AXOL

axoCells™ human iPSC-derived motor neurons + microglia + astrocytes co-culture (unaffected), cells, media and supplement kit

User guide

Contents

This protocol and its associated products are for research use only.

Products are not for diagnostic or therapeutic use and are not to be administered to humans or animals.

Important

The cell culture media provided by Axol DOES NOT contain antibiotics or antifungal agents. Axol does not recommend the use of antimicrobial agents such as penicillin, streptomycin and amphotericin. Antimicrobial agents should not be necessary if proper aseptic technique is adopted.

Reagents:

This table shows reagents needed for setting up co-culture with motor neurons, microglia and astrocytes. For media composition when using only two cell types (motor neurons and astrocytes OR motor neurons and microglia), refer to APPENDIX 2.

- **ax0185** media only kit: axoCells™ human iPSC-derived motor neuron co-culture media and supplement kit
- **ax0192** media and cells kit: axoCells™ human iPSC-derived motor neurons + microglia co-culture (unaffected), cells media and supplement kit
- **ax0193** media and cells kit: axoCells™ human iPSC-derived motor neurons + astrocytes co-culture (unaffected), cells media and supplement kit
- **ax0194** media and cells kit: axoCells™ human iPSC-derived motor neurons + microglia + astrocytes co-culture (unaffected), cells media and supplement kit

Axol kit ax0194:

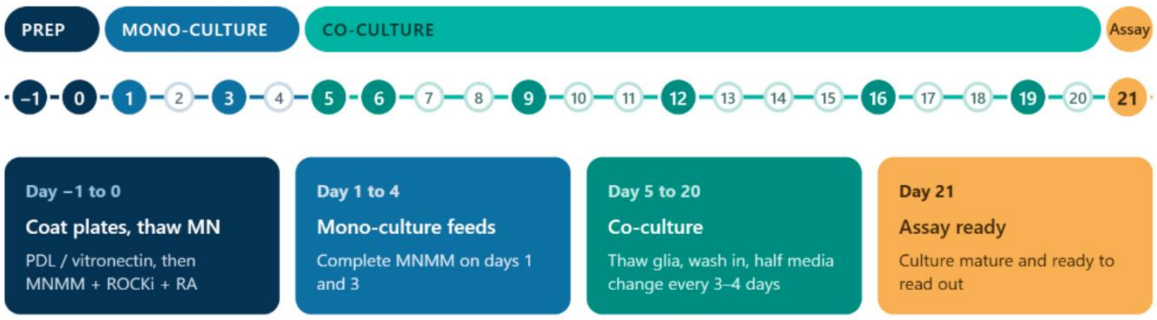
Reagent	Supplier	Catalogue number
Motor neuron maintenance media (MNMM)	Axol	ax0072
CNTF	Axol	ax139888-5ug
BDNF	Axol	ax139800-10ug
GDNF	Axol	ax139855-10ug
Microglia Maintenance Media Supplement B	Axol	ax0060b
iPSC derived motor neurons - unaffected	Axol	ax0076
iPSC derived microglia – unaffected	Axol	ax0664
iPSC derived astrocytes - unaffected	Axol	ax0704

Provided by other suppliers:

Reagent	Supplier	Catalogue number
Poly-D-Lysine	ThermoFisher	A3890401
D-PBS (-/-)	any	any
Vitronectin (VTN-N)	ThermoFisher	A400457
Compound E	Abcam	Ab142164

Reagent	Supplier	Catalogue number
Retinoic acid	Sigma-Aldrich	R2625
Y-27632 (ROCKi)	Focus biomolecules	10-2301
Human serum albumin	any	any
Heregulin	Peprotech	100-03

Quick View Process:



Schematic representation of the process to generate co-cultures over 21 days. A more detailed timeline of the process is described in Appendix 1.

Reagent preparation and vessel coating:

Coating plates:

1. Dilute the liquid poly-D-lysine 1:1 in sterile D-PBS to create a 0.05mg/ml working solution (0.5x).
2. Add 300 µL per cm^2 of 0.5x PDL solution (0.05 mg/mL) to the culture vessel. Incubate for 60 mins at 37°C, remove the solution and thoroughly rinse the surface twice with sterile D-PBS.
3. Remove D-PBS and allow the surface to dry for at least two hours at RT before proceeding onto Vitronectin coating.
4. Dilute the Vitronectin stock solution in D-PBS (without calcium or magnesium) to make 1x working solution of 10µg/ml. e.g. perform a 1:90 dilution by adding 111.11µl of Vitronectin to 10ml of D-PBS
5. Coat the surface of your culture vessel with 300µL per cm^2 of Vitronectin 1x working
6. Incubate overnight at 37°C, 5% CO2.
7. Do not wash the culture vessel after coating with Vitronectin

8. Remove Vitronectin immediately before adding cells. Do not let the Vitronectin coating dry out before seeding the motor neurons

Preparation of basal media:

9. Once thawed, motor neuron maintenance media (MNMM) requires supplementation with Compound E
10. Prepare Compound E by creating a stock concentration of 2mM in DMSO.
11. Add compound E directly to the MNMM to create a final concentration of 0.2µM (20µl added into 200ml of media, this can be done by adding directly to the bottle).
12. MNMM+CompE can be aliquoted and stored at -20°C for 1 month. Once thawed, media can be kept at 4°C for up to a week.

Complete motor neuron maintenance media (MNMM for mono-culture):

Thaw an aliquot of MNMM+CompE and supplement with the following reagents. Media should be made fresh on the day when media changes are required. CNTF, BDNF and GDNF are reconstituted in 0.05% HSA.

Growth factor	Stock concentration	Final concentration	For 20ml of media
CNTF	5µg/ml	10ng/ml	40µl
BDNF	10µg/ml	5ng/ml	10µl
GDNF	10µg/ml	10ng/ml	20µl
Retinoic acid	1mM	0.5µM	10µl

Complete co-culture maintenance media:

Thaw an aliquot of MNMM+CompE and supplement with the following reagents. Media should be made fresh on the day when media changes are required. Media composition for use of only two cell types can be found in APPENDIX 2.

Growth factor	Stock concentration	Final concentration	For 20ml media
CNTF	5µg/ml	10ng/ml	40µl
BDNF	10µg/ml	5ng/ml	10µl
GDNF	10µg/ml	10ng/ml	20µl
Retinoic acid	1mM	0.5µM	10µl

Growth factor	Stock concentration	Final concentration	For 20ml media
Microglia Maintenance media Supplement B	100µg/ml	100ng/ml	20µl
Heregulin	10µg/ml	10 g/ml	20µl

Motor neuron media for plating cells

13. MNMM+CompE requires supplementing with retinoic acid and ROCKi before thawing cells.
14. Prepare retinoic acid by creating a stock concentration of 1mM in DMSO
15. Prepare ROCKi by creating a stock concentration of 10mM in sterile water
16. On day of use, prepare plating media by adding retinoic acid to create a final concentration of 0.1µM (NOTE: this is different from the concentration in complete MNMM shown above), and ROCKi to a final concentration of 10µM.

Cell thaw and culture methodology:

Thawing and plating motor neuron progenitors

17. Prepare coating of culture vessels as described above (1-8)
18. Thaw MNMM and prepare as described above, supplementing with retinoic acid and ROCKi (13-16).
19. Thaw a vial (or multiple if needed) of motor neuron cells in a water bath set to 37°C until only a small amount of ice remains. Ensure not to agitate the vial
20. Spray the vial with 70% ethanol and transfer into a biological safety cabinet
21. Once completely thawed, use a P1000 pipette to transfer cells into a 15mL conical tube containing 8ml of plating media.
22. Centrifuge cells at 200 *xg* for 5min at RT
23. Carefully aspirate the supernatant and gently resuspend the pellet in 1ml of plating media, pipetting up and down ~10 times to generate a single cell suspension. If several vials are thawed, pool before counting and dilute accordingly to ensure an accurate cell count (~1-3 million cells/ml)
24. Perform a cell count
25. Seeding density for co-culture use is recommended at 100k cells/cm2 (32,000 cells/well of a 96wp).
26. Remove the coating solution and add cells resuspended in media to the culture vessel. For a 96wp, add 100µl per well.
27. Incubate at 37°C, 5% CO_2 for 24h, then replace the culture media by removing ~90µl of spent media, and adding 190µl of fresh Complete MNMM for a final well volume of 200µl of Complete MNMM (removing ROCKi). Ensure to leave ~10% volume in the wells when replacing media to prevent cells from peeling.

28. Perform half media changes every 2-3 days. Example schedule can be found in Appendix 1.

Thawing microglia and astrocytes to add to motor neurons – day 5

29. If only one additional cell type (microglia or astrocytes) are to be added, refer to APPENDIX 2 for media composition
30. Astrocytes are seeded at 15,500-31,000 cells/cm^2 (5000 - 10000 cells/well of a 96wp) and microglia at ~24,000-50,000 cells/cm^2 (7,500 – 15,000 cells/well of a 96wp). Calculate how many vials of cells will be needed to add to motor neurons
    NOTE: co-culture has been optimised using these cell densities. However, these can be further optimised by end user depending on end goal.
31. Prepare Motor neuron media for plating cells (13-16). This will be used as thawing media for astrocytes and microglia.
32. Thaw the vials of microglia and astrocytes in a water batch set to 37°C until only a small amount of ice remains. This can be performed in parallel or staggered.
33. Spray with ethanol and transfer into a biological safety cabinet. Using a P1000 transfer the content of the microglia and astrocyte vials into separate 15ml conical tube containing 8ml of MNMM with ROCKi.
34. Centrifuge at 200 *xg* for 5min, remove supernatant and resuspend in 1ml of thawing media ensuring to break up the pellet and generate single cells. Perform a cell count.
35. For a 96 well plate, prepare each cell type at the corresponding density to be added in 50µl per well. For example, for 10 wells at 15,000 cells/well, you need 150,000 microglia in 500µl. Then 50µl of cell suspension is added per well.
    Note: calculate for 15% more wells than needed to ensure enough volume to add to all wells
36. Remove 100µl of spent media from the motor neuron plate.
37. Add 50µl of astrocyte cell suspension and 50µl microglia cell suspension separately to desired wells. If only one cell type is being added, then add the cells plus an additional 50µl of MNMM+ROCKi so the final volume added is 100µl
38. Place plate in an incubator at 37ºC, 5% CO_2 for 24h
39. The next day, gently perform 3x half washes with fresh complete co-culture maintenance media.
40. Remove 100µl of media from the cells, add 100µl of fresh media. Repeat another two times for a total of 3x half washes. This step is to ensure the Rocki is reduced to a low concentration whilst helping to reduce the chance of peeling.
    Note: keeping 200uµ per well helps reduce the disturbance to the neurons.
41. Perform a half media change every 3-4 days. Cells are considered assay ready by day 21.

APPENDIX 1:

Example timeline of cell culture

Day	Media	Action
-1	PDL/vitronectin	Coat plates
0	MNMM + ROCKi + RA	Thaw MN in plating media
1	Complete MNMM (mono-culture)	90% media change Add 200µL of media to the wells.
2		
3	Complete MNMM (mono-culture)	Half media change
4		
5	MNMM with ROCKi	Thaw microglia and astrocytes
6	Complete co-culture media	3x half media washes
7		
8		
9	Complete co-culture media	Half media change
10		
11		
12	Complete co-culture media	Half media change
13		
14		
15		
16	Complete co-culture media	Half media change
17		
18		
19	Complete co-culture media	Half media change
20		
21	Assay ready	

APPENDIX 2:

Media composition when only adding microglia OR astrocytes

Microglia only added (ax0192):

Growth factor	Stock concentration	Final concentration	For 20ml media
CNTF	10µg/ml	10ng/ml	20µl
BDNF	10µg/ml	5ng/ml	10µl
GDNF	10µg/ml	10ng/ml	20µl
Retinoic acid	1mM	0.5µM	10µl
Microglia Maintenance media Supplement B	100µg/ml	100ng/ml	20µl

Astrocytes only added (ax0193):

Growth factor	Stock concentration	Final concentration	For 20ml media
CNTF	5µg/ml	10ng/ml	40µl
BDNF	10µg/ml	5ng/ml	10µl
GDNF	10ug/ml	10ng/ml	20µl
Retinoic acid	1mM	0.5µM	10µl
Heregulin	10µg/ml	10ng/ml	20µL

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Contact our technical support team at support@axolbio.com