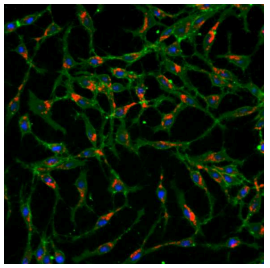
Feb 14, 2024 Version 1

LUHMES culturing and differentiation protocol V.1



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Mallory Wright[1], William Buchser[2], ckremitz[2], jwaligor[2], bachman@wustl.edu[2]
[1]Washington University, Saint Louis. McDonnell Genome Institute (MGI); [2]Washington University in St. Louis

WashU FIVE @ MGI

Mallory Wright
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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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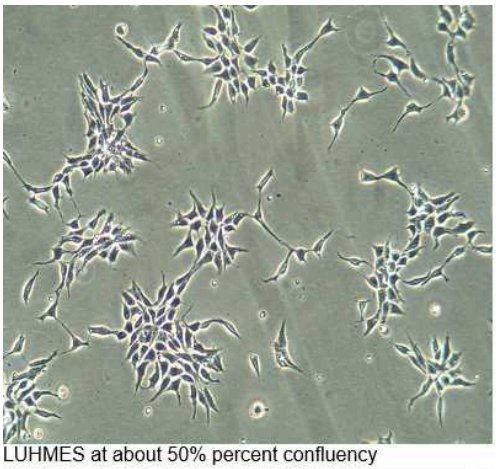
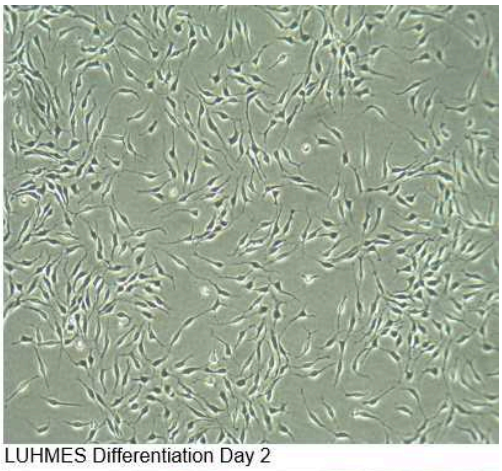
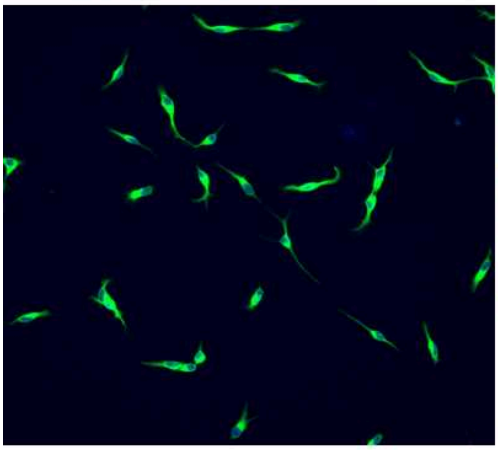
Abstract



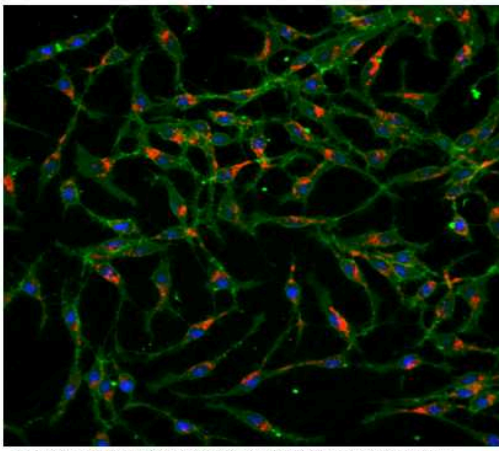
LUHMES at about 50% percent confluency



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

	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
	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

	Reagents	Note	Stock Concentration	Working Concentration	Final Solution Volume = 20mL
	Human Recombinant LIF (50ug vial)	Reconstitute: Add 500ul of nuclease free water to 50ug vial. *2 weeks or at -20°C to -80°C for up to 3 months	0.1ug/mL	10ng/mL	2uL
	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	Reconstitue: 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
- Poly-L- Ornithine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A-004-C
- Fibronectin human plasma,liquid, 0.1% (Solution), **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**F0895-1MG
- Trypsin/edta Solution (TE) **Thermo Scientific Catalog #**R001100
- Absorbic Acid **STEMCELL Technologies Inc. Catalog #**72132
- Human Recombinant BDNF **STEMCELL Technologies Inc. Catalog #**78005
- 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
- Tgf beta 3 (human) Recombinant Protein **Invitrogen - Thermo Fisher Catalog #**RP8600

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

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

3 Dilute Poly-L-Ornithine solution to 50ug/mL in sterile water.
(Add 500 µL of PLO for every 500 µL sterile water)

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

5 Rinse 3 times with bio-grade water.

6 Allow the flask to air dry for 15 minutes uncapped and stand upright in the hood. (turn on UV)

7 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

8 Slowly thaw the fibronectin solution at 2–8 °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

9 Dilute the Fibronectin in sterile Hank’s Balanced Salt Solution (HBSS).
Add 2 µL Fibronectin to 998 µL HBSS

10 Place fibronectin coated flask in incubator for 3 hours

11 Rinse 3 times with HBSS.

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

LUHMES growth media

13 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.

14
- 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 last

LUHMES Passaging (~ every 2-3 days)

15 Remove media and rinse with DPBS

16 Add 4mL of the pre-warmed .025% Trypsin/EDTA
- Trypsin/edta Solution (TE) **Thermo Scientific Catalog #**R001100

and place in the incubator for 3 minutes.

17 Neutralize trypsin with 6mL of pre-warmed DMEM/F12

18 Transfer cells to 15mL tube and centrifuge for 7min at 1200 RPM

19 Discard the supernatant and resuspend in 1mL and use a 5mL pipette

20 Triturate cells only 1 or 2 times before seeding.

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

Cryopreservation

22 Label 2mL cryovials with the date, name, FIV#, Qbench number, passage number and cell type and add about 1.5 million cells per vial with 1mL freezing media.

Freezing Media:

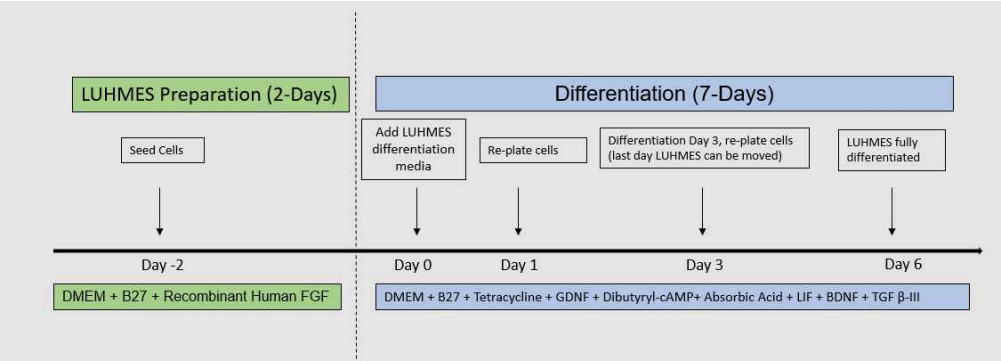
- 7mL LUHMES Media
- 4uL B-fgf (40ng)
- 2mL FBS (20%)
- 1mL DMSO (1%)

LUHMES Differentiation

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

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

25 Replace LUHMES differentiation media with fresh differentiation media every day (neurons become mature in 7 days)



LUHMES Differentiation Timeline

Protocol references

ATCC LUHMES CRL-2927