

VERSION 2

MAR 21, 2024

OPEN ACCESS



**Protocol Citation:** Mallory Wright, William J Buchser, Colin Kremitzki, Serena Elia, Graham Bachman, emanuel gerbi, Jason Waligorski, Nicholas Tu, Lina Mohammed Ali 2024. CRISPR/Cas9-Mediated Knockdown in LUHMES Cells: Nucleofection and Validation Protocol. [protocols.io](https://protocols.io/view/crispr-cas9-mediated-knockdown-in-luhmes-cells-nuc-dazg2f3w) <https://protocols.io/view/crispr-cas9-mediated-knockdown-in-luhmes-cells-nuc-dazg2f3w> Version created by [Mallory Wright](#)

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

**Created:** Mar 21, 2024

**Last Modified:** Mar 21, 2024

**PROTOCOL integer ID:** 97032

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

## 🌐 CRISPR/Cas9-Mediated Knockdown in LUHMES Cells: Nucleofection and Validation Protocol V.2

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### 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

## 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 Nucleocuvette (100uL)	Lonza Nucleocuvette 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

- ☒ Cas9 (Nuclear Localized Signaling) purified protein **Macro labs (Berkeley) Catalog #1111** Materials
- ☒ 1XPBS **Fisher Scientific Catalog #10-010-023** Materials
- ☒ Alt-R CRISPR-Cas9 sgRNA,10 **Integrated DNA Technologies, Inc. (IDT)** Materials
- ☒ trypsin-EDTA (TE) **Fisher Scientific Catalog #R001100** Step 4
- ☒ Lysis Buffer 1X Working solution **Takara Bio Inc. Catalog #G35013** Step 18
- ☒ 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

## Nucleofection Protocol

- Maintain cell confluency between 70–85% to optimize Nucleofection efficiencies; optimal results typically occur with cells in the logarithmic growth phase.
- Coat a new 6-well plate freshly with poly-L-ornithine and fibronectin to facilitate LUHMES attachment.
- 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% CO<sub>2</sub>.
- Rinse LUHMES with 1XPBS, then add ☒ trypsin-EDTA (TE) **Fisher Scientific Catalog #R001100** to dissociate the cells, using 🧴 4 mL **3m** for a T-75 flask, and incubate for ⌚ 00:03:00
- Centrifuge 🌀 1200 rpm for ⌚ 00:05:00, then carefully discard the supernatant **5m**
- Re-suspend cells with 🧴 5 mL of 1X PBS and count cells.
- After cell counting, distribute the cells into individual 🧴 15 mL 15mL tubes according to the number of samples.  
Add approximately 🧴 0 mL per single cuvette and 500,000 cells per well in a nucleocuvette strip.
- 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	

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.

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 media, and incubate.

- 16 A typical analysis time is  00:00:00 post-nucleofection.

## Nucleofection Validation

- 17 24 hours after nucleofection, rinse the cells using 1X PBS, apply TE dissociation solution, allow a ⌚ 00:03:00 incubation period, transfer to 15 mL tube, and then centrifuge. 3m
- 18  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
- 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 #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 $\mu$ M	0.125 $\mu$ L	2.5 $\mu$ L
Reverse Primer (100uM)	0.5 $\mu$ M	0.125 $\mu$ L	2.5 $\mu$ L
MyTaq (DNA Polymerase)	2X	12.5	250 $\mu$ L
DMSO	5%	1.25 $\mu$ L per reaction.	25 $\mu$ L
Water (ddH <sub>2</sub> O)	amount depends on the remaining volume after adding the other components	9 $\mu$ L	180 $\mu$ L
Template DNA	need ~100 ng per 25uL reaction	2 $\mu$ L	2 $\mu$ L
Total amount	25 $\mu$ L		





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 10X buffer in a beaker
- Microwave for  00:01:15 or until completely dissolved.
- Add Sybr Safe dye 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.
- Let the gel sit undisturbed for about  00:30:00 to solidify.

### 27 Prepare Samples:

- Remove combs carefully to prevent well damage
- Add  5  $\mu$ L of DNA ladder to one well for size reference
- Add  5  $\mu$ L of each DNA sample to the wells. Ensure proper labeling to keep track of the samples.

### 28 Run the Gel:

28m

- Connect wires to matching color ports on the power supply (red to red, black to black)
- Set voltage to 170V and run gel for  00:28:00

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.