

LUHMES (lund human mesencephalic) culturing and differentiation protocol V.2

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DOI:

dx.doi.org/10.17504/protocols.io.kxygx36ykg8j/v2

Protocol Citation: Mallory Wright, William J Buchser, ckremitz, jwaligor, bachman@wustl.edu 2024. LUHMES (lund human mesencephalic) culturing and differentiation protocol.

protocols.io
<https://dx.doi.org/10.17504/protocols.io.kxygx36ykg8j/v2> Version created by [Mallory Wright](#)

MANUSCRIPT CITATION:

ATCC

<https://www.atcc.org/products/crl-2927#product-references>

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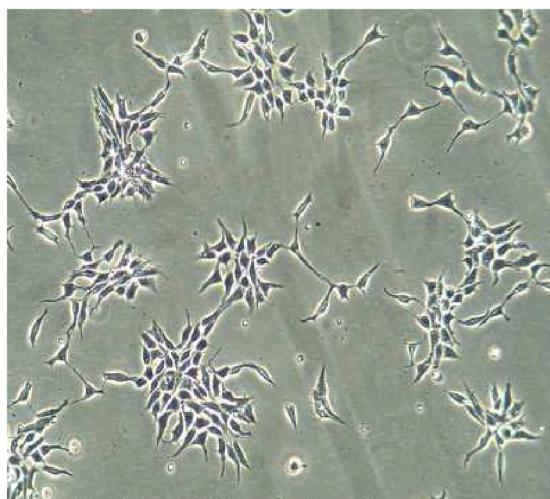
Protocol status: Working
We use this protocol and it's working

Created: Feb 14, 2024

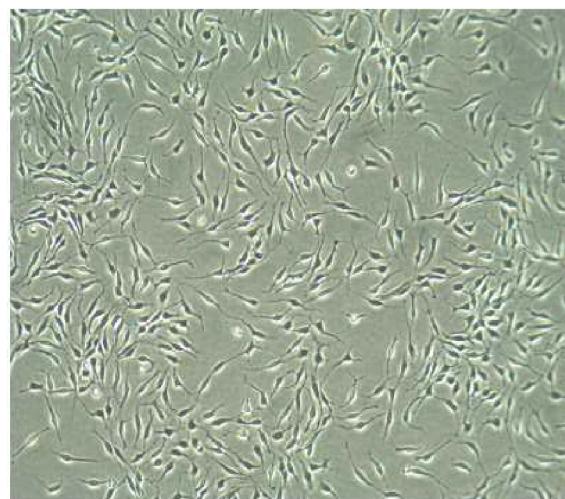
Last Modified: Feb 14, 2024

PROTOCOL integer ID: 95260

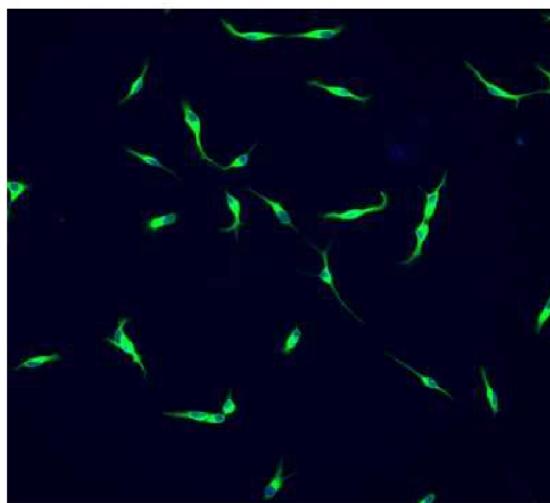
ABSTRACT



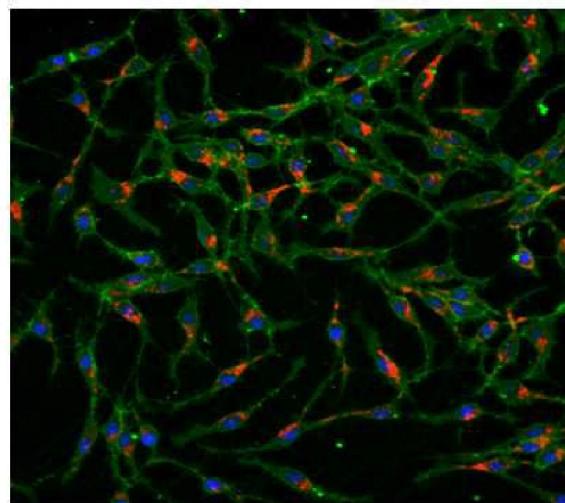
LUHMES at about 50% percent confluence



LUHMES Differentiation Day 2



LUHMES Differentiation day 4 with MAP2/rabbit Ab



LUHMES Differentiation day 2. Stained with Cell mask orange, lyso-tracker deep red and Hoechst

MATERIALS

| A | B | C | D |
|--|---|---------------------|------------------------------|
| Reagents | Stock Concentration | Final Concentration | Final Solution Volume = 20mL |
| DMEM/F12 | x | x | 19,792uL |
| B27 | 50X | 1X | 200ul |
| Recombinant Human FGF basic (100ug) store at -20C | 100ug/mL Reconstitute: add 1000ul PBS to 100ug vial of fgf | 40ng/mL | 8uL |
| Penicillin Streptomycin (10,000 U/mL) | X | X | 20uL |

LUHMES Growth Media

| Reagents | Note | Stock Concentration | Working Concentration | Final Solution Volume = 20mL |
|--|---|---------------------|-----------------------|------------------------------|
| DMEM/F12 | | X | X | 19,568uL |
| B27-supplement | | 100X | 1X | 200uL |
| Dibutyryl cAMP MW: 491.4 g/mol Mass: 100mg | Reconstitute: Add 1.56mL of PBS to 100 mg Vial | 100mM | 1mM | 200uL |
| Ascorbic Acid (500mg Vial) | Reconstitute: Add 14.195 mL PBS | 200mM | 0.2mM | 20uL |

| Reagents | Note (pH 7.2) to 500 mg of abs orbi c aci d | Stock Concentration | Working Concentration | Final Solution Volume = 20mL |
|------------------------------------|--|---------------------|-----------------------|------------------------------|
| Human Recombinant LIF (50ug vial) | Rec ons titut e: Add 500 ul of nuc leas e free wat er to 50u g vial. *2 wee ks or at -20° C to -80° C for up to 3 mo nth s | 0.1ug/mL | 10ng/mL | 2uL |
| Human Recombinant BDNF (10ug vial) | Rec ons titut e: Add 100 ul to vial. cen trifu ged bef ore ope nin | 0.1 mg/mL | 20ng/mL | 4uL |

| Reagents | Note | Stock Concentration | Working Concentration | Final Solution Volume = 20mL |
|---|---|---------------------|-----------------------|------------------------------|
| Human Recombinant GDNF (10ug vial) | Reconstitute: Add 100 ul of nuclease free water to 10ug vial of GDNF. Make 5ul aliquots Store aliquots at -20°C | 0.1mg/mL | 20ng/mL | 4uL |
| Tetracycline MW: 480.91 g/mol Mass: 500mg | Reconstitute: Add 50 mL of biograde water to 500 mg of tetracyclin | 10mg/mL | 1ug/mL | 2uL |

| | Reagents | Note e. Stor e in -20 | Stock Concentration | Working Concentration | Final Solution Volume = 20mL |
|--|---------------------------------|-----------------------------------|---------------------|-----------------------|------------------------------|
| | TGF β -III (10ug vial) | Storage Conditions 4° C | 0.25 mg/mL | 20ng/mL | 0.25 mg/mL |

LUHMES Differentiation Media

- ☒ Tgf beta 3 (human) Recombinant Protein **Invitrogen - Thermo Fisher Catalog #RP8600**
- ☒ Human Recombinant LIF **STEMCELL Technologies Inc. Catalog #78055**
- ☒ Dibutyryl-cAMP **STEMCELL Technologies Inc. Catalog #73884**
- ☒ Tetracycline Hydrochloride **Thermo Scientific Catalog #A39246**
- ☒ Human Recombinant GDNF **STEMCELL Technologies Inc. Catalog #78058**
- ☒ Human Recombinant BDNF **STEMCELL Technologies Inc. Catalog #78005**
- ☒ Absorbic Acid **STEMCELL Technologies Inc. Catalog #72132**

PROTOCOL MATERIALS

-  Poly-L- Ornithine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A-004-C** Step 1
-  Tetracycline Hydrochloride **Thermo Scientific Catalog #A39246** Materials
-  DMEM/F12 **Thermo Fisher Scientific Catalog #11320033** Step 12
-  Dibutyryl-cAMP **STEMCELL Technologies Inc. Catalog #73884** Materials
-  Absorbic Acid **STEMCELL Technologies Inc. Catalog #72132** Materials
-  Fibronectin human plasma,liquid, 0.1% (Solution), **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F0895-1MG**

Step 6

-  Recombinant Human FGF basic/FGF2/bFGF (145 aa) Protein, CF **R&D Systems Catalog #3718-FB**

Step 12

-  Trypsin/edta Solution (TE) **Thermo Scientific Catalog #R001100** Step 15
-  Human Recombinant LIF **STEMCELL Technologies Inc. Catalog #78055** Materials
-  Human Recombinant GDNF **STEMCELL Technologies Inc. Catalog #78058** Materials
-  Tgf beta 3 (human) Recombinant Protein **Invitrogen - Thermo Fisher Catalog #RP8600**
- Materials
-  Penicillin Streptomycin (10,000 U/mL) **Gibco - Thermo Fischer Catalog #15140122**
- Step 12
-  B-27 Supplement (50X) **Thermo Fisher Scientific Catalog #17504044** Step 12
-  Human Recombinant BDNF **STEMCELL Technologies Inc. Catalog #78005** Materials

LUHMES coating protocol

- 1  Poly-L- Ornithine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A-004-C**
 - $[M]$ 0.1 mg/mL Stock concentration
 - $[M]$ 50 μ g/ μ L Working concentration

Thaw an aliquot of Poly-L-Ornithine solution at room temperature.

- 2 Dilute Poly-L-Ornithine solution to 50ug/mL in Nuclease-free water

Add  500 μ L of PLO for every  500 μ L Nuclease-free water

- 3 Add 7mL of the 50ug/mL PLO to a T-75 overnight at RT.

- 4 Rinse flask 3 times with bio-grade water.
- 5 Allow the flask to air dry for 15 minutes uncapped and standing upright in the hood. (turn on UV)
- 6  Fibronectin human plasma,liquid, 0.1% (Solution), **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F0895-1MG**

Size: 100uL, Storage Temperature: -20 °C in aliquots

 - **[M]** 1 mg/mL Stock Concentration
 - **[M]** 2 µg/µL Working concentration

Thaw an aliquot of Poly-L-Ornithine solution at room temperature.

Note

Do not vortex or shake vigorously to resuspend the fibronectin. This will cause the fibronectin to “crash” out of solution, which is irreversible
- 7 Dilute the Fibronectin in sterile Hank’s Balanced Salt Solution (HBSS).

Add  2 µL Fibronectin to  998 µL HBSS
- 8 Place fibronectin coated flask in incubator for 3 hours
- 9 Rinse 3 times with HBSS.

10 Air dry for 15 minutes and add LUHMES growth media.

LUHMES growth media

11 Change (pre-warmed) media every 1-2 days

Note

LUHMES are sensitive to changes in the media pH and oxidative stress. Always use fresh DMEM/F12 because the HEPES buffer in DMEM is subject to photooxidation upon exposure to light and produces hydrogen peroxide.

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☒ DMEM/F12 **Thermo Fisher Scientific Catalog #11320033**

☒ Penicillin Streptomycin (10,000 U/mL) **Gibco - Thermo Fischer Catalog #15140122**

☒ B-27 Supplement (50X) **Thermo Fisher Scientific Catalog #17504044**

☒ Recombinant Human FGF basic/FGF2/bFGF (145 aa) Protein, CF **R&D Systems Catalog #3718-FB**

- Add recombinant human FGF to media after seeding cells

LUHMES Passaging (~ every 2-3 days)

13

Remove media and rinse with DPBS

14

Add fresh culture media to flask for at least 15 minutes before seeding cells to allow media to reach normal pH



15

Add 4mL of the pre-warmed .025% Trypsin/EDTA and place in the incubator for 3 minutes.

☒ Trypsin/edta Solution (TE) **Thermo Scientific Catalog #R001100**

- 16 Neutralize trypsin with 6mL of pre-warmed DMEM/F12
- 17 Transfer cells to 15mL tube and centrifuge for 5 minutes at 1200 RPM
- 18 Discard the supernatant and resuspend in 1mL LUHMES growth media
- 19 use a 5mL pipette to Triturate cells only 1 or 2 times before seeding.

Cryopreservation

- 20 Label 2mL cryovials with the date, name, FIV#, Qbench number, passage number and cell type.

Add about 1.5 million cells per vial with 1mL freezing media.

Freezing Media:

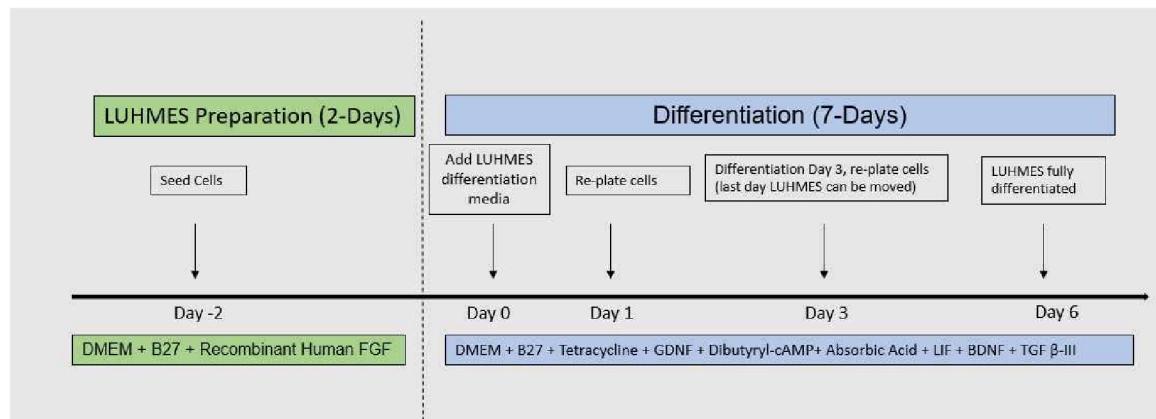
- 7mL LUHMES Media
- 4uL B-fgf (40ng)
- 2mL FBS (20%)
- 1mL DMSO (1%)

LUHMES Differentiation

- 21 Day 0, when the LUHMES are 80% confluent, add differentiation media and incubate overnight.

22 Day 1, replate cells onto a new poly-l-ornithine and fibronectin-coated plate. Replate cells at a density of 1 million cells per T-75 flask or ~ 400,000 per well of a 6-well.

23 Replace LUHMES differentiation media every day while differentiation (neurons become mature in 7-days)



LUHMES Differentiation Timeline