BE Sensor CRISPR Library Cloning

Amplification → Insert preparation → Ligation → Electroporation

I – Amplification

BC Fwd Primer	BC Fwd Primer Sequence	BC Rev Primer	BC Rev Primer Sequence
F1	AGGCACTTGCTCGTACGACG	R1	TTAAGGTGCCGGGCCCACAT
F2	GTGTAACCCGTAGGGCACCT	R2	GTCGAAGGACTGCTCTCGAC
F3	CAGCGCCAATGGGCTTTCGA	R3	CGACAGGCTCTTAAGCGGCT
F4	CTACAGGTACCGGTCCTGAG	R4	CGGATCGTCACGCTAGGTAC
F5	CATGTTGCCCTGAGGCACAG	R5	AGCCTTTCGGGACCTAACGG
F6	GGTCGTCGCATCACAATGCG	R6	CGTCACATTGGCGCTCGAGA
n=36 possible F_R barcode combinations			

****NOTE TO ROSHAN: LIBRARIES PROVIDED USE F1-R1 PRIMERS**

1. **Reconstitution of lyophilized OLS library:**

- Resuspend the pellet to a final concentration of 1ng/uL of TE Buffer pH 8.0 or QIAGEN EB Buffer.
- Incubate at RT for ~1hr and periodically vortex to ensure complete resuspension (or put in a shaking block at RT).
- Nanodrop and aliquot (typically n=10 aliquots per OLS library) and store these at -20°C.
- If cloning libraries right away, prepare *fresh* serial dilutions until getting a diluted stock at 1ng/µL (see below).

2. **Amplification details/notes:**

- We typically perform n=4 PCR reactions per pool of ~1000 gRNAs (an excess, but allows for plenty of backup insert for subsequent sub-cloning of libraries into different destination vectors, if desired, or to repeat any that fail QC).
- All PCR reactions should be set up in a PCR hood following standard procedures.

- Always include a water-only control for every primer set to assess non-specific amplification and/or contamination.

3. **PCR reaction conditions** (Note: mastermix number depends on the size of your library)

Item	Concentration	Amount (uL)	Master Mix	MM amount (uL)
Oligo pool	1 ng/uL	1	-	-
Fwd primer	10 uM	1.5	6	9
Rev primer	10 uM	1.5	6	9
NEBNext	2X	25	6	150
H2O	-	21	6	126
Total		50	6	294

*The oligo pool should be added outside of the PCR room, preferably in a bench or room that is not used routinely for cloning gRNAs. Never bring oligos or oligo pools into the PCR room.

4. **Cycling conditions** (Named “CRISPR library cloning 25 cycles” under the library cloning folder in the Mastercycler 1 in Bay 1)

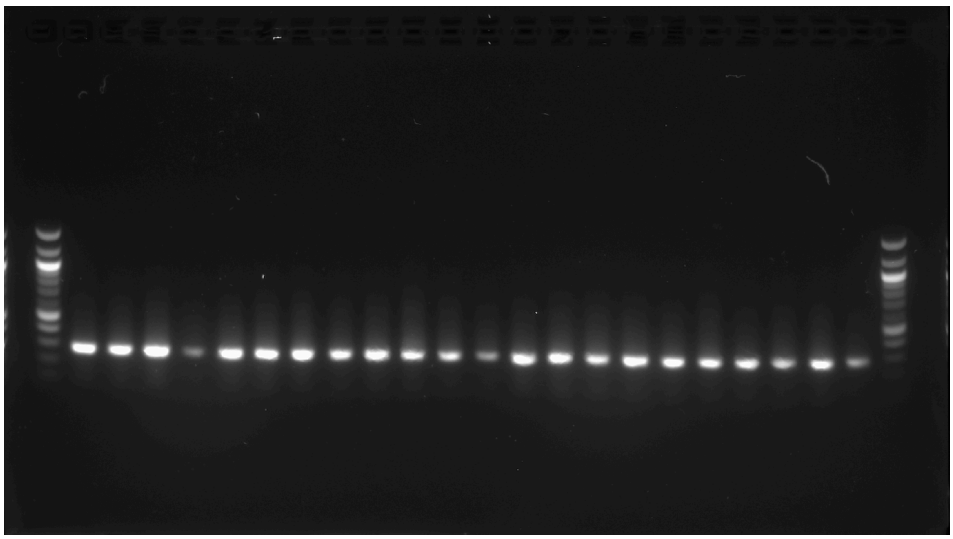
Temperature (°C)	cycle no.
98	30 s
98	30 s
53	30 s
72	30 s
72	5 min
4	keep

note: [GO TO STEP 2 x 18 total cycles] Go to step 2 x 24 cycles (note: the # of cycles can be varied and has been tested and optimized; anywhere from 10-24 cycles is fine; less is usually better to

minimize potential over-amplification but I haven't found this to be an issue; in fact, most libraries I've cloned have been done w/ 24 cycles; if doing 10 cycles, I would run 8 rxns per pool)

5. **PCR purification**

- Pool up to four 50µL reactions per pool and PCR purify using a single QIAGEN column and standard QIAGEN PCR purification protocol.
- Add 10µL of 3M NaOAC pH 5.2 for every 5 volumes of PB used per 1 volume of PCR reaction (e.g. 200µLpooled rxns need 1mL of PB + 10µL NaOAC).
- Elute in 50µL of pre-warmed (55°C) EB.
- Run 5µL of each purification in a gel (it should look like the gel below).



II - Insert and vector preparation

6. **Insert preparation** (Note: mastermix number depends on the size of your library)

Item	Amount (uL)	Master Mix	MM amount (uL)
Purified PCR product	45	8	360
rCutSmart NEB buffer	6	8	48
Esp3I NEB enzyme	3	8	24
EcoRI_HF NEB enzyme	3	8	24
H2O	3	8	24
Total	60	8	480

- Digest at 37°C for 4 hours

7. **Insert purification**

- Pool up to 4 reactions per pool and PCR purify using a single QIAGEN column.
- Add 10µL of 3M NaOAC pH 5.2 for every 5 volumes of PB used per 1 volume of PCR reaction.
- Elute in 30µL of pre-warmed (55°C) EB.

8. **Backbone preparation** (Note: mastermix number depends on the size of your library)

Item	Amount (uL)	Master Mix	MM amount (uL)
Backbone vector	5 ug	10	50 ug
rCutSmart NEB buffer	5	10	50
Esp3I NEB enzyme	2.5	10	25
EcoRI_HF NEB enzyme	2.5	10	25
H2O	to 50	10	to 500
Total	50	10	

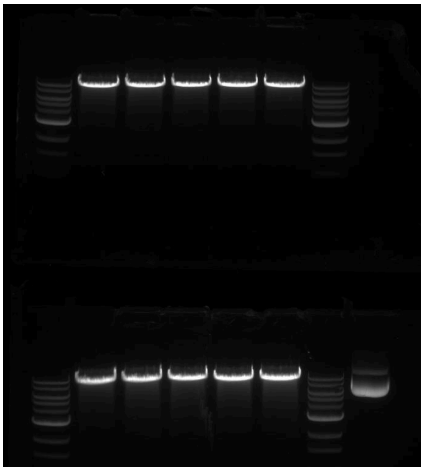
Note: Buffer should be 10% of the reaction volume

- Incubate at 55 C for at least 2hr (or at 37C if using Esp3I from NEB)

9. **Backbone dephosphorylation using CIP**

- Cool down digest reaction to 37°C or RT
- Add 1µL of NEB CIP
- Incubate for at least 30min at 37°C

10. **Run cut backbone in 1% agarose gel to gel-purify cut vector backbone**



11. Gel purification of backbone

- Excise vector backbone (largest linear band in the gel)
- Place gel fragment in a clean, labeled eppendorf tube
- Add 500µL of Buffer QG (for a typical gel slab)
- Incubate at 55°C for at least 10 minutes, or until the gel fragment completely dissolves
- Add 170µL of Isopropanol and mix by inverting a few times
- Transfer ~800µL of this mix to a purple gel extraction column
    - *note: since DNA yield for gel extractions is typically quite low, multiple rxns for the same backbone can be combined into a single purple gel extraction column to concentrate the DNA (these columns can hold up to 10ug DNA for all constructs smaller than 10kb)*
- Spin at max RPM for 1 minute
- Discard flowthrough and put column back Add 500uL of Buffer QG to the column
- Spin at max RPM for 1 minute
- Discard flow-through in the sink and put the column back into the recovery tube.
- Add 700 uL of Buffer PE (w/ EtOH).
- Spin at max RPM x 1 min.
- Discard flow-through in the sink and put the column back into the recovery tube.
- Spin empty column at max RPM x 1 min.
- Transfer the empty (and dry) column into a clean eppendorf tube.
- Add 30-50 uL of EB (pre-warming at 55 C increases yield) to the middle of the column and incubate at RT for 5 minutes.
- Spin at max RPM x 1min.
- Discard column, spec plasmid prep, and store labeled eppendorf tube in - 20 C.

III - Ligation

12. Ligation of insert to cut backbone (perform as many ligations as your digested vector allows, I do 10 ligations minimum (usually it is the limiting factor)

Note: high concentration; #M0202M 2,000,000 units/ml

Item	Concentration	Amount (uL)	Master Mix	MM amount (uL)
Cut and dephosphorylated backbone	50 ng/uL	6	10	60
Cut insert	1 ng/uL	10	10	100
T4 ligase buffer	10X	2	10	20
T4 ligase	-	1	10	10
H2O	-	1	10	10
Total		20	10	200

- Incubate at 16°C overnight

13. Precipitation of ligation reactions

- Pre-spin PhaseLock tubes at max speed for 5 min.
- Pool 5 ligations per pool and complete to 300µL using water.
- Add 300µL of equilibrated Phenol (no Chloroform or Isoamyl alcohol; ensure you pipet from bottom phase).
- Mix and extract using PhaseLock tubes (spin at max speed for 5min).
- After spin, take the top 250µL watery phase, add 25µL 3M NaOAC pH 5.2, 750µL ice cooled EtOH and 1.5µL Pellet paint (Novagen).
- Mix and store in -20°C (overnight or longer). Can also do -80°C for 2hrs.
- Spin down (13K RPM for 30 min at 4°C).
- Discard supernatant and add ~1mL of 70% EtOH.
- Spin down (13K RPM for 5 min at 4°C).
- Repeat for another 70% EtOH wash.
- Dry and resuspend in pre-warmed EB (55°C) (2.5µL of EB per 5 precipitated reactions).

IV - Electroporation

14. Bacterial electroporation

- Dry 10 cm/15 cm LB-Amp / LB-Carb plates at 37°C for at least 6 hrs until completely dry.
- Pre-chill cuvettes at -20°C / -80°C throughout the day and store at -20°C when ready.
- Thaw electrocompetent cells (e.g. Lucigen Endura ElectroCompetent Cells; #60242-2) on ice and per transformation (typically 1 transformation per precipitated ligation reaction, and 1 of these for 1 pool of ~1000 gRNAs).
- Add 5µL of precipitated ligation to 50uL bacteria (generally 10% of bacteria volume is good)
- Incubate on ice for 10min.
- Transfer bacteria to pre-chilled cuvette (~55µL) (wipe sides of cuvette w/ kim-wipe before electroporating to remove condensation).
- Electroporate (manual setting, 2.00kV, aim for at least 5.2 msec).
- Rapidly quench w/ ~945µL pre-warmed (37°C) SOC or LB.
- Recover at 37°C x 1hr in a bacterial shaker.

15. Plating (Dilution plates)

- Set up serial dilution plates (10E2 - 10E6) by taking 10µL of bacteria and diluting in 990µL SOC/LB (initial 10E2 dilution) and then serially dilute (10µL bacteria + 90µL SOC/LB) until obtaining the 10E6 dilution.
- Plate 100µL dilutions into 10cm pre-warmed plates (make sure these are dry).
- Spread thoroughly using 4 glass beads per plate until dry.
- Incubate inverted at 37°C overnight for 16hrs.
- Count colonies next day; ideal representation = 10,000X.
- SEND FOR SEQUENCING

16. Plating (Library plates)

- Plate ~240µL of bacteria per 15cm plate (divide in 4 spots in the plate).
- Spread thoroughly using 4 glass beads per plate until dry.
- Incubate inverted at 37°C O/N for 16hrs.

17. Scraping

- Prepare at least 250mL of fresh LB-Amp per four 15cm plates.

- Add 20mL of LB-Amp per 15cm plate.
- Scrape using cell lifters.
- Transfer to 1L flask.
- Repeat steps 1-4.
- Complete to 250mL with fresh LB-Amp.
- Shake at 37°C for at least 2hrs (up to 4hrs).
- Spin bacteria and freeze pellets or proceed to large-scale maxipreps.