

Paired Guide Design and Construction for Cas-CLOVER

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Two Main Options for Designing Guide-RNA Pairs for Cas-CLOVER

Option #1: Designing guide-RNA pairs for Cas-CLOVER

A variety of open-source gRNA design tools are available online for you: platforms that accommodate gRNA designs for paired Cas9 Nickase or single Cas9, such as [CRISPOR](#) and [CHOPCHOP](#) work well for CasCLOVER designs.

Option #2: Designing guide-RNA pairs for Cas-CLOVER manually

Using a variety of molecular biology software's to visualize and annotate sequences, a user can easily identify paired guides with a set of parameters.



mCherry

CRISPOR: Designing Guide-RNA Pairs for Cas-CLOVER

Pros:

- Allows for no genome to be selected
- Provides efficiency scoring based on two different promoters
- Displays inefficiency and high GC warnings

Cons:

- Cas9 single guides ONLY

No need for selecting a genome unless listed

Name Project

Copy and Paste Target Sequence

CRISPOR ([citation](#)) is a program that helps design, evaluate and clone guide sequences for the CRISPR/Cas9 system. [CRISPOR Manual](#)
Sep 2022: RIP Jean-Stéphane Joly 1967-2022 [Full list of changes](#)

Step 1

Planning a lentiviral gene knock-out screen? Use [CRISPOR Batch](#)

Sequence name (optional):

Enter a single genomic sequence, < 2300 bp, typically an exon

[Clear Box](#) - [Reset to default](#)

Paste here the genomic - not a cDNA - sequence of the exon you want to target. The sequence has to include the PAM site for your enzyme of interest, e.g. NGG. Maximum size 2300 bp. If you only have a cDNA, please BLAST or BLAT the cDNA first to find the right exon sequence for CRISPOR.

Text case is preserved, e.g. you can mark ATGs with lowercase.

Instead of a sequence, you can paste a chromosome range, e.g. chr1:11,130,540-11,130,751

Step 2

Select a genome

We have 917 genomes, but not yours? Search [NCBI assembly](#) and send a GCF_/GCA_ID to [CRISPOR support](#).

Step 3

Select a Protospacer Adjacent Motif (PAM)

See [notes on enzymes](#) in the manual.

SUBMIT

CasCLOVER uses deactivated SpCas9

CRISPOR Results: Viewing and Downloading Guide-RNAs for Cas-CLOVER

Interactive

Many useful download formats

No Genome selected, specificity scoring is deactivated
Note: There are no predicted off-targets below and all specificity scores are shown in red as their score is 0.
 Your input sequence is 711 bp long. It contains 139 possible guide sequences.
 Shown below are their PAM sites and the expected cleavage position located -3bp 5' of the PAM site.
 Click on a match for the PAM NGG below to show its 20 bp-long guide sequence. (Need help? Look at the [CRISPOR manual](#))
 Colors **green**, **yellow** and **red** indicate high, medium and low specificity of the PAM's guide sequence in the genome.

Input sequence not in genome, cannot show genome variants.

Position	0	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200		
Sequence	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCCGCCCTACGAGGGCACCCAGACCGCCAAGTGAAGGTGACCAAGGGTGGCCCTGCCCCTCGCCTTGCCTGGGACATCCTGTCCCTCAGT			---TGG	---AGG		---AGG	---TGG	---AGG	CCG---	CCA---	---AGG	---AGG	CCC---	CCC---	CCA---	---AGG	CCA---	---AGG	CCC---	CCT---	CCT---	CCC---
			CCA---					---AGG	---GGG	---CGG		---AGG	---GGG	CCG---	CCC---	CCA---	CCG---	---AGG	---GGG	---TGG	CCC---	CCC---	
								---GGG				---AGG	---GGG	CCT---	---AGG	CCG---	---AGG	---GGG	---TGG	CCT---	---GGG	CCT---	

Download for: [SerialCloner \(free\)](#) - [ApE \(free\)](#) - [GenomeCompiler](#) - [Benchling](#) - [SnapGene](#) - [Genelous](#) - [Vector NTI](#) - [LaserGene](#) - [Genbank](#) - [FASTA](#)

Predicted guide sequences for PAMs

Note: As there is no genome that can be used to get flanking sequence for your sequence, efficiency scores 50bp from the start or the end of your sequence cannot be calculated and are shown as "--". If needed, extend the input sequence and retry.

Ranked by default from highest to lowest specificity score ([Hsu et al., Nat Biot 2013](#)). Click on a column title to rank by a score.
 If you use this website, please cite our [paper in NAR 2018](#). Too much information? Look at the [CRISPOR manual](#).

Download as Excel tables: [Guides / Guides, all scores / Off-targets / Saturating mutagenesis assistant](#)

Sequence Positions (+/- strand) with PAM

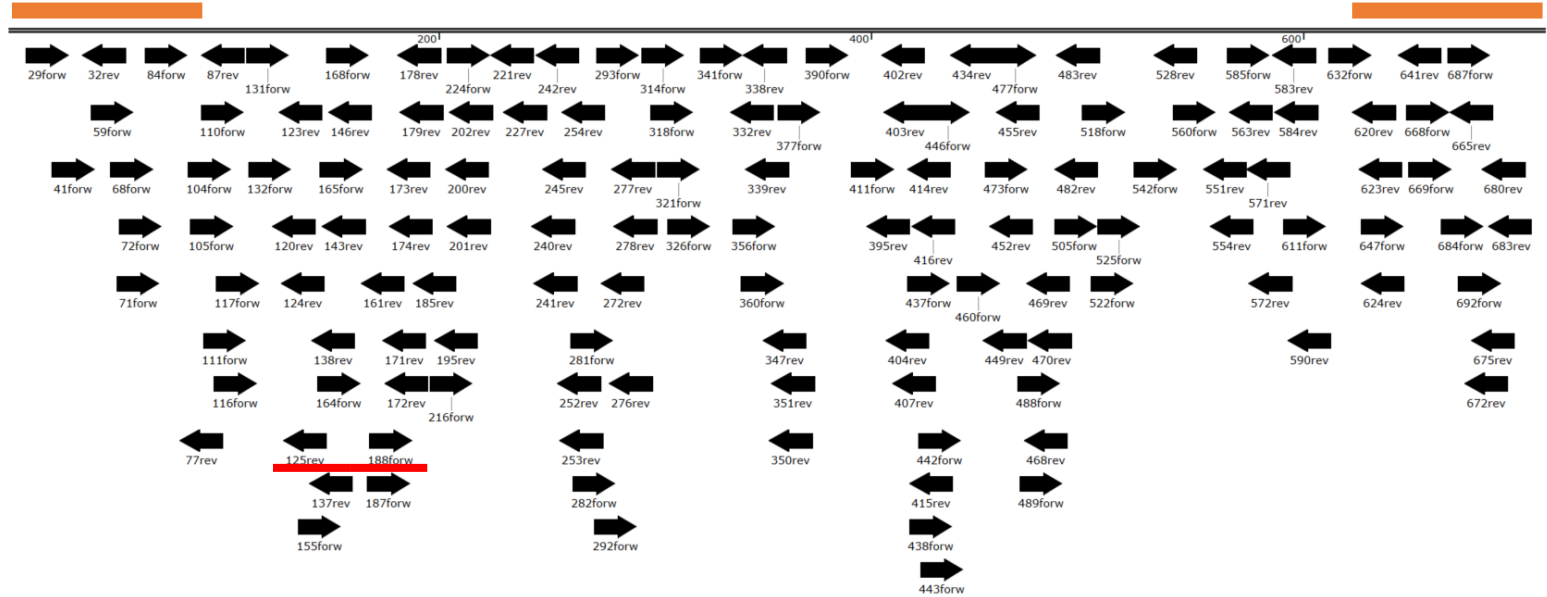
Position/ Strand	Guide Sequence + PAM + Restriction Enzymes	MIT Specificity Score	CFD Spec. score	Predicted Efficiency	Outcome	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score
	<input type="checkbox"/> Only G- <input type="checkbox"/> Only GG- <input type="checkbox"/> Only A-			Show all scores Doench '10 Mor-Mateos	Out-of-Frame Lindel		No exons. No match, no chrom filter
29 / fw	CAAGGGCGAGGAGGATAAICA TGG	No matches	No matches	--	--	Not found	

CRISPOR Results: Viewing Potential Excel and SnapGene gRNAs

Excel Table Download of Target

	A	B	C	D
1	# Name	Perfect Day		
2	# Sequenc	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCC		
3	# Genome	noGenome		
4	# PAM	NGG		
5	# Position	?		
6	# Version	CRISPOR 5.01, 2023-02-21T17:43:11CET		
7	# Results	http://crispor.org/crispor.py?batchId=8jX8Ck4Zh1S3rrNuSune		
8	#guidel	targetSeq	Doench '16-Score	Moreno-Mateos-Score
10	29forw	CAAGGGCGAGGAGGATAACATGG	NotEnoughFlankSeq	NotEnoughFlankSeq
11	32rev	GCGCATGAACCTCCTTGTATGATGG	NotEnoughFlankSeq	NotEnoughFlankSeq
12	41forw	GGATAACATGGCCATCATCAAGG	NotEnoughFlankSeq	NotEnoughFlankSeq
13	59forw	CAAGGAGTTCATGCGGTTCAAGG	50	50
14	68forw	CATGCGCTTCAAGGTGCACATGG	53	37
15	71forw	GCGCTTCAAGGTGCACATGGAGG	63	61
16	72forw	Cgcttcaagggtgcacatggaggg	51	41
17	77rev	CTGGAACTCGTGGCGGTTCAAGG	46	56
18	84forw	CACATGGAGGGCTCGGTGAAAGG	50	64
19	87rev	CGCCCTCGATCTCGAATCGTGG	58	30
20	104forw	CGGGCCAGAGTTCGAGATCGAGG	60	63
21	105forw	GGCCACGAGTTCGAGATCGAGGG	89	34
22	110forw	CGAGTTCGAGATCGAGGGCGAGG	53	81
23	111forw	GAGTTCGAGATCGAGGGCGAGGG	65	59
24	116forw	CGCATCGAGGGCGAGGGCGAGG	43	57
25	117forw	GAGATCGAGGGCGAGGGCGAGGG	54	66
26	120rev	TCTGGGTGCCCTCGTAGGGGCGG	55	67
27	123rev	CGGTCTGGGTGCCCTCGTAGGGG	59	72
28	124rev	GCGGTCTGGGTGCCCTCGTAGGG	40	79
29	125rev	GGCGGTCTGGGTGCCCTCGTAGG	41	80
30	131forw	GGGCGAGGGCGGCGCCCTACGAGG	52	75
31	132forw	GGCGAGGGCGGCGCCCTACGAGGG	56	71
32	137rev	CACCTTCAGCTTGGCGGCTCTGGG	32	71
33	138rev	TCACCTTCAGCTTGGCGGCTCTGG	31	33
34	143rev	CTTGGTCACCTTCAGCTTGGCGGG	53	56
35	146rev	ACCCTTGGTCACCTTCAGCTTGG	46	29
36	155forw	CACCCAGACCGCCAAAGCTGAAGG	55	40
37	161rev	GAAGGGCAGGGGGCCACCCCTTGG	40	70
38	164forw	CGCCAAGCTGAAGGTGACCAAGG	61	45
39	165forw	GCCRAAGCTGAAGGTGACCAAGGG	65	67
40	168forw	AAGCTGAAGGTGACCAAGGGTGG	59	69
41	171rev	TGTCCAGGGCGAAGGGCAGGGGG	53	49
42	172rev	ATGTCCAGGGCGAAGGGCAGGGG	41	63
43	173rev	GATGTCCAGGGCGAAGGGCAGGG	51	56
44	174rev	GGATGTCCAGGGCGAAGGGCAGGG	39	44
45	178rev	GACAGGATGTCCAGGGCGAAGGG	56	72
46	179rev	GGACAGGATGTCCAGGGCGAAGG	49	43
47	185rev	CTGAGGGGACAGGATGTCCACAGG	56	67
48	187forw	GTGGCCCGCTGCCCTTCGCCCTGG	28	29
49	188forw	TGGCCCGCTGCCCTTCGCCCTGGG	43	56

SnapGene Download of Target



Regions to avoid selecting gRNAs

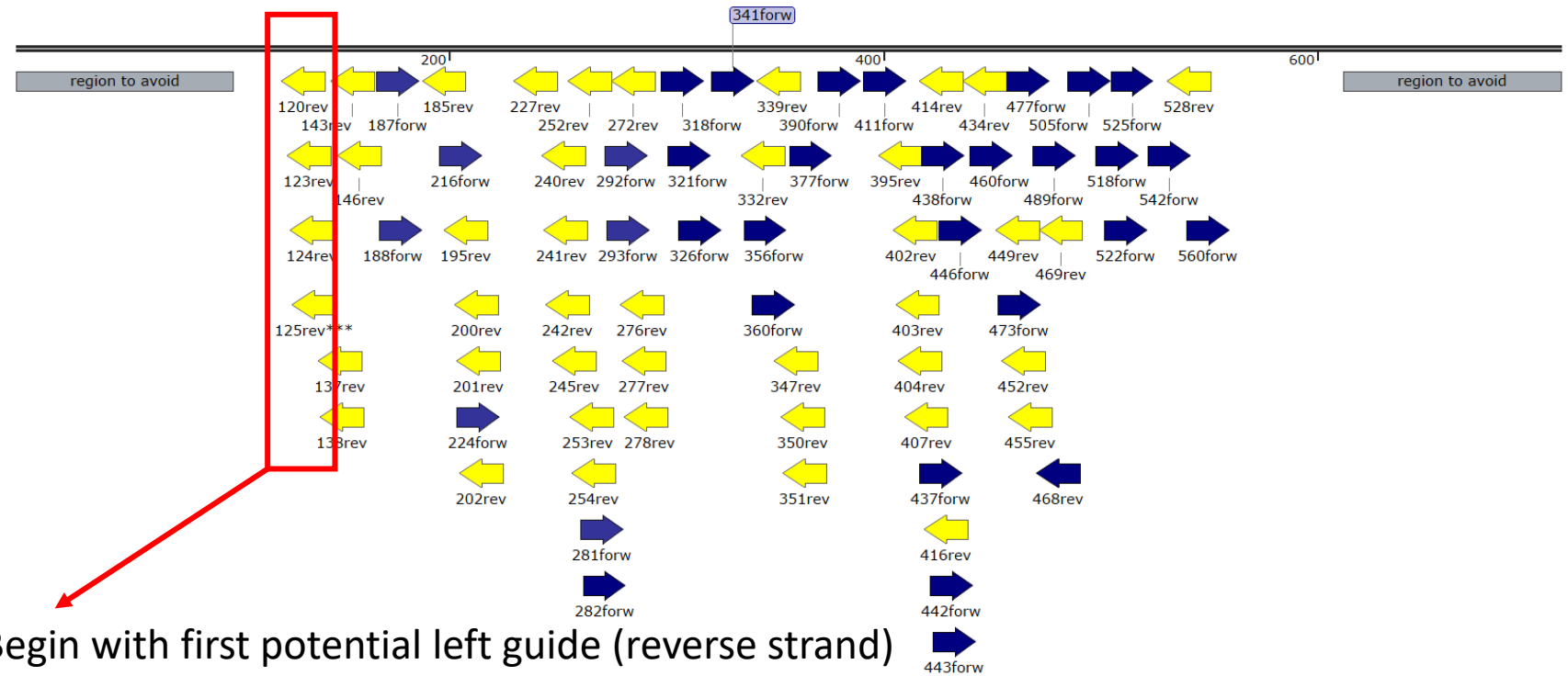
- 100bp region on the 5' and 3' of the target
- This will make analysis much easier if you are looking at the specific target edits
- This note can be discarded if only observing phenotype

CRISPOR Results: Filtering Potential Cas-CLOVER gRNAs

Filtered Excel Table Download of Target

	A	B	C	D
1	# Name	Perfect Day		
2	# Sequenc	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCC		
3	# Genome	noGenome		
4	# PAM	NGG		
5	# Position	?		
6	# Version	CRISPOR 5.01, 2023-02-21T17:43:11CET		
7	# Results	http://crispor.org/crispor.py?batchId=8jX8Ck4Zh1S3rrNuSune		
8	#guidel	targetSeq	Doench '16-Score	Moreno-Mateos-Score
10	29forw	CAAGGGCGAGGAGGATAACATGG	NotEnoughFlankSeq	NotEnoughFlankSeq
11	32rev	GCGCATGAACCTCCTTGATGATGG	NotEnoughFlankSeq	NotEnoughFlankSeq
12	41forw	GGATAACATGGCCATCATCAAGG	NotEnoughFlankSeq	NotEnoughFlankSeq
13	59forw	CAAGGAGTTCATGCGGTTCAAGG	50	50
14	68forw	CATGCGCTTCAAGGTGCACATGG	53	37
15	71forw	GCGCTTCAAGGTGCACATGGAGG	63	61
16	72forw	CgctTCAAGGTGCACATGGAGGG	51	41
17	77rev	CTGGAACTCGGTGGCGGTTCAAGG	46	56
18	84forw	CACATGGAGGGCTCGGTGAACGG	50	64
19	87rev	CGCCCTCGATCTCGAATCTGTTGG	58	30
20	104forw	CGGGCCAGGAGTTCGAGATCGAGG	60	63
21	105forw	GGCCACGAGTTCGAGATCGAGGG	89	34
22	110forw	CGAGTTCGAGATCGAGGGCGAGG	53	81
23	111forw	GAGTTCGAGATCGAGGGCGAGGG	65	59
24	116forw	CGAGATCGAGGGCGAGGGCGAGG	43	57
25	117forw	GAGATCGAGGGCGAGGGCGAGGG	54	66
26	120rev	TCTGGGTGCCCTCGTAGGGGCGG	55	67
27	123rev	CGGTCGGGTGCCCTCGTAGGGG	59	72
28	124rev	GCGGTCTGGGTGCCCTCGTAGGG	40	79
29	125rev	GGCGGTCTGGGTGCCCTCGTAGG	41	80
30	131forw	GGGCGAGGGCGGCCCTACGAGG	52	75
31	132forw	GGCGAGGGCGGCCCTACGAGGG	56	71
32	137rev	CACCTTCAGCTTGGCGGTCTGGG	32	71
33	138rev	TCACCTTCAGCTTGGCGGTCTGGG	31	33
34	143rev	CTTGGTCACCTTCAGCTTGGCGGG	53	56
35	146rev	ACCCTTGGTCACCTTCAGCTTGG	46	29
36	155forw	CACCCAGACCGCCAAAGCTGAAGG	55	40
37	161rev	GAAGGGCAGGGGGCCACCCCTTGG	40	70
38	164forw	CGCCAAGCTGAAGGTGACCAAGG	61	45
39	165forw	GCCRAAGCTGAAGGTGACCAAGGG	65	67
40	168forw	AAGCTGAAGGTGACCAAGGGTGG	59	69
41	171rev	TGTCCAGGGCGAAGGGCAGGGGG	53	49
42	172rev	ATGTCCAGGGCGAAGGGCAGGGGG	41	63
43	173rev	GATGTCCAGGGCGAAGGGCAGGG	51	56
44	174rev	GGATGTCCAGGGCGAAGGGCAGGG	39	44
45	178rev	GACAGGATGTCCAGGGCGAAGGG	56	72
46	179rev	GGACAGGATGTCCAGGGCGAAGG	49	43
47	185rev	CTGAGGGGACAGGATGTCCACAGG	56	67
48	187forw	GTGGCCCCCTGCCCTTCGCCCTGG	28	29
49	188forw	TGGCCCCCTGCCCTTCGCCCTGGG	43	56

SnapGene Download of Target



Begin with first potential left guide (reverse strand)

Initial Parameters

- 1) GC Content <80%
- 2) Efficiency → Highest possible (may not be applicable)
- 3) Location → Avoid 5' and 3' 100bp regions for analysis

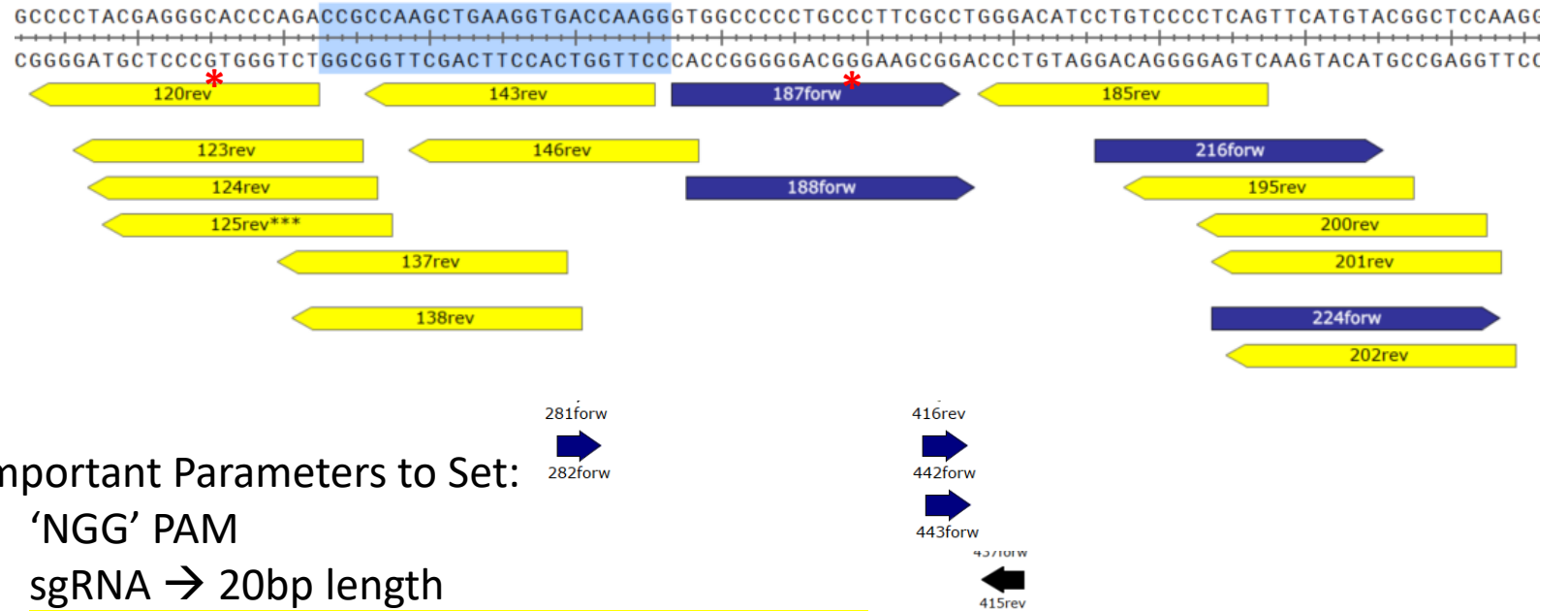
CRISPOR Results: Manually Finding gRNAs within Cas-CLOVER Parameters

Filtered Excel Table Download of Target

	A	B	C	D
1	# Name	Perfect Day		
2	# Sequenc	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCC		
3	# Genome	noGenome		
4	# PAM	NGG		
5	# Position	?		
6	# Version	CRISPOR 5.01, 2023-02-21T17:43:11CET		
7	# Results	http://crispor.org/crispor.py?batchId=8jX8Ck4Zh1S3rrNuSune		
8				
#guidel	targetSeq	Doench '16-Score	Moreno-Mateos-Score	
10	29forw	CAAGGGCGAGGAGGATAACATGG	NotEnoughFlankSeq	NotEnoughFlankSeq
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12	41forw	GGATAACATGGCCATCATCAAGG	NotEnoughFlankSeq	NotEnoughFlankSeq
13	59forw	CAAGGAGTTCATCGCGTTCAGGG	50	50
14	68forw	CATCGCGTTCAGGGTGCACATGG	53	37
15	71forw	GCGCTTCAGGGTGCACATGGAGG	63	61
16	72forw	CgctTCAGGGTGCACATGGAGGG	51	41
17	77rev	CTGGAACTCGGTGGCGGTTGACGG	46	56
18	84forw	CACATGGAGGGCTCGGTGACCGG	50	64
19	87rev	CGCCCTCGATCTCGAATCTCGTGG	58	30
20	104forw	CGGGCCAGAGTTGAGATCGAGG	60	63
21	105forw	GGCCACGAGTTCGAGATCGAGGG	89	34
22	110forw	CGAGTTCGAGATCGAGGGCGAGG	53	81
23	111forw	GAGTTCGAGATCGAGGGCGAGGG	65	59
24	116forw	CGACATCGAGGGCGAGGGCGAGG	43	57
25	117forw	GAGATCGAGGGCGAGGGCGAGGG	54	66
26	120rev	TCTGGGTGCCCTCGTAGGGGCGG	55	67
27	123rev	CGGTCTGGGTGCCCTCGTAGGGG	59	72
28	124rev	GCGGTCTGGGTGCCCTCGTAGGG	40	79
29	125rev	GGCGGTCTGGGTGCCCTCGTAGG	41	80
30	131forw	GGGCGAGGGCGGCCCTACGAGG	52	75
31	132forw	GGCCAGGGCGGCCCTACGAGGG	56	71
32	137rev	CACCTTCAGCTTGGCGGTCTGGG	32	71
33	138rev	TCACCTTCAGCTTGGCGGTCTGG	31	33
34	143rev	CTTGTACCTTCAGCTTGGCGGG	53	56
35	146rev	ACCCTTGGTCACTTCAGCTTGG	46	29
36	155forw	CACCCAGACCGCCAAAGCTGAAG	55	40
37	161rev	GAAAGGGCAGGGGGCCACCCCTGG	40	70
38	164forw	CGCCAAGCTGAAGGTGACCAAGG	61	45
39	165forw	GCCRAAGCTGAAGGTGACCAAGGG	65	67
40	168forw	AAGCTGAAGGTGACCAAGGGTGG	59	69
41	171rev	TGTCCAGGGCGAAGGGCAGGGGG	53	49
42	172rev	ATGTCCAGGGCGAAGGGCAGGGG	41	63
43	173rev	GATGTCCAGGGCGAAGGGCAGGG	51	56
44	174rev	GGATGTCCAGGGCGAAGGGCAGG	39	44
45	178rev	GACAGGATGTCCAGGGCGAAGGG	56	72
46	179rev	GGACAGGATGTCCAGGGCGAAGG	49	43
47	185rev	CTGAGGGGACAGGATGTCCACAG	56	67
48	187forw	GTGGCCCTCGCCCTTCGCCCTGG	28	29
49	188forw	TGGCCCTCGCCCTTCGCCCTGGG	43	56

The space between the left (yellow) and right (blue) guides must be within the 11-36bp range. Try to stay within 15-28 for highest success

Spacer region = 24bp



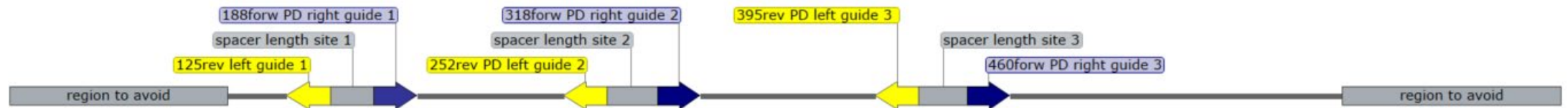
Important Parameters to Set:

- 'NGG' PAM
- sgRNA → 20bp length
- Cas-CLOVER allows for a very flexible spacer length between guides (11bp - 36bp)

CRISPOR Results: Manually Finding gRNAs within Cas-CLOVER Parameters

At Demeetra, we try to identify guide pairs from the 5' end to the 3' end. We will pick three guide pairs that target regions spaced apart from each other. Therefore, after the user has found their first guide pairs, start moving your way down the target sequence

The final guide pair selection should look like this where the target sites are throughout the target sequence and all spacer lengths are within optimal range.



Left guide sequence (bottom strand read right to left)

Site 1: GGCGGTCTGGGTGCCCTCGT

Site 2: AGGACAGCTTCAAGTAGTCG

Site 3: GGGCCGTCGGAGGGGAAGT

Right guide sequence (top strand read left to right)

Site 1: TGGCCCCCTGCCCTTCGCCT

Site 2: CGCGTGATGAACTTCGAGGA

Site 3: GCTGGGAGGCCTCCTCCGAG

CHOPCHOP: Designing Guide-RNA Pairs for Cas-CLOVER

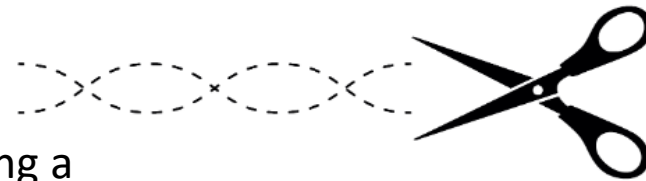
Pros:

- CRISPR/Cas9 Nickase option
- Self-complementarity scores
- Can set many different parameters

Cons:

- Have to select a genome
- Does not display any inefficiencies
- Cas9 Nickase guide pair results are not always consistent
- Few Cas Enzymes

CHOPCHOP



No need for selecting a genome unless listed

Target:
RefSeq/ENSEMBL/gene name or genomic coordinates.

In:
Add new species.

Using:
Change default PAM and guide length in Options.

For:
Presets can be adjusted in Options.

Click 'paste target' and then copy and paste target sequence

Paste Target Options Reset Options

Find Target Sites!

CHOPCHOP: Setting Parameters for Guide-RNA Pairs for Cas-CLOVER



Target: In: Using: For:

Paste Target Options Reset Options

Find Target Sites!

Click on options

Important Parameters to Set:

- 'NGG' PAM
- sgRNA → 20bp length
- Cas-CLOVER allows for a very flexible spacer length between guides (11bp - 36bp)

Target: In: Using: For:

sgRNA length without PAM:

PAM-3':
 NGG NAG NGA NRG (R = A or G) NNAGAAW (W = A or T) NNNNGMTT (M = A or C) NNGRRT (R = A or G)
 Custom PAM:

Method for determining off-targets in the genome:
 Off-targets with up to mismatches in protospacer (Hsu et al., 2013)
 Off-targets may have no more than mismatches in the protospacer seed region (Cong et al., 2013)

Efficiency score:
 Doench et al. 2014 - only for NGG PAM
 Doench et al. 2016 - only for NGG PAM
 Chari et al. 2015 - only for NGG and NNAGAAW PAM's in hg19 and mm10
 Xu et al. 2015 - only for NGG PAM, but can be used with other PAMs
 Moreno-Mateos et al. 2015 - only for NGG PAM
 G20

5' requirements for sgRNA:
 GN or NG
 GG
 No requirements

Self-complementarity (Thyme et al.):
 Check for self-complementarity
 I intend to replace the leading nucleotides with "GG"
 Check for complementarity versus backbone:

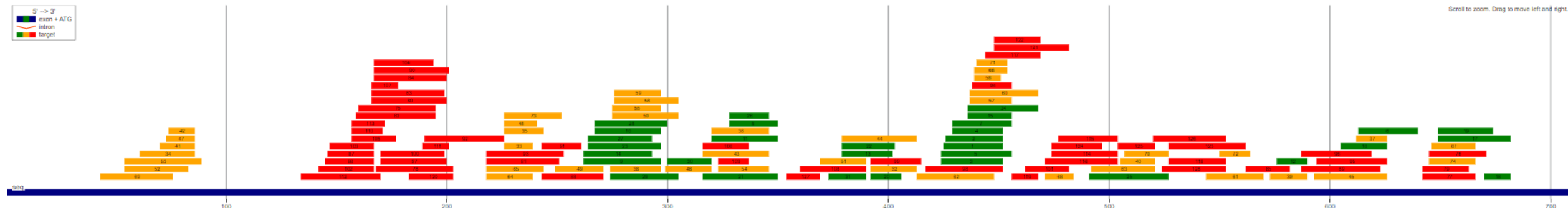
Standard backbone (AGGCTAGTCCGT) Extended backbone (AGGCTAGTCCGT,ATGCTGGAA) Custom backbone:

Distance between guides: to:

Max distance between offtargets:

CHOPCHOP: Viewing and Downloading Guide-RNA Pairs for Cas-CLOVER

fastalinput.fa



Download results:

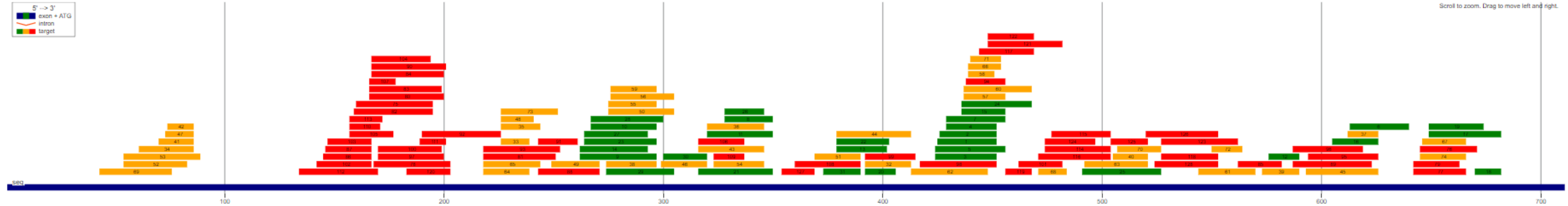
[View in UCSC genome browser](#)

Rank	Target sequence	Genomic location	Cluster	Off-target pairs	Off-targets MM0	Off-targets MM1	Off-targets MM2	Off-targets MM3	Restriction sites	Best ID
1	CCCTCCGACGGCCCGTAAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGGGGTACATCCGCTCGGAG	seq:403	75	0	0/0	0/0	0/0	0/0	BtgI:10 MboII:14,17 BbsI:12 BsaJI:10 StuI:24 PflMI:16 NcoI:10 BseYI:16 BslI:16 StyI:10	1
2	CCTCCGACGGCCCGTAAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGGGGTACATCCGCTCGGAG	seq:404	76	0	0/0	0/0	0/0	1/0	BtgI:9 MboII:16 BbsI:11 BsaJI:9 StuI:23 PflMI:15 NcoI:9 BseYI:15 BslI:15 StyI:9	2
3	GCATTACGGGCGCTCGAGGGGAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGGGGTACATCCGCTCGGAG	seq:402	73	0	0/0	0/0	0/0	1/0	BtgI:11 MboII:15,18 BbsI:13 BsaJI:11 StuI:25 PflMI:17 NcoI:11 BseYI:17 BslI:17 StyI:11	3
4	CCGACGGCCCGTAAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGGGGTACATCCGCTCGGAG	seq:407	77	0	0/0	0/0	0/0	2/0	BtgI:6 StuI:20 PflMI:12 BsaJI:6 NcoI:6 BseYI:12 BslI:12 StyI:6	5
5	GCATTACGGGCGCTCGAGGGGAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGAGCGGATGACCCCGAGGACGG	seq:402	74	0	0/0	0/0	0/0	1/1	BtgI:11 MboII:15,18 BbsI:13 BsaJI:11 StuI:25 PflMI:17 NcoI:11 BseYI:17 BslI:17 BseRI:18 StyI:11	7
6	CCAATTGATGTTGACGTTGACATCACCTCCCAACAGGAGTACACCATCGTGGAAACAGTACGAACGC	seq:591	120	0	0/0	0/0	0/0	0/0	BslI:18 BsaXI:22	8
7	CCGACGGCCCGTAAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGAGCGGATGATACCCCGAGGACGG	seq:407	78	0	0/0	0/0	0/0	2/1	BtgI:6 BseYI:12 PflMI:12 BsaJI:6 StuI:20 NcoI:6 BseRI:13 BslI:12 StyI:6	9
8	CCACGCCCGCTCCTCGAAGTTCGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCT	seq:306	57	0	0/0	0/0	0/0	0/1	HpyCH4III:6 PleI:10 MlyI:10 BstNI:13 BsaJI:11 Tsp45I:6 BseRI:9 StyD4I:11 ScrFI:13 PspGI:11 HinfI:16	10
9	CCCCGCCGACATCCCGACTACTTGAAGCTGTCTTCCCCGAGGGCTTCAAGTGGGAGCCGCTCCTCGAAGTTCATCACGCG	seq:240	36	0	0/0	0/0	0/0	3/1	BsoBI:16 AvaI:16 HpyAV:21 BsaJI:17	11
10	CCGACATCCCGACTACTTGAAGCTGTCTTCCCCGAGGGCTTCAAGTGGGAGCCGCTCCTCGAAGTTCATCACGCG	seq:245	39	0	0/0	0/0	0/0	3/1	BsoBI:11 AvaI:11 HpyAV:16 BsaJI:12	13
11	CCGTCCTCGAAGTTCATCACGCGGGCTGGTACCCTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCT	seq:298	53	0	0/0	0/0	0/0	1/1	HpyCH4III:14 PleI:18 MlyI:18 BstNI:21 BsaJI:19 Tsp45I:8,14 HphI:20 BstEII:8 BseRI:17 StyD4I:19 ScrFI:21 PspGI:19 HinfI:24	14
12	CCTACAAGCCCAAGAAGCCCGTCAAGCTGCCCGGCCCAACTTGCAGTGTGACGTTGTAG	seq:554	113	0	0/0	0/0	1/0	3/0	ApeKI:4 TseI:4 MspAII:4 NciI:9 PvuII:4 StyD4I:7 Fnu4HI:5 ScrFI:9	15
13	GGACGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCCCAACTTCCCTCCGACGGCCCGTAAATGCA	seq:357	64	0	0/0	0/0	0/0	2/0	ApeKI:6 TseI:6 BanI:13 Fnu4HI:7,12 NlaIV:15	16
14	CCCCGCCGACATCCCGACTACTTGAAGCTGTCTTCCCCGAGGGCTTCAAGTGGGAGCCGCTCCTCGAAGTTCATCACGCGCTCC	seq:240	35	0	0/0	0/0	0/0	3/2	BsoBI:16 AvaI:16 HpyAV:21 BsaJI:17	17
15	CCCCGTAAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGAGCGGATGATACCCCGAGGACGG	seq:414	79	0	0/0	0/0	0/0	4/1	BseRI:6 StuI:13 BseYI:5	19
16	CCCCGCCCTACAACGTCAACATCAAGTTGGACATCACCTCCCAACACGATGGTGTAGTCTCGTTG	seq:583	118	0	0/0	0/0	0/0	5/0	HphI:5	20

This is an interactive results page with many viewing and downloading options

CHOPCHOP: Viewing and Downloading Guide-RNA Pairs for Cas-CLOVER

fastalinput.fa



Download results:

[View in UCSC genome browser](#)

Rank	Target sequence	Genomic location	Cluster	Off-target pairs	Off-targets MM0	Off-targets MM1	Off-targets MM2	Off-targets MM3	Restriction sites	Best ID
1	CCCTCCGACGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGGGGTACATCCGCTCGGAG	seq:403	75	0	0/0	0/0	0/0	0/0	BtgI:10 MboII:14,17 BbsI:12 BsaJI:10 StuI:24 PflMI:16 NcoI:10 BseYI:16 BslI:16 StyI:10	1
2	CCTCCGACGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGGGGTACATCCGCTCGGAG	seq:404	76	0	0/0	0/0	0/0	1/0	BtgI:9 MboII:16 BbsI:11 BsaJI:9 StuI:23 PflMI:15 NcoI:9 BseYI:15 BslI:15 StyI:9	2
3	GCATTACGGGCGCTCGAGGGGAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGGGGTACATCCGCTCGGAG	seq:402	73	0	0/0	0/0	0/0	1/0	BtgI:11 MboII:15,18 BbsI:13 BsaJI:11 StuI:25 PflMI:17 NcoI:11 BseYI:17 BslI:17 StyI:11	3
4	CCGACGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGGGGTACATCCGCTCGGAG	seq:407	77	0	0/0	0/0	0/0	2/0	BtgI:6 StuI:20 PflMI:12 BsaJI:6 NcoI:6 BseYI:12 BslI:12 StyI:6	5
5	GCATTACGGGCGCTCGAGGGGAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGAGCGGATGACCCCGAGGACGG	seq:402	74	0	0/0	0/0	0/0	1/1	BtgI:11 MboII:15,18 BbsI:13 BsaJI:11 StuI:25 PflMI:17 NcoI:11 BseYI:17 BslI:17 BseRI:18 StyI:11	7
6	CCAATTGATGTTGACGTTGAGACATCACCTCCCAACGAGGACTACACCATCGTGGAAACAGTACGAACGC	seq:591	120	0	0/0	0/0	0/0	0/0	BslI:18 BsaXI:22	8
7	CCGACGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGAGCGGATGACCCCGAGGACGG	seq:407	78	0	0/0	0/0	0/0	2/1	BtgI:6 BseYI:12 PflMI:12 BsaJI:6 StuI:20 NcoI:6 BseRI:13 BslI:12 StyI:6	9
8	CCACGCCGCCCTCGAAGTTCGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCT	seq:306	57	0	0/0	0/0	0/0	0/1	HpyCH4III:6 PleI:10 MlyI:10 BstNI:13 BsaJI:11 Tsp45I:6 BseRI:9 StyD4I:11 ScrFI:13 PspGI:11 HinfI:16	10
9	CCCCGCCGACATCCCGACTACTTGAAGCTGTCTTCCCCGAGGGCTTCAAGTGGGAGCCGTCTCGAAGTTCATCACGCG	seq:240	36	0	0/0	0/0	0/0	3/1	BsoBI:16 AvaI:16 HpyAV:21 BsaJI:17	11
10	CCGACATCCCGACTACTTGAAGCTGTCTTCCCCGAGGGCTTCAAGTGGGAGCCGTCTCGAAGTTCATCACGCG	seq:245	39	0	0/0	0/0	0/0	3/1	BsoBI:11 AvaI:11 HpyAV:16 BsaJI:12	13
11	CCGTCCTCGAAGTTCATCACGCGGGCTGGTACCCTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCT	seq:298	53	0	0/0	0/0	0/0	1/1	HpyCH4III:14 PleI:18 MlyI:18 BstNI:21 BsaJI:19 Tsp45I:8,14 HphI:20 BstEII:8 BseRI:17 StyD4I:19 ScrFI:21 PspGI:19 HinfI:24	14
12	CCTACAAGCCCAAGAAGCCCGTCAGCTGCCCGGCCCAACTTGATGTTGACGTTGTAG	seq:554	113	0	0/0	0/0	1/0	3/0	ApeKI:4 TseI:4 MspAII:4 NciI:9 PvuII:4 StyD4I:7 Fnu4HI:5 ScrFI:9	15
13	GGACGCGGAGTTCATCAAGGTGAAGCTGCGCGGCCACCAACTTCCCTCCGACGGCCCGTAATGCA	seq:357	64	0	0/0	0/0	0/0	2/0	ApeKI:6 TseI:6 BanI:13 Fnu4HI:7,12 NlaIV:15	16
14	CCCCGCCGACATCCCGACTACTTGAAGCTGTCTTCCCCGAGGGCTTCAAGTGGGAGCCGTCTCGAAGTTCATCACGCGCTCC	seq:240	35	0	0/0	0/0	0/0	3/2	BsoBI:16 AvaI:16 HpyAV:21 BsaJI:17	17
15	CCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCCTCCCTCGAGCGGATGACCCCGAGGACGG	seq:414	79	0	0/0	0/0	0/0	4/1	BseRI:6 StuI:13 BseYI:5	19
16	CCCCGCCCTACAACGTCACATCAAGTTGGACATCACCTCCCAACACGATGGTGTAGTCTCGTTG	seq:583	118	0	0/0	0/0	0/0	5/0	HphI:5	20



Target Sequence includes left and right guides (blue and red, respectively) with the spacer region in between

This is an interactive results page with many viewing and downloading options

CHOPCHOP: Downloading and Filtering Guide-RNA Pairs for Cas-CLOVER

Remember Region to avoid ↓

IMPORTANT - CHOPCHOP results include the PAM sequence!

***Sequences are 5' to 3' and INCLUDE the PAM

Since Sequences are 5' to 3' you will notice that the left guide will begin with 'CCN' →

Sometimes you may see sequences that are not 'NGG' or 'CCN' →

We can now map these on SnapGene!

Rank	Left Guide	Spacer Region	Right Guide	Genomic location
69	CCTTGATGATGGCCATGTTATCC	AGTTCATGCGCTTCAAGGTGCACATGGAGGGCT	CCGTGAACGGCCACGAGTTTCGAG	21
52	CCATCATCAAGGAGTTCATGCGC	TTCAAGGTGCACATGGAGGGCTCCGTGAA	CCTCGATCTCGAACTCGTGGCCG	32
53	CCATCATCAAGGAGTTCATGCGC	TTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCA	CCTCGCCCTCGATCTCGAACTCG	32
34	CCTTGAAGCGCATGAACTCCTTG	TGCACATGGAGGGCTCCGTGAACGG	CCACGAGTTCGAGATCGAGGGCG	39
41	CCATGTGCACCTTGAAGCGCATG	AGGGCTCCGTGAACGG	CCACGAGTTCGAGATCGAGGGCG	48
47	GCGCTTCAAGGTGCACATGGAGG	GCTCCGTGAACGG	CCACGAGTTCGAGATCGAGGGCG	51
42	CCCTCCATGTGCACCTTGAAGCG	CTCCGTGAACGG	CCACGAGTTCGAGATCGAGGGCG	52
112	CCCTCGTAGGGGCGGCCCTCGCC	CACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGG	CCCCCTGCCCTTCGCCTGGGACA	112
102	CCGCCCTACGAGGGCACCCAGA	CCGCCAAGCTGAAGGTGACCAAGGG	CCCAGGCCAAGGGCAGGGGGCCA	120
86	CCCCTACGAGGGCACCCAGACCG	CCAAGCTGAAGGTGACCAAGGG	CCCAGGCCAAGGGCAGGGGGCCA	123
87	CCCTACGAGGGCACCCAGACCGC	CAAGCTGAAGGTGACCAAGGG	CCCAGGCCAAGGGCAGGGGGCCA	124
103	CCTACGAGGGCACCCAGACCGCC	AAGCTGAAGGTGACCAAGGG	CCCAGGCCAAGGGCAGGGGGCCA	125
105	CCTTCAGCTTGGCGGTCTGGGTG	TGACCAAGGGTGGCCCTG	CCCTTCGCCTGGGACATCCTGTC	135
110	CCTTCAGCTTGGCGGTCTGGGTG	TGACCAAGGGTGGC	CCCCTGCCCTTCGCCTGGGACAT	135
113	CCTTCAGCTTGGCGGTCTGGGTG	TGACCAAGGGTGGCC	CCCTGCCCTTCGCCTGGGACATC	135
82	CACCTTCAGCTTGGCGGTCTGGG	ACCAAGGGTGGCCCTGCCCTTCGCCTGGGACATC	CCGTACATGAACTGAGGGGACAG	137
75	TCACCTTCAGCTTGGCGGTCTGG	CCAAGGGTGGCCCTGCCCTTCGCCTGGGACATC	CCGTACATGAACTGAGGGGACAG	138
80	CCTTGGTCACCTTCAGCTTGGCG	GTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTC	CCCTCAGTTCATGTACGGCTCCA	144
83	CCTTGGTCACCTTCAGCTTGGCG	GTGGCCCCCTGCCCTTCGCCTGGGACATCCTGT	CCCTCAGTTCATGTACGGCTCC	144
107	CCTTGGTCACCTTCAGCTTGGCG	GTGGCCCCCTGC	CCTTCGCCTGGGACATCCTGTCC	144
84	CCCTTGGTCACCTTCAGCTTGGC	TGGCCCCCTGCCCTTCGCCTGGGACATCCTGTC	CCCTCAGTTCATGTACGGCTCCA	145
90	CCCTTGGTCACCTTCAGCTTGGC	TGGCCCCCTGCCCTTCGCCTGGGACATCCTGTC	CCTCAGTTCATGTACGGCTCCA	145
104	CCCTTGGTCACCTTCAGCTTGGC	TGGCCCCCTGCCCTTCGCCTGGGACAT	CCTGTCCCTCAGTTCATGTACG	145
78	CCAAGCTGAAGGTGACCAAGGGT	GGCCCCCTGCCCTTCGCCTGGGACATCCTGTCC	TCAGTTCATGTACGGCTCCAAGG	146
97	CCACCCCTGGTCACCTTCAGCTT	CCCCCTGCCCTTCGCCTGGGACATCCTGTC	CCCTCAGTTCATGTACGGCTCCA	148
100	CCACCCCTGGTCACCTTCAGCTT	CCCCCTGCCCTTCGCCTGGGACATCCTGT	CCCTCAGTTCATGTACGGCTCC	148
120	CCAAGGGTGGCCCCCTGCCCTC	GCCTGGGACATCCTGTCC	TCAGTTCATGTACGGCTCCAAGG	161

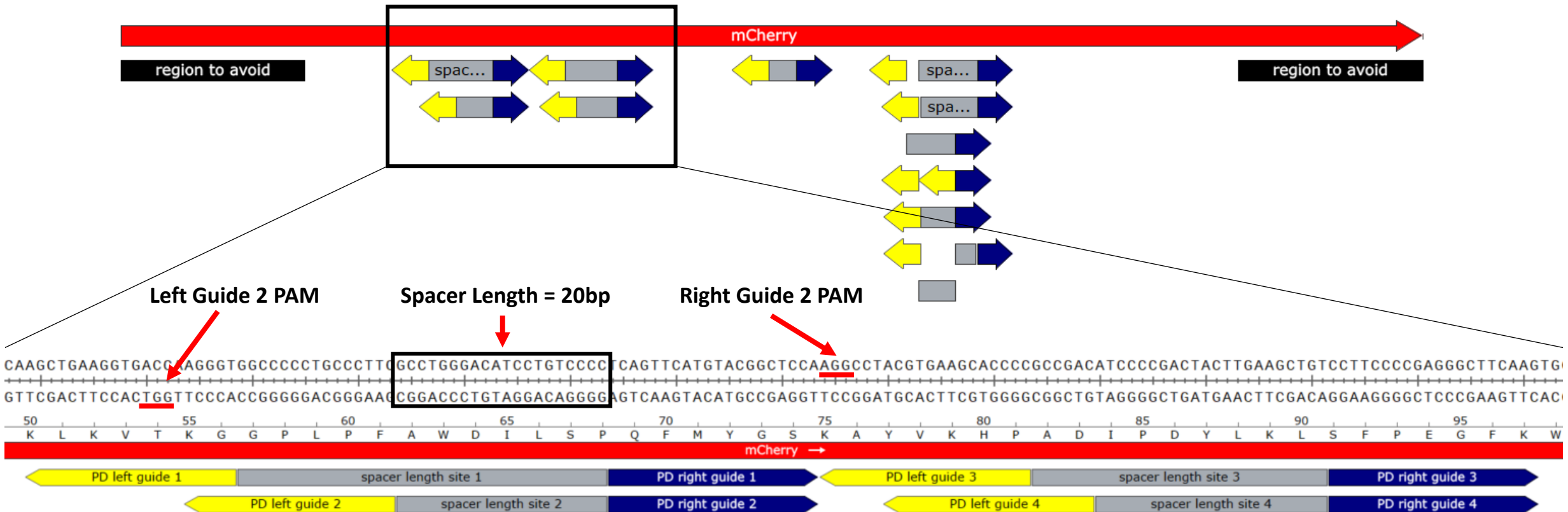
Excel Table Download of Perfect Day Target

Although CHOPCHOP results in 128 potential sgRNA pairs, the user will have to manually filter results depending on parameters set → **11 Final sgRNA pairs**

CHOPCHOP: Viewing and Selecting Guide-RNA Pairs for Cas-CLOVER

Take the results from Excel and import them into SnapGene → After the importing you will have a refined version like below.

	104	CCCTTGGTACACCTTCAGCTTGGC	IGGCCCCCTGCCCTTCGCCTGGGACAT	CCTGTCCCCTCAGTTCATGTACG	145
PD site 1	78	CCAAGCTGAAGGTGACCAAGGGT	GGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCC	TCAGTTCATGTACGGCTCCAAGG	146
	97	CCACCCCTTGGTCACCTTCAGCTT	CCCCCTGCCCTTCGCCTGGGACATCCTGT	CCCTCAGTTCATGTACGGCTCCA	148
	100	CCACCCCTTGGTCACCTTCAGCTT	CCCCCTGCCCTTCGCCTGGGACATCCTGT	CCCTCAGTTCATGTACGGCTCC	148
PD site 2	120	CCAAGGGTGGCCCCCTGCCCTTC	GCCTGGGACATCCTGTCCCC	TCAGTTCATGTACGGCTCCAAGG	161
	111	CCAGGCGAAGGGCAGGGGGCCAC	GACATCCTGTCC	CCTCAGTTCATGTACGGCTCCAA	167
	92	CCCAGGCGAAGGGCAGGGGGCCA	ACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGG	CCTACGTGAAGCACCCCGCCGAC	168



CHOPCHOP: Designing Guide-RNAs for Cas-CLOVER

CHOPCHOP can be used just like CRISPOR to identify single guide RNAs for use with Cas-CLOVER



Target Paste fasta entry here.

In [Add new species.](#)

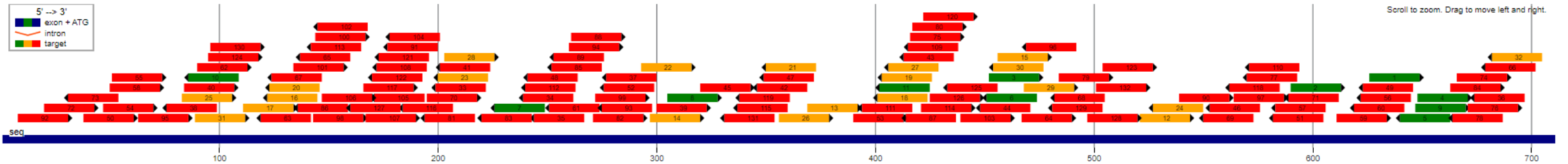
Using Change default PAM and guide length in Options.

For Presets can be adjusted in Options.

CHOPCHOP: Viewing and Downloading Guide-RNAs for Cas-CLOVER

CHOPCHOP can be used just like CRISPOR to identify single guide RNAs for use with Cas-CLOVER

fastalInput.fa



Download results:

[View in UCSC genome browser](#)

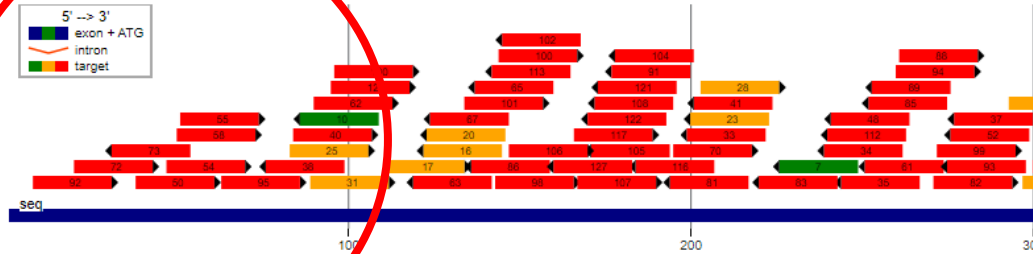
Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	CAACGAGGACTACCCATCGTGG	seq:627	+	55	0	0	0	0	0	73.83
2	CTACAACGTCAACATCAAGTTGG	seq:591	+	40	1	0	0	0	0	71.94
3	CTCCGAGCGGATGTACCCCGAGG	seq:453	+	70	0	0	0	0	0	67.34
4	GAACAGTACGAACGCGCCGAGGG	seq:649	+	60	0	0	0	0	0	67.16
5	GCGTTCGTACTGTTCCACGATGG	seq:641	-	55	1	0	0	0	0	67.53
6	CTCGGGGTACATCCGCTCGGAGG	seq:452	-	70	2	0	0	0	0	68.51
7	GTCGGCGGGGTGCTTCACGTAGG	seq:227	-	70	0	0	0	0	0	64.17
8	GAACTTCGAGGACGGCGCGTGG	seq:306	+	70	0	0	0	0	0	63.33
9	GGAACAGTACGAACGCGCCGAGG	seq:648	+	65	0	0	0	0	0	61.93
10	CGCCCTCGATCTCGAACTCGTGG	seq:87	-	65	1	0	0	0	0	58.50
11	TGCATTACGGGCGTCGGAGGG	seq:403	-	65	1	0	0	0	0	49.57
12	CGGCGGCCACTACGACGCTGAGG	seq:522	+	75	0	0	0	0	1	66.36
13	ATCTACAAGGTGAAGCTGCGCGG	seq:370	+	50	0	0	0	0	1	66.22
14	CGCGTGATGAACTTCGAGGACGG	seq:298	+	55	0	0	0	0	1	64.73
15	GAGCGGATGTACCCCGAGGACGG	seq:457	+	65	1	0	0	0	1	63.34

CHOPCHOP: Downloading and Filtering Guide-RNAs for Cas-CLOVER

The user will manually filter out guide choices by set parameters

*Regions to avoid

fastinput.fa



We can now map these on SnapGene!

Initial Parameters

- 1) GC Content <80%
- 2) Efficiency → Highest possible (may not be applicable)
- 3) Location → Avoid 5' and 3' 100bp regions for analysis
- 4) Self-Complementarity → lowest possible

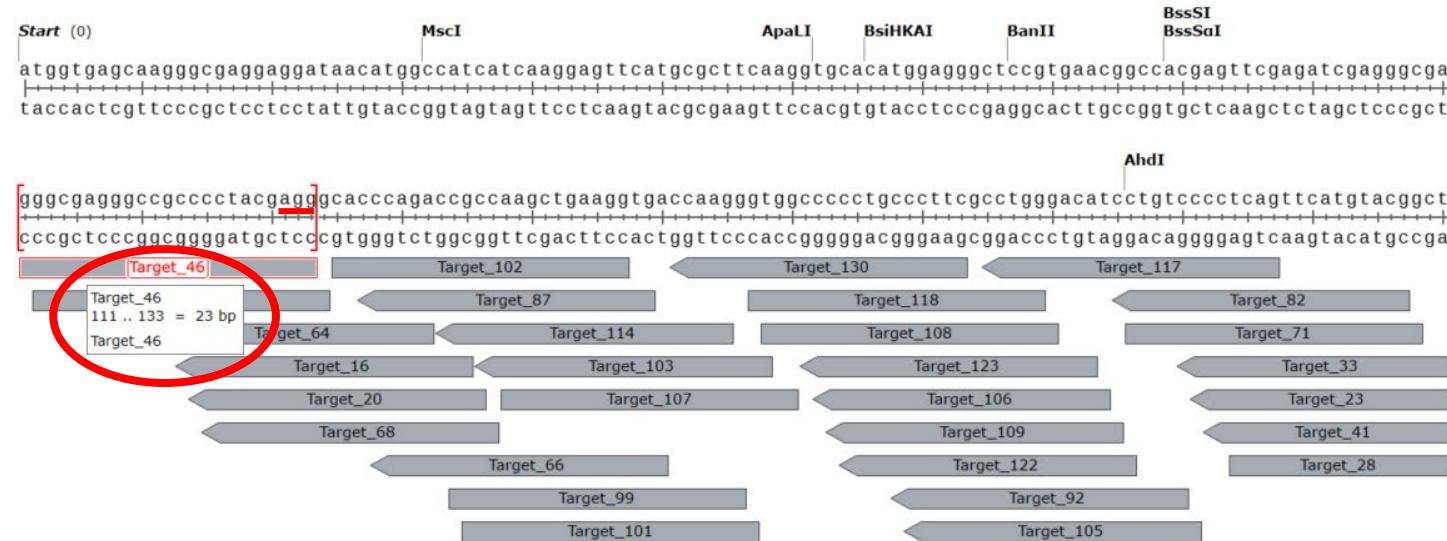
Begin with first potential left guide (reverse strand)
 - The highlighted are the first top two left guides

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	Efficiency
92	CAAGGGCGAGGAGGATAACATGG	9	+	55	0	46.7
72	GGATAACATGGCCATCATCAAGG	24	+	45	0	60.62
73	GCGCATGAACTCCTTGATGATGG	32	-	50	0	57.08
50	CAAGGAGTTCATGCGCTTCAAGG	39	+	50	0	50.38
54	CATGCGCTTCAAGGTGCACATGG	48	+	55	0	52.7
58	GCGCTTCAAGGTGCACATGGAGG	51	+	60	0	62.8
55	CGCTTCAAGGTGCACATGGAGGG	52	+	55	0	51.08
95	CACATGGAGGGCTCCGTGAACGG	64	+	60	5	56.24
38	CTCGAACTCGTGGCCGTTACGG	77	-	60	4	45.62
25	CGGCCACGAGTTCGAGATCGAGG	84	+	65	1	60.07
40	GGCCACGAGTTCGAGATCGAGGG	85	+	60	1	69.4
10	CGCCCTCGATCTCGAACTCGTGG	87	-	65	1	58.5
31	CGAGTTCGAGATCGAGGGCGAGG	90	+	65	1	52.59
62	GAGTTCGAGATCGAGGGCGAGGG	91	+	60	1	64.75
124	CGAGATCGAGGGCGAGGGCGAGG	96	+	75	0	43.2
130	GAGATCGAGGGCGAGGGCGAGGG	97	+	70	0	53.89
130	GAGATCGAGGGCGAGGGCGAGGG	97	+	70	0	53.89
63	TCTGGGTGCCCTCGTAGGGGCGG	120	-	70	2	54.73
16	CGGTCTGGGTGCCCTCGTAGGGG	123	-	70	1	58.51
20	GCGGTCTGGGTGCCCTCGTAGGG	124	-	70	1	39.58
67	GGCGGTCTGGGTGCCCTCGTAGG	125	-	75	0	41.22
101	CACCCAGACCGCCAAGCTGAAGG	135	+	65	0	54.81
86	CACCTTCAGCTTGGCGGTCTGGG	137	-	60	0	32.29
65	TCACCTTCAGCTTGGCGGTCTGG	138	-	60	0	30.65
113	CTTGTCACCTTCAGCTTGGCGG	143	-	55	0	53.38
98	CGCCAAGCTGAAGGTGACCAAGG	144	+	60	0	60.96
100	GCCAAGCTGAAGGTGACCAAGGG	145	+	55	0	65
102	ACCCTTGGTCACCTTCAGCTTGG	146	-	55	0	45.57
106	AAGCTGAAGGTGACCAAGGGTGG	148	+	55	0	59.23
127	GAAGGGCAGGGGGCCACCCTTGG	161	-	75	1	40.15
107	TGGCCCCCTGCCCTTCGCCTGGG	168	+	75	0	42.63

CHOPCHOP: Downloading and Filtering Guide-RNAs for Cas-CLOVER

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	Efficiency
92	CAAGGGCCGAGGAGGATAACATGG	9 ±	55	0	46.7	
72	GGATAACATGGCCATCATCAAGG	21 ±	45	0	60.62	
73	GCGCATGAACCTTGATGATGG	32 -	50	0	57.08	
50	CAAGGAGTTCATGCGCTCAAGG	39 ±	50	0	50.38	
54	CATGCGCTTCAAGGTGCACATGG	48 ±	55	0	52.7	
58	GCGCTTCAAGGTGCACATGGAGG	51 ±	60	0	62.8	
55	GCGCTTCAAGGTGCACATGGAGGG	52 ±	55	0	51.08	
95	CACATGGAGGGCTCCGTGAACGG	64 ±	60	5	56.24	
38	CTCGAACTCGTGGCCGTTACGG	77 -	60	4	45.62	
25	CGGCCACGAGTTCGAGATCGAGG	84 ±	65	1	60.07	
40	GGCCACGAGTTCGAGATCGAGGG	85 ±	60	1	69.4	
10	CGCCCTCGATCTCGAACTCGTGG	87 -	65	1	58.5	
34	CGAGTTCGAGATCGAGGGCCGAGG	90 ±	65	1	52.59	
62	GAGTTCGAGATCGAGGGCCGAGGG	91 ±	60	1	64.75	
124	CGAGATCGAGGGCCGAGGGCCGAGG	96 ±	75	0	43.2	
130	GAGATCGAGGGCCGAGGGCCGAGGG	97 ±	70	0	53.89	
130	GAGATCGAGGGCCGAGGGCCGAGGG	97 ±	70	0	53.89	
63	TCTGGGTGCCCTCGTAGGGGCGG	120 -	70	2	54.73	
16	CGGTCTGGGTGCCCTCGTAGGGG	123 -	70	1	58.51	
20	GCGGTCTGGGTGCCCTCGTAGGG	124 -	70	1	39.58	
67	GGCGGTCTGGGTGCCCTCGTAGG	125 -	75	0	41.22	
101	CACCCAGACCGCCAGCTGAAGG	135 +	65	0	54.81	
86	CACCTTCAGCTTGGCGGTCTGGG	137 -	60	0	32.29	
65	TCACCTTCAGCTTGGCGGTCTGG	138 -	60	0	30.65	
113	CTTGGTCACCTTCAGCTTGGCGG	143 -	55	0	53.38	
98	CGCCAAGCTGAAGGTGACCAAGG	144 +	60	0	60.96	
100	GCCAAGCTGAAGGTGACCAAGGG	145 +	55	0	65	
102	ACCCTTGGTCACCTTCAGCTTGG	146 -	55	0	45.57	
106	AAGCTGAAGGTGACCAAGGGTGG	148 +	55	0	59.23	
127	GAAGGGCAGGGGCCACCCCTTGG	161 -	75	1	40.15	
107	TGGCCCCCTGCCCTTCGCCTGGG	168 +	75	0	42.63	

CHOPCHOP allows you to download results to a GenBank file which will allow you to import the guides into SnapGene...But beware CHOPCHOP includes the PAM sequence in their guides!



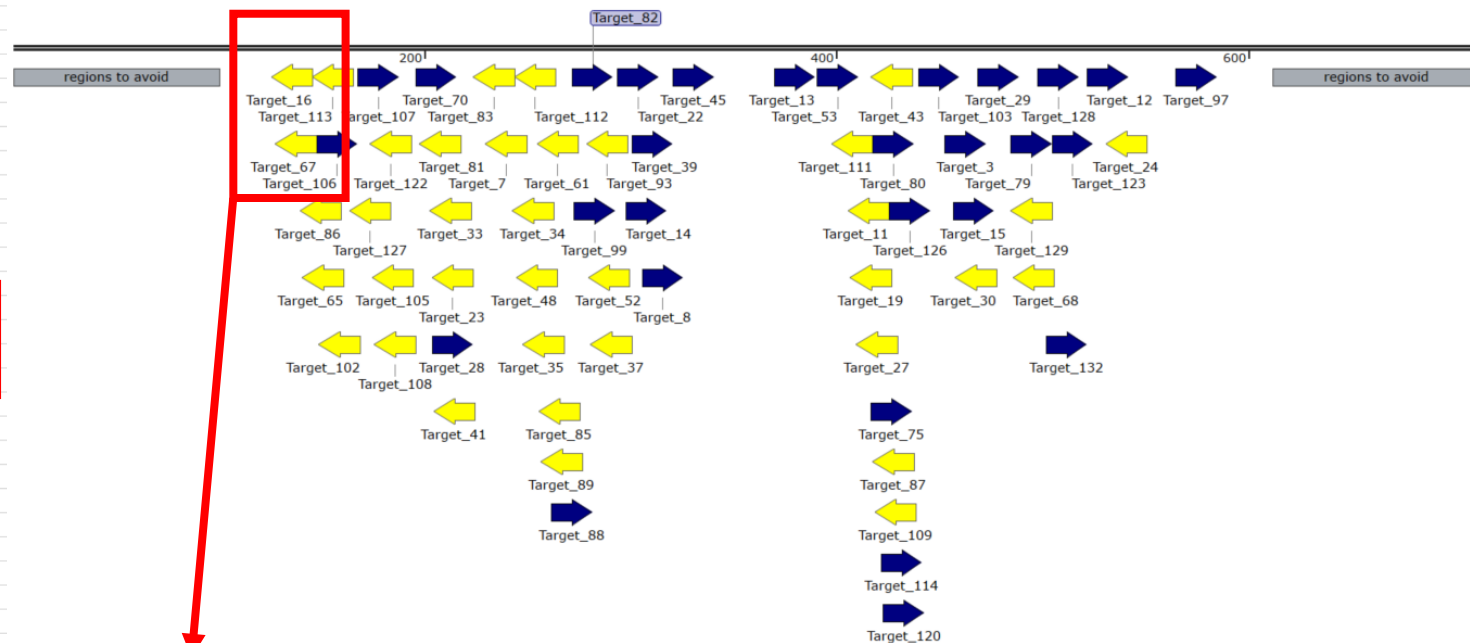
Initial Parameters

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- 4) Self-Complementarity → lowest possible

CHOPCHOP: Downloading and Filtering Guide-RNAs for Cas-CLOVER

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	Efficiency
92	CAAGGGCCGAGGAGGATAACATGG	9 ±	55	0	46.7	
72	GGATAAACATGGCCATCATCAAGG	21 ±	45	0	60.62	
73	GCCCATGAACTCCTTGATGATGG	32 -	50	0	57.08	
50	CAAGGAGTTCATGCGCTTCAAGG	39 ±	50	0	50.38	
54	CATGCGCTTCAAGGTGCACATGG	48 ±	55	0	52.7	
58	GCGCTTCAAGGTGCACATGGAGG	51 ±	60	0	62.8	
55	GCTTCAAGGTGCACATGGAGGG	52 ±	55	0	51.08	
95	CACATGGAGGGCTCCGTGAACGG	64 ±	60	5	56.24	
38	CTCGAACTCGTGGCCGTTACGG	77 -	60	4	45.62	
25	CGGCCACGAGTTCGAGATCGAGG	84 ±	65	1	60.07	
40	GGCCACGAGTTCGAGATCGAGGG	85 ±	60	1	69.4	
10	CGCCCTCGATCTCGAACTCGTGG	87 -	65	1	58.5	
34	CGAGTTCGAGATCGAGGGCCGAGG	90 ±	65	1	52.59	
62	GAGTTCGAGATCGAGGGCCGAGGG	91 ±	60	1	64.75	
124	CGAGATCGAGGGCCGAGGGCCGAGG	96 ±	75	0	43.2	
130	GAGATCGAGGGCCGAGGGCCGAGGG	97 ±	70	0	53.89	
129	GAGATCGAGGGCCGAGGGCCGAGGG	97 -	70	0	53.89	
63	TCTGGGTGCCCTCGTAGGGCGG	120 -	70	2	54.73	
16	CGGTCTGGGTGCCCTCGTAGGGG	123 -	70	1	58.51	
20	GCGGTCTGGGTGCCCTCGTAGGG	124 -	70	1	39.58	
67	GGCGGTCTGGGTGCCCTCGTAGG	125 -	75	0	41.22	
101	CACCCAGACCGCCAAGCTGAAGG	135 +	65	0	54.81	
86	CACCTTCAGCTTGGCGGTCTGGG	137 -	60	0	32.29	
65	TCACCTTCAGCTTGGCGGTCTGG	138 -	60	0	30.65	
113	CTTGGTCACCTTCAGCTTGGCGG	143 -	55	0	53.38	
98	CGCCAAGCTGAAGGTGACCAAGG	144 +	60	0	60.96	
100	GCCAAGCTGAAGGTGACCAAGGG	145 +	55	0	65	
102	ACCCTTGGTCACCTTCAGCTTGG	146 -	55	0	45.57	
106	AAGCTGAAGGTGACCAAGGGTGG	148 +	55	0	59.23	
127	GAAGGCGAGGGGCCACCTTGG	161 -	75	1	40.15	
107	TGGCCCCCTGCCCTTCGCCTGGG	168 +	75	0	42.63	

Reminder: The space between the left (yellow) and right (blue) guides must be within the 11-36bp range. Try to stay within 15-28 for highest success

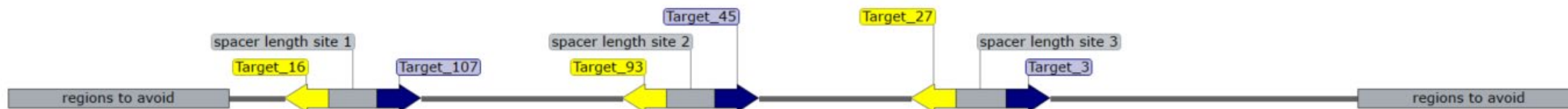


Begin with first potential left guide (reverse strand)
 - The highlighted are the first top two left guides

CHOPCHOP Results: Manually Finding gRNAs within Cas-CLOVER Parameters

At Demeetra, we try to identify guide pairs from the 5' end to the 3' end. We will pick three guide pairs that target regions spaced apart from each other. Therefore, after the user has found their first guide pairs, start moving your way down the target sequence

The final guide pair selection should look like this where the target sites are throughout the target sequence and all spacer lengths are within optimal range.



Left guide sequence (bottom strand read right to left)

Site 1: CGGTCTGGGTGCCCTCGTAG

Site 2: GCTCCCACTTGAAGCCCTCG

Site 3: CTTCTGCATTACGGGGCCGT

Right guide sequence (top strand read left to right)

Site 1: TGGCCCCCTGCCCTTCGCCT

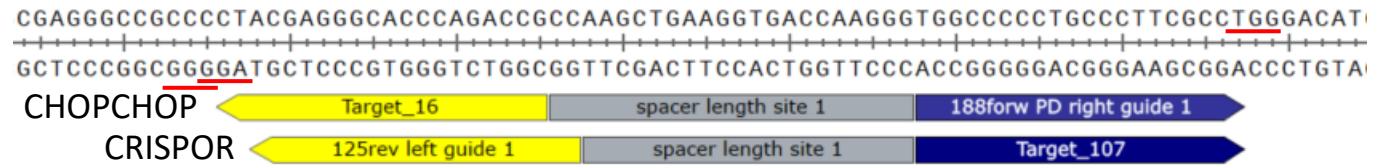
Site 2: CGGCGTGGTGACCGTGACCC

Site 3: CTCCGAGCGGATGTACCCCG

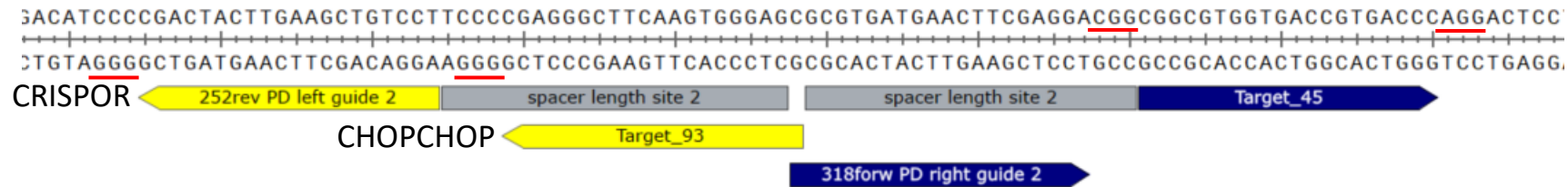
CRISPOR and CHOPCHOP Yield Similar Results for gRNAs within Cas-CLOVER Parameters

— PAM Sequences

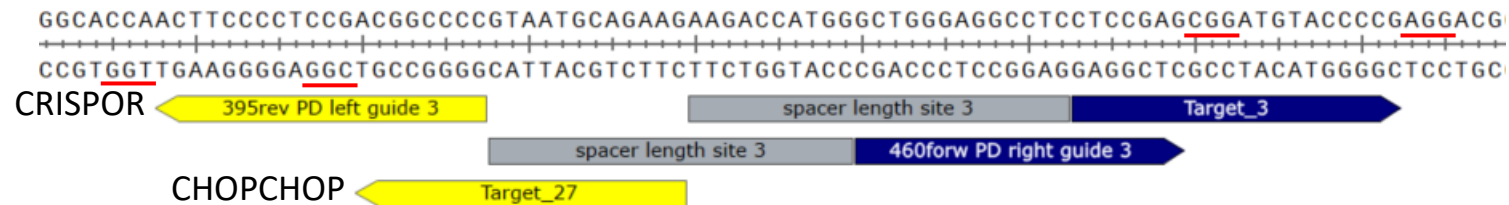
PD Site 1



PD Site 2



PD Site 3



The two guide-RNAs should be designed in the PAMs-out orientation, separated by a spacer region of 15 – 28 nucleotides. You simply need to ensure that the gRNA pairs are in the PAMs-out orientation and the targeted sequences are separated by 15 – 28 nucleotides. The recruited Clo51 nuclease domains dimerize and this introduces a single double-stranded break in this spacer region between the two gRNA target sites.

Manually Designing sgRNA Pairs for Cas-CLOVER

- 1) In the users molecular cloning program of choice, import the target sequence as a DNA file.
- 2) Search for 'GG' in your target sequence → there should be a plethora of hits

711 bp (DNA)

5' ATGGT GAGCAA GGG CGA GGAGGATAACAT GGCCATCATCAA GGAGTTCATGCGCTTCAA GGTGCACAT GGA GGGCTCCGTGAAC GGCCACGAGTTCGAGATCGA GGGCGA GGGCGA GGGCCGCCCTACG
3' TACCACTCGTTCCCGCTCCTCTATTGTACC GG TAGTAGTTCCTCAAGTACGCGAAGTTCACGTGTACCTCCCGA GGCACTTGCC GG TGCTCAAGCTCTAGCTCCCGCTCCCGCTCCC GGCGGGATGC

1 5 10 15 20 25 30 35 40
M V S K G E E D N M A I I K E F M R F K V H M E G S V N G H E F E I E G E G E G R P Y
mCherry

region to avoid

260
A GGG CACCCAGACCCGCAAGCTGAA GG TGACCAA GGG TGG CCCCCTGCCCTTCGCCCT GGG ACATCCTGTCCCCTCAGTTCATGTAC GGCTCCAA GG CCTACGTGAAGCACCCCGCCGACATCCCGACTA
3' TCCCGT GGG TCT GGC GG TCGACTTCCACT GG TCCCAACC GGGGGAC GGG AAGC GG ACCCTGTA GGACA GGGG AGTCAAGTACATGCCGA GG TCC GGATGCACCTTCGT GGGGG GG CTGTA GGGG CTGAT

45 50 55 60 65 70 75 80 85
E G T Q T A K L K V T K G G P L P F A W D I L S P Q F M Y G S K A Y V K H P A D I P D Y
mCherry

390
CTTGAAGCTGTCCTTCCCGA GGG CTTCAAGT GGG AGCGCGTGATGAACTTCGA GGAC GG GG CGT GG TGACCGTGACCCA GGACTCCTCCCTGCA GGAC GG CGAGTTCATCTACAA GG TGAAGCTGCGC
3' GAACTTCGACA GGA GGGG CTCCCGAAGTTCACCCTCGGCACTACTTGAAGCTCCTGCCGCCGACCCT GGCACT GGG TCCCTGA GGA GGG ACGTCTGCCGCTCAAGTAGATGTTCCACTTCGACGG

90 95 100 105 110 115 120 125 130
L K L S F P E G F K W E R V M N F E D G G V V T V T Q D S S L Q D G E F I Y K V K L R
mCherry

520
GGCACCAACTTCCCTCCGAC GG CCCCCTAATGCAGAAGAAGACCAT GGG CT GGG A GG CCTCCTCCGAGC GGATGTACCCGA GGAC GG CGCCCTGAA GGG CGAGATCAAGCAGA GG CTGAAGCTGAA GG
3' CCGT GG TTGAA GGGG A GG TGCC GGGG CATTACGTCTTCTCT GG TACCCGACCCTCC GGA GG A GG CTGCGCTACAT GGGG CTCTGCCGC GGG ACTTCCCGCTCTAGTTCGTCTCCGACTTCGACTTCC

135 140 145 150 155 160 165 170 175
G T N F P S D G P V M Q K K T M G W E A S S E R M Y P E D G A L K G E I K Q R L K L K
mCherry

650
AC GG GG CCACTACGACGTGA GG TCAAGACCACCTACAA GG CCAAGAAGCCGTGCAGCTGCC GG CGCCTACAACGTCAACATCAAGTT GG ACATCACCTCCCAACAACGA GG ACTACACCATCGT GGA
3' TGCCGCC GG TGATGCTGCGACTCCAGTTC GG TGATGTTCC GG TCTTC GGG CAGTGCAGC GGG CCGC GGATGTTGCAGTTGTAGTTCACCTGTAGT GGA GGG TGTGCTCCTGATGT GG TAGCACCT

175 180 185 190 195 200 205 210 215
D G G H Y D A E V K T T Y K A K K P V Q L P G A Y N V N I K L D I T S H N E D Y T I V E
mCherry

region to avoid

3' ACAGTACGAACGCGCCGA GGG CCGCACTCCACC GG C GG CAT GGACGAGCTGTACAAGTAG
5' TGTCATGCTTGCGC GGCTCCC GG GG TGA GG GG CCGCCGTACCTGCTCGACATGTTTCATC

* Find DNA sequence: GG 144 matches Previous Next

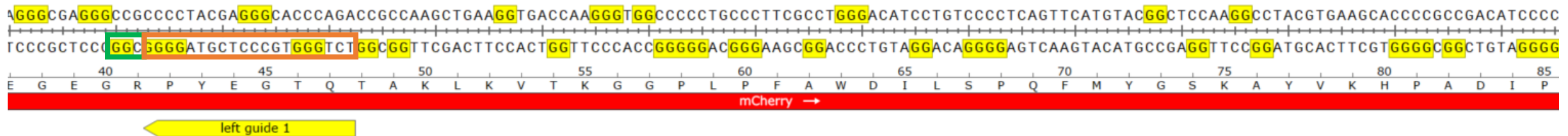
Map Sequence Enzymes Features Primers History Description Panel

Manually Designing sgRNA Pairs for Cas-CLOVER

- Avoid the 100bp 5' and 3' regions if sequencing analysis will be performed
- Begin on the reverse strand to identify your first potential left guide (see green boxes)

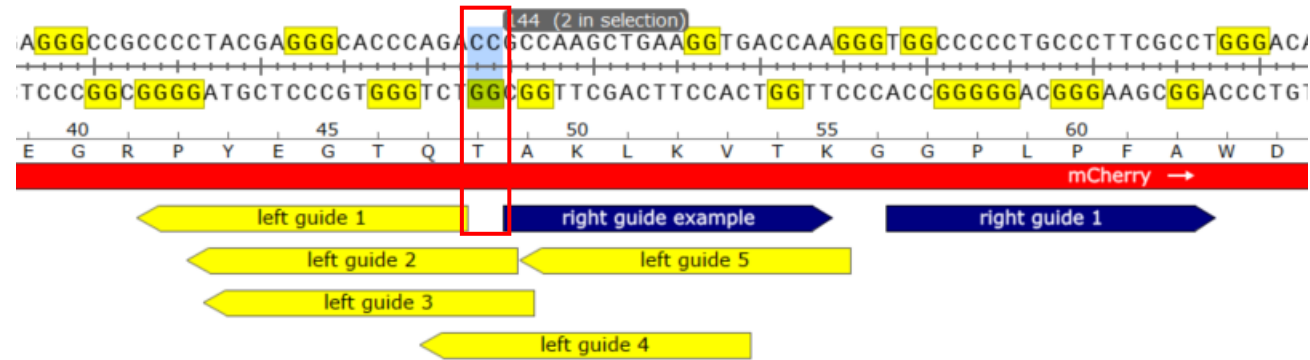


- From the PAM site, identify a 20bp guide sequence and annotate



Manually Designing sgRNA Pairs for Cas-CLOVER

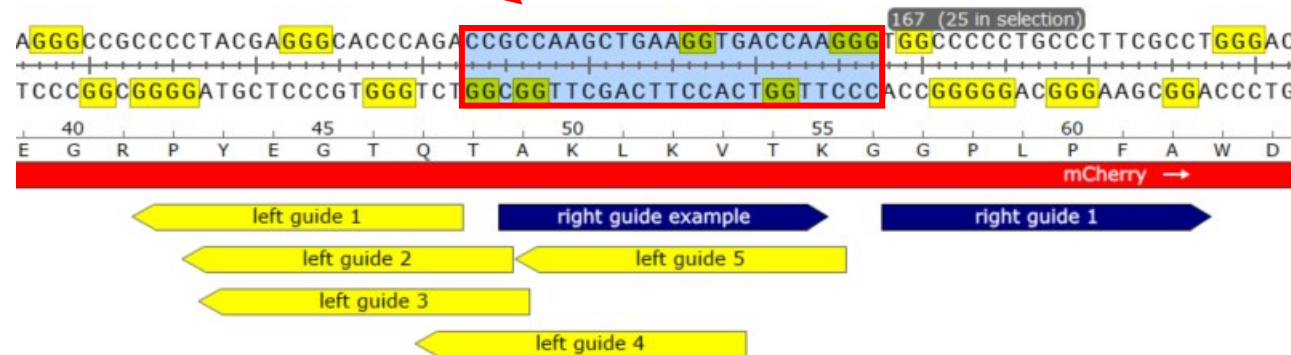
- 6) Regions with a lot of PAM sites are good areas to focus on and then keep scanning downstream for the first potential right guide → The distance between the left guide boundary to the right guide PAM must be more than 30bps (20bp right guide sequence will leave a 10bp spacer length)



Spacer length between left guide 1 and right guide 1 is 25bps which is within the optimal range for Cas-CLOVER, however, there are multiple choices well within range for Cas-CLOVER

Left guide 3 sequence
(bottom strand read right to left)

Site 1:
GCGGTCTGGGTGCCCTCGTA



Right guide 1 sequence (top strand read left to right)

Site 1:
TGGCCCCCTGCCCTTCGCCT

Production of sgRNA Pairs for Cas-CLOVER

Option #1: Synthetic guide synthesis

If the user will be using a non-plasmid based system (mRNA, protein, etc.) then ordering synthetic guides from providers such as Synthego which will convert the DNA target sequence to RNA and automatically add the SpCas9 scaffold (Bottom left picture)

Option #2: Plasmid-based sgRNA expression

Order oligos for both the left and right guide sequences and assemble via Golden Gate Assembly (Bottom right picture)

SYNTHEGO

CRISPRevolution sgRNA EZ Kit

Please enter your item details below. Each kit includes 1 tube of sgRNA, 1 tube of Tris-EDTA Buffer, and 1 tube of Nuclease-free Water.

Tubes Plates

Kit #1 (sgRNA)

RNA Label

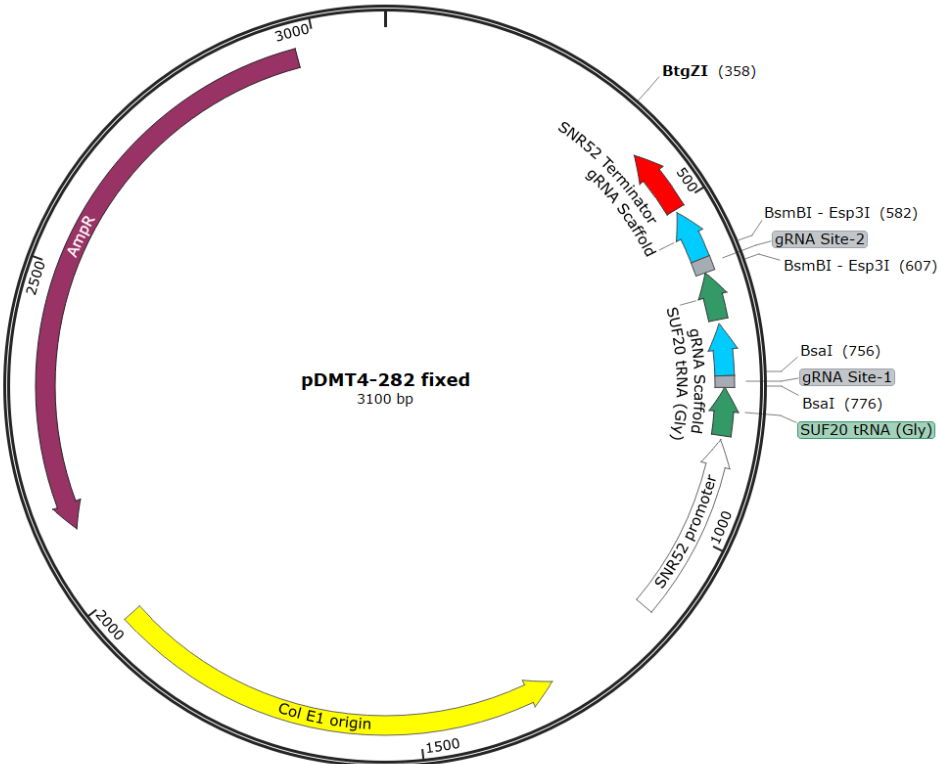
Guaranteed Yield

sgRNA Target Sequence

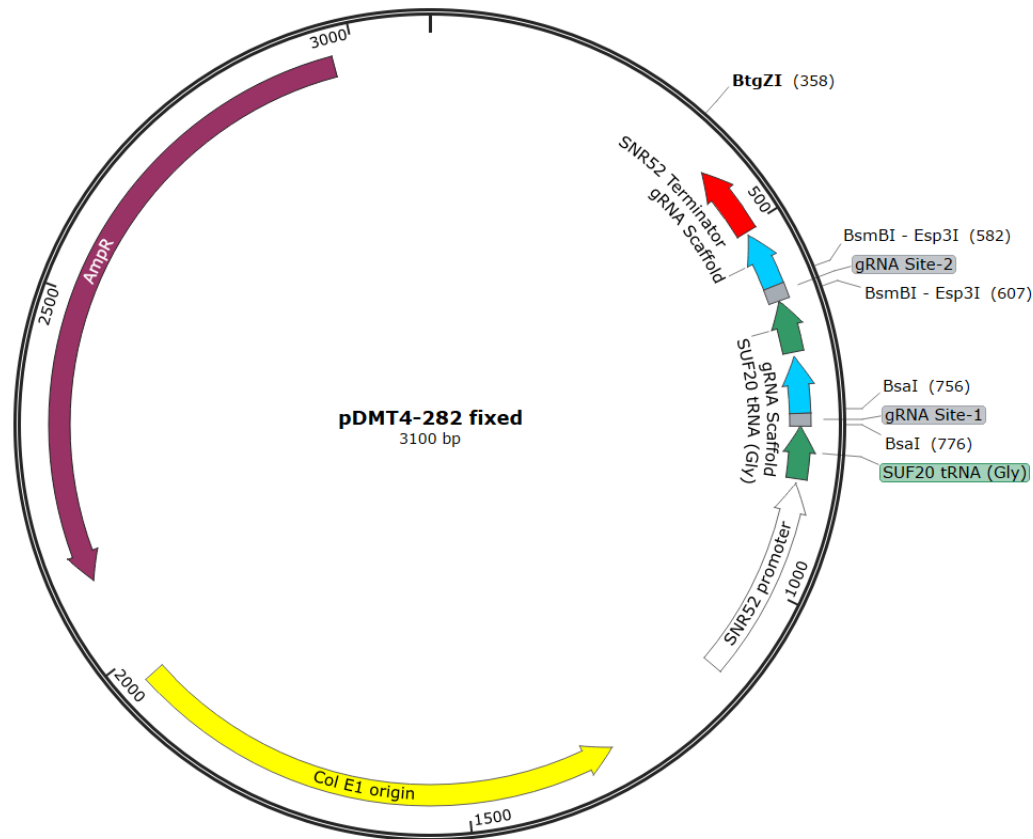
Enter the 17-23 nucleotide genome targeting sequence in 5' to 3' order and do not include the PAM sequence. We will automatically add an 80-mer SpCas9 scaffold to create a single guide RNA.

Modifications

Chemical modifications provide superior editing in most cell types, including primary cells and stem cells.



Cloning of sgRNA Pairs for Cas-CLOVER



Golden Gate Assembly (GGA)
Destination Plasmid → pDMT4-282

Contains:
GGA sites (BsaI and BsmBI)
Dual guide cassette driven by SNR52 promoter
E.Coli components (AmpR, ori)

At Demeetra, we start with the left guide cloning first.

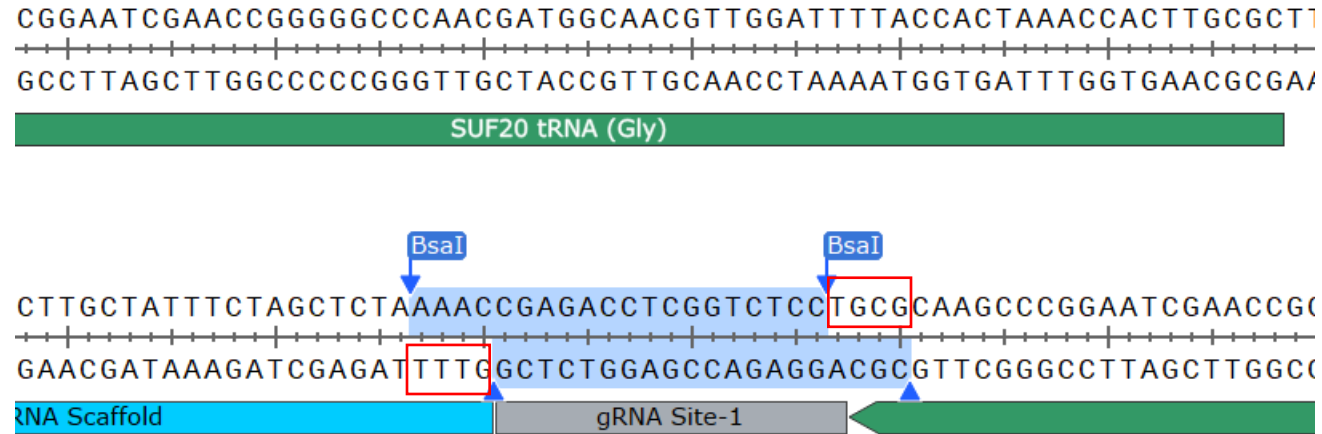
Left guide sequence (bottom strand read right to left)

Site 1: GGCGGTCTGGGTGCCCTCGT

Site 2: AGGACAGCTTCAAGTAGTCG

Site 3: GGGGCCGTCGGAGGGGAAGT

Cloning of sgRNA Pairs for Cas-CLOVER



Note how BsaI will cleave, the overhangs will be used in the design of the oligos for building the guides. Let's start with **Site 1**. The user will order two oligos for each guide, anneal them, and assemble.

Oligo 1 (**bottom** strand 5' to 3')

cgcaGGCGGTCTGGGTGCCCTCGT

Oligo 2 (**top** strand 5' to 3')

aaacACGAGGGCAGCCAGACCGCC

Annealed oligos

aaacACGAGGGCAGCCAGACCGCC
 TGCTCCCGTGGGTCTGGCGG**acgc**

Left guide sequence (bottom strand read right to left)

Site 1: GGCGGTCTGGGTGCCCTCGT

Site 2: AGGACAGCTTCAAGTAGTCG

Site 3: GGGGCCGTCGGAGGGGAAGT

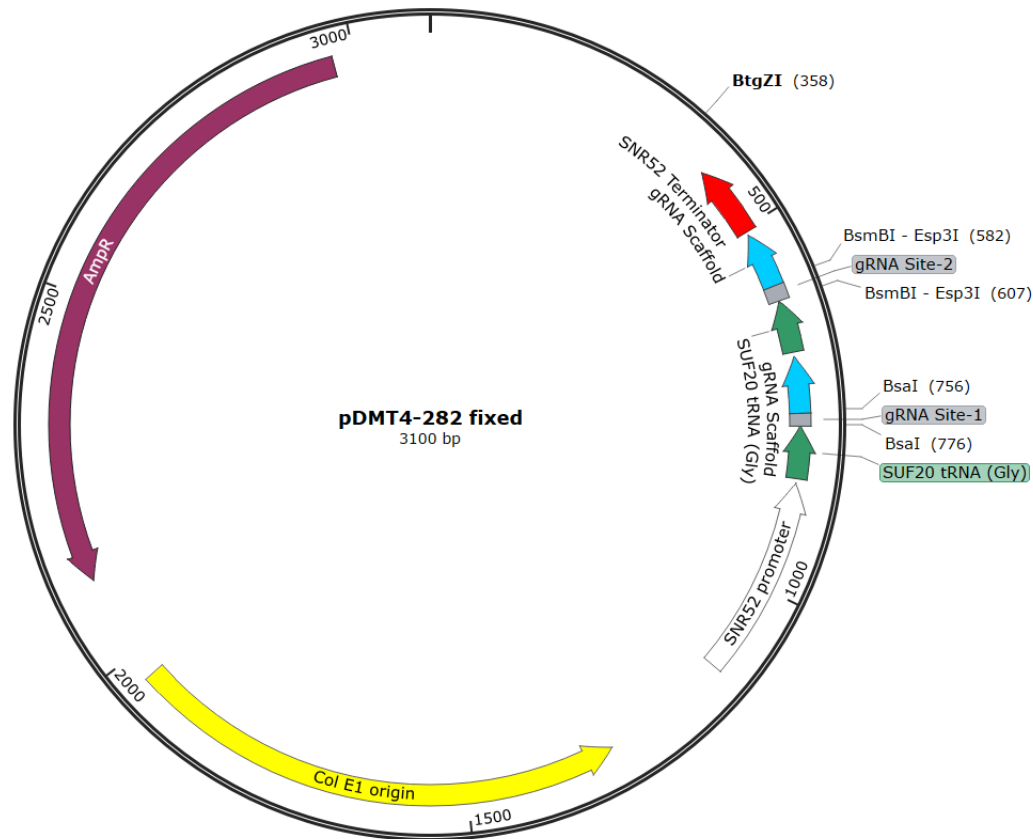
Cloning of sgRNA Pairs for Cas-CLOVER

The screenshot displays a cloning software interface. On the left, two oligos are defined: 'leftguide1 sense' (5' cgc aGGCGGTCTGGGTGCCCTCGT) and 'leftguide1 antisense' (5' aaacACGAGGGCACCCAGACCGCC). Below them is a sequence alignment showing the sense strand (5' to 3') and the antisense strand (3' to 5') with a 28 bp overlap. On the right, a plasmid map is shown with three regions: a blue 'gRNA Scaffold', a grey 'PD LG Site 1', and a green 'SUF20 tRNA (Gly)'. Red boxes highlight the 'aaac' sequence in the antisense oligo and the 'tgcg' sequence in the plasmid map, indicating the target site for cloning.

- After annealing the oligos together, Golden Gate Assembly is performed to insert a single guide into the base vector:
 - Cuts at the Bsa1 sites
 - Ligates in the guide fragment
 - Orientation dictated by the overhangs
- In current system, each oligo fragment is cloned into the base vector individually
- After GGA, reaction is transformed into E.coli
- Colonies are prepped and sequenced to verify guide sequence and correct orientation

Upon proper assembly, the Left guide for Site 1 will be correctly orientated and will have replaced the left guide site in the GGA destination plasmid. Now the user will move on to the Right guide for Site 1.

Cloning of sgRNA Pairs for Cas-CLOVER



Golden Gate Assembly (GGA)
Destination Plasmid → pDMT4-282

Contains:
GGA sites (BsaI and BsmBI)
Dual guide cassette driven by SNR52 promoter
E.Coli components (AmpR, ori)

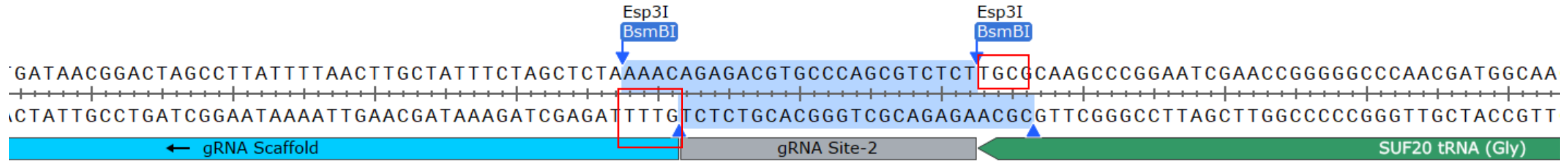
Right guide sequence (top strand read left to right)

Site 1: **TGGCCCCCTGCCCTTCGCCT**

Site 2: **CGCGTGATGAACTTCGAGGA**

Site 3: **GCTGGGAGGCCTCCTCCGAG**

Cloning of sgRNA Pairs for Cas-CLOVER



Note how BsmBI will cleave, the overhangs will be used in the design of the oligos for building the guides.

For Right Guide Site 1, the assembly is more straight forward. The user will order two oligos for each guide, anneal them, and assemble.

Oligo 1 (**top** strand 5' to 3')

cgcaTGGCCCCCTGCCCTTCGCCT

Oligo 2 (**bottom** strand 5' to 3')

aaacAGGCGAAGGGCAGGGGGCCA

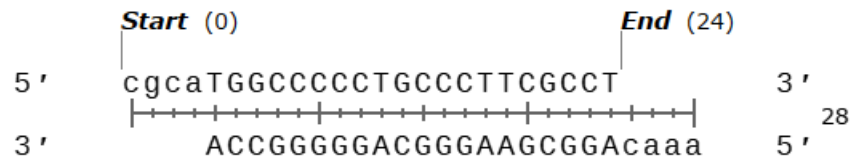
Right guide sequence (top strand read left to right)

Site 1: **TGGCCCCCTGCCCTTCGCCT**

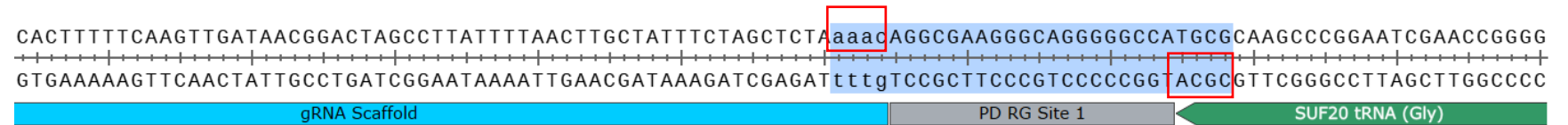
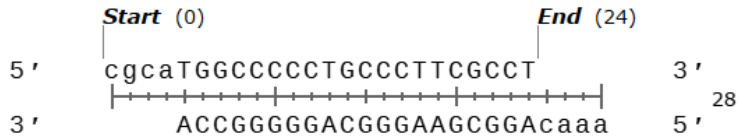
Site 2: CGCGTGATGAACTTCGAGGA

Site 3: GCTGGGAGGCCTCCTCCGAG

Annealed Oligos



Cloning of sgRNA Pairs for Cas-CLOVER



- After annealing the oligos together, Golden Gate Assembly is performed to insert a single guide into the base vector:
 - Cuts at the BsmB1 sites
 - Ligates in the guide fragment
 - Orientation dictated by the overhangs
- In current system, each oligo fragment is cloned into the base vector individually
- After GGA, reaction is transformed into E.coli
- Colonies are prepped and sequenced to verify guide sequence and correct orientation

Cloning the Guide Cassette for Cas-CLOVER

Upon proper assembly, the Right AND Left guide for Site 1 will be correctly orientated and will have replaced the right and left guide sites in the GGA destination plasmid. Now the user is ready to clone the entire cassette via HiFi cloning into their plasmid that also has Cas-CLOVER (Assembly techniques may vary depending on destination plasmids).

