Arrayed CRISPR Multi-guide Library Nucleofection Using RNPs in Immortalized Cell Lines

Developed by EditCo

Introduction

EditCo's Arrayed CRISPR Screening Libraries are used for loss-of-function screening applications and are amenable with a wide variety of assays. Each target gene is knocked out using a multi-guide approach, in which up to 3 chemically modified synthetic sgRNAs induce a fragment deletion. EditCo libraries for human and mouse genomes are available. Standard libraries are delivered in a 384-well plate format (with each well containing 150 pmol of multi-guide sgRNA) and custom libraries are delivered in a 96-well plate format (with each well containing 1.5 nmol of multi-guide sgRNA).

This protocol describes how to deliver ribonucleoprotein (RNP) complexes that consist of purified Cas9 nuclease duplexed with chemically modified synthetic single guide RNA (sgRNA) to immortalized adherent or suspension cells. RNP delivery is accomplished using the Lonza 4D Nucleofector™ unit with 16-well Nucleocuvette™ Strips or the 96-well Nucleofector™ Kit. Although optimized for HEK293 (human embryonic kidney 293 cells), this protocol is applicable to other easy to transfect immortalized cell lines (e.g., A549, U2OS, HeLa, CHO, MCF-7).

Chemically modified sgRNAs are designed to resist degradation by exonucleases and prevent innate intracellular immune cascades that can lead to cell death. This protocol is designed for EditCo's multi-guide sgRNA libraries, but may be used to transfect one sgRNA per gene. Optimization using EditCo's human TRAC multi-guide sgRNA or mouse Rosa26 positive control is highly recommended (see Materials Required table).

Abbreviations:

CRISPR: clustered regularly interspaced short palindromic repeats
Cas9: CRISPR associated protein 9
sgRNA: single guide RNA
RNP: ribonucleoprotein
PCR: polymerase chain reaction
ICE: Inference of CRISPR Edits
FACS: fluorescence-activated cell sorting
TE: Tris EDTA
PBS: phosphate-buffered saline
GFP: green fluorescent protein

Material	Ordering Information
Arrayed library (chemically modified multi-guide sgRNA)	EditCo, available here
Cas9 2NLS nuclease (*S. pyogenes*)	EditCo, available as an add-on
Positive control sgRNA: Human • Transfection Optimization Kit (Multi-guide): Positive Control Multi-guide sgRNA (mod), Human TRAC (1.5 nmol), PCR & sequencing primers, nuclease-free water, nuclease-free Tris- EDTA buffer, & Cas9 2NLS (*S. pyogenes*, 300 pmols, 20 µM, 162 ug/nmol) or • Positive Control Multi-guide sgRNA (mod), Human TRAC Mouse • Positive Control, Mouse Rosa26, mod-sgRNA (1 nmol) (one sgRNA sequence (not multi-guide))	EditCo, available as an add-on
PCR & sequencing primers (See Table on page 5)	Multiple vendors
Nuclease-free 1X TE buffer	Multiple vendors (e.g., Thermo Fisher Scientific, Catalog #AM9849)
Nuclease-free water	Multiple vendors (e.g, Thermo Fisher Scientific)
Thermal microplate sealer	Agilent, PlatLoc
Plate seals	Peelable aluminum, Agilent #24210-001
Transfection control (optional)	Recommended: pMAXGFPTM (Lonza)
Normal growth medium	Multiple vendors (cell-type dependent)
96-well PCR plates	Multiple vendors (e.g., Thermo Fisher Scientific)
96-well cell culture plate	Multiple vendors (e.g, Thermo Fisher Scientific)
TrypLE Express or preferred dissociation reagent	Multiple vendors (e.g, Thermo Fisher Scientific)
1X PBS, cell culture grade	Multiple vendors (e.g., Thermo Fisher Scientific)
Cell counter	Multiple vendors (e.g., Thermo Fisher Scientific)
Microcentrifuge tubes	Multiple vendors (e.g., Eppendorf)
4D-Nucleofector™ System with X Unit	Lonza
4D-Nucleofector™ X Kit S (32 RCT) or 96-well Nucleofector™ Kit (specific for cell type)	Lonza

Note: All protocols outlined have been validated using materials mentioned in this manual. Materials other than the ones outlined in our manual may require additional optimization by the user.

General Guidelines

- Wearing gloves and using nuclease-free tubes and reagents are recommended in order to avoid RNase contamination.

Always maintain sterile technique, and use sterile consumables and filter pipette tips.
Synthetic sgRNA should be dissolved in sterile TE buffer and diluted to a working concentration using nuclease-free water. Please see the Arrayed CRISPR Screening Libraries Quick Start Guide for instructions on how to properly rehydrate your library.

- Use cells that have been passaged at least once after being thawed from a cryofreeze. Use cells at the lowest passage number possible.
- The GFP transfection efficiency (Transfection control in the Suggested Controls table below) should be assessed using fluorescence microscopy 48-72 hours post-transfection. This step can be skipped after establishing the percentage of GFP positive cells. This optimization should be done once for each cell type.
- All EditCo and Nucleofector™ reagents should be stored according to the manufacturer's recommendations.
- RNPs can be formed directly in the Nucleofector™ solution.
- RNP complexes are stable at room temperature for up to 1 hour and may be stored at 4°C for up to one week.

  Note: RNPs stored at 4°C may become susceptible to contamination from microbial growth after long periods of time.
- This protocol is optimized for either Lonza 16-well Nucleocuvette™ Strips or 96-well Nucleocuvette™. The wells in the Lonza 16-well Nucleocuvette™ are identical to the 96-well Nucleocuvette™ and optimization conditions are interchangeable between the two formats.

Visit EditCo.com/resources for analysis and clonal expansion protocols.

Optimization Guidelines

It is critical to optimize transfection conditions using your particular cell type in order to maximize editing efficiency and increase the likelihood of successful knockouts. It is also important to validate and optimize your assay to ensure your readout is robust and reliable. Please see the recommendations below.

Editing Efficiency

This protocol is meant to provide a starting point for your CRISPR editing experiments and has been optimized for nucleofection of 1 x 10^5 HEK293 cells using a 3:1 sgRNA to Cas9 ratio, but it is important to test a range of ratios in the cell type you plan to use for screening. You can also optimize several other conditions for each cell type and plate format you plan to use for your screening:

- Ratio of sgRNA:Cas9
- Total pmols of RNP per reaction
- The number of cells per reaction
- Nucleofection program*
- Type of Nucleofection Solution

  * For specific nucleofection settings for your cell type, we suggest consulting the Lonza Nucleofector™ cell and transfectiondatabase, available online at: knowledge.lonza.com.

For EditCo's human libraries, we recommend our positive control multi-guide sgRNA, human TRAC. This positive control results in robust and consistently high editing efficiencies across a range of cell types. For EditCo's mouse libraries we recommend using mouse *Rosa26*. See the Positive Control Information table (page 5) for more information.

Functional/ Phenotypic Assay

A functional assay is typically conducted with arrayed CRISPR screens to correlate the effect of each gene perturbation on an aspect of cellular function, morphology, or physiology. It is crucial to optimize your chosen assay in order to ensure that it robustly identifies phenotype(s) of interest. This can be done using assay-specific positive and negative controls (see the Suggested Controls table on page 5). In addition, it is important to optimize the time between CRISPR editing and the assay to ensure a clear phenotypic signal can be measured. The assay-specific positive control can be used for this purpose.

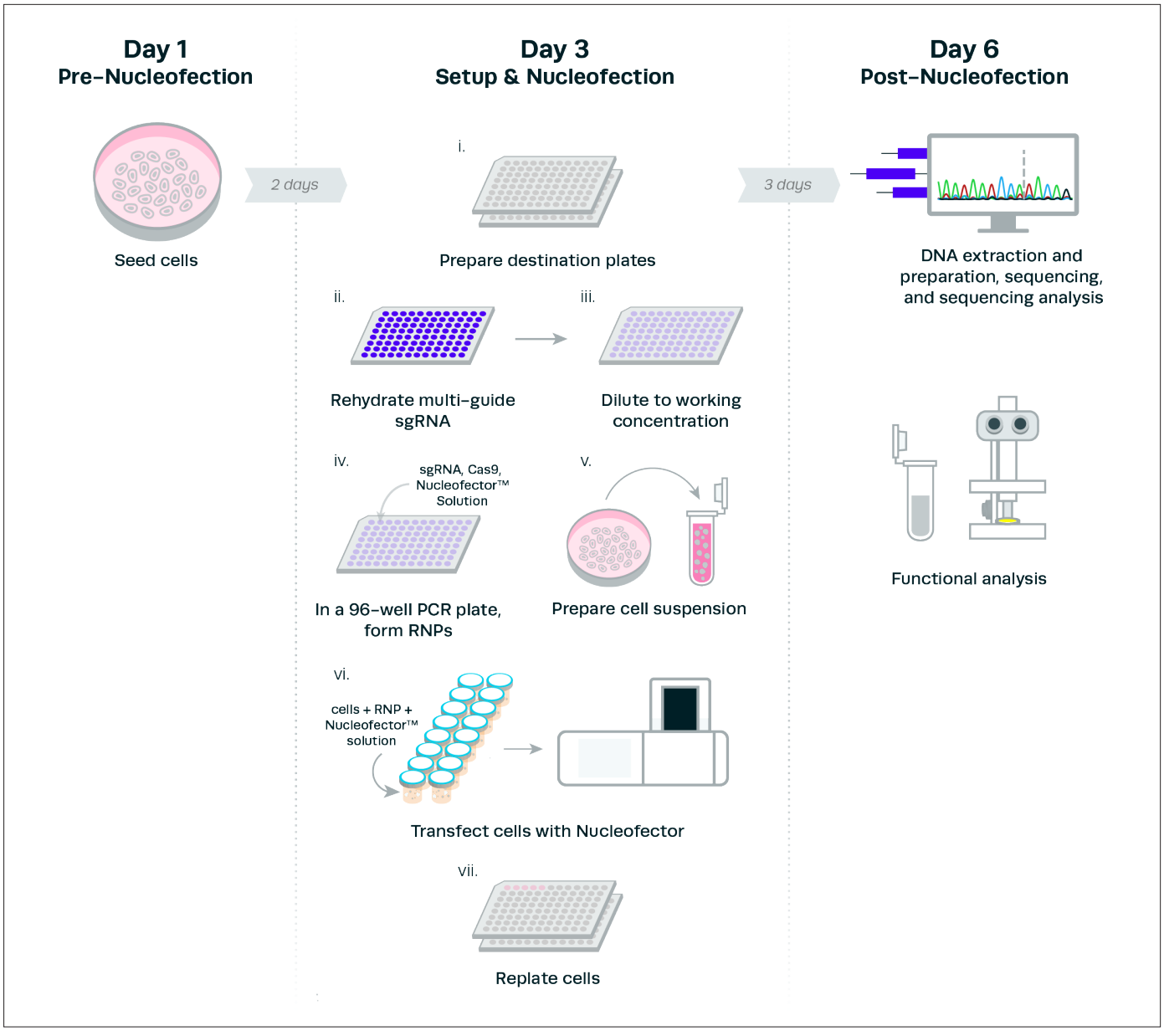
Suggested Controls

Control	Description	Purpose
Editing positive control	Validated sgRNA that has high gene editing efficiency (e.g., TRAC multi-guide sgRNA for human libraries)	Used to identify optimal transfection conditions for maximum genotypic editing in the cell type of interest. Ensures experimental procedure is working.
Editing negative control	Transfection with no sgRNA and no Cas9	Establishes a baseline phenotype (used as a wild type comparison) and accounts for background cellular response and cell viability following Lipofectamine treatment.
User's assay-specific positive control	Subset of genes known to induce a phenotype of interest for the user's desired assay when knocked out	Correlates genotypic editing efficiency with the phenotype of interest.
User's assay-specific negative control	Subset of genes known not to induce a phenotype when knocked out (e.g., AAVS1)	Correlates genotypic editing efficiency with the absence of phenotype of interest.

Editing Positive Control Information

Genome	Positive control sgRNA & Primers	Sequence
Human	Multi-guide positive control sgRNA targeting human TRAC	sgRNA 1: 5'-CUCUCAGCUGGUACACGGCA-3' sgRNA 2: 5'-GAGAAUCAAAAUCGGUGAAU-3' sgRNA 3: 5'-ACAAAACUGUGCUAGACAUG-3'
Human	TRAC PCR forward primer	5'- TCAGGTTTCCTTGAGTGGCAGG - 3'
Human	TRAC PCR reverse primer	5'- TAAGGCCGAGACCACCAATCAG - 3'
Human	TRAC Sanger Sequencing primer	5'-CTGGCCGTGAACGTTCACTGAAATCATGGC-3'
Mouse	Single guide sgRNA targeting mouse *Rosa26*	5'-ACTCCAGTCTTTCTAGAAGA-3'
Mouse	*Rosa26* PCR forward primer	5'-GAGGCGGATCACAAGCAATA-3'
Mouse	*Rosa26* PCR reverse primer	5'-GGGAGGGGAGTGTTGCAATA-3'
Mouse	*Rosa26* Sanger Sequencing primer (use forward primer)	5'-GAGGCGGATCACAAGCAATA-3'

Timeline


Day 1
Pre-Nucleofection
Seed cells
2 days
Day 3
Setup & Nucleofection
i.
Prepare destination plates
ii.
Rehydrate multi-guide sgRNA
iii.
Dilute to working concentration
iv.
sgRNA, Cas9, Nucleofector™ Solution
In a 96-well PCR plate, form RNPs
v.
Prepare cell suspension
vi.
cells + RNP + Nucleofector™ solution
Transfect cells with Nucleofector
vii.
Replate cells
3 days
Day 6
Post-Nucleofection
DNA extraction and preparation, sequencing, and sequencing analysis
Functional analysis

Protocol

Before You Begin

Optimize Nucleofection Conditions for desired cell line

This protocol describes transfecting cells with the Lonza 4D Nucleofector™ system. Prior to editing cells the optimal Lonza buffer and electroporation pulse code must be determined. We recommend testing 8–192 electroporation conditions per cell line with RNPs composed of 180 pmoles positive control sgRNA and 20 pmoles Cas9. The number of conditions testing varies based on how difficult the cell line is to transfect and available recommendations from Lonza. We recommend reducing the sgRNA:Cas9 ratio and scaling down the amount of RNP concentrations only after the optimal conditions are determined

Suggested Nucleofection Optimization Controls

Control	Description	Purpose
Mock	No Cas9, no sgRNA	Wild type sequence for comparison with experimental and other negative controls. Controls for toxicity from RNP, cell death from electroporation, or possible viability issues associated with editing the specific gene of interest
RNP negative control	Cas9 complexed with a non-targeting sgRNA or no sgRNA	Ensures that there are no false positives due to contamination (no effect expected= wild type).
RNP positive control	sgRNA that has validated high editing efficiency	Ensures that all reagents, protocol, and equipment are functioning (effect expected). Used to optimize transfection conditions for a particular cell type.
Transfection	pMAXGFPTM vector	Assess transfection efficiency (without the use of RNPs).

Note: Positive and transfection controls must be ordered separately.

1. Pre-Nucleofection

1.1. Seed Cells

a. Subculture cells 2-3 days before nucleofection and seed them in an appropriately sized vessel so that they are 70-80% confluent on the day of transfection. Each nucleofection reaction will require 1 x 10^5 cells.

Note: As a general rule, it is recommended to use cells at the lowest passage number possible.

2. Setup & Nucleofection

2.1. Prepare Destination Plates

a. Dispense 100 µL of pre-warmed normal growth medium in each well of two 96-well cell culture plates. After nucleofection, the cells will be split into two wells on the duplicate plates. The cells on the first plate will be lysed and processed to analyze editing efficiency. The cells on the second plate will be cultured for use in assays, banking, and/or single-cell cloning. Consider whether you plan to culture control cells on thesecond plate.

2.2. Rehydrate Multi-guide sgRNA to Storage Concentration

Note: EditCo's multi-guide sgRNA libraries are available in 96-well and 384-well plates and ship dry at ambient temperature. Rehydrated sgRNA at concentrations >20 µM should be stored at -20°C. Under these conditions, sgRNA will be stable for up to 3 years if not repeatedly thawed. For concentrations <20 µM, we recommend storage at -80°C. All plates are heat-sealed before shipment.

a. Centrifuge the plate for 5-10 minutes at 500-1000 x g to ensure that RNA collects at the bottom of each well.
b. Remove the seal by firmly holding the plate in place on a table and then gently pulling the seal off starting at one corner.
c. Add nuclease-free 1X TE buffer to desired wells according to the plate format and sgRNA volume of your library (see Table 1 for 384-well and 96-well plate formats).

Table 1: sgRNA storage concentrations for 384-well and 96-well plate formats

Plate Format	sgRNA/well (pmols)	Nuclease-free 1X TE buffer (µl)	Storage Concentration (µM)
384-well	150	30	5
96-well	1500	15	100
	3000	30	100

d. Re-seal plates with a thermal-based heat sealer. EditCo recommends sealing plates using the Agilent PlateLoc Thermal Microplate Sealer at 160°C for 2.8 seconds.
e. Let sit overnight at 4°C to fully rehydrate dried sgRNA.
f. When ready to use the sgRNA, let the plate warm to room temperature for 15 minutes to prevent condensation on the seal.
g. Vortex sealed plate at low speed for 30 seconds to mix.
h. Centrifuge plate or 5-10 minutes at 500-1000 x g.
i. Remove the seal by firmly holding the plate in place on a table and then gently pulling the seal off starting at one corner to prevent cross-contamination.

For further details on resuspension and storage refer to our Screening Libraries Quick Start Guide.

Note: If not being used immediately, dissolved sgRNA should be stored at -20 °C. Under these conditions, the sgRNA is stable for 3 years if not repeatedly thawed.

2.3. Dilute sgRNA to working stock concentration

If multi-guide sgRNA storage concentration is 100 µM, dilute multi-guide to working stock (5 µM) in a 96-well PCR plate.

a. In a 96-well PCR plate, create a 5 µM working solution for each sgRNA by adding 1µL of the rehydrated sgRNA (100µM) to 19 µL 1x nuclease-free TE buffer.
b. Vortex to ensure that the sgRNA is fully mixed.

2.4. Assemble Ribonucleoprotein (RNP) Complexes (3:1 sgRNA to Cas9 ratio)

a. Ensure Cas9 2NLS is at a concentration of 20 µM (20 pmol/µl; 3.22 mg/ml).

Note: Cas9 2NLS from EditCo is at a concentration of 20 µM and does not require further dilution.

b. Make sure that the entire supplement is added to the Nucleofector™ Solution. The ratio of Nucleofector™ Solution to supplement is 4.5:1.
c. Label and add the reagents to a sterile and nuclease-free PCR plate or similar, in the order shown in the w/table on page 9.

Note: The table on page 9 also includes reagents for positive, negative, and transfection controls. The sgRNA: Cas9 ratio in the table is 3:1, but you may need to experimentally determine the optimal sgRNA:Cas9 ratio for your cell type or experiment. EditCo recommends sgRNA:Cas9 ratios between 3:1 and 9:1. The RNPs should be formed directly in Nucleofector™ Solution (at room temperature).

Reagents	Controls				Experimental
	Transfection (GFP)	Mock	Negative (Cas9 only)	Positive control sgRNA	Target-specific sgRNA
Nucleofector™ Solution + Supplement	19 µl	20 µl	19.75 µl	16.75 µl	16.75 µl
pmaxGFP vector (1 µg/µl)	1 µl	-	-	-	-
sgRNA (5 µM)	-	-	-	3 µl	3 µl
Cas9 (20 µM)	-	-	0.25 µl	0.25 µl	0.25 µl
Total volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

d. Incubate RNPs for 10 minutes at room temperature. Keep at room temperature for up to 1 hour for use, store at 4°C for up to one week. If immediately transfecting cells, a 5 µl cell suspension (prepared in section 2.4 below) will be added to the 20 µl of pre-complexed RNPs for a total transfection volume of 25 µl per reaction.

2.5. Prepare Cells

Note: For suspension cells, re-suspend in growth medium and mix well. Skip steps a and b below and proceed to step c.

a. Aspirate cell culture media and wash cells 1-2 times with appropriate volume of 1X PBS.
b. Add appropriate amounts of TrypLE Express, or preferred dissociation reagent, and incubate the cells at 37°C with 5% CO_2 for 5 minutes, or until they detach from the plate completely. Do not shake or hit the flask to dislodge cells, as this may lead to clumping and inaccuracies in cell counting and inefficient ransfection.
c. Neutralize the dissociation reaction with at least 2 volumes of normal growth medium and pipette gently to form single cell suspension.
d. Count the cells to determine the cell density.
e. Prepare a cell suspension

Example: For a cell suspension for 18 transfections (16 reactions + 2 extra), each with 1 x 10^5 cells, centrifuge 1.8 x10^6 cells at 200 x g for 10 minutes, aspirate medium and resuspend the cell pellet carefully in 90 µl of Nucleofector™ Solution (2.0 x 10^4 cells/µl).

2.6. Prepare Cell/RNP Solution

a. For each reaction, add 5 µl of cell suspension to 20 µl of pre-complexed RNP for a total transfection volume of 25 µl.

b. Transfer all 25 µl of cell-RNP solution to Nucleocuvette™ strips and click the lid into place.

Note: The total transfection volume (with cell suspension) to be transferred to the Nucleocuvette™ strips can be adjusted to 20 µl to match Lonza's recommendations. Optimize the total volume of RNP complexes by keeping the concentrations of guide RNA and Cas9 proportional to that recommended in the Table above.

c. Gently tap the Nucleocuvette™ Vessels on the benchtop to make sure the sample covers the bottom of the cuvette and ensure that there are no bubbles in the cuvette.

Note: While pipetting, the cell suspension needs frequent/gentle agitation to prevent the cells from settling. Work quickly, but carefully, and avoid leaving cells in Nucleofector™ Solution for longer than 15 minutes. Avoid bubble formation.

2.7. Transfect Cells

a. Program the Nucleofector with appropriate program code determined during optimization (example: CM-130 for HEK293).

b. Place the Nucleocuvette™ Vessel with the closed lid into the retainer of the 4D-X Core unit. Check for proper orientation of the Nucleocuvette™ Vessel. Larger cutout is the top (A1 and A2) and the smaller cutout is the bottom (H1 and H2).

c. Press “Start” on the display of the core unit.

d. After run completion, the screen should display a green “+” over the wells that were successfully transfected. Remove the cuvette strips from the Core unit.

Note: Some cell types require a 10-minute incubation at room temperature after nucleofection. Please consult the optimized Lonza protocol to see if this is a necessary step for your cell line.

2.8. Add Recovery Medium

a. Carefully resuspend the cells in each well of the Nucleocuvette™ with 75 µl of pre-warmed growth medium, and mix gently by pipetting up and down (~3 times) to ensure an even distribution of cells in suspension.

2.9. Plate Cells

a. Retrieve the two 96-well cell culture plates prepared in step 2.1.

b. Of the 100 µl cell suspension (for each nucleofection reaction), transfer 50 µl to the first pre-warmed 96-well plate and pipette mix 5x to evenly plate the cells. This plate is for genomic analysis. Transfer the other 50 µl to the second pre-warmed plate and pipette mix 5x for assays/clonal expansion.

c. Incubate the cells in a humidified 37°C with 5% CO_2 incubator.

d. Replace medium after 24 hours.

Note: Maintain the second plate by replacing medium and splitting as necessary until clonal expansion, assays, or banking.

3. Post-Nucleofection

3.1. Genomic Analysis

a. Design PCR primers compatible with Inference of CRISPR Edits (ICE) analysis.
b. 72 hours post nucleofection, isolate DNA, PCR target region, and Sanger-sequence amplicons following the guidelines provided in our Genotyping protocol.
c. Conduct Inference of CRISPR Edits (ICE) analysis on Sanger sequences to determine editing efficiency.

3.2. Functional Analysis

a. Perform the desired assay.

Please visit EditCo.com/resources for protocols on clonal expansion and protein assessment. Nextgeneration sequencing, FACS, Western blot, or functional assays may also be conducted.

Troubleshooting Guide

Problem	Possible Cause(s)	Recommended Solutions
No cells on plate	Loss of cells during pelleting/removing supernatant before Nucleofection	Use caution when aspirating supernatant.
	Cells left in Nucleofection cuvette	After Nucleofection, add medium to the cuvette and pipette up and down multiple times to ensure cells are not left in the bottom of the cuvette.
Uneven distribution of cells between reactions	Non-uniform cell suspension	Ensure that the cell suspension is mixed thoroughly before adding to pre-complexed RNPs, and continue to gently agitate the suspension to avoid settling.
	Cells left in Nucleofection cuvette	After Nucleofection, add medium to the cuvette and pipette up and down multiple times to ensure cells are not left in the bottom of the cuvette and that the concentration of cells is uniform.
	Cells not mixed enough after Nucleofection	
Low viability	Cell culture conditions were suboptimal	Cells should be viable and in culture for several passages. Avoid excessive cell densities or high cell confluencies as this may decrease cell viability post-nucleofection.
	Cells were damaged by harvesting procedure or through handling	Avoid harsh conditions during cell harvesting, especially centrifugation at high speed or overexposure to trypsin. Pipette cells smoothly.
	Cells were in Nucleofector Solution too long	Transfer cells immediately into pre-warmed medium as recommended in the optimized protocol. Avoid leaving cells in Nucleofector Solution for longer than 15 minutes.
Low transfection efficiency	Cell number	The optimal number of cells per transfection reaction varies between cell types. Optimize cell number using the GFP plasmid provided in the 4D Nucleofector Kit.
	sgRNA:Cas9 ratio	Optimize ratio of sgRNA:Cas9 using a positive control sgRNA in your cell type.
	Wrong Nucleofector Solution/program for cell type	Use the recommended Lonza Nucleofector solution and program for your cell type. If Lonza does not have an optimized protocol and established solution for your cell type, it is recommended to do a transfection optimization using GFP plasmid prior to editing.
Low editing efficiency	Genotyped too early	We recommend waiting 2-3 days before genomic DNA extraction for complete CRISPR/Cas9 editing.
	Low transfection efficiency	See above
	Primers designed sub-optimally for ICE	Primer design being too close to the cut site for optimal ICE analysis. If this is the case, ICE will return low model fit (R2) value. Please refer to our ICE Knockout Analysis Protocol.
	Sanger sequencing quality	Quality of the Sanger Sequenced .ab1 files was too low, if this is the case, ICE will return low model fit (R2) value. Please refer to our ICE Knockout Analysis Protocol.

Representative Data

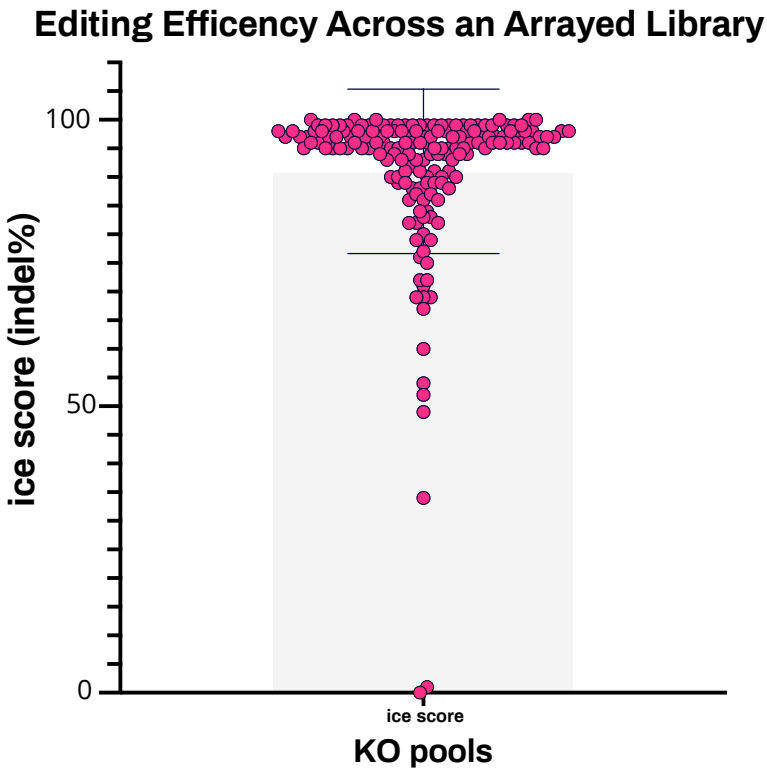


Figure 1. High knockout efficiencies across a range of targets using an arrayed multi-guide library in an immortalized cell line. Knockout efficiencies of 170 gene targets in A549 cells. Cells were transfected with RNPs (15 pmoles : 5 pmoles multi-guide sgRNA to Cas9) via nucleofection. Three days post-transfection, genomic DNA was extracted, PCR-amplified at the target site, and Sanger sequenced. Editing efficiencies were analyzed using EditCo's Inference of CRISPR Edits (ICE) tool.

Additional Information

For an up-to-date list of all protocols and other resources, please visit this link.

For technical assistance, contact our Scientific Support Team at technicalsupport@editco.bio.

For common FAQs, please visit this link.