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Lonza Rockland, Inc.
www.lonza.com
biotechserv@lonza.com
Tech Service: 800-521-0390
Customer Service: 800-638-8174
Document # 18153-0807-7
Rockland, ME 04841 USA

MDE® Gel Solution

Protocols for SSCP and Heteroduplex Analyses

Introduction

Detection of DNA sequence heterogeneity is important not only for detection of mutations, but also for detection of DNA polymorphisms for high resolution gene mapping. Currently, most mutation detection techniques rely on comparisons between small fragments of PCR† amplified DNA. Single-stranded conformation polymorphism (SSCP) analysis is the most commonly used gel-based mutation detection method. SSCP relies on conformational intrastrand differences in DNA of different sequence. Heteroduplex (HDX) analysis separates DNA based on conformational differences between double-stranded heteroduplex DNA and homoduplex DNA.

MDE® Gel Solution is a polyacrylamide-like matrix that has a high sensitivity to DNA conformational differences. This gel's unique structure causes DNA separation to occur on the basis of both size and conformation, thus increasing the probability of detecting sequence differences from as low as 15% in standard polyacrylamide gels, to approximately 80%. In addition, the time required for optimization of the electrophoretic conditions is greatly reduced when compared to polyacryiamide gels.

To do SSCP analysis, DNA radiolabeled during PCR with ^{32}P is first denatured at 95°C in a denaturing formamide buffer, snap cooled on ice, then run on a nondenaturing MDE® Gel that has been cast in a standard sequencing apparatus. Under these conditions, each individual strand will form conformational differences which cause the DNA strands to migrate differentially within the matrix. Ideally, each strand of DNA will have a unique mobility and consequently separate from others in the MDE® Gel.

Heteroduplex analysis is dependent on conformational differences in double stranded DNA. In this technique, two PCR products in equal quantities (e.g., from wild and mutant DNA samples) are combined in a nondenaturing buffer. The DNA is melted at 95°C, then slowly cooled to room temperature. During the cooling process, the complementary DNA strands anneal, and the noncomplementary DNA strands also reanneal to form heteroduplex DNA. The mismatch in the heteroduplex DNA causes it to have a different flexibility or three-dimensional shape than homoduplex DNA, and as a result, the mobility of heteroduplex DNA will be less than that of the homoduplex DNA.

The factors determining the most appropriate technique to use are: the DNA fragment size, the GC content of the DNA fragment and the overall purpose of the investigation. SSCP analysis works best with fragments 300 bp or less in length, whereas HDX analysis works well for fragments from 200 bp-600 bp in length. SSCP is very powerful in determining if there are sequence differences between DNA fragments, whether the alleles are heterozygous or homozygous. In contrast, HDX analysis is simplest when looking for sample heterozygosity.

The following guidelines may also help you decide the best method for your needs.

- SSCP is most effective for DNA with a relatively high GC content. SSCP analysis of DNA with intermediate GC content will be facilitated by cooling the gel to 4°C during electrophoresis.
- The maximum size of DNA used for SSCP analysis is directly proportional to its GC content. With low GC content, the maximum DNA fragment size is less than 200 bp.
- Heteroduplex analysis is more effective with DNA that has a low GC content. Longer fragments may differentiate better than shorter fragments.

A related method simultaneously examines heteroduplex and SSCP DNA. This relies on the finding that if excess DNA is added to denaturation buffer, homo-and heteroduplex DNA will also form. All of these fragments can then be separated on the same gel (often in the same lane). The advantage of this method is that there is a lower probability of missing a true polymprophism.

Whichever technique is chosen, separation on a MDE® Gel will provide superior results when compared with standard polyacrylamide gels.

Precautions

Wear gloves, eye protection, lab coat, and use all safety precautions routinely used when handling acrylamide solutions. Please read the Material Safety Data Sheet (MSDS) for this product prior to use.

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SSCP Analysis with MDE® Gel Solution

Guidelines for Preparation of PCR† Samples

This procedure is suitable for SSCP samples in the 150 bp to 300 bp size range. DNA strands outside this range may be more difficult to resolve.

It is critical to use PCR conditions which minimize unwanted products, as these can result in artifact bands which interfere with the recognition of SSCP bands. You should isolate DNA samples and perform PCR according to standard methods. The tips below will help you get the best results for SSCP analysis:

- Use only highly purified, salt-free template DNA. The presence of excess salts may alter the hybridization temperature of the primer, thus decreasing the PCR efficiency.
- Use primers with no partial mismatches in the target sequence.
- Optimize reagent and primer concentrations for each amplification reaction.
- Determine thermal cycle settings which eliminate non-specific priming.
- Use the minimum number of PCR cycles to obtain a sufficient quantity of DNA; 30 cycles or fewer for 100 ng of genomic DNA.

The products of amplification reactions are routinely evaluated for purity by electrophoresis in highly resolving agarose gels, such as NuSieve® GTG®, NuSieve® 3:1 or MetaPhor® Agarose products. These artifacts will be detected in MDE® Gels and may interfere with the identification of SSCP bands. Therefore, it is critical to use PCR conditions which minimize unwanted side products. PCR artifact products which differ by 10 to 20 base pairs from the expected product can be resolved on the NuSieve® Agarose gels (4%). PCR artifact products which differ by 4 bp to 12 bp from the expected product can be resolved on a 4% MetaPhor® Agarose gel.

Gel Preparation and Pouring

Assemble glass plates according to manufacturer's instructions. Use 0.4 mm spacers of uniform thickness.

MDE® Gel Solution is supplied as a 2X liquid concentrate. Table 1 lists the components required for 100 ml of 0.5X prepared MDE® Gel Solution. Adjust volumes accordingly.

TABLE 1
Preparation of a 0.5X MDE® Gel Solution (100 ml volume)

Component	Amount
MDE® Gel Solution (2X)	25 ml
AccuGENE® 10X TBE Buffer	6 ml
Deionized water (fill to)	100 ml
TEMED	40 µl
10% APS (prepare fresh)	400 µl

1. Place the specified quantity of the first three components into a clean beaker and mix gently by swirling. The addition of 5% to 10% glycerol to the gel solution is sometimes helpful in resolving SSCP bands.
2. Filter the solution through Whatman® #1 filter paper or a Nalgene® cellulose acetate filter (≤ 0.45 µm).
3. Degas the solution for 5 minutes to remove bubbles.
4. Add the specified amounts of TEMED and 10% APS.
5. Mix by inversion.
6. Pour the gel solution into the plates using the standard procedure for acrylamide.
7. Insert comb (in an inverted position if using a sharkstooth comb)
8. Allow the gel to polymerize for at least 60 minutes at room temperature.
   NOTE: We do not recommend storing the gel overnight. Prolonged storage has been known to result in disadhesion of MDE® Gels from the glass plates.
9. Gently remove the comb, and rinse the wells or top surface of the gel with buffer.
10. Mount the gel cassette onto the sequencing apparatus according to the manufacturer's instructions.
11. Prepare sufficient quantity of running buffer to fill both anodal and cathodal chambers by diluting 10X TBE stock to 0.6X.

Electrophoresis

1. After thermal cycling, add 1 µl of the PCR product to 9 µl of stop solution (95% formamide; 10 mM NaOH; 0.25% bromophenol blue and 0.25% xylene cyanol).
2. Heat to 94°C for 3 minutes and place denatured DNA in an ice water bath.
3. Rinse the top of the gel thoroughly with running buffer.
4. Reinsert sharkstooth comb so the teeth just touch the gel.
5. Load 1 µl-3 µl of sample.
6. Run the samples through the MDE® Gel at 6-8 watts constant power for 14 hours at room temperature.
   We recommend running the following control samples on your gels. Run a molecular weight standard. If the standard is not separating as it should, this can indicate problems with electrophoresis conditions. Also run a non-denatured sample. This will indicate the purity of your PCR product.

GelStar® Nucleic Acid Stain

If you wish to detect the DNA non-radioactively, you can detect the SSCP bands with GelStar® Nucleic Acid Stain (Lonza Catalog No 50535), which can detect DNA at quantities less than 100 pg. Protocols for staining SSCP gels are included with the product or can be obtained by contacting Technical Service Department. To prevent gel swelling when staining MDE® Gels, 50% glycerol may be added to the staining solution.

If you wish to silver stain, several staining kits are commercially available. Call our Technical Service Department.

Autoradiography

The DNA should be labeled during the PCR reaction following standard procedures. When the run is complete, turn off the power supply, disconnect electrodes and remove the plates from the apparatus.

Allow the plates to cool briefly before separating. Transfer the gel onto Whatman® 3MM filter paper.

Place the paper on a flat surface with the gel side up and cover with plastic wrap.

Dry the gel and expose to X-ray film using standard techniques.

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Troubleshooting for SSCP Analysis

Symptoms	Causes	Solutions
Fuzzy Bands	PCR† reactions not optimized	Run control, non-denatured PCR products on MDE® Gel. There should be only a single, clear band.
	Buffer breakdown	There should be no precipitate in the TBE stock solution. Prepare fresh if precipitate is present.
	Samples overheated	Do not heat samples to 100°C. Heat samples at 94°C in formamide stop solution for 3 minutes, then place denatured DNA in ice water bath.
	Gel not polymerized properly	Use fresh ammonium persulfate.
	Samples not fully denatured	Follow denaturation protocol.
	Wrong loading buffer	Use recommended loading buffer. Other formamide loading buffers without NaOH can cause loss of resolution for some samples.
SSCP not working (no band shift)	Conditions not optimized	SSCP relies on differential folding of samples based on sequence. For this to occur, conditions must be optimized for this particular sample. Temperature, gel glycerol content and wattage (heat) may all affect the folding of the strands. These parameters sometimes have to be optimized through a trial and error process.
Many bands in SSCP sample appear	PCR not optimized	If there is not a single PCR product, many bands will appear. Run a non-denatured control on the SSCP gel.
	Running at too low a temperature	DNA strands with a high GC content can form several stable conformation variants, particularly with low temperature (4°C) running conditions.
	Incomplete denaturing	Reannealed PCR products may be present following denaturing. Follow denaturing protocol.
Gel sticking or swelling to plates	Silanization problems	Strip plates by soaking in 2 N NaOH for 30 minutes, then resilanize one plate only.
Smiling or frowning	Heat dissipation problems	Use buffer-backed or an aluminum plate-backed sequencing system.
	Gel not of even thickness	The pressure of gel clamps should be on the center of side spacers. Do not place clamps across the bottom of the gel when casting. This may cause the bottom of the gel to be thinner in the middle, leading to field distortions.

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SSCP Analysis with MDE® Gel Solution on the ABI Prism® 377 DNA Sequencer

Guidelines for Preparation of PCR Samples

It is critical to use PCR† conditions which minimize unwanted products, as these can result in artifact bands which interfere with the recognition of SSCP bands. You should isolate DNA samples and perform PCR according to standard methods, but the tips below will help you get the best results:

- Use only highly purified, salt-free template DNA. The presence of excess salts may alter the hybridization temperature of the primer; thus decreasing the PCR efficiency.
- Use fluorescent-labeled forward and reverse primers with no partial mismatches in the target sequence.
- Optimize reagent and primer concentrations for each amplification reaction.
- Determine thermal cycle settings which eliminate non-specific priming.

Gel Preparation and Pouring

Ensure the glass plates are thoroughly clean and dust free. Glass plates can accumulate fluorescent contaminants from many sources including detergents, marker pens, ethanol and other solvents, poor quality water, and hands or gloves. These invisible contaminants can seriously affect data collection.

Assemble glass plates according to manufacturer's instructions.

MDE® Gel Solution is supplied as a 2X liquid concentrate. Table 2 lists the components required for 40 ml of 0.5X prepared MDE® Gel Solution. This volume is sufficient for plates with a 36-cm well-to-read distance. Decrease the volumes proportionately for smaller sized plates.

TABLE 2
Preparation of a 0.5X MDE® Gel Solution (40 ml Volume)

Component	Amount
MDE® Gel Solution (2X)	10 ml
AccuGENE® 10X TBE Buffer	2.4 ml
Glycerol (optional)	2.0 g
Deionized water (fill to)	40 ml
TEMED	25 µl
10% APS (prepare fresh)	200 µl

1. Place the specified quantity of the first three components into a clean beaker and mix gently by swirling.
2. Filter through a 0.2 µm filter.
3. Add the APS and TEMED and swirl gently to avoid introducing bubbles.
4. Immediately cast the gel and allow to polymerize for 2 hours before electrophoresing.
5. Mount the gel cassette onto the sequencing apparatus according to the manufacturer's instructions.
6. Gently remove the comb.
7. Rinse the wells or top surface of the gel with buffer.
8. Prepare sufficient quantity of running buffer to fill both anodal and cathodal chambers by diluting 10X TBE stock to 1.0X.

Electrophoresis

After thermal cycling, add 1 µl of the PCR product to 3 µl of deionized formamide, 0.5 µl GeneScan® size standard,0.5 µl of 100 mM NaOH and 0.5 µl of Blue dextran EDTA (50 mM EDTA containing 50 mg/ml Blue dextran), heat to 90°C for 2 minutes and immediately place denatured DNA in ice water bath. Load the sample volume appropriate for the type of comb used.

Do not pre-run the gel for more than a few seconds. Pre-running is not necessary for SSCP analysis. If you decide to use the ABI Prism® chiller apparatus, follow use instructions provided by ABI.

For a 36 cm gel, run at 2140 volts limiting, 200 watts and 60 mA for 6-11 hours at 30°C. Run 12 cm plates, run at 750 Volts limiting 200 watts and 60 mA for 2-4 hours at 30°C. Analyze the results with the GeneScan® software.

Heteroduplex Analysis with MDE® Gel Solution
Guidelines for preparation of PCR samples for heteroduplex analysis

It is critical to use PCR† conditions which minimize unwanted products, as these can result in artifact bands which interfere with the recognition of heteroduplex bands. You should isolate DNA samples and perform PCR according to standard methods, but the tips below will help you get the best results:

- Use only highly purified, salt-free template DNA. The presence of excess salts may alter the hybridization temperature of the primer, thus decreasing the PCR efficiency.
- Use primers with no partial mismatches in the target sequence.
- Optimize reagent and primer concentrations for each amplification reaction.
- Determine thermal cycle settings which eliminate non-specific priming.
- Use the minimum number of PCR cycles to obtain a sufficient quantity of DNA; 30 cycles or fewer for 100 ng of genomic DNA.

The products of amplification reactions are routinely evaluated for purity by electrophoresis in highly resolving agarose gels, such as NuSieve® GTG®, NuSieve® 3:1 or MetaPhor® Agarose products. PCR artifacts will be detected in MDE® Gels and may interfere with the identification of heteroduplex bands. Therefore, it is critical to use PCR conditions which minimize unwanted side products. PCR artifact products which differ by 10 to 20 base pairs from the expected product (size range 200 bp to 600 bp) can be resolved on NuSieve Agarose gels (4%). PCR artifact products which differ by 4 bp to 12 bp from the expected product can be resolved on a MetaPhor Agarose gel (4%).

Gel Preparation and Pouring

Glass plates must be clean and free of dried gel and soap residues. To remove residues, apply ethanol to both plates and wipe dry.

Ensure that the gel will not stick to the glass plates by treating one plate with Gel Slick® Solution (Lonza Catalog No.50640) or similar anti-stick product. (If plates were previously silanized, the coating must be removed completely prior to Gel Slick Solution application.)

Assemble glass plates according to manufacturer's instructions. We recommend using a sequencing sized gel with 1 mm spacers. If large DNA mobility shifts are found, it may be possible to use smaller sized gels.

MDE® Gel Solution is supplied as a 2X liquid concentrate. (A 40 cm x 20 cm x 1 mm gel requires 80 ml of the prepared 1X gel solution.) Prepare the right amount for your size plates by adjusting each component in Table 3 proportionately.

TABLE 3

Preparation of 1X MDE® Gel Solution (100 ml volume)

Component	Amount
MDE® Gel Solution (2X)	50 ml
AccuGENE® 10X TBE Buffer	6 ml
Urea (optional)*	15 g
Deionized water (fill to)	100 ml
TEMED	40 µl
10% APS (prepare fresh)	400 µl

*The inclusion of 15% (w/v) urea (a non-denaturing concentration) in the MDE® Gel sometimes helps to minimize band broadening and the appearance of doublets in the homoduplex wild-type control.

1. Place the specified quantity of the first three components into a clean beaker.
2. Mix gently by swirling.
3. If desired, filter the solution through Whatman® #1 filter paper or a Nalgene® cellulose acetate filter (≤0.45 µm).
4. Degas the solution for 5 minutes to remove bubbles.
5. Add the specified amounts of TEMED and l0% APS
6. Mix the solution by gently swirling.
7. Pour the gel solution into the plates using the standard procedure for acrylamide.
8. Insert comb (a well-forming comb is preferable to a sharkstooth comb for 1.0 mm gels).
9. Allow the gel to polymerize for at least 60 minutes at room temperature. We do not recommend storing the gel overnight. Prolonged storage has been known to result in disadhesion of MDE® Gels from the glass plates.
10. Remove the comb, and rinse the wells with buffer.
11. Mount the gel cassette onto the sequencing apparatus according to the manufacturer's instructions.
12. Prepare a sufficient quantity of 0.6X running buffer to fill both anodal and cathodal chambers by diluting 10X TBE stock.

Electrophoresis

1. After thermal cycling, inactivate the DNA polymerase by adding EDTA to a final concentration of 5 mM (1 µl of 0.5 M EDTA per 100 µl reaction). If you are testing for homozygous mutant alleles, mix equal amounts of amplified wild-type DNA and test sample DNA.
2. Heat the reaction mixture for 3 minutes at 94°C.
3. Slowly cool (1°C/minute) the reaction mixture to 37°C.
4. Add 1 µl Triple Dye Loading Buffer (Lonza Catalog No.50632) for each 5 µl of sample and mix well.
5. Rinse the wells thoroughly with running buffer, and then load the samples.
6. Load at least one lane with 5 µl of Heteroduplex Control DNA (regardless of the size of the gel). The control DNA

is supplied in Triple Dye Loading Buffer ready for use.

7. Load at least one lane of Lonza 50 bp to 2500 bp DNA marker (Lonza Catalog No. 50631) or another appropriate DNA size marker, prepared in Triple Dye Loading Buffer as described above. To test the heating and reannealing steps, run one wild/wild PCR[†]product which has not been heated and cooled, and a known wild/wild fragment that has been heated and cooled. These should both give single bands.
8. Run the gel at a constant voltage of 20 V/cm. For a 40 cm plate the power supply would be set for 800 volts. For a fast run, set voltage to 30-35 V/cm. For a 40 cm plate the power supply would be set for 1250-l400 volts. The run time is directly proportional to PCR fragment size. Table 4 can be utilized as a guide in determining optimal run time.

TABLE 4

Dye migration in 1X MDE® Gels (0.6X TBE)

Dye	Comigration Point
Orange G	Ion front
Bromophenol blue	70 bp
Xylene cyanol	230 bp

Ethidium Bromide Staining

1. After the run is completed, remove one of the glass plates.
2. Leave the MDE® Gel adhered to the other plate to facilitate handling during the staining and destaining steps.
3. Stain 5 to 15 minutes at room temperature in 1µg/ml ethidium bromide solution (made in 0.6X TBE or H_20).
4. Destain the gel for 10 to 15 minutes in 0.6X TBE buffer. Destaining for up to 1 hour may be necessary to completely eliminate background that could obscure faint bands.
5. Invert the plate (gel side down) on a UV-transilluminator to visualize bands.
6. For easier handling, cut the part of the gel containing the area of interest. Place the gel on UV-transilluminator to visualize the DNA bands.

GelStar® Nucleic Acid Stain or Silver Staining

If you are dissatisfied with the strength of the bands you detect with ethidium bromide, you may wish to detect the bands with GelStar® Nucleic Acid Stain (Lonza Catalog No. 50535), which can detect DNA at quantities less than 100 pg. To prevent gel swelling when staining MDE® Gels, 50% glycerol should be added to the staining solution.

If you wish to silver stain, several staining kits are commercially available. Call your local Lonza representative.

Analysis of Results

The Heteroduplex Control DNA lane should contain two bands: a slower moving heteroduplex DNA and a faster moving homoduplex band (heteroduplex bands often run as a single band). Homozygous normal or mutant samples are expected to migrate as a single band (homoduplex). Depending on the mutation, the PCR products of heterozygous DNA will form up to four bands: mutant/mutant, normal/normal homoduplexes, plus two mutant/normal heteroduplexes. When using ethidium bromide staining, bands may appear broader since this staining technique requires more DNA to be loaded on the gel.

Troubleshooting for Heteroduplex Analysis

Symptoms	Causes	Solutions
Fuzzy bands	PCR† reactions not optimized	Run control, melted and unmelted non-heteroduplexed PCR products on MDE® Gel. There should be only a single, clear band for each control.
	Wrong loading buffer	Use only a sucrose based, non-denaturing loading buffer. Glycerol or Ficoll® loading buffer can produce fuzzy bands.
	Buffer breakdown	There should be no precipitate in the TBE stock solution. Prepare fresh if precipitate is present.
	Sample overheating	Boiling the samples to form heteroduplexes can cause the DNA to degrade. Heat samples to 94°C for 3 to 5 minutes, then cool slowly **(**1°C/min*)* to 37°C.
	Gel polymerization problems	Use fresh ammonium persulfate.
	Unstable heteroduplex	Add 15% urea to the gel which can sometimes help decrease conformation band fuzziness. This may be a result of stabilizing the heteroduplex, but the exact mechanism is not known.
Faint ethidium bromide	Not enough DNA	Check the markers. If these stain well, and the PCR staining do not stain well, more DNA is required
No heteroduplex seen	Too short a migration distance	Fragments ≤300 bp should travel at least 25 cm in the MDE® Gel. Fragments >300 bp should travel at least 30 cm in the MDE® Gel.
	Check the heteroduplex	If this does separate, the problem could be with the sequence that is analyzed. The suspected mismatches should be in the middle of the PCR fragment. Some sequences may not be suitable for heteroduplex analysis. Consider SSCP analysis.
Gel Swelling	Gel not adhered to plate	Over silanization or silanization of both plates can cause this. Strip plates in 2 N NaOH for 30 minutes and resilanize one plate.
Bands smiling or frowning	Uneven heat distribution	Use buffer-backed or aluminum-backed sequencing gel systems. Decrease voltage to reduce heat generation.
Worm-like pattern on gel	Gel disadhesion from glass plate	After casting, gels should not be stored overnight. Gels stored longer than necessary can disadhere from the glass plates, resulting in a worming pattern.
Bands appear as smears	If using radioactivity, over-exposure of gel to the film can cause the appearance of smearing.	Expose gel to film for shorter period of time.

Ordering Information

Catalog No.	Description	Size
50620	MDE® Gel Solution	250 ml
50622	MDE® Heteroduplex Kit	250 ml

Related Products

GelStar® Nucleic Acid Gel Stain
Gel Slick® Solution
DNA Marker
SYBR® Green I Gel Stain
AccuGENE® TBE Buffer

For more information contact Technical Service at (800) 521-0390 or visit our website at www.lonza.com

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†The PCR process may be covered by one or more third-party patents.

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