

Loss of Heterozygosity

This method was successful in our lab using prostate tissue and for our specific objectives. Investigators must be aware that they will need to tailor the following protocol for their own research objectives and tissue under study.

This method is used to detect genomic DNA deletions in tumor cells. For a more detailed discussion of applying this approach to microdissected samples, see [Allelic Loss Studies](#) in [Prostate MP at NCI](#).

1. Reagents

1. DNA sample (see [Processing of Microdissected Tissue - DNA-based Analysis](#))
2. Proteinase K (Sigma)
3. Proteinase K buffer (0.05 M tris-HCL, 0.001 M EDTA, 1% Tween 20, 0.1 mg/ml proteinase K, pH 8.0)
4. Ampli Taq Gold Buffer (Perkin Elmer)
5. dNTP mixture (Perkin Elmer)
6. Primers
7. DEPC-treated H₂O
8. Ampli Taq Gold Polymerase (Perkin Elmer)
9. α -³²P dCTP, 6000 Ci/mmol (NEN Dupont)
10. Formamide, 99% (Fluka)
11. Bromophenol blue-Xylene cyanole (Sigma), reconstituted as directed
12. Gel Mix-6 sequencing gel solution (Life Technologies)
13. Ammonium persulfate (Biorad)
14. 10X TBE buffer (0.89 M Tris Base, 0.89 M Boric Acid 0.02M Disodium EDTA) (Advanced Biotechnologies)
15. Acrylease (Stratagene)
16. Glass cleaner (e.g., Windex, Glass Plus)
17. 95% ethanol

2. Equipment

1. Thermal cycler (MJ Research)

2. Sequencing gel electrophoresis apparatus (Gibco BRL)
3. High voltage power supply
4. Gel dryer (Life Technologies)
5. Glass plates, 31.0 x 38.5 cm (Life Technologies)
6. 0.4 mm spacers (Life Technologies)
7. Casting boot (Life Technologies)
8. Shark tooth comb (Life Technologies)
9. Small clamps
10. Whatman blotting paper, 3 mm thickness
11. Kodak Biomax MR or AR film
12. Film cassette (Amersham Life Science)
13. Film processor

3. Time Requirements

1. Gel preparation: 1.5 - 2 hours. Polymerization requires 1 hour, but may stand overnight.
2. LOH reactions: 2 .5 hours (approximately 1 hour for set-up, 1.5 for PCR)
3. High-resolution denaturing polyacrylamide gel electrophoresis: 1-3 hours. Twenty minutes for set-up. Electrophoresis time varies according to product size.
4. Gel drying: 1 hour
5. Autoradiography: 1 hour-2 days

4. Methods

TIP: Investigators must be especially careful when using this methodology to analyze archival tissue specimens. Formalin fixation in particular results in DNA that is difficult to amplify and often produces inconsistent PCR results, including artifactual allelic loss and poor amplification of large products. Therefore, when this technique is used to analyze archival samples, it is highly recommended that replicate experiments (multiple independent dissections, triplicate PCR reactions, etc.) be used to verify results.

A: LCM and Proteinase K Treatment

1. Obtain microdissected cells using the [LCM procedure](#).

TIP: The number of cells needed to successfully perform the assay varies depending on the quality and processing conditions of the tissue samples. One thousand cells is recommended as a good starting point.

2. Suspend approximately 1000 microdissected cells in 20 μ l proteinase K buffer.
3. Incubate overnight at 37°C.

B: Prepare the Glass Plates

TIP: Use Accuwipes for cleaning purposes, as they will not leave lint behind and are non-abrasive.

1. Clean glass plates twice with glass cleaner.
2. Repeat using 95% EtOH.
3. Spray small plate with Acrylease.
4. Spread Acrylease evenly using a circular motion.
5. Buff dry.
6. Quickly assemble the plates without touching the clean surface.
7. Place 0.4 mm spacers on the edges of the larger plate.
8. Place the smaller glass plate on top of the larger plate and spacers.
9. Secure the plates with a casting boot (tape or clamps may be substituted for the casting boot).

C: Polymerize the Gel

TIP: Acrylamide is a neurotoxin. Be sure to wear gloves and a labcoat when working with this substance.

1. Add 480 μ l of 10% ammonium persulfate to 75 ml of Gel-mix-6.
2. Mix by inversion.
3. Hold the nozzle of the bottle at the corner of the gel cast.
4. Hold the gel cast at a 45° angle to the bench and pour the gel between the plates. If bubbles get trapped between the plates, remove them by tapping the outside of the plates or by tipping the plates upright.
5. Insert the straight side of the comb approximately 1 cm into the gel. If bubbles are introduced at this point, remove the comb and use the teeth of the comb to sweep out small bubbles.
6. Clamp the top of the plates together.
7. Allow the gel to polymerize for at least one hour.

TIP: The gel can be left to polymerize overnight. However, if bubbles appear, the gel has begun to separate from the plates. To minimize separation, wrap the gel in plastic film and store at 4°C until use.

D: PCR Reaction

TIP: Investigators must be especially careful when using this methodology to analyze archival tissue specimens. Formalin fixation in particular results in DNA that is difficult to amplify and often produces inconsistent PCR results, including artifactual allelic loss and poor amplification of large products. If this technique is to be utilized for analysis of archival samples, we highly recommend that replicate experiments (multiple independent dissections, triplicate PCR reactions, etc.) be used to verify results.

1. Remove reagents from the freezer before beginning the procedure.
 - Thaw thoroughly before use.
 - Prepare all reactions on ice.
 - Prepare the reduced cytosine mixture prior to beginning the LOH reaction setup.
 - Vortex all reagents, with the exception of Taq Gold Polymerase before beginning the PCR reaction setup.
2. Prepare 320 µl reduced nucleotide mixture:

Reduced Nucleotide Mix	
10 µl	dATP, 10 mM
10 µl	dGTP, 10 mM
10 µl	dTTP, 10 mM
2.0 µl	dCTP, 10 mM
288 µl	DEPC-treated H ₂ O

3. Aliquot 1 µl of each DNA sample into a separate PCR tube and set aside.

TIP: DNA that is recovered from microdissected samples and "semi-purified" using a one-step proteinase K buffer will sometimes produce "non-specific" PCR products in addition to the true alleles. Moreover, larger alleles will sometimes amplify much less well than smaller alleles. Thus, normal-cell DNA recovered from the same tissue section as the tumor DNA serves as the best control for determining the presence or absence of allelic loss.

4. Prepare sufficient volume of the reaction mixture in a separate tube for all reaction tubes:

Reaction mixture/reaction tube	
1.0 μ l	Taq Buffer
0.8 μ l	Reduced nucleotide mixture
0.2 μ l	Forward primer, 20 μ M
0.2 μ l	Reverse primer, 20 μ M
6.6 μ l	DEPC treated H ₂ O
0.1 μ l	α - ³² P dCTP
0.1 μ l	AmpliTaq Gold Polymerase
	Total volume = 9 μ l

5. Thoroughly mix the reaction mixture by pipetting and dispense 9 μ l of the reaction mixture into each tube containing DNA sample.

TIP: Be sure to mix the LOH reaction mixture with the DNA sample by pipetting. This is especially critical for DNA from microdissected samples that has been processed through a one-step proteinase K-based "purification."

6. Cap the reaction tubes and place them in a thermal cycler.
7. Cycle the reactions according to T_m of the specific primer set.
8. After PCR, remove the samples from the thermal cycler and dispense 2 μ l of formamide/dye solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanole) into each reaction tube.
9. Store reactions at 4°C until the gel is ready for loading.

TIP: Investigators may want to consider the "touchdown" procedure for PCR by Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS: "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucl Acids Res 19:4008, 1991. Advantages include:

- Much cleaner bands, since by starting with a high annealing temperature of 66 degrees and lowering 1 degree every cycle, the first PCR products are the most specific ones.
- The exact same protocol can be used for all primers.

E: Finalize Gel Preparation

1. Remove the gel from the casting boot.
2. Push the spacers into the gel until they are flush with the smaller glass plate to prevent the buffer from leaking during electrophoresis (spacers tend to get pushed out of the gel during polymerization).
3. Place the gel in the sequencing apparatus and close the buffer release valve.
4. Pour 500 ml of 0.5X TBE buffer in the upper chamber and 500 ml of 1X TBE buffer in the lower chamber.
5. Remove the comb and clear bubbles from the loading area with a pipette.
6. Insert the teeth of the comb approximately 1 mm into the gel.
7. Pre-heat the gel at 1700 volts for 15-20 minutes.

F: Gel Loading

1. Remove the samples from the freezer.
2. Denature the samples in a thermal cycler at 95°C for 5 mins.
3. Remove samples from the thermal cycler and immediately place on ice, with an ice pack on top of the samples, for 1 min.
4. Turn off the power supply.
5. Adjust comb if it has been pushed out of the gel during pre-heating.
6. Load 4 µl of each sample per well.

TIP: It is best to skip lanes to avoid contamination caused by leaking between the wells.

7. Run the gel at 1700 volts for 1-2 hours (running time based on PCR product size).

G: Separate the Gel

1. Turn off the power supply.
2. Drain buffer chambers (buffer must be disposed of in a liquid radioactive waste carboy).
3. Remove the gel from the sequencing apparatus.
4. Separate the plates by removing the spacers and inserting the tips of two thin spatulas in their place.
5. Gently lift the spatulas until the top plate separates from the lower plate and gel.
6. Place Whatman paper on the gel.

7. Slowly peel the Whatman paper and gel off the glass plate.
8. Cover the gel with plastic wrap and dry on a gel dryer for 1 hour.

H: Autoradiography

1. Remove the plastic wrap from the gel.
2. Place the gel in an autoradiography cassette.
3. Expose film 1 hour-2 days using Kodak BioMax MR or AR film.

TIP: Use MR film for maximum resolution of bands. An intensifying screen is useful when analyzing PCR products from small numbers of microdissected cells.

References

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