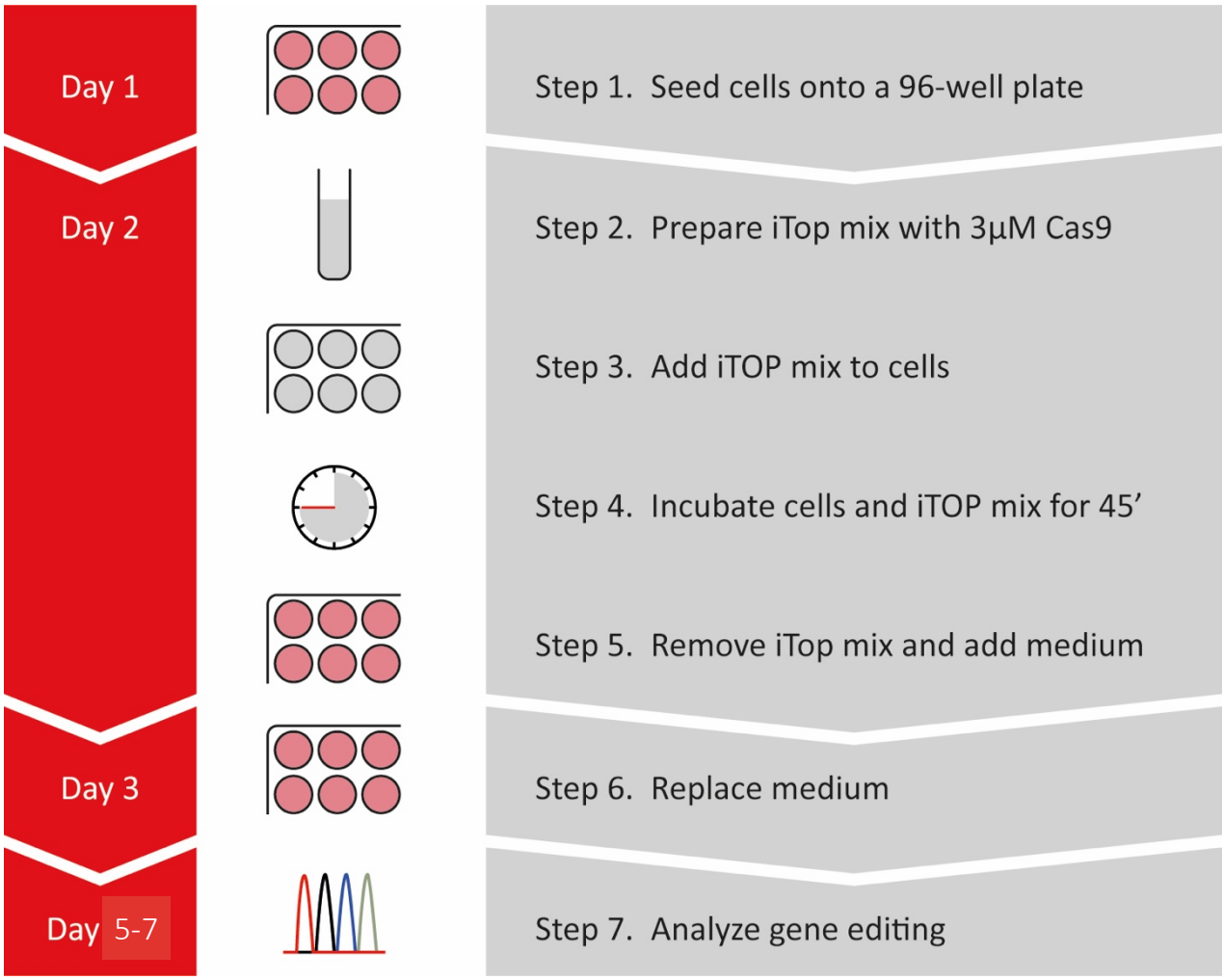
User Guide

iTOP Technology® User Guide

Delivery of CRISPR-Cas9 ribonucleoprotein complexes into adherent cells



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Introduction

iTOP is an intracellular delivery technology based on a combination of small molecule compounds which drive the transfer of extracellularly applied molecules, such as Cas9 and gRNA into the cell. The iTOP Technology ® Reagent A is a proprietary formulation for the introduction of Cas9 nuclease and gRNA into adherent eukaryotic cells. It is non-viral and it does not contain lipid particles. The iTOP Technology ® Reagent A is a high-throughput-friendly, and cost-effective alternative to electroporation with ease of scalability.

The iTOP technology introduces Cas9 and gRNA into adherent cells, resulting in genome-editing efficiencies of ±55-85%, and therefore eliminating the need for extensive selection to obtain edited cells.

Key features are:

- High editing efficiencies
- High throughput — an ideal delivery solution for a 96-well format
- Low cell toxicity — less cells needed to initiate your experiment
- Low costs — whether cost per reaction or initial investment for material/equipment

Unlike CRISPR plasmid or Cas9 mRNA, using Cas9 protein provides superior cleavage efficiency in various cell types. It eliminates the need for transcription or translation, removes the risk of genomic integration, and is cell-cycle independent.

Kit contents & storage

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Reagent	Storage upon arrival	Manufacturer, cat #
iTOP Technology ® Reagent A	-20°C	NTrans Technologies BV, NTT-A-001

Additional reagents required

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Reagent	Suggested Manufacturer, cat #
RNase decontamination solution	Sigma, R2020
70% ethanol	General laboratory supplier
0.25% Trypsin-EDTA	Gibco/Lifetechnologies, 25200072
Dulbecco's phosphate-buffered saline (DPBS)	Sigma, D8537
Matrigel	Corning, 356231
DMEM, high glucose GlutaMAX for HEK-293 & HeLa	Gibco/Lifetechnologies, 31966-021
DMEM/F12 GlutaMAX culture media for ARPE-19	Gibco/Lifetechnologies, 31331-028
Fetal Bovine Serum	General laboratory supplier
Penicillin/Streptomycin	Life technologies, 15140122
Nuclease-free water (not DEPC-treated)	Thermo/Ambion, AM9932
Single or dual gRNA	Synthego, IDT or other supplier
Cas9 protein, 10mg/ml	IDT (1081058), Aldevron (9212) or other supplier

Safety Information

The iTOP Technology ® Reagent A is for Research Use Only and not for *in-vivo* applications. Read MSDS for more information.

Important Guidelines

- Use of low-passage, healthy cells result in highest editing efficiencies.
- Subculture freshly thawed cells for at least 2 weeks before iTOP procedure.
- Cell confluency at the time of iTOP is critical.
- Cells should be 50 – 70% confluent on the day of iTOP.
- iTOP should be performed 24 hours after seeding the cells to achieve optimal results.
- Stability of the iTOP-Cas9/gRNA mix: Use the iTOP mix within 1 hour after its preparation.

Protocol

Day 1: Plating cells for iTOP

Step 1. Seed cells

Only for weakly adherent cells like HEK293: before seeding, coat a 96-well plate with Matrigel

a. Coat the required number of wells of a 96-well plate with Matrigel (1:100 dilution in DPBS) according to manufacturer's instructions.
b. Aspirate the Matrigel solution before plating cells (as in step h.).

Plate HEK293, ARPE-19 or HeLa cells in a 96-well plate.

c. Aspirate medium from a 6-cm dish of cells and wash once with 4 mL of DPBS.
d. Aspirate DPBS and add 0.5 mL of 0.25% Trypsin-EDTA.
   Incubate at 37°C until cells detach from the plate (this takes a few minutes and is cell type dependent). Monitor detachment under the microscope to avoid over-trypsinization.
e. Neutralize trypsin by adding 1 mL of culture medium.
f. Resuspend cells by pipetting ±5 times and transfer to a 5 mL tube.
   Make sure cells are resuspended well and no clumps remain.
g. Count the trypsinized cells.
h. Plate an appropriate number of cells per well, so that they are 50% confluent 24 hours later.

NOTE: It is recommended to make several cell dilutions to ensure ±50% confluency on the day of iTOP to reach optimal results. We recommend making dilutions between 10.000-20.000 cells/well. For an example of optimal confluency on the day of iTOP for HEK293, see figure 2.

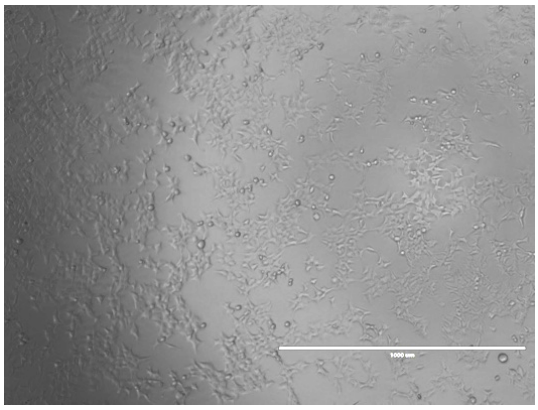

Figure 2: Optimal confluency of HEK293 cells on the day of iTOP (magnification: 4x).

Day 2: iTOP-CRISPR/Cas9 delivery

Step 2. Prepare the iTOP mix.

a. Thaw iTOP reagent A and guide RNA on ice.
b. Thaw the recombinant Cas9 protein according to manufacturer's instructions.
c. Clean all components and materials first with RNase decontamination solution and then with 70% ethanol before placing them in a sterile RNase-free workstation.
d. Prepare guide RNA. A final concentration of 3 µM in the iTOP mix is optimal.

- Single gRNA.
  Resuspend RNA oligos in nuclease-free water and determine their molar concentration. Before use, heat the RNA oligos to 70°C for 10 minutes and cool them quickly by placing them on ice for 5 minutes.
- Dual gRNA.
  Resuspend crRNA and tracrRNA in nuclease-free water, and determine their molar concentration. Make a working solution mix of crRNA and tracrRNA by combining them in the same molar ratio. Before use, anneal the crRNA and tracrRNA by heating them at 95˚C for 5 minutes followed by a cool down step for 15-20 minutes at RT.

Per iTOP (per well of a 96-well plate), prepare the following iTOP mix, shown in the table below.
NOTE: Add the components in the order that is mentioned on the table below. Keep the iTOP mix at room temperature.

iTOP mix	
Nuclease-free water	x µL (until 50µl final volume)
iTOP reagent A	30 µL
Cas9	2.5 µL of a 10mg/ml stock (final conc. 3µM)
gRNA	y µL (final conc. 3µM)
Total	50 µL

e. Calculate the required volumes according to the table.
- Mix iTOP reagent A with x µL nuclease-free water. Nuclease-free water is added to adjust the final volume of the iTOP mix to 50 µL.
- Add Cas9 and gRNA to a final concentration of 3µM. 3 µM Cas9 corresponds with adding 2.5 µL of a 10 mg/ml (62 µM) Cas9 stock to the iTOP mix.

Step 3. Remove culture medium and add iTOP mix to cells

Make sure the components are mixed thoroughly. Aspirate culture media from the cells and add 50 µL of the iTOP mix or the control iTOP mix.
NOTE: Add the mix by pipetting it slowly against the wall of the well (!). HEK293 cells are known to detach easily.

Step 4. Incubate cells and iTOP mix

Incubate at 37˚C for 45 minutes.

Step 5. Remove iTOP mix from cells

- After 45 minutes, *completely* and *slowly* remove the iTOP mix from the cells using a multichannel pipette. Aspirate the leftovers, if present.
- Gently add 200 µL of culture medium by pipetting against the wall of the wells.

Do not wash the cells at this stage!

Day 5-7: Analysis

Step 6. Analysis

The next day, replace the medium. Repeat this on a daily basis until analysis (e.g. FACS, ICE, TIDE).

Technical Support

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Reference

1. D'Astolfo et al. 2015, Cell 161: 674-690

Limited product warranty and licensing information

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