

## Preparation of bisulfite padlock probes from Agilent's oligos.

(NongLuk Plongthongkum, last modified: 4/23/2012)

### Materials

Reagents and equipments	Sources	Catalog number
IQ SYBR Green Supermix (2X)	BIO-RAD	170-8880
KAPA SYBR Fast Universal qPCR Master Mix (2X)	KAPA Biosystems	KK4602
GlycoBlue (15mg/mL)	Ambion	AM9516
3M NaOAC pH 5.2 or 5.5		
100% EtOH		
Qiaquick PCR Purification Kit	QIAGEN	28106
Lambda Exonuclease	NEB	M0262L
USER	NEB	M5505L
DpnII	NEB	R0543M
Nanosep	PALL	ODM02C34
1.5 mL Eppendorf LoBind tube	Eppendorf	
Needle 22G	BD	
0.5 mL tube		
6%TBE-Urea gel 1.0mm, 12 well	Invitrogen	EC68652
6%TBE_Urea gel, 1.5mm, 2D well	Invitrogen	EC6869
SYBR Gold (10,000x concentrate)	Invitrogen	S11494
2x TBE-Urea sample buffer	Invitrogen	LC6876
10 bp DNA ladder	Invitrogen	10821-015
Low DNA mass ladder	Invitrogen	10068-013

### 1. Expansion PCR

#### Primer sequences

Please see Supplementary text and figures

<http://www.nature.com/nmeth/journal/v9/n3/extref/nmeth.1871-S1.pdf>

Components	Volume (µl)	Final Conc.
20nM Agilent Oligos	2.00	0.2nM
100uM AP1V6	0.80	400nM
100uM AP2V6	0.80	400nM
2x IQ SYBR Green Supermix	100.00	1X
H <sub>2</sub> O	96.40	
Total	200.00	

### Real-time PCR profile

95°C 2min -> (95°C 30sec -> 54°C 45sec-> 72°C 45sec) x15-16 -> 72°C 3min -> 15°C hold

Note: The number of cycles should be monitored with a real-time thermalcycler to prevent over-amplification. The reactions should be terminated right before the amplification curves plateau.

- Purify amplified amplicons with Qiaquick column (2 columns) and elute with 50 µl EB buffer each
- Measure DNA concentration with Nanodrop and dilute the 1<sup>st</sup> round amplicons to 10nM with H<sub>2</sub>O

## 2. Production PCR

Components	Volume (µl)	X reaction mix
10nM 1 <sup>st</sup> round amplicons	0.20	
100uM pAP1V61U	0.40	
100uM AP2V6	0.40	
2x KAPA SYBG MM	50.00	
H <sub>2</sub> O	49.00	
Total	100.00	Aliquot 100µl reaction mix to each tube

## Real-time PCR profile

95°C 30sec -> (95°C 3sec -> 55°C 30sec -> 60°C 20sec) x15-16 -> 60°C 2min → 15°C hold

## 3. EtOH precipitation

(2.5 volume of 100% EtOH, 1/300 volume of Glycoblue, 0.1 volume of 3M NaOAc pH 5.2 or 5.5)

- Transfer amplified amplicon into 15mL tube (maximum volume 2mL)
- Add glycoblue, 3M NaOAc, and 100% EtOH
- Mix well and leave at -80°C for at least 30 min
- Precipitate by centrifugation at 3,000rpm for 30min, at 4°C
  - Note: the speed of centrifugation could be varied depends on the centrifuge model
- Discard spnt. and add 800ul of cold 75% EtOH to each tube.
- Use 1mL clean pipette tip with the pre-cut tip to transfer DNA pellet to 1.5 mL tube.
- Spin the tube at 14,000 rpm for 5 min.
- Discard the spnt. and air-dry the pellet for 3-5 min in the hood.
- Resuspend DNA with 150ul ddH<sub>2</sub>O (may incubate the DNA at 37°C for 15 min) or just mix well by pipetting up and down.

## 4. Re-purification of amplified-amplicon with Qiaquick column

- Add 5x or 6x volume of PB buffer to each tube

- Transfer 700  $\mu$ l of the mixture to each Qiaquick column
- Spin at 14,000 rpm for 1 min. Discard the spnt.
- Add 750  $\mu$ l of PE buffer to each tube.
- Spin at 14,000 rpm for 1 min. Discard the spnt.
- Spin the empty column at 14,000 rpm for 1 min.
- Transfer the column to 1.5 mL tube.
- Add 40  $\mu$ l of EB buffer to each tube (might warm EB buffer at 55°C to enhance the yields).
- Elute the DNA by spinning at 14,000 rpm for 1 min.
- Pool the purified amplicons into the same tube.
- Measure DNA concentration by Nanadrop and calculate the total yields.

### 5. Lambda exonuclease digestion to generate ssDNA

- Incubate ~20 $\mu$ g of purified amplicon with 10ul of lambda exonuclease in 1X lambda exonuclease reaction buffer in total volume 150ul and incubate at 37°C for 1h.
- Purify the single-strand amplicons with Qiaquick columns and elute with 50 $\mu$ l EB buffer.
- Measure concentration with Nanodrop.

### 6. Removal of amplification adapters

- Incubate ~5-8 $\mu$ g of single-strand amplicons with 5ul USER in total volume 80 $\mu$ l at 37°C for 1h.
- Anneal the USER-digested DNA to 5.88uM RE-DpnII-V6 guide oligos in 1X DpnII buffer in total volume 100 $\mu$ l. Incubate the reaction on thermocycler with the following thermalcycling program.

#### Program

94°C 2min -> cool down to 20°C -> 37°C 3min

- Add 5 $\mu$ l of DpnII and incubate at 37°C for 2h.
- Add 100 $\mu$ l of reaction mixture containing 5 $\mu$ l of USER and 1X DpnII buffer and further incubate at 37°C for 2h.
- Heat inactivate enzyme at 75°C for 20min.
- Purify USER/DpnII-digested DNAs with Qiaquick column.

### 7. Probe purification by PAGE-size selection

2 $\mu$ g of the USER/ DpnII-digested DNAs are purified in Invitrogen 2D TBE-urea gel

#### Probe-loading dye mixture

Components	Volume ( $\mu$ l)
USER/DpnII-digested DNA probe (2 $\mu$ g)	A
2x TBE-urea loading dye	50.00
H <sub>2</sub> O	50 – A
Total volume	100.00

**DNA ladder-loading dye mix**

Components	Volume ( $\mu$ l)
10-bp DNA ladder (1 $\mu$ g/ $\mu$ l)	1.00
2x TBE-urea sample buffer	5.00
H <sub>2</sub> O	4.00
Total volume	10.00

- Mix 2 $\mu$ g of USER/DpnII-digested DNA with 2x TBE-urea sample buffer in 1:1 ratio in the final volume  $\sim$ 100 $\mu$ l in PCR tube.
- For DNA ladder, mix 1  $\mu$ g of 10-bp DNA ladder in 1x TBE-Urea sample buffer in the final volume 10  $\mu$ l.
- Pre-run TBE-urea (TBU) gel at 200 volts for 10-15 min. At the same time, heat DNA and DNA ladder-loading dye mixture at 75°C for 8-10 min.
- Quick cool on ice at least 1 min.
- Flush the well with 1 mL pipette to remove residual urea.
- Quickly load the probe mixture along with 10 bp DNA ladder.
- Run at 200 volts for 30 min in 0.5X TBE buffer.
- Remove the gel from the cassette and stain in  $\sim$  25-50 mL 0.5XTBE buffer with 1X SYBR Gold (add 2.5-5  $\mu$ l SYBR Gold to 25-50 mL 0.5X TBE buffer)
- Stain for 5 min on the shaker.
- Place stained gel on a clean Saran wrap on the UV box with the long wavelength.
- Locate the position approx. 102 bp according to 10-bp DNA ladder. Use scalpel to cut the gel as close as to the band and minimize exposing the gel to UV light.
- Move the gel slide to the a clean area and chop the gel piece to small pieces with scalpel and transfer to prepared tube (one gel goes to two tubes)
- Centrifuge the tube at 15,000 rpm, for 3 min at RT.
- Transfer the gel remaining in 0.5 mL tube to 1.5 mL tube below with pipette tip.
- Add 400 – 500  $\mu$ l of 1X TAE buffer.
- Shake vigorously on vortex for 45-60 min at 37°C.
- Centrifuge at 15,000 rpm for 3 min at RT.
- Transfer the clear spnt. to Nanosep column and centrifuge at 15,000 rpm for 3 min.
- Transfer the lower layer to Nanosep again, but try to avoid the large chunk of gel. Centrifuge with the same speed and time.
- Transfer spnt to fresh 1.5 mL tube (volume  $\sim$  400 $\mu$ l/ tube)
- Precipitate with 2.5 volume of 100% EtOH, 1/300 volume of Glycoblue, 0.1 volume of 3M NaOAc pH 5.2 as previously described.
- Mix well and place the tube at -80°C for at least 30 min.
- Centrifuge 10,000 rpm for 20 min at 4°C.
- Discard spnt. and wash with 600  $\mu$ l 75% cold EtOH as previously described.
- Dry the DNA pellet and dissolve with  $\sim$ 10-15  $\mu$ l H<sub>2</sub>O/tube.
- Pool the dissolved probe to the same tube and mix well.
- Estimate DNA concentration using 6% TBE-urea gel (1mm, 12 well)

**Tubes preparation for PAGE-size selection**

- Place 0.5 mL tube into 1.5 mL DNA LoBind tube.
- Punch 0.5 mL tube with 22G needle
- Treat the tube under UV light for 15 min if possible