Genotyping

Primer Design, DNA Extraction, and PCR Amplification

Developed by EditCo

Introduction

After you have performed a CRISPR experiment, you can evaluate the genotype of the edited cells with either Next Generation Sequencing (NGS) or Sanger sequencing.

For single-guide knockouts and knock-in CRISPR edits, if you want to analyze genotypes using NGS, we recommend using CRISPResso. NGS primer sequences are provided in the QC report for your project or you can reach out to technicalsupport@editco.bio and they will provide you with NGS primer sequences.

Alternatively, you can analyze single-guide, multi-guide, and knock-in CRISPR edits using EditCo's Inference of CRISPR Edits (ICE) tool, which relies on Sanger sequencing. Notably, EditCo's ICE tool is currently the only publicly available option for analyzing multi-guide derived CRISPR edits. Inference of CRISPR Edits (ICE) tool is a free online software that analyzes Sanger sequencing data to determine editing efficiency. Prior to using ICE, edited and control DNA samples must be prepared for Sanger sequencing. This protocol provides instructions for how to design primers, isolate genomic DNA, and perform PCR. The instructions can be used for knockout experiments that utilize one sgRNA (or cr:tracr) per target or multiple sgRNAs per target (i.e., EditCo's multi-guide design). This protocol can also be used for knock-in experiments. If you need the Sanger sequencing primers you can reach out to technicalsupport@editco.bio. Please note that the Sanger primer recommendations are computed using standard bioinformatic algorithms. They are not validated functionally by EditCo.

Positive Controls: EditCo recommends using a positive control sgRNA to optimize your transfection conditions for your cell type. Positive control individual sgRNAs (targeting human RELA and CDC42BPB) are available in EditCo's Controls Kit and positive control multi-guide sgRNA (targeting human TRAC) is available in EditCo's Transfection Optimization Kit (Multi-guide). The latter kit should only be used for knockout experiments. All controls can also be purchased individually along with SpCas9 here.

Postive Control	Species	sgRNA Format	PCR Primers	Recommended to Optimize	EditCo Product
RELA	Human	Individual-guide	Provided, also included below	Knockout or Knock-in	Controls Kit, Individual control
CDC42BPB	Human	Individual-guide	Provided, also included below	Knockout or Knock-in	Controls Kit, Individual control
TRAC	Human	Multi-guide	Provided, also included below	Knockout	Transfection Optimization Kit, Individual control
Rosa26	Mouse	Individual-guide	Provided, also included below	Knockout or Knock-in	Individual control

Note: CRISPR-Cas9 often does not affect mRNA expression, thus qRT-PCR or other techniques that analyze mRNA transcript levels cannot determine if CRISPR-Cas9 editing has occurred.

Materials Required

Material	Ordering Information
Target-specific PCR primers (forward & reverse)	Multiple vendors
Target-specific sequencing primer*	Multiple vendors
Positive control PCR primers (forward & reverse)	Recommended: Controls Kit (Indvidual-guide) or Transfection Optimization Kit (Multi-guide), EditCo, available at checkout Mouse *Rosa26*: Multiple vendors, Not supplied with purchase
Positive control sequencing primer	Recommended: Controls Kit (indvidual guide) or Transfection Optimization Kit (Multi-guide), EditCo, available at checkout Mouse *Rosa26*: Multiple vendors, Not supplied with purchase
QuickExtract™ DNA Extraction Solution	Epicentre, Catalog #QE09050
AmpliTaq Gold™ 360 Master Mix	Thermo Fisher, Catalog # 4398881
PCR-grade water	Multiple vendors (e.g., Millipore Sigma, Catalog # 3315959001)
Thermal cycler	Multiple vendors
PCR tubes	Multiple vendors
PCR Purification Kit (optional)	Multiple vendors

* If both forward or reverse PCR primers are more than 150 bp from the cut site, we recommend ordering an additional sequencing primer.

Protocol

1. Setup Prior to PCR

The table below has primer information for EditCo's products. EditCo will recommend primer sequences that meet stringent design criteria. However, recommended primer pairs may not be suitable for every cell line/type. To design your own primers, please refer to Step 1.1 below.

EditCo Product	Primer Information
Gene Knockout Kit	A single amplicon (400-800 bp in length) can be used to assess the editing efficiency of all experimental sgRNAs provided in the Gene Knockout Kit. Primer sequences are provided with the order.
Arrayed gRNA Libraries	A single amplicon (400-800 bp in length) can be used to assess the editing efficiency of all experimental sgRNAs provided in the Gene Knockout Kit. Primer sequences are provided with the order.
Engineered Cells	Primer sequences are provided with the order.

1.1. Design Primers

You will need to design two sets of PCR primers (forward & reverse) and a sequencing primer. Design PCR primers for your target using the recommended guidelines below to ensure optimal amplification and subsequent detection. It is recommended to design at least two primer pairs. A primer design software tool (e.g., Primer3) may assist in your design. Order primers from your preferred DNA oligonucleotide provider.

A. PCR primers

1. Select ~ 500 bp of genomic DNA on each side of the guide sequence. For multi-guide samples, select ~ 500 bp of genomic DNA on each side the furthest upstream and downstream guides.
2. Design the forward and reverse primers at least 150 bp from the guide RNA cut-site to allow for optimal sequencing quality across the edit. If using multi-guide, design the forward and reverse primers at least 150 bp from the outermost guide RNA cut-sites.
3. For efficient amplification of PCR product amenable to ICE analysis after sequencing, design the primers to yield amplicon lengths between 400-800 bp (optimal length is ~500 bp).
4. For best results, use primers with a melting temperature (Tm) of ~60°C (range: 55-65°C)
5. Design primers that are 18–22 bp in length and have 45–60% GC content.
6. Tools such as Primer-Blast can be used to check for off-target amplification.

B. Sequencing primers

Design nested DNA primers for sequencing using a design software tool (e.g., Primer3). Make sure to enter the amplicon sequence as a template and that you select "sequencing" as task/application/use.

C. EditCo's positive control primers

EditCo includes positive control primers in two kits used for transfection optimization. The Controls Kit contains primers for human RELA and human CDC42BPB. The Transfection Optimization Kit contains primers for human TRAC (see table below). The controls can also be purchased individually, in this instance only primer sequences are provided.

Postive Control	Sequence
Human *RELA* PCR primers	Forward: 5'-TTCTAGGGAGCAGGTCCTGACT-3' Reverse: 5'-TCCTTTCCTACAAGCTCGTGGG-3'
Human *RELA* sequencing primer	5'-AGTACAGAGGCCCAGACATCCAA-3'
Human *CDC42BPB* primers	Forward: 5'-GCGCCCTGACGGACTGGCCGA-3' Reverse: 5'-GGAGGGCAAGGAGGGATGAAAA-3'
Human *CDC42BPB* sequencing primer	5'-GGAGGGCAAGGAGGGATGAAAA-3'
Human *TRAC* (multi-guide) PCR primers	Forward: 5'- TCAGGTTTCCTTGAGTGGCAGG - 3' Reverse: 5'- TAAGGCCGAGACCACCAATCAG - 3'
Human *TRAC* (multi-guide) sequencing primer	5'-CTGGCCGTGAACGTTCACTGAAATCATGGC-3'
Mouse *Rosa26* PCR primers	Forward: 5'- GAGGCGGATCACAAGCAATA - 3' Reverse: 5'- GGGAGGGGAGTGTTGCAATA - 3'
Mouse *Rosa26* sequencing primer	Either PCR primer

Note: The PCR band size for human *RELA* is 595 bp, human *CDC42BPB* is 477 bp, human *TRAC* is 667 bp and mouse *Rosa26* is 355bp.

1.2. Optimize PCR Conditions

The PCR instructions in this protocol (Step 3) have been optimized to generate a single amplicon of the correct size when using EditCo's positive controls (e.g., human RELA, TRAC, or mouse Rosa26) forward and reverse genotyping primer mix (10 µM) with AmpliTaq Gold™ 360 Master Mix.

It is highly recommended to optimize the annealing temperature and amount of genomic DNA using PCR primers for your target until a single band of the correct size is obtained for the wild type sample. Generally, optimal annealing temperature (Ta) is between 1 and 5°C below the lowest Tm value of the primer pair, and the optimal amount of genomic DNA is between 10 and 100 ng. We recommend optimizing PCR conditions using genomic DNA extracted by the same extraction buffer that will be used during analysis. For regions with high GC, the addition of a GC enhancer or 3% DMSO may improve ability to PCR amplify the desired fragment.

1.3. Prepare Cells

Ensure cells are healthy post-transfection. Allow cells to grow to ~80% confluency before isolating DNA. At least 100,000 cells will be needed for genomic DNA extraction (Step 2). If analyzing a EditCo Knockout Cell Pool, thaw cells according to the Immortalized, iPS or Primary T Cells Quick Start Guides.

2. Extract Genomic DNA

1. Aspirate medium from vessel containing cells for genomic DNA extraction.
2. Add 50 µl of QuickExtract™ DNA Extraction Solution to cells.
3. Tilt plate to pool lysate. Pipette up and down or scrape each well with a clean pipette, and transfer all 50 µl of lysate to a PCR tube.
4. Place tubes in a thermal cycler and run the following program:

Temperature	Time
68°C	15 minutes
95°C	10 minutes
4°C	HOLD

5. After genomic DNA extraction protocol, proceed immediately to Genomic PCR-Amplification (step 3) or store lysates at -20°C for up to 1 week (-80°C for longer periods). Do not allow lysates to stay at 4°C for more than 2 hours.

3. PCR Amplification

It is necessary to PCR-amplify the genomic region targeted by your gRNAs prior to Sanger sequencing. It is recommended that at least two primer pairs be designed in case one of them fails (Step 1.1) and that the annealing temperature and amount of genomic DNA be optimized until a single PCR product of the correct size is observed for the wild type sample (Step 1.2).

Note: The PCR instructions below have been optimized to generate a single amplicon of the correct sizes when using EditCo's positive control forward and reverse primers with AmpliTaq Gold 360 2X Master Mix.

3.1. Set up & Run PCR

The determination of editing efficiency using ICE requires a comparison of a wild type amplicon (wt) and the respective amplicon generated from the edited population of cells (edited). The wild type amplicons for the positive control and the target gene should be generated from the "Positive control: unedited" and "Target: unedited" conditions, respectively. It is good practice to include a PCR negative control (without template DNA) to ensure there is no DNA contamination in your reagents.

1. Prepare primers to their appropriate working dilutions (10 µM forward,10 µM reverse).
2. Briefly vortex extracted genomic DNA.
3. The table below can be used as a guide to prepare each PCR condition using PCR-grade water, 2X AmpliTaq Gold 360 Master Mix, positive control primers or target gene primers, and the appropriate genomic DNA.

Condition	PCR negative control	Positive control: unedited	Positive control: edited	Target: unedited	Target: edited
PCR primers	Target fwd/rev	Positive control fwd/rev	Positive control fwd/rev	Target fwd/rev	Target fwd/rev
Genomic DNA	None	Unedited positive control gene	Edited positive control gene	Unedited target gene	Edited target gene

4. The table below can be used as a guide to prepare each PCR condition using PCR-grade water, 2X AmpliTaq Gold 360 Master Mix, positive control primers or target gene primers, and the appropriate genomic DNA.

Reagent	Volume per reaction (µl)	Final Concentration
2X AmpliTaq Gold 360 master mix	25	1x
Forward primer (10 µM)	0.5	0.1 µM
Reverse primer (10 µM)	0.5	0.1 µM
Genomic DNA	2	-
UltraPure water	22 (24 for PCR neg control)	-
Total volume	50 µl	-

* If using a different Taq Polymerase adjust reaction volumes and PCR thermocycling conditions according to manufacturer.

5. Run the PCR in a thermocycler with the following program:

Stage	Temperature	Time	Cycles
Enzyme activation	95°C	10 minutes	1X
Denature	95°C	30 seconds	40X
Anneal	55°C *	30 seconds	
Extend	72°C	1 minute/kb	
Final extension	72°C	7 minutes	1X
Hold	4°C	HOLD	1X

* The annealing temperature may need to be optimized based on the target gene primers used.

3.2. Verify Amplicons & Prepare for Sanger Sequencing

1. Following amplification, run the PCR product on a 1% agarose gel to verify the amplification generates a single band of the correct size. If a single band of the correct size is not present in all unedited samples, re-optimize the PCR conditions including primers, annealing temperature, and amount of genomic DNA until you obtain a clean amplicon of the expected size.

2. PCR-purify amplicons prior to sequencing following the recommendations of the sequencing service provider (purification is offered by some sequencing companies).

3. Submit samples for Sanger sequencing:
   i. For transfection optimization: positive control (unedited) & positive control (edited)
   ii. For target gene knockout or knock-in: target (unedited) & target (edited)

4. Use EditCo's free Inference of CRISPR Edits (ICE) online tool to analyze editing efficiency. Please see EditCo's ICE Knockout Analysis and ICE Knock-in Analysis protocols.

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.