Which are the best CRISPR-CAS9 strategies?

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Table of contents

Introduction

The CRISPR-CAS9 system may have opened Pandora's box, but it is also definitely the cornucopia of genome editing.

We can do what we want in the genome: settle a mutation, correct a mutation, insert a fluorescent tag to a protein, add an exogenous gene, delete an endogenous function, suppress a cis-regulatory region, add a reporter.... I might just not have enough imagination!

The main challenge is to define a good strategy, taking in account the specifics of the project and being aware of the corresponding limitations.

The principle of the CRISPR-CAS9 has already been exposed numerous times. The protein CAS9 (from the bacteria Streptococcus pyogenes) is an endonuclease that generates a Double Stranded Break (DSB). Its activity is targeted by one single small RNA, called guide RNA (gRNA or sgRNA). The DSB is repaired by the cell (via the NHEJ pathway) leading to knock-out (KO) of the site targeted by the gRNA molecule.

In this overview, I won't go into details of the structure, the mechanism, or history, in order to stay focused on the biotechnology and the concerns of end-users from a practical point of view.

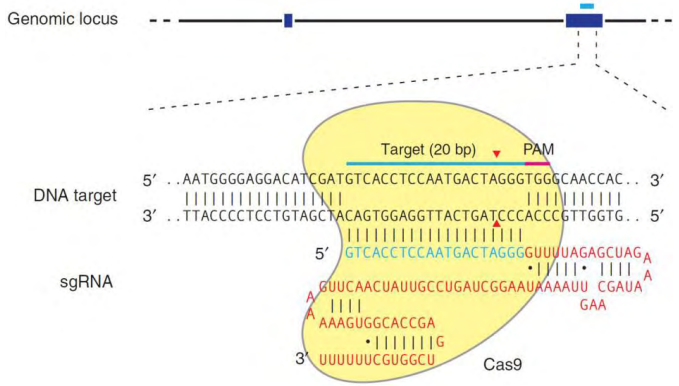


Figure 1: CRISPR-CAS9 system is 1 guide RNA with the CAS9 endo-

A minimal approach for knock-out

The minimal strategy is to transfect a plasmid encoding for the gRNA specific for the target site and the CAS9 mRNA coding for the endonuclease.

For cells compatible with transfection (30% transfection efficiency would be a minimum), it is a simple way to generate a targeted KO. The all-in-one plasmid pCRISPR-CG01 (*Figure 2*) is an excellent example since it has also a convenient mCherry reporter to control the transfection in fluorescence.

There are pre-optimized transfection reagents and the broad spectrum PolyJet to help with the system delivery into your cells. These reagents respect this cheap approach as they are also very competitively priced.

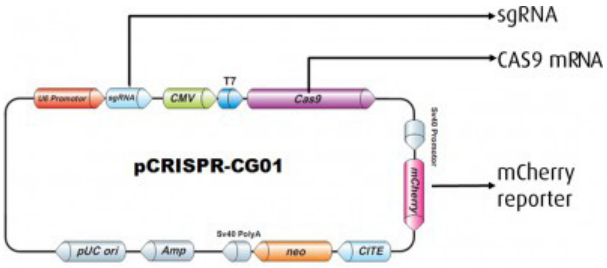


Figure 2: All-in-one plasmid to deliver the CRISPR-CAS9 system into cells.

IndelCheck: screening clones for knock-out

Since the CRISPR-CAS9 efficiency depends on the gRNA, it may be wise to compare efficiency of 3 gRNA for the same target site.

For 1 or more gRNA, how can you assess the CRISPR-CAS9 efficiency and specificity? Consider using T7 Endonuclease I assays on the target site for the efficiency and on the potential off-target site to monitor the possible non-specific events.

The principle is simple. CRISPR-CAS9 generates DSB that are repaired (by NHEJ) and so leads to mutated version of the site (indel mutation, in red on figure 3). Indel can be insertion (In) or deletion (Del). Through denaturation and re-annealing, mismatches can appear only in presence of indels. Then the T7 endunuclease I can cut such mismatches and so reveal the indels on electrophoresis gel.

This analysis is focused on the selected site by PCR. If the amplified site is the targeted site, the result is the CRISPR-CAS9 efficiency. If the amplified site is a potential target-off site, the specificity is checked.

Pratically, DNA extraction can be done in 5 minutes with QuickExtract and then one could use the Indelcheck, which contains all the required reagents for the assays including the PCR kit and the T7 endonuclease I.

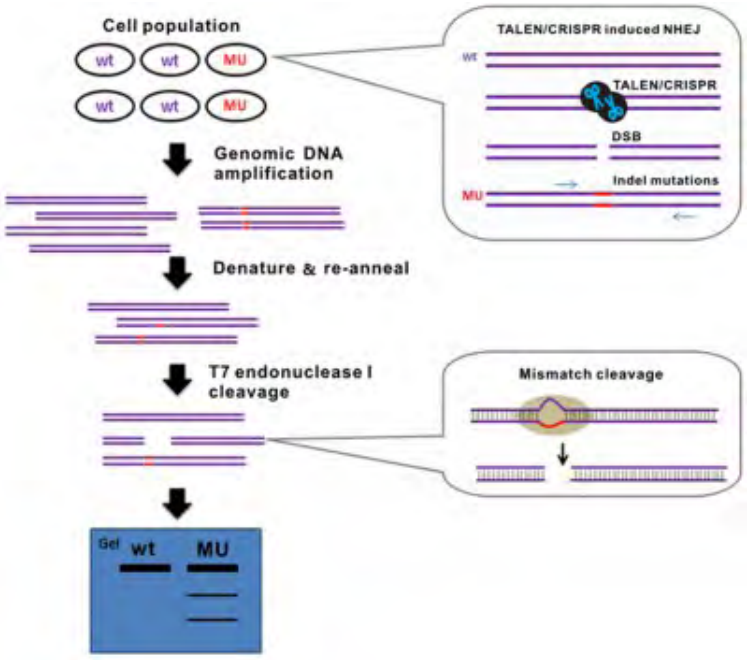


Figure 3: T7 endonuclease I assay to check the indels on selected sites

The number of rounds to hold on for until complete KO

Delivery of the system into the cells (a plasmid transfection at minimum), then clone isolation and IndelCheck analysis constitutes one round.
This can be repeated if needed.

How many rounds? The rule is hard to fix - the last round is when there is a complete knock out of the gene on its alleles.

From diploid cells it is quite easy, a knock out on both alleles can be done in one round, if not, then just one supplementary round should be enough.

For multiple ploidy, it can be a real challenge. For this reason, it's highly recommended to work with diploid cells whenever possible.
Typically, HeLa cells are not a good idea.

The Donor that gives a lot

What is a Donor? It is a DNA template with homologous regions (blue in figure 4) upstream and downstream to the target site. It triggers a repair of the DSB by homologous recombination (HR). This repair leads to the insertion of the Donor sequences (that are between the 2 homologous regions) into the genome.
It's very accurate. That way, you can add a tag in frame to an ORF.

Thus, a Donor is definitely required for knock-in. It includes notably the following projects:

- Adding a tag to an ORF
- Correcting or adding a single base mutation
- Inserting a new construct in the genome for a cell-based assay or protein production

Be aware that without a donor (NHEJ on figure 4), you should grow the cells in order to isolate clonal events and a part of these clones will be wild type. The percentage of this useless part depends on the guide RNA efficiency for the target site and the transfection efficiency.

So I would insist, especially without a Donor, that testing the efficiency of several guide RNA can be useful, and monitoring the transfection is required. Actually, I recommend that you use a Donor even for knock-out and not only for knock-in.

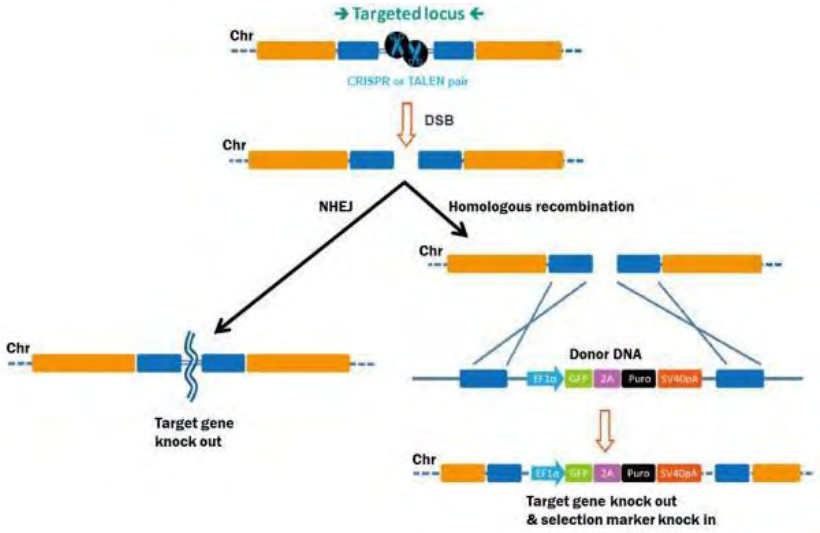


Figure 4: Double stranded break repair by homologous recombination

Donors change the strategy. As illustrated in Figure 4, it can bring eGFP and Puromycin and so allows FACS enrichment and selection.

With a Donor, you're sure to work only on cells that are modified on at least one allele. You eliminate the wild type cells. Thus, you could even start by polyclonal culture. Clonal isolation can be done later if needed.

For diploid cells, a second Donor would give you the possibility to select double allele modification in one round. Figure 5 illustrates an example of 2 donors allowing selection with Neomycin and Puromycin. In that particular case, polyclonal is far enough. A quick control of the possible off-target sites would ensure to get isogenic cell line.

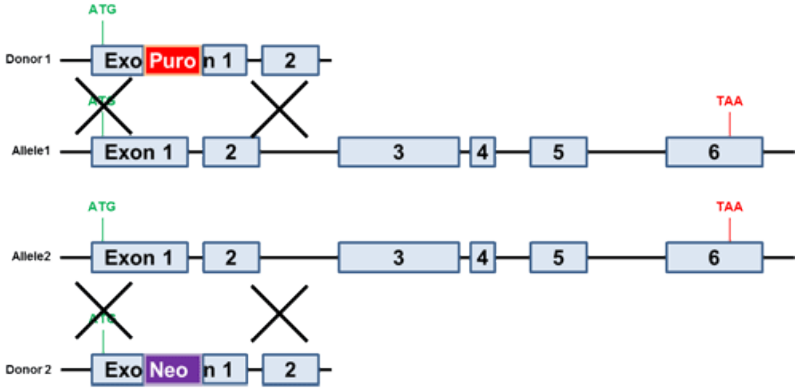


Figure 5: 2 Donors for 1 round knock-out on diploid cells

CRE-LOX recombination serving CRISPR-CAS9

A Donor can be a quite basic plasmid. Nevertheless, with LoxP sites it brings some other possibilities (Figure 6, in orange). First of all, after allele modifications it is possible to remove the cassette of selection with the LoxP sites in order to re-use the same Donor for another round.
It can be useful when you have no choice but to work with multiploid cells!

Here again, polyclonal rounds are possible before making the effort of monoclonal establishment.
To induce the CRE-Lox recombination, I would suggest transfection with CRE mRNA.

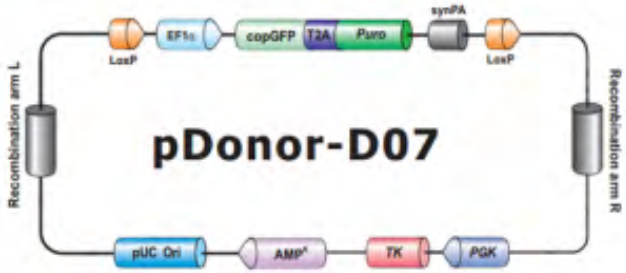
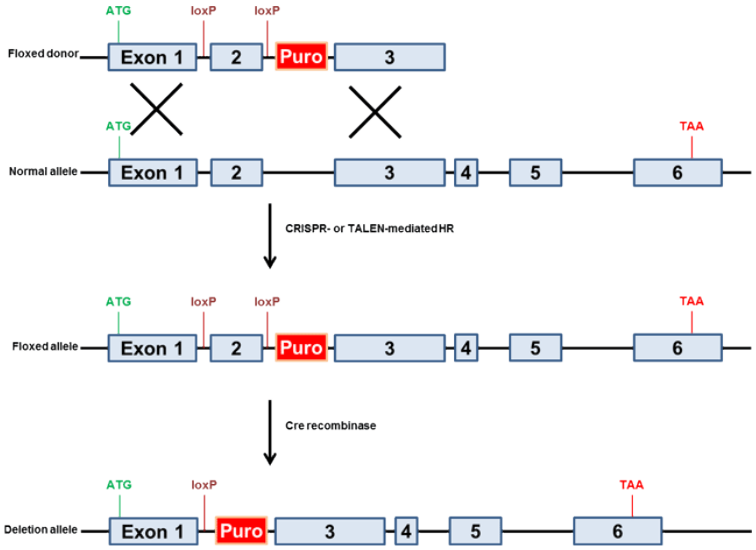


Figure 6: Donor plasmid with LoxP sites



Furthermore, a Donor with LoxP sites also allows a conditional KO. For some genes, it is impossible to establish a total KO cell line. But it is possible to modify all alleles so that LoxP sites are inserted. Of course it is possible only with a dedicated donor and its corresponding selection (Puromycin in the case of Figure 7).

As far as I know, today this is the unique way to induce a KO. Nevertheless, I should warn you that the CRE recombination depends on the CRE mRNA transfection and it cannot be 100% efficient.

Figure 7: Genome editing to prepare a conditional KO induced by CRE

Summary of CRISPR-CAS9 strategies depending on the Donor

Strategy	Advantage	Limitation
No Donor	Cheapest	Monoclonal analysis required at the 1st round With possibly a lot of negative clones High transfection efficiency required May require to screen numerous cells
1 Donor	Selection or FACS enrichment	Benefit limited at the 2nd round if needed
2 Donors	Only 1 round selection for diploide	Benefit reduced for more than 2 alleles
1 Donor with loxP	Re-use the donor for several rounds Conditional KO	CRE-loxP recombination not at 100%

Figure 8: Overview of options depending on the Donor.

The Nick solution against target-off

According to Smith, *et al.* (2014) and Veres *et al.* (2014), there is no significant CRISPR-CAS9 off-target in stem cells. Some authors mention in their discution that the off-target effects may be overstated. Still, it is something to keep under control at least *a posteriori* by analysis of the potential off-target site. I should also mention that it is also possible to drop the risk of off-target to almost zero using the Nickase version of the CAS9.

CAS9 makes a DSB. A mutated version of the CAS9 (D10A), called 'Nickase' can make a single stranded break (a nick). One single nick can't help with genome editing. Nevertheless, if we target 2 Nickases using 2 different gRNA, and close enough, we can generate 2 nicks that mimic the DSB. This is illutrated in Figure 9. Then this pair of Nickase/gRNA can benefit of the repair by HR with the Donor and by NHEJ without Donor as previously shown.

It has been shown that pair of targeted Nickases can cut with the same efficiency as the wild type version and the off-target sites can be reduced up to x1500 times. The pair of Nickase/gRNA can be delivered by plasmid as previously. The strategies in the overiew on the previous page are also valid for the Nickase but with a pair of gRNA in place of 1 single gRNA.

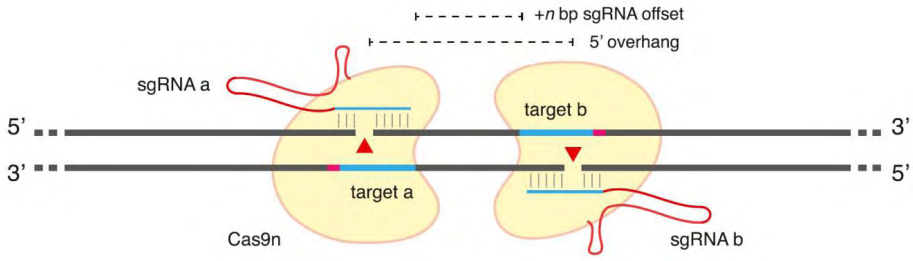


Figure 9: Targeted pair of Nickases to reinforce the specificity

Challenge of delivery into cells

When transfection is not possible to deliver the CRISPR-CAS9, lentivirus is an option.

Unfortunately, due to the packaging of the lentiviral particles, they are limited in size. Thus it is not possible to have an all-in-one lentivirus solution expressing CAS9 and the gRNA. They must be delivered from separate lentiviral particles.

As you can imagine, a strategy with Donor is very tricky since it would mean a triple transduction. And actually, the insert of the Donor should be smaller than 8kb because of the same packaging limitation.

That is why, persisting with optimisation of transfection and the use of Donor for selection may be sometime preferable. For example, with 10% of transfection on diploid cells, a strategy with a Donor and selection may be a reasonable attempt. Lentivirus may be better if transduction can be far more efficicent (>70%), since it may be not possible to have a Donor and so a selection.

In conclusion, before jumping on a lentivirus option, accurate evaluation of the transduction and transfection efficiency is required. And of course the more the allele number has to be modified, the more difficult the project will be.

Loss of function screening

What about KO of numerous genes to study their role for the cell? It could be several or 1 single gene KO per well, in both case intend to 1 single KO in each cell. Here, the strategy is to deliver a gRNA library to a cell line expressing stably the Cas9. The process is quite simple : plate the Cas9-expressing cells, add the gRNA by transfection (plasmid) or transduction (lentiviral particules) and then make your specific screening (Figure 10).

The screening could be based on apoptosis monitoring, cell invasion assay, or even based on a promotor reporter to find genes involved in a pathway (for example activation of ER stress).

Thanks to CRISPR-CAS9 and notably the Safe-Harbor strategy (that I will cover soon in another post on Being bio-reactive), it is possible to establish cell line expressing CAS9 without any hit in the genome.

Note - such human cell lines for H1299, HEK293T, HeLa and A549 are available through tebu-bio, who also provide barcoded gRNA libraries as pool. Libraries with individually arrayed clones are available upon request too.

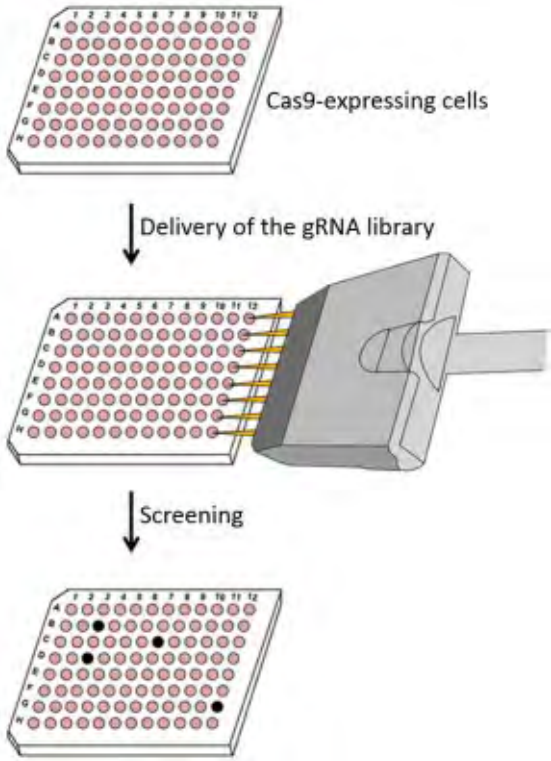


Figure 10: Preparing plate cells with KO genes for screening.

Without vector for a therapeutic perspective

To generate transgenic animal models such as mouse, vectors are avoided when possible. They may affect the genome integrity, and there are other means to deliver the CRISPR-CAS9 system. Similarly, with a non-vector approach, stem cells could benefit from the genome editing capacity of the CRISPR-CAS9 without risk for the genome integrity. This is of great interest for cell-based therapy. Indeed, in principle we could take stem cells from a patient, correct the mutation in cause and then re-implant the corrected stem cells.

A successful strategy is to use Cas9 mRNA combined with the specific gRNA. It is becoming a real standard in mouse transgenesis via cytoplasmic injections. For cell transfection, I would suggest as starting point 500ng of sgRNA/Cas9-mRNA (with a ratio 1:1 in ng) per well for 24-well plate. Nevertheless, transfection should be optimised depending on the cells and the ratio could be adjust since there is no well defined rule today.

Messenger RNA production for *in vivo* translation is a real challenge (see how to face it by yourself in one of my previous posts) which is why I suggest ready-to-use Cas9 mRNA. This messenger has been codon-optimised and its UTR (UnTranslated Regions) have been fine-tuned to ensure the best Cas9 protein synthesis in Mammalian. Of course it also has a capping and poly-A tail (120A). The DNase treatment is also reinforced. The most popular is the version fully modified with 5meC and Psi. The corresponding Cas9 protein has 2 NLS (Nuclear Localization Signal) that improve the efficiency of the CRISPR-CAS9 system. In other words, it is designed for Mammalian genome editing. Thus, a simple injection (for transgenesis) or a simple transfection in stem cells allow genome editing.

gRNA production by *in vitro* transcription (IVT) is not the same challenge. In this case, the T7-FlashScribe transcription kit is an efficient and cheap option. Nevertheless, we also provide ready-to-use gRNA produced by IVT or even from chemical synthesis. Thus researchers don't need to invest time and effort in RNA production.

The Donor, when needed, can be single stranded oligonucleotide in order to avoid random hit in the genome. Unfortunately, such donor is too short and cannot allow a selection. Nevertheless, transgenesis is not compatible with selection and it may be not very wise to use selection marker in stem cells, especially for therapeutic purposes.

About the author

Dimitri Szymczak (PhD) is a specialist in Genomics, Transcriptomics and RNA/DNA related research. Building on his experience as a Researcher and Teacher in genetics, he now helps scientists by advising optimal, dedicated solutions for their projects (miRNA, CRISPR-CAS9 system, mRNA delivery...)

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