CRISPR Cas9 FAQ

We were wondering if there is percent knockout that you could guarantee? Also, if we are unable to get knockout with any of the 3 targets can you provide others?

Based on our experience, more than 90% constructs we designed will achieve complete knockout in 20-90% cells. Most constructs will lead to complete knockout in >50% transduced and selected cells. Mounting evidence suggests that sgRNA is much better than shRNA and siRNA. No significant knockout even with 3 constructs will only happen in rare cases.

If none of the sgRNA constructs designed for use with Cas9 Nuclease can achieve reasonable knockout, we will design another set of 3 constructs for free. This is a one time replacement, and cells must be monitored. Please provide Surveyor assay evidence. Simply design primers to PCR amplify the targeted sequence and digest with T7E.

Do you have any experience using your system on long non-coding RNAs?

The system can be used to knock out long non-coding RNAs or miRNAs or any short pieces of DNA by using a pair of sgRNAs. However, the efficiency will be much lower than knocking out protein-coding genes, at ~1%. Thus, single cell cloning is required. We may design 3 sgRNAs targeting the start and the end of lncRNA (3x2 contructs). You may have 3x3 combinations for knocking out the targeted lncRNA and PCR screening will lead to identification of the best combination. You may then perform single cell cloning after transduction of the 2 best sgRNAs. We expect that in 1-5% clones, the lncRNA will be completely knocked out.

What is the suggested culture conditions?

Please consult the optimized culture condition for the particular cells you are working with as well as follow the standard protocol for lentiviral transduction.

What are the sgRNA guidelines for replacements?

abm guarantees that at least one out of the three sgRNA Lentiviral constructs purchased in a set designed to be used with Cas9 Nuclease will result in complete gene knockout in over 50% of cells at 1 week after successful transduction and drug selection due to frameshift mutation. The gene knockout efficiency can be quickly examined by Surveyor assay and confirmed by Sanger sequencing or other functional assays. If in extremely rare cases that significant knockout was not achieved, we would provide a one-time replacement of three new constructs with alternative sgRNA sequences. To qualify for this replacement, customers must provide Surveyor assay data. Before sending your inquiry, please make sure you have optimized your experiments as far as possible, this includes (where applicable) increasing your MOI (up to 10) and the

duration of infection (up to 72 hours), and carrying out clone screening before assaying for knockout. Please also provide data to show that a reporter lentivirus was used to show that you have optimized the MOI to use on your target cell line.

For vector transfection, you will need to prove that there is good enough vector transfection by qPCR on Cas9 or Puro for the 'All-in-One' vectors, qPCR on Neomycin for constructs containing only the sgRNA, or successful puromycin/neomycin selection. In addition, please provide Surveyor Assay or Sanger Sequencing data on at least 20 isolated clones.

abm limits its obligation and liability for the success of this technology to providing one replacement of any sgRNA lentivector product only. The replacement set will not be covered by the same guarantee. If these constructs are also considered to be ineffective then it is most likely due to unknown reasons.

What is the difference between using the Cas9 nuclease vs. the nickase? Is one better than the other for genome editing?

Generally, you would use the nuclease to make a double-stranded break, which results in non-homologous end-joining. This will result in a few bp deletion and is ideal if for example your goal is to punch a small hole in the active site of an enzyme. You can consider the nickase to trigger homologous recombination from a second "donor" plasmid. For example this can be used for inserting a resistance cassette into your gene of interest, and then selecting for the knockout cells.

If you would like to use Nickase for knockdown, we can design pairs of sgRNA for you if two units of either the individual sgRNA or the set is ordered. The knockdown efficiency may be lower due to the need for TWO targeted single stranded breaks, but it will also be more specific. However, our sgRNA design has improved with a shorter target sequence which has been shown to reduce off-target effects so the nuclease can be used with a single sgRNA effectively.

What is the expected time for both siRNA viruses and sgRNA viruses to show an efficient knock down? Are there any differences?

siRNA lentiviral plasmids usually take 72 hours post transfection. For siRNA lentiviruses, it will also be around 72 hours post transduction but please note that a drug selection step will also need to be included.

For sgRNA lentiviruses, it usually takes approximately 1 week to observe knockout. For sgRNA adenoviruses 1 week will also be a good starting point.

Note that these are general guidelines only and may vary depending on your target cells as well as experimental parameters.

Can the sgRNA constructs designed for use with Nickase also be used for Nuclease?

Yes.

Can we use only one sgRNA construct with Cas9 nickase? Would these sgRNAs target the same exon or would they target two separate coding regions?

Cas9 nickase is a modification of the Cas9 nuclease. Cas9 Nickase only creates single stranded breaks from each guide RNA, thus two guide RNAs that have target sequences in very close proximity to each other are required in order to generate the double stranded break that the wild type Cas9 nuclease makes.

If you are interested in two sets of sgRNAs (i.e. 3 pairs of sgRNAs, total 6 constructs), we can put in a special request to have them designed against different exons if you wish. A custom quotation can be provided upon request. Alternatively if you order one pair (i.e. 1 pair of sgRNA, total 2 constructs), then they will be designed on the same exon as they need to be targeting the same region in order to create the double stranded nicks.

What is the frequency of off targets?

We "predict" the off target effects by using software to determine matches to other parts of the genome. We typically select target sequences that return a "0" value for off-target matches.

If genome editing is detected in diploid or triploid cells by sanger sequencing, what kind of results is the best in order to confirm to make a homo knockout cell?

For Sanger Sequencing, the best way may be to PCR the target region in question, clone, and sequence multiple clones. If you do not detect wild type sequence in your clones, then you can consider them homo knockout cells. However, if you do detect a mixture of genome edited and wild type clones, then you have a heterozygous knockout. Please note this is a guideline only and the end user will have to determine the suitability of their data based on their experimental parameters.

If genome editing is detected in diploid or triploid cells by surveyor assay, what kind of results is the best in order to confirm to make a homo knockout cell?

If you are generating KO, you don't need a homozygous mutation. Frame shift biallelic mutation should do that. It is not very common to have the same mutation in both alleles by CRISPR. So successful genome editing in 1 allele is observed and not in the other, the surveyor assay will still show digestion. If successful genome editing in both alleles are observed (likely to be different mutations), you will also see digestion in the surveyor assay. The best way to confirm homo knockout is to then do Sanger Sequencing on these clones to confirm whether it is single allele knockout or biallele knockout. Please note this is a guideline only and the end user will have to determine the suitability of their data based on their experimental parameters.

Which method is the best for detecting knockout cell (Surveyor assay, Sanger sequencing or western blot)?

You may do a quick screen with a Surveyor assay first (using our CRISPR Genomic Cleavage Detection Kit, Cat# G932), then expansion of clones from single cells followed by Sanger sequence validation to verify the desired mutation on all alleles is the most rigorous approach for confirming complete gene knockout. Please note this is a guideline only and the end user will have to determine the suitability of their data based on their experimental parameters.

What is the sequence of U6 Forward Sequencing Primer in pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro?

Sequencing Primer:
5'--TACGTCCAAGGTCGGGCAGGAAGA--3' in the U6 promoter region

Can we switch the U6 promoter for an EF1a promoter to express the sgRNA?

RNA pol III promoters, such as U6 and H1, are commonly used to express small RNAs such as siRNAs, miRNAs and sgRNAs. We do not recommend using EF1a to drive the expression of the sgRNA as it is untested and may not work. Most publications state using U6 or H1 promoters to drive the sgRNA expression.

Does abm's Cas9 contain a NLS sequence?

Yes, the Cas9 in our system will contain the NLS sequence.

For our All-In-One lentivectors/viruses, there is an N-terminal SV40 NLS and a C-terminal Nucleoplasmin NLS on the Cas9 Nuclease.

How can I design primers for validation of knockout?

The best way for showing knockout is to design primers in the genomic region, similar to those you would design for the surveyor assay, and use those primers to PCR amplify the genomic region targeted for editing and clone the fragment into a vector for plasmid DNA sequencing. Sequencing will confirm the exact mutation that arose from the genome editing.

If the target gene for the CRISPR product is a long non coding RNA, can I expect to see a knockout of the whole gene, or just part of it? This question is relevant since we need to design primer for screening.

For long non coding RNAs, we suggest using a pool of the viruses. This way, the knockout could occur in two or more different ways:
1) If the cell gets infected with a single CRISPR lentivirus, then the gene will get cleaved at 1 location, creating an insertion/deletion spanning anywhere from 1-20nt (on average). This could be enough to knockout its function, or it might not.

2) If the cell gets infected with 2 CRISPR lentiviruses, then the gene may be cleaved at the 2 locations, creating a much larger deletion, enhancing the effect of the knockout.