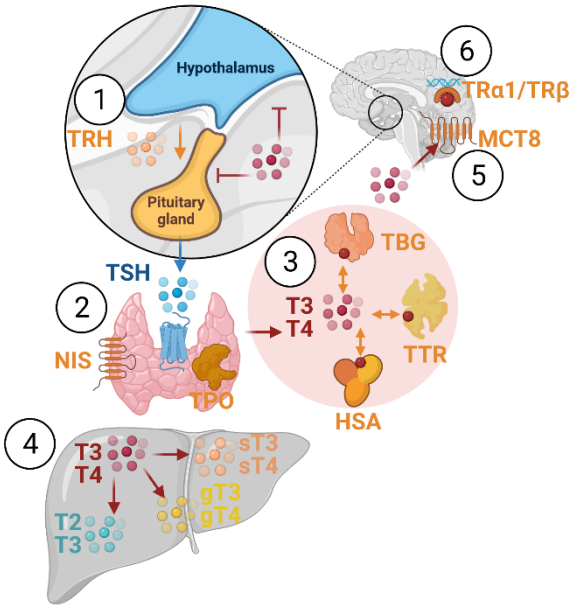
European Commission

STANDARD OPERATING PROCEDURE

for solubility testing, used in the thyroperoxidase (TPO) inhibition assay based on oxidation of Amplex UltraRed (AUR-TPO), version 2.0

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

Fant K.



2023

Joint Research Centre

This Standard Operating Procedure (SOP) has been prepared within the context of a collaboration agreement 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. It aims to provide evidence-based scientific support to the European policymaking process. The contents of this publication do not necessarily reflect the position or opinion of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

This SOP is part of a series of 4 SOPs used to perform the "Thyroperoxidase Inhibition Assay based on Amplex UltraRed (AUR-TPO)":

1. SOP "Thyroperoxidase Activity Assay with Amplex UltraRed (AUR-TPO)" v1.0 (used in Part1 of the validation study)
2. **SOP "Solubility determination by visual inspection" v2.0** (used in Part 1 and Part 2 of the validation study)
3. SOP "Culture of FTC-238 and FTC-238/hrTPO cells" v3.0 (used in Part 1 and Part 2 of the validation study)
4. SOP "Thyroperoxidase (TPO) extract preparation" v1.0 (used in Part 1 and Part 2 of the validation study)

SOP "Thyroperoxidase Activity Assay with Amplex UltraRed (AUR-TPO)" has been updated after Part 1, the other SOPs were considered complete to be used also in Part 2.

The method was developed by US EPA and subsequently implemented by the EU-NETVAL test facility RISE (Sweden) within the validation study.

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Dokumentnr.	Informationstyp	Omfattning	Grupp	Författare
17783	Instruktion	4.Metodspecifikt	General cell lab procedures	Kristina Fant
Utgåva	Process		Godkänt	Godkännare
2.0	Provning		2021-02-15	Benny Lyvén

Solubility determination by visual inspection

Aim and use of this SOP

This standard operating procedure describes chemical solubility determination based on visual inspection, applicable to e.g. SOP KM 17050 "*Transactivation assay for the detection of compounds with (anti)androgenic potential using AR-CALUX® cells*" and SOP KM 20077 *"Thyroperoxidase Activity Assay with Amplex UltraRed (AUR-TPO)"*.

The purpose is to assess stock solution solubility in solvent, with observation of stability upon dilution in medium, including incubation equivalent to the conditions of assay cell culture/test item exposure. For SOP KM 17050, the dilution factor is 500x for interim dilution and 1000x for complete dilution, and the incubation time is 24 hours. For SOP KM 20077, the dilution factor is 62.5x for interim dilution and 500x for complete dilution, and the incubation time is 30 minutes for the main assay and 24 hours for the cytotoxicity control assay. Other methods may use dilution factors and incubation times as appropriate.

For accurate handling of potentially hazardous test, reference and control items, refer to the laboratory's internal procedures.

Definitions and Abbreviations

Three trial concentrations are prescribed, starting with the stock solution corresponding to the highest test concentration, one concentration 10x lower, and one concentrations selected in between. In the event of insolubility at the lowest evaluated concentration, further dilutions may be considered.

For test methods where the maximal test concentration of the stock solution is 50 mg/mL (e.g., AR-CALUX), the following three trial concentrations are suggested (abbreviated as C50, C15, C5):

C50	Solvent stock solution concentration: 50mg/mL Medium 1000-fold dilution concentration: 50µg/mL
C15	Solvent stock solution concentration: 15mg/mL Medium 1000-fold dilution concentration: 15µg/mL
C5	Solvent stock solution concentration: 5mg/mL Medium 1000-fold dilution concentration: 5µg/mL

When the maximal test concentration of the stock solution is 100 mM (e.g. AUR-TPO), the following three trial concentrations are prescribed (abbreviated as C100, C30 and C10):

C100	Solvent stock solution concentration: 100 mM Buffer 500-fold dilution concentration: 200 µM
C30	Solvent stock solution concentration: 30 mM Buffer 500-fold dilution concentration: 600 µM
C10	Solvent stock solution concentration: 10 mM Buffer 500-fold dilution concentration: 20 mM

Depending on the intended test method for the dissolved chemicals, the tested concentrations may be altered.

Principle

The test chemical is dissolved in appropriate solvent and assessed for solubility by visual inspection for signs of turbidity due to solid particulate or liquid droplet suspension. Thereafter

the test chemical is diluted in appropriate assay medium/buffer according to the test method SOP and assessed for stability. Stability determination in medium, where observation of dissolution may be obscured by foaming, is assisted by centrifugation to detect any insoluble suspension as a sediment deposit. Solubility in medium/buffer is also checked by microscope examination of a droplet.

An overview (flowchart) of the procedure is included in Annex 1.

Equipment

- Balance (Balance resolution minimum 0.1 mg)
- Vortex mixer
- Ultrasonic water bath
- Thermal water bath
- Incubator, 37 °C, humidified, 5 % CO_2
- Centrifuge with fixed head for spin at 10,000g
- Microscope, for observation of clarity/turbidity of medium/buffer droplets
- Cell culture microplate (96-well flat-bottom, preferably seal wrapped individually to minimise interference from dust etc.) for observation of sample medium/buffer droplets
- Clear glass vials (at least 5 mL size) with caps, for test item weighing and stock solutions
- Test tubes (clear plastic, 15 mL size) with caps, for medium/buffer dilutions and incubation
- Test tubes (clear plastic, 50 mL size) with caps, for medium/buffer handling
- Centrifuge tubes (conical, clear plastic, 1.5mL size or similar)
- Micro-pipettes (ranges: 2 – 20 µL; 10 – 100 µL; 100 – 1000 µL)
- Pipette (5 mL)
- Polyfoam floating tube racks (suitable for the glass vials)

Medium, Chemicals and Reagents

- Complete assay medium or buffer suitable for the intended test method, specified by the test method SOP.
    - For test method AR-CALUX, Complete assay medium 1× AR-CALUX, specified by SOP KM 17050, should be used.
    - For the AUR-TPO assay, Potassium phosphate buffer (0.2M, pH 7.4) should be used in the first step, and for test items that show an activity also Complete assay medium 1×FTC CTG should be evaluated, c.f. SOP KM 20077.
- Solvent appropriate for the intended test method.
    - For test method AR-CALUX, allowed solvents are specified by SOP KM 17050.
    - For test method AUR-TPO, allowed solvents are specified by SOP KM 20077.

  DMSO is the preferred solvent for both AR-CALUX and AUR-TPO, however in the event of insolubility other solvents such as water may be evaluated.

Method

The method is illustrated in a flowchart in Appendix 1, using the example of the AR-CALUX test method.

Solubility in solvent (stock solutions)

Note: Soluble stock solutions should be used for medium stability testing within 24 hours.

1. Add appropriate solvent to a known amount of test item (SOP KM 17182) in a clear glass vial at a volume sufficient for visual inspection of solubility, starting at the upper concentration C50 or C100 (suggested minimum weight: 25 mg, suggested minimum volume: 0.5 mL). For a swift work flow, it is recommended to prepare several test items together as a series.

   The chemical weight and solvent volume are calculated according to:

$$\text{Volume solvent [µL]} = \frac{\text{Weight chemical [mg]} * 1000}{\text{Concentration required [mg/mL]}}$$

2. Record the test item weight(s) and solvent volume(s), preferably using the "Solubility study log" template.
3. Vortex mix
   - 3.1 Vortex mix for 1 minute (repeating, if appropriate) with visual check for dissolution against a suitable background illumination/contrast. A black background is recommended for effective observation of white suspensions. Microscopic evaluation might also be performed.
   - 3.2 If the test item is already soluble, indicate the vortex time and result, preferably in the study log, and set the solution aside for stability determination in medium.
4. Ultrasonic immersion (for crystal disaggregation)
   - 4.1 If not completely soluble after vortex mixing, immerse the vials in the ultrasonic water bath for 15 minutes, supported in a polyfoam floating tube rack.
   - 4.2. Repeat the vortex mix for 10 seconds to ensure homogeneity, with visual check for dissolution against a suitable background illumination/contrast. Microscopic evaluation might also be performed.
   - 4.3. If the test item is now soluble, indicate the sonication time and result, preferably in the study log, and set the solution aside for stability determination in medium.
5. Thermal immersion (to accelerate kinetic delay)
   - 5.1. If not completely soluble after sonication, immerse the vials in the thermal water bath at 37°C for 30 minutes, retained in the same polyfoam rack. NB: Also allow the mixture to cool for at least 30 minutes, checking for possible recrystallization, and note observations preferably in the study log.
   - 5.2. Repeat the vortex mix for 10 seconds to ensure homogeneity, with visual check for dissolution against a suitable background illumination/contrast. Microscopic evaluation might also be performed.
   - 5.3. If the test item is now soluble, indicate the warming time and result, preferably in the study log, and set the solution aside for stability determination in medium.
6. Solution standing (to ensure complete dissolution)
   - 6.1. If the test item is evidently soluble but visible traces of undissolved material remain, allow the solution to stand at room temperature for 1 hour (approx.) to complete the dissolution.
   - 6.2. Repeat the vortex mix for 10 seconds to ensure homogeneity, with visual check for dissolution against a suitable background illumination/contrast. Microscopic evaluation might also be performed.

6.3. If the test item is now satisfactorily soluble, indicate the standing time (approx.) and result, preferably in the study log, and set the solution aside for stability determination in medium.

7. Stock solution insoluble at C50/C100

7.1 If the test item persists as insoluble at concentration C50/C100, then solubility is attempted at the intermediate concentration C15/C30, preparing fresh stock solution.

7.2 Repeat the above procedure (steps 1 – 6) using re-weighed test item, appropriate for concentration C15/C30, recording weight(s) and volume(s) , preferably in the study log.

8. Stock solution insoluble at C15/C30

8.1 If the test item persists as insoluble at the intermediate concentration C15/C30, then solubility is attempted at the lower concentration C5/C10, preparing fresh stock solution.

8.2 Repeat the above procedure (steps 1 – 6) using re-weighed test item, appropriate for concentration C5/C10, recording weight(s) and volume(s), preferably in the study log.

9. If the test item is not soluble in the preferred solvent, even at C5/C10, either continue the dilution series or repeat the above steps using the alternative solvent(s) (specified by the test method). Further dilutions than C5/C10 may be appropriate. Record results, preferably in the study log.

10. Print the available records (e.g. the Solubility study log, if used) when finished. Data should be provided for stock solution preparation and solubility (including, if applicable, confirmation of the duration of vortex mixing, sonication, warming and standing). This is included in the available Solubility study log template. It also allows space for conclusions (e.g., C50 / C15 / C5 soluble, or C5 insoluble) and comments.

Stability in medium (with incubation)

Note 1: For the AR-CALUX method, stability in medium is determined for interim (500-fold) and full (1000-fold) dilutions, including two time points for the latter:

1) pre-incubation (time zero), and
2) post-incubation (24 hours).

The observation at 500-fold dilution is for information only.

Effective solubility is the highest concentration (C50, C15, C5) at 1000-fold dilution where no precipitation is observed at either time point.

Other methods may use other dilution factors and incubation times as specified by the test method description.

Note 2: Although test items are generally soluble in solvent at C50, precipitation frequently occurs on aliquot addition to medium/buffer (dilution 500/1000-fold, pre/post-incubation). Therefore, medium/buffer stability testing of <u>all</u> soluble stock solution concentrations (in parallel) is standard procedure.

Thus (AR-CALUX):
- if stock solution is C50 soluble: medium stability is tested at C50, C15 and C5;
- if stock solution is C15 soluble: medium stability is tested at C15 and C5;
- if stock solution is C5 soluble only: medium stability is tested at C5 and possibly also at lower concentrations.

or (AUR-TPO):

- if stock solution is C100 soluble: buffer stability is tested at C100, C30 and C10;
- if stock solution is C30 soluble: buffer stability is tested at C30 and C10;
- if stock solution is C10 soluble only: buffer stability is tested at C5 and possibly also at lower concentrations.

1. Warm a sufficient volume of medium/buffer (10 mL per test item and concentration, and 10 mL extra for a blank) to 37°C in the thermal water bath.
2. Pipette an appropriate amount of stock solution of the highest available soluble concentration (determined according to the procedure above) to ~5 mL medium/buffer in 15 mL clear plastic tubes (e.g. 10 µL stock solution in 5 mL for 500-fold dilution) and vortex mix for 10 seconds.
3. Dilute the remaining stock solution (by simple addition of solvent) to the next lower concentration(s):

   For C15/C30 stock solution, dilute the C50/C100 stock 3.33-fold.

   For C5/C10 stock solution, dilute the C15/C30 stock 3-fold.

Dilution volumes are calculated according to:

$$\text{Volume}^{1} \text{ of solvent to add } [\mu\text{L}] = \text{Final Volume } [\mu\text{L}] - \text{Initial Volume } [\mu\text{L}]$$

where:

$$\text{Final Volume } [\mu\text{L}] = \text{Dilution Factor} * \text{Initial Volume } [\mu\text{L}]$$

The calculations are incorporated in the ”Solubility study log”. If this template is not used, the calculations have to be recorded and archived.

4. Record the total and added solvent volumes, preferably in the “Solubility study log”.
5. Repeat the medium/buffer dilution (step 2) for the lower concentration(s) of stock solution, prepared consecutively (steps 3 – 4) as applicable.
6. Prepare an extra tube containing 10 mL medium/buffer only, as reference blank.
7. Transfer 950 µL aliquot samples (including a blank) to 1.5 mL clear plastic conical vials, for solubility determination (500-fold medium dilution) assisted by centrifugation.
8. Pipette 50 µL aliquot samples (including a blank) to a 96-well flat-bottom microplate, for solubility determination (500-fold medium dilution) assisted by microscopy.

Note 3: For convenience, it is recommended to prepare the samples for centrifugation and microscopy together, enabling simultaneous observation.

9. Centrifuge the vials (950 µL aliquots) at 10,000 g for 5 minutes at room temperature.
10. Examine the microplates (50 µL aliquots) under the microscope (at e.g. 20X magnification) with reference to the blank, checking for occurrence of undissolved material, recording the solubility observations in the study log. It is recommended to also save at least one image per test chemical.
11. Check the centrifuge vials for occurrence of deposited precipitate (visible as a small speck or pellet) indicative of insolubility, alongside the blank for comparison, recording the solubility observations, preferably in the study log. For effective observation of a white deposit, a contrasting black background is recommended.

[1] rounded to nearest integer (µL).

12. Complete the dilution (1000-fold) by doubling the medium/buffer volume (4 mL) remaining in the tube and vortex mix again for 10 seconds.
13. Repeat steps 7 – 11 for pre-incubation solubility determination at the full dilution (1000-fold medium dilution, time zero for AR-CALUX, 500-fold buffer dilution , time zero for AUR-TPO).
14. Incubate the full dilutions (with tube caps loosened, blank included) under conditions and for a time equivalent to the conditions and duration of the assay test item exposure.

    AR-CALUX: 24 hours in a cell culture incubator at 37°C and 5 % CO_2 under humidified atmosphere.

    AUR-TPO: 30 minutes at 37°C for the main assay, 24 hours in a cell culture incubator at 37°C and 5 % CO_2 under humidified atmosphere for the cytotoxicity control assay.
15. Repeat the vortex mixing (10 seconds) and centrifugation (1000 µL aliquots) with check for deposited precipitate, as post-incubation solubility determination (24 hours) recording the results, preerably in the study log.
16. Repeat the microscope examination (50 µL aliquot) as supplementary final solubility determination (24 hours) recording the results in the study log. It is recommended to also save at least one microscope image per test chemical.
17. From the two observations of the full dilutions, pre- and post-incubation, note the effective solubility result, preferably in the study log. Effective solubility is the highest concentration (e.g. C50, C15, C5) at 1000-fold dilution where **no precipitation is observed at either time point**.
18. Print the recorded observations (e.g. the Solubility study log, if used) when finished. This includes raw data for medium dilution stability with incubation, and overall conclusions on solubility (e.g., explicit soluble concentration).

Results

Solubility testing is normally performed as part of another study, in conjunction with some test method for biological safety. At least the following documents should be included in the archived folder:

- A report containing a summary and conclusion of the study (see below for more details) with specific details and conclusions regarding the solubility testing
- Study plan for the project including details regarding the solubility testing
- Details regarding equipment and chemicals
- Microscope images acquired during the work
- Solubility study log
- Other information relevant for the work

Report

The report shall contain at least:

- A summary of the study
- Conclusions drawn from the study

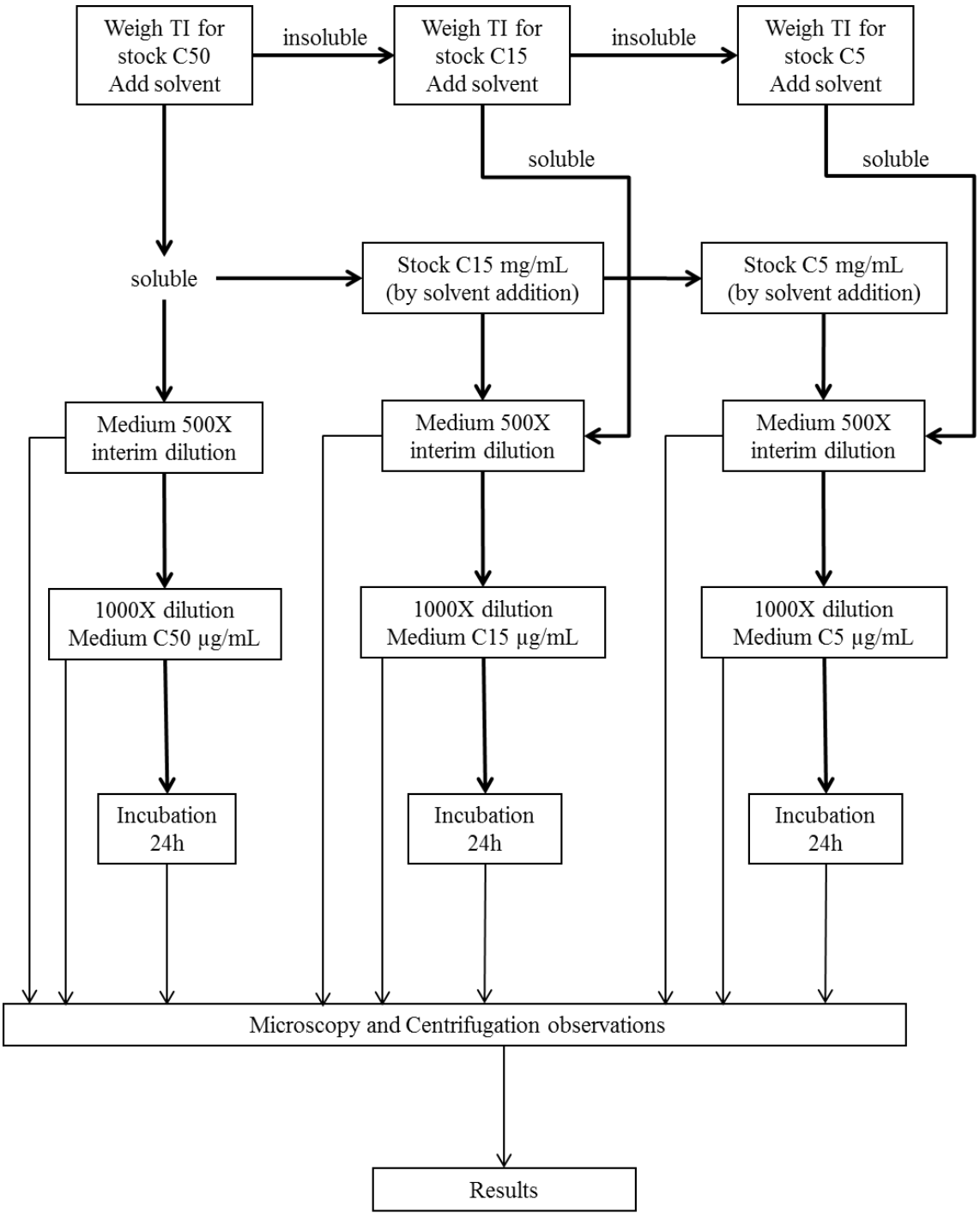
Revision history of SOP

Edition*	Date	Author	Short information about the changes
1.0	2021-02-15	Kristina Fant	Made SOP more general, to more easily be applied to the AUR-TPO assay.

* Edition of the SOP which is subject to revision. Once the changes are approved the SOP will obtain a new (higher) edition number.

APPENDIX 1

Flowchart: solubility determination by visual inspection



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