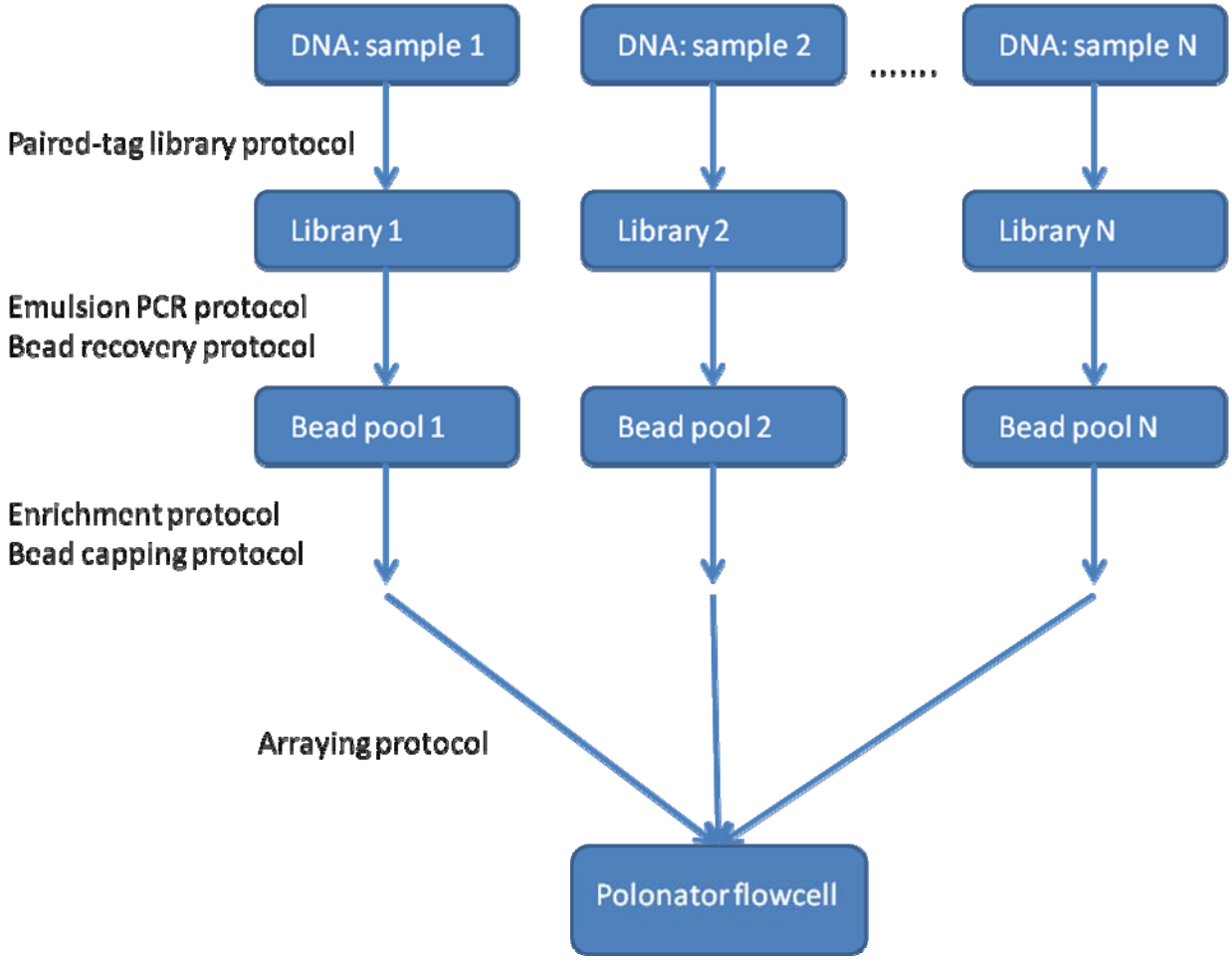
Polony Sequencing Protocols
Church Lab
12-12-2007

This document provides the following protocols for Polony sequencing :

1. Paired-tag library construction
2. Emulsion PCR / recovery
3. (optional) bead enrichment
4. Bead capping
5. Arraying
6. Aminosilanation of coverglass

The basic sequencing workflow is as follows:



CONSTRUCTION OF A SHOTGUN PAIRED-TAG GENOMIC LIBRARY

Polony genomic resequencing is generally performed on a shotgun paired-tag library (Shendure et al., 2005). Each library molecule is 135 bp in length, and has two 17- to 18-bp paired genomic tags separated and flanked by common sequences.
The key step in this protocol is the circularization of randomly sheared and size-selected genomic DNA around a synthetic insert-linker. This links sequences that are separated on the genome by a defined distance distribution.

Materials

Genomic DNA
Buffer EB (Qiagen)
End-It DNA end repair kit (Epicentre), including:
- 10× buffer
- 10× ATP
- 10× dNTP mix
- Enzyme mix

10× PCR buffer without $MgCl_2$ (Invitrogen)
50 mM $MgCl_2$
100 mM dATP
5 U/μl *Taq* DNA polymerase
20 mg/ml glycogen
Oligonucleotides:
- 100 μM T30-T: 5′-phosphorylated-GTCGGAGGCCAAGGCGGCCGTACGT CCAACT-3′ (purified by HPLC)
- 100 μM T30-B: 5′-phosphorylated-GTTGGACGTACGGCCGCCTTGGCC TCCGACT -3′ (purified by HPLC)
- 1 mM N6 oligonucleotides: 5′-NNNN*N*N-3′ (*signifies phosphorothioate linkage; IDT)
- 100 μM FDV-B: 5′-ATCACCGACTGCCCATAGAGAGGAAAGCGGAGGC GTAGTGGTT-3′ (purified by HPLC)
- 100 μM FDV-T: 5′-AACCACTACGCCTCCGCTTTCCTCTCTATGGGCAGT CGGTGAT-3′ (purified by HPLC)
- 100 μM RDV-B: 5′-AGAGAATGAGGAACCCGGGGCAGTT-3′ (purified by HPLC)
- 100 μM RDV-T: 5′-AACTGCCCCGGGTTCCTCATTCTCT-3′ (purified by HPLC)

Quick ligation kit (NEB), including:
- 2× Quick ligation buffer
- Quick T4 DNA ligase

20 U/μl Exonuclease I (NEB)
100 U/μl Exonuclease III (NEB)
TE buffer, pH 8.0 (*APPENDIX 2*)
25 mM dNTP mix (25 mM each nucleotide)
10× RepliPhi phi29 reaction buffer (Epicentre Technologies)
100 U/μl RepliPhi phi29 polymerase (Epicentre)
10× NEBuffer 4 (NEB)
1.6 mM *S*-adenosylmethionine (SAM; diluted from 32 mM stock in 1× NEBuffer 4; NEB)
2 U/μl *Mme*I (NEB)
2000 U/μl T4 DNA ligase and 10× buffer (NEB)
40% (w/v) polyethylene glycol 8000 (PEG) in H_2O
10× NEBuffer 2 (NEB)
10 U/μl *E. coli* DNA polymerase I (NEB)
5 U/μl Platinum *Taq* DNA polymerase (Invitrogen)

Qiaquick columns (Qiaquick PCR cleanup kit; Qiagen)
NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies)
Microcon-30 column (Millipore)
Thermal cycler

Additional reagents and equipment for DNA quantitation (*APPENDIX 3D*), phenol/chloroform extraction and ethanol precipitation (*UNIT 2.1A*), and agarose and polyacryamide gel electrophoresis (*UNITS 2.5A & 2.7*)

Prepare genomic DNA for circularization

1. Shear 15 μg genomic DNA to desired size distribution.

   The Hydroshear (http://www.genomicsolutions.com) is the recommended instrument for this purpose since, when the manufacturer's instructions are followed, it produces a population of fragments having a relatively tight size distribution and ends that can be efficiently blunted. The authors typically shear DNA at a concentration of ~60 ng/μl.

2. Purify DNA on Qiaquick columns (≤10 μg per column) as per manufacturer's instructions.

   Use as few columns as possible.

3. To repair the DNA ends (i.e., blunt them following shearing), combine the DNA with the components of the End-It DNA end repair kit as follows:

   10 μg column-purified, sheared genomic DNA in buffer EB
   25 μl 10× buffer
   25 μl 10× ATP
   25 μl 10× dNTP mix
   5 μl enzyme mix
   H_2O to a final volume of 250 μl.

   Incubate 1 hr at room temperature.

4. Purify DNA on a Qiaquick column per manufacturer's instructions, except elute with 90 μl buffer EB.

5. Quantitate DNA using a NanoDrop ND-1000 spectrophotometer (see *APPENDIX 3D*).

   Concentration should be ~100 ng/μl.

6. Incubate 15 min at 70°C to eliminate any residual enzyme activity.

7. Add an adenosine tail by combining the following components:

   2.2 μg end-repaired DNA
   10 μl 10× PCR buffer without $MgCl_2$
   6 μl 50 mM $MgCl_2$
   0.5 μl 100 mM dATP
   0.5 μl 5 U/μl *Taq* DNA polymerase
   H_2O to 100 μl.

   Incubate 30 min at 70°C then cool to 4°C. Transfer to ice.

   In the A-tailing reaction, Taq polymerase adds a single A to the 3′ ends of the blunted DNA in a template-independent fashion.

8. Extract DNA from the reaction mixture with phenol/chloroform and precipitate in ethanol using 20 μg glycogen as carrier (*UNIT 2.1A*). Resuspend pellet in 40 μl buffer EB.

   Glycogen should be used as a carrier in all ethanol precipitation steps in this protocol.

9. Separate the sheared, A-tailed DNA on a 6% polyacrylamide gel with TBE buffer (*UNIT 2.7*). Stain with ethidium bromide.

   For Invitrogen precast gels, mix 20 μl DNA with 5 μl of 5× loading buffer (Novex) and load across two 0.5-cm lanes.

10. Cut out the desired bands and extract DNA using the crush-and-soak method (*UNIT 2.7*). Precipitate in ethanol using glycogen as a carrier, and resuspend in 10 μl buffer EB.

    For a paired-tag library with an average intertag distance of 1 kb, excise and pool gel bands centered at 1 kb with a total width of no more than 500 bp. Exposure of the DNA to UV light during visualization should be minimized.

11. Quantitate the size-selected DNA by separating on an agarose or polyacrylamide gel (*UNIT 2.5A or 2.7*) and comparing the brightness of the smear to that of a known amount of a molecular weight standard.

    The concentration should be ~20 to 40 ng/μl.

Circularize genomic material around synthetic oligonucleotides

12. Anneal oligonucleotides T30-T and T30-B in a thermal cycler by combining equal amounts and heating to 95°C for 10 min and then slowly cooling to room temperature over the course of 1 hr.

    The final concentration of the T30 insert-linker should be 100 μM. T30 has T 3′ overhangs designed to be complementary to the A 3′ overhangs of the genomic DNA.

13. Ligate genomic DNA fragments in the presence of annealed T30 using the Quick ligation kit as follows:

    170 ng size-selected DNA (0.25 pmol at ~1 kb)
    0.8 μl 1 μM annealed T30 (0.8 pmol)
    40 μl 2× Quick ligation buffer
    4.0 μl Quick T4 DNA ligase
    H_2O to a final volume of 80 μl.

    Incubate reaction 10 min at room temperature then heat-inactivate 10 min at 65°C. Use the heat-inactivated reaction mixture directly in the next step without further purification.

    The ligation should be performed under conditions favoring formation of monomeric recombinant circles (i.e., genomic DNA–T30). To this end, T30 should be present at a three-fold molar excess to genomic fragments.

14. Eliminate all noncircularized material by exonucleolysis as follows:

    1.0 μl 20 U/μl Exonuclease I
    0.1 μl 100 U/μl Exonuclease III
    80 μl heat-inactivated reaction mix
    TE buffer, pH 8.0, to a final volume of 90 μl.

    Incubate 45 min at 37°C, and then heat-inactivate 20 min at 80°C. Use this reaction mixture directly in the next step without further purification.

Amplify circular DNA

15. Prepare the master mix for hyper-branched rolling-circle amplification of the circular DNA:

    12 μl 25 mM dNTP mix
    30 μl 10× RepliPhi phi29 reaction buffer
    15 μl 1 mM N6 oligonucleotides
    213 μl H_2O
    30 μl circularized DNA.

    Split into 6 tubes of 50 μl each.

16. Denature material by heating to 95°C for 5 min, then anneal by rapidly cooling to 4°C. Add 2.5 μl phi29 polymerase to each tube, keeping on ice. Incubate overnight at 30°C.

17. Pool amplified circular DNA into a single tube. Purify DNA using a Microcon-30 column according to the manufacturer's instructions, washing with 1 ml TE buffer, pH 8.0. Wash the membrane several times to maximize recovery.

18. Resuspend the DNA using 750 μl buffer EB, preheated to 50°C. Quantitate the DNA using a NanoDrop ND-1000 spectrophotometer.

    Concentration should be ~230 ng/μl.

Release paired tags

19. Digest the amplified DNA with *Mme*I to release the 70-bp T30-linked paired tags:

    40 μg amplified circular DNA
    100 μl 10× NEBuffer 4
    1.6 μl of 1.6 mM SAM
    60 μl *Mme*I (2 U/μl)
    H_2O to 1000 μl.

    Split into eight tubes of 125 μl each on ice, and then incubate at 37°C for 30 min.

    MmeI cuts 18 to 19 bp from its recognition site, which is 1 bp from the start of the genomic fragment. The released fragments contain the common T30 sequence flanked by two 17- to 18-bp tags of genomic DNA.

20. Immediately extract the DNA with phenol/chloroform and precipitate with ethanol using glycogen as a carrier. Resuspend pellet in a total volume of 80 μl TE buffer, pH 8.0.

21. Purify the 70-bp paired-insert library as in steps 9 and 10, resuspending precipitated DNA in 20 μl TE buffer, pH 8.0.

    The sample should be split across four 0.5-cm lanes of a precast 6% polyacrylamide gel in TBE buffer.

22. Quantitate the gel-purified DNA by electrophoresis (step 11).

    DNA concentration should be ~12 ng/μl.

Add emulsion PCR adapters

23. To perform the blunting reaction (to remove 2-nt 3′ overhangs from the *Mme*I digestion), combine the DNA with components of the End-It DNA end repair kit as follows:

    8.5 μl paired-insert library (~100 ng DNA)
    1.25 μl 10× buffer
    1.25 μl 10× ATP
    1.25 μl 10× dNTP mix
    0.25 μl enzyme mix.

    Incubate at room temperature for 45 min.

24. Put on ice and add TE buffer, pH 8.0 to bring volume to 50 µl. Extract the DNA with phenol/chloroform and precipitate in ethanol using 20 µg glycogen as carrier. Resuspend DNA in 8 µl TE, pH 8.0.

25. Prepare emulsion PCR amplification primer sequences by annealing oligonucleotides FDV-T and FDV-B and oligonucleotides RDV-T and RDV-B as in step 12.

    The final concentration of each oligonucleotide should be 50 µM. FDV and RDV are not 5′-phosphorylated, so they will not ligate to one another.

26. Prepare the ligation reaction mixture by mixing the following components on ice:

    8.0 µl blunt-ended library DNA (~100 ng, 2 pmol)
    1.0 µl 50 µM FDV (50 pmol)
    1.0 µl 50 µM RDV (50 pmol)
    2.5 µl 10× T4 DNA ligase buffer (~0.5×)
    21.1 µl 40% PEG
    12.3 µl H_2O.

27. Mix on ice, then add 2.0 µl of 2000 U/µl T4 DNA ligase and incubate overnight at 16°C to ligate primer sequences to the library molecules.

    After ligation, several species will be present: FDV-insert-FDV, FDV-insert-RDV, and RDV-insert-RDV.

28. Increase volume to 100 µl with TE buffer, pH 8.0. Extract the DNA with phenol/chloroform, precipitate in ethanol using glycogen as a carrier, and resuspend in 10 µl buffer EB.

29. Purify the 135-bp FDV-RDV paired-insert library as in steps 9 and 10, resuspending the precipitated DNA in 10 µl TE buffer, pH 8.0.

30. Assemble on ice the reaction mixture used to remove nicks in double-stranded library molecules by nick translation:

    10.0 µl library DNA
    0.5 µl 25 mM dNTP mix
    2.5 µl 10× NEBuffer 2
    1.0 µl 10 U/µl *E. coli* DNA polymerase I
    11.0 µl H_2O.

    Incubate 30 min at 16°C.

31. Increase volume to 100 µl with TE buffer, pH 8.0. Extract the DNA with phenol/chloroform, precipitate in ethanol using glycogen as a carrier, and resuspend in 10 µl TE buffer, pH 8.0.

Amplify FDV/RDV-adapted library

32. Prepare the master mix for PCR amplification (for eight tubes of 50 µl each):

    50 µl 10× PCR buffer without $MgCl_2$
    4.0 µl 25 mM dNTP mix
    15 µl 50 mM $MgCl_2$
    426 µl H_2O
    1.0 µl 100 µM FDV-T
    1.0 µl 100 µM RDV-T
    2.0 µl 5 U/µl Platinum *Taq* DNA polymerase.

33. Split the master mix into eight tubes of 50 µl each, and add 0.5 µl FDV/RDV-adapted library material to each tube.

34. Carry out PCR using the following amplification cycles:

Initial step:	2 min	94°C	(denaturation)
12 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	90 sec	72°C	(extension).

The number of cycles is kept low to minimize PCR amplification bias. Due to suppression PCR effects (Siebert et al., 1995), FDV/FDV- and RDV/RDV-adapted molecules are not amplified.

35. Purify DNA using Qiaquick columns according to the manufacturer's instructions for PCR cleanup.

36. Purify the amplified 135-bp FDV-RDV paired-insert library as in steps 9 and 10, resuspending the precipitated DNA in 10 µl TE buffer, pH 8.0.

When excising the 135-bp library band, be careful to avoid contamination by marker DNA, if used.

37. Quantitate the final DNA library by electrophoresis (step 11).

The concentration should be ~2 ng/µl.

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Emulsion PCR Protocol
Church Lab
11-26-07

This protocol is for emulsification and thermal cycling of one 96-well microwell 'plate' of emulsion, 50ul per well. Each plate is sufficient for 1 unenriched 1.5cm array; for an enriched array, 2.5 plates are needed. Titrate library DNA to yield 40% amplified beads.

Aqueous phase:

- 96 ul 10x PCR buffer without MgCl2 (Invitrogen)
- 360 ul 50 mM MgCl2
- 135 ul 25 mM(each) dNTP mix
- 6.0 ul 2 mM PR1R-S
- 60 ul MyOne beads in TE, pre-loaded w/ forward primer
- 54 ul hot-start Taq (5U/ul, Denville)
- 1.0 ul template DNA at appropriate concentration
- 248 ul dH2O

Oil phase:

- 4.01 ml Tegosoft DEC (Degussa)
- 1.10 ml light mineral oil (Sigma #5904-500ml)
- 385 ul ABIL WE09 (Degussa)

For aqueous phase, add all components except Taq, mix well, then add Taq and mix well again. For oil phase, add all components to a 50 ml polypropylene falcon tube and vortex well to mix. Add aqueous phase, cap tube, and vortex well for 5 min. Distribute mix into 96-well polypropylene plate @ approx 50ul per well.

Thermal cycle as follows:
1. 10' 94C
2. 15 sec 94C
3. 30 sec 57C
4. 75 sec 70C
5. Goto 2, 119 more times
6. 2 min 70C
7. Forever 4C

Emulsion breaking protocol
Church Lab
11-26-2007

This protocol is performed on a 96-well microwell plate of emulsion following PCR, ~50ul of emulsion per well.

1. Using a multichannel pipette add 100 uL of isopropanol to each well.
2. Pipette up and down at least 10 times
3. Using the multichannel pipette pool the isopropanol/PCR mixes into a reservoir
4. Transfer the pooled mixture into a 15 mL polypropylene falcon tube
5. Vortex for 1 min
6. Spin for 30 sec at 4000 rpm in table-top centrifuge
7. Remove supernatant by decanting
8. Add 5 mL of isopropanol and resuspend the pellet via pipetting. Pipette for at least 1 min. Be sure no clumps are visible.
9. Add 5 mL of isopropanol
10. Vortex for 30 sec
11. Spin for 30 sec at 4000 rpm in table-top centrifuge
12. Remove supernatant making sure that you remove as much of the isopropanol as possible.
13. Resuspend the pellet in 6 mL of NXS. Pipette for ~ 1 min using pipetaid. The goal here is to make sure that the pellet is VERY well resuspended. There cannot be any yellowish/orangeish clumps (Even on the side of the tube). This is absolutely critical.
14. Vortex for 30 sec
15. Spin for 30 sec at 3000 rpm in table-top centrifuge
16. Remove supernatant using magnetic particle separator
17. Resuspend in 1 mL of NXS and pipette for ~1 minute and then transfer to a 1.5 mL siliconized eppendorf
18. Vortex for 30 sec
19. Spin for 30 sec at 1000 g.
20. Use magnet (Dynal MPC) to remove supernatant
21. Resuspend in 1 mL TE
22. Mix by pipetting for at least 30 sec
23. Spin for 30 sec at 1000 g
24. Use magnet to remove supernatant
25. Resuspend in 500 uL TE
26. Use magent to remove supernatant
27. Resuspend in 500 uL 0.1 M NaOH
28. Incubate on labquake at RT for 5 min
29. Wash 1x with 500 uL 0.1 M NaOH
30. Wash 2x with 500 uL TE
31. Resuspend in 30 uL of TE

*NXS: 10mM Tris pH 7.5, 1mM EDTA pH 8.0, 100mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) SDS

Bead Capping Protocol
Church Lab
01-09-07

The purpose of this protocol is to attach a 'capping' oligonucleotide to every 3' end on the bead – unextended forward ePCR primers and 3' template ends. The cap we use is an amino group, which blocks any further ligation and also allows for coupling directly to the glass coverslip through a dual-NHS moiety (BS3).

1. Perform 'bridging oligo' hybridization in 1.5mL silicon tube:
   - 180ul 6x SSPE with 0.01% Triton X-100
   - 3.6ul 1mM PR1F5-CAP-TMPL -- 5'-GTG AGC TTC GTC TGC CCC GGG TTC-3'
   - 3.6ul 1mM PR1R5-CAP2-TMPL – 5'-GTG AGC TTC GTC CAT AGA GAG GAA AGC G-3'

Resuspend (meaning remove the TE beads are suspended in) single-stranded, (optionally) enriched beads in this solution and incubate for 10' at 56C, pipet to mix, then 10' at RT on Labquake.

2. Wash 2x with 200ul TE

3. Prepare ligation mix:
   - 93ul dH2O
   - 4ul 1mM 5Phos-9N-3NH -- /5Phos/NNNNNNNNN/3AmM/
   - 100ul 2x Quick Ligase Buffer (NEB)
   - 4ul 1mM PR1F5-NHCAP -- /5Phos/CG AAG CTC AC/3AmM/
   - 4ul 1mM PR1F5A-NHCAP -- /5Phos/ACGA AGC TCA C/3AmM/
   - 4ul T4 DNA ligase (2KU/ul)

Remove all liquid from beads and resuspend in this solution. Incubate on Labquake for 1 hour at RT.

4. Place solution on magnet and wait for beads to pellet against back of tube. This will take time because the solution is very viscous.

5. Remove liquid and add 200ul TE. Vortex, spin to bring contents to bottom of tube, place on magnet (Dynal MPC), and remove all liquid.

6. Add 200ul NX, vortex, spin briefly, and remove all liquid on magnet.

7. Wash 2x with 200ul TE as in 6.

8. Transfer to new tube, wash 1x w/ 200ul TE, then resuspend in 30ul TE.

9. To array beads, wash 3x in 50 ul PBS, then resuspend in 35 ul PBS.
10. Prepare BS3 solution (2.86 mg BS3 -- Bis(Sulfosuccinimidyl) suberate – in 1 ml PBS).

11. Remove liquid from beads with MPC, quickly resuspend in 35 ul BS3 solution, and pipette into inlet port of correct chamber on flowcell. It should only take several seconds to resuspend the beads and transfer them to the flowcell chamber.

12. Leave flowcell upside-down on benchtop, un-disturbed, for 1 hour. During this time, the beads will settle to the glass and be covalently coupled to it by the BS3.

13. Flush each flowcell chamber with 100ul TE to remove un-attached beads.

*NX: 10mM Tris pH 7.5, 1mM EDTA pH 8.0, 100mM NaCl, 1% (v/v) Triton X-100

Aminosilane treatment of coverslips
Church Lab
12-10-2007

This protocol is used to wash and aminosilane-treat the rectangular coverslips used in polony sequencing. It is critical that the protocol be followed exactly as written to ensure 1) no fluorescent contamination is present on the glass, and 2) coverslip is uniformly amino-functionalized.

1. Load coverslips into rack (available from flowcell supplier)
2. Immerse coverslips in solution of 0.5% Triton X-100 in dH2O. Wash by manually agitating the rack for ~1 minute.
3. Pour all liquid off and wash thoroughly under running water to remove all detergent. Rinse with dH2O.
4. Pour off all liquid and dry by immersion in 100% EtOH. Remove from EtOH and allow all ethanol to evaporate (aided by use of a Dust-off compressed air canister if possible).
5. Prepare 2% aminopropyl triethoxysilane (Pierce) solution in dry acetone in a glass beaker. Pipette with glass – the solution will react with polystyrene.
6. Immerse rack of completely dry (and ethanol-free) coverslips in silane solution for 30 seconds. Use the handle to manually agitate the rack.
7. Remove and immediately immerse in fresh dry acetone rinse for 1 minute. Use the handle to manually agitate the rack.
8. Remove rack and blow-dry partially with compressed air (to remove the bulk of the acetone).
9. Completely air-dry and store under vacuum at RT.
10. When cleaning beakers containing acetone and aminopropyltriethoxysilane, be sure to rinse with fresh acetone to remove all traces of aminopropyltriethoxysilane. If present, it will form a white precipitate upon evaporation of acetone.

Bead Enrichment
Church Lab
12-10-2007

The purpose of this protocol is to enrich for amplified beads from a pool containing ~40% amplified beads (the rest of which are unamplified). The protocol is similar to that in the Redbook except for the volumes used and the ratio of capture beads to amplified ePCR beads. When performing this on a sample to be arrayed in a gel-less array, **at least 2.5 plates of beads amplified to 40% should be used**.

>Bind beads (enough to enrich 3 plates):

- 540ul SVP-30-5 spherotech 3um streptavidin polystyrene beads, washed 2x and resuspended in original volume in TE (beads are 0.67e8/ml, want ratio of 2-3 capture beads per amplified ePCR bead)
- 10.8ul 1mM PR1-BIOXL (unpurified, 5'-biotinTEG-CGTACCCCGCTTGGTCTTTCTCCCGTACCCCGCTTGGTCTTTCTCCCTGCCCCGGGTTCCTCATTCTCT-3')
  Incubate for 1 hour at RT. Wash 3x in B&W buffer and resuspend in 60ul B&W buffer (5 mM Tris HCl pH 7.5, 0.5 mM EDTA pH 8.0, 1.0 M NaCl). ***Use 20ul per plate per enrichment****.*

>Resuspend plate of ePCR beads in 20ul TE. To perform enrichment,

- Add 20ul capture beads from above and incubate at 56C for 10 min
- Prepare 300ul 60% v/v glycerol in dH2O. Solution must be prepared fresh.
- Add 40ul capture-ePCR bead mix to top of glycerol. Immediately centrifuge at >= 16,000rcf for 1 min at RT
- Pipette off all supernatant, being careful to not disturb the pellet. Transfer this supernatant (containing ePCR-capture bead complexes) to a new siliconized tube
- Add 1ml dH2O and vortex. Centrifuge for 1 min at >= 16,000 rcf
- Place on magnet, and remove most liquid (being careful to not aspirate any beads)
- Add 200ul 0.1M NaOH, and vortex
- Place on magnet and wait until solution turns pure white (all ePCR beads will now be against the back of the tube facing the magnet)
- Remove liquid and wash 2x w/ 200ul 0.1M NaOH, then transfer to a new tube and wash 2x w/ TE. Resuspend in 20ul TE. If enriching more than 1 plate, beads can be pooled into a single tube when transferred after final wash with 0.1M NaOH.

Polony Sequencing
Fluorescent Nonamer Sequences
12-10-2007

All nonamers are synthesized by IDT on the 1 umol scale, with HPLC purification. Specify N:25:25:25:25 for all degenerate bases. Average yield is ~30 nmol for Cy3, Cy5, and TxRed, ~100 nmol for FAM.

Minus 1 nonamers	5' Cy5-NNNNNNNNT
	5' Cy3-NNNNNNNNA
	5' TexasRed-NNNNNNNNC
	5' 6FAM-NNNNNNNNG
Minus 2 nonamers	5' Cy5-NNNNNNNTN
	5' Cy3-NNNNNNNAN
	5' TexasRed-NNNNNNNCN
	5' 6FAM-NNNNNNNGN
Minus 3 nonamers	5' Cy5-NNNNNNTNN
	5' Cy3-NNNNNNANN
	5' TexasRed-NNNNNNCNN
	5' 6FAM-NNNNNNGNN
Minus 4 nonamers	5' Cy5-NNNNNTNNN
	5' Cy3-NNNNNANNN
	5' TexasRed-NNNNNCNNN
	5' 6FAM-NNNNNGNNN
Minus 5 nonamers	5' Cy5-NNNNTNNNN
	5' Cy3-NNNNANNNN
	5' TexasRed-NNNNCNNNN
	5' 6FAM-NNNNGNNNN
Minus 6 nonamers	5' Cy5-NNNTNNNNN
	5' Cy3-NNNANNNNN
	5' TexasRed-NNNCNNNNN
	5' 6FAM-NNNGNNNNN
Plus 1 nonamers	5' Phos-TNNNNNNNN-Cy5
	5' Phos-ANNNNNNNN-Cy3
	5' Phos-CNNNNNNNN-TexasRed
	5' Phos-GNNNNNNNN-6FAM
Plus 2 nonamers	5' Phos-NTNNNNNNN-Cy5
	5' Phos-NANNNNNNN-Cy3
	5' Phos-NCNNNNNNN-TexasRed
	5' Phos-NGNNNNNNN-6FAM
Plus 3 nonamers	5' Phos-NNTNNNNNN-Cy5

	5' Phos-NNANNNNNN-Cy3
	5' Phos-NNCNNNNNN-TexasRed
	5' Phos-NNGNNNNNN-6FAM
Plus 4 nonamers	5' Phos-NNNTNNNNN-Cy5
	5' Phos-NNNANNNNN-Cy3
	5' Phos-NNNCNNNNN-TexasRed
	5' Phos-NNNGNNNNN-6FAM
Plus 5 nonamers	5' Phos-NNNNTNNNN-Cy5
	5' Phos-NNNNANNNN-Cy3
	5' Phos-NNNNCNNNN-TexasRed
	5' Phos-NNNNGNNNN-6FAM
Plus 6 nonamers	5' Phos-NNNNNTNNN-Cy5
	5' Phos-NNNNNANNN-Cy3
	5' Phos-NNNNNCNNN-TexasRed
	5' Phos-NNNNNGNNN-6FAM