Methods in Molecular Biology (2016) 1341: 321–343
DOI 10.1007/7651_2015_213

Published online: 12 March 2015

Generation of a Knockout Mouse Embryonic Stem Cell Line Using a Paired CRISPR/Cas9 Genome Engineering Tool

Rahel Wettstein*, Maxime Bodak*, and Constance Ciaudo

Abstract

CRISPR/Cas9, originally discovered as a bacterial immune system, has recently been engineered into the latest tool to successfully introduce site-specific mutations in a variety of different organisms. Composed only of the Cas9 protein as well as one engineered guide RNA for its functionality, this system is much less complex in its setup and easier to handle than other guided nucleases such as Zinc-finger nucleases or TALENs.

Here, we describe the simultaneous transfection of two paired CRISPR sgRNAs-Cas9 plasmids, in mouse embryonic stem cells (mESCs), resulting in the knockout of the selected target gene. Together with a four primer-evaluation system, it poses an efficient way to generate new independent knockout mouse embryonic stem cell lines.

Keywords: CRISPR/Cas9, CRISPR-Cas9 paired design, mESCs: mouse embryonic stem cells, (Homozygous) knockout, Genome engineering

1 Introduction

Mouse embryonic stem cells (mESCs) can be derived from the inner cell mass of the blastocyst at 3.5 days post-coïtum (dpc). In appropriate culture conditions (e.g., BMP/LIF), they retain their pluripotent state and are able to proliferate indefinitely. Three proteins form the core factors essential for the maintenance of stem cell pluripotency, namely Oct4 (Pou5f1), Sox2, and Nanog (1, 2). Their complex network of interaction with each other or with additional pluripotency factors and transcription factors provides a fragile balance between stemness and the possibility for cell fate specification via differentiation (3). Stem cells harbor an immense developmental potential as they can differentiate into three germ layers and eventually all tissue types. From a medical point of view, these abilities of stem cells, together with technical progress, open up new possibilities for the investigation of genetic diseases. A better understanding of stem cells in biology is a key step

*These authors contributed equally to this work

in the development of personalized medicine (4). Several stem cell lines have been established from embryonic and adult tissues, but the mESCs are a comparatively easy to handle model suited to address very diverse questions in biology and medical science.

In the past years, a number of genome engineering tools such as Zinc-finger nucleases and TALENs have been used to introduce targeted modifications in the genome. Lately, CRISPR/Cas9 has emerged as a new tool and proven to be an easy and cost-effective application (5).

Although interspersed repeats were first mentioned in 1987 (6), the term "Clustered Regularly Interspersed Short Palindromic Repeats," shortly CRISPR, emerged only in 2002 when Jansen and Mojica used the term to describe genomic loci, containing repeated DNA sequences interspersed by nonrepetitive elements in microbes (7). Recently, CRISPRs have been more closely and intensively investigated and were identified as a defence system used by bacteria against foreign DNA, derived from pathogens such as viruses (8). Thanks to a better understanding of the system, scientists have been able to adapt the CRISPR/Cas9 defence system for genome engineering in a wide range of organisms. Many publications proved this system to be an effective and efficient tool to generate targeted genome modifications (9, 10). Among the three types of the CRISPR system found in bacteria, type II CRISPR/Cas9 from *Streptococcus pyogenes* is the most suitable for genome editing and therefore also most investigated. It consists of a CRISPR-associated protein 9 (Cas9) and two RNAs—CRISPR RNA (crRNA) and trans-acting CRISPR RNA (tracrRNA)—which are partially complementary to each other and form a duplex. The crRNA and tracrRNA can be fused to form a single guide RNA (sgRNA), simplifying the use of the CRISPR/Cas9 as a genome engineering tool (11, 12). The system gains its specificity from the sgRNA, which is complementary to a sequence in the target genome, and the so-called protospacer adjacent motif (PAM), a defined 3 nt sequence at the 3′ end of the target DNA sequence (13–15). The sgRNA guides the Cas9 protein to its site of action where it recognizes the PAM and cleaves the DNA using its two catalytic subunits HNH and RuvC, inducing a double-strand break (DSB) (16). Then, DSBs caused by CRISPR/Cas9 will be repaired by the cell either using nonhomologous end joining (NHEJ), resulting in small insertions/deletions at the ligation site, or by homology-directed repair (HDR), requiring a template for perfect repair. Generally, NHEJ is preferred over HDR in case of CRISPR/Cas9 DSBs but the repair mode can be guided toward HDR by the use of a modified Cas9 protein, which only nicks the DNA instead of cleaving it (10). Recent modifications of the Cas9 protein allow a broader employment of the CRISPR/Cas9 system going beyond genome editing. The use of catalytically inactive

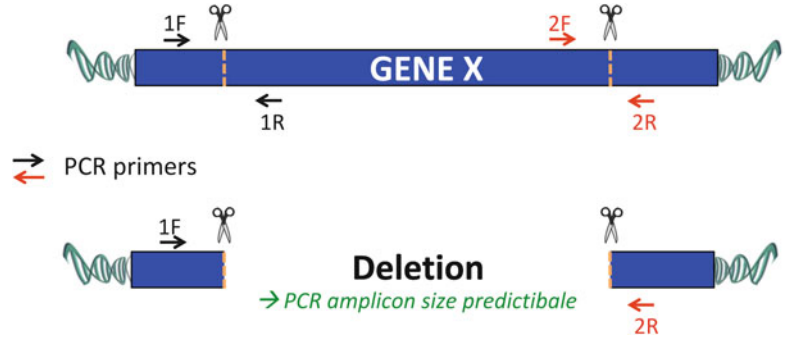

Fig. 1 Overview of the paired CRISPR/Cas9 strategy. In this protocol, specific CRISPR/Cas9 constructs are designed and used in pairs in order to induce two cuts in the genomic DNA involving the generation of a deletion. Then specific PCR primers on both side of each CRISPR/Cas9 target site are designed. By combining the forward primer of the upstream CRISPR/Cas9 target site (called 1F) and the reverse primer of the downstream CRISPR/Cas9 target site (called 2R), it is possible to distinguish the "wild type" amplicon, resulting from the amplification of the wild type allele, from the "deleted" amplicon, shorter, resulting from the amplification of the deleted allele. This PCR allows the assessment of the efficiency of CRISPR/Cas9 pairs as well as the screen of the independent clones by distinguishing wild type, heterozygous and homozygous mutant clones from each other

Cas9 (dCas9) fused to activator or inhibitor attracting molecules allows specific endogenous gene regulation posing an alternative to conventional RNA interference (RNAi) (17, 18). Fused to fluorescent molecules, dCas9 can also serve as an imaging tool, visualizing specific loci on the genome (19). Additionally, O'Connell and colleagues found a way to guide Cas9 also to ssRNA, opening up new possibilities to study RNA function (20). To date several online tools, such as e-CRISP (21) and Feng Zhang Lab's target finder (22), have been published, simplifying the design of sgRNAs and prediction of possible off-target effects (23–25). These tools allow rapid and efficient generation of CRISPR target sites resulting in genome scale screening libraries (26).

In our studies, we employed a combination of two CRISPR sgRNAs targeting a selected gene together with an easily handled four primer-evaluation system (*see* Fig. 1). By cutting the target gene twice in close proximity, a piece of the DNA is cut out, creating a deletion in the gene of interest, rendering it nonfunctional (27). Even though deletions up to 1 Mb can be achieved (28), careful positioning of the two sgRNA target sites and the verification primers allow easy detection of successful deletions of around 2 kb by PCR as well as distinction between homo- and heterozygous mutants. The specific steps of this protocol are described in the following sections.

2 Materials

2.1 Design of Paired CRISPR/Cas9 sgRNAs and PCR Primers

1. Genomic DNA sequences (*see* **Note 1**).
2. sgRNA CRISPR design software (*see* **Note 2**).
3. PCR primer design software (*see* **Note 3**).

2.2 Construction of Specific CRISPR/Cas9 Vectors Using the pSpCas9 (BB)-2A-GFP Plasmid

1. pSpCas9(BB)-2A-GFP (Addgene plasmid ID: 48138), also called pX458 (29).
2. BpiI (BbsI) enzyme, 10 U/μL and its related buffer G (Thermo Scientific).
3. Agarose powder (Invitrogen).
4. Ethidium Bromide Solution 10 mg/mL (Bio-Rad).
5. 6× Loading dye (Thermo Scientific).
6. 2-Log DNA Ladder (0.1–10.0 kb) (New England BioLabs).
7. Electrophoresis DNA power supply and horizontal electrophoresis cell (Bio-Rad).
8. UV revelation device for DNA electrophoresis gel.
9. QIAquick® Gel extraction Kit (Qiagen).
10. T4 DNA ligase and its associated 10× T4 DNA Ligase Buffer (Thermo Scientific).
11. One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen).
12. Lysogeny Broth (LB) medium (BD).
13. Autoclaved water.
14. Ampicillin (Applichem). Prepare 100 mg/mL stock solution by diluting 100 mg of Ampicillin powder into 1 mL of autoclaved water. This stock solution must be stored at −20 °C.
15. Ampicillin agar plates (Ampicillin at 0.1 mg/mL final concentration).
16. GeneJET Plasmid Miniprep Kit (Thermo Scientific).
17. Pure Link™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen).

2.3 Efficacy Assay of Paired Designed CRISPR/Cas9 Constructs

1. mESC proliferative medium: Dulbecco's Modified Eagle Media (DMEM) (Invitrogen) containing 15 % of a special selected batch of fetal bovine serum (FBS; Life Technologies) tested for optimal growth of mESCs, 1,000 U/mL of LIF (Millipore), 0.1 mM of 2-β-mercapto-ethanol (Life Technologies), 0.05 mg/mL of streptomycin, and 50 U/mL of penicillin (Sigma).
2. mESC differentiation medium: DMEM (Invitrogen) containing 10 % of FBS (Life Technologies), 0.1 mM of 2-β-mercapto-ethanol (Life Technologies), 0.05 mg/mL of streptomycin and 50 U/mL of penicillin (Sigma). This medium is LIF-free.

3. 0.2 % gelatin solution and gelatin-coated flask/plate. Prepare 500 mL of 0.2 % gelatin solution: dissolve 1 g of gelatin from porcine skin (Sigma) with 500 mL of autoclaved water, mix well and then autoclave the solution. Mouse ESCs grow on gelatin-coated support in the absence of feeder cells. To gelatin-coat a flask or plate, pour 1–2 mL of 0.2 % gelatin solution on the bottom of the flask, distribute homogeneously (the entire surface must be covered), incubate minimum 5 min at room temperature, and finally remove the gelatin (*see* **Note 4**).
4. Cell culture incubator: mESCs grow at 37 °C in 8 % CO_2.
5. Cell culture media: 0.05 % Trypsin containing EDTA (Life Technologies) and PBS pH 7.4 (1×) without $CaCl_2$ and $MgCl_2$ (Life Technology).
6. pX458 empty: Unmodified pSpCas9 (BB)-2A-GFP (pX458) vector at 500 ng/μL.
7. Specific CRISPR/Cas9 constructs: pSpCas9(BB)-2A-GFP vectors containing specific sgRNA (Obtained from Section 3.2) at 500 ng/μL.
8. Lipofection reagents: Opti-MEM® I reduced Serum (1×) (Life Technologies) (*see* **Note 5**) and Lipofectamine™ 2000 reagent (Life Technologies).
9. GenElute™ Blood Genomic DNA kit, Miniprep (Sigma).
10. PCR primers at a concentration of 10 μM.
11. Compounds for PCR reactions: 5× Green GoTaq® reaction buffer (Promega), dNTP mix 10 mM each (Thermo Scientific), GoTaq® G2 Polymerase (Promega), and autoclaved water.
12. Agarose powder (Invitrogen).
13. Ethidium Bromide Solution 10 mg/mL (Bio-Rad).
14. 2-Log DNA Ladder (0.1–10.0 kb) (New England BioLabs).
15. Electrophoresis DNA power supply and horizontal electrophoresis cell.
16. UV revelation device for DNA electrophoresis gel.

2.4 Lipofection of Cells with the Selected Combinations of Two Specific CRISPR/Cas9 Constructs and Isolation of Single Cells in 96-Well Plates

Items 1–8 are identical to Section 2.3.

9. Fluorescence-activated cell sorting (FACS) device/Moflo (Beckman Coulter) (*see* **Note 6**).
10. Dimethyl Sulfoxide (Sigma).

2.5 Genomic DNA Extraction from 96-Well Plates, PCR Screening and Identification of Potential Candidates

1. 96-well plates containing mESCs from Section 2.4.
2. Autoclaved water.
3. Lysis buffer: 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5 % SDS, 10 mM NaCl. Prepare 10 mL of 1 M Tris–HCl pH 7.5: weight 1.57 g of Tris–HCl (Applichem), dissolve it in 10 mL of autoclaved water and adjust the pH to 7.5. To prepare 50 mL of lysis buffer mix: 500 µL of 1 M Tris–HCl pH 7.5, 0.146 g of EDTA, 0.25 g of SDS, and 0.029 g of NaCl, complete to 50 mL with autoclaved water and mix. This buffer can be stored at room temperature.
4. Proteinase K (Applichem). Prepare a stock solution at 20 mg/mL: dilute 20 mg of proteinase K in 1 mL of autoclaved water. This stock solution must be stored at −20 °C.
5. Precipitation solution: 1.5 M NaCl. To prepare 15 mL of precipitation solution dissolve 1.31 g of NaCl in 15 mL of autoclaved water. This buffer can be stored at room temperature.
6. Ice cold EtOH 100 %.
7. EtOH 70 %.
8. 96-well PCR plates (Thermo Scientific).
9. PCR primers at a concentration of 10 µM.
10. Compounds for PCR reactions: 5× Green GoTaq® reaction buffer (Promega), dNTP mix 10 mM each (Thermo Scientific), GoTaq® G2 Polymerase (Promega).
11. Agarose powder (Invitrogen).
12. Ethidium Bromide Solution 10 mg/mL (Bio-Rad).
13. 2-Log DNA Ladder (0.1–10.0 kb) (New England BioLabs).
14. Electrophoresis DNA power supply and horizontal electrophoresis cell.
15. UV revelation device for DNA electrophoresis gel.

3 Methods

Many of the following procedures were adapted from a publicly available protocol by E.P. Nora and E. Heard (27).

3.1 Design of Paired CRISPR/Cas9 sgRNAs and PCR Primers

In this section, it is described how to design specific sgRNAs using two different online tools: the e-CRISP software (21) and the Feng Zhang lab's Target Finder (22). As previously mentioned in Section 1, a lot of CRISPR sgRNA design tools are now available, and any tools can be used as long as they take into account the prediction of off-targets (30).

Each sgRNA design software gives potential CRISPR target sites using a specific algorithm; in order to increase the chance to hit

genomic DNA, it is advised to use two distinct tools and to design two sgRNAs with each one (*see* **Note 2**).

In this protocol, the plasmid pSpCas9 (BB)-2A-GFP (pX458) (29) is used as vector and requires to add specific overhangs to the designed sgRNAs. Other vectors are available, and if another vector than pX458 is planned to be used, make sure to add the correct overhangs to insert the sgRNAs into the corresponding vector.

1. Delimit your genomic sequence of interest (*see* **Note 7**). It is advised to paste the DNA sequence of interest into a text editor of choice (e.g., Word) for further easy localization and handling of sgRNAs and PCR primer sequences.
2. Design sgRNAs using the e-CRISP online bioinformatics tool (21). Go to www.e-crisp.org and choose "De-novo". Then select "Mus musculus (Mouse, GRCm38.75)" as model organism using the drop-down list. Next, select "Enter Target Sequence" and enter the sequence of interest (FASTA format) in the corresponding box. Finally click on "Start sgRNA search". The website will provide a ranked list of potential sgRNAs, with a specificity score, an annotation score and their positions on the sequence of interest.
3. Choose two sgRNAs, which are within a 1–2 kb size range region (*see* **Note 8**) and locate them on your sequence of interest created at **step 1**.
4. Design sgRNAs using the Feng Zhang lab's Target Finder (22). Go to http://crispr.mit.edu/ and fill the required field: search name and e-mail address. Then select "mouse" as target genome and paste your sequence of interest into the corresponding box. Notice that a maximum of 250 bp can be pasted inside this box. It is advised to perform several rounds of research of 250 bp inside the 1–2 kb region delimited at **step 3**. Finally click on "Submit". The complete analysis takes 15 min in average, when the job is completed click on "Guides & off targets". The website will provide a ranked list of potential sgRNAs with a quality score, the number of off-target sites with the corresponding number and position of mismatches.
5. Choose two sgRNAs, which are within a 1–2 kb size range region delimited at **step 3** (*see* **Note 8**), and locate them on your sequence of interest created at **step 1**.
6. Design specific PCR primers using a PCR primer online software (*see* **Note 3**). For each sgRNA: design PCR primers in a region comprising at least 300 bp upstream and downstream of the sgRNA target site. Set up the parameters to amplify amplicons around 500–600 bp, at a melting temperature of 60 °C (*see* **Note 9**). Identify their positions on the sequence of interest.
7. Determine the expected PCR amplicon sizes for the deletion PCR. For each sgRNA couple combination (four distinct sgRNAs means six different combinations), determine the

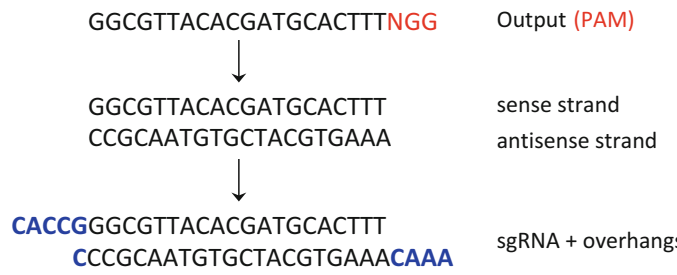

Fig. 2 sgRNA modifications for insertion into the pSpCas9(BB)-2A-GFP vector. sgRNAs have to be modified to assure a proper ligation with the digested vector backbone. The PAM sequence (*red*) needs to be removed in the first step and the sense strand complemented with its corresponding antisense. The ends need to be modified according to the restriction enzyme used for the vector. For BpiI (BbsI), this corresponds to a "CACCG" 5′ overhang of the sense strand and "CAAA" 5′/"C" 3′ overhang of the antisense strand (*blue*)

expected size for the deletion PCR product (*see* Fig. 1) knowing that the CRISPR/Cas9 complex cleaves the genomic DNA 3 nucleotides before the PAM motif (16).

8. Preparation for the ordering of sgRNAs and PCR primers. For each sgRNA: remove the PAM motif from the sequence, this is the sense strand (*see* **Note 10**). Then write the corresponding complement sequence, this is the antisense strand. Add a "CACCG" overhang on the 5′ of the sense strand. Flank a "CAAA" overhang on the 5′ and a "C" overhang on the 3′ of the antisense strand (*see* Fig. 2) (*see* **Note 11**). Order the sgRNAs and the PCR primers (*see* **Note 12**).

3.2 Construction of Specific CRISPR/Cas9 Vectors Using the pSpCas9(BB)-2A-GFP Plasmid

In this protocol, the pSpCas9(BB)-2A-GFP plasmid is used, also known as pX458 (Addgene plasmid ID: 48138). This plasmid allows the expression of a Cas9 nuclease fused with GFP. The plasmid pX458 is used at a concentration of 500 ng/μL.

1. Digest 1 μg of pX458 plasmid with BpiI (BbsI). Prepare two reactions, one containing the restriction enzyme and one negative control (uncut pX458 plasmid), as follows:

Stock	For one digestion reaction (μL)	For one negative control (μL)
Buffer G	2	2
BpiI (BbsI)	1	0
pX458 plasmid (500 ng/μL)	2	2
H_2O	15	16
Total (μL)	20	20

And incubate 1 h at 37 °C.

2. Prepare a 1 % Agarose gel with an Ethidium Bromide final concentration of 0.1 μg/mL. Add 4 μl of 6× loading dye to each sample and load the totality of the digestion reaction on the gel. Run the gel at 100 V for 45 min. Use the negative control as reference to excise the linearized plasmid and purify it using the QIAquick® Gel Extraction Kit. The linearized pX458 plasmid can be stored at −20 °C.
3. Generate the double stranded sgRNAs. Mix 8 μL of sense and antisense strand from one sgRNA in one PCR tube (one PCR tube per sgRNA design). Put the PCR tubes into a thermo cycler and run the following program:

Step 1: 94 °C for 4 min
Step 2: 70 °C for 10 min
Step 3: 37 °C for 20 min

4. Ligate the double-stranded sgRNAs into the BbsI digested vector. Additionally to the samples, prepare one negative control reaction that does not contain double stranded sgRNA. For one ligation reaction, mix the following reagents:

Stock	For one ligation reaction (μL)	For one negative control (μL)
Double-stranded sgRNA (annealed oligos)	2	0
Linearized pX458 plasmid (1 μg)	1	1
10× T4 DNA ligase buffer	2	2
T4 DNA ligase	1	1
H_2O	4	6
Total (μL)	10	10

Incubate overnight at room temperature.

5. Amplify the ligation products. For one ligation reaction: thaw one aliquot One Shot® TOP10 Chemically Competent *E. coli* on ice, then add 5 μL of ligation product to the competent cells, mix gently and incubate 15–30 min on ice. Heat shock bacteria 30 s at 42 °C in a water-bath and immediately put them back on ice. Then add 1 mL of room temperature LB without antibiotics and incubate 1 h at 37 °C under agitation (200 rpm). Centrifuge bacteria at 400 × *g* for 5 min, remove and discard 900 μl of supernatant, resuspend the bacteria pellet in the remaining 100 μl and plate on ampicillin agar plates

(one plate per condition). Finally incubate the plates overnight at 37 °C (*see* **Note 13**).

6. Validation of the cloning procedure. The day after, for each plate (except for the negative control plate), pick three single independent colonies and inoculate each of them into one bacterial culture tube containing 3 mL of LB with ampicillin at a final concentration of 0.1 mg/mL. Incubate at 37 °C under agitation (200 rpm) overnight. The day after, use the 2 mL of bacterial culture to extract plasmids for each condition using the GeneJET Plasmid Miniprep Kit, and keep the rest of the bacterial culture tubes at 4 °C. Send purified plasmids for sequencing using the following primer: 5′-GACTATCATAT GCTTACCGT–3′. Analyze the sequencing results and determine which bacterial clones present a correct sgRNA sequence incorporated into the pX458 vector (*see* **Note 14**).
7. Amplify the correct specific CRISPR/Cas9 constructs. Take out the bacterial culture tubes corresponding to the bacterial clones expressing the correct CRISPR/Cas9 constructs (determined at **step 6**). For each sample, prepare 1 L Erlenmeyer containing 200 mL of LB with ampicillin at a final concentration of 0.1 mg/mL. Then, pipet the 1 mL bacterial culture left at **step 6** into the corresponding 1 L Erlenmeyer. Incubate the 1 L Erlenmeyer at 37 °C under agitation (200 rpm) overnight. The day after, extract plasmid using the Pure Link™ HiPure Plasmid Filter Maxiprep Kit. Measure the DNA concentration of the samples and dilute them in autoclaved water to reach a final concentration of 500 ng/μL. Store the diluted DNA at –20 °C (*see* **Note 15**).

3.3 Efficacy Assay of Paired Designed CRISPR/Cas9 Constructs

For this part of the protocol, lipofection reactions are performed in 6-well plates (1 well per condition). There are eight different conditions: six different combinations of two specific CRISPR/Cas9 constructs and two negative controls. One negative control corresponds to cells transfected with the empty pSpCas9(BB)-2A-GFP and is referred in the protocol as "pX458 empty". The other negative control corresponds to cells transfected with PBS (1×) instead of DNA and is referred in the protocol as "Mock".

Mouse ESCs have been plated the day before, as cells must be between 50 and 70 % of confluence for the lipofection reaction.

1. Change the medium of the cells. Remove the mESC proliferative medium, and wash the cells with 2 mL of PBS (1×). Then add 2 mL of mESC proliferative medium without antibiotics (the antibiotics can inhibit the lipofection reaction). For each condition, prepare two 1.5 mL Eppendorf tubes labeled A and B.

2. For each A tube: put 240 μL of Opti-MEM® (1×) and then add 10 μL of Lipofectamine™ 2000 reagent. Mix gently by pipetting up and down and incubate 5 min at room temperature under the hood without light (*see* **Note 5**). During this time, prepare the B tube of each condition as follows:

Stock	For one reaction (μL)	Empty pX458 (μL)	Mock (μL)
Opti-MEM® (1×)	242	246	246
CRISPR/Cas9 construct 1 (500 ng/μL)	4	0	0
CRISPR/Cas9 construct 2 (500 ng/μL)	4	0	0
pX458 empty (500 ng/μL)	0	4	0
PBS (1×)	0	0	4
Total (μL)	250	250	250

3. For each condition: mix A and B gently by pipetting up and down. Then incubate 20 min at room temperature under the hood without light (*see* **Note 5**). Finally put the lipofection mix (500 μL) on each well and mix gently by pipetting up and down. Put the cells back in the incubator.
4. After 6–8 h, remove the medium from the cells, wash them with 2 mL of PBS (1×) and add 2 mL of new mESC proliferative medium for all conditions.
5. Change the medium of the cells 24 h after lipofection.
6. Harvest the cells 48 h after lipofection. For each well: aspirate the medium and wash once with 2 mL PBS (1×) then add 0.5 mL of 0.05 % Trypsin containing EDTA and incubate 5 min at 37 °C. Add 2 mL of mESC differentiation medium, pipet up and down and flush the well several times to detach all the cells. Then collect the cells in a 15 mL falcon tube and spin the cells 5 min at 180 × *g*. Aspirate the supernatant and resuspend the cell pellet in 1 mL PBS (1×). Then, transfer to a 1.5 mL Eppendorf tube. Finally spin the cells again 5 min at 180 × *g*, aspirate the supernatant and keep the cell pellet. Cell pellets must be kept on ice. If the following step in not performed the same day, store the cell pellets at −80 °C.
7. Extract DNA from cell pellets following the manual of the GenElute™ Blood Genomic DNA kit, Miniprep (Sigma). Measure DNA concentrations and dilute samples with autoclaved water to reach a DNA concentration of 50 ng/μL for all the samples (*see* **Note 16**).

8. Prepare the PCR tubes. For each of the CRISPR/Cas9 DNA samples, label three PCR tubes: 1F + 1R, 2F + 2R, and 1F + 2R. Then prepare for each tube: a corresponding "empty pX458" control containing "empty pX458" DNA, a corresponding "Mock" control containing "Mock" DNA, and a negative control PCR reaction containing water instead of DNA. This represents a total of 12 PCR tubes per combination of two specific CRISPR/Cas9 constructs (*see* Fig. 3).
9. Prepare PCR reactions as follows:

Stock	For one PCR reaction (μL)	For one negative control PCR reaction (μL)
5× Green GoTaq® reaction buffer	4	4
dNTP mix 10 mM each	0.4	0.4
Primer-F 10 μM	0.5	0.5
Primer-R 10 μM	0.5	0.5
GoTaq® G2 Polymerase	0.1	0.1
Sample's DNA (50 ng/μL)	1	0
H_2O	13.5	14.5
Total (μL)	20	20

10. Run the following PCR program:

Step 1: 94 °C for 5 min
Step 2: 94 °C for 30 s
Step 3: X°C for 30 s
Step 4: 72 °C for Y seconds
Step 5: GOTO step 2, 34 times
Step 6: 72 °C for 5 min
Step 7: 12 °C HOLD

X corresponds to the melting temperature of the primers, which have been designed previously. Y is specific for each primer combination and corresponds to the elongation time depending on the amplicon size that has been determined previously (Section 3.1, **step 7**) (*see* **Note 17**).

11. Prepare a 2 % Agarose gel with an Ethidium Bromide final concentration of 0.1 μg/mL. For each condition forecast 13 slots (*see* Fig. 3). Then load the gel with 10 μL of the PCR reactions. Store the rest at 4 °C (*see* **Note 18**). Reserve one slot

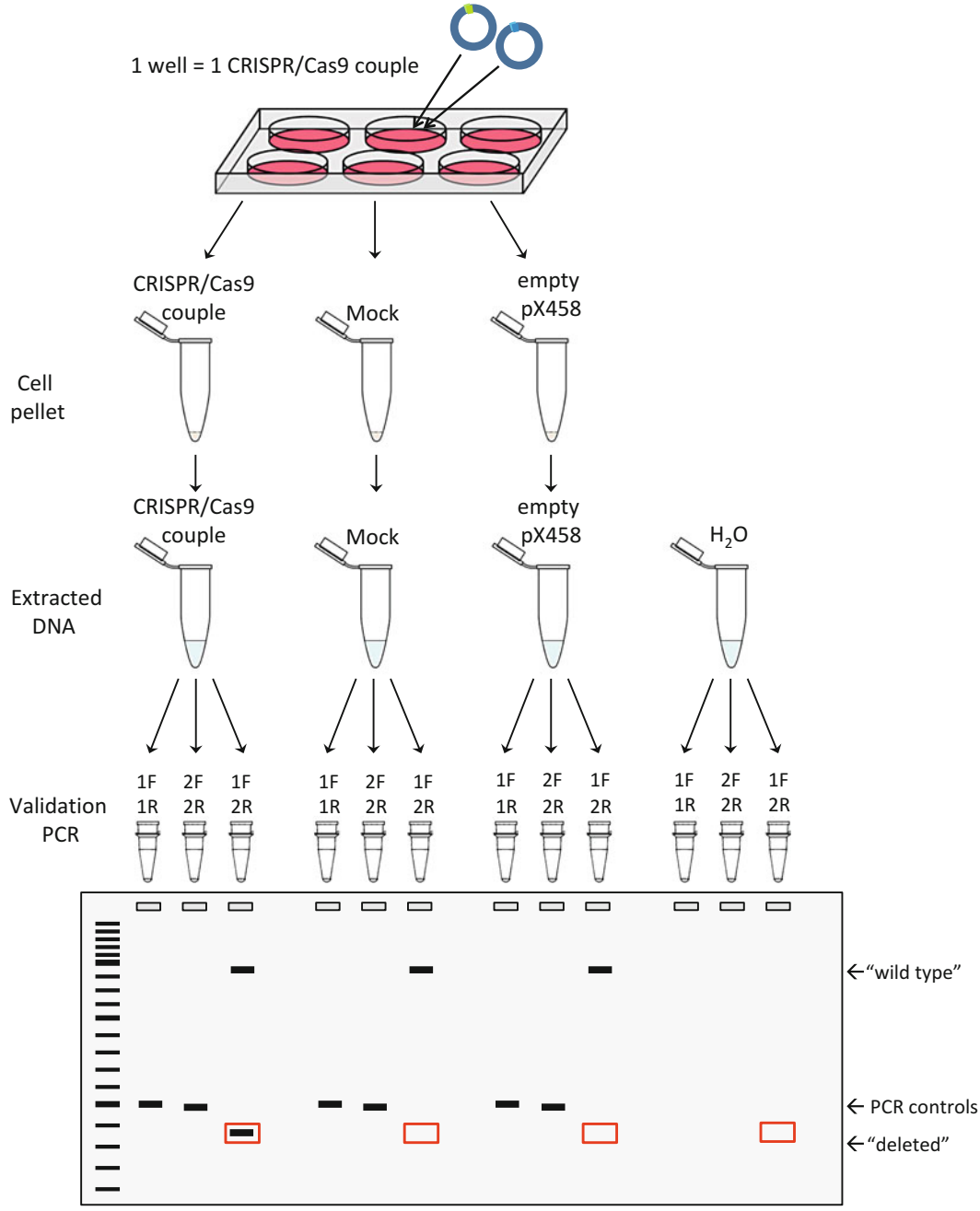

Fig. 3 PCR screen for CRISPR/Cas9 couple performance assessment. The figure shows consecutive steps to analyse one CRISPR/Cas9 couple in mixed population including empty pX458 vector-, mock-, and water-control (all steps, including controls have to be repeated for each CRISPR/Cas9 couple tested with the corresponding primers). 1F and 1R refer to specific primers flanking the sgRNA target site 1; 2F and 2R refer to specific primers of sgRNA target site 2. 1F + 1R as well as 2F + 2R are used as PCR primer control. Combination of the primers 1F and 2R allows the detection of the deletion induced by the paired CRISPR/Cas9 system. For the sample, two different scenarios are possible: (i) only one large amplicon corresponding to the “wild type” amplicon can be observed meaning that the deletion event did not occur, or (ii) one large and one small amplicon corresponding to the “wild type” and the “deleted” amplicon, respectively, can be observed, meaning that the deletion event occurred. The mock and empty pX458 controls must only display the “wild type” amplicon. No bands should be detected in the water control

to load 2.5 μL of 2-Log DNA Ladder. Run the electrophoresis (eg. 110 V for 1 h). Then reveal the Agarose gel using a UV device for DNA electrophoresis gel.

12. Analyze the results. Select the couples for which a band at the expected size appears for the deletion PCR but not for the "empty pX458" control, not for the "Mock" control, and not for the negative control PCRs (*see* Fig. 3). Only these couples will be used for the continuation of the protocol.

3.4 Lipofection of Cells with the Selected Combinations of Two Specific CRISPR/Cas9 Constructs and Isolation of Single Cells in 96-Well Plates

For this part of the protocol, lipofection reactions are performed in 6-well plates (1 well per condition). There are four different conditions: the two best combinations of two specific CRISPR/Cas9 constructs (determined in the previous step) and two negative controls: "empty pX458" and "Mock" (previously described in Section 3.3).

Here GFP expression is used as criteria to select the cells expressing the specific sgRNA pSpCas9(BB)-2A-GFP constructs. GFP expressing cells are FACS sorted and single cell distributed into 96-well plates (1 cell per well) in order to obtain single independent clones. Selecting only cells expressing GFP should increase the number of positive candidates with the specific knockout. The two negative controls are used to calibrate the cell sorter as the "Mock" control does not contain cells expressing GFP and the "empty pX458" does.

If no cell sorting device is available, an alternative method is proposed in Section 4 (*see* **Note 6**).

In this part of the protocol, 96-well plates are used, which implies a lot of pipetting steps that can be facilitated by the use of a multichannel pipette.

Mouse ESCs have been plated on 6-well plates the day before, as cells must be between 50 and 70 % of confluence for the lipofection reaction.

1. Transfect mESCs with the best combinations of two specific CRISPR/Cas9 constructs. Perform four lipofection reactions: one for each of the two best combinations of two specific CRISPR/Cas9 constructs (determined in the previous Section 3.3) and two negative controls: "empty pX458" and "Mock". For the lipofection procedure, follow exactly the **steps 1–5** described in Section 3.3.
2. Collect and sort the cells 48 h after lipofection. First, prepare eight gelatin-coated 96-well plates (50 μL of 0.2 % gelatin solution per well for minimum 5 min), remove the gelatin and add 200 μL of mESC proliferative medium per well. Then, for each well of the 6-well plate: aspirate the medium, wash the cells with 2 mL PBS (1×) then add 0.5 mL of 0.05 % Trypsin containing EDTA, and incubate 5 min at 37 °C. Add

2 mL of mESC differentiation medium, pipet up and down and flush the well several times to detach all the cells. Then collect the cells in a 15 mL falcon tube and spin the cells 5 min at 180 × *g*. Aspirate the supernatant and well resuspend the cell pellet in 1 mL of PBS (1×) + 2 % FBS. Keep the cells on ice until the sorting.

3. Sort the cells. Use the "empty pX458" cells and the "Mock" cells to calibrate the machine and sort 1 cell per well of the 96-well plates. For each "combination of two specific CRISPR/Cas9 constructs" cell sample, fill four 96-well plates. Then put the 96-well plates back in the incubator. If no cell sorting device is available, an alternative method is proposed in Section 4 (*see* **Note 6**).
4. Wait until the cells grow. If the medium's color changes, remove the medium and put 200 μL of fresh mESC proliferative medium per well. On average, it takes 15 days to observe a color change of the medium.
5. Regroup the clones from one "combination of 2 specific CRISPR/Cas9 constructs" on the same 96-well plate. For each combination: examine the plate under a microscope and identify full wells. Then pick all these clones into a new gelatin-coated 96-well plate containing 100 μL of mESC proliferative medium per well. For each clone: remove the medium, wash with 100 μL of PBS (1×), add 50 μL of 0.05 % Trypsin containing EDTA, incubate 5 min at 37 °C, then add 200 μL of mESC proliferative medium per well, pipet up and down and transfer everything into a well of the new 96-well plate.
6. Change medium 24 h after.
7. Wait until more than 50 % of the plate is around 70 % of confluence. Change medium if necessary. Then split each 96-well plate into two 96-well plates: one labeled as "freezing plate," the other one as "DNA plate". For one plate: prepare two new gelatin-coated 96-well plates containing 100 μL of mESC proliferative medium per well. Then for each well: remove the medium, wash with 100 μL of PBS (1×), add 50 μL of 0.05 % Trypsin containing EDTA. Then incubate 5 min at 37 °C, then add 100 μL of mESC proliferative medium per well, pipet up and down and transfer: 50 μL into a well of the "freezing plate" and 50 μL into a well of the "DNA plate".
8. Change medium 24 h after.
9. Wait until the cells grow. If the medium's color changes, remove it and put 200 μL of fresh mESC proliferative medium per well. When more than 50 % of the plate is around 50 % of confluence, the "freezing plate" can be frozen and the "DNA plate" is ready for DNA extraction.

10. Freeze cells in 96-well plates. For one well of a "freezing plate": remove the medium, wash with 100 µL of PBS (1×), add 25 µL of 0.05 % Trypsin containing EDTA, incubate 5 min at 37 °C, add 25 µL of FBS, and then add 50 µL of FBS + 20 % DMSO, pipet up and down. Finally seal the 96-well plate using parafilm, wrap the 96-well plate with tissues and store it at −80 °C.
11. Prepare 96-well plates for DNA extraction. For one well of a "DNA plate": remove the medium, wash with 100 µL of PBS (1×), then the DNA extraction can be performed or the plate can be sealed with parafilm and stored at −80 °C.

3.5 Genomic DNA Extraction from 96-Well Plates, PCR Screening and Identification of Potential Candidates

In this part of the protocol, DNA is extracted from the cells directly from 96-well plates. This implies a lot of pipetting steps, which can be facilitated by the use of a multichannel pipette.

1. If the cell plates have been stored at −80 °C, let them slowly thaw on ice. Otherwise directly proceed to the **step 2** with the samples.
2. Prepare 10 mL of complete lysis buffer. Add Proteinase K to lysis buffer at 1 mg/mL final concentration by mixing 500 µL of Proteinase K stock solution at 20 mg/mL with 9.5 mL of lysis buffer.
3. Lyse the cells. Add 50 µL of complete lysis buffer to each well, wrap plates with tissues and incubate at 60 °C, 4 h to overnight.
4. Extract DNA. Cool down plates on ice during 5 min, add 10 µL precipitation solution to each well and mix by pipetting. Then add 150 µL ice cold 100 % EtOH to each well, mix by pipetting and spin the plate 30 min at 4 °C at 2,500 × *g*. Invert plate to decant liquid and blot excess liquid on paper towels. Add 150 µL of 70 % EtOH to each well, mix by pipetting and spin the plate 15 min at room temperature at 2,500 × *g*. Again, invert plate to decant liquid and blot excess liquid on paper towels. Then, leave the plate open to dry completely at room temperature.
5. Resuspend DNA. Add 200 µL of autoclaved water to each well and incubate plates at least 1 h at 37 °C to resuspend DNA. These plates, designated as "undiluted DNA" plates, can be stored at −20 °C.
6. Dilute DNA. Prepare a 1/10 DNA dilution plate for each plate by distributing 45 µL of autoclaved water in new 96-well plates and adding 5 µL of "undiluted DNA" (*see* **Note 19**). These plates, designated as "diluted DNA" plates, can be stored at −20 °C.
7. Perform deletion PCR. For each plate, add 2 µL of "diluted DNA" to a 96-well PCR plate and prepare two master mixes,

one for each specific combination of primers for deletion PCR (corresponding to the CRISPR/Cas9 construct combination), as follows:

Stock	For one PCR reaction (μL)	Mix for 100 PCR reactions (μL)
5× Green GoTaq® reaction buffer	4	400
dNTP mix 10 mM each	0.4	40
Primer-F 10 μM	0.5	50
Primer-R 10 μM	0.5	50
GoTaq® G2 Polymerase	0.1	10
Sample's DNA at 50 ng/μL	1	–
H_2O	13.5	1350
Total (μL)	20	–

Add 19 μL of the master mix to the corresponding 96-well PCR plate containing DNA and mix by pipetting (*see* **Note 20**). Do not forget to perform a negative control for each master mix by mixing 19 μL of the master mix and 1 μL of autoclaved water.

8. Put the 96-well PCR plate tubes into a thermo cycler and run the following PCR program:

Step 1: 94 °C for 5 min
Step 2: 94 °C for 30 s
Step 3: X °C for 30 s
Step 4: 72 °C for Y seconds
Step 5: GOTO step 2, 34 times
Step 6: 72 °C for 5 min
Step 7: 12 °C HOLD

X corresponds to the melting temperature of the primers, which have been determined previously. Y is specific for each primer combination and corresponds to the elongation time depending on the amplicon size that has been determined previously (Section 3.1, **step 7**) (*see* **Note 17**).

9. Prepare a 2 % Agarose gel with an Ethidium Bromide final concentration of 0.1 μg/mL. Then load the gel with 10 μL of the PCR reactions. Store the rest at 4 °C (*see* **Note 18**). Reserve one slot to load 2.5 μL of 2-Log DNA Ladder and run the electrophoresis at 110 V for 1 h.

10. Analysis of the results. Identify the clones that present only the deletion band.
11. Thaw the candidate clones. As the freezing medium contains DMSO, which is toxic for the cells, once the "freezing plate" is taken out of the −80 °C, all the following steps have to be performed as fast as possible. For each candidate clone, transfer the cells from one well of the 96-well plate to one well of a 12-well plate.

    Gelatin-coat the number of 12-well plates you need according to the number of candidates clones you identified at **step 20**, then put 1 mL of mESC proliferative medium per well. It is advised to thaw at least 12 candidate clones, if possible. Take the "freezing plate" out of the −80 °C and add 200 μL of mESC proliferative medium to each candidate clone well, wait around 3 min until the medium is completely thawed. Then for each candidate clone, pipette up and down and transfer the entire content of one well of the 96-well plate to one well of a 12-well plate. When one 12-well plate is completed, put it in the cell incubator. From this point, candidate clones can be amplified and further tests for knockout confirmation can be performed at mRNA and protein level. The rest of the 96-well plate can be discarded.

4 Notes

1. Genomic DNA sequences are provided by the UCSC Genome Bioinformatics website (http://genome.ucsc.edu/).
2. Numerous sgRNA design tools are now available (29). However, another way to select sgRNAs is to choose already designed sgRNAs. For instance, Koike-Yusa's laboratory established a sgRNA library of 87′897 sgRNAs targeting 19′150 mouse protein-coding genes. The sgRNA sequences are provided in the supplementary data of the corresponding article (26).
3. To design specific PCR primers, the Primer-BLAST online software was used (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (31).
4. Gelatin-coated flasks or plates can be prepared in advance: pour few mL of 0.2 % gelatin on the bottom of the flask, distribute the gelatin homogeneously and keep them in the incubator (not more than 2 days to avoid evaporation). Five minutes before usage, take the plate out of the incubator and redistribute the gelatin homogeneously and do not forget to remove the gelatin solution before to add the cells.

5. Opti-MEM® I reduced Serum (1×) (Life Technologies) is a light sensitive product and must be kept from direct light exposure. It is advised to turn off the light of the cell culture hood during its use and wrap the bottle in aluminum foil for protection.
6. If FACS device is not available, an alternative method consists of diluting the cells and picking clones by "hand". Forty-eight hours after lipofection of the selected combination of two specific CRISPR/Cas9 constructs (Section 3.4, **step 2**), for each well of the 6-well plate: aspirate the medium, wash the cells with 2 mL PBS (1×) then add 0.5 mL of 0.05 % Trypsin containing EDTA, and incubate 5 min at 37 °C. Add 2 mL of mESC differentiation medium, pipet up and down and flush the well several times to detach all the cells. Then sample some of this cells suspension and assess its cellular concentration (number of cells per mL). In a new 15 mL Falcon tube, collect the right volume of cell suspension to have 2,000 cells and spin the cells 5 min at 180 × *g*. Aspirate the supernatant, well resuspend the cell pellet in 10 mL of mESC proliferative medium and transfer everything to a 10 cm gelatin-coated cell culture dish. Wait till cells proliferate. It should take around 1 week until clones can be seen by eye. During this waiting time, change the cell medium on a daily basis. Then for each condition process the following steps:
   (a) Prepare one gelatin-coated 96-well plate with 50 μL of 0.2 % gelatin solution per well (do not remove the gelatin) and one 96-well plate with 20 μL of 0.05 % Trypsin containing EDTA per well.
   (b) Take out the 10 cm dish containing the cells from the incubator and transfer the medium into a 15 mL Falcon tube. Then gently wash the 10 cm dish once with 5 mL of PBS 1(×).
   (c) Using a 10 μL pipette, take 10 μL of 0.05 % Trypsin from the first well of the first column on the 96-well plate containing the Trypsin. Select a colony on the 10 cm dish, place the pipette tip on the colony, pipet up and down until the colony comes off (around five times) and release the content of the pipette tip into the first well of the first column on the 96-well plate containing the Trypsin.
   (d) Repeat step (c) for all 8 wells of the first column on the 96-well plate containing the Trypsin. Be careful to not let the 10 cm dish dry more than 5 min, otherwise the cells will dry out and die. Filling one column of the 96-well plate must not take more than 5 min.

(e) Once one column is filled, transfer the medium from the 15 mL Falcon tube collected at step (b) to the 10 cm dish, and place the dish back in the incubator. Remove the gelatin from the first column of the gelatin-coated 96-well plate prepared at step (a). Then add 200 μL of mESC proliferative medium to each well of the first column of the 96-well plate containing the trypsinized cells, pipet up and down, transfer everything to the first column of the gelatin-coated 96-well plate and put this plate in the incubator. Be careful to not leave the cells more than 5 min in the 0.05 % Trypsin containing EDTA. Verify the presence of cells in the well with a microscope.

(f) Repeat steps (b–e) until the entire 96-well plate is filled. After step (e), it is advised to let the 10 cm dish containing the clones at least 5 min in the incubator to let the cells recover before a new round of picking. During these 5 min, the procedure can be performed on the other 10 cm dish (from the other specific CRISPR/Cas9 construct combinations). The two 10 cm dishes can be used alternatingly to avoid wasting time.

(g) Change the medium 48 h after (200 μL of mESC proliferative medium per well). Then change medium whenever the medium's color changes.

Then, go back to the protocol and pursue with Section 3.4, **step 7**.

7. It is advised to design your sgRNAs in order to target the gene in an exonic region ensuring the deletion is propagated to the mature RNA and does not accidentally get spliced out. It is also beneficial if the deletion is located close to the transcription start site as this avoids production of RNA fragments, which could have unknown side effects.

8. Choosing two sgRNAs, which are within a 1–2 kb size range region is not necessary but better for the PCR screening step, as this will allow to amplify the "wild type" PCR amplicon and the "deleted" PCR amplicon in the same PCR reaction. This will allow a quick detection and distinction between homozygote deleted mutants, heterozygote deleted mutants, and wild-type clones. It is possible to delete larger genomic regions. However, it is commonly observed that the larger the deletion is, the less efficient is the procedure (28).

9. PCR primers are designed in a way that they all have the same melting temperature. This makes them easier to combine for the PCR screening step.

10. Be careful, the sgRNA output sequence from the e-CRISP online bioinformatics tool includes the PAM motif. When modifying the sgRNAs by adding the specific BbsI overhangs and ordering the oligos, make sure that the PAM sequence has been removed.
11. The overhangs added to the sgRNAs make them compatible with the BpiI (BbsI) digested pX458 vector used in this protocol. In case it is planned to use another vector with different restriction site(s), do not forget to adapt the modifications of the sgRNAs according to the needs.
12. PCR primers and sgRNAs were ordered from Sigma. Basic desalting purification of primers and sgRNAs is sufficient enough for this protocol.
13. After overnight incubation at 37 °C, compare the number of colonies present on the negative control and sample plates. If there are no colonies on the sample plates, it could be due to a problem during the sgRNA annealing. Prepare a 5 % Agarose gel with an Ethidium Bromide final concentration of 0.1 μg/mL and load 5 μL of annealed sgRNAs (generated at Section 3.2, **step 3**) supplemented with 1 μL of 6× loading dye, next to 5 μl of single-stranded sgRNA (sense or antisense) supplemented with 1 μL of 6× loading dye. Then run the gel at 100 V for 1 h and verify the successful annealing by comparing the bands' sizes between annealed and nonannealed sgRNAs.
14. Serial Cloner (version 2-6-1) software was used to analyze sequencing results.
15. In order to save specific CRISPR/Cas9 constructs, it is possible to make glycerol stocks of the bacteria clones expressing the correct constructions. For each bacterial clone, take 750 μL of the 200 mL bacterial suspension (before to perform plasmid extractions using the Pure Link™ HiPure Plasmid Filter Maxiprep Kit) and mix it with 250 μL of 100 % autoclaved glycerol into a screw cap cryotube. Vortex and store the glycerol stocks at −80 °C.
16. If one (or several) sample(s) is (are) at a concentration lower than 50 ng/μL, equilibrate all the samples to the lowest sample concentration. Adjust the calculations to perform PCR reactions with 50 ng of DNA per reaction.
17. Concerning the elongation time of the deletion PCR (1F + 2R), choose an elongation time long enough to amplify the "wild type" amplicon. As this PCR reaction is performed on DNA extracted from a mixed population (transfected and nontransfected), if the CRISPR/Cas9 complex cleaved genomic DNA, two bands should be observed on the gel: one corresponding to the "wild type" amplicon and one lower in size, corresponding to the "deleted" amplicon.

18. Keeping 10 μL of the PCR reactions allow to have a backup in case of something goes wrong during the gel electrophoresis.
19. Be careful, the DNA solutions from the "undiluted DNA" plates are really viscous. Pipet slowly.
20. It is possible to run a positive control for this PCR by setting up a PCR reaction using DNA extracted from cells, which have been transfected with the corresponding combination of two specific CRISPR/Cas9 constructs (Section 3.3, **step 7**); or by loading the Agarose gel with the corresponding 10 μL of PCR reactions left at Section 3.3, **step 11**.

Acknowledgments

We thank the Epigenesys network for access to its protocol database. We thank the Bourc'his laboratory and specifically Dr. Maxim Greenberg for technical assistance and discussions. We thank Dr. Tobias Beyer and the Ciaudo laboratory for the critical reading of the manuscript and for fruitful discussions.

CRISPR reagents are available to the academic community through Addgene (http://www.addgene.org/).

This work was supported by a core grant from ETH-Z (supported by Roche) and SNF (31003A_153220). M.B. is supported by a PhD fellowship from the ETH-Z foundation (ETH-21 13-1).

References

1. Martello G, Smith A (2014) The nature of embryonic stem cells. Annu Rev Cell Dev Biol 30:647–675. doi:10.1146/annurev-cellbio-100913-013116
2. Boyer LA, Mathur D, Jaenisch R (2006) Molecular control of pluripotency. Curr Opin Genet Dev 16:455–462. doi:10.1016/j.gde.2006.08.009
3. Beyer TA, Narimatsu M, Weiss A et al (2013) The TGFβ superfamily in stem cell biology and early mammalian embryonic development. Biochim Biophys Acta 1830:2268–2279. doi:10.1016/j.bbagen.2012.08.025
4. Murry CE, Keller G (2008) Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 132:661–680. doi:10.1016/j.cell.2008.02.008
5. Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157:1262–1278. doi:10.1016/j.cell.2014.05.010
6. Ishino Y, Shinagawa H, Makino K et al (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol 169:5429–5433
7. Jansen R, Embden JDA, Van Gaastra W, Schouls LM (2002) Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol 43:1565–1575. doi:10.1046/j.1365-2958.2002.02839.x
8. Barrangou R, Fremaux C, Deveau H et al (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712. doi:10.1126/science.1138140
9. Mali P, Yang L, Esvelt KM et al (2013) RNA-guided human genome engineering via Cas9. Science 339:823–826. doi:10.1126/science.1232033.RNA-Guided
10. Cong L, Ran FA, Cox D et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823. doi:10.1126/science.1231143
11. Deltcheva E, Chylinski K, Sharma CM, Gonzales K (2011) CRISPR RNA maturation by

trans -encoded small RNA and host factor RNase III. Nature 471:602–607. doi:10.1038/nature09886.CRISPR

12. Jinek M, Chylinski K, Fonfara I et al (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821. doi:10.1126/science.1225829
13. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Almendros C (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology 155:733–740. doi:10.1099/mic. 0.023960-0
14. Deveau H, Barrangou R, Garneau JE et al (2008) Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. J Bacteriol 190:1390–1400. doi:10.1128/JB.01412-07
15. Semenova E, Jore MM, Datsenko KA et al (2011) Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. Proc Natl Acad Sci U S A 108:10098–10103. doi:10.1073/pnas.1104144108
16. Jinek M, Jiang F, Taylor DW et al (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 343:1247997. doi:10.1126/science.1247997
17. Gilbert LA, Horlbeck MA, Adamson B et al (2014) Genome-scale CRISPR-mediated control of gene repression and activation. Cell 159:647–661. doi:10.1016/j.cell.2014.09.029
18. Kearns NA, Genga RMJ, Enuameh MS et al (2014) Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells. Development 141:219–223. doi:10.1242/dev.103341
19. Tanenbaum ME, Gilbert LA, Qi LS et al (2014) A protein-tagging system for signal amplification in gene expression and fluorescence imaging. Cell 159:635–646. doi:10.1016/j.cell.2014.09.039
20. O'Connell MR, Oakes BL, Sternberg SH et al (2014) Programmable RNA recognition and cleavage by CRISPR/Cas9. Nature. doi:10.1038/nature13769
21. Heigwer F, Kerr G, Boutros M (2014) E-CRISP: fast CRISPR target site identification. Nat Methods 11:122–123. doi:10.1038/nmeth.2812
22. Hsu PD, Scott DA, Weinstein JA et al (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31:827–832. doi:10.1038/nbt.2647
23. Bae S, Park J, Kim J-S (2014) Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics 30:1473–1475. doi:10.1093/bioinformatics/btu048
24. Montague TG, Cruz JM, Gagnon JA et al (2014) CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res 42:401–7. doi:10.1093/nar/gku410
25. Xiao A, Cheng Z, Kong L et al (2014) CasOT: a genome-wide Cas9/gRNA off-target searching tool. Bioinformatics 30:1180–1182. doi:10.1093/bioinformatics/btt764
26. Koike-Yusa H, Li Y, Tan E-P et al (2014) Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nat Biotechnol 32:267–273. doi:10.1038/nbt.2800
27. Nora EP, Heard E (2011) Engineering genomic deletions and inversions in mouse ES cells using custom designed nucleases 1–15, Epigenesys website, protocol #62 (http://www.epigenesys.eu/images/stories/protocols/pdf/20130507072445_p62.pdf).
28. Canver MC, Bauer DE, Dass A et al (2014) Characterization of genomic deletion efficiency mediated by CRISPR/Cas9 in mammalian cells. J Biol Chem. doi:10.1074/jbc.M114.564625
29. Ran FA, Hsu PD, Wright J et al (2013) Genome engineering using the CRISPR-Cas9 system. Nat Protoc 8:2281–2308. doi:10.1038/nprot.2013.143
30. Marx V (2014) Gene editing: how to stay on-target with CRISPR. Nat Methods 11:1021–1026. doi:10.1038/nmeth.3108
31. Ye J, Coulouris G, Zaretskaya I et al (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:134. doi:10.1186/1471-2105-13-134