DNA-In® *CRISPR*

"Optimization Protocol for CRISPR/CAS9 Plasmids"

Overview

DNA-In® CRISPR Transfection Reagent is a chemically defined compound and 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. DNA-In® CRISPR Transfection Reagent is designed to deliver Cas9 expression vectors or Cas9/sgRNA expression vectors such as PX458 at high efficiency. It is highly recommended that each cell type be optimized for the amount of DNA and DNA-In® CRISPR that gives the highest percent cells transfected. Once the % cells transfected and the total amount of DNA that does not cause toxicity under your plating conditions are established, then a HDR DNA construct can be mixed with the Cas9/sgRNA vector to determine the optimal mixture ratio needed to achieve repair. The amount of plasmid DNA/DNA-In® CRISPR 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 cells in the presence of serum at a cell density of 50% to 70%. High levels of expression can be achieved using the amount of DNA-In® CRISPR and DNA recommended in the following protocol, however high expression of Cas9 can be very toxic to cells.

For best results, it is important to empirically determine the optimal amount of DNA and DNA-In® CRISPR for any given cell line. **If toxicity is observed, reducing the amount of DNA may reduce toxicity** while still maintaining high levels of expression and % cells transfected. **Some Cas9 expression vectors have very low GFP expression and antibody staining for GFP is recommended to evaluate % cells transfected.**

Storage & Stability

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

Materials

- DNA-In® CRISPR Reagent; Transfection qualified DNA; Cells 50 to 70% confluent
- Opti-MEM® I[1] Reduced Serum Medium (*not supplied*)

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® CRISPR and DNA should be determined empirically. (*See section Optimization and Scale-Up*).
- **DNA Concentrations** - Cytotoxicity is greatly influenced by the quality and amount of DNA used in forming transfection for a given 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 **two (2) different DNA amounts matrixed with DNA-In® CRISPR over a 3-4-fold range**. For example, in a **24-well format**, we suggest setting up complexing reactions with **0.25 and 0.5 µg DNA per 12.5 µl** of serum-free medium. For each DNA amount, **add 0.5, 1.0, 1.5, 2.0, and 3.0µL DNA-In® CRISPR. Confluencey of cells will determine how much reagent is needed.**
- As controls, include 'Reagent alone' and 'DNA alone' added to cell-containing wells. Proving that the transfection reagent is not toxic without DNA is critical to the understanding of how these reagents perform. Cas9 over expression can be very toxic to cells.

The lowest amount of reagent and DNA that provides adequate expression should be used. **Some Cas9 vectors have very low GFP expression and antibody staining for GFP or Cas9 is recommended to evaluate the % cells transfected.**

Transfection Protocol

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 table next page).

A. Day Before Transfection - Cell Plating

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 0.5 ml of medium per well.

B. Day of Transfection – Reagent Preparation:

1. Thaw DNA at room temperature.
2. Allow the **DNA-In® CRISPR** 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:** Primary cells and transfection sensitive cell types may require 1**µg** 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® CRISPR** to Tubes A, B, C and D respectfully. After addition of DNA-In® CRISPR, flick tube gently to mix. Some cell types (e.g. primary cells and other transfection sensitive cell types) may require **0.5µl of DNA-In® CRISPR.**
7. Incubate 10 minutes at room temperature.
8. Bring DNA /DNA-In® CRISPR 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.

Optimization and Scale-Up

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 % cell transfected is desired then increase the amount of DNA and DNA-In® CRISPR. If the results from the above show toxicity reduce the amount of DNA. **If 1.0 µl of DNA-In® CRISPR is the optimal volume of reagent and non-toxic or if 1.0 µl of DNA-In® CRISPR is toxic, then DNA-In® CRISPR can be diluted in water and reduced amounts of DNA can be used or both to optimize. Reducing the volume of DNA/ DNA-In® CRISPR complexes added to the cells can also be tried (25µl of complex vs 50µl) if toxicity is encountered.**

Table 1 - Recommended quantities for transfecting CRISPR DNA (>8kb plasmids) in various plate formats.

Culture Plate	Relative Surface Area (cm^2/well)	Volume of Complete Medium	Volume of DNA / DNA-In® CRISPR Complex	Recommended *Start amount* of DNA	Amount of DNA for *Optimization*	Volume of DNA-In® CRISPR
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 cell type.