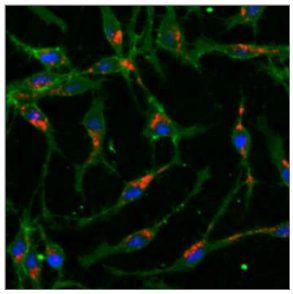

VERSION 1

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We use this protocol and it's working

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CRISPR/Cas9-Mediated Knockdown in LUHMES Cells: Nucleofection and Validation Protocol V.1

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ABSTRACT

This protocol uses a CRISPR RNP complex and nucleofection to precisely knock out genes. LUHMES cells, derived from human fetal mesencephalic tissue, offer a valuable model for studying dopaminergic dysfunction, featuring stimulus-induced dopamine release, relevant electrophysiological traits, and unique dopaminergic markers validating their phenotypic relevance.

LUHMES Cell Line: (ATCC Catalog Number: CRL-2927)
Nucleofection kit: P3 Primary Cell 4D-Nucleofector X Kit S Catalog #V4XP-3032

MATERIALS

	A	Lonza Single Nucleocuvette (100uL)	Lonza Nucleocuvette strip (20uL)
	pmaxGFP Vector (0.5ug/uL)	Add 4uL	Add 1.2 uL
	Nucleofection solution (P3)	82 uL	16.4 uL
	Nucleofection supplement (P3)	18 uL	3.6 uL
	Amount of cells	~ 1.3 million	~500,000 cells

P3 Primary Cell 4D-Nucleofector X Kit S **Lonza Catalog #**V4XP-3032

Alt-R S.p. Cas9 Nuclease/Nickase **Integrated DNA Technologies, Inc. (IDT)**

Cas9 (Nuclear Localized Signaling) purified protein **Macro labs (Berkeley) Catalog #**1111

1XPBS **Fisher Scientific Catalog #**10-010-023

Ribonucleoprotein components(RNP): gRNA + Cas9 + 1XPBS

	Components	Stock Concentration	Lonza Single Nucleocuvette (100uL)	Lonza Nucleocuvette strip (20uL)	Final Concentration
	gRNA	100 uM	6 uL	1.2 uL (120 pmol)	4.8 µM
	Cas9 (IDT)	62 uM	Add 8.5 µL	Add 1.7 uL	4.216 µM
	*2nd option: Cas9 (Berkely)	40 uM	Add 10.5 µL	Add 2.635 µL	4.216 µM
	1X PBS	1X	Add 10.5 uL (IDT Cas9) or 8.5 uL (Berkley cas9)	Add 2.1 uL (IDT Cas9) or 1.2uL (berkley Cas9)	
	Total volume per Nucleocuvette:		125 uL	25 uL	

*Adding 1.7µL of Alt-R Cas9 enzyme from a 62 µM stock introduces 105.4 pmol into the RNP mixture. With 8.5 µL from the same stock, it introduces 527 pmol. The final Alt-R Cas9 concentration in a 125 µL volume is approximately 4.216 µM.

PROTOCOL MATERIALS

- Lysis Buffer 1X Working solution **Takara Bio Inc. Catalog #**635013 Step 16
- P3 Primary Cell 4D-Nucleofector X Kit S **Lonza Catalog #**V4XP-3032 Materials
- Alt-R S.p. Cas9 Nuclease/Nickase **Integrated DNA Technologies, Inc. (IDT)** Materials
- Cas9 (Nuclear Localized Signaling) purified protein **Macro labs (Berkeley) Catalog #**1111 Materials
- 1XPBS **Fisher Scientific Catalog #**10-010-023 Materials
- trypsin-EDTA (TE) **Fisher Scientific Catalog #**R001100 Step 4

Nucleofection Protocol

1 Maintain a cell confluency of 70–85%. Higher cell densities may lead to lower Nucleofection efficiencies.

2 Coat a new 6-well plate freshly with poly-L-ornithine and fibronectin to facilitate LUHMES attachment.

3 Following the coating process, add LUHMES growth media to the 6-well plate and pre-incubate/equilibrate the plates in a humidified incubator set at 37°C with 5% CO2.

4 Rinse LUHMES cells with 1XPBS, then add trypsin-EDTA (TE) **Fisher Scientific Catalog #**R001100 to dissociate the cells, using 4 mL in a T-75 flask, and incubate for 3 minutes.

5 Spin at 1200 revolutions per minute (rpm) in a centrifuge for 5 minutes, then carefully discard the supernatant

6 Re-suspend cells with 5 mL of 1X PBS.

7 Following cell counting, aliquot cells into separate 15 mL tubes, adding around 1.3 million cells per cuvette and 500,000 cells per well in a nucleocuvette strip

8 Centrifuge the samples a second time at a low speed (90xg) for 10 minutes, ensuring minimal cell agitation before nucleofection.

9 During centrifugation, prepare RNP complex while setting up Nucleofector 4D. Choose plate layout, program, and cell type (e.g., single cuvette or strip). Employ LUHMES program CA-137.

Ribonucleoprotein components (RNP): gRNA + Cas9 + 1XPBS				
	Stock Concentration	Lonza Single Nucleocuvette (100uL)	Lonza Nucleocuvette strip (20uL)	Final Concentration
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*2nd option Cas9 (Berkely)	40 uM	Add 10.54 µL	Add 2.635 µL	4.216 µM
1X PBS	1X	Add 10.5 uL (IDT Cas9) or 8.5uL (if using using Berkley Cas9)	Add 2.1 uL (IDT Cas9) or 1.2 uL (if using using Berkley Cas9)	
Total volume per Nucleocuvette		125 uL	25 uL	

RNP Complex

10 Carefully re-suspend each cell pellet in room temperature 4D-Nucleofection Solution + supplement.

	Lonza Single Nucleocuvette (100uL)	Lonza Nucleocuvette strip (20uL)
pmaxGFP Vector (0.5ug/uL)	Add 4uL	Add 1.2 uL
Nucleofection solution (P3)	82 uL	16.4 uL
Nucleofection supplement (P3)	18 uL	3.6 uL
Amount of cells	~1.3 million	~500,000 cells

11 Add cells to the cuvette, ensuring the sample covers the bottom of the cuvette. Gently tap to distribute evenly and avoid bubbles.

12 Place the nucleocuvettes in the nucleofector cuvette holder, ensuring proper alignment

13 Replace the existing media with warmed fresh media, followed by an incubation period of 10 to 15 minutes at room temperature or in an appropriate incubator. Avoid any disturbance to the cells post-nucleofection.

14 A typical analysis time is 24-hours post-nucleofection.

Nucleofection Validation

15 24 hours after nucleofection, rinse the cells using 1X PBS, apply TE dissociation solution, allow a 3-minute incubation period, transfer to a 15 mL tube, and then centrifuge.

16 Lysis Buffer 1X Working solution **Takara Bio Inc. Catalog #**635013

Re-suspend the cells in 1X lysis buffer, gently triturating approximately 5 times to ensure efficient cell membrane disruption and DNA extraction.

Note

Excessive trituration can result in increased shearing of DNA

17 Add cell suspension to a 1.5 mL snap cap tube

18 Add the 1.5 mL snap cap tube to a 65 °C heat block for 15 minutes to disrupt cellular structures, denature proteins, and aid DNA extraction.

19 Follow with a second heat treatment at 95 °C for 3 minutes to further denature DNA

20 Add the Snap cap tube to fridge over night in 4 °C

PCR Protocol

21 Utilize a nanodrop device to determine the nucleic acid concentration of your bulk sample.

22

Prepare the PCR reaction mix in a sterile microcentrifuge tube by combining the following components:

- **Template DNA:** The DNA you wish to amplify, need ~100 ng of DNA per 25uL reaction
- **Forward and Reverse Primers:** Short DNA sequences that bind to the start and end of the target DNA region you want to copy.
- **MyTaq Red Mix (Meridian Life Science Catalog #CSA-01178):** A ready-to-use PCR master mix containing DNA polymerase, dNTPs, buffer, and a red dye.
- **DMSO (Dimethyl Sulfoxide):** Added to enhance PCR specificity and amplification of GC-rich templates

	Components	Concentrations	1	20
	Forward Primer (100uM)	0.5 µM	0.125 µL	2.5 µL
	Reverse Primer (100uM)	0.5 µM	0.125 µL	2.5 µL
	MyTaq (DNA Polymerase)	2X	12.5	250 uL
	DMSO	5%	1.25 µL per reaction.	25 µL

	Components	Concentrations	1	20
	Water (ddH2O)	amount depends on the remaining volume after adding the other components	9 uL	180 uL
	Template DNA	need ~200 ng per 25uL reaction	2 uL	2 uL
	Total amount	25 uL		

PCR1 components