USER GUIDE

DNA Fragment Analysis by Capillary Electrophoresis

Publication Number 4474504
Revision B

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About This Guide

IMPORTANT! Before using the products described in this guide, read and understand the information in the "Safety" appendix in the documents provided with each product.

Revision history

Revision	Date	Description
A	August 2012	New document.

Purpose

This guide is intended for customers who plan, conduct, and troubleshoot fragment analysis applications.

This guide is for use by novice and experienced users who perform automated fragment analysis with any of these instruments:

- Applied Biosystems® 3500 or 3500xL Genetic Analyzers (3500 Series instruments)
- Applied Biosystems® 3730 or 3730*xl* DNA Analyzers (3730 Series instruments)
- Applied Biosystems® 3130 or 3130*xl* Genetic Analyzers (3130 Series instruments)
- 310 Genetic Analyzers (310 instruments)

Prerequisites

This guide assumes that:

- Thermo Fisher Scientific genetic analyzers and other instruments for which Thermo Fisher Scientific provides installation service have been installed by a Thermo Fisher Scientific technical representative.
- Thermo Fisher Scientific reagents are used.

Structure of this guide

Chapter	Subject
Introduction	
1	Introduction to Fragment Analysis
2	Experimental Design
Core processes in fragment analysis	
3	Optimizing PCR
4	Optimizing Capillary Electrophoresis
5	Data Analysis with GeneMapper® Software and Peak Scanner™ Software
Types of fragment analysis	
6	Microsatellite Analysis
7	Single Nucleotide Polymorphism (SNP) Genotyping
8	Fingerprinting
9	Relative Fluorescence Quantitation (RFQ)
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Introduction to Fragment Analysis

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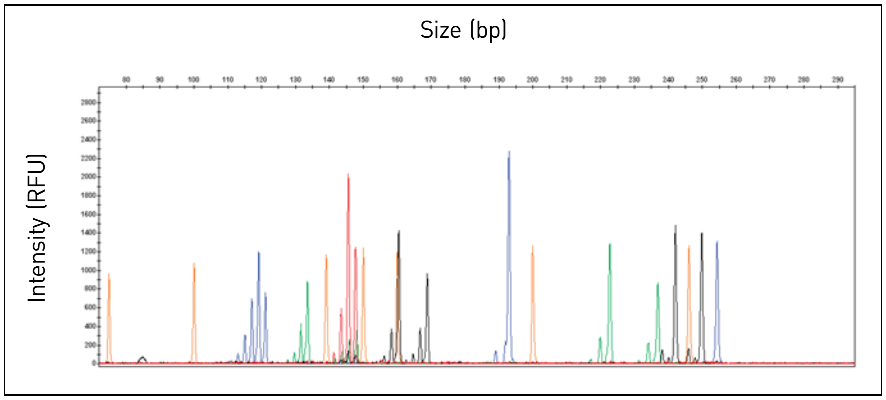
Fragment analysis versus sequencing...what is the difference?

Fragment analysis

Fragment analysis using Thermo Fisher Scientific products involves:

- Labeling fragments with fluorescent dyes. Multiple different colored fluorescent dyes can be detected in one sample. One of the dye colors is used for a labeled size standard present in each sample. The size standard is used to extrapolate the base-pair sizes of the sample product peaks.
- Amplifying the labeled fragments using polymerase chain reaction (PCR) on a thermal cycler.
- Separating the fragments by size using capillary electrophoresis.
- Analyzing the data using software to determine:
  - **Size**: The analysis software uses the size standard in each sample to create a standard curve for each sample. It then determines the relative size of each dye-labeled fragment in the sample by comparing fragments with the standard curve for that specific sample.
  - **Genotype**: The analysis software assigns allele calls based on user-defined makers (loci).

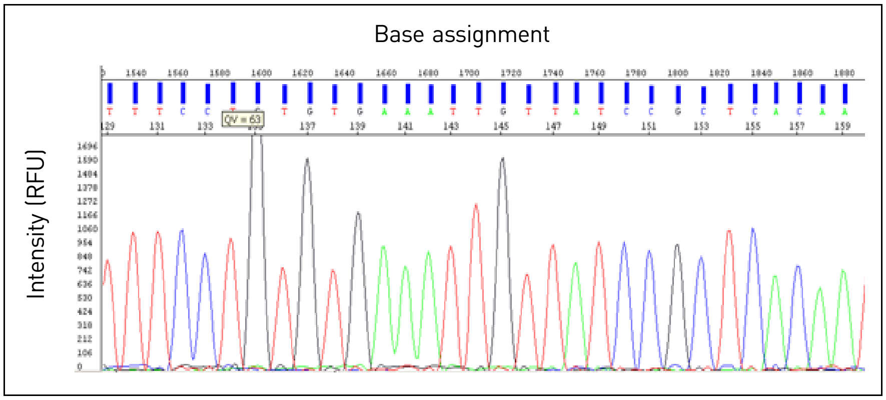
Figure 1 Fragment analysis – fluorescently labeled fragments are separated and sized



Sequencing

Sequencing is the determination of the base-pair sequence of a DNA fragment by the formation of extension products of various lengths amplified through PCR. For more information, refer to the *DNA Sequencing by Capillary Electrophoresis | Chemistry Guide* (Pub. no. 4305080).

Figure 2 Sequencing – fluorescently labeled nucleotides are separated and base-called



What can I do with fragment analysis?

Types of applications

- **Microsatellite (STR) analysis** (see Chapter 6, “Microsatellite Analysis”)

  Microsatellite markers (loci), also known as short tandem repeats (STRs), are polymorphic DNA loci consisting of a repeated nucleotide sequence. In a typical microsatellite analysis, microsatellite loci are amplified by PCR using fluorescently labeled forward primers and unlabeled reverse primers. The PCR amplicons are separated by size using electrophoresis. Applications include:

  - Linkage mapping
  - Animal breeding
  - Human, animal, and plant typing
  - Pathogen sub-typing
  - Genetic diversity
  - Microsatellite instability
  - Loss of Heterozygosity (LOH)
  - Inter-simple sequence repeat (ISSR)
  - Multilocus Variant Analysis (MLVA)

- **SNP Genotyping** (see Chapter 7, “Single Nucleotide Polymorphism (SNP) Genotyping”)

  A Single Nucleotide Polymorphism (SNP) marker consists of a single base pair that varies in the known DNA sequence, thereby creating up to four alleles or variations of the marker. Applications include:

  - SNaPshot® Multiplex Kit

- **Fingerprinting** (see Chapter 8, "Fingerprinting")

  Several AFLP®-based technologies use restriction enzyme length polymorphism and polymerase chain reaction (PCR) to generate a fingerprint for a given sample, allowing differentiation between samples of genomic DNA based on the fingerprint. Applications include:
  - Microbial genome typing
  - Animal or plant genome typing
  - Creation of genetic maps of new species
  - Genetic diversity and molecular phylogeny studies
  - Establishment of linkage groups among crosses
- **Relative Fluorescence** (see Chapter 9, "Relative Fluorescence Quantitation (RFQ)")

  Relative fluorescence applications compare peak height or area between two samples. Common techniques include:
  - Qualitative Fluorescence (QF) PCR
  - Quantitative Multiplex PCR of Short Fluorescent Fragments (QMPSF)
  - Multiplex Ligation-dependent Probe Amplification (MLPA)

  Applications include:
  - LOH in tumor samples
  - Copy Number Variation (CNV)
  - Aneuploidy detection

Applications described in this guide

The applications in this guide are identified as one of the following:

Category	Description
Thermo Fisher Scientific-supported	Thermo Fisher Scientific has tested and validated this protocol on the instrument system specified. The technical support and field application specialists have been trained to support this protocol.
Thermo Fisher Scientific-demonstrated	Thermo Fisher Scientific has tested this protocol but has not validated for the instrument system specified. Certain components of the protocol workflow such as reagent kits and other protocols for preparation of reagents may not be available through Thermo Fisher Scientific. Supporting documentation such as application notes may be available from Thermo Fisher Scientific and/or third parties. Limited support is available from Thermo Fisher Scientific.
Customer-demonstrated	Thermo Fisher Scientific has not tested this protocol. However, at least one customer or third party has reported successfully performing this protocol on the instrument system specified. Thermo Fisher Scientific cannot guarantee instrument and reagent performance specifications with the use of customer-demonstrated protocols. However, supporting documentation from Thermo Fisher Scientific and/or third parties may be available and Thermo Fisher Scientific may provide basic guidelines in connection with this protocol.

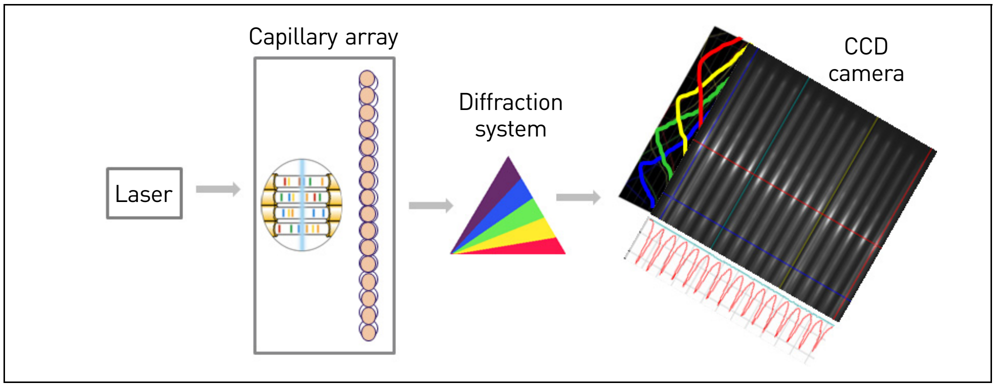
What is capillary electrophoresis?

Capillary electrophoresis (CE) is a process used to separate ionic fragments by size. In Thermo Fisher Scientific CE instrumentation, an electrokinetic injection is used to inject DNA fragments from solution and into each capillary.

During capillary electrophoresis, the extension products of the PCR reaction (and any other negatively charged molecules such as salt or unincorporated primers and nucleotides) enter the capillary as a result of electrokinetic injection. A high voltage charge applied to the sample forces the negatively charged fragments into the capillaries. The extension products are separated by size based on their total charge.

The electrophoretic mobility of the sample can be affected by the run conditions: the buffer type, concentration, and pH; the run temperature; the amount of voltage applied; and the type of polymer used.

Shortly before reaching the positive electrode, the fluorescently labeled DNA fragments, separated by size, move across the path of a laser beam. The laser beam causes the dyes attached to the fragments to fluoresce. The dye signals are separated by a diffraction system, and a CCD camera detects the fluorescence.



Because each dye emits light at a different wavelength when excited by the laser, all colors, and therefore loci, can be detected and distinguished in one capillary injection.

The fluorescence signal is converted into digital data, then the data is stored in a file format compatible with an analysis software application.

Fragment analysis workflow

Phase		Technology	Thermo Fisher Scientific products used
1.	Isolate DNA	Depends on sample source and application	DNA isolation methods depend on your starting DNA source. Refer to guidelines for your application for information on isolating DNA.
2.	Purify DNA	Depends on sample source and application	Go to **www.lifetechnologies.com** for advice on the appropriate product to use.
3.	Quantify DNA	Dye-labeling and fluorometric detection	Qubit® Fluorometer and Quantitation Kit, go to **www.lifetechnologies.com/qubit**.
4.	PCR amplification	Dye-labeling and amplification of fragments using a thermal cycler	• Veriti® Thermal Cycler: – 96-well – 384-well • GeneAmp® PCR System 9700: – Dual 96-well – Dual 384-well – Auto-Lid Dual 384-well • 2720 Thermal Cycler
5.	Capillary electrophoresis	Separation of fragments based on size using a genetic analyzer	• 3500/3500xL Genetic Analyzer (3500 Series instrument) • 3730/3730*xl* Genetic Analyzer (3730 Series instrument) • 3130/3130*xl* Genetic Analyzer (3130 Series instrument) • 310 Genetic Analyzer (310 instrument)
6.	Data analysis	Sizing and optional genotyping	• GeneMapper® Software
		Sizing	• Peak Scanner™ Software (available free-of-charge on **www.lifetechnologies.com**) Use this software with data generated on 3730 Series, 3130 Series, and 310 instruments. It is not compatible with data generated on the 3500 Series instrument, which performs fragment sizing during data collection.

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Experimental Design

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Experimental design considerations

Consider the following questions when designing your experiment:

- What sequences and markers (loci) are you investigating? (not applicable for AFLP® studies)
- Which enzyme is appropriate for your experiment? (see "DNA polymerase enzymes" on page 22)
- What is the expected allele distribution? (determine from published literature or from your own design and empirical testing)
- What labeling method will you use? (see "Fluorescent labeling methods" on page 25)
- Do fragment sizes overlap? (see "Compensating for overlapping fragment sizes" on page 28)
- Will you evaluate one target per reaction (singleplex) or multiple targets per reaction (multiplex)? (see "Singleplexing versus multiplexing" on page 26)
- What factors affect the design of your primers? (see "Primer design guidelines" on page 29)
- Which dye sets are compatible with your genetic analyzer and are appropriate for the number of markers of interest? (see "Dye sets" on page 41 and "Singleplexing versus multiplexing" on page 26)
- Which size standard is appropriate for the fragment size range and dye labels of your samples? (see "Size standards" on page 42)

DNA polymerase enzymes

Overview

For most applications, AmpliTaq Gold® DNA Polymerase is the enzyme of choice. However, Thermo Fisher Scientific supplies a number of PCR enzymes that have been optimized for specific needs as listed below. Go to **www.lifetechnologies.com** for other available enzymes.

Note: AmpFℓSTR®, AFLP®, and SNaPShot® kits include the appropriate DNA polymerase for the application.

Table 1 PCR enzymes supplied by Thermo Fisher Scientific

DNA polymerases	Description
AccuPrime™ *Taq* DNA Polymerase System	Provides reagents for amplification of nucleic acid templates with antibody-mediated hot-start for improved PCR specificity over other hot-start DNA polymerases. Platinum® anti-*Taq* DNA polymerase antibodies inhibit polymerase activity, providing an automatic hot-start, while a thermostable accessory protein enhances specific primer-template hybridization during every cycle of PCR. This combination improves the fidelity of *Taq* DNA Polymerase by two-fold and is ideal for high-throughput screening and multiplex PCR. AccuPrime™ *Taq* DNA Polymerase broadens primer annealing temperatures, giving you optimal performance between 55°C and 65°C. Applications: Multiplex PCR, TOPO TA Cloning®, allele-specific amplifications.
AccuPrime™ *Taq* DNA Polymerase High Fidelity	Amplifies nucleic acid templates using antibody-mediated hot-start, a blend of *Taq* DNA Polymerase and proofreading enzyme, and AccuPrime™ accessory proteins for improved PCR fidelity, yield, and specificity over other hot-start DNA polymerases. This enzyme provides: • The highest specificity and yield for the most robust PCR amplification • 9-fold higher fidelity than *Taq* DNA polymerase alone • Minimal optimization steps, even with non-optimized primer sets • Efficient amplification of targets over a broad size range up to 20 kb High fidelity is achieved by a combination of Platinum® anti-*Taq* DNA polymerase antibodies that inhibit polymerase activity, providing an automatic "hot-start", and the proofreading (3'-5´ exonuclease activity) enzyme *Pyrococcus* species GB-D. The thermostable AccuPrime™ accessory proteins enhance specific primer-template hybridization during every cycle of PCR, preventing mispriming and enhancing PCR specificity and yield.
AmpliTaq® DNA Polymerase	For general use in PCR. AmpliTaq® DNA Polymerase is a recombinant form of Taq DNA polymerase obtained by expressing a modified Taq DNA polymerase gene in an *E. coli* host. Similar to native Taq DNA polymerase, the enzyme lacks endonuclease and 3'-5' exonuclease (proofreading) activities, but has a 5'-3' exonuclease activity.
AmpliTaq® DNA Polymerase, LD	Low concentrations of *E. coli* DNA contamination, thus is better suited for amplifying DNA of bacterial origin. AmpliTaq® DNA Polymerase, LD (Low DNA), is the same enzyme as AmpliTaq® DNA Polymerase; however, the LD formulation has undergone a further purification process. The purification step insures that false-positive PCR products will be effectively minimized when amplifying bacterial sequences. AmpliTaq® DNA Polymerase, LD is especially useful for low-copy number amplifications.

DNA polymerases	Description
AmpliTaq Gold® DNA Polymerase	Use in most applications because it yields PCR fragments of high specificity. AmpliTaq Gold® DNA Polymerase is a chemically modified form of AmpliTaq® DNA Polymerase. It provides the benefits of hot-start PCR (that is, higher specific product yield, increased sensitivity, and success with multiplex PCR) without the extra steps and modifications of experimental conditions that make hot-start impractical for high-throughput applications. AmpliTaq Gold® DNA Polymerase is delivered in an inactive state. A pre-PCR heating step of 10 to 12 minutes at 95°C, which can be programmed into the thermal cycling profile, activates the enzyme. For low-template copy number amplifications, step-wise activation of AmpliTaq Gold® DNA Polymerase, or time-release PCR, can be useful.
GeneAmp® Gold Fast PCR Master Mix	Allows PCR to be finished in ~40 minutes.
Platinum® Multiplex PCR Master Mix	Designed specifically for endpoint multiplex PCR. It supports easy multiplexing with minimal optimization. Amplifies up to 20 amplicons in a single reaction. Amplifies products from 50 bp to 2.5 kb. The performance of the Platinum® Multiplex PCR Master Mix over a wide range of amplicon sizes permits the amplification of templates from 50 bp to 2.5 kb, greatly enhancing workflow flexibility. Coupled with its 20-plex capability and absence of primer dimers, it not only provides a high-throughput solution but also boasts high specificity through fewer non-specific primer binding events, and hence less reaction and primer waste.
Platinum® *Pfx* DNA Polymerase	Ideal for amplification of DNA fragments for high-fidelity PCR applications. High fidelity is provided by a proprietary enzyme preparation containing recombinant DNA Polymerase from *Thermococcus* species *KOD* with proofreading (3´t´ exonuclease) activity. Platinum® antibody technology provides a simple, automatic hot-start method that improves PCR specificity. PCR_x Enhancer Solution is included for higher primer specificity, broader magnesium concentration, broader annealing temperature, and improved thermostability of Platinum® *Pfx* DNA Polymerase. The PCR_x Enhancer Solution also helps optimize PCR of problematic and/or GC-rich templates. Platinum® *Pfx* provides: • 26 times higher fidelity than *Taq* DNA polymerase • Amplification of fragments up to 12 kb • Room temperature reaction assembly Applications: Amplification of DNA from complex genomic, viral, and plasmid templates; and RT-PCR. Unit Definition: One unit incorporates 10 nmoles of deoxyribonucleotide into acid-precipitable material in 30 minutes at 74°C.
SuperScript® III Reverse Transcriptase (RT)	Proprietary mutant of SuperScript® II RT that is active at 50°C and has a half-life of 220 minutes, providing increased specificity with Gene-Specific Primers (GSPs) and the highest cDNA yield of all RTs. It is ideal for RT-PCR of a specific gene or generating cDNA from total or poly (A)+ RNA sample. Like SuperScript® II, it synthesizes a complementary DNA strand from single-stranded RNA, DNA, or an RNA:DNA hybrid. SuperScript® III RT is genetically engineered by the introduction of point mutations that increase half-life, reduce RNase activity, and increase thermal stability. Applications: array labeling, cDNA libraries, RT-PCR, primer extension, and 3´ and 5´ RACE. Purified from *E. coli*.

Derivatives of *Tth* DNA polymerase

Thermo Fisher Scientific supplies two modified forms of Thermus thermophilus (*Tth*) DNA polymerase:

- *rTth* DNA Polymerase is obtained by expression of a modified form of the *Tth* gene in an *E. coli* host.
- *rTth* DNA Polymerase, XL (Extra Long), provides the same features as *rTth* DNA Polymerase for target sequences from 5 to 40 kb. An inherent 3'-5' exonuclease activity allows for the correction of nucleotide misincorporations that might otherwise prematurely terminate synthesis.

Enzyme characteristics

Table 2 Enzyme characteristics

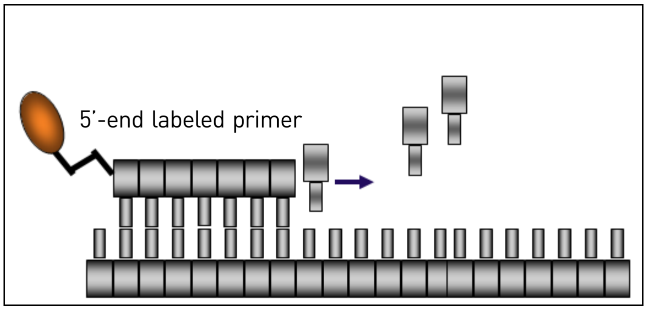
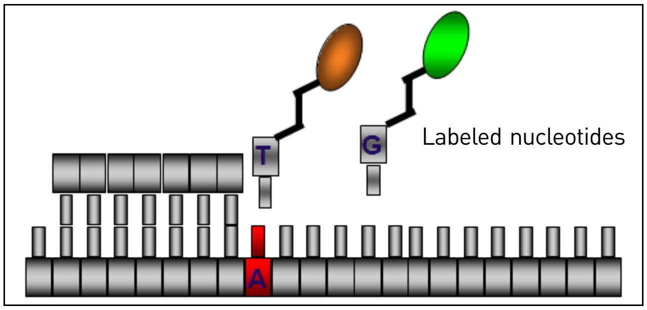
Characteristics	Recommended enzyme
High specificity	AccuPrime™ *Taq* DNA Polymerase
High sensitivity	AmpliTaq Gold® DNA Polymerase
High fidelity	Platinum® *Pfx* DNA Polymerase
High temperatures	AmpliTaq® DNA Polymerase
Multiplex PCR	Platinum® Multiplex PCR Master Mix
Amplification of low-copy number template	• AmpliTaq Gold® DNA Polymerase • AmpliTaq® DNA Polymerase • AmpliTaq® DNA Polymerase LD (for bacterial sequences)
High specificity at high ionic strength	• AmpliTaq Gold® DNA Polymerase • AmpliTaq® DNA Polymerase
Amplification of extra-long fragments (>5 kb)	*rTth* DNA Polymerase, XL
Pre-PCR conversion to cDNA	SuperScript® III Reverse Transcriptase
Extra cycles	AmpliTaq® DNA Polymerase
High magnesium ion concentration	• AmpliTaq Gold® DNA Polymerase • AmpliTaq® DNA Polymerase

Fluorescent labeling methods

IMPORTANT! With any labeling technique, use only Thermo Fisher Scientific dyes. Thermo Fisher Scientific provides spectral calibration matrix standards that have been optimized for our dye sets.

Other dyes (or mixed isomers of dyes) have variable emission spectra and require a spectral calibration generated for the specific dyes to correct for the spectral overlap between the dyes. You are responsible for obtaining the appropriate spectral calibration reagents and for optimizing custom dye sets.

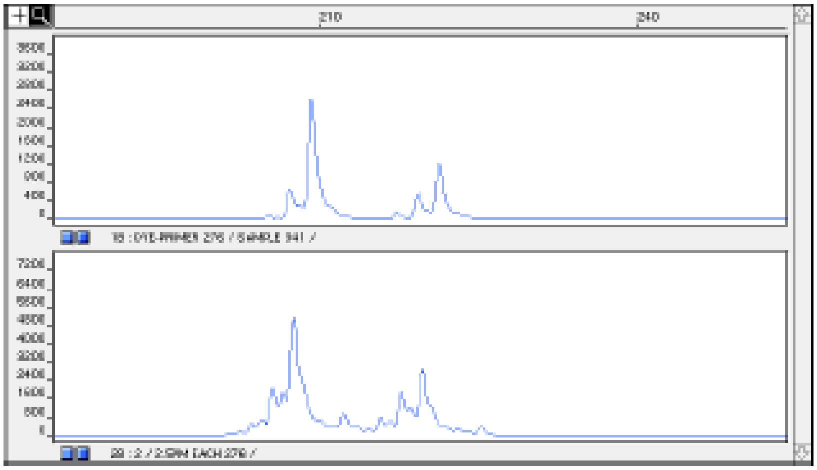
Table 3 Fluorescent labeling methods

5'-end labeled primer incorporated during the PCR primer-annealing step	**Fluorescent dye-labeled dUTPs or dCTPs ([F]dNTPs incorporated during the PCR primer-extension step†**
• Most commonly used in microsatellite analysis • **Higher precision:** Different fluorophores have different mobilities. DNA fragments with the same 5'-end primer and fluorophore have comparable electrophoretic mobility, and yield sharper fragment peaks because 5'-end primer labeling yields 1:1 incorporation (that is, one fluorophore-to-one DNA fragment). • **More consistent quantitation:** Every peak in an electropherogram is made up of multiple DNA fragments of equal size in base pairs. When using 5'-end primer labeling, every DNA fragment contributes a single fluorophore to the total signal of a peak, and thus the peak area is proportional to the number of DNA molecules. • **Distinct strands:** By attaching different fluorophores to the forward and reverse primers, it is possible to distinguish between the peaks corresponding to each strand, and between residual double-stranded products. • **Lower sensitivity:** Because of 1:1 incorporation (that is, one fluorophore-to-one DNA fragment), it yields a lower signal than [F]dNTP-labeled fragments.	• Most commonly used in SNP analysis • **Lower precision:** Fragments labeled with [F]dNTPs tend to produce broader peaks that often appear to be split because variable numbers of [F]dNTPs are incorporated during PCR in variable positions on both strands. • **Less consistent quantitation:** A variable number of fluorophores are attached to each DNA fragment in a population. The average number of attached fluorophores depends upon the fragment base composition and length and upon the ratio of [F]dNTPs to dNTPs added to the reaction mixture. Therefore, it is not advisable to compare peak areas between fragments labeled with [F]dNTPs for relative quantitation studies. • **Higher sensitivity:** Because most fragments contain multiple fluorophores, a given number of [F]dNTP-labeled fragments will produce a higher signal when compared to the same number of 5'-end labeled fragments. The increased signal strength allows you to use smaller reaction volumes and fewer amplification cycles during PCR. • **Low cost:** You can add [F]dNTPs to any PCR. You do not need to order or synthesize fluorescently labeled primers before each PCR and you can use [F]dNTPs with your existing primer sets.
	

† Post-PCR end-labeling with [F]dNTPs using Klenow is an alternative to labeling during PCR (Iwahana *et al.*, 1995; Inazuka *et al.*, 1996). You can also label with [F]dNTPs using traditional techniques such as random priming or nick translation.

The following figure compares the results obtained using 5'-end labeled primers and [F]dNTPs. The 5'-end labeled primers give better resolution, but [F]dNTPs result in higher peaks. Note also the unincorporated fluorescently labeled nucleotides in the [F]dNTP-labeled sample.

Figure 3 Comparison of 5'-end labeled primers (top panel) and [F]dNTP-labeled primers (bottom panel)



Singleplexing versus multiplexing

Singleplexing

Singleplexing is a PCR technique in which a single target is amplified in a reaction tube. This technique uses only one primer pair in each reaction and does not require as much optimization as multiplexing. However, singleplexing increases the cost and time per analysis.

Multiplexing

Multiplexing is a PCR technique in which multiple DNA targets are amplified in the same reaction tube. Multiplexing uses multiple primer pairs in each reaction, and requires optimization to ensure primer pairs are compatible.

Thermo Fisher Scientific fluorescent multi-color dye technology allows multiplexing. Alleles for overlapping loci are distinguished by labeling locus-specific primers with different colored dyes. Multicomponent analysis separates the different fluorescent dye colors into distinct spectral components.

Benefits	Potential limitations
• Simplifies PCR setup • Increases throughput • Decreases cost per amplification	• Primer-oligomer formation • Loss of specificity • Decreased yield of specific products • Can require significant optimization

Multiplexing (pooling) strategies

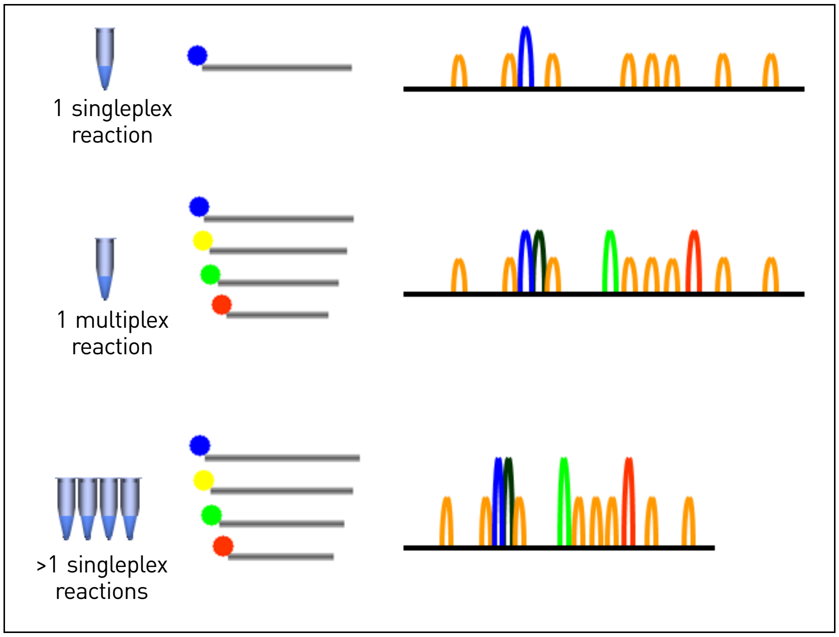
Strategies

Multiplexing strategies include:

- Pooling samples after PCR

  Note: It is generally easier to pool the products from individually amplified, fluorescently labeled primer pairs than to optimize a multiplex PCR containing multiple fluorescently labeled primer pairs. Because primer efficiencies vary, it may be necessary to add different amounts of each individually amplified PCR product to a pool to achieve similar peak heights. Fluorescence intensity from each individual dye may also vary.

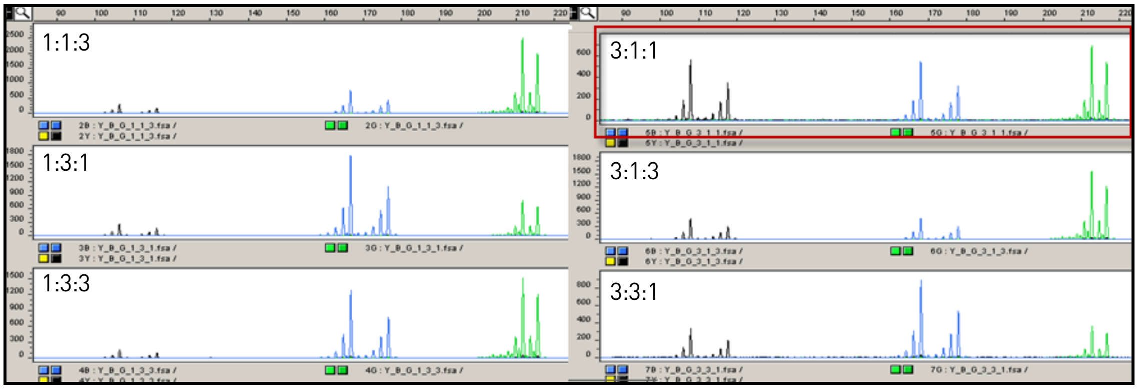
- Amplifying multiple products in a single PCR reaction. Options for pooling in a PCR reaction are illustrated in the following figure (the orange peaks are the size-standard peaks).



If you pool samples after PCR:	If you amplify multiple PCR products:
• This strategy is simpler and more flexible, but pooling products from multiple singleplex PCR reactions often increase the salt concentration in the loaded samples, which can cause unwanted downstream effects (see "Desalting" on page 190). • If PCR product sizes overlap, use different color dyes so they separate during electrophoresis (see "Dyes" on page 36). • Use a combination of dyes that can be detected using one spectral matrix (one spectral calibration). • Optimize sample concentration to optimize signal intensity for each dye (see "Optimizing signal intensity" on page 77).	• Optimize primers: – Use different dyes to label multiplex primers of similar lengths. – Primers cannot contain large regions of complementarity. – Primers should have similar melting temperatures (T_m). • Before performing the PCR, perform a preliminary check for primer compatibility and test the pairs for successful co-amplification. • Optimize conditions for primers in singleplex reactions before using them in multiplex reactions to ensure the primers are suitable for your experiment.

Adjusting pooling ratios

To ensure signal balance between dyes in a multiplexed sample, adjust pooling ratios as needed. The figure below shows the effect of different pooling ratios on signal balance. In this example, a pooling ratio of 3:1:1 yields balanced signal for the three dyes.



Multiplex design software

Software applications are available to assist with the design of multiplex PCR (Holleley and Geerts, 2009).

Multiplexing guidelines

Compensating for overlapping fragment sizes

If the sizes of different fragments overlap, you can do the following to differentiate between them:

- Label overlapping products with different dyes.
- Leave the following number of base pairs between the known size ranges:
  - Microsatellite applications: 15 to 20 base pairs
  - SNaPshot® applications: 8 to 10 base pairs
- Use different primer sites to alter the PCR-product fragment lengths.
- Load overlapping products in different wells or separate capillary injections (runs).

Enzyme choice

The high specificity of AmpliTaq Gold® DNA Polymerase typically permits amplifying with elevated Mg^{2+} concentrations for increased yield.

Primer quality

Because reagents (such as dNTPs) are often limiting during multiplex PCR, using high quality primers is particularly important. For example, the decreased specificity (and thus the increased reagent consumption) of one pair of degraded PCR primers can affect the entire multiplex reaction. Although you can compensate for a degraded pair of primers to some extent by increasing the concentration of the other primer pairs, the increased cost per reaction and the decreased reproducibility over time do not justify this short-term solution.

When buying or making primers, make sure that they are length-purified and that they are free of contaminants.

Primer-pair concentrations

Typically, start out with equal concentrations for all primer pairs.

It is often necessary to adjust the concentration of primer pairs in the multiplex reaction until the peak heights are relatively even. Increase the primer-pair concentration for fragments showing weak amplification. Decrease the primer-pair concentration for fragments showing significantly greater than average amplification.

Primer-pair compatibility

With either single or multiplex PCR, evaluate primers for compatibility. Avoid excessive regions of complementarity among the primers. Also, select or design primers with similar melting temperatures (T_m).

After identifying compatible primer pairs, test and evaluate pairs in singleplex reactions before attempting any multiplex reactions. You will often need to optimize reaction conditions and, occasionally, you will need to redesign the primers.

Troubleshooting multiplex PCR

Consider amplifying separately any primer pair that fails to amplify after its concentration is increased.

To eliminate interfering background peaks, try:

- Swapping primer pairs between different multiplex reactions
- Removing primer pairs from the multiplex reaction

Primer design guidelines

Primer design criteria

- Optimum length: 17 to 25 nucleotides
- Optimum T_m: 55 to 65°C

  Using primers with similar T_m values makes it possible to find thermal cycling parameters that are optimal for both primers in a primer pair. The T_m of a reaction is influenced by base composition, concentrations of Mg^{2+} and K^+ ions in the mixture, and cosolvents.

  Based on the T_m, calculate the annealing temperature:

  T_a (°C)= $T_m - 5$

  The “2+4 Rule”: $T_m = [(A+T) \times 2 + (G+C) \times 4]$
- Avoid:
  - Primer-dimers
  - Hairpins
  - Secondary structures
  - Secondary binding sites

Primer design software

Thermo Fisher Scientific offers OligoPerfect™ Designer available at **www.lifetechnologies.com**.

Factors affecting T_m and primer annealing

Primer annealing is influenced by:

- Primer/template base composition
- Primer/template base order
- Primer or template secondary structure

Effects of base composition

G-C bonds contribute more to the stability (increased melting temperature) of primer/template binding than do A-T bonds.

To ensure stable annealing of primer and template while avoiding problems with the internal secondary structure of primers or long stretches of any one base, select primers with a 40% to 60% G+C content.

Note: Designing primers based on T_m and primer length to avoid primer-dimers and gapped-duplex structures is more important than designing primers based on actual percent G+C content.

Effects of base order

Two primer/template complexes with identical content may have different T_m values because base order influences the overall annealing stability. You can determine the exact effect of base order on complex stability using the base-pairing energies listed in Table 4 (adapted from Salser, 1978). Larger negative values represent more stable interactions between the template and primer.

Table 4 Base-pairing energies (kcal/dinucleotide pair)

5' Nucleotide	3' Nucleotide			
	A	C	G	T
A	–1.2	–2.1	–2.1	–1.8
C	–2.1	–4.8	–3.0	–2.1
G	–2.1	–4.3	–4.8	–2.1
T	–1.8	–2.1	–2.1	–1.2

Example: Consider the two sequences: 3-GAC-5' and 3'-CGA-5'. The sequence 3-GAC-5' contained within a primer would contribute –4.2 kcal to the binding energy (–2.1 kcal [3-GA-5'] + –2.1 kcal [3-AC-5'] = –4.2 kcal). However, if the G and C are next to each other, as in 3-CGA-5', the contribution increases to –6.4 kcal (–4.3 kcal [3-CG-5'] + –2.1 kcal [3-GA-5'] = –6.4 kcal).

Note: Although a G-C dinucleotide at the 3' end of the primer can stabilize the template-primer binding complex when using thermostable enzymes such as AmpliTaq® DNA Polymerase, a 3' G-C can also lead to false priming if you do not optimize PCR conditions (Topal and Fresco, 1976).

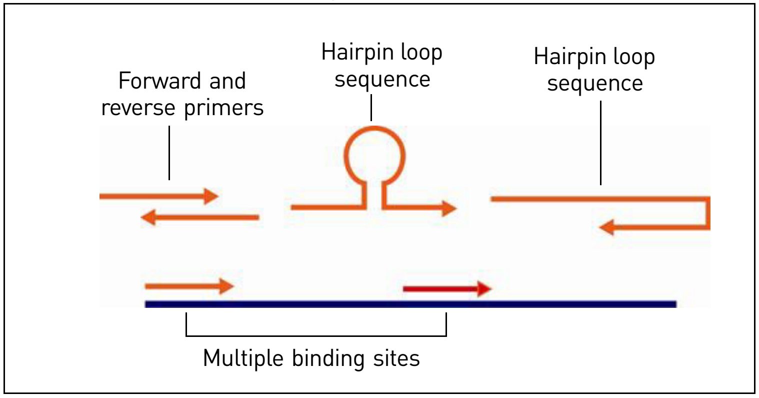
Effects of primer secondary structure

Strings of Gs and Cs can form internal, non-Watson-Crick base pairs (Sarocchi *et al.*, 1970) that disrupt stable primer annealing. Although this anomalous behavior is difficult to predict, a good general rule is to avoid runs of more than three consecutive Gs in primers.

However, a short run of Gs at or near the 5' end of a primer will not disrupt the stability of primer-template complexes because 5' positioning does not lead to involvement in disruptive secondary structures (for example, primer-dimer or duplex loops).

Similarly, self-complementary sequences within the primer can lead to the formation of hairpin structures that disrupt stable primer binding to template. A stable hairpin can form with just four G+C base pairs in the stem and three bases in the loop (Summer *et al.*, 1985) (Figure 4).

Figure 4 Secondary structures in primers



Effects of template secondary structure

Primers do not bind effectively to target sequences with known secondary structures. For example, hairpin structures are often found in regions of high G+C content or in RNA sequences. If you must design primers to a specific target region with the potential for hairpin formation, you may try addition of DMSO to your reaction or other commercially available kits for difficult template amplification.

Selective amplification

Amplification of the desired target sequence requires minimizing primer binding to secondary sites in the DNA and to other primers.

Note: This applies to template genomic DNA. The probability of binding to secondary sites is lower for low-complexity templates, such as plasmid DNA.

Ideally, the binding of the primer to the desired template region:

- Is strongest at the 5' end.
- Generally requires a higher, more negative value (to maximize base-pairing energy) than
  –9.8 kcal/mole at the 3' end (see Table 4 on page 30).

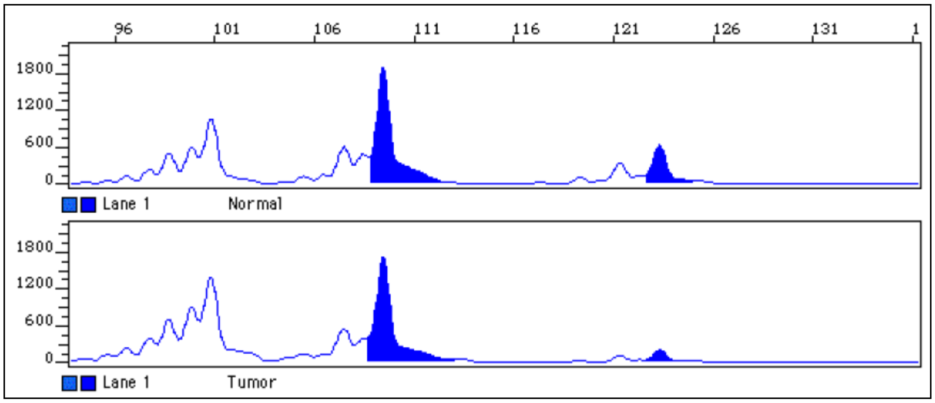
As a general rule, binding at the 3’ end should be weaker than –9.8 kcal/mole.

Preferential amplification

When alleles differ in size by ten or more base pairs you may observe preferential amplification of shorter PCR products over longer ones (Walsh *et al.*, 1992). This will also occur when amplifying low copy-number DNA or DNA isolated from paraffin-embedded tissues. Figure 5 on page 32 is an example of preferential amplification of the D5S346 marker. In both the normal (top panel) and tumor (bottom panel) samples, the peak height of the larger 124-base pair (bp) fragment is much lower than that of the smaller 110-bp fragment.

IMPORTANT! Preferential amplification can decrease the accuracy of relative quantitation measurements.

Figure 5 Example of preferential amplification of the D5S346 marker. In both the normal (top panel) and tumor (bottom panel) samples, the peak height of the larger 124-bp fragment is much lower than that of the smaller 110-bp fragment.



Non-specific amplification

Polymerases require only the binding of the nucleotides at the 3' end to begin elongation. If the 3' nucleotides bind strongly to random regions of the genome (perhaps because of a 3' G+C), any template sequences complementary to the 3' end are amplified. In this case, specificity is lost because the entire primer does not specifically target the genomic region of interest.

Self-complementary sequences within the primer can lead to the formation of hairpin structures that decrease binding specificity (as well as disrupt binding stability). Nucleotides in the hairpin structure are not available for binding of the target sequence. The available nucleotides can be thought of as forming a "smaller," and therefore less specific, primer.

Conversely, if binding is strongest at the 5' end, the typical binding event on the template DNA begins at the 5' end. Polymerases, however, cannot begin elongation until the 3' end binds. Therefore, the entire primer is used to distinguish among target sequences.

Also, when performing a computer-assisted search to evaluate binding to secondary sites in the target DNA, consider the potential for "gapped duplex" formation. A gapped duplex can form when the primer and target are completely complementary except for a single base (Miller, Kirchoff *et al.*, 1987; Miller, Wlodawer *et al.*, 1987).

Note: Binding to secondary sites can also involve the formation of stable non-Watson-Crick base pairs (Topal and Fresco, 1976). Stable base-pairing is most likely to occur between G and T, but A-C and G-A pairs can also be stable (Hunter, 1986). All software programs have difficulty modeling these sorts of interactions.

Minimizing binding to other primers

Complementary sequences between two primers, especially at the 3' ends, can lead to the formation of product artifacts arising from amplified primer-dimers and primer-oligomers. Avoid primers with inter-complementary regions between members of a primer pair or pairs.

Post-amplification manipulations

Adding extensions that are not complementary to the template at the 5′ end of the primer can facilitate a variety of useful post-amplification manipulations of the PCR product without adversely affecting yield. Examples include 5' extensions that contain restriction sites, universal primer binding sites, or promoter sequences.

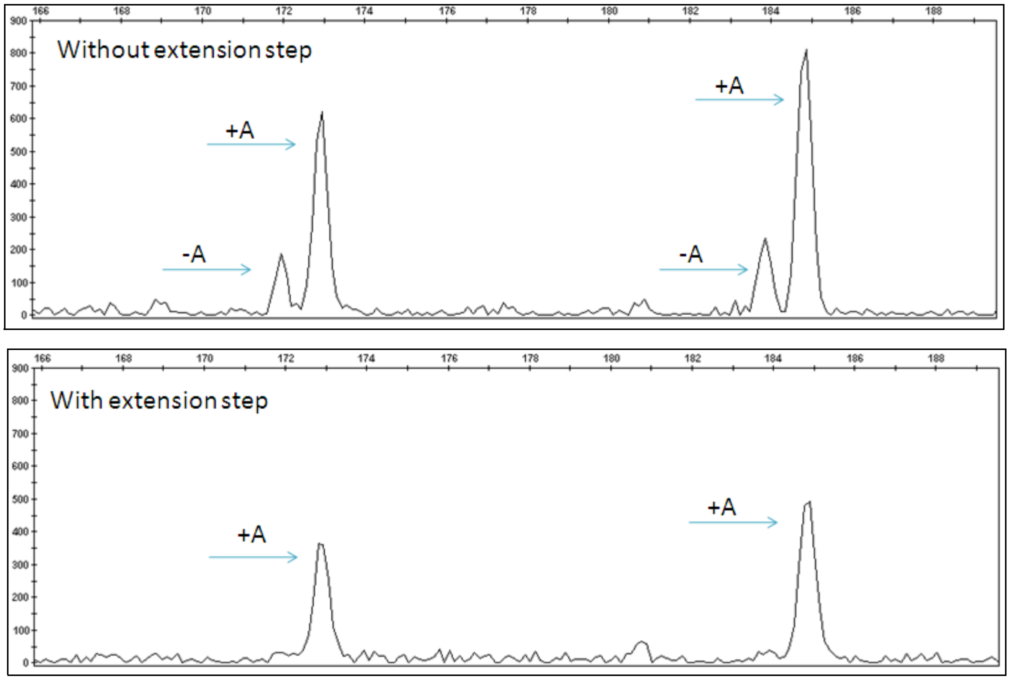
Addition of 3' A nucleotide by Taq polymerase

The AmpliTaq® and AmpliTaq Gold® DNA Polymerases, like many other DNA polymerases, catalyze the addition of a single nucleotide (usually an adenosine) to the 3' ends of the two strands of a double-stranded DNA fragment. This non-template complementary addition results in a denatured PCR product that is one nucleotide longer than the target sequence. A PCR product containing the extra nucleotide is referred to as the plus-A form.

Incomplete 3' A nucleotide addition

Because 3' A nucleotide addition rarely goes to completion without a long extension step at the end of thermal cycling (that is, only a fraction of the fragments receive the extra nucleotide), single-base ladders often form, creating peak patterns that analysis software might not interpret correctly (Figure 6). The resulting allele calls can be inconsistent, incorrect, or missing entirely.

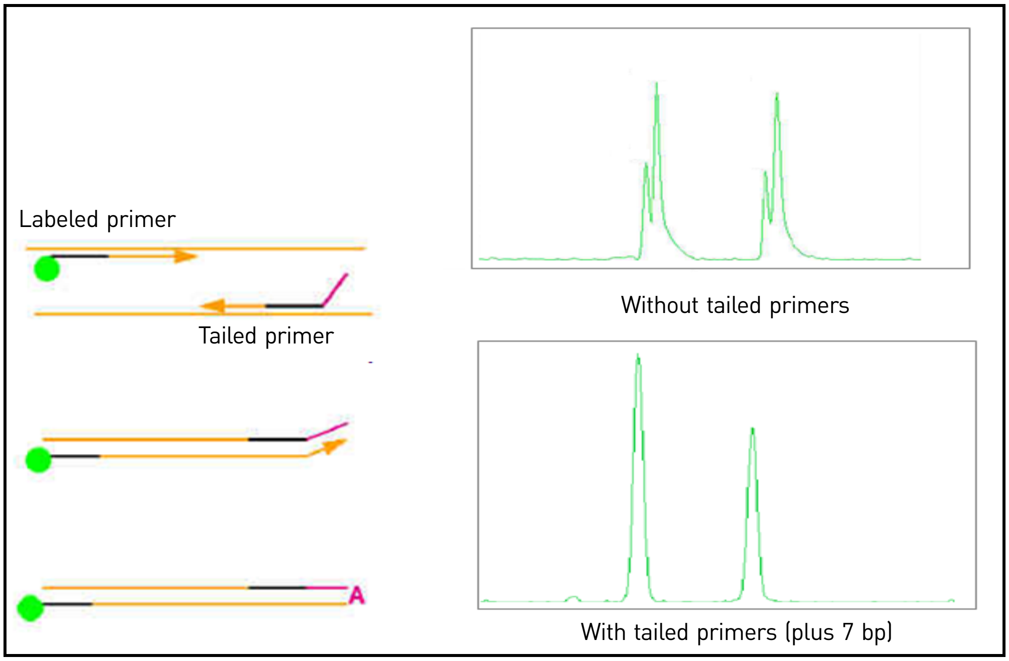
Figure 6 Split peaks resulting from incomplete 3' A nucleotide addition



Avoiding incomplete 3' A nucleotide addition

Modify	Considerations
Thermal cycling conditions	Increasing the time spent between 60 and 72°C promotes 3' A nucleotide addition. Decreasing the time spent between 60 and 72°C inhibits 3' A nucleotide addition. To use this method effectively, determine the optimal thermal cycling conditions for each marker in each set of reaction conditions. Promoting 3' A nucleotide addition has proven to be more successful than removing 3' A. Residual polymerase activity at room temperature (or even at 4°C) is often sufficient to catalyze enough 3' A nucleotide addition to create genotyping problems. Many protocols increase the final extension step to 30 to 45 minutes to promote 3' A nucleotide addition.
Magnesium ion concentration	Increasing the magnesium ion concentration promotes 3' A nucleotide addition. Decreasing the magnesium ion concentration inhibits 3' A nucleotide. In general, optimizing the magnesium ion concentration is best used in conjunction with other strategies. If you choose to maximize 3'A nucleotide addition, consider using AmpliTaq Gold® DNA Polymerase at 2.5 mM $MgCl_2$.
"Tail" the 5' end of the reverse primer	Brownstein *et al.* (1996) found that adding additional nucleotides (a "tail") to the 5' end of the reverse PCR primer either promoted or inhibited 3' A nucleotide addition to the (forward) labeled strand, depending on the sequence of the added nucleotides (Figure 7 on page 35). Magnuson *et al.* (1996) noticed a correlation between tail sequence and the amount of 3' A nucleotide addition. In particular, they found that adding a single G to the 5' end of the reverse PCR primer generally resulted in almost complete 3' A nucleotide addition. Therefore, using a tail to promote 3' A nucleotide addition can consistently yield a pattern that analysis software can identify. Reverse-primer tailing has advantages compared to other methods because it: • Works well under diverse reaction conditions • Does not require additional experimental steps Go to **www.lifetechnologies.com** for information on ordering tailed primers.

Figure 7 Tailed primers and 3' A nucleotide addition



Note: In general, the most reliable strategy is to promote 3' A nucleotide addition by modifying thermal cycling conditions and Mg_2^+ concentration, and (if necessary) by tailing the reverse primer.

Enzymatic treatment

Ginot *et al.* (1996) used T4 DNA polymerase to remove the 3' A overhangs from treatment-pooled PCR products.

Although effective, this method has serious limitations because it requires:

- A post-PCR enzymatic treatment step
- Titrating each lot of T4 DNA polymerase to determine optimal enzyme concentrations and treatment times

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IMPORTANT! Excess T4 treatment can cause PCR product degradation. Insufficient treatment will not remove the 3′ overhangs and can make some alleles more difficult to genotype.

Start optimization experiments with 0.5 to 1 unit of T4 DNA polymerase in 10 µL of pooled PCR product. Incubate at 37°C for 30 minutes.

Dyes

IMPORTANT! We recommend using only Thermo Fisher Scientific dyes. Thermo Fisher Scientific provides spectral calibration reagents that have been optimized for our dye sets.

Other dyes (or mixed isomers of dyes) have variable emission spectra and also require a spectral calibration generated for the specific dyes in use to correct for the spectral overlap between the dyes. You are responsible for obtaining the appropriate spectral calibration reagents and for optimizing custom dye sets.

Dyes and chemical forms

Thermo Fisher Scientific dyes are available in multiple chemical forms. Some forms are supplied coupled to primers and others you can use to label custom primers or fragments. Each form has distinct advantages and disadvantages depending upon the intended application and your laboratory setup.

You can analyze phosphoramidite-labeled fragments with NHS-ester-labeled fragments, but you should not combine [F]dNTP-labeled fragments with any other labeling method.

Table 5 Dye chemical forms

Chemical form	Purpose	Available dyes
NHS-esters	Post-synthesis 5'-end labeling of oligonucleotides containing a 5' Aminolink2	NED™[†], TAMRA™[‡], ROX™ SNaPshot® Kit dyes: dR110, dR6G, dTAMRA™[‡], dROX™
Phosphoramidite reagents	Preparing custom, 5'-end labeled primers directly on any Thermo Fisher Scientific DNA synthesizer[§]	6-FAM™, HEX™, TET™, NED™[†], VIC®[†], PET®[†]
[F]dNTPs	Simple internal fluorescent labeling of multiple nucleotides during PCR amplification	R6G, R110, ROX™, TAMRA™[‡]
Labeled primers in reagent kits	Microsatellite and human identification applications	5-FAM™[††], JOE™[††], 6-FAM™, HEX™, TET™, NED™[†], VIC®[†], PET®[†]
Labeled size standard	Generating the sizing curve to size unknown sample fragments	TAMRA™[‡], ROX™, LIZ®

[†] NED™, VIC®, and PET dye-labeled primers are available only in kits or through the Thermo Fisher Scientific Custom Oligo Service. Contact your Thermo Fisher Scientific representative or visit our website for information on how to order custom-labeled oligonucleotides.

[‡] Matrix standards for spectral calibration available for the 310 instrument only.

[§] For information about synthesizing labeled oligonucleotides, contact your Thermo Fisher Scientific representative.

[††] 5-FAM™ and JOE™ are available only as labeled primers in certain reagent kits.

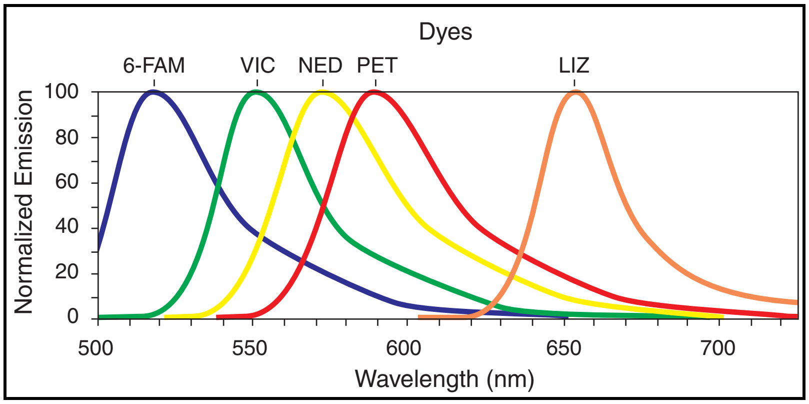
Multicomponent analysis with fluorescent dyes

Fluorescent-dye labeling enables you to analyze multiple independent markers (loci) in the same capillary injection by using different dye colors in addition to size to distinguish between markers.

During data collection on our genetic analyzers, the fluorescence signals are separated by diffraction grating according to wavelength and projected onto a CCD camera in a predictably spaced pattern.

Although each dye emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the dyes (Figure 8). To correct for spectral overlap, the software applies a multicomponent matrix. A multicomponent matrix is created when you perform a spectral calibration for a dye set using a matrix standard (for more information, see "Dye sets" on page 41 and "Understanding spectral calibration" on page 84).

Figure 8 Emission spectra of dyes



Factors that affect dye signal

Fluorescent dyes have the following characteristics:

- **Emission spectrum**: The intensity of emitted light (fluorescence) as a function of the wavelength of the emitted light.
- **Absorption (excitation) spectrum**: The intensity of emitted light as a function of the wavelength of the exciting light.
- **Absorption (excitation) efficiency**: A measure of the probability that a dye will absorb light of a certain wavelength, as a percentage of the probability of absorption at the wavelength of maximum absorption.
- **Quantum yield**: The probability that its excited state will emit a photon as it decays back to the ground state.

The ability of the instrument to detect a dye signal depends upon:

- The absorption efficiency of the dye at the wavelengths of light emitted by the laser
- The laser/light source
- The quantum yield of the dye
- The dye concentration

The emission and absorption wavelengths of a dye depend upon:

- The chemical structure of the dye
- The physical environment, including:
  - Buffer pH and concentration
  - Polymer composition
  - Whether the DNA it is attached to is single- or double-stranded

Although altered by the physical environment, the wavelengths of maximum emission and absorption for each dye always lie within a small wavelength range.

Emission and absorption (excitation) wavelengths and relative intensities

The maximum fluorescence absorption and emission wavelengths are listed below for Thermo Fisher Scientific NHS-esters, dye phosphoramidites, and [F]dNTP-based dyes. (The actual maximum absorption and emission wavelengths may differ from the listed values because of the influence of the physical environment upon the dye.)

The intensity of emitted fluorescence is different for each dye, and you must optimize sample concentration to account for differences in dye signal strength.

Examples:

- 6-FAM™dye emits a stronger signal than NED™dye. Therefore, to generate signals of equal intensity, you must load approximately three times as much NED™ dye-labeled fragments as 6-FAM™dye-labeled fragments.
- VIC® dye emits a stronger signal and is more stable than HEX™ dye. Use VIC® dye for weak amplicons.

Table 6 Dye Absorption max, emission max, and relative intensities

Dye	Absorption Max	Emission Max	Relative Intensity	Intensity (not to scale)
5-FAM™	494 nm	530 nm	100 RFU	●
6-FAM™	494 nm	522 nm	100 RFU	●
TET™ (310 only)	521 nm	538 nm	100 RFU	●
VIC®	538 nm	554 nm	100 RFU	●
JOE™	528 nm	554 nm	50 RFU	•
HEX™	535 nm	553 nm	50 RFU	•
LIZ®	638 nm	655 nm	50 RFU	•
NED™	546 nm	575 nm	40 RFU	•
TAMRA™	560 nm	583 nm	25 RFU	·
ROX™	587 nm	607 nm	25 RFU	·
PET®	558 nm	595 nm	25 RFU	·

Points to consider when selecting dyes for custom primers

When you order custom primers, you specify the dyes for labeling. Based on the dyes you specify, you must use the appropriate dye set to perform a spectral calibration (described in "Dye sets" on page 41).

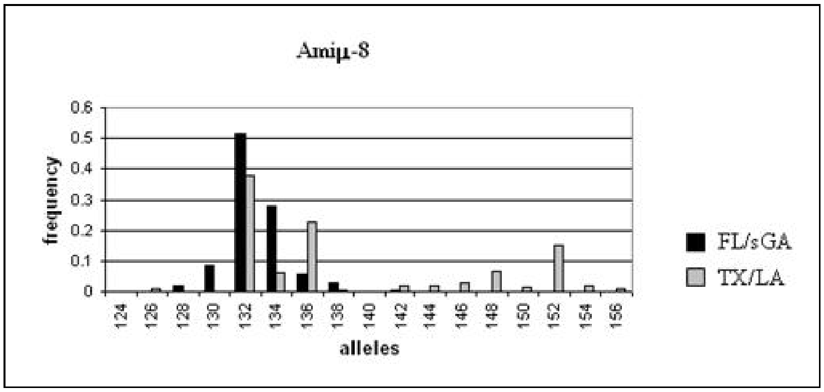
Consider the following when selecting dyes:

- One dye is needed for the size standard (red or orange).
- Using 5 dyes provides 33% greater throughput than using 4 dyes.
- Use the most intense dyes for PCR products with low recovery rate (from lower to higher intensity: Blue > Green > Yellow > Red) (see "Emission and absorption (excitation) wavelengths and relative intensities" on page 38).
- Use less-intense dyes for PCR product with good recovery rate.
- Select dyes with absorption maxima that are as far apart as possible to avoid overlap and for easier generation of matrix/spectral calibration (see "Emission and absorption (excitation) wavelengths and relative intensities" on page 38).
- Consider the relative dye intensities and sample concentration (see "Emission and absorption (excitation) wavelengths and relative intensities" on page 38).

Example: selecting dyes

The following figure shows the marker range (allele distribution) and allele frequencies for the alligator microsatellite locus Amiμ-8 for samples taken from Florida/South Georgia and Texas/Louisiana.

Figure 9 Allele distribution for alligator marker Amiμ-8 in two populations (124 to 156 bp)

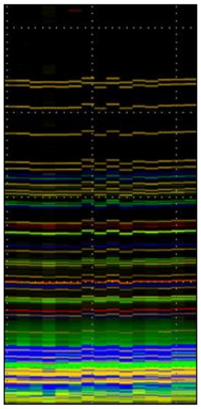


Using a hypothetical set of 10 markers as an example:

- For each marker, determine the expected allele distribution, either from published literature or from empirical testing.
- Determine the dyes that are appropriate for the range of each marker of interest.

- For this hypothetical set of markers, you might select 6-FAM™, VIC®, PET®, NED™ dyes, and LIZ® dye for the size standard (see the table below). This group of dyes corresponds to the G5 dye set, so you would also need the DS-33 matrix standard for spectral calibration (see Table 7 on page 41 for the matrix standard that corresponds to each dye set).

  Note that different dyes can be used for similar fragment lengths, and the same dye can be used for fragments of different lengths.

Locus	Marker Range	Dye	Capillary electrophoresis array view of example 10-marker panel
Marker 1	90–104	6-FAM™	
Marker 2	112–146	VIC®	
Marker 3	119–177	PET®	
Marker 4	117–202	NED™	
Marker 5	156–190	6-FAM™	
Marker 6	221–253	6-FAM™	
Marker 7	234–282	NED™	
Marker 8	260–342	VIC®	
Marker 9	311–327	6-FAM™	
Marker10	340–380	NED™	

Dye sets

Dye sets and matrix standards

A dye set corresponds to the group of dyes you select for labeling (described in the previous section). You use the matrix standard that corresponds to the dye set (shown below) to perform a spectral calibration. This calibration prepares the instrument for detection of the dyes with which your primers are labeled. For information on spectral calibration, see “Understanding spectral calibration” on page 84.

Table 7 Dye set and matrix standard components

	Dye Set (ROX™, LIZ®, and TAMRA™ dyes are reserved for the size standard)					
Dye Set	**E5**	**D**	**D**	**F**	**G5**	**C**[†]
Matrix standard	**DS-02**	**DS-30**[‡][§]	**DS-31**[§]	**DS-32**	**DS-33**[§]	**DS-34**
Blue	dR110	6-FAM™	6-FAM™	5-FAM™	6-FAM™	6-FAM™
Green	dR6G	HEX™	VIC®	JOE™	VIC®	TET™
Yellow	dTAMRA™	NED™	NED™	NED™	NED™	HEX™
Red	dROX™	ROX™	ROX™	ROX™	PET®	TAMRA™
Orange	LIZ®	—	—	—	LIZ®	—

[†] Used on 310 instruments only.

[‡] Can be used for custom-labeling primers.

[§] DS-30 versus DS-31: VIC® dye emits a stronger signal and is more stable than HEX™ dye. Use VIC® dye for weak amplicons.

The kits available from Thermo Fisher Scientific use the dye sets listed below.

Table 8 Dye sets and matrix standards for kits and genotyping applications

Application	**Dye Set**	**Matrix Standard**
SNaPshot® Primer Focus®, SNaPshot® Multiplex	E5	DS-02
Custom oligos	D	DS-30
Custom oligos, Plant and Microbial AFLP®, Bovine and Canine Stockmarks®	D	DS-31
Stockmarks®, AFLP®	F	DS-32
Equine Stockmarks®, custom oligos, AmpFℓSTR®	G5	DS-33

Creating a custom dye set

IMPORTANT! We recommend using only Thermo Fisher Scientific dyes. Thermo Fisher Scientific provides spectral calibration reagents that have been optimized for our dye sets.

Non-Thermo Fisher Scientific dyes (or mixed isomers of dyes) have variable emission spectra and also require a spectral calibration generated for the specific dyes in use to correct for the spectral overlap between the dyes. You are responsible for obtaining the appropriate spectral calibration reagents and for optimizing custom dye sets to ensure the dye labels do not affect PCR efficiency.

However, the 3500 Series, 3730 Series, and 3130 Series instruments do support custom dye sets. For information, see Chapter 4, “Optimizing Capillary Electrophoresis” on page 67.

Size standards

Functions of a size standard

Each unknown sample is mixed with size standard before electrophoresis and run together in the same capillary with the same conditions. Size standards perform two functions:

- **Allow sizing of sample peaks**. A size curve is generated for each sample. Because the sizes (in bp) of the size-standard peaks are known, the sizes of sample peaks are determined through a relative comparison of migration speeds during electrophoresis. The uniform spacing of size-standard fragments ensures precise sizing throughout the sizing range.

  IMPORTANT! Because the called size for a fragment can differ from its actual size, compare the allele calls instead of the fragment size.

  IMPORTANT! Use the same size standard, instruments, and instrument conditions for all samples in a study. Using different size standards, instruments, or instrument conditions may shift the sizing of DNA fragments.

  Precision, or reproducibility, is the measure of instrument ability to generate the same size consistently for a given fragment.

  For more information, see “Precise versus accurate sizing” on page 90, “Guidelines for consistent sizing” on page 90, and “How the GeneMapper® Software performs sizing” on page 98.

- **Correct for injection-to-injection variations** that result in differences when comparing the same DNA fragments from different capillaries, runs, and instruments.

  When comparing fragment size across injections, ensure that data is analyzed with the same sizing method and the same size-standard definition.

Size-standard peak intensity

For optimum performance, the signal intensity of the size-standard peaks should be lower than or equal to the signal intensity of the sample peaks. For more information, see “Balancing size-standard and sample-peak intensities” on page 77.

Selecting a GeneScan™ size standard

Select a size standard with at least two fragments smaller and larger than your unknown sample fragments, and with a dye that is compatible with the dyes used for labeling primers.

Expected marker length	LIZ® Size Standard, 5-dye chemistry				ROX™ Size Standard, 4-dye chemistry			
	GS120 LIZ®† (page 47)	GS500 LIZ®‡ (page 50)	GS600 LIZ®‡ (page 45)	GS1200 LIZ®‡ (page 47)	GS350 ROX™ (page 49)	GS400HD ROX™ (page 49)	GS500 ROX™‡ (page 50)	GS1000 ROX™ (page 51)
≤120 bp	✓	—	—	—	✓	—	—	—
≤400 bp	—	✓	✓	—	✓	✓	✓	—
≤500 bp	—	✓	✓	—	—	—	✓	✓
≤600 bp	—	—	✓	—	—	—	—	—
≤1000 bp	—	—	—	—	—	—	—	✓
≤1200 bp	—	—	—	✓	—	—	—	—

† Used with SNaPshot® Multiplex Kit.

‡ For denaturing and non-denaturing applications.

Peaks not used for sizing

Some size standards include peaks that are not used for sizing. These peaks are denoted with a "*" in the following figures. These peaks can be used as an indicator of precision within a run.

Preparing a size standard

1. Vortex to mix the contents of each size-standard tube thoroughly, then centrifuge briefly to collect the liquid at the bottom of the tube.
2. Optimize the ratio of sample-to-size standard and Hi-Di™ formamide using the values listed below as a starting point.

Components	3500 Series, 3730 Series, and 3130 Series instruments	310 instrument
Sample	0.5 µL per reaction	0.5 µL per reaction
Size standard	0.5 µL per reaction	0.5 µL per reaction
Hi-Di™ Formamide†	9.0 µL per reaction	11.0 µL per reaction

† Hi-Di™ Formamide (Part no. 4311320) is purchased separately from the size standard.

3. Create a master mix of the size standard and formamide.
4. Add samples and master mix to tubes or wells.
5. Heat the reaction mix for 3 to 5 minutes at 95°C. Immediately chill on ice for 2 to 3 minutes, then load samples.

IMPORTANT! After size standards are mixed with formamide, run immediately. Signal will decrease significantly if left at room temperature for >1 day or at 2 to 8°C for >5 days. Plates can be stored at –20°C for up to 1 week.

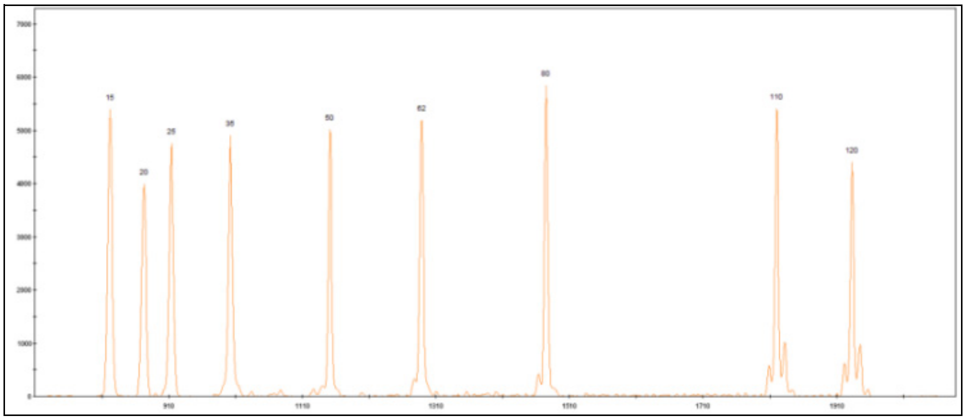
GeneScan™ 120 LIZ® Size Standard

Range: 15 to 120 bp under denaturing conditions

GeneScan™ 120 LIZ® denatured fragment lengths (nt): 9 fragments		
15	35	80
20	50	110
25	62	120

This single-stranded size standard was designed to provide accurate sizing of short DNA fragments. Therefore, it is particularly useful for SNP analysis. All fragments have been optimized under a wide variety of run conditions.

Figure 10 GeneScan™ 120 Size Standard run under denaturing conditions



GeneScan™ 500 LIZ® Size Standard

Range: 35 to 500 bp under denaturing conditions

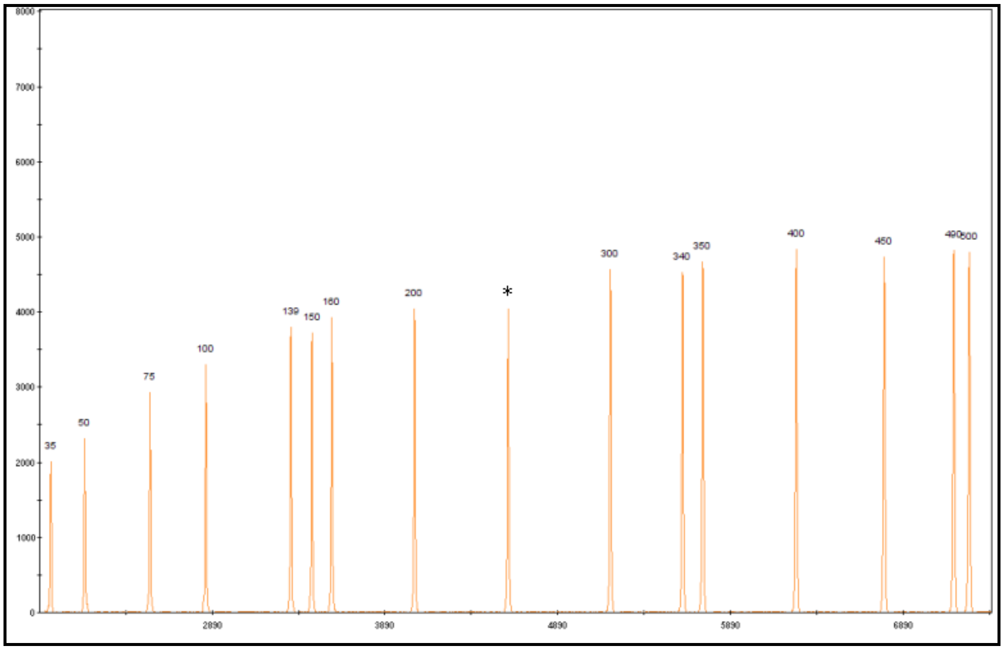
This size standard is recommended for analysis of tri- and tetranucleotide microsatellite loci, which can often exceed 400 bp in length.

GeneScan™ 500 LIZ® denatured fragment lengths (nt): 16 fragments			
35	139	250†	400
50	150	300	450
75	160	340	490
100	200	350	500

† Do not use this fragment for sizing. See "Peaks not used for sizing" on page 43 for information.

Only one strand of the double-stranded DNA fragments in this size standard is labeled. The unlabeled strand does not interfere with peak detection of the labeled strand when run under denaturing conditions.

Figure 11 GeneScan™ 500 LIZ® Size Standard run under denaturing conditions



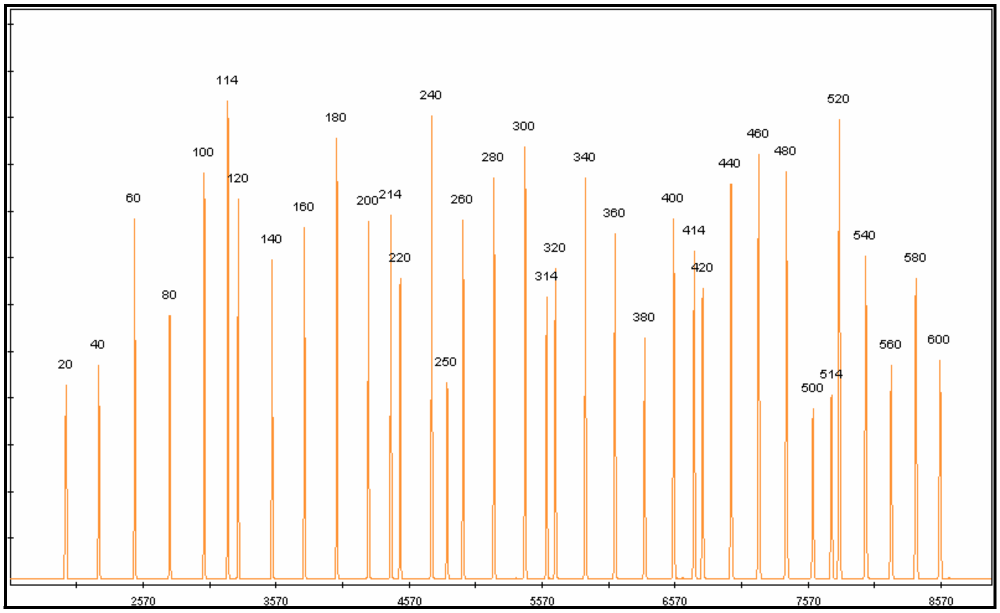
GeneScan™ 600 LIZ® and GeneScan™ 600 LIZ® v2.0 Size Standards

Note: The GeneScan™ 600 LIZ® and GeneScan™ 600 LIZ® v2.0 Size Standards contain the same peaks. The GeneScan™ 600 LIZ® v2.0 Size Standard can be used for normalization on 3500 Series instruments.

Range: 20 to 600 bp under denaturing conditions

GeneScan™ 600 LIZ® denatured fragment lengths (nt): 36 fragments					
20	120	220	314	414	514
40	140	240	320	420	520
60	160	250	340	440	540
80	180	260	360	460	560
100	200	280	380	480	580
114	214	300	400	500	600

Figure 12 GeneScan™ 600 LIZ® Size Standard fragments run under denaturing conditions



Optimizing the 3130 Series instrument run module

The run modules provided with these instruments may need to be optimized for use with the GeneScan™ 600 LIZ® Size Standard. Add 100 seconds to the run time if needed.

Optimizing the 310 instrument run module

The run modules provided with these instruments have not been optimized for use with the GeneScan™ 600 LIZ® Size Standard. Add 200 seconds to the run time before using this size standard.

GeneScan™ 1200 LIZ® Size Standard

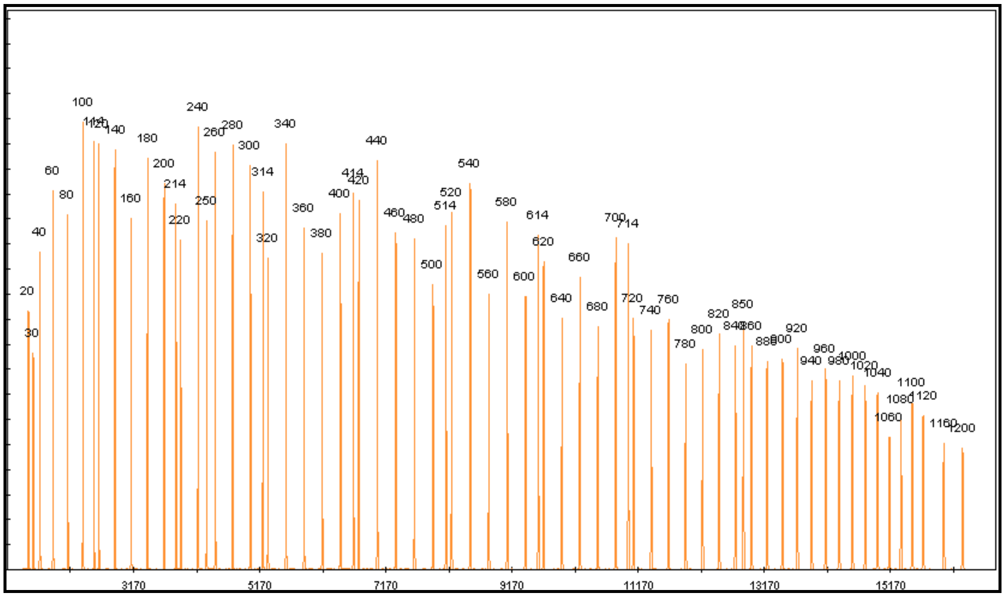
Range: 20 to 1200 bp under denaturing conditions

GeneScan™ 1200 LIZ® Size Standard denatured fragment lengths (nt): 68 fragments			
20	280	560	850
30	300	580	860
40	314	600	880
60	320	614	900
80	340	620	920
100	360	640	940
114	380	660	960
120	400	680	980
140	414	700	1000
160	420	714	1020
180	440	720	1040
200	460	740	1060
214	480	760	1080
220	500	780	1100
240	514	800	1120
250	520	820	1160
260	540	840	1200

The high fragment density (68 fragments) yields greater sizing precision, and landmark fragments allow easy peak-pattern identification during data analysis.

This size standard is ideal for BAC fingerprinting, T-RFLP, VNTR, STR, and many other DNA fragment analysis applications.

Figure 13 GeneScan™ 1200 LIZ® Size Standard run under denaturing conditions



Downloading 3130 instrument run modules from our website

Updated run modules for the 3130 Series instrument and the GeneScan™ 1200 LIZ® Size Standard are available on our website. Before using the downloaded run modules, adjust as described below. Further optimization may be necessary.

36-cm array with POP-4® polymer	50-cm array with POP-4® polymer
• Decrease run voltage to 8000 volts • Increase run time to 6000 seconds	• Decrease run voltage to 12,000 volts • Increase run time to 6000 seconds

Downloading 3730 instrument run modules from our website

Updated run modules for the 3730 Series instrument and the GeneScan™ 1200 LIZ® Size Standard are available on our website. Before using the downloaded run modules, adjust as described below. Further optimization may be necessary.

50-cm array
• Decrease run voltage to 8000 volts • Increase run time to 6200 seconds

GeneScan™ 350 ROX™ Size Standard

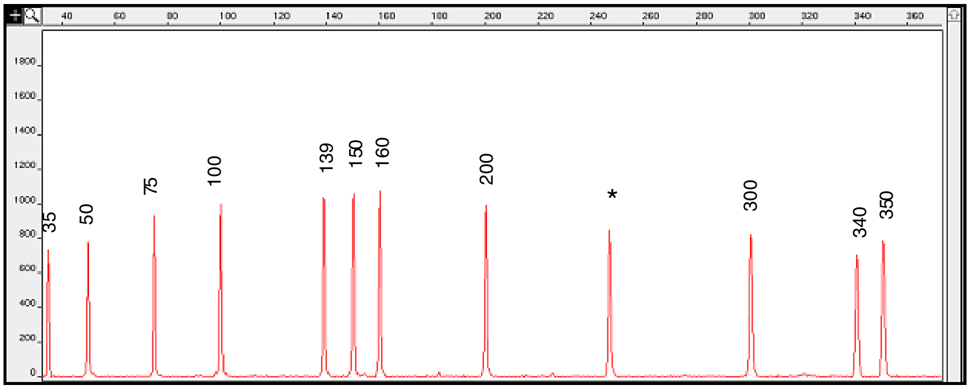
Range: 35 to 350 bp under denaturing conditions

GeneScan™ ROX™ 350 denatured fragment lengths (nt): 12 fragments		
35	139	250†
50	150	300
75	160	340
100	200	350

† Do not use this fragment for sizing. See "Peaks not used for sizing" on page 43 for information.

Only one strand of the double-stranded DNA fragments in this size standard is labeled. The unlabeled strand does not interfere with peak detection of the labeled strand when run under denaturing conditions.

Figure 14 GeneScan™ 350 Size Standard run under denaturing conditions



GeneScan™ 400HD ROX™ Size Standard

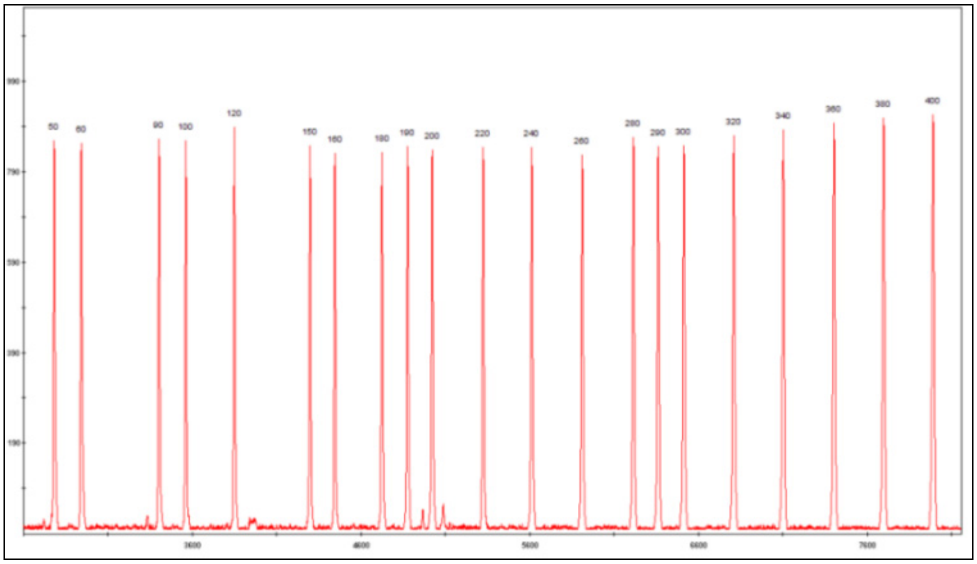
This size standard uses ROX™ dye. The high density of marker bands in this standard makes it particularly useful for microsatellite analysis.

Range: 50 to 400 bp under denaturing conditions

GeneScan™ ROX™ 400HD denatured fragment lengths (nt): 21 fragments			
50	160	260	360
60	180	280	380
90	190	290	400
100	200	300	—
120	220	320	—
150	240	340	—

Only one strand of the double-stranded DNA fragments in this size standard is labeled. The unlabeled strand does not interfere with peak detection of the labeled strand.

Figure 15 GeneScan™ 400HD Size Standard run under denaturing conditions



GeneScan™ 500 ROX™ Size Standard

Range: 35 to 500 bp under denaturing conditions

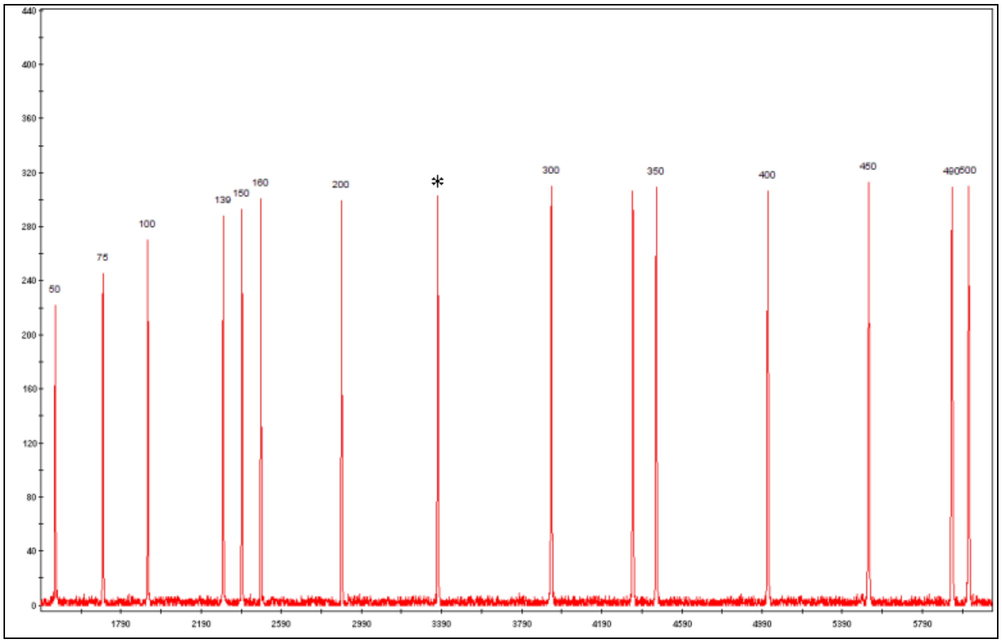
This size standard is recommended for analysis of tri- and tetranucleotide microsatellite loci, which can often exceed 400 bp in length.

GeneScan™ 500 ROX™ denatured fragment lengths (nt): 16 fragments			
35	139	250†	400
50	150	300	450
75	160	340	490
100	200	350	500

† Do not use this fragment for sizing. See "Peaks not used for sizing" on page 43 for information.

Only one strand of the double-stranded DNA fragments in this size standard is labeled. The unlabeled strand does not interfere with peak detection of the labeled strand when run under denaturing conditions.

Figure 16 GeneScan™ 500 ROX™ Size Standard run under denaturing conditions



GeneScan™ 1000 ROX™ Size Standard

Range:

- 100 to 900 bp under non-denaturing conditions
- 100 to 539 bp under denaturing conditions (verified with POP-4® polymer only)

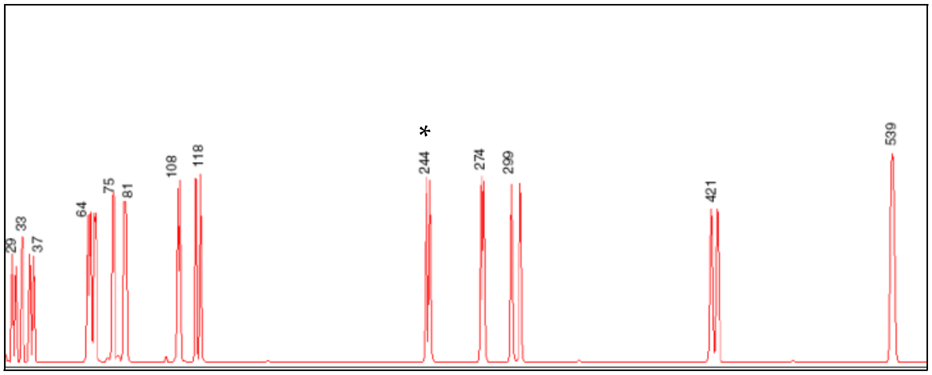
GeneScan™ 1000 ROX™ non-denatured fragment lengths[†] (nt): 17 fragments			
47	93	292	695
51	99	317	946
55	126	439	—
82	136	557	—
85	262[‡]	692[‡]	—

[†] If run under denaturing conditions (Figure 17 on page 52), fragments run 18 nucleotides shorter than the lengths listed above and some or all of the peaks appear split.

[‡] Do not use this fragment for sizing. See "Peaks not used for sizing" on page 43 for information.

Both strands of the GeneScan™ 1000 ROX™ Size Standard fragments are labeled and are used for non-denaturing applications.

Figure 17 GeneScan™ 1000 ROX™ Size Standard run under denaturing conditions. Fragments run 18 nt shorter than lengths obtained under non-denaturing conditions (see the table on the previous page). Under denaturing conditions, sizing is not accurate above 539 nt, therefore the and 674-nt (corresponds to the 692-nt) peak is not shown in the figure.



Note: Under denaturing conditions, the two strands of this doubly labeled size-standard migrate at different rates, appearing as split peaks. To ensure sizing precision and a reliable size-standard definition, you must define one peak from each split peak pair in the size-standard definition.

Ordering custom primers

You can obtain custom 5'-end labeled primers from the Thermo Fisher Scientific Custom Oligo Service. For information, see our website.

Order labeled and unlabeled primer pairs for the markers of interest.

Testing the primers and optimizing conditions with test DNA panel

Testing

Before using primers in an analysis, test the primers and optimize sample preparation, PCR, and electrophoresis conditions.

Create a panel of test DNA samples to ensure that expected alleles are detected for each marker. Use DNA samples that are representative of your overall study to capture as much allelic variation as possible. CEPH Individual 1347-02 Control DNA is available from Thermo Fisher Scientific and can be used in your test DNA panel.

Test DNA panel guidelines:

- Include 8 to 16 samples
- Use samples of good quality that are well-quantified
- Use equal concentrations of DNA samples

Note: If optimization of signal intensity is necessary for a given sample, inject the sample multiple times using a range of injection parameters.

Order unlabeled primers for the markers of interest and optimize amplification conditions on your DNA test panel. You may need to optimize a variety of parameters including annealing temperature, and variables such as magnesium concentration and primer concentration to ensure that the primers work under universal conditions. Bands are visualized on agarose gels with ethidium bromide staining.

Optimizing conditions

The intensity of emitted fluorescence is different for each dye, and you must optimize sample concentration to account for differences in dye-signal strength. For example, to generate signals of equal intensity, you must load approximately three times as much NED™ dye-labeled fragments as 6-FAM™dye-labeled fragments.

For more information, see:

- Chapter 3, "Optimizing PCR" on page 55
- Chapter 4, "Optimizing Capillary Electrophoresis" on page 67

3

Optimizing PCR

This chapter contains general information for PCR. For application-specific information on PCR, see the application chapters later in this guide.

This chapter covers:

- Safety information 55
- Isolating, purifying, quantifying, and storing DNA 55
- Handling primers 57
- Using control DNA. 57
- Reaction volumes and plate types 58
- Reagent concentrations 59
- Preventing competing side reactions: hot-start PCR. 60
- Thermal cycling parameters Veriti® Thermal Cyclers 60
- Thermal cycling parameters 9700 Thermal Cyclers 62
- Optimizing thermal cycling parameters 63
- Avoiding contamination 64

Safety information

IMPORTANT! For every chemical, read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Note: For the SDSs of chemicals not distributed by Thermo Fisher Scientific, contact the chemical manufacturer.

Isolating, purifying, quantifying, and storing DNA

Isolating DNA

DNA isolation methods depend on your starting DNA source. Refer to guidelines for your application for information on isolating DNA.

IMPORTANT! **DO NOT FREEZE BLOOD SAMPLES** before DNA isolation. Freezing can lyse red blood cells, and increase the concentration of PCR inhibitors in DNA samples.

Purifying DNA

The quality, accuracy, and amplified length of a DNA fragment can be significantly affected by characteristics of the sample itself and the method used for purification.

IMPORTANT! The success of AFLP® analysis is particularly dependent upon the quality of DNA.

Select a method based on the sample source or tissue type, how it was obtained from its source, and how it was handled or stored before purification. Go to **www.lifetechnologies.com** for the latest information on DNA purification.

Quantifying DNA

For PCR with custom primers, optimize DNA concentration for your application. Concentration may range from 10 to 100 ng of purified DNA per reaction.

It is almost always necessary to dilute PCR amplification products before adding them to the sample tube. Typically, the required dilution is 1:3 to 1:80 (PCR product-to-distilled, deionized water or Hi-Di™ Formamide).

Begin by optimizing PCR run conditions for your specific application. Then run a dilution series on your instrument to determine the optimal dilution. Alternatively, run 1 µL of PCR product on a mini-gel. If, after ethidium bromide staining, the product signal is visible but not oversaturated, try a 1:10 dilution.

After determining the optimal dilution ratio, you can use the same dilutions for subsequent analyses because PCR yields should be fairly consistent. Any changes to the PCR conditions or the primer design may require different dilutions.

Note: Different dyes emit different fluorescent intensities. Therefore, PCR product concentrations may need optimization depending on relative fluorescence intensity during electrophoresis. See “Emission and absorption (excitation) wavelengths and relative intensities” on page 38.

Storing prepared DNA before or after PCR

Store the prepared samples at –20 °C to 4 °C until you perform capillary electrophoresis.

Handling primers

Reconstituting and diluting primers

Primers are commonly shipped in a lyophilized state. The units of a lyophilized primer are given as a mass, in picomoles.

To create a stock of primers or probe, reconstitute the primer or probe in sterile 1✕ TE buffer (1mM Tris, 0.1mM EDTA, pH 8.0) or sterile, nuclease-free water.

Quantifying primers

Measure the primer quantity with a spectrophotometer using a primer-specific absorption coefficient.

Storing primers

- –20°C to –80°C for stock solution (undiluted, keep concentration as high as possible)
- +4°C for working solution, diluted appropriately (up to one month)

Using control DNA

Purpose of control DNA

- Serves as a positive control for troubleshooting PCR amplification

  Control DNA allows you to distinguish between problems with the sample DNA (the control DNA amplifies but samples do not) and problems with reagents, thermal cyclers, or protocols (the control DNA does not amplify).
- Allows you to monitor sizing precision

  Because the control DNA is not used to calculate the sizing curve, you can use the sizes obtained during different capillary injections to verify that sizing precision (reproducibility) is within acceptable limits.
- Allows you to correlate the fragment sizes that are obtained in different runs or on different instruments.

Guidelines for use

- Amplify at least one control DNA sample in every PCR run.
- Include at least one injection of amplified control DNA during every series of capillary runs. Use one control injection for every variation in the electrophoresis parameters.

CEPH 1347-02 Control DNA

CEPH Individual 1347-02 Control DNA is available for human studies from Thermo Fisher Scientific (Part no. 403062).

Reaction volumes and plate types

Reaction volumes

Reaction volumes for Thermo Fisher Scientific PCR thermal cyclers are 5 to 100 µL.

Using small amounts of template

Although reaction tubes usually do not need to be sterilized or siliconized, use autoclaved tubes when amplifying with quantities (approximately 150 to 500 pg) of starting DNA template.

Autoclaved PCR tubes are available from Thermo Fisher Scientific (see "Thermal cyclers and accessories" on page 193).

Plate types

Table 9 Reaction plates for each thermal cycler

Thermal Cycler	Block Format	Reaction Plate Type	Networking capability	Uses
Veriti® 96-Well Thermal Cycler	0.1 mL or 0.2 mL Alloy VeriFlex™ Blocks	Standard 0.2 mL and Fast 0.1 mL 96-well formats	Yes	10 to 80 µL medium/high throughput. VeriFlex Blocks™ provide "better than gradient" PCR optimization.
Veriti® 384-Well Thermal Cycler	0.02 mL aluminum single block	384-well plate	Yes	5 to 20 µL high throughput, small sample volume.
Dual 96-Well GeneAmp® PCR System 9700	2 aluminum 0.2 mL 96-well blocks	96-well, 0.2 mL format	No	10 to 100 µL high throughput, small sample volume.
Dual 384-Well GeneAmp® PCR System 9700	2 aluminum 0.02 mL 384-well blocks	384-well, 0.02 mL format	No	5 to 20 µL high throughput, small sample volume.
Auto-Lid Dual 384- Well GeneAmp® PCR System 9700	2 aluminum 0.02 mL 384-well blocks	384-well plate	No	5 to 20 µL high throughput, small sample volume with robotic capability.
2720 Thermal Cycler	0.2 mL aluminum single block	96-well, 0.2 mL format	No	Ideal for basic PCR using 0.2 mL reaction tubes or 96-well reaction plates.

Reagent concentrations

The following factors can affect overall yield of specific DNA target sequences:

- dNTP concentration
- Magnesium ion concentration
- Primer concentration
- Template concentration
- Enzyme concentration

dNTP concentration

In the standard GeneAmp® PCR protocol, the concentration of each deoxynucleoside triphosphate (dNTP) is 200 µM.

In most cases, lower dNTP concentrations do not significantly affect the yield of PCR amplification product and will increase the fidelity of the PCR amplification product. However, for efficient base incorporation, keep the four dNTP concentrations balanced and above the estimated K_m of each dNTP (10 to 15 µM).

Some applications might require higher dNTP concentration (especially when dNTP analogues are used). However, excess dNTPs decrease enzyme fidelity.

Magnesium ion

DNA polymerases require free magnesium ion in solution for activity. For most PCR amplifications, you can relate product yield and specificity as well as enzyme fidelity to the free magnesium ion (Mg^{2+}) concentration:

$[\text{free } Mg^{2+}] = [\text{total } Mg^{2+}] - [\text{total dNTP}] - 2[\text{EDTA}]$

In general, an increase in free magnesium concentration leads to an increase in product yield but a decrease in specificity and fidelity. To identify the magnesium concentration that gives the best compromise between yield and specificity, in the presence of 800 µM total dNTP concentration, run a $MgCI_2$ reaction series in 50-µM increments over the range from 100 to 400 µM $MgCI_2$.

Template concentration

The concentration of template in a sample can affect the success of PCR amplification. Too much template promotes non-specific binding of primers to secondary sites or changes the pH of the reaction mix. Too little template can result in poor yields, especially if the template is degraded.

Even very low template concentrations (10 copies) are often sufficient for successful PCR amplification.

If the starting sample is DNA, you can use up to 20,000 copies of the target to start optimization experiments. In general, this translates to:

- 1 to 5 ng of cloned template
- 200 pg to 1 ng of genomic DNA

Start optimization experiments with less genomic DNA if starting sample is limited. With clean, good-quality genomic DNA, 500 to 1000 pg of starting sample is typically sufficient.

Enzyme concentration

For most PCR applications, 2.0 to 2.5 units of AmpliTaq Gold® DNA Polymerase is recommended for each 100-µL reaction volume.

Note: To avoid the inaccuracies involved in pipetting 0.5-µL amounts of enzyme into each reaction, prepare a fresh master mix of reagents and add the enzyme.

Preventing competing side reactions: hot-start PCR

When to use

Consider using the hot-start technique whenever you need to improve the specificity and sensitivity of your PCR amplifications. Loss of specificity and sensitivity are often caused by competing side reactions, which usually occur during the pre-PCR setup period. (A common competing side reaction involves the amplification of non-target sequences in background DNA due to mispriming or to primer oligomerization.)

Limitations and alternatives

The hot-start technique is cumbersome. If you have high-throughput needs, switching to AmpliTaq Gold® DNA Polymerase will give the same benefits as performing the hot-start technique, without the need for using wax barriers or opening reaction tubes. If you are already using AmpliTaq Gold® DNA Polymerase, performing the hot-start technique will not improve the specificity and sensitivity of PCR amplification.

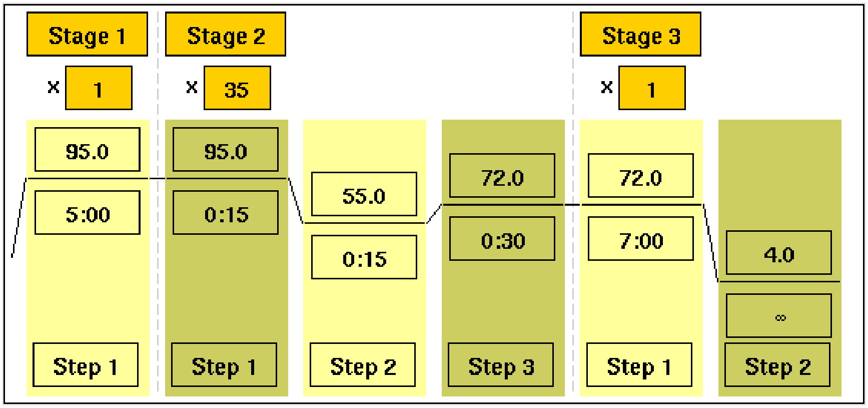
Components necessary for amplification must be kept separately so that critical reactants do not mix until reaching a temperature sufficiently high to suppress primer self-annealing or annealing to non-target sequences.

Note: Although manual hot-start PCR can increase specificity and yield, it is inconvenient and you can encounter reproducibility and contamination problems.

Thermal cycling parameters Veriti® Thermal Cyclers

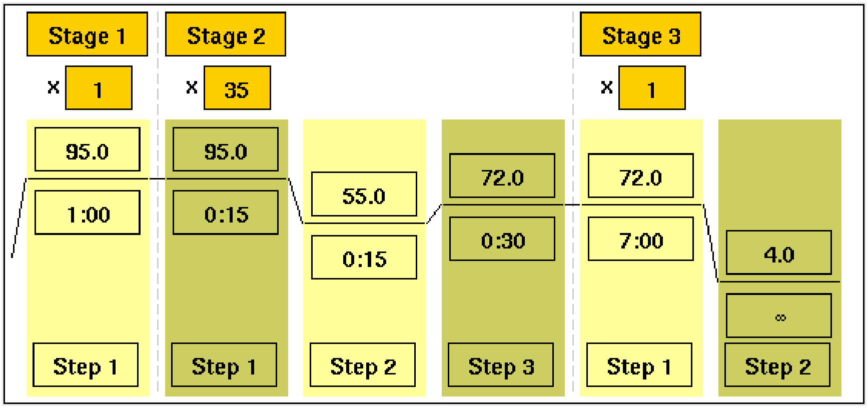
AmpliTaq_Gold

Use this profile for hot-start PCR in place of labor-intensive methods such as manual hot-start or wax-bead-mediated hot-start techniques. The Hot-start technique helps to minimize the formation of primer-dimers or non-specific products, thereby increasing specificity and sensitivity of PCR. This profile specifies a pre-PCR heat step for activation of AmpliTaq Gold® DNA Polymerase.



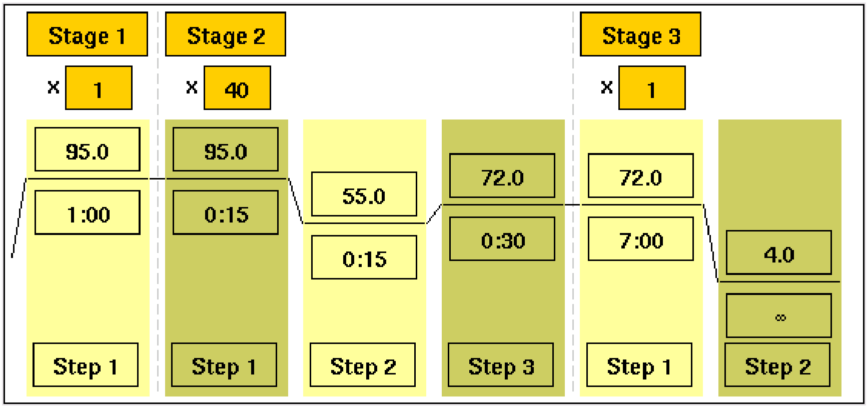
General PCR

Use this profile for standard PCR.


Stage 1
× 1
95.0
1:00
Step 1
Stage 2
× 35
95.0
0:15
Step 1
55.0
0:15
Step 2
72.0
0:30
Step 3
Stage 3
× 1
72.0
7:00
Step 1
4.0
∞
Step 2

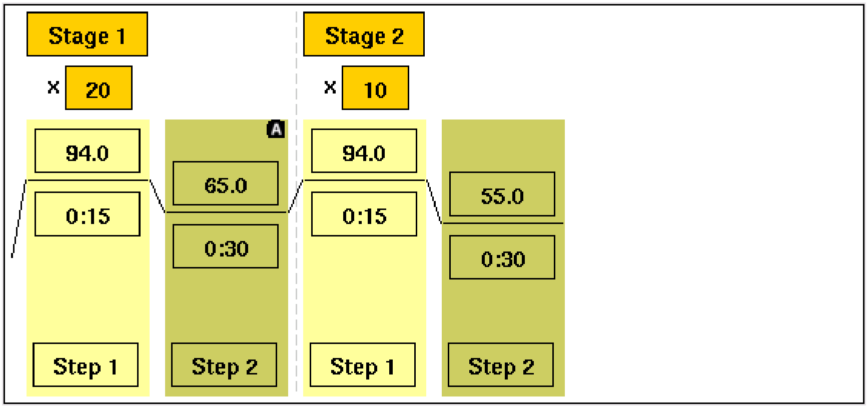
Time-release PCR

Use this profile with AmpliTaq Gold® DNA Polymerase. This method minimizes the pre-PCR activation step and adds a minimum of 10 additional cycles, allowing for slow activation of the enzyme during cycling. This provides a simple method where polymerase activity increases more slowly as product accumulates, improving specificity.


Stage 1
× 1
95.0
1:00
Step 1
Stage 2
× 40
95.0
0:15
Step 1
55.0
0:15
Step 2
72.0
0:30
Step 3
Stage 3
× 1
72.0
7:00
Step 1
4.0
∞
Step 2

Touchdown PCR

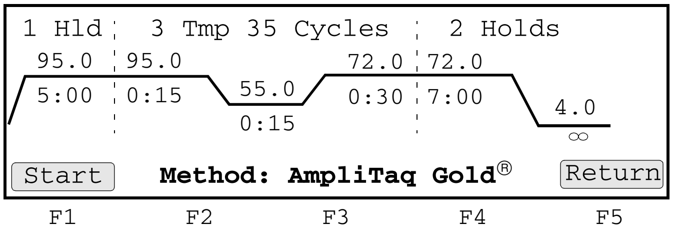
Use this profile if the optimal annealing temperature is not known. This method incrementally decreases the annealing temperature in early cycles to maximize the yield of specific products.


Stage 1
× 20
94.0
0:15
Step 1
A
65.0
0:30
Step 2
Stage 2
× 10
94.0
0:15
Step 1
55.0
0:30
Step 2

Thermal cycling parameters 9700 Thermal Cyclers

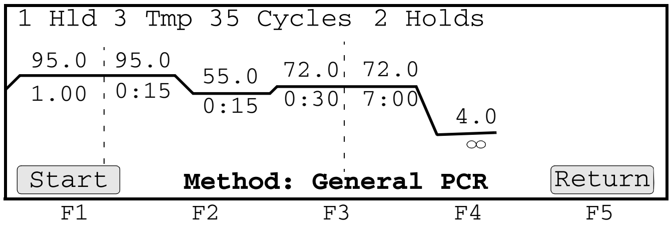
AmpliTaq_Gold

Use this profile for hot-start PCR in place of labor-intensive methods such as manual hot-start or wax-bead-mediated hot-start techniques. The hot-start technique helps to minimize the formation of primer-dimers or non-specific products, thereby increasing specificity and sensitivity of PCR. This profile specifies a pre-PCR heat step for activation of AmpliTaq Gold® DNA Polymerase.


1 Hld
3 Tmp 35 Cycles
2 Holds
95.0
5:00
95.0
0:15
55.0
0:15
72.0
0:30
72.0
7:00
4.0
∞
Start
Method: AmpliTaq Gold®
Return
F1
F2
F3
F4
F5

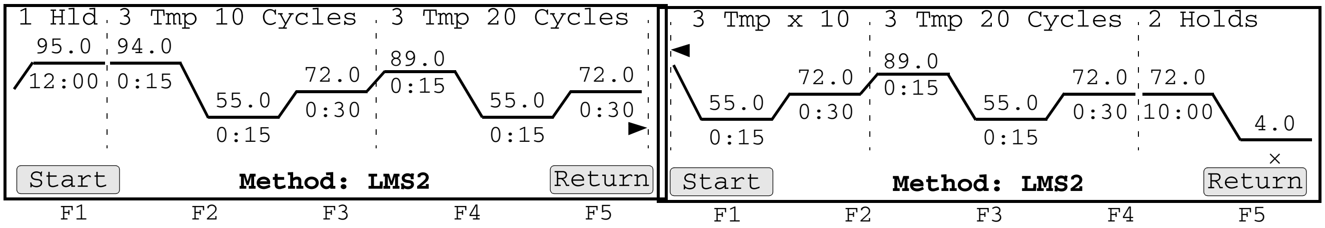
General PCR

Use this profile for standard PCR.


1 Hld 3 Tmp 35 Cycles 2 Holds
95.0
1.00
95.0
0:15
55.0
0:15
72.0
0:30
72.0
7:00
4.0
∞
Start
Method: General PCR
Return
F1
F2
F3
F4
F5

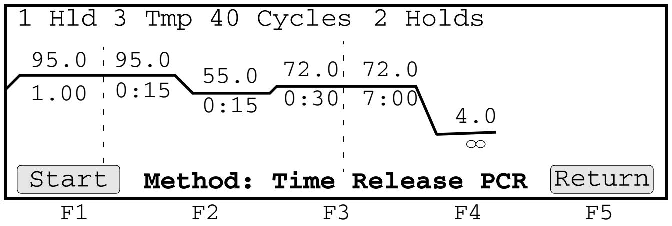
LMS2

Use this profile with Linkage Mapping Set primers. Linkage Mapping Set primers are for analysis of select microsatellite loci from the Généthon human linkage map.


1 Hld
3 Tmp 10 Cycles
3 Tmp 20 Cycles
95.0
12:00
94.0
0:15
55.0
0:15
72.0
0:30
89.0
0:15
55.0
0:15
72.0
0:30
Start
Method: LMS2
Return
F1
F2
F3
F4
F5
3 Tmp x 10
3 Tmp 20 Cycles
2 Holds
55.0
0:15
72.0
0:30
89.0
0:15
55.0
0:15
72.0
0:30
72.0
10:00
4.0
×
Start
Method: LMS2
Return
F1
F2
F3
F4
F5

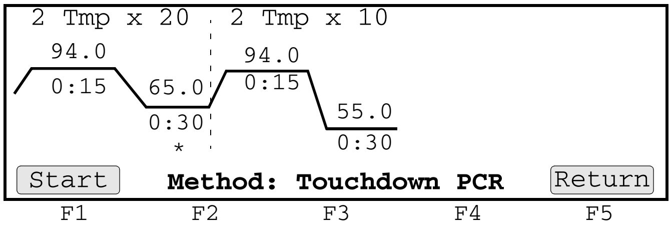
Time Release PCR

Use this profile with AmpliTaq Gold® DNA Polymerase. This method minimizes the pre-PCR activation step and adds a minimum of 10 additional cycles, allowing for slow activation of the enzyme during cycling. This provides a simple method where polymerase activity increases more slowly as product accumulates, improving specificity.


1 Hld 3 Tmp 40 Cycles 2 Holds
95.0
1.00
95.0
0:15
55.0
0:15
72.0
0:30
72.0
7:00
4.0
∞
Start
Method: Time Release PCR
Return
F1
F2
F3
F4
F5

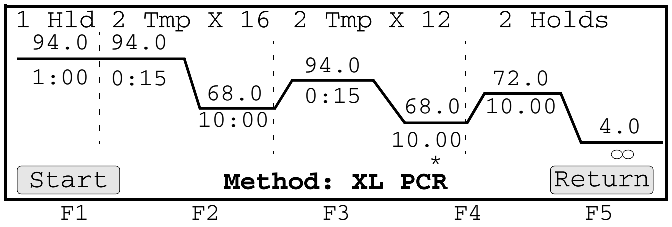
Touchdown PCR

Use this profile if the optimal annealing temperature is not known. This method incrementally decreases the annealing temperature in early cycles to maximize the yield of specific products.


2 Tmp x 20
2 Tmp x 10
94.0
0:15
65.0
0:30
*
94.0
0:15
55.0
0:30
Start
Method: Touchdown PCR
Return
F1
F2
F3
F4
F5

XL PCR

Use this profile for amplification of 5 to 40 kb PCR products using *rTth* DNA Polymerase, XL and unique reaction conditions. By providing longer templates, XL PCR complements technologies for rapid, long-range PCR. More complete genes can be amplified in one reaction from known expressed sequences, allowing more introns to be spanned. You can use XL PCR to amplify the control target (a 20.8-kb product from Lambda DNA) supplied in the kit.


1 Hld
2 Tmp X 16
2 Tmp X 12
2 Holds
94.0
1:00
94.0
0:15
68.0
10:00
94.0
0:15
68.0
10.00
*
72.0
10.00
4.0
∞
Start
Method: XL PCR
Return
F1
F2
F3
F4
F5

Optimizing thermal cycling parameters

Optimizing temperature

Six independent temperature blocks are available for the Veriti® Thermal Cycler. Each block provides precise control over thermal cycling parameter optimization. For information, refer to our website.

To find the optimal thermal cycling parameters, perform a series of runs varying the annealing or denaturation temperatures in 2°C increments.

Note: Do not vary more than one parameter at a time.

Annealing temperature change	Positive effects	Negative effects
Decreased	Increased PCR product yield	Increased amplification of non-specific products (background)
Increased	Increased PCR specificity	Reduced PCR yield

Guidelines

The following table summarizes the effects of modifying temperature control parameters on PCR performance.

Change in thermal cycling parameter	Effect on PCR performance
Increase denaturation temperatures (up to 96°C)	• Can be necessary to allow denaturation, especially with G+C-rich templates • Can also cause template degradation by depurination
Decrease annealing temperatures	Can increase yield, but can reduce specificity
Increase annealing temperatures	Increases specificity, but can reduce yield
Set the denaturation, annealing, and extension step to at least: • 15 seconds (preferably 30 seconds) with the GeneAmp® PCR System 9700 • 45 seconds using thin-walled tubes with the DNA Thermal Cycler	Allows samples to reach thermal equilibrium at each stage
Use the autoextension (or AutoX) function of a thermal cycler to allow longer extension times in later cycles†	Increases yield by allowing complete extension of PCR product in later cycles

† For most applications, an extension temperature of 72°C is effective and rarely requires optimization. In the two-temperature PCR process, the combined annealing/extension step temperature should range from 60 to 70°C.

Avoiding contamination

PCR protocols are extremely sensitive to contaminants in the DNA. Although many protocols describe "simple" or "fast" extraction or purification methods, carefully evaluate any changes or improvements in extraction or purification methods. (Also, be sure that the physical and chemical condition of the sample itself are adequate for the intended labeling and assay methods.)

PCR setup work area

IMPORTANT! These items should never leave the PCR Setup Work Area.

- Calculator
- Gloves, disposable
- Marker pen, permanent
- Microcentrifuge
- Microcentrifuge tubes, 1.5-mL, or 2.0-mL, or other appropriate clean tube (for Master Mix preparation)
- Microcentrifuge tube rack
- Pipette tips, sterile, disposable hydrophobic filter-plugged
- Pipettors
- Tube decapper, autoclavable
- Vortex

Amplified DNA work area

IMPORTANT! Place the thermal cyclers in the Amplified DNA Work Area.

You can use the following systems:

- Veriti® 96-Well Thermal Cycler
- GeneAmp® PCR System 9700 with the Silver 96-Well Block
- GeneAmp® PCR System 9700 with the Gold-plated Silver 96-Well Block

Avoiding contamination from the environment

To avoid general contamination, take the following precautionary measures:

- Change pipet tips between samples.
- Use filter-plugged pipet tips.
- Clean any work-contaminated surface using a cloth soaked with 50% bleach.

  IMPORTANT! Before cleaning the sample block of a thermal cycler, refer to the instrument user guide for the proper procedure.

- Close sample tubes when not using them.
- Always run a no-DNA negative control.

  A negative control contains no template DNA, only primers and the DNA diluent (usually water or buffer).
- Aliquot reaction reagents to minimize the number of times you use a stock solution.

Avoiding PCR product carryover

PCR product carryover is the contamination of an unamplified sample with previously amplified DNA.

Why carryover is a particular concern

PCR product carryover is a particular concern because amplified PCR product is an ideal template for subsequent amplifications of that same target.

A single PCR amplification produces a large number of copies (as many as 10^{13}). The inadvertent transfer of even a tiny volume or aerosol of amplified product can significantly contaminate unamplified samples. Contamination can result in false-positives and the detection and amplification of the contaminating sequence instead of the target sequence.

Precautionary measures

- Use positive-displacement pipettes or filter-plugged pipette tips.
- Physically separate reactions before and after amplification.
- Handle pre- and post-PCR solutions with separate sets of:
  - Pipettes
  - Pipette tips
  - Microcentrifuge tubes
  - Gloves
- Use AmpErase® UNG in reaction mixtures to prevent the subsequent reamplification of dU-containing PCR products.

For more information

Thermo Fisher Scientific supplies the GeneAmp® PCR Carryover Prevention Kit (Part no. N808-0068) and AmpErase® UNG (Part no. N808-0096) to ensure that PCR products are not reamplified in subsequent PCR amplifications.

4

Optimizing Capillary Electrophoresis

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Safety information

For safety and biohazard guidelines, refer to the “Safety” section in the user guide for your instrument. For every chemical, read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Note: For the SDSs of chemicals not distributed by Thermo Fisher Scientific, contact the chemical manufacturer.

Overview

Note: Perform capillary electrophoresis under well-controlled conditions with standard operating procedures. We recommend using a dedicated instrument platform for an experiment to minimize random error due to sizing imprecision.

This chapter contains general information for capillary electrophoresis. For application-specific information on capillary electrophoresis, see the application chapters later in this guide.

Thermo Fisher Scientific Genetic Analyzers

Table 10 Thermo Fisher Scientific Genetic Analyzers (capillary electrophoresis technology)

Instrument	Number of capillaries	Capillary array length	Polymer type	Sample capacity	Data Collection Software version
3500	8	36[†] and 50 cm	POP-7™ POP-4®[†] POP-6™	96-well plates and 8-tube strips	3500 Series Software v1.0 or later
3500xL	24			96- and 384-well plates and 8-tube strips	
3730	48	36 and 50 cm	POP-7™ POP-6™	96- and 384-well plates	Data Collection Software v3.0 or v3.1 or later
3730*xl*	96				
3130	4	22, 36, and 50 cm	POP-7™ POP-4® POP-6™	96- and 384-well plates	Data Collection Software v3.0 or v3.1 or later
3130*xl*	16				
310	1	47 cm	POP-4® POP-6™	Up to 48 or 96 sample tubes	310 Data Collection Software

[†] 3500 Series instruments: 36-cm capillary arrays and POP-4® polymer are used for HID applications only.

For more information on instruments, see “Instrument documentation” on page 199.

Overview of run modules

A run module contains electrophoresis parameters, such as oven temperature, detector cell temperature, ramp rate, injection time and voltage, and run time or run voltage. Each run module provided with the Data Collection Software has been optimized for a specific instrument, polymer, capillary, and sample configuration (run modules for the 3500 Series instrument are embedded in the instrument protocols). Some settings, such as injection time/voltage or run time, may need to be optimized for your instrument and application.

Updated versions of run modules for your instrument may be available on our web site.

Using controls

To simplify troubleshooting, run controls with every run for multicapillary instruments or each set of runs on 310 instruments.

3500 Series instruments

The 36-cm capillary arrays and POP-4® polymer are used for HID applications only.

Run modules

Table 11 3500 Series instrument run modules

Run modules type and run modules name	Configuration		23 hours Throughput†			Performance			
							Sizing Precision§		
	Capillary length	Polymer	Run Time	3500	3500xL	Range‡	50 bp-400 bp	401 bp-600 bp	601 bp-1200 bp
Fragment analysis FragmentAnalysis50_POP7	50 cm	POP-7™	≤40 min	≥280	≥840	≤40 to ≥520	<0.15	<0.30	NA††
Fragment analysis FragmentAnalysis50_POP6	50 cm	POP-6™	≤100 min	≥112	≥336	≤20 to ≥550	<0.15	<0.30	NA††
Long fragment analysis LongFragAnalysis50_POP7	50 cm	POP-7™	≤125 min	≥88	≥360	≤40 to ≥700	<0.15	<0.30	<0.45
HID HID36_POP4	36 cm	POP-4®	≤35 min	≥312	≥936	≤60 to ≥400	<0.15	NA††	NA††
HID HID36_POP7	36 cm	POP-7™	≤26 min	≥424	≥1272	≤60 to ≥400	<0.15	NA††	NA††
SNaPshot® SNaPshot50_POP7	50 cm	POP-7™	≤30 min	≥376	≥1104	≤40 to ≥120	<0.50	NA††	NA††

† Throughput (samples/day): The total number of samples run in 23 hours (0.5 hour for user interaction and 0.5 hours for warm-up time).

‡ Resolution range: The range of bases over which the resolution (peak spacing interval divided by the peak width at half-max in a GeneScan™ 600 LIZ® or GeneScan™ 1200 LIZ® Size Standard sample sized with a third order fit) is ≥1. The table shows the resolution range in ≥90% of samples.

§ Sizing precision: Standard deviation of sizes for one allele in the DS-33 install standard sized with the GeneScan™ 600 LIZ® Size Standard v2.0 across multiple capillaries in the same run. For one injection to pass, 100% of the alleles in that injection must meet the intra-run sizing precision specifications. The table shows the sizing precision of 100% of alleles in ≥90% of samples.

†† Not applicable because of the size of the fragments collected in the run.

Performance

Table 12 3500 Series instrument resolution, largest fragment, and sizing

General		Sizing Precision of 100% of Alleles in ≥90% of Samples			Multirun Sizing of 100% of Alleles in ≥90% of Samples		
Resolution Range in ≥90% of Samples	**Largest Fragment Collected in ≥90% of Samples**	**50–400 bp**	**401–600 bp**	**601–1,200 bp**	**50–400 bp**	**401–600 bp**	**601–1,200 bp**
≤40 to ≥520	≥600	<0.15	<0.30	NA	<1 bp	<2 bp	NA
≤20 to ≥550	≥600	<0.15	<0.30	NA	<1 bp	<2 bp	NA
≤40 to ≥700	≥1,200	<0.15	<0.30		<1 bp	<2 bp	<3 bp
≤60 to ≥400	≥420	<0.15	NA	NA	<1 bp	NA	NA
≤60 to ≥400	≥420	<0.15	NA	NA	<1 bp	NA	NA
≤40 to ≥120	≥120	<0.50	NA	NA	<1 bp	NA	NA

Dye sets and matrix standards

Table 18 on page 86 lists the dye sets and matrix standards each instrument.

Creating a custom dye set

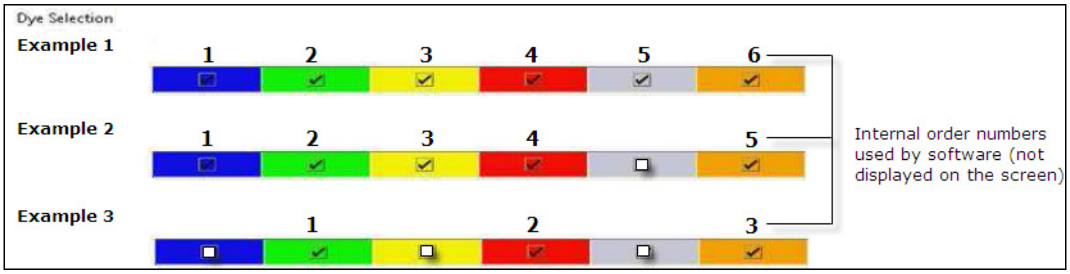
IMPORTANT! We recommend using only Thermo Fisher Scientific dyes. Thermo Fisher Scientific provides calibration reagents that have been optimized for our dye sets.

Non-Thermo Fisher Scientific dyes (or mixed isomers of dyes) have variable emission spectra and require a spectral calibration generated for the specific dyes to correct for the spectral overlap between the dyes. You are responsible for obtaining the appropriate spectral calibration reagents and for optimizing custom dye sets to ensure the dye labels do not affect PCR efficiency.

1. In the DyeSet library, click **Create**.
2. Enter a dye set name.
3. Select a chemistry and the AnyDye dye set template.
4. Select the dye colors to use and set the calibration peak order:

a. Select the dye colors to use, which specifies the order number of the dye used internally by the software. Note that when you deselect a dye, the order number of the dye used internally by the software changes. The examples below are for a 3500 Series instrument with 6-dye support, but the logic applies to 4 and 5 dyes.

  - In Example 1 with all dyes selected, internal order number is Blue (1), Green (2), Yellow (3), Red (4), Purple (5), Orange (6).
  - In Example 2 with the Purple dye deselected, internal order number is Blue (1), Green (2), Yellow (3), Red (4), Orange (5) - the internal order number of Orange changes to 5.
  - In Example 3 with the Blue, Yellow, and Purple dyes deselected, internal order number is Green (1), Red (2), Orange (3) - the internal order number of Green changes to 1, Red changes to 2, and Orange changes to 3.

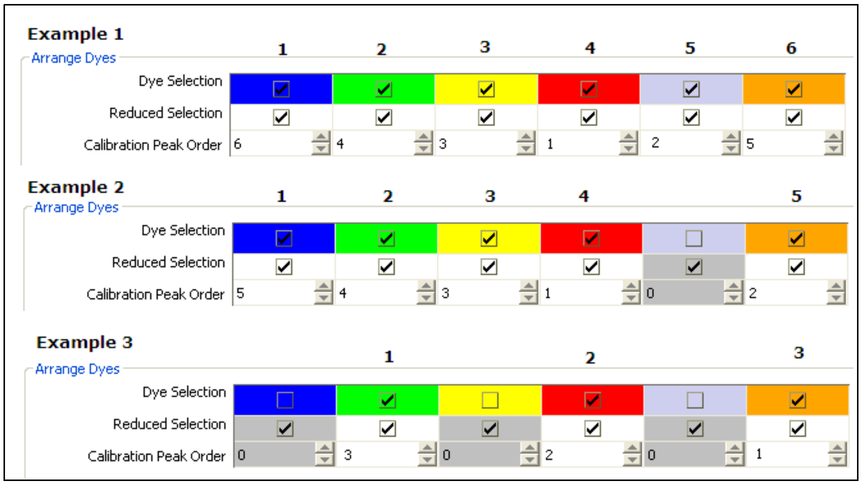

Dye Selection
Example 1
1 2 3 4 5 6
Example 2
1 2 3 4 5
Example 3
1 2 3
Internal order numbers used by software (not displayed on the screen)

b. Specify the order of the peaks in the calibration standard you are using. Use the internal order number of the dye based on the dyes selected.

IMPORTANT! The Calibration Peak Order fields do not correspond to the dye colors displayed above the Calibration Peak Order fields.

  - In Example 1 on the next page, if the order of the peaks in the calibration standard you are using is Orange, Red, Yellow, Blue, Green, Purple, specify for Calibration Peak Order: 6 (Orange), 4 (Red), 3 (Yellow), 1 (Blue), 2 (Green), 5 (Purple).
  - In Example 2 if the order of the peaks in the calibration standard you are using is Orange, Red, Yellow, Blue, Green, specify for Calibration Peak Order: 5 (Orange), 4 (Red), 3 (Yellow), 1 (Blue), 2 (Green).

- In Example 3 if the order of the peaks in the calibration standard you are using is Orange, Red, Green, specify for Calibration Peak Order: 3 (Orange), 2 (Red), 1 (Green).Expand the Parameters section, then specify remaining settings.



Example 1

Arrange Dyes

	1	2	3	4	5	6
Dye Selection	☑	☑	☑	☑	☑	☑
Reduced Selection	☑	☑	☑	☑	☑	☑
Calibration Peak Order	6	4	3	1	2	5

Example 2

Arrange Dyes

	1	2	3	4		5
Dye Selection	☑	☑	☑	☑	☐	☑
Reduced Selection	☑	☑	☑	☑	☑	☑
Calibration Peak Order	5	4	3	1	0	2

Example 3

Arrange Dyes

		1		2		3
Dye Selection	☐	☑	☐	☑	☐	☑
Reduced Selection	☑	☑	☑	☑	☑	☑
Calibration Peak Order	0	3	0	2	0	1

5. Perform a spectral calibration using the AnyDye dye set.
6. Create an instrument protocol that specifies the custom dye set, then specify the instrument protocol in an assay.

For more information

Refer to the specification sheet for your instrument to select a combination of capillary array and polymer that provide the required resolution (see "Instrument documentation" on page 199).

3730 Series instruments

Note: Because of the close proximity of capillaries on the 96-capillary 3730*xl* instrument, we recommend using the 48-capillary 3730 instrument for fragment analysis.

Note: Sizing precision may be lower than expected for fragments <50 bp run on 3730/3730*xl* instruments with POP-7™ polymer.

Run module and performance

Note: If you use GeneScan™ 1200 LIZ® Size Standard, download a new run module and optimize it before use. See "Downloading 3730 instrument run modules from our website" on page 48.

Table 13 3730 Series instrument run module

Fragment analysis run modules	Resolution	Runs/ day	3730 Analyzer		3730*xl* Analyzer	
			Samples/ day	Genotypes/ day	Samples/ day	Genotypes/ day
Fragment Analysis	Up to 500 bp resolution with 0.15 bp sizing resolution	44	4224†	84,508	2112	42,254†

† 20 genotypes/sample.

Refer to the specification sheet for your instrument to select a combination of capillary array and polymer that provide the required resolution (see "Instrument documentation" on page 199).

Dye sets and matrix standards

Table 18 on page 86 lists the dye sets and matrix standards each instrument.

Creating a custom dye set

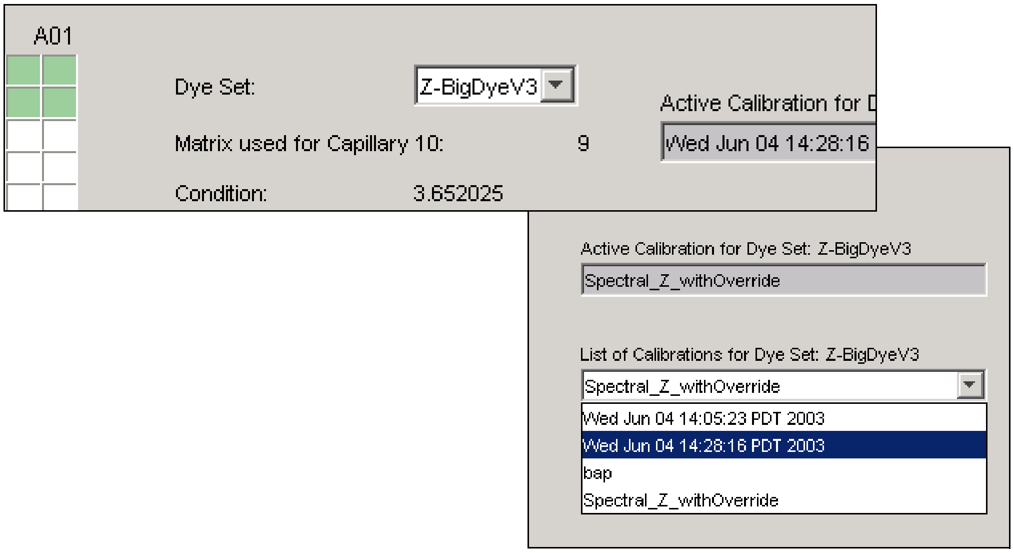
IMPORTANT! We recommend using only Thermo Fisher Scientific dyes. Thermo Fisher Scientific provides calibration reagents that have been optimized for our dye sets.

Non-Thermo Fisher Scientific dyes (or mixed isomers of dyes) have variable emission spectra and require a spectral calibration generated for the specific dyes to correct for the spectral overlap between the dyes. You are responsible for obtaining the appropriate spectral calibration reagents and for optimizing custom dye sets to ensure the dye labels do not affect PCR efficiency.

The software assumes the following dye color order: blue, green, yellow, red, orange.

1. In the navigation pane of the Data Collection Software, click **GA Instruments > ga3730 *or* ga3130 > Protocol Manager**.
2. In the Instrument Protocols pane, click **New**. The Protocol Editor opens.
3. In the Protocol Editor, create a spectral protocol for the Any4Dye or Any5Dye dye set, specifying the appropriate protocol parameters.
4. Create a Spectral Plate Record using the newly created Spectral Instrument Protocol.
5. Perform a spectral calibration.

6. Set the custom dye set calibration as the active spectral calibration:
   a. In the tree pane of the Data Collection software, click **GA Instruments > ga3130xl** or **ga3130** > *instrument name* > **Spectral Viewer**.
   b. In the Dye Set drop-down list, select the custom dye set.
   c. In the List of Calibrations for Dye Set drop-down list, select the spectral calibration you want to use. The spectral profile and raw data is displayed.



A01

Dye Set: Z-BigDyeV3

Matrix used for Capillary 10: 9

Condition: 3.652025

Active Calibration for D

Wed Jun 04 14:28:16

Active Calibration for Dye Set: Z-BigDyeV3

Spectral_Z_withOverride

List of Calibrations for Dye Set: Z-BigDyeV3

Spectral_Z_withOverride

Wed Jun 04 14:05:23 PDT 2003

Wed Jun 04 14:28:16 PDT 2003

bap

Spectral_Z_withOverride

   d. Click **Set**.
   e. Create an instrument protocol that specifies the custom dye set.

For more information

Refer to the specification sheet for your instrument to select a combination of capillary array and polymer that provide the required resolution (see “Instrument documentation” on page 199).

3130 Series instruments

Run modules and performance

Note: If you use GeneScan™ 1200 LIZ® Size Standard, download a new run module and optimize it before use. See “Downloading 3130 instrument run modules from our website” on page 48.

Table 14 3130 Series instrument run modules and resolution

Fragment analysis run modules	Array length	Polymer	Run time	24-hr throughput†		Resolution	Performance SD‡
				3130 Analyzer GT†	3130*xl* Analyzer GT†		
Fragment Analysis 22_POP4	22 cm	POP-4®	20 min	5,760	23,040	400 bp	0.50
SNP22_POP4	22 cm	POP-4®	15 min	3840§	15,360§	120 bp	0.50
Fragment Analysis 36_POP7	36 cm	POP-7™	35 min	3280	13,120	500 bp	0.15
Fragment Analysis 36_POP4	36 cm	POP-4®	45 min	2560	10,240	500 bp	0.15
HID Fragment Analysis 36_POP4	36 cm	POP-4®	45 min	2560	10,240	500 bp	0.15
SNP36_POP4	36 cm	POP-4®	30 min	3840	15,360	120 bp	0.15
Fragment Analysis 50_POP7	50 cm	POP-7™	50 min	2240	8,960	500 bp	0.15
Fragment Analysis 50_POP4	50 cm	POP-4®	65 min	1760	7,040	500 bp	0.15
Fragment Analysis 50_POP6	50 cm	POP-6™	90 min	1280	5,120	500 bp	0.15

† 20 genotypes/injection.
‡ Standard deviation: 1 base pair (bp) resolution at 99.99% accuracy.
§ 10 genotypes/injection.

Refer to the specification sheet for your instrument to select a combination of capillary array and polymer that provide the required resolution (see “Instrument documentation” on page 199).

Dye sets and matrix standards

Table 18 on page 86 lists the dye sets and matrix standards each instrument.

Creating a custom dye set

IMPORTANT! We recommend using only Thermo Fisher Scientific dyes. Thermo Fisher Scientific provides calibration reagents that have been optimized for our dye sets.

Non-Thermo Fisher Scientific dyes (or mixed isomers of dyes) have variable emission spectra and require a spectral calibration generated for the specific dyes to correct for the spectral overlap between the dyes. You are responsible for obtaining the appropriate spectral calibration reagents and for optimizing custom dye sets to ensure the dye labels do not affect PCR efficiency.

The procedure for creating custom dye sets is the same as the procedure for the 3730 Series instruments. “Creating a custom dye set” on page 73.

For more information

Refer to the specification sheet for your instrument to select a combination of capillary array and polymer that provide the required resolution (see “Instrument documentation” on page 199).

310 instruments

Run modules and performance

Note: If you use GeneScan™ 600 LIZ® Size Standard, optimize the run module before use. See "Optimizing the 310 instrument run module" on page 46.

Table 15 310 instrument run module

Fragment analysis run modules	Resolution	Samples/day		Genotypes/day
GS STR POP4	1 base detection up to 250 bases with 0.15 SD	4-dye	>57	720 genotypes†
	2 base detection 250 to 350 bases with 0.3 SD	5-dye	>57	960 genotypes‡

† 15 genotypes/run.

‡ 20 genotypes/run.

Refer to the specification sheet for your instrument to select a combination of capillary array and polymer that provide the required resolution (see "Instrument documentation" on page 199).

Dye sets and matrix standards

Table 18 on page 86 lists the dye sets and matrix standards each instrument.

Optimizing sample loading concentration

Note: Sample overloading can clog capillaries.

- Optimize the ratio of sample-to-size standard and Hi-Di™ formamide using the values listed below as a starting point.

Components	3500 Series, 3730 Series, and 3130 Series instruments	310 instrument
Sample	0.5 µL per reaction	0.5 µL per reaction
Size standard	0.5 µL per reaction	0.5 µL per reaction
Hi-Di™ Formamide†	9.0 µL per reaction	11.0 µL per reaction

† Hi-Di™ Formamide (Part no. 4311320) is purchased separately from the size standard.

- If you anticipate an extremely high sample concentration, run dilutions of the sample. If the signal is too strong, you can further dilute the sample or you can decrease the sample injection time and/or injection voltage.
- If the signal is too weak, first try increasing the signal by increasing the sample injection time or voltage.
- To optimize the signal intensity for a given sample, inject the same sample multiple times using a range of injection parameters.

  If the signal intensity is still too weak or the resolution is poor, concentrate the sample.

If the signal intensity is too low after concentration, see "Desalting" on page 190.

- Different dyes emit different fluorescence intensities. Therefore, concentrations of PCR products may have to be increased or decreased depending on relative fluorescence intensity during electrophoresis. (See "Emission and absorption (excitation) wavelengths and relative intensities" on page 38).

Optimizing signal intensity

Optimal detection ranges

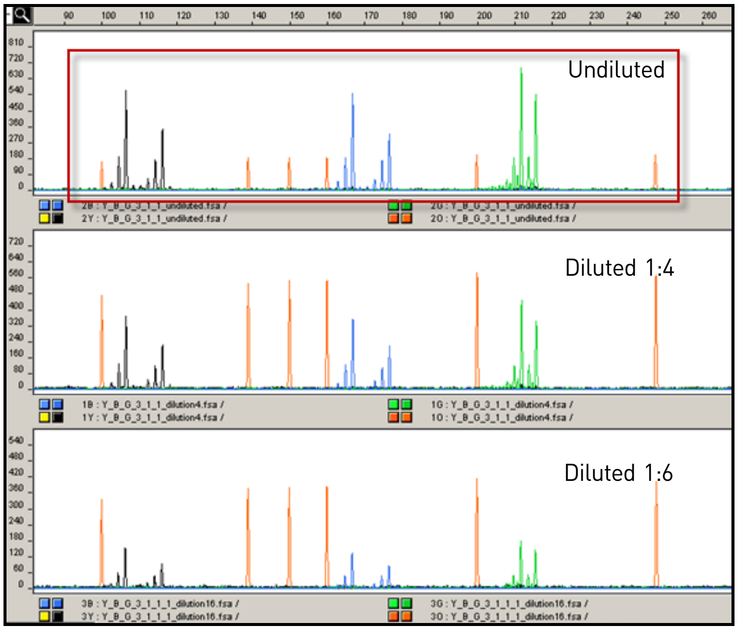
Thermo Fisher Scientific genetic analyzers can convert a limited range of fluorescence signal into digital values. For optimal results, ensure the signal intensities are within the ranges listed below.

Table 16 Signal intensity ranges and fluorescence saturation

Instrument	Recommended signal intensity range	Fluorescence saturation
3500 Series	175–10,000 RFU	30,000 RFU
3730 Series	150–10,000 RFU	30,000 RFU
3130 Series	150–4,000 RFU	8000 RFU
310	150–4000 RFU	8000 RFU

Balancing size-standard and sample-peak intensities

The intensity of size-standard peaks should be 30 to 100% of the intensity of sample peaks. Dilute samples before preparing capillary electrophoresis reactions to balance the signal intensities. In the example below, the undiluted sample yields the correct size standard-to-peak intensity ratio.



Size-standard peaks should be 30 to 100% of the intensity of sample peaks

If signal intensity is above the detection range

When the signal intensity of a peak is too high, the instrument cannot measure the true value of the signal and consequently cannot compensate for the spectral overlap between the dyes. As a result, artifact peaks called "bleed-through" or "pull-up" peaks can appear beneath the sample peaks. These pull-up peaks can corrupt both automated sizing (because extra peaks are present in the size-standard dye color) and the analysis of samples (because the size standard is present in each sample).

If signal intensity is high, you can:

- Dilute the template before PCR
- Dilute the amplified sample before adding to formamide
- Decrease the sample injection time and/or injection voltage

If signal intensity is below the detection range

When signal is too low, the signal-to-noise ratio is also low and makes it difficult to discriminate between sample peaks and common background fluctuations.

If signal intensity is low, you can:

- Increase the sample injection time or injection voltage
- Increase the volume of template added to the PCR reaction

Minimizing signal intensity variation

To minimize signal intensity variations, consider the ionic strength of samples and consumables. The amount of DNA injected is inversely proportional to the ionic strength of the solution. Note the following:

- Samples high in salt result in poor injections.
- PCR reactions vary in efficiency, therefore some reactions may result in higher ionic concentration post-amplification.
- Conductivity of the solvent used for injection affects the sample injection and can cause variation in peak height.

  The recommended injection solvent, Hi-Di™ Formamide, is highly deionized formamide, formulated with a stabilizer. Storage of Hi-Di™ Formamide is important in maintaining the quality and conductivity of the solvent. See "Hi-Di™ Formamide storage" on page 82.

For more information, see "Irregular signal intensity troubleshooting" on page 164.

Optimizing electrokinetic injection parameters

Electrokinetic injection parameters affect data quality, run-to-run precision in sizing, and reproducibility in the amount of sample loaded. Optimize parameters to inject sufficient DNA to yield peaks of adequate height (that is, data with a good signal-to-noise ratio) while maintaining the resolution and precision required by the application.

The Data Collection Software includes run modules with preset values for injection times and voltages that have been optimized for specific instrument/polymer/capillary length configurations. These values are adequate for many applications. However, consider modifying the injection parameters if the run modules yield signal that is too strong or too weak or if the resolution is poor. (The maximum recommended injection time is 30 seconds and the maximum possible injection voltage is 15 kV.)

Definition of resolution

The resolution, R_s, of two peaks in an electropherogram is defined as:

$$R_s = \frac{|P_1 - P_2|}{0.5 \times (W_1 + W_2)}$$

where P_1 and P_2 are the peak positions measured below the peak apex and W_1 and W_2 are the peak widths measured at half peak maximum.

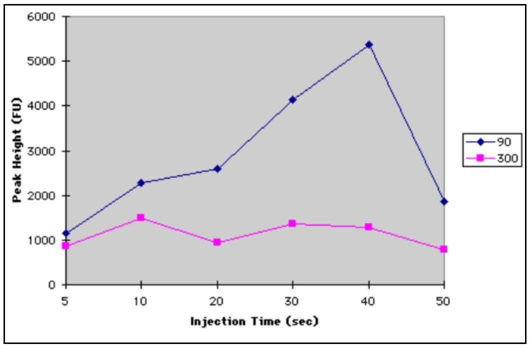
An R_s value of 1 corresponds to fragments that can be discriminated by one nucleotide.

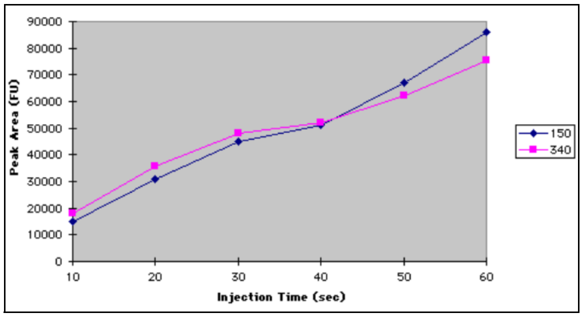
Optimizing injection time

Injection time affects signal intensity and resolution.

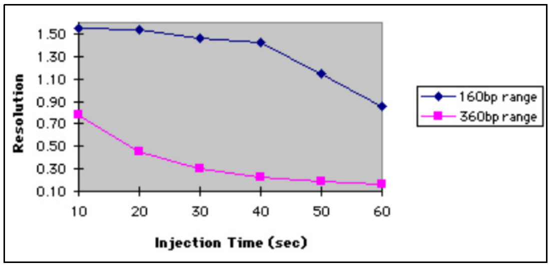
Note: Salt concentration can also affect signal intensity and resolution. If adjusting injection time and voltage does not provide adequate signal strength, you may need to concentrate the sample or desalt the sample ("Desalting" on page 190).

<table>
<tr><th>Attribute</th><th>Effect of injection time</th></tr>
<tr><td>Signal intensity</td><td>Signal intensity (as measured both by peak height and by peak area) typically increases linearly with increasing injection time. However, an *n*-fold increase in injection time does not result in an *n*-fold increase in peak height. In the examples below, no improvement is seen after 10 seconds for the larger fragment. The signal decreases dramatically after 40 seconds for the smaller fragment. As the injection time increases, the resolution decreases, leading to increasing peak widths and decreasing peak heights




</td></tr>
<tr><td>Resolution</td><td>Increasing the injection time decreases the resolution. As shown below, the negative effect on resolution is more pronounced for larger fragments.

The decrease in resolution results from an increase in peak width (as opposed to a decrease in peak separation).

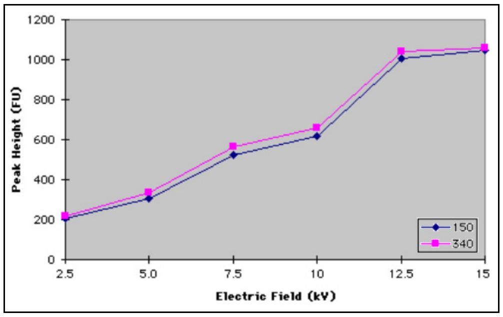

</td></tr>
</table>

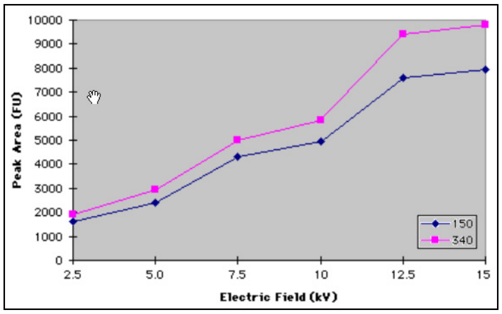
Optimizing injection voltage

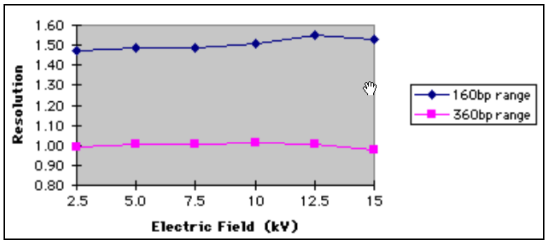
Injection voltage affects signal strength. However, lower voltages, which produce lower currents, are often preferable because injection timing is more accurate. Accurate timing ensures reproducibility in sample loading.

Note: Salt concentration can also affect signal intensity and resolution. If adjusting injection time and voltage does not provide adequate signal strength, you may need to concentrate the sample or desalt the sample ("Desalting" on page 190).

<table>
<tr><th>Attribute</th><th>Effect of injection voltage</th></tr>
<tr><td>Signal intensity</td><td>Peak height and peak area increase linearly with increasing injection voltage. The figures below show the effect of increasing the injection voltage from 53 V/cm to 319 V/cm on peak height and peak area, respectively, for two different-sized fragments.




</td></tr>
<tr><td>Resolution</td><td>Injection voltage has little effect on peak resolution.


</td></tr>
</table>

Optimizing electrophoresis conditions

Optimizing electrophoresis conditions (run time, run voltage, run temperature) can greatly improve data quality, run-to-run precision, and/or throughput. Optimize settings appropriate for:

- Range of fragment lengths
- Required degree of resolution
- Type of genetic analysis you will be performing (for example, denaturing or non-denaturing conditions)

The settings in the run modules provided are set to ensure the following:

- Detection of all fragments in the typical size range for the application
- Acceptable run times
- Acceptable resolution

Optimizing run time

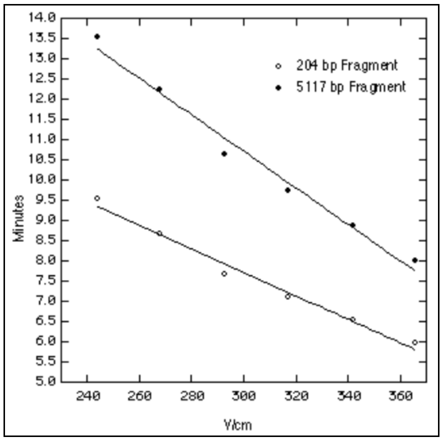
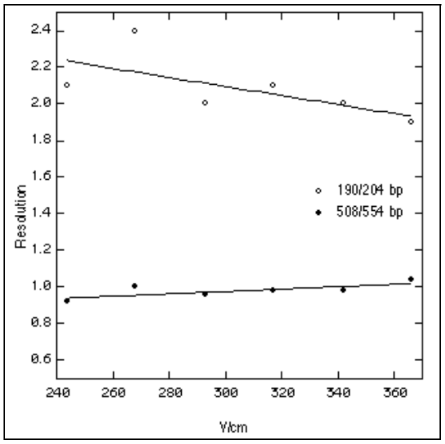
Perform trial runs to determine the minimum acceptable run time for a given run voltage. To ensure that you collect sufficient data to perform analysis, set the electrophoresis run time approximately 10% higher than the migration time of the largest fragment of interest.

The largest fragment of interest is often a size-standard peak that is needed for sizing the largest sample fragments of interest. The set of size-standard peaks that GeneMapper® Software uses to generate the sizing curve can vary with the size-calling method. In general, be sure to include the two size-standard peaks immediately smaller than the smallest fragment and the two size-standard peaks immediately larger than the largest sample fragment of interest, or modify the size-standard definition to eliminate the peaks that are not present.

Note: For faster run times, you can also increase the run voltage. However, a higher run voltage can decrease the resolution.

Optimizing run voltage

Run voltage can affect migration rates and resolution because it affects the speed at which samples migrate through the capillary. If they migrate too quickly, the samples do not optimally separate.

Attribute	Effect of run voltage
Migration rates	Higher run voltages yield faster run times but can affect the resolution. 
Resolution	In general, resolution is better at lower run voltages. 

Optimizing run temperature

Perform non-denaturing applications at lower temperatures (27 to 42°C).

Protocols for denaturing applications use POP-4® or POP-7™ polymer with optimized run temperatures. Altering the run temperature can affect migration rates and resolution.

Other factors that affect electrophoresis

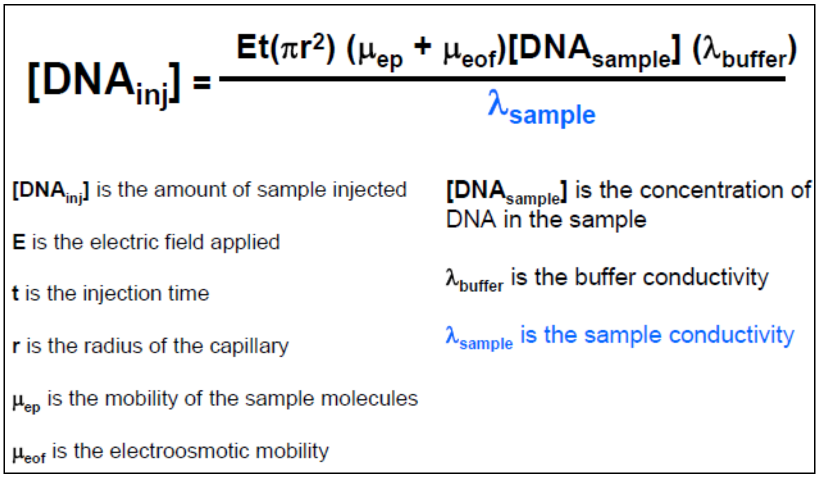
Laboratory temperature and humidity

Maintain the laboratory temperature between 15 to 30°C. After the instrument is set up and in operation, the laboratory temperature should not fluctuate more than ±2°C. The instrument can tolerate up to 80% non-condensing relative humidity. Avoid placing it near heaters or cooling ducts.

Salt concentration, ionic strength, and conductivity

Salt anions compete with negatively charged DNA for entry into the capillary during electrokinetic injection. As the salt concentration of a sample increases, less DNA will enter the capillary, decreasing the fluorescence signal. Excess salt can also precipitate the DNA in the sample tube in the presence of formamide.

The amount of DNA injected is inversely proportional to the ionic strength of the solution (Butler *et. al.*)



$$[DNA_{inj}] = \frac{Et(\pi r^2)\,(\mu_{ep} + \mu_{eof})[DNA_{sample}]\,(\lambda_{buffer})}{\lambda_{sample}}$$

$[DNA_{inj}]$ is the amount of sample injected

E is the electric field applied

t is the injection time

r is the radius of the capillary

μ_{ep} is the mobility of the sample molecules

μ_{eof} is the electroosmotic mobility

$[DNA_{sample}]$ is the concentration of DNA in the sample

λ_{buffer} is the buffer conductivity

λ_{sample} is the sample conductivity

Hi-Di™ Formamide storage

CAUTION! Mixing Hi-Di™ Formamide with water generates formic acid.

Proper handling and storage of Hi-Di™ Formamide is critical. For quality results:

- Aliquot the contents from the original bottle into one-time use, 1.5-mL or smaller tubes.
- Minimize exposure to air and freeze/thaw cycles.

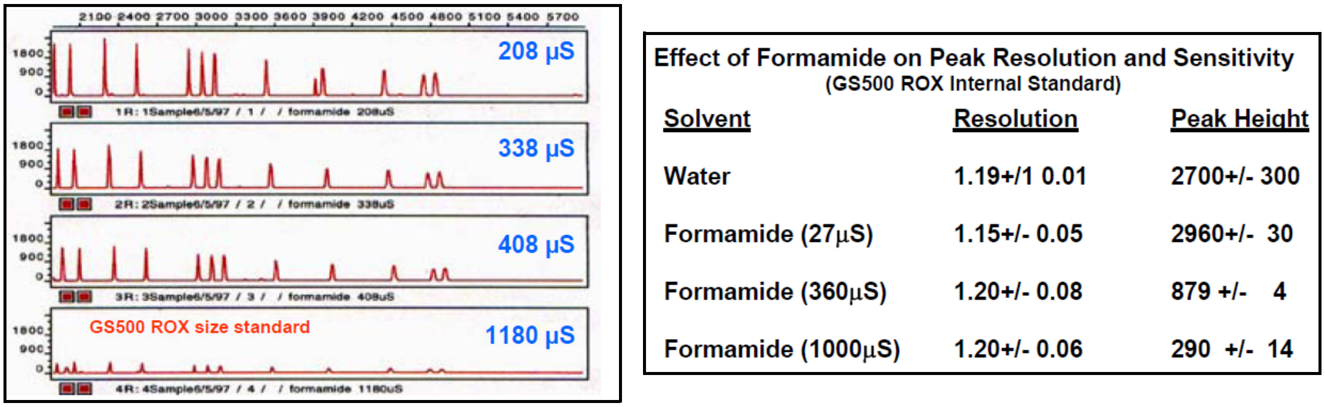
  IMPORTANT! Do not freeze/thaw more than two times. Excessive freeze/thaw cycles or storage at 2 to 8°C for more than 1 week causes hydrolysis into formic acid and formate. Formate ions migrate preferentially into the capillary during electrokinetic injection causing a loss of signal intensity.

- Ensure that you do not contaminate Hi-Di™ Formamide when setting up samples.

- Store for up to 3 months at –15 to –25°C.
- Store for up to 1 week at 2 to 8°C.

The figure below illustrates the variation in conductance in different quality formamide solutions.

Figure 18 Effect of formamide quality on conductance



Effect of Formamide on Peak Resolution and Sensitivity
(GS500 ROX Internal Standard)

Solvent	Resolution	Peak Height
Water	1.19+/1 0.01	2700+/- 300
Formamide (27µS)	1.15+/- 0.05	2960+/- 30
Formamide (360µS)	1.20+/- 0.08	879 +/- 4
Formamide (1000µS)	1.20+/- 0.06	290 +/- 14

Improperly stored Hi-Di™ Formamide can cause a variety of electrophoresis problems. For information, see "Hi-Di™ formamide" on page 159.

Polymer handling and characteristics

IMPORTANT! Do not leave polymer on the instrument more than seven days. Polymer left on the instrument for more than seven days causes a loss of resolution. Avoid actions that introduce bubbles or particles into the polymer. Dust in the polymer can cause data spikes.

To minimize bubbles and particles in polymer:

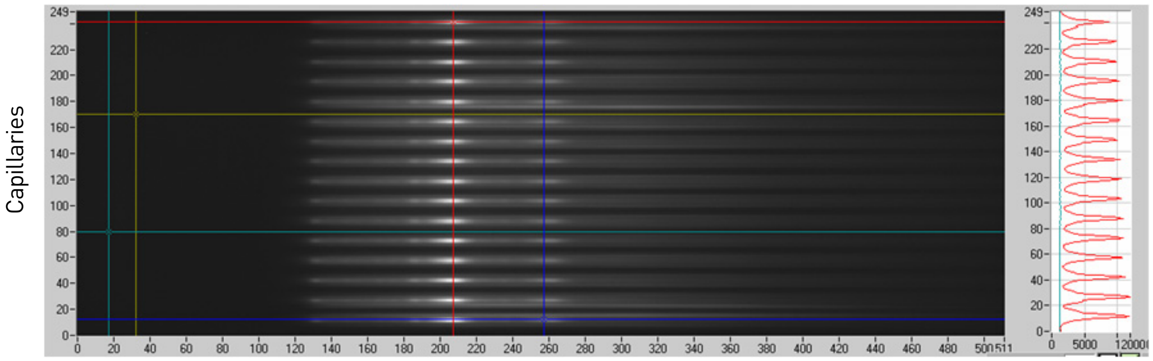
- Close the polymer cap during storage to minimize exposure of the polymer to air.
- Clean the polymer delivery system with deionized water.
- Discard capillaries that are exposed to dust or are dried out.
- Change the buffer and water and discard the waste daily or before each set of runs.

Table 17 Polymer characteristics

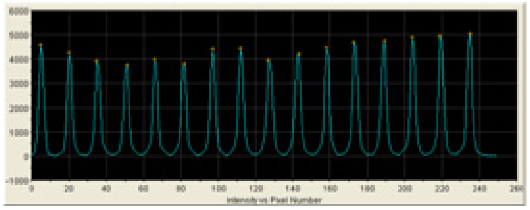
Instrument	Polymer characteristics
POP-4® Polymer	Less viscous, fast runs
POP-6™ Polymer	More viscous, slow runs
POP-7™ Polymer (not for use on 310 instruments)	Less viscous, fast runs

Understanding spatial calibration

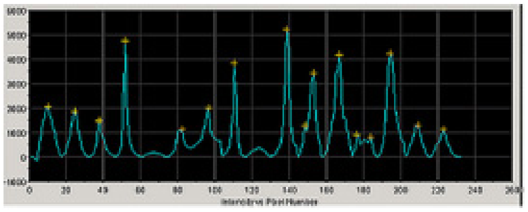
A spatial calibration maps each capillary location to the CCD camera, which detects the signal for each capillary.


Capillaries
CCD camera positions
249
220
200
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
500
511
5000
12000

Good spatial calibration



Bad spatial calibration



Spatial calibration maximizes data quality and accuracy.

Refer to the instrument user guide for instructions on performing a spatial calibration. See “Instrument documentation” on page 199 for document part numbers.

Perform a spatial calibration when you:

- Install or replace a capillary array
- Temporarily remove the capillary array from the detection block
- Move the instrument
- Open the detection block

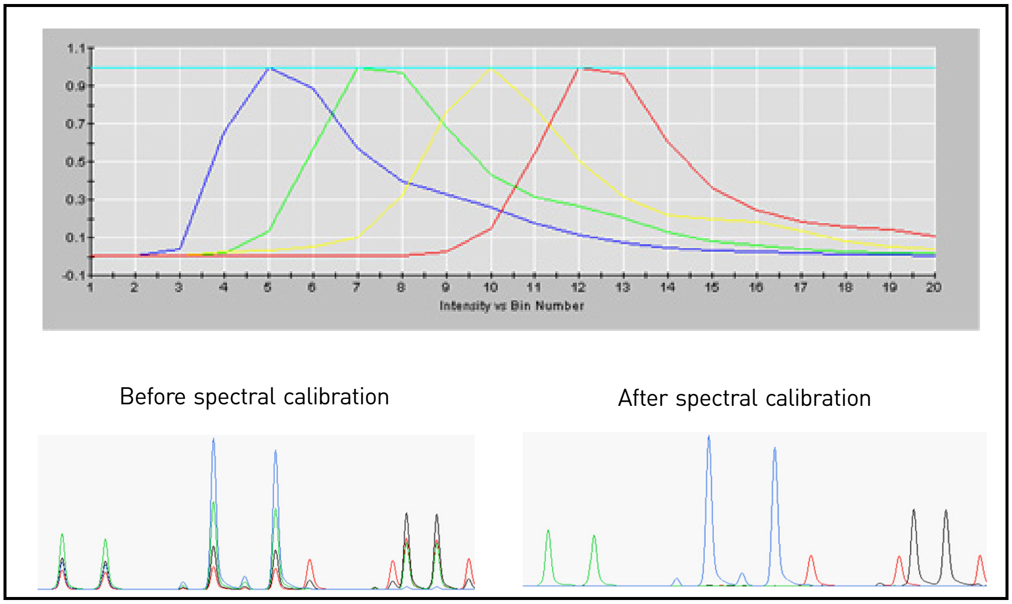
Understanding spectral calibration

IMPORTANT! Always visually examine the data generated by a spectral calibration. Accepting a calibration with poor data will yield inaccurate results when you run samples.

For information on creating a custom dye set for spectral calibration, see the instrument sections earlier in this chapter.

Spectral calibration

A spectral calibration (or matrix on 310 instruments) allows the software to distinguish between dyes by subtracting out the spectral overlap between the different dyes (for more information, see "Multicomponent analysis with fluorescent dyes" on page 36).



Before spectral calibration

After spectral calibration

Spectral calibration matrix standards are available from Thermo Fisher Scientific in premixed form for all Thermo Fisher Scientific instruments and dye sets (see Table 18 on page 86).

The values in a matrix generated by a spectral calibration are unique for each instrument, for each dye set, and for each specific set of run conditions. The Data Collection Software applies the values in the matrix to the sample data to perform multicomponent analysis: the separation of the dye fluorescence in the raw data from the instrument to the data stored in the sample files.

Note: For 310 instruments, you must manually create and apply the matrix file in the GeneMapper® Software.

Refer to the instrument user guide for your instrument for instructions on performing a spectral calibration or creating a 310 matrix.

When to perform

Perform a spectral calibration run:

- When you use a new dye set on the instrument
- When you change the capillary array or polymer
- For each combination of capillary array length and each dye set that you use
- After the laser or CCD camera has been realigned/replaced by a service engineer
- If you observe a decrease in the quality of raw or analyzed data (for example, pull-up and/or pull-down peaks with a distinct pattern)

Dye set and matrix standards for spectral calibration

The table below lists the appropriate dye set and matrix calibration standard combinations for each instrument. For the dye sets for applications, see Table 8 on page 41.

Table 18 Matrix standards (see page 197 for part numbers)

Matrix standard	Dye set	3500	3730/ 3730*xl*†	3130/ 3130*xl*	310
DS-33	G5	Yes	Yes	Yes	Yes
DS-02	E5	Yes	Yes	Yes	Yes
DS-32	F	Yes	Yes	Yes	Yes
DS-30	D	Yes	Yes	Yes	Yes
DS-31	D	Yes	Yes	Yes	Yes
DS-34	C	No	No	No	Yes
AnyDye	Custom dye set you create	Yes	Yes	Yes	No

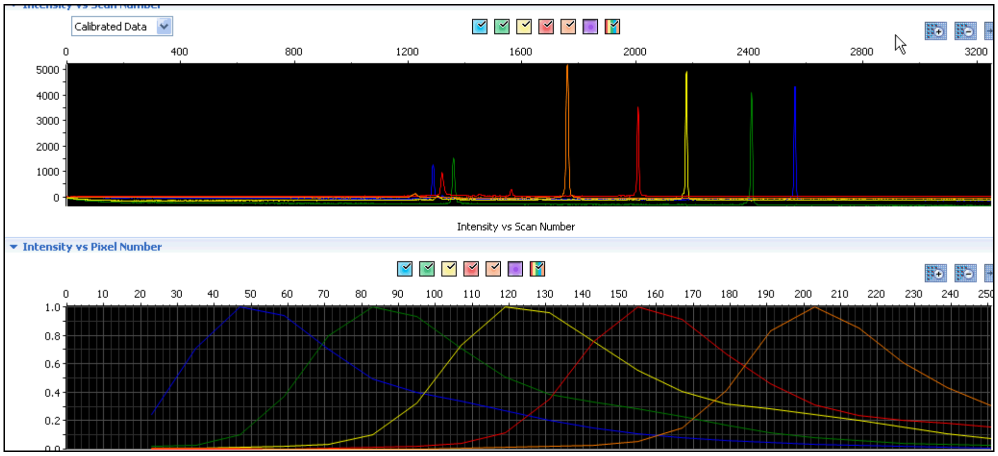
† Because of the close proximity of capillaries on the 96-capillary 3730*xl* instrument, we recommend using the 48-capillary 3730 instrument for fragment analysis. For best results, use the G5 dye set with reduced cross-talk (RCT) configuration.

Evaluating the calibration results

Use the following criteria to evaluate the data:

- **Q Value or Quality Value** – Measures the consistency between the final matrix and the data from which it was computed. A Q value of 1.0 indicates high consistency and that no pull-up or pull-down peaks were detected.
- **Condition Number** – Represents the amount of overlap between the dye peaks in the emission spectra of the dyes in a dye set. A Condition Number of 1.0 (lowest possible value) indicates there is no overlap in a dye set. The condition number increases with increasing peak overlap.
- **Spectral profile** – Shows the emission spectra of the dyes.
- **Raw data** – The emission image shows distinct fluorescence emission maxima, one for each dye (Figure 19).

Figure 19 Example output from a spectral calibration using a matrix standard


Calibrated Data
0
400
800
1200
1600
2000
2400
2800
3200
5000
4000
3000
2000
1000
0
Intensity vs Scan Number
Intensity vs Pixel Number
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
1.0
0.8
0.6
0.4
0.2
0.0

Q Value and Condition Number ranges

Matrix standard	Dye set	Quality value	Maximum Condition Number
User-provided	AnyDye	0.8 (default)	–
DS-30	D	0.95	–
DS-33	G5	0.95	13.5
DS-32	F	0.95	8.5
DS-31	D	0.95	–
DS-02	E5	0.95	6

Troubleshooting spectral calibration

A poor or incorrect matrix results in too much or too little subtraction of dye spectral overlap during data analysis. Each causes a recognizable electropherogram anomaly:

- Bleed-through peaks, also called pull-up peaks (caused by too little subtraction)
- Elevated inter-peak baseline (caused by too much subtraction)

If the spectral calibration fails, or if the quality of a passing calibration is not acceptable, try one or more of the following:

- Ensure that you used fresh, properly prepared and vortexed matrix standard. Old, improperly prepared, or insufficiently vortexed matrix standard can cause low signal intensity.
- Check instrument status for any run errors.
- Verify the correct run module was used. Correct as needed and repeat the run.
- Check the freshness and preparation of reagents.
- Check for possible contamination of matrix standards.
- Make sure that there are no bubbles in the sample wells.
- Verify that all peaks were detected.

  A slow-running system can partially or completely cut off the blue peak. Increase the run time (instruments other than the 3500 series) or change reagents if needed and repeat the run.

For troubleshooting the spectral calibration, refer to the instrument user guide for your instrument.

Understanding the matrix file (310 instruments only)

Purpose of a matrix

The most intense fluorescence emitted by a fluorescently labeled dye falls within a small wavelength-detection range. However, some fluorescence emission in the detection ranges of the other dyes will always occur. You create a matrix that compensates for overlap by subtracting out, in the detection range of each dye, the portion of the signal due to fluorescence from other dyes.

Run each relevant dye matrix standard separately to determine the proportional amount of fluorescence that is emitted in all dye detection regions. Always create a new matrix if run conditions change (such as the pH or polymer type and concentration).

Virtual Filter Set C

Virtual Filter Set C/dye set DS-34 is used on 310 instruments. The emission maximum of 6-FAM™ dye, the recommended blue dye for the Virtual Filter Set C, is very close to the laser wavelength of 514.5 nm. Thus, the window for collected blue light-intensity data is offset to longer wavelengths and does not contain the emission maximum of 6-FAM™ dye. The emission maximum of 6-FAM™ dye is also very close to the detection region for the green TET™ dye. Matrix files made for Virtual Filter Set C are especially susceptible to minor changes in run conditions. If you are using Virtual Filter Set C, watch for evidence of matrix problems and create a new matrix as soon as problems appear.

Factors affecting matrix quality

- Aging reagents
- Buffer type and concentration
- Polymer type
- Denaturing or non-denaturing conditions
- Run temperature

5

Data Analysis with GeneMapper® Software and Peak Scanner™ Software

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Overview

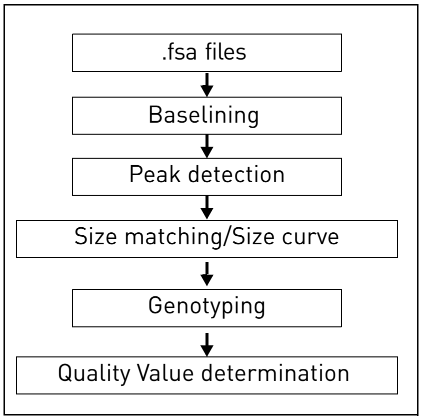
This chapter contains general information for data evaluation. For application-specific information on data evaluation, see the application chapters later in this guide.

GeneMapper® Software analyzes the data collected on Thermo Fisher Scientific genetic analyzers to size and genotype DNA fragments. You can also use the GeneMapper® Software data to perform relative quantitation (for more information, see Chapter 9, "Relative Fluorescence Quantitation (RFQ)" on page 143).

Peak Scanner™ Software, can be used for preliminary sizing.

How the software processes data

Both GeneMapper® Software and Peak Scanner™ Software perform analysis on original .fsa files generated by the Data Collection Software.



Precise versus accurate sizing

When evaluating sizing, consider two metrics:

- **Precision (reproducibility)** – The measure of the ability to generate the same size consistently for a given fragment obtained under the same conditions.
- **Accuracy** – The measure of the ability to generate fragment sizes that are close to the actual size as determined by sequencing.

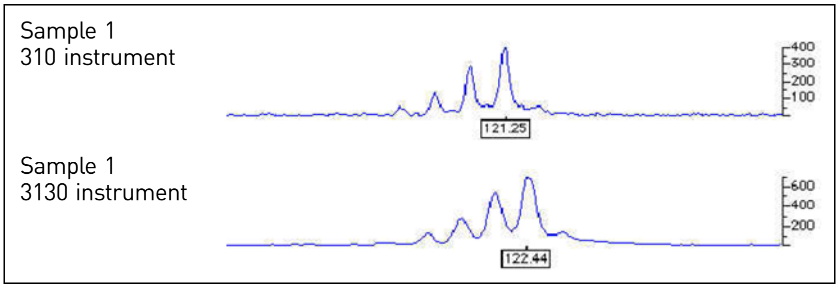
The size of a DNA fragment is altered by the dye with which it is labeled, and each Thermo Fisher Scientific dye has a different size. Therefore, a fragment with a known size may be sized differently when run using Thermo Fisher Scientific dyes and instruments. Although this size may not be "accurate" when compared to the actual size, it will be precise when compared to other fragments run under the same conditions.

Note the following:

- Sizing differences between various types of polymer are more apparent for sequences <50 base pairs (bp).
- Smaller fragments (<50 bp) run on POP- 7™ polymer on 3730/3730*xl* instruments may have slightly lower sizing precision.

Relative sizing

The size of a fragment is calculated based on the size standard with which it co-migrates. Dye-labeled DNA fragments can yield different sizes when run with a different instrument, polymer, capillary array length, or size standard as shown below. High precision is important in relative sizing.



Guidelines for consistent sizing

- Use the same sizing method for all injections.

  To verify, check the analysis method in the GeneMapper® Manager or in the Size Match Editor window.
- Use the same size standard for all samples in a run. You may need to modify the size-standard definition of individual samples.

  To verify, overlay the size-standard peaks from all injections or display the sizing curve for each sample file.
- Verify that all defined size-standard peaks are present in the Size Match Editor.

  Variable run conditions can occasionally cause size-standard peaks not to be detected, for example if a run is too fast, too slow, or if the signal intensity of some of the peaks is too low.
- Use an Analysis Range that includes all the scans (or data points) where size-standard peaks occur in the raw data of each sample.

  To verify, check the analysis method in the GeneMapper® Manager.

Autoanalysis and manual analysis (GeneMapper® Software only)

The GeneMapper® Software provides two analysis options:

- **Autoanalysis** – The software applies an analysis method, size standard, and (optionally) panel to the fragment analysis files immediately after Data Collection Software collects the data from the instrument. The analysis settings are saved in the GeneMapper® Software project. You can review the analyzed data using GeneMapper® Software.
- **Manual Analysis** – You obtain the fragment analysis files from the computer connected to the instrument. If the Data Collection Software and the GeneMapper® Software are installed on the same computer, you can import the data files into the GeneMapper® Software. If the Data Collection Software and the GeneMapper® Software are installed on different computers, move or copy the files to another computer that has GeneMapper® Software installed. To perform analysis, you manually apply the analysis parameters to the fragment analysis files in the GeneMapper® Software.

GeneMapper® Software features

Feature	Description
Autoanalysis	Yes, with the corresponding Data Collection Software version
Applications	Amplified fragment length polymorphism (AFLP®), loss of heterozygosity (LOH), microsatellite genotyping, and SNaPshot® genotyping. Analysis of large fragments (~1200 bp), BAC fingerprinting, genetic fingerprinting, multilocus variant analysis (MLVA), Fragile X assays, biodefense, T/B-cell clonality assay, bird sex identification, microsatellites, VNTRs, T-RFLP.
Regulatory compliance	Security and audit features to help users meet 21 CFR Part 11 requirements
Report	• Report Manager tools for customized report generation • Customization of the project auto-saving frequency
Analysis	• Definition of a linearity range in the analysis methods • Process Quality Values (PQVs) for automated evaluation
Sizing methods	Least Squares, Cubic Spline, Local Southern, and Global Southern
Instrument software	• Supports data generated on 3500 Series, 3730 Series, 3130 Series, and 310 instruments. • GeneMapper® Software v4.1 and later includes the ability to record and reapply the Size Standard Normalization factor calculated in 3500 Series Data Collection Software
Use environment	• Multiuser, client-server deployment • Remote auto-analysis and command line operation
Support	Fully supported by Thermo Fisher Scientific

Peak Scanner™ Software Features

Overview

Peak Scanner™ Software is a nucleic-acid-sizing software that identifies peaks and fragment sizes for application-specific capillary electrophoresis assays. This software allows you to annotate data with functions such as labeling, merging, and splitting peaks. The software stores all editing and analysis data in the original .fsa data files generated on Thermo Fisher Scientific genetic analysis instruments.

Peak Scanner™ Software is available free-of-charge on **www.lifetechnologies.com**.

Note: Thermo Fisher Scientific does not support Peak Scanner™ Software.

Use this software with data generated on 3730 Series, 3130 Series, and 310 instruments. It is not compatible with data generated on the 3500 Series instrument, which performs fragment sizing during data collection.

Features

- Import and analyze fragment analysis sample files (.fsa) from all currently supported Thermo Fisher Scientific genetic analyzers
- Analyzed data (sizing information) is written back to the sample files (.fsa)
- Ability to organize the sample files in a project
- Simultaneous viewing of raw and analyzed data
- Large fragment sizing up to 1200 bp
- Ability to define the expected linear range in large fragment size standards where non-linearity might be expected
- Expanded feature set for editing peaks that includes labeling, merging, and splitting peaks
- Customizable sizing table
- Ability to overlay sizing curves on analyzed data
- Ability to display and print plots in thumbnail view
- Lightweight software application with easy installation
- Ability to archive projects with sample files and associated reference data (analysis methods, size standards and so on) for data sharing purposes

Workflow

Step	GeneMapper® Software	Peak Scanner™ Software
Set up a run file	**1.** Create a new project. **2.** Click to add samples to the project.	Import Sample files (drag and drop or "add files" function.)
Define analysis parameters	In the Samples tab, specify the analysis parameters for the samples in the project: **1.** In the Analysis Method column, select or create an analysis method depending on the application used (AFLP®, Microsatellite, SNaPshot®) **2.** In the Panel column, select **None** or **Project Specific Panel**. **3.** In the Size Standard column, select the appropriate size standard.	Choose appropriate size standard and analysis method.
Analyze	Analyze the data by clicking the green arrow to analyze the samples in the project.	Analyze the data by clicking the green arrow to analyze the samples in the project.
Review the data	**1.** (Optional) In the Genotypes tab, review the Process Quality Value (PQV) columns (BD, BIN, CC, LPH, OBA, OS, PHR, SHP, SP, SPA, SPU, and XTLK). **2.** Review the size quality and sizing data: In the Samples tab, examine the Size Quality (SQ) scores and the size standards. **3.** Modify sizing or analysis parameters if necessary. **4.** Display the samples and genotypes plots. **5.** (Optional) Save/print/export the results.	**1.** View sizes in sizing table and label and/or edit peaks. **2.** Save project and print or export results if necessary. **3.** Check sizing quality. **4.** (Optional) Modify sizing or analysis parameters if necessary.

For a list of the GeneMapper® Software documents available, see , "Documentation and Support" on page 199.

GeneMapper® Software peak detection settings

Peak Amplitude Thresholds

Only peaks with heights that exceed the peak amplitude threshold values for a dye color are detected.

Smoothing

Smoothing optimizes peak size and can reduce the number of false peaks detected:

- None (default) applies no smoothing. None is useful if the data display sharp, narrow peaks of interest.
- Light provides the best results for typical data. Light smoothing slightly reduces peak height in the electropherogram. It does not affect tabular data.
- Heavy is useful for data from slower runs that display broad peaks or to avoid the detection of sharp edges. This selection may reduce peak size or eliminate narrow peaks in the electropherogram. It does not affect tabular data.

Baseline Window

The Baseline Window adjusts the baselines of all detected dye colors to the same level for an improved comparison of relative signal intensity and helps to eliminate noise from the baseline.

- If the Baseline Window value is too low, the baseline approaches the peaks and the data display shorter peaks.
- If the Baseline Window value is too high, the baseline is too low and the data display elevated and possibly not baseline-resolved peaks.

Min. Peak Half Width

The Min. Peak Half Width setting specifies the smallest full width at half maximum for peak detection.

Use a low value if the data display narrow peaks.

If the value is high, noise spikes are ignored.

Polynomial Degree and Peak Window Size parameters

Use the Polynomial Degree and the Peak Window Size settings to adjust the sensitivity of the peak detection. You can adjust these parameters to detect a single base pair difference while minimizing the detection of shoulder effects or noise.

Sensitivity increases with larger polynomial degree values and smaller window size values. Conversely, sensitivity decreases with smaller polynomial degree values and larger window size values.

The peak detector calculates the first derivative of a polynomial curve fitted to the data within a window that is centered on each data point in the analysis range.

Using curves with larger polynomial degree values allows the curve to more closely approximate the signal and, therefore, the peak detector captures more peak structure in the electropherogram.

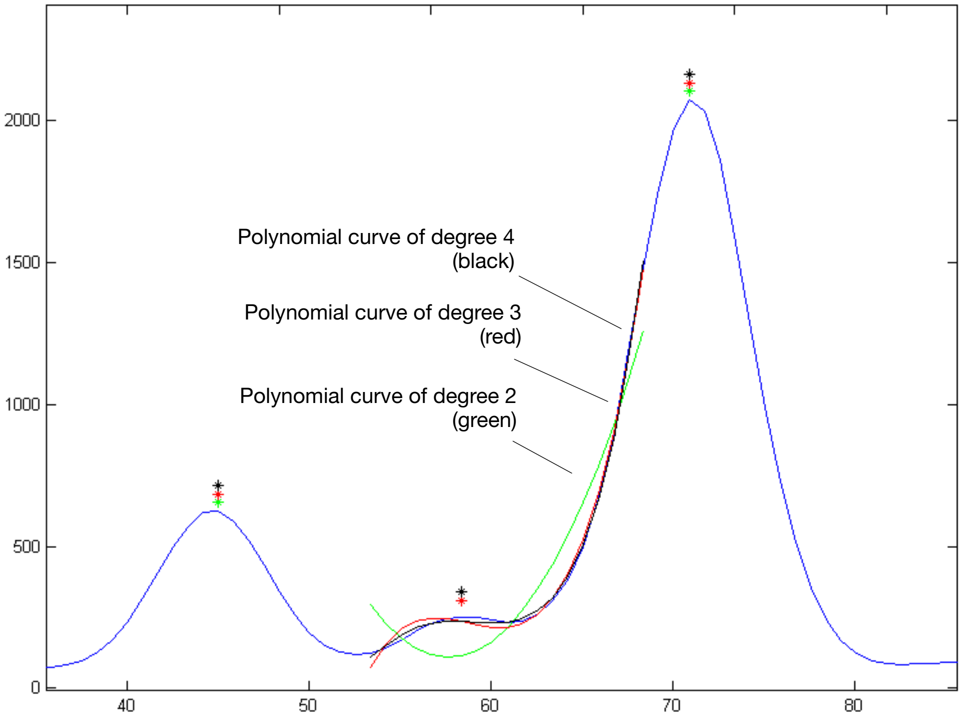
The peak window size sets the width (in data points) of the window to which the polynomial curve is fitted to data. Higher peak window size values smooth out the polynomial curve, which limits the structure being detected. Smaller window size values allow a curve to better fit the underlying data.

Function	Polynomial Degree Value	Window Size Value
Increase sensitivity	Higher	Lower
Decrease sensitivity	Lower	Higher

Effects of varying the Polynomial Degree

The figure below shows peaks detected with a window size of 15 data points and a polynomial curve of degree 2 (green), 3 (red), and 4 (black). The diamonds represent a detected peak using the respective polynomial curves.

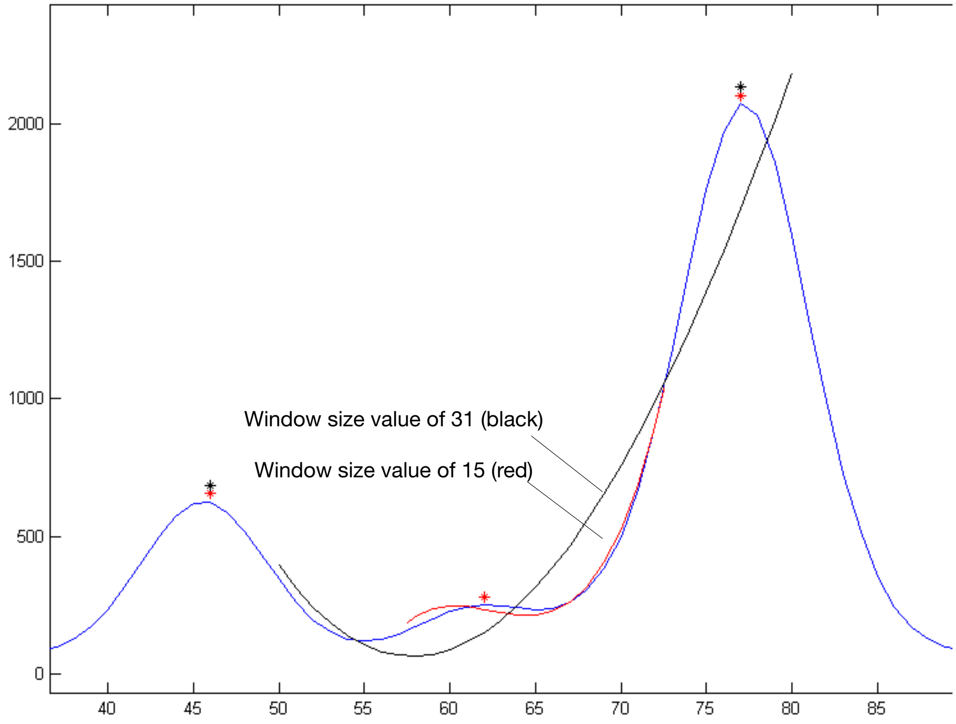
Note that the smaller trailing peak is not detected using a degree of 2 (green). As the peak detection window is applied to each data point across the displayed region, a polynomial curve of degree 2 could not be fitted to the underlying data to detect its structure.



Effects of Increasing the Window Size Value

In the figure below, both polynomial curves have a degree of 3 and the window size value was increased from 15 (red) to 31(black) data points.

As the cubic polynomial is stretched to fit the data in the larger window size, the polynomial curve becomes smoother. Note that the structure of the smaller trailing peak is no longer detected as a distinct peak from the adjacent larger peak to the right.

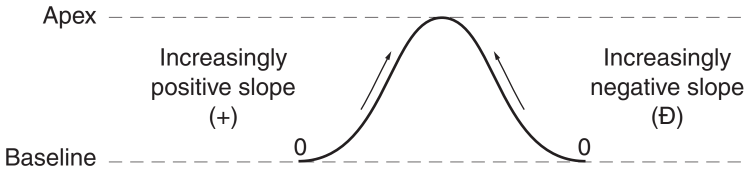


GeneMapper® Software peak start and end settings

The Slope Threshold for Peak Start and Slope Threshold for Peak End parameters adjust the start and end points of a peak.

The values assigned to these parameters can be used to better position the start and end points of an asymmetrical peak, or a poorly resolved shouldering peak to more accurately reflect the peak position and area.

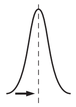
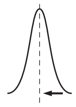
In general, from left to right, the slope of a peak increases from the baseline up to the apex. From the apex down to the baseline, the slope decreases negatively until it returns to zero at the baseline (see the figure below).


Apex
Increasingly positive slope (+)
Increasingly negative slope (Ð)
Baseline
0
0

Note the following:

- For typical or symmetrical peaks, use a value of zero.
- For asymmetrical peaks, select values other than zero to better reflect the beginning and end points.
- A value of zero does not affect the sizing accuracy or precision of an asymmetrical peak.

Note: The size of a detected peak is the calculated apex between the start and end points of a peak. Peak size does not change based on start and end settings.

To move the...	Then...	Example
Start point of a peak closer to its apex	Change the Slope Threshold for Peak Start value from zero to a positive number.	
End point of a peak closer to its apex	Change the Slope Threshold for Peak End value to a more negative number.	

How the GeneMapper® Software performs sizing

The GeneMapper® Software and the Peak Scanner™ Software use settings in the analysis method to size samples.

This section provides a brief description of the sizing process. For more information, see the *GeneMapper® Software Reference and Troubleshooting Guide* (Pub. no. 4403673).

Size-standard definitions

During sizing, the software compares the size of observed fragments for the size standard in the sample to the expected fragment sizes listed in the size-standard definition used for analysis.

The GeneMapper® Software includes several size-standard definitions. You can also create your own size-standard definition files or download updated size-standard definitions from our website.

Data from 3500 Series instruments can be analyzed with a size-standard definition that specifies normalization if the data was collected with a normalization standard.

Step 1: Size matching

Size matching uses ratio matching, based on relative height and distance of neighboring peaks. It then derives quality values statistically by examining the similarity between the theoretical (from the size-standard definition) and actual (observed) fragment patterns (see the figure on the next page).

To complete this step successfully, the analysis software must match at least three peaks.

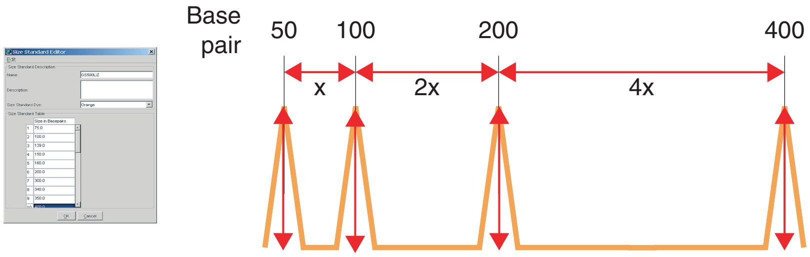
The software ignores anomalous peaks that do not match the expected patterns. The software constructs a best-fit curve using the data points of each size-standard fragment detected. A comparison between the sizes calculated from the best-fit curve and the matched peaks from the size-standard definition using the array of numbers is performed. Size-matching (and subsequent sizing) fails if significant differences in peak patterns are found, if no match can be made based on the expected patterns, or if all peaks are not found.

Because the software uses ratio-matching (looks for the expected number of alleles and expected peak patterns instead of specific data points), it is not necessary to define new size-standard definitions to accommodate migration shifts.

Determines the expected peak spacing and height ratios

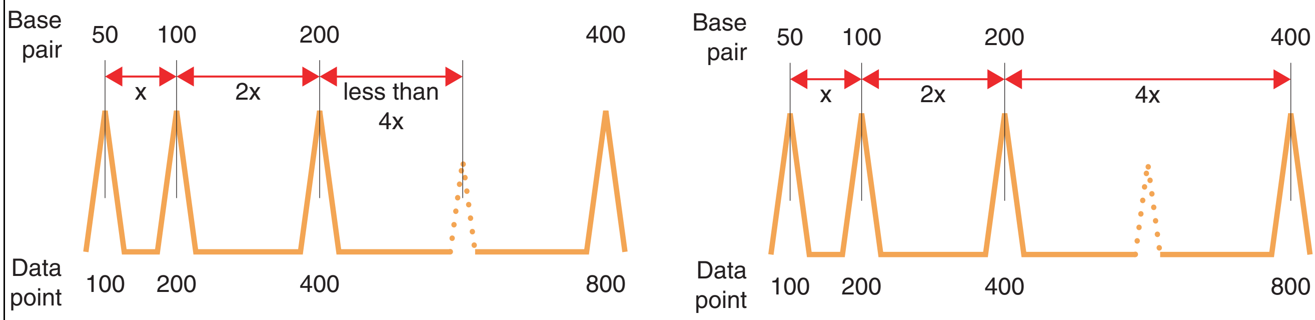
Uses the list of sizes from the Size Standard definition.

Note: The values used are for example only and do not reflect typical size standard values.


Base pair
50
100
200
400
x
2x
4x

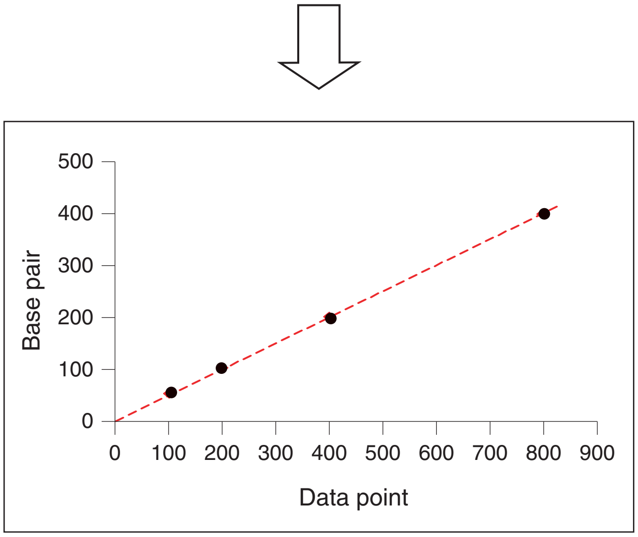
Evaluates peaks in the size standard data

Ignores peaks that do not meet expected pattern (dotted peak).


Base pair
50
100
200
400
x
2x
less than 4x
Data point
100
200
400
800
Base pair
50
100
200
400
x
2x
4x
Data point
100
200
400
800

Plots the sizing curve

Uses peaks that meet expected pattern



Step 2: Sizing curve and sizing

To generate the size-calling curve, the software plots the actual data points of the size standard against the expected size of each size standard peak. The size-calling method determines how the size-calling curve is generated and used to size each sample.

Factors that affect sizing

- The sizing method, size-standard definition, or size standard used to generate the sizing curve
- Well-to-read or time-to-read differences
- Electrophoresis conditions, such as run temperature, voltage, or the denaturing ability of the separation matrix
- Polymer type (POP-4®, POP-6™, POP-7™) and concentration
- Capillary length (22 cm, 36 cm, or 50 cm)
- Instrument model due to differences in instrument configuration

GeneMapper® Software sizing methods

The sizing methods available in classic and advanced modes in the analysis method of the GeneMapper® Software are:

- Least Squares
- Cubic Spline Interpolation
- Local Southern
- Global Southern

Global methods, which generate the best-fit curve from all matched fragments in the size standard, are less affected than local methods by anomalies in the run times of single size-standard fragments. Does not normalize capillary-to-capillary.

Local methods, which generate the best-fit curve from nearby size-standard data points, are less affected by changes in the electrophoresis conditions or in the analysis range. (A change in the analysis range changes the subset of size-standard fragments that is available for generating the sizing curve.) Normalizes capillary-to-capillary.

Least Squares method

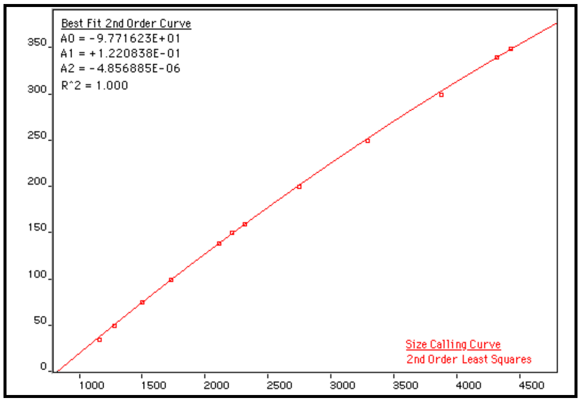
Advantages

Both Least Squares methods (2nd Order and 3rd Order) use regression analysis to build a best-fit sizing curve. This curve compensates for any fragments that may run anomalously. As a result, this method normally results in the least amount of deviation for all the fragments, including the size standards and the samples. Depending on whether you choose the 2nd or 3rd Order Least Squares Method in the Analysis Parameters dialog box, the resulting size curve is either a quadratic or a cubic function. The software uses the known standard fragments and the associated data points to produce a sizing curve based on Multiple Linear Regression.

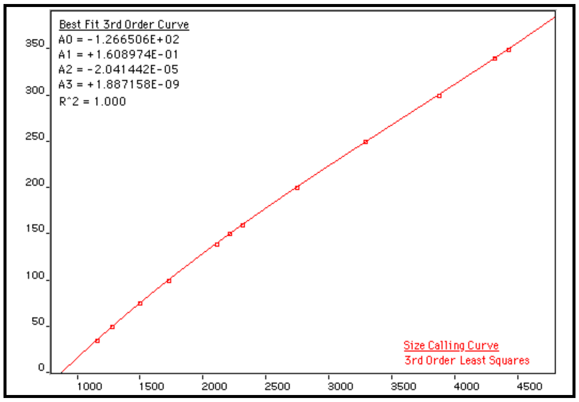
In the following figures, you can see that in nearly all instances the mobility of an individual DNA fragment is coincident with the best curve fit of the entire data set. Stated differently, the mobility of most DNA fragments is strictly length-dependent. This method automatically compensates for fragments that run anomalously. GeneMapper® Software calculates a best-fit least squares curve for all samples, regardless of the sizing method you choose. The curve is black in the Standard Sizing Curve window.

Note: All of the graphs in this section were generated using GeneScan™ Software v3.7.1. These results are similar to results obtained when you use GeneMapper® Software v3.5 and higher.

2nd-Order Least Squares sizing curve



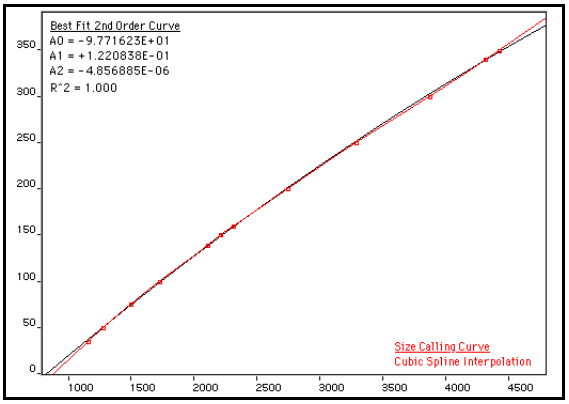
3rd-Order Least Squares sizing curve



Cubic Spline Interpolation method

The Cubic Spline method forces the sizing curve through all the known points of the selected size standard. Although this enforcement produces exact results for the values of the standards themselves, it does not compensate for standard fragments that may run anomalously.

Cubic Spline interpolation sizing curve



Possible local sizing inaccuracy

Mobility of any DNA fragment can be affected by its sequence, and by secondary and tertiary structure formation. If any internal size-standard fragment has anomalous mobility, the Cubic Spline method may exhibit local sizing inaccuracy. For example: Assume that a standard fragment is close in molecular length to an unknown sample fragment. Assume further that the standard fragment runs anomalously. The Cubic Spline method assigns the official value to this standard fragment, even though it may be slightly incorrect. The size of the unknown fragment is then likely to be calculated incorrectly as well.

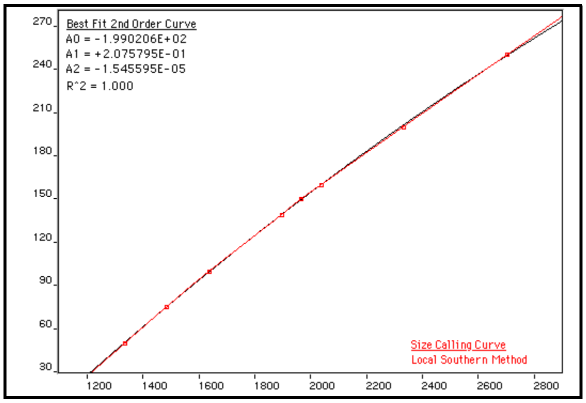
Note: This method does not determine the amount of sizing accuracy error.

Local Southern method

The Local Southern method determines the sizes of fragments by using the reciprocal relationship between fragment length and mobility, as described by E. M. Southern (1979).

IMPORTANT! For the Local Southern Method to work, you must have at least two size-standard fragments smaller than your smallest unknown fragment and two size-standard fragments larger than your largest unknown fragment. If you do not, a second order least squares curve extrapolation will be used to derive the size curve, instead of the method specified in the analysis method.

Local Southern sizing curve



Local Southern Method equation

L = [c/(m-m0)] + L0

The equation attempts to describe the reciprocal relationship between the mobility, m, and the length, L0, of the standard fragments.

How the Local Southern method works

This method, which is similar to the Cubic Spline method, uses the four fragments closest in size to the unknown fragment to determine a best fit line value. Only the region of the size standard near the fragment of unknown length is analyzed.

Note: Size estimates may be inaccurate if any of the size-standard fragments run anomalously.

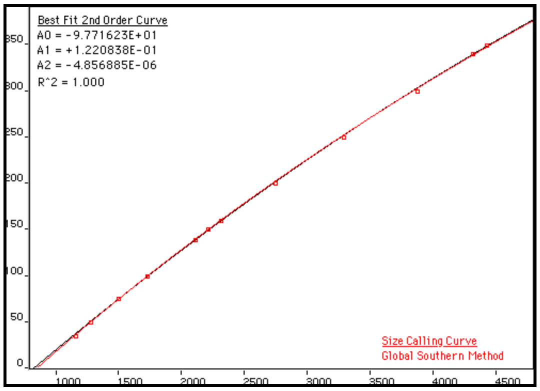
This is how the Local Southern method works:

1. The fitting constants of the curve are calculated for each group of three neighboring points on the standard. A separate curve is created for each set of three points.
2. A curve is then created by using three standard points (two points below and one point above the fragment) and a fragment size is determined.
3. Another curve is created by looking at an additional set of three points (one point below and two points above the fragment) and another value is assigned.
4. The two size values are averaged to determine the unknown fragment length.

Global Southern method

This method is similar to the Least Squares method in that it compensates for standard fragments that may run anomalously. The method creates a best-fit line through all the available points, and then uses values found on that line to calculate the fragment values.

Global Southern sizing curve



Global Southern method equations

Equation	Description
$L = [c/(m - m0)] + L0$	Attempts to describe the reciprocal relationship between the mobility, m, and the length, L0, of the standard fragments.
$\sum i\{Li - [c/((mi - m0) + L0)]\}^2$	The fitting constants L0, m0, and c are calculated by a least-squares fit to minimize the left side quantity.

How the Global Southern method works

All points in the standard are weighted equally and the curve is not constrained to pass through any specific point. The software can analyze a large range of fragment sizes with this method.

For best results, use a size standard that has at least two peaks smaller than the smallest fragment of interest and at least two peaks larger than the largest fragment of interest.

Evaluating data quality

Note: For detailed information on quality value determination, see the *GeneMapper® Software Reference and Troubleshooting Guide v4.1*(Pub. no. 4403673).

Examining PQVs

The GeneMapper® Software displays Process Quality Values (PQVs) in the Samples or Genotypes tab of the Project window.

Symbol	Definition	Default Range
🟩	Pass: The sample or genotype passed the PQV test.	0.75 to 1.0
⚠	Check: A possible problem exists for the sample or genotype.	0.25 to 0.75
🛑	Low Quality/Fail: There is a strong possibility that a problem exists for the sample or genotype.	0.0 to 0.25

Review the SQ (Sizing Quality) and GQ (Genotype Quality) results for each sample. Many of the PQVs can affect the GQ result.

Note: If the SQ PQV is 🛑, the sample is not sized or genotyped, and the GQ PQV is 🛑.

We recommend examining all samples that produce ⚠ (Check) or 🛑 (Low Quality) SQ flags.

For information on configuring and interpreting PQVs, refer to the *GeneMapper® Software Reference and Troubleshooting Guide v4.1*(Pub. no. *4403673*).

For information on troubleshooting SQ ⚠/🛑 results, see “Checking data quality” on page 153.

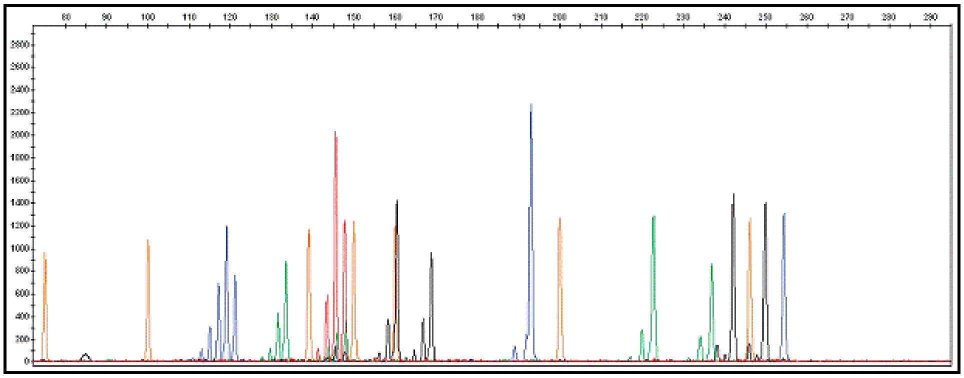
Criteria for a good electropherogram

Figure 20 illustrates an electropherogram that meets the following criteria:

- Peak heights are ≥50 RFU (peaks <50 RFU are considered to be noise).

  Ideal peak heights are:
  - 3500 Series instruments: ≥175 RFU
  - 3730 Series, 3130 Series, and 310 instruments: ≥150 RFU
- Peaks are sharp with no shoulders or splits.
- The peaks corresponding to different color dyes may not be of equal intensity, but the data for the less intense colors should be clearly resolvable at higher magnification.
- All expected peaks are detected.
- Peaks are sized properly (see “Size standards” on page 42).
- If you are genotyping, samples are accurately genotyped.
- Results are reproducible.

Figure 20 Example of a good electropherogram



If electropherograms do not meet the criteria above, see Chapter 11, "Troubleshooting" on page 151.

Examining peak definitions

To examine how GeneMapper® Software has defined a peak, select **View ▸ Show Peak Positions**. The peak positions, including the beginning, apex, and end of each peak, are tick-marked in the electropherogram.

Comparing data

- Use the same GeneScan™ size standard labeled with the same dye for all samples in a single study.
- Compare peak areas, heights, and sizes in nucleotide bases only if fragments are labeled with the same dye. For more information, see "Precise versus accurate sizing" on page 90 and "Relative sizing" on page 90.
- Compare only data that is collected under the same conditions (capillary array length, polymer type and electrophoretic run conditions) for the same study because these conditions affect the relative size of the fragment.

6

Microsatellite Analysis

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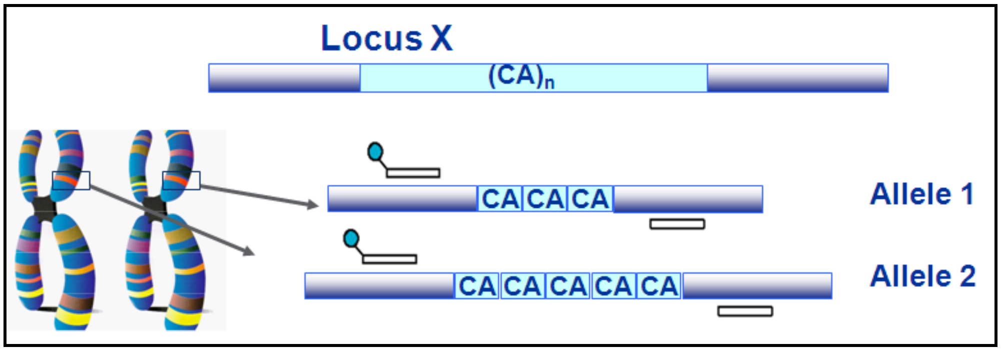
Overview of microsatellite analysis

Microsatellite markers, also called short tandem repeat (STR) markers, are polymorphic DNA loci that contain a repeated nucleotide sequence. Each repeat unit can be 2 to 7 nucleotides in length, and alleles differ by the number of repeats. The number of nucleotides per repeat unit is the same for a majority of repeats within a microsatellite locus.

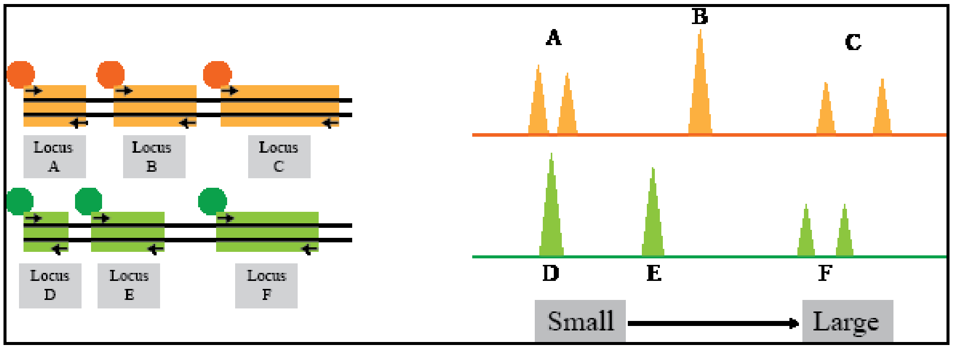


Microsatellite markers are also known as:

- Short tandem repeats (STRs)
- Simple sequence repeats (SSRs)
- Variable number tandem repeats (VNTRs)

Principle of the analysis

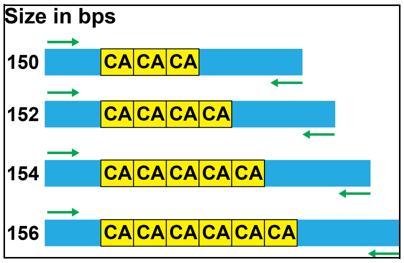
Microsatellite analysis is the separation of fluorescently labeled fragments using forward and reverse primers and determination of the relative size of the fragments.



A PCR primer pair consists of two oligonucleotides (forward and reverse primers), typically 15 to 30 nucleotides long. Each primer hybridizes to its respective complementary strand of the DNA template such that the primer pair flanks the region of interest. Based on the application, one or both of the primers may be labeled with a fluorescent dye.

The number of repeat units at a microsatellite locus may differ, so alleles of many different lengths are possible at each locus. The microsatellite marker in the figure below contains a dinucleotide repeat. When PCR is performed using primers that flank the region of interest, PCR fragments of different sizes are generated based on the length of the dinucleotide repeat.

Figure 21 Different repeats lead to PCR fragments of different length (arrows indicate forward and reverse primers)



Advantages of using microsatellite markers (loci) in genetic studies

Several features of microsatellites and their corresponding set of alleles make them ideal for use in genetic studies:

- They are present in large numbers.
- They are relatively evenly spaced throughout the genome and often physically situated near or within genes.
- They show a varying, but relatively high mutation rate relative to non-microsatellite loci:
  - Mitochondrial DNA evolves 5 to 10 times faster than single-copy nuclear DNA
  - Microsatellites evolve 100 to 1000 times faster than single-copy nuclear DNA

  The mutation rate of microsatellite loci is 10^{-2} to 10^{-6} events per locus per generation (Wan, *et al.*, 2004). The rate is believed to be different depending on the number of nucleotides in the repeated unit (Eckert and Hile, 2009).

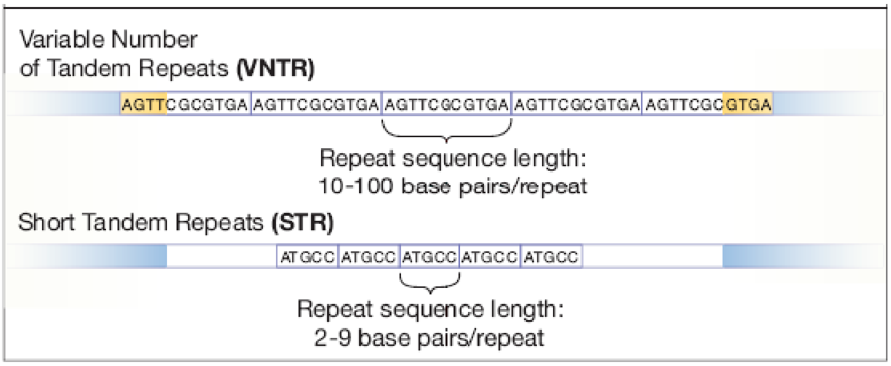
- Their alleles are inherited in a Mendelian manner and are stable over multiple generations.
- Their alleles can be unique to specific populations.
- Detailed data on allelic variation, number of repeats, and allelic frequencies are widely available for a large number of microsatellite markers.
- The small size of microsatellite loci improves the chance of obtaining a result, particularly for samples containing very low amounts of DNA and/or degraded DNA.
- The small size range of microsatellite loci makes them ideal candidates for co-amplification while keeping all amplified alleles smaller than 350 base pairs. Many microsatellite loci can therefore be typed from a single PCR.
- Microsatellite alleles have discrete sizes, allowing for simplified interpretation of results.
- PCR-based tests are rapid, giving results in 24 hours or less.
- PCR-based tests are easy to standardize and automate, ensuring reproducible results.

Microsatellite motifs and distribution

STRs typically contain 2 to 7 nucleotide repeats.

VNTRs contain 10 to 100 nucleotide repeating motifs.

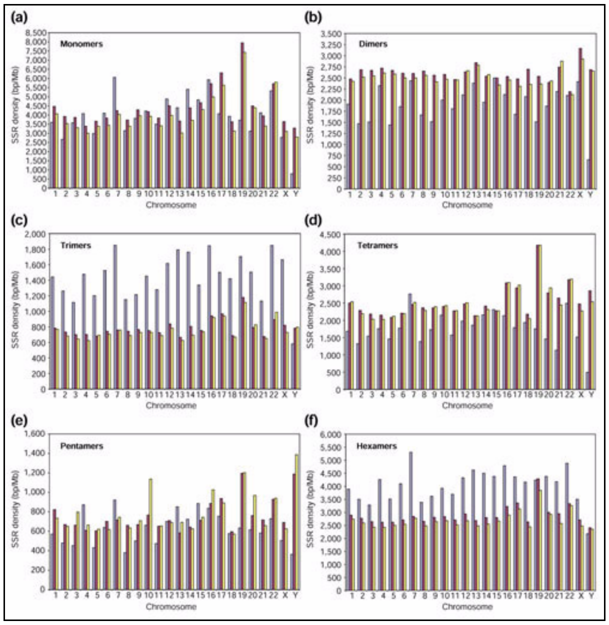
Figure 22 STRs compared to VNTRs


Variable Number
of Tandem Repeats (VNTR)
AGTTCGCGTGA AGTTCGCGTGA AGTTCGCGTGA AGTTCGCGTGA AGTTCGCGTGA
Repeat sequence length:
10-100 base pairs/repeat
Short Tandem Repeats (STR)
ATGCC ATGCC ATGCC ATGCC ATGCC
Repeat sequence length:
2-9 base pairs/repeat

Often, the length of the repeating unit correlates with its frequency within a genome. For example, in the human genome, mononucleotide repeats are the most common form of microsatellites found, and pentanucleotide and hexanucleotide repeats are the least common (Ellegren, 2004).

However, the frequency of a repeating unit can vary across a particular chromosome as shown in the following figure.

Figure 23 SSR density in exonic, intronic and intergenic regions on individual human chromosomes: (a) monomers (b) dimers; (c) trimers; (d) tetramers; (e) pentamers; (f) hexamers. Blue bars, exons; red bars, introns; yellow bars, intergenic regions (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC151303/figure/F2/ Copyright 2003, Subramanian *et al.*; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.)



Applications

The large selection of highly informative markers has made microsatellite analysis a widely accepted tool for the following types of studies:

- Linkage mapping studies
- Association studies
- Population studies
- Parentage analysis
- Breeding

Custom microsatellite assays are often used for:

- Cancer progression analysis
- Phylogenetic studies
- Genome scans for an organism where commercial marker panels are not available
- Population genetics studies
- Paternity testing
- Parentage analysis for selective breeding

Instrument and consumable recommendations

This is a “Thermo Fisher Scientific-supported” protocol.

- Thermal cycler: 2720, Veriti®, or GeneAmp® 9700
- Genetic analyzer: 3500 Series, 3130 Series, or 310 instrument
- Polymer and capillary array: see “Run modules” on page 69 for the polymer and capillary array length combinations supported on each instrument
- 600 LIZ® Size Standard
- DS-33 G5 dye set
- AmpliTaq® and AmpliTaq Gold® DNA Polymerases are typically used for microsatellite analysis. Like other DNA polymerases, these polymerases may catalyze the addition of a single nucleotide (usually A, adenosine) to the 3′ ends of the two strands of a double-stranded DNA fragment. For more information, see “Addition of 3' A nucleotide by Taq polymerase” on page 33 and Witmer *et al.*, 2003.

IMPORTANT! Throughout a set of experiments, use the same equipment, run parameters, polymers, dyes, and so on. Consistent conditions are required to avoid mobility shifts that interfere with accurate interpretation of data.

Experiment and primer design recommendations

- Identify the markers for your study by examining the existing scientific literature for a specific marker or cross-species marker, or by following a microsatellite development protocol (Fleischer and Lowe, 1995; Kandpal *et. al.*, 1994).
- The discovery and random naming of new microsatellite markers across different organisms at multiple institutions has led to inconsistent nomenclatures for microsatellites. For more information on nomenclature standards for specific genomes, go to the nomenclature website of the institution, a few of which are listed below.
  - Human: **https://iris.ucl.ac.uk**
  - Rat/mouse: **http://rgd.mcw.edu/**
  - Fly: **http://flybase.org/**
- Design primers so the range of amplicon lengths across markers in the study spans is within the size-standard fragment range, with two size-standard peaks preceding the smallest fragment of interest and two size-standard peaks following the largest fragment of interest.
- Use 5′-end labeled primers. The success of microsatellite analysis depends upon the ability to detect small mobility differences. The reproducible sizing and sharp peaks obtained when using the 5′-end labeling method are crucial to the success of this application.
- If you plan to multiplex, design primers with similar annealing temperatures ~60°C.
- Use reverse-primer tailing on one primer in each set of primers to help differentiate between peaks made by the forward and reverse DNA strands and to promote A addition. See “Tail” on page 34 for more information.

- Based on sample DNA concentration, robustness of the PCR, and/or peak heights observed in capillary electrophoresis, determine whether you need to dilute the PCR products. Dilutions can range from undiluted to 1:20 in water. You can pool the diluted PCR products if desired.
- Dye-labeled PCR products must be mixed in different ratios because each dye has a slightly different fluorescence signal strength (see “Emission and absorption (excitation) wavelengths and relative intensities” on page 38).
- To avoid inaccuracies associated with pipetting small volumes, prepare a master mix of reagents. Prepare sufficient master mix for at least one extra reaction volume.
- Store the master mix in the dark at 2 to 6°C for up to 1 month, or at –15 to –25°C for longer.
- A typical reaction may include: 1 µL of each PCR product and 0.5 µL of the GeneScan™ size standard in 8.5 µL of Hi-Di™ formamide (for denaturing applications) or distilled, deionized water (for non-denaturing applications).
- Master-mix reagents are optimized for capillary electrophoresis, and differ depending on the capillary electrophoresis instrument you use.

Workflow

1. Select primers and size standards:
    a. Design and order primers for a microsatellite application.
    b. Optimize amplification conditions with microsatellite markers on test DNA.
    c. Order dye-labeled primers.
2. PCR
3. Capillary electrophoresis
4. Data analysis

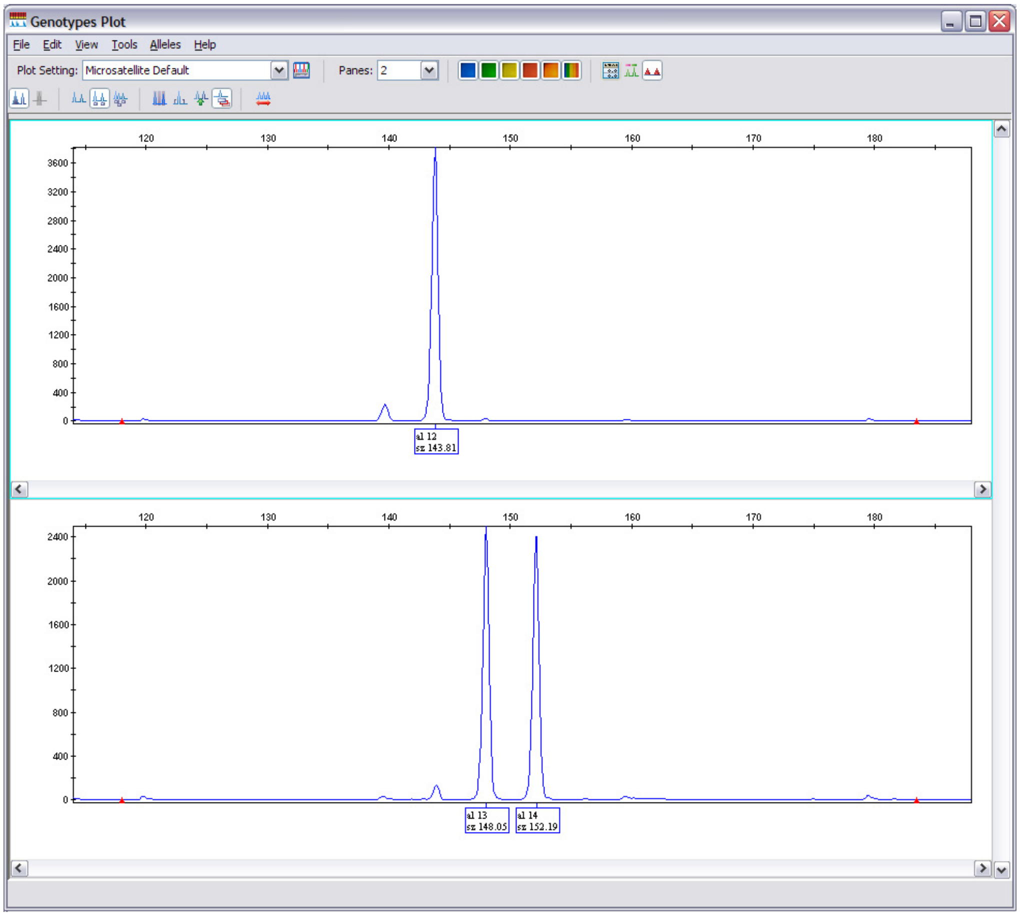
Data analysis

The GeneMapper® Software includes a Microsatellite Default analysis method that you can use as a starting point for analysis.

Figure 24 on page 113 shows a typical microsatellite electropherogram from the GeneMapper® Software.

The number of repeats for a given locus may vary, resulting in alleles of differing lengths. The following figure shows two different FAM™dye-labeled human dinucleotide loci (from the GeneMapper® Software tutorial dataset) from two individuals. The top panel illustrates a DNA sample that is homozygous at both loci (a single major peak is observed at each locus), the bottom panel shows a DNA sample that is heterozygous at the same loci (two major peaks are observed at each locus).

Figure 24 Example of microsatellite analysis of two samples by capillary electrophoresis. Samples have different genotypes as shown by the different peaks for the same marker.



Plot Setting: Microsatellite Default

Panes: 2

Genotypes Plot
File
Edit
View
Tools
Alleles
Help
120
130
140
150
160
170
180
3600
3200
2800
2400
2000
1600
1200
800
400
0
al 12
sz 143.81
2400
2000
1600
1200
800
400
0
al 13
sz 148.05
al 14
sz 152.19

Common problems with microsatellite analysis

The most commonly encountered problems during microsatellite analysis are:

- Poor or non-specific amplification. See “Optimizing PCR” on page 55 and “PCR troubleshooting” on page 176.
- Incomplete 3´ A nucleotide addition. See “Incomplete 3' A nucleotide addition” on page 33.
- Stutter. See the next section.

See Chapter 11, “Troubleshooting” on page 151 for more information on stutter peaks and plus A products.

Identifying stutter products in microsatellite analysis

Overview

During the PCR amplification of di-, tri-, and tetranucleotide microsatellite loci, minor products that are 1 to 4 repeat units shorter than the main allele are produced. The minor product peaks are referred to as “stutter” peaks. Stutter peaks may be caused by polymerase slippage during elongation (Hauge and Litt, 2003; Murray and Lai, 2003).

Stutter peaks appear as multiple lower peaks that precede the true allele peak. These stutter peaks differ in size from the true allele peak by multiples of the length of the repeat unit. The number of peaks and their intensities are proportional to the length of the repeat and the number of repeats in the PCR product (Shinde *et. al.* 1993). Shorter repeat units (di- or tri-, for ex.) generate more stutter, and dinucleotide repeats tend to generate more stutter peaks than trinucleotide repeats.

Stutter peaks can also be caused by off-scale data. For more information, see "Evaluating data with stutter" on page 117.

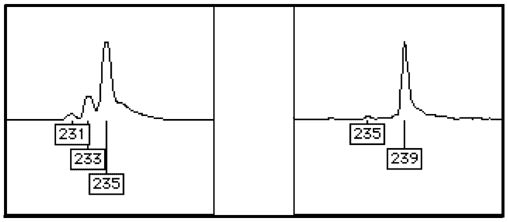
GeneMapper® Software is optimized to filter out stutter peaks.

Estimating the amount of stutter

You can estimate the percent stutter by calculating the ratio of the combined heights of the stutter peaks with the height of the true allele peak. Note the following:

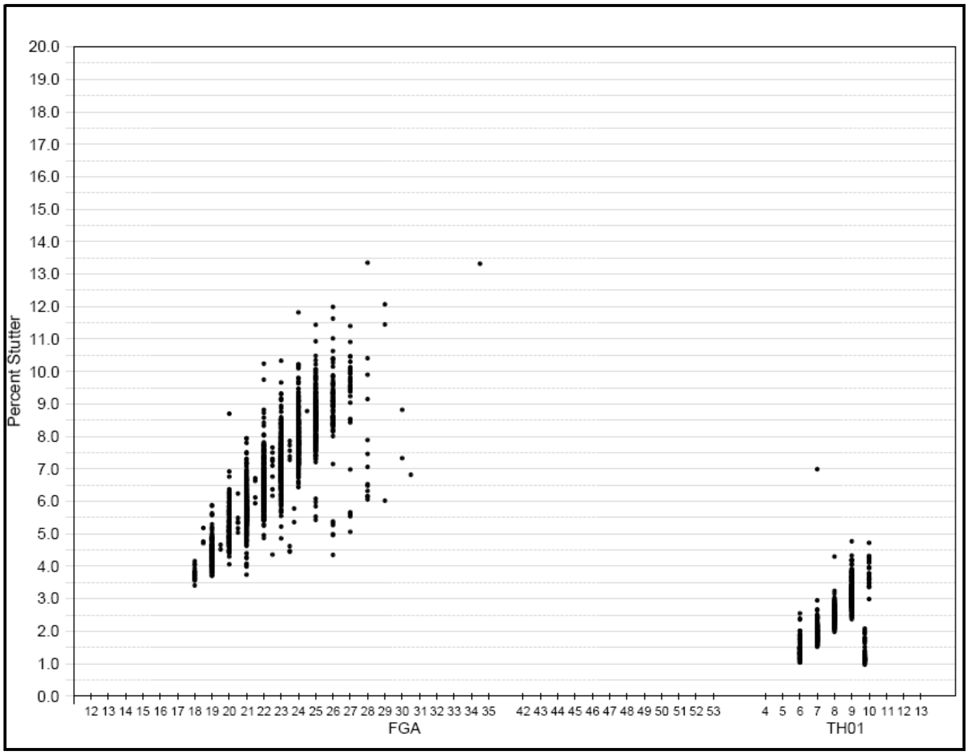
- The longer the repeat unit, the less stutter product produced. For microsatellite loci with the same number of repeat units, the percent stutter is greater for dinucleotide microsatellite loci than it is for trinucleotide microsatellite loci, and so on (Walsh *et al.*, 1996).

  The figure below illustrates the greater stutter in dinucleotide (left) as compared to tetranucleotide (right) repeat loci. Each locus is homozygous, with the largest peak in each figure representing the "true" allele.



- The percent stutter increases with increasing allele length (that is, with increasing number of repeat units). However, if some of the repeats are partial repeats, you may not see the proportionate increase in percent stutter.

Figure 25 Stutter percentages for the FGA and TH01 loci. (Black data points indicate loci labeled with NED™ dye.)



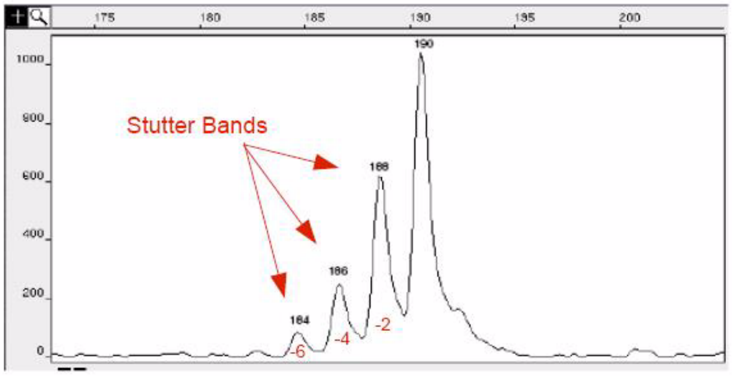
Dinucleotide repeats

Successful amplification of dinucleotide repeat markers yields allele peaks and associated stutter peaks within a maximum range of eight base pairs from the allele peak. In addition, the number of allele peaks depends on whether the individual tested is a heterozygote or homozygote.

Dinucleotide repeats in a homozygous individual

The GeneMapper® Software electropherogram of a dinucleotide repeat marker from a homozygous individual (190 bp, 190 bp) is shown in the following figure.

Figure 26 Stutter peaks in a dinucleotide repeat electropherogram (homozygote)



Typical pattern for a dinucleotide repeat marker from a homozygous individual

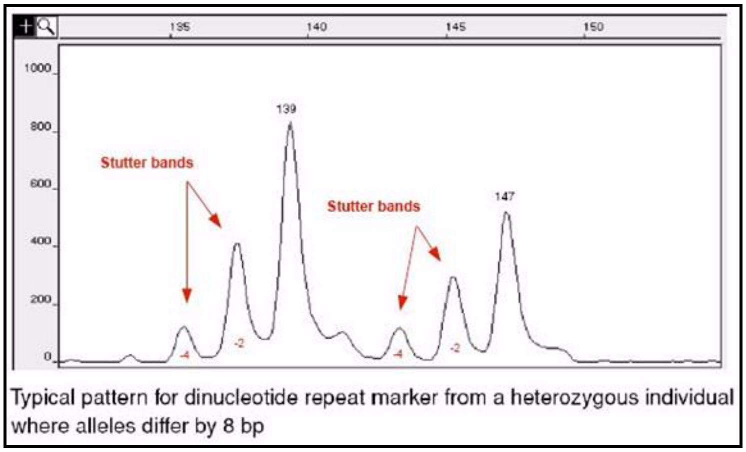
The peaks at 188 bp, 186 bp, and 184 bp show the typical 2-bp stutter pattern seen with dinucleotide repeats. They represent the –2 bp, –4 bp, and –6 bp stutter peaks from the true 190-bp true allele peak.

Dinucleotide repeats in a heterozygous individual (8 bp)

The GeneMapper® Software electropherogram of a dinucleotide repeat marker from a heterozygous individual (139 bp, 147 bp) is shown in the following figure. Allele sizes differ by 8 bp.

The 2-bp stutter peak to the left of each allele peak is always of lower intensity than the allele peak itself. The larger 147-bp allele peak is of lower intensity than the smaller 139-bp allele. In heterozygotes, the higher molecular weight allele (that is, the allele peak further to the right in electropherograms) often produces a fluorescence signal of lower intensity than the lower molecular weight allele, suggesting a less efficient amplification of the larger fragment. This phenomenon could also be caused by preferential injection of the smaller fragments.

Figure 27 Stutter peaks in a dinucleotide repeat electropherogram (heterozygote 8 bp)

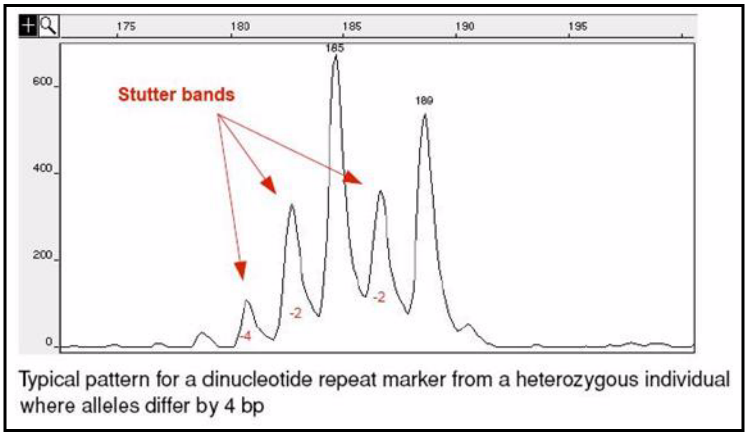


Typical pattern for dinucleotide repeat marker from a heterozygous individual where alleles differ by 8 bp

Dinucleotide repeats in a heterozygous individual (4 bp)

The GeneMapper® Software electropherogram from a dinucleotide repeat marker of a heterozygous individual (185 bp, 189 bp) is shown in the following figure. Allele sizes differ by 4 bp.

Figure 28 Stutter peaks in a dinucleotide repeat electropherogram (heterozygote 4 bp)



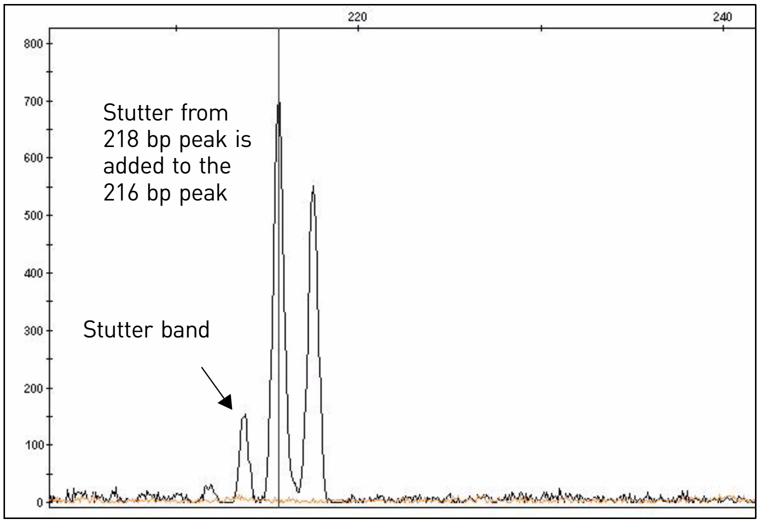
Typical pattern for a dinucleotide repeat marker from a heterozygous individual where alleles differ by 4 bp

When the difference between the allele sizes is ≤4 bp, a shift occurs in the height ratio between the two allele peaks (compare the two preceding figures). The fluorescence signal from the –4 bp stutter of the 189-bp allele is added to the signal from the 185-bp allele.

Dinucleotide repeats in a heterozygous individual (2 bp)

The GeneMapper® Software electropherogram from a dinucleotide repeat marker of a heterozygous individual (216 bp, 218 bp) is shown in the following figure. Allele sizes differ by 2 bp.

Figure 29 Stutter peaks in a dinucleotide repeat electropherogram (heterozygote 2bp)



When the difference between the allele sizes is ≤2 bp, the fluorescence signal from the –2 bp stutter of the larger base pair allele does not appear as a separate stutter peak. It is added to the signal of the smaller base pair allele.

Evaluating data with stutter

The multipeak pattern seen with stutter peaks can complicate analysis, particularly for samples with two or more alleles that are close in size. For example, small peaks in a position that is one repeat unit smaller than the true allele can be interpreted either as a stutter peak or as an allele in a minor component of a mixed sample. The possible presence of stutter peaks makes precise quantitation especially important, to allow the GeneMapper® Software filtering algorithm to interpret the peak pattern accurately.

The percent stutter for a given allele is reproducible and does not depend on the quantity of input DNA or the number of loci amplified during multiplex PCR. The relative reproducibility of percent stutter is important for a few reasons:

- In many cases, you can adjust the Peak Amplitude Threshold in the analysis method of the GeneMapper® Software to filter out stutter peaks and detect only true allele peaks. Fore more information, refer to the *GeneMapper® Software Getting Started Guide: Microsatellite Analysis* (Pub. no. 4403672).
- Amplifications with an abnormally high percent stutter can indicate mixed samples or some other problem with PCR amplification or electrophoresis.

Is stutter a real problem?

Stutter, once understood, does not pose a real problem for microsatellite analysis and can aid in allele calling by:

- Distinguishing true allele peaks from non-specific PCR products. Non-specific PCR products are not associated with stutter peaks.
- Identifying alleles that fall far outside the reported allele range. The percent stutter is often specific to a particular locus. You can sometimes identify alleles that fall far outside the previously reported range on the basis of percent stutter.

For more information

See "Microsatellite applications" on page 200.

7

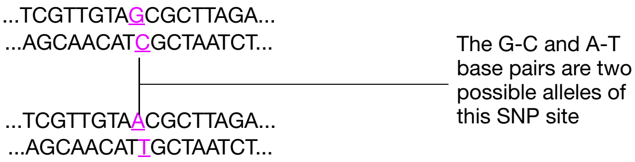
Single Nucleotide Polymorphism (SNP) Genotyping

- Overview of SNP genotyping 119
- SNaPshot® Multiplex System. 120

Overview of SNP genotyping

Overview

A Single Nucleotide Polymorphism (SNP) marker consists of a single base pair that varies in the known DNA sequence, thereby creating up to four alleles or variations of the marker.



The G-C and A-T base pairs are two possible alleles of this SNP site

SNP markers occur in the human genome at a frequency of about 1 in every 1000 bp, with a total number of over 10 million SNP markers distributed evenly over the 3 billion bps of the human genome. They have been shown to be responsible for differences in genetic traits, susceptibility to disease, and response to drug therapies. SNP markers are excellent genetic markers to construct high-resolution genetic maps.

SNP markers can be genotyped by a variety of methods. Thermo Fisher Scientific products support the following methods:

- Single-base extension
- Shifted Termination Assay (STA) primer extension

Applications (SNP)

Some applications of SNP genotyping include:

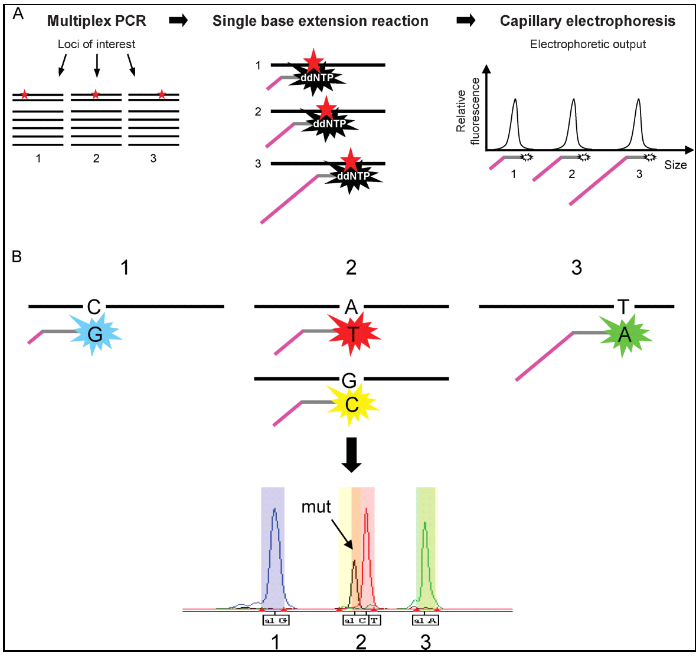
- Study of mutations implicated in various cancers
- Genetic disease research
- Mitochondrial DNA investigations
- Scrapie susceptibility in sheep
- Loss of heterozygosity
- Assess performance in food animal production,
- Differentiate drug and non-drug forms of *Cannabis*

SNaPshot® Multiplex System

Components

The SNaPshot® Multiplex System investigates up to ten SNP markers simultaneously by using PCR amplification, then dideoxy single-base extension of an unlabeled primer, and then capillary electrophoresis. After electrophoresis and fluorescence detection, the alleles of a single marker appear as different colored peaks at roughly the same size in the electropherogram plot. The size of the different allele peaks will vary slightly due to differences in molecular weight of the dyes.

Figure 30 Overview of the SNaPshot® kit assay


A
Multiplex PCR
Single base extension reaction
Capillary electrophoresis
Loci of interest
1
2
3
ddNTP
Electrophoretic output
Relative fluorescence
Size
B
1
2
3
C
G
A
T
G
C
T
A
mut
a1 G
a1 C T
a1 A

Components of the system are:

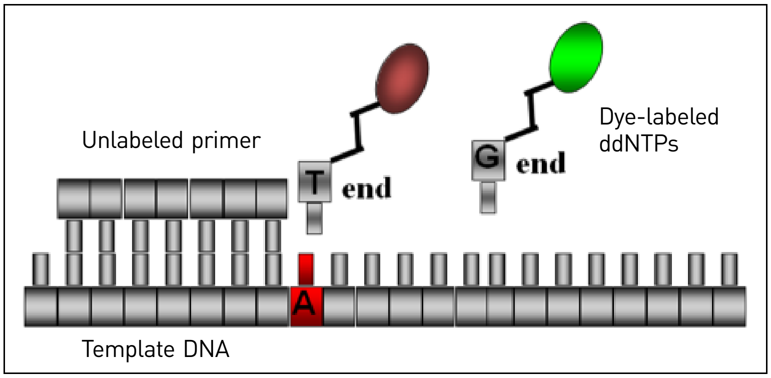
- **SNaPshot® Multiplex Kit** – Includes SNaPshot® Multiplex Ready Reaction Mix, control primer mix, and control template.
- **SNaPshot® Primer Focus® Kit** – Designed to determine the approximate fragment sizes generated by various primers before SNP genotyping (critical if two oligonucleotides produce overlapping signals when run simultaneously) and enables the setting of tight loci-windows in GeneMapper® Software.
- **GeneScan™ 120 LIZ® Size Standard** – Five-dye size standard that is designed for reproducible sizing of small fragment analysis data generated with the SNaPshot® Multiplex Systems. It accurately sizes samples ranging from 20 to 120 nucleotides (nt). When used with GeneMapper® Software, the GeneScan™ 120 LIZ® Size Standard eliminates the need for manual genotyping.
- **Matrix Standard Set DS-02** – Used for spectral calibration.
- **GeneMapper®** Software – Genotype analysis for data generated with SNaPshot® Multiplex Systems.

Additionally, the SNaPshot® Primer Focus® Kit allows rapid assessment of potential SNP oligonucleotides. You can preview all potential single-base extension products and calculate the mobility rate for each allele. After assessing this data, you can determine the optimal combination of SNP markers for multiplexing. After you determine the multiplex format, you can use the reference data created with the Primer Focus Kit to establish markers and bin sets in the GeneMapper® Software, and reduce the time required to define and edit bins manually.

Principle of the analysis

In the single-base extension technique, a unlabeled primer is designed to anneal to the sequence adjacent to the SNP site. After the primer anneals, the single-base extension occurs by the addition of the complementary dye-labeled ddNTP (dye terminator) to the annealed primer. Each of the four ddNTPs is fluorescently labeled with a different color dye (Figure 31).

Figure 31 Single-base extension with dye-labeled ddNTPs



The addition of ddNTPs yields marker fragments for the different SNP alleles that are all the same length, but vary by color.

After electrophoresis and fluorescence detection, the alleles of a single marker appear as different colored peaks at roughly the same size in the electropherogram plot. The size of the different allele peaks will vary slightly due to differences in molecular weight of the dyes.

Advantages

- Uses unlabeled user-defined primers that are customized for your target
- Offers multiplexing capability (up to 10-plex, regardless of their positions on the chromosome or the amount of separation from neighboring SNP loci)
- Sensitive allele frequency detection (5%)
- Compatible with all Thermo Fisher Scientific genetic analyzers
- Automated analysis using specific GeneMapper® Software data analysis module

Additionally, the SNaPshot® kit can be used for a variety of other applications:

- BAC fingerprinting
- DNA methylation

Applications (SNaPshot®)

- Low-to-medium throughput linkage and association studies
- Single-locus fragment analysis

- Screen and confirm SNPs
- Screen for prion gene mutation

Screen and confirm SNPs

The SNaPshot® Multiplex System includes a variety of SNaPshot® Multiplex Kits used for SNP screening and validation. Each kit offers a one-tube single-base extension/termination reagent to label DNA fragments.

Screen for Prion gene mutations

The single base-pair sensitivity of the SNaPshot® Multiplex System enables you to accurately screen samples for codon differences in prion genes. Prion diseases are caused by abnormally folded isoforms of host-encoded proteins. Use the SNaPshot® Multiplex System to screen for SNPs in the genes that code for these proteins. For instance, polymorphisms at codons 136, 154 and 171 of the PrP gene in sheep and goats can lead to abnormally folded isoforms of the protein product to result in scrapie.

Instrument and consumable recommendations

- Thermal cycler: Veriti®, or GeneAmp® 9700 (for fast thermal cyclers, use a 1°C/second ramp rate), 2720
- Genetic analyzer: 3500 Series, 3730 Series, 3130 Series, or 310 instruments
- Polymer and capillary array: see “Run modules” on page 69 for the polymer and capillary array length combinations supported on each instrument
- GeneScan™ 120 LIZ® Size Standard
- DS-02 dye set

IMPORTANT! Throughout a set of experiments, use all the same equipment, run parameters, polymers, dyes, and so on. Consistent conditions are required to avoid mobility shifts that interfere with accurate interpretation of data.

Experiment and primer design recommendations

This is a "Thermo Fisher Scientific-supported" protocol.

- Minimum primer length is 23 nt, however it is strongly recommended that primers shorter than 36 nt be tested before multiplexing.
- HPLC purification of primers longer than 30 nt is recommended. Heterogeneous primer populations will lead to major analysis issues.
- Each primer should have 23 nt complimentary to the gDNA sequence.
- Use 5′ tails to create different length primers.
- Add poly (dGACT) to generate a size difference of at least 4 to 6 nt.
- Primers can be complimentary to the (-) strand of the DNA if the (+) strand is difficult to assay.
- Always run a negative control (no template DNA) when evaluating a new primer.

Workflow

1. Design primers.
2. Prepare template by PCR of target, then clean-up
3. Prepare SNaPshot® reactions
4. Post-extension by PCR, then clean-up
5. Capillary electrophoresis
6. Analyze data

Data analysis

The GeneMapper® Software includes a SNaPshot® Default analysis method that you can use as a starting point for analysis.

For more information

For documents and publications, see "SNP applications" on page 201.

An extensive list of publications demonstrating the utility of the SNaPshot® Multiplex System is available at **www.lifetechnologies.com**.

For ordering information, see "SNaPshot® Kits" on page 198.

8

Fingerprinting

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- Inter-simple sequence repeat (ISSR) PCR 137

Overview

DNA fingerprinting is a technique that is used to identify patterns that occur in genetic markers. These fingerprints are specific to particular organisms. A number of techniques are available for fingerprinting.

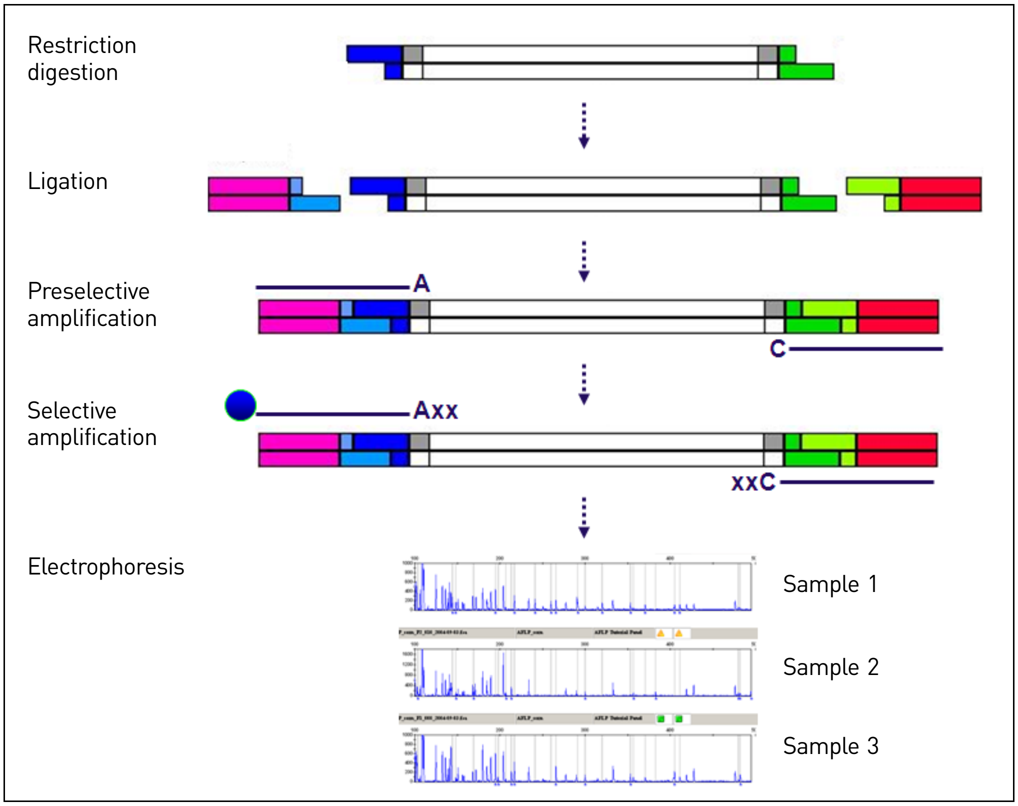
Amplified fragment length polymorphism (AFLP®) Analysis

Amplified fragment length polymorphism (AFLP®) is a mapping technique used to visualize polymorphisms in genomic DNA. The AFLP® system combines the restriction fragment length polymorphism (RFLP) technique and polymerase chain reaction (PCR) to generate a large number of amplified restriction fragments from prepared, genomic DNA. When separated by electrophoresis, the samples yield unique band patterns that, when visualized by southern blot or fluorescence-based fragment analysis, can be used for high-resolution genotyping, polymorphism detection, or cladistics (Savelkoul *et al*. 1999).

Principle of the analysis

The AFLP® procedure involves digesting genomic DNA to produce a population of restriction fragments, ligation of priming sites, then amplified by PCR. (Goel *et al.* 2006.) It is sometimes considered a variation of random amplified polymorphic DNA (RAPD).

Figure 32 AFLP® analysis



AFLP® is possible because the abundant complexity in eukaryotic genomic DNA means that it is statistically likely that enough restriction fragments will be short enough to successfully produce PCR amplicons that yield a unique “fingerprint” profile.

Advantages

The power of AFLP® analysis derives from its ability to quickly generate large numbers of marker fragments for any organism, without prior knowledge of the genomic sequence. In addition, AFLP® analysis requires only small amounts of starting template and can be used for a variety of genomic DNA samples.

Applications

Fingerprints, or AFLP® band patterns, can be used for many purposes. For example, AFLP® analysis is often used in plant research where fingerprints can be compared to determine the plant variety or to compare the similarities between different plant varieties.

Thermo Fisher Scientific provides kits for performing AFLP® on microbes and plants, and reagents that are useful for performing AFLP® on other organisms. Some additional applications for AFLP® analysis include:

- Molecular diversity studies (Zhao *et al.* 2006 and Johnson *et al.* 2005.)
- Phylogeny studies (Goel *et al.* 2006.)
- Breeding (Zhao *et. al.*, *ibid.*)
- Backcross studies (Johnson *et al.* and Goel *et al.*, *ibid.*)
- Mapping of cloned fragments in bacterial and yeast artificial chromosomes (BACs and YACs) (Serra, *et al.* 2006, Naimuddin, *et al.* 2004)
- Identifying new species or subspecies (Johnson *et al. ibid.*, and Savelkoul, *et al.* 1999.)

The AFLP® kits available from Thermo Fisher Scientific are optimized for plants and microbes. However, they are an excellent starting point for custom AFLP experiments on other organisms (such as fish). Contact your Thermo Fisher Scientific field applications specialist for more information on using Applied Biosystems®AFLP kits to conduct experiments in organisms other than plants or microbes.

Instrument and consumable recommendations

This is a “Thermo Fisher Scientific-supported” protocol.

- Thermal cycler: Veriti® (standard mode only), GeneAmp® 9700, 2720
- Genetic analyzer: 3500 Series, 3730 Series, 3130 Series, or 310 instruments
- Polymer: see “Run modules” on page 69 for the polymer and capillary array length combinations supported on each instrument
- Capillary array:
  - 3500 Series instruments: 50-cm
  - 3730 Series, 3130 Series, and 310 instruments: 36-cm
- GeneScan™ 500 ROX™ Size Standard (included in kits)
- DS-32 Matrix Standard (Dye Set F)

IMPORTANT! Throughout a set of experiments, use all the same equipment, run parameters, polymers, dyes, and so on. Consistent conditions are required to avoid mobility shifts that interfere with accurate interpretation of data.

Experiment and primer design recommendations

DNA extraction and purification

Because AFLP® analysis requires only a small amount of DNA (50 to 500 ng, ideally 10 to 100 ng), DNA purification is critical.

We recommend the following kits for extracting DNA for AFLP analysis:

- Plant Analysis: DNAzol® Reagent or PureLink® Genomic Plant DNA Purification Kit
- Microbial Analysis: PureLink® Genomic DNA Mini Kit

Restriction

In AFLP® experiments on genomes of unknown content, determine whether or not your genomic DNA restricts properly with EcoRI and MseI enzymes.

In general, the Regular Plant Genome Kit modules should produce quality genetic fingerprints with genomes of $5x10^8$ to 6×10^9 base pairs, and the Small Plant Genome Kit modules with genomes of $5x10^7$ to 5×10^8 base pairs.

Empirical guidelines suggest that if the G+C content of the genome is >65%, MseI will not give a significant number of fragments. Optimal results are obtained with MseI when the G+C content is <50%. EcoRI also tends to produce more fragments in G+C- poor genomes. In cases where an organism's G+C content is unknown, the effectiveness of the restriction enzymes must be determined empirically.

Primers

For the selective amplification step, the primers that target the EcoRI/A binding site are fluorescently labeled at the 5' end. The primers that target the MseI/C binding site are unlabeled.

You may need to optimize to identify primer combinations that generate sufficient unique marker fragments for a study.

For example, the Plant Mapping Kits contain eight selective forward primers and eight reverse primers labeled with the 5-FAM™, NED™, and JOE™ dye-labeled fluorophores (Dye Set F). The possible combinations of forward and reverse primers provides 128 possible primer combinations that have been tested across several crop genomes, facilitating identification of the optimal pair(s) for a given organism without having to design, synthesize, or perform quality control tests of custom primers.

The Appendix of the *AFLP® Plant Mapping Protocol* (Pub. no. 4303146) shows primer combinations that have been successfully used for a variety of plant species and the *AFLP® Microbial Fingerprinting Protocol* (Pub. no. 402977) shows primer combinations that have been successfully used for a variety of microbial organisms. (Note that if your organism of interest does not appear in the list, you can still conduct experiments by choosing primers from the most closely related species that is available.)

In general, the strategy with AFLP® analysis is to generate informative fragments, or enough fragments so that individuals are distinguishable. However, too many fragments complicate the analysis, so you must empirically determine the optimum number of fragments needed for adequate discrimination. As a general rule, it is best to have between 50 and 200 peaks as the “fingerprint” after amplification.

Workflow

1. Restriction digestion
2. Ligation
3. Preselective amplification
4. Selective amplification
5. Capillary electrophoresis
6. Data analysis

Data analysis

The GeneMapper® Software includes an AFLP® Default analysis method that you can use as a starting point for analysis.

This method contains analysis parameters for pattern recognition of fragments across samples to generate a fingerprint for every sample. This method can be used to analyze any type of data from fragment length polymorphism assays such as AFLP or T-RFLP. Features of the software useful for analysis include:

- Ability to generate a panel (the collection of markers) from sample files that have been added to a project.
- Sizing Quality and Genotyping Quality values flag poor quality samples enabling easy identification and decrease manual review.
- Automatic generation of final marker genotypes in a standard binary format where "1" represents the presence of a given fragment while "0" represents the absence of the corresponding fragment.

Up to four profiles are expected for each sample because:

- Both the forward and reverse PCR primers may be fluorescently labeled
- Two restriction enzymes are used

Generate panels and binsets using the AFLP Default analysis method. You can then routinely analyze data using this panel.

A change in the fragment profile can be indicated by the absence of a peak as well as a reduction in the height of a peak when comparing different samples.

The following two figures are examples of typical and polymorphic AFLP® reactions.

Figure 33 Typical electropherogram of an AFLP reaction

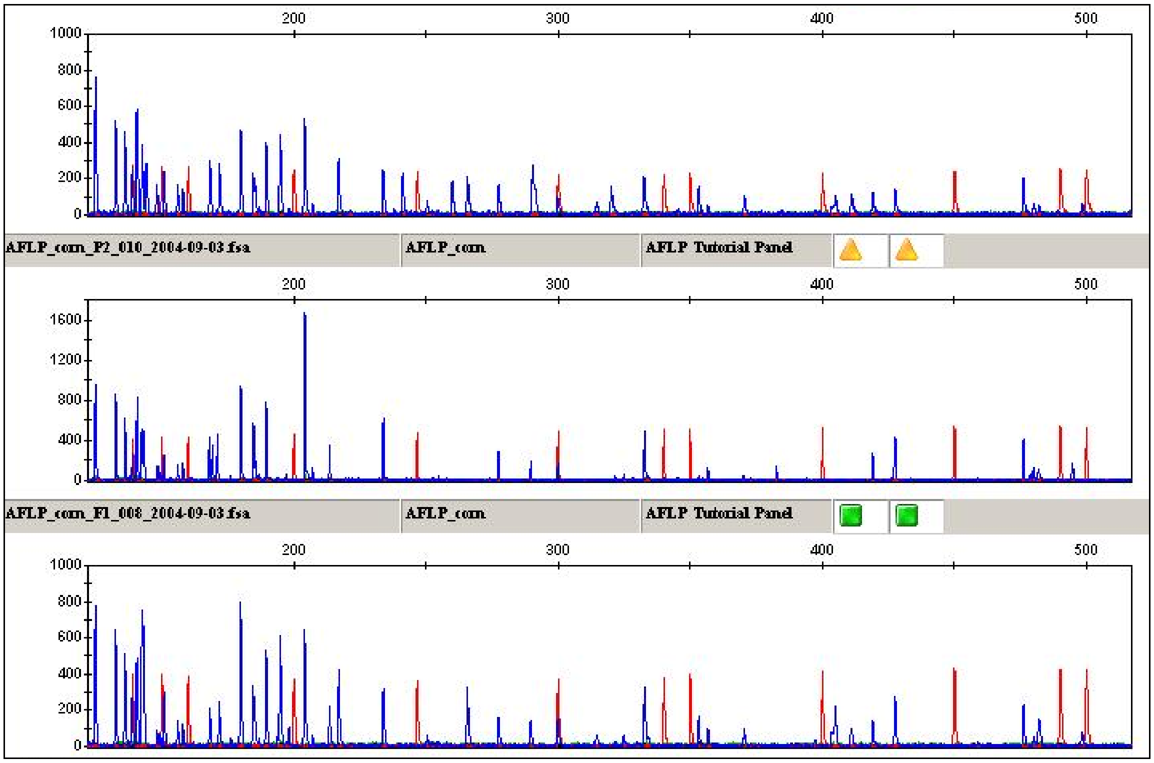
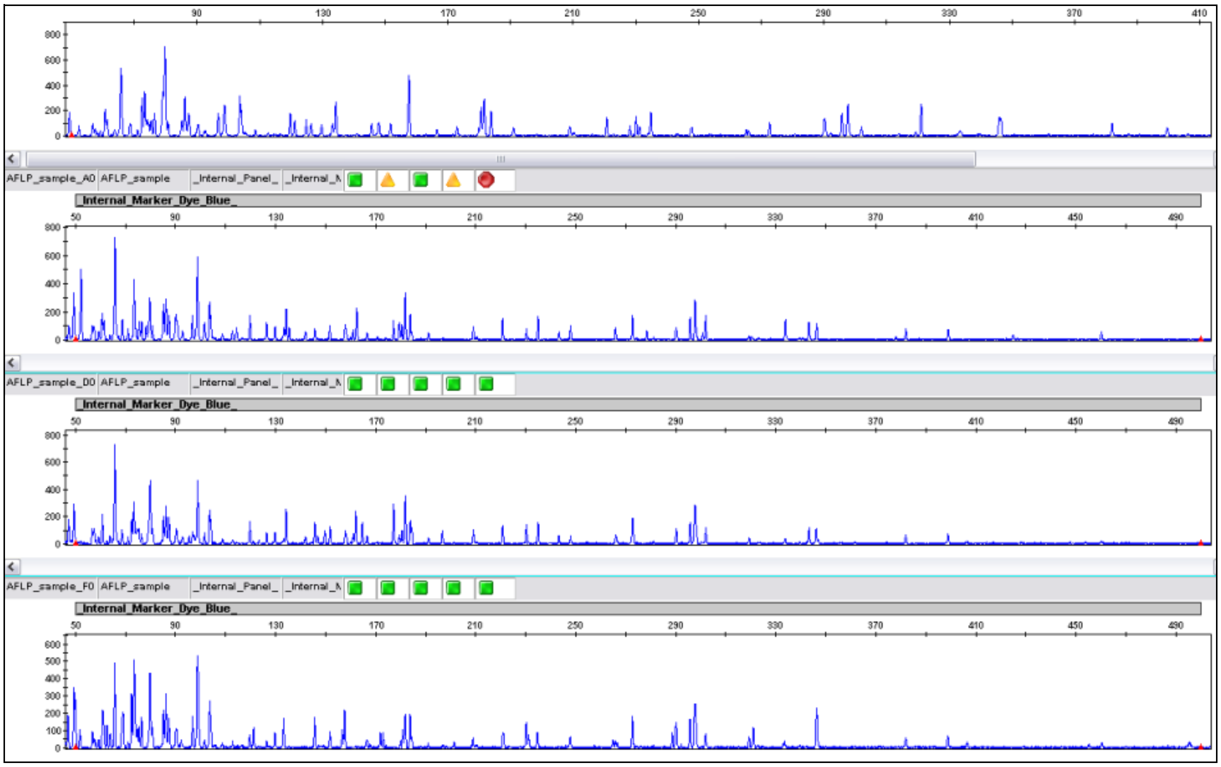
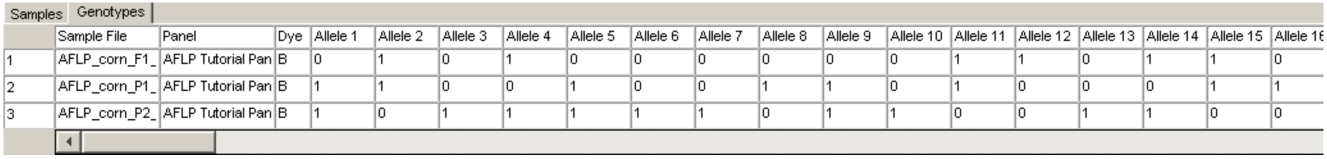

200
300
400
500
1000
800
600
400
200
0
AFLP_corn_P2_010_2004-09-03.fsa
AFLP_corn
AFLP Tutorial Panel
200
300
400
500
1600
1200
800
400
0
AFLP_corn_F1_008_2004-09-03.fsa
AFLP_corn
AFLP Tutorial Panel
200
300
400
500
1000
800
600
400
200
0

Figure 34 Polymorphic AFLP peaks


90 130 170 210 250 290 330 370 410
0 200 400 600 800
AFLP_sample_A0 AFLP_sample _Internal_Panel_ _Internal_N
_Internal_Marker_Dye_Blue_
50 90 130 170 210 250 290 330 370 410 450 490
AFLP_sample_D0 AFLP_sample _Internal_Panel_ _Internal_N
_Internal_Marker_Dye_Blue_
AFLP_sample_F0 AFLP_sample _Internal_Panel_ _Internal_N
_Internal_Marker_Dye_Blue_
0 100 200 300 400 500 600

These peak patterns are automatically converted to a table of binary marker genotypes (Figure 35), which can be exported and analyzed for similarity and generation of dendrograms using a statistical software package or other downstream analysis software for this type of clustering analysis.

Figure 35 AFLP genotypes in GeneMapper® Software


Samples
Genotypes

	Sample File	Panel	Dye	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8	Allele 9	Allele 10	Allele 11	Allele 12	Allele 13	Allele 14	Allele 15	Allele 1
1	AFLP_corn_F1_	AFLP Tutorial Pan	B	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	0
2	AFLP_corn_P1_	AFLP Tutorial Pan	B	1	1	0	0	1	0	0	1	1	0	1	0	0	0	1	1
3	AFLP_corn_P2_	AFLP Tutorial Pan	B	1	0	1	1	1	1	1	0	1	1	0	0	1	1	0	0

For more information

For documents and publications, see "AFLP® applications" on page 200.

For ordering information, see "Ordering Information" on page 193.

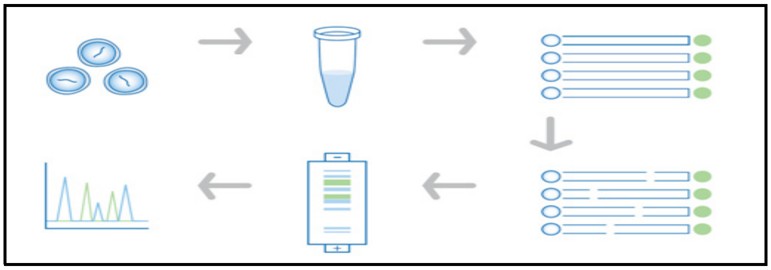
Terminal restriction fragment length polymorphism (T-RFLP)

Overview

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a mapping technique used to study complex microbial communities based on variation in the 16S rRNA gene (Osborn and Moore *et.al.*). It is culture-independent, rapid, sensitive, and reproducible and does not require genomic sequence information.

Principle of the analysis

In T-RFLP analysis, fluorescently labeled DNA is digested with restriction enzymes that have 4 base-pair recognition sites. This step generates fluorescently labeled terminal restriction fragments. The fragments in the digest are then separated by capillary electrophoresis. Profiles can then be compared between samples, or matched to a database of known species.



Applications

- Examine microbial community structure and community dynamics in response to changes in different environmental parameters or to study bacterial populations in natural habitats.
- Study of complex microbial communities in diverse environments such as soil (Derakshani and Lukow *et.al.*), marine and activated sludge systems (Eschenhagen and Schuppler *et al.*)
- Characterize oral bacterial flora in saliva in healthy subjects versus patients with periodontitis (Sakamoto and Takeuchi *et al.*).
- Preliminary screening of microorganisms before analysis using Applied Biosystems® MicroSEQ® Microbial identification kits.

Instrument and consumable recommendations

This is a “Thermo Fisher Scientific-demonstrated” protocol.

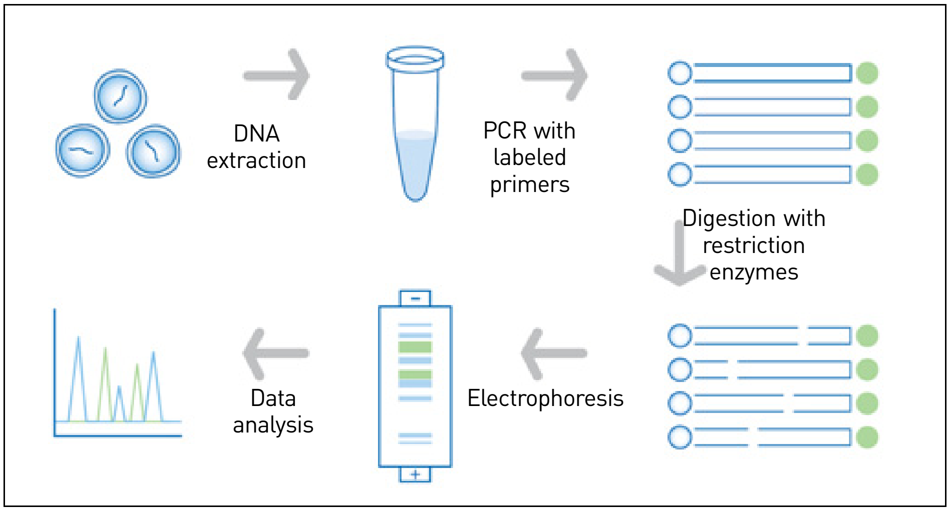
- Thermal cycler: Veriti®, GeneAmp® 9700, 2720
- Genetic analyzer: 3500 Series, 3730 Series, 3130 Series, and 310 instruments
- Polymer: see “Run modules” on page 69 for the polymer and capillary array length combinations supported on each instrument
- GeneScan™ 600 LIZ® Size Standard
- DS-33 Matrix Standard (Dye Set G)

IMPORTANT! Throughout a set of experiments, use all the same equipment, run parameters, polymers, dyes, and so on. Consistent conditions are required to avoid mobility shifts that interfere with accurate interpretation of data.

Experiment and primer design recommendations

Follow the recommendations for AFLP® analysis. See "Experiment and primer design recommendations" on page 127.

Workflow



1. DNA isolation and purification.
2. PCR amplification and restriction enzyme digestion.
3. Separation and detection of the digested products via electrophoresis.
4. Analysis of data to generate the fragment profile for each sample.
5. Clustering analysis based on the profile of samples from step 4.

Data analysis

T-RFLP analysis uses the same data analysis technique as AFLP. See "Data analysis" on page 129.

For more information

For documents and publications, see "AFLP® applications" on page 200.

For ordering information, see "Ordering Information" on page 193.

Bacterial Artificial Chromosome (BAC) fingerprinting

Overview

BAC fingerprinting provides an efficient and cost-effective method of characterizing large genomic fragment libraries for genome sequencing, positional cloning, and physical mapping efforts. Restriction endonuclease digestion of BAC clones followed by fluorescent-dye labeling can be used to generate a profile or fingerprint. Overlap between fingerprints are subsequently used to assemble contiguous sequences (contigs) in the construction of whole-genome physical maps. Physical maps are important resources for genome sequencing efforts, positional cloning, comparative genomics, and to determine the size and structure of genomes.

The SNaPshot® Multiplex Kit (Luo *et al.*) provides an effective, easy, and cost-effective solution for high-throughput BAC fingerprinting.

Principle of the analysis

In BAC fingerprinting analysis using the SNaPshot® Multiplex Kit, BAC clones are subjected to restriction-endonuclease to generate fragments of various lengths that end in A, C, G, or T. The SNaPshot® chemistry then labels the fragments with the corresponding bases by single-base extension to create a distinct DNA fragment pattern or "fingerprint" for each clone. The clones are then mapped based on the order of the overlapping parts of "fingerprints" with other clones of the same genome.

Figure 36 SNaPshot restriction fragment labeling

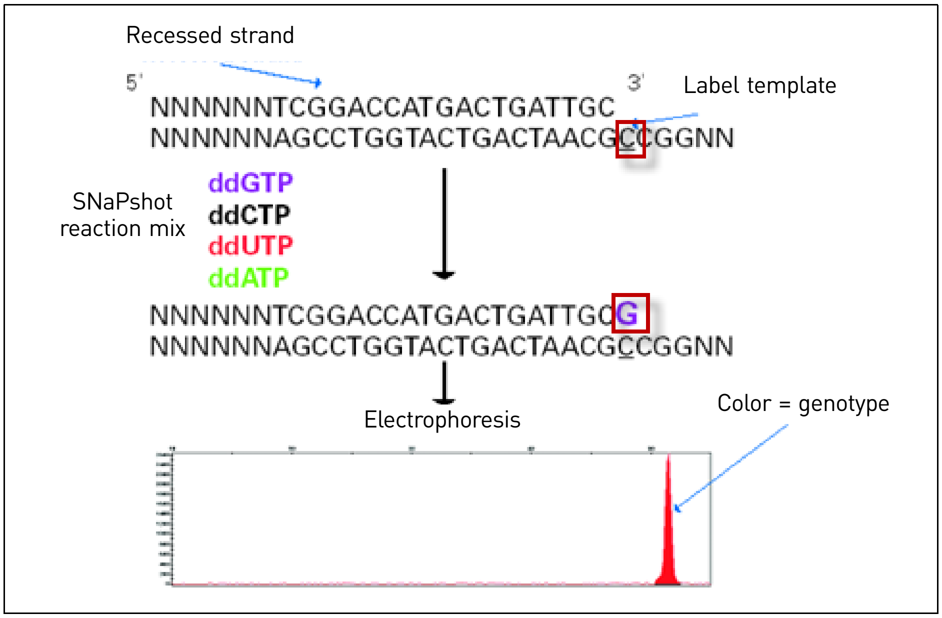

Recessed strand
5'
3'
Label template
NNNNNNTCGGACCATGACTGATTGC
NNNNNNAGCCTGGTACTGACTAACGCCGGNN
SNaPshot reaction mix
ddGTP
ddCTP
ddUTP
ddATP
NNNNNNTCGGACCATGACTGATTGCG
NNNNNNAGCCTGGTACTGACTAACGCCGGNN
Electrophoresis
Color = genotype

Table 19 Example of possible six-base cutters for restriction endonucleases and dyes used in the SNaPshot® Multiplex Kit

Restriction endonuclease	Restriction site	ddNTP	Fluorescent dye	Restriction fragment color
EcoRI	GˉAATTC	A	dR6G	Green
BamHI	GˉGATCC	G	dR110	Blue
XbaI	TˉCTAGA	C	dTAMRA™	Yellow
XhoI	CˉTCGAG	T	dROX™	Red
HaeIII	GGˉCC	None	—	—

Applications

With BAC fingerprinting, you can create whole-genome physical maps that are important resources for:

- Genome sequencing
- Positional cloning
- Comparative genomics

Instrument and consumable recommendations

This is a "Thermo Fisher Scientific-demonstrated" protocol.

IMPORTANT! BAC fingerprinting is based upon pattern recognition; therefore, data analysis is focused on relative size and distribution. We recommend using a dedicated instrument platform to minimize low random error caused by sizing imprecision.

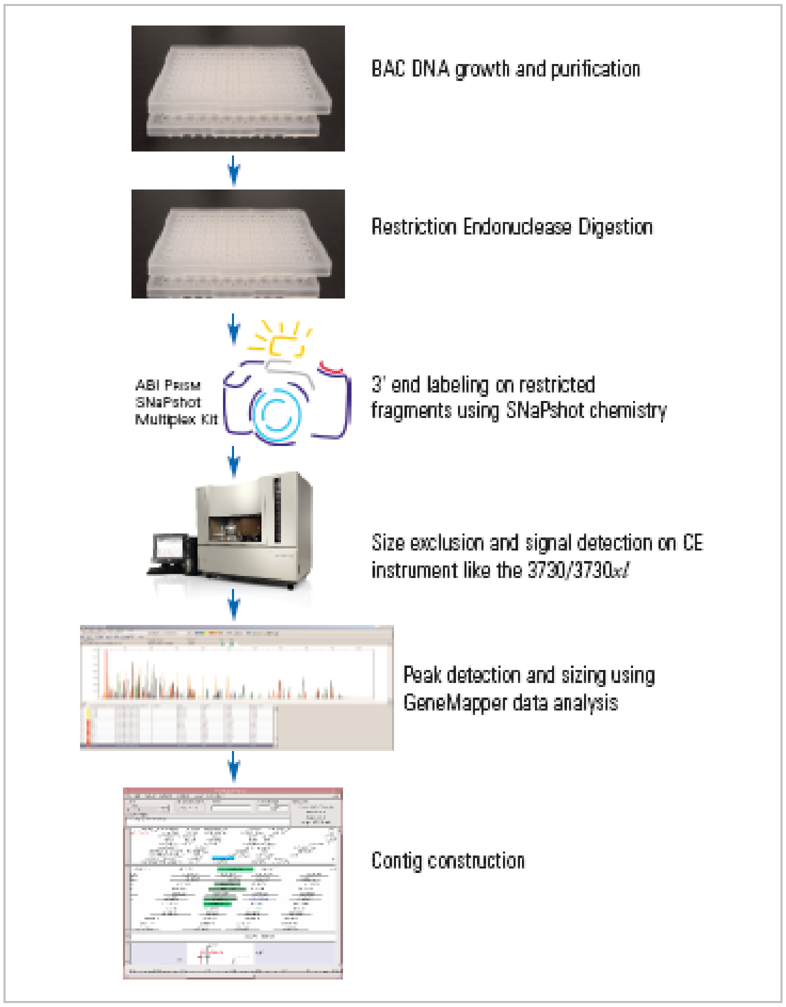
- Thermal cycler: Veriti®, GeneAmp® 9700, 2720
- Genetic analyzer: 3500 Series, 3730 Series, 3130 Series, and 310 instruments
- Polymer: see "Run modules" on page 69 for the polymer and capillary array length combinations supported on each instrument
- Capillary array:
  - 3500 Series instruments: 50-cm
  - 3730 Series, 3130 Series, and 310 instruments: 36-cm
- GeneScan™ 120 LIZ® Size Standard
- DS-33 Matrix Standard (Dye Set G5)

IMPORTANT! Throughout a set of experiments, use all the same equipment, run parameters, polymers, dyes, and so on. Consistent conditions are required to avoid mobility shifts that interfere with accurate interpretation of data.

Experiment and primer design recommendations

- Protocols may differ based on the kind of restriction endonucleases and the BAC DNA purification kits that are used.
- Enzymatic digestion and SNaPshot® reagent labeling can be performed in one tube or in separate reactions.

Workflow


BAC DNA growth and purification
Restriction Endonuclease Digestion
ABI Prism SNaPshot Multiplex Kit
3' end labeling on restricted fragments using SNaPshot chemistry
Size exclusion and signal detection on CE instrument like the 3730/3730*xl*
Peak detection and sizing using GeneMapper data analysis
Contig construction

1. Selective bacterial growth of single colonies.
2. BAC purification by restriction-endonuclease digestion.
3. Restriction-endonuclease digestion of the BAC clones with several different enzymes.
4. SNaPshot®-reagent labeling of fragments. The dye-labeled primers are bound to the BAC fragments based on the overhangs left by the restriction enzymes (see Table 19 on page 133).
5. Post-extension clean-up of the clones (not shown in diagram).
6. Capillary electrophoresis.
7. Data analysis.
8. Contig construction.

Data analysis

See “BAC applications” on page 200.

For more information

For documents and publications, see "BAC applications" on page 200.

For ordering information, see "Ordering Information" on page 193.

High coverage expression profiling (HiCEP)

Overview

The high coverage expression profiling (HiCEP) method of fragment analysis was developed to address the shortcomings in gene expression profiling and to provide a sensitive method for detecting a large proportion of transcripts in both known and unknown genes, with a low false-positive rate.

As an AFLP®-based gene expression profiling method, the HiCEP method does not require sequence information and has a reduced rate of false positives with a high degree of detection of both coding and noncoding transcripts. After HiCEP analysis, fragments of interest can be purified and cloned from agarose gels and sequenced to identify the transcripts. If whole-genome sequence information for the organism under study is known, the fragments of interest can be identified by bioinformatic prediction using the sequence information available from public databases and the restriction enzyme recognition sites used in the HiCEP workflow.

Principle of the analysis

HiCEP is an AFLP®-based method. The analysis involves digesting genomic DNA to produce a population of restriction fragments. Priming sites are then ligated onto the ends of the restriction fragments so that they can be amplified by PCR (Goel *et al.* 2006).

Applications

Fingerprinting

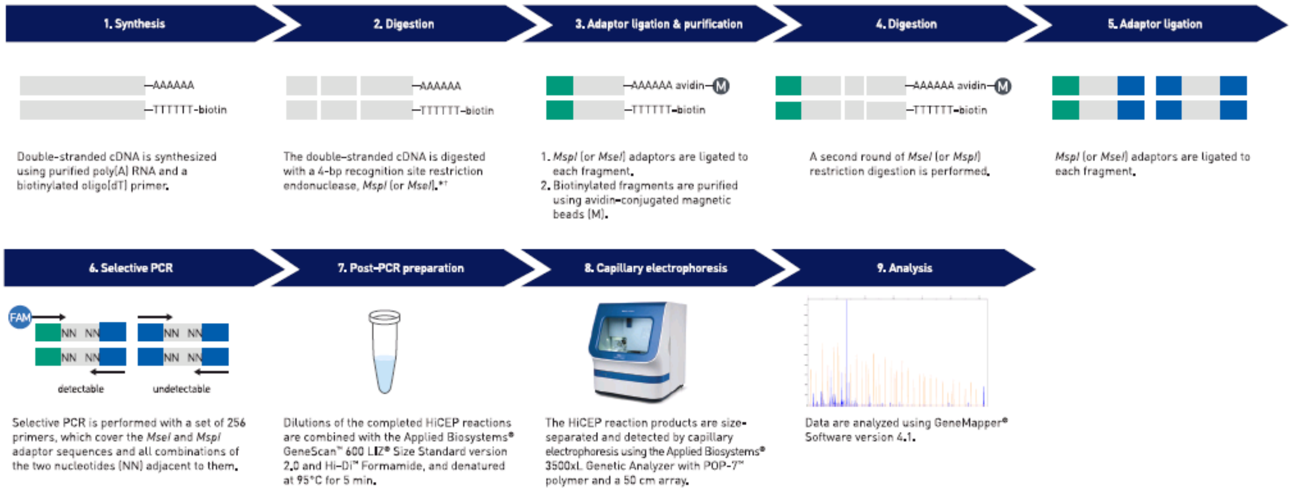
Recommendations

This is a "Customer-demonstrated" protocol. For information, refer to "HiCEP applications" on page 200.

Workflow

1. Synthesis
2. Digestion
3. Adaptor ligation and purification
4. Digestion
5. Adaptor ligation
6. Selective PCR
7. Post-PCR preparation
8. Capillary electrophoresis
9. Data analysis

Figure 37 HiCEP workflow



1. Synthesis: Double-stranded cDNA is synthesized using purified poly(A) RNA and a biotinylated oligo(dT) primer.

2. Digestion: The double-stranded cDNA is digested with a 4-bp recognition site restriction endonuclease, *MspI* (or *MseI*).*†

3. Adaptor ligation & purification:
1. *MspI* (or *MseI*) adaptors are ligated to each fragment.
2. Biotinylated fragments are purified using avidin-conjugated magnetic beads (M).

4. Digestion: A second round of *MseI* (or *MspI*) restriction digestion is performed.

5. Adaptor ligation: *MspI* (or *MseI*) adaptors are ligated to each fragment.

6. Selective PCR: Selective PCR is performed with a set of 256 primers, which cover the *MseI* and *MspI* adaptor sequences and all combinations of the two nucleotides (NN) adjacent to them.

7. Post-PCR preparation: Dilutions of the completed HiCEP reactions are combined with the Applied Biosystems® GeneScan™ 600 LIZ® Size Standard version 2.0 and Hi-Di™ Formamide, and denatured at 95°C for 5 min.

8. Capillary electrophoresis: The HiCEP reaction products are size-separated and detected by capillary electrophoresis using the Applied Biosystems® 3500xL Genetic Analyzer with POP-7™ polymer and a 50 cm array.

9. Analysis: Data are analyzed using GeneMapper® Software version 4.1.

* The use of a 4-bp recognition site restriction enzyme generates small fragments, which is important for improving subsequent PCR amplification efficiencies.

† If *MspI* is used for the first digestion, *MseI* is used for the second digestion, and vice versa.

For more information

For documents and publications, see "HiCEP applications" on page 200.

For ordering information, see "Ordering Information" on page 193.

Inter-simple sequence repeat (ISSR) PCR

Overview

Inter-simple sequence repeat (ISSR) PCR is a fast and inexpensive genotyping technique with a wide range of uses, including the characterization of genetic relatedness among populations. ISSR PCR is a genotyping technique based on variation found in the regions between microsatellites.

In addition to the use of long fragments for accurate analysis, this technique provides additional benefits over agarose gels. The increased sensitivity of Thermo Fisher Scientific genetic analyzers over traditional analysis methods routinely allows the detection of an order of magnitude more peaks, and this increased resolution results in better discrimination between individuals being compared in the populations.

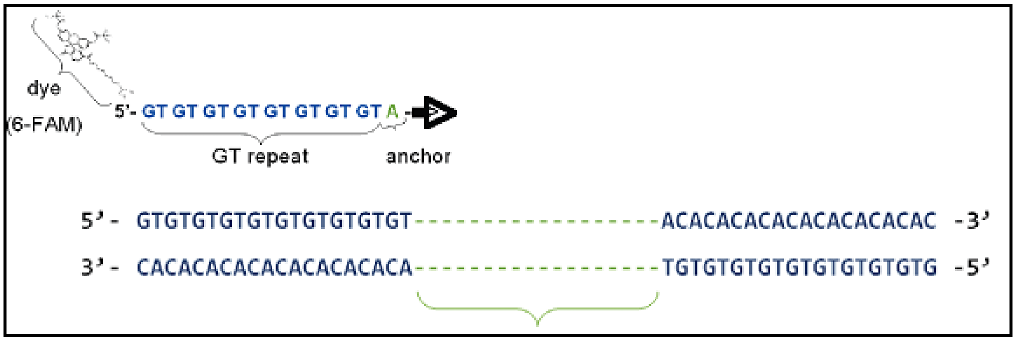
However, the primers that are designed to anneal to the di- or trinucleotide repeats can lack specificity in PCR and are a major contributor to a lack of reproducibility. Also, the lack of complexity of the ISSR primers can lead to nonspecific amplification, particularly if coupled to poor-quality gDNA extraction methods and suboptimal PCR amplification conditions.

Principle of the analysis

ISSR PCR uses a single fluorescently labeled primer to target the region between identical microsatellites (Figure 38). An ISSR PCR primer comprises three parts:

- A fluorescent tag
- Eight dinucleotide repeat units (or 6 trinucleotide-repeat units)
- One or more anchor nucleotides designed with a dual purpose: to target the end of a microsatellite region and to prevent primer dimerization. More than 100 primers have been developed for use in ISSR techniques (UBC Primer Set 9, 2006 catalog).

Figure 38 Example inter-simple sequence repeat (ISSR) region


dye
(6-FAM)
5'- GT GT GT GT GT GT GT GT A
GT repeat
anchor
5'- GTGTGTGTGTGTGTGTGTGT------------------ACACACACACACACACACAC -3'
3'- CACACACACACACACACACA------------------TGTGTGTGTGTGTGTGTGTG -5'

Because ISSRs are dominant markers, the amplified regions in an ISSR PCR are scored as diallelic. Between individuals within a population, changes in the amplified products can arise through structural changes to the region (insertions or deletions) or the loss of primer binding sites.

Advantages

- Faster and requires a lower startup investment than other genotyping methodologies such as AFLP® and RFLP.
- Several studies have compared AFLP® and ISSR results and have found ISSR preferable because of the reduced number of protocol steps required and the smaller amounts of DNA consumed.
- Less expensive and less time-consuming than microsatellite-based genotyping.
- No need to clone and characterize microsatellites.
- Capillary electrophoresis delivers significantly higher resolution than traditional agarose gel electrophoresis, thus increasing the amount of information obtained from each experiment.

Applications

ISSR has been used to investigate many plant and animal species in the following techniques:

- Genetic fingerprinting (Blair and Panaud *et. al.* 1999)
- Gene tagging (Ammiraju and Dholakia *et. al.* 2001)
- Detection of clonal variation (Leroy and Leon 2000)
- Cultivar identification (Wang and Wu *et. al*. 2009)
- Phylogenetic analysis (Gupta and Souframanien *et. al*. 2008)
- Detection of genomic instability (Anderson and Brenner *et. al.* 2001)
- Assessment of hybridization (Wolfe and Xiang *et. al.* 1998)

The versatility of this genotyping technique makes ISSR useful for researchers interested in diverse fields such as conservation biology and cancer research.

Recommendations

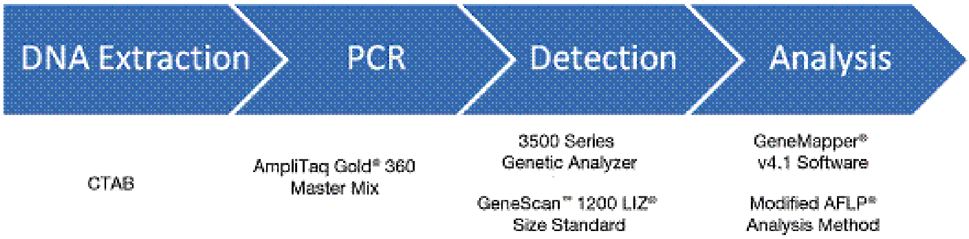
This is a “Customer-demonstrated” protocol. For information, refer to “ISSR applications” on page 200.

Experiment and primer design considerations

- Cetyltrimethyl ammonium bromide (CTAB) gDNA isolation delivers high and consistent amplification (Doyle and Doyle, 1993).
- In DNA amplification, primers and PCR master mixes should be tested for robustness and consistency when amplifying ISSR targets in both species. Subsequently thermal cycling conditions can be refined, with particular focus on primer annealing temperature and primer annealing time.

For additional information on optimization, refer to “ISSR Genotyping of Endangered Plants Using an Optimized Workflow” on page 200.

Workflow

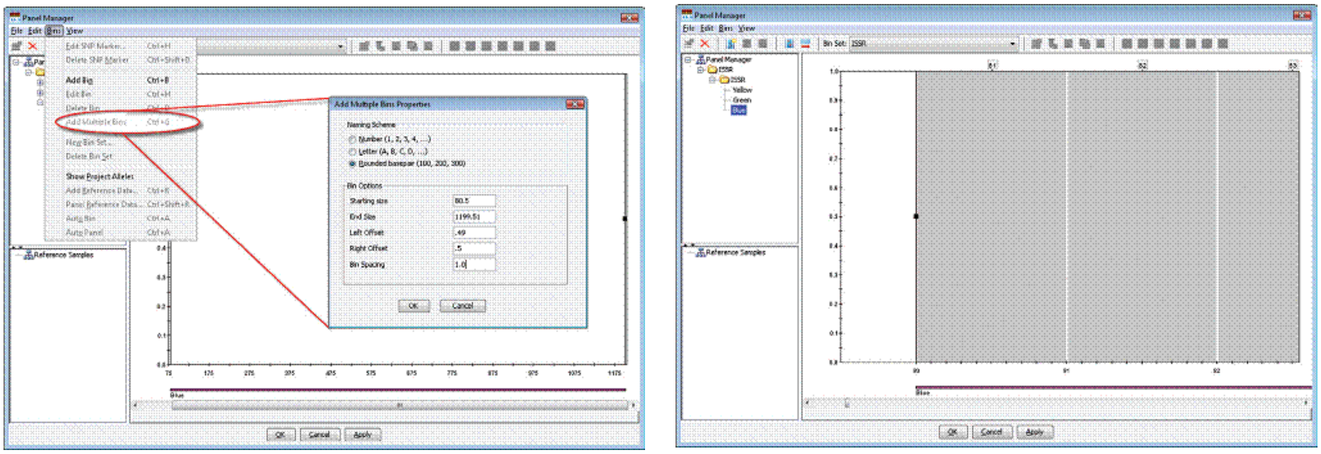

DNA Extraction
PCR
Detection
Analysis
CTAB
AmpliTaq Gold® 360 Master Mix
3500 Series Genetic Analyzer
GeneScan™ 1200 LIZ® Size Standard
GeneMapper® v4.1 Software
Modified AFLP® Analysis Method

Data analysis

In the GeneMapper® Software:

- In the Panel Manager, create a panel for each dye color (primer) with bins, centered at whole base pairs, one base pair wide covering the entire range of 80 to 1200 bp (Figure 39).
- In the GeneMapper® Manager, modify the AFLP Analysis Method (Figure 40).
- This method detects peaks above a minimum peak height as an allele and applies a binary label of either 1 or 0 for the presence of a peak in a particular bin.

Figure 39 Creating multiple ISSR bins and example of multiple bins centered at whole base pairs for the blue marker in an ISSR Panel


Panel Manager
File Edit Bins View
Add Bin Ctrl+B
Edit Bin Ctrl+H
Delete Bin
Add Multiple Bins Ctrl+G
New Bin Set...
Delete Bin Set
Show Project Alleles
Auto Bin Ctrl+A
Auto Panel Ctrl+A
Reference Samples
OK
Cancel
Apply
Bin Set: ISSR
ISSR
Yellow
Green
Blue

Add Multiple Bins Properties

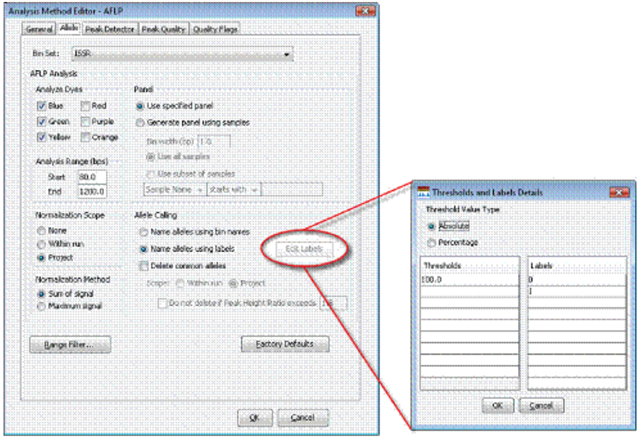
Naming Scheme

○ Number (1, 2, 3, 4, ...)
○ Letter (A, B, C, D, ...)
● Rounded basepair (100, 200, 300)

Bin Options

Starting size: 80.5
End Size: 1199.51
Left Offset: .49
Right Offset: .5
Bin Spacing: 1.0

Figure 40 Example of the Allele tab settings for an ISSR analysis method



Analysis Method Editor - AFLP

General | Allele | Peak Detector | Peak Quality | Quality Flags

Bin Set: ISSR

AFLP Analysis

Analyze Dyes

☑ Blue ☐ Red
☑ Green ☐ Purple
☑ Yellow ☐ Orange

Analysis Range (bps)

Start 80.0
End 1200.0

Panel

● Use specified panel
○ Generate panel using samples
Bin width (bp) 1.0
● Use all samples
○ Use subset of samples
Sample Name | starts with

Normalization Scope

○ None
○ Within run
● Project

Normalization Method

● Sum of signal
○ Maximum signal

Allele Calling

○ Name alleles using bin names
● Name alleles using labels
☐ Delete common alleles
Scope: ○ Within run ● Project
☐ Do not delete if Peak Height Ratio exceeds

Edit Labels

Range Filter... | Factory Defaults | OK | Cancel

Thresholds and Labels Details

Threshold Value Type

● Absolute
○ Percentage

Thresholds	Labels
100.0	0
	1

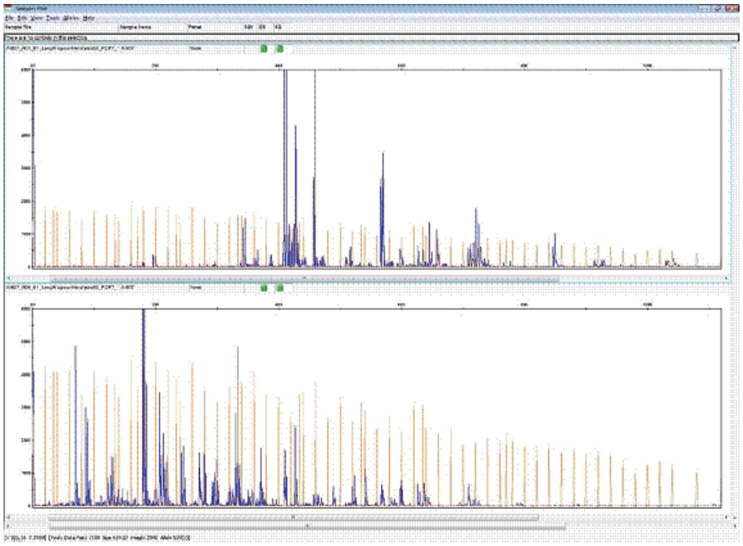
OK | Cancel

Example analysis

ISSR was used to compare two samples: *Agave shawii shawii* from Rosarito and *Agave shawii shawii* from Border.

The figure below shows the distinct peak patterns of these two individuals.

Figure 41 Example ISSR data



Agave shawii shawii from Rosarito

Agave shawii shawii from Border

After analyzing the data in the GeneMapper® Software, genotypes were exported and evaluated using a spreadsheet program to:

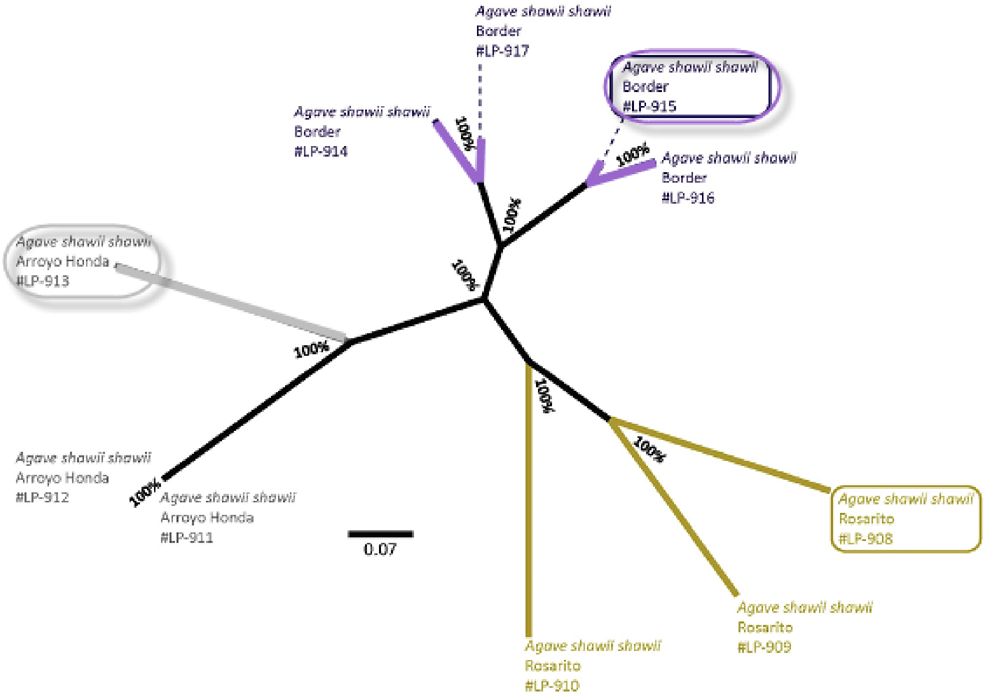
- Assess the consistency of genotyping for four replicate ISSR PCR reactions for each primer analyzed.
- Calculate the alleles shared between the replicates.

Only those alleles with 100% concordance were scored as true alleles and used in subsequent phylogenetic analyses.

True allele data for each individual for each primer were concatenated into a single list of binary states. The binary data were then analyzed using the phylogenetic software MrBayes (Huelsenbeck and Ronquist; Ronquist and Huelsenbeck).

The phylograms generated from the MrBayes software is shown below. It indicates with high confidence that three distinct populations of *Agave shawii shawii* (also known as Shaw's Agave) existed.

Figure 42 Phylogram generated using MrBayes software shows three distinct populations of Agave. Individuals collected from Rosarito, Arroyo Honda, and Border are shown in gold, grey, and purple, respectively. Highlighted individuals correspond to the data presented in Figure 41 on page 140. Nodes in phylogram with posterior probability values above 95% are considered to be informative in Monte Carlo Markov Chain (MCMC) Bayesian analysis (MrBayes).


Agave shawii shawii
Border
#LP-917
Agave shawii shawii
Border
#LP-915
Agave shawii shawii
Border
#LP-914
100%
100%
Agave shawii shawii
Border
#LP-916
100%
Agave shawii shawii
Arroyo Honda
#LP-913
100%
100%
100%
100%
Agave shawii shawii
Arroyo Honda
#LP-912
100%
Agave shawii shawii
Arroyo Honda
#LP-911
0.07
Agave shawii shawii
Rosarito
#LP-908
Agave shawii shawii
Rosarito
#LP-909
Agave shawii shawii
Rosarito
#LP-910

For more information

For documents and publications, see "ISSR applications" on page 200.

For ordering information, see "Ordering Information" on page 193.

9

Relative Fluorescence Quantitation (RFQ)

- Overview 143
- Experiment and primer design recommendations 144
- LOH workflow 145
- Data analysis 145
- For more information. 146
- Microsatellite Instability (MSI) and Replication Error (RER). 146

Overview

Relative fluorescence quantitation (RFQ) is a technique used in a variety of fragment analysis applications to compare peak heights across samples.

Relative fluorescence applications compare peak height or area between two samples. Common techniques include:

- Qualitative Fluorescence (QF) PCR
- Quantitative Multiplex PCR of Short Fluorescent Fragments (QMPSF)
- Multiplex Ligation-dependent Probe Amplification (MLPA)

Principle of the analysis

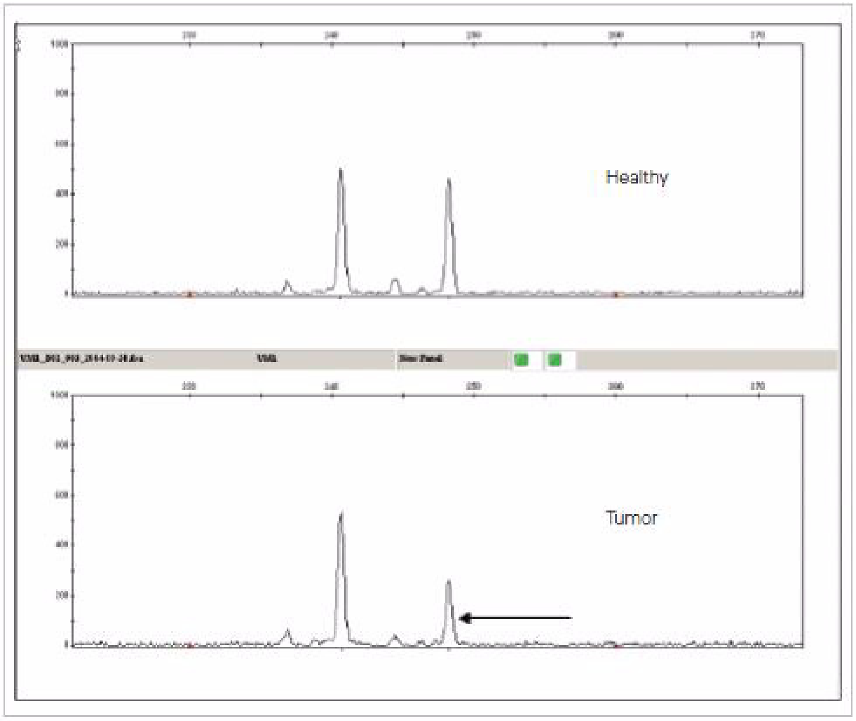
The data for an RFQ experiment can be obtained with microsatellite or AFLP® analysis.

Peak height or peak area can be used to compare differences in the same marker across multiple samples. However, you may see a difference in results depending on whether peak height or peak area is used.

IMPORTANT! Variations in signal intensity adversely affects results in RFQ experiments. For information on minimizing variation, see "Optimizing signal intensity" on page 77.

As an example microsatellite RFQ experiment, the figure below shows an electropherogram of a microsatellite marker in DNA from a healthy and tumor sample. The reduced peak height in the tumor sample indicates potential loss of heterozygosity (LOH) in the sample.

Figure 43 Example electropherogram of healthy and tumor samples



Applications

- Screening for loss of heterozygosity (LOH) using microsatellites or Single Nucleotide Polymorphisms (SNPs)
- Aneuploidy assays
- Detection of large chromosomal deletions
- Multiplex ligation-dependant probe amplification (MLPA)

Experiment and primer design recommendations

Recommendations

- Do not use internally labeled ([F]dNTP-labeled) fragments in quantitative experiments. Variations in the per-fragment number of labeled nucleotides and the increased peak spreading with this method make relative quantitation unreliable.
- For more information, see “Microsatellite Analysis” on page 107, and “Amplified fragment length polymorphism (AFLP®) Analysis” on page 126.

Minimizing signal intensity variation

To minimize variations, consider the ionic strength of samples and consumables. The amount of DNA injected is inversely proportional to the ionic strength of the solution:

- Samples high in salt result in poor injections. PCR reactions vary in efficiency, therefore some reactions may result in higher ionic concentration post-amplification.
- Conductivity of the solvent used for injection will affect the sample injection and can cause variation in peak height.

For more information, see:

- "Optimizing signal intensity" on page 77
- "Irregular signal intensity troubleshooting" on page 164
- "Relative fluorescence quantitation applications" on page 200

IMPORTANT! Preferential amplification can decrease the accuracy of relative quantitation measurements. For more information, see "Preferential amplification" on page 31.

LOH workflow

1. Design the primers and select the primer dye set.
2. PCR:
   - Run two DNA samples from each individual, for example:
     - One from normal tissue (N)
     - One from tumor tissue (T)

     Note: Some normal tissue contaminating the tumor tissue sample is typical.
   - Run 3 to 4 independent injections for each sample (N and T) to obtain sufficiently accurate quantitative estimates for subsequent data analysis.
   - Run control DNA:
     - Amplify at least one control DNA sample for every round of PCR amplification.
     - Run at least one injection of amplified control DNA for every set of microsatellite markers used.
     - Run at least one injection of amplified control DNA whenever you change the capillary or electrophoresis conditions.
3. Capillary electrophoresis.
4. Data analysis.

Data analysis

Precise peak detection

Optimize peak detection parameters to ensure precise peak detection. For more information, see "Data Analysis with GeneMapper® Software and Peak Scanner™ Software" on page 89.

If noise peaks are detected, increase the Minimum Peak Half Width or use a stronger smoothing option when analyzing noisy data.

Determining relative quantities

You can determine the relative quantities of two 5'-end labeled fragments by comparing the corresponding peak areas or peak heights on a GeneMapper® Software or Peak Scanner™ Software electropherogram.

Assess the reproducibility of peak height and area for each new analysis. Note the following:

- Use area for slow migrating or wide peaks at high concentration.
- Use height for sharp peaks at low concentration.
- There is a linear relationship between the migration time and the reproducibility. As the migration time increases, the peak width and area increase. Therefore, fast migrating peaks result in higher reproducibility as measured by the peak area. However, improved reproducibility calculated using peak height has been observed as the migration time increases (Shihabi and Hinsdale, 1995).

Determining relative number of molecules

To determine the relative number of molecules of two different-sized fragments, calculate the ratio of respective peak areas or heights. Make sure to compare peak area to peak area or peak height to peak height:

- If two fragments are similar in size, compare peak heights, especially if the peaks overlap slightly. If the peaks are well defined, peak area and peak height will give similar results. If the peaks are irregularly shaped or have shoulders, peak heights will often give better results than peak areas.
- If two fragments differ greatly in size, compare peak areas because large peaks tend to spread considerably more than small peaks.

For more information

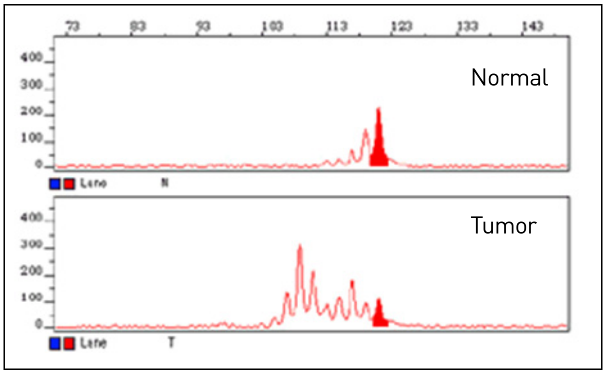
For documents and publications, see “LOH applications” on page 200.

For ordering information, see “Ordering Information” on page 193.

Microsatellite Instability (MSI) and Replication Error (RER)

Microsatellite instability (MSI) describes the reduced fidelity during the replication of repetitive DNA often occurring in tumor cells. It is thought to be caused by strand slippage during DNA replication due to mutations in DNA mismatch repair genes. MSI leads to the appearance of multiple alleles at microsatellite loci. Replication error (RER) is usually defined as MSI at multiple microsatellite markers or loci. The appearance of numerous extra alleles at lower molecular weights in the tumor sample (Figure 44 bottom panel) indicates significant genomic instability.

Figure 44 Microsatellite instability identified by lower peak signal intensity in the tumor sample



The technique for detecting RER involves comparing microsatellite alleles after PCR amplification in normal and tumor samples from the same host. You calculate a raw "RER score" using an algebraic formula that quantifies the relative strength of the stutter peaks in the two samples after normalizing for differences in PCR efficiency.

While both microsatellite instability and loss of heterozygosity are indicative of cancerous tissue, if an electropherogram shows RER at a given marker location, an LOH calculation for that allele region is complicated or even invalid (Canzian *et al.* 1996). We do not recommend LOH calculations in regions that show clear signs of RER.

10

Additional Applications

- DNA methylation 149

DNA methylation

The study of methylation/epigenetics is emerging as an important component of cancer research. In a typical assay to detect methylation, bisulfite treatment of DNA deaminates non-methylated cytosine and converts it to uracil while methylated cytosine remains unreactive. The subsequent step of PCR amplification converts uracil bases to thymine. Use the SNaPshot® system to quantitatively detect the base differences in treated and untreated samples to learn the methylation status of the samples.

For more information, see "Methylation applications" on page 200.

11

Troubleshooting

- Troubleshooting workflow 152

Refer to the following sections for troubleshooting solutions and information on how each component of the system can affect data:

- Checking data quality 153
- Running controls to isolate a problem. 156
- Sample issues 158
- Reagent and consumable issues 159
- Instrument and ambient condition issues. 160

Refer to the following sections for symptom troubleshooting information:

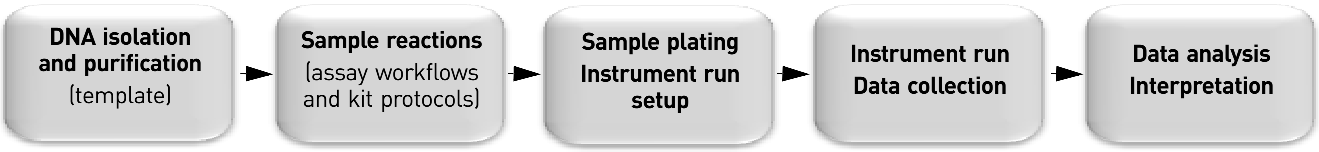
- Symptoms you may observe 162
- Irregular signal intensity troubleshooting 164
- Migration troubleshooting 168
- Abnormal peak morphology troubleshooting 169
- Extra peaks troubleshooting 172
- PCR troubleshooting 176
- Irregular baseline troubleshooting. 178
- Instrumentation troubleshooting 180
- Sizing or Size Quality (SQ) troubleshooting. 182
- GeneMapper® Software troubleshooting 187
- Preamplification gel troubleshooting 190

Refer to the following sections for procedures to solve issues:

- Desalting 190
- Evaluating 310 Genetic Analyzer multicomponent matrix quality 191

Troubleshooting workflow

Problems with data can be caused during any step of the experiment.


DNA isolation and purification (template)
Sample reactions (assay workflows and kit protocols)
Sample plating Instrument run setup
Instrument run Data collection
Data analysis Interpretation

When troubleshooting, follow this workflow to identify the problem.

1. Make sure you understand the basics of the experiment:
    - The chemistry
    - Labeling of the samples
    - How the genetic analyzer collects data
    - How the data analysis software performs sizing and peak detection

   Review the experiment for errors in primer design, sample quantitation and purification, pipetting problems, software preference settings and other common mistakes.
2. Examine the data. Evaluate the problem as specifically as possible:
    - Is it a problem with the sample peaks, the baseline, or the peaks of only one color?
    - Look for patterns: Does the problem exist in all parts of the run or does it affect only DNA fragments of a certain length? in a specific capillary? in a certain area of the plate? multiple runs?
    - Is the problem visible in raw data? analyzed data? log files?

   Continue to refine the description of the problem as specifically and thoroughly as possible.
3. In general, check first for the issues that can be resolved most easily. Review:
    - Data quality
    - Analysis settings
    - Data collection
    - Experimental setup

For more troubleshooting information, see your instrument and software user guides and the documents listed in “Documentation and Support” on page 199.

Checking data quality

Sizing Quality (SQ) PQV description

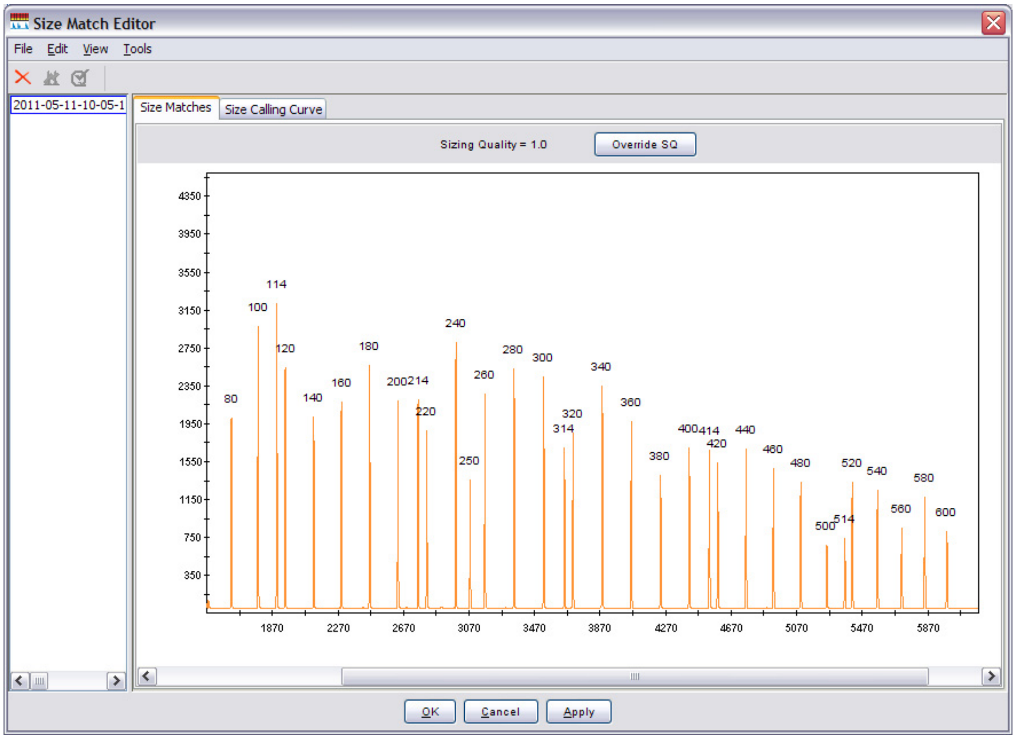
The GeneMapper® Software SQ PQV reflects the similarity between the fragment pattern defined by the size-standard definition and the actual distribution of size-standard peaks in the sample data. The metric of the Sizing Quality test is a combination of several values which measure the success of the algorithms that:

- Identify and eliminate primer peaks based on peak shape
- Perform size matching (ratio matching)
- Make a size-calling curve using the sizing method specified in the analysis method. (for more information, see "GeneMapper® Software sizing methods" on page 100)

Checking samples with ⚠ yellow and 🛑 red SQ samples

Review the data of the size standards that fail the SQ PQV as described below. For more information, see "Sizing or Size Quality (SQ) troubleshooting" on page 182.

1. In the Samples tab of the GeneMapper® window, click (Analysis ▸ Low Quality to Top) to sort the data so that the samples that produced errors appear at the top of the table.
2. In the Samples tab, select the rows for the sample(s) that display ⚠ (Check) or 🛑 (Fail) in the SQ column.
3. Click (Analysis ▸ Size Match Editor) to view the sizing information for the selected sample(s).


Size Match Editor
File Edit View Tools
2011-05-11-10-05-1
Size Matches
Size Calling Curve
Sizing Quality = 1.0
Override SQ
4350
3950
3550
3150
2750
2350
1950
1550
1150
750
350
80
100
114
120
140
160
180
200214
220
240
250
260
280
300
314
320
340
360
380
400414
420
440
460
480
500
514
520
540
560
580
600
1870
2270
2670
3070
3470
3870
4270
4670
5070
5470
5870
OK
Cancel
Apply

4. In the Navigation Pane of the Size Match Editor, select a sample file to display the sizing data for the associated sample.

5. Review the data for the following qualities:
    - **Signal strength** – The signal strength (peak height) of all peaks must exceed the Peak Amplitude Thresholds defined in the analysis method used to analyze the data.
    - **Correct size calls/labels** – All peaks listed in the size-standard definition must be correctly identified by the software. The labels above the peaks must be in sequential order from left to right, low to high.
    - **Evenness of signal strength** – All peaks should have relatively uniform signal strengths.

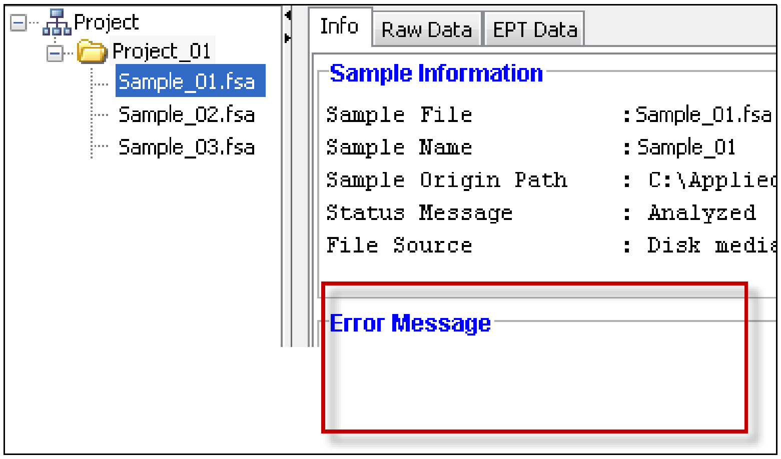
    Note: To magnify the plot of the Size Matches tab, drag the mouse cursor (🔍) across a region of the x- or y-axis.

Examining the raw data for 🔴 red SQ

Data for 🔴 SQ samples is displayed only in the Raw Data tab (see "Examine the sample info, raw data, and EPT trace" below).

Examine the sample info, raw data, and EPT trace

1. In the Project window, select a sample in the navigation pane.



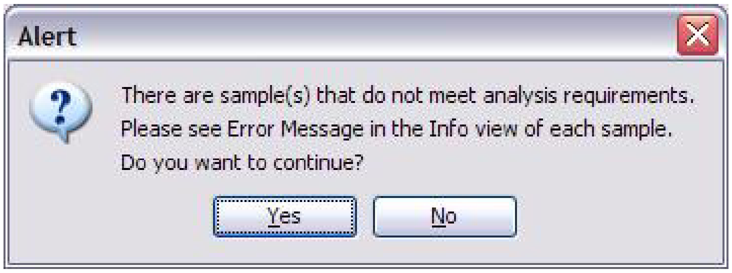
Sample Information

Sample File : Sample_01.fsa
Sample Name : Sample_01
Sample Origin Path : C:\Applied
Status Message : Analyzed
File Source : Disk medi

Error Message

2. Check for error messages.

    Note: If this error message is displayed at any time when you are using the software, check the Info tab to determine the error.



Alert

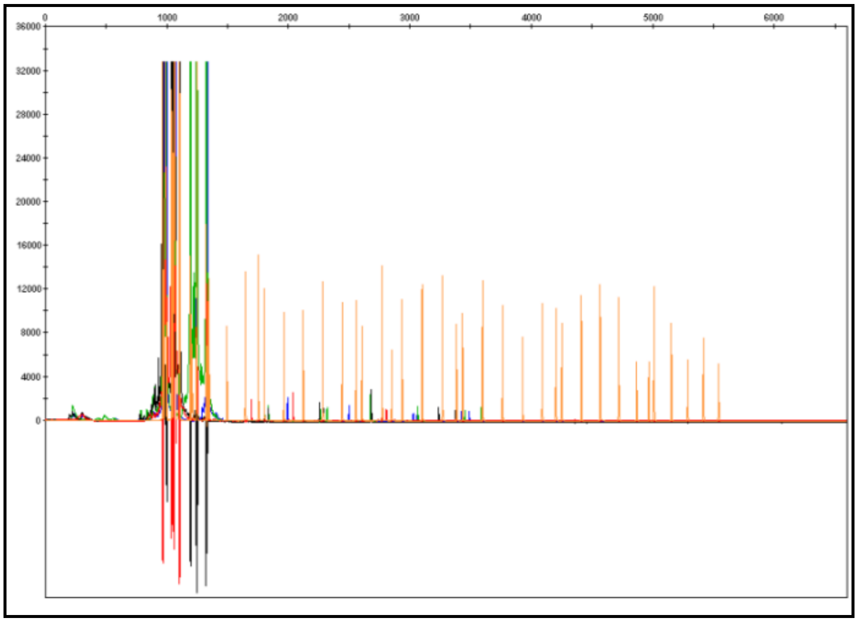
There are sample(s) that do not meet analysis requirements.
Please see Error Message in the Info view of each sample.
Do you want to continue?

3. Review the sample information. Ensure that the correct analysis settings and data collection setting were used.

4. Click the **Raw Data** tab.

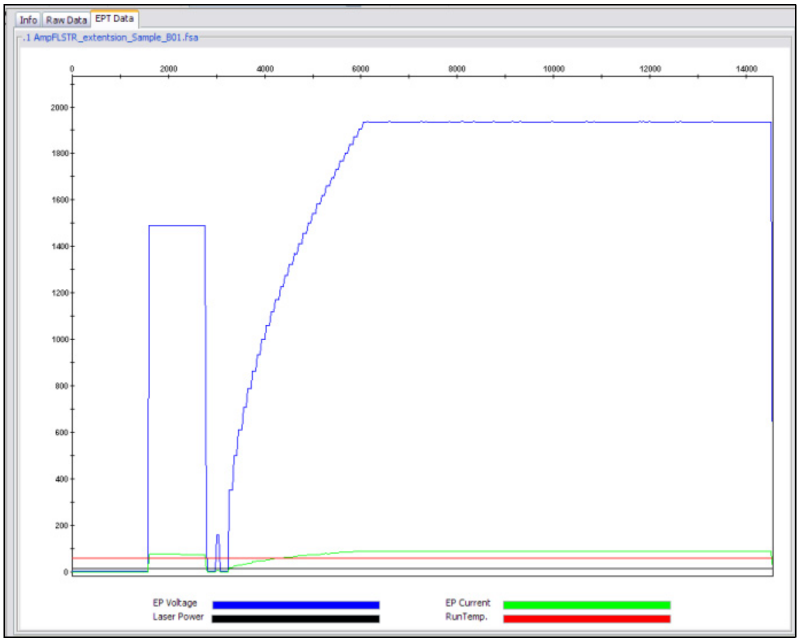
   Note: The Raw Data tab is the only place in the software in which you can view:
   - Negative baselines
   - Run data for 🛑 SQ samples

   The example below illustrates good-quality raw data for multiplexed microsatellite data.



5. Click the **EPT Data** tab. Review the current, voltage, temperature, and power throughout the electrophoresis run. Large fluctuations in the values can result in poor quality data.

   The example below illustrates a good-quality EPT trace. The values for the trace may differ depending on the run module used, but the shape of the trace should be similar to the example below.


Info
Raw Data
EPT Data
..1 AmpFLSTR_extentsion_Sample_B01.fsa
0
2000
4000
6000
8000
10000
12000
14000
2000
1800
1600
1400
1200
1000
800
600
400
200
0
EP Voltage
Laser Power
EP Current
RunTemp.

Note: Trace colors differ between the Data Collection Software and the GeneMapper® Software.
For more information, see "Instrumentation troubleshooting" on page 180

Running controls to isolate a problem

To simplify troubleshooting, Thermo Fisher Scientific recommends that you run controls with every run for multicapillary instruments or each set of runs on 310 instruments.

In addition to controls included in each run, you can run size standards, installation standards, agarose gels, or DNA template controls when additional troubleshooting is required.

Size standard

1. Perform a run with only size standard, using one of the default run modules that are provided with the software:
   a. Vortex the size standard for 1 minute.
   b. Add to each well of a plate:

3500 Series, 3730 Series, and 3130 Series instruments	310 instruments
• 0.5 uL of size standard • 9.5 uL of fresh Hi-Di™ Formamide	• 0.5 uL of size standard • 11.5 uL of fresh Hi-Di™ Formamide

   c. Run the plate.
2. Examine the peak morphology:
   - If the peak morphology changes, for example, peaks become broader, are tailing, or are below 50 RFU, then the problem may be in the instrument, reagents, or Hi-Di™ Formamide.
   - If the peak profiles for the size standard alone are sharp and very well defined, add your product to the same wells and re-run.
   - If the peak morphology then changes, for example peaks become broader, show tailing, are less than 50 RFU, then contamination may be contributing to the problem.

   Note: The size standard peak heights are affected by the presence of sample because the sample introduces salt and competes for entry into the capillary during injection.
   If the size quality fails in the presence of sample, it indicates a problem with the PCR product, for example, it may contain too much salt.

Installation standard

Installation standards contain pooled PCR products amplified from microsatellite loci present in CEPH individual 1347.

To run the installation standard:

- For all platforms, you can load the installation standard as a regular run and view the results in the GeneMapper® Software. For instructions, refer to the appropriate instrument user guide.
- Alternatively, for a 3500 Series instrument, you can run a performance check which produces a report quantifying each peak (refer to your instrument user guide).

Thermo Fisher Scientific currently supplies the following installation standards for its capillary electrophoresis instruments (see “Installation standards” on page 197 for part numbers):

If you use...	Then use...	Which uses these dyes:
GeneScan™ 600 LIZ® Size Standard v2.0	DS-33 GeneScan™ Installation Standards	6-FAM™, VIC®, NED™, and PET® dyes
GeneScan™ 500 LIZ® Size Standard	DS-33 GeneScan™ Installation Standards	6-FAM™, VIC®, NED™, and PET® dyes
GeneScan™ 500 ROX™	DS-30 GeneScan™ Installation Standards	6-FAM™, HEX™, and NED™ dyes

Agarose gel

Run the PCR product through an agarose gel if the electropherogram shows miscellaneous unexpected peaks which may be due to unincorporated product. Results from the gel will help to determine if your sample is contaminated.

DNA template control

You can use a DNA template control (for example, CEPH 1347-02 Control DNA useful for human-target primers) as a process control to ensure that sample preparation, PCR, and electrophoresis yield the expected results.

The results can help you determine whether failed reactions are caused by poor template quality, problems with the control, or problems with the primers:

1. Run an agarose gel to separate the PCR products.
2. Run control primer with control template to eliminate contaminated reagents as a possible cause.
3. Run control template with your primers to eliminate your primers as a possible cause.
4. Run control primers with template to eliminate template as a possible cause.

Possible cause if the control fails	Action
Incorrect PCR thermal cycling conditions	Choose correct temperature control parameters (refer to your instrument user guide).
Pipetting errors	Calibrate pipettes, attach tips firmly, and check technique.
Combined reagents not spun to bottom of tube	Place all reagents in bottom of tube. Spin briefly after combining.

Possible cause if the control fails	Action
Combined reagents left at room temperature or on ice for extended periods of time	Put tubes in block immediately after reagents are combined.
Restriction digest incomplete (AFLP applications)	Repeat the restriction-ligation. Make sure that the volume of enzyme added does not cause the amount of glycerol to be >5%, which can lead to EcoRI* (star) activity.

Sample issues

Sample concentration

If the sample concentration is too low, the signal-to-noise ratio may be too low to discriminate between sample peaks and background fluctuations.

If the sample DNA concentration is too high, signal intensity may be off-scale or saturated and can cause:

- Split peaks
- Raised baseline
- Pull-up peaks which can affect sizing and accuracy of genotypes

Adjust sample concentration to ensure signal intensity is within the recommended range for your instrument:

Instrument	Recommended signal level	Fluorescence saturation
3500 Series	175–10,000 RFU	30,000 RFU
3730 Series	150–10,000 RFU	30,000 RFU
3130 Series	150–4000 RFU	8000 RFU
310	150–4000 RFU	8000 RFU

If necessary, dilute PCR products (before including the size standard in the reagent mix) so that the final allele peak height falls into the recommended range for the instrument.

Sample contamination

Sample contamination can mimic a degraded capillary. You can determine if the capillary issue is caused by sample contamination by running a size standard and formamide only (see “Running controls to isolate a problem” on page 156).

Salt concentration

Salt anions compete with negatively charged DNA for entry into the capillary during electrokinetic injection. As the salt concentration of a sample increases, less DNA will enter the capillary, decreasing the fluorescence signal. Excess salt can also precipitate the DNA in the sample tube in the presence of formamide.

Reagent and consumable issues

IMPORTANT! For every chemical, read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Note: For the SDSs of chemicals not distributed by Thermo Fisher Scientific, contact the chemical manufacturer.

Laboratory water

Poor-quality laboratory water systems and cleaning reagents can adversely affect PCR efficiency, sample resolution, and signal intensity.

PCR reagents

Expired PCR reagents can cause decreased DNA template concentration.

Hi-Di™ formamide

Improperly stored Hi-Di™ Formamide can cause:

- Incomplete denaturation of both the size standard and sample peaks
- Altered pH of the loading solution
- Tailing peaks
- Artifacts
- Decreased signal

Ensure that you do not contaminate Hi-Di™ Formamide when setting up samples.

For more information, see "Hi-Di™ Formamide storage" on page 82.

Polymer

Degraded or expired polymer, or polymer that is left at ambient temperature for >7 days, can cause:

- Reduced capillary array life (the number of runs per array)
- Reduced resolution due to increased conductivity (often caused by the hydrolysis of urea in the polymer)
- Low current
- Artifact peaks from degraded polymer
- Reduced sizing precision:
  - Sizing differences between various types of polymer are more apparent for sequences <50 bp.
  - Fragments <50 bp run on 3730/3730*xl* instruments with POP- 7™ polymer may have slightly lower sizing precision.

Polymer that is left at ambient temperature for extended periods of time can cause microbubbles in the pump.

Cold polymer can cause bubbles.

Ensure that polymer is at room temperature. Allow polymer to equilibrate to room temperature and pressure. Loosen the lid seal at least 30 to 60 minutes before use. Do not leave the lid off the polymer bottle, as dust may contaminate stock, causing spikes in data.

Note: Do not shake polymer or introduce bubbles.

Size standard

Sizing quality issues can be caused by a degraded or improperly stored size standard. A size standard can be degraded by using improperly stored Hi-Di Formamide (see“Hi-Di™ Formamide storage” on page 82).

Ionic buffer strength (not applicable to 3500 Series instruments)

Conductivity changes in the buffer affect the run current and can cause the following:

- Decreased sample resolution
- Slower than expected migration of size-standard peaks
- Low current

Possible causes of buffer issues:

- Water impurities
- High salt concentration
- Expired or incorrectly stored buffer

Instrument and ambient condition issues

Capillary array

Degraded capillary arrays can cause:

- Decreased sample resolution
- Broad, lagging peaks

Possible causes of degraded capillary array performance:

- Capillary array life is exceeded
- Capillary array is left idle or dries out
- Poor quality DNA or water or degraded Hi-Di™ formamide has introduced contaminants that ultimately affect the current flow through the capillaries
- Water wash is not performed as recommended, or contaminated water is used for the wash
- Clogged capillary

  If the same capillary always fails, run Hi-Di™ Formamide blanks, then an installation standard or size standard as controls through the capillary (see “Running controls to isolate a problem” on page 156).

  Note: Sample contamination can mimic a degraded capillary. You can determine if the capillary issue is caused by sample contamination by running clean DNA samples or the size standard alone as a control.
- Bubbles in the capillaries
- Arcing

Pump: large bubbles

Large bubbles can affect all or many of the samples in a run.

Large bubbles in the pump or blocks can affect the current and can cause the following:

- No current when voltage is applied (the flow of ions is blocked by the bubble)
- A run stops during initialization if the instrument detects unstable current
- “Leak detected” error message as the air bubble compresses when the plunger moves down to fill the array

- Arcing
- Early loss of resolution on certain capillaries

If large bubbles are present, you can usually see them in the upper polymer block, near the array end. Refer to the instrument user guide for information on removing bubbles.

Pump: small bubbles

Small micro-bubbles usually only affect single capillaries.

Micro-bubbles in the polymer path can cause:

- Current fluctuations or no current
- Decreased resolution

Possible causes of small bubbles in the pump:

- Polymer is:
  - Not at room temperature. Allow polymer to equilibrate to room temperature and pressure. Crack open lid seal for at least 30 to 60 minutes before use. Do not leave the lid off the polymer bottle, as dust may contaminate stock, causing spikes in data.
  - Newly installed
- Valves, syringes, or the array port are not screwed tightly in place

Refer to the instrument user guide for information on removing bubbles.

Pump: polymer leaks

Polymer leaks can cause:

- Formation of crystals which introduce contaminants that can affect the conditions of your run. If the pump is not adequately pushing polymer through the array, the array can clog or become contaminated.
- Loss of resolution

Autosampler misalignment

Autosampler misalignment can cause consistent failures in the same wells or rows of a plate.

Temperature/ humidity

Drastic changes in room temperature and humidity can cause distinct changes in migration.

Matrix/spectral Issues

Incorrect matrix/calibration, or using different conditions to calibrate than you do to run samples can cause the following:

- Raised baseline
- Negative peaks
- Peaks under peaks (especially if the highest peak is not off scale)
- Multiple dye colors being detected as one dye color (has been observed when running 5-dye samples with a 4-dye matrix)

Symptoms you may observe

Irregular signal intensity

Symptom	See page
No signal or low signal	164
Signal intensity is too high or oversaturated	166
Ski-slope peak pattern	166
Decreased signal	167
Size-standard signal and sample signal are not balanced	168

Migration issues

Symptom	See page
Size-standard peaks are not migrating as expected during a normal run time	168
Sizing precision is low	168

Abnormal peak morphology

Symptom	See page
Poor resolution	169
Loss of resolution	169
Broad, lagging peaks	170
Tailing peaks	170
Uneven peak heights in dyes in multiplexed sample	171
Sudden loss of signal in all samples	171
Multiple dye colors are detected as one dye color	171

Extra peaks

Symptom	See page
Extra peaks	172
Pull-up peaks	173
Data spikes	174
Split peaks	175
Many small extraneous peaks appearing next to a high-intensity peak	175

PCR

Symptom	See page
Poor priming resulting in weak signal	176
Amplified DNA concentration is lower than expected	176
PCR inhibition	176
Contamination with exogenous DNA	176
Poor amplification, nonspecific amplification	177

Symptom	See page
Poor amplification, nonspecific amplification in restriction-ligation experiments (AFLP only)	177
Hairpin secondary structures in PCR primers	177
Primer/dimer formation	178

Irregular baseline

Symptom	See page
Constant elevated signal in raw data	178
Baseline “waterfall”	178
Noisy baseline	179
Adequate signal strength with noisy data	179

Instrumentation

Symptom	See page
Low or fluctuating current	180
Drop-off of current signal	181
Current too high	180
Instrument has stopped running and red light is on. There are black marks inside the instrument.	181

Sizing or size quality

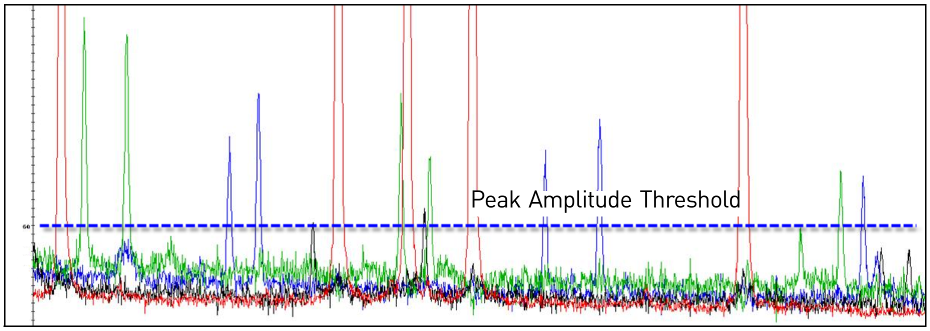
Symptom	See page
No sample plot is displayed for a sample with the error “No sizing data”	183
Size Match Editor does not display peak data	183
Missing size-standard peaks	183
Smaller size-standard peaks are not labeled	184
Larger size-standard peaks are not present in trace	184
Extra peaks in size-standard trace	185
Sizing failures occur in a regular pattern (the same wells fail repeatedly)	186
Noise peaks are detected as size-standard peaks	186
Size call inaccurate for known DNA sample	186

GeneMapper® Software

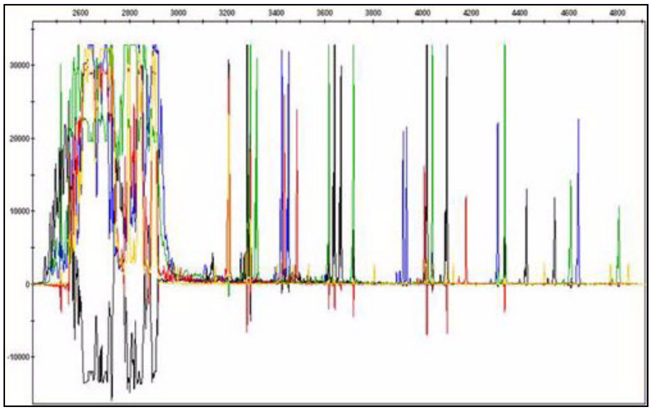
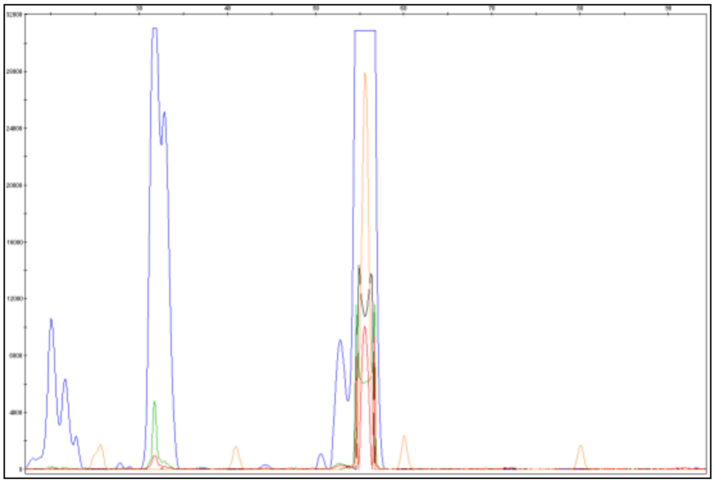
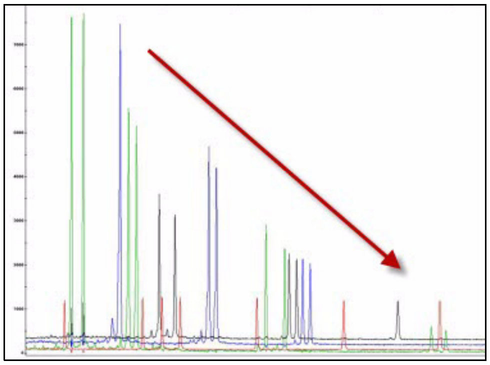
Symptom	See page
GeneMapper® Software error message	187
Error Message: The bin set in the analysis method does not match the panel used for analysis.	187
al?” label or alleles are not falling within bins	188
Allele not labeled	188

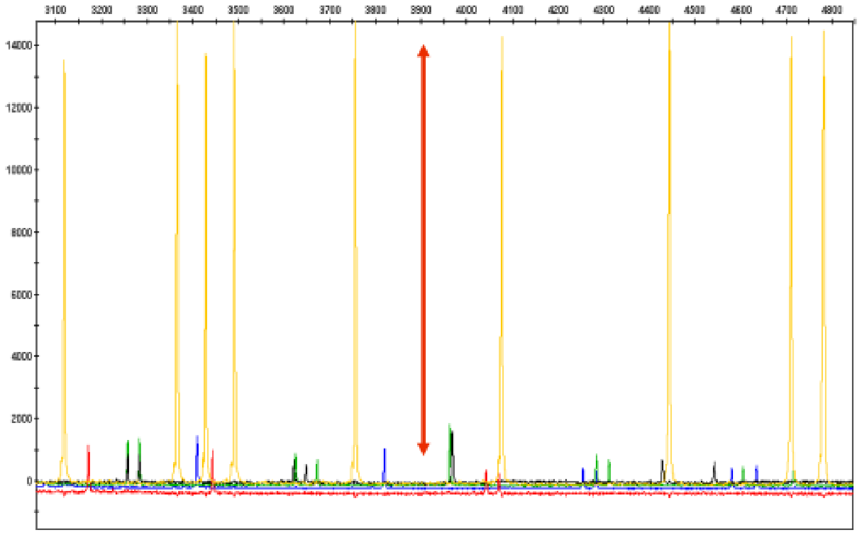
Symptom	See page
Data not sorted by name	188
When adding samples to a project, the expected data files are not listed in the Add Samples to Project dialog box	188
Genotypes tab is grayed	189
Two peaks do not separate and are detected as one peak	189

Irregular signal intensity troubleshooting

Symptom	Possible cause	Action
No signal or low signal		
	Poor or non-specific amplification.	See "PCR troubleshooting" on page 176. See "Non-specific amplification" on page 32 for more information.
	PCR inhibition.	See "PCR troubleshooting" on page 176.
	Sample was prepared with water instead of Hi-Di™ Formamide.	Prepare the sample with Hi-Di™ Formamide.
	Degraded or incorrectly stored Hi-Di™ Formamide.	Use fresh, properly stored Hi-Di™ Formamide. See "Hi-Di™ Formamide storage" on page 82.
	Air bubble at bottom of sample tube.	Centrifuge the plate before running.

Symptom	Possible cause	Action
No signal or low signal *(continued)*	High salt concentration.	Salt preferentially injects smaller fragments and inhibits injection of larger fragments, so the majority of salt may have been injected in the first injection. Re-inject the sample.
		If signal intensity does not increase, see “Desalting” on page 190.
	Injection time too low.	See “Optimizing electrokinetic injection parameters” on page 78.
	Sample concentration too low.	See “Optimizing sample loading concentration” on page 76.
	Sample volume too low.	• Sample volume must be ≥10 µL for 3500 Series, 3730 Series, and 3130 Series instruments. • Sample volume must be ≥12 µL for 310 instruments.
	Autosampler is misaligned.	• 3500 Series, 3730 Series, and 3130 Series instruments: Fill wells with 0.5 µL size standard and 9.5 µL sample, then re-inject. If the signal is still missing, contact Thermo Fisher Scientific • 310 instruments: Recalibrate autosampler.

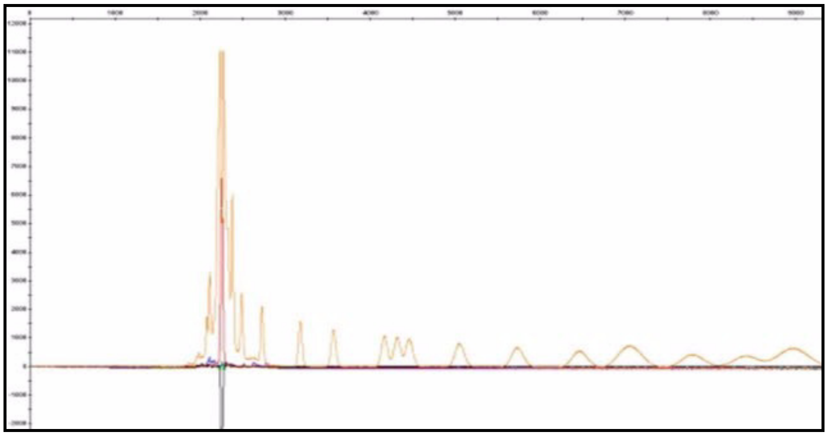
Symptom	Possible cause	Action
Signal intensity is too high or saturated	Raw data: pull-down peaks  Zoomed view of figure above 	
	Sample concentration too high.	• Decrease sample concentration. • Decrease injection time.
“Ski slope” peak pattern of sample peaks but size standard peak heights do not decrease		
	Sample concentration too high in amplification step or insufficient primer is present.	Optimize ratio of DNA template and primer.

Symptom	Possible cause	Action
Decreased signal	Degraded or improperly stored Hi-Di™ Formamide.	Use fresh, properly stored Hi-Di™ Formamide. See "Hi-Di™ Formamide storage" on page 82.
	Expired or incorrectly stored reagents.	Use fresh reagents.
	Degraded primers.	Store unused primers at –15 to –25°C. Do not expose fluorescent dye-labeled primers to light for long periods of time.
Size-standard signal and sample signal are not balanced		
	Size-standard concentration is too high.	Although the data is still sized properly, decrease size-standard concentration to balance peaks in future runs (see "Size-standard peak intensity" on page 42).

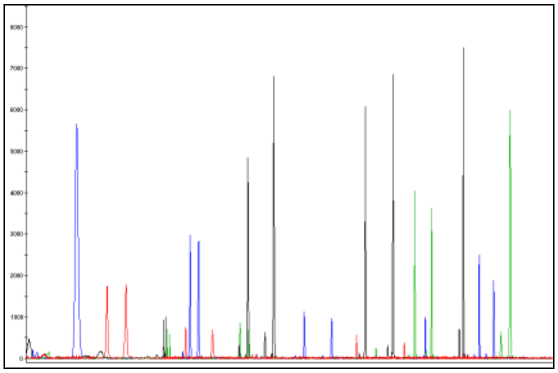
Migration troubleshooting

Symptom	Possible Cause	Action
Size-standard peaks are not migrating as expected during a normal run time	• Poor quality sample. • Degraded or frozen polymer. • Water used to dilute buffe.r • Poor-quality formamide. • Fluctuations in ambient temperature and/or humidity. • Incorrect oven temperature. • Old array or capillary. • Contaminants. • Low ionic buffer strength.	Prepare fresh buffer (not applicable to 3500 Series instruments).
	Incorrect capillary length (Length to Detector) or run module was selected.	Specify correct capillary length or run module.
	Variation in ambient temperature causes faster or slower migration rates.	Ensure ambient temperature is stable.
Sizing precision is low	Analyzing small fragments <50 bp.	• Sizing differences between various types of polymer are more apparent for sequences <50 bp. • Fragments <50 bp run on 3730/3730*xl* Series instruments with POP- 7™ polymer may have slightly lower sizing precision than expected.

Abnormal peak morphology troubleshooting

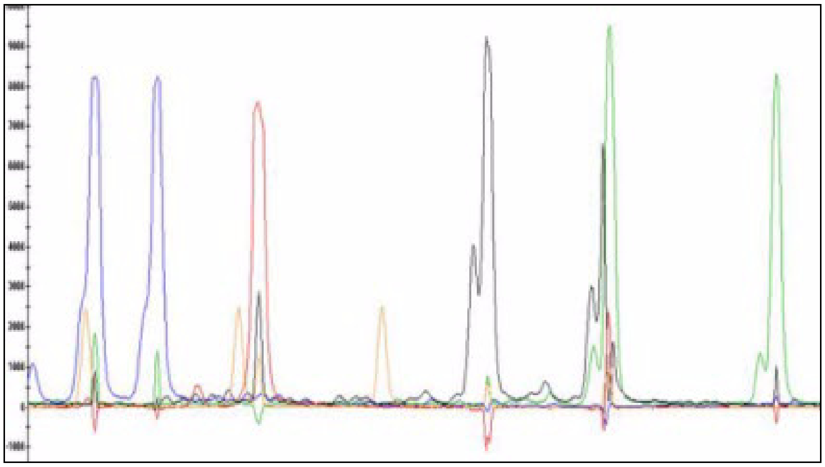
Symptom	Possible cause	Action
Poor resolution	High sample concentration.	Dilute the sample before adding to formamide/ size-standard mix.
	Injection time and/or voltage too high.	See “Optimizing electrokinetic injection parameters” on page 78.
	Wrong capillary array and/or polymer used.	Use appropriate capillary array or polymer for your application.
	Incomplete strand separation due to insufficient heat denaturation.	Make sure the samples are heated at 95°C for 3 to 5 minutes, then immediately placed on ice for 2 to 3 minutes before loading.
Loss of resolution		
	Pump, polymer block, or septa contaminated with chemicals during cleaning.	**1.** Perform a water wash. Refer to the instrument user guide for information. **2.** Replace polymer, buffer, septa and water/waste with fresh materials.
	Incomplete replacement of polymer between runs.	**1.** Check the polymer delivery system for leaks, looking for residue in and around the polymer block area. **2.** Check the pin valve for signs of arcing on the tip. Black markings within the block channel are also a sign of an arcing event. **3.** Check for polymer in the anode buffer jar. If you see evidence of a leak, retighten connections, then run the sample again.
	Sample or reagent is contaminated.	Use fresh samples and reagents.

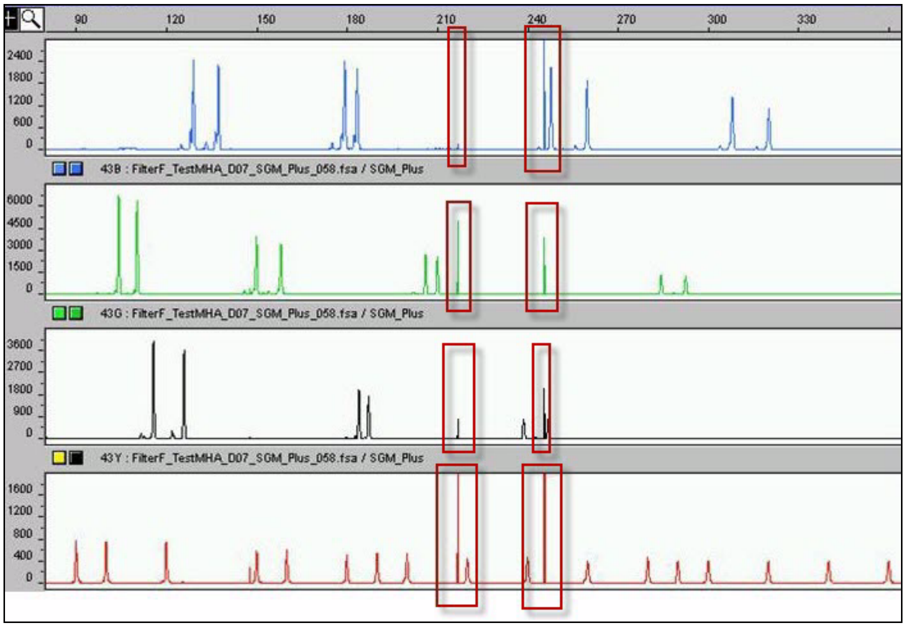
Symptom	Possible cause	Action
Loss of resolution *(continued)*	High salt concentration.	Salt preferentially injects smaller fragments and inhibits injection of larger fragments, so the majority of salt may have been injected in the first injection. Re-inject the sample.
		If signal intensity does not increase, see “Desalting” on page 190.
	Bubbles or debris in polymer path.	Remove bubbles or clean the polymer path. Refer to the instrument user guide for information.
	Capillary array degrading.	**1.** Perform a water wash through the polymer delivery system. Refer to the instrument user guide for information. **2.** Replace the capillary/array. **3.** Run a size standard. **4.** If the problem is present in the size standard, replace reagents, then run your samples again.
	Samples are degraded because they have been sitting in the instrument >24 hours.	Run samples as soon as possible after preparation.
	Expired or degraded polymer, Hi-Di™ Formamide, buffer, or water.	Replace the reagent, then run your samples again. Use fresh, properly stored Hi-Di™ Formamide. See “Hi-Di™ Formamide storage” on page 82.
	Instrument current problem.	See “Instrumentation troubleshooting” on page 180.
	Use of non-Thermo Fisher Scientific reagents.	**1.** Perform a water wash on all components of the system using the wizard in Data Collection Software. **2.** Replace reagents with Thermo Fisher Scientific products.
Broad, lagging peaks	Old or clogged capillary array.	Replace the capillary array or flush the capillary array with polymer.
Tailing peaks	Degraded or improperly stored Hi-Di™ Formamide.	Use fresh, properly stored Hi-Di™ Formamide. See “Hi-Di™ Formamide storage” on page 82.

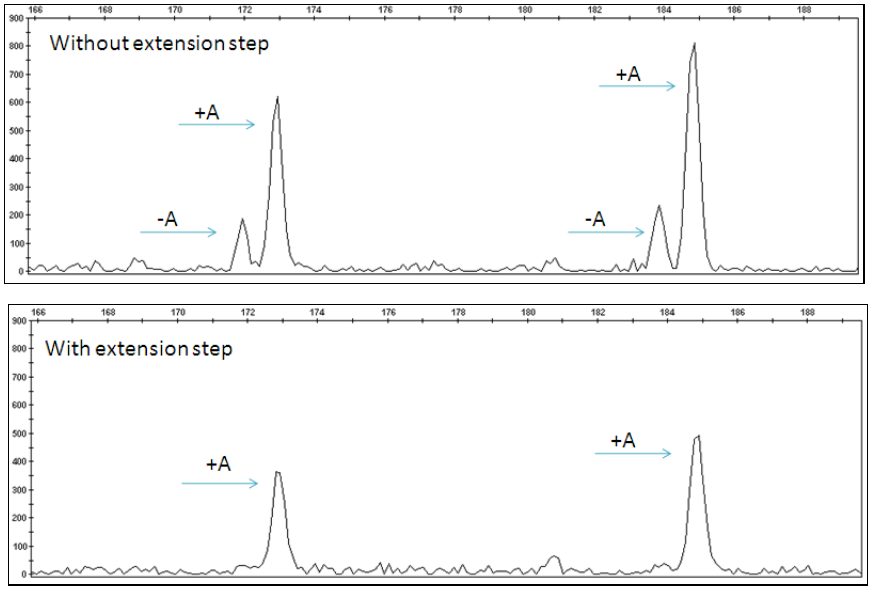
Symptom	Possible cause	Action
Uneven peak heights in dyes in multiplexed sample		
	Sample preparation issues.	Optimize sample preparation and PCR.
	Preferential amplification of PCR products.	see "PCR troubleshooting" on page 176.
	Selection of dyes is not optimal (for example, a low-intensity sample peak is labeled with a low-intensity dye).	Select appropriate dye. For information, see "Points to consider when selecting dyes for custom primers" on page 39.
	Concentration of some samples is too high.	• Adjust the pooling ratio before PCR (see "Multiplexing (pooling) strategies" on page 27). • If overall concentration is too high, dilute pooled samples with deionized water before PCR.
		Increasing the $MgCl_2$ concentration of some samples can reduce the disparity in peak heights, but may also increase the amplification of non-specific products (background).
Sudden loss of signal in all samples	Instrument laser power or current problem.	Check laser power and current in the EPT window (see "Examine the sample info, raw data, and EPT trace" on page 154).
Multiple dye colors are detected as one dye color	5-dye samples were run with a 4-dye matrix.	Repeat run with correct dye set.

Extra peaks troubleshooting

Symptom	Possible cause	Action
Extra peaks	Pull-up or "cross-talk" due to saturated data in a dye color (for example, a high intensity blue peak can create pull up peaks in other colors). See "Signal intensity is too high or saturated" on page 166.	Decrease sample concentration during PCR or when preparing samples for electrophoresis.
	Degraded PCR products.	Repeat PCR.
	Stutter peaks.	See "Identifying stutter products in microsatellite analysis" on page 113.
	Incomplete restriction or ligation (AFLP applications only).	Extract the DNA again and repeat the restriction-ligation.
	Sample is not denatured.	Make sure the samples are heated at 95°C for 3 to 5 minutes, then immediately placed on ice for 2 to 3 minutes before loading.
	Hairpin secondary structures are present in PCR primers.	See "PCR troubleshooting" on page 176.
	Non-specific primer peaks.	
	Primer/dimer peaks.	See "Primer design guidelines" on page 29.
	Artifact peaks.	See "Pull-up peaks from a sample appear in the red or orange dye signal, and are detected as size-standard peaks due to over-saturation of sample-peak signal." on page 185.
	Sample or reagent contamination.	Use fresh sample or reagent.
	Contamination with exogenous DNA.	Use appropriate techniques to avoid introducing foreign DNA during laboratory handling.
	Renaturation of denatured samples.	Load the sample immediately following denaturation, or store it on ice until ready.

Symptom	Possible cause	Action
Pull-up peaks		
	Incorrect or poor-quality matrix or spectral calibration.	Run a new spectral calibration.
		Edit the instrument protocol to specify the correct spectral calibration.
	Wrong matrix or spectral use for analysis of 310 instrument data.	Reanalyze with the correct matrix in the GeneMapper® Software.
	Offscale, saturated signal in primary peak caused by high sample concentration.	• Decrease sample concentration. • Decrease injection time.
	Polymer on instrument >7 days, degraded polymer contaminants.	Perform warm water wash(es) and replace polymer. If the problem persists, replace the capillary array.

Symptom	Possible cause	Action
Data spikes	 90 120 150 180 210 240 270 300 330 2400 1800 1200 600 0 43B : FilterF_TestMHA_D07_SGM_Plus_058.fsa / SGM_Plus 6000 4500 3000 1500 0 43G : FilterF_TestMHA_D07_SGM_Plus_058.fsa / SGM_Plus 3600 2700 1800 900 0 43Y : FilterF_TestMHA_D07_SGM_Plus_058.fsa / SGM_Plus 1600 1200 800 400 0	
	Bubbles, dried polymer, or dust in the capillary array migrate past the camera.	**1.** Flush the water trap. Refer to the instrument user guide for information. **2.** Check for bubbles and run the bubble wizard if any are visible. Clean all connections and tubing around the instrument pump. **3.** Check the polymer bottle, the area around the pump lines, and the array port for crystals. If present, warm the polymer gently to 30°C with gentle mixing, then refill the pump and array with the polymer. **4.** If the problem persists, perform a water wash and replace the polymer.

Symptom	Possible cause	Action
Split peaks		
	Plus A and/or minus A peaks due to: • Too many fragments for A-addition. • Suboptimal PCR conditions and/or suboptimal primer design.	Repeat the experiment with a lower initial template concentration.
		Modify the experiment to: • Increase addition of A: – Add a final extension step of 60 minutes at 72°C. – Increase Mg^{2+} concentration – Use ABI PRISM® Tailed Primer Pairs • Remove A by enzymatic treatment (T4 DNA polymerase) For more information, see "Avoiding incomplete 3' A nucleotide addition" on page 34.
Many small extraneous peaks appearing next to a high-intensity peak	Background signal is above Minimum Peak Height value.	Adjust the setting in analysis method.
	High sample concentration. (Extraneous peaks represent non-specific DNA comigrating with main fragment peak.)	Dilute the sample.
	Sample concentration is too high.	Decrease the injection time or injection voltage. See "Optimizing electrokinetic injection parameters" on page 78.

PCR troubleshooting

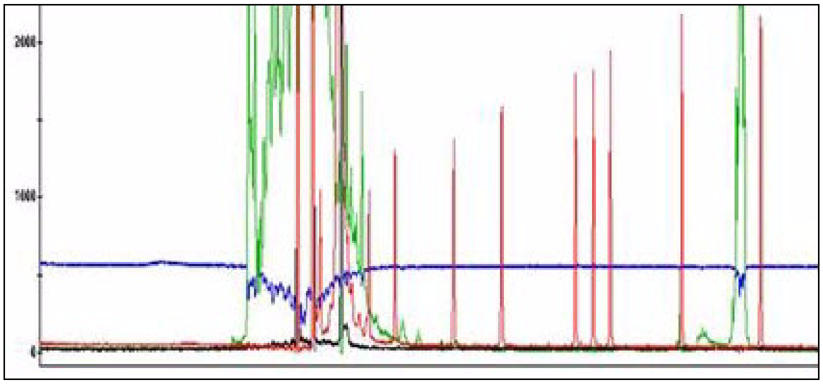
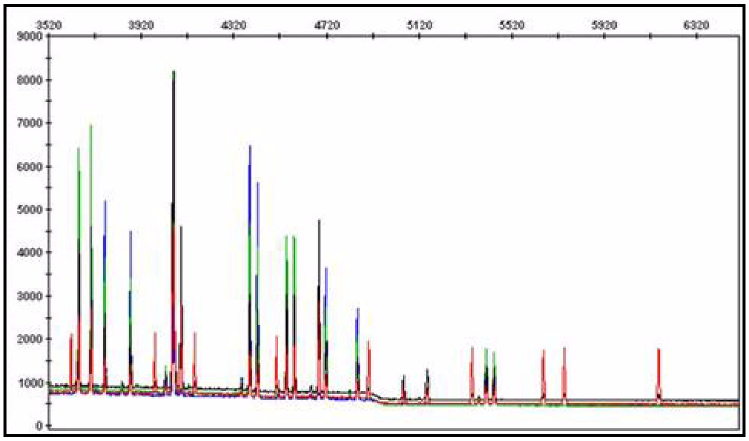
Symptom	Possible Cause	Action
Poor priming resulting in weak signal	Melting temperature is too low due to low G+C content and/or short primer length.	Evaluate primer design.
	Secondary structure of the primer, particularly at the 3' end.	
	Secondary structure of the template in the region of hybridization.	
	Insufficient [F]dNTPs added to PCR reaction.	Reamplify using more [F]dNTPs or examine the efficiency of the PCR.
Amplified DNA concentration is lower than expected	Amplification cycle setting is too low.	Add 3 to 5 cycles.
	Low $MgCl_2$ concentration.	Increase the $MgCl_2$ concentration.
	Low affinity of the primer to the template.	Decrease the annealing temperature 2 to 3°C at a time. Background signal may increase.
	Low sample concentration.	Increase sample concentration.
	Inhibitors in template.	Purify template (see “Purifying DNA” on page 56).
	Thermal cycler malfunction.	Troubleshoot the thermal cycler problem. Refer to the thermal cycler user guide for information.
	PCR reagents are contaminated or expired.	Use fresh PCR reagents.
	Degraded primers.	Store unused primers at –15 to –25°C. Do not expose fluorescent dye-labeled primers to light for long periods of time.
PCR inhibition	• Sample contains hemoglobin, heparin, polyphenol (plant), polysaccharides. • Extraction introduced inhibitors (chloroform, phenol, EDTA, detergents (SDS), xylol, ethanol, bromophenol blue).	Dilute the sample before amplification to reduce the amount of PCR inhibitors.
Contamination with exogenous DNA	Carryover.	Use appropriate techniques to avoid introducing foreign DNA during laboratory handling. For more information, see “Avoiding contamination” on page 64,

Symptom	Possible Cause	Action
Poor amplification, non-specific amplification	Poor-quality or degraded DNA template.	Use fresh template.
		Run an agarose gel to check sample concentration and quality.
		If DNA is stored in water, check water purity.
	Insufficient or excess template DNA.	Use recommended amount of template DNA. Run an agarose gel to check sample concentration and quality.
	PCR inhibitors in the DNA sample (binding proteins, salts that carry over from poor DNA extractions).	Use different extraction procedures.
	Incorrect thermal cycling parameters.	Check protocol for correct thermal cycling parameters.
	Incorrect pH.	Use correct concentration of DNA and buffer.
	Tubes loose in the thermal cycler.	Push reaction tubes firmly into contact with block before first cycle.
	Third-party or non-PCR tube type used.	Use GeneAmp® Thin-Walled Reaction Tubes with Caps with Thermo Fisher Scientific thermal cyclers.
	Primer concentration too low.	Use recommended primer concentration.
	Primer design.	See "Non-specific amplification" on page 32.
Poor amplification, non-specific amplification in restriction-ligation experiments (AFLP only)	Incomplete restriction-ligation (in experiments involving restriction-ligation) due to insufficient or insufficiently active ligase.	**1.** Test the ligase activity with control DNA. **2.** Repeat restriction-ligation with a higher concentration of ligase (in Weiss units). **Note:** 1 Weiss unit = 67 cohesive-end ligation units If the problem persists, repeat the restriction-ligation with fresh enzymes and buffer. Use an agarose gel to check the reaction results. Refer to the AFLP® Plant and Microbial Protocols for more information (see "AFLP® applications" on page 200).
	TE0.1 is buffer not properly made, or contains too much EDTA.	Add the appropriate amount of $MgCl_2$ solution to amplified reaction. Remake the TE0.1 buffer.
	Insufficient enzyme activity.	Repeat the experiment with the recommended amount of restriction enzyme, ligase, and AmpliTaq® DNA Polymerase. **Note:** 1 Weiss unit = 67 cohesive-end ligation units.
Hairpin secondary structures in PCR primers	Primer design.	See "Primer design guidelines" on page 29.

Symptom	Possible Cause	Action
Primer/dimer formation	• MgCl2 concentration. • Annealing temperature in the PCR.	See "Optimizing PCR" on page 55.
	Primer design.	See "Primer design guidelines" on page 29.
	Too much primer added to reaction.	Prepare new reaction.
	Primer over-amplification due to insufficient or poor-quality template.	Prepare new reaction.

Irregular baseline troubleshooting

Excessive noise or an elevated baseline affects both sizing and genotyping results.

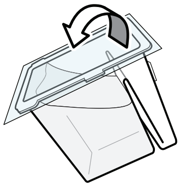
Symptom	Possible Cause	Action
• Constant elevated signal in raw data • "Waterfall" (most common on 310 instruments)	Baseline "waterfall"  	
	Contamination from marker pen ink (if you used a marker to label the plate or the heat seal).	Prepare new plate.

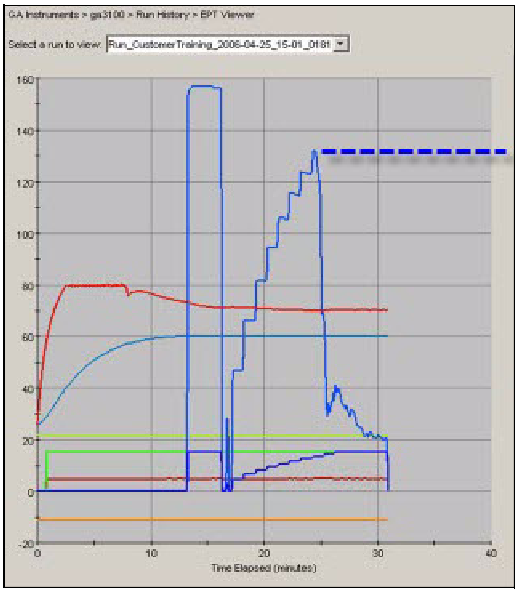
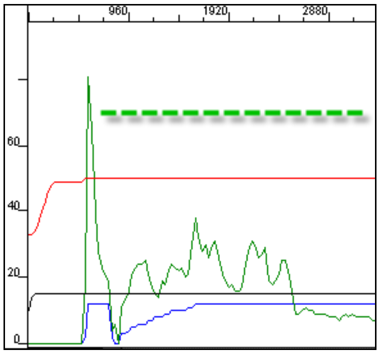
Symptom	Possible Cause	Action
(continued) • Constant elevated signal in raw data • "Waterfall" (most common on 310 instruments)	Contamination from water used to make buffer, wash reservoirs/septa, for sample injection, for any sample-prep steps, or for water wash.	Use fresh water.
	Instrument contamination.	Refer to the instrument user guide for troubleshooting information.
	Improperly filled/leaky connections, tubing, or polymer block.	
	Spectral/matrix calibration issue.	Use correct matrix standard (see "Dye sets and matrix standards" on page 41).
		Specify the correct dye set in the instrument protocol.
		Ensure the correct dye set was selected in spectral calibration.
		Apply the correct matrix file (310 instruments only).
	Polymer on instrument >7 days, polymer degraded or precipitated.	Perform warm water wash(es) and replace polymer. If the problem persists, replace the array.
	Arcing/electronic noise.	Remove bubbles. Refer to the instrument user guide for information.
Noisy baseline	Amplification of non-specific products during PCR.	See "PCR troubleshooting" on page 176.
	Degraded or incorrectly stored Hi-Di™ Formamide can cause low signal and degraded products.	Use fresh, properly stored Hi-Di™ Formamide. See "Hi-Di™ Formamide storage" on page 82.
	Capillary is contaminated.	Perform a water wash.
	Weak or low signals and/or an elevated baseline.	See "Irregular signal intensity troubleshooting" on page 164.
	High salt concentration.	Salt preferentially injects smaller fragments and inhibits injection of larger fragments, so the majority of salt may have been injected in the first injection. Re-inject the sample.
		If signal intensity does not increase, see "Desalting" on page 190.
	Electrical noise	Contact Thermo Fisher Scientific.
Adequate signal strength with noisy data	Secondary hybridization site is present on primer, which results in many extra peaks.	Evaluate primer design.
	Impure primer. You may see a shadow sequence of N-1.	HPLC-purify the primer.

Instrumentation troubleshooting

Some data quality issues are not sample-related, but are caused by settings or conditions used for the instrument run.

Note: The color of the current trace varies between versions of Data Collection Software.

Symptom	Possible Cause	Action
Low or fluctuating current	Expired or incorrectly stored buffer and/or polymer.	Use fresh buffer and polymer.
	Bubbles in polymer.	Remove bubbles. Refer to the instrument user guide for information.
	Anode buffer jar (3130 Series, 3730 Series, or 310 instruments) buffer is not above required level.	Fill the anode buffer jar to the required level.
	ABC (3500 Series instruments) buffer is not above required level.	• For opened containers: Pipet buffer from the overflow chamber to the main chamber. • For unopened containers: Invert the ABC, then tilt it slightly to make sure most of the buffer is in the larger side of the container. There should be less than 1 mL of the buffer remaining in the smaller side of the container. 
Fluctuating current	Arcing caused by bubbles.	Remove bubbles. Refer to the instrument user guide for information.
Current too high	Decomposition of urea in the polymer.	Use fresh polymer.
	Incorrect buffer formulation (most likely too concentrated) (not applicable to 3500 Series instruments).	Use correctly prepared buffer.
	Arcing to conductive surface on the instrument.	Ensure that the ambient temperature is 15 to 30°C and the humidity is <80%. Check for excessive condensation on the instrument.

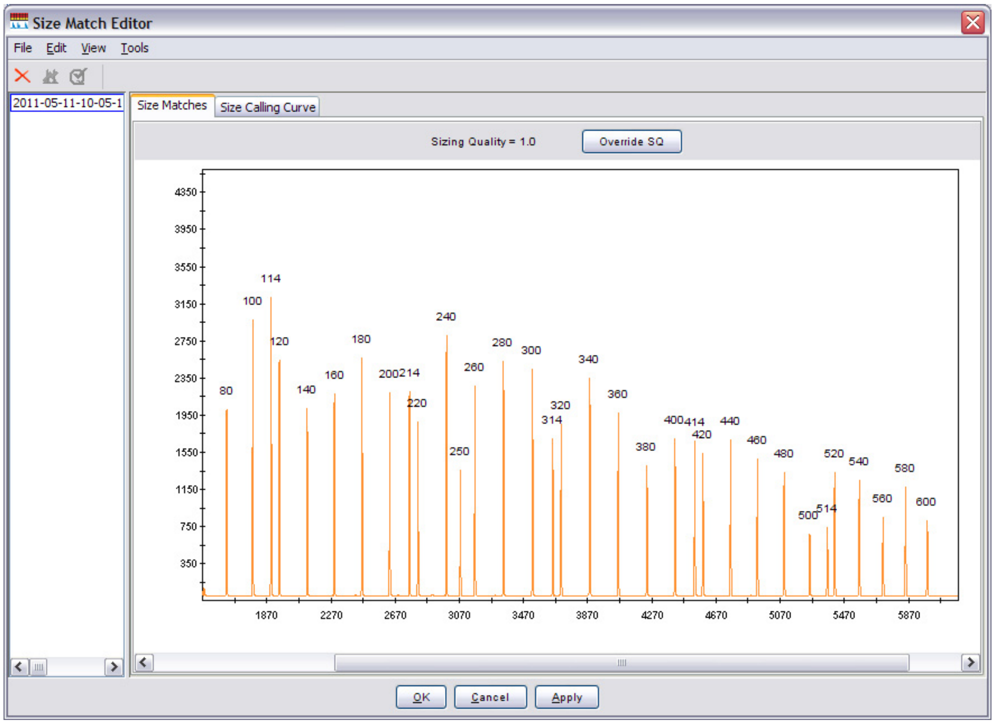
Symptom	Possible Cause	Action
Drop-off of current signal	Data Collection Software EPT view (current trace is blue)  GA Instruments > ga3100 > Run History > EPT Viewer 160 140 120 100 80 60 40 20 0 -20 0 10 20 30 40 Time Elapsed (minutes) Select a run to view: Run_CustomerTraining_2006-04-25_15-01_0181	Current signal should be horizontal
	Air bubble in lower polymer block.	Remove bubbles. Refer to the instrument user guide for information.
	Clogged capillary (caused by sample overloading).	Flush the capillary array with polymer.
Instrument has stopped running and red light is on. There are black marks inside the instrument.	Arcing to conductive surface on the instrument.	Ensure that the ambient temperature is 15 to 30°C and the humidity is <80%. Check for excessive condensation on the instrument.
	Arcing caused by bubbles in polymer.	Remove bubbles from polymer.
	GeneMapper® Software EPT view (current trace is green)  	Current signal shape should resemble the example shown in "Examine the sample info, raw data, and EPT trace" on page 154

Sizing or Size Quality (SQ) troubleshooting

Viewing the size-standard definition

Sizing issues can occur if the peaks detected do not match the peaks listed in the size-standard definition, for example, if additional peaks are detected as size-standard peaks, or if size-standard peaks are not detected.

To view the peaks detected in the size standard and the peaks listed in the size-standard definition for a sample, select the sample, then click (Analysis ▸ Size Match Editor) to view the sizing information for the selected sample(s).


Size Match Editor
File Edit View Tools
2011-05-11-10-05-1
Size Matches
Size Calling Curve
Sizing Quality = 1.0
Override SQ
4350
3950
3550
3150
2750
2350
1950
1550
1150
750
350
1870
2270
2670
3070
3470
3870
4270
4670
5070
5470
5870
80
100
114
120
140
160
180
200
214
220
240
250
260
280
300
314
320
340
360
380
400
414
420
440
460
480
500
514
520
540
560
580
600
OK
Cancel
Apply

If the expected peaks are not detected, your first course of action should be to determine the cause of the peak detection issue and resolve the issue.

If you want to use the data even if the size standard data is of lower quality, you can modify the size-standard definition (described below) to eliminate or add peaks to improve the size standard quality result.

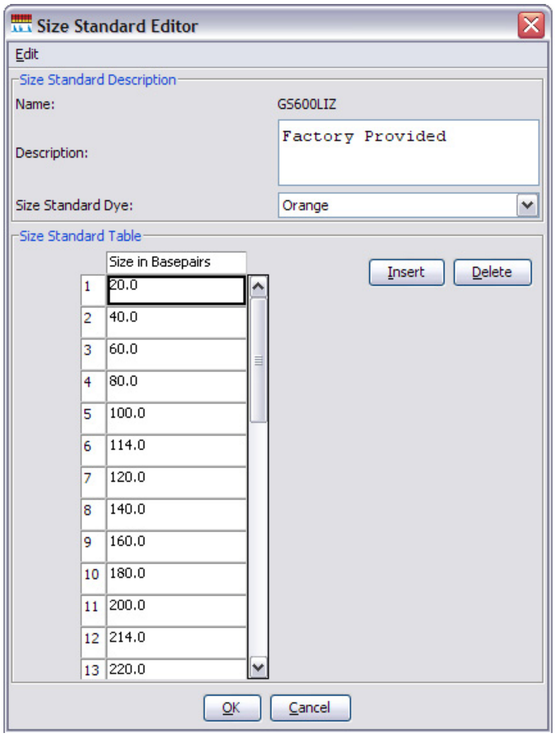
Note: Data for ● SQ samples is viewable only in the Raw Data view.

For more information on techniques for improving sizing accuracy on Thermo Fisher Scientific genetic analyzers, refer to Rosenblum *et al.* (1997) and Wenz *et al.* (1998).

Modifying the size-standard definition

1. In the Project Window, select **Tools** ▸ **GeneMapper Manager**.
2. Select the **Size Standard** tab, then select the size-standard definition used to analyze the data.
3. Select **Save As** and name the new size-standard definition.

4. Open the new size-standard definition and add or remove peaks as needed.



Size Standard Description

Name: GS600LIZ

Description: Factory Provided

Size Standard Dye: Orange

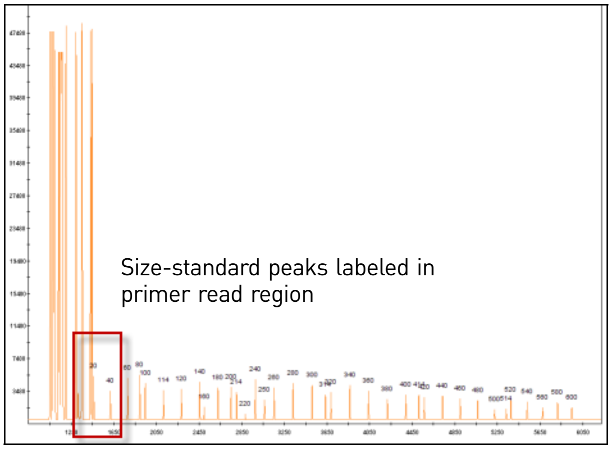
Size Standard Table

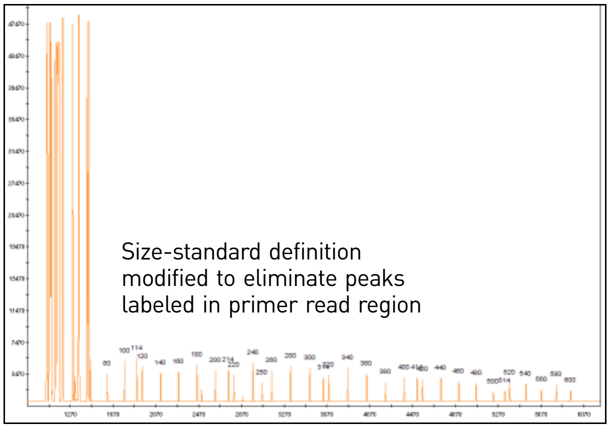
	Size in Basepairs
1	20.0
2	40.0
3	60.0
4	80.0
5	100.0
6	114.0
7	120.0
8	140.0
9	160.0
10	180.0
11	200.0
12	214.0
13	220.0

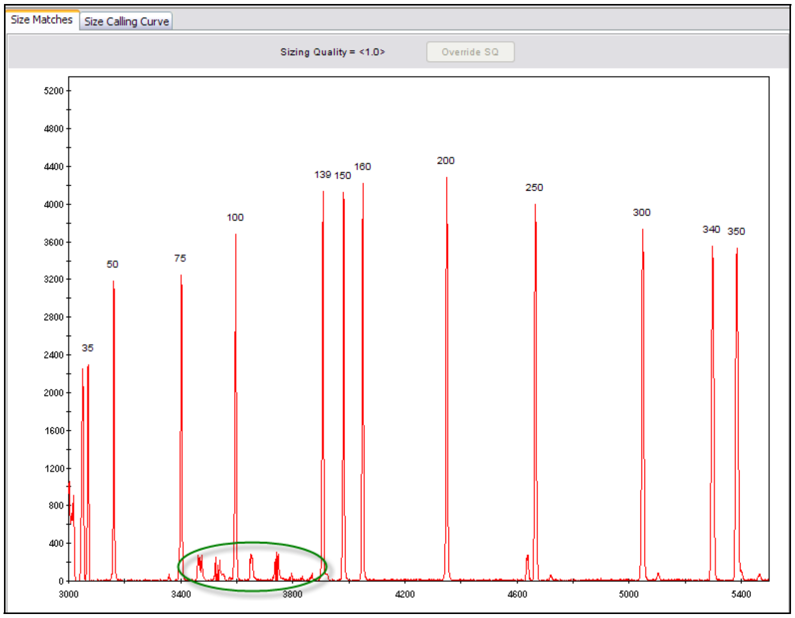
Troubleshooting

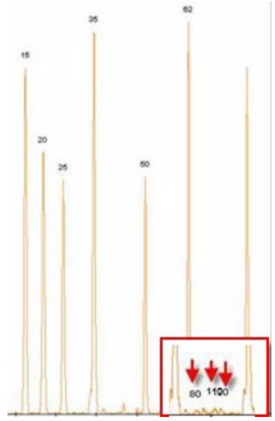
Symptom **SQ ⚠ or 🛑 and...**	**Possible Cause**	**Action**
No sample plot is displayed for a sample with the error "No sizing data"	A plot is not displayed if sizing fails.	See "Sizing or Size Quality (SQ) troubleshooting" on page 182. **Note:** You can view data for failed sizing in the Raw view (see "Examine the sample info, raw data, and EPT trace" on page 154).
Size Match Editor does not display peak data	Incorrect Size Standard Dye specified in size-standard definition.	Verify that the correct dye and fragment sizes are specified in the Size Standard definition. Select, modify, or create correct size-standard definition as needed (see "Modifying the size-standard definition" on page 182).
Missing size-standard peaks	The fragment sizes of the size-standard definition do not match the positions of the detected peaks.	
	The Peak Amplitude Threshold of the dye color associated with the size standard is set too high or low in the analysis method.	Adjust the analysis method so that the peak detection threshold associated is greater than the height of the noise signal. See "GeneMapper® Software peak detection settings" on page 94. **IMPORTANT!** We do not recommend decreasing the threshold below 50 RFU.
	Expired or degraded size standard.	Use fresh size standard.

Symptom **SQ ⚠ or 🛑 and...**	**Possible Cause**	**Action**
Missing size-standard peaks *(continued)*	Incorrect concentration of size standard in sample loading reagent.	Increase the concentration of size standard added to subsequent runs.
	Size-standard peaks are not migrating as expected during a normal run time.	See "Migration troubleshooting" on page 168.
	Incorrect injection settings (for example, the injection time is too short).	Review the injection settings of the run module for errors.
	High salt concentration.	Salt preferentially injects smaller fragments and inhibits injection of larger fragments, so the majority of salt may have been injected in the first injection. Re-inject the sample.
		If signal intensity does not increase, see "Desalting" on page 190.
Smaller size-standard fragments are not labeled	Size standard peak and primer peak are in the same read region (see figure below).	Modify the size standard-definition and remove the size-standard peak that overlaps with the primer peak.
		Change the analysis range in the Advanced Peak Detection Algorithm field in the Peak Detector tab of the analysis method. Select **Partial Range** and select a starting data point that eliminates the primer peaks from analysis.
Larger size-standard peaks are not present in trace	Run time too short.	Increase run time.
	Late start caused by a reagent issue, a blocked capillary, or high sample concentration.	Use fresh polymer.
	A non-Thermo Fisher Scientific size standard was used.	Use a Thermo Fisher Scientific size standard.





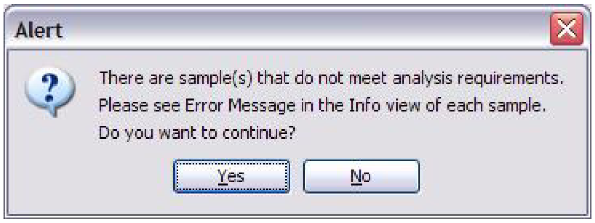
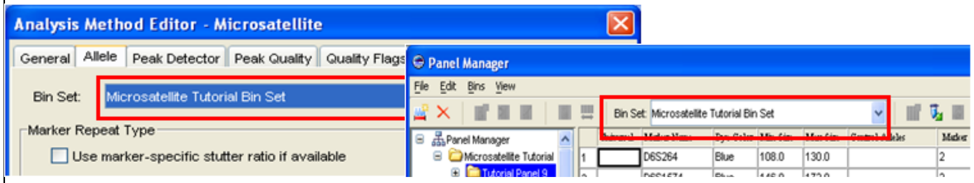
Symptom **SQ ⚠ or 🛑 and...**	**Possible Cause**	**Action**
Extra peaks in size-standard trace	 Size Matches Size Calling Curve Sizing Quality = <1.0> Override SQ 5200 4800 4400 4000 3600 3200 2800 2400 2000 1600 1200 800 400 0 35 50 75 100 139 150 160 200 250 300 340 350 3000 3400 3800 4200 4600 5000 5400	
	Pull-up peaks from a sample appear in the red or orange dye signal, and are detected as size-standard peaks due to over-saturation of sample-peak signal.	• Decrease sample concentration. • Decrease injection time.
	Spectral calibration performed with the incorrect matrix standard for the dye set.	Perform a spectral calibration with the correct matrix standard for the dye set (see Table 18 on page 86).
	Spectral calibration is from a different array or has not been run within the last 3 months.	If the peak heights that cause the pull-up peak are not near the saturation limits of the instrument, repeat the spectral calibration.
	The signal in a neighboring capillary is very strong and creating a bleed-through peak.	Decrease the sample concentration. Decrease the injection time.
	Degraded size standard. A size standard can be degraded by sitting at room temperature for >24 hours or using improperly stored Hi-Di Formamide.	Use fresh size standard and fresh, properly stored Hi-Di™ Formamide. See "Hi-Di™ Formamide storage" on page 82.
	Wrong size-standard definition was used for the analysis.	Re-analyze with correct size-standard definition.
	Degraded or incorrectly stored Hi-Di™ Formamide.	Use fresh, properly stored Hi-Di™ Formamide. See "Hi-Di™ Formamide storage" on page 82.

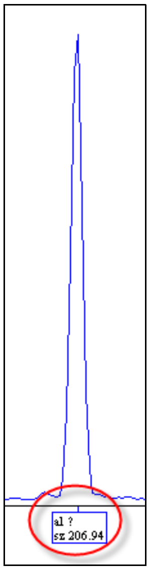
Symptom **SQ ⚠ or 🔴 and...**	**Possible Cause**	**Action**
Sizing failures occur in a regular pattern (the same wells fail repeatedly)	• Electrophoresis or pipetting error. • Defective capillaries/arrays. • Autosampler is misaligned.	Refer to the instrument user guide for information on troubleshooting capillaries/arrays.
Noise peaks are detected as size-standard peaks	 Noise peaks	
	Contaminated sample.	Prepare new sample.
		Use fresh, properly stored Hi-Di™ Formamide. See “Hi-Di™ Formamide storage” on page 82.
	The Peak Amplitude Threshold of the dye color associated with the size standard is set too high or low in the analysis method.	Adjust the analysis method so that the peak detection threshold associated is greater than the height of the noise signal. See “GeneMapper® Software peak detection settings” on page 94. **Note:** You can adjust the threshold if you want to examine the data. However, we recommend that you rerun with increased size-standard concentration.
Size call inaccurate for known DNA sample	Incorrect size standard was added to sample.	Repeat with the correct size standard.
	Size standard peaks are not sized correctly.	Examine the size standard peaks and compare the sizes to the size standard definition. See “Viewing the size-standard definition” on page 182.
	Migration issues.	See “Migration troubleshooting” on page 168.

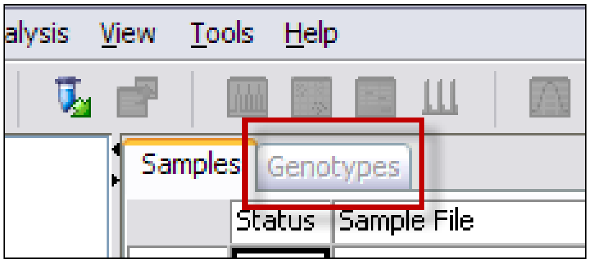
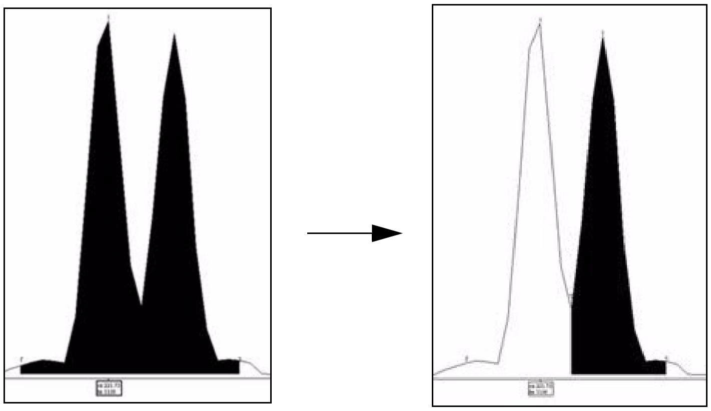
GeneMapper® Software troubleshooting

Some problems with data can be caused by the settings used to analyze the data. If peak height, morphology, and number of expected peaks are acceptable, but the sample fails sizing, it may be caused by analysis method settings that are not optimized for your application.

For additional troubleshooting information, refer to the GeneMapper® Software Help and the *GeneMapper® Software Reference and Troubleshooting Guide* (Pub. no. 4403673).

Symptom	Possible Cause	Action
GeneMapper® Software error message	 Alert There are sample(s) that do not meet analysis requirements. Please see Error Message in the Info view of each sample. Do you want to continue? 	
	Generic message - the error for each sample may be different.	View the Info tab (see "Examine the sample info, raw data, and EPT trace" on page 154). For information on resolving the error, refer to the *GeneMapper® Software Reference and Troubleshooting Guide*.
Error Message: The bin set in the analysis method does not match the panel used for analysis.	The bin set in the analysis method does not match the panel used for analysis.	Modify the Bin Set selection on the Allele tab in the analysis method.
	 Analysis Method Editor - Microsatellite Bin Set: Microsatellite Tutorial Bin Set Marker Repeat Type ☐ Use marker-specific stutter ratio if available Panel Manager Bin Set: Microsatellite Tutorial Bin Set 	

Symptom	Possible Cause	Action
“al?” label or alleles are not falling within bins		
	Allele is migrating at a different rate than expected.	See “Migration troubleshooting” on page 168.
	Bin is not defined for the allele.	Modify the binset to define the allele.
Allele not labeled	Peak is detected as stutter.	Decrease the Stutter ratio in the analysis method. If the problem persists, mask the stutter peak by labeling the allele as a mono peak: In the Panel Manager, change the marker repeat # to 1 for the marker in question.
	Peak intensity is below the Peak Amplitude Threshold in the analysis method.	Adjust the Peak Amplitude Threshold in the analysis method.
Data not sorted by name	The default setting in GeneMapper® Software sorts the data by status rather than sample name.	To resort the data: • Select **Edit ▸ Sort**, or • Shift+click the column header to sort by Clicking once sorts in ascending order, clicking twice sorts in descending order.
When adding samples to a project, the expected data files are not listed in the Add Samples to Project dialog box	Data was not collected as expected.	Ensure the following on the Data Collection Software computer: • The instrument completed the run(s) and data are visible in Data Collection Software. • Sample files were extracted successfully (3130 Series or 3730 Series instruments). • The run folder was created and saved on the instrument computer. • The correct number of *.fsa sample files were created within the run folder.

Symptom	Possible Cause	Action
Genotypes tab is grayed	 alysis View Tools Help Samples Genotypes Status Sample File	
	No panel specified before analysis.	Specify a panel in the Project window before analysis.
Two peaks do not separate and are detected as one peak		
	The software is detecting two separate peaks as one.	Adjust analysis method Peak Detector settings: • Polynomial degree: A higher setting increases the sensitivity of the curve-fitting process. A lower setting decreases it. • Peak Window size value: A lower setting increases the sensitivity of the curve-fitting process. A higher setting decreases it. Decrease the peak window size to 13 and re-analyze. For more information, see "GeneMapper® Software peak detection settings" on page 94.

Preamplification gel troubleshooting

Symptom	Possible Cause	Action
No DNA smear on agarose gel (AFLP® only)	Incomplete digestion of DNA.	Check that enzymes are not expired, incubation temperature settings are correct, and so on. Redigest DNA.

Desalting

Impact of high salt concentration

Samples high in salt result in poor injections and low signal intensity. You may be able to compensate for decreased signal intensity by:

- Re-injecting the sample. Salt preferentially injects smaller fragments and inhibits injection of larger fragments, so the majority of salt may have been injected in the first injection.
- Increasing the injection time and/or injection voltage.

If reinjecting and increasing the injection parameters does not improve signal intensity, desalt and/or concentrate the samples.

Do not increase sample concentration by evaporating the samples without performing a desalting step. Doing so increases the salt concentration and prevents complete denaturation of the DNA which causes decreased signal strength.

Eliminating salt concentration as the cause

To determine if salt concentration is causing low signal intensity, run the size standard alone (see “Running controls to isolate a problem” on page 156). If you see:

- Resolution loss with the size standard, troubleshoot instrument/reagent issue. Refer to the instrument user guide for information.
- No resolution loss with the size standard, add sample to the well containing the size standard and run again. If resolution decreases, desalt the sample.

Desalting

To desalt your sample(s), try one of the following:

- **NucAway™ Spin Columns**—NucAway™ Spin Columns remove unincorporated nucleotides and salts after probe synthesis reactions. Rehydrate the column, centrifuge to remove the interstitial fluid, add the sample to the top of the column, and centrifuge again.
- **Amicon Centricon-100 Microconcentrator** (or Centricon-30 for fragments smaller than 130 bp)
- **Ethanol precipitation** of the pooled PCR product, followed by resuspension in distilled, deionized water. Refer to *Molecular cloning* (Sambrook, Fritsch, and Maniatis 1989) for protocols.
- **Sample dialysis on a filter membrane**:
  a. Float a Millipore® VS filter (Millipore Part no. VSWP 02500), shiny side up, on top of 50 mL of deionized, autoclaved water in a 50-mL conical plastic tube.
  b. Carefully spot ~15 µL of sample on top of the filter, using an appropriate pipette.

c. Dialyze the sample for 20 minutes.

d. Using a pipette, very carefully remove the sample and dilute.

Evaluating 310 Genetic Analyzer multicomponent matrix quality

Purpose of the multicomponent matrix

The multicomponent matrix compensates for the overlap of different dye colors by subtracting out, in each dye's detection range, the portion of the signal due to fluorescence from other dyes.

Factors affecting matrix quality

- Reagent quality/freshness
- Buffer type and concentration
- Polymer type
- Denaturing or non-denaturing conditions
- Run temperature

When to create a new matrix

When you create a matrix, you must run each dye matrix standard separately to determine the proportional amount of fluorescence that is emitted in all four detection regions.

Because the emission spectra of the dyes vary with the physical environment (such as the pH or polymer type and concentration), create a new matrix if run conditions change.

If you observe any of the symptoms listed in the table on the next page, create a new matrix. You can apply the new matrix to old samples and reanalyze the data.

Virtual Filter Set C

Matrix files made for Virtual Filter Set C are especially susceptible to minor changes in run conditions because of the emission maximum of 6-FAM™ dye (the recommended blue-displaying dye for this filter set):

- It is very close to the laser wavelength of 514.5 nm. Thus, the window for collected blue light-intensity data is offset to longer wavelengths and does not contain the emission maximum of 6-FAM™ dye.
- It is also very close to the detection region for the green-displaying TET™ dye.

If you are using Virtual Filter Set C, watch for evidence of matrix problems and create a new matrix as soon as problems appear

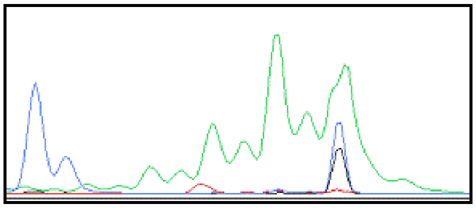
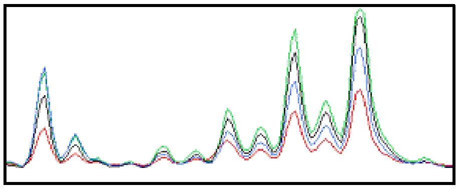
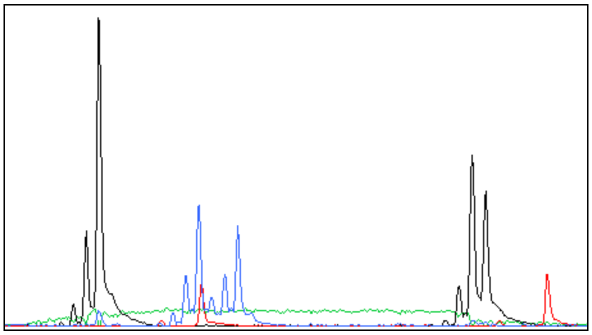
Identifying matrix problems

A poor or incorrect matrix results in too much or too little subtraction of dye spectral overlap during data analysis. Each causes a recognizable electropherogram anomaly:

- Bleedthrough peaks, also called "pull-ups", are caused by too little subtraction

  Bleedthrough peaks are small peaks of one color lying directly under a large peak of another color even though there is no PCR product corresponding to the smaller peak.
- Elevated interpeak baseline is caused by too much subtraction

The table on the next page contains examples of the symptoms listed above.

Symptom	Possible Cause	Action
Pull-up peaks (too little matrix subtraction)		
	The matrix was made with the wrong dyes or filter set.	Create a new matrix with the correct dye and filter set. See "Dye sets" on page 41.
	The signal from a large peak is off-scale because of sample overloading. In the raw data, the peak showing pull-up is off-scale. 	Keep peak heights between approximately 150 and 4000 RFU. If sample data is off-scale, do one of the following: • Rerun the samples using a shorter injection time. • Dilute and rerun the samples. • Create a new matrix with the correct dye and filter set. See "Dye sets" on page 41.
Elevated interpeak baseline (too much matrix subtraction)		
	Too much matrix subtraction	Create a new matrix.

Ordering Information

- Thermal cyclers and accessories 193
- Genetic analyzers and consumables 194
- GeneScan™ size standards 197
- Matrix standards for spectral calibration 197
- Installation standards 197
- Reagent kits 198
- Other user-supplied materials 198

Thermal cyclers and accessories

Item	Source
Veriti® 96-Well Thermal Cycler	4375786
GeneAmp® PCR System 9700 with the Silver 96-Well Block	N8050001
GeneAmp® PCR System 9700 with the Gold-plated Silver 96-Well Block	4314878
Silver 96-Well Sample Block	N8050251
Gold-plated Silver 96-Well Sample Block	4314443
MicroAmp® Autoclaved Reaction Tubes with Caps, 0.2 mL	N8010612
GeneAmp® Autoclaved Thin-walled Reaction Tubes with Domed Caps, 2000 tubes	N8010537
GeneAmp® Autoclaved Thin-walled Reaction Tubes with Domed Caps, 1000 tubes	N8010611
MicroAmp® 96-Well Tray	N8010541
MicroAmp® Reaction Tube with Cap, 0.2-mL	N8010540
MicroAmp® 8-Tube Strip, 0.2-mL	N8010580
MicroAmp® 8-Cap Strip	N8010535
MicroAmp® 96-Well Tray/Retainer Set	403081
MicroAmp® 96-Well Base	N8010531
MicroAmp® Clear Adhesive Film	4306311
MicroAmp® Optical Adhesive Film	4311971
MicroAmp® Optical 96-Well Reaction Plate	N8010560

Genetic analyzers and consumables

Item†	**Source**
Applied Biosystems® 3500 or 3500xL Genetic Analyzer	Contact your local Thermo Fisher Scientific sales representative
Applied Biosystems® 3500xL Genetic Analyzer	
3130 or 3130*xl* Genetic Analyzer	
3730 or 3730*xl* DNA Analyzer	
310 Genetic Analyzer	
3500/3500xL Analyzer materials	
Anode Buffer Container (ABC)	4393927
Cathode Buffer Container (CBC)	4408256
Septa Cathode Buffer Container, 3500 Series	4410715
Conditioning Reagent	4393718
Capillary Array, 36 cm, 8 Capillary, 3500 Genetic Analyzers	4404683
Capillary Array, 50 cm, 8 Capillary, 3500 Genetic Analyzers	4404685
Capillary Array, 36 cm, 24 Capillary, 3500xL Genetic Analyzers	4404687
Capillary Array, 50 cm, 24 Capillary, 3500xL Genetic Analyzers	4404689
GeneScan™ Size Standards	See "GeneScan™ size standards" on page 197.
Matrix Standard Kits	See "Matrix standards for spectral calibration" on page 197.
POP-4® Polymer (960 Samples), 3500/3500xL Genetic Analyzers	4393710
POP-4® Polymer (384 Samples), 3500/3500xL Genetic Analyzers	4393715
POP-6™ Polymer (960 Samples), 3500/3500xL Genetic Analyzers	4393712
POP-6™ Polymer (384 Samples), 3500/3500xL Genetic Analyzers	4393717
POP-7™ Polymer (960 Samples), 3500/3500xL Genetic Analyzers	4393710
POP-7™ Polymer (384 Samples), 3500/3500xL Genetic Analyzers	4393715
8-Tube Retainer and Base Set (Standard) for 3500/3500xL Genetic Analyzers	4410231
8-Strip Septa for 3500/3500xL Genetic Analyzers	4410701
96-well Retainer and Base Set (Standard) 3500/3500xL Genetic Analyzers	4410228
96-Well Septa for 3500/3500xL Genetic Analyzers	4412614
3730/3730*xl* Analyzer materials	
Capillary Array, 50 cm, 48 Capillary, 3730 for Genetic Analyzers	4331250
Capillary Array, 36 cm, 48 Capillary, 3730 for Genetic Analyzers	4331247
Capillary Array, 50 cm, 96 Capillary, 3730*xl* for Genetic Analyzers	4331246
Capillary Array, 36 cm, 96 Capillary, 3730*xl* for Genetic Analyzers	4331244
GeneScan™ Size Standards	See "GeneScan™ size standards" on page 197.

Item†	**Source**
Matrix Standard Kits	See "Matrix standards for spectral calibration" on page 197.
POP-7™ Polymer (28 mL), 3730/3730*xl* Genetic Analyzers	4363929
POP-7™ Polymer (140 mL), 3730/3730*xl* Genetic Analyzers	4335615
POP-7™ Polymer (280 mL), 3730/3730*xl* Genetic Analyzers	4363935
POP-7™ Polymer (840 mL), 3730/3730*xl* Genetic Analyzers	4335611
MicroAmp® Optical 96-Well Reaction Plate	N8010560
Hi-Di™ Formamide	4311320
Reservoir Septa	4315932
Running Buffer, 10×	402824
96-Well Plate Septa	4315933
3130/3130*xl* Analyzer materials	
96-Well Plate Septa	4315933
Reservoir Septa	4315932
Capillary Array, 80 cm, 4 Capillary, 3130 Genetic Analyzers	4333465
Capillary Array, 50 cm, 4 Capillary, 3130 Genetic Analyzers	4333466
Capillary Array, 36 cm, 4 Capillary, 3130 Genetic Analyzers	4333464
Capillary Array, 22 cm, 4 Capillary, 3130 Genetic Analyzers	4333463
Capillary Array, 80 cm, 16 Capillary, 3130*xl* Genetic Analyzers	4319899
Capillary Array, 50 cm, 16 Capillary, 3130*xl* Genetic Analyzers	4315930
Capillary Array, 36 cm, 16 Capillary, 3130*xl* Genetic Analyzers	4315931
Capillary Array, 22cm, 16 Capillary, 3130*xl* Genetic Analyzers	4319898
GeneScan™ Size Standards	See "GeneScan™ size standards" on page 197.
Matrix Standard Kits	See "Matrix standards for spectral calibration" on page 197.
POP-4® Polymer (7000 µL), 3130/3300*xl* Genetic Analyzers	4352755
POP-4® Polymer (3500 µL), 3130/3300*xl* Genetic Analyzers	4363752
POP-6™ Polymer (7000 µL), 3130/3300*xl* Genetic Analyzers	4352757
POP-6™ Polymer (3500 µL), 3130/3300*xl* Genetic Analyzers	4363783
POP-7™ Polymer (7000 µL), 3130/3300*xl* Genetic Analyzers	4352759
POP-7™ Polymer (3500 µL), 3130/3300*xl* Genetic Analyzers	4363785
Running Buffer, 10×	402824
MicroAmp® Optical 96-Well Reaction Plate	N8010560
3100/3100-*Avant* Genetic Analyzer Autosampler Plate Kit, 96-well	4316471
Hi-Di™ Formamide	4311320
310 Analyzer materials	

Item[†]	Source
Capillary Array, 47-cm, 310 DNA Analyzer capillary array	402839
Capillary Array, 61-cm, 310 DNA Analyzer capillary array	402840
96-well tray adaptor (for 9700 thermal cycler trays)	4305051
GeneScan™ Size Standards	See “GeneScan™ size standards” on page 197.
Matrix Standard Kits	See “Matrix standards for spectral calibration” on page 197.
0.5 mL sample tray	5572
MicroAmp® 8-tube strip, 0.2-mL	N8010580
MicroAmp® 96-well base (holds 0.2-mL reaction tubes)	N8010531
MicroAmp® 96-well full plate cover	N8010550
MicroAmp® 96-well tray/retainer set	403081
POP-4® Polymer (5000 µL), 310 Genetic Analyzers	402838
POP-6™ Polymer (5000 µL), 310 Genetic Analyzer	402837
Hi-Di™ Formamide	4311320
Running Buffer, 10✕	4335643
Genetic Analyzer Septa Retainer Clips for 96-tube Sample Tray	402866
Genetic Analysis Sample Tubes (0.5-mL)	401957
Septa for 0.5-mL Sample Tubes	401956

[†] For the Safety Data Sheet (SDS) of any chemical not distributed by Thermo Fisher Scientific, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

GeneScan™ size standards

LIZ® dye-labeled size standards (5-dye chemistry)	Part no.	ROX™ dye-labeled size standards (4-dye chemistry)	Part no.
GeneScan™ 120 LIZ®	4324287	GeneScan™ 350 ROX™	401735
GeneScan™ 500 LIZ®†	4322682	GeneScan™ 400HD ROX™	402985
GeneScan™ 600 LIZ®	4366589	GeneScan™ 500 ROX™†	401734
GeneScan™ 600 LIZ® v2.0†	4408399	GeneScan™ 1000 ROX™‡	401098
GeneScan™ 1200 LIZ®†	4379950	—	—

† For denaturing and non-denaturing applications.

‡ For non-denaturing applications only.

Matrix standards for spectral calibration

Dye sets have been tested and optimized for the instrument, except where noted.

Instruments	Dye Set part numbers						
	DS-02	DS-20†	DS-30	DS-31	DS-32	DS-33	DS-34
3500 Series	4323014	Not tested	4345827†	4345829†	4345831	4345833	Not tested
3730 Series	4323014‡	Not tested	4345827†	4345829†	4345831†	4345833	Not tested
3130 Series	4323014	Not tested	434445827	4345829	4345831	4345833	Not tested
310	4323050	401114	401546, 402996	401546, 402996, 4313939	4312131	4318159	401546

† Dye primer matrix standards.

‡ We have tested this dye set but have not optimized for this instrument.

Installation standards

Installation standard	Source	For use with
DS-33 GeneScan™ Installation Standards	4376911	GeneScan™ 600 LIZ® Size Standard v2.0 (6-FAM™, VIC®, NED™, and PET® dyes)
DS-33 GeneScan™ Installation Standards	4330397	GeneScan™ 500 LIZ® Size Standard (6-FAM™, VIC®, NED™, and PET® dyes)
DS-30 GeneScan™ Installation Standards	4316144	GeneScan™ 500 ROX™ (6-FAM™, HEX™, and NED™ dyes)

Reagent kits

Item[†]	Source
AFLP® Kits	
AFLP® I Selective Primer Kit	4303050
AFLP® II Selective Primer Kit	4303051
AFLP® EcoRI Ligation/Amplification Module	402941
AFLP® MseI Ligation/Amplification Module	402942
AFLP® Amplification Core Mix Module	402005
SNaPshot® Kits	
SNaPshot® Multiplex Kit	Contact your local representative
SNaPshot® Primer Focus® Kit	4329538
GeneAmp® EZ *rTth* RNA PCR Kit	N808-0178
GeneAmp® PCR Carryover Prevention Kit	N808-0068
AmpErase® UNG	N808-0096

[†] For the Safety Data Sheet (SDS) of any chemical not distributed by Thermo Fisher Scientific, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Other user-supplied materials

Item[†]	Source
Hi-Di™ Formamide, 25-mL	4311320
Aerosol resistant pipette tips	MLS
Microcentrifuge tubes	MLS
Pipettors	MLS
Tape, labeling	MLS
Tube, 50-mL Falcon	MLS
Tube decapper, autoclavable	MLS
Deionized water, PCR grade	MLS
Vortex	MLS
Millipore® VS filter	Millipore Part no. VSWP 02500
CEPH Individual 1347-02 Control DNA	403062

[†] For the Safety Data Sheet (SDS) of any chemical not distributed by Thermo Fisher Scientific, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Documentation and Support

The publication numbers in this section are for the latest product versions available at the time of publication. For documentation for newer product versions, go to **www.lifetechnologies.com**.

Instrument documentation

Document	Publication number
Applied Biosystems® *3500/3500xL Genetic Analyzer User Guide (3500 Series Software v1.0)*	4401661
Applied Biosystems® *3500/3500xL Specification Sheet*	106SP11-01
Applied Biosystems® *3730 DNA Analyzer Getting Started Guide*	4359476
Applied Biosystems® *3730/3730xl DNA Analyzer Specification Sheet*	106SP08-01
Applied Biosystems® *3130/3130xl Genetic Analyzers Getting Started Guide*	4352715
Applied Biosystems® *3130/3130xl Specification Sheet*	106SP07-03
310 Genetic Analyzer User Guide	4317588
310 Genetic Analyzer Specification Sheet	903565
Veriti® Thermal Cycler User Guide	4375799
GeneAmp® PCR System 9700 User Guide	4303481

GeneMapper® Software documentation

Document	Publication number
GeneMapper® Software Getting Started Guide: AFLP® System Analysis	4403620
GeneMapper® Software Getting Started Guide: Loss of Heterozygosity (LOH) Analysis	4403621
GeneMapper® Software Getting Started Guide: Microsatellite Analysis	4403672
GeneMapper® Software Getting Started Guide: SNaPshot® Kit Analysis	4403618
GeneMapper® Software Reference and Troubleshooting Guide	4403614
GeneMapper® Software Installation and Administration Guide v4.1	4403614
GeneMapper® Software Reference and Troubleshooting Guide v4.1	4403673
GeneMapper® Software Quick Reference Guide v4.1	4403615

Peak Scanner Software documentation

Document	Publication number
Peak Scanner Software Reference Guide	4382253
Peak Scanner Software Quick Reference Card	4383719

Application documentation

Document	Publication number
AFLP® applications	
GeneMapper® Software Getting Started Guide: AFLP® System Analysis	4403620
AFLP® Microbial Fingerprinting Protocol	402977
AFLP® Plant Mapping Protocol	4303146
Aneuploidy applications	
Aneuploidy Detection by QF-PCR of STR Markers on the Applied Biosystems® *3500xL Genetic Analyzer*	106AP28-01
BAC applications	
BAC Fingerprinting on the Applied Biosystems® *3730/3730xl DNA Analyzer*	107AP04-01
Sizing of Large DNA Fragments Generated by BAC Fingerprinting on Capillary Electrophoresis System	106AP25-01
HiCEP applications	
High coverage gene expression profiling on the Applied Biosystems® *3500xL Genetic Analyzer*	CO15884
ISSR applications	
ISSR Genotyping of Endangered Plants Using an Optimized Workflow	106AP31-01
LOH applications	
GeneMapper® Software Getting Started Guide: Loss of Heterozygosity (LOH) Analysis	4403621
Relative Fluorescent Quantitation on CapillaryElectrophoresis Systems: Screening for Loss of Heterozygosity in Tumor Samples on the Applied Biosystems® *3130 Series Genetic Analyzers with GeneMapper® Software v3.7*	106AP15-01
Methylation applications	
Cells-to-CpG™ Bisulfite Conversion Kit (50) Protocol	4448998
Advances in Capillary Electrophoresis-Based Methods for DNA Methylation Analysis	106BR14-01
Three Optimized Workflows for CpG Island Methylation Profiling	106AP24-01
Microsatellite applications	
GeneMapper® Software Getting Started Guide: Microsatellite Analysis	4403672
Fact sheet: Microsatellite Analysis on the Applied Biosystems® *3130 Series Genetic Analyzers*	106MI61-01
Uniparental disomy (UPD) analysis of chromosome 15	CO18249
Relative fluorescence quantitation applications	
Aneuploidy Detection by QF-PCR of STR Markers on the Applied Biosystems® *3500xL Genetic Analyzer*	106AP28-01

Document	Publication number
Relative Fluorescent Quantitation on Capillary Electrophoresis Systems: Screening for Loss of Heterozygosity in Tumor Samples on the Applied Biosystems® *3130 Series Genetic Analyzers with GeneMapper® Software v3.7*	106AP15-01
SNP applications	
GeneMapper® Software Getting Started Guide: SNaPshot® Kit Analysis	4403618
User Bulletin: Using the SNaPshot® Multiplex System with the POP-7™ Polymer on Applied Biosystems® *3730/3730xl DNA Analyzers and 3130/3130*	4367258
SNaPshot® Multiplex Kit Quick Reference Card	4323975
Demonstration of SNP Genotyping Using a Single Nucleotide Extension Method and the 3500 Series Genetic Analyzer	CO12863
Using the SNaPshot® Multiplex System with the POP-7™ Polymer on Applied Biosystems® *3730/3730xl DNA Analyzers and 3130/3130xl Genetic Analyzers*	4367258

Obtain SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Thermo Fisher Scientific, contact the chemical manufacturer.

Obtain support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

References

Ammiraju, J.S.S., Dholakia, B.B., Santra, D.K. *et al*. 2001. Identification of inter simple sequence repeat (ISSR) markers associated with seed size in wheat. *Theor Appl Genet.* 102:726–732.

Andersen, P.S., Jespersgaard, C., Vuust, J. *et al*. 2003.Capillary electrophoresis-based single strand DNA conformation analysis in high-throughput mutation screening. *Hum Mutat.* 21:455–465.

Anderson, G.R., Brenner, B.M., Swede, H. *et al*. 2001. Intrachromosomal genomic instability in human sporadic colorectal cancer measured by genome-wide allelotyping and inter-(simple sequence repeat) PCR. *Cancer Res.* 61:8274–8283.

Blair, M.W., Panaud, O., McCouch, S.R. 1999. Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerprinting in rice (*Oryza sativa* L.). *Theor Appl Genet.* 98:780–792.

Canzian, F. Salovaara, R., Hemminki, A. *et al*. 1996. Semiautomated Assessment of Loss of Heterozygosity and Replication Error in Tumors. *Cancer Res.* 56:3331–3337.

Davis, L.M., Glenn, T.C., Strickland, D.C. *et al*. 2002. Microsatellite DNA analyses support an east-west phylogeographic split of American alligator populations. *J Exp Zool.* 294:352–72.

Derakshani, M., Lukow, T. and Liesack, W. 2001. Novel bacterial lineages at the (sub) division level as detected signature nucleotide targeted recovery of 16s rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Applied Environmental Microbiology.* 67(2):623–631.

Doyle, J.J., Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull.* 19:11–15.

Ellegren, H. 2004. Microsatellites: simple sequences with complex evolution. *Nat Rev Genet.* 5:435–45.

Eschenhagen, M., Schuppler, M., and Roske, I. 2003. Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents. *Water Res.* 37(13):3224–3232.

Fleischer, R.C. and Lowe, S. 1995. Construction and screening of microsatellite-enrichedgenomic libraries. In Ferraris, J. and Palumbi, S. (eds), *Molecular Zoology: Advances, Strategies and Protocols.* Wiley-Liss, NY, 459–468.

Goel, S., Chen, Z., Akiyama, Y., Connor, J.A., *et al*. 2006. Comparative physical mapping of the apospory-specific genomic region in two apomictic grasses: *Pennisetum squamulatum* and *Cenchru sciliaris*. *Genetics.* 173(1):389–400.

Gupta, S.K., Souframanien, J., and Gopalakrishna, T. 2008. Construction of a genetic linkage map of black gram, *Vigna mungo* (L.) Hepper, based on molecular markers and comparative studies. *Genome.* 51:628–637.

Hauge, X.Y., and Litt, M. 1993. A study of the origin of 'shadow bands' seen when typing dinucleotide repeat polymorphisms by the PCR. *Hum Mol Genet.* 2:411–415.

Holleley, C.E., and Geerts, P.G., 2009. Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *BioTechniques* 46:511-517.

Huelsenbeck, J.P., and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17:754–755.

Inazuka, M., Tahira, T., and Hayashi, K. 1996. One-tube post-PCR fluorescent labeling of DNA fragments. *Genome Res.* 6:551–557.

Inazuka, M., Wenz, H.M., Sakabe, M., Tahira, T., and Hayashi, K. 1997. A streamlined mutation detection system: multicolor post-PCR fluorescence labeling and single-strand conformational polymorphism analysis by capillary electrophoresis. *Genome Res.* 7:1094–1103.

Iwahana, H., Adzuma, K., Takahashi, Y., Katashima, R., Yoshimoto, K., and Itakura, M. 1995. Multiple fluorescence-based PCR-SSCP analysis with postlabeling. *Genome Res.* 4:275–282.

Johnson, E.L., Zhang, D., Emche, S.D. *et al*. 1994. Construction of libraries enriched for sequence repeats and jumping clones, and hybridization selection for region-specific markers. *Proc Natl. Acad. Sci. USA* 91:88–92.

Lai, Y. and Sun, F., The relationship between microsatellite slippage mutation rate and the number of repeat units. 2003. *Molecular Biology and Evolution.* 20(12):2123–2131.

Leroy, X.J., and Leon, K. 2000. A rapid method for detection of plant genomic instability using unanchored-microsatellite primers. *Plant Mol Biol Rep.* 2000. 18:283a–283g.

Luo, M.C. *et al.*, High-throughput fingerprinting of bacterial artificial chromosomes using the SNaPshot® labeling kit and sizing of restriction fragments by capillary electrophoresis, *Genomics* 82 (2003) 378–389.

Molecular Cloning. 1987. Second Edition. Cold Spring Harbor Laboratory, NY: Cold Spring Harbor Laboratory Press.

Murray, V., Monachawin, C., and England, P.R. 1993. The determination of the sequences present in the shadow bands of a dinucleotide repeat PCR. *Nucleic Acids Research.* 21:2395–2398.

Orita, M., Iwahana, H., Kanazawa, H. *et al*. 1989. Inter- and Intra-specific Variation among Five Erythroxylum Taxa Assessed by AFLP. *Proc Natl Acad Sci USA* 86:2766–2770.

Osborn, A. M., Moore, R.B., and Timmis, K.N. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology*. 2(1): 39–50.

Naimuddin, M. and Nishigaki, K. 2004. Genome analysis technologies: Towards species identification by genotype. *Briefings in Functional Genomics and Proteomics* 1(4):356–371.

Ronquist, F., and Huelsenbeck, J.P. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.

Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I. and Benno, Y. 2003. Application of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis. *J Med Microbiol*. 52:79–89.

Savelkoul, P.H.M., Aarts, H.J.M., de Haas, J. *et al*. 1999. Amplified-fragment length polymorphism analysis: the state of an art. *J Clin Microbiol.* 37(10)3083–3091.

Serra, R., Cabanes, F.J., Perrone, G., Venancio, A., Mule, G., and Kozakiewicz, Z. 2006. *Aspergillus ibericus:* a new species of section Nigri isolated from grapes. *Mycologia* 98(2)295–306.

Shihabi, Z. K. and Hinsdale, M. E. 1995. Some variables affecting reproducibility in capillary electrophoresis. *Electrophoresis.* 16: 2159–2163.

Shinde, D., Lai, Y., Sun, F., and Arnheim, N. 2003. Taq DNA polymerase slippage mutation rates measured by PCR and quasi-likelihood analysis: (GA/GT)n and (A/T)n microsatellites. *Nucleic Acids Res.* 31:974–980.

Topal, M.D., and Fresco, J.R. 1976. Complementary base pairing and the origin of substitution mutations. *Nature* 263:285–289.

UBC Primer Set 9, 2006. www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/Primers_Oct2006.pdf

Wan Q-H., Wu, H., Fujihara, T., and Fang, S-G. 2004. Which genetic marker for which conservation genetics issue? *Electrophoresis.* 25:2165–2176.

Wang, H.Z., Wu, Z.X., Lu, J.J. *et al*. 2009. Molecular diversity and relationships among *Cymbidium goeringii* cultivars based on inter-simple sequence repeat (ISSR) markers. *Genetica* 136:391–399.

Witmer, P. D., Doheny, K.F., Adams, M.K., Boehm, C.D. *et al*., 2003. The development of a highly informative mouse simple sequence length polymorphism (SSLP) marker set and construction of a mouse family free using parsimony analysis. *Genome Res.* 13:485–491.

Wolfe, A.D., Xiang, Q-Y., and Kephart, S.R. 1998. Assessing hybridization in natural populations of *Penstemon (Scrophulariaceae)* using hypervariable inter simple sequence repeat markers. *Mol Ecol.* 71:1107–1125.

Zhao, H., Bughrara, S.S., and Oliveira, J.A. 2006. Genetic diversity in colonial bentgrass (*Agrostis capillaris* L.) revealed by EcoRI-MseI and PstI-MseI AFLP markers. *Genome.* 49(4):328–335.

Glossary

allele	One specific sequence of a locus. Different alleles of a single locus will have slightly different DNA sequences.
amplicon	The product of a PCR reaction. Typically, an amplicon is a short piece of DNA.
association studies	Studies that interrogate a dense set of markers to identify associations between those markers and the loci of interest. Association studies are more powerful than linkage mapping studies for the detection of weak-susceptibility loci.
backcross	A genetic cross where an individual from the F1 generation is mated to an individual with the genotype of one of the parents.
bin	In GeneMapper® Software, the expected location of an individual peak or allele, defined by a base-pair range and a color. You typically define a bin for each possible allele associated with a marker.
binset	In GeneMapper® Software, a set of bins that you specify in the analysis method.
contig	In BAC fingerprinting, a contiguous sequence of DNA created by assembling overlapping sequenced fragments of a chromosome. A group of clones representing overlapping regions of the genome.
diploid	Having two copies of every chromosome.
dye set	The term "dye set" corresponds to: • The physical dye set used for labeling fragments. • The software selection you make that identifies the dye colors in the dye set, the order of the dye peaks in the dye set, and spectral analysis parameters for the dyes.
electrophoresis	The act of applying an electrical field to a porous substrate in order to separate molecules by size or shape. For example, nucleic acids like DNA and RNA are negatively charged and are thus attracted to a positive charge and will move toward it through a matrix like a polymer or agarose gel. The strength of the electrical field and charge on the molecules and the size of the pores in the matrix determine the speed at which different molecules in the starting mixture migrate. In general, smaller molecules migrate faster than larger molecules because they are less obstructed as they travel through the matrix.
emission spectrum	The intensity of emitted light (fluorescence) as a function of the wavelength of the emitted light.

excitation efficiency	The probability that it will absorb light of a certain wavelength, as a percentage of the probability of absorption at the wavelength of maximum absorption.
excitation spectrum	The intensity of emitted light as a function of the wavelength of the exciting light.
filter set	Physical filters that separate dye signals in older gel-based capillary electrophoresis instruments. Newer Thermo Fisher Scientific instruments use a diffraction grating.
fingerprint	A characteristic pattern of peaks or bands after an amplification or digest of genomic DNA that can be used to identify an individual.
genetic mapping	Analysis of the progeny from genetic crosses to determine the relative position of chromosomal locations.
Linkage mapping	A meiotic mapping technique which searches for linkage between a marker and the disease gene within several generations of affected families. These studies examine the inheritance of markers in both affected and unaffected members of the family and then determine whether particular markers are physically close to the disease gene of interest. Linkage mapping can be used to scan the entire genome relatively easily.
ISSR PCR	Inter-simple sequence repeat PCR. See "Inter-simple sequence repeat (ISSR) PCR" on page 137.
locus	A location on a chromosome. The term is sometimes used more narrowly to describe the location of a gene or genetic marker. (Plural: loci). See also Marker.
marker	Any observable genetic characteristic (for example, gene, phenotype, microsatellite sequence, SNP) that can be used to identify a genetic location. To be useful in genetic studies, a marker must be present in different forms to allow researchers to distinguish between individuals. One marker represents one locus and one primer pair. Defined by size range of expected alleles and dye (color) attached to primer.
microsatellites	Highly repetitive simple sequence repeats of 2 to 7 base pairs, also called short tandem repeats (STRs). Microsatellites fall into the broader category referred to as variable number of tandem repeats (VNTRs), which also includes minisatellites.
minisatellites	Highly repetitive simple sequence repeats ranging from approximately 10 to 30 base pairs. Minisatellites fall into the broader category referred to as variable number of tandem repeats (VNTRs), which also includes microsatellites.
panel	In GeneMapper® Software, a collection of expected size ranges and dye colors (markers) associated with each primer pair.
phylogenetic studies	Studies to determine evolutionary relationships between organisms (for example, species or populations).
polymerase	An enzyme that catalyzes polymerization. DNA and RNA polymerases build single-stranded DNA or RNA (respectively) from free nucleotides, using another single-stranded DNA or RNA as the template.
polymorphic locus	A locus with more than one allele that is commonly found in a population.

population study	A study that types genetic markers on a large population to identify associations between a marker and a phenotype.
primer	A short single strand of DNA that serves as the priming site for DNA polymerase in a PCR reaction.
pull-up peaks	Artifact peaks in one or more dye colors that are caused by high or saturated signal intensity in another dye color.
quantum yield	The probability that a dye in its excited state will emit a photon as it decays back to the ground state.
restriction enzymes	Endonucleases that cleave the phosphate backbone of double-stranded DNA at highly specific sequences called restriction sites.
template	In PCR, the nucleic acid molecule that provides the sequence to amplify. For example, genomic DNA can be the template for a PCR reaction that amplifies a specific region within the genome.
Variable Number Tandem Repeat (VNTR)	Any genes whose alleles contain different numbers of tandemly repeated oligonucleotide sequences.

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05 April 2014