Screen of Cas9 modified cell culture cells

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This collection is meant to give an overview about how to effectively use verified sgRNAs to create genetically engineered cell culture cells (NHEJ or HDR). It is a collection of thoughts and workflows and will be updated as soon as recent developments occur (it isn't and most probably will never be complete), i.e. recommendations and suggestions by users are very welcome.

General considerations:

- The starting point for all applications is to have at least one verified sgRNA preferably by Surveyor assay, since this is the gold standard and the only quantifiable assay.

- Take into account the ploidity of your test cell lines and that all eventual donors are ready to use.

- It is recommended that the genomic target sequence in your cell line of choice is re-sequenced. This is just to avoid presence of any kind of SNP in a critical position in comparison to the hg38 assembly to which the design is directed. By personal experience: using 293T as a tester line I hardly ever saw a SNP effect, indicative that the 293T genomic is well conserved. One event at WIMM used sgRNAs targeting HeLa and presence of 2 SNPs blocked cutting.

- sgRNA expressing plasmids should be highly purified (Qiagen Mini or Midiprep kit), plasmid donors should be highly supercoiled (Qiagen Midi or Maxiprep kit), and ssODN (IDT Ultramers) should be readily dissolved to 10μM in water for TF of cells, dialysed to injection buffer on floating membranes for zygote injection (see separate protocol).

- Transfection conditions for the target cell line should have been already established and optimised prior to starting the initial experiment. Important: addition of ssODN to the transfection mixes massively changes the requirement of the amount of transfection reagent and must be tested beforehand.

- For your consideration: if you need to have lentiviral delivery of your Cas9 and sgRNA cassettes, try to use an inducible system to avoid prolonged action of RGEN complexes as discussed above.

(1) Standard procedure for cells that can be cloned:

- Cells are transfected with optimised conditions and are allowed to recover for at least 2 days, better 3 days (will significantly increase the survival rate, based on experience from Thomas Riffelmacher/Katja Simon).
- In case cells do tolerate cloning procedures, cells will be single-cell sorted by FACS based on the fluorescent marker that is coupled to Cas9-expression by a 2A peptide (e.g. either pX458 or the Genome Engineering Services proprietary pX458-Ruby). After the initial three days in culture the FP is still sufficiently expressed. Note: the overall transfection efficiency does not really matter; a total of 200 to 500 cells are enough (just calculate back from the TF efficiency of your cell line).
- Cells should be initially sorted into suitable plates. The choice of those is very much dependent on your cell line. Test if cells survive cloning better in either 96 well flat-bottom, V-bottom or small volume Terasaki plates to allow for good clonal expansion. The generally observed mortality ranges very broad from 10% up to 90% and is very much cell line dependent (should be tested before). Sort enough cells to have at least 50 surviving clones available for screen.
- Some cells require more than 48h recovery time after transfection to allow for survival after FACS isolation. In general: FP is still detectable after 72hrs, why not wait that long…
- Test conditions in which your single clones do survive best. This can range from daily medium change, addition of selective growth factors or cytokines that make the cells grow better, use of conditioned medium in case secreted factors are vital, addition of extra FCS and so forth. In general: anything that makes your cells grow better: do/add it.
- Let cells expand and transfer to 96 well flat bottom plates, and prior to screen to 48/24 well plates. It can take up to 4 weeks to obtain confluent 24 well plates.
- **IMPORTANT**: Make sure to have a life backup plate before screening. When this plate is confluent, freeze down the individual clones to make sure you retain a vital backup. If you are confident with the technology, cells can be frozen down as well directly in 48/96 well plates (note: if those do not recover you lose your clones and must start from scratch).
- Go ahead with screening for your genomic modification (see below).

(2) Suggested procedures for cells that can NOT be cloned:

- You would need to have a stably FP-marked cell line that will allow discrimination between WT and Cas9 transfected cells. My suggestion is to generate a marked "feeder population" with either eGFP, mNG, BFP, Ruby lentiviral backbones that will allow Cas9/sgRNA transfected (pX458 or pX458-Ruby, opposite label to the feeder population) singly sorted cells to feel happy. Produce a high-titer supernatant and transduce your cells of choice to make them stably express the FP of choice (again: a different FP to the one you will be using for sgRNA delivery). Sort in bulk and allow cells to recover before using them as "feeder" or "companion" cells.
- If applicable, seeding single cells onto inactivated (irradiation, mitomycin) feeder cells will save you generation of the Lentivirus transduced feeders. This depends on your cell line.
- This population of marked sister/feeder/companion cells (FP or replication-incompetent) later serves to overcome the problem of maintaining sorted single cells.
- The suggested procedure avoids transducing your to-be-engineered cells with a lentiviral integration. Random integration will always be a threat to produce unwanted side-effects. Moreover, stable Lenti-Cas9/sgRNA integration will prolong exposure of cells to Cas9 and sgRNA, ultimately having the consequence to increase the amounts of off-target effects over time.
- Transfect WT target cells with an appropriate pX458-eGFP/Ruby/sgRNA construct and allow cells to recover for 2-3 days as stated above (again, 3 days will significantly increase the survival rate).
- Sort cells by FACS based on the fluorescent marker that is coupled to Cas9-expression and add single cells into pools of lentivirally marked/replication incompetent feeder cells (here: Lenti-dsRed/mChery/Ruby for Cas9-2A-eGFP and Lenti-eGFP/YFP for Cas9-2A-Ruby). Seed an sufficient amount of feeder/helper cells allowing for optimum growth of the sorted single cell clone positive for Cas9/sgRNA expression (10^3 to 10^4 cells should be sufficient, but really depends on your cell line).
- Keep cells in culture until a significant amount of genome-engineered cells is available (worth while testing the amount of non-FP cells over time in a pilot experiment) and re-sort each clonal expansion *de novo* into strictly non-FP expressing clones (based on SC/FS and FP exclusion). Nota bene: all cells that still have the Cas9-coupled FP have undergone random plasmid insertion and should be discarded (on a personal communication, Bill Skarnes reported a high frequency of plasmid integration when targeting mouse ESCs). Those cells will be sorted in batch and in a sufficient amount to allow Feeder-free propagation.
- Expand those cells and prior to screen transfer to 48/24 well plates.
- Go ahead with screening for your genomic modification.

(3) Screening for NHEJ-mediated KO

In order to prove the modification status in all alleles you finally must have bona fide sequencing evidence. For initial screening, several methods are available to reduce the amount of clones to be sequenced. In case money is no issue and time is short a MySeq run will solve all issues. However, there are more economic solutions. **IMPORTANT**: always compare at least three independently generated cell lines to be able to exclude unspecific effects generated by off-target cleavage by Cas9 or by eventual lentivirus-integration. Best laboratory practice is to compare independent clones generated with different sgRNAs. If they behave the same it is pretty much impossible that the off-target effect will be responsible for your phenotype.

A **HRMA** (High Resolution Melting Analysis) as a primary screen. This requires access to LightCycler (Nerlov lab) or the BioRad qPCR machines (Tudor Fulga/Tatjana Sauka-Spengler). Procedure: gDNA-isolation, PCR amplification of the region of interest and test of melting properties in defined HRMA protocols. Once a good protocol has been established for your amplicon, good and fast way to detect SNPs in the amplified material (using clonal material the interpretation of the melting curve results will be relatively straightforward). Any kind of shift in melting temperature will be an easy-to-obtain indication that your target has been modified. Pitfalls: you will not be able to detect clones that have been identically modified on both alleles (this might be rare, but you will still miss it). Important note: HRMA is by no means a quantitative assay, and having cell lines with multi-copy alleles might be tricky to interpret (long discussions with Roche). For final confirmation use standard sequencing as outlined in (3-B).

B **Sanger sequencing** as primary and verification screen. Procedure: gDNA-isolation, PCR amplification of the region of interest and submission to sequencing. Note: you will get back overlapping sequencing reads in case of mono-allelic modifications (WT vs. mutation) or in case of diverse genome engineering events on both alleles (2 different kinds of mutation), and this might be difficult to interpret. This is especially true in case your target is present in more than 3 copies (not many tumour lines really are fully diploid). The gold standard to circumvent this issue is to sub-clone the PCR-fragment into a suitable backbone (TOPO_TA, pGEM-T, pZero-II), transform the reaction, pick 10-15 clones (the higher the suspected ploidity the more clones need to be picked) and make Minipreps (this is supposed to yield individual single clone DNA) and submit all to sequencing. Half of the clones in a diploid line should represent each allele. Immediate analysis is possible using the TIDE software package from Bas van Staensel at NKI Amsterdam (https://tide.nki.nl).

C **Western blotting**: this is possible if you have a good antibody available detecting your target protein. Procedure: Isolation of cell pellets from sufficiently enough cells to be able to detect it by the screen (probably a 48 to 24 well plate will be required) and test for absence or presence of your full-length target protein.

(4) Screening for NHEJ-mediated deletions.

Two sgRNAs (or pairs thereof) are applied that work more or less equally well in Surveyor assays flanking the target site. Due to the nature of the modification the screen is pretty straightforward. Nota bene: it seems that addition of a bridging oligo does NOT increase efficiency. Also here: always compare at least three independently generated cell lines to be able to exclude unspecific effects generated by Cas9 off-target cleavage.

(A) Simple **one-step PCR** will identify all allelic combinations irrespective of cell ploidity. Procedure: gDNA-isolation, PCR amplification of the region of interest using a 5' and 3' specific primer set that amplifies about 200bp upstream and downstream of the deletion (leave space, since sometimes NHEJ can create greater deletions around the initial cut site and you might not be picking up the full spectrum of deletions). Amplicons of the shorter size are favourably amplified and represent successfully deleted clones. Screen for your desired outcome: heterozygous or pan-allelic deletion simply by looking at the PCR gel. All clones that are up to your desired criteria: submit the amplicon for sequencing and determine the correct deletion.

Of note: more recent analyses have shown that parts of the WT-amplicon in fact are not WT but excised, inverted and re-inserted gDNA, and as such a full knock-out. It is very much worthwhile including a second set of primers into the screen that will allow discrimination of orientation of the WT band.

(5) Screening for HDR-mediated small insertions (SNPs, Tags, LoxP).

The donor template usually is a ssODN composed of <200bp (preferably asymmetric sODNs are applied according to Richardson *et al*., 2016, NBT), flanking the desired short change by 70-90bp homology arms, and will insert mutations inactivating sgRNA re-binding, and preferably co-insert a de novo restriction site for screening purposes. IMPORTANT: several reports show that usually the central part of the ssODN is incorporated flawless, but that end insertion (5' and 3') is prone to errors. All cell lines generated in this way **must** be verified by full-length sequencing of the locus. Also here, compare at least 3 independent cell lines to ensure absence of off-target effects.

(A) Making use of the inserted **tag and its generated size difference** by PCR methodology. Procedure: gDNA-isolation, PCR amplification of the region of interest with all primers annealing outside the repair template. Modified alleles by tag insertion can be detected by the size difference of tag versus WT genomic.

(B) Making use of **a novel restriction site** has been co-inserted that is not present within the amplified genomic DNA fragment. After PCR, bands are cleaned and subjected to digestion. Since all cells are/should be clonal, the outcome is restricted to three results: fully WT (i.e. undigested), a heterozygous insertion (WT and both digestion fragments will be visible in an equal molar ratio if diploid, bit more tricky if >3 alleles are present), or a homozygous targeting event (only the digested bands are visible). Any mix or non-molar ratio of bands is indicative of a non-clonal origin in diploid cells, or of presence of a third to fourth copy of your target gene.

(C) If nothing but a single **SNP** needs to be inserted (**sgRNA inactivation permissive**) and no chance of REN insertion is available, the same screening procedure as for NHEJ-mediated mutagenesis applies (part 3). *Nota bene*: HRMA is only a reliable method for SNP insertions and not for tag-insertions, since larger modifications will go unnoticed by the re-annealing detection chemistry.

(D) If nothing but a **single SNP** needs to be inserted and even sgRNA inactivation is not permissive, GE services suggest to change the donor and not use ssODNs. It would be advisable to use a larger donor that will allow the SNP target site to be un-severed, and that will insert sgRNA inactivating mutations and/or de novo REN insertion at a position decently up- or downstream of the SNP in an inconspicuous locus. In worst case, very large cassettes will need to be used. The general screening principle as described in 5-B applies, however, with much larger amplicons (more difficult). Using HRMA can only be applied if it can be certified that the donor plasmid has been lost (preferably you could mark it with a FP expression cassette and select for non-fluorescent clones.

Of note: several recent projects of this nature used the asymmetric ssODN approach and in many instances a successful HHR was observed although the protospacer was still intact. I guess it boils down to when there is this window of opportunity. Encouraging enough: if you have no other choice it is well worth having a go.

(6) Screening for HDR-mediated large insertions.

The donor template usually is a plasmid with homology arms > 500bp flanking the desired cassette. Such plasmid has the potential (limited, though possible) to randomly insert into the genomic DNA, and the ultimate verification of the cell line should involve a Southern blot screen with internal and external probes (gold-standard test) and a ddPCR mediated copy number determination.

IMPORTANT: You must have a strategy to control for random insertion events. The simplest way will make use of a Southern-based screen using co-inserted de novo RENs. More complex/expensive scenarios can involve DNA-DNA-FISH or genomic sequencing on a MySeq platform. If performing Southern blots I would recommend using external 5' and 3' probes (detection of proper insertion on both targeting arms) and with a cassette specific internal probe (allowing to pick up random insertion events. Of note: it is crucial to select restriction enzymes with a rather frequent genomic cutting or to control transfer of DNA very well.

(A) **PCR designed to use outside in and insert-specific primers**. Procedure: gDNA-isolation, PCR amplification of the region of interest using specific primer sets that have an external and an internal (cassette based) annealing. Big disadvantage: there is no good positive control except you construct it (costly). Additionally, plasmid only can give you false positive signals. Hence, I would strongly recommend specificity tests in which cell lysates prepared after transfection with donor plasmid only (w/o Cas9 and w/o sgRNA) are compared to the test samples. Other difficulty: this procedure is prone to false negatives if the amplicon is very long due to extensive homology arms.

(B) **PCR amplification over the region of interest**. gDNA-isolation, PCR amplification over the region of interest using 5' and 3' primers with external annealing. This is much more tricky to establish (routinely >3kb genomic DNA amplicons), but if, it is well possible the obtained size difference (if available) clearly identified modified versus non-modified genomic DNA. Extensive pre-testing using different long-range PCR kits is very much advisable. Difficulty: highly prone to false negatives if amplicon is very long.

(C) **PCR and detection with internal de novo RENs**. Same issues as in part 6-B, however, if cut is successful will give you a higher level of confidence. This approach works even in case that WT and inserted cassettes do not significantly differ in size (given eventual deletions). The digest can identify the genotype as heterozygous of homozygous. Prone to false negatives if amplicon is very long.

(D) ***In vivo* read out**. Best opportunity. If the tag to be inserted is expressed from a promoter that is expressed in your cell line, you can set up an easy screen based on reporter-expression (eGFP by simple fluorescence microscopy or tags by Western-blotting). This is a powerful initial screen and should be completed by genomic sequencing (correct insertion, mono- vs. bi-allelic insertion, random insertions etc.).

(E) **Internal PCR**. This can be done only under strictly controlled conditions, since minute amounts of donor plasmid will yield false positive results. Ensure the plasmid is lost after growing up single cell clones (must be controlled by both, PCR and by loss of associated FP expression) and use an internal primer approach annealing to inserted cassette and surrounding genomic DNA or flank the insert by primers, in which case the WT will give a smaller fragment and the properly inserted cassette will have the additional length of the inserted tag → easy discrimination of WT vs. HET vs. homozygously modified. This most likely will not give false negative results (based on a rather small amplicon), but the risk of false positive signals is high. This approach critically relies on the fact that the donor plasmid has been lost over time (else you will have 100% positive clones, indicative that this approach will not work).