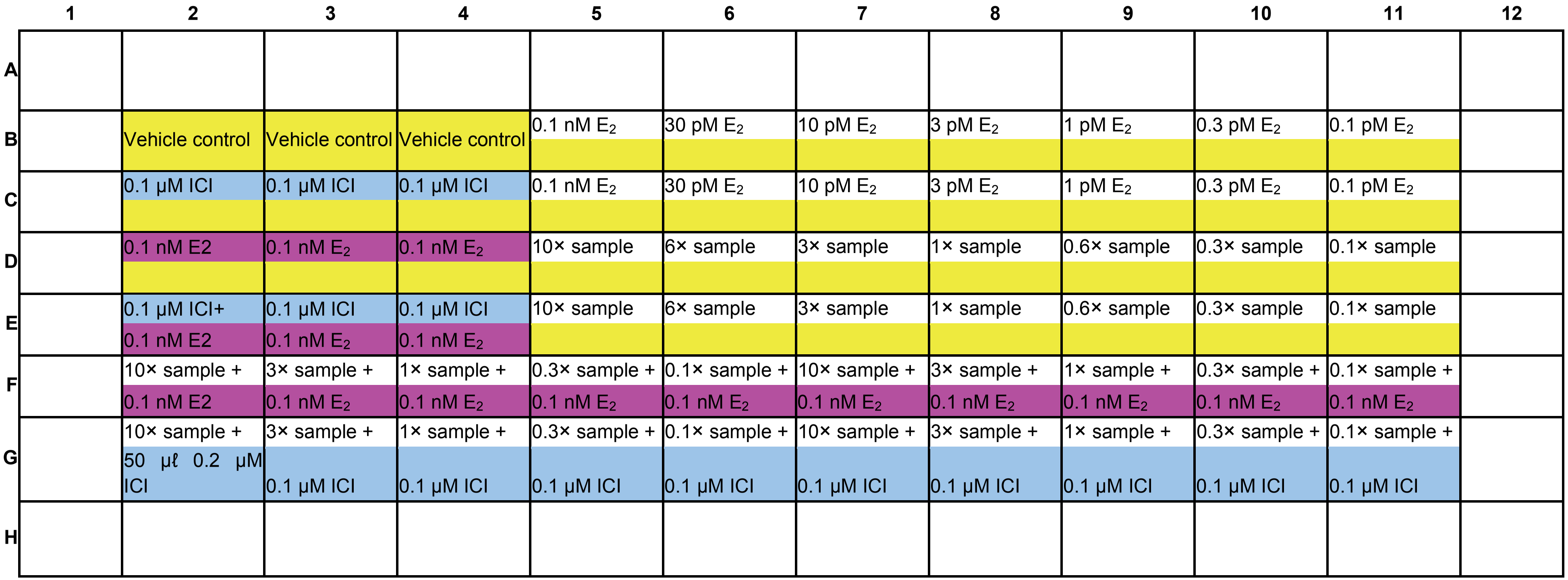
Note:

- Ethanol (vehicle) concentrations in the final dilutions of controls or test substances may not exceed 0.2%.
- During the extraction procedure, samples are concentrated 1000×, but are diluted 100× 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 an eppendorf tube, allow to evaporate and resuspend in medium containing 0.2% vehicle control. E.g. to obtain a final concentration equal to 10× 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.
- Where possible, dosing plates should be kept in a separate incubator and not with tissue culture flasks containing cells.
- Dosing plates should not be stacked on one another to avoid cross-contamination.



	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	100 μℓ VC	100 μℓ VC	50 μℓ 0.2 nM E_2 +50 μℓ VC	50 μℓ 60 pM E_2 +50 μℓ VC	50 μℓ 20 pM E_2 +50 μℓ VC	50 μℓ 6 pM E_2 +50 μℓ VC	50 μℓ 2 pM E_2 +50 μℓ VC	50 μℓ 0.6 pM E_2 +50 μℓ VC	50 μℓ 0.2 pM E_2 +50 μℓ VC	Medium/ HBSS
C	Medium/ HBSS	50 μℓ 0.2 μM ICI +50 μℓ VC	50 μℓ 0.2 μM ICI +50 μℓ VC	50 μℓ 0.2 μM ICI +50 μℓ VC	50 μℓ 0.2 nM E_2 +50 μℓ VC	50 μℓ 60 pM E_2 +50 μℓ VC	50 μℓ 20 pM E_2 +50 μℓ VC	50 μℓ 6 pM E_2 +50 μℓ VC	50 μℓ 2 pM E_2 +50 μℓ VC	50 μℓ 0.6 pM E_2 +50 μℓ VC	50 μℓ 0.2 pM E_2 +50 μℓ VC	Medium/ HBSS
D	Medium/ HBSS	50 μℓ 0.2 nM E_2 +50 μℓ VC	50 μℓ 0.2 nM E_2 +50 μℓ VC	50 μℓ 0.2 nM E_2 +50 μℓ VC	20× sample +50 μℓ VC	12× sample +50 μℓ VC	6× sample +50 μℓ VC	2× sample +50 μℓ VC	1.2× sample +50 μℓ VC	0.6× sample +50 μℓ VC	0.2× sample +50 μℓ VC	Medium/ HBSS
E	Medium/ HBSS	50 μℓ 0.2 μM ICI 50 μℓ 0.2 nM E_2	50 μℓ 0.2 μM ICI 50 μℓ 0.2 nM E_2	50 μℓ 0.2 μM ICI 50 μℓ 0.2 nM E_2	20× sample +50 μℓ VC	12× sample +50 μℓ VC	6× sample +50 μℓ VC	2× sample +50 μℓ VC	1.2× sample +50 μℓ VC	0.6× sample +50 μℓ VC	0.2× sample +50 μℓ VC	Medium/ HBSS
F	Medium/ HBSS	20× sample + 50 μℓ 0.2 nM E_2	6× sample + 50 μℓ 0.2 nM E_2	2× sample + 50 μℓ 0.2 nM E_2	0.6× sample + 50 μℓ 0.2 nM E_2	0.2× sample + 50 μℓ 0.2 nM E_2	20× sample + 50 μℓ 0.2 nM E_2	6× sample + 50 μℓ 0.2 nM E_2	2× sample + 50 μℓ 0.2 nM E_2	0.6× sample + 50 μℓ 0.2 nM E_2	0.2× sample + 50 μℓ 0.2 nM E_2	Medium/ HBSS
G	Medium/ HBSS	20× sample + 50 μℓ 0.2 μM ICI	6× sample + 50 μℓ 0.2 μM ICI	2× sample + 50 μℓ 0.2 μM ICI	0.6× sample + 50 μℓ 0.2 μM ICI	0.2× sample + 50 μℓ 0.2 μM ICI	20× sample + 50 μℓ 0.2 μM ICI	6× sample + 50 μℓ 0.2 μM ICI	2× sample + 50 μℓ 0.2 μM ICI	0.6× sample + 50 μℓ 0.2 μM ICI	0.2× sample + 50 μℓ 0.2 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 3a: 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	Vehicle control	Vehicle control	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	
C		0.1 μM ICI	0.1 μM ICI	0.1 μM ICI	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	
D		0.1 nM E2	0.1 nM E_2	0.1 nM E_2	10× sample	6× sample	3× sample	1× sample	0.6× sample	0.3× sample	0.1× sample	
E		0.1 μM ICI+ 0.1 nM E2	0.1 μM ICI 0.1 nM E_2	0.1 μM ICI 0.1 nM E_2	10× sample	6× sample	3× sample	1× sample	0.6× sample	0.3× sample	0.1× sample	
F		10× sample + 0.1 nM E2	3× sample + 0.1 nM E_2	1× sample + 0.1 nM E_2	0.3× sample + 0.1 nM E_2	0.1× sample + 0.1 nM E_2	10× sample + 0.1 nM E_2	3× sample + 0.1 nM E_2	1× sample + 0.1 nM E_2	0.3× sample + 0.1 nM E_2	0.1× sample + 0.1 nM E_2	
G		10× sample + 50 μℓ 0.2 μM ICI	3× sample + 0.1 μM ICI	1× sample + 0.1 μM ICI	0.3× sample + 0.1 μM ICI	0.1× sample + 0.1 μM ICI	10× sample + 0.1 μM ICI	3× sample + 0.1 μM ICI	1× sample + 0.1 μM ICI	0.3× sample + 0.1 μM ICI	0.1× sample + 0.1 μM ICI	
H												

Figure 3b: An example of the final concentrations in the wells

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 PBS, at room temperature, using a 25 mℓ serological pipette and discard PBS.
4. Add 25 µℓ lysis buffer to each well using a multichannel pipette in order to lyse the cells.
5. 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.
6. Thaw the lysed cells at 37°C while gently shaking on a plate warmer/shaker.
7. 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).

Calculation and reporting of results for environmental samples

1. Transfer raw data to Excel.
2. Convert 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 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. Transform FI values to log and express values as a percentage of 0.1 nM E_2.
4. Transfer data to Graphpad Prism (version 4), with E_2 concentrations as X-values and log 10% max values as Y-values.
5. Log transform X-values.
6. Fit the E_2 standard curve (sigmoidal function, variable slope) and determine X-values for all unpaired Y-values.
7. 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.

Calculating and reporting the half maximal effective concentration (EC50), relative potency (RP) and relative induction efficiency (RIE) values 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. Transform FI values to log and express 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 log 10% max values 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:

$RIE = \text{Max RLU chemical}/\text{max RLU } E_2 \times 100$

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:

$RP = EC_{50} \text{ of } E_2/EC_{50} \text{ of sample} \times 100$

Fang et al. (2000)

RP gives an indication of the potency of a test chemical compared to the positive control (positive control = 100%), for 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.

Limitations of the bioassay

- 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.

Recommendations

This assay can be used in place of the ER-CALUX as it is less expensive, but equally sensitive (Leusch, 2008). The assay is rapid and can be conducted in 96 well plates. Consistent results are produced which makes it suitable for high throughput screening. The cell line is freely available from ATCC.

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Recommended reading

DAVIS JM editor. Basic cell culture. 2nd edition. New York; Oxford University Press; 2006.

E-Screen for Estrogenicity

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Anti-hERα	Antibodies against human estrogen receptor alpha
ATCC	American Type Culture Collection
BM Blue POD	BM Blue peroxidase substrate (3,3’-5,5’-Tetramethylbenzidine ready to use solution
BSA	Bovine serum albumin
C_{18}-SPE	C_{18} (Octadecyl), Solid phase extraction columns
DES	Diethylstilbestrol (synthetic estrogen)
DMSO	Dimethyl sulfoxide (DMSO) is the chemical compound with the formula $(CH_3)_2SO$.
DNA	Deoxyribonucleic acid
E-Screen	Estrogenicity screen
E_2	17β-Estradiol
EDs	Endocrine disruptors
EE	Ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
ERE	Estrogen response element
FBS	Fetal bovine serum
GLP	Good laboratory practice
GWRC	Global Water Research Coalition (www.globalwaterresearchcoalition.net)
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
hERα	Human estrogen receptor alpha
hERβ	Human estrogen receptor beta
LDH	Lactate dehydrogenase
MCF-7 (ATCC)	Human Breast Adenocarcinoma Cell Line (ATCC stock)
MCF-7 (BUS)	Human Breast Adenocarcinoma Cell Line (BUS stock)
nm	Nano meter
nM	Nano molar
OD	Optical density
RPMI-1640	Medium formulation was developed at Roswell Park Memorial Institute
v/v	Volume to volume
w/v	Weight to volume
XTT	Tetrazolium salt: Sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate

INTRODUCTION

Several *in vitro* bioassays (Zacharewski, 1997) are used as rapid primary screens to detect xenobiotic estrogenic activity (Odum et al., 1998). Environmental estrogens (manmade and natural compounds) (ligands) are known to bind and activate the nuclear bound estrogen receptor (ER). Three receptor sub-types (ERα and ERβ1 and ERβ2), known to mediate the estrogen response are expressed in several tissues in the body (Esterhuyse, 2008; Fu et al., 2008; Swart & Pool, 2009). The general principle is that the ligand-nuclear receptor complex bind to a specific estrogen response element (ERE) sequence on DNA and initiate transcription of a downstream gene that will code for a specific protein. Estrogens are important regulators of growth and differentiation of normal cells (for example, the normal mammary gland), however, altered expression of genes involved in the cell cycle could lead to the mitogenic effects known from breast cancer (Moggs et al., 2005). MCF-7 is a human breast cancer cell line that predominantly express ERα (also some ERβ) (Bursztyka et al., 2008) and have been widely used to screen for endocrine disruptors (EDs) with estrogenic activity (EScreen) (Soto et al., 1992; Soto et al., 2004). The estrogen sensitive proliferation response of the MCF-7 cells is used in this assay. Although the proliferation response could be quantified by counting the cells, instead of counting the cells secondary endpoints directly related to cell number variation can be used (Rasmussen & Nielsen, 2002; Swart & Pool, 2009).

The aim of this protocol is to describe the application of the MCF-7 proliferation assay for estrogenicity screening, using several secondary endpoints related to MCF-7 cell proliferation, for example, XTT activity, cellular Lactate Dehydrogenase (LDH) enzyme (protein) expression and ERα (estrogen receptor-α) protein expression (Swart, 2008; Swart & Pool, 2009).

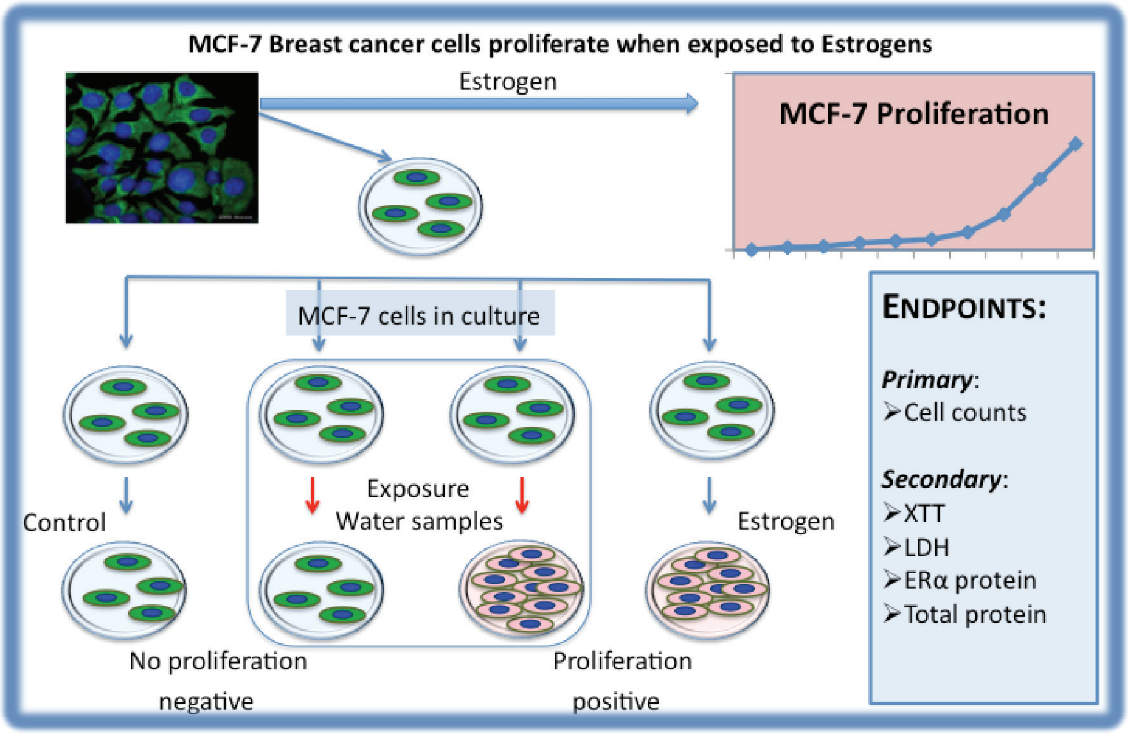

Figure 1: Schematic di

LITERATURE REVIEW (AVAILABLE AS SP 29/11 ON WRC WEB SITE)

SAMPLE COLLECTION (REFER TO MANUAL 1)

EXTRACTION PROCEDURE FOR WATER SAMPLES

See previous section (Aneck-Hahn, Van Zijl and De Jager, this report) for the procedure used to extract organics from water samples using solid phase extraction (SPE).

For the validation of the MCF-7 estrogenicity assay, the hydrophobic content of environmental water samples were extracted on C_{18}-SPE columns (Anatech) using a modified method previously described by Swart and Pool (2007). **In short**: C_{18}-SPE columns are pre-washed with 4 mℓ of solvent mixture (40% hexane, 45% methanol and 15%, 2- propanol), followed by another wash with 4 mℓ of ethanol. The column is washed with one column volume of reverse osmosis water after which the water sample is applied onto the column. The column is subsequently air-dried. The bound hydrophobic substances are eluted with solvent mixture (see above). The eluate is dried under a stream of air (or inert nitrogen gas) and then reconstituted to 1/1000th of the original sample volume with DMSO. The samples are ready to be stored at -20°C until further use.

Note: It is recommended that the water samples should be acidified (using hydrochloric acid (HCL) to pH 3 to prevent microbial degradation and facilitate effective extraction.

Potential test solutions

Extractions from

- Chemical compounds (serial dilutions)
- Waste water treatment effluents (serial dilutions)
- Drinking water samples
- Environmental water samples (serial dilutions)

Preparation of standards and exposure solutions (chemicals)

Stock solutions can be prepared using several methods. Although it is recommended that stock solutions be prepared in estrogen-free water (Millipore MilliQ system prepared) without the use of solvents, for example using a liquid-liquid or solid-liquid saturators (Kahl et al., 1999), solvents including analytical grade ethanol or DMSO are commonly used. The final solvent concentration must never exceed 1% (v/v). It is accepted that DMSO at this concentration does not affect cells. DMSO is preferred as the primary solvent because it has been shown that ethanol and isopropyl alcohol, despite good solvent properties, may have proliferation enhancing effects (Imhof & Molzer, 2008).

Estrogen positive controls

For the estrogen positive controls, 17β-Estradiol (E_2; 98% purity), 17β-Ethinylestradiol (EE; 98% purity; or Diethylstilbestrol (DES; 98% purity) can be used.

Solvent negative controls

When using solvents to deliver chemicals to the test water, then a solvent negative control should be included. Similar to test solutions, the final solvent concentration must never exceed 1% (1/100 dilution) (v/v).

Water negative controls

When solvent free stock solutions are used, exposures are done against a dilution water control (artificial water).

REQUIREMENTS

Acquisition of the cell line

The MCF-7 cells can be obtained from American Type Culture Collection (ATCC, catalogue number HTB-22), Manassas, USA.

Information and contact details:
Website: http://www.atcc.org
E-mail: tech@atcc.org
Tel: +703 365 2700
Fax: +703 365 2750

Permits

- The requirements for obtaining cells from ATCC can be found on their website (http://www.atcc.org).
- An import permit is required from the Department of Health, Pretoria, RSA.

Information and contact details:
Tel: 012 312 0816 Fax: 012 323 0093
Physical address: Hallmark Building, 266 Vermeulen Street, Pretoria
Postal address: Private Bag X828, Pretoria, 0001

Staff training (technical skills)

- Good laboratory practice.
- Practical biochemistry techniques.
- Introductory course in Immunology (Theory and Practical).
- Training in cell culture techniques.
- Training in ELISA techniques.
- Basic Statistics (Basic analysis of variance regression techniques).
- Statistical software use and interpretation of outputs.

Laboratory (test environment)

- Analytical Biochemistry laboratory.
- Dedicated cell culture laboratory.

Equipment

- 12 Channel pipette (10 -100 μℓ)
- CO_2 incubator
- Electronic balance
- Refrigerated Centrifuge with swing-out rotor
- Laminar flow Cabinet
- Fumehood
- Freezer (-20°C)
- Freezer (-80°C)
- Refrigerator (4°C)
- Inverted microscope
- Plate warmer-shaker
- Microplate (ELISA) reader
- Millipore Milli-Q ultrapure water system
- Oven
- pH-meter
- Pipettes (1-10 μℓ, 20-200 μℓ, 200-1000 μℓ)
- Vortex mixer
- Waterbath
- Laboratory oven
- Autoclave
- Hemacytometer

Consumables / Materials

Glassware

- Schott bottles

- Erlenmeyer flasks
- Glass tubes

Materials

- C_{18}-SPE Columns (www.anatech.co.za)
- Eppendorf tubes
- Sterile pipette tips
- 96-well microplates
- Tin foil
- Nitrile gloves
- Tissue culture flasks

Reagents and Bio-assay kits

17β-Estradiol (E_2)	Sigma-Aldrich
Anti-hERα protein antibodies	Santa Cruz Biotechnology (www.scbt.com)
Antibiotic / antimycotic solution	Cat. No. A5955, Sigma- Aldrich
BM Blue peroxidase soluble substrate	Roche Applied Science (www.roche-applied-science.com)
Bovine Serum Albumin (BSA)	Cat. No. A4379, Sigma-Aldrich
Bradford Reagent	Cat. No. B6916, Sigma-Aldrich
Diethylstilbestrol (DES)	Sigma-Aldrich
DMSO	Sigma-Aldrich
Ethinylestradiol (EE)	Sigma-Aldrich
Fetal bovine serum (FBS)	Sigma-Aldrich
Horseradish peroxidase conjugated anti-mouse immunoglobulin	Cat. No. NX-A931, AEC Amersham International
L-Glutamine powder	Cat. No. 90114C, Sigma-Aldrich
LDH Cytotoxicity detection Kit (LDH)	Cat. No. 11644793001 or 04744934001, Roche Applied Science (www.roche-applied-science.com)
MCF-7 whole cell lysate	www.abcam.com
Phosphate Buffer Solution	Sigma-Aldrich
Paraformaldehyde	Cat. No. 158127, Analytical Grade, Sigma-Aldrich
RPMI-1640	Cat. No. R8758, Sigma-Aldrich
RPMI-1640 (modified)	Cat. No. R7509, Sigma-Aldrich
Serum Replacement solution	Cat. No. S2640, Sigma-Aldrich
Trypsin-EDTA (10×)	Cat. No. T4174, Sigma-Aldrich
XTT Cell Proliferation Kit (XTT)	Cat. No. 11465015001, Roche Applied Science (www.roche-applied-science.com)

Software

Microsoft Excel
SigmaStat
Statistica 8

PROCEDURES

Cell culture reagents

RPMI-1640 with L-glutamine and phenol red and RPMI-1640 modified without L-glutamine and phenol red (Estrogen deprived culture medium) is available from Sigma Aldrich. Glutamax (L-glutamine), fetal bovine serum (FBS), antibiotic-antimycotic solution, phosphate buffered saline (PBS) tablets, serum replacement solution, trypsin in ethylene-diamine-tetra-acetic acid (trypsin-EDTA) solution and 17β-Estradiol are available from Sigma-Aldrich. Estradiol is prepared as a 1 mg/mℓ stock solution in DMSO. Estradiol standards and environmental samples are diluted 1:1000 (v/v) in culture medium.

MCF-7 experimental cell culture

- *MCF-7 maintenance medium (Full medium)* (prepared by adding 10 mℓ FBS and 1 mℓ antibiotic – antimycotic solution to 100 mℓ RPMI-1640 medium containing phenol red and L-glutamine) (Sigma-Aldrich, Cat. No. R8758) (store at 4°C).
- *MCF-7 serum (estrogen) deprived medium*: (prepared by adding 1 mℓ antibiotic – antimycotic solution, 1 mℓ glutamax and 2 mℓ serum replacement solution to 100 mℓ RPMI-1640 without L-glutamine and phenol red) (Sigma-Aldrich, Cat. No. R7509) (store at 4°C).

MCF-7 Cell culture and exposure

Stock cultures of MCF-7 cells should be maintained according to recognized cell culture protocols (Freshney, 2005), previously described for MCF-7 cells (Soto et al., 1992; Soto et al., 1995; Zhao et al., 2008). General procedures regarding thawing of frozen cells, subculturing and preparation of stock cultures are describe in a previous section (Van Zijl, Aneck-Hahn & De Jager, this report).

Assay procedure

Step 1: From the stock, MCF-7 cells are suspended into maintenance full medium (RPMI-1640 medium containing phenol red and L-glutamine and supplemented with 10% (v/v) FBS and 1/100 diluted antibiotic-antimycotic solution) to a concentration of 5 × 105 cells / mℓ.

Step 2: From this cell suspension, dispense (seed) 200 µℓ per well (10 000 cells/well) in a 96-well flat bottom tissue culture plate (Nunc, AEC-Amersham).

Step 3: Allow cells to adhere to the wells for a minimum of 5 hours (up to 24 hours).

Step 4: Decant the medium.

Step 5: Rinse the wells twice with PBS pre-heated to 37°C.

Step 6: Add estrogen deprived RPMI-1640 medium (200 µℓ per well).

Step 7: Culture for 48 hours to deplete estrogen levels

Step 8: Replace medium with 200 µℓ estrogen deprived RPMI 164 medium containing 1% v/v of the 17β-Estradiol standards, water extracts or DMSO controls.

Step 9: Culture cells for 48 hours

Endpoints

Cytotoxicity

Although cytotoxicity may simply implicate cell death, it may be an oversimplified definition since metabolic alteration could also affect the viability of cells. Most viability assays rely on the breakdown in cell membrane and the subsequent measurement of the uptake or release of a dye (Freshney, 2005). Using the same principle of damage to a normally impermeable membrane and the release of the enzyme lactate dehydrogenase (LDH) from the cytosol into the supernatant of such damaged cells, the degree of cytotixicity can be monitored. LDH in the supernatant is measured using the instructions of a LDH cytotoxicity detection kit (Roche Applied Science).

In short: Following the final 48 hour culture period, 100 µℓ of the culture supernatants are transferred to an optically clear 96-well flat bottom plate. LDH reaction mixture is prepared according to the manufacturer's instructions immediately before use. To determine the LDH activity, 100 µℓ of LDH reaction mixture is added to 100 µℓ of 1:2 diluted culture medium (diluted with estrogen deprived medium) and gently tapped to mix.

The absorbance is immediately measured at 492 nm to obtain a background reading. The plate is then incubated in the dark for 30 min at room temperature, where after the absorbance at 492 nm is again measured.

Total Cellular Lactate Dehydrogenase (LDH) Determination

Total LDH activity is determined as an indicator of total cell number or proliferation. In order to obtain total LDH levels, culture supernatant is removed from the exposed cells after the 48-hour incubation period. Estrogen deprived RPMI-1640 medium containing 2% (v/v) Triton X-100 is added at 200 µℓ/well. The plate is then incubated on a shaker for 10 min at 300 rpm after which the cell lysates are assayed for LDH.

LDH reaction mixture is prepared according to the manufacturer's instructions immediately before use. To determine the LDH activity, 100 µℓ of LDH reaction mixture is added to 100 µℓ of 1:10 cell lysate preparation (Abcam MCF-7 whole cell lysate, diluted with estrogen deprived RPMI-1640 medium) in an optically flat clear 96-well bottom plate. The absorbance is then immediately measured at 492 nm to obtain a background reading. The plate is incubated in the dark for 30 min at room temperature; where after the absorbance at 492 nm is again measured. Absorbance readings must be corrected by subtracting the background reading from the absorbance of the specific sample.

Total Protein Determination

Following the 48-hour culture period of the MCF-7 cells, culture supernatants are replaced with 100 µℓ of a 1 M NaOH solution. The 96 well plates are allowed to gently shake at room temperature for 30 minutes. A multi-pipet is used to gently detach all cells after which the appropriate volume of cell lysate is transferred to clear 96 well plates. Total protein yield of the cell lysate is determined according to the Bradford method (Bradford, 1976) using Bovine Serum Albumin (BSA) (Sigma-Aldrich) as a standard protein

Cell Proliferation Assay (XTT procedure)

The cell proliferation assay (XTT) is performed using a kit obtained from Roche Applied Sciences, according to the manufacturer's instructions. This assay is based on the cleavage of the yellow tetrazolium salt, XTT, to form an orange formazan dye by metabolic active cells. Therefore, the conversion occurs in viable cells. An increase in the number of viable cells will result in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This increase directly correlates to the amount of orange formazan dye formed. This assay can be used for both cytotoxic assessments and cell proliferation studies.

In short: Cells are grown in a 96-well microplate described in the previous section. XTT reaction mixture is prepared according to the manufacturer's instructions immediately before use. To determine the XTT activity, 50 µℓ of XTT reaction mixture is added to the each well after the final cell culture incubation period (48 hours). The absorbance is immediately measured at 492 nm to obtain a background reading. The plate is now incubated in the dark for 2 hours, where after the absorbance is again measured.

hERα ELISA

Following the culture of MCF-7 cells, medium was carefully removed from the wells and cells fixated to the tissue culture plate as previously described by Maggiolini et al. (2002). Previous studies have shown that hERα protein expression is upregulated by estrogen deprivation (Villalobos et al., 1995). In maintenance medium (containing estrogen) the expression of hERα is therefore relatively high.

The quantification of hERα protein, using a direct ELISA represents an additional biomarker for cell proliferation. However, in the case of hERα it is expected that the expression will be a reciprocal phenomenon (deFazio et al., 1992). The optimization and validation of this ELISA is described in Swart & Pool (2009).

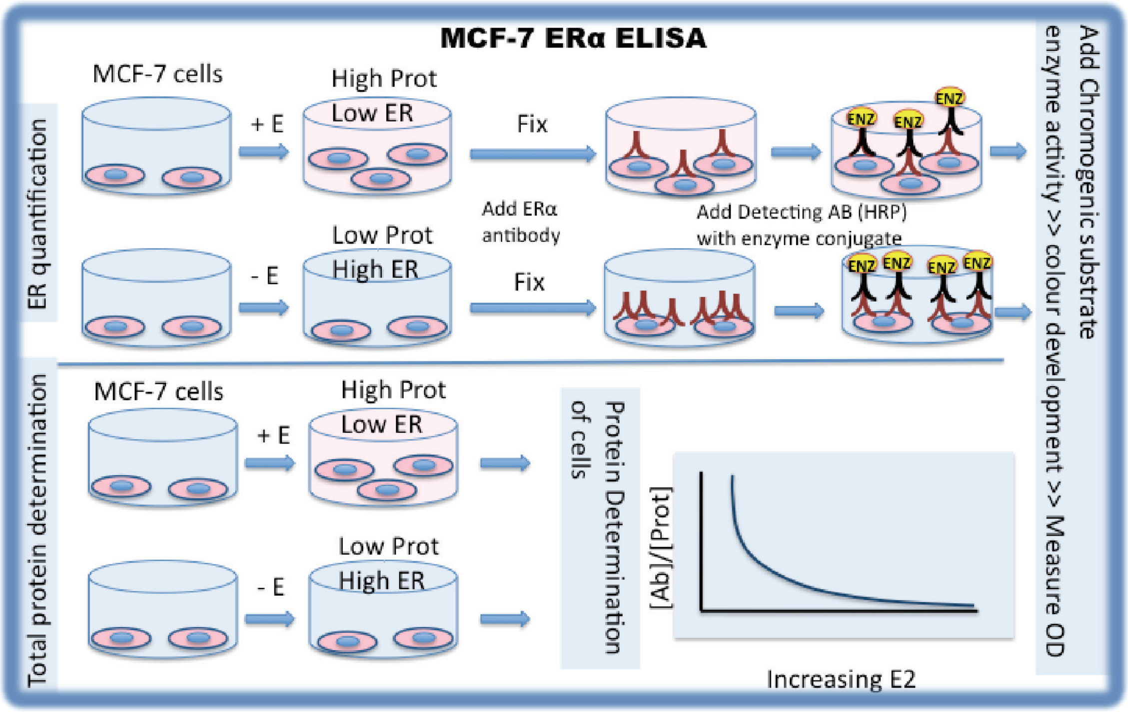

Figure 2: Principle of the ER-ELISA used in this bioassay.
MCF-7 cells are cultured and the expression of ERα protein measured using specific anti-hERα antibodies along with a detecting antibody with a conjugated enzyme. Enzymatic activity on the chromatogenic substrate result in a direct relationship of ER concentration and colour development (measured as optical density (OD)). Total cellular protein is measured in a parallel incubation to account for the cellular proliferation. Results are expressed as hERα OD/mg cellular protein.

Note: Duplicate cell cultures are done and Total Protein determinations done on these used to express the antibody concentration as a ratio of the Total Protein concentration. Total Protein concentration is a measure of cell proliferation (hERα OD/mg cell protein)

Assay procedure

Step 1: 200 µℓ of 2% v/v paraformaldehyde (AR grade) in PBS is dispensed in all the wells.
Step 2: The plate is then incubated for 45 minutes at room temperature.
Step 3: The paraformaldehyde solution is replaced with 200 µℓ of 3% v/v H2O2 in methanol and incubated for another 45 minutes.
Step 4: After fixation, wells are washed twice with PBS.
Step 5: Protein adsorption sites in the well are then blocked with 3% w/v low fat milk powder in PBS for 1 hour at room temperature with gently shaking.
Step 6: Anti-hERα (Santa Cruz Biotechnology, INC.) is diluted 1/200 in saline containing 0.3% w/v milk powder and dispensed at 50 µℓ per well.
Step 7: The plate is then incubated for 2 hours at room temperature.
Step 8: The wells are then washed 4 times with 200 µℓ PBS.
Step 9: Horseradish peroxidase conjugated anti-mouse immunoglobulin (AEC-Amersham International) is diluted 1/2500 with PBS containing 1% w/v human serum albumin and 0.01% v/v Tween and dispensed at 50 µℓ per well.
Step 10: Plates are incubated for another hour and the same wash procedure followed thereafter.
Step 11: BM Blue soluble peroxidase substrate is heated to 37°C and dispensed at 50 µℓ per well.
Step 12: Plates are incubated at room temperature for 30 minutes followed by the addition of 50 µℓ per well of stop solution (0.5 M H2SO4).
Step 13: The optical density is lastly determined at 450 nm. All ELISA readings must be corrected for background. Background controls are wells that receive all procedures except that the anti-hERα step is replaced by an incubation step with 0.3% (w/v) milk powder in saline.

Step 14: Results are expressed as hERα OD/mg cell protein, calculated using the formula:

hERα OD/mg cell protein = (OD experimental well – OD background control) / cellular protein (mg).

In order to compare the results of multiple plates with each other, ELISA results of the experimental wells being analyzed are expressed as a percentage of the hERα OD/mg protein obtained for the 0.1 nM E_2 exposure control used on all 96 well plates (normalized against a 0.1 nM E_2 standard).

Limits of detection, validations and standard curves

Standard curves can be constructed by plotting E_2 concentrations (nM E_2) on the X-axis (independent variable) with endpoint data, for example, Total LDH (OD 450 nm), XTT (OD 492 nm) or hERα OD/mg protein (% of 0.1nM E_2) or on the Y-axis (dependent variable). A linear model is fitted to the points and the hERα OD/mg protein or Total LDH (OD 450) used to read the corresponding E_2 concentration from the plots or calculate the corresponding E_2 concentrations using the linear regression function.

Cell proliferation (XTT assay) and Total LDH assay

A direct linear correlation with an R^2 value of 0.9 exists between the concentrations of E_2 exposed MCF-7 cells and total LDH levels. The linear range for the total LDH assay was between 0.1 and 1000 nM E_2. They also reported that the total LDH levels obtained from MCF-7 cells exposed to a dilution series of E_2 were correlated (R^2 =1) with the hERα ELISA OD/mg protein (Swart, 2008; Swart & Pool, 2009).

The total LDH levels obtained for MCF-7 cells exposed to a dilution series of E_2 between 1 and 1000 nM E_2 were correlated with XTT activity and total protein yield (Swart, 2008). A good correlation between total LDH and XTT activity with an R^2 value of 0.93 were obtained. A similar good correlation between total LDH and total protein yield were also obtained with an R^2 value of 0.97 (Swart and Pool 2009; Swart 2008).

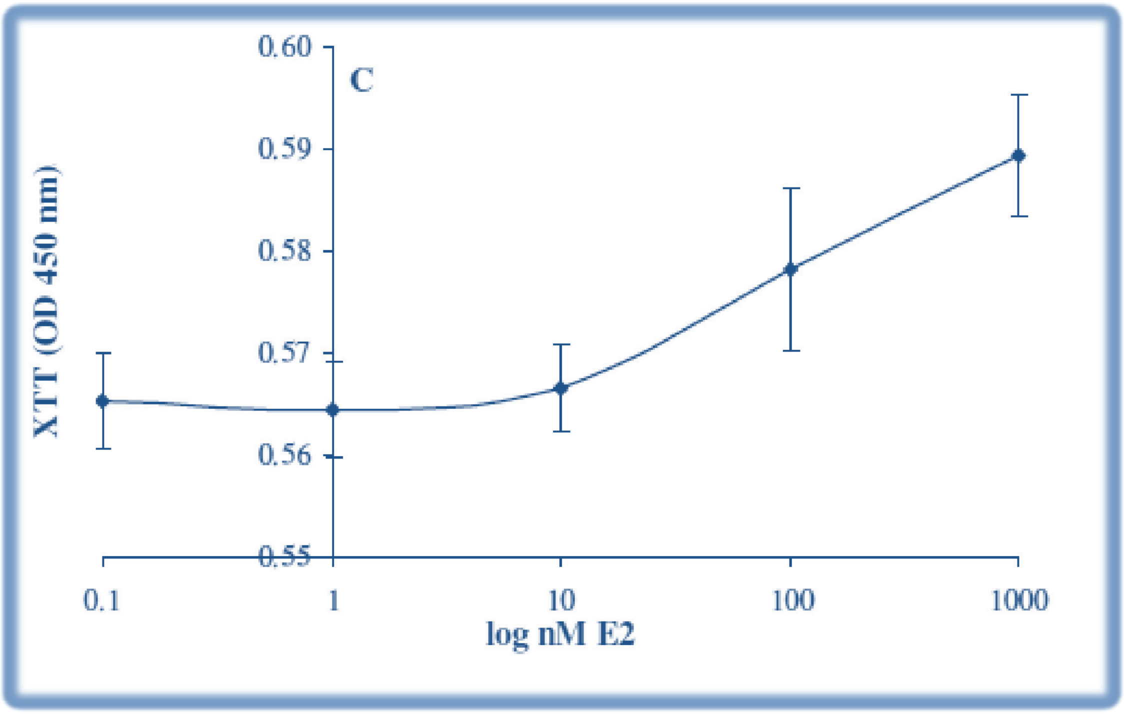

Figure 3: Dose responsiveness of MCF-7 cells to E_2: XTT activity as indicator of cell proliferation (Swart, 2008).

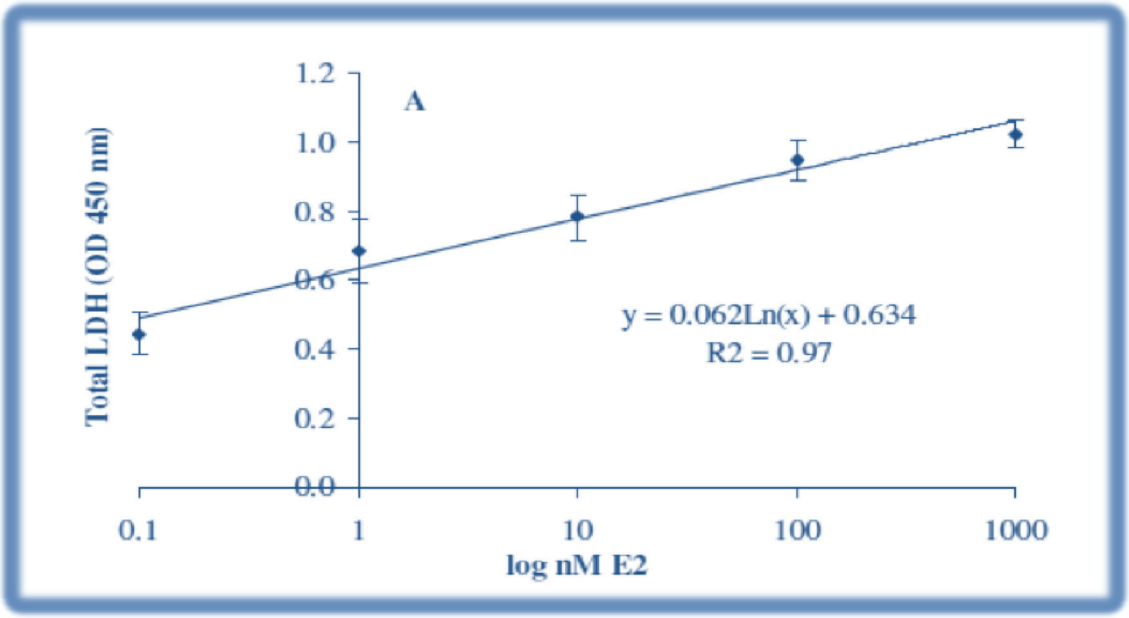

Figure 4: **Dose responsiveness of MCF-7 cells to E_2: Total LDH as indicator of cell proliferation (Swart, 2008).**

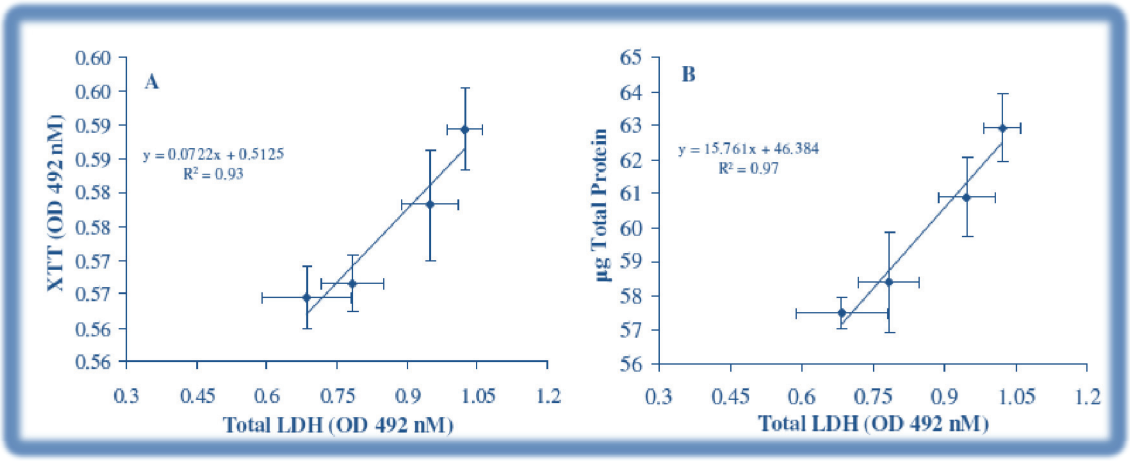

Figure 5: **Correlation of Total LDH activity with XTT activity (A) and Total protein yield (B). Vertical and horizontal error bars represent the standard deviation of the mean for ten replicates (Swart, 2008)**

The hERα ELISA

The hERα ELISA was validated by Swart & Pool (2009) and they reported that the lowest concentration of E_2 that result in a significant reduction in hERα OD/mg protein compared to the Control was 1 nM E_2. The range of the assay was between 1-1000 nM E_2.

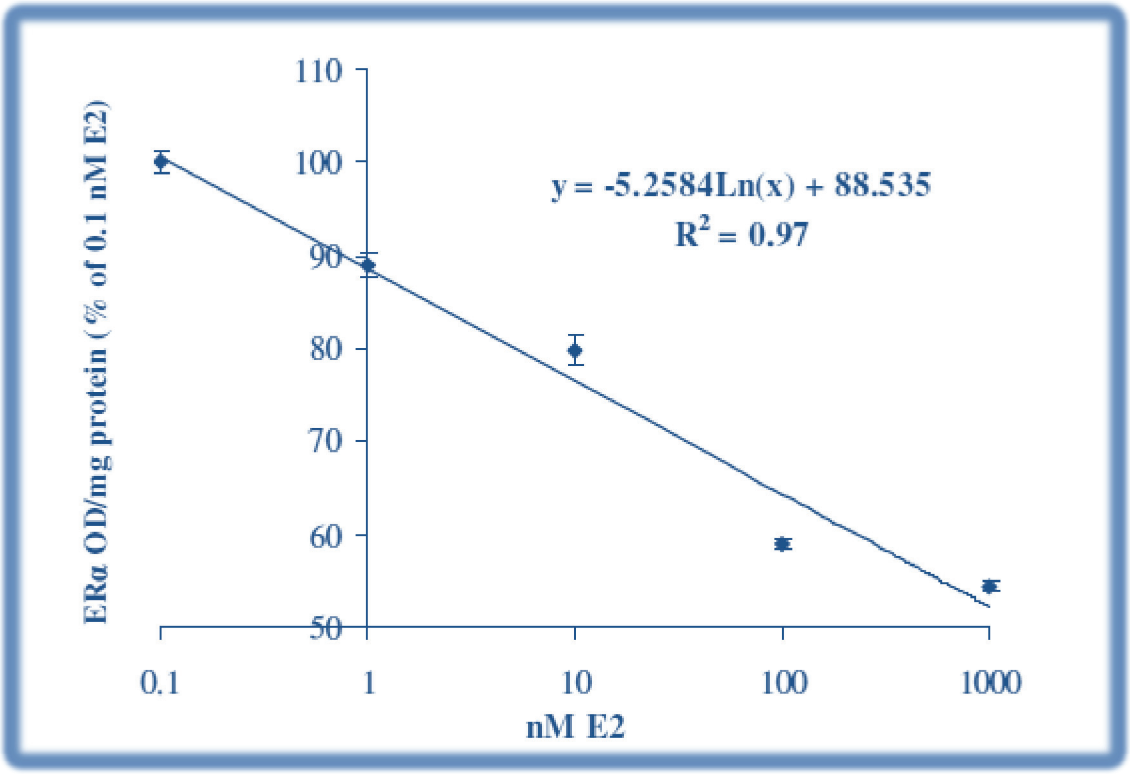

Figure 6: hERα-protein expression in MCF-7 cells in response to exposure to varied E_2 concentrations. hERα-protein levels are expressed as the hERα OD/mg protein as a percentage of the 0.1 nM E_2 exposure. Vertical bars represent the standard deviation of the mean for ten replicates (Swart, 2008; Swart & Pool, 2009).

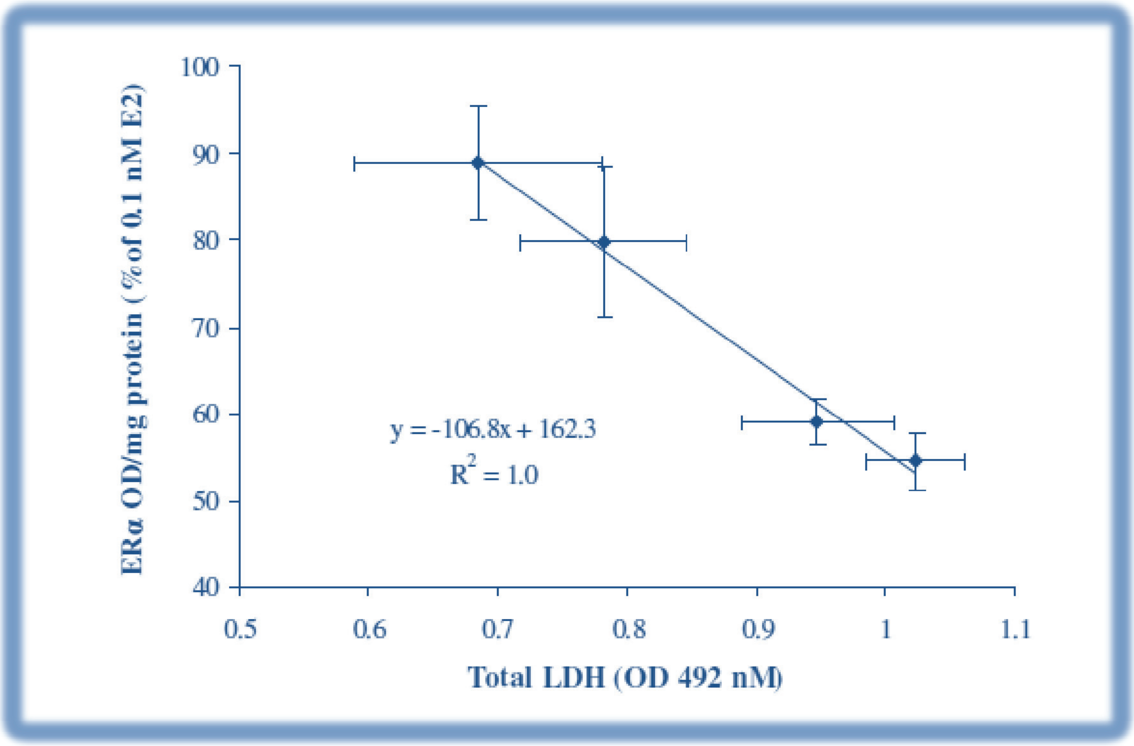

Figure 7: Correlation of hERα ELISA and Total LDH activity. Vertical and horizontal error bars represent the standard deviation of the mean of ten replicates (Swart, 2008; Swart & Pool, 2009).

Data analyses

Before testing for significant group effects (different dilutions or different sites or combinations of both), data should be tested for normality (Shapiro-Wilk test) and equal variance/variance homogeneity (Levene test). If the data conform to the assumptions of parametric testing (normal distribution and homogeneity of variance), an Analysis of variance (ANOVA) can be used to test for significant ($P < 0.05$ significance level) variation relative to a Control group (water and/or vehicle). Subsequently, a multiple comparison test (Bonferroni adjusted Fisher's Least Significant difference test, Tukey's test or Dunnett's test) can be used to identify the experimental groups that differ significantly from the Control group. When a "solvent control" was included, the latter must be compared to the "water control" using a test for two groups, a t-Test or Mann-Whitney test. If not significant ($P > 0.05$), the water and solvent controls may be combined. If statistically significant ($P < 0.05$), then the water control must be discarded and the solvent control data used in all further analyses. If data are found not to conform to the assumptions for parametric testing, it could be transformed (Log_{10}) to obtain normality and homogeneity of variance. Otherwise, results must be evaluated by using a non-parametric ANOVA (Kruskal-Wallis ANOVA of ranks) followed by a Multiple Comparison versus Control group test (Dunnett's Test).

Limitations of the Bio-assay

It has been shown that the proliferation response of MCF-7 cells to estrogens may vary between laboratories, ranging from 2-fold up to 10-fold above the hormone-free controls (Rasmussen & Nielsen, 2002). This may be the result of varying test regimes followed by the different laboratories. Odum et al. (1998) warned that MCF-7 cells have several drawbacks that need careful consideration when screening for estrogenicity.

For example, varying sensitivity of clones, the passage number, the serum batch used, the lack of xenobiotic metabolism in these cells, background rate of proliferation and non-estrogenic proliferation of cells when exposed to compounds. Comparative studies have shown that the MCF-7 BUS subline is the most responsive cell line (Villalobos et al., 1995; Schaffer et al., 1999; Rasmussen & Nielsen, 2002). Andersen et al. (1999) showed that standardized protocols can limit this inter-laboratory variation, therefore underlining the importance of such standardization. Although the MCF-7 cell line is mostly used, the T44-D cell line has shown to be equally sensitive (Villalobos et al., 1995; Leusch, 2008).

In the comparative performance evaluation conducted by the GWRC Leusch 2008, the E-Screen performed well, although in the "ease of use" and "time to results" sections this assay was ranked rather low when compared to the other assays (see Table 18 in (Leusch, 2008). Swart and Pool (2009) showed that both these concerns could be addressed.

Examples of environmental water studies

Water samples were collected from a pristine river site (mountain stream), and a sewage treatment works. The hydrophobic content of the water samples were extracted as outlined above. Following the culturing of MCF-7 cells as described; supernatant samples were collected and used for LDH activity testing (cytotoxic activity), total protein yield, XXT activity as well as Total cellular LDH activity following the exposure.

Conclusion and recommendations

One of the aims of the current modification of the E-Screen protocol was simplify this well-known estrogenicity screen. The culturing of MCF-7 cells in a 96-well format using chemically defined serum replacement factors, instead of FBS, was validate by Swart (2008). Normally, in E-Screen protocols the culture medium includes human serum of FBS. In the current assay, instead of going through all the effort, time and costs involved in the estrogen-stripping of FBS with charcoal and dextran, the use of commercially available serum replacement solution, supplemented with E_2 was validate (Swart & Pool, 2009). This validation studies confirmed the value (specifically in inter-laboratory comparisons) of using serum replacement solution, eliminating the variation associated with FBS because of herd-herd variation.

The measurement range for E_2 using Total LDH as secondary biomarker for estrogenicity in the E-Screen assay varies between 100 ρM E_2 through 1000 nM E_2 (Swart, 2008). Previous studies reported detection limits of the E-Screen ranging between 30 ρM (Soto et al., 1992) and 1 ρM (Korner et al., 1999). The Total LDH assay is 100 times more sensitive that Total protein of XTT activity as biomarkers for cell proliferation.

The quantification of hERα protein as biomarker for estrogenicity compared well with the use of Total LDH activity (Swart, 2008). The hERα-protein expression allow for detection of estrogenicity in the range between 1 and 1000 nM for E_2.

In the modified protocol, the hERα-protein ELISA is performed on cultured and fixated MCF-7 cells in a 96-well plate and does not require protein preparation of cultured cells and therefore considered well-suited for high throughput *in vitro* screening (Swart, 2008; Swart & Pool, 2009; Swart et al., 2009).

In summary, breast cancer proliferation assays (E-Screen using MCF-7 cell lines) provide a sensitive measure of estrogenicity and complement receptor-binding assays by providing a further dimension to estrogenicity evaluation. The E-Screen is a reproducible tool for identification of estrogenic compounds. This assay can be used in a 96-microwell plate setup and secondary endpoints are successfully used to quantify cell proliferation as a response to estrogenic stimulation. Using these secondary quantification assays allow for “high throughput” screening. The modified E-Screen assay as presented here (according to protocols outlined in Swart (2008) and Swart & Pool (2009) is less time consuming, easy to use and sensitive, therefore exhibiting all the characteristics of a valuable tool to assess environmental estrogenic activity on a routine basis.

Important, however, that prior training in several aspects, including GLP, several specialized biochemical techniques and cell culture work as well as the use of specialized equipment must be ensured before attempting to use this assay. Odum et al. (1998) pointed out that the uncontrolled use of the E-Screen might lead to the detection of false positive and false negative results.

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Fish VTG Screen for Estrogenicity

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BM Blue POD	BM Blue peroxidase substrate (3,3'-5,5'-Tetramethylbenzidine ready to use solution
BSA	Bovine serum albumin
DES	Diethylstilbestrol (synthetic estrogen)
DMSO	Dimethyl sulfoxide (DMSO) is the chemical compound with the formula $(CH_3)_2SO$.
E_2	17β-Estradiol
EDs	Endocrine disruptors
EDC	Endocrine disrupting contaminants
EE	Ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
ENZ	Enzyme
ER	Estrogen receptor
FBS	Fetal bovine serum
GLP	Good laboratory practice
GnRH	Gonadotropic releasing hormone
GtH	Gonadotropic hormone
H_2SO_4	Sulphuric acid
HRP	Horseradish peroxidise
IgG	Immunoglobulin G
kDa	Kilo Daltons (measure of molecular weight)
MS222	
MT-juv	Mozambique Tilapia juveniles
NaCl	Sodium chloride
OD	Optical density
RO	Reverse osmosis
v/v	Volume:volume
VTG	Vitellogenin (yolk precursor protein)
w/v	Weight:volume
WBH	Whole body homogenate

INTRODUCTION

Vitellogenin (VTG) is a high molecular weight phospholipoglycoprotein (250-600 kDa) that is produced and secreted by the liver of oviparious vertebrates as the precursor to several egg yolk proteins (Wallace & Bergink, 1974; Ho et al., 1980; Arukwe & Goksoyr, 2003). Vitellogenin synthesis is controlled by female hormone, 17β-Estradiol (E_2) produced in the gonads. The production and release of E_2 in the liver is under the control of the hypothalamic-anterior pituitary gland complex. Gonadotropin-releasing hormones (GnRH) from the hypothalamus reach the anterior pituitary gland via a portal blood connection and control the production and release of gonadotropin hormones (GtH). Gonadotropins reach the ovaries through the general blood circulation, controlling the synthesis and release of female hormone, 17β-Estradiol (E_2) (Arukwe & Goksoyr, 2003; Rocha & Rocha, 2006). The circulating E_2, when taken up by hepatocytes, binds to intra-cellular estradiol receptors (ER) leading to the increased transcription of the VTG gene(s) (and upregulation of VTG mRNA). From the hepatocytes, VTG (the yolk precursor) enters the blood circulation and upon reaching the ovary is taken in by the growing oocytes through receptor-mediated endocytosis (Matozzo et al., 2008). Within the oocyte, VTG is degraded to the yolk proteins that will eventually serve as nutrition to the growing embryos or larvae (see Figure 1).

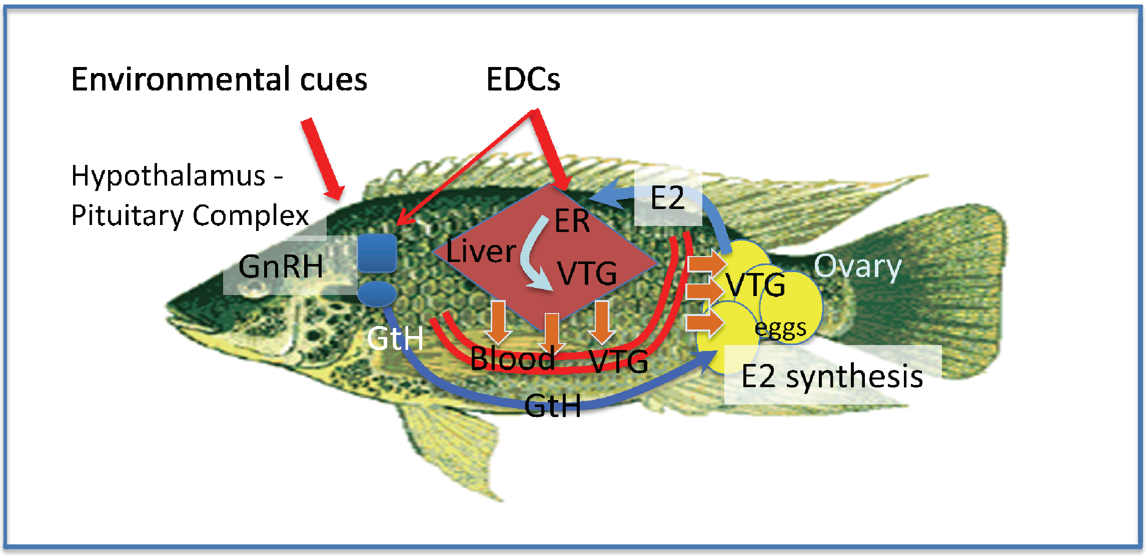

Figure 1: Diagramme to show the vitellogenin (VTG) control pathway in fish. VTG production in the liver under the control of Estradiol (E_2) is utilized as the biomarker for estrogenic activity.

Mature female fish will normally be exposed to endogenous estrogens (controlled by exogenous environmental cues and therefore produce high levels of VTG in contrast to the low VTG levels in males and juveniles (low endogenous estrogen levels). VTG is mostly only measurable/detectable in the plasma of reproductive active adult female fish, but potentially also in males and juveniles (immature) when exposed to exogenous estrogens or estrogenic compounds (Kime et al., 1999; Zhang et al., 2005). The up-regulation of hepatic VTG expression and subsequent accumulation in the plasma of juvenile and adult male fish (Purdom et al., 1994; Tyler et al., 1999) has been utilized as in vivo bioassays for estrogenicity screening (Sumpter & Jobling, 1995; Rose et al., 2002; Hiramatsu et al., 2006; Davis et al., 2007; Harris et al., 2007).

Although the use of juvenile fish may make it necessary to measure VTG in whole body homogenates (WBH), the advantage of ensuring low background estrogenic activity, especially when sex is mis-identified, is important (Van Wyk & Pool 2007). When using juvenile fish in exposure studies, additional endpoints for assessing estrogenic activity may be added. For example, if exposure takes place inside the sex determination developmental window (as early as 20 days after fertilization), the expression of the VTG mRNA gene (Zhang et al., 2005; Esterhuyse et al., 2009), gonadal histology and information on biased sex ratios (Andersen et al., 2003; Esterhuyse, 2008) can also be used to assess estrogenicity.

Although VTG bioassays are available for several small fish species from around the world, the Mozambique Tilapia (Oreochromis mossambicus) has been selected for use in locally developed VTG bioassays. Oreochromis mossambicus, is extensively used in aquaculture (Trewavas, 1983) and similar to the fathead minnow, medaka and zebrafish is easy to culture, tolerate a wide range of water-quality conditions and importantly, is native to Southern Africa. Locally, two important endpoints for estrogenicity, namely, quantitative expression of VTG mRNA in the liver (Esterhuyse et al., 2009) and the quantification of circulating VTG in the blood have recently been developed and validated (Swart & Pool, 2009; Swart et al., 2009). Since the 20 days post fertilization (dpf) fish is small, both approaches successfully utilize whole body homogenates (WBH).

In this bioassay we describe the standard protocol to quantify the VTG concentration in juvenile Mozambique Tilapia after exposure to either environmental water or man-made chemicals. Using a sensitive ELISA method (enzyme linked immunosorbent assay), antigens (in this case the VTG lipoprotein complex) or antibodies (anti-VTG antibodies) can be detected in a variety of biological samples. It can be used in a quantitative way (with a standard curve) or qualitatively (without a standard curve). Quantitative detection of proteins is mostly done by using one of two approaches, either a competition ELISA or a sandwich ELISA (Crowther 2001) (see Figure 2). In the Mozambique Tilapia VTG ELISA, the VTG lipoprotein complex is detected with a specific anti-VTG antibody (commercially available Sea bream anti-VTG polyclonal antibodies) in a competition-type ELISA.

Note: Inherent in the understanding of the ELISA method is the definition of the terms, **antigens** and **antibodies.** An **antigen (e.g. VTG)** is a substance, when introduced into the body (by injection), that elicits an **antibody (anti-VTG) response. Antibodies,** a group of glycoproteins, termed immunoglobulins (Igs) are produced by white blood cells (lymphocytes). **Specific antibodies** are raised by injecting small amounts of **antigen** into a host animal and later harvesting and selecting specific **antibodies** against the **antigen.** Because **antibodies** may be specific for a **particular antigen (e.g. VTG),** these antibodies (**anti-VTG antibodies**) can be used to detect the **antigen (VTG)** in an ELISA.

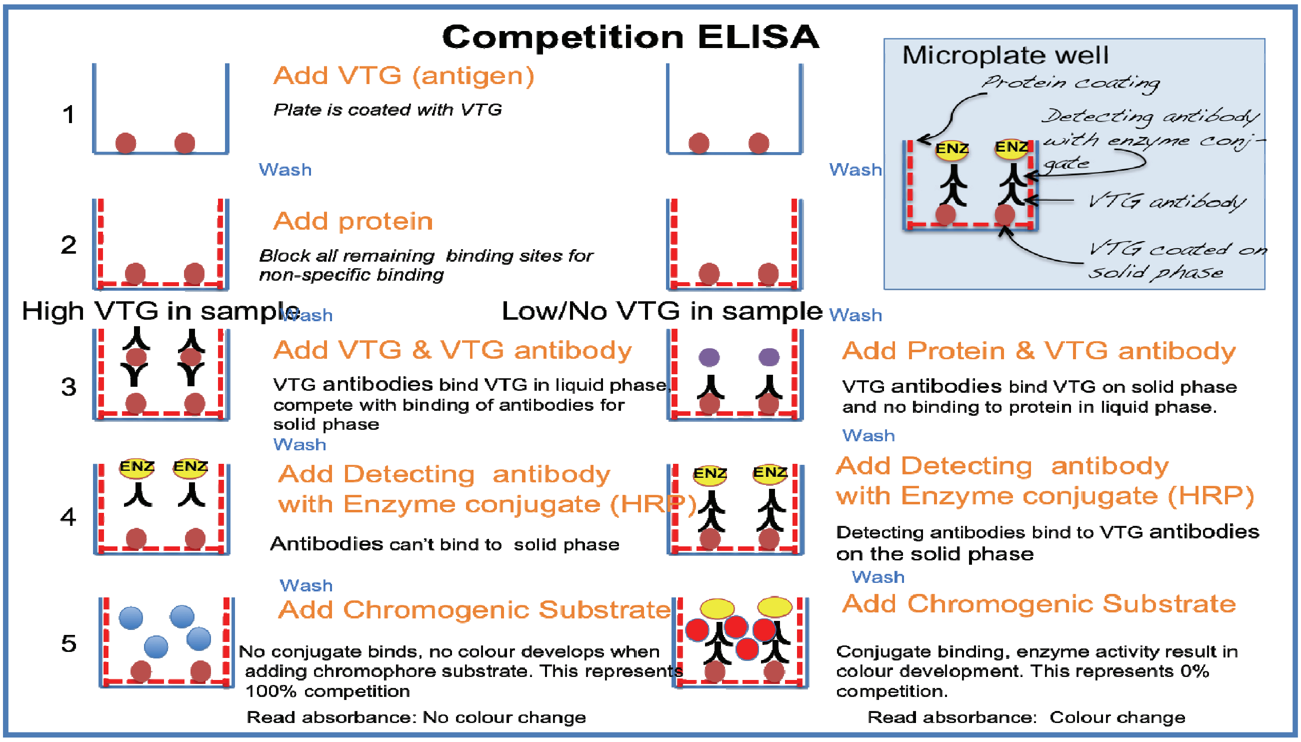

Figure 2: **Diagrammatic representation of the VTG competition ELISA protocol used here.**

LITERATURE REVIEW (AVAILABLE AS SP 29 ON WRC WEB SITE)
SAMPLE COLLECTION (REFER TO MANUAL 1)

EXPOSURE PREPARATION

Potential test solutions

- Serial dilutions of chemical compounds (single or mixed)
- Waste water treatment effluents (serial dilutions)
- Drinking water samples (serial dilutions)
- Environmental water samples (serial dilutions)
- Use of un-extracted water samples
- Exposure to specific chemicals or mixtures of chemicals

Preparation of exposure solutions from stock solutions

Stock solutions can be prepared using several techniques (Kahl et al., 1999). Although it is recommended that stock solutions be prepared in water without the use of solvents, for example, using a liquid-liquid or solid-liquid saturators (Kahl et al., 1999), solvents, including analytical grade ethanol or DMSO (OECD, 2004), are commonly used. The final solvent concentration, according to OECD recommendations (OECD, 2004; OECD, 2006), must never exceed 0.01% (v:v).

Extraction procedures of water samples and using extracts in exposures (see protocol described by Aneck-Hahn et al., and Van Wyk et al., this volume).

CONTROLS

Estrogen positive controls

For the estrogen positive controls, 17β-Estradiol (E_2; 98% purity, SIGMA), 17β-Ethinylestradiol (EE_2; 98% purity; SIGMA or Diethylstilbestrol (DES; 98% purity, SIGMA) can be used.

Solvent negative controls

When using solvents to deliver chemicals to the test water, then a solvent negative control should be included. Similar to test solutions, the final solvent concentration must never exceed 0.01% (v:v).

Water negative controls and exposure water

When solvent-free stock solutions are used, exposures are done against a dilution water control (artificial water). (Basic recipe: To 2.5 ℓ RO water add 0.625 g NaCl, and 0.2 g NaHCO3).

REQUIREMENTS

Acquisition of juvenile fish

Mozambique Tilapia (*O. mossambicus*) juveniles (MT-juv), 14-20 days post fertilization (dpf) swim-up fry are used in exposure experiments (Swart, 2008) 2008; Esterhuyse (Esterhuyse, 2008; Esterhuyse et al., 2009).

In-house breeding of juvenile tilapia

The Mozambique Tilapia (*O. mossambicus*) is a maternal mouth brooder. Males construct nests and females may produce up to 2000 eggs at a time (Trewavas, 1983; Van Dyk, 2006; Esterhuyse, 2008). Breeding can be done successfully in the laboratory (Esterhuyse, 2008).

Adult breeding stock of *O. mossambicus* can be obtained from Aquastel PTY LTD, South Africa) (http://www.sun.ac.za/aquastel) and maintained in aquaria with water under constant aeration and filtered through activated charcoal. Water temperature must be maintained at 27°C (±1°C) (see Summary Table below for water quality parameters). Fish are fed once daily with Tilapia pellets (AquaNutro, South Africa) (http://www.aquanutro.com). The light regime must be set to a 14:10 light:dark cycle. Offspring production is monitored daily in this mouth brooding species. Females carrying eggs in their mouths are removed from the breeding aquaria into culturing tanks. Each brooding female must be kept alone until the offspring reached the swim-up fry stage, at which time the adult females are removed and re-introduced into the breeding tank. Each batch of offspring is reared separately in the same water conditions as for breeding stock. Animals at the appropriate developmental stage can then be collected. It is best to use at least three different breeding pairs to generate offspring.

Obtaining tilapia juveniles from commercial breeders

Juvenile fish (MT-juv) (similar maternal batch) must be obtained from a reliable commercial breeder (Aquastel) or from an in-house breeding programme (Esterhuyse, 2008).

On upon arrival, MT-juv must be transferred to holding tanks (10 ℓ) and maintained in aged tap water or bore-hole water filtered by activated charcoal filters, water temperature controlled to a mean temperature of 27°C and a light:dark regime of 14:10 (L:D). Fish are fed daily with crushed commercial tilapia pellets (AquaNutro, South Africa). After an initial acclimation period of 24 hours, MT-juv individuals of similar size are randomly selected and transferred to exposure tanks.

Note: When ordering tilapia juveniles specifically ask for juveniles that were not treated with hormones.

Permits

If tilapia fish will be collected from natural water resources, or transported between laboratories or kept in captivity, researchers should enquire at the local Nature Conservation authorities about permit requirements.

Ethical clearance

All vertebrate animals are protected by law in South Africa (Animals Protection Act No. 71 of 1962) (South African Medical Research Council, (2004), and it could therefore be an offence in terms of this law to conduct exposure experiments without justification and permission. Most institutions have an ethical review process that is normally coordinated by an Ethics Committee for Research on Animals (ECRA). It is the responsibility of the researcher/project manager to submit such an application to the institutional ECRA. Since fish will be exposed to known controls and unknown chemicals, followed by sacrifice using overdose euthanasia or mechanical means, ethical aspects must be considered and approved (Borski & Hodson, 2003). All procedures must conform to the ethical guidelines of The South African Medical Council, SABS, the ethical guidelines of the research or academic institution (ECRA) where the exposures and sampling will be done and a recognized Ichthyology Society, for example American Society of Ichthyologists and Herpetologists (ASIH) (http://www.asih.org).

Staff training

- Fish breeding and husbandry.
- Good laboratory practice.
- Introductory course to Immunology (Theory and Practical).
- Training in basic Biochemistry laboratory techniques.
- Training in ELISA techniques.
- Basic Statistics (Basic analysis of variance, ANOVA and linear and non-linear regression techniques).
- Use of Statistical software and interpretation of outputs.

Laboratory facilities

- Exposures: Environmental Controlled Aquarium room (wet laboratory with temperature and light control). Activated Charcoal filtered water, compressed air and temperature control (ambient and water).

- Analytical: Clean and sterile workbench in an analytical Biochemistry Laboratory.

Equipment
- Sonicator / homogenizer
- Plate Reader (spectrophotometer)
- Refrigerated centrifuge
- Ultra-cold Refrigerator (-80°C)
- pH meter
- Electronic balances, top loader (nearest 0.01 g) and chemical balance (nearest 0.0001 g)
- Dissolved oxygen analyser
- Fumehood
- Refrigerator (4°C)
- Freezer (-20°C)
- Millipore Milli-Q ultrapure water system
- In-line Activated Charcoal filters
- Compressed air supply
- pH-meter
- Pipettes (1-10 µℓ, 20-200 µℓ, 200-1000 µℓ)
- Vortex mixer
- Waterbath
- Laboratory oven

Consumables
- Glassware
  - ➢ Schott Bottles
  - ➢ Erlenmeyer flasks
  - ➢ Glass tubes
  - ➢ Glass bottles (5 ℓ)
- Materials
  - ➢ Eppendorf tubes
  - ➢ Cryo-tubes
  - ➢ Sterile pipette tips
  - ➢ 96-well microplates
  - ➢ Nitrile gloves

Reagents and Bioassay kits

17β-Estradiol (E_2)	Sigma-Aldrich
Anti-rabbit IgG horseradish peroxidase conjugate	Cat. No. W4011, Promega (http://www.promega.com)
Aprotinin	Cat. No. A1153; Sigma-Aldrich
AquaNutro, tilapia food	http://www.aqanutro.com
Aquastel	http://www.sun.ac.za
Benzocaine	Cat. No. E1501: Sigma-Aldrich
BM Blue peroxidase soluble substrate	Roche Applied Science (www.roche-applied-science.com)
Bovine Serum Albumin (BSA)	Cat. No. A4379, Sigma-Aldrich
Bradford Reagent	Cat. No. B6916, Sigma-Aldrich
Diethylstilbestrol (DES)	Sigma-Aldrich
DMSO	Sigma-Aldrich
Ethinylestradiol (EE)	Sigma-Aldrich
Fetal bovine serum (FBS)	Sigma-Aldrich
Hibitane solution	Chlorhexidine gluconate is an antiseptic effective against a wide range of bacteria, yeasts, some fungi and viruses.
Horseradish peroxidase conjugated anti-mouse immunoglobulin	Cat. No. NX-A931, AEC Amersham International
MS222	Tricaine: 3-aminobenzoic acid ethyl ester, methanesulfonate salt: Cat. NO. A5040; Sigma-Aldrich
Neptune Liquid soap	Bell Products, www.bellchemicals.com,
PMSF (Phenyl-methyl-sulphonyl-fluoride)	Cat. No. P7626; Sigma-Aldrich
Protease inhibitor	Sigma-Aldrich
RNALater	Ambion: http://www.ambion.com/techlib/resources/RNAlater/
Sea bream anti-VTG antibody	www.caymanchem.com or BioSense Laboratories, www.biosense.com

Computer software

- Microsoft Office Software (MS Excel)
- SigmaStat (SPSS Science: http://www.spss.com)
- Statistica 8 (http://www.statsoft.co.za)

Experimental procedures

Juvenile Fish Exposure System

Two basic exposure systems are possible. The static renewal system utilizes glass bottles or tanks with the content either not being replaced during the exposure period, static renewal system or where the content is replaced at intervals within the exposure period, semi-static renewal system.

Alternatively, exposures can be done in tanks with a continuous flow-through of exposure water during the exposure period (Barnhoorn, 1996; Nussey, 1998; Brion et al., 2004; Esterhuyse, 2008; Thorpe et al., 2008). The advantage of the latter system being that the exposure concentration of the chemicals in solution remains stable and not deplete through time as result of uptake by the fish in the closed system.

Static or semi-static renewal exposure system (current protocol)

For the semi-static renewal approach (water change on day 4) two different renewal procedures may be followed. Test solutions can be prepared in duplicate, clean vessels and juveniles transferred into the replacement vessels, or the juvenile fish are retained in the test vessels and a proportion (50-66%) of the test water is changed (OECD, 2006). For example, water is replaced on day four of a 7-8 day juvenile exposure period.

Prior to experiments, all glass containers must be washed thoroughly (e.g. Neptune Liquid soap, Bell Products, www.bellchemicals.com, and Hibitane solution), followed by a rinse with water (distilled or subjected to reverse osmosis (RO) and charcoal filtered). Tanks are then stripped with 100% methanol and again rinsed with RO filtered water. Tanks are filled with the required amount of water (environmental, aged / de-chlorinated tap water or artificial water) and aerated at 27°C for 48 hours. Artificial water can be prepared using reverse-osmosis (RO) water with the addition of salts (see OECD Guidelines 203; 210). General water quality must be monitored and controlled to values shown in Table 1.

Flow-through exposure system (future protocol)

Preferably, exposures should be done in tanks with a *continuous flow-through* of exposure water during the exposure period (Barnhoorn 1996; Esterhuyse 2008; Brion et al., 2004; Nussey 1998; Thorpe et al., 2008). The protocol for exposing juvenile *O. mossambicus* in a flow-through system needs detailed validation.

Experimental design

MT-juvs (n = 10-20) are exposed to test solutions (natural water, effluents, chemicals in solution) for a period of nine days to a dilution water control and/or a solvent water control as well as an estrogen positive control (n = 10-20 fish per treatment). Chemical screening (individual chemicals or as mixtures) as well as environmental samples (including serial dilutions) can be used in a similar manner. All experimental fish are sampled at the end of the exposure period.

Sampling and storage of fish samples

Juvenile sacrifice

MT-juvs are sacrificed in a lethal dose (500 mg/ℓ) of either MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt: SIGMA) buffered in to pH 7.2 (1M NaOH) or Benzocaine (ethyl-p-aminobenzoate) (100 mg/ℓ, 0,01%) (stock 10 g dissolved in acetone in 100 mℓ water).

Morphometrical measurements

Fish measurements, to the nearest 1 mm, can be done using a stereomicroscope (Leica EZ4D) and digital image analysis software (Leica Microsystems (Switzerland) Limited). The wet weight of fish is determined with a precision chemical balance (Ohaus Precision Scale) to the nearest 0.01 g.

Plasma samples and whole body homogenates

Plasma samples

Blood can be collected by cardiac puncture using a heparinised syringe or capillary tubes. Alternatively, blood can be collected from the tail using heparinised capillary tubes after cutting the tail with a sterilized scalpel blade.

Whole body homogenates

All the following steps must be carried out on ice.

Sonication (Swart 2008): Protein extract buffer (0.9% (w/v) NaCl containing 0.01% (v/v) Phenylmethylsulphonylfluoride (PMSF)) to prevent proteolysis is added to the juvenile fish at a weight to volume ratio of 1 g wet weight per 10 mℓ extraction buffer. Samples are then sonicated (Omni-Ruptor 400; Omni International INC.) at 40% power, for 15 seconds, 5 second bursts followed by 1 min incubation on ice. Cell rests are removed by centrifugation at 12000 × g for 10 minutes at 4˚C. Cell pellets are discarded and the supernatants are aliquoted (0.5 mℓ per cryo-tubes) and stored at -80˚C until further use.

Homogenization: Two protocols are described

a) Method used by Rose et al. (2002): Juvenile samples are frozen in liquid nitrogen or taken from -80°C storage and each frozen fish crushed separately in a morter filled with liquid nitrogen. The homogenate must be weighed, and four volumes of homogenate buffer (50 nM Tris-HCl, pH 8.0, 0.02% aprotinin, 0.1 nM PMFS) added. The homogenates are then centrifuged at 50 000 × g for 60 min at 3°C. The supernatant is then transferred into 3 × 0.05 mℓ eppendorf tubes and stored at -80°C.

b) Method recommended in OECD (2007) guideline: Use 10× the tissue weight of ice-cold homogenisation buffer (50 nM Tris-HCl pH 7.4, 1% Protease inhibitor cocktail (Sigma, P8340)): 12 mℓ Tris-HCl pH7.4 + 120 µℓ Protease inhibitor cocktail). The homogenization buffer must be used on the same day of mixing. The samples are kept on ice until centrifugation at 4°C at 50 000 × g for 30 min. Dispense portions of 50 µℓ supernatant into at least two tubes. The tubes are stored at -80°C until use (OECD, 2007).

Note:
All dissection boards, instruments must be rinsed and cleaned (96% ethanol) between handling of each single fish to prevent contamination.

BIOCHEMICAL DETERMINATIONS

Total protein concentration

Total protein concentration of the samples is measured according to the method of Bradford (1976) using bovine Serum Albumin (BSA) as a standard protein (Sigma). Follow the detailed description supplied with the kit.

VTG mRNA expression

At the end of an exposure, MT-juv fish can be preserved for additional VTG mRNA quantitative gene expression studies to compliment the VTG ELISA data. Following sacrifice as describe above, juveniles are preserved in RNAlater (Ambion, Applied Biosystems). This is a stabilization solution that rapidly penetrates most fresh tissues and stabilizes and protects the RNA. Cut the head (behind the pectoral fin) and tail (behind the dorsal fin or cloaca) from the fish and submerge body in 5-10 volumes of RNAlater. Samples can be stored at room temperature for a week, or at 4°C for one month. If samples are to be frozen, incubate these samples overnight at 4°C, remove supernatant and then store samples indefinitely at -20°C or -80°C until RNA extraction. These samples will now be available for quantitative determination of mRNA VTG copies (Esterhuyse et al., 2009).

Histopathology and sexual differentiation

Additional material can be preserved following the exposure period to conduct a histopathological study or determine the sex of the individuals. The head and tail can be removed in the same manner as described above. The body (carcass) including the gonads, is transferred to Bouin's fixative or 10% buffered formalin (Bancroft and Stevens 1977). The volume of the fixative should be at least 10 times the volume of the tissues. The preserved tissue can be stored at room temperature until routine histological methods can be applied to study the histology and histopathology of the developing gonads.
For histopathological approach see Van Dyk (2006).

Vitellogenin (VTG) determination

Vitellogenin (VTG) is determined using a VTG competition ELISA (See Figure 2 for diagramme) for Mozambique Tilapia (*O. mossambicus*) developed and validated by Swart (2008) and Swart & Pool (2009).

PROCEDURE

Anti-VTG antibody

In this ELISA, a polyclonal anti-VTG antibody can be obtained from Cayman Chemical Co (Catalog number: 170150). This antibody was produced from purified VTG of E_2 treated Gilthead Sea bream (*Sparus aurata*) (obtained from Cayman Chemical Co.; www.caymanchem.com or Biosense Laboratories; www.biosense.com). This antibody recognizes VTG from several fish species, including *O. mossambicus* (Swart and Pool 2009).

The anti-VTG antibody recognizes three VTG isoforms (molecular weights; 200 kDa, 140 kDa, 130 kDa). During optimization, a VTG antiserum dilution of 1:1 000 was chosen for setting up the competition ELISA (Swart & Pool, 2009).

Sample dilution

The dilution factor suggested by Swart and Pool (2009) is 1:2 000. The VTG standard (produced in the EJ Pool laboratory) is serially diluted in dilution buffer (100 mℓ 0.9% NaCl + 2 g BSA + Tween 20 1 mℓ). An assay control and standard are applied in duplicate and samples in triplicate.

Step by step ELISA procedure

- Step 1 (Coating of micro-titer plates): Nunc-Immuno Maxisorp® plates (Nalge Nunc, Denmark) are used for all ELISA assays. Plates are coated overnight at 4°C with 50 µℓ per well of Mt-VTG (1 µg/mℓ) (VTG antigen) diluted in 0.9% NaCl (saline).
- Step 2 (Wash): At the end of the incubation period plates were decanted and washed four times with saline.
- Step 3 (Blocking and washing): Following the wash procedure, the remaining adsorption sites are blocked (to prevent the anti- VTG primary antibody from binding to the plate) by dispensing 0.2 mℓ of a high protein block solution (0.9% saline containing 2% v/v human serum albumin [HSA]) per well and the plates are then incubated for one hour at room temperature.
- Step 4 (Wash): The same wash procedure as earlier was followed. Tap the plate dry.
- Step 5 (Add samples): Add 50 µℓ of the diluted standards or sample solution to the wells. Immediately after dispensing the standards and samples into wells, the anti-VTG antibody diluted 1/1000 in blocking buffer is added to the sample/antigen containing plates at 50 µℓ per well.
- Step 6 (Incubation): Incubated plates for three hours on a horizontal shaking platform (500 rpm) at room temperature.
- Step 7 (Wash): Using the same wash procedure (see above) wash plates seven times and dry by tapping.
- Step 8 (Add detecting antibody conjugate, enzyme attached): Anti-rabbit IgG-horseradish peroxidase conjugate (anti-r-IgG-HRPO) is diluted 1/5000 with saline containing 1% (v/v) HSA and 0.1% (v/v) Tween and dispensed at 50 µℓ per well. The conjugated antibody detects the amount of primary VTG antibody bound to the solid phase VTG.

Note:
Sodium azide is an inhibitor of horseradish peroxidase. Do not include sodium azide in buffers or wash solutions, if an HRP-labeled conjugate will be used for detection.

- Step 9 (Incubate): Plates must be incubated for 1 hour at room temperature.
- Step 10 (Wash): Following incubation, the washing procedure is again repeated seven times.
- Step 11 (Add substrate): BM Blue (a chromogenic substrate for peroxidase, POD), soluble substrate is heated to 37˚C, dispensed at 100 µℓ per well and allows to incubate for 20 minutes at room temperature. The reaction is terminated with the addition of 50 µℓ stop solution (0.5 M H_2SO_4) to each of the wells.
- Step 12 (Determine OD): The absorbance (OD) is read at 450 nm using a plate reader. A standard curve is drawn using the OD readings obtained for the VTG standards and the concentration of the samples was read of this curve.

Note: Competitive ELISAs yield an inverse curve, where higher values of antigen (VTG) in the samples or standards yield a lower amount of colour change

Limits of detection

The Mozambique Tilapia VTG ELISA was validated by Swart and Pool (2009) and they reported a limit of detection of 80 ng/mℓ VTG. The working range of the ELISA is broad (80 ng/mℓ - 5.4 µg/mℓ VTG), making it suitable for VTG quantification at low estrogenic activity as well as at potent (high) estrogenic activity. Repeatability has been confirmed by reporting relatively low inter- and intra-assay variation, the inter-assay variation was 8.4% at 50% binding and 2.9% at 90% binding respectively. Intra-assay variation was 1.1% at 50% binding and 3.4% at 90% binding (Swart 2008; Swart and Pool 2009).

Calculations

The percentage of maximum binding is calculated for each standard and sample dilution using the following equation (Rose et al., 2002).

% of maximum = [(standard or sample OD)/(OD at maximal binding)] x% of maximum = [(standard or sample OD)/(OD at maximal binding)] × 100

Standard curves

A standard curve is constructed by plotting "percentage of maximum" on a logarithmic Y-axis against the standard concentrations (µg/mℓ VTG) on the X-axis. A linear model is fitted to the points and the "percentage of maximum" binding of the unknown samples used to read the VTG concentration from the plot or calculate the VTG concentrations using the linear regression function (Swart and Pool; Mourot and Le-Bail, 1995).

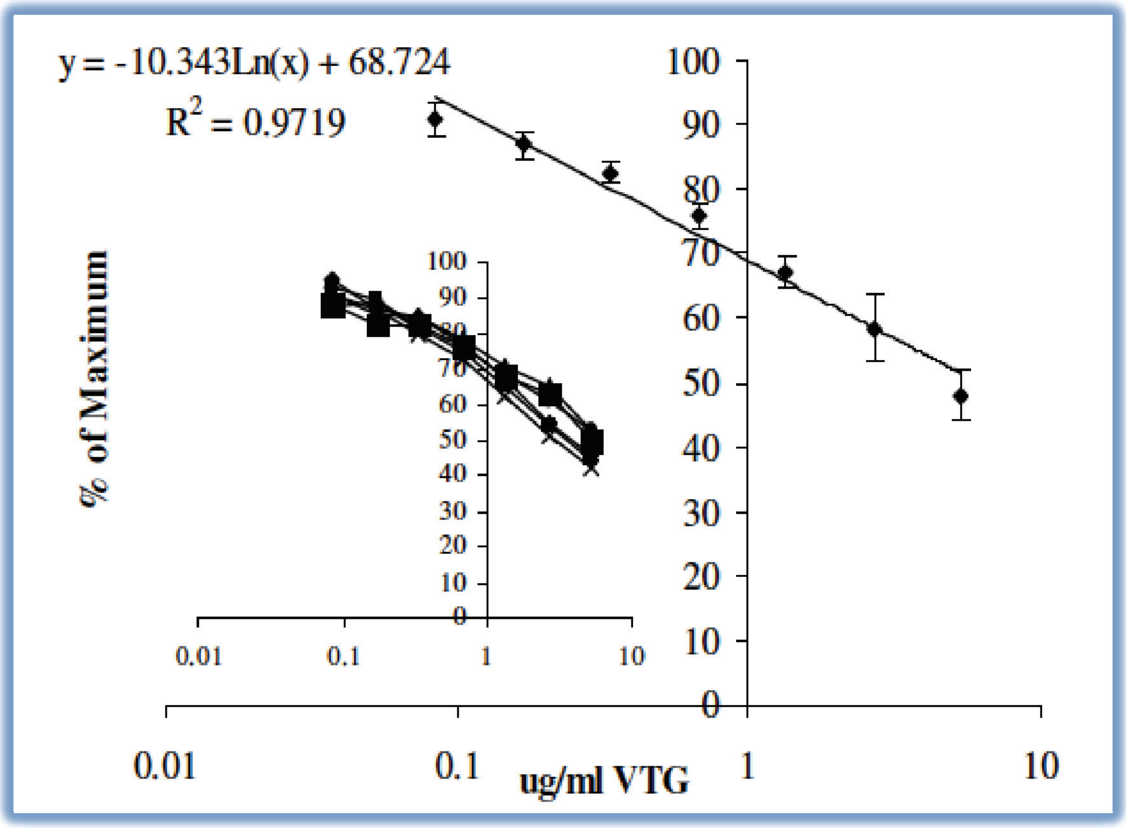

Figure 3: The tilapia VTG competition ELISA standard curve. VTG standard (In-house standard produced in the EJ Pool laboratory) diluted from 84.1 ng/mℓ to 5.4 µg/mℓ VTG. Six replicates were done.

Data analyses

Following the correction of the VTG concentrations for the dilution employed during the assay, the data are ready for statistical analysis. Before testing for significant group effects (different dilutions or different sites or combinations of both), data should be tested for normality (Shapiro-Wilk test) and equal variance/variance homogeneity (Levene test). If the data conform to the assumptions parametric testing (normal distribution and homogeneity of variance), an Analysis of variance (ANOVA) can be used to test for significant ($P < 0.05$ significance level) variation relative to a control group (water and/or vehicle). Usually a multiple comparison test (Bonferroni adjusted Fisher's Least Significant difference test) can be used to identify the experimental groups that differ significantly from the Control group. When a "solvent control" was included, the latter must be compared to the "water control" using a Mann-Whitney test. If not significant ($P > 0.05$), the water and solvent controls may be combined. If statistically significant ($P < 0.05$), then the water control must be discarded and the solvent control data used in all further analyses. If data are found not to conform to the assumptions for parametric testing, it could be transformed (Log10) to obtain normality and homogeneity of variance. Otherwise, results can be evaluated by using a non-parametric ANOVA (Kruskal-Wallis ANOVA of ranks) followed by a Multiple Comparison versus Control group test (Dunn's Method).

Rose et al. (2002) suggested the use of logistic regression analysis to determine the relation between percentage of responding fish and external E_2 or EE_2 concentrations to reflect on effective concentrations, in particular LOEC (lowest effective concentration). To do this they categorized individuals, as responsive if the VTG concentration was higher than twice the highest VTG concentration observed in control fish. They assumed that for a group of fish exposed to estrogenic compounds, the percentage of responding fish equals the probability of a response in an individual fish. From the fitted logistic regression, the EC10, EC50 and EC90 for E_2 can be extrapolated.

Limitations of the Bio-assay

- Time consuming and labour intensive.
- Relatively expensive.
- Animal ethics clearance needed.
- Restricted by the availability of juvenile fish.
- Dedicated aquarium facilities needed.
- In house breeding and fish husbandry require tilapia aquaculture skills/knowledge.

Conclusion and recommendations

The shortfall of in vitro exposure assays is that they do not take into account bioaccumulation and bio-metabolism of the compound and they exclude all metabolic effects that are not mediated via the estrogen receptor. Nielsen (2008) pointed out that the use of results obtained from in vitro assays for human risk assessment might be problematic.

A validated VTG ELISA for Mozambique Tilapia (Oreochromus mossambicus) described above, is considered a sensitive in vivo bioassay for estrogenicity testing. The tilapia VTG ELISA is a useful tool to detect estrogenic activity, not only following in vivo exposures of juvenile or adult male tilapia to environmental samples or samples spiked with chemicals, but also a tool to be used along with in vitro assays (for example, primary hepatocyte cultures and ex vivo liver slice cultures).

Outside the laboratory, in the field situation, VTG concentrations in field-caught tilapia (juveniles, adult males and females) can also be determined to assess the impact of estrogenic activity in natural water sources.

Important, however, that prior training in several aspects, including fish husbandry, GLP and several specialized biochemical techniques as well as the use of specialized equipment must be ensured before attempting to use this assay. It is also important to realize that prior to fish exposure experiments ethical clearance will be needed and before fish are removed for testing or breeding from natural aquatic systems, a collecting permit is needed from the local conservation agency.

TABLE 1: Summary of test parameters for juvenile Mozambique tilapia (*O. mossambicus)* vitellogenin assay.

Test species	*Oreochromis mossambicus*
Stage	20 dpf
Age criteria	Days post fertilization
Exposure period	7-8 days
Exposure regime	Static or Static-renewal (change water on day 4)
Density	~100 specimens per 10 ℓ
Replication	100 samples per tank, Two tanks per exposure substance
Dilution water/ lab control	Charcoal filtered water containing buffering salt, NaCl (iodated sea salt, 2.5 g containing 0.004 g iodine per 100 g salt).
Exposure vessel	12 ℓ glass aquaria, containing 10 ℓ of test/control water. Aquaria are washed once with Neptune, then rinsed 3× with RO water, then sprayed with Methanol, and finally rinsed 5× with RO water.
Mortality rate in controls	< 10%
Feeding	Tilapia pellets, ground finely (AquaNutro, SA)
Light regime	14:10, L:D
Water temperature	27°C +/- 1°C (must not differ by more than ± 1°C between test vessels at any one time during the exposure period and maintained within a range of 2°C within the temperature ranges specified for the test species).
pH	6.5-8.5 (The inter-replicate/inter-treatment differential should not exceed 0.5
Dissolved Oxygen	> 80% air saturation
Total hardness	Between 10 and 250 mg CaC03 per litre
Analytical chemistry sample schedule	At 0 hrs and at 24 hrs

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Guidelines for Chemical Analysis of Endocrine Disrupter Chemicals in Water Resources

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This report was written as part of WRC project K8/920

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GUIDELINES FOR CHEMICAL ANALYSIS OF EDC COMPOUNDS

This section deals with the period from the time the samples enter the laboratory to the final submission of the results.

In this section, only the analytical procedures of organic compounds listed in the list of priority EDCs developed by the WRC (Volume 2) will be described. These compounds, their appropriate extraction, clean-up and instrumental analysis are given in Table 1.

1 EXPLANATION OF TERMINOLOGY USED

Sample	A representative portion of the substance that requires analysis.
Standard	An authentic sample of the organic compound to be analysed normally obtained from the manufacturer. The purity should be provided.
Standard solution (Stock solution)	A solution made by adding an appropriate solvent to an accurate mass/volume of the standard in a volumetric flask. The concentration depends on the limit of detection.
Working solution	Dilutions of the standard solution used to draw a standard curve.
Standard curve	A mathematical function drawn by plotting different concentration values of the working solution against instrumental results.
Internal standard	A standard curve obtained by adding working solution concentration to a matrix before extraction, thus compensating for possible matrix effect and loss during extraction and clean up.
Blank	A sample of the same matrix as samples to be analysed, but known to be free from contamination going through the same extraction and clean-up procedure. In the case of water analysis, distilled water is usually used.
Field blank	A sample from the same area and matrix but not necessarily free from contamination. This sample is used to determine matrix effect on extraction and clean-up procedures.
Recovery test	A recovery test is done by adding known quantities of standard to a sample and analysing it concurrently with the samples.
Drinking water	Water suitable for human consumption that will have no detrimental effect over a life time. This is usually purified water from a water purification system. (In South Africa drinking water may also be understood as borehole or spring water or even surface water).
Potable water	Purified water suitable for human consumption and for sale in containers.
Surface water	Water exposed to atmosphere usually from rivers, streams and dams.
Ground water	Water taken beneath ground level: can be from boreholes or wells.
Environmental water	Water taken from surface, wetlands estuaries, rivers, streams and dams.
Borehole water	Water taken from borehole.
Sediment	Suspended matter that settles at bottom of water.

Soil	Upper layer of earth.
Biological tissue	Tissue taken from a living organism, such as fat, liver, muscle etc.
Plasma	The fluid portion of whole blood obtained by centrifuging or sedimentation.
Serum	The liquid left after clotting of blood, fibrinogen having been removed.
Sewerage influent	Water and waste going into a water purification plant.
Sewerage effluent	Water coming out of a water purification plant.
Lowest limit of detection LOD*	The lowest limit where a compound may be detected by a specific analytical method.
Quantification limit LOQ**	The lowest limit where a value can be calculated with confidence.

* Can be calculated as 3x the noise level of the baseline of the chromatogram
OR Can be experimentally estimated from the injection of serially diluted standard solutions or extracts of fortified water samples until the signal to noise ratio (s/n) reaches the value of three
OR The lowest point on a standard curve

** LOQ set at three times the limit of detection.

2 GENERAL PRINCIPLES AND QUALITY CONTROL

1. A quality control document should be filled in on submission of the samples. This document should accompany the samples through the entire analytical process (refer to Document A).
2. Avoid usage of plastic and Teflon ware at all times.
3. Wash all glassware for sample storage, filtration and extraction of water samples in chromic acid and rinsed once in methanol and twice in ethanol.
4. Use separate glass volumetric flasks for standard solutions. Calibrate them once and verify them in the laboratory once a year. Do not clean in a dishwasher.
5. Calibrate measuring instrumentation regularly. (Balances, pH meters, thermometers, GCs and HPLC detectors).
6. Check distilled laboratory water quality daily and replace filters regularly.
7. Use HPLC grade solvents when available.
8. Always use authenticated standards for standard solutions and recovery tests. Replace when expiry dates are reached.
9. Store standards in a separate facility away from samples.
10. A standard curve, recovery test, blank and field blank should be run concurrently with each batch of samples.
11. Maintain an ambient temperature of 23°C ± 2°C at all times.
12. Work in fume cupboards equipped with extraction fans when using dangerous solvents.
13. Instrumental analysis and extraction/clean-up procedures should be done in separate rooms to avoid contamination.

DOCUMENT A: QC DOCUMENT FOR LABORATORY ANALYSIS

Date of submission		**Sampling date**	
Name and address of person/organisation submitting samples			
Description of samples (water, sediment, biota)			
Number of samples submitted			
Containers in which samples were submitted (glass, plastic, etc.)			
Condition of samples (temperature, state of decomposition, etc.)			
Tests required			
Identification numbers of samples (given by submitter)	**Laboratory number of samples**		
Comments			
Signature of person submitting samples		**Signature of person receiving samples**	

3 SOURCES OF STANDARDS, SOLVENTS, CHEMICALS AND INSRUMENTATION

Standards		
17β-Estradiol	Sigma-Aldrich Steraloids Inc.	P/N E8875
Estrone	Sigma-Aldrich Steraloids Inc.	P/N E3201
Estriol	Sigma-Aldrich Steraloids Inc.	P/N E1149
Ethinylestradiol	Sigma-Aldrich Steraloids Inc.	P/N 28586-2
Nonylphenol	Sigma-Aldrich	P/N 29085-8
4-*t*-Octylphenol	Sigma-Aldrich	P/N 290832
Bisphenol A	Sigma-Aldrich	P/N 239658
Benzyl butyl phthalate (BBP)	Sigma-Aldrich	P/N 308501
Tetrabromobisphenol A (TBBPA)	Sigma-Aldrich	P/N 330396
Dieldrin	Supelco ChemService	P/N PS76
α-Endosulfan	Supelco ChemService	P/N PS81-1
β-Endosulfan	Supelco ChemService	P/N PS81-2
p,p’ DDT	Supelco ChemService	P/N PS699
	Dr. Ehrenstorfer	
PDE-47 and PDE-99	Industrial Analytical (Pty. Ltd.)	
PBDE Mix	Institute for Fisheries Research The Netherlands	
Solvents		
Acetone	Merck, Darmstadt, Germany	
Acetonitrile	Merck, Darmstadt, Germany	
Chloroform	Merck, Darmstadt, Germany	
Dichloromethane	Merck, Darmstadt, Germany	
Diethylether	Merck, Darmstadt, Germany	
Ethanol HPLC grade	Sigma-Aldrich	Cat No 270741
n-Hexane	Merck, Darmstadt, Germany	
Isopropanol	Merck, Darmstadt, Germany	
Methanol AR	Merck, Darmstadt, Germany	
Methanol HPLC grade	Riedel de Haën	Cat No 34860
Methanol HPLC grade	Acros organics	
Petroleum Ether (bp 60-80°)		
Veterinary drug standards		
Synthetic hormones		
Diethylstilbesterol (DES)	Sigma-Aldrich	
Ethynylestradiol	Sigma-Aldrich	
17β-Estradiol	Sigma-Aldrich	
Androgenic steroids	Dr. Ehrenstorfer	
Trenbolone	Dr. Ehrenstorfer	
Nortestosterone	Steraloids	
Methyltestosterone		
Estrogenic action		
Zeranol		
Natural hormones	Sigma-Aldrich	
17α-Estradiol	Sigma-Aldrich	
Testosterone	Sigma-Aldrich	
Progesterone		
Beta agonists		
Clenbutarol		
	Sigma-Aldrich Corp. St. Louis, MO,USA Sigma-Aldrich Chemicals,Oakville, ON, Canada	

Salbutanol	Steraloids Inc. Newport, RI, USA	
Mabutanol	Dr. Ehrenstorfer Gmbh, Augsburg, Germany	
Zilpaterol	Acros organics, Fairlawn, NI, USA	
Solid phase Cartridges		
Oasis HLB	Waters Corp., Milford, MA, USA	P/N 18600115
Bakerbond Speedisk^{TM} Octadecyl-bonded silica (C_{18}XF) 50 mm	J.T. Baker, Deventer, The Netherlands	
Silica Cartridges:	500 mg, 10 mℓ Sopachem nv The Netherlands	
NH_2-cartridge	100 mg, 1 mℓ, Sopachem nv The Netherlands	
Chromabond C_{18} ec Cartridge	Macherey-Nagel	Cat No 730014
C_{18} cartridge (Supelco)	Supelco, Bellafonte, PA, USA	
Silica gel 70-230 mesh	Industrial Analytical (Pty) Ltd.	
Activated charcoal 50-100 mesh	Industrial Analytical (Pty) Ltd.	
Sep-Pak Environmental tC18 plus 900 mg	Waters Corp., Milford, MA, USA	
Superclean ENVI-18 500 mg	Waters Corp., Milford, MA, USA	
C_{18} SPE column	Anatech	
Filters, Vials and accessories		
Glass wool filter	Macherey-Nagel	Cat No 000904
47 mm Sterile filter 0.45 micron	Osmonics MicronSep	Cat No E04WG047S1
Whatman GF/C 47 mm	Merck, Darmstadt, Germany	
Whatman SP	Merck, Darmstadt, Germany	
MaxRecovery vails	Waters Corp	P/N 186000326c
Cellulose filter disks	Dionex, Sunnyvale, CA,USA	
Small Instrumentation		
12 port Visiprep SPE vacuum manifold	Supelco Bellafonte, PA, USA	P/N 57030-U
Speed disk^{TM} Extraction Station	J.T. Baker, Deventer The Netherlands	
Solid Phase Extraction Unit (SPU)	VWR, Darmstadt, Germany	
Rotavapor	Buchi, Flawil, Switzerland	

Centrifugal evaporation System	Gyrovap, Howe and Co., London, England	
IKA Ultra Turrax ®T25	Staufen, Germany	
ASE 200 system	Dionex, Sunnyvale, CA, USA	
Turbovap®LV evaporator	Zymark Co., Hoptkinson, MA, USA	
Freeze dryer Christ LMC-2	Germany	
Pulverisette 5	Frisch GmbH, Idar-Oberstein, Germany	

4 PREPARATION OF SAMPLES

4.1 Water

1. Water should ideally be analysed as soon as possible after sampling (according to Noppe et al., organic compounds degrade within 2 weeks even when protected against biodegradation). Samples should be taken in clean amber glass bottles previously rinsed with ethanol. If amber bottles are not available, clear bottles may be wrapped in black plastic bags to protect against light.
2. To protect the water samples from microbial degradation a few drops of 0.02% Sodium azide should be added.
3. Should water contain solid matter filter through a glass fibre filter.
4. The pH of all water samples should be adjusted to < 3 by adding formic acid.
5. Transport and store the samples at 4°C.

4.2 Sewerage effluent

1. Sewerage effluent should ideally be analysed as soon as possible after sampling. Samples should be taken in clean glass amber bottles previously rinsed with ethanol. If amber bottles are not available, clear bottles may be wrapped in tinfoil to protect against light.
2. To protect the effluent samples from microbial degradation a few drops of 0.02% Sodium azide should be added.
3. Should water contain solid matter homogenise with an Ultra Turrax and filter through a glass fibre filter.
4. The pH of all effluent samples should be adjusted to < 3 by adding formic acid.
5. Transport and store the samples at 4°C.

4.3 Soil and sediment

1. Mix sample thoroughly in a glass, ceramic or stainless steel container. Avoid any contact with plastic or Teflon.
2. Determine moisture content of sample.
3. Dry sample by freeze drying (Christ LV-2, Germany).
4. Pulverise and homogenise sample thoroughly using a Pulverisette.

4.4 Biological tissue

1. Add dry ice to the fish tissue and meat tissue in a blender and blend until a homogeneous powder is formed.
2. Leave until sample reaches ambient temperature.

4.5 Blood products

If a laboratory undertakes the analysis of blood or blood product samples, a certified medical analyst must be employed.

1. Serum and plasma are treated the same.

2. Determine the lipid concentration of the serum/plasma (Rylander et al., 2006).
3. Add enough ethanol or any other organic water miscible solvent to the sample to precipitate the protein.
4. Centrifuge at high speed and use an aliquot of the top layer.

5 EXTRACTION AND CLEAN-UP

5.1 Water

Several methods are described for extraction and clean-up of micro components in water, sediment and biological tissue. These methods include:

ACE	Activated Carbon Extraction (Okonkwo, 2005).
LLE	Liquid-Liquid Extraction (SABS). WRC Report No 1402/1/08.
LP	Liquid Partitioning (SABS/CSIR).WRC Report No 1402/1/08.
MAE	Microwave Assisted Extraction (CSIR).WRC Report No 1402/1/08.
OTT	Open Tubular Trapping (Ortner and Rohwer, 1999).
SBSE	Stirr Bar Sorptive Extraction (Kolahgar et al., 2002).
SFE	Super Critical Fluid Extraction (Naude et al., 1998).
SOE	Soxhlet Extraction (Naude, 1997).
SPE	Solid Phase Extraction (Sabik et al., 2000).
SPME	Solid Phase Micro Extraction (Sabik et al., 2000).

Only the most often used procedures will be described in this volume.

It is possible to determine most of the chemicals by multi residue methods. The chemicals of concern are given in table1 together with appropriate extraction and clean-up procedures.

CODE

LLE: Liquid-liquid extraction, SPE: Solid phase extraction, ACE: Activated Carbon Extraction, SE: Soxhlet extraction, LP: Liquid partitioning.

GC: Gas chromatograph, ECD: Electron Capture Detector
NPD: Nitrogen Phosphorus Detector, FPD: Flame Photometric Detector
MS: Mass Spectrometer, LC: Liquid chromatograph, UVD: Ultra Violet Detector, DAD: Diode Array Detector, FLD: Fluorescence Detector, FID: Flame Ionisation Detector

* The class of compounds can be done in one multi residue method.
** These are members of Triazine Family of compounds and can be analysed in one multi residue method.
[a] Requires Pre column derivatization.

TABLE 1: LIST OF COMPOUNDS, RECOMMENDED EXTRACTION PROCEDURES AND INSTRUMENTATION ANALYSIS

Compound class	**Extraction and clean-up**			**Instrumentation**
	Water	**Soil and sediment**	**Biological tissue**	
Insecticides				
_**Organochorines**_* DDT, DDE, DDD Dieldrin, Aldrin, Endrin α-Endosulfan, β-Endosulfan, Endosulfan-sulphate Heptachlor, Heptachlor epoxide Lindane (γ-BHC)	LLE, SPE, ACE	SE	SE, LP	GC-ECD, GC-MS ELIZA GC-MS-MS LC-MS-MS
**Organophosphates******* Azinfos-methyl Chlorpirifos Parathion	LLE, SPE, ACE	SE	SE LP	GC-NPD GC-FPD GC-MS
**Pyrethroids******* Cypermethrin Deltamethrin	LLE, SPE	SE	SE, LP	LC-DA
**Carbamates******* Aldicarb Carbaryl	LL, SPE	SE	SE, LP	LC-FLD [a]
Herbicides 2,4-D Acetochlor Alachlor Amitrole Atrazine** Diuron Linuron Metribuzin Propazine ** Simazine** Terbuthylazine**	SPE	SE	SE	GC-MS LC-MS-MS
Fungicides Benomyl Carbendazim[1] Cyhexatin Fentin hydroxide Metiram Procymidone Vinclozolin*	SPE	SE	SE	LC-MS-MS
PCBs	LLE SPE	SE	SE	GC-ECD GC-MS
Alkylphenols * *p*-Nonyl phenol Nonylphenol ethoxilates *p*-Octylphenol Octyl phenol ethoxilates	LLE SPE	SE	SE	GC-ECD GC-MS LC-MS LC-DAD

Compound class	Extraction and clean-up			Instrumentation
	Water	Soil and sediment	Biological tissue	
Veterinary drugs* ***Synthetic hormones*** Diethylstilbesterol (DES) Ethynylestradiol 17β-Estradiol ***Androgenic steroids*** Trenbolone Nortestosterone Methyltestosterone ***Estrogenic action*** Zeranol ***Natural hormones*** 17α-Estradiol Testosterone Progesterone ***Beta agonists*** Clenbutarol Salbutanol Mabutanol Zilpaterol	SPE	LP-SPE	LP-SPE	GC-MS GC-MS-MS LC-MS LC-MS-MS
Hormones* 17β-Estradiol Estriol Estrone 17α-Ethinylestradiol	LOE-SPE	LP-SPE	LP-SPE	GC-MS GC-MS-MS LC-MS LC-MS-MS
Plasticizers* Bisphenol A *Di-n-butyl phthalate* Dicyclohexyl phthalate Diethyl phthalate Diethylhexyl adipate Dihexyl phthalate Dipentyl phthalate Dipropyl phthalate	SPE	LP-SPE	LP-SPE	GC-FID GC-MS
Fire retardants Polybromobiphenyl ethers	LLE, SPE	SE		GC-FID GC-MS
Other Benzo(a) perene n-butyl benzene Styrene Dichlorophenol *(Dye intermediate)* Benzophenone *(Raw material in medical products)* 4- nitrotoluene *(2,4-dinitrotoluene intermediate)* Octachlorostyrene *(Byproduct of organochlorine compounds)*	SPE			
Dioxins **Dibenzofuranes**				

* Same Extraction and clean-up can be used in a multi residue process, but different instrumental analysis will be required for the different classes of compounds.

** These compounds belong to the Triazine class of compounds and be analysed in one multi residue method.

[1] Benomyl breaks down to carbendazim rapidly. Therefore carbendazim was included.

[a] Requires Post column derivatization.

A LC-MS-MS method is available to determine 46 pesticides in positive ion mode and 14 in negative ion mode with minimum sample clean up and extraction (www.agilent.com/chem)

Detection limits

Detection limits in EDC studies are determined by the level at which the specific compound has effect on one or more of the hormone systems. Some EDCs are active at a million times lower than 17β-Estradiol (Activity 0.03 ng/ℓ). The implication of this is that the required detection limits for the majority EDCs are in the µg/ℓ range. Exceptions are hormones that need to be determined at ng/ℓ, Dioxins, PCBs and Dibenzo furans that also need to be determined at very low levels. Some detection limits are given in Table 2.

TABLE 2: LOWEST LIMITS OF DETECTION CALCULATED ON POTENCY OF EDC COMPOUNDS

Compound	Detection Limit
17β-Estradiol	**0.03 ng/ℓ**
Estriol	**0.04 ng/ℓ**
17α-Ethinylestradiol	**0.03 ng/ℓ**
Estrone	**0.03 ng/ℓ**
***p*-Nonylphenol**	**0.2 µg/ℓ**
***p*-Octylphenol**	**0.05 µg/ℓ**
BBP	**3.0 µg/ℓ**
DBP	**3.0 µg/ℓ**
DEHP	**3.0 µg/ℓ**
DDT	**30 µg/ℓ**
DDE	**30 µg/ℓ**
Methoxychlor	**0.03 µg/ℓ**
PCB	**0.003 µg/ℓ**

5.1.1 Liquid-liquid extraction from water

Suitable for extraction of polar and semi polar compounds such as insecticides, PCBs, Alkylphenols

Apparatus

Separating funnels 2 ℓ volume with glass stoppers and taps
Round bottomed flasks with glass stoppers 250 mℓ and 10 mℓ
Small sample bottles 2 mℓ
500 mℓ measuring cylinders
50 mℓ measuring cylinders
Glass funnels
Glass Pipettes 1 mℓ and 2 mℓ
Whatman SP filter paper
Rotavap apparatus for solvent evaporation

Standard solutions (Stock solutions)

Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in appropriate solvent (for GC analysis hexane is recommended and for HPLC analysis methanol). Store at 4°C in the dark when not in use.

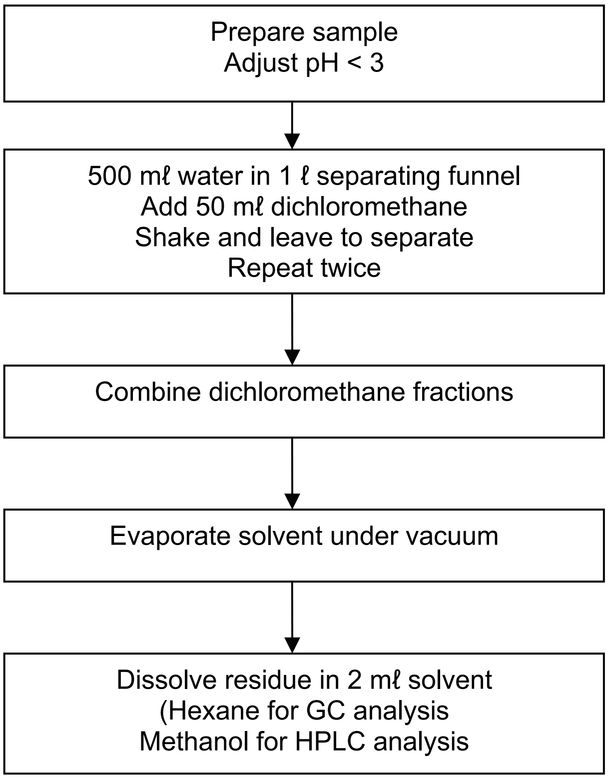
Working solutions

Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (for GC analysis hexane is recommended and methanol for HPLC analysis).

Procedure

1. Use distilled water for a blank and recovery tests.
2. Measure in duplicate 500 mℓ water into a 2 ℓ separating funnel.
3. Add appropriate standard solution to recovery sample.
4. Add 50 mℓ dichloromethane. Shake well and leave to separate.
5. Filter bottom layer (Dichloromethane layer) through a Whatman SP filter paper into a 250 mℓ flat round bottomed flask.
6. Repeat steps 4 and 5 twice. Combine Dichloromethane fractions.

7. Place flask with combined dichloromethane fractions on a Rotavap apparatus and remove solvent under vacuum until approximately 5 mℓ remains. Water bath temperature must not exceed 35°C.
8. Wash residue over in 10 mℓ round bottomed volumetric flask and remove solvent under a gentle stream of nitrogen.
9. Reconstitute residue in 2 mℓ hexane and use for GC analysis.



SCHEMATIC REPRESENTATION OF LIQUID-LIQUID EXTRACTION

5.1.2 Solid phase extraction from water

Suitable for extraction and clean-up of non-polar, semi-polar and polar compounds such as pesticides, alkylphenols, PCB, natural and synthetic hormones, veterinary drugs and industrial compounds.

Apparatus
Measuring cylinders 500 mℓ and 1 ℓ
Solid phase extraction unit / Visiprep or similar device
Vacuum pump
Solid Phase Extraction Cartridges
Glass distilled water
Test tubes 10 mℓ, with glass stoppers
Evaporation equipment
Fibreglass filters
Pipette 1 mℓ and 2 mℓ
Flat round bottomed flask 100 mℓ

Solvents
Methanol
Ethanol
Acetone
Dichloromethane

n-Hexane
Isopropanol
Diethyl ether
Methyl-*t*-butyl ether (MTBE)

Standard solutions (Stock solutions)
Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in appropriate solvent (for GC analysis hexane is recommended and for HPLC analysis methanol). Store at 4°C in the dark when not in use.

Working solutions
Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (for GC analysis hexane is recommended and methanol for HPLC analysis).

TABLE 3: GENERAL PRINCIPLES OF SOLID PHASE EXTRACTION PROCEDURE

STEPS	PURPOSE
Prepare sample Adjust pH < 3 using formic acid Filter sample if necessary or centrifuge if sample is cloudy and only water phase needs to be analysed	To deconjucate organic compounds. At low pH bases are ionised and acid and neutral compounds are neutral. Acidic compounds bind strongly to matrix proteins. To break these interactions acidification is needed. Filtration is needed if sample contains too much solid material, however when environmental water is used for drinking purposes filtration is not recommended.
Select appropriate cartridge Refer to Table 4	To select a cartridge compatible with the structure of the chemical class to be analysed. Reverse phase cartridges are most commonly used in most applications.
Condition/equilibrate with suitable solvent or mixture of solvents	Prepares sorbent for use. Normally a solvent such as Methanol is used because it mixes well with water and organic solvents.
Load sample Up to 1 ℓ sample	Compounds are retained on the sorbent. Care must be taken that cartridge does not run dry during this process. The speed should not exceed 10 mℓ/min to avoid breakthrough.
Wash/Rinse (Methanol)	To wash through last of loaded sample and to prepare cartridge for elution of active compounds.
Dry cartridge/Rinse (Isopropanol)	Water does not mix with organic solvents and the cartridges must be dried to prepare them for elution with organic solvents. This step can be eliminated by rinsing the cartridge with a small volume (1 mℓ) of isopropanol.
Elute Suitable organic solvent (Dichloromethane or Hexane/Ethylacetate or MTBA or any other polar organic solvent)	Elute active compounds from cartridge. Select suitable solvent for chemical class.
Concentrate eluent	Concentrate sample either using an evaporation apparatus or a gentle stream of nitrogen.
Reconstitute sample	Reconstitute sample in suitable solvent for instrumental analysis.

NOTES

1. Use glass distilled water for blank and recovery tests.
2. Select appropriate SPE cartridge for application (see Table 4).
3. Condition cartridge with appropriate solvent mix.
4. Load sample (do not exceed 1 ℓ). Maintain a constant flow rate of not more than 10 mℓ/min to avoid breakthrough. Do not let cartridge/disk run dry during loading (it would result in bad absorption and unrepeatable results).
5. Dry cartridge with a gentle flow of nitrogen or rinse with 2 mℓ isopropanol.
6. Rinse with appropriate solvent mix to remove unwanted residues.

7. Elute with appropriate solvent (normally a polar organic solvent/solvent mix).
8. Collect eluent in flat round bottomed flask.
9. Remove solvent under vacuum using a Rotavap until almost dry. Do not exceed temperature above 35°C (Volatile compounds such as DDT will be lost).
10. Remove rest of solvent under gentle stream of nitrogen.
11. Reconstitute residue in appropriate solvent for instrumental analysis (normally Hexane for GC analysis and Methanol for LC analysis).

TABLE 4: RECOMMENDED SPE CARTRIDGES FOR SOLID PHASE EXTRACTION

Compound class	Recommended cartridge	Supplier
Insecticides ***Organochorines**** DDT, DDE, DDD Dieldrin, Aldrin, Endrin α-Endosulfan, β-Endosulfan, Endosulfan-sulphate Heptachlor, Heptachlor epoxide Lindane (γ-BHC) ***Organophosphates**** Azinfos-methyl Chlorpirifos Parathion ***Pyrethroids**** Cypermethrin Deltamethrin	Oasis® HLB Glass Cartridge 5cc/200 mg Part No 186000683 Or Chromabond C_{18} ec Cartridge Cat no 730 034 Or C_{18} cartridge (Supelco) Or Bakerbond Speedisk™ Octadecyl-bonded silica (C_{18}XF) 50 mm	Waters Corp., Milford, MA, USA Macherey-Nagel Supelco, Bellafonte, PA, USA J.T. Baker, Deventer, The Netherlands
Carbamates* Aldicarb Carbaryl Carbofuran Methiocarb Methomyl Propoxur	Oasis® HLB Glass Cartridge 6cc/200 mg Part No WAT 06202	Waters Corp., Milford, MA, USA
Herbicides ***Acidic Herbicides*** 2,4 D 2,4,5 T Dinoseb Pichloram ***Triazines*** Atrazine Propazine Simazine Terbutylazine	Oasis® MAX Cartridge 6cc/150 mg Part No 186000369 Bakerbond SPE cartridges 200 mg SDB	Waters Corp., Milford, MA, USA J.T. Baker, Deventer, The Netherlands
Veterinary drugs ***Synthetic hormones*** Diethylstilbesterol (DES) Ethynylestradiol 17β-Estradiol ***Androgenic steroids*** Trenbolone Nortestosterone Methyltestosterone ***Estrogenic action*** Zeranol	Oasis® HLB Glass Cartridge 6cc/200 mg Part No WAT 06202	Waters Corp., Milford, MA, USA

Compound class	Recommended cartridge	Supplier
Natural hormones 17α-Estradiol Testosterone Progesterone ***Beta agonists*** Clenbutarol Salbutanol Mabutanol Zilpaterol		
Hormones* 17β-Estradiol Estriol Estrone 17α-Ethinylestradiol	Oasis® HLB Glass Cartridge 6cc/200 mg Part No WAT 06202 Or Bakerbond Speedisk™ Octadecyl-bonded silica (C $_{18}$XF) 50 mm Silica Cartridges 500 mg / 10mℓ	Waters Corp., Milford, MA, USA J.T. Baker, Deventer, The Netherlands Sopachem nv The Netherlands
Industrial compounds ***Phthalates*** Dimethyl phthalate Diethyl phthalate (DEP) Dibutyl phthalate (DBP) Benzylbutyl phthalate (BBP) Bis(ethylhexyl) phthalate (BEHP) Dioctyl phthalate	Oasis® HLB Glass Cartridge 5cc/200 mg Part no 186000683	Waters Corp., Milford, MA, USA
Phenols *p*-Nonyl phenol Octyl phenol Bisphenol A	Oasis® HLB Glass Cartridge 5cc/200 mg Part No 186000683	Waters Corp., Milford, MA, USA
	Or Chromabond C_{18} ec Cartridge Cat no 730 034	Macherey-Nagel
PCBs	Oasis® HLB Glass Cartridge 5cc/200 mg Part No 186000683	Waters Corp., Milford, MA, USA
	Or Chromabond C_{18} ec Cartridge Cat no 730 034	Macherey-Nagel
	Or C_{18} cartridge (Supelco)	Supelco, Bellafonte, PA, USA
	Or Bakerbond Speedisk™ Octadecyl-bonded silica (C $_{18}$XF) 50 mm	J.T. Baker, Deventer, The Netherlands Supelco

Some chemical classes require additional clean-up and derivatization before instrumental analysis can be conducted. These chemical classes include carbamates and hormones and will be dealt with under the section 7 Multi Residue Methods.

5.1.3 Activated carbon extraction from water

Suitable for extraction of organochlorine pesticides especially the DDT family from environmental and drinking water

Apparatus

Measuring cylinders 500 mℓ and 1 ℓ
Solid phase extraction unit / Visiprep or similar device
Vacuum pump
Muffle furnace
Desiccators
Glass columns 10 mℓ
Glass distilled water
Test tubes 10 mℓ, with glass stoppers
Evaporation equipment
Fibreglass filters
Acid washed fibre glass
Pipette 1 mℓ and 2 mℓ
Flat round bottomed flask 100 mℓ
Filter paper Whatman no1

Chemicals

Activated charcoal (50-100 mesh) AR
Silica gel (70-230 mesh) AR
Anhydrous sodium sulphate AR

Solvents

n-Hexane
Dichloromethane
Acetone
Methanol
Isopropanol

Standard solutions (Stock solutions)

Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in appropriate solvent (for GC analysis hexane or acetone is recommended). Store at 4°C in the dark when not in use.

Working solutions

Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (for GC analysis hexane is recommended).

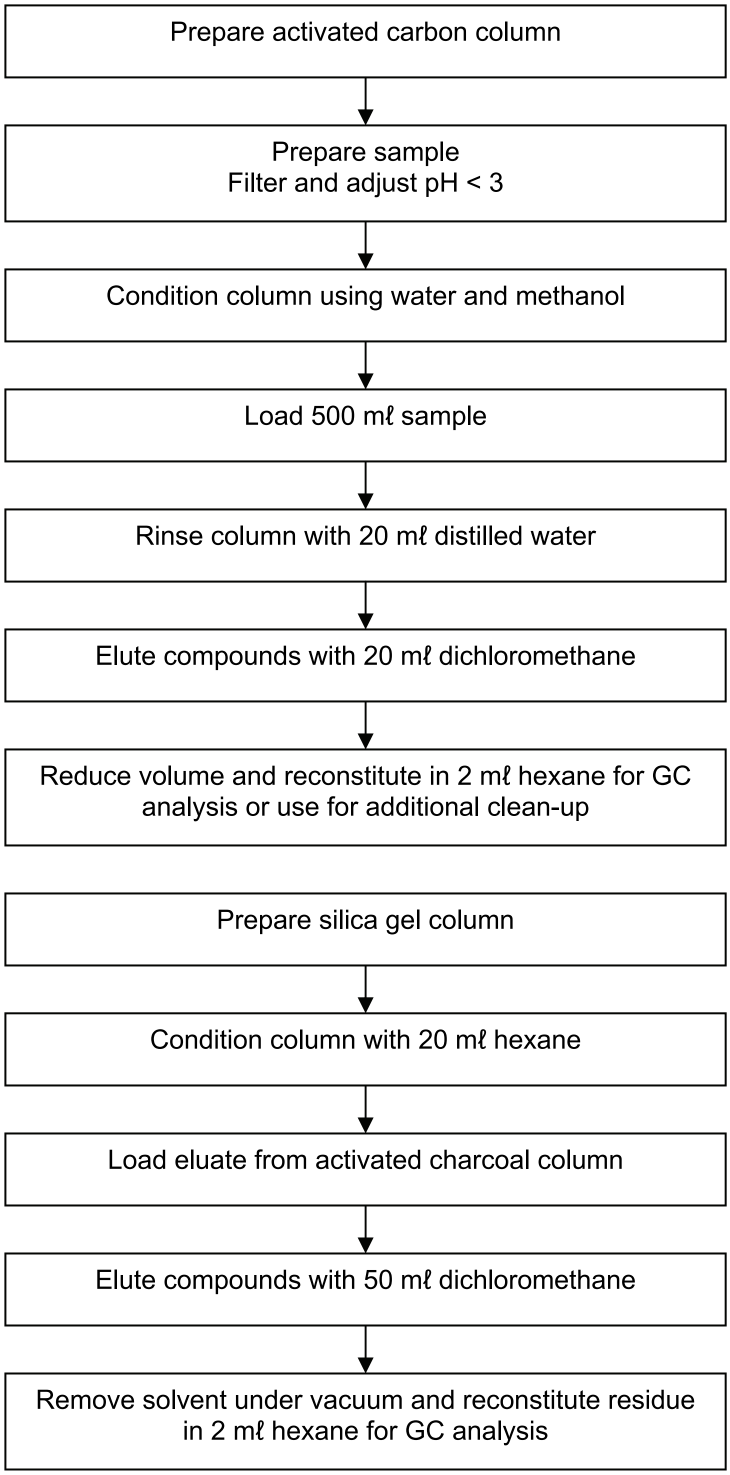
Procedure

1. Pre-heat activated charcoal and anhydrous sodium sulphate in a muffle furnace between 350-400°C for 4 hours. Place in desiccator to cool.
2. Prepare sample. Adjust water sample pH < 3 and filter through glass fibre filter if necessary to remove solid particles.
3. Pack activated charcoal columns as follows:
   Place small plug of acid washed glass fibre in bottom of column.
   Add 10-15 g activated charcoal. Tap lightly in order to settle the charcoal.
   Put another plug of fibre glass (or cotton wool) on top of charcoal.
4. Activate the charcoal column by passing 5 mℓ methanol followed by 10 mℓ double distilled water through the column at flow rate of 10 mℓ/min.
5. Load column with 500 mℓ sample. Do not exceed flow rate over 10 mℓ/min. Apply vacuum if necessary.
6. Rinse column with 10 mℓ double distilled water followed by 2 mℓ isopropanol.

7. Elute compounds from column with 20 mℓ dichloromethane and pass through filter paper containing a few grammes of anhydrous sodium sulphate.
8. Reduce volume of eluate to almost dry using an evaporation apparatus (Rotavap). Temperature of water bath should not exceed 35°C. Remove last bit of solvent under a gentle flow of nitrogen.
9. Reconstitute residue in 2 mℓ *n*-Hexane for GC analysis or in 10 mℓ *n*-hexane when additional clean-up is needed.

Additional clean-up

10. Dry silica gel at 300°C for 4 hours. Leave to cool in desiccator.
11. Weigh out 10 g silica gel and make slurry of silica gel in hexane (2% v/m).
12. Pack slurry in glass column using hexane. Do not let column run dry.
13. Add ca. 0.5-1 cm^3 anhydrous sodium sulphate on top of silica gel.
14. Wash column with 20 mℓ hexane.
15. Load extracted solutions to column and let it run through without applying vacuum.
16. Elute compounds with 50 mℓ dichloromethane.
17. Remove solvent under vacuum using a Rotavap until almost dry. Keep water bath temperature below 35°C.
18. Remove last bit of solvent under a gentle flow of nitrogen.
19. Reconstitute residue in 2 mℓ hexane and use for GC analysis.



SCHEMATIC REPRESENTATION OF ACTIVATED CARBON EXTRACTION AND CLEAN-UP FOR ORGANOCHLORINE PESTICIDES IN WATER

5.2 Biological tissue

5.2.1 Liquid partitioning used for extraction and clean-up from biological tissue

Suitable for extraction and clean-up of pesticides, hormones, PCBs and veterinary drugs in biological tissue (liver, kidneys, muscle and whole aquatic animals such as fish).

Apparatus
Laboratory balance
Ultra Turrax macerator
Witts filtration apparatus
Rotavap evaporation apparatus
Vacuum pump
Thick walled glass thimble 100 mℓ
Separating funnels 250 mℓ
Glass funnels
Filter paper Whatman no1

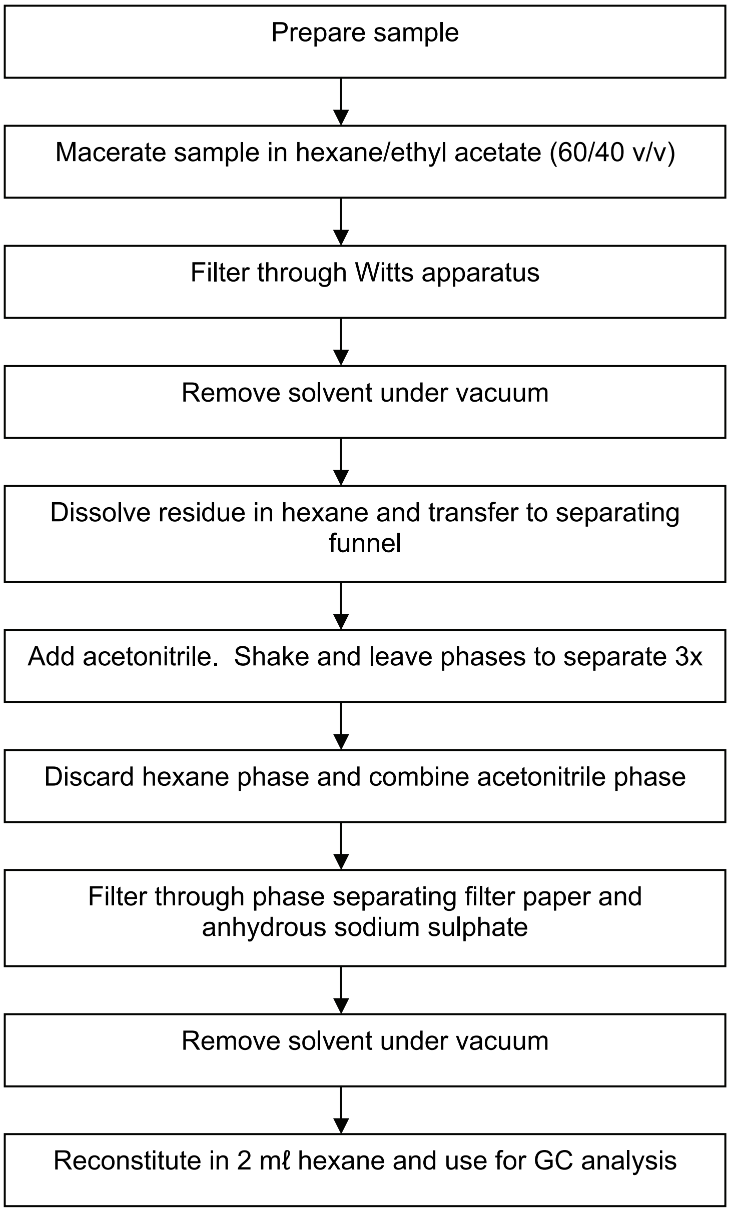
Solvents
Hexane
Ethyl acetate
Acetonitrile

Chemicals
Anhydrous sodium sulphate

Standard solutions (Stock solutions)
Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in appropriate solvent (for GC analysis hexane or acetone is recommended 0. Store at 4°C in the dark when not in use.

Working solutions
Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (for GC analysis hexane is recommended).

Procedure
1. Accurately weigh out 0.5-2 g of prepared sample in a thick walled glass thimble (see section 4.2).
2. Add 20 mℓ hexane/ethyl acetate (60:40; v:v).
3. Macerate with an UltraTurrax apparatus until a homogeneous consistency is achieved.
4. Filter through Whatman no1 filter paper using a Witts apparatus and vacuum.
5. Wash filter paper with additional 20 mℓ of hexane/ethyl acetate mixture.
6. Transfer filtrate into a 250 mℓ separating funnel. Rinse round bottomed flask (from Witts apparatus) with additional 10 mℓ hexane/ethyl acetate mixture.
7. Remove solvent under vacuum in a Rotavap apparatus until almost dry. Take care that water bath temperature does not exceed 35°C. Remove last bit of solvent under a gentle stream of nitrogen.
8. Dissolve residue in 10 mℓ hexane and wash over into a 250 mℓ separating funnel with 20 mℓ hexane. *(Alternatively this solution can now be used for SPE clean-up see section 5.1.2).*
9. Add 20 mℓ hexane saturated acetonitrile to separating funnel. Shake well and leave for phases to separate.
10. Discard the hexane phase. Repeat twice more. Combine acetonitrile phases and filter through a Whatman PS filter paper on which a small amount of anhydrous sodium sulphate was placed (to remove any remaining traces of water). Repeat this process two more times.
11. Remove solvent under vacuum in a Rotavap apparatus until almost dry. Take care that water bath temperature does not exceed 35°C. Remove last bit of solvent under a gentle stream of nitrogen.
12. Reconstitute residue in 2 mℓ hexane for GC analysis.



SCHEMATIC REPRESENTATION OF LIQUID PARTITIONING EXTRACTION AND CLEAN-UP FOR BIOLOGICAL TISSUE SAMPLES

5.2.2 Soxhlet extraction for biological tissue

Suitable for extraction of pesticides, hormones, PCBs and Alkyl phenols from biological tissue such as fat, muscle, liver, kidney and reproductive organs.

Apparatus

Soxhlet extraction unit
Extraction thimbles 50 mℓ
Rotavap or other evaporation apparatus
Round bottomed flasks 250 mℓ
Mortar and pestle
Pipette 2 mℓ and 10 mℓ
Whatman SP filter paper

Solvents

Acetonitrile
Hexane

Chemicals

Anhydrous sodium sulphate

Standard solutions (Stock solutions)

Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in appropriate solvent (for GC analysis hexane or acetone is recommended). Store at 4°C in the dark when not in use.

Working solutions

Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (for GC analysis hexane is recommended).

Procedure

1. Prepare sample (see section 4.4).
2. Accurately weigh out 1-2 g sample in a porcelain mortar.
3. Add enough anhydrous sodium sulphate and mix with pestle until a fine powder is obtained.
4. Transfer quantitatively to extraction thimble.
5. Place in Soxhlet apparatus and percolate with 100 mℓ acetonitrile over night. Leave to cool and remove water cooler section.
6. Filter solvent through Whatman SP filter paper to remove any remaining water.
7. Remove solvent under vacuum in a Rotavap apparatus. Do not let water bath temperature rise above 35°C.
8. Remove last bit of solvent under a gentle stream of nitrogen.
9. Reconstitute residue in 2 mℓ hexane and use for instrumental analysis or in 10 mℓ should additional clean-up be necessary. (*Follow instructions for SPE extraction described in section 5.1.2).*

5.3 Soil and sediment

5.3.1 Soxhlet extraction from soil and sediment

Suitable for extraction of pesticides, hormones, PCBs and Alkyl phenols from soil and sediment

Apparatus

Soxhlet extraction unit
Extraction thimbles 50 mℓ
Rotavap or other evaporation apparatus
Round bottomed flasks 250 mℓ
Pipette 2 mℓ and 10 mℓ
Whatman SP filter paper

Solvents

Petroleum ether
Hexane

Chemicals

Anhydrous sodium sulphate

Standard solutions (Stock solutions)

Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in appropriate solvent (for GC analysis hexane or acetone is recommended and methanol for HPLC). Store at 4°C in the dark when not in use.

Working solutions

Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (for GC analysis hexane is recommended and for HPLC analysis methanol).

Procedure

1. Prepare sample (see section 4.3).
2. Accurately weigh out 5 g sample.
3. Transfer quantitatively to extraction thimble.
4. Place in Soxhlet apparatus and reflux with 100 mℓ hexane/petroleum ether (50:50 v:v) over night. Leave to cool and remove water cooler section.
5. Filter solvent through Whatman SP filter paper to remove any remaining water.
6. Remove solvent under vacuum in a Rotavap apparatus. Do not let water bath temperature rise above 35°C.
7. Remove last bit of solvent under a gentle stream of nitrogen.
8. Reconstitute residue in 2 mℓ hexane or 2 mℓ methanol and use for instrumental analysis or in 10 mℓ should additional clean-up be necessary. (*Should additional clean-up be necessary follow instructions for SPE extraction described in section 5.1.2).*

5.3.2 Accelerated solvent extraction

Suitable for extraction of pesticides, hormones, PCBs and Alkyl phenols from soil and sediment

Apparatus

Accelerated Solvent Extraction unit ASE 200 (Dionex) equipped with 11 mℓ stainless steel extraction cells.
Cellulose filter discs
Rotavap or other evaporation apparatus
Round bottomed flasks 100 mℓ
Pipette 2 mℓ

Solvents

Acetone
Methanol

Standard solutions (Stock solutions)

Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in appropriate solvent (for GC analysis hexane or acetone is recommended and methanol for HPLC). Store at 4°C in the dark when not in use.

Working solutions

Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (for GC analysis hexane is recommended and for HPLC analysis methanol).

Procedure

1. Prepare sample (see section 4.3).
2. Accurately weigh out 5 g sample.
3. Transfer quantitatively to extraction cells with cellulose filter discs.
4. Follow instructions of manufacturer using following parameters:
   Use acetone/methanol (1:1) for two cycles.
   Oven temperature: 100°C.
   Pressure: 2000 psi.
   Oven heat up time: 5 min.
   Static time: 5 min.
   Purge time: 60 sec.
   Flush volume: 60% of extraction cell volume.
5. Remove solvent under a gentle stream of nitrogen.
6. Reconstitute residue in 2 mℓ hexane or 2 mℓ methanol and use for instrumental analysis or in 10 mℓ should additional clean-up be necessary (*should additional clean-up be necessary follow instructions for SPE extraction described in section 5.1.2).*

5.4 Serum and plasma

If a laboratory undertakes the analysis of blood or blood product samples, a certified medical analyst must be employed.

Suitable for extraction and clean-up of pesticides, PCBs, hormones, veterinary drugs and alkylphenols.

Apparatus

Centrifuge
Pipettes 5 mℓ and 1 mℓ

Solvents

Ethanol
Methanol
Hexane

Standard solutions (Stock solutions)

Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in appropriate solvent (for GC analysis hexane or acetone is recommended and methanol for HPLC). Store at 4°C in the dark when not in use.

Working solutions

Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (for GC analysis hexane is recommended and for HPLC analysis methanol).

Procedure

1. Serum and plasma are treated the same.
2. Determine the lipid concentration of the serum/plasma.
3. Add enough Ethanol or any other organic solvent to the sample to precipitate the protein.

4. Centrifuge at high speed and use an aliquot of the top layer.
5. Proceed with solid phase extraction as described in section 5.1.2

Note: For hormone analysis and other analysis that require derivatization see section 7 Multi Residue Methods.

6 INSTRUMENTAL DETECTION

6.1 Gas chromatography

Suitable for any compound or class of compounds that are sufficiently volatile and stable at high temperature.

Apparatus

For use of determining the compounds in the priority list of EDC compounds the following is recommended.

Any instrument equipped with a facility for a capillary column and a split/split less injector. Select the appropriate detector from Table 5.

TABLE 5: RECOMMENDED DEECTOR FOR SPECIFIC CLASSES OF COMPOUNDS

Compound type	Recommended detector
Compounds with high carbon content such as phthalates hydrocarbons poly aromatic hydrocarbons	Flame Ionisation Detector (FID)
Compounds containing halogen or oxygen atoms such as organochlorine pesticides (OCs), PCBs	Electron capture Detector (ECD)
Compounds containing phosphorous and/or nitrogen atoms such as organophosphorous pesticides (OPs)	Nitrogen phosphorous Detector (NPD)
Compounds containing sulphur atoms such as certain pesticides	Flame Photometric Detector (FPD)

Columns

Columns should be selected to suit the specific application and properties of the compounds to be analysed (see Table 6).

TABLE 6: PROPERTIES AND RECOMMENDED APPLICATIONS OF GC COLUMNS

Column Type	Properties	Application
Non polar OV1, DB5,*	Separates by boiling point Very stable	Hydrocarbons, pesticides** (OPs), PCBs, hormones, phthalates
Medium polar OV 1701, DB17**	Separates on boiling point and polarity Medium stability to high temperature	Organochlorine pesticides, hormones, veterinary drugs
Polar DB210	Separates on polarity	Very polar or very large molecules

** Most commonly used column. Not recommended for OC pesticides because it does not separate two critical pairs.

* Best column for GC-MS application

A mass spectrometer (MS) can also be utilised as a detector and is the recommended detector for confirmation of the identity of a specific compound.

TABLE 7: COLUMN PARAMETERS

Column type	Column length	Inner diameter	Film thickness
Capillary	25-30 m	0.2-0.25 mm	0.25-0.5 micron
Macrobore	15-25 m	0.5 mm	0.5 micron

Temperature settings

Each method requires its own set of temperature settings. Temperature programming is often necessary for both inlet and column to obtain optimum separation. The Table below reflects common parameters.

TABLE 8: TYPICAL TEMPERATURE SETTINGS

Injector	Column	Detector
200-250°C	180-200°C	250-300°C

Gas flow

Helium or high purity nitrogen is recommended for separation. Gas flow rate is very important. For capillary columns a flow rate of 3 mℓ/min is optimum with make-up gas flow in the detector at 30 mℓ/min. Nitrogen is usually used as make-up gas. High purity hydrogen and oxygen (medical air) are needed for FID and FPD detectors (follow manufacturer's recommendations).

6.2 High Performance Liquid Chromatography (HPLC)

Suitable for large and/or very polar compounds or classes of compounds or compounds not easily separated and detected by GC.

HPLC is a very versatile technique and combinations of columns (stationary phases), detectors, temperature variations and mobile phases provide the analyst to separate and detect virtually any organic compound.

Apparatus

Any apparatus equipped with a single or binary pump, a manual or automatic inlet system.

Detectors

Ultra violet detector (UV)
Florescence detector (FLD)
Diode array detector (DAD)
Mass spectrometer (MS)

Columns

Normal phase (Silica gel)
Reverse phase (C_8 or C_{18} columns)
Special columns developed for a specific purpose (Oasis HLB, C_{18}XAD)

Mobile Phase

Each application has its own mobile phase (see sections 7 and 8).

7 MULTI RESIDUE METHODS

7.1 Organochlorine-, Organo phosphate pesticides and Pyrethroids

Organochlorines: DDT, DDE, DDD, Heptachlor, Heptachlor epoxide, Dieldrin, Aldrin, Endrin. Endosulfan, BHC, Lindane

Organophosphates: Parathion, Malathion, Azinphos-methyl, Chlorpirifos

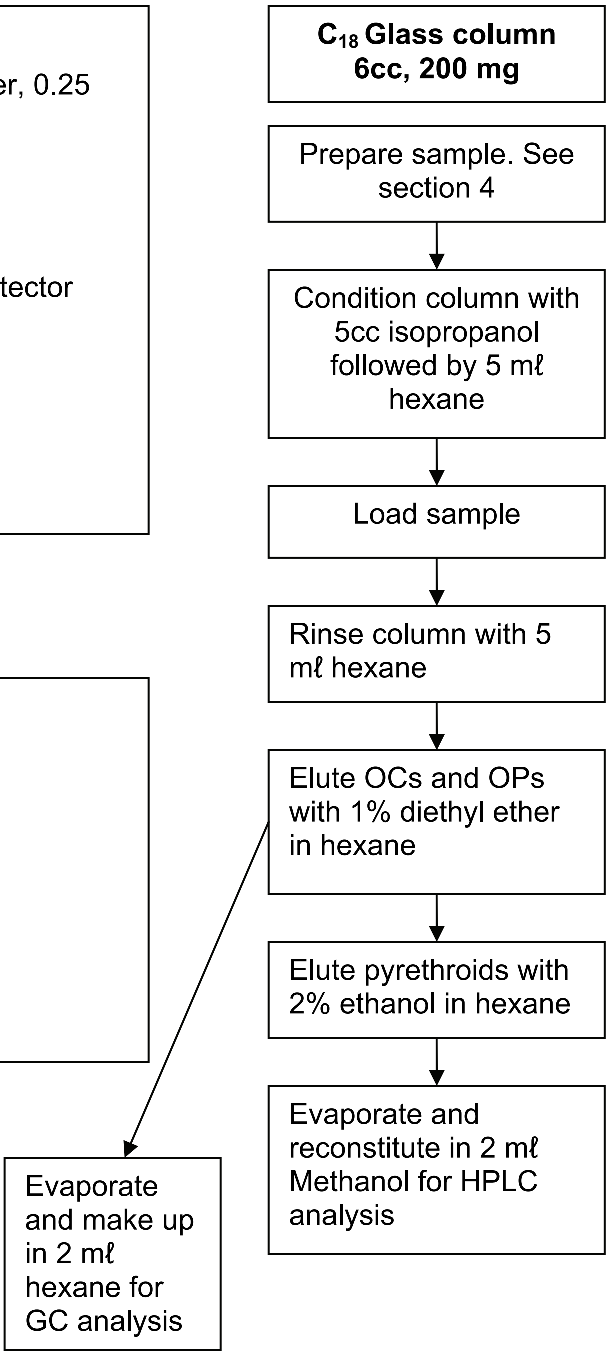
Pyrethroids: Deltamethrrin, Cypermethrin

GC conditions

Column:	DB 1701 30 m, 0.2 mm diameter, 0.25 micron film thickness
Gas flow:	3 mℓ/min Helium 30 mℓ/min make up nitrogen
Temperature:	Inlet 250°C, Column 180°C, Detector 280°C
Detector:	ECD for OCs NPD for OPs
Injection volume:	1 µℓ

HPLC conditions

Column:	C_{18} 150 x 2 mm, 4 micron
Mobile Phase:	Methanol : water
Flow rate:	2 mℓ/min
Detector:	UV
Wave length:	254, 360
Injection volume:	20 µℓ

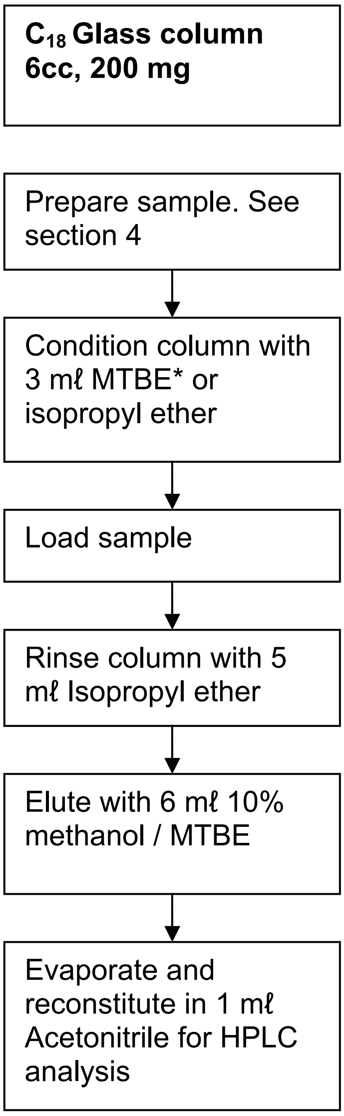


7.2 Carbamate pesticides by HPLC

Compounds: Aldicarb sulfoxide, Aldicarb sulfone, Oxamyl, Methomyl, Aldicarb, Propexur, Carbofuran, Carbaryl, Methiocarb

HPLC conditions

Column:	Waters carbamate analysis 150 x 3.9 mm, 4 micron
Mobile Phase A:	Water
Mobile Phase B:	Methanol
Mobile Phase C:	Acetonitrile
Flow rate:	1.5 mℓ/min
Detector:	Fluorescence after post column derivitisation
Injection volume:	25 μℓ



C_{18} Glass column 6cc, 200 mg

Prepare sample. See section 4

Condition column with 3 mℓ MTBE* or isopropyl ether

Load sample

Rinse column with 5 mℓ Isopropyl ether

Elute with 6 mℓ 10% methanol / MTBE

Evaporate and reconstitute in 1 mℓ Acetonitrile for HPLC analysis

7.3 Triazine herbicides

7.3.1 In drinking water by HPLC: (Waters Method)

Compounds: Atrazine, Simazine, Terbutylazine, Propazine

HPLC conditions

Column:SymmetryShield ™ RP$_8$ 150 x 3.9 mm, 5 micron

Mobile Phase A: 5 mM Phosphate buffer pH 6.7 / Acetonitrile 85:15

Mobile Phase B: Acetonitrile

Gradient: Time Profile

Min	%A	%B
0	100	0
2	100	0
25	30	70

Flow rate: 1 mℓ/min

Detector: UV 214 nm (0.02 AUFS)

Injection volume: 75 μℓ

Oasis HLB glass 3cc 60 mg

Prepare sample. See section 4

↓

Condition column with 3 mℓ Methanol 3 mℓ water

↓

Load 75 mℓ sample @ 4 mℓ/min

↓

Rinse column with 1 mℓ water

↓

Elute with 2 mℓ methanol

↓

Evaporate and reconstitute in 2 mℓ 20% Methanol / water

7.3.2 *Triazine Herbicides in soil, sediment and biological tissue: by HPLC and GC-EI-MS-MS*
(Belgian Method)

Compounds: Atrazine, Simazine, Propazine, Terbutylazine

HPLC conditions

Column: SymmetryShield ™ RP$_8$ 150 x 3.9 mm, 5 micron

Mobile Phase A:	5 mM Phosphate buffer pH 6.7 / Acetonitrile 85:15
Mobile Phase B:	Acetonitrile

Gradient:

Time	Profile	
Min	%A	%B
0	100	0
2	100	0
25	30	70

Flow rate:	1 mℓ/min
Detector:	UV 214 nm (0.02 AUFS)
Injection volume:	75 µℓ

GC-EI-MS-MS

Instrument:	Trace GC coupled to PolarisQ quatrupole ion trap MS
Column:	BPX-35 capillary fused silica 25 m x 0.22 mm ID, 0.25 µm film thickness
Temperature settings:	
Column:	150°C (2 min), ramped at 6°C/min to 280°C for 5 min
Injector:	250°C
Ion source:	200°C
Transfer line:	285°C
Injector:	Split-splitless: (split flow 20 mℓ/min, splitless time 1 mℓ/min)
Injection volume:	1 µℓ
Carrier gas:	Helium
Flow rate:	1 mℓ/min
Spectra:	EI mode, 70 eV

Filament emission current 250 µA

Bakerbond SPE 200 mg SDB*

Prepare sample. See section 4. Adjust pH 4-

↓

Condition column with 5 mℓ Ethyl acetate. Leave to dry

↓

Rinse with Methanol and ultra pure water pH 4

↓

Load 1 ℓ water or extract from ASE in case of soil, sediment or biological tissue

↓

Rinse with 2 mℓ isopropanol

↓

Elute with 6 mℓ Ethyl acetate

↓

Evaporate and reconstitute in 100 µℓ Ethyl acetate for GC-EI- MS-MS analysis or reconstitute in 10 mℓ Methanol for HPLC analysis

7.4 Acidic Herbicides in water by HPLC (Waters method)

Compounds: 2,4D, 2,4,5T, Dinoseb, Dicamba, 4-nitrophenol

HPLC conditions

Column:	SymmetryShield ™ RP$_8$ 150 x 3.9 mm, 5 micron
Mobile Phase A:	13 mM Phosphate buffer pH 3.4
Mobile Phase B:	Acetonitrile
Gradient:	

Time	Profile	
Min	%A	%B
0	85	15
8	70	30
15	70	30
30	40	60
35	10	90

Flow rate:	1 mℓ/min
Detector:	UV 250 nm (0.015 AUFS)
Injection volume:	75 µℓ

Oasis HLB glass 3cc 60 mg

Prepare sample. See section 4. Adjust pH2 with H_3PO_4

↓

Condition column with 3 mℓ 10% Methanol / MTBE

↓

Rinse 3 mℓ methanol
Rinse 2 mℓ water

↓

Load 75 mℓ sample @ 4 mℓ/min

↓

Rinse column with 1 mℓ water

↓

Elute with 2 mℓ 10% methanol / MTBE

↓

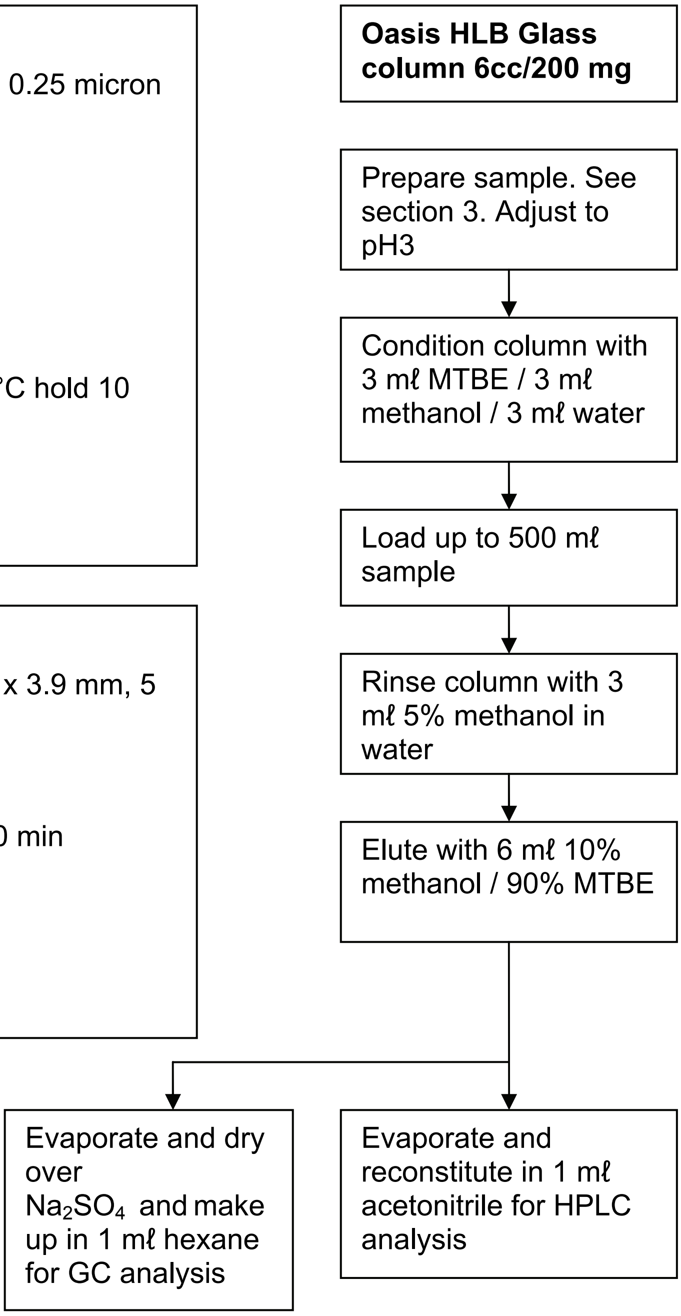
Evaporate to 0.2 mℓ and reconstitute in 0.5 mℓ with water

7.5 Plastisizers in water by GC and HPLC

Compounds: Bisphenol A, Di-n-butyl phthalate, Dicyclohexyl phthalate, Diethyl phthalate, Diethylhexyl adipate, Dihexyl phthalate, Dipentyl phthalate, Dipropyl phthalate.

GC conditions	
Column:	DB 5 30 m, 0.2 mm diameter, 0.25 micron film thickness
Gas flow:	3 mℓ/min Helium 30 mℓ/min make up nitrogen
Temperature:	Inlet 250°C
Detector:	300°C
Column:	50°C initial, 30°C /min to 120°C hold 10 min
Detector:	FID
Injection volume:	1 µℓ

HPLC conditions	
Column:	SymmetryShield ™ RP_8 150 x 3.9 mm, 5 micron
Mobile Phase:	A: Water; B: acetonitrile
Gradient:	Linear: 50% B to100% B in 10 min
Flow rate:	0.8 mℓ/min
Detector:	UV
Wave length:	196 nm (0.03AUF)



Oasis HLB Glass column 6cc/200 mg

Prepare sample. See section 3. Adjust to pH3

Condition column with 3 mℓ MTBE / 3 mℓ methanol / 3 mℓ water

Load up to 500 mℓ sample

Rinse column with 3 mℓ 5% methanol in water

Elute with 6 mℓ 10% methanol / 90% MTBE

Evaporate and dry over Na_2SO_4 and make up in 1 mℓ hexane for GC analysis

Evaporate and reconstitute in 1 mℓ acetonitrile for HPLC analysis

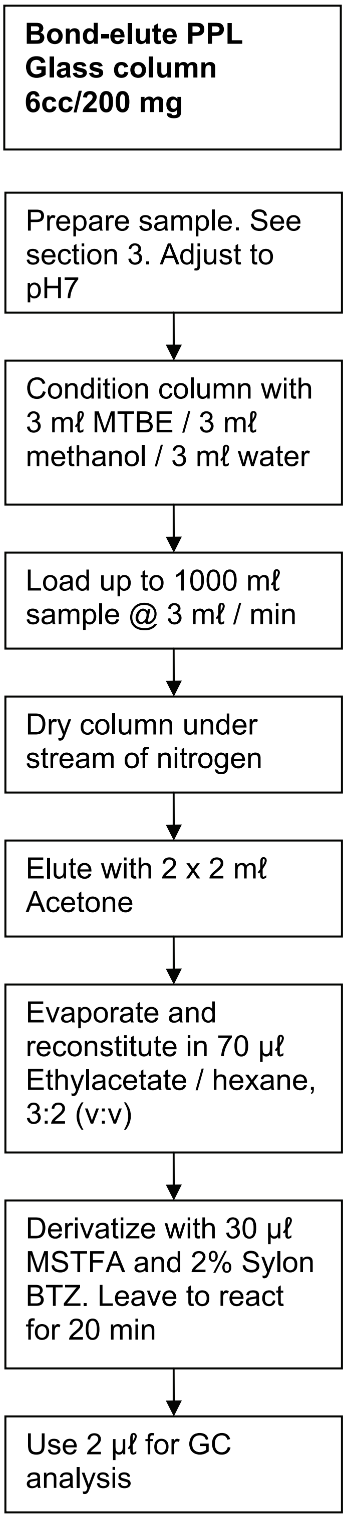
7.6 Natural and synthetic hormones in water

7.6.1 GC-MS method

(German method developed by Technolgie Zentrum Wasser "TZW")
Compounds: 17β-Estradiol, estrone, estriol, ethinylestradiol (LOD: 0.3 ng/ℓ)

GC conditions

Apparatus:	Finnigan ITS 40
Column:	DB 5 MS 30 m x 0.33 mm diameter, 0.25 micron film thickness
Gas flow:	3 mℓ/min Helium 30 mℓ/min make up nitrogen
Temperature:	Inlet 250°C,
Detector:	300°C
Column:	50°C initial, 16°C / min to 180° 5°C / min to 290°C hold 10 min
Transfer line:	220°C
Detector:	Ion trap MS
Injection volume:	2 µℓ split/split less for 1.5 min



Bond-elute PPL Glass column 6cc/200 mg

Prepare sample. See section 3. Adjust to pH7

Condition column with 3 mℓ MTBE / 3 mℓ methanol / 3 mℓ water

Load up to 1000 mℓ sample @ 3 mℓ / min

Dry column under stream of nitrogen

Elute with 2 x 2 mℓ Acetone

Evaporate and reconstitute in 70 µℓ Ethylacetate / hexane, 3:2 (v:v)

Derivatize with 30 µℓ MSTFA and 2% Sylon BTZ. Leave to react for 20 min

Use 2 µℓ for GC analysis

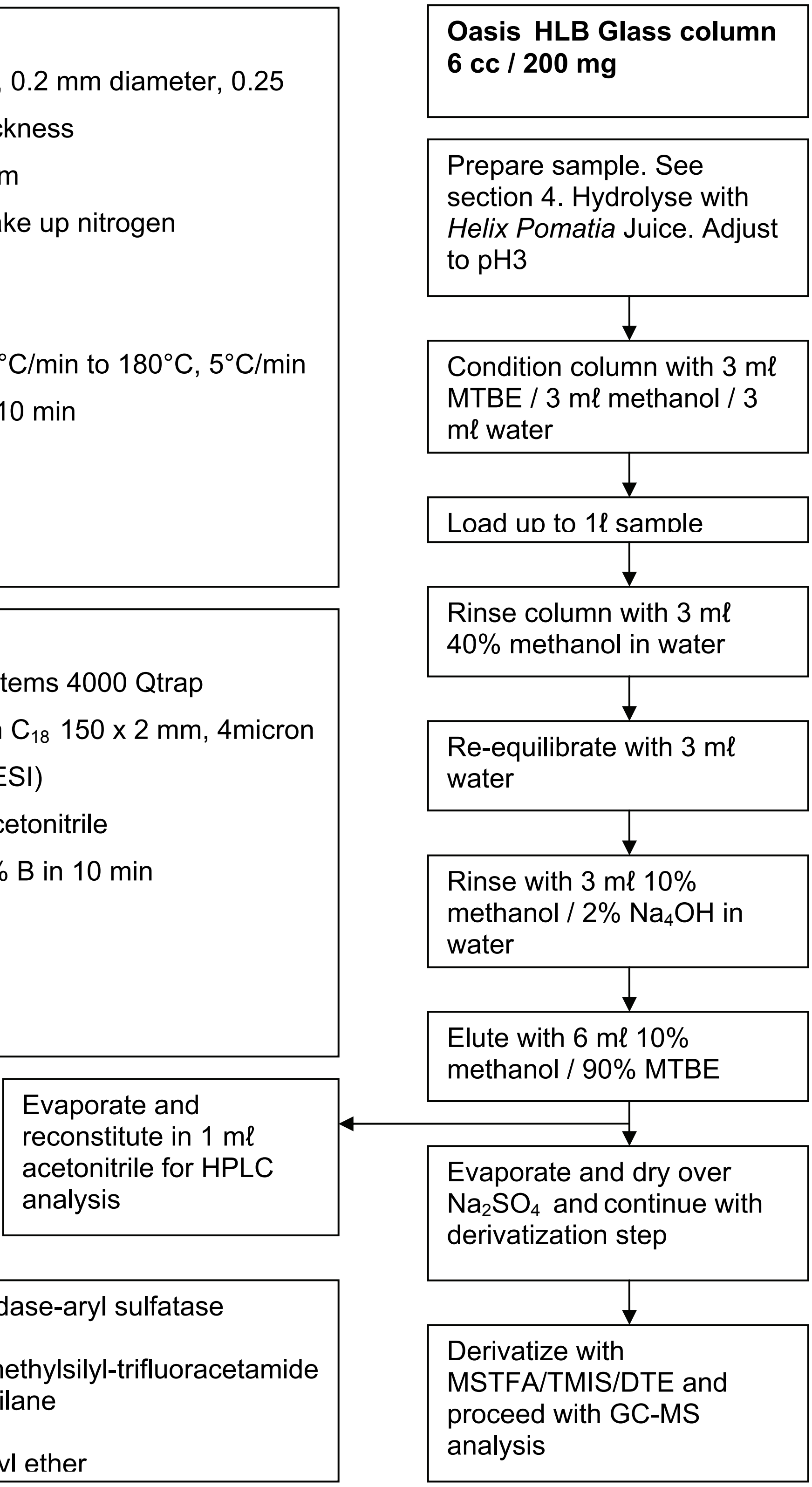
7.6.2 Natural and synthetic hormones and veterinary drugs in water by GC-MS and LC-MS-MS developed by the food and drug assurance (FDA) laboratory in RSA.

GC conditions

Column:	DB 1701 30 m, 0.2 mm diameter, 0.25 micron film thickness
Gas flow:	3 mℓ/min Helium 30 mℓ / min make up nitrogen
Temperature:	Inlet 250°C,
Detector:	300°C
Column:	50°C initial, 16°C/min to 180°C, 5°C/min to 290°C hold 10 min
Transfer line:	220°C
Detector:	GC-MS
Injection volume:	1 µℓ

HPLC conditions

Apparatus:	Applied Biosystems 4000 Qtrap
Column:	Synergi Fusion C_{18} 150 x 2 mm, 4micron
Ionisation:	Electrospray (ESI)
Mobile Phase:	A: Water; B: acetonitrile
Gradient:Linear:	50% B to 100% B in 10 min
Flow rate:	0.8 mℓ/min
Detector:	LC-MS-MS
Injection volume:	20 µℓ



Oasis HLB Glass column 6 cc / 200 mg

Prepare sample. See section 4. Hydrolyse with *Helix Pomatia* Juice. Adjust to pH3

Condition column with 3 mℓ MTBE / 3 mℓ methanol / 3 mℓ water

Load up to 1ℓ sample

Rinse column with 3 mℓ 40% methanol in water

Re-equilibrate with 3 mℓ water

Rinse with 3 mℓ 10% methanol / 2% Na_4OH in water

Elute with 6 mℓ 10% methanol / 90% MTBE

Evaporate and reconstitute in 1 mℓ acetonitrile for HPLC analysis

Evaporate and dry over Na_2SO_4 and continue with derivatization step

Derivatize with MSTFA/TMIS/DTE and proceed with GC-MS analysis

Helix Pomatia: β-glucuronidase-aryl sulfatase

MSTFA: n-Methyl-n-trimethylsilyl-trifluoracetamide
TMIS: Trimethyliodosilane
DTE: Dithiothereitol
MTBE: Methyl-tert-butyl ether

7.7 Polybrominated diphenyl ethers (PBDEs) by GC-ECD

This method was developed by Technical University of Tshwane for determination of 15 PBDE congeners in leachate, but the method should also be suitable for water analysis (Odusanya et al., 2009).

<u>GC conditions</u>

Column:	ZB 5 30 m, 0.25 mm diameter, 0.25 micron film thickness
Gas flow:	3 mℓ/min Helium 30 mℓ/min make up nitrogen
Temperature:	Inlet 250°C
Detector:	300°C
Column:	90°C 1 min, 30°C/min to 210°C, 10°C/min to 290°C hold 10 min
Detector:	ECD
Injection volume:	1 μℓ

Liquid-liquid extraction

Extract 100 mℓ leachate 3x with 20 mℓ petroleum ether (60-80°C bp).

Combine extracts and add a small amount of anhydrous sodium sulphate.

Reduce volume to 2 mℓ by bubbling nitrogen through solution.

Proceed with silica gel column clean-up.

Preparation of Silica gel column

Dry silica gel overnight at 180°C.

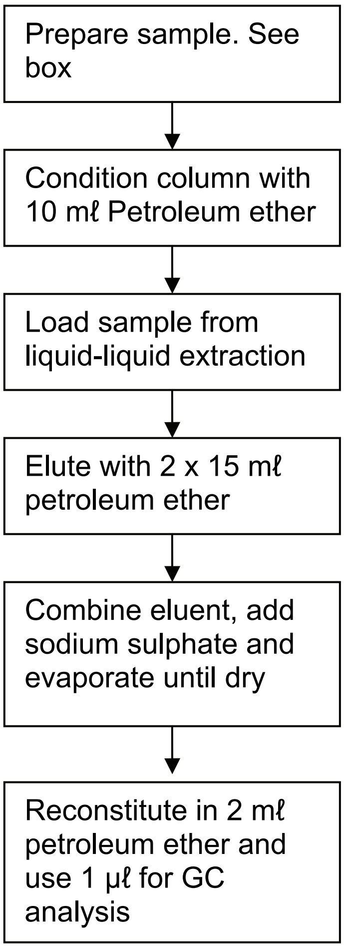
Activate by adding 10% double distilled water and stir for 15 min.

Weigh out 6 gm and make a slurry with petroleum ether (60-80°C bp).

Pack column under gravity force.

Place ca. 0.5 cm^3 sodium sulphate on top.

Silica Gel Glass column 6 gm in 30 cm x 8 mm i.d.



Prepare sample. See box

Condition column with 10 mℓ Petroleum ether

Load sample from liquid-liquid extraction

Elute with 2 x 15 mℓ petroleum ether

Combine eluent, add sodium sulphate and evaporate until dry

Reconstitute in 2 mℓ petroleum ether and use 1 μℓ for GC analysis

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8 ELISA (Enzyme Linked Imminosorbent Assay) TECHNIQUES

ELISA techniques are very specific. Normally a separate kit is needed for each compound, although it is possible to analyse for a specific group of compounds, such as the triazines with a single kit.

8.1 Natural and synthetic hormones, 17β-Estradiol, estrone and estriol in sewerage effluent

Apparatus

Measuring cylinders 500 mℓ and 1ℓ
Solid phase extraction unit / Visiprep or similar device
Vacuum pump
Solid Phase Extraction Cartridges
Glass distilled water
Test tubes 10 mℓ, with glass stoppers
Evaporation equipment
Fibreglass filters
Pipettes
Incubator
ELISA Plates (96 well)
ELISA plate reader
ELISA KIT:
    Estrone (cat no DB 52051, IBL, Germany)
    17β-Estradiol (cat no RE 52041, IBL, Germany)
    Estriol (cat no BM 52011, IBL, Germany)

Solvents

Methanol
Ethanol
n-Hexane
Isopropanol

Standard solutions (Stock solutions)

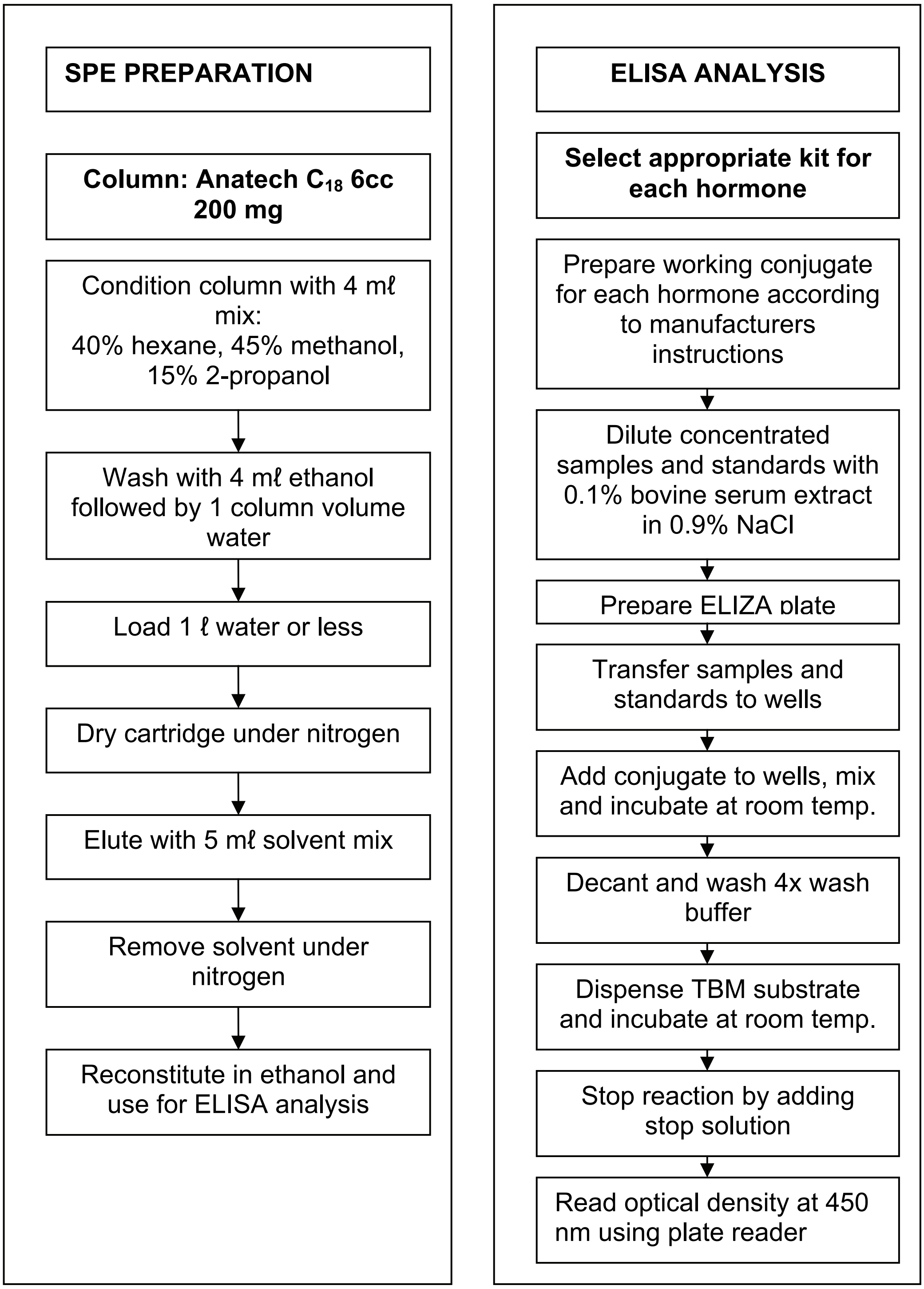
Accurate weigh out appropriate quantity of standard (usually 10x the required detection limit) in a 10 mℓ volumetric flask. Dissolve in ethanol.

Working solutions

Place Stock solutions in room for 30 min or until it reaches ambient temperature. Make appropriate dilutions to obtain a 5 point standard curve with appropriate solvent (ethanol).

Procedure

1. Use glass distilled or reverse osmosis water for blank and recovery tests.
2. Select appropriate SPE cartridge for application (Anatech C_{18}).
3. Condition cartridge with 4 mℓ solvent mix (40% hexane, 45% methanol, 15% 2-propanol).
4. Wash with 4 mℓ ethanol followed by one column volume of reverse osmosis water.
5. Load sample (do not exceed 1 ℓ). Maintain a constant flow rate of not more than 10 mℓ/min to avoid breakthrough. Do not let cartridge/disk run dry during loading (it would result in bad absorption and unrepeatable results).
6. Dry cartridge with a gentle flow of nitrogen.
7. Elute with 5 mℓ solvent mix.
8. Collect eluent in 10 mℓ test tube.
9. Remove solvent under gentle stream of nitrogen. Do not exceed temperature above 35°C (Volatile compounds such as DDT will be lost).
10. Reconstitute residue in appropriate solvent for ELISA analysis in 1/1000 of original sample volume.
11. Proceed with ELISA analysis according to instructions supplied by manufacturers of the ELISA kits.



Schematic representation of ELISA analysis of hormones: General procedure
Compounds: 17β-Estradiol LOD: 0.3 ng/ℓ), Estrone (LOD: 0.3 ng/ℓ), Estriol (3 ng/ℓ).

8.1.1 ELISA analysis for estrone

1. Prepare working conjugate solution by mixing 100 µℓ estrone-biotin, 100 µℓ avidin peroxidase conjugate and 9.8 mℓ assay buffer. Mix and incubate at room temperature for at least 20 min.
2. Add to ELIZA plate.
3. Dilute concentrated (1/1000) water extracts 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% NaCl.
4. Assay the diluted (100x concentrated) extract directly according to the instructions of the manufacturer.
5. Use ELISA kit for Estrone (DB52051, IBL, Germany) and all solvents and reagents supplied in the kit.
6. Remove the microtiter place strips precoated with rabid anti-estrone from strip holder and fix firmly in ELISA plate.
7. Transfer samples and standards (25 µℓ per well) to the wells.
8. Add working conjugate (100 µℓ per well).
9. Mix by tapping the plate.
10. Incubate the ELISA plate for 1 hour at room temperature.
11. Wash the plate 4x with wash buffer (300 µℓ per well).
12. Dispense TBM substrate (150 µℓ per well).
13. Incubate plate for 15 min at room temperature.
14. Stop the reaction by adding stop solution (50 µℓ per well).
15. Measure optical density at 450 nm using a plate reader.

8.1.2 ELISA analysis for 17β-Estradiol

1. Dilute concentrated (1/1000) water extracts 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% NaCl.
2. Assay the diluted (100x concentrated) extract directly according to the instructions of the manufacturer.
3. Use ELISA kit for 17β-Estradiol (RE52051, IBL, Germany) and all solvents and reagents supplied in the kit.
4. Remove the microtiter place strips precoated with rabid anti-estradiol from strip holder and fix firmly in ELISA plate.
5. Transfer samples and standards (25 µℓ per well) to the wells.
6. Add estradiol-horseradish conjugate to all the wells (200 µℓ per well).
7. Mix by tapping the plate.
8. Incubate the ELISA plate for 120 min at room temperature.
9. Decant solution from wells and wash the plate 3x with wash buffer (300 µℓ per well).
10. Dispense TBM substrate (150 µℓ per well).
11. Incubate plate for 15 min at room temperature.
12. Stop the reaction by adding stop solution (100 µℓ per well).
13. Measure optical density at 450 nm using a plate reader.

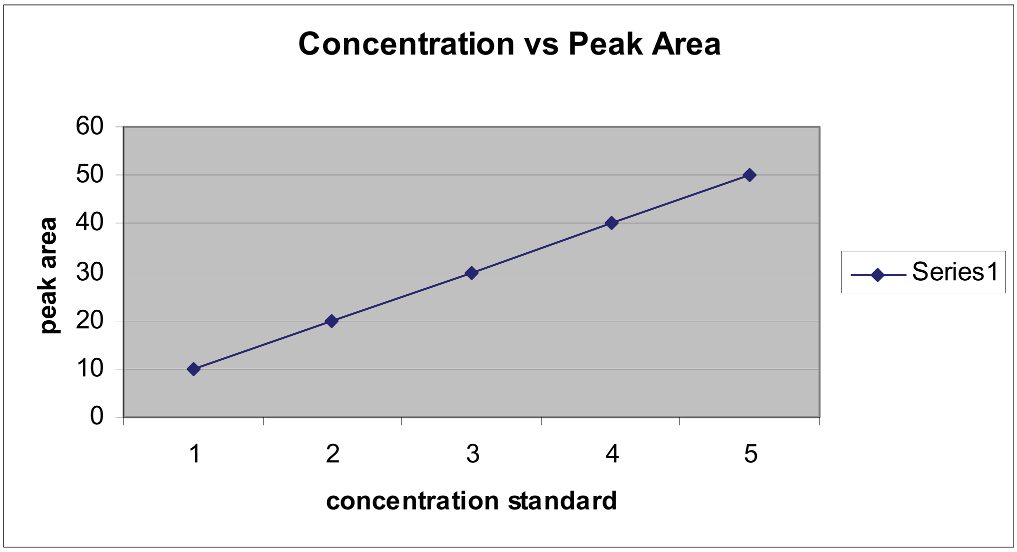
8.1.3 ELISA analysis for Estriol

1. Dilute concentrated (1/1000) water extracts 1/10 using 0.1% (w/v) bovine serum albumin in 0.9% NaCl.
2. Assay the diluted (100x concentrated) extract directly according to the instructions of the manufacturer
3. Use ELISA kit for 17β-Estradiol (BM52011, IBL, Germany) and all solvents and reagents supplied in the kit.
4. Remove the microtiter place strips precoated with rabid anti-estradiol from strip holder and fix firmly in ELISA plate.
5. Transfer samples and standards (10 µℓ per well) to the wells.
6. Add estriol-horseradish conjugate to all the wells (100 µℓ per well).
7. Mix by tapping the plate.
8. Incubate the ELISA plate for 1 hour at room temperature.
9. Decant solution from wells and wash the plate 4x with wash buffer (300 µℓ per well).
10. Dispense TBM substrate (150 µℓ per well).
11. Incubate plate for 30 min at room temperature.
12. Stop the reaction by adding stop solution (100 µℓ per well).
13. Measure optical density at 450 nm using a plate reader.

9 CALCULATION

9.1 Calculation using an external standard

1. Dilute stock standards with appropriate solvent to obtain a 5 point standard curve at levels of detection.
2. Conduct recovery test by adding concentration of authentic standards (one at detection limit and one 10x higher) to distilled water and running them concurrently with the samples.
3. Inject samples and standards alternatively into instrument.
4. Plot concentration of standards against peak area to obtain standard curve (normally a straight line).

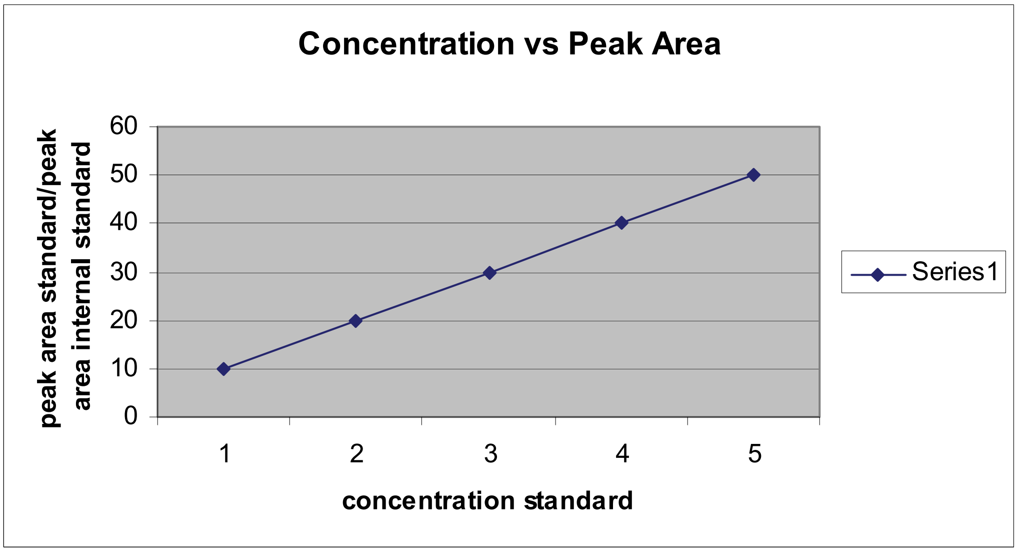


5. Use function: y = mx+c to determine concentration of compound, taking dilution into account.

FIGURE 1: STANDARD CURVE USING EXTERNAL STANDARD

9.2 Calculation by using an internal standard (Water only)

1. Dilute stock standards with appropriate solvent by transferring different volumes of standard into 10 mℓ volumetric flasks. Transfer a fixed volume of a concentrated compound similar to the compound of interest to the volumetric flask and make up to volume to obtain a 5 point standard curve at levels of detection.
2. No recovery test is necessary because the internal standard compensates for losses during extraction.
3. Inject samples and standards alternatively into instrument.
4. Plot concentration of standards against peak area of standard divided by peak area of internal standard to obtain 5 point standard curve (normally a straight line).

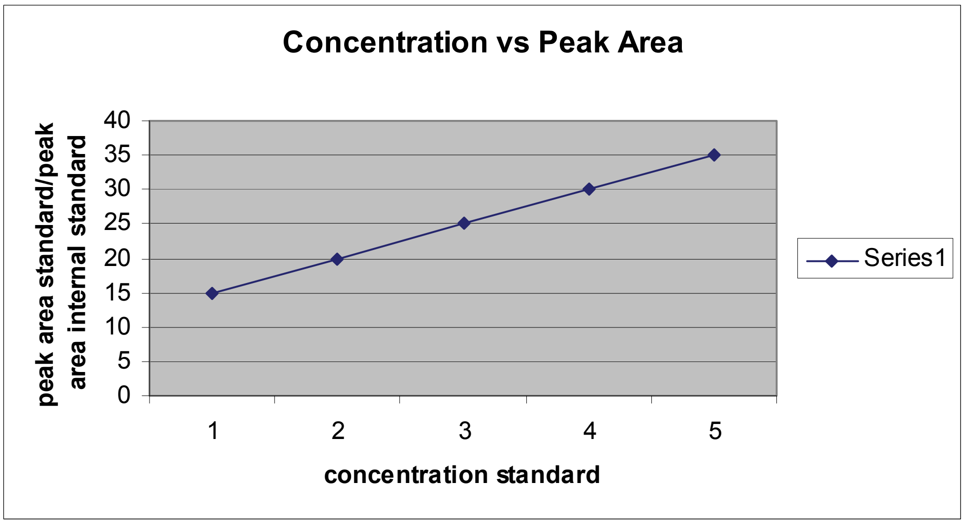


5. Use function: y = mx+c to determine concentration of compound taking dilution into account.

FIGURE 2: STANDARD CURVE USING INTERNAL STANDARD

9.3 Calculation using standard addition (Use for biological tissue)

1. Dilute stock standards with appropriate solvent by transferring different volumes of standard into 10 mℓ volumetric flasks. Transfer a fixed volume of a concentrated compound similar to the compound of interest to the volumetric flask and make up to volume to obtain a 5 point standard curve at levels of detection. Make up to volume with extract of matrix obtained by extraction and clean up (see section 5).
2. No recovery test is necessary because the internal standard will compensate for losses during extraction and matrix effect.
3. Inject samples and standards alternatively into instrument.
4. Plot concentration of standards against peak area of standard divided by peak area of internal standard to obtain 5 point standard curve (normally a straight line).



5. Use function: y = mx+c to determine concentration of compound taking dilution into account. (Line will not go through 0)

FIGURE 3: STANDARD CURVE USING STANDARD ADDITION

9.4 Calculation for ELISA procedure

1. Use standards supplied with specific kit.
2. Make dilutions for standard curve with ethanol.
3. Apply standards and samples on same plate.
4. Plot optical density against concentration.
5. Calculate concentrations using appropriate function and taking dilutions into account.

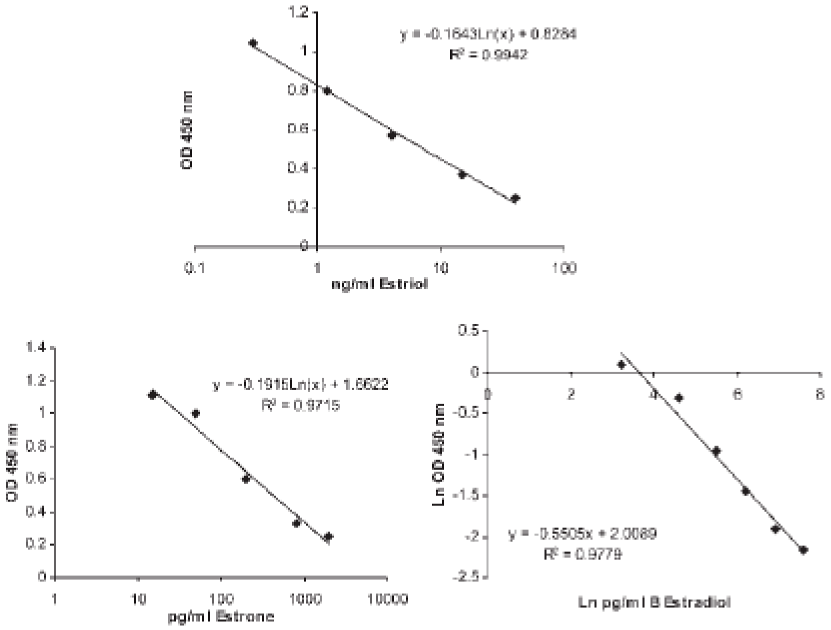


FIGURE 4: STANDARD CURVES TO CALCULATE ESTRONE, ESTRIOL AND 17B-ESTRADIOL IN SEWERAGE EFFLUENT

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