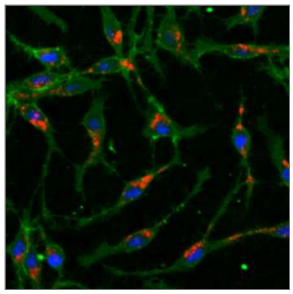

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We use this protocol and it's working

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CRISPR/Cas9-Mediated Knockdown in LUHMES Cells: Nucleofection and Validation Protocol V.3

Mallory Wright[1], William J Buchser[2], Colin Kremitzki[3], Serena Elia[3], Graham Bachman[3], emanuel gerbi[3], Jason Waligorski[4], Nicholas Tu[5], Lina Mohammed Ali[5]

[1]Washington University, Saint Louis. McDonnell Genome Institute (MGI); [2]Washington University in St. Louis; [3]Washington University School of Medicine; [4]Washington University School of Medicine @FIVE; [5]McDonnell Genome Institute

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

ABSTRACT

Utilizing a CRISPR RNP complex and nucleofection, this protocol enables precise gene knockout. LUHMES cells, sourced from human fetal mesencephalic tissue, provide a valuable model for investigating dopaminergic dysfunction. They exhibit stimulus-induced dopamine release, pertinent electrophysiological traits, and distinctive 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

Keywords: LUHMES, Ribonucleoprotein Complex (RNP), Neurodegenerative disease models, Gene knockout, Neuroscience, CRISPR /Cas9, Gel electrophoresis, PCR

MATERIALS

Alt-R CRISPR-Cas9 sgRNA,10 **Integrated DNA Technologies, Inc. (IDT)** , Length 100

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

	Lonza Single Nucleocurvette (100uL)	Lonza Nucleocurvette strip (25 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
pmaxGFP Vector (0.5ug/uL) (optional positive control)	Add 4 uL	Add 1.2 uL

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

Components	Stock Concentration	Lonza Single Nucleocuvette (100uL)	Lonza Nucleocuvette strip (20uL)	Final Concentration
gRNA (IDT)	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
1X PBS	1X	Add 10.5 uL	Add 2.1 uL	
Total volume of RNP Complex:		25 uL	5 uL	

For a second Cas9 option, use Cas9 NLS (Berkeley) at 40 µM:

- Add 10.54 µL to a single nucleocuvette.
- Add 2.635 µL to a cuvette strip to achieve 4.216 µM.

*By adding 1.7µL of Alt-R Cas9 enzyme from a 62 µM stock, 105.4 pmol is introduced into the RNP mixture. The inclusion of the RNP Complex into the nucleofection solution along with the supplement yields a final concentration of 4.216 uM.

*Adding 8.5 µL from the same stock introduces 527 pmol into the RNP complex. The final Alt-R Cas9 concentration in a 125 µL volume is approximately 4.216 µM.

PROTOCOL MATERIALS

trypsin-EDTA (TE) **Fisher Scientific Catalog #**R001100 Step 4

Lysis Buffer 1X Working solution **Takara Bio Inc. Catalog #**635013 Step 18

Nucleofection Protocol

1 Maintain cell confluency between 70–85% to optimize Nucleofection efficiencies; optimal results typically occur with cells in the logarithmic growth phase.

2 Coat a new 6-well plate freshly with poly-L-ornithine (50ug/mL) and fibronectin (2ug/mL) to facilitate LUHMES attachment.

3 Add 2 mL of the 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 with 5 mL 1XPBS, then add trypsin-EDTA (TE) **Fisher Scientific Catalog #**R001100 to dissociate the cells, using 4 mL for a T-75 flask, and incubate for 00:03:00 3m

5 Centrifuge 1200 rpm for 00:05:00, then carefully discard the supernatant 5m

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

7 After cell counting, distribute the cells into individual 15mL tubes according to the number of samples.

Add approximately 1 million cells per single cuvette and 500,000 cells per well in a nucleocuvette strip.

8 Centrifuge the samples a second time at a low speed 90 x g for 00:10:00, ensuring minimal cell agitation before nucleofection. 10m

9 While centrifuging, prepare the RNP complex as outlined in the table below and configure the Nucleofector 4D accordingly. Select the plate layout (e.g., single cuvette or strip), program CA-137, and specify the cell type.

Ribonucleoprotein components (RNP): gRNA + Cas9 + 1XPBS				
	Stock Concentration	Lonza Single Nucleocuvette (100uL)	Lonza Nucleocuvette strip (20uL)	Final Concentration
1. gRNA	100 uM	6 uL	Add 1.2 uL (120 pmol)	4.8 µM
2. Cas9 (IDT)	62 uM	Add 8.5 µL	Add 1.7 uL	4.216 µM
3. 1X PBS	1X	Add 10.5 uL	Add 2.1 uL	
Total volume in RNP Complex		25 uL	5 uL	

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

	Lonza Single Nucleocuvette (100uL)	Lonza Nucleocuvette strip (20uL)
Nucleofection supplement (P3)	18 uL	3.6 uL
Nucleofection solution (P3)	82 uL	16.4 uL
pmaxGFP Vector (0.5ug/uL) (optional positive control)	Add 4 uL	Add 1.2 uL

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

12 For each single nucleocuvette, add 25 µL of the RNP Complex, or for each well of a strip, add 5 µL of the RNP complex.

13 Insert the nucleocuvettes into the nucleofector cuvette slot, ensuring they are properly aligned, then press start.

14 Following nucleofection, immediately add an additional 500 µL of pre-warmed media to each individual single cuvette or 50 uL to a curvette strip. 15m

Maintain cell stability by avoiding any disturbance for at least 00:15:00 at room temperature 2 °C or within an incubator

15 Transfer the cells to the previously coated flask or plate containing pre-warmed LUHMES growth media, and incubate.

16 A typical analysis time is 24-Hours post-nucleofection.

Nucleofection Validation

17 24 hours after nucleofection, rinse the cells using 5 mL 1X PBS, apply TE dissociation solution, allow a 00:03:00 incubation period, transfer to a 15 mL tube, and then centrifuge 1200 rpm, 00:05:00 8m

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

Re-suspend the cells in 100 µL 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

19 Add cell suspension to a 1.5 mL snap cap tube

20 Add the 1.5 mL snap cap tube to a 65 °C heat block for 00:15:00 to disrupt cellular structures, denature proteins, and aid DNA extraction. 15m

21 Follow with a second heat treatment at 95 °C for 00:03:00 3m

22 Add the Snap cap tube to fridge Overnight in 4 °C 3m

PCR Protocol

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

24

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 25 µL 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 #**BIO-25047**):** 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

	Components	Concentrations	1	20
	DMSO	5%	1.25 µL per reaction.	25 µL
	Water (ddH2O)	amount depends on the remaining volume after adding the other components	9 uL	180 uL
	Template DNA	need ~100 ng per 25uL reaction	2 uL	2 uL
	Total amount	25 uL		

PCR1 components

Running an Agarose Gel for DNA Electrophoresis

35m

25 Prepare Agarose Gel: 6m 15s

- Measure 1 g of agarose.
- Dissolve in 70 mL of 0.5X TBE buffer in a beaker
- Microwave for 00:01:15 or until completely dissolved.
- Add SybrSafe dye at 1 µL for ever 10 mL of 0.5X TBE Buffer to the agarose solution for staining the DNA bands.
- Cool for about 00:05:00

26 Pour Gel and Insert Combs: 30m

- Pour the cooled agarose into a gel tray.
- Insert combs to create wells for loading samples.
- Remove any bubbles in gel
- Let the gel sit undisturbed for about 00:30:00 to solidify.

27 Prepare Samples:

-Remove combs
- Add 5 µL of DNA ladder 100bp (Gold bio)
-Add 5 µL of each DNA sample to the wells. Ensure proper labeling to keep track of the samples.

28 Run the Gel: 25m

-Connect wires to matching color ports on the power supply (red to red, black to black)
-Set voltage to 170V, 400mA and run gel for 00:25:00

Monitor gel progress as DNA fragments move through agarose: smaller ones faster, longer ones slower.

29 Once complete:

- Carefully remove the gel from the casting tray and place it in a gel imaging system.
- Visualize the DNA bands under UV light.
- The Sybr Safe dye will fluoresce upon binding to DNA, allowing for the visualization of DNA fragments.

Analyze the gel to identify the presence of contaminants, estimate the size of amplified DNA fragments, and confirm whether they match the expected sizes based on primer design.

30 Last, perform PCR2 to incorporate barcodes and then submit the samples for next-generation sequencing.