SureScore™
Genotyping Kit

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SureScore™ Genotyping Kit

For identifying single nucleotide polymorphisms (SNPs) in DNA samples

Catalog no. T6000

Invitrogen™
life technologies

www.invitrogen.com
tech_service@invitrogen.com

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Introduction

Overview

SureScore™ is an ELISA-based scoring assay for detecting single nucleotide polymorphisms (SNPs) in genomic DNA samples (Nikiforov *et al*, 1994). It relies on the inherent ability of DNA polymerase to distinguish SNPs in a hapten-labeled, single-base primer extension reaction. SureScore™ assays can be used for a variety of experimental purposes, including screening, investigating specific SNP panels, evaluating small sets of SNPs and samples, analyzing point mutations, and assay development for systems utilizing SureScore™ technology.

To identify a particular SNP using SureScore™, first you design a capture primer corresponding to the DNA sequence immediately adjacent to the SNP and attach it to the well bottoms of the SureScore™ plate. Then you amplify the sequence surrounding the SNP in a standard PCR reaction, digest the PCR product to produce a single-stranded template, and hybridize the template to the capture primer. Finally, you extend the capture primer by a single hapten-labeled nucleotide corresponding to the SNP base and detect the base using a colorimetric reaction. (See the workflow on the next page.)

There are six SureScore™ Genotyping Kits (see page 3), each containing reagents specific for scoring a particular type of SNP. Each kit contains a different extension mix—A/G, T/C, A/C, T/A, G/C or T/G—and three Control Templates for use as positive controls. The type of kit you use will depend on the SNP you are evaluating and which DNA strand will be queried for the SNP.

Each SureScore™ Genotyping Kit allows you to analyze up to 96 samples in a single SureScore™ strip-well plate. Plates can be processed manually or using commonly available equipment, such as a multi-channel pipettor, plate reader, and/or plate washer. The SureScore™ Complete Genotyping Kit contains enough components for scoring all six types of SNPs.

Note

You must design the PCR Primers and SNP-IT™ Capture Primers for your SNPs of interest **before** selecting a kit. The SureScore™ Primer Design Web site (www.invitrogen.com/SureScore) is recommended for this purpose. See pages 7–9 for more information.

Analysis

SureScore™ plates can be read visually, or you can use a plate reader to measure the optical density (OD) of each well. If you are using a plate reader, SureScore™ Data Analysis Software (see page 23) automates the process of genotype calling. Using the software, you can automatically identify genotypes from the OD readings, generate scatter plots, and display and print results.

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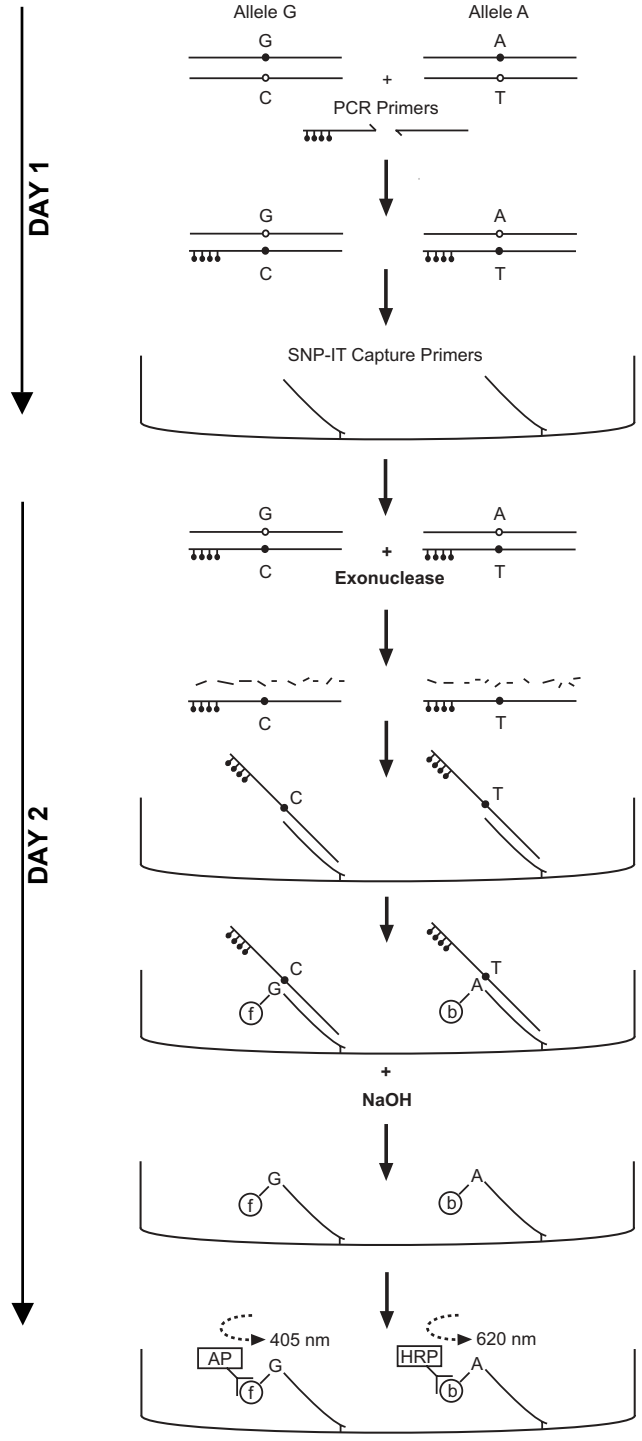
SureScore™ Workflow

First, design and generate your PCR Primers and SNP-IT™ Capture Primers as described on page 7. Then follow the workflow shown below.

Because attaching the SNP-IT™ Capture Primer to the plate requires a 12-to-16 hour incubation period, we recommend performing Steps 1 and 2 in the afternoon of Day 1 and the remaining steps on Day 2.



1. From DNA samples, amplify the region surrounding the SNP of interest using a PCR reaction with one phosphorothioate-modified PCR Primer and one unmodified PCR Primer.

2. Attach the SNP-IT™ Capture Primers to the well bottoms of the SureScore™ 96-strip-well plate. Incubate 12–16 hours.

3. Digest the PCR product with Exonuclease. The phosphorothioate modification protects one strand, generating a single-stranded template.

4. Hybridize the single-stranded template to the SNP-IT™ Capture Primers in the SureScore™ plate. The immobilized SNP-IT™ Capture Primer serves as the template for primer extension.

5. Extend the SNP-IT™ Capture Primers by one labeled terminating nucleotide.

6. Add NaOH to remove the template DNA strand.

7. Detect each labeled nucleotide using a colorimetric reaction. Antibody conjugates of alkaline phosphatase and horseradish peroxidase are used to identify the extended base.

Kit Contents and Storage

Overview

There are six individual SureScore™ Genotyping Kits—one for each possible SNP. In addition, a SureScore™ Complete Genotyping Kit includes components for analyzing all six SNPs.

Kit contents are listed on the following page.

Kit Name	Catalog Number
SureScore™ A/G Genotyping Kit	T6000-01
SureScore™ T/C Genotyping Kit	T6000-02
SureScore™ A/C Genotyping Kit	T6000-03
SureScore™ T/A Genotyping Kit	T6000-04
SureScore™ G/C Genotyping Kit	T6000-05
SureScore™ T/G Genotyping Kit	T6000-06
SureScore™ Complete Genotyping Kit	T6000-07

Continued on next page

Kit Contents and Storage, Continued

Individual Genotyping Kits

Each individual SureScore™ Genotyping Kit contains one 96-strip-well plate and enough reagents to analyze approximately 100 DNA samples.

In the table below, the 10X Extension Mix and Control Templates are unique for each kit (see the second table below for details). The remaining reagents/materials are the same for all kits:

Reagent/Material	Quantity	Storage
Specific to each kit (see following table for details):		
10X Extension Mix	1 tube	-20°C
Control Template XX	225 µl	-20°C
Control Template YY	225 µl	-20°C
Control Template XY	225 µl	-20°C
Common to all kits:		
Exonuclease	2000 units	-20°C
Control Capture Primer	20 µl	-20°C
96-Strip-Well Plate	1	Room temp.
20X Wash Buffer	55 ml	+4°C
Attachment Buffer	10 ml	+4°C
Exonuclease Buffer	5 ml	+4°C
Hybridization Solution	5 ml	+4°C
Extension Dilution Buffer	5 ml	+4°C
Detection Complex Dilution Buffer	10 ml	+4°C
Detection Complex I	10 µl	+4°C
Detection Substrate I	15 ml	+4°C
Detection Complex II	10 µl	+4°C
Detection Substrate II	15 ml	+4°C

Reagent	A/G Kit	T/C Kit	A/C Kit	G/C Kit	T/A Kit	T/G Kit
10X Extension Mix	A/G	T/C	A/C	G/C	T/A	T/G
Control Template XX	GG	CC	CC	CC	AA	GG
Control Template YY	AA	TT	AA	GG	TT	TT
Control Template XY	AG	TC	AC	GC	TA	TG

Complete Genotyping Kit

The SureScore™ Complete Genotyping Kit contains all six SureScore™ Individual Genotyping Kits listed above, enabling you to screen 96 DNA samples for each possible SNP. Quantities are as listed above times six.

Storage Conditions

The 10X Extension Mix, Control Templates, Exonuclease, and Control Capture Primer are boxed together for storage at –20°C. The remaining reagents are boxed together for storage at +4°C.

Note: The 10X Extension Mix is *light sensitive*. To maintain stability, keep this reagent stored at -20°C in its original light protective vial.

With proper storage, kits have a shelf life of 6 months.

Accessory Products

The following products for use in the PCR reaction are available separately from Invitrogen:

Product	Quantity	Cat. no.
Platinum® *Taq* DNA Polymerase	100 rxns	10966-018
	250 rxns	10966-026
	500 rxns	10966-034
	5,000 rxns	10966-083
10 mM dNTP Mix	100 µl	18427-013

Product Qualification

Assays were performed using the protocols in this manual and the results were measured with a plate reader. Clustering analysis of positive and negative controls was performed using SureScore™ Data Analysis Software.

Additional Materials

PCR Primers and Capture Primers

You must design and order the PCR Primers and SNP-IT™ Capture Primers separately, based on the target sequence for your SNP(s) of interest. Invitrogen's SureScore™ Primer Design Web site (www.invitrogen.com/SureScore) is recommended this purpose (see page 7).

Additional Equipment and Reagents

The following required equipment and reagents are not included in the kits:

- 96-well microtiter plates (for PCR reactions)
- 37° C incubator
- Rotating orbital platform shaker or vortex with plate adaptor
- Thermostable DNA polymerase (see ordering information on previous page)
- Thermocycler
- Sodium Hydroxide (0.1 N NaOH) (ACS grade)
- Pipettes (20 µl, 200 µl, 1000 µl)
- Pipette tips
- Aerosol resistant tips for PCR
- Deionized water

Analysis Software

SureScore™ Data Analysis Software can be used to analyze SNP data from a plate reader. SureScore™ customers can download this software for free from www.invitrogen.com/SureScore. See page 23 for details.

Recommended Equipment

The following equipment is highly recommended if more than three strip-wells are being processed at a time:

- 8- or 12-channel pipettor
- 96-well plate reader with 405 nm and 620–650 nm filters for measuring optical density.

The following equipment is also recommended for high-volume applications:

- 96-well plate washer

Primer Design

Introduction

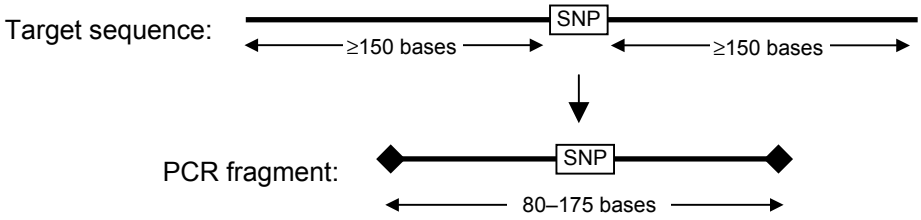
Before proceeding with the SureScore™ protocol, you must design and order the PCR Primers and SNP-IT™ Capture Primer for the specific target sequence surrounding each SNP of interest. The Invitrogen SureScore™ Primer Design Web site (www.invitrogen.com/SureScore) is recommended for designing primers for use with SureScore™ kits. However, you can use any primer design program to create your SureScore primers.

The required amount of each primer will depend on the number of target samples you are screening. See the following page for recommended concentrations.

Target Sequence Specifications

For accurate primer design, the target sequence must:

- Include at least 150 flanking bases both upstream and downstream of the SNP of interest (i.e., the total sequence length must be ≥301 bp).
- Be at least 95% known (i.e., no more than five "N" bases per 100 bases of sequence).
- Contain no more than one "N" base within 25 bases on either side of the SNP.
- Have a GC content of between 40% and 65%.
- Be of high quality (see the note on PHRED scores below).
- Result in a PCR fragment of between 80-175 bp in length.



Information on PHRED, a common DNA quality measurement, can be found at the following Web site: http://www.genome.washington.edu. DNA that has a score of PHRED 20 or higher should work well in this application (e.g., ~99% accuracy).

Masking of repeats in the target sequence is also highly recommended. You can block repetitive elements during primer design by inserting "N" bases in their place. Make sure that no "N" bases are located within 25 bases upstream or downstream of the SNP.

Primer Preparation

Primers should be desalted, deprotected, and resuspended in purified water. In addition, primers with phosphorothioate modifications and/or C3 linkers should be reverse-phase (cartridge) purified.

Continued on next page

Using the SureScore™ Primer Design Web Site

To design your primers, we recommend using the Invitrogen SureScore™ Primer Design Web site at www.invitrogen.com/SureScore. This site provides an easy-to-use tool for designing all primers used in the protocol. (Note that you can use any primer design program to create your SureScore primers.)

This site requires on-line registration. After registration, you can log onto the site using your e-mail address and a password.

After log-in, follow the detailed instructions on the Web site for submitting the target sequence containing your SNP of interest. The Web site will automatically generate one or more recommended primer sequences for the upper and lower PCR Primers and the SNP-IT™ Capture Primer and display them in a table, as shown in the example below:

SNP Name	SNP Type	Rcmd. Ta	Primer Type-Mod.	Amplicon Length	Sequence
SNP1 Design 1	GT	55.0	PCRU PCRL-S SNPU	170	CGATGCGAGACGATCCGC GATTTGATGCATACTTACATATACGATAAAGA AATTATATTXTAGCATAAACTTAAT
SNP1 Design 2	CA	55.0	PCRU-S PCRL SNPL	170	CGATGCGAGACGATCCGC GATTTGATGCATACTTACATATACGATAAAGA ATATAXGATAAAGATAATATGCTCGTC

The columns in the table are described below:

SNP Name—The SNP name you supplied with the submitted sequence.

SNP Type—The SNP bases used in the extension reaction. Note that these will depend on which DNA strand the SNP-IT™ Capture Primer is modeled on (upper or lower). Use this to select the appropriate SureScore™ kit (see page 9).

Rcmd Ta—The recommended annealing temperature of the PCR primers.

Primer Type-Mod.—PCRU is the upper PCR primer. PCRL is the lower PCR primer. "-S" indicates which PCR primer has the phosphorothioate modification. SNP is the SNP-IT™ Capture Primer, flagged with a U or L, depending on which DNA strand it's modeled on.

Amplicon Length—The length of the resulting PCR product.

Sequence—The sequences of the three primers. These correspond to the primers as listed in the Primer Type-Mod. column. An "X" in the sequence indicates that a C3 linker is recommended to reduce secondary structure that may lead to template-independent extension.

Phosphorothioate Modification

When ordering your primers, you must specify a phosphorothioate modification on the appropriate PCR primer (i.e., the primer flagged with "-S" by the Primer Design Web Site).

Specify four phosphorothioates on the 5' end of the primer.

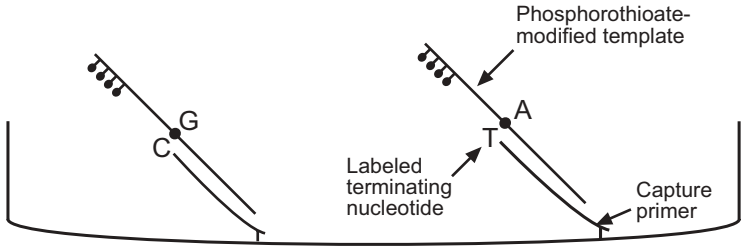
Amounts of Primer

Type of primer	Amount
PCR	0.1-0.5 µM of each PCR Primer per PCR reaction
SNP-IT™ Capture	0.25 µM of Capture Primer in a 50 µl solution per well

Selecting a SureScore™ Genotyping Kit

Introduction

Select the appropriate SureScore™ Genotyping Kit based on your SNP of interest and your primer designs (page 7). The name of each Genotyping Kit indicates which bases will be incorporated as labeled terminating nucleotides on the SNP-IT™ Capture Primer. These terminating nucleotides are used to identify the SNP of interest, as shown in the diagram below:



NOTE: For each SNP, the SNP-IT™ Capture Primer you design will be based on either the upper (sense) DNA strand or the lower (antisense) DNA strand, and this will determine the type of SureScore™ Genotyping Kit you select. Therefore, you should design your primers *before* selecting a kit.

Kit Selection Guide

Use the table below to select the appropriate kit:

SNP Bases	SNP-IT™ Capture Primer is identical to:	Use:
A/G	Upper DNA strand	A/G kit
	Lower DNA strand	T/C kit
T/C	Upper DNA strand	T/C kit
	Lower DNA strand	A/G kit
A/C	Upper DNA strand	A/C kit
	Lower DNA strand	T/G kit
T/A	Upper DNA strand	T/A kit
	Lower DNA strand	T/A kit
G/C	Upper DNA strand	G/C kit
	Lower DNA strand	G/C kit
T/G	Upper DNA strand	T/G kit
	Lower DNA strand	A/C kit

Labeled Nucleotides in Each Kit

In each kit, the "X" and "Y" nucleotides are labeled as shown below:

	Labeled Nucleotide	
Kit	X (405 nm)	Y (620 nm)
A/G	G	A
T/C	C	T
A/C	C	A
T/A	A	T
G/C	C	G
T/G	G	T

Controls

Introduction

The following section describes the control wells for each SureScore™ Assay.

Positive Controls

One Control Capture Primer and three corresponding Control Templates are supplied with each SureScore™ Genotyping Kit.

The Control Capture Primer is attached to positive control wells on the SureScore™ 96-strip-well plate. The Control Templates provide examples of homozygous XX, homozygous YY, and heterozygous XY signals in the detection step (see the previous page for a table identifying the X and Y labeled nucleotides in each kit).

Negative Controls

We recommend two negative controls for each unique experimental SNP-IT™ Capture Primer used in an assay, and one negative control for the Control Capture Primer:

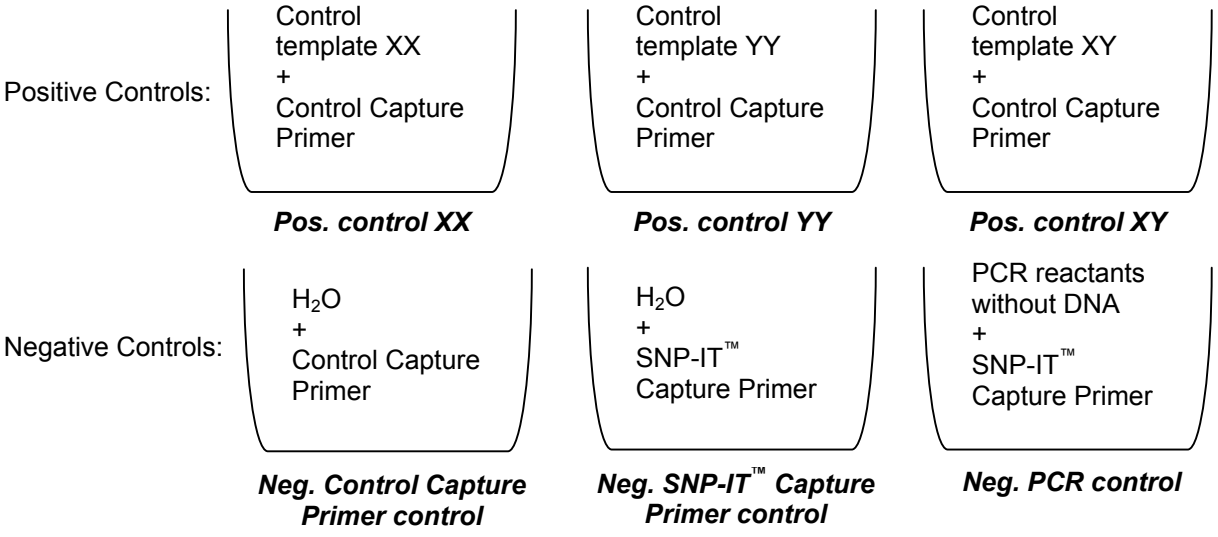
The **negative PCR control** is used to detect contamination during PCR set-up. You set up negative PCR control wells in the PCR plate, then transfer the reaction mix to corresponding wells in the SureScore™ plate. Negative PCR control wells should contain all the PCR reactants *except* genomic DNA.

The **negative SNP-IT™ Capture Primer control** is used to detect self-annealing and self-extension of the SNP-IT™ Capture Primer. Set up this control well in SureScore™ plate; it contains only water and the SNP-IT™ Capture Primer.

The **negative Control Capture Primer control** is used to detect self-annealing and self-extension of the Control Capture Primer (see previous page). Set up this control well in SureScore™ plate; it contains only water and the Control Capture Primer.

Control Wells

The diagrams below summarize the control wells in each SNP assay:



Genomic DNA

Obtaining Genomic DNA Using Buccal Swabs

The following procedure is a general-purpose method for obtaining genomic DNA samples using buccal swabs. Note that the method developed by Truett *et al* may provide higher yields for some applications (Truett *et al*, 2000).

1. Briefly rub a sterile swab against the inside of the cheek and place in 400 µl of DNAzol® Reagent* (Catalog no. 10503) for one minute.
2. Precipitate the DNA with 200 µl of 100% ethanol.
3. Centrifuge gently at ~4,000 × *g* for 2–3 minutes at +4°C.
4. Wash with 70% ethanol and dry for 10 seconds.
5. Resolubilize in ~200 µl of TE, 20 mM Tris-Cl, and 1 mM EDTA at pH 8.5 to a concentration of ~1 ng/µl. (If desired, adjust to a desired pH with Hepes buffer according to the manufacturer's suggested protocol.)

The solubilized DNA is ready for PCR amplification.

*DNAzol® is a registered trademark of Molecular Research Center, Inc.

Genomic DNA Quantity

The recommended quantity of input DNA for the PCR reaction is 2-to-50 ng/50 µl of PCR reaction (assuming ~36 cycles of PCR.) However, this concentration can vary, depending on the particular SNP, the target sequence being amplified, and the PCR Primer design. If, during the detection step, you observe a weak signal for a particular sample, you may want to run the SureScore™ assay again for that sample using more genomic DNA.

Genomic DNA Quality

Use high quality genomic DNA in the PCR reaction. We recommend using DNA that has a PHRED score of 20 or higher (e.g., ~99% accuracy). Information on PHRED, a common DNA quality measurement, can be found at the following Web site: http://www.genome.washington.edu.

The DNA should not contain any PCR inhibitors.

Before You Begin...

SureScore™ Plate Layout

Before proceeding, we recommend that you create a SureScore™ plate layout that designates the different well types and the samples that they contain. This is useful for tracking the position of samples and controls when they are transferred from the PCR plate to the SureScore™ plate. A layout function is included in the SureScore™ Data Analysis Software, as shown below:

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1.1	S2.1	S3.1	S4.1	P1.1	P2.1	P3.1	P4.1	B	B	B	B
B	S1.2	S2.2	S3.2	S4.2	P1.2	P2.2	P3.2	P4.2	B	B	B	B
C	S1.3	S2.3	S3.3	S4.3	P1.3	P2.3	P3.3	P4.3	B	B	B	B
D	S1.4	S2.4	S3.4	S4.4	B	B	B	B	B	B	B	B
E	S1.5	S2.5	S3.5	S4.5	B	B	B	B	B	B	B	B
F	S1.6	S2.6	S3.6	S4.6	B	B	B	B	B	B	B	B
G	N1.1	N2.1	N3.1	N4.1	B	B	B	B	B	B	B	B
H	N1.2	N2.2	N3.2	N4.2	B	B	B	B	B	B	B	B

Coding

[SUMPLE_TYPE][SNP_NUM].[SAMPLE_NUM]

P - Positive Control

N - Negative Control

S - Samples

B - Blank

Recommendations and Tips

Plates:

- Remove any unused strips from the SureScore™ strip-well plate before the assay and save them for future assays.
- Label each strip in the strip-well plate for identification in case it becomes dislodged from the plate holder. This is particularly important if you invert the plate to remove reagents.
- Do not substitute standard 96-well plates for the special 96-strip-well plates provided with these kits. Use of standard 96-well plates in the SureScore™ assay may significantly alter experimental results.
- If you are processing two or more plates at the same time, be careful to process the plates in the same order at each step and avoid substantial time lags between reagent addition/removal from the wells.

Wash Steps:

- Do not skip any wash steps. Skipping wash steps can adversely affect the quality of the results.
- Use an automated plate washer if you are processing multiple plates simultaneously.

Reagents:

- Do not thaw frozen reagents using 37°C water baths.
- Return all stock reagents to their recommended storage conditions immediately after use.

Plate Reading:

- For best results, read plates immediately after the 30-minute color development incubation period.

Step 1: PCR Amplification of Target Sequence

Overview

In this step, you amplify genomic DNA samples using a PCR reaction with a thermostable DNA polymerase (preferably hot start) and the phosphorothioate-modified primer and unmodified primer designed for your target region of interest. **We recommend that you perform this step the day before performing Steps 3–8.**

Materials Supplied by the User

You will need the following reagents and equipment for this step:

- Thermocycler
- Thermostable DNA polymerase (see ordering information on page 5)
- PCR buffer
- Mg^{++} (if required for your polymerase)
- dNTP mix (see ordering information on page 5)
- Autoclaved, distilled water
- Genomic DNA (~0.1–1.0 ng/µl of PCR reaction; see page 11).
- PCR Primers designed for your SNP of interest

Primer Preparation

You must design and create your PCR Primers separately, as described on page 7.

Note

The use of dyes in the PCR reaction has not been validated or tested, and is not recommended. Additives such as detergents and other chemicals (e.g., DMSO) may also interfere with the output of this assay. However, bovine serum albumin (BSA) is acceptable to use during PCR amplification.

PCR Plate Layout

We recommend that you lay out your PCR plate in the same configuration as the SureScore™ 96-strip-well plate (see page 12). This will minimize confusion when you transfer DNA from the PCR plate to the SureScore™ plate in Step 4 (page 18).

Positive PCR Controls

Wells designated for positive controls should be left **completely empty** during the PCR reaction. The positive Control Templates will be added to the PCR plate *after* the PCR reaction, to avoid contamination.

Negative PCR Controls

Wells designated for negative PCR controls should contain all the PCR reactants *except* genomic DNA. The solution from these wells will be added to the designated negative PCR control wells in the SureScore™ 96-strip well plate to detect potential contamination during the PCR set-up.

Continued on next page

Step 1: PCR Amplification of Target Sequence, Continued

Amount of PCR Product

The SureScore™ assay requires only 15 µl of PCR product per well. The 50 µl PCR reaction provided below as an example allows some excess for verification by gel electrophoresis. You may also want to keep some PCR product in reserve in case you need to rerun the assay.

PCR product should produce a clear, distinct band of 80–175 bp on the gel.

Choosing a Thermostable Polymerase

You can use any thermostable DNA polymerase in your PCR reaction. We recommend using Platinum® *Taq* DNA Polymerase (see ordering information on page 5), as described in the following example protocol.

Setting Up the PCR Reaction

The procedure described below is suggested as a general guideline and starting point when using Platinum® *Taq* DNA Polymerase in a PCR amplification. Optimal reaction conditions (incubation times and temperatures, concentration of polymerase, primers, Mg, and DNA) will vary depending on the thermostable polymerase you are using, primers, target sequence, etc.

The reaction size may be scaled up or down.

1. Set up a 50 µl PCR reaction:

10X PCR Buffer	5.0 µl
10 mM dNTP Mix	1.0 µl
50 mM $MgCl_2$	1.5 µl
Forward PCR Primer (10 µM)	1.0–2.0 µl
Reverse PCR Primer (10 µM)	1.0–2.0 µl
Genomic DNA (2–20 ng)	≥1.0 µl
Platinum® *Taq* (5 units/µl)	0.5 µl
Autoclaved, distilled water	to 50.0 µl

2. Perform ~35 cycles of PCR amplification as follows:

1 cycle	94°C for 1 minute
35 cycles	94°C for 30 seconds
	60°C for 30–60 seconds
	72°C for 2 minutes
1 cycle	72°C for 12 minutes

   These reaction parameters are provided as a guideline only. Use parameters suitable for your primers and samples. Be sure to include at least a 10-minute extension after the last cycle to ensure that all PCR products are full-length.

3. Maintain the reaction at 4°C after cycling.

PCR Plate Storage

If necessary, PCR plates may be frozen at –20°C following amplification. Be sure to store PCR products in a separate location away from genomic samples and PCR reagents to avoid cross-contamination of samples.

Step 2. SureScore™ Plate Preparation

Overview

In this step, you prepare the SureScore™ 96-strip-well plate. Attach the positive Control Capture Primer and the experimental SNP-IT™ Capture Primers to the appropriate wells, as designated in your plate layout.

The attachment incubation period is 12–16 hours. **Therefore, we recommend that you perform this step in the late afternoon of the day before performing Steps 3–8.**

Labeling the Strips

We recommend that you number and label the strips in case they become accidentally dislodged from the plate during the washing steps.

Recommended Control Wells

See page 10 for detailed information on controls.

At least three wells per SureScore™ Genotyping Kit should be designated for positive controls: one for Control Template XX, one for Control Template YY, and one for Control Template XY.

At least one well per SureScore™ Genotyping Kit should be designated as a negative control for the Control Capture Primer.

At least two wells per experimental SNP-IT™ Capture Primer should be designated for negative controls: one for the SNP-IT™ Capture Primer alone, and one for the SNP-IT™ Capture Primer and the product of the negative PCR control reaction.

Preparing the Capture Primers

The Control Capture Primer comes ready-to-use in each SureScore™ kit.

You must design and create your experimental SNP-IT™ Capture Primers separately, as described on page 7.

Continued on next page

Preparing the 96-Strip-Well SureScore™ Plate

In this procedure, you attach the experimental SNP-IT™ Capture Primers and the Control Capture Primer to the appropriate wells in the 96-strip-well plate.

1. Briefly spin the Control Capture Primer tube in a microcentrifuge.

2. Dilute the Control Capture Primer to a final concentration of 0.0625 µM in Attachment Buffer. See the table below for control primer volume and dilution guidelines:

# of Positive Control Wells	Control Capture Primer Volume (10 µM)	Attachment Buffer Volume
3 wells	2.0 µl	320 µl
6 wells	3.0 µl	480 µl
9 wells	4.0 µl	640 µl
12 wells	5.0 µl	800 µl

   Dilutions can be made in standard polypropylene tubes. Invert tubes 10 times to mix (or gently vortex). We recommend that you make these solutions fresh for every assay and keep them on ice until they are added to the plate.

3. Dilute the experimental SNP-IT™ Capture Primers to a final concentration of 0.25 µM in Attachment Buffer. See the following table for SNP-IT™ Capture Primer volume and dilution guidelines:

# Strips/Wells	Experimental Primer Volume (e.g., 100 µM stock solution)	Attachment Buffer Volume
3 strips/24 wells	4.5 µl	1.8 ml
6 strips/48 wells	9.0 µl	3.6 ml
9 strips/72 wells	13.5 µl	5.4 ml
12 strips/96 wells	18.0 µl	7.2 ml

   Note that the table provides an example for a 100 µM stock solution of primer. Your primer concentrations may vary and calculations should be adjusted accordingly.

   Dilutions can be made in standard polypropylene tubes. Invert tubes 10 times to mix (or gently vortex). We recommend that these solutions be made fresh for every assay and that they are kept on ice until you add them to the plate.

4. Add 50 µl of the Control Capture Primer attachment mixture to each positive control well and the negative Control Capture Primer control well(s). Tap the sides of the plates or strips to ensure that the bottom of each well is uniformly covered with primer solution.

5. Add 50 µl of the experimental SNP-IT™ Capture Primer attachment mixture to each experimental well and each negative SNP-IT™ Capture Primer control well. Set up at least two negative control wells for each experimental SNP-IT™ Capture Primer (see page 10). Tap the sides of the plates or strips to ensure that the bottom of each well is uniformly covered with primer solution.

6. Cover the plate securely with aluminum foil and then plastic wrap, and incubate for 12–16 hours at 37° C. Double wrapping is necessary to minimize evaporation from the plate.

Step 3: Exonuclease Treatment

Overview

In this step, you add each type of Control Template (XX, YY, and XY) to the appropriate positive control wells of the PCR plate, and then digest the PCR-amplified template and the Control Template with Exonuclease. The non-phosphorothioate-modified DNA strands are digested, producing single-stranded DNA for the single-base primer extension reaction.

Digestion with Exonuclease

1. Spin Exonuclease briefly in a microcentrifuge before use.
2. Dilute Exonuclease 1:55 in Exonuclease Buffer. See the following table for dilution guidelines:

# Strips/Wells	Volume of Exonuclease	Volume of Exonuclease Buffer
3 strips/24 wells	9.0 µl	491.0 µl
6 strips/48 wells	18.0 µl	982.0 µl
9 strips/72 wells	27.0 µl	1473.0 µl
12 strips/96 wells	36.0 µl	1964.0 µl

   Note that volumes in the table include some excess to compensate for typical losses during the procedure.

   The dilution can be made in a standard polypropylene tube. Invert the tube 10 times to mix (or *gently* vortex). We recommend that you make this solution fresh for every assay and keep it on ice until use.

3. If the PCR reaction is more than 15 µl, transfer 15 µl of PCR product from each well of the PCR plate to a new plate.
4. Three Control Templates (XX, YY, and XY) are included with each SureScore™ Genotyping Kit (see page 10). Add 15 µl of each Control Template to the appropriate positive control well on the new plate.
5. Add 15 µl of the diluted Exonuclease to each well, including wells containing the positive and negative controls. Mix by pipetting up and down three times.
6. Cover the PCR plate with aluminum foil to prevent evaporation and incubate at room temperature for one hour.

Note: During the one-hour incubation period, proceed to steps 1–3 of the **Hybridization protocol** (see next page). It is *very important* to add hybridization solution to the Exonuclease-treated samples **immediately after** the one-hour incubation.

Step 4: Hybridization

Overview

In this step, the single-stranded target sequence is hybridized to the SNP-IT™ Capture Primer attached to the wells in the SureScore™ plate.

Hybridizing the SNP-IT™ Capture Primer and Control Capture Primer

Steps 1–3 below should be performed *during* the 1-hour incubation period specified at the end of the **Exonuclease Treatment** protocol (previous page).

1. Remove the hybridization solution from the refrigerator and allow it to warm up to room temperature. The hybridization solution precipitates at 4ºC. Invert the bottle several times to mix and wait until all precipitates have dissolved before use.

2. Prepare the 1X wash solution by diluting 20X wash buffer with deionized water. See the following table for dilution guidelines:

# Strips/Wells	Volume of 20X Wash Buffer	Volume of Deionized Water
3 strips/24 wells	8.0 ml	152.0 ml
6 strips/48 wells	16.0 ml	304.0 ml
9 strips/72 wells	24.0 ml	456.0 ml
12 strips/96 wells	32.0 ml	608.0 ml

3. Remove the SureScore™ 96-strip well plates from the incubator after the 12-to-16-hour incubation period (specified on page 16). Wash each well *three times* with 200 µl of 1X wash solution. Remove the wash solution *as completely as possible* after each wash. We recommend removing the liquid by aspiration or by vigorous tapping of the plate upside-down on paper towels.

4. After the PCR plate has incubated for one hour at room temperature (as specified on the previous page), *immediately* add 15 µl of hybridization solution to each of the Exonuclease-treated DNA samples, including the controls. **Do not remove the Exonuclease solution before adding the hybridization solution.** Pipet up and down to mix thoroughly.

5. Transfer 30 µl of solution from each well of the PCR plate to the corresponding wells on the SureScore™ strip-well plate: PCR-amplified target sequence in the experimental wells, Control Template in the positive control wells, and negative PCR control product in the negative PCR control wells.

Note: Avoid contamination of the negative SNP-IT™ Capture Primer control wells and the negative Control Capture Primer control wells with solution from the PCR plate.

6. Cover the SureScore™ strip-well plate and incubate at room temperature for one hour.

7. After incubation, wash each well *three times* with 200 µl 1X wash solution. Remove the wash solution *as completely as possible* after each wash. We recommend removing the liquid by aspiration or by vigorous tapping of the plate upside-down on paper towels.

8. If necessary, the SureScore™ plate strip wells can be filled with 200 µl of 1X wash solution and left at 4º C for a few hours.

Step 5: Extension of the SNP-IT™ Capture Primers

Overview

In this step, DNA polymerase and labeled terminating nucleotides are added to the SureScore™ plate wells, extending the SNP-IT™ Capture Primers by a single nucleotide.

Performing the Extension Reaction

1. Thaw the 10X Extension Mix for each SNP type in the plate at room temperature and spin briefly in a microcentrifuge. Note that the 10X Extension Mix contains light sensitive reagents and should be keep out of the light as much as possible.

2. Prepare a 1X working solution for each Extension Mix by diluting 1:10 in extension dilution buffer. See the following table for dilution guidelines:

# Strips/Wells	Volume of 10X Extension Mix	Volume of Extension Dilution Buffer
3 strips/24 wells	90.0 µl	810.0 µl
6 strips/48 wells	180.0 µl	1.6 ml
9 strips/72 wells	270.0 µl	2.4 ml
12 strips/96 wells	360.0 µl	3.2 ml

   This table takes into account the extra volume of final reagents needed to compensate for typical losses during the procedure.

   The dilution can be made in a standard polypropylene tube. Invert the tube 10 times to mix (or *gently* vortex). We recommend that you make this solution fresh for every assay and keep it on ice and out of the light until use.

3. Add 30 µl of the 1X extension solution to each well in the SureScore™ strip-well plate, cover the plate in plastic or aluminum foil to prevent evaporation, and incubate at room temperature for 30 minutes. The specified extension incubation temperature and time are optimal and should not be changed.

4. Wash each well *three times* with 200 µl of 1X wash solution. Remove the wash solution *as completely as possible* after each wash. We recommend removing the liquid by aspiration or by vigorous tapping of the plate upside-down on paper towels.

Step 6: Denaturation

Overview

In this step, you use NaOH to remove the template DNA strand, leaving only the extended SNP-IT™ Capture Primer bound to the plate. This reduces potential background signal in the assay.

Denaturing the Template DNA

1. Add 100 µl of 0.1 N NaOH to each well, then remove. It is not necessary to leave NaOH in the plate for any length of time for it to be effective.
2. Wash each well *three times* with 200 µl of 1X wash solution. Remove the wash solution *as completely as possible* after each wash. We recommend removing the liquid by aspiration or by vigorous tapping of the plate upside-down on paper towels.

Step 7: Detecting 405 Signals

Overview

In this step, you detect the presence of the "X" labeled terminating nucleotide (see page 9) on the extended SNP-IT™ Capture Primer using an indirect colorimetric assay. The color of this reaction is yellow, and can be detected with a plate reader at a 405 nm wavelength.

Detecting 405 Signals

1. Spin the tube containing Detection Complex I briefly in a microcentrifuge before use.
2. Dilute Detection Complex I 1:1500 using Detection Complex Dilution Buffer. Because assays involving 6 strips/48 wells or less require very small volumes of this reagent, we recommend using a two-step process to achieve the final 1:1500 dilution:
   - 2.1 Dilute 1:10 by mixing 2 µl Detection Complex I with 18 µl Detection Complex Dilution Buffer.
   - 2.2 Dilute this dilution 1:150 according the following table:

# Strips/Wells	Volume of Detection Complex I	Volume of Detection Complex Dilution Buffer
3 strips/24 wells	6.0 µl of 1:10 dilution	900.0 µl
6 strips/48 wells	12.0 µl of 1:10 dilution	1.8 ml
9 strips/72 wells	1.8 µl of stock	2.7 ml
12 strips/96 wells	2.4 µl of stock	3.6 ml

3. Add 30 µl of diluted Detection Complex I to each well of the SureScore™ strip-well plate. Cover the plate with plastic wrap or aluminum foil to prevent evaporation, and incubate at room temperature for 30 minutes.
4. Wash each well *six times* with 200 µl 1X Wash Solution. Remove the wash solution *as completely as possible* after each wash. We recommend removing the liquid by aspiration or by vigorous tapping of the plate upside-down on paper towels.
5. Add 100 µl of undiluted Detection Substrate I to each well. Cover the plate and incubate at room temperature for 30 minutes on a rotary shaker. The shaker speed should provide sufficient mixing without splashing. Monitor the wells containing XX and XY Control Template for development of a **yellow color**. Note that different wells may have different color intensities.
6. Read the plate visually or using a plate reader at a 405 nm wavelength. **For best results, plates should be read immediately following the 30-minute incubation period.** The plate reader must be able to read plates with the footprint of Nunc™ 96-well plates.
7. After you have read the plate, wash each well *three times* with 200 µl 1X Wash Solution. Remove the wash solution *as completely as possible* after each wash. We recommend removing the liquid by aspiration or by vigorous tapping of the plate upside-down on paper towels.

Step 8: Detecting 620 Signals

Overview

In this step, you detect the presence of the "Y" labeled terminating nucleotide (see page 9) on the extended SNP-IT™ Capture Primer using an indirect colorimetric assay. The color of this reaction is blue, and can be detected with a plate reader at a 620–650 nm wavelength.

Detecting 620 Signals

1. Spin the tube containing Detection Complex II briefly in a microcentrifuge before use.
2. Dilute Detection Complex II 1:4000 using Detection Complex Dilution Buffer. Because assays involving 6 strips/48 wells or less require very small volumes of this reagent, we recommend using a two-step process to achieve the final 1:4000 dilution:
   - 2.1 Dilute 1:20 by mixing 2 µl Detection Complex II with 38 µl Detection Complex Dilution Buffer.
   - 2.2 Dilute this dilution 1:200 according the following chart:

# Strips/Wells	Volume of Detection Complex II	Volume of Detection Complex Dilution Buffer
3 strips/24 wells	4.5 µl of 1:20 dilution	900.0 µl
6 strips/48 wells	9.0 µl of 1:20 dilution	1.8 ml
9 strips/72 wells	13.5 µl of 1:20 dilution	2.7 ml
12 strips/96 wells	18.0 µl of 1:20 dilution	3.6 ml

3. Add 30 µl of diluted Detection Complex II to each well of the SureScore™ strip-well plate, cover the plate with plastic wrap or aluminum foil to prevent evaporation, and incubate at room temperature for 30 minutes.
4. Wash each well *six times* with 200 µl 1X Wash Solution. Remove the wash solution *as completely as possible* after each wash. We recommend removing the liquid by aspiration or by vigorous tapping of the plate upside-down on paper towels.
5. Add 100 µl of undiluted Detection Substrate II to each well. Cover the plate and incubate at room temperature for 30 minutes on a rotary shaker. The shaker should provide sufficient mixing without splashing. Monitor the wells containing YY and XY Control Template for development of a **blue color**. Note that different wells may have different color intensities.
6. Read the plate visually or on a plate reader at a 620–650 nm wavelength. **This reaction substrate degrades quickly over time, and the plates should be read *immediately* following the 30-minute incubation period.** The plate reader must be able to read plates with the footprint of Nunc™ 96-well plates.

Data Analysis

SureScore™ Data Analysis Software

The SureScore™ Data Analysis Software automates the process of analyzing SureScore™ data collected by a plate reader. This software is intended solely for research use by Invitrogen's SureScore™ customers, and can be downloaded for free from the Web site listed below.

To use the software, first you read the optical density (OD) values of the wells in the plate at each wavelength (405 nm and 620–650 nm). Then you copy and paste the data directly from the plate reader software into the SureScore™ Data Analysis Software. The software is a Microsoft® Excel workbook with built-in calculation functions to define high/low cutoff values for genotype calling, automatically identify the genotype of each sample, create scatter plots, perform cluster analysis, and generate data reports.

To download the software, go to www.invitrogen.com/SureScore and follow the download instructions. See the SureScore™ Data Analysis Software manual for additional information.

Troubleshooting

Problem	Possible Cause	Potential Solution
No signal observed in the samples AND in the positive controls	Procedures were not followed accurately	Repeat the assay procedures, following all steps as described.
	Hybridization buffer was not used, or another buffer was substituted	Repeat the assay using the hybridization buffer.
	Extension did not occur	Confirm that the extension reaction was performed at room temperature for 30 minutes.
		Confirm that the appropriate extension buffer was freshly prepared, correctly diluted, and kept away from light. Repeat the assay with freshly prepared buffer.
	SNP-IT™ Capture Primers failed to bind to the SureScore™ plates	Ensure that the SNP-IT™ Capture Primers were diluted to the appropriate concentration in attachment buffer. This buffer is specially formulated to facilitate noncovalent attachment of the primer to the plates.
No signal in the samples, but the positive controls worked	PCR products were placed in the wrong SureScore™ strip-well plate wells	Repeat the assay.
	Wrong extension mix used	Confirm that you are using the correct extension mix for your SNP and primers, and repeat the assay.
	Incomplete Exonuclease digestion	Ensure that the enzyme is still active by assaying for the loss of double-stranded PCR product by gel electrophoresis after digestion by Exonuclease. Then repeat the assay.
	Wrong template strand was digested	Confirm that the correct primer was phosphorothioated and protected from Exonuclease digestion, and repeat the assay.
	PCR failed	Check PCR reactions using gel electrophoresis to detect 80–175-bp DNA fragments prior to running the SureScore™ assay. If the products are not readily visible, try optimizing the PCR reagents and conditions.
	DNA quality is poor	Ensure that the template DNA is of high purity by checking the A_{260}/A_{280} ratio, and check the overall integrity of the genomic DNA samples by gel electrophoresis to evaluate for possible degradation. Then repeat the assay.

Continued on next page

Troubleshooting, Continued

Problem	Possible Cause	Potential Solution
Both the negative PCR controls and negative SNP-IT™ Capture Primer controls produce a strong background signal	NaOH wash was skipped or performed incorrectly.	NaOH removes template DNA from the SNP-IT™ Capture Primer, leaving only the extended SNP-IT™ Capture Primer and reducing background signal. Repeat the assay, making sure to perform this step correctly.
	Insufficient or improper washing of plates	Repeat the assay. Be sure to perform all the wash steps and remove as much wash solution as possible from the wells between steps. We strongly recommend the use of aspirator.
	Template independent background signal. This is usually due to complementarity between the 3′ end of the SNP-IT™ Capture Primer and another region of the primer, leading to self-extension of the SNP-IT™ Capture Primer and associated signal production.	Although the genotype data/clusters may still look relatively good in some of these cases, it is a good idea to redesign the SNP-IT™ Capture Primers, preferably for the opposite strand. This will ensure full accuracy of the genotyping data.
Only the negative PCR control gives a positive signal	DNA contamination	Repeat the PCR reaction with fresh reagents and a new stock of DNA.
Signal is observed for only one color in the assay	Detection reagent was improperly diluted or has degraded.	Make sure that all detection reagents are properly diluted and have been kept away from light, and repeat the detection step.
	A colorimetric detection step was skipped or not performed properly.	Repeat the assay and ensure that all steps are followed correctly.
Some samples work well while others do not show any signal or give low signal	Poor sample quality	Check the PCR product of the failed samples using gel electrophoresis. If no band is visible, check on the purity of the failed samples by measuring the A_{260}/A_{280}. If necessary, re-purify the DNA by phenol/chloroform extraction or any alternate method. If possible, obtain new DNA from the source. If a PCR band is visible, adjust the amount of input DNA in the SureScore™ assay. Note that the amount of input DNA required is *highly* dependent on the individual SNP. Some SNPs may require a higher concentration of input DNA than recommended in the protocol, while others may require a lower concentration.
Weak signal is observed in both samples and positive controls	Insufficient washing of the plates	Repeat the assay. Be sure to perform all the wash steps and remove as much wash solution as possible from the wells between steps. We strongly recommend the use of an aspirator.

Frequently Asked Questions

How much DNA template do I need in the PCR reaction?	As a starting point, we recommend using between 2 and 20 ng of template DNA per 50 µl of PCR reaction. This is also dependent on the number of PCR cycles. We have observed that 10 ng/50 µl for 36 cycles works well in most cases. However, the concentration of template DNA that is necessary for successful PCR can vary widely, depending on the specific PCR Primers utilized and the target DNA sequence being amplified. Additionally, it is important that the purity of the template DNA is good. This DNA should also be devoid of any potential PCR inhibitors.
Should I run the assay if I do not see a band for the PCR product on the gel?	It is always prudent before running the SureScore™ assay to ensure that the appropriate sized PCR-amplified product is present. In those cases where no PCR product can be visualized by gel electrophoresis, you should use appropriate controls to determine whether the PCR reaction failed. In some tests, no PCR product was visualized by electrophoresis but proper controls indicated that the PCR reactions were successful, and the PCR product still worked well in the SureScore™ assay. This indicates that some SNPs require very little sample for the SureScore™ assay to be successful. If you decide to proceed with PCR product that cannot be visualized, you should include another sample that you know will work as an additional positive control in the SureScore™ assay.
How long are the SureScore™ plates stable after the SNP-IT™ Capture Primers have been attached to the wells?	It is preferable to use freshly prepared SureScore™ plates whenever possible for the assay. However, storage of SureScore™ plates with attached primers is possible using the following protocol: • Wash wells three times with 100 µl/well of 1X Wash Buffer. • Wash wells once with 30 µl of 1X TE/10% Isopropanol solution. Remove as much liquid as possible from wells. • Place plate in 50ºC oven for 30 minutes. • Ensure that wells are completely dry before placing into foil or plastic bags containing a desiccant pack and seal. Plates may be stored at room temperature for up to one year in the sealed bag.
How long can I keep the plates after each colorimetric detection step before I actually analyze the results?	We strongly recommend that you analyze the results immediately after the color development step to ensure that the most accurate genotype calls can be made. The yellow reaction product is relatively stable and can be analyzed up to 2 hours after substrate incubation. However, the blue reaction product is much less stable and should be scored *immediately* without exception.
What is the optimum number of samples for the SureScore™ assay?	We recommend using at least 20 samples for unconfirmed SNPs to obtain a sufficient number of data points. This number can be reduced if internal sample controls (samples with known genotypes) are available and run in the assay. These samples can then be used as a reference for assigning genotypes to the unknown samples.

Continued on next page

Frequently Asked Questions, Continued

What if one of the clusters seems to be shifting into another or the clusters are very close to each other? **OR** **What if homozygous controls look like heterozygotes in the assay?**	Both observations are usually due to template-dependent noise. This happens when the SNP-IT™ Capture Primer anneals to more than one site on the single-stranded PCR product. This leads to multiple extensions occurring at different sites and often produces a significant level of background in the SureScore™ assay. This is commonly observed as a shift towards heterozygotes in one or more of the genotype clusters. Since this is template specific, it is usually best to choose an alternate design for the assay primers.
What if I see a split cluster on either axis in the scatter plot?	This usually occurs if an alternate SNP site is present in the single-stranded target sequence, in the region complementary to the SNP-IT™ Capture Primer. This potential single-base mismatch at the alternate SNP site may cause inefficient SNP-IT™ Capture Primer/template hybridization in some of the samples. Even though the extension step occurs, the signal is weaker in these samples and will show up as a distinct cluster in the scatter plot. This can be overcome by redesigning the SNP-IT™ Capture Primer for the opposite template strand on the other side of the SNP, so that the alternate SNP site is avoided altogether at the SNP-IT™ Capture Primer annealing step.

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Technical Service

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Corporate Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
tech_service@invitrogen.com

Japanese Headquarters:
Invitrogen Japan K.K.
Nihonbashi Hama-Cho Park
Bldg. 4F
2-35-4, Hama-Cho, Nihonbashi
Tel: 81 3 3663 7972
Fax: 81 3 3663 8242
E-mail: jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tel (Toll Free): 0800 5345 5345
Fax: +44 (0) 141 814 6117
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