



Amplifluor® SNPs HT Genotyping System for Assay Development

Catalog No. S7907

Amplifluor® SNPs HT Genotyping System for FAM-SR

Catalog No. S7908

Amplifluor® SNPs HT Genotyping System for FAM-JOE

Catalog No. S7909

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Introduction

Using This Manual

This manual is intended for use with all Amplifluor® SNPs Genotyping Systems for DNA genotyping. The Amplifluor® SNPs Genotyping Systems can be used with several instrumentation platforms, including standard thermocyclers and platereaders as well as the ABI PRISM® 7700 Sequence Detection System and the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems®). The Amplifluor® SNPs Genotyping System for Assay Development is used to validate instrumentation set-up and serves as a positive control when developing SNP screening protocols. The control reagents include DNA samples of known genotype and a primer mixture optimized for genotyping rs#962, a G/T type SNP in the human NUDEL gene which encodes for an endooligopeptidase enzyme. Once a specific SNP screening protocol has been developed, the Amplifluor® SNPs HT Genotyping Systems should be used for high throughput (HT) applications.

Protocols and experimental examples to support the general use of these products in endpoint detection are detailed in this manual. If problems with protocol development are encountered, please contact Technical Service at <http://www.millipore.com/reply/form/techservice> or (800) 221-1975.

Background

Single nucleotide polymorphisms (SNPs) are positions in genomic DNA where a single nucleotide difference occurs between individuals. SNPs are the most abundant DNA markers with an estimated frequency of 1 SNP in every 1000 bases. There is much interest in characterized SNPs due to the application of pharmacogenomic studies to the design and metabolism of drugs. SNPs can occur in both coding and noncoding regions of the genome and can cause non-conservative amino acid changes or altered gene regulation. These changes can have long-term effects that result in disease or affect response to medication.

The development of a one step, PCR-based, universal detection method, the Amplifluor® SNPs Genotyping System, simplifies high throughput analysis for the genotyping of characterized SNPs. The Amplifluor® SNPs Genotyping System is a highly sensitive, homogeneous fluorescent assay. It can be adapted to virtually any sample that can be analyzed by allele-specific PCR. Amplifluor® technology uses energy transfer (ET) primers that generate fluorescent PCR products, permitting SNP typing in a single step. The SNPs Genotyping Systems are designed as multiplex assays that detect both alleles in a single reaction tube with two differently labeled Amplifluor® SNPs Primers.

Product Overview

Principles of the Technique

The Amplifluor® UniPrimer™ System

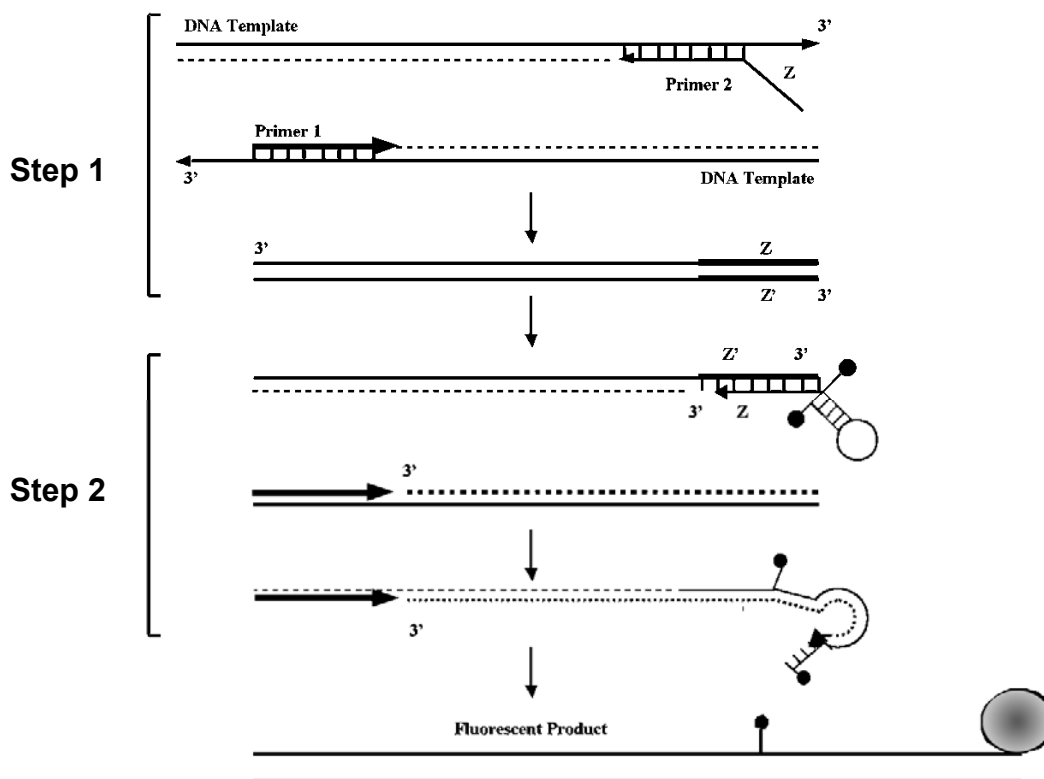
Amplifluor® SNPs Genotyping Systems utilize the Amplifluor® Universal Primer (UniPrimer™) System (9). Amplifluor® Universal Detection systems offer a closed-tube fluorometric detection format for the detection of nucleic acids in endpoint and real-time applications. This patented technology facilitates high throughput analysis and reduces the chances of false-positives due to carry-over amplicon contamination (9-11).

Amplifluor® Universal Systems are based on energy transfer from an excited fluorophore to a complexed acceptor moiety that results in quenching of the fluorescence. This quenching is accomplished by linking the fluorophore and the acceptor 4-(dimethylamino)azo benzene sulfonic acid (DABSYL) to an oligonucleotide primer. The optimized design of the UniPrimers™ results in a large increase in fluorescent emission only upon incorporation into the amplification products produced during each PCR cycle. Unincorporated primers have low fluorescence, eliminating the need to purify the PCR reaction products prior to quantitation.

Each UniPrimer™ consists of a different 3' primer sequence and a 5' hairpin region that is labeled with a unique energy transfer pair. The primer sequences enable the UniPrimers™ to work with target specific unlabeled primers to the researcher's target specific sequence. The target specific primers are synthesized with a 5' tail sequence identical to the 3' region of a particular UniPrimer™, allowing it to hybridize to the PCR product (see Figure. 1, beginning of Step 2.)

During PCR, the incorporated UniPrimer™ serves as a template for DNA polymerization. This process involves the displacement and replication of the hairpin sequence catalyzed by DNA polymerase. The fluorophore is located at the 5' base of the primer and the quencher is linked to the nucleotide that is complementary to the 5' base. Efficient fluorescence quenching occurs when the primer is in this hairpin conformation. Upon incorporation into the double-stranded product, the hairpin structure unfolds and disrupts the energy-transfer between the fluorophore and the quencher. The fluorescent signal produced during each PCR cycle directly correlates to the amount of amplified DNA generated (9).

Figure 1. Amplifluor® UniPrimer™ System.

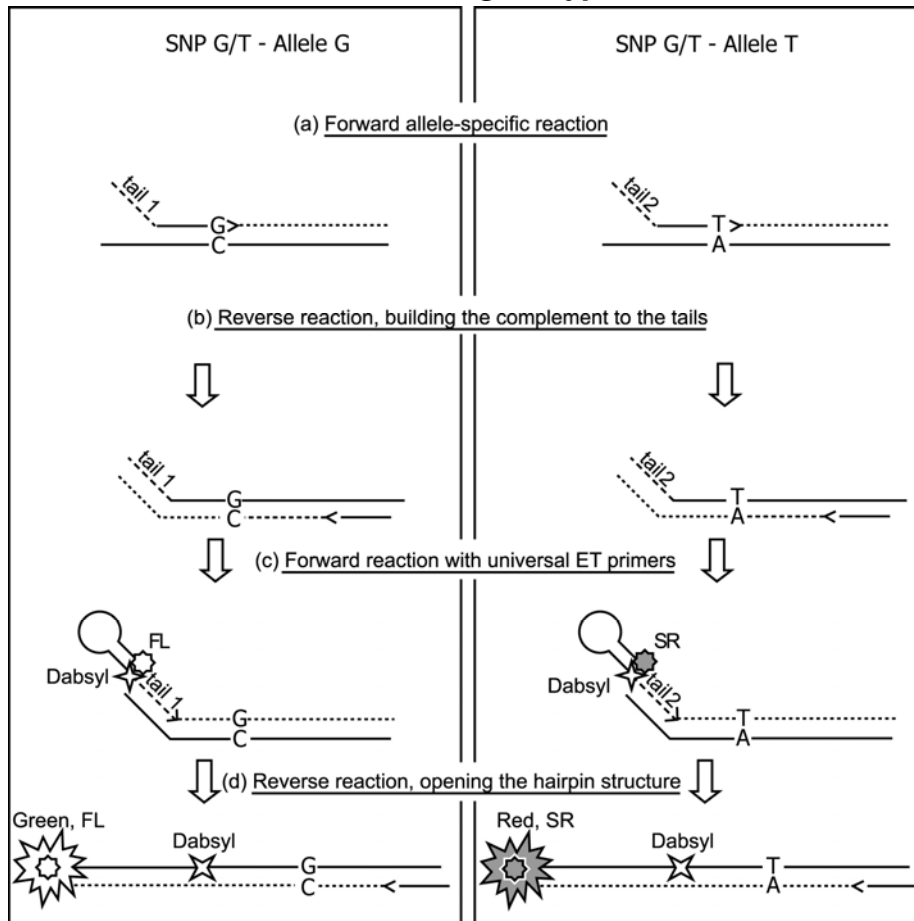


In Step 1 of the reaction, the tailed primer 2 anneals to the target DNA and is extended. Primer 1 anneals to the extended primer 2 and the complementary sequence is synthesized, including the sequence complementary to Z (Z'). In Step 2 of the reaction, the UniPrimer™ anneals to the (Z') sequence and is extended. Primer 1 anneals to the extension product of the UniPrimer™. During DNA polymerization, the UniPrimer™ hairpin structure is displaced and the fluorescent signal is generated. The observed fluorescent signal correlates directly to the amount of amplification product.

Amplifluor® SNPs Genotyping System

The Amplifluor® SNPs Genotyping System is a single tube system utilizing two Amplifluor® SNPs Primers and three unlabeled primers. The pair of Amplifluor® SNPs Primers are labeled either with fluorescein (FAM) and sulforhodamine (SR) or with FAM and a 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE). A single common reverse primer and two SNP specific primers (12, 13) are designed to amplify across the SNP, each with a 5' tail that corresponds to one of the two Amplifluor® SNPs Primers. As SNP-specific PCR products are generated, the primer sequence of the Amplifluor® SNPs Primers hybridizes to the complement of the tail sequence on the newly generated PCR product and, in conjunction with the common reverse primer, amplifies the product further. Depending on which base is present in the target sequence, a FAM or a JOE signal is generated. (When the Amplifluor® SNPs Primers labeled with FAM and SR are used, the signal produced is either FAM or SR.) Heterozygotes result in signals from both fluorophores. The Amplifluor® SNPs Primers and the common reverse primer are used in a 10-fold excess over the SNP specific tailed primers to ensure high yield of the fluorescent product. The same Amplifluor® SNPs Primers can be used for scanning many different SNPs since the discrimination of each SNP only requires the user to have three unmodified oligonucleotides synthesized (14).

Figure 2. Amplifluor® SNPs Detection of an G/T genotype.



(a) Allele-specific primers anneal to the target and are elongated by Taq Polymerase. Each primer has a unique 5' tail sequence that is identical to the priming domain of one of the Amplifluor® SNPs Primers. (b) The common reverse primer anneals and is elongated by Taq Polymerase resulting in synthesis of the tail sequence complement. (c) Amplifluor® SNPs Primers anneal specifically to the products of the reverse reaction and are elongated by Taq Polymerase. (d) During PCR, the hairpin structures of the Amplifluor® SNPs Primers are unfolded and fluorescent signal are generated. FL, Fluorescein; SR, Sulforhodamine.

Several Amplifluor® SNPs Genotyping Systems are available. The Amplifluor® SNPs Genotyping System for Assay Development is designed for new users. This kit contains the FAM-, JOE-, and SR- labeled Amplifluor® SNPs Primers, a Control Primer Mix (containing a common reverse primer and two tailed primers specific for typing rs#962, a common G/T type SNP in humans), and control templates containing GG, GT or TT SNP types. This kit can be used to learn the Amplifluor® SNP genotyping technology and can also assist in the testing of custom primers for the SNP of interest. The Amplifluor® SNPs HT Genotyping Systems are designed for users accustomed to the Amplifluor® System and SNP genotyping technology. The Amplifluor® SNPs HT Genotyping System FAM-SR kit contains only the FAM- and SR-labeled Amplifluor® SNPs Primers, dNTPs and reaction buffer (labeled as Reaction Mix S or Reaction Mix S+). In the Amplifluor® SNPs HT Genotyping System FAM-JOE, the JOE-labeled Amplifluor® SNPs Primer® replaces the SR-labeled Amplifluor® SNPs Primer®

Materials Provided

Table 1: Amplifluor® SNPs Genotyping System for Assay Development (S7907)

Cat# S7907: 200 reactions (10µl volume) Store at -20°C		
<u>Component</u>	<u>Item No.</u>	<u>Volume</u>
20X Amplifluor® FAM SNPs Primer, 5.0 µM stock	90441	100 µL
20X Amplifluor® SR SNPs Primer, 5.0 µM stock	90428	100 µL
20X Amplifluor® JOE SNPs Primer, 5.0 µM stock	90471	100 µL
20X Control Primer Mix	90464	100 µL
10X Reaction Buffer S	90469	200 µL
10X Reaction Buffer S-plus (with reference dye)	90470	200 µL
dNTP Mix (2.5 mM each)	90468	160 µL
Control template, GG type, 25 ng/µL	90465	30 µL
Control template, CC type, 25 ng/µL	90466	30 µL
Control template, GC type, 25 ng/µL	90467	30 µL

Table 2: Amplifluor® SNPs HT Genotyping System FAM-SR (S7908)

Cat# S7908: 5000 reactions (10µl volume) Store at -20°C		
<u>Component</u>	<u>Item No.</u>	<u>Volume</u>
20X Amplifluor® FAM SNPs Primer, 5.0 µM stock	90386	2.5 mL
20X Amplifluor® SR SNPs Primer, 5.0 µM stock	90387	2.5 mL
10X Reaction Buffer S	90388	5.0 mL
dNTP Mix (2.5 mM each)	90389	4.0 mL

Table 3: Amplifluor® SNPs HT Genotyping System FAM-JOE (S7909)

Cat# S7909: 5000 reactions (10µl volume) Store at -20°C		
<u>Component</u>	<u>Item No.</u>	<u>Volume</u>
20X Amplifluor® FAM SNPs Primer, 5.0 µM stock	90386	2.5 mL
20X Amplifluor® SR SNPs Primer, 5.0 µM stock	90439	2.5 mL
10X Reaction Buffer S-plus (with reference dye)	90440	5.0 mL
dNTP Mix (2.5 mM each)	90389	4.0 mL

Materials Required But Not Supplied

PCR Reagents

- Antibody-mediated, hot start Taq polymerase
- Molecular Biology Grade water (DNase- and RNase-Free)

Note: *Titanium™ Taq (BD Clontech®), Platinum® Taq (Invitrogen®) and JumpStart™ Taq (Sigma®) have been successfully used with the Amplifluor® SNPs Genotyping Systems. However, other polymerases not listed may or may not work with Amplifluor® SNPs Primers. Contact Technical Service at <http://www.millipore.com/reply/form/techservice> or (800) 221-1975 for more detailed information regarding enzyme selection.*

Sample Purification Reagents and Methods

The following QIAamp™ DNA blood kits from Qiagen, Inc. are recommended for DNA sample preparation: catalog numbers 51104, 51106, 51183, 51185, 51192, 51194, 51161, or 51162. The concentration of the DNA sample to be used in the PCR reactions should be 1-10 ng/μL. For each PCR reaction, it is recommended to use 5 ng total DNA.

Instrumentation

- Validated thermocycler with heated lid feature (see page 11).
- Validated detection instrument, either a fluorescence plate reader or an ABI PRISM® Sequence Detection System (7700 or 7900HT).
 - The Amplifluor® SNPs Genotyping Systems (S7907, S7908) were developed using the PE Wallac Victor 1420 Fluorescence Plate Reader with optically clear microplates and seals. The plate reader was equipped with fluorescein excitation/emission filters (485 + 7 nm/ 535 + 15 nm) and sulforhodamine excitation/emission filters (585 + 10 nm/ 620 + 4 nm). Measurements were taken through the top of the tube or well. Additional information regarding PCR plates and platereaders can be found in Sec.VIII. Appendix.
 - The ABI PRISM® 7700 and 7900HT Sequence Detection Systems (Applied Biosystems®) with optically clear microplates and seals, were used during development of the Amplifluor® SNPs Genotyping Systems (S7907 and S7909). For information regarding instrument setup, refer to the ABI PRISM® 7700 Sequence Detection System Manual, Chapter 4 (Setup and Operation for Allelic Discrimination) or to ABI PRISM® 7900HT Sequence Detection System Manual, Chapter 4 (End-Point Analysis).
 - The choice of detection instrument will determine which Red label is used on one of the Amplifluor® SNPs Primers. Sulforhodamine (SR) label is used with plate readers and JOE label is used with PRISM® instruments.

Storage and Stability

All kit components may be stored at -20°C (short term storage) or at -80°C (long term storage). Since the Amplifluor® SNPs Primers (-FAM, -SR, -JOE) are light sensitive, dark storage is recommended. The Amplifluor® SNPs Primers may be divided into convenient aliquots and wrapped in foil to minimize exposure to light. Avoid frequent freezing and thawing of Amplifluor® reagents.

Beginning Considerations

This section outlines matters to be considered before using the Amplifluor® technology.

Genomic DNA

1. Most SNPs can be genotyped with 1-5 ng of high quality genomic DNA per reaction with Amplifluor®.
2. The following QIAamp™ DNA blood kits from Qiagen, Inc. are recommended for sample preparation: catalog numbers 51104, 51106, 51183, 51185, 51192, 51194, 51161 or 51162.

Reference DNA Samples

1. Purified reference DNA samples with known genotypes can aid in testing primers designed to new SNP targets. It is advisable to utilize a standard panel of DNA.
2. The M24PDR multi-ethnic DNA sample panel is available from the Coriell Institute for Medical Research and is composed of the following DNAs, by NA#: 15029, 15036, 15215, 15223, 15245, 15224, 15236, 15510, 15213, 15221, 15227, 15385, 15590, 15038, 15056, 15072, 15144, 15216, 15226, 15242, 15268, 15324, 15386, 15594.

Primer Design

1. Details on the considerations for Amplifluor primer design are presented on page 13. For generation of a SNP primer SNP primer design software can be used. However, the appropriate Amplifluor tail sequence (see page 18) should be appended at the 5' end of your SNP primer. The complete design of all your primers should be checked against the design guidelines before send out for synthesis.
2. Standard desalted oligonucleotides should be used for Amplifluor® SNP genotyping assays.

Taq Polymerase

1. Antibody-mediated “hot start” Taq Polymerases have been shown to be optimal for Amplifluor® reactions. Titanium® Taq (BD Clontech), Platinum® Taq (Invitrogen) and JumpStart™ Taq (Sigma) have been successfully used with the Amplifluor® SNPs Genotyping Systems.
2. The 10X Reaction Mix (buffer) provided with the Amplifluor® reagents should be used for PCR and not the buffer provided with the Taq Polymerase.
3. Other polymerases, in particular AmpliTaq Gold®, may not work with Amplifluor® primers. Please contact Technical Service at <http://www.millipore.com/reply/form/techservice> or (800) 221-1975 for more detailed information regarding enzyme selection.

Control SNP Primer Mix

1. The primers for the control SNP rs#962, a G/T type SNP in the human NUDEL gene which encodes for an endooligopeptidase enzyme.
2. The 20X Control Primer Mix was assembled as described in the Protocol section of this manual. The primer sequences (5' to 3') are:
Green-tailed: GAAGGTGACCAAGTTCATGCTTCACGTGGCCCCCGACG
Red-tailed: GAAGGTCCGGAGTCAACGGATTCACGTGGCCCCCGACT
Common Reverse: TCCACAGTGGTCTAGGAGACTT

Qualification of Instrumentation

Thermocycler

1. When using 384- or 1536-well plates, adjust the temperature of the heated lid to 85°C. Do not use the high lid temperatures for these plate formats.
2. Verify that the thermocycler is set for the fastest ramp time.
3. Preheat the thermocycler to 96°C prior to adding samples for PCR.

Detection with Plate Readers

1. When using fluorescence plate readers, use the Amplifluor® SNP Primers labeled with FAM and SR and the 10X Reaction Buffer Mix S provided in the Amplifluor® kit.
2. Most fluorescence plate readers can be used to detect Amplifluor® reactions as long as they utilize appropriate and acceptable filter sets or laser wavelengths. (See Table 4)

Table 4: Wavelengths for Fluorescent Labels

<u>Amplifluor® Label</u>	<u>Excitation</u>	<u>Emission</u>
FAM	485 + 7 nm	535 + 15 nm
Sulforhodamine	585 + 10 nm	620 + 4 nm

Detection with ABI PRISM® Instrumentation

1. When using the ABI PRISM® instruments, use the Amplifluor® SNPs Primers labeled with FAM and JOE and the 10X Reaction Mix S-plus (contains passive reference dye) provided in the Amplifluor® kit.
2. Refer to the Genotyping with ABI PRISM® Instrumentation section of Sec. VI for specific instructions on the use of the PRISM® 7700 and 7900HT for allele discrimination.
3. The ABI PRISM® 7000 has also been used successfully with a simple programming modification. Contact Technical Service at <http://www.millipore.com/reply/form/techservice> or (800) 221-1975 for more detailed information.

Data Analysis

1. Scatter plots can be generated from fluorescence measurements made by appropriate instruments in many spreadsheet programs.
2. EMD Millipore provides a Microsoft Excel macro, the AssayAuditor, that aids in generating genotype calls from scatter plots. This can be found on the company website <http://millipore.com/userguides/tech1/mcproto177>.

Primer Design

The Amplifluor® SNPs Genotyping method is based on the principles of competitive allele-specific PCR (12-13). Because of the universal nature of the detection system, it is necessary to design 3 unlabeled primers specific for each SNP studied. Design of these primers can be complicated by the many parameters typically considered in PCR as well as the additional parameters critical to the Amplifluor® system. The considerations for design are included in this

Guidelines for Primer Design

Considerations to take into account for primer design are described below. Optimization of this reaction is simplified by considering the following guidelines in the order presented: the direction of amplification (choice of target strand), the length of primers and the position of the common reverse primer. The location of the common primers is more flexible and can be more easily used to help optimize the PCR reaction. The design and synthesis of several primer sets is suggested since standard oligonucleotide synthesis is relatively inexpensive. For example, synthesizing primer sets for both target strands is recommended when difficult sequences are encountered. This can save time, especially for problematic sequences. Examples of primer design are presented in Table 5.

When designing SNPs assays, it is optimal if no other SNP is in close proximity to the one to be studied. If the primers overlap another SNP, the assay may fail. Generally, multiple SNPs located in close proximity do not present a problem (see Primer Design for Multiple SNPs in One Location below for guidelines). To check whether additional SNPs are located near the SNP of interest, always perform a BLAST SNP search of the db SNP database before beginning assay design.

Direction of Amplification

Relative to the orientation of the target strand, allele-specific amplification is performed in either the 5' or 3' direction from the SNP. Many SNPs are typed in either direction with equal efficiency. With two types of SNPs, A/G (T/C) and G/T (C/A), the 3'-terminal bases of the allele-specific primers differ depending on the target strand and direction of amplification (1).

Note: DNA sequence is normally shown in the 5' to 3' direction. The sequence of the allele-specific primers is identical to the target sequence shown if the primer position is 5' to the SNP. The primer sequence will be complementary to the target sequence if positioned 3' to the SNP.

Target Strand Considerations

1. Estimate the G + C content of the target strand 20-25 bases 5' and 3' to the SNP. If the G + C content is uneven, position the allele-specific primer so that the G + C content is closer to 50%. Keep the G + C content between 30 and 75%, but 50% is preferable. If the G + C content is high in either orientation, choose a target strand so that the allele-specific primer contains more C's than G's.
2. Avoid inverted repeats (palindromes). If there are palindromes of 4 bp or longer that border the SNP, select the opposite orientation for the design of the allele-specific primer. Palindromes positioned at the 3' end of the primer can cause PCR artifacts. A 6 bp or longer palindrome that is not adjacent to the SNP may reduce PCR efficiency.

Example: The sequence surrounding the C/T SNP is 5'...GGC(C/T) ACCTT. A primer for the C-allele would end with a 4 bp palindrome ...GGCC-3. If the allele-specific primer is assigned to the other strand, it would not have palindromes on the 3'-ends (i.e., ...AAGGTA-3' and ...AAGGTG-3').

3. If both target strands seem equally usable, inspect the DNA sequence for the placement of the reverse primer. Avoid amplification of regions that contain G – C rich islands (>20 bp), since that may reduce PCR efficiency. Avoid target strands that contain repeated elements or A – T rich areas. Select the strand that contains the least problematic sequences. If efficient PCR appears to be possible for both target strands design and test primer sets for both strands.

Note: If two or more SNPs are located in close proximity, it may be possible to combine assays for both by using the same common primer. In this case, the assays should be designed in the same amplification direction. If the SNPs are located such that a single primer covers both, see *Primer Design for Multiple SNPs in One Location* below for guidelines.

Length of Allele-specific Primers

Allele-specific primers should be 17-25 nucleotides in length depending on the base composition. Primers that have higher G + C content and/or end with a 3'-G or C are more efficient and can be shorter (Compare #s 3, 6, 7, 9 and 10 with #s 4, 5, 11, 16, 18 and 21 in Table 5.). The average T_m of the primers should be around 65°C. There is no need to introduce additional mismatches into the allele-specific primers (refs 14-19).

Select primer sequences that are 17-25 base long for one of the alleles and determine T_m using primer design software. If T_m is higher than 65°C, remove bases from the 5' end of the primer. Avoid primers shorter than 17 bases. If the T_m is lower than 65°C, add bases to the 5'-end of the primer but avoid primers longer than 25 bases. If these problems are difficult to avoid, select the allele-specific primers using the other strand.

Selection of Common Reverse Primers

Generally, smaller amplicons are amplified more efficiently. However, the primers in Table 5 have distances from 1 to 157 bases between the 3'-end of the reverse primer and the SNP with no observable effects on PCR efficiency. Longer amplicon sizes may require a longer extension time. There are two standard approaches for considering primer composition: selecting G + C-rich 3'-ends for more efficient PCR or selecting A + T-rich 3'-ends for more specific PCR. The latter approach is preferred since the likelihood of mispriming is higher when using primers with G + C-rich 3'-ends (20). To compensate for less stable A/T-rich 3'-ends, the common reverse primers should be made longer with higher T_m than the allele-specific primers (Table 5). Software for PCR primer selection can be used or the primers may be chosen manually. The manual method is explained below.

Considerations for Common Primer Selection

1. Inspect the target strand for the position of the common reverse primer by locating the first of two or three consecutive A-T and/or T-A base pairs. Design the common primer so it is 20-25 bases long with its 3' end at the first A-T (T-A) base pair.

2. Check primer T_m using any oligonucleotide primer design software. It should preferably be in the range 65-75°C. If the G + C content of the sequence is >50%, a primer with a higher T_m is preferable. If it is below 50%, select a primer with a lower T_m .
3. Check the primer for palindromes (palindromes 6 bases and longer should be avoided) and self-annealing sequences that could potentially form dimers at the 3'-end. The 3'-end of the primer should not form duplexes greater than 2 bp. If 3' dimer overlaps or palindromes are found, go to the next step.
4. Check if the primer 3' dimer overlap can be corrected. For example, if the primer ends with a ...TTT-3', and it overlaps with the sequence ...AAA... inside the primer, simply remove one of the T's. A repeat of primer analysis in Step 2 should be done. If the 3' overlap cannot be corrected or a palindrome of 6 or more bases is present, choose another primer.
5. If the DNA sequence of interest contains G + C or A + T repeats, or other unfavorable sequences for PCR, design two or more common primers and select the primer with the best PCR performance. Illustrative examples can be found in Table 5. For example, the sequences near SNP No. 15 and No. 20 have low G + C content. Two primers were tried for each of these SNPs and in each case, only one common reverse primer was successful.

Table 5. Examples of Successful Primers for Allele Discrimination using Amplifluor®

No	Gene symbol/ Genebank access. #	dbSNP rs# or ref.	SNP Type ^a	Allele-specific tailed (forward) primers Sequence ^b	Length/ % _(G+C) ^c	Td (°C) ^d	Sequence	Common primer Sequence	Length/ % _(G+C) ^c	Td (°C) ^d
1	CKK L29399	(21)	A/G	(tail 1)-GGCTGCTCTTAAATAGCCCG (tail 2)-GGCTGCTCTTAAATAGCCCA	22/54 22/50	70 68	GTTACTGCAGTCTGGGTCA	GTTACTGCAGTCTGGGTCA	20/55	64
2	DRD1 X55758	4532	A/G	(tail 1)-CDT-GACCCATTCCTGCTTA (tail 2)-T-GACCCATTCCTGCTTG	21/52 20/55	66 67	ACAGGCAGTGAGGATACGAACA	ACAGGCAGTGAGGATACGAACA	22/50	67
3	DRPP32 AC025531	734645	A/G	(tail 1)-GCCACCTTTGGCTGATG (tail 2)-GCCACCTTTGGCTGATA	18/61 18/56	66 63	TCCCTGTTCCCGGTTTTCTTGAT	TCCCTGTTCCCGGTTTTCTTGAT	23/48	73
4	HTR2A S78723	6306	A/G	(tail 1)-GGCTAGAAAACAGTATGCTCCG (tail 2)-GGTAGAAAACAGTATGCTCCA	23/48 23/44	65 63	TCTGGACACAAAACACTGTTGGCTT	TCTGGACACAAAACACTGTTGGCTT	24/46	71
5	DRD3 AC023645	6280	A/G	(tail 1)-D-GGCATCTCTGAGTCACTGAGTA (tail 2)-D-GGCATCTCTGAGTCACTGAGTG	24/46 24/50	66 69	GCAGTAGGAGGAGGGCATAAGTA	GCAGTAGGAGGAGGGCATAAGTA	21/52	61
6	CHRNA5 AC027228	(22)	A/G	(tail 1)-CATTGGAAAGCTGGCTCA (tail 2)-CATTGGAAAGCTGGCTCG	18/56 18/61	66 64	TCACAGACCTCACGGACATCATT	TCACAGACCTCACGGACATCATT	23/48	69
7	CHRNA7 AC056803	885073	A/G	(tail 1)-AGCCTTGGTAGTCCCA (tail 2)-AGCCTTGGTAGTCCCG	17/59 17/65	61 64	AGACAAGACACCAAAACCACCTT	AGACAAGACACCAAAACCACCTT	22/46	65
8	CYP17 M19489	743572	T/C	(tail 1)-D-GCCACAGCTCTTCTACTCCACT (tail 2)-D-GCCACAGCTCTTCTACTCCACC	23/52 24/54	68 73	GGCAGGGCCACCTTCTCTT	GGCAGGGCCACCTTCTCTT	21/62	72
9	ABCD1 LM000033	11803	T/C	(tail 1)-D-GCCAAAGAGGGGTCA (tail 2)-D-GCCAAAGAGGGGTCA	17/53 17/59	60 61	GAAAAGGCACACGGTGGCGGT	GAAAAGGCACACGGTGGCGGT	21/62	76
10	DRD2 X51645	6275	T/C	(tail 1)-CCGACCCGTCGCCACCAC (tail 2)-CCGACCCGTCGCCACCAT	17/76 17/71	69 68	CTTGGGGTGGTCTTTGGCAT	CTTGGGGTGGTCTTTGGCAT	20/55	69
11	HTR2A S71229	6313	T/C	(tail 1)-GCT-CTACAGTAATGACTTAACTCC (tail 2)-D-CTACAGTAATGACTTAACTCT	25/40 23/30	62 53	CACAGAAAAGTTGGTTCCGATT	CACAGAAAAGTTGGTTCCGATT	22/46	68
12	HTR2A S78723	6311 ^e	T/C	(tail 1)-D-CRAGTGTGTGAGTGTCT (tail 2)-D-CRAGTGTGTGAGTGTCC	21/57 20/60	65 66	TCTGGACACAAAACACTGTTGGCTT	TCTGGACACAAAACACTGTTGGCTT	24/46	71
13	HTR1B M81590	6296	C/G	(tail 1)-D-CCGGATCTCCTGTGTGTG (tail 2)-D-CCGGATCTCCTGTGTGTG	21/52 21/52	65 64	GCGGCCATGAGTTTTCTTCTTT	GCGGCCATGAGTTTTCTTCTTT	21/48	68
14	HTR2C X80763	6318	C/G	(tail 1)-D-GGGTCAACAGAAATATCAG (tail 2)-D-GGGTCAACAGAAATATCAC	21/48 20/45	62 60	TGCACCTAATGGCCCTATTGGTTT	TGCACCTAATGGCCCTATTGGTTT	24/42	72
15	F5 L32757	6019	C/G	(tail 1)-D-GGATGCTCAAGGGCTTATG (tail 2)-D-GGATGCTCAAGGGCTTATC	20/50 20/50	66 65	ACAGGACTTCTTGGGCCCTACTTT	ACAGGACTTCTTGGGCCCTACTTT	23/48	68
16	CHRNA5 M83712	1042500	C/G	(tail 1)-GCTJ-CGATTTCTATTCGCTACATTACAAC (tail 2)-D-CGATTTCTATTCGCTACATTACAAG	27/41 25/36	70 66	TCAACAACCTCACGGACATCATT	TCAACAACCTCACGGACATCATT	24/42	70
17	MAOA M68840	6323	G/T	(tail 1)-D-GACAGTCCCATTTGGAAGG (tail 2)-D-GACAGTCCCATTTGGAAGT	20/55 20/50	68 65	GCGATCCCTCCGACCCTTGACT	GCGATCCCTCCGACCCTTGACT	20/62	72
18	GRIN2B G06785	890	G/T	(tail 1)-AGGTAGCTTTTCCCAAAC (tail 2)-AGGTAGCTTTTCCCAAACG	19/42 19/47	58 62	AGAGCCTCTTTTCCCTTTTCCACAA	AGAGCCTCTTTTCCCTTTTCCACAA	21/43	63
19	DRD2 M29066	6278	G/T	(tail 1)-D-CCTTGGCCCTAGCCACC (tail 2)-D-CCTTGGCCCTAGCCACCCT	21/71 20/65	77 73	GCCAGCATGTGGCTGTGAGAA	GCCAGCATGTGGCTGTGAGAA	21/57	71
20	CYP1A2 M31664	762551	G/T	(tail 1)-D-CCATCTACCATCGCTCTGT (tail 2)-D-CCATCTACCATCGCTCTGG	21/52 21/57	67 70	AGGCTCCTTTCCAGCTCTCAGAT	AGGCTCCTTTCCAGCTCTCAGAT	23/52	70
21	CHRN2 AF077186	(23)	A/C	(tail 1)-D-ATAACAGGCACAGTTGGCCAC (tail 2)-D-ATAACAGGCACAGTTGGCCAA	21/48 21/43	64 64	CCAGTCAAAAGGCTTGCACAAA	CCAGTCAAAAGGCTTGCACAAA	22/50	71

22	CYP17 M63871	6163	A/C	(tail 1- <u>I</u>)-CTTGGTGCCGATACGAACA (tail 2- <u>I</u>)-CTTGGTGCCGATACGAACC	20/50 20/55	67	CAAGTACCCCAAGAGCCTCCTGT	23/56	71
23	no gene AC002525	363	A/T	(tail 1- <u>I</u>)-GGGTTTTGAGGCTTCCTTGTT (tail 2- <u>I</u>)-GGGTTTTGAGGCTTCCTTGTA	22/46 22/46	70	ACCCGTTGTCGGCTGTGGATTT	21/54	75
24	HTRSA X81411	6320	A/T	(tail 1)-CCCAGAGATGGATTTACCA (tail 2)-CCCAGAGATGGATTTACCT	19/47 19/47	60	TCGTCTTTGCCCGAGGGCTGTGGTT	23/56	77
25	CHRN82 U62437	1126885	A/T	(tail 1- <u>I</u>)-CTCCATGCTCTTTACCCCT (tail 2- <u>I</u>)-CTCCATGCTCTTTACCCCA	20/50 20/50	64	TCCATCCTCCACCCCAACACTA	21/52	67
26	Unknown G49226	4422 ^d	GC/CG	(tail 1- <u>I</u>)-GCAGACACTGGGTTTCGC (tail 2- <u>II</u>)-GCAGACACTGGGTTTCCCG	19/58 20/55	67	AGGTCACTTCCCTAAAGGGGCAT	21/48	65

Comments to the Table:

- SNP types are given with respect to the 3'-terminal bases in the allele-specific primers.
- Bases in the priming domain that are shared with the Amplifluor primer sequence bases are underlined. Note, that in this case the underlined base is part of the "tail" sequence, not an extra base in the primer domain. However, these bases were included into the % (G+C) and Td calculations.
- All numbers are rounded to integers.
- Calculated using the nearest neighbor method in Oligo 5.0 software.
- The "tailed" oligos for this SNP overlap with the SNP rs#6306 (#4, this Table). That position was made 50% A+50% G in these oligos, as indicated by "F" symbol. The same reverse primer had been used in both assays.
- rs#4422 is an insertion (-/G) type SNP.

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Assigning Tails to the Allele-Specific Primers

The choice of a Green or Red Amplifluor® Primer tail sequence for a given allele is arbitrary when considered on the basis of PCR efficiencies since they are essentially equal. However, there may be cases in which there are advantages to putting one tail or another on a particular allele-specific primer. These are discussed below and in the next section. The allele-specific primer needs to be synthesized with either the green tail sequence or the red tail sequence on the 5' end. A high quality primer synthesis should only require desalting after deprotection.

Green Amplifluor® Tail

5'-GAA GGT GAC CAA GTT CAT GCT – allele-specific primer X –3'

Red Amplifluor® Tail

5'-GAA GGT CGG AGT CAA CGG ATT – allele-specific primer Y –3'

When adding the tails, it is sometimes possible to “share” bases between the 5'-ends of the allele-specific primers and the 3'-ends of the Amplifluor® SNPs Primers. Base sharing reduces the length of the tailed primers and may help reduce differences in the PCR efficiencies. An example is shown below (Table 5, #2):

Green Amplifluor® Primer (tail sequence)

5'...GAAGGTGACCAAGTTCATGCT

flanking genomic sequence Allele-specific Primer Sequence

5'.....agg act tcc tgg aaa tct gac TGACCCCTATTCCCTCTTA-3'

Tailed allele-specific primer with 2 shared bases (underlined)

5'...GAAGGTGACCAAGTTCATGCIGACCCCTATTCCCTCTTA-3'

Red Amplifluor® Primer (tail sequence)

5'...GAAGGTCGGAGTCAACGGATT

flanking genomic sequence Allele-specific Primer Sequence

5'.....agg act tcc tgg aaa tct gacTGACCCCTATTCCCTGCTTG-3'

Tailed primer with 1 shared base (underlined)

5'GAAGGTCGGAGTCAACGGATIGACCCCTATTCCCTGCTTG-3'

In this example, as in example No. 12 (Table 5), base sharing with the Green Primer tail allowed for partial compensation of the unequal primer efficiencies between the 3'-A (or T)- and 3'-G (or C)-terminal allele-specific primers by adding an extra base pair. However, such compensation is not always possible (see Sec. IV. *Primer Design, Checking the Primer Design*).

If the Red tail can share more bases than the Green tail (or vice-versa) with the genomic/primer sequence, assign the Red tail (with the most shared bases) to the primer with the 3' end of A or T. This is only applicable if the SNP is a A/G, A/C, T/C or T/G SNP. If the SNP is a C/G or A/T SNP, the Red tail can be assigned to either allele-specific primer sequence.

To help verify that the primers adhere to the design guidelines presented above, analyze the designed primer sequences with any general primer design software and measure the amount of self-annealing and palindromes. If there is sufficient 3'-end dimers possible, switch the Red and Green tails between primers and reanalyze for self-annealing. If the junction between the tail and the primer creates palindromes of 6 base pairs or longer, insert a single T between the tail and primer sequences.

Primer Design for Multiple SNPs in One Location

If two SNPs are located in close proximity and it is desirable to screen both, select the direction of amplification that is most appropriate for both. If the allele-specific primer for one of the SNPs has to overlap the other SNP, randomize this position when designing the allele-specific primers. The same common primer can be used in both assays. Examples are given in Table 5 (No. 4 and 12).

Checking Primer Design

A Review of Criteria and Design Strategies

The primers should be designed and evaluated by any general primer design software in the order discussed previously. The allele-specific sequence that will be assigned to the tailed sequences should be selected first. The common reverse primer should then be chosen. After the tails have been assigned to the allele-specific primers, it is important to check them by the criteria discussed. Again, any general primer design software should be used in the analysis. However, empirical testing after primer synthesis will be necessary.

Design Considerations

1. Avoid 3' terminal dimers (complementary base pair overlaps), greater than 2 base pairs, that create an internally recessed 3' end. This includes a primer with itself (homodimer) or with another primer (heterodimer).
2. Switching tails between primers can sometimes eliminate primer-dimer problems. Sharing nucleotides is not as important as avoiding primer dimers. For example, with SNP No.8 and No.11 in Table 5, the compensation for the unequal primer efficiencies due to the 3' end of the allele-specific primers was possible as described. However, it was not done due to anticipated 3' terminal dimer problems. The 3' C primer was assigned the tail that created the greatest amount of sharing instead. If "sharing" at the junction of the tail and allele-specific primer(s)

causes a problem, avoid sharing and add a spacer of one or more T's between the tail and the allele-specific primer. If the reverse primer is likely to cause the problem, select another reverse primer.

3. If one or both tailed primers are likely to cause primer-dimers even with the previously suggested strategies, try extending the 3' end of the primer by one more base across the SNP. If the extra base is an A or a T, this may increase selectivity. Polymerase priming is greatly reduced when the extended primer's penultimate 3' nucleotide is mismatched (eg., X primer on Y allele), but the primer specificity may be reduced.
4. Avoid or reduce the amount of repeated sequences and strings of 5 or more tandem G's if possible. Sometimes shortening the primer from the 5' end can eliminate these problems. Changing the target strand may also help.
5. Keep the tailed allele-specific primers 38 to 45 nucleotides in length. The common reverse primers may be made longer to compensate for allele-specific primers that have less than optimal efficiencies.
6. Keep the T_m of the target specific sequences (both tailed primers and common primer) at approximately 65°C.
7. Keep the G + C content of the primers around 50%.

Protocols

The Protocols section describes experimental design and procedures for using the Amplifluor® SNP technology. The first section discusses the Amplifluor® SNPs Genotyping System for Assay Development for new users. It includes a detailed protocol which introduces the user to the Amplifluor® SNPs system from reaction set-up to amplification using known DNA samples provided by Chemicon® and several DNA samples of the researchers choosing. It is recommended that these controls be run first to qualify your instrumentation and PCR procedures. Researchers typing SNPs in a large number of samples are referred to the final section, *Amplifluor® SNPs Genotyping System for High Throughput Screening*.

Amplifluor® SNPs Genotyping Systems for Assay Development

Experimental Design and Parameters for Running Control Templates

Experimental Design

The experiment will consist of 9 reactions: 3 No Target Controls, 3 DNA samples of unknown genotype and 3 control DNA samples (Table 6).

Table 6: Experimental Design for Assay Development Kit

<u>Tube Number</u>	<u>Template Type</u>	<u>Template Conc. (ng/rxn)</u>
A1	0	0
A2	0	0
A3	0	0
B1	GG Control	2.5
B2	TTControl	2.5
B3	GT Control	2.5
C1	Unknown #1	2.5
C2	Unknown #2	2.5
C3	Unknown #3	2.5

Assay Steps

1. Remove all reagents needed for amplification except Taq Polymerase from -20°C storage. Place control template tubes and DNAs of unknown genotype in a separate rack and thaw remaining reagents in a PCR-designated hood in a template-free PCR room (recommended).
2. Program thermocycler with conditions described in Table 7. Start the program and pause the thermocycler at 96°C to preheat the block for a true “hot start”.

Table 7: PCR Program

<u>Step</u>	<u>#Cycles</u>	<u>Time</u>	<u>Temp.</u>
Preheat Block			96°C
Denaturation	1	4 min	96°C
Amplification - Step 1	20	10-15 sec* 5 sec 10 sec	96°C* 55-60°C* 72°C
Amplification - Step 2	22-24	10-15 sec* 20 sec 40 sec	96°C 55°C 72°C
Extension	1	3 min	72°C
Hold	1	Hold	20°C

Note: * indicates that this parameter can be adjusted for individual assay optimization. For example, SNPs located in areas with higher G + C content may require longer denaturation times at 96°C.

3. In sterile 1.5 mL microcentrifuge tubes, prepare 1:10 dilution of the control templates using dH₂O as the diluent (Table 8). The initial concentration of each control template provided is 25 ng/μL. Dilute DNAs of unknown genotype in a similar manner. Template dilutions can be kept at room temperature but must be used within the day.

Table 8: Dilutions of the Control Templates

<u>Tube #</u>	<u>FinalTemplate Concentration</u>	<u>Template Solution</u>	<u>dH₂O</u>
1	25 ng/μL	30 μL Control Template	0 μL
2	2.5 ng/μL	5 μL Tube 1	45 μL

- Transfer 2 μL of dH₂O to A1 – A3 (No Target Controls).
- Transfer 2 μL of each template dilution according to the well designations in Table 6.
Note: Reactions should be done using either an optically clear plate and plate seal or optically clear strip cap tubes (see Sec.II. Kit Components, Material Required but Not Supplied).
- Once thawed, mix all reagents thoroughly and spin down briefly before preparing the amplification cocktail mixture (Table 9). The cocktail is assembled using the 20X Control Primer Mix for twelve 10 μL reactions (9 reactions plus 3 to ensure sufficient cocktail for all reactions) at room temperature in a sterile 1.5 mL microcentrifuge tube. Add all reagents in the order presented in the Table 9. Mix the reagent mix with a pipet prior to dispensing into the samples.

Table 9: Amplification Cocktail Preparation

<u>Reagent</u>	<u>1 Rxn</u>	<u>15 Rxn</u>
dH ₂ O	4.6 μL	69 μL
20X Amplifluor SNP™ FAM Primer	0.5 μL	7.5 μL
20X Amplifluor SNP™ SR Primer*	0.5 μL	7.5 μL
20X Control Primer Mix	0.5 μL	7.5 μL
10X Reaction Mix S	1.0 μL	15 μL
dNTPs (2.5 mM each)	0.8 μL	12 μL
Taq (5 U/μL)	0.1 μL	1.5 μL
Total	8.0 μL	120 μL

***Note:** If using the PRISM® 7700 or 7900HT, the 20X Amplifluor® SNP JOE Primer must be substituted for the 20X Amplifluor® SNP SR Primer, and the 10X Reaction Mix S-plus must be used instead of the 10X Reaction Mix S

- Add 8 μL of the cocktail mixture to the PCR tubes, beginning with the No Target Controls.
- Seal the reaction vessels with an optically clear seal.
- Collect the reaction mix to the bottom of the tubes by quickly spinning the tubes in a bench top centrifuge.
- Place the plate into the preheated thermocycler (or PRISM® 7700 or 7900HT) and resume the PCR program.
- At the completion of the amplification reaction, place the reactions into a tube holder rack with base plate having the No Target Controls on the left. If using a plate reader, select the option to save the fluorescence of FAM and SR in columns. This is not necessary if using the PRISM® 7700 or 7900HT.
- Plot and analyze the data as described in Sec.VI. *Data Analysis*.

Testing the System with Primers for the Specific SNP of Interest

Experimental Design

The experimental design will be determined by the number of samples to be typed. Only primers specific for the XY type SNP of interest will be used. Include at least three No Target controls and three reference DNA samples (XX, XY, YY) in your experimental design. If needed, the Kit Control Templates can serve as positive DNA controls.

Preparation of the 20X Primer Mix

Prepare a 20X SNP-specific primer mix from the stock solutions of the primers as described below.

The 20X SNP-specific primer mix contains 0.5 μM solution of the green tailed allele-specific primer, 0.5 μM solution of the red tailed allele-specific primer and 7.5 μM solution of the common reverse primer in water. It is recommended to make 1 mL of each 20X SNP-specific primer mix. Depending on the concentration of each primer stock, dilute appropriately to achieve the proper concentration of primers. Refer to Sec. VIII *Appendix* for determination of initial primer concentrations and examples in preparation of alternative 20X SNP specific primer mixes used in Sec. VII *Troubleshooting*.

The recommended storage conditions for 20X SNP-specific primer mix are -20°C for over 1 week or $+4^{\circ}\text{C}$ for immediate use.

Assay Steps

1. Remove all reagents needed for amplification except Taq Polymerase from -20°C storage. Place control template tubes and DNAs of unknown genotype in a separate rack and thaw remaining reagents in a PCR-designated hood in a template-free PCR room (recommended).
2. Program thermocycler with conditions described in Table 7. Start the program and pause the thermocycler at 96°C to preheat the block for “hot start”.
3. The concentrations of the references and unknown sample DNAs should be 1-10 $\text{ng}/\mu\text{L}$ *. If dilution of the DNA is necessary, dilute in sterile microcentrifuge tubes using sterile dH_2O as the diluent. Dilutions can be kept at room temperature but must be used within the day.

**Note: High quality DNA samples can be reliably typed for most SNPs using only 1 ng of DNA per reactions. However, samples of lesser quality may require additional PCR cycles for a reliable reading. The kit is capable of correctly typing samples utilizing only 400 pg of DNA per reaction in less than 3 hours.*

4. Transfer 2 μL of dH_2O or DNA samples to the reaction vessel according to your Experimental Design.
5. Prepare the amplification cocktail at room temperature using Table 10. Add the reagents in the order presented. Mix and spin down.

Table 10: Amplification Cocktail Preparation

<u>Reagent</u>	<u>1 Rxn</u>	<u>1.1 x n^a Rxns</u>
dH ₂ O	4.6 µL	(4.6 x 1.1n) µL
dNTPs (2.5 mM each)	0.8 µL	(0.8 x 1.1n) µL
20X Amplifluor® SNP FAM Primer	0.5 µL	(0.5 x 1.1n) µL
20X Amplifluor® SNP SR Primer*	0.5 µL	(0.5 x 1.1n) µL
20X SNP Specific Primer Mix	0.5 µL	(0.5 x 1.1n) µL
10X Reaction Mix S*	1.0 µL	(1.0 x 1.1n) µL
Taq (5 U/µL)	0.1 µL	(0.1 x 1.1n) µL
Total	8.0 µL	(8.0 x 1.1n) µL

^an is the number of reaction from Experimental Design. In order to prepare 10% extra cocktail, “n” is multiplied by 1.1.

***Note:** If using the PRISM® 7700 or 7900HT, the 20X Amplifluor® SNPs JOE Primer must be substituted for the 20X Amplifluor® SNPs SR Primer and the 10X Reaction Mix S-plus must be used instead of the 10X Reaction Mix S.

6. Preheat the thermocycler to 96°C.
7. Transfer 8 µL Amplification Cocktail into each well beginning with the No Target Controls.
8. Seal the reaction vessels with an optically clear cover.
9. Collect the reaction mix at the bottom of the tubes by quickly spinning the tubes in a bench top centrifuge.
10. Place the plate into the preheated thermocycler and resume the PCR program.
11. After PCR is completed, measure the fluorescence of the reactions and proceed to Sec. VI. *Data Analysis*.

Amplifluor® SNPs Genotyping System for High Throughput Screening

Experimental Design and Parameters for 384-Well Format

Experimental Design

The experimental design will be determined by the number of samples to be typed. Only primers specific for the SNP of interest will be used. Construct a table identifying the well and sample designations. Include three no target controls and three reference DNA samples (XX, XY, YY) in your experimental design.

Assay Steps

Follow the general PCR conditions and procedures discussed in Sec. V. Protocol, Assay Development. Below is an abbreviated list of the assay steps required for this type of assay.

1. Prepare 20X Primer Mix.

2. Set thermocycling parameters. When using 384- or 1536-well plates, adjust the heated lid temperature to 85°C. Do not use high lid temperatures for these plate formats.
3. Dilute DNA samples between 1-10 ng/μL in dH₂O.
4. Transfer 2 μL dH₂O or DNA samples to the 384-well reaction vessel.
5. Prepare the amplification cocktail at room temperature using Table 11. Add the reagents in the order presented. Mix and spin down.

Table 11: Amplification Cocktail Preparation

<u>Reagent</u>	<u>1 Rxn</u>	<u>1.1 x n^a Rxns</u>
dH ₂ O	1.3 μL	(1.3 x 1.1n) μL
dNTPs (2.5 mM each)	0.4 μL	(0.4 x 1.1n) μL
20X Amplifluor® SNP FAM Primer	0.25 μL	(0.25 x 1.1n) μL
20X Amplifluor® SNP SR Primer*	0.25 μL	(0.25 x 1.1n) μL
20X Specific Primer Mix	0.25 μL	(0.25x 1.1n) μL
10X Reaction Mix S*	0.5 μL	(0.5 x 1.1n) μL
Taq (5 U/μL)	0.05 μL	(0.05 x 1.1n) μL
Total	3.0 μL	(3.0 x 1.1n) μL

^an is the number of reaction from Experimental Design. In order to prepare 10% extra cocktail “n” is multiplied by 1.1.

***Note:** If using the PRISM® 7700 or 7900HT, the 20X Amplifluor® SNPs JOE Primer must be substituted for the 20X Amplifluor® SNPs SR Primer and the 10X Reaction Mix S-plus must be used instead of the 10X Reaction Mix S. For certain hot start Taq polymerases, the volume of enzyme used in the cocktail preparation can be reduced from 0.05 μl to 0.025 μl.

6. Preheat the thermocycler to 96°C.
7. Aliquot 3 μl Amplification Cocktail into the wells beginning with No Target Controls.
8. Place the plate into the preheated thermocycler and resume the PCR program.
9. Detect the fluorescence and perform data analysis (Figure 5).

Data Analysis

Genotyping with Thermocycler/Plate Reader

Overview

Performing PCR using the control reagents in Amplifluor® SNPs Genotyping System for Assay Development is recommended in order to become familiar with the technology. To begin analyzing the data, it is advisable to obtain a graphical representation of the data that should resemble the scatter plot in Figure 3. The sensitivity of the assay should be calculated (as demonstrated in Assay Sensitivity) in order to assess the signal that the instrumental set-up will be able to measure. In order to type unknown SNPs, data should be processed using the AssayAuditor spreadsheet. Scatter plots and allele calls can also be generated by appropriate instruments using various programs.

Assay Sensitivity

In order for a measurement to be considered significant, the signal should be at least 5 standard deviations above background for both the FAM and SR emissions. By performing the following calculations using the signals obtained in the Control SNP Assay (Table 12 and Figure 3), the number of standard deviations above background can be obtained.

1. Calculate the average fluorescent signal RFUAvg and standard deviation (Std) of the No Target Controls (NTC), as shown below:

$$\text{RFU}_{\text{Avg}} = \text{NTC}_1 + \text{NTC}_2 + \text{NTC}_3 / 3$$
$$\text{Std} = \sqrt{\{\text{NTC}_1 - \text{RFU}_{\text{Avg}}\}^2 + \{\text{NTC}_2 - \text{RFU}_{\text{Avg}}\}^2 + \{\text{NTC}_3 - \text{RFU}_{\text{Avg}}\}^2 / (3 - 1)}$$

For the example below,

The FAM RFUs for NTC 1-3 are 10952, 9916 and 9992, respectively, with an $\text{RFU}_{\text{Avg}} = 10287$ and an $\text{Std} = 577$.

The sulforhodamine RFUs for NTC 1-3 are 18801, 17589 and 18273, respectively, with an $\text{RFU}_{\text{Avg}} = 18221$ and a $\text{Std} = 608$.

Table 12: Data in Excel Worksheet

<u>Template Type</u>	<u>Description</u>	<u>FAM</u>	<u>SR</u>
0	NTC	10952	18801
0	NTC	9916	17589
0	NTC	9992	18273
GG	Control	55463	20118
TT	Control	10822	52673
GT	Control	29048	33139
Unknown #1	NA15213	53282	19341
Unknown #2	NA15215	11388	55381
Unknown #3	NA15029	30296	35232

2. Calculate the difference between the signals of the individual Controls and RFU_{Avg} of the No Target Controls and divide by the corresponding Std value. Using the data in Table 12 as an example:

The FAM signals for GG and GT type templates are 55463 and 29048, respectively. Then the sensitivity, S, would be

$$S = (55463-10287)/577 = 78.3 \text{ for GG}$$

$$S = (29048-10287)/577 = 32.5 \text{ for GT}$$

The sulforhodamine signals for TT and GT types templates are 52673 and 33139, respectively.

$$S = (52673-18221)/608 = 56.7 \text{ for TT}$$

$$S = (33139-18221)/608 = 24.5 \text{ for GT}$$

3. In this example, the lowest observed signal, SR in the GT template is significant since it exceeds the background by almost 7 standard deviations.

Note: If the sensitivity is lower than expected, put the plate into the thermocycler and perform 3-5 more cycles of PCR using the same program. Omit pre-heating at 96°C.

Using the AssayAuditor Spreadsheet for the Assignment of Genotype

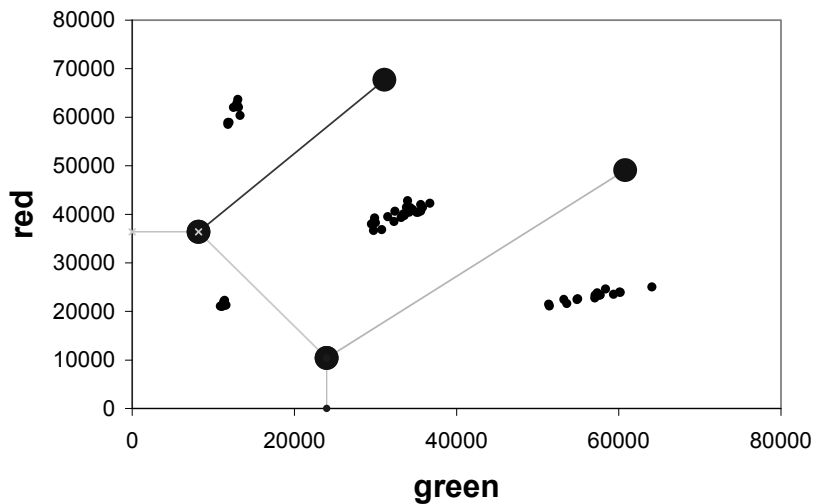
After the fluorescent measurements are imported, the AssayAuditor Spreadsheet will assign a genotype for each sample. These measurements will be pasted into the appropriate columns in the spreadsheet. A scatter plot will be displayed and boundaries can be adjusted to define the 3 clusters of signal generated by the homozygous and heterozygous samples. When boundaries are finalized, the assigned genotypes are displayed.

To use the AssayAuditor Spreadsheet:

1. Transfer the plate reader measurements into an Excel Spreadsheet.
2. Go to <http://millipore.com/userguides/tech1/mcproto177> and download the Excel Macro.
3. Follow the instructions on the spreadsheet.

Figure 3: Output from AssayAuditor Spreadsheet.

A



B

<u>Sample</u>	<u>Red (1)</u>	<u>Green (1)</u>	<u>Genotype</u>	<u>Red (2)</u>	<u>Green (2)</u>
NTC	24278	10652	Background	21288	11570
NTC	21092	10872	Background	21560	11320
NTC	21040	11088	Background	21986	11284
NTC	21238	11452	Background	22264	11414
NA15029	38123	29658	Heterozygote	36660	29768
NA15036	38013	29502	Heterozygote	39221	29908
NA15215	58511	11804	Red Allele Homozygote	58935	11806
NA15223	39531	31522	Heterozygote	40988	34583
NA15245	40460	35292	Heterozygote	40682	35580
NA15224	39741	33557	Heterozygote	40454	34171
NA15236	38519	32281	Heterozygote	42789	33975
NA15510	40614	32399	Heterozygote	39353	33175
NA15213	21096	51451	Green Allele Homozygote	21492	51361
NA15221	21638	53594	Green Allele Homozygote	22466	53234
NA15227	62003	12506	Red Allele Homozygote	62827	12928
NA15385	40086	33457	Heterozygote	41980	35588
NA15590	22792	57056	Green Allele Homozygote	23486	59363
NA15038	23308	57074	Green Allele Homozygote	23306	57530
NA15056	22458	54865	Green Allele Homozygote	23820	57358

NA15072	41460	33829	Heterozygote	40442	33887
NA15144	38267	30018	Heterozygote	36846	30792
NA15216	58949	11948	Red Allele Homozygote	60340	13294
NA15226	22596	54949	Green Allele Homozygote	23904	60182
NA15242	23382	57706	Green Allele Homozygote	25021	64098
NA15268	63684	13028	Red Allele Homozygote	62051	13108
NA15324	24609	58377	Green Allele Homozygote	23972	60104
NA15386	40382	35094	Heterozygote	42272	36712
NA15594	41374	34301	Heterozygote	41408	35818

Twenty-four DNAs from the Coriell M24PDR panel were assayed in duplicate and evaluated using the Control Primer Mix. Panel A displays the scatter plot generated using the “AssayAuditor For End-Point” (found on “Quadrants” spreadsheet) with the separation lines positioned correctly. Panel B shows the input fluorescent data (found on “End-Point PCR Data” spreadsheet) and the resulting sample genotype (found on “OutPut” spreadsheet) for the Coriell M24PDR panel performed in duplicate.

Note: *In Figure 3 Panel A, the scatter plot is generated using an Excel spreadsheet with the above fluorescent data.*

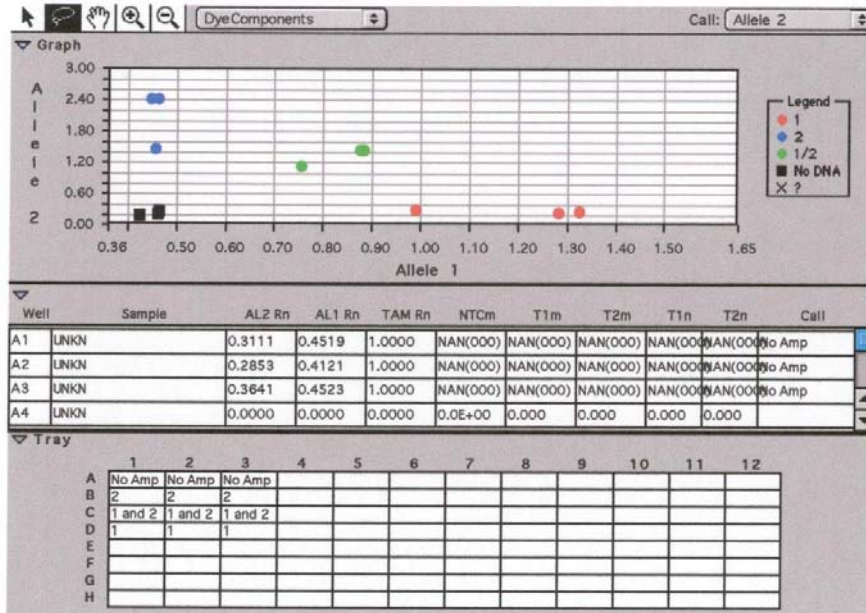
Genotyping with ABI Instrumentation

Overview

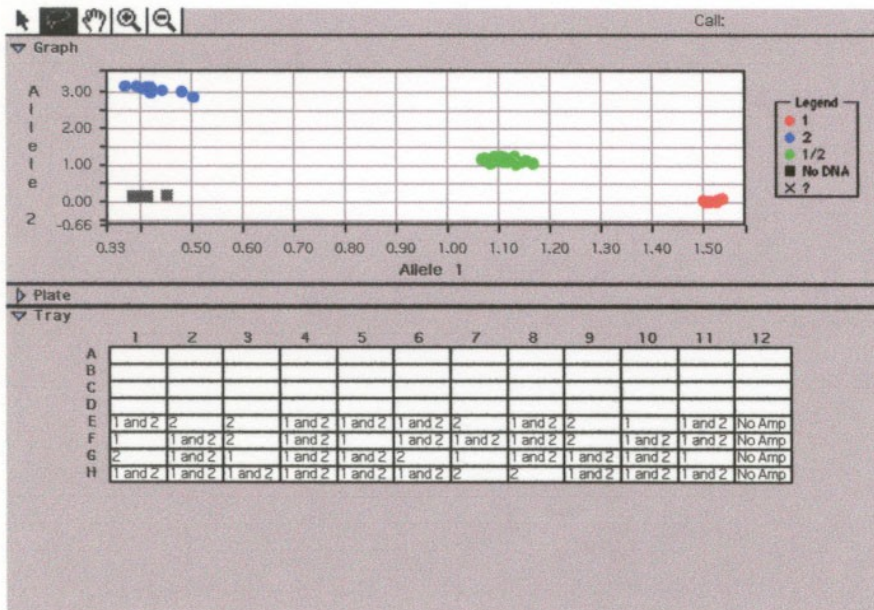
Performing PCR using the control reagents in Amplifluor® SNPs Genotyping System for Assay Development is recommended in order to become familiar with the technology. It is expected that the scatter plot will resemble Figure 4 when analyzing the kit control templates. Data obtained from the kit controls, researcher’s reference samples and unknown SNP samples should be examined in a similar fashion.

Figure 4. Analysis of experimental DNAs using the ABI PRISM® 7700.

A



B



*Panel A: Analysis of G/C type SNP on the human HTR2C gene (rs#6318). Signals from the homozygous CC and GG genotypes are distributed along the X and Y axis respectively, while signals from the heterozygous GC genotypes are located approximately on the bisector. Panel B: Analysis of a G/C type SNP on the human CYP1B1*2 gene (rs#1056836). Signals from the homozygous CC and GG genotypes are distributed along the X and Y axis respectively, while signals from the heterozygous GC genotypes are located approximately on the bisector.*

Allelic Discrimination Using the ABI PRISM® 7700.

Refer to the ABI PRISM® 7700 Sequence Detection System Manual Chapter 4 (Setup and Operation for Allelic Discrimination) for analysis of data. An abbreviated protocol for data analysis follows:

1. Reading the Results Using the ABI PRISM® 7700.
If you have used a PCR machine other than ABI PRISM® 7700 Sequence Detection System, place the PCR plate into the Prism® 7700 and close the lid.
 2. Instrument Setup.
Open the Sequence Detector, version 1.6.3 (or later).
Under **File**, open **New Plate**. Choose **Allelic Discrimination** in **Plate type** and **7700** in **Instrument type**. Click on OK.
In **Sample Type** thumbwheel, select **UNKN** for all your samples.
In **Sample Type** thumbwheel, click on **Sample Type Set Up** and deactivate TAMRA Quencher box. Select the FAM/JOE dye combination. (FAM/TET combination is usually set by default.)
 3. Reading the plate.
Click on **Show Analysis** button, then on the green **Post-PCR Read** button. **Progress** pop-up menu opens up then closes automatically.
Under **Analysis**, select **Analyze** (note, that after this step the sample symbols will turn into open circles) and close the **Event Log** pop-up menu that opens up.
Under **Analysis**, select **Allelic Discrimination**.
In **Graphical view selector** thumbwheel, select **Dye Components**.
Select **Plus Zoom** tool in **Graphical** tools menu and zoom in the area where you see the dots on the graph by clicking approximately in the center of the dot cluster or by framing the cluster using the mouse click and drag. The dots should be arranged in 4 clusters (if all possible allelic types are present in your samples); see Figure 7 (B).
Using **Group Selector** tool from the **Graphical** Tools menu, group the clusters and call the alleles, using the Call Selector.
Save your data.
- Note:** The ABI PRISM® 7700 assigns JOE as Allele 1 and FAM as Allele 2. Your NTC cluster should be in the lower left of the graph, the Allele 1 cluster in the lower right, the Allele 2 cluster in the upper left, and the Allele 1,2 (heterozygote) cluster in between the two homozygote clusters.
4. Data printout.
The allelic types will show in **Tray** view under the graph and you can print out the whole **Allelic Discrimination** viewer after an appropriate **Page Setup** is done. Also, you can choose **Experimental Report** in **View** menu and print it out separately.

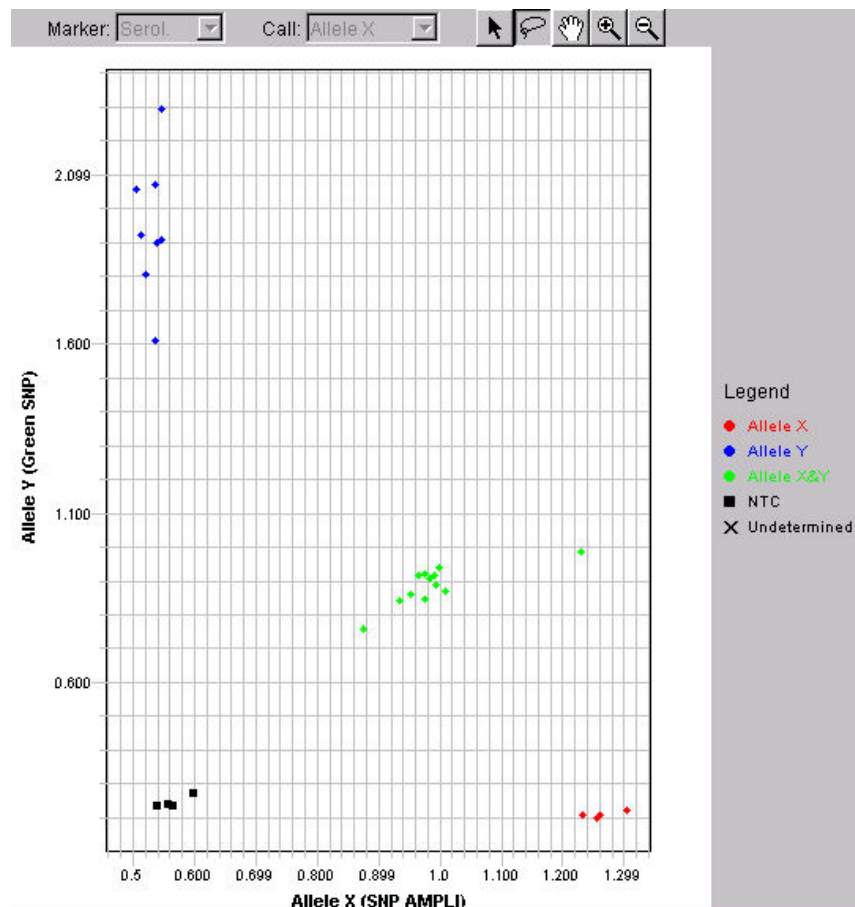
Allelic Discrimination Using the ABI PRISM® 7900HT.

First, refer to the ABI PRISM® 7900HT Sequence Detection System Manual, Section 4 (End-Point Analysis) for analysis of data. Some important guidelines to follow when configuring the ABI PRISM® 7900HT Instrument and Sequence Detection System software to use with Amplifluor® SNPs Genotyping System are listed below.

Note: It is important to note that the ABI PRISM® 7900HT instrument does not provide the option to thermally cycle allelic discrimination plate documents (see “Programming the Method” on page 3-12). If you would like to perform both thermal cycling and the plate read using the 7900HT instrument, you must first run the plate as a real-time document and then as an allelic discrimination plate document (see “About Allelic Discrimination Methods” on page 3-13 for notes on the procedure).

The throughput of the ABI PRISM® 7900HT Sequence Detection System can be increased by dividing the workload between the 7900HT instrument and several thermal cyclers. Since the allelic discrimination experiments are end-point runs, the 7900HT instrument does not collect data during the PCR. As a result, the thermal cycling of the allelic discrimination plates can be performed on a conventional thermal cycler and then transferred to the 7900HT instrument for an end-point read and analysis.

Figure 5: Analysis of a G/C type SNP on the human CYP1B1*2 (Val⁴³²Leu) gene (rs#1056836) using the ABI PRISM® 7900HT Sequence Detection System.



Signals from the homozygous genotypes are distributed along the X and Y axis, while the heterozygous genotypes are located approximately on the bisector.

Template Set-Up

Once the Sequence Detection System software is launched, you will need to create a **Plate Document Template** and configure it properly with the appropriate **Detector** and **Locus** assignments. Refer to **Plate Document Setup and Basic Operation**, beginning on pages 3-5, in the ABI PRISM® 7900HT User Manual.

In the template setup for allelic discrimination you will:

- a. Create a new plate document as outlined in the manual.
- b. Configure and apply detectors to the plate document. Here you will assign **FAM** and **JOE** as the appropriate **Reporter Dyes**, and the **Quencher Dyes** from the drop down list should be set to **Non-Fluorescent**. (Make sure that the Reporter Dyes correspond to your Locus assignments, (e.g. Allele 1, see pages 3-9).
- c. Configure the plate document with a **Passive Reference**. Refer to pages 3-12. To select the Passive Reference for the plate document, always select **ROX** from the **Passive Reference** pop-up list when using the Amplifluor® SNPs Genotyping System on the ABI PRISM® 7900HT.
- d. Create a method for the plate document. See pages 3-13.
- e. Apply samples to the plate document as outline in the manual.

Once the plate document template is created and saved as an SDS file, prepare and run the plate. Data can then be **Analyzed** (see page 4-10) when the run is complete. Results are then **Viewed** (see page 4-12) and **Called** (see page 4-13). Page 4-18 provides **Post Analysis Options** (e.g. Printing, Exporting data, etc.) for the run data.

Genotyping With Other Instrumentation

The ABI PRISM® 7000 as well as numerous fluorescent plate readers can be used with Amplifluor®. However, modifications to the instrument setup may be required in order to obtain accurate data. Please inquire with Technical Service at <http://www.millipore.com/reply/form/techservice> or (800) 221-1975 for further information.

Troubleshooting

If a researcher's SNP assays are not performing as expected, it is often helpful to use the Control SNP DNA and primer mix to determine if the problem is sample, reagent, SNP, or instrument related.

A. Buffer modifications for challenging DNA sequences.

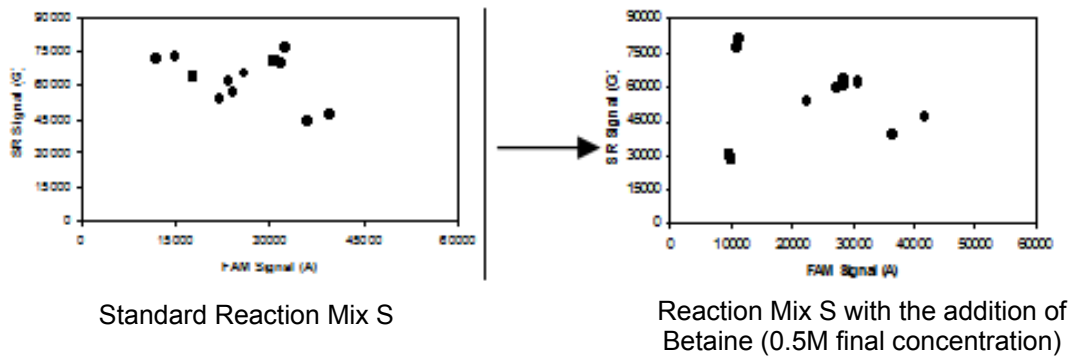
Most Amplifluor® SNP genotyping assays will work in standard buffer (Reaction Mix S or Reaction Mix S-Plus). However, there may be some particularly high GC or AT rich sequence flanking the SNP of interest that can be accurately genotyped with a simple buffer change. The betaine added to the PCR reaction is monohydrate and is most often used at 0.25 M, 0.5 M, and 1 M final concentration.

General Buffer Change Rules:

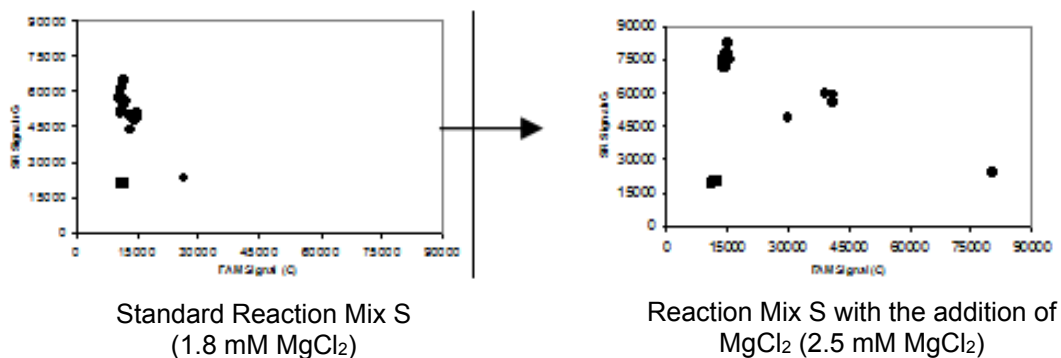
GC rich sequences (>60-90% GC) – Add Betaine to standard buffer

AT rich sequences (65-90%) – Increase MgCl₂ (2.5-3.0 mM)

Example 1: GC Rich: 100 bases flanking the SNP are 80% GC.



Example 2: AT Rich: 100 bases flanking the SNP are 75% AT.



B. Control Assays: Minimal or low signal observed in reactions

1. Potential problem: No initiation of PCR or low amplification efficiency

Recommendations:

- a. Confirm addition of all the kit reagents. Were the correct amounts of the 10X Reaction Mix S or 2.5 mM dNTP Mix added? Was Taq polymerase included?
- b. Do not add polymerase or any other reagents that contain a dye.
- c. dUTP can inhibit Amplifluor® reactions under certain conditions. Check with Technical Service at <http://www.millipore.com/reply/form/techservice> or (800) 221-1975 for more detailed information.
- d. Check the thermocycler/ABI PRISM® for the correct PCR parameters. Are the parameters set as indicated in Table 6?
- e. Determine the optimal annealing temperature. It may need to be determined empirically (50°C to 60°C) for each thermocycler.
- f. Check list of recommended polymerases for use with the Amplifluor® SNPs Genotyping System.
- g. Optimize the amplification conditions for the thermocycler being used. Thermocyclers vary in ramp rate (the time required to heat or cool the block to a given temperature) which affects PCR efficiency. Assay sensitivity for low target concentrations (< 1000 copies) is improved with efficient PCR.
- h. Increase the number of amplification cycles in the second period of cycling (22-24 cycles). The increased number of cycles will enhance signals provided the No Target Control signal is low and stable.
- i. Use 0.2 mL thin wall PCR tubes or PCR plates. The amplification conditions using the Perkin-Elmer Gene-Amp Model 9700 and MJ Research PTC-100® were optimized using Sorrenson plates (VWR Cat. #53550-036). Amplification conditions for the ABI PRISM® 7700 or ABI PRISM® 7900HT were optimized using ABgene® plates (Thermo-Fast 96, Non-Skirted, Natural, AB-0600), and Robbins Scientific PCR Plate Sealers (Cat No. 1044-39-4). Thin wall tubes (ABI® Cat #N801-0933 and #N801-0935) also work in the assay. Amplification using thick wall tubes may need substantial optimization.

2. Potential Problem: Instrumentation

Recommendations for a standard thermocycler/plate reader:

- a. Confirm that the tubes or plates are optically clear.
- b. The tubes or plate may not be correctly aligned or may be in the wrong type of holder. Check which holders are suitable or reset instrument's reading alignment and home position.
- c. There may be condensation on caps or sealer. Spin down by brief centrifugation. Allow samples to equilibrate to room temperature before reading.
- d. If the samples are excited and monitored from below, the light beam may be too wide and is scattering on the conical bottom of tubes or plate wells. Use the appropriate tubes or, if possible, adjust the light guide to make the light beam narrower. The reflective surface of a clear microtiter plate that is used for chromogenic ELISA assays may scatter too much light if read from the bottom.
- e. Excitation light source, power supply or detector may be failing and noisy. Perform diagnostics and/or contact manufacturer.
- f. The light sampling (counting) time may be incorrect. Make sure the sampling time (amount of time emission light is collected) is long enough or is not set to zero.
- g. The wavelength settings, excitation source or filters may be inappropriate. Optimal excitation/emission wavelength for fluorescein-labeled Amplifluor® SNPs Primer is 495/516 nm. Excitation light in the range of 480-495 nm is generally adequate. Monitoring of emission at wavelengths between 515-535 nm is in range. The optimal excitation/emission for sulforhodamine-labeled Amplifluor® SNP Primer is 600/620 nm. A range of 585 to 600 nm should be adequate. Monitoring of emission between 615 to 635 nm is in range. The optimal excitation/emission wavelength for a JOE-labeled Amplifluor® SNP Primer is 535/555 nm. Light sources can be diodes, lasers, tungsten halogen and xenon lamps. The lasers in these types of instruments have specific wavelengths that usually cannot be adjusted. Consult the instrument's optical specifications.
- h. The instrument settings may be inappropriate. Check aperture widths, gain and dampening adjustments. The first two may need to be increased while dampening, if any, needs to be decreased.
- i. The instrument setting for fluorescein can be checked by adding 5 µL of 1M NaOH to the PCR reaction mixture in the tube or well. It doesn't matter if it was already thermocycled. There should be a large increase in fluorescence after this is done. Alternatively, free fluorescein dye (Sigma-Aldrich, Cat No. F 245-6) in 25 µL of 20 mM Tris-HCl, pH 8, 50 mM NaCl, 2 mM MgCl₂ can be used. Free sulforhodamine dye in the same buffer can also be used to check the instrument (Sigma, 28,491-2). A significant signal above background (tube or plate with buffer only) should be seen with 50 to 100 fmols of dye.

Refer to user manual and manufacturer for these instruments.

C. Control Assays: High fluorescent signals observed in all reactions, including No Target Control

1. Potential problem: Amplicon or target contamination

Recommendations:

- a. Analyze negative controls and a few samples by electrophoresis to verify the presence of amplicon in negative controls.
- b. Use fresh aliquots of all reagents including sterile water.
- c. Only use pipets and racks dedicated to setting up reactions. Do not use pipets and accessories that have been exposed to amplicon and that have been used to load gels for amplicon analysis. Follow the recommendations for Lab Set-up and Precautions.
- d. Isolate the contaminated stock by running PCR of no target controls with cocktails prepared with various stock solutions.
- e. Decontaminate surfaces, pipets and racks.
- f. Change gloves and tips frequently.
- g. Do not keep a volume of water for the reactions greater than 1-2 mL.
- h. Change location of PCR set-up.

2. Potential problem: Primer-dimer formation

Recommendations:

- a. Analyze No Target Controls, control template and samples by electrophoresis. Primer-dimer bands are visualized as ~ 90 base pair fragments or a faint, low M.W. smear.
- b. Place PCR reactions in thermocycler block preheated to 96°C.
- c. Use a recommended hot start polymerase.
- d. Prepare the PCR cocktail and reactions at room temperature. Do not store on ice.
- e. Minimize the preparation time before thermocycling.
- f. Decrease the number of cycles.
- g. Increase the annealing temperature but try not to exceed 65°C.
- h. Determine if the common reverse primer is involved in the artifact formations, as this primer is the easiest to alter. Run three to five No Target Controls as described in Experimental Setup, omitting the reverse primer from the primer mixture. If no artifacts are formed, redesign the reverse primer.
- i. Redesign tailed primers. Make these primers shorter or expand them one additional base across the single nucleotide polymorphism. The latter approach is especially helpful if the extra base added is A or T.

3. Potential problem: Amplifluor® primers have been degraded or denatured

Recommendations:

- a. Change aliquots of reagents including polymerase.
- b. Make sure to use sterile technique and autoclaved, deionized water that has a very low metal content (check quality by conductivity/resistivity measurements).
- c. Avoid working with nucleases near PCR set-up or with pipets that have been exposed to nucleases.
- d. Make sure additives to cocktail are nuclease-free (eg., certain grades of BSA can have high nuclease contamination) and do not cause an acidic pH change.
- e. High pH and denaturants will cause high Amplifluor® fluorescence and severely inhibit the PCR reaction. Make sure alkaline solutions or denaturants were not inadvertently added.
- f. Polymerases with high exonuclease activities, particularly 3'→5', can degrade primers and amplicon and are not generally suitable for PCR. Check that a recommended polymerase was used in the reaction.

4. Potential problem: Instrumentation

Recommendations (platereader):

- a. Check that the appropriate excitation/emission settings or filters were used. Make sure the excitation light is in the range of 480 to 495 nm and the emission range is around 515 to 535 nm for fluorescein. Excitation/emission ranges for sulforhodamine are 585 – 600/ 615 – 630 nm.
- b. Check that there is no overlap of bandwidths between excitation/emission filters or settings. If the emission window overlaps with the excitation window or is too close to it, an unacceptable amount of excitation light will bleed through the emission window into the detector and cause very high readings that are not related to the Amplifluor® primers. The highest wavelength value of the excitation bandwidth should be approximately 20 to 25 nm below the lowest value of the emission bandwidth. For example, an excitation bandwidth for fluorescein could be set at 485 nm +/- 10 nm (i.e., 475 to 495 nm) and an emission of 535 nm +/- 15 nm (520 to 550 nm). Wider windows have a greater amount of transmitted light. This can increase signal strength but also show more scattering and stray light. Since this kind of strategy often requires directing or collecting the light off of the peak excitation/emission values, an increased bandwidth can make up for some of the lost efficiency. Certain plate readers, such as Molecular Devices® Gemini™, have monochromators and do not require the user to consider bandwidth spacing in the manner discussed since the excitation/emission values can be entered with better than 2 nm accuracy and cross talk is minimal.
- c. Confirm that the apertures and sensitivity settings are appropriate. If the apertures are opened too wide and/or sensitivity is set too high, the system will be swamped. Decrease the aperture and/or readjust sensitivity.

Refer to user manual and manufacturer for these instruments.

D. Researcher's system has minimal or no signal in all reactions

1. Potential Problem: Low PCR efficiency.

Recommendation: Run additional PCR cycles following the procedures described below:

- a. Create a PCR program with the following parameters: 96°C/10 seconds, 55°C/20 seconds and 72°C/40 seconds for 3 cycles.
- b. Start the program to pre-heat the block, stop the program when the block temperature is 96°C, place the samples into the block, wait 5 seconds and start the program again.
- c. Measure sample fluorescence. If the signal is weak, repeat step 2 until a strong signal is observed.
- d. An increase in both sample and No Target Controls fluorescence after 30 or more PCR cycles in the second period of cycling may indicate that the primers are too short. Redesign primers following the same steps in Sec. IV. Primer Design.
- d. Some assays may require an increase in the initial denaturation time and the cycling denaturation temperature and time. Perform the initial denaturation for 5 min and increase the temperature 96°C. Set the cycling denaturation parameters for 15 – 20 seconds at 95°C.
- e. Also, check the size of the amplicon. If it is more than 250 nucleotides, increase the incubation at 72°C step of the PCR program to 60 seconds.
- f. PCR efficiency can be increased by increasing the Mg²⁺ concentration up to 2.0-2.5 mM, particularly with primers and targets having low G + C content. Assays with Chemicon® control DNAs and primers should be run in parallel with the SNP of interest. If the fluorescence units in the experimental samples are significantly lower than those obtained from the Chemicon® control assays, 3-6 additional amplification cycles may be added to the thermocycling protocol and/or the MgCl₂ concentration increase up to 2.5 mM.

E. Researcher's assay has fluorescent signal of both colors in all reactions including No Target Controls

1. Potential Problem: Primer design failure.

Recommendation:

Determine which of the primers is causing the problem. Run several No Target Control reactions, each excluding one of the unlabeled primers. Redesign the primer causing the problem. If both the allele-specific primers cause the problem make these primers shorter.

F. Researcher's assay only shows one fluorescent signal in all the samples

1. Potential Problem: Not all the primers were added to the reaction cocktail.

Recommendations:

- a. Make sure both allele-specific primers were added.
- b. Make sure both Amplifluor® primers were added. If both were not added, No Target Controls will show abnormally low background for one Amplifluor® primer.

2. Potential Problem: Only one of the alleles is present in the samples.

Recommendation:

The minor allele frequency of SNPs vary significantly, from less than 5% to more than 50%. The allelic distribution among the population is uneven. Try to scan more samples or abandon the SNP.

G. Significant signal crossover in homozygotes

1. Potential Problem: Tailed primers are too long.

Recommendation:

Re-design the tailed primers by removing several bases from the 5'-ends of the allele-specific primer domain. Check the new design for 3'-end overlaps using primer design software as described in Sec. IV. Primer Design.

Note: Sometimes changing only one of the primers is sufficient; e.g., if the FAM background increases in the SR- or JOE- generating homozygote, but not vice versa, shorten only the allele specific portion of the green tailed primer.

2. Potential Problem: Ramp rate of the annealing step is too rapid.

Recommendation:

The ABI PRISM® 9700 thermocycler has a rapid ramp rate and may need adjustment to allow maximum amplification of the template. Reducing the ramp rate of the annealing step (55°C) to 40-50% of the maximum will make the ramp rate roughly equal to that of MJ Research PTC-100®, which has a fixed ramp rate.

H. High amount of scatter in data in control and/or researcher's assay

1. Potential Problem: Experimental method and design

Recommendation:

- a. Perform duplicates or triplicates. Use extra care when pipetting solutions containing low amounts of target.
- b. Frozen stocks of target, primers or buffer are not completely thawed or mixed when used. Thaw all solutions completely and briefly mix before using.

2. Potential Problem: Instrumentation

Recommendations:

- a. Refer to Sec. VII. *Troubleshooting, Control DNA System: Minimal or low signal observed in reaction.*
- b. To eliminate cross-talk between tubes and wells, use masked tube holders.
- c. The tube caps may be deformed by thermocycler lid. Do not secure lid too tightly. Carefully transfer reactions to new tubes or plate in a safe area.
- d. Check to see if the optics are dirty or the filters/grating are misaligned. Contact the instrument manufacturer for guidance.
- e. If using an oil overlay*, make sure the oil is clean, clear and in one continuous layer above the aqueous reaction mixture. If the oil is only partially covering the mixture, add more. Make sure there are no emulsions.

***Note:** *The use of oil overlays is not recommended unless absolutely necessary.*

Related Products

Amplifluor® Universal Detection System

Amplifluor® UniPrimer™ Fluorescein (S7903, S7905) contains only the UniPrimer™, making this an appropriate choice for high throughput applications after assay development.

Table 13: Amplifluor® Universal System Product Offering

<u>Description</u>	<u>Catalog #</u>	<u>Quantity</u>
Amplifluor® UniPrimer™ Fluorescein	S7905	1000 rxns
Amplifluor® UniPrimer™ Fluorescein	S7903	10000 rxns

Appendix

A. PCR Plates, Seals and Instrument Compatibility

Plate Readers/Thermocyclers

PCR plates and seals must be compatible with both your PCR block and fluorescence plate reader. Skirted or semi-skirted plates and/or low height (15 mm) may not fit in some PCR blocks and/or plate reader platforms. Plates with frosted wells, such as Bio-Rad iQ™ PCR plates (Cat #223-9441), should be used only with plate readers that send and detect light through the top of the wells or tubes. Plates made with transparent colorless wells, such as Sorrenson Multi PCR Plates (VWR Cat. #53550-036) or MARSH/ABgene® Plate (Cat #AB0600), can be used with plate readers that send and monitor light from either the top or bottom. Top reading instruments require a transparent cover, such as Robbins Scientific Cycle Seal Plate Sealer (Robbins Cat. #1044-39-4). This seal is used only with PCR plates that have rimmed wells, such as those mentioned above. With bottom reading plate readers, any other PCR compatible seals and covers can be used.

ABI PRISM® 7700 and 7900HT

PCR plates and seals must be compatible with both your PCR block and the PCR block of the ABI PRISM® 7700 and 7900HT instruments, especially if you are using the thermal cycling capacity of other PCR instruments. Skirted or semi-skirted plates and/or low height (15 mm) may not fit into the block of the ABI PRISM® 7700 while the ABI PRISM® 7900HT Instrument requires a skirted plate. Plate compatibility should be checked before performing any analysis. PCR plates made with transparent colorless wells, such as MARSH/ABgene® Plate (Cat #AB0600) can be used and will require a transparent cover, such as Robbins Scientific® Cycle Seal Plate Sealer (Robbins Cat. #1044-39-4). This seal is used only with PCR plates that have rimmed wells, such as those mentioned above.

B. Determination of DNA and Primer Concentrations

1. Estimate the concentration of the reference DNA controls by
 $(50 \times 10^{-6} \text{g}) \times (\text{OD}_{260}) \times (\text{dilution factor}) = \text{g DNA/mL}$
Dilute the DNA to 50 ng/μL by dividing your stock DNA concentration grams/μL by 50 ng. This is the dilution fold. Dilute the DNA to 5 and 0.5 ng/μL as indicated in Table 4.
2. Estimate the molar concentration of the SNP specific primers by

$$\frac{\text{OD}_{260} \times (\text{Dilution Factor})}{\text{OD}^* \times (\text{M.W.})} = [\text{Primer}]$$

***Note:** This conversion factor can be obtained using several oligonucleotide primer design programs. It generally ranges between 30 to 33 OD₂₆₀ per mg per mL.

C. Preparing the 20X Primer Mix

Prepare a 20X SNP specific primer mix from the stock solutions of the primers as follows:

The 20X SNP specific primer mix contains 0.5 μM solution of the green tailed allele-specific primer (Vgt), 0.5 μM solution of the red tailed allele-specific primer (Vrt), and 7.5 μM solution of the common reverse primer (Vc) in water. Alternatively, TE (10 mM Tris:0.1 mM EDTA) can be used to dilute primer stocks and mixes. It is recommended to make 1 ml of each 20X SNP specific primer mix. Depending on the concentration of each primer stock, dilute appropriately to achieve the proper concentration of primers.

For example, if Vgt, Vrt, and Vc stock solutions are 50 μM , 40 μM , and 250 μM concentrations, respectively:

10.0 μL	Vgt stock primer (50 μM)
12.5 μL	Vrt stock primer (40 μM)
30.0 μL	Vc stock primer (250 μM)
<u>947.5 μL</u>	<u>water</u>
1000 μL	Total 20X SNP specific primer mix

Note: If the assay is biased towards one allele, i.e. the heterozygous cluster is positioned too close to one of the homozygous clusters; prepare a new adjusted 20X SNP specific primer mix.

For example, if the heterozygous cluster is shifted towards the green allele homozygous cluster, make the following adjusted 20X SNP specific primer mix which contains 0.4 μM Vgt primer, 0.6 μM Vrt primer, and a 7.5 μM solution of the common reverse primer (Vc) primer.

8.0 μL	Vgt stock primer (50 μM)
15.0 μL	Vrt stock primer (40 μM)
30.0 μL	Vc stock primer (250 μM)
<u>947.0 μL</u>	<u>water</u>
1000 μL	Total adjusted 20X SNP specific primer mix

20X SNP Specific Primer Mix storage conditions:	-20°C for over 1 week
	+4°C for immediate use

D. Laboratory Setup and Precautions

PCR Environment

One of the most important considerations when performing PCR is the environment where the initial reaction mixtures are set up. The optimal working environment partitions the assay procedures in two areas. There should be a physical separation of the preparative area from the PCR amplification and analysis area. The ideal set up employs separate rooms. If the same room must be used, then an

assay station set up should be adopted with a clear division between the preparation area and the PCR amplification/analysis area. It is mandatory that no amplified products or equipment exposed to the amplified products enter the preparative area.

Precautions

The following precautions should be followed in all steps of the assay protocol:

- a. Always wear gloves and change them frequently.
- b. Use high quality, molecular biology grade dH₂O for all solutions.
- c. Keep the assay solutions (10X Reaction Mix S and S-plus, 2.5 mM dNTPs and primers) separate from DNA Targets and amplified DNA.
- d. Always use a designated set of pipets exclusively for the PCR setup and DNA Targets and always use aerosol resistant tips.
- e. Decontaminate the PCR tube racks and workstation with 10% bleach and UV irradiation and after each use.
- f. Let all reagents equilibrate to room temperature and mix thoroughly before use.
- g. Work quickly after enzymes have been added.
- h. Always use a preheated PCR block for PCR reactions.
- i. Be aware that the optimal number of PCR cycles can vary with some PCR machines. If the signal is not intense, run 1-5 more cycles of PCR and read plate again. Use this technique to optimize the number of cycles for your laboratory's PCR machine.

References

Internet Sites

1. EMD Millipore Company web site available at:
<http://millipore.com/userguides/tech1/mcproto177>
2. Applied Biosystems technical support
3. dbSNP at NCBI available at: <http://www.ncbi.nlm.nih.gov/SNP/index.html>

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3. Weiss, K., and J. Terwilliger. 2000. How many diseases does it take to map a gene with SNPs? *Nature Genetics* 26:151.
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