CRISPR/Cas9 to silence long non-coding RNAs

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Abstract: Knock-out (KO) of long non-coding RNAs (lncRNAs) enables functional characterization of this still poorly described group of transcripts. One of the most efficient and simplest methods to achieve complete KO of a lncRNA is by employing CRISPR/Cas gene editing. As most lncRNAs are not well annotated, their individual functional regions are not defined, and the majority of the transcripts are not affected by single nucleotide mutations. Therefore, CRISPR/Cas KO is more challenging for lncRNAs as compared to KO of protein coding genes. Strategies for lncRNAs KO include complete removal of the entire gene, removal of the promoter and transcriptional start site, abolishing exon-exon junctions, or removing the transcriptional termination site. Here, we describe the methodology to perform CRISPR/Cas9 KO of lncRNAs *in vitro* using electroporation as the method of transfection of pre-synthesized single guide RNAs (sgRNAs) and Cas9 enzyme.

Keywords: Long non-coding RNA, CRISPR/Cas9, knock-out, gene editing, electroporation.

1. Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins (Cas) is a genome editing technique, established in 2012, that uses RNA molecules to guide nucleases to specific target sites in the DNA [1-3]. The system is a highly specific method for DNA cleavage and allows researchers to create specific modifications to a genomic area of interest. The method is based on the adaptive bacterial immune system, where CRISPR and the endonuclease family Cas, make up a defense mechanism against viruses and endogenous plasmids [4-6]. The CRISPR arrays are unique genomic regions in the bacteria where viral DNA and plasmid DNA sequences from previous invasions have been incorporated, and the locus is transcribed into crispr RNAs (crRNAs) [7]. The crRNA is characterized by a 20 nucleotide (nt) complementary sequence to the invading viral or plasmid DNA. In experimental research, this region is designed to be complementary to the intended cut site in the target gene. After processing, the mature crRNA binds, via a complementary spacer, to a trans-activating crRNA (tracrRNA), which is either transcribed from the same locus, or from a region in close proximity upstream of the locus [2]. The tracrRNA has the recognition sequence necessary to form a complex with the Cas endonuclease [3]. There are three major types of CRISPR-Cas systems, each characterized by signature genes: Cas3 in type I systems, Cas9 in type II, and Cas10 in type III [8,9]. The Cas genes are organized in operons under the control of a single promotor, and characterized by their use of dual-RNAs for site-specific DNA cleavage [6,3]. There are 93 known Cas protein families, and multiple and highly diverse Cas proteins have been identified [8]. Cas9 is one of the most commonly used Cas enzymes, and is a dual RNA-guided endonuclease that creates site-specific double-stranded breaks in the target DNA. Cas9 activity requires both a seed sequence in the crRNA as well as a protospacer adjacent motif (PAM) containing a guanine dinucleotide, 5'-NGG-3', where N is any nucleotide. Cas12 is another commonly used Cas enzyme. In contrast to Cas9, Cas12 targets PAM sequences rich in thymine (5′-TTN-3′) [10]. Another emerging Cas enzyme in research is Cas13. In contrast to Cas9 and Cas12, Cas13 targets RNA sequences and requires a guide RNA of approximately 64 nt in length [11]. Recently, a hypercompact CRISPR/Cas system was discovered by Pausch *et al*. in huge bacteriophages [12]. The newly discovered CRISPR associated enzyme CasΦ is about half the size of Cas9 and Cas12, and targets a wider range of genetic sequences. This is a promising finding that could lead

to more efficient delivery of the CRISPR/Cas components into cells, and to a broader application of the CRISPR/Cas method for gene editing [12]. The CRISPR/Cas technique for genome editing has shown great potential for applications in biomedical research, biotechnology, and in innovative therapies for diseases [13-16], and multiple clinical trials are currently being conducted [17].

***Info box**: The term **gRNA** is used to describe all CRISPR guide RNA formats, while **sgRNA** is used to describe a guide RNA that is made up of the combination of the crRNA and the tracrRNA elements into a single RNA molecule.*

Long non-coding RNAs (lncRNAs) are a class of RNA molecules with no protein coding potential which exceed 200 nt in length [18]. LncRNAs have multiple described functions: guides for proteins or other RNA molecules to specific DNA or RNA regions/sequences; enhancers or inhibitors of transcription; scaffolding RNAs that assemble ribonucleoprotein complexes at specific cellular sites; decoys that inhibit protein or other RNA molecules' function by sequestering or by blocking protein binding sites; or modifiers that can allosterically change proteins or other RNA molecules [19,20]. Even though great effort has been put into investigating the function of this large class of genes, most lncRNAs are still poorly characterized. We consider that one of the best method for studying the functionality of lncRNAs is to perform *in vitro* and *in vivo* knock-out (KO). A simple method to achieve complete KO of a lncRNA is by CRISPR/Cas gene editing. In comparison to CRISPR/Cas KO of other gene types, like protein coding genes, CRISPR/Cas KO of lncRNAs is harder to achieve because of one important challenge: most lncRNA genes are not well characterized and the functional regions of the transcripts are not known. For this reason, it is often necessary to KO the complete lncRNA gene in order to study the functionality of a given lncRNA. In most cases, this will lead to the additional challenge of avoiding alterations of nearby genes, as most classes of lncRNAs are located in close proximity or even overlapping with a protein coding or another non-coding gene.

In this methods section we will give a detailed description of how to perform CRISPR/Cas9 KO of a lncRNA in the laboratory using pre-synthesized sgRNAs and Cas9 with electroporation as the transfection method. In addition, we will give a guide on how to plan and design a KO experiment for different lncRNAs. Cell culturing, flow

sorting, DNA isolation, and PCR will only be described in general, as the methods will depend on the cell culture of interest and the availability of kits, reagents, and platforms at the institution where the experiment will be conducted.

2. Materials

2.1. Electroporation agent

Cell Line Nucleofector™ Kit V (Lonza) (VVCA-1003).

2.2. sgRNA

CRISPRevolution sgRNA EZ Kit (1.5 nmol) (Modified) (Synthego): sgRNA should be diluted to 100 uM in Low TE buffer upon arrival, aliquoted in separate RNase free tubes (2.0 µl per tube), and stored at -20° C.

2.3 Cas9

Cas9 2NLS Nuclease – 300 pmol (Synthego).

2.4. Electroporation apparatus

Any electroporation apparatus compatible with the Nucleofector™ Kit V is suitable for the CRISPR/Cas9 silencing of lncRNAs. For each cell line, a different electroporation program is recommended.

2.5. Cell culturing reagent

Normal cell culturing reagents and materials are used for the CRISPR/Cas9 silencing of lncRNAs. For the expansion of cells after electroporation, a conditioned growth media is necessary. Prepare conditioned growth medium by sterile filtering growth medium used to culture the cell using a 0.2 µm filter. The culture medium should have been cultivated with the cells for at least 48 hours and should not be more than 1 month old at the time of use.

2.6 Flow sorting to establish single cell colonies

Flow tubes with filtering cap.

96-well plates.

Propidium iodide.

2.7. PCR screening

DNA isolation kit.

PCR kit.

Specific PCR primers (see section for tips on how to design the primers).

3. Methods

For generation of a lncRNA KO cell line, a dual sgRNA strategy will be utilized. Here, the two sgRNAs will generate two unique double DNA breaks within the genomic region of the lncRNA. The sgRNAs will target upstream and downstream of the desired target sequence, allowing a relegation of the two broken ends with the loss of the genomic sequence in between.

3.1 Establish a knock out strategy

1. Foremost, it is important to get an overview of the genomic organization of the target lncRNA and its residing genes. Annotations of lncRNAs are still poor, and often the annotations will include a large number of potential transcriptional isoforms with different start and termination sites within the gene region. Essential information that will affect the KO strategy includes:

i. Buildup of gene: gene and transcripts length, number of annotated transcript isoforms, number of exon-intron junctions, location and number of transcriptional start and termination sites;

ii. Genomic location of lncRNA and location of overlapping or neighboring genes;

iii. Validity of promoter region for the lncRNA.

2. Examine the genomic buildup of the lncRNA, by using the UCSC Genome browser (genome.ucsc.edu) or Ensembl (ensembl.org). Both databases include previously annotated lncRNAs. If the target lncRNA is a novel transcript, information regarding the gene buildup will not be included in the above-mentioned databases. Regardless of whether the lncRNA is a previously annotated lncRNA or a novel transcript, the precise organization of the transcript should be further validated.

3. Validate the transcript by using *in silico* RNA-Seq data analysis. As many lncRNAs are tissue specifically expressed, use RNA-Seq data from tissue with the same origin as the cell line in which lncRNA KO will be performed. Use the RNA-Seq data to examine from which genomic regions RNA transcription is initiated and terminated and the location of exons and introns. If the precise organization of the transcript cannot be validated by RNA-Seq data, conduct 3' and 5' RACE and Sanger sequencing of the entire lncRNA transcript to identify the exact DNA region that is being covered by the transcribed lncRNA.

4. After validation of the transcript, choose the most suitable lncRNA KO strategy: (a) remove the entire gene, (b) remove the promoter and transcriptional start site, (c)

remove the transcriptional termination site, or (d) abolish exon-intron junctions (Figure 1). Some of the strategies may only cause a destabilization of the lncRNA (exon and termination) and will therefore not generate a complete KO cell line, but instead a stable knock-down (KD) cell line (Note 1).

3.2 Identification of target region for CRISPR/Cas9

1. After identifying a desired genomic region for KO, use the genomic location of the desired region as an input parameter for target analysis. Analyze the two target regions (upstream and downstream region), separately. Starting with the upstream region, investigate a region of ±500 nt covering the ideal target site in order to get sufficient numbers of targets. For target analysis, use the web tool CHOPCHOP (chopchop.cbu.uib.no). Input parameters are genomic region and species. The format for the genomic region is: chrX:Y-Z, where X=chromosome, Y=lower genomic region for KO, and Z=upper genomic region for KO. The format is case sensitive, and only lower-case letters are used. Additional inputs include: for “Using” use “CRISPR/Cas9” and for “For” use “knock-out”.
2. Submit these input parameters, and the web tool will generate a ranked list of targets with the most optimal target regions listed at the top. Here, target areas are selected based on their rank and their genomic location. The Cas9 enzyme has a very high specificity, so 3 nt difference should not be considered as off targets.
3. After identification of a target region for the upstream region, do the same procedure for the downstream region.
4. Export the two identified target sequences, and remove the PAM sequences (the three last nucleotides corresponding to NGG) before using the sequences to order the sgRNAs (named synthetic sgRNA) from the Syntego homepage (synthego.com).

3.3 Design of PCR primers for validation of clones

1. Design specific PCR primers for amplification of a region corresponding to 200-500 nt up- and down-stream of the two cut sites. Design a forward primer 200-500 nt upstream of the upstream cut site, and a reverse primer 200-500 nt downstream of the downstream cut site. This will give a 400-1000 nt large PCR product if the genomic region of interest has been removed (Figure 2).
2. In addition, if the KO region is >1 kb, include internal primers (primers located in the region to be removed) giving a PCR product of approximately the same size as your

KO PCR product (Figure 2). In order to separate the two products, it is recommended that the two products have a difference in size of about 100-200 nt.

3.4 Cultivation of cells and collection of conditioned media

1. Cultivate the cells in normal growth media. With every media change and splitting, the old media that has been cultivated with the cells (not including trypsin or PBS) is transferred to a 50 ml falcon tube and placed at 4^{o}C. The used media, termed conditioned media, will be used during single cell seeding. The later step requires a total of 106 ml conditioned media.
2. On the day of the transfection (Section 3.5), the cells should be at 50-80% confluent. The transfection protocol only requires the use of 100,000 cells, so cells can be cultivated in a small cell culture flask, but pre cultivation in larger flasks are encouraged for collection of sufficient conditioned media.

3.5 Transfection of sgRNAs and Cas9

1. To a 12-well plate, add 450 µl complete growth media to two wells and place the plate in the cell incubator.
2. Prepare the Nucleofector mix by combining 43 µl of Nucleofector solution with 9.5 µl Nucleofector supplement.
3. In a 1.5 ml tube, add 1.8 µl of each sgRNA, 2.0 µl Cas9, and 19.4 µl Nucleofector mix to a total volume of 25 µl. Incubate the solution at room temperature for a minimum of 10 min, but not for more than 1 hour.
4. Split the cells and transfer the split cells to a falcon tube. Take 1 ml aliquot of the cell suspension and transfer to a 1.5 tube. From this tube do a cell counting. The numbers of cells should be in the range of 100,000-500,000 cells per ml. If the cell number is not within this range, dilute the cell suspension and recount the cells. Exact cell number is highly important for the success of the transfection, so care should be taken to prevent errors in cell number.
5. Transfer 100,000 cells to a new 1.5 ml tube and spin down the cells at 100 g for 10 min. The supernatant is carefully removed so not to disrupt the cell pellet. It is important that all liquid is removed. To ensure complete removal of cell media, first use a 1 ml pipette to remove 90% of the media, before changing to a 100 µl pipette and do the same. Finally, use a 10 µl pipette to remove the remaining liquid. When removing the

liquid, be sure not to tilt or do rapid movements of the tube. If the cell pellet is disrupted, do a 5 min spin at 100 g before continuing removing the liquid.

6. Carefully resuspend the cells in 25 µl Nucleofector mix, and transfer the cells to the 1.5 ml tube containing the sgRNA-Cas9 mix (mix from step 3.5.3.). Mix by careful pipetting. Continue immediately with the electroporation, as cells should not be kept in the Nucleofector solution for more than 15 min before cells are resuspended in cell media (step 3.5.9).

7. Transfer the cell mix to an electroporation tube using the included plastic pipettes in the Nucleofector kit. Be careful not to introduce bubbles when placing the solution in the electroporation tube as this will disrupt the electroporation. Bubbles can be removed by gently knocking the electroporation tube onto the bench or by pipetting up the liquid before pipetting it out again into the tube.

8. Place the electroporation tube in the electroporation apparatus and electroporated using a defined electroporation program (**Table 1**). For instructions on how to program the electroporation apparatus, please refer to the instruction manual of the apparatus.

9. After the electroporation, 50 µl of growth medium is added to the cell suspension in the electroporation tube, before the cells are seeded out equally into the two wells on the 12-well plate containing 450 µl of warm growth medium from step 3.5.1. (50 µl of the cell mix is added to each well). Be sure to transfer all of the cell mixture (Note 2).

10. Change growth medium after 24 hours.

11. 72 hours after electroporation, harvest cells from one of the two wells and perform DNA isolation. Follow the same procedure as for genotype screening (Step 3.7.6, expected result is identical as for a heterozygous KO clone).

12. The remaining cells are left to recover for an additional 1-5 days, until they have reached a 10-30% confluence. Do not keep the cells longer before performing single cell seeding.

3.6 Seeding of single cell colonies

1. On the day of the single cell seeding, it is recommended to include wild-type cells (same cell line as the KO cells) in addition to the CRISPR/Cas9 KO cells, in order to use the wild-type cells for gating for flow sorting. Wild-type cells do not need to be sorted into 96-well plates.

2. Prepare six 96-well plates for the flow sorting. In each well, pipette 200 µl of single cell cultivation media. To make single cell cultivation media, take 106 ml of 0.2 µm

sterile filtered conditioned media and mix with 26 ml FBS. If the cells are grown with specific supplements (like insulin), add an equal amount of supplement as to normal growth media to the single cell cultivation media. Place the 96-well plates in the cell incubator.

3. Split the cells from the remaining 12-well and spin down the cells at 100 g for 10 min. Carefully remove the supernatant. Now, excess media can be present, as this will not affect the following procedures. Resuspend the cells in 500 µl warm PBS (This is a higher dilution than for normal flow, as the cell number is low and for sorting into 96-well plates additional time is required for changing position of the wells).

4. Pipette the cells through a filter cap on a flow tube to separate clusters of cells into single cells. Here, pipetting should be done in a steady but gentle manner. Too gentle and the cell suspension will not go through the filter, and too hard will cause disruption of cells. Immediately move onto flow sorting.

5. Add Propidium iodide staining just before flow sorting for the separation of dead and live cells.

6. Sort into each well on the six 96-well plates a single cell. Use the lowest flow rate applicable for flow sorting, in order to allow for sufficient time for the machine to move between the wells on the 96-well plate without losing too many cells.

7. Place the 96-well plates into the cell culture cabinet and leave the cells for 1 week. Do not examine cells or do excessive moving of the plates during this period (Note 3).

3.7 Expansion of single cell colonies and genotype screening

1. Expansion of the single cells can take between 1-4 weeks before they are ready to be transferred to a larger well. No media change should be conducted during this period. If no experience with single cell seeding of the cell line used is available, perform the first screening of colonies 1 week after single cell sorting (Note 4).

2. Take one of the six plates and examine the wells with a normal cell culture microscope. Mark wells where cell colonies can be visualized. The ideal magnification is so that the entire 96-well is visualized without the need to move the plate. The colonies should have reached a cell number of >200 cells, or >40% confluence. For cell lines which has a very low recommended splitting ration (like 1:2 and 1:3), it would be recommended to wait for the cells to become more confluent than the above recommended confluence. However, the cells should not be kept in the 96-well plates for more than 4 weeks before they are split. If no colonies have reached the desired

cell number or confluence, move the plate back into the incubator. Continue with a 2-5 days interval to examine the identified colonies on the same plate as first examined, until the desired cell number or confluence has been reached (Note 5).

3. At the time when the first colonies have reached a number of >200 cells or a confluence of >40%, split the colonies that have reached these numbers or confluence and transfer them to a 24-well to allow further expansion of the colonies. To split the colonies, remove the cell culture media and wash with warm PBS. Add 50 µl of Trypsin. Leave the trypsin on until the cells have loosened. This might take up to 45 min as the cells often are solidly attached to the plastic. Be sure to use fresh Trypsin, as to reduce the time the trypsin needs to be on the cells. If the cells have not detached after >45 min, use a 200 µl pipette and pipette up and down the trypsin with a fast release of the trypsin directly above the cell colony in order to loosen the cells by a strong flow of liquid. Repeat this 4-5 times and transfer the trypsin to a 24-well. Examine the 96-well in the microscope and evaluate if the cells are still present in the 96-well or of they have been moved to the 24-well. If the cells are still present, add new trypsin and wait 15 min before repeating this step.

4. Continue screening the rest of the 96-well plates and transfer colonies that have reached high enough cell numbers or confluence to 24-wells. Be careful not to mix colonies or to get cross contamination of colonies. From this time point, 96-wells with identified single cell colonies should be screened every 2-5 days in order to identify colonies with desirable confluence.

5. After cells have reached a high confluence (>80%) in the 24-well plates, split the cells and transfer ¾ of the cell suspension to a 12-well and ¼ of the cell suspension is collected for DNA isolation.

6. Isolate DNA from the clone, and perform PCR with the predesigned PCR primers. Identification of homozygous WT, heterozygous KO, or homozygous KO is done according to Figure 2 (Note 6 and Note 7).

7. Continue cultivating and expanding the preferred colonies (Note 8).

Notes:

1. The methods for KO should be prioritized in the following manner: a) full gene, b) promoter, c) termination, d) exon junctions. For KO of entire genes, the gene length will be important as genes larger than 20 KB should be avoided as the efficiency of KO decreases with increased length. In addition, for full length gene KO, the entire lncRNA

gene needs to be located in a region that does not overlap with other genes. For the three other strategies, all need to be evaluated based on neighboring genes and how deletions of the specific regions might affect the neighboring genes expression. Identification of strong promoter regions can be examined using the UCSC Genome Browser.

2. Successful electroporation is usually accompanied with partial to high cell death. If no cell debris can be observed immediately after electroporation, electroporation was not successful. Further cell death will be visualized at media change after 24 hours.

3. If a low amount of 96-well plates were filled with cells during flow sorting, the following reasons could explain this: insufficient trypsin digestion of cell suspension to generate single cell suspension, high loss of cells during preparation, or wrong flow rate during sorting.

4. If no single cell colonies are generated, the cell line might not be viable when seeded as single cells into a 96-well plate. Before performing KO, a test seeding should be performed on WT cells. Perform single cell sorting of WT cells into 96-wells and estimate number of viable single cells after 3 weeks. Clones should be viable and proliferating in >10% of the wells for the cell line to be appropriate for KO.

5. During the expansion of the colonies, there is a high risk of bacterial contamination, the wells need to be handled with precaution and should be taken out of the incubator only when necessary.

6. No heterozygous or homozygous clones: electroporation was not successful or unknown mutations are present in the sgRNA region of the lncRNA. Hence, we recommend before the start of the experiment the screening of the lncRNA region in all cell lines in which KO is desired.

7. If there is a presence of a large population of heterozygous clones (>50%), the genomic region could be highly important for survival of the cells and not allowing for a complete removal of the genomic area. Alternatively, the genomic region could be amplified in the cell line, thereby decreasing the likelihood for removal of all genomic copies of the gene.

8. Additional methods to verify the establishment of complete KO include Northern blotting, RT-qPCR, and FISH.

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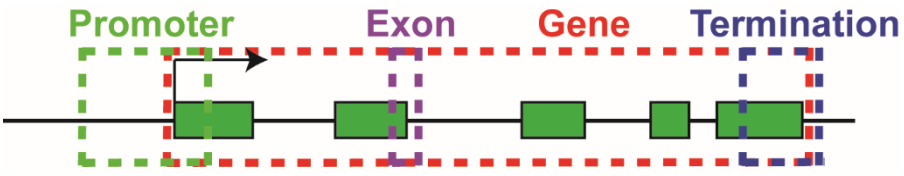

Figure 1: **Strategies for long non-coding RNAs (lncRNAs) knock out (KO).** (a) Gene: complete removal of the entire genomic region where the lncRNA transcript resides is the most efficient and secure method for lncRNA KO. However, this often implies removal of a larger genomic area, which might have additional unintended off target effects. Of major importance, the region must not contain additional genes, as these genes will also be affected. (b) Promoter: removal of the promoter and transcriptional start site will prevent transcription of the lncRNA. The method will not completely remove the gene, but only affect initiation of transcription. The promoter region should be examined, as not to be used by neighboring genes. (c) Termination: removing the termination site will cause a destabilization of the lncRNA. If the lncRNA is a polyadenylated transcript, removal of the termination site will prevent polyadenylation. Some lncRNAs have alternative stabilizing structures located at their 3' ends, and these will also be affected by the KO. (d) Exon: removal of an exon-intron junction might destabilize the lncRNA transcript, leading to a degradation of the transcript. The method will not completely remove the transcript and will only cause a knock down (KD) of the lncRNA. The method can also result in a new stable transcript with an inclusion of the intron. This method, as for termination, will only cause a KD of the transcript, and will not generate a complete KO.

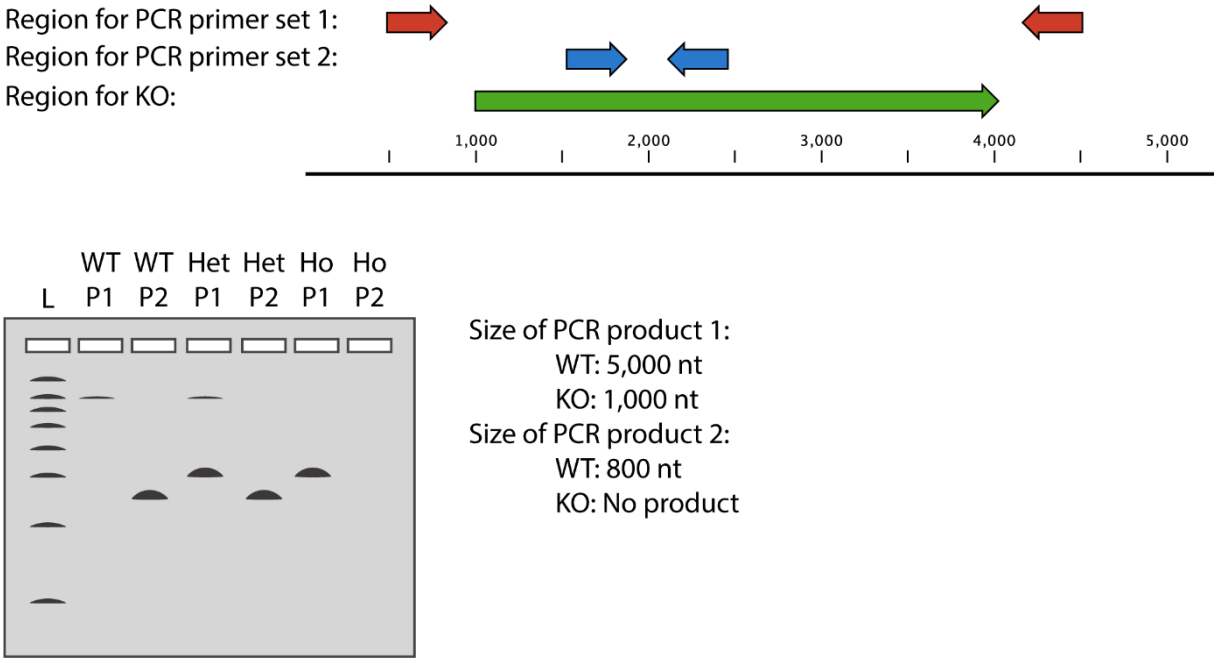

Figure 2: For genotype screening of generated knock out (KO) clones, PCR screening is performed. As PCR is more efficient for PCR products <3000 nt in length, KO strategies of larger DNA fragments will require two PCR primer sets. When using two primer sets, homozygous (Ho) wild type (WT), heterozygous (Het) KO, or Ho KO are identified based on the combination of the two PCR products; Ho KO only PCR product by primer set 1, Het KO PCR product from both primer sets, Ho WT only PCR product by primer set 2.

Table 1. Examples of electroporation programs recommended for CRISPR/Cas9 KO in different cell lines with specific characteristics (according to our personal experience). Further suggestions for electroporation program can be acquired form knowledge.lonza.com.

Cell line characteristic	Example	Electroporation program
Large epithelial cells	MCF7, T-47D, HN30, HN31	P-020
Small epithelial cells	KM12SM, COLO320, HCT116	D-032
Normal suspension cells	MEC1, BM52	X-005
Small suspension cells	JURKAT	X-001