

LUHMES culturing and differentiation protocol V.1

Mallory Wright¹, William J Buchser², ckremitz², jwaligor², bachman@wustl.edu²

¹Washington University, Saint Louis. McDonnell Genome Institute (MGI);

²Washington University in St. Louis

Washington University FIVE @ MGI

Mallory



Mallory Wright

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

VERSION 1

FEB 14, 2024

OPEN  ACCESS



DOI:

dx.doi.org/10.17504/protocols.io.kxygx36ykg8j/v1

Protocol Citation: Mallory

Wright, William J Buchser,
ckremitz, jwaligor,
bachman@wustl.edu 2024.

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differentiation protocol.

protocols.io

<https://dx.doi.org/10.17504/protocols.io.kxygx36ykg8j/v1>

MANUSCRIPT CITATION:

ATCC

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

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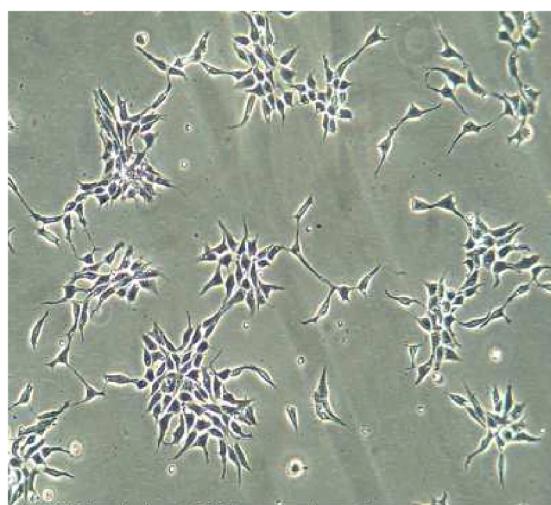
Protocol status: Working
We use this protocol and it's working

Created: Feb 14, 2024

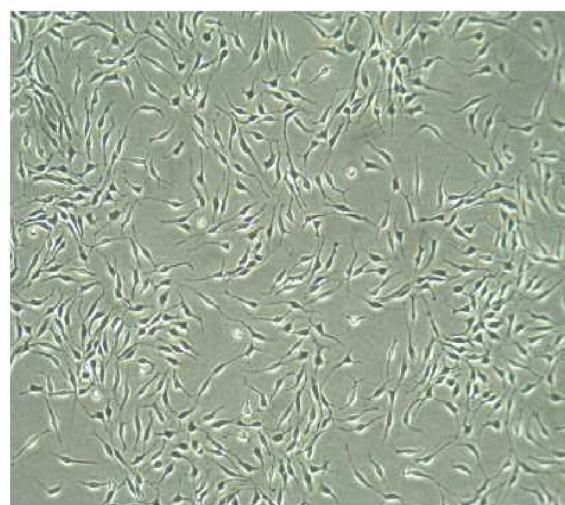
Last Modified: Feb 14, 2024

PROTOCOL integer ID: 95208

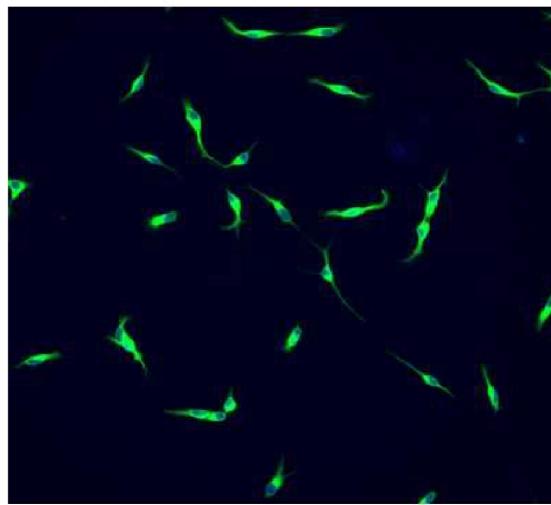
ABSTRACT



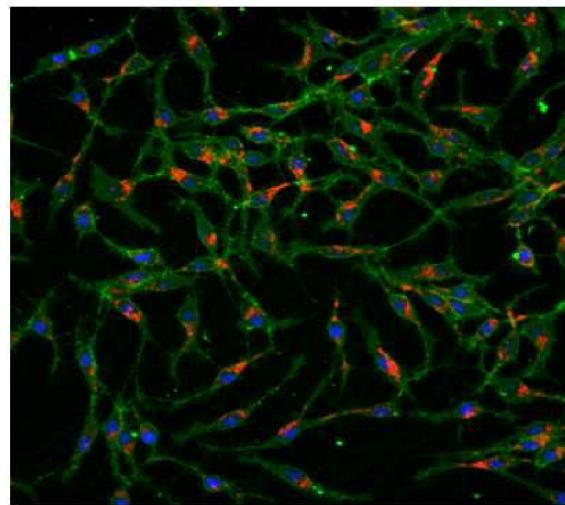
LUHMES at about 50% percent confluence



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

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

Materials

 DMEM/F12 **Thermo Fisher Scientific Catalog #11320033** Step 14

 B-27 Supplement (50X) **Thermo Fisher Scientific Catalog #17504044** Step 14

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

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

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

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

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

Step 14

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

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

Step 14

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

Step 7

 Trypsin/edta Solution (TE) **Thermo Scientific Catalog #R001100** Step 16

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

 Dibutyryl-cAMP **STEMCELL Technologies Inc. Catalog #73884** 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 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 \ddagger 500 μL of PLO for every \ddagger 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: 100 μL , Storage Temperature: -20°C in aliquots

- $[\text{M}]$ 1 mg/mL Stock Concentration
- $[\text{M}]$ 2 $\mu\text{g}/\mu\text{L}$ Working concentration

- 8 Slowly thaw the fibronectin solution at 2–8 $^\circ\text{C}$.

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 \ddagger 2 μL Fibronectin to \ddagger 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 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 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

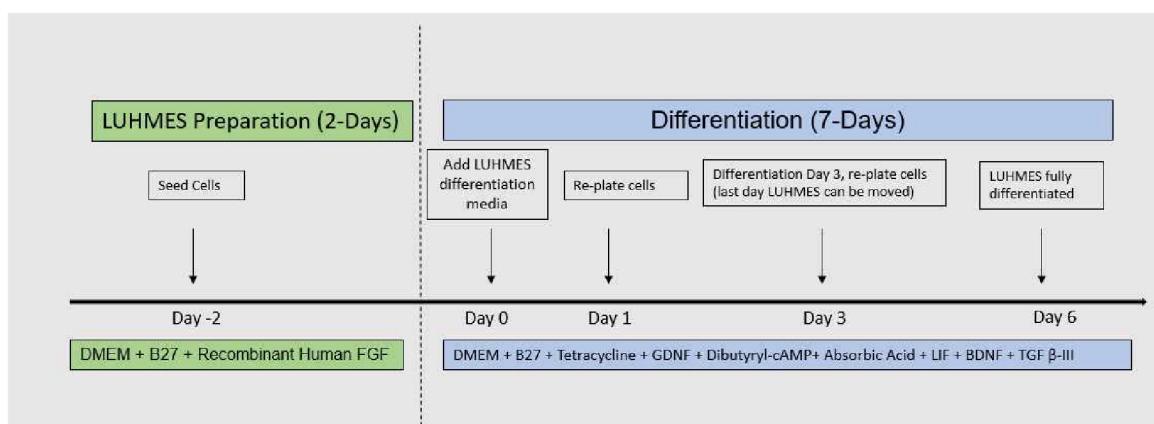
- 22 Label 2mL cryovials with the date, name, FIV#, Qbench number, passage number and cell type and add about 1.5 million cells per vial with 1mL freezing media.

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