Pin-point™ Base Editing Platform

Technical Manual

Pin-point platform for base editing with mRNA and synthetic guide RNA

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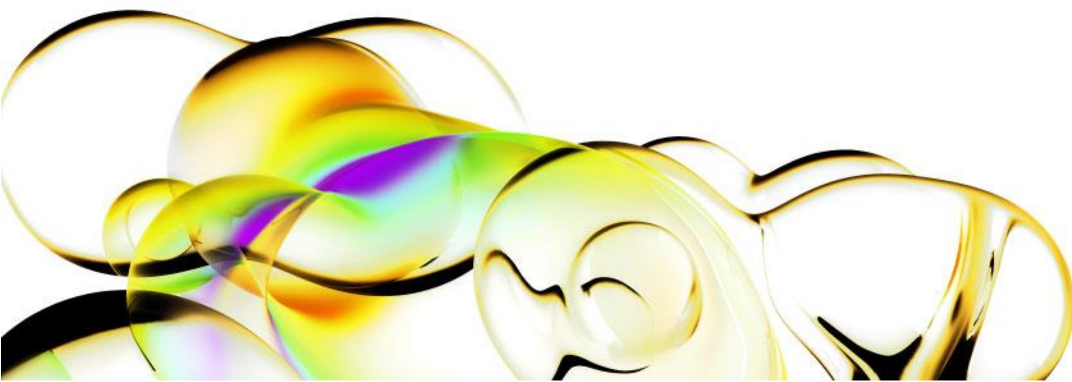

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1. Introduction to the Pin-point base editing platform

CRISPR-based technologies provide powerful tools for gene editing and modulation (Mali, 2014; Sampson, 2014). Base Editing is a CRISPR-based technology that has been developed for precise and efficient genome editing without the induction of double-strand DNA cleavage. Cytidine base editors (CBEs) enable recruitment of cytidine deaminase enzymes to a DNA target site, specifically enabling conversion of C-G base pairs to T-A base pairs (Komor, 2016; Nishida, 2016). This ability to make specific nucleotide changes can be harnessed to then produce gene knockouts through introduction of premature stop codons (Billon, 2017) or disruption of splice donor and splice acceptor sites (Webber, 2019), while avoiding DNA double-strand breaks and imprecise indel formation.

The Pin-point base editing platform was developed to efficiently recruit base-modifying enzymes through the use of RNA aptamers (Collantes, 2021). Pin-point base editing system effectively induces target specific nucleotide changes without the formation of DNA double-strand breaks or indels. This system consists of three components: [1] a nuclease-defective "nickase" nCas9 that only cuts or "nicks" a single strand of DNA, fused to uracil glycosylase (UGI) inhibitor (Komor, 2016), [2] a cytidine deaminase base editor (Rat APOBEC) fused to an aptamer binding protein, and [3] an aptameric single guide RNA (sgRNA) that recruits both the nCas9 and the aptamer-deaminase fusion to a specific DNA target site (Figure 1). Delivery of these three components into a mammalian cell induces highly specific levels of C-G to T-A base conversion, suitable for cell and gene therapy applications involving either single amino-acid point mutations or for functional gene knockout.

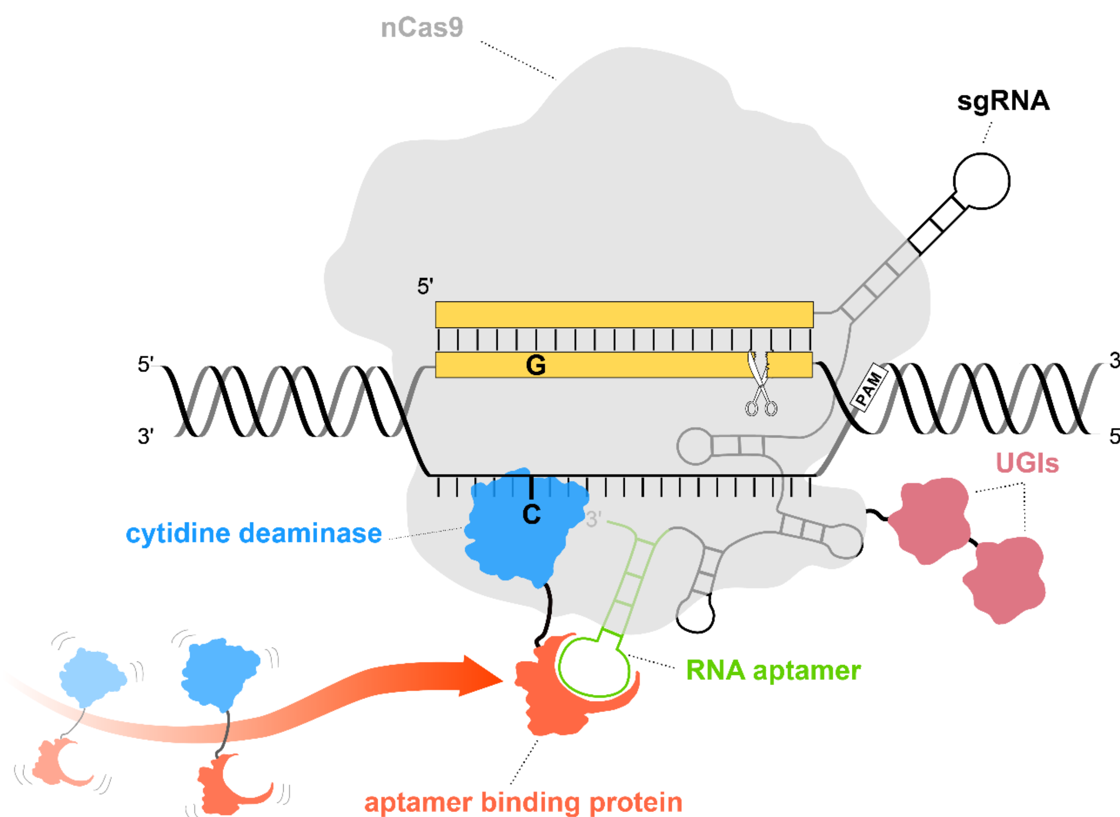

Figure 1. Illustration of Pin-point base editing system. The utilization of nCas9 (light gray) ensures that no DNA double-strand breaks occur, and DNA damage response pathway is not triggered. Pin-point sgRNA contains an aptamer (green) that is used to recruit deaminase (blue) via aptamer binding protein (orange) to perform base editing.

2. Pin-point mRNA and synthetic guide RNA for base editing

The Pin-point immuno-oncology reagents workflow includes all three of the critical Pin-point system components required for base editing in mammalian cells: an mRNA for translation of a mammalian codon-optimized nCas9, an mRNA for translation of a mammalian codon-optimized deaminase with aptamer binding protein fusion, and an aptameric Pin-point synthetic sgRNA. All three components are electroporated into mammalian cells to enable the gene editing reaction. Figure 2 summarizes the general experimental workflow.

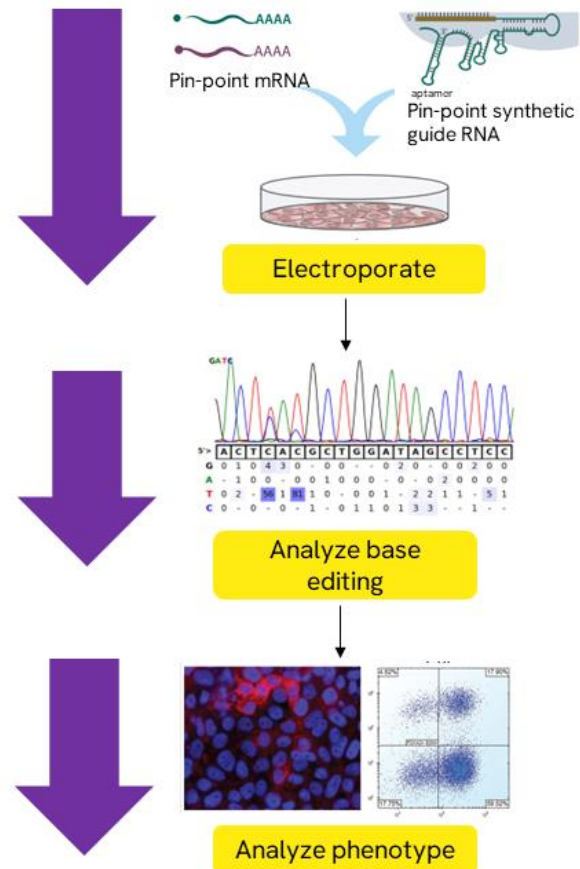

Figure 2. Base editing workflow using Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Pin-point synthetic sgRNA. All three components are electroporated into a specific cell type of interest. Base editing may then be observed using analysis primers to amplify and sequence the base editing region of interest. A phenotype may be analyzed to assess target protein knockout.

Pin-point nCas9 mRNA

The Pin-point nCas9 mRNA contains a human codon-optimized version of the *S. pyogenes* nickase Cas9 (D10A) with a 5' and 3' nuclear localization signal (NLS), fused to UGI. The nCas9 mRNA is *in vitro* transcribed, capped using CleanCap® AG, and polyadenylated for translation and nuclear localization of the protein.

Pin-point Rat APOBEC mRNA

The Pin-point Rat APOBEC mRNA contains a human codon-optimized version of an aptamer binding protein fused to a cytidine deaminase base editor enzyme with a 5' NLS. The Rat APOBEC mRNA is *in vitro* transcribed, capped using CleanCap® AG, and polyadenylated for translation and nuclear localization of the protein.

Pin-point synthetic sgRNA

The Pin-point synthetic sgRNA is a synthetically produced 128-nucleotide chimeric oligo: fusing the crRNA, tracrRNA, and aptamer regions. It is chemically modified for nuclease resistance on both 5' and 3' ends of the molecule.

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3. Guidelines for electroporation of Pin-point base editing components

Successful electroporation of Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Pin-point synthetic sgRNA requires careful optimization of delivery conditions for each cell type of interest. The protocol below assumes experimental conditions have been optimized as recommended, and is for electroporation of both mRNA components and a single sgRNA in mammalian cells.

Materials required for base editing using Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA and Pin-point synthetic sgRNA

- Pin-point nCas9 mRNA (2µg/µl), (20 µg; Cat# PNP12579) (100 µg; Cat# PNP12577) (500 µg; Cat# PNP12581)
- Pin-point Rat APOBEC mRNA (2µg/µl), (20 µg; Cat# PNP12580) (100 µg; PNP12578) (500 µg; PNP12582)
- Pin-point synthetic sgRNA
    - TRAC (Cat# PNP-02000-01); Targeting sequence: TTCGTATCTGTAAAACCAAG
    - CD52 (Cat# PNP-02200-01); Targeting sequence: CTCTTACCTGTACCATAACC
    - PDCD1 (Cat# PNP-02300-01); Targeting sequence: CACCTACCTAAGAACCATCC
- Pin-point Non-targeting Control synthetic sgRNA
    - NTC#1 (Cat# PNP-01000-01)
    - NTC#2 (Cat# PNP-01000-02)
- Pin-point custom synthetic sgRNA (Optional)

Additional materials required

Electroporation experiments require standard cell culture reagents and instruments appropriate for the maintenance of cells. The following additional materials are required but not supplied:

- Neon™ Transfection System (ThermoFisher Cat #MPK5000) or Neon™ Nxt Electroporation system (ThermoFisher Cat #NEON1)
- Electroporation reagents (buffer, Neon pipette tips)
- Multi-well tissue culture plates or tissue culture dishes
- Antibiotic-free complete medium: Cell culture medium (including serum and/or supplements) recommended for maintenance and passaging of the cells of interest without antibiotic
- Phosphate-buffered saline (PBS)
- 10 mM Tris pH 7.4, nuclease free buffer (Tris buffer) solution (Dharmacon, Cat #B-006000-100) for synthetic guide resuspension

4. General protocol for electroporation of Pin-point base editing components in HEK293T cells

The following is a general protocol using electroporation to deliver Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Pin-point synthetic sgRNA into HEK293T cells. Exact reagents, amounts and parameters for electroporation should be empirically determined through careful optimization in cells of interest, in accordance with electroporation instrument manufacturer's recommendations. The protocol below describes delivery conditions in HEK293T cells in a 96-well plate format using the Neon transfection or electroporation system and is given for illustrative purposes only. Reagent volumes can be calculated for including replicate samples as necessary and 10% excess. Three different types of samples are recommended for a base editing experiment (see Table 1). All steps of the protocol should be performed in a biosafety cabinet cell culture hood using sterile technique.

Cell Plating

Optimal cell number for plating will vary with growth characteristics of specific cells and should be determined empirically.

1. Trypsinize and count cells.
2. Plate cells to achieve 70–80% confluence the next day. For example, plate 3 x 10^6 HEK293T cells in a 10 cm dish.
    a. Cell densities greater than 80% may reduce electroporation efficiency.
3. Incubate cells at 37 °C in 5% CO_2 overnight.

Electroporation

Table 1. Recommended samples for a base editing experiment using the Pin-point platform

Sample elements	Purpose
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and gene-specific Pin-point synthetic sgRNA	Base editing sample: nCas9 and Rat APOBEC mRNA directed by guide RNAs for base editing in a gene of interest
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Non-targeting control guide RNA	Negative control: nCas9 and Rat APOBEC mRNA without targeting guide RNAs, provides a baseline to compare experimental conditions
Non-electroporated	No treatment control sample: confirmation of cell viability

1. Prepare 96-well plates by transferring 100 µL of pre-warmed appropriate cell culture medium to the number of wells required for each sample in the experiment. Pre-incubate/equilibrate by placing at 37°C in 5% CO_2 while preparing samples.
2. Prepare guide RNA samples for electroporation (see Table 2).
    a. Prepare a 100 µM synthetic sgRNA stock solution by adding the appropriate volume of Tris Buffer to the sgRNA. Verify the RNA concentration using UV spectrophotometry at 260 nm and adjust the volume necessary to obtain 100 µM.

For multiplexing applications with synthetic sgRNA, it is recommended that the stock solutions be re-suspended at 200-400 µM and then a working stock be made at 100 µM.

3. Prepare the Rat APOBEC mRNA for electroporation (see Table 2).
    a. Prepare a 200 ng/µL stock of Rat APOBEC mRNA by diluting in nuclease-free water.

Table 2. Recommended working stocks and final amounts of Pin-point base editing components for one electroporation (50,000 cells in 10 µL reaction).

Pin-point component	Working stock*	Final amount per reaction
nCas9 mRNA	2 µg/µL	1 µg
Rat APOBEC mRNA	200 ng/µL	100 ng
synthetic sgRNA	100 µM	6 µM

* nCas9 mRNA and Rat APOBEC mRNA shipped at 2µg/µl .

It is recommended to prepare a working stock of the Rat APOBEC mRNA in nuclease-free water to reduce the risk of pipetting error from the higher concentration stock. Prepare only enough working stock for the current experiment as we do not recommend long term storage at the lower concentration.

4. Prepare each sample to be electroporated in a 1.7 mL microcentrifuge tube by mixing sgRNA, nCas9 mRNA, Rat APOBEC mRNA, and buffer R. Appropriate volumes (µL) are outlined in Table 3, corresponding to one well per sample.

We recommend preparing the mRNA and sgRNA mixtures in advance and keeping on ice. Add Buffer R to the mixture shortly before the electroporation.

Table 3. Preparing electroporation samples for Pin-point base editing experiment in a 96-well plate format.

Sample	Buffer R	sgRNA (100 µM)	nCas9 mRNA (2 µg/µL)	Rat APOBEC mRNA (200 ng/µL)	Cell suspension in buffer R (50,000 cells)
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and gene specific Pin-point synthetic sgRNA	3.4 µL	0.6 µL	0.5 µL	0.5 µL	5 µL
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Non-targeting control guide RNA	3.4 µL	0.6 µL	0.5 µL	0.5 µL	5 µL
Non-electroporated	5 µL	0 µL	0 µL	0 µL	5 µL

Volumes (µL) are for a single well of a 96-well plate for HEK293T cells to be electroporated. It is recommended to prepare sufficient sample volumes for the total number of replicates and to account for pipetting error. Exact reagent amounts for electroporation in other cell types should be empirically determined through careful optimization prior to experimentation.

5. Harvest cells and collect 50,000 cells for each sample and centrifuge at 100–400 × g for 5 minutes at room temperature.
6. Aspirate medium from the cell pellet, wash once with phosphate-buffered saline (PBS) by adding buffer to gently resuspend cells, and repeat centrifugation.
7. Aspirate PBS from the cell pellet and resuspend the cell pellet with Buffer R for a final concentration of 1 x 10^7 cells/mL.
8. Gently mix components and using a Neon Pipette and 10 µL electroporation tips, pipette 10 µL of the sample, ensuring there are no air bubbles in the pipette.
9. Electroporate sample following the manufacturer's protocol and using the following conditions: 1150V, 20 ms, 2 pulses.
10. Pipette electroporation cell mixture into the pre-incubated medium in one well of a 96-well plate.
11. Incubate cells at 37 ^{o}C in 5% CO_2 for a total of 48 to 72 hours post electroporation; proceed with base editing analysis.

5. Guidelines for analyzing base editing with Pin-point analysis primers

The following protocol is an example of cell lysis and thermal cycling conditions for base editing analysis. For direct cell lysis, cells were treated for base editing in a 96-well plate format.

Materials required for PCR amplification and sequence verification of base editing with Pin-point platform

Pin-point analysis primers

- Pin-point Human TRAC Control Forward Primer (Cat# PNP-002001-01-10)
  GGGGATATGCACAGAAGCTGC
- Pin-point Human TRAC Control Reverse Primer (Cat# PNP-002002-01-10)
  CTCAGAGCTTAGGATGCACCC
- Pin-point Human CD52 Control Forward Primer (Cat# PNP-002201-01-10)
  AAGCTGCTACCAAGACAGCC
- Pin-point Human CD52 Control Reverse Primer (Cat# PNP-002202-01-10)
  CAGGTTTCTCTCAGGGCAGC
- Pin-point Human PDCD1 Control Forward Primer (Cat# PNP-002301-01-10)
  GGCACCCTCCCTTCAACCT
- Pin-point Human PDCD1 Control Reverse Primer (Cat# PNP-002302-01-10)
  CTCCAGACCCCTCGCTCC

  Additionally, custom primers can be ordered from our website.

Cell Lysis and PCR

1. Aspirate cell culture media (for adherent cells), or spin cells down and aspirate media (for suspension cells). Lyse cells by adding 100 uL of 1X Phusion High-Fidelity buffer (Thermo Fisher Scientific) with additives (see Table 4).

Table 4. Preparing cell lysis reaction

Reagent	Volume per sample (µL)	Final Concentration
5X Phusion HF Buffer	20	1x
Proteinase K (~ 20mg/mL)	5	~ 1 mg/mL
RNase A (10 mg/mL)	5	0.5 mg/mL
Water, nuclease free	70	N/A

2. Seal the 96-well plate with a plate seal to minimize evaporation and cross contamination. Incubate for 15–30 minutes at 56 °C, followed by deactivation for 5 minutes at 96 °C. Briefly centrifuge to collect liquid at bottom of wells.
3. Set up a 50 µL PCR for each primer set, for each sample to be analyzed (see Table 5).

Table 5. Preparing PCR for amplification of base editing region of interest

Reagent	Volume per sample (µL)	Final Concentration
5X Phusion HF Buffer	10	1x
Forward primer (50 µM)	0.5	500 nM
Reverse primer (50 µM)	0.5	500 nM
dNTPs (10 mM)	1	200 µM each
Phusion Hot Start II High-fidelity DNA polymerase (2 U/µL)	1	0.04 U/µL
Cell lysate	1 – 5	N/A
Water, nuclease free	up to 50	N/A

4. Gently mix, centrifuge plate, and run PCR samples (see Table 6).

Table 6. Thermal cycling conditions

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	3 min	1
Denature	98 °C	10 s	10
Touchdown annealing	72 °C – 1 °C/cycle	15 s	
Extension	72 °C	30 s	
Denature	98 °C	10 s	25
Annealing	62 °C	15 s	
Extension	72 °C	30 s	
Final extension	72 °C	10 min	1

5. PCR product (2–5 µL) can be run on a 2% agarose gel to confirm amplification.
6. Submit samples for sequencing with the provider of your choice. We recommend sending for Sanger sequencing using the same Forward and Reverse Primers used in the PCR product generation.

In some instances (different cell types, donors., etc.) manual design of 2-3 additional analysis primer sets may be required to succesfully PCR amplify and sequence the base edited region of interest.

Data Analysis

Multiple programs are available to assist in the analysis of base editing to quantify levels of target C to T conversion. Two examples for analysis of base editing from Sanger Sequencing include:

- BEAT: https://hanlab.cc/beat (Xu, 2019)
- EditR: https://moriaritylab.shinyapps.io/editr_v10 (Kluesner, 2018)

Additional Tips

- If the PCR results in a faint band or if there is low final product quantity, consider replacing 5X Phusion HF Buffer with 5X Phusion GC Buffer, or supplementing the reaction with 5% DMSO in order to optimize the PCR.
- The number of cells used/lysed should be considered for analysis purposes; a minimum of 1000 cells (2000 alleles) is needed to assess complex indel formation, however lower amounts can be used for base editing. In the event of low cell density, caution should be taken to avoid underrepresentation.
- Sanger sequencing should be used to quickly interrogate base editing efficiency.
- Next Gen Sequencing (NGS) can be used to get more reliable and in-depth data on the target locus and guide-dependent off-target sites; we recommend designing primers for paired-end short read sequencing (300PE or 250PE) for NGS analysis.

6. General protocol for multiplex base editing in stimulated primary human T cells using Pin-point mRNA and synthetic guide RNA

The following is a general protocol using electroporation to deliver Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and a multiplex of up to three independent Pin-point synthetic sgRNA sequences into T cells (CD3+ Pan T Cells) (Figure 3). Exact reagents, amount, and parameters for electroporation should be empirically determined through careful optimization in the cells of interest in accordance with the electroporation instrument manufacturer's recommendations. The protocol below describes delivery conditions in human T cells using the Neon transfection or electroporation system (Thermo Fisher Scientific) and is given for illustrative purposes only. Reagent volumes can be calculated for including replicate samples as necessary.

Different types of samples are recommended for a multiplex base editing experiment (Table 7). All steps of the protocol should be performed in a biosafety cabinet using sterile technique. A sample assay schedule is provided as a guide for a multiplex base editing workflow in T cells (Figure 3).

Table 7. Recommended samples for a multiplex base editing experiment using Pin-point platform

Sample elements	Purpose
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Multiple gene specific Pin-point synthetic sgRNAs	Multiplex base editing sample: nCas9 and Rat APOBEC mRNA
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Individual gene specific Pin-point synthetic sgRNA (one sample per each sgRNA involved in the multiplex experiment)	Single base editing samples: nCas9 and Rat APOBEC mRNA directed by guide RNA for base editing in the gene of interest
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Non-targeting control guide RNA	Negative control: nCas9 and Rat APOBEC mRNA with non-targeting guide RNAs, provides a baseline to compare experimental conditions.
Non-electroporated	No treatment control sample: confirmation of cell viability

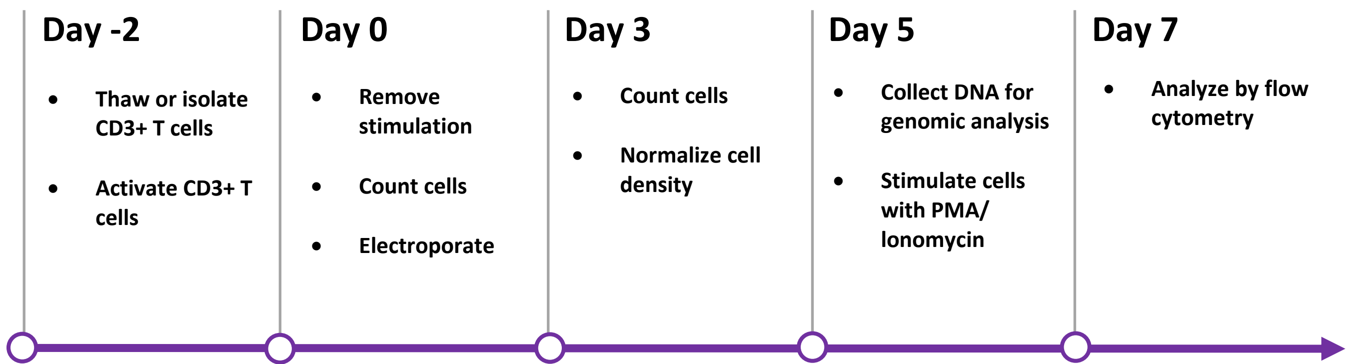

Figure 3. Example workflow for multiplex base editing in T cells that includes assessing base editing and protein knockout for: CD52, PDCD-1, and TRAC.

T cell isolation and activation prior to electroporation

1. CD3+ Pan T cells can be isolated from peripheral blood mononuclear cells using commercially available negative selection kits or can be acquired as cryopreserved cells. Quality control of the isolated cells by flow cytometry is recommended using the following markers: CD3, CD4, CD8, CD25.
2. Post isolation, activate CD3+ T cells for 48 hours with Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fisher Scientific) in media supplemented with IL-2 according to the manufacturer's instructions.

T cell electroporation

1. Preparation of cells
    a. Remove Dynabeads Human T-Activator CD3/CD28 from the culture according to the manufacturer's instructions and count cells. Keep the cells at 37 °C and 5% CO_2 while preparing the electroporation reagents.
    b. Prepare antibiotic-free complete T cell medium supplemented with the appropriate cytokines (IL-2, IL-7, and IL-15), allowing for 500 µL per electroporation reaction.
    c. Prepare post-electroporation tissue culture plates by adding the appropriate volume of cell media to each recipient well. (Appropriate volume determined by size of tissue culture plates; e.g 500 µL/well in a 24-well plate for a standard 250,000 cell electroporation reaction)
    d. Incubate the tissue culture plate(s) at 37°C and 5% CO_2 for a minimum of 30 minutes prior to electroporation to pre-warm the medium.

Table 8. Recommended working stocks and final concentration of Pin-point base editing components for one electroporation in T cells (250,000 cells in 10 µL reaction).

Pin-point component	Working stock*	Final amount per reaction
nCas9 mRNA	2 µg/µL	1.56 µg
Rat APOBEC mRNA	1 µg/µL	0.222 µg
sgRNA single-plex	200 µM	2 µM
sgRNA multi-plex (up to 3 sgRNAs)	200 µM	2 µM per guide, 6 µM total

* nCas9 mRNA and Rat APOBEC mRNA shipped at 2µg/µl . See note about working stock preparation

It is recommended to prepare a 1 µg/µL working stock of the Rat APOBEC mRNA in nuclease-free water to reduce the risk of pipetting error from the higher concentration stock. Prepare only enough working stock for the current experiment as we do not recommend long term storage at the lower concentration.

2. Prepare the sgRNAs for electroporation (see Table 8)
    a. Calculate the number of reactions required and include a minimum of 10% excess volume.
    b. The concentration of each sgRNA in the electroporation mix is 2 µM sgRNA (0.1 µl of 200 µM stock sgRNA in a 10 µL electroporation mix), see Tables 8 and 9.
    c. If performing triplex editing (three targets), use 0.1 µL of each of the three sgRNA per 10 µL reaction.
    d. Mix the calculated volumes of sgRNAs and maintain the compositions on ice until ready for electroporation.
3. Prepare the nCas9 and Rat APOBEC mRNA mix (see Table 8)
    a. Calculate the number of reactions required for the experiment and include a minimum of 10% excess volume; each 10 µL electroporation reaction will require a total of 1 µL mRNA mix. The mRNA mix is composed of 1.56 µg nCas9 and 0.222 µg Rat APOBEC mRNAs per reaction, see Tables 8 and 9.
    b. Transfer mRNA aliquots from the –80 °C freezer directly onto wet ice.
    c. Thaw mRNA on ice and mix the calculated volumes for the nCas9 and Rat APOBEC mRNAs.
    d. Keep mRNA mix on ice until ready for electroporation.
4. Prepare cells
    a. Calculate the total number of samples needed for the experiment and divide the total number of electroporations into subsets of 6-10 reactions, which will be processed together. This is to avoid prolonged exposure of cells to the Buffer R which will lead to increased toxicity.
    b. Calculate the number of cells required for each subset, using 250,000 cells per electroporation, including 10% excess.
    c. Transfer the required number of cells to a centrifuge tube.
    d. Pellet the cells at 400 x g for 5 minutes at room temperature.
    e. Aspirate the supernatant to waste.
    f. Flick gently to dissociate the cell pellet.
    g. Dilute the cell solution with PBS to wash.
    h. Pellet the cells at 400 x g for 5 minutes at room temperature.

During this centrifugation wait step, finalize the preparation of mRNA and sgRNAs.

    i. After T cell centrifugation, carefully remove the supernatant from the pelleted cells.
    j. Resuspend the cell pellet in Buffer R at a final concentration of 5×10^7 cells/mL; each 5 µL electroporation reaction will contain 250,000 cells.
5. Final mix of components and electroporation

We recommend preparing the mRNA and sgRNA mixtures in advance and keeping on ice. Add Buffer R to the mixture shortly before the electroporation.

    a. As outlined in Table 9, prepare each sample to be electroporated in a 0.6 mL microcentrifuge tube by mixing Buffer R, sgRNAs, nCas9, and Rat APOBEC mRNA mix. Appropriate volumes (µL) are corresponding to one electroporation.
    b. Add 5 µL of the cell suspension in Buffer R (250,000 cells) per reaction.
    c. Proceed immediately to electroporation following the manufacturer's protocol and using the following conditions: 1600 V, 10 ms, 3 pulses.
    d. Pipette electroporation cell mixture into the pre-incubated medium in one well of a 24-well plate.
    e. Once electroporations have been completed, incubate the electroporated cells and controls at 37°C and 5% CO_2 for 72 hours, being careful not to agitate the plates during the hours immediately post-electroporation.

Table 9. Preparing electroporation samples for base editing experiment using Pin-point platform in T cells.

Sample	Buffer R	sgRNA (200 µM)	nCas9 mRNA (2 µg/µL)	Rat APOBEC mRNA (1 µg/µL)	Cell suspension in buffer R (250,000 cells)
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and multiple gene-specific Pin-point synthetic sgRNAs.	3.6 µL	0.1 µL of each sgRNA (0.3 µL total when using 3 sgRNA)	0.78 µL	0.22 µL	5 µL
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and individual gene specific Pin-point synthetic sgRNA (one sample per each sgRNA involved in the multiplex experiment).	3.9 µL	0.1 µL	0.78 µL	0.22 µL	5 µL
Pin-point nCas9 mRNA, Pin-point Rat APOBEC mRNA, and Non-targeting control guide RNA.	3.6 – 3.9 µL	0.1 µL as a control for the singleplex or 0.3 µL as control for the multiplex	0.78 µL	0.22 µL	5 µL
Non-electroporated	5 µL	0 µL	0 µL	0 µL	5µL

Volumes are per single sgRNA to be electroporated. It is recommended to prepare sufficient sample volumes for the total number of replicates and to account for pipetting error. Exact reagent amounts for electroporation in other cell lines should be empirically determined through careful optimization prior to experimentation

6. Post electroporation
    a. We recommend checking cell viability 3 days following the electroporation and adjusting cell culture density to maintain approximately 100,000 – 300,000 cells/mL.
    b. At 5 days post electroporation, collect DNA for genomic analysis following Section 5: Guidelines for analyzing base editing with Pin-point analysis primers (Page 6).

Considerations for flow cytometry read-out to assess functional gene knockout

1. Flow cytometry read-out is performed 7 days post electroporation.
    a. We recommend optimizing the antibody panel for the detection of the three surface markers and finalizing compensation matrix for the selected fluorophores ahead of the experiment.

Table 10. Flow cytometry antibody panel

Target	Description	Provider	Cat. No.
TCR a/b	TCRa/b-BV785 antibody	Biolegend	306742
CD52	CD52-PE antibody	BD Pharmingen™	562945
PD1 (CD279)	PD1-APC antibody	Biolegend	329908

2. As a proxy for TRAC knockout, we recommend measuring the surface level of TCR a/b (T cell receptor a/b).
3. While TCR a/b, CD52 is regularly expressed on the surface of T cells, PD1 is present only on the membrane of exhausted T cells. For this reason, to evaluate the levels of functional knockout for PD1 we recommend stimulating T cells before the flow cytometry readout. Stimulation is not required for functional surface marker readout for TCRa/b, and CD52.
    a. To increase PD1 levels on the surface, T cells can be stimulated using Dynabeads Human T-Activator CD3/CD28 or by phorbol myristate acetate (PMA)-Ionomycin treatment. PD1 surface expression has displayed donor-specific behavior in our experience, therefore unedited controls are required for normalization.
    b. Stimulation with Dynabeads Human T-Activator CD3/CD28 should only be performed in the absence of TRAC knockout. When performing simultaneous PD1 and TRAC knockout, PMA-Ionomycin stimulation is recommended.

   c. If stimulation is desired, PMA-Ionomycin treatment can also be used for TRAC, and CD52, but some surface antigens are known to internalize or be affected by the treatment. Therefore, non-edited control cells are paramount for accurate interpretation of results. Additionally, PMA-Ionomycin adversely affects the way cells take up DAPI and should be considered when using this as a live-dead stain.

4. Additional tips and recommendations
   a. In single gene knockout experiments, do not stimulate cells for flow cytometry readout for TCRa/b, and CD52, but stimulate them with Dynabeads Human T-Activator CD3/CD28 for measurement of PD1.
   b. In multiple gene knockout experiments that do not include PD1, do not stimulate cells for flow cytometry readout.
   c. In multiple gene knockout experiments that include PD1, stimulation is required for functional surface marker readout. Stimulation can be via Dynabead Human T-Activator CD3/CD28 if the TCRa/b is intact or by PMA-Ionomycin treatment when TRAC is knocked out.
5. Protocol for PMA-Ionomycin Stimulation
   a. T cells are stimulated with PMA-Ionomycin 48 hours prior to flow cytometry read-out. The later the read-out time point is, the more effective the stimulation will be.
   b. Prepare PMA-Ionomycin stimulation medium by adding PMA and Ionomycin to the T cell medium at 50 ng/mL and 250 ng/mL, respectively.
   c. 100,000 – 300,000 cells are resuspended in 100 µl of stimulation medium in a 96-well plate.
   d. Incubate the stimulated and non-stimulated cultures for 48 hours at 37°C and 5% CO_2.

7. Appendix

Stability and Storage

Pin-point nCas9 and Rat APOBEC mRNA

Pin-point mRNA reagents are shipped on dry ice for overnight domestic delivery or priority international for delivery outside of the U.S. Upon receipt, mRNA should be stored at –80 °C. Each thaw can reduce functionality, so freeze-thaws should be avoided as much as possible. At first thaw, aliquot mRNA stock or working solution into RNase-free tubes at convenient volumes and store at -80 °C to minimize the number freeze-thaws prior to each experiment.

Pin-point synthetic sgRNAs

Pin-point synthetic sgRNA reagents are shipped as dried pellets at room temperature (23 °C). The reagents are stable for at least four weeks. Upon receipt, synthetic guide RNAs should be stored at –20 °C to –80 °C. Under these conditions, the reagents are stable for at least two years. Always resuspend RNA in nuclease-free solutions, such as Tris buffer. In solution and stored at –20 °C, the aliquoted reagents are stable for at least one year.

Pin-point analysis primers

Pin-point analysis primer reagents are shipped as dried pellets at room temperature (23 °C). Under these conditions, they are stable for at least four weeks. Upon receipt, primers should be stored at –20 °C to –80 °C. Under these conditions, the reagents are stable for at least two years. In solution and stored at –20 °C, the aliquoted reagents are stable for at least one year.

8. Frequently Asked Questions

What is the editing window for the Pin-point system?

The Pin-point base editing system uses the cytidine deaminase Rat APOBEC1, which can convert C-G base pairs in the editing window to T-A base pairs. In published base editing systems, the canonical APOBEC1 activity window is positions 4–8 at the PAM-distal end of the protospacer (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7898459). The base targeted by pre-designed sgRNAs is on the product page, written as the base and the position, counting from the 5’ end of the target sequence. For example “C4” means the targeted base is the cytosine at position 4, if the PAM was positions 21–23.

Can I multiplex Pin-point sgRNAs?

Yes – we have used up to 3 sgRNAs in-house to knock out 3 genes simultaneously.

What is the best way to confirm base editing has taken place?

Base editing can be confirmed at the DNA level by sequencing the target site. For pre-designed Pin-point sgRNAs, PCR and sequencing primers are available for purchase. Knockout at the protein level can be confirmed by functional assay.

How are the Pin-point base editing components shipped and stored?

sgRNAs are shipped as dried pellets at room temperature. Pin-point rat APOBEC mRNA and Pin-point nCas9 mRNA are shipped on dry ice. All components should be stored at -80°C upon receipt. Upon first thaw, please consider aliquoting the mRNA and resuspending sgRNA into smaller volumes to limit the number of freeze-thaw cycles.

Are there positive controls for the Pin-point system?

The 3 pre-designed gene targeting sgRNAs available have been validated in-house to produce a functional knockout at the protein level.

What is the Pin-point sgRNA sequence?

The targeting sequence can be found on the product page. The sequence of the full sgRNA is confidential.

Are the Pin-point sgRNAs modified?

Pin-point sgRNAs have modifications on both 5’ and 3’ ends of the molecule to increase nuclease resistance. Pin-point sgRNAs also include an aptamer to recruit the cytosine deaminase base editor, which is fused to an aptamer binding protein.

Can I use Pin-point sgRNA with Edit-R Cas9?

Pin-point sgRNAs should not be used with Edit-R Cas9 for DNA cutting. For knockout, please consider using pre-designed Edit-R sgRNAs, or designing a custom sgRNA with the CRISPR design tool.

Can I use Edit-R sgRNAs with Pin-point Rat APOBEC mRNA and Pin-point nCas9 mRNA?

The Pin-point guide RNA contains an aptamer sequence to recruit the deaminase. Therefore, to conduct a base editing experiment, you must use a Pin-point sgRNA.

Can I use Pin-point reagents for simultaneous knock-in and knockout?

For knockout alongside HDR-mediated knock-in applications, Pin-point sgRNAs can be multiplexed with a pair of custom-designed Edit-R sgRNAs. This allows for simultaneous targeted knock-in via introduction of a staggered dsDNA break by the nCas9 component of the Pin-point system when an appropriate donor template is provided. This feature enables complex multiplex knock-in and knockout genome editing outcomes.

Can I use the Pin-point system in non-mammalian cells?

We use the Pin-point system in human cell lines and primary cells in-house, but use in other species has not been tested. The Pin-point mRNAs contain a mammalian codon-optimized nCas9, and a mammalian codon-optimized deaminase and aptamer binding protein fusion. Base editing with the Pin-point system requires that the guide RNA targets the desired edit site. Therefore, custom sgRNAs may be required for base editing in species other than human.

Which deaminases are available in the Pin-point system?

The Pin-point system is modular: the sgRNA contains an aptamer to recruit an aptamer binding protein, which is fused to the cytosine deaminase, Rat APOBEC. The same sgRNA also recruits nCas9.

Which formats of the Pin-point system are available?

We offer Pin-point Rat APOBEC mRNA, Pin-point nCas9 mRNA, and synthetic Pin-point single guide RNA.

If you have questions or comments, please reach out to Scientific Support.

9. References

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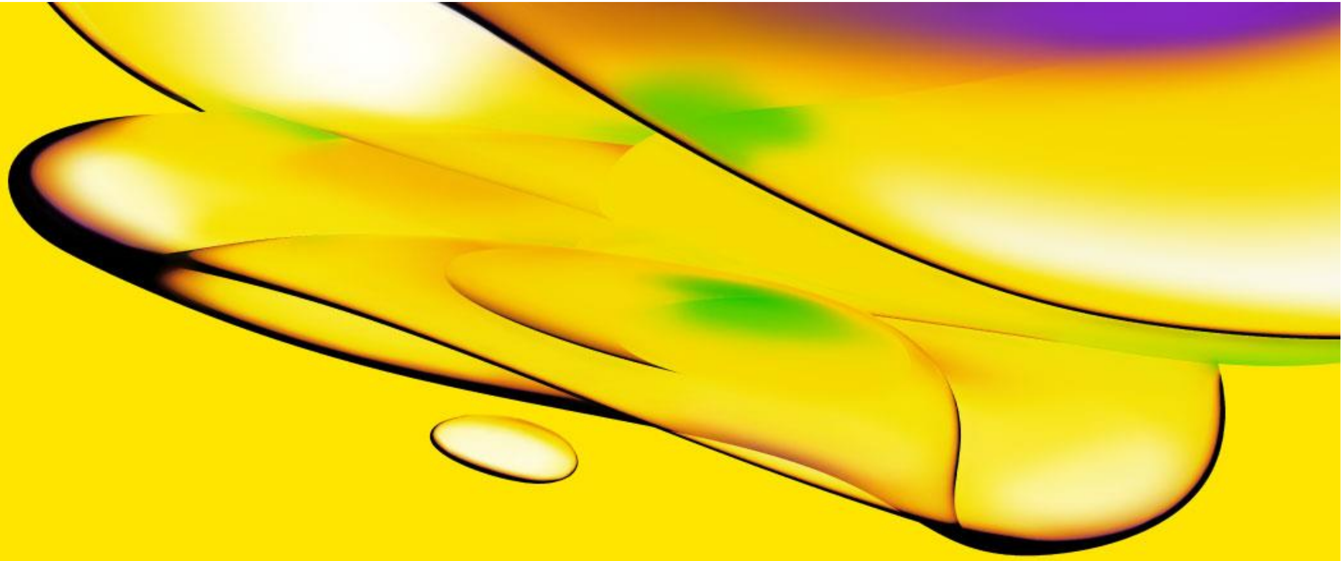

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