

# MIDAScan™ AT Mutation Detection Kit

Cat #: 4072-100-K

Heteroduplex Mutation Analysis for  
Detection of C/G to T/A Transitions  
and C/G to A/T Transversions in DNA

## Contents

<u>Section</u>	<u>Page</u>
I. Introduction	2
II. Materials Supplied in Kit	2
III. Additional Materials Required but Not Supplied	2
IV. Protocol	2
A. Amplicon Preparation	2
B. Labeling	3
C. Heteroduplex Formation and Cleavage	3
Procedure I	3
Procedure II	4
Procedure III	4
D. Electrophoretic Separation	5
E. MIDAScan Control Sequences	5
V. References	5
VI. Cleavage Products of Control Sequences	6
VII. Reagent and Buffer Composition	8
VIII. Troubleshooting Guide	10

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## I. Introduction

C/G to T/A or A/T transitions/transversions represent the most common somatic point mutations in prokaryotic and eukaryotic DNA (1). The MIDAScan™ Mutation Detection Kit accurately detects and localizes C to T, C to A and C to G point mutations and single nucleotide polymorphisms in sequences of DNA up to 500 base pairs in length without the high background normally associated with mutation detection technologies employing bacteriophage resolvases. In the MIDAScan approach (see Figure 1, p. 6), regions of genomic DNA or cDNA of interest are amplified by PCR, mixed together, denatured, and annealed to form heteroduplexes. The amplified DNA is treated with the DNA mismatch repair enzymes MutY and TDG. MutY recognizes A/G mismatches and cuts the strand containing the A. TDG recognizes T/G mismatches and cuts the strand containing the T. At G/G mismatches, TDG cuts both strands, but not simultaneously, leaving a nick in the DNA. Both enzymes cut only at single base mismatches and not at bubbles, cruciforms, or sites of 2 or more base deletions. Thus, unlike bacteriophage resolvases, reannealing artifacts arising during heteroduplex formation are not substrates for these enzymes. Output is gel-based, with clear and unambiguous banding patterns. Cleavage products can be resolved in less than 10 minutes on a rapid denaturing polyacrylamide gel and detected by autoradiography following a 2 hour exposure. The formation of cleavage products indicates the presence of a mutation, and the size of the fragments localizes the mutation site. Finally, the specificity of the DNA repair enzyme helps to identify the mutated base change.

## II. Materials Supplied in Kit

<b>Component</b>	<b>Concentration</b>	<b>Amount Provided</b>	<b>Storage Temp.</b>	<b>Catalog Number</b>
<i>E. coli</i> MutY Enzyme	1 Unit/μl	100 μl	-20°C	4072-100-01
Thermostable TDG Protein	1 Unit/μl	100 μl	-20°C	4072-100-02
10X REC™ Buffer 4	10X	500 μl	4°C	3900-500-04
MIDAScan Control Sequence A	1X	20 μl	-20°C	4075-020-01
MIDAScan Control Sequence B	1X	20 μl	-20°C	4075-020-02
3X Alkaline Loading Buffer	3X	500 μl	4°C	4017-500
T4 Polynucleotide Kinase	5 Units/μl	25 μl	-20°C	9500-25-05
10X Kinase Buffer	10X	100 μl	-20°C	9500-25-01
Distilled water	1X	3.75 ml	-20°C	4667-50-05

## III. Additional Materials Required but not Supplied

1-20 μl pipettor	Thermostable DNA polymerase, nucleotides, templates and primers
10-100 μl pipettor	PCR product purification kits
Thermocycler for PCR	Power supply
Film cassettes	Electrophoresis apparatus (Novex QuickPoint™ recommended)
X-ray film	Microcentrifuge
Film developing setup	Disposables: 0.5 μl and 1.5 μl microcentrifuge tubes, pipet tips
γ- <sup>32</sup> P-ATP	Nucleotide Removal Kit (Qiagen Inc., QIAquick, Cat# 28304)

## IV. Protocol

### A. Amplicon Preparation

MIDAScan utilizes the DNA repair enzymes MutY and TDG to cleave at A/G, and T/G or G/G base mismatches, respectively, within DNA heteroduplexes formed between PCR amplicons from a known reference sample and test sample. We recommend that the reference and sample PCR amplicons be gel purified from an agarose gel prior to MIDAScan analysis. DNA gel extraction kits from Qiagen (Cat# 28704) and Life Technologies (Cat# 11456-019) work well for this purpose. If an aliquot of the PCR reaction shows a single band of the correct size on an agarose gel, then the amplicons need only be cleaned up with PCR purification kits (Qiagen, Cat# 28104 or Life Technologies, Cat# 11458-015) to remove unincorporated primers and deoxynucleotides. The purified amplicons should be dissolved in 25-50 μl of 10 mM Tris-Cl (pH 8.0) or another low ionic strength buffer with a final concentration of 50 ng/μl or greater. A distinct band of ethidium bromide stained DNA should be observed when 2 μl of the amplicon are run on an agarose gel.

## B. Labeling

In order to detect all possible mutations, both the reference and the sample amplicon must be radiolabeled. We provide two purified control amplicons in this kit (MIDAScan™ Sequences A and B) and we highly recommend that you use these controls to familiarize yourself with the assay. Perform the following labeling procedure in a 0.5 ml tube:

Purified amplicon, 50 ng/μl (reference, sample, or MIDAScan Amplicon A or B)	2-5 μl
10X T4 Polynucleotide Kinase Buffer	2 μl
γ- <sup>32</sup> P-ATP (3000-6000 Ci/mmole)	5 μl
T4 Polynucleotide Kinase	1 μl
dH <sub>2</sub> O	to 20 μl

Incubate at 37°C for 30 minutes, 75°C for 10 minutes, and then hold at 4°C. Remove unincorporated γ-<sup>32</sup>P-ATP with QIAquick Nucleotide Removal Kit (Qiagen Inc., Cat# 28304) or QIAquick PCR Purification Kit (Qiagen Inc., Cat# 28104). Follow the instructions provided by the manufacturer and recover the <sup>32</sup>P-labeled amplicon, which should now be free of unincorporated γ-<sup>32</sup>P-ATP. Measure the number of CPMs/μl and adjust the volumes to a constant number of CPMs/μl between samples. The final concentrations of the labeled amplicons are now about 5 ng/μl and should have approximately 10,000 cpm/μl.

Alternatives to radioactive detection include the incorporation of FAM (6-carboxyfluorescein) on the 5'-end of the forward PCR primer and/or TET (4,7,2',7'-tetrachloro-6-carboxyfluorescein) on the 5'-end of the reverse PCR primer. Cleavage products are analyzed on an automated DNA sequencer with fluorescence detection.

## C. Heteroduplex Formation and Cleavage

At least three procedures are possible, designated Procedure I, Procedure II, and Procedure III, each providing varying levels of mutation information. In each procedure, the controls for the heteroduplexes are the corresponding homoduplexes, both treated with DNA repair enzyme. Any band that appears in the heteroduplex lanes and not in the homoduplex lanes must arise from a point mutation in the sample or test amplicon.

### Procedure I:

In 0.5 ml tubes mix:

	1	2	3	4	5	6
<sup>32</sup> P-sample/test amplicon	x μl	--	x μl	x μl	--	x μl
<sup>32</sup> P-reference amplicon	--	y μl	y μl	--	y μl	y μl
10X REC Buffer 4	2 μl	2 μl	2 μl	2 μl	2 μl	2 μl
dH <sub>2</sub> O	to 19 μl	to 19 μl	to 19 μl	to 19 μl	to 19 μl	to 19 μl

Where x and y are volumes required for 20,000 cpm of sample and reference amplicons, respectively. Place the tubes in a thermocycler. Heat to 95°C for 5 minutes, ramp the temperature slowly down to 65°C over a 5 minute period, and hold at 65°C for 30 min. Cool to room temperature or to 4°C. The samples are now ready for treatment with MutY or TDG. Lanes 1, 2, 4, and 5 are the homoduplex controls for the heteroduplexes in lanes 3 and 6. Note that both strands in the heteroduplexes will be labeled and all possible cleavage products will be observed.

Add the MutY and TDG to the tubes as follows:

	1	2	3	4	5	6
<i>E. coli</i> MutY	1 μl	1 μl	1 μl	--	--	--
Thermostable TDG Protein	--	--	--	1 μl	1 μl	1 μl

Incubate the tubes with MutY and Thermostable TDG protein at 37°C and 65°C, respectively, for 60 minutes. Stop the reactions by adding 10 μl of 3X Alkaline Loading Buffer to each tube. The samples are now ready for denaturing polyacrylamide gel electrophoresis.

### **Procedure II:**

An even simpler approach is to follow the procedure described above, but digest the heteroduplexes with MutY and TDG in the same tube. Set up the following tubes:

	<b>1</b>	<b>2</b>	<b>3</b>
<sup>32</sup> P-sample amplicon	x $\mu$ l	--	x $\mu$ l
<sup>32</sup> P-reference amplicon	--	y $\mu$ l	y $\mu$ l
10X REC Buffer 4	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l
dH <sub>2</sub> O	to 18 $\mu$ l	to 18 $\mu$ l	to 18 $\mu$ l

Where x and y are volumes required for 20,000 cpm of sample and reference amplicons, respectively. Place the tubes in a thermocycler. Heat to 95°C for 5 minutes, ramp the temperature slowly down to 65°C over a 5 minute period, and hold at 65°C for 30 min. Cool to 4°C or to room temperature. The samples are now ready for treatment with MutY or TDG. Lanes 1, and 2, are the homoduplex controls for the heteroduplexes in lane 3. Add the MutY, followed by TDG to the tubes as follows:

	<b>1</b>	<b>2</b>	<b>3</b>
<i>E. coli</i> MutY	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l
Thermophilic TDG Enzyme	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l

Incubate the tubes at 37°C for 45 minutes in the presence of MutY. Then add the Thermostable TDG enzyme and continue the incubation at 65°C for 45 minutes in a thermocycler. Stop the reactions by adding 10  $\mu$ l of 3X Alkaline Loading Buffer to each tube. The samples are now ready for denaturing polyacrylamide gel electrophoresis.

### **Procedure III:**

This procedure provides the most information about the type and location of the mutations in your test sample. Set up the following tubes:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>
<sup>32</sup> P-sample amplicon	x $\mu$ l	x $\mu$ l	x $\mu$ l	x $\mu$ l	--	--	--	--
<sup>32</sup> P-reference amplicon	--	--	--	--	y $\mu$ l	y $\mu$ l	y $\mu$ l	y $\mu$ l
Unlabeled sample amplicon	1 $\mu$ l	--	1 $\mu$ l	--	--	1 $\mu$ l	--	1 $\mu$ l
Unlabeled reference amplicon	--	1 $\mu$ l	--	1 $\mu$ l	1 $\mu$ l	--	1 $\mu$ l	--
10X REC Buffer 4	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l
dH <sub>2</sub> O	to 19 $\mu$ l	to 19 $\mu$ l	to 19 $\mu$ l	to 19 $\mu$ l	to 19 $\mu$ l	to 19 $\mu$ l	to 19 $\mu$ l	to 19 $\mu$ l

Where x and y are volumes required for 20,000 cpm of sample and reference amplicons, respectively. Place the tubes in a thermocycler. Heat to 95°C for 5 minutes, ramp the temperature slowly down to 65°C over a 5 minute period, and hold at 65°C for 30 min. Cool to 4°C or to room temperature. Add MutY and TDG to the tubes as follows:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>
<i>E. coli</i> MutY	1 $\mu$ l	1 $\mu$ l	--	--	1 $\mu$ l	1 $\mu$ l	--	--
Thermophilic TDG Protein	--	--	1 $\mu$ l	1 $\mu$ l	--	--	1 $\mu$ l	1 $\mu$ l

Incubate the tubes with MutY and Thermostable TDG protein at 37°C and 65°C, respectively, for 60 minutes. Stop the reactions by adding 10  $\mu$ l of 3X Alkaline Loading Buffer to each tube. The samples are now ready for denaturing polyacrylamide gel electrophoresis. Note that one strand in each heteroduplex is radiolabeled and the other strand is unlabeled. This procedure was followed to generate the autoradiograph shown in Figure 3, Page 8.

## D. Electrophoretic Separation

### 1. Electrophoresis in a Standard Sequencing Apparatus

Separate the cleavage products on a 6-8% polyacrylamide sequencing gel containing 8 M urea and 0.5X-1X TBE. Use a well-forming comb rather than a sharktooth comb for superior banding patterns. Prepare the gel and pre-run for 15-30 minutes at 50-100 watts, constant power, until the temperature of the gel reaches 50°C or above.

Heat the samples at 95°C for 5 minutes and rapidly cool to room temperature. Centrifuge the tubes briefly to collect all the sample contents to the bottom of the tubes.

Turn off the power supply, rinse the wells with upper electrode buffer and load 5-10 µl of the denatured samples in each well. Depending on your requirements, you may wish to include a sequencing ladder or MIDAScan Sequences A and B, which will generate a series of well-defined cleavage products of known size.

Perform the electrophoresis at 50 watts, constant power, or at a power level sufficient to maintain the gel temperature between 50°C and 65°C. Run the gel until the bromophenol blue dye reaches within 2 cm of the bottom of the gel.

Dry the gel and expose to X-ray film with an intensifying screen at -80°C for 2 hours. Develop the film and examine for cleavage products in the heteroduplex lanes. Note that the optimal exposure time may vary. It is not necessary to dry the gel. However, the optimal exposure time will be longer and the bands will not be as sharp.

### 2. Electrophoresis in the Novex QuickPoint™ Mini-Sequencing Apparatus

We highly recommend that you perform the electrophoresis step in the Novex QuickPoint rapid nucleic acid separation cell (Cat# E19700, phone number 1-800-456-6839) and pre-made QuickPoint gel (Cat# QP9731). Follow the manufacturer's instructions and load 1 µl of each sample per well. Run at 1200 volts, constant voltage, until the bromophenol blue is about 5 mm from the bottom of the gel (caution: the electrophoresis will take less than 10 min). Separate the glass plates, fix the gel in 10% acetic acid, 10% alcohol for 10 minutes, wash the gel with deionized water for 10 minutes, and dry the gel with a hair dryer. Expose the gel to X-ray film as described above.

## E. MIDAScan Control Sequences

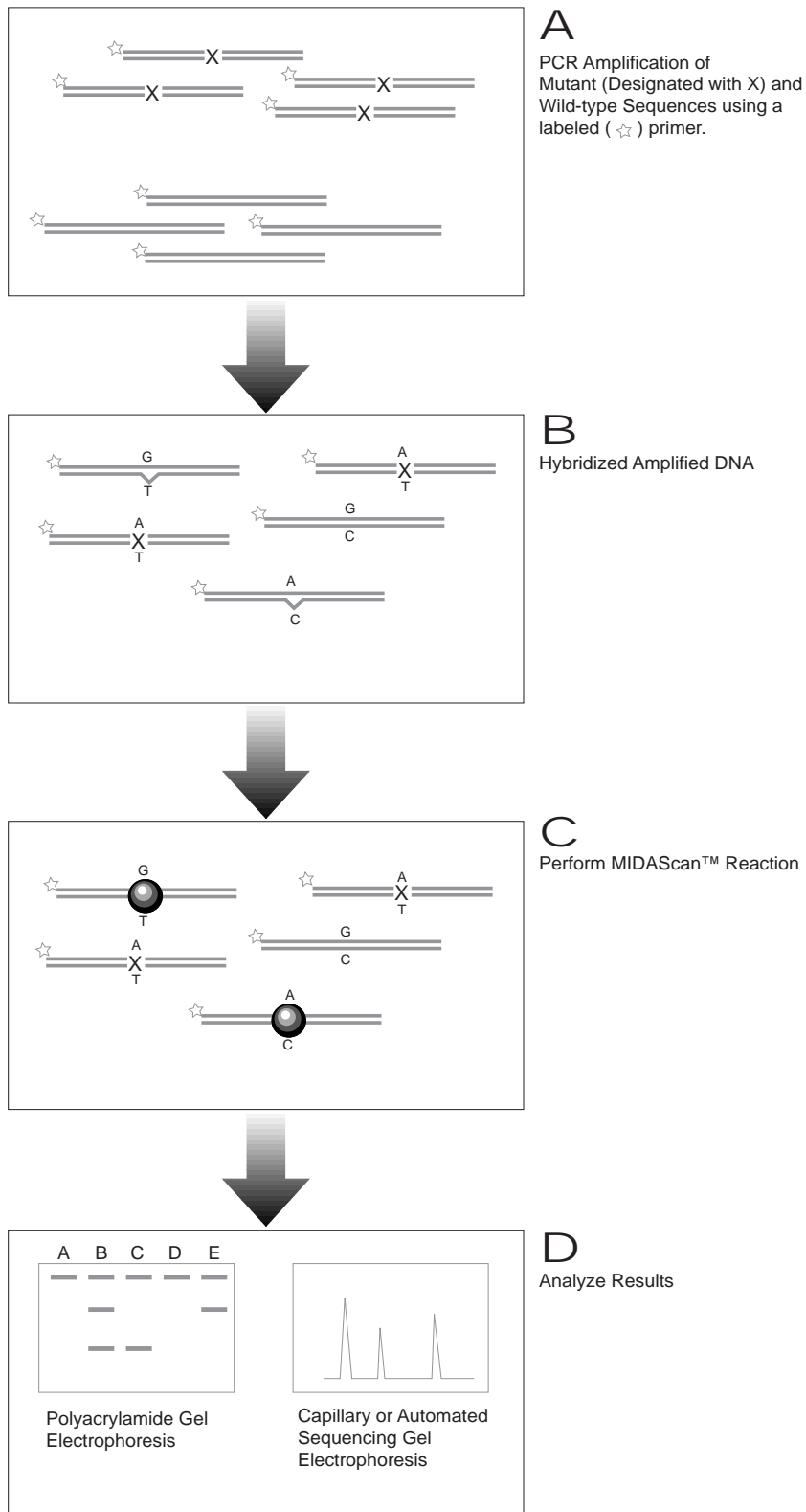
We encourage you to initially work with the MIDAScan Control Sequences A and B to gain familiarity with the assay. These control amplicons will provide you with a series of cleavage products of known size that can substitute as a sequencing ladder for approximating the position of the mutations in your experimental samples. These amplicons are 270 bp in length and have a common sequence except at the highlighted base pairs (see Figure 2, page 7). When the MIDAScan amplicon sequences A and B are mixed together, denatured and reannealed, two heteroduplexes are generated with a series of base mismatches regularly spaced by 20 base pairs. MutY cleaves at A/G mismatches and cuts the strand with the A. TDG cleaves at T/G mismatches and cuts the strand with the T. TDG also cleaves at G/G mismatches and cuts one strand or the other. The predicted cleavage products are listed in Table I, p. 7.

For up to 25 lanes on the Novex QuickPoint, or 10 lanes on a regular sequencing apparatus, label 5 µl of each MIDAScan Amplicon Sequence A and B and follow the heteroduplex analysis protocol described above. A typical banding pattern on the Novex QuickPoint separation system is shown in Figure 3, p. 8.

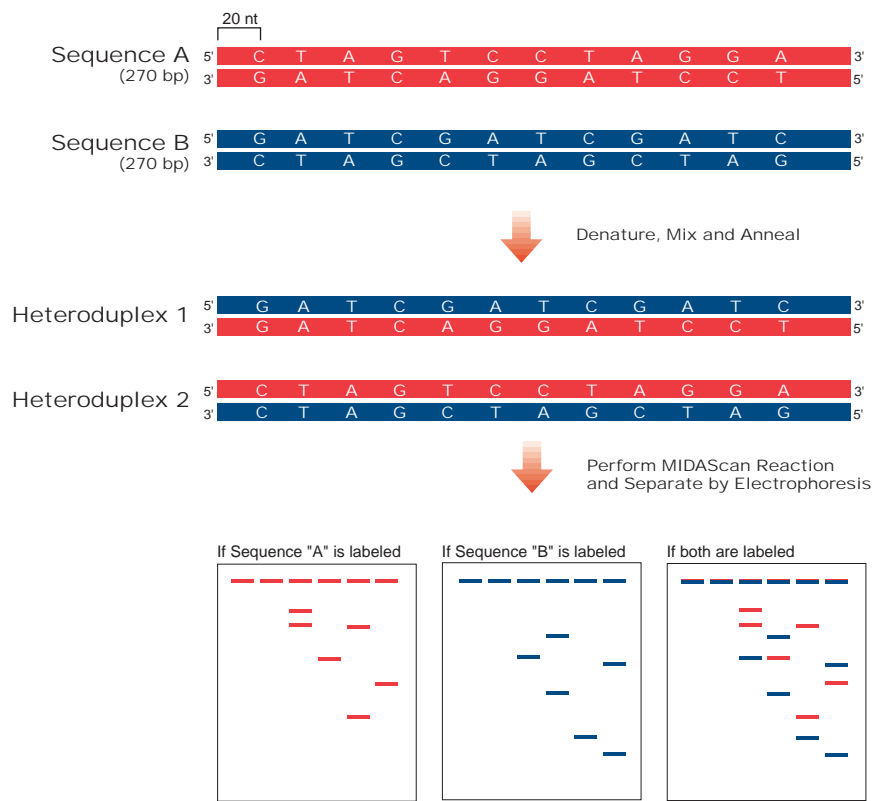
## V. References

1. Hsu I.C. 1997. Use of MutY and Thymine Glycosylase to Detect Point Mutations in "Detection of Mutations and Polymorphisms in DNA", CRC Press, p.195-205.

**Figure 1.** Heteroduplex analysis of point mutations in DNA. Labeled wild type and mutant amplicons are mixed together, denatured, and reannealed to form heteroduplexes. The enzymes MutY and TDG recognize A/G, and T/G and G/G mismatches in the heteroduplexes, resulting in the formation of cleavage products which are resolved by denaturing polyacrylamide gel electrophoresis.



**Figure 2.** Sequences of the MIDAScan Controls and resultant heteroduplexes. The nucleotides listed below are the unique bases in each control sequence and are regularly spaced by 20 bases.



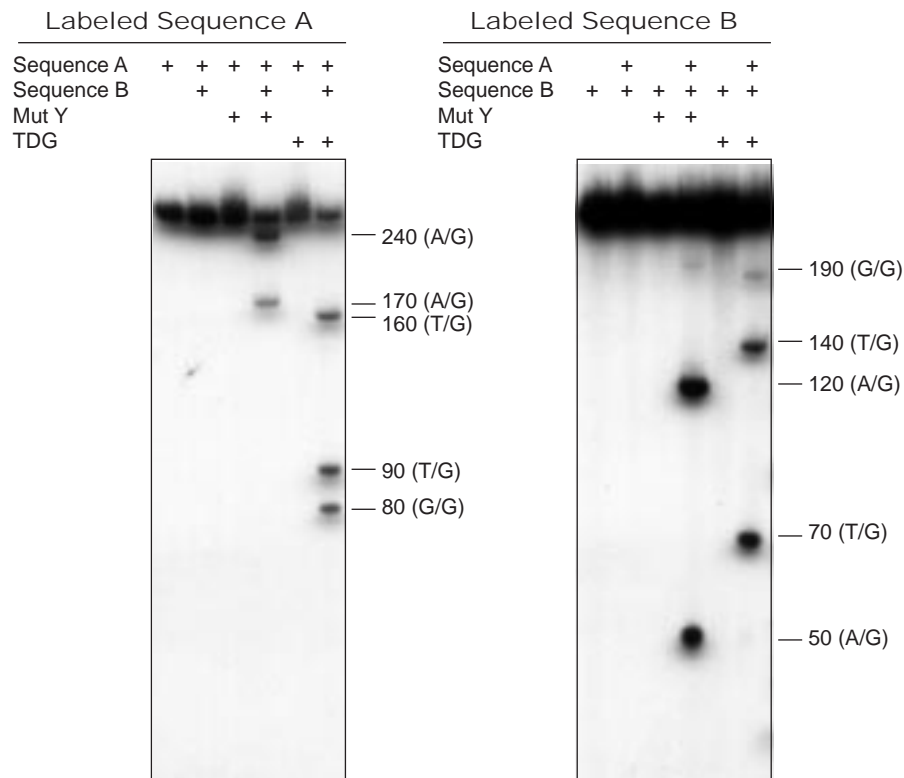
**Table I.** MIDAScan™ Sequences A and B Cleavage Products

Enzyme	Procedure I	Cleavage Products		
		Procedure II	Heteroduplex 1	Heteroduplex 2
			Procedure III	
<i>E. coli</i> MutY	50 bp (A/G)	20 bp (G/G)	170 bp (A/G)*	240 bp (A/G)*
	120 bp (A/G)	50 bp (A/G)	120 bp (A/G)**	50 bp (A/G)**
	170 bp (A/G)	70 bp (T/G)		
	240 bp (A/G)	80 bp (T/G)		
		90 bp (G/G)		
Thermophilic TDG Protein	20 bp (G/G)	20 bp (A/G)	90 bp (T/G)*	80 bp (G/G)*
	70 bp (T/G)	140 bp (T/G)	250 bp (G/G)*	160 bp (T/G)*
	80 bp (T/G)	160 bp (T/G)	20 bp (G/G)**	70 bp (T/G)**
	90 bp (G/G)	170 bp (A/G)	140 bp (T/G)**	190 bp (G/G)**
	140 bp (T/G)	190 bp (G/G)		
	160 bp (T/G)	240 bp (A/G)		
	190 bp (G/G)	250 bp (G/G)		
250 bp (G/G)				

\* 5'-<sup>32</sup>P-MIDAScan Sequence A + Unlabeled MIDAScan Sequence B

\*\* 5'-<sup>32</sup>P-MIDAScan Sequence B + Unlabeled MIDAScan Sequence A

**Figure 3.** Heteroduplex cleavage analysis of MIDAScan Amplicon Sequences A and B. Cleavage products were resolved on a Novex QuickPoint minisequencing gel in less than 10 minutes. Besides the 20 bp cleavage product generated by the TDG protein on Heteroduplex 2 with labeled MIDAScan Sequence B and unlabeled Sequence A (last lane on the right panel), all the predicted bands are accounted for. The 20 bp cleavage product has migrated off the gel, but is observed on the larger standard sequencing gels. The 250 bp cleavage product generated by TDG with labeled MIDAScan Sequence A (last lane on the left panel) can be seen on the autoradiograph itself.



## VI. Reagent and Buffer Composition

<u>Component</u>	<u>Cat. #</u>	<u>Volume</u>	<u>Storage</u>	<u>Composition</u>
10X REC Buffer 4	3900-500-04	500 $\mu$ l	4°C or -20°C	100 mM HEPES-KOH (pH 7.4), 1 M KCl, 100 mM EDTA
MIDAScan Sequence A	4075-020-01	20 $\mu$ l	-20°C	100 ng/ $\mu$ l in 10 mM Tris-Cl (pH 8.0)
MIDAScan Sequence B	4075-020-02	20 $\mu$ l	-20°C	100 ng/ $\mu$ l in 10 mM Tris-Cl (pH 8.0)
3X Alkaline Loading Buffer	4017-500	500 $\mu$ l	4°C or -20°C	300 mM NaOH, 95% Formamide, 0.2% Bromophenol Blue
10X Kinase Buffer	9500-25-01	100 $\mu$ l	-20°C	700 mM Tris-Cl (pH 7.6), 100 mM MgCl <sub>2</sub> , 50 mM DTT
Distilled water	9500-25-03	2 ml	4°C or -20°C	

## ***E. coli* MutY**

<b>Catalog Number:</b>	4072-100-01
<b>Source:</b>	Purified from <i>E. coli</i> containing the recombinant <i>E. coli</i> MutY gene.
<b>Activity:</b>	1.0 Unit/ $\mu$ l
<b>Total Activity:</b>	100 Units
<b>Protein Conc.:</b>	0.2 mg/ml
<b>Total Protein:</b>	20 mg
<b>Specific Activity:</b>	5,000 Units/mg
<b>Unit Definition:</b>	One unit of enzyme cleaves 1 pmole of an oligonucleotide duplex containing an A/G mismatch in 1 hour at 37°C. Only the strand with the A is cleaved.
<b>Specificity:</b>	MutY recognizes A/G, and A/8oxoG mismatches in duplex DNA and cleaves the strand with the A. The opposite strand is not cleaved. Our MutY enzyme exhibits AP endonuclease activity.
<b>Storage Buffer:</b>	10 mM HEPES-KOH, pH 7.4, 100 mM KCl, 1 mM EDTA, and 50% (v/v) glycerol.
<b>Storage:</b>	Store the enzyme at -20°C in a frost-free freezer.

## **Thermophilic TDG Protein**

<b>Catalog Number:</b>	4072-100-02
<b>Source:</b>	Purified from <i>E. coli</i> containing a recombinant plasmid harboring the TDG gene.
<b>Activity:</b>	1.0 Unit/ $\mu$ l
<b>Total Activity:</b>	100 Units
<b>Protein Conc:</b>	20 $\mu$ g/ml
<b>Total Protein:</b>	2 $\mu$ g
<b>Specific Activity:</b>	50,000 Units/mg
<b>Unit Definition:</b>	One unit of enzyme cleaves 1 pmole of an oligonucleotide duplex containing a T/G mismatch in 1 hour at 65°C. Only the strand with the T is cleaved.
<b>Specificity:</b>	TDG enzyme recognizes T/G, and G/G mismatches in duplex DNA and cleaves the strand with the T, or one G strand or the other, respectively.
<b>Storage Buffer:</b>	10 mM HEPES-KOH, pH 7.4, 100 mM KCl, 1 mM EDTA, 0.1 mg/ml BSA, 50% (v/v) glycerol.
<b>Storage:</b>	Store the enzyme at -20°C in a frost-free freezer.

## **T4 Polynucleotide Kinase**

<b>Catalog Number:</b>	9500-25-05
<b>Source:</b>	Purified from <i>E. coli</i> containing a recombinant plasmid harboring the T4 Polynucleotide Kinase gene.
<b>Activity:</b>	10 Units/ $\mu$ l
<b>Total Activity:</b>	250 Units
<b>Unit Definition:</b>	One Richardson Unit is the amount of enzyme required to catalyze the production of 1 nmole of acid-insoluble <sup>32</sup> P in 30 minutes at 37°C.
<b>Specificity:</b>	T4 Polynucleotide Kinase catalyzes the transfer and exchange of the $\gamma$ - <sup>32</sup> P from ATP to the 5'-hydroxyl terminus of single and double-stranded DNA and RNA, and nucleoside 3'-monophosphates.
<b>Storage Buffer:</b>	10 mM Tris-Cl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 0.1 $\mu$ M ATP, and 50% glycerol.
<b>Storage:</b>	Store the enzyme at -20°C in a frost-free freezer.

## VII. Troubleshooting Guide

Problem	Cause	Action
Test samples <u>and</u> control sequences A and B show no cleavage products	Denaturation of the DNA and/or reannealing is not taking place.  Enzymes are inactive.	Ensure that the denaturing temperature is 94°C or greater. Follow the reannealing conditions carefully.  The enzymes are inherently very stable. However, they should be stored at -20°C. Obtain fresh enzyme.
Control sequences A and B display correct cleavage products but the Test samples fail to cut.	Carryover of PCR contaminants into the test samples.  Deletions of two or more bases in the test sample.  The test sample may have a wild type sequence or a point mutation which generates a mismatch in the heteroduplex that is not recognized by MutY or TDG.	Gel purify amplicons or clean with Qiagen or Life Technology PCR purification kits.  MutY and TDG recognize A/G, T/G, and G/G single base mismatches only. Try resolvases or SSCP analysis.  Try SSCP analysis to detect mutations or sequence the test sample amplicon.
High background in test samples.	PCR not optimized. Presence of spurious bands such as primer dimers.	Optimize PCR reactions, including higher annealing temperatures and hotstart PCR. Gel purify amplicons if spurious bands cannot be removed. Optimize choice of primers.
Poor radiolabeling of amplicons.	High salt or EDTA in the amplicons which may inhibit T4 polynucleotide kinase. Primer contamination.	Use Qiagen or Life Technologies PCR purification kits to clean up the amplicons. Dissolve the DNA in 10 mM Tris-Cl, pH 8.0 prior to labeling. Repeat amplicon purification if necessary.
Bands on the autoradiograph appear as sharp points or dots	A sharktooth comb was used.	Use a well-forming comb.
All test samples show a broad smear just below the full length PCR product.	Reannealing of the DNA strands is taking place in the gel. The DNA may not have been sufficiently denatured prior to electrophoresis.	The urea concentration in the gel should be at least 8 M and the gel temperature should be maintained above 50°C.  Increase the denaturing temperature and denaturing time to 95°C and 5 min, respectively. Note that the 3X Alkali Loading Buffer contains 300 mM NaOH and should keep the DNA strands separated once denaturation has occurred.