

# The RNAi Consortium (TRC)

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### TRC Laboratory Protocols

Protocol Title: **One Step PCR Preparation of Samples for Illumina Sequencing**

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### Brief Description:

This protocol describes the one step PCR process to generate Illumina sequencing readout for pooled screening. In general, we use this single step PCR process for negative selection genome-scale screens to increase PCR throughput. If the amount of available gDNA is limiting, e.g. for screens involving small custom pools and positive selection screens, please refer to the nested PCR protocol.

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### 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 desired representation goals. For genome-scale screens, we target ~200 average reads/ hairpin (e.g. 100,000 shRNA library requires a minimum of  $2 \times 10^7$  reads).  
For smaller pools (<10,000 shRNAs) we increase the read representation to ~500-1000 average reads/hairpin.
- B. The total amount of gDNA used for each sample should reflect the desired sequencing depth. Use a constant of  $6.6 \mu\text{g gDNA}/1 \times 10^6$  cells to estimate the total amount of gDNA required. (e.g. genome-scale screen using 100,000 shRNA library at a 300 cell/shRNA representation requires a minimum of  $198 \mu\text{g gDNA}$ ).
- C. For each 100 $\mu\text{l}$  PCR reaction limit the mass of gDNA to 10 $\mu\text{g}$  and no more than 40% of total volume (40 $\mu\text{l}$ ). Determine the total number of independent PCR reactions required at this scale to achieve the desired representation.
- D. The 5' and 3' PCR primers include the P5 and P7 Illumina adapter sequences; therefore this PCR product can be directly sequenced with no additional ligation steps.
- E. Multiplexing of PCR samples in a single Illumina lane is made possible by using a unique 5' bar coded primer(s) for each sample. Each sample/pool of samples to be run on a single lane must have at least 12 unique bar codes to aid in the cluster calling portion of the sequence process. If you are only sequencing 1 PCR sample, then the PCR should include 12 independent PCR reactions, each using a different 5' barcoded primer. These products can then be pooled together into one sample for sequencing.

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- F. PCR primers and sequencing primers are different for the constitutive human U6 backbones (pLKO.1 and pLKO\_TRC005), inducible human U6 promoter backbone (IPTG/Lac Inducible), and human H1 promoter backbones (Dox/TetR inducible).
- G. Multiplexing of PCR samples can only be performed for samples with the same backbone type (constitutive, IPTG inducible, Dox inducible).

### Part 1. One Step 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)

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

1. Create a master mix for the number of reactions needed.

	<u>Per 100ul rxn</u>	<u>22 rxns</u>
10X buffer	10	220
dNTPs	8	176
5uM 3' Universal Primer	10	220
TaKaRa exTaq	1.5	33
H2O	up to 50µL	451

2. Aliquot 90 ul of master mix to NTC control. Add 10ul 5'barcode primer (5uM). Seal tube.

3. Add gDNA/H2O (40ul /rxn.) to correct volume of master mix,  
eg for gDNA that is at 0.5 ug/ml concentration requires 20 rxns

Add 400 ul gDNA  
400 ul H2O  
1000ul Master Mix

Mix and aliquot 90 ul into each PCR tube

4. Add 10 uL 5' BC primer (5uM) to each reaction (See Appendix A for sequences)

5. Run One Step Illumina PCR Protocol:

95°C 5min	} 28 cycles
<b>94°C 30sec</b>	
<b>58°C 30sec</b>	
<b>72°C 20 secs</b>	
72°C 5min	

6. After cycle is complete, if sample with identical 5' BC primer was split over multiple wells, pool PCR products prior to quantification.

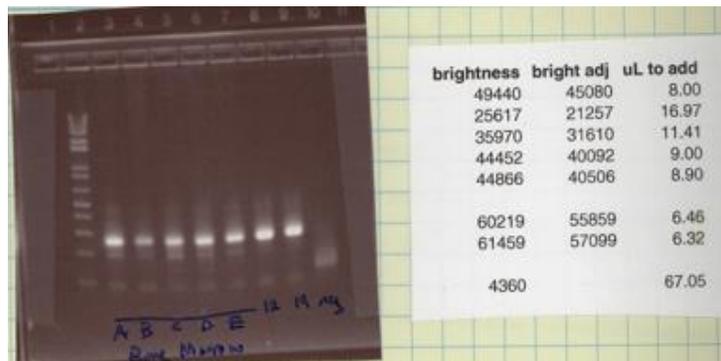
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### Part 2. Pooling and Purification of Sequencing sample

#### 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 0.8-1.0% 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 (Cat # 19066)
7. Elute in final vol of 60 ul EB
8. OD using nano-drop

#### Illumina Sequencing:

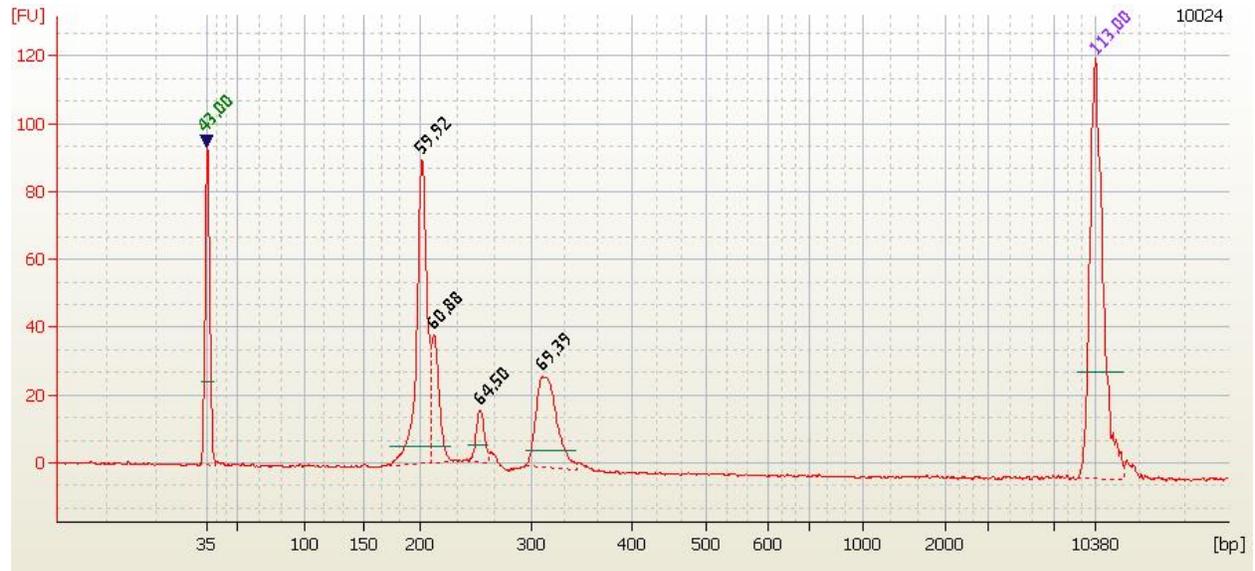
Need to submit 1-2 ug (~15-50 ng/ul) PCR product per Illumina lane, along with custom sequencing primer (HPLC pure)-(See Appendix B for sequences).

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

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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.

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**Appendix A. Primer Sequences**

3' Universal PCR primer:

5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTTGTGGATGAATACTGCCATTTGTCTC-3'

5' 6nt Bar-coded PCR primer : [Constitutive vectors (pLKO.1 and pLKO-TRC005)]

5' -AATGATACGGCGACCACCGACCGTAACTTGAAAGTATTTCGATTTCTGGCTTATATATCNNNNNNAAAGGAC-3'

5' 6nt Bar-coded PCR primer : [IPTG/LacO inducible vectors (pLXI-TRC9##)]

5' -AATGATACGGCGACCACCGACGTAAGTAACTTGAAAGTAAATTGTGAGCGCTCACAAATTATATATCNNNNNNAAAGGAC-3'

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

**Appendix B. Custom Illumina Sequencing Primer Sequences**

Constitutive pLKO.1 and pLKO-TRC005 vectors

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

CCGTAACCTTGAAAGT/i6diPr/TTTCGATTTCTTGGCTTT/i6diPr/T/i6diPr/TATC  
[Working] = 10 uM

IPTG/LacO inducible pLXI-TRC9## vectors

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

CGTAACCTTGAAAGT/i6diPr/TTGTGAGCGCTCACAAATT/i6diPr/T/i6diPr/TATC  
[Working] = 10 uM

**Revision Notes:**