INSTRUCTIONS

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Pierce Primary Neuron Isolation Kit

MAN0016221
Rev A.0
Pub. Part No. 2162549.0

88280

Number	Description
88280	**Pierce Primary Neuron Isolation Kit,** contains sufficient reagents to isolate primary neurons from 50 pairs of embryonic mouse/rat cortexes or 150 pairs of embryonic mouse/rat hippocampi. Also contains reagents to support the culture of primary cortical and hippocampal neurons.

Kit Contents:

Neuron Culture Module (88280X), store at 4°C:

Neuronal Culture Medium, 500mL

Hanks' Balanced Salt Solution (HBSS without Ca^{2+}, Mg^{2+}), 500mL

Neuron Isolation Module (88280Y), store at -20°C:

Neuronal Isolation Enzyme (with papain), lyophilized, 5 vials

Neuronal Culture Medium Supplement (50X), 10mL

Glutamine Supplement (100X), 5mL

Neuronal Growth Supplement (1000X), 0.5mL

Storage: Upon receipt, store Product 88280X at 4°C and Product 88280Y at -20°C. Product 88280X is shipped with an ice pack. Product 88280Y is shipped on dry ice.

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Introduction

The Thermo Scientific™ Pierce™ Primary Neuron Isolation Kit provides a simple, reliable and convenient method for the isolation and culture of primary neurons from embryonic mouse/rat cerebral cortex or hippocampus. The kit consists of unique tissue-specific dissociation reagents and a validated protocol to ensure a high yield of viable and functional neurons when used by both experienced and non-experienced users. The fully optimized culture reagents are designed to provide optimal growth conditions for maintaining highly pure primary neurons in either short- or long-term cultures.

Primary neurons isolated and cultured using the Pierce Primary Neuron Isolation Kit can be maintained in culture for up to four weeks. They are appropriately polarized, develop extensive axonal and dendritic arbors, express neuronal and synaptic

markers, and form numerous, functional synaptic connections (Figure 1). They can be used as a model system for molecular and cellular biology studies of neuronal development and function, especially for visualizing the subcellular localization of endogenous or over-expressed proteins and protein trafficking.[1] In combination with knockout or transgenic technologies, primary neurons isolated and cultured with the Pierce Primary Neuron Isolation Kit may serve as a biologically relevant system for preclinical drug discovery, neurotoxicity testing and predictive disease modeling.

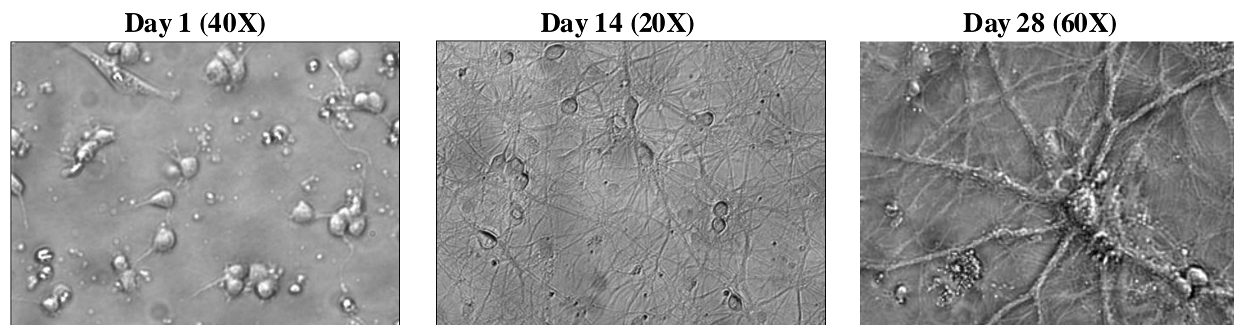

Figure 1. Primary cortical neuron cultures at different developmental stages in culture. Phase-contrast images of mouse cerebral cortical cultures after 1 day, 14 days and 28 days. Cultures are plated at a density of 2×10^5 cells per 12-well plate. After one day in culture, neurons start to differentiate and one or several short neurites extending from the cell body can be observed; at Day 14 and Day 28, an extensive, intertwined network of axons and dendrites has developed.

Important Product Information

- For best cell yield and viability, always isolate neurons from freshly dissected tissues. The dissection and plating of neurons should take no more than three hours.
- Euthanize mice or rats in accordance with the Guide for the Care and Use of Laboratory Animals.[2]
- Perform all tissue digestion, cell manipulations and media handling using sterile technique in a laminar flow cell culture hood to minimize contamination of the isolated neurons.
- Pierce Primary Neuron Isolation Kit does not contain poly-D-lysine-coated chamber slides/dishes. Use commercially available poly-D-lysine-treated culture vessels or prepare poly-D-lysine-treated chamber slides/dishes before cell isolation using the coating method described in Additional Information, pg. 6.

Additional Materials Required

- Heat-inactivated fetal bovine serum (FBS) (e.g., Thermo Scientific™ Hyclone™ FBS)
- Penicillin-streptomycin (pen/strep) (e.g., Thermo Scientific™ Hyclone™ Pen/Strep Solution)
- Cerebral cortex or hippocampus freshly dissected from mouse/rat embryos taken from a euthanized pregnant mouse/rat (E17-19)
- Poly-D-lysine (Sigma) or poly-D-lysine-coated culture vessels (e.g., Nunc™ 6-well culture plate)
- 37°C heat block or incubator
- 37°C tissue culture incubator with humidified, 5% CO_2 atmosphere
- Laminar flow cell culture hood
- Hemocytometer or automated cell counter
- Trypan blue stain (e.g., Thermo Scientific™ Hyclone™ Trypan Blue)

Material Preparation

Note: After media supplementation, media is stable for approximately one month when stored at 4°C.

Serum-supplemented Neuronal Culture Medium	Determine the amount of medium required based on experimental conditions (see Table 2, pg. 5 for guidelines). In a sterile bottle, add heat-inactivated FBS (10% final concentration), Glutamine Supplement (1X final concentration) and pen/strep (1% final concentration) to desired volume of Neuronal Culture Medium. Pre-warm medium to 37°C before use.
Serum-free Neuronal Culture Medium	Determine the amount of medium required based on experimental conditions (see Table 2, pg. 5 for guidelines). In a sterile bottle, add Neuronal Culture Medium Supplement (1X final concentration), Glutamine Supplement (1X final concentration) and pen/strep (1% final concentration) to desired volume of Neuronal Culture Medium. Pre-warm medium to 37°C before use.

Procedure for Cortical or Hippocampal Neuron Isolation

A. Enzyme Digestion of Neural Tissue

Note: For the medium and buffer removal steps, it is critical to carefully remove the buffer/medium without disturbing the tissue. For best results, use a pipette and 1000µL tip. Do not aspirate using a vacuum flask.

Note: Equilibrate HBSS to 4°C before use.

1. Reconstitute the Neuronal Isolation Enzyme (with papain) by adding 2.5mL of HBSS to one of the vials. Mix gently for 5 minutes or until completely dissolved. Keep enzyme solution on ice.

   Note: 2.5mL reconstituted Neuronal Isolation Enzyme (with papain) is sufficient for preparing 10 pairs of mouse/rat cortexes or 30 pairs of mouse/rat hippocampi.

   Note: Reconstituted Neuronal Isolation Enzyme (with papain) can be stored at -20°C for 6 months and is stable for up to two freeze-thaw cycles. The enzyme solution expires one week following preparation if stored at 4°C. Use of the reconstituted Neuronal Isolation Enzyme (with papain) after one week may result in poor performance.

2. Place freshly dissected cortexes or hippocampi into separate 1.5mL sterile microcentrifuge tubes. Immediately add 500µL ice cold HBSS.

   Note: For best results, use one microcentrifuge tube containing one pair of mouse/rat cortex or 3 pairs of hippocampi.

3. Gently remove HBSS using a pipette to the level of the tissue (450µL). Add 0.2mL reconstituted Neuronal Isolation Enzyme (with papain) to each tube. Incubate at 37°C in a cell culture incubator for 25-30 minutes.
4. Gently remove the Neuronal Isolation Enzyme (with papain) solution and wash tissue twice with 500µL HBSS.
5. Add 0.5mL pre-warmed Serum-supplemented Neuronal Culture Medium to each tube. Break up the tissue by pipetting up and down 15-20 times using a sterile 1000µL pipette tip. Avoid air bubbles when pipetting.

   Note: Disrupting the tissue by pipetting improves cell yield. However, pipetting too vigorously can result in cell damage.

6. After the tissue is primarily a single-cell suspension, add 1.0mL pre-warmed Serum-supplemented Neuronal Culture Medium to each tube to bring the total volume to 1.5mL.
7. Combine individual cell suspensions for determination of cell concentration and cell viability.

B. Cell Yield and Viability Determination

1. Mix 25µL of single-cell suspension obtained in Section A, Step 7, with 25µL of 0.4 % trypan blue stain in a 1.5mL microcentrifuge tube.
2. Immediately transfer 10µL trypan blue-stained cell suspension to each of two hemocytometer counting chambers.

3. Count both the total number of cells and the number of stained (blue) cells in the center square in each hemocytometer microscopic grid.

   Cell concentration (cells/mL) = number of cells × dilution factor × 10^4

   Example: If 120 cells in a square, then $120 \times 2 \times 10^4 = 2.4 \times 10^6$ cells/mL

   Cell yield = cell concentration × volume of cell suspension, obtained in Section A, Step 7

   Viability (%) = [(total cell counted – total stained cells)/total cell counted] × 100%

   Typical cell yields and viabilities from different tissue types are shown in Table 1.

4. If using an automated cell counter, determine cell yield and viability according to the manufacturer's instructions.

Table 1. Cell yield and viability from a typical isolation.

Tissue Type	Yield (cells/mL)	Viability (Trypan Blue Exclusion)
Mouse cortical neuron (one pair in 1.5mL cell suspension)	4.5×10^6	94%
Mouse hippocampal neuron (three pairs in 1.5mL cell suspension)	3.6×10^6	93%
Rat cortical neuron (one pair in 1.5mL cell suspension)	4.0×10^6	94%
Rat hippocampal neuron (three pairs in 1.5mL cell suspension)	4.0×10^6	96%

C. Plating and Culturing Isolated Neurons

Note: Determine the desired plating density for the cultured neurons based on the intended downstream study. Table 2 shows recommended cell densities for culturing low- (sparse), medium- and high-density neurons, respectively.[3] Figure 2 provides images of cells plated at the different densities.

1. Pipette the appropriate cell suspension volume into each well of the culture vessel (see Table 2, pg. 5):

   Cell suspension volume/well = [required cell density × growth area (cm^2)]/cell concentration (cells/mL from Section B, Step 3, above)

   Example: For a 12-well Nunc plate, a single well is approximately $3.5cm.^2$ If the cell count is 4×10^6 cells/mL, add 22µL, 44µL or 220µL of cell suspension to each well to obtain low-, medium- or high-density cultures, respectively.

2. Add Serum-supplemented Neuronal Culture Medium to each well to bring the total volume to the recommended level.

   Example: For the 12-well Nunc plate example in Step 1, add 778µL, 756µL or 580µL Serum-supplemented Neuronal Culture Medium to each well to bring the total volume to 0.8mL.

3. Place the dishes/chamber slides in a 5% CO_2 incubator and incubate at 37°C for 24 hours.

4. After 24 hours, replace the Serum-supplemented Neuronal Culture Medium with an equivalent volume of Serum-free Neuronal Culture Medium.

5. Incubate cultures at 37°C in a 5% CO_2 incubator.

6. At Day 3, add Neuronal Growth Supplement (1000X) to each well for a 1X final concentration.

   Note: Neuronal Growth Supplement reduces non-neuronal cell contamination and maintains neurons at high purity during the culture period. Neuron purity in culture at Day 7 is expected to be over 90% when using the recommended dilution of Neuronal Growth Supplement.

Example: For a 35mm culture dish containing 2.0mL Serum-free Neuronal Culture Medium, add 2.0µL Neuronal Growth Supplement. For small volumes, dilute the Neuronal Growth Supplement 10-fold in HBSS before use to avoid pipetting errors. Do not store diluted Neuronal Growth Supplement.

7. One week after plating, and every week thereafter, feed the cultures by replacing one-fourth of the volume with fresh Serum-free Neuronal Culture Medium containing Neuronal Growth Supplement.

Table 2. Recommended seeding densities for commonly used culture vessels.*

Nunc Culture Dishes/Chamber Slides	Well Diameter (mm)	Approximate Growth Area (cm^2)	Medium Volume (mL)	Total Number of Cells Required to Seed Each Well		
				Low Density† (2.5×10^4 cells/cm^2)	Medium Density† (5.0×10^4 cells/cm^2)	High Density (2.5×10^5 cells/cm^2)
35mm dish	35	9.0	2.0	2×10^5	5×10^5	2×10^6
6-well plate	35	9.6	2.0	2×10^5	5×10^5	2×10^6
12-well plate	22	3.5	0.8	1×10^5	2×10^5	1×10^6
24-well plate	16	1.8	0.5	5×10^4	1×10^5	5×10^5
48-well plate	11	1.1	0.3	2.5×10^4	5×10^4	2.5×10^5
96-well plate	4.3	0.14	0.1	5×10^3	1×10^4	5×10^4
4-well chamber slide	NA	1.8	0.5	5×10^4	1×10^5	5×10^5
8-well chamber slide	NA	0.8	0.2	2×10^4	4×10^4	2×10^5

*Low- and medium-density cultures may be better suited for imaging, while a high-density culture may be appropriate for biochemical assays or protein extraction.

†Additional medium may be required for low- and medium-density cultures.

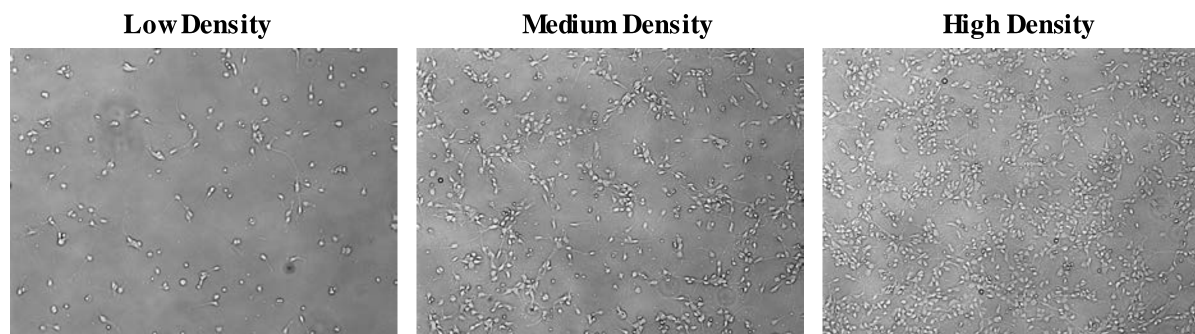

Figure 2. Mouse hippocampal cultures plated at different densities (low, medium and high) contain different numbers of neurons. Phase-contrast images of mouse hippocampal cultures after 2 days. Cultures are plated into poly-D-lysine-coated 6-well plates at a density of 2×10^5 cells (low), 5×10^5 cells (medium) and 2×10^6 cells (high) per well. Images are taken at 10X magnification.

Troubleshooting

Problem	Possible Cause	Solution
Low yield/low viability	Insufficient dissociation	Use freshly reconstituted Neuronal Isolation Enzyme (with papain)
		Ensure digested tissue is fully disrupted by pipetting
	Over-digestion	Do not incubate longer than 35 minutes
		Ensure that Neuronal Isolation Enzyme (with papain) is reconstituted to the recommended concentration
Poor cell attachment after 24 hours	Culture plates are not coated properly	If coating your own plates, follow the coating method recommended (see Additional Information, pg. 6)
		Check the concentration of poly-D-lysine. Do not use expired poly-D-lysine
	Cells are dead	Do not pipette too vigorously when disrupting digested tissue
Slow cell growth	Medium and/or supplement stored incorrectly, or expired medium and/or supplements	Check the recommended storage conditions. Confirm that the products were stored properly
		Do not use expired medium and/or supplements
Low cell purity	Did not use cell growth supplement	Add Neuronal Growth Supplement at recommended concentration at Day 3 after seeding cells

Additional Information

A. Method for Preparation of Poly-D-Lysine-Coated Chamber Slides/Dishes

Note: For best results, always use freshly prepared poly-D-lysine-coated chamber slides/dishes. Perform the preparation in a laminar flow cell culture hood to minimize contamination.

1. Prepare a 1.0mg/mL stock solution of poly-D-lysine in HBSS.

   Example: Add 5mL HBSS to 5mg poly-D-lysine (Sigma). Poly-D-lysine may then be aliquoted and stored at -20°C for 12 months. Reconstituted poly-D-lysine is stable for up to three freeze-thaw cycles.
2. Dilute poly-D-lysine working solution to a concentration of 10µg/mL (1:100 dilution in HBSS) and coat chamber slides/dishes with the poly-D-lysine working solution using the medium volume recommended in Table 2, pg. 5).
3. Incubate chamber slides/dishes with poly-D-lysine working solution at room temperature for 1-2 hours.
4. Aspirate poly-D-lysine working solution to dryness with a vacuum flask and wash chamber slides/dishes three times with HBSS using the medium volume recommended in Table 2, pg. 5.
5. Dry chamber slides/dishes in a laminar flow cell culture hood for 1 hour before adding Serum-supplemented Neuronal Culture Medium.

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Related Thermo Scientific Products

88283	**Neuronal Culture Medium**
88284	**Hanks' Balanced Salt Solution (HBSS, without Ca^{2+}, Mg^{2+})**
88285	**Neuronal Isolation Enzyme (with Papain)**
88286	**Neuronal Culture Media Supplement**
88287	**DMEM for Primary Cell Isolation**
88281	**Pierce Cardiomyocyte Isolation Kit**
88288	**Cardiomyocyte Isolation Enzyme 1 (with Papain)**
88289	**Cardiomyocyte Isolation Enzyme 2 (with Thermolysin)**
88282	**Pierce Mouse Embryonic Fibroblast Isolation Kit**
88290	**Mouse Embryonic Fibroblast Isolation Enzyme (with Papain)**
87793	**Syn-PER™ Synaptic Protein Extraction Reagent**
87792	**N-PER™ Neuronal Protein Extraction Reagent**
87790	**Subcellular Protein Fractionation Kit for Tissue**
78510	**T-PER™ Tissue Protein Extraction Reagent**

References

1. Silva, R.F.M, *et al.* (2006) Dissociated primary nerve cell cultures as models for assessment of neurotoxicity. *Toxicology Letters* **163:**1-9.
2. National Research Council of the National Academies (2011) The guide for the care and use of laboratory animals. *The National Academies Press.* Eighth Edition.
3. Ivenshitz, M. and Segal, M, (2009) Neuronal Density Determines Network Connectivity and Spontaneous Activity in Cultured Hippocampus. *J Neurophysiol* **104:**1052-1060.

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