Reference Guide – Designing CRISPR-Cas9 RNA Oligonucleotides for the No-Amp Targeted Sequencing Procedure

Introduction

The purpose of this document is to provide technical guidance on how to generate and select CRISPR RNA oligonucleotides (crRNA) that are compatible with the PacBio *Procedure and Checklist, No-Amp Targeted Sequencing Utilizing the CRISPR-Cas9 System*.

CRISPR-Cas9 RNA oligonucleotides are comprised of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). When annealed together or synthesized as one continual construct they makeup guide RNA (gRNA) or single-guide RNA (sgRNA), respectively. Regardless of the guide RNA type, the tracrRNA enables gRNA to be complexed with Cas9 nuclease. At the same time, the crRNA provides the targeting function of the gRNA-Cas9 complex to enable double-stranded digestion at a specific genomic location.

The PacBio procedure referenced above makes use of gRNA as opposed to sgRNA. The tracrRNA oligo is comprised of a universal sequence that does not require customization. It is the crRNA that requires customization for a specific target. The No-Amp procedure uses two gRNAs to excise the target region from genomic DNA (gDNA).

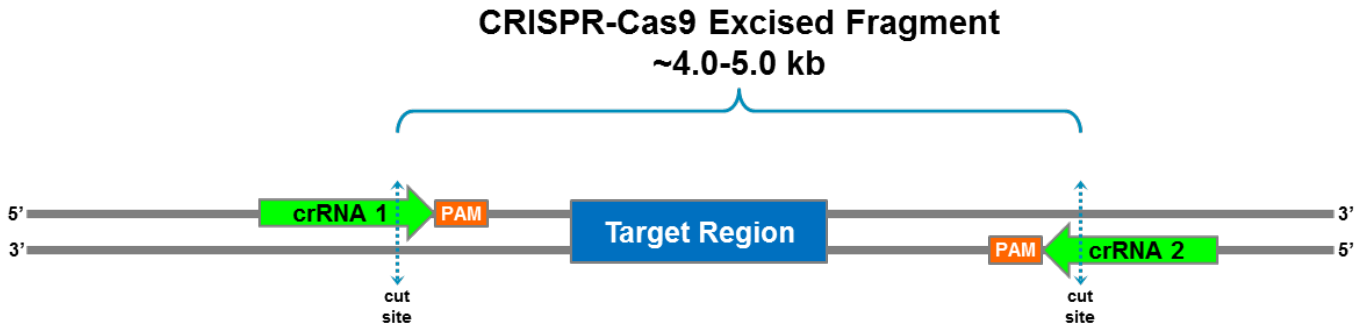

Figure 1. A depiction of a generic No-Amp targeted sequencing assay design. Various aspects of this illustration will be addressed in the design steps that follow. Note that the two flanking crRNA binding sites must be upstream or 5' to the target region with the 3-bp protospacer adjacent motif (PAM) sites closest to the target region necessitating a trans configuration for the crRNAs. The PAM site is a required targeting element located immediately 3' of the crRNA design in the target sequence, not in the crRNA design sequence.

This targeted sequencing method was developed for analysis of HTT, FMR1, ATXN10, and C9orf72 repeat loci but is readily applicable to other targets. The guidance below covers the design process for the ATXN10 repeat locus (as an example).

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Design Steps

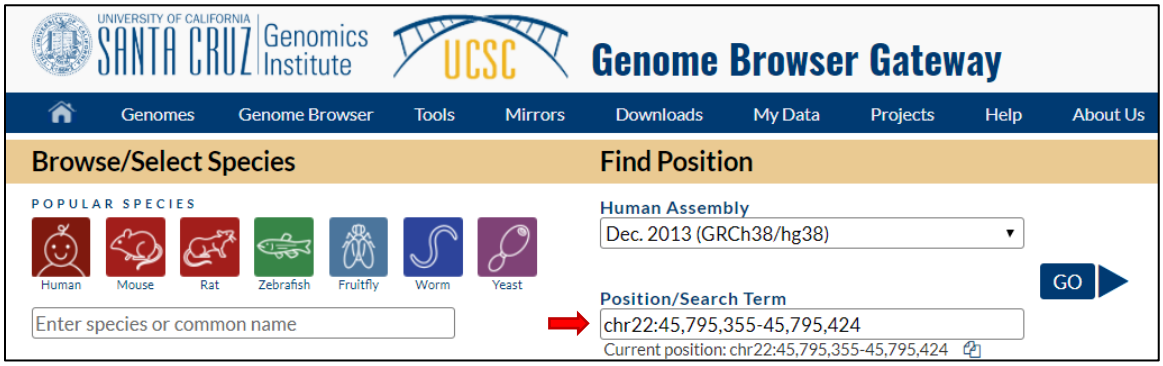
There are 4 steps to designing a pair of crRNAs for a particular target region:

1. Retrieve gDNA sequence with common SNPs identified
2. Generate crRNA designs
3. Select crRNA designs
4. Test crRNA designs

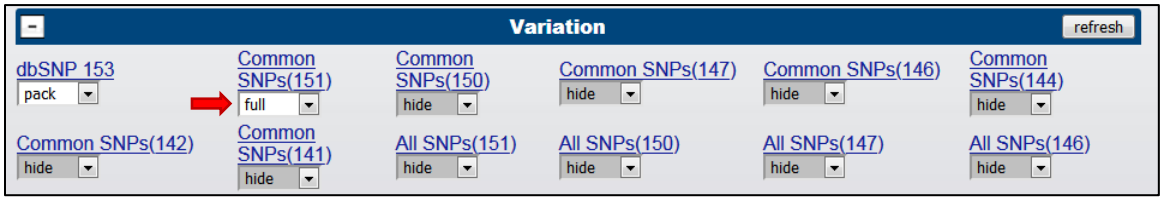
Retrieve gDNA Sequence with Common SNPs Identified

In this step, 5,000 bp of gDNA sequence containing the target region is retrieved in preparation for generating crRNA designs. Use a retrieval tool capable of marking common SNPs in the sequence. Common SNPs located within the crRNA design sequence or PAM site may alter the binding efficiency of the crRNA in the gRNA-Cas9 complex, ultimately reducing the number of on-target sequencing reads. The following instructions use the UCSC Genome Browser website (https://genome.ucsc.edu/).

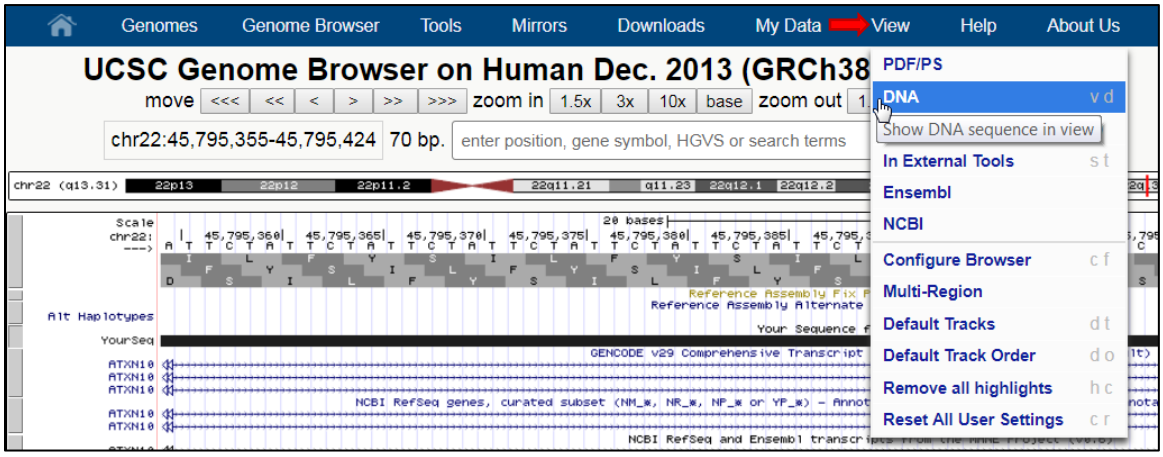
1. Identify the location of the target region. Here the target is the ATXN10 repeat locus in Chromosome 22 with ATTCT repeat motif (chr22:45,795,355-45,795,424; GRCh38/hg38). Use the latest reference assembly build available.
2. On the UCSC Genome Browser website, enter the chromosome and coordinates of the region of interest in the **Position/Search Term** field as shown below and click the **Go** button.



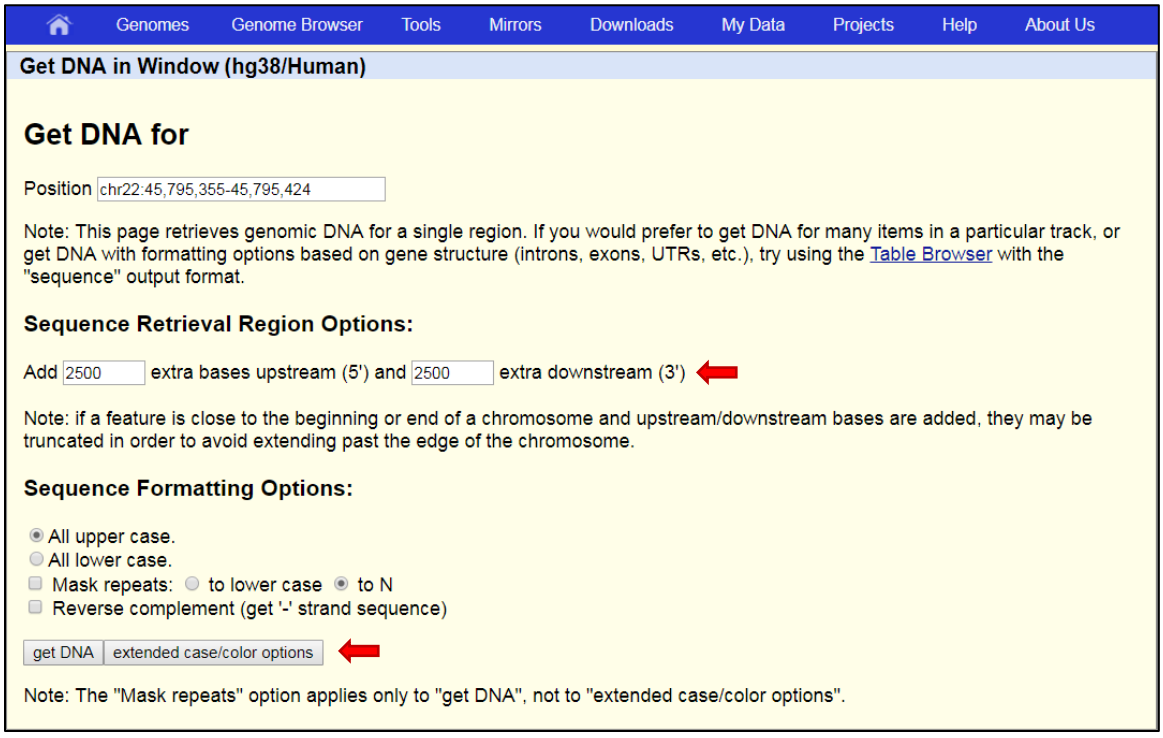
3. In the next window, scroll down to the **Variation** options section and make sure at least one of the **Common SNPs** option is set to full.



4. In the same window, choose **DNA** under the **View** menu at the top of the webpage.

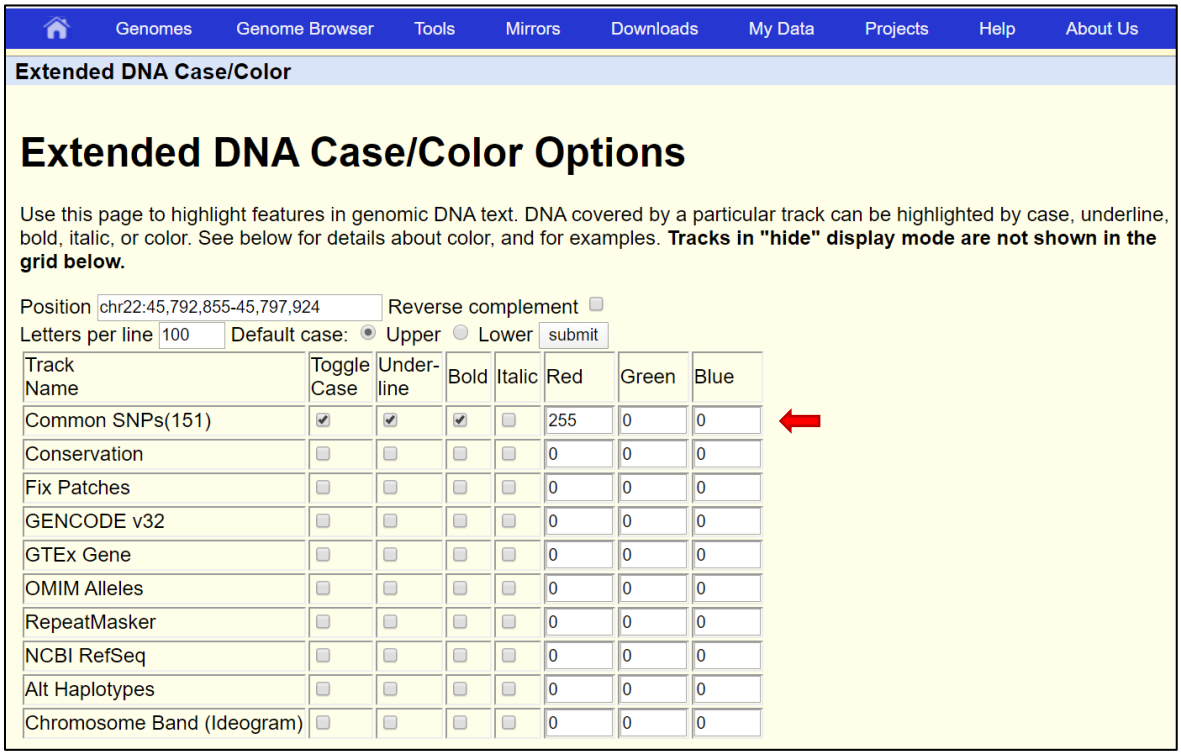


5. Retrieve 5,000 bases surrounding the target region (2,500 bp on each side). In the next window, enter 2,500 bp into the fields for extra upstream and downstream bases under the **Sequence Retrieval Region Options** section.



6. Click the **Extended Case/Color Options** button to advance to the next webpage and enable marking of SNPs in the sequence.

7. Select the desired SNP marking attributes and retrieve the sequence by clicking the **Submit** button.



8. Copy the resulting target sequence into a text editing program (e.g. Microsoft Word) and mark the region of interest (highlighted yellow below). Note that common SNPs are marked as instructed above.

>chr22:45792855-45797924
TCGACATTTTTACTGACTTTGTTAAATTCTTCTAAACTTGCATTGGCTGTTATACCTACATAgGGAGCCACATGAAGATCATAGATAAGGTGAAT
CAAATGATTTACATGTTTACTGACAAGAGCTTTTTTAGTTCCACCCAAATGCTTTCTCCTAATAGCCCAGCCAGGCCTTTTGCAGGCTCTGCAGT
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ATTAAAAAGATAATAAGAGAATAGCATAAACAACT

Generate crRNA Designs

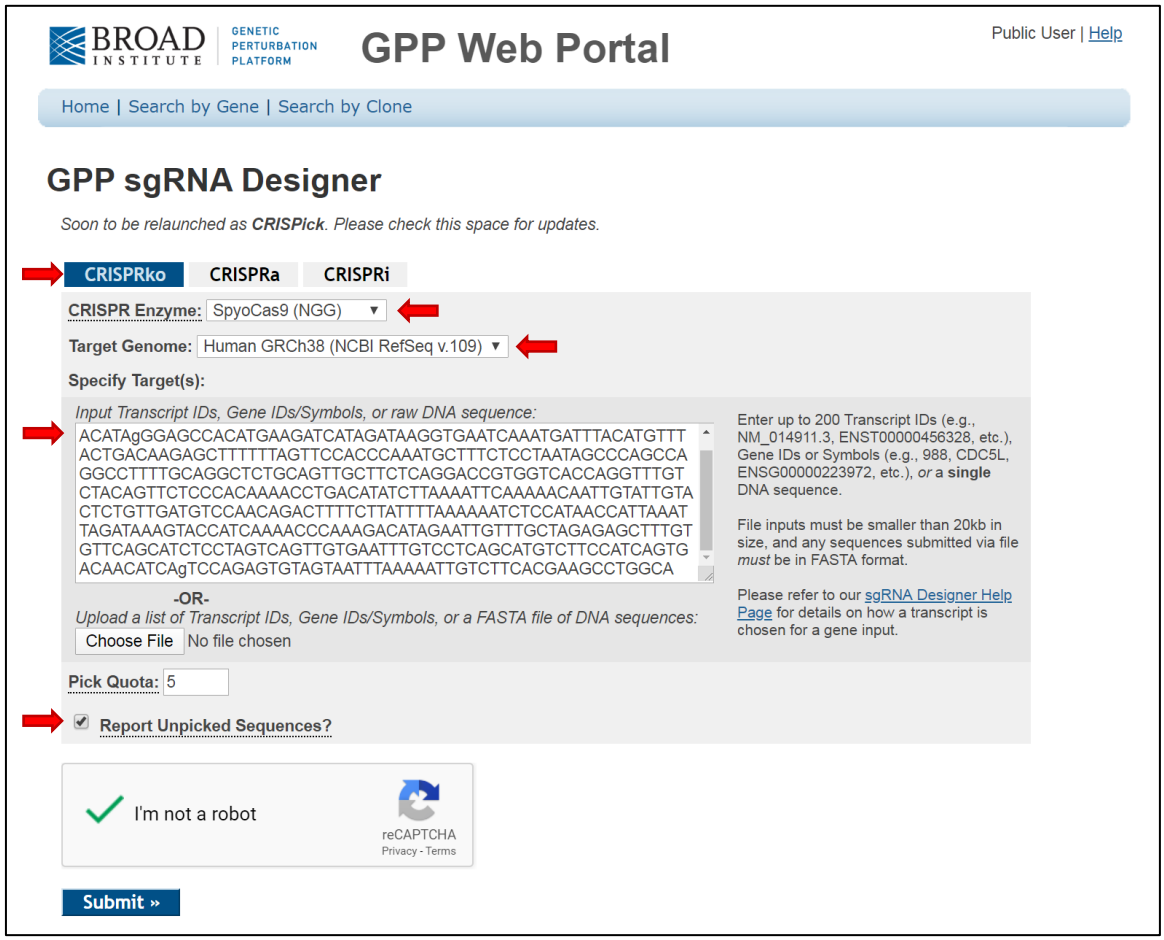
In this step, the retrieved sequence is submitted to an online CRISPR-Cas9 design tool to generate several crRNA designs. Note that many websites use the terms crRNA and gRNA interchangeably, but, as explained in the introduction, crRNA is the targeting component of gRNA. Also, at times a distinction isn't made between gRNA and sgRNA. As pointed out in the introduction, gRNA is constructed by annealing separate crRNA and tracrRNA oligos together and sgRNA is formed by synthesizing the crRNA and tracrRNA as one continuous oligonucleotide.

The following instructions use the GPP sgRNA Designer webtool on the Broad Institute GPP Web Portal website (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). Designs generated by this webtool are compatible with the No-Amp targeted sequencing procedure.

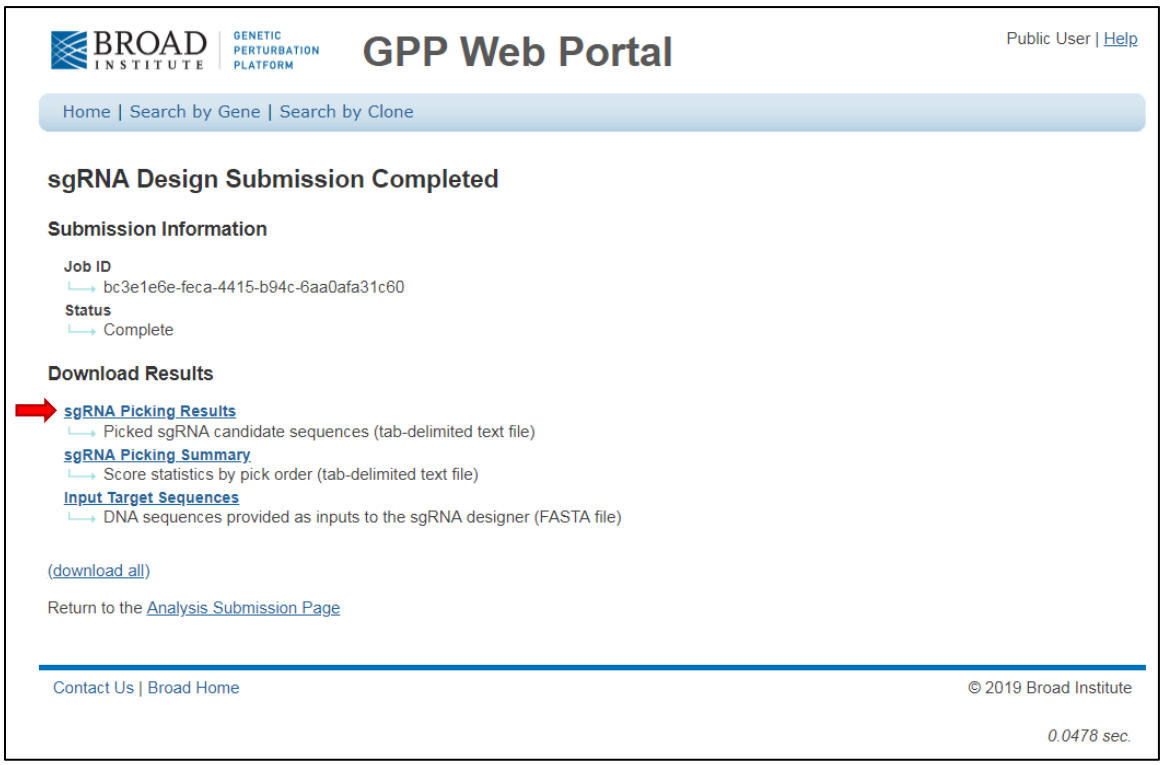
1. Select the first 500 bp from the beginning of the retrieved target sequence and submit to the GPP sgRNA Designer webtool. Despite the "sgRNA" reference in the website screen capture below, it is crRNA designs that are generated.

   Make sure the **CRISPRko** tab is active and that **SpyoCas9 (NGG)** is selected in the **CRISPR Enzyme** pull-down field. The No-Amp procedure uses Cas9 nuclease from *S. pyogenes* and is associated with the specific PAM sequence, NGG.

   Under the **Target Genome** pull-down field choose the latest build. Make sure the **Report Unpicked Sequences?** box is checked. Click the **Submit** button to generate results after verifying you are not a robot.



2. Download the “sgRNA Picking Results” file in the next window by clicking the link.



3. Repeat Steps 1 through 2 in this section for the last 500 bp at the end of the retrieved target sequence. In the next section, crRNA designs flanking the target region will be selected from the first 500 bp of sequence and last 500 bp of sequence. This will generate a 4-5 kb fragment containing the target region after CRISPR-Cas9 digestion.

Select crRNA Designs

In this step, crRNA designs are selected from the output of a CRISPR-Cas9 design tool based on orientation and uniqueness. They are then mapped to the gDNA target sequence obtained earlier in the design process to determine the presence of SNPs in the designs. Final design selections are then made for testing. Following the previous section, the “sgRNA Picking Results” files generated separately for the first and last 500 bp of the 5 kb target sequence are used below.

Note: This reference guide uses the “Combined Rank” order to select top-ranking crRNA designs. The GPP sgRNA Designer webtool applies additional selection criteria listed in the “Picking Notes” column to re-order the top crRNA designs in the “Pick Order” column. These additional ranking filters are, in general, not applicable to No-Amp crRNA designs with the exception of “Off-target Match” flags in the “Picking Notes” column which potentially indicate poor crRNA design specificity.

1. Open the “sgRNA Picking Results” files (tab-delimited text file) in Microsoft Excel and save each as an Excel Workbook (.xlsx file extension).
2. Each table is sorted by the “Combined Rank” column by default as shown in Tables 1 and 2 below in the next step. Only the columns to aid in mapping the designs to the 5 kb target sequence are shown, and other columns have been hidden. Again, “sgRNA Sequence” as appears in the table is synonymous with “crRNA Sequence”.
3. Identify crRNA designs compatible with the No-Amp procedure.

   Orientation of the crRNA design relative to the target sequence is **critically** important to the No-Amp targeted sequencing procedure. The gRNA-Cas9 complex cuts 3-bp upstream of the associated PAM site. After cleavage, the gRNA-Cas9 complex remains associated with the DNA on the 5’ side of the cut site, resulting in low or no read coverage of the associated DNA.

   Compatible crRNA designs must have 3’ ends directed toward the target region as shown in the figure below. Fragments generated with the correct crRNA design orientation will not be encumbered by the presence of the gRNA-Cas9 complex in the ligation step following CRISPR-Cas9 digestion.

Figure 2. Illustration of four possible locations for crRNA designs for a generic target region. The two flanking crRNA designs for any target region must be located on opposite strands with the 3’ end of each design closest to the target region. This will also place the PAM site in-between the crRNA design and the target region. Designs crRNA1 and crRNA2 are compatible with the No-Amp targeted sequencing procedure whereas designs crRNA 3 and crRNA 4 are not (shown crossed-out) as the 5’ ends of these designs are closest to the target region with the PAM sites not adjacent to the target region.

In both tables below, the rows containing designs with compatible orientations are labeled in italicized, bolded text. Compatible “forward” designs generated for the first 500 bp of sequence before the target region are located on the sense strand (Table 1). Compatible “reverse” designs generated for the last 500 bp after the target region are located on the antisense strand (Table 2).

Table 1. *Forward crRNA designs from the first 500 bp of the 5 kb ATXN10 sequence.*

Orientation	sgRNA Cut Position (1-based)	sgRNA Sequence	PAM Sequence	Combined Rank
sense	***85***	***CCACATGAAGATCATAGATA***	***AGG***	***1***
antisense	218	GGGAGAACTGTAGACAAACC	TGG	2
sense	***59***	***ATTGGCTGTTATACCTACAT***	***AGG***	***3***
antisense	209	GTAGACAAACCTGGTGACCA	***CGG***	***4***
sense	***203***	***GCAGTTGCTTCTCAGGACCG***	***TGG***	***5***
antisense	74	CCTTATCTATGATCTTCATG	TGG	6
antisense	464	ATTTTTAAATTACTACACTC	***TGG***	***7***
sense	***165***	***TTTCTCCTAATAGCCCAGCC***	***AGG***	***8***
antisense	409	GGACAAATTCACAACTGACT	***AGG***	***9***
sense	***60***	***TTGGCTGTTATACCTACATA***	***GGG***	***10***
sense	***41***	***AAATTCTTCTAAACTTGCAT***	***TGG***	***11***
antisense	327	TTTATCTAATTTAATGGTTA	TGG	12
antisense	247	TTTGAATTTTAAGATATGTC	AGG	13
antisense	362	GCAAACAATTCTATGTCTTT	GGG	14
antisense	61	CTTCATGTGGCTCCCTATGT	AGG	15

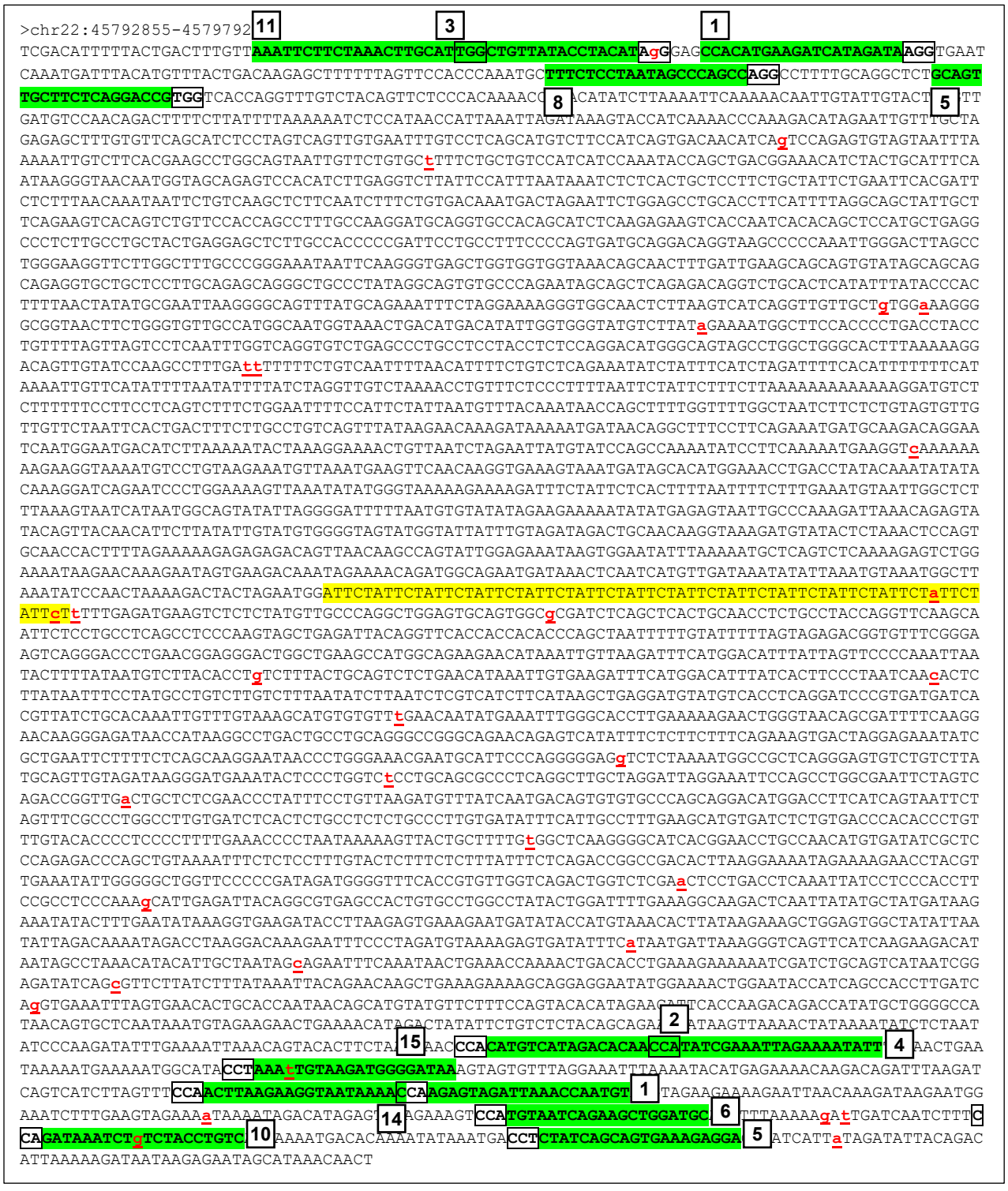
Table 2. *Reverse crRNA designs from the last 500 bp of the 5 kb ATXN10 sequence.*

Orientation	sgRNA Cut Position (1-based)	sgRNA Sequence	PAM Sequence	Combined Rank
antisense	***224***	***ACATTGGTTTAATCTACTCT***	***TGG***	***1***
antisense	***40***	***ATGGTTGTGTCTATGACATG***	***TGG***	***2***
sense	119	CATACCTAAATTGTAAGATG	GGG	3
antisense	***59***	***AATATTTTCTAATTTCGATA***	***TGG***	***4***
antisense	***425***	***TCCTCTTTCACTGCTGATAG***	***AGG***	***5***
antisense	***327***	***GCATCCAGCTTCTGATTACA***	***TGG***	***6***
sense	270	AGAATTAACAAAGATAAGAA	***TGG***	***7***
sense	204	TCTTAGTTTCCAACTTAAGA	AGG	8
antisense	***39***	***TGGTTGTGTCTATGACATGT***	***GGG***	***9***
antisense	***376***	***GACAGGTAGACAGATTTATC***	***TGG***	***10***
sense	435	ACCTCTATCAGCAGTGAAAG	AGG	11
sense	334	AAGTCCATGTAATCAGAAGC	TGG	12
sense	308	AAAATAAAATAGACATAGAG	TGG	13
antisense	***202***	***GTTTTATTACCTTCTTAAGT***	***TGG***	***14***
antisense	***112***	***TTATCCCCATCTTACAATTT***	***AGG***	***15***

In addition to being located on the appropriate strand, designs are further selected by their uniqueness following the combined rank order (green shaded cells in Tables 1 and 2 above). For example, in Table 1, designs with combined ranks 1, 3, 5 and 8 all occupy unique 20-bp positions on the sense strand and are all highlighted as potential designs. However, Design 10 almost entirely overlaps with Design 3; therefore, preference is given to Design 3. Lower ranked designs with minimal overlap with higher ranked designs are considered as a potential design as is the case with Design 11 which overlaps with Design 3 by 2 bp.

4. Map the selected crRNA designs that are both properly oriented and unique to the 5 kb target sequence in order to determine the presence of common SNPs in crRNA and downstream PAM sequences.

   The sequence below shows the five forward designs highlighted in green in Table 1 and the eight reverse designs highlighted in green in Table 2. PAM sites are enclosed within a box.



5. Select a final set of designs for testing which do not contain common SNPs. Carefully inspect all designs for the presence of common SNPs in the crRNA sequence and downstream PAM sequence. Of the forward designs for ATXN10, Design 3 has a SNP in the PAM site. Of the reverse designs for ATXN10, designs 10 and 15 have a single SNP in the crRNA sequence. All other designs present valid selections. Because not all crRNAs are equivalent in practice, choose several crRNA designs to be tested.

   We recommend testing the top 3 compatible designs as ranked by combined rank order from the forward (sense) and reverse (antisense) flanking orientations. For the ATXN10 target above, this would be Designs 1, 5 and 8 from the forward flanking design set and Designs 1, 2 and 4 from the reverse flanking design set.

Test crRNA Designs

It is advised to order and test several designs before choosing a final crRNA design set for a particular target. The crRNA designs can be tested with the No-Amp procedure or with a plasmid construct containing the flanking crRNA design sequences. This is a more efficient and economical testing model for a small number of targets as CRISPR-Cas9 digestion products are analyzed directly by agarose gel electrophoresis instead of sequencing.

We recommend ordering crRNA oligos from Integrated DNA Technologies (IDT) under the product offering “Alt-R® CRISPR-Cas9 crRNA”. The universal 67mer tracrRNA used in conjunction with crRNA to form gRNA is also ordered from IDT under the product offering “Alt-R® CRISPR-Cas9 tracrRNA”. These RNA oligos contain chemical modifications to protect from degradation by cellular RNases.

Revision History (Description)	Version	Date
Initial Release.	01	August 2019
Updated SNPs information on Page 2, selection criteria for crRNA Designs on Page 8 (‘Combined Rank’ order used), Updated Tables 1 and 2 on Page 9 and the figure on Page 10.	02	April 2020