



PROTOCOL

CRISPR nuclease editing using CCBio Super Knock-in Cocktail

Efficient, precise knock-in of point mutations or gene fragments **using lipofection**



Table of Contents

A. Overview	3
A.1 Symbols	3
B. Preparing your edit	4
B.1 Choose your editing strategy	4
B.2 Material requirements for CRISPR genome editing	4
B.3 Consumables checklist	5
B.4 Experimental timeline	6
B.4.1 Cell Seeding	6
B.5 General advice before starting the experiment	6
B.6 Before you begin — Checklist	7
C. Step by step procedure	8
C.1 Preparation of editing components	8
C.1.1 CRISPR gRNA preparation	8
C.1.2 Prepare the RNP complex	9
C.1.3 Combine editing components	9
C.1.4 Prepare the Lipid RNP mix	10
C.2 Preparation of culture media	10
C.3 Preparation of cells for lipofection	11
C.4 Transfection of RNP complex into cells	11
C.4.1 Reverse transfection	11
C.4.2 Forward transfection	12
C.5 Analysis and expectations	12



A. Overview

The CCBio Super Knock-in Cocktail is a two-component formulation designed to transiently enhance homology-directed repair (HDR) efficiency, enabling precise knock-in of point mutations and gene fragments with up to 93% efficiency while minimizing unwanted off-target editing.

For highly efficient precise genome editing, we recommend delivering a ribonucleoprotein (RNP) complex consisting of recombinant Cas9 nuclease in complex with a synthetic guide RNA (crRNA:tracrRNA duplex or sgRNA). This approach provides high editing efficiency across most target sites and avoids common issues associated with plasmid-based CRISPR delivery, such as inconsistent nuclease expression levels and unintended incorporation of DNA constructs into the genome.

This protocol describes RNP assembly with addition of CCBio Super Knock-in Cocktail components and delivery into mammalian cells via lipofection. General guidance for adapting the workflow to different cell types is provided.



If you are using electroporation as a delivery method, please refer to the corresponding protocol.



Go to
protocol >>

A.1 Symbols



Indicates a part of the protocol in which components of the CCBio Super Knock-in Cocktail are used.

● ● ● Colored bullets indicate the cap color of the reagent to be added.

Note icons:



Attention / Caution



Product suggestion



Plasmid integration



B. Preparing your edit

B.1 Choose your editing strategy

For most applications, we recommend RNP-based editing due to significantly higher efficiency and reproducibility. Preassembly ensures Cas9 is already loaded with guide RNA upon entering the cell. Alternatively, plasmid-based delivery can be used (delayed timing due to expression).*

B.2 Material requirements for CRISPR genome editing

Healthy cells

Cell health is critical for efficient editing. Use cells with a low passage number and ensure they are in active growth phase at the time of transfection. For sensitive cell types such as iPSCs, we recommend using Accutase for dissociation and supplementing recovery media with ROCK inhibitor (Y-27632) to minimize cell death post-lipofection.

Guide RNA

- Option 1 - Two-part guide RNA (crRNA with tracrRNA): The crRNA contains the 20 nt target-specific spacer sequence and must be duplexed with a tracrRNA to form the active guide RNA recognized by *S. pyogenes* Cas9. The crRNA and tracrRNA are annealed by heating to 95°C and cooling to room temperature prior to RNP assembly.
- Option 2 - Single guide RNA (sgRNA): As an alternative to the 2-part system, a covalently fused sgRNA can be used without a separate tracrRNA. This can simplify preparation and is compatible with the same RNP assembly workflow. Use without tracrRNA.

Recombinant CRISPR nuclease

The CRISPR nuclease is delivered as recombinant protein and combined with the assembled gRNA to form a ribonucleoprotein (RNP) complex prior to lipofection. For Cas12a-based edits, use the corresponding crRNA without tracrRNA.

Several CRISPR nucleases are available. We recommend Cas9 and its optimized variants:

- Cas9: Widely used and suitable for many applications.
- HiFi-Cas9: Increased specificity and reduced off-target activity.
- Cas9 nickase: Induces a single-strand break, when used with two gRNAs a staggered double-strand break is induced and HDR can be promoted; off-targets are reduced.



*Plasmid delivery requires a different lipofection reagent and different amounts. Details can be found in our general CRISPR protocol.



Go to
protocol >>



For optimal editing efficiency, we recommend using the CCBio Super UniGuide as the tracrRNA component.



Go to
product >>



Donor DNA template

Precise knock-in via homology-directed repair (HDR) requires a donor template carrying your desired edit flanked by homology arms:

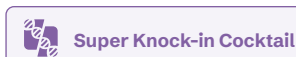
- ssDNA: Recommended for point mutations and small edits
- dsDNA (plasmid or PCR donor): For larger insertions

If you want to knock out a gene by introducing indels, a donor DNA template is not needed.

Lipofection system

CRISPR components are delivered into cells as a preformed RNP complex via lipofection. Lipofection parameters should be optimized for your specific cell type following the manufacturer's recommendations.

CCBio Super Knock-in Cocktail



The Super Knock-in Cocktail consists of two complementary enhancers that work at distinct steps of the editing workflow to increase precise knock-in efficiency:

- Enhancer A: Added to the RNP complex immediately before lipofection to improve editing outcomes at the delivery stage.
- Enhancer B: Added to the culture media post-lipofection and maintained for 48 hours to support HDR during the recovery phase.

Genotyping and analysis tools

After a recovery period of 1-5 days (see experimental timeline), edited cells can be analysed to confirm successful knock-in. Recommended approaches include Sanger or next-generation sequencing of the target locus, TIDE or ICE analysis for estimating editing efficiency (where indels are expected to predominate), and restriction fragment analysis where applicable.

B.3 Consumables checklist

- ☐ CCBio Super Knock-in Cocktail (● Enhancer A, ● Solution A and ● Enhancer B)
- ☐ Opt.1 tracrRNA (CCBio Super UniGuide) and crRNA / Opt.2 sgRNA
- ☐ Cas9/Cas12a
- ☐ Donor DNA
- ☐ Lipofection kit (for example: Opti-MEM, Lipofectamine RNAiMAX)
- ☐ Nuclease-free water
- ☐ Tris-EDTA (TE) buffer, pH 7.5-8



For general CRISPR-Cas9 genome editing guidance, including crRNA and donor DNA design, see our general CRISPR protocol.



Go to
protocol >>



- ☐ Phosphate buffered saline (PBS)
- ☐ Cell dissociation reagent (Accutase, Trypsin, etc.)
- ☐ Cell culture media
- ☐ Y-27632 (ROCK inhibitor, 10 μ M, recommended when working with iPSC)
- ☐ HEPES buffer (20 mM)
- ☐ KCl (150 mM)

B.4 Experimental timeline

Table 1: Timeline for a CRISPR experiment using lipofection

	Day	Step
For all edits	-2 / -1*	Seed cells
	0	Electroporation
For indels	1 – 3	Recovery and expansion
	2 – 5	Genotyping / Analysis
For knock-ins	3 – 5	Recovery and expansion
	5 – 7	Genotyping / Analysis



* The day cells are seeded depends on the growth speed of your cells. Adjust transfection day accordingly.

B.4.1 Cell Seeding

Depending on the cell type, cells can be dissociated and plated 1-2 days before the editing experiment. Transfection will then be carried out as a forward transfection. Another option is to do the cell dissociation and transfection on the same day. This is the reverse lipofection approach. For iPSCs we recommend the dissociation of the cells right before the lipofection (reverse lipofection).

• Adherent Cells

Aim for 60–80% confluence on the day of lipofection, or follow the recommendation for your cell type, as cells outside the logarithmic growth phase often show lower editing efficiency.

One editing reaction corresponds to one well of a 96-well plate, seeded with 2×10^4 cells. Make sure to prepare enough wells for the number of reactions planned, plus controls. For larger plate formats, scale all components as shown in Table 2.

• Suspension Cells

Ensure cells are in log-phase growth with an approximate density of $\sim 0.5\text{--}1 \times 10^6$ cells/mL. Cells are harvested from this culture and resuspended to 2×10^5 cells/mL for transfection (see section C.3).



B.5 General advice before starting the experiment

- Prepare enough cells for your experiment, including coated plates if necessary. This protocol is optimized for 2×10^4 cells per reaction in a 96-well format.
- Prepare enough media and pre-warm to 37°C (see section C.2 for further information on media preparation).
- Check if all necessary components are in stock when planning your experiment.
- Make sure lyophilized DNA and RNA are dissolved and aliquoted.
- Pulse spin down reagents before opening reaction tubes.
- Always store CRISPR reagents at -20°C.
- Take care when working with RNA oligonucleotides and apply general RNA protection measures (e.g. use RNase-free tubes and tips, avoid repeated freeze-thaw cycles).
- For first-time CRISPR experiments, include appropriate controls such as a validated positive control gRNA and a non-targeting negative control gRNA.

Table 2: Plate formats and scaling of the editing reaction. One editing reaction corresponds to one well. All volumes scale with the format.

Plate format*	96-well	48-well	24-well	12-well
Cells per well	2×10^4	6×10^4	1.2×10^5	2.4×10^5
Cell suspension per well	100 μ L	300 μ L	600 μ L	1.2 mL
Lipid RNP mix per well	50 μ L	150 μ L	300 μ L	600 μ L
Total volume per well	150 μ L	450 μ L	900 μ L	1.8 mL



*Larger plate formats are intended for scale-up rather than routine use.

Given volumes are suggestions and scale with plate format. Depending on the cell type and lipofection system used, titration of cell number, RNP and lipofection reagent may be necessary to reach optimal efficiency.

B.6 Before you begin — Checklist

- ☐ Confirm enough healthy cells
- ☐ Resuspend gRNA
- ☐ Prepare donor DNA
- ☐ Prepare CRISPR nuclease for use
- ☐ Warm media
- ☐ Include controls



C. Step by step procedure

C.1 Preparation of editing components

C.1.1 CRISPR gRNA preparation

Assemble a two-part gRNA complex by combining crRNA and tracrRNA, allowing the oligos to anneal and form an active guide complex. Alternatively, sgRNA can be used without hybridization.

1. Resuspend each oligo in TE buffer. ¹ We suggest a final concentration of 200 μ M.
2. If using sgRNA, proceed to section C.1.2.
3. Mix equal amounts of crRNA (200 μ M) with tracrRNA (200 μ M) in a sterile microcentrifuge tube.²

Table 3: Example Cas9 crRNA: tracrRNA duplex formation

Component	Amount
200 μ M crRNA component of gRNA	2 μ L
200 μ M tracrRNA component of gRNA	2 μ L
Total volume	4 μ L

4. Heat the mixture at 95°C for 2 minutes.
5. Remove from heat and cool to room temperature (15-25°C) on the bench top for 5 minutes.
6. You do not need to purify after annealing.
7. Dilute the gRNA to a 1 μ M working stock using TE buffer.¹

Table 4: Example gRNA working stock dilution

Component	Amount
100 μ M gRNA	2 μ L
TE buffer ¹	198 μ L
Total volume	200 μ L

8. For an optimized starting condition use 1.5 μ L gRNA (1 μ M) per lipofection reaction.



¹ When using CCBio Super UniGuide use ● Buffer A for resuspension.

² No tracrRNA is required for Cas9 edits using sgRNA or edits with Cas12a crRNA.

Critical: Do not repeatedly freeze-thaw RNA.



For optimal Cas9 editing efficiency, use the CCBio Super UniGuide as the tracrRNA component of hybridized gRNAs.



Go to
product >>



C.1.2 Prepare the RNP complex

- Mix the hybridized gRNA (or sgRNA) with a CRISPR nuclease to form an RNP complex. The RNP is assembled using an equimolar ratio of CRISPR nuclease and gRNA.
For lipofection, an equimolar ratio of gRNA and CRISPR nuclease is sufficient, as lipid complexes provide a more controlled delivery environment than electroporation.
- Prepare a CRISPR nuclease working buffer (20 mM HEPES, 150 mM KCl, pH 7.5).
- Depending on your cell type, you may use Opti-MEM medium or 1× PBS instead of CRISPR nuclease working buffer.
- Dilute CRISPR nuclease to a working concentration of 1 μ M in CRISPR nuclease working buffer, Opti-MEM, or 1× PBS.
- Combine these components to form an RNP. The amounts given are per editing reaction, i.e. per well of a 96-well plate; scale according to Table 2 for other formats:

Table 5: Example RNP complex formation

Component	Amount
gRNA (1 μ M)	1.5 μ L
Diluted CRISPR nuclease (1 μ M)	1.5 μ L
Opti-MEM medium	5 μ L
Total volume	8 μ L

- Incubate at room temperature for 20–30 minutes to assemble the RNP. During this step, start preparing cell dissociation (see section C.3).

C.1.3 Combine editing components



- All editing components will be prepared in a 1.5 mL microcentrifuge tube.
- For knock-in or substitution of a few base pairs, we recommend using a ssDNA donor. Other applications might require a dsDNA donor.
- Dilute the ssDNA donor (recommended stock concentration is 200 μ M) to a 1 μ M working concentration in TE buffer.
- Add 1.5 μ L of the ssDNA donor (1 μ M) to the RNP complex. The amount might be adjusted for dsDNA donor.
- Resuspend ● Enhancer A in 20 μ L of ● Solution A to make a 100 μ M solution. Add 0.6 μ L to the RNP complex.
- Add Opti-MEM medium to reach a total volume of 25 μ L.



Important: consider donor design improvements for your intended edit.

Please refer to our general CRISPR protocol for specific donor DNA design guidelines.



Go to
protocol >>



Table 6: RNP and oligo mix

Component	Amount
RNP complex	8 μ L
ssDNA donor (1 μ M)	1.5 μ L
● Enhancer A	0.6 μ L
Opti-MEM medium	14.9 μ L
Total volume	25 μ L

C.1.4 Prepare the Lipid RNP mix

- Dilute the lipofection reagent (RNAiMAX) in a separate tube with Opti-MEM. For iPSCs we recommend starting with 1.2 μ L RNAiMAX + 23.8 μ L Opti-MEM (the amount of RNAiMAX can be modified for higher efficiency).
- Incubate for 5 minutes at room temperature.
- Combine the diluted transfection reagent with the RNP and oligo mix.

Table 7: Lipid RNP mix

Component	Amount
RNP and oligo mix	25 μ L
RNAiMAX Opti-MEM mix	25 μ L
Total volume	50 μ L

- Mix gently and incubate at room temperature for 25-30 minutes.
- During incubation, proceed to sections C.2 and C.3 to prepare the media and the cells.

C.2 Preparation of culture media



Super Knock-in Cocktail

- Dilute ● Enhancer B (2000 $\times$ stock in DMSO) in cell culture medium to a 1 $\times$ final concentration. For example, add 5 μ L of ● Enhancer B to 10 mL of medium.
- For iPSCs, adding ROCK inhibitor (Y-27632) at a final concentration of 10 μ M is recommended to help prevent cell death in the first 24 hours.
- On the day of transfection, make sure to adjust the amount of ● Enhancer B in the medium (1.5 $\times$ more of ● Enhancer B when using the volumes suggested here) because the transfection mix will dilute the cell media. For a 96-well plate prepare 10 mL me-



dium and add 7.5 μL of ● Enhancer B. The medium will be diluted by the transfection complex to reach a 1 $\times$ final concentration in the well.

- As HDR predominantly occurs during the first cell divisions after editing, we recommend leaving ● Enhancer B on the cells for 48 hours to achieve maximum efficiency. A daily media change with fresh ● Enhancer B is optional, depending on the cell line.



A transient reduction in cell viability can be expected to occur during the first 24–48 hours.

C.3 Preparation of cells for lipofection



Super Knock-in Cocktail

Depending on the cell type, cells can be dissociated and plated the day before the editing experiment or on the same day right before the lipofection. For iPSCs we recommend the dissociation of the cells right before the lipofection.

- Wash cells and prepare a single-cell suspension. A single-cell state is critical for efficient delivery of CRISPR components.
- To generate a single-cell suspension, add cell dissociation reagent to the cells and incubate for 6 minutes at 37°C. We recommend Accutase dissociation reagent especially when working with sensitive cells like iPSCs.
- Stop dissociation reaction by adding medium.
- Gently triturate (~20 times) until a uniform single-cell suspension is obtained; we recommend checking under the microscope that no cell clumps remain.
- After dissociation, wash cells in PBS and centrifuge for 3–5 minutes at 200–300 $\times$ g.
- Resuspend the pellet in medium containing ● Enhancer B at 1.5 $\times$ concentration to a density of $\sim 2 \times 10^5$ cells/mL.

C.4 Transfection of RNP complex into cells



Super Knock-in Cocktail

Reverse transfection (C.4.1) is generally recommended for suspension cells, iPSCs, or screening workflows, whereas forward transfection (C.4.2) is often suitable for robust adherent cell lines.

C.4.1 Reverse transfection

- After the 25–30 minutes incubation time, add 50 μL of the lipid RNP mix to each well of a 96-well tissue culture plate.
- Add 100 μL of diluted cell suspension (2×10^4 cells/well) to the wells of the 96-well tissue culture plate containing the lipofection complex (final concentration of RNP will be 10 nM, ● Enhancer B will be at 1 $\times$ concentration).



- Keep the transfection mix on the cells for ~24 hours. If your cells require daily media change, then replace with fresh medium containing ● Enhancer B at 1× concentration for further 24 hours.

C.4.2 Forward transfection

- Seed cells 1-2 days in advance so that each well of the 96-well plate contains approximately 2×10^4 cells at the time of transfection. On the day of transfection, perform a medium change and add 100 µL of fresh medium containing ● Enhancer B at 1.5× concentration.
- After the 25-30 minutes incubation time, add 50 µL of the lipid RNP mix directly into the media of the growing cells in the 96-well tissue culture plate.
- Keep the transfection mix on the cells for ~24 hours. If your cells require daily media change, then replace with fresh medium containing ● Enhancer B at 1× concentration for further 24 hours.

C.5 Analysis and expectations

As the outcome of a CRISPR edit is very dependent on the cell line and the target site, it is difficult to give a general expected outcome. We have tested the CCBio Super Knock-in Cocktail across cell lines and target sites and find that it generally increases the editing efficiency. Especially for human iPSCs, for which this protocol has been optimized, we achieve precise knock-in efficiencies up to 93%.

To analyse the editing efficiencies, harvest the cells and extract their DNA. This can be done as soon as 72 hours after transfection. However, recovery time of the cells after the edit can vary. A simple option for this is to use the QuickExtract solution. If edited cells should be further cultivated, it might be useful to have one well for harvesting and analysis and one well for propagation.

To assess the correct incorporation of your intended edit and to gauge the efficiency, several options exist:

- PCR target site amplification (~300 bp around cut site) and sequencing (Sanger or next-generation sequencing)
- Mismatch cleavage assay (T7EI or Surveyor)
- Fluorescence-based methods for detection in flow cytometry

For all methods it might be useful to include an untreated control for sequence comparison. In our general CRISPR protocol we explain in detail the different methods, their advantages and disadvantages. Also, we highlight several quality controls including off-target assessment, copy-number loss due to large deletions or loss of heterozygosity.



See our general CRISPR protocol for editing methods, their pros and cons, and key quality controls,



Go to
protocol >>

