

# SequaGel<sup>®</sup> MD

MUTATION DETECTION

- **Point Mutation Analysis**
- **SSCP Analysis**
- **Heteroduplex Analysis**

SequaGel MD permits minor mutational differences in DNA sequences to be detected as a high resolution relative mobility ( $R_f$ ) shift. SequaGel MD is a proprietary formulation, supplied as a 2X stock, designed to resolve such sequence related differences by SSCP (Single Strand Conformational Polymorphism)<sup>5,7</sup> and Heteroduplex Analysis<sup>4</sup>. DNA mutations, or sequence modifications, are readily associated with specific disease states<sup>1</sup>. Since a variation

of a single nucleotide in a sequence may indicate a significant genetic anomaly, an extremely sensitive method to analyze these mutations is necessary.

Two principle methods of analyzing conformational differences have been developed. National Diagnostics' SequaGel MD can be applied to both of these methods as follows:

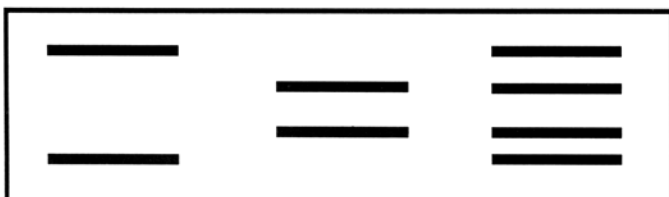
**SSCP Analysis** has been demonstrated to be effective in detecting polymorphisms of single base differences<sup>3,6</sup>. In brief, the method involves:

1. Amplification of a discrete sequence of DNA by PCR\*.
2. Denaturation (separation) of the double stranded PCR product.
3. Snap cooling of the denatured DNA to maximize self-annealing of individual strands.
4. Analysis of the differences in relative mobility ( $R_f$ ) of the single strands by electrophoresis in National Diagnostics' SequaGel MD. See figure below.

SequaGel MD is designed to run SSCP samples in the 100-300 nucleotide range at a 0.5X concentration. Small fragments may be better resolved by using higher concentration (0.75X) gels. Larger fragments may be better resolved by running lower concentration (0.4X) gels.

**FIGURE 1: SSCP ANALYSIS**

Wild Type Control    Mutant Sample    Wild Type+Mutant



**Heteroduplex Analysis** is an effective method of sequence mutation analysis which compares double stranded heteroduplex chain formation to a standard homoduplex. In brief, the method involves:

1. Amplification of a discrete sequence of DNA by PCR.
2. Denaturation of a mixture of homoduplex control fragment with homoduplex test sample fragment.
3. Reannealing of the mixture of strands creating homoduplex and heteroduplex strands.
4. Analysis of the differences in relative mobility ( $R_f$ ) of the resulting double strands by electrophoresis in National Diagnostics' SequaGel MD. See figure below.

SequaGel MD is designed to run heteroduplex analysis on DNA fragments up to 900 bases at a 1X concentration. The proprietary formulation of National Diagnostics' SequaGel MD results in clear, sharp bands, and a larger shift in mobility of the heteroduplex relative to the homoduplex.

**FIGURE 2: HETERODUPLEX ANALYSIS**

Wild Type Control    Mutant Sample    Wild Type+Mutant



\*Heteroduplexes

# Instructions for SequaGel<sup>®</sup> MD Heteroduplex Analysis

## Preparation of Working Solutions

### 1.0X SequaGel MD:

- A. To cast a 0.8 to 1.0 mm thick gel ( $\geq 40$  cm vertical gel recommended), combine the following in an Erlenmeyer flask:

SequaGel MD (2X)	50 mL
10X TBE	6 mL
15% (w/v) Urea (optional)	15 g

Fill to 100 mL with deionized water and mix thoroughly.

The solution may be filtered through Whatman<sup>®</sup>#1 filter paper or Nalgene<sup>®</sup>cellulose acetate filter ( $\leq 0.45$   $\mu\text{m}$  if necessary). Urea may assist in the formation of more distinct bands during electrophoresis, and reduces the formation of doublets in homoduplex controls.

- B. Add the following to the solution, and swirl gently:

TEMED	40 $\mu\text{L}$
10% aqueous, freshly prepared APS (Ammonium Persulfate)	400 $\mu\text{L}$

- C. Using standard acrylamide procedure, pour the gel solution into the plates, insert the comb, and allow to

polymerize at room temperature for a minimum of 60 minutes. Attach the gel cassette to the electrophoresis apparatus.

### RUNNING BUFFER

- D. Prepare 0.6X running buffer by diluting 60 mL of 10X TBE stock to 1 L with deionized water. Prepare an adequate amount to fill both anode and cathode chambers.

### STAINING:

- E. Stain using 0.6X TBE containing 1  $\mu\text{g}/\text{mL}$  of ethidium bromide. Water should not be used in place of the TBE, as the gel will swell when placed in water.

*Warning: Ethidium Bromide has been shown to be a carcinogen, and should be used and disposed of appropriately.*

- F. Silver staining may be used to increase band visibility. National Diagnostics' Protostain (Order No. EC-710) has been proven to be 10 times more sensitive than ethidium bromide while eliminating the toxicity involved when staining with ethidium bromide.

## Method of Use

1. PCR\* conditions should be optimized to obtain the desired PCR product in order to simplify 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.
2. After PCR thermal cycling, add EDTA to a final concentration of 5 mM (1  $\mu\text{L}$  of 0.5 M EDTA per 100  $\mu\text{L}$  water) to inactivate the *Taq* DNA Polymerase. Mix equivalent quantities of wild type and sample PCR-amplified DNA.
3. Heat at 95°C for 3 minutes, then during a 20-30 minute period slowly cool the mixture to room temperature. The use of a thermocycler can facilitate this step.
4. Add 1  $\mu\text{L}$  Triple Dye Loading Buffer (provided in kit) per 5  $\mu\text{L}$  of sample and mix thoroughly.
5. Rinse the wells with running buffer and load the samples in the 1.0X SequaGel MD gel. One lane should consist of the Heteroduplex Control DNA which is supplied in the Triple Dye Loading Buffer ready-to-use (provided in kit). Another lane should consist of an appropriate DNA size marker.
6. Run the gel 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 800 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

7. When the electrophoretic run is complete, remove the gel cassette and disassemble. Leave the gel adhered to one glass plate, and stain the gel with ethidium bromide solution for 5 to 15 minutes (Refer to Step E, Preparation of Working Solutions). Silver staining (National Diagnostics' Protostain, Order No. EC-710) may be used to improve band visualization.
8. The gel should be destained in 0.6X TBE buffer for 10 to 15 minutes. It may be necessary to destain for an hour to reduce background staining for optimal visualization of faint bands.
9. 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.

\*The PCR process is covered by U.S. patents owned by Hoffmann-LaRoche Inc.

# Instructions for SequaGel® MD SSCP Analysis

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## Preparation of Working Solutions

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### 0.5X SequaGel MD:

- A. To cast a 0.4 mm thick gel ( $\geq 40$  cm vertical gel recommended), combine the following in an Erlenmeyer flask:

SequaGel MD (2X)	25 mL
10X TBE	6 mL

Fill to 100 mL with deionized water and mix thoroughly.

- B. Add the following to the solution, and swirl gently:

TEMED 40  $\mu$ L

10% aqueous, freshly prepared APS 400  $\mu$ L

(Ammonium Persulfate)

- C. Using standard acrylamide procedure, pour the gel solution into the plates, insert the comb (inverted if using a sharktooth comb), and allow polymerization at room temperature for a minimum of 60 minutes. Attach the gel cassette to the electrophoresis apparatus.

### RUNNING BUFFER:

- D. Prepare 0.6 X running buffer by diluting 60 mL of 10X TBE stock to 1 L with deionized water. Make sufficient buffer to fill both anode and cathode chambers.

### SILVER STAINING

- E. Silver staining (National Diagnostics' Protostain, Order No. EC-710) may be used to visualize the DNA as an alternative to radiolabeling the sample. Silver staining is highly sensitive and therefore may be used for smaller samples, such as those used in SSCP Analysis.

## Method of Use

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1. PCR conditions should be optimized to obtain the desired PCR product in order to simplify SSCP 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. If radiolabeling is going to be utilized instead of silver staining, it is recommended that end-labeled primers be used, or  $\alpha$ - $^{32}$ P dNTP may be included in the PCR amplification.
2. After PCR thermal cycling, 1  $\mu$ L of PCR product should be added per 10  $\mu$ L of SSCP Stop Solution (provided in 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.
3. Rinse the gel wells with running buffer. The sharktooth comb should be reinserted so that it just touches the surface of the gel, and 1 to 3  $\mu$ L of the sample should be loaded.
4. At room temperature, run the gel at a constant power of 6-8 Watts for 14 hours.
5. When the electrophoretic run is complete, turn off the power supply, disconnect the electrodes, remove the gel cassette from the apparatus, and allow the plates to cool before prying apart.
6. If the DNA was not radiolabeled, National Diagnostics' Protostain Silver Stain Kit may be used for visualization.
7. 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.

These steps should be followed when performing SSCP reactions on SequaGel MD. If further information and suggestions are needed to optimize these procedures, refer to Dean and Gerrard, 1991, and Sheffield, *et al.*, 1993<sup>2,8</sup>.

## Result Analysis

### SSCP

As shown in Figure 1, the control denatured DNA will run as two bands in the SequaGel<sup>®</sup> 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. Because of this, double-stranded DNA should also be run on the gel in an adjacent lane.

### HETERODUPLEX

As shown in Figure 2, 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 "kink") 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.

## System Components

SequaGel MD SSCP Kit Order No. EC-846	1 Kit
•SequaGel MD, 200 mL; SSCP Stop Solution (1.2 mL)	
SequaGel MD Heteroduplex Kit Order No. EC-847	1 Kit
•SequaGel MD, 200 mL; Triple Dye Loading Buffer (1.2 mL);	
SequaGel MD Monomer Solution Order No. EC-845	200 mL (1-3) 200 mL (4 +)
Triple Dye Loading Buffer (6X) Order No. EC-855	1.2 mL
10X TBE Buffer Order No. EC-860	1 Liter

### For Additional Information / Order Placement:

**TOLL FREE:** (800) 526-3867  
**GEORGIA:** (404) 699-2121  
**FAX:** (404) 699-2077

**U.K.:** 441 482 646022  
441 482 646020  
**FAX** 441 482 646013



305 Patton Drive  
Atlanta, Georgia 30336

Unit 4, Fleet Business Park  
Itlings Lane  
Hessle, Hull  
HU13 9LX, England

The SequaGel MD may be stored at room temperature.  
Other system components must be stored at 2-8°C.

### References

- Danenberg PV, Horikishi TM, Volkenandt M, Danenberg K, Lenz HJ, Shea CCL, Kicker AP, Simoneau A, Jones PS, Bertinao JR (1992). "Detection of Point Mutations in Human DNA by Analysis of RNA Conformation Polymorphisms." *Nucleic Acids Res.* 20: 573-579.
- Dean M, Gerrard B. (1991). "Helpful Hints for the Detection of Single-stranded Conformation Polymorphisms." *BioTechniques.* 10: 332-333.
- Hzuka M, Mashiyama S, Oshimura M, Sekiya T, Jayashi K. (1991). "Cloning and Polymerase Chain Reaction Single-Strand Conformation Polymorphism Analysis of Anonymous *Alu* Repeats on Chromosome 11." *Genomics.* 12: 139-146.
- Nagamine CM, Chan K, Lau YFC. (1989). "A PCR Artifact: Generation of Heteroduplexes." *Am. J. Hum. Genet.* 45: 337-339.
- Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. (1988). "Detection of Polymorphisms of Human DNA by Gel Electrophoresis as Single-Strand Conformation Polymorphisms." *Proc. Natl. Acad. Sci.* 86: 2766-2770.
- Orita M, Sekiya T, Hayashi K. (1990). "DNA Sequence Polymorphisms in *Alu* Repeats." *Genomics.* 8: 271-278.
- Orita M, Suzuki Y, Sekiya T, Hayashi K. (1989). "Rapid and Sensitive Detection of Point Mutations and Genetic Polymorphisms Using polymerase Chain Reaction." *Genomics.* 5: 874-879.
- Sheffield VC, Beck JS, Kwitek AE, Sandstrom DW, Stone EM. (1993). "The Sensitivity of Single-Strand Conformation Polymorphism Analysis for the Detection of Single Base Substitutions." *Genomics.* 16: 325-332.