Preparing sgRNA-sensor construct for Next Generation Sequencing (NGS)

- Extract gDNA from 5M cells using “Genomic DNA extraction from cells using the QIAGEN DNeasy Blood & Tissue Kit”.
- Perform 1 PCR reaction for every 1000 sgRNA in your library to maintain ≥1000X representation.
- Ex: 16 PCRs (it is easier to use a number divisible by 4, see below) for a 17, 000 sgRNA library
    - Overall the more PCRs into which you divide your gDNA the better
- Divide your total volume of gDNA into the number of PCR determined above.
    - If you extracted gDNA from 1000X equivalent of cells, then you want to amplify all of it
- To amplify plasmid libraries use 1-10ng of plasmid DNA
- Perform PCR reactions using Q5 High Fidelity 2X Master Mix (NEB #M0429S).

Component	**50uL Reaction**	**Master Mix (X36) X40**
Q5 High-Fidelity 2X Master Mix	25 uL	1000 uL
10 uM Forward Primer	2.5 uL	100 uL
10 uM Reverse Primer	2.5 uL	100 uL
Template DNA	20 uL	20 uL each
H2O	-	
Total	**50 uL**	**1200 uL**

For master mix: pipette 30uL to each PCR tube

Trono.BR applicable PCR1 primers (adds the partial illumina adapter)

Primer	**Sequence**
Sensor_PCR1_F	CGCTCTTCCGATCTCTAGCGTTCGAGTTAGGAATT
Sensor_PCR1_R	CTGAACCGCTCTTCCGATCTTTGTGGAAAGGACGAAACACC

- Cycle conditions (25x CYCLES)!!!:

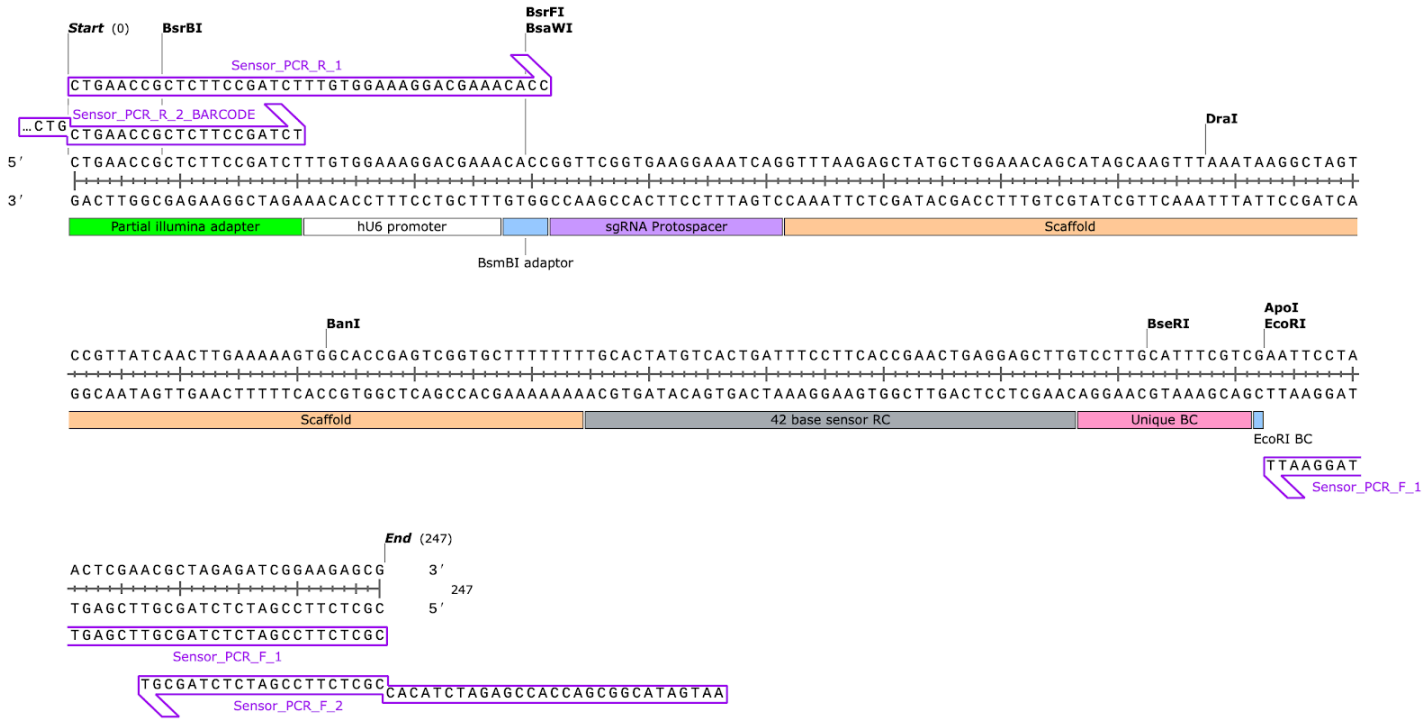
Temperature (°C)	cycle no.
98	30 s
98	10 s
64	30 s
72	30 s
72	2 min
4	keep

NOTE: for plasmid libraries use only 10x cycles to avoid recombination

Named “Sensor-PCR1” under Grace’s folder in thermocycler 1.

- Pool up to 4 PCR reactions and purify using PCR purification kit (QIAquick PCR Purification Kit) according to manufacturer’s protocol and elute in 50uL of EB buffer.
- Add 10uL of 6X loading dye to each sample eluted.
- Run samples on a 1% agarose gel alongside a 100bp ladder.
- Gel purify the correctly sized band using (Qiagen Gel Extraction Kit) and elute in 30uL of EB buffer.
- Measure DNA concentration using the NanoDrop 2000 (ThermoFisher).

Sensor-PCR1 product



- Use 10ng of PCR1 product to perform 4 PCR reactions using Q5 2X

- Perform 1 PCR reaction for every 10,000 sgRNAs in your library
    - In most cases 1 or 2 PCR2 reactions is more than enough, as very little DNA is actually required for sequencing

Component	50uL Reaction	Master Mix (X4) X5
Q5 High-Fidelity 2X Master Mix	25 uL	125 uL
10 uM Forward Primer	2.5 uL	12.5 uL
10 uM Reverse BARCODED Primer	2.5 uL	12.5 uL
Template DNA (10ng/uL)	1 uL	1 uL each
H2O	19 uL	95 uL
Total	**50 uL**	**250 uL**

Note: USE DIFFERENT BARCODED PRIMER FOR EACH SAMPLE

PCR2 primers bind the illumina partial adaptors in add unique sample barcodes as well as i7 and i5 anchor sequences required for NGS

Primer	**Sequence**
Sensor_PCR2_F	AATGATACGGCGACCACCGAGATCTACACCGCTCTTCCGATCTCTAGCGT
Sensor_PCR2_R_BARCODE	CAAGCAGAAGACGGCATACGAGATNNNNNNNNCCTGCTGAACCGCTCTTCCGATCT

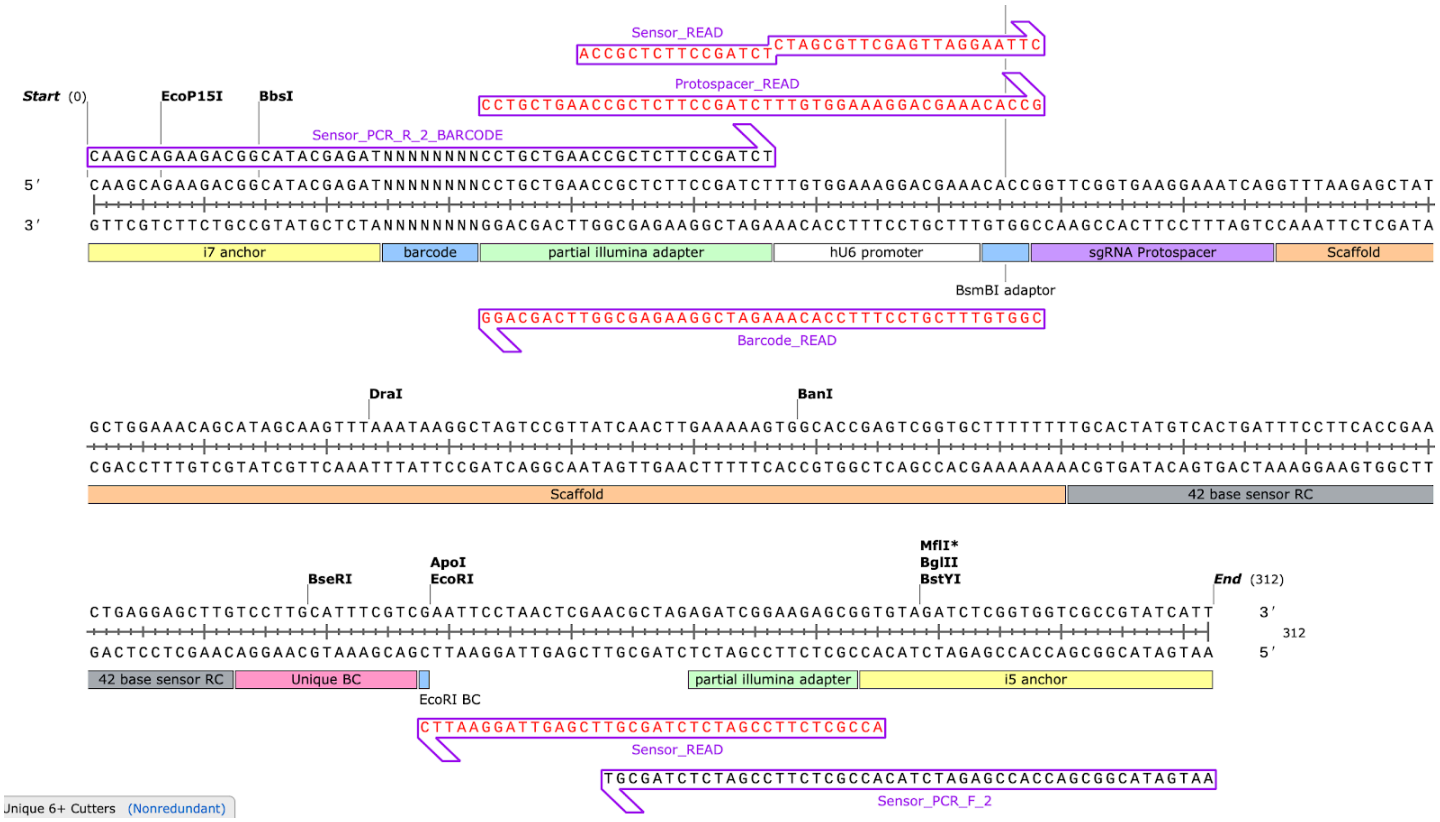
- Cycle conditions (10x CYCLES)!!!:

Temperature (°C)	cycle no.
98	2 min
98	10 s
70	30 s
72	30 s
72	2 min
4	keep

Named “Sensor-PCR2” under Grace’s folder in thermocycler 1.

- Pool up to 4 PCR reactions and purify using PCR purification kit according to manufacturer’s protocol and elute in 50uL of EB buffer.
- Add 10uL of 6X loading dye to each sample eluted.
- Run samples on a 1% agarose gel alongside a 100bp ladder.
- Gel purify the correctly sized band and elute in 30uL of EB buffer.
- Measure DNA concentration using the NanoDrop.

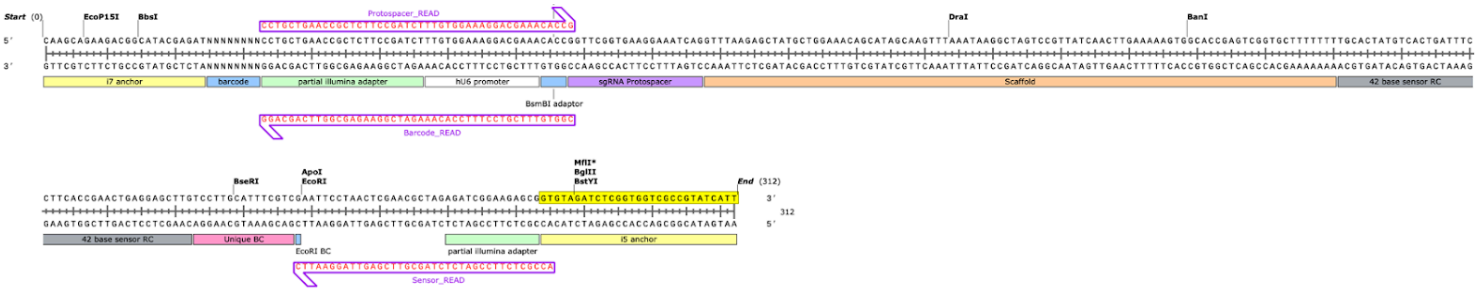
Sensor-PCR2 product



- Submit with the appropriate primers.

Examples:

Primer	Sequence
Protospacer_Read	CCTGCTGAACCGCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
Sensor_Read	ACCGCTCTTCCGATCTCTAGCGTTCGAGTTAGGAATTC
Barcode_Read	CGGTGTTTCGTCCTTTCCACAAAGATCGGAAGAGCGGTTCAGCAGG


Start (0)
EcoP15I
BbsI
Protospacer_READ
DraI
BanI
5'
3'
i7 anchor
barcode
partial illumina adapter
hU6 promoter
sgRNA Protospacer
Scaffold
42 base sensor RC
BsmBI adaptor
Barcode_READ
BseRI
ApoI
EcoRI
MfiI*
BglII
BstYI
End (312)
312
42 base sensor RC
Unique BC
EcoRI BC
partial illumina adapter
i5 anchor
Sensor_READ