

# MPRA Extended Methods

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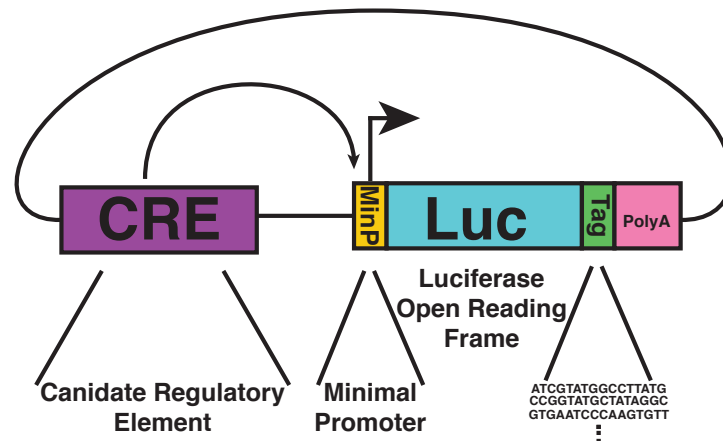
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# 1 Introduction

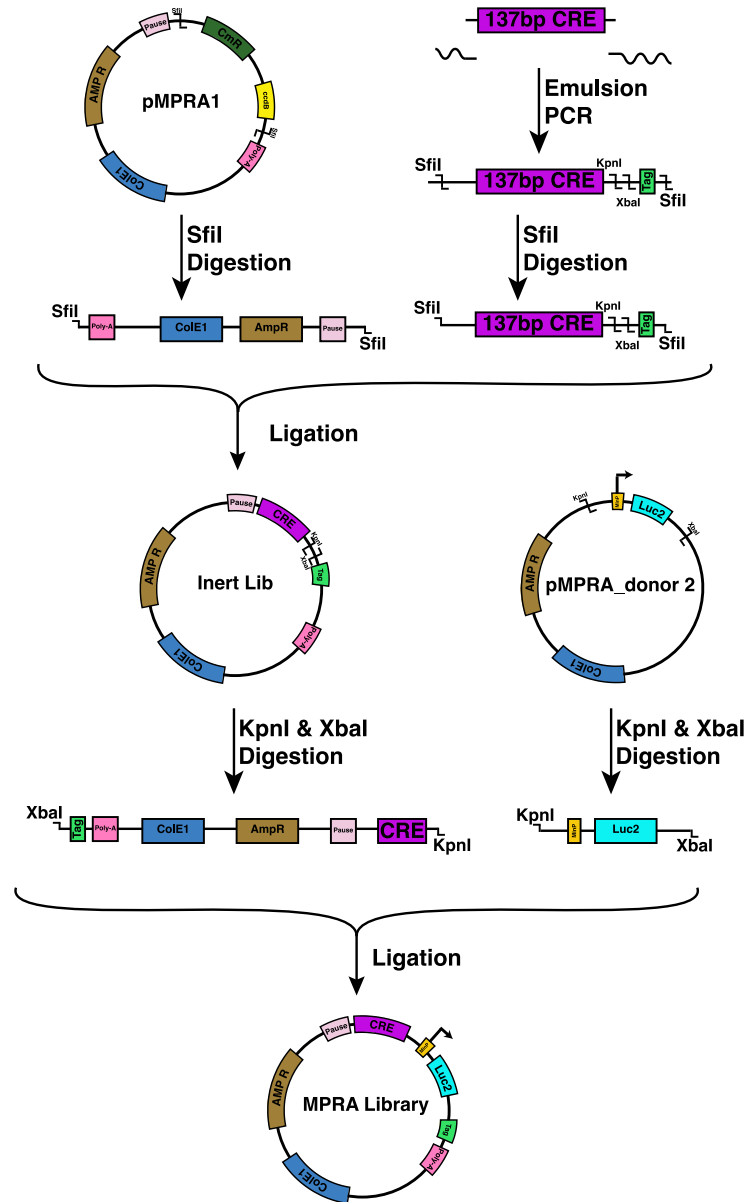
## 1.1 Method Overview



Extended Methods Figure 1: Mechanism of Enhancer Interrogation

- The MPRA assay relies on measuring candidate enhancers through their transcriptional capacities in parallel. A Candidate Response Element is cloned into a 5' orientation to a MinP driven ORF with a 16 random base pair tag between the ORF and poly-A tail. Utilizing the proportion of counts assigned to an individual transcript relative to that of the template DNA activity is measured as a fold enrichment.

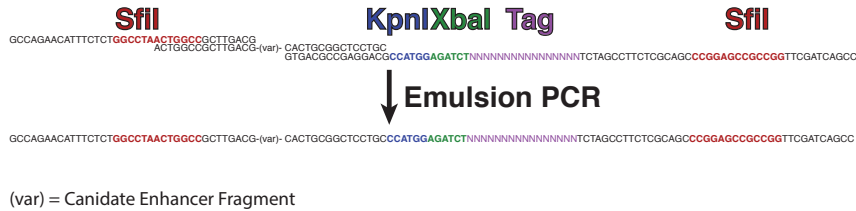
## 1.2 Library Construction Overview



Extended Methods Figure 2: MPRA library construction

- The basic schematic of MPRA library construction above includes an emulsion PCR tailing reaction (table 7) and two cloning steps to create a competent library containing an active ORF driven by MinP.

### 1.3 Emulsion PCR Overview



Extended Methods Figure 3: Tailing emulsion PCR

- The SfiI cloning arms are added to the distal ends to clone into the MPRA backbone vector, while the KpnI and XbaI sites lie in between the candidate enhancer and tag sequence. These sites are used to directionally clone in our Promoter-ORF complex.

## 2 Library Design

### 2.1 Fragments

- Fragment size is limited by synthesis capacity. The maximum insert size is the maximum synthesis length minus 30 base pairs to account for incorporation of flanking amplification primers.

### 2.2 Library Primers

- The first set of primers is un-tailed  
Forward: 5' - ACTGGCCGCTTGACG - 3'  
Reverse: 5' - GCAGGAGCCGCAGTG - 3'
- The second set primers has tails used to add restriction sites and tags to synthesized fragments.
- The Forward primer adds SfiI (GGCCNNNNNGGCC) cloning site:  
5' - GCCAGAACATTTCTCTGGCCTAACTGGCCGCTTGACG - 3'
- The Reverse primer adds SfiI (GGCCNNNNNGGCC), KpnI (CCATGG), XbaI (AGATCT), and a 16 bp tag sequence:

CCGACTAGCTTGGCCGCCGAGGCCGACGCTTCCGATCTNNNNNNNNNNNNNNNNNTCTAGAGGTACCGCAGGAGCCGCAGTG

- The reverse primer tag sequences are made by IDT and each N base pair is hand mixed with all nucleotides having an equal proportion of incorporation. The resulting Primer library is purified via HPLC (PAGE

has low yield and could skew representation, standard desalting would allow truncated primer products to contaminate primer pool).

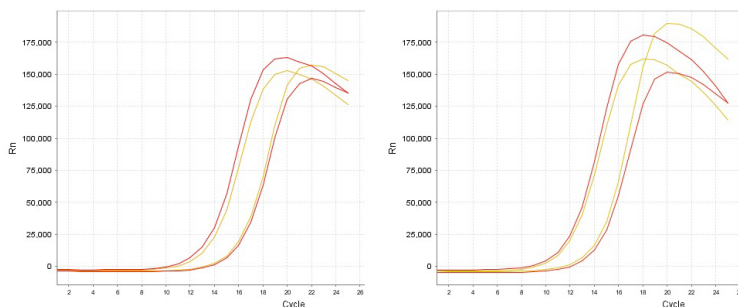
### 3 Library QC and Initial Amplification

The synthesized library needs to be PCR amplified to complement the synthesized single strand and to select only full length synthesis product for the emulsion PCR. The goal is to amplify the library with as few PCR cycles as possible to minimize amplification biases. To reach this goal, primer and library concentration in the PCR mix must be dialed in. Too much primer results in primer dimers, too few primer leads to no or incomplete amplification, too much library leads to over-saturation of the PCR. RT qPCR and Bioanalyzer analyses are used to estimate the needed concentrations.

Once primer and library concentration are dialed in, run a full-length, small input qPCR to estimate optimal amplification cycle number. Then run a qPCR with the determined cycle number using a few observation wells (SYBR Green added) and the rest of the library in the remaining wells (without SYBR Green).

#### 3.1 Single Reaction

- Observe library amplification with fluorescent SYBR Green in full-length qPCR



Extended Methods Figure 4: Initial qPCR library amplification: left, Human; right, Chimp

- **PCR mix:**

Reagent	Volume	30x
2x NEB Master Mix	10 $\mu$ l	300 $\mu$ l
Water	6.55 $\mu$ l	196.5 $\mu$ l
Fwd + Rev primers (10 $\mu$ mol/ $\mu$ l)	1.25 $\mu$ l	37.5 $\mu$ l
10x SYBR Green	1.2 $\mu$ l	-
DNA 1:10 dilution	1 $\mu$ l	-
Final Volume	20 $\mu$ l	600 $\mu$ l

Extended Methods Table 1: Initial library PCR.

- **Low cycle qPCR library amplification setup**

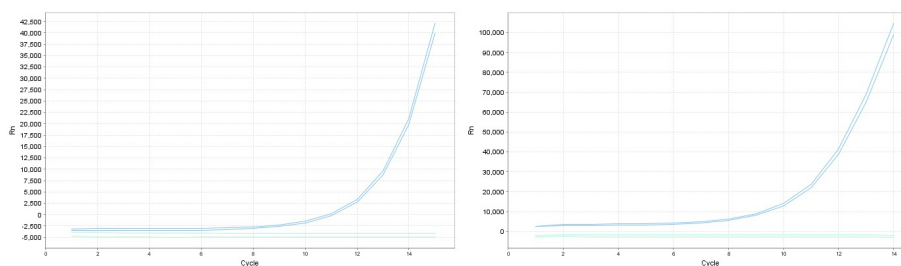
- Pipette out 17.8  $\mu$ l into 7 wells on qPCR plate.
- Add SYBR Green and DNA to 5 wells (fluorescent reporter wells).
- Add SYBR Green and water in place of DNA to 2 wells (control wells).
- Add water instead of SYBR Green to the remaining 23-reaction master mix.
- Pipette out 20 wells to collect for purification.

- **PCR conditions:**

Stage	Temperature	Time	Cycles
Stage 1	98°C	30 Sec	1 Cycle
	98°C	10 Sec.	
Stage 2	62°C	30 Sec.	X Cycles
	72°C	30 Sec.	
Stage 3	72°C	5 Min.	1 Cycle

Extended Methods Table 2: qPCR amplification conditions.

- Amplification cycles are determined empirically to stop amplification in the log phase.



Extended Methods Figure 5: Left: Human, Right: Chimp



- Bead Purify with 2x volume ratio. Elute in 50  $\mu\text{l}$  Qiagen EB.

## 4 Emulsion PCR

### 4.1 Reaction Mix

- **Emulsion**

Reagent	Volume	16x
Emulsion Component 1	220 $\mu\text{l}$	3520 $\mu\text{l}$
Emulsion Component 3	60 $\mu\text{l}$	960 $\mu\text{l}$
Emulsion Component 2	20 $\mu\text{l}$	320 $\mu\text{l}$
Final Volume	300 $\mu\text{l}$	4800 $\mu\text{l}$

Extended Methods Table 3: Emulsion component mixture.

- Add components in order, use a wide bore tip for Emulsion Component 2.
- Incubate at 4°C for 30 minutes on wet ice.
- **Aqueous PCR Mix:**

Reagent	Volume	16x
2x NEB Master Mix	25 $\mu\text{l}$	400 $\mu\text{l}$
Water	19 $\mu\text{l}$	304 $\mu\text{l}$
Fwd + Rev primers (5 $\mu\text{mol}/\mu\text{l}$ )	2.5 $\mu\text{l}$	40 $\mu\text{l}$
BSA (10 mg/ml)	2 $\mu\text{l}$	32 $\mu\text{l}$
DNA	1 $\mu\text{l}$	16 $\mu\text{l}$
q5 Pol	0.5 $\mu\text{l}$	8 $\mu\text{l}$
Final Volume	50 $\mu\text{l}$	800 $\mu\text{l}$

Extended Methods Table 4: Aqueous PCR component mixture.

- Add entire volume to pre-chilled emulsion mix and vortex for 5 minutes on high at 4°C.
- **PCR Conditions:**

Stage	Temperature	Time	Cycles
Stage 1	98°C	30 sec.	1 Cycle
Stage 2	98°C	20 sec.	15 Cycles
	72°C	10 sec.	
Stage 3	72°C	15 sec.	1 Cycle
	72°C	5 min.	

Extended Methods Table 5: PCR conditions for library insert amplification.

- Each PCR reaction is 50  $\mu\text{l}$  per well.

- **Cleanup:**
- Pool all reactions and add 1 ml of 2-butanol. Vortex thoroughly.
- Use kit provided spin columns.

## 4.2 Full Scale Reaction

- **Emulsion:**

Reagent	Volume
Emulsion Component 1	3520 $\mu$ l
Emulsion Component 3	960 $\mu$ l
Emulsion Component 2	320 $\mu$ l
Final Volume	4800 $\mu$ l

Extended Methods Table 6: Emulsion component mixture.

- Add components in order, use a wide bore tip for Emulsion Component 2.
- Incubate at 4°C for 30 minutes on wet ice.
- **Aqueous PCR Mix:**

Reagent	Volume
2x NEB Master Mix	400 $\mu$ l
Water	319 $\mu$ l
Fwd + Rev primers (5 $\mu$ mol/ $\mu$ l)	40 $\mu$ l
BSA (10 mg/ml)	32 $\mu$ l
DNA	1 $\mu$ l
q5 Pol	8 $\mu$ l
Final Volume	800 $\mu$ l

Extended Methods Table 7: Aqueous PCR component mixture.

- Add entire volume to pre-chilled emulsion mix and vortex for 5 minutes on high at 4°C.
- **PCR Conditions:**

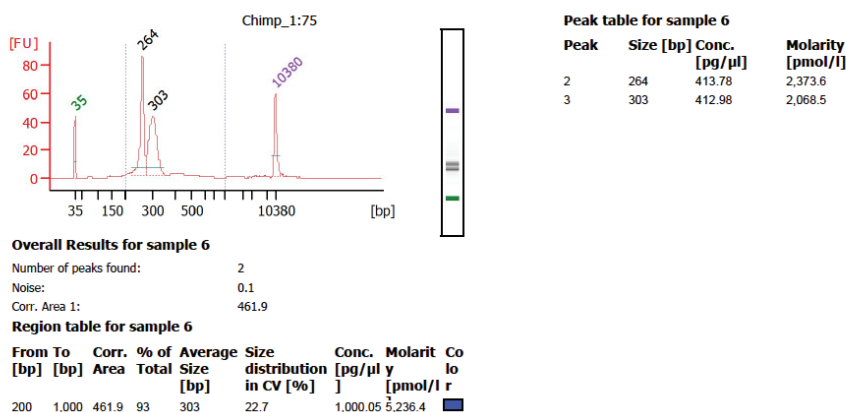
Stage	Temperature	Time	Cycles
Stage 1	98°C	30 sec.	1 Cycle
	98°C	20 sec.	
Stage 2	72°C	10 sec.	15 Cycles
	72°C	15 sec.	
Stage 3	72°C	5 min.	1 Cycle

Extended Methods Table 8: PCR conditions for library insert amplification.

- Each PCR reaction is 50  $\mu$ l per well (96 wells total).
- **Cleanup:**
- Pool all reactions and add 13.7 ml of 2-butanol. Vortex thoroughly.
- Use kit provided spin columns. Condense all volume over 3–4 columns to concentrate eluted library.

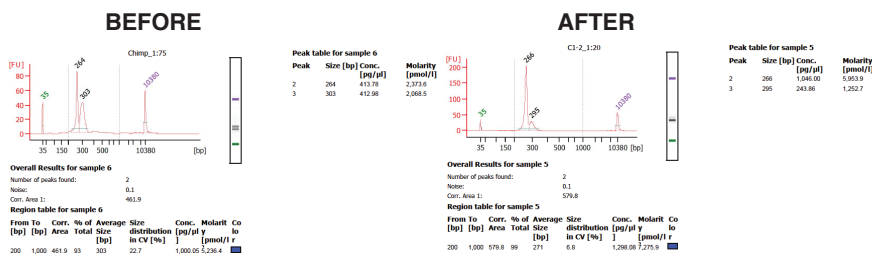
### 4.3 Size Select Library to Remove Slippage Products

- Slippage on the random tag sequence causes the production of additional products to arise from the emulsion PCR.



Extended Methods Figure 6: Extra large molecular weight slippage product ~300 bp.

- Run libraries in a 2% agarose Pippin Prep gel on Pippin Prep DNA Size Selection System to enrich for the correct 265 bp size product.



Extended Methods Figure 7: Tailed libraries before and after size selection.

## 5 Inert Library Cloning

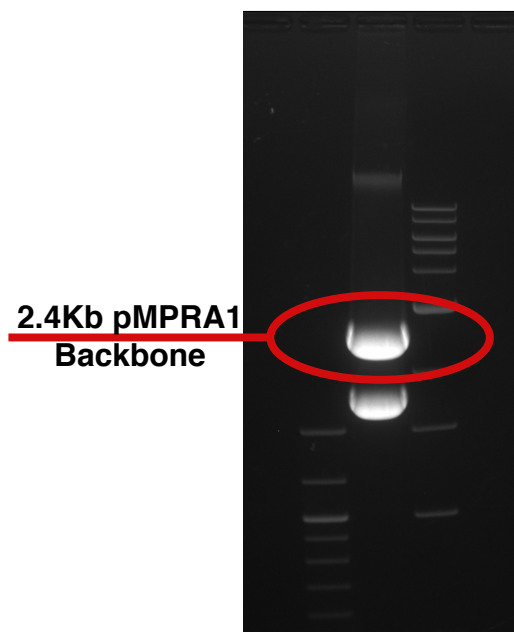
### 5.1 SfiI Digests

- Digest libraries and pMPRA1 vector over night at 37°C.

Reagent	Library	pMPRA1 Vector
DNA, at least:	200 ng	700 ng
10x Cutsmart buffer	X $\mu$ l	X $\mu$ l
SfiI	2 $\mu$ l/10 $\mu$ l	2 $\mu$ l/10 $\mu$ l
NcoI	-	2 $\mu$ l/10 $\mu$ l
AclI	-	2 $\mu$ l/10 $\mu$ l
XmaI	-	2 $\mu$ l/10 $\mu$ l
Final Volume	X $\mu$ l	X $\mu$ l

Extended Methods Table 9: Initial library digest setup.

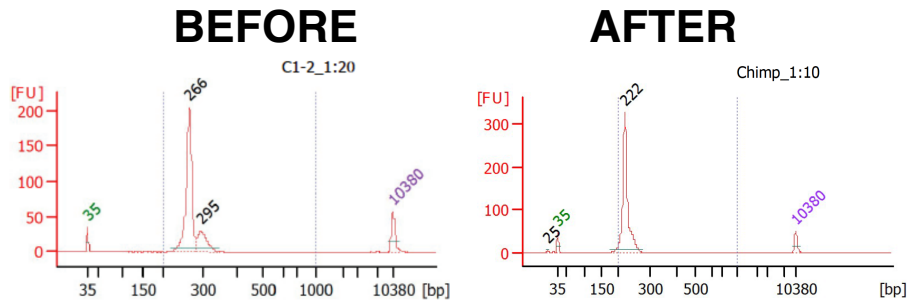
- Add 2  $\mu$ l CIP, 1  $\mu$ l Cutsmart buffer and 7  $\mu$ l water to the pMPRA1 vector digest. Incubate at 37°C for 1 hour.
- Clean up fragment library digests with MinElute columns. Elute in 8  $\mu$ l EB buffer.
- The plasmid digest yields a 2.4 Kb band while all other bands are less than 600 bp. Use 0.5x AmpureXP beads to size select the DNA. This avoids a gel extraction.



Extended Methods Figure 8: Gel extract pMPRA1 backbone to isolate from insert.

## 5.2 Library Ligation

- Before setting up the ligation reaction confirm that the fragment libraries have been completely digested on both ends by running a Bioanalyzer high sensitivity chip on them.



Extended Methods Figure 9: The 266 bp library fragments should lose ~40 bp after digest.

- Ligation reactions are performed at a fragment to backbone ratio of 3:1. Backbone input mass is 340 ng, making this particular library input mass 103 ng (corresponding to 3x backbone copy number). 2000 U of ligase is used per reaction. Ideally each reaction should yield at least 5,000,000 CFU. Reactions were setup as described in Table 10.

Reagent	Amount
Water	? $\mu$ l
Fragment library	103 ng
Vector backbone	340 ng
10x Ligation buffer	X $\mu$ l
Ligase (400 U/ $\mu$ l)	5 $\mu$ l
Total Volume	X $\mu$ l

Extended Methods Table 10: Initial library ligation.

- Ligations incubated for 16 hours at 16°C in a thermocycler with 20–100  $\mu$ l per tube.

## 5.3 Library Transformation

- Ligations should be cleaned up with 1x room temperature AMPure XP beads. Add 1 volume of beads to the ligation reaction. Incubate for 5 minutes. Apply magnet for 5 minutes.
- Remove supernatant and wash twice with 200  $\mu$ l 80% ethanol. Air dry for 10–15 minutes and elute in 10  $\mu$ l EB.

- Label all LB-Amp plates (100 µg/ml) plates and place in 37°C incubator to pre-warm.
- Label tubes for a tenfold dilution series and fill with recovery media according to the serial dilution table below:

Dilution	1:10	1:100	1:1,000	1:5,000	1:10,000*	1:50,000	1:100,000*
Recovery media	90 µl	90 µl	90 µl	40 µl	90 µl	40 µl	90 µl
Cell volume	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl

Extended Methods Table 11: Serial dilution schema for ligation and transformation efficiency.

*\*These dilutions are made from the preceding ten-fold dilution not the half fold dilution.*

- Place 5 electroporation cuvettes per 20 µl recombination reaction and a 1.5 ml Eppendorf tube on ice for each transformation reaction.
- Thaw cells on watery ice, make sure the water has time to come down to 4°C, mix cells by gently flicking the side of the tube. **DO NOT VORTEX!**
- Pipette 20 µl of thawed cells into the Eppendorf tube on ice with a **wide bore** pipette tip.
- Pipette 2 µl of DNA into the 20 µl of cells and tap tube to mix.
- Incubate on ice for 10 minutes.
- Take 21 µl of the cells with a **wide bore** pipette tip and place in the center of the cuvette. Tap the cuvette gently on the table to get the cells to fall into the bottom and rid the cells of any air bubbles.
- Place into electroporator arm and ensure that the metal cuvette is dry with a chem-wipe. Be sure not to warm the cuvette with your hand.
- Electroporate at 200 Ω, 25 µF, 2.0 kV, and immediately place the cuvette back on ice.
- Place 1 ml of **room temperature** recovery into the cuvette.
- Use a **wide bore** 1 ml pipette tip to mix the cells once and then remove the cells slowly while tilting the cuvette on its side to ensure the cuvette well is drained. Place the cells into a 15 ml falcon round-bottom tube.
- Loosely fasten the cap to allow gas exchange and incubate at 37°C and 225 RPM for 1 hour.
- Pool all reactions and remove 10 µl. Dilute the cells according to Table 11. Plate two plates with 10 µl of each dilution.
- For every transformation, seed a **dense range** of 500 ml of LB-Amp in 2.5 l flasks. Allowing for a bit more than 20% variation, I chose 10, 15, 23, 36, 57, 90, 140, 220, 350, 550, 870, 1370, and 2150 µl.

- Grow inoculations at 37°C and 225 rpm for 5–11 hours and stop each inoculation at an  $OD_{600} = 0.95\text{--}1.2$ . Check OD after 5 hours and from then on in regular intervals.
- First, remove 800  $\mu\text{l}$  and transfer to a cryotube. Add 200  $\mu\text{l}$  anhydrous glycerol, vortex to mix and store at  $-80^\circ\text{C}$  to generate glycerol stocks.
- Pellet cells at 4500 rpm for 15 minutes at  $4^\circ\text{C}$  and freeze pellets at  $-20^\circ\text{C}$ .

## 5.4 Plasmid Library Purification

- Plasmid library is purified with HiSpeed Qiagen Maxi kit (12662).

## 5.5 Estimate Complexity

- Calculate the concentration of competent transformants using the diluted series of plates equation:

$$CFU\text{per}\mu\text{seeded} = \frac{AvgCFU \times DilutionFactor}{10 \mu\text{l}}$$

- For a 52,000–104,000 element library, 80 tags per candidate enhancer is the target.
- Therefore for a 52,000 element library, total complexity is:

$$4,200,000 \approx CFU\text{per}\mu\text{seeded} \times \mu\text{seeded}$$

- Dilutions should be within 25% of your targeted complexity. It is better to err on the large end, because it is still possible to lose complexity afterwards. If you did not hit a satisfying complexity, repeat the transformation.

## 5.6 QC Inert Library

- Equation to calculate total number of independent ligated transformants:

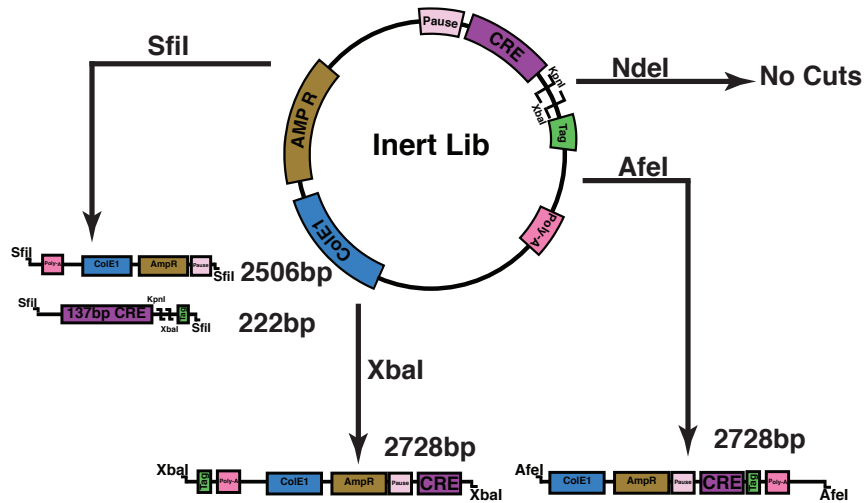
$$TotalComplexity = CFU \times DilutionFactor \times \frac{VolumeofCellsCultured(\mu\text{l})}{10\mu\text{l}}$$

- Set up four different restriction digests of the purified inert library as described in table 12.

Reagent	SfiI	XbaI	AfeI	NdeI
Water	10 $\mu\text{l}$	10 $\mu\text{l}$	10 $\mu\text{l}$	10 $\mu\text{l}$
DNA (100 ng/ $\mu\text{l}$ )	5 $\mu\text{l}$	5 $\mu\text{l}$	5 $\mu\text{l}$	5 $\mu\text{l}$
10x Buffer	2 $\mu\text{l}$	2 $\mu\text{l}$	2 $\mu\text{l}$	2 $\mu\text{l}$
SfiI	3 $\mu\text{l}$	- $\mu\text{l}$	- $\mu\text{l}$	- $\mu\text{l}$
XbaI	- $\mu\text{l}$	3 $\mu\text{l}$	- $\mu\text{l}$	- $\mu\text{l}$
AfeI	- $\mu\text{l}$	- $\mu\text{l}$	3 $\mu\text{l}$	- $\mu\text{l}$
NdeI	- $\mu\text{l}$	- $\mu\text{l}$	- $\mu\text{l}$	3 $\mu\text{l}$
Total volume	20 $\mu\text{l}$	20 $\mu\text{l}$	20 $\mu\text{l}$	20 $\mu\text{l}$

Extended Methods Table 12: Inert library digests.

- Run out all four digests on a 1% agarose gel.
- If library is correctly constructed it will digest according to Figure 5.6.

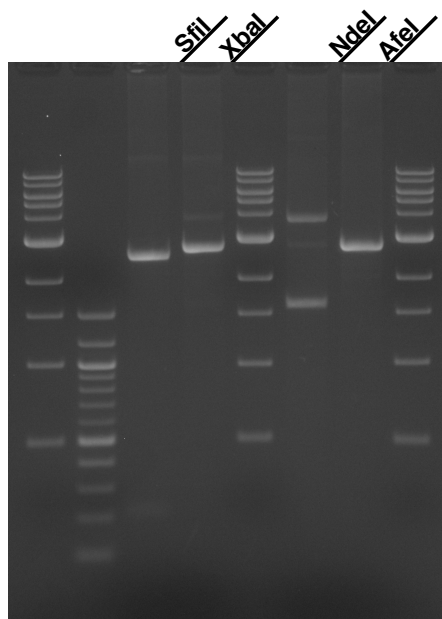


**Because the tag is random, there will be minor products for every enzyme**

Extended Methods Figure 10: Library QC digest schematic

- Digestion gel should look similar to Figure 5.6.





Extended Methods Figure 11: Library QC digest

## 6 Library Sequencing

### 6.1 PCR Amplification of Inert Library

- Dilute primers to 100  $\mu\text{M}$ , then mix forward and reverse primers to a final working concentration of 10  $\mu\text{M}$ .
- Setup PCR reactions as follows:

Reagent	1x	10x
5X Q5 buffer	10 $\mu\text{l}$	100 $\mu\text{l}$
5X GC enhancer	10 $\mu\text{l}$	100 $\mu\text{l}$
DMSO	2.5 $\mu\text{l}$	25 $\mu\text{l}$
Primers (10 $\mu\text{M}$ )	2.5 $\mu\text{l}$	25 $\mu\text{l}$
dNTPs (25 mM)	0.4 $\mu\text{l}$	4 $\mu\text{l}$
Library DNA (3 ng/ $\mu\text{l}$ )	0.5 $\mu\text{l}$	5 $\mu\text{l}$
Q5 polymerase	0.5 $\mu\text{l}$	5 $\mu\text{l}$
Water	22.25 $\mu\text{l}$	236 $\mu\text{l}$
Final Volume	50 $\mu\text{l}$	500 $\mu\text{l}$

Extended Methods Table 13: Volumes for library PCR reactions.

- Run 10 PCR reactions under the following conditions:

Stage	Temperature	Time	Cycles
Stage 1	98°C	30 sec.	1 Cycle
	98°C	10 Sec.	
Stage 2	66°C	20 Sec.	18 Cycles
	72°C	20 Sec.	
Stage 3	72°C	5 Min.	1 Cycle

Extended Methods Table 14: PCR conditions for library insert amplification.

- Pool the 10 PCR reactions and cleanup with 1.4x AMPureXP Beads or Qiagen PCR Purification Kit. Elute in 30 µl EB.
- Check size fraction and concentration on Bioanalyzer.

**The following steps are done by the sequencing center:**

## 6.2 dA-Tailing

- Combine the following:

Reagent	Volume
End-repaired DNA	42 µl
10x NEBNext end repair reaction buffer	5 µl
Klenow Fragment (3'-5' exo-)	3 µl
Final volume	50 µl

Extended Methods Table 15: dA tailing reaction.

- Incubate at 37°C for 30 minutes.
- Clean up with Qiagen QIAQuick PCR Purification kit. Elute in 25 µl EB

## 6.3 Adaptor Ligation

- Combine the following:

Reagent	Volume
dA-Tailed DNA	25 µl
5x NEBNext Quick Ligation Reaction Buffer	10 µl
15 µM NEBNext adaptors	10 µl
Quick T4 DNA ligase	5 µl
Final volume	50 µl

Extended Methods Table 16: Adaptor ligation reaction.

- Incubate at 20°C for 15 minutes.
- Clean up with Qiagen QIAQuick PCR Purification kit. Elute **twice** in 25 µl EB (final volume = 50 µl)

## 6.4 Indexing PCR Amplification

- Dilute PCR primers to a final working concentration of 50  $\mu\text{M}$ .
- Setup PCR reactions as follows:

Reagent	1x	5x
2x NEB High Fidelity	12.5 $\mu\text{l}$	62.5 $\mu\text{l}$
Library DNA	1 $\mu\text{l}$	5 $\mu\text{l}$
F-Primer (10 $\mu\text{M}$ )	1.25 $\mu\text{l}$	6.25 $\mu\text{l}$
R-Primer (10 $\mu\text{M}$ )	1.25 $\mu\text{l}$	6.25 $\mu\text{l}$
Water	9 $\mu\text{l}$	45 $\mu\text{l}$
Final vlume	25 $\mu\text{l}$	125 $\mu\text{l}$

Extended Methods Table 17: Volumes library PCR reactions.

- Run 4 PCR reactions under the following conditions:

Stage	Temperature	Time	Cycles
Stage 1	98°C	30 Sec.	1 Cycle
Stage 2	98°C	10 Sec.	10 Cycles
	65°C	30 Sec.	
	72°C	30 Sec.	
Stage 3	72°C	5 Min.	1 Cycle

Extended Methods Table 18: PCR conditions for library insert amplification.

- Pool the 4 PCR reactions and cleanup with 0.9x AMPureXP beads. Elute in 20  $\mu\text{l}$  EB.
- Check size and concentration on Bioanalyzer.
- Sequence 2x250 bp at high depth according estimated complexity in 5.6 dope in 5% PhiX to add complexity for cluster generation.

## 7 Cloning Promoter/ORF into Inert Vector

### 7.1 KpnI and XbaI Library Digests

- Digest inert vector libraries with KpnI over night at 37°C as described in Table 19.

Reagent	Volume
Plasmid library	15 $\mu\text{l}$
Buffer	2 $\mu\text{l}$
KpnI	3 $\mu\text{l}$
Total volume	20 $\mu\text{l}$

Extended Methods Table 19: Inert library digest with KpnI.

- Cleanup the digestion with Qiagen Minelute column.
- Digest inert vector libraries with XbaI over night at 37°C as described in Table 20.

Reagent	Volume
Plasmid library	15 $\mu$ l
Buffer	2 $\mu$ l
XbaI	3 $\mu$ l
Total volume	20 $\mu$ l

Extended Methods Table 20: Inert library digest with XbaI.

- Add 1  $\mu$ l CutSmart Buffer, 3  $\mu$ l CIP, 4  $\mu$ l water and incubate 45 minutes at 37°C.
- Cleanup the digestion with Qiagen Minelute column.

## 7.2 KpnI and XbaI ORF Digests

- Digest donorMPRA 2 plasmid with KpnI and XbaI over night at 37°C as described in Table 21.

Reagent	Volume
Plasmid library	21 $\mu$ l
Buffer	3 $\mu$ l
XbaI	3 $\mu$ l
KpnI	3 $\mu$ l
Total volume	20 $\mu$ l

Extended Methods Table 21: DonorMPRA 2 digestion

- Run reaction on 0.8% agarose gel and gel purify the lower 1790 bp band.
- Cleanup the gel extraction with 1x AMPureXP beads.

## 7.3 Library Ligation

- Ligate 500 ng of inert plasmid library to 650 ng of MinP/Luc2 ORF (2:1 Insert:Vector) as follows (Table 22):

Reagent	Volume
Digested inert plasmid library	X $\mu$ l = 500 ng
Digested MinP/Luc2 ORF	X $\mu$ l = 650 ng
10x ligase buffer	2 $\mu$ l
T4 DNA ligase (400 U/ $\mu$ l)	5 $\mu$ l
Water	X $\mu$ l
Total volume	20 $\mu$ l

Extended Methods Table 22: MPRA library ligation

- Incubate ligation reaction at 16°C for 16 hours in a thermocycler.
- Clean up ligation reaction with 1x AMPureXP beads and elute in 20  $\mu$ l EB.

## 7.4 Library Transformation

- Label all LB-Amp plates (100  $\mu$ g/ml) plates and place in 37°C incubator to pre-warm.
- Label tubes for a tenfold dilution series and fill with recovery media according to the serial dilution table below:

Dilution	1:10	1:100	1:1,000	1:5,000	1:10,000*	1:50,000	1:100,000*
Recovery Media	90 $\mu$ l	90 $\mu$ l	90 $\mu$ l	40 $\mu$ l	90 $\mu$ l	40 $\mu$ l	90 $\mu$ l
Cell Volume	10 $\mu$ l	10 $\mu$ l	10 $\mu$ l	10 $\mu$ l	10 $\mu$ l	10 $\mu$ l	10 $\mu$ L

Extended Methods Table 23: Serial dilution schema for ligation and transformation efficiency.

*\*These dilutions are made from the preceding ten-fold dilution not the half fold dilution.*

- Place 10 electroporation cuvettes per 20  $\mu$ l recombination reaction and 10 1.5 ml Eppendorf tubes on ice for each transformation reaction.
- Thaw cells on watery ice, make sure the water has time to come down to 4°C, mix cells by gently flicking the side of the tube. **DON'T VORTEX!**
- Pipette 20  $\mu$ l of thawed cells into the Eppendorf tube on ice with a **wide bore** pipette tip.
- Pipette 2  $\mu$ l of DNA into the 20  $\mu$ l of cells and tap tube to mix.
- Incubate on ice for 10 minutes.
- Take 21  $\mu$ l of the cells with a **wide bore** pipette tips and place in the center of the cuvette. Tap the cuvette gently on the table to get the cells to fall into the bottom and rid the cells of any air bubbles.
- Place into electroporator arm and ensure that the metal cuvette is dry with a chem-wipe. Be sure not to warm the cuvette with your hand!
- Electroporate at 200  $\Omega$ , 25  $\mu$ Fd, 2.0 kV, and immediately place the cuvette back on ice.
- Place 1 ml of **room temperature** recovery into the cuvette.
- Use a **wide bore** 1 ml pipette tip to mix the cells once and then remove the cells slowly while rotating the cuvette on its side to ensure the cuvette well is drained. Place the cells into a 15 ml falcon tube.
- Loosely fasten the cap to allow gas exchange and incubate at 37°C and 225 RPM for 1 hour.

- Pool all reactions and remove 10  $\mu$ l and dilute the cells according to table 23. Plate two plates with 10  $\mu$ l of each dilution.
- For every 2 transformations, seed a 500 ml culture of LB-Amp in 2.5 l flasks (5 cultures total).
- Grow colonies at 37°C and 225 rpm for 8–11 hours **until OD = 1.5–2.0**.
- Pellet cells at 4500 rpm for 15 minutes at 4°C.

## 7.5 Plasmid Library Purification

- Resuspend all pellets together and purify with Qiagen EndoFree Mega (or 4 replicates of a Maxi) Kit.
- Add one additional centrifugation after neutralization of pellet lysis; 17,900 x g for 10 minutes at 4°C.
- Elute in half the recommended amount of elution buffer.

## 7.6 Estimate Transformation Efficiency

- Calculate total transformants utilizing the diluted series of plates equation:

$$CFU\text{per}\mu\text{lseeded} = AvgCFU \times DilutionFactor \times \frac{TotalVolumeofCells\mu\text{l}}{10\mu\text{l}}$$

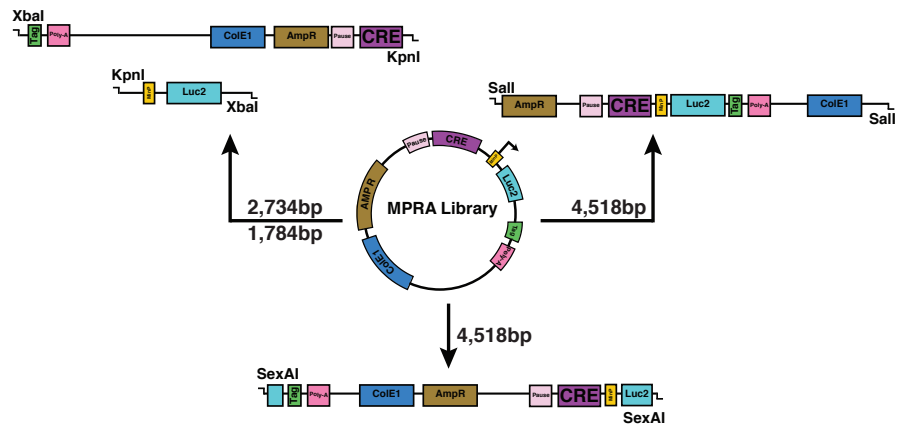
## 7.7 QC Competent Library

- Perform diagnostic digests as explained in Table 24. Incubate at 37°C overnight.

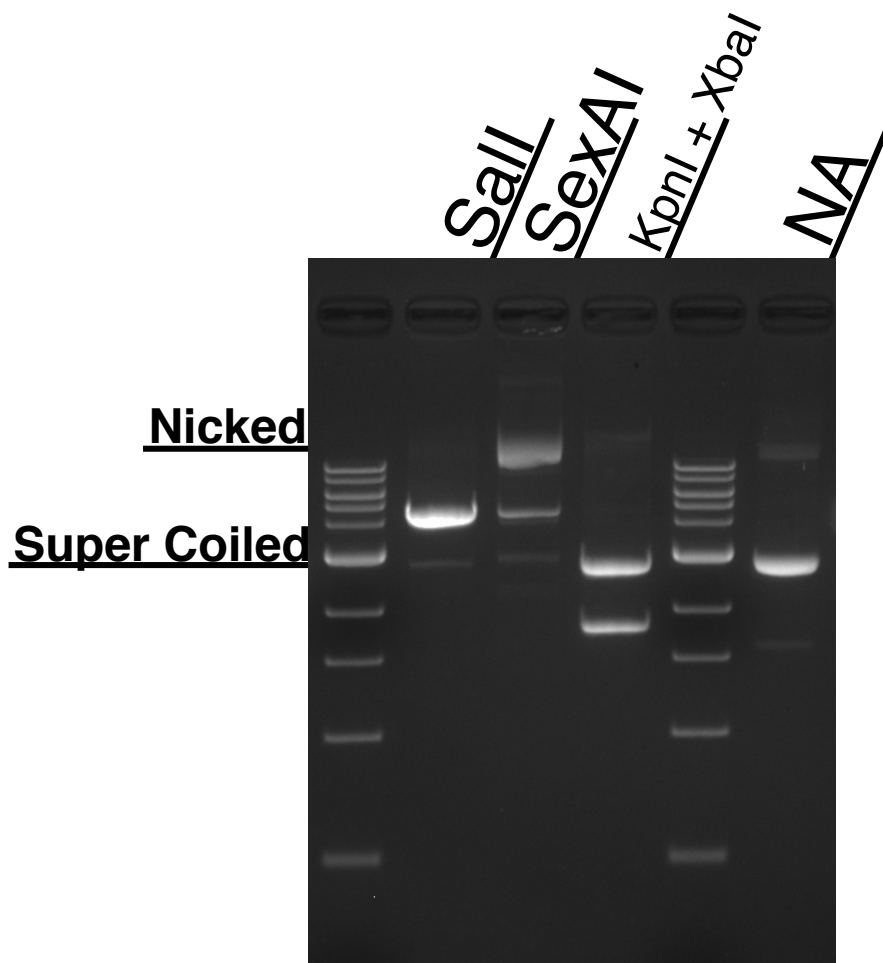
Reagent	KpnI/XbaI	SexAI	SalI
Water	8.5 $\mu$ l	11.5 $\mu$ l	11.5 $\mu$ l
DNA(200 ng/ $\mu$ l)	3.5 $\mu$ l	3.5 $\mu$ l	3.5 $\mu$ l
10x Buffer	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l
KpnI	3 $\mu$ l	- $\mu$ l	- $\mu$ l
XbaI	3 $\mu$ l	- $\mu$ l	- $\mu$ l
SexAI	- $\mu$ l	3 $\mu$ l	- $\mu$ l
SalI	- $\mu$ l	- $\mu$ l	3 $\mu$ l
Total volume	20 $\mu$ l	20 $\mu$ l	20 $\mu$ l

Extended Methods Table 24: Competent library digests.

- The expected band sizes are shown in Figure 12.



Extended Methods Figure 12: Competent library QC digest expected results



Extended Methods Figure 13: Competent library QC digest results

- Nicked and super-coiled variants may escape digestion therefore they can be identified by running non-digested plasmid in an alternate lane. In Figure 7.7, the super-coiled is around the same size as the backbone with MinP and luciferase removed (~2.7 kb) and SexAI appears to be low in activity.

## 7.8 Barcode Sequencing of Competent Library

- PCR conditions for bar-code sequencing of competent library:



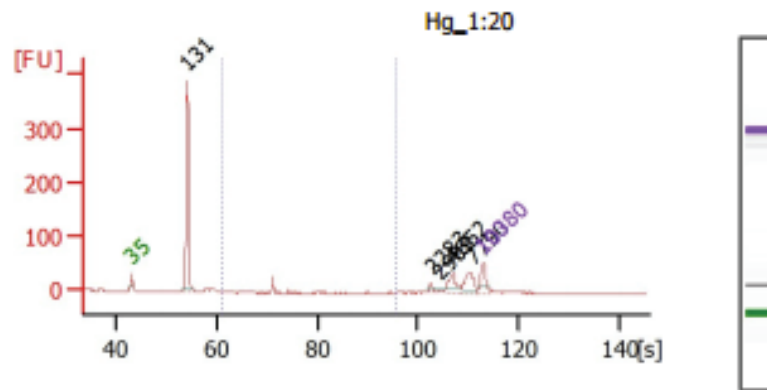
Reagent	1x	9x
2x NEB High Fidelity	25 $\mu$ l	225 $\mu$ l
Library DNA (15 ng/ $\mu$ l)	1 $\mu$ l	9 $\mu$ l
Fwd & Rev primer (10 $\mu$ M)	2.5 $\mu$ l	22.5 $\mu$ l
Water	21.5 $\mu$ l	193.5 $\mu$ l
Final Volume	50 $\mu$ l	450 $\mu$ l

Extended Methods Table 25: Volumes barcode PCR reactions.

Stage	Temperature	Time	Cycles
Stage 1	98°C	30 Sec.	1 Cycle
Stage 2	98°C	10 Sec.	12 Cycles
	65°C	30 Sec.	
	72°C	30 Sec.	
Stage 3	72°C	2 Min.	1 Cycle

Extended Methods Table 26: PCR Conditions for barcode amplification.

- Purify with Qiagen min elute and check for target amplicon size around 131 bp.
- Check size on bioanalyzer high sensitivity DNA chip.



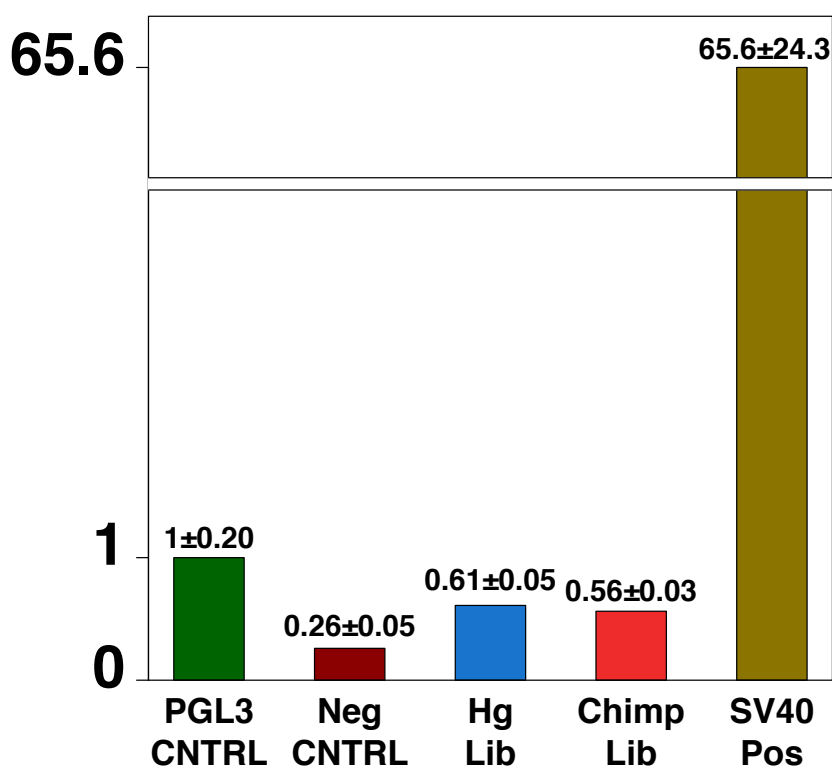
Extended Methods Figure 14: Bar-code containing amplicons

- Expected size with 5 degenerate bases on the 5' primer is 131 bp. Sequence amplicons 2x 150 bp.

## 8 Competent Library Characterization

### 8.1 Library Activity

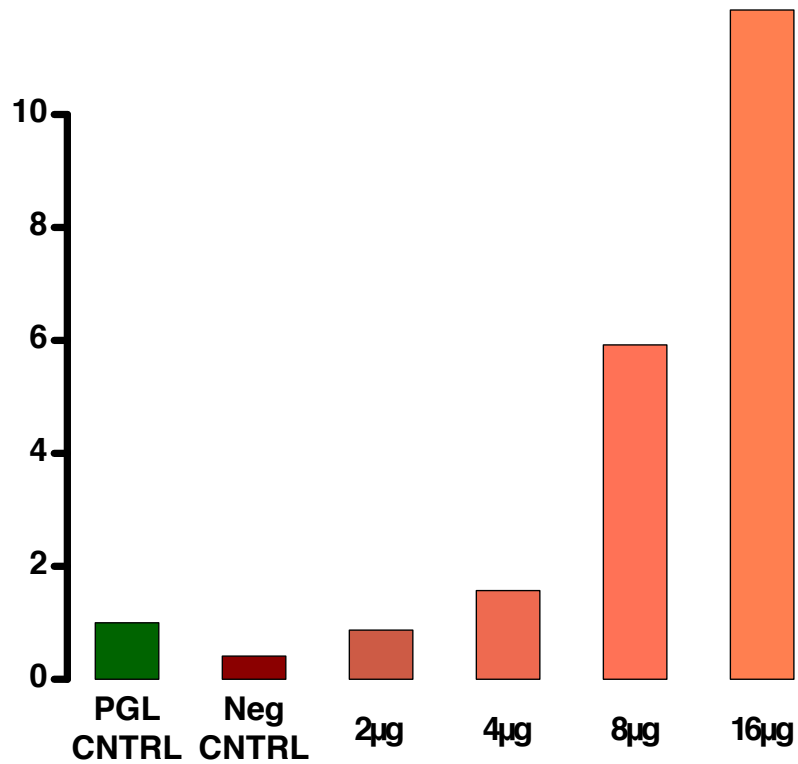
- Four co-transfections for each condition were performed at 2  $\mu\text{g}$  of designated plasmid and 10 ng of PGL 4.72 Renilla luciferase per 2 million cells. 1 ml of media was added to each cuvette and 250  $\mu\text{l}$  were plated per well on a six-well plate for 24 hours.
- Luciferase assays were performed with Promega Dual Luciferase Assays (E1910) and read on a Promega Luminometer (GM3500).



Extended Methods Figure 15: Competent Library Transfection

### 8.2 Optimize Library Amount to Transfect

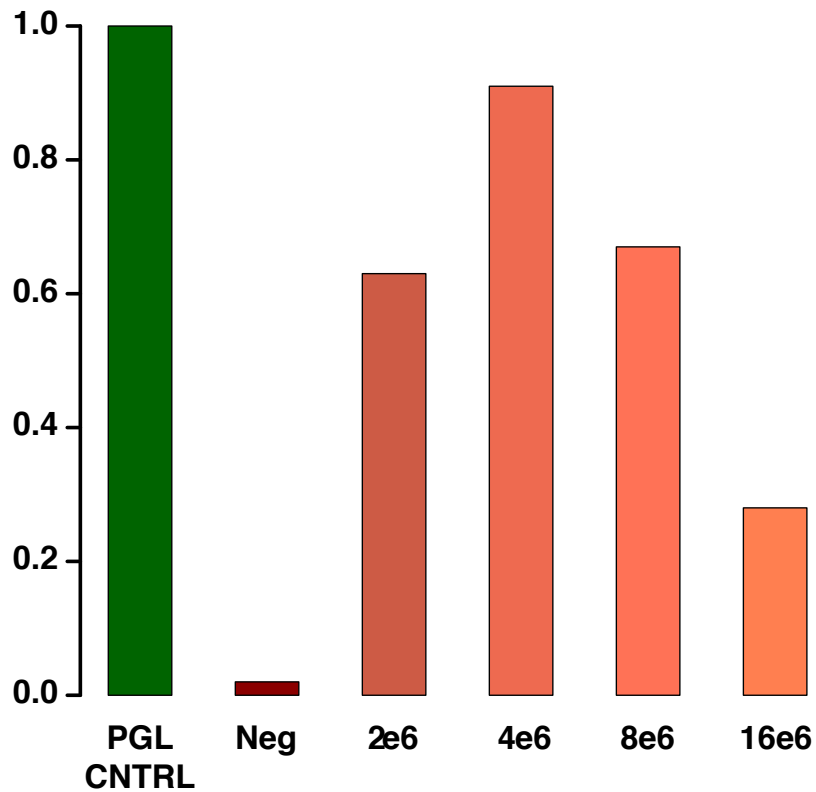
- Libraries were co-transfected with 2, 4, 8, and 16  $\mu\text{g}$  of human plasmid library and 15 ng of 4.72 Renilla luciferase per 2 million cells. 1 ml of media was added to each cuvette and 250  $\mu\text{l}$  were plated per well on a six-well plate for 24 hours.
- Luciferase assays were performed with Promega Dual Luciferase Assays (E1910) and read on a Promega Luminometer (GM3500).



Extended Methods Figure 16: Transfection of increasing amounts of library.

### 8.3 Optimize Amount of Cells to Transfect

- Libraries were co-transfected with 2, 4, 8, and 16 million cells, 16 µg plasmid library per 2 million cells, and 20 ng of 4.72 Renilla Luciferase per 2 million cells. 1 ml of media was added to each cuvette and 250 µl were plated per well on a six-well plate for 24 hours.
- Luciferase assays were performed with Promega Dual Luciferase Assays (E1910) and read on a Promega Luminometer (GM3500).



Extended Methods Figure 17: Transfection of Increasing Amounts Cells

- Note: PGL Control seems extremely active here. Around 10 fold more active than previously, as evidence by extremely low negative control activity.

## 9 Library Transfection

### 9.1 Harvest the cells

- Wash a T150 flask of cells with 10 ml PBS(-), aspirate PBS.
- Pipette 8 ml pre-warmed Accutase into T150 flask and incubate for 5 minutes at 37°C.
- Tap the flask to dislodge cells and neutralize with 16 ml of complete media, pipette into a falcon tube and spin down cells at 200 x g for 4 minutes.
- Resuspend pellet in 5–10 ml of complete media.
- Count cells to quantify concentration.

## 9.2 Transfection Conditions

- Pipette 2 x 20 million cells into 10 ml falcon tubes and spin down at 200 x g for 4 minutes.
- Aspirate media completely and pipette 2 x 160 µg of plasmid library on top of the cell pellet.
- Add 2 x 500 µl of Amaxa mNSC Reagent with Supplement 1 added and gently pipette to resuspend the cells.
- Gently resuspend the cell pellet and transfer to 2 x 5 Amaxa cuvettes.
- Electroporate the sample using pre-defined protocol **A-033**, and immediately add 1 ml of complete media to recover after each transfection.
- Use the pipette provided by Amaxa to transfer recovered cells into one falcon tube for both of the 5 x transfection rows.

## 9.3 Plating Transfected Cells

- **Before transfection:** Coat two 150 cm<sup>2</sup> flasks each with 12 ml of coating media and one aliquot of matrigel for 1 hour to over night.
- Wash with 20 ml PBS(-), and add 30 ml of growth media. Leave plate in cell incubator to equilibrate.
- Add half of the pooled recovered cell volume to each equilibrated 150 cm<sup>2</sup> flask and grow for 6 hours before harvesting.

# 10 Post Transfection Processing

## 10.1 Harvest the cells

- Wash cells with 20 ml PBS(-).
- Pipette 10 ml pre-warmed Accutase into each 150 cm<sup>2</sup> flask and incubate for 6 minutes at 37°C.
- Tap the dish to dislodge cells and neutralize with 12 ml complete media, pipette into a 50 ml falcon tube and spin down cells at 200 x g for 4 minutes.
- Resuspend pellet in 10 ml PBS(-).
- Remove 5% of the total volume and place into a 1.5 ml Eppendorf tube for plasmid DNA library recovery. The remaining 95% are used for RNA extraction.
- Spin both the 10 ml falcon tube and the Eppendorf tube down at 200 x g for 4 minutes.

## 10.2 Genomic DNA Purification

- Add 40  $\mu$ l 1 M DTT per 1 ml of RLT buffer (Allprep kit).
- Aspirate PBS from the 1.5 ml Eppendorf tube and add 350  $\mu$ l of previously prepared lysis buffer (RLT + DTT).
- Pipette to resuspend the cells and vortex vigorously to lyse cells.
- Spin the Eppendorf tube down and then add the entire volume to a Qiagen Allprep DNA/RNA Mini Kit column.
- Spin at 8150 x g for 30 seconds. **Proceed to Section 10.3 until instructed to return.**
- Wash column with 500  $\mu$ l of buffer AW1 and spin at 8150 x g for 30 seconds.
- Wash column with 500  $\mu$ l of buffer AW2 and spin at full speed for 2 minutes.
- Place in 1.5 ml Eppendorf tube, pipette 80  $\mu$ l of EB onto the column, and incubate for 1 minute.
- Elute by spinning at 8150 x g for 1 minute.
- Pipette another 80  $\mu$ l of EB onto the column and incubate for 1 minute.
- Elute a second time by spinning at 8150 x g for 1 minute.

## 10.3 RNA Purification

- Add 40  $\mu$ l 1 M DTT per 1 ml of RLT buffer and resuspend pellet in 600  $\mu$ l of RLT lysis buffer plus DTT per 10 million cells transfected. Pipette to mix.
- Vortex well and pipette the solution onto a QiaShredder column in increments of 600  $\mu$ l per column. Spin at max speed for 2 minutes.
- Add one volume of 70% EtOH to the flow through and pipette the combined solution onto an RNeasy Column in increments of 650  $\mu$ l. Spin at 8500 x g for 1 minute, this should equate to two spins per sample given this volume.
- Add 350  $\mu$ l of RW1 buffer to the column and spin at 8500 x g for 30 seconds.
- Digest DNA on column by adding 10  $\mu$ l pre-aliquoted DNase I to 70  $\mu$ l RDD buffer and pipetting all 80  $\mu$ l directly onto the column. Incubate at room temperature with lid closed for 15 minutes. **While digesting the DNA return to Section 10.2 and finish the genomic DNA purification.**
- Add 350  $\mu$ l of RW1 buffer to the column and spin at 8500 x g for 30 seconds.
- Add 500  $\mu$ l of buffer RPE to the column and spin at for 30 seconds.
- Add 500  $\mu$ l of buffer RPE to the column and spin at for 2 minutes.

- Place column in new collection tube and spin at full speed for 1 minute to dry.
- Place column in another new collection tube and pipette 30  $\mu\text{l}$  of RNase free water directly onto the column, wait 1 minute, and spin at 8500 x g.
- Pipette another 30  $\mu\text{l}$  of RNase free water directly onto the column, wait 1 minute, and spin at 8500 x g.
- Pool total RNA from the same transfections together and remove 2  $\mu\text{l}$  aliquots of DNA for gel and nano-drop analysis.

#### 10.4 Second DNase Treatment

- Prepare a second DNase digestion as described in Table 27. Scale as necessary up to 200  $\mu\text{l}$ , then split into different tubes.

Reagent	1x	2x
RNA	Y $\mu\text{l}$	Y $\mu\text{l}$
Water	X $\mu\text{l}$	X $\mu\text{l}$
Buffer RDD	10 $\mu\text{l}$	20 $\mu\text{l}$
DNaseI	2.5 $\mu\text{l}$	5 $\mu\text{l}$
Final Volume	100 $\mu\text{l}$	200 $\mu\text{l}$

Extended Methods Table 27: Second DNase digestion mix.

- Incubate ad room temperature for 20 minutes.

#### 10.5 Plasmid Library Enrichment from Purified DNA

- **Perform while second digest is running in Section 10.4.**
- Add 300  $\mu\text{l}$  ERC buffer to 20–100  $\mu\text{l}$  of pDNA.
- Add 10  $\mu\text{l}$  NaOAC (3M). Vortex to mix and spin down.
- Add total sample to QIAprep Spin Miniprep column. Spin at 17,900 x g for 1 minute.
- Add 500  $\mu\text{l}$  of PB buffer to column and spin at 17,900 x g for 1 minute.
- Add 750  $\mu\text{l}$  of PE buffer to column and spin at 17,900 x g for 1 minute.
- Transfer column to new tube and spin at 17,900 x g for 1 minute to dry column.
- Place in 1.5 ml Eppendorf tube, add 30  $\mu\text{l}$  EB onto the column, and incubate for 1 minute.
- Elute enriched pDNA by spinning at 17,900 x g for 1 minute.
- Pipette another 30  $\mu\text{l}$  EB onto the column and incubate for 1 minute.
- Elute a second time by spinning at 8150 x g for 1 minute.

## 10.6 RNA Clean-up

- Add 350  $\mu\text{l}$  of RLT buffer for every 100  $\mu\text{l}$  of DNase-treated RNA.
- Add 250  $\mu\text{l}$  of 96–100% RNase free EtOH for every 100  $\mu\text{l}$  of DNase-treated RNA.
- Pipette up to 700  $\mu\text{l}$  onto an RNeasy Mini Kit column and spin at 8500 x g for 30 seconds. Repeat as necessary up to column limit of 100  $\mu\text{g}$ .
- Pipette 500  $\mu\text{l}$  of RPE onto column and spin at 8500 x g for 30 seconds.
- Pipette 500  $\mu\text{l}$  of RPE onto column and spin at 8500 x g for 2 minutes.
- Place column in new collection tube and spin at full speed for 1 minute to dry.
- Place column in another new collection tube and add 30  $\mu\text{l}$  of RNase free water directly onto the column. Wait 1 minute and spin at 8500 x g.
- Pipette another 30  $\mu\text{l}$  of RNase free water directly onto the column, wait 1 minute, and spin at 8500 x g.
- Pool total RNA from the same transfections and remove 2  $\mu\text{l}$  aliquots of DNA for gel and nano-drop analysis.

## 10.7 mRNA Purification using Dynabeads Kit (if Necessary)

- This step is usually not recommended due to unnecessary, high loss of RNA.
- Dilute 1–5  $\mu\text{g}$  of total RNA to 50  $\mu\text{l}$  with RNase free water.
- Pipette 20  $\mu\text{l}$  of resuspended beads in a **separate tube**.
- Add 100  $\mu\text{l}$  of binding buffer to the beads and mix by pipetting gently.
- Place beads on magnet for 2 minutes.
- Remove supernatant, then remove tube from the rack and wash with another 100  $\mu\text{l}$  of binding buffer. Mix gently by pipetting, place on magnet for 2 minutes, remove supernatant, remove tube from the rack.
- Resuspend the beads in 50  $\mu\text{l}$  binding buffer and add 50  $\mu\text{l}$  of total RNA. Mix gently by pipetting.
- Heat the entire reaction at 65°C for 5 minutes and then hold at 4°C or on ice for 3 minutes to denature secondary structure.
- Resuspend beads gently by pipetting.
- Incubate at room temperature for 5 minutes.
- Resuspend beads gently by pipetting.
- Incubate at room temperature for 5 minutes.



- Place tube on magnet for 2 minutes and discard supernatant.
- Remove tube from the rack and wash with 200  $\mu$ l washing buffer. Mix by pipetting and place the tube on the magnet for 2 minutes. Discard supernatant.
- Repeat the previous step a second time.
- Resuspend beads in 50  $\mu$ l of Tris, mix by pipetting, and transfer to thin wall 0.2 ml PCR tube.
- Heat sample in a thermocycler to 80°C for 2 minutes, then hold at 25°C to elute. Remove the samples when they reach 25°C.
- Add 50  $\mu$ l binding buffer to each sample to allow the RNA to bind to the same beads. Mix by pipetting.
- Incubate at room temperature for 5 minutes.
- Resuspend beads gently by pipetting.
- Incubate at room temperature for 5 minutes.
- Place tube on magnet for 2 minutes and discard supernatant.
- Remove the tube from the rack and wash with 200  $\mu$ l washing buffer, mix by pipetting then place the tube on the magnet for 2 minutes and discard supernatant.
- Spin down tube then place back on magnet and remove the remaining supernatant with a 20  $\mu$ l pipette.
- Remove tube from the rack and resuspend beads in 17  $\mu$ l Tris, mix by pipetting and transfer to thin wall 0.2 ml PCR tube.
- Heat sample in a thermocycler to 80°C for 2 minutes, then hold at 25°C to elute. Immediately remove samples when they reach 25°C and place on the magnetic rack for 2 minutes.
- Collect the enriched mRNA and remove 2  $\mu$ l aliquots of DNA for gel and nano-drop analysis.

## 10.8 RNA QC

- Run an Agilent Eukaryotic RNA Pico chip with samples of 1:100 and 1:1000 dilutions of extracted and digested RNA. If mRNA enrichment was performed, run lanes of mRNA and a 1:10 dilution of mRNA.

## 10.9 cDNA Synthesis

- First strand synthesis performed with Invitrogen SSIII (18080-400). Reaction size scaled 2.5 x as follows.

Reagent	Volume
Annealing buffer	2.5 $\mu$ l
Oligo dT	2.5 $\mu$ l
Custom primer	2.5 $\mu$ l
mRNA (no more than 5 $\mu$ g)	12.5 $\mu$ l
Final volume	20 $\mu$ l

Extended Methods Table 28: Volumes for a single oligo dT annealing reaction.

- Incubate 20  $\mu$ l annealing reaction in DNase/RNase free PCR tube at 65°C for 5 minutes. Immediately place on ice.
- Add 2x buffer and SSIII enzyme to each reaction and incubate at 50°C for 2.5 hours in a thermocycler with the lid set to 60°C.

Reagent	Volume
Annealing reaction	20 $\mu$ l
2x buffer	25 $\mu$ l
SSIII enzyme	5 $\mu$ l
Final volume	50 $\mu$ l

Extended Methods Table 29: Volumes for a single cDNA synthesis reaction.

## 10.10 cDNA Second Strand Synthesis

- Add DEPC treated water, 10x buffer, and enzyme mix (NEB E6111) to each reaction. Pipette 50  $\mu$ l aliquots into 0.2 ml PCR tubes and incubate at 16°C for 2.5 hours in a thermocycler with the lid left unheated.

Reagent	Volume
1st strand reaction	50 $\mu$ l
DEPC water	120 $\mu$ l
10x buffer	20 $\mu$ l
Enzyme mix	10 $\mu$ l
Final volume	200 $\mu$ l

Extended Methods Table 30: Volumes for a single cDNA synthesis reaction.

- Clean up reactions with Qiagen Minelute columns, try to keep volume as small as possible to help in PCR amplification.

## 11 qPCR

### 11.1 Preparation

- Primer stocks: All primers are kept at 100  $\mu$ M, mix 10  $\mu$ l forward with 10  $\mu$ l reverse, and 80  $\mu$ l for 100  $\mu$ l of 10  $\mu$ M working stock.
- Dilute 2  $\mu$ l of cDNA 10-fold, and 100-fold.

- Dilute 2  $\mu\text{l}$  of pDNA 10-fold, 100-fold, and 1,000-fold.
- Serial dilute plasmid standard out fresh each time from  $1 \times 10^8$  copies /  $\mu\text{l}$  to  $1 \times 10^2$  copies /  $\mu\text{l}$ .

## 11.2 Master Mix

- All samples and standards are run in triplicate with an additional fourth reaction included to compensate for pipette error.
- Factor in 2–4 additional reactions into the master mix to account for pipette error as the master mix is very viscous.
- Include a water and genomic DNA control as well.
- Once total number of samples are accounted for make master mix as follows:

$$Samples_{total} = Samples \times 4 + 4$$

Reagent	Volume/Rxn	Scale to volume
2x Power Syber master mix	10 $\mu\text{l}$	$\times Samples_{total}$
DEPC water	8 $\mu\text{l}$	$\times Samples_{total}$
10 $\mu\text{M}$ F+R primer mix	1 $\mu\text{l}$	$\times Samples_{total}$
Sample	1 $\mu\text{l}$	NA
Final volume	20 $\mu\text{l}$	$19\mu\text{l} \times Samples_{total}$

Extended Methods Table 31: qPCR master mix.

- Pipette 76  $\mu\text{l}$  into each well along the D or E rows of a 96-well Fast Optical qPCR Plate (Applied Biosystems 4346906).
- Add 4  $\mu\text{l}$  of sample to each well.
- Use the multi-channel P200 pipette to add 20  $\mu\text{l}$  of the 80  $\mu\text{l}$  reaction into the three wells above (Row D) or below (Row E).

## 11.3 Sample qPCR Plate Layout

- Below is a sample qPCR plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1e8	1e7	1e6	1e5	1e4	1e3	1e2					
B	1e8	1e7	1e6	1e5	1e4	1e3	1e2					
C	1e8	1e7	1e6	1e5	1e4	1e3	1e2					
D	<b>Copy # Standard</b>											
E							<b>pDNA</b>	<b>cDNA</b>	<b>Cntrl</b>			
F							1:100	1:1000	1:10	1:100	GM	Water
G							1:100	1:1000	1:10	1:100	GM	Water
H							1:100	1:1000	1:10	1:100	GM	Water

**Master Mix**

AOD 10uL/rxn	X	<u>56</u>	rxns.	=	<u>560</u>	uL
H2O 8uL/rxn	X	<u>56</u>	rxns.	=	<u>448</u>	uL
Prim 1uL/rxn	X	<u>56</u>	rxns.	=	<u>56</u>	uL

Extended Methods Figure 18: Sample Layout of qPCR Plate

### 11.4 Barcode PCR Amplification

- Generic PCR Master-Mix for Barcode-Seq:

Reagent	Volume
2x NEB Master Mix	10 µl
Water	x µl
F+R primers (10 M)	1.25 µl
DNA (20 million copies)	x µl
Final volume	20 µl

Extended Methods Table 32: Barcode PCR master mix.

- Generic PCR Conditions for Barcode-Seq:

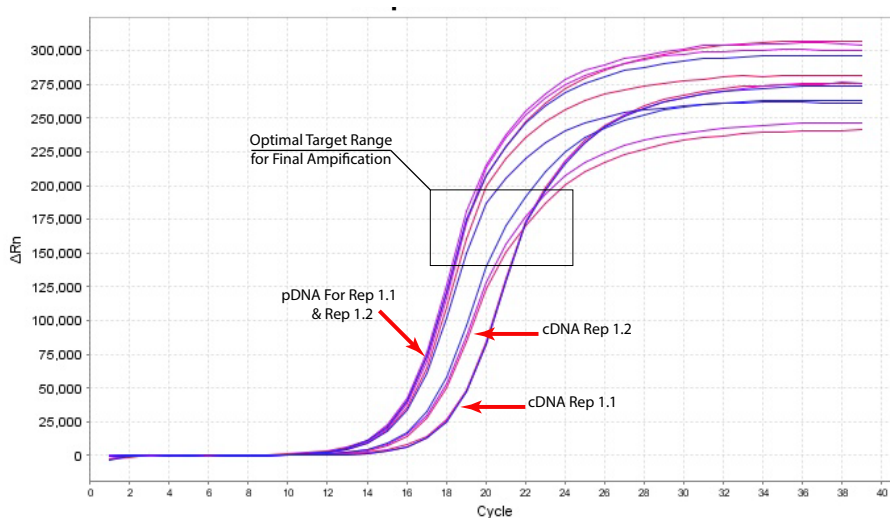
Stage	Temperature	Time	Cycles
Stage 1	98°C	30 Sec.	1 Cycle
Stage 2	98°C	10 Sec.	X Cycles
	65°C	20 Sec.	
	72°C	30 Sec.	
Stage 3	72°C	2 Min.	1 Cycle

Extended Methods Table 33: PCR conditions for sample barcode amplification.

- Before you can amplify the cDNA or pDNA fractions for sequencing you need to figure out the optimal amount of PCR cycles for however many template copies you want to seed for each 20  $\mu\text{l}$  PCR reaction.
- Observation of amplification per-cycle (for 40 cycles) of the 1:10 dilutions with SYBR green under actual PCR conditions is needed as follows:

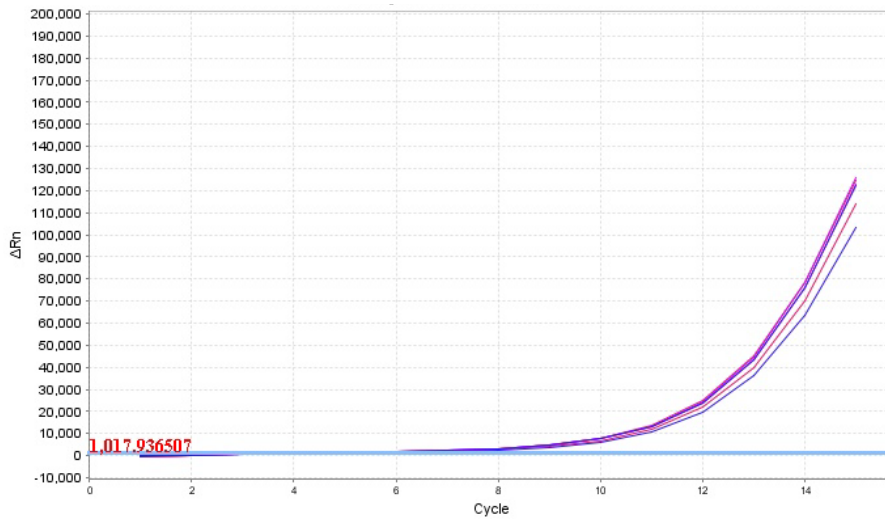
Reagent	Volume
2x NEB master mix	10 $\mu\text{l}$
Water	6.55 $\mu\text{l}$
F+R primers (10 $\mu\text{mol}/\mu\text{l}$ )	1.25 $\mu\text{l}$
10x SYBR Green	1.2 $\mu\text{l}$
DNA	1 $\mu\text{l}$
Final volume	20 $\mu\text{l}$

Extended Methods Table 34: Barcode qPCR master mix.



Extended Methods Figure 19: SYBR Green amplification

- Repeat amplification with undiluted pDNA. Take the cycle number around the desired range and subtract four cycles for the dilution change. Round up, and add one cycle to be safe. In the case of Figure 19 15 cycles seems sufficient.



Extended Methods Figure 20: pDNA undiluted SYBR Green amplification

- Figure 20 is just on the lower end of optimal, but will work.
- Next step is to run out wells from both SYBR reactions on a 2% agarose gel and then purify separate wells with a Qiagen Minelute column and run them on an Agilent DNA High Sensitivity chip. This will confirm that there are no off target effects and that there will be enough product produced at the fluorescent intensity in Figure 20.
- Generic PCR Conditions for pDNA Barcode-Seq:

Stage	Temperature	Time	Cycles
Stage 1	98°C	30 Sec.	1 Cycle
	98°C	10 Sec.	
Stage 2	65°C	20 Sec.	17 Cycles
	72°C	30 Sec.	
Stage 3	72°C	2 Min.	1 Cycle

Extended Methods Table 35: PCR conditions for sample barcode amplification.

- Generic PCR Conditions for cDNA Barcode-Seq:

Stage	Temperature	Time	Cycles
Stage 1	98°C	30 Sec.	1 Cycle
	98°C	10 Sec.	
Stage 2	65°C	20 Sec.	18 Cycles
	72°C	30 Sec.	
Stage 3	72°C	2 Min.	1 Cycle

Extended Methods Table 36: PCR conditions for sample barcode amplification.

- PCR products are submitted to Illumina sequencing. Add 5% phi-X to help with sequence heterogeneity issues.

## 12 Reagents

### 12.1 Consumables

Reagent	Provider	Product number
Accutase, StemPro	Gibco	A11105-01
Agarose	American Bio	AB00972-00500
AMPureXP SPRI Beads	Beckman Coulter	A63880
BSA (20 mg/ml)	NEB	B9000S
2-Butanol	Acros Organics	10770-0010
Cells, MegaX DH10B T1 <sup>R</sup> Electrocomp	Thermo Fischer Scientific	C6400-03
Cuvettes for electroporation, 0.1 cm	Bio-Rad	1652089
DMSO	American Bio	AB03091-00100
DNaseI, RNase-free set	Qiagen	79254
dNTPs	NEB	N0446S
Ethanol, 70%	American Bio	AB04010-00500
Ethanol, 200 proof	American Bio	AB00515-00500
Ethidium Bromide	Sigma	E1510-10ML
Glycerol, anhydrous	American Bio	AB00751-00500
LB/Amp plates, 100 µg/ml	Recombinant Thechnologies	Stockroom
Ligase, T4 DNA (400 U/µl)	NEB	M0202M
Master Mix High Fidelity 2x	NEB	M0541L
NaOAC, 3M	American Bio	AB13168-01000
PBS(-) (i.e., w/o MgCl & CaCl)	Gibco	14190-144
PGL 3 Control Vector	Promega	E174A
PGL 4.72 Renilla Luciferase Vector	Promega	E690A
Phosphatase, Alkaline (CIP)	NEB	M0290L
Pippin Prep gels, 2% agarose	Sage Science	CSD2010
Q5 High fidelity polymerase	NEB	M0491L
qPCR Plates, fast optical	Applied Biosystems	4346906
SYBR Green	Life Technologies	S7563
Water, DEPC	American Bio	AB02128-00500

Extended Methods Table 37: Primary consumable reagents.

## 12.2 Kits

Provider	Kit	Product number
Agilent	Bioanalyzer Eukaryotic Pico Kit	5067-1513
Agilent	Bioanalyzer High Sensitivity DNA	5067-4626
CHIMER <sub>x</sub> /EUR <sub>x</sub>	Micellula Emulsion PCR Kit	3600-02
Invitrogen	Super Script III cDNA kit	18080-400
Lonza	Amaya Mouse NSC Nucleofector Kit	VPG-1004
NEB	NEBNext Library Kit (Seq center)	E6040L
NEB	HighSeq Indexing Primers (Seq center)	E7335L
Promega	Dual Luciferase Assay Kit	E1910
Qiagen	AllPrep Mini DNA/RNA (for DNA)	80204
Qiagen	Endofree Mega Kit (before transfection)	12381
Qiagen	HiSpeed Maxi Kit	12662
Qiagen	Minelute	28004
Qiagen	QIAprep Spin Miniprep Kit	27106
Qiagen	QIAshredder	79654
Qiagen	Reaction Clean-up (ERC Buffer is in here)	28206
Qiagen	RNeasy Mini Kit (for mRNA)	74104
Thermo Fisher	Dynabeads mRNA Purification Kit	61006

Extended Methods Table 38: Primary kit reagents.

## 12.3 Specialized Equipment

- Bio-Rad Gene Pulser
- Lonza Nucleofector 2b
- Applied Biosystems Step One Plus qPCR
- Agilent 2100 Bioanalyzer
- Promega Luminometer GM3500
- Pippin Prep with 2% gel cassettes

## 13 Primer Sequences

### 13.1 Initial Low Cycle Library Primers

- Forward: 5' - ACTGGCCGCTTGACG - 3'
- Reverse: 5' - GCAGGAGCCGACGTG - 3'

### 13.2 Library Bar-coding Primers

- Forward: 5' GCCAGAACATTTCTCTGGCCTAACTGGCCGCTTGACG 3'
- Reverse: 5' CCGACTAGCTTGGCCGCCGAGGCCGACGCTCTTCCGATCTNNNNNNNNNNNNNNNTC-TAGAGGTACCGCAGGAGCCGACGTG 3'



### 13.3 Inert TagSeq Library Primers

- Forward:5' - (N:25252525)(N)(N)(N)(N)CAGGTGCCAGAACATTTCTCT - 3'
- Reverse:5' - TTATCATGTCTGCTCGAAGCGG - 3'

### 13.4 Competent TagSeq Primer

- The 3' primer falls down of custom cDNA primer, allows for longer PCR product
- Forward:5' -(N:25252525)(N)(N)(N)(N)CAAGAAGGGCGGCAAGAT - 3'
- Reverse:5' - TTATCATGTCTGCTCGAAGCGG - 3'

### 13.5 Barcode-Seq Primers (Targets cDNA)

- The 3' primer falls upstream of custom cDNA primer!!
- Forward:5' -(N:25252525)(N)(N)(N)(N)CGAGGTGCCTAAAGGACTG- 3'
- Reverse:5' - CCGACGCTCTTCCGATCT - 3'

### 13.6 Reverse Transcription Primer

- 5' -CCGACTAGCTTGGCCGC- 3'

### 13.7 Transcript qPCR Primers (targets Luciferase)

- Forward:5' -AACACCCCAACATCTTCGAC- 3'
- Reverse:5' -TCTCGGTCATGGTTTTACCG- 3'

### 13.8 Backbone qPCR Primers

- Forward:5' -ATTTGGTATCTGCGCTCTGC- 3'
- Reverse:5' -TTTGCCGGATCAAGAGCTAC- 3'

### 13.9 Experimental TagSeq Primer

- Forward:5' -(N:25252525)(N)(N)(N)(N)CGAGGTGCCTAAAGGACTG- 3'
- Reverse:5' -CCGACGCTCTTCCGATCT- 3'