

MPRA Protocol

December 9, 2016

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1 Introduction

1.1 Method Overview

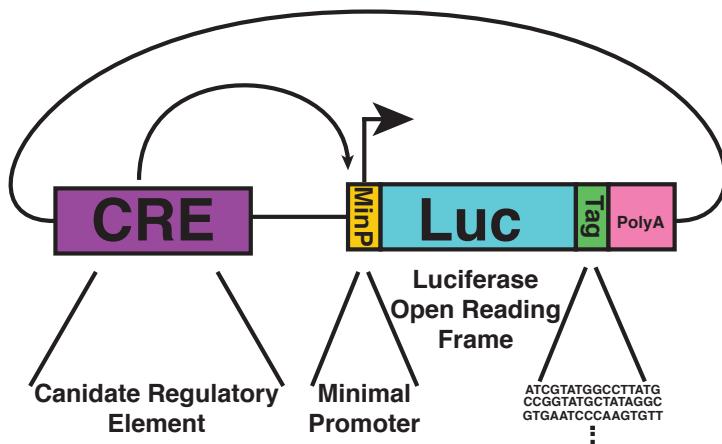


Figure 1: Mechanism of Enhancer Interrogation

- 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

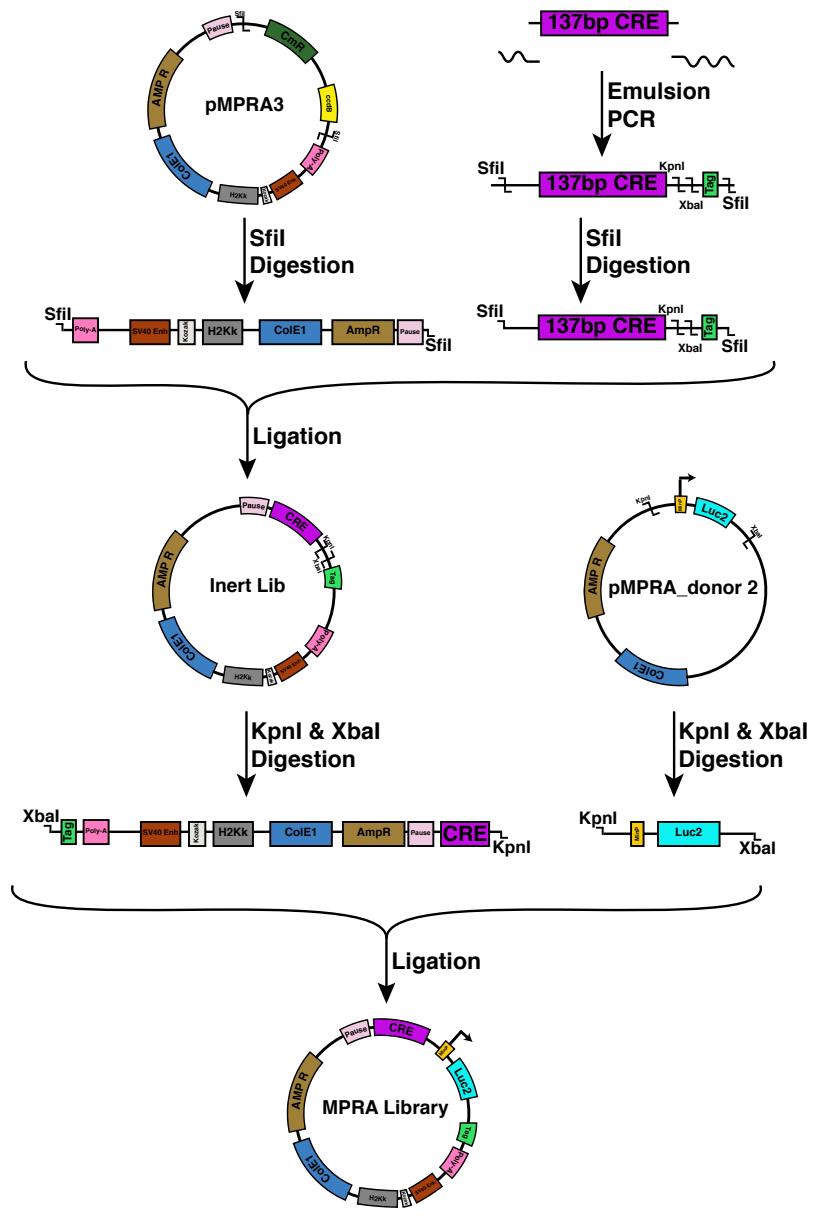


Figure 2: MPRA Library Construction

- 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

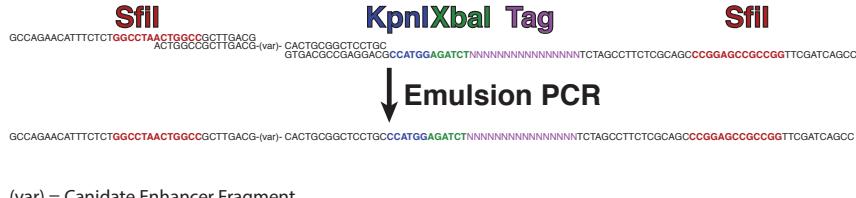


Figure 3: Tailing Emulsion PCR

- The SwiI 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 sequencing 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

- First set of primers are un-tailed
 - Forward: 5' - GCCAGAACATTCTCT - 3'
 - Reverse: 5' - GCAGGAGCCGCAGTG - 3'
- Second set primers utilize tails to add restriction sites and tags to synthesized fragments.
- The Forward primer adds SwiI (GGCCNNNNNGGCC) cloning site:
5' - GCCAGAACATTCTCT**GGCCTAA**CTGGCCGCTTGACG - 3'
- The Reverse primer adds SwiI (GGCCNNNNNGGCC), KpnI(CCAGTGG), XbaI(AGATCT), and a 16bp tag sequence:

CCGACTAGCTT**GGCGCCGAGGCCGACG**CTTCCGATCTNNNNNNNNNNNNNTAGAGGTACCGCAGGAGCCGCAGTG

- 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 Preliminary Amplification

3.1 Single Reaction

- Small Cycle qPCR Library Amplification
- Observe library amplification with fluorescent SYBR Green qPCR reporting on every cycle

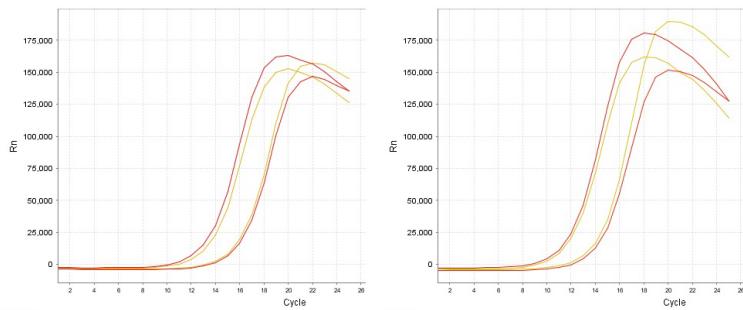


Figure 4: Initial qPCR Library Amplification Left: Human, Right: Chimp

- PCR Mix:

Reagent	Volume	30x
2x NEB Master Mix	10µL	300µL
Water	6.55µL	196.5µL
Fwd + Rev Primers (10µmol/µL)	1.25µL	37.5µL
10x SYBR Green	1.2µL	-µL
DNA 1:10 dilution	1µL	-µL
Final Volume	20µL	600µL

Table 1: Initial Library PCR.

- Pipette out 17.8µL 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 and DNA to the 23 reaction Master Mix
- Pipette 20 wells to collect for purification

- **PCR Conditions:**

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

Table 2: qPCR Amplification Conditions.

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

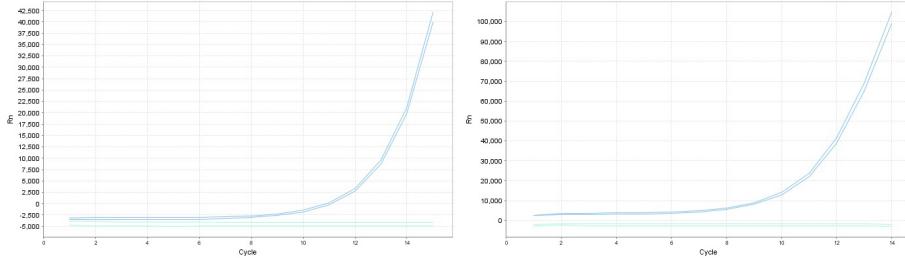


Figure 5: Left: Human, Right: Chimp

- Bead Purified 2x Volume ratio. Elute in 50 μ L qiagen EB

4 Emulsion PCR (CHIMERx: 3600-02)

4.1 Reaction Mix

- **Emulsion:**

Reagent	Volume	16x
Emulsion Component 1	220	3520 μ L
Emulsion Component 3	60	960 μ L
Emulsion Component 2	20	320 μ L
Final Volume	300 μ L	

Table 3: Emulsion Component Mixture.

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

- **Aqueous PCR Mix:**

Reagent	Volume	16x
2x NEB Master Mix	25µL	400µL
Water	19µL	304µL
Fwd + Rev Primers (5µmol/µL)	2.5µL	40µL
BSA(10mg/mL)	2µL	32µL
DNA	1µL	16µL
q5 Pol	0.5µL	8µL
Final Volume	50µL	800µL

Table 4: Emulsion Component Mixture.

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

- **PCR Conditions:**

Stage	Temperature	Time	Cycles
Stage 1	98C	30 Sec	1 Cycle
Stage 2	98C	20 Sec.	15 Cycles
	72C	10 Sec.	
	72C	15 Sec.	
Stage 3	72C	5 Min.	1 Cycle

Table 5: PCR Conditions for Library Insert Amplification.

- Each PCR reaction is 50µL per-well.
- **Cleanup:**
 - Pool all reactions and add 1mL of 2-butanol. Vortex thoroughly.
 - Use kit provided spin columns

4.2 Full Scale Reaction

- **Emulsion:**

Reagent	Volume
Emulsion Component 1	3520µL
Emulsion Component 3	960µL
Emulsion Component 2	320µL
Final Volume	300µL

Table 6: Emulsion Component Mixture.

- Add components in order, use a wide bore tip for Emulsion Component 2.

- Incubate at 4C for 30 minutes on wet ice.

- **Aqueous PCR Mix:**

Reagent	Volume
2x NEB Master Mix	400 μ L
Water	319 μ L
Fwd + Rev Primers (5 μ mol/ μ L)	40 μ L
BSA(10mg/mL)	32 μ L
DNA	1 μ L
q5 Pol	8 μ L
Final Volume	800 μ L

Table 7: Emulsion Component Mixture.

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

- **PCR Conditions:**

Stage	Temperature	Time	Cycles
Stage 1	98C	30 Sec	1 Cycle
Stage 2	98C	20 Sec.	15 Cycles
	72C	10 Sec.	
	72C	15 Sec.	
Stage 3	72C	5Min.	1 Cycle

Table 8: PCR Conditions for Library Insert Amplification.

- Each PCR reaction is 50 μ L per-well (96 wells total).

- **Cleanup:**

- Pool all reactions and add 13.7mL 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

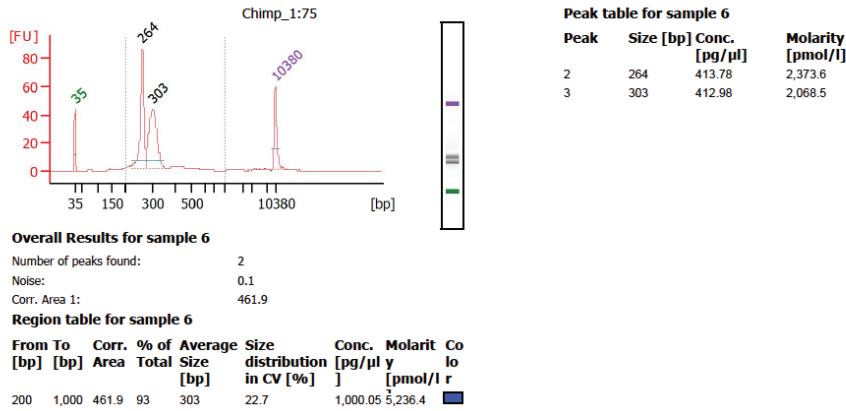


Figure 6: Extra large molecular weight slippage product ~300bp

- Libraries were ran in a 2% Agarose pipjen prep gel on Pippin Prep DNA Size Selection System to enrich for the correct 265bp size product.

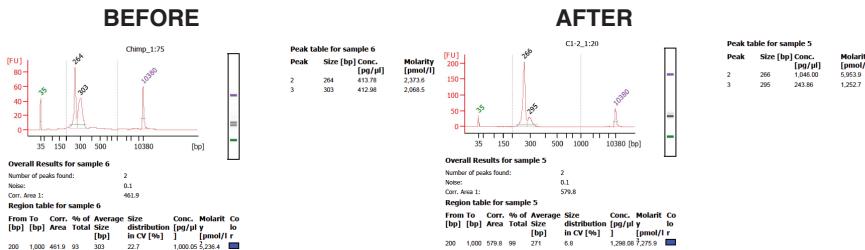


Figure 7: Tailed Libraries Before and After size selection

5 Inert Library Cloning

5.1 SfiI Digests

- Libraries and pMPRA1 vector were digested over night at 37C. Digests were made as described in Table 9.

Reagent	Human Lib	Chimp Lib	pMPRA1 Vector
DNA	25µL	25µL	12µL
Cutsmart Buf	3µL	3µL	2µL
Water	0µL	0µL	2µL
SfiI	2µL	2µL	4µL
Final Vol	30µL	30µL	20µL

Table 9: Initial Library Digests.

- Add 2µL CIP, 1µL Cutsmart Buffer and 7µL Water to the pMPRA1 vector digest. Incubate at 37C for one hour.
- Clean up Fragment Library digests with Min Elute columns. Elute in 8µL EB buffer.
- Run out pMPRA1 digest on a 1% agarose gel. Excise larger backbone band and gel purify.
- *Alternativley you can digest the pMPRA 1 pasmid with SfiI, NcoI, AclI, and XmaI. This yeilds a 2.4Kb band and all other bands are less than 600bp. Use AmpureXP beads to size select and CIP the eluted DNA. This allows you to avoid the gel extraction*



Figure 8: Gel Extract pMPRA1 Backbone to isolate from insert

- Purify backbone though second purification with 1X beads. Elute in EB.

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.

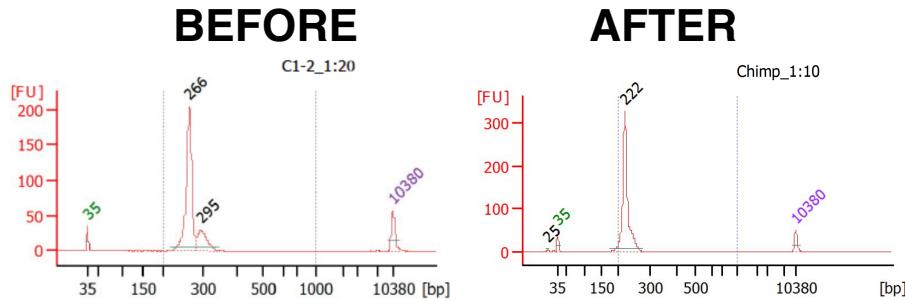


Figure 9: The 266bp library fragments should lose ~40 base pairs after digest

- Ligation reactions are performed a Fragment to backbone ratio of 3:1. Backbone input mass is 340ng, making this particular library input mass 103ng. 2000 units of ligase is used per-reaction. Ideally each reaction should yield 5,000,000CFU. Reactions were setup as described in table in Table 10.

Reagent	Human	Chimp	Combined
Water	- μ L	3.48 μ L	- μ L
Fragment Lib	- μ L	5.80 μ L	- μ L
Vector (91.5ng/ μ L)	3.72 μ L	3.72 μ L	7.44 μ L
10x Buffer	2 μ L	2 μ L	4 μ L
Ligase (400U/ μ L)	5 μ L	5 μ L	10 μ L
Total Volume	20 μ L	20 μ L	40 μ L

Table 10: Initial Library Digests.

- Ligations incubated for 16 hours at 16C in a thermocycler. 20 μ L per-tube.

5.3 Library Transformation

- Ligations should be cleaned up with room temperature AMPure XP beads. Add 20 μ L beads per-20 μ L ligation reaction. Incubate 5 minutes. Apply magnet for 5 minutes.
- Remove supernatant and wash twice with 200 μ L 80% ethanol. Air dry for 10-15 minutes and elute in 10 μ L EB per-20 μ L ligation reaction.
- Label all LB-AMP plates (100ug/mL) plates and place in 37C 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	90µL	90µL	90µL	40µL	90µL	40µL	90µL
Cell Volume	10µL	10µL	10µL	10µL	10µL	10µL	10µL

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

*These dilutions are made from the proceeding ten-fold dilution not the half fold dilution

- Place 5 electroporation cuvettes per-20µL recombination reaction and a 1.5mL eppendorf tube on ice for each transformation reaction
- Thaw cells on watery ice, make sure the water has time to come down to 4C, mix cells by gently flicking the side of the tube. **DON'T VORTEX**
- Pipette 20µL of thawed cells into the eppendorf tube on ice with a **wide bore** pipette tip
- Pipette 2µL of DNA into the 20µL of cells and tap tube to mix
- Incubate on ice for 10min
- Take 21µL 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 ensue that the metal cuvette is dry with a chem-wipe. Be sure not to warm the cuvette with your hand
- Electroporate at 200Ω, 25µFd, 2.0kV, and immediately place the cuvette back on ice
- Place 1mL of **room temperature** recovery into the cuvette
- Use a **wide bore** 1mL 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 15mL falcon tube
- Loosely fasten the cap to allow gas exchange and incubate at 37C & 225RPM for 1hr
- Pool all reactions and remove 10µL and dilute the cells according to table 23. Plate two plates with 10µL of each dilution
- For every transformation, **seed a range** of 500mL of LB-Amp in 2.5L flasks.
- Grow colonies at 37C & 225RPM for 8–11hr. **UNTIL OD = 0.95-1.2**
- Pellet cells at 4500rpm for 15 minutes at 4C

5.4 Plasmid Library Purification

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

5.5 Estimate Complexity and Blend Libraries

- Calculate the concentration of competent transformants utilizing the diluted series of plates equation

$$CFU \text{ per } \mu\text{L Seeded} = \frac{\text{Avg CFU} \times \text{Dilution Factor}}{10 \mu\text{L}}$$

- 80 tags per candidate enhancer is the target for a 52,000-104,000 element library
- Therefore for a 52,000 element library:

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

- If one dilution is not within 20% of your targeted complexity then you can blend libraries together to equal the correct complexity. Blending should be done with library **masses** proportional to the complexity seeded. This allows for roughly equivalent molecular molarity of any given CRE-Tag molecular.

5.6 QC Inert Library

- Equation to calculate total number of independent ligated transformants:

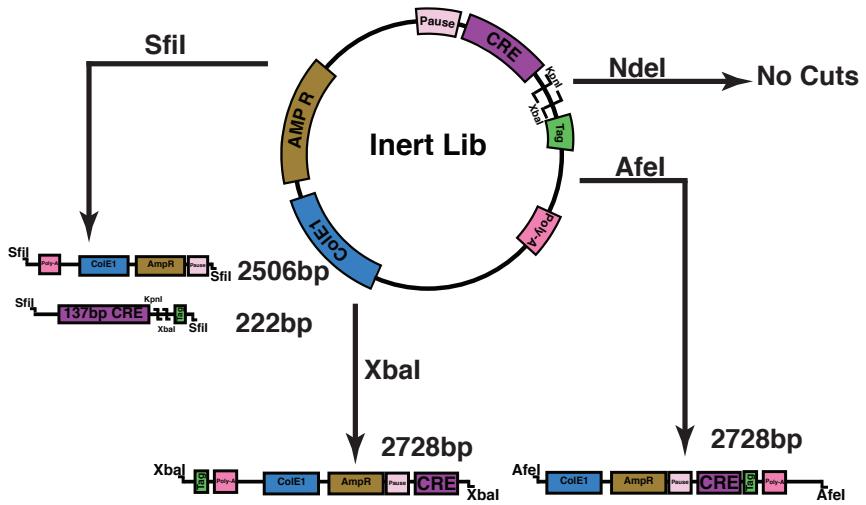
$$Total Complexity = CFU \times Dilution Factor \times \frac{Volume of Cells Cultured (\mu\text{L})}{10 \mu\text{L}}$$

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

Reagent	SfiI	XbaI	AfeI	NdeI
Water	10 μL	10 μL	10 μL	10 μL
DNA (100ng μL)	5 μL	5 μL	5 μL	5 μL
10x Buffer	2 μL	2 μL	2 μL	2 μL
SfiI	3 μL	- μL	- μL	- μL
XbaI	- μL	3 μL	- μL	- μL
AfeI	- μL	- μL	3 μL	- μL
NdeI	- μL	- μL	- μL	3 μL
Total Volume	20 μL	20 μL	20 μL	20 μL

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 10.



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

Figure 10: Library QC Digest schematic

- Digestion gel should look similar to figure 11.

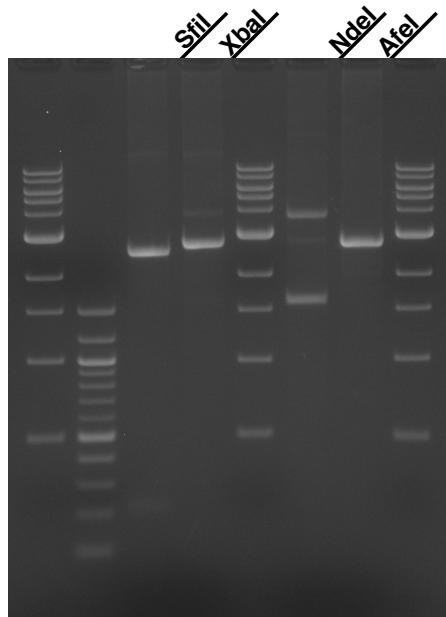


Figure 11: Library QC Digest

6 Library Sequencing

6.1 PCR Amplification Off Inert Library

- Dilute primers to 100uM, then mix forward and reverse primers to a final working concentration of 10uM
- Setup PCR reactions as follows:

Reagent	1x	10x
5X Q5 Buffer	10µL	100µL
5X GC Enhancer	10µL	100µL
DMSO	2.5µL	25µL
Primers (10uM)	2.5µL	25µL
dNTPs (25uM)	0.4µL	4µL
Library DNA (3ng/µL)	0.5µL	5µL
Q5 Polymerase	0.5µL	5µL
Water	22.25µL	236µL
Final Volume	50µL	500µL

Table 13: Volumes Library PCR Reactions.

- Run 10 PCR reactions under the following conditions:

Stage	Temperature	Time	Cycles
Stage 1	98C	30 sec.	1 Cycle
Stage 2	98C	10 Sec.	
	68C	20 Sec.	18 Cycles
	72C	20 Sec.	
Stage 3	72C	5Min.	1 Cycle

Table 14: PCR Conditions for Library Insert Amplification.

- Pool the 10 PCR reactions and cleanup with 1.4x AMPureXP Beads. Elute in 30µL EB.
- Check size fraction and concentration in Bioanalyzer

6.2 dA-Tailing

- Combine the following:

Reagent	Volume
End Repaired DNA	42µL
10x NEBNext End Repair Reaction Buffer	5µL
Klenow Fragment (3' - 5' exo-)	3µL
Final Volume	50µL

Table 15: dA Tailing Reaction.

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

6.3 Adaptor Ligation

- Combine the following:

Reagent	Volume
dA-Tailed DNA	25 μ L
5x NEBNext Quick Ligation Reaction Buffer	10 μ L
15uM NEBNext Adaptors	10 μ L
Quick T4 DNA Ligase	5 μ L
Final Volume	50 μ L

Table 16: Adaptor Ligation Reaction.

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

6.4 Indexing PCR Amplification

- Dilute PCR primers to a final working concentration of 50uM
- Setup PCR reactions as follows:

Reagent	1x	5x
2x NEB High Fidelity	12.5 μ L	62.5 μ L
Library DNA	1 μ L	5 μ L
F-Primer (10uM)	1.25 μ L	6.25 μ L
R-Primer (10uM)	1.25 μ L	6.25 μ L
Water	9 μ L	45 μ L
Final Volume	25 μ L	125 μ L

Table 17: Volumes Library PCR Reactions.

- Run 4 PCR reactions under the following conditions:

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

Table 18: PCR Conditions for Library Insert Amplification.

- Pool the 4 PCR reactions and cleanup with 0.9x AMPureXP Beads. Elute in 20 μ L EB.
- Check size and concentration in Bioanalyzer
- Sequence 2x250bp 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 37C as described in table 19

Reagent	Volume
Plasmid Lib	15 μ L
Buffer	2 μ L
KpnI	3 μ L
Total	20 μ L

Table 19: Inert Library KpnI Digest.

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

Reagent	Volume
Plasmid Lib	15 μ L
Buffer	2 μ L
XbaI	3 μ L
Total	20 μ L

Table 20: Inert Library KpnI Digest.

- Add 1 μ L CutSmart buffer, 3 μ L CIP, 4 μ L water and incubate 45 minutes at 37C
- 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 37C as described in table 21

Reagent	Volume
Plasmid Lib	21 μ L
Buffer	3 μ L
XbaI	3 μ L
KpnI	3 μ L
Total	20 μ L

Table 21: DonorMPRA 2 Digestion

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

7.3 Library Ligation

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

Reagent	Volume
Digested Inert Plasmid Lib	X μ L = 500ng
Digested MinP/Luc2 ORF	X μ L = 650ng
10x Ligase Buffer	2 μ L
T4 DNA Ligase (400U/ μ L)	5 μ L
Water	X μ L
Total Volume	20 μ L

Table 22: MPRA Library Ligation

- Incubate ligation reaction at 16C for 16 hours in a thermocycler
- Clean up ligation reaction with 1x AMPureXP beads and elute in 20 μ L EB

7.4 Library Transformation

- Label all LB-AMP plates (100ug/mL) plates and place in 37C 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 μ L	90 μ L	90 μ L	40 μ L	90 μ L	40 μ L	90 μ L
Cell Volume	10 μ L						

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

*These dilutions are made from the proceeding ten-fold dilution not the half fold dilution

- Place 10 electroporation cuvettes per-20 μ L recombination reaction and 10 1.5mL eppendorf tubes on ice for each transformation reaction

- Thaw cells on watery ice, make sure the water has time to come down to 4C, mix cells by gently flicking the side of the tube. **DON'T VORTEX**
- Pipette 20 μ L of thawed cells into the eppendorf tube on ice with a **wide bore** pipette tip
- Pipette 2 μ L of DNA into the 20 μ L of cells and tap tube to mix
- Incubate on ice for 10min
- Take 21 μ 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 ensue 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 μ Fd, 2.0kV, and immediately place the cuvette back on ice
- Place 1mL of **room temperature** recovery into the cuvette
- Use a **wide bore** 1mL 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 15mL falcon tube
- Loosely fasten the cap to allow gas exchange and incubate at 37C & 225RPM for 1hr
- Pool all reactions and remove 10 μ L and dilute the cells according to table 23. Plate two plates with 10 μ L of each dilution
- For every 2 transformations, seed a 500mL culture of LB-Amp in 2.5L flasks.
- Grow colonies at 37C & 225RPM for 8–11hr. **UNTIL OD = 1.5-2.0**
- Pellet cells at 4500rpm for 15 minutes at 4C

7.5 Plasmid Library Purification

- Re-suspend all pellets together and purify with Qiagen EndoFree Mega Kit
- Add one additional centrifugation after neutralization of pellet lysis; 17,900xg for 10 minutes at 4 degrees

7.6 Estimate Transformation Efficiency

- Calculate total transformants utilizing the diluted series of plates equation

$$CFU \text{ per } \mu\text{L Seeded} = AvgCFU \times DilutionFactor \times \frac{TotalVolumeofCells\mu\text{L}}{10\mu\text{L}}$$

7.7 QC Competent Library

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

Reagent	KpnI/XbaI	SexAI	SalI
Water	8.5 μ L	11.5 μ L	11.5 μ L
DNA(200ng μ L)	3.5 μ L	3.5 μ L	3.5 μ L
10x Buffer	2 μ L	2 μ L	2 μ L
KpnI	3 μ L	- μ L	- μ L
XbaI	3 μ L	- μ L	- μ L
SexAI	- μ L	3 μ L	- μ L
SalI	- μ L	- μ L	3 μ L
Total Volume	20 μ L	20 μ L	20 μ L

Table 24: Competent Library Digests.

- The Expected band sizes are shown in Figure 7.7

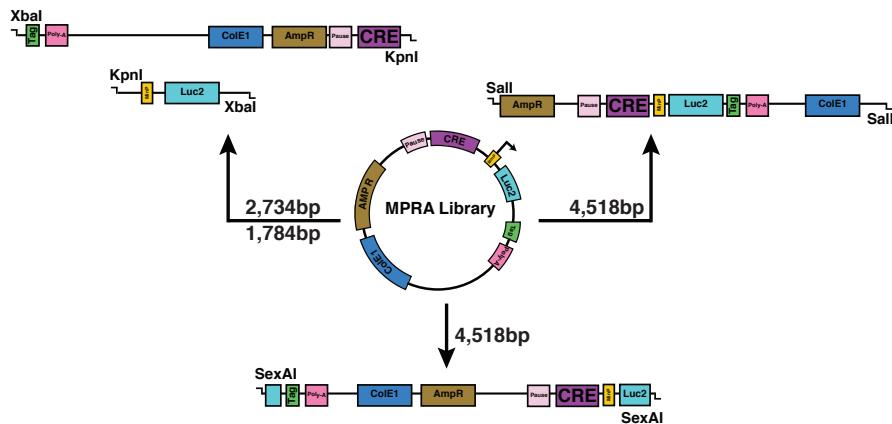


Figure 12: Competent Library QC Digest Expected Results

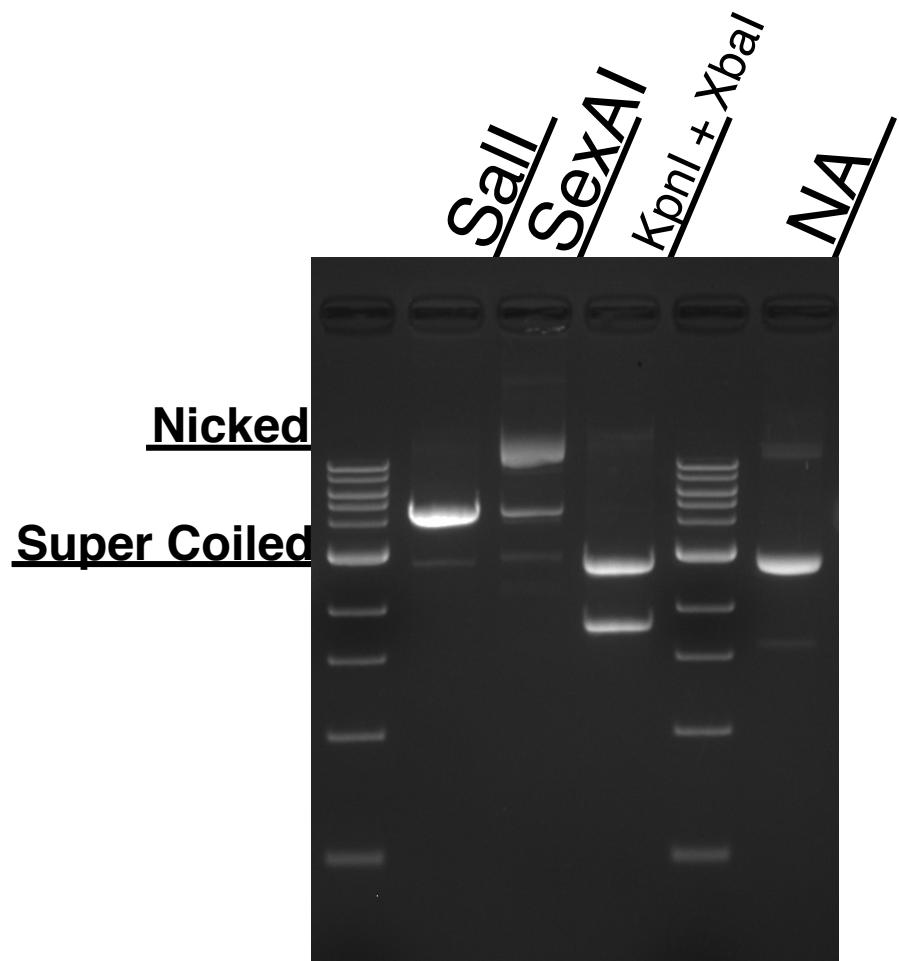


Figure 13: Competent Library QC Digest Results

- 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 (2.7Kb) 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 μ L	225 μ L
Library DNA(15ng/ μ L)	1 μ L	9 μ L
F+R-Primer (10uM)	2.5 μ L	22.5 μ L
Water	21.5 μ L	193.5 μ L
Final Volume	50 μ L	450 μ L

Table 25: Volumes Barcode PCR Reactions.

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

Table 26: PCR Conditions for Barcode Amplification.

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

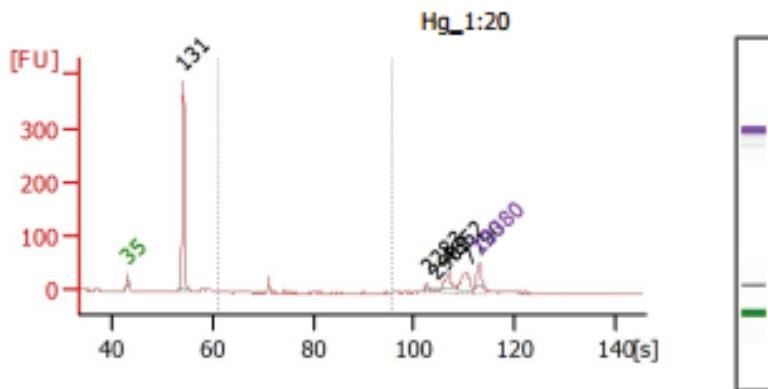


Figure 14: Bar-code containing amplicons

- Expected size with 5 degenerate bases on the 5' primer is 131 base pairs.
Sequence amplicons 2x150bp

8 Competent Library Characterization

8.1 Library Activity

- Four Co-Transfections for each condition were preformed at 2 μ g of designated plasmid and 10ng of PGL 4.72 Renilla Luciferase per-2 million cells. One mL of media was added to each cuvette and 250 μ L were plated per-well on a six well plate for 24hours.
- Luciferase assays were preformed with Promega Dual Luciferase Assays (E1910) and read on a Promega Luminometer(GM3500)

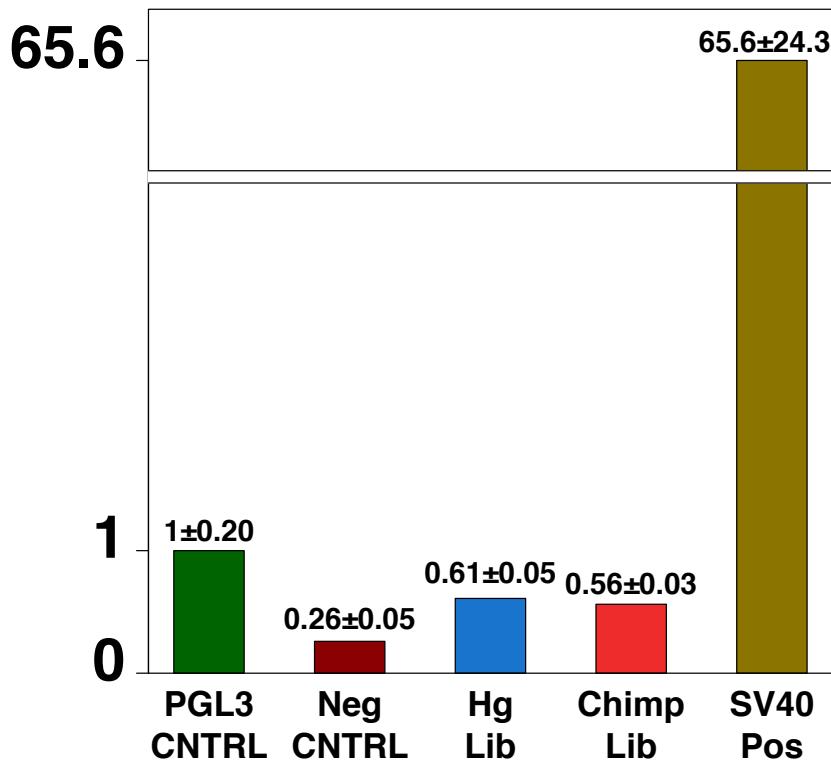


Figure 15: Competent Library Transfection

8.2 Optimize Library Amount to Transfect

- Libraries were C0-transfected with 2, 4, 8, and 16 μ g of human plasmid library and 15ng of 4.72 Renilla Luciferase per-2 million cells. One mL of media was added to each cuvette and 250 μ L were plated per-well on a six well plate for 24hours.
- Luciferase assays were preformed with Promega Dual Luciferase Assays (E1910) and read on a Promega Luminometer(GM3500)

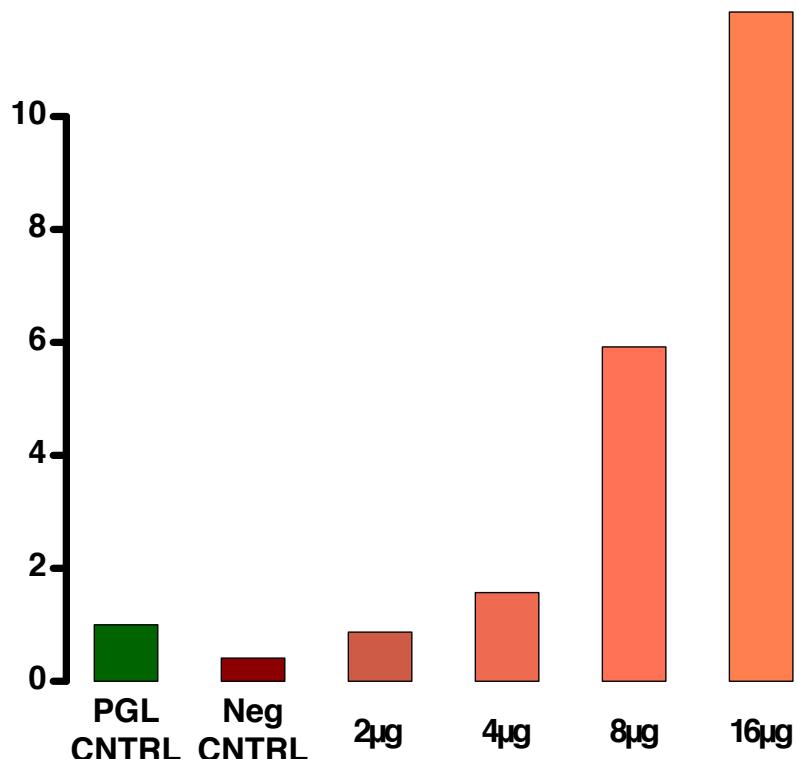


Figure 16: Transfection of Increasing Amounts of Library

8.3 Optimize Amount of Cells to Transfect

- Libraries were C0-transfected with 2, 4, 8, and 16 million cells, 16 μ g plasmid library per- 2 million cells, and 20ng of 4.72 Renilla Luciferase per-2 million cells. One mL of media was added to each cuvette and 250 μ L were plated per-well on a six well plate for 24hours.
- Luciferase assays were preformed with Promega Dual Luciferase Assays (E1910) and read on a Promega Luminometer(GM3500)

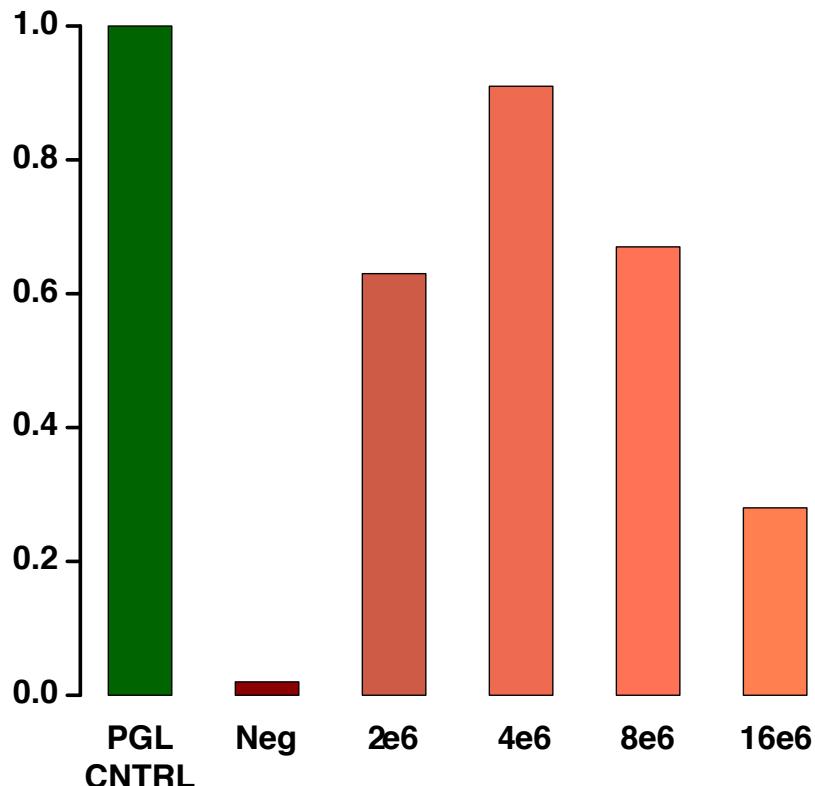


Figure 17: Transfection of Increasing Amounts Cells

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

9 Library Transfection

9.1 Harvest the cells

- Wash cells with 10mL (T-75) PBS (-) MgCl (-) CaCl, aspirate PBS
- Pipette 5mL pre-warmed Acutase into T-75 flask and incubate for 5 minutes at 37°C
- Tap the flask to dislodge cells and neutralize with 6mL of complete media, pipette into a falcon tube and spin down cells at 200xg for 4 minutes
- Aspirate off fluid and re-suspend the pellet in 5-10mLs of complete media
- Count cells to quantify concentration

9.2 Transfection Conditions

- Pipette 40 Million cells a 10mL falcon tube and spin down at 200xg for 4 minutes
- Aspirate off media completely and pipette 320µg of plasmid library on top of the cell pellet
- Add 1mL of Amaxa mNSC Reagent with Supplement 1 added and gently pipette to re-suspend the cells
- Gently re-suspend the cell pellet and transfer to 10 Amaxa cuvettes
- Electroporate the sample using pre-defined protocol **A-033**, and immediately add 1mL of complete media to recover after each transfection
- Use the pipette provided by Amaxa to add recovered cells to a falcon tube

9.3 Plating Transfected cells

- **Before Transfection:** Coat two $150cm^2$ flasks each with 12mL of coating media and 1 aliquot of matrigel for 1hr - Over Night
- Wash with 20mL of PBS (-) MgCl, (-) CaCl, aspirate off, and add 30mL of growth media. Leave plate in cell incubator to equilibrate
- Add half of the pooled recovered cell volume to each equilibrated $150cm^2$ flask and grow for 6 hours before harvesting

10 Post Transfection Processing

10.1 Harvest the cells

- Wash cells with 20mL PBS (-) MgCl (-) CaCl, aspirate PBS
- Pipette 10mL pre-warmed Acutase into each $150cm^2$ flask and incubate for 6 minutes at $37^\circ C$
- Tap the dish to dislodge cells and neutralize with 12mL of complete media, pipette into a 50mL falcon tube and spin down cells at 200xg for 4 minutes
- Aspirate off fluid and re-suspend the pellet in 10mLs of PBS (-) MgCl (-) CaCl.
- Remove 5% of the total volume and place into a 1.5mL eppendorf tube for plasmid library recovery
- Spin both the 10mL falcon tube and the eppendorf tube down at 200xg for 4 minutes

10.2 Genomic DNA Purification

- Add 40 μ L 1M DTT per 1mL of RLT buffer and re-suspend pellet in 600 μ L of RLT lysis buffer plus DTT per-10 Million cells transfected. Pipette to mix.
- Aspirate off the PBS from the 1.5mL eppendorf tube and add 350 μ L of previously prepared lysis buffer
- Pipette to re-suspend the cells and vortex to vigorously to lyse cells
- Spin the eppendorf tube down and then add the entire volume to a Qiagen DNA/RNA 2 and 1 genomic DNA column
- Spin at 8150xg for 30 seconds. **Proceed to section 10.3 until instructed to return.**
- Wash column with 500 μ L of buffer AW1 and spin at 8150xg for 30 seconds
- Wash column with 500 μ L of buffer AW2 and spin at full speed for 2 minutes
- Place in 1.5mL eppendorf tube and Pipette 80 μ L of EB onto the column and incubate for one minute
- Elute by spinning at 8150xg for 1 minute
- Pipette another 80 μ L of EB onto the column and incubate for one minute
- Elute a second time by spinning at 8150xg for 1 minute

10.3 RNA Purification

- Add 40 μ L 1M DTT per 1mL of RLT buffer and re-suspend pellet in 600 μ 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 μ 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 RNaseeasy Column in increments of 650 μ L. Spin 8500xg for 1 minute, this should equate to two spins per sample given this volume.
- Add 350 μ L of RW1 buffer to the column and spin at 8500xg for 30s.
- Digest DNA on column by adding 10 μ L pre-aliquoted DNase I to 70 μ L RDD buffer and pipetting all 80 μ L directly onto the column. Incubate at room temperature with lid closed for 15 minutes. **While the digesting DNA return to Section and finish the genomic DNA purification**
- Add 350 μ L of RW1 buffer to the column and spin at 8500xg for 30s.
- Add 500 μ L of buffer RPE to the column and spin at for 30s.

- Add 500 μ 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 μ L of RNase free water directly onto the column wait one minute then spin at 8500xg
- Pipette another 30 μ L of RNase free water directly onto the column wait one minute then spin at 8500xg
- Pool total RNA from the same transfections together and remove 2 μ 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 μ L, then split into different tubes.

Reagent	1x	2x
RNA	Y μ L	Y μ L
Water	X μ L	X μ L
Buffer RDD	10 μ L	20 μ L
DNaseI	2.5 μ L	5 μ L
Final Volume	100 μ L	200 μ L

Table 27: Second DNase Digestion Mix.

- Incubate ad room temperature for 20 minutes

10.5 Plasmid Library Enrichment from Purified DNA

- Preform while second digest is occurring in Section 10.4
- Add 300 μ L ERC buffer to 20-100 μ L of pDNA
- Add 10 μ L NaOAC (3M). Vortex to mix and spin down
- Add total sample to QiaPrep Spin Miniprep column (27106). Spin at 17900xg for 1 minute
- Add 500 μ L of PB buffer to column and spin at 17900xg for 1 minute
- Add 750 μ L of PE buffer to column and spin at 17900xg for 1 minute
- Transfer column to new tube and spin at 17900xg for 1 minute to dry column
- Place in 1.5mL eppendorf tube and Pipette 30 μ L of EB onto the column an incubate for one minute
- Elute enriched pDNA by spinning at 17900xg for 1 minute
- Pipette another 30 μ L of EB onto the column an incubate for one minute
- Elute a second time by spinning at 8150xg for 1 minute

10.6 RNA Clean-up

- Add 350 μ L of RLT buffer for every 100 μ L of DNase treated RNA.
- Add 250 μ L of 96-100% RNase free EtOH for every 100 μ L of DNase treated RNA.
- Pipette up to 700 μ L onto an RNAeasy Mini-prep column and spin at 8500xg for 30s. Repeat as necessary up to column limit of 100 μ g.
- Pipett 500 μ L of RPE onto column and spin at 8500xg for 30s.
- Pipett 500 μ L of RPE onto column and spin at 8500xg 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 μ L of RNase free water directly onto the column wait one minute then spin at 8500xg
- Pipette another 30 μ L of RNase free water directly onto the column wait one minute then spin at 8500xg
- Pool total RNA from the same transfections together and remove 2 μ L aliquots of DNA for gel and nano-drop analysis

10.7 mRNA Purification (If Necessary)

- Dilute 1-5 μ g of total RNA to 50 μ L with RNase free water
- Pipette 20 μ L of re-suspended beads in a **Separate Tube**
- Add 100 μ L of binding buffer to the beads and mix by gently pipetting
- Place beads on Magnet for 2 minutes
- Remove supernatant, and then remove the tube from the rack and wash with another 100 μ L of binding buffer mix gently by pipetting, 2 minutes magenet, remove supernatant, remove the tube from the rack.
- Re-suspend the beads in 50 μ L binding buffer and then add 50 μ 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.
- Re-suspend beads gently by pipetting
- Incubate at room temperature for five minutes.
- Re-suspend beads gently by pipetting
- Incubate at room temperature for five minutes.
- Place tube on magnet for 2 minutes and discard the supernatant.

- Remove the tube from the rack and wash with 200 μ L washing buffer, mix by pipetting then place the tube on the magnet for 2 minutes and discard the supernatant.
- Repeat the previous step a second time
- Re-suspend the beads in 50 μ L of Tris, mix by pipetting and transfer to thin wall 0.2mL PCR tube.
- Heat sample in a thermocycler at 80°C for 2 minutes, then hold at 25°C to elute. Remove the samples when they reach 25°C
- Add 50 μ L binding buffer to each sample to allow the RNA to bind to the same beads. Mix by pipetting.
- Incubate at room temperature for five minutes.
- Re-suspend beads gently by pipetting
- Incubate at room temperature for five minutes.
- Place tube on magnet for 2 minutes and discard the supernatant.
- Remove the tube from the rack and wash with 200 μ L washing buffer, mix by pipetting then place the tube on the magnet for 2 minutes and discard the supernatant.
- Spin down tube then place back on magnet and remove the remaining supernatant with a 20 μ L pipette
- Remove teh tube from the rack and re-suspend the beads in 17 μ L of Tris, mix by pipetting and transfer to thin wall 0.2mL PCR tube.
- Heat sample in a thermocycler at 80°C for 2 minutes, then hold at 25°C to elute. Immediately remove the samples when they reach 25°C and place on the magnetic rack for 2 minutes.
- Collect the enriched mRNA and remove 2 μ 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 preformed run lanes of mRNA and a 1:10 dilution of mRNA.

10.9 cDNA Synthesis

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

Reagent	Volume
Annealing Buffer	2.5µL
Oligo dt	2.5µL
mRNA	15µL
Final Volume	20µL

Table 28: Volumes for a Single Oligo dt Annealing Reaction.

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

Reagent	Volume
Annealing Reaction	20µL
2x Buffer	25µL
SSIII Enzyme	5µL
Final Volume	50µL

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 50µL aliquots into 0.2mL PCR tubes and incubate at 16C for 2.5 hours in a thermocycler with the lid left unheated.

Reagent	Volume
1st Strand Reaction	50µL
DEPC Water	120µL
10x Buffer	20µL
Enzyme mix	10µL
Final Volume	200µL

Table 30: Volumes for a Single cDNA Synthesis Reaction.

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

11 qPCR

11.1 Preparation

- Primer Stocks: All Primers are kept at 100uM, mix 10µL forward with 10µL reverse, and 80µL for 100µL of 10uM working stock
- Dilute 2µL of cDNA 10-fold and 100-fold
- Dilute 2µL of pDNA 10-fold, 100-fold, and 1,000-fold

- Serial dilute plasmid standard out fresh each time from 1×10^8 copies/ μL to 1×10^2 copies/ μL

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 μL	$\times Samples_{total}$
DEPC Water	8 μL	$\times Samples_{total}$
10uM F+R Primer Mix	1 μL	$\times Samples_{total}$
Sample	1 μL	NA
Final Volume	20 μL	$19\mu\text{L} \times Samples_{total}$

Table 31: qPCR Master Mix.

- Pipette 76 μL into each well along the D or E rows of a 96-well Fast Optical qPCR Plate (Applied Biosystems 4346906)
- Add 4 μL of sample to each well
- Use the multi-channel P200 pipette to pipette 20 μL of the 80 μL 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	Copy # Standard											
E							pDNA	cDNA	Cntrl			
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

$$\begin{array}{lll}
 \text{AOD } 10\mu\text{L}/\text{rxn} & X & \frac{56}{\text{rxns.}} = \frac{560}{\mu\text{L}} \\
 \text{H2O } 8\mu\text{L}/\text{rxn} & X & \frac{56}{\text{rxns.}} = \frac{448}{\mu\text{L}} \\
 \text{Prim } 1\mu\text{L}/\text{rxn} & X & \frac{56}{\text{rxns.}} = \frac{56}{\mu\text{L}}
 \end{array}$$

Figure 18: Sample Layout of qPCR Plate

11.4 Bar-code PCR Amplification

- Generic PCR Master-Mix for Barcode-Seq:

Reagent	Volume
2x NEB Master Mix	10 μ L
Water	x μ L
Fwd + Rev Primers (10 μ mol/ μ L)	1.25 μ L
DNA	x μ L
Final Volume	20 μ L

Table 32: Barcode PCR Master-mix.

- Generic PCR Conditions for Barcode-Seq:

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

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 μ 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 μ L
Water	6.55 μ L
Fwd + Rev Primers (10 μ mol/ μ L)	1.25 μ L
10x SYBR Green	1.2 μ L
DNA	1 μ L
Final Volume	20 μ L

Table 34: Barcode qPCR Master-mix.

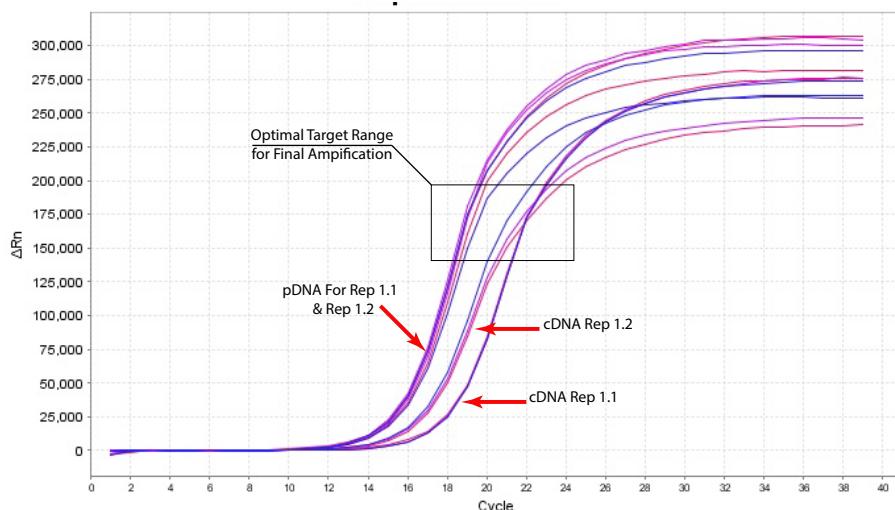


Figure 19: SYBR Green Amplification

- 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 11.4 15 cycles seems sufficient.

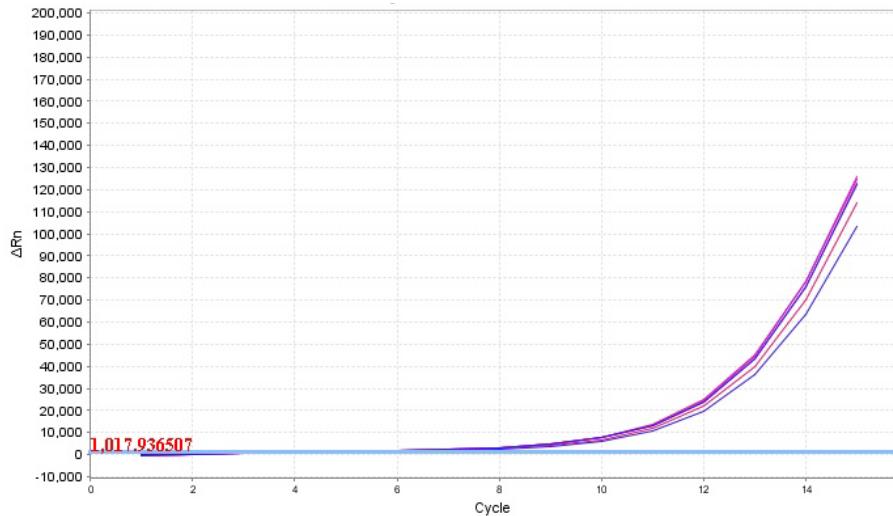


Figure 20: pDNA Undiluted SYBR Green Amplification

- Figure 11.4 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 also that there will be enough product produced at the fluorescent intensity in Figure 11.4.
- Generic PCR Conditions for pDNA Barcode-Seq:

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

Table 35: PCR Conditions for Sample Barcode Amplification.

- Generic PCR Conditions for cDNA Barcode-Seq:

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

Table 36: PCR Conditions for Sample Barcode Amplification.

12 Reagents

12.1 Consumables

Provider	Reagent	Product Number
American Bio	DEPC Water:AB02128-00500	
NEB	High Fidelity 2x Master Mix	M0541L
Life Technologies	SYBR Green	S7563
Beckman Coulter	AMPureXP SPRI Beads	A63880
NEB	BSA (20mg/mL)	B9000S
NEB	Q5 High fidelity Polymerase	M0491L
Acros Organics:2-Butanol	10770-0010	
Sage Science	2% Agarose pippen prep gels	CSD2010
NEB	CIP	M0290L
NEB	400U/textmu L DNA T4 Ligase	M0202M
American Bio	70% Ethanol	AB04010-00500
American Bio	200 Proof Ethanol	AB00515-00500
Recombinant Thecnologies	100textmu g/mL LB/Amp plates	Stockroom
Bio-Rad	Electroporation Cuvettes 0.1cm	1652089
American Bio	Agarose	AB00972-00500
Sigma	Ethidium Bromide	E1510-10ML
American Bioanalytical	DMSO	AB03091-00100
NEB	dNTPs	N0446S
Promega	PGL 4.72 Renilla Luciferase Vector	E690A
Promega	PGL 3 Control Vector	E174A
Gibco	PBS Mg/Cl -	14190-144
Gibco	StemPro Acutase	A11105-01
Qiagen	DNAseI	79254
American Bio	3M NaOAC	AB13168-01000
ThermoFischerScientific	MegaX DH10B	C6400-03
Applied Biosystems	Fast Optical qPCR Plates	4346906

Table 37: Primary Consumable Reagents.

12.2 Kits

Provider	Kit	Product Number
CHIMERx/EURx	Micellula Emulsion PCR Kit	3600-02
Agilent	Bioanalyzer Eukaryotic Pico Kit	5067-1513
Agilent	Bioanalyzer High Sensitivity DNA	5067-4626
Qiagen	Minelute	28004
Qiagen	Reaction Clean-up (ERC Buffer is in here)	28206
Qiagen	HiSpeed Maxi Kit	12662
Qiagen	Endofree Mega Kit	12381
Qiagen	QiaShredder	79654
Qiagen	RNeasy Mini Kit	74104
NEB	NEBNext Library Kit	E6040L
NEB	HighSeq Indexing Primers	E7335L
Promega	Dual Luciferase Assay Kit	E1910
Qiagen	AllPrep Mini DNA/RNA	80204
Invitrogen	Super Script III cDNA kit	18080-400

Table 38: Primary Kit Reagents.

12.3 Specialized Equipment

- Bio-Rad Gene Pulser
- Amaxa Nucleofector
- Applied Biosystems Step One Plus qPCR
- Agilent 2100 Bioanalyzer
- Promega Luminometer GM3500

13 Primer Sequences

13.1 Initial Low Cycle Library Primers

- Forward: 5' - GCCAGAACATTCTCTGGCTTAACGGCCGTTGACG - 3'
- Reverse: 5' - GCAGGAGCCGCAGTG - 3'

13.2 Library Bar-coding Primers

- Forward: 5' GCCAGAACATTCTCTGGCTTAACGGCCGTTGACG - 3'
- Reverse: 5' CCGACTAGCTTGGCCGCCAGGGCCGACCGCTTCCGATCTNNNNNNNNNNNNNNNTCTAGAGGTACCGCAGGAGCCGCAGTG - 3'

13.3 Inert TagSeq Library Primers

- Forward: 5' - (N:25252525)(N)(N)(N)CAGGTGCCAGAACATTCTCT - 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' - TTATCATGTCTGCTCGAACGCGG - 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' - CCGACGCTCTCCGATCT - 3'

13.6 Reverse Transcription Primer

- 5' -CCGACTAGCTTGGCCGC- 3'

13.7 Transcript qPCR Primers: targets Luciferase

- Forward:5' -AACACCCCCAACATCTTCGAC- 3'
- Reverse:5' -TCTCGGTCATGGTTTACCG- 3'

13.8 Backbone qPCR Primers

- Forward:5' -ATTGGTATCTGCGCTCTGC- 3'
- Reverse:5' -TTGCCGGATCAAGAGCTAC- 3'