DNA-In® *Stem* Transfection Reagent

MTI-GlobalStem

Overview

DNA-In® Stem Reagent is a formulation of chemically defined compounds, and is completely free of animal-derived components. **The protocol provided below has been optimized to achieve the highest number of cells transfected in a population (%CT), without toxicity**. Higher expression levels can be obtained later by addition of more DNA if required. It is recommended that the first set of experiments be done using a GFP reporter system to optimize percent cells transfected with DNA-In® Stem Reagent. The amount of plasmid DNA/DNA-In® Stem Reagent complex that is added to cells is a critical factor in determining percent cells transfected, level of expression, and cellular toxicity. This reagent has been optimized for intracellular delivery of DNA into Stem cells in the presence of serum or low protein medium at a cell density of 60% to 80%. High levels of expression can be achieved using the amount of DNA-In® Stem Reagent and DNA amounts recommended in the following protocol. For best results, it is important to empirically determine the optimal amount of DNA and DNA-In® Stem Reagent for any given cell type. **If toxicity is observed, reducing the amount of DNA may reduce toxicity** while still maintaining high levels of expression and % cell transfected.

Storage & Stability:

- DNA-In® Stem Reagent is shipped at room temperature. Store at 4°C. DO NOT FREEZE!

Materials:

- DNA-In® Stem Reagent
- Opti-MEM®I[1] Reduced Serum Medium (*not supplied*)
- Plasmid pMTICAG-GFP (10µg/ml in sterile tissue culture grade PBS without Mg^{2+}/Ca^{2+}) – *supplied only with 0.1 ml SKU*
  Note: The EF1-alpha and CAG promoters are recommended over the CMV promoter for a much stronger reporter for assays.
- Vitronectin (*recommended for ES & iPSC culture, not supplied*) - Cat# A14700, ThermoFisher Scientific
- PluriQ™ G9 Base Medium (*recommended for ES & iPSC culture, not supplied*) - Cat# GSM-9001, MTI-GlobalStem

IMPORTANT NOTES – **Before You Start**

- **Antibiotics** - Do not add antibiotics to medium during transfection as this leads to cell death.
- **Transfection Optimization** - The optimal concentrations of DNA-In® Stem Reagent and DNA should be determined empirically for each cell line (*see section Optimization and Scaling).*
- **DNA Concentrations** - Cytotoxicity is greatly influenced by the amount of DNA present and the optimal amount should be determined. The lowest concentration which provides adequate expression should be used. **If toxicity is observed, reduce the amount of DNA used**. In general lowering the amount DNA does not reduce the % cells transfected.
- ** **Stem Cell Culture Conditions** - Culture conditions have a significant effect on the efficiency of transfection of iPS and hES cells. To obtain the highest % cell transfected with iPS and hES cell it is highly recommended that cell culture conditions that promote formation of a monolayer vs. clumps of cells are followed. Stem cells passaged with **EDTA** prior to culture on Vitronectin (VTN-N) or Geltrex®, in PluriQ™ G9 Medium (GSM-9001) or mTeSR medium can be successfully transfected at high efficiency (>80% cell transfected). **To avoid cell aggregates that form large colonies,** do not let cells over grow, be gentle to split cells to very tiny colonies of only 2-4 cells, and quickly plate micro colonies to promote a monolayer of cells. Plating density should be such that cells are not over grown 24 hours after transfection.
- **See** p.3 for CRISPR/Cas9 Transfection protocol.

I. Quick Start Protocol – 24 well Plate Format

This protocol is written for transfection of cells in a 24-well plating format. It may be adapted to other formats by scaling the volumes up or down to fit the format used (*See Optimization & Scale-Up Protocol on p.2*;

A. Day Before Transfection - Cell Plating Preparation

Approximately 24 hours before transfection, cells should be plated such that the cell density is approximately 50-70% confluent at the time of transfection in complete medium without antibiotics. For a 24-well plate format, cells should be plated in 500µl of medium per well.

B. Day of Transfection – Transfection Reagent Preparation

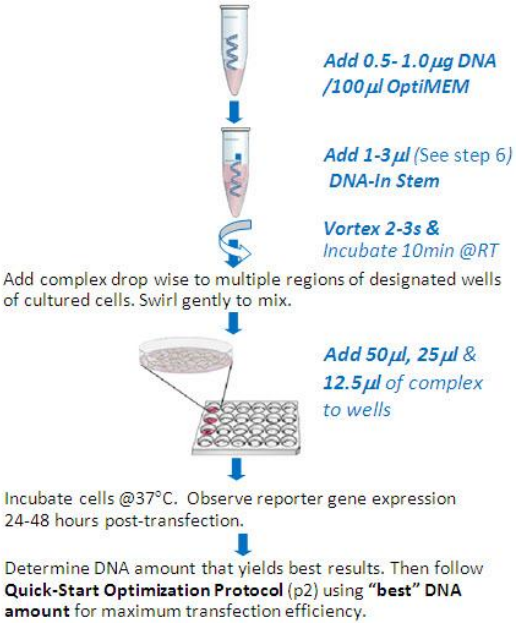
1. Thaw DNA at room temperature.
2. Allow the DNA-In® Stem Transfection Reagent to reach room temperature.
3. Mix the reagent by inversion of the tube several times.

C. Day of Transfection – General Transfection Protocol

4. To a sterile tube add 100µl of provided DNA or add 100µl of Opti-MEM®I medium pre-warmed to room temperature.
5. Add 0.5-1.0µg of plasmid DNA to medium containing tube and mix.
6. Add the following volumes of DNA-In® Stem Reagent:
   - ❖for Neural Stem Cells – 1µl
   - ❖for Meschenchymal Stem Cells – 3µl
   - ❖for iPSC-Derived Stem Cells – 1.5µl
   - ❖for Human Embryonic Stem Cells –3µl

Quick Start Protcol Schematic

*** Use 100µl of supplied DNA for the first experiment*



7. Incubate the complexing mixture at room temperature for 10-15 minutes.
8. To each of 3 cell-containing wells, add 12.5µl, 25µl, and 50µl of the Complex to the 500µl of existing medium.*
9. Mix thoroughly, but gently. Return cells to incubator.
10. Change media 24 hours post-transfection.
11. After an appropriate length of incubation, typically 24-48 hours, measure the transfection efficiency using an assay appropriate for the reporter gene used.

NOTES:

❶ *Neural stem cells and iPS cells typically require lower amounts of DNA-In® Stem Reagent & DNA in the transfection complex. By contrast, mesenchymal stem cells commonly need more DNA for optimal transfection efficiency. Adjust the amount of DNA and DNA-In® Stem Reagent accordingly for the desired expression.*

❷ *Further optimization can be achieved using the amount of DNA that gave the highest % cell transfected with no toxicity.*

****Test all the above volumes of complex on cells. This titration is used to determine the optimal amount of DNA required.*** 50µl of complex is equivalent to 1.0µl DNA-In® Stem and 250ng DNA.

II. Quick-Start *Optimization and Scale-Up Protocol*

Results from the "Quick Start Protocol" will guide this optimization process. If the best result was obtained with the 25µl addition of complex (250ng DNA) this would indicate that optimization experiments should center on 0.25µg DNA per 50µl of Opti-MEM with this particular cell type. Titrate various amounts of the DNA-In® Stem with 0.250µg of DNA. Different DNA constructs may require more or less DNA depending on function. If toxicity was observed with the 12.5µl addition, reduce the amount DNA-In™ Stem. If no toxicity was observed with the 50µl of complex then adding more DNA and DNA-In® Stem can be consider to increase expression or for higher % cells transfected.

Table 1 - Recommended quantities for transfecting DNA in various plate formats.

Culture Plate	Relative Surface Area (cm^2/well)	Volume of Complete Medium	Volume of DNA / DNA-In® Stem Complex	Recommended *Start Volume* of DNA	Amount of DNA for *Optimization*	Volume of DNA-In® Stem
96-well	0.2x	100µl	10µl	0.05µg	0.05-0.2µg	0.0.05 -0.3µl
48-well	0.4x	200µl	20µl	0.1µg	0.1-0.4µg	0.1-0.6µl
24-well	1x	500µl	50µl	0.25µg	0.125-1.0µg	0.5-2µl
6-well	5x	2.5ml	250µl	1.55µg	1-5µg	2.5-10µl

The amount of DNA used in forming transfection complexes determines toxicity for a particular cell type. Optimization involves determining the optimal amount of DNA along with the best reagent to DNA ratio. Generally, as a starting point, we recommend examining at least **four (4) different DNA amounts** over an 8- to 10-fold range **matrixed with DNA-In® Stem Reagent over a 4-fold range**. For example, in a **24-well format**, we suggest setting up complexing reactions with **0.125, 0.25, 0.5** and **1.0µg DNA per 50µl** of serum-free medium. For each DNA amount, **add 0.5, 0.75, 1.0, 1.5,** and **2.0µl of DNA-In® Stem Reagent**. As controls, include 'Reagent alone' and 'DNA alone' added to cell-containing wells.

Do not make DNA/DNA-In® Stem Reagent complexes in volumes smaller than 20µl nor handle individual components in volumes of less than 1µl. DNA-In® Stem Reagent may be diluted in Opti-MEM®I immediately before use, if needed. Diluted reagent is not stable to storage and should be discarded.

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This protocol is written to deliver Cas9 expression vectors or Cas9/sgRNA expression vectors such as PX458 at high efficiency into stem cells using DNA-In Stem Transfection Reagent. Culture conditions have a significant effect on the efficiency of transfection of iPS and hES cells. To obtain the highest % cell transfected with iPS and hES cell it is highly recommended that cell culture conditions that promote formation of a **monolayer** vs. clumps of cells are followed. Cells passaged with EDTA prior to culture on vitronectin (VTN-N) - *recommended* or Geltrex®, in PluriQ™ G9 Medium (GSM-9001) or mTeSR medium can been successfully transfected at high efficiency (>80% cell transfected). **To avoid cell aggregates that form large colonies,** do not let cells overgrow, be gentle to split cells to very tiny colonies of only 2-4 cells, and quickly plate micro-colonies to promote a monolayer of cells. Plating density should be such that cells are not over grown 24 hours after transfection.

A. Day Before Transfection

Approximately 24 hours before transfection, stem cells in complete medium without antibiotics. For a 24-well plate format, cells should be plated in 0.5 ml of medium per well.

B. Day of Transfection – Reagent Preparation:

1. Thaw DNA at room temperature.
2. Allow the **DNA-In® Stem** to reach room temperature.
3. Mix the reagent by vortexing.

C. Day of Transfection – General Transfection Protocol

4. To a sterile tube add 50**µl** Opti-MEM®I medium pre-warmed to room temperature. Add 2**µg** of DNA to be transfected into medium containing tube and mix. ***NOTE:*** *Some stem cells may require* ***1µg*** *of* ***DNA.***
5. Aliquot 12.5**µl** of DNA solution in to 4 different sterile microfuge tubes. Mark tubes A, B, C, and D.
6. Add (**0.5**) **1.0, 1.5, 2.0, and 3.0µl** of **DNA-In® Stem** to Tubes A, B, C and D respectfully. After addition of DNA-In® Stem flick tube gently to mix. ***NOTE****: Some stem cells may require* ***0.5µl*** *of* ***DNA-In® Stem.***
7. Incubate 10 minutes at room temperature.
8. Bring DNA /DNA-In® Stem solution to 50**µl** with OptiMEM. Mix gently.
9. Add 50**µl** of transfection complexes to 0.5 ml of media with cells and swirl to evenly distribute transfection complexes to cells.
10. Incubate cells 16-24 hours and change media.

III. CRISPR/Cas9 *Optimization and Scale-Up Protocol*

Establishing the highest % cells transfected with the least amount of toxicity is the purpose of the above Transfection protocol. If no toxicity was observed and higher expression and % cells transfected is desired then increase the amount of DNA and DNA-In® Stem. If the results from the above show toxicity reduce the amount of DNA. **If 1.0 µl of DNA-In® Stem is the optimal volume of reagent and non-toxic or if 1.0 µl of DNA-In® Stem is toxic, then DNA-In® Stem can be diluted in water and reduced amounts of DNA can be used or both to optimize. Reducing the volume of DNA/ DNA-In® Stem complexes added to the stem cells can also be tried (25µl of complex vs 50µl) if toxicity is encountered.**

Table 1 - Recommended quantities for transfecting plasmid DNA (>6kb) in various plate formats.

Culture Plate	Relative Surface Area (cm^2/well)	Volume of Complete Medium	Volume of DNA: DNA-In® Stem Complex	Recommended *Start amount* of DNA	Amount of DNA for *Optimization*	Volume of DNA-In® Stem
96-well	0.2x	100µl	12.5µl*	0.1µg	0.05-0.2µg	0.1-0.6µl
24-well	1x	500µl	12.5µl	0.5µg	0.125-0.5µg	0.25-3µl
12-well	2x	1.0ml	25µl	1.0µg	0.25-1.0µg	0.5-6µl
6-well	5x	2.5ml	62.5µl	2.5µg	0.625-2.5µg	2.5-15µl

* For 96 well plates complexing should be done with the 12.5µl complexing volume vs. 2.5µl to avoid evaporation. Bring volume to final volume 50µl and add 5ul -10µl to cells depending on stem cell type.

For more detailed instructions, other tips and troubleshooting, please see our website at *www.mti-globalstem.com*

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