

# The RNAi Consortium (TRC)

## Broad Institute

### TRC Laboratory Protocols

Protocol Title: **Nested PCR Preparation of Samples for Illumina Sequencing**

Current Revision Date: **11/10/2012**

RNAi Platform, Broad Institute, [trc\\_info@broadinstitute.org](mailto:trc_info@broadinstitute.org)

### Brief Description:

This protocol describes the nested PCR process to generate Illumina sequencing readout for pooled screening. In general, we use nested PCR for screens where gDNA is limiting, e.g. screens involving small custom pools, or any positive selection screens.

### Table of Contents:

- Notes
- Materials
- Part 1: Primary PCR
- Part 2: Secondary PCR
- Part 3. Pooling and Purification of Sequencing sample
- Appendix A. Primary Primer Sequences.
- Appendix B. Secondary Primer Sequences.
- Appendix A. Primary Primer Sequences.
- Appendix C. Custom Illumina Sequencing Primer Sequences
- Revision Notes

### Materials:

TaKaRa exTaq (Millipore, Cat# RR001A)

Qiagen Gel purification kit (Qiagen, Cat# 28704)

### Notes:

- A. This procedure can be scaled up or down as appropriate to cover the necessary representation goals. For genome scale screens, we target ~ 200 average reads/ hairpin (eg 100,000 shRNA library required a minimum of  $2e7$  reads)  
For smaller pools (<10,000 shRNAs we increase the read representation (~500 - 1000 average reads/hairpin).
- B. The amounts of total gDNA for each sample should reflect the sequencing depth. We use a constant of 6.6 ug gDNA/1e6 cells as a proxy for calculating the required amount of total gDNA required. (eg genome scale screen using 50,000 shRNA library at a 200 cell/shRNA representation requires a minimum of 66 ug gDNA)
- C. For each 100ul primary PCR reaction we limit the amount of gDNA to 10ug or 20% volume (20ul). We adjust the total number of independent PCR reactions to get to the desire representation scale.
- D. We use a nested PCR process to amplify hairpin sequences from gDNA. The primary PCR uses the same primer pair for all TRC vector backbones. However the secondary PCR and eventual sequencing primer is different for the constitutive human U6 backbones (pLKO.1 and pLKO\_TRC005), inducible human U6 promoter backbone (Lac Inducible) , and human H1 promote backbones (TetR inducible)
- E. Multiplexing of secondary PCR samples in a single Illumina lane is possible by using several independent 5' bar coded primers during the secondary PCR step. However, in planning for pooling of samples to run in

# The RNAi Consortium (TRC)

## Broad Institute

a single Illumina lane, you must have at least 12+ independent bar codes to aid in the cluster calling portion of the sequence process. If you are only sequencing 1 sample, then the secondary PCR should include 12 independent reactions of the same primary PCR, each with a different barcoded 5' primer that will be pooled together.

- F. The 5' and 3' secondary PCR primers already incorporate the P5 and P7 Illumina sequences. This PCR product can be directly sequenced with no additional ligation steps

### Part 1. Primary PCR

Determine the amount of gDNA required to maintain representation of the initial pool (See notes (B) section)

Determine the number of reactions (100ul) necessary to amplify target gDNA (See notes (C) section)

1. Create a master mix for the number of reactions (below is mix for single rxn. Increase as appropriate)  
10µL 10X buffer  
8µL dNTPs  
10µL 5uM primary PCR Primer Mix  
1.5µL TaKaRa exTaq  
H2O up to 80µL
2. Add gDNA/H2O (20ul /rxn.) to correct vol of master mix, and aliquot into as many PCR tubes as necessary (100ul final vol.)

3. Run Primary Illumina PCR Protocol:

95°C 5min	} 15 cycles
<b>94°C 30sec</b>	
<b>65°C 30sec</b>	
<b>72°C 20 secs</b>	
72°C 5min	

4. Pool all reaction using the same gDNA template prior to secondary PCR Step.

### Part 2. Secondary PCR

Use at least 12 independent barcoded 5' primers for each lane of sequencing. (See notes (D-F) section)

To calculate the number of secondary PCR reaction necessary to maintain representation use the following:

Roundup(# hairpins in pool / 10000).

For example 50,000 shRNA pool requires a minimum of 5 100 ul reactions

1. Create a master mix for each 5' Bar-coded primer, create a 100uL reaction with master mix as follows:

7.5 µL	10X buffer
8 µL	dNTPs
10 µL	5uM common 3' primer
3.75 µL	DMSO
1.5 uL	TaKaRa exTaq
30 uL	primary PCR mix
H2O up to 90 µL	

2. Add 10 uL independent 5' BC primer (5uM) to each reaction (See Appendix A for sequences)
3. Split the 100 uL reaction into 2 X 50µL PCR tubes
4. Run Secondary Illumina PCR Protocol:

# The RNAi Consortium (TRC)

## Broad Institute

95°C 5min  
94°C 30sec  
58°C 30sec  
72°C 20 secs  
72°C 5min

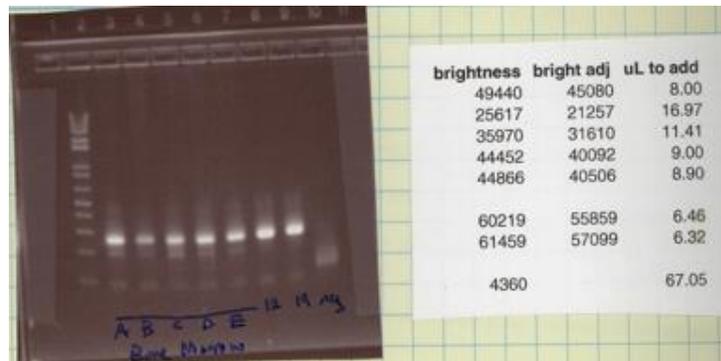
} 15 cycles

5. Pool both 50 uL reaction for same 5' BC primer prior to test gel electrophoresis.

### Part 3. Pooling and Purification of Sequencing sample

#### Gel Quantification and multiplexing of PCR reactions:

1. Run 5 ul of each independent 5' barcoded primer sample on a 2% agarose gel (all samples to be pooled in same Illumina lane should be run on the same agarose gel if possible)
2. Use imager to quantify amount of 200-220 bp product in each lane (See below)



Your imager should have a tool to quantify the intensity of the bands, in which case measure this for each band

3. Calculate amount of PCR product to pool based on relative intensity. If you desire to have each PCR product sequenced to the same depth calculate volume from each PCR sample to give equal relative abundance of PCR product.
4. Gel purify pooled PCR sample using a 2% agarose gel (use multiple wells in necessary)
5. Use Qiagen Gel purification kit to purify sample (use multiple columns as appropriate based on mass of agarose gel).
6. Follow kit protocol, with the following exception: Prior to PE wash, wash column with 1 volume Qiagen PB buffer.
7. Elute in final vol of 60 ul EB
8. OD using nano-drop

# The RNAi Consortium (TRC) Broad Institute

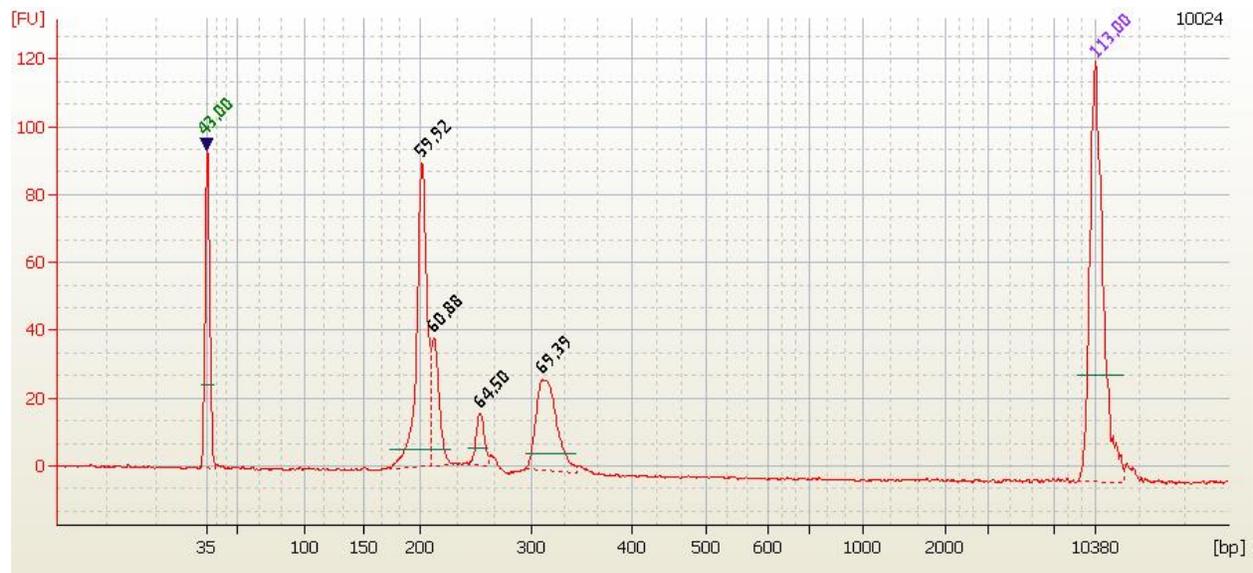
## Illumina Sequencing:

Need to submit 1-2 ug (~15-50 ng/ul) PCR product per Illumina lane, along with custom sequencing primer (HPLC pure)-(See Appendix C for sequences).

**Note to Illumina sequencing facility :** The amount of purified PCR product to load in a Hi-Seq lane should be empirically determine for each independent sequencing facility. However, a good starting point would be to load 9-12 pM of / Hi Seq lane.

## Agilent QC (optional):

You can run a small amount of your purified Illumina sample on Agilent and compare to the following:



Note that there are 3 peaks (and the relative amount of each will vary). These are due to the conformation changes inherent to a short piece of DNA that contains a sizeable hairpin.

# The RNAi Consortium (TRC)

## Broad Institute

### Appendix A. Primary Primer Sequences

#### Primary PCR Primer Sequences:

Used for all constitutive

5' primer: AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTTCG

3' primer: CTTAGTTTGTATGTCTGTTGCTATTATGTCTACTATTCTTTCCC

And for inducible shRNA backbone pLKO derivative vectors

5' primer: CATTATCGTTTCAGACCCACCTCCCAACC

3' primer: CTTAGTTTGTATGTCTGTTGCTATTATGTCTACTATTCTTTCCC

### Appendix B. Secondary Primer Sequences

#### Secondary Primer Sequences for constitutive pLKO.1 and pLKO-TRC005 vectors:

3' Universal PCR primer:

5'-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTTGGATGAATACTGCCATTTGTCTC-3'

5' 6nt Bar-coded PCR primer :

5' -AATGATACGGCGACCACCGACCGTAACTTGAAAGTATTTTCGATTTCTGGCTTATATATC**NNNNNN**AAAGGAC-3'

[Historical 5' 4nt Bar-coded PCR primer: No longer in use]

5'-AATGATACGGCGACCACCGAGAAAGTATTTTCGATTTCTGGCTTATATATCTTGTGG**ANNNN**ACGA-3'

#### Secondary Primer Sequences for Lac Inducible vectors:

3' Universal PCR primer:

5'-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCGGATGAATACTGCCATTTGTCTCGAGG-3'

5' 6nt Bar-coded PCR primer :

5' -AATGATACGGCGACCACCGACCGTAACTTGAAAGTAATTGTGAGCGCTCACAATTATATATC**NNNNNN**AAAGGAC -3'

**Bold** nt's can be altered to generate bar-codes within the primer sequence.

### Appendix C. Custom Illumina Sequencing Primer Sequences

#### Constitutive pLKO.1 and pLKO-TRC005 vectors

**(HPLC or PAGE Purification-must!)**

CCGTAACCTTGAAAGT/i6diPr/TTTCGATTTCTGGCTTT/i6diPr/T/i6diPr/TATC

[Working] = 10 uM

#### Lac inducible pLXI vectors

**(HPLC or PAGE Purification-must!)**

CGTAACCTTGAAAGTA/i6diPr/TTGTGAGCGCTCACAATT/i6diPr/T/i6diPr/TATC [Working] = 10 uM

**Revision notes:**