The electroporation machine may not be functioning properly. In this case re-calibration may be needed. If local electricity supply is not stable, connect the electroporation machine to an uninterrupted power supply (UPS) to stabilize the current. In addition, different target cells may require different voltage to be effective.

I have initial high transfection efficiency, but very few iPSC colonies. How can I increase the reprogramming efficiency?
Reprogramming is largely dependent on cell type and concentration of miR-302 (Lin et al., NAR 2011). Make sure puromycin selection is performed. Wait 3~4 days after Puromycin selection before transferring colonies to Feeder-Free Medium. If you switch cells to Feeder-Free Medium too soon, cells may detach and form aggregates which may reduce iPSC survival. We recommend culturing iPSC colonies under feeder-containing conditions to improve reprogramming efficiency.

How can I isolate an iPSC colony to produce a cell line?
A two- to eight-cell stage iPSC colony is the best choice to isolate for the formation of a single iPSC line. Cell isolation can be performed under an inverted microscope using a micromanipulator in conjunction with a micro-injector/holder. Alternatively, you can select colonies with pipette and very sharp eyes. Fluorescence activated cell sorting (FACS) can also be used to isolate iPSC colonies.

How do I ensure the quality of mir-302 plasmid?
Store the kit at -80°C freezer to maintain its quality. The plasmid is stable for one year under this condition. Avoid repeated freeze-thaw cycles of the plasmid. Due to the structural complexity of the miR-302 cluster, amplification of the plasmid may cause mutations. If you would like to sequence the plasmid to insure its quality, please contact Mello Biotech for primer information.

Refer to the FAQ section of our website (http://www.mellobiotech.com/faq.html) and Lin et.al. (2011) *Nucleic Acids Research* 39: 1054-1065 for additional information on miR-302/367 reprogramming.

References

1. Lin et al., (2008) MiR-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. *RNA*, 14: 2115–2124.
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3. Lin et al., (2011) Regulation of somatic cell reprogramming through inducible mir-302 expression. *Nucleic Acids Res*., 39: 1054-1065.
4. Moyoshi et al., (2011) Reprogramming of mouse and human cells to pluripotent using mature microRNAs. *Cell Stem Cell*, Vol 8: 633-638.
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6. Subramanyam et al., (2011) Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nat. Biotech*, 29: 443-448.
7. Card et al., (2008) Oct4/Sox2-regulated miR-302 targets cyclin D1 in human embryonic stem cells. Mol. Cell Biol. 28: 6426–6438.

Notice to Purchaser

CAREFULLY READ THE FOLLOWING TERMS AND CONDITIONS BEFORE OPENING THIS KIT. OPENING THIS PACKAGE INDICATES YOUR ACCEPTANCE OF THESE TERMS AND CONDITIONS. IF YOU DO NOT AGREE WITH THEM, YOU SHOULD PROMPTLY RETURN THE PACKAGE UNOPENED AND YOUR MONEY WILL BE REFUNDED.

1. Upon using this kit, the users agree to the terms of the Notice to Purchaser and government regulations.
2. All usage of this kit is subject to the US and PCT patent laws.
3. This kit is to not be used for human cloning or generation of hybrids.

D-105-302-PR

mello biotech

mirPS® Electroporation Kit User Manual

Catalog Numbers:

M-105-302-CM
M-106-302-CM
M-107-302-CM
M-108-302-CM

Stable for six months when stored at 4°C

Introduction

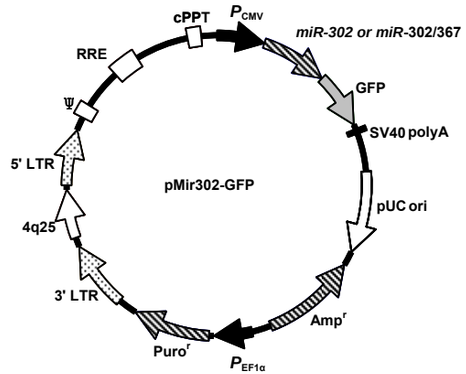

Figure 1

The **miR-302 Electroporation Expression Kit** is designed for intracellular delivery and expression of the miR-302 microRNA family (miR-302b, c, a and d pre-microRNA cluster, with or without miR-367). As shown in Figure 1, expression of the miR-302 pre-microRNA is cytomegalo virus (CMV) promoter-driven and directly linked to GFP expression, allowing easy identification of transfected cells. In addition, the plasmid includes a puromycin resistance gene for selection of target cells.

The miR-302 family is expressed most abundantly in early human embryonic stem (hES) cells and quickly decreases after cells differentiate. Transgenic delivery of miR-302s into human skin, hair follicle and kidney epithelial cells, as well as prostate cancer, breast cancer, liver cancer, and melanoma cells has been shown to orchestrate the reprogramming of somatic cells to induced pluripotent stem (iPS)-like cells (References 1, 2 and 3). The miR-302-reprogrammed iPS cells, namely mirPS cells, not only express many key hES cell markers, such as Oct4, Sox2 and Nanog, but also have a highly demethylated genome similar to hES cells. Microarray analyses revealed that genome-wide gene expression patterns between the mirPS and hES H1 and H9 cells share over 86%–92% similarity. Under molecular guidance in vitro, these mirPS cells can differentiate into many distinct tissue cell types derived from all three germ layers. Therefore, miR-302 can replace Oct4, Sox2 and Nanog for simpler and more efficient generation of iPS cell lines.

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Electroporation Kit

For delivery of miR-302 (only) or miR-302/367 into mammalian cells.

Included in this kit:
miR-302 or miR-302/367 Plasmid
Control Plasmid
Hypoosmolar Buffer
Isoosmolar Buffer

Additional Equipment and Reagents Required:

- Mammalian cells with appropriate culture medium
- Collagenase I / trypsin-EDTA solution (4 : 1) (optional)
- Electroporator
- Sterile cuvettes (1mm gap)
- Cell culture incubator at 37°C under 5% CO_2
- 6-well tissue culture dishes
- Feeder-Free Culture Medium (M-800-011)
- 1 mg/mL puromycin stock

PROTOCOL

Step 1—Cell Preparation:
This electroporation kit can be used for adherent cells, suspension cell, or fresh tissues.
For most cell types use 20,000 to 200,000 cells per reaction.

Adherent cells

1. Collect cells from a 70-90% confluent tissue culture dish by incubating with Trypsin for ~1 minute.
2. Observe cells for detachment, and then deactivate the Trypsin with fresh tissue culture media or Trypsin inhibitor.
3. Spin cells at 180xg for 5 minutes.
4. Discard the supernatant and wash the cell pellet with 1 mL sterile PBS. Count cells and collect approximately 20,000-200,000 cells (times the numbers of reactions). Transfer the cells to a new tube and spin cells at 180xg for 5 minutes.
5. Discard the supernatant and resuspend the cell pellet in hypo- or iso osmolar electroporation buffer—use 200ul per reaction.
6. Incubate cell suspension on ice for 10 minutes prior to electroporation.

Suspension cells

1. Collect cells from a 70-90% confluent tissue culture flask.
2. Spin cells at 180xg for 5 minutes.
3. Discard the supernatant and wash the cell pellet with 1 mL sterile PBS. Count cells and collect approximately 20,000-200,000 cells (times the numbers of reactions). Transfer the cells to a new tube and spin cells at 180xg for 5 minutes.
4. Discard the supernatant and resuspend the cell pellet in hypo- or iso osmolar electroporation buffer—use 200ul per reaction.
5. Incubate cell suspension on ice for 10 minutes prior to electroporation.

Fresh tissues

1. Incubate ~1-2 mm^3 of fresh tissue in 500 uL fresh culture media containing 4 mg/ml collagenase I for 1 to 6 hours at 37°C. Manually dissociate cells.
2. Spin cells at 180xg for 5 minutes.
3. Discard the supernatant and wash the cell pellet with 1 mL sterile PBS. Count cells and collect approximately 20,000-200,000 cells (times the numbers of reactions). Transfer the cells to a new tube and spin cells at 180xg for 5 minutes.
4. Discard the supernatant and resuspend the cell pellet in hypo- or iso osmolar electroporation buffer—use 200ul per reaction.

*Hypoosmolar electroporation buffer is suitable for use with most mammalian cell types. However, if cells are significantly damaged in 100% hypoosmolar electroporation buffer, incubate the cells in a mixture of hypo-/iso-osmolar electroporation (9:1) buffer for 10 minutes. If cells still sustain significant damage, increase the volume ratio of iso-electroporation buffer in the mixture till 90% of the cells remain intact. This final ratio will be the hypo-/iso-*osmolar *electroporation buffer for the cells of interest.*

5. Incubate cell suspension on ice for 10 minutes prior to electroporation.

Step 2—Electroporation:
The following protocol was optimized using the Multiporator from Eppendorf.

1. For each electroporation reaction, prepare a sterile microcentrifuge tube.
2. Add 10 uL plasmid (control, miR-302, or miR-302/367 plasmid) for each reaction.
3. Add 200 uL cell suspension and mix gently.
4. Transfer the cell mixture into a sterile electroporation cuvette (2 mm gap).
5. Place cuvette into electroporator and perform electroporation at 300-600V for 100-150 microseconds.

Electroporation conditions and plasmid delivery may vary depending on the design of your electroporation apparatus and cell type. To test for the optimum setting, first use the control plasmid, which expresses a green fluorescent protein (EGFP). Perform electroporation at voltages starting from 300, 400, 450, 500, to 600 volts for 150 µsec. Try additional voltages depending on cell type. Two to three days after electroporation, check the cells for GFP expression under an inverted fluorescent microscope. The setting with the most abundant green fluorescent cells is the best choice for future electroporation experiments.

6. Allow cells to rest for **30 seconds** before transferring them to one well of a 6-well tissue culture dish, containing cell culture medium.
7. Transfer cells to a tissue culture incubator (set at 37°C with 5% CO_2). For best results, limit the time and frequency that cells are out of the incubator.
8. Two to three days after electroporation, check the cells for GFP expression under an inverted fluorescent microscope

Step 3—Selection of Positive Cells:
The working concentrations of puromycin for mammalian cell lines range from 0 to 10 µg/ml. It is recommended that an initial experiment be done using untransfected cells to determine the optimal concentration of puromycin required.

1. Once GFP expression has been observed, puromycin selection may be used to remove any non-transfected cells. Observe cells; if a large percentage of non-transfected cells remain, increase the puromycin concentration and treat for and additional 24-48 hours or until population is relatively pure.
2. Once iPSC colonies are larger, select colonies (either manually with a pipette or with a micromanipulator) and transfer them to a new tissue culture dish containing Feeder-Free Culture Medium (Mello Bio cat. no. M-800-011). Allow colonies to grow to desired size.

Troubleshooting

Does the miR-302/367 plasmid integrate into the genome?
Integration depends on the delivery methods. Liposomal transfection is transient in nature, and it should not integrate into the genome. Transfection by electroporation should not cause integration. However, under conditions of high voltage or long duration, some integration (<10%) may occur. Lentiviral infection leads to integration.

How can I improve the transfection efficiency?
GFP should be observed within 1~2 days after transfection. Some cells are difficult to transfect. Low transfection efficiency may be the result of multiple factors including:

- DNase contamination
- Inappropriate cell density at the time of electroporation
- Inadequate incubation time and electroporation condition
- Poor cell condition/old cells

If you are experiencing problems with electroporation: try different voltage, pulse frequencies and durations. Multiple transfections (every 3 days) may be necessary for difficult cell lines. Also, some cell lines, such as fibroblasts have higher transfection efficiencies when a square-wave electroporator is used.

What is the morphology of a miR-302-induced pluripotent stem cell?
The iPSCs are spherical cells with loose nuclei and are surrounded by abundant cytoplasm.

Why does electroporation have no effect on my test cells?

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