Neuron/Astrocyte/Microglia 96-well Tri-culture Protocol (v10.0)

CONTENTS

- One vial cryopreserved human neurons (multiple options)
- One vial cryopreserved human astrocytes (BrainXell BX-06XX-XX-XX)
- One vial cryopreserved human microglia (BX-09XX-XX-XX)
- BrainFast Motor #BX-2100 or Glut #BX-2300 or GABA #BX-2400 (neuron type specific) formerly known as Seeding Supplement
- BrainFast Astro (BrainXell #BX-2600) formerly known as Astrocyte Supplement
- BrainFast D4 (BrainXell #BX-2040) formerly known as Day 4 Supplement
- BrainFast SK (BrainXell #BX-2020) formerly known as Supplement K

STORAGE

- Immediately transfer the cryovials of cells to a liquid or vapor nitrogen storage system.
- BrainFast supplements should be stored at -20°C (6 months) or -80°C (18 months). Return vial to -20°C between each time of use to maintain stability.

ADDITIONAL MATERIALS NEEDED

- DMEM/F-12 Medium, +L-glutamine +HEPES (Thermo Fisher Scientific #11330032)
- Neurobasal Medium (Thermo Fisher Scientific #21103049)
- B-27 Supplement (Thermo Fisher Scientific #17504044)
- N-2 Supplement (Thermo Fisher Scientific #17502048)
- GlutaMAX Supplement (Thermo Fisher Scientific #35050061)
- Geltrex (Thermo Fisher Scientific #A1413201) or Cultrex (R&D Systems #3432-001-01)
- Chemically Defined Lipid Concentrate (Thermo Fisher Scientific #11905031)
- M-CSF (Thermo Fisher Scientific #300-25)
- IL-34 (Thermo Fisher Scientific #200-34)
- Ascorbic Acid (MilliporeSigma #A8960)
- PDL-Coated 96-Well Plates (Refer to BrainXell Culture Plates PDL Coating Protocol v10.0)

OPTIONAL MATERIALS

- BDNF (Thermo Fisher Scientific #450-02)
- GDNF (Thermo Fisher Scientific #450-10)
- If culturing spheroids, we recommend either PrimeSurface 3D Culture Spheroid (ULA) plates (S-Bio #MS-9096WZ) or Nunclon Sphera 96-Well U-Shaped-Bottom Microplate (Thermo Fisher Scientific #174925). Successful cultures have resulted when seeding between 100 and 10,000 cells/well.

PROCEDURE

Day 0 Seeding Preparation

1. Gather the Basal Medium components according to the recipe (see Media Compositions section below).
    a. Note that BDNF, GDNF, IL-34, and M-CSF are lyophilized powders. Follow the manufacturer's instructions for reconstitution and long-term storage. We recommend creating stock solutions of 10 µg/mL BDNF and 10 µg/mL GDNF if they will be added to the culture medium.
    b. We recommend reconstituting ascorbic acid in DMEM/F-12 medium to create a 200 mM stock solution; See "Preparation of 200 mM Ascorbic Acid" at end of this document for important information.
2. Working in a cell culture hood (biological safety cabinet), combine all Basal Medium components in an appropriately sized sterile container. Allow the Basal Medium to equilibrate to room temperature for at least 15 minutes. Do not warm the medium in a 37°C water bath. Culture plates should also be at room temperature prior to use.
3. Prepare a pre-diluted Cultrex solution: add 495 µL of **cold** DMEM/F12 into a 55 µL aliquot of frozen Cultrex taken directly from the -80 freezer. Mix to dissolve and store at 4°C until it is time to seed the plate (Step 16).
4. Prepare a labeled 50-mL conical tube for each cell type that will be thawed: add 3 mL of Basal Medium to each tube.
5. Prepare a labeled microcentrifuge tube for each cell type: add 25 µL of Trypan Blue solution for cell counting.

Day 0 Seeding of Neurons and Astrocytes

A general recommended seeding ratio of neurons to astrocytes is 2:1 to 6:1. Other ratios may be suitable depending on individual research needs.

6. Remove the cryovials of neurons and astrocytes from liquid nitrogen storage and place in a 37°C water bath. To minimize contamination, avoid submerging the caps. Gently swirl the vials within the bath to increase the rate of thawing.
7. As soon as the last of the ice melts, which will take ~75-90 seconds, remove the cryovials from the water bath. Disinfect the vials by spraying them with 70% ethanol before transferring them to the cell culture hood.
8. Slowly add 500 µL of Basal Medium to the first cryovial of neurons at a rate of ~2-3 drops/sec using a P1000 pipette. This process should take about 10 seconds.
9. Gently transfer all contents from the neuron cryovial to the corresponding 50-mL tube. Rinse the cryovial with 1 mL additional Basal Medium to collect residual cells and transfer to the same 50-mL tube. Repeat steps 8 and 9 for the thawed cryovial of astrocytes using the second prepared 50-mL tube.
10. Centrifuge the tubes of neurons and astrocytes at 1115 rpm (200xg) for 5 mins.
11. Carefully aspirate the supernatant from the first tube, leaving the cell pellet, and resuspend the cells in 950 µL fresh Basal Medium. Mix thoroughly by gently pipetting up and down using a P1000 pipette. Using a clean pipette tip, repeat for the second tube.
12. Based on the number of *Viable Cells/Vial* listed on each Certificate of Analysis (CoA), resuspend cells to a concentration of 1.0×10^6 live cells/mL by slowly adding additional Basal Medium to the existing ~1 mL in each tube.
    a. Ex: 6.7 million live cells per CoA will be resuspended to a total volume of 6.7 mL.
13. Count the resuspended cells: gently swirl each conical tube and pipette up and down 3-5 times to ensure cells are evenly suspended in medium. Transfer 25 µL of the cell suspension to the matching labeled

microcentrifuge tube prefilled with 25 µL Trypan Blue solution and pipette up and down a few times to mix. Count the number of viable and dead cells using a hemocytometer. Determine the live cell concentration (live cells/mL) and viability for each cell suspension.

14. Calculate the volumes of each cell type needed to make the final mixed seeding suspension. For a 96-well plate, an initial combined seeding density of 30,000 – 40,000 viable cells per well is recommended (~95,000 – 125,000 viable cells/cm^2). Dilute the cells to the desired seeding concentration using the calculation table below as an example. A general recommended seeding ratio of neurons to astrocytes is in the range of 2:1 to 6:1, but other ratios may be suitable depending on user preference.

    Example calculations for seeding (1) 96-well plate at a combined 40,000 cells/well with a 3:1 ratio of neurons to astrocytes:

Actual Neuron Live Cell Concentration	Target Seeding Density	Total Seeding Volume Needed	**Volume of Neuron Suspension**
1.1x10^6 viable cells/mL *(Step 13)*	30,000 viable cells per 100 µL/well = *300,000/mL*	11 mL *(Seeding 1 plate)*	**Add: 3 mL** *300,000/mL x 11mL / 1.1x10^6 cells/mL*

Actual Astrocyte Live Cell Concentration	Target Seeding Density	Total Seeding Volume Needed	**Volume of Astrocyte Suspension**	**Volume of Basal Medium**
1.2x10^6 viable cells/mL *(Step 13)*	10,000 viable cells per 100 µL/well = *100,000/mL*	11 mL *(Seeding 1 plate)*	**Add: 0.917 mL** *100,000/mL x 11mL / 1.2x10^6 cells/mL*	**Add: 7.083 mL** *(11mL – 3 mL neurons – 0.917 mL astrocytes)*

15. In a new sterile 50-mL tube: add the calculated volumes of cell suspension for each cell type and add enough Basal Medium to reach 11 mL total of the mixed cell suspension.
16. Per the Seeding Medium recipe, add in the appropriate volumes of each required BrainFast supplement (1:1000) and pre-diluted Cultrex from Step 3 (1:100) to the desired volume of seeding suspension.
    a. Example: add 11 µL of BrainFast Motor, 11 µL of BrainFast Astro, and 110 µL pre-diluted Cultrex solution to 11 mL total of neuron/astrocyte mixed cell suspension.
17. Using a 5-mL serological pipette, slowly mix the suspension to ensure a homogeneous suspension and seed 100 µL/well of the Seeding Medium onto a PDL-coated 96-well plate using a multi-channel pipette or liquid handler. Throughout the seeding process, be careful not to move or agitate the plate as this may lead to uneven attachment.
18. After seeding, let the plate rest for 10 minutes before moving it to allow the cells to settle to the bottom of the well. After 10 minutes, very gently transfer the plate to a humidified incubator at 37°C with 5% CO_2. The day of cell plating (today) is designated as Day 0.

    **Note: The entire thawing and plating process should not exceed 1 hour. Post-thaw viability and overall cell health could be severely impacted and lead to an unsuccessful culture if the whole process is too long.*

Day 1 Medium Replacement

19. On Day 1 (24 hours after seeding), prepare fresh Day 1 Medium.
20. Carefully remove all 100 µL medium/well and gently replace with 100 µL/well Day 1 Medium. Complete one row or column at a time to ensure the wells do not dry out during the medium replacement process.

Day 4 Medium Addition

21. On Day 4 (96 hours after seeding), prepare fresh Day 4 Medium.
22. Gently add 100 µL/well of Day 4 medium to the entire plate for a total of 200 µL/well.

Day 7 Seeding of Microglia

23. Gather the components for the Day 7 MG Seeding Medium according to the recipe below and mix components in an appropriately sized sterile container. Allow the medium to equilibrate to room temperature for at least 15 minutes. Do not warm the medium in a 37°C water bath.
24. Prepare one 50-mL conical tube: add 3 mL of Day 7 MG Seeding Medium.
25. Prepare a microcentrifuge tube: add 25 µL of Trypan Blue solution for cell counting.
26. Remove the cryovial of microglia from liquid nitrogen storage and place in a 37°C water bath. To minimize contamination, avoid submerging the caps. Gently swirl the vials within the bath to increase the rate of thawing.
27. As soon as the last of the ice melts, which will take ~75-90 seconds, remove the cryovial from the water bath. Disinfect the vial by spraying it with 70% ethanol before transferring it into the cell culture hood.
28. Slowly add 500 µL of Day 7 MG Seeding Medium to the cryovial at a rate of ~2-3 drops/sec using a P1000 pipette. This process should take about 10 seconds.
29. Gently transfer all contents from the cryovial (~1mL total) to the prepared 50-mL conical tube. Rinse the cryovial with 1 mL additional MG Seeding Medium to collect residual cells and transfer to the 50-mL tube.
30. Centrifuge the tube of microglia at 1115 rpm (200xg) for 5 mins.
31. Carefully aspirate the supernatant, leaving the cell pellet, and resuspend cells in 950 µL fresh Day 7 MG Seeding Medium. Mix thoroughly by gently pipetting up and down using a P1000 pipette.
32. Based on the CoA value (*Viable Cells/Vial*), resuspend the cells to a concentration of $1.0x10^6$ live cells/mL by slowly adding additional Day 7 MG Seeding Medium to the existing ~1 mL in the tube.
    a. Ex: 2.3 million live cells per CoA will be resuspended to a <u>total</u> volume of 2.3 mL.
33. Count the cells: gently swirl the conical tube and pipette up and down 3-5 times to ensure cells are evenly suspended in medium. Transfer 25 µL of the microglia suspension to the microcentrifuge tube prefilled with 25 µL Trypan Blue solution and pipette up and down a few times to mix. Count the number of viable and dead cells using a hemocytometer. Determine the live cell concentration (live cells/mL) and viability. Remember to calculate in your dilution factor.
34. Calculate the volumes needed to make the final microglia seeding suspension. It is recommended to use a seeding density equal to the astrocyte density, but your final ratios may vary depending on individual assay needs. Tri-culture totals > 50,000 live cells/well may appear clustered due to the higher density.
    a. Dilute the cells in Day 7 MG Seeding Medium to the desired seeding concentration using the calculation table below as an example.

Example of cell dilution calculations for addition of microglia to (1) <u>96-well</u> plate.

Actual Live MG Cell Concentration	Target Seeding Density	Total Seeding Volume Needed	**Volume of Microglia Suspension**	**Volume of MG Seeding Medium**
$1.1x10^6$ viable cells/mL *(Step 33)*	10,000 viable cells per 100 µL/well = *100,000 cells/mL*	11 mL *(Seeding 1 plate)*	**Add: 1 mL** *<u>100,000/mL x 11mL</u>* *$1.1x10^6$ cells/mL*	**Add: 10 mL** *(11mL – 1mL)*

35. Carefully remove 100 µL/well of spent medium from each well and add in 100 µL/well of microglia seeding suspension.

36. 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 9 and Onward Medium Changes

37. On Day 9 and onward, prepare Maintenance Medium according to the recipe below by adding the required reagents to room temp Basal Medium stock.
38. Change half the medium (100 µL/well) twice weekly (on Day 9, 12, 15, etc.) using freshly prepared Maintenance Medium.
39. Tri-cultures can be maintained viable and adherent in culture under the above conditions for at least 3 weeks post-seeding.

Tri-Culture Media Compositions

Step 2		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Basal Medium	1	DMEM/F12 Medium	1X	0.5X	48 mL	96 mL	240 mL
	2	Neurobasal Medium	1X	0.5X	48 mL	96 mL	240 mL
	3	B-27 Supplement	50X	1X	2 mL	4 mL	10 mL
	4	N-2 Supplement	100X	1X	1 mL	2 mL	5 mL
	5	GlutaMAX	200 mM	0.5 mM	250 µL	500 µL	1.25 mL
	6	Ascorbic Acid Solution (Step1b)	200 mM	0.2 mM	100 µL	200 µL	500 µL
		Basal Medium stock can be stored for up to 3 weeks at 4°C.					

Note: Addition of BDNF and GDNF is optional. If added, recommended final medium concentrations are 10 ng/mL BDNF and 10 ng/mL GDNF.

Step 16		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Seeding Medium	1	**Neuron/Astrocyte Cell Suspension** (Step 15)	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	BrainFast (Motor, Glut, or GABA)	1000X	1X	11 µL	22 µL	55 µL
	3	BrainFast Astro	1000X	1X	11 µL	22 µL	55 µL
	4	Pre-diluted Cultrex*	1.5mg/mL	15 µg/mL	110 µL	220 µL	550 µL

* Cultrex must be pre-diluted according to step 3 in the protocol and added to the final seeding suspension right before seeding the plate.

Step 19		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 1 Medium	1	**Basal Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	BrainFast (Motor, Glut, or GABA)	1000X	1X	11 µL	22 µL	55 µL
	3	BrainFast Astro	1000X	1X	11 µL	22 µL	55 µL
	4	BrainFast SK	1000X	1X	11 µL	22 µL	55 µL

Step 21		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 4 Medium	1	**Basal Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	BrainFast Astro	1000X	1X	11 µL	22 µL	55 µL
	3	BrainFast D4	1000X	1X	11 µL	22 µL	55 µL
	4	BrainFast SK**	1000X	1X	11 µL	22 µL	55 µL

** BrainFast SK addition is recommended in Day 4 Medium for all cultures containing cortical GABAergic neurons but is optional in Day 4 Medium for other neuronal subtypes cultured 7 days or less. Please contact support@brainxell.com for further assistance if needed.

Step 23		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Day 7MG Seeding Medium	1	**Basal Medium**	**1X**	**1X**	**20 mL**	**40 mL**	**100 mL**
	2	Chemically Defined Lipid Mix	1X	0.005X	100 µL	200 µL	500 µL
	3	M-CSF	100 µg/mL	20 ng/mL	4 µL	8 µL	20 µL
	4	IL-34	100 µg/mL	100 ng/mL	20 µL	40 µL	100 µL

Step 37		Component	Stock Conc.	Final Conc.	1 Plate Volume	2 Plate Volume	5 Plate Volume
Maintenance Medium	1	**Basal Medium**	**1X**	**1X**	**11 mL**	**22 mL**	**55 mL**
	2	Chemically Defined Lipid Mix	1X	0.005X	55 µL	110 µL	550 µL
	3	M-CSF	100 µg/mL	20 ng/mL	2.2 µL	4.4 µL	11 µL
	4	IL-34	100 µg/mL	100 ng/mL	11 µL	22 µL	55 µL

Preparation of 200 mM Ascorbic Acid Solution

Additional Sterile Materials Required

50-mL Conical Tubes, 0.22 micron Filter, Amber Microcentrifuge Tubes

Critical Information

- Ascorbic Acid must be added *into* the DMEM/F12 (not vice versa) to ensure it dissolves.
- The rate of Ascorbic Acid addition to DMEM/F12 is critical. If added too rapidly, the chemical will fail to go into solution.
- Only mix by inversion. Undissolved Ascorbic Acid will adhere to pipette tips and other implements.
- Ascorbic Acid is pH, temperature, and light sensitive.

Steps

1. Aliquot 17.2 mL of DMEM/F12 into a 50-ml tube.
2. Weigh 1.0 gram of Ascorbic Acid using a separate vessel.
3. **Critical Step**: Gradually transfer Ascorbic Acid ***into*** the tube of DMEM/F12 (no more than 0.2 gram at a time, waiting 10 seconds between each addition).
4. Secure the cap on the tube and invert to mix.
5. Allow the Ascorbic Acid to dissolve into the solution:

a. Place in a 37°C water bath for 30 minutes, inverting the tube every 10 minutes to mix.

b. After 30 minutes check to see if Ascorbic Acid is fully dissolved. If not, invert tube to mix and place capped tube at 4°C overnight.

c. After overnight storage, invert to mix and confirm Ascorbic Acid is completely dissolved.

6. Filter the fully dissolved Ascorbic Acid Solution using a Steriflip filter or equivalent 0.22 µm sterile filter.
7. Aliquot into amber 1.5mL Eppendorf tubes; recommended volumes of 110µl or 550µl.
8. Maintain frozen aliquots at -20°C for up to 6 months. Aliquots are single use. Do not refreeze.