General CRISPR/Cas9 Considerations

General information: We clone our sgRNAs into backbones that can be selected for eGFP (pX458), mRuby (pX458-Ruby) or Puro (pX459.2). In case you need LV delivery for hard-to-transfect cell lines, guides are individually tested in plasmids and then re-cloned into FP co-expressing LVs (dual guides are cloned as GeneART cassettes). FACS is used for enrichment of doubly transfected cells, and the FACS facility knows how to enrich for Ruby positive cells (signal is generally a bit weaker than eGFP but this is not of concern for FACS enrichment).

Important points before starting:

1. In case deletion of the target gene is detrimental for cell growth or proves to be essentiual, it will be hard or impossible to obtain homozygous deleted clones.
2. The ploidy of the target cell line is important to be able to judge the expected outcome (mono/multi-allelic targeting, full KO/HDR). ATCC and COSMIC are good sources of reference. Eventually several rounds of targeting with different sgRNAs might be required for cells with a chromosome count >3.
3. A dual-sgRNA deletion strategy allows a very simple screening strategy (plain PCR-over) and this is independent of the allelic situation. We observe a very high percentage of PCR'ed "WT" bands to be excised & re-inserted in inverse orientation, i.e. *bona fide* null alleles.
4. NHEJ and HDR are competing pathways that are active at different time points of the CDC and generally HDR is much harder to achieve.
5. HDR or NHEJ over WT is difficult to achieve. If WT is required, make sure to have an appropriate strategy in place (to be discussed project-specifically), e.g. by offering WT and mutated donors at the same time or by retargeting the NHEJ'ed allele with custom secondary sgRNA and donors.
6. For N- or C-terminal tagging, sgRNAs will target the 5'/3' UTRs to leave the coding exons intact in case that the NHEJ over HDR ratio is severely shifted towards the NHEJ pathway. Larger than expected deletions (Owens et al., NAR, 2019) need to be considered and screened for.
7. If possible, link your modification to a *de novo* REN for ease of screening (PCR and digestion).
8. Sequence your target region prior to engineering (especially primary cells and hiPSC samples) to uncover non-annotated SNPs.
9. The repair of DSBs by either HDR and NHEJ is a cell line intrinsic process and varying degrees of efficiency are observed between different cell lines (i.e. cannot be controlled for).
10. Not all sgRNAs do cut efficiently and not at the same level in different cell types. The underlying chromatin has a big influence, and as such we recommend to test at least three guides per target. The ON-target validation in a test cell line is a very important parameter, however, transferal to your cell line of choice in 1:1 is not possible.

Workflow: (**a**) Validation of reagents by GE services, (**b**) Transfection of cell lines by empirically determined protocols, (**c**) Minimum 48h and better 72h of recovery, (**d**) Single cell isolation by FACS-ing Ruby/eGFP double positive cells (preferably into round bottom, V-bottom or Terasaki wells; eventually use of conditioned medium or higher amounts of FCS to keep cells happy; all to be empirically determined), (**e**) Clonal outgrowth (variable time and depends on the cell line; typically 10-50% of cells survive; aim for having a maximum of 50-100 clones to screen for), (**f**) Preparation of maintenance and gDNA plates, (**g**) gDNA preparation and screen for absence of target exon by multiplex PCR, (**h**) Sanger sequencing of all alleles, (**i**) Further validation (see below). More information on model-development, screening and detailed protocols can be found on the WIMM internal Genome Engineering website.

Reagent choice (to be discussed on a per-project basis). (**A**) Delivery of Cas9 in *plasmid* has a longer persistence of Cas9 and as such a higher chance for accumulation of OFF-target hits.

However, plasmids enable coupling Cas9 to a selectable marker (any FP, Puro, Hygro, Bla, G418). Cas9, HiFi-Cas9, nCas9, xCas9, BE3, BE4max, ABE7.x, CRISPRi, CRISPRa, Prime Editors and any other variant you are interested in can be immediately used or generated. (**B**) Delivery of Cas9 and mRNA as an RNP works immediately and reagents are usually degraded within 24h. This is the least OFF-target prone event and seems very efficient throughout a wide variety of cell lines. sygRNA should be ordered as full length, 5' chemically protected RNA and is available at about 70-80 GBP per 2nmol (e.g. (e.g. MERCK, IDT, Horizon or Synthego). Cas9 protein is available from several suppliers, and it should be used only as NLS-tagged version. Caveats: so far no good way for enrichment of transfected cells available (atto-555 labeled sygRNA or eGFP-Cas9 give in our experience relatively high levels of false positive enrichment). RNPs can only deliver Cas9, eCas9, HiFi Cas9 or nCas9, no functional xCas9 or Cas9-bases editors are available in a functional format yet (01/2020). (**C**) For hard to transfect cells (predominantly primary cells) we can assemble lentiviral particles (all in one, Cas9-2A-FP and hU6-sgRNA at usually $\leq 5x10^8$ TU/ml, WIMM Virus Facility). Of note: any LV that integrates into the host genome will create extended Cas9 and sgRNA expression and as such will trigger higher levels of OFF-targeting. We can create non-integrating LVs or use dox-inducible systems.

Off-targeting: In general, GE services recommends using WT *S.p.* Cas9 since observed off-target frequencies prove to be low when isolated clones have been analyzed. The current Gold standard recommends generating ≥ 3 independent cell lines preferably with different sgRNAs. If all of the generated cell lines behave similarly in the experimental setup it is generally accepted that the observed phenotype is not due to OFF-targeting. Targeted sequencing of the most likely off-target regions can be done but is mostly hand-waving. The ultimate verification can be obtained by global unbiased technology platforms such as Circle-Seq or Digenome-Seq, however, due to cost and labor involved those are rather applied in clinically relevant applications only.

Must do functional validation: (**A**) Cloning of deleted alleles and verification by Sanger Sequencing, (**B**) Western Blot and verification of absence of target protein in whole clone extracts (HET and WT clones serve as "littermate" controls), (**C**) Since NHEJ events have been shown to frequently produce larger than expected deletions (up to 20%, Owens et al., NAR, 2019), GE recommends to use long range PCR approaches for verification. This is to avoid having false positive/negative results generated by loss of primer binding sites, (**D**) Rearrangements do occur when cells use the DSB pathways, and events as larger inversions, duplications and translocations can occur and need to be screened for with appropriate methods (PCR or capture based technologies), (**E**) A relatively easy and simple procedure is to generate several independent cell lines (preferably using different sgRNAs) and cross-compare their behavior. After all clones underwent functional validation, they are tested in the experimental model of choice for their responses. HET and WT clones from the same experiment are perfect controls that will be obtained in the screening process, anyhow. All clones have seen the same set of reagents and if they behave like un-edited cells there is a very good chance that no effect can be attributed to whatever kind of OFF-targeting.

Additional (recommended) layers of functional validation: (**F**) We recommend performing ddPCR analyses of all verified clones that have been generated as a KO or KI. Copy counting of the to-be-deleted region clearly identifies heterozygous and homozygous deletions in comparison to "littermate" clones. ddPCR can identify random insertion events of donor cassettes that might not been picked up by outside/inside PCR screening, (**G**) Targeted locus amplification (TLA) is a 4C based technology that is able to identify the sequence of the inserted fragment, the proper insertion sites of 5' and 3' arms as well as potential random insertions with the exact insertion site in base pair resolution. Characterization and analysis of clones treated with relatively short donors (<20kb) can be done by Cergentis for less than 1700 GBP in less than 6 weeks turnaround time, full analysis included.