96-well GABAergic Neuron Assay (BX-0400, 0450, and 0700)

CONTENTS

- One vial of 5 million viable cryopreserved human neurons (500 µL)
- Neuron Seeding Supplement at 1000X
- Neuron Day 4 Supplement at 1000X
- Supplement K at 1000X

Immediately transfer the vial of neurons from dry ice to liquid nitrogen. Transfer the vials of supplements to a -20°C freezer. Once thawed, the supplements can be stored at 4°C for up to one week.

ADDITIONAL MATERIALS NEEDED

- DMEM/F12 Medium (Life Technologies #11330-032)
- Neurobasal Medium (Life Technologies #21103-049)
- B27 Supplement (Life Technologies #17504-044)
- N2 Supplement (Life Technologies #17502-048)
- GlutaMAX (Life Technologies #35050-061)
- Geltrex (Life Technologies #A1413201)
- BDNF (Peprotech #450-02)
- GDNF (Peprotech #450-10)
- TGF-β1 (Peprotech #100-21C)
- PDL-Coated 96-Well Plates

PROCEDURE

Thawing and Seeding the Neurons

1. Gather the components for the Seeding Medium according to the recipe below. Note that BDNF, GDNF, and TGF-β1 are lyophilized powders. Follow the manufacturer's instructions for reconstitution. We recommend creating stock solutions of 10 µg/mL for BDNF, 10 µg/mL for GDNF, and 1 µg/mL for TGF-β1.
2. Working in a cell culture hood (biological safety cabinet), combine all components in an appropriately sized sterile container. For preparation of the Geltrex, add cold DMEM/F12 directly to an aliquot of frozen Geltrex to yield a 1:10 dilution. For example, if aliquots of Geltrex have a volume of 100 µL, add 900 µL of cold DMEM/F12. Immediately place this mixture at 4°C to allow the Geltrex to thaw and dissolve before adding the appropriate amount to the Seeding Medium. Allow the Seeding Medium to equilibrate to room temperature for at least 15 minutes. Do not warm the medium in a 37°C water bath.
3. Remove the cryovial from the liquid nitrogen and place in a 37°C water bath. To minimize contamination, avoid submerging the cap. Gently move the vial within the bath to increase the rate of thawing.
4. As soon as the last of the ice melts, which will take ~75-90 seconds, remove the vial from the water bath. Disinfect the vial by spraying it with 70% ethanol and transfer it to the cell culture hood.
5. Slowly add 500 µL of seeding medium to the vial at a rate of ~1 drop/s using a 1 mL pipette tip. This process should take about 30 seconds.
6. Gently transfer all contents (~1mL total) from the vial to a new sterile 50 mL conical tube.

7. To collect any residual cells, gently add another 1 mL of seeding medium to the vial and then transfer to the conical tube.
8. Slowly add an additional 3 mL of seeding medium to the 50 mL conical tube using a 10 mL serological pipette. Gently swirl the conical tube while adding the medium. This process should take about 1 minute.
9. To count the cells, gently swirl the conical tube again and remove 10 µL from the cell suspension. Count the number of viable cells per mL with a hemocytometer using the trypan blue exclusion method to identify dead/viable cells.
10. The recommended seeding density is 25,000 – 40,000 viable cells/well for a 96-well plate (~80,000 – 125,000 viable cells/cm^2). Use the following equation to determine the volume of cell suspension needed for each 96-well plate: volume of cell suspension needed (mL) = (3.0 – 4.8x10^6 cells)/(viable cells per mL).
11. In a separate 50 mL conical tube, add the calculated volume of cell suspension needed, and then add enough medium to obtain a final volume of 12 mL. For example, if the volume of cell suspension needed is 2 mL, combine 2 mL of cell suspension with 10 mL of medium.
12. Mix completely and then plate 100 µL/well (25,000 – 40,000 cells/well) onto a PDL-coated 96-well plate using a multi-channel pipettor or liquid handler. Throughout the seeding process, be careful not to move or agitate the plate as this may lead to uneven attachment.
13. After seeding, do not immediately transfer the plate to the incubator. Leave it in the hood for 15 minutes to allow the cells to settle to the bottom of the well. After 15 minutes, very gently transfer the plate to a humidified incubator at 37°C with 5% CO_2. Day of cell plating is designated as Day 0.

    *Note: Entire thawing and plating process should not exceed 2 hours, post-thaw viability and overall cell health will be severely impacted and lead to an unsuccessful culture.

Day 4 Medium Addition

1. On Day 4 (96 hours after seeding), prepare fresh Day 4 Medium (see recipe below).
2. Gently add 100 µL/well to the entire plate for a total volume of 200 µL/well.

Day 7 and Onward Medium Changes

1. Change half the medium (100 µL/well) twice weekly (on Day 7, 11, 14, 18, etc.) using Maintenance Medium (see recipe below).
2. The neurons mature rapidly and can be maintained viable and adherent in culture under the above conditions for at least 3 weeks post-seeding.

Media Compositions

		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Seeding Medium	1	DMEM/F12 Medium	1X	0.5X	9.5 mL	19 mL	47.5 mL
	2	Neurobasal Medium	1X	0.5X	9.5 mL	19 mL	47.5 mL
	3	B27 Supplement	50X	1X	400 µL	800 µL	2 mL
	4	N2 Supplement	100X	1X	200 µL	400 µL	1 mL
	5	GlutaMAX	200 mM	0.5 mM	50 µL	100 µL	250 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL	40 µL	100 µL
	9	Geltrex	15 mg/mL	15 µg/mL	200 µL (of 1:10)	400 µL (of 1:10)	1 mL (of 1:10)
	10	Seeding Supplement	1000X	1X	20 µL	40 µL	100 µL
	11	Supplement K	1000X	1X	20 µL	40 µL	100 µL

		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 4 Medium	1	DMEM/F12 Medium	1X	0.5X	9.6 mL	19.2 mL	48 mL
	2	Neurobasal Medium	1X	0.5X	9.6 mL	19.2 mL	48 mL
	3	B27 Supplement	50X	1X	400 µL	800 µL	2 mL
	4	N2 Supplement	100X	1X	200 µL	400 µL	1 mL
	5	GlutaMAX	200 mM	0.5 mM	50 µL	100 µL	250 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL	40 µL	100 µL
	9	Day 4 Supplement	1000X	1X	20 µL	40 µL	100 µL
	10	Supplement K	1000X	1X	20 µL	40 µL	100 µL

		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Maintenance Medium	1	DMEM/F12 Medium	1X	0.5X	9.6 mL	19.2 mL	48 mL
	2	Neurobasal Medium	1X	0.5X	9.6 mL	19.2 mL	48 mL
	3	B27 Supplement	50X	1X	400 µL	800 µL	2 mL
	4	N2 Supplement	100X	1X	200 µL	400 µL	1 mL
	5	GlutaMAX	200 mM	0.5 mM	50 µL	100 µL	250 µL
	6	BDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	7	GDNF	10 µg/mL	10 ng/mL	20 µL	40 µL	100 µL
	8	TGF-β1	1 µg/mL	1 ng/mL	20 µL	40 µL	100 µL