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Generation of genetic knockouts in myeloid cell lines using a lentiviral CRISPR/Cas9 system

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Running Head: Lentiviral CRISPR/Cas9 in myeloid cells

Abstract

CRISPR/Cas9-based gene targeting allows deletion of a gene of interest from cultured cell lines. Due to difficulty in transiently transfecting haematopoetic cells with components required for this process, we have adopted a lentiviral system for delivery of the CRISPR/Cas9 components into myeloid cell lines. Here, we detail the process of knocking out genes from pools of cultured myeloid cells using this CRISPR/Cas9 system and describe methods of validating these knockout pools.

Key Words

CRISPR, lentivirus, myeloid, THP-1, immortalised BMDM, CRISPR validation

1. Introduction

Clustered Regularly-Interspersed Small Palindromic Repeats (CRISPR) are found within the genome of many bacterial and archaeal species. The spacers between these repeats are homologous to regions of bacteriophage DNA. Transcripts produced from these loci were found to act with the Cas family of proteins as a highly specific prokaryotic immune mechanism against phage infection ***(1)***. This system has been successfully adapted by the biotechnology sector for targeted gene disruption and has revolutionized genetic manipulation of both *in vitro* and *in vivo* models of disease ***(2–5)***. Most biotechnology applications use a type-II CRISPR/Cas system, originating from *Streptococcus pyogenes*. This system is comprised of a single endonuclease, Cas9 and small guide RNAs (sgRNAs), which carry a short sequence homologous to a region of the target gene (analogous to the spacer region in the endogenous prokaryotic system) followed by a region of secondary structure that allows interaction with Cas9 (analogous to the repeat regions in the endogenous prokaryotic system). These components are delivered into target cells either via direct transfection, or by transfection of DNA vectors encoding one or both components.

This protocol describes the use of CRISPR/Cas9 targeting to knock-out genes from cultured myeloid cells, using the human monocyte cell line THP-1 and immortalized murine bone-marrow-derived macrophages (iBMDM) as examples. The major hurdle encountered in deleting genes from myeloid cells is the difficulty in transfecting DNA into haematopoetic cell types. Myeloid cells are not only poorly transfectable using lipid-based transfection agents, but they are also prone to potent cytokine and cell-death responses induced through intracellular nucleic acid-sensing receptors such as AIM2 and STING. We have therefore adopted a lentiviral system developed by Aubrey *et al.* (2015) for delivery of CRISPR components into haematopoetic cells ***(6)***. This third-generation lentiviral system comprises a

mCherry-tagged vector that constitutively expresses Cas9 (FUCas9Cherry) and an eGFP-tagged vector, which under the control of a doxycycline-inducible promoter, carries the Cas9-binding region of the sgRNA immediately downstream of restriction sites allowing for the introduction of oligonucleotides homologous to the gene of interest (FgH1tUTG). Being a lentiviral system, these vectors are able to circumvent sensing by nucleic acid-specific pattern recognition receptors and integrate into the genomic DNA of transduced cells allowing for maintenance of stable cell lines. These cells constitutively express Cas9 and can be triggered for permanent knockout of the gene of interest by doxycycline induction of sgRNA expression (*see* **Figure 1**).

We also briefly describe a number of techniques for validating successful knockout of the targeted gene and their respective advantages and disadvantages. The techniques covered include western blotting and quantitative or semi-quantitative PCR. A third validation technique, next-generation sequencing of genomic DNA is described in Aubrey *et al.* (2015) ***(6)***.

2 Materials

2.1 Making Lentivirus in 293T cells

1. 293T cells.
2. DMEM cell culture medium: Dulbecco's Modified Eagle Medium supplemented with 100 U/mL Penicillin, 100 U/mL Streptomycin, 2 mg/mL L-Glutamine (DMEM) and 10% Fetal Bovine Serum (FBS).
3. 100 mm cell culture dishes.
4. The CRISPR component vectors required for this protocol, FUCas9Cherry and FgH1tUTG, are available from Addgene as plasmid numbers #70183 and #70182, respectively (*see* **Note 1**).
5. Third generation lentiviral packaging vectors pMDL, pRES-REV and pVSVg.
6. Lipofectamine 2000 (Invitrogen).
7. optiMEM (Gibco).
8. Cell culture facility including incubator at 37ºC, 5-10% CO_2.

2.2 Lentiviral transduction of CRISPR components into myeloid cell lines

1. Inverted fluorescence microscope equipped with laser module capable of exciting eGFP (~488 nm) and/or mCherry (~561 nm).
2. Myeloid cell line (e.g. THP-1 or iBMDM).
3. Cell culture medium: DMEM or Roswell Park Memorial Institute 1640 medium supplemented with 100 U/mL Penicillin, 100 U/mL Streptomycin, 2 mg/mL L-Glutamine (RPMI 1640) and 10% Fetal Bovine Serum (FBS).
4. 6-well tissue culture cluster plates.
5. 10 mL Syringes.
6. 0.45 μm Syringe filters.

7. 4 mg/mL Polybrene (Hexadimethrine Bromide) diluted in sterile nuclease-free H_2O.
8. Centrifuge with plate adapters.
9. FACS sorter capable of exciting eGFP (Blue laser ~488 nm, 530/30 filter) and mCherry (Yellow-Green laser ~561 nm, 615/30 filter).

2.3 Doxycycline-induction of CRISPR targeting

1. Cell culture medium: DMEM or Roswell Park Memorial Institute 1640 medium supplemented with 100 U/mL Penicillin, 100 U/mL Streptomycin, 2 mg/mL L-Glutamine (RPMI 1640) and 10% Fetal Bovine Serum (FBS).
2. 1 mg/mL doxycycline diluted in sterile nuclease-free H_2O.
3. 1x Dulbecco's Phosphate-buffered saline (D-PBS).

2.4 Validation of gene disruption

2.4.1 Western Blotting

1. Ice.
2. 1x D-PBS.
3. 1x RIPA buffer: 1% Triton X-100, 0.1% Sodium dodecyl sulfate (SDS), 1% Sodium Deoxycholate, 150mM NaCl, 10mM Tris-HCl (pH 7.5), sterile nuclease-free H_2O supplemented with protease inhibitors (Roche 25x cOmplete protease inhibitor cocktail).
4. Bench-top centrifuge capable of maintaining samples at 4ºC.
5. 4x SDS sample buffer: 40% glycerol, 50% 500 mM Tris-HCl (pH 7.5), 80mg/mL SDS powder, 0.01% Bromophenol blue, 30.8 mg/mL Dithiothreitol (DTT) powder, sterile nuclease-free H_2O.
6. Heat-block at 95ºC.
7. Standard SDS-PAGE running apparatus and buffers (e.g Bis- or Tris- Acrylamide gels, gel tank, power pack, MES or MOPS running buffer).

8. Standard western blot transfer apparatus and buffers (e.g. transfer tank, transfer cassette, Whatman paper, nitrocellulose or polyvinylidene difluoride (PVDF) membrane, Western Transfer buffer, power pack).
9. Antibody specific for the protein encoded by the CRISPR-targeted gene and respective HRP-conjugated secondary antibody.
10. Western blot developing equipment (e.g. chemiluminescent substrate, film or digital developing system).

2.4.2 Quantitative or Semi-Quantitative RT-PCR

2.4.2.1 RNA extraction and reverse transcription

1. Column-based RNA-miniprep kit.
2. Small volume spectrophotometer for measuring nucleotide concentration (e.g. NanoDrop).
3. 10 mM dNTPs.
4. 500 μg/mL Oligo(dT)$_{15}$ primers.
5. PCR thermocycler.
6. 5x First Strand Buffer.
7. 0.1 M DTT.
8. 40 U/μL RNasin ribonuclease inhibitor.
9. 200 U/μL Superscript III reverse transcriptase.
10. Nuclease-free H_2O.

2.4.2.2 Semi-quantitative PCR (sqPCR)

1. 10x *Taq* Buffer.
2. 10 mM dNTPs.
3. 100 μM Primers specific for cDNA of the CRISPR-targeted gene and a housekeeping control (*see* **Note 2**).

4. 5 U/µL *Taq* DNA Polymerase.
5. PCR Thermocycler.
6. Standard DNA gel electrophoresis equipment (e.g. TAE or TBE running buffer, agarose, gel tray, gel combs, gel tank, power pack).
7. UV-excitable DNA-binding dye (e.g. ethidium bromide or a non-cytotoxic alternative such as GelRed or RedSafe).
8. Plastic container large enough to hold one or more agarose gel(s).
9. Aluminium Foil.
10. Orbital Shaker.
11. UV DNA gel imager (e.g. BIO-RAD UV GelDoc).
12. Nuclease-free H_2O.

2.4.2.3 Quantitative PCR (qPCR)

1. 2x SYBR Green PCR Master Mix (+ROX).
13. 100 µM primers specific for cDNA of the CRISPR-targeted gene and a housekeeping control (*see* **Note 2**). Primer pairs should be combined and diluted to create a 10 µM primer mix: 10 µL forward primer + 10 µL reverse primer stock + 80 µL H_2O.
2. 384-well qPCR plates.
3. Plate seals & roller (or a solid piece of plastic such as a ruler or tip-box lid).
4. qPCR machine (e.g. the Life Technologies Viia7 system).

3 Methods

3.1 Making lentivirus in 293T cells

1. Plate $3x10^6$ 293T cells in a 100 mm dish (8 mL DMEM) and incubate at 37ºC for 12-18 h.
2. Prepare transfection mixtures for making lentivirus (*see* **Note 3**).
   - Tube 1:
     - 10 µg FUCas9Cherry or FgH1tUTG (or 5 µg of each if infecting simultaneously)
     - 5 µg pMDL
     - 2.5 µg pRES-REV
     - 3 µg pVSVg
     - OptiMEM to a final volume of 250 µL
   - Tube 2:
     - 26.7 µL Lipofectamine 2000
     - 223.3 µL OptiMEM
   - Incubate tubes at room temperature for 5 min and then combine the contents of Tube 1 and Tube 2 (final volume 500 µL). Flick or pipette up and down to mix, do not vortex.
   - Incubate at room temperature for 25 min before adding transfection mixture to 293T cells in a drop-wise manner.
3. Incubate overnight at 37ºC.
4. Aspirate supernatant and replace with 6 mL target cell media (e.g. for infecting THP-1 use RPMI, for iBMDM use DMEM). Incubate overnight at 37ºC.

3.2 Lentiviral transduction of CRISPR components into myeloid cell lines

1. Plate 5x10^{5} target cells in 500 µL cell culture medium per well in a 6-well plate. (*see* **Table 1**, **Note 4** & **Note 5**).
2. Harvest the viral supernatant from 293T cells and filter through a 0.45 µm syringe filter. Replace with 6 mL fresh target cell culture medium; return 293T cells to incubator overnight in order to repeat infection the following day if necessary (*see* **Note 6**).
2. Add 3 mL of freshly harvested and filtered viral supernatant per well of target cells (final volume 3.5 mL). Supplement each well with 8 µg/mL Polybrene (*see* **Note 7**).
3. Spin-infect the myeloid cells by centrifugation at 840 x g for 3 h at 32ºC (*see* **Note 8**).
4. Incubate overnight at 37ºC.
5. Check transduction efficiency using an inverted fluorescent microscope or flow cytometry. If efficiency is good (>40%) sort fluorescent cells directly into warm medium + 20% FCS (*see* **Note 9**).
6. Plate sorted cells so they are not too sparse (*see* **Note 10**) and allow them to expand by incubation at 37ºC until sufficient numbers are obtained for downstream applications (*see* **Note 11**).

3.3 Doxycycline-induction of CRISPR targeting

1. Following expansion, separate a portion of the sorted cells for doxycycline-induction, in order to stimulate expression of the sgRNA for CRISPR-based targeting of the gene of interest (*see* **Note 11**).
2. Supplement cells with 1 µg/mL doxycycline and incubate at 37ºC for 72 h.
3. At 72 h, wash cells 1x in D-PBS and plate in a fresh flask in medium without doxycycline.
4. Incubate for at least a further two days before assessing gene knockout (*see* **Note 12** & **Note 13**).

3.4 Validation of gene disruption

Depending on availability of resources, a number of approaches can be taken to validate Non-Homologous End Joining (NHEJ) activity at the targeted locus as described below (*see* **Note 14**). The advantages and disadvantages of each method presented, as well as, next generation sequencing, are also presented in **Table 2**.

3.4.1 Western Blotting

1. Wash $1x10^6$ cells in 1 mL D-PBS (*see* **Note 14** & **Note 15**).
2. Lyse cells in 100 µL 1x RIPA buffer on ice for 30 min.
3. Centrifuge lysate at 17,000 x g for 20 min at 4ºC to pellet the insoluble fraction.
4. Harvest the top 75 µL of the cell lysate and add to 25 µL SDS sample buffer.
5. Boil for 15 min at 95ºC.
6. Separate proteins in lysate by running 25-30 µL lysate on a SDS-PAGE.
7. Transfer to nitrocellulose or PVDF membrane by electrophoretic transfer.
8. Blot with antibody specific to the protein encoded by the targeted gene.

3.4.2 Quantitative or Semi-Quantitative RT-PCR

CRISPR induces double stranded breaks that result in frameshift mutations. This may not always cause insertion of a STOP codon and nonsense mediated decay of the message. Instead, in-frame deletion of a part of the transcript may result, leading to expression of a truncated or non-functional protein product. Amplifying mRNA-derived cDNA with a common reverse primer and two distinct forward primers can differentiate these outcomes (*see* **Note 2** and **Figure 2**). The ability of the designed primers to amplify the targeted sequence can be visualised by semi-quantitative PCR or measured by quantitative PCR.

3.4.2.1 RNA extraction and reverse transcription

1. Lyse dox-induced and non-dox-induced cells and extract RNA using a commercial RNA mini-prep kit according to kit specifications (e.g. lyse 10^5-10^6 cells with 350 µL

lysis buffer). Cell lysates can be stored at -80ºC (*see* **Note 14** & **Note 15**). Elute into the lowest recommended volume (e.g. 30 µL).

2. Determine the yield of RNA in elution using a spectrophotometer. Purified RNA can be at -80ºC for up to 6 months
3. Prepare reverse transcription-PCR (RT-PCR) for synthesizing cDNA.

   Reaction #1 (13 µL total volume):

   - 1 µL dNTPs
   - 1 µL Oligo(dT)$_{15}$ primers
   - RNA (normalize all samples to a consistent concentration, ideally 1-2 µg)
   - make up total volume to 13 µL with nuclease-free H_2O

   Incubate in PCR thermocycler at 65ºC for 5 min (hold at 12ºC)

   Reaction #2 (20 µL):

   - 4 µL 5x Buffer
   - 1 µL DTT
   - 1 µL RNase inhibitor
   - 1 µL Superscript III Reverse transcriptase
   - 13 µL reaction #1

   Incubate in PCR thermocycler at 50ºC for 1 h, followed by 70ºC for 15 min (hold at 4ºC).

4. cDNA can be stored at -20ºC.

3.4.2.2 Semi-quantitative PCR (sqPCR)

1. Using two sets of primers (*see* **Note 2**), amplify the CRISPR-targeted region of the gene of interest and a housekeeping control (e.g. GAPDH) from the prepared cDNA.

   50 µL reaction:

   - 5 µL *Taq* Buffer

- 1 μL dNTPs
- 2.5 μL forward primer (10 μM)
- 2.5 μL reverse primer (10 μM)
- 0.125 μL *Taq* DNA Polymerase
- 1 μL cDNA, 37.9μL nuclease-free H_2O

Thermocycler conditions:

Initial Denaturation: 95ºC, 3 min

Denaturation: 95ºC, 30 s

Annealing: (primer melting temperature)ºC, 30 s

Extension: 68ºC, 1 min per kilobase

2. PCR amplify **the same** samples 3 times (*see* **Note 16**):
   - 15 cycles
   - An additional 10 cycles (25 cycles)
   - An additional 10 cycles (30-35 cycles)
3. After each round of amplification, load 10μL of each sample on a 2% agarose **without** ethidium bromide or equivalent (*see* **Note 17**) and run for 35 min at 100 Volts.
4. To ensure even staining of DNA bands across the gels, soak gels in running buffer (TAE or TBE) + 0.05 μL/mL UV excitable DNA-binding dye for 15-20 min in the dark with gentle agitation (*see* **Note 18**).
5. Expose each gel using a UV gel imager and compare the intensity of the bands amplified from the cDNA of interest between samples and against the bands amplified using the housekeeping control primers.

3.4.2.3 Quantitative PCR (qPCR)

1. In a 384-well plate prepare qPCR reactions **in duplicate** for the CRISPR-targeted region of the gene of interest and a housekeeping control. Use two different forward primers as described for semi-quantitative PCR in section 3.4.2.2 (*see* **Note 2**).

   10 μL reaction:

   - 5 μL SYBR Green PCR Master Mix
   - 0.26 μL 10 μM primer mix
   - 2 μL cDNA
   - 2.74 μL nuclease-free H_2O

2. Make up standards **in duplicate** (1.0x-0.001x of a wild type sample) for each cDNA being amplified (including housekeeping control).
3. Put on plate seal and pulse plate in a centrifuge for 30 sec to settle contents to the bottom of the wells.
4. Properly seal plate using a roller or a solid piece of plastic such as ruler.
5. Run samples on a real-time PCR machine.

4 Notes

1. Instructions for designing and ordering gene specific oligonucleotides and ligating into FgH1tUTG can be found in Aubrey *et al.* (2015) ***(6)***. The Optimized CRISPR design site (crispr.mit.edu) ranks genes by predicted specificity for the input sequence, where possible, we choose guides with a score of 85 or above. The most effective guide design strategy is to design one guide targeting the most N-terminal exon possible and another targeting an exon that encodes a functional domain of the protein. This increases the chance of inactivating the targeted protein if a frameshift mutation that disrupts the entire open reading frame is not induced.
2. The first forward primer should be designed so that its 3' end anneals to the CRISPR cut site (3 bp 5' of the PAM on the template strand, *see* **Figure 1**) and the second, to be used in a separate reaction, designed 50-100 bp upstream. If CRISPR targeting was successful, and results in nonsense-mediated decay, both primers should be unable to bind (*see* **Figure 2**). If CRISPR targeting was successful, but nonsense-mediated decay does not occur, the second primer should still be able to bind to the upstream sequence. In this latter scenario, if the CRISPR-induced genetic lesion is sufficiently different to the forward primer, it should be unable to anneal to the mutated sequence. We recommend using plasmid-borne cDNA of the target gene as a positive control for primer efficacy.
3. It is recommended to first create cells stably expressing Cas9 that can then be transduced with FgH1tUTG carrying sgRNAs to different genes of interest, however if time is limited, both FUCas9Cherry and FgH1tUTG can be introduced simultaneously.

4. If cells are non-adherent, such as THP-1 monocytes, this step may be performed on the same day as infection. If cells are adherent, such as iBMDMs, they should be plated the evening before infection.
5. Successful transfection of HEK 293Ts can be assessed using a fluorescent microscope to observe $mCherry^+$ or $eGFP^+$ cells. 90-95% HEK 293T should be positive for the fluorophore on the vector being used. mCherry expression can be difficult to detect using a conventional inverted fluorescence microscope, so flow cytometry may be used as a more powerful alternative.
6. Some cell types are able to withstand two rounds of spin infection on subsequent days to increase the rate of infection. For example, THP-1 monocytes can be subjected to a second round of infection with little impact on cell viability, however iBMDMs fare better with only one round of infection. If a second round of infection is performed, always return cells to virus-free medium before returning to the incubator, as two subsequent nights in the presence of polybrene and virus often results in loss of cell viability.
7. Do not put polybrene on cells without virus as this leads to rapid and potent cell death.Check the morphology of the cells following the spin infection. There should be some stress evident due to the infection process, however if excessive cell death has occurred replace viral supernatant with fresh medium. While changing the medium will allow optimal recovery, it may also result in reduced rates of infection.
8. Gate on a medium population as many myeloid cell types (e.g. iBMDMs) have a tendency to silence integrated lentiviral sequences that are highly repeated (more fluorescence = more copies of integrated virus).
9. THP-1 cells in particular need to be plated at a density of at least $2x10^5$ cells/mL.

10. We usually expand up to 1.0-1.5x10^7 cells. For THP-1 or iBMDM, we seed 2x T150 flasks, each with 1x10^6 cells. One flask will be doxycycline-induced for 72 h and the other will be left without doxycycline as controls; after 72 h each flask should be close to confluence, providing target gene knockout has no detrimental effect on proliferation or survival. The remaining cells are suspended in 4-5 mL FBS + 10% DMSO and frozen in 1 mL cryovials in a cell freezing container at -80ºC overnight. Cells may be stored at -80ºC for up to 6 months or in liquid nitrogen for longer periods.
11. This allows the cells time to both turnover any remaining protein encoded by the targeted gene (complete protein turnover may require longer depending on the gene targeted) and to recover from any metabolic effects of the doxycycline treatment ***(7)***.
12. As this protocol leads to gene knockout in >90% of cells, we tend to continue from this point with the mixed pool in order to control for clonal effects such as *de novo* mutations and epigenetic or metabolic variation between monoclonal cell populations. This method also avoids delays due to the slow growth rate and poor survival of many myeloid cell types when cultured from a single cell. Disadvantages include persistence of low levels of target gene expression at the population level within the pool and outgrowth of those cells retaining the targeted gene if knockout is likely to result in a proliferative defect. If these phenomena are incompatible with the experiments to be performed, single-cell clones should be isolated by FACS sorting or limiting dilution. Multiple (4 or 5) clones of each knockout must be carried forward to allow for clonal effects on cell phenotype. For further details on working with clonal populations of CRISPR-targeted myeloid cells, refer to Schmidt *et al.* (2016) ***(8)***.
13. To control for effects of doxycycline treatment, potential spontaneous expression of the sgRNA vector (FgH1tUTG can be "leaky" in some cell types) and phenotypic

differences in the pool following sorting, validation of cell lines and initial phenotypic assays should be carried out on the knockout population, the same population without doxycycline treatment and a Cas9-only population with and without doxycycline. The appropriate control population(s) to carry forward may differ depending on cell type, genes knocked out or the exact assays being performed.

14. If lysing adherent cells, such as iBMDM, we find that a greater yield of protein, RNA or DNA is obtained if the cells are first scraped or trypsinised from the culture dish before lysis.
15. The exact number of PCR cycles required to amplify a band from the gene of interest will differ depending on its baseline expression levels. We have found the protocol described to be a good preliminary method for identifying the optimal number of PCR cycles for each gene (usually between 20-35 cycles). GAPDH was used as a housekeeping control and was detected from THP-1 monocytes at 15-20 cycles.
16. The electrophoretic current will form a gradient of ethidium bromide or other DNA-binding dyes down the agarose gel, preventing even staining of amplified bands.
17. If high background staining is an issue, the gels can be destained with water for 5 min, however this may also reduce the signal from faint bands.
18. This is particularly troublesome in cases when attempting to blot for loss of a protein that is part of a family whose members are of similar molecular weights and contain similar epitopes allowing antibody cross-reactivity (e.g. the inflammatory caspases)

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Table 1. Protocol variations for working with human THP-1 monocytes (THP-1) or murine immortalized bone marrow-derived macrophages (iBMDM)

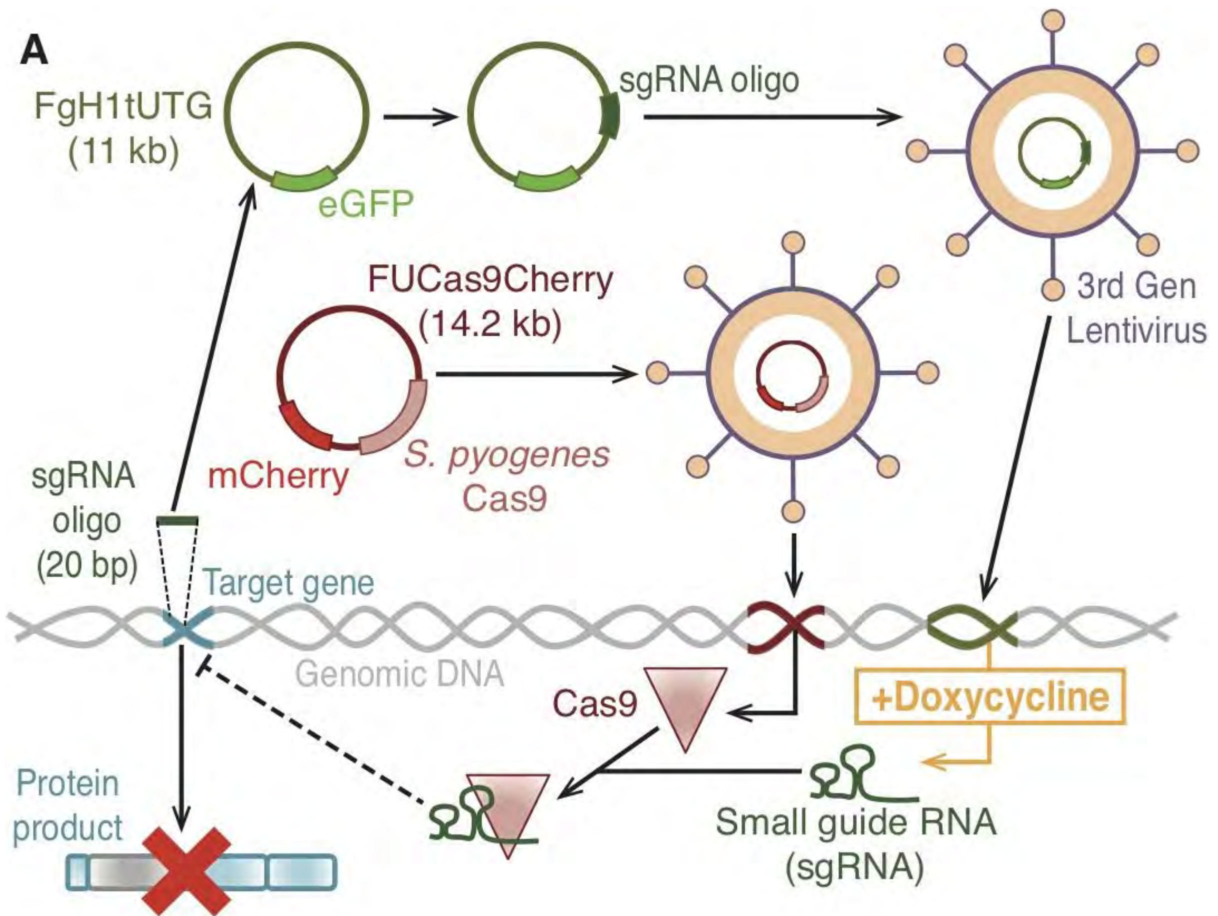
Myeloid Cell line:	**Culture conditions:**	**CRISPR protocol variations:**
THP-1 (human monocytes)	• Suspension cells • Maintain at $\leq 1 \times 10^6$ cells/mL in RPMI 1640 + 10% FCS	• Can handle two spin infections
iBMDM (murine macrophages)	• Adherent cells • Maintain at $\leq 1.5 \times 10^6$ cells/mL in DMEM + 10% FCS	• Single spin infection only • Be wary of silencing of CRISPR components over time, particularly following freeze-thaw. eGFP and mCherry levels should be monitored by flow cytometry following thaw and on a weekly basis during continuous culture. FACS sorting should be employed to re-enrich the population if the proportion of $eGFP^+$ or $mCherry^+$ cells drops below 85%.

Table 2. Advantages and disadvantages of knockout validation techniques

Validation Technique	**Western blotting**	**Quantitative PCR or Semi-quantitative PCR**	**Next-generation sequencing**
Advantages	Only technique that allows validation of knockout at the protein level.	Validates the loss of target-gene function at a post-transcriptional level in the absence of a specific antibody. Distinguishes between mutations that lead to nonsense mediated decay of mRNA and those that retain message viability, but may result in altered amino acid sequence and loss of protein function. sqPCR does not require specialty equipment.	Only technique that provides information on the exact mutation created by CRISPR/Cas9 targeting. Multiplexing allows analysis of many cell lines at once.
Disadvantages	Dependent on the availability of a validated antibody specific to the protein being detected (*see* **Note 19**).	Provides no information on the effect of the induced mutation on protein expression.	Provides no information on the effect of the induced mutation on mRNA or protein expression. Dependent on access to next generation sequencing facilities.

Figure 1. Delivery of CRISPR/Cas9 components into myeloid cells by lentiviral transduction

(A) A 20 bp oligonucleotide homologous to the targeted gene is cloned into the eGFP-tagged FgH1tUTG vector to create a doxycycline-inducible small guide RNA (sgRNA). This is delivered by lentiviral transduction into target cells, alongside a mCherry-tagged vector (FUCas9Cherry) encoding the Cas9 endonuclease from *Streptococcus pyogenes*. The lentiviruses integrate into the host genome allowing for selection of stable cell lines based on eGFP or mCherry fluorescence. Cas9 is constitutively expressed, whereas the sgRNA is inducible by treatment with doxycycline. (B) The sgRNA will interact with Cas9 via a stem-loop structure and guide it to the target gene, where Cas9 induces a double stranded break 3 bp upstream of the PAM motif (5'-NGG-3') on the template strand. This leads to an error-prone DNA-repair process (Non-Homologous End Joining, NHEJ), which most often causes frameshift mutations and disruption of protein expression and/or function.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(14.2 kb)
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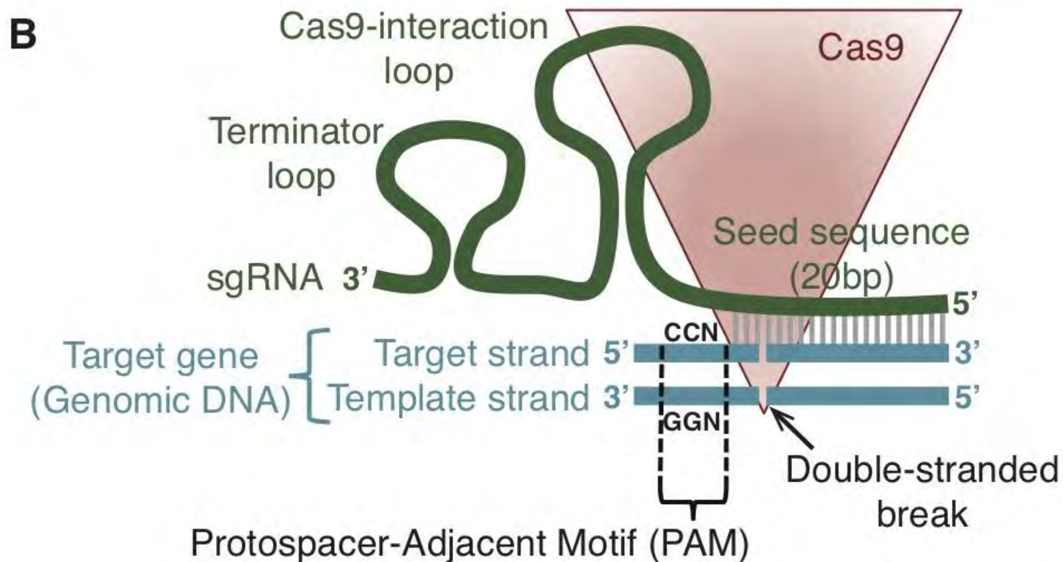
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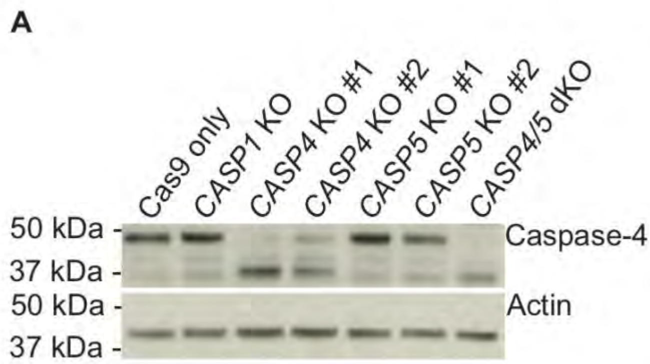
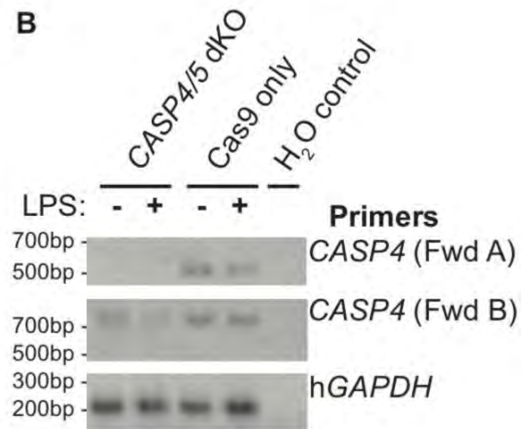
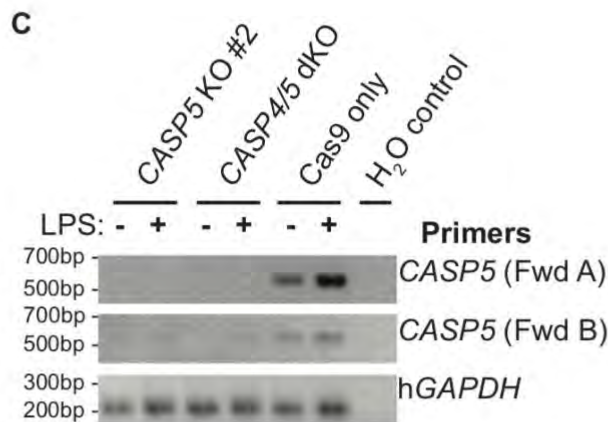
Cas9-interaction
loop
Cas9
Terminator
loop
Seed sequence
(20bp)
sgRNA 3'
5'
CCN
Target gene
(Genomic DNA)
Target strand 5'
3'
Template strand 3'
5'
GGN
Double-stranded
break
Protospacer-Adjacent Motif (PAM)

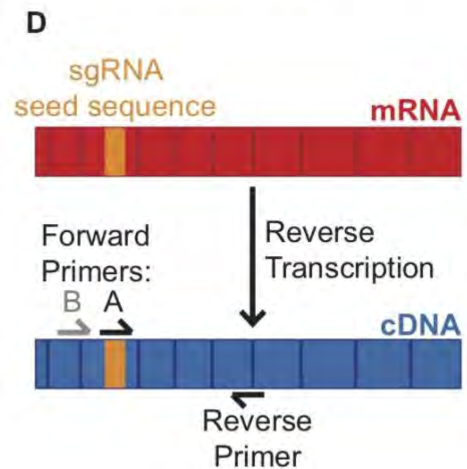
Figure 2. CRISPR-targeting of a gene does not always result in complete ablation of the protein product

CRISPR/Cas9 targeting of caspase-4 (*CASP4*) using two distinct sgRNAs did not result in nonsense mediated decay of the mRNA. (A) Using a caspase-4-specific antibody (Flamy-1, Adipogen), a truncated protein product could be detected (~37 kDa) by western blotting in *CASP4* and *CASP4*/*5*-targeted THP-1 monocytes. (B) *CASP4* cDNA synthesized from total mRNA from *CASP4*/*5*-targeted THP-1 monocytes was also detected by semi-quantitative PCR. Bands were amplified using a forward primer specific for a region upstream of the *CASP4* CRISPR target site (Fwd B), but not a forward primer specific to the site itself (Fwd A). This suggests that while deletion of a region of *CASP4* occurred, the truncated gene could still be expressed. (C) Targeting of caspase-5 (*CASP5*) resulted in complete loss of *CASP5* mRNA as neither set of *CASP5*-specific primers were able to amplify bands from *CASP5* or *CASP4*/*5*-targeted THP-1 cells, suggesting nonsense mediated decay of this message had occurred. (D) Schematic of semi-quantitative PCR strategy for validation of CRISPR knockout at the mRNA level. Adapted from Baker *et al.* (2015) with permission of John Wiley & Sons, Inc. ***(9)***


A
Cas9 only
CASP1 KO
CASP4 KO #1
CASP4 KO #2
CASP5 KO #1
CASP5 KO #2
CASP4/5 dKO
50 kDa
37 kDa
Caspase-4
50 kDa
37 kDa
Actin


B
CASP4/5 dKO
Cas9 only
H2O control
LPS: - + - +
Primers
700bp
500bp
CASP4 (Fwd A)
700bp
500bp
CASP4 (Fwd B)
300bp
200bp
hGAPDH


C
CASP5 KO #2
CASP4/5 dKO
Cas9 only
H2O control
LPS: - + - + - +
Primers
700bp
500bp
CASP5 (Fwd A)
700bp
500bp
CASP5 (Fwd B)
300bp
200bp
hGAPDH


D
sgRNA
seed sequence
mRNA
Forward
Primers:
B A
Reverse
Transcription
cDNA
Reverse
Primer