

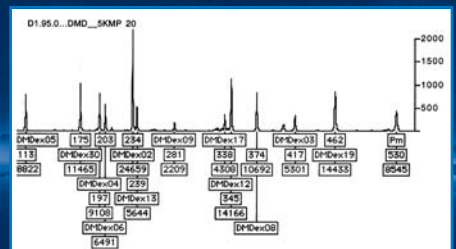
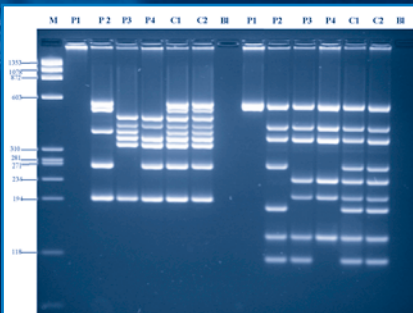
Molecular Diagnosis of Genetic Diseases

Second Edition

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Mutation Scanning for the Clinical Laboratory

DHPLC

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1. Introduction

This chapter describes the Transgenomic WAVE® DNA Fragment Analysis System with DNA Sep® Technology, as used in our molecular genetics diagnostic laboratories for the detection of unknown mutations. Four software packages are currently available: WAVEmaker 3.4, 4, 4.1, and most recently, Navigator. Because versions 4 and 4.1 are similar, version 4 is presented here. Denaturing high-performance liquid chromatography (DHPLC), also known as TMHA (temperature-modulated heteroduplex analysis) provides a rapid, automated scanning method for mutations, whether known or unknown. Mutations are visualized as a characteristic pattern of peaks corresponding to the mixture of heteroduplexes and homoduplexes formed when wild-type and mutant DNA fragments are hybridized (*I*; **Fig. 1**).

Individuals who are heterozygous for a mutation or polymorphism have a 1 : 1 ratio of wild-type and mutant DNA. A mixture of hetero- and homoduplexes is formed when the polymerase chain reaction (PCR) product containing the mutation is heated to 95°C and slowly cooled. To form these molecular species, the DNA from individuals who have two mutant alleles (homozygous mutation) must be mixed with the corresponding wild-type DNA fragment and hybridized. After this treatment, a sample will contain a mixture of hetero- and homoduplexes. However, DNA from homozygous wild-type individuals will form only one species, the homoduplex wild-type, unless mixed as described previously.

The WAVE nucleic acid analysis platform is based upon ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC), and can be

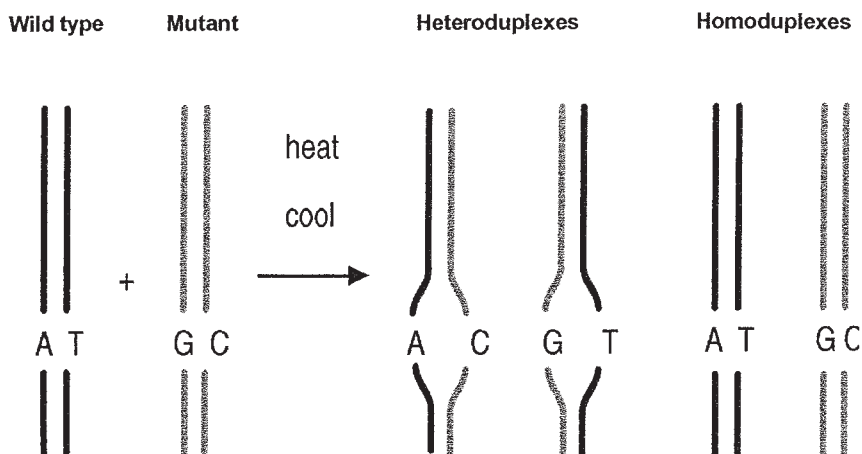


Fig. 1. Formation of a 1:1 mixture of heteroduplexes and homoduplexes through hybridization.

operated in three modes, as determined by the temperature at which separation is performed (**Fig. 2**).

The process has two phases—a stationary phase consisting of a DNA Sep[®] column packed with alkylated nonporous polystyrene-divinyl benzyl C18 and a mobile phase of chemicals including acetonitrile (ACN), (an organic co-solvent that is used to selectively elute samples) and the ion-pairing agent triethylammonium acetate (TEAA), which ensures that the DNA interacts with the stationary phase. Under non-denaturing conditions (<50°C) double-stranded DNA (dsDNA) will be eluted according to size with the WAVE buffer system (**Fig. 2A**). Essentially, the DNA interacts electrostatically with the ammonium ion of the TEAA on the basis of size. The larger the DNA, the more interactions it has with TEAA. Once it is associated with TEAA, it effectively has a hydrophobic outer coating that allows it to interact with the hydrophobic DNASep column, and the number of bonds formed are determined by the DNA fragment size. The TEAA acts as a bridging molecule, and the alkyl chains of the TEAA interact with the hydrophobic surface of the DNASep matrix. Elution of DNA from the DNASep matrix occurs through the use of the hydrophobic eluant ACN, which reduces the hydrophobic interaction between the stationary phase and the alkyl chains of the bridging molecules. As a gradient (a constantly increasing concentration) of ACN is run through the column, DNA fragments of increasing size are eluted. Under non-denaturing conditions (50°C), this separation is size-dependent but not sequence-dependent (**Fig. 2A**; *see Note 1*).

At fully denaturing temperatures, single-stranded molecules (RNA or DNA) can be separated (**Fig. 2C**). This mode can be used for oligonucleotide quality

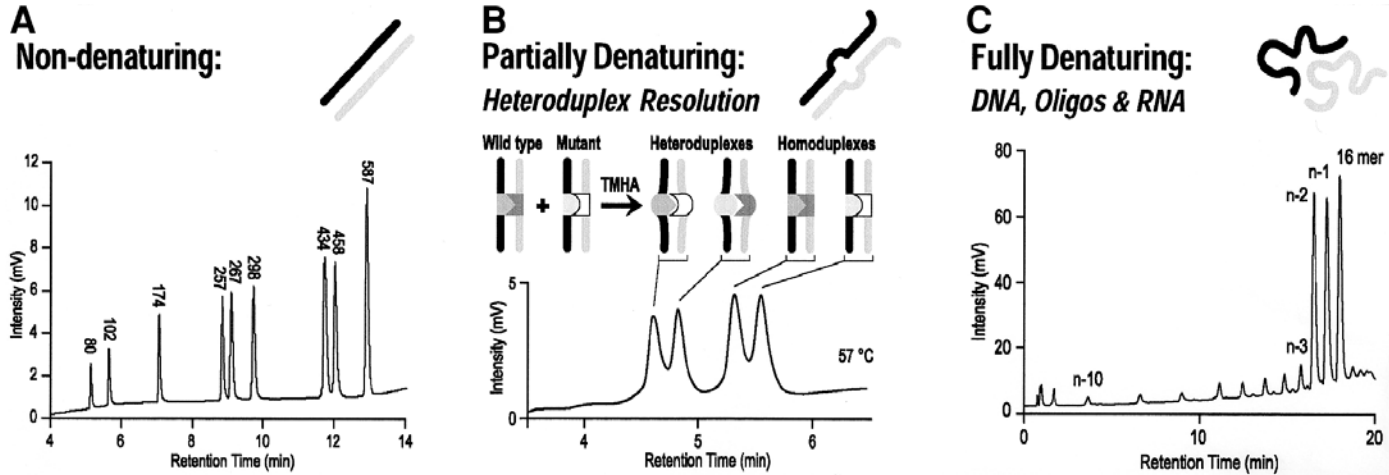


Fig. 2. Three modes of operation of the WAVE Nucleic Acid System. WAVE traces of: (A) a pUC18 *Hae*III digest, (B) a sample heterozygous for an A to G transition in a 209-bp *DYS271* nucleotide sequence, (C) the separation profile of 14-, 15-, and 16-mer oligonucleotides.

control and purification. Oligos can be effectively separated from failure peaks using a fragment collector (*see Note 2*).

The separation of fragments under partially denaturing conditions is the focus of this chapter. Denaturing and hybridizing a sample containing two products that differ by as little as one nucleotide results in the formation of two homoduplexes and two heteroduplexes. These may be visualized as four distinct peaks (**Fig. 2B**), but any difference in peak profile compared with a homozygote wild-type control is indicative of a mutation (*see Note 3*).

At a column temperature determined by use of the melting algorithm WAVEmaker software (Transgenomic Ltd., Crewe, UK), DHPLC separates the DNA molecules, with the heteroduplexes eluting first (**Fig. 2B**). This is the result of bubble formation in the dsDNA duplex, which reduces the number of charges accessible for electrostatic interactions between the DNA bases and the ion-pairing layer of the column stationary phase. The charge density of the DNA molecule is reduced within the single-stranded region.

The major procedures involved in DHPLC mutation detection include: PCR primer design (*see Note 4*); oil-free PCR (*see Note 5*); heteroduplex formation; melting profile determination of the fragment; method preparation, which includes setting of the separation gradient; making a new project, which includes sample sheet preparation, sample running, and sample analysis. When optimized DHPLC provides a reported sensitivity of 94–100% for the detection of heterozygous sequence variants, comparing well with full-sequence analysis (2–9) (*see Note 6*). DHPLC has been shown to detect mosaic mutations that account for only a few percent of alleles in a PCR product and that remain undetected by direct sequence analysis (10). The system is gel-free and can be mostly automated, since samples are transferred directly from the PCR cyclor to the WAVE platform.

2. Materials

2.1. PCR Amplification

1. DNA template ~50 ng per reaction (*see Note 7*).
2. Optimase™ Polymerase (Transgenomic Ltd., Crewe, UK) Amplitaq Gold™ (Perkin-Elmer, Foster City, CA) (*see Note 8*).
3. GeneAmp® 10X PCR buffer 11 (Perkin-Elmer) (*see Note 9*) which contains 100 mM Tris-HCl pH 8.3, 500 mM KCl. Store at –20°C for long-term storage and 4°C for daily use or short-term storage.
4. 25 mM MgCl₂ solution (Perkin-Elmer). Store at –20°C long term and 4°C short term (*see Note 10*).
5. 100 mM stocks of deoxyadenosine triphosphate (dATP), thymidinetriphosphate (TTP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP) stored at –20°C. Dilute to yield a 2-mM mix for daily use and store at 4°C (Promega, Madison, WI).

6. Primer stocks at 100 μM stored at -20°C . Working stocks of 10 μM stored at 4°C . (ThermoHybaid, Interactiva Division, Germany. <http://www.thermoHybaid.com>).
7. Thermal cycler allowing oil-free operation and 96-well plate format, e.g., 9600 (Perkin-Elmer) or PTC 225 Tetrad (GRI, Essex, UK).
8. Microtiter plates or tube strips. Our plates consist of either 8 or 12 0.2-mL PCR tubes (Strip-ease, GRI) held in a MicroAmp[®] 96-well tray/retainer system (Perkin-Elmer) or 96-well halfskirt plates (Axygen, Thistle Scientific, UK) sealed with adhesive PCR film (ABgene, Surrey, UK).

2.2. DHPLC and Data Analysis

1. WAVE[®] DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE) (*see Note 11*).
2. DNASep[®] cartridges (Transgenomic Ltd.) (*see Note 12*).
3. In-line filters (Transgenomic Ltd.) (*see Note 13*).

2.3. Water and Buffers (*see Note 14*)

The buffer solutions for the WAVE should be made to analytical chemistry standards, using quality-assured glassware. For health and safety reasons, always wear nitrile gloves when making buffers, and use a fumehood when available. **Caution:** for safety and disposal, *see Note 16*. Whenever possible, use water of the highest quality, e.g., Millipore (Millipore Co, Bedford, MA), since transition ions shorten the column's life. The minimum standard is HPLC-grade water (Sigma Aldrich Co Ltd., Poole, UK). Before use, the Duran 1-L or 2-L bottles (Sigma Aldrich Co Ltd.) we use for buffers are washed out with Millipore water (to remove any transition ions), especially if the glassware has been autoclaved.

1. Buffer A: 0.1 M triethyl ammonium acetate (TEAA) for ion pairing (Transgenomic Ltd.). Store 2 M stock at 4°C .
2. Buffer B: 0.1 M TEAA and 25% acetonitrile (ACN) (Sigma Aldrich Co Ltd.) for producing the gradient.
3. Buffer C: 75% ACN (Sigma Aldrich Co Ltd.) for cleaning the column.
4. Buffer D: 8% ACN for washing the needle.

2.4. Quality Control (*see Note 15*)

1. A pUC18 HaeIII digest (Transgenomic Ltd). Store at -20°C .
2. The mutation detection control, DYS271. This is a 209-bp heterozygous locus of a G > A transversion (Transgenomic Ltd.). Store at -20°C .

3. Methods

3.1. PCR Amplification (*Table 1*)

Carry out the PCR reactions in a total vol of 25 μL , or 50 μL if more than two injections.

Table 1
Reagent Volumes for a Single PCR Reaction

	X1 reaction
Gene Amp 10X buffer 11 or Optimase reaction buffer 10X	2.5
2 mM deoxynucleotide 5' triphosphate (dNTP) mix	2.5
25 mM MgCl ₂	2.5
10 μM Primer—Forward	1.0
10 μM Primer—Reverse	1.0
AmpliTaq Gold™ or Optimase	0.1
Sterile distilled water	10.4
50 ng DNA	5.0

Prepare a master mix based on the volumes listed here, allowing for 10% possible pipetting wastage, and remembering to allow for the equivalent number of wild-type samples if mixing is necessary, as when screening males for an X-linked condition.

Amplify the samples under appropriate conditions, including an initial denaturation step at 95°C for 15 min, and then 35 cycles of denaturation, annealing, and extension followed by a final extension at 72°C for 10 min (*see Note 5*).

For a full plate, aliquot the master mix into each vial of a 12-vial strip-ease (GRI, Essex, UK) (*see Subheading 2.1.8.*).

Add primers as appropriate, and distribute the mix using a multichannel pipetter. Patient DNA can then be added in a similar way, allowing 50 ng DNA per well. Include normal, mutation-positive, and “no DNA” controls in the setup design.

3.2. Heteroduplex Formation

Directly before analysis, heat the PCR products, and then cool slowly to promote heteroduplex formation: heat for 5 min at 95°C, then reduce temperature at 1.5°C per cycle for 45 cycles. To detect homozygous mutant DNA and X-linked mutations in males, mix the patient's sample with the corresponding wild-type PCR product in a ratio of approx 1 : 1 prior to the heteroduplex step. It is advisable to first check all PCR products on a gel to ensure that there are no failures.

The total volume for reaction may need to be increased if there are more than three injections per sample, as approx 5–8 μL are required per injection. Plates may be subject to evaporation loss during the run. This can be minimized by adjusting the cooling of the chiller block of the autosampler. Alternatively,

“easy pierce” adhesive foil to cover the plates (ABgene, Surrey, UK) (*see Note 17*). Subsequently, the plates can be resealed with adhesive plastic film (ABgene) if a sample is required for further analysis.

3.3. Separation Gradient Determination

For gradient optimization, you will need about 50 μL of the wild-type PCR product of length 60–500 basepairs (bp), prepared under the same conditions as those used for diagnostic samples. Mutant samples are not needed for this optimization.

3.4. Size and Purity Determination

Determine the fragment size and purity of the PCR product under non-denaturing conditions. It must provide a single sharp elution peak of the expected size. If more than one peak is present, or a peak with a shoulder, then there is more than one fragment of DNA in the sample, and the PCR protocol may need to be optimized.

3.5. Selection of Gradient for Mutation Detection

The recommended gradient for mutation detection is a slope of 2% increase in buffer B per min. The universal gradient illustrated in **Table 2** can be used for unknown sequences or any sample within the size range that is amenable to the technique, although it has not been optimized for speed of analysis. Run times can be reduced by starting the clean off at a lower percentage of buffer B. This optimization is performed by the WAVE[®] system utility software. Run the gradient initially at non-denaturing conditions of 50°C.

3.6. Separation Temperature

Typically, at the T_m (the temperature at which fragments have an average helicity of 75%), mutations are detected at a retention time of 0.75–1 min earlier than the elution of the fragment under non-denaturing conditions (50°C) using the universal gradient (equivalent to 1.5–2% less buffer B).

3.7. DHPLC and Data Analysis

There are three main sections to the WAVEmaker software:

1. D-7000 Admin: This is the filing system containing application folders in which data is stored. Make a new application folder every month and copy methods across from other folders. The programs will not run if more than one folder is open. The authors store application folders in the “D” drive.
2. D-7000 HSM: This is the main program, which is icon-driven and can be followed easily in a stepwise fashion.

Table 2
Universal Gradient for Mutation Detection
on the WAVE® System

Time (min)	%A	%B	Flow (mL/min)
0.0	65	35	0.9
0.1	60	40	0.9
16.1	28	72	0.9
16.2	0	100	0.9
16.7	0	100	0.9
16.8	85	15	0.9
18.0	85	15	0.9

3. WAVEmaker: This is used for designing methods before importing the new method into D-7000 HSM (this is done by the operator in version 3.4, but is automatically imported by the software in version 4).

For new users of WAVEmaker 4.0 in our laboratory, we have designed a stepwise program of operation, although a more detailed training protocol in procedures and machine operation will be given by the manufacturers on machine purchase.

3.7.1. Making a New Project in WAVEmaker 4.0

1. Import a new sequence: Import your sequence as a text file, which WAVEmaker changes to a .DNA file. Save the DNA file in your sequence folder. It is then ready to be used in a project. Import your sequence from disk by going to:

File → Open DNA sequence → Select your sequence from your disk

WAVEmaker will say that the file will be renamed “*your sequence .DNA.*” Click on OK. Your sequence now appears in the drop down list under the “*Sequence file*” box in the project. Now save your sequence as a .DNA file in your sequence folder. Go to:

*File → Save DNA → Name your sequence and change → Save in your sequence as the “Save as type” box to sequence folder
 Sequence file (*.DNA)*

Your sequence is now saved as a .DNA file in your sequence folder.

2. Using a previously saved sequence: Sequences previously saved in your sequence folder as a .DNA file are ready to use in a project. To open the sequence in the new project, go to:

File → Open DNA sequence → Select your sequence from your sequence folder

Your sequence will now appear in the drop down list under the “*Sequence file*” box in the project (see **Note 18**).

3.7.2. Melting Profile

1. Making a melting profile (**Fig. 3–5**): Once you have your sequence in the drop down list under the “*Sequence file*” box, click on the sequence name to make it appear in the “*Sequence file*” box. To check that the sequence is correct and to calculate the T_m of your fragment (denoting 75% helicity), click on the “*Sequence*” button on the left of the screen. You will now see your sequence displayed in the text box. The T_m for your fragment can be seen to the right of the text box.

To see a melting profile of your fragment, click on the “*Melting*” button on the left of the screen and then click on “*Calculate*” on the right of the top box. This will show you “*helical fraction vs temp.*” To see a melting profile of your fragment at a given temperature, click on “*Calculate*” to the right of the lower box. The temperature range of the profile can be changed in the boxes above the “*Calculate*” button (see **Note 19**).

2. To print out a melting profile: Print out the melting profile for your fragment by going to:

File → *Print report*

You will then see a preview of your report. Click on the “*print*” button to print out your report.

3.7.3. Method

1. To make a new method: Open your sequence from the drop down list under the “*Sequence file*” box. The name of your sequence will now appear in the box. Enter the application type (usually “mutation”) either by clicking on the appropriate circle or clicking on “*Mutation*” on the drop down list under the “*Application type*” box. The default values calculated for your fragment are then shown in the boxes.

Click on the “*Gradient*” button on the left of the screen to display the gradient information. Change the default settings displayed for use with (or without) the accelerator, which will decrease the run time for each sample from 6.8 min to 5.6 min (see **Table 3**). To do this, go to the menu at the top of the screen and click on:

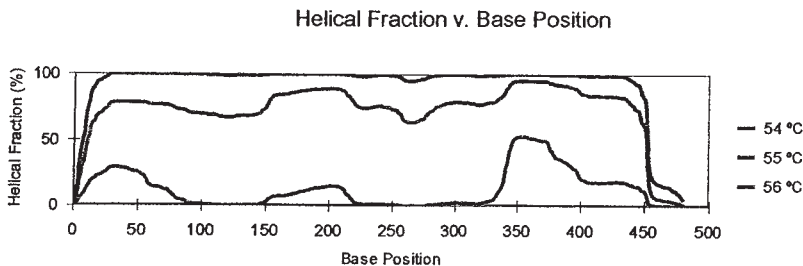
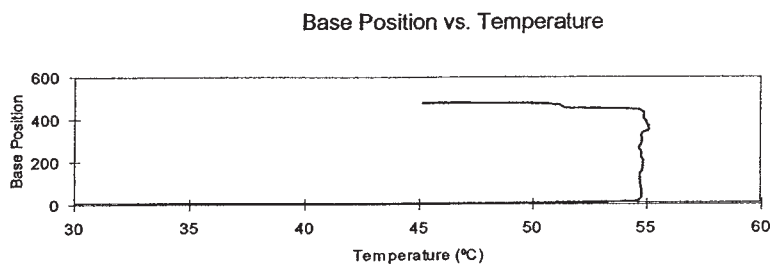
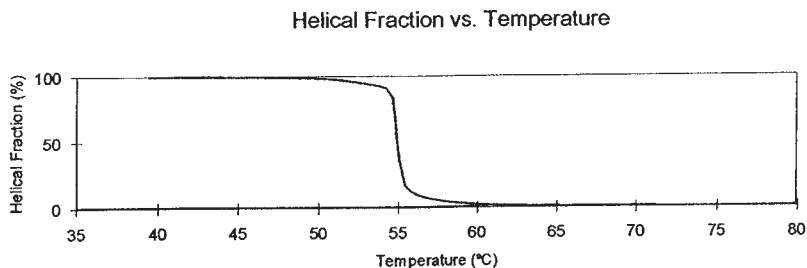
Project → *Project options* → *Then click on the “Gradient” tab*

Check the “*Update changes to all samples*” and “*Save project options as default*” boxes. Click on “*OK*.” This should change the run time to 5.6 min on the gradient display (with accelerator) and 6.8 min (without accelerator).

Default values are shown in the oven temp (–) and time shift (0) boxes. If the “*Oven temp*” and “*Time shift*” boxes need changing, click on the relevant box and enter the value you want followed by “*Enter*.” You should see the red and blue lines on the gradient profile shift when you press “enter.” It is important that

Melting Curves Report

Project: C:\Program Files\Transgenomic\WAVEMaker 4.0\Sequences\Rachel\Wave Round 2\Untitled.wmp
 Sequence Name: Ac6054-1
 Sequence Desc:



```
CTGATCAATCAGACAAGACAAGAAAGGTCCTCCTGAACTGGAAAAGGAGAAGTCAAATTTATTCCTTTGCGATGATATGATCTCATATTTGGAAATACCTTAAGA
CTCTATCAAAAATTTGGAACTGATAAAGCAAATTAAGTAAAGTTGCAGGATACAAAATCAACATACGAAAATCAGTAGCATTCTGTATGCCAACAGCAGACAATCGA
AAAAAATTTAAAGTAAGCCATTITGCAATAGCAACAAATAAAATTAATACTTATGAGTTAACCAAAGAACGAAATGACCTCTACAAGGAAAACATAAAAACTGATA
AAAGAAATTTAAGAGGACACACACAGAAATGGAAAAGATAGGCCATGTTTATGGATTGGAAGAATCGATATGTTAAAAATGTTGATCACTATCCAAAGCAATCTGCAGAT
TCAATGCAATCCCTATTAATAATACAAATGACATCTTCACA
```

Fig. 3. Melting curves report for a 480-bp DNA fragment showing a single melting domain with a predicted T_m of 55°C.

you see the change on the gradient profile in order for it to be saved correctly in your method.

If all the data for your method is correct, save it in your method folder. Go to:

File → **Save method as** → **Name your method** → **Save in your method folder**

Melting Curves Report

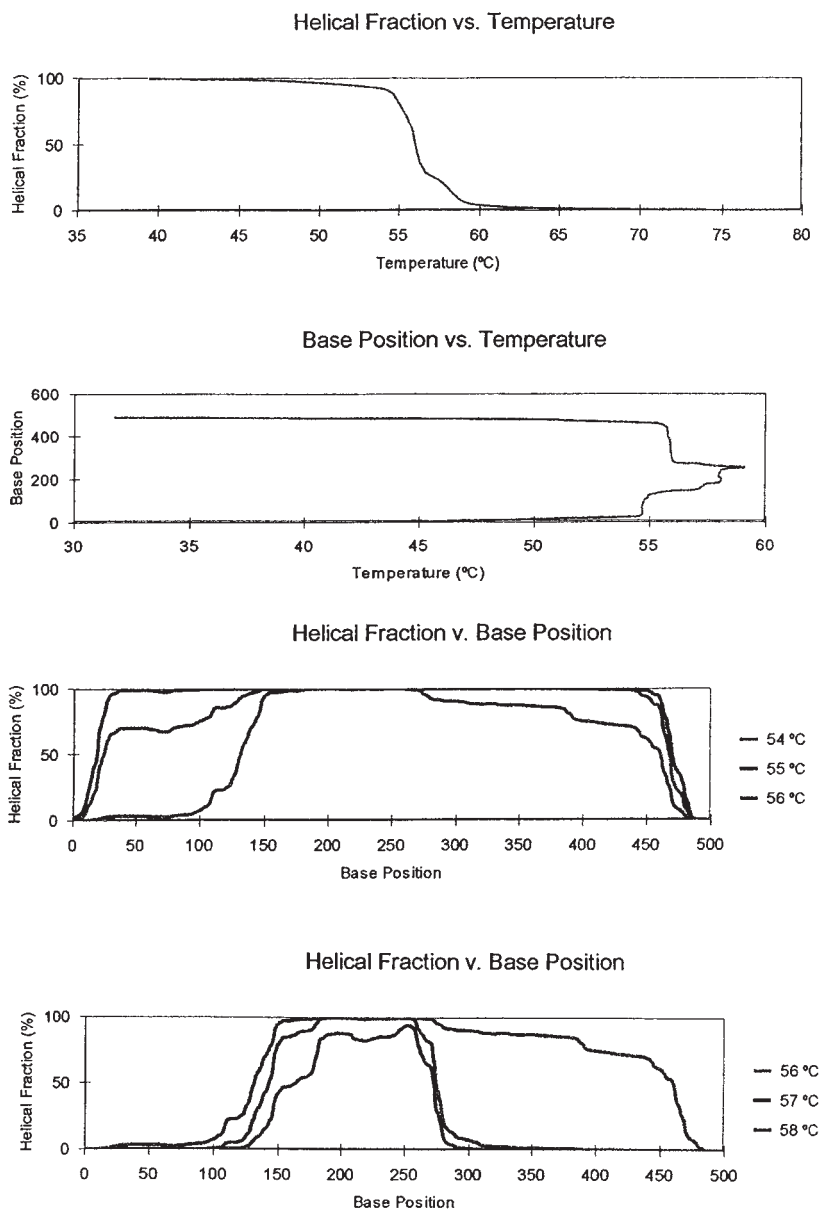


Fig. 4. Melting curves report for a 492-bp DNA fragment showing two melting domains with predicted T_m of 55°C and 58°C to accommodate a GC-rich domain. A T/G transversion at base position 123 was detected in this fragment at the predicted T_m of 55°C (Fig. 5).

Table 3
Gradient Settings for Mutation Detection

	With Accelerator	Without Accelerator
Caution level	Normal	Normal
Drop for loading	5% B	5% B
Loading duration	0.1 min	0.1 min
Gradient duration	4.0 min	4.0 min
Clean duration	0.1 min	0.5 min
Equilibration duration	0.9 min	2.0 min

Methods are usually named “Your fragment@temp” e.g., “Exon 6@57.” Your method name is displayed in the “*Method name*” box and your method saved in your method folder.

2. To open a previously saved method: Open a method already saved in your method folder by going to:

File → *Open method* → *Select your method from your method folder*

Your method appears in the drop down list below the “*Method name*” box.

3.7.4. Project

1. To make a new project: You can open your previously saved sequence and method (if not already open) by going to:

File → *Open DNA sequence/method* → *Select your sequence/method from the appropriate folder*

The method data for your fragment is displayed in the boxes. Click on the “*Sample table*” tab. Your method data is displayed in the top line of the sample table. To enter the same method for additional samples, highlight the top line and drag it down to highlight the number of samples you need. Move the cursor into the green highlighted area and right click on the mouse. When the menu is displayed, click on “*Fill*” and wait.

Eventually, your method data will be shown in all of the lines you have selected. The vial numbers of the samples may need changing if you are not starting at the beginning of a plate. Enter your sample names, typing the name in the “*Sample name*” box or copy and paste from a previous project.

Check that all the data for each sample is correct for your method. Repeat the process for any additional methods to be run in the same project. If not using an accelerator, a 45-min column wash at 0.9 mL/min with buffer C (75% ACN) is included at the end of each run series followed by the low flow settings (A and B 50:50, at 0.5 mL/min).

Chromatogram Report

Project: E:\Rachel\February\AL9048-1.wmp
 Application Folder:
 Data Path: C:\Wn32App\HSM\February

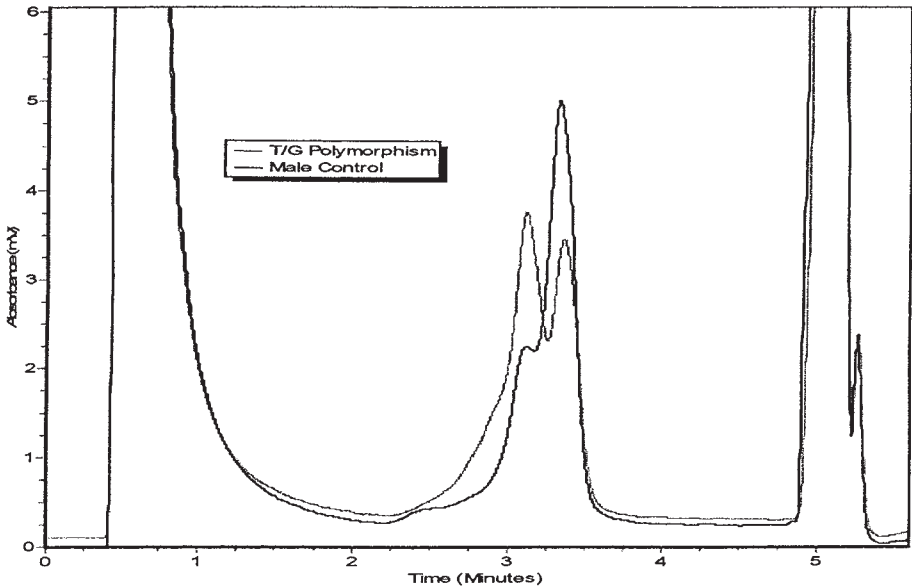


Fig. 5. WAVE trace of a T/G transversion (double peak) found at T_m 55°C in the 492-bp fragment of Fig. 4. A normal male control (single homoduplex peak) is superimposed for comparison. Peaks at the beginning and end of the trace represent the injection peak and accelerator peak, respectively. The mutation was characterized by fluorescent sequencing using an ABI 377. Data courtesy of Rachel Wycherley, National Genetics Reference Laboratory (Wessex) UK.

Once your sample table is complete, check that the default “*Application folder*” and “*Column name*” displayed at the bottom of the screen are correct. Save your project by going to:

File → **Save project as** → **Name your project** → **Save in your project folder**

Projects are usually named “Gene fragment@temp date”—e.g., “APCExon6@57 28.02.01.” It is important that you put the date at the end of your project name if projects are archived by month.

2. To open a previously saved project: If your project has been saved in your project folder, go to:

File → *Open project* → *Select your project from your project folder*

When you click on “*Open project*” WAVEmaker will ask if you want to “*Save the current project?*” If you have just opened WAVEmaker and this is a blank project, then click on “*No*” or you will save the blank project in place of your completed one. If this happens, your original project cannot be retrieved.

When you next select a project from your project folder, the method and sample table will normally be displayed as previously saved, but these should be checked before starting a run.

3.7.5. To Run Samples

1. Starting a run: Before starting, check that all buffer and wash solution bottles are at least half full. Always wash the injection valve and needle and purge liquid lines according to the manufacturer’s instructions. Once your project has been opened/saved, go to the bottom of the sample table and click on “*Run samples*” to start the new run. The estimated run time will be displayed at the bottom right portion of the screen. Once the run has started, the display will change from the sample table to the monitor view, where you will see the result as your samples are being run.
2. To alter a run once started: It is possible to change your method data while the run is in progress. To do this, go to the sample table and click on the sample you want to change. Fill in the new data, and your run will be altered accordingly. Once a sample is in the process of running, its record will turn gray on the sample table, and changes cannot be made.
3. To stop a run once it is started: If you need to stop or pause your run once it has started, go to the sample table view and click on “*Pause table.*” Doing this means, you will lose your result for the sample in progress if it is not complete. Your run can now either be restarted by clicking on “*Resume table*” or stopped by clicking on “*Abort.*”

3.7.6. Results

1. To view results while WAVEmaker is running: You can look at and print out your results while a run is in progress. To do this change from the monitor view to the results view by clicking on the “*Results*” tab at the top of the screen. You have a choice of display settings for your results, and you can either see them in a single window or as two smaller windows. To change the display from two windows (default) to one, click on the first of three buttons showing a peak and a number. This button has a number one on it, and is just above the list of samples. To show your results, just double click on the sample you want to see from the list on the left of the window. The result is then displayed in the window.
2. To change the display settings: If you want to change the display settings for the window (e.g., to alter the axes or to add a title), move the cursor onto the window and right click the mouse. On the menu that appears, click on “*Chart.*” You can now change any of the settings. Once done, click on “*Apply*” to get a preview of

- the changes and then **“OK.”** If you change the axes settings, then you must check the **“Save axes range as default”** box to apply the changes to all of your results.
3. To display more than one result: It is possible to see more than one result displayed in the same window. To do this, the **“Show all”** box just above the upper results window must be checked. Once this is done, go to the list of samples and double click on the ones that you want to display. The gap between each result displayed can be changed by moving the cursor onto the result window, right clicking on the mouse, and then choosing **“Chart”** from the menu. You will need to change the **“Trace axes offset”** setting to make the gap between individual results larger or smaller.
 4. To review the results one at a time: Your results can be seen as a review using the up and down arrows rather than double clicking on each sample name. To do this check the **“Review box”** above the upper result window. To review your results, double click on the first sample you want to display in the result window, and then use the up and down arrows to scroll through your results.
 5. To print out results: When your results are ready to be printed out, go to:

File → print report

This will show you a preview of your report before you print it out. To print the report, click on the **“Print”** button. Your sample table and gradient information can also be printed out in the same way as your results. To do this, check the appropriate box on the left side of the screen and print as before.

3.7.7. Archiving

At the end of each month, it is a good idea to archive your projects for that particular month into a monthly folder. This is made easier when you have named your projects using the date that they were run.

3.7.8. A WAVE System Report

Printing out a WAVE system report may be useful if there is a problem with the WAVE as it plots pressure vs temperature for the pump. Go into D-7000 HSM. Click on the **“blue filing cabinet”** icon on the left of the screen and select the appropriate application folder. Click on the **“chromatogram”** icon (also on the left of the screen). This gives you a list of samples you have run in that application folder. Click on the sample you want to print out a report for. You should see another window with the name of that sample in. Click on the **“recalculate”** button at the bottom of the screen followed by the **“modify report”** button. This should show you a print preview of your WAVE system report.

4. Notes

1. This mode works well for quantitative work comparing peak height or area ratios for duplication/deletion detection (**11**).

2. There are a range of columns and buffers available (Transgenomic Ltd.) for high purification work (Transgenomic application Note 103).
3. In practice, a four-peak pattern is seldom seen, as the homo- and heteroduplex elution profiles associated with many mutations overlap. A distinct mutant pattern is nevertheless usually seen when the fragment-specific T_m (temperature at which ~75% helical) is selected for analysis and at temperatures corresponding to ~70–90% melt.
4. Many operators will choose to use primer sets that were originally designed for gel SSCP/heteroduplex analysis. However, consideration should be given to the predicted fragment melting profiles.

For the detection of unknown mutations, it is probably best to select fragments of 150–450 bp (although single-base mutations have been detected in 1.5-kb fragments using the technique described here). If the sequence is known, construct the melting profile using appropriate software. Locate primers near the high points on this profile so that GC-rich regions are at the ends of fragments rather than in the middle. Avoid the inclusion of regions that differ by more than 15°C in the same fragment. If this is unavoidable, it may be necessary to substitute dGTP with N⁷-dGTP (7-deaza-2'-deoxyguanosine 5'-triphosphate) to lower the melting temperature of GC-rich regions, or exceptionally, to include a short GC clamp of three or four bases. Once the approximate locations of primers have been determined, more detailed primer design can begin.

The use of primers with non-template tails such as universal sequencing primers or T7 promoters should be avoided, although it may be possible to use these with *Pfu* polymerase (Stratagene, La Jolla, CA) or Optimase (Transgenomic Ltd., Crewe, UK). The recommended primer melting temperature T_m is 56°C. The difference in T_m between Primer 1 and 2 should be less than 1°C. The difference in T_m between primer and template should be about 25°C. Any possible primer dimers should be less stable than the 3'-pentamer by at least 5 kcal/mol. Any primer self-annealed loops should have a T_m of less than 12°C. Primers should be of high purity without failure sequences. To avoid degradation, it is preferable to store primers in Tris-HCl (pH 8.0) buffer rather than in pure water. Many web sites are now available for effective primer design—e.g., Primer 3 software program (http://www.genome.wi.mit.edu/genome_software/other/primer3/htm and www.mutationdiscovery.com).

5. The PCR must be oil-free. Although the authors use standard PCR procedures, “touch down” PCR may be used to minimize misprimed products. This is particularly recommended by the manufacturers if using *Pfu* polymerase (Tables 4 and 5).
6. Although elution profiles are consistent for a specific mutation, different mutations may share indistinguishable profiles. Sequencing is advised for characterization of variants. DHPLC and sequencing analyses provide an excellent two-tiered approach to mutation analysis. However, specific mutation analysis can be achieved by DHPLC using primer extension methods (12).
7. High template concentrations can affect the WAVE trace.

Table 4
Protocol for PCR Amplification with Pfu

Protocol for Pfu	Initial	Touchdown	Main amplification	Final
Denaturation	95°C, 45 s	94°C, 45 s	94°C, 45 s	
Annealing		63°C, -0.5°C per cycle for 14 cycles to 56.5°C, 45 s	56°C, 45 s	
Extension		72°C, 2 min	72°C, 2 min	72°C, 10 min, finally perform heteroduplex step
Cycles	1	14	20	1

Table 5
Protocol for PCR Amplification with Optimase or TaqGold™

Protocol for Taq or TaqGold™	Initial	Touchdown	Main amplification	Final
Denaturation	95°C, 5 min (15 min for TaqGold™)	94°C, 30 s	94°C, 30 s	
Annealing		63°C, -0.5°C per cycle for 14 cycles to 56.5°C, 1 min	56°C, 1 min	
Extension		72°C, 1 min	72°C, 1 min	72°C, 5 min, finally perform heteroduplex step
Cycles	1	14	20	1

8. The recent introduction of Optimase (Transgenomic, Ltd.) with its own proof-reader is giving favorable results in our labs. Amplitaq Gold (Perkin-Elmer) works well for smaller fragments up to 300 bp, yielding fewer artefacts than standard Taq polymerase or Hot Start Taq. Although we do not use Pfu polymerase to generate PCR products for DHPLC in our laboratories, this enzyme minimizes PCR-induced mutations (Taq can cause 15% of dsDNA fragments to contain one or more mutation). As a rule of thumb, manufacturers recommend the use of Taq only if cycle number \times base pairs < 8000 . Pfu is expensive, and some laboratories use a

Pfu: Taq Gold mix to minimize cost. Optimase appears to be a cheaper, effective alternative. The use of a proofreading enzyme is recommended for fluorescence work on the WAVE, and the increased sensitivity of fluorescence (100×) can introduce artefacts if using Taq polymerase.

9. If other buffer systems are used containing additional reagents such as detergents, it is important to check first with DHPLC column manufacturers (Transgenomic) to ensure that they are compatible with the column packing, otherwise column life can be seriously curtailed. In our experience, dimethyl sulfoxide (DMSO) and 7 deaza-2-dGTP do not cause problems.
10. PCR amplification items 2, 3, and 4 can be purchased together as a kit from Perkin-Elmer.
11. The integrated system is comprised of an autosampler, pump, column oven, DNASep[®] column, in-line degasser, UV detector, WAVE utility software 3.4, 4.0, 4.1, or Navigator computer system, monitor, and color printer. Optional extras include a fragment collector for isolating individual peaks for further analysis or sequencing (Transgenomic Application note 104), an accelerator for reducing the time of sample throughput—e.g., from 6.8–5.6 min. The accelerator may be a useful addition for high-throughput work but it needs careful optimizing and current methods must be modified to ensure that peaks are captured in the smaller window that is provided. An additional facility is a fluorescence detector for increased sensitivity (×100). Currently, only a single-channel detector is available, but development of multichannel systems will allow sample multiplexing and thus higher throughput, although at extra cost for fluorescently tagged primers. The latest high-throughput WAVE[®] system (3500 HT) has a 2 × 96-well capacity sample plates with 2 × 384-well sample capacity, and incorporate a wide bore column, allowing an increase of standard flow rate from 0.9 mL/min to 1.5 mL per min, reducing the run time per sample to approx 3.2 min. The new WAVE-MED is a cheaper and currently slower machine using a single gradient and disposable column.
12. Enzymes, multivalent ions (such as Mg²⁺) and larger DNA fragments are present in all PCR samples. They are injected together with the PCR product on the WAVE[®] DNA Fragment Analysis System. They are usually eluted from the DNASep cartridge during the washing step with 100% buffer B (0.1 M TEAA, 25% ACN). However, some of those substances can accumulate on the surface of the separation matrix over time, causing back pressure and a loss of separation performance. The DNASep cartridge can withstand pressures of up to 3600 psi, and usually lasts for 4000–6000 injections or considerably more if treated carefully.
13. Replace the in-line filters every 1500 injections, and keep a log. It is also important to keep a log of injection numbers and the column life to highlight problems. Any maintenance carried out by the user should also be recorded.
14. The most common source of error is in the preparation of the buffers. Prepare these carefully, using volumetric flasks rinsed in HPLC water. The authors use water from a Millipore Elix 3 reverse osmosis system (Millipore Co., Bedford, MA). Water is taken from this and then polished by taking through a Simplicity 185

(Millipore) column for immediate buffer preparation. The quality of the water deteriorates if it is not used immediately. Ready-made buffers may now be purchased from Transgenomics, Ltd. This is recommended and eliminates in-house preparation variables.

Buffers A, B, and D must be changed weekly, and buffer C every 3 wk if not used. We find it convenient to keep a WAVE logsheet for each month, including date; buffer change; A B C D; QC run; injection number; notes.

15. In accordance with Best Practice Guidelines (**13**) mutation standards should be run weekly and after any system change for good internal quality control. If a QC test fails (that is the resolution and sensitivity has dropped appreciably), try an 80°C column hot wash. If this fails, an 80°C reverse hot wash with buffer C.

For the 80°C column wash, set the oven to 80°C and run 100% buffer C through for 30 min at 0.9 mL/min. Set the oven back to 50°C, allow to cool, and then run buffers A and B 50:50 at 0.9 mL/min for at least 3 h to equilibrate. Return to low flow and run two blank samples, then a pUC18 digest plus the mutation standard. If there is no improvement, try the reverse hot wash. First switch off the pump and reverse the orientation of the column, then proceed as previously by setting the oven at 80°C and running at 100% buffer C for 30 min at 0.9 mL/min flow and set the oven back to 50°C. Once the oven has cooled to about 60°C, return the column to its original orientation and equilibrate as before. Run through two blanks followed by the rest (pUC18 digest and mutation standard). If there is still no improvement, remove the in-line filter, replace with a plastic union joint, and run standards. If the problem is resolved, replace with a new in-line filter. Oven calibration may be suspect if DYS271 heterozygous/homozygous peak distances are not equivalent. If the problem remains, change the column and/or call/E-mail the Transgenomic Help Desk:

Tel: 1 (888) 233-WAVE
Fax: 1 (402) 733-1932
E-mail: custserv@transgenomic.com
or UK E-mail: help.desk@transgenomic.co.uk

16. TEAA and particularly ACN are hazardous chemicals, and safety procedures as outlined in the manufacturer's instructions should be observed. Ideally, buffers should be prepared in a fume cabinet. A relatively large volume of waste buffer accumulates. To collect the waste, we use 25-L containers (placed under the machine bench) provided by a local disposal firm that provides containers (and labels), and collect them every 2–3 mo. The waste contents and quantities (to the L) must be recorded for disposal. Many hospital departments already use disposal contractors, and it is usually possible to link to an existing service. Many WAVE® users rely on a well ventilated laboratory to remove ACN fumes. We also cover the opening of the waste container with parafilm "M" (Sigma Aldrich Co Ltd.). Ideally, the waste could be vented to a fumehood. As an alternative, we are currently attempting to improve our present collection system by introducing a carbon filter to vent the waste containers.

17. A number of different types of plate covers are available. Blockages with foil plugs or build up of adhesive on the needle can cause a problem. It is advisable to wipe the needle at least every 1000 injections with IPA or equivalent to remove adhesive. Transgenomic (Crewe, UK) have trialed a number of covers, and can advise as appropriate. Sharp needles are available from the manufacturer, and may alleviate the problem.
18. Once you are familiar with WAVEmaker 4, there are a number of shortcuts you can use when you are making your project by clicking on the icons at the top of the screen. For example, instead of going to “File → Open DNA sequence” and then looking for your sequence, you can just click on the appropriate icon and it will bring up the window to look for your sequence straight away. To print a melting profile, just click on the printer icon and the print preview will come up. You can also save a sequence or method in the same way.
19. A melting profile produced in the top graphic: *helical fraction vs temperature* that has an S-shaped curve melting over 4–6 degrees is likely to have one major melting domain or multiple domains close together (**Fig. 3**). Melting over a wider range or a differently shaped profile suggests the presence of multiple distinct melting domains (**Fig. 4**).

The bottom graphic (**Figs. 3, 4**) shows the calculated T_m of each individual base-pair (bp) in the sequence plotted against bp position. This can also be viewed with bp position plotted on the Y axis and temperature on the X axis to check the melting temperature of the major domains. If only one domain is present, analysis at the T_m will usually be appropriate. If more than one domain exists, but these are separated by no more than 3°C, a temperature at or close to the mean T_m will usually suffice. Domains more than 3°C apart may require analysis at more than one temperature when screening for unknown mutations, and these temperatures can be derived from the graphic. For fragments with one domain or domains less than 3°C apart, select the “trial function” and enter values around the T_m (e.g., $\pm 1^\circ\text{C}$) and press “calculate.” For fragments with more than one domain, start at 1°C lower than the lowest melting domain and evaluate the helical fraction across the fragment at progressively higher temperatures. Although a helical fraction of 75% is ideal, this will rarely be maintained along the whole fragment but a temperature associated with a helical fraction of 60–90% should be identified. Large fragments (>500 bp) are likely to have more than one melting domain, and it may be best to redesign primers or cut the fragment with a restriction enzyme.

If more than one temperature is used, we usually choose $>1^\circ\text{C}$ intervals, using as few temperatures as possible. The melting temperature of the ends of the fragment are irrelevant, as the first 20 bases are primer. Mutations near the primers are usually seen easily, since they will be into the first major domain. When a small part of a fragment has a significantly higher melt domain than the remainder, it is advisable to select a temperature that is 1°C lower than the predicted temperature for that domain. If the running temperature differs from T_m , a time shift of generally ± 0.5 min per 1°C will be required to elute the fragment at the correct time.

It is worth noting that melting profiles prepared with WAVEmaker 4.0 or above may differ from those prepared for the same fragment with WAVEmaker 3.4. Versions 4.0 and above are recommended.

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