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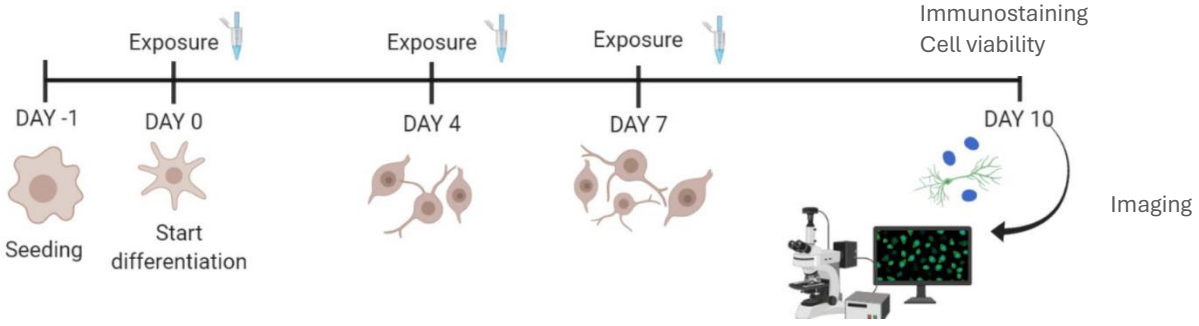
Method: Neurite outgrowth-Neuronal differentiation of C17.2 in response to RAR, RXR and PPARδ

Originally isolated from cerebellum of a 4-days old mouse, C17.2 is a multipotent neural progenitor cell line immortalized by avian myc oncogene transduction. When cultured in serum free medium containing N2 supplements and due to their autocrine function, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are secreted by the cells in culture and differentiation occurs spontaneously. After 10 days, a mixed culture of neurons and other cell types is obtained. The method provides information on endocrine dependence of the cellular transition from the progenitor stage into post-mitotic neurons, and on the subsequent morphological changes part of the differentiation process. Indeed, in order to assess endocrine disruption induced developmental neurotoxicity (ED-DNT), the cells are exposed to the test items in combination with RAR, RXR or PPARδ agonist or antagonist. Their effects are characterised thanks to the measurements of neuronal outgrowth and branching, by evaluation of their influence on the proportion of neurones after 10 days and by assessing cells viability.

Principle of the method

After 10 days of exposure (with repetition of exposure every 3.5 days) with test items, neurite outgrowth (neurite length) and branching (number of branches from the cell body) are evaluated by immunofluorescence microscopy of βII tubulin-GFP stained neurons as well as the percentage of neurons in culture (number of GFP labeled cells in relation to the total number of cells in culture). Cell viability is assessed simultaneously by an Alamar blue test.



Biological Endpoint and Endpoint Measurements:

- Neurite outgrowth
- Branching
- Percentage of neurons in culture
- Cell viability

Figure 1: Test items exposure protocol of C17.2 cells. Exposure starts one day after plating (day 0) and it is repeated every 3.5 days. After 10 days of exposure assays are conducted.

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Readouts:

- Neurite outgrowth and branching are evaluated by **immunofluorescence microscopy** (βII tubulin-GFP stained neurons).
- Percentage of neurons in culture (number of GFP labeled cells in relation to the total number of cells in culture).
- Cell viability (Alamar blue testing)

Protocol overview

After testing the test item alone (Tier 1), results obtained after 10 days guide the testing mode for Tier 2 as followed:

- A test item having an effect, i.e. either **decreasing** neurite outgrowth (or branching) and/or the ratio of neurons in culture, or **increasing** neurite outgrowth is suspected to act as an **agonist** and should be tested in agonist mode in Tier 2 (in presence of 3 receptors antagonists AGN193109 for RAR, HX531 for RXR and GSK3787 for Pparδ).

- A test item showing no effect on neurite outgrowth or branching is suspected to act as an **antagonist** and should be tested in antagonist mode in Tier 2 (in presence of 3 receptors agonists Retinoic acid for RAR, Bexarotene for RXR and GW0742 for Pparδ).

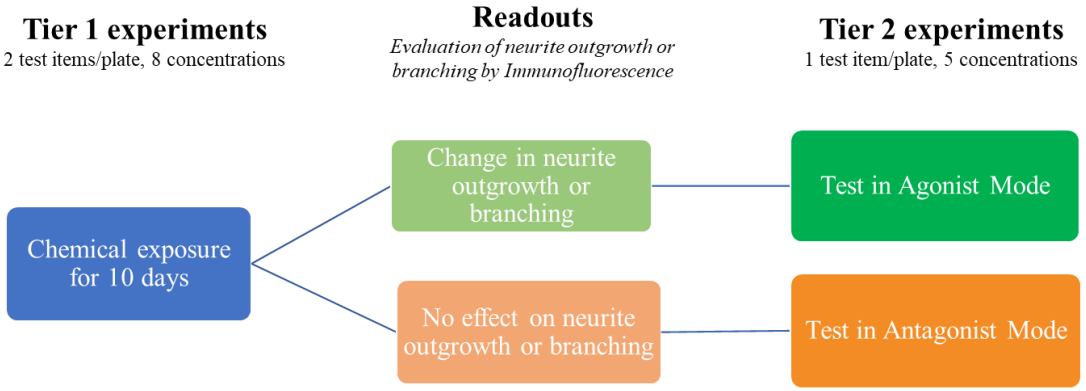

Figure 2 : Experimental process overview

Experimental design

- A preliminary test is required once before starting, using 6 reference items => Test 6 reference items, 2 per plate = Three 96 wells plates.
- Tier 1: 2 chemicals tested by plate
- Tier 2: 1 chemical tested by plate, in agonist or antagonist mode

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Tier 1 plate plan:

Figure 3 : Tier 1 : Two test items (blue and orange) are tested per plate, each at 8 concentrations, in triplicate. DMSO 0.1% is used as solvent control (white) and Rotenone 125 mM as positive control (yellow). To avoid edge effects, outer wells are filled with PBS.

Tier 2 plate plans:

A) AGONIST MODE

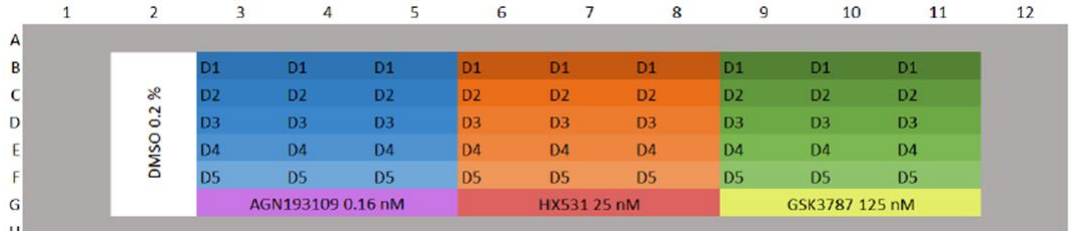

Figure 4 : Tier 2 : One test item per plate, at 5 concentrations, in triplicate. DMSO 0.2% is used as solvent control (white). Controls for RAR, RXR and PPARδ antagonism are included in the plate (purple : RAR antagonist, red : RXR antagonist and yellow : PPARδ antagonist).To avoid edge effects, outer wells are filled with PBS.

B) ANTAGONIST MODE

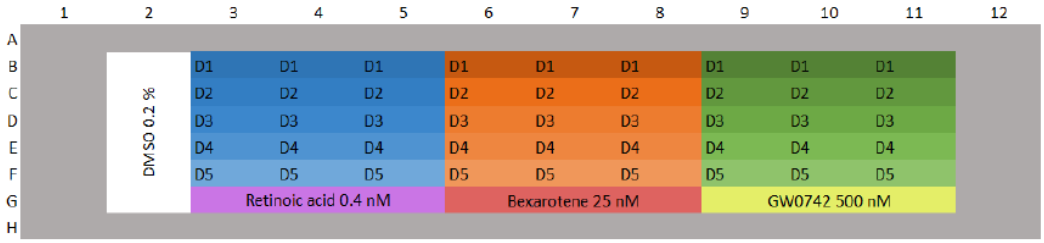

Figure 5 : Tier 2 : One test item per plate, at 5 concentrations, in triplicate. DMSO 0.2% is used as solvent control (white). Controls for RAR, RXR and PPARδ agonism are included in the plate (purple : RAR agonist, red : RXR agonist and yellow : PPARδ agonist).To avoid edge effects, outer wells are filled with PBS.

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Validation design:

- **Phase I (Transferability phase):** 3 test items to be tested with 3 independent valid runs of each one (first, test items alone (Tier 1), then in agonist or antagonist mode (Tier 2)).
- **Phase II (blind ring test):** 24 test items to be tested with 3 independent valid runs of each one first, test items alone (Tier 1), then in agonist or antagonist mode (Tier 2).
- In addition to the experimental operations, the partner lab will have to participate to a training organised in the Dev Lab facilities; participate to 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.

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ANNEX 1

Necessary equipment and materials

Reagents	Equipment

• DMEM, high glucose, no pyruvate, no L-glutamine, no phenol red
• DMEM:F12, HEPES, no phenol red
• L-glutamine, 200 mM
• Sodium pyruvate 100 mM
• N2 supplements, 100x
• PBS, pH 7.4, without Ca2+ and Mg2+
• TrypLE™ Express enzyme (1X), no phenol red, or equivalent
• Dimetyl sulfoxide ≥99.5% (DMSO)
• Poly-L-Lysine solution 0.01% sterile filtered
• AlamarBlue™ cell viability reagent
• Hoechst 33342 staining solution
• Formaldehyde solution 4%, buffered, pH 6.9
• Triton X-100
• MP Biomedicals™ Albumin, Bovine, IVD Grade, 98%
• Anti-beta III Tubulin antibody
• Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)
• Retinoic acid ≥98%
• AGN 193109 ≥98%
• Bexarotene ≥99%
• HX531 ≥98%
• GW0742 ≥98%
• GSK3787 ≥98%
• Horse serum (HS)
• Fetal bovine serum (FBS)
	• **Key elements:**
	  ○ **Fluorescence microscope or ImageXpress® Micro 4 High-Content Imaging System**
	  ○ **Imaging software**
	• Incubator, 37°C, humidified 5%CO2
	• Centrifuge
	• Laminar flow hood biosafety level 2 or higher
	• Inverted phase contrast microscope
	• Water bath
	• Hemocytometer, cell counter or similar
	• Spectrophotometer

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ANNEX 2 : Required information for the Offer

In this offer, please provide the following information: financial conditions linked to the validation of the assay (consumables, workforce). In addition, specify in this offer the following logistic information: contingency plan and description of the quality system set up in your lab.

Method of interest	*"Neurite outgrowth-Neuronal differentiation of C17.2 in response to RAR, RXR and PPARδ"*
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 its understanding of the project.* *The availability of key equipment should be mentioned..*
Starting date and planning	*Expected planning for phase I and phase II, including experimental phases 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".* *Provide a separate estimate for each phase (transferability, ring trial).* *Note that Pepper's support is fungible between work and consumables.).*

To apply

Please sent your offer to : Philippe.hubert@ed-pepper.eu and to Andrea.RIVERO-ARZE@ed-pepper.eu before May 30th 2024.

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

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ANNEX 3

References

1. Evan Y.Snyder DLD, Christopher Walsh, Susan Arnold-Aldea, Erika A. Hartwieg, Constance L. Cepko. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell. 1992;68(1):33-51.

2. Lundqvist J, Andaloussi-Lilja JE, Svensson C, Dorfh HG, Forsby A. Optimisation of culture conditions for differentiation of C17. 2 neural stem cells to be used for in vitro toxicity tests. Toxicology in Vitro. 2013;27(5):1565-9.

3. Lundqvist J, Svensson C, Attoff K, Forsby A. Altered mRNA expression and cell membrane potential in the differentiated C17. 2 cell model as indicators of acute neurotoxicity. Applied In Vitro Toxicology. 2017;3(2):154-62.

4. Attoff K, Gliga A, Lundqvist J, Norinder U, Forsby A. Whole genome microarray analysis of neural progenitor C17.2 cells during differentiation and validation of 30 neural mRNA biomarkers for estimation of developmental neurotoxicity. PLoS One. 2017;12(12):e0190066.

5. Attoff K, Kertika D, Lundqvist J, Oredsson S, Forsby A. Acrylamide affects proliferation and differentiation of the neural progenitor cell line C17.2 and the neuroblastoma cell line SH-SY5Y. Toxicol In Vitro. 2016;35:100-11.