

Cellomics[®] Neurite Outgrowth Kits

High-Content Screening Reagents

1818.1

Number	Description
K07-0001-1	Neurite Outgrowth Kit, sufficient materials for 5 × 96 wells
R01-0513-1	Neurite Outgrowth Kit, sufficient materials for 50 × 96 wells

Kit Contents:	K0700011	R0105131
Neurite Outgrowth primary antibody, mouse	75 µl	830 µl
DyLight™ 488-Conjugated Goat Anti-Mouse)	72 µl	1 ml
Hoechst Dye	2 x 30 µl	745 µl
Wash Buffer (10X)	100 ml	--
Neurite Outgrowth Buffer (10X)	100 ml	--
Thin Plate Seal Assembly	7/pack	--

Storage: Upon receipt store all kit components at 4°C. Keep vial containing DyLight 488-conjugated Goat Anti-Mouse IgG and Hoechst Dye protected from light. Allow buffers to warm to room temperature before use. See the **Solution Preparation** section for storage and stability of prepared solutions.

Warning: Please completely read these instructions and the accompanying material safety data sheets before using this product. The Cellomics Reagents are not for diagnostic use in humans or animals.

Introduction

The Neurite Outgrowth HCS Reagent Kit enables quantification of neurons, neuronal like cells, and neurite outgrowth from a heterogeneous population of live cells growing on standard high-density microplates. The cells can either be neuronal-like cell models, such as PC12 or Neuroscreen™-1 Cells, or primary brain cultures that contain a subpopulation of neurons. The kit includes a primary antibody specific for neuron cell bodies and neurites and DyLight[®] 488-conjugated Secondary Antibody. Cell nuclei are identified by DNA-specific Hoechst Dye.

Neurons and neuronal-like cells extend processes known as neurites. A major focus for drug discovery is identifying compounds that affect the growth of neurites. Drugs that promote nerve growth would aid treatment in a wide variety of diseases and traumas that result in neuropathy and nerve injury. Examples of areas that would benefit from neuronal regeneration include spinal cord injuries; neuropathy resulting from diseases such as diabetes; stroke; and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.

The Neurite Outgrowth HCS Reagent Kit is based on immunofluorescence using a validated antibody that specifically labels both neurites and neuronal cell bodies from a wide range of mammalian species. Specific identification of neurons and their neurites from mixed populations of cells such as primary brain cultures is also possible (Figure 1). The Neurite Outgrowth Kit reagents, in combination with the ArrayScan HCS Reader and the (Extended) Neurite Outgrowth BioApplication software enable automated plate handling, focusing, cell image acquisition, analysis, and neurite outgrowth quantification. For a more detailed description of the image processing algorithm, see the Neurite Outgrowth or Extended Neurite Outgrowth BioApplication Guide that accompanies the software.

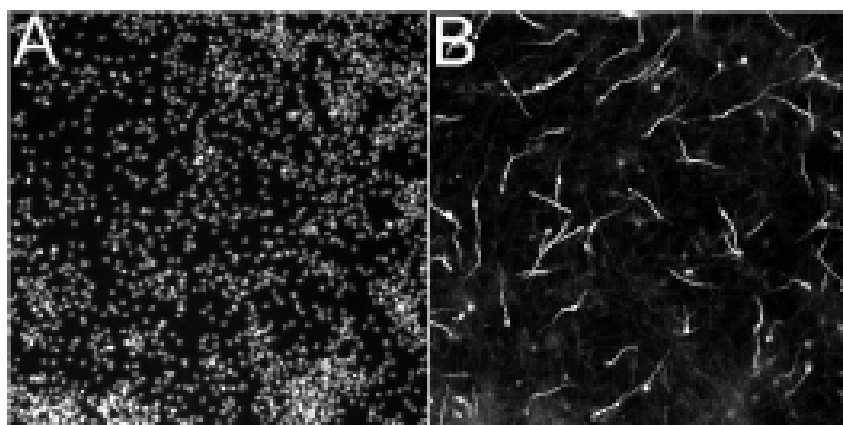


Figure 1. Images of a primary human brain culture labeled with Neurite Outgrowth HCS reagents. Panel A: Cell nuclei are labeled with Hoechst Dye. Panel B. Neuronal cells and their neuritis in the same optical field are labeled with the fluorophore-conjugated secondary antibody.

Additional Materials Required

- Nerve Growth Factor (NGF) (BD, Product No. 356004)
- Formaldehyde (37%) (Sigma, Product No. F1268)
- Clear-bottom Microplates: For PC12 cells, use collagen IV-coated plates (BD, Product No. 354429); for NS-1 cells, use collagen-I coated plates (BD, Product No. 356407)

Note: For the screening size kit, Wash Buffer and Neurite Outgrowth Buffer are available separately (please call customer service for more information).

Cell Preparation Information

Note: The protocol for NS-1 cells is the same as the protocol for PC12 cells except when indicated.

- For **PC12 cells**, culture cells in F12K Nutrient Mixture, Kaighn's (F12K; GIBCO, Product No. 21127-022) containing the following supplements (=F12K Complete Medium) 15% horse serum (HyClone, Product No. SH30074.03) 2.5% fetal calf serum (HyClone, Product No. SH30070.03) 1% penicillin/streptomycin (BioWhittaker, Product No. 17-602E) 1% L-glutamine (BioWhittaker, Product No. 17-605E)
- For **NS-1 cells**, culture cells in RPMI 1640 (RPMI; BioWhittaker, Product No. 12-702Q) containing the following supplements (=RPMI Complete Medium): 10% horse serum (HyClone, Product No. SH30074.03), 5% fetal calf serum (HyClone, Product No. SH30070.03) 1% penicillin/streptomycin (BioWhittaker, Product No. 17-602E) 1% L-glutamine (BioWhittaker, Product No. 17-605E)
- Split cells when they reach 70-80% confluency (every 3-4 days) at a dilution of 1:5.
- Harvest cells with Trypsin-Versene mixture (BioWhittaker, Product No. 17-161F). For PC12 cells, dilute into F12K Complete Medium. For NS-1 cells, dilute into RPMI Complete Medium.
- Determine cell density. Adjust cells to 2×10^4 cells/ml with F12K Complete Medium with or without 1 μ g/ml NGF for PC12 cells; for NS-1 cells, use RPMI Complete Medium with or without 200 ng/ml NGF.
- Add 100 μ l (=2,000 cells) of the cell suspension to each well of a collagen IV-coated 96-well microplate for PC12 cells; for NS-1 cells use collagen I-coated 96-well microplate.
- Incubate for 7 days at 37°C, 10% CO₂, replacing the medium with new F12K Complete Medium containing 1 μ g/ml NGF every 3-4 days; incubate NS-1 cells for 3-4 days at 5% CO₂; no medium exchange is necessary.

Neurite Outgrowth Kit Protocol

A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml of 10X Wash Buffer to 180 ml of ultrapure water. Store this solution at 4°C for up to 7 days.
1X Neurite Outgrowth Buffer	Add 20 ml 10X Neurite Outgrowth Buffer to 160 ml ultrapure water, adjust pH to 7.2, and bring to a final volume of 200 ml with ultrapure water. Store this solution at 4°C for up to 7 days.
Fixation/Hoechst Solution	Add 2.2 ml 37% formaldehyde and 11 µl of Hoechst Dye to 19.8 ml of 1X Wash Buffer. Warm to 37°C before use. Prepare just before each assay.
Primary Antibody Solution	Add 13.75 µl of Neurite Outgrowth Primary Antibody antibody to 11 ml of 1X Neurite Outgrowth Buffer. Prepare just before each assay.
Secondary Antibody Staining Solution	Add 12 µl Secondary Antibody to 6 ml of 1X Neurite Outgrowth Buffer. Prepare just before each assay.

B. Procedure

Note: This protocol requires ~2.5 hours to perform once compound incubation has been completed.

1. Add test compounds to plates prepared in Cell Preparation Section. Compounds may be added either before cell and neurite growth or after neurite outgrowth has occurred.
2. Aspirate medium and add 200 µl pre-warmed Fixation/Hoechst Solution to each well. Incubate in fume hood at room temperature for 20 minutes. Pre-warming fixative is critical to maintaining cell integrity; low-velocity fluid dispensing is recommended.
3. Aspirate Fixation/Hoechst Solution and wash three times with 200 µl of 1X Neurite Outgrowth Buffer.
4. Aspirate Neurite Outgrowth Buffer and add 100 µl of Primary Antibody Solution and incubate for 1 hour
5. Aspirate Primary Antibody Solution and wash three times with 200 µl of 1X Neurite Outgrowth Buffer.
6. Aspirate Neurite Outgrowth Buffer and add 50 µl of Secondary Antibody Solution and incubate for 1 hour, protected from light.
7. Aspirate Secondary Antibody Solution and wash twice with 200 µl of 1X Neurite Outgrowth Buffer.
8. Aspirate Neurite Outgrowth Buffer and wash twice with 200 µl of 1X Wash Buffer-M, leaving buffer from the final wash in wells.
9. Seal plate and evaluate on the ArrayScan HCS Reader.
10. Store plate at 4°C in the dark.

Additional Information

A. Dose Response Curves

The dose response curve is plotted using the percentage of cells with neurite outgrowth (i.e. Neurite Outgrowth Index) versus NGF concentration (Figure 2A). The dose response curve is plotted using the percentage of cells with neurite outgrowth versus dopamine concentration (Figure 2B).

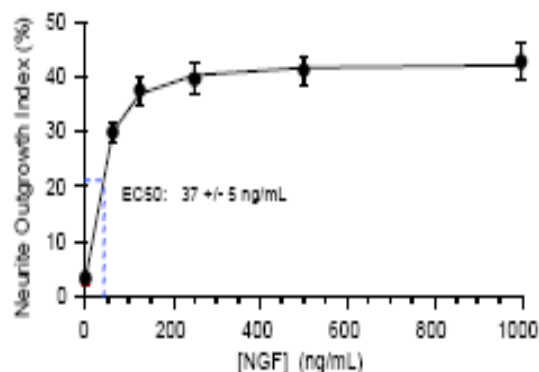


Figure 2A. Dose response curve of NGF in PC12 cells. PC12 cells were grown on collagen IV-coated 96-well plates for 7 days in the presence of different concentrations of NGF. Each data point is the mean result from 16 wells, and error bars are the standard deviations. The EC_{50} for NGF induced neurite outgrowth for PC12 cells calculated from this graph is 37 ± 5 ng/ml.

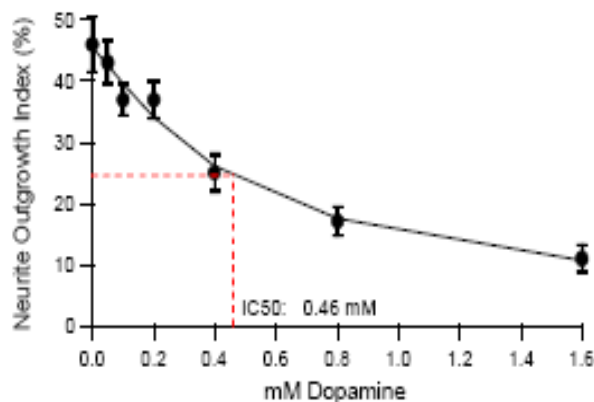


Figure 2B. Dose response curve of dopamine in PC12 cells. PC12 cells were grown on collagen IV-coated plates for 7 days in the presence of $1 \mu\text{g/ml}$ NGF. Varying concentrations of dopamine were added for 3 hours. Each data point is the mean result from 8 wells, and error bars are the standard deviations. The IC_{50} for dopamine toxicity to neurites from this graph is 0.46 mM.

B. Microscope Information

Cells prepared and labeled according to these instructions can be used and analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization may be required when using slides, coverslips or multi-well chamber slides. Use image-processing software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are as follows:

DyLight 488 Conjugates = 495/519 nm

Hoechst Dye = 350/461 nm

C. Recommendations for Automation

- **Plating Cells:** To improve the uniformity and throughput of plating cells, use a liquid handling system such as Thermo Scientific Multidrop[®] Combi or WellMate[®] Dispensers.
- **Dead Volumes:** Every piece of automation instrumentation has a non-recoverable dead volume associated with it. Be aware of these dead volumes, priming volumes and rinsing volumes when calculating your reagent requirements.
- **Nonspecific Binding:** Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.
- **Mixing:** Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.
- **Cell Washing:** Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.
- **Incubation:** Minimize the time when plates with live cells are out of a controlled CO_2 environment. For best results, use an automated incubator to deliver plates to a pipetting deck.
- **Exposure:** Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.

- Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed. Some suggested starting volumes are listed in Table 1.

Table 1. Suggested volumes to use for different cell culture plates.

<u>Kit Component</u>	<u>96-Well Plates</u> (μ l/well)	<u>384-Well Plates</u> (μ l/well)	<u>24-Well Plates</u> (μ l/well)
Fixation Solution	100	25	400
1X Wash Buffer	100	25	400
1X Neurite Outgrowth Buffer	100	25	400
Antibody Solution	50	12.5	200
Staining Solution	50	12.5	200
1X Wash Buffer (final wash)	150	37.5	200

Compatible BioApplication Software Modules

S50-0007-2 or S50-20007-1 **Neurite Outgrowth BioApplication**

S50-0020-2 or S50-2020-1 **Extended Neurite Outgrowth BioApplication**

References

- Taylor, D.L., *et al.* (2007). High content screening: A powerful approach to systems cell biology and drug discovery. *Method Mol Biol* **356**. Humana Press, Totowa, N.J.
- Zhang, J.H., *et al.* (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* **4**:67-73.

Thermo Scientific Cellomics Reagent Kits are developed and manufactured at the same Thermo Fisher Scientific Inc. facility as Pierce Protein Research Products and are supported by Pierce Technical Support (see contact information in page footer). These kits are part of the Cellomics Total Solution Platform for HCS, which also includes Cellomics ArrayScan and other HCS Instrumentation, BioApplication Image Analysis Software and High-Content Informatics. For more information, visit www.thermo.com/cellomics or call 800-432-4091 (toll free) or 412-770-2500.

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