applied biosystems

TaqMan® SNP Genotyping Assays

USER GUIDE

Single-tube assays

for use with:

TaqMan® Predesigned SNP Genotyping Assays

TaqMan® Drug Metabolism Enzyme Genotyping Assays

TaqMan® Custom SNP Genotyping Assays

Publication Number MAN0009593

Revision B.0

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Manufacturer: Multiple Life Technologies Corporation manufacturing sites are responsible for manufacturing the products associated with the workflow covered in this guide.

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The information in this guide is subject to change without notice.

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Revision history: Revision history of Pub. No. MAN0009593

Revision	Date	Description
B.0	29 September 2017	• Updates to supported instruments, required materials, and document delivery information • Added information about TaqMan® QSY™ probes • Updates to analysis options • Corrections to instrument accessories • Updates to Troubleshooting • Updated to the current template, with associated updates to warranty, trademarks, and logos
A.0	30 January 2014	New document

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

TaqMan® SNP Genotyping Assay overview

Applied Biosystems™ TaqMan® SNP Genotyping Assays use TaqMan® 5′-nuclease chemistry for amplifying and detecting specific polymorphisms in purified genomic DNA samples. Each assay allows genotyping of individuals for a single nucleotide polymorphism (SNP). All assays are developed using our bioinformatics assay design processes that apply heuristic rules that are deduced from both manufacturing and assay performance data.

Each TaqMan® SNP Genotyping Assay contains:

- Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest
- Two TaqMan® minor groove binder (MGB) probes with nonfluorescent quenchers (NFQ):
  - One VIC™-labeled probe to detect Allele 1 sequence
  - One FAM™-labeled probe to detect Allele 2 sequence

MGB probes at the 3' end bind to the DNA helix minor groove, improving hybridization-based assays by stabilizing the MGB probe-template complex. This increased binding stability allows the use of probes as short as 13 bases for superior allelic discrimination and assay design flexibility. All MGB probes also include a nonfluorescent quencher (NFQ) that virtually eliminates background fluorescence and provides excellent signal-to-noise ratio for superior assay sensitivity.

TaqMan® Assays QPCR Guarantee (see "TaqMan® Assays qPCR Guarantee" on page 68) ensures performance and satisfaction with results that are obtained with all Predesigned TaqMan® SNP Genotyping Assays and TaqMan® Drug Metabolism Genotyping Assays (DME).

Context sequence representation

Reporter dye information for the TaqMan® SNP and DME Genotyping Assays are represented in the assay context sequence. The context sequence is the nucleotide sequence surrounding the SNP site and is provided in the (+) genome strand orientation relative to the NCBI reference genome. The SNP alleles are included in brackets, where the order of the alleles corresponds to the association with probe reporter dyes, where [Allele 1 = VIC™ dye / Allele 2 = FAM™ dye].

If the context sequence is `...XXXXX[A/B]XXXXX...`:

- A allele *always* represents the VIC™ dye
- B allele *always* represents the FAM™ dye

Table 2 Example allele-to-dye associations

Context sequence clip	SNP alleles	VIC™-associated allele	FAM™-associated allele
`...CCCACCCTTC[G/A]ACACTATTAC...`	`[G/A]`	G	A
`...ATTAACCCAT[T/C]AGTGATGGGG...`	`[T/C]`	T	C
`...AAGCAACTAT[G/A]TTCATAACTT...`	`[G/A]`	G	A
`...AACCGTGTGA[T/C]GGCAGTGATT...`	`[T/C]`	T	C

The context sequence for your assay is provided in the Assay Information File, available at **thermofisher.com/taqmanfiles**.

Compatible master mixes

All TaqMan® SNP Genotyping Assays are optimized for use with TaqMan® Genotyping Master Mix and require only three components for PCR.

- 1–20 ng purified genomic DNA per well (final concentration: ≥0.2 ng/µL)
- 20X, 40X, or 80X TaqMan® SNP Genotyping Assay (depending on product and assay scale)
- TaqMan® Genotyping Master Mix, or another compatible master mix

Table 3 Compatible master mixes for use with TaqMan® SNP Genotyping Assays

Master mix	Guidelines for use
TaqMan® Genotyping Master Mix	Recommended master mix for use with DNA samples not prepared with TaqMan® Sample-to-SNP™. Use standard thermal cycling conditions only.
TaqMan® GTXpress™ Master Mix[1]	Recommended master mix for use with DNA samples prepared with TaqMan® Sample-to-SNP™. Fast thermal cycling conditions are not recommended for TaqMan® DME SNP Genotyping Assays due to the overall longer length of many DME amplicons.
TaqMan® Universal Master Mix II	TaqMan® Genotyping Master Mix is recommended over TaqMan® Universal Master Mix II due to overall better performance and stronger signal.
TaqPath™ ProAmp™ Master Mix	Recommended for samples with PCR inhibitors such as heparin and hematin. Not recommended for use with DME and CFTR TaqMan® SNP Genotyping Assays due to high background signal associated with some sensitive assays in these collections.

[1] Provided with the TaqMan® Sample-to-SNP™ Kit

Predesigned TaqMan® SNP Genotyping Assays

The Predesigned TaqMan® SNP Genotyping Assays collection includes millions of genome-wide human assays:

- Common 1,000 Genome SNPs—The 1,000 Genomes Project created the largest and most detailed catalog of human variation and genotypes.
- HapMap SNPs—SNPs genotyped by the HapMap Project across several populations to develop a haplotype map of the human genome.
- Coding SNP assays—Assays for the detection of informative and putative functional, nonsynonymous cSNPs in gene-coding regions.

The library also includes over 10,000 predesigned mouse assays. For targets of interest that are not covered by the predesigned assays collections, Custom TaqMan® SNP Genotyping Assays are available (see "CustomTaqMan® SNP Genotyping Assays" on page 9).

Predesigned TaqMan® SNP Genotyping Assays are made-to-order, available in multiple scales, and available in the following types:

- **Validated**—Validated assays are tested using genomic DNA from 45 individuals from each of four ethnic populations (African American, Caucasian, Chinese, and Japanese). The minor allele frequency (MAF) for validated assays is >5% in at least one test population. MAF data is available on the product web page and in the order Assay Information Files (AIF).
- **Functionally Tested**—Functionally Tested assays meet minimum functionality criteria in testing of 20 genomic DNA samples from three ethnic populations (African American, Caucasian, and Asian) and both genders. Minor allele frequency is not calculated. To ensure assay amplification and sample clustering, all human SNP genotyping assays undergo functional testing before shipment of the first order. Quality of repeat orders is verified by mass spectrometry.
- **Qualified**—Qualified Assays are pre-tested using cell line and tissue genomic DNA and/or synthetic templates to meet specific functionality requirements.
- **DME**—See "TaqMan® Drug Metabolism Genotyping Assays" on page 9.

See **thermofisher.com/taqmansnp** for more information.

TaqMan® Drug Metabolism Genotyping Assays

TaqMan® Drug Metabolism Genotyping Assays (DME Genotyping Assays) offer a comprehensive collection of inventoried assays that are optimized for genotyping SNPs, insertions and deletions (indels), and multi-nucleotide polymorphisms (MNPs) in drug metabolism-related genes. TaqMan® DME Genotyping Assays are available in small-scale and are inventoried for fast availability.

TaqMan® DME Genotyping Assays detect potentially causative polymorphisms in 221 drug metabolism enzyme and associated transporter genes. Assays are tested using genomic DNA from 45 individuals from each of four ethnicities. The minor allele frequency for each population is available, but rare and mutant alleles may not be present in the test DNA.

See **thermofisher.com/taqmandme** for more information.

Custom TaqMan® SNP Genotyping Assays

Create Custom TaqMan® SNP Genotyping Assays by submitting confidential target sequences to our secure assay design pipeline using the Custom TaqMan® Assay Design Tool (**thermofisher.com/taqmansnpdesign**). All human SNP genotyping assays are functionally tested using 20 unique genomic DNA samples to ensure assay amplification and sample clustering. Custom assays are designed, synthesized, formulated, optimized, and quality control tested. See **thermofisher.com/taqmancustomsnp** for more information.

Custom TaqMan® SNP Genotyping Assays allow you to:

- Perform genotyping studies with any possible SNP in any organism.
  For example, AGTTCATCCATGGTCA ▶ AGTTCATACATGGTCA, annotated as AGTTCAT[C/A]CATGGTCA.
- Detect indels of up to six bases for genotyping studies.
  For example, AGTTCATCCATGGTCA ▶ AGTTCATGGTCA, annotated as AGTTCAT[CCAT/*]GGTCA.
- Detect MNPs of up to six bases for genotyping studies.
  For example, AGTTCATCCATGGTCA ▶ AGTTCATATATGGTCA, annotated as AGTTCAT[CC/AT]ATGGTCA.

For maximal efficiency in a multiplex format, Custom TaqMan® SNP Genotyping Assays are available with TaqMan® probes using a QSY™ quencher. QSY™ probes can be ordered with FAM™ and VIC™ dyes and with ABY™ and JUN™ dyes, allowing amplification of up to four targets in a single reaction. These reporter dyes are optimized to work together with minimal spectral overlap for optimal performance. The QSY™ quencher is fully compatible with probes that have MGB quenchers. See **thermofisher.com/multiplexqpcr** for compatible master mixes and ordering information.

SNP primer and probe sequences can also be submitted directly to the Custom TaqMan® Assay Design Tool to manufacture a known assay sequence.

Assay format, size, and storage

Predesigned assays: single-tube

For the list of available Predesigned TaqMan® SNP Genotyping Assays, visit **thermofisher.com/taqmansnp**.

Search the assay database by:

- Gene name
- Gene symbol
- SNP ID
- Assay ID
- Genomic location

Table 4 Predesigned TaqMan® SNP Genotyping Assays: single-tube

Scale	Number of reactions			Assay mix formulation	Cat. No.	
	384-well[1]	96-well Fast[2]	96-well[3]		Human	Non-human[4]
Small	1,500	750	300	40X	4351379	4351384
Medium	5,000	2,500	1,000	40X	4351376	4351382
Large	12,000	6,000	2,400	80X	4351374	4351380

[1] 5-µL reaction volume
[2] 10-µL reaction volume
[3] 25-µL reaction volume
[4] Non-human TaqMan® Predesigned SNP Genotyping Assays are designed to amplify and detect specific polymorphisms in specified non-human genomic DNA samples.

DME assays: single-tube

For the list of inventoried TaqMan® DME Genotyping Assays, visit **thermofisher.com/taqmandme**.

Table 5 TaqMan® DME Genotyping Assays: single-tube

Scale	Number of reactions		Assay mix formulation	Cat. No.	
	384-well[1]	96-well[2]		Human	Non-human[3]
Small	750	150	20X	4362691	—

[1] 5-µL reaction volume
[2] 25-µL reaction volume
[3] Non-human TaqMan® Predesigned SNP Genotyping Assays are designed to amplify and detect specific polymorphisms in specified non-human genomic DNA samples.

TaqMan® DME Genotyping Assays can also be ordered through the DME Index (**thermofisher.com/taqmandme**). The DME Index contains a comprehensive list of the DME assays with annotations that include:

- The gene to which the polymorphism location is mapped
- Allele nomenclature (from public allele nomenclature sites, when available)
- Polymorphism (for example, A/G)
- Context sequence
- Amino acid change (if applicable)
- Thermo Fisher Scientific MAF data

Custom assays: single-tube

Custom TaqMan® SNP Genotyping Assays are designed, synthesized, and receive analytical quality control based on sequence information supplied. All information supplied is secure and confidential. For more information, visit **thermofisher.com/taqmancustomsnp**.

Note: For detailed instructions on how to design and order Custom TaqMan® SNP Genotyping Assays, consult the *Custom TaqMan® Assays Design and Ordering Guide* (Pub. No. 4367671). Custom SNP Assays are designed using the Custom Assay Design Tool (CADT)(**thermofisher.com/taqmansnpdesign**). This tool may also be used to upload assays that have already been designed outside the system.

Table 6 Custom TaqMan® SNP Genotyping Assays: single-tube

Scale	Number of reactions			Assay mix formulation	Cat. No.	
	384-well[1]	96-well Fast[2]	96-well[3]		Human	Non-human[4]
Small	1,500	750	300	40X	4331349	4332077
Medium	5,000	2,500	1,000	40X	4332072	4332075
Large	12,000	6,000	2,400	80X	4332073	4332076

[1] 5-µL reaction volume
[2] 10-µL reaction volume
[3] 25-µL reaction volume
[4] Non-human TaqMan® Predesigned SNP Genotyping Assays are designed to amplify and detect specific polymorphisms in specified non-human genomic DNA samples.

Plate products and formats

For projects that require analyzing many samples or for evaluating samples for many SNP assays can be ordered in plated formats.

Table 7 TaqMan® SNP Genotyping Assays plate products and formats

Format	Source
OpenArray™ plates[1]	**thermofisher.com/openarray**
96- or 384-well plates (fast or standard)	**thermofisher.com/customplating**

[1] For use with the QuantStudio™ 12K Flex Real-Time PCR System.

Shipment contents

Single-tube shipments include:

- Dispatch Note (Packing List)—Contains your order information, a list of the assays that were ordered, and the quantity of the assays shipped.
- Assay tube(s)—Each TaqMan® SNP Genotyping Assay tube is identified with a label and a 2-D barcode.
- Data Sheet—Provides a summary of the assays in your shipment, the assay details, and the storage conditions.

Plate shipment contents depend on the assay format. Regardless of format, each shipment includes:

- Assay plate(s) identified with a label, including 2-D barcodes, on the bottom.
- Data Sheet—Provides a summary of the assays in your shipment, the assay details, and the storage conditions.

Go to **thermofisher.com/taqmanfiles** to download the following supporting documents.

- Assay information files (AIFs)
- User Instruction Documents (Protocols, User Guides, and Quick Reference Cards)
- Certificates of Analysis
- Safety Data Sheets

For detailed information about the shipment and assay information files (AIFs), see *Understanding Your Shipment* (Pub. No. MAN0017153).

Storage and stability

Note: For plate formats, see the documentation included with your shipment for storage and stability information.

- Store single-tubeTaqMan® SNP Genotyping Assays at –25 to –15°C in the dark.

  IMPORTANT! Protect TaqMan® Assays from direct exposure to light. Excessive exposure to light may affect the fluorescent probes.

- Do not perform more than 10 freeze-thaw cycles. If you expect to freeze-thaw TaqMan® Assays more than three times, consider aliquoting to minimize the number of freeze-thaw cycles.
- We recommend diluting the 40X and 80X predesigned and CustomTaqMan® SNP Genotyping Assays to a 20X working stock (see "Dilute, then dispense aliquots of TaqMan® SNP Genotyping Assays" on page 13).
- TaqMan® SNP Genotyping Assays are stable for up to 5 years past their manufacturing date. The manufacturing date is printed on each assay tube or plate.

Dilute, then dispense aliquots of TaqMan® SNP Genotyping Assays

We recommend diluting TaqMan® SNP Genotyping Assays to a 20X working stock, then dispensing aliquots into tubes for routine use, to minimize freeze-thaw cycles and exposure to light.

1. Dilute 40X or 80X TaqMan® SNP Genotyping Assays to a 20X working stock with 1X TE buffer.

   Note: 1X TE buffer composition: 10-mM Tris-HCl, 1 mM EDTA, pH 8.0, in DNase-free, sterile-filtered water.

2. Vortex, then centrifuge the mixture.

3. Store multiple aliquots of the TaqMan® SNP Genotyping Assays at –25 to –15°C in the dark.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Table 8 Kits for genomic DNA extraction

Item	Sample type	Source
Kits for genomic DNA extraction	Select the appropriate kit for your sample type.	thermofisher.com/gdnaprep
TaqMan® Sample-to-SNP™ Kit[1]	• Buccal samples • Blood • Cells cultures • Plant samples • Tissue	4403081

[1] We recommend pre-amplifying all samples prepared with the TaqMan® Sample-to-SNP™ Kit when using OpenArray™ for subsequent genotyping.

Table 9 Supported instruments

Item	Block modules	Source
Veriti™ Thermal Cycler[1]	96-well, 96-well Fast, 384-well, and 8-strip tubes	Contact your local sales office.
GeneAmp™ PCR System 9700[1]	96-well, 2 × 96-well, 2 × 384-well, and 8-strip tubes	
ProFlex™ PCR System[1]	3 × 32-well, 96-well, 2 × 96-well, 2 × 384-well, 2 × Flat, and 8-strip tubes	
SimpliAmp™ Thermal Cycler[1]	96-well and 8-strip tubes	
7500 Real-Time PCR System	96-well and 8-strip tubes	
7500 Fast Real-Time PCR System	96-well and 8-strip tubes	
7900HT Fast Real-Time PCR Instrument	96-well, 96-well Fast, and 384-well	
StepOne™ Real-Time PCR System/StepOnePlus™ Real-Time PCR System	StepOne™ 48-well and 8-strip tubes	
ViiA™ 7 Real-Time PCR System	96-well, 96-well Fast, 384-well, and 8-strip tubes	
QuantStudio™ 3 Real-Time PCR System	96-well, 96-well Fast, and 8-strip tubes	
QuantStudio™ 5 Real-Time PCR System	96-well, 96-well Fast, 384-well, and 8-strip tubes	
QuantStudio™ 6 Flex Real-Time PCR System	96-well, 96-well Fast, 384-well, and 8-strip tubes	

Item	Block modules	Source
QuantStudio™ 7 Flex Real-Time PCR System	96-well, 96-well Fast, 384-well, and 8-strip tubes	Contact your local sales office.
QuantStudio™ 12K Flex Real-Time PCR System	96-well, 96-well Fast, 384-well, OpenArray™, and 8-strip tubes	

[1] If a thermal cycler is used for PCR amplification, the optional pre-read and the post-read must be performed separately on a real-time PCR system in order to detect and record fluorescent signals.

Table 10 Equipment, tubes, plates and consumables

Item	Source
Equipment	
Centrifuge with plate adapter	MLS
Microcentrifuge	MLS
Adjustable pipettors	MLS
Laboratory mixer (vortex or equivalent)	MLS
Tubes, plates, and other consumables	
RNase-Free Microfuge Tubes (1.5 mL)	AM12400
Plastics consumables[1]	**thermofisher.com/plastics**
Pipette tips	**thermofisher.com/pipettetips**
Disposable gloves	MLS

[1] Click **Plastics selection guide** to select the appropriate plates and seals for use with your instrument.

Table 11 Reagents for PCR

Item	Source
Master mix, one of the following[1]:	
TaqMan® Genotyping Master Mix (2X)	4371355
TaqMan® Sample-to-SNP™ Kit[2]	4403081
TaqMan® Universal Master Mix II	4440038
TaqMan® GTXpress™ Master Mix	4401892
TaqPath™ ProAmp™ Master Mix	A30866
Additional reagents	
Nuclease-Free Water (not DEPC-Treated)	AM9938

[1] See "Compatible master mixes" on page 8

[2] TaqMan® Sample-to-SNP™ Kit contains all necessary reagents for sample preparation and amplification, including TaqMan® GTXpress™ Master Mix.

Workflow

Start with high-quality gDNA, quantified 18

▼

Set up the PCR reactions: wet DNA method (page 19)	OR	**Set up the PCR reactions: dried-down DNA method (page 21)**

▼

(Recommended) Perform a pre-PCR plate read (page 22)

▼

Perform PCR (page 22)

▼

Perform the post-PCR plate read and analysis (page 23)

2 Methods

Procedural guidelines

- Store TaqMan® SNP Genotyping Assays frozen and away from light until use. Excessive exposure to light may affect the fluorescent probes.
- Do not perform more than 10 freeze-thaw cycles.
- To ensure optimal analysis and troubleshooting of the TaqMan® SNP Genotyping Assays, prepare an optical reaction plate containing the following for each assay.

Table 12 Recommended reaction types for each assay

Reaction type	Component
Test sample	DNA samples with unknown genotype at the polymorphism of interest
No-template control[1]	DNase-free water
(Optional) Positive control	DNA sample with known genotype at the polymorphism of interest

[1] Use two no-template controls per assay to allow the genotyping algorithm to correct for background signal from fluorescent probes and to enable the detection of DNA contamination.

- Mix thoroughly after adding reagents to DNA samples to avoid stratification of the reagents and/or air bubbles in the well, which can lead to "stringy" clusters (see Appendix A, “Troubleshooting“).
- This procedure is optimized for TaqMan® Genotyping Master Mix. Use the appropriate thermal cycling conditions for your assay type and the master mix used.

Table 13 Alternative thermal cycling conditions

Assay or master mix	Thermal cycling conditions
TaqMan® DME Genotyping Assays	*(Recommended)* Use standard mode thermal cycling conditions and a longer extension time with additional cycles due to the longer average amplicon lengths.
TaqMan® Genotyping Master Mix	Use standard mode thermal cycling conditions only.
Other compatible master mix[1]	Use the protocol that is provided with the master mix. For TaqMan® DME Genotyping Assays, use standard mode thermal cycling conditions only.

[1] See “Compatible master mixes“ on page 8.

- See “Good laboratory practices for PCR and RT-PCR“ on page 63 for additional guidelines.

Before you begin

Extract and purify genomic DNA

Extract and purify genomic DNA (gDNA) according to standard practices. For recommended kits and ordering information, see Table 8 on page 14.

Quantify the sample gDNA

Use one of the following methods to quantify sample gDNA before using TaqMan® SNP Genotyping Assays:

- UV/Vis spectrophotometry [A_{260} /A_{280} measurement (see "UV spectroscopy" on page 62)
- NanoDrop™ spectrophotometry
- RNase P method (see "Absolute quantification" on page 63)
- Qubit™ 3.0 Fluorometer (Cat. No. Q33216) with Qubit™ Assay Tubes (Cat. No. Q32856) and Qubit™ dsDNA HS Assay Kit (Cat. Nos. Q32851 and Q32854)

See "DNA quantification" on page 61 for more information.

Prepare assays, DNA samples, and master mix

- Dilute the 40X or 80X Predesigned and Custom TaqMan® SNP Genotyping Assays to a 20X working stock solution (see "Dilute, then dispense aliquots of TaqMan® SNP Genotyping Assays" on page 13).

  Note: TaqMan® DME Genotyping Assays are supplied at a 20X concentration.

- Before each PCR run:
  - Resuspend the assay by vortexing, then centrifuge the tube briefly.
  - *(For wet DNA only)* Resuspend thawed frozen DNA samples by vortexing, then centrifuge the tubes briefly.
  - Thoroughly mix the TaqMan® Genotyping Master Mix by swirling the bottle.

Set up the PCR reactions

Select the DNA delivery method

TaqMan® SNP Genotyping Assays can be used with either wet or dried-down DNA. Drying down the gDNA in plates is the most convenient method for an experiment that requires multiple plates using the same gDNA or for multiple experiments that use the same gDNA.

Note: For both methods, it is recommended to use a calibrated, positive-displacement pipette to minimize contamination and error.

Table 14 DNA delivery methods

Method	Description	Experimental uses
Wet DNA delivery	1. SNP reaction mix is dispensed to an optical reaction plate. 2. gDNA is delivered to the final reaction mix. **Note:** In this method the DNA is already dissolved in buffer or water and is used as a component in the final reaction.	Large number of DNA templates tested on few SNP targets.
Dried-down DNA delivery	1. gDNA is applied to the bottom surface of an optical reaction plate. 2. DNA sample is dried down completely by evaporation. 3. SNP reaction mix is added and the DNA disperses in the final reaction mix.	• Low DNA concentration results in large sample volumes (2–5 µL) to run the assay. • Limited number of DNA templates tested repeatedly on different SNP targets. • Large number of DNA samples prepared in plates, dried down, and stored before use.

Set up the PCR reactions: wet DNA method

IMPORTANT! This procedure is optimized for TaqMan® Genotyping Master Mix. See Table 13 on page 17 for alternative master mixes and plate and cycling conditions.

Prepare the reaction mix: wet DNA method

1. Calculate the number of reactions to be performed for each assay, including recommended controls (see Table 12 on page 17).

   Note: Multiple assays can be run on one reaction plate. Controls for each assay must be included on the same plate.

2. Combine the following components for the number of reactions required, plus 10% overage.

Table 15 Reaction mix for wet DNA method

Component	384-well (5-µL reaction)	96-well Fast[1] (10-µL reaction)	96-well (25-µL reaction)
2X TaqMan® Master Mix	2.50 µL	5.00 µL	12.50 µL
20X Assay Working Stock	0.25 µL	0.50 µL	1.25 µL
Nuclease-Free Water	—	—	—
Total volume per well	**2.75 µL**	**5.50 µL**	**13.75 µL**

[1] When using TaqMan® Genotyping Master Mix, use Fast reaction plates only on the QuantStudio™ 6 and 7 Flex, the QuantStudio™ 12K Flex, ViiA™ 7, or the 7500 Fast Real-Time PCR System and only with Standard thermal cycling conditions.

3. Vortex briefly to mix.

4. Centrifuge briefly to bring the reaction mix to the bottom of the tube and eliminate air bubbles.

Prepare the reaction plate: wet DNA method

1. Dilute each DNA sample, including controls, in Nuclease-Free Water to deliver 1–20 ng per well.

IMPORTANT! All wells using the same assay must contain similar amounts of sample. A final concentration of at least 0.2 ng/µL is recommended.

2. Add the appropriate volume of reaction mix to each well of the reaction plate (see Table 15 on page 20).

3. Seal the plate with adhesive film, then centrifuge briefly to bring the reaction mix to the bottom of the well and eliminate air bubbles.

   A non-optical seal can be used for this step.

4. Remove adhesive film from the plate, then add the appropriate volume of sample or control to the wells. Be sure to include wells for use as no template controls.

Reaction plate	DNA sample volume
MicroAmp™ Optical 384-Well Reaction Plate	2.25 µL
MicroAmp™ Fast Optical 96-Well Reaction Plate	4.50 µL
MicroAmp™ Optical 96-Well Reaction Plate	11.25 µL

IMPORTANT! Ensure that no cross-contamination occurs between wells.

5. Seal the plate with adhesive film, then centrifuge briefly to bring the reaction mix to the bottom of the well and eliminate air bubbles.

IMPORTANT! Use a MicroAmp™ Optical Film Compression Pad when using:

- MicroAmp™ Optical 96-Well Reaction Plate on the 7900HT Real-Time PCR Instrument
- MicroAmp™ Optical 96-Well Reaction Plate or MicroAmp™ Optical 384-Well Reaction Plate on the GeneAmp™ PCR System 9700

Set up the PCR reactions: dried-down DNA method

IMPORTANT! This procedure is optimized for TaqMan® Genotyping Master Mix. See Table 16 on page 21 for alternative master mixes and plate and cycling conditions.

Prepare the reaction mix: dried-down DNA method

1. Calculate the number of reactions to be performed for each assay, including recommended controls (see "Prepare assays, DNA samples, and master mix" on page 18).

   Note: Multiple assays can be run on one reaction plate. Controls for each assay must be included on the same plate.

2. Combine the following components for the number of reactions required, plus 10% overage.

   Table 16 Reaction mix for dried-down DNA method

Component	384-well (5-µL reaction)	96-well Fast[1] (10-µL reaction)	96-well (25-µL reaction)
2X TaqMan® Master Mix	2.5 µL	5.00 µL	12.50 µL
20X Assay Working Stock	0.25 µL	0.50 µL	1.25 µL
Nuclease-Free Water	2.25 µL	4.50 µL	11.25 µL
Total volume per well	**5.0 µL**	**10.0 µL**	**25.00 µL**

[1] When using TaqMan® Genotyping Master Mix, use Fast reaction plates only on the QuantStudio™ 6 and 7 Flex, the QuantStudio™ 12K Flex, ViiA™ 7, or the 7500 Fast Real-Time PCR System and only with Standard thermal cycling conditions.

3. Vortex briefly to mix.

4. Centrifuge briefly to bring the reaction mix to the bottom of the tube and eliminate air bubbles.

Prepare the reaction plate: dried-down DNA method

1. Add 2–5 µL of sample or control (1–20 ng of purified gDNA or no-template control consisting of Nuclease-Free Water) to each well of the reaction plate.
2. Dry the samples completely by evaporation at room temperature in a dark, amplicon-free location. Cover the plate with a lint-free tissue while drying.

   IMPORTANT! Do not accelerate drying by heating the plate. Heating the plate can cause poor gDNA recovery.

3. Add the appropriate volume of reaction mix to each well of the reaction plate (see Table 16 on page 21).
4. Seal the plate with optical adhesive film, then centrifuge briefly to bring the reaction mix to the bottom of the well and eliminate air bubbles.

   IMPORTANT! Use a MicroAmp™ Optical Film Compression Pad when using:

   - MicroAmp™ Optical 96-Well Reaction Plate on the 7900HT Real-Time PCR Instrument
   - MicroAmp™ Optical 96-Well Reaction Plate or MicroAmp™ Optical 384-Well Reaction Plate on the GeneAmp™ PCR System 9700

(Recommended) Perform a pre-PCR plate read

A pre-PCR plate read records the background fluorescence of each well of the plate before PCR. During the post-PCR plate read, the pre-read fluorescence is subtracted from the post-read fluorescence to account for pre-amplification background fluorescence, ensuring accurate results. If no pre-read data is available, no pre-read background subtraction is performed.

See the appropriate instrument user guide for instructions on how to use the system software to perform the pre-PCR plate read and analysis.

Perform PCR

IMPORTANT! This procedure is optimized for TaqMan® Genotyping Master Mix. See Table 13 on page 17 for alternative master mixes and plate and cycling conditions.

Note: *(Optional)* Perform PCR in real-time experiment mode. Real-time PCR allows observation of genotyping data over time to evaluate the accuracy of genotype calls by observing the location of a given sample relative to others throughout all cycles. If the quantity of DNA added is too high, resulting in merged genotyping clusters, the experiment can be reanalyzed at fewer cycles to recover genotyping accuracy.

See the appropriate instrument user guide for more information.

1. Program the instrument using the following conditions:

Step	Predesigned and Custom TaqMan® SNP Genotyping Assays			TaqMan® DME Genotyping Assays[1]		
	Temp.	Length	Cycles	Temp.	Length	Cycles
Polymerase activation	95°C	10 minutes	HOLD	95°C	10 minutes	HOLD
Denaturation	95°C	15 seconds	40	95°C	15 seconds	50
Annealing/ extension	60°C	1 minute		60°C	90 seconds	

[1] TaqMan® DME Genotyping Assays have a longer extension time with additional cycles due to the longer average amplicon lengths.

2. Select **Standard** run mode in the plate document or experiment file.

   IMPORTANT! Use only Standard Mode thermal cycling conditions with TaqMan® Genotyping Master Mix.

3. Enter the reaction volume.

Reaction plate	Reaction volume
MicroAmp™ Optical 384-Well Reaction Plate	5 µL
MicroAmp™ Fast Optical 96-Well Reaction Plate	10 µL
MicroAmp™ Optical 96-Well Reaction Plate	25 µL

4. Load the reaction plate, then start the run.

Perform the post-PCR plate read and analysis

Fluorescence measurements collected during the post-PCR plate read are used to plot the reporter signal normalized to the fluorescence signal of ROX™ (R_n) for each sample well. This data is used to then determine the genotypes present in the DNA samples.

To analyze data for allelic discrimination or genotyping:

1. Create a post-PCR plate document or experiment file.
2. Perform a post-PCR plate read on a real-time PCR instrument.

   Note: If no pre-read data is available, no pre-read background subtraction is performed.

3. Analyze the experimental data using the following:

Software	Features
Real-time instrument software	• Instrument software • View real-time trace data to aide genotype calling Data analysis varies depending on your real-time PCR system. See the instrument User Guide for more information.
TaqMan® Genotyper Software	• Desktop software • Create studies • Overlay data from multiple plates See "Data analysis with TaqMan® Genotyper Software" on page 24.
Thermo Fisher Cloud Genotyping Application	• Cloud software • Create studies • Overlay data from multiple plates • View real-time trace data to aide genotype calling See "Data analysis with the Thermo Fisher Cloud Genotyping Application" on page 25.

Note: TaqMan® Genotyper Software and the Thermo Fisher Cloud Genotyping Application use algorithms that yield more accurate genotype calls and quality control functionality than instrument analysis software.

4. Make automatic or manual allele calls. See "Allelic discrimination plots" on page 26 for more information.
5. Verify allele types.

Data analysis with TaqMan® Genotyper Software

TaqMan® Genotyper Software is a standalone software application that can be used to analyze raw data from genotyping experiments created on an Applied Biosystems™ Real-Time PCR system.

The TaqMan® Genotyper Software can be used to:

- Create a study:
  - Import multiple experiments into a single study
  - Import assay information files (`*.txt` or `*.xml`) to update assay information
  - Define analysis settings
  - Import Supplementary Sample Information (SSI) files to update sample information such as gender or population
  - Import reference panel files to add reference samples to a study
- Generate a study template, then use the study template to create new studies. The software analyzes the data according to the analysis settings that were defined in the study template.
- Analyze the study data using one of two call methods:
  - **Autocalling**—The software algorithm is used to call the data points.
  - **Classification Scheme**—You define the cluster boundaries that are used to call the data points.

- View the study results; for example, a summary of the Quality Control (QC) statistics at the study level, assay level, experiment level, and sample level.
- Export the following data:
  - Analysis results
  - Analysis settings
  - Audit trails
- Transfer studies from one TaqMan® Genotyper Software application to another.
- Ensure data security. The security feature allows you to:
  - Set security parameters to manage users (set up user accounts and assign user roles)
  - Track changes to the data

For more information on using TaqMan® Genotyper Software, see the *TaqMan® Genotyper Software Getting Started Guide* (Pub. No. 4448637).

Data analysis with the Thermo Fisher Cloud Genotyping Application

The Thermo Fisher Cloud Genotyping Application is a stand-alone software application that can be used to analyze raw data from genotyping experiments that are created on an Applied Biosystems™ real-time PCR system.

Use the Thermo Fisher Cloud Genotyping Application to:

- Create a study:
  - Import multiple experiments into a single study.
  - Import data from probes that are labeled with FAM™, VIC™, ABY™, or JUN™.
  - Import assay information files (`*.txt` or `*.xml`).
  - Define the analysis settings.
  - Import Supplementary Sample Information (SSI) files to update sample information such as gender or population.
  - Import reference panel files to add reference samples to a study.
- Analyze study data using one of two call methods:
  - **Autocalling**—The software algorithm is used to call the data points.
  - **Classification Scheme**—User-defined cluster boundaries are used to call the data points.
- Analyze a subset of data by creating analysis groups with a subset of data files or samples. Each analysis group has its own set of analysis settings and analysis results. The analysis group table displays the analysis status for each group.
- View real-time data, including amplification plots, multi component plots, and traces of individual samples for a given assay by cycle in allelic discrimination plots.
- View the study results. For example, a summary of the Quality Control (QC) statistics at the study level, assay level, experiment level, and sample level.
- Export the following data:
  - Analysis results
  - Analysis settings
  - Genotype call settings
  - Flag settings
  - Genotype matrix of samples vs. assays

  - QC by assays showing the assay call rates and flag statistics
  - QC by samples showing the sample call rates and flag statistics
- Transfer or share studies from the Thermo Fisher Cloud Genotyping Application with another user.

See the Thermo Fisher Cloud Genotyping Application Release Notes (**https://apps.thermofisher.com/app-gt-web/src/release-notes.html**) for more information.

Allelic discrimination plots

TaqMan® Genotyper Software, the Thermo Fisher Cloud Genotyping Application, and real-time PCR instrument software plot the results of the allelic discrimination data as a plot of Allele 1 (VIC™ dye) versus Allele 2 (FAM™ dye). The allelic discrimination (AD) plot, also known as a *cluster plot* or *scatter plot*, represents each sample well as an individual point on the plot.

A typical AD plot shows Homozygote clusters, a Heterozygote cluster, and the no-template controls. The points in each cluster are grouped closely together and each cluster is located well away from the other clusters. See Figure 1 and Table 17 on page 27 for expected cluster locations in an AD plot.

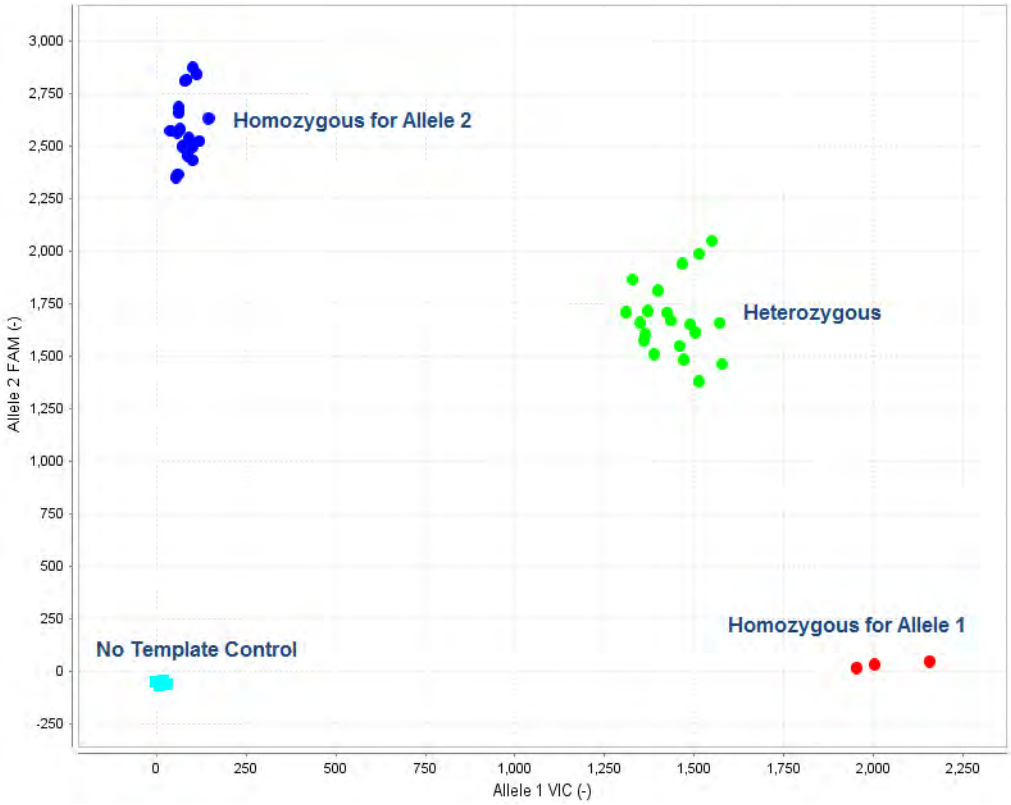


Figure 1 Typical allelic discrimination plot

Table 17 Cluster assignments in an allelic discrimination plot

Content of samples	Location in AD plot
Allele 1 (homozygote), labeled with VIC™ dye	Lower right corner
Allele 2 (homozygote), labeled with FAM™ dye	Upper left corner
Alleles 1 and 2 (heterozygote)	Approximately midway between Allele 1 and Allele 2 clusters
No-template control	Bottom left corner
Undetermined	Anywhere outside the regions described above
No amplification	With NTC cluster in the bottom left corner

Unexpected distributions in allelic discrimination plots can result from:

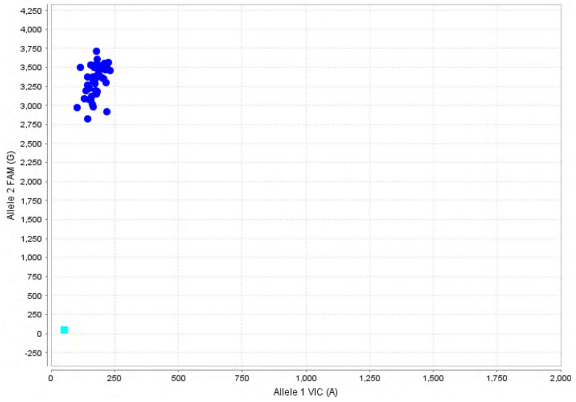
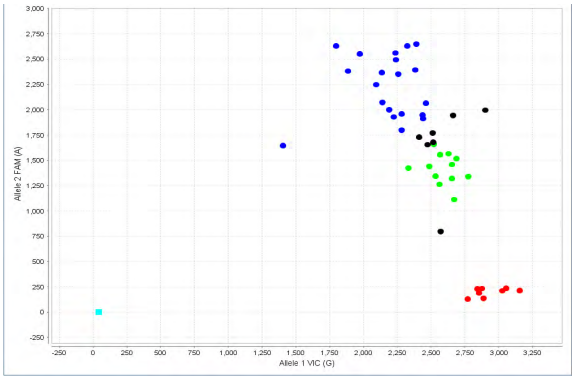
- Genetic characteristics of the assay and/or the sample
- Sample preparation errors
- Assay preparation errors
- Instrument setup and maintenance errors
- Data analysis (software) errors

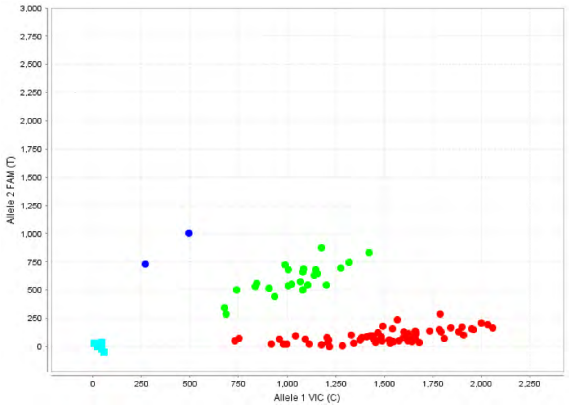
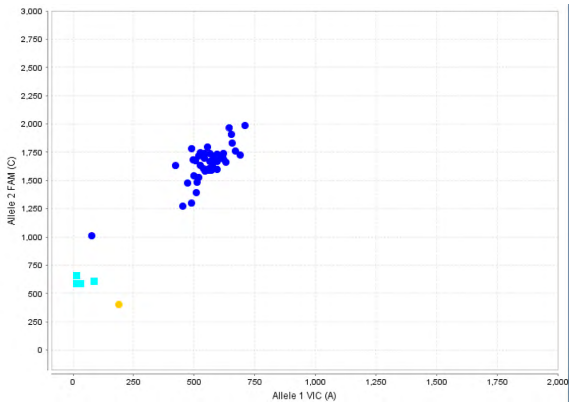
See Appendix A, "Troubleshooting" for more information.

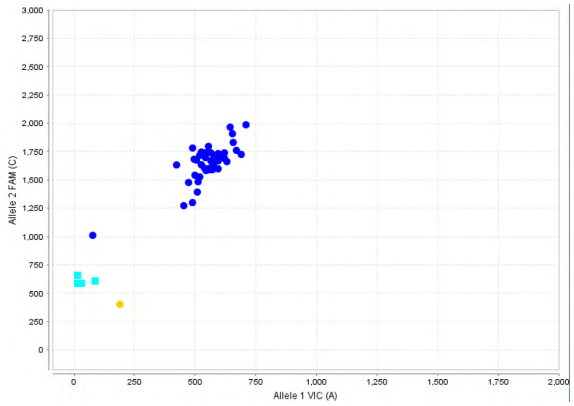
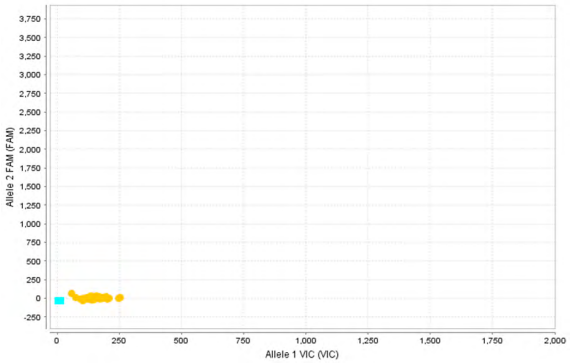
Troubleshooting

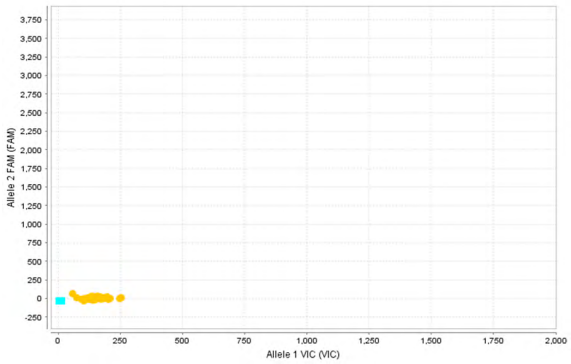
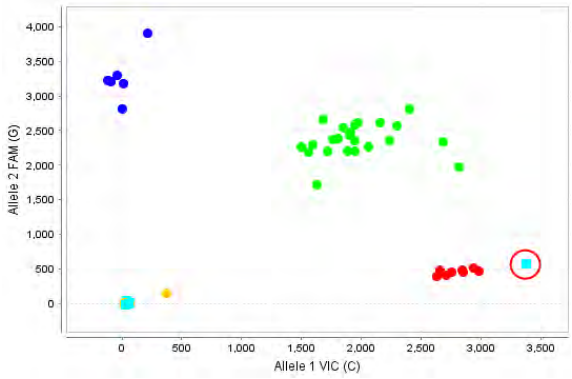
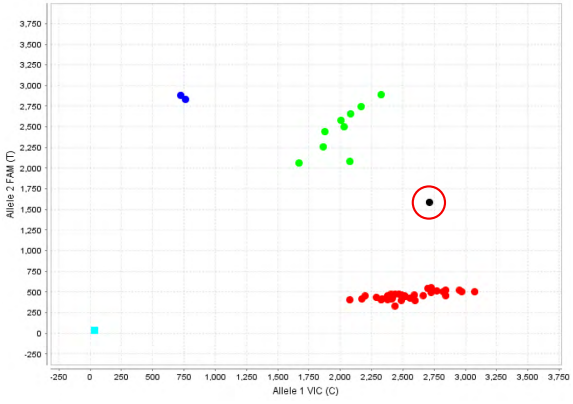
Troubleshooting the AD plot

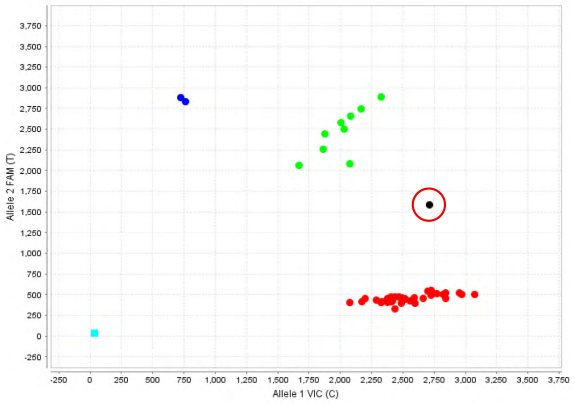
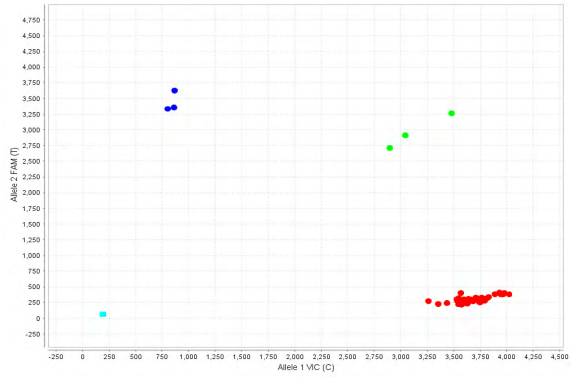
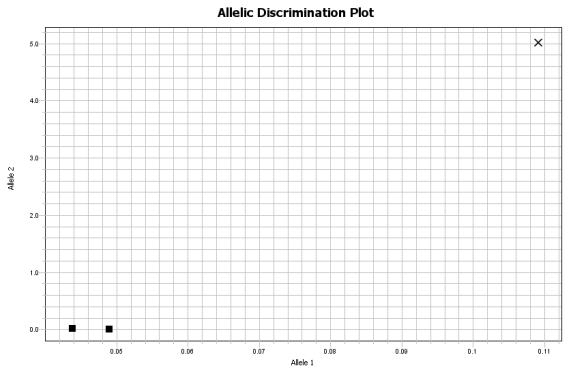
See "Allelic discrimination plots" on page 26 for more information about expected cluster locations in an AD plot .

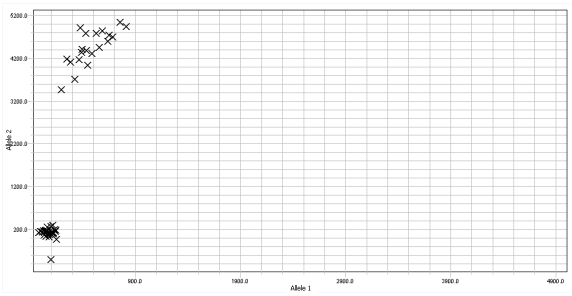
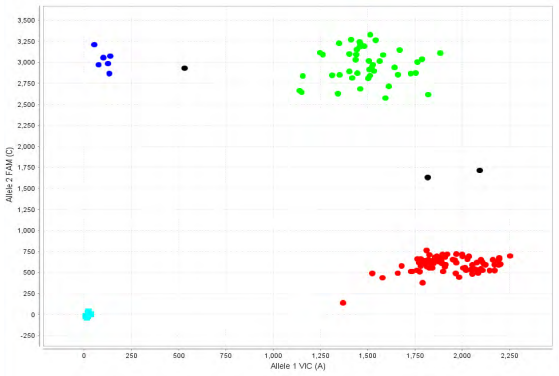
Observation	Possible cause	Recommended action
There is only one cluster present 	*Genetic characteristics of the assay and/or the samples*	
	The MAF is too low for the sample size from the tested population.	See "Low allele frequency" on page 36.
The clusters are cloudy or diffuse 	*Sample preparation errors*	
	Sample DNA is degraded.	See "Degraded DNA" on page 45.
	There are PCR inhibitors in the sample.	See "PCR inhibitors in sample" on page 47.
	Assay preparation errors	
	ROX™ dye was not present in the PCR Master Mix.	See "Using a master mix without ROX™ dye" on page 48.
	There was evaporation from the reaction well.	See "Evaporation from the reaction well" on page 49.
	There were pipetting errors.	See "Pipetting errors" on page 49.
	Instrument set-up and maintenance errors	
	ROX™ dye was not designated as the reference dye.	See "ROX™ dye not designated as a passive reference" on page 55.
	If only one cluster is present, the allelic discrimination plot was incorrectly scaled.	See "Single cluster assay" on page 55.

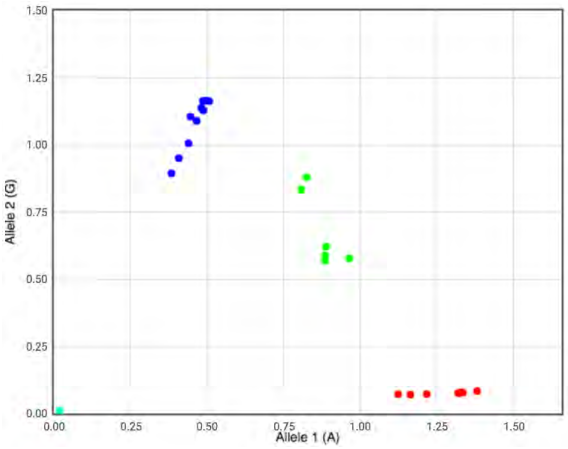
Observation	Possible cause	Recommended action
There are trailing clusters  	*Sample preparation errors*	
	Sample DNA is degraded.	See "Degraded DNA" on page 45.
	There are PCR inhibitors in the sample.	See "PCR inhibitors in sample" on page 47.
	Assay preparation errors	
	Insufficient DNA was added to the well.	See "Insufficient DNA added to the reaction well" on page 48.
	DNA concentration is highly variable.	Use the same concentration of DNA for each sample. See "Quantify the sample gDNA" on page 18.
	The reagents were mishandled or are expired.	See "Reagents mishandled or expired" on page 47.
	ROX™ dye was not present in PCR master mix.	See "Using a master mix without ROX™ dye" on page 48.
	There was evaporation from the reaction well.	See "Evaporation from the reaction well" on page 49.
	There were pipetting errors.	See "Pipetting errors" on page 49.
	There was inefficient mixing and/or insufficient centrifugation.	See "Inefficient mixing or centrifugation" on page 50.
	Instrument set-up and maintenance errors	
	The thermal cycler is poorly calibrated.	See "Instrument calibration" on page 52.
	Data analysis (software) errors	
	ROX™ dye was not designated as the reference dye.	See "ROX™ dye not designated as a passive reference" on page 55.
Some samples cluster with the NTCs  	*Genetic characteristics of the assay and/or the samples*	
	There is an extra SNP under the probe or the primer.	See "Additional SNP present under a probe or primer" on page 39.
	The individual sample has two deletion alleles.	See "Deletion alleles in an individual" on page 38.
	The SNP is triallelic.	See "SNP is triallelic" on page 42.

Observation	Possible cause	Recommended action
Some samples cluster with the NTCs 	The SNP is on the Y chromosome and some samples are female. **Note:** A Y chromosome SNP assay will only have just one or two homozygous clusters, and no heterozygous cluster.	See "Gene on the Y chromosome" on page 45.
	Sample preparation errors	
	Sample DNA is degraded.	See "Degraded DNA" on page 45.
	There are PCR inhibitors in the sample.	See "PCR inhibitors in sample" on page 47.
	Assay preparation errors	
	There was evaporation from the reaction well.	See "Evaporation from the reaction well" on page 49.
	DNA or reagent was not added to the well.	See "DNA or assay reagent not added to the reaction well" on page 48.
	Insufficient DNA was added to the well.	See "Insufficient DNA added to the reaction well" on page 48.
	There were pipetting errors.	See "Pipetting errors" on page 49.
	There was inefficient mixing and/or insufficient centrifugation.	See "Inefficient mixing or centrifugation" on page 50.
All samples cluster with the NTC 	*Sample preparation errors*	
	Sample DNA is degraded.	See "Degraded DNA" on page 45.
	There are PCR inhibitors in the samples.	See "PCR inhibitors in sample" on page 47.
	Assay preparation errors	
	The reagents were mishandled or are expired.	See "Reagents mishandled or expired" on page 47.
	DNA or reagent was not added to the wells.	See "DNA or assay reagent not added to the reaction well" on page 48.
	Insufficient DNA was added to the wells.	See "Insufficient DNA added to the reaction well" on page 48.
	There were pipetting errors.	See "Pipetting errors" on page 49.
	Instrument set-up and maintenance errors	

Observation	Possible cause	Recommended action
All samples cluster with the NTC 	Annealing temperature on the thermal cycler was too high or too low.	Perform several PCR reactions with decreasing and increasing annealing temperatures, then select the conditions that amplify the target of interest.
	Annealing temperature on the thermal cycler was too high or too low for the primers or probes due to poor calibration.	See "Instrument calibration" on page 52.
	The polymerase was not efficiently activated due to incorrect thermal cycler conditions.	See "Perform PCR" on page 22.
	The polymerase was not efficiently activated due to poor calibration of the thermal cycler.	See "Instrument calibration" on page 52.
NTCs generate high fluorescence signals that cluster with samples instead of close to the origin 	*Assay preparation errors*	
	The reagents were mishandled or are expired.	See "Reagents mishandled or expired" on page 47.
	The NTC was contaminated.	See "Good laboratory practices for PCR and RT-PCR" on page 63.
	Instrument set-up and maintenance errors	
	The block was contaminated.	See "Routine instrument maintenance" on page 51.
	Data analysis (software) errors:	
	The passive reporter dye was assigned incorrectly.	See "ROX™ dye not designated as a passive reference" on page 55.
The sample (or samples) did not cluster with the specific genotype 	*Genetic characteristics of the assay and/or the samples*	
	There is an extra SNP under the primer or the probe.	See "Additional SNP present under a probe or primer" on page 39.
	The SNP is triallelic or tetra-allelic.	See "SNP is triallelic" on page 42.
	There is a copy number polymorphism.	See "Gene has a copy number polymorphism" on page 41.
	Sample preparation errors	
	Sample DNA is degraded.	See "Degraded DNA" on page 45.
	Assay preparation errors	

Observation	Possible cause	Recommended action
The sample (or samples) did not cluster with the specific genotype 	There was evaporation from the sample well.	See "Evaporation from the reaction well" on page 49.
	There were pipetting errors.	See "Pipetting errors" on page 49.
	There was more than one sample in the well.	See "More than one sample in the well" on page 50.
	There was inefficient mixing and/or insufficient centrifugation.	See "Inefficient mixing or centrifugation" on page 50.
	Instrument set-up and maintenance errors	
	The block was contaminated.	See "Routine instrument maintenance" on page 51.
The samples are not in the Hardy-Weinberg equilibrium (the expected ratios of each genotype are not seen) 	*Genetic characteristics of the assay and/or the samples*	
	There is a copy number polymorphism.	See "Gene has a copy number polymorphism" on page 41.
	The gene is on the X chromosome.	See "Gene on the X chromosome" on page 44.
	It is a non-random population.	No action. Non-random population are not expected to follow Hardy-Weinberg equilibrium.
	Data analysis (software) errors	
	The detectors and markers, or assays, were set up incorrectly.	See "No assay or marker assigned to the well" on page 54.
Some (or all) of the data is missing (no data points are shown on the AD plot) 	*Data analysis (software) errors*	
	No marker was assigned to the sample.	See "No assay or marker assigned to the well" on page 54.
	Omit was checked for the well or wells with missing data.	See "Omit option checked in Real Time PCR instrument software" on page 54.

Observation	Possible cause	Recommended action
Some or all the alleles are not called (X is shown on the AD plot) 	*Data analysis (software) errors*	
	The NTC task was not assigned to the NTC wells.	See "NTCs not assigned" on page 57.
	The autocall option was not selected.	See "Autocall is not selected" on page 54.
	The sample only has two clusters, but the two-cluster calling option was not selected (the software cannot assign alleles).	See "Two cluster calling not selected" on page 54.
	The sample only has one cluster (the software cannot assign alleles).	See "Single cluster assay" on page 55.
	The sample was an outlier too far off the scale for alleles to be called for other samples.	See "Outlier too far off scale" on page 56.
There are more than 3 clusters 	*Assay preparation errors*	
	More than one assay was included in a well.	See "More than one assay in the well" on page 51.
	Genetic characteristics of the assay and/or the samples	
	There is an extra SNP under the probe or the primer.	See "Additional SNP present under a probe or primer" on page 39.
	There is a copy number polymorphism.	See "Gene has a copy number polymorphism" on page 41.
	The SNP is triallelic or tetra-allelic.	See "SNP is triallelic" on page 42.
	Data analysis (software) errors	
	Several assays were run but only one marker was assigned.	See "Too many alleles called in AD plot" on page 58.

Observation	Possible cause	Recommended action
There are vector clusters (the sample data has two clusters at the same angle) 	*Genetic characteristics of the assay and/or the samples*	
	There is an extra SNP under the probe or the primer.	See "Additional SNP present under a probe or primer" on page 39.
	There is a copy number polymorphism.	See "Gene has a copy number polymorphism" on page 41.
	Sample preparation errors	
	Samples are not in equal quantity due to degraded DNA.	See "Degraded DNA" on page 45.
	Samples are not in equal quantity due to inaccurate DNA quantitation.	See "DNA quantification" on page 61.

Troubleshooting the AD plot: genetic characteristics of the assay and/or the samples

Unexpected distributions in allelic discrimination plots can result from genetic characteristics of the assay and/or the samples, including:

- Low allele frequency (page 36)
- Deletion alleles in an individual (page 38)
- Additional SNP present under a probe or primer (page 39)
- Gene has a copy number polymorphism (page 41)
- SNP is triallelic (page 42)
- Gene on the X chromosome (page 44)
- Gene on the Y chromosome (page 45)

Low allele frequency

Only one or two clusters can occur in the AD plot, as shown in Figure 2, when the minor allele occurs at a low frequency in the population being studied.

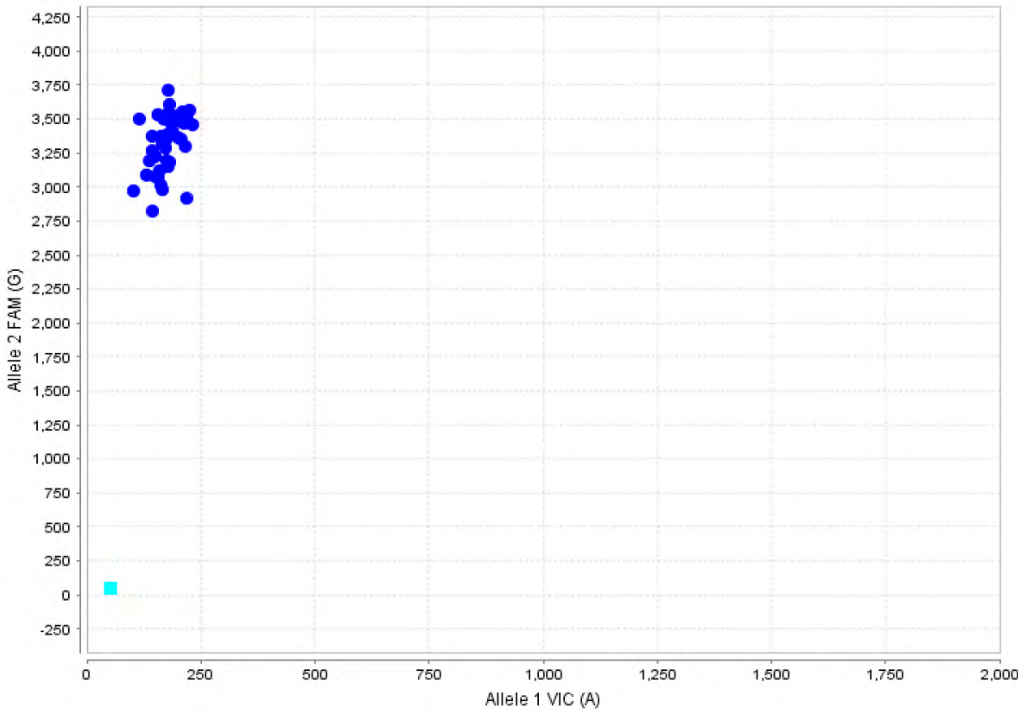


Figure 2 Allelic discrimination plot shows a single cluster (in addition to the NTCs).

What to do

To determine if the size of your sample population is sufficient to detect the minor allele of interest:

1. Find the MAF for your assay on the TaqMan® SNP Genotyping Assays page at **thermofisher.com/taqmansnp**, which is frequently updated.

   Alternatively, the MAF is in the Assay Information File that is distributed with the assays. Allele frequency data can also be found using the public SNP identifier, from public websites such as:

   a. The dbSNP index at **http://www.ncbi.nlm.nih.gov/SNP/index.html**
   b. The 1,000 Genomes project at **www.1000genomes.org**

2. Using the Hardy-Weinberg Equilibrium equation, determine if the minor allele is detectable for a sample the size of your test population (see "Example calculation" on page 37).

   In the Hardy-Weinberg Equilibrium equation, $q^2 + 2qp + p^2 = 1$, the expected genotype frequencies are q^2, $2qp$, and p^2, where q and p represent the allele frequencies.

   The values for q^2, $2qp$, and p^2 correspond to the fraction of a population that would be homozygous for the minor allele (qq), heterozygous (qp), and homozygous for the major allele (pp), respectively.

3. Multiply your sample size by the fraction for each allele to determine the number of individuals with each genotype that you expect to see.

If your sample size is small, you might not be able to detect rare alleles.

Example calculation

For an SNP with a MAF of 5% (0.05), the predicted frequencies are 0.0025 q:q, 0.095 q:p, and 0.9025 p:p.

If you test 20 genomic DNA samples from this population, you might expect:

- Homozygotes for the minor *allele—0.0025 × 20 = 0.05*, no individuals
- *Heterozygotes—0.095 × 20 = 1.9*, about 2 individuals
- Homozygotes for the major *allele—0.9025 × 20 = 18.05*, about 18 individuals

To detect one homozygote for the minor allele, it would take a sample size of approximately 400 individuals ($Sample\ Size = 1/MAF^2$)

Discussion

Many assays in TaqMan® SNP and DME Assays collection show low or 0 for the minor allele frequency. Many of these SNPs are believed to be functional polymorphisms that can occur at low frequencies, depending on the population you are studying. The MAF indicates the frequency of the less-frequent allele in a population. Traditionally, only the minor allele frequency is reported. The major allele frequency is calculated as *1 - MAF*. From the MAF, you can calculate how large the sample population should be to detect a specific allele. The lower the frequency of the minor allele, the larger the sample size that is required to detect the allele.

Deletion alleles in an individual

When an individual does not have the gene or the portion of the gene that contains the SNP of interest, the data point in the allelic discrimination plot will either:

- Appear as a homozygote of the allele that is present (where there is one deletion allele)
- Cluster with the NTCs (when there are two deletion alleles)

If an individual sample consistently clusters with the NTCs for a particular assay, it may indicate the individual has a deletion allele. There are documented occurrences of copy number variation and deletion alleles in the genes CYP2A6 , GSTM1 , GSTT1 , SULT1A1 and CYP2D6 in the TaqMan® DME Genotyping Assays collection.

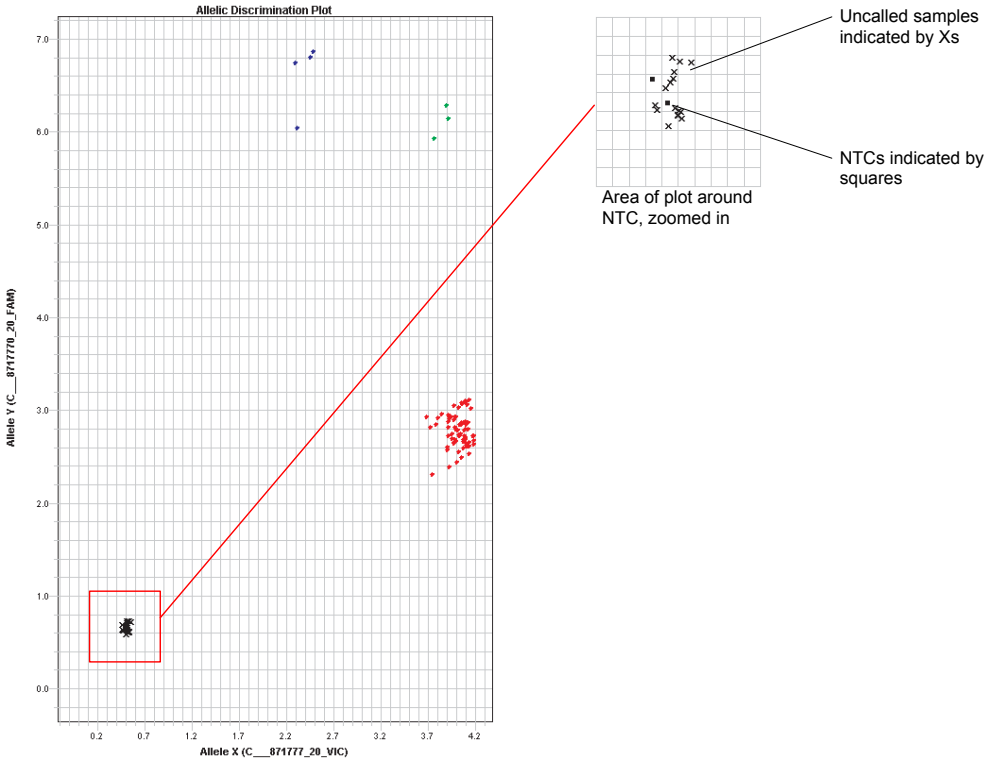


Figure 3 Allelic discrimination plot showing a deletion allele for the assay C__8717770_20.

What to do

1. Evaluate the overall assay performance:
   - Do the assay results appear in tight clusters?
   - Do the clusters have good separation?
2. Repeat the experiment. If the same sample(s) consistently cluster with the NTC while other samples show fluorescence, a deletion allele may be present.
3. Examine the sample's performance in other assays to rule out problems caused by this particular sample, such as sample impurity or degradation.

4. Perform a literature search for documentation reporting the presence of deletion alleles for the gene.
5. Perform analysis using a TaqMan® Copy Number Assay (Cat. No. 4400291) on all samples to confirm the sample has a deletion allele to rule out assay interference caused by a SNP present in the individual's DNA, perform comparative sequencing on the subjects to identify any undocumented SNPs.

   To locate the copy number assay of interest, visit **thermofisher.com/taqmancnv**.

Additional SNP present under a probe or primer

A non-target SNP under a primer or probe can result in off-cluster data. The location of the non-target SNP under the primer or probe, as well as the MAF, influences the extent to which the cluster pattern is atypical. The number of individuals exhibiting this pattern depends on the allele frequency of the non-target SNP. A SNP under a probe can result in an outlier that falls between the heterozygote and one of the homozygotes (an angle cluster), due to a lack of amplification of the sample when there is an additional polymorphism under the primer (Figure 4). The presence of a polymorphism under a primer generally leads to lower PCR efficiency.

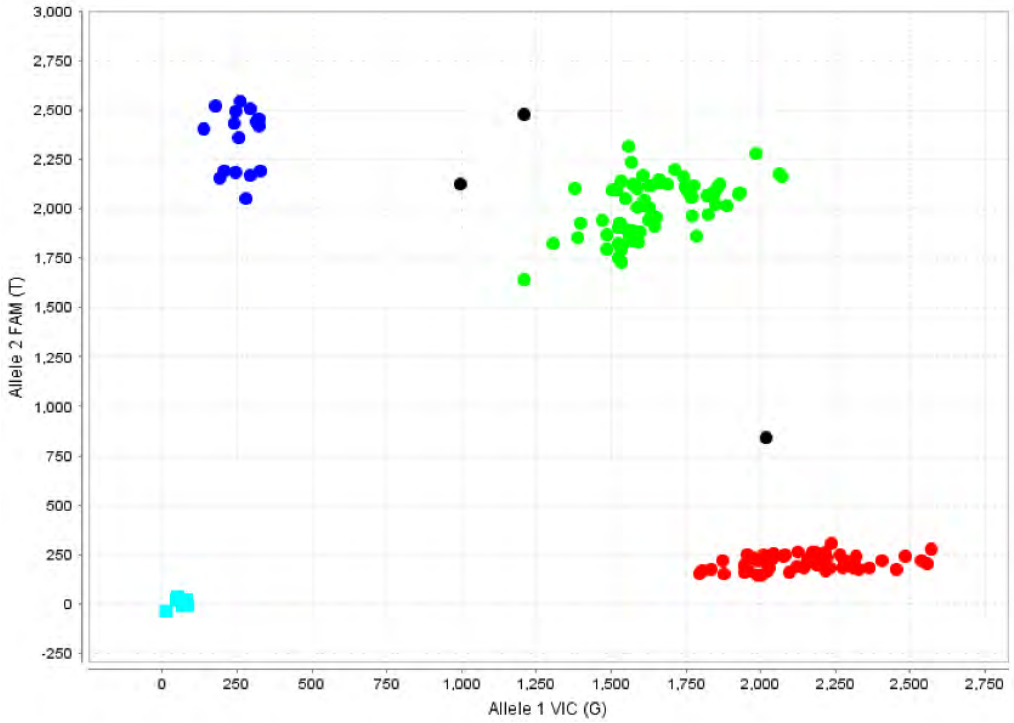
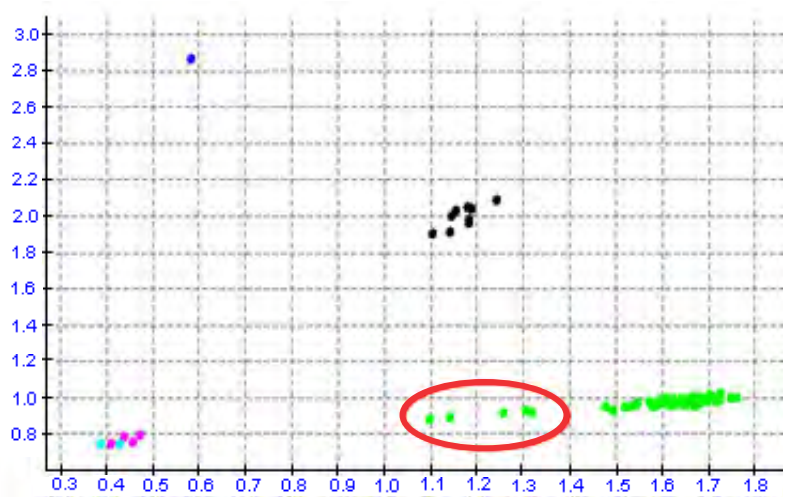


Figure 4 Additional SNP under a primer. The points in black between the green and red clusters constitute angle clusters.

A SNP under a probe can also result in an outlier that has the same angle as a cluster but trails behind the main cluster (a vector cluster). You may see points that are in agreement with a cluster but trail behind the main cluster (a "vector cluster") when

there is an additional SNP under the probe (Figure 5). The presence of a SNP under a probe leads to lower fluorescence intensity.



SNP under probe circled in red. The points trailing behind the main clusters are vector clusters.

What to do

To confirm the presence of another SNP under the probe or primer:

1. Repeat the experiment, then evaluate overall assay performance.
   - Do the assay results appear in tight clusters?
   - Do clusters have good separation?
2. Verify the presence of the outlier.
3. Examine the sample's performance in other assays to rule out problems caused by this particular sample, such as sample impurity or degradation.
4. Search the public databases, such as dbSNP, to see if the additional SNP has been discovered.
5. Perform comparative sequencing on the subjects to identify any undocumented SNPs present under the primer or probe. The presence of extra SNPs may cause angle clusters or vector clusters.

Discussion

Our assay design process included many checks to assure that primers and probes were not designed over significant polymorphisms other than the intended SNP target. However, the growing number of SNPs discovered in studies of different ethnic populations make it possible that some of the primers and/or probes in the TaqMan® SNP and DME Genotyping Assays may overlap currently unknown polymorphisms in certain populations. In some rare cases, there are some assays where primers and probes are located over SNPs or other polymorphisms due to the close proximity of the two SNPs. For these assays, the vector cluster falls in line with samples of the same genotype, but the reduced PCR efficiency causes a reduction in signal intensity.

Gene has a copy number polymorphism

A copy number polymorphism for a gene may or may not appear as an anomaly in the allelic discrimination plot.

- If an individual is homozygous with more than three copies of the gene and each copy has the same genotype, the data will most likely appear in the homozygous cluster.
- If an individual is heterozygous with an odd number of copies and the copies have different genotypes, then the data will probably fall between the clusters for the heterozygote (T:A) and the homozygote (A:A).

Several of the genes included in the TaqMan® Genotyping and DME Assays are known to have copy number polymorphisms: CYP2D6, GSTM1, GSTT, CYP2E1, and CYP2A6.

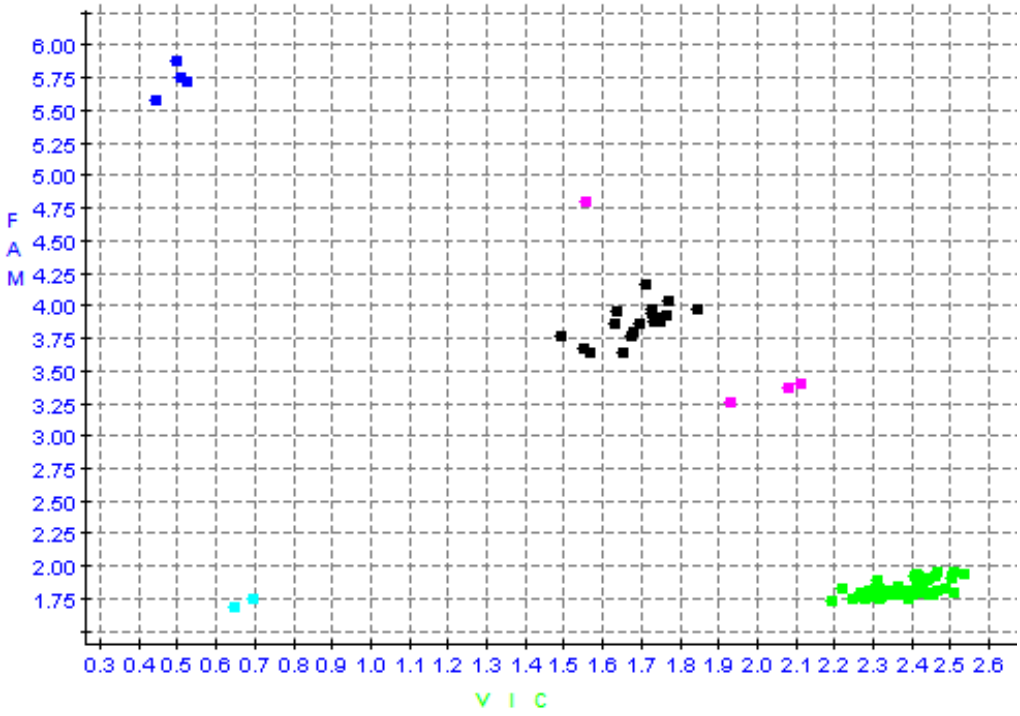


Figure 5 Allelic discrimination plot for assay to CYP2D6*2A -1584C>G (C__32407252_30), showing samples with a copy number polymorphism.

What to do

1. Evaluate overall assay performance
   - Do the assay results appear in tight clusters?
   - Do clusters have good separation?
2. Repeat the experiment to confirm the presence of the off-cluster sample.
3. Examine the sample's performance in other assays to rule out problems caused by this particular sample, such as sample impurity or degradation.
4. Perform a literature search for documentation of copy number polymorphisms for the gene.
5. Perform comparative sequencing on the subjects to identify any undocumented SNPs present under the primer or probe; extra SNPs may cause angle clusters.

6. Perform analysis using a TaqMan® Copy Number Assay (Cat. No. 4400291) on all samples to determine the copy number for the gene in which the polymorphism resides.
    - The Copy Number Assay of interest can be found at **thermofisher.com/taqmancnv**.
    - A Custom TaqMan® Copy Number Assay can be designed if a pre-designed TaqMan® Copy Number Assay does not exist for a target of interest.
    - A TaqMan® Copy Number Assay must be run in duplex with a reference assay. Reference assays are commercially available for human and mouse analysis. If working with other species, you need to identify and use your own reference assay target.

Discussion

Data points for samples from homozygous individuals with extra copies of a gene will generally cluster with the homozygous cluster. Data points for heterozygous individuals with copy number polymorphisms may appear as outliers such as a fourth or fifth cluster between the heterozygote cluster and one of the homozygous clusters. Since a copy number variation may not present itself in all individuals, a gene dosage assay should be performed on all samples to determine which individuals carry extra copies of the gene.

SNP is triallelic

When an SNP is triallelic, you may see outlier samples in the allelic discrimination plot, although the samples may not be well separated from the main clusters. These situations are best confirmed by running replicate plates. We provide pairs of TaqMan® SNP Genotyping Assays to several important DME and CFTR gene variants that are triallelic (see the *Pharmacogenomics Experiments Application Guide* (Pub. No. MAN0009612) and the *Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Genotyping Experiments User Guide* (Pub. No. MAN0014405).

What to do

1. Evaluate overall assay performance: Are there consistent outlier samples?
2. Examine the sample performance in other assays to rule out problems that are caused by this particular sample, such as sample impurity or degradation.
3. Perform comparative sequencing on the subjects to verify the presence of more than two alleles.
4. Repeat the experiment. If the same samples are consistently in the same outlier space (away from the NTCs, the heterozygotes, and the homozygotes) your gene may be triallelic.
5. Check the literature for the SNP in question. There may be newly reported polymorphisms that are described in the literature. Calculate the allele frequencies for your plate and compare them to the literature to confirm your results agree with the literature.

Discussion

If an SNP is triallelic, you might see six clusters (three homozygotes and three heterozygotes) instead of the typical pattern of three clusters (two homozygotes and one heterozygote). Figure 6 and Table 18 show the possible genotypes that are associated with SNP rs2032582 in the ABCB1 gene that is known to have three alleles in several populations. Note that the alleles reported by the ABCB1 SNP assays are given in the (+) strand genome orientation whereas the ABCB1 gene maps to the (–) genome strand. Thus, the reported SNP assay alleles and the SNP cDNA annotations are the reverse complement of one another.

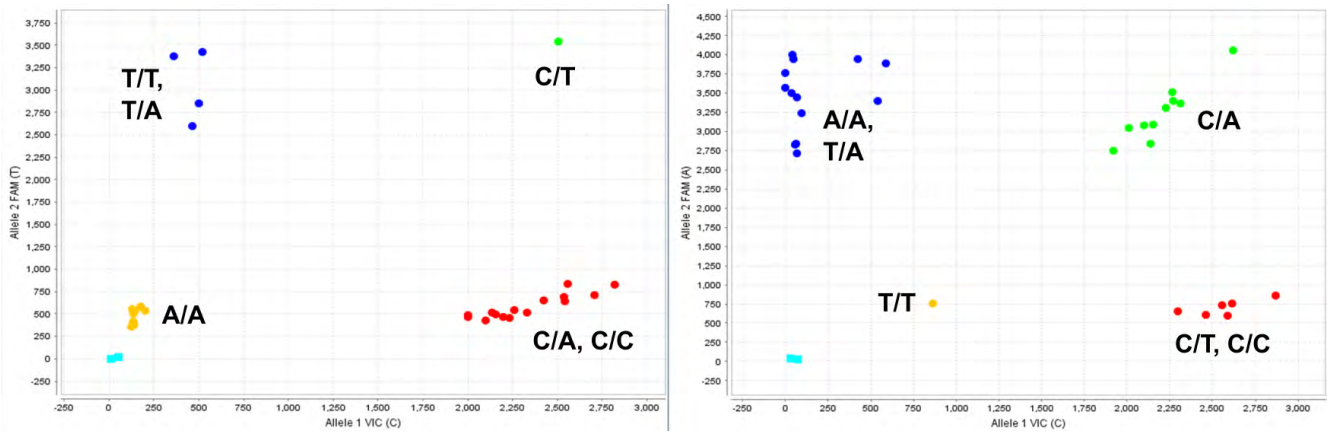


Figure 6 Allelic discrimination plot for triallelic SNP rs2032582. (Left) ABCB1 c.3095G>A assay (C_11711720D_40) to the plus strand C wild type and T mutant allele. (Right) ABCB1 c.3095G>T assay (C_11711720C_30) to the C wild type and A mutant allele.

Table 18 Translation table for the ABCB1 c.3095G>T/A triallelic SNP rs2032582 assays

ABCB1 c.3095G>T/A	C/T assay C_11711720D_40	C/A assay C_11711720C_30
G/G	C/C	C/C
G/A	C/T	C/C
A/A	T/T	noamp
G/T	C/C	C/A
T/A	T/T	A/A
T/T	noamp	A/A

Gene on the X chromosome

When a gene is on the X chromosome and the population being studied is made up of both males and females, the genotype frequencies of the samples do not correspond to the predicted autosomal Hardy-Weinberg frequencies. For a sample population composed of a mixture of males and females, the number of heterozygotes will be noticeably lower than predicted by the Hardy-Weinberg equilibrium equation. None of the males should be heterozygous because males have only one X chromosome.

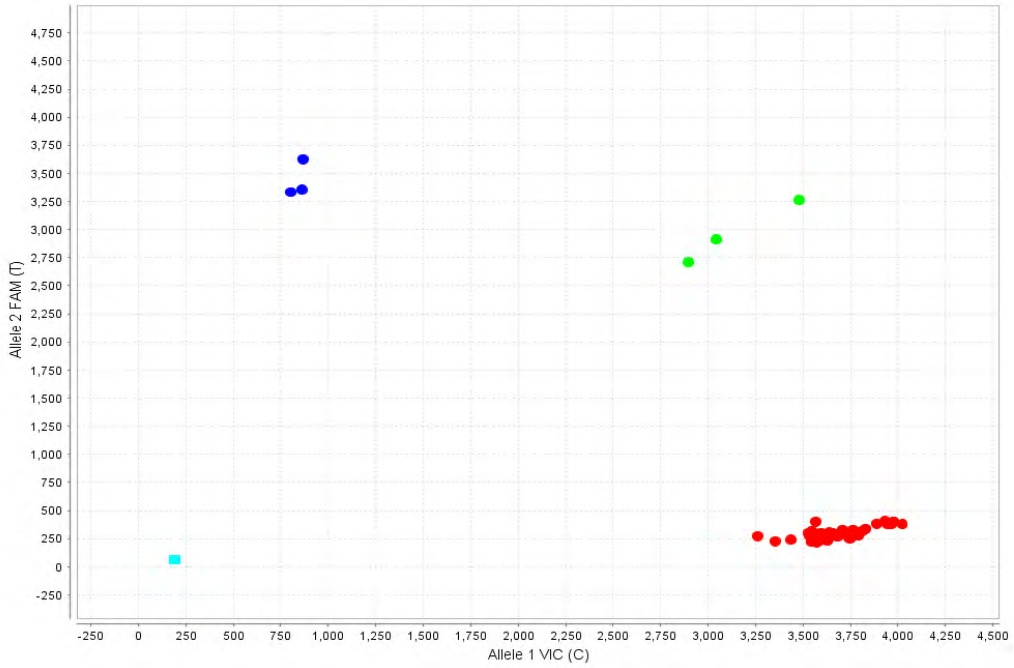


Figure 7 Allelic discrimination plot with a small number of heterozygotes for a gene on the X chromosome

What to do

1. Check the AIF included with the assay or the TaqMan® SNP or DME Genotyping Assays pages (**thermofisher.com/taqmansnp** or **thermofisher.com/taqmandme**) to determine if the assay is for a target located on the X chromosome.
2. Check your results by gender.

Discussion

When a SNP is located on the X chromosome, only the females in the population can be heterozygous. Males, with only one X chromosome, will always be homozygous. Depending upon the minor allele frequency, you may see males in only one of the two homozygous forms.

Gene on the Y chromosome

When a gene is on the Y chromosome and the population is made up of both males and females, female samples will appear with the NTC samples. This result does not reflect assay performance but is due to the fact that female samples will not amplify. As result, only two clusters will be visible because males have only one Y chromosome. In Figure 8, female samples are yellow.

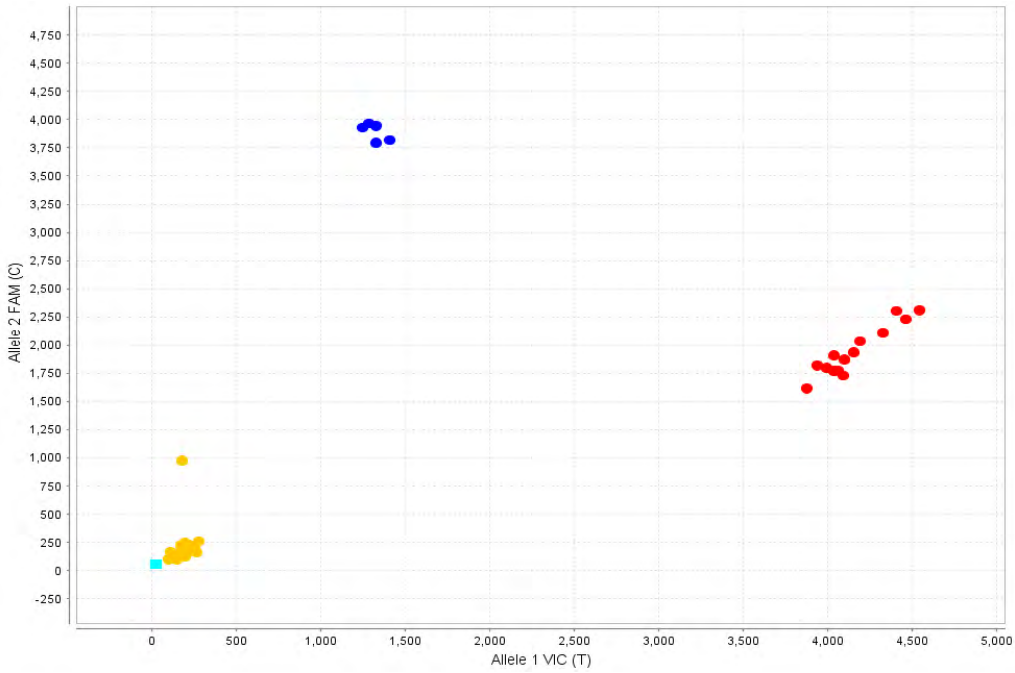


Figure 8 Allelic discrimination for a gene on the Y chromosome

Troubleshooting the AD plot: sample preparation

Unexpected distributions in allelic discrimination plots can result from sample preparation errors, including:

- Degraded DNA (page 45)
- PCR inhibitors in sample (page 47)
- DNA quantification (page 61)

Degraded DNA

Degraded DNA can affect PCR efficiency due to the presence of fewer template copies. Degradation can result from:

- Using very old DNA samples
- Using DNA extracted from formalin-fixed paraffin embedded samples
- Freezing and thawing DNA samples repeatedly
- Leaving DNA samples at room temperature
- Exposing DNA samples to heat, physical shearing, or UV light in ambient lighting
- Purifying DNA samples inefficiently so residual nucleases remain

What to do

- Run an agarose gel to determine if your DNA is degraded. Look for a tight band of high molecular weight. Smearing indicates degraded DNA. Figure 9 illustrates DNA degraded by heat.

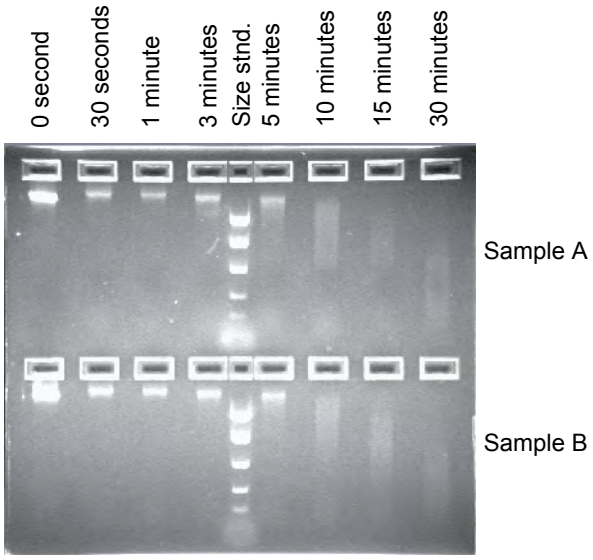


Figure 9 Agarose gel stained with ethidium bromide showing two samples of human gDNA subjected to heating at 99°C for 0 to 30 minutes.

- If the DNA is substantially degraded, use more caution in interpreting your results. If possible, consider repeating the assay using freshly prepared genomic DNA samples.
- For future experiments, follow the sample storage guidelines in Table 19.

Discussion

Because the average size of the DNA in a degraded sample approaches the size of the target sequence, the amount of PCR product generated is reduced because there are fewer intact templates in the size range necessary for amplification.

Table 19 Recommendations for sample storage conditions to minimize DNA degradation.

Tissue type	Storage conditions
Buccal tissue	Store between –15°C and –25°C
Tissue	Immediately place tissue in liquid nitrogen and store at –80°C or Freeze and store between –15° and –25°C
Blood	• Whole blood: store between –15°C and –25°C • Buffy coat: store between –15°C and –25°C and thaw at room temperature before use

PCR inhibitors in sample

Potential PCR inhibitors can originate from the tissue source of the DNA sample or from the purification method. Inhibitors originating from the cell include heparin, proteins, and heme. Inhibitors originating from DNA preparation include phenol, proteases, detergents (SDS), and salts (EDTA and citrate).

The presence of polymerase inhibitors can decrease PCR efficiency, leading to:

- Trailing clusters
- Nonamplification such that some (or all) samples cluster with the NTCs

What to do

- Dilute the sample and run the assay with the diluted sample. If the inhibition decreases, then it is likely there are PCR inhibitors in the sample.
- Repurify the sample and run the assay again.
- Consider using a master mix more tolerant to inhibitors such as TaqPath™ ProAmp™ Master Mix.

Discussion

Inhibition of PCR is always possible when DNA is extracted from tissue and/or blood samples.

Because DNA purification method that is used to prepare gDNA can affect the success of PCR, the selected method must minimize degradation and remove inhibitors. One method for evaluating DNA purity is to calculate the A_{260} /A_{280} ratio. In addition, absorbance at 230 nm can indicate the presence of phenol. See "UV spectroscopy" on page 62 for more information.

Troubleshooting the AD plot: assay preparation

Unexpected distributions in allelic discrimination plots can result from assay preparation errors, including:

Reagents mishandled or expired

The use of mishandled or expired reagents may result in:

- Some or all samples clustering with the NTCs
- Trailing clusters
- Weak overall reaction (weak signals)

What to do

Perform the assay again with newly prepared reagents. Follow the Assay and Master Mix Considerations for reagent storage and handling.

Assay consideration

- Store TaqMan® Assays between –15 and –25°C when they are not in use.
- Minimize freeze-thaw cycles to no more than ten cycles. Too many freeze thaw cycles can cause cleavage of the dye from the probe.
- Limit the assay exposure to light. The fluorescent dyes are susceptible to photo-bleaching. Photo-bleaching can result in a lower overall signal for the reaction.

TaqMan® Genotyping Master Mix considerations

- Store TaqMan® Genotyping Master Mix at 2–8°C.
- Make sure the master mix is thoroughly mixed prior to use.

Using a master mix without ROX™ dye

The use of a PCR Master Mix that does not contain ROX™ dye (or a similar passive reference) can cause:

- Trailing clusters
- Undetermined data (an "X" instead of a called allele in the AD plot)
- Diffuse clusters

What to do

Use a TaqMan® Genotyping Master Mix which includes ROX™ dye.

Discussion

ROX™ dye is a passive reference dye that improves the precision of the results by compensating for small fluorescent fluctuations, such as bubbles and small well-to-well variations.

Our analysis software for allele discrimination and genotyping experiments will not call the alleles when ROX™ dye (or another passive reference) is not present.

See “ROX™ dye not designated as a passive reference“ on page 55 for more information.

DNA or assay reagent not added to the reaction well

When gDNA or one of the assay reagents is not added to the reaction well, no PCR amplification takes place and the sample clusters with the NTCs.

What to do

Perform the assay again, making sure to:

- Follow the TaqMan® SNP Genotyping Assay protocol exactly.
- Pipette carefully.
- Mix thoroughly.

Insufficient DNA added to the reaction well

When insufficient gDNA is added to the reaction well, no PCR amplification takes place and the sample clusters with the NTCs.

What to do

Perform the assay again, making sure to:

- Quantitate your DNA accurately (see “Quantify the sample gDNA“ on page 18).
- Follow the TaqMan® SNP Assays protocol as recommended, adding 1–20 ng of purified genomic DNA (ensuring final concentration no less than 0.2 ng/µL).
- Pipette carefully.
- Mix thoroughly.

Evaporation from the reaction well

Evaporation of your reaction can occur if the reaction plates are not properly sealed, leading to:

- Outliers (mild/moderate evaporation)
- Trailing clusters (moderate evaporation)
- Samples clustering at the NTC (extreme evaporation)

What to do

1. Check the location of the wells for the problem calls. Evaporation can most often occur around the edges of the plate.
2. Check the seals of the optical adhesive cover for leaks.
3. If there are leaks, perform the assay again.

Use a MicroAmp™ Adhesive Film Applicator (Cat. No. 4333183) to thoroughly seal the cover. Make sure to run the applicator over the edges of the seal.

Discussion

Evaporation can occur if your plate is not properly sealed. As evaporation occurs, the water in the reaction decreases, causing the signals from the reporter and ROX™ dyes to increase due to increased concentration of the dyes. The degree of evaporation influences the assay results:

- Mild — If the PCR reaction is not affected, the ROX dye can compensate for the increased signals and the assay will work correctly.
- Mild to moderate — You may see outlier samples. Depending on the number of wells affected, the plot may show only a few outliers or it may show a trailing cluster.
- Extreme evaporation occurring early in the reaction — The PCR reaction fails and the samples cluster with the NTC.

Pipetting errors

Pipetting errors can cause inconsistent delivery of reagents or sample to the wells, which can cause:

- Trailing clusters
- Some (or all) samples clustering with the NTCs
- Cloudy or diffuse clusters
- One or more samples that do not cluster with a specific allele (i.e. an outlier)

What to do

- Improve pipetting precision, using the following techniques:
  - Calibrate and clean the pipettors regularly.
  - Pipette larger volumes (no less than 5 µL) for greater accuracy and precision.
  - Reduce the number of pipetting steps whenever possible.
  - Increase the consistency of the pipetting method (such as using robotic pipetting).
  - See the manufacturer instructions for the correct method of dispensing liquid volumes accurately from the pipettor. For example, some pipettors are designed to deliver the designated volume at the first plunger stop, so *blowing out* the remaining volume can cause errors.
- Check your pipetting process by preparing a replicate plate (same assay and sample over a plate) to be sure that results are reproducible.

Inefficient mixing or centrifugation

Insufficient mixing or centrifugation can cause trailing clusters in the AD plot.

What to do

Rerun the assay, mixing the samples well (by pipetting up and down a few times) and performing the centrifugation steps as described in the protocol. Centrifuging the samples ensures that the contents of the sample well are pooled at the bottom of the well, allowing for the most efficient PCR reaction and the most accurate endpoint read.

Assay has high background fluorescence

Some assays have higher levels of background fluorescence than others which can cause:

- The position of the NTCs moving away from the origin of the allelic discrimination plot
- The position of the homozygous cluster moving towards the heterozygous clusters

What to do

If the clusters are well separated from each other, TaqMan® Genotyper Software or the Thermo Fisher Cloud Genotyping Application can autocall the clusters. You can also manually call the clusters.

More than one sample in the well

Sometimes samples are inadvertently mixed together due to poor lab technique, resulting in an outlier.

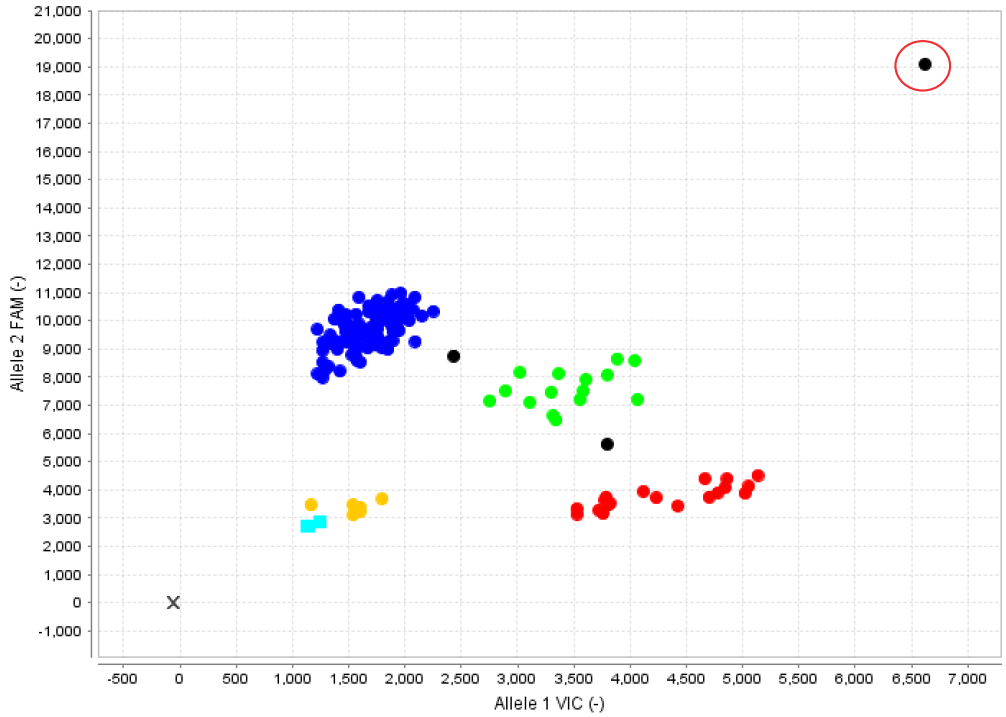


Figure 10 Allelic discrimination plot with different samples in the same well (outlier sample circled in red)

What to do

Perform the assay again making sure that two samples are not combined in the same wells.

More than one assay in the well

Sometimes assays are inadvertently mixed together due to poor lab technique, resulting in more than 3 clusters in a plot.

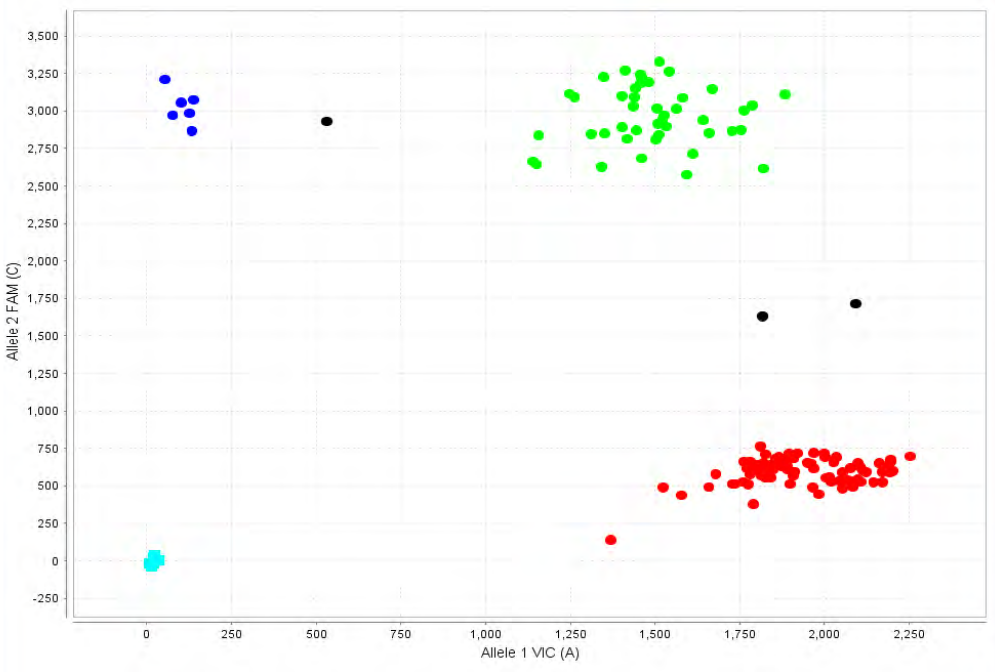


Figure 11 Allelic discrimination plot with different assays in the same well

What to do

Perform the assay again making sure that two assays are not combined in the same wells.

Troubleshooting the AD plot: instrument set-up and maintenance

For best results, make sure to calibrate and maintain your instrument as recommended.

Improper instrument set-up and maintenance can result in unexpected distributions in allelic discrimination plots, including:

- Trailing clusters
- Some or all samples cluster with the NTCs
- Cloudy or diffuse clusters
- High signal for the NTCs in one or more of the sample wells

Routine instrument maintenance

To ensure optimal performance of your thermal cycler or real-time PCR system, we strongly recommend that you perform routine maintenance. Maintenance schedules vary by instrument; refer to your relevant instrument manual for details.

Instrument calibration

We recommend that you regularly calibrate your thermal cycler or real-time PCR system to ensure optimal performance. Calibration varies by instrument. See your specific instrument user guide for details.

Types of calibration

ROI calibration

ROI calibration allows the Real Time PCR Instrument Software to map the position of the wells on the sample block so that, during instrument operation, the software can associate increases in the fluorescence with specific wells of the reaction plates.

Background calibration

The background calibration measures the ambient fluorescence that is generated from background electrical signals, sample blocks, water inside consumables, and from the consumables themselves. This calibration enables the Real Time PCR Instrument Software to eliminate background signal from the fluorescent samples, thus increasing the precision of the instrument.

Pure dye spectra calibration

The pure dye spectra calibration enables the instrument to distinguish the fluorescent dyes being used in the system. The Real Time PCR Instrument Software uses the spectral data from a set of pure dye standards to process the raw spectral data it receives after each run.

Instrument verification run

The test verifies that the instrument can generate a standard curve and its ability to calculate the quantities of two unknowns. This test requires an RNase P Verification Plate that contains pre-loaded reagents that create a standard curve with known copy numbers and two unknowns (also with known copy numbers).

Troubleshooting the AD plot: data analysis (software)

For complete instructions for setting up, running and analyzing TaqMan® SNP Genotyping Assays experiments, see the user manual for your instrument or software.

Unexpected distributions in allelic discrimination plots can result from data analysis errors, including:

- "Empty allelic discrimination plots" on page 53
- "No alleles called in the AD plot" on page 54
- "Homozygous allele frequencies reversed" on page 57
- "Too many alleles called in AD plot" on page 58

Empty allelic discrimination plots

Incorrectly creating and/or selecting the detector and marker can result in an empty allelic discrimination plot even when the assay chemistry is successful.

When the allelic discrimination plot is empty, causes include:

- "No assay or marker assigned to the well" on page 54
- "Omit option checked in Real Time PCR instrument software" on page 54
- "ROX™ dye not designated as a passive reference" on page 55

No assay or marker assigned to the well

An assay or a marker must be assigned to each well before the Real Time PCR Instrument Software, the TaqMan® Genotyper Software, or the Thermo Fisher Cloud Genotyping Application can analyze the plate and obtain results.

What to do

If you have a post-read plate that appears to have no data:

1. Check to see if an assay or a marker is assigned.
2. If no assay or marker is assigned, assign one and reanalyze the data.

Omit option checked in Real Time PCR instrument software

If there is a red X in the plate document for a well, the **Omit Well** option may have been checked for this well.

The **Omit Well** option removes the selected well from the analysis. Consult your instrument user manual for information on how to check and uncheck **Omit well** options.

No alleles called in the AD plot

To assist with cluster calling, the Real Time PCR Instrument Software can autocall the data. When autocalling fails, you will see "X" on the plot rather than the symbol for called alleles. There are some instances where the software will not autocall the data:

- "Autocall is not selected" on page 54
- "Two cluster calling not selected" on page 54
- "Single cluster assay" on page 55
- "ROX™ dye not designated as a passive reference" on page 55
- "Outlier too far off scale" on page 56
- "NTCs not assigned" on page 57

Autocall is not selected

If the Autocall is not enabled in the software analysis settings, the Allelic Discrimination Plot displays an X (Undetermined) for each sample. Default analysis settings vary depending on your real-time PCR system. See the instrument User Guide for more information on how to use **autocall** options.

Two cluster calling not selected

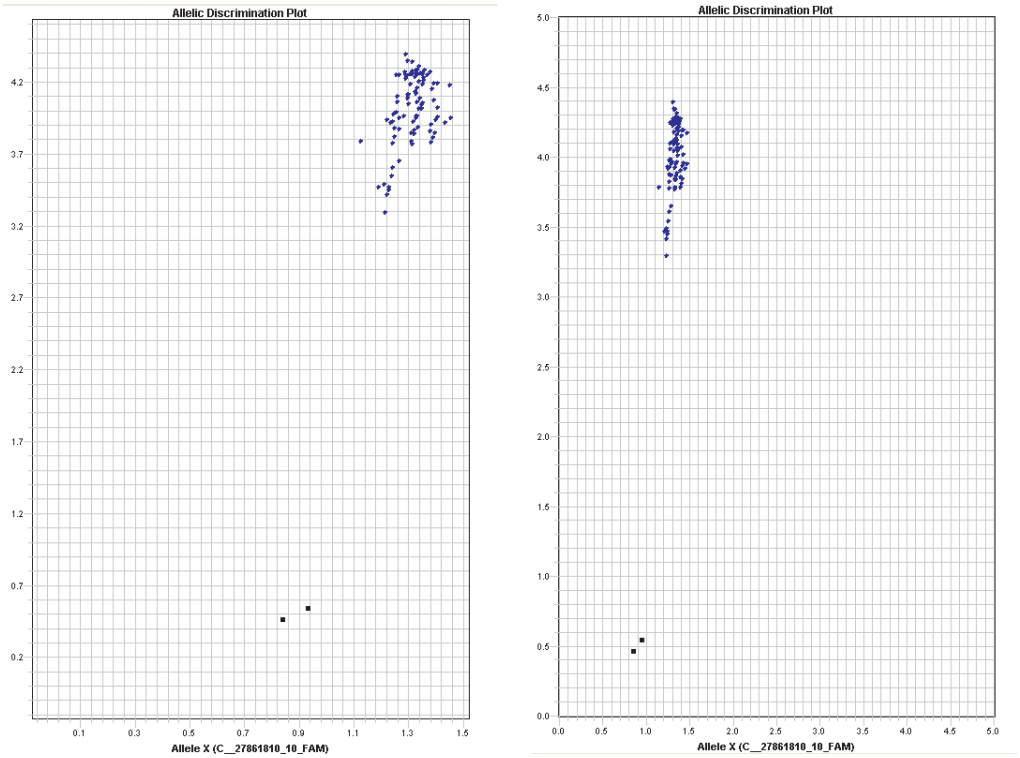
On an instrument other than a QuantStudio™ instrument, the **2 cluster calling** option in the Real Time PCR Instrument Software must be selected if the software is to successfully autocall plates when only two clusters are detected. Detection of only two clusters can happen if the MAF is low and/or you ran too few samples to detect all three genotypes. If this is the case, you must select the two cluster calling option and re-analyze the plate.

Single cluster assay

The Real Time PCR Instrument Software cannot autocall single cluster assays. For many assay/sample combinations, a single cluster assay is the correct result. For example, a single cluster assay can be correct for a SNP with a very low MAF, such as many of the TaqMan® DME Genotyping Assays (see "Low allele frequency" on page 36).

Use TaqMan® Genotyper Software or theThermo Fisher Cloud Genotyping Application to assign genotype calls to single clusters. However, if these applications do not assign a genotype to the cluster and if you believe that the single cluster is correct, manually call the alleles. You may need to rescale the plot before you can call the alleles accurately. For complete instructions for plot rescaling, see the user manual of your instrument or software.

Figure 12 shows an example of a single cluster plot before and after rescaling.



Single cluster before rescaling

Single cluster after rescaling

Figure 12 Allelic discrimination plot before and after rescaling.

ROX™ dye not designated as a passive reference

When ROX™ dye is not designated as a passive reference, some of the Real Time PCR Instruments will not autocall the alleles while others may exhibit trailing clusters in the allelic discrimination plots. ROX™ dye is a passive reference that improves the precision of the results by compensating for small fluorescent fluctuations, such as bubbles and small well-to-well variations that occur in the plate. We recommend TaqMan® Genotyper Software or the Thermo Fisher Cloud Genotyping Application to assign genotype calls when no passive reference is used. We also recommend using TaqMan® Genotyping Master Mix that contains ROX™ as a passive reference dye to

normalize for well-to-well variations in volume. Consult your specific instrument manual for more information on ROX™ designation for genotyping applications.

Outlier too far off scale

If you have an assay that shows clustering around the NTCs, you may want to look for data from an outlier sample. In some cases, the software scales to include the outlier giving the other samples the appearance of clustering around the NTC. If you remove the outlier from the analysis, the software rescales the data and the analysis can proceed.

For more details on how to remove outlier, refer to the user manual of your instrument or software.

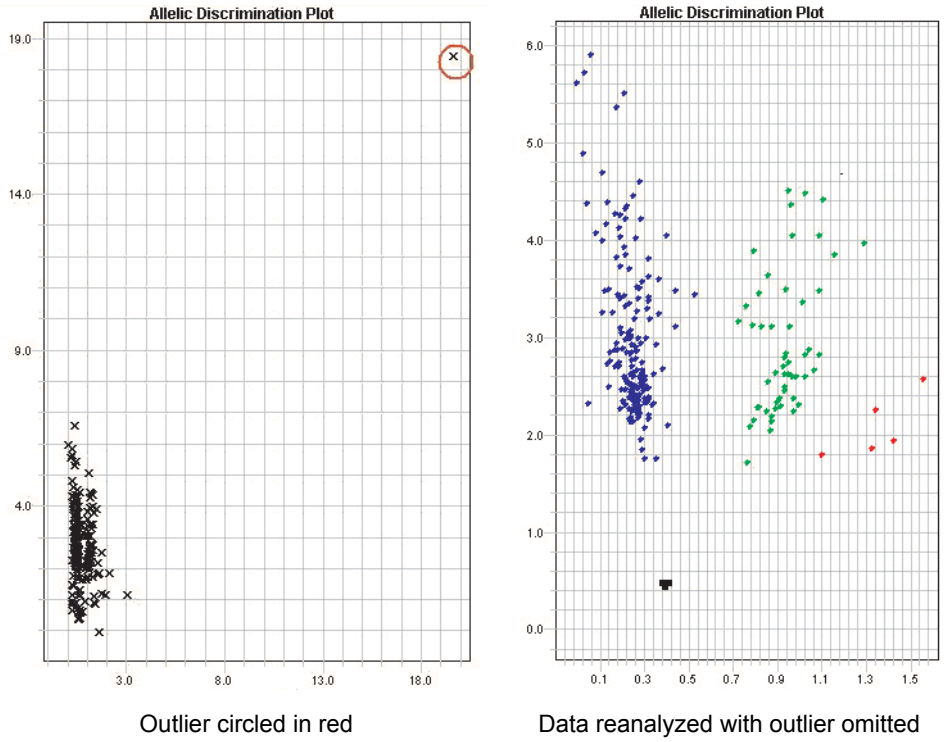


Figure 13 Data analyzed with and without outlier included.

What to do

Remove the outlier(s) using the **omit well** function in your Real Time PCR Instrument software and re-analyze. The program will adjust the scaling.

NTCs not assigned

If the wells containing the NTCs are not assigned with the NTC task in the software, the software may not call the alleles.

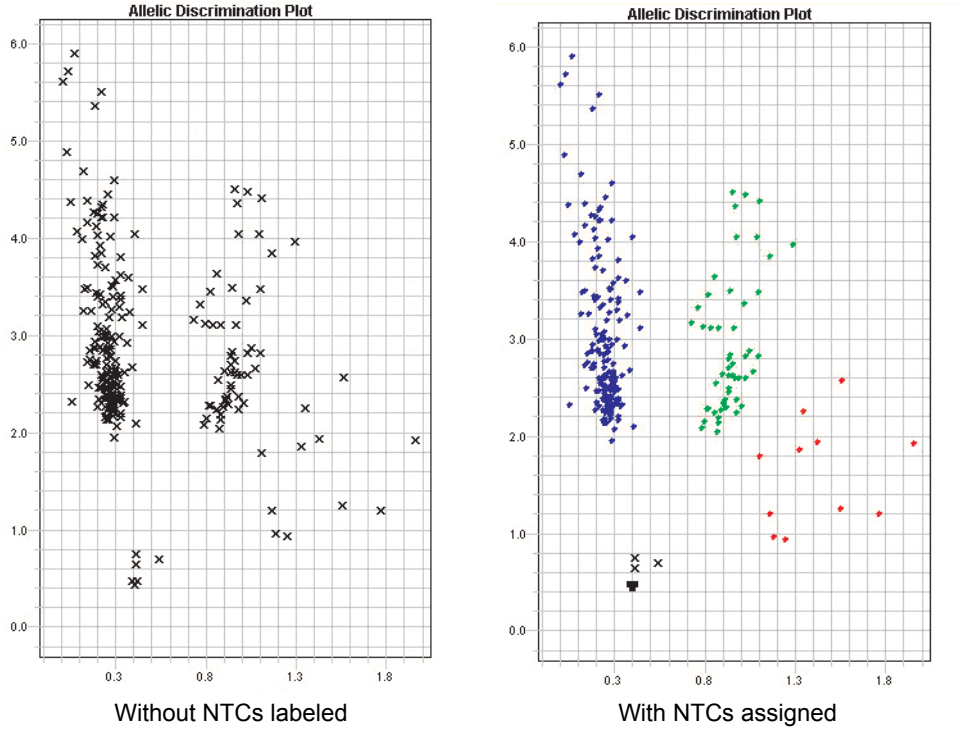


Figure 14 Data analyzed with and without the NTCs assigned.

What to do

In some instances where the data is diffuse and software does not autocall, labeling the NTC wells with the NTC task provides a point of reference for the software, improving clustering and autocalling.

Assign the NTC task to the NTC wells in the plate and re-analyze the data. Refer to the instruction manual appropriate for your software.

Note: NTCs are not required for autocalling.

Homozygous allele frequencies reversed

Your observed major and minor allele frequencies for homozygotes are reversed from those predicted by the Hardy-Weinberg Equilibrium equation. For example, for a SNP with a MAF of 5% (0.05), the predicted frequencies are 0.0025 q:q, 0.095 q:p, and 0.9025 p:p. If the allele frequencies are reversed, you see 0.9025 q:q, 0.095 q:p, and 0.0025 p:p.

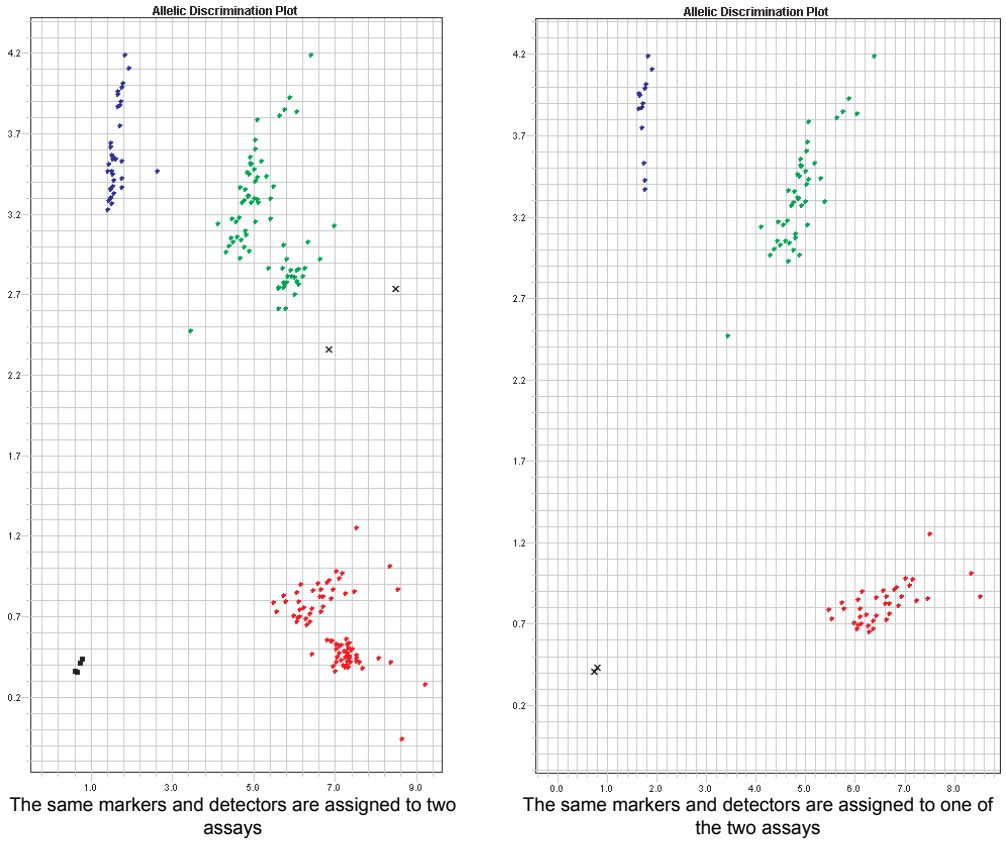
Reporter dye assigned incorrectly to the allele in the detector

In the software, you must set up two detectors and one marker in order for the alleles to be called. The detector defines which dye is assigned to the allele. If you inadvertently assigned the dyes to the wrong alleles when you created the detectors, the observed frequencies will be the reverse of those predicted from the Hardy-Weinberg equation.

Note: The AIF included with your order contains the correct allele-dye assignments.

Too many alleles called in AD plot

In the software, you must set up one marker for each assay run on the plate. Running more than one assay per marker can result in more than three clusters in the allelic discrimination plot.



The same markers and detectors are assigned to two assays

The same markers and detectors are assigned to one of the two assays

Figure 15 Allelic discrimination plots showing two assays assigned to one detector and marker.

What to do

1. Create markers and detectors for each assay on the plate. Refer to the instructions in your instrument manual appropriate for your software.
2. Assign each marker to the correct assays.
3. Re-analyze your data.

Multiple assays may be run on a single plate, however it is essential that each assay is assigned its own marker. Each assay has its own unique run characteristics. Running two assays with the same marker name may result in genotyping miscalls and the appearance of assay failure.

B

Supplemental information

TaqMan® SNP Genotyping Assays chemistry overview

TaqMan® MGB and QSY™ probes

TaqMan® MGB probes consist of target-specific oligonucleotides with:

- A reporter dye at the 5′ end of the probe:
  - VIC™ dye for Allele 1 probe
  - FAM™ dye for Allele 2 probe
- A non-fluorescent quencher (NFQ) dye at the 3′ end of the probe.
- A minor groove binder (MGB) at the 3′ end of the probe that:
  - Increases the melting temperature (T_m) without increasing probe length.
  - Allows for the design of shorter probes.

TaqMan® QSY™ probes consist of target-specific oligonucleotides with:

- A reporter dye at the 5′ end of the probe.
- A QSY™ quencher at the 3′ end of the probe

Two TaqMan® QSY™ probes can be used in multiplex with two MGB probes to analyze two SNPs per well or more than two alleles per well.

About the 5' nuclease assay

Note: The following figures are general representations of PCR with TaqMan® MGB probes and TaqMan® SNP Genotyping Assays. The sequence regions are not necessarily drawn to scale. The principals of the assay are the same for TaqMan® QSY™ probes.

The 5' nuclease assay process takes place during PCR amplification. It occurs in every cycle and does not interfere with the exponential accumulation of product.

For the PCR amplification, genomic DNA is introduced into a reaction mixture consisting of master mix, forward and reverse primers, and two TaqMan® probes Figure 17.

Figure 16 Legend

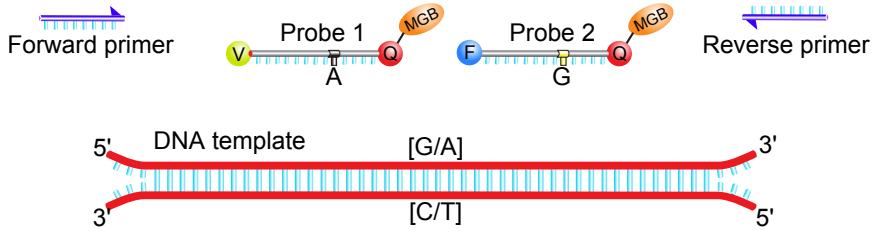


Figure 17 TaqMan® SNP Assay components and DNA template

During the PCR, the forward and reverse primers anneal to complementary sequences along the denatured cDNA template strands (Figure 18). The TaqMan® probe anneals specifically to a complementary sequence between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer.

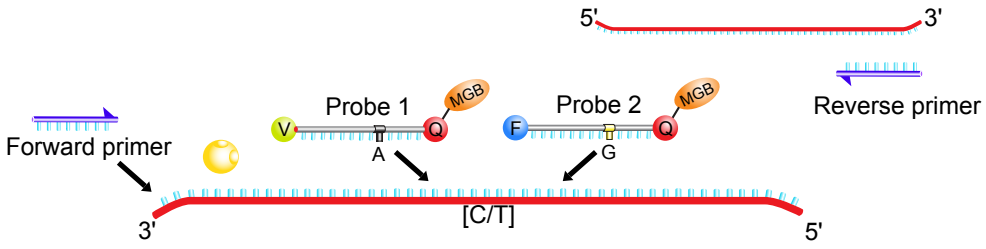


Figure 18 Primers and probes anneal to the denatured DNA template

During polymerization, the DNA polymerase only cleaves probes that hybridize to the target sequence. Cleavage separates the reporter dye from the probe. The separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter (Figure 19). The increase in fluorescence occurs only if the target sequence is complementary to the probe and amplified during PCR. Because of these

requirements, nonspecific amplification is not detected, and the fluorescence signal indicates which alleles are in the sample (Table 20).

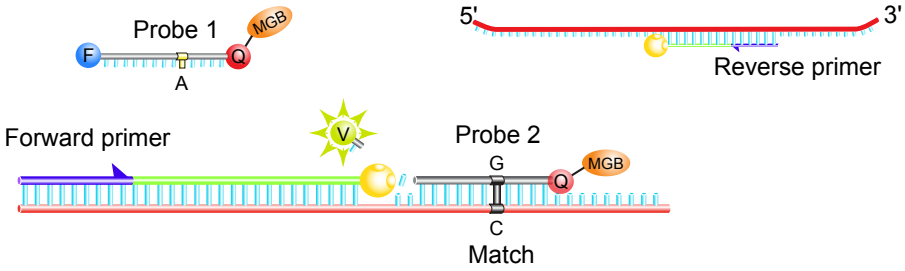


Figure 19 Polymerization and signal generation

Table 20 Correlation between fluorescence signals and sample sequence.

Fluorescence signals	Sample genotype
VIC™ signal	Homozygosity for Allele 1
FAM™ signal	Homozygosity for Allele 2
VIC™ and FAM™ signals	Heterozygosity Allele 1-Allele 2

Mismatches between probes and target sequences

The probes in SNP Genotyping Assays are designed so their hybridization to off-target template is highly reduced by even single nucleotide mismatches between a probe and the target sequence. Poor probe hybridization reduces the amount of reporter dye cleaved from the quenched probe. Furthermore, AmpliTaq Gold™ DNA Polymerase is more likely to displace a mismatched probe without cleaving it. Each of these factors minimizes the production of nonspecific fluorescence signals from off target templates.

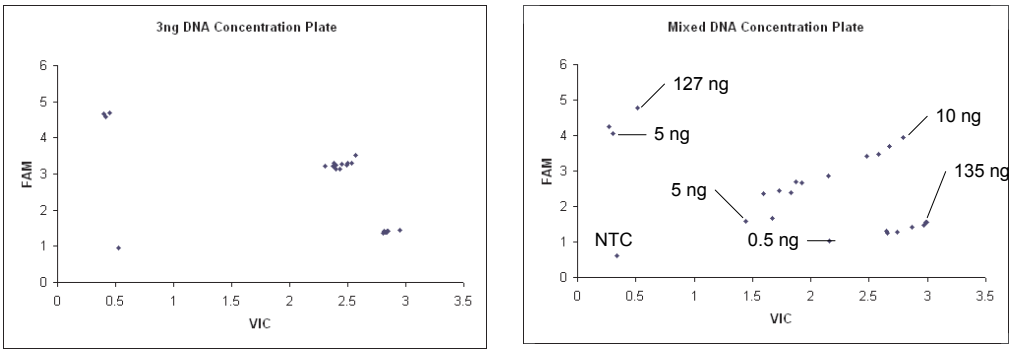
DNA quantification

In an assay and/or study, gDNA concentration uniformity leads to accurate, robust, and reproducible results and ensures efficient use of valuable samples. Precise handling and quantitative measurements before running an assay can prevent errors without waste of reagents and samples.

Variability in gDNA concentrations in TaqMan® SNP Genotyping Assays can lead to experimental anomalies that can affect interpretation of genotyping results, including:

- Trailing clusters
- Some (or all) samples clustering with the NTCs
- Cloudy or diffuse clusters
- A sample (or samples) does not cluster with a specific allele (i.e. is an outlier)

Figure 20 shows how variability in gDNA can affect interpretation of genotyping results.



All samples have 3 ng gDNA

Samples with gDNA ranging from 0.5 to 127 ng

Figure 20 Allelic discrimination plots for TaqMan® DME Genotyping Assay C__1204092_20.

To ensure accurate results:

- Always perform your own concentration measurements before using any genomic DNA (gDNA), even commercially prepared DNA.
- Use the recommended amount of gDNA, 1–20 ng per sample per assay.
- Always use the same quantity of gDNA for all samples of an assay on a plate.

There are numerous methods for quantitating genomic DNA, including:

- UV spectroscopy
- Absolute quantification
  - TaqMan® RNase P method
  - SYBR™ Green assay
- Fluorometric analysis

We recommend quantifying gDNA using UV spectroscopy or absolute quantification using the TaqMan® RNase P method.

UV spectroscopy

UV spectroscopy can be used to quantitate gDNA by reading sample absorbance at 260 nm (A_{260}). The A_{260} is most accurate when using pure nucleic acid and is most useful for DNA in microgram to nanogram quantities. Proteins, particles in the solution, and aromatic chemicals can affect the reading. Samples are usually concurrently read at 280 nm, to determine the concentration of contaminating proteins. The A_{260} /A_{280} ratio is used to determine purity of a DNA sample (see "PCR inhibitors in sample" on page 47).

The effective read range of UV spectroscopy is 0.1 to 0.999, which corresponds approximately to 4 ng/µL to 50 ng/µL of gDNA. Values above or below are outside the linear range for concentration determination. To ensure accurate quantitative results, gDNA samples should be diluted so that the A_{260} reading is between 0.1 and 0.999.

Absolute quantification

Absolute quantification measures the total amount of amplifiable gDNA. This technique requires the creation of a standard curve using gDNA samples of known quantities. The standard samples must be pre-quantified and confirmed using an independent method such as spectrophotometry or fluorometry. The unknown samples are compared to the known samples for quantification.

Absolute quantification method	Description
TaqMan® RNase P	TaqMan® RNase P is a highly accurate 5' nuclease assay that detects and quantifies genomic copies of the human RNase P gene. TaqMan® DNA Template Reagents (Cat. No. 401970) and TaqMan® RNase P Detection Reagents Kit (Cat. No. 4316831) allow for convenient means to quantify gDNA. See *RNase P Quantification for Genotyping Experiments* (Pub. No. MAN0014349) for more information.
SYBR™ Green assay	SYBR™ Green dye binds to double-stranded DNA (dsDNA). Quantification with theSYBR™ Green assay is less specific than TaqMan® RNase P because the dye binds to any dsDNA whereas TaqMan® reagent chemistry targets a specific DNA sequence. The SYBR™ Green method requires melt curve analysis to ensure the specificity of the assay.

For either technique, run the standard curve and unknown samples on the same plates in the real-time PCR instrument.

Fluorometric analysis

You can quantify DNA by fluorometric analysis using the Qubit™ 3.0 Fluorometer or various intercalating dyes.

Good laboratory practices for PCR and RT-PCR

When preparing samples for PCR or RT-PCR amplification:

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA*Zap*™ Solutions (Cat. No. AM9890).

C

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

D Documentation and support

Related documentation

PDF versions of this guide and the documents listed in this section are available at **thermofisher.com/support**.

Note: To open the user documentation, use the Adobe™ Reader™ software available from **www.adobe.com**.

Table 21 Product web pages

Product	Web page
TaqMan® SNP Genotyping Assays	thermofisher.com/taqmansnp
TaqMan® Drug Metabolism Genotyping Assays	thermofisher.com/taqmandme
Custom TaqMan® SNP Genotyping Assays	thermofisher.com/taqmancustomsnp

Table 22 Product documentation

Document	Pub. No.
TaqMan® Genotyping Master Mix Protocol	4371131
TaqMan® Genotyping Master Mix Quick Reference	4371130
Custom TaqMan® Assays Design and Ordering Guide	4367671
Pharmacogenomics Experiments Application Guide	MAN0009612
Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Genotyping Experiments User Guide	MAN0014405
TaqMan® Genotyper Software Getting Started Guide	4448637
TaqMan® Genotyper Software Quick Reference	4448638
Custom Primers and TaqMan® Probes shipped at ambient temperature reduce environment impact and retain their quality and stability (White Paper)	090071

Table 23 Instrument documentation

Document	Pub. No.
Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Allelic Discrimination Assays	4347822
Applied Biosystems™ ViiA™ 7 Real-Time PCR System Getting Started Guide	4441434
Applied Biosystems™ ViiA™ 7 Real-Time PCR System User Guide: Calibration, Maintenance, Networking, and Security	4442661
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR Systems Genotyping Experiments Getting Started Guide	4376786
QuantStudio™ 12K Flex Real-Time PCR System: OpenArray™ Experiments User Guide	4470935
QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide	4470050
QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide	4489822
Applied Biosystems™ 7900HT Fast Real-Time PCR System Allelic Discrimination Getting Started Guide	4364015
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407

TaqMan® Assays qPCR Guarantee

Only the following assays are covered by the TaqMan® Assay qPCR Guarantee: predesigned TaqMan® SNP Genotyping Assays and TaqMan® Drug Metabolism Genotyping Assays. Without limiting the foregoing, custom SNP Assays are expressly excluded from the TaqMan® Assay qPCR Guarantee. Visit **thermofisher.com/taqmanguarantee** for detailed information about the TaqMan Assay qPCR Guarantee.

Customer and technical support

Visit **thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support

- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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