Designing and Constructing CRISPR/Cas9

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Designing a CRISPR/Cas9 for your *Glycine max* gene of choice
(Steps 1-3 credit Justin Anderson ande9112@umn.edu)
Note: All of our CRISPR/Cas9 information can be found at:
http://stuparlab.cfans.umn.edu/protocols/crisprcas9-glycine-max/

1) Find your gene
    a) Go to www.arabidopsis.org/
    b) Search your gene of interest in the top right corner i.e. RNA Polymerase
    c) Click on a locus (AT#G#####)
    d) Scroll down to the "Protein Data" section and click on the name (AT#G#####)
    e) Scroll down to the "Sequence" section and copy the entire amino acid sequence of your gene (including the numbers is fine)
2) Use BLAST to find your gene's Soybean version
    a) Go to www.phytozome.net/
    b) Click on the large box with the list of species
    c) Click on the little box next to *Glycine max*
    d) Under "2. Choose a tool:" expand "BLAST search"
    e) Select "proteome" instead of "genome: masked"
        — The bar under proteome should say BLASTP- protein query to protein db
    f) Paste your amino acid sequence from part one into the Query sequence box (the numbers can be included and will not affect the BLAST)
    g) Click "submit" to begin the BLASTp search
3) Pick a target gene from the BLAST results
    a) Browse the list of BLAST results for a corresponding gene
    b) Click the box "Gene Page" to open the details about this gene
    c) Confirm the description, domain, or annotations fit your original interests
    d) Open the "Sequences" tab near the top
    e) Expand "Genomic sequence" to view the genomic context
    f) The color key is in the upper right, Blue indicates CDS
    g) Copy the entire genomic sequence
4) Use your Glyma to find CRISPR/Cas9 target sites
    a) Go to http://cfans-pmorrell.oit.umn.edu/CRISPR/
    b) Enter your Glyma number (v1.1 or v2.1a of the genome assembly) or sequence into the text box and click submit
    c) Once submitted scroll down to find your designed CRISPR target region (this site is designed to target GN19NGG recognition sites only)
    d) Pick a target site with a unique five to six base pair restriction enzyme that cuts between 15-19 bp for forward CRISPRS and 4-8bp for reverse compliment CRISPR sites
    e) If you are going to use Stupar Lab destination vectors order the displayed target oligos from your favorite site

5) Order you plasmids from Addgene.org (skip if you already order them)
   a) Go to http://www.addgene.org/CRISPR/
   b) In the right side of the page there will be an orange box with the title "CRISPR Depositing Labs" click the Robert Stupar link
   c) You will find a list of plasmids available, you MUST select the pBlu gRNA and one or more of the destination vectors

Name	Backbone	Promoter	Non-bacterial selection	Bacterial Selection	DoubleNLS	Digestion Enzyme
pBlu gRNA	pBluescript KS(+)	U6	NA	Ampicillin	NA	BbsI
Cas9 MDC32	PMDC32	2x35S	Hygromycin	Kanamycin	no	EcoRI
Cas9 MDC123	PMDC123	2x35S	Bar cassette	Kanamycin	no	EcoRI
G10 Cas9 MDC123	PMDC123	G10	Bar cassette	Kanamycin	yes	EcoRI

   d) Order selected plasmids and proceed to assembly
6) Assembly Round 1
   a) Dilute your target oligos and anneal at 50C for 6 hours

Annealing target oligos	Concentration	amount
Forward target oligo	100ng/ul	10ul
Revese target oligo	100ng/ul	10ul
PCR buffer	10X	2.2ul
Total		22.2ul
Anneal at 50C for 6 hours		

   b) Digest pBlu gRNA with Bbsi (New England Biolabs # R0539S)

Digestion with BbsI	amount
water	7ul
NEB cutsmart buffer	2ul
vector (1 microgram)	10ul
BbsI (5,000 units/ml)	1ul
Total	20ul
Digest at 37C for 5 hrs then heat inactivate for 15 minutes	

   c) Run the product from step 6b on a gel and extract 3500 base pair band

d) Ligation of target oligos and BbsI cut pBlu/gRNA (NEB #M0202S)

Ligation of target oligos and Bbsi cut pBlu gRNA	
Vector	2ul
Target oligos	2ul
T4 ligase buffer	2ul
T4 ligase	1ul
H20	up to 20ul
Total	20ul
Ligate overnight at 16C	

e) Clone into Dh5 alpha (Life technologies #18258-012)
   i) Add 5ul of ligation to comp cells on ice
   ii) Heat shock at 42C for 30 seconds
   iii) Place cells immediately back on ice
   iv) Add 250 ul LB broth
   v) Grow cells for 30min-1hr at 37C
   vi) Plate 40-80 ul on LB agar with Ampicilli 100mg/L resistance grow overnight
   vii) Pick three colonies into 15ml tubes and grow in 3ml LB Amp 100mg/L overnight

f) Miniprep and Sequence
   i) Extract DNA from ecoli using a miniprep kit (Qiagen # 27104)
   ii) Sequence DNA using T3 primer: aattaaccctcactaaaggg

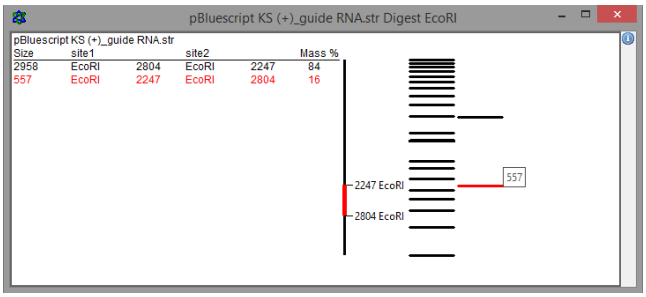
7) Assembly Round 2

a) Digest sequenced pBlu/gRNA/insert and destination vector of choice using EcoRI (NEB # R0101S)

Digestion with EcoRI	**pBlu/gRNA/insert**	**Destination Vector**
Water	7ul	7ul
NEB cutsmart buffer	2ul	2ul
Vector/insert	10ul	10ul
EcoRI	1ul	1ul
Total	20ul	20ul
Digest at 37C for 5 hrs then heat inactivate for 15 minutes		

b) CIP treat destination vector (NEB # M0289S)
   i) Add 2.5 ul of Antarctic Phosphatase Reaction Buffer directly to the ligation
   ii) Add 1 ul of Antarctic Phosphatase
   iii) Incubate at 37C for 1 hour
   iv) Incubate at 70C for 5 minutes

c) Run EcoRI cut pBlu/gRNA/insert on a gel



d) Cut out 557 bp band using gel extraction kit (Qiagen # 28704)

e) Ligation of gRNA insert and CIP treated destination vector

Ligation of gRNA and Destination vector	
Destination vector	1ul
gRNA fragment (EcoRI cut)	4ul
T4 ligase buffer	2ul
T4 ligase	1ul
H20	up to 20ul
Total	20ul
Ligate overnight at 16C	

f) Clone ligation into Dh5 alpha with Colony PCR

i) Add 5ul of ligation to competent cells on ice

ii) Heat shock at 42C for 30 seconds

iii) Place cells immediately back on ice

iv) Add 250 ul LB broth

v) Grow cells for 30min-1hr at 37C

vi) Plate entire solution on LB agar with Kanamycin100mg/L resistance grow overnight

vii) Pick three colonies (MAKING SURE TO REPEAT PIPETTE INTO YOUR COLONY PCR BEFORE PUTTING YOUR PIPPETTE TIP INTO YOUR 15ml TUBE) and grow in 3ml LB Kanamycin 100mg/L overnight

Colony PCR	
Qiagen HS+ MM	10ul
primers F+R (10um)	3ul
water	7ul
one colony	repeat pipette
Step1: 95C for 5 min	
Step 2: 95C for 45 seconds	
Step 3: 52C for 45 Seconds	
Step 4: 72C for 1 minute	
Step 5: go back to step 2 for 30 cycles	
Step 6: 72C for 5 min	

g) Run colony PCR product on a gel to screen for present absent 550bp gRNA band

h) Miniprep gRNA positive 15 ml tubes

You have now completed CRISPR/Cas9 the next step is to either transform the vector yourself or send it the facility of your choice.