

Preparation of bisulfite padlock probes from LC Sciences' oligomix.

(NongLuk Plongthongkum, last modified: 4/21/2012)

Materials

Reagents and equipments	Sources	Catalog number
KAPA SYBR Fast 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
Nt.AlwI	NEB	R0627S
Nb.BsrDI	NEB	R0648S
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 (ul)	Final Conc.
20nM LC Sciences Oligoes	10.00	1nM
100uM eMIP_CA_F	0.80	400nM
100uM eMIP_CA_R	0.80	400nM
2x KAPA SYBG MM	100.00	1X
H ₂ O	88.40	
Total	200.00	

Real-time PCR profile

95°C 30sec -> (95°C 5sec -> 52°C 60sec-> 72°C 30sec) x5 -> (95°C 5sec -> 60°C 30sec-> 72°C 30sec) x10-12 -> 72C 2min -> 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 ul EB buffer each
- Measure DNA concentration with Nanodrop and dilute the 1st round amplicons to 10nM with H₂O

2. Production PCR

Components	Volume (ul)	X reaction mix
10nM 1 st round amplicons	0.20	
100uM eMIP_CA_F	0.40	
100uM eMIP_CA_R	0.40	
2x KAPA SYBG MM	50.00	
H ₂ O	49.00	
Total	100.00	Aliquot 100ul reaction mix to each tube

Real-time PCR profile

95°C 30sec -> (95°C 5sec -> 60°C 30sec -> 72°C 20sec) x13-15 -> 72°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 -80C 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 ddH₂O (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 ul of the mixture to each Qiaquick column
- Spin at 14,000 rpm for 1 min. Discard the spnt.
- Add 750 ul 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 ul of EB buffer to each tube (might warm EB buffer at 55C 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. Nicking endonuclease digestion

- Incubate 2-4ug of amplified amplicon with 10ul of Nt.AlwI (10U/ul) in total volume 200ul at 37°C for 1hr.
- Heat inactivate enzyme at 80°C for 20 min.
- Add 10ul Nb.BrsDI (10U/ul) at 65°C for 1hr.
- Purify of the nicked DNA with Qiaquick columns and elute with 50ul EB buffer.
- Measure concentration with Nanodrop.

6. Probe purification by PAGE-size selection

2ug of nicked DNA is purified in Invitrogen 2D TBE-urea gel

Probe-loading dye mixture

Components	Volume (ul)
Nicked DNA probe (2 ug)	A
2x TBE-urea loading dye	50.00
H ₂ O	50 – A
Total volume	100.00

DNA ladder-loading dye mix

Components	Volume (ul)
10-bp DNA ladder (1 ug/ul)	1.00
2x TBE-urea sample buffer	5.00
H ₂ O	4.00
Total volume	10.00

- Mix 2ug of nicked DNA with 2x TBE-urea sample buffer in 1:1 ratio in the final volume ~100ul in PCR tube.
- For DNA ladder, mix 1 ug of 10-bp DNA ladder in 1x TBE–Urea sample buffer in the final volume 10ul.
- 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 a long 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 ~ 25-50 mL 0.5XTBE buffer with 1X SYBR Gold (add 2.5-5 ul 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. 70 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 ul 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 ~ 400ul/ 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 4C.
- Discard spnt. and wash with 600 ul 75% cold EtOH as previously described.
- Dry the DNA pellet and dissolve with ~10-15 ul H₂O/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