

Oligopaints Synthesis Protocol

Updated 7/31/13

Part 1 – PCR amplification

We recommend setting up 10 ml of PCR master mix and aliquoting it into a 96-well plate, 100 μ l per well. This protocol is scaled for a starting amount of 10 ml; simply scale the volumes in the later sections accordingly if you start with a different volume of PCR.

In our hands, this typically results in a final yield of 1000 – 3000 pmol of ssDNA FISH probe. This should be sufficient for 50-150 20 pmol hybridizations if using fixed tissue culture cells or metaphase spreads, or 10-30 100 pmol hybridizations on whole-mount tissue sections. Yield will scale linearly with the volume of the initial PCR; thus, if you only need ~10 20 pmol hybridizations 2 ml of PCR should be plenty.

Reagents:

200 μ M unlabeled “R” primer stock
200 μ M labeled “F” primer stock
10X PCR buffer (KAPA Buffer A or equiv.)
10 mM dNTP mix
Molecular biology grade ddH₂O
100 pg/ μ l complex DNA library
Taq DNA polymerase (e.g. KAPA Taq)

PCR Master Mix:

Per 100 μ l of reaction

10 μ l 10x buffer
2 μ l 10 mM dNTP mix
0.5 μ l 200 μ M “R” unlabeled primer
0.5 μ l 200 μ M “F” labeled primer
1 μ l 20 pg/ μ l complex DNA library
1 μ l KAPA Taq
85 μ l ddH₂O

“Touch-up” PCR steps (ONLY IF STARTING WITH 60mer OLIGOS):

95°C 5:00

95°C 0:30

50°C 0:30

72°C 0:15

Repeat 2x (3 cycles total with annealing T of 50°C)

Move to the below program

PCR Program for 74mers

95°C 0:30

60°C 0:30

72°C 0:15

Repeat 39x (40 cycles total with annealing T of 60°C)

72°C 5:00

End

Part 2 – DNA precipitation of PCR products

Reagents:

Molecular biology grade glycogen, 20 mg/ml (Thermo #R0561)

4 M ammonium acetate (Sigma A1542)

Ice-cold 100% ethanol

Ice-cold 70% (v/v) ethanol in ddH₂O

1. Pool the cycled PCR reactions and collect in a 50 ml conical tube

We find the quickest way to do this is to use a multichannel pipette and a reagent reservoir in a PCR or tissue culture hood.

2. Add 38 µl of glycogen, 1 ml of 4M ammonium acetate, and 25 ml of ice-cold 100% ethanol. Vortex vigorously
3. Transfer to 2 ml eppendorf tubes, 2 ml each (~18 tubes)
4. Incubate at -80°C for 35' or at -20°C for at least 2 hours
5. Spin at max speed for 1 hour at 4°C
6. Aspirate off the supernatant, taking care not to disturb the pellet
7. Add 1350 µl of ice-cold 70% ethanol to each tube
8. Spin at max speed for 30 minutes at 4°C
9. Aspirate off the supernatant, taking care not to disturb the pellet
10. Air dry the pellets for 15' on a 42°C heat block
11. Add 40 µl of ddH₂O to each tube
12. Incubate at 37°C for 30-60 minutes

13. Proceed to the digestion step directly, else store at 4°C

Part 3 – Nicking Endonuclease Digestion and Concentration

Reagents:

NEB buffer 2

Nb.BsrDI (NEB R0648)

1. Pool the precipitated PCR products into one eppendorf tube (~720 µl)
2. Add 10 µl ddH₂O, 90 µl buffer 2, and 80 µl Nb.BsrDI
3. Vortex the digestion mix and split into PCR strip-tubes, 25 µl per tube (~36 total)
4. Run the following program in a thermocycler: 65°C for 4 hours -> 80°C for 20 minutes -> 4°C

The digestion products can be left at 4°C or concentrated via precipitation immediately

5. Pool the digestion reactions (~900 µl), and split 2 x 450 µl into 2 ml eppendorf tubes
6. To each, add 2 µl of glycogen, 50 µl of 4M ammonium acetate, and 1350 µl of ice-cold 100% ethanol. Vortex vigorously
7. Incubate for 35' at -80°C or at -20°C for at least 2 hours
8. Spin at max speed for 1 hour at 4°C
9. Aspirate off the supernatant, taking care not to disturb the pellet
10. Add 1350 µl of ice-cold 70% ethanol to each tube
11. Spin at max speed for 30 minutes at 4°C
12. Aspirate off the supernatant, taking care not to disturb the pellet
13. Air dry the pellets for 15' on a 42°C heat block
14. Add 60 µl of ddH₂O to each tube

15. Incubate at 37°C for 30-60 minutes
16. Proceed to the electrophoresis step directly, else store at 4°C

Part 4 – Electrophoresis, Gel Extraction, and Recovery

Reagents:

2X TBE-Urea sample buffer (Bio-Rad 161-0768)
Low Range DNA ladder (Thermo #SM1211)
Ultra Low Range DNA ladder (Thermo #SM1203)
15% TBE-Urea polyacrylamide gel (Bio-Rad 345-0092)
0.4 M ammonium acetate

We have had success with the Bio-Rad Criterion cell and gels, but any equivalent set-up should work

1. Microwave 1L of 1X TBE buffer for 3.5 minutes on 'high.'
2. *The TBE should be hot but not boiling (~60-65 °C). We microwave in a glass 1 L bottle, which will be too hot to touch with bare skin but can be handled carefully with a latex or nitrile glove. If the buffer is too hot, the plastic gel casing will crack.*
3. Add an empty gel to the gel box and fill with heated buffer. Run the gel (pre-loading) at a constant 20 W while the samples are being prepared (~30 mins)

It is essential that the gel is sufficiently hot to prevent renaturation from occurring (55-60 °C). Renaturation will result in the appearance of apparent high-molecular weight smears.
4. Pool the precipitated digestion products (~120 µl) and add an equivalent volume of 2X TBE-Urea sample buffer (120 µl) and vortex
5. Split into 40 µl aliquots in PCR strip tubes
6. Add 10 µl of each DNA ladder to separate PCR strip tubes. To each, add 10 µl of 2X TBE-Urea sample buffer and mix
7. Denature the digest products and ladders at 95°C for 5 minutes in a thermocycler, then transfer directly to ice
8. Just before loading, blast the urea out of the sample wells with a P1000 or syringe
9. Pipette the samples and ladders into the gel wells

10. Run the gel for ~15-30 minutes at a constant 20 W

We typically run the gel such that the orange G marker is at the very bottom of the gel (OK to run it off). In our set up, the labeled 53 bp band co-migrates with the xylene cyanol FF band.

11. Remove the gel from its plastic casing and stain with ethidium bromide for 5 minutes

12. Remove the ethidium bromide stain and de-stain with ddH₂O for 5 minutes

13. Visualize the gel on a UV box. Cut out the 53 bp band using a razor blade

14. Add each gel slice to a 2 ml eppendorf containing 600 µl of 0.4 M ammonium acetate

15. Incubate overnight at 55°C with shaking

The more shaking the better – a heated vortexer (e.g. Eppendorf Thermomixer) is best, but a shaking incubator such as those used for bacteria and yeast culture is also sufficient

16. The next day, draw up the fluid from each tube using a pipette and transfer to a fresh 2 ml eppendorf

17. Add 13.5 µl of glycogen and 1350 µl of ice-cold 100% ethanol to each tube and vortex vigorously

18. Incubate for 35' at -80°C or at -20°C for at least 2 hours

19. Spin at max speed for 1 hour at 4°C

20. Aspirate off the supernatant, taking care not to disturb the pellet

21. Add 1350 µl of ice-cold 70% ethanol to each tube

22. Spin at max speed for 30 minutes at 4°C

23. Aspirate off the supernatant, taking care not to disturb the pellet

24. Air dry the pellets for 15' on a 42°C heat block

25. Add 10 µl of ddH₂O to each tube

26. Incubate at 37°C for 30-120 minutes

27. Quantify probe yield by spectrophotometry using a fluorometer or Nanodrop (microarray setting) on the fluorophore (i.e. read out pmoles/ μ l of fluor while noting ssDNA concentration and use the fluor reading to determine how much probe to add for FISH). FISH tends to work well with 10-30 pmoles/slide.