Protocol for Cloning Promoter Library into pLibacceptorV2

1. Library Order Prep
    a. Agilent provides a precipitated Oligo Library
        i. Resuspend in 100uL EB
        ii. Mix well to ensure all DNA is precipitated

2. Promoter Library Amplification
    a. Amplify library using qPCR, find out how many cycles are needed. **Does not add barcode.** Perform this PCR in **3 parallel replicates**
        i. Kapa Library Amplification Kit

	1X	3x
H20	10.5 uL	31.5 uL
KAPA SYBR Fast	12.5 uL	37.5 uL
10 uM Primers	1 uL	3 uL
Library (undiluted)	1 uL	3 uL

        ii. Use primers that amplify specific library but do not barcode
        iii. Primers used: (fwd)_____ & (rev)_____
        iv. Thermal Cycles

Temp	Time
98°	45''
98°	20''
60 °	30''
72° (Measure after)	30''
Repeat steps 2-4 x19 (20 total)	
72° (Measure after)	1'

    b. Amplify library using Q5 polymerase in thermal cycler
        i. Perform in **5** technical replicates
        ii. Perform using same number of cycles
            1. Number of Cycles? _____

    c. Gel extract correct sized fragment
        i. Pour Gel
            1. 2%, 200mL, gel in long casting mold with 15-well comb @ 100 volts until lower dye (pink?) is 75% through the gel (1-1.5hrs) take care not to let the gel run for too long
            2. Expected band is 230bp so run alongside 100bp ladder and 50bp ladder. Leave empty lanes on either side of your samples to ease gel extraction process

ii. Band extraction
    1. Remove as much gel as possible (ideally under 20mg)

iii. Elute in 10uL each, combine in same tube.

    1. Concentration: _____(ng/uL)

3. Barcoding Library
    a. Repeat (a) above with barcoding primers
        i. Primers used: (fwd)_____ & (rev)_____
        ii. **Dilute Gel extraction product to 1ng/uL and use as template**
    b. Repeat (b) above with barcoding primers
        1. Number of Cycles? _____
    c. PCR Cleanup of combined products
        i. Elute in 12uL each, combine in same tube

        1. Concentration: _____(ng/uL)

4. Digest Library Ends
    a. Digest ends of Library to make them suited for cloning into pLibacceptorv2
        i. Digestion Protocol

	1x	3x
H20	Up to 50uL Vol:_____	Up to 150uL Vol:_____
(10x) Cutsmart Buffer	5uL	15uL
DNA	333ng Vol:_____	1ug Vol:_____
SbfI-HF	1uL	3uL
XhoI	1uL	3uL

        ii. 1.5hrs @ 37 degrees

    b. PCR Clean-up (Zymo Clean and Concentrate)
        i. Concentrate Library DNA
        ii. Elute in 12uL Nuclease-Free H20
            1. Concentration: _____(ng/ul)

5. Plasmid Digestion
    a. Digest Vector (pLibacceptorV2) to clone in library
        i. Plasmid should be from a PCR-Purified after maxi-prep

	1x	3x
H20	Up to 50uL Vol:_____	Up to 150uL Vol:_____
(10x) Cutsmart Buffer	5uL	15uL
DNA	666ng Vol:_____	2ug Vol:_____
SbfI-HF	1uL	3uL
SalI-HF	1uL	3uL
rSAP	1uL	3uL

        ii. Digest 1.5 Hrs @ 37degrees
    b. Zymo Cleanup
        i. Combine and elute in 15uL Nuclease-Free H20
            1. Concentration: _____(ng/ul)

6. pLib Ligation
    a. Ligate barcoded promoter into plasmid vector (3:1 insert:vector molar ratio)
        i. Must do no insert control reaction, make sure there is minimal background

	Full-Ligation	No Insert Ctrl
H20	Up to 50uL Vol:_____	Up to 50uL Vol:_____
(2x) T7 Ligation Buffer	25uL	25uL
Insert (Promoter Library)	40ng Vol:_____	XXXXXX
Vector (Digested pLibacceptorV2)	350ng Vol:_____	350ng Vol:_____
T7 Ligase (3Million Units/mL)	2uL	2uL

        ii. Leave on bench @RT for 1 hour
    b. Zymo Cleanup of Ligation
        i. Elute in 7uL Nuclease-Free H20
    c. Drop Dialysis
        i. 10-15 minutes

        ii. Concentration: _____(ng/ul)

7. Transformation
    a. Transform 3x with Full-Ligation, 1x with NIC
    b. DH5alpha electrocompetent cells come in 100uL aliquots, use 75uL for 3x Full-Ligation Transformations, 25uL for NIC transformation
    c. Follow electrotransformation protocol
    d.
    e. Scrape plates, inoculate overnight, make 10x glycerol stocks, Maxiprep-Wizard (After allowing for 24 hours of growth on plates, scrape undiluted plate and resuspend in 6mL LB. Inoculate 800 million cells in 450 mL LB + kanamycin (25 ug/mL) overnight @30C. (based on OD600, Agilent cell calculator))
    f. OD of 1:100 Dilution? _____ Cells/mL
    g. Volume of 1:100 Dilution inoculated? _____mL

	10^-4 Dilution	10^-3 Dilution	Undiluted
Number of Colonies			

==========

Cloning RiboJ::sfGFP into pLib

1. Amplify RiboJ::sfGFP
    a. Perform in triplicate to ensure sufficient DNA yield

	1x	3x
H20	22uL	66uL
Q5 Polymerase MM	25uL	75uL
10uM GU99 + GU100	2uL	6uL
Template @ 1ng/uL (dTopo:RibosfGFP)	1uL	3uL

    b. 30s Elongation, anneal at 60C, 25 Cycles

2. Zymo Cleanup
    a. Pool together PCRs and cleanup altogether.
    b. Elute in 17uL Nuclease-Free H20
        i. Concentration: _____(ng/uL)

3. Digest RiboJ::sfGFP

	1x	3x
H20	Up to 50uL Vol:_____	Up to 150uL Vol:_____
(10x) Cutsmart Buffer	5uL	15uL
DNA	500ng Vol:_____	1.5ug Vol:_____
BsaI-HF	1uL	3uL
NcoI-HF	1uL	3uL

   a. Digest 1.5hr @ 37C

4. Zymo Cleanup
   a. Pool together Digests and cleanup together
   b. Elute in 12uL Nuclease-Free H20
      1. Concentration: _____(ng/ul)

5. Digest pLib

	1x	3x
H20	Up to 50uL Vol:_____	Up to 150uL Vol:_____
(10x) Cutsmart Buffer	5uL	15uL
DNA	666ng Vol:_____	2ug Vol:_____
NheI-HF	1uL	3uL
BsaI-HF	1uL	3uL
rSAP	1uL	3uL

   a. Digest 1.5hrs @ 37C
   b. Also, perform single digests for NheI/BsaI/SalI, run all on gel alongside Double-digest and undigested

6. Zymo Cleanup
   a. Pool Digests Before Cleanup
   b. Elute in 12uL Nuclease-Free H20

      i. Concentration: _____

7. Ligate RiboJ::sfGFP into pLib
    a. (3:1 insert:vector molar ratio)

	Full-Ligation	No Insert Ctrl
H20	Up to 50uL Vol:_____	Up to 50uL Vol:_____
(2x) T7 Ligation Buffer	25uL	25uL
Insert (RiboJ::sfGFP, Digested)	160ng Vol:_____	XXXXXX
Vector (Digested pLibacceptorV2)	350ng Vol:_____	350ng Vol:_____
T7 Ligase (3Million Units/mL)	2uL	2uL

    b. Leave on bench @RT for 1hr
    c. Zymo Cleanup of Ligation
        i. Elute in 7uL Nuclease-Free H20
    d. Drop Dialysis
        i. 10-15 minutes

        i. Concentration: _____

8. Transformation
    a. Transform 3x with Full-Ligation, 1x with NIC
    b. DH5alpha electrocompetent cells come in 100uL aliquots, use 75uL for 3x Full-Ligation Transformations, 25uL for NIC transformation
    c. Follow electrotransformation protocol
    d. Scrape plates and inoculate
    e. Scrape plates, inoculate overnight, make 10x glycerol stocks, Maxiprep-Wizard
    f. Scrape plates, inoculate overnight, make 10x glycerol stocks, Maxiprep-Wizard (After allowing for 24 hours of growth on plates, scrape undiluted plate and resuspend in 6mL LB. Inoculate 800 million cells in 450 mL LB + kanamycin (25 ug/mL) overnight @30C. (based on OD600, Agilent cell calculator))
    g. Density of 1:100 Dilution? _____ cells/mL
    h. Volume of 1:100 Dilution inoculated? _____mL

	10^-4 Dilution	10^-3 Dilution	Undiluted
Number of Colonies			

Transform pLib_sfGFP into Landing pad strain
(fLP3 for Looping TFs, LP5 for UP element variants)

2. Digest pLib_sfGFP
    a. Removes background (pLibacceptorV2/pLib)

	1x	3x
H20	Up to 50uL Vol:_____	Up to 150uL Vol:_____
(10x) Cutsmart Buffer	5uL	15uL
DNA	1ug Vol:_____	3ug Vol:_____
SalI-HF	.5uL	1.5uL
NheI-HF	.5uL	1.5uL

    b. Incubate 1hr @ 37C
    c. Do single digests with SalI, NheI, DraIII next to undigested to see how much of each plasmid species is present
    d. Zymo Cleanup
        i. Pool Digests and cleanup
        ii. Elute in 15uL Nuclease-Free H20
    e. Drop Dialysis
        i. 10-15 min
            1. Concentration: _____

3. Transform LP strain
    a. May need to adjust volume of cells:
        i. Start with 1uL DNA in 80uL cells, if arc time is above 5, add 10uL cells for every .2 away. If arc time below 4.5, add 10uL water to 80uL cells for every .2 away
    b. Scrape plates, inoculate overnight, make 20x glycerol stocks
    (After allowing for 24 hours of growth on plates, scrape undiluted plate and resuspend in 6mL LB. Inoculate 800 million cells in **200 mL** LB + kanamycin (25 ug/mL) overnight @30C. (based on OD600, Agilent cell calculator

    c. OD of 1:100 Dilution? _____ Cells/mL
    d. Volume of 1:100 Dilution inoculated? _____mL

	10^-4 Dilution	10^-3 Dilution	Undiluted
Number of Colonies			

Landing Pad Integration (For Libraries)
Kosuri Lab

- Day 1: Grow glycerol stock of Landing pad strain w/Integration Plasmid overnight (ON) in 200mL LB + Kan (25ug/ml) @30C

- Day 2: Reinoculate culture with LB +Ara (Recommend start at 3pm)
    - Inoculate 200-400M cells from overnight in 250mL LB + .2% Arabinose + 25(ug/ml) Kan @30C for 24 hours
        - This step integrates the library into the LP

- Day 3: Grow ON to exponential phase and heat-cure (Recommend start at 3pm)
    - Pre-warm 4 LB + 25(ug/mL) Kan plates @42C
    - Inoculate 800M cells of induced overnight in 80mL LB + 25(ug/mL) Kan **@42C**
        - Grow cells to OD .3-.7 (~1.5hrs)
    - Plate 200M cells from log-phase culture on two plates, grow **16hrs @42C**
        - Use remaining plates for 10^{-5} & 10^{-6} dilutions
        - 42C heat-cures plasmids

	10^-6 Dilution	10^-5 Dilution	Undiluted
Number of Colonies			

- Day 4: Scrape Plates and reinoculate overnight
    - Make sure plates have enough colonies for > 10x library coverage
    - Scrape plates in 6mL LB and combine 5mL of each cell suspension in 15mL falcon tube
    - Prepare 10^{-1}& 10^{-2} dilutions of suspension, measure OD of 10^{-2} and inoculate 400M cells from 10^{-1} dilution in 200mL LB + Kan(25ug/mL). Grow overnight @37C.
        - OD of 1:100 Dilution? _____ Cells/mL
        - Volume of 1:100 Dilution inoculated? _____mL

Day 5: Make glycerol stocks of your integrated library and enjoy

    - To validate integration, plate integrated library on LB + Kan(25ug/mL) & grow @ 30C overnight. Perform colony PCR using primers flanking integration site.

Electrotransformation Protocol

1. Before anything, warm 6 plates at 37C (takes 2 hours to dry, if you forget this, abandon experiment)
2. Preparation
    a. Thaw 3x tubes of electrocompetent cells per transformation
        i. Gently flick after 5 min, then return on ice for 5 min (thaw 10 min total)
    b. Put 3x electrocuvettes (brown caps) on ice per transformation
    c. Prepare 1ml of SOC in a culture tube for each tube, leave in 30degree incubator
3. Add plasmid DNA to cells
    a. Add 1uL of ~50-100ng/uL to each tube of cells
        i. Measure concentration after drop dialysis
    b. Flick gently 6x times (to mix)
4. Load into electocuvettes
    a. Eject up to the first stop
        i. 2nd stop adds bubbles
    b. remove bubbles
        i. tap bottom of cuvette onto table
        ii. centrifugal arm spin
5. Electroporation (shock plasmid into cells)
    a. Wipe down sides of cuvettes
    b. Load 1mL of SOC from culture tube into p1000
    c. Perform shock
        i. Set genepulser to bacteria & time(ms)
            1. Time refers to arc-time
        ii. Desire arc time of 4.5-5.1
    d. Slowly add the SOC to electrocuvette, resuspend, and transfer to culture tube
    e. Recover 45min-1hr@30degrees in shaking incubator
        i. Be sure to color-code separate transformations
6. Plate cells
    a. During wait, prepare 5x tubes with 270uL LB per transformation
        i. For dilutions
        ii. Make sure LB is not contaminated (cloudy)
    b. Dilutions
        i. Combine replicate transformations (3ml total)
        ii. Add 30uL(1% total cells, or 10^-2) to 270uL LB
            1. Continue dilution series and plate 300uL 10^-3, 10^-4, 10^-5
                a. First tube is 10^-2

7. Scraping
    a. Make sure library has sufficient coverage (i.e. plate is covered by a lawn of *E. coli*
    b. Pour 6 mL LB onto plate
    c. Prepare sterile glass spreader
        i. Pour ~10mL 95-100% ethanol into empty vessel, a petri dish is good for this
        ii. Dip glass spreader into ethanol vessel and pass through a bunsen burner flame
            1. The glass spreader will catch fire, sterilizing it and evaporating the ethanol
    d. Using a glass spreader, “rake” up bacteria into the liquid. Be careful not to apply too much pressure
    e. Once all colonies suspended in LB, mix using a P1000
    f. Transfer up to 5 ml of LB into 5 mL falcon tube
    g. Dilute 500uL of Scrape in 4.5mL LB (10^-1 dilution)
    h. Dilute 500uL of previous dilution in another 4.5mL LB (now a 10^-2 dilution)
    i. Measure OD of 10^-2 dilution (Should be between .3 and .8)
        i. Use http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp to determine how many cells per mL in 10^-2 dilution
    j. Inoculate 800Million cells (Should be a few mL) from 10^02 dilution into 450mL LB + Kanamycin (Make sure to use a 1Liter flask!). Grow at 30 degrees for 16-20 hours
    k. Next day...
        i. Take 10mL of overnight culture, set aside and prepare 10 glycerol stocks as soon as possible
        ii. Add 10mL of 50% glycerol to 10mL of overnight culture, aliquot into 10x cryotubes, set on ice for 5min and transfer to -80 box