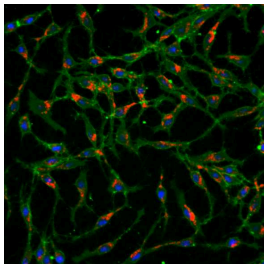
Feb 15, 2024 Version 3

LUHMES (lund human mesencephalic) culturing and differentiation protocol V.3



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Manuscript citation:
ATCC https://www.atcc.org/products/crl-2927#product-references

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

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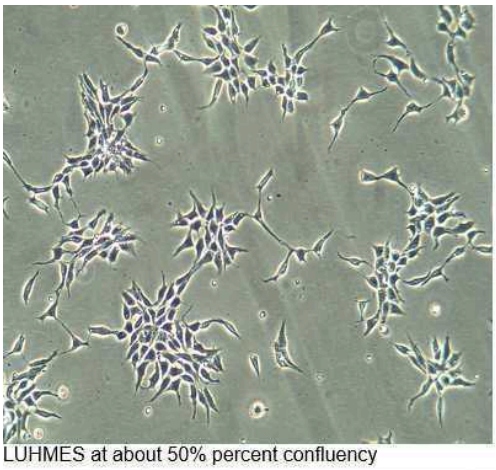
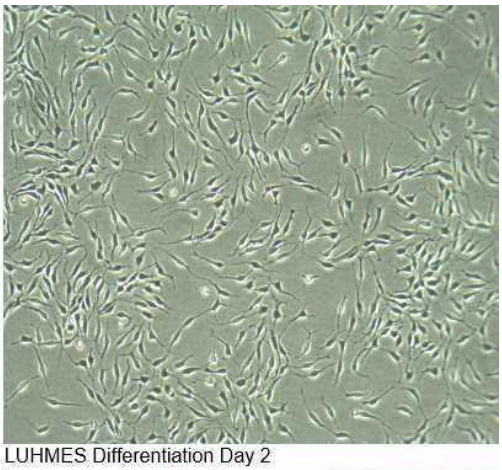
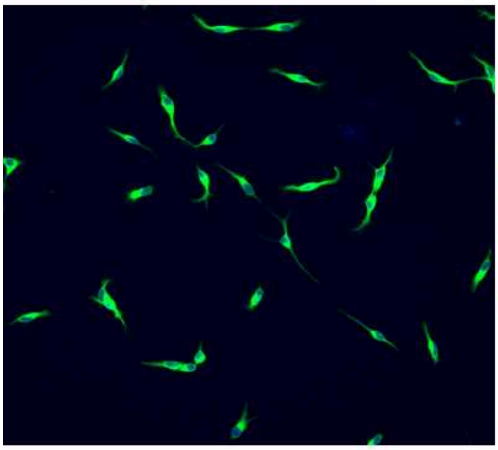
Abstract



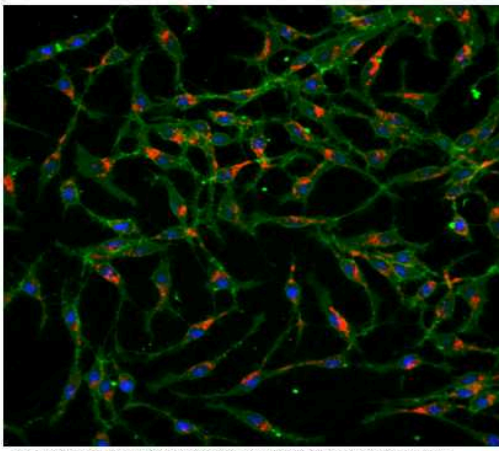
LUHMES at about 50% percent confluency



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

	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
	DibutyrylcAMP MW: 491.4 g/mol Mass: 100mg	Reconstitute: Add 1.56mL of PBS to 100mg Vial	100mM	1mM	200uL
	Ascorbic Acid (500mg Vial)	Reconstitute: Add 14.195mL PBS (pH 7.2) to 500mg of absorbic acid	200mM	0.2mM	20uL
	Human Recombinant LIF (50ug vial)	Reconstitute: Add 500ul of nuclease free water to 50ug vial. *2 weeks	0.1ug/mL	10ng/mL	2uL

	Reagents	Note	Stock Concentration	Working Concentration	Final Solution Volume = 20mL
		or at -20°C to -80°C for up to 3 months			
	Human Recombinant BDNF (10ug vial)	Reconstitute: Add 100ul to vial. centrifuged before opening, Do Not Vortex	0.1 mg/mL	20ng/mL	4uL
	Human Recombinant GDNF (10ug vial)	Reconstitute: Add 100ul 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 50mL of bio-grade water to 500mg of tetracycline. Store in -20	10mg/mL	1ug/mL	2uL
	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
- Tetracyline 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

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

Penicillin Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Scientific** **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

Fibronectin human plasma,liquid, 0.1% (Solution), **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**F0895-1MG

Tgf beta 3 (human) Recombinant Protein **Invitrogen - Thermo Fisher** **Catalog #**RP8600

Dibutyryl-cAMP **STEMCELL Technologies Inc.** **Catalog #**73884

Tetracyline Hydrochloride **Thermo Scientific** **Catalog #**A39246

Human Recombinant GDNF **STEMCELL Technologies Inc.** **Catalog #**78058

Human Recombinant BDNF **STEMCELL Technologies Inc.** **Catalog #**78005

Human Recombinant LIF **STEMCELL Technologies Inc.** **Catalog #**78055

Absorbic Acid **STEMCELL Technologies Inc.** **Catalog #**72132

LUHMES coating protocol

1 Poly-L- Ornithine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A-004-C

- [M] 0.1 mg/mL Stock concentration
- [M] 50 µg/µL 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 µL of PLO for every 500 µL Nuclease-free water

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

4 Rinse flask 3 times with Nuclease-free 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

Storage: –20 °C in aliquots

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

Thaw an aliquot of Fibronectin solution at 5°C .

> 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 fresh 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.

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

Penicillin Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Scientific 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

See material section for media concentration information

LUHMES Passaging (~ every 2-3 days)

13 Remove media and rinse with DPBS

14 Add fresh culture media to newly coated 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.

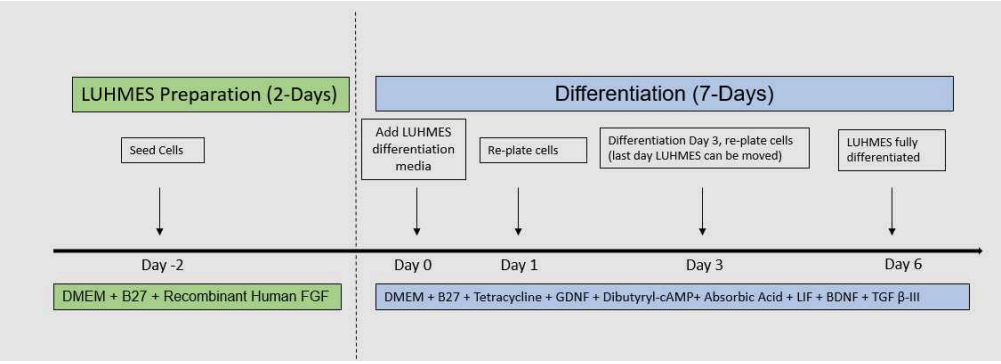
Freezing Media:
- 7mL LUHMES Media
- 4uL B-fgf (40ng)
- 2mL FBS (20%)
- 1mL DMSO (1%)

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 differentiating. LUHMES become mature dopamine-like neuron in 7-days.



LUHMES Differentiation Timeline

See material section for media concentration information

Protocol references

ATCC LUHMES CRL-2927