1) Vector Preparation- sg 1371 w/Blp1 Ef1α- puro t29 BFP (602 ng/uL)

a) Digest 5 ug of vector with Thermo Scientific FastDigest BstX1 and Blp1 for 1h at 37ºC. Set up reaction as follows:

<u>100 uL Reaction</u>

- **Water, 76.7 uL**
- **Fast Digest Green Buffer, 10 uL**
- **Vector, 8.3 uL (5 ug)**
- **Blp1, 2.5 uL**
- **BstX1, 2.5 uL**

If you plan to use the vector often I recommend setting up multiple reactions (4 to 8).

b) While digest is incubating, pour 100 mL 0.8% agarose gel and use wide gel combs. When digest is finished incubating load the digest, and run your gel at 120V for ~1h. Stain gel with SYBRsafe (~10 to 15min), and use safe imager blue light transilluminator to excise digested vector.

c) Gel purify excised vector with the QIAquick MinElute gel extraction protocol.

2) Insert Preparation: PCR

a) Set up 3, 100 uL or 6, 100 uL reactions and an additional "NO template" negative control. While 6, 100 uL reactions is excessive you will have plenty of back-up insert.

<u>50 uL reaction</u>

- **Water, 35.9 uL**
- **5X Phusion HF Buffer, 10 uL**
- **DMSO (100%), 1.5 uL**
- **dNTPs (100 mM), 0.1 uL**
- **P(F) 100 uM, 0.25 uL**
- **P(R) 100 uM, 0.25 uL**
- **Template (0.05 pM/uL), 1 uL**
- **HF Phusion, 1 uL**

<u>PCR conditions</u>

- **98ºC, 30s**
- **15 cycles**
  - **98ºC, 15s**
  - **56ºC, 15s**
  - **72ºC, 15s**
- **72ºC, 10min**
- **7ºC hold**

b) Combine reactions. Run 5 uL of combined reactions on a 20% acrylamide gel, at 200V for 45min-1h.

c)While gel is running purify the remaining ~295 uL of PCR on one Qiagen Minelute column as follows:

- **Add 4X sample volume of PB to combined PCR. Add 1/100th volume 3M NaOAc.**
    - **Combined PCR= ~300 uL, Add 1.2 mL PB and 15 uL NaOAc**
- **Once PCR is bound to column, Wash 2X w/ 750 uL of PE, and one dry spin**
- **Elute in 15 uL of EB, wait 5 min, spin**

Your recovery should be between ~0.75 ug to 1.5ug (3,100 uL rxns), ~1.5ug to 3ug (6, 100 uL rxns). If your recovery is low you will need to optimize your PCRs before proceeding with the digest.

d) At this point the gel should be finished. **The expected insert size is ~86bp.** *Once you have verified your Insert PCR is correct (you see a nice bright band at 86bp), you can proceed and digest your insert.*

3) Insert Digest:

*a) Digest 1 ug of insert***.** *Incubate Digest at 37ºC for 1 h-18h. Following digest insert will be ~33bp.*

<u>30 uL Digest</u>

- **Insert, 1 ug**
- **3uL FastDigest green buffer**
- **Bstx1, 1 uL**
- **Blp1, 1uL**
- **Bring reaction to 30 uL with water**

b) Gel purification

- *Run 15-20 uL of digest on 20% acrylamide gel.*
- *While gel is running prepare tubes and tips for rapid extraction (see protocol below). Pierce the bottom (tip) of a 0.5 ml nonstick tube with an 18.5 gauge needle and place it inside a 1.5 ml nonstick tube. Use a clean blade or clean scissors to cut the tips off of p1000 pipette tips.*

- *Stain gel with SYBRsafe (10-15min), and excise insert using blue light transilluminator. Change blades between samples to avoid cross contamination. Place excised gel piece inside 0.5mL nonstick tube prepared above, and proceed with Rapid Extraction.*

c) Rapid Extraction

- Pierce a 0.5mL nonstick tube with a 18.5 gauge needle and place inside a 1.5 mL nonstick tube
- Excise gel piece and place inside
- Spin tubes at 20,000 x g for 3 min. This will force the gel through the hole, and gel will crush into tiny pieces. Check for gel pieces in small tube. Residual pieces should be transferred to large tube. If you cannot tap the residual pieces use a pipette tip.
- Add 200 uL of water to gel pieces, and incubate for 10 min at 70ºC
- Vortex gel slurry for 30s and use cut p1000 tips to transfer gel mixture to Costar Spin-X columns.
- Spin tubes for 3 min at 20,000 x g to recover the elution mixture free of gel debris.
- Transfer eluate to a new 1.5 mL nonstick tube
- Isopropanol Precipitation
    - **200 uL of eluate (insert is here)**
    - **1.5 uL-2uL of glycoblue (this will help you visualize your pellet)**
    - **25 uL 3M NaOAc or 3M NaCl**
        - Mix Well (invert 10-15X)
- Add 0.75 mL Isopropanol, mix well
- -30ºC, 30 min to 18h or -80ºC, 30 min
- Pellet: Spin 30 min at 20,000 x g at 4ºC , remove supernatant
- Wash pellet 2X with ice cold 80% EtOH
- Air Dry
- Resuspend pellet in 15 uL water or Qiagen EB

d) If you nanodrop your sample you will observe a contamination peak at 230. This is from the glycoblue. I generally observe a nanodrop concentration of ~20-25 ng/uL with glycoblue, and without glycoblue ~10-15 ng/uL. From Qubit, a fluorometer, (DNA high sensitivity assay) I observe ~0.5-2 ng/uL. As the Qubit is more sensitive I use these concentrations. Proceed to ligation.

4) Ligation

Set up reaction at a molar ratio of 2:1 (vector:Insert). As a first pass, you may want to set up 3 conditions (vector:insert), 1:2, 1:1, 2:1. Adding too much insert can cause sgRNA concatemers. I observed concatemers at a ratio of 1:3.

a) <u>20 uL ligation</u>

- **Vector, 500ng**
- **Insert, 0.9 ng**
- **Ligase buffer, 2 uL**
- **T4 ligase, 1 uL**
- **Bring reaction to 20 uL with water**
- **16h at 16ºC in thermal cycler**

Also run a (-) insert control/no insert control with vector only

b) <u>Ligation Reaction Clean-Up: EtOH Precipitation</u>

Removing the excess salt in the ligation reaction will help improve electroporation conditions.

<u>EtOH Precipitation</u>

- Bring 20 uL ligation reaction to 200 uL with water.
- 1.5 uL-2uL of glycoblue (this will help you visualize your pellet)
- 1/10 reaction volume of 3M NaOAc, 20 uL. Mix Well.
- Add 3X reaction volume of 100% EtOH, 600 uL. Invert 10X to mix.
- Place at -30ºC, 30 min to 18h or -80ºC, 30 min
- Pellet: Spin 30 min at 20,000 x g at 4ºC , remove supernatant
- Wash pellet 2X with ice cold 80% EtOH
- Air Dry
- Resuspend pellet in 20 uL of water

<u>5) Test Transformation</u>

a) Do a test transformation for (+) insert and (-) insert control

- Add 0.5uL ligation to 20 uL DH5α, and gently tap 3X to mix.
- Incubate on ice, 15min to 30min
- Heat-shock 30s @ 42ºC
- Incubate on ice for 2 min
- Add 180 uL S.O.C, LB, or recovery media. Recover at 37ºC for 30 min

- Plate 160 uL on pre-warmed plates. Also plate 16 uL(diluted 10X)
- Incubate O/N at 37ºC

b) Scrape plates with 1.5 mL of LB and purify plasmid (mini-prep). Transform again, and plate.

c) Sequence 10-20 colonies from the second transformation. If 60-80% of the sequences are perfect and match the library it is safe to proceed with the large-scale transformation.

6) Large-Scale Transformation

a) Before starting, Plan out the number of plates you will need. You want 1 plate per 3666.6 oligos for roughly 30 fold coverage at 100,000 colonies per plate. I recommend making a spreadsheet as follows:

The large plates we describe below are from Fisher
No Divider X6023; cat# NC9372402

# of oligos on 90k chips	# of plates for 90k chips	# of plates for 90k chips rounded up	ul MegaX for 90k chips	uL 10% glycerol	# Cuvettes	Recovery Media (uL)
17444.318	4.75762777505046	5	100	100	3	3000
13234.78	3.60955108274696	4	75	75	2	2400
13276.81	3.6210140184367	4	75	75	2	2400
6265.739	1.70886897943599	2	50	50	2	1200
16137.652	4.40125784105166	5	100	100	3	3000
12432.007	3.39060901107293	4	75	75	2	2400
11208	3.05678285059728	3	75	75	2	1800

b) Thaw Mega-X cells on ice (this will take ~30min), pre-warm large carbenicillin plates (75% of normal concentration) at 37ºC, pre-chill 10% glycerol on ice, and pre-chill cuvettes on ice.

- In a 1.5 mL eppendorf tube, mix Mega-X and ~100 ng of ligation per 20 uL of MegaX. Tap gently 3X to mix
    - From a 20 uL ligation I observe ~15 ng/uL (from Qubit)
- Incubate on ice for 30 min
- Add pre-chilled 10% glycerol to the MegaX-Ligation mix, and transfer to a prechilled cuvette

- Electroporate at 2.0 kV, 200 ohms, 25 uF in 0.1 cm cuvette (Gene Pulser Xcell, Bio Rad)
- Following electroporation add 600 uL of recovery media or S.O.C. to the cuvette and transfer to a cell culture tube. Shake at 250 rpm at 37ºC for 1.5h.
    - You will be plating 600 uL/plate. It is a good idea to rinse the cuvette twice with recovery media. If you are only plating one plate, rinse the cuvette twice with 300 uL. Gel loading tips are also helpful.
- Plate 600 uL per plate.
- Incubate at 37ºC for 18h

7) Scrape Plates

Add 15-25 mL LB for the initial scrape. Transfer to a clean collection tube. Add an additional 5-15 mL LB and transfer to collection tube. If you are scraping multiple plates, place your collection tube on ice. Once you are finished scraping, spin at 4,000 rpm for 20 min. The pellet should be tight. Pour off media and freeze pellet or proceed with plasmid preparation.

8) Plasmid Preparation

Qiagen or Sigma midi, maxi, mega or giga plasmid prep kits. Make sure columns are dry prior to the final elution. Wet columns will give a low recovery.

9) Prepare Samples for Illumina Sequencing

Primers for use with single or paired end sequencing. Compatible with both Hiseq 2500 and Hiseq 4000.

Crispri TSS (Index) paired end 5'

aatgatacggcgaccaccgaGATCTACACGATCGGAAGAGCACACGTCTGAACTCCA
GTCAC**GCCAAT**gcacaaaaggaaactcaccct

purple= adapter; blue= illumina primer site for sequencing; **black=** barcode;
green= complements sg casette for enrichment

Crispr i TSS Common paired end 3'

caagcagaagacggcatacgaGATCGACTCGGTGCCACTTTTTC

purple= illumina adapter; black= complements sg for enrichment

Excel files of our primer sequences and different barcodes are available upon request.

a) <u>Index PCRs (run 3, 100 ul reactions for column purification/ run 1, 100 uL reaction if you plan to purify with SPRI):</u>

- **Library, ~100 ng**
- **5X Phusion HF Buffer, 20 uL**
- **DMSO (100%), 3 uL**
- **dNTPs (100 mM), 0.1 uL**
- **P(Index) 100uM, 0.25 uL**
- **P(Common) 100uM, 0.25 uL**
- **HF Phusion, 1 uL**
- **Bring reaction volume to 100 uL with water**

<u>PCR conditions</u>

- **98ºC, 30s**
- **15 cycles**
  - **98ºC, 15s**
  - **56ºC, 15s**
  - **72ºC, 15s**
- **72ºC, 10min**
- **7ºC hold**

After PCR is complete combine reactions in a 1.5 mL nonstick tube. Proceed with SPRIbead purification.

b) <u>SPRIbead purification</u>

Following the index PCR the library is ~264 bp. For purifying with SPRIbeads you will need 1.5 mL nonstick tubes, DynaMag 2 magnet (life tech), 80% EtOH (make fresh), and Qiagen EB.

- *Add 0.65X reaction volume SPRI beads (195 uL)*
  - *Pipette mix 15X*
  - *Incubate RT for 5 min*

    - *Place on magnet stand 5 min or until supernatant becomes clear*
        - *>300 bp on beads, < 300 bp in supernatant*
    - *Transfer supernatant to new tube. Keep this fraction as your library is here.*
- *Rebind. Add 1.0X your initial reaction volume SPRI*
    - *Pipette mix 15X*
    - *Incubate at RT for 5 min*
    - *Place on magnet stand for 5 min or until clear*
        - *150-300 bp on beads*
    - *Keep beads. Your library is here.*
    - *Wash beads. Add 1mL 80% EtOH. Incubate at RT 2min. Remove EtOH. Repeat for a total of two washes.*
    - *Air Dry. Beads will change from a glossy texture to matte (non-glossy) and will look dry. This can take from 5 to 15 min.*
    - *Elute 50 uL of Qiagen EB*

c) <u>Sample Validation</u>

1) Nanodrop
    - expect: 30 ng/uL-300 ng/uL
    - useful for making dilutions for running bioanalyzer

2) Qubit
    - useful for making dilutions for bioanalyzer
    - useful when pooling libraries

3) Agilent Bioanalyzer
    - high sensitivity kit
    - load 400 pg
    - You should see a nice peak at ~274bp.

4) Pool Samples for submission

5) Quantify
    - You want high quality data. The Bioanalyzer tells us how clean our illumina library is (we see nice peak at 274bp). Next we want to load the correct concentration onto the illumina platform. Typically sequencing centers will perform a qPCR to determine the best concentration to load samples, and obtain the best cluster density.

You will need to provide your core facility with our custom sequencing primer, **oCrispri TSS seq V2: gtgtgttttgagactataaGtatcccttggagaaCCAcctTGTTGG**