Genomic Mismatch Scanning in Yeast

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Brown Lab protocol, modified by Maitreya Dunham and Matt Brauer of the Botstein lab for use with USB enzymes.

A. **Preparation of genomic DNA from yeast (Qiagen protocol)** 3-4 days from colony

Large amounts of high-quality genomic DNA are critical to the success of the procedure.

Yields 100-200 μg DNA.

B. Restriction digest of genomic DNA 5-6 hours

1. Retain 5-10 μL DNA for checking DNA quality and progress of digest.
2. Digest minimum of 20 μg each parent and 40 μg offspring DNA.
3. Each digest should be 0.2 mg/mL DNA, with 5 U/ug PstI, NEB buffer 3, BSA.
4. Incubate 37˚C 2hrs.
5. Add 0.5X volume PstI originally added.
6. Incubate 37˚C 2 hrs. May be stored indefinitely at –20˚C.
7. Check digest by running 1 μL pre- and 2 μL post-digest DNA on 0.8% gel.
8. Phenol/chloroform extract; chloroform extract, and NaOAc/EtOH precipitate digests. Dissolve in 100 μL TE. (Can store at 4˚C O/N.)

C. Methylation of parent (or ancestor) DNA 5 hours minimum

1. To 200 μg DNA add:
    i. 100 μL 10x dam methylase buffer;
    ii. 2.5 μL SAM;
    iii. H_2O to 900 μL;
    iv. 100 μL dam methylase.

   Mix well.
2. Incubate at 37˚C for 4 hr, spiking reaction with 2.5 μL SAM every hour.
3. Phenol/chloroform extract; chloroform extract, and NaOAC/EtOH precipitate digests. Dissolve in 100 μL TE. (Can store at 4˚C O/N.)
4. Quantify DNA concentration by spectrophotometer.
5. Perform MboI, DpnI and Sau3A digests on both methylated and unmethylated DNA to assess quality/extent of methylation. *Sau3A should cut all species; MboI will cut only unmethylated DNA; DpnI will cut only methylated DNA.*

D. Hybridization (FPERT) 2 days minimum

1. Mix 20 μg methylated parent 1 with 20 μg offspring and 20 μg methylated parent 2 with 20 μg offspring. Bring volume to 200 μL with water.
2. Add 14 μL 5 N NaOH to denature. Mix rapidly. Incubate at RT for 15', mixing every 5'.
3. Neutralize reaction with 29 μL 3M MOPS buffer. pH should be 8.5. Check with pH paper and adjust if not 8.5.
4. Split into 2 aliquots, in 1.5 mL screw-cap tubes.
5. To each tube add:
    i. 32 μL formamide;
    ii. 45 μL H_2O;
    iii. 200 μL 2xFPERT buffer.

   Mix well.
6. Add 150 μL phenol to each tube. If solution is clear, continue to add phenol 25 μL at a time until solution becomes cloudy.
7. Agitate at RT for 12-24 hrs (yeast) or 48 hrs (human).
8. Extract each tube with 150 μL chloroform. Keep top (aqueous) layer.
9. Extract with 400 μL chloroform. Keep top (aqueous) layer.
10. Split combined aqueous fractions into two equal volumes and add 1 mL EtOH to each.
11. Incubate –80˚C 30'; spin 4˚C max speed, 30'.
12. Carefully remove S/N. Wash pellet with 70% EtOH and air-dry.
    Note: Pellet may be a clear, viscous liquid. If so, dissolve pellet in 0.5 mL 70% EtOH. Spin max speed10'. Repeat 1x or until viscous pellet is fully dissolved after spin, and all that remains is "typical" salt/DNA precipitate.
13. Dissolve each pellet in 45 μL 1xTE. Pool each set of hybrids into 1 tube each.
14. Quantify [DNA] by A_{260}, 1:25 dilution. Spectra have a peak at 230 nm and a shift of the 260 peak upwards.

E. Selection of heterohybrids (DpnI/MboI/ExoIII digest)

1. To 20 μg each heterohybrid DNA add:
    i. 20 μL 10x NEB Buffer 3;
    ii. 1x TE to 195 μL;
    iii. 2 μL 20 U/μL DpnI;
    iv. 1.6 μL 25 U/μL MboI.

   Mix well.
2. Incubate at 37˚C for 1 hr.
3. Spin down condensation and keep on ice.
4. Prepare ExoIII mix (per reaction):
    i. 539.4 μL H_2O;
    ii. 60 μL 10x ExoIII Buffer;

iii. 0.6 μL 100 U/μL ExoIII.

5. Add 600 μL mix to digested heterohybrids. *Do this step as quickly as possible.*
6. Incubate 37˚C for 15'.
7. Stop the reaction by adding 8 μL 0.5 M EDTA, 80 μL 8M LiCl to each tube.
8. Add 30 μL SSAM, well mixed.
9. Incubate at RT for 10', mixing every 2'.
10. Spin down SSAM in picofuge, 1'.
11. Divide S/N between two 0.45 μ Millipore filters.
12. Spin in microfuge, 1000 rpm, for 3'.
13. Add 1 μL linear acrylamide to each tube and mix well.
14. Add 900 μL EtOH to each tube and mix well.
15. Incubate at –80˚C for 30'.
16. Spin at maximum RPM, 4˚C, 30'.
17. Wash with 70% EtOH and air-dry pellet.
18. Resuspend each pellet in 26 μL 1x TE.
19. Quantify DNA concentration by spectrophotometer.

F. Mismatch-specific nicking (MutHLS digest)

1. Bring 1 μg DNA to 25 μL with 1x TE. Add:
   i. 5 μL 10x ENH;
   ii. 3 μL PPD*;
   iii. 0.5 μL 100 mM ATP;
   iv. 10 ng MutH;
   v. 100 ng MutL;
   vi. 2.5 μg MutS;
   vii. H_2O to 50 μL total.

**Before using add 2.5 μL 0.1 M DTT and 25 μL 10 mg/mL BSA (not acetylated) to 225 μL PPD.*

Also note that the concentration of MutHLS are dependent on the activity of your enzymes. These concentrations are for use with USB enzymes and may not be completely optimal.

2. Incubate at RT for 20'. Stop the reaction by heating to 80˚C for 5' and placing on ice.

G. Depletion of nicked duplex (ExoIII digest)

1. For each reaction mix 5 μL 10x ExoIII buffer, 45 μL H_2O and 0.2 μL 100 U/μL ExoIII. Add to MutHLS-digested DNA.

2. Incubate at 37˚C for 10'.
3. Stop the reaction by adding 1 μL 0.5 M EDTA, 5 μL 8M LiCl to each tube.
4. Add 5 μL SSAM, well mixed.
5. Incubate at RT for 10', mixing every 2'.
6. Spin down SSAM in picofuge, 1'.
7. Transfer S/N to 0.45 u Millipore filter.
8. Spin in microfuge, 1000 rpm, for 3'.
9. Add 1 μL linear acrylamide and mix well.
10. Add 240 μL EtOH and mix well.
11. Incubate at –80˚C for 30'.
12. Spin at maximum RPM, 4˚C, 30'.
13. Wash with 70% EtOH and air-dry pellet.
14. Resuspend each pellet in 15 μL 1x TE. Check concentration.

The resulting DNA can be labeled and hybed by your preferred method. My methods are available under Protocols on the Dunham Lab website, *genomics.princeton.edu/dunham*. If you have trouble getting enough labeled material, you can amplify the DNA. This may increase the noise, though.

Reagents and supplies

General
Phenol, buffer-saturated, pH 7-8
Chloroform
EtOH, absolute and 70%
3M NaOAc
Linear acrylamide

B.
PstI

C.
dam methylase, SAM
DpnI
MboI

D.
5 N NaOH
3 M MOPS acid

2xFPERT buffer	100 mL
4 M Sodium thiocyanate	32.4 g
20 mM Tris-HCl pH 7.9	2 mL 1 M
0.2 mM EDTA	40 μL 0.5 M

filter sterilize

E.
DpnI
MboI
ExoIII
0.5 M EDTA
8 M LiCl
SSAM

F.
MutH
MutL
MutS
PPD (20 mM KPi pH 7.4, 50 mM KCl, 0.1 mM EDTA pH 8)
10xENH (500 mM Hepes pH 8, 200 mM KCl, 40 mM $MgCl_2$, 10 mM DTT)
100 mM ATP
0.1 M DTT
0.2 10 mg/mL BSA (not acetylated)

G.
ExoIII
0.5 M EDTA
8 M LiCl
SSAM