**Transient and stable transfection of mouse ES cells**

**using the Neon electroporation system**

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**Sources**: Neon transfection system manual (Life Technologies MPK50000)

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**OVERVIEW**

Day 0 Passage cells so they are ready to split again 24h later

Day 1 Electroporate

Day 2 Change medium and/or assay expression

Day 3 Subclone and/or assay expression

*For creating stable clonal lines:* Day 13-16 Pick single clones

**MATERIALS AND REAGENTS**

* Either 0.1 or 1 million mESCs per transfection
* mESC medium, PBS 1X
* Optional: antibiotic (e.g. puromycine, blasticidin, geneticin, hygromycin)
* DNA to transfect
* DNA for positive transfection control (e.g. pmax GFP plasmid)
* Neon transfection kit (MPK1025 10µL format or MPK10025 for 100µL tip format)

**GENERAL GUIDLINES**

**10µL electroporation tip format:**

* 1. million mESCs and 3 µg DNA maximum

Use when not many transfected cells are needed.

Example of applications: transient expression of CRE or Flp before subcloning without selection, establishing optimal transfection parameters…

**100µL electroporation tip format**

1 million mESCs and 30 µg DNA maximum

Use when a lot of transfected are needed.

Example of applications: establishing stable knock-in lines

CRITICAL POINT: Each cell type has specific electroporation conditions. Before starting it is imperative to test transfection efficiencies. Refer to the Neon manual from Life technologies for guidance as to how to determine optimal transfection parameters.

**To isolate single clones after TRANSIENT expression (e.g. transient CRE or FLP expression…)**

At day 1 Use a 10µL tip with 0.1 million cells and 250ng-500ng each expression plasmid.

If more cells are needed you can use a 100µL tip but you will need to dilute cells at day 3 to have a 1/100, 1/1000 and 1/10.000 dilution plates.

**To isolate stable KNOCK-IN clones (e.g. using Cas9 or random integration)**

At day 1 Use a 100µL tip with 1 million cells, 15-20µg of donor plasmid and 2.5-5µg each transient expression plasmid (e.g. Cas9/sgRNA constructs). Do not exceed 30µg total DNA.

**Isolating a stable POLYCLONAL pool**

Sometime one wants to work with a polyclonal pool of stable integrant. If this is the case follow protocol until day 3 but reseed the cells in a 6-well plate and add antibiotic selection. You may have to repeat 1:1 passaging a couple of times before the population looks healthy enough to proceed with expansion.

**REAGENT SETUP**

* Prepare plasmid/BAC DNA by Midi or Maxi prep. Maximum DNA volume is 1/10th of electroporaction tip. When creating knock-ins best is to have targeting constructs >5µg/µL. **DO NOT** use DNA from minipreps.
* Final antibiotic concentration to use when creating stable cell lines:

Geneticin (“neo”): 200 µg/mL

Puromycin: 1 µg/mL

Hygromycin: 200 µg/mL

Blasticidin: 10 µg/mL

CRITICAL POINT: These concentrations work for the E14 mESC line. Other lines may have different requirement. When working with a new cell line it always a good idea to first perform a killing curve. Work with the lowest concentration that kills all non-transfected cells.

**PROCEDURE**

**Day1**

1. Gelatinize a 6-well plate. You will need one cell per transfection.
2. Place the DNA to be transfected in 1.5mL tubes. Do not exceed 1/10th volume of electroporation tip. Have one tube per transfection.
3. Add R solution to DNA   
   10µL tip format: use 12µL R solution  
   100µL tip format: use 120 µL R solution
4. Add 3 mL of Electrolyte solution to the sterile electroporation tube and place it on the device

10µL tip format: use solution E  
100µL tip format: use solution E2

FYI: electroporation tubes (not tips!) can be used 10 times even with different cells or DNA

1. Wash mESCs with PBS 1X once and detach using TRYPLE/trypsin mESCs for 7-10 minutes
2. Resuspend gently with mESC medium
3. Count cells.
4. Distribute 1 transfection worth of cells in sterile 1.5mL Eppendorf. Use one tube per transfection. Spin 950rpm 5 minutes.  
   10µL tip format: use 0.1 million cells  
   100µL tip format: use 0.1 million cells

TROUBLESHOOTING: each tip can be use twice. If a lot of cells are needed it is OK to transfect more cells with the same DNA with the same tip (at least twice is OK).

FYI: The R solution for resuspension can be quite toxic to mESCs. When doing many transfections you will have better survival if cells site in PBS rather than R.

1. Aspirate mESC medium and replace with 1mL PBS 1X. Spin 950rpm 5 minutes.
2. Replace the gelatin by **5mL** of mESC medium in the 6-well plate.
3. Aspirate PBS from the first tube of cells. Pipet up the R+DNA mix and use it to resuspend cells   
   10µL tip format: use 12µL R solution  
   100µL tip format: use 120 µL R solution
4. Using the Neon pipet pick one electroporation tip and pipet up the DNA+cell mixture
5. Fit the Neon pipet in the electroporation tube on the Neon device and electroporate

For E14 mESCs use **1400V, 10ms, 3 pulses**

1. Remove the Neon pipet and eject gently the cells in the 6-well plate containing 5mL of mESC medium. Disperse the cells while pipetting down so they don’t end up just on one side
2. Repeat steps 11 to 14 for remaining electroporations. Move cells to incubator promptly.

**Day2**

1. Change medium.
2. *Optional: assay cells for expression of transfected constructs*

FYI: If transfected DNA carries an expression cassette expression can be detected after 6-8hrs and peaks around 24-48hrs (depending on the type of cassette).

**Day3**

1. *Optional: assay cells for expression of transfected constructs*
2. **When establishing stable clonal lines** proceed to subcloning: Gelatinize three 10cm dishes. Then srypsinize cells 10 minutes, Resuspend with mESC medium. Pipet gently but thoroughly with a P1000 tip in order to obtain a single-cell suspension.   
     
   Replace gelatin in 10cm dishes with 9 mL of mESC medium. If antibiotic selection is need add proper antibiotic in the 10cm dish at this stage.

Put 1mL of trypsinized cells in the first plate.

Pipet up and down a couple of times with a 10mL pipet (this is the undiluted plate) and transfer 1mL in the second plate.   
Pipet up and down a couple of times with a 10mL pipet (this is the 1/10 plate) and transfer 1mL in the second plate.   
Pipet up and down a couple of times with a 10mL pipet (this is the 1/100 plate) and transfer 1mL in the third plate.

Change medium daily until single clones are visible and proceed to manual picking and expansion.