Gene Knockout Kit

Thank you for choosing Gene Knockout Kit for your cell-based human or mouse knockout experiment! This kit includes multi-guide sgRNA that has been strategically designed to knock out your human or mouse protein-coding gene of interest. The sgRNAs jointly cause a fragment deletion at the targeted genomic locus, consequently disrupting gene function.

Before using this kit, we highly recommended optimizing transfection conditions in your particular cell type using a positive control sgRNA. For human genes, we suggest our human TRAC multi-guide sgRNA or our Transfection Optimization Kit. For mouse genes, we recommend our single guide *Rosa26* sgRNA (see Additional Materials Required). Proper optimization will ensure that you achieve a robust knockout with your target-specific sgRNA supplied in this kit.

Materials Provided

Quantity	Name	Description	Storage
1.5 nmol 3 nmol 5 nmol 10 nmol	Target specific multi-guide sgRNA	1-3 chemically modified sgRNA(s) in 1 tube (2ml); molecular weight of an average 100-nt sgRNA is 32 µg/nmol Human (Fisher Scientific P/N # AB1389) Mouse (Thomas Scientific P/N # 1149J78)	–20°C for up to 3 years if not repeatedly thawed.
1.5 ml	Nuclease-free Tris-EDTA Buffer (1X TE buffer)	10 mM Tris, 1 mM EDTA (pH 8.0) Thomas Scientific P/N # 1149J78	Room temperature
1.5 ml	Nuclease-free water	Thomas Scientific P/N # 1149J78	Room temperature

Note: The 2ml tubes for Human guides will have clear caps (AB1389) whereas green capped 2 ml tubes will be sent out for Water, Buffer, and Mouse guides (1149J78).

Additional Materials Required

Material	Description	Ordering Information
SpCas9 2NLS nuclease	Wild type SpCas9 from *S. pyogenes* (20 µM, 162 µg/nmol)	EditCo, available at checkout as an add-on
Positive control sgRNA: Human Transfection Optimization Kit (Multi-guide) or Positive Control, Human *TRAC*, multi-guide mod-sgRNA Mouse Positive Control, Mouse *Rosa26*, mod-sgRNA	• Chemically modified multi-guide sgRNA targeting human *TRAC* (1.5 nmol) • PCR & sequencing primers • Nuclease-free water • Nuclease-free Tris-EDTA buffer • SpCas9 2NLS (*S. pyogenes*, 300 pmols, 20 µM, 162 µg/nmol) Chemically modified multi-guide sgRNA targeting human *TRAC* (1.5 nmol) Chemically modified sgRNA targeting mouse *Rosa26* (1 nmol), 1 guide sequence (*not* multi-guide)	EditCo, available at checkout as an add-on

Rosa26* positive control is one sgRNA sequence (*not*** multi-guide)

Note: Additional materials are listed in EditCo's transfection protocols (nucleofection and lipofection) and in the ICE Knockout Analysis protocol.

Positive Control Primer Information

Genome	Primer	Sequence
Human	*TRAC* PCR primer	Forward: 5'- TCAGGTTTCCTTGAGTGGCAGG - 3' Reverse: 5'- TAAGGCCGAGACCACCAATCAG - 3'
Human	*TRAC* Sequencing primer	5'-CTGGCCGTGAACGTTCACTGAAATCATGGC-3'
Mouse	*Rosa26* PCR primer*	Forward: 5'-GAGGCGGATCACAAGCAATA-3' Reverse: 5'-GGGAGGGGAGTGTTGCAATA-3'

Note: Human *TRAC* primers (PCR & sequencing) are included in the Transfection Optimization Kit. Otherwise, all primers must be purchased separately. The Forward or Reverse *Rosa26* PCR primers can be used for sequencing.

Workflow Schematic

6 DAYS

Optimization of Transfection

Transfection Using Positive Control species specific gRNA

PCR & Sanger Sequencing

ICE Analysis

6 DAYS

Knockout of Experiment

Transfection Using Target Multi-guide gRNA

PCR & Sanger Sequencing

ICE Analysis

2-8 WEEKS

Clonal Isolation of Knockout (optional)

Limiting Dilution

Clonal Expansion

Step 1: Rehydrate the Multi-guide sgRNA and Quantify

EditCo's multi-guide sgRNA ships dry at ambient temperature and remains stable for up to two weeks at <30°C. However, we recommend that you **store sgRNA at –20°C immediately** upon arrival. Please store dried sgRNAs at –20°C for long-term storage (up to 3 years, if not repeatedly thawed).

Be sure to work in an RNase-free and in sterile environment and use sterile, filter pipette tips throughout the entire protocol.

1. Briefly centrifuge the tube containing dried multi-guide sgRNA to ensure the pellet is collected at the bottom.

2. For all cell lines and primary cells: carefully rehydrate multi-guide sgRNA (up to 3 sgRNAs/tube) in nuclease-free buffer (1X TE buffer*; provided) and pulse vortex for 30 seconds to ensure complete mixing. The following table states the recommended amount of nuclease-free 1X TE buffer for four different starting quantities of multi-guide sgRNA. The final concentration of the sgRNA will be 100 µM (100 pmol/µl).

Multi-guide sgRNA (nmols)	Nuclease-free 1X TE Buffer* (µl)	Storage Concentration (µM)
1.5	15	100
3	30	100
5	50	100
10	100	100

*TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Note: For microinjection, it is critical to ***only*** hydrate and dilute sgRNA in a nuclease-free 1X microinjection buffer (e.g., 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0; not provided).

3. Pulse vortex for 30 seconds to ensure that the sgRNA is fully resuspended. If you notice any remaining pellet or precipitant, then gently mix with a pipette or vortex ***until the sgRNA is completely dissolved***. If the sgRNA is not fully reconstituted, you can let the tube sit up to 72 hours at 4°C to fully rehydrate dried sgRNA before use.

4. Rehydrated sgRNA should be stored at –20°C. Under these conditions, sgRNA will be stable for up to 3 years. We recommend storing sgRNA at a concentration of 100 µM rather than in more dilute concentrations.

Quantification of the sgRNA

Each guide in this kit is present in an equimolar concentration. The quantity of material present (printed on the tube) is measured by UV absorbance spectroscopy at a wavelength of 260 nm prior to dehydration. Upon rehydration and prior to experimental use, it is best practice to verify RNA concentration using a sensitive UV absorbance spectroscopy instrument. We recommend using a non-absorbing liquid such as water for UV measurements. For 100 µM samples, please make a 200X dilution, and add 1 µl sampling volume + 199 µl water. Please note that lower sampling volumes will increase potential error in the measurement.

Calculate the concentration of sgRNA using the Beer-Lambert Equation below:

$C = A/(\varepsilon * p) * \text{Dilution Factor}$

Where:
- C = Concentration (M)
- ε = Extinction coefficient (M^{-1} cm^{-1}) (found on the QC report)
- p = pathlength of light (cm) (specific to the instrument)
- A = Blank Adjusted Absorbance
- Dilution Factor is the factor by which the sample was diluted

Convert the UV obtained concentration to yield (mole) if needed, by multiplying the concentration and the resuspension volume. Small variations between the printed value and what you measure are normal.

If the guide concentration is higher than 100 µM after quantification, please correct the resuspension volume so that the final concentration is equal to or less than 100 µM and re-quantify.

Example

5 µl of resuspended sgRNA from a 100 µl volume well was taken out and mixed with 195 µl of water in a UV plate. With a 40X dilution factor, we recorded an unadjusted absorbance at 260 nm of 0.348 and a blank diluent well absorbance of 0.051. The machine's pathlength is 0.52 cm. The sgRNAs have an average extinction coefficient, ε = 1010436.667 M^{-1} cm^{-1}, per the QC document, to determine the total concentration from absorbance.

$C = A/(\varepsilon * p) * \text{Dilution Factor}$

$C = (0.348 - 0.051) /(1010436.667\ M^{-1}\ cm^{-1} * 0.52\ cm) * 40$

$C = 0.000022610\ M$

C= 22.610 µM

Converting to Yield (if needed)

Yield = C * Volume of well

Yield = (22.610 µM *100 µl)/1000 = **2.261 nmoles of total sgRNA** in the well prior to sampling

You may instead multiply by the expected volume in the well after sampling to determine the updated yield. In this case, it would be Yield = (22.610 µM *95 µl)/1000 = **2.147 nmoles**

Step 2: Dilute the Multi-guide sgRNA

1. Depending on the application, the multi-guide sgRNA may be used directly at the rehydration concentration (100 µM) in 1X TE buffer or diluted to a working stock using nuclease-free water in a sterile microcentrifuge tube. EditCo's RNP nucleofection protocol requires a concentration of 30 µM multi-guide sgRNA (see Example Dilution below). For EditCo's lipofection protocol, a working concentration of 3 µM is needed whereas the Neon electroporation protocol requires 30 µM working concentration.

   Example Dilution: Add 6 µl of 100 µM multi-guide sgRNA to 14 µl of nuclease-free water to make a total volume of 20 µl of 30 µM multi-guide sgRNA (30 pmol/µl).

2. Use diluted sgRNA immediately or store at –20°C for up to 3 years (if not repeatedly thawed).

Step 3: Optimize Conditions & Transfect Cells

We highly recommend that you optimize transfection conditions using positive controls in your particular cell type prior to using your target-specific multi-guide sgRNA. For optimizing conditions for human cell lines, EditCo offers a Transfection Optimization Kit which contains our positive control multi-guide sgRNA targeting human *TRAC*. For mouse cell lines, we recommend using our positive control single guide sgRNA targeting mouse *Rosa26* to optimize your conditions (see Additional Materials Required on pg 2). We recommend forming ribonucleoprotein (RNP) complexes for your genome editing experiments to maximize editing efficiency and reduce off-target effects.

Choose either EditCo's electroporation, lipofection, or nucleofection protocols to be used with this kit. All protocols are available at editco.com/resources.

Step 4: Analyze Knockout Efficiency

Following the purchase of our Gene Knockout Kit, EditCo provides the guide sequences and suggested PCR and sequencing primers to genotype the region in the genome the kit targets. Using these primers to assess how your CRISPR experiments worked, we recommend you to follow our Genotyping protocol. The protocol walks through how to design primers, extract DNA, and perform PCR. Once you have your PCR product, you can send the sample for sanger sequencing.

EditCo's Inference of CRISPR Edits (ICE) is a free online tool that provides an easy quantitative assessment of genome editing using Sanger sequencing data. The software compares the sequence traces of amplicons generated from genomic DNA isolated from both the experimental (CRISPR edited) and control (unedited) pools of cells.

For instructions on how to analyze the editing efficiency using EditCo's Inference of CRISPR Edits (ICE) tool, please see EditCo's ICE Knockout Analysis protocol for knockout CRISPR assessments. To generate knockout clones, see our Limiting Dilution and Clonal Expansion for iPS Cells or Limiting Dilution and Clonal Expansion for Immortalized Cells depending on the cell line you are using.

Representative Data

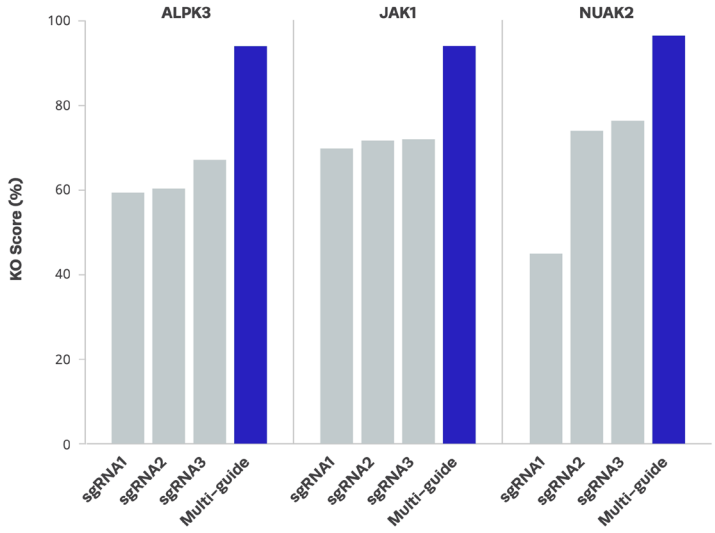

Figure 1. Multi-guide sgRNA achieves high knockout efficiencies.

Three single guide RNAs were designed for each of three human genes (*ALPK3, JAK1, NUAK2*) and introduced individually (sgRNA1, sgRNA2, sgRNA3) and together (multi-guide). On average, the multi-guide sgRNA performed 50.8%, 32.1%, and 48.2% better than individual guides for *ALPK3, JAK1*, and *NUAK2*, respectively. HEK293 cells (for *ALPK3*) and MCF7 cells (for *JAK1* and *NUAK2*) were transfected with ribonucleoproteins (RNPs) via nucleofection. The region around each target was PCR-amplified, Sanger-sequenced, and analyzed using Inference of CRISPR Edits (ICE) analysis. Knockout (KO) Score refers to the percentage of sequences that result in a putative knockout (frameshift-inducing indels and 21+ bp fragment deletions).

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.