Paired Guide Design and Construction for Cas-CLOVER

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Demeetra

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.

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

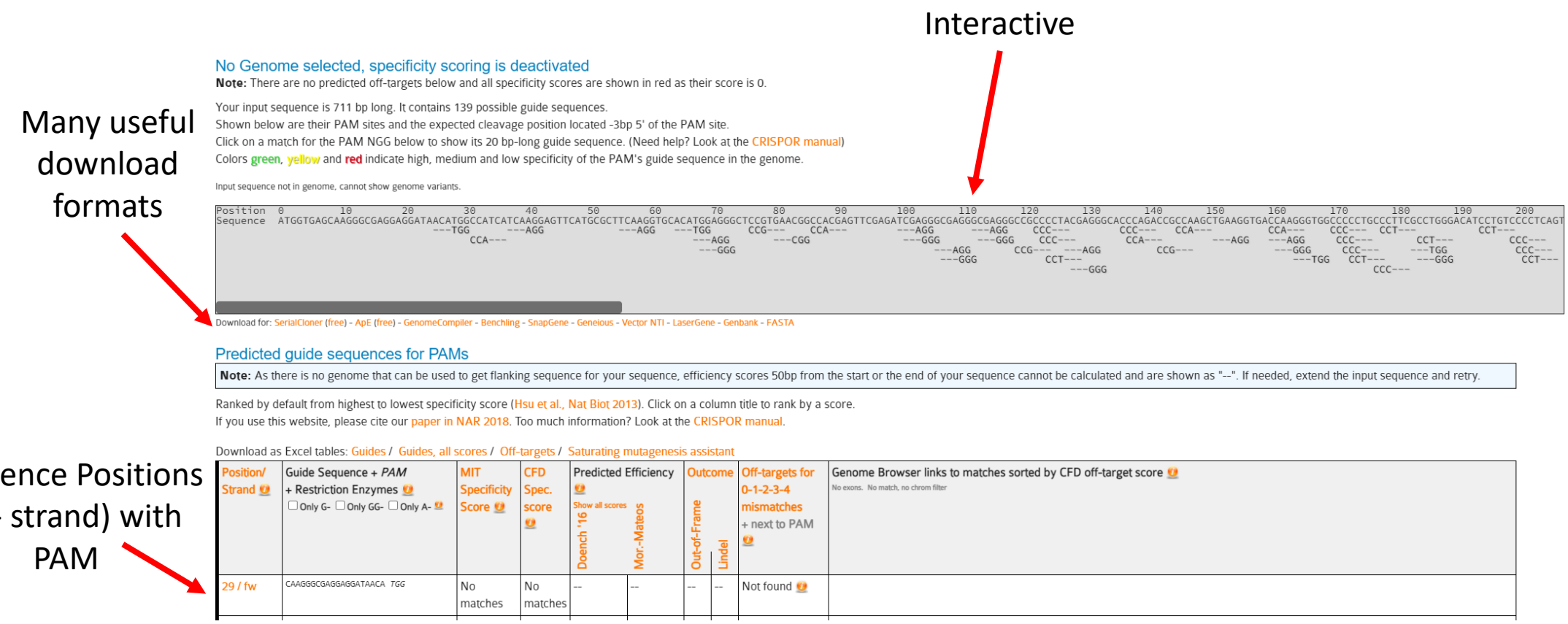
CasCLOVER uses deactivated SpCas9

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

Interactive

Many useful download formats

Sequence Positions (+/- strand) with PAM



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.

Download for: SerialCloner (free) - ApE (free) - GenomeCompiler - Benchling - SnapGene - Geneious - 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

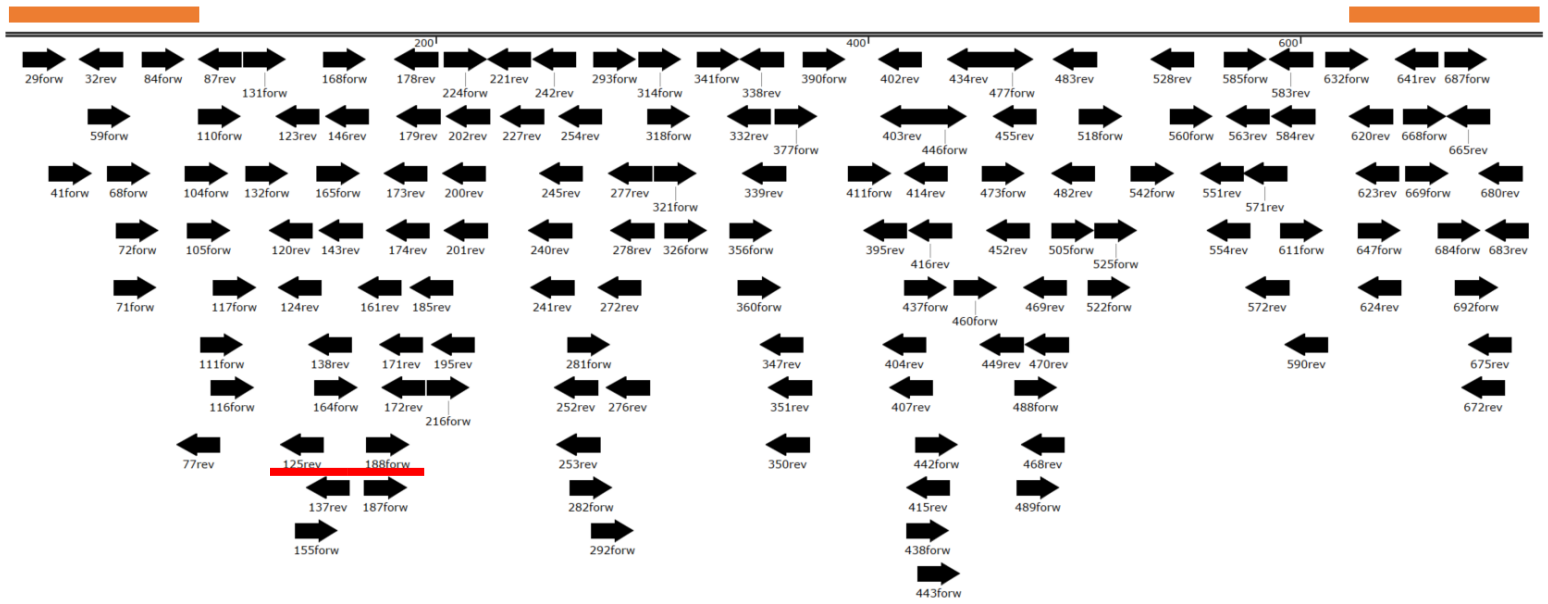
Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes (Only G- / Only GG- / Only A-)	MIT Specificity Score	CFD Spec. score	Predicted Efficiency (Show all scores) Doench '16	Mor.-Mateos	Outcome Out-of-Frame	Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score (No exons. No match, no chrom filter)
29 / fw	CAAGGGCGAGGAGGATAACA *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	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCG		
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=8jX8Ck4Zh1S3rrNu5une		
8				
9	#guideId	targetSeq	Doench '16-Score	Moreno-Mateos-Score
10	29forw	CAAGGGCGAGGAGGATAACATGG	NotEnoughFlankSeq	NotEnoughFlankSeq
11	32rev	GCGCATGAACTCCTTGATGATGG	NotEnoughFlankSeq	NotEnoughFlankSeq
12	41forw	GGATAACATGGCCATCATCAAGG	NotEnoughFlankSeq	NotEnoughFlankSeq
13	59forw	CAAGGAGTTCATGCGCTTCAAGG	50	50
14	68forw	CATGCGCTTCAAGGTGCACATGG	53	37
15	71forw	GCGCTTCAAGGTGCACATGGAGG	63	61
16	72forw	CGCTTCAAGGTGCACATGGAGGG	51	41
17	77rev	GTCGAACTCGTGGCCGTTCACGG	46	56
18	84forw	CACATGGAGGGCTCCGTGAACGG	56	64
19	87rev	CGCCCTCGATCTCGAACTCGTGG	58	30
20	104forw	CGGCCACGAGTTCGAGATCGAGG	60	63
21	105forw	GGCCACGAGTTCGAGATCGAGGG	69	34
22	110forw	CGAGTTCGAGATCGAGGGCGAGG	53	61
23	111forw	GAGTTCGAGATCGAGGGCGAGGG	65	59
24	116forw	CGAGATCGAGGGCGAGGGCGAGG	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	GGGCGAGGGCCGCCCCTACGAGG	52	75
31	132forw	GGCGAGGGCCGCCCCTACGAGGG	56	71
32	137rev	CACCTTCAGCTTGGCGGTCTGGG	32	71
33	138rev	TCACCTTCAGCTTGGCGGTCTGG	31	33
34	143rev	CTTGGTCACCTTCAGCTTGGCGG	53	56
35	146rev	ACCCTTGGTCACCTTCAGCTTGG	46	29
36	155forw	CACCCAGACCGCCAAGCTGAAGG	55	40
37	161rev	GAAGGGCAGGGGGCCACCCTTGG	40	70
38	164forw	CGCCAAGCTGAAGGTGACCAAGG	61	45
39	165forw	GCCAAGCTGAAGGTGACCAAGGG	65	67
40	168forw	AAGCTGAAGGTGACCAAGGGTGG	59	69
41	171rev	TGTCCCAGGCGAAGGGCAGGGGG	53	49
42	172rev	ATGTCCCAGGCGAAGGGCAGGGG	41	63
43	173rev	GATGTCCCAGGCGAAGGGCAGGG	51	56
44	174rev	GGATGTCCCAGGCGAAGGGCAGG	39	44
45	178rev	GACAGGATGTCCCAGGCGAAGGG	56	72
46	179rev	GGACAGGATGTCCCAGGCGAAGG	49	43
47	185rev	CTGAGGGGACAGGATGTCCCAGG	56	67
48	187forw	GTGGCCCCCTGCCCTTCGCCTGG	28	29
49	188forw	TGGCCCCCTGCCCTTCGCCTGGG	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	ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCG		
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=8jX8Ck4Zh1S3rrNu5une		
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9	#guideId	targetSeq	Doench '16-Score	Moreno-Mateos-Score
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12	41forw	GGATAACATGGCCATCATCAAGG	NotEnoughFlankSeq	NotEnoughFlankSeq
13	59forw	CAAGGAGTTCATGCGCTTCAAGG	50	50
14	68forw	CATGCGCTTCAAGGTGCACATGG	53	37
15	71forw	GCGCTTCAAGGTGCACATGGAGG	63	61
16	72forw	CGCTTCAAGGTGCACATGGAGGG	51	41
17	77rev	GTCGAACTCGTGGCCGTTCACGG	46	56
18	84forw	CACATGGAGGGCTCCGTGAACGG	56	64
19	87rev	CGCCCTCGATCTCGAACTCGTGG	58	30
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21	105forw	GGCCACGAGTTCGAGATCGAGGG	69	34
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23	111forw	GAGTTCGAGATCGAGGGCGAGGG	65	59
24	116forw	CGAGATCGAGGGCGAGGGCGAGG	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	GGGCGAGGGCCGCCCCTACGAGG	52	75
31	132forw	GGCGAGGGCCGCCCCTACGAGGG	56	71
32	137rev	CACCTTCAGCTTGGCGGTCTGGG	32	71
33	138rev	TCACCTTCAGCTTGGCGGTCTGG	31	33
34	143rev	CTTGGTCACCTTCAGCTTGGCGG	53	56
35	146rev	ACCCTTGGTCACCTTCAGCTTGG	46	29
36	155forw	CACCCAGACCGCCAAGCTGAAGG	55	40
37	161rev	GAAGGGCAGGGGGCCACCCTTGG	40	70
38	164forw	CGCCAAGCTGAAGGTGACCAAGG	61	45
39	165forw	GCCAAGCTGAAGGTGACCAAGGG	65	67
40	168forw	AAGCTGAAGGTGACCAAGGGTGG	59	69
41	171rev	TGTCCCAGGCGAAGGGCAGGGGG	53	49
42	172rev	ATGTCCCAGGCGAAGGGCAGGGG	41	63
43	173rev	GATGTCCCAGGCGAAGGGCAGGG	51	56
44	174rev	GGATGTCCCAGGCGAAGGGCAGG	39	44
45	178rev	GACAGGATGTCCCAGGCGAAGGG	56	72
46	179rev	GGACAGGATGTCCCAGGCGAAGG	49	43
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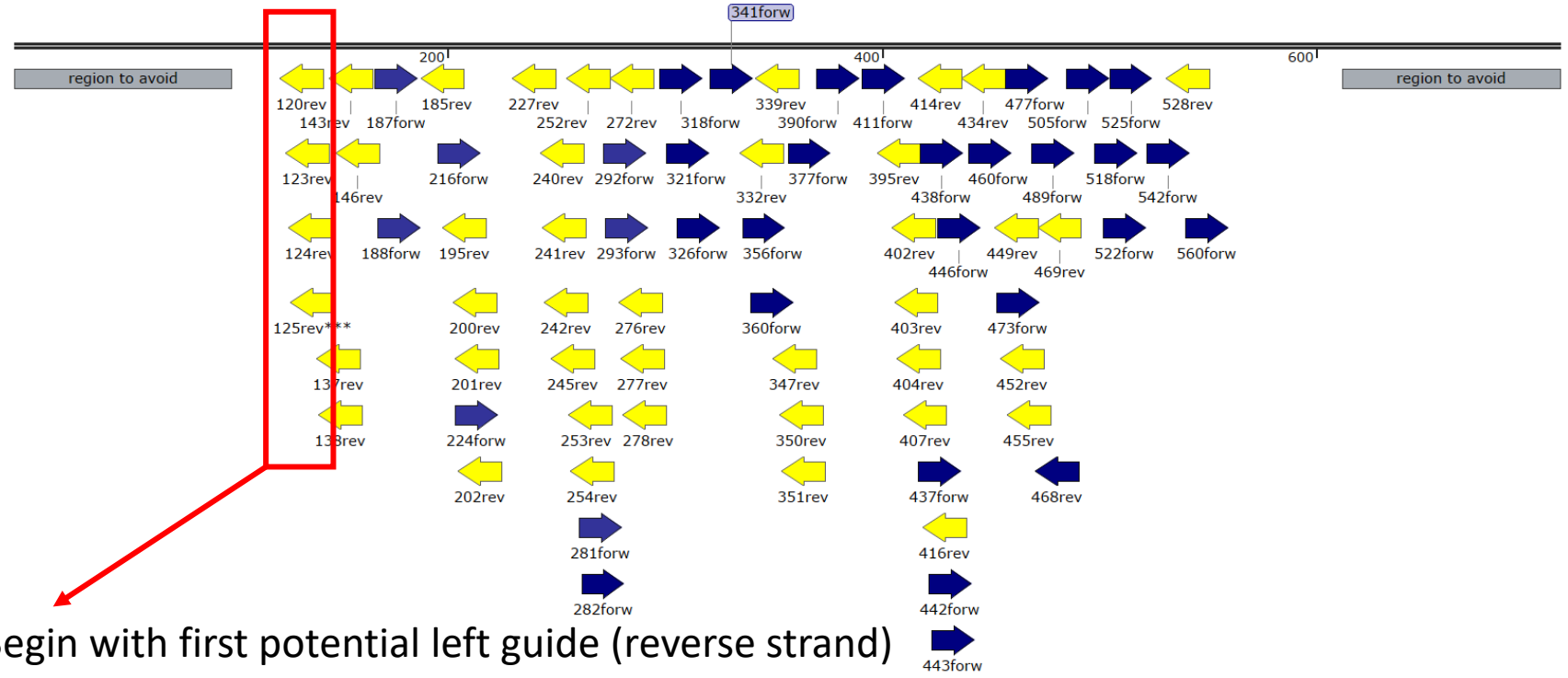

Begin with first potential left guide (reverse strand)

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

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22	110forw	CGAGTTCGAGATCGAGGGCGAGG	53	61
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24	116forw	CGAGATCGAGGGCGAGGGCGAGG	43	57
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29	125rev	GGCGGTCTGGGTGCCCTCGTAGG	41	80
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31	132forw	GGCGAGGGCCGCCCCTACGAGGG	56	71
32	137rev	CACCTTCAGCTTGGCGGTCTGGG	32	71
33	138rev	TCACCTTCAGCTTGGCGGTCTGG	31	33
34	143rev	CTTGGTCACCTTCAGCTTGGCGG	53	56
35	146rev	ACCCTTGGTCACCTTCAGCTTGG	46	29
36	155forw	CACCCAGACCGCCAAGCTGAAGG	55	40
37	161rev	GAAGGGCAGGGGGCCACCCTTGG	40	70
38	164forw	CGCCAAGCTGAAGGTGACCAAGG	61	45
39	165forw	GCCAAGCTGAAGGTGACCAAGGG	65	67
40	168forw	AAGCTGAAGGTGACCAAGGGTGG	59	69
41	171rev	TGTCCCAGGCGAAGGGCAGGGGG	53	49
42	172rev	ATGTCCCAGGCGAAGGGCAGGGG	41	63
43	173rev	GATGTCCCAGGCGAAGGGCAGGG	51	56
44	174rev	GGATGTCCCAGGCGAAGGGCAGG	39	44
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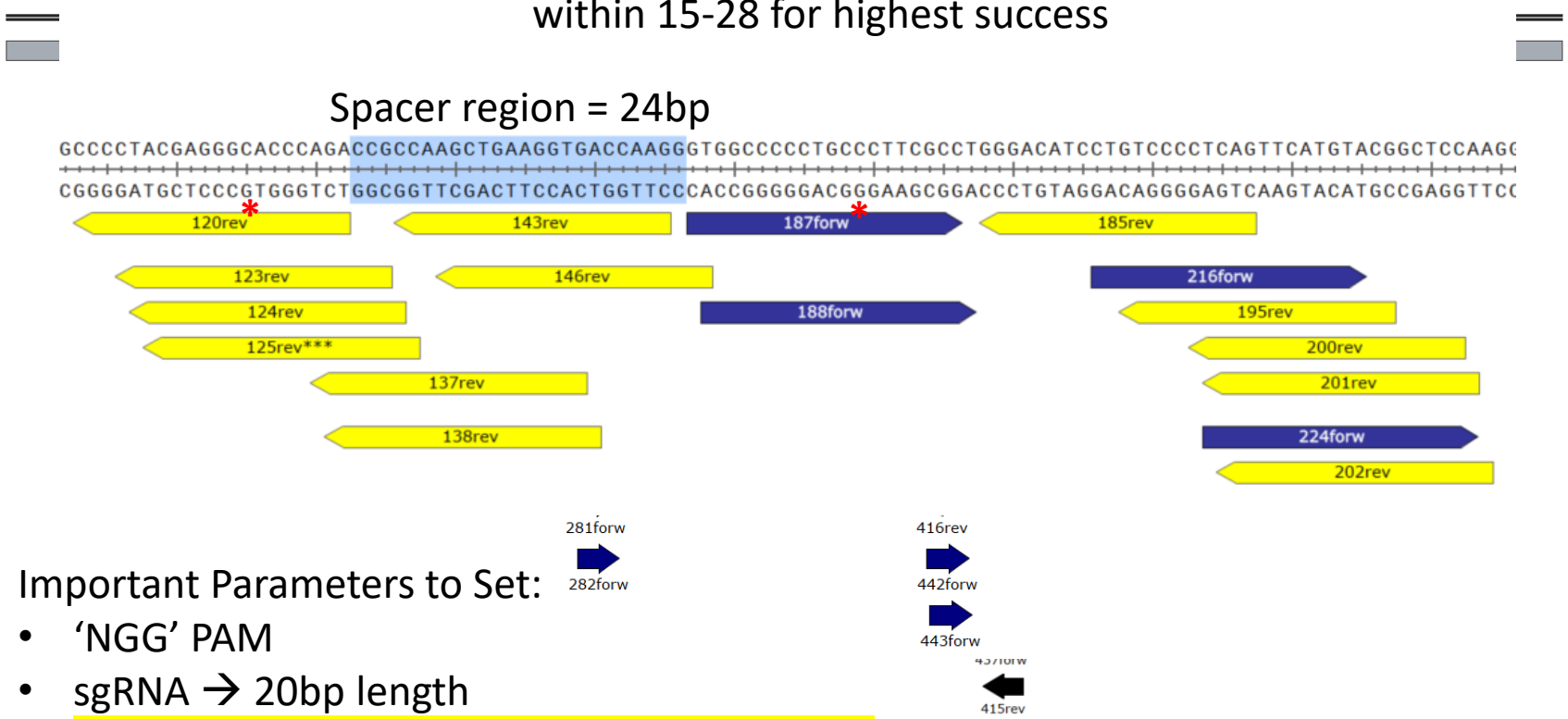
48	187forw	GTGGCCCCCTGCCCTTCGCCTGG	28	29
49	188forw	TGGCCCCCTGCCCTTCGCCTGGG	43	56

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



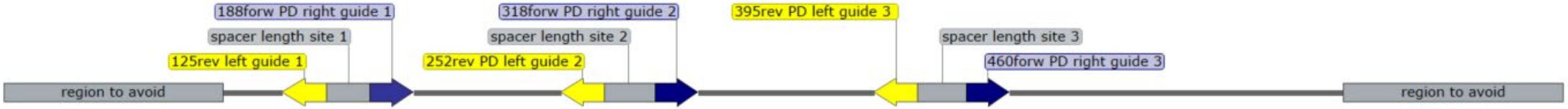
Important Parameters to Set:

- '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: GGGGCCGTCGGAGGGGAAGT

Right guide sequence (top strand read left to right)
Site 1: TGGCCCCCTGCCCTTCGCCT
Site 2: CGCGTGATGAACTTCGAGGA
Site 3: GCTGGGAGGCCTCCTCCGAG

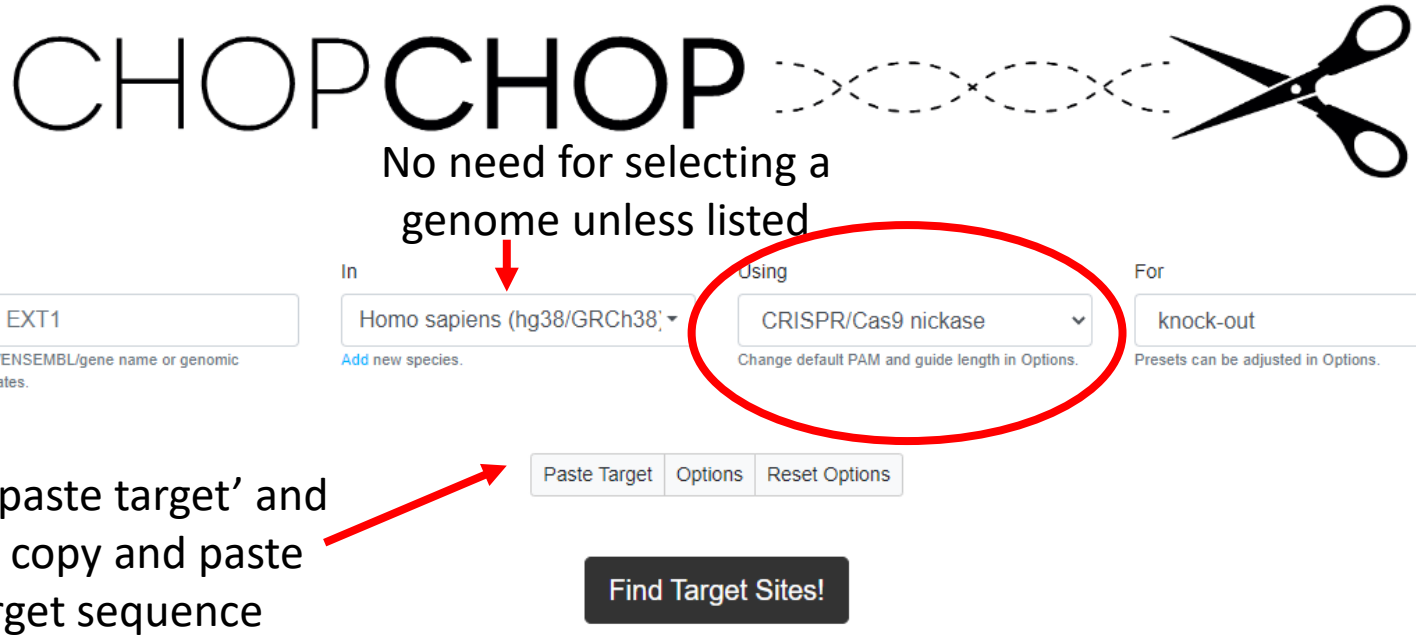
CHOPCHOP: Designing Guide-RNA Pairs for Cas-CLOVER

Pros:

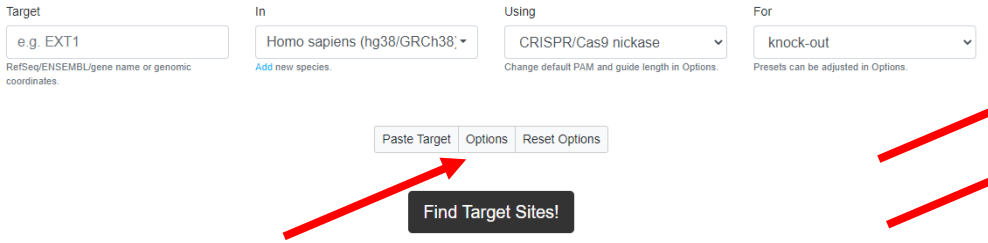
- CRISPR/Cas9 Nickase option
- Self-complementarity scores
- Can set many different parameters

Cons:

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



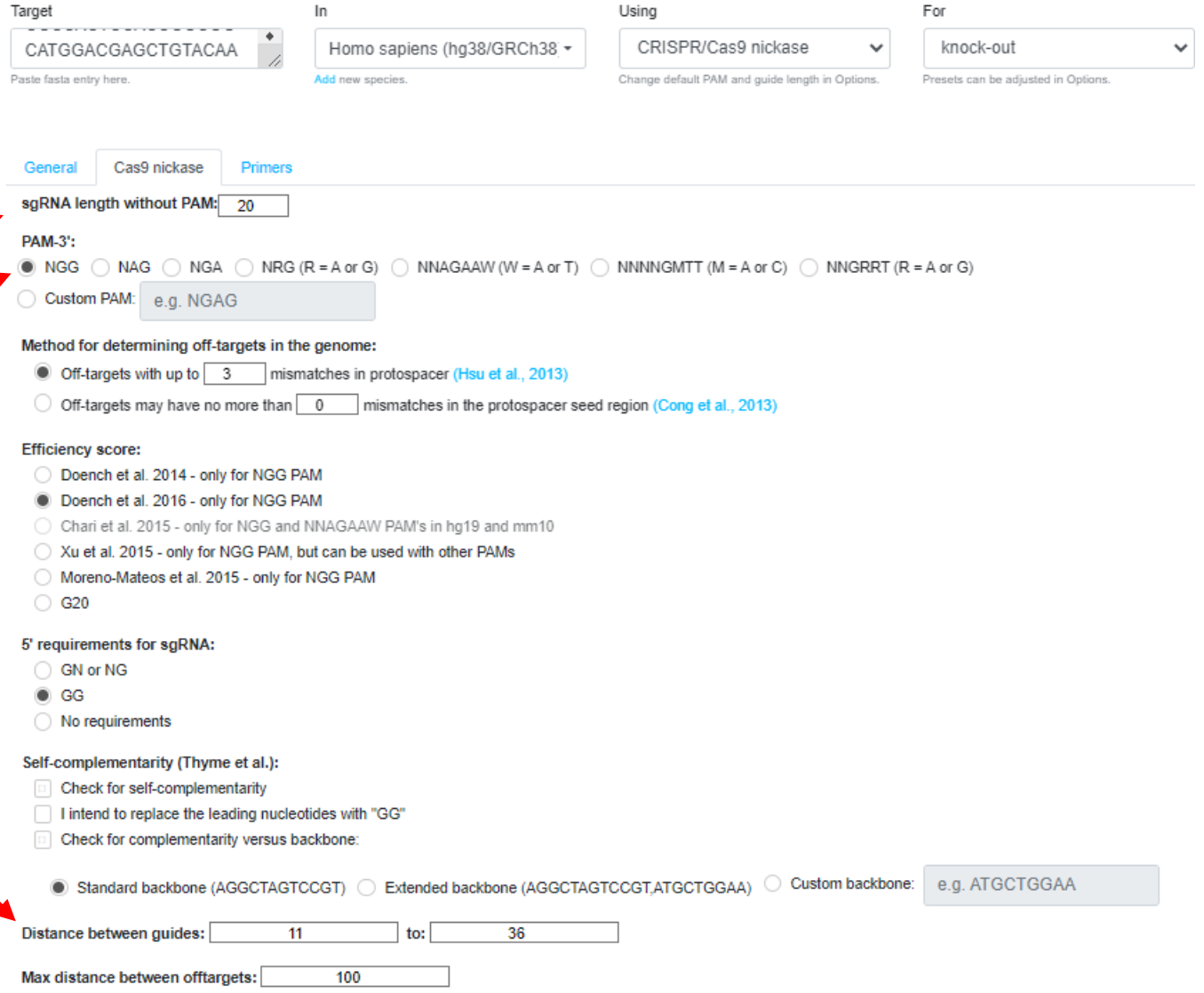
CHOPCHOP: Setting Parameters for Guide-RNA Pairs for Cas-CLOVER



Click on options

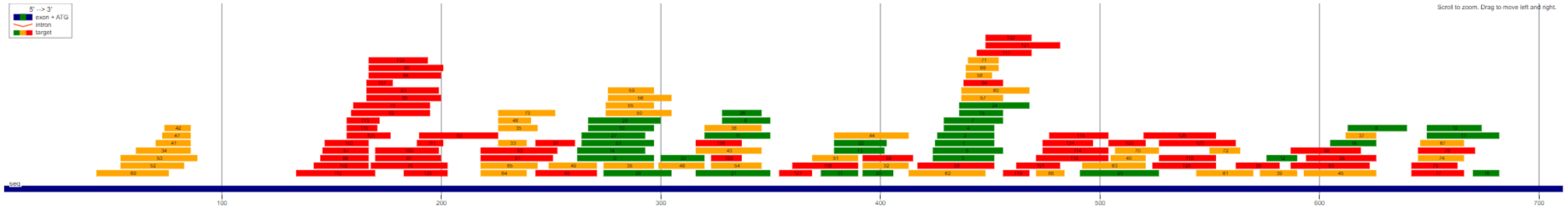
Important Parameters to Set:

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



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

fastaInput.fa



Download results: Please select one

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	CCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCCTCGGGGTACATCCGCTCGGAG	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	CCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCCTCGGGGTACATCCGCTCGGAG	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	GCATTACGGGGCCGTCGGAGGGGAGAAGAAGACCATGGGCTGGGAGGCCTCCCTCGGGGTACATCCGCTCGGAG	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	CCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCCTCGGGGTACATCCGCTCGGAG	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	GCATTACGGGGCCGTCGGAGGGGAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGG	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	CCAACTTGATGTTGACGTTGTAGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGC	seq:591	120	0	0/0	0/0	0/0	0/0	BslI:18 BsaXI:22	8
7	CCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGG	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	CCACGCCGCCGTCCTCGAAGTTCTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCT	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	CCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCCGTCCTCGAAGTTCATCACGCG	seq:240	36	0	0/0	0/0	0/0	3/1	BsoBI:16 AvaI:16 HpyAV:21 BsaJI:17	11
10	CCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCCGTCCTCGAAGTTCATCACGCG	seq:245	39	0	0/0	0/0	0/0	3/1	BsoBI:11 AvaI:11 HpyAV:16 BsaJI:12	13
11	CCGTCCTCGAAGTTCATCACGCGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCT	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	CCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCCAACTTGATGTTGACGTTGTAG	seq:554	113	0	0/0	0/0	1/0	3/0	ApeKI:4 TseI:4 MspA1I:4 NciI:9 PvuII:4 StyD4I:7 Fnu4HI:5 ScrFI:9	15
13	GGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCA	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	CCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGCCTCGAAGTTCATCACGCGCTCC	seq:240	35	0	0/0	0/0	0/0	3/2	BsoBI:16 AvaI:16 HpyAV:21 BsaJI:17	17
15	CCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGG	seq:414	79	0	0/0	0/0	0/0	4/1	BseRI:6 StuI:13 BseYI:5	19
16	CCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACCACGATGGTGTAGTCCTCGTTG	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

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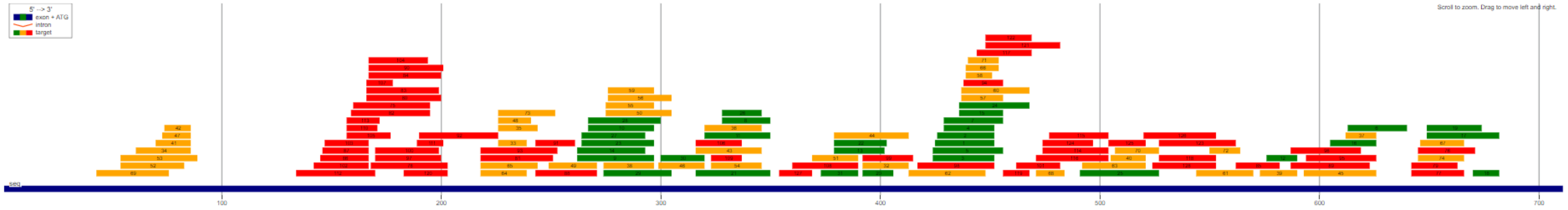

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1	CCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCCTCGGGGTACATCCGCTCGGAG	seq:403	75	0	0/0	0/0	0/0	0/0	BtgI:10 MboII:14,17 BbsI:12 BsaJI:10 StuI:24 PflMI:16 NcoI:10 BseYI:16 BslI:16 StyI:10	1
2	CCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCCTCGGGGTACATCCGCTCGGAG	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	GCATTACGGGGCCGTCGGAGGGGAGAAGAAGACCATGGGCTGGGAGGCCTCCCTCGGGGTACATCCGCTCGGAG	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	CCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCCTCGGGGTACATCCGCTCGGAG	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	GCATTACGGGGCCGTCGGAGGGGAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGG	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	CCAACTTGATGTTGACGTTGTAGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGC	seq:591	120	0	0/0	0/0	0/0	0/0	BslI:18 BsaXI:22	8
7	CCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGG	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	CCACGCCGCCGTCCTCGAAGTTCTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCT	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	CCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCCGTCCTCGAAGTTCATCACGCG	seq:240	36	0	0/0	0/0	0/0	3/1	BsoBI:16 AvaI:16 HpyAV:21 BsaJI:17	11
10	CCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCCGTCCTCGAAGTTCATCACGCG	seq:245	39	0	0/0	0/0	0/0	3/1	BsoBI:11 AvaI:11 HpyAV:16 BsaJI:12	13
11	CCGTCCTCGAAGTTCATCACGCGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCT	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	CCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCCAACTTGATGTTGACGTTGTAG	seq:554	113	0	0/0	0/0	1/0	3/0	ApeKI:4 TseI:4 MspA1I:4 NciI:9 PvuII:4 StyD4I:7 Fnu4HI:5 ScrFI:9	15
13	GGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCA	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	CCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGCCTCGAAGTTCATCACGCGCTCC	seq:240	35	0	0/0	0/0	0/0	3/2	BsoBI:16 AvaI:16 HpyAV:21 BsaJI:17	17
15	CCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGG	seq:414	79	0	0/0	0/0	0/0	4/1	BseRI:6 StuI:13 BseYI:5	19
16	CCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACCACGATGGTGTAGTCCTCGTTG	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

IMPORTANT - CHOPCHOP results **include** the PAM sequence!

Remember Region to avoid

***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	CCGTGAACGGCCACGAGTTCGAG	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	CCGCCCCTACGAGGGCACCCAGA	CCGCCAAGCTGAAGGTGACCAAGGG	CCCAGGCGAAGGGCAGGGGGCCA	120
86	CCCCTACGAGGGCACCCAGACCG	CCAAGCTGAAGGTGACCAAGGG	CCCAGGCGAAGGGCAGGGGGCCA	123
87	CCCTACGAGGGCACCCAGACCGC	CAAGCTGAAGGTGACCAAGGG	CCCAGGCGAAGGGCAGGGGGCCA	124
103	CCTACGAGGGCACCCAGACCGCC	AAGCTGAAGGTGACCAAGGG	CCCAGGCGAAGGGCAGGGGGCCA	125
105	CCTTCAGCTTGGCGGTCTGGGTG	TGACCAAGGGTGGCCCCCTG	CCCTTCGCCTGGGACATCCTGTC	135
110	CCTTCAGCTTGGCGGTCTGGGTG	TGACCAAGGGTGGC	CCCCTGCCCTTCGCCTGGGACAT	135
113	CCTTCAGCTTGGCGGTCTGGGTG	TGACCAAGGGTGGCC	CCCTGCCCTTCGCCTGGGACATC	135
82	CACCTTCAGCTTGGCGGTCTGGG	ACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATC	CCGTACATGAACTGAGGGGACAG	137
75	TCACCTTCAGCTTGGCGGTCTGG	CCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATC	CCGTACATGAACTGAGGGGACAG	138
80	CCTTGGTCACCTTCAGCTTGGCG	GTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTC	CCCTCAGTTCATGTACGGCTCCA	144
83	CCTTGGTCACCTTCAGCTTGGCG	GTGGCCCCCTGCCCTTCGCCTGGGACATCCTGT	CCCCTCAGTTCATGTACGGCTCC	144
107	CCTTGGTCACCTTCAGCTTGGCG	GTGGCCCCCTGC	CCTTCGCCTGGGACATCCTGTCC	144
84	CCCTTGGTCACCTTCAGCTTGGC	TGGCCCCCTGCCCTTCGCCTGGGACATCCTGTC	CCCTCAGTTCATGTACGGCTCCA	145
90	CCCTTGGTCACCTTCAGCTTGGC	TGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCC	CCTCAGTTCATGTACGGCTCCAA	145
104	CCCTTGGTCACCTTCAGCTTGGC	TGGCCCCCTGCCCTTCGCCTGGGACAT	CCTGTCCCCTCAGTTCATGTACG	145
78	CCAAGCTGAAGGTGACCAAGGGT	GGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCC	TCAGTTCATGTACGGCTCCAAGG	146
97	CCACCCTTGGTCACCTTCAGCTT	CCCCCTGCCCTTCGCCTGGGACATCCTGTC	CCCTCAGTTCATGTACGGCTCCA	148
100	CCACCCTTGGTCACCTTCAGCTT	CCCCCTGCCCTTCGCCTGGGACATCCTGT	CCCCTCAGTTCATGTACGGCTCC	148
120	CCAAGGGTGGCCCCCTGCCCTTC	GCCTGGGACATCCTGTCCCC	TCAGTTCATGTACGGCTCCAAGG	161

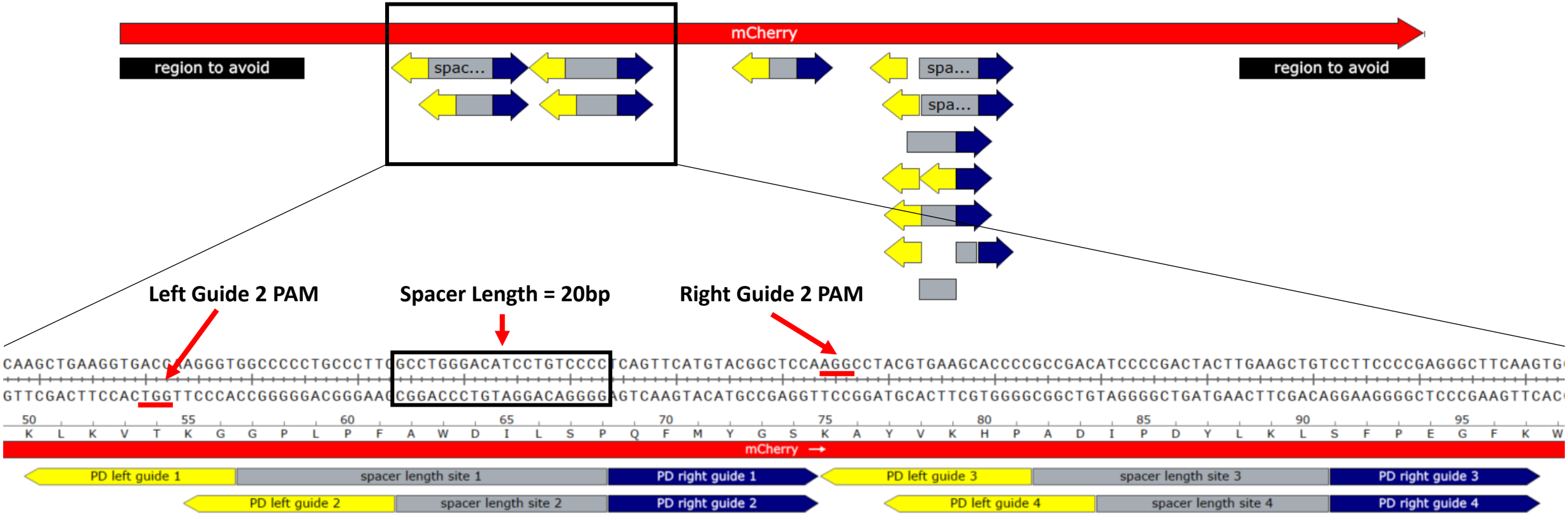
Excel Table Download of Perfect Day Target

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

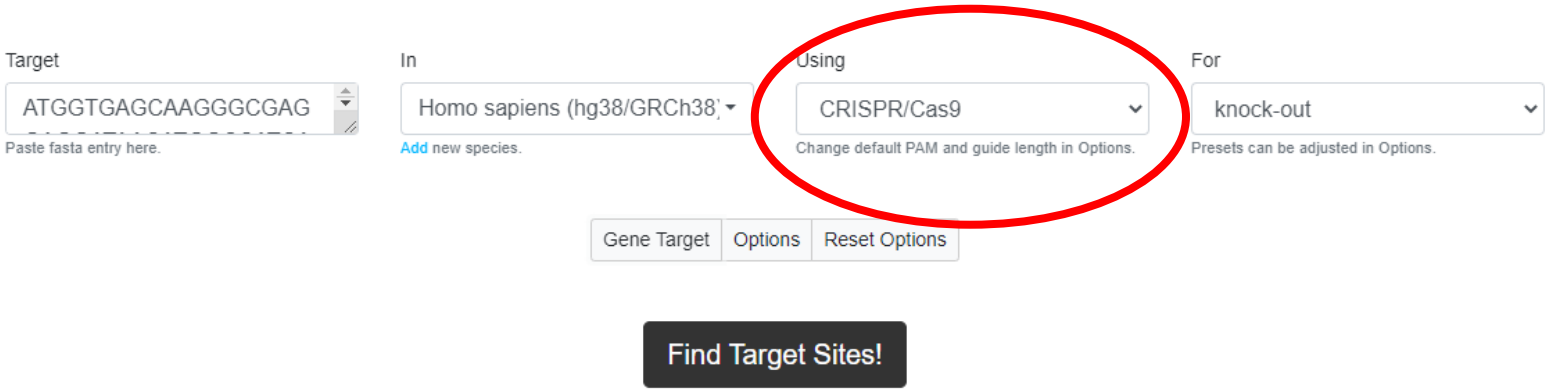
Take the results from Excel and import them into SnapGene → After the importing you will have a refined version like below.

	104	CCCTTGGTCACCTTCAGCTTGGC	TGGCCCCCTGCCCTTCGCCTGGGACAT	CCTGTCCCCTCAGTTCATGTACG	145
PD site 1	78	CCAAGCTGAAGGTGACCAAGGGT	GGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCC	TCAGTTCATGTACGGCTCCAAGG	146
	97	CCACCCTTGGTCACCTTCAGCTT	CCCCCTGCCCTTCGCCTGGGACATCCTGTC	CCCTCAGTTCATGTACGGCTCCA	148
	100	CCACCCTTGGTCACCTTCAGCTT	CCCCCTGCCCTTCGCCTGGGACATCCTGT	CCCCTCAGTTCATGTACGGCTCC	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

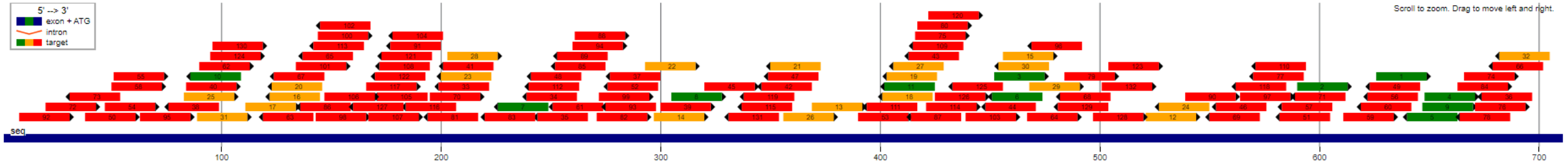
CHOPCHOP can be used just like CRISPOR to identify single guide RNAs for use with Cas-CLOVER



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

fastaInput.fa



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	CAACGAGGACTACACCATCGTGG	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	GAACTTCGAGGACGGCGGCGTGG	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	TGCATTACGGGGCCGTCGGAGGG	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

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~~	~~21~~	~~+~~	~~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~~	~~CTCGAACTCGTGGCCGTTCACGG~~	~~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	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	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~~	~~CAAGGGCGAGGAGGATAACATGG~~	~~9~~	~~+~~	~~55~~	~~0~~	~~46.7~~
~~72~~	~~GGATAACATGGCCATCATCAAGG~~	~~21~~	~~+~~	~~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~~	~~CTCGAACTCGTGGCCGTTCACGG~~	~~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	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	GAAGGGCAGGGGGCCACCCTTGG	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!

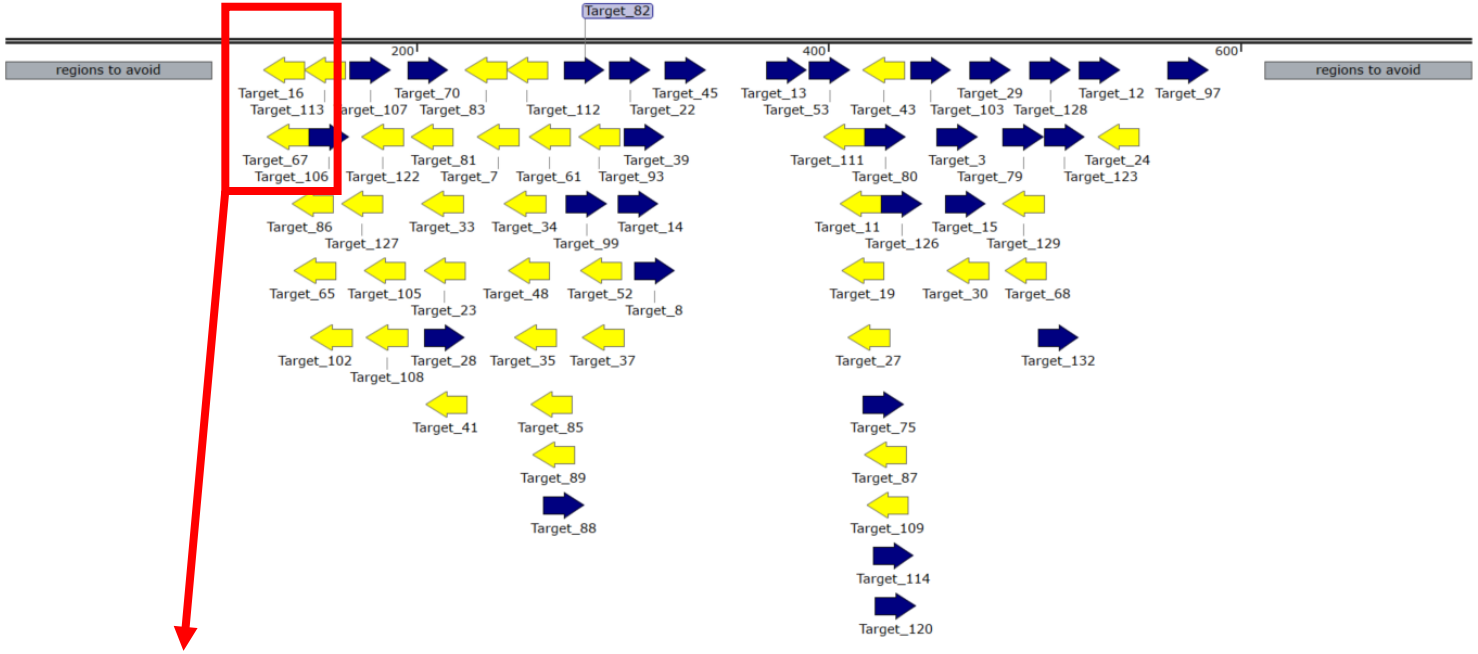
Initial Parameters

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

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

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	Efficiency
92	CAAGGGCGAGGAGGATAACATGG	9	+	55	0	46.7
72	GGATAACATGGCCATCATCAAGG	21	+	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	CTCGAACTCGTGGCCGTTCACGG	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
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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	GAAGGGCAGGGGGCCACCCTTGG	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

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.

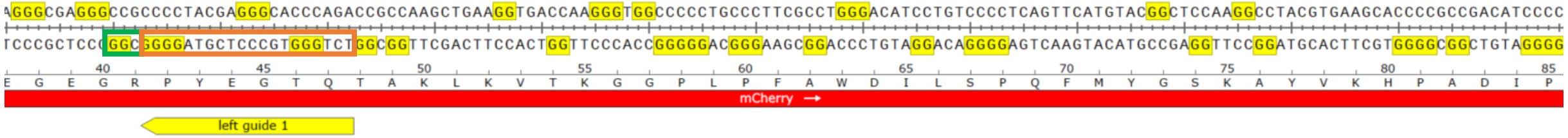
Manually Designing sgRNA Pairs for Cas-CLOVER

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

Manually Designing sgRNA Pairs for Cas-CLOVER

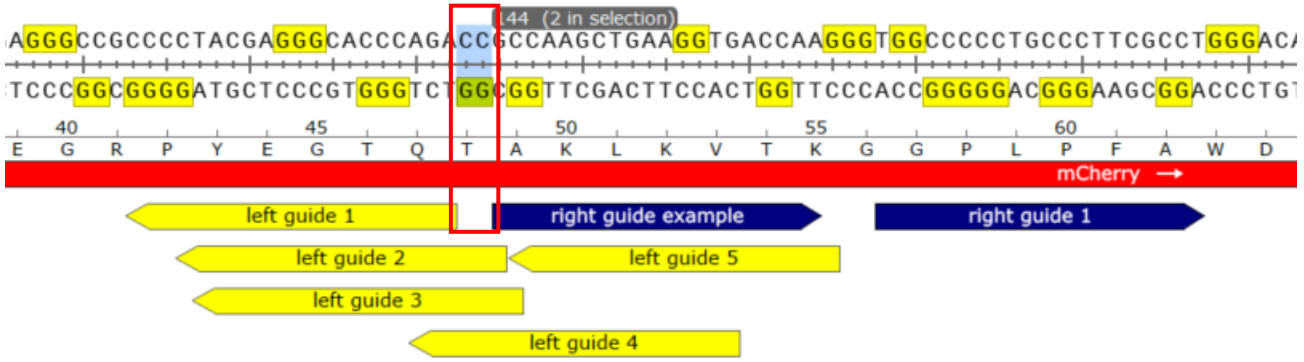
3) Avoid the 100bp 5′ and 3′ regions if sequencing analysis will be performed
4) Begin on the reverse strand to identify your first potential left guide (see green boxes)

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



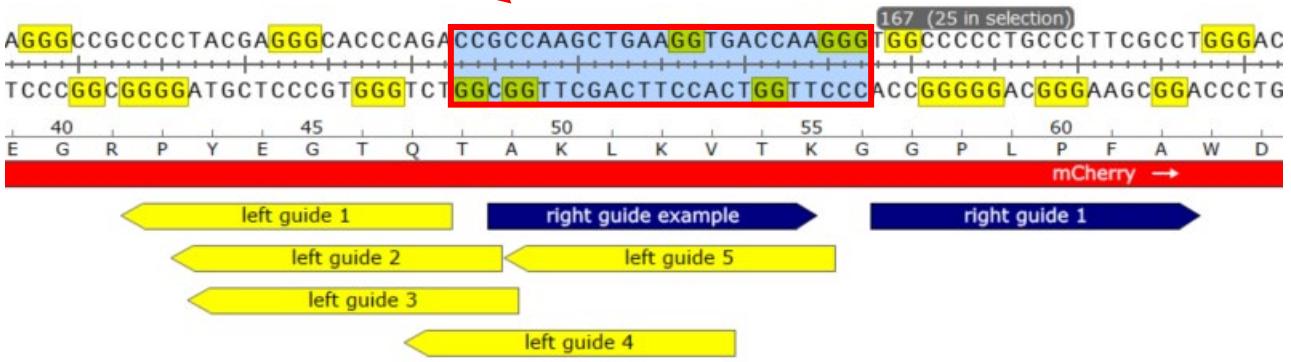
Manually Designing sgRNA Pairs for Cas-CLOVER

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

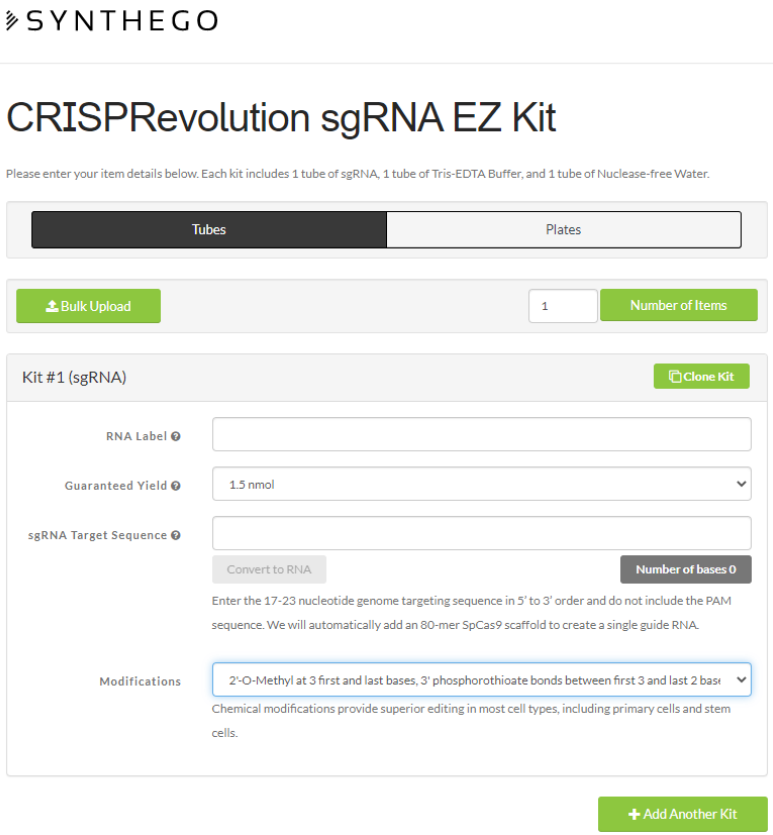
Production of sgRNA Pairs for Cas-CLOVER

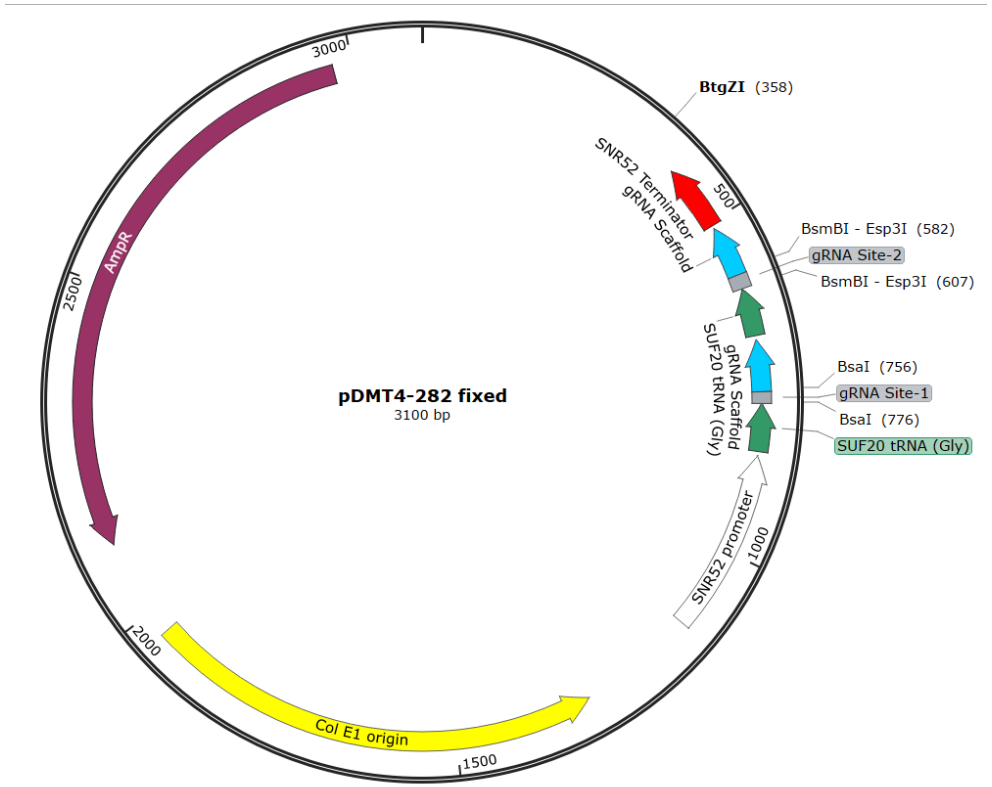
Option #1: Synthetic guide synthesis

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)

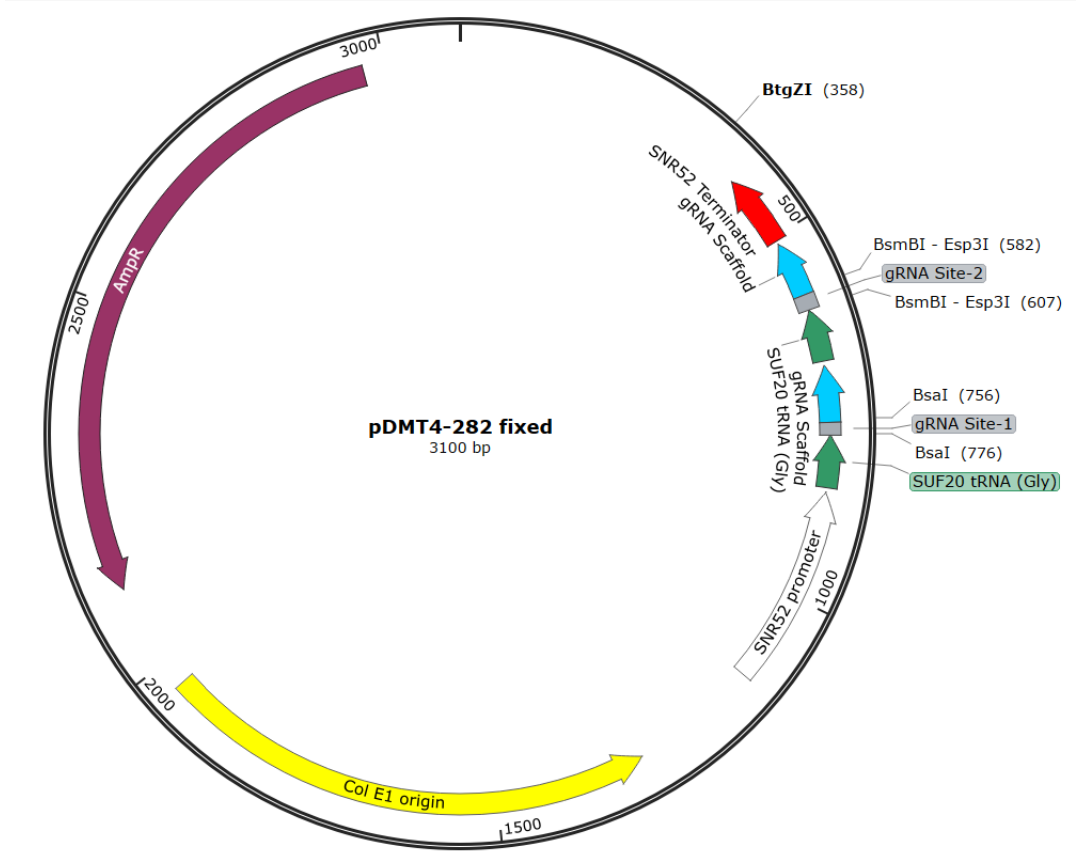
Option #2: Plasmid-based sgRNA expression

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





Cloning of sgRNA Pairs for Cas-CLOVER



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

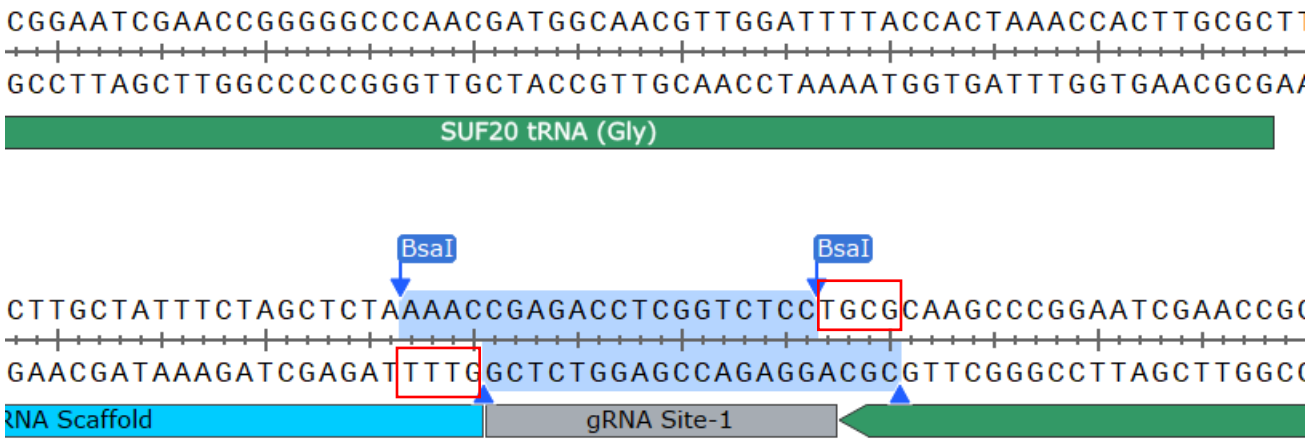
Contains:
GGA sites (BsaI and BsmBI)
Dual guide cassette driven by SNR52 promoter
E.Coli components (AmpR, ori)

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

Demeetra

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’)

aaacACGAGGGCACCCAGACCGCC

Annealed oligos

aaacACGAGGGCACCCAGACCGCC

TGCTCCCGTGGGTCTGGCGGacgc

Left guide sequence (bottom strand read right to left)

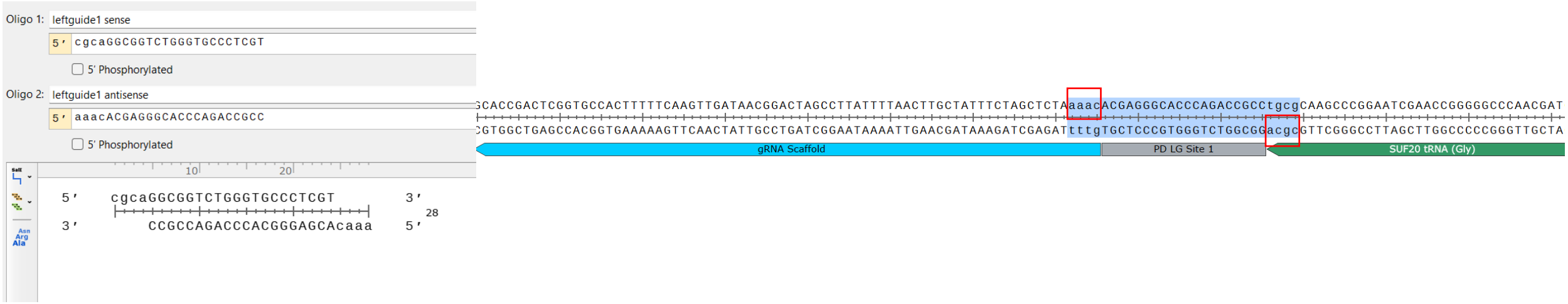
Site 1: GGCGGTCTGGGTGCCCTCGT

Site 2: AGGACAGCTTCAAGTAGTCG

Site 3: GGGGCCGTCGGAGGGGAAGT

Demeetra

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 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.

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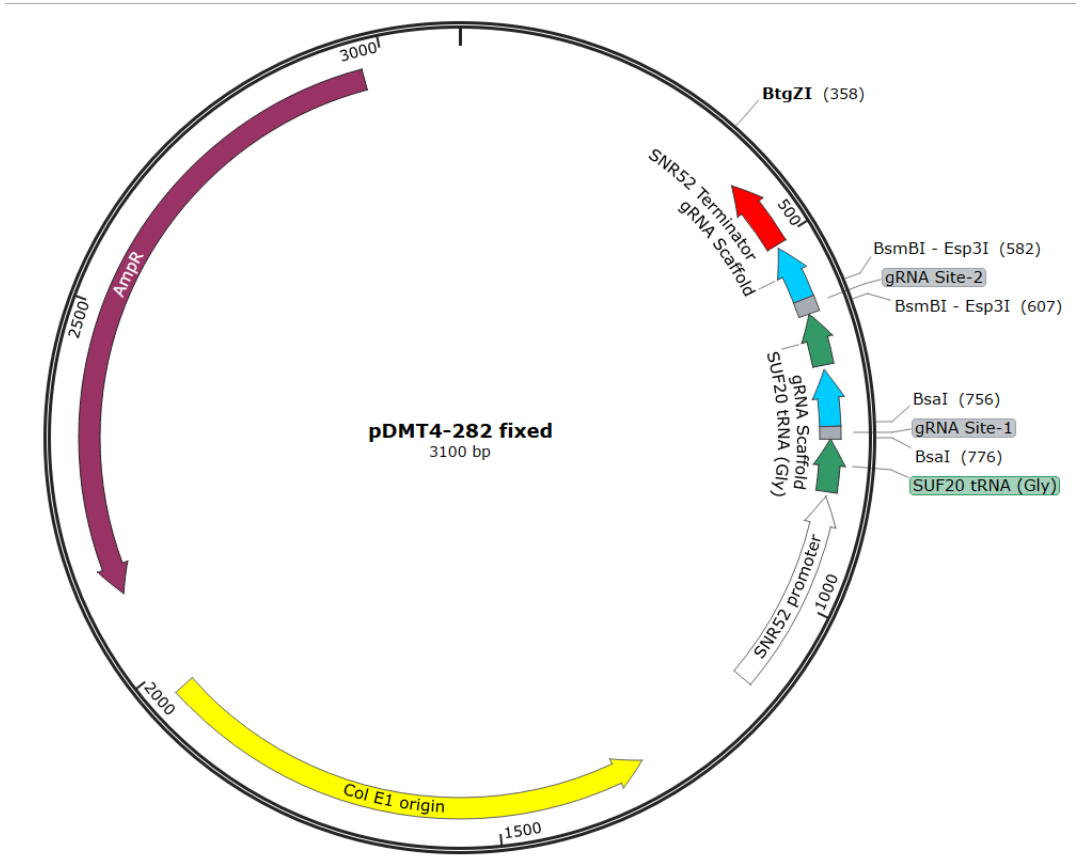
Cloning of sgRNA Pairs for Cas-CLOVER



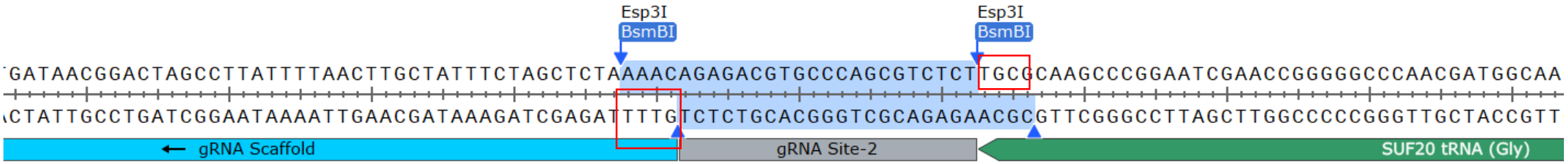
Golden Gate Assembly (GGA)
Destination Plasmid → pDMT4-282

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

Demeetra

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.

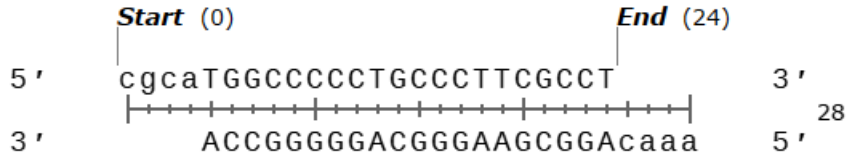
Oligo 1 (**top** strand 5' to 3')

cgcaTGGCCCCCTGCCCTTCGCCT

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

aaacAGGCGAAGGGCAGGGGGCCA

Annealed Oligos



Right guide sequence (top strand read left to right)

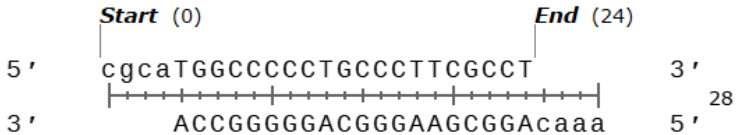
Site 1: **TGGCCCCCTGCCCTTCGCCT**

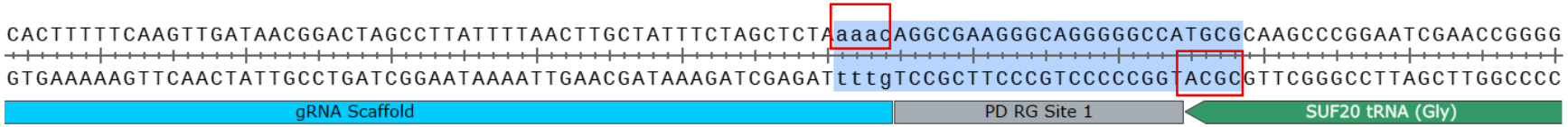
Site 2: CGCGTGATGAACTTCGAGGA

Site 3: GCTGGGAGGCCTCCTCCGAG

Demeetra

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

Demeetra

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).

Demeetra