**Lang Lab CRISPR Protocol**

*Modified by R. Vignogna from Brian Metzger, based off Laughery et al. 2015. Yeast.*

1. **pGIL094 Miniprep** (this is the Cas9 plasmid)
   1. Grow pGIL094 overnight in 5mL LB + amp
   2. Miniprep plasmid and nanodrop

**Note: pGIL094 is maintained in dam-/dcm- cells, necessary for digestion step**

1. **pGIL094 Digestion**
   1. Combine ~1μg pGIL094, 1μL BCLI, 1μL SWAI, and 5μL Buffer 3.1 (NEB) in a 50μL reaction
   2. Digest at 25°C for 6 hours and then 50°C for 6 hours
   3. Add 1μL of rSAP and incubate at 37°C for 1 hour
   4. Use Zymo clean and concentrator kit (elute 2-3 times in 10μL of elution buffer)
   5. Qubit to get concentration and confirm digestion on gel

**\*\*Check before performing steps 1&2 as we may have cut plasmid stocked\*\***

1. **gRNA Hybridization** (start in the early afternoon so ligation can occur overnight)
   1. Design gRNAs for yeast genome at wyrickbioinfo2.smb.wsu.edu/crispr.html
   2. Use 1μL of 100mM each primer, 1μL 10x T4 Ligase Buffer, and 0.5μL T4 PNK in a 10μL reaction
   3. Incubate at 37°C for 30 minutes, 95°C for 5 minutes, then decrease 1°C every minute to 25°C
2. **Ligation**
   1. Use ~100ng of digested pGIL094 vector (from step 2) with 2μL of 1/20 diluted hybridized gRNA primers
   2. Combine with 2.5μL T4 Ligase Buffer and 1μL T4 Ligase in a 25μL reaction
   3. Incubate overnight at 16°C, followed by heat inactivation at 65°C for 10 minutes
3. **Transformation in Bacteria** (first thing in the morning after ligation)
   1. Thaw DH5α cells on ice for ~5 minutes (50μL aliquot of cells per transformation)
   2. Add 2-10μL of ligation product.Gently flick tube to mix. **Do not pipette up and down**
   3. Incubate on ice for 30 minutes
   4. Heat shock at 42°C for 20 seconds
   5. Place on ice for 5 minutes
   6. Add 950μL of SOC and incubate for 1 hour at 37°C while shaking/rolling
   7. Plate 100μL on LB + amp and grow overnight at 37°C

**Note: useful to perform positive control with pUC plasmid**

1. **Colony PCR Screen** (this step may be skipped)
   1. Select half a colony and suspend in 50μL of lysis buffer\* in a microcentrifuge tube
   2. Incubate at 90°C for 10 minutes
   3. Spin tubes at max speed for 10 minutes
   4. Use 4μL of supernatant for PCR
   5. Confirm PCR on gel

\*Lysis Buffer: 1% Triton X-100, 20mM Tris HCl (pH 8.0), 2mM EDTA (pH 8.0)

1. **Miniprep – For colonies that confirm in PCR Screen**
   1. Grow 2-5 positive colonies overnight in 5mL LB + amp at 37°C
   2. Miniprep plasmid and nanodrop
   3. Sequence miniprep using primer RCV\_Primer\_2676 (forward) or RCV\_Primer\_5352 (reverse) to confirm gRNA insertion
   4. After sequencing confirms insertion, prepare a glycerol stock
2. **Design Repair Fragment**
   1. Obtain repair fragment through our universal gBlock protocol
3. **Transform in Yeast**
   1. Grow overnight culture
   2. In the morning, dilute culture 1:100 (100μL into 10mL YPD)
   3. Grow ~6 hours until mid-log
   4. Transfer culture to a 15mL falcon tube
   5. Spin for 5 minutes at 2000rpm. Decant media
   6. Resuspend cells in 1mL H2O. Transfer to a microfuge tube and spin for 1 minute at top speed
   7. Aspirate H2O and repeat wash 3 times with 1mL H2O
   8. Wash twice with 1ml LiOAc Mix
   9. Resuspend cells in 70μL LiOAc Mix. Add 10μL of carrier DNA. This should yield a volume of ~100 ul; separate equally into two tubes of ~50 each
   10. Add 10μL of repair fragment and 10uL of Cas9/gRNA plasmid. Vortex briefly
   11. Add 360μL (6-fold volume) of PEG Mix and incubate 30° C for 30 min
   12. Add 47μL of DMSO (final concentration ~10%).
   13. Heat shock for 15 min at 42° C
   14. Sediment cells for 3 minutes at 2000 rpm in microcentrifuge
   15. Resuspend in 100μL of H2O and plate to selective media (SC -ura/-his, if using our gBlock protocol)
4. **Removing Plasmids**
   1. After transformants grow, inoculate into 5mL YPD and grow overnight
   2. Plate cultures onto 5FOA plates to select for removal of Cas9/gRNA plasmid
   3. Select colonies and inoculate into 5mL YPD and grow overnight
   4. Plate cultures to YPD and select colonies for removal of repair plasmid by replica plating to SC -his
   5. Select colonies that grow on YPD but not on SC -his, grow overnight in 5mL YPD
   6. Create a glycerol stock

**Lang Lab gBlock Protocol**

*To construct a repair template for use in our CRISPR protocol*

1. **pGIL092 Miniprep** (the gBlock will be ligated into this vector)
   1. Grow pGIL092 overnight in 5mL LB + amp
   2. Miniprep and nanodrop
2. **pGIL092 Digest**
   1. Combine ~1μg pGIL092, 1μL BamHI, 1μL SbfI-HF, and 5μL Cutsmart Buffer in a 50μL reaction
   2. Digest at 37°C for ~6 hours
   3. Add 1μL of rSAP and incubate at 37°C for 1 hour
   4. Run product on a gel and size select for the double-digested vector (~5100bp)
   5. Gel extract and purify
   6. Nanodrop to get concentration

**\*\*Check before performing steps 11&12! We may have cut plasmid stocked\*\***

1. **gBlock Design**
   1. Design a gBlock repair template to have ~400bp of homology to your gene of interest, centered around your gRNA target site. The gBlock should have the mutation(s) you want to introduce and, for high efficiency, a synonymous mutation in the gRNA target site. Ideally, your mutation is in the gRNA target site.

**gRNA target site mutations introduced via the repair fragment will prevent Cas9 from continually cutting, preventing the generation of toxic DNA breaks**

* 1. Also, design the ends of the gBlock to have the following sequences:

The gBlock should start with the following sequence:

5’ GACGTATGAAGCAAGGAGTCGTACGGTCAGGCAGAATAGGCCTGCAGG 3’

and the gBlock should end with the following sequence:

5’ GGATCCCAACTGGTAGCTCCAGTCAGCTACGCGTTTATGTCCTAGC 3’

**These allow amplification using common primers and digestion/ligation into pGIL092 vector**

* 1. When gBlocks arrive from IDT:
     1. Centrifuge tubes for 3-5 seconds
     2. Add 1X TE Buffer to a final concentration of 10ng/μL **(Do not use H2O)**
     3. Vortex briefly
     4. Incubate at 50°C for 20 minutes, vortex briefly, centrifuge
     5. Store in fridge for short term, avoid freeze/thaw cycles

1. **gBlock Amplification**
2. PCR amplify gBlock fragments using a high-fidelity polymerase
3. Use our common primers. We have two “sets”:
   * 1. RCV\_Universal\_Fwd and RCV\_Universal\_Rev
     2. RCV\_Global\_Fwd and RCV\_Global\_Rev

**Both sets should work. Try the Universal set and if that doesn’t work try the Global set.**

1. PCR cleanup kit your product and confirm PCR on a gel
2. **gBlock Digest**
3. Combine ~1μg purified PCR product, 1μL BamHI, 1μL SbfI-HF, and 5μL Cutsmart Buffer in a 50μL reaction
4. Digest at 37°C for ~6 hours
5. Use Zymo clean and concentrator kit (elute 2-3 times in 10μL of elution buffer)
6. Qubit to get concentration
7. **Ligation**
8. Use a vector:insert ratio between 1:1 – 1:10 and combine with 2.5μL T4 Ligase Buffer and 1μL T4 Ligase in a 25μL reaction
9. Incubate overnight at 16°C, followed by heat inactivation at 65°C for 10 minutes
10. Confirm ligation on gel (optional)
11. **Transformation in Bacteria**
12. Thaw DH5α cells on ice for ~5 minutes (50μL aliquot of cells per transformation)
13. Add 2-10μL of ligation product.Gently flick tube to mix. **Do not pipette up and down**
14. Incubate on ice for 30 minutes
15. Heat shock at 42°C for 20 seconds
16. Place on ice for 5 minutes
17. Add 950μL of SOC and incubate for 1 hour at 37°C while shaking/rolling
18. Plate 100μL on LB + amp and grow overnight at 37°C
19. **Miniprep**
    1. Grow 2-5 positive colonies overnight in LB + amp at 37°C
    2. Miniprep plasmid and nanodrop
    3. Sequence miniprep using pGIL092\_forward\_SbfI or pGIL092\_reverse\_BamHI to confirm gBlock insertion and sequence fidelity
    4. After sequencing confirms insertion, prepare a glycerol stock

**Oligos**

RCV\_Primer\_2676 5’ CTG TAG GTC AGG TTG CTT TC 3’ Backbone of pGIL094 (forward)

RCV\_Primer\_5352 5’ CGT TCG AAA CTT CTC CGC AG 3’ Backbone of pGIL094 (reverse)

RCV\_Universal\_Fwd 5’ GTA CGG TCA GGC AGA ATA GG 3’ Universal gBlock sequence

RCV\_Universal\_Rev 5’ CTG ACT GGA GCT ACC AGT TG 3’ Universal gBlock sequence

RCV\_Global\_Fwd 5’ GAC GTA TGA AGC AAG GAG TC 3’ Universal gBlock sequence

RCV\_Global\_Rev 5’ GCT AGG ACA TAA ACG CGT AG 3’ Universal gBlock sequence

pGIL092\_forward\_SbfI 5’ CTT AAC CCA ACT GCA CAG AAC 3’ Backbone of pGIL092

pGIL092\_reverse\_BamHI 5’ CTG TGC GGT ATT TCA CAC CG 3’ Backbone of pGIL092

**gBlock Sequences**

5’ GACGTATGAAGCAAGGAGTCGTACGGTCAGGCAGAATAGGCCTGCA˅GG 3’ Beginning of gBlock

RCV\_Global\_Fwd RCV\_Universal\_Fwd SbfI

5’ G˅GATCCCAACTGGTAGCTCCAGTCAGCTACGCGTTTATGTCCTAGC 3’ End of gBlock

BamHI RCV\_Universal\_Rev RCV\_Global\_Rev

**Alternative Names of Plasmids (on Addgene)**

pGIL094 = pML104 (Plasmid #67638)

pGIL092 = pHU (Plasmid #64172)