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

Mallory Wright<sup>1</sup>, William J Buchser<sup>2</sup>, ckremitz<sup>2</sup>, jwaligor<sup>2</sup>, bachman@wustl.edu<sup>2</sup>, serenaelia@wustl.edu<sup>2</sup>

<sup>1</sup>Washington University, Saint Louis. McDonnell Genome Institute (MGI);

<sup>2</sup>Washington University in St. Louis

Washington University FIVE @ MGI

Mallory



Mallory Wright

Washington University, Saint Louis. McDonnell Genome Institu...

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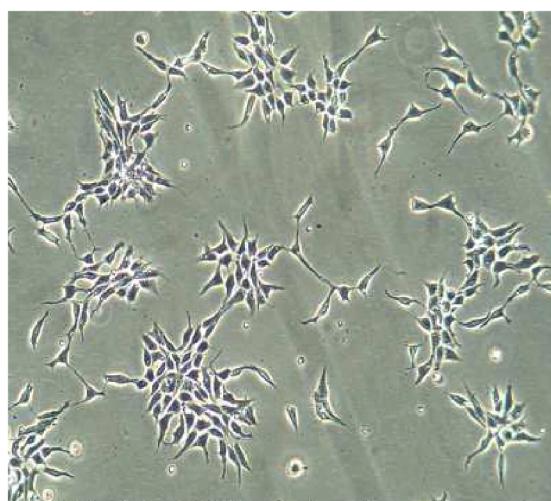
**Protocol status:** Working  
We use this protocol and it's working

**Created:** Feb 15, 2024

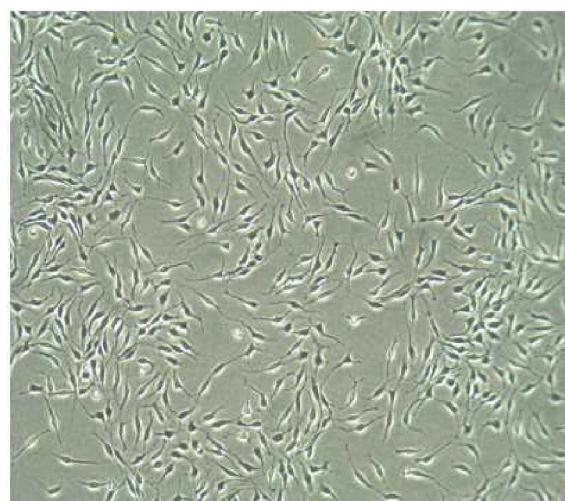
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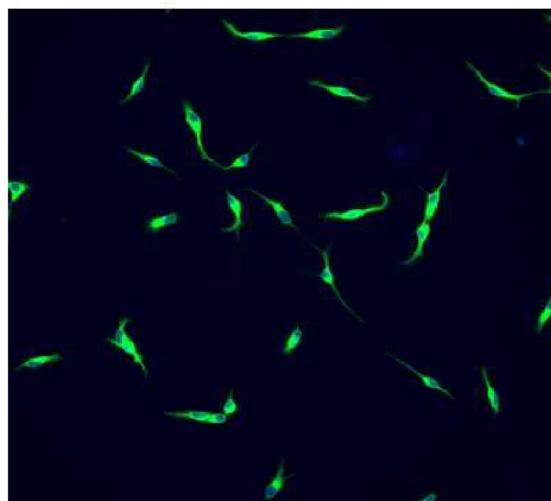
## ABSTRACT



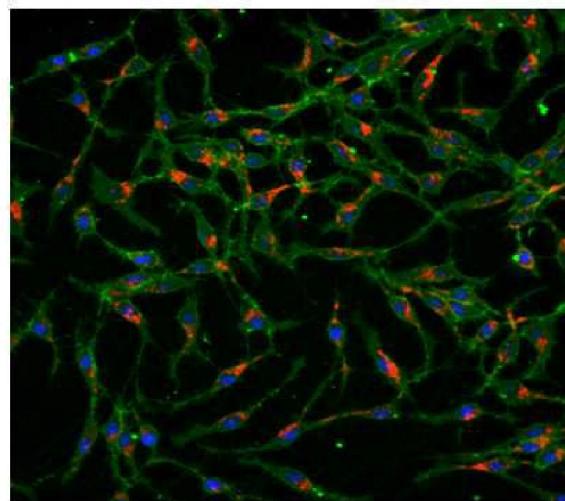
LUHMES at about 50% percent confluence



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

	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 (pH	200mM	0.2mM	20uL

Reagents	Note	Stock Concentration	Working Concentration	Final Solution Volume = 20mL
	7.2) to 500 mg of abs orbi c aci d			
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 trifug ed bef ore ope nin g,	0.1 mg/mL	20ng/mL	4uL

	Reagents	Note Do Not Vor tex	Stock Concentration	Working Concentration	Final Solution Volume = 20mL
	Human Recombinant GDNF (10ug vial)	Rec ons titut e: Add 100 ul of nuc leas e free wat er to 10u g vial of GD NF. Ma ke 5ul aliq uot s Stor e aliq uot s at -20° C	0.1mg/mL	20ng/mL	4uL
	Tetracycline MW: 480.91 g/mol Mass: 500mg	Rec ons titu e: Add 50 mL of bio- gra de wat er to 500 mg of tetr acy clin e.	10mg/mL	1ug/mL	2uL

	Reagents	Note Store 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

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

Step 12

 B-27 Supplement (50X) **Thermo Fisher Scientific Catalog #17504044** Step 12

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

Step 12

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

Step 6

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

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

 Tetracycline Hydrochloride **Thermo Scientific Catalog #A39246** Materials

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

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

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

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

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

Materials

## LUHMES coating protocol

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

-  0.1 mg/mL Stock concentration
-  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  $\Delta$  2 µL Fibronectin to  $\Delta$  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 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

\*\*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.

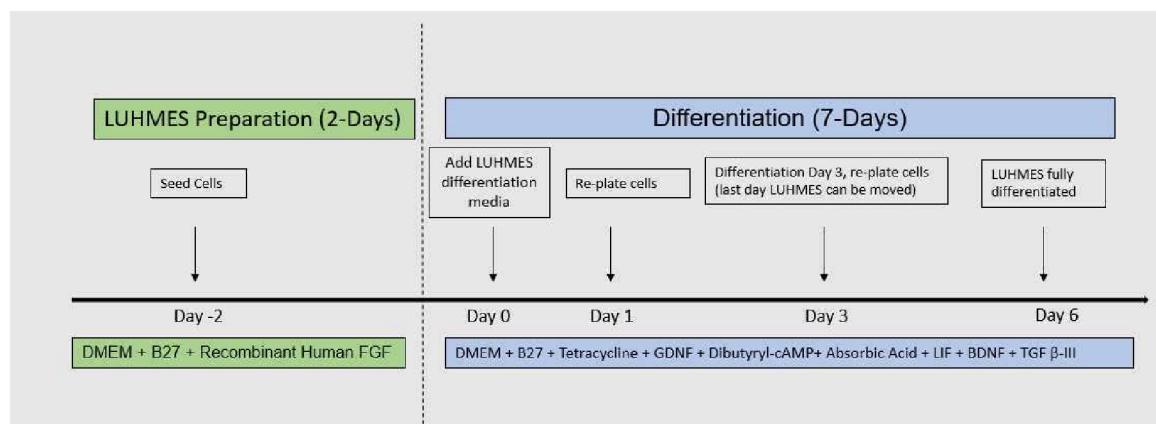
Freezing Media:

- 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\*\*