CRISPR

Synthetic single guide RNA (sgRNA)

Application Guide

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Package Contents and Storage Conditions

Package Contents

3 nmol purified and sequence verified lyophilized sgRNA

1.5ml Nuclease-free 1X TE buffer (Tris-EDTA, pH8.0)

1.5ml Nuclease –free water

Storage Conditions

sgRNA oligos are dried down prior to shipping. They are stable in this format for several weeks at room temperature, but it is best practice to store any undissolved RNA oligos at -20 °C. Dissolved RNA should be stored at -20 °C. Under these conditions, RNA will be stable for at least one year.

Related products

Cas9 protein: http://origene.com/Protein/TP790148/CAS9.aspx

Positive or Negative sgRNA control

sgRNA overview

Guide RNAs can be generated through several techniques: produced as part of a plasmid or vector via cloning; through a process known as *in vitro* transcription (IVT) or chemically synthesized as RNA oligonucleotides. Each of these gRNA formats present the researcher with certain benefits and constraints. For example, producing plasmid-based gRNAs is time-consuming and is impractical when generating a large number of guides. In addition, the use of plasmid gRNAs results in constant expression in the cell, leading to a higher level of off-target effects and the possibility that plasmid DNA can be incorporated into the host genome. Similarly, IVT-generated gRNAs are also time-consuming to produce and are not easily scalable beyond a few guides. Since the IVT process involves many steps, and relies on enzymes to transcribe RNA from DNA, the purity of the resulting gRNAs can be highly variable and their quality relies on the skill of

the researcher. This variability can lead to inconsistencies in gene editing efficiency between replicates and unwanted off-target effects. In addition, IVT-derived gRNAs cannot be chemically modified - a significant disadvantage as cell types that are challenging to edit, such as stem cells, have been shown to require chemically-modified gRNAs for effective CRISPR editing (Hendel et al., 2015). Using synthetic guide RNA (sgRNA) can easily solve this problem and greatly simplify and expedite your experimental workflow.

Chemically synthesized sgRNAs provide the benefits of having a high level of purity and a low level of variability between batches. The purity and consistency of synthetic gRNA enables a high-level of reproducibility between experimental CRISPR replicates. Furthermore synthetic gRNAs can be chemically modified, which is critical when editing particular cell types, such as stem cells (Hendel et al., 2015), or certain genomic targets that prove otherwise challenging to edit. OriGene offers 2'-O-methyl analogs and 3' phosphorothioate internucleotide linkages at the first three 5' and 3' terminal RNA residues. These modifications provide protection against exonuclease activity and immune responses.

Experimental Workflow

The first step in a CRISPR experiment is to identify your CRISPR target sequences and design guide RNAs. It is difficult to fully predict how well a guide RNA will work. We recommend that you try 3 or 4 different guides to find out which sequences have the best editing efficiencies.

When designing for a complete knockout of a gene, it is best practice to choose targets within early coding regions of a common exon. When generating a knockout, it is preferred to introduce an indel as close to the 5′ end of the coding region as possible. This will have the highest likelihood of creating a nonsense mutation or loss-of-function frameshift. Regardless of application, guide RNAs with minimal off-target effects are preferred.

OriGene offers a CRISPR Design Tool for knockouts optimized for use with *Streptococcus pyogenes* Cas9. To generate guides, select your genome and gene of interest, and we'll provide recommended guides. You can use the following OriGene Design Tool to design gRNA for your knock out project:

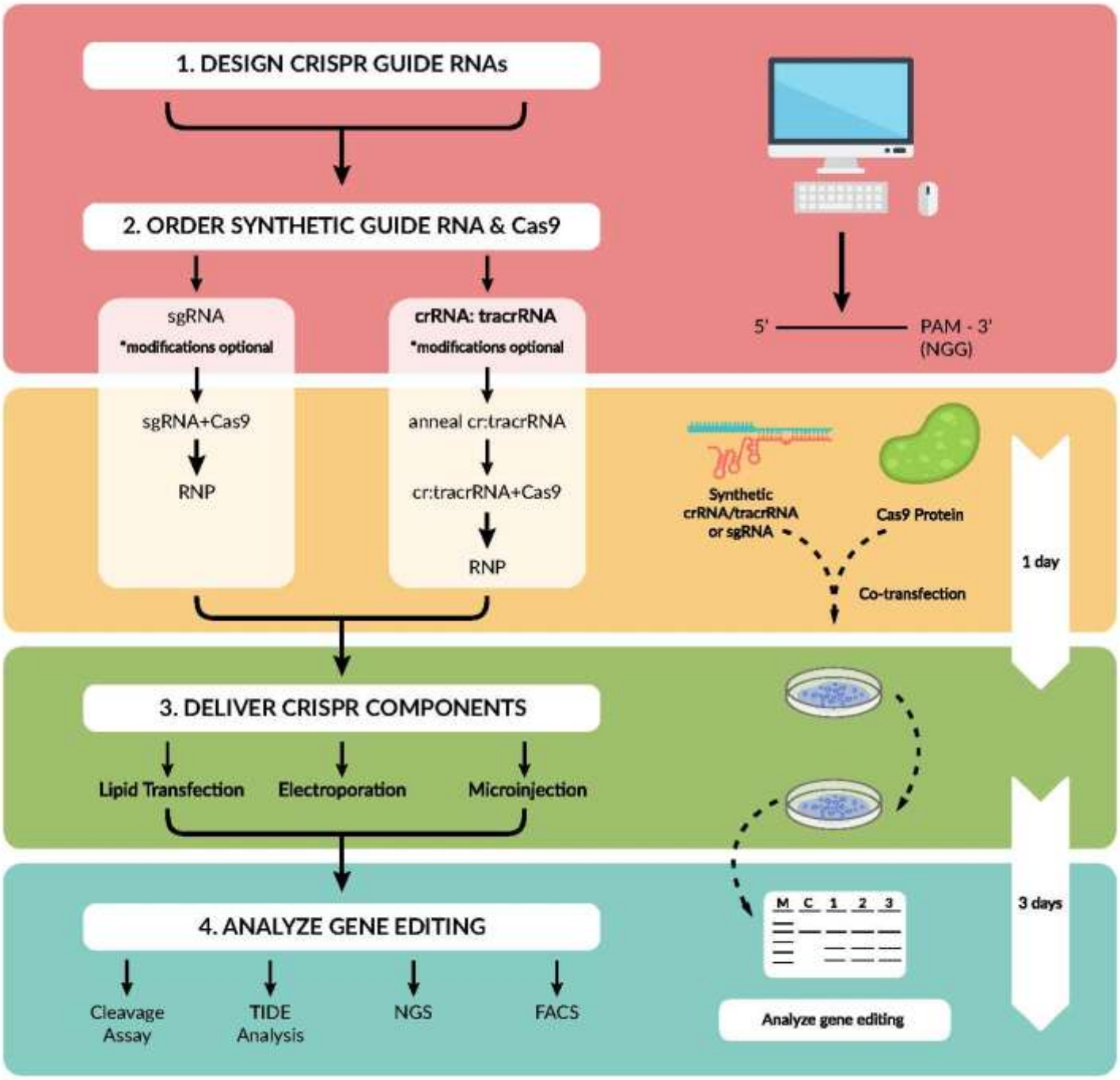
http://origene.com/CRISPR-CAS9/sgRNA/OriGene_Design.aspx.

After designing your gRNA, you can order the sgRNA with the custom SKU# and Cas9 protein (SKU# TP790148) from OriGene. These two components can form a ribonucleoprotein (RNP) complex and get introduced into the target cells by different techniques. The most commonly used techniques include: lipid transfection, electroporation and microinjection. After transforming cells with CRISPR components using the appropriate method, they can be left to grow or divide.

The final step of the workflow is to analyze the CRISPR editing efficiency. We recommend the use of a number of commonly used techniques, including: cleavage assay, TIDE analysis, NGS, Site-Seq and FACS. A description of these techniques and specific recommendations are discussed in the following section.

Please refer to Figure 1 for our recommended workflow on the next page.

Figure 1: sgRNA CRISPR experimental workflow.



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Product Application

Dissolve sgRNA

Be sure to work in an RNase-free environment.

Before you start, we recommend that users dissolve sgRNA to concentrations based upon the labeled amount. The value printed on the tube represents the quantity of material present as measured by OD260.

1. Briefly centrifuge your tubes or plates containing single guide RNA (sgRNA) oligos to ensure that the dried RNA pellet is collected at the bottom.

2. Carefully dissolve RNA in the provided nuclease-free 1X TE Buffer (Tris-EDTA, pH8.0) to an appropriate stock concentration.

Recommended protocol: For sgRNA (3nmol), add 20µl of nuclease-free 1X TE buffer for a final concentration of 150µM (150pmol/µl).

3. Dissolved sgRNA should be stored at -20 °C. Under these conditions, sgRNA will be stable for at least one year.

Dilute sgRNA

1. To make a working stock, add 10µl of 150µM sgRNA oligo to 40µl of the provided nuclease-free water to make a total of 50µl of 30µM (30pmol/µl) sgRNA. This will be your working stock.

2. Use diluted sgRNA immediately or store at -20 °C for up to three months.

Choice of Transfection Method

The most appropriate delivery technique for CRISPR components depends upon the cell type you are using. There are three primary methods for delivering CRISPR components into cells: lipid-based transfection, electroporation and microinjection.

Please see Table 1 for a list of recommended delivery methods for commonly used cell types. Detailed protocols for several delivery methods can be found in the following sections.

Table 1: Delivery formats of CRISPR components to the cell.

[1] e.g., Lipofectamine etc.
[2] e.g., Thermo Neon, Lonza Nucleofection etc.

Cell Type	Lipid Transfection[1]	Electroporation[2]	microinjection	Chemical Modifications Recommended?
HEK 293/T	X			May Provide Benefit
U2	X			May Provide Benefit
CHO-K1	X			May Provide Benefit
Stem Cells (iPSC, hPSC)		X		Yes
HeLa		X		May Provide Benefit
K562		X		Yes
A549		X		Yes
Jurkat		X		May Provide Benefit
Prokaryotes		X		Yes
Yeast		X		Yes
Plant (e.g. protoplasts)		X		May Provide Benefit
Embryo (Mouse, Zebrafish, *C. elegans*, etc.)			X	May Provide Benefit

For commonly used adherent cell lines, such as HEK293/T, U20S and CHO, lipid-based transfection reagents, such as Lipofectamine™, are most optimal for delivery of CRISPR components. Generally speaking, the use of optimized lipid nanoparticles for

transfection is very cost effective and gentler on cells than electroporation. Additionally, lipid-based transfection uses liquid reagents, making it more amenable to transformation scalability. We recommend the use of the Thermo Fisher CRISPRMAX™ kit, which has been optimized for delivery of RNP-format CRISPR components into cells (Yu et al., 2016).

While lipid-based transfections can be used for a wide-variety of cell types, they can perform poorly or be completely ineffective for challenging to transform cell lines. In particular, suspension cells such as K562, Jurkat, plant and primary cells, including T-cells and stem cells, may not transform well using lipid-based transfection. In addition, prokaryotic and yeast cells cannot be transformed this way. For these cell types, electroporation is recommended. Several electroporation systems exist, and each has its benefits. For example, the Lonza Nucleofector™ system utilizes a combination of cell-type specific solutions and electroporation cuvettes to achieve maximum transformation rates. In addition, Lonza provides cell-type specific transformation protocols for over 600 cell types (including primary cells and stem cells), and the system can be scaled to various reaction volumes - utilizing 100µl cuvettes, 20µl electroporation strips or 96-well plates. Another popular electroporation system is the Thermo Neon™ system, which utilizes a 10µl volume electroporation "tip", which generates a more uniform electric field, and allows samples to be directly, and sterilely, transferred directly into tissue culture vessels, such as 96-well plates. For electroporation, we recommend the use of either the Lonza Nucleofector™ system or the Thermo Neon™ system - and leave it up to the CRISPR researcher to decide which system may function best for their particular cell type.

For delivery of CRISPR components directly into embryonic-stage organisms, microinjection is the preferred method. This includes the use of CRISPR to generate transgenic mice, zebrafish or *C. elegans*. In this format, CRISPR components should be delivered using the RNP format in an appropriate microinjection buffer, and injected

directly into the embryonic cell. Sometimes, a dye can be used to visualize the microinjection mixture.

Given these recommendations, it may still be the case that experimental optimization will need to be performed in order to determine the best transformation method for CRISPR components into your cell type. With more and more types of cells being edited using CRISPR every day, this is to be expected.

Lipofection of Cas9/sgRNA (RNP) complexes for CRISPR/Cas9 genome editing (Thermo CRISPRMAX™ Kit)

Background

This protocol describes how to transfect cultured cells with ribonucleoprotein (RNP) complexes that consist of purified Cas9 nuclease duplexed with synthetic sgRNA using the Lipofectamine™ CRISPRMAX™ Transfection kit.

Materials Required

- sgRNA & Cas9 nuclease (SKU# TP790148)
- Lipofectamine™ CRISPRMAX™ Transfection Reagent with Cas9 PLUS™
- Cell counter
- Normal growth medium
- Opti-MEM™ Reduced Serum Medium
- Tissue culture plates
- Microcentrifuge tubes

Important Considerations

- Wearing gloves and using nuclease-free tubes and reagents is recommended in order to avoid RNase contamination.
- All reagents should be stored according to the manufacturer's recommendations.

- This protocol is optimized for use with commonly used cell lines, such as HEK293T, A549, U2OS, HeLa, CHO, MCF-7. It may be necessary to experimentally optimize this protocol for a particular cell line.
- Successful transfection is critically dependent on cell density. It may be necessary to experimentally optimize cell seeding densities in order to determine the most appropriate level of confluence for transfection. For fast growing cells, seed fewer cells. Cell seeding is based on the rate of cell growth. Suggested starting cell numbers are listed in the protocol below.
- Use cells at lowest passage number possible.
- sgRNA should be dissolved in an appropriate buffer and diluted to a working concentration using nuclease-free water.
- Cas9 nuclease (SKU# TP790148, 20µM) can be diluted in Opti-MEM™ I Reduced Serum Medium in order to achieve a working concentration for each plate volume.
- OriGene recommends Cas9:sgRNA ratios between 1:3 and 1:9 for RNP formation.
- RNP complexes are stable at room temperature for up to 2 hours, and at 4°C for several weeks. Note that RNPs stored at 4°C may become susceptible to contamination from microbial growth after long periods of time.
- RNP complexes are formed in Opti-MEM™ I Reduced Serum Medium and can be added directly to cells in culture medium irrespective of antibiotics. Following transfection, it is not necessary to remove RNP complexes or to add or change media.
- Always maintain sterile technique, and use sterile, filter micropippetor tips.

Experimental Outline

1. Seed cells so that they are 30-70% confluent for transfection.
2. Prepare RNP complexes (sgRNA + Cas9 nuclease) in Cas9 Plus™ Reagent.
3. Prepare RNP/transfection reagent complex.
4. Add the RNP/transfection reagent complex to cells.
5. Incubate for 2-3 days.
6. Perform an appropriate genomic editing assay (e.g., PCR followed by genomic cleavage detection and/or TIDE analysis; Next-Gen Sequencing).

Protocol

It is critical to add reagents in the order recommended below. For amounts of Cas9 nuclease and synthetic guide RNA, a recommended range is given; it may be necessary to experimentally optimize these volumes and ratios for RNP formation for each cell type. Prepare the RNP complexes with the Lipofectamine™ Cas9 Plus™ Reagent and Opti-MEM™ I Reduced Serum Medium in a separate tube before adding into diluted CRISPRMAX™ Reagent.

Reaction volumes are for EACH WELL and should be scaled up proportionally for the number of wells to be used.

Pre-transfection

Seed cells so that they are 30-70% confluent on the day of transfection.

For 96-well culture, use 0.7 – 2 x 10^4 cells/well and 100µl growth medium/well.

For 24-well culture, use 0.42 – 1.2 x 10^5 cells/well and 500µl growth medium.

For 6-well culture, use 2.1 – 6 x 10^5 cells/well and 2ml growth medium/well.

Day 1

1. Prepare RNPs in a microfuge tube:

Cells	Opti-MEM™	Cas9	sgRNA	Lipofectamine™ Cas9 Plus™ Reagent
96 well	5µl	0.5 – 5pmol	0.5 – 5pmol	0.17µl
24 well	25µl	1 – 20pmol	1 – 20pmol	1µl
6 well	125µl	10 – 30pmol	10 – 30pmol	5µl

* You may need to experimentally determine the optimum amounts of Cas9 nuclease and guide RNA. In this example, a 1:1 ratio of Cas9 nuclease:sgRNA is used for the RNP formation. OriGene recommends Cas9:gRNA ratios between 1:3 and 1:9 for RNP formation.

2 Incubate RNPs for 5-10 minutes at room temperature.

3. In a separate tube, dilute CRISPRMAX™ Reagent in Opti-MEM™ I Reduced Serum Medium:

Cells	Opti-MEM™	Lipofectamine™ CRISPRMAX™ Transfection Reagent
96 well	5µl	0.3µl
24 well	25µl	1.5µl
6 well	125µl	7.5µl

4. Add the RNP complex mixtures directly to the diluted Lipofectamine™ CRISPRMAX™ Transfection Reagent tube, and mix well by pipetting up and down.
5. Incubate for 5-10 minutes at room temperature. **Do need exceed 30 minutes.**
6. Add the RNP complex-CRISPRMAX mixture to cells:
7. Incubate cells for 2-3 days at 37°C.

Days 2-4

8. Incubate the cells for 2-3 days in a humidified 37°C/5% CO2 incubator.
9. Perform an appropriate assay to determine editing efficiency (e.g., PCR followed by genomic cleavage detection assay and/or TIDE analysis; Next-Gen Sequencing).

Electroporation of Cas9/sgRNA (RNP) Complexes for CRISPR/Cas9 Genome Editing (Thermo Neon™ System)

Background

This protocol describes how to transfect cultured cells with ribonucleoprotein (RNP) complexes that consists of purified Cas9 nuclease duplexed with synthetic sgRNA using the Thermo Fisher Neon™ Electroporation system.

Materials Required

- sgRNA and Cas9 nuclease (SKU# TP790148)
- ThermoFisher Neon Transfection System
- ThermoFisher Neon Transfection System 10 µl Kit (MPK1025)
- Cell counter
- Normal growth medium
- TrypLE Express or preferred cell dissociation reagent
- 1X PBS, cell culture grade
- Tissue culture plates
- Microcentrifuge tubes

Important Considerations

Working with RNA and RNPs

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

- All reagents should be stored according to the manufacturer's recommendations.
- sgRNA should be prepared prior to Electroporation.
- RNPs can be formed directly in Neon Resuspension R Buffer. Use Resuspension Buffer R for all cell types, except primary suspension blood cells (use Resuspension Buffer T).

Optimized Protocols

- For specific electroporation settings for your cell type, we suggest consulting the Thermo Fisher Neon™ cell type guide, available online at: www.thermofisher.com/us/en/home/life-science/cell-culture/transfection/transfection---selection-misc/neon-transfection-system/neon-protocols-cell-line-data.html.
- Optimization of editing efficiency for a specific cell type will require empirically determining the number of cells required, amount of Cas9 and ratio of Cas9:gRNA. This guide is meant to provide a starting point for your CRISPR editing experiments.

Controls

- Mock transfection (cells transfected without Cas9 or guide RNA to identify potential toxicity from RNP, cell death from electroporation or possible viability issues with the specific gene being edited)
- Cas9 complexed with a non-targeting sgRNA or no guide RNA (a negative control to be used to compare against when assessing editing efficiency)
- Optional transfection control: pMAX GFP

Protocol

1. Pre-Electroporation

1.1. Subculture cells 2-3 days before electroporation and seed cells in the appropriately sized vessel so that they are 70-80% confluent on the day of transfection. Each transfection reaction will require approximately 100,000 - 200,000 cells, dependent on cell type (refer to Neon® Transfection System Protocols and Cell Line Data for more information).

1.2. Just prior to electroporation, pre-warm 1ml of normal growth medium in each well of a 12-well cell culture plate, per transfection reaction.

2. Electroporation

2.1. Assemble RNP complexes in Resuspension R Buffer, adding reagents in the order shown below. OriGene recommends Cas9:gRNA ratios between 1:3 and 1:9 for RNP formation. Below is an example experiment using a 1:9 gRNA to Cas9 ratio:

	Experimental gRNA1	Experimental gRNA2	Experimental gRNA3	Non-targeting gRNA	Mock Transfection
µl Resuspension R Buffer	**3.5**	**3.5**	**3.5**	**3.5**	**6**
µl gRNA (30pmol/µl)	**3**	**3**	**3**	**3**	**-**
µl Cas9 (20pmol/µl)	**0.5**	**0.5**	**0.5**	**0.5**	**1**
Total Volume (µl)) RNP	**7**	**7**	**7**	**7**	**7**

Incubate RNPs for 10 minutes at room temperature and keep on ice until ready to use.

2.2. Aspirate cell culture media and wash cells 1-2 times with appropriate volume of 1X PBS.

2.3. Add appropriate amount of TrypLE Express and incubate the cells 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.

2.4. Neutralize the dissociation reaction with at least 2x normal growth medium.

2.5. Count the cells to determine cell density.

2.6. Transfer 1-2e+06 cells to a sterile microfuge tube. (One tube of cells should be enough for about 10 transfections). Centrifuge cells for 5 minutes at 500 x g. Aspirate media.

2.7. Wash the cells once with 1X PBS and repeat the centrifugation. Aspirate PBS.

2.8. Resuspend the cell pellet in 50µL of Resuspension Buffer R. Avoid storing the cell suspension for more than 15–30 minutes at room temperature, which reduces cell viability and transfection efficiency.

2.9. Add 5µL of cells to each pre-complexed RNP tube.

2.10. Aspirate 10µL of RNP/cell mixture to a 10µL Neon tip.

2.11. Electroporate using cell line optimized conditions (refer to Neon® Transfection System Protocols and Cell Line Data for more information).

2.12. Immediately transfer cells to pre-warmed 12-well plate.

3. Post Electroporation

3.1. Incubate the cells for 2-3 days in a humidified 37°C/5% CO2 incubator.

3.2. Perform an appropriate assay to determine editing efficiency (e.g., PCR followed by genomic cleavage detection assay and/or TIDE analysis; Next-Gen Sequencing).

Electroporation of Cas9/sgRNA (RNP) complexes for CRISPR/Cas9 genome editing (Lonza Nucleofection™ System)
Background

This protocol describes how to transfect cultured cells with ribonucleoprotein (RNP) complexes that consist of purified Cas9 nuclease duplexed with sgRNA using the Lonza 4D Nucleofector™ unit with 16-well Nucleocuvette™ Strips.

Materials Required

- sgRNA and Cas9 Nuclease
- Lonza 4D-Nucleofector™ System with X Unit
- 4D-Nucleofector® X Kit S (32 RCT) specific for cell type
- Cell counter
- Normal growth medium
- TrypLE Express or preferred cell dissociation reagent
- 1X PBS, cell culture grade
- Tissue culture plates
- Microcentrifuge tubes

Important Considerations

Working with RNA and RNPs

- Wearing gloves and using nuclease-free tubes and reagents is recommended in order to avoid RNase contamination.
- All OriGene and Nucleofector™ reagents should be stored according to the manufacturer's recommendations.
- sgRNA should be prepared prior to Nucleofection.
- RNPs can be formed directly in Nucleofector™ solution.
- RNP complexes are stable at room temperature for up to 2 hours, and at 4°C for several weeks. Note that RNPs stored at 4°C may become susceptible to contamination from microbial growth after long periods of time.

Optimized Protocols

- For specific electroporation settings for your cell type, we suggest consulting the Lonza Nucleofector™ cell and transfection database, available online at bio.lonza.com/6.html.

- Optimization of editing efficiency for a specific cell type will require empirically determining the number of cells required, amount of Cas9 and ratio of Cas9:gRNA. This guide is meant to provide a starting point for your CRISPR editing experiments.

Controls

- Mock transfection: cells transfected without Cas9 or guide RNA to identify potential toxicity from RNP, cell death from electroporation or possible viability issues with the specific gene being edited)
- Cas9 complexed with a non-targeting sgRNA or no guide RNA (a negative control to be used to compare against when assessing editing efficiency)
- *Optional transfection control: pMAX GFP*

Protocol

1. ***Pre-Nucleofection***

1.1. Subculture cells 2-3 days before Nucleofection and seed cells in the appropriately sized vessel so that they are 70-80% confluent on the day of transfection. Each Nucleofection reaction will require ~150,000 cells.

Set Up (Day of Nucleofection)

1.2. Pre-warm 1ml of normal growth medium in each well of a 12-well cell culture plate, per nucleofection reaction.

1.3. Program the Nucleofector™.

Make sure that the entire Nucleofector™ Supplement is added to the Nucleofector™ Solution (Solution to Supplement ratio is 4.5:1) and that the mixture is not more than 3 months old.

2. ***Nucleofection***

2.1. Assemble RNP complexes in Nucleofector™solution, adding reagents in the order shown below. OriGene recommends Cas9:gRNA ratios between 1:3 and 1:9 for RNP formation. Below is an example experiment using a 1:9 gRNA to Cas9 ratio. Note: Cas9:gRNA ratios may need to be determined empirically to achieve optimal editing efficiency.

Incubate RNPs for 10 minutes at room temperature and keep on ice until ready to use.

	Experimental gRNA1	**Experimental gRNA2**	**Experimental gRNA3**	**Non-targeting gRNA**	**Mock Transfection**
µl Nucleofector Solution	**23**	**23**	**23**	**23**	**29**
µl gRNA (30pmol/µl)	**6**	**6**	**6**	**6**	**-**
µl Cas9 (20pmol/µl)	**1**		**1**	**1**	**1**
Total Volume (µl) RNP	**30**	**1 30**	**30**	**30**	**30**

2.2. Aspirate cell culture media and wash cells 1-2 times with 1X PBS.

2.3. Add appropriate amount of TrypLE Express and incubate the cells 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.

2.4. Neutralize the dissociation reaction with at least 2x normal growth medium.

2.5. Count the cells to determine cell density.

2.6. For each reaction, aliquot 150,000 cells into a 1.5mL microcentrifuge tube. Centrifuge cells at 90 x *g* for 8-10 minutes at room temperature. The cell pellets will not be packed tightly so care is required when removing supernatant.

2.7. Resuspend the cell pellet in the 30µL of RNP complex in Nucleofector Solution. Work quickly, but carefully, and avoid leaving cells in Nucleofector Solution for longer than 15 minutes. Avoid bubble formation.

2.8. Transfer all 30µL of cell-RNP solution to Nucleocuvette™ strips and click the lid into place.

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

2.10. Place the Nucleocuvette™ Vessel with 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).

2.11. Press “Start” on the display of the core unit (protocol was programmed in Step 1.4, above).

2.12. 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 suggest a 10 minute incubation at room temperature after Nucleofection is complete. Please consult the optimized Lonza protocol for your cell line to see if this is a necessary step.

2.13. Carefully resuspend the cells in each well of the Nucleocuvette™ with 70µl of pre-warmed growth media, and mix gently by pipetting up and down 2-3 times.

2.14. Transfer all 100µl to the pre-warmed 12-well plate, or desired culture system.

3. ***Post Nucleofection***

3.1. Incubate the cells for 2-3 days in a humidified 37°C/5% CO2 incubator.

3.2. Perform an appropriate assay to determine editing efficiency (e.g., PCR followed by genomic cleavage detection assay and/or TIDE analysis;

Analyze Gene Editing

Following transformation of CRISPR components into your cell type, and subsequent outgrowth or cell division of cell cultures or embryos, it is critical to genotype cells in order to determine the efficiency of the CRISPR events and identify CRISPR-edited clones for isolation and propagation. Typically, a genetic analysis is performed on a subset of transformed cells in order to assess CRISPR-Cas9 editing efficiency and to identify cultures that contain edited cells. Here we will discuss several types of genetic analysis, in addition to more indirect methods, such as protein detection. For CRISPR-edited embryos, a phenotypic readout may be utilized in conjunction with a genetic analysis.

In general, there are two ways to consider CRISPR genotyping methods. These can be classified as low vs. high throughput, and biased vs. unbiased analysis methods. Throughput reflects the ease, speed and suitability of a technique for CRISPR genotyping. Either type of detection method may be unbiased or biased in nature. If a sequencing approach is used, these labels indicate whether the sequencing is targeted to specific regions in the genome based on an algorithmic prediction or not. Specifically, unbiased assays search for evidence of CRISPR editing across the entire genome. In a biased assay, only bioinformatically predicted regions are analyzed (e.g., predicted on- and off-targeted regions). Table 2 below summarizes CRISPR genotyping methods.

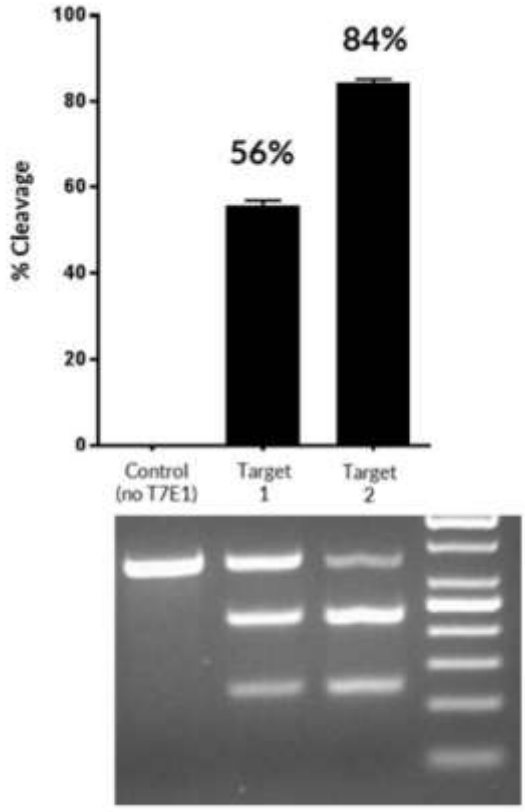
Table 2. An overview of CRISPR genotyping techniques for validating genome editing outcomes

Scope	Low Throughput	High Throughput
Biased	Surveyor / T7E1 mismatch cleavage assay Sanger sequencing / TIDE analysis	Targeted deep amplicon sequencing Site-Seq
Unbiased	None Available	Whole genome sequencing Guide-Seq Digenome-Seq Circle-Seq BLESS

A common and relatively easy method for analyzing CRISPR editing is the use of mismatch cleavage assays that rely on T7 endonuclease I (T7E1). These endonucleases cleave double-stranded DNA wherever there are mismatches, which are formed following CRISPR-Cas9-mediated DSBs and subsequent Indel formation. First, the targeted region is amplified using Polymerase Chain Reaction (PCR) and the resulting amplicons are incubated with T7E1. Fragments are then analyzed by gel electrophoresis and band intensity measured using gel analysis software to determine the percentage of cleaved/uncleaved DNA. This can give a rough estimation of editing efficiency and demonstrate that CRISPR-Cas9 mediated cleavage has taken place.

However, this assay provides no sequence information and is not considered to be very sensitive; the limit of detection is likely close to 5% (Fu et al. 2013, Vouillot et al. 2015). When performing this assay, it is critical to include a PCR amplicon on the gel that has not been incubated with T7E1. The T7E1 assay is considered a biased assay since they rely on simple visualization of gel fragments and are not truly quantitative.

Figure 2: Typical T7E1 cleavage assay result of two CRISPR genome edited targets, amplified by PCR, on an agarose gel. Mismatches (formation of Indels) are identified by the presence of smaller bands which indicate cleavage by the T7E1 enzyme of the PCR product. Cleaved PCR products are analyzed using gel-band intensity software to calculate the percentage of the total PCR product that has been cleaved (% Indel formation).



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In conjunction with a T7E1 cleavage assay, a Tracking of Indels by Decomposition (TIDE) sequencing analysis can also be performed on the PCR amplicons of the edited region. Critically, a PCR amplicon of the same region from unedited cells (not given CRISPR components) must also be obtained. Both PCR amplicons are sequenced using traditional Sanger sequencing, and the resulting DNA sequencing chromatogram files are uploaded to the online TIDE web tool (Brinkman et al., 2014). The web tool utilizes an algorithm that accurately reconstructs the spectrum of Indels from the sequence traces, which will contain mixed peaks due to the formation of Indels at the editing site. The web tool then reports the identity of the mutations and their frequencies in a graphical and tabular output. Like the T7E1 assay, TIDE analysis is a rough estimate of the true CRISPR editing efficiency of a cell population, and may under- or over-estimate actual editing efficiency. In addition, can be performed in most laboratories without access to special equipment, since Sanger sequencing is very inexpensive and can be outsourced overnight. However, Sanger sequencing has a lower detection limit of 50-20% (although this has been improved in some studies) (Davidson et al., 2012, Tsiatis et al., 2010). PCR of edited regions and subsequent sequencing and TIDE analysis are considered biased approaches because only regions targeted for editing are analyzed.

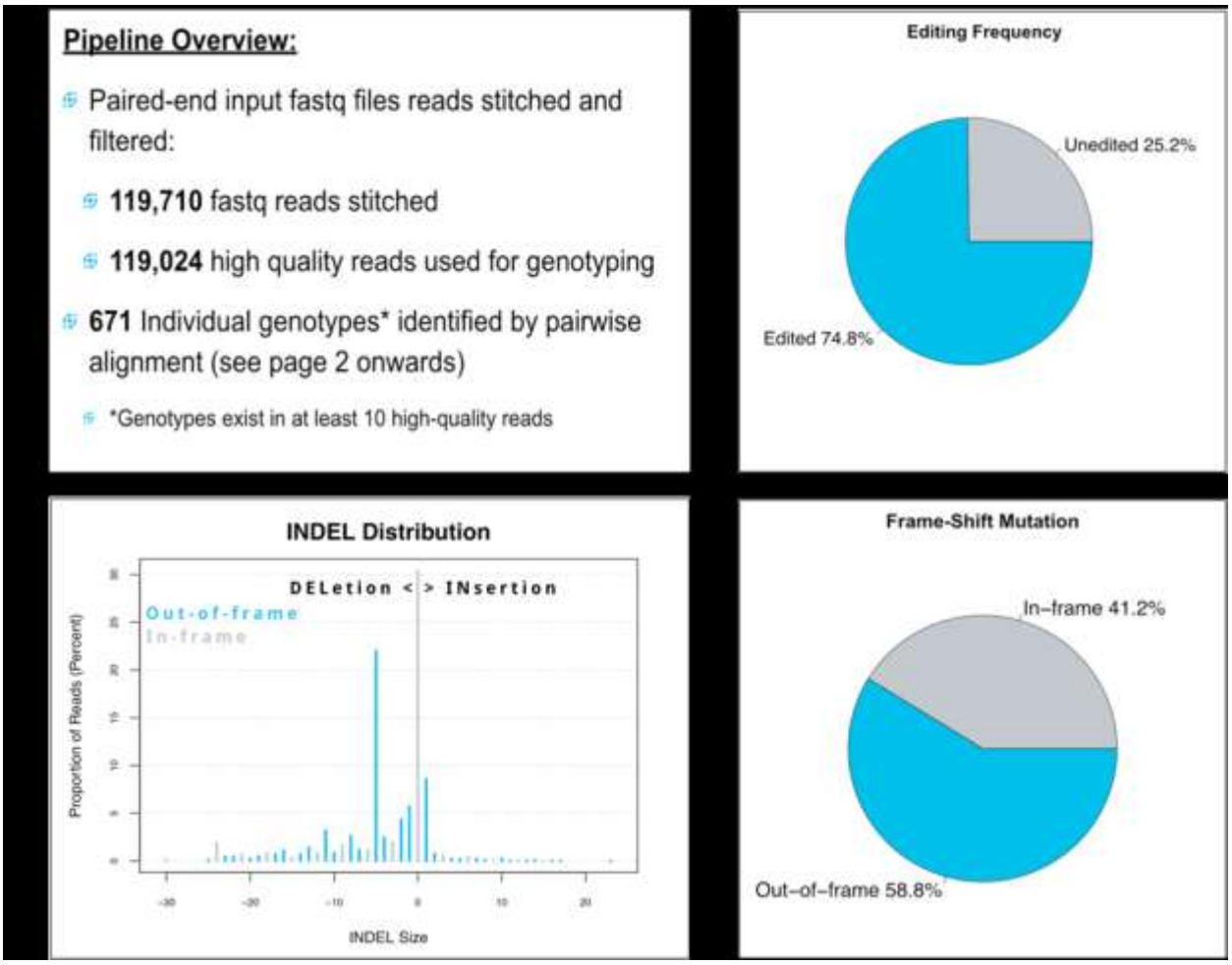
Figure 3: Typical TIDE analysis of a CRISPR-edited genomic region. The edited region was amplified using PCR and sequenced. TIDE analysis was then used to determine the percentage of Indel formation after deconvoluting mixed peaks in the DNA chromatograms, compared to a wild-type sequence.

A high throughput, and more accurate method for analyzing CRISPR editing is the use of Next-Generation Sequencing (NGS). The primary advantage of NGS is that it provides a picture of not only on-target cleavage but also of off-target effects throughout the genome. However, if an unbiased approach such as Whole genome sequencing (WGS) is employed by comparing pre-editing sequencing with CRISPR-edited sequence, it is challenging to deconvolute what (if any) mutations besides those targeted by CRISPR are truly off-target effects or baseline mutations unless the model is genotyped before and after CRISPR editing. A more practical, but biased approach is to perform NGS on targeted amplicons throughout the genome that may have a high probability for off-target effects. In addition, target amplicon deep sequencing is much less expensive than WGS.

An alternative to NGS are unbiased cell-based analyses such as Guide-Seq (Tsai et al., 2015). Guide-Seq is a genome-wide method of identifying DSBs by sequencing and can detect sites with cleavage activity as low as 0.1%. Guide-seq relies on erroneous NHEJ-mediated DNA repair to capture co-introduced blunt-ended double stranded oligonucleotides (dsODNs) at CRISPR/Cas9-induced breakpoints within the genome. These oligonucleotides display a high frequency of insertion at DSBs caused by Cas9 and in effect tag the edited loci for subsequent amplification and deep sequencing.

An effective combination of the aforementioned methods is Site-Seq. This is a biased, high throughput technique used for assessing and validating the size, frequency and identities of CRISPR mutations at on- and off-target sites. By combining targeted amplicon sequencing with bioinformatic prediction, Site-Seq provides a quantitative report of guide cutting efficiency and specificity, and the size, frequency and distribution of indel mutations within the sample population. Site-seq is offered as a service by Desktop Genetics. Figure 4 below shows an example of a Site-seq analysis and the reports generated when using this service.

Figure 4. An example of a Site-Seq Guide Characterization report.



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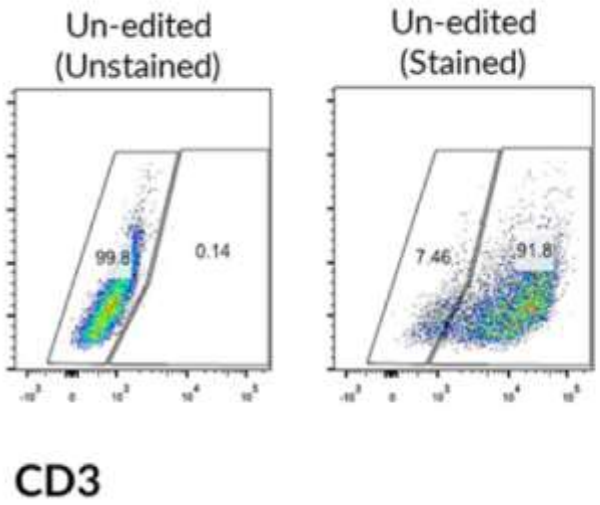
If the gene target that is being edited using CRISPR produces a cell surface receptor or exterior facing membrane protein, then fluorescence-activated cell sorting (FACS) can be used to sort cell populations that have been edited or have not been edited. An example of this is shown in Figure 5 below. Although this method can quite accurately report the percentage of the cell population that have a successful CRISPR edit, they also provide no sequence information regarding the edit itself or off-target effects.

Figure 5: FACS analysis of CD34+ cells edited using CRISPR. Using CRISPR, the CD3 cell surface receptor gene was knocked out, using either a chemically modified 2-piece crRNA:tracrRNA or a chemically modified single sgRNA. Using the crRNA:tracrRNA pair (box3), 46.7% of cells showed knockdown of the CD3 receptor, while with the sgRNA (box4), 91% of cells showed knockdown of CD3. Box 1 shows un-edited cells that have not been stained. Box 2 shows un-edited cells that have been stained for CD3.



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