Double Nickase Plasmid Transfection

PROTOCOL

Santa Cruz Biotechnology, Inc.

PHASE 1: Double Nickase Transfection

This protocol is recommended for a single well from a 6-well tissue culture plate. Adjust cell and reagent amounts proportionately for wells or dishes of different sizes.

- In a 6-well tissue culture plate seed 1.5 x 10^5 - 2.5 x 10^5 cells in 3 ml of antibiotic-free standard growth medium per well, 24 hours prior to transfection. Grow cells to a 40–80% confluency. Initial cell seeding and cell confluency after 24 hours are determined based on the rate of cell growth of the cells used for transfection. Healthy and subconfluent cells are required for successful KO and HDR Plasmid transfection.

NOTE: The optimal Plasmid DNA:**UltraCruz® Transfection Reagent** (sc-395739) ratio should be determined experimentally beginning with 1 µg of Plasmid DNA and between 5-15 µl of UltraCruz® Transfection Reagent. Once the Transfection Reagent volume is optimized to minimize cell toxicity, Plasmid DNA concentrations can vary between 1-3 µg per well. If the optimal UltraCruz® Transfection Reagent volume is 10 µl, then Plasmid DNA concentrations ranging from 1-3 µg/10 µl should be tested. For example, test Plasmid DNA/UltraCruz® Transfection Reagent amounts: 1 µg/10 µl, 2 µg/10 µl, and 3 µg/ 10 µl. The appropriate amount of Plasmid DNA/UltraCruz® Transfection Reagent complex used per well should be tested to determine which amount provides the highest level of transfection efficiency.

NOTE: If transfecting more than one plasmid (i.e., CRISPR/Cas9 KO Plasmid with HDR Plasmid), mix Plasmid DNA at equivalent ratios.

- Prepare the following solutions:

  Solution A: For each transfection, dilute 1–3 µg of Plasmid DNA into **Plasmid Transfection Medium** (sc-108062) to bring final volume to 150 µl. Pipette up and down to mix. Let stand for 5 minutes at room temperature.

  Solution B: For each transfection, dilute 5–15 µl of **UltraCruz® Transfection Reagent** (sc-395739) with enough **Plasmid Transfection Medium** (sc-108062) to bring final volume to 150 µl. Pipette up and down to mix. Let stand for 5 minutes at room temperature.

NOTE: Do not add antibiotics to the **Plasmid Transfection Medium** (sc-108062).

- Add the Plasmid DNA solution (Solution A) dropwise directly to the dilute UltraCruz® Transfection Reagent (Solution B) using a pipette. Vortex immediately and incubate for no less than 20 minutes at room temperature.
- Prior to transfection, replace media with fresh antibiotic-free growth medium. Add the 300 µl Plasmid DNA/UltraCruz® Transfection Reagent Complex (Solution A + Solution B) dropwise to well.
- Gently mix by swirling the plate.
- Incubate the cells for 24–72 hours under conditions normally used to culture the cells. No media replacement is necessary during the first 24 hours post-transfection. Add or replace media as needed 24–72 hours post-transfection.

PHASE 2: Selection

- After incubation, screen for GFP-positive cells (using your preferred method) to select for successfully-transfected cells.
- Proceed with standard growth medium containing puromycin (selective medium) to complete screening for successfully transfected cells.

The working puromycin concentration for mammalian cell lines ranges from 1–10 µg/ml. Prior to using the **puromycin antibiotic** (sc-108071), titrate the selection agent to determine the optimal concentration for target cell line. Use the lowest concentration that kills 100% of non-transfected cells in 3–5 days from the start of puromycin selection.

It may be necessary to expand the transfected cells into a larger vessel 24–72 hours post-transfection and prior to puromycin selection. For adherent cells, use standard growth medium to re-plate in a larger vessel. Once cells are adherent, aspirate and replace medium with selective medium containing puromycin at the appropriate concentration.

- Select cells for a minimum of 3–5 days. Approximately every 2–3 days, aspirate and replace with freshly prepared selective medium.
- Cells may be assayed at this point.
- For cells transfected with Double Nickase Plasmid, assay cells 24–72 hours after transfection step, and/or after puromycin selection.

PHASE 3: Cell Assay

Complete phenotypic and/or genotypic analysis may require isolation of single cell colonies to confirm complete allelic knockouts.

- For protein analysis, change media to standard growth medium 3 days prior to cell lysis. To lyse adherent cells, aspirate media, rinse cells with PBS, scrape and centrifuge cells at low speed to obtain a cell pellet. For suspension cells, transfer the culture to a centrifuge tube and centrifuge cells at low speed to obtain a cell pellet. Wash once with PBS and centrifuge again. For 100% confluent HEK 293 or HeLa cells, add 100 µl of **RIPA Lysis Buffer System** (sc-24948) to the pellet. For other cell lines or confluencies, the amount of RIPA Lysis Buffer System to use should be determined experimentally. Sonicate or shear cells. Incubate sample on ice for 10 minutes, vortex, and incubate again for 10 minutes on ice. Spin cell lysate at 10000 RPM for 20 minutes at 4° C. Use the **BCA Protein Assay Kit** (sc-202389) to determine protein concentration.
- For RT-PCR analysis isolate RNA using the method described by P. Chomczynski and N. Sacchi (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156–159) or a commercially available RNA isolation kit.

References: PMID: 24157548, PMID: 23287718

RECOMMENDED SUPPORT PRODUCTS

PRODUCT	CAT. #	DESCRIPTION	AMOUNT
UltraCruz® Transfection Reagent	sc-395739	Delivers CRISPR Activation Plasmid into cells with minimal cell toxicity; enables highly efficient DNA transfection in a variety of cell lines including HeLa, A549, Jurkat and NIH/3T3.	0.2 ml
Plasmid Transfection Medium	sc-108062	Reduced-serum medium suitable for addition to CRISPR Activation Plasmid and Plasmid Transfection Reagent immediately prior to cell transfection; modification of Eagle's Minimal medium, buffered with HEPES and sodium bicarbonate, and supplemented hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, growth factors and phenol red.	20 ml
Control Double Nickase Plasmid	sc-437281	Control Scrambled gRNA Double Nickase Plasmid is a negative control for experiments using a target-specific Double Nickase Plasmid; encodes a non-targeting scrambled gRNA that does not recognize any DNA sequence. The Cas9n/gRNA complex will not bind DNA nor cleave genomic DNA.	20 µg
Puromycin dihydrochloride	sc-108071	Selection and maintenance of cells transfected with the puromycin-N-acetyl-transferase (pac) gene.	25 mg

Double Nickase Plasmid support reagents are optimal for successful delivery of Santa Cruz Biotechnology, Inc.'s Double Nickase Plasmid into mammalian cells. Amounts listed above are based on use of 6-well plates.