User Guide

User Guide for the IDT® Surveyor® PLUS Mutation Detection Kit for WAVE® and WAVE HS Systems

This guide applies to catalog numbers: **706035, 706030, 706031**

www.IDTDNA.com/Surveyor

Revision: 1

For Research Use Only

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Introduction

IDT Surveyor PLUS Mutation Detection Kits use a mismatch-specific DNA endonuclease to scan for known and unknown mutations and polymorphisms in heteroduplex DNA. Surveyor Nuclease, the key component of the kit, is an endonuclease that cleaves DNA with high specificity at sites of base-substitution mismatch and other distortions[1]. 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[1].

Surveyor Nuclease has been shown to be a robust and reproducible tool for mutation detection[1, 32, 37]. It has been used to detect accurately a variety of mutations and polymorphisms in the human, mammalian, bacterial, and plant genomes.

Examples of use of Surveyor Nuclease include:

- germline mutations in human genes:
    - ATRX for X-linked mental retardation[7]
    - ABCC6 associated with pseudoxanthoma elasticum[31]
    - HBB gene linked to β-thalassemia[38]
    - genes involved in uracil catabolism[8, 9]
- mtDNA and nuclear gene mutations associated with respiratory chain defects[10-12, 33]
- mutations associated with kidney disease[5, 40, 41]
- somatic mutations in:
    - EGFR gene in human tumors[13-19]
    - JAK2 tyrosine kinase gene in patients with myeloproliferative disorders[20, 21]
    - variety of genes in patients undergoing radiotherapy[22]
    - p53 gene in squamous-cell carcinomas[23] and hematological malignancies[22]
    - KIT tyrosine kinase receptor gene in various malignancies[25, 26]
    - PKC412 tyrosine kinase receptor in mast cell leukemia[27]
    - LKB1 tumor suppressor in lung cancer[36]
    - VHL gene in renal tumours[34]
    - hCDC4 gene in patients with AML[29]
- mutations in drug-resistant genes of *Mycobacterium tuberculosis*[5, 6] and in Group II introns of bacterial rRNA genes[29]
- induced point mutations in barley[4, 39] and error-free clones generated from a plant cDNA library by PCR-based cloning[30]

The Surveyor PLUS 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. Since the introduction of the original kit, we have made several technical advances that improve the performance of Surveyor Nuclease and these are included in this updated version of the kit. We now provide digestion reaction conditions for DNA amplified by PCR using most commercially available DNA polymerases; these reaction conditions increase the amounts of mismatch cleavage products generated by Surveyor Nuclease from DNA containing genetic variations.

The updated kit also contains an Enhancer Cofactor and a new thermostable Enhancer, Surveyor Enhancer W2 that replaces Enhancer W. Enhancer W2 and its cofactor are superior to Enhancer W in improving the signal to noise ratio in Surveyor Nuclease reactions, due to improved suppression of background.

Note: Washing procedures specifically recommended and described in this User Guide are for the Surveyor PLUS 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 all of the control experiments outlined in the section *Control Experiments—Using Control C and Control G Plasmid DNA.*

If you have further questions or need assistance, please contact IDT Customer Care at:

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Surveyor PLUS Mutation Detection Kit Components

The kit is available in three sizes as follows:

Component	25-Reaction Kit (706035) Amount Provided	100-Reaction Kit (706030) Amount Provided	1000-Reaction Kit (706031) Amount Provided
Surveyor Nuclease W	0.03 mL	4 x 0.03 mL	1.0 mL
Surveyor Enhancer W2	0.03 mL	4 x 0.03 mL	1.0 mL
Enhancer Cofactor	0.25 mL	2 x 0.25 mL	2 x 1.5 mL
0.15 M $MgCl_2$	0.25 mL	3 x 0.25 mL	2 x 1.5 mL
Stop Solution	0.25 mL	3 x 0.25 mL	2 x 1.5 mL
Control C	0.01 mL	0.01 mL	0.01 mL
Control G	0.01 mL	0.01 mL	0.01 mL

Store all components at –20°C.

Detecting Mutations with the IDT Surveyor PLUS Mutation Detection Kit

An Overview

Mutation detection and confirmation with Surveyor Nuclease involves 4 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, due to the presence of one or more mismatches, is indicated by the presence of additional peaks. The retention time of the cleavage products indicate the size of the fragments and therefore the location of the mismatch or mismatches.

Factors Affecting the Quality of Results

The following factors influence the quality of results when using Surveyor Nuclease.

- **The quality of the genomic DNA to be amplified.**

  High quality DNA (from fresh or frozen cells or tissue) should be used. The DNA should have a concentration of >5 ng/µL as determined by absorbance at 260 nm, have an absorbance ratio at 260/280 nm of >1.7 and be >90% DNA (i.e. free of most tRNA and rRNA contamination as judged by appearance on an agarose gel). Store DNA samples at –20°C.

  If the DNA template is extracted from paraffin-embedded tissue, several additional precautions can be taken. The extracted DNA can be treated with uracil DNA glycosylase to prevent amplification of DNA fragments containing deaminated C residues[46]. Often a high percentage of the A_{260} adsorbing material extracted from paraffin-embedded tissue is not amplified well during PCR. Using a larger amount of starting DNA, e.g. ~50 ng versus 10 ng, will often help to produce a reasonable amplification product.

- **The quality of the PCR amplified DNA.**

  PCR should produce a sufficiently high yield (>15 ng/µL) of a SINGLE amplified species of the correct size. We strongly recommend the use of a proofreading DNA polymerase (such as Transgenomic Optimase® Polymerase) to reduce the amount of base misincorporation during PCR (which leads to the generation of 'false' mutations and spurious Surveyor Nuclease cleavage fragments). Similarly, non-specific PCR fragments can be interpreted as mutations and can mask Surveyor Nuclease mismatch cleavage products. If possible, a reference DNA should be digested with Surveyor Nuclease and run to exclude spurious background by visual comparison of chromatogram profiles (see for example Figures 3 and 5). Primer-dimers should be strenuously avoided as their presence 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.**

  Whenever possible, test and reference PCR products should be hybridized in equal proportion to maximize the amount of heteroduplex DNA available for digestion.

- **Suppression of DNA nicking.**

  Surveyor Nuclease nicks double-stranded DNA at random matched sites, which produces background during extended incubations[32]. This activity is suppressed by Surveyor Enhancer W2 and its cofactor without otherwise negatively affecting the reaction. Surveyor Nuclease Enhancer W2 and cofactor are included in this kit.

- **The composition of the PCR buffer.**

  Commercially available PCR buffers vary dramatically in content and the contents are often not defined by suppliers. A few buffers are NOT compatible with Surveyor Nuclease due to pH or the presence of additives, surfactants or other proprietary ingredients. **Surveyor Nuclease reaction conditions have been defined for a large number of different PCR buffers that improve signal intensity significantly (1.5–4 fold, depending upon the buffer) while maintaining low background.**

These new reaction conditions are included in the protocols described in this User Guide (see *Step 3—Treatment with Surveyor Nuclease* and *Appendix B: Surveyor Nuclease Reaction Conditions*).

- **Signal to Noise ratio.**
  The signal to noise ratio is generally high enough to detect mutations present at a low percentage of the total DNA template; it is possible to detect 1% to 20% mutant DNA depending upon the particular DNA amplicon, its size, the number and type(s) of mutation(s) and the analysis platform. Figures 2 and 3 show detection of heteroduplex present in homoduplex at a ratio of 1 in 128 (0.8% heteroduplex) with a WAVE HS system and Figures 4 and 5 show detection at a ratio of 1 in 64 (1.8% heteroduplex) with a WAVE UV system. Figures 6 and 7 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 that can be estimated relative to the DNA size marker.

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*.

Examples of Results

Examples of results obtained using the Surveyor PLUS Mutation Detection Kit for WAVE and WAVE HS Systems are shown in Figures 1 through 5 below. In these examples, the four step process outlined in the Overview section was followed carefully. 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 1 shows Surveyor Nuclease digestion products from a 405 bp DNA fragment amplified from heterozygous mutant and normal human genomic DNA. The mutant fragment contains a G>A mutation 116 bp from one end. Digestion products were analyzed on a WAVE HS system.

Figure 2 shows WAVE HS System analysis of Surveyor Nuclease digestion products from mixtures of heteroduplex and homoduplex DNA representing the level of mutation detection in pooled genomic DNAs.

Figure 3 shows the WAVE HS digestion product profiles from Figure 2 for homoduplex and the 1:64 and 1:128 heteroduplex/homoduplex ratios superimposed with the fluorescence signal axis expanded. Surveyor Nuclease digestion products derived from heteroduplex mixed with homoduplex at a ratio of 1:128 can clearly be seen in the chromatograms.

Figures 4 and 5 show the sensitivity of WAVE UV analysis of the same Surveyor Nuclease-digested heteroduplex/homoduplex mixtures analyzed in Figures 3 and 4, respectively. The mismatch could be detected down to a ratio of heteroduplex to homoduplex of 1:64 with UV detection.

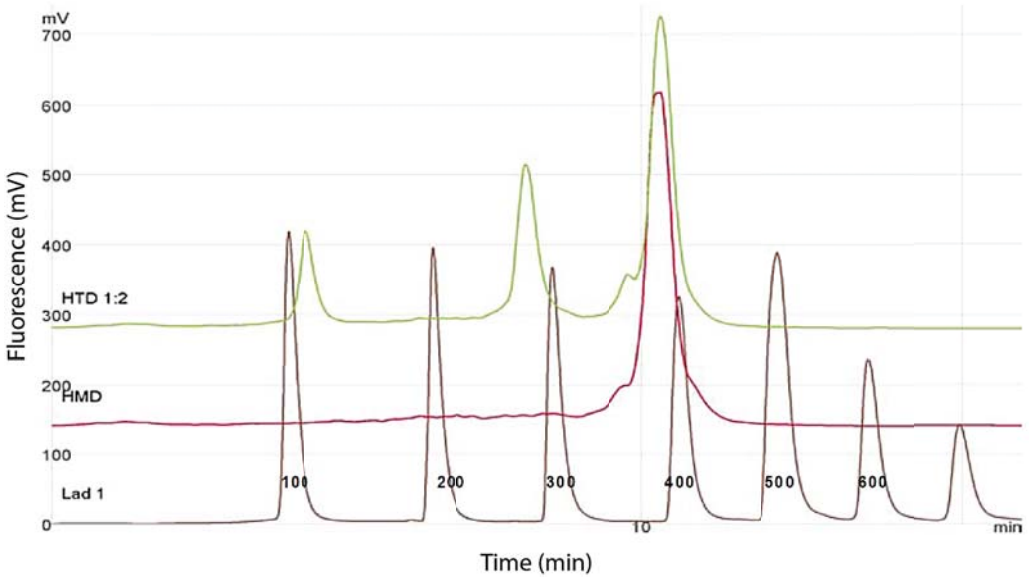

Figure 1. Surveyor Nuclease digestion products of an amplicon derived from exon 28 in the ABCC6 gene of human genomic DNA. The 405 bp amplicon was PCR amplified with Optimase Polymerase. Approximately 600 ng of unpurified amplicon DNA were self-hybridized, digested with Surveyor Nuclease and analyzed by WAVE HS HPLC as described in the Step-by-Step Instructions. The mutant was heterozygous for a single mutation (G>A; fragments were 116 + 289 bp long). Digestion products from self-hybridized mutant (green, top) and normal control DNA (red, middle) are shown. Marker (100 bp DNA Ladder) is shown at the bottom.

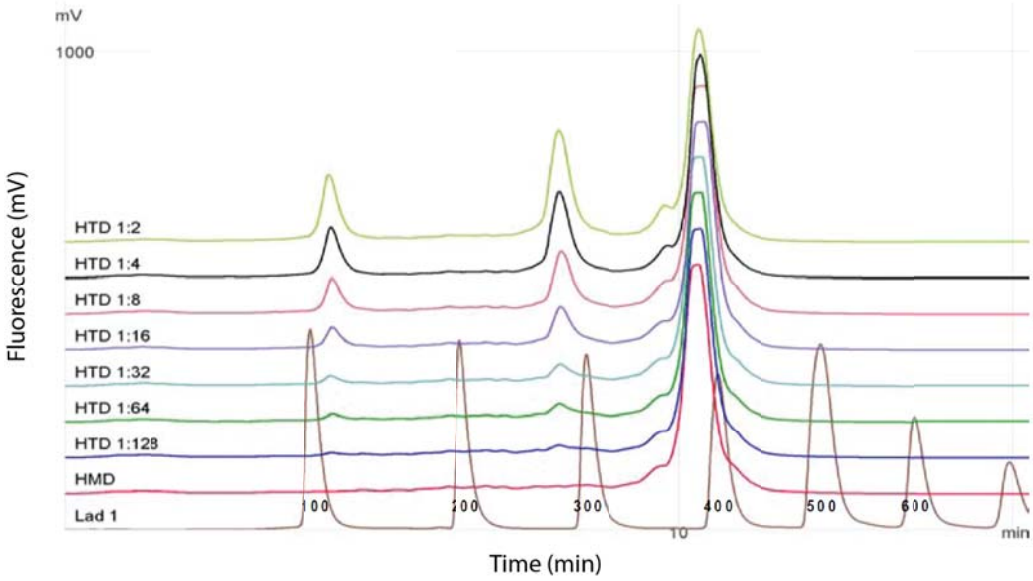

Figure 2. Surveyor Nuclease digestion of heteroduplex DNA present in decreasing amounts in homoduplex DNA. Human target DNA shown in Figure 1 was amplified from both mutant and normal DNA with Optimase Polymerase. Wild-type and mutant allele amplicons were mixed in the ratios indicated (HTD) and 600 ng of each mixture were hybridized, digested with Surveyor Nuclease and analyzed by WAVE HS HPLC as described in the Step-by-Step Instructions. Digestion products 116 + 289 bp long formed by cutting at the single-base mismatch are visible down to a ratio of heteroduplex to homoduplex of 1:128. Homoduplex (HMD) is shown near the bottom. Marker (100 bp DNA Ladder) is shown at the bottom.

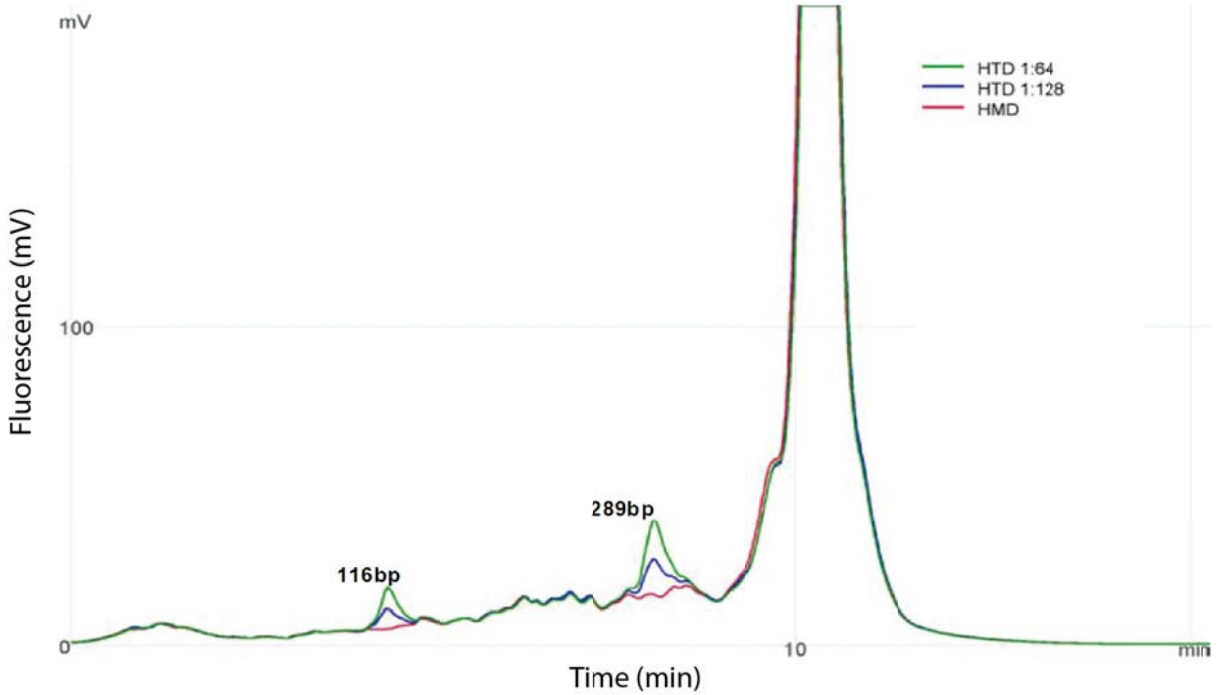

Figure 3. Superimposed WAVE profiles show differences in small proportion of heteroduplex: homoduplex DNA. The 1:64 (green) and 1:128 (blue) heteroduplex/homoduplex digestion profiles in Figure 2 are shown superimposed on the homoduplex digestion profile (red). The fluorescence signal axis has been expanded compared to Figure 2.

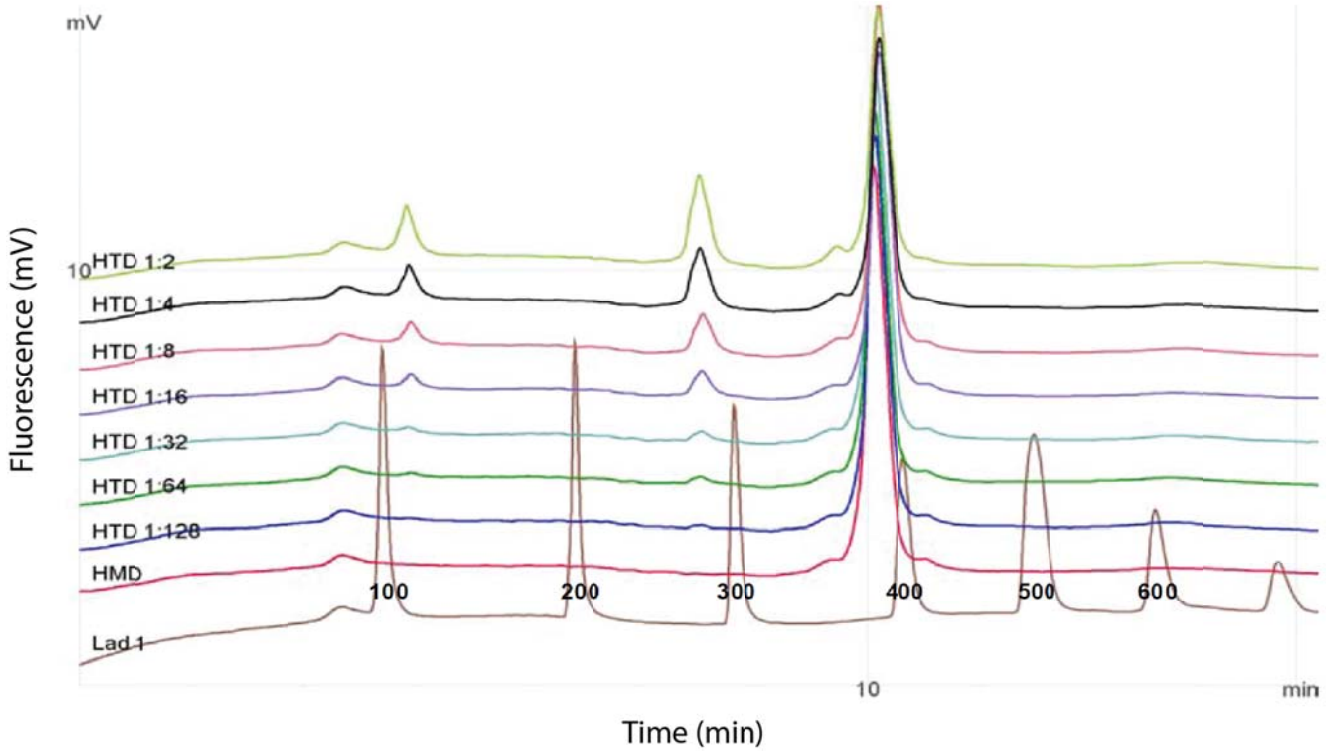

Figure 4. Surveyor Nuclease digestion of heteroduplex DNA present in decreasing amounts in homoduplex DNA. The digested DNAs analyzed by WAVE HS HPLC in Figure 2 were analyzed by HPLC on a WAVE UV system. Digestion products 116 + 289 bp long, formed by cutting at the single-base mismatch, are visible down to a ratio of heteroduplex to homoduplex of 1: 64. Homoduplex (HMD) is shown near the bottom. Marker (100 bp DNA Ladder) is shown at the bottom.

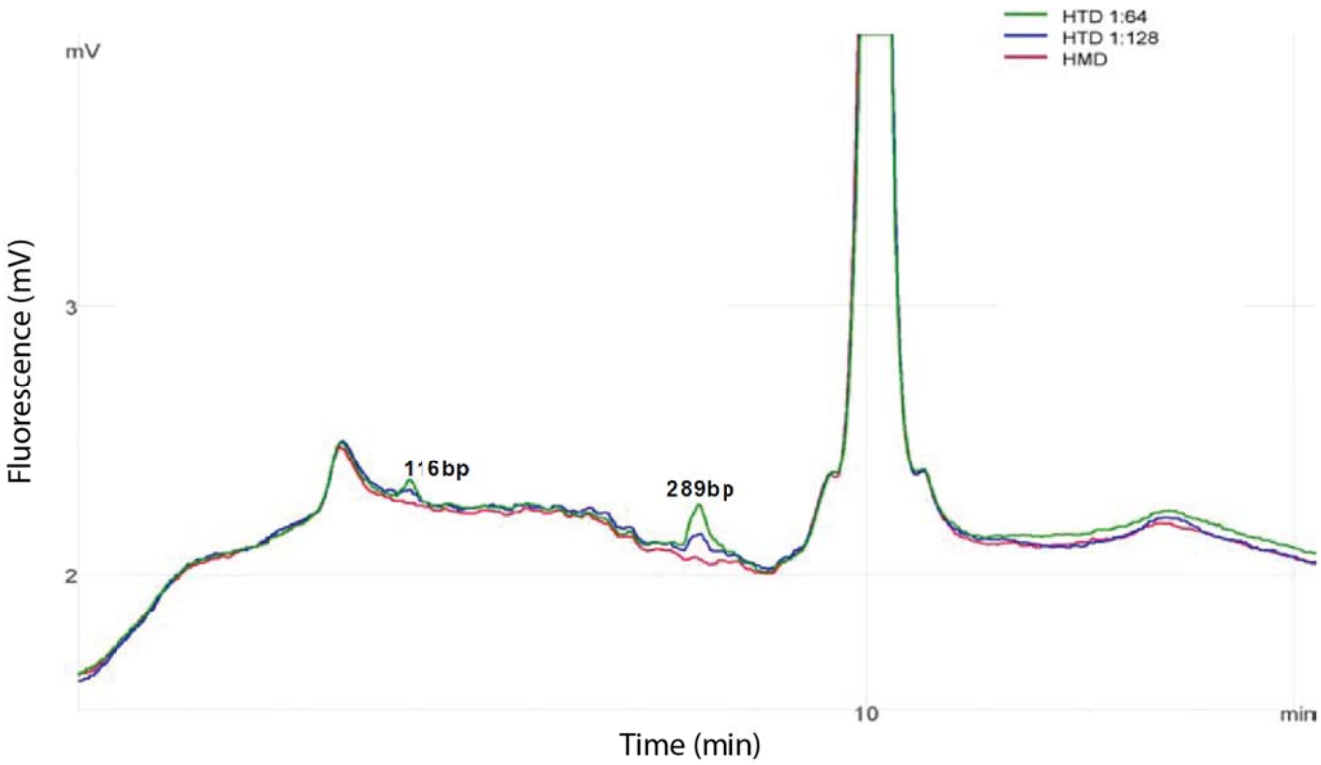

Figure 5. Sensitivity of WAVE UV analysis of Surveyor Nuclease-digested heteroduplex/homoduplex mixtures. The 1:64 (green) and 1:128 (blue) heteroduplex/homoduplex digestion profiles in Figure 4 are shown superimposed on the homoduplex digestion profile (red). The absorbance signal axis has been expanded compared to Figure 4.

Step-by-Step Instructions

Detecting Mutations with Surveyor Nuclease

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

In general, processing of samples should be carried out from start to finish as described in this User Guide. If processing of a sample is stopped before completion of all steps, the DNA should be stored at –20°C until the next step is carried out. However, exposure of any frozen sample to repeated freeze-thaw cycles should be avoided and storage at –20°C of PCR amplified DNA or Surveyor Nuclease digestion products for extended periods (>1 week) should be avoided.

Step 1—PCR Amplification of Reference and Test Samples

THIS STEP IS CRITICALTO 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).**
- **Your PCR product is essentially free of primer-dimer artifacts.**

The first step in the process is to prepare the PCR 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.
- Optimize your PCR conditions carefully. The PCR amplicon should appear as a single sharp peak or band of the expected size when analyzed by WAVE HPLC or agarose gel electrophoresis. Mispriming during PCR amplification can result in the formation of spurious DNA fragments that produce increased background during Surveyor Nuclease digestion. Use primers that are at least 20 nucleotides long (oligomers 25–35 nucleotides in length 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 nonspecific 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

In order to detect a homogeneous mutation in a test sample the PCR product must be hybridized with a wild-type reference PCR product to generate mismatches for Surveyor Nuclease cleavage. Both test sample and wild-type reference DNA are amplified with the same primers. 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 source or because it contains a pool of fragments derived from genetically different homozygous or heterozygous sources. 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 1% and 2% for analysis on the WAVE HS and WAVE System, respectively. After hybridization, retain some of the hybridized PCR product as an undigested reference (control).

Preparing PCR Products

This protocol was developed using Optimase Polymerase. Optimase is a proof-reading enzyme that has been developed specifically for SNP and mutation discovery applications using the WAVE and WAVE HS Systems. Other proofreading enzymes should work as long as the PCR is optimized according to this guide. Optimase Polymerase is not included in the Surveyor PLUS 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 2–5 µL aliquots of each product by electrophoresis in a 2% (w/v) agarose gel, prepared with high-resolution agarose 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 6X 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.
5. As long as the DNA polymerase buffer is compatible with Surveyor Nuclease, the amplified DNA can be used without further purification. Alternatively, the DNA can be concentrated by ethanol precipitation as follows. Transfer the reaction mixtures to suitable microcentrifuge tubes, 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 micropipetter, 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°C. 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.

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. 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 Cytosine base in lieu of the Guanine base (underlined). Primer sequences are underlined at the 5′ and 3′ end of the amplicon sequence.

ACACCTGATCAAGCCTGTTCATTTGATTACCAGAGAGACTGTCATGATCCACATGGAGGGAAGGACATGTGTG
TTGCTGGAGCCATTCAAAATTTCACATCTCAGCTTGGCCATTTCCGCCATGGAACATCTGATCGTCGATATAA
TATGACAGAGGCTTTGTTATTTTTATCCCACTTCATGGGAGATATTCATCAGCCTATGCATGTTGGATTTACA
AGTGATATGGGAGGAAACAGTATAGATTTGCGCTGGTTTCGCCACAAATCCAACCTGCACCATGTTTGGGATA
GAGAGATTATTCTTACAGCTGCAGCAGATTACCATGGTAAGGATATGCACTCTCTCCTACAAGACATACAGAG
GAACTTTACAGAGGGTAGTTGGTTGCAAGATGTTGAATCCTGGAAGGAATGTGATGATATCTCTACTAGCGCC
AATAAGTATGCTAAGGAGAGTATAAAACTAGCCTGTAACTGGGGTTACAAAGATGTTGAATCTGGCGAAACTC
TGTCAGATAAATACTTCAACACAAGAATGCCAATTGTCATGAAACGGATAGCTCAGGGTGGAATCCGTTTATC
CATGATTTTGAACCGAGTTCTTGGAAGCTCCGCAGATCATTCTTTGGCG

PCR amplification of 2 µL of each DNA solution in a 50 µL reaction should produce >25 ng/µL of a 633 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 amount of the user's amplified DNA that optimally matches the recommended amount of Surveyor Nuclease S in Appendix A, and the expected amount and appearance of digestion products on an agarose or polyacrylamide gel.

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 Ta = 65°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 and different amounts of a DNA mass ladder [e.g., 100 bp DNA Ladder from New England BioLabs] on a 2% high resolution agarose gel or a 10% polyacrylamide gel and compare the band intensities with those of the DNA ladder to establish the DNA concentration. See *Appendix B: Agarose Gel Electrophoresis* or *Appendix C: Polyacrylamide Gel Electrophoresis* for details. 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 (15 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 30 µL of Control C homoduplex in a separate tube.

4. Digest hybridized Control G/C and Control C homoduplex with Surveyor Nuclease S. Follow the instructions in ***Step 3—Treatment with Surveyor Nuclease*** to identify the Surveyor Nuclease reaction parameters, i.e. requirements for additional MgCl2 and/or enzyme, that match the PCR DNA polymerase/buffer formulation used to prepare the DNA substrate. Further optimization can be achieved by carrying out a pilot study matching amount of Control C and Control G/C to the recommended amount of Surveyor Nuclease. An example of this for Optimase Polymerase PCR products is shown in Table 1. Set up 6, 0.5 mL reaction tubes on ice with the indicated amounts of PCR product in Table 1 and the amount of Surveyor Nuclease and additional MgCl2 determined as described in Step 3. Incubate the tubes at 42°C for 60 min. Add 1/10th volume of Stop Solution.

5. Analyze the digested DNA in each reaction mixture as described in ***Step 4—Analysis of DNA Fragments***. Load all the samples on the gel. Program the autosampler to inject 6, 12, and 24 µL from tube number 1 and 4, 2 and 5, and 3 and 6, respectively.

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

Step 2—DNA Duplex Formation

In this step, sample and reference DNA are mixed and hybridized to form heteroduplexes. Make sure to take along sample and/or reference DNA alone in separate tubes to serve as controls. The use of a heated-lid thermocycler is recommended—it is important to carefully follow the protocol shown here.

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

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 25–80 ng/µL (**ideally 50 ng/µL**). About 200–400 ng of hybridized DNA is recommended for treatment with Surveyor Nuclease S, so that **each tube should contain at ≥200 ng total DNA**.
   - Heterogeneous test sample DNA that does not require the addition of wild-type, or reference DNA should still undergo the following hybridization procedure to ensure the formation of cleavable mismatches.

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

Temperature	Time	Temperature ramp
95°C	10 min	
95°C to 85°C		(–2.0°C/s)
85°C	1 min	
85°C to 75°C		(–0.3°C/s)
75°C	1 min	
75°C to 65°C		(–0.3°C/s)
65°C	1 min	
65°C to 55°C		(–0.3°C/s)
55°C	1 min	
55°C to 45°C		(–0.3°C/s)
45°C	1 min	
45°C to 35°C		(–0.3°C/s)
35°C	1 min	
35°C to 25°C		(–0.3°C/s)
25°C	1 min	
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 in equal quantities of the two PCR products to generate the heteroduplex. 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°C.

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 and mix.

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

In this step, the heteroduplex test sample DNA from Step 2 is cleaved by the Surveyor Nuclease, along with necessary reference DNA and controls.

Optimized Mg^{2+} concentration is essential to Surveyor Nuclease function. If a different PCR DNA polymerase is used to amplify the DNA, consult the table in Appendix A for appropriate Surveyor Nuclease reaction conditions to digest the DNA. If the DNA polymerase you are using is not listed in the Appendix A table, consult the instructions in Appendix A to select the appropriate reaction conditions.

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

Set up Surveyor Nuclease reactions for Control G and Control C duplexes as follows:

Tube No.	Volume Required (µL)					
	Hybridized Control G/C	Hybridized Control C	0.15 M $MgCl_2$	Surveyor Enhancer S	Surveyor Nuclease S	Stop Solution (Add after incubation)
1	-	6	0.6	1	1	0.9
2	-	12	1.2	1	1	1.5
3	-	18	1.8	1	1	2.2
4	6	-	0.6	1	1	0.9
5	12	-	1.2	1	1	1.5
6	18	-	1.8	1	1	2.2

Set up Surveyor Nuclease reactions for experimental sample DNA as follows:

1. The volumes of 0.15 M $MgCl_2$ Solution required are calculated based upon the volume (V) of the PCR product used.

2. For each digestion, add the following components in the order shown to a nuclease-free 0.2 mL tube (kept on ice):

Component	**Amount**
Hybridized DNA[1]	200–400 ng
0.15 M $MgCl_2$ Solution[2]	$1/10^{th}$ volume
Surveyor Enhancer S	1 µL
Surveyor Nuclease S	1 µL

[1]DNA volume should be 8–40 µL

[2]This additional magnesium is required even if PCR buffer also contains some magnesium

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

4. Incubate at 42°C for 60 min.

    - **We recommend staring with a 60 min incubation time at 42C. In some cases, reaction time can be reduced to 20 minutes. Increasing reaction time beyond 60 min will increase DNHA degradation due to exonuclease activity of the Surveyor enzyme**

Add 1/10th volume of Stop Solution and mix. Store the digestion products at –20°C if not analyzed immediately.

Note:

- DNA prepared with some DNA polymerase/buffer formulations requires double the amount of Surveyor Nuclease S (2 µL; see APPENDIX A table). The amount of Enhancer S used should be kept at 1 µL in all cases.

- To reduce the number of manipulations, MgCl2, Surveyor Enhancer S and Nuclease S can be mixed and a single pipetting can be done. This 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.

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 300 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 Type:	DS Multiple Fragments
Oven Temperature:	50°C
Min/100 bp:	2.5
Number of Segments:	5
Base Pair Minimum:	30 bp
Base Pair Maximum:	Amplicon Size + 50 bp
Clean Duration:	0.5
Equilibrium Duration:	0.9
Flow rate:	0.9 mL/min
Dyes:	WAVE-HS 1
Clean Type:	Active
Injection Type:	All
Sequence Context:	None
Amplicon:	None
Eluent A:	WAVE Optimized® Buffer A
Eluent B:	WAVE Optimized Buffer B
Detection:	260 nm for UV; 492 nm Ex and 526 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:

- Every 100 injections of Surveyor Nuclease digests should be followed by a HOT WASH. To perform a HOT WASH, at 80°C pump 100% Solution D (75% ACN) for 15 minutes, followed by 50%/50% buffer A/B for 30 minutes 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.

Surveyor Nuclease **digestion of hybridized Control G/C PCR** products gives rise to two cleavage products, 217 and 416 bp in size, which are clearly distinguishable by WAVE analysis as shown in Figures 6 and 7. Analysis of different amounts of substrate digested with the recommended amount of 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 6 and 7, the digestion and injection conditions for tube 6 gave the best results. The amounts of digestion products produced in the 6, 12 and 24 µL digestion of Control G/C substrate were approximately proportional to the amount of substrate present. Under the reaction conditions used, 1 µL of Surveyor Nuclease W contained sufficient mismatch cutting activity to continue to cut the mismatches in 1200 ng substrate during a 60 minute incubation. 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 5–10 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.

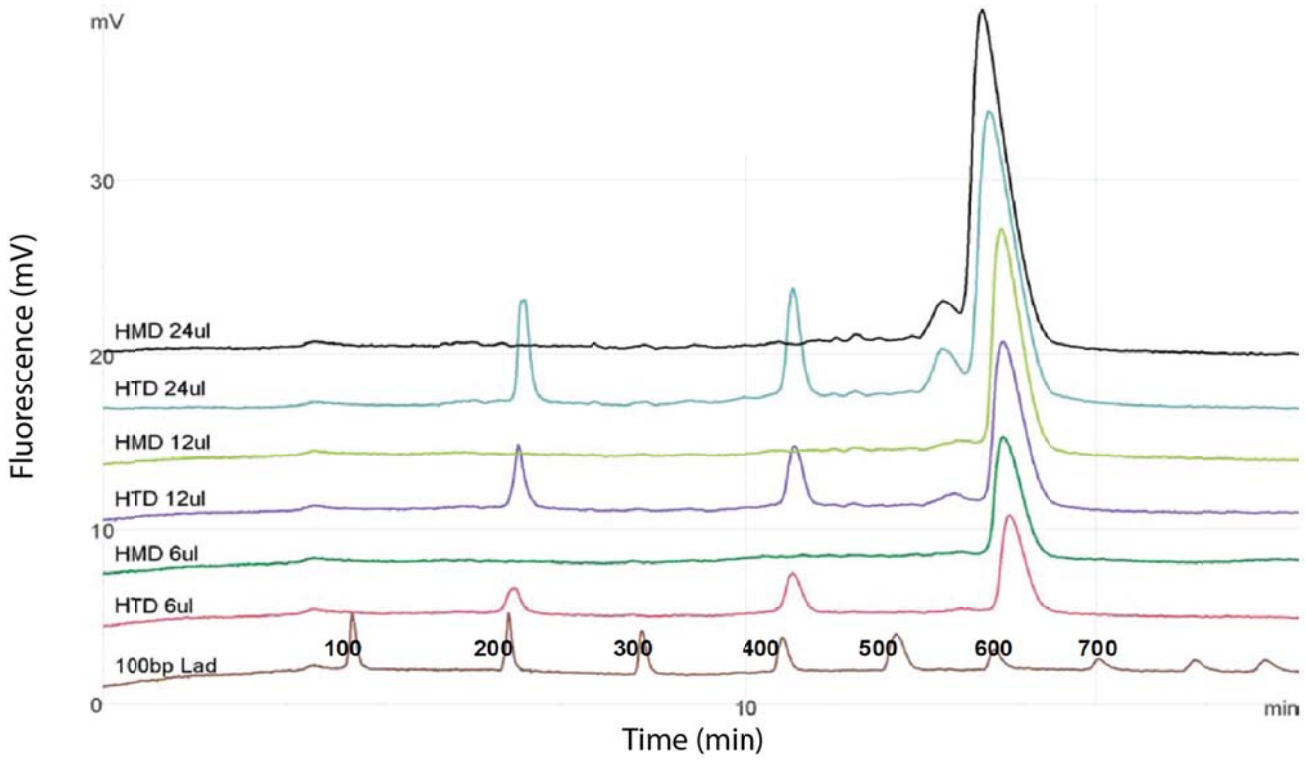

Figure 6. **Surveyor Nuclease digestion products of self-annealed Control C homoduplex (HMD) and Control G/C homoduplex/heteroduplex (HTD).** The 633 bp amplicons were PCR amplified with Optimase Polymerase from 2 µL of Control G and Control C. Control G/C homoduplex/heteroduplex was formed by hybridizing 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 1200 ng) was digested with 1 µL of Surveyor Nuclease W, 1 µL of Surveyor Enhancer W2, 1/10th volume 0.15 M $MgCl_2$ and 1/10th volume Enhancer Cofactor for 60 min at 42°C. Surveyor Nuclease digestion products [180 (6 µL), 400 (12 µL), and 850 ng (24 µL)] were analyzed using the WAVE System run under non-denaturing conditions at 50°C with UV detection. Transgenomic 100 bp Ladder DNA was run as a marker. The 217 and 416 bp cleavage products expected from the Control G/C heteroduplex are clearly visible. Also visible in all three chromatograms is the full-length 633 bp homoduplex.

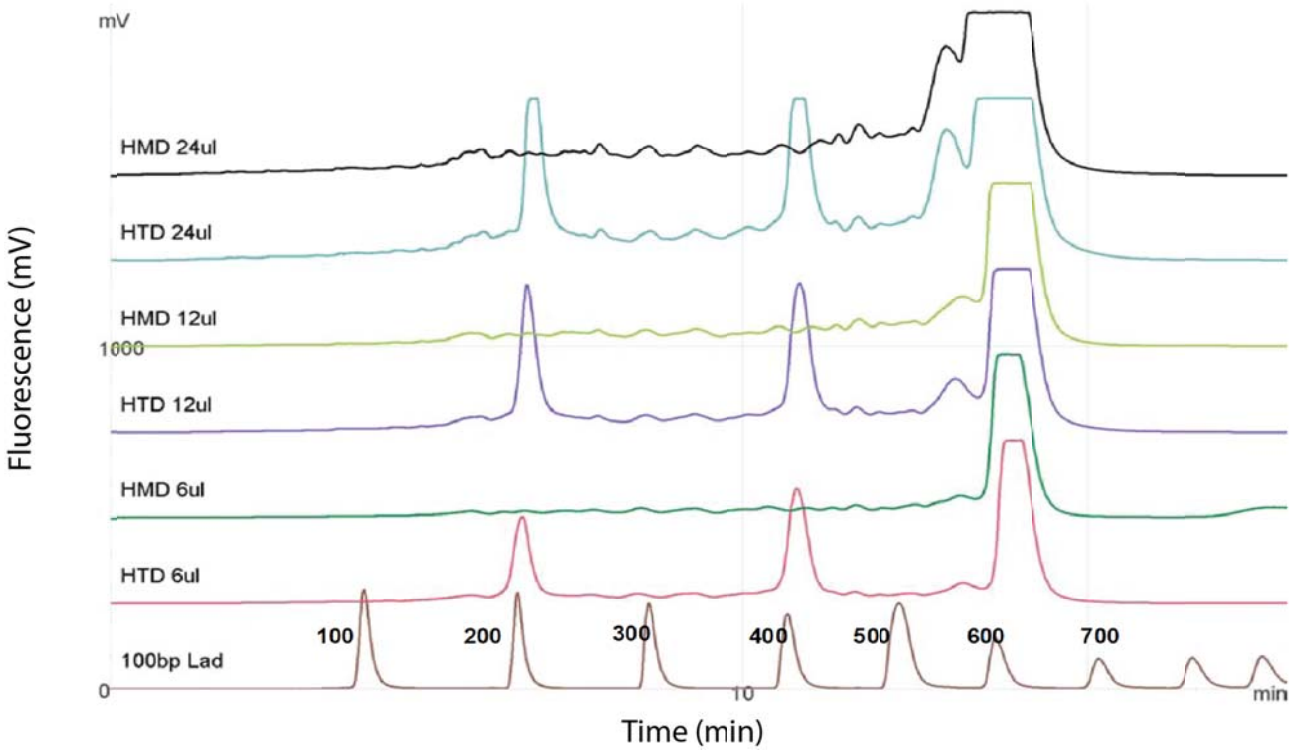

Figure 6. **Surveyor Nuclease digestion products of self-annealed Control C homoduplex (HMD) and Control G/C homoduplex/heteroduplex (HTD).** The 633 bp amplicons were PCR amplified with Optimase Polymerase from 2 µL of Control G and Control C. Control G/C homoduplex/heteroduplex was formed by hybridizing 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 1200 ng) was digested with 1 µL of Surveyor Nuclease W, 1 µL of Surveyor Enhancer W2,1/10th volume of 0.15 M $MgCl_2$ and 1/10th volume of Enhancer Cofactor for 60 min at 42°C. Surveyor Nuclease digestion products [180 (6 µL), 400 (12 µL) and 850 ng (24 µL)] were analyzed using the WAVE System run under non-denaturing conditions at 50°C and equipped with a Fluorescence Detector and a High-Sensitivity Accessory for post-column DNA intercalation with fluorescent dye. Transgenomic 100 bp Ladder DNA was run as a marker. The 217 and 416 bp cleavage products expected from the Control G/C heteroduplex are clearly visible. Also visible in all three chromatograms is the full-length 633 bp homoduplex. Flat-top peaks were produced by injection of amounts of DNA that saturated the instrument detector.

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 10X 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:

IF	THEN
You want to use Optimase ProtocolWriter™ to design a PCR protocol	1. Access www.mutationdiscovery.com. 2. On MutationDiscovery.com, click on **ProtocolWriter** under Optimase. The ProtocolWriter entry window appears. 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. Calculate the Tm for your primers using the IDT OligoAnalyzer® tool at www.idtdna.com/SciTools. Enter the primer sequences and the exact PCR conditions into the tool. 2. The T_a is estimated as 5°C below the lowest T_m of the PCR primer pair. Optimization of primer T_m may be necessary. 3. Proceed to step 4

TIP: Please use the Optimase MasterMix Calculator at http://www.mutationdiscovery.com to prepare a master mix for your PCR reaction.

4. Use the following program for a heated-lid thermocycler:

94°C	2 min	1 Cycle
94°C T_a°C 72°C	30 s 30 s 30 s per 250 bp	30 Cycles
72°C	5 min	1 Cycle
4°C	∞	Hold

5. Analyze 2–5 µL aliquots of each product by electrophoresis in a 2% (w/v) agarose gel, prepared with high-resolution agarose 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 6X 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 2/3rd 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 250–300 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 15–80 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 micropipetter, 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: Surveyor Nuclease Reaction Conditions

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. Consult Table B1 in this section for the Surveyor Nuclease reaction conditions recommended for use with several commercial DNA polymerase/buffer formulations. If your DNA polymerase/buffer formulation is not in the table use the following recommendations.

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 $MgCl_2$; for Optimase Polymerase the 1X PCR buffer consists of 10 mM Tris-HCl, 75 mM KCl and 1.5 mM $MgCl_2$. 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 10–20 mM Tris-HCl or Tris-SO_4 (pH 8.8 to 9.3), 50–75 mM KCl, 1–3 mM $MgCl_2$ or $MgSO_4$, 0.1%–1% (v/v) nonionic detergent and BSA or gelatin. $(NH4)_2SO_4$ at 10–20 mM is also acceptable as long as the KCl concentration is ≤50 mM. PCR additives such as DMSO (5% v/v), glycerol (10% v/v), betaine (1 M) and 1X PCR_x Enhancer (Invitrogen) **are acceptable at or below the concentrations listed. If any of these additives are present in the PCR reaction mixture at higher concentrations than those listed, Surveyor Nuclease mismatch cutting activity will be inhibited.**

If an additive is present at an inhibitory concentration, we suggest cleaning up the PCR product. Use ethanol precipitation (see *Preparing PCR Products* section) or a commercial PCR clean-up column before the hybridization step. The DNA should then be placed in a buffer compatible with the hybridization and Surveyor Nuclease digestion steps, such as 10 mM Tris-HCl (pH 8.8), 15 mM $MgCl_2$, and 50 mM KCl. After hybridization in this optimal buffer, digest the DNA with 1 µL of Surveyor Nuclease W, 1 µL of Surveyor Enhancer W2 and $1/10^{th}$ volume of Enhancer Cofactor at 42°C for 60 min.

If the DNA polymerase/buffer formulation is not listed in the table and the manufacturer does not reveal the contents of the PCR buffer, there are several options. Clean up the DNA and proceed as described in the paragraph immediately above. Alternatively, carry out pilot Surveyor Nuclease digestion studies with Control G/C or genomic DNA containing a known mismatch amplified with your DNA polymerase to establish empirically the best digestion conditions. Digest the hybridized PCR product in 1X PCR buffer with and without additional Mg^{2+} with 1 or 2 µL of Surveyor Nuclease, 1 µL of Enhancer W2 and $1/10^{th}$ volume of Enhancer Cofactor for 60 min at 42°C (see *Step 3—Treatment with Surveyor Nuclease* and *Control Experiments—Using Control G and Control C Plasmid DNA*).

Note: Injection of 1X PCR buffers other than those used with Optimase, Maximase™, or T-Taq DNA Polymerases can rapidly degrade the performance of DNASep and DNASep HT cartridges. The cartridges are not warranted for injection of such samples.

NOTE: Information about competitor products is included for guidance only and should in no way be construed or implied as a endorsement or recommendation.

Table B1 Surveyor Nuclease Reaction Conditions Recommended for use with Various PCR DNA Polymerase/Buffer Formulations

Enzyme	Optimal Surveyor Nuclease Reaction Conditions[a]
Transgenomic T-Taq DNA Polymerase	1 µL SNW/Add Mg^{2+}
Transgenomic Optimase Polymerase	1 µL SNW/Add Mg^{2+}
Transgenomic Maximase DNA Polymerase	1 µL SNW/Add Mg^{2+}
ABI AmpliTaq® DNA Polymerase	1 µL SNW**/Add Mg^{2+}**[b]
ABI GeneAmp® Fast PCR Master Mix	1 µL SNW/Add Mg^{2+}
Bio-Rad iProof™ HF DNA Polymerase + HF Buffer	1 µL SNW/Add Mg^{2+}
Denville Hot Start Taq	1 µL SNW/Add Mg^{2+}
Epicentre FailSafe™ PCR 2X PreMix A through L	1 µL SNW**/Add Less Mg^{2+}**[c]
GeneChoice® AccuPol™ DNA Polymerase	1 µL SNW/Add Mg^{2+}
Invitrogen Platinum® Taq DNA Polymerase	1 µL SNW/Add Mg^{2+}
NEB Taq DNA Polymerase	1 µL SNW/Add Mg^{2+}
NEB Phusion™ High-Fidelity DNA Polymerase + HF Buffer	1 µL SNW/Add Mg^{2+}
Novagen NovaTaq™ DNA Polymerase	1 µL SNW/Add Mg^{2+}
Roche Taq DNA Polymerase	1 µL SNW/Add Mg^{2+}
Roche FastStart Taq DNA Polymerase	1 µL SNW/Add Mg^{2+}
Roche FastStart Taq DNA Polymerase + Q Solution	1 µL SNW/Add Mg^{2+}
Roche Expand High Fidelity^{PLUS} PCR System	1 µL SNW/Add Mg^{2+}
Sigma JumpStart™ Taq DNA Polymerase	1 µL SNW/Add Mg^{2+}
Stratagene SureStart™ Taq DNA Polymerase	1 µL SNW/Add Mg^{2+}
Stratagene PfuTurbo® Hotstart DNA Pol.	1 µL SNW/Add Mg^{2+}
Stratagene PfuUltra™ Hotstart DNA Pol.	1 µL SNW/Add Mg^{2+}
Takara Taq™ DNA Polymerase	1 µL SNW/Add Mg^{2+}
Takara Ex Taq™ DNA Polymerase	1 µL SNW/Add Mg^{2+}

Enzyme	Optimal Surveyor Nuclease Reaction Conditions[a]
ABI GeneAmp® High Fidelity PCR Kit	2 µL SNW/Add Mg^{2+}
Invitrogen Platinum Taq DNA Polymerase High Fidelity	2 µL SNW/Add Mg^{2+}
Novagen KOD XL DNA Polymerase	2 µL SNW/Add Mg^{2+}
Promega GoTaq® DNA Polymerase	2 µL SNW/Add Mg^{2+}
Qiagen HotStarTaq® DNA Polymerase	2 µL SNW/Add Mg^{2+}
Qiagen HotStarTaq DNA Polymerase + Q-Solution	2 µL SNW/Add Mg^{2+}
Qiagen ProofStart® DNA Polymerase	2 µL SNW/Add Mg^{2+} (**Not Recommended**)[d]
Qiagen ProofStart DNA Polymerase + Q-Solution	2 µL SNW/Add Mg^{2+} (**Not Recommended**)
Roche Expand™ High Fidelity PCR System	2 µL SNW/Add Mg^{2+}
Stratagene PfuUltra™ II Fusion HS Polymerase	2 µL SNW/Add Mg^{2+}
Takara E2TAK™ DNA Polymerase	2 µL SNW/**Add Less Mg^{2+}**
Takara PrimeSTAR® HS DNA Polymerase	2 µL SNW/Add Mg^{2+} (**Not Recommended**)
Bio-Rad iProof HF DNA Polymerase + GC Buffer	1 µL SNW/Add No Mg^{2+}[f]
Eppendorf HotMaster™ Taq DNA Polymerase	1 µL SNW/Add No Mg^{2+}
Invitrogen Platinum Pfx DNA Polymerase	1 µL SNW/Add No Mg^{2+} (**Not Recommended**)
Invitrogen Platinum Pfx DNA Polymerase + PCRx Enhancer	1 µL SNW/Add No Mg^{2+} (**Not Recommended**)
NEB Phusion High-Fidelity DNA Polymerase + GC Buffer	1 µL SNW/Add No Mg^{2+}
Sigma AccuTaq™ LA DNA Polymerase	1 µL SNW/Add No Mg^{2+}
Takara LA Taq™ DNA Polymerase	1 µL SNW/Add No Mg^{2+}

[a] All reactions should contain the indicated volume of Surveyor Nuclease W (SNW), $1/10^{th}$ volume Enhancer Cofactor and 1 µL Surveyor Enhancer W2

[b] Add Mg^{2+} unless indicated otherwise add $1/10^{th}$ volume of 0.15 M $MgCl_2$ to reactions as described in Step 3

[c] FailSafe PCR PreMixes contain high Mg^{2+} concentrations; add only $1/20^{th}$ volume of 0.15 M $MgCl_2$ to reactions

[d] Not Recommended—reaction conditions could not be found that produced acceptable amounts of mismatch digestion products with acceptable background; the reaction conditions specified were the best identified

[e] E2TAK DNA Polymerase 1X PCR reaction buffer appears to contain a high Mg^{2+} concentration; add only $1/40^{th}$ volume of 0.15 M $MgCl_2$ to reactions

[f] Add No Mg^{2+} do not add any 0.15 M $MgCl_2$ to these reactions

Appendix C: Troubleshooting

Effective use of the Surveyor PLUS 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 PLUS Mutation Detection Kit and how to resolve them.

Problem 1—Low PCR yield or no PCR product

POSSIBLE CAUSE	SOLUTION
Not enough template and/or too few cycles	Increase the template concentration and/or add more PCR cycles.
Suboptimal PCR parameters	Do one or more of the following: • Decrease the annealing temperature in increments of 2°C. • Increase the extension time. For Optimase Polymerase, use 30 sec per 250 bp. • Adjust the Mg^{2+} concentration
Suboptimal DNA polymerase for target	Use a "hot-start" PCR DNA polymerase. Increase the amount of DNA polymerase.

Problem 2—Multiple PCR products

POSSIBLE CAUSE	SOLUTION
Poor primer design	Redesign primers to improve specificity, T_m, and GC content.
Annealing temperature too low	Increase the annealing temperature in increments of 2°C.
Extension time too long	Reduce the extension time. For Optimase Polymerase, use 30 sec per 250 bp.
Cycle number too high	Reduce cycle number in increments of 2.
Suboptimal PCR conditions	Use "hot-start" PCR and/or touchdown PCR or perform nested PCR.

Problem 3—No cleavage products observed upon analysis after Surveyor Nuclease treatment of known heteroduplex

POSSIBLE CAUSE	SOLUTION
Proportion of mismatch target too low	Mix equal amounts of test and reference DNA before annealing.
Cleavage site too close to PCR product end	Redesign the primer set to move the target site away from ends.
Inactive Surveyor Nuclease	Perform the Control reaction to verify enzyme performance.
Too little enzyme	Increase the amount of Surveyor Nuclease 2-fold and repeat digestion.
Too little substrate	Concentrate the PCR products by ethanol precipitation before annealing.
Incompatible 1X PCR buffer	Consult Appendix B for suggested courses of action

Problem 4—High background after Surveyor Nuclease treatment

POSSIBLE CAUSE	SOLUTION
Suboptimal hybridization step	Do the following: 1. Make sure the DNA concentration is in the range of >25 ng/µL to <50 ng/µL. 2. Repeat the hybridization step, taking care to cool the annealing mixture slowly. 3. Add 1X PCR reaction buffer to precipitated products before annealing. 4. Use sufficient sample volume (>20 µL) during hybridization in a water bath. 5. Add 1/10 volume 0.5 M KCl to PCR product contained in low salt 1X PCR reaction buffer
Errors introduced by PCR enzyme	Use a high fidelity PCR enzyme, such as Optimase Polymerase.
Incompatible 1X PCR buffer	Consult Appendix B for suggested courses of action
Too much Surveyor Nuclease	Reduce the Surveyor Nuclease 2 fold and repeat digestion.
DNA amount too low	Increase the DNA amount to at least 400 ng of substrate per 1 µL of Surveyor Nuclease W used.
Nonspecific PCR products	Optimize the PCR parameters to increase specificity. Always use an appropriate substrate as a control to identify background.
Enhancer W2 has lost activity	Increase the amount of Enhancer W2 2-fold and repeat digestion.

References

1. Qiu P, Shandilya H, et al. (2004) Mutation detection using Surveyor nuclease. Biotechniques, 36(4):702–707.
2. Scaffino MF, Pilotto A, et al. (2004) Heteroduplex detection with a plant DNA endonuclease for standard gel electrophoresis. Transgenics, 4:157–166.
3. Mitani N, Tanaka S, Okamoto Y (2006) Surveyor nuclease-based genotyping of SNPs. Clin Lab, 52(7–8):385–396.
4. Caldwell DG, McCallum N, et al. (2004) A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare L*.). Plant J, 40(1):143–150.
5. Shi R, Otomo K, et al. (2006) Temperature-mediated heteroduplex analysis for the detection of drug-resistant gene mutations in clinical isolates of *Mycobacterium tuberculosis* by denaturing HPLC, SURVEYOR nuclease. Microbes Infect, 8(1):128–135.
6. Shi RR, Du CM (2006) Analysis of ethambutol drug-resistance gene mutation in Mycobacterium tuberculosis by specific endonuclease. Zhonghua Jie He He Hu Xi Za Zhi, 29(10):702–704.
7. Wada T, Fukushima Y, Saitoh S (2006) A new detection method for ATRX gene mutations using a mismatch-specific endonuclease. Am J Med Genet A, 140(14):1519–1523.
8. Thomas HR, Ezzeldin HH, et al. (2007) Genetic regulation of dihydropyrimidinase and its possible implication in altered uracil catabolism. Pharmacogenet Genomics, 17(11):973–987.
9. Thomas HR, Ezzeldin HH, et al. (2008) Genetic regulation of beta-ureidopropionase and its possible implication in altered uracil catabolism. Pharmacogenet Genomics,18(1):25–35.
10. Bannwarth S, Procaccio V, Paquis-Flucklinger V (2005) Surveyor Nuclease: a new strategy for a rapid identification of heteroplasmic mitochondrial DNA mutations in patients with respiratory chain defects. Hum Mutat, 25(6):575–582.
11. Bannwarth S, Procaccio V, Paquis-Flucklinger V (2006) Rapid identification of unknown heteroplasmic mutations across the entire human mitochondrial genome with mismatch-specific Surveyor Nuclease. Nat Protoc, 1(4):2037–2047.
12. Bannwarth S, Procaccio V, et al. (2007) Rapid identification of mitochondrial DNA (mtDNA) mutations in neuromuscular disorders by using surveyor strategy. Mitochondrion, 8(2):136-45.
13. Jänne PA, Borras AM (2006) A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. Clin Cancer Res, 12(3 Pt 1):751–758.
14. Jänne PA, Johnson BE (2006) Effect of epidermal growth factor receptor tyrosine kinase domain mutations on the outcome of patients with non-small cell lung cancer treated with epidermal growth factor receptor tyrosine kinase inhibitors. Clin Cancer Res, 12(14 Pt 2):4416s–4420s.
15. Engelman JA, Mukohara T, et al. (2006) Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. J Clin Invest, 116(10):2695–2706.
16. Jackman DM, Yeap BY, et al. (2006) Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. Clin Cancer Res, 2(13):3908–3914.
17. Giaccone G, Gallegos Ruiz M, et al. (2006) Erlotinib for frontline treatment of advanced non-small cell lung cancer: a phase II study. Clin Cancer Res, 12(20 Pt 1):6049–6055.
18. Jackman DM, Holmes AJ, et al. (2006) Response and resistance in a non-small-cell lung cancer

patient with an epidermal growth factor receptor mutation and leptomeningeal metastases treated with high-dose gefitinib. J Clin Oncol, 24(27):4517–4520.

19. Marchetti A, Felicioni L, Buttitta F (2006) Assessing EGFR Mutations. N Engl J Med, 354(5):526–527.
20. Sattler M, Walz C, et al. (2006) A sensitive high-throughput method to detect activating mutations of Jak2 in peripheral-blood samples. Blood 107(3):1237–1238.
21. Jamieson CH, Gotlib J, et al. (2006) The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. Proc Natl Acad Sci U S A, 103(16):6224–6229.
22. Ho AY, Atencio DP, et al. (2006) Genetic predictors of adverse radiotherapy effects: the Gene-PARE project. Int J Radiat Oncol Biol Phys, 65(3):646–655.
23. Poeta ML, Manola J, et al. (2007) TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med 357(25):2552–2561.
24. Mitani N, Niwa Y, Okamoto Y (2007) Surveyor nuclease-based detection of p53 gene mutations in haematological malignancy. Ann Clin Biochem, 44(Pt 6):557–559.
25. Mitsuhashi T, Nakayama M, et al. (2007) KIT-negative undifferentiated endometrial sarcoma with the amplified epidermal growth factor gene showing a temporary response to Imatinib mesylate. Ann Diagn Pathol, 11(1):49–54.
26. Nakayama M, Mitsuhashi T. et al. (2006) Immunohistochemical evaluation of KIT expression in sarcomas of the gynecologic region. Int J Gynecol Pathol, 25(1):70–76.
27. Gotlib J, Berubé C, et al. (2005) Activity of the tyrosine kinase inhibitor PKC412 in a patient with mast cell leukemia with the D816V KIT mutation. Blood, 106(8):2865–2870.
28. Nowak D, Mossner M, et al. (2006) Mutation analysis of hCDC4 in AML cells identifies a new intronic polymorphism. Int J Med Sci, 3(4):148–151.
29. Conlan LH, Stanger MJ, et al. (2005) Localization, mobility and fidelity of retrotransposed Group II introns in rRNA genes. Nucleic Acids Res, 33(16):5262–5270.
30. Qiu P, Shandilya H, Gerard GF (2005) A method for clone confirmation using a mismatch-specific DNA endonuclease. Mol Biotechnol, 29(1):11–18.
31. Shi Y1, Terry SF, et al. (2007) Development of a rapid, reliable genetic test for pseudoxanthoma elasticum. J. Molec. Diagnostics 9, 105–112.
32. Gerard GF, Shandilya H, Qiu, P, Shi Y, Lo J. (2006) Genetic variance detection using Surveyor Nuclease. In Genetic Variance Detection. In: Hecker KH (editor) *Technologies for Pharmacogenomics.* DNA Press. p 95–129.
33. Pagniez-Mammeri H, Lombes A, et al. (2009) Rapid screening for nuclear genes mutations in isolated respiratory chain complex I defects. Mol Genet Metab, 96(4):196–200.
34. Nickerson ML1, Jaeger E, et al. (2008) Improved identification of von Hippel-Lindau gene alterations in clear cell renal tumors. Clin Cancer Res, 14(15):4726–4734.
35. Tan YC, Blumenfeld JD, et al. (2009) Novel method for genomic analysis of PKD1 and PKD2 mutations in autosomal dominant polycystic kidney disease. Hum. Mutat. 30(2):264–273.
36. Koivunen JP, Kim J, et al. (2008) Mutations in the LKB1 tumour suppressor are frequently detected in tumours from Caucasian but not Asian lung cancer patients. Br J Cancer, 99(2):245–

52.

37. Tsuji T, Niida Y (2008) Development of a simple and highly sensitive mutation screening system by enzyme mismatch cleavage with optimized conditions for standard laboratories. Electrophoresis 29(7):1473–83.
38. Hung CC, Su YN, et al. (2008) Comparison of the mismatch-specific endonuclease method and denaturing high-performance liquid chromatography for the identification of HBB gene mutations. BMC Biotechnol. 8:62 doi: 10.1186/1472-6750-8-62.
39. Talamè V, Bovina R, et al. (2008) TILLMore, a resource for the discovery of chemically induced mutants in barley. Plant Biotechnol J, 6(5):477–485.
40. Voskarides K, Makariou C, et al. (2008) NPHS2 screening with SURVEYOR in Hellenic children with steroid-resistant nephrotic syndrome. Pediatr Nephrol, 23(8):1373–1375.
41. Voskarides K, Deltas C (2009) Screening for mutations in kidney-related genes using SURVEYOR nuclease for cleavage at heteroduplex mismatches. J Mol Diagn, (4):311–318.
42. Kuang Y, Rogers A, et al. (2009) Noninvasive detection of EGFR T790M in gefitinib or erlotinib resistant non-small cell lung cancer. Clin Cancer Res, 15(8):2630–2636.