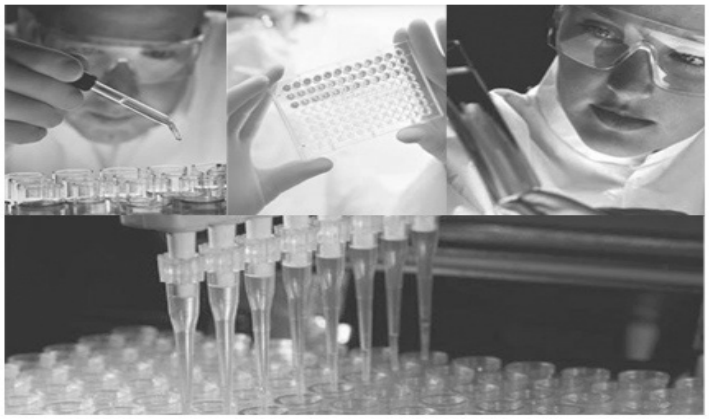
national diagnostics



Mutation Detection

SequaGel® *MD*

For analyzing conformational differences in large numbers of DNA samples

- Point Mutation Analysis
- SSCP Analysis
- Heteroduplex Analysis

SequaGel® *MD*

Native DNA PAGE gels can be used to detect small mutational differences between DNA molecules. In heteroduplex analysis—for double stranded DNA—the basis of separation is the conformational difference arising from the bending of the rodlike double helix caused by small mismatches between the strands. In SSCP analysis, single stranded DNA molecules are fractionated based on the compactness of their folded structure.

Heteroduplex Analysis

In heteroduplex analysis, control DNA is denatured and allowed to anneal with denatured sample DNA. The renatured products are analyzed on a gel optimized to resolve conformational differences, such as National Diagnostics' SequaGel MD. If the sample DNA is not identical to the control DNA, multiple bands are observed. The fastest migrating band is the homoduplex control and/or the homoduplex sample. Heteroduplexes with mismatches migrate more slowly.

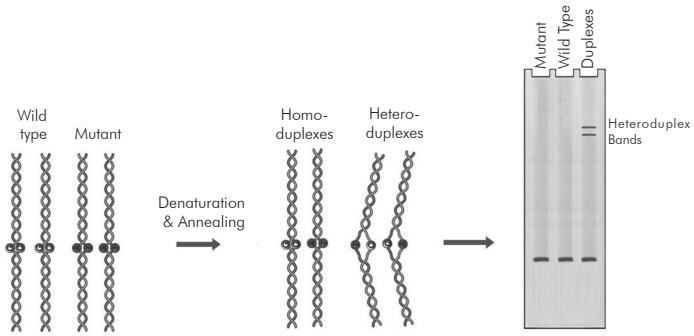


Figure 1. Heteroduplex Analysis. Annealing of mutant DNA to wild type probe gives duplexes with one or more mismatched bases (heteroduplexes). Mismatching causes the double helix to take on a conformation which retards its mobility during electrophoresis.

Instructions for Heteroduplex Analysis

GEL PREPARATION

1. Preparation of Working Solutions:

   To cast a 0.8 to 1.0 mm thick gel (>40 cm vertical gel recommended) combine the following in an Erlenmeyer flask:
   50 ml SequaGel MD
   6 ml 10X TBE
   15 g urea (optional)
   Fill to 100 ml with deionized water and mix thoroughly. Urea may assist the formation of more distinct bands during electrophoresis and reduces the formation of doublets in homoduplex controls.

2. Casting the gel:

   Add the following to the solution, and swirl gently:
   40 µl TEMED
   400 µl freshly prepared 10% ammonium persulfate
   Treat one plate from the gel cassette with Glass Free to facilitate later disassembly of the cassette. Using standard acrylamide procedure, pour the gel solution into the cassette, insert the comb, and allow to polymerize at room temperature for a minimum of 60 minutes. Attach the gel cassette to the electrophoresis apparatus, and fill the upper and lower chambers with 0.6X TBE.

SAMPLE PREPARATION

1. PCR amplification:

   PCR conditions should be optimized for the desired PCR product before heteroduplex analysis. It is recommended that the minimum number of PCR cycles be used on a purified, salt-free template, and that reagent and primer concentrations be optimized.

   After PCR thermal cycling, add EDTA to a final concentration of 5 mM (1µl of 0.5 M EDTA per 100 µl reaction) to inactivate the T_{aq} DNA Polymerase.

2. Hybridization:

   Mix equivalent quantities of wild type and sample PCR-amplified DNA. Heat at 95°C for 3 minutes. Then, over a 20-30 minute period, slowly cool the mixture to room temperature. Using a thermocycler can facilitate this step.

ELECTROPHORESIS

1. Add 1 µl Triple Dye Loading Buffer (provided in kit) per 5 µl of sample and mix thoroughly.

2. Rinse the wells with running buffer and load the samples in the 1.0X SequaGel MD gel. One lane should consist of control homoduplex DNA, and one of the sample homoduplex DNA. This will allow the detection of non-heteroduplex artifacts on the gel. Another lane should consist of an appropriate DNA size marker.

3. Run the gel in 0.6X TBE at a constant voltage of 20 V/cm, as determined by the length of the gel. For a 40 cm gel, set the power supply to 860 V. Approximate run times can be estimated from the chart below:

Fragment Size	Run Time (800V)	Volt X Hours
200 bp	14.0 hours	11,200
250 bp	14.5 hours	11,600
300 bp	16.5 hours	13,200
500 bp	20.0 hours	16,000
700 bp	25.0 hours	20,000
900 bp	30.0 hours	24,000

4. When the electrophoretic run is complete, remove the gel from the apparatus, and carefully remove one plate from the gel. Stain with ethidium bromide or silver stain.

STAINING

Stain using 0.6X TBE containing 1 µg/ml of ethidium bromide. Water should not be used in place of the TBE, because the gel will swell when placed in water. Stain for 15-30 minutes. For maximum sensitivity, destaining in 0.6X TBE for up to 30 minutes may be required.

To visualize ethidium bromide stained bands, cover the gel with plastic wrap and place the plate with the gel side down on a UV-transilluminator. It may assist in handling and visualization to cut out the gel region containing the bands of interest.

WARNING: ETHIDIUM BROMIDE HAS BEEN SHOWN TO BE A CARCINOGEN AND SHOULD BE DISPOSED OF PROPERLY.

Silver staining may be used to increase band visibility.

SSCP Analysis

In SSCP analysis samples are denatured with heat and then rapidly cooled. Rapid cooling favors self-annealing because insufficient time is allowed for complementary strands to collide and orient for duplex formation. The renatured samples are analyzed on a gel opposite the control DNA. As with heteroduplex analysis, all fragments must be the same length. Mutant samples will show a mobility different from the control DNA. The gel matrix used must be optimized for the resolution of DNA conformers of the same length. Various combinations of acrylamide/bis-acrylamide are mentioned in the literature, at ratios from 29 to 1 to 50 to 1, and at percentages from 4 to 8. National Diagnostics' SequaGel MD is optimized to provide superior results in both heteroduplex and SSCP analysis.

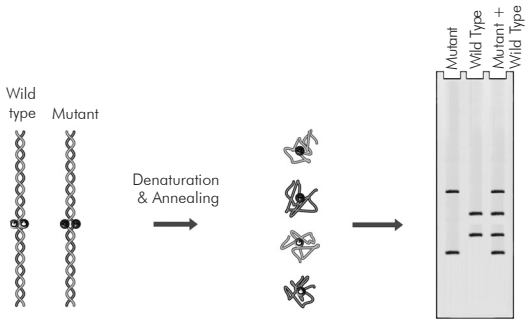


Figure 2. SSCP Analysis (Single Strand Conformational Polymorphism). Single point mutations can cause major differences in the folded form of single stranded DNA. These differences can be detected as differences in electrophoretic mobility.

Instructions for SSCP Analysis

GEL PREPARATION

1. Preparation of Working Solutions:
   To cast a 0.4 mm thick gel (>40 cm vertical gel recommended) combine the following in an Erlenmeyer flask:
   25 ml SequaGel MD
   6 ml 10X TBE
   Fill to 100 ml with deionized water and mix thoroughly.

   Prepare 0.6X running buffer by diluting 60 ml of 10X TBE stock to 1 L with deionized water.

2. Casting the gel:
   Add the following to the gel solution, and swirl gently:
   40 µl TEMED
   400 µl freshly prepared 10% APS
   Using standard acrylamide procedures, pour the gel solution into the cassette, insert the comb (inverted if using a sharkstooth comb), and allow polymerization at room temperature for a minimum of 60 minutes. Attach the gel cassette to the electrophoresis apparatus.

SAMPLE PREPARATION

1. PCR amplification:

   PCR conditions should be optimized for desired PCR product before SSCP analysis is undertaken. It is recommended that the minimum number of PCR cycles be used on a purified, salt-free template, and that reagent and primer concentrations be optimized. If radiolabeling is going to be utilized instead of silver staining, end-labeled primers may be used, or an α-^{32}*P dNTP may be included in the PCR amplification.*

2. After PCR thermal cycling, 1 µl of PCR product should be added per 10 µl of SCCP Stop Solution (provided with SequaGel MD kit). To denature the sample DNA, this solution should be heated to 94°C for 2 minutes. The vials should then be placed immediately into an ice slurry to rapidly cool the solution.

ELECTROPHORESIS

1. Rinse the gel wells with running buffer. The sharkstooth comb should be reinserted so that it just touches the surface of the gel, and 1 to 3 µl of the sample should be loaded.

2. Run the gel at a constant power of 6-8 watts for 14 hours.

3. If the DNA was radiolabeled, transfer the gel to Whatman 3MM filter paper, place on a flat surface, and cover with plastic wrap. Dry the gel and, using standard technique, expose to X-ray film. Silver staining may be used for detection. Ethidium bromide is not effective at detecting single stranded DNA.

Result Analysis

HETERODUPLEX

As shown in Figure 1, homoduplex DNA species—either normal or mutant—are expected to run as a single band. If the sample DNA does not contain a mutation the heteroduplex species will have 100% complementation and will therefore run as one band, equivalent to the normal homoduplex DNA. However, if a mutation exists in the sample DNA, the heteroduplex species will not be 100% complementary and will have some conformational distortion (often a "bubble" or a "kick") which alters migration through the gel and therefore will result in separate heteroduplex bands. Often non-complementary heteroduplex DNA will migrate slower in the gel and appear as one or two fainter bands above the homoduplex species.

SSCP

As shown in Figure 2, the control denatured DNA will run as two bands in the SequaGel MD gel, as each complementary strand will independently fold to a unique conformation. If a denatured DNA sample is mutated two bands will also result on the gel, although these bands will have shifted mobilities compared to the control DNA. If the sample DNA is homologous to the control, it will migrate to the same positions as the control DNA. At times, more than two bands may result in any lane if more than one conformation is optimal for the single-strand species. A fainter band may be observed in all lanes which results from the reannealing of the complementary DNA strands. Due of this, double-stranded DNA should also be run on the gel in an adjacent lane.

Ordering Information

SequaGel® MD

SequaGel MD SSCP Kit [SequaGel MD, 200mL; SSCP Stop Solution (1.2 mL)]	1 kit	EC-846
SequaGel MD Heteroduplex Kit [SequaGel MD, 200mL; Triple Dye Loading Buffer (1.2 mL)]	1 kit	EC-847
SequaGel MD Monomer Solution	200 ml	EC-845

Running and Loading Buffers

Triple Dye Loading Buffer (6X)	1.2 ml	EC-855
10X TBE Buffer	1 Liter	EC-860

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