User Guide for the Transgenomic SURVEYOR™ Mutation Detection Kit for WAVE® and WAVE® HS Systems
For technical support and/or technical tips, please contact Transgenomic Technical Support:

US – Toll Free (888) 233-9283 or 402-452-5400
UK – +44 1670-732-992
Europe – +33 1-30-68-90-00

E-mail: support@transgenomic.com
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Introduction

Transgenomic SURVEYOR™ Mutation Detection Kits use a new mismatch-specific plant DNA endonuclease to scan for known and unknown mutations and polymorphisms in heteroduplex DNA. SURVEYOR Nuclease, the key component of the kit, is a member of the CEL family of plant endonucleases that cleave DNA with high specificity at sites of base-substitution mismatch and other distortions. These DNA endonucleases cut both strands of a DNA heteroduplex on the 3’-side of the mismatch site. Insertion/deletion mismatches and all base-substitution mismatches are recognized, but the efficiency of cleavage varies with the sequence of the mismatch.

DNA endonucleases from celery have been used to detect accurately a variety of mutations and polymorphisms in the human BRCA1 gene. Other applications include high-throughput screening of induced point mutations (TILLING) in Arabidopsis, Lotus, and zebrafish, screening for SNPs in inbred rat strains, and scanning of large regions of bacterial genomic DNA for mutations and polymorphisms. SURVEYOR Nuclease has been used to verify the presence of known mutations in a number of genes in human peripheral blood DNA, to carry out screening for induced point mutations in barley and to screen for error-free clones generated from a plant cDNA library by PCR-based cloning.

The SURVEYOR Mutation Detection Kit for WAVE® and WAVE® HS Systems has been designed to cleave DNA fragments at mismatched sites for subsequent analysis by ion-pairing reverse-phase HPLC using the WAVE and WAVE HS Systems.

Note: Washing procedures specifically recommended and described in this User Guide for the SURVEYOR Mutation Detection Kit for WAVE and WAVE HS Systems and the use of DNASEp® and DNASEp® HT Cartridges are different from those used for standard WAVE DHPLC procedures. Please follow the specific recommendations in this manual to maintain optimum performance of your WAVE or WAVE HS System.

TO USE THIS KIT SUCCESSFULLY, WE STRONGLY RECOMMEND THAT YOU READ THIS MANUAL THOROUGHLY AND CAREFULLY FOLLOW THE INSTRUCTIONS AND GUIDELINES PROVIDED. FIRST TIME USERS SHOULD PERFORM THE CONTROL EXPERIMENTS OUTLINED IN THE SECTION ON USING CONTROL C AND CONTROL G PLASMID DNA.

If you have further questions or need assistance, please contact Transgenomic Technical Support Hotline/Help Desk:

US – Toll Free (888) 233-WAVE (9283) or 402-452-5400
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Europe - +33 1-30-68-90-00

Technical Support E-mail: support@transgenomic.com
SURVEYOR Mutation Detection Kit Components

The kit is available in two sizes:
25-Reaction Kit (Catalog No. 706035)
100-Reaction Kit (Catalog No. 706030)

<table>
<thead>
<tr>
<th>Component</th>
<th>25-Reaction Kit (706035)</th>
<th>100-Reaction Kit (706030)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SURVEYOR Nuclease W</td>
<td>0.03 mL</td>
<td>4 x 0.03 mL</td>
</tr>
<tr>
<td>SURVEYOR Enhancer W</td>
<td>0.03 mL</td>
<td>4 x 0.03 mL</td>
</tr>
<tr>
<td>10X SURVEYOR Reaction Buffer</td>
<td>0.75 mL</td>
<td>0.75 mL</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>0.25 mL</td>
<td>3 x 0.25 mL</td>
</tr>
<tr>
<td>Control C</td>
<td>0.02 mL</td>
<td>0.02 mL</td>
</tr>
<tr>
<td>Control G</td>
<td>0.02 mL</td>
<td>0.02 mL</td>
</tr>
<tr>
<td>H2O</td>
<td>2 x 1 mL</td>
<td>6 x 1 mL</td>
</tr>
</tbody>
</table>

Store all components at –20 °C.

Detecting Mutations with the Transgenomic SURVEYOR Mutation Detection Kit

An Overview

Mutation detection and confirmation with SURVEYOR Nuclease involves four steps:

**Step 1** - Prepare PCR amplicons from mutant (test) and wild-type (reference) DNA.

**Step 2** - Mix equal amounts of test and reference DNA; hybridize them by heating and cooling the mixture to form hetero- and homoduplexes.

**Step 3** - Treat the annealed heteroduplex/homoduplex mixture with SURVEYOR Nuclease. The reference DNA alone, treated similarly, serves as a background control.

**Step 4** - Analyze the DNA fragments with the WAVE or WAVE HS System. The formation of new cleavage products indicates the presence of a mutation, while their size indicates the location of the mismatch.
The combination of these four steps is outlined in the flow chart in Figure 1.

**Figure 1.** A schematic representation of mutation detection using SURVEYOR Nuclease.
Examples of Results

Examples of results obtained using the SURVEYOR Mutation Detection Kit for WAVE and WAVE HS Systems are shown in Figures 2 and 3 below. In these examples, the four-step process outlined in the flowchart on the previous page was followed carefully.

**Figure 2.** SURVEYOR Nuclease digestion products of amplicons derived from 129/Sv and C57BL/6J mouse DNA. The 275-bp amplicons were PCR amplified with Optimase® Polymerase. Amplified 129/Sv DNA was annealed alone or in combination with C57BL/6J DNA. Approximately 600 ng of unpurified amplicon DNA were digested by adding 1 µL of SURVEYOR Enhancer W and 1 µL of SURVEYOR Nuclease W to the DNA and incubating for 20 min at 42°C. After adding Stop Solution, a portion (400 ng) of the digestion products was analyzed by HPLC on a WAVE HS system. The amplicon contained a single SNP (C/G; fragments were 80 + 195 bp long). Digestion products from cross-hybridized DNA and 129/Sv DNA alone are shown in black and red, respectively. Marker (100-bp DNA Ladder; New England BioLabs) is shown in green at the top.

SURVEYOR Nuclease W in combination with a WAVE or WAVE HS system can be used to identify single and multiple mutations in fragments <1,000 bp long. Mutations can also be found in amplified, pooled genomic DNAs. Figure 2 shows SURVEYOR Nuclease digestion products from 275-bp DNA amplified from C57BL/6J mouse DNA and annealed to DNA amplified from 129/Sv mouse DNA. The fragment contains a G/C SNP 80 bp from one end. Digestion products were analyzed on a WAVE HS system.
Figure 3. SURVEYOR Nuclease digestion of heteroduplex DNA present in decreasing amounts in homoduplex DNA. Mouse SNP target DNA shown in Figure 2 was amplified from both mouse strains with Optimase Polymerase. Heteroduplex/homoduplex mixtures were formed at the ratios indicated and 600 ng of each mixture were digested with 1 µL of SURVEYOR Enhancer W and 1 µL of SURVEYOR Nuclease W for 20 min at 42 °C. After adding Stop Solution, a portion (400 ng) of the digestion products was analyzed by HPLC on a WAVE HS system. Digestion products 80 + 195 bp long formed by cutting at a SNP are clearly visible down to a ratio of heteroduplex to homoduplex of 1 in 32. Homoduplex is shown in light blue. Marker (100-bp DNA Ladder; New England BioLabs) is shown in orange at the top.

Figure 3 shows WAVE HS System analysis of SURVEYOR Nuclease digestion products from mixtures of heteroduplex and homoduplex DNA representing the sensitivity of mutation detection in pooled genomic DNAs. SURVEYOR Nuclease digestion products derived from heteroduplex mixed with homoduplex at a ratio of 1 to 32 can still be seen in the chromatogram.
Factors Affecting the Quality of Results

The two key factors influencing the quality of results are signal and noise (background).

Signal

The magnitude of the signal depends upon

- **The quality of the PCR amplified DNA.**
  The presence of high concentrations of primer-dimers in PCR products dramatically inhibits SURVEYOR Nuclease cleavage at mismatch sites. Examine each amplified DNA product before digestion by gel electrophoresis or WAVE HPLC to be sure it is a single species of the expected size.

- **The relative proportion of mutant (test) to wild-type (reference) DNA in the hybridized sample.**
  Test and reference PCR products should be hybridized in equal proportion to maximize the amount of heteroduplex DNA available for digestion, whenever possible. After hybridization of equal amounts of test and reference DNA, on the average half of the resulting DNA molecules will reanneal as homoduplexes and the other half as heteroduplexes; the heteroduplex population will contain two distinct heteroduplexes, each representing approximately 25% of the total population.

- **The efficiency of heteroduplex cleavage.**
  SURVEYOR Nuclease cleaves heteroduplexes with efficiencies that can vary over a broad range. However, each substrate with a mismatch will have at least one heteroduplex species that will be efficiently cleaved at an ideal rate for analysis as described here.

- **Protection of DNA ends.**
  SURVEYOR Nuclease also has 5’-exonuclease activity that attacks the ends of double-stranded DNA, causing chromatography peaks to broaden. This activity can be partially suppressed by the presence of SURVEYOR Nuclease Enhancer without otherwise negatively affecting the reaction. SURVEYOR Nuclease Enhancer is included in this kit.
Noise (Background)

- The fragment pattern obtained by digesting heteroduplex DNA can also reveal the presence of fragments derived from PCR artifacts, e.g. primer-dimers, products from primer mis-priming, and errors introduced by the DNA polymerase itself. A nearly identical background should be present in the digestion pattern of reference DNA, and this background can be identified by visual comparison of test and reference digestion patterns (see Figures 2 and 3). When the quality of the PCR product is poor, the background after SURVEYOR Nuclease digestion can reach a level high enough to obscure the signal. Examine each amplified DNA product before digestion by gel electrophoresis or WAVE HPLC to be sure it is a single species of expected size. If it is not, optimize the PCR conditions until you obtain good quality PCR product. To reduce background due to PCR errors, use a proofreading DNA polymerase such as Optimase Polymerase (Transgenomic Catalog Numbers 703005, 703030 or 703045).

- SURVEYOR Nuclease has 5'-exonuclease activity that gives rise to non-specific background products as digestion time is increased and as the ratio of enzyme to DNA is increased. The SURVEYOR Mutation Detection Kit has been designed to set up optimal reaction conditions that keep this background to a minimum.

- Reaction conditions for SURVEYOR Nuclease recommended in the published literature and in an early version of this kit’s User Guide required dilution of the DNA substrate into a reaction mixture containing a 1X reaction buffer. Subsequent research has shown that digestion of substrate DNA directly in the 1X PCR reaction buffer without any further dilution reduces background and actually results in an improved signal to noise ratio. The signal to noise ratio is generally high enough to detect mutations present at a low percentage of the total mutant and wild-type DNA: 2.5% to 20% mutant DNA depending upon the particular DNA amplicon, its size, the number and type(s) of mutation(s) and the analysis platform. Figure 3 shows detection of heteroduplex present in homoduplex at a ratio of 1 in 32 (3.1% heteroduplex). Figures 4 and 5 show the digestion products generated with homoduplex and heteroduplex Control DNA (included in this kit) fractionated by ion-pairing reverse phase HPLC under non-denaturing conditions using the WAVE and WAVE HS System, respectively. The mutation-specific cleavage products are clearly seen as two new peaks eluting with the expected sizes.

**Note:** If you are a first time user, perform the experiments in the section Control Experiments – Using Control G and Control C Plasmid DNA after reading and understanding the section Step-by-Step Instructions.
Step-by-Step Instructions
Detecting Mutations with SURVEYOR Nuclease

This section provides detailed instructions for the detection of mutations using the SURVEYOR Mutation Detection Kit for WAVE and WAVE HS Systems.

The step-by-step process described can be stopped subsequent to completion of any step. DNA should be stored at –20 ºC until the next step is carried out.

Step 1 — PCR Amplification of Reference and Test Samples

THIS STEP IS CRITICAL TO THE SUCCESS OF THE SURVEYOR NUCLEASE DIGESTION. DO NOT PROCEED UNTIL:

- Your PCR yield is sufficiently high (>15 ng/µL).
- Your PCR product has low background (preferably a single species of the correct size).

The first step in the process is to prepare the amplified DNA samples.

Several factors must be considered carefully in preparing PCR amplified DNA to be used as substrate for SURVEYOR Nuclease analysis. Primer placement and amplified product quality and yield are crucial to obtaining good results. The following should be considered:

- Amplified DNA fragments in the size range of 200 to 1,000 bp are most effectively resolved from potential digestion products on the WAVE system. Place primers at least 50 bp outside the region of interest to ensure cleavage products are longer than 70 bp, since cleavage too close to the end of a large substrate produces a cleavage product not easily resolved from undigested substrate.

- For a new PCR amplicon, the PCR parameters must be optimized carefully. The PCR amplicon should appear as single sharp peak or band of the expected size when analyzed by WAVE HPLC or agarose gel electrophoresis. Mis-priming during PCR amplification can result in the formation of spurious DNA fragments that produce increased background during SURVEYOR Nuclease digestion. This places a premium on the careful design of primers and optimization of PCR conditions. Use primers that are at least 20 nucleotides long (25- to 35-oligomers are preferred), and have a G-C content of 45-60%. If possible, use a high-fidelity DNA polymerase to minimize the introduction of errors that will result in higher background. If after careful design of primers and optimization of PCR conditions non-specific PCR products persist, consider using a hot-start DNA polymerase, a touchdown PCR protocol and/or a second amplification with nested primers.

- Both the amount and concentration of DNA in a SURVEYOR Nuclease reaction mixture influence the efficiency and specificity of SURVEYOR Nuclease digestion. For the amount of enzyme recommended for use in a reaction mixture (1 µL of SURVEYOR Nuclease W), 600 ng of substrate at 50 ng/µL is optimal. If DNA product yield is <15 ng/µL, consider a second amplification with nested primers. Alternatively concentrate the DNA by ethanol precipitation and dissolve the DNA pellet in a smaller volume of 1X PCR buffer to increase DNA concentration.
**Amplification of Homogeneous DNA Populations**

A PCR product from a test sample that is homogeneous for a mutation must be hybridized with a PCR product from a wild-type reference sample in order to generate mismatches for SURVEYOR Nuclease cleavage. Both test sample and wild-type reference DNA are amplified with the same primer pair. Test sample and wild-type reference PCR products are then mixed in a 1:1 ratio to maximize the formation of heteroduplexes during hybridization.

**Amplification of Heterogeneous DNA Populations**

A DNA sample can be heterogeneous either because it is derived from a heterozygous individual, or because it contains a pool of fragments derived from distinctly different homozygous individuals. Such heterogeneous samples can be PCR amplified and hybridized without mixing them with a wild-type reference DNA. The proportion of mutant to wild-type DNA in the population should be above 2.5% for analysis on the WAVE and WAVE HS System. After hybridization, use only a portion of the PCR product for SURVEYOR Nuclease digestion. Retain the remainder of the hybridized PCR product as an undigested reference (control).

Continue with the *Preparing PCR Products* section.

**Preparing PCR Products**

To prepare PCR products we recommend Optimase Polymerase because it has higher fidelity than other thermostable DNA polymerases. Optimase Polymerase is a proof-reading enzyme that has been developed specifically for SNP and mutation discovery applications using the WAVE and WAVE HS Systems. Optimase Polymerase (Catalog number 703005 or 703030) is not included in the SURVEYOR Mutation Detection Kits, but can be purchased separately from Transgenomic.

If you are using Optimase Polymerase, please refer to Appendix A: Preparing PCR Products using Optimase Polymerase. If you are not using Optimase Polymerase, perform the PCR amplification of test sample and wild-type (reference) DNA using a high-fidelity thermostable DNA polymerase according to the manufacturer’s instructions, and follow the steps below.

1. To ensure compatibility of the PCR components with a WAVE or WAVE HS System, consult the PCR Considerations for Successful Mutation Detection chapter in Volume I — Getting Started on the WAVE System of the WAVE System Operator’s Guide.

2. Analyze 2- to 5-µL aliquots of each product by electrophoresis in a 2% agarose gel, prepared with high-resolution agarose such as Transgenomic TransOneK™ Agarose (Catalog No. 556001) and cast in 1X TBE [89 mM Tris-Borate (pH 8.3), 1 mM EDTA] + 0.2 µg/mL ethidium bromide. Add 1/6 volume of a 6 X loading dye buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50% (w/v) sucrose, 0.15% (w/v) bromophenol blue] or your loading dye buffer of choice to the aliquot and mix. Run the gel in 1X TBE at 5 V/cm until the bromophenol blue has run 2/3 of the length of the gel. Run several different amounts of a mass DNA ladder, such as a 100 bp DNA Ladder (New England BioLabs, Beverly, MA), as a reference.

3. Visualize the DNA bands using a UV transilluminator at 250 to 300 nm and photograph the gel.

4. Use the ladder to estimate the concentration of the amplified DNA by visual inspection. If a single band is visible in each sample, proceed; if not, consider optimizing the PCR further as already described in this section. The DNA concentration is ideally ~50 ng/µL, but should be in the range of 15 to 80 ng/µL.
The amplified DNA can be used without further purification. Alternatively, the DNA can be concentrated by ethanol precipitation. To precipitate DNA, transfer the reaction mixtures to microcentrifuge tubes that can be centrifuged at high speed. Add 2.5 volumes of ethanol and store the tubes at –20 ºC for 30 min. Centrifuge the tubes at 13,000 rpm for 10 min in a microcentrifuge. Carefully remove the ethanol with a micro-pipetter, being sure not to disturb the invisible pellet on the tube sidewall and bottom. Concentrated PCR products are suspended in 1X PCR buffer. Estimate the DNA concentration on an agarose gel as described above.

Alternatively, evaluate the quality and concentration of each DNA product by WAVE HPLC analysis at 50 ºC. The PCR product should appear as a single peak. A DNA mass ladder run in parallel in different amounts can be used to establish a relationship between peak area and DNA mass during UV WAVE analysis. Estimate the DNA concentration in each sample.

Once you have prepared the PCR products, continue with Step 2 – DNA Hybridization.

Step 2 — DNA Hybridization

Hybridize the test and reference DNA to form hetero- and homoduplexes. Hybridize the reference DNA alone to form a reference control. The use of a heated-lid thermocycler is recommended.

Because amplified PCR products are hybridized and digested with SURVEYOR Nuclease directly in 1X PCR buffer, careful consideration must be given to the 1X PCR buffer composition.

Read the manufacturer’s literature to determine the salt concentration of the 1X PCR buffer before carrying out the hybridization step. The salt concentration in the PCR product solution should be in the range of 50 to 75 mM to ensure that complete annealing of complementary DNA strands takes place. Reaction buffers used for most PCR DNA polymerases do not require additional salt. For example a PCR buffer for a Taq polymerase is typically 10 mM Tris-HCl, 50 mM KCl and 1.5 mM MgCl2; for Optimase Polymerase the 1X PCR buffer consists of 10 mM Tris-HCl, 75 mM KCl and 1.5 mM MgCl2. Therefore PCR products amplified with these buffers can be annealed directly without the addition of KCl.

However, if the PCR product was prepared with a low salt PCR buffer, sufficient 0.5 M KCl should be added to adjust the final KCl concentration to be within the range of 50 to 75 mM. Please note that KCl concentrations above 75 mM inhibit the SURVEYOR Nuclease; therefore it is critical that KCl be added only when it is required.

Read the manufacturer’s literature to determine the other constituents of the 1X PCR buffer before carrying out the SURVEYOR Nuclease digestion step. The constituents of most 1X PCR buffers support efficient digestion of heteroduplex DNA by SURVEYOR Nuclease. These include 10 to 20 mM Tris-HCl or Tris-SO4 (pH 8.3 to 9.3), 50 to 75 mM KCl, 1 to 3 mM MgCl2 or MgSO4, 0.1% to 1% nonionic detergent, and BSA or gelatin. (NH4)2SO4 at 10 to 20 mM is also acceptable as long as the KCl concentration is ≤50 mM. PCR additives such as DMSO (>5%), glycerol (>10%), betaine (>1 M), and 1X PCRx Enhancer (Invitrogen) inhibit SURVEYOR Nuclease above the concentrations listed. If any of these or other additives are present in the PCR reaction mixture, they must be removed before the PCR product is treated with SURVEYOR Nuclease.
If additives are present, we suggest using ethanol precipitation to clean up the PCR product before the hybridization step (see Preparing PCR Products section). The precipitated DNA should be dissolved in a 1X PCR buffer compatible with the hybridization and SURVEYOR Nuclease digestion steps, such as 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, and 50 mM KCl. If the manufacturer does not reveal the contents of the PCR buffer, precipitate the DNA product and dissolve it in a compatible PCR buffer.

If you are using a thermocycler, go to the Performing Heteroduplex Formation using a Thermocycler section below. If your thermocycler cannot be programmed appropriately for hybridization or if it lacks a heated lid, go to the Performing Heteroduplex Formation without a Thermocycler section below.

Performing Heteroduplex Formation using a Thermocycler

To perform heteroduplex formation using a thermocycler:

1. Mix equal amounts of test sample and reference PCR products in a 0.2-mL tube. Place reference DNA alone in a separate 0.2-mL tube. For efficient annealing the final volume should be at least 10 µL.

   Note the following:
   • The concentration of test sample DNA and wild-type reference DNA should be in the range of 15 to 80 ng/µL and ideally 50 ng/µL. About 600 ng of hybridized DNA is recommended for treatment with SURVEYOR Nuclease W, so that each tube should contain at least 600 ng total DNA.
   • Heterogeneous test sample DNA is hybridized by itself.

2. Place the tube in a thermocycler and run the following program:

   95 °C                      2 min
   95 ºC to 85 ºC        (-2 ºC/s)
   85 ºC to 25 ºC        (-0.1 ×C/s)
   4°C                         Hold

   The product is now ready to be treated with SURVEYOR Nuclease for heteroduplex analysis. Continue with Step 3 — Treatment with SURVEYOR Nuclease.

Performing Heteroduplex Formation without a Thermocycler

To perform heteroduplex formation without a thermocycler:

1. Mix equal masses of the two PCR products intended to generate the heteroduplex as above. Set up reference DNA in a separate tube as above.

2. Incubate the mixture at 95 °C for 5 min in a 1-liter beaker filled with 800 mL of water and then allow the water to come to < 30 ºC.

   Note: Because of evaporation of liquid at the tube bottom and condensation under the tube lid, the volume in a tube should be ≥20 µL so that sufficient volume is present to prevent the concentrations of constituents in the mixture from changing substantially during the hybridization step.

3. Spin the tube contents to the bottom of the tube.

   The product is now ready to be treated with SURVEYOR Nuclease for heteroduplex analysis. Continue with Step 3 — Treatment with SURVEYOR Nuclease.
Step 3 — Treatment with SURVEYOR Nuclease

Once you have prepared the test sample/reference hetero/homoduplex mixtures and hybridized reference control, you must treat them separately with SURVEYOR Nuclease.

Two alternative digestion protocols are described:

**Direct Addition Protocol** — direct addition of SURVEYOR Nuclease to annealed DNA in 1X PCR buffer.

**Standard 1X Buffer Protocol** — dilution of annealed DNA into a 60-µL reaction mixture containing 1X SURVEYOR Nuclease Reaction Buffer followed by digestion with SURVEYOR Nuclease.

**Note the following:**

- The first protocol consistently helps to reduce background from digestion products during WAVE HPLC analysis, but in some cases also reduces the amount of SURVEYOR Nuclease mismatch cleavage products observed. More often than not, signal to noise is improved.

- The second method sometimes produces greater amounts of SURVEYOR Nuclease mismatch cleavage products.

- We recommend use of the direct addition protocol. Digestion in 1X SURVEYOR Nuclease Reaction Buffer should be used instead of direct addition digestion only if difficulty is encountered in obtaining sufficient SURVEYOR Nuclease mismatch cleavage products from DNA samples containing known mutations with the direct addition protocol.

**Direct Addition Protocol**

Be sure the test sample/reference hetero/homoduplex mixtures and hybridized reference control are in appropriate 1X PCR buffer (for details see Step 2 – DNA Hybridization).

1. Digest the hetero/homoduplex DNA samples and any reference DNA in separate tubes.

2. For each digestion, add the following components in the order shown to a nuclease-free 0.2-mL tube (kept on ice):
   - 600 ng (8 to 25 µL) hybridized DNA
   - 1 µL SURVEYOR Nuclease Enhancer W
   - 1 µL SURVEYOR Nuclease W

3. Mix by vortexing gently, by agitation or by aspiration/expulsion in a pipette tip using a micro-pipetter.

4. Incubate at 42 ºC for 20 min.

5. Add 1/10 volume of Stop Solution and mix. Store the digestion products at −20 ºC if not analyzed immediately.
Note the following:

• To reduce the number of manipulations, SURVEYOR Enhancer W and Nuclease W can be mixed at 1:1 and a single 2-µL pipetting can be done. The mixture should be used immediately after preparation and should not be stored, since reducing agent in the Enhancer storage buffer will inactivate SURVEYOR Nuclease over time.

• When a heterogeneous DNA sample is analyzed, a portion of the hybridized heterogeneous DNA is not digested with SURVEYOR Nuclease and is run as a control in Step 4 — Analysis of DNA Fragments.

Standard 1X Buffer Protocol

1 Digest the hetero/homoduplex DNA samples and any reference DNA in separate tubes.

2 For each digestion, add the following components in the order shown to a nuclease-free 0.2-mL tube (kept on ice):
   • H₂O provided in the kit to bring the final volume to 60 µL
   • 6 µL 10 X SURVEYOR Nuclease Reaction Buffer
   • 600 ng (≤ 25 µL) hybridized DNA
   • 1 µL SURVEYOR Enhancer W
   • 1 µL SURVEYOR Nuclease W

3 Mix by vortexing gently, by agitation or by aspiration/expulsion in a pipette tip using a micro-pipetter.

4 Incubate at 42 ºC for 20 min.

5 Add 6 µL of Stop Solution and mix. Store the digestion products at –20 ºC if not analyzed immediately

Note the following:

• If necessary, a master mix can be prepared on ice containing all components except DNA or SURVEYOR Nuclease. Add aliquots of this mix to tubes (kept on ice) containing the appropriate missing component and mix them, and then incubate them at 42 ºC. The master mix should be used immediately after preparation and should not be stored.

• Volumes of unpurified DNA ≥25 µL in a 60-µL reaction mixture inhibit SURVEYOR Nuclease cleavage. If necessary, concentrate the DNA by ethanol precipitation. If smaller amounts of DNA (<600 ng) are used in the SURVEYOR Nuclease digestion, the amount of SURVEYOR Nuclease W and SURVEYOR Nuclease Enhancer W used should be decreased proportionately. Cleavage products can be detected using the WAVE HS System even when only 10 ng of hybridized Control G/C DNA is digested in a 20-µL reaction volume.

• When a heterogeneous DNA sample is analyzed, a portion of the hybridized heterogeneous DNA is not digested with SURVEYOR Nuclease and is run as a control in Step 4 — Analysis of DNA Fragments.

Continue with Step 4 – Analysis of DNA Fragments
Step 4 — Analysis of DNA Fragments

To analyze SURVEYOR Nuclease digestion products on the WAVE or WAVE HS System:

1. Transfer the 0.2-mL tube with the SURVEYOR Nuclease digestion mixture to the Autosampler of a WAVE or WAVE HS System. Digests of control or undigested control DNA, and digests of mixtures of heteroduplexes and homoduplexes need to be analyzed in parallel. A sizing standard such as a 100-bp DNA ladder should also be run in parallel.

2. Set up methods to inject 200 to 500 ng of SURVEYOR Nuclease digested DNA into a DNASep® or DNASep® HT Cartridge. Injection volumes exceeding 22 µL may require a 500-µL syringe in the Autosampler.

3. Run the following program

   - Application: Double-strand sizing – Multiple Fragments
   - Oven Temperature: 50 ºC
   - Flow rate: 0.9 mL/min
   - Gradient: From 37 % B to 67% B @ 2.5 min/100 bp
   - Run Time: 17.3 min
   - Eluent A: WAVE Optimized Buffer A
   - Eluent B: WAVE Optimized Buffer B
   - Detection: 260 nm for UV; 495 nm Ex and 637 nm Em for Fluorescence Detection (using High Sensitivity Detection with the WAVE HS system)

4. Run the samples.

5. Analyze the chromatograms.

Washing Procedure for DNASep® and DNASep® HT Cartridges Used to Analyze SURVEYOR Nuclease Digests

When injecting SURVEYOR Nuclease digests onto DNASep® and DNASep® HT Cartridges in a WAVE System, the recommended cleaning option is either ACTIVE CLEAN or FAST CLEAN. A NORMAL CLEAN is not sufficient.

Note the following:

- After every 100 injections of SURVEYOR Nuclease digests should be followed by a HOT WASH. To perform a HOT WASH, pump 100% Solution D (75% ACN) for 15 minutes, followed by 50%/50% buffer A/B for 30 minutes at 80 ºC through the system. This HOT WASH should also be performed at the end of each batch of SURVEYOR Nuclease digest runs irrespective of whether 100 injections have been reached or not, e.g. when temporary cessation of cartridge use or a change to DHPLC analysis is anticipated.

- After every 500 injections of SURVEYOR Nuclease digests the in-line filter must be changed. The in-line filter should also be changed if the pressure becomes too high (>2300 PSI for DNASep and >1500 PSI for DNASep HT Cartridge).

- After every 500 injections a REVERSE HOT WASH should be performed. Refer to Performing a REVERSE HOT WASH on a DNASep or DNASep HT Cartridge section below.
WARNING! Failure to follow these procedures will lead to high column pressure and deteriorating column performance.

Performing a REVERSE HOT WASH on a DNASep or DNASep HT Cartridge

To perform a REVERSE HOT WASH:

1. Remove the DNASep Cartridge or DNASep HT Cartridge and reinsert it in the reverse orientation in the flow path.

2. Remove the in-line filter and substitute it with a union during the REVERSE HOT WASH.

3. Pump 100% Solution D (75% ACN) for 30 minutes at 80 °C through the system at a flow rate of 0.9 mL/min.

4. Pump 50%/50% buffer A/B for 1 hour at 80 °C through the system at a flow rate of 0.9 mL/min.

5. Remove the union and insert a new in-line filter.

This REVERSE HOT WASH should be performed without the in-line filter. Insert a new in-line filter after the REVERSE HOT WASH and run the cartridge in the reverse direction for the next 500 injections.

Control Experiments — Using Control G and Control C Plasmid DNA

Two plasmid DNAs, Control G and Control C, are provided in the SURVEYOR Mutation Detection Kit for WAVE and WAVE HS Systems. These two control DNAs are plasmids with inserts that differ at a single base pair. They are provided in separate vials, each at a concentration of 5 ng/µL; forward and reverse primers needed for PCR amplification are already combined with the plasmid templates. The sequence of the PCR product for Control G is shown below. Control C differs from Control G because it has a C in lieu of the G (underlined). Primer sequences are underlined at the 5’ and 3’ end of the amplicon sequence.

```
ACACCTGATCAAGCCTGTTCATTTGATTTAC
CAGAGAGACTGTCATTGATCCACAT
GGAGGGGAAGGACATGTGTGTGTGCTGAGCCATTCAAAATTTCCACATCTCGCTGTG
GCCATTTCGCCATGGAACATCTGATCGATCGATATATAATATAGACAGAGGCTTGT
ATTTTATCCACTTCAATGGAGATATATTTCATCAGCCTATGATGTGGATTTACA
AGTIGATATGGAAGAGGAAAGCATAGATATTGTCGTGTTTGCGCTGCTGTGTTCT
CCACAAATCCAAACC
TGCACCATGTTGGGATAGAGAGATTATTTACTACAGCTGACGAGATATACCATG
TAAAGGATATGCAACTCTCTCTCTCTACAAAGACATACAGAGGAACTTTACAGAGGTAG
TGGTTGCAAGATGCTGGAAATCCTGGGAAGGAAATGTGATGATATATCTCTACTAGCGCA
ATAAGATATGCTAAGGAGATATAAACATGCTTGAACTGGGAGGTACAAAGATGT
GTAATCTGGCAGAACACTCTGTCAGATATACATCTTCAACACAAGAATGCGCAATTC
ATGAAACGGATACGCTGCAGGATGGGAAATCCTGGCAATCTTGATTTGATTTGAAAGGAGT
TTGGAACAGCTCGAGATTTCTTCTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
ACAGACATACAGAGGAACTTTACAGAGGTAGT
```

PCR amplification of 2 µL of each DNA solution in a 50-µL reaction should produce >25 ng/µL of a 632-bp PCR product. Sufficient DNA is provided to perform ten PCR reactions with each control.

Control G and Control C plasmids can be used to troubleshoot the PCR amplification, hybridization, and SURVEYOR Nuclease digestion steps of the SURVEYOR Nuclease Kit.
WE STRONGLY RECOMMEND THAT FIRST TIME USERS PERFORM THE CONTROL EXPERIMENTS OUTLINED BELOW.

Successful completion of the control experiments will give the user an appreciation for: the yield and quality of PCR product obtained with their amplification system, the optimal amount of SURVEYOR Nuclease and their amplified DNA to use in a reaction, and the expected amount and appearance of digestion products in a WAVE HPLC analysis.

Use Control G and Control C as follows.

1 Amplify 2 µL of Control G and 2 x 2 µL of Control C DNA in separate 50-µL reactions using a proofreading DNA polymerase and the PCR program described in Appendix A: Preparing PCR Products using Optimase Polymerase (Step 4) where $T_a = 65 \, ^\circ \text{C}$ and the 72 °C extension time is appropriate for the polymerase used, e.g. 1 min for Optimase Polymerase.

2 After amplification, analyze a 2-µL aliquot of each amplified DNA to determine product quality and yield as described in Step 1 – PCR Amplification of Reference and Test Samples. The yield with Optimase Polymerase is in the range of 25-80 ng/µL.

3 Hybridize Control G- and Control C-amplified DNA in equal amounts (25 µL of each if the amplified DNA concentrations are ≥40 ng/µL) as described in Step 2 – DNA Hybridization. This produces a population of molecules containing 50% homoduplex, 25% heteroduplex with a C/C mismatch, and 25% heteroduplex with a G/G mismatch. Also self anneal 50 µL of Control C homoduplex in a separate tube.

4 Digest hybridized Control G/C and Control C homoduplex with 1 µL of SURVEYOR Nuclease W and 1 µL of SURVEYOR Enhancer W by direct addition. In order to empirically determine the best conditions for digesting your DNA with SURVEYOR Nuclease W, set up 6, 0.5-mL reaction tubes on ice with the components listed in Table 1. Incubate the tubes at 42 °C for 20 min. Add 1/10 volume of Stop Solution.

5 Analyze the digested DNA in each reaction mixture as described in Step 4 – Analysis of DNA Fragments. Program the autosampler to inject 6, 12, and 23 µL from tube number 1 and 4, 2 and 5, and 3 and 6, respectively.

---

**Table 1 – Reaction Tube Contents for Control Experiments**

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>Volume Required (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hybridized Control G/C</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
</tr>
</tbody>
</table>
SURVEYOR Nuclease digestion of hybridized Control G/C PCR products gives rise to two cleavage products, 217 and 415 bp in size, which are clearly distinguishable by WAVE analysis as shown in Figures 4 and 5. Analysis of different amounts of substrate digested with SURVEYOR Nuclease W provides an opportunity to establish the optimal ratio of enzyme to DNA with DNA amplified with your PCR enzyme and the optimal amount of DNA to inject. The optimal conditions produce the maximum amount of cleavage products while maintaining low background. In Figures 4 and 5, the digestion and injection conditions for tube 5 gave the best results. The increase in the amounts of digestion products produced in the 12-µL versus 6-µL digestion of Control G/C substrate (tube 5 versus tube 4) was proportional to the amount of substrate present (600 ng versus 300 ng). However this was not true when the amounts of products produced in the 24-µL versus 12-µL digestions were compared. The digestion products in the 24-µL digestion decreased. This is due to several factors. First, 1 µL of SURVEYOR Nuclease W is not sufficient to completely digest 1,200 ng of Control G/C in 20 min. Second, injection of ~1 µg of DNA at ~50 ng/µL results in some of the DNA passing directly through the DNASep HT column and appearing in the injection peak at the beginning of the run.

Keep in mind that PCR amplification of a plasmid template such as Control G or C will result in higher yields of DNA product than amplification of a comparable amount of genomic DNA template. Using 5 to 10 times more genomic DNA (50–100 ng) will give comparable yields of PCR product. This should be taken into consideration in preparing and digesting DNA amplified from genomic DNA.
Figure 4. SURVEYOR Nuclease digestion products of self-annealed Control C homoduplex (green lines) and Control G/C homoduplex/heteroduplex (red lines). The 632-bp amplicons were PCR amplified with Optimase Polymerase from 2 µL of Control G and Control C. Control G/C homoduplex/heteroduplex was formed by hybridizing equal amounts of Control G and Control C homoduplex PCR product and contains homoduplexes and C/C and G/G mismatched heteroduplexes. DNA (300 ng, 600 ng, and 1,200 ng) was digested with 1 µL of SURVEYOR Nuclease W and 1 µL of SURVEYOR Enhancer W for 20 min at 42 ºC. SURVEYOR Nuclease digestion products (230, 470, and 970 ng) were analyzed using the WAVE System run under non-denaturing conditions at 50 ºC. New England BioLabs 100 bp Ladder DNA was run as a marker (black line). The 217- and 415-bp cleavage products expected from the Control G/C heteroduplex (red line) are clearly visible. Also visible in all three chromatograms are the full-length 632-bp homoduplex and a smaller shoulder resulting in part from the exonuclease activity of SURVEYOR Nuclease.
Figure 5. SURVEYOR Nuclease digestion products of self-annealed Control C homoduplex (green lines) and Control G/C homoduplex/heteroduplex (red lines). The 632-bp amplicons were PCR amplified with Optimase Polymerase from 2 µL of Control G and Control C. Control G/C homoduplex/heteroduplex was formed by hybridizing equal amounts of Control G and Control C homoduplex PCR product and contains homoduplexes and C/C and G/G mismatched heteroduplexes. DNA (300 ng, 600 ng, and 1,200 ng) was digested with 1 µL of SURVEYOR Nuclease W and 1 µL of SURVEYOR Enhancer W for 20 min at 42 ºC. SURVEYOR Nuclease digestion products (230, 470, and 970 ng) were analyzed using the WAVE System run under non-denaturing conditions at 50 ºC and equipped with a Fluorescence Detector and a High-Sensitivity Accessory for post column DNA intercalation with fluorescent dye. New England BioLabs 100 bp Ladder DNA was run as a marker (black line). The 217- and 415-bp cleavage products expected from the Control G/C heteroduplex (red line) are clearly visible. Also visible in all three chromatograms are the full-length 632-bp homoduplex and a smaller shoulder resulting in part from the exonuclease activity of SURVEYOR Nuclease. Note also the improved baseline and the magnitude of the signal compared to the profile obtained by UV detection.
Appendix A: Preparing PCR Products using Optimase Polymerase

To prepare PCR products using Optimase Polymerase:

1. Isolate test sample and wild-type (reference) DNA by standard methods.
2. Add the following components to each of the two 0.2-mL tubes (kept on ice). One tube will be used for test sample DNA and the other for reference DNA:
   - Sterile, deionized water sufficient to bring the final volume to 50 µL
   - 5 µL 10 X Optimase Polymerase Buffer
   - Test sample or reference DNA (10 ng plasmid DNA or 100 ng genomic DNA)
   - 4 µL dNTPs (2.5 mM each of dTTP, dATP, dCTP and dGTP; final concentration of each dNTP is 0.2 mM)
   - 15 picomoles sense primer (~120 ng of a 25 mer)
   - 15 picomoles antisense primer
   - 1 µL Optimase Polymerase (2.5 units)
3. Set up PCR amplification using one of the following methods:

<table>
<thead>
<tr>
<th>IF</th>
<th>THEN</th>
</tr>
</thead>
</table>
3. Enter the appropriate information and click the Develop PCR Protocol button. The PCR protocol appears for a heated-lid thermocycler. Perform PCR.  
4. Go to step 5. |

| You want to calculate the annealing temperature | 1. Determine the annealing temperature ($T_a$) by calculating the $T_m$ for each primer using the following equation:  
$$T_m = 63.728 + (0.41 \times \%GC) - (600/\text{length})$$  
%GC = percentage GC of the primer  
length = length of the primer in nucleotides  
$T_a$ is the average of the two primers $T_{ms} + 3 ^\circ C$  
2. Go to step 4. |

**TIP:** Please use the Optimase MasterMix Calculator at [http://www.mutationdiscovery.com](http://www.mutationdiscovery.com) to prepare a master mix for your PCR reaction.
4 Use the following program for a heated-lid thermocycler:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C</td>
<td>2 min</td>
<td>x1 Cycle</td>
</tr>
<tr>
<td>94 °C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>T_a °C</td>
<td>30 s</td>
<td>x 30 Cycles</td>
</tr>
<tr>
<td>72 °C</td>
<td>30 s per 250 bp</td>
<td></td>
</tr>
<tr>
<td>72 °C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>4 °C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

5 Analyze 2- to 5-µL aliquots of each product by electrophoresis in a 2% agarose gel, prepared with high-resolution agarose such as Transgenomic TransOneK Agarose (Catalog No. 556001) and cast in 1X TBE [89 mM Tris-Borate (pH 8.3), 1 mM EDTA] + 0.2 mg/µL ethidium bromide. Add 1/6 volume of a 6 X loading dye buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50% (w/v) sucrose, 0.15% (w/v) bromophenol blue] or your loading dye buffer of choice to the aliquot and mix. Run the gel in 1X TBE at 5 V/cm until the bromophenol blue has run 2/3 of the length of the gel. Run several different amounts of a mass DNA ladder, such as a 100 bp DNA Ladder (New England BioLabs, Beverly, MA), as a reference.

6 Visualize the DNA bands using a UV transilluminator at 250 to 300 nm and photograph the gel.

7 Use the ladder to estimate the concentration of the amplified DNA by visual inspection. If a single band is visible in each sample, proceed; if not, consider optimizing the PCR further as already described in Step 1 – PCR Amplification of Reference and Test Samples. The DNA concentration is ideally ~50 ng/µL, but should be in the range of 15 to 80 ng/µL.

The amplified DNA can be used without further purification. Alternatively, the DNA can be concentrated by ethanol precipitation. To precipitate DNA, transfer the reaction mixtures to microcentrifuge tubes that can be centrifuged at high speed. Add 2.5 volumes of ethanol and store the tubes at –20 °C for 30 min. Centrifuge the tubes at 13,000 rpm for 10 min in a microcentrifuge. Carefully remove the ethanol with a micro-pipetter, being sure not to disturb the invisible pellet on the tube sidewall and bottom. Concentrated PCR products are suspended in 1X PCR buffer. Estimate the DNA concentration on an agarose gel as described above.
Appendix B: Troubleshooting

Effective use of the SURVEYOR Mutation Detection Kit depends upon successful completion of a number of steps. One of the most critical is PCR amplification that must result in the production of specific, uniform-sized DNAs in sufficient quantity to be detected after hybridization and cleavage. Also critical is matching the amount of DNA and SURVEYOR Nuclease used. If you are a first-time user, you should process the control DNAs provided through all the steps as described in Control Experiments – Using Control G and Control C Plasmid DNA.

The Control DNAs should be used also to troubleshoot various steps in the procedure.

This appendix section covers a list of issues that you might encounter when using the SURVEYOR Mutation Detection Kit and how to resolve them.

Problem 1 – Low PCR yield or no PCR product

<table>
<thead>
<tr>
<th>POSSIBLE CAUSE</th>
<th>SOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not enough template and/or too few cycles</td>
<td>Increase the template concentration and/or add more PCR cycles.</td>
</tr>
<tr>
<td>Suboptimal PCR parameters</td>
<td>Do one of the following:</td>
</tr>
<tr>
<td></td>
<td>• Decrease the annealing temperature in increments of 2 °C.</td>
</tr>
<tr>
<td></td>
<td>• Increase the extension time. For Optimase Polymerase, use 30 sec per 250 bp.</td>
</tr>
<tr>
<td>Suboptimal DNA polymerase for target</td>
<td>Use a “hot-start” PCR DNA polymerase.</td>
</tr>
<tr>
<td></td>
<td>Increase the polymerase concentration.</td>
</tr>
</tbody>
</table>

Problem 2 – Multiple PCR products

<table>
<thead>
<tr>
<th>POSSIBLE CAUSE</th>
<th>SOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor primer design</td>
<td>Redesign the primers to improve specificity, Tₘ and GC content.</td>
</tr>
<tr>
<td>Annealing temperature too low</td>
<td>Increase the annealing temperature in increments of 2 °C.</td>
</tr>
<tr>
<td>Extension time too long</td>
<td>Reduce the extension time. For Optimase Polymerase, use 30 s per 250 bp.</td>
</tr>
<tr>
<td>Cycle number too high</td>
<td>Reduce cycle number in increments of 2.</td>
</tr>
<tr>
<td>Suboptimal PCR conditions</td>
<td>Use a “hot-start” PCR and/or touchdown PCR or perform nested PCR.</td>
</tr>
</tbody>
</table>
### Problem 3 – No cleavage products observed upon analysis after SURVEYOR Nuclease treatment of known heteroduplex

<table>
<thead>
<tr>
<th>POSSIBLE CAUSE</th>
<th>SOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of mismatched target too low</td>
<td>Mix equal amounts of test and reference DNA before annealing.</td>
</tr>
<tr>
<td>Cleavage site is too close to PCR product end</td>
<td>Redesign the primer set to move the target site away from ends.</td>
</tr>
<tr>
<td>Inactive SURVEYOR Nuclease</td>
<td>Perform the Control reaction to verify enzyme performance.</td>
</tr>
<tr>
<td>Too little enzyme</td>
<td>Increase the amount of SURVEYOR Nuclease two-fold and repeat digestion.</td>
</tr>
<tr>
<td>Too little substrate</td>
<td>Concentrate the PCR product by ethanol precipitation before annealing.</td>
</tr>
</tbody>
</table>

### Problem 4 – High background after SURVEYOR Nuclease treatment

<table>
<thead>
<tr>
<th>POSSIBLE CAUSE</th>
<th>SOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suboptimal hybridization step</td>
<td>Do the following:</td>
</tr>
<tr>
<td></td>
<td>1  Make sure the DNA concentration is in the range of &gt;25 ng/µL to &lt;50 ng/µL.</td>
</tr>
<tr>
<td></td>
<td>2  Repeat the hybridization step, taking care to cool the annealing mixture slowly.</td>
</tr>
<tr>
<td></td>
<td>3  Add 1X PCR reaction buffer to precipitated products before annealing.</td>
</tr>
<tr>
<td></td>
<td>4  Use sufficient sample volume (&gt;20 µL) during hybridization in a water bath.</td>
</tr>
<tr>
<td></td>
<td>5  Add 1/10 volume 0.5 M KCl to PCR product contained in low salt 1X PCR reaction buffer.</td>
</tr>
<tr>
<td>Errors introduced by PCR enzyme</td>
<td>Use a high fidelity PCR enzyme, such as Optimase Polymerase.</td>
</tr>
<tr>
<td>Incubation time too long</td>
<td>Reduce the SURVEYOR Nuclease digestion time by 5 min increments.</td>
</tr>
<tr>
<td>Too much SURVEYOR Nuclease</td>
<td>Reduce the SURVEYOR Nuclease two-fold and repeat digestion.</td>
</tr>
<tr>
<td>DNA amount too low</td>
<td>Increase the DNA amount to at least 400 ng of substrate per 1 µL of SURVEYOR Nuclease W used.</td>
</tr>
<tr>
<td>Nonspecific PCR products</td>
<td>Optimize the PCR parameters to increase specificity. Always use an appropriate substrate as a control to identify background.</td>
</tr>
<tr>
<td>Enhancer has lost activity</td>
<td>Increase the amount of Enhancer two-fold and repeat digestion.</td>
</tr>
</tbody>
</table>
References


Corporate Headquarters

Transgenomic, Inc.
12325 Emmet Street
Omaha, NE 68164, USA
Phone: +1 (888) 233-9283* or +1 (402) 452-5400
Fax: +1 (402) 452-5401
E-mail: info@transgenomic.com

*In North America only

Europe

Transgenomic Ltd.
Z.A. de la Clef Saint Pierre
10B, Rue Blaise Pascal
78990 ELANCOURT
France
Phone: +33 (0) 1 30 68 90 00
Fax: +33 (0) 1 30 68 90 01

Technical Support E-mail: support@transgenomic.com

www.transgenomic.com

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