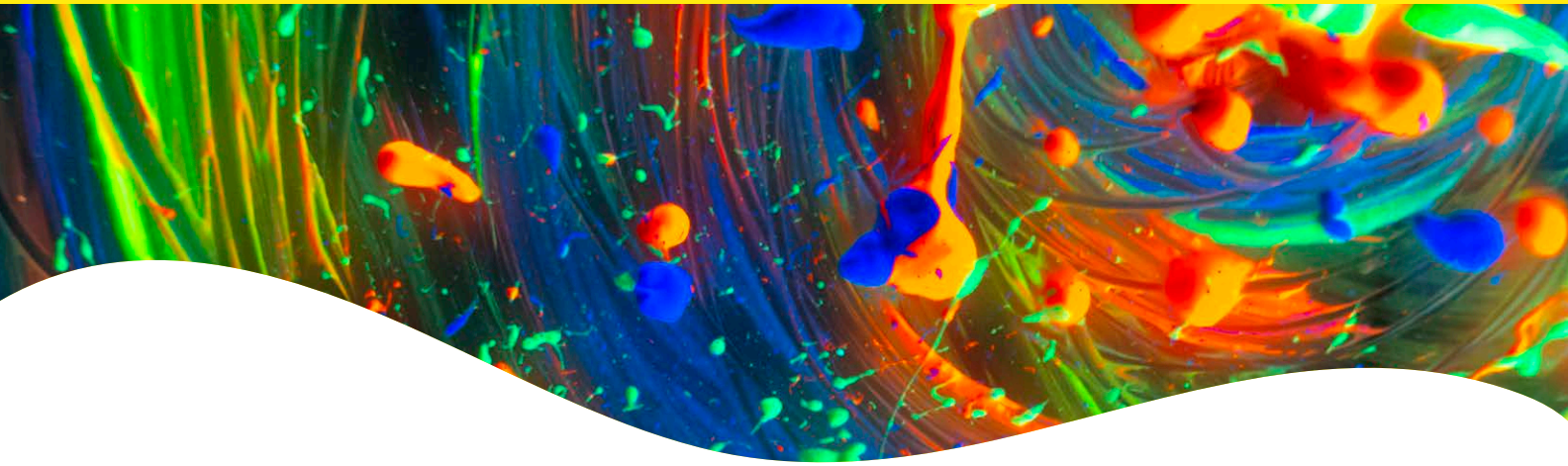
PhenoVue Neuronal Differentiation Staining Kit



Overview

In neuroscience, more physiologically-relevant cell models, such as human or rodent primary cell models, IPS-derived neuronal cells, or co-cultured cells in 2D or 3D, are essential to better recapitulate pathophysiological states underlying disease onset.

Therefore, the accurate qualification of neuronal cell models is critical, especially when long and complex differentiation processes are required, e.g., iPSC -derived cells. To this end, the PhenoVue™ neuronal differentiation staining kit combines specific mature neuron markers, which allows multiplexing immunostaining and the ability to quantify the percentage of progenitors versus differentiated neurons in the cell population.

PhenoVue neuronal differentiation staining kit includes four fluorescent markers:

- Nestin is a protein predominantly expressed by stem/progenitor cells, playing an important function in progenitors' cell division. This marker tends to switch off with neuronal maturation.
- B3-tubulin is a protein predominantly expressed in neurons. Its expression increases with neuronal maturation and is maintained at latest maturation stages.
- Actin is a protein that polymerizes to form the actin cytoskeleton, commonly used to assess cell morphology.
- PhenoVue Hoechst 33342 nuclear stain.

Each component has been extensively validated and carefully optimized, ensuring maximum spectral separation with no spectral overlap. Furthermore, the specificity of each reagent has been extensively ensured, making this kit a straightforward solution to qualify the neuronal differentiation process and the subsequent neuronal cell population.

Product information

Product name	Part no.	Number of vials per unit	Shipping conditions
PhenoVue neuronal differentiation staining kit	PNDIF11	9	Dry ice

Kit contents	Format	Packaging*	Storage
PhenoVue Hoechst 33342 nuclear stain	Liquid (H_2O)	1 vial of 70 µL (500X)	2-8 °C or below Protect from light
PhenoVue Fluor 555 – Phalloidin (600X)	Desiccated	1 vial of 60 µL (600X)	-16 °C or below Protect from light
PhenoVue anti-nestin antibody (100X)	Liquid	1 vial of 100 µL (100X)	-16 °C or below Protect from light
PhenoVue anti-B3 tubulin antibody (100X)	Liquid	1 vial of 100 µL (100x)	-16 °C or below Protect from light
PhenoVue Fluor 488 - Rat anti-mouse IgG1 antibody highly cross-adsorbed	Liquid	1 vial of 200 µL (50X)	-16 °C or below Protect from light
PhenoVue Fluor 647 - Rat anti-mouse IgG2A antibody highly cross-adsorbed	Liquid	1 vial of 200 µL (50X)	-16 °C or below Protect from light
PhenoVue paraformaldehyde, 4% solution	Liquid	1 vial of 25 mL (1X)	2-8 °C or below Protect from light
PhenoVue permeabilization, 0.5% Triton X-100 solution	Liquid	1 vial of 25 mL (5X)	2-8 °C or below Protect from light
PhenoVue dye diluent A (5X)	Liquid	1 vial of 8 mL (5X)	16 °C or below Protect from light

*Amount of reagents provided is sufficient for 1 x 384 well microplate using the recommended concentrations.

Storage and stability

- After receiving, store PhenoVue permeabilization 0.5% Triton X-100 solution and PhenoVue paraformaldehyde, 4% solution reagents at 2–8 °C, protected from light. Other reagents can be stored together at ≤ -16 °C or separately between ≤ -16 °C to 2-8 °C protected from light, as indicated in the table above. Avoid repeated freeze / thaw cycles.
- Allow the reagents to warm up to room temperature for 30 min before opening the vials and reconstitution. Aliquoted reagents must be stored at -16 °C or below and are stable for 6 months.
- After thawing, the PhenoVue dye diluent A (5x) may contain some aggregates which will not impair the product and image quality. If aggregate removal is preferred, the PhenoVue dye diluent A (5x) can be filtered (0.22 µm filter) prior to dilution. The resulting PhenoVue dye diluent A 1X must be stored at 2-8 °C for no more than 2 days.
- The stability of these products is guaranteed until the expiration date provided in the certificate of analysis, when stored at recommended and protected from light.

Spectral and photophysical properties

Product name	Maximum excitation wavelength (nm)	Maximum emission wavelength (nm)	Common filter set	Quantum yield (Φ)	Epsilon* (ε in $M^{-1}.cm^{-1}$ at λ max)	Brightness (Φ x ε)
PhenoVue Hoechst 33342	357**	455**	DAPI	dsDNA: 0.38 ssDNA: 0.22	43,000	Nd
PhenoVue Fluor 488	495	520	FITC	92%	73,000	65,320
PhenoVue Fluor 555	555	570	Cy3	10%	155,000	15,500
PhenoVue Fluor 647	650	670	Cy5	30%	240,000	72,000

* In PBS pH 7.4 ** In methanol with 0.2 M HCl
Nd: not determined.

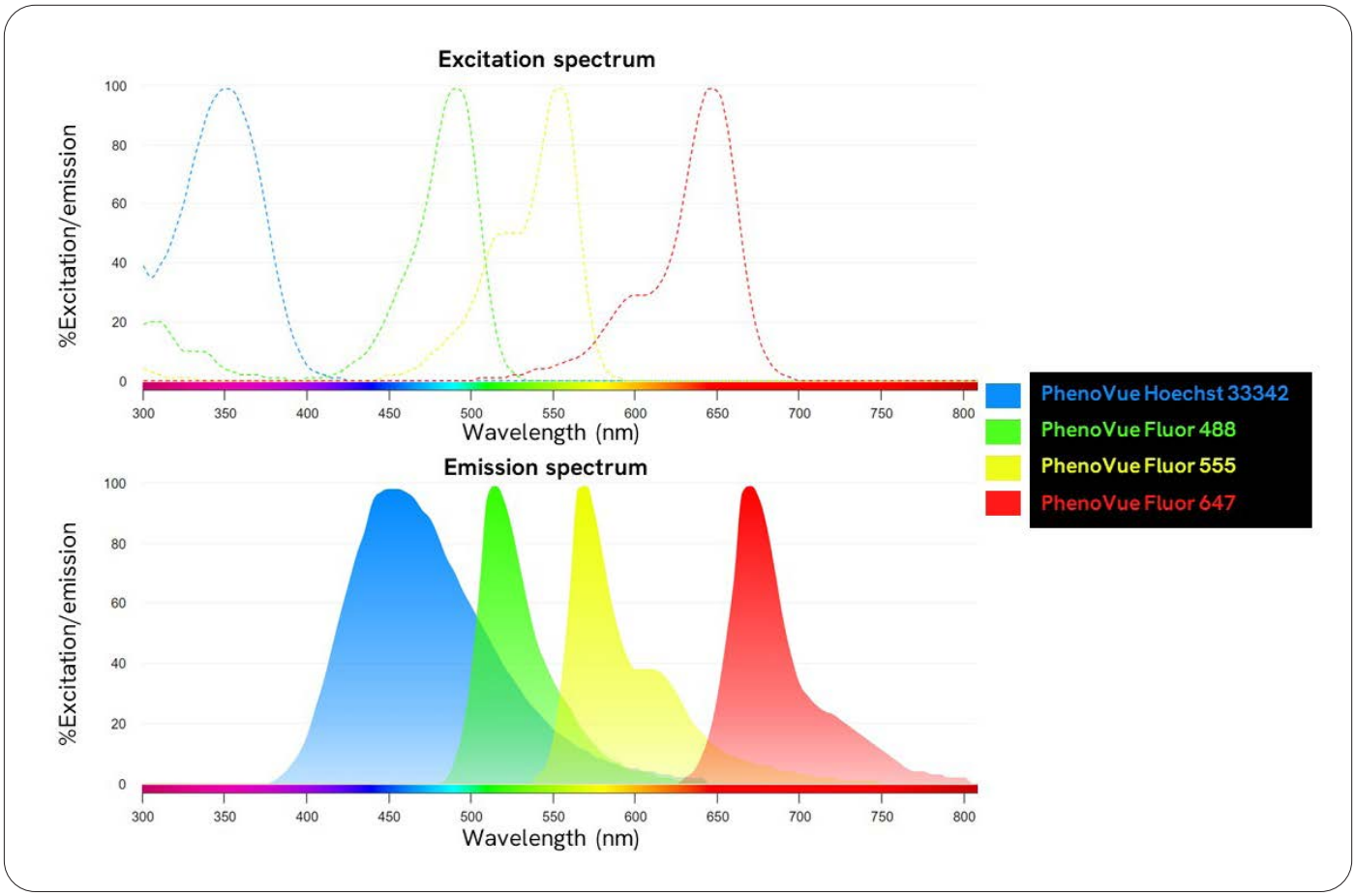


Figure 1: Excitation and emission spectrum associated with the PhenoVue neuronal differentiation staining kit components.

Other materials and reagents not provided

Reagents or consumables	Usage
PBS	Washing buffer and diluent for PhenoVue permeabilization 0.5% Triton X-100 solution
Distilled H_2O	Dilution of PhenoVue dye diluent A (5X)
Anhydrous DMSO	Reconstitution of PhenoVue Fluor 555 – Phalloidin (600X)
PhenoPlate™ 384-well or 96-well	Cell plating, stimulation, staining and imaging
Aluminum single-tab foil	Plate sealing to protect fluorescent probes from light

Reagent reconstitution and preparation of staining solutions

	Reagents	Reconstitution / preparation of stock solution	Preparation of staining solution
Buffers	PhenoVue paraformaldehyde, 4% solution	Ready to use	Ready to use
	PhenoVue permeabilization 0.5% Triton X-100 solution	Dilute 5 times in PBS to give a ready to use 1X buffer	Ready to use permeabilization buffer
	*PhenoVue dye diluent A (5X)	Dilute 5 times in distilled H_2O to give a ready to use 1X buffer	Ready to use for dilution of other reagents and as Saturation buffer
Staining solution 1	PhenoVue anti-nestin antibody (100X)	100X liquid stock solution provided	Dilute stock solution 100 times in diluent A to give a 1x staining solution
	PhenoVue anti-B3 tubulin antibody (100X)	100X liquid stock solution provided	Dilute stock solution 100 times in diluent A to give a 1x staining solution
Staining solution 2	PhenoVue Hoechst 33342 nuclear stain	500X liquid stock solution provided (1 mg/mL)	Dilute stock solution 500 times in diluent A to give a 1x staining solution
	PhenoVue Fluor 555 – Phalloidin (600X)	Reconstitute with 60 µL DMSO to give a 600X liquid stock solution	Dilute stock solution 600 times in diluent A to give a 1x staining solution
	PhenoVue Fluor 488 - Rat anti-mouse antibody highly cross-adsorbed	50X liquid stock solution provided	Dilute stock solution 50 times in diluent A to give a 1x staining solution
	PhenoVue Fluor 647 - Rat anti-mouse antibody highly cross-adsorbed	50X liquid stock solution provided	Dilute stock solution 50 times in diluent A to give a 1x staining solution

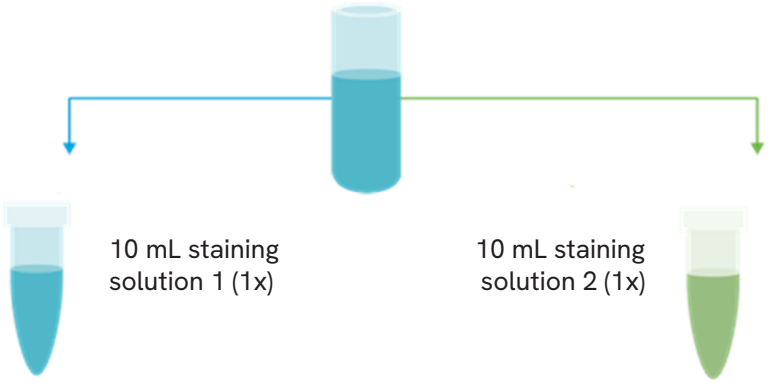
* PhenoVue dye diluent A 1x is used as staining solution diluent and as saturation buffer.

- **Prepare stock solution of buffers and diluent:**
  - **Fixative solution:** PhenoVue paraformaldehyde, 4% solution is ready-to-use.
  - **Permeabilization solution:** Dilute stock solution of PhenoVue permeabilization 0.5% Triton X-100 solution 5 times in PBS to give a 0.1% PhenoVue permeabilization Triton X-100 solution.
  - **Saturation and staining diluent solution:** as described in the table above, dilute stock solution of PhenoVue dye diluent A (5x) 5 times in distilled H_2O to give a 1x solution.
- **Prepare the staining solutions in PhenoVue dye diluent A (1x) as described in the table above:** (protect stock and staining solutions from light)
  - **Staining solution 1:** Intended to be used on fixed, permeabilized and saturated cells and includes:
    - PhenoVue anti-nestin antibody
    - PhenoVue anti-B3 tubulin antibody
  - **Staining solution 2:** Staining solution intended to be used after incubation of the staining solution 1 and includes:
    - PhenoVue Hoechst 33342 nuclear stain
    - PhenoVue Fluor 555 - Phalloidin
    - PhenoVue Fluor 488 - Rat anti-mouse IgG1 antibody highly cross-adsorbed
    - PhenoVue Fluor 647 - Rat anti-mouse IgG2a antibody highly cross-adsorbed

Example preparation of staining solutions

The following example describes the preparation of 10 mL staining solution 1 and 10 mL of staining solution 2, sufficient for 1 x 384-well plate (or 2 x 96-well plates).

Prepare 20 mL 1x PhenoVue dye diluent A
To 16 mL ddH_2O, add 4 mL PhenoVue dye diluent A (5x)



To 9.8 mL PhenoVue dye diluent A (1x)

- Add 100 µL of PhenoVue anti-nestin antibody (100x)
- Add 100µL of PhenoVue anti-B3 tubulin antibody (100x)

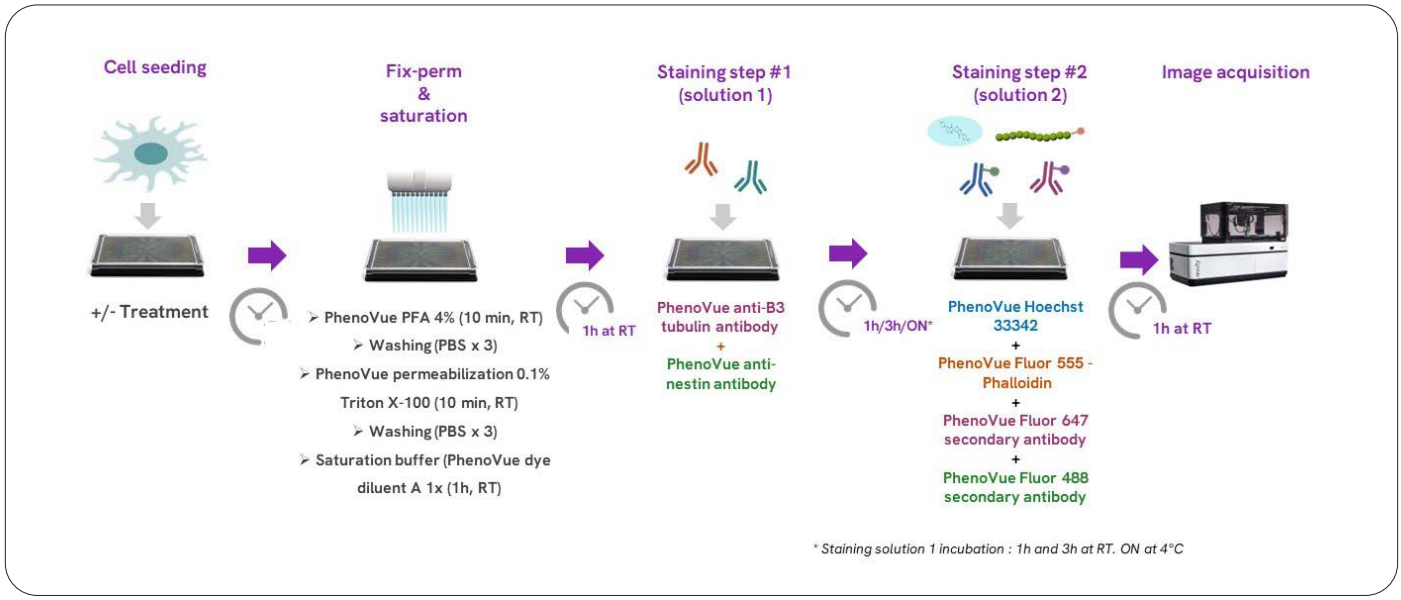
10 mL staining solution 1 (1x)

10 mL staining solution 2 (1x)

To 9.564 mL PhenoVue dye diluent A (1x)

- Add 20 µL of PhenoVue Hoechst 33342 nuclear stain (500x)
- Add 16.7 µL of PhenoVue Fluor 555 - Phalloidin (600x)
- Add 200 µL of PhenoVue Fluor 488 - Rat anti-mouse antibody highly cross-adsorbed (50x)
- Add 200 µL of PhenoVue Fluor 647 - Rat anti-mouse antibody highly cross-adsorbed (50x)

Experimental workflow



Protocols

Cell culture

Seed cells in PhenoPlate 384-well imaging microplates* (or any other convenient cell culture vessels). Incubate in the appropriate cell culture conditions, usually 37 °C, 5% CO_2 until 50-70% confluency.

Fixed-cell imaging

Rinse briefly in phosphate-buffered saline (PBS) then proceed with cell fixation.

1. **Fixation:** Add 25 µL ready-to-use PhenoVue paraformaldehyde 4% solution for 10 min at room temperature.
2. **Washing:** Wash three times with PBS.
3. **Permeabilization:** Add 25 µL PhenoVue permeabilization solution diluted at 0.1% Triton X-100 in PBS for 10 min at room temperature.
4. **Washing:** Wash three times with PBS for 5 min.
5. **Saturation:** Incubate with 25 µL PhenoVue dye diluent A 1x for 1h at room temperature.
6. **Primary antibody incubation:** Remove saturation buffer and add 25 µL per well of primary antibodies solution (staining solution 1). Incubate for at least 1h at room temperature.
7. **Washing:** Wash three with PBS for 5 min.
8. **Staining solution:** Add 25 µL per well of staining solution 2 and incubate for 1h at room temperature protected from light.
9. **Washing:** Wash three times with PBS for 5 min.
10. **Acquire images on an imaging device.**

Tips

- Reagent concentrations have been carefully optimized to limit fluorescence crosstalk on Revvity's high content screening instruments. Thus, increasing them may increase crosstalk.
- Comfortable fluorescence intensities are achieved after 1 hour incubation of primary antibodies at room temperature. Overnight incubation at 4 °C can be performed as well.
- Using 384-well microplates, we recommend centrifuging the plate for 1 min (500 g) at room temperature between each step to allow reagents settle in the bottom of the wells.
- PhenoVue Fluor 555 – Phalloidin reconstitution: avoid methanol and other alcohol-based or aqueous solvents. It is preferable to use anhydrous DMSO which preserves the integrity of actin filaments, enabling brighter staining intensity.

* PhenoPlate 96-well microplates may also be used. Adjust the cell density accordingly and increase the corresponding volumes by 2-fold.

- Staining can be performed on human, mouse and rat cellular models, except the nestin antibody which is validated on human models.
- **To gain flexibility**, PhenoVue Fluor 555 - Phalloidin can be replaced by your primary antibody of choice or any other compatible fluorescent stains. In that case, important considerations follow:
  - Primary PhenoVue mouse anti-nestin antibody is an IgG1, whereas primary PhenoVue mouse anti-B3 tubulin antibody is an IgG2a.
  - Secondary PhenoVue Fluor 488 - Rat anti-mouse IgG1 antibody and secondary PhenoVue Fluor 647 - Rat anti-mouse IgG2a antibody are used to recognize their respective primary antibody. They are highly cross-adsorbed, mouse and isotype specific (Figure 7).
  - Do not replace PhenoVue Fluor 555 - Phalloidin with primary mouse IgG1 or IgG2a antibodies. Privileged primary anti-rabbit antibody or other species.
  - Do not use a secondary anti mouse or anti-rat antibody, privileged secondary rabbit antibody or other species.

Recommendations for acquisition settings

- The PhenoVue neuronal differentiation staining kit enables multiplexing four colors simultaneously. If using a Revvity high content screening system, we recommend the following acquisition settings (depending on the instrument), to obtain high fluorescent signal as well as best image quality:

HCS instruments		PhenoVue Hoechst 33342	PhenoVue Fluor 488	PhenoVue Fluor 555	PhenoVue Fluor 647
Opera Phenix® Plus 5 lasers	Excitation laser	375	488	561	640
	Emission filters	435-480	500-550	570-630	650-760
Opera Phenix Plus 4 lasers	Excitation laser	405	488	561	640
	Emission filters	435-480	500-550	570-630	650-760
Operetta CLS 4 or 8 LED – 1600 & 1601	Excitation LED (filters)	370 (355-385)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filters	430-500	500-550	570-650	655-760

Safety information

Chemical reagents are potentially harmful, please refer to the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Applications

- High-content analysis / high-content screening
- Imaging microscopy

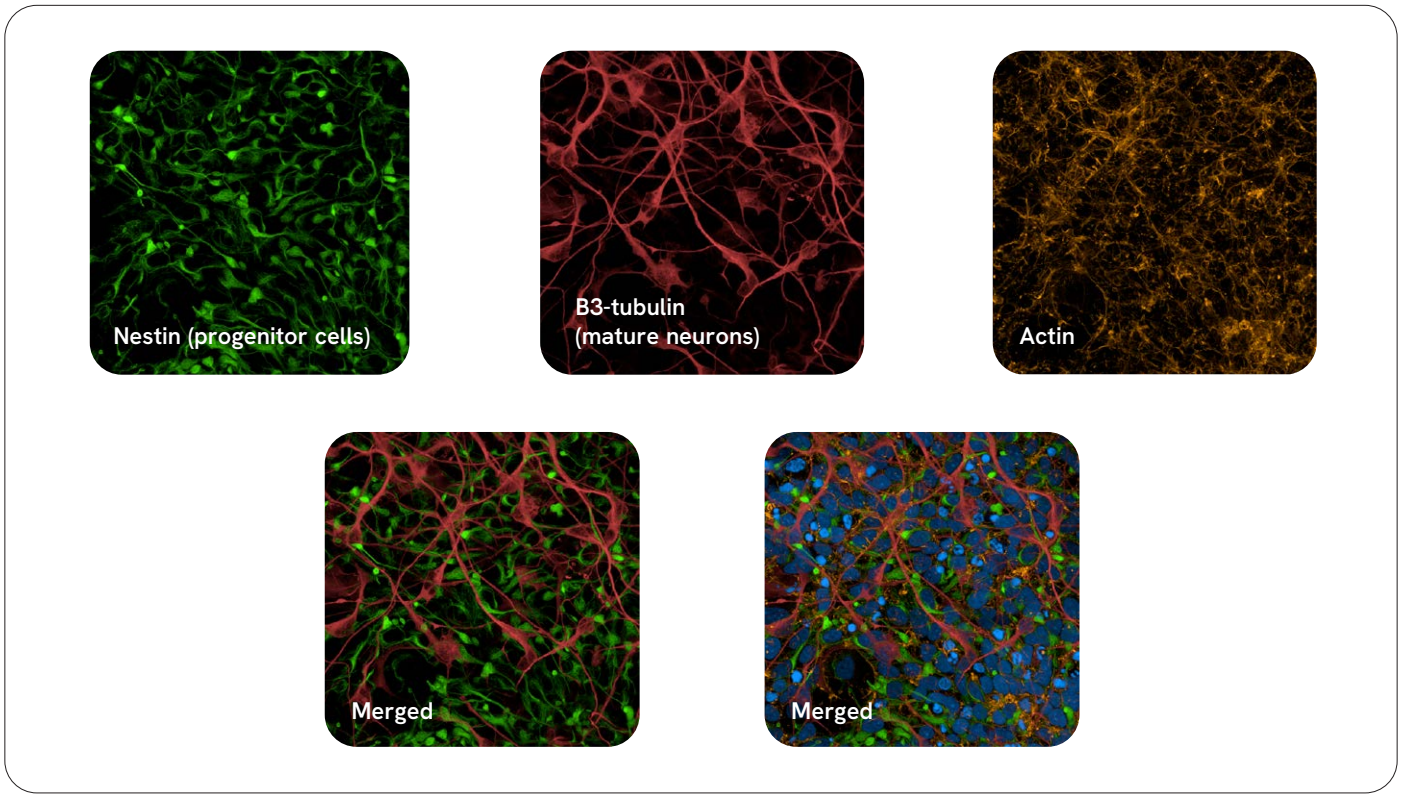


Figure 1: iPSC-derived human cortical neurons were seeded in PhenoPlate 96-well microplates (40,000 cells/well) and incubated at 37 °C, 5% CO_2. Seven days post-seeding, cells were fixed, permeabilized, saturated and stained using the PhenoVue neuronal differentiation staining kit (protocol described above). Images were acquired on the Operetta CLS (8 LED) high-content analysis system with the 63X water objective.

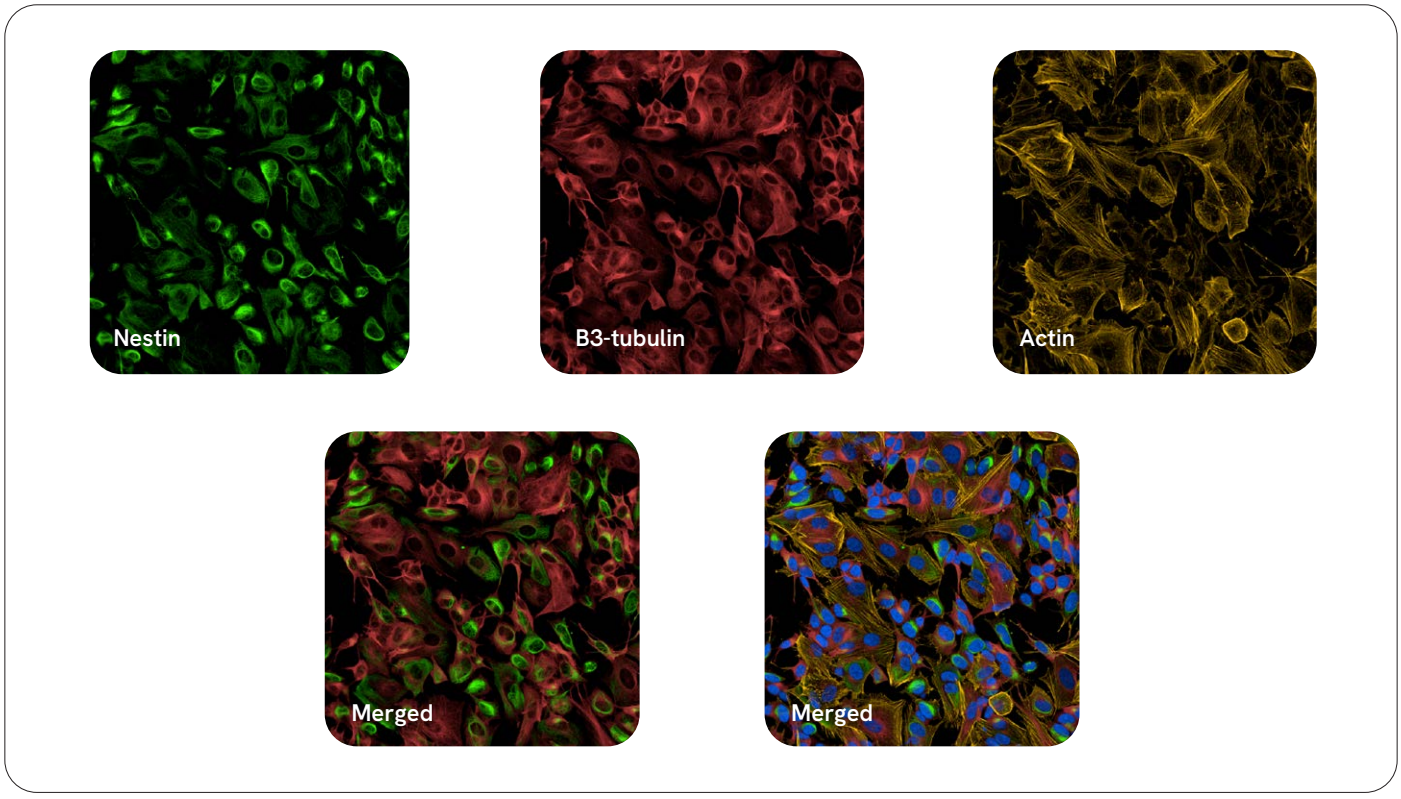


Figure 2: Neuroblastoma SHSY5Y cells were seeded in PhenoPlate 96-well microplates (75,000 cells/well) and incubated at 37 °C, 5% CO_2 for 24h. After 24h, cells were fixed, permeabilized, saturated and stained using the PhenoVue neuronal differentiation staining kit (protocol described above). Images were acquired on the Opera Phenix Plus (5 lasers) high-content screening system with the 63X water objective.

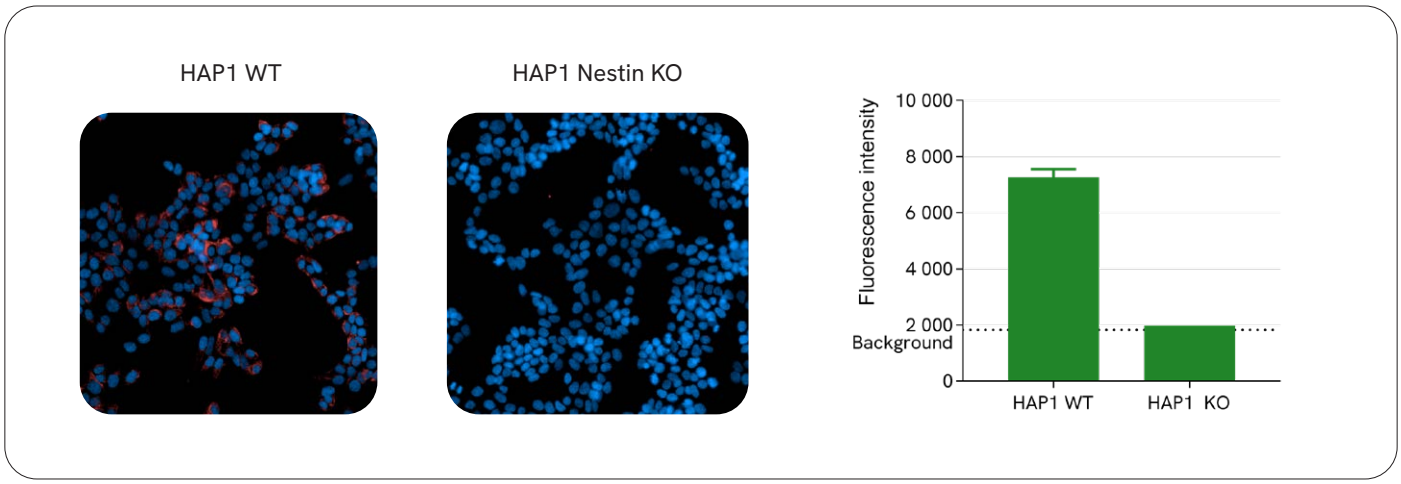


Figure 3: HAP1 WT and HAP1 Nestin KO were stained with PhenoVue anti-nestin antibody and PhenoVue Fluor 647 - Goat anti-mouse highly cross adsorbed antibody. As expected, no fluorescent signal was detected in the Nestin KO cells, validating the specificity of PhenoVue anti-nestin antibody.

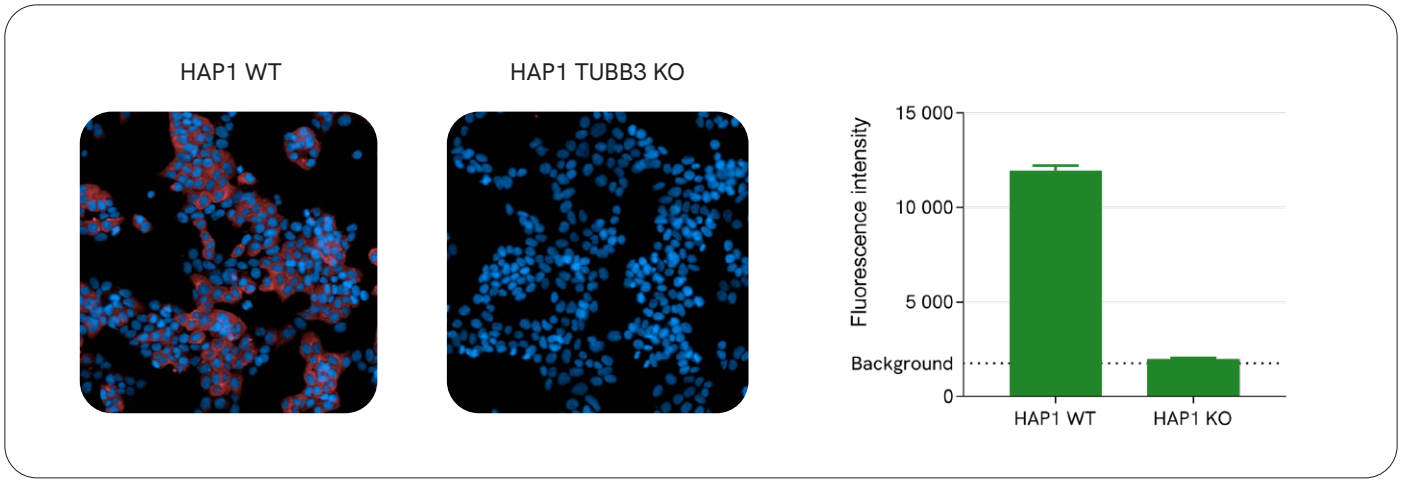


Figure 4: HAP1 WT and HAP1 TUBB3 KO were stained with PhenoVue anti-B3 tubulin antibody and PhenoVue Fluor 647 - Goat anti-mouse highly cross adsorbed antibody. As expected, no fluorescent signal was detected in the TUBB3 KO cells, validating the specificity of PhenoVue anti-B3 tubulin antibody.

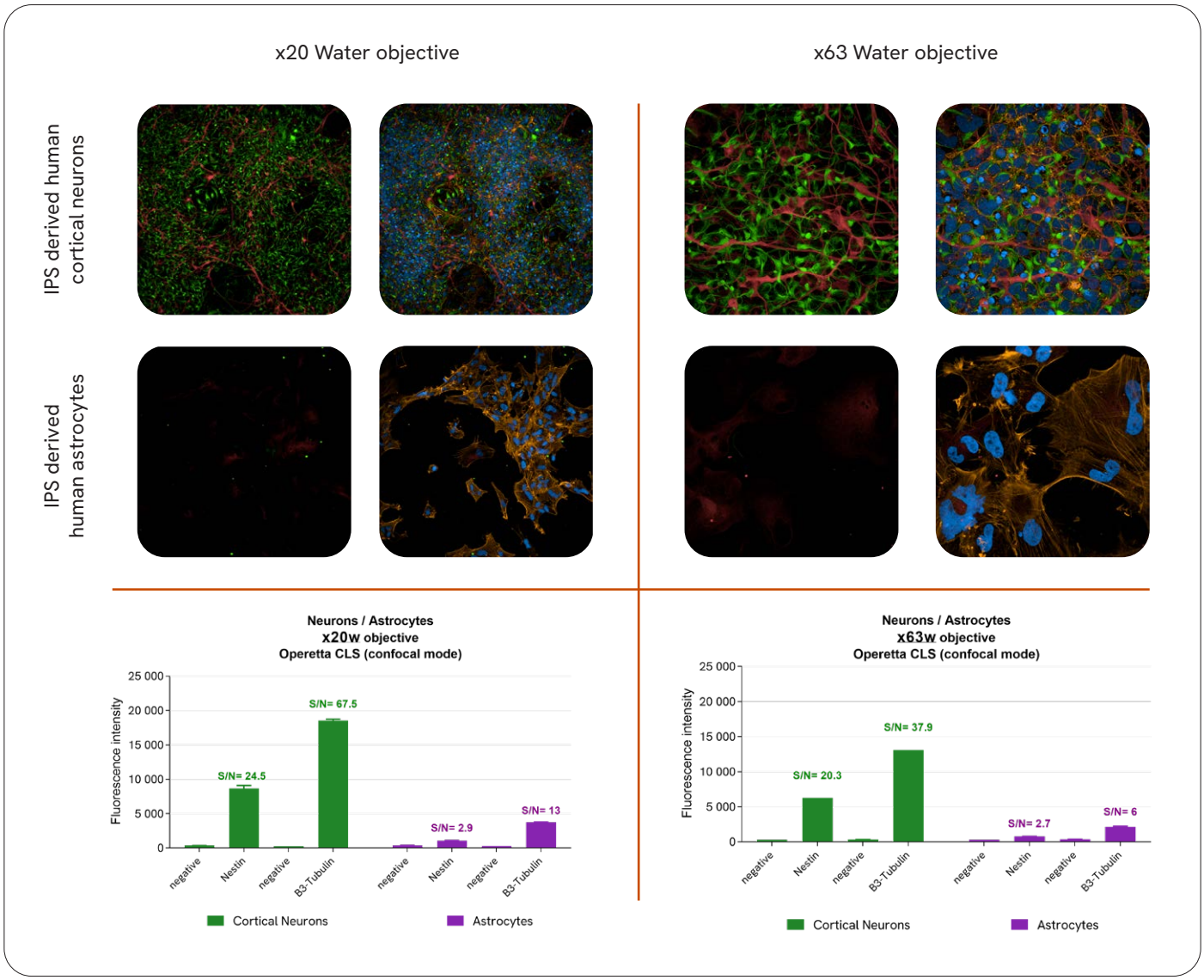


Figure 5: iPSC-derived human cortical neurons (40,000 cells/well) or iPSC-derived human astrocytes (30,000 cells/well) were seeded in PhenoPlate 96-well microplates and incubated at 37 °C, 5% CO_2. Respectively 7 or 4 days post-seeding, cells were fixed, permeabilized, saturated and stained using the PhenoVue neuronal differentiation staining kit (protocol described above; primary antibodies were incubated 3h at RT). Images were acquired on the Operetta CLS (8 LED) high-content analysis system with the 20X water objective and the 63X water objective.

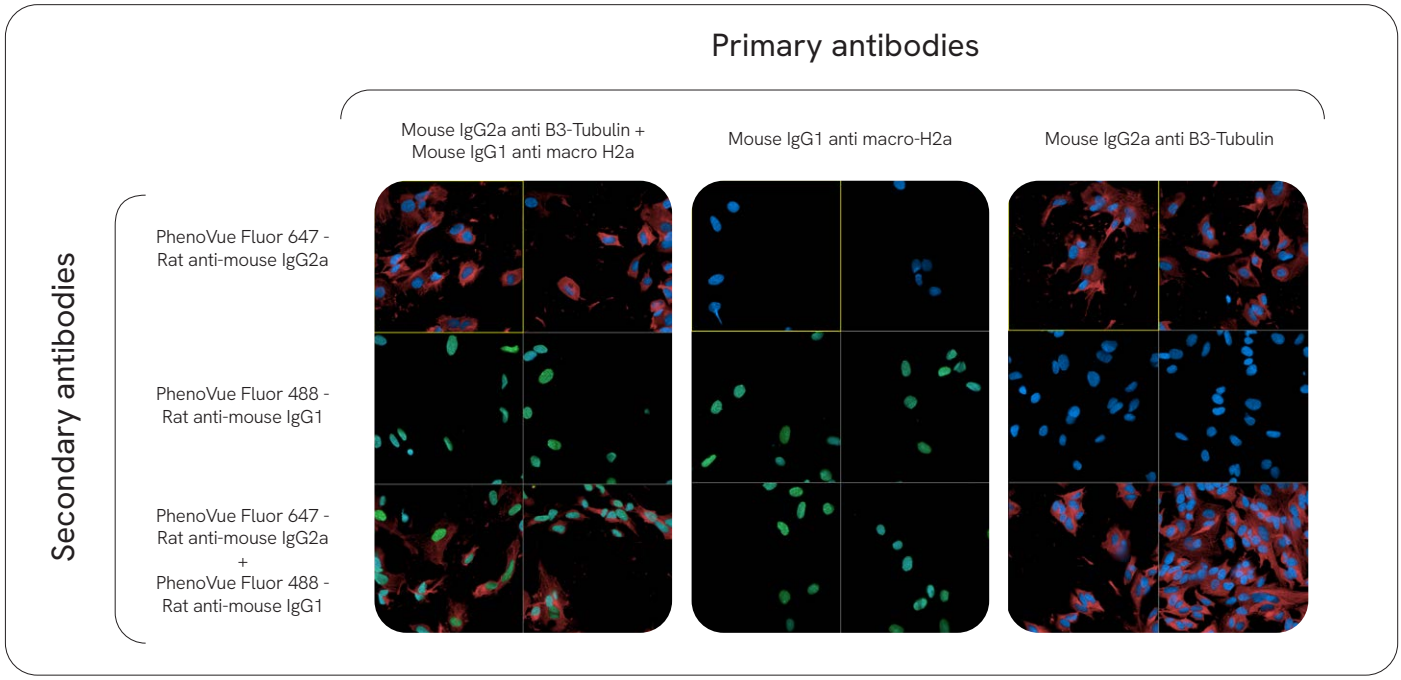


Figure 6: Neuroblastoma SHSY5Y cells were seeded in PhenoPlate 96-well microplates (75,000 cells/well) and incubated at 37 °C, 5% CO_2 for 24h. After 24h, cells were fixed, permeabilized, saturated and incubated overnight with PhenoVue anti-B3 tubulin mouse IgG2a, anti-mouse macro H2 IgG1 either alone or mixed. Cells were then stained with the PhenoVue rat anti-mouse IgG1 and IgG2a. Images were acquired on the Operetta CLS (8 LED) high-content analysis system with the 63X water objective and the 63X water objective. These results demonstrate the isotype specificity recognition obtained with of PhenoVue rat anti-mouse IgG1 and IgG2a.

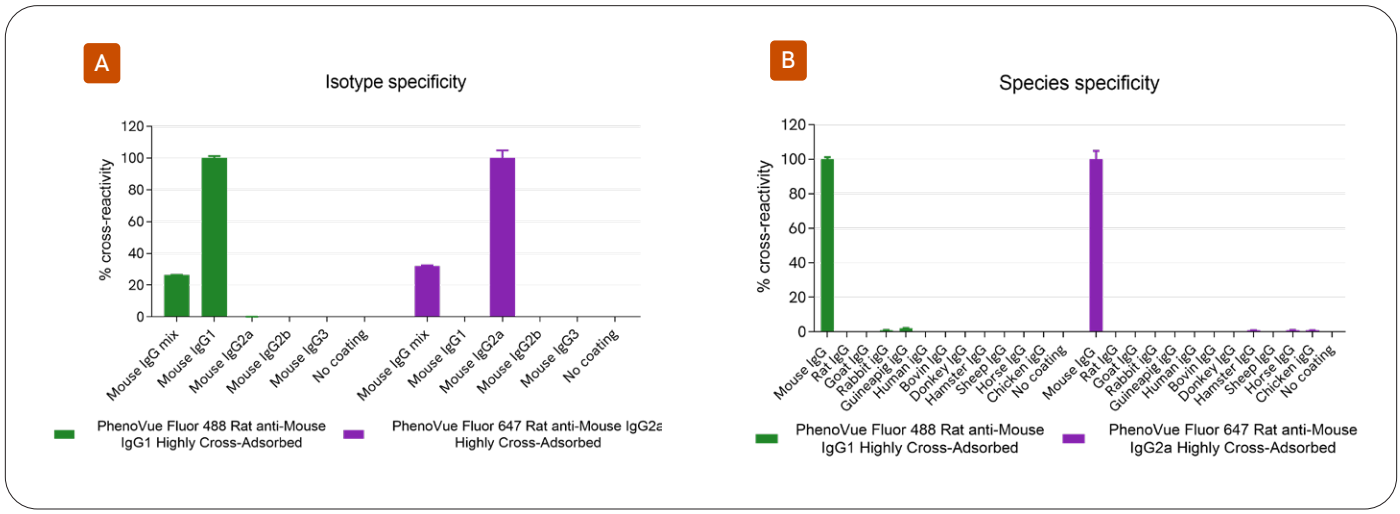


Figure 7: F-LISA experiments: different IgG isotypes (A) or rat IgG species (B) were used to coat a 96-well microplate, then incubated with PhenoVue Fluor 488 – Rat anti-mouse IgG1 and PhenoVue Fluor 647 – Rat anti-mouse IgG2a (5 µg/mL). Fluorescence intensity was measured on an EnVision multimode plate reader. These results confirm the high specificity of these two secondary antibodies, allowing the flexibility to add the immunostaining of another target of interest alongside the PhenoVue neuronal differentiation staining kit reagents (see "Tips" for details on restrictions).

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