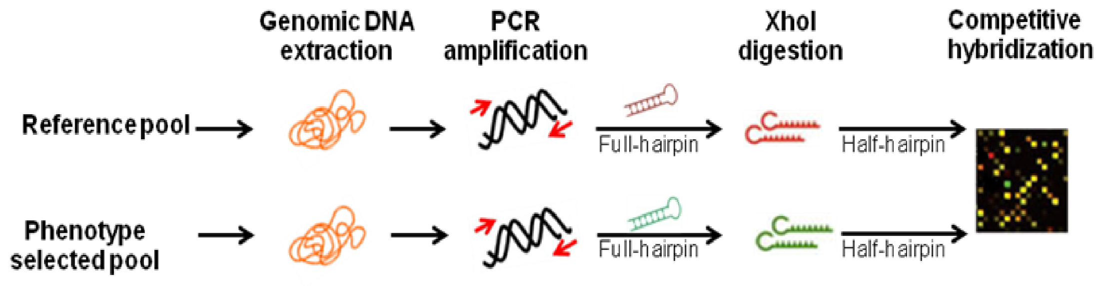
Protocol of sample preparation for pooled shRNA half-hairpin microarray



【PCR amplification】

Step 1 – Target/Puro Enrichment

(1-1) PCR

* gDNA (total 130 μg= about ~500x oversampling of 82k library)	X μl
**Puro F primer (100 μM)(5'-TTCACCGAGGGCCTATTTCCCATG)	6.5 μl
**Puro R primer (100 μM) (5'-CGTGAGGAAGAGTTCTTGCAGCTC)	6.5 μl
10X ExTaq Buffuer (TaKaRa)	65 μl
dNTP (2.5 mM each)(TaKaRa)	52 μl
Ex-Taq (Cat.No.KA4101AA TaKaRa)	3.25 μl
DMSO	40 μl
ddW	650-Xμl
Total	650 μl

> Aliquot 100μl of PCR mixture to 7 wells attached strip caps PCR tube.

(1-2) PCR profile

94°C x 5min → [94°C x 30sec → 55°C x 30sec → 72°C x 1.5min] x 15 cycles → 72°C x 10min

(1-3) Concentration

Qiagen PCR purification Kit (Cat. No. 28106)

> Pool 650 µl of PCR product together and expand with 5x volume of Buffer PB (3.25ml).

> Add 600 µl each to three Qiagen MinElute columns (total 3 columns), spin at full speed for 1 min, discard the flow through

> Repeat once, until the all PCR product input to column.

> Wash each column with 750 µl of Buffer PE

> Elute with 10 µl of ddW for each column, and pool 30 µl together.

> Store the Target/Puro enriched PCR product at -20^{0}C.

Step2 – Half-hairpin Probe Generation

(2-1) TRC-PCR-I

Target/Puro enriched template	3.25 µl
Cy5 (control)/Cy3 (experiment) labeled F primer (100 µM)	6.5 µl
(5'-AATGGACTATCATATGCTTACCGTAACTTGAA)	
Universal unlabeled R primer (100 µM)	6.5 µl
(5'-TGTGGATGAATACTGCCATTTGTCTCGAGGTC)	
10X ExTaq Buffuer	32.5 µl
dNTP (2.5 mM each)	26 µl
Ex-Taq	6.5 µl
ddW	243.75 µl
Total	350 µl

> Aliquot 50µl of PCR mixture to 7 wells attached strip caps PCR tube.

(2-2) PCR profile

95°C x 5min → [94°C x 30sec → 50°C x 30sec → 72°C x 1min] x 35 cycles → 72°C x 10min

(2-3) TRC-PCR-II **(without DNA template !!)**

Cy5 (control)/Cy3 (experiment) labeled F primer (100 μM)	6.5 μl
Universal unlabeled R primer (100 μM)	6.5 μl
10X ExTaq Buffuer	32.5 μl
dNTP (2.5 mM each)	26 μl
Ex-Taq	6.5 μl
ddW	243.75 μl
Total	254 μl

> Add 50μl of PCR mixture to previous tube, total 100ul/well

(2-4) PCR profile

95°C x 7min → 55°C x 2min → 72°C x 60min

(2-5) XhoI digestion

PCR product	600 μl
10x NEB buffer 4	80 μl
10x NEB BSA	80 μl
XhoI (NEB)	25 μl
ddW	15 μl
Total	800 μl

> Digest at 37°C overnight, covered with aluminum foils

(2-6) Concentration

Qiagen PCR purification Kit (Cat. No. 28106)

> Add 5x volume of Buffer PB (4ml) to the 800μl digested PCR product , and mix well.

> Add 750μl each to 3 Qiagen MinElute columns, spin at full speed for 1 min, discard the flow

through .

> Repeat the previous step.

> Wash each column with 750μl of Buffer PE, spin at full speed for 1 min, and discard the flow Through.

> Spin at full speed for another 3 min.

> Transfer the column to a clean 1.5ml microcentrifuge tube.

> Elute with 10μl of ddW for each column, and pool 30 μl concentrated PCR product together.

(2-7) Gel purification

> Add 6μl 6x DNA loading dye to the 30 μl concentrated PCR product

> Gel purification using a 2% DNA agarose gel (in 1x TAE solution) running with 100V,~120min

> Cut the lower band (~100bp size) with an attempt to minimize the gel size (Cy5 quenches EtBr).

> Extract DNA out using Qiagen gel purification kit (<u>don't add 2-propanol!</u>) (Cat. No. 28706), elute with 30 μl of ddW.

> Check concentration with using Nanodrop (with "Microarray")

- ✧ Cy5-probe (control) usually has a concentration of 60~90 ng/μl, Cy5 intensity ~0,6-0.7
- ✧ Cy3-probe (experiment) is 1.5-fold more concentrated, Cy3 intensity ~0.8-1.0

(Repeat probe preparation if concentration is less than 45 ng/μl or signal intensity less than 0.6)

【Note】

The input amount of genomic DNA for stage I-PCR

~For example~

82,000 shRNA * 500 replicates = 41,000,000 copies

41,000,000 copies / (333 copeis/ng)= 123,123 ng =~130 μg

(at least $2.1*10^7$ cells for gDNA extraction)

Primer information

The primers used in this protocol are all synthesized by IDT (Integrated DNA Technologies, Inc)

Scale: HPLC purification

Store: -20^0C in dark (especially for Cy3/Cy5-labeled primer!)