Simultaneous Detection of Multiple Point Mutations Using Allele-Specific Oligonucleotides

This approach can be used to screen one gene for many allelic mutations or to screen several loci for several allelic mutations each. In the basic protocol, pools of radiolabeled allele-specific oligonucleotide (ASO) probes are hybridized to dot blots containing polymerase chain reaction (PCR)-amplified DNA products generated from one or more loci (Fig. 9.4.1). Because tetramethyl ammonium chloride (TMAC) is added to the hybridization solution, the melting temperature of each oligonucleotide is independent of G-C content and oligonucleotides of the same length can be hybridized simultaneously. The pooled probes will give a positive hybridization signal from any PCR-amplified DNA sample containing a sequence complementary to any of the ASOs in the pool of oligonucleotide sequences. If many PCR-amplified samples are spotted onto a single filter, multiple individuals can then be screened simultaneously for many mutant sequences. This multiple ASO hybridization technique is appropriate only for circumstances when hybridization with any one of the pooled probes is expected to be uncommon. The support protocol describes the removal of radiolabeled probe DNA from the filter in order to reuse the filter for further screening. An example highlighted in the Commentary details the use of this protocol for studying the cystic fibrosis transmembrane conductance regulator (CFTR) gene.

SCREENING PCR-AMPLIFIED DNA WITH MULTIPLE POOLED ASOs

BASIC PROTOCOL

This approach is particularly powerful when used to screen for rare alleles, but is not appropriate for screening commonly occurring alleles. It works well for detecting point mutations and small deletions/insertions. PCR-amplified DNA samples that test positive are then rescreened individually using single allele-specific oligonucleotides (ASOs) to identify which mutation-specific ASO hybridizes. The mutant PCR-amplified DNA can also be screened using an ASO corresponding to the normal gene sequence to determine whether the individual is heterozygous or homozygous for the mutant allele.

The following steps describe radiolabeling ASOs using T4 polynucleotide kinase, preparing dot blots of PCR-amplified DNA, and hybridizing pooled radiolabeled ASOs to dot blots.

Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **SUPPLIERS APPENDIX**.*

10× T4 polynucleotide kinase buffer (see recipe)
200 mM DTT
10 µM allele-specific oligonucleotide (ASO), prepared just before use by diluting 100 µM stock
10 mCi/ml [γ-^{32}P]dATP (~3000 Ci/mmol)
10 U/µl T4 polynucleotide kinase
25 mM EDTA
Denaturing solution (see recipe)
25 ng/µl PCR-amplified products from genes of interest (see Critical Parameters)
2× SSC (*APPENDIX 2*)
TMAC hybridization solution (see recipe)
TMAC wash solution (see recipe), room temperature and 52°C

Contributed by Barbara Handelin and Anthony P. Shuber

Dot-blot apparatus
Nylon membrane: e.g., Biotrans+ (ICN Biomedicals) or Biodyne (Pall)
Whatman 3MM filter paper
80°C oven
Sealable bags
52°C shaking water bath
X-Omat AR film (Eastman Kodak)

Additional reagents and equipment for PCR (*UNITS 7.1 & 9.3*; *CPMB UNIT 15.1*) and preparing dot blots, (*CPMB UNIT 2.9B*)

CAUTION: ^{32}P and TMAC are hazardous; see *APPENDIX 2A* for guidelines on handling, storage, and disposal.

Label the ASO with ^{32}P

1. For each ASO to be labeled, prepare the following labeling reaction:

   10 µl H_2O
   2 µl 10× T4 polynucleotide kinase buffer
   1 µl 200 mM DTT
   3 µl 10 µM ASO
   3 µl 10 mCi/ml [γ-^{32}P] ATP
   1 µl 10 U/µl polynucleotide kinase.

   Incubate 1 hr at 37°C.

   See Critical Parameters for important tips on design of probes to be used in the labeling reaction. The specific probes which are to be used would, of course, be determined by the genetic locus of interest.

2. Stop the reaction with 80 µl of 25 mM EDTA.

Prepare dot blot of PCR-amplified DNA samples

3. Prepare the dot-blot apparatus according to the manufacturer's instructions.

   If necessary, clean the dot-blot apparatus because the apparatus must be clean and dry to obtain adequate suction.

4. Estimate the size of membrane that will be necessary to contain the number of samples to be analyzed and cut the membrane to the appropriate size to fit the dot-blot apparatus. Mark the dry membrane in asymmetric corners so that it can be reoriented after hybridization.

   The membrane may be marked with a pen (e.g., Sharpie extra-fine-point marker) or by cutting a corner.

5. Wet the membrane by floating it on water (it should wet immediately), then submerge it briefly. Place the membrane on the dot-blot apparatus gasket and assemble the manifold—do not apply vacuum until just before loading the samples.

6. For each sample to be dotted onto the membrane, add 50 µl denaturing solution to a 1.5-ml microcentrifuge tube. Next, add 8 µl PCR-amplified DNA from the gene of interest and mix by vortexing.

   As many as eight PCR-amplified DNA fragments (products) can be combined in a single tube and co-dotted onto the membrane (see Critical Parameters). PCR-amplified DNA from a multiplex PCR (using 8 µl containing 200 ng of each amplification product; see UNIT 9.3) can be used.

   For each ASO included in the hybridization cocktail (see below), include a PCR-amplified DNA from an individual who is a known homozygote or heterozygote for that mutation (as a positive control). Include PCR-amplified DNA from an individual who does not have the mutation and a "no-DNA" PCR sample as negative controls.

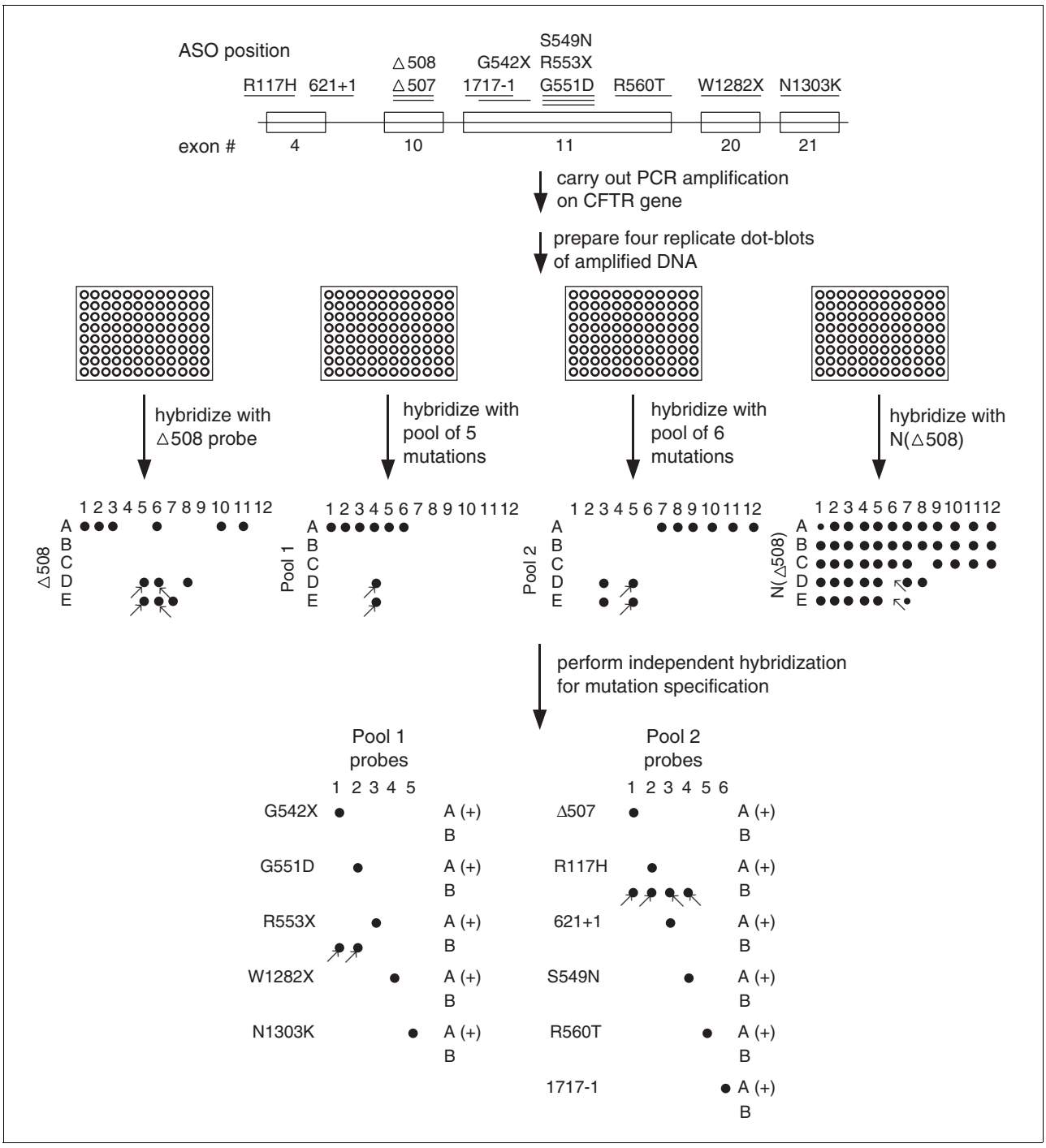

Figure 9.4.1 Detection of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene using allele-specific oligonucleotides.

7. When all samples have been added to denaturing solution, apply the vacuum to the dot-blot apparatus. Add the entire volume of each sample to a well, avoiding bubbles on the filter. Prepare Whatman 3MM filter paper saturated with 2× SSC.

   A common mistake at this point is to place a sample in the wrong well. Use bromphenol blue in the denaturing solution to keep track of sample loading.

8. With the vacuum still on, remove the upper block of the apparatus, quickly remove the membrane, and place it onto Whatman 3MM filter paper saturated with 2× SSC. Let sit for 2 min.

9. Fix the DNA onto the membrane by placing it for 15 min in an 80°C oven. Rewet the membrane in water and transfer it to a sealable bag for hybridization.

   The filters may be temporarily stored by keeping in a dark place, at room temperature.

Hybridize ^{32}P-labeled ASOs to dot blots and autoradiograph

10. Add an appropriate amount of hybridization solution containing the ^{32}P-labeled ASO pooled probes to each bag. Incubate 2 hr to overnight, with shaking, in 52°C water bath.

    For example, for a standard 96-well format membrane, use 10 ml of hybridization solution. The optimal concentration of each ASO probe should be determined empirically, but should be in the range of 0.03 to 0.15 pmol/ml of hybridization solution. To reduce background hybridization, add ≥20-fold excess unlabeled ASO corresponding to the "normal" allele.

    This protocol has been optimized for 17-mer ASO probes.

11. Remove the membrane from the bag and wash with vigorous agitation as follows: 20 min in 200 ml TMAC wash solution, room temperature, followed by 20 min in 300 ml TMAC wash solution, 52°C.

12. Blot membranes on Whatman 3MM filter paper to dry and expose to X-Omat AR film 1 hr to overnight at −70°C.

SUPPORT PROTOCOL

STRIPPING OLD PROBES AND REHYBRIDIZATION

Filters can be stripped of previously hybridized probe and rehybridized several times, depending on the amount of DNA (control and experimental) on the filters. If the signal-to-noise ratio allows reading of the appropriate hybridization signals from positive- and negative-control DNA, and the amount of experimental and control DNA on the filter is equivalent, then the experimental results can be interpreted.

Additional Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **SUPPLIERS APPENDIX**.*

Hybridized membrane (first basic protocol)
TMAC wash solution (see recipe), freshly prepared and prewarmed to 15°C above previous hybridization temperature
Agitating water bath, 15°C above previous hybridization temperature

CAUTION: Radiolabeled hybridized membranes and TMAC are hazardous; see *APPENDIX 2A* for guidelines on handling, storage, and disposal.

1. Add previously hybridized membrane to 300 ml prewarmed TMAC wash solution in a washing dish. Wash 1 hr with vigorous agitation in a water bath at 15°C above previous hybridization temperature.

2. Remove filter from the wash and blot dry on Whatman 3MM filter paper.

3. Autoradiograph to confirm that the previously hybridized probe has been removed.

4. Hybridize filters as described in steps 10 to 12 of the basic protocol, or store dry at room temperature (they can be stored indefinitely in resealable bags).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Denaturing solution

500 mM NaOH
2.0 M NaCl
25 mM EDTA (prepare freshly)
0.0001% (w/v) bromphenol blue (add just before use)
Prepare fresh

T4 polynucleotide kinase buffer, 10×

700 mM Tris·Cl, pH 7.6
100 mM $MgCl_2$
50 mM spermidine
Store frozen

Tetramethylammonium chloride (TMAC) solution

Dissolve 657.6 g TMAC (mol. wt. = 109.6) in 1 liter H_2O. Filter solution through Whatman no. 1 filter paper and determine the precise concentration by measuring the refractive index (n) of a three-fold diluted solution. The molarity (M) of the diluted solution = 53.6 (n − 1.331) and the molarity of the stock solution = 3 × M. Store TMAC at room temperature in brown bottles.

CAUTION: *TMAC is hazardous; see* APPENDIX 2A *for guidelines on handling, storage, and disposal.*

TMAC hybridization solution

6 ml TMAC solution (see recipe; ~3 M final)
600 µl 10% (w/v) SDS (0.6% final)
20 µl 0.5 M EDTA (1 mM) final
1 ml 0.1 M Na_3PO_4, pH 6.8 (10 mM final)
1 ml 50× Denhardt solution (*APPENDIX 2*; 5× final)
40 µl 10 mg/nl yeast RNA (40 µg/ml final)
1.34 ml H_2O

CAUTION: *TMAC is hazardous; see* APPENDIX 2A *for safety guidelines.*

TMAC wash solution

60 ml TMAC solution (see recipe; ~3 M final)
200 µl 0.5 M EDTA (1 mM final)
10 ml 0.1 M Na_3PO_4, pH 6.8 (10 mM final)
6 ml 10% (w/v) SDS (0.6% final)
23.8 ml H_2O

CAUTION: *TMAC is hazardous; see* APPENDIX 2A *for safety guidelines.*

COMMENTARY

Background Information

Review of current human molecular genetics literature reveals that the paradigm of sickle cell anemia, in which one mutation accounts for essentially 100% of the cases of sickle cell phenotype, is the exception rather than the rule. As associations between genes and disease phenotypes are discovered and the mutations that confer the disease phenotype are determined, a new rule is rapidly being established: DNA diagnosis of genetic diseases typically requires analysis of many alternative mutations, each of which confers the disease phenotype.

Mutation analysis using ASO probes is direct and is less subject to errors or misinterpretation than other techniques for detecting specific sequences; i.e., if specific hybridization of the probe is observed, the complementary se-

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