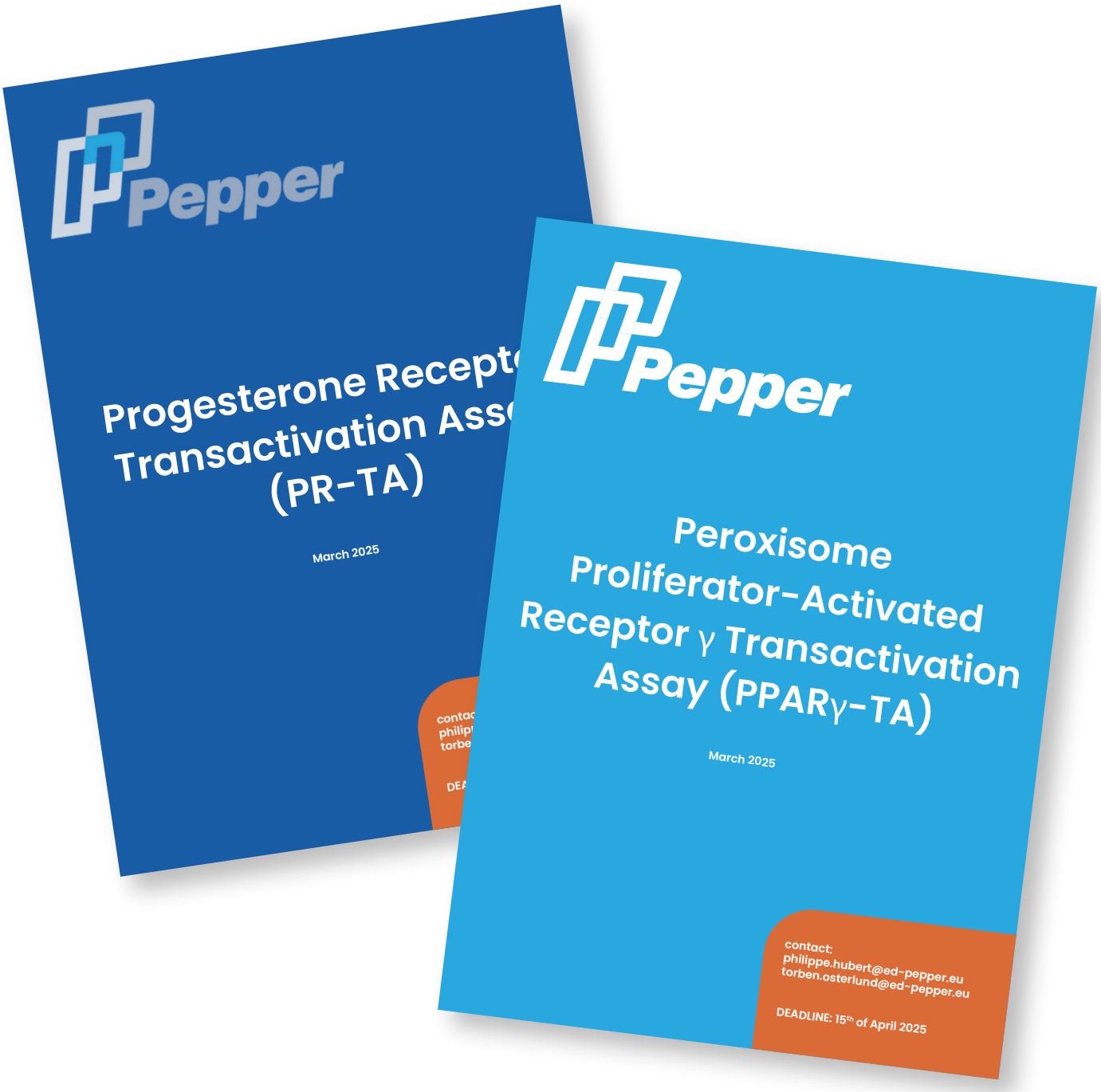
# 2 New Methods in call for “testing laboratories”

The application processes for the two methods are independent. Labs may apply for one or both methods separately.



# Peroxisome Proliferator-Activated Receptor γ Transactivation Assay (PPARγ-TA)

March 2025

contact:
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torben.osterlund@ed-pepper.eu

DEADLINE: 20th of April 2025

# Peroxisome Proliferator-Activated Receptor γ Transactivation Assay (PPARγ-TA)

## References

Garoche C, Boulahtouf A, Grimaldi M, Toporova L, den Broeder MJ, Legler J, Bourguet W, Balaguer P. “Interspecies differences in activation of Peroxysome proliferator-activated receptor γ by pharmaceutical and environmental chemicals”. Environ Sci Technol 2021. DOI: https://doi.org/10.1021/acs.est.1c04318

Léger T, Balaguer P, Le Hégarat L, Fessard V. Fate and PPARγ and STATs-driven effects of the mitochondrial complex I inhibitor tebufenpyrad in liver cells revealed with multi-omics. J Hazard Mater. 2023 Jan 15;442:130083. DOI: https://doi.org/10.1016/j.jhazmat.2022.130083

## Summary of the method

The peroxisome proliferator-activated receptor γ (PPARγ) is a well-known regulator of adipocyte differentiation, immune responses, proliferation of different tissues, and general metabolism including fat storage. PPARγ has been the target of drug discovery projects for a long period. Among approved medicines are the glitazones which enhance insulin sensitivity. There are numerous other metabolites and xenobiotics that are able to interfere with binding to PPARs. However, due to the obligatory heterodimerization of PPAR with the retinoid X receptor (RXR), it is complicated to distinguish effects caused by PPAR ligands from effects caused by RXR ligands. Moreover, dysregulation of PPARγ signaling is associated with the development of several tumors.

This cell-based in vitro method measures the specific activation or inhibition of chemical substances of the human PPARγ through regulation a luciferase reporter gene. Similar to other transactivation assays, PPARγ-TA relies on the binding and activation of chemicals to PPARγ to induce the luciferase. This can be turned into an antagonistic assay using rosiglitazone as inducing agonist that may be competed by test chemicals.

## Readout and Result

The assay is based on HeLa cells expressing the DNA binding domain (DBD) of the yeast transcription factor GAL4 fused to the ligand binding domain (LBD) of human PPARγ along with the responsive element of GAL4 fused to luciferase. This allows for transactivation assessment of PPARγ by measuring the luciferase activation. This enables the identification of PPARγ agonists and, in the presence of a reference agonist, also of PPARγ antagonists.

Below are the outlines of the workflow, the data interpretation and plate designs.

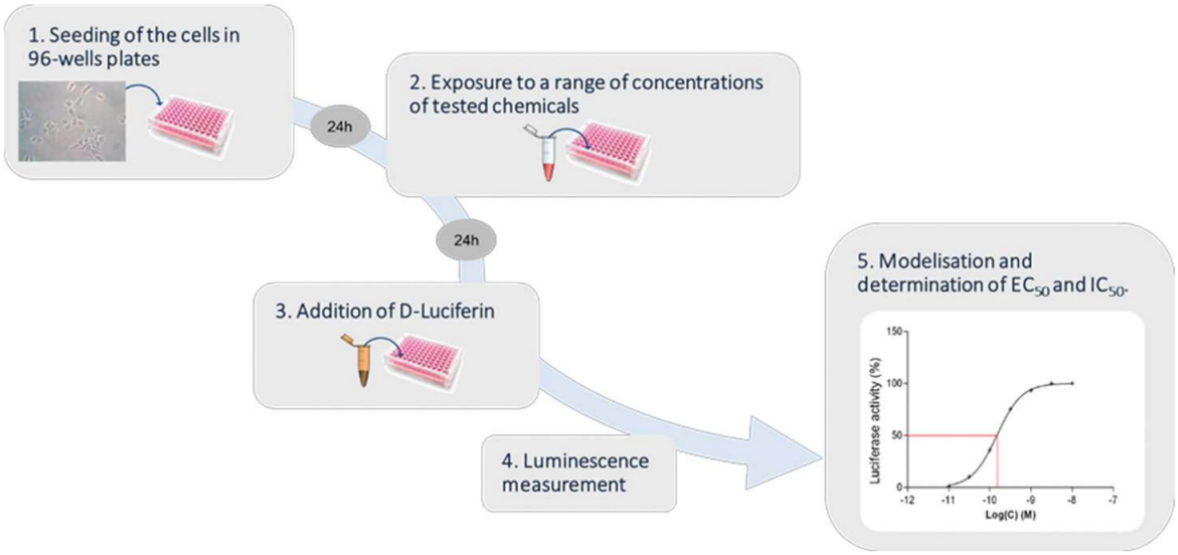


***Figure 1: Workflow of the PPARγ-TA**. First, cells are seeded and incubated in a 96-well plate for 24 hours. Afterwards, cells are treated with a test compound for 24 hours. Thereafter, D-Luciferin is added, and Luminescence is measured.*

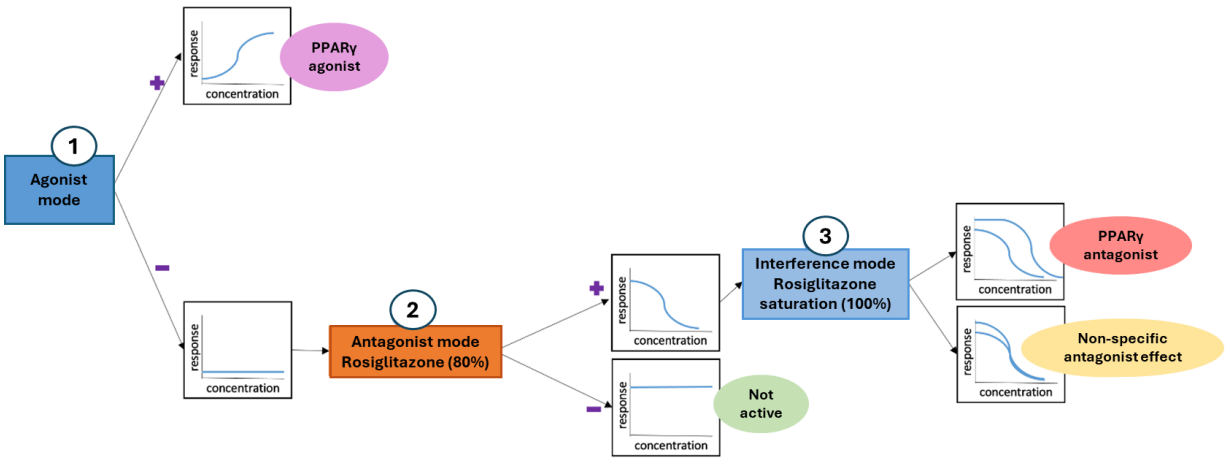


***Figure 2: Prediction model of the PPARγ-TA.** Substances eliciting an effect in agonism mode (1) are treated as PPARγ agonists. Substances not eliciting an effect in agonism mode are tested in antagonism mode (2). Substances that are neither active in agonism nor in antagonism mode are non-active. Substances showing an effect in antagonism mode are tested in interference mode (3) to distinguish between PPARγ antagonists and substances interfering with luciferase activity.*

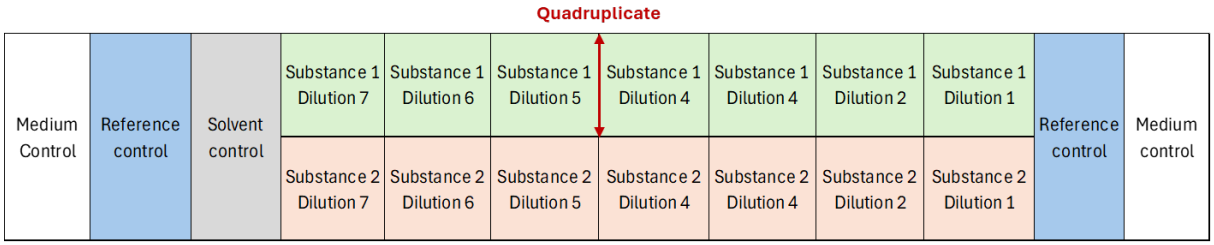


***Figure 3*: Plate layout for agonism mode** *(96-well plate). 2 test items are tested per plate in 7 concentrations and quadruplicates. Rosiglitazone is used as reference control.*

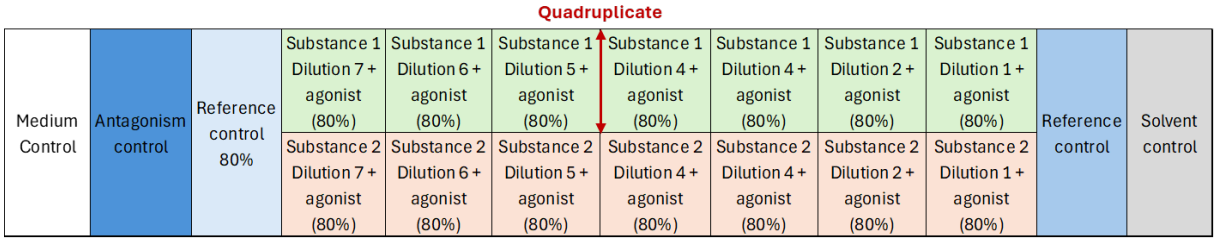


***Figure 4: Plate layout for antagonism mode*** *(96-well plate). 2 test items are tested per plate in 7 concentrations and quadruplicates. Rosiglitazone is used as reference control. A lower concentration Rosiglitazone is used to achieve 80% response. Antagonism control includes a co-exposure with Rosiglitazone at 80% response with a reference antagonist (T0070907).*

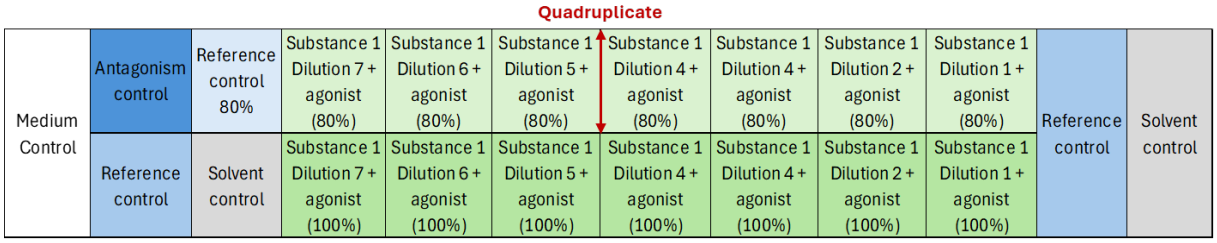


***Figure 5: Plate layout for interference*** *mode (96-well plate). 1 test item is tested in antagonism (upper part) and interference (lower part) per plate in 7 concentrations and quadruplicates. Rosiglitazone is used as reference control. Different concentrations for Rosiglitazone are applied to achieve 80% and 100% response. Antagonism control includes a co-exposure with Rosiglitazone at 80% response with a reference antagonist (T0070907).*

## ANNEX 1: Summary of validation steps

**Transferability phase:**

- 3 modes: PPARγ agonism, PPARγ antagonism, Interference (Interference will be tested for substances showing an effect in antagonism mode).
- 3 **independent, valid runs** (biological replicates) per chemical. This corresponds to 3 plates that contain different cell culture passages and are treated independently from each other.
- 2 Chemicals can be tested in parallel on the same plate for agonism or antagonism. Interference mode is performed alongside antagonism mode with the same substance on the same plate. Several plates can be run in parallel in a day.
- A total of 4 substances will be tested: 1 positive control per mode (agonism and antagonism),1 negative control and 1 interferent.
- 7 concentrations per substance will be screened (in quadruplicates).
- Cytotoxicity is measured for each plate.

**Phase II:**

**Dose-range finding:**

- An initial dose-range finding experiment must be performed for each chemical to identify the maximum soluble non-toxic concentration and potentially identify a dose-response relationship.

**Transcriptional activation assay:**

- 3 modes will be tested: PPARγ agonism, PPARγ antagonism, Interference (Interference will be tested for substances showing an effect in antagonism mode).
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    - 2 Chemicals can be tested in parallel on the same plate for agonism or antagonism. Interference mode is performed alongside antagonism mode with the same substance on the same plate. Several plates can be run in parallel in a day.
- Every time experiments with test chemicals are performed, a run with the corresponding agonism or antagonism control must be performed.

- 30 target substances (blinded) will be tested.
- 7 concentrations per substance will be screened (in quadruplicates).
- Cytotoxicity is measured for each plate.

**Additional responsibilities:**

- In addition to the experimental operations, the partner laboratory will have to participate in regular online meetings with other labs and Pepper.
- For each phase, a study plan, raw and processed data and a report will be sent to Pepper.

## ANNEX 2: Consumables and necessary equipment

| Consumables | Necessary equipment (should be present in the lab) |
|---|---|
| • DMEM/F-12 with phenol red (e.g. Gibco 31331-028)<br>• DMEM/F-12 without phenol red (e.g. Gibco 21041-025)<br>• Fetal bovine serum (FBS) (e.g. Eurobio CVFSVF00)<br>• Penicillin/streptomycin (e.g. Gibco 15070-63)<br>• Geneticin (e.g. Invivogen ant-gn)<br>• Puromycin (e.g. Sigma P8833)<br>• D-luciferin (e.g. Perkin Elmer 122799)<br>• Dimethyl sulfoxide (DMSO) (e.g. Sigma-Aldrich D2650)<br>• Activated charcoal Norit (e.g. Sigma 97876)<br>• Dextran (e.g. Sigma D8821)<br>• Trypsin (e.g. trypsin-EDTA (Gibco 15400054)<br>• Rosiglitazone (CAS-nr.: 122320-73-4)<br>• T0070907 (CAS-nr.: 313516-66-4)<br>• Triton X-100<br>• Cytotoxicity kit (i.e. MTT, Alamar blue, Neutral red)<br>• 96-well plates flat bottom white polystyrene with microclear bottom (e.g. Greiner Bio-One 655098, CellStar) | • Luminometer (e.g. MicroBeta Wallac luminometer (Perkin-Elmer). Reading of microplates from above.<br>• Incubator, 37°C, humidified 5% $CO_2$<br>• Laminar flow hood biosafety level 2 or higher<br>• Water bath<br>• Hemocytometer, cell counter or similar<br>• Centrifuge |

## ANNEX 3: Required information for the offer

In this offer, please provide the following information:

- Financial conditions linked to the validation of the assay (consumables, workforce).
- Contingency plan (including any changes about the workforce, work continuity during holidays, ...).
- Description of the quality assurance system set up in your lab.

| | |
|---|---|
| **Method of interest** | *"Peroxisome proliferator-activated receptor γ transactivation assay (PPARγ-TA)"* |
| **Date of the proposal** | *dd-mm-yyyy* |
| **Laboratory Name and address** | |
| **Contact person information [name(s), email(s) and phone number(s)]** | |
| **Description of the work** | *The way in which the laboratory intends to carry out the work must be described in brief, but in sufficient detail to demonstrate the understanding of the project and method. The availability of key equipment should be mentioned.* |
| **Starting date and planning** | *Expected planning for phase I and phase II, including experimental work as well as reporting.* |
| **Laboratory quality assurance** | *QA guaranties of the laboratory must be described (GLP or other processes).* |
| **Contingency plan (to ensure continuity of the work)** | *Indicate how the continuity of work is ensured (e.g. illness, holidays of chief investigator and key persons; response to materials delivery delays...)* |
| **Apply for funding.** | *Indicate the financial support you need to participate in the validation as a "test laboratory".*<br>*Provide a separate estimate for each phase (transferability, ring trial).*<br>*Note that Pepper's support is exchangeable between work and consumables.).* |

**To apply**

Please send your offer to Phillipe.hubert@ed-pepper.eu and to Torben.osterlund@ed-pepper.eu until April 20th 2025.

If you have questions, please do not hesitate to contact us.

# Progesterone Receptor Transactivation Assay (PR-TA)

March 2025

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DEADLINE: 20th of April 2025

# Progesterone Receptor Transactivation Assay (PR-TA)

## References

Grimaldi M, Boulahtouf A, Toporova L, Balaguer P. “Functional profiling of bisphenols for nuclear receptors”. Toxicology (2019) 420:39. DOI: https://doi.org/10.1016/j.tox.2019.04.003

Neale P, Grimaldi M, Boulahtouf A, Leusch F, Balaguer P. “Assessing species-specific differences for nuclear receptor activation for environmental water extracts”. Water Research (2020) 85:116247. DOI: https://doi.org/10.1016/j.watres.2020.116247

Garoche C, Aït-Aïssa S, Boulahtouf A, Creusot N, Hinfray N, Bourguet W, Balaguer P, Brion F. “Human and Zebrafish Nuclear Progesterone Receptors Are Differently Activated by Manifold Progestins”. Environ Sci Technol (2020) 54:9510. DOI: https://doi.org/10.1021/acs.est.0c02056

Kocour Kroupová H, Grimaldi M, Šauer P, Bořík A, Zálohová K, Balaguer P. “Environmental water extracts differentially activate zebrafish and human nuclear progesterone receptors”. Sci Total Environ (2023) 859:160232. DOI: https://doi.org/10.1016/j.scitotenv.2022.160232

## Summary of the method

Progesterone is important for various processes and bodily functions including pregnancy, neuroprotection, behavioral development, bone formation, and metabolism. Often this is in collaboration with other hormones. Moreover, disruption of progesterone receptor (PR) signaling is associated with adverse outcomes such as an increased risk of developing hormone-dependent cancers.

This in vitro method measures the potential of chemical substances to activate or inhibit the PR through regulation of a luciferase reporter gene. Similar to other transactivation assays, PR-TA relies on the binding and activation of chemicals to PR to induce the luciferase. In addition, the method can be turned into an antagonistic assay using progesterone that may be competed by test chemicals.

## Readout and Result

The assay is based on the human osteosarcoma cells (U2OS) stably expressing the human progesterone receptor (hPR) together with a luciferase reporter construct. This stable model allows transactivation assessment of hPR by measuring luciferase activation. This enables the identification of PR agonists and, in the presence of a reference agonist, also of PR antagonists.

Below are the outlines of the workflow, the data interpretation and plate designs.

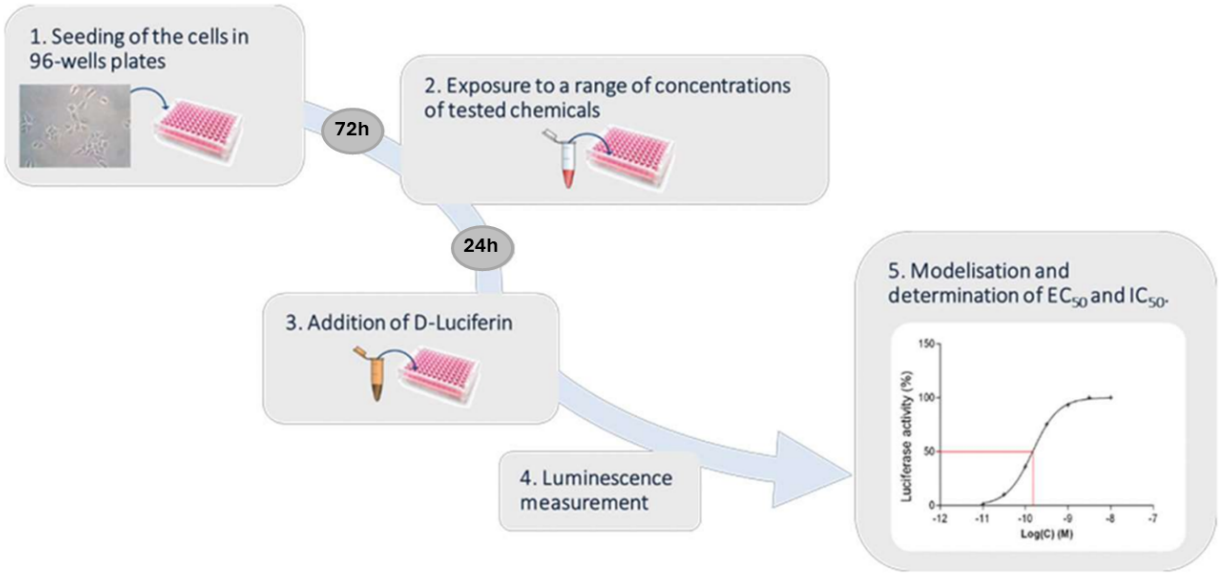
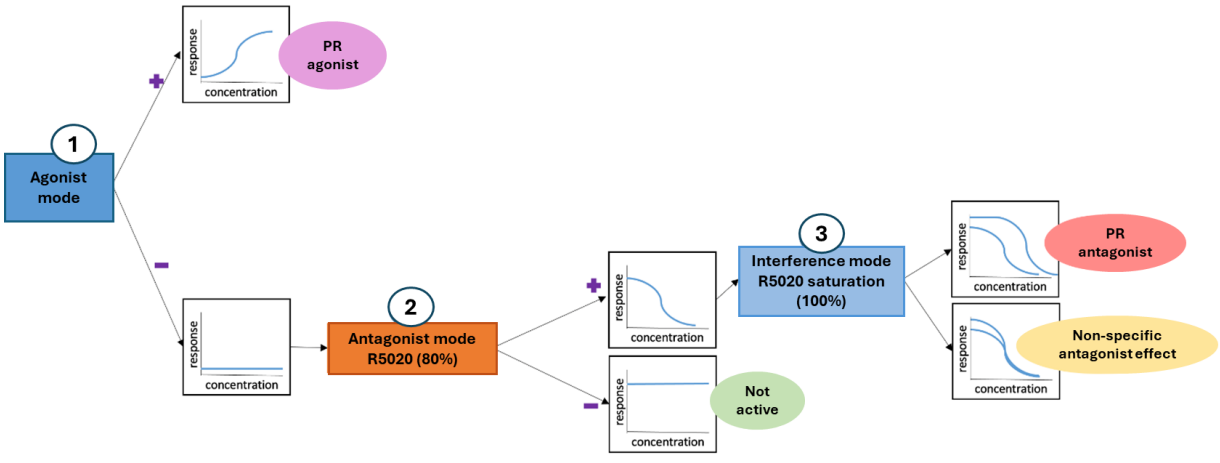



***Figure 1: Workflow of the PR-TA***. *First, cells are seeded and incubated in a 96-well plate for 72 hours. Afterwards, cells are treated with a test compound for 24 hours. Thereafter, D-Luciferin is added, and Luminescence is measured.*

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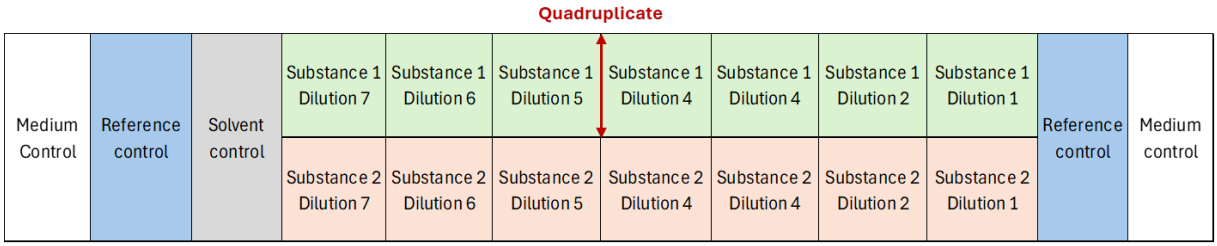


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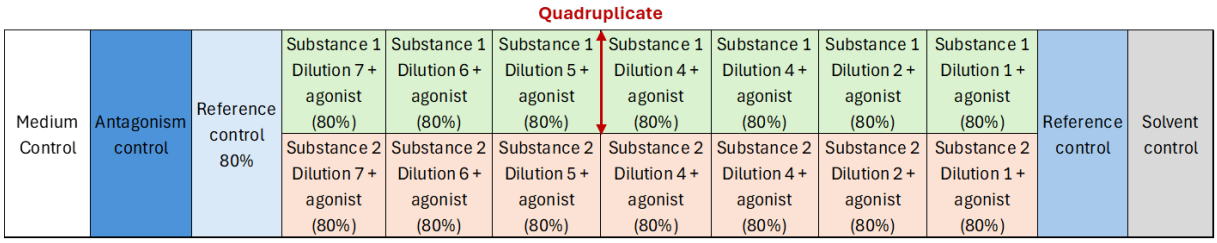


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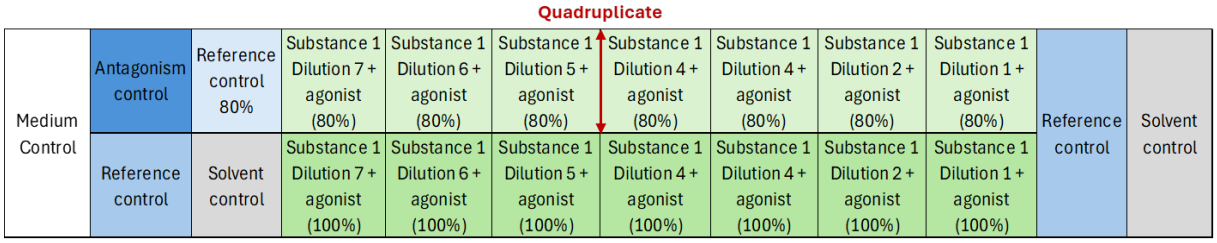


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