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Gene Knock-in by CRISPR/Cas9 and Cell Sorting in Macrophage and T Cell Lines

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TITLE:

Gene Knock-in by CRISPR/Cas9 and Cell Sorting in Macrophage and T Cell Lines

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knock-in; CRISPR/Cas9; *Rosa26*; macrophage; T cell; hACE2

SUMMARY:

This protocol uses fluorescent reporters and cell sorting to simplify knock-in experiments in macrophage and T cell lines. Two plasmids are used for these simplified knock-in experiments, namely a CRISPR/Cas9- and DsRed2-expressing plasmid and a homologous recombination donor plasmid expressing EBFP2, which is permanently integrated at the *Rosa26* locus in immune cells.

ABSTRACT:

Functional genomics studies of the immune system require genetic manipulations that involve both deletion of target genes and addition of elements to proteins of interest. Identification of gene functions in cell line models is important for gene discovery and exploration of cell-intrinsic mechanisms. However, genetic manipulations of immune cells such as T cells and macrophage cell lines using CRISPR/Cas9-mediated knock-in are difficult because of the low transfection efficiency of these cells, especially in a quiescent state. To modify genes in immune cells, drug-resistance selection and viral vectors are typically used to enrich for cells expressing the CRIPSR/Cas9 system, which inevitably results in undesirable intervention of the cells. In a

previous study, we designed dual fluorescent reporters coupled to CRISPR/Cas9 that were transiently expressed after electroporation. This technical solution leads to rapid gene deletion in immune cells; however, gene knock-in in immune cells such as T cells and macrophages without the use of drug-resistance selection or viral vectors is even more challenging. In this article, we show that by using cell sorting to aid selection of cells transiently expressing CRISPR/Cas9 constructs targeting the *Rosa26* locus in combination with a donor plasmid, gene knock-in can be achieved in both T cells and macrophages without drug-resistance enrichment. As an example, we show how to express human ACE2, a receptor of SARS-Cov-2, which is responsible for the current Covid-19 pandemic, in RAW264.7 macrophages by performing knock-in experiments. Such gene knock-in cells can be widely used for mechanistic studies.

INTRODUCTION:

Immune cells are critical for defense against pathogens. Both innate and adaptive immunity are required for clearance of infectants and maintenance of tissue homeostasis[1,2]. Cell line models are essential tools for understanding the molecular fundamentals of the mammalian immune system; they are used in *in vitro* functional assays, such as those modeling human T cell activation, and in determining the function of genetic factors in activating or dampening immune responses[3,4]. It is important to note that the mammalian immune system is enormously heterogeneous and, equally important, a huge number of molecules control the differentiation, migration, and function of a given cell type[5,6].

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 genome editing tools allow for genetic manipulation of specific cell types, which facilitates functional annotation of genes in a precise manner[7,8]. Several published protocols have described the delivery of CRISPR/Cas9 in the form of Cas9-guide RNA complexes known as a ribonucleoproteins (RNPs) in HEK293 cells, Jurkat cell lines, primary T cells[9,10], macrophages[11,12,13], stem cells[14], and others[15,16]. In these protocols, gene tagging is usually achieved by fusing a fluorescent tag to endogenous proteins[17,18]. However few attempts have been made to use dual fluorescent reporters, which are compatible with single cell sorting, to facilitate knock-in experiments[19,20], particularly in immune cells.

In-depth mechanistic analyses aimed at understanding the functions of a novel genetic factor in immune cells generally require cell-type specific deletion of a gene, genetic rescue experiments, and ideally identification of its interactors. Even though methods for optimization of genetic deletion of genes in immune cells have been published[9,15,21], far fewer methods have been reported for introducing knock-in alleles with versatile functions to understand the immune response. Therefore, in this protocol we aim to describe in detail an efficient and highly reproducible protocol to express a protein of interest (POI) at the safe harbor locus *Rosa26* in both human and murine immune cell lines. We designed a two-color reporter system to enrich for cells transfected with plasmids expressing CRISPR/Cas9 (DsRed2) and a recombinant DNA template (EBFP2), which can be isolated by cell sorting. Following this protocol, we obtained multiple knock-in lines of the human T cell line Jurkat and murine macrophage RAW264.7 for functional analyses of poorly studied proteins.

As an example, we show in this protocol how to obtain knock-in RAW264.7 macrophages stably expressing human ACE2 (a receptor of SARS-Cov-2)[22]. Because innate immune cells are involved in the pathogenesis of Covid-19[23,24] and human ACE2 is regarded as a major receptor required for viral entry into cells before replication, macrophages with knock-in of human ACE2 can serve as a useful tool for mechanistic studies of viral multiplication inside macrophages. In parallel, we also present an example of knock-in of a gene at the human *ROSA26* locus to express the RASGRP1 protein, which was fused at its amino terminus with an affinity Twin-Strep-tag (OST). T cells are key target cells in immune therapies, and an increasing number of studies have focused on manipulation of their responsiveness to cancer[25,26]. As Rasgrp1 is known to be a key signaling molecule downstream of the T cell receptor and its interactors are not well elucidated[27], the OST-RASGRP1 knock-in model provides the foundation for identifying interactors regulating the response of T cells to tumors and infection. Taken together, these tools can be used for Covid-19 studies and the discovery of novel molecules interacting with Rasgrp1.

PROTOCOL:

1. Design and Plasmid Construction of sgRNAs Targeting *Rosa26* Locus

1.1 Design guide RNAs around the desired insertion site

1.1.1 Ensure that the insertion site for mouse *Rosa26* (hereafter designated as *mRosa26*) knock-in experiments is located in the first intron of *mRosa26*; this site has been used in previous studies[28,29]. For knock-in experiments in human cells, ensure that the insertion site resides in the human *ROSA26* locus (hereafter referred as *hROSA26*), which has been identified as the human homolog of *mRosa26*[30].

1.1.2 Use the genomic sequences of mouse and human species obtained from Mouse Genome Informatics (http://www.informatics.jax.org/) and Ensembl genome browser (http://www.ensembl.org/index.html), respectively. Copy 50 base pairs (bp) of the genomic sequence of the *mRosa26* or *hROSA26* locus on each side flanking the desired insertion site.

1.1.3 Design guide RNAs using the online web tool CRISPOR (http://crispor.tefor.net/)[31]. Paste the 100 bp of input sequence from 1.1.2. Select two guide RNAs with a high specificity score, a high efficiency score, and low off-target activity. In addition, choose RNA guides with higher Doench scores and avoid those labeled as "Inefficient"[32].

NOTE: Alternative CRISPR design tools are also valuable and publicly available, for instance the Benchling CRISPR design tool (https://benchling.com/crispr). To increase the frequency of CRISPR/Cas9-mediated knock-in mutated cells, select two guide RNAs close to the desired insertion site when possible[33].

1.1.4 For the *mRosa26* locus, use two adjacent guide RNAs designated as mR26-sg1 (5'-CTCCAGTCTTTCTAGAAGAT -3') and mR26-sg2 (5'- CGCCCATCTTCTAGAAAGAC -3') (**Figure 1A**). For the *hROSA26* locus, use two adjacent guide RNAs, namely hR26-sg1 (5'-

GGCGATGACGAGATCACGCG -3') and hR26-sg2 (5'- AATCGAGAAGCGACTCGACA -3') (**Figure 1B**).

NOTE: Genome editing activities of mR26-sg1 and mR26-sg2 and those of hR26-sg1 and hR26-sg2 were evaluated in RAW264.7 cells and Jurkat cells, respectively (See **Supplementary File, Figure S1**).

1.2 Cloning of CRISPR expression vectors containing sgRNA targeting the *Rosa26* Locus

1.2.1 Synthesize forward and reverse oligos for one guide RNA (See **Supplementary File**).

NOTE: It is preferable to add a 'G' nucleotide to the start of the guide sequence, which helps with expression driven by the U6 promoter. For example, to clone the aforementioned mR26-sg1, the forward oligo is CACC**G**CTCCAGTCTTTCTAGAAGAT and the reverse oligo is AAACATCTTCTAGAAAGACTGGAG**C**. The additional G in the forward oligo and its complement in the reverse oligo are indicated in bold.

1.2.2 Clone synthetic oligos corresponding to the guide RNAs into the all-in-one CRISPR expression vector pX458-DsRed2 generated in our previous work[21] (**Figure 1C**) using the general protocol for gRNA cloning developed by Zhang's Lab (https://www.addgene.org/crispr/zhang/); however, skip the gel purification step and purify the digested vector using a PCR purification kit (see **Table of Materials**).

NOTE: In previous protocols, gel purification of the BbsI-digested vector was performed; however, this step is time-consuming. In the present protocol, the digested product is purified using a PCR purification kit and used directly for the downstream ligation reaction, which generally produces positive colonies with comparable efficiency.

1.2.3 Transform 10 µL of the ligation product (step 1.2.2) into 50 µL of *Escherichia coli* DH5α competent cells according to the manufacturer's instructions. Randomly pick three colonies from an LB agar plate with ampicillin (100 µg/mL) and inoculate each into 3 mL of LB medium with ampicillin for culturing overnight.

1.2.4. Use 1 mL of the overnight bacterial culture for Sanger sequencing and keep the rest at 4 °C until the construct is confirmed to be correct: pDsR-mR26-sg1 and pDsR-mR26-sg2 for *mRosa26* locus knock-in, and pDsR-hR26-sg1 and pDsR-hR26-sg2 for the *hROSA26* locus.

1.2.5 Prepare a high concentration of plasmid DNA (approximately 2 µg/µL) with a maxiprep kit for purification of transfection-grade plasmid (see **Table of Materials**).

2. Design and Construction of Targeting Vectors as Homologous Recombination Templates

2.1 Design a homology-directed repair template

2.1.1 Design a targeting vector to constitutively express the POI using an expression cassette

optimized from a previous study[29], which includes a CAG hybrid promoter, an AscI restriction site permitting cloning of the cDNA of the POI, an IRES-EBFP2 reporter, and a bovine growth hormone polyadenylation (bGH-polyA) signal (**Figure 1B** and **Supplementary File**).

2.1.2 Design homology arms (HAs) that share homology with the genomic sequences of the mRosa26 or hROSA26 locus. Include ~1 kb of the 5' HA corresponding to the upstream genomic sequence from the desired insertion site to the left of the expression cassette. Similarly, include ~1 kb of the 3' HA corresponding to the downstream genomic sequence from the insertion site to the right of the expression cassette.

NOTE: The expression cassette is inserted as close as possible to the CRISPR/Cas9 cleavage sites[34]. To avoid re-cutting of the targeted allele by CRISPR/Cas9, incorporate nucleotide changes into the protospacer-adjacent motif (PAM) sequence (5'-NGG-3') in the targeting vector[34,35].

2.1.3 To allow linearization of the targeting vector, insert a unique EcoRI site just upstream of the 5' HA and a unique BamHI site just downstream of the 3' HA.

2.1.4 Have a commercial vendor synthesize the targeting vectors and designate them as pKR26-iBFP and pKhR26-iBFP for *mRosa26* and *hROSA26* knock-in, respectively.

2.2 Construct a targeting vector as a homology-directed repair template

2.2.1 To express the POI, obtain the cDNA sequence (coding sequence) from the Ensembl genome browser and choose the transcript possessing the longest protein sequence. For example, the cDNA of the human *ACE2* gene is ACE2-202 ENST00000427411.2 and the cDNA of human *RASGRP1* gene is RASGRP1 ENSG00000172575.

2.2.2 Synthesize the cDNA of the POI with the help of a commercial vendor. It is also possible to clone the cDNA via PCR amplification (not described here). Place the sequence ***GGCGCGCC***<u>ACC</u> (5' - 3'), which includes an AscI restriction site (italics and bold) and Kozak consensus translation initiation site (underlined), immediately before the ATG initiation codon of the cDNA and another AscI restriction site (5'- GGCGCGCC -3') after the stop codon of the cDNA.

2.2.3 Digest the synthesized sequence with AscI and purify using a PCR purification kit designed for purifying DNA fragments after restriction digestion, following the manufacturers' instructions (see **Table of Materials**).

2.2.4 Ligate the purified cDNA insert into the AscI-linearized backbone vector pKR26-iBFP or pKhR26-iBFP using T4 DNA ligase following the manufacturer's instructions.

2.2.5 Verify the final targeting vector, pKR26-POI-iBFP or pKhR26-POI-iBFP, by sequencing. Use maxiprep to purify the verified constructs according to the manufacturer's protocol.

2.2.6 Digest the targeting vector using either EcoRI or BamHI and purify the digestion product

using a PCR purification kit according to the manufacturer's instructions. Store the linearized targeting vector (~0.8 µg/µL) at -20 °C until electroporation.

3. Electroporation of Macrophage and T Cell Lines

3.1 Prepare cell cultures for electroporation

3.1.1 Prepare Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS) as complete growth medium for Jurkat T cells. Prepare Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS for culturing RAW264.7 macrophages. Supplement all complete growth media with 100 U/mL penicillin and 100 µg/mL streptomycin (Pen/Strep) except for media used for post-transfection incubation before cell sorting.

3.1.2 Perform subculturing of RAW264.7 and Jurkat T cells according to the supplier's instructions (See **Table of Materials**). When subculturing RAW264.7 cells, use trypsin-EDTA solution (0.25%) to detach cells. Use FBS supplemented with 10% (v/v) DMSO as a cryopreservation medium for RAW264.7 and Jurkat cells.

3.1.3 Collect cells at 250 × g for 5 min for RAW264.7 macrophages and 90 × g for 8 min for Jurkat T cells. Then wash with 5–10 mL of 1× DPBS (without Ca^{2+} or Mg^{2+} ions). Remove the DPBS.

3.1.4 Resuspend the cell pellet using 2 mL of 1× DPBS. Use 10 µL of cells and mix with an equal volume of 0.2% trypan blue to estimate the cell count and viability.

NOTE: Ensure that the cell culture has >90% viability on the day of transfection.

3.1.5 For a single knock-in experiment, perform electroporation with 10 µL nucleofection tips with five repetitions. Calculate the volume needed for 2.0×10^6 cells and pellet the cells by centrifugation. Wash the cell pellet again with 1× DPBS as described in step 3.1.3.

NOTE: When using 10 µL nucleofection tips, 4.0×10^5 cells are needed per electroporation. Accordingly, prepare at least 2.0×10^6 cells for one knock-in experiment.

3.1.6 Prepare a 24-well plate with 0.5 mL of complete growth medium (prepared in step 3.1.1) per well without Pen/Strep and prewarm in a 37 °C incubator.

3.2 Electroporation of CRISPR/Cas9 components and the targeting vector

3.2.1 Turn on the electroporation system. Use electroporation parameters optimized in a previous study[21]: 1,400 V/20 ms/2 pulses for RAW264.7 macrophages and 1350 V/20 ms/2 pulses for Jurkat T cells.

3.2.2 Accounting for sample loss due to pipetting, prepare a 55 µL electroporation mixture in a sterile 1.5 mL microcentrifuge tube containing 2.5 µg of each CRISPR/Cas9 vector, 2.4 µg of the

linearized targeting vector, and the Resuspension Buffer R.

NOTE: To save time, the electroporation mixture can be prepared during centrifugation (step 3.1.5).

3.2.3 Resuspend 2.0×10^6 cells (prepared in step 3.1.5) in the 55 µL electroporation mixture from step 3.2.2.

3.2.4 Aspirate the cell/electroporation mixture from step 3.2.3 using a 10 µL nucleofection tip with a pipette.

NOTE: During pipetting, avoid introducing air bubbles, which may cause electroporation failure.

3.2.5 Add the sample to a tube filled with 3 mL of Buffer E from the electroporation kit.

3.2.6 Apply the electroporation parameters for the two cell types as described in step 3.2.1.

3.2.7 Transfer the sample into one well of the 24-well plate with prewarmed medium from step 3.1.6.

3.2.8 Repeat steps 3.2.4–3.2.7 for the other four repetitions as well as for the targeting vector only and CRISPR expression vector only controls.

NOTE: Change the nucleofection tip and tube when switching to a different cell type/plasmid DNA.

3.2.9 Culture the transfected cells for 48–72 h to allow for recovery after electroporation and expression of CRISPR/Cas9 components prior to flow cytometry analysis or fluorescence-activated cell sorting (FACS). Examine the expression of DsRed2 using a fluorescent microscope equipped with a red channel at 24 h post-transfection.

NOTE: A benefit of monitoring DsRed2 fluorescent cells is that the efficiency of electroporation can be estimated and the suitability for further cell sorting can be predicted.

4. Cell Sorting to Isolate Putative Knock-in Cells

4.1 Before FACS sorting, wash the transfected cells once with complete growth medium. Pellet cells by centrifugation as described in step 3.1.3 and resuspend the pellet in 500 µL of fresh medium.

4.2 Set the cell sorter (located in a biosafety cabinet) with an 85 µm nozzle and low flow rate. Select the “single cell” mode for sorting and test the single cell sorting efficiency and precision by sorting 20–30 cells onto the cover of 96-well microplate. One droplet localized at the center of each well indicates proper setup of the instrument.

4.3 Transfer the cell suspension from step 4.1 into sterile FACS tubes and add SYTOX Red Dead Cell Stain to a final concentration of 1 nM.

4.4 Analyze samples on the cell sorter. SYTOX Red and EBFP2 are excited by a 405 nm laser and detected by the V450/BV421 and APC channels, respectively. DsRed2 is excited by a 488 nm laser and detected by the PE channel. Use non-transfected cells and EBFP2- and DsRed2-single positive cells as controls for spectral compensation.

4.5 Sort 10 single cells into each well of a 96-well microplate containing 150 µL per well of pre-warmed complete growth medium. Use round bottom microplates for culturing suspension cell lines such as Jurkat T cells and flat bottom microplates for adherent RAW264.7 macrophages.

NOTE: Sorting of 10 cells per well is an optimized strategy for improving cell survival and minimizing the number of 96-well microplates that need to be seeded and the number of samples that need to be screened by flow cytometry and genotyping; if sorting only one cell per well, seeding of more microplates is required to increase the chance of obtaining correctly edited cells.

5. Screening and Validation of Positive Knock-in Cells

5.1 Screening for candidate knock-in cells by flow cytometry

5.1.1 Incubate the cells from step 4.5 for 10–15 days and add complete growth medium every 3 days to replace liquid lost through evaporation. For Jurkat T cells, transfer cells grown in the round bottom 96-well plate to a flat bottom plate to optimize cell proliferation.

5.1.2 Transfer the expanded sorted cells to 48-well plates for further expansion.

5.1.3 When cells are close to confluence, use half of the culture to screen for EBFP2 expression by flow cytometry (**Figure 3**). Normally, half of the culture in a 48-well plate after confluent growth equates to 0.5–1.0 × 10^5 cells, which is adequate for analysis of one sample by flow cytometry.

5.1.4 Transfer the remaining cells to 24-well plates for further proliferation.

5.1.5 Keep the candidate cell populations possessing a high percentage of EBFP2-positive cells for further genotyping experiments.

NOTE: Because 10 cells are sorted into each well of the 96-well microplate, it is possible that the knock-in cells will grow with cells that do not express the knock-in gene. Therefore, it is normal to perform an additional round of cell sorting to separate the EBFP2-positive cells from the negative cells.

5.2 Screening for candidate knock-in cells by PCR and sequencing

5.2.1 Collect 2 × 10^5 candidate cells. Extract the genomic DNA using a DNA prep kit (See **Table of Materials**).

5.2.2 To verify that precise homology-directed repair (HDR) has occurred at the m*Rosa26* locus rather than at random sites in the genome of the candidate knock-in cells, perform PCR with primers spanning each side of the HAs (**Figure 4**). Choose one primer located in the genomic region outside of the targeting vector (external oligo) and another primer located inside the targeting vector (internal oligo).

NOTE: A similar design is used to verify HDR at the *hROSA26* locus. PCR primers can also be easily designed for detecting the insertion of the POI sequence. In addition, the wild-type and knock-in allele genotypes can be simultaneously detected using three-primer PCR (See **Supplementary File, Figure S2**).

5.2.3 Clone the positive PCR products using a fast-cloning kit (see **Table of Materials**). Transform the reaction mixture into DH5α competent cells.

5.2.4 Randomly pick 8–10 individual bacterial colonies per transformation and sequence the PCR products from step 5.2.3 by Sanger sequencing.

5.3 Validation of positive knock-in cells by immunoblot analysis and affinity purification

NOTE: Candidate cells are subjected to immunoblot analysis for validation of insertion of the POI, or affinity purification (AP) for studies of protein-protein interaction.

5.3.1 Perform immunoblot analysis according to antibody manufacturer's instructions. See **Table of Materials** for information regarding the use of primary antibodies and secondary antibodies.

5.3.2 Subject the lysates of the knock-in cells expressing the OST-tagged POI to AP using Strep-Tactin Sepharose beads following the manufacturer's instructions (see **Table of Materials**).

5.3.3 Detect chemiluminescence using an imager.

REPRESENTATIVE RESULTS:

Following the protocol described above to perform knock-in experiments at the *mRosa26* locus using murine RAW264.7 macrophages, we designed a targeting vector to express human ACE2, a receptor for the SARS-Cov-2 virus (**Figure 2A**). Using a similar design, we generated human Jurkat T cells with knock-in of the OST-tagged RASGRP1 fusion protein (**Figure 2C**). After transfection of three plasmids, two of which were used for expression of CRISPR/Cas9 (DsRed2; pDsR-mR26-sg1 and pDsR-mR26-sg2 for *mRosa26* knock-in; pDsR-hR26-sg1 and pDsR-hR26-sg2 for the *hROSA26* knock-in) and another used as a DNA template for homologous recombination (EBFP2), double positive cells expressing two fluorescent reporters were sorted into a 96-well plate. Of the RAW264.7 cells sorted using the single cell sorting mode, 0.91% were DsRed2$^+$

EBFP2$^+$, but 10 cells were collected into each well (**Figure 2B**). The Jurkat T cells had a higher transfection efficiency, and the percentage of double positive cells was 7.91% in this representative experiment, demonstrating successful knock-in of the OST-RASGRP1 fusion protein (**Figure 2D**).

In the next step, flow cytometry was used to screen for the candidate wells with EBFP2-positive cells. Representative histograms showed that there were obvious EBFP2-positive populations (**Figure 3**). It is notable that the knock-in cells did not express DsRed2 when flow cytometry was performed two weeks after cell sorting.

For discrimination of precise knock-ins and random insertions, genomic DNA from the EBFP2-positive cells was further tested by performing PCR with primers recognizing genomic sequence external to the HAs of the targeting vector and the specific region inside the expression cassette (**Figure 4A** and **4B**). Sequence analysis of these PCR products confirmed the occurrence of HDR at the *mRosa26* target site (**Figure 4C**). The same genotyping strategy can be applied to validate the precise insertion of the expression cassette in the *hROSA26* locus of Jurkat T cells.

To obtain a pure population of hACE2-expressing RAW264.7 cells, we further sorted the cells and validated the presence of the fluorescent reporters following expansion using wild-type cells as controls (**Figure 5A**). The expanded cells were EBFP2 positive, and the hACE2 protein was readily detectable in murine RAW264.7 macrophages (**Figure 5B** and **5C**). Similarly, we validated the expression of fluorescent reporters and the OST-RASGRP1 protein in human Jurkat T cells after screening and a second round of cell sorting (**Figure 6A** and **6B**). In addition, affinity purification of the OST-RASGRP1 protein was performed using commercially available beads. We found a higher amount of RASGRP1 protein in the total cell lysates of knock-in Jurkat cells than in those of wild-type cells; RASGRP1 knockout Jurkat cells were used as controls (**Figure 6C**). After purification using the OST tag, only the knock-in samples had detectable RASGRP1 (**Figure 6D**).

FIGURE AND TABLE LEGENDS:

Figure 1. Gene targeting strategy for generating *mRosa26*/*hROSA26* knock-in cell lines overexpressing a protein of interest. (A) *mRosa26*-specific guide RNA targeting sequences and the protospacer adjacent motifs (PAMs) in the desired insertion site are indicated in blue and red letters, respectively. Cas9 normally cleaves 3–4 bp upstream of the PAM sequence, which is indicated by green arrows. The targeting vector is used as a template for homology-directed repair (HDR) leading to the precise insertion of the expression cassette in the mouse or human genome. Each of the 1 kb sequences upstream and downstream of the desired target site are used as 5' and 3' homology arms (HAs) in the targeting vector. The HAs are separated by an expression cassette consisting of a CAG promoter, the cDNA sequence of the protein of interest (POI) with an OST-tag, an IRES-EBFP2 reporter, and a bGH-polyA signal (pA). The restriction site AscI is used for cloning of the POI, and EcoRI and BamHI are used for linearization of the targeting vector. The strategy for CRISPR/Cas9-mediated knock-in at the *hROSA26* locus is similar. **(B)** Diagram of the human *ROSA26* locus. The hR26-sg1 and hR26-sg2 targeting sequences are indicated in blue letters and the corresponding PAM sequences in red. **(C)** Schematic for the all-in-one CRISPR expression vector pX458-DsRed2, which contains a human U6-driven sgRNA

expression cassette and Cas9-T2A-DsRed2 fluorescent reporter cassette. Two BbsI restriction sites allow for the cloning of the guide RNA. U6, human U6 RNA polymerase III promoter; sgRNA, a chimeric single-guide RNA; CBh, a chicken β-actin hybrid promoter; NLS, nuclear localization signal; T2A, Thosea asigna virus 2A self-splicing peptide; bGH-pA, bovine growth hormone polyadenylation signal.

Figure 2. Single cell sorting of RAW264.7 and Jurkat cells transfected with CRISPR/Cas9 vectors and targeting vectors for homologous recombination. Targeting vectors used for stable gene expression in RAW264.7 **(A)** and Jurkat **(C)** cells. Representative flow cytometric plots of mouse RAW264.7 macrophages **(B)** and human Jurkat T cells **(D)** transfected with CRISPR/Cas9 vectors expressing the DsRed2 reporter and the targeting vector expressing the EBFP2 reporter. Cells co-expressing DsRed2 and EBFP2 were subjected to cell sorting and cultured for expansion; numbers adjacent to outlined areas indicate the percentage of cells in each gate, and non-transfected cells were used as a negative control.

Figure 3. Screening of knock-in cells by detection of EBFP2 expression. Flow cytometric analysis of $EBFP2^+$ and $DsRed2^+$ RAW264.7 macrophages **(A)** and Jurkat T cells **(B)** 14 days after electroporation. In the histograms, fluorescence intensity (FI) of either $EBFP2^+$ or $DsRed2^+$ cells is displayed on the X-axis and the count of events in each fluorescence channel is displayed on the Y-axis. **(A)** The expression of EBFP2 and hACE2 was driven by the same promoter at the *Rosa26* locus in RAW264.7 murine macrophages. **(B)** In Jurkat cells, OST-RASGRP1 expression is linked to EBFP2 expression at the human *ROSA26* locus. At 14 days post-electroporation, flow cytometric analysis revealed nearly zero $DsRed2^+$ cells among the sorted cells. Wild-type (WT) cells were used as a negative control, and KI stands for knock-in cells.

Figure 4. Screening for candidate knock-in cells by PCR and sequencing. (A) Strategy for generating hACE2 knock-in cells. The positions of PCR primers used to distinguish precise HDR and random insertion are indicated by green arrows. **(B)** PCR genotyping of five candidate cells (#4, #15, #22, #25, and #43) that were identified by flow cytometry screening for EBFP2 expression as exemplified in **Figure 3**, showed that both the 5' junction (1472 bp) and 3' junction (1472 bp) spanning the homology arms were correct. M, DNA ladder; WT, the wild-type RAW264.7 control; H_2O, negative control. **(C)** Sanger sequencing of the PCR products from **B** revealed successful knock-in of the hACE2-expression cassette into the *mRosa26* locus without mutations.

Figure 5. Validation of successful knock-in of the hACE2 expression cassette into the *mRosa26* locus in RAW264.7 macrophages. (A) WT RAW264.7 macrophages were used as a negative control for flow cytometric analysis. **(B)** To separate the EBFP2-positive cells from the negative cells, an additional round of cell sorting was performed to obtain a population consisting of nearly 100% $EBFP2^+$ knock-in cells, designated as hACE2 KI cells. DsRed2 expression was also examined to ensure that the CRISPR/Cas9 plasmid was not integrated into the genome of RAW264.7 macrophages. In the histograms, fluorescence intensity (FI) of either $EBFP2^+$ or $DsRed2^+$ cells is displayed on the X-axis and the number of events in each fluorescence channel is displayed on the Y-axis. **(C)** Detection of hACE2 expression by immunoblot analysis using rabbit anti-human

ACE2 monoclonal antibody. Expression of hACE2 was observed in cells from multiple wells (#4, #15, #22, #25, and #43). WT RAW264.7 macrophages were used as a negative control and GAPDH was used as the loading control.

Figure 6. Validation of successful knock-in of the OST-RASGRP1 expression cassette into the *hROSA26* locus in Jurkat T cells. (A) WT Jurkat T cells were used as a negative control for flow cytometric analysis. **(B)** $EBPF2^+$ subpopulations of Jurkat cells were enriched by additional rounds of cell sorting and expanded; these cells were designated as OST-RASGRP1 KI cells. The knock-in cells were analyzed by flow cytometry and did not keep the DsRed2-expressing vector. In the histograms, the fluorescence intensity (FI) of either $EBFP2^+$ or $DsRed2^+$ cells is displayed on the X-axis and the count of events in each fluorescence channel is displayed on the Y-axis. **(C)** Detection of OST-RASGRP1 expression in two independent knock-in cells (#1 and #2) by immunoblot analysis using anti-RASGRP1 antibody. WT Jurkat and RASGRP1-knockout Jurkat cells were used as controls and β-actin was used as the loading control. **(D)** OST-mediated affinity purification was used to validate the expression of OST-RASGRP1 using RASGRP1 knockout cells as a negative control. Immunoblot analysis of equal amounts of proteins from cell lysates that were either subjected to affinity purification on Strep-Tactin Sepharose beads (Affinity purification) or directly analyzed (Total lysates) and probed with RASGRP1 or GAPDH (loading control) antibodies.

Supplemental File: Supporting figures, table, and sequences

DISCUSSION:

In our experiments, we demonstrated how to perform knock-in editing in immune cells from construct design to knock-in cell screening and validation using human Jurkat T cells and murine RAW264.7 macrophages as examples. Both T cell and macrophage cell lines are resistant to transfection[36,37]; however, the problem of low efficiency of CRISPR/Cas9 delivery can be overcome with the aid of fluorescent reporters coupled with cell sorting. This protocol is suitable for gene rescue experiments and protein-protein interaction experiments, but it cannot be applied to the study of regulatory DNA sequences such as binding sites of transcription factors because the protocol was developed for knock-in modification of the *Rosa26* locus.

Dual fluorescent reporters aided CRISPR/Cas9 knock-in editing

We successfully applied a dual fluorescent reporter system to transiently express independent sets of CRISPR/Cas9 vectors in immune cells, which resulted in deletion of large fragments of DNA in previous studies[21]. We designed a CRISPR/Cas9 targeting tool using the DsRed2 fluorescent protein as a reporter and an additional spectrally distinct fluorescent protein to track delivery of the DNA template for knock-in modification. To make knock-in allele modification feasible, we used the EBFP2 fluorescent protein reporter, which has no spectral spillover with the RFP (DsRed2 in our case), to monitor transfection of the donor DNA serving as the template during homologous recombination. To optimize the cassette expressing the POI and fluorescent reporter, an IRES sequence was introduced to obtain independent proteins. In our previous study, we noted that the residual amino acids from the P2A or T2A linker remaining after post-transcriptional cleavage affected protein localization on the cell surface. The IRES sequence does

not leave such residual amino acids. As described in a previous study, the IRES gave rise to higher levels of the fluorescent reporter, following expression of the Cas9 protein driven by CAG promoter[38].

All-in-one CRISPR/Cas9 plasmid

Various formats and combinations of the CRISPR/Cas9 system have been described in previous studies, such as Cas9 transfection as an mRNA or protein together with chemically synthesized sgRNAs[39]. CRISPR/Cas9 RNP complexes have also been delivered into mammalian cells; this strategy offers the advantages of earlier onset of nuclease activity and a shorter half-life; however, labeling the RNPs is less cost effective compared with constructing all-in-one plasmid. In our previous studies, we found that using single cell sorting to isolate those rare cells expressing CRISPR/Cas9 (DsRed2 positive) and the knock-in protein (EBFP2 positive) is far less complicated than using RNP delivery, and it is easy to prepare the plasmids. It is true that this protocol relies on single cell sorting. But our protocol is easy to perform and yields successful knock-in modifications with high reproducibility.

Expression of a POI from the *Rosa26* locus

There are multiple reports in the literature describing methods for tagging endogenous proteins with fluorescent reporters using CRISPR/Cas9 editing[40,41,42]. The advantages of endogenous tagging are that it is feasible to determine subcellular localization and perform *in vivo* tracking of the endogenous protein. However, problems may be encountered if it is not possible to design an appropriate CRIPSR guide RNA at the endogenous locus. Here we developed an alternative knock-in method by incorporating POI-IRES-EBFP2 into the genomic safe harbor locus *Rosa26*, which overcomes the limitations of finding appropriate guides for positioning endogenous tags.

We summarize a few key points that need to be considered during the experiments to ensure technical reproducibility. First, the cell sorter needs to have a 405 nm violet laser for excitation of EBFP2 and a 488 nm blue laser or alternatively a 561 nm yellow laser for excitation of DsRed2. With such configurations, EBFP2 and DsRed2 can be detected with no spectral spillover, which may lead to false positive results. In our experiments, the proportion of $DsRed2^{+}$ $EBFP2^{+}$ double positive cells was as low as 0.9%; therefore, gating of the proper population was essential for the success of the experiments. A second round of sorting was performed to gate the $EBFP2^{+}$ positive cells, followed by PCR validation. In addition, for knock-in protein detection, it is preferable to introduce a protein tag such as OST or another type of tag. Knockout of the gene prior to *Rosa26* locus knock-in experiments provides a good opportunity to assess whether the antibody has desirable specificity. When the antibody specificity is not sufficient, detection of the knock-in protein should be performed following pulldown via the protein tag. Finally, during FACS screening of the cells expressing the knock-in allele, the intensity of EBFP2 can be used to assess whether two copies or one copy of the knock-in allele is present.

Applications

In this protocol we described the knock-in modification of RASGRP1, a key molecule involved in T cell activation[27]. We first obtained RASGRP1-knockout Jurkat cells which can be used for loss-of-function studies, and we generated additional Jurkat cells expressing OST-RASGRP1 at the

hROSA26 locus. Jurkat cells are the most used human cell line for studying T cell biology[43]. Because of the success of immunotherapy in preventing T cell exhaustion in cancer patients, immunologists and cancer biologists have great interest in modifying Jurkat cells for functional studies of candidate molecules. It is also noteworthy that the Jurkat T cell line is commonly used for dissection of signaling pathways, but there are limitations to using this cell line, as Jurkat cells are poor producers of IFN-γ upon stimulation[44]. Previous studies used both endogenous locus modification[45] and genetic engineering at the *hROSA26* locus[46] to perform knock-in experiments in human Jurkat cells. Both strategies have their own advantages; by modifying the endogenous locus the protein is presumably expressed at a "physiological" level. Knock-in modification at the *hROSA26* locus produces predictable results because alternative splicing of mRNA is avoided, and the abundance of the modified protein is also readily detectable. Other genomic safe harbors, such as the adeno-associated virus site 1 (*AAVS1*) and chemokine (CC motif) receptor 5 (*CCR5*)[47,48] deserve more exploration.

In our previous study, when Vav1-OST was expressed at higher levels at the mouse *Rosa26* locus in RAW264.7 cells, which are very frequently used macrophage cells, we were able to detect its interaction with lowly expressed Vav3 molecules because of the high levels of bait protein and high efficiency of OST affinity purification[13]. We also described knock-in experiments to establish a macrophage cell line stably expressing hACE2, a receptor for SARS-CoV-2, in which abundant expression is guaranteed. In the single cell RNA sequencing database, murine Ace2 is expressed in lung macrophages, and the genetic cellular model expressing hACE2 we developed could be useful for studies of macrophages during SARS-CoV-2 infection.

Other considerations

This protocol is designed to identify knock-in cells that express a POI with aid of flow cytometric analyses of a fluorescent reporter, in our case the EBFP2 reporter. However, when surface labeling antibodies detectable by FACS are available for a surface protein[49], it is not necessary to use the reporter system[50]. The T cell and macrophage cell lines, as well as the examples of OST-tagged proteins used for interactome studies, are mainly used for signaling studies, and the majority of these signaling molecules are localized in the cytosol or nuclei of cells. Thus, a fluorescent reporter may be needed for the identification of desirable knock-in cells.

It is important to point out that this protocol was developed for cell lines, and application to primary immune cells such as T cells, monocytes/macrophages was not validated. Because of the limited capacity of the cells to proliferate, we do not recommend this protocol for use with primary immune cells. As the fluorescent reporter EBFP2 was expressed under the same promoter as the knock-in gene or POI using an IRES element, we did not observe cells that expressed the fluorescent reporter in the absence of the knock-in gene. We suspect that the recovery of the fluorescent protein-expressing cells is highly dependent on the success of homologous recombination. As reported in a previous study, it is tedious to sort, expand, and identify the correct knock-in cells by single cell sorting when the knock-in efficiency is very low[42], which also explains why we needed to sort the cells in bulk to improve the success rate.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

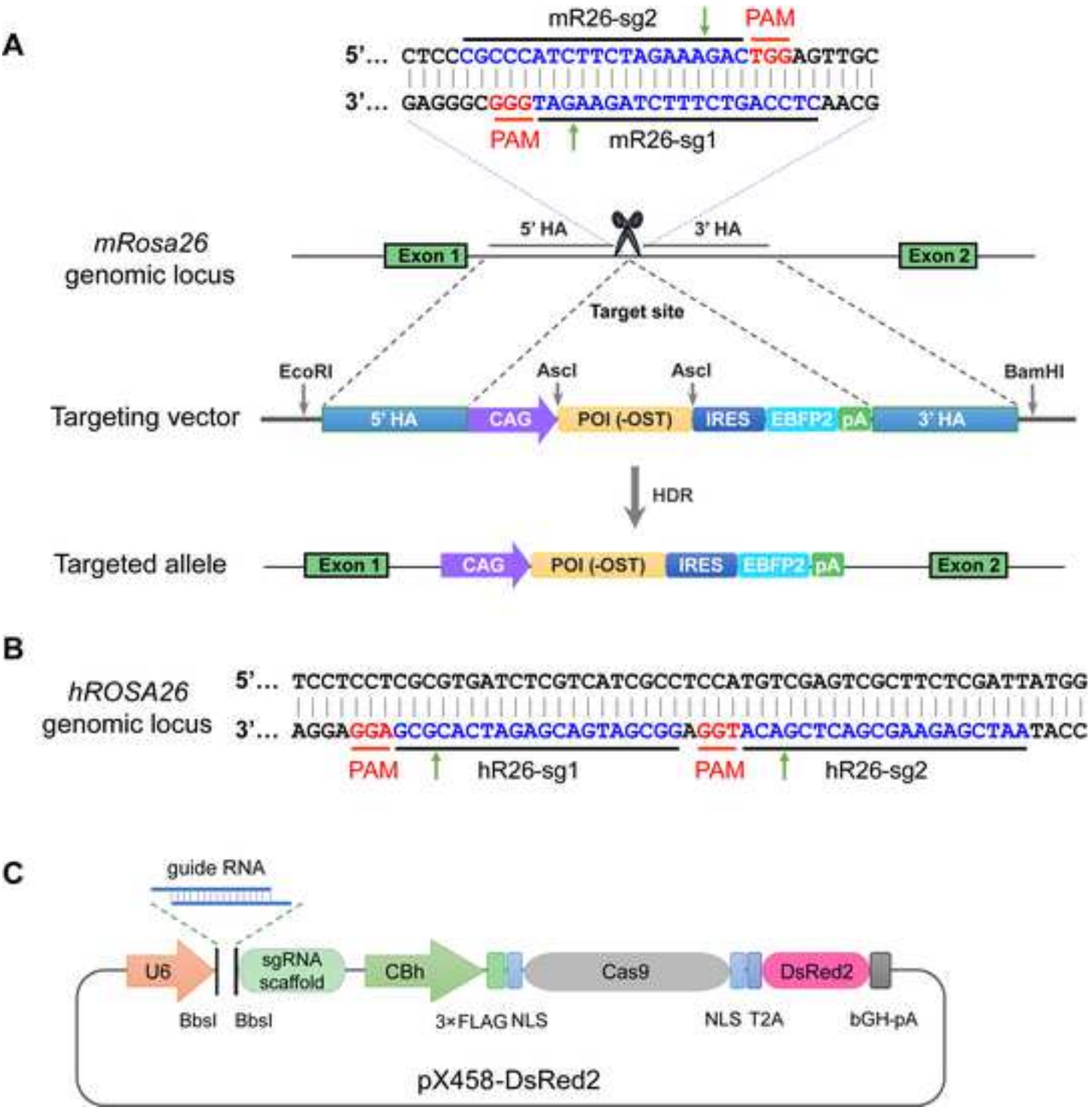

Figure 2

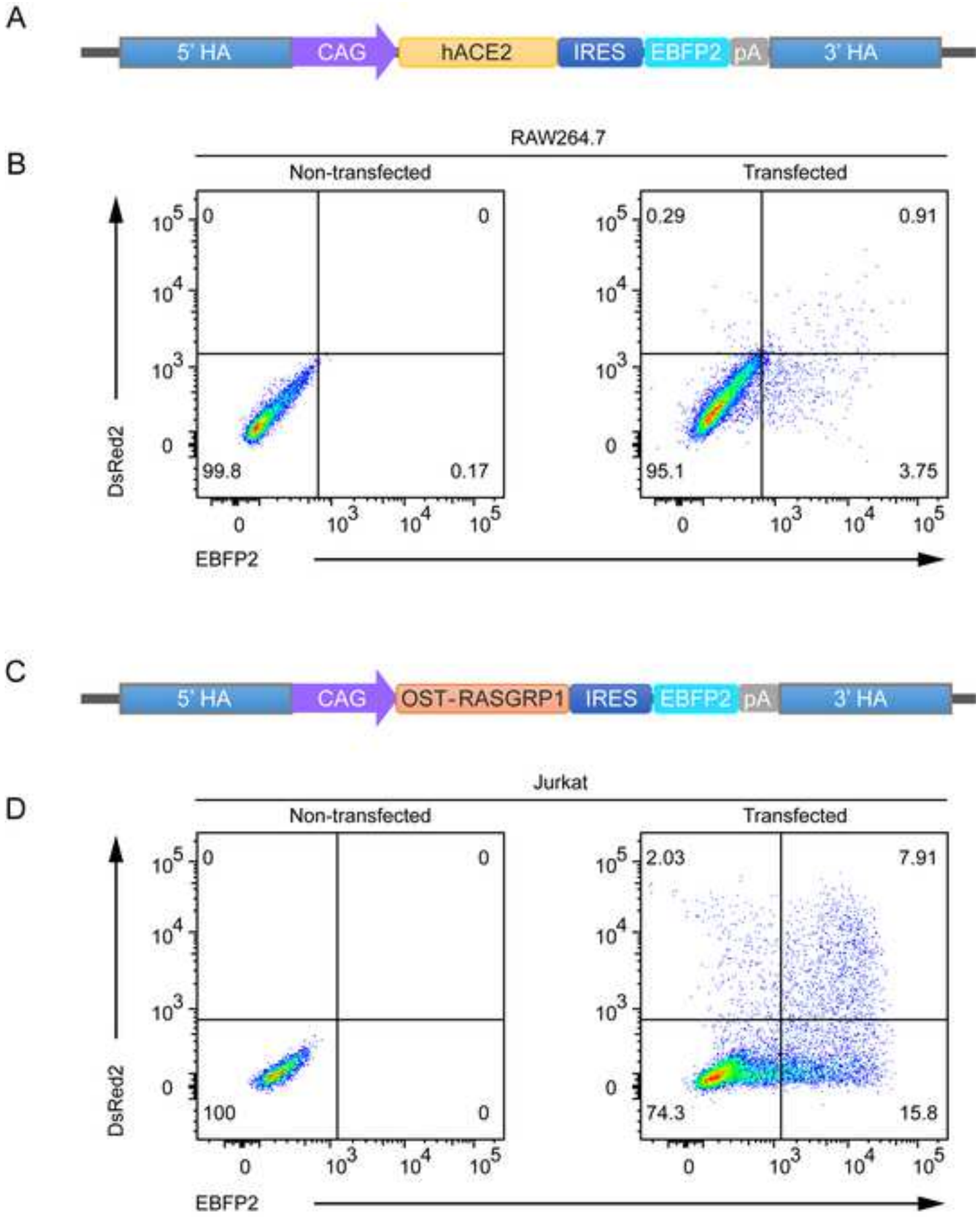

Figure 3

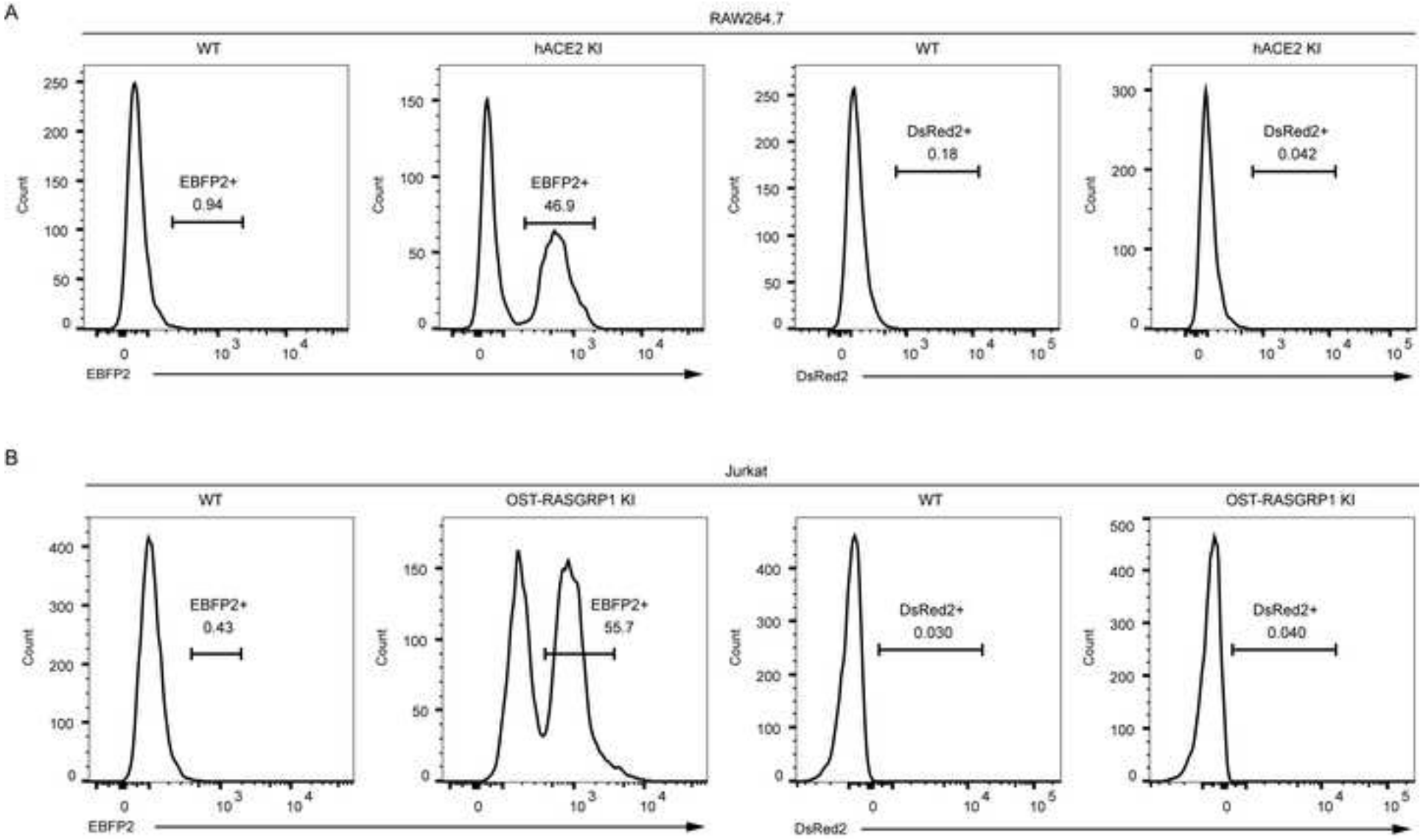

Figure 4

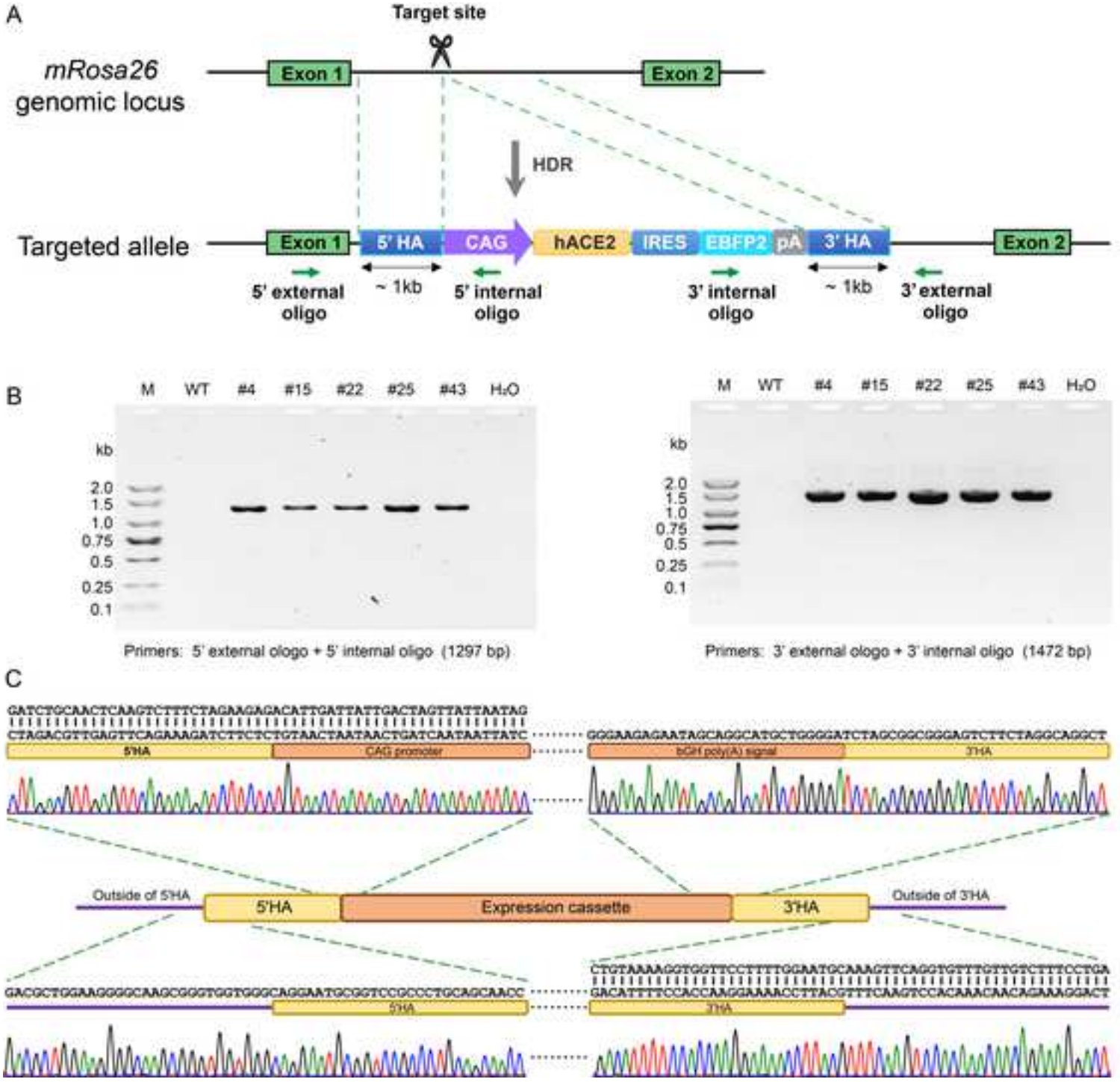

Figure 5

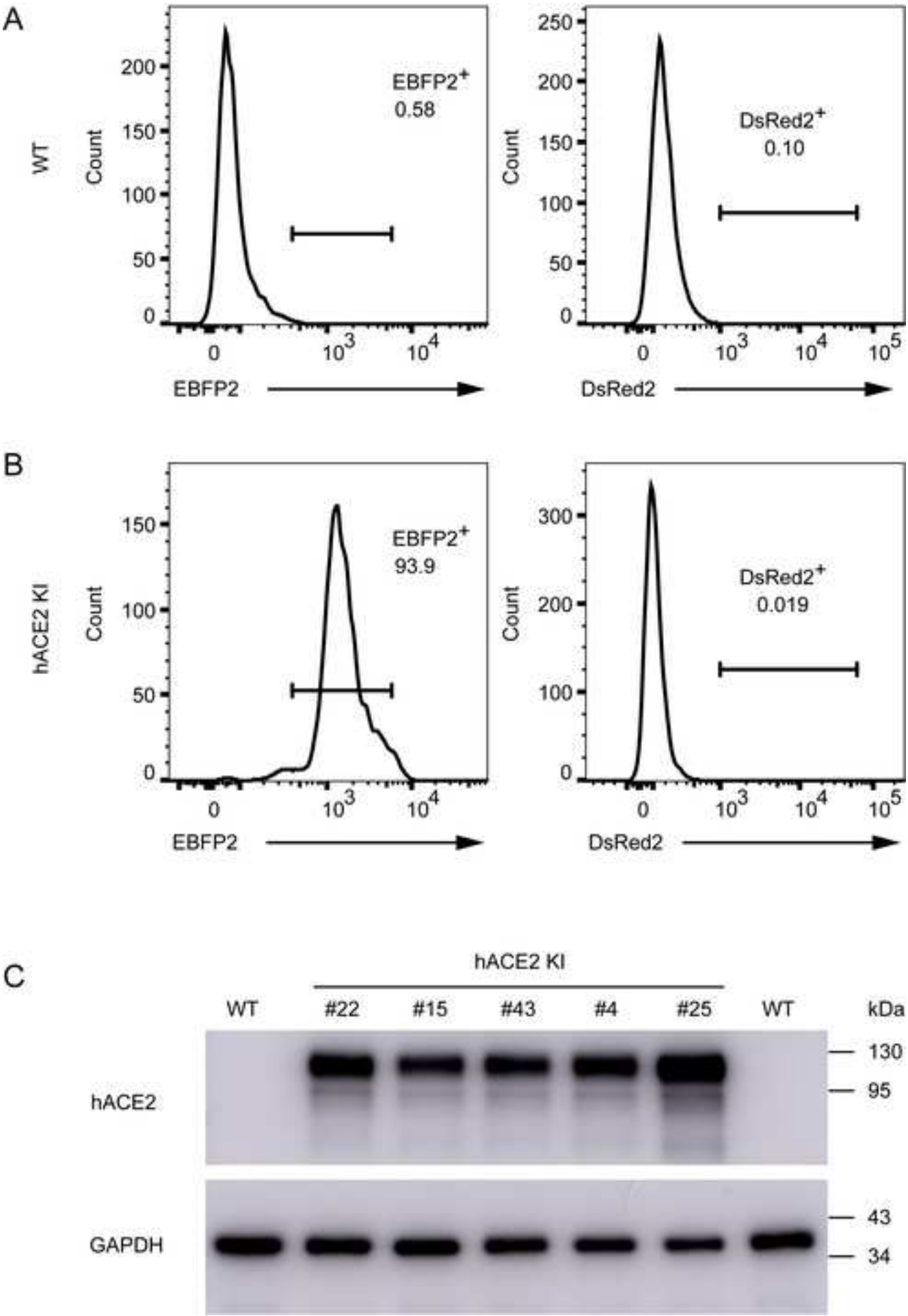

Figure 6

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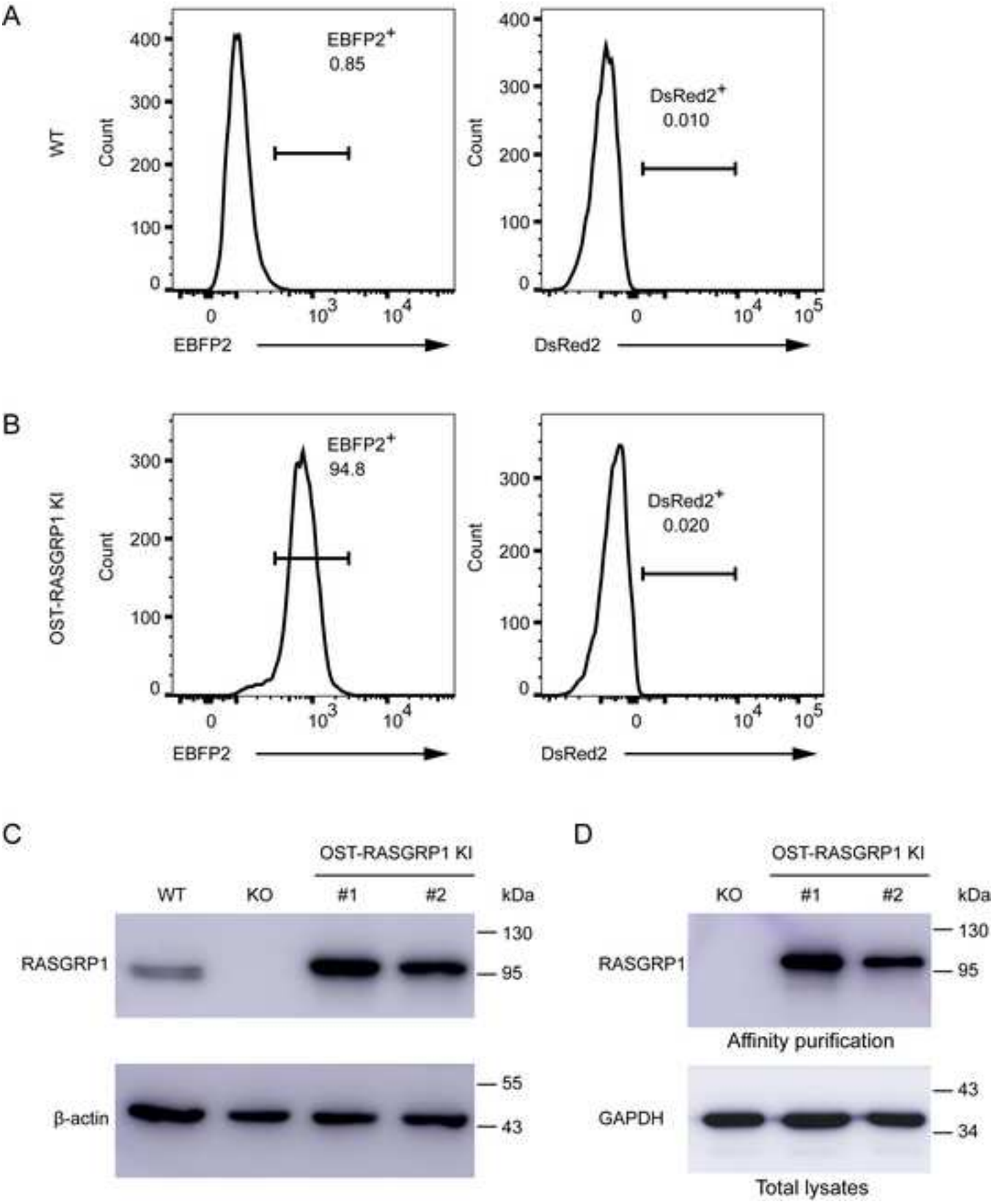

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Changes to be made in the text.

1. The editor has formatted the manuscript to match the journal's style. Please retain.
2. Please address specific comments marked in the manuscript.
3. Please address all the reviewers' comments as well.

Author response: Thank you. We've addressed all specific comments in the revised manuscript.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.
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Author response: Thank you. We've redone the video and tried our best to improve the video quality.

Once done please ensure that the video is no more than 15 min length. Please uplaod the revised video at https://www.dropbox.com/request/5QVtGMegtJ3xayiPGmkZ?oref=e

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have addressed all my questions and comments. Therefore, I have no major concerns.

Author response: Thank you.

Reviewer #4:

Manuscript summary:

In this manuscript, authors explain a method for a 2-plasmid system to facilitate CRISPR-mediated knock-in. While the method can be reproduced, some details are still missing (see below) that would make execution much easier to perform for independent groups.

Major remarks:

Please consider having the manuscript copy-edited by an English native. Some phrases and tenses used are not common, or do not fortify the message of the manuscript.

Author response: Thank you. We have revised the manuscript.

Minor remarks:

Introduction

* Where applicable, please note that cell lines have been used, as Jurkat and RAW cells are not primary cells (i.e. line 67, 70).

Author response: Thank you. We have made revisions. Please refer to Line 798 and 821 of the revised manuscript.

Line 55 - please add specific references for Cas9-mediated genome editing in primary target cells (T cells and macrophages). For T cells, one of the following two papers would be good: https://doi.org/10.1002/cpim.69 or https://doi.org/10.1084/jem.20171626.

Author response: Thank you. We have added the literatures in the revised manuscript. Please refer to Line 65 to 665 of the revised manuscript.

Line 60 - a recent paper by the Doudna laboratory explored the use of virus-like particles to perform gene knock-out and simultaneous gene transfer, see https://doi.org/10.1016/j.celrep.2021.109207, it would be good to incorporate this into the introduction.

Author response: Thank you. We have added the literatures in the revised manuscript. Please refer to Line 7069 of the revised manuscript (Reference 20).

Line 64 - also here the addition of especially the JEM paper mentioned earlier would be good (https://doi.org/10.1084/jem.20171626).

Author response: Thank you. We have added new literatures in the revised manuscript. Please refer to Line 754 of the revised manuscript (Reference 9).

Protocol

Line 105: a note on other CRISPR design tools would be very valuable, for instance the Benchling CRISPR design tool.

Author response: Thank you. We have added the new tool in the revised manuscript. Please refer to Line 123~~2~~ of the revised manuscript.

Line 122: please add details if not performed according to manufacturer's instructions, or mention that manufacturer's instructions were followed.

Furthermore, please also add purification method used to purify digested plasmid required for next step on line 124, as this information is missing but crucial.

Author response: Thank you. We've made revisions (step 1.2.2-1.2.4) and added new citation from Dr. Zhang's Lab.

In addition, we added a "Note" to describe the method for~~to~~ purifying digested plasmid ~~also followed their protocol~~. Please refer to Line 147 to 150~~8~~ of the revised manuscript.

Line 124: please add details if not performed according to manufacturer's instructions, or mention that manufacturer's instructions were followed.

Author response: Thank you. We have made revisions (step 1.2.2, Line 142~~1~~ to 150~~49~~ of the revised manuscript).

Line 126: please provide detailed information regarding transformation (how many microliters/vector with how many bacteria).

Author response: Thank you. We have made revisions (step 1.2.3, Line 152~~1~~ to 155~~4~~ of the revised manuscript).

Line 133: please provide manufacturer of the kit.

Author response: Thank you. We provided the Plasmid Maxi Kit (Qiagen, 12163) in the **Table of Materials** ~~.~~(Line 161 to 162 of the revised manuscript).

Line 150: add reference to the figure/supplementary file that describes this.

Author response: Thank you. We have added new references with illustrations of introducing mutations to prevent donor DNA re-cut by Cas9. Please refer to Line 180~~79~~ of the revised manuscript (Reference 34 and 35).

Line 154: If possible, please deposit the plasmids in Addgene depository and mention in the manuscript.

Author response: Thank you. We're happy to share the plasmids and will deposit them after submitting the revised manuscript, since it involved lots of communications with Addgene and it takes time. We've experienced the procedures when we submitted a work with two new constructions pX458-DsRed2 and pX458-ECFP (https://www.addgene.org/Yinming_Liang/). We will proceed with the deposition of the plasmids used in this protocol as we did before.

Line 164: please provide which bases need to be added for addition of AscI restriction site for completion.

Author response: Thank you. We have provided the DNA sequence for addition of AscI restriction site as well as the Kozak consensus translation initiation site. Please refer to step 2.2.2 of the revised manuscript (Line 197~~6~~ to ~~19~~2009~~9~~).

Line 168/168: please add details if not performed according to manufacturer's instructions, or mention that manufacturer's instructions were followed.

Furthermore, please also add purification method used to purify digested plasmid required for next steps, as this information is missing but crucial.

Author response: Thank you. We have made revisions (step 2.~~-~~2.3). Please refer to Line 2021~~1~~ to 2043~~3~~ of the revised manuscript.

Line 216: if possible, please restate to mention that "DsRed2 expression can be assessed via fluorescence microscopy imaging, or flow cytometry. Authors employ ZOE fluorescent cell imaging." Or something similar.

Author response: Thank you. We've made revisions and substituted ZOE with general term. Please refer to Line 2887~~7~~ to 2898~~8~~ of the revised manuscript.

Line 223: please add the harvesting as a step for completeness.

Author response: Thank you. We have added more information. Please refer to Line 2965~~5~~ to 2976~~6~~ of the revised manuscript.

Line 231: please mention these controls further upstream, as they also need to be prepared and treated similarly (e.g. electroporation without any DNA, or subculturing the cells in the same vessel [24-well format in this case]). If researchers do not do this and only find out at point 4.4, it's too late.

Author response: Thank you. We have revisions for this purpose. Please refer to step 3.2.8 of the revised manuscript (Line ~~280~~ 281 to ~~283~~284).

Line 249: there is a typo ("well").

Author response: Thank you. We've made the revision. Please refer to Line 3276~~6~~ of the revised manuscript.

Discussion

Line 426: Please discuss that while Jurkat cells are often used for studying cell signaling, they are notoriously bad at producing IFN-g, and are in general not suitable for studying primary T cell effector mechanisms. For that aim, studying primary cells is more useful. For instance, for studying the regulation of IFN-g production, the use of CRISPR-edited primary human T cells is required and has already been performed in literature (e.g. https://publons.com/publon/10.1002/eji.201948458).

Author response: Thank you very much. We have added this important information to the discussion section. Please find the revised info from Line 5676~~6~~ to 5698~~8~~.

Reviewer #5:

The submission has been significantly improved since the first round of revision. As the method of CRISPR/Cas9-induced gene knock-in became a standard way of genetic manipulation in many laboratories around the world, it is of common interest to have it presented in Jove format. I have only a few minor comments.

Minor Comments:

Comments to the video

0:08 - 0:30 and 11:26 - 11:57 - In the introductory and concluding parts of the video the sound seems distorted (echo?). Could it be improved with a sound mixing software?

2:46-3:30 - The video loses quality to the point of not being readable (2:56-3:06). It should be corrected.

8:17, 8:30 - Certain steps shown in the video could be cut out, if the length of the video was an issue. For example, showing the transfer of the samples through the biosafety gate (as indicated by the timestamps) is not necessary. Not every laboratory uses it in FACS rooms and it is not an essential step of the procedure. Similarly, the video could shorten breaks without the voiceover, or even speed the voiceover up, if the length of the video was an issue.

Author response: Thank you. We have redone the video and tried our best to improve the video quality.

Comments to the text

1. Although, there is nothing wrong with the authors approach to perform the cell sorting twice, alternative approaches would be similarly feasible. For example, keeping the cells in the bulk, until the Cas9 protein is no longer expressed and then sorting on EBFP2. Lack of expression of Cas9 plasmid would mean that, EBFP2-containing plasmid should be gone too, and only successful knock-in cells would still express EBFP2.

If this technique was applied to primary cells, working with the bulk might be the only feasible option. Therefore, it would be interesting to know the rate of false-positive cells in the bulk.

Performing proper limiting dilution cloning (with one cell per well) should be a feasible option as well. It is true that the number of surviving clones would be lower, but if the goal is to establish only a few clonal cell lines, this approach could actually save some time, as the heterogeneity of the population would occur less often. It could always be scaled up by seeding more plates.

However, it is worth noting that generating clonal cell lines is not without its drawbacks (PMID: 31889332). Cell lines are highly heterogeneous, and generating mutated clones directly from the bulk is very risky as they might show very high variation in the studied parameter. It is difficult then to concur if the observed differences are the result of mutation or just a clonal heterogeneity. Therefore, it is important to first generate wild-type mother-clones, which could be later used as the basis for any mutated cell line and as a corresponding wild-type control. This is another argument why adjusting the authors' protocol for the bulk analysis might be a more practical approach.

It would be helpful for the reader if the authors made that kind of "notes" throughout the protocol or in the discussion, highlighting the possible improvements/adjustments of the

method.

Author response: Thank you. This protocol aims to improve the practice of doing knock-in in immune cell lines. We have not been able to use this protocol to do primary cell knock-in, and we do not recommend applying such a method to do knock-in experiments in primary T cell or monocyte because the sorted cells might have limited capacity in expansion. The fluorescent reporter was expressed under the same promoter of the knock-in gene. In our cases the driving promoter is CAG and IRES is used to express fluorescent reporter following expression of knock-in gene. We have not yet found the results that the cells express fluorescent reporter in the absence of the knock-in gene which is termed protein of interest in our manuscript. Indeed, for some of the cells the fluorescent protein expression wells can be rare after sorting when the success rate of homologous recombination is very low. Such low efficiency was also observed in literature, which may explain why we need to sort the cells in bulk to improve success rate. We added such information in the discussion section of the revised manuscript. Please find the revision from Line 5998 to 6087.

2. The method described by the authors is not particularly novel and a multitude of its alternatives are described in the literature. It might be useful for the reader if these adjustments to the approach were mentioned in the protocol.

- introducing a stably expressed fluorescent protein only for the sake of sorting would not work for the fluorescent microscopy experiments. Alternative approach is to use truncated versions of common surface markers (which should not be present in a given cell line), which can be recognized by common antibodies and used for sorting purpose (PMID: 21653320).
- using CRISPR/Cas9 knock-in to fluorescently tag endogenously expressed proteins (PMID: 28814507).
- using CRISPR/Cas9 knock-in to introduce in a certain locus a new variant of the same protein or another transcript, which should be similarly regulated (PMID: 28225754) - this is the most similar approach to the one described by the authors, but there are still certain differences, which make it worth mentioning. For example, if the protein is expressed on the surface, fluorescent tag is unnecessary. In addition, it is important to note that the readers can introduce mutations or other alterations to the repair sequence in order to obtain their specific goal.

Author response: Thank you very much. We have added this important point into the discussion section in the revised manuscript. Please find the revision from Line 590 to 597.

3. The figures have generally low resolution/quality. Smaller fonts (for example Fig. 3) or sequencing results (for example Fig.4) are barely readable. Would it be possible to export them in a vector graphic format?

Author response: Thank you. We've improved the resolution/quality of Figures 3 and 4.

4. Western blot results miss the indication of the molecular size of the given bands.

Author response: Thank you. We've added the important information on band sizes in the revised Figures 5 and 6.

5. The supplementary materials for reviewers might actually also be informative for the other readers, as they present alternative methods of the validation of the results. The authors might

consider putting it as well in the submission's supplementary file. Some gels in the supplementary material for reviewers 1B and C contain mixed sample order.

Author response: Thank you. We've put the Supplementary materials (for reviewers) 1 and 2 into the submission's **Supplementary File**. Please refer to Line 1310 ~~0~~ to 1332 ~~2~~ concerning Figure S1, and Line 357 to 36059 ~~59~~ o concerning Figure S2, accordingly. Meanwhile, we've corrected the improper sample order in the **Supplementary File**, Figure S2B-C of the new submission.

Gene knock-in by CRISPR/Cas9 and cell sorting in macrophage and T cell lines

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Figure S1.

To evaluate the cleavage activities of guide RNAs, electroporation of 4.0 × 10^5 cells was performed in the format one 10 µL nucleofection tip with 0.5 µg of each CRISPR expression vectors in the absence of a targeting vector. 48-72 h post-transfection, 1000 single cells express both DsRed2 and EBFP2 fluorescent reporters were sorted into a total 50 µL of lytic reaction with 25 µL 2 × lysis buffer (100 mM KCl, 20 mM Tris-HCl pH 9.0, 0.2% Triton X-100, and 0.8 mg/mL proteinase K) for isolating genomic DNA. Samples were incubated at 56 °C for 15 min and subsequently at 94 °C for 5 min. 1 µL of the crude extraction was used as template in a 10 µL of fPCR using 5'-fluorescein-amidite (FAM)-labeled forward primers and non-labeled reverse primers. 200~400 bp of PCR amplicons spanning the CRISPR target sites were designed. To test the activities of mR26-sg1 and mR26-sg2, Fm-FAM (5' - 3'): TAAGGGAGCTGCAGTGGAGTA; Rm (5' - 3'): CCCGACAAAACCGAAAATCTGT. To test the activities of hR26-sg1 and hR26-sg2, Fh-FAM (5' - 3'): GGAGTGCCGCAATACCTTTATG; Rh (5' - 3'): TGCATAAAATCAGCCCCAGGT. PCR products were subjected to capillary array electrophoresis (CAE) using an ABI 3730 DNA analyzer[1]. Perform data analysis by GeneMapper software v3.1, which enables determination of the positions and areas of the peaks, indicating the lengths and relative amounts of PCR products, respectively[2].

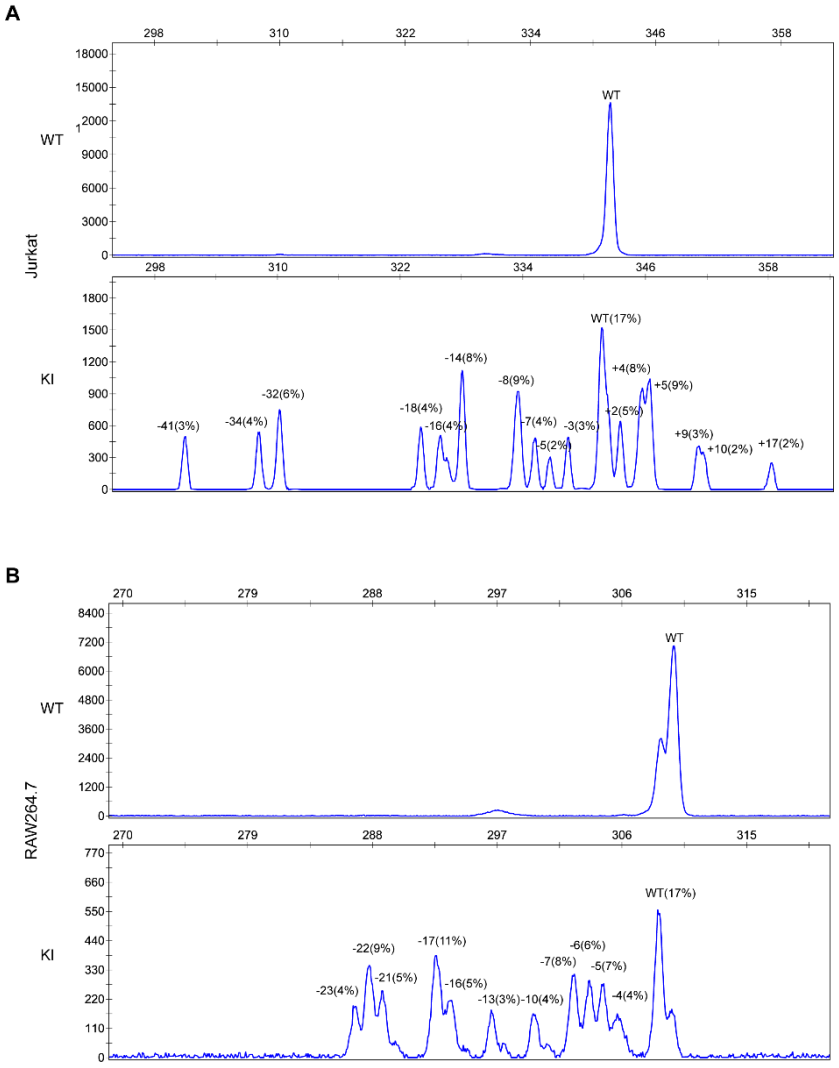

Figure S1. Analysis of on-target activity by fluorescence PCR and capillary array electrophoresis (fPCR-CAE). CRISPR-editing efficiency is evaluated by the frequencies of small insertions and/or deletions (Indel) resulted from the nonhomologous end joining (NHEJ) pathways. Over 80% cells are edited by CRISPR/Cas9 system in both RAW264.7 and Jurkat cells. **(A)** For RAW264.7 macrophages transfected with pDsR-mR26-sg1 and pDsR-mR26-sg2, crude extracts containing genomic DNA of DsRed2^{+}EBFP2^{+} cells were analysis by fPCR-CAE using Fm-FAM and Rm. Genomic DNA of the WT RAW264.7 cells was used as control with a predicted size of 308 bp. **(B)** For Jurkat T cells transfected with pDsR-hR26-sg1 and pDsR-hR26-sg2, genomic DNA of DsRed2^{+}EBFP2^{+} cells were analysis by fPCR-CAE using Fh-FAM and Rh. The predicted PCR band testing the WT Jurkat control cells has size of 342 bp. The size and frequency of Indels are indicated near selected peaks.

References:

1. Velasco, E. et al. Heteroduplex analysis by capillary array electrophoresis for rapid mutation detection in large multiexon genes. *Nature Protocols.* **2** (1), 237-246 (2007).
2. Lonowski, L. A. et al. Genome editing using FACS enrichment of nuclease-

expressing cells and indel detection by amplicon analysis. *Nature Protocols.* **12** (3), 581-603 (2017).

Figure S2.

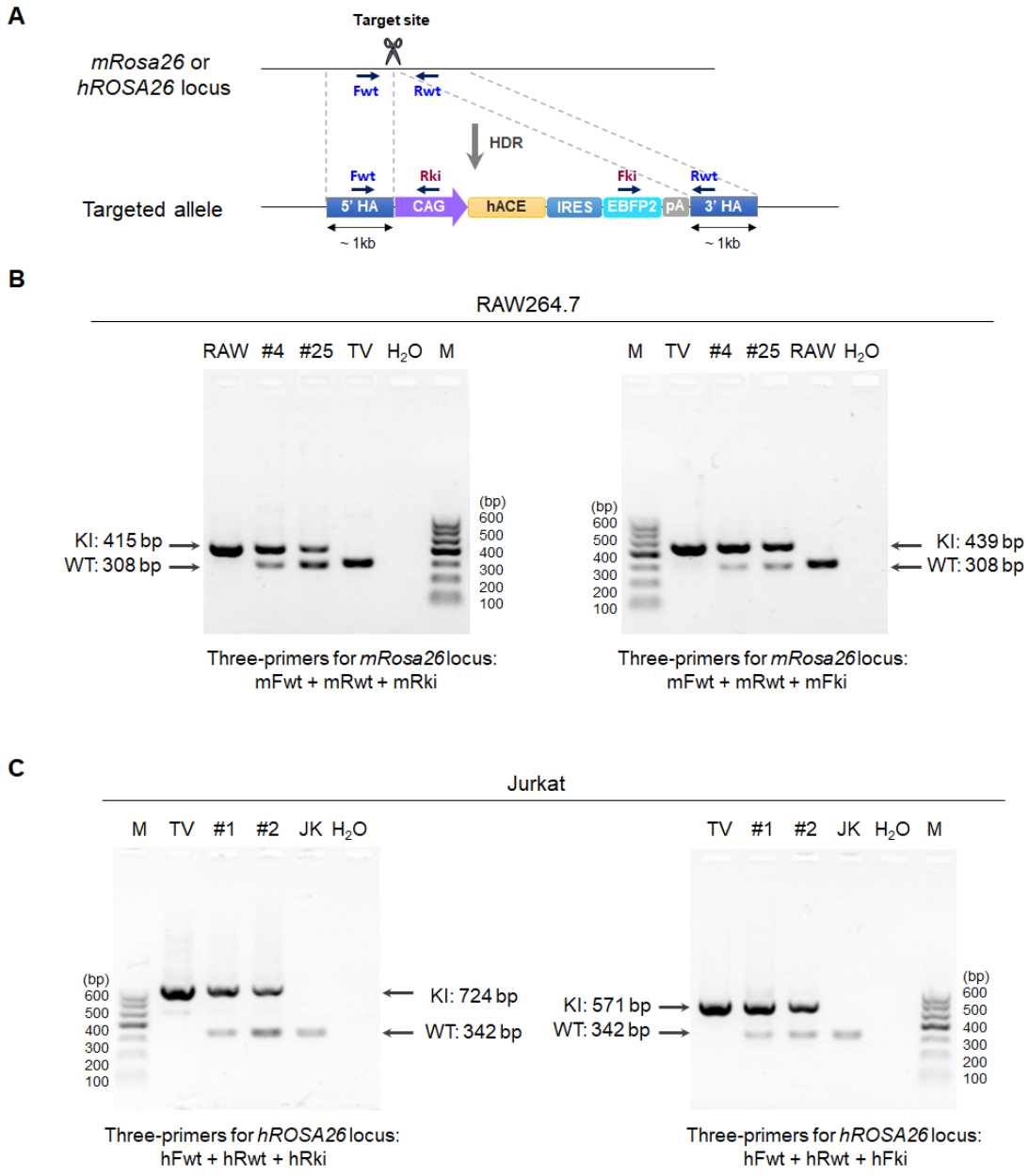

Figure S2. Three-primers PCR for simultaneous detection of the wild-type allele and the targeted allele. (A) The position of PCR primers is depicted in the diagram. Three-primers PCR for detection of *mRosa26* and *hROSA26* knock-in are designed in the same manner. **(B)** Genomic DNA of two candidate knock-in RAW264.7 cells (#4 and #25) was analysis by PCR reaction with either mFwt/mRwt/mRki primers (left) or mFwt/mRwt/mFki primers (right). The 308 bp of PCR products correspond to wild-type (WT) and the 415 bp/439bp of PCR amplicons correspond to the targeting vector (TV) positive control which suggesting the occurrence of

insertion of the targeting vector. **(C)** Genomic DNA of two candidate knock-in Jurkat (#1 and #2) are amplified by PCR with either hFwt/hRwt/hRki primers (left) or hFwt/hRwt/hFki primers (right). Both WT allele (342 bp) and the targeted allele (724 bp of region spanning the junction of 5'HA and CAG promoter and 571 bp region spanning the junction of EBFP2 and 3'HA) are present in the candidate knock-in cells. M, DNA ladder. The primers used were (5' – 3'):
mFwt, TAAGGGAGCTGCAGTGGAGTA;
mRwt, CCCGACAAAACCGAAAATCTGT;
mFki, GAGCAAAGACCCCAACGAGA;
mRki, CCAAGTGGGCAGTTTACCGTA;
hFwt, GGAGTGCCGCAATACCTTTATG;
hRwt, TGCATAAAATCAGCCCCAGGT;
hFki, GACTTCAAGGAGGACGGCAA;
hRki, GGCTATGAACTAATGACCCCGT.

Supplementary Table:

Primers used for plasmid construction and validation of knock-in allele in *mRosa26* locus.

Primer name	Sequence (5' - 3')	Description
mR26-sg1f	CACC**G**CTCCAGTCTTTCTAGAAGAT	construction of pDsR-mR26-sg1
mR26-sg1r	AAACATCTTCTAGAAAGACTGGAG**C**	
mR26-sg2f	CACC**G**CGCCCATCTTCTAGAAAGAC	construction of pDsR-mR26-sg2
mR26-sg2r	AAACGTCTTTCTAGAAGATGGGCG**C**	
hR26-sg1f	CACCGGCGATGACGAGATCACGCG	construction of pDsR-hR26-sg1
hR26-sg1r	AAACCGCGTGATCTCGTCATCGCC	
hR26-sg2f	CACC**G**AATCGAGAAGCGACTCGACA	construction of pDsR-hR26-sg2
hR26-sg2r	AAACTGTCGAGTCGCTTCTCGATT**C**	
5' external oligo	GTGGAGCCGTTCTGTGAGAC	primers spanning 5'HA for verifying precise HDR at the *mRosa26* locus
5' internal oligo	CCAAGTGGGCAGTTTACCGT	
3' internal oligo	GAGCAAAGACCCCAACGAGA	primers spanning 3'HA for verifying precise HDR at the *mRosa26* locus
3' external oligo	AGGTCCTGAAGAAGCTTGGC	

Sequence information:

>expression cassette sequence

GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCC
GCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATG
ACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGC
CCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCG
CCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTAT
TACCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATTTTGTAT
TTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGCGCGCGCCAGGCGGGGCGGGGC
GGGGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCGGCGCGCTCCGAAA
GTTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGGCCCTATAAAAAGCGAAGCGCGCGGCGGGCGGGAGTCGC
TGCGCGCTGCCTTCGCCCCGTGCCCCGCTCCGCCGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGT
TACTCCCACAGGTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCGCTTGGTTTAATGACGGCTT
GTTTCTTTTCTGTGGCTGCGTGAAAGCCTTGAGGGGCTCCGGGAGGGCCCTTTGTGCGGGGGGAGCGGCTCGG
GGGGTGCGTGCGTGTGTGTGTGCGTGGGGAGCGCCGCGTGCGGCTCCGCGCTGCCCGGCGGCTGTGAGCGCT
GCGGGCGCGGCGCGGGGCTTTGTGCGCTCCGCAGTGTGCGCGAGGGGAGCGCGGCCGGGGGCGGTGCCCCG
CGGTGCGGGGGGGGCTGCGAGGGGAACAAAGGCTGCGTGCGGGGTGTGTGCGTGGGGGGGTGAGCAGGGG
GTGTGGGCGCGTCGGTCGGGCTGCAACCCCCCCTGCACCCCCCTCCCCGAGTTGCTGAGCACGGCCCGGCTTCG
GGTGCGGGGCTCCGTACGGGGCGTGGCGCGGGGCTCGCCGTGCCGGGCGGGGGGTGGCGGCAGGTGGGGG
TGCCGGGCGGGGCGGGGCCGCCTCGGGCCGGGGAGGGCTCGGGGGAGGGGCGCGGCGGCCCCCGGAGCGC
CGGCGGCTGTCGAGGCGCGGCGAGCCGCAGCCATTGCCTTTTATGGTAATCGTGCGAGAGGGCGCAGGGACT
TCCTTTGTCCCAAATCTGTGCGGAGCCGAAATCTGGGAGGCGCCGCCGCACCCCCTCTAGCGGGCGCGGGGCG
AAGCGGTGCGGCGCCGGCAGGAAGGAAATGGGCGGGGAGGGCCTTCGTGCGTCGCCGCGCCGCCGTCCCCTT
CTCCCTCTCCAGCCTCGGGGCTGTCCGCGGGGGGACGGCTGCCTTCGGGGGGGACGGGGCAGGGCGGGGTTC
GGCTTCTGGCGTGTGACCGGCGGCTCTAGAGCCTCTGCTAACCATGTTCATGCCTTCTTCTTTTTCCTACAGGGC
GCGCCccccccccctaacgttactggccgaagccgcttggaataaggccggtgtgcgtttgtctatatgttattttccaccatattgccgtcttttg
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GCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCG
CACCATCTTCTTCAAGGACGACGGCACCTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTG
AACCGCATCGAGCTGAAGGGCGTCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAAC
TTCAACAGCCACAACATCTATATCATGGCCGTCAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCA
CAACGTGGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTG
CTGCTGCCCGACAGCCACTACCTGAGCACCCAGTCCGTGCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA
TGGTCCTGCTGGAGTTCCGCACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAActgtgccttcta
gttgccagccatctgttgtttgcccctccccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcat
cgcattgtctgagtaggtgtcattctattctgggggtggggtggggcaggacagcaagggggaggattgggaagagaatagcaggcatgctgg

gga

NNN: CAG prometer
GGCGCGCC: AscI restriction site
nnn: internal ribosome entry site (IRES)
NNN: EBFP2
nnn: bGH poly(A) signal

>5'HA sequence of plasmid pKR26-POI-iBFP

CAGGAATGCGGTCCGCCCTGCAGCAACCGGAGGGGGAGGGAGAAGGGAGCGGAAAAGTCTCCACCGGACGC
GGCCATGGCTCGGGGGGGGGGGGGCAGCGGAGGAGCGCTTCCGGCCGACGTCTCGTCGCTGATTGGCTTCTT
TTCCTCCCGCCGTGTGTGAAAACACAAATGGCGTGTTTTGGTTGGCGTAAGGCGCCTGTCAGTTAACGGCAGCC
GGAGTGCGCAGCCGCCGGCAGCCTCGCTCTGCCCACTGGGTGGGGCGGGAGGTAGGTGGGGTGAGGCGAGC
TGGACGTGCGGGCGCGGTCGGCCTCTGGCGGGGCGGGGGAGGGGAGGGAGGGTCAGCGAAAGTAGCTCGC
GCGCGAGCGGCCGCCCACCCTCCCCTTCCTCTGGGGGAGTCGTTTTACCCGCCGCCGGCCGGGCCTCGTCGTCT
GATTGGCTCTCGGGGCCCAGAAAACTGGCCCTTGCCATTGGCTCGTGTTCGTGCAAGTTGAGTCCATCCGCCGG
CCAGCGGGGGCGGCGAGGAGGCGCTCCCAGGTTCCGGCCCTCCCCTCGGCCCCGCGCCGCAGAGTCTGGCCG
CGCGCCCCTGCGCAACGTGGCAGGAAGCGCGCGCTGGGGGCGGGGACGGGCAGTAGGGCTGAGCGGCTGCG
GGGCGGGTGCAAGCACGTTTCCGACTTGAGTTGCCTCAAGAGGGGCGTGCTGAGCCAGACCTCCATCGCGCAC
TCCGGGGAGTGGAGGGAAGGAGCGAGGGCTCAGTTGGGCTGTTTTGGAGGCAGGAAGCACTTGCTCTCCCAA
AGTCGCTCTGAGTTGTTATCAGTAAGGGAGCTGCAGTGGAGTAGGCGGGGAGAAGGCCGCACCCTTCTCCGG
AGGGGGGAGGGGAGTGTTGCAATACCTTTCTGGGAGTTCTCTGCTGCCTCCTGGCTTCTGAGGACCGCCCTGG
GCCTGGGAGAATCCCTTCCCCCTCTTCCCTCGTGATCTGCAACTCaAGTCTTTCTAGAAGA

>3'HA sequence of plasmid pKR26-POI-iBFP

GGCGGGAGTCTTCTaGGCAGGCTTAAAGGCTAACCTGGTGTGTGGGCGTTGTCCTGCAGGGGAATTGAACAGG
TGTAAAATTGGAGGGACAAGACTTCCCACAGATTTTCGGTTTTGTCGGGAAGTTTTTTAATAGGGGCAAATAAG
GAAAATGGGAGGATAGGTAGTCATCTGGGGTTTTATGCAGCAAAACTACAGGTTATTATTGCTTGTGATCCGCC
TCGGAGTATTTTCCATCGAGGTAGATTAAAGACATGCTCACCCGAGTTTTATACTCTCCTGCTTGAGATCCTTACT
ACAGTATGAAATTACAGTGTCGCGAGTTAGACTATGTAAGCAGAATTTTAATCATTTTTAAAGAGCCCAGTACTT
CATATCCATTTCTCCCGCTCCTTCTGCAGCCTTATCAAAAGGTATTTTAGAACACTCATTTTAGCCCCATTTTCATT
TATTATACTGGCTTATCCAACCCCTAGACAGAGCATTGGCATTTTCCCTTTCCTGATCTTAGAAGTCTGATGACTC
ATGAAACCAGACAGATTAGTTACATACACCACAAATCGAGGCTGTAGCTGGGGCCTCAACACTGCAGTTCTTTT
ATAACTCCTTAGTACACTTTTTGTTGATCCTTTGCCTTGATCCTTAATTTTCAGTGTCTATCACCTCTCCCGTCAGG
TGGTGTTCCACATTTGGGCCTATTCTCAGTCCAGGGAGTTTTACAACAATAGATGTATTGAGAATCCAACCTAAA
GCTTAACTTTCCACTCCCATGAATGCCTCTCTCCTTTTTCTCCATTTATAAACTGAGCTATTAACCATTAATGGTTT
CCAGGTGGATGTCTCCTCCCCCAATATTACCTGATGTATCTTACATATTGCCAGGCTGATATTTTAAGACATTAAA
AGGTATATTTCATTATTGAGCCACATGGTATTGATTACTGCTTACTAAAATTTTGTCATTGTACACATCTGTAAAA
GGTGGTTCCTTTTGGAATGC

>5'HA sequence of plasmid pKhR26-POI-iBFP

TTTGTTACGTTGGGAGGGAAAGGGGTGGCTGGATGCAGGCGGGAGGGAGGCCCGCCCTGCGGCAACCGGAG

GGGGAGGGAGAAGGGAGCGGAAAATGCTCGAAACCGGACGGAGCCATTGCTCTCGCAGAGGGAGGAGCGCT
TCCGGCTAGCCTCTTGTCGCCGATTGGCCGTTTCTCCTCCCGCCGTGTGTGAAAACACAAATGGCGTATTCTGGT
TGGAGTAAAGCTCCTGTCAGTTACGCCGTCGGGAGTACGCAGCCGCTTAGCGACTCTCGCGTTGCCCCCTGGGT
GGGGCGGGTAGGTAGGTGGGGTGTAGAGATGCTGGGTGTGCGGGCGCGGCCGGCCTCCTGCGGCGGGAGG
GGAGGGTCAGTGAAATCGGCTCTGGCGCGGGCGTCCTCCCACCCTCCCCTTCCTTCGGGGGAGTCGGTTTACCC
GCCGCCTGCTTGTCTTCGACACCTGATTGGCTGTCGAAGCTGTGGGACCGGGCCCTTGCTACTGGCTCGAGTCT
CACATGAGCGAAACCACTGCGCGGGGCGCGGGGGTGGCGGGGAGGCGGGCGTTGGTACGGTCCTCCCCGAG
GCCGAGCGCCGCAGTGTCTGGCCCCGCGCCCCTGCGCAACGTGGCAGGAAGCGCGCGCTGGAGGCGGGGGC
GGGCTGCCGGCCGAGACTTCTGGATGGCGGCGGCCGCGGCTCCGCCCCGGGTTCCCACCGCCTGAAGGGCGA
GACAAGCCCGACCTGCTACAGGCACTCGTGGGGGTGGGGGAGGAGCGGGGGTCGGTCCGGCTGGTTTGTGG
GTGGGAGGCGCTTGTTCTCCAAAAACCGGCGCGAGCTGCAATCCTGAGGGAGCTGCGGTGGAGGAGGTGGAG
AGAAGGCCGCACCCTTCTGGGCAGGGGGAGGGGAGTGCCGCAATACCTTTATGGGAGTTCTCTGCTGCCTCCC
GTCTTGTAAGGACCGCCCTGGGCCTGGAAGAAGCCCTCCCTCCTTTCCTCaTCGCGTGATCT

>3'HA sequence of plasmid pKhR26-POI-iBFP

CGTCATCGCCTCtATGTCGAGTCGCTTCTCGATTATGGGCGGGATTCTTTTGCCTAGGCTTAAGGGGCTAA
CTTGGTCCCTGGGCGTTGCCCTGCAGGGGAGTGAGCAGCTGTAAGATTTGAGGGGCGACTCCGATTAGTT
TATCTTCCCACGGACTAGAGTTGGTGTCGAGGTTATTGTAATAAGGGTGGGGTAGGGAAATGGAGCTTA
GTCATTCACCTGGGGCTGATTTTATGCAACGAGACTGCGGATTATCACTACTTATCATTTTTGGAGCATTTT
TCTAGAGACAGACATAAAGCATGATCACCTGAGTTTTATACCATTTGAGACCCTTGCTGCACCACCAAAGT
GTAGCATCAGGTTAAATCTTAATAGAAAAATTTTAGCTTTTGCTTGAGAAACCAGTGCTTCCCTCCCTCACC
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CTTACCCAGCAGTGCTTTTATTCCTCCCTAGTTCACGTTCTTAAATGTTTATCTTGATTTTCATTTTATCCTTT
TTCCTTAGCTGGGATTCTGTCCCTGACCGTCTTCACAGTCCAGGTGATCTTGACTACTGCTTTACAGAGAAT
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AACTGATCTCAGATGCCTGTGTGATAGCTTCCAAATTGCTGTCTCTGTCTTTAGTGTTATCTTTTGATCCATC
TTACATCTTGTTAGGATGATTGTCCTAAAGGAAGATAGAGCATGAAAATGACAGGTGAAACTCCATT

Note: Nucleotide changes (underlined lowercase) in the homology arms are introduced to destroy the PAM sequence for avoiding re-cut the targeted allele by CRISPR/Cas9.

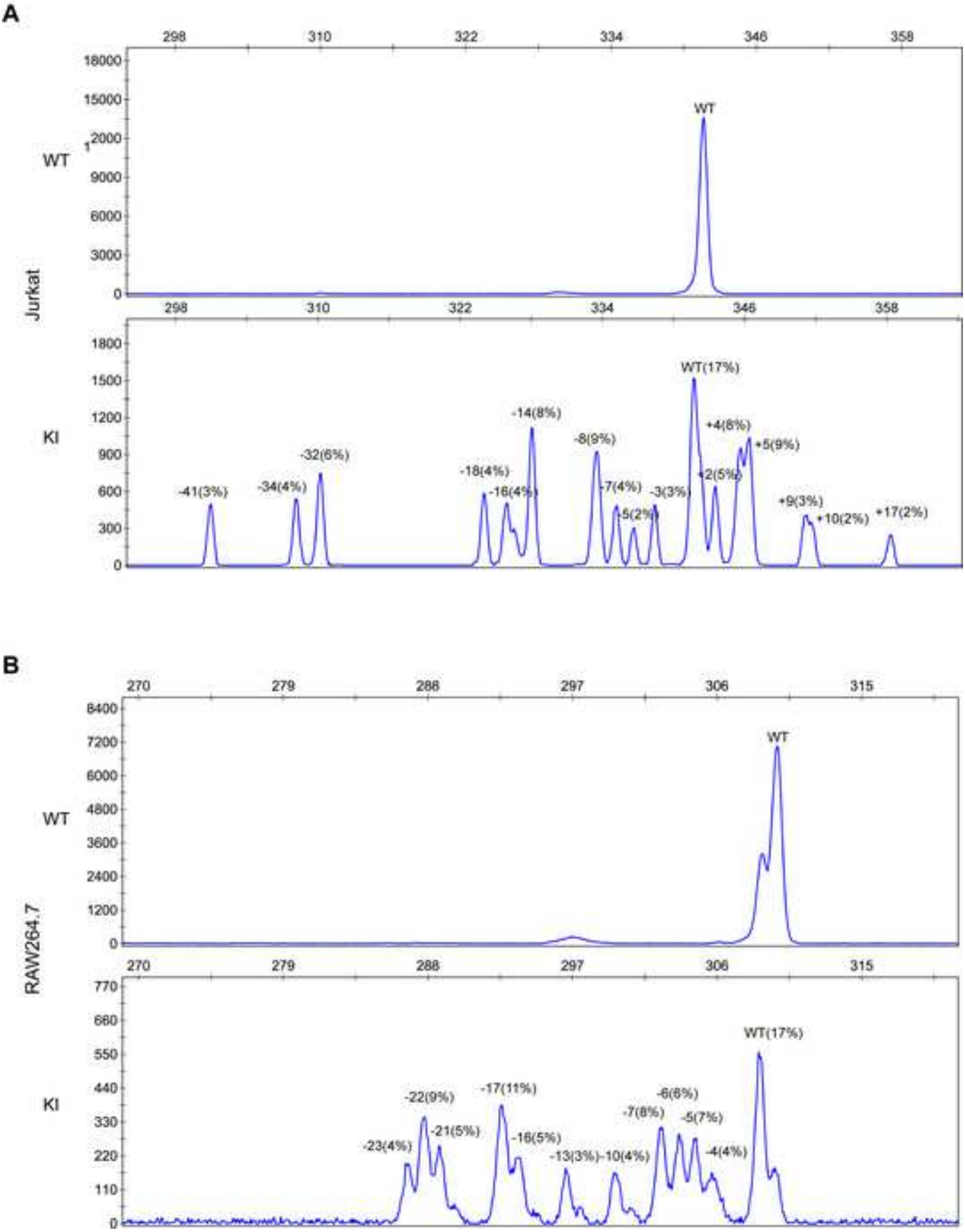
>OST tag with linker sequence

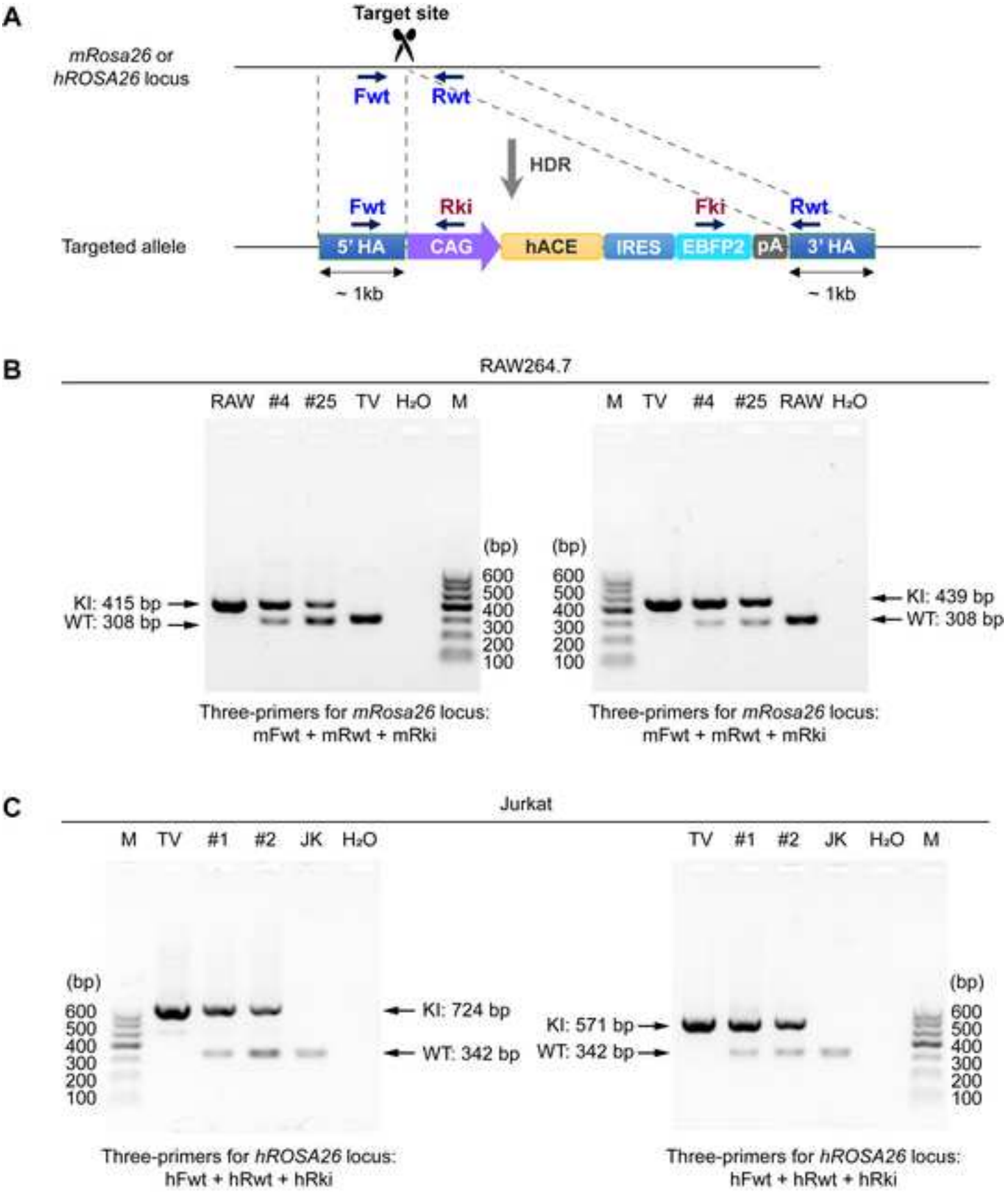
ATGTGGAGCCACCCGCAGTTCGAGAAAGGTGGAGGTTCAGGAGGTGGATCGGGAGGTGG
ATCGTGGAGCCACCCGCAGTTCGAAAAAGGTTCCGGG

NNN: OST tag
NNN: GSG linker

Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);62328_R3_Figure S1.tif





Supplementary Table:

Primers used for plasmid construction and validation of knock-in allele in *mRosa26* locus.

Primer name	Sequence (5' - 3')	Description
mR26-sg1f	CACCGCTCCAGTCTTTCTAGAAGAT	construction of pDsR-mR26-sg1
mR26-sg1r	AAACATCTTCTAGAAAGACTGGAGC	
mR26-sg2f	CACCGCGCCCATCTTCTAGAAAGAC	construction of pDsR-mR26-sg2
mR26-sg2r	AAACGTCTTTCTAGAAGATGGGCGC	
hR26-sg1f	CACCGGCGATGACGAGATCACGCG	construction of pDsR-hR26-sg1
hR26-sg1r	AAACCGCGTGATCTCGTCATCGCC	
hR26-sg2f	CACCGAATCGAGAAGCGACTCGACA	construction of pDsR-hR26-sg2
hR26-sg2r	AAACTGTCGAGTCGCTTCTCGATTC	
5' external oligo	GTGGAGCCGTTCTGTGAGAC	primers spanning 5'HA for verifying precise HDR at the *mRosa26* locus
5' internal oligo	CCAAGTGGGCAGTTTACCGT	
3' internal oligo	GAGCAAAGACCCCAACGAGA	primers spanning 3'HA for verifying precise HDR at the *mRosa26* locus
3' external oligo	AGGTCCTGAAGAAGCTTGGC	