CRISPR Genome Editing Made Easy Through the CHOPCHOP Website

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The design of optimal guide RNA (gRNA) sequences for CRISPR systems is challenged by the need to achieve highly efficient editing at the desired location (on-target editing) with minimal editing at unintended locations (off-target editing). Although laboratory validation should ideally be used to detect off-target activity, computational predictions are almost always preferred in practice due to their speed and low cost. Several studies have therefore explored gRNA-DNA interactions in order to understand how CRISPR complexes select their genomic targets. CHOPCHOP (*https://chopchop.cbu.uib.no/*) leverages these developments to build a user-friendly web interface that helps users design optimal gRNAs. CHOPCHOP supports a wide range of CRISPR applications, including gene knock-out, sequence knock-in, and RNA knock-down. Furthermore, CHOPCHOP offers visualization that enables an informed choice of gRNAs and supports experimental validation. In these protocols, we describe the best practices for gRNA design using CHOPCHOP. © 2021 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Design of gRNAs for gene knock-out
Alternate Protocol 1: Design of gRNAs for dCas9 fusion/effector targeting
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Basic Protocol 4: Design of gRNAs for knock-in using Cas9 nickases

Keywords: Cas9 • CRISPR • CRISPRi/a • gene knock-out • genome editing • guide design • knock-down • knock-in • nickase • RNA targeting

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INTRODUCTION

The CRISPR system allows for programmable targeting of genomes and transcriptomes, and has thus been repurposed for a range of applications in biological systems. At its core, CRISPR is a modular two-part system requiring a guide RNA (gRNA) and a Cas protein.

The gRNA is a sequence of ~100-nt that consists of two parts: one that interfaces with the Cas protein and one that guides the complex to its target. The latter is a recognition site of ~20 bp that contains the complementary sequence of its genomic or transcriptomic target. In addition, most Cas proteins require a specific sequence adjacent to the target. This is referred to as a protospacer adjacent motif (PAM) for DNA or a protospacer flanking sequence (PFS) for RNA. It is typically specific for each Cas protein. For example, the CRISPR system harvested from *Streptococcus pyogenes* uses a Cas9 protein requiring an NGG PAM motif. When the Cas protein recognizes its preferred PAM in the genome, the adjacent sequence can base-pair with the 20-bp recognition site of the gRNA. If base-pairing succeeds, the Cas protein will create a double-strand break (DSB) in the genomic DNA. Even though the complementary target of the gRNA's recognition site may be unique in the genome, base-pairing and PAM recognition are not perfect. Thus, there is a possibility that a gRNA will guide the Cas protein to off-target locations, and that DSBs will occur even when bulges and mismatches are present in these sites. Achieving efficient target recruitment and DSB generation at the intended locus is very important, but higher on-target efficiency often results in higher off-target frequency. Thus, computational tools are needed that can assess and balance high on-target efficiency and low off-target frequency.

CHOPCHOP (*https://chopchop.cbu.uib.no/*) was one of the first tools created to design and visualize gRNAs (Montague, Cruz, Gagnon, Church, & Valen, 2014). It was designed to be simple to operate without the need for expert knowledge but to be highly customizable for expert users. To this end, it provides preset options optimized for specific CRISPR applications and updates them regularly to include the most recent scientific advances. For expert users, it also provides a long and growing list of options for customizing how gRNAs are selected and ranked. Here, we explain the basic use of CHOPCHOP step by step, focusing on the most common pitfalls and errors users may experience. All protocols will result in a ranked list of gRNAs for a target of interest, visualized in a user-friendly interface. The results can be downloaded in multiple formats.

Strategic Planning explains how to make the best use of the CHOPCHOP output. It outlines general considerations for selecting an experimental mode that tailors CHOPCHOP towards your needs. Specifically, we highlight the need for your own evaluation of CHOPCHOP ranking and the need for validation. Basic Protocol 1 introduces the most basic use of CHOPCHOP to design a gRNA to create a DSB at a specific genomic location for a knock-out. Alternate Protocol 1 applies the same targeting principles for recruiting Cas9 effectors. A Support Protocol explains how to target transgenic or plasmid sequences. Basic Protocol 2 describes the use of the relatively recently discovered Cas13 to target RNA for knock-down. Basic Protocol 3 outlines a protocol for knock-in of specific sequences with homology or microhomology arms using homology-directed repair (HDR). Alternative Protocol 2 shows how to perform knock-in using non-homologous end joining (NHEJ) when this is preferred over HDR. Finally, Basic Protocol 4 describes knock-in using the nickase system, where two Cas proteins are recruited to the target site, thus minimizing off-target events at the expense of efficiency.

STRATEGIC PLANNING

CHOPCHOP aims to simplify gRNA design for CRISPR. It runs on a web browser, requires very few input parameters, provides genomes and annotations for hundreds of organisms, and provides an output with visualization of PCR primers and restriction enzymes sites. To use CHOPCHOP efficiently and correctly, some strategic planning of the experiment and expected outcomes is necessary. Here, we highlight the most

important considerations for basic gRNA design and give a brief overview of advanced options.

Before using CHOPCHOP, it is important to define your aim (e.g., knock-out of a specific gene) and the required experimental procedure. Depending on these choices, you will select the correct Cas protein and the application. These selections will load default presets that tailor the gRNA design and results output towards your specific needs. Experienced users can adjust these presets under "Options" (see Critical Parameters and the various protocols for specific examples).

It is important to familiarize yourself with your target of interest. Although CHOPCHOP provides an output that takes gene structure into account, prior knowledge ensures that you make the best use of the presented results. Before using CHOPCHOP, it is advised to study the gene for features important for the experimental outcome. Some considerations include:

What is more important in my experiment: achieving the best on-target efficiency or minimizing off-targets?

CHOPCHOP prioritizes off-targets first and then on-target efficiency.

Is it important to target one specific genetic locus or can a wider region be targeted?

Can multiple gRNAs be used in parallel?

One must remember that multiple gRNAs can cause very large deletions and chromosomal aberrations.

Is it likely that SNPs are present in the targeted region?

SNP databases can be checked if this is of concern.

Do I want to target one isoform of a gene or all possible isoforms?

For a knock-out, where are the functionally relevant domains of the protein?

For effector targeting, which regions would result in the largest effect (e.g., on expression)?

Could certain features of the target reduce the chances of a positive outcome?

For example, CpG islands can reduce on-target efficiency. It is possible to import results from CHOPCHOP to the UCSC Genome Browser to review such features.

For a knock-in, which repair strategy should be used (HDR vs. NHEJ)?

HDR is precise and efficient in cell cultures, but very limited in vivo and in non-reproducing cells. NHEJ can be more error-prone, but can be used in vivo and in difficult cell types such as neurons.

Would a nickase approach be a valuable alternative to a single-gRNA knock-in?

Nickases are thought to produce less off-target mutations, as single-strand breaks (SSBs) are efficiently repaired and the chance of having two SSBs through the two chosen gRNAs in close proximity is rather low. For more information, see Basic Protocol 4.

CHOPCHOP is designed to make CRISPR editing easy, but cannot replace proper experimental design or ensure that gRNAs are perfectly matched to the needs of the specific experiment. It is a good idea to familiarize yourself with your target and any potential overlapping genomic features. CHOPCHOP enables off-target searching, but does not scan for features that could affect neighboring genes or elements (e.g., overlapping genes, insulator elements, or enhancers). It is in the best interest of the experimenter to choose from the ranked results to find one that best fits the needs of the experiments.

Considerations for Gene Knock-outs

Important questions that should be considered are:

When targeting a region close to the start codon, are there in-frame start codons downstream (highlighted in green in CHOPCHOP) that could initiate and translate a truncated protein?

Are the most important functional domains downstream of the gRNA target site?

Could alternative splicing compensate for an introduced mutation?

Could the target site disrupt splicing?

Are any SNPs present in or close to the edit site?

Considerations for dCas9 Fusion/Effector Targeting

Targeting of Cas9 effectors follows the same principles as Cas9 targeting for genome editing, but the choice of target is typically different. In these experiments, it is often necessary to target a very specific region (e.g., a promoter, enhancer, or splice site), and screening with restriction enzymes and primers is not applicable. Instead, the location and efficiency of gRNA binding are the only considerations of importance. It is therefore advised to define the location and the acceptable window of recruitment around the desired locus before starting. To ensure efficient recruitment to the desired locus, one can consider using multiple gRNAs spaced a minimum a of ∼50 bp apart (Ran et al., 2013).

Considerations for Knock-ins

Knock-in strategies are summarized in Figure 1 and can be achieved by (1) using Cas9 to create DSBs (Fig. 1A) that are repaired by HDR or NHEJ, or (2) using Cas9 nickase to create pairs of SSBs (nicks; Fig. 1B), thereby increasing chances for HDR. Both options are presented in the protocols below.

Knock-ins using HDR

Knock-in with Cas9 and gRNAs follows the same principles as Cas9 targeting for genome editing, but additional parameters need to be used. One key aspect to consider when deciding which repair technique to use (HDR or NHEJ) is whether the modification will need to take place in dividing or nondividing cells. HDR is restricted to the S and G2 phases of the cell cycle, and thus unsuitable for knock-ins in nondividing cells (Nami et al., 2018). We describe how to design a knock-in on CHOPCHOP using either HDR (see Basic Protocol 3) or NHEJ (see Alternate Protocol 2). For HDR, the donor sequence has long homology arms (Fig. 1C,D) that allow for more accurate DSB repair.

An important point to note is that CRISPR enzymes will continue to cleave DNA if the gRNA target site/PAM site remains intact. This could be problematic when a specific mutation or sequence is introduced. We recommend that the template be designed to block further Cas9 targeting after the initial DSB is repaired. This is commonly done by mutating either the PAM sequence or the gRNA seed sequence.

Knock-ins using Cas9 nickases

The three most important parameters for this experiment are the choice of homology arms, the choice of nickase (D10A or N863A; Mali et al., 2013), and the optimal spacing of gRNAs (Ran et al., 2013). D10A Cas9 mutants are considered to perform slightly better in generating knock-ins compared to N863A mutants. In either case, the gRNAs should target both DNA strands and should be designed in "PAM-out" configuration, facing away from each other. These parameters are set by default for the CHOPCHOP nickase design and substantially restrict the choice of possible gRNA pairs. The distance between the two gRNAs is optimal between 25 and 100 bp, but can be adjusted
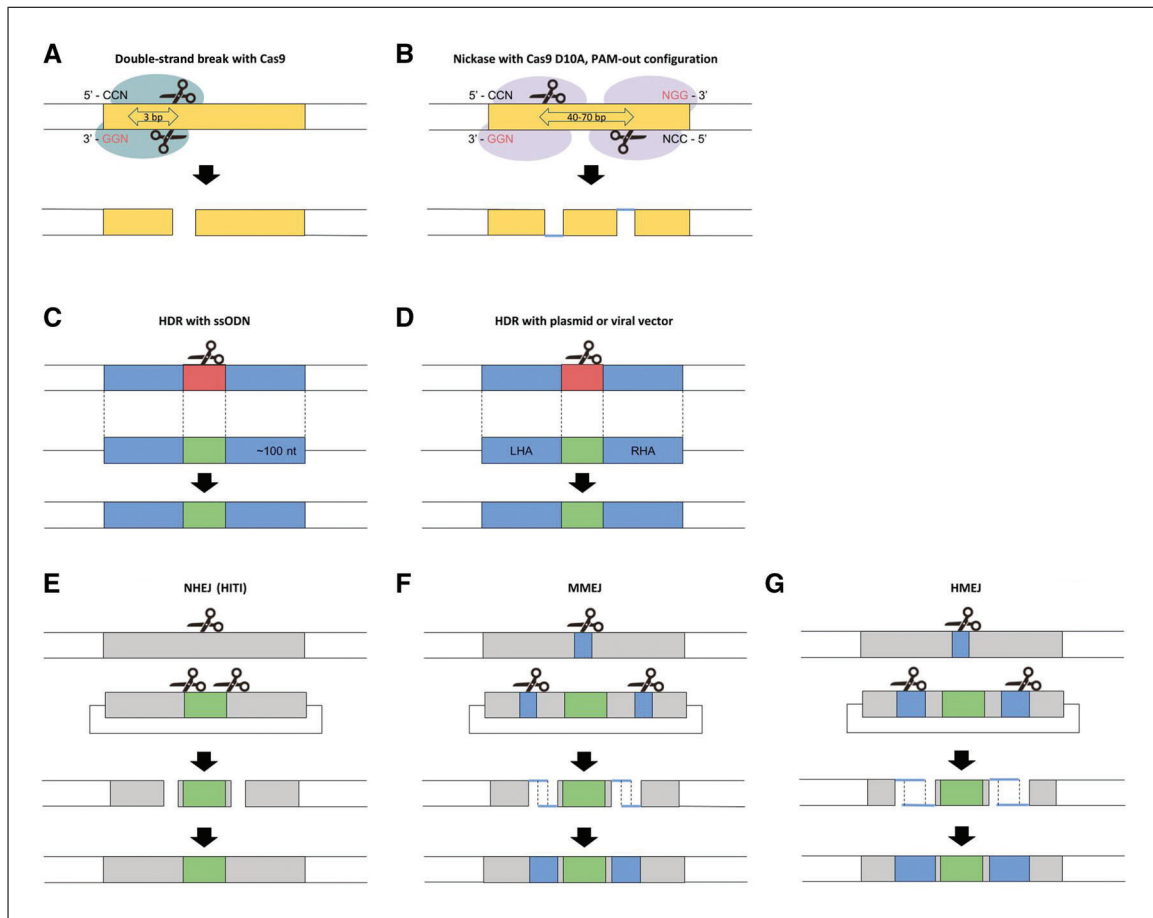
Figure 1 Overview of knock-in strategies. (**A**) Cas9 (green) creates a double-strand break ∼3 bp downstream of the gRNA's PAM. These genomic lesions can be used for knock-in the strategies C-G. (**B**) When using nickases, two gRNA targets in close vicinity are used. In this example, Cas9 RuvC mutant D10A (purple) produces a SSB (nick) on the targeting strand only. When two gRNA targets fall within an optimal distance (40-70 bp) and are positioned in the preferred PAM-out configuration, the two SSBs are recognized by the cellular DSB repair mechanism and can be exploited for knock-in strategies C, D, F, and G. (**C,D**) Gene correction by homology-directed repair (HDR) using a ssODN (**C**) or a plasmid or viral DNA vector (**D**) to provide the DNA donor. The former uses short homology arms (∼100 nt) whereas the latter generally uses longer homology arms (∼800 nt, LHA and RHA = left and right homology arms). (**E-G**) Knock-in methods using non-homologous end joining (NHEJ). (**E**) Donor plasmids in homology-independent targeted integration (HITI) lack homology arms, but include Cas9 cleavage sites flanking the donor sequence. (**F**) In microhomology-mediated end joining (MMEJ), an alternative NHEJ pathway is used involving annealing between identical microhomologous sequences (>2 bp) flanking the break. (**G**) Homology-mediated end joining (HMEJ) can use the HDR or NHEJ pathway to direct repair. The HMEJ construct contains homology arms similar to those used for HDR (∼800 nt) as well as CRISPR targets flanking the donor DNA similar to NHEJ. Figure adapted from Jang, Song, Hwang, & Bae (2020).

manually (Mali et al., 2013; Ran et al., 2013). For good overviews about these experimental considerations, see Internet Resources.

Validation

It is prudent to consider how you will validate your experiment. Primers flanking the targeted genomic region are useful for several validation approaches, and by default CHOPCHOP reports a set of primer pairs for each gRNA using Primer3 (Untergasser et al., 2012). Depending on your preferences, however, you might want to optimize the primer design parameters before searching for a gRNA. This can easily be accomplished by navigating to "Options" → "Primers". For knock-out and other genetic ablation strategies, a common approach for evaluating experiment efficiency uses restriction enzymes to target the sequence around the expected mutation. To aid with this, CHOPCHOP highlights
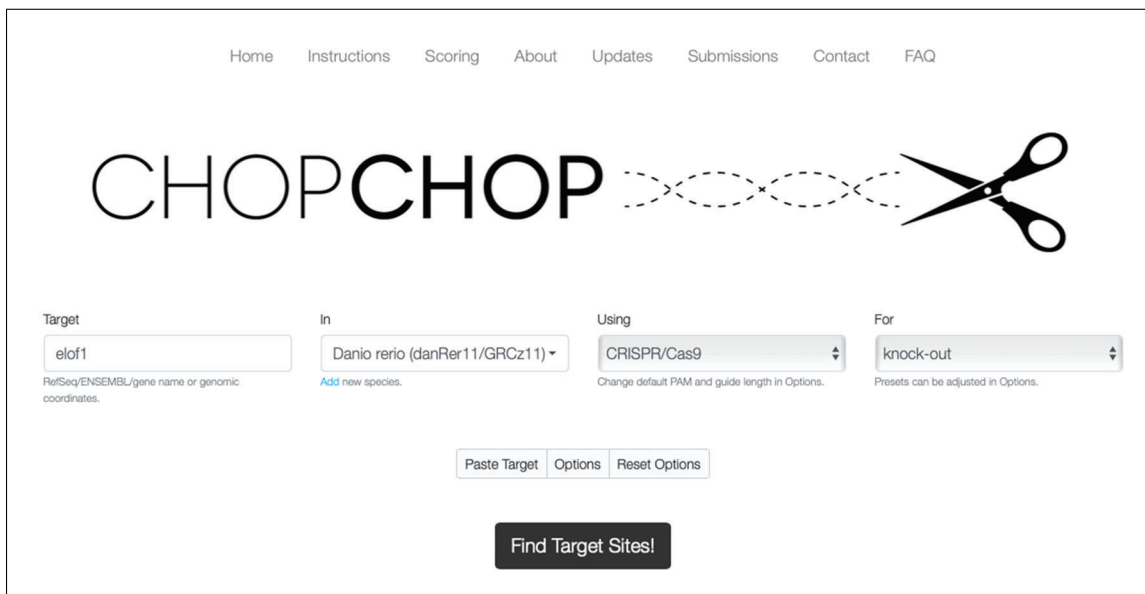
Figure 2 Home screen of CHOPCHOP website. Top: Links for additional information on advanced options ("Instructions"), how gRNA ranking is performed ("Scoring"), and general information about the tool ("About," "Updates"). Help and additional requests can be accessed through "Submission," "Contact" or "FAQ". Center: Main fields for gRNA design where one specifies the gene/transcript of interest ("Target") and selects the genome ("In") and the planned experiment ("Using," "For"). Immediately below, additional options can be specified using the grey buttons. Bottom: To perform the search, press the black "Find Target Sites!" button.

restriction enzyme sites around the gRNAs. The user can adjust the settings for these under "Options" → "General" → "Restriction Enzymes". Together, these options empower the user to select gRNAs that are both efficient and amenable to validation.

DESIGN OF gRNAs FOR GENE KNOCK-OUT

Due to its versatility, efficiency, and precision, the CRISPR system has superseded previous tools to modify genomic information (Gaj, Sirk, Shui, & Liu, 2016). The most common application is creating gene knock-outs through the introduction of double-strand breaks followed by repair through the NHEJ pathway. This protocol provides a basic outline of the considerations and steps necessary for the use of CHOPCHOP when targeting Cas9 to genes. For additional considerations, see Strategic Planning.

Materials

Computer, tablet, or phone
Modern browser with javascript enabled (e.g., Chrome, Firefox, Safari)

Perform search

1. Go to *https://chopchop.cbu.uib.no*.

2. Enter the gene of interest in the "Target" field.

    As an example, we use the zebrafish gene elof1 (Fig. 2). To avoid possible confusion due to ambiguous nomenclature, we recommend using RefSeq or ENSEMBL gene ID and not gene names when possible.

3. Choose the genome of interest in the "In" field.

    Here, we use the genome build Danio rerio (danRer11/GRCz11). The dropdown menu provides genomes from common model organisms as well as many others. Some organisms are supported with multiple genome versions. If your preferred organism is not present, click "Add new species" for a custom request.

elof1

5' → 3'
exon + ATG
intron
target

NM_200366

Scroll to zoom. Drag to move left and rig

14,498,200   14,498,000   14,497,800   14,497,600   14,497,400   14,497,200   14,497,000   14,496,800   14,496,600   14,496,400

Download results: Please select one

View in UCSC genome browser

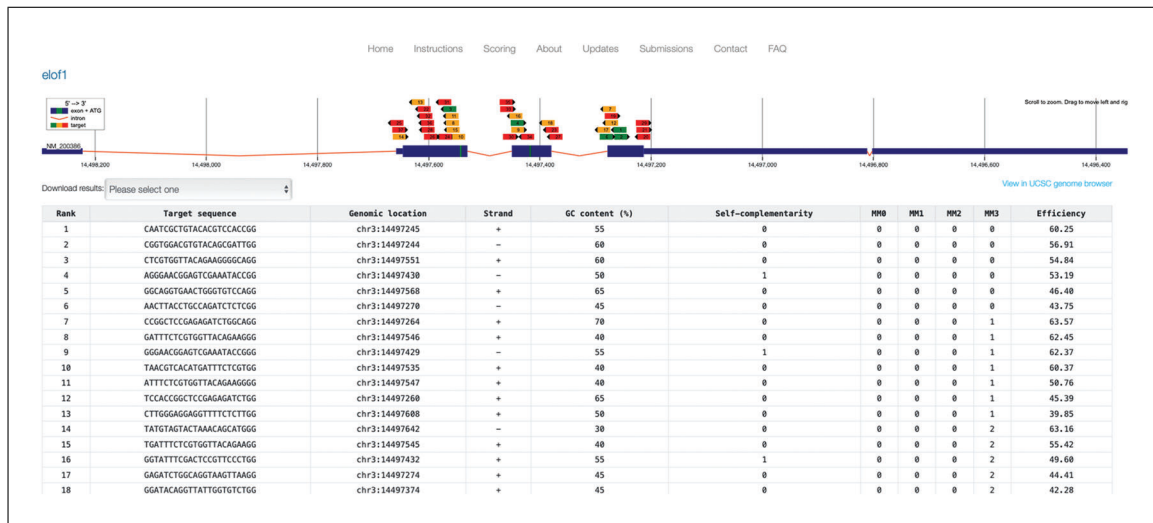| Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	CAATCGCTGTACACGTCCACCGG	chr3:14497245	+	55	0	0	0	0	0	60.25
2	CGGTGGACGTGTACAGCGATTGG	chr3:14497244	−	60	0	0	0	0	0	56.91
3	CTCGTGGTTACAGAAGGGCAGG	chr3:14497551	+	60	0	0	0	0	0	54.84
4	AGGGAACGGAGTCGAAATACCGG	chr3:14497430	−	50	1	0	0	0	0	53.19
5	GGCAGGTGAACTGGGTGTCCAGG	chr3:14497568	+	65	0	0	0	0	0	46.40
6	AACTTACCTGCCAGATCTCTCGG	chr3:14497270	−	45	0	0	0	0	0	43.75
7	CCGGCTCCGAGAGATCTGGCAGG	chr3:14497264	+	70	0	0	0	0	1	63.57
8	GATTTCTCGTGGTTACAGAAGGG	chr3:14497546	+	40	0	0	0	0	1	62.45
9	GGGAACGGAGTCGAAATACCGGG	chr3:14497429	−	55	1	0	0	0	1	62.37
10	TAACGTCACATGATTTCTCGTGG	chr3:14497535	+	40	0	0	0	0	1	60.37
11	ATTTCTCGTGGTTACAGAAGGGG	chr3:14497547	+	40	0	0	0	0	1	50.76
12	TCCACCGGCTCCGAGAGATCTGG	chr3:14497260	+	65	0	0	0	0	1	45.39
13	CTTGGGAGGAGGTTTTCTCTTGG	chr3:14497608	+	50	0	0	0	0	1	39.85
14	TATGTAGTACTAAACAGCATGGG	chr3:14497642	−	30	0	0	0	0	2	63.16
15	TGATTTCTCGTGGTTACAGAAGG	chr3:14497545	+	40	0	0	0	0	2	55.42
16	GGTATTTCGACTCCGTTCCCTGG	chr3:14497432	+	55	1	0	0	0	2	49.60
17	GAGATCTGGCAGGTAAGTTAAGG	chr3:14497274	+	45	0	0	0	0	2	44.41
18	GGATACAGGTTATTGGTGTCTGG	chr3:14497374	+	45	0	0	0	0	2	42.28

Figure 3 General results screen for gene knock-out using the gene *elof1* in the *Danio rerio* genome danRer11 as an example. On the top is the genome view of the gene with identified gRNAs. This view can be toggled left/right and zoomed in/out. The gene model is drawn with the name of each isoform. Thick blue boxes represent exons, thin blue boxes represent untranslated regions, and green lines indicates in-frame start codons. gRNAs are displayed as arrows, with colors matching the scoring. Directionality is indicated by black arrowheads. Immediately below the genome view are options to download all identified gRNA targets (left) or view the results in the UCSC Genome Browser (right). On the bottom is a ranked list of all gRNA targets found, with all features necessary to make an informed decision. Clicking on a gRNA in the list or the graphical representation opens a detailed results view (Fig. 4).

4. Choose "CRISPR/Cas9" in the "Using" field.

5. Choose "knock-out" in the "For" field.

6. *Optional:* Modify the default search based on needs using the "Options" button. For example:

> Select a specific gene feature to target (e.g., 5′-UTR or coding region) under "Options" → "General" → "Target specific region of gene".
> Specify a preferred set of restriction enzymes to validate mutations under "Options" → "General" → "Restriction enzymes".
> Specify preferences for primer design under "Options" → "Primers".
>
> *For information about other important options, see Critical Parameters.*
>
> *Targeting effector proteins for chosen genetic loci follows the same ranking logic, but different "Options" for target locus definition (see Alternate Protocol 1).*

7. Press "Find Target Sites!".

Interpret results

8. In the genome locus view of the results (Fig. 3, top), identify a gRNA that fits your purpose.

> *One can drag to navigate left and right along the genomic locus and scroll to zoom in on a specific genomic region.*

a. Consider your experimental needs. For example, choose a gRNA that targets splice sites, regions downstream of in-frame ATGs, or exons of important domains.

b. Consider the number of off-targets based on color coding (green indicates almost no off-targets, orange indicates some off-targets, and red indicates many off-targets).
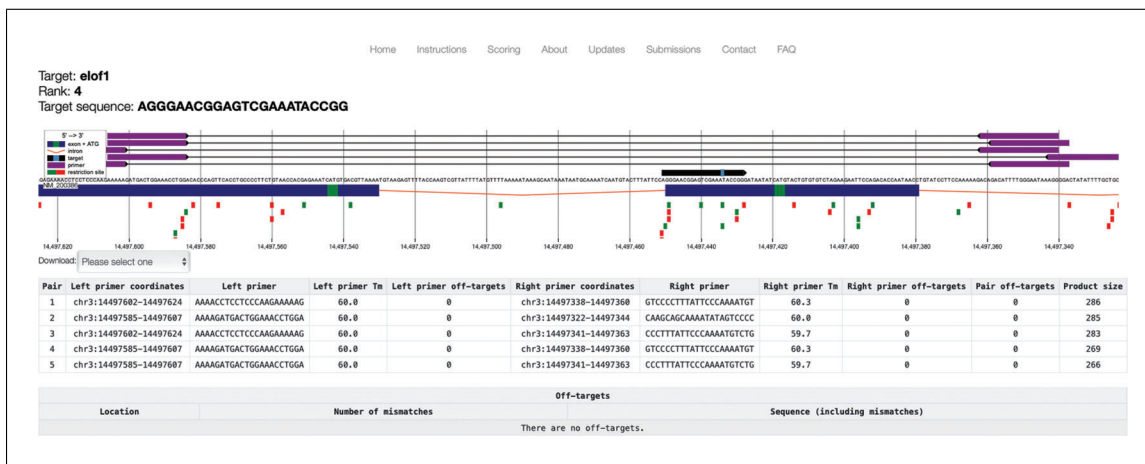
Figure 4 Detailed results view for gRNA rank 4 from Figure 3. The top-left corner summarizes details about the specific gRNA hit. Below this is a genome view of the specific target site. This view can be toggled left/right and zoomed in/out. Proposed primer pairs for validation are shown at the top (violet). Immediately below is the gRNA location and directionality (black arrow) with precise dsDNA cut site (light blue). Restriction enzyme sites that can used for mutation validation are also shown (red, green). The genome view is followed by the list of proposed primer pairs with their features, and a list with possible genome off-targets, including location, sequence, and possible mismatches (highlighted in red). In this example, no off-targets were found.

This may change with future updates, but is specifically described under the "Scoring" link.

    c. Consider the directionality of binding (indicated by black arrowheads).

    d. Consider placement of in-frame ATG start codons (green lines) to avoid re-initiation.

9. In the table view (Fig. 3, bottom), sort by clicking the header of any column.

The default ranking is based on the score. The score is defined primarily by number of off-targets, with efficiency as the second-most important feature. Depending on your preferences, the results can be sorted based on efficiency only, ignoring possible off-target effects.

10. Review all listed features of your preferred gRNAs. Make sure that the GC content fits your needs and that self-complementarity is not too high.

Self-complementarity represents 4-mer based complementary regions inside the gRNA and its scaffold which impede efficiency due to RNA folding. Low/high GC content is also known to lower gRNA efficiency. These features are supplementary and the efficiency score already accounts for these for many of the default models.

11. *Optional:* Download the complete list of proposed targets by choosing a preferred format in the "Download results" box (Fig. 3, top-left). Make sure your browser allows pop-up windows to open.

12. *Optional:* Transfer results to the UCSC Genome Browser by clicking on the link (Fig. 3, top-right).

This allows you to view the gRNA in relation to many other genomic features annotated in UCSC.

13. When a suitable candidate is found, click on the graphical representation or the table entry to open a detailed view.

By default, the detailed page will open in a new browser window, allowing detailed comparison of multiple candidates.

14. Inspect the detailed view (Fig. 4) for important considerations.

As an example, we use the gRNA ranked no. 4. The top of the detailed view lists the target, rank, and sequence of the gRNA. The target is shown in black, with the expected genome edit site highlighted in light blue.

a. Consider primer pairs that can be used for validation experiments (shown in violet).

   In the table view, each primer pair is listed with coordinates, sequences, melting temperatures, and product size. This information can be downloaded in Genbank or .tsv format.

b. Consider restriction sites (green and red) boxes.

   Moving the cursor over a restriction site will display the name of the enzyme. Before choosing a gRNA that modifies a restriction site, verify that the enzyme can be retrieved from your local source, and that it does not cut too often within the PCR product, making analysis impossible. To be used efficiently for experiment validation, the restriction site should be as close as possible to the expected mutation site.

c. Consider off-targets.

   Any possible off-targets are listed at the bottom of the table view, with genomic location and possible off-target mismatches highlighted in red (there are no off-targets in this example).

15. Once a preferred target has been found, copy the target sequence from the top left corner of the results page.

    Be aware that the displayed sequence includes the PAM sequence. The gRNA should NOT include the PAM, so the sequence must be modified before ordering. In our example in Figure 4, the target sequence is AGGGAACGGAGTCGAAATACCGG and the gRNA sequence is AGGGAACGGAGTCGAAATAC.

DESIGN OF gRNAs FOR dCas9 FUSION/EFFECTOR TARGETING

The CRISPR/Cas9 system can be used not only for creating breaks in the genome, but also to recruit effectors to specific genomic locations by fusing effector domains to Cas9 (Adli, 2018). The superior target specificity and the modular setup of the CRISPR system make it an ideal tool for fusion protein targeting. In these approaches, the Cas9 protein is mutated to be enzymatically inactive and not produce DNA breaks. This so-called dead Cas9 (dCas9) is fused with an effector domain targeted to a specific genomic location. These effectors can be transcription activators (Konermann et al., 2015), repressors (Yeo et al., 2018), fluorescent tags (Ye, Rong, & Lin, 2017), or other proteins (Berckman & Chen, 2019; Lei et al., 2017; Xu et al., 2016; Ribeiro, Ribeiro, Barreto, & Ward, 2018; Tsui et al., 2018; Vora, Tuttle, Cheng, & Church, 2016). Combining these Cas9 effectors with specific gRNAs will elicit the desired effect at the targeted genomic locus and not elsewhere.

1. Follow steps 1-4 as in Basic Protocol 1.

2. Select "activation" or "repression" in the "For" field.

   For effectors that do not fall into these categories, choose either of the two and adjust the options for target definition (see Basic Protocol 1, step 6) according to your needs.

3. Define a specific genomic range for recruiting the effector under "Options" → "General".

   Default for activation is 300 bp upstream and 0 bp downstream of the annotated TSS. Default for repression is 200 bp upstream and 200 bp downstream of the annotated TSS. These definitions can be adjusted based on your needs.

4. *Optional:* Modify other advanced options under "Options" (see Strategic Planning).

5. Press "Find Target Sites!" and proceed with interpretation as in Basic Protocol 1.

   Although the results display and interpretation are similar to Basic Protocol 1, the considerations for selecting a good gRNA differ. Here, position is typically most important (e.g., adjacent to or overlapping the promoter). A thorough understanding of possible alternative promoters and surrounding chromatin features (including insulators) is essential to making the right choice. Additionally, it can be advantageous to choose an array of gRNAs that are spaced throughout the promoter to maximize the desired effect. Finally, one should be prepared to accept lower-ranked gRNAs, as promoters present smaller targets with more limited options. These features can complicate the design of a highly specific and efficient gRNA.

SUPPORT PROTOCOL

DESIGN OF gRNAs FOR TARGETING TRANSGENIC OR PLASMID SEQUENCES

In the specific cases of targeting transgenic sequences in living organisms or designing gRNAs against plasmids, the user provides the sequence to search for targets and the choice of genome only applies to the off-target search.

1. Prepare the target sequence (plasmid or transgenic sequence) as a single FASTA file.

   CHOPCHOP considers only the first sequence for multi-FASTA files. Thus, a separate search is required for each sequence.

2. Go to *https://chopchop.cbu.uib.no*.

3. Press the "Paste Target" button (Fig. 2).

4. Copy the FASTA entry (including header) into the "Target" field.

5. Choose a genome for off-target search in the "In" field.

   The dropdown menu provides genomes of most relevant model organisms, including E. coli strains. If your experiment is done in vitro with pure DNA, choose a random genome and ignore the off-target results. Adjust ranking by clicking on the "Efficiency" column twice to sort results by the highest predicted editing efficiency.

6. Proceed as in Basic Protocol 1 beginning with step 4.

BASIC PROTOCOL 2

DESIGN OF gRNAs FOR RNA TARGETING

Design for RNA targeting with Cas13 (formerly C2C2) has proven useful for applications such as viral detection (Freije et al., 2019; Gootenberg et al., 2017) and temporary alteration of pathogenic mutations (Cox et al., 2017). CHOPCHOP supports identification of optimal gRNAs for targeting RNA through its RNA knock-down mode. In this mode, off-targets are identified not on the genome, but rather the transcriptome. CHOPCHOP still optimizes for GC content, self-complementarity, and off-targets, but also optimizes for local RNA structure. In these cases, the PAM sequence is replaced by its RNA equivalent, the protospacer flanking sequence (PFS).

Perform search

1. Visit the website *https://chopchop.cbu.uib.no*.

2. Enter the gene or transcript name or ID in the "Target" field.

   As an example, we use the human gene ext1. We recommend Ensembl/GENCODE gene IDs in this mode, as it is highly dependent on the annotations.

3. Choose the genome of interest in the "In" field.

   Here, we use the genome build Homo sapiens (hg38/GRCh38).

4. Choose "CRISPR/Cas13" in the "Using" field.

   By default, "knock-down" will appear in the "For" field.

5. *Optional:* Modify Cas effector settings under "Options" → "Cas13".

6. *Optional:* Modify general options under "Options" → "General" (see Critical Parameters).

7. Press the "Find Target Sites!" button.

Interpret results

8. In the genome locus view of the results (Fig. 3, as in Basic Protocol 1), identify a gRNA that fits your purpose.

   a. Consider the number of off-targets based on color coding (green indicates almost no off-targets, orange indicates some off-targets, and red indicates many off-targets).

   This may change with future updates, but is specifically described under the "Scoring" link.

   b. Consider the "Constitutive" column of the results table to determine whether a guide targets all isoforms of the gene.

   The constitutive column can have value 0 or 1, where 1 means the guide is targeting all isoforms of the gene. The columns IsoformsMM0 to IsoformsMM3 show which isoforms of the gene are being targeted by gRNA, with increasing number of mismatches. In our example, all three isoforms are targeted by the gRNA with 0 mismatch, indicated also by constitutive value 1.

   c. Consider the MM0, MM1, MM2, and MM3 columns.

   These count how many isoforms of the gene are targeted by a gRNA with an increasing number of mismatches (from 0 to 3). These columns do not count off-targets that belong to the family of isoforms of the targeted gene; these can be found in the IsoformMM0-MM3 columns.

   d. Consider the local structure score.

   Local structure influences on-target efficiency, as Cas13 binds preferentially to unstructured regions. The CHOPCHOP local structure score is computed using RNAfold from the ViennaRNA package. Accessibility in the RNA is calculated in windows of 70 nt and indicates the probability that a given nucleotide position in the transcript is unpaired and therefore accessible to a gRNA. For each target, we take the mean probability across each position. The smaller the value, the less likely there is secondary structure in the transcript at that gRNA target site.

9. In the table view, sort the gRNAs by clicking on any column header.

   Rank is defined primarily by number of off-targets, with local structure as the second-most important feature. Depending on your preferences, the results can be sorted based on local structure only, ignoring possible off-target effects.

10. Review all listed features of your preferred gRNAs. Make sure that the GC content fits your needs and that self-complementarity is not too high.

11. *Optional:* Download the complete list of proposed targets by choosing a preferred format in the "Download results" box. Make sure your browser allows pop-up windows to open.

12. *Optional:* Transfer results to the UCSC Genome Browser by clicking on the link.

    This allows you to view the gRNA in relation to many other genomic features annotated in UCSC.

13. When a suitable candidate is found, click on the graphical representation or results table entry to open a detailed view.

By default, the detailed page will open in a new browser window, allowing comparison of multiple candidates in more detail.

14. Inspect the detailed view for a full list of off-targets.

15. Once a preferred gRNA has been identified, copy the sequence next to "Guide (no PFS)" and use it when ordering gRNAs.

This sequence is displayed in the upper left of the detailed view, under the target sequence.

DESIGN OF gRNAs FOR SEQUENCE KNOCK-IN

The CRISPR/Cas9 system is commonly used to insert new sequences into a locus of interest. CRISPR knock-in allows site-specific gene engineering via programmable nuclease-induced DNA DSBs and subsequent repair by cellular repair pathways (both HDR and NHEJ). In order for the machinery to introduce a knock-in, a donor sequence must be provided. For additional considerations, see Strategic Planning.

Perform search

1. Go to *https://chopchop.cbu.uib.no*.

2. Enter the gene of interest in the "Target" field.

As an example, we use the zebrafish gene elof1.

3. Choose the genome of interest in the "In" field.

Here, we use the genome build Danio rerio (danRer11/GRCz11).

4. Choose "CRISPR/Cas9" in the "Using" field.

5. Choose "knock-in" in the "For" field.

6. *Optional:* Modify the default search based on needs using the "Options" button (see Strategic Planning and Critical Parameters).

7. Press "Find Target Sites!".

Interpret results

8. Identify a gRNA that fits your purpose and open the detailed view (see Basic Protocol 1, steps 8-13).

The detailed view is shown in Figure 5.

9. Inspect the detailed view for important considerations as described (see Basic Protocol 1, step 14).

10. Use the toggles (Fig. 5) to adjust the cut site respective to the PAM and the length of homology arms (20-800 bp).

Cas9 cuts 3 bp from the PAM sequence, but this offset varies for each Cas protein. Users should confirm the offset for the Cas of choice.

If longer arms are desired (up to 2 kb), the size of the flanking sequence must be changed in the initial search ("Options" → "General" → "Displayed flanking sequence length in detailed view").

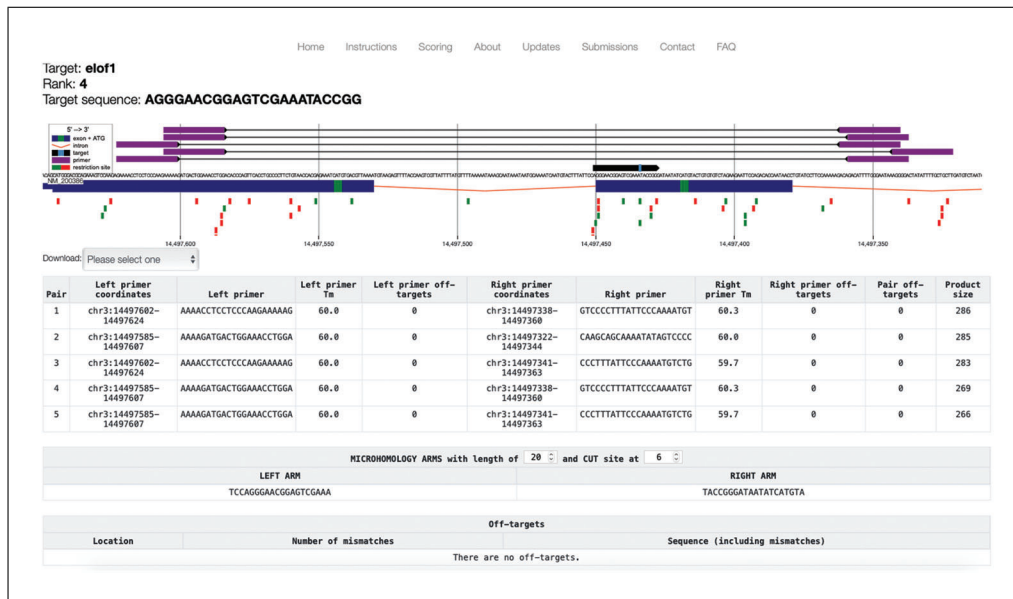*When choosing the HDR method and the length of homology arms, the following should be considered:*

Figure 5 Detailed results view for gRNA rank 4 for a knock-in experiment using the gene *elof1* in the *Danio rerio* genome danRer11. This is similar to the view for knock-out in Figure 4, and a detailed description can be found there. For knock-in experiments, the crucial parameters of homology arm length and cut site can be directly toggled and viewed on the results page. These parameters can be used to retrieve the homology arms of interest for your experiment.

> *HDR with single-stranded DNA oligonucleotides (ssODNs) is preferred for correction of one or a few mutations due to its low cost (thanks to the small size of sdODNs: 90-220 nt) and the relatively high associated editing efficacy. This uses homology arms of ∼100 bp. This strategy is easily applied at the cellular level, but not with in vivo corrections due to the lack of a delivery method.*
>
> *HDR for in vivo applications can be prepared as plasmids carrying the desired sequence flanked by long homology arms (typically 800 bp). This allows for precise insertion of large constructs at specific loci.*
>
> *Homology-mediated end joining (HMEJ) uses homology arms of ∼800 bp as well as CRISPR targets flanking the donor DNA, similar to those used for NHEJ (see Alternate Protocol 3). HMEJ allows target integration and has the highest reported efficiency among all existing strategies in cultured cells, animal embryos, and in vivo tissues. This new combination strategy allows repair to be mediated by either NHEJ or HDR, depending on the cell type.*

11. Once a preferred target has been found, copy the target sequence from the top left corner of the results page.

> *Be aware that the displayed target sequence includes the PAM, which should not be included in the gRNA sequence when ordered. In Figure 4, the target sequence is AGGGAACGGAGTCGAAATACCGG and the gRNA sequence is AGGGAACGGAGTC-GAAATAC.*

DESIGN OF gRNAs FOR KNOCK-IN USING NON-HOMOLOGOUS END JOINING

NHEJ relies not on DNA sequence homology, but rather ligation of broken DNA ends (Cubbon, Ivancic-Bace, & Bolt, 2018). This can be leveraged by introducing a plasmid containing a donor DNA construct to be integrated in the genomic DNA. In eukaryotes, NHEJ occurs predominantly in G1 phase and is inhibited during mitosis to prevent undesirable chromosome fusions at telomeres (Nami et al., 2018; Rees & Liu, 2018; Suzuki et al., 2016). NHEJ is an error-prone pathway that often creates insertions or deletions

of nucleotides (indels). Indels can cause frameshifts in protein coding regions, leading to truncation and inactivity after translation. This is the repair pathway used for knock-outs when no donor sequence is introduced.

For an NHEJ knock-in, the gRNA is designed as normal to target the site of interest. The donor DNA, however, is designed with flanking sites identical to the target so that Cas9 cleaves the genomic target sequence at one location and the donor plasmid at two locations (Fig. 1E-G). This releases a donor sequence between the flanking sites that contains the desired insert. The sequence should also lack homology arms so that repair of the Cas9-induced genomic DSB cannot occur through the HDR pathway. Instead, cleavage generates blunt ends at the target site and at both ends of the donor sequence. The linearized donor DNA sequence can then be used for repair by the NHEJ pathway, allowing for its integration into the genomic target site. Once incorporated into the genome, the donor DNA disrupts the Cas9 target sequence and prevents further Cas9-induced DSBs. If the target DSB is rejoined by error-free NHEJ without donor DNA insertion, the Cas9 target sequence will remain intact and a second round of Cas9 cutting will ensue.

1. Follow steps 1-9 as in Basic Protocol 3.

2. Unless HMEJ or MMEJ is desired, design your template with your donor DNA *without* homology arms but with the same Cas9 target sequence. Remember to also include PAM sequences. We recommend following one the following for design of donor sequences:

   a. For homology-independent targeted insertion (HITI), flank the donor sequence by one (for circular donor) or two (for linear donors) gRNA target sites.

   A third identical sequence in the reverse orientation is in the target locus in which the donor DNA is to be pasted. This allows for knock-in in the correct orientation, as intact gRNA target sequence persists if inserted in the reverse orientation (Suzuki & Izpisua Belmonte, 2018; Suzuki et al., 2016).

   b. For microhomology-mediated end joining (MMEJ), flank the donor sequence by the two microhomology arms. To avoid promoter interference, ensure that integration is in the opposite orientation of the endogenous gene.

   MMEJ relies on 5-25 bp of sequence complementarity (microhomology) between DNA strands. It can occur in most of the cell cycle, which leads to higher knock-in efficiency than HDR knock-in (Nakamae et al., 2017). The short arm lengths make it very easy to add in a single PCR without amplification from genomic DNA. MMEJ can perform seamless knock-in without carrying any of the target sequence from the gRNA. It requires three gRNAs, and the donor vector must include two different gRNA target sites.

DESIGN OF gRNAs FOR KNOCK-IN USING Cas9 NICKASES

Whether using HDR or NHEJ, knock-in approaches relying on DSB repair can have adverse effects on off-target loci. To reduce the risk of off-target genome edits and increase the chance of specific knock-in sequence insertions, one approach is to introduce SSBs flanking the site of insertion. Single nicks are usually efficiently corrected through cellular repair mechanisms without the introduction of mutations; however, two nicks in close vicinity can activate DSB repair pathways that can be leveraged for HDR-mediated knock-in (see Basic Protocol 3; Mali et al., 2013). This strategy can be implemented using Cas9 nickases (Trevino & Zhang, 2014). Cas9 nickases have one of the nuclease domains inactivated. The Cas9 D10A mutant cuts the DNA strand complementary to the gRNA (Fig. 1C), whereas the Cas9 N863A mutant cuts the opposite DNA strand. By using either of the nickases and designing a pair of gRNAs at optimal distance (Ran et al., 2013), one can exploit HDR-driven knock-in insertion. This strategy has the advantage of only allowing knock-in if both gRNAs introduce nicks at the same time on the same DNA.
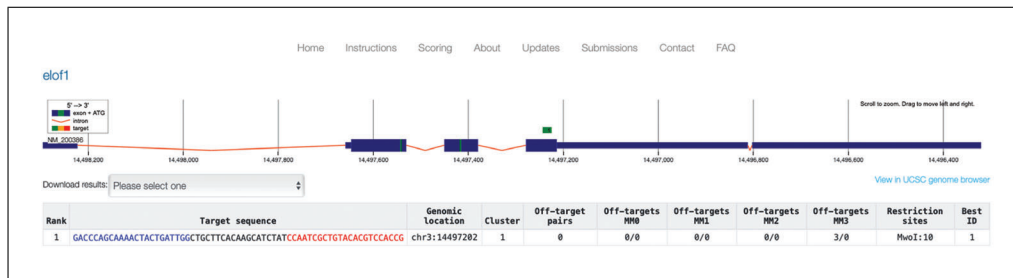
Figure 6 Results view for a Cas9 nickase knock-in experiment on zebrafish *elof1*. This view is similar to Figure 3, and a detailed description can be found there. In the knock-in approach, the "Target sequence" column of the table view shows gRNA pairs in blue and red.

Any single gRNA cut event (whether on- or off-target) is efficiently repaired, reducing the risk of off-target mutations. For additional considerations, see Strategic Planning.

1. Follow steps 1-3 as in Basic Protocol 3.

2. Choose "CRISPR/Cas9 nickase" in the "Using" field.

3. Choose "knock-in" in the "For" field.

4. *Optional:* Modify the default search based on your needs using the "Options" button. To modify the optimal distance between two gRNAs (and possible off-targets shown), go do "Options" → "Cas9 nickase" → "Distance between guides".

   This can be useful to restrict the results to only optimal matches or to allow possible suboptimal matches that fit closer to your desired genome locus. For details on optimal distance between guides, see Strategic Planning.

5. Press "Find Target Sites!".

6. In the genome locus view (Fig. 6), identify a gRNA pair that fits your purposes.

   The two gRNA targets are color-coded in blue and red (Fig. 6). CHOPCHOP only displays pairs with correct orientation and distance. CHOPCHOP only considers PAM-out configurations to be effective in HDR-driven knock-in experiments (Mali et al., 2013). The most important consideration is having an optimal distance between the gRNA DNA lesions (how to change this is outlined in step 4). However, genomic locus restrictions might make it necessary to adjust the predefined distance offset.

   The number of off-target hits for any single gRNA is less important in these experiments, as SSBs are expected to be efficiently repaired. This makes the on-target efficiency and gRNA pair distance the most important features for choosing the correct target pairs.

   For additional details on choosing gRNAs, see Basic Protocol 1. The cut site offset from the PAM should only be adjusted when an enzyme other than Cas9 is used.

7. After clicking on your preferred target, adjust the HDR homology arms according to your needs (Fig. 7).

   For additional details, see Basic Protocol 3. The cut site offset from the PAM should only be adjusted when an enzyme other than Cas9 is used.

COMMENTARY

Background Information

CHOPCHOP was one of the first tools for computational design of guide RNAs for CRISPR genome editing (Montague et al., 2014) and is now one of the most cited tools for design of gRNAs. Over time, support has been added for an increasing number of genomes, and the tool has been updated to stay current with developments in the field. CHOPCHOP supports a range of Cas proteins, targeting modes, and efficiency scoring methods (Labun, Montague, Gagnon, Thyme, & Valen, 2016, 2019).

Figure 7 Detailed results view for gRNA pair from Figure 6. This view is similar to Figure 4, and a detailed description can be found there. Note that the pair of gRNAs is shown in blue and red on the gene view and in the sequence at the top of the page. As for other knock-in experiments (Fig. 5), the homology arm sequences can be optimized and retrieved in the results view.

Critical Parameters

CHOPCHOP offers a large number of options. Most will not be of use for many users and are therefore hidden under the "Options" button on the home page. Nonetheless, users are advised to look through those options and familiarize themselves with the default settings so that results can be fine tuned if needed.

Each query on the website is required: genome/annotation ("In"), protein ("Using"), and specific application ("For"). CHOPCHOP applies a set of default parameters for each application (e.g., knock-out, knock-in, nanopore enrichment). If the settings are changed, they are saved to reflect the latest query run by the user. They can be returned to default by clicking the "Reset Options" button. This is also useful when you encounter an error on the website and need a fresh start for testing purposes.

CHOPCHOP currently supports more than 300 genomes. If your genome of interest is not provided or you prefer to use your own genomic sequence, you can submit your genome and annotations to the authors. For details, see the "Submissions" or "Add new species". Alternatively, to avoid waiting for the genome update, a local version of CHOPCHOP is available at *https://bitbucket.org/valenlab/chopchop* (requires basic bioinformatics skills). Local installation also supports bulk designs for many genes at once, unlike the main website where each query has to be run separately.

CHOPCHOP provides a list of the most popular PAM for the respective CRISPR protein selected. It also supports any custom PAM, written with IUPAC code. The PAM can be configured under "Options". For example, to use *Staphylococcus aureus* Cas9 targeting 21-bp-NNGRRT, select "CRISPR/Cas9" under "Using", to go "Options" → "Cas9", and change "PAM-3′" to NNGRRT and "sgRNA length without PAM" to 21. Similarly, for the alternative PAM located on the 5′ end of the gRNA, select "CRISPR/Cpf1 or CasX" and adjust the "5′-PAM" under "Options" → "Cpf1".

When targeting genes with multiple isoforms (e.g., ATF3 on hg38 has six isoforms), CHOPCHOP will by default compute the intersection of all isoforms and scan it for gRNAs. The intersection mode ensures that all isoforms will be targeted, but restricts the regions searched to exons that are common to all isoforms. If this is too restrictive, one can switch to the union mode under "Options" → "General" → "Isoform consensus determined by". The union mode will take the region of all exons of all isoforms of a gene. Although this is the most inclusive approach, it does not guarantee that the designed gRNA will target all of the isoforms at the same time, because exons represented in only one isoform are considered. In cases where annotations are ambiguous (e.g., isoforms are not on the same chromosome), CHOPCHOP will return an error. In that case, specific isoform of the

desired gene target (specified as a transcript ID) should be used.

In addition to gene name or gene identifiers, FASTA input genomic coordinates are also supported using the "Paste Input" button. This is provided in the form of chromosome:start-end (e.g., chr1:4512312-4512512). In cases where CHOPCHOP does not find a gene/isoform name, you can instead use sequence or location inputs. For multi-FASTA files, CHOPCHOP makes use of only the first sequence entry. When using FASTA input from transcripts, it is important to realize that, if exons are concatenated, gRNAs that cross over exon-exon borders will be reported. Therefore, one must make sure that the selected guide maps to the genome. When using FASTA from a sequence that is also in the genome, it is expected that the gRNA will have one perfect off-target at the position of the target site. To address this, CHOPCHOP will by default subtract one zero mismatch off-target for each gRNA. This can be adjusted by going to "Options" → "General" → "Fasta input" and checking "Color scoring should ignore one off-target without mismatches".

Troubleshooting

The PAM sequence (e.g., NGG) is always included in the general results table under "Target sequence" (hence, it is not named "gRNA sequence"). When ordering gRNA for your experiment, the PAM must be excluded so that only the gRNA sequence is ordered.

Finding working gene names depends on the annotations, and CHOPCHOP does not yet support auto-complete. If your gene is not found in our database of all genes, CHOPCHOP will output the following error: "Error 501: Your gene Id: yourgeneid has not been found in our database". Additionally, examples of gene names will be listed, e.g., SLC41A3 (ENST00000512470), VPS28 (ENST00000642606). In that case, consult Ensembl or RefSeq gene sets or genome browsers to find an identifier for your gene of interest.

Generally, CHOPCHOP includes instructions on what to do in case of an error. When an error is encountered, do not be discouraged by the red color. Simply read the error message and follow the instructions provided.

Time Considerations

CHOPCHOP deliver results for relatively short gene queries quickly: typically at most a couple of minutes. Additionally, CHOPCHOP keeps queries and their results for a couple days and returns results immediately when possible. If many users use the website at the same time, however, long waiting times may result: as long as an hour, depending on the size of the genes, the analyses performed, and demand during peak hours. In this case, you can bookmark the results page and return at a later date. Results will be waiting under the same link.

Author Contributions

Kornel Labun: Conceptualization, Writing-original draft, Writing-review & editing, Maximilian Krause: Writing-original draft, Writing-review & editing, Yamilla Torres Cleuren: Writing-original draft, Writing-review & editing, Eivind Valen: Funding acquisition, Writing-review & editing

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing not applicable – no new data generated.

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