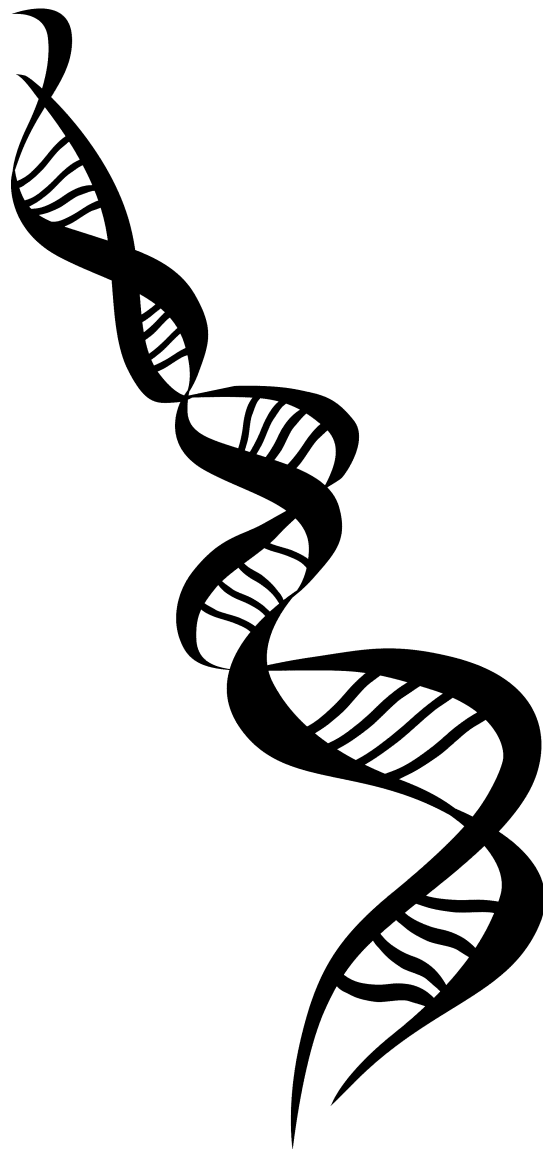


PCR Considerations for Successful Mutation Detection

5



The polymerase chain reaction (PCR) is used to generate DNA products for mutation detection analysis on the Transgenomic WAVE[®] System. Due to the sensitivity of the WAVE System in detecting mutations in DNA amplicons, a number of guidelines should be followed to ensure that PCR products generated are relatively free from polymerase-induced errors and spurious products. In addition, the polymerase mixtures need to be free of chemicals that are incompatible with the DNASep[®] Cartridge and the DNASep[®] HT Cartridge. Production of amplicons suitable for mutation analysis on the WAVE System is affected by a number of variables, including:

- Amplicon Design
- Primer Design and Purity
- Template DNA Source and Purity
- Polymerase Fidelity and PCR Component WAVE System Compatibility
- Thermocycling conditions

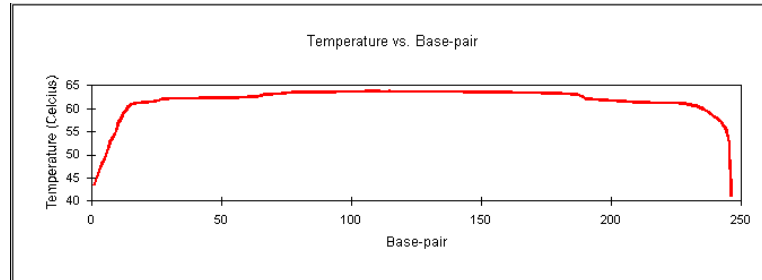
The following recommendations are intended as guidelines for producing better PCR products for analysis on the WAVE System for mutation detection. Due to the large number of commercially available PCR reagents, primer design packages and template isolation protocols, this information might not include all compatible protocols and reagents. For more information or to obtain Application Support Services, contact Transgenomic Technical Support.

Amplicon Design

Amplicon design is one critical factor in successful mutation detection on the WAVE System. Optimizing amplicon design prior to conducting PCR can significantly reduce analysis time and increase mutation detection efficiency. If the sequence of the gene or fragment to be analyzed is known, the task of amplicon design is made significantly easier with Transgenomic's WAVEMAKER[™] Software. Sequences can be copied or entered into the software (refer to the appropriate section in the *WAVEMAKER Software Manual* for specific information) and amplicons (generally ranging from 150 – 700 bp) with fairly uniform melt domains can be chosen for further analysis with a primer-design software. Mutation detection in larger amplicons is also possible. However, large fragments often contain multiple melt domains that require screening at multiple temperatures.

A well-designed amplicon ideally should have a single melt domain (Figure 5-1, Panel A), and the entire fragment should melt, e.g. change of the percent helical fraction of the fragment from 100 to 0% within a temperature interval of 5°C (Panel B). This allows fragments to be analyzed at a minimum number of temperatures.

Panel A



Panel B

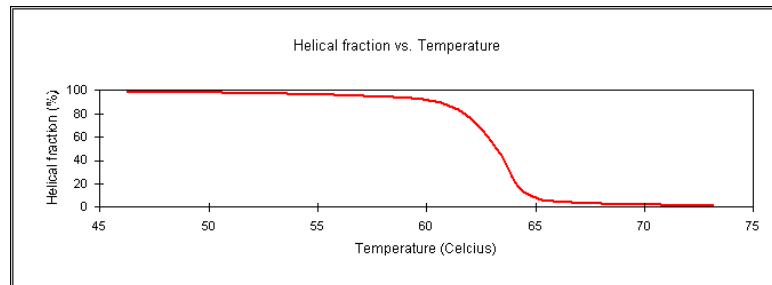
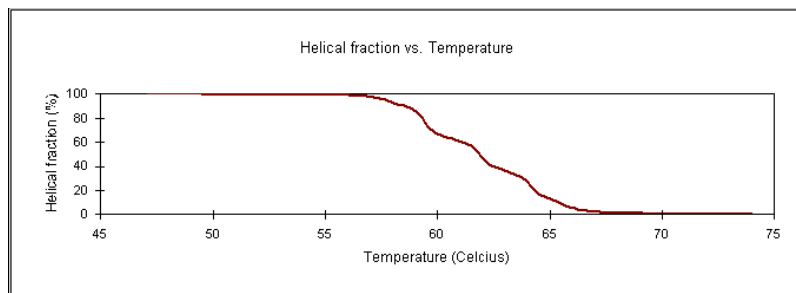


Figure 5-1. Melting profile of a 248 bp fragment. Predicted percent helicity of the fragment is shown on the Y axis and melting temperature on the X axis of panel A. Panel B shows predicted melting temperature on the Y axis and base pair of the fragment on the X axis.

Fragments with melt domains greater than 5°C apart should be avoided when possible, and might need to be redesigned using short GC clamps or smaller amplicons. An example of a large fragment with multiple melt domains and domains that are more than 5°C apart is shown in Figure 5-2.

Panel A



Panel B

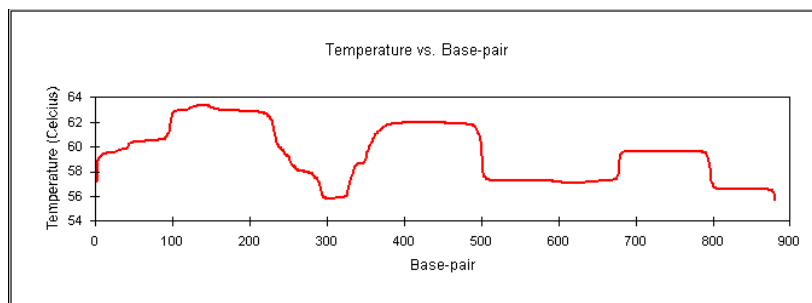


Figure 5-2. Melting profile of a 888 bp fragment. Predicted percent helicity of the fragment is shown on the Y axis and melting temperature on the X axis of panel A. Panel B shows predicted melting temperature on the Y axis and base pair of the fragment on the X axis.

Notice the 'stair-step' pattern and the wide melt curve in Panel A. Multiple melt domains are observed along the length of the fragment in panel B. A fragment like this one would require analysis at multiple temperatures to ensure detection of mutations along the entire length of the fragment. Optimized amplicon designs for many genes are available from Transgenomic's mutationdiscovery.com Web site. Custom amplicon design services are available from Transgenomic's Application Support group.

Amplicon Design Guidelines

- Typically 150 to 700 bp
- Single melt domain if possible (< 5°C melting range for entire fragment)

Primer Design

Once the target sequence for amplicon design is determined, the sequence should be entered into a primer design program to pick a suitable primer set for PCR. Generally accepted criteria for PCR primer design should be observed, as should the following guidelines:

- Primers ideally should be no closer than 30 - 50 bp from the end of the sequence to be analyzed for mutations (e.g. intron/exon boundary)
- Primers should be 18 - 30 bp in length
- Primers should contain 45 - 60% GC
- The T_m difference between primers in a pair ideally should be less than 2°C
- Avoid primer self-complementarity (e.g. hairpin loops)
- Avoid complementarity between primers (e.g. primer dimers)
- Avoid primer sequences that contain runs of three or more nucleotides of the same base (e.g. GGGG)
- Avoid the use of primers with non-templated tails such as universal sequencing primers, M13- or T7-primers, etc. Refer to the note in the *Calculating Primer Melting Temperature (T_m)* section.

Calculating Primer Melting Temperature (T_m)

The T_m is the temperature at which 50% of the primer is melted. The following equation has been modified for the specific conditions provided by the Transgenomic Optimase™ Polymerase 10X buffer (containing 1.5 mM MgSO₄). Because buffer conditions can vary significantly between different polymerases, we recommend that the following equation be used to calculate the T_m of primers used in PCR with Optimase Polymerase:

$$T_m = 63.72 + 0.41(\% G + C) - (600/n)$$

Where n is the number of bases in the oligonucleotide and (% G+C) is the combined percentage of guanines and cytosines in the oligonucleotide.

The following equation using the specific monovalent cation concentration should be used for other enzyme buffer combinations:

$$T_m = 81.5 + (16.6 * \log_{10} [\text{cat}^+]) + 0.41(G+C\%) - (600/n)$$

Where [cat⁺] is the monovalent cation concentration, n is the number of bases in the oligonucleotide and (% G+C) is the combined percentage of guanines and cytosines in the oligonucleotide.

Note: When calculating the T_m of primers with GC clamps, the length and GC content entered in the equation should be that of the template-specific portion of the oligonucleotide to calculate the initial annealing temperature used for PCR. However, because the tailed PCR primers anneal to the PCR product at a significantly higher T_m than to the original template, fully optimized thermocycling conditions are necessary.

Primer Purity

Oligonucleotides (primers) should be purchased from a reputable vendor. Although many oligonucleotide manufacturers routinely provide excellent primers, synthesis failures occur frequently and often go undetected. Transgenomic suggests analyzing oligonucleotides on the WAVE System prior to use in PCR reactions.

Oligonucleotides with significant failure products or misincorporated bases can produce non-specific products during PCR and make the detection of mutations more difficult or impossible. Analysis of oligonucleotides on the WAVE System with WAVEMAKER Software is automated. Simply select the application type “OLIGO,” (labeled or unlabeled), and the size range of the oligonucleotide to be analyzed. If you have further questions, contact Transgenomic Technical Support. Figure 5-3 shows a chromatogram with multiple primer peaks (A), an example of a poor synthesis, and a single primer peak (B), an example of a pure synthesis product.

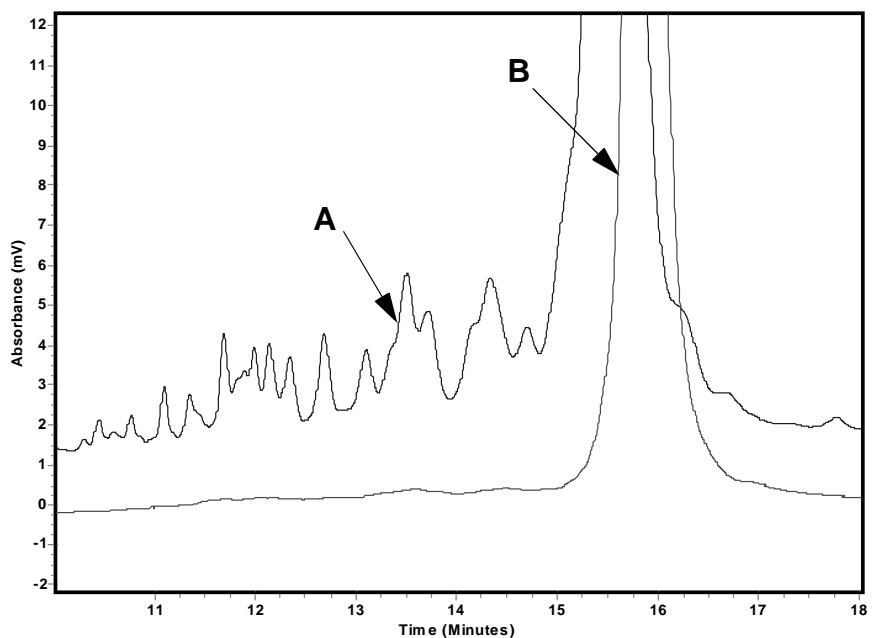


Figure 5-3. Chromatogram A shows an impure oligonucleotide with contaminating failure sequences and *n-1* products that can affect PCR product purity. Chromatogram B is the same oligonucleotide purified on the WAVE System without significant contaminant products.

Template DNA Source and Purity

Once the amplicon has been designed and primer sets have been designed, ordered and analyzed for purity, it is necessary to set up the PCR reaction. An important part of the PCR reaction is the template or source DNA that is to be used for the amplification reaction. Although the template DNA makes up a small portion of the total PCR reaction, purity of the template can dramatically affect the results of the PCR reaction and the lifetime of the separation cartridge.

DNA from virtually any source, for example blood or tissue, can successfully be used as a template in PCR. However, the success in terms of yield, and reliability in terms of fidelity, are highly dependent on the way the DNA was isolated and stored. Success also depends on such factors as specific sequence effects or degradation of the DNA. Transgenomic recommends the use of a DNA extraction procedure/kit that utilizes a salting-out method. Template DNA isolation methods utilizing spin columns, chaotropic salts, organic extraction procedures, or “dirty” preparations in which cellular debris or organic compounds have not been removed, are NOT compatible with the separation cartridge, and void the cartridge warranty if additional purification steps are not performed. Organic extraction methods (e.g. phenol/chloroform extractions) must be followed by chloroform/isoamyl back-extraction and subsequent ethanol precipitation and wash.

Note: An excellent resource for common nucleic acid extraction and concentration procedures is *Molecular Cloning: a laboratory manual*. J. Sambrook, E.F. Fritsch, and T. Maniatis – 2nd ed. (1989), Appendix E: Commonly Used Techniques in Molecular Cloning, E-10 – E-14.

Spin column purifications or other protocols utilizing chaotropic salts (e.g. guanidinium isothiocyanate) must be followed with an ethanol precipitation and wash. Transgenomic’s warranty does not replace separation cartridges that are contaminated by incompatible template DNA preparations. Please call Transgenomic Technical Support with any questions regarding specific DNA isolation protocols.

Template Purification

The template DNA should be as pure as possible for PCR. This not only helps to ensure the quality of the PCR product, but also helps avoid contamination of the separation cartridge that reduces the quality of subsequent analyses. Reagents commonly used in the purification of DNA, such as proteases (e.g. Proteinase K), solvents, SDS, nucleases, phenol, guanidinium, etc., are inhibitors of DNA polymerase activity and also might show incompatibility with the DNASep Cartridge or the DNASep HT Cartridge. If these reagents are used, it is essential to carry out a final ethanol precipitation and wash step to remove most of these contaminants prior to PCR. Elution buffers from DNA extraction kits can adversely affect PCR reactions as well. Excess EDTA, isopropanol, or iso-amyl alcohol can inhibit PCR reactions, and need to be removed prior to PCR.

Template Source

The template source also can have an adverse effect on PCR. DNases and polysaccharides can be present in intact tissue. Heme can be carried over in DNA extraction from blood, and inhibit PCR. PCR inhibitors often can be removed efficiently by phenol/chloroform extraction. Again, a chloroform/isoamyl alcohol back-extraction followed by a final ethanol precipitation and wash step is required to remove excess phenol.

Polymerase Fidelity and PCR Component WAVE System Compatibility

Due to the sensitivity of the WAVE System for detecting mutations in DNA, including polymerase-induced errors, Transgenomic recommends the use of a high-fidelity DNA polymerase, such as Transgenomic's Optimase™ Polymerase. Although a variety of variables, such as Mg²⁺ concentration, presence of inhibitors, annealing temperature, cycling conditions, template/primer concentration and so on, can affect PCR product quality and need to be optimized, the inherent fidelity of the polymerase used is the ultimate limiting factor for the fidelity of polymerization.

Another important consideration for choosing a polymerase is the composition of the storage buffers and 10X reaction buffers. Many additives common to commercially available polymerase mixtures and reaction buffers can have a negative influence on resolution or even damage the separation cartridges. It is important to choose a polymerase mixture, such as Optimase Polymerase, that is fully compatible with the WAVE System and separation cartridges.

The use of a polymerase preparation compatible with the WAVE System ensures consistent results and extends the life of the separation cartridge, minimizing the need for remedial procedures, such as special or extra washes. Figure 5-4 shows the retention time variability as a result of an alternative polymerase buffer compared to the Optimase Polymerase buffer.

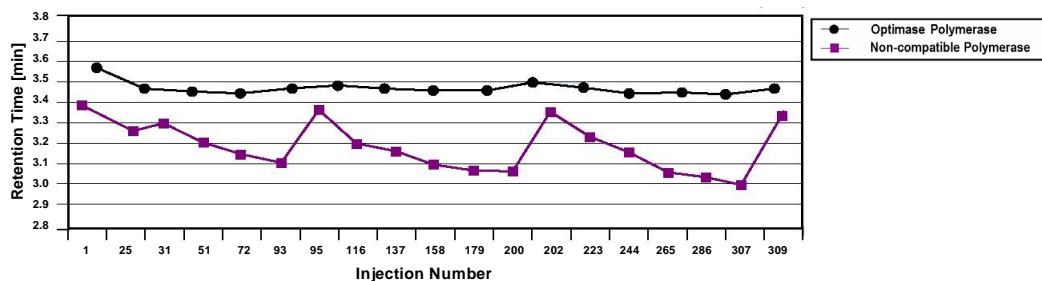


Figure 5-4. The Optimase Polymerase 10X Buffer and an alternative polymerase 10X buffer were diluted using 18 MegaOhm water to the concentration specified by Transgenomic and the manufacturer. Standard 5 μ l aliquots were injected onto the DNASep Cartridge and eluted using a 6.2 minute gradient (2% Buffer B per minute) at 56°C. A WAVE™ Mutation Standard/Control Sample was run after every 20 injections using a 5 μ l injection volume and recommended gradient procedures at 56°C. The retention times plotted represent the first heteroduplex peak. A standard cartridge wash procedure using 75% acetonitrile at 60°C was carried out after every 100 injections of 10X buffer.

Figure 5-5 shows compatibility data from the DNASep Cartridge when used with the Transgenomic polymerase buffer system, demonstrating the reproducibility that is possible over extended injection numbers.

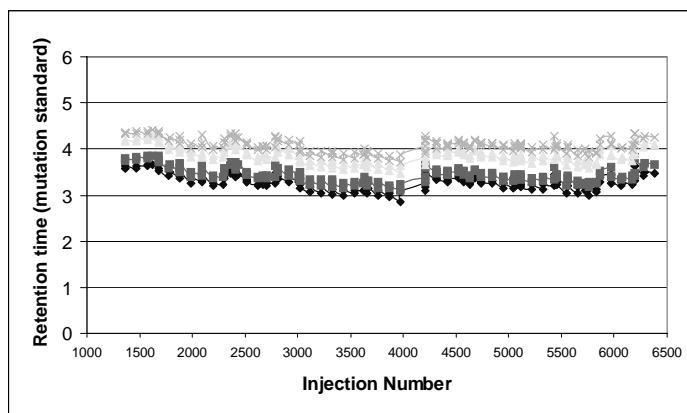


Figure 5-5. The Optimase Polymerase 10X Buffer was diluted using 18 MegaOhm water to the concentration specified by Transgenomic. Standard 5 μ l aliquots were injected onto the DNASep Cartridge and eluted using a 6.2 minute gradient (2% Buffer B per minute) at 56°C. WAVE™ Mutation Standard/Control Sample injections and cartridge wash procedures were conducted as described in Figure 5-4. The retention times plotted represent the two heteroduplex and two homoduplex peaks.

PCR Component Compatibility

The following PCR components cause loss of resolution and/or degradation of the separation cartridges, or acceptable concentrations have not yet been determined. **Avoid these components.**

WARNING! Use of any of the following PCR components voids your separation cartridge warranty.

- Unidentified “proprietary” ingredients such as “stabilizers”, “enhancers” or “additives”
- Bovine serum albumin (BSA)
- Autoclaved water
- Mineral oil
- Formamide
- Template DNA extracted/purified in a manner not consistent with the recommendations in this chapter
- Proteinase K

The following PCR components cause loss of resolution and/or degradation of the separation cartridges. These components **should not be used** in final reaction concentrations of greater than 1%:

- High molecular weight stabilizers such as polyethylene glycol (PEG)
- Detergents including, but not limited to:
 - Triton X100
 - NP40
 - Tween 20
 - SDS/SLS

The following PCR components should not exceed the final reaction concentrations listed, and **might require additional cartridge cleaning**. Contact Transgenomic Technical Support for more information.

Component Maximum Final Concentration

- Glycerol 2%
- DMSO 10%
- Betaine 1.25 to 2.5 M.

The effect of using reaction mixtures containing multiple components (each one individually in acceptable concentrations) from the lists above is unknown. Contact Transgenomic Technical Support for additional recommendations.

Please refer to the Warranty Information provided with the separation cartridge for additional requirements for extended cartridge performance.

Generalized Reaction Conditions

When setting up PCR conditions for new reactions, there are certain aspects that always should be optimized to ensure high fidelity polymerization and adequate product yield. With the purchase of Transgenomic Optimase Polymerase, a *User's Guide to PCR for the WAVE[®] Nucleic Acid Fragment Analysis System* and a *Quick Guide to PCR and Reaction Optimization* are provided. The manufacturers of other polymerases should provide their own guidelines for optimization of reaction conditions for their enzymes.

Reaction conditions are presented for a 50µl reaction volume.

Optimase Polymerase	1µl (2.5 units)
Forward Primer	0.4 to 0.6µM
Reverse Primer	0.4 to 0.6µM
Reaction buffer	5µl of 10x stock solution
Template DNA	100 to 150 ng (Human Genomic DNA)
dNTPs	200µM each
MgSO ₄	1.5mM
H ₂ O	to 50µl

Thermocycling Conditions

Just as the concentration of individual PCR reaction components needs to be optimized for each template and primer set, so do the thermocycling conditions. The number of cycles used for PCR is a balance between the yield required from the reaction and the need to preserve optimum reaction conditions. As PCR proceeds, the conditions in the reaction change because dNTPs are polymerized to form the PCR product. The polymerase is slowly denatured and the relative concentrations of different components change. In general, the absolute number of errors incorporated in each cycle increases with increasing cycle number throughout the course of the PCR. Therefore, it is prudent to use the minimum number of cycles required to achieve sufficient product yield.

Examples are provided below for a typical Three-Step PCR reaction as well as Touchdown PCR. These methods are recommended as the starting point from which to optimize most reactions.

T_a is the annealing temperature. (T_a = 3°C above the average of forward primer T_m and reverse primer T_m. Ideally the difference between the T_m values for the individual primers in a pair should not be more than 2°C.)

Standard Three-Step Method (Cycling Conditions)

- 1 95°C for two minutes
- 2 95°C for 30 seconds
- 3 T_a for one minute
- 4 72°C for one minute/500 bp of amplicon length
- 5 Repeat steps 2 through 4, 20 to 30 times
- 6 72°C for five minutes
- 7 Hold at 4°C

Touchdown Method (Cycling Conditions)

- 1 95°C for two minutes
- 2 95°C for 30 seconds
- 3 T_a + 8°C (- 0.5C /cycle) for one minute
- 4 72°C for one minute/500 bp of amplicon length
- 5 Repeat steps 2 through 4, 15 times
- 6 95°C for 30 seconds
- 7 T_a for one minute
- 8 72°C for one minute
- 9 Repeat steps 5 through 7, 19 times

10 72°C for five minutes

11 Hold at 4°C

Post PCR product purification is not recommended prior to analysis on the WAVE System.

Heteroduplex Formation

Because the PCR product is intended for heteroduplex analysis by DHPLC on the WAVE System, a final heteroduplex-formation step should be carried out at the end of the reaction. A basic protocol for this denaturation/annealing step would be 95°C for five minutes, followed by a slow reduction in temperature (at a rate of $-0.1\text{C}/4\text{ sec}$) until a temperature of 25°C is reached. This step provides the conditions for the formation of hetero- and homoduplex products required for mutation analysis based on the presence of heteroduplexes.

Non-denaturing Analysis of PCR Products

Non-denaturing analysis of PCR products at 50°C is necessary to determine the size, yield and quality of products prior to mutation detection analysis on the WAVE System. A PCR product should appear as a single, clean peak at 50°C (Figure 5-6). Refer to the *WAVE MAKER™ Software Manual* for instructions on predicting sizing gradients for single or multiple fragments.

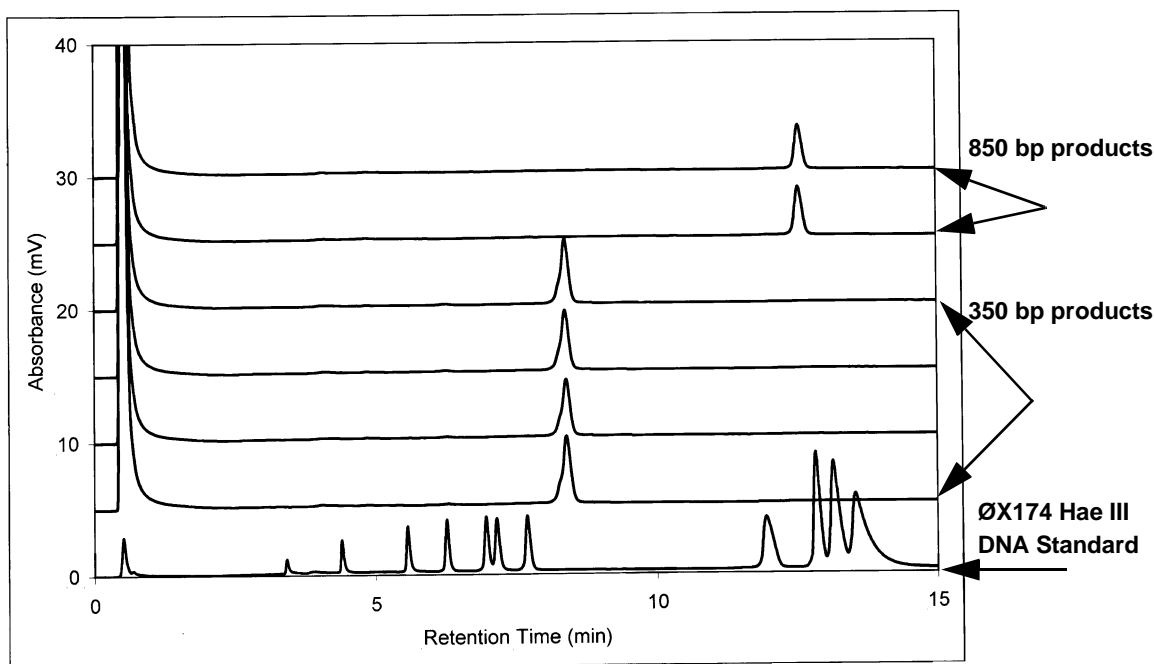


Figure 5-6. Non-denaturing analysis of PCR products at 50°C demonstrates product size, purity and yield. The ØX174/HaeIII standard was run to confirm the size of the fragments. This standard contains 11 fragments of sizes 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1353 base pairs.

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