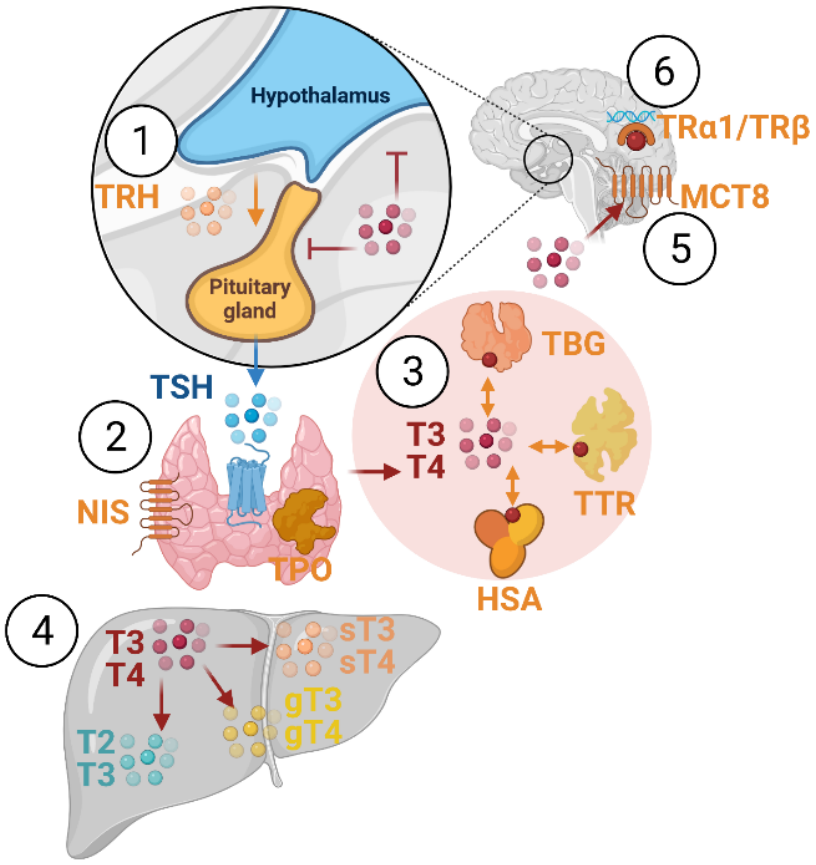
STANDARD OPERATING PROCEDURE

for the thyrotropin-stimulating hormone (TSH) receptor activation assay based on cAMP measurement, version 1.0

EURL ECVAM validation study of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system



2021

This Standard Operating Procedure (SOP) has been prepared within the context of a collaboration agreement signed in 2019 with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling.

The SOP provided was used during both Part 1 and Part 2 of the validation study. It cannot be excluded that later versions exist.

The published method (see references in the SOP) from Santini, Dimida and Tonacchera of the University of Pisa, Italy was implemented, optimised and experimentally assessed by EU-NETVAL laboratory 'National Institute of Public Health', Czech Republic.

Contact information EU-NETVAL laboratory

Marketa Dvorakova, MSc., Ph.D.

National Institute of Public Health, Centre of Toxicology and Health Safety
NRL for Experimental Immunotoxicology

Šrobárova 49/48,
100 00 Prague 10,
Czech Republic

e-mail: marketa.dvorakova@szu.cz

Thyrotropin-stimulating hormone (TSH) receptor activation based on cAMP measurement

Standard Operating Procedure (S.O.P.)

Version 1

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Authors:

Marketa Dvorakova, MSc., Ph.D.
National Institute of Public Health, Centre of Toxicology and Health Safety
NRL for Experimental Immunotoxicology
Šrobárova 49/48, 100 00 Prague 10, Czech Republic
Tel:
e-mail: marketa.dvorakova@szu.cz

Nominated laboratory in the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) coordinated by the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

Method Name: TSH receptor mediated activation non-radioactive
Method Number: 1b
Block 1: Central regulation
Short Name (ACRONYM): TSH

Method Developer(s)
M. Dvorakova, National Institute of Public Health, NRL for Experimental Immunotoxicology (CZ), EU-NETVAL Facility (SOP development).

Test system supplier(s)
S. Costagliola, Université libre de Bruxelles, Bruxelles (B) (test system developer).

Reference(s)

For the test method development:

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1 RATIONALE, ADVANTAGES AND LIMITATIONS, APPLICABILITY DOMAIN, ENDPOINT, EXPERIMENTAL TEST SYSTEMS, BASIC PROCEDURE, DISCUSSION, SAFETY

1.1 Rationale

The human Thyroid-Stimulating Hormone Receptor (hTSHR) is a G-protein-coupled receptor, involved in cell signaling through activation of the adenylate cyclase upon binding of the endogenous hormone(thyrotropin or TSH). The activation of adenylate cyclase results in the increase of 3',5'- cyclic adenosine monophosphate (cAMP). The increase of cAMP in thyroid follicular cells results in increased thyroid hormone (TH) production. Chemicals with the potential of mimicking the activity of TSH may disrupt the thyroid signaling, which may result in altered thyroid hormone levels, followed by adverse effects *in vivo*. This method allows *in vitro* prediction whether chemicals used at soluble, non-cytotoxic concentrations exhibit an agonist potential to disrupt thyroid functions via the human thyrotropin receptor (hTSHR) mediated signaling pathway. Chinese hamster ovary cells (CHO - K1) transfected with the gene of hTSHR (CHO-K1 hTSHR cells - clone JP-09, hereafter referred as JP09 cells) are responsive to TSH, in terms of adenylate cyclase activation related to the increase of cAMP. This non-radioactive method should identify chemicals that interfere with the normal hTSHR-mediated increase of cAMP by interacting with the hTSHR.

1.2 Advantages and limitations, applicability domain

The method can be performed in a high throughput 96-well format for rapid screening of substances. The method requires culture of adherent cells using aqueous media and water-based vehicle (buffer) during the exposure to test items. All chemicals or mixtures soluble in medium or DMSO may be evaluated at soluble and non-cytotoxic concentrations. If any other solvent is used, care should be taken that any solvent is not used at cytotoxic concentration and does not interfere with the functional assay and measurement of cAMP. Interfering samples or solvents should be excluded from analysis, as they fall outside the applicability domain. The increased cAMP may be measured directly in the cell culture supernatant using standardized ELISA kits and the standard concentrations should cover the range of concentrations encountered during the analysis of test samples. The assay should not be used as a stand-alone test for final prediction of adverse biological effects on thyroid functions.

1.3 Endpoint, endpoint value and quantification

Endpoint: concentration-dependent increase in cAMP production, detected by colorimetric analysis.
Material used for quantification: cell culture supernatant
Exposure time: min. 12 hours
Endpoint Value: unit of relative absorbance intensity.
Endpoint quantification method: commercial colorimetric cAMP detection ELISA kits.

1.4 Test system

Test system, as per given references, is described below:

CHO-K1 cells transfected with human TSH-receptor, clone JP-09 (CHO-K1 TSHR, JP-09), hereafter referred as JP09 cells, developed at University Libre de Bruxelles (Chinese Hamster Ovary cells co-transfected with the hTSHR-pSVL expression shuttle and a pSV2NEO neomycin resistance gene vector) are used as the test system for detection of specific agonist interactions towards hTSHR. Cells may be obtained from the test system developer: S. Costagliola (scostag@ulb.ac.be), Université libre de Bruxelles, Bruxelles.

1.5 Control cell line

CHO-K1 cells (ATCC® CCL-61) is the control cell line, which does not express the hTSHR, and allows test system characterization and control of selection. Stocks are to be prepared by the test laboratory from seed cells obtained from a reliable cell bank.

1.6 Basic procedure of the functional agonist assay

For each test item for which the cytotoxicity is not known, a cell viability dose response test should be run prior the functional assay to identify the concentration showing minimally 75% viability (≥CV75) to be used in the functional assay as the highest tested concentration. The wider cell viability dose response test should be performed using a higher dilution factor such as 10. For the functional assay, a narrow range of concentrations (e.g. logarhitmic), starting with the ≥CV75 is to be used. The functional assay consists of pre-culture of the test system (JP09 cells), exposure to the test item(s) and TSH (used as the reference item), followed by detection of cAMP increase in the cell culture supernatant. Detection of cAMP increase is performed with the use of commercial standardized ELISA non-radioactive kit CA200 (Merck), the Direct cAMP Enzyme Immunoassay, non-Acetylated Version. The assay is designed for a 96 well plate layout and allows testing of max. 2 Test Items at 8 concentrations. Solvent control (1%) and reference item (0.0003 – 0.003 – 0.03 – 0.3 – 3 – 15 – 24 mIU/ml TSH) are run in parallel in the same plate. After min. 12h exposure, the samples (cell culture supernatant) are collected and used for measurement for cAMP production. The positive result is evaluated in case 1.2 increase of cAMP is detected at min. 2 (non-consecutive) non-cytotoxic concentrations comparing to the values of the Vehicle control (if diluted in the Exposure Vehicle with IBMX) or Solvent control (if diluted in a Solvent), depending on the material used for test item dilutions.

1.7 Safety

Reagents should be treated as possible mutagens, handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with each product for information on specific components. Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures. All culture and chemical exposure procedures must be performed under sterile conditions.

2 DEFINITIONS / ABBREVIATIONS

abbreviation	in full
cAMP	adenosine 3', 5'-cyclic monophosphate
≥CV75	concentration leading to cell viability of min. 75 % cell viability
ELISA	Enzyme-Linked ImmunoSorbent Assay
CHO-K1	Chinese Hamster Ovary cells (clone K1, CCL-61) (ATCC® CCL-61)
JP09	Chinese Hamster Ovary cells (clone K1, CCL-61) (ATCC® CCL-61) transfected with human TSH-Receptor, clone JP-09
DMSO	dimethyl sulfoxide
F-12K	Kaighn´s modification of Ham´s F-12 medium
hTSHR	human thyrotropin (Thyroid Stimulating Hormone) Receptor
IBMX	3-Isobutyl-1-methylxanthine
KRH	Krebs-Ringer-HEPES (KRH buffer)
O/N	overnight
PBS	phosphate buffered saline
RT	room temperature
SC	solvent control
SOP	standard operating procedure
TI	test item
TSH	thyrotropin

3 MATERIAL

3.1 Technical equipment

- Freezer -20°C and –80°C
- Refrigerator +4°C
- Laminar flow
- CO_2 incubator 37 +/- 1˚C and 5% CO_2
- Glassware, plasticware for the preparation of reagents and buffer solutions
- Gas burner
- Centrifuge
- Vacuum aspirator
- Heating plate
- Plate shaker, orbital shaker
- Micro plate reader (absorbance, compatible with cAMP detection kit)
- T25, T75 culture flasks
- Cryovials
- 96-well plates with flat bottom
- Pipettor
- Sterile pipettes (1, 5, 10 ml)
- Automated Pipettes (1 – 10 - 50 - 100 – 200 – 1000 - 5000 µl)
- Multichannel pipettes (1 – 10 – 50 – 100 – 200 µl)
- Sterile pipette tips (1 – 10 - 50 - 100 – 200 – 1000 - 5000 µl)
- Conical tubes 50, 15 ml
- Centrifuge tubes 2 ml, micro-centrifuge tubes 0.2, 0.5 ml
- Vacuum filters (0.45 µm)
- Blotting tissues
- Sealing tapes
- Ultrapure water
- cAMP detection ELISA kits (standard calibration curve approx. 0 – 200 pmole cAMP/ml)

3.2 Reagents

Reagents from other suppliers can be used, as far as they provide equal results, as demonstrated by meeting the acceptance criteria.

No.	Reagent	CAS	Description	Supplier	Catalogue No.
1	PBS	-	Phosphate buffer saline without Mg^{2+}/Ca^{2+}	Biochrom	L1825
2	F-12K Medium	-	Kaighn´s modification of Ham´s F-12 medium	ATCC® LGC STANDARDS	ATCC® 30-2004
3	FBS (*animal derived material*)	-	Fetal Bovine Serum	ATCC® LGC STANDARDS	30-2020™
4	G418	108321-42-2	Geneticin, selection antibiotics	Merck	A1720-1G
5	Penicilin / Streptomycin	-	Penicillin / Streptomycin solution with 10,000 units penicillin and 10 mg streptomycin per mL in 0.9% NaCl, 0.1 µm filtered, BioReagent, suitable for cell culture;used to maintain sterile conditions during cell culture. Concentration: 100x	Merck	P0781
6	Trypsin/ EDTA (*Trypsin: animal derived material*)	-	0.25% Trypsin / 0.02% EDTA	Merck	59428C-100ML
7	IBMX	28822-58-4	3-Isobutyl-1-methylxanthine used as phosphodiesterase inhibitor	Merck	I5879-250MG
8	DMSO	67-68-5	Dimethyl sulfoxide 100% Purity 99,9 % ACS Reagent used as the solvent and solvent control at final tested concentration [1%].	Merck	472301
9	TSH	9002-71-5	Thyroid stimulating hormone, Thyrotropic hormone from human pituitary. Agonist of hTSHR. Purity: ≥ 95% by weight. Immunopotency: ≥ 6 IU/mg	Merck	T9265-5UG (1 vial)
10	MTT	298-93-1	Thiazolyl Blue Tetrazolium Bromid	Merck	M5655-1G
11	SDS	151-21-3	Sodium dodecyl sulfate	Merck	436143
12	DPBS, 10x		Dulbecco's Phosphate Buffered Saline 10x, 100 ml	Merck	D1408-100ML

3.3 Media and Solutions

	Medium / Solution	Material	Preparation	Storage / Expiration
1	Complete culture medium (CHO-K1 cells)	F-12K Medium 10% FBS 1% Penicilin / Streptomycin	Add: 50 ml FBS 5 ml of Penicilin/ Streptomycin into 500 ml of F-12K medium.	4 weeks, 4°C
2	Selection medium (JP09 cells)	Complete culture medium 400 mg/l Geneticin (G418)	Add: 200 mg geneticin (G418) into 500 ml of complete culture medium.	4 weeks, 4°C
3	Freezing medium	Complete culture medium 10% DMSO	Add: 0.1 ml of DMSO into 0.9 ml of complete culture medium	Freshly prepared
4	Krebs-Ringer-HEPES buffer (KRH buffer)	NaCl, KCl, MgSO4, CaCl2, KH2PO4, HEPES, glucose, BSA, sterile demineralized water	Dissolve: 124 mM NaCl, 5 mM KCl, 1.25 mM MgSO4, 1.45 mM CaCl2, 1.25 mM KH2PO4, 25 mM HEPES, 8 mM glucose, 0.5 g/l BSA, pH 7.4 in 1 L of sterile demineralized water.	RT, protect from light / 24 months
5	IBMX 0.5 M Stock solution	IBMX 100% DMSO	Dissolve: 111.15 mg of IBMX in 1 ml of 100% DMSO. Vortex properly. Prepare 50 µl aliquots. Freeze at -20°C.	- 20°C / 6 months
6	Exposure Vehicle with IBMX: KRH buffer supplemented with 0.5 mM IBMX	KRH buffer 99.9 ml IBMX 0.5 M 0.1 ml	1. Add slowly 0.1 ml of 0.5 M IBMX stock solution to to 1.9 ml of KRH buffer, preferably in a glass tube. 2. Vortex properly. 3. If precipitate is formed, heat carefully the content in the glass tube for approx. 5-10 sec. over the gas burner flame, gently shake by hand until the precipitate dissolves and the solution is completely clear with no signs of turbidity or precipitate. Do not boil. 4. Transfer the content from the glass tube (2 ml) into a pipetting reservoir with previously added 98 ml of KRH buffer. 5. Mix properly with a multichannel pipette (at least 10 up / down strokes).	Freshly prepared
7	TSH [300 mIU/ml] Stock solution	TSH Exposure Vehicle with IBMX	1. Reconstitute 1 vial with 5 µg of TSH (Sigma, T9265) in 100 µl of the Exposure Vehicle with IBMX. 2. Vortex properly. After reconstitution, the bioactivity of the TSH stock solution should be 300 mIU/ml.	- 20°C / 6 months
8	DPBS 1x	DPBS 10x sterile demineralized water	Add 1 ml of DPBS 10x into 9 ml of sterile demineralized water.	Freshly prepared
9	MTT stock solution	MTT DPBS 1x	1. Dissolve 10 mg of MTT s in 2 ml of DPBS 1x. 2. Filter-sterilize the solution through 0.45 µm filter.	Freshly prepared

10	MTT working solution	MTT stock solution Exposure Vehicle with IBMX	Dissolve 2 ml of MTT stock solution in 18 ml of Exposure Vehicle with IBMX.	Freshly prepared
11	Lysis solution for MTT assay	SDS Acetic Acid DMSO	10 g of SDS and 0.4 ml of Acetic Acid are dissolved in 99.6 ml of DMSO.	RT, 3 months

3.4 Controls, reference material

	Function	Material	Preparation	Storage / Expiration
1.	Vehicle control (VC)	Exposure Vehicle with IMBX	KRH buffer supplemented with 0.5 mM IBMX; 0.1 ml of 0.5 M IBMX are added into 99.9 ml of KRH buffer	Freshly prepared
2.	Solvent control (SC)	1% Solvent (default solvent: 100% DMSO)	2 µl of 100% DMSO are added into 0.198 ml of Exposure Vehicle with IBMX. Final concentration of DMSO [%]: 1.	Freshly prepared
3.	Reference material (TSH)	TSH	Final tested concentrations [mIU/ml]: 24 -15 – 3 - 0.3–0.03–0.003–0.0003 Stable concentration of 3 mIU/ml may be used for intra-assay validation as the stable concentration of the positive control.	Freshly prepared

3.5 Solubility, cell viability dose response test, post-exposure cytotoxicity test

Solubility: proper solubility of a test item in the Selection medium, the default solvent (100% DMSO) or a selected appropriate solvent should be verified by centrifugation and visual microscopic inspection. Initially, a test item is diluted up to the maximum concentration of 1 mg/ml, or 10 mM by vortexing for 30s in the Selection medium and 100% DMSO. If any precipitate or cloudiness is observed, proper solubility is further promoted by stirring, vortexing, mixing, heating or sonication. In case of permanent insufficient solubility (observed as a persistent precipitate / cloudiness), a test item is diluted to lower concentrations, preferably in the Selection medium, or 100% DMSO (as the default solvent), in order to find the highest soluble concentration (HSC). In case of permanent insufficient solubility (observed as a persistent precipitate / cloudiness), a test item is diluted in another more appropriate selected solvent. The highest soluble concentration (HSC), preferably in the Selection medium, will be used in the cell viability dose response test as the highest tested concentration. If a test item is soluble only in a solvent (and not in the Selection medium), the HSC should be diluted 100x prior testing in order to achieve the highest non-cytotoxic concentration of the solvent (HSC/100).

Cell viability dose response test: a preliminary cell viability dose response test should be performed prior the functional assay to observe whether a test item exhibits any problems in mitochondrial activity or cytotoxicity towards JP09 cells and to determine the range of concentrations suitable to be used in the functional test. The concentration, showing minimally 75% of cell viability should be used as the highest concentration used in the functional assay (≥CV75), using a higher dilution factor such as 10. Cytotoxicity of a test item towards JP09 cells should be tested using the standardized in vitro basal cytotoxicity assays to determine either cell proliferation or metabolism (e.g. MTT [3-(4,5,dimethylthiazol-2yl)2,5-diphenyl tetrazolium bromide] (*note: simple endpoints such as total protein may underpredict the toxicity of certain test substances by including protein from dead cells*). In general, 11 decimal dilutions (D2-D11) starting from the

highest soluble concentration (HSC) should be used in the first run of the cell viability dose response test (see details in point 4.1). If a test item is diluted in 100% DMSO (or another appropriate selected solvent), cytotoxicity of the solvent must be excluded. Therefore, the HSC of a test item diluted in the solvent should be further diluted 100x prior testing (HSC/100). If cytotoxicity of a test item (or the solvent) is observed and the concentration showing at least 75% viability (≥CV75) is not identified in the first run of the cell viability dose response test, the test must be repeated with lower dilutions, including the dilution of the selected solvent. Cytotoxic chemical (DMSO, 50%) is used in order to evaluate the influence of cytotoxicity on cAMP production.

Post-exposure cytotoxicity test:
Standardized in vitro basal cytotoxicity assay to determine either cell viability or metabolism should be performed under the same exposure conditions as the functional assay in order to obtain data for normalisation of cAMP levels produced during the functional assay.

3.6 Quality check of reagents

- Visual and microscopic inspection of solubility must be performed, dilutions with any precipitate excluded.
- Contamination must be excluded as observed visually or microscopically.

3.7 Test systems maintenance

The procedure below describes the cell culture of adherent cells. Cell viability, date of subculture, subculture intervals, morphology, passage number should be documented. The work with cells is performed in aseptic conditions under a laminar hood. For general subculture, the cell bank guidance (e.g. ATCC) is recommended.

3.7.1 Thawing of JP09 cells

- 1 cryovial with JP09 cells (min. 1.5 x 10^6 cells in 1ml) is thawed at least 10 days before performing the functional assay from the liquid nitrogen and placed in a warm water bath (37°C). Care should be taken in order to avoid submerging the cryovial into the water.
- As soon as the content is melted, the whole content from the cryovial (1ml of suspension) is gently resuspended in 14 ml of the selection medium (pre-warmed at 37°C) preapplied in a T75 flask. The flask is marked as “JP09, P0”.

Note: After thawing, cells are naturally under stress and higher loss of cells may be expected. Therefore, cell count marked on the cryovial from the day of freezing is documented for P0. Subsequently, the cell count / viability at each passage is documented, starting at passage number P1.

Cells are incubated at 37 ±1 °C, 5% CO2 and approx. 95 % relative humidity for 24h. If humidity cannot be monitored, distilled water is kept in a water container inside the incubator according to the manufacturer´s guidance.

- 24h after seeding, the adherence, confluence and morphology of cells is microscopically inspected.
- Selection medium is aspirated and 15 ml of the same type of fresh medium is applied to the flasks.
- The cells are incubated in culture flasks to reach 80 - 90% confluence (next 24 – 48h).
- When 80 - 90% confluent, the medium is aspirated and the cells are passaged (split ratio 1:4 – 1:8).
- The cells are counted using a cell counter when passaged, starting at passage 1 (P1).
- If a cell counter is not available, a hemocytometer or Bürker cell counting chamber may be used.

3.7.2 Passaging of JP09 cells in T75 flasks

- Cells are washed twice with 10 ml PBS (pre-warmed at 37°C).
- PBS is aspirated and approx. 1 ml of 0.25% Trypsin / 0.02% EDTA (37±1°C) is added to the flask.
- Cells are incubated (at 37±1°C) until detachment is observed (approx. 5 min).
- After detachment, 4 ml of selection medium (37±1 °C) is added.
- Cells are gently rinsed off the flask by repeated pipetting strokes.
- Cell suspension is counted, cell count and viability is documented.
- 4.5 ml of JP09 / CHO-K 1 cell suspension is discarded or used on the day of the functional assay.

- 0.5 ml of cell suspension is left in the T75 culture flask
  (split ratio is approx. 1:4 – 1:8, depending on passage number).
- 14 ml of selection medium is added to the T75 flasks and the cell suspension is gently resuspended.
- Passage number, date of passage, cell count and viability are documented and marked on the flask.

3.7.3 Incubation

- Cells are incubated in an incubator (37±1ºC , 5% CO2, approx. 95% humidity) until 80-90 % confluent.
- When reaching 80-90 % confluence, the passaging step is repeated.
- Cells are passaged usually three times a week (Monday / Wednesday / Friday).
- It is recommended for the functional assay to use JP09 cells in a logarhithmic phase of growth (approx. at passage numbers 6 – 20). For long-term continual cultivation, complete culture media should be used.
- The JP09 cells should be cryopreserved / used for the functional assay after a sufficient selection, observed as a decrease of viability in CHO-K1 cells, used as the selection control (see details in point 3.7.4).

3.7.4 Sufficient selection

- At least three passages in the selection medium are required to select JP09 cells prior freezing or performing the functional assay.
- Parallel cultivation of CHO-K1 cells in a T25 flask with 5 ml of selection media may be used for microscopic inspection of selection.
- Sufficient selection is observed microscopically by comparison of T25 flask with CHO-K1 cells and T75 flask with JP09 cells. In the T25 flask with CHO-K1 cells, significant decrease of cell viability should be observed (see details in point 3.7.5).

3.7.5 Acceptance criteria for using the cells in the functional assay

- Uniform morphology, 80 – 90 % confluence, homogenous monolayer and 90 % viability should be observed for JP09 cells (see Fig. 1).

Morphology of cells

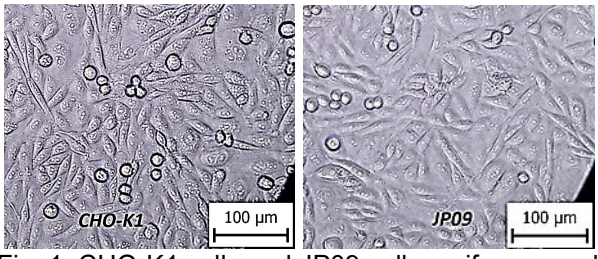


Fig. 1: CHO-K1 cells and JP09 cells: uniform morphology, approx. 90 % confluence, homogenous monolayer, sterile culture.

Decrease of cell viability, CHO-K1 cells after sufficient selection

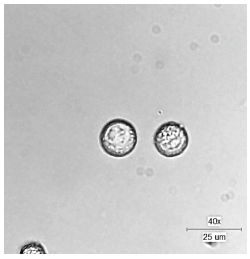


Fig. 2: CHO-K1 selection control, P2. Sufficient selection (at least 1 week after thawing) observed as cell death (non-adhered dead rounded cells).

3.7.6 Seeding density into 96-well plates

Recommended seeding density: min. 7 x 10^4 cells/well (0.1 ml volume of cell suspension of density 7 x 10^5 cells/ml).

3.7.7 Cryopreservation

- A cryovial is marked with the cell line code (JP09 / CHO-K1), passage number, date of freezing.
- A freezing container is cooled in the refrigerator to 4 - 8˚C.
- Cells are detached with 1 ml of trypsin as previously described.
- Cells are passaged (gently rinsed off the flask) with 4 ml of complete culture medium.
- Cells in the suspension are counted. Cell count is marked on the cryovial and documented.
- Cells are centrifuged at approx. 200 x g for 5 min.
- Supernatant is carefuly aspirated.
- The cell pellet is resuspended in 1 ml of freezing medium.
- The resuspended cell pellet is transferred to the cryovial.
- The cryovial is placed into the freezing container previously cooled to 4 - 8˚C.
- The cryovial in the freezing container is frozen to -80±1 °C for 24 h in a deep freezer.
- After 24 h the cryovial is transferred to liquid nitrogen vapor phase for longer storage.

3.7.8 Quality check of test systems

- Viability of JP09 cells used in the functional assay must be documented prior treatment.
- Mycoplasma check (e.g. with the use of colorimetric or PCR kits) should be performed at least 1 x year.
- Contamination (bacteria, fungi, yeast) must be excluded as observed by pH indicator in the media or microscopically. Contaminated culture must be discarded.

4 PROCEDURES

4.1 Plate Layouts

Prepare the Plate Layout for each assay.

1. Plate layout for the cell viability dose response test (PLATE 1)
2. Plate layout for the functional assay (PLATE 2)
3. Plate layout for the cAMP detection using ELISA kit (PLATE 3).

PLATE 1: **Suggested plate layout for the cell viability dose response test.**
Max. 4 Test Items, duplicates, 11 dilutions (D2-D11) from the Highest Soluble Concentration. If the test item is diluted in a solvent, the HSC is diluted 100x diluted to the final non-cytotoxic concentration (HSC/100)

P1	1	2	3	4	5	6	7	8	9	10	11	12
A	Selection medium	Test Item 1 D11	Test Item 1 D10	Test Item 1 D9	Test Item 1 D8	Test Item 1 D7	Test Item 1 D6	Test Item 1 D5	Test Item 1 D4	Test Item 1 D3	Test Item 1 D2	Test Item 1 HSC (HSC/100)
B	Selection medium	Test Item 1 D11	Test Item 1 D10	Test Item 1 D9	Test Item 1 D8	Test Item 1 D7	Test Item 1 D6	Test Item 1 D5	Test Item 1 D4	Test Item 1 D3	Test Item 1 D2	Test Item 1 HSC (HSC/100)
C	Selection medium	Test Item 2 D11	Test Item 2 D10	Test Item 2 D9	Test Item 2 D8	Test Item 2 D7	Test Item 2 D6	Test Item 2 D5	Test Item 2 D4	Test Item 2 D3	Test Item 2 D2	Test Item 2 HSC (HSC/100)
D	Selection medium	Test Item 2 D11	Test Item 2 D10	Test Item 2 D9	Test Item 2 D8	Test Item 2 D7	Test Item 2 D6	Test Item 2 D5	Test Item 2 D4	Test Item 2 D3	Test Item 2 D2	Test Item 2 HSC (HSC/100)
E	Solvent control	Test Item 3 D11	Test Item 3 D10	Test Item 3 D9	Test Item 3 D8	Test Item 3 D7	Test Item 3 D6	Test Item 3 D5	Test Item 3 D4	Test Item 3 D3	Test Item 3 D2	Test Item 3 HSC (HSC/100)
F	Solvent control	Test Item 3 D11	Test Item 3 D10	Test Item 3 D9	Test Item 3 D8	Test Item 3 D7	Test Item 3 D6	Test Item 3 D5	Test Item 3 D4	Test Item 3 D3	Test Item 3 D2	Test Item 3 HSC (HSC/100)
G	Solvent control	Test Item 4 D11	Test Item 4 D10	Test Item 4 D9	Test Item 4 D8	Test Item 4 D7	Test Item 4 D6	Test Item 4 D5	Test Item 4 D4	Test Item 4 D3	Test Item 4 D2	Test Item 4 HSC (HSC/100)
H	Solvent control	Test Item 4 D11	Test Item 4 D10	Test Item 4 D9	Test Item 4 D8	Test Item 4 D7	Test Item 4 D6	Test Item 4 D5	Test Item 4 D4	Test Item 4 D3	Test Item 4 D2	Test Item 4 HSC (HSC/100)

PLATE 2: **Suggested plate layout for the functional assay.**
Max. 3 Test items, triplicates, 8 concentrations (≥CV75 – C8).
Reference item: TSH, max. 7 concentrations
(0.0003 – 0.003 – 0.03 – 0.3 – 3 – 15 – 24 mIU/ml).

P 2	1	2	3	4	5	6	7	8	9	10	11	12
A	Exposure Vehicle with IBMX (Vehicle control)	Exposure Vehicle with IBMX (Vehicle control)	Exposure Vehicle with IBMX (Vehicle control)	TSH 24 mIU/ml	TSH 24 mIU/ml	TSH 24 mIU/ml	Test Item 1 C8	Test Item 1 C8	Test Item 1 C8	Test Item 2 C8	Test Item 2 C8	Test Item 2 C8
B	Solvent control	Solvent control	Solvent control	**Cytotoxic chemical (DMSO 50%)**	**Cytotoxic chemical (DMSO 50%)**	**Cytotoxic chemical (DMSO 50%)**	Test Item 1 C7	Test Item 1 C7	Test Item 1 C7	Test Item 2 C7	Test Item 2 C7	Test Item 2 C7
C	TSH 0.0003 mIU/ml	TSH 0.0003 mIU/ml	TSH 0.0003 mIU/ml	**EMPTY**	**EMPTY**	**EMPTY**	Test Item 1 C6	Test Item 1 C6	Test Item 1 C6	Test Item 2 C6	Test Item 2 C6	Test Item 2 C6
D	TSH 0.003 mIU/ml	TSH 0.003 mIU/ml	TSH 0.003 mIU/ml	**EMPTY**	**EMPTY**	**EMPTY**	Test Item 1 C5	Test Item 1 C5	Test Item 1 C5	Test Item 2 C5	Test Item 2 C5	Test Item 2 C5
E	TSH 0.03 mIU/ml	TSH 0.03 mIU/ml	TSH 0.03 mIU/ml	**EMPTY**	**EMPTY**	**EMPTY**	Test Item 1 C4	Test Item 1 C4	Test Item 1 C4	Test Item 2 C4	Test Item 2 C4	Test Item 2 C4
F	TSH 0.3 mIU/ml	TSH 0.3 mIU/ml	TSH 0.3 mIU/ml	**EMPTY**	**EMPTY**	**EMPTY**	Test Item 1 C3	Test Item 1 C3	Test Item 1 C3	Test Item 2 C3	Test Item 2 C3	Test Item 2 C3
G	TSH 3 mIU/ml	TSH 3 mIU/ml	TSH 3 mIU/ml	**EMPTY**	**EMPTY**	**EMPTY**	Test Item 1 C2	Test Item 1 C2	Test Item 1 C2	Test Item 2 C2	Test Item 2 C2	Test Item 2 C2
H	TSH 15 mIU/ml	TSH 15 mIU/ml	TSH 15 mIU/ml	**EMPTY**	**EMPTY**	**EMPTY**	Test Item 1 ≥CV 75	Test Item 1 ≥CV 75	Test Item 1 ≥CV 75	Test Item 2 ≥CV 75	Test Item 2 ≥CV 75	Test Item 2 ≥CV 75

PLATE 3: **Suggested plate layout for the cAMP ELISA.**

All samples need to be tested in the same kit of the same catalogue no. and the same Lot with the same standard calibration curve.

P 3	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	STD1	STD5	TSH 0.0003 mIU/ml	TSH 0.0003 mIU/ml	TSH 0.0003 mIU/ml	Test Item 1 C8	Test Item 1 C8	Test Item 1 C8	Test Item 2 C8	Test Item 2 C8	Test Item 2 C8
B	BLANK	STD1	STD5	TSH 0.003 mIU/ml	TSH 0.003 mIU/ml	TSH 0.003 mIU/ml	Test Item 1 C7	Test Item 1 C7	Test Item 1 C7	Test Item 2 C7	Test Item 2 C7	Test Item 2 C7
C	TA	STD2	Exposure Vehicle with IBMX (Vehicle control)	TSH 0.03 mIU/ml	TSH 0.03 mIU/ml	TSH 0.03 mIU/ml	Test Item 1 C6	Test Item 1 C6	Test Item 1 C6	Test Item 2 C6	Test Item 2 C6	Test Item 2 C6
D	TA	STD2	Exposure Vehicle with IBMX (Vehicle control)	TSH 0.3 mIU/ml	TSH 0.3 mIU/ml	TSH 0.3 mIU/ml	Test Item 1 C5	Test Item 1 C5	Test Item 1 C5	Test Item 2 C5	Test Item 2 C5	Test Item 2 C5
E	NSB	STD3	Exposure Vehicle with IBMX (Vehicle control)	TSH 3 mIU/ml	TSH 3 mIU/ml	TSH 3 mIU/ml	Test Item 1 C4	Test Item 1 C4	Test Item 1 C4	Test Item 2 C4	Test Item 2 C4	Test Item 2 C4
F	NSB	STD3	Solvent control	TSH 15 mIU/ml	TSH 15 mIU/ml	TSH 15 mIU/ml	Test Item 1 C3	Test Item 1 C3	Test Item 1 C3	Test Item 2 C3	Test Item 2 C3	Test Item 2 C3
G	Bo	STD4	Solvent control	TSH 24 mIU/ml	TSH 24 mIU/ml	TSH 24 mIU/ml	Test Item 1 C2	Test Item 1 C2	Test Item 1 C2	Test Item 2 C2	Test Item 2 C2	Test Item 2 C2
H	Bo	STD4	Solvent control	**Cytotoxic chemical (DMSO 50%)**	**Cytotoxic chemical (DMSO 50%)**	**Cytotoxic chemical (DMSO 50%)**	Test Item 1 ≥CV 75	Test Item 1 ≥CV 75	Test Item 1 ≥CV 75	Test Item 2 ≥CV 75	Test Item 2 ≥CV 75	Test Item 2 ≥CV 75

4.1.1 DAY 0 – cell viability dose response test - Monday

1. One 96-well plate is prepared.
2. 100 µl, (approx. 7 x 10^4 cells/well) of JP09 cell suspension in the selection medium are seeded in each well.
3. PLATE 1 is incubated O/N (16 hours +/- 2 hours, 5% CO2, 95% humidity, 37± 1 ºC).

4.1.2 DAY 1 – cell viability dose response test – Tuesday

1. Maximum of 4 test items may be tested in duplicates at 11 dilutions (D2-D11), starting with the highest soluble concentration (HSC, if the test item is diluted in the Selection medium, or HSC/100, if the test item is diluted in a solvent).
2. The highest soluble concentration (HSC) of the test items is determined according to solubility procedure (for details see point 3.5).
3. 100 ml of Selection medium is equilibrated to 37°C.
4. A test item is serially diluted in the Selection medium. If poorly soluble in the Selection medium, the test item is diluted in a selected solvent, preferably in 100% DMSO. If poorly soluble in 100% DMSO, the test item is diluted in another appropriate selected solvent (see point 3.5).
5. Every well in PLATE 1 is microscopically inspected for homogeneous confluence prior next step.
6. Selection medium from DAY 0 / DAY 1 O/N incubation is aspirated from all wells.
7. If a test item is diluted in the Selection medium, serial (decimal) dilutions (D2-D11) of a test item are directly applied to the appropriate wells, starting with the highest soluble concentration (HSC). Selection medium is used as the Vehicle control (see the Plate Layouts, PLATE 1, point 4.1). Slow pipetting is used, whereas the tip must not touch the bottom of the well.
8. If a test item cannot be diluted in the Selection medium, hence it is diluted in a selected solvent (preferably in 100% DMSO as the default solvent), 198 µl of the Selection medium are pre-applied to all appropriate wells. 2 µl of a test item serially diluted in the solvent are added to the appropriate wells to achieve final serial dilutions (D2 – D11) and non-cytotoxic concentrations of the solvent, starting with the the Highest Soluble Concentration, 100x diluted (HSC/100) (see the Plate Layout, PLATE 1, point 4.1 and point 3.5). 1% selected solvent in the Selection medium is used as the solvent control (see the Plate Layouts, PLATE 1, point 4.1). Slow pipetting is used, whereas the tip must not touch the bottom of the well.
9. 2 µl of the selected solvent are added to the appropriate wells in order to achieve the highest non-cytotoxic concentration (e.g., 1% of DMSO, or 1% of the selected solvent).
10. Careful pipetting is used, whereas the tip must not touch the bottom of the well.
11. Every well in PLATE 1 is microscopically inspected for homogeneous confluence. PLATE 1 is incubated O/N (16 hours +/- 2 hours, 5% CO2, 95% humidity, 37± 1 ºC).

4.1.3 DAY 2 – cell viability dose response test – Wednesday

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is performed:

MTT assay:

1. MTT stock solution, MTT working solution and the Lysis solution for MTT assay are prepared according to the procedure mentioned above (see Table 3.3).
2. Selection medium with dilutions of test items is aspirated from PLATE 1.
3. 200 µl of MTT working solution is applied to all wells.
4. The plate is incubated 3 hours (37 °C, 5% CO2) in the incubator.
5. After incubation, the MTT working solution is aspirated.
6. 100 µl of Lysis solution for MTT assay is applied to all wells.
7. The plate is incubated 15 min on the (orbital) shaker at room temperature in order to dissolve the formazan crystals. No precipitate should be observed. In case of precipitate, the wells need to be mixed with gentle pipette strokes. Changing tips is essential between the wells.
8. Absorbance is measured at 570 nm.

4.1.4 DAY 2 – functional assay – Wednesday

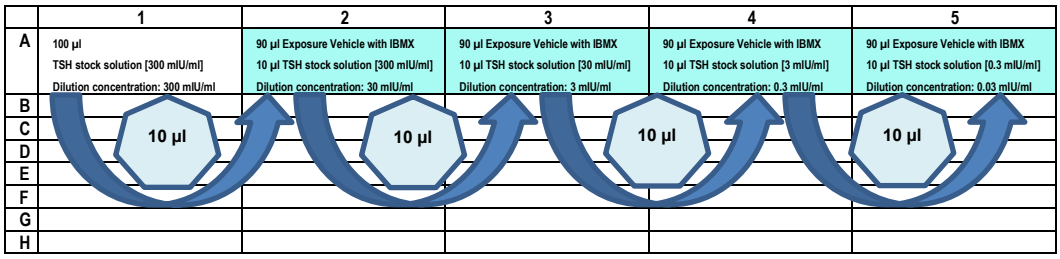
1. One 96-well plate is prepared for the functional assay
   (see the Plate Layouts, PLATE 2 / PLATE 3, point 4.1).
2. 100 µl, (approx. 7 x 10^4 cells/well) of JP09 cell suspension in selection medium are seeded in each well.
3. PLATE 2 is incubated O/N (16 hours +/- 2 hours, 5% CO2, 95% humidity, 37± 1 ºC).

4.1.5 DAY 3 – functional assay – Thursday

1. **Test Items dilutions**
   Depending on the solubility and cytotoxicity, previously identified on DAY 2, non-cytotoxic serial dilutions (e.g. logarhitmic) of test items are prepared either in the Exposure vehicle with IBMX or in 100% DMSO. The Exposure vehicle with IBMX is used in the functional assay instead of the Selection medium in order to prevent low concentrations of cAMP that may be present in cell culture media and to prevent degradation of cAMP by the activity of phosphodiesterases**. Separate dilution plate for test items is recommended to be used. Logarhitmic dilutions (instead of wide decimal dilutions used in the cell viability dose response test), starting with the concentration showing minimally 75% of cell viability (≥CV75) are to be tested in the functional assay.
   *** RPMI medium may contain >350 fmol/µl cAMP*

2. **Positive control / Reference item dilutions**
   1 aliquot of TSH stock solution [300 mIU/ml] is freshly prepared or thawed and equilibrated to RT and vortexed. Working dilutions of TSH are prepared in the Exposure Vehicle with IBMX.
   Working dilutions are stronger (0.03 - 0.3 - 3 – 30 - 300 mIU/ml) than the final tested concentrations used in the functional assay (0.0003 - 0.003 - 0.03 - 0.3 – 3).
   A separate dilution plate is recommended to be used for the preparation of working dilutions.
   Changing tips is essential between the dilutions. Example of dilution procedure is recommended below:

Suggested plate layout for a separate dilution plate (TSH dilutions):



1. 90 µl of Exposure Vehicle with IBMX is applied to wells A2-A5 in the separate dilution plate (blue wells).
2. 100 µl of TSH stock solution [300 mIU/ml] is applied to the well A1 in the dilution plate.
   The volume in the well A1 is mixed with 5 pipette strokes.
   This will result in dilution concentration 300 mIU/ml in the well A1.
3. 10 µl of TSH stock solution [300 mIU/ml] from the well A1 is applied to the well A2.
   The volume in the plate is mixed with 5 pipette strokes.
   This will result in dilution concentration 30 mIU/ml in the well A2.
4. With a new pipette tip, 10 µl of TSH dilution [30 mIU/ml] from the well A2 is applied to the well A3.
   The volume in the plate is mixed with 5 pipette strokes.
   This will result in dilution concentration 3 mIU/ml in the well A3.
5. With a new pipette tip, 10 µl of TSH dilution [3 mIU/ml] from the well A3 is applied to the well A4.
   The volume in the plate is mixed with 5 pipette strokes.
   This will result in dilution concentration 0.3 mIU/ml in the well A4.
6. With a new pipette tip, 10 µl of TSH dilution [0.3 mIU/ml] from the well A4 is applied to the well A5.
   The volume in the plate is mixed with 5 pipette strokes.
   This will result in dilution concentration 0.03 mIU/ml in the well A5.

TSH dilutions should be used within 60 minutes.

3. **Application of the Vehicle control, Solvent control, Reference Item and Test Items**

The Vehicle control, Solvent control, Reference Item and Test Item dilutions are applied according to the plate layout for PLATE 2 (see point 4.1). Maximum of 2 test items at 8 concentrations may be tested in triplicates (1 concentration = 1 triplicate) in PLATE 2. Maximum of 7 concentrations of the Reference Item (TSH) may be tested in triplicates in PLATE 2.

1. Selection medium is aspirated from all wells.
2. Cells are washed with 200 µl of KRH buffer 3 times.
3. After the last wash, cells are equilibrated in the KRH buffer for 30 min.
4. 25 ml of the Exposure Vehicle (with IBMX) is prepared in a reagent pipetting reservoir (see Table 3.3).
5. KRH buffer is aspirated from the wells. Plate is blotted on a blotting paper.
6. Using a multichannel pipette with 200 µl tips:
   - 200 µl of the Exposure Vehicle (with IBMX) is applied into wells A1-A3 (Vehicle control).
   - 198 µl of the Exposure Vehicle (with IBMX) is applied into wells in rows B, C, D, E, F, G, columns 1-3.
   - 190 µl of the Exposure Vehicle (with IBMX) is applied into wells H1-H3.
   - 184 µl of the Exposure Vehicle (with IBMX) is applied into wells in row A, columns 4-6.
   - 100 µl of the Exposure Vehicle (with IBMX) is applied into wells in row B, columns 4-6.

   Slow pipetting is used, whereas the tip must not touch the bottom of the well.
7. If a test item is diluted in the Exposure Vehicle with IBMX, 200 µl of serial test item dilutions from a separate dilution plate are directly applied to the appropriate wells, to achieve final dilutions, starting with the concentration showing minimally 75% of cell viability (≥CV75). Slow pipetting is used, whereas the tip must not touch the bottom of the well. Changing tips is essential between the samples and dilutions.
8. If a test item is diluted in a solvent (e.g., 100% DMSO), 198 µl of the Exposure Vehicle with IBMX are applied into all appropriate wells left for test items diluted in 100% DMSO. Slow pipetting is used, whereas the tip must not touch the bottom of the well.

9. Using a pipette with 0.5 – 10 µl tips:
   - 2 µl of 100% DMSO is applied into wells B1-B3. This will result in final concentration of DMSO 1%.
   - With a new pipette tip, 2 µl of TSH dilution [0.03 mIU/ml] from the well A5 in the separate dilution plate is applied into wells C1 – C3 in PLATE 2. This will result in final concentration of TSH 0.0003 mIU/ml.
   - With a new pipette tip, 2 µl of TSH dilution [0.3 mIU/ml] from the well A4 in the separate dilution plate is applied into wells D1 – D3 in PLATE 2. This will result in final concentration of TSH 0.003 mIU/ml.
   - With a new pipette tip, 2 µl of TSH dilution [3 mIU/ml] from the well A3 in the separate dilution plate is applied into wells E1 – E3 in PLATE 2. This will result in final concentration of TSH 0.03 mIU/ml.
   - With a new pipette tip, 2 µl of TSH dilution [30 mIU/ml] from the well A2 in the separate dilution plate is applied into wells F1 – F3 in PLATE 2. This will result in final concentration of TSH 0.3 mIU/ml.
   - With a new pipette tip, 2 µl of TSH stock solution [300 mIU/ml] from the well A1 in the separate dilution plate is applied into wells G1 – G3 in PLATE 2. This will result in final concentration of TSH 3 mIU/ml.
   - With a new pipette tip, 10 µl of TSH stock solution [300 mIU/ml] from the well A1 in the separate dilution plate is applied into wells H1 – H3 in PLATE 2. This will result in final concentration of TSH 15 mIU/ml.
   - With a new pipette tip, 16 µl of TSH stock solution [300 mIU/ml] from the well A1 in the separate dilution plate is applied into wells A4 – A6 in PLATE 2. This will result in final concentration of TSH 24 mIU/ml.
   - With a new pipette tip, 100 µl of 100%DMSO will be applied into the wells B4 – B6. This will result in final concentration of DMSO 50%.
   - With a new pipette tip, 2 µl of Test items dilutions in a selected solvent solvent (to 100x stronger concentrations) are applied into the appropriate wells in PLATE 2. Changing tips is essential between the samples and dilutions.

As a result, the PLATE 2 was prepared as follows:

Vehicle control: 200 µl of the Exposure Vehicle with IBMX were applied to all appropriate wells.
Solvent control: for every test item diluted in 100% solvent (e.g., 100% DMSO), 198 µl of the Exposure Vehicle with IBMX were applied to all appropriate wells. 2 µl of 100% solvent were added to the appropriate wells to achieve final tested concentration 1% (e.g., 1% DMSO).
TSH (reference item): 198 µl of the Exposure Vehicle with IBMX were applied to wells in rows C, D, E, F, G, in columns 1, 2, 3. 2 µl of TSH dilutions from the separate dilution plate were applied into triplicate wells in rows C, D, E, F, G, in columns 1, 2, 3. 190 µl of the Exposure Vehicle (with IBMX) were applied to wells in row H1-H3. 10 µl of TSH dilution from the separate dilution plate (well A1) were applied into the triplicate wells H1-H3. 184 µl of the Exposure Vehicle with IBMX were applied to wells A4-A6. 16 µl of TSH dilution from the separate dilution plate (well A1) were applied into the triplicate wells A4-A6.
Cytotoxic chemical: 100 µl of the Exposure Vehicle (with IBMX) were applied into wells in row B, columns 4-6. 100 µl of 100% DMSO were applied into the wells B4 – B6.
Test Items diluted in the Exposure Vehicle with IBMX: serial dilutions of a test item diluted in the Exposure Vehicle with IBMX were directly applied to the appropriate wells, to achieve final dilutions, starting with the concentration showing minimally 75% of cell viability (≥CV75) (see the Plate Layout, PLATE 2).
Test Items diluted in a solvent: 198 µl of the Exposure Vehicle (with IBMX) were applied to all appropriate wells. 2 µl of a test item serially diluted in 100% solvent (e.g. 100% DMSO) were added to the appropriate wells to achieve final serial dilutions and non-cytotoxic concentration of the solvent (e.g., 1% DMSO), starting with the concentration showing minimally 75% cell viability (≥CV75).

PLATE 2 is incubated O/N (16 hours +/- 2 hours, 5% CO2, 95% humidity, 37± 1 ºC).
PLATE 2 may be covered with a plate sealer to prevent evaporation.

Collection of Samples from PLATE 2 and they transfer into a SAMPLE PLATE
After incubation, 200 µl of the cell culture supernatant (Exposure Vehicle with IBMX) is removed from the PLATE 2 and transferred to another 96 well plate (SAMPLE PLATE) to the same appropriate wells using a multichannel pipette. Changing tips between columns is inevitable. Samples are used for cAMP quantification using ELISA (see below).
If not used immediately, samples may be stored in -20˚C for longer storage. In case of longer storage in -20˚C, the SAMPLE PLATE must be sealed with a plate sealer, covered with a lid, and placed into a Zip-lock bag.

Post-exposure cytotoxicity test:
After collection of supernatant from PLATE 2, a standardized in vitro basal cytotoxicity assay to determine either cell viability or metabolism may be performed in PLATE 2 in order to obtain data for normalisation of cAMP levels produced during the functional assay.

4.1.6 DAY 4 - detection of extracelular cAMP - Friday

cAMP increase is measured using commercial standardized ELISA non-radioactive kit CA200 (Merck), the Direct cAMP Enzyme Immunoassay, non-Acetylated Version, 2,000 pmol/ml. Specific procedure described in the Technical documentation provided with the kit used must be strictly followed. Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipette tips and other items that are used in the assay with bare hands.

Reagent Preparation
Standards can be made up in either glass or polypropylene tubes. Avoid polystyrene tubes.

1. Allow all reagents to warm to room temperature.
2. Prepare cAMP Standards according to the procedure in the technical documentation of the kit used.
   cAMP Standards (Non-Acetylated Version, 2,000 pmol/ml) – Allow the Cyclic AMP Standard to warm to room temperature. Label five 12 ´ 75 mm tubes 1 through 5. Pipette 900 ml of 0.1 M HCl into tube 1 and 750 ml of 0.1 M HCl into tubes 2–5. Add 100 ml of the Cyclic AMP Standard to

tube 1. Vortex thoroughly. Add 250 ml of tube 1 to tube 2 and vortex thoroughly. Continue for tubes 3 through 5. The concentration of cAMP in tubes 1 through 5 will be 200, 50, 12.5, 3.12, and 0.78 pmole/ml, respectively. Diluted standards should be used within 60 minutes of preparation.

3. **1´ Wash Buffer** – Prepare 1´ Wash Buffer by diluting 10 ml of the Wash Buffer Concentrate provided with the kit with 90 ml of deionized water. 1´ Wash Buffer can be stored at room temperature for 3 months.

Sample Handling:
The Direct cAMP Enzyme Immunoassay is compatible with cAMP samples that have been treated with hydrochloric acid to stop endogenous phosphodiesterase activity. Samples in this matrix can be read directly without evaporation or further treatment. The supernatants may be assayed directly with the EIA Direct cyclic AMP kit, Catalog Number CA200 (Merck) ***(note: phosphodiesterase inhibitor IBMX is used in the Exposure Vehicle, thus the cell culture supernatants from the functional assay may be used directly in the Direct cAMP Enzyme Immunoassay when diluted in 0.1 M HCl ≥ 1:10 – 1:20 to fit the standard curve).***

Assay Procedure, Non-Acetylated version
(repetition of important steps procedure as described in the technical documentation for kit CA200, Merck).
Allow all reagents to warm to room temperature for at least 30 minutes before opening.

1. Equilibrate the kit CA200, all reagents and samples to RT.
   The kit CA200 (Merck) use break-apart multiwell strips. Determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal. Store unused wells at 2–8 °C.
2. Pipette 50 µl of the Neutralizing Reagent into each well, except the TA and Blank wells.
3. Pipette 100 ml of 0.1 M HCl into the NSB and the Bo (0 pmole/ml Standard) wells.
4. Pipette 100 ml of Standards 1 through 5 into the appropriate wells.
5. **Transfer and dilution of samples directly in PLATE 3:** pre-pipette the desired amount of 0.1 M HCl (90 µl when diluting the samples 1:20, 80 µl when diluting the samples 1:10), then apply the samples (10 µl when diluting the samples 1:20 and 20 µl when diluting the samples 1:10). The samples are transferred as triplicates to the appropriate wells according to the Plate Layout for PLATE 3 (see point 4.1), using a multichannel pipette with 3 tips. The samples are transferred directly from PLATE 2 if used directly after the functional assay, or from the SAMPLE PLATE if collected for long-term storage (see point 4.1.4). *Note: the recommended dilution factor is ≥ 1:10 to fit the standard calibration curve. However, the dilution factor may differ according to the potency of the tested item to induce the increase of cAMP. All wells from the functional assay (excluding the empty wells) represent unique samples in which cAMP level is measured in the ELISA assay.*
6. Pipette 50 ml of 0.1 M HCl into the NSB wells.
7. Pipette 50 ml of blue cAMP-Alkaline Phosphatase Conjugate into each well except the TA and Blank wells.
8. Pipette 50 ml of yellow cAMP EIA Antibody into each well, except the Blank, TA (total activity), and NSB (non specific binding) wells. **Note: Every well used should be Green in color except the NSB wells which should be Blue. The Blank and TA wells are empty at this point and have no color.**
9. Incubate the plate at room temperature for 2 hours on a plate shaker at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
10. Empty the contents of the wells and wash by adding 200 ml of 1´ Wash Buffer to every well.
    Repeat the wash 2 more times for a total of 3 washes.
11. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer. Note: Prior to addition of substrate, ensure there is no residual Wash Buffer in the wells. Any remaining Wash Buffer in the wells may cause variation in assay results.
12. Add 5 ml of the blue cAMP-Alkaline Phosphatase Conjugate to the TA wells.
13. Add 200 ml of the p-Nitrophenyl Phosphate Substrate Solution to every well. Incubate at room temperature for 1 hour without shaking.
14. Add 50 ml of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
15. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader cannot be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Results
It is recommended the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic curve fitting program such as "AssayZap" (www.biosoft.com).
The concentration of cAMP can be calculated as follows:

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:
   Average Net OD = Average Bound OD – Average NSB OD

2. Calculate the binding of each pair of standard wells
   as a percentage of the maximum binding wells (Bo), using the following formula:
   Percent Bound = Net OD / Net Bo OD x 100

3. Using the Logit-Log paper plot Percent Bound (B/Bo) versus Concentration of cAMP for the standards. Approximate a straight line through the points. The concentration of cAMP in the unknowns can be determined by interpolation.

The intensity of yellow color is inversely proportional to the concentration of cAMP in either the standards or the samples. Reversed axis values and ascending curve-shape may be used in order to better demonstrate the increase of cAMP.

Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

Average Net OD = Average Bound OD – Average NSB OD

Calculate the binding of each pair of standard wells
as a percentage of the maximum binding wells (Bo), using the following formula:

Percent Bound = Net OD / Net Bo OD x 100

4.2 Data evaluation, interpretation of results, acceptance criteria

In cell-based assays, a threshold of 20 % reflects the general value of biological variability.

A test item is predicted as a "positive thyroid disruptor in vitro"
if it induced, compared to the average values of the Solvent control (or, of the Vehicle control, if the test item was diluted in the Exposure Vehicle with IBMX without the use of any solvent), ≥ 1.2 fold increase in cAMP production at least at two (even non-consecutive) concentrations.

A test item is predicted as a "potentially weak thyroid disruptor in vitro"
if it induced, compared to the average values of the Solvent control (or, of the Vehicle control, if the test item was diluted in the Exposure Vehicle with IBMX without the use of any solvent), ≥ 1.2 fold increase in cAMP production at least at one tested concentration.

A test item is predicted as a "negative thyroid disruptor in vitro"
if it did not induce, compared to the average values of the Solvent control (or, of the Vehicle control, if the test item was diluted in the Exposure Vehicle with IBMX without the use of any solvent), ≥ 1.2 fold increase in cAMP production at least at one tested concentration.

5 ACCEPTANCE CRITERIA:

- The average induction of the positive control (TSH, 3 mIU/ml) should be > 1.2 fold compared to the value of the Vehicle control.
- Dose-response curve of the reference item (TSH, 24 - 15 – 3 - 0.3 – 0.03 – 0.003 – 0.0003 mIU/ml) has to be observed.