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Neurite Outgrowth Assay Kit (3 µm)

12 Tests

Catalog No. NS220

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

Proper neuronal migration and establishment of circuitry are key processes for nervous system functioning. During development, neurons extend numerous processes that differentiate into dendrites and axons. These processes, also termed neurites, are critical for communication between neurons.

The characterization of neurite formation, maturation and collapse/resorption is an area of intense interest, since these cellular processes are essential for interconnection of neuronal cell bodies. Neurites are particularly interesting in relation to neuropathological disorders, neuronal injury/regeneration, and neuropharmacologic research and screening. Nerve transection in the mammalian central nervous system (CNS) was once believed to be irreversible, however, it has recently become apparent that the inability of damaged nerve fibers to regenerate is an active process under the control of molecules able to inhibit and repulse growing neurites [1,2,3,4]. Therefore, major efforts in CNS drug discovery research are focused on the identification of compounds that affect neurite outgrowth. However, the study of neurites is hampered by difficulties associated with isolating and purifying these minute organelles. Currently available methods for measuring neurite outgrowth consist of manual microscopic examination of individual cells or measurement of total fluorescence from a labeled neuronal cell population using a fluorescence plate reader. The disadvantages of the first method include labor intensiveness and subjectivity; the second method is unable to discriminate between fluorescently-labeled neuronal cell bodies and neurites.

Consequently, the lack of a means to isolate and purify sufficient neurite material, and the lack of a uniform and highly reproducible method for neurite quantification, has impeded the understanding of the role of these organelles in development, injury and disease states.

The method for measuring neurite outgrowth in Chemicon's NS220 Neurite Outgrowth Assay kit has been developed for characterization of neurite formation, composition and behavior in response to chemical agents and growth conditions. Means are also suggested for recovery of purified neurite preparations. What makes this method unique is the convenient production, observation and quantification of neurites in a standard (24-well) format.

This product differs from our previous NS200 Neurite Outgrowth Quantification Assay kit in that an improved assay protocol is included and Millicell tissue culture inserts from Millipore are now used. These changes result in less background and a greater signal to noise ratio, along with easier visualization of cell bodies and neurites.

Application

CHEMICON's NS220 Neurite Outgrowth Assay Kit (3 µm) utilizes microporous tissue culture insert technology from Millipore and is based on the use of Millicell cell culture inserts (chambers) containing a permeable membrane with 3-µm pores at the base. The Millicell inserts utilized in this kit are appropriate for cells projecting neurites of up to 3 µm diameter, e.g. N1E-115 cells [5], Dorsal Root Ganglia and Schwann cells [6]. The inserts are designed to fit into a receiver vessel, which when in use contains differentiation media in contact with the bottom of the insert membrane. The permeable membranes allow for discrimination between neurites and cell bodies, as projecting neurites will pass easily through the pores but cell bodies will not. Therefore, by inducing neurites to traverse these membrane pores, a purified population of neurites is located on the underside of the membrane, distal to the surface on which neural cell bodies are deposited.

The assay is a simple, efficient, and versatile system for the quantitative determination of compounds that influence neurite formation and repulsion. With this system, it is possible to screen numerous biological and pharmacological agents simultaneously, directly evaluate adhesion and guidance receptor functions responsible for neurite extension and repulsion, as well as analyze gene function in transfected cells. The microporous filter allows for biochemical separation and purification of neurites and cell bodies for detailed molecular analysis of protein expression, signal transduction processes and identification of drug targets that regulate neurite outgrowth or retraction processes. Reagents and materials supplied in the NS220 Neurite Outgrowth Assay Kit are sufficient for 12 tests.

Note: the 3 µm pore size inserts in this kit are unsuitable for use with PC12 cells. Due to the relatively small diameter of PC12 cell bodies [5], some of these cells may pass though 3 µm pores and obscure the results. Chemicon's NS225 Neurite Outgrowth Assay Kit (1 µm) has been specifically designed for and validated with PC12 cells.

Kit Components

1. Neurite Outgrowth Plate Assembly, 3 µm: (Part No. 2007256) 1 x 24-well plate containing 12 x Millicell hanging inserts with 3 µm pore size membranes.
2. Neurite Stain Solution: (Part No. 90242) One 20 mL bottle.
3. Neurite Stain Extraction Buffer: (Part No. 90243) One 20 mL bottle.
4. Neurite Outgrowth Assay Plate: (Part No. 2007255) Two 24-well plates.
5. Cotton Swabs: (Part No. 10202) 50 swabs.
6. Forceps: (Part No. 10203) One each.

Materials Required But Not Provided

1. Desired extracellular matrix membrane coating protein, *e.g.*, bovine collagen I or mouse laminin.
2. Tissue culture instruments/supplies (including 37ºC incubator, growth media, flasks/plates, detachment buffer, etc.) for cell type of interest.
3. Differentiation media/factors for cell type of interest (*e.g.*, nerve growth factor, serum-free media).
4. 1X phosphate buffered saline (PBS) with Ca^{2+}/Mg^{2+}.
5. Inverted microscope for neurite visualization on membrane inserts.
6. Parafilm and spectrophotometer for neurite stain extraction and quantification.
7. Protein lysis buffer (optional)

Storage

When stored at 2-8º C, the kit components are stable up to the expiration date. Do not freeze or expose to elevated temperatures. Discard any remaining reagents after the expiration date.

Assay Instructions

Note: The procedure described has been optimized for use with N1E-115 cells. Users may adjust the priming and differentiation conditions to meet the needs of their own experimental system.

1. Prior to initiation of the assay, culture cells in growth media until ~60-70% confluent in culture flasks/plates appropriate for the cell type.

2. For neural cell differentiation and neurite extension to occur, cells must first be "primed" by specific external stimuli to induce cessation of proliferation and increased tubulin production. For example, for N1E-115 cells, growth media should be replaced with serum-free differentiation media for 24 hours. N1E-115 differentiation media may consist of: DMEM with L-glutamine + 1% penicillin/ streptomycin + 0.1% bovine serum albumin (BSA).

3. Following cell "priming" and prior to initiation of the neurite outgrowth assay, prepare the underside of the insert membrane surface (opposite the top membrane surface on which cells are deposited) by extracellular matrix (ECM) protein coating, for induction of neurite outgrowth away from the cell bodies. Prepare fresh ECM protein at a desired working concentration in 1X phosphate buffered saline (PBS, with Ca^{2+}/Mg^{2+}), *e.g.*, 10 µg/mL laminin for N1E-115 cells. Add 400 µL of sterile protein solution to the bottom of an empty well of the 24-well plate included in the Neurite Outgrowth Plate Assembly. Place a Millicell insert (provided) into the well containing protein solution and allow to coat the underside of the membrane for 2 hours at 37ºC. For negative controls, membranes may be coated with a BSA solution rather than with ECM protein. See Figure 1.

4. While membranes are being coated, detach cells from culture flasks/plates. Cells may be more resistant to detachment due to some degree of neurite extension during "priming." Resuspend in differentiation media at a concentration of 100,000-200,000 cells per 100 µL (1-2 x 10^6 cells/mL). Following coating, remove each insert from its coating solution (rinsing not required) and place into a new well of the Neurite Outgrowth Plate Assembly 24-well plate containing 600 µL of differentiation media. Add 100 µL of cell suspension onto the top of the membrane and allow to sit at room temperature for ~15 minutes to provide for even cell distribution before transferring to a 37ºC incubator for culture. Allow neurites to extend to the underside of the membrane for 48 hours at 37ºC. See Figure 1. If it is necessary to change the culture media during this extension period, replace the volume below and above the membrane with 600 µL and 100 µL of fresh media, respectively. At all times when adding or

removing media from the top of the membrane, be careful to not excessively disrupt the cell layer or puncture the membrane with pipette tips, etc.

5. Following the neurite extension period, remove each insert from its culture well, gently pipette off the liquid from the top of the membrane (Note: always remove liquid from the top of the membrane prior to placement in new solutions, now and throughout), and place into 800 µL of 1X PBS in a well of a 24-well Neurite Outgrowth Assay Plate. PBS-containing wells may be emptied and re-used for all subsequent PBS rinses. Allow the insert to sit briefly to rinse, then transfer to a new well containing 400 µL of -20ºC methanol. Allow the membrane to fix for 20 minutes at room temperature. Rinse again briefly in 800 µL of fresh PBS.

6. Staining of neurites for visualization and quantification:

   Following fixation, place the insert into 400 µL of Neurite Stain Solution in a new well of a Neurite Outgrowth Assay Plate. Allow neurites to stain for 15-30 minutes at room temperature, then rinse in 800 µL of fresh PBS. For most complete cell body/neurite separation and accurate/reproducible quantification, it is **essential** to remove all cell bodies from the top of the membrane (particularly at the edge), as well as any stray stain that may be present on the inner or outer walls of the insert. To do this, gently remove cell bodies from the top of the membrane by wiping with the flattened tip of a cotton swab (to flatten, press swab tip firmly against a hard surface). Firmly run the swab around the inner wall of the insert, while simultaneously using a slow twirling motion of the swab to gently wipe the top of the membrane. The swab may be moistened with PBS, as the presence of PBS from the moistened swab aids in the removal of cell bodies that may be more difficult to access at the membrane edges. Repeat this procedure with fresh cotton swabs and PBS rinses as necessary to remove any stain not localized to the underside of the membrane. Swabbed, dry inserts may be held in empty wells of a Neurite Outgrowth Assay Plate. Stained neurite extensions may be visualized on intact inserts in a 24-well plate using an inverted microscope focused on the underside of the membrane (Figure 2). For quantification, for each insert place a 100-200µL drop of Neurite Stain Extraction Buffer onto a piece of Parafilm that has been taped to a flat surface. Position the underside of the membrane over the drop of Extraction Buffer so that the entire membrane surface is covered. Allow the stain to extract for 5 minutes at room temperature. Completely collect buffer from around the insert, including the volume left on the Parafilm surface, liquid inside the insert (*i.e.*, on the top membrane surface), and any liquid adhering to the underside of the membrane (may be blotted from the insert onto the Parafilm surface). Remove the buffer to a

96-well plate (or cuvette) and quantify neurite extension on a spectrophotometer by reading absorbance at 562 nm (Figures 3 & 4).

7. (OPTIONAL) Protein isolation from neurites:

   In lieu of neurite staining, protein may be isolated from extended neurites. Following methanol fixation and rinsing in PBS, remove cell bodies from the top of the membrane as described in Step 6, being as thorough as possible despite the absence of cell body staining/visualization. For each insert, place a 100-200µL drop of protein lysis buffer (*e.g.*, 1% SDS) onto a piece of Parafilm that has been taped to a flat surface. Position the underside of the membrane over the drop of buffer so that the entire membrane surface is covered. Neurites may also be removed from the membrane with a cell scraper and placed back into the drop of lysis buffer. Completely collect buffer on the Parafilm, inside the insert (*i.e.*, on the top membrane surface) and any liquid adhering to the underside of the membrane. Following lysis, samples may be boiled and used for SDS-PAGE or Western blot.

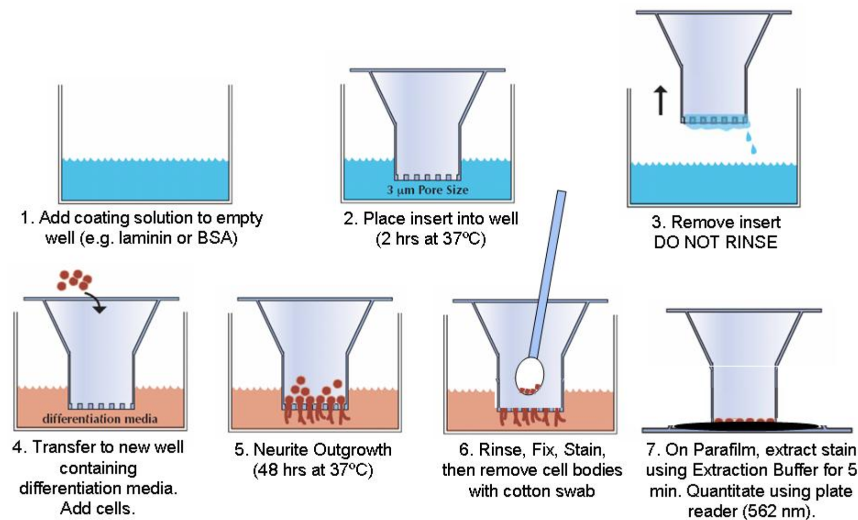

Figure 1. Overview of NS220 Neurite Outgrowth Assay Protocol.

Example Results

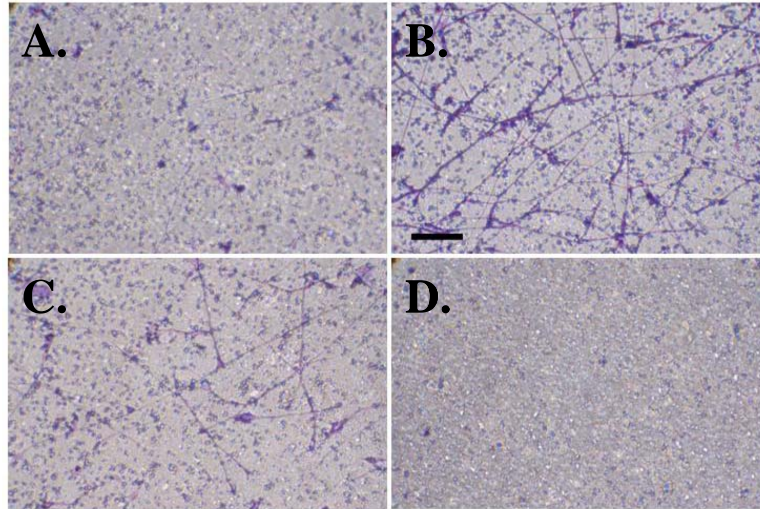

Figure 2. Experiment showing stimulation and inhibition of neurite outgrowth from N1E-115 cells in response to a range of conditions. A. Negative Control. BSA-coated inserts (2 mg/mL), serum-free media; B. Laminin-coated inserts (10 µg/mL), serum-free media; C. Laminin-coated inserts (10 µg/mL), serum free media plus 25 ng/mL nocodazole, a potent inhibitor of neurite extension; D. Laminin-coated inserts (10 µg/mL), serum free media plus 500 ng/mL nocodazole. Scalebar represents 50 µm. In this experiment, N1E-115 cells were primed for 24 hours, seeded at 150,000 cells/well, allowed to extend neurites for 48 hours, then stained with Neurite Stain Solution for 30 minutes. Nocodazole-treated samples were allowed to extend in serum-free differentiation media for 24 hours, then replaced with serum-free differentiation media plus nocodazole for an additional 24 hours.

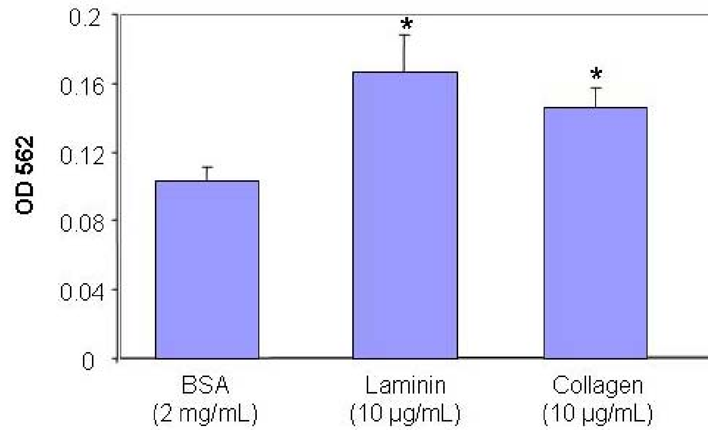

Figure 3. Experiment showing stimulation of neurite outgrowth from N1E-115 cells in response to a range of membrane coating substrates. Neurite outgrowth was significantly stimulated in Millicell inserts coated with the ECM proteins laminin or collagen (each 10 µg/mL) compared to BSA controls (*p<0.05; n=3). In this experiment, N1E-115 cells were primed for 24 hours, seeded at 150,000 cells/well, allowed to extend neurites for 48 hours, then stained with Neurite Stain Solution for 30 minutes. .100 µL of Neurite Stain Extraction Buffer was used for stain extraction. OD_{562} values were normalized to Stain Extraction Buffer.

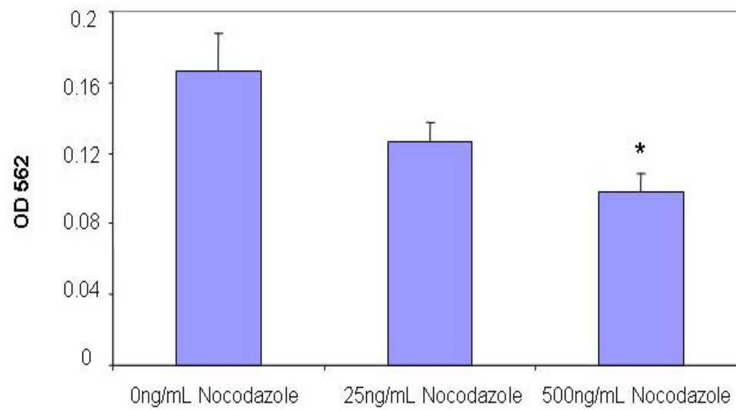

Figure 4. Experiment showing inhibition of neurite outgrowth from N1E-115 cells in response to the potent microtubule depolymerizing agent nocodazole. Neurite outgrowth was inhibited in cells exposed to 25 ng/mL or 500 ng/mL nocodazole compared to control (*p<0.05; n=3). In this experiment, N1E-115 cells were primed for 24 hours, seeded at 150,000 cells/well, allowed to extend neurites for 48 hours, then stained with Neurite Stain Solution for 30 minutes. 100 µL of Neurite Stain Extraction Buffer was used for stain extraction. OD_{562} values were normalized to Stain Extraction Buffer. Nocodazole-treated samples were allowed to extend in serum-free differentiation media for 24 hours, then replaced with serum-free differentiation media plus nocodazole for an additional 24 hours.

Troubleshooting

The number of cells seeded onto inserts may be increased to augment absorbance signal. Note that increasing cell number may also increase the "background" of negative control (no extension) samples.

If using the provided membrane inserts in 24-well plates other than those included in this kit, it may be necessary to adjust the volumes utilized, due to differences in well depths between plate manufacturers. In many cases, it may be necessary to increase coating, culture, fixation and staining volumes.

References

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Warranty

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