OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE 4XX:

The Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals

INTRODUCTION

1. The OECD Endocrine Disruption Testing and Assessment Task Force (EDTA) of the Test Guidelines Programme initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals (1)(2). In 2002, the EDTA established the OECD conceptual framework for testing and assessment of potential endocrine disrupting chemicals comprising five levels, each level corresponding to a different level of biological complexity (3). The Transcriptional Activation (TA) assay described in this Test Guideline is a level 2 *in vitro* assay, providing mechanistic information. The validation study of the Stably Transfected Transactivation Assay (STTA) using the hER-HeLa-9903 (HeLa) cell line to detect estrogenic agonist activity mediated through human estrogen receptor alpha (hERα) demonstrated the relevance and reliability of the assay for its intended purpose (4). This guideline was developed for using the hER-HeLa-9903 cell line to detect estrogenic activity mediated by hERα.

2. *In vitro* TA assays using the reporter gene technique are screening assays that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptor and the androgen receptor (AR) (5)(6)(7)(8), and have been proposed for the detection of estrogenic transactivation regulated by the ER (9)(10)(11). TA assays are based upon the production of a reporter gene product induced by a chemical, following the ligand-receptor binding and subsequent downstream transcriptional activation. This TA assay is therefore a tool that can evaluate the ability of a chemical to activate agonist ERα responses, and so can be utilised for ERα screening and prioritisation purposes.

3. Definitions used in this Test Guidelines are set out in Annex 1.

PURPOSE OF THE TEST

4. To provide information on hERα-mediated agonist activity of test compounds for screening and prioritization purposes.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. Estrogen agonists act as ligands for ERs, and may activate or inhibit the transcription process of estrogen reactive genes. By disrupting endocrine systems, this interaction may have the potential to trigger adverse health hazards by disrupting endocrine systems. This Test Guideline describes an assay that evaluates transcriptional activation mediated by the hERα. This process is considered to be one of the key mechanisms of possible endocrine disruption related health hazards, although there are also other important endocrine disruption mechanisms. These include the actions mediated via other nuclear receptors and enzymes, the entire metabolic activation or deactivation, distribution to target tissues, and clearance from the body. This Test Guideline exclusively addresses the agonist mechanisms mediated by the transcriptional activation of the hERα and therefore it has not been directly extrapolated to the complex *in vivo* estrogenic situation. Furthermore this guideline does not address hERα antagonist mechanisms.

6. This test method is specifically designed to detect hERα mediated transcriptional activation. However, non-receptor mediated luminescence signals have been reported at phytoestrogen concentrations higher than 1 μM due to the over activation of the luciferase reporter gene (12)(13). While the dose response curve indicates that true activation of the ER system occurs at lower concentrations, luciferase expression obtained at high concentrations of phytoestrogens needs to be examined carefully in such stably transfected TA assay systems.

PRINCIPLE OF THE TEST

7. The TA assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The assay is used to signal binding of the estrogen with a ligand. Following ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene. Luciferin is a

substrate that is transformed by the luciferase enzyme to bioluminescence and can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available kits.

8. The test system provided in this guideline is based on the HeLa cell line, which is derived from a human cervical tumor with stably inserted constructs: the hERα expression construct (full-length) and a firefly luciferase reporter construct bearing five tandem repeats of a vitellogenin Estrogen-Responsive Element (ERE) driven by a mouse metallothionein promoter TATA element. Accordingly, the transcriptional activation assay using this stably transfected HeLa cell line can provide the hERα-mediated transcriptional activation of a test chemical.

PROCEDURE

Performance Standard and laboratory proficiency

Performance Standards

9. *Positive and negative controls:* Prior to and during the study, the responsiveness of the test system should be verified using the appropriate dose(s) of a reference estrogen: 17β-estradiol (E2; CAS 50-28-2), a weak estrogen (17α-estradiol; CAS 57-91-0) and a negative compound (corticosterone; CAS 50-22-6). Acceptable historical range values are given in Table 1. These demonstrate that the appropriate concentration response curve is obtained within the range of acceptable variation, such that each experiment can be accepted. If this is not the case, the experimental design should be modified.

Table 1. Acceptable range values of concurrent control chemicals for the STTA assay.

	Concentration Test range	**Acceptable range**			
		logEC50	**logPC50**	**logPC10**	**Hill-slope**
17β-Estradiol (E2)	10^{-14} – 10^{-8} M	-10.16 - -12.86	-10.16 - -12.75	<-11	1.711 - 0.462
17α-Estradiol	10^{-12} – 10^{-6} M	-8.02 – -9.86	-7.76 – -9.99	-8.78 – -11.17	3.136 – 0.490
Corticosterone	10^{-9} – 10^{-3}M	-	-	-	-

10. With respect to the quality control of the assay, the fold induction corresponding to the PC10 value should be greater than 1+2SD of the fold induction value of the VC (vehicle control).

11. *Fold-induction:* ([average of PC] / [average of VC]) should be greater than four-fold. This criterion is established based on the reliability of the endpoint values historically and recently (between four and 30 fold). However fold induction values greater than 30 would not be problematic with respect to the generation of reliable EC50, PC 10 and PC 50 values.

12. *Proficiency chemicals:* Prior to testing chemicals in the ER TA assay, the responsiveness of the test system should be confirmed by testing at least twice, the reference chemicals given in Table 2.

Table 2. List of reference chemicals

Compound	**CAS no**	**Class**
Diethylstilbestrol (DES)	56-53-1	Strong positive
17α-Ethynyl estradiol (EE_2)	57-63-6	Strong positive
Hexestrol	84-16-2	Strong positive
Genistein	446-72-0	Strong positive
Estrone	53-16-7	Strong positive
Butyl paraben	94-26-8	Strong positive
Nonylphenol	-	Strong positive
Di butyl phthalate (DBP)	84-74-2	Positive
Atrazine	912-24-9	Negative

CELL LINES

13. The HeLa cell line should be used for the assay. The cell line can be obtained from Sumitomo Chemicals Co, and will also be available from a public source [Will be decided before the guideline is adopted].

14. Monitoring for mycoplasma infection is mandatory, and should be conducted at least every two to three months and at least four times a year, for the same batch. New cells should always be checked using PCR (polymerase chain reaction) on arrival at the test laboratory.

For laboratories with multiple cell lines in house, more frequent monitoring will be necessary to avoid cross contamination. Only cells characterised as mycoplasma-free should be used in testing.

CELL CULTURE AND PREPARATION OF ASSAY PLATE

15. Cells should be maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red, supplemented with a 10% dextran-coated-charcoal-treated fetal bovine serum (DCC-FBS), in a CO_2 incubator (5% CO_2) at 37˚C. Cells can be expanded at $0.4 \times 10^5 – 1 \times 10^5$ cells/mL. Cells should be suspended with 10% FBS-EMEM and plated into a well of microplate at a density of 1×10^4 cells/100 µL/well. Then the cells should be pre-incubated in a 5% CO_2 incubator at 37˚C for 3 hrs before the chemical exposure. The plastic-ware should be free of estrogenic activity.

16. To maintain the integrity of the response, the cells should be grown more than one passage from the frozen stock and should not be cultured over an appropriate passage level, which for the HeLa cell line would be less than three months.

17. To monitor the stability of the stably transfected cell line, the investigator should compare the results (EC50, PC50 and PC10) of E2, 17α -estradiol and corticosterone (or E2 only) that are measured at least in one run each day of the assay, with that of the historical data generated by each laboratory.

18. The recommended volume of media required for each well is 150 µL after addition of 50 µL of test chemical solution serially diluted with 500 µL of media by adding 1.5 µL of test chemical diluted with the solvent.

19. The preparation of 10% DCC-FBS is attached as Annex 2.

VEHICLE

20. Test substance should be dissolved in a solvent that solubilizes that test substance and is miscible with the cell medium. Water, ethanol (95% to 100%) and dimethylsulfoxide (DMSO) are suitable vehicles. If DMSO is used the level should not exceed 0.1% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with assay performance

PREPARATION OF CHEMICAL

21. Generally, the test chemical should be dissolved in DMSO, and serially diluted with the same solvent at a common ratio of 1:10 in order to prepare stock solutions for dilution with media.

Cytotoxicity and solubility.

22. For the purpose of this assay, cytotoxicity is defined as a reduction in the number of cells present in the well at the end of the exposure period when compared to the concurrent solvent control. For ER agonists, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data. Cytotoxicity should be evaluated using techniques that consider total cell growth (*e.g.*, by measuring levels of ATP [adenosine-tri-phosphate], by production of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], or through visual inspection of cell density and cell morphology). The HeLa control cell line, designed for inducing constitutive luciferase induction driven by the thymidine kinase (TK) promoter in the HeLa cell line, has been developed for sensitive cytotoxicity assessment. However, it should be noted that the non-specific luciferase induction is also observed in this cell line for some chemicals.

Considerations for range finding

23. A preliminary test should be carried out to determine the appropriate concentration range of chemical to be tested, and to ascertain whether the test chemical may have any cytotoxicity and solubility problems. Chemicals need to be first tested up to maximum of 1 mM (11) (but if preferred can also be tested up to the limit of solubility/cytotoxicity, although this generally does not exceed 1 mM).

24. The concentration range-setting should start with serial doses of 1 mM, 100 µM, 10 µM, 1 µM, 100 pM etc, with each concentration being carefully inspected for cloudiness, or precipitate. If no precipitate is observed, the test chemical can be assessed for cytotoxicity using an appropriate cytotoxicity test.

25. Should the results of the cytotoxicity test show that the concentration caused 20% inhibition of cell viability, this result is regarded as cytotoxic, such that concentrations at or above the cytotoxic concentration should be excluded from evaluation.

CONTROL SUBSTANCES

26. 17β-estradiol (E2), 17α-estradiol and corticosterone should be used as the control substances and a complete concentration response curve in the test concentration range provided in Table 1 should be run in every experiment. DMSO at the same level of concentration used for E2 and test chemicals should be used as a concurrent vehicle control.

CHEMICAL EXPOSURE

27. Each test chemical diluted in DMSO should be added in serial dilutions to the wells to achieve final serial concentrations as determined by the preliminary range finding test (typically in a series of 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-5}M -10^{-11})) for triplicate testing. Test samples and control substances can be assigned according to the table 3, as an example;

Table 3. Example of plate dose assignment of test chemicals in the assay plate including the control chemicals*

	Test Chemical 1			**Corticosterone**			**17α-Estradiol**			**E2**		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	conc 1 (10 µM)	→	→	1 mM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	100 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 µM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	1 nM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (DMSO); PC: Positive control (1 nM E2)

*: The control chemicals should be tested at least once in each experiment. If cells come from different sources (*e.g.*, different passage number, different lot, etc.) are used in the same experiment, the control chemicals should be tested for each source of cells.

28. The edge effects should be confirmed as appropriate and if the edge effects are suspected, plate layout should be change to avoid such effects, for example, the plate layout excluding the edge wells can be employed.

29. Positive control wells treated with 1 nM of E2 that can produce maximum induction and vehicle control wells treated with DMSO alone should be prepared on every assay plate.

30. After adding the chemicals, the assay plates should be incubated in a CO_2 incubator for 20-24 hours to induce the reporter gene products.

31. Special considerations will need to be applied to those compounds that are highly volatile, in such cases nearby control wells may generate false positives, and this should be seen in divergence from expected and historical control values.

32. It is highly recommended that the definitive screening assay is replicated at least twice on different days, rather than performing all tests during a single day.

LUCIFERASE ASSAY

33. A commercial luciferase assay reagent (*e.g.*, Steady-Glo® Luciferase Assay System (Promega, E2510, or equivalents) or a standard luciferase assay system (Promega, E1500, or equivalents) can be used for the assay, as long as the criteria for the performance standard is met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. The luciferase reagent can be applied following the manufacturer instructions.

ANALYSIS OF DATA

34. The luminescence signal data should be processed and the average for the concurrent vehicle control wells should be calculated. The value for each test well should be subtracted by the average value of the concurrent vehicle control and divided by the average value of the positive control wells to obtain individual relative transcriptional activity to positive control in each plate. Then, the average transcriptional activity should be calculated for each concentration of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs.

Decision criteria

35. Data interpretation criteria are shown in Table 4. Positive results will be noted by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% or 10% of control values are reached accomplishes both of these goals. In general, when (i) PC50 values can be calculated in a minimum of two replicate experiments, a test chemical is considered as strong agonist in hERα mediated

transcriptional activation, (In this case, dose responsiveness should be considered); (ii) at least PC10 values can be calculated in replicate experiments, a test chemical is considered as a positive agonist in hERα mediated transcriptional activation; and (iii) at least PC10 values cannot be calculated in replicate experiments, the test chemical is considered negative for ER agonist activity.

Table 4. Positive/negative decision criteria

Strong positive	If a PC50 is obtained in two of two or two of three replicate runs.
Positive	If a PC50 is not obtained but at least a PC10 is obtained in two of two or two of three replicate runs.
Negative	If the result fails to achieve a PC10 in two of two or two of three replicate runs.

36. The results should be based on two (or three) independent runs. If two runs give identical results, it is not necessary to conduct a third run. To be acceptable, the results should:

- Meet the performance standard requirements
- Confirm that the observed luciferase-activity is an ER-specific response, using ER antagonist, if EC50, PC50 or PC10 is obtained,
- Be reproducible

EC50 and maximum induction considerations

37. The full concentration response curve is required for the calculation of the EC50, but this may not always be achievable or practical due to limitations of the test concentration range (for example due to cytotoxicity or solubility problems). However, as the EC50 and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, these parameters should be reported where possible to calculate. For the calculation of EC50 and maximum induction level, appropriate statistical software should be used.

38. If Hill's logistic equation is applicable to concentration response data, the EC50 should be calculated by the following equation:

Y=Bottom + (Top-Bottom)÷(1+10^((LogEC50-X)*Hill-Slope))

Where:

X is the logarithm of concentration; and,

Y is the response and Y starts at the Bottom and goes to the Top with a sigmoid shape.

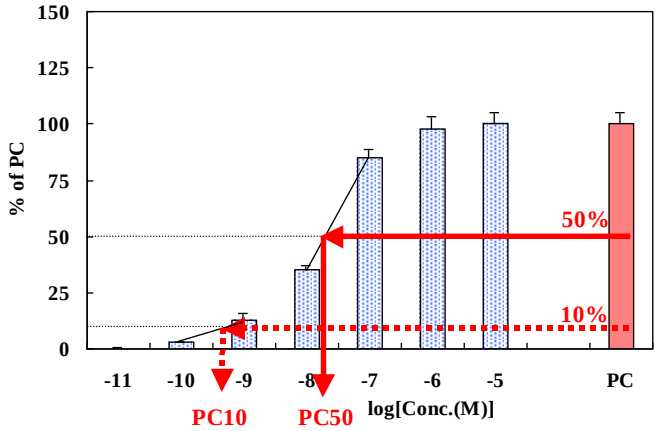
39. The PC50 and PC10 values should be calculated where possible for each test chemical. These PC values are defined as the concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the average of positive control response in each plate.

40. The PCx value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a PCx value. Where the data points lying immediately above and below the PCx value have the coordinates (a,b) and (c,d) respectively, then the PCx value may be calculated using the following equation:

log[PCx] = log[c]+(x-d)/(d-b)

41. Descriptions of PC values are provided in Figure 1, below;

Figure 1. Example on how to derive PC-values. The PC (Positive control; 1 nM of E2) is included on each assay plate.



Description of PC10 and PC50

42. The calculations of PC10 and PC50 can be made by using the attached spreadsheet (Appendix 2).

43. It should be sufficient to obtain PC10 or PC50 values at least twice. However, should the resulting base-line curve for data in the same dose range show variability with an unacceptably high CV, the data may not be considered reliable and the source to the high variability should be identified.

44. If quenching is suspected, this could be tested for by washing the plate contents prior to luciferase signal measurement, then testing for cytotoxicity or antagonism. Comparison with the original test result may indicate if the resultant signal is a consequence of quenching, or not.

TEST REPORT

45. The test report should include the following information:

Testing facility:

- Responsible personnel and their study responsibilities.

Test substance:

- identification data and CAS RN, if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance.

Solvent/Vehicle:

- characterisation (nature, supplier and lot.);
- justification for choice of solvent/vehicle;
- solubility and stability of the test substance in solvent/vehicle, if known.

Cells:

- type and source of cells;

- number of cell cultures;
- number of cell passages, if applicable;
- methods for maintenance of cell cultures, if applicable.

Test conditions:

cytotoxicity data and solubility limitations should be reported, also;

- composition of media, CO_2 concentration;
- concentration of test chemical;
- volume of vehicle and test substance added;
- incubation temperature and humidity;
- duration of treatment;
- cell density during treatment;
- positive and negative controls;
- length of expression period;
- Luciferase assay reagents (Product name, supplier and lot.);
- criteria for considering tests as positive, negative or equivocal.

Reliability check:

- Fold inductions for each assay plate.
- Actual logEC50, logPC50, logPC10 and Hillslope values for concurrent control chemicals.

Results:

- Raw and normalised data of luminescent signals;
- Concentration-response relationship, where possible;
- PC50 and/or PC10 values, as appropriate;
- EC50 values, if appropriate;
- statistical analyses, if any.

Discussion of the results

Conclusion

LITERATURE

1. OECD (1998). Report of the First Meeting of the OECD Endocrine Disrupter Testing and Assessment (EDTA) Task Force, 10th-11th March 1998, [ENV/MC/CHEM/RA(98)5].
2. OECD (2001). Summary record of the 3rd meeting of the Validation Management Group on Screening and Testing of Endocrine Disrupters for Mammalian Effects (VMG-3(MAM)) 26-27 March, 2001. [ENV/JM/TG/EDTA/M(2001)1/REV1].
3. OECD (2002). OECD conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals [Available at: http://www.oecd.org/dataoecd/17/33/23652447.doc]
4. CERI (2006). Draft validation report of TA assay using HeLa-hER-9903 to detect estrogenic activity
5. Takeyoshi, M., Yamasaki, K., Sawaki, M., Nakai, M., Noda, S. and Takatsuki, M. (2002). The efficacy of endocrine disruptor screening tests in detecting anti-estrogenic effects downstream of receptor-ligand interactions. Toxicol. Lett., 126, 91-98.
6. Jefferson, W.N., Padilla-Banks, E., Clark, G. and Newbold (2002). Assessing estrogenic activity of phytochemicals using transcriptional activation and immature mouse uterotrophic responses. J. Chromat. B., 777, 179-189.
7. Sonneveld, E., Riteco, J.A., Jansen, H.J., Pieterse, B., Brouwer, A., Schoonen, W.G. and van der Burg, B. (2006). Comparison of *in vitro* and *in vivo* screening models for androgenic and estrogenic activities. Toxicol. Sci., 89, 173-187.
8. Escande, A., Pillon, A., Servant, N., Cravedi, J.P., Larrea, F., Muhn, P., Nicolas, J.C., Cavailles, V. and Balaguer, P. (2006). Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. Biochem. Pharmacol., 71, 1459-1469.
9. Gray, L.E. Jr. (1998). Tiered screening and testing strategy for xenoestrogens and antiandrogens. Toxicol. Lett., 102-103, 677-680.
10. EDSTAC (1998) Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final report. Available at: [http://www.epa.gov/scipoly/oscpendo/pubs/edspoverview/finalrpt.htm]
11. ICCVAM (2003). ICCVAM Evaluation of *In Vitro* Test Methods for Detecting

Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. Available at: [http://iccvam.niehs.nih.gov/methods/endocrine.htm#fineval]

12. Escande, A., Pillon, A., Servant, N., Cravedi, J.P., Larrea, F., Muhn, P., Nicolas, J.C., Cavailles, V. and Balaguer, P. (2006). Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. Biochem. Pharmacol., 71, 1459-1469.
13. Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B. and Gustafsson, J.A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology, 139, 4252-4263.
14. OECD (1982). OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. Available at: [http://www.oecd.org/findDocument/0,3354,en_2649_34381_1_1_1_1_1,00.html]

ANNEX 1

DEFINITIONS

Anti-estrogenic activity is the capability of a chemical to suppress the action of 17β-estradiol mediated through estrogen receptors.

Cytotoxicity is defined as a reduction in the number of cells present in the well at the end of the exposure period when compared to the concurrent solvent control.

Dose is the concentration of test substance applied. For the Transcriptional Activation assay, "mol" unit is used as the concentration in the reaction medium.

Estrogenic activity is the capability of a chemical to act like estradiol 17β-estradiol mediated though estrogen receptors. hERα specific estrogenic activity can be detected in this Test Guideline.

Negative result is a chemical that does not induce at least a PC10 in replicate experiments.

PC10 is the concentration of a test chemical that increases the measured activity in an agonist assay to 10% of the maximum activity induced by the appropriate reference compound.

PC50 is the concentration of a test chemical that increases the measured activity in an agonist assay to 50% of the maximum activity induced by the appropriate reference compound.

Positive result is a chemical that induces at least a PC10 in replicate experiments.

Validation is a scientific process designed to characterize the operational requirements and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose according to the OECD Guidance Document No.34 [ENV/JM/MONO(2005)14].

ANNEX 2

Preparation of Serum treated with Dextrane Coated Charcoal (DCC)

1. The treatment of serum with dextrane-coated charcoal (DCC) is a generally used methodology for the removal of estrogenic compounds from serum that is added in cell medium in order to exclude the biased response associated with residual estrogens in serum.

Components

2. The following materials and equipments will be required;

Materials

Activated charcoal

Dextrane

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)

Sucrose

1 M HEPES buffer solution (pH 7.4)

Ultrapure water produced by a filter system

Equipments

Autoclaved glass container (size should be adjusted as appropriate)

General Laboratory Centrifuge (that can set temperature at 4°C.)

Procedure

3. The following procedure is adjusted for the use of 50 mL centrifuge tubes.

[Day-1] Prepare 1 liter of dextran coated charcoal suspension with ultrapure water containing 1.5 mM of $MgCl_2$, 0.25 M sucrose, 2.5 g of charcoal, 0.25 g of dextran and 5 mM of HEPES and stir it at 4°C, overnight.

[Day-2] Dispense the suspension in 50 mL centrifuge tubes and centrifuge at 10,000 rpm, 4°C for 10 minutes. Remove supernatant and store the half of the charcoal sediment 4°C for the use on Day-3. Suspend the charcoal with unfrozen fetal bovine serum (FBS) at 42°C and transfer into the autoclaved glass container such as an Erlenmeyer flask. Stir this suspension gently at 4°C, overnight.

[Day-3] Dispense the suspension with FBS into centrifuge tubes for centrifugation at 10,000 rpm, 4°C for 10 minutes. Collect FBS and transfer into new charcoal sediment that is stored on Day-2. Suspend the charcoal sediment and stir gently at 4°C, overnight.

[Day-4] Dispense the suspension for centrifugation at 10,000 rpm, 4°C for 10 minutes and sterilize the supernatant. This DCC treated FBS should be stored at -20°C