**Generation of the sgRNA-Cas9-YFP from the pSAG1::CAS9-U6::sgUPRT vector from Sibley’s lab** (vector: [**http://www.addgene.org/54467/**](http://www.addgene.org/54467/)**)**

**A. Design the primer containing the specific sgRNA sequence and the sequence allowing the annealing on the vector**

1) Find a **NGG** in your target DNA region of interest. Be careful, use the GT1 or RH DNA sequences if available instead of the ME49 (which is Pru).

2) Design your **sgRNA** of 20 nucleotides length preceding your PAM.

Note: the PAM sequence should not be included as a part of the sgRNA. The PAM sequence is located in the genomic target site, immediately following the 20-bp target sequence, but it is not a part of the sgRNA. Only the 20-bp target sequence is used to construct the sgRNA.

3) If possible find a G at the first position of your sgRNA (**underlined G**).

Note: For efficient U6 transcription of sgRNA, a G is preferred at the 5' position, which corresponds with the first base of the 20-bp guide sequence. For guide sequences that do not begin with a G, we recommend adding an additional G to the 5' of the guide sequence, resulting in a 21-bp guide sequence (5'-GNNNNNNNNNNNNNNNNNNNN-3', where the 20 Ns correspond to the 20-bp genomic target sequence immediately upstream of the 5'-NGG PAM). The addition of a 5' G does not alter the specificity of the sgRNA or affect the efficiency of Cas9 cleavage (Ran et al., Nature Protocols 2013)

4) BLAST on the genome to be sure there are no off-targets

**Example:**

Target DNA region

**TTCCTTCTTTGCTGAAACAACCAGGCTCGCCGTGCTCTCTGCATCATGGCGATTGGAAAAGGTACTTTT**

**PAM: NGG**

**sgRNA: GCTCTCTGCATCATGGCGAT (N= 20 nt)**

Primer design

gRNA-Fw: **GCTCTCTGCATCATGGCGAT**GTTTTAGAGCTAGAAATAGC

(N20 is your gene specific gRNA sequence)

gRNA-Rv: AACTTGACATCCCCATTTAC (4883) primer to use for the mutagenesis

These steps are done now with the **Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (**<http://grna.ctegd.uga.edu/>), using the following settings:

RNA guided nuclease selection:

pCas9: 20nt gRNA, NGG PAM on 3’ end

optional search:

Microhomology search: (?) off

Conserved region search: (?) off

genome:

T. gondii GT1 ToxoDB-32

**Mutagenesis:**

For the PCR follow the mutagenesis NEB kit instruction

**Protocol**

**Step I: Exponential Amplification (PCR)**  
1. Assemble the following reagents in a thin-walled PCR tube.

|  |  |  |
| --- | --- | --- |
|  | **12.5 μl RXN** | **FINAL CONC.** |
| Q5 Hot Start High-Fidelity 2X Master Mix | 6.25 μl | 1X |
| 10 μM Forward Primer | 0.625 μl | 0.5 μM |
| 10 μM Reverse Primer | 0.625 μl (4883) | 0.5 μM |
| Template DNA (1–25 ng/μl) | 1 μl | 1-25 ng |
| Nuclease-free water | 4 μl |  |

2. Mix reagents completely, then transfer to a thermocycler.  
  
3. Perform the following cycling conditions:

|  |  |  |
| --- | --- | --- |
| **STEP** | **TEMP** | **TIME** |
| Initial Denaturation | 98°C | 30 seconds |
| 25 Cycles | 98°C | 10 seconds |
| 56°C | 30 seconds |
| 72°C | 5 minutes |
| Final Extension | 72°C | 2 minutes |
| Hold | 16°C |  |

**Step II: Kinase, Ligase & DpnI (KLD) Treatment**  
1. Assemble the following reagents:

|  |  |  |
| --- | --- | --- |
|  | **VOLUME (5ul)** | **FINAL CONC.** |
| PCR Product | 1 μl |  |
| 2X KLD Reaction Buffer | 2.5 μl | 1X |
| 10X KLD Enzyme Mix | 0.5 μl | 1X |
| Nuclease-free Water | 1 μl |  |

2. Mix well by pipetting up and down and incubate at room temperature for 5-10 minutes.

**Step III: Extensive DpnI degestion**

add to the tubes:

39 μl of ddH2O

5 μl of cutsmart buffer

1 μl of DpnI

Incubate for 2 hours at 37°C

**Step IV: Transformation**  
1. Thaw a tube of Competent *E. col*i cells on ice.  
2. Add the 5 μl of the KLD mix from Step II to the tube of thawed cells. Carefully flick the tube 4-5 times to mix. Do not vortex.  
3. Place the mixture on ice for 20 minutes.  
4. Heat shock at 42°C for 30 seconds.  
5. Place on ice for 5-10 minutes.  
6. Plate on Amp LB agar

**Step V: screen colonies by miniprep and digestion**

1. Pick 2-3 colonies for miniprep

2. Do a SacI digestion on 1-2 μl of the miniprep

Pattern should be 5953+3077+645 bp

**Step VI: sequencing**

1. Send for sequencing using M13 reverse

3. If OK start a midiprep to transfect 15-20 μg of the vector

**Transfection**

- Transfect 1/3 of a 6cm dish with 15-20 μg of your gRNA.

- If you are co-transfecting another vector, your gRNA sequence should be ABSOLUTELY absent of your vector.

- If you are co-transfecting another vector, you should try to use at least 20x more than your gRNA (in terms of copy number).

- If you don’t provide DNA template to repair, you should rather go in RH than Ku80 (see Lourido’s paper).

**FACS**

- You should syringe your parasites at least once. No need to change culture medium or to concentrate the parasites (note that the serum is slightly auto-fluorescent in the red channel)

- 5-10 parasites per well in your 96 well plate is a good ratio.

- It is possible to clones different strains in the same 96 well plate! It will save some cells.

- There will be soon other CRISPR/Cas9 vectors in DsRed, YFP, and mCherry.