## Casellas Lab CRISPR Protocol

The "CRISPR sgRNA design" presentation complements this protocol with useful illustrations of steps.

Design and test 3-4 CRISPRs per targeted genomic region. Choose the CRISPR with the highest frequency of cutting, as assessed via the T7 Endonuclease assay, for actual genome editing experiment.

Updated Rules for sgRNA design.

- a) 18-mer guide sequences are more sensitive to mismatches than a 20-mer sgRNA. Consequently, a shorter sgRNA induces low or undetectable off-targets with 1 or 2 mismatches while 20-mer sgRNAs cause higher levels of mutagenesis at off-targets with 4 or 5 mismatches (5).
- b) sgRNA should target the non-transcribed strand (11).
- c) sgRNA should target the earliest coding exons when possible (11).
- d) SEED sequences of guide should be unique (6).
- e) Target GGG PAM sequences may be ideal for conventional Cas9(6).
- f) GC content of sgRNA should be within 40 to 60% (11).
- g) For 20-mer sgRNA, the four last 3' NTs should be : 5'-....NNN-R<sub>(17)</sub>-[R/Y]<sub>(18)</sub>- R<sub>(19)</sub>-R<sub>(20)</sub>-3' (11).

CRISPR sgRNA Design.

- 1. For strategies based on homologous recombination, select a genomic target from 23 to 250 nucleotides around the expected cutting site (sense strand or non-coding strand of genic sequence).
- 2. Verify that the selected target sequence is unique in the genome (e.g. use BLAT tool on UCSC genome browser and use Mappability or Repeat Masker tracks in browser).
- In the CRISPR web tool: http://crispr.ybzhao.com enter "task description", name and email address in "Information" section, select "Target sgRNA Size", "PAM type", designate target genome, and enter genomic sequence (Figure 1).
- 4. Once the CRISPR web tool has finished calculating scores for all discovered guide sequences, you will get a link to the result page by your email.

sgRNA Online <sup>1.0 Beta</sup>		Example							
' <b>sgRNA Online'</b> is an o xCa9 3.7.	online tool that ide	ntifies efficient sgRNAs for CRISPR editing of mouse and human genomes. It supports both classic Cas9 and							
Information									
Task description (*)	geneA								
Your name (*)	name								
Email (*)	email@nih.gov								
Parameters									
PAM type	classic Cas9 (PA	M: NGG)							
Target sgRNA Size 20bp 18bp									
Target genome	Mouse (mm10)	\$							
Sequence(s):	AGTATCTGCCG	CAGACGGAGCGAGGCGGCGGCGGCGGGGGGGGGGGGGG							
	<ul><li> If your sequ</li><li>Sequence r</li></ul>	to find optimized sgRNA in multiple sequences, please paste sequences in fasta format; Example ence(s) include repeated region/sequence, please mask them with "N"s, which will largely reduce the time cost; equirements: a) Max sequence number: 10; b) Min sequence length >=23; c) Max sequence length <= 300; al sequence number : 1, min length of sequence: 200, max length of sequence: 200.							
		Submit							
2018 This web server is maintained by Casellas Lab at NIH/NIAMS Developed & Designed by Yongbing Zhao									

Figure 1. CRISPR web tool Input page at http://crispr.ybzhao.com

	na npio	data, re	ferenced id: 6ffc5bee490e0e	2dee3353a1665a755	2				
ormati	lon	Summar	y Result Download						
	Sequen	e: exa	mplel(127 bp)						
	GTCGC	TCGTCG	GAGCTGCAGGGACCGGCGCGAGCG	AGTGCTGGACTGTTTGT	GCAGGGCTCCGAGGGGACCCATGTGGCTCA	GGGTGGCTAN	GGGGGCAATGCTGCGTCGTCGT	GTTTTTTGGGGGG	
	Potent	al gui	ide sgRNA number: 71						
				Cu La	sgRNA # 1				
	•		Sequence	sgRNJ	A score: 95				
	#1	95	GGGCAATGCTGCGTCGTCGTAGT		ontent: 65% nd: Forward				
	#2	94	CCARARARCTACGACGACGCAGC	Poter	ntial off-target sites: 66 (Mi				
	#3	92	OCTOCOTCOTCOTACTTTTTCC	Oligo	os to order: FWD: caccGGGCAATG	CTGCGTCGTCG	T REV: aaacACGACGACGCA	GCATTGCCC	
	#4	90	AGGGGGCAATGCTGCGTCGTCGT			h			
	#5	89	AAACTACGACGACGCAGCATTGC	Top	<pre>20 off-target sites were shown Sequence</pre>	Score	Mismatch	Genomic locus	Genomic annotation
	#6	89	CCCCCAAAAAACTACGACGACGC	#1	GGGCggcGCcGCGTCGTCGT CGT	0.51	4MMs [5:6:7:10]	chr18 38418891 +	Ndfip1 TSS upstream +2kb,Ndfip1 exon
	#7	87	AGTCCAGCACTCGCTCGCGCCGG	#2	GGGacATGLTLCGTCGTCGT COT	0.47	400ts [4:5:9:11]	chr8 119874183 -	- 1
	#8	84	GTCCCTGCAGCTCCGACGAGCGA	#3	AGGCAAAGCAGgGTCGTCGT GGT	0.45	49998 [1:7:10:12]	chr17 86347110 -	
	#9	84	AACAGTCCAGCACTCGCTCGCGC	*3	CAGCARTGCTOCCTOSTOST TGA	0.35	4994s [1:2:6:13]	chr17 50086767 -	
	#10	83	GACCGGCGCGAGCGAGTGCTGCA						
	#11	83	GTCCAGCACTCGCTCGCGCCGGT	#5	GAACAATGCTGAGTCGACGT GGC	0.29	400ts [2:3:12:17]	chr7 55988953 +	
	#12		CGGTCCCTGCAGCTCCGACGAGC	#6	GGeCAAgGaTGCeTCGTCGT AGA	0.23	4MMs [3:7:9:13]	chr13 40800542 +	
	#13		GCGTCGTCGTAGTTTTTTGGGGGG	#7	aGGaAAgGCTGCGTCGTgGT GGT	0.21	4MMs [1:4:7:18]	chr13 107793431 -	
	#14		GGACCGGCGCGAGCGAGTGCTGG	#8	GGeCAcTGaTGCGTCGTCtT CGA	0.18	4MMs [3:6:9:19]	chr10 93162133 -	Mir1931 TSS upstream +2kb
	#14		TCGCTCGCGCCGGTCCCTGCAGC	*3	GGECAATGGAGCGTCGTGGT GGA	0.17	4MMa [3:9:10:18]	chr3 122440975 +	
				#10	GGatAATGCTGgGTCGTgGT AGG	0.15	4MMs [3:4:12:18]	chr3 86889856 -	
	#16		TGCGTCGTCGTAGTTTTTTGGGG	#11	GG&CAATGgTGgGTCGTCeT GGG	0.15	4MMs [3:9:12:19]	chr3 10357387 -	
	#17		CTGCGTCGTCGTAGTTTTTTGGG	#12	GGGCgAcGgTGCGTCGTaGT CGG	0.12	4MMa [5:7:9:18]	chr1 171065434 -	Mir6546 TSS upstream +2kb
	#18	77	C7AAGGGGGGCAATGCTGCGTCGT	#13	GGGCgAcGgTGCGTCGTaGT CGG	0.12	4MM8 [5:7:9:18]	chr1 171080683 -	
	#19	76	AGCCACATGGGTCCCCTCGGAGC	#14	GOGCgAcGgTGCGTCGTaGT COG	0.12	4MMs [5:7:9:18]	chr1 171073069 -	
	#20	75	GGCGCGAGCGAGTGCTGGACTGT	#15	GOGaeATGCTGCGTCagCGT GOG	0.1	4MMs [4:5:16:17]	chr17 29389361 +	
	#21	75	CGACGCAGCATTGCCCCCTTAGC	#16	GGGCAgaGCTGgGTCGTCeT ACC	0.09	4MM8 [6:7:12:19]	chr11   107881324  -	
	#22	72	TGCAGGGACCGGCGCGAGCGAGT	#17	GGGCAAgGgcGCGTCcTCGT GGG	0.09	4MMs [7:9:10:16]	chr17 34197773 +	Pamb8 TSS upstream +2kb
	#23	72	GTCGCTCGTCGGAGCTGCAGOGA	#18	AGGCAATGCTtqGTCtTCGT TOC	0.07	4MMs [1:11:12:16]	chr7 52027194 -	
	#24	71	GAGCTGCAGGGACCGGCGCGAGC	#19	GGGCLCTGCTGCGTCLTCGA TOG	0.06	40018 [5:6:16:20]	chr12 32849634 -	
	#25		GCTGCAGGGACCGGCGCGAGCGA	#19		0.00	[ararrarea]	and ye   sea a losa   -	

Figure 2. CRISPR web tool "Result" page.

- 5. In "Result" tab, you can find off target information for each sgRNA candidates.
- 6. Click "Download" tab to save all information .

## Choose a sgRNA.

- 7. In "Result" tab, choose guide sequences with 1) the least number of offtargets, 2) high PAM Score, 3) 40-60% GC content, and 4) high Cas9 Loading score if possible.
- 8. Verify that the final guide candidates have a unique genomic binding site (e.g. by Blast).
- 9. Select the "Result" tab (third tab) and utilize off-target data to determine if any true off-targets of guide may be detrimental to later experiments.
- 10. Order oligos of candidate guides as given in the "Forward/Reverse oligo primer" column.

Note# 1: The number of off-targets given per each guide represents the number of off-targets identified by the CRISPR web tool that have a functional NGG PAM motif and 100% SEED homology to its respective guide. These are "true off-targets".

Note# 2: The "Result" tab (third tab) identifies the guide sequence and guide number for each putative off-target. It designates the sequence, genome location (for target genome in UCSC browser), and strand for each off-target. In addition, it also designates if the off-target is in a genic (by UCSC gene code) or intergenic region, the number of mismatches its SEED sequence has with respect to its target guide, and if the off-target is a "true" off-target.

## Cloning of the sgRNA into the px330 vector

Addgene plasmid number for px330 used here: #42230. Alternatively, a px330\_pgk\_puro plasmid may be used if selection is desired.

sgRNA linkers phosphorylation

- Anneal primers by adding equal molar amounts of each primer to a single Ep. Tube (≈0.6nmoles), briefly vortex, spin and incubate for 10 minutes at 95 (thermomixer).
- 2. Immediately place into water bath (preheated at 85) and turn it off to allow slow annealing of the primers. Leave the reaction overnight or until it reaches room temp.
- 3. Use the following reaction to phosphorylate the annealed primers to be used as linkers in upcoming ligations:
  - a. use 6  $\mu$ l of annealed primers ( $\approx$ 100 picomoles)
  - b. add 1  $\mu l$  T4 ligase buffer
  - c. add 1  $\mu$ l T4 polynucleotide kinase
  - d. up to  $10\mu$ l of H<sub>2</sub>O
- 5. Incubate for 1 hr at 37°C.

px330 plasmid preparation

- 1. Digest 5 μ2222px330 vector in 50 μl volume reaction with 50 U of BbsI (NEB) overnight.
- 2. Add 1  $\mu$ l of CIP to the digestion mix with the px330 vector and incubate at 37°C for 90 min.
- 3. Purify the digested vector with PCR purification column from Qiagen or Zymogen.

Ligation of sgRNA into px330 vector

- 1. Ligate 50 picomoles of annealed primers with 10 ng of Bbsi digested px330 vector.
  - a. add  $1 \mu$ l of T4 ligase buffer
  - b. add 1  $\mu$ l of T4 ligase
  - c. up to  $10\mu l$  of  $H_2O$
- 2. Incubate overnight at 16 °C.

Confirm successful cloning via sequencing.

Sequencing Primer for CRISPR imaging: CRISPR\_imaging\_seq REV: 5'-CTAATGCATGGCGGTAATACG-3'

Sequencing Primer for CRISPR genome editing: px330\_CP\_rev : 5'-TATTGGCGTTACTATTGACGTCAATG-3' T7E1+ampligase\* assay to test sgRNA efficiency

\* T7 has high degree of non-specificity depending on the template. Ampligase repairs non-specific cleavage of T7 to a certain extent. Always include non-targeted sample to compare.

- 1. Transfect your favorite cells with and without (WT, control) the CRISPR vector.
- 2. 48 hours later, harvest genomic DNA using Qiagen Blood and DNeasy kit.
- 3. From the gDNA, amplify a 500bp fragment around the CRISPR binding site by PCR (use 100 ng of gDNA as template in 50 ul with Phusion polymerase).
- 4. Verify amplification by running 5  $\mu$ l on a gel.
- 5. Purify the PCR product with Zymogen Clean and Concentrator kit and elute with 10-15 ul of 0.1xEB.
- 6. Measure OD.
- 7. Prepare 800 ng DNA in 20  $\mu$ l reaction of 1x ampligase buffer (2  $\mu$ l 10x ampligase buffer, DNA volume, and up to 18 ul dH<sub>2</sub>O).
- 8. Anneal using thermocycler: 95 °C for 5 min with 100% ramp rate, 95-85 °C at -2 °C/s (85°C at time 0 ramp rate at 80%), 85-25 °C at -0.1 °C/s (25°C at time 0 at ramp rate 3.7%); hold at 4 °C (4% at 100% ramp rate).
- 9. Take 10 μl annealed DNA +2 μl ampligase + 1μl T7E1, incubate 40 min at 37°C.
- 10. Run on ≥2% agarose gel and verify additional bands in mutant wells compared to wildtype wells.

## **References & Further Reading**

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