

TaqMan® SNP Genotyping Assays

TaqMan® Predesigned SNP Genotyping Assays, TaqMan® Custom SNP Genotyping Assays, and TaqMan® Drug Metabolism Enzyme Genotyping Assays

Catalog Number 4351379, 4351384, 4351376, 4351382, 4351374, 4351380, 4362691, 4331349, 4332077, 4332072, 4332075, 4332073, and 4332076

Publication Number MAN0009593

Revision A.0

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

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

DISCLAIMER

LIFE TECHNOLOGIES CORPORATION AND/OR ITS AFFILIATE(S) DISCLAIM ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT. TO THE EXTENT ALLOWED BY LAW, IN NO EVENT SHALL LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

Important Licensing Information

These products may be covered by one or more Limited Use Label Licenses. By use of these products, you accept the terms and conditions of all applicable Limited Use Label Licenses.

TRADEMARKS

The trademarks mentioned herein are the property of Life Technologies Corporation and/or its affiliate(s) or their respective owners. TaqMan[®] and AmpliTaq Gold[®] are registered trademarks of Roche Molecular Systems, Inc., TaqMan[®] used under permission and license. DNazol[®] is a registered trademark of Molecular Research Center, Inc. Oragene[™] is a trademark of DNA Genotek, Inc. NanoDrop[®] is a registered trademark of NanoDrop Technologies, LLC. Microsoft[®] Excel[®] is a registered trademark of Microsoft Corporation.

© 2014 Life Technologies Corporation. All rights reserved.

Contents

About This Guide	7
Revision history	7
Purpose of the guide	7
User attention words	7
■ CHAPTER 1 Product Information	8
Product information	8
TaqMan [®] Predesigned SNP Genotyping Assays	8
TaqMan [®] Drug Metabolism Enzyme Genotyping Assays	9
TaqMan [®] Custom SNP Genotyping Assays	9
Product overview	9
Product part numbers	10
Product properties	10
Assay contents	11
Context sequence representation	11
Shipment contents	11
About the Assay Information File	12
About the Drug Metabolism Enzyme Assay Index	13
Storage and handling recommendations	13
Storage and stability	13
Perform the dilution of SNP Genotyping Assays	13
Workflow	14
Ordering Assays	14
TaqMan [®] Predesigned SNP and DME Genotyping Assays	14
TaqMan [®] Custom SNP Genotyping Assays	15
Plated formats	16
96- or 384-well plates	16
OpenArray [®] Plates	16
User supplied materials	16
For sample preparation	16
To perform the PCR	17

■	CHAPTER 2	Genotyping experiment overview	20
	Workflow		20
	Genotyping Assay overview		20
	Assay setup guidelines		21
	Reagents and samples preparation		21
	Set up the PCR reactions		21
	Prepare the reaction mix		21
	Prepare the DNA samples		23
	Perform the PCR		26
	Post-PCR plate read and analysis		27
	Overview		27
	Analyze data using TaqMan [®] Genotyper [™] Software		28
	Resources for data analysis		29
■	APPENDIX A	Troubleshooting	30
	About the allelic discrimination plot		30
	Characteristics of a good allelic discrimination plot		30
	Troubleshooting the AD plot		31
	Unexpected patterns in AD plots		31
	Genetic issues		38
	Low allele frequency		39
	Null alleles in an individual		41
	Additional SNP present under the probe or primer		42
	Gene has a copy number polymorphism		44
	SNP is triallelic or tetra-allelic		46
	Gene on the X chromosome		47
	Gene on the Y chromosome		48
	Sample preparation problems		48
	Degraded DNA		48
	PCR inhibitors in sample		51
	Inaccurate DNA quantitation		52
	Assay problems		54
	Reagents mishandled or expired		54
	Using a master mix without ROX [™] dye		55
	DNA or assay reagent not added to the reaction well		55
	Insufficient DNA added to the reaction well		55
	Evaporation from the reaction well		56
	Pipetting errors		56
	Inefficient mixing or centrifugation		57
	Assay has high background fluorescence		57
	More than one sample in the well		57
	Instrument troubleshooting		58

Routine instrument maintenance	58
Instrument calibration	58
Types of calibration	59
Troubleshooting software problems	59
Empty allelic discrimination plots	59
No alleles called in the AD plot	60
Homozygous allele frequencies reversed	63
Too many alleles called in AD plot	63
■ APPENDIX B Overview of the TaqMan[®] SNP Genotyping Assays ...	65
Overview of the assay components	65
About the probes	65
Basics of 5' nuclease assay	66
PCR principles	66
Mismatches between probes and target sequences	67
Data analysis	67
■ APPENDIX C About the Drug Metabolism Enzyme genes	68
Introduction to the Drug Metabolism Enzyme genes	68
Drug Metabolism Enzyme classifications	68
Allele nomenclature	69
Assay development and testing	70
Bioinformatics evaluation and design	70
Development of the thermal cycler method	70
Wet testing of assays	72
Other assays for the DME genes	73
TaqMan [®] DME Assays for genotyping triallelic SNPs	73
■ APPENDIX D How to order TaqMan[®] SNP Genotyping Assays	76
How to order TaqMan [®] Predesigned SNP and Drug Metabolism Genotyping Assays	76
Assay part numbers	76
Life Technologies website	76
Quick order	77
How to order TaqMan [®] Custom SNP Genotyping Assays	77
Assays part numbers	77
Considerations for choosing human SNP Genotyping Assays	78
Review of the target sequence	78
Custom TaqMan [®] Assay Design Tool	78
■ APPENDIX E About the Assay Information File (AIF)	80
About the Assay Information File (AIF)	80

For LIMS users	81
AIF and tube contents	81
Explanation of the AIF Columns	81
■ APPENDIX F User supplied materials and equipment	87
■ APPENDIX G Good laboratory practices for PCR/RT-PCR	91
■ APPENDIX H Safety	92
Chemical safety	93
Biological hazard safety	94
Documentation and support	95
Obtaining documentation	95
TaqMan [®] Assays QPCR Guarantee	96
Obtaining SDSs	96
Obtaining support	97
Limited product warranty	97
References	
Index	101

About This Guide

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

Revision history

Revision	Date	Description
A.0	January 2014	New document

Purpose of the guide

The *TaqMan[®] SNP Genotyping Assays User Guide* provides:

- overview of the TaqMan[®] Predesigned, Custom SNP, and Drug Metabolism Enzyme Genotyping Assays.
- general guidelines for ordering assays.
- procedures for using TaqMan[®] Predesigned, Custom SNP, and Drug Metabolism Enzyme Genotyping Assays.
- data analysis guidelines for interpreting results.
- troubleshooting information.

User attention words

Two user attention words may appear in this document. Each word implies a particular level of observation or actions as described below:

Note: Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! Provides information that is necessary for proper instrument operation or accurate chemistry kit use.



Product Information

Product information

The 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 Life Technologies robust bioinformatics assay design process relying on a pipeline using heuristic rules deduced from both manufacturing and assay performance data.

These assays use TaqMan[®] minor groove-binding (MGB) probes for superior allelic discrimination, improved signal-to-noise ratios, and design flexibility.

TaqMan[®] Assays QPCR Guarantee (see “TaqMan Assays QPCR Guarantee” on page 96) ensures performance and satisfaction with the results obtained with all predesigned TaqMan[®] SNP and Drug Metabolism Enzyme (DME) Genotyping Assays.

TaqMan[®] Predesigned SNP Genotyping Assays

The TaqMan[®] Predesigned SNP Genotyping Assays in the Life Technologies collection include over 4.5 million genome-wide human assays:

- 3.5 million HapMap SNPs – SNPs genotyped by the HapMap Project across several populations. This project (<http://hapmap.ncbi.nlm.nih.gov>) defines common patterns of human genetic variation and provides tools, including haplotypes maps and tag SNPs, to aid genetic association studies.
- 160,000 validated assays – Highly tested assays to common SNPs having at least a 5% minor allele frequency in at least one of the test populations. Validated assays were tested with 2-4 ethnic populations [90 Coriell cell line DNAs (45 from African American origins and 45 from Caucasian origins) and 90 DNAs from Chinese and Japanese samples, obtained from a collaborator]. The minor allele frequency (MAF) data is published on the product assay details page on our web site and in the order Assay Information Files (AIF).
- Over 126,000 coding region assays – Assays for the detection of informative and putative functional, nonsynonymous cSNPs in gene-coding regions.

The Life Technologies library also includes 10,000 predesigned mouse assays. For targets of interest not covered by our predesigned assays collections, TaqMan[®] Custom SNP Genotyping Assays are available (see “TaqMan Custom SNP Genotyping Assays” on page 9).

Each TaqMan[®] Predesigned SNP Genotyping Assay in the Life Technologies collection includes two allele-specific TaqMan[®] MGB probes containing distinct fluorescent dyes and a PCR primer pair to detect specific SNP targets. Assays are made-to-order, available in multiple scales, and manufactured upon ordering. Human assays undergo functional testing on a panel of 20 genomic DNA samples from

Coriell cell lines before shipment upon first order. Within the panel, there are 8 female and 12 male samples. Of this set of 20 DNAs, there are 7 Caucasian, 7 African American, and 6 Asian descent samples. Quality of repeat orders are verified by mass spectrometry and concentration measurement.

TaqMan[®] Drug Metabolism Enzyme Genotyping Assays

TaqMan[®] Drug Metabolism Enzyme (DME) Genotyping Assays offer a comprehensive collection of assays optimized for genotyping SNPs, insertions and deletions (indels), and multi-nucleotide polymorphisms (MNPs) in drug metabolism related genes.

The TaqMan[®] DME Genotyping Assays collection of 2,700 assays detect potentially causative polymorphisms in 221 drug metabolism enzyme and associated transporter genes. For more information on DME genes and assay development, see Appendix C, “About the Drug Metabolism Enzyme genes”.

TaqMan[®] DME Genotyping Assays are inventoried for fast availability.

TaqMan[®] Custom SNP Genotyping Assays

Create your own assays by submitting confidential target sequences to our secure assay design pipeline using the TaqMan[®] Custom Assay Design Tool (CADT). All human SNP genotyping assays are functionally tested on 20 unique DNA samples to ensure assay amplification and sample clustering. These custom assays are designed, synthesized, formulated, optimized, and quality control tested.

TaqMan[®] Custom 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.

SNP assay sequences can also be submitted directly into the CADT to manufacture a specific known assay sequence.

Product overview

SNP Genotyping Assays contain VIC[®] dye-labeled probe, FAM[™] dye-labeled probe, and two target-specific primers. TaqMan[®] probes incorporate MGB technology at the 3' end to deliver superior allelic discrimination. The MGB molecule binds to the DNA helix minor groove, improving hybridization based assays by stabilizing the MGB probe-template complex. This increased binding stability permits the use of probes as short as 13 bases for improved mismatch discrimination and greater flexibility when designing assays for difficult or variable sequences. All MGB probes also include a non-fluorescent quencher (NFQ) that virtually eliminates the background fluorescence and provides excellent signal-to-noise ratio for superior assay sensitivity.

Product part numbers

For detailed information on how to order, see Appendix D, “How to order TaqMan[®] SNP Genotyping Assays”.

Table 1 Ordering information for TaqMan[®] Predesigned SNP, Drug Metabolism Enzyme (DME), and Custom SNP Genotyping Assays

Product	Scale	Number of reactions		Assay mix formulation	Cat. no.	
		384-well	96-well		Human	Non-human ^[1]
Predesigned SNP	Small	1500	300	40X	4351379	4351384
	Medium	5000	1000	40X	4351376	4351382
	Large	12,000	2400	80X	4351374	4351380
DME	Small	750	150	20X	4362691	—
Custom SNP	Small	1500	300	40X	4331349	4332077
	Medium	5000	1000	40X	4332072	4332075
	Large	12,000	2400	80X	4332073	4332076

^[1] Non-human TaqMan[®] Predesigned SNP Genotyping Assays are designed to amplify and detect specific polymorphisms in purified mouse genomic DNA samples

For storage and handling of the TaqMan[®] Genotyping assays, see “Storage and stability” on page 13.

Product properties

All TaqMan[®] Predesigned SNP, DME, and Custom 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/ μ L)
- 20X, 40X, or 80X TaqMan[®] Genotyping Assay (depending on product and assay scale)
- 2X TaqMan[®] Genotyping Master Mix

Note: TaqMan[®] Genotyping Assays are also compatible with Universal Master Mix II and Sample-to-SNP[™] GTXpress[™] Master Mix reagents. Note that standard PCR conditions are recommended for DME Assays over Fast PCR conditions (see the DME Assay thermal cycling conditions in “Perform the PCR” on page 26).

Most assays use universal reagent concentrations and thermal cycling condition and require only one PCR amplification step with an endpoint reading to obtain results. For DME assays, the recommended PCR protocol includes a longer extension time with additional cycles to take into account the typically longer amplicon lengths (see Appendix C, “About the Drug Metabolism Enzyme genes”).

Assay contents

TaqMan® Pre-designed and Custom SNP Genotyping Assays (40X and 80X) and TaqMan® DME Genotyping Assays (20X) contain:

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

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

For example, the sequence clip in Table 2 would result in the following allele-to-dye association:

- Line 1: VIC® dye associated with the G allele, FAM™ dye associated with the A allele
- Line 2: VIC® = T, FAM™ = C
- Line 3: VIC® = G, FAM™ = A
- Line 4: VIC® = T, FAM™ = C

Table 2 Sequence clips from an Assay Information File showing the targeted SNP in square brackets

	Context Sequence
Line 1	GAGGGGAGGCGGCTGCCACCCCTTC [G/A] ACACTATTACCCATCGGTCA CTTGT
Line 2	GATACTGCTTTTCTATTAACCCAT [T/C] AGTGATGGGGTCAGAAGGCT GAGGC
Line 3	TTCCACAAGTTCTCAAAGCAACTAT [G/A] TTCATAACTTAATCTCTCTT TTTTT
Line 4	ATTGACATCTGTATAAACCGTGTGA [T/C] GGCAGTGATTTAGTAACTTT TTGTC

Shipment contents

Each TaqMan® SNP Genotyping Assay shipment consists of:

- One tube for each TaqMan® assay ordered. Each tube is identified with a label on the side and a 2-D bar code on the bottom
- A copy of the Safety Data Sheet (SDS)

- A CD-ROM containing documents relevant to the type of genotyping assays (Table 3)

Table 3 Documents included in the CD-ROM

Predesigned SNP	DME	Custom SNP
<ul style="list-style-type: none"> - TaqMan[®] SNP Genotyping Assays Protocol (.pdf) - Assay Information File (AIF){.txt, .xml, and .html} - Understanding Your Shipment Document (.pdf) - Datasheet (.pdf) - Safety Data Sheet (SDS) (.pdf) - Certificate of Analysis (CofA) (.pdf) 	<ul style="list-style-type: none"> - TaqMan[®] SNP Genotyping Assays Protocol (.pdf) - Assay Information File (AIF){.txt and .xml} - Understanding Your Shipment Document (.pdf) - Product Insert (.pdf) 	<ul style="list-style-type: none"> - TaqMan[®] SNP Genotyping Assays Protocol (.pdf) - Assay Information File (AIF){.txt, .xml, and .html} - Understanding Your Shipment Document (.pdf) - Datasheet (.pdf) - Safety Data Sheet (SDS) (.pdf) - Certificate of Analysis (CofA) (.pdf) - Custom TaqMan[®] Assays Design and Ordering Guide (.pdf)

About the Assay Information File

The Assay Information File (AIF) contains information about each ordered TaqMan[®] SNP Genotyping Assay.

Predesigned and DME Assays AIFs contain genomic information about the polymorphism including:

- Gene name
- Chromosomal location
- Allele frequency (if available)
- Context sequence (with nucleotide variation in square brackets)
- Reporter dye concentrations
- Dye-SNP allele associations

Custom SNP Assay AIFs provide:

- Assay concentration
- Forward and reverse primer sequences
- Probe sequences
- Reporter dye concentrations

For more information about the AIF, see Appendix E, "About the Assay Information File (AIF)".

About the Drug Metabolism Enzyme Assay Index

The TaqMan[®] Drug Metabolism 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)
- Phenotype (from PharmGKB)
- Life Technologies MAF data

To download the TaqMan[®] Drug Metabolism Index, go to: <http://www.lifetechnologies.com/taqmandme>

Storage and handling recommendations

Storage and stability

- Store the SNP Genotyping Assays at –15 to –25 °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 the Assays to minimize the number of freeze-thaw cycles.
- Life Technologies recommends diluting the 40X and 80X TaqMan[®] Predesigned and Custom SNP Genotyping Assays to a 20X working stock (see “Perform the dilution of SNP Genotyping Assays” on page 13).
- TaqMan[®] SNP Assay products are stable for up to 5 years past their manufacturing date.

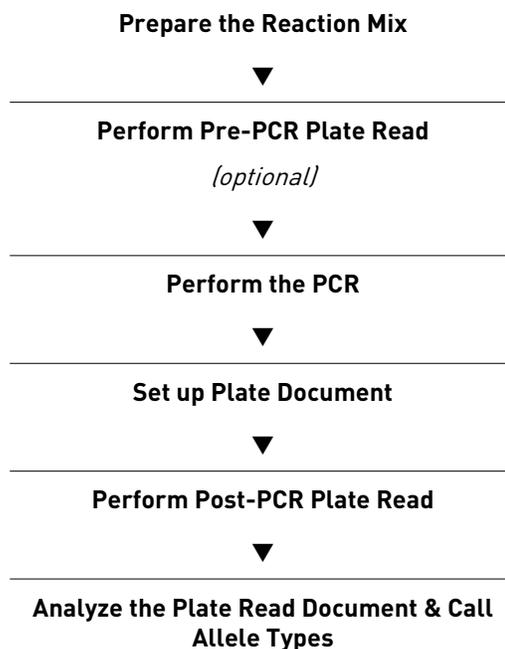
Perform the dilution of SNP Genotyping Assays

Life Technologies recommends that the SNP Genotyping Assays be diluted to a 20X working stock, then aliquoted for routine use to minimize freeze-thaw cycles and the Assays exposure to light.

1. Dilute 40X or 80X SNP Genotyping Assay 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 SNP Genotyping Assays at –15 to –25 °C in the dark.

Workflow

The following workflow illustrates an overview of a genotyping experiment using TaqMan[®] SNP Genotyping Assays.



Ordering Assays

TaqMan[®] Predesigned SNP and DME Genotyping Assays

For the list of available TaqMan[®] Predesigned SNP Assays and inventoried TaqMan[®] DME Genotyping Assays, visit www.lifetechnologies.com.

The database of over 4.5 million available assays can be searched by:

- Gene name
- Gene symbol
- SNP ID
- Assay ID
- Allele nomenclature (available for DME Assays for which this information has been mapped)
- Genomic location

TaqMan[®] DME Genotyping Assays can also be ordered by selecting assays from the DME Index (available at <http://www.lifetechnologies.com/taqmandme>). Selected list of assays can be ordered by batch query on the TaqMan[®] SNP Genotyping Assay search page or by entering the assay IDs and the product part number into the Quick Order tool.

TaqMan[®] Custom SNP Genotyping Assays

Note: All assays are predesigned and optimized and sold with the TaqMan[®] Assays QPCR guarantee (see “TaqMan Assays QPCR Guarantee” on page 96 for details). For more information on ordering TaqMan[®] Predesigned SNP and DME Genotyping Assays, consult “How to order TaqMan Predesigned SNP and Drug Metabolism Genotyping Assays” on page 76.

Note: For detailed instructions on how to design and order TaqMan[®] Custom 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). This tool may also be used to upload assays that have already been designed outside the system.

Life Technologies designs, synthesizes, and performs analytical quality-control on Custom TaqMan[®] SNP Assays based on sequence information supplied. All information supplied is secure and confidential.

After selecting the sequences to study, access the Custom TaqMan[®] Assay Design Tool (CADT) on the Life Technologies website to enter target sequences, import sequence information from a file, or search the Life Technologies database for sequences. Please note that target sequences must be prepared before submitting for design and that upfront bioinformatics work must be done before SNPs can be submitted to the CADT:

- Sequences must be accurate. The CADT doesn't verify that a sequence can be found within a genome.
- Ensure that the allele frequency of the SNP is sufficiently high to provide useful data for the size of the study.
- Mask neighboring SNPs with Ns.
- Mask repeat sequences with Ns.
- BLAST or BLAT search sequences to ensure their uniqueness within the genome
- The maximum size of an indel or MNP is 6 bases.

After selecting the target sequences, submit the sequences to Life Technologies for custom designs. Selection must specify whether the target is human (functional testing performed) or non-human (no functional testing). Please note that, under certain circumstances, human targets should be submitted as non-human targets. Human SNP targets that should be ordered as non-human assays include: Y-chromosome, mitochondrial, and cDNA sequences; these assays often fail functional testing.

The selected assays can be ordered directly from the CADT. Life Technologies will manufacture, package, and ship the TaqMan[®] Assays. Alternatively, primer and probe sequences can be submitted directly to CADT for synthesis. Assays are shipped only if they pass the following analytical quality control criteria:

- Each primer and probe is individually tested by mass spectroscopy to verify the accuracy and the yield of the resulting synthesized oligonucleotide.
- Assays must meet probe and primer yield specifications, as performed by mass spectroscopy.
- Human SNP Genotyping Assays are tested using 20 human genomic DNA samples for 3 populations. To pass this functional test, amplification must occur and at least one allelic discrimination cluster (heterozygous or homozygous, compared to no template controls) must be generated. Non-human SNP Genotyping Assays are not functionally tested.

Note: Custom SNP Assays are not covered by the TaqMan[®] Assays QPCR guarantee (see “TaqMan Assays QPCR Guarantee” on page 96 for details). For more information on ordering TaqMan[®] Custom SNP Genotyping Assays, consult “How to order TaqMan Custom SNP Genotyping Assays” on page 77.

Plated formats

For projects that require analyzing a large number of samples or for evaluating samples for a large number of SNPs, assays may be ordered in plated formats.

96- or 384-well plates

TaqMan[®] SNP Assays can be ordered pre-plated into Fast or standard 96- or 384-well plates or TaqMan[®] Array Cards through the TaqMan[®] Custom Plating Service. For more information, visit the TaqMan[®] Custom Plating Service at <http://www.lifetechnologies.com/customplating>.

OpenArray[®] Plates

TaqMan[®] OpenArray[®] Genotyping Plates enable the highest sample throughput for mid-density genotyping: >110,000 genotypes in a single day with the QuantStudio[™] 12K Flex Real-Time PCR System. For more information, visit the TaqMan[®] OpenArray[®] Genotyping Technology web page at <http://www.lifetechnologies.com/openarray>.

User supplied materials

For sample preparation

For genomic DNA extraction, we recommend one of the following kits.

Table 4 Recommended kits for genomic DNA extraction

Sample Type	Name	Cat. no.
Blood & Serum	MagMAX [™] -DNA Multi-Sample Kit	4413021 (96-well format) and 4413020 (50 single preps)
	DNAzol [®] BD Reagent	10974-020
	ChargeSwitch [®] gDNA 50–100 µL Blood Kit	CS11000
	GeneCatcher [™] gDNA 3–10 mL Kit	CS21110
	PureLink [®] Genomic DNA Purification Kit	K1820-01, K1820-02, and K1821-04
Buccal cells	MagMAX [™] -DNA Multi-Sample Kit	4413021 (96-well format)
Saliva	MagMAX [™] -DNA Multi-Sample Kit (using Oragene [™] -preserved saliva as input)	4413021 (96-well format) and 4413020 (50 single preps)
FFPE tissue samples	RecoverAll [™] Total Nucleic Acid Isolation Kit for FFPE	AM1975

Sample Type	Name	Cat. no.
	MagMAX™ FFPE DNA Isolation Kit	4463578
	PureLink® FFPE RNA Isolation Kit	K156002
Fresh-frozen tissue samples	PureLink® Genomic DNA Purification Kit	K1820-01, K1820-02, and K1821-04
	DNAzol® Reagent	10503-027
Cell lines	MagMAX™ -DNA Multi-Sample Kit	4413021 (96-well format) and 4413020 (50 single preps)
	PureLink® Genomic DNA Purification Kit	K1820-01, K1820-02, and K1821-04
	DNAzol® Reagent	10503-027

Alternatively, sample preparation and amplification reagents compatible with TaqMan® SNP, Custom SNP and DME Genotyping Assays are included in TaqMan® Sample-to-SNP™ Kits (for ordering information, see Appendix F, “User supplied materials and equipment”). However, Life Technologies recommends pre-amplifying all samples prepared with TaqMan® Sample-to-SNP™ Kits when using OpenArray® for subsequent genotyping. Contact Life Technologies for more information on how to order TaqMan® Pre-Amplification Pools and reagents.

Note: Due to the longer length of the amplicons in DME assays, it is not recommended to run samples processed with TaqMan® Sample-to-SNP™ Kits using Fast PCR conditions. Use instead the DME Assay thermal cycling conditions described in “Perform the PCR” on page 26.

TaqMan® Sample-to-SNP™ Kits can be used to extract genomic DNA from the following samples:

- Blood
- Blood cards
- Cell culture suspension
- Buccal swab
- Rat or mouse tail
- Tissue
- Hair with follicle
- Leaf punch or needle
- Seed chip
- FFPE tissue

To perform the PCR

PCR amplification can be performed with any of the instruments in Table 5 and Table 6. However, 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 the fluorescent signals generated by TaqMan® probe cleavage.

Table 5 Supported thermal cyclers

Thermal Cycler ^[1]	Modules
Veriti [®] Thermal Cycler	96-well, 96-well Fast, and 384-well
9700 Thermal Cycler	96-well and 384-well
9800 Fast Thermal Cycler	96-well
ProFlex [™] PCR system	96-well, 2×96-well, and 2×Flat

^[1] Genotyping required post-read using a Real-Time PCR system listed in Table 6

Table 6 Supported Real-Time PCR systems

Real-Time PCR Systems	Block Modules
7500 System Standard	96-well
7500 Fast System	96-well
7900HT Fast System	96-well, 96-well Fast, and 384-well
StepOne [™] /StepOnePlus [™] System	StepOne [™] 96-well Fast
ViiA [™] 7 System	96-well, 96-well Fast, and 384-well
QuantStudio [™] 6 Flex Real-Time PCR System	96-well, 96-well Fast, and 384-well
QuantStudio [™] 7 Flex Real-Time PCR System	96-well, 96-well Fast, 384-well, and Array Card
QuantStudio [™] 12K Flex Real-Time PCR System	96-well, 96-well Fast, 384-well, Array Card and OpenArray [®]

Table 7 Consumables

Item	Cat. no.
1.5-mL Microcentrifuge tubes	AM12400
Reaction plates and accessories for Real-Time PCR system	For ordering information, see Appendix F, "User supplied materials and equipment"

Table 8 Reagents

Reagent	Volume	Cat. no.
2X TaqMan [®] Genotyping Master Mix ^[1]	1 mL	4371353
	10 mL	4371355
	10 mL	4381656
	50 mL	4371357
	2 × 50-mL	4381657
Nuclease-free water	100 mL	AM9938

^[1] 2X TaqMan[®] Genotyping Master Mix is the recommended master mix for use with DNA samples not prepared with TaqMan[®] Sample-to-SNP[™]

Alternatively, TaqMan[®] SNP, Custom SNP and DME Genotyping Assays are also compatible with:

- GTXpress[™] Master Mix (a component of the TaqMan[®] Sample-to-SNP[™] kit; for ordering information, see Appendix F, “User supplied materials and equipment”)
- Universal Master Mix II

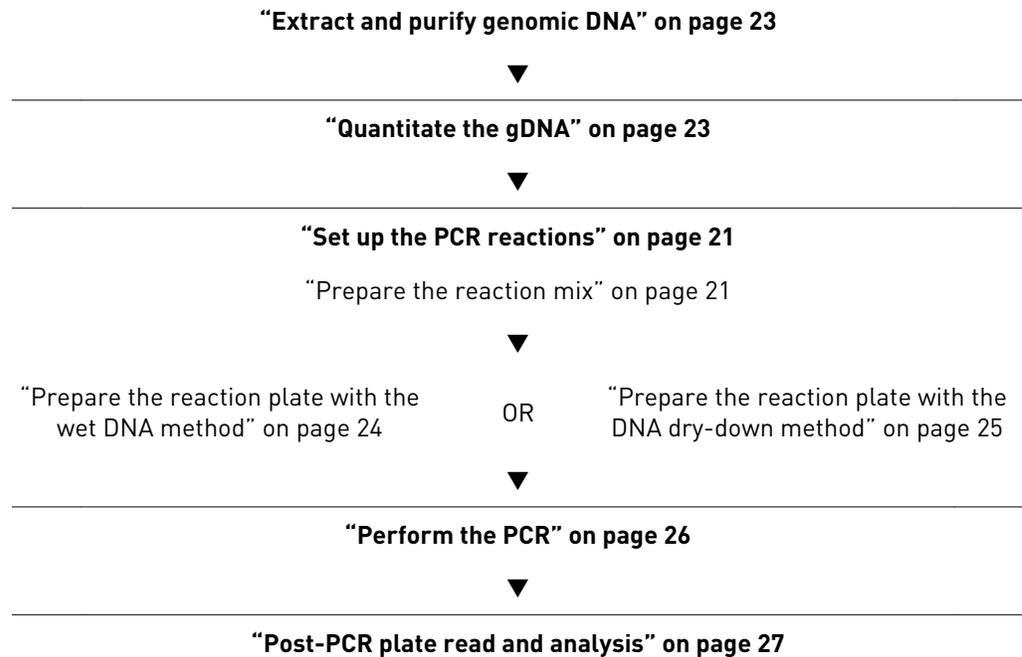
Note: It is not recommended to use Fast thermal cycling conditions for TaqMan[®] DME Genotyping assays. Use instead the DME Assay thermal cycling conditions described in “Perform the PCR” on page 26.

2

Genotyping experiment overview

Workflow

The following workflow illustrates the steps of a genotyping experiment using TaqMan[®] SNP Genotyping Assays.



Genotyping Assay overview

In the first step of a TaqMan[®] Genotyping Assay experiment, AmpliTaq Gold[®] DNA polymerase from the TaqMan[®] Genotyping Master Mix amplifies target DNA using sequence-specific primers.

TaqMan[®] MGB probes from the Genotyping Assay provide a fluorescence signal for the amplification of each allele.

PCR amplification requires one to:

1. “Prepare the reaction mix” on page 21.
2. “Prepare the DNA samples” on page 23.
3. “Prepare the reaction plate with the wet DNA method” on page 24 or “Prepare the reaction plate with the DNA dry-down method” on page 25.

4. "Perform the PCR" on page 26.

See Appendix G, "Good laboratory practices for PCR/RT-PCR" for general instructions on avoiding false positive amplifications.

Assay setup guidelines

To ensure optimal analysis and troubleshooting of the TaqMan[®] SNP Genotyping Assays, it is recommended to prepare an optical reaction plate containing the following for each assay:

- DNA samples with unknown genotype at the polymorphism of interest
- (*Strongly recommended*) Two no template controls (NTCs)
Use DNase-free water. Life Technologies strongly recommends using at least two NTCs per assay to:
 - Orient the VIC[®] -dye and/or FAM[™] -dye clusters to an origin
 - Enable the detection of DNA contamination on a given set of plates
- (*Optional*) DNA controls with known genotype at the polymorphism of interest

Reagents and samples preparation

- Keep all TaqMan[®] Genotyping Assays protected from light, in the freezer, until ready to use. Excessive exposure to light may affect the fluorescent probes
- Minimize freeze-thaw cycles
- Dilute the 40X or 80X Predesigned SNP or Custom SNP Genotyping Assay to a 20X working stock solution (see "Perform the dilution of SNP Genotyping Assays" on page 13)
Note: TaqMan[®] DME Genotyping Assays are supplied at a 20X concentration.
- Prior to use:
 - Thoroughly mix TaqMan[®] Genotyping Master Mix by swirling the bottle
 - Resuspend SNP Genotyping Assay by vortexing, then centrifuge the tube briefly
 - (*For wet DNA only*) Resuspend the thawed frozen samples by vortexing, then centrifuge the tubes briefly
 - Prepare the reaction mix for each assay before transferring it to the optical reaction plate for thermal cycling
 - Mix thoroughly after adding the reagents to the 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").

Set up the PCR reactions

Prepare the reaction mix

The reaction mix is composed of 20X, 40X, or 80X TaqMan[®] Genotyping Assay, TaqMan[®] Genotyping Master Mix, and nuclease-free water.

The recommended final reaction volume per well is 5 μ L for a 384-well plate, 10 μ L for a 96-well Fast plate, and 25 μ L for a 96-well plate (see Table 9 and Table 10).

1. Calculate the number of reactions to be performed for each assay, including recommended controls (see "Reagents and samples preparation" on page 21).

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

- Calculate the total volume of each component needed for each assay, using the following table.

Note: Prepare excess volume to account for pipetting errors.

IMPORTANT! Be sure to choose the appropriate DNA delivery method (wet or dry) according to the experiment type you wish to perform.

Table 9 Preparation of the reaction mix for wet DNA method

Component	384-Well Plate (5 μ L reaction)	96-Well Fast Plate (10 μ L reaction)	96-Well Plate (25 μ L reaction)
2X TaqMan [®] Master Mix	2.50 μ L	5.00 μ L	12.50 μ L
20X Assay Working Stock	0.25 μ L	0.50 μ L	1.25 μ L
Nuclease-free water	—	—	—
Total volume per well	2.75 μ L	5.50 μ L	5.00 μ L

Table 10 Preparation of the reaction mix for dried-down DNA method

Component	384-Well Plate (5 μ L reaction)	96-Well Fast Plate (10 μ L reaction)	96-Well Plate (25 μ L reaction)
2X TaqMan [®] Master Mix	2.50 μ L	5.00 μ L	12.50 μ L
20X Assay Working Stock	0.25 μ L	0.50 μ L	1.25 μ L
Nuclease-free water	2.25 μ L	4.50 μ L	11.25 μ L
Total volume per well	5.00 μ L	10.00 μ L	25.00 μ L

Note the following:

- When using TaqMan[®] Genotyping Master Mix, use fast reaction plates only on the QuantStudio[™] 6K, 7K or 12K Flex 96-well Fast block, ViiA[™] 7 96-well Fast Block, 7500 Fast Real-Time PCR System, or 9800 Fast Thermal Cycler and only with Standard thermal cycling conditions.
- TaqMan[®] SNP Genotyping Assays are also compatible with Universal Master Mix II and GTXpress[™] Master Mix (provided with the TaqMan[®] Sample-to-SNP[™] kit). However, TaqMan[®] Genotyping Master Mix is preferable to Universal Master Mix II for purified DNA because it will overall provide better performance and stronger signal.

- Due to the overall longer length of many TaqMan[®] DME Assay amplicons, Fast cycling conditions are not recommended if using GTXpress[™] Master Mix.
3. Swirl the bottle of 2X TaqMan[®] Genotyping Master Mix gently to mix the contents.
 4. Vortex and centrifuge the 20X Assay Working Stock, then mix briefly.
 5. Pipette the required volumes of 2X TaqMan[®] Genotyping Master Mix, 20X Genotyping Assay mix, and (for the dry-down method only) nuclease-free water into a sterile tube.
 6. Cap the tube.
 7. Vortex the tube briefly to mix the components.
 8. Centrifuge the tube briefly to spin down the contents and to eliminate air bubbles from the solution.

Prepare the DNA samples

Extract and purify genomic DNA

The genomic DNA (gDNA) should be extracted and purified according to standard practices. We recommend the gDNA extraction and purification kits listed in Table 4. Alternatively, the TaqMan[®] Sample-to-SNP[™] Kit can also be used (refer to the kit protocol for assay setup; see Table 23 for ordering information).

Quantitate the gDNA

We recommend quantifying the amount of gDNA in samples before using TaqMan[®] SNP Genotyping Assays.

Use a reliable method, such as:

- UV/Vis spectrophotometry [A_{260}/A_{280} measurement (Haque, 2003)]
- NanoDrop[®] spectrophotometry
- Qubit[®] 2.0 Fluorometer (Cat. no. Q32866) with Qubit[®] Assay Tubes (Cat. no. Q32856) and Qubit[®] dsDNA HS Assay Kit (Cat. no. Q32854)
- RNase P method (see “Absolute Quantitation” on page 53)

Methods for adding DNA

TaqMan[®] SNP Genotyping assays can be used with either wet or dried-down DNA. If the experiment requires multiple plates using the same gDNA or if the same gDNA is used in several experiments, drying down the gDNA in the plates is the most convenient method to have the samples ready for use at any time. The methods for assays using both wet and dried-down DNA are described here.

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

Table 11 DNA delivery methods

Method	Description	Experimental uses
Wet DNA delivery	<ol style="list-style-type: none"> 1. SNP reaction mix is aliquoted to an optical reaction plate. 2. gDNA is delivered to the final reaction mix. <p>Note: In this method, the liquid used to resuspend the DNA is used as a component of the final reaction.</p>	Large number of DNA templates tested on a limited number of SNP targets.
Dried-down DNA delivery	<ol style="list-style-type: none"> 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. 	<p>This method can be used in either of these conditions:</p> <ul style="list-style-type: none"> • Low DNA concentration results in large sample volumes (2 to 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.

Prepare the reaction plate with the wet DNA method

1. Dilute each DNA in nuclease-free water in order 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. Pipette the reaction mix (see “Prepare the reaction mix” on page 21) into each well of the reaction plate. Use the appropriate volumes listed in Table 9.
3. Cover the plate with MicroAmp[®] Optical Adhesive Film.
4. Centrifuge the plate briefly to spin down the contents and eliminate any air bubbles from the solutions.
5. Dilute 1–20 ng of each purified gDNA sample into nuclease-free water.
6. Pipette samples into the plate:
 - a. Remove the clear adhesive film from the plate.
 - b. Into each well of the plate, pipette one control or DNA sample, using the volumes listed in the following table. 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! Be sure that no cross-contamination occurs from well to well.

7. Cover the plate with MicroAmp[®] Optical Adhesive Film or MicroAmp[®] Optical Caps if you are using a MicroAmp[®] Optical 96-well plate. Seal the plate with a MicroAmp[®] Adhesive Film Applicator.

IMPORTANT! Use a MicroAmp[®] Optical Film Compression Pad when using MicroAmp[®] Optical Adhesive Film with a:

- MicroAmp[®] Optical 96-well plate on the 7900HT Real Time PCR System or 9800 thermal cycler
- MicroAmp[®] Optical 96- or 384-well plate on the GeneAmp[®] PCR System 9700

8. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.

Prepare the reaction plate with the DNA dry-down method

1. Pipette and dry the sample:
 - a. Pipette one control or sample (1-20 ng of purified gDNA or no template control consisting of nuclease-free water) in each well of a MicroAmp[®] Optical 96- or 384-Well Reaction Plate. Use a calibrated, positive-displacement pipettor to minimize contamination and error.

IMPORTANT! Use sample volumes of 2-5 µL to minimize drying time.

- b. Dry down 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! In the case of gDNA, do not accelerate drying by heating the plate. Heating the plate may cause poor gDNA recovery.

2. Pipette the reaction mix (see “Prepare the reaction mix” on page 21) into each well of the reaction plate. Use the appropriate volumes listed in Table 10.

- Cover the plate with MicroAmp[®] Optical Adhesive Film. Seal the plate with a MicroAmp[®] Adhesive Film Applicator.

IMPORTANT! Use a MicroAmp[®] Optical Film Compression Pad when using MicroAmp[®] Optical Adhesive Film with a:

- MicroAmp[®] Optical 96-well plate on the 7900HT Real Time PCR System or 9800 thermal cycler
 - MicroAmp[®] Optical 96- or 384-well plate on the GeneAmp[®] PCR System 9700
-

- Centrifuge the plate briefly to spin down the contents and eliminate any air bubbles from the solutions.

Perform the PCR

Refer to the appropriate instrument user guide for help with programming the thermal cycling conditions or with running the plate.

- Use the thermal cycling conditions specified in the following table:

Step	Predesigned SNP and Custom SNP			DME		
	Temp.	Duration	Cycles	Temp.	Duration	Cycles
AmpliAmp [®] Gold [®] , UP, Enzyme 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	

Note: TaqMan[®] DME Genotyping Assays use a 90 second extension time and 50 cycles. These conditions are for optimal performance due to the longer average amplicon size of TaqMan[®] DME Assays compared to that of TaqMan[®] SNP Genotyping Assays.

IMPORTANT! These conditions are optimized for use only with TaqMan[®] Genotyping Assays on the instruments and reaction plates specified in Table 5 and Table 6. Because of differences in ramp rates and thermal accuracy, you may need to adjust thermal cycling settings if you use a thermal cycler that is not listed.

- In the plate document in the case of real-time PCR systems, select the **Standard** mode thermal cycling setting.

Note: If using GTXpress™ Master Mix, follow the protocol provided with the Master Mix.

IMPORTANT! TaqMan® Genotyping Master Mix is not for use with Fast Mode thermal cycling conditions. If you use TaqMan® Genotyping Master Mix on the 7500 Fast or ViiA™ 7 96-Well Fast Block, or the 9800 Fast Thermal Cycler, use only Standard Mode thermal cycling conditions.

3. Set the reaction volume according to the following table.

Reaction Plate	Reaction Volume
MicroAmp® Optical 384-Well Reaction Plate	5.00 µL
MicroAmp® Fast Optical 96-Well Reaction Plate	10.00 µL
MicroAmp® Optical 96-Well Reaction Plate	25.00 µL

4. Load the reaction plate in the thermal cycler, then start the run.

Post-PCR plate read and analysis

Overview

After PCR amplification, perform a post-PCR plate read on a real-time PCR instrument. Using the fluorescence measurements made during the plate read, the real-time PCR instrument software plots R_n values based on the fluorescence signals from each well, then determines which alleles are in each sample.

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

The general process for analyzing data for allelic discrimination or genotyping involves:

- Creating and setting up a post-PCR plate read document
- Performing a post-PCR plate read on a real-time PCR instrument
- Analyzing the experiment
- Making automatic or manual allele calls
- Verifying allele types

Note: Refer to the allelic discrimination or genotyping section of the appropriate instrument user guide for instructions on how to use the system software to perform the post-PCR plate read and analysis. For more information, see Appendix A, “Troubleshooting”.

Life Technologies real-time instrument software plots the results of the allelic discrimination data as a scatter plot of Allele 1 (VIC® dye) versus Allele 2 (FAM™ dye). Each well of the 96-well or 384-well reaction plate is represented as an individual point on the plot.

Variation in clustering due to the genotype of the target allele is shown in Figure 1.

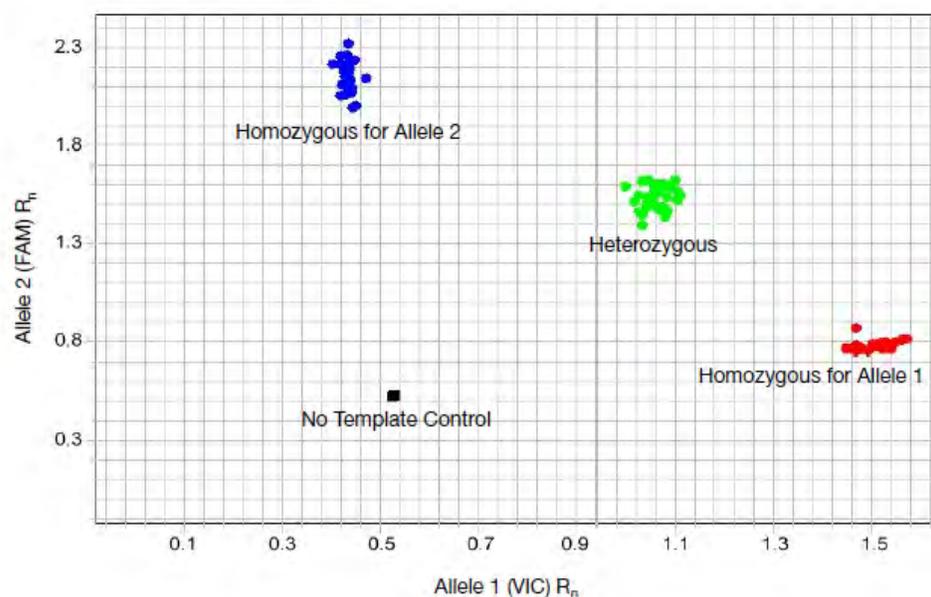


Figure 1 Example of an allelic discrimination plot

Analyze data using TaqMan[®] Genotyper[™] Software

TaqMan[®] Genotyper[™] Software is a standalone software application that can be used to analyze raw data from genotyping experiments created on a Life Technologies 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
 - Set 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
- 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, consult *TaqMan[®] Genotyper[™] Software Getting Started Guide* (Pub. no. 4448637).

Resources for data analysis

Data analysis varies depending on the instrument. Refer to the following documents for more information about analyzing your data:

- The appropriate instrument user guide (see “Obtaining documentation” on page 95)
- *TaqMan[®] Genotyper Software Getting Started Guide* (Pub. no. 4448637)
- Life Technologies website for a variety of tutorials on how to analyze data from TaqMan[®] Genotyping Assays (see “Obtaining support” on page 97)



Troubleshooting

About the allelic discrimination plot

Problems with sample preparation, running the assay, the instrument, and/or software can give rise to atypical results in the allelic discrimination plot, also known as a *cluster plot*, a *scatter plot*, or an *AD plot*. The genetic characteristics of the assay and/or the sample can also lead to unexpected results in the allelic discrimination plot.

Characteristics of a good allelic discrimination plot

An allelic discrimination plot is shown in Figure 2. Ideally, an AD plot shows one, two, or three clusters and, near the origin, the no template controls (NTCs), described in Table 12. The points in each cluster are grouped closely together and each cluster is located well away from the other clusters.

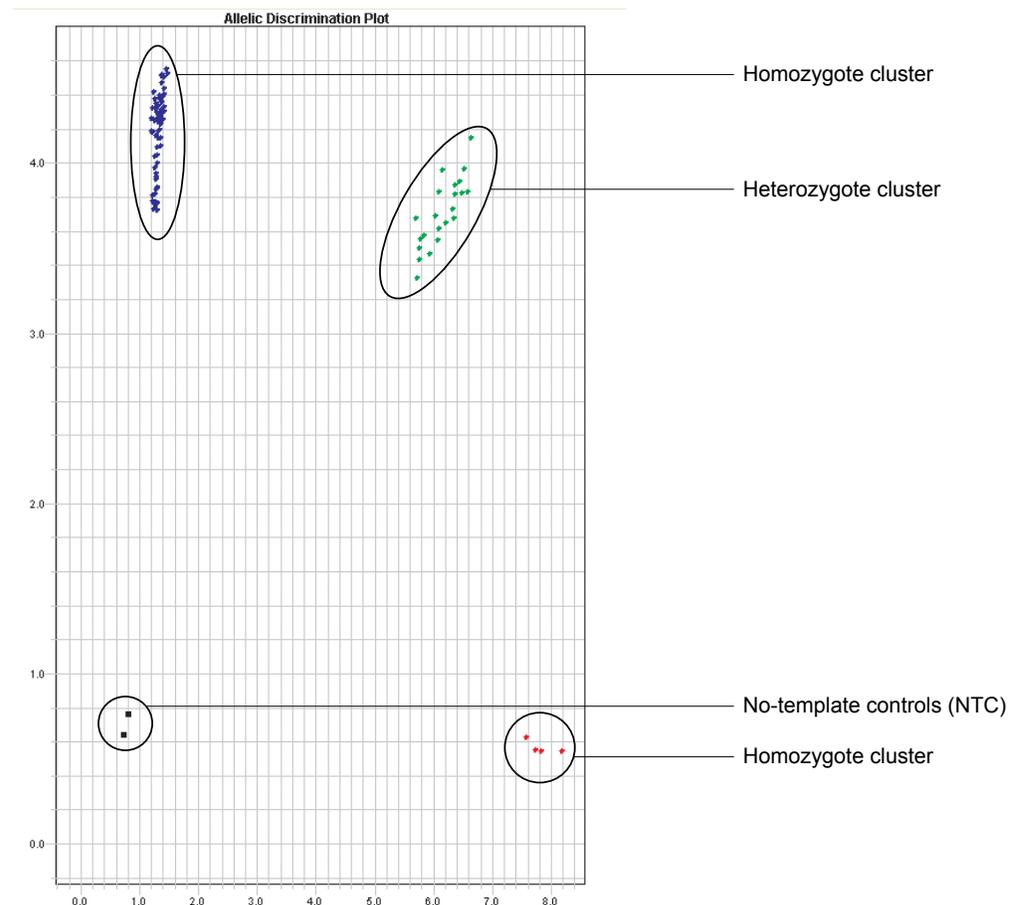


Figure 2 Typical 3-cluster allelic discrimination plot

Table 12 Assignment of clusters in an allelic discrimination plot

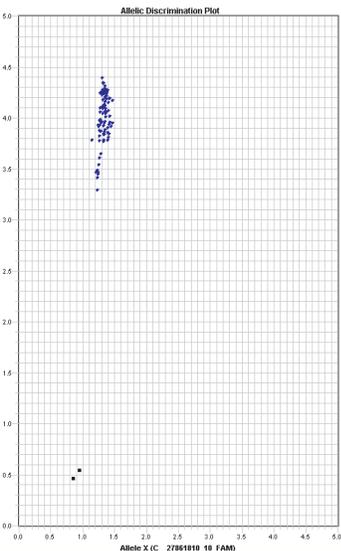
Content of samples	Location in AD plot
Allele X (homozygote), labeled with VIC [®] dye	Lower right corner
Allele Y (homozygote), labeled with FAM [™] dye	Upper left corner
Alleles X and Y (heterozygote)	Approximately midway between Allele X and Allele Y clusters
No Template Control (NTC)	Bottom left corner
Undetermined	Anywhere outside the regions described above
No amplification	With NTC cluster in the bottom left corner

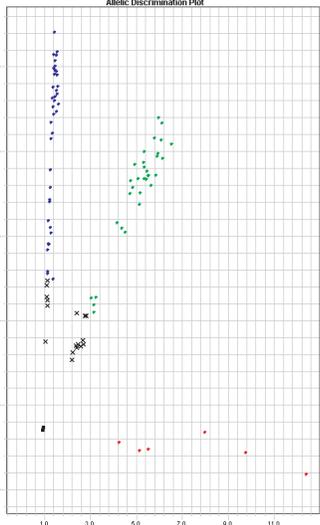
Troubleshooting the AD plot

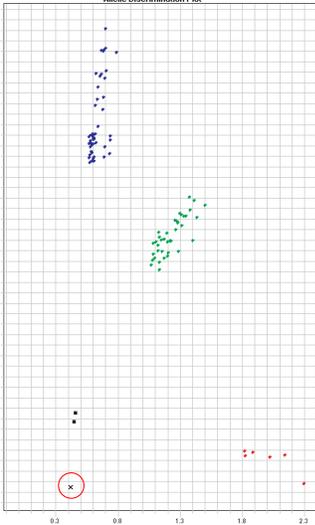
Unexpected patterns in AD plots

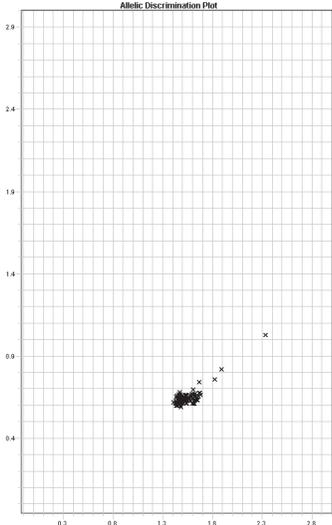
AD plots do not always present a three-cluster pattern for various reasons, including genetic reasons. However, other issues such as sample preparation and/or assay issues may also cause unexpected patterns.

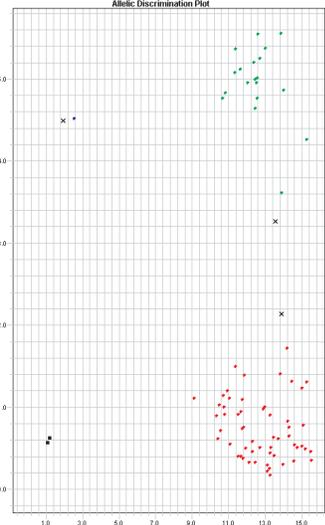
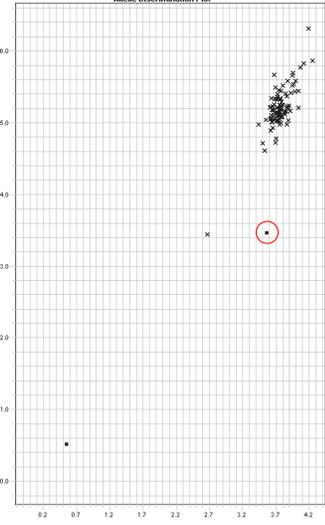
The following table summarizes unexpected patterns observed in AD plots and indicates the pages in this manual where a discussion of these patterns and their causes can be found.

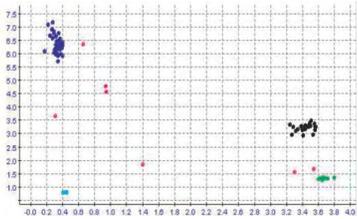
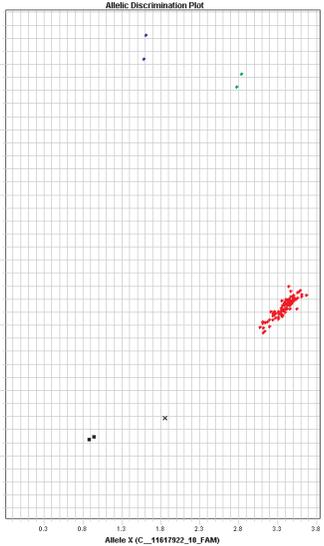
Observation	Possible cause	Recommended action
Only one or two clusters present 	<i>Genetic Reasons:</i> MAF is too low for sample size from the tested population	See "Low allele frequency" on page 39

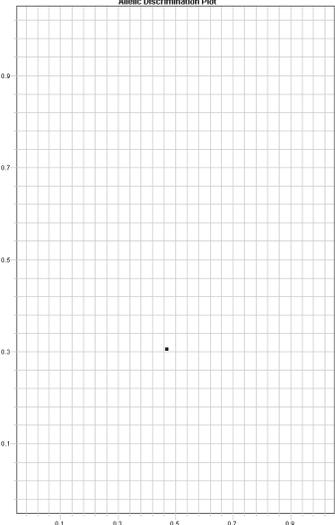
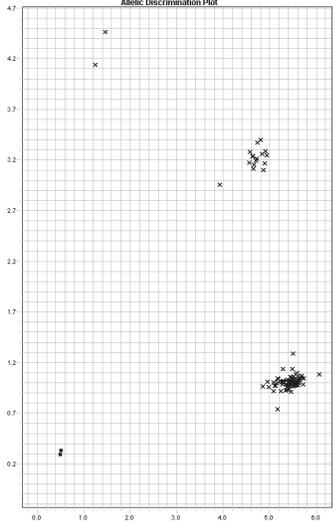
Observation	Possible cause	Recommended action
<p>Trailing clusters</p> 	<i>Sample Preparation Problems:</i>	
	Samples are not in equal quantity due to degraded DNA	See “Degraded DNA” on page 48
	Samples are not in equal quantity due to inaccurate DNA quantitation	See “Inaccurate DNA quantitation” on page 52
	PCR inhibitors in sample	See “PCR inhibitors in sample” on page 51
	<i>Assay Problems:</i>	
	Reagents mishandled or expired	See “Reagents mishandled or expired” on page 54
	ROX™ dye not present in PCR master mix	See “Using a master mix without ROX dye” on page 55
	Evaporation from the reaction well	See “Evaporation from the reaction well” on page 56
	Pipetting errors	See “Pipetting errors” on page 56
	Inefficient mixing and/or insufficient centrifugation	See “Inefficient mixing or centrifugation” on page 57
	<i>Instrument Problems:</i>	
	Thermal cycler poorly calibrated	See “Instrument calibration” on page 58
	<i>Software Problems:</i>	
	ROX™ dye not designated as the reference dye	See “ROX dye not designated as reference” on page 61

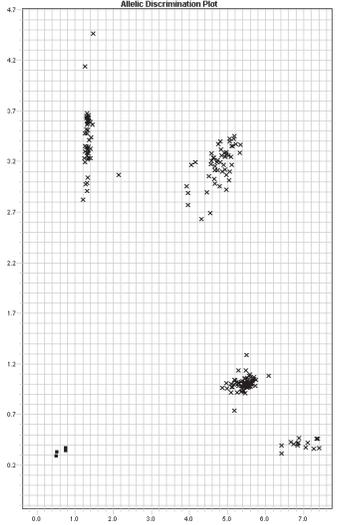
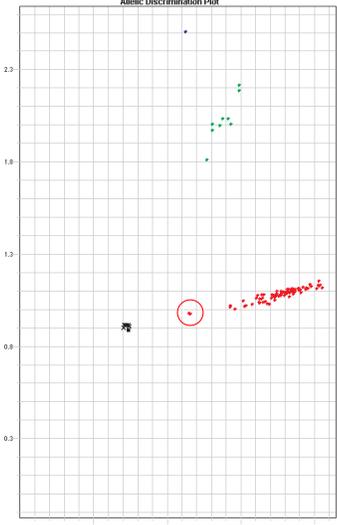
Observation	Possible cause	Recommended action
Some samples cluster with the NTCs 	<i>Genetic Reasons:</i>	
	Individual sample has two null alleles	See “Null alleles in an individual” on page 41
	SNP is triallelic	See “SNP is triallelic or tetra-allelic” on page 46
	SNP is on the Y chromosome and some samples are female	See “Gene on the Y chromosome” on page 48
	<i>Sample Preparation Problems:</i>	
	Samples are not in equal quantity due to degraded DNA	See “Degraded DNA” on page 48
	Samples are not in equal quantity due to inaccurate DNA quantitation	See “Inaccurate DNA quantitation” on page 52
	PCR inhibitors in sample	See “PCR inhibitors in sample” on page 51
	<i>Assay Problems:</i>	
	Evaporation from the reaction well	See “Evaporation from the reaction well” on page 56
	DNA or reagent not added to the well	See “DNA or assay reagent not added to the reaction well” on page 55
	Insufficient DNA added to the well	See “Insufficient DNA added to the reaction well” on page 55
	Pipetting errors	See “Pipetting errors” on page 56
	Inefficient mixing and/or insufficient centrifugation	See “Inefficient mixing or centrifugation” on page 57

Observation	Possible cause	Recommended action
<p>All samples cluster with the NTC</p> 	<i>Sample Preparation Problems:</i>	
	Samples are not in equal quantity due to degraded DNA	See “Degraded DNA” on page 48
	Samples are not in equal quantity due to inaccurate DNA quantitation	See “Inaccurate DNA quantitation” on page 52
	PCR inhibitors in sample	See “PCR inhibitors in sample” on page 51
	<i>Assay Problems:</i>	
	Reagents mishandled or expired	See “Reagents mishandled or expired” on page 54
	DNA or reagent not added to the well	See “DNA or assay reagent not added to the reaction well” on page 55
	Insufficient DNA added to the well	See “Insufficient DNA added to the reaction well” on page 55
	Pipetting errors	See “Pipetting errors” on page 56
	<i>Instrument Problems:</i>	
	Annealing temperatures on the thermal cycler were too high or too low for the primers or probes due to poor calibration	See “Instrument calibration” on page 58
	AmpliAq Gold® polymerase was not efficiently activated due to incorrect thermal cycler method	See “Perform the PCR” on page 26
	AmpliAq Gold® polymerase was not efficiently activated due to poor calibration of the thermal cycler	See “Instrument calibration” on page 58

Observation	Possible cause	Recommended action
<p>Cloudy or diffuse clusters</p> 	<p><i>Sample Preparation Problems:</i></p> <p>Samples are not in equal quantity due to degraded DNA</p> <p>Samples are not in equal quantity due to inaccurate DNA quantitation</p> <p>PCR inhibitors in sample</p> <p><i>Assay Problems:</i></p> <p>ROX™ dye not present in PCR Master Mix</p> <p>Evaporation from the reaction well</p> <p>Pipetting errors</p> <p><i>Instrument Problems:</i></p> <p>ROX™ dye not designated as the reference dye</p> <p>If only one cluster is present, allelic discrimination plot incorrectly scaled</p>	<p></p> <p>See “Degraded DNA” on page 48</p> <p>See “Inaccurate DNA quantitation” on page 52</p> <p>See “PCR inhibitors in sample” on page 51</p> <p></p> <p>See “Using a master mix without ROX dye” on page 55</p> <p>See “Evaporation from the reaction well” on page 56</p> <p>See “Pipetting errors” on page 56</p> <p></p> <p>See “ROX dye not designated as reference” on page 61</p> <p>See “Single cluster assay” on page 60</p>
<p>NTCs generate high fluorescence signals that cluster with samples rather than close to the origin</p> 	<p><i>Assay Problems:</i></p> <p>Reagents mishandled or expired</p> <p>Contamination due to poor laboratory practices</p> <p><i>Instrument Problems:</i></p> <p>Block contaminated</p> <p><i>Software Problems:</i></p> <p>Reporter dye assigned incorrectly</p>	<p></p> <p>See “Reagents mishandled or expired” on page 54</p> <p>See Appendix G, “Good laboratory practices for PCR/RT-PCR”</p> <p></p> <p>See “Routine instrument maintenance” on page 58</p> <p></p> <p>See “ROX dye not designated as reference” on page 61</p>

Observation	Possible cause	Recommended action
<p>Sample (or samples) did not cluster with specific allele</p>  <p>Note: In this plot:</p> <ul style="list-style-type: none"> • Homozygotes are blue and green • Heterozygotes are black • NTCs are light blue • Outliers are pink 	<i>Genetic Reasons:</i>	
	Additional SNP under primer	See “Additional SNP present under the probe or primer” on page 42
	SNP is triallelic or tetra-allelic	See “SNP is triallelic or tetra-allelic” on page 46
	Copy number polymorphism	See “Gene has a copy number polymorphism” on page 44
	<i>Sample Preparation Problems:</i>	
	Samples are not in equal quantity due to degraded DNA	See “Degraded DNA” on page 48
	Samples are not in equal quantity due to inaccurate DNA quantitation	See “Inaccurate DNA quantitation” on page 52
	<i>Assay Problems:</i>	
	Contamination due to poor laboratory practices	See Appendix G, “Good laboratory practices for PCR/RT-PCR”
	Evaporation from the sample well	See “Evaporation from the reaction well” on page 56
	Pipetting errors	See “Pipetting errors” on page 56
	More than one sample in the well	See “More than one sample in the well” on page 57
	Inefficient mixing and/or insufficient centrifugation	See “Inefficient mixing or centrifugation” on page 57
<i>Instrument Problems:</i>		
Block contaminated	See “Routine instrument maintenance” on page 58	
<p>Samples not in Hardy-Weinberg equilibrium (expected ratios of each genotype not seen)</p> 	<i>Genetic Reasons:</i>	
	Copy number polymorphism	See “Gene has a copy number polymorphism” on page 44
	Gene is on X chromosome	See “Gene on the X chromosome” on page 47
	<i>Software Problems:</i>	
Detectors and markers set up incorrectly	See “No marker assigned to the well” on page 60	

Observation	Possible cause	Recommended action
<p>Some (or all) data is missing (no data point shown on AD plot)</p> 	<i>Software Problems:</i>	
	No marker assigned to sample	See “No marker assigned to the well” on page 60
	May have checked Omit for the missing well(s)	See “Omit option checked in Real Time PCR instrument software” on page 60
<p>Some (or all) alleles not called (X is shown on the AD plot)</p> 	<i>Software Problems:</i>	
	NTC task not assigned to NTC wells	See “NTCs not assigned” on page 62
	Autocall option not selected	See “Autocall is not selected” on page 60
	Sample only has two clusters, but 2-cluster calling option not selected (software cannot assign alleles)	See “Two cluster calling not selected” on page 60
	Sample has only one cluster (software cannot assign alleles)	See “Single cluster assay” on page 60
	Outlier sample too far off scale for alleles to be called for other samples	See “Outlier too far off scale” on page 61

Observation	Possible cause	Recommended action
<p>More than 3 clusters</p> 	<i>Genetic Reasons:</i>	
	Additional SNP under probe	See “Additional SNP present under the probe or primer” on page 42
	Copy number polymorphism	See “Gene has a copy number polymorphism” on page 44
	SNP is triallelic or tetra-allelic	See “SNP is triallelic or tetra-allelic” on page 46
	<i>Software Problems:</i>	
	Several assays were run but only one marker was assigned	See “Too many alleles called in AD plot” on page 63
<p>Vector clusters (sample data has two clusters at the same angle)</p> 	<i>Genetic Reasons:</i>	
	Additional SNP under probe	See “Additional SNP present under the probe or primer” on page 42
	Copy number polymorphism	See “Gene has a copy number polymorphism” on page 44
	<i>Sample Preparation Problems:</i>	
	Samples not in equal quantity due to degraded DNA	See “Degraded DNA” on page 48
Samples not in equal quantity due to inaccurate DNA quantitation	See “Inaccurate DNA quantitation” on page 52	

Genetic issues

Genetic issues may cause atypical or unexpected assay results, including:

- “Low allele frequency” on page 39
- “Null alleles in an individual” on page 41
- “Additional SNP present under the probe or primer” on page 42
- “Gene has a copy number polymorphism” on page 44
- “SNP is triallelic or tetra-allelic” on page 46

- “Gene on the X chromosome” on page 47
- “Gene on the Y chromosome” on page 48

Use this section to determine the reasons for unexpected or atypical genetic results.

Low allele frequency

Only one or two clusters can occur in the AD plot, as shown in Figure 3, when the minor allele occurs at a very low frequency in the population being studied.

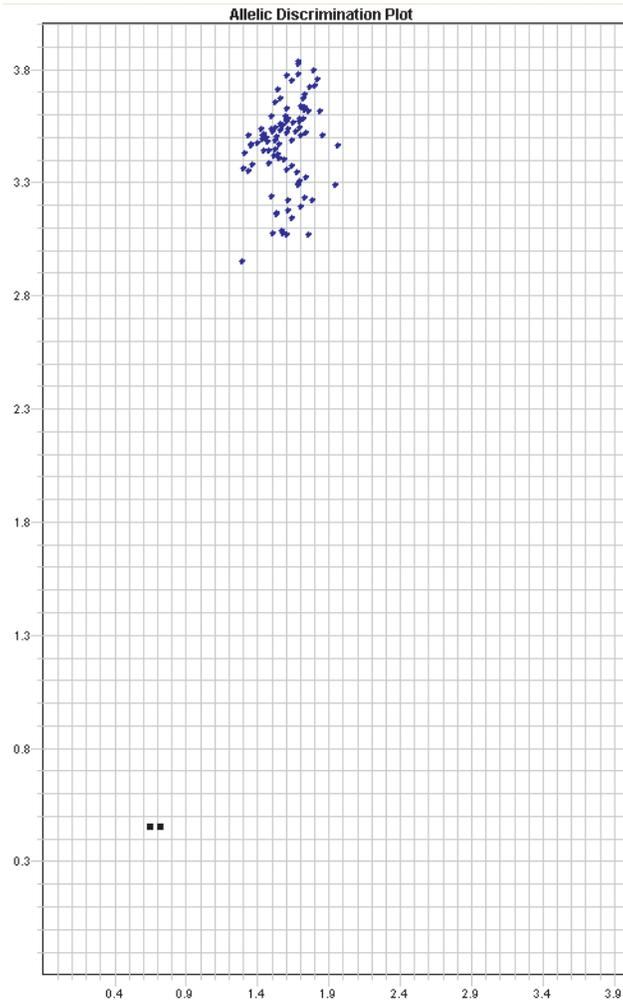


Figure 3 Allelic discrimination plot showing a single cluster (in addition to the NTCs)

What to do

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

1. Find the MAF for your assay on the TaqMan[®] SNP Genotyping Assays page at www.lifetechnologies.com, which is frequently updated.
Alternatively, the MAF can be found in the Assay Information File distributed with the assays. Allele frequency data can also be found using the public SNP identifier, from public websites such as:
 1. dbSNP at <http://www.ncbi.nlm.nih.gov/SNP/index.html>
 2. the HapMap project at <http://www.hapmap.org>

- 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 40).

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 given population that would be homozygous for the minor allele (qq), heterozygous (qp), and homozygous for the major allele (pp), respectively.

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

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

Example calculation

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

- Homozygotes for the minor allele - $0.0025 \times 20 = 0.05$, no individuals
- Heterozygotes - $0.095 \times 20 = 1.9$, about 2 individuals
- Homozygotes for the major allele - $0.9025 \times 20 = 18.05$, 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

Several of the 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 which may occur at very low frequencies, depending on the population you are studying. Many functional polymorphisms occur at low frequencies (Wong, 2003). 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 needs to be to detect a specific allele. The lower the frequency of the minor allele, the larger the sample size required to detect the allele.

The allele frequencies for validated TaqMan[®] SNP and DME Genotyping Assays were calculated for four populations:

- African American
- Caucasian
- Chinese
- Japanese

Some of the assays are for polymorphisms that may:

- Not occur in some populations
- Have very low minor allele frequencies

An example is assay ID C__11703892_30 for gene ALDH2 (rs671). In the Caucasian/African American test populations, the minor allele frequency was 0 but in the tested Chinese/Japanese populations it was 20-30%.

During development of TaqMan[®] SNP and DME Genotyping Assays, a sample size of 45 people was tested in each of the four populations. This sample size provides 95% confidence that alleles with a minor allele frequency of 5% will be detected. Therefore, the sample sizes of the populations tested for the DMEs were too small to detect allele frequencies less than 5%.

Null 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 individual has a "null allele." The data point in the allelic discrimination plot from such an individual will either:

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

If an individual sample consistently clusters with the NTCs for a particular assay, it may indicate the individual has a null allele. There are documented occurrences of null alleles in the genes CYP2A6 (Topcul, 2002), GSTM1 (Smits, 2003), GSTT1 (Bolt, 2006; Thier, 2006; Cho, 2005; Rebbeck, 1997), and CYP2D6 (Luo, 2005; Zanger, 2004) in TaqMan[®] Drug Metabolism Genotyping Assays collection.

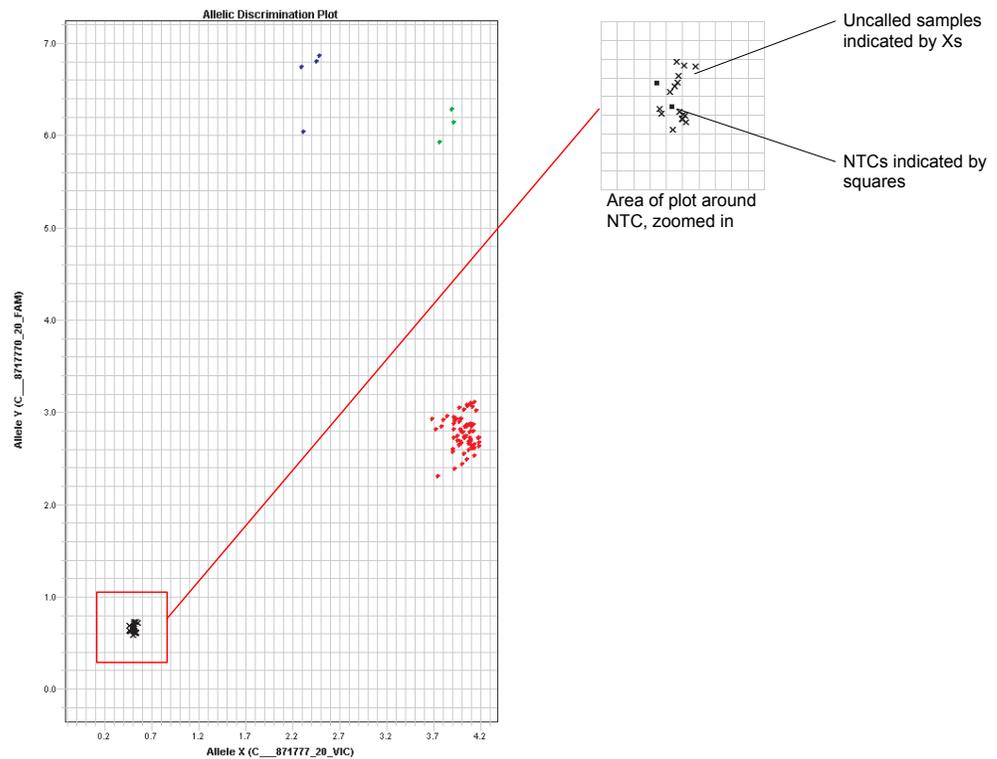


Figure 4 Allelic discrimination plot showing a null allele for the assay C__8717770_20

What to do

1. Evaluate the overall assay performance:
 - Do the assay results appear in tight clusters?
 - Do the clusters have good separation?
1. Repeat the experiment. If the same sample(s) consistently cluster with the NTC while other samples show fluorescence, a null allele may be present.

2. Examine the sample's performance in other assays to rule out problems caused by this particular sample, such as sample impurity or degradation.
3. Perform a literature search for documentation reporting the presence of null alleles for the gene.
4. Perform analysis using a TaqMan[®] Copy Number Assay (Cat. no. 4400291) on all samples to confirm the sample has a null 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.

Note: To locate the copy number assay of interest, find them in the Assays search page at www.lifetechnologies.com.

Additional SNP present under the probe or primer

A non-target SNP under a primer or probe may 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. You may see additional clusters ("angle clusters") or a lack of amplification of the sample when there is an additional polymorphism under the primer (Figure 5). The presence of a polymorphism under a primer generally leads to lower PCR efficiency.

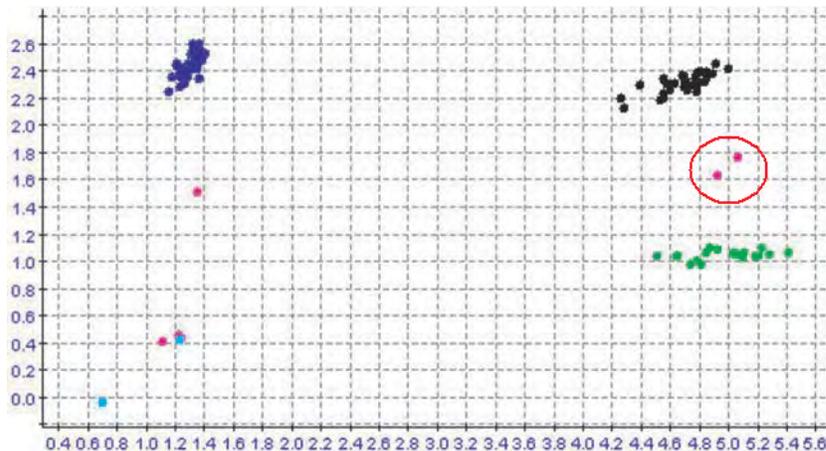


Figure 5 SNP under primer. The points in pink between the black and green cluster constitute an angle cluster.

A SNP under a probe can result in an outlier that falls between the heterozygote and one of the homozygotes (an angle cluster) or 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 6). The presence of a SNP under a probe leads to lower fluorescence intensity.

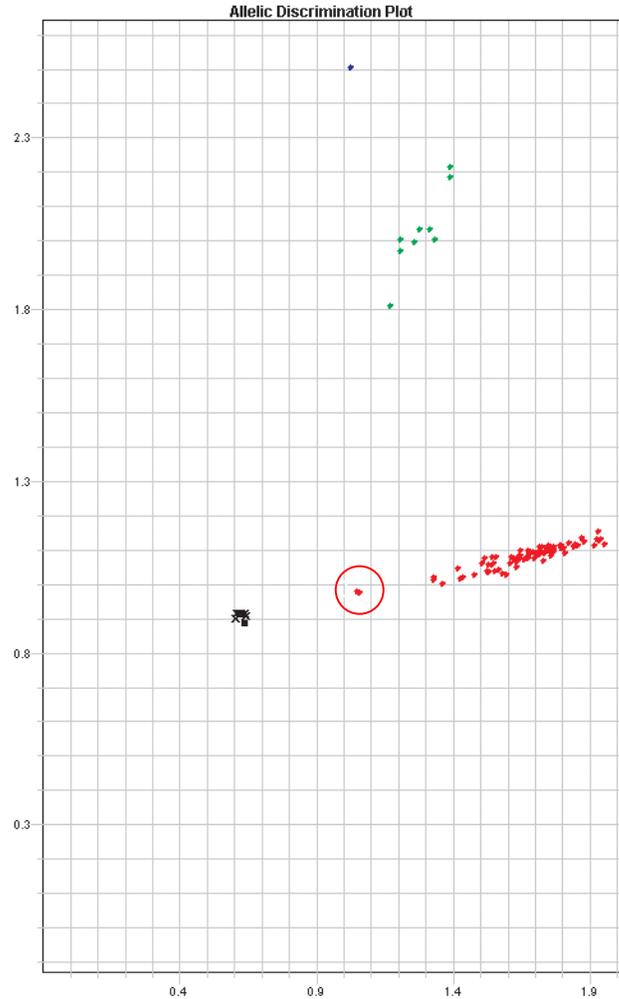


Figure 6 SNP under probe circled in red. The red cluster is a vector cluster.

What to do

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

1. Repeat the experiment and 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

Life Technologies assay design process included many checks to assure that primers and probes were not designed over polymorphisms other than the intended SNP

target. However, the growing number of SNPs discovered in studies of different ethnic populations make it likely 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 (Ouahchi, 2006; Wilkinson, 2005), GSTM1 (Ouahchi, 2006), GSTT (Ouahchi, 2006), CYP2E1 (Liew, 2005), and CYP2A6 (Ouahchi, 2006; Oscarson, 2001; Rao, 2000; Xu, 2002).

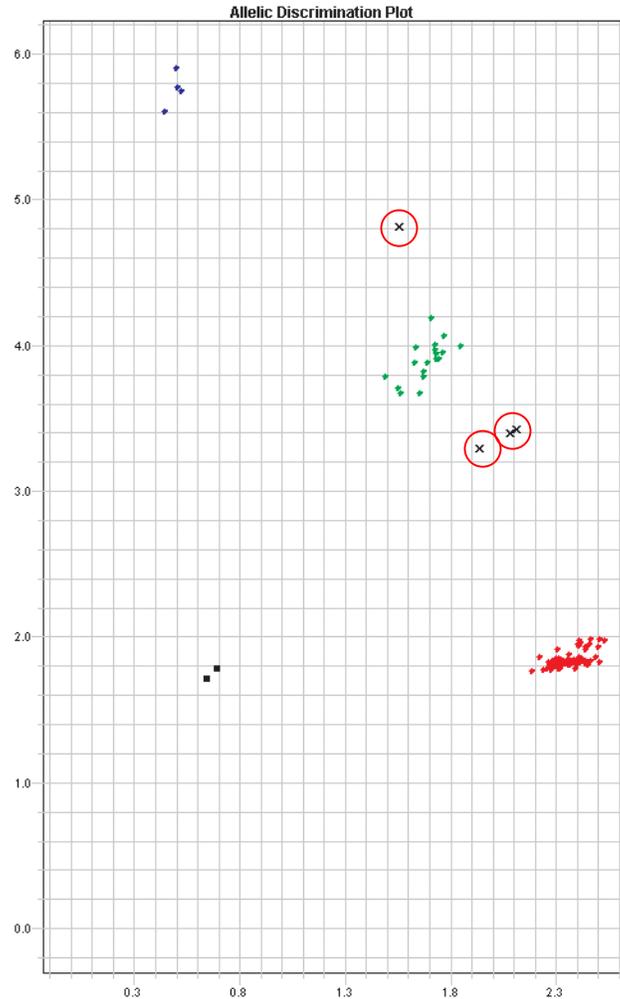


Figure 7 Allelic discrimination plot for CYP2D6, showing samples with a copy number polymorphism (circled in red) for assay C__32407252_30

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.

Note the following:

- The Copy Number Assay of interest can be found on the Assays search page at www.lifetechnologies.com.
- A Custom TaqMan[®] Copy Number Assay can be designed if a pre-designed Copy Number Assay does not exist for a target of interest.
- Copy Number Assays 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 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 or tetra-allelic

When a SNP is triallelic or tetra-allelic, you may see outlier samples in the allelic discrimination plot, although the samples may not be well separated from the main clusters. You may also see more than three clusters. These situations are best confirmed by running replicate plates. Life Technologies did not include known triallelic SNPs in the predesigned TaqMan[®] SNP Genotyping Assays collection. However, Life Technologies provides pairs of assays to several important DME gene variants that are triallelic SNPs (see “TaqMan DME Assays for genotyping triallelic SNPs” on page 73 for more information).

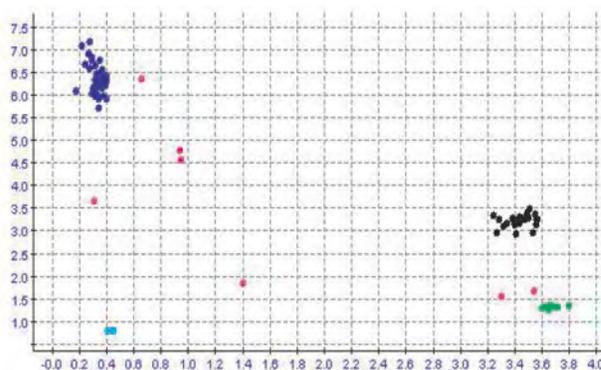


Figure 8 Allelic discrimination plot for a triallelic gene (additional clusters shown in pink)

What to do

1. Evaluate overall assay performance: Are there consistent outlier samples?
2. Examine the sample's performance in other assays to rule out problems 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 located 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 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 a SNP is triallelic, you might see six clusters (three homozygotes and three heterozygotes) rather than the typical pattern of three clusters (two homozygotes and one heterozygote). If a SNP is tetra-allelic, the possible cluster pattern can be more complicated. Figure 9 shows an assay created for the SNP rs2032582 in the ABCB1 gene which is known to have three alleles in several populations.

In the triallelic example in Figure 9, the following alleles are present:

Table 13 Possible genotypes in a triallelic SNP

Bases in DNA	Possible Homozygotes	Possible Heterozygotes
Original SNP: G, T	GG and TT	GT
Triallelic SNP: G, T, A	GG, TT, and AA	AT, GT, and GA

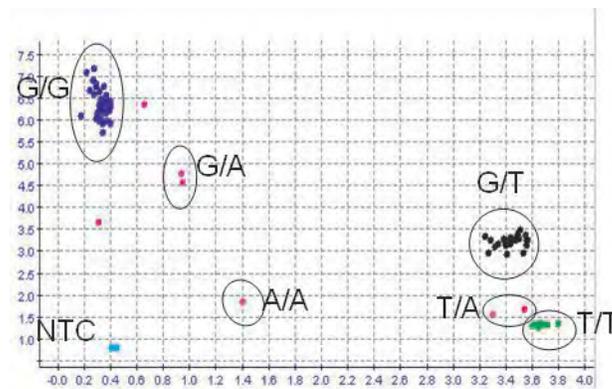


Figure 9 Allelic discrimination plot for a triallelic SNP with genotype for each cluster shown

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.

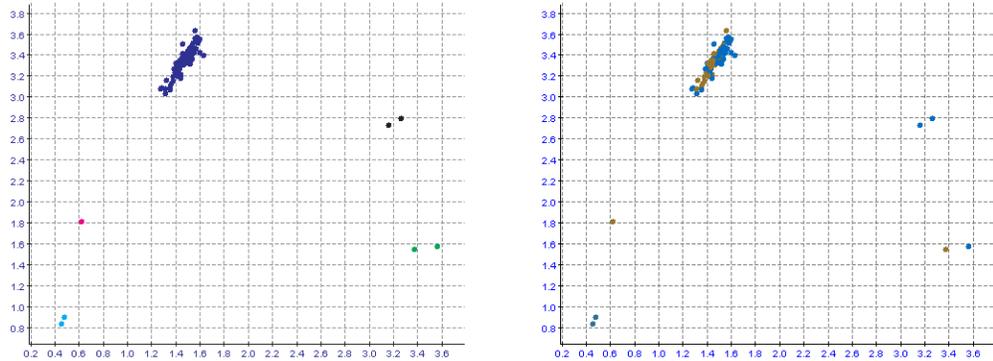


Figure 10 Allelic discrimination plot with a small number of heterozygotes for assay C__11617922_10 for SNO rs6324 in the MAOB gene (colored by gender on the right panel: blue – female, brown – male)

What to do

1. Check the AIF included with the assay or the TaqMan[®] SNP or DME Genotyping Assays page at www.lifetechnologies.com 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. Note that there are no male heterozygotes in Figure 10.

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 2 clusters will be visible because males have only one Y chromosome.

Sample preparation problems

Problems with preparing genomic DNA for the assay may include:

- “Degraded DNA” on page 48
- “PCR inhibitors in sample” on page 51
- “Inaccurate DNA quantitation” on page 52

Degraded DNA

Degraded DNA can affect PCR efficiency due to the presence of fewer template copies, which will affect the success of TaqMan[®] SNP Genotyping Assay. 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 or physical shearing

- 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 11 illustrates DNA degraded by heat. 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.

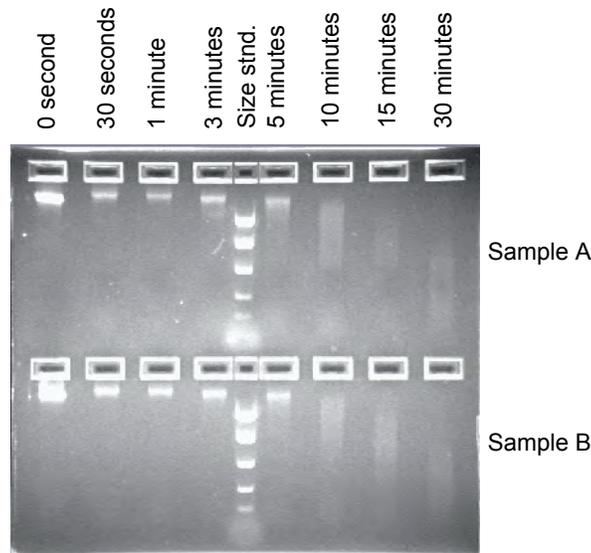


Figure 11 Agarose gel stained with ethidium bromide showing two samples of human gDNA subjected to heating at 99°C for 0 to 30 minutes.

- For future experiments, follow the sample storage guidelines in Table 14.

Discussion

As 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. An example of degradation by heating is illustrated in Figure 12. You can expect similarly poor assay results for gDNA degraded by other causes.

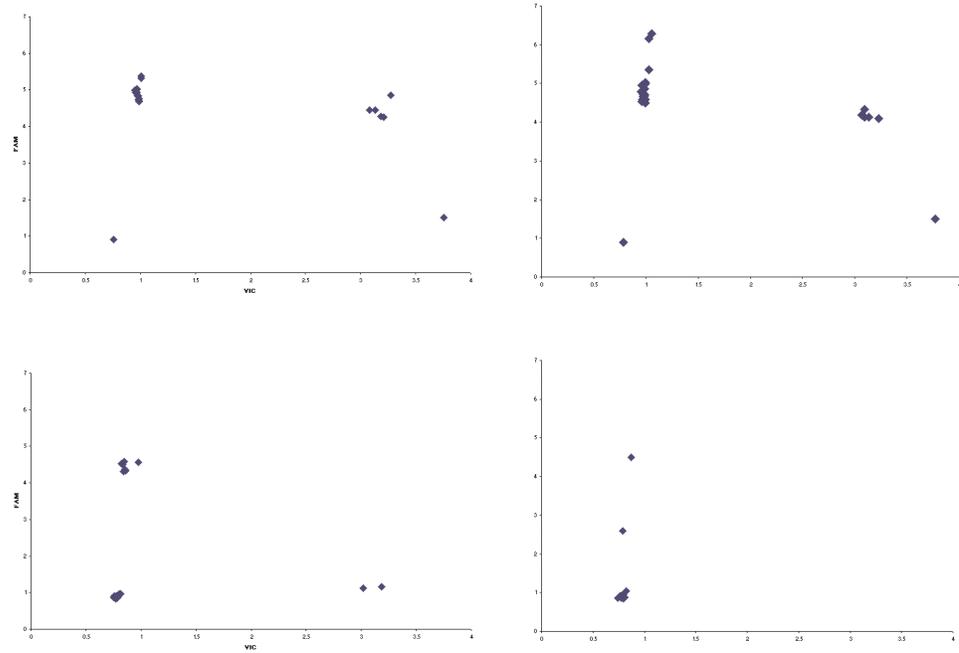


Figure 12 Allelic discrimination plot showing the effects of DNA degradation caused by heating

Factors that affect DNA degradation include tissue preservation methods, exposure to UV radiation, temperature, pH, and salt concentration of the environment (Dean, 2001). There are many sources of gDNA including fresh capillary blood, buccal scrapes, solid organ biopsies, and paraffin embedded tissue.

Table 14 Recommendations for sample storage conditions to minimize DNA degradation

Tissue Type	Storage Conditions
Buccal tissue	Store frozen between -15°C and -25°C
Tissue	Immediately place tissue in liquid nitrogen and store at -80°C or Freeze and store between -15°C and -25°C
Blood	<ul style="list-style-type: none"> Whole blood: store frozen between -15°C and -25°C Buffy coat: store frozen 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. Examples of inhibitors originating from the cell include heparin (Holodniy, 1991), proteins, and heme (Akane, 1994; DeFranchis, 1998). Examples of inhibitors originating from DNA preparation are phenol (Katcher, 1994), proteases, detergents (SDS), and salts.

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

1. 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.
2. Re-purify the sample and run the assay again.

Discussion

Inhibition of PCR is always possible when DNA is extracted from tissue and/or blood samples. Life Technologies examined the effects of hematin on the TaqMan[®] SNP and DME Genotyping Assays. Hematin was added to each well of the assay at one of three concentrations (0.25 μ M, 0.50 μ M, and 1.00 μ M), except for the wells containing the control samples. The results are shown in Figure 13.

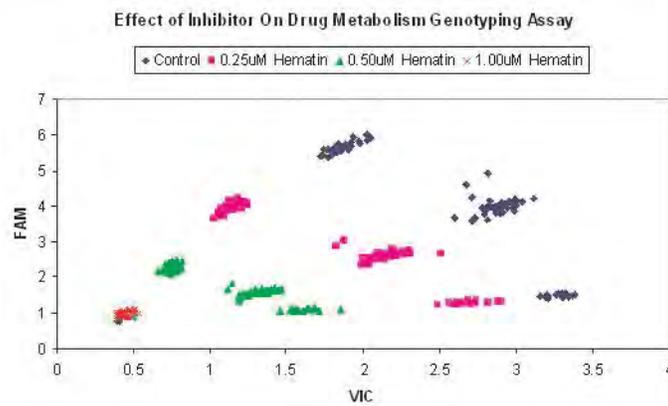


Figure 13 PCR inhibition as a function of hematin concentration

PCR inhibition effects begin at 0.25 μ M hematin. Assay performance is severely compromised at 0.50 μ M hematin, and although signal strength is significantly lowered, it is still possible to call genotypes. Assay performance is entirely inhibited at 1.00 μ M, where cleaving of the probes is nonexistent, resulting in no fluorescence.

The DNA purification method used to prepare gDNA can affect the success of PCR (Maaroufi, 2004). The selected method must minimize degradation and remove inhibitors. One method for assessing DNA purity is to calculate the A_{260}/A_{280} ratio. In addition, absorbance at 230 nm can indicate the presence of phenol (Gallagher, 1994).

In Life Technologies laboratories, A_{260}/A_{280} ratios between 1.8 and < 2.0 , indicate that the gDNA samples are pure enough to use for TaqMan[®] SNP and DME Genotyping Assays. The effective read range of UV spectroscopy is 0.1 to 0.999, which corresponds approximately to 4 ng/ μ L to 50 ng/ μ L of gDNA. Values above or below that range are invalid absorbance readings. To ensure accurate quantitative results,

gDNA samples should be diluted so that the A_{260} reading is between 0.1 and 0.999. Dilution factor and the diluents used must be recorded.

Most plates and cuvettes have minimum working volumes, and the gDNA sample used for the quantitative measurement will be discarded. It is therefore necessary to ensure having enough gDNA to use this method and still leave sufficient DNA for the study.

Inaccurate DNA quantitation

Problems with DNA quantitation manifest themselves as:

- 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)

What to do

- Always perform your own concentration measurements before using any genomic DNA (gDNA), even commercially prepared DNA
- Use the recommended amount of gDNA, 3 to 20 ng per sample per assay
- Always use the same quantity of gDNA for all samples of an assay on a plate

Discussion

The amount of gDNA is critical to the success of the assays. Within an assay and/or study, gDNA concentration uniformity leads to accurate, robust, and reproducible results and ensures efficient use of valuable samples. Variability in gDNA concentrations can lead to experimental anomalies that may affect interpretation of genotyping results, as shown in Figure 14. Precise handling and quantitative measurements before running an assay can prevent possible errors without waste of reagents and samples.

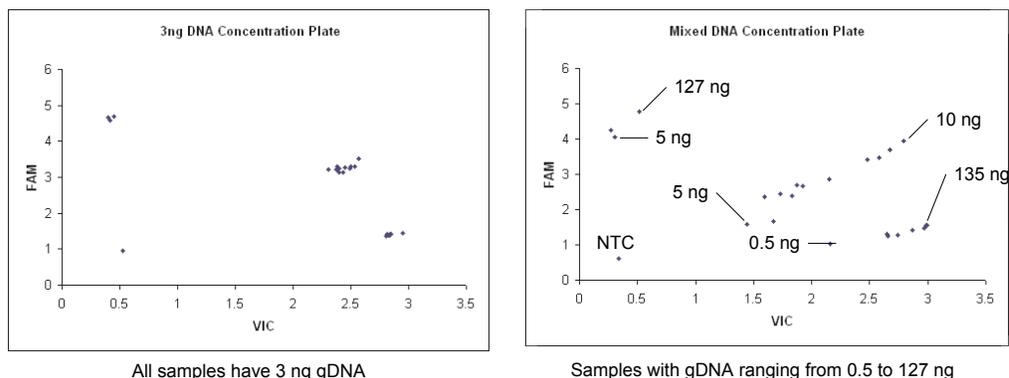


Figure 14 Allelic discrimination plots for TaqMan[®] DME Genotyping Assay C__1204092_20

Commercially purchased DNA comes with concentration information, but it is good practice to confirm DNA concentrations. Life Technologies has found that DNA concentrations listed for commercially available gDNA can be quite different from in-house measurements.

There are numerous methods for quantitating genomic DNA, including:

- UV spectroscopy

- Absolute quantitation
- Fluorometric analysis

Life Technologies recommends quantitating gDNA using UV spectroscopy or absolute quantitation using the TaqMan® RNase P method.

UV spectroscopy

UV Spectroscopy is the most widely used method for quantifying all types of DNA; however, the consumable reagents used in the process vary greatly. A spectrophotometer must be set up correctly for the reagents and equipment used.

Optical plastic cuvettes and plates have different background constants than quartz cuvettes and plates; consult the instrument's manual for ways to determine the background constant. Also, be cautious of the diluents used with the gDNA samples. They too can have differing properties and may affect the final results.

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 quantities (Gallagher, 1994). 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 51).

The effective read range of UV spectroscopy is 0.1 to 0.999, which corresponds approximately to 4 ng/ μ L to 50 ng/ μ L 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 to 0.999.

Remember to record the dilution factor and the diluents used. Most plates and cuvettes have minimum working volumes, and the gDNA sample used for the quantitative measurement will be discarded. Ensure that you have enough gDNA to use this method.

Absolute Quantitation

Absolute quantitation 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-quantitated and validated using an independent method such as spectrophotometry or fluorometry. The unknown samples are compared to the known samples for quantitation.

Two well-known techniques for absolute quantitation are:

- TaqMan® assay chemistry (RNase P)
- SYBR® Green assay

Absolute quantitation using TaqMan® assay chemistry is a highly accurate technique for quantifying DNA. TaqMan® DNA Template Reagents (Cat. no. 401970) and TaqMan® RNase P Detection Reagents (Cat. no. 4316831) allow for convenient means to quantify gDNA. They include pre-diluted and validated standards at five concentrations: 0.6 ng/ μ L, 1.2 ng/ μ L, 3.0 ng/ μ L, 6.0 ng/ μ L, and 12.0 ng/ μ L. Dilute or aliquot to the appropriate range for your samples.

SYBR® Green is a dye which binds only to double-stranded DNA (dsDNA). Quantitation with SYBR® Green assay chemistry is less specific than TaqMan® assay

chemistry 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 verify the specificity of the assay.

For either technique, be sure to run the standard curve and unknown samples on the same plates in the Real Time PCR instrument.

Fluorometric analysis

You can quantitate DNA by fluorometric analysis using various intercalating dyes. These are summarized in Table 15.

Table 15 Dyes used for fluorometric DNA quantitation

Dye	Features
Hoechst 33285	<ul style="list-style-type: none"> • More sensitive than spectrophotometric measurements due to low affinity for RNA • Base composition of DNA can affect readings because the dye binds preferentially to 5'-AT-3'-rich DNA
Ethidium bromide	<ul style="list-style-type: none"> • Not base composition sensitive • Binds to RNA • Capable of detecting ng levels of DNA • Ideal for relatively pure DNA with a high 5'-GC-3' content
PicoGreen [®]	<ul style="list-style-type: none"> • Can quantitate as little as 25 pg/mL to 1000 ng/mL of dsRNA

Assay problems

Problems with preparing the assay may include:

- "Reagents mishandled or expired" on page 54
- "Using a master mix without ROX dye" on page 55
- "DNA or assay reagent not added to the reaction well" on page 55
- "Insufficient DNA added to the reaction well" on page 55
- "Evaporation from the reaction well" on page 56
- "Pipetting errors" on page 56
- "Inefficient mixing or centrifugation" on page 57
- "Assay has high background fluorescence" on page 57
- "More than one sample in the well" on page 57

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 to –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 between 2 and 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
- Some or all data is undetermined (There is 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.

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

See also “ROX dye not designated as reference” on page 61

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 “Quantitate the gDNA” on page 23)
- Follow the TaqMan[®] SNP Assays protocol as recommended, adding between 1 to 20 ng of purified genomic DNA (ensuring final concentration no less than 0.2ng/ μ L)
- 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 an adhesive seal 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 samples that do not cluster with a specific allele (i.e. an outlier)

What to do

- Improve pipetting precision, as follows:
 - Calibrate and clean the pipettors regularly
 - Pipette larger volumes (no less than 5 μ L) 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)
- Consult the manufacturer about 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 residual volume may cause errors.
- Validate your pipetting process by preparing a replicate plate (same assay and sample over a plate) to be sure 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, the Real-Time PCR Instrument Software 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.

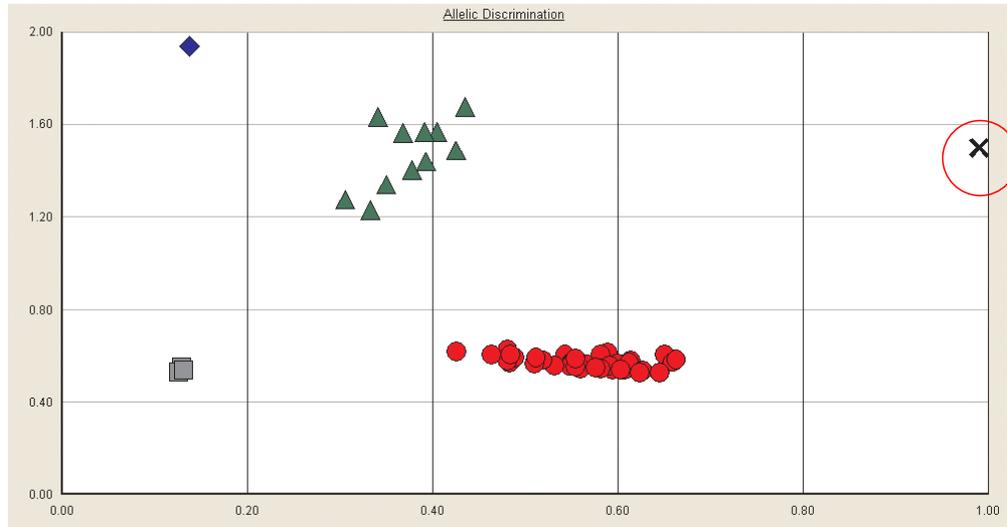


Figure 15 Allelic discrimination plot with different samples in the same well (outlier sample circled in red)

What to do

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

Instrument troubleshooting

To eliminate poor thermal cycler performance, ensure that you maintain instruments according to the recommendations provided in the relevant instrument manuals.

For best results, make sure to calibrate and maintain your instrument as recommended by Life Technologies. Poor thermal cycler performance can result in:

- 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, Life Technologies strongly recommends that you perform routine maintenance. Maintenance schedules vary by instrument, refer to your relevant instrument manual for details.

Instrument calibration

To ensure optimal performance of your thermal cycler or Real Time PCR System, Life Technologies strongly recommends that you regularly calibrate your thermal cycler. Calibration varies by instrument, refer to your specific instrument manual 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 software problems

Three conditions in allelic discrimination plots may be caused by problems that occurred during software analysis. Most of these problems can be eliminated during software setup.

Allelic Discrimination plot problems due to software fall into four broad categories, each of which is further discussed below.

- “Empty allelic discrimination plots” on page 59
- “No alleles called in the AD plot” on page 60
- “Homozygous allele frequencies reversed” on page 63
- “Too many alleles called in AD plot” on page 63

This section highlights common data analysis issues that can lead to undetermined, ambiguous, or incorrect genotypes. For complete instructions for setting up, running and analyzing TaqMan[®] Genotyping Assay experiments, refer to the user manual for your instrument or software and follow the steps provided.

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 marker assigned to the well” on page 60

- “Omit option checked in Real Time PCR instrument software” on page 60
- “ROX dye not designated as reference” on page 61

No marker assigned to the well

A marker must be assigned to each well before the Real Time PCR Instrument Software 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 a marker is assigned.
2. If no 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 60
- “Two cluster calling not selected” on page 60
- “Single cluster assay” on page 60
- “ROX dye not designated as reference” on page 61
- “Outlier too far off scale” on page 61
- “NTCs not assigned” on page 62

Autocall is not selected

The software will not autocall unless the **autocall** option is selected. If **autocall** is not selected, the allelic discrimination plot shows all the calls as X. Refer to your instrument manual for instructions on how to use **autocall** options.

Two cluster calling not selected

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 39).

If you believe 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, refer to the user manual of your instrument or software.

Figure 16 shows an example of a single cluster plot before and after rescaling.

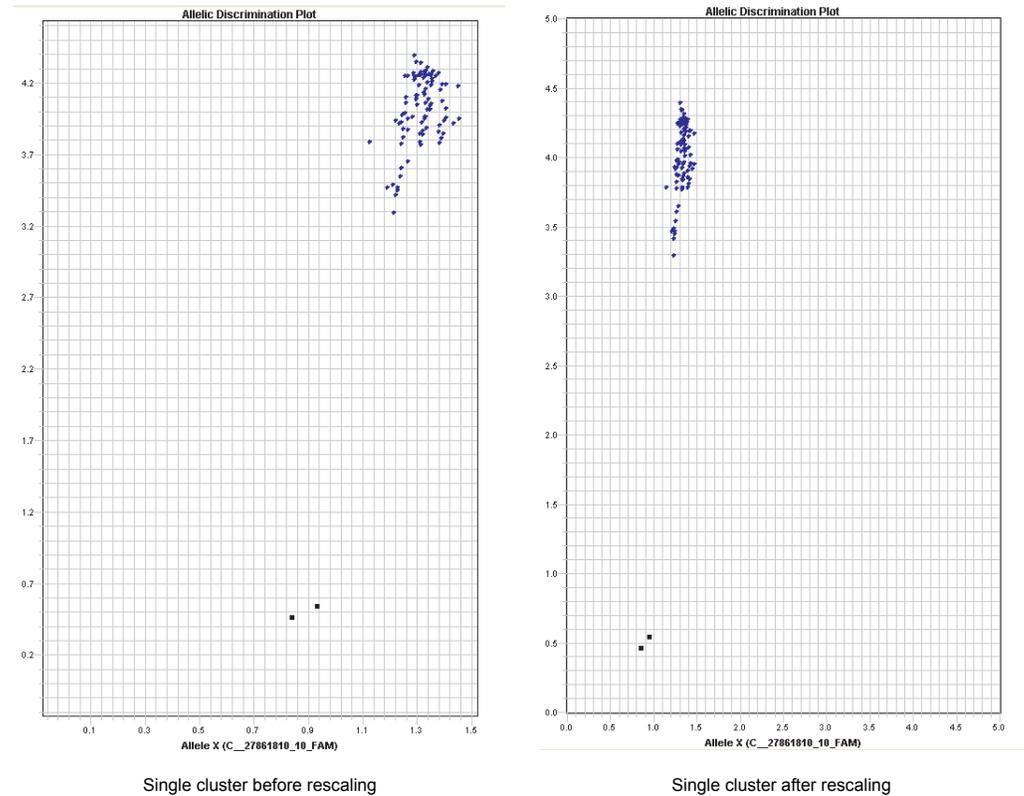


Figure 16 Allelic discrimination plot before and after rescaling

ROX[™] dye not designated as reference

When ROX[™] dye is not designated as a 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. Life Technologies recommends using TaqMan[®] Genotyping Master Mix that contains ROX[™] as a passive reference dye. 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.

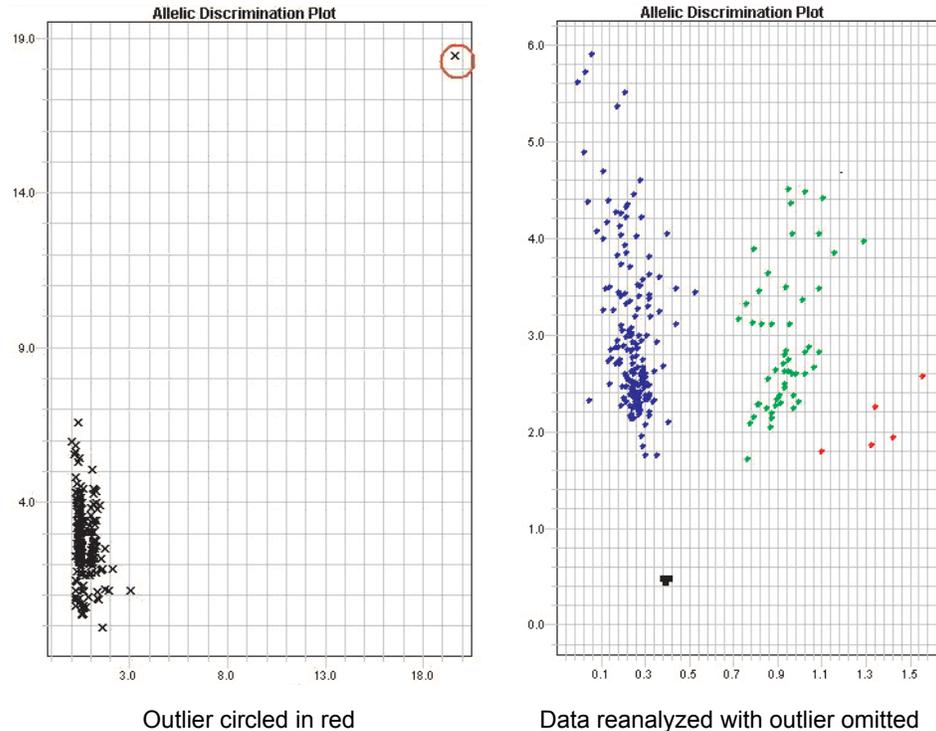


Figure 17 Data analyzed with and without outlier included

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.

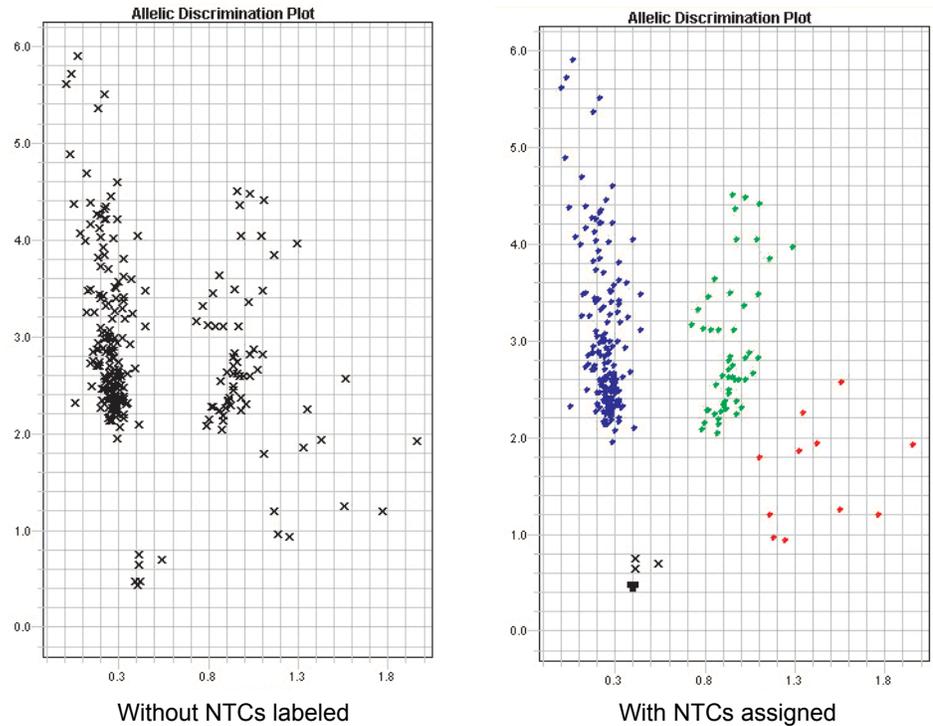


Figure 18 Data analyzed with and without the NTCs assigned

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.

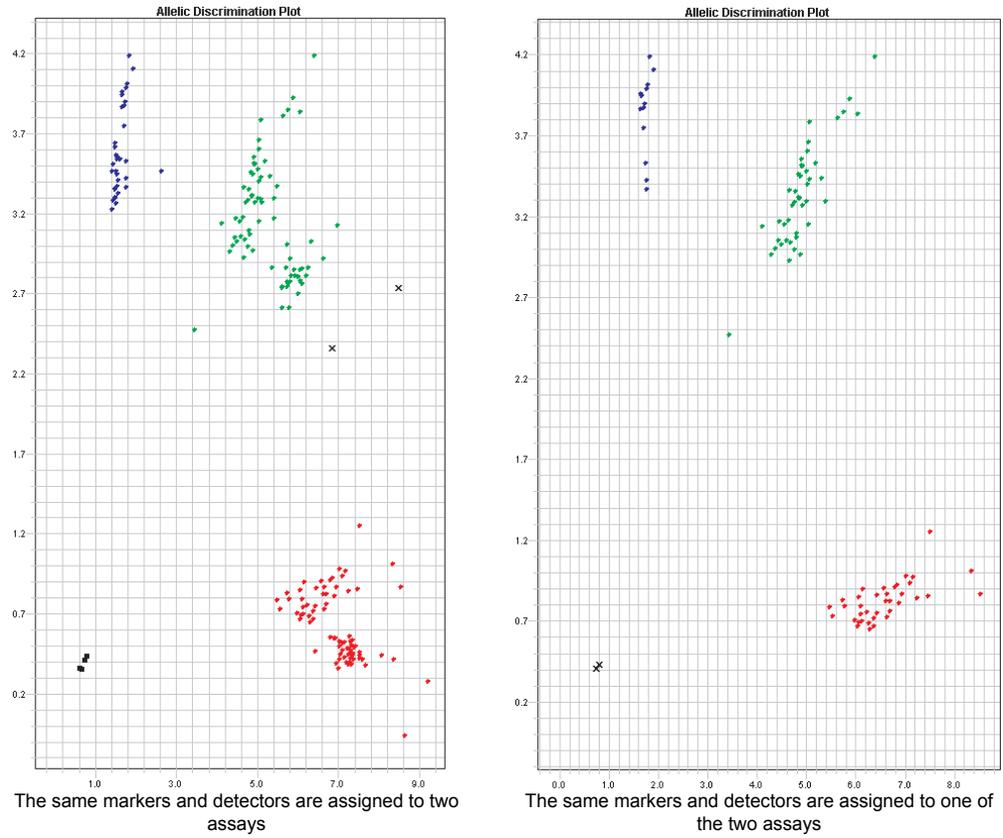


Figure 19 Allelic discrimination plots showing two assays assigned to one detector and marker

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.



Overview of the TaqMan[®] SNP Genotyping Assays

Overview of the assay components

TaqMan[®] SNP Genotyping Assays allow for genotyping of nucleotide polymorphisms using the 5' nuclease assay for amplifying and detecting specific alleles in purified genomic DNA samples.

Each assay genotypes individuals for a specific polymorphism. Each TaqMan[®] Genotyping Assay contains two primers for amplifying the sequence of interest and two TaqMan[®] MGB probes for allele detection. The presence of two probes in each reaction allows for genotyping two possible variant alleles at the polymorphic site in a DNA target sequence. The genotyping assay determines the presence or absence of a polymorphism based on the change in fluorescence of the dyes associated with the probes (see Table 16).

About the probes

The TaqMan[®] MGB Probes consist of target-specific oligonucleotides with:

- A reporter dye is linked to the 5'-end of each probe:
 - VIC[®] dye for Allele 1 probe
 - FAM[™] dye for Allele 2 probe
- A minor groove binder (MGB), which increases the melting temperature (T_m) without increasing probe length (Afonina, 1997; Kutuyavin, 1997), thus allowing the design of shorter probes. Shorter probes result in greater differences in T_m values between matched and mismatched probes
- A non-fluorescent quencher (NFQ) at the 3'-end of the probe.

Table 16 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

Basics of 5' nuclease assay

PCR principles

- Genomic DNA is introduced into a reaction mixture consisting of TaqMan® Genotyping Master Mix, forward and reverse primers and two TaqMan® MGB Probes.
- Each TaqMan® MGB Probe anneals specifically to a complementary sequence, if present, between the forward and reverse primer sites. When the probe is intact, the proximity of the quencher dye to the reporter dye suppresses the reporter fluorescence.
- As shown in Figure 20, the exonuclease activity of AmpliTaq Gold® DNA Polymerase cleaves only probes hybridized to the target. Cleavage separates the reporter dye from the quencher dye, increasing fluorescence by the reporter.

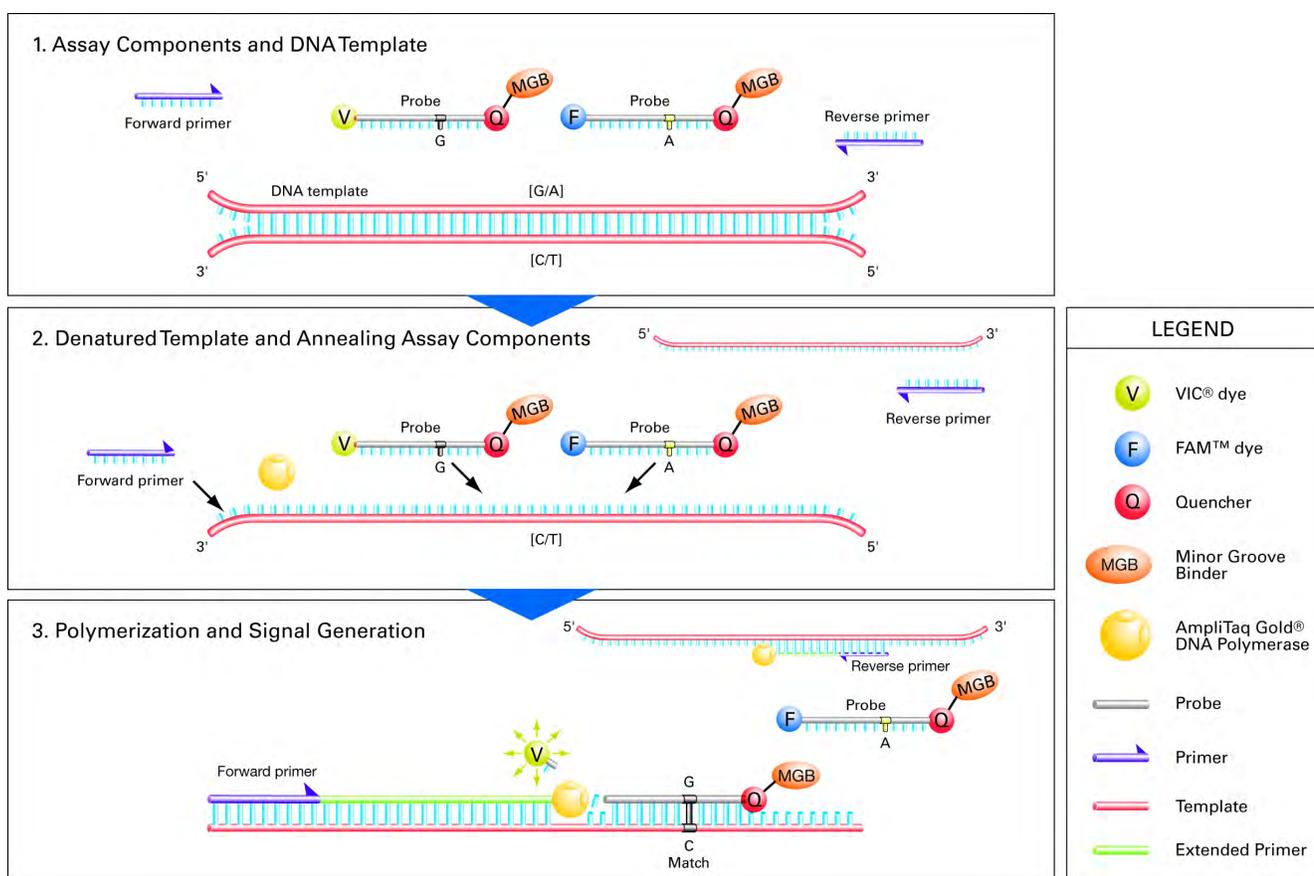


Figure 20 TaqMan® Reagents detection process

The increase in fluorescence occurs only if the amplified target sequence is complementary to the probe. Thus, the fluorescence signal generated by PCR amplification indicates which alleles are in the sample.

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.

Data analysis

After generating an endpoint read on a real-time PCR instrument, the data is plotted by the instrument software [conventionally, VIC® dye (allele 1) on the X-axis and FAM™ dye (allele 2) on the Y-axis].

The instrument analysis software allows for auto-calling genotypes, minimizing manual intervention. In addition, Life Technologies real-time instrument **.eds** files can be loaded into TaqMan® Genotyper™ Software for data analysis. For instructions on how to use TaqMan® Genotyper™ Software, consult the *TaqMan® Genotyper™ Software Getting Started Guide* (Pub. no. 4448637).

Note: TaqMan® Genotyper™ Software is available for free download at www.lifetechnologies.com.



About the Drug Metabolism Enzyme genes

Introduction to the Drug Metabolism Enzyme genes

Drug metabolizing enzymes (DMEs) are proteins involved in the biotransformation, metabolism, and/or detoxification of endogenous and foreign compounds (for example, naturally occurring compounds like prostaglandins, drugs and environmental agents). Genes that code for these enzymes are often referred to as “DME genes.” Polymorphisms in the DME genes may influence the rate of foreign compound metabolism and/or excretion among individuals, thereby potentially affecting foreign compound efficacy and/or toxicity. Several of these polymorphisms may affect drug efficacy and toxicity (Eichelbaum, 2006; Roses, 2004).

For example, there are over 100 known variants of the CYP2D6 gene. Some of these variations result in a complete loss of enzymatic activity while some of the variants only reduce catalytic activity (<http://www.cypalleles.ki.se/cyp2d6.htm>).

TaqMan[®] Drug Metabolism Genotyping Assays are designed to detect polymorphisms such as SNPs, indels, and MNPs in the genes that code for the drug metabolism enzymes and drug transporters. The terms “SNP” and “polymorphism” will be used interchangeably throughout the rest of this appendix and refer to all of these polymorphisms.

Drug Metabolism Enzyme classifications

The products of DME genes are Phase I and Phase II drug metabolism enzymes, transmembrane transporters, and other gene products thought to be involved in the metabolism of endogenous compounds and xenobiotics in humans.

- **Phase I enzymes** – Involve the hydrolysis, oxidation, or reduction of xenobiotic compounds. These enzymes either create or expose a functional group (such as a hydroxyl group or carboxyl group) on the target small molecule. Examples of Phase I enzymes are the cytochrome P450 gene super-family and the alcohol dehydrogenase gene family. Four members of the P450 super-family, CYP3A, CYP2D6, CYP2C19, and CYP2C9, account for almost 50% of metabolism of commonly used drugs (Wilkinson, 2005).
- **Phase II enzymes** – Involve the conjugation of various cofactors to functional groups on small molecules that are often exposed or created by a Phase I reaction. Phase II reactions include glucuronidation, acetylation, and sulfation. Phase II reactions often increase the hydrophilicity of a compound, thereby enhancing its excretion from the body. This class includes the UDPglucuronosyl transferase (UGT) family that metabolizes morphine and thiopurine S-methyltransferase (TMPT) that metabolizes Captropril (Shastry, 2006).
- **Drug transporters** – Expressed in liver, kidney, and the intestines, involved in the elimination of xenobiotics from the body. This class of protein includes organic



anion transporters, organic cation transporters, peptide transporters, and nucleoside transporters. Many of these proteins exhibit broad substrate specificity which can lead to significant drug-drug interactions at the transporter level. Examples of transporters are: the multidrug resistance-associated protein MRP1 (ABCC1), ATP-dependent transporters like MDR1 (ABCB1; P-glycoprotein), organic anion transporters such as hOAT1 (SLC22A6) (Bleasby, 2005), and multidrug resistance protein MRP2 (ABCC2) (Szakács, 2004).

Allele nomenclature

Allele nomenclature for the polymorphisms in the DME genes follows an international system created to describe polymorphisms in DNA and protein sequences (den Dunnen, 2001). When available, Life Technologies provides the allele nomenclature for the SNP being interrogated by a particular TaqMan[®] Drug Metabolism Genotyping Assay. The allele nomenclature indicates the gene name, the allele designation, and the base changes associated with the polymorphism. Because this nomenclature is used in the Life Technologies website for categorizing the TaqMan[®] Drug Metabolism Genotyping Assays, you can search for an assay using the allele nomenclature for the SNP of interest.

An example of this nomenclature is CYP1A1*1C, g.-3229G>A, which describes a polymorphism in a cytochrome P450 gene, where:

- “CYP1A1” indicates the HUGO gene name
- “*1C” indicates the allele from the Human Cytochrome P450 Nomenclature Committee (<http://www.cypalleles.ki.se/>)
- “g” indicates that the mapping position comes from the genomic DNA reference sequence (“c” indicates cDNA)
- “-3229” indicates the nucleotide position relative to the first base of the start codon on the genomic DNA reference sequence
- “G>A” indicates the nucleotide or base change

Note: The order of the alleles does not reflect frequency and/or a wild-type versus mutation designation, because they can differ between populations.

The allele nomenclature can indicate one SNP or multiple SNPs associated with a haplotype. The TaqMan[®] Drug Metabolism Genotyping Assays are designed to identify a single polymorphism within an “allele,” which is generally a haplotype. To investigate an allele from the common allele nomenclature sites, you may need several TaqMan[®] Drug Metabolism Genotyping Assays.

Allele nomenclature was gathered from the following sites:

- The human cytochrome P450 (CYP) allele nomenclature committee web site: <http://www.cypalleles.ki.se/>
- The arylamine N-acetyltransferase (NAT) nomenclature web site: <http://nat.mbg.duth.gr/>
- The home page of the committee mediating the naming of UDP glucuronosyltransferase: http://www.pharmacogenomics.pha.ulaval.ca/cms/ugt_alleles/

Note: Because not all the genes or polymorphisms included in TaqMan[®] Drug Metabolism Genotyping Assays have been curated on a nomenclature site, not all of them are associated with public allele nomenclature. For some very important variants in *Very Important Pharmacogenes* (VIP), this nomenclature can be found on the Pharmacogenomics Knowledge Base web site: <http://www.pharmgkb.org>.



Assay development and testing

This section provides general information about how Life Technologies designed and tested the TaqMan[®] Drug Metabolism Genotyping Assays.

Bioinformatics evaluation and design

Life Technologies performed extensive bioinformatics to ensure that all SNPs in the TaqMan[®] Drug Metabolism Genotyping Assays were properly mapped to the human genome assembly. The most current mapping information is available on the TaqMan[®] Drug Metabolism Genotyping Assays page on the Life Technologies website: <http://www.lifetechnologies.com/taqmandme>.

1. 221 genes were selected based on their roles in drug metabolism and transport. A complete list of genes can be found in the TaqMan[®] Drug Metabolism Genotyping Assay Index, available on the Life Technologies web site.
2. Polymorphisms associated with DME genes were identified from multiple sources, including dbSNP, HapMap, collaborators, public allele nomenclature sites and Life Technologies proprietary database.
3. For each potential assay, the polymorphism and 300 bases on either side of it were mapped to the human genome. A polymorphism was considered to be successfully mapped when it was aligned to only one location in the genome.
4. Polymorphisms were selected for inclusion into the TaqMan[®] Drug Metabolism Genotyping Assays collection based on their location on the reference genome. SNPs in coding regions (missense, nonsense, silent polymorphisms) as well as SNPs in splice site acceptor regions, splice site donor regions, and the 5'UTR were chosen. Additional intronic polymorphisms were included only when these were available from the public allele nomenclature web sites.
5. For all polymorphisms, repetitive regions, non-target polymorphisms, and putative polymorphisms within 300 bases on either side of the SNP of interest were masked to avoid assays with potential polymorphisms under probes and primers.
6. The masked sequence was sent to the Life Technologies proprietary TaqMan[®] Assay Design Software.
7. The resulting assay design was mapped to the genome again, using the BLAST database search algorithm, to ensure the primers and probes are specific for the target polymorphism.
8. If the assay design mapped with sufficient specificity, it was synthesized and submitted for wet lab testing (see "Wet testing of assays" on page 72). Assays that failed wet lab testing were resubmitted to the assay design software to generate alternative assay designs.

Development of the thermal cycler method

To address the homology issues within the DME genes and to ensure specificity of these assays, Life Technologies created assay designs that resulted in amplicon lengths greater, in general, than those for other TaqMan[®] Genomic Assay product lines. The average amplicon length for the TaqMan[®] Drug Metabolism Genotyping Assays is 160 base pairs, compared to 100 base pairs for other Life Technologies TaqMan[®] genotyping products. Wet testing of these longer amplicons showed that the Rn values (normalized signal) were often lower for these longer amplicons, affecting cluster separation. To address this issue, Life Technologies developed a new thermal cycler method. The development of this new thermal cycler method is in the following sections.



Determination of the optimal number of cycles

A representative group of assays were subdivided by amplicon length into three groups: short (<100 bases), medium (100-110 bases) and long (>110 bases). The normalized signals for each set of assays, performed at 40 and 50 cycles with a 60 second extension, were compared. At 50 cycles, the average signal for both the VIC[®] and FAM[™] dyes increased dramatically for the long amplicons. For these assays, 50 cycles produced better genotyping data than the traditional 40 cycles.

Determination of the optimal extension time

To further improve assay performance, Life Technologies investigated longer extension times. Three different extension times (60, 90 and 120 seconds) for 50 cycles were investigated. The data produced by these experiments indicated that the 90 second extension showed significant increases in the normalized signal; the average signal increased for both the VIC[®] and FAM[™] dyes. No significant increases were observed with the 120 second extension.

Clustering was also analyzed, and the group of assays with the longest amplicons showed the most improvement in tightness and angle separation of the genotype clusters. Tightness and angle separation are determined algorithmically; tightness is the distance of individual points from each other and angle separation measures the distance between clusters.

Figure 21 demonstrates the effects of increased number of cycles and longer extension time on the assays based on amplicon length.

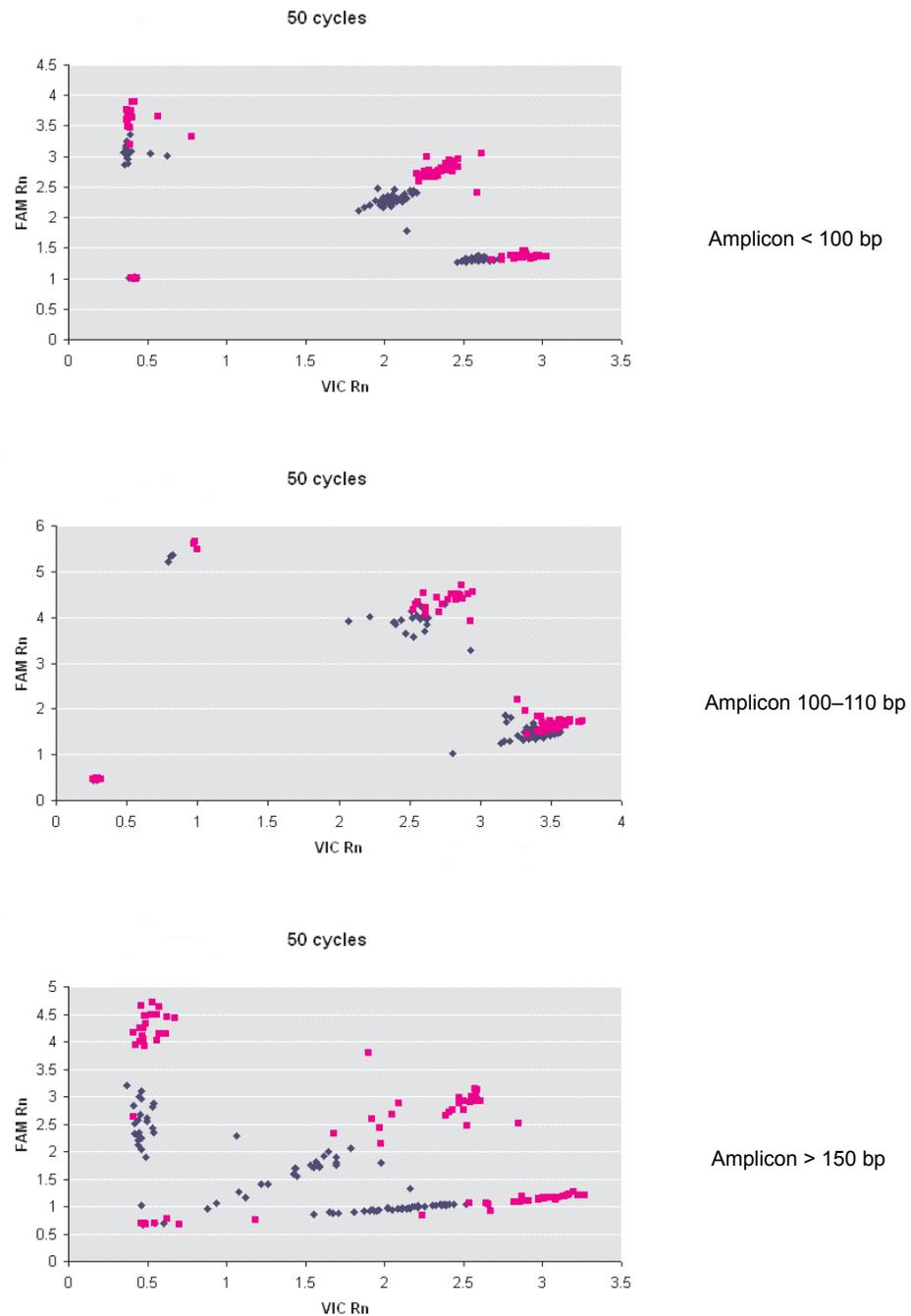


Figure 21 Allelic distribution plots for short, medium, and long amplicon assays for 60- (dark blue) and 90-second (pink) extension times

Wet testing of assays

Stock DNA samples were obtained from Coriell Cell Repositories (<http://ccr.coriell.org>). Dilutions of the stock DNA were quantitated by the RNase P method. Each stock DNA was then diluted with water to yield a 1 ng/μL working stock. 3 μL (3 ng total DNA) of each DNA was delivered to the wells of a 384-well microtiter plate. Each plate was dried overnight and the plates were sealed and stored for up to a year prior to use.



Reaction mixes were prepared for 5 µL reactions. PCR was performed using GeneAmp® PCR System 9700, as described in “Perform the PCR” on page 26 with the only difference that the Universal Master Mix was used instead of the TaqMan® Genotyping Master Mix. Endpoint data were collected using the Life Technologies 7900HT Real-Time PCR System.

All assays were wet-tested on duplicate plates, containing African American and Caucasian DNA samples. Each plate contained DNA samples from:

- 45 African Americans
- 45 Caucasians
- 3 No template controls (water)

Each assay was run twice to confirm performance. Parameters used to analyze the assays included: cluster signal, tightness, angle separation, and Hardy-Weinberg equilibrium.

If good performance was observed, assays were run on a proprietary set of DNA samples from Chinese and Japanese populations to generate allele frequencies for those populations. (Each set containing 45 samples was provided as part of a collaboration. These sets are not publicly available.)

If an assay performed poorly, it was submitted to the assay design software for redesign. Due to limitations in the technology and/or the type of polymorphism, an assay could not be developed for every polymorphism in the drug metabolism enzyme genes.

Other assays for the DME genes

221 genes were included in TaqMan® Drug Metabolism Genotyping Assay set. Genes that are not part of the TaqMan® Drug Metabolism Genotyping Assay collection may be found in the Life Technologies TaqMan® SNP Genotyping Assays product line. The TaqMan® Drug Metabolism Genotyping Assays include polymorphisms found in coding regions, splice junctions, or regulatory elements for the DME genes. Additional intronic SNPs were included only if evidence from the public allele nomenclature web sites was identified.

If your SNP of interest is not available as a TaqMan® Drug Metabolism Genotyping Assay, it may be available as a Life Technologies TaqMan® SNP Genotyping Assay. This pre-designed assay collection includes over four million assays. Search for the gene of interest at the website <http://www.lifetechnologies.com/taqmandme>.

TaqMan® DME Assays for genotyping triallelic SNPs

Several important DME gene variants are triallelic SNPs wherein 3 bases occur at the same genomic location (see Table 17). Triallelic SNP targets can be interrogated using a pair of TaqMan® assays. Each assay contains one probe for the major SNP allele, which is labeled with the same reporter dye in both assays (e.g. VIC® dye), and one probe for one of the minor alleles, which is labeled with the second reporter dye (e.g. FAM™ dye). To generate accurate sample genotypes, the two assays must be run independently on the same panel of samples, and the resulting allelic discrimination plots must be analyzed in concert, comparing the expected cluster positions from both assays to a map of the true sample genotypes. Sample genotypes can be assigned with the help of a spreadsheet program and a chart of the expected genotypes (see “Example: ABCB1 c.3095G>T/A triallelic SNP rs2032582 assays” on page 74) or the AlleleTyper™ Software can be used to translate the results.



Refer to the *TaqMan® Drug Metabolism Genotyping Assays for Triallelic SNPs Application Note* (Pub. no. 135AP01-01 for more details on manual analysis of triallelic SNPs using 2 DME SNP Assays or to the *AlleleTyper™ Software User Guide* (Pub. no. 4469874) for information on having the genotypes assigned automatically by this software.

Table 17 TaqMan® DME Assays for genotyping triallelic SNPs

Gene	rsSNP ID	TaqMan® DME Assay	Allele name	Assay alleles
ABCB1	rs2032582	C_11711720C_30	N/A	C/A
		C_11711720D_40	N/A	C/T
CYP2C9	rs7900194	C__25625804_10	CYP2C9*8,c.449G>A	A/G
		C_25625804D_20	CYP2C9*27,c.449G>T	T/G
CYP2D6	rs5030865	C_30634117C_20	CYP2D6*8,g.1758G>T	A/C
		C_30634117D_30	CYP2D6*14,g.1758G>A	T/C
CYP1A1	rs41279188	C_30634152C_70	CYP1A1*5,g.2461C>A	G/T
		C_30634152D_80	CYP1A1*9,g.2461C>T	G/A
CYP2C8	rs72558195	C_72650009C_10	CYP2C8*7,c.556C>T	G/A
		C_72650009D_20	CYP2C8*8,c.556C>G	G/C

Note: On the Life Technologies Assay Search results pages, the annotation for the DME assays is tied to the SNP ID and not the SNP alleles. Therefore, both assays for a triallelic SNP will be assigned the same allele nomenclature. Refer to the Important Information and/or the context sequence to determine which alleles are interrogated by each assay.

Example: ABCB1 c.3095G>T/A triallelic SNP rs2032582 assays

C_11711720C_30 ABCB1_c.3095	G>T	TATTTAGTTTGACTCACCTTCCCAG [C/A]ACCTTCTAGTTCTTTCTTA TCTTTC
C_11711720D_40 ABCB1_c.3095	G>A	TATTTAGTTTGACTCACCTTCCCAG [C/T]ACCTTCTAGTTCTTTCTTA TCTTTC

After running paired assays for triallelic SNPs in separate reactions on the same gDNA samples, examine the cluster plots in your real-time instrument software or TaqMan® Genotyper™ Software:

1. Heterozygotes – Any sample called as a heterozygote is a true heterozygote for the reported alleles in a given assay.
2. Homozygotes – Samples running in or near a FAM™ or VIC® homozygous cluster can be either a true homozygote for the reported allele, or can be a heterozygote for the FAM™ or VIC® reported allele and for the unreported SNP allele in a given assay. Samples carrying just one reported allele may or may not run together with those carrying two reported alleles; i.e. cluster splitting may or



may not be notable. If samples close to a homozygote cluster are called as undetermined, manually call these as homozygotes.

3. Noamp – Weak or No amplification. Samples that are homozygous for the unreported allele can exhibit weak amplification, relative to other samples carrying reported alleles, due to nonspecific activity of the assay probes.

Table 18 Example of a translation table (ABCB1 c.3095G>T/A triallelic SNP rs2032582 assays)

Genotype	C/A Assay	C/T Assay
	c.3095G>T, A893T	c.3095G>A, A893S
ABCB1 c.3095G>T/A	C_11711720C_30	C_11711720D_40
G/G	C/C	C/C
G/A	C/C	C/T
A/A	noamp	T/T
G/T	C/A	C/C
T/A	A/A	T/T
T/T	A/A	noamp

Note: The alleles reported by the ABCB1 SNP assays are given in the plus strand genome orientation whereas the ABCB1 gene maps to the minus genome strand. Thus the reported SNP assay alleles and the SNP cDNA annotations are the reverse complement of one another.



How to order TaqMan[®] SNP Genotyping Assays

How to order TaqMan[®] Predesigned SNP and Drug Metabolism Genotyping Assays

Assay part numbers

Use the TaqMan[®] Predesigned SNP Genotyping Assay part numbers that correspond to the type of assay and the number of reactions needed.

Table 19 Ordering information for TaqMan[®] Predesigned SNP and Drug Metabolism Enzyme (DME) Genotyping Assays

Product	Scale	Number of reactions		Assay mix formulation	Cat. no.	
		384-well	96-well		Human	Mouse
Pre-designed SNP	Small	1500	300	40X	4351379	4351384
	Medium	5000	1000	40X	4351376	4351382
	Large	12,000	2400	80X	4351374	4351380
DME	Small	750	150	20X	4362691	—

Life Technologies website

To order one of the 4.5 million available assays from the Life Technologies website:

1. Go to the **Assay Search Tool** at www.lifetechnologies.com to view the available TaqMan[®] Predesigned SNP and Drug Metabolism Enzymes Genotyping Assays.
2. Select **SNP Genotyping** for the type of experiment and **All SNP Genotyping** for the type of assay. DME assays can be found by selecting either **All SNP Genotyping** or **Drug Metabolism Assays**.
3. Choose the **Species** of interest.
4. Enter your **Target Information**. You can search by:
 - Gene name
 - Gene symbol
 - RefSeq accession numbers



- SNP ID
 - Assay ID
 - Allele nomenclature (available for Drug Metabolism Assays for which this information has been mapped)
 - Genomic location
5. Select **Search**.
From the search result page, you will be supplied with a list of assays that match your search criteria, including information on annotation for assays and SNPs.
 6. Select the assays to add to your shopping cart.
Note: You must be logged on to add assays to your shopping cart.
 7. View your shopping cart to select assay scales and complete your order.

Quick order

If you have already chosen the TaqMan® Predesigned SNP or Drug Metabolism Enzyme Genotyping Assay needed or you have selected TaqMan® Drug Metabolism genotyping Assays from the DME Index (available at <http://www.lifetechnologies.com/snpanhme>), you can order using the Quick Order tool.

1. Go to www.lifetechnologies.com.
2. Ensure you are signed in to your account.
3. Select **Quick Order** from the top Menu bar.
4. Enter the part number, Assay ID(s), and the quantity.
5. Click **Add to Cart** and proceed to checkout.

How to order TaqMan® Custom SNP Genotyping Assays

Assays part numbers

Use the TaqMan® Custom SNP Genotyping Assay part numbers that correspond to the type of assay and the number of reactions needed.

Table 20 Ordering information for TaqMan® Custom SNP Genotyping Assays

Product	Scale	Number of reactions		Assay mix formulation	Cat. no.	
		384-well	96-well		Human	Non-human
Custom SNP	Small	1500	300	40X	4331349	4332077
	Medium	5000	1000	40X	4332072	4332075
	Large	12,000	2400	80X	4332073	4332076

Considerations for choosing human SNP Genotyping Assays

Life Technologies performs a functional test on all human SNP Genotyping Assays. Genomic DNAs (gDNAs) from 20 unrelated individuals (from 3 populations and both sexes) are amplified under universal conditions with the SNP Genotyping Assay to test for amplification and clustering. Human SNP Genotyping Assays that fail this test are not shipped. Consequently, if you expect SNP Genotyping Assays to human targets to fail the functional test, order the nonhuman SNP Genotyping Assays.

Failures can occur for the following reasons:

- For human cDNA sequences, the test fails because intronic sequences prevent primer or probe binding, or separate assay component binding sites and prevent efficient amplification because of longer amplicon size.
- For human Y-chromosome-specific sequences, the test fails because >90% of the samples in the test must amplify to pass, and the female samples in the functional test will not amplify because they do not contain a Y chromosome.

Review of the target sequence

The success of your TaqMan® Custom SNP Assay depends largely on the quality of the sequence data that you submit for the design process. Refer to the *Custom TaqMan® Assays Design and Ordering Guide* (Pub. no. 4367671) for detailed instructions on designing your TaqMan® Custom SNP Genotyping Assay.

After you select your target sequence, verify that it meets criteria for:

- Biological Significance
- Allele frequency
- Length
- Accuracy
- Uniqueness

Select an appropriate target site and review your sequence for polymorphisms and sequence repeats.

Custom TaqMan® Assay Design Tool

1. Access the **Custom TaqMan® Assay Design Tool** at www.lifetechnologies.com.
2. Enter or edit sequence information within the **Order Custom SNP Genotyping Assays** link in the **Custom TaqMan® Assay Design Tool**. Multiple sequences may be entered.
 - Sequences must be entered in the 5' to 3' direction
 - Sequence length must be between 61 and 5000 nucleotides
 - Sequences must only be composed of A, C, G, T, or N for masked neighboring SNPs
 - Targeted SNPs, indels, or MNPs up to 6 bases must be entered between brackets in the format [Allele1/Allele2] or by using IUPAC code letters for two SNP bases without brackets. Deletions are denoted by an asterisk (*).

Note: Ensure successful design and performance by reading *TaqMan® Custom Assays Design and Ordering Guide* (Pub. no. 4367671).

3. Click on **Check Format** to ensure data input meets requirements.
4. Select **Submit for Assay Design**.



Alternatively, if the assay sequences are already known, the primer and probe sequences for manufacturing can directly be entered:

1. Click on the **Enter Custom Primer/Probe pairs** button.
2. Enter or edit the sequence information.
3. Select the **Species** of interest.
4. Check format and submit.

After the sequences are submitted for assay design, you can either wait for the TaqMan® Custom Assay Design Tool to complete your design or you can close your browser and return to your order at a later time. After receiving your sequence, Life Technologies sends you an e-mail to confirm that your sequences have been submitted. The email contains a link to your design job in the TaqMan® Custom Assay Design Tool that you can use to check the status of your job.

5. Select the row that corresponds to your design job in the **Design Details** section of the **Select Assays** tab to check on the status of your submission

For each design job, the **Design Details** table displays:

- Batch ID - The ID assigned to the submission by Life Technologies.
- Submitted - The date that you submitted the sequence information.
- Status - The status of the design: Completed or Pending.
- Details- The assays in each batch that passed, failed, or were not designed

6. Select the assay size or scale and the quantity in the drop-down lists in the **Size** and the **Quantity** columns for each assay to order.

7. Click **Add All** to add all assays to the order, or alternatively click **Add** next to each assay to select them individually.

8. Click **Order Now** to proceed to checkout.

You will have the option to review your order before finalizing it. In the Review Order section, you can:

- Delete an Assay
- Change the size of a selected Assay
- Change the quantity of a selected Assay
- Add more items in your shopping cart
- Select the conditioning of the Assays:
 - Individual tubes
 - Other plating options (see “Plated formats” on page 16)

Note: Once a TaqMan® SNP Custom Assay has been designed, it can be re-ordered using Quick Order with its part number and Assay ID (see “Quick order” on page 77).



About the Assay Information File (AIF)

This appendix describes the assay information included in each shipment of TaqMan[®] SNP Genotyping Assays.

About the Assay Information File (AIF)

The CD-ROM received with each TaqMan[®] SNP Genotyping Assay order contains an Assay Information File.

The Assay Information File (AIF):

- Is identical to AIFs of other TaqMan[®] Genotyping Assay products
- Includes the number of the bar code of the plate in which the assays are shipped
- Is provided in three convenient formats:
 - a tab-delimited `.txt` format that can be opened in Excel or similar spreadsheet programs
 - a browser-readable `.html` format (not available for TaqMan[®] DME Genotyping Assays)
 - a `.xml` file that can be opened with a `.xml` viewer
- Includes up to 57 data fields (depending on the type of assay and the file format; see Table 21)

AIF can be used to:

- Identify the assay included in each tube
- Locate each tube in the assay rack
- Provide the 2-D bar code for each tube
- Determine assay IDs
- Identify the chromosome location of the SNP
- View the minor allele frequencies in Caucasian, African American, Japanese, and Chinese populations, when available (for Predesigned SNP and Drug Metabolism Enzyme Genotyping Assays only)
- View the context sequence (for Predesigned SNP and Drug Metabolism Enzyme Genotyping Assays only)
- View the assay sequence (for Custom SNP Genotyping Assays only)



For LIMS users

The AIF on the CD-ROM is named so a LIMS system can automatically upload it. The AIF name has the following format:

Assay Type_xx_yy.txt, where:

- Assay Type refers to TaqMan® Predesigned SNP, Drug Metabolism or Custom SNP Genotyping Assay
- xx is the order number (up to 10 characters)
- yy is the plate ID number (up to 10 characters)

AIF and tube contents

To determine the contents of each assay tube, match the Assay ID on the tube label with the values of the Assay ID and Well Location columns in the AIF. The Well Location column indicates the plate rack position for the assay in the corresponding row of the AIF.

Explanation of the AIF Columns

The table below describes the AIF columns.

Note: Because the information in the AIF varies by product line, not all the fields are populated for all assay types. Refer to the *Understanding your shipment* document included in your order for a description of the AIF columns pertinent to your assay. The format of the AIF where the fields can be found is indicated in the following table in the rightmost column.

Table 21 Description of the AIF columns

Columns	Example	Description	AIF File Format
Customer Name	University of X	Name of the customer's organization or institution	.txt .html
Order Number	185185185	Customer sales order number unique to an order	.txt
Sales Order Number	185185185	Customer sales order number unique to an order	.html
Contact Name	John Doe	Name of the customer	.html
XML Template Version	2.0	Version of the template in which the AIF is created	.html



Appendix E About the Assay Information File (AIF)
Explanation of the AIF Columns

Columns	Example	Description	AIF File Format
Assay Name	MyAssay	For custom assays only, provided by the customer	.txt .html
Expiration Date	28-AUG-2018	Date when the product expired and should no longer be used	.html
Annotation Sources	NCBI	Databases used to annotate the AIF	.html
Ship Date	15-JAN-13	Date when the product was packaged for shipment	.txt .html
Delivery Number	See packing slip (e.g. 4181727)	Shipment unique number (see packing slip for details)	.txt
Part Number	4351379	Product number used for ordering the assay	.txt .html
Product Type	TaqMan [®] SNP Genotyping Assay Service	Product type indicated by the part number	.txt .html
Assay ID	C__123456789_10	Unique identifier for the assay	.txt .html
Lot Number	654321	Unique identifier for the manufacturing batch	.txt .html
Plate Type	96-position tube rack v1	Type of container in which the assay is shipped	.txt
Rack or Array Type	96-position tube rack v1	Type of container in which the assay is shipped	.html
Plate ID	1234567	Bar Code ID of the box in which the assay is shipped	.txt
Rack or Array ID	1234567	Bar Code ID of the box in which the assay is shipped	.html
Vial Type	10-digit bar coded tube	Type of tube containing the assay	.txt .html
Vial ID	0004696076	Unique 2-D bar code of the assay tube	.txt .html



Columns	Example	Description	AIF File Format
Well Location	B02	Well location of the assay in the associated bar coded plate	.txt
Tube/Well Position	B02	Well location of the assay in the associated bar coded plate	.html
Assay Mix Concentration	40X	Concentration of the SNP Genotyping Assay mix [primers and probe(s)]	.txt .html
Forward Primer Name	AssayID_F	For custom assays only	.txt
Forward Primer Sequence	TGGAGAAGCACCGTGAACC	For custom assays only	.txt .html
Forward Primer Concentration (µM)	36	Concentration (in µM) of the forward primer	.txt .html
Reverse Primer Name	AssayID_R	For custom assays only	.txt
Reverse Primer Sequence	CTCTCCCAGACCCACTTTT	For custom assays only	.txt .html
Reverse Primer Concentration (µM)	36	Concentration (in µM) of the reverse primer	.txt .html
Reporter 1 Name	AssayID_V	For custom assays only	.txt
Reporter 1 Sequence	CCCCCAAGGACCTC	For custom assays only	.txt .html
Reporter 1 Dye	VIC®	Dye label for reporter 1	.txt .html
Reporter 1 Concentration (µM)	8	Concentration (in µM) of reporter 1	.txt .html
Reporter 1 Quencher	NFQ	Quencher used for reporter 1 of the assay	.txt .html
Reporter 2 Name	AssayID_M	For custom assays only	.txt
Reporter 2 Sequence	CCCCCAAGGACCTC	For custom assays only	.txt .html
Reporter 2 Dye	FAM™	Dye label for reporter 2	.txt .html



Appendix E About the Assay Information File (AIF)
Explanation of the AIF Columns

Columns	Example	Description	AIF File Format
Reporter 2 Concentration (μM)	8	Concentration (in μM) of reporter 2	.txt .html
Reporter 2 Quencher	NFQ	Quencher used for reporter 2 of the assay	.txt .html
Context Sequence	...AGCTA[T/A]CAGTC...	Nucleotide sequence surrounding the SNP site. SNP alleles are bracketed. The allele order corresponds to the association with reporter dyes, where [Allele1 = VIC®/Allele2 = FAM™]. For Predesigned SNP and Drug Metabolism Enzyme assays only.	.txt .html
Design Strand	Forward	Strand used to design the TaqMan® probe	.txt
Category	Chromosome 12	Chromosome on which the SNP is found	.txt
Category ID	Chr12	Chromosome on which the SNP is found	.txt
Group	D12S345-D12S1663	Microsatellite markers associated with the SNP	.txt
Group ID	D12S345	Microsatellite markers associated with the SNP	.txt
Entrez Gene ID	45241185	Entrez Gene database ID of the gene in which the SNP is found	.html
Transcript Accession	NM_005957.4	Accession number of the transcript in which the SNP is found	.html
Location on Transcript or Gene	3583	Location of the SNP in the gene or transcript in which it is found	.html
Gene Symbol	IRAK4	Entrez Gene database symbol for associated gene	.txt .html
Gene Name	Interleukin-1 receptor	Entrez Gene database gene name	.txt .html



Columns	Example	Description	AIF File Format
Chromosome	12	Chromosome on which the SNP is found	.txt .html
Species	Homo sapiens	Organism for which the assay was designed	.txt .html
Target Exons	N/A	N/A	.txt
NCBI Gene Reference	NM_001735	NCBI transcript ID detected by the assay	.txt
NCBI SNP Reference	rs17611	Reference ID from NCBI dbSNP	.txt .html
Medline Reference	N/A	N/A	.txt
Celera ID	hCV11720402	Celera Discovery System (CDS) unique SNP ID	.txt
Celera SNP ID	hCV11720402	Celera Discovery System (CDS) unique SNP ID	.html
Cytogenic Band	9q34	Cytoband location of the target sequence	.txt .html
SNP Type	Missense	SNP mutation type (Acceptor splice site, donor splice site, intergenic/unknown, intron, missense mutation, nonsense mutation, silent mutation, UTR 3', or UTR 5')	.txt .html
Minor Allele Freq Caucasian	0.48	As calculated by SNP genotyping at Life Technologies – Caucasian	.txt .html
Minor Allele Freq African American	0.14	As calculated by SNP genotyping at Life Technologies – African American	.txt .html
Minor Allele Freq Japanese	0.24	As calculated by SNP genotyping at Life Technologies – Japanese	.txt .html
Minor Allele Freq Chinese	0.36	As calculated by SNP genotyping at Life Technologies – Chinese	.txt .html
Celera Assembly Build Number	N/A	N/A	.txt



Appendix E About the Assay Information File (AIF)
Explanation of the AIF Columns

Columns	Example	Description	AIF File Format
Location on Celera Assembly	N/A	N/A	.txt
NCBI Assembly Build Number	37	NCBI assembly version from which the coordinate position is obtained	.txt .html
Location on NCBI Assembly	59971795	Location on the chromosome on the NCBI genome assembly (as referenced)	.txt .html
Amino Acid Change	H1146Q	Change in amino acid sequence due to the SNP	.html
HapMap Minor Allele Freq - YRI	0.09	As calculated by HapMap Project – Yoruba in Ibadan, Nigeria	.html
HapMap Minor Allele Freq - CEU	0.31	As calculated by HapMap Project – Utah residents with Northern and Western European ancestry from the CEPH collection	.html
HapMap Minor Allele Freq - CHB	0.49	As calculated by HapMap Project – Han Chinese in Beijing, China	.html
HapMap Minor Allele Freq - JPT	0.36	As calculated by HapMap Project – Japanese in Tokyo, Japan	.html
Amplicon Size	N/A	N/A	.html



User supplied materials and equipment

The tables below list the reaction plates and accessories available for Life Technologies real-time PCR systems.

Table 22 Recommended consumables to used with Life Technologies real-time PCR systems

Real-Time PCR System	Reaction Plates and Accessories
7500 System Standard 96-Well Block Module	<ul style="list-style-type: none"> • MicroAmp® Optical 96-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> – 500 plates (Cat. no. 4326659) – 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Optical Film Compression Pad, 5 pads (Cat. no. 4312639) • MicroAmp® Optical 8-Cap Strip, 300 strips (Cat. no. 4323032) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)
7500 Fast System Fast 96-Well Block Module	<ul style="list-style-type: none"> • MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> – 200 plates (Cat. no. 4366932) – 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Optical 8-Cap Strip, 300 strips (Cat. no. 4323032) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)

Real-Time PCR System	Reaction Plates and Accessories
<p>ViiA™ 7 System or QuantStudio™ 6K, 7K, or 12K Flex System 384-Well Block Module</p>	<ul style="list-style-type: none"> • MicroAmp® Optical 384-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> - 1000 plates (Cat. no. 4343814) - 500 plates (Cat. no. 4326270) - 50 plates (Cat. no. 4309849) • MicroAmp® Optical 384-Well Reaction Plate, 1000 plate (Cat. no. 4343370) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)
<p>ViiA™ 7 System or QuantStudio™ 6K, 7K, or 12K Flex System Standard 96-Well Block Module</p>	<ul style="list-style-type: none"> • MicroAmp® Optical 96-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> - 500 plates (Cat. no. 4326659) - 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Optical Film Compression Pad, 5 pads (Cat. no. 4312639) • MicroAmp® Optical 8-Cap Strip, 300 strips (Cat. no. 4323032) • MicroAmp® Snap-On Optical Film Compression Pad, for use with the automation accessory (Cat. no. 4333292) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)
<p>ViiA™ 7 System or QuantStudio™ 6K, 7K, or 12K Flex System Fast 96-Well Block Module</p>	<ul style="list-style-type: none"> • MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> - 200 plates (Cat. no. 4366932) - 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)
<p>StepOnePlus™ System</p>	<ul style="list-style-type: none"> • MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> - 200 plates (Cat. no. 4366932) - 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)

Real-Time PCR System	Reaction Plates and Accessories
7900HT/7900HT Fast System 384-Well Block Module	<ul style="list-style-type: none"> • MicroAmp® Optical 384-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> - 1000 plates (Cat. no. 4343814) - 500 plates (Cat. no. 4326270) - 50 plates (Cat. no. 4309849) • MicroAmp® Optical 384-Well Reaction Plate, 1000 plate (Cat. no. 4343370) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)
7900HT/7900HT Fast System Standard 96-Well Block Module	<ul style="list-style-type: none"> • MicroAmp® Optical 96-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> - 500 plates (Cat. no. 4326659) - 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Optical Film Compression Pad, 5 pads (Cat. no. 4312639) • MicroAmp® Optical 8-Cap Strip, 300 strips (Cat. no. 4323032) • MicroAmp® Snap-On Optical Film Compression Pad, for use with the automation accessory (Cat. no. 4333292) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)
7900HT/7900HT Fast System Fast 96-Well Block Module	<ul style="list-style-type: none"> • MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> - 200 plates (Cat. no. 4366932) - 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Adhesive Film Applicator (Cat. no. 4333183)

Table 23 Optional Master Mix reagents

Product	Volume	Cat. no.
TaqMan® Sample-to-SNP™ Kit ^[1]	5 mL Sample prep, 1 mL PCR	4403313
	200 mL Sample prep, 10 mL PCR	4403081
	20 mL Sample prep, 10 mL PCR	4403083
	20 mL Sample prep, 50 mL PCR	440387

Product	Volume	Cat. no.
TaqMan [®] GTXpress [™] Master Mix	400 reactions	4403311
	4000 reactions	4401892
TaqMan [®] GTXpress [™] Master Mix	20,000 reactions	4401890
	40,000 reactions	4401857

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



Good laboratory practices for PCR/RT-PCR

When preparing samples for PCR/RT-PCR amplification:

- Wear clean gloves and a clean lab coat (not previously worn while handling amplified PCR/RT-PCR products or used during sample preparation).
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
 - Sample preparation and PCR/RT-PCR setup.
 - PCR/RT-PCR amplification and analysis of PCR/RT-PCR products.
- Do not bring amplified PCR/RT-PCR products into the PCR/RT-PCR setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying PCR/RT-PCR 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 DNAZap™ Solutions (Cat. no. AM9890).



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, and 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. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may 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/bmb15/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
-

Documentation and support

Obtaining documentation

Portable document format (PDF) versions of this guide and the documents listed in this section are available on the Life Technologies website at www.lifetechnologies.com/support.

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

Table 24 Product documentation

Document	Publication number
TaqMan® Genotyping Master Mix Protocol	4371131
TaqMan® Genotyping Master Mix Quick Reference Card	4371130
Custom TaqMan® Assays Design and Ordering Guide	4367671
TaqMan® Genotyper™ Software Getting Started Guide	4448637
TaqMan® Genotyper™ Software Quick Reference Card	4448638
TaqMan® Assays Shipped at Ambient Temperature Reduce Environment Impact and Retain Their Quality and Stability	090071

Table 25 Instrumentation documentation

Document	Publication number
Applied Biosystems® 7300/7500/7500 Fast Real-Time PCR System Allelic Discrimination Getting Started Guide	4347822
Applied Biosystems® ViiA™ 7 Real-Time PCR System Getting Started Guide	4441434
Applied Biosystems® ViiA™ 7 Real-Time PCR System User Guide	4442661
Applied Biosystems® StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Genotyping Experiments	4376786
Applied Biosystems® QuantStudio™ 12K Flex Real-Time PCR System Getting Started Guide	4470935

Document	Publication number
Applied Biosystems® QuantStudio™ 12K Flex Real-Time PCR System User Guide	4470542
QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide	MAN0007993
Applied Biosystems® 7900HT Fast Real-Time PCR System Allelic Discrimination Getting Started Guide	4364015

TaqMan® Assays QPCR Guarantee

Only the following assays are covered by the TaqMan® Assay QPCR Guarantee: predesigned SNP Genotyping Assays and Drug Metabolism Enzyme Genotyping Assays. Without limiting the foregoing, custom SNP Assays are expressly excluded from the TaqMan® Assay QPCR Guarantee. Life Technologies makes the following guarantee to the customer on each assay delivered to the customer:

- Quality - Each assay is manufactured with high quality to enable customer to obtain reproducible results from lot-to-lot.
- Performance - Each assay is designed with a robust algorithm enabling superior sensitivity, specificity, and accuracy. Assay's performance specifications are set based on purified, high quality samples. Assay's performance may vary depending on the type and quality of sample.
- Content - Each assay is part of the largest collection of qPCR primer and probe sets.
- Results - Each Assay will enable you to obtain data you can trust.

If any Assay does not meet the above guarantee, Life Technologies at its option will either replace it at no cost or credit your account with the purchase price of such assay. For assay purchased in a multi-assay arrayed format (for example, TaqMan® Array Cards or Plates), Life Technologies reserves the right to replace or provide credit for each assay individually and is not obliged to replace or provide credit for the entire card or plate. Customer may use such credit to purchase other assay products from Life Technologies. Replacement or credit of the purchase price as set forth above is Life Technologies sole obligation and customer's sole and exclusive remedy with respect to failure of the assays to meet the TaqMan® Assay QPCR Guarantee.

Visit www.lifetechnologies.com for full information on TaqMan® Assays QPCR Guarantee or refer to *Gene Expression Assay Performance Guaranteed With the TaqMan® Assays QPCR Guarantee Program* (Pub. no. CO13369).

Note: For additional documentation, see "Obtaining support" on page 97.

Obtaining SDSs

Safety Data Sheets (SDSs) are available from www.lifetechnologies.com/support.

Note: For the SDSs of chemicals not distributed by Life Technologies Corporation, contact the chemical manufacturer.

Obtaining support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

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.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

References

- Afonina, I., Zivarts, M., Kutuyavin, I., et. al. 1997. *Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder*. *Nucleic Acids Res.* 25:2657-2660.
- Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., and Kimura, K. 1994. *Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification*. *J. Forensic Sci.* 39:362-372.
- Bleasby, K., Hall, L.A., Perry, J.L., Mohrenweiser, H.W., and Pritchard, J.B. 2005. *Functional Consequences of Single Nucleotide Polymorphisms in the Human Organic Anion Transporter hOAT1 (SLC22A6)*. *J. Pharmacol. and Exp. Therapeutics* 314:923-931.
- Bolt, H. M. and Thier, R. 2006. *Relevance of the deletion polymorphisms of the glutathione S-transferases GSTT1 and GSTM1 in pharmacology and toxicology*. *Current Drug Metabolism*, 7:613-628.
- Cho, H.J., Lee, S.Y., Ki, C.S., and Kim J.W. 2005. *GSTM1, GSTT1 and GSTP1 polymorphisms in the Korean population*. *J. Korean Med. Sci.* 20:1089-92.
- Dean, M. and Ballard, J.W.O. 2001. *Factors affecting mitochondrial DNA quality from museum preserved *Drosophila simulans**. *Entomologia Experimentalis et Applicata*. 98:279-283.
- DeFranchis, R., Cross, N.C.P., Foulkes, N.S., and Cox, T.M. 1998 *A potent inhibitor of Taq DNA polymerase copurifies with human genomic DNA*. *Nucleic Acids Res.* 16:10355.
- den Dunnen, J., Antonarakis, S. 2001 *Nomenclature for the description of human sequence variations*. *Human Genetics* 109:121-124.
- Eichelbaum, M., Ingelmann-Sundberg, M., and Evans W.E. 2006 *Pharmacogenomics and Individualized Drug Therapy*. *Annual Review of Medicine* 57:119-137.
- Gallagher, S.R. 1994. *Quantitation of DNA and RNA with Absorption and Fluorescence Spectroscopy*. *Current Protocols In Molecular Biology*, Vol. 3. John Wiley & Sons, pp. A3.D.1-A3.D3.
- Holodniy, M., Kim S., Katzenstein, D., Konrad, M., Groves, E., and Merigan, T.C. 1991. *Inhibition of human immunodeficiency virus gene amplification by heparin*. *J. Clin. Microbiol.* 29:676-679.
- Katcher, H.L., and Schwartz, I. 1994. *A distinctive property of Tth DNA polymerase: Enzymatic amplification in the presence of phenol*. *Biotechniques* 16:84-92.
- Kutyavin, I.V., Lukhtanov, E.A., Gamper, H.B., and Meyer, R.B. 1997. *Oligonucleotides with conjugated dihydropyrroloindole tripeptides: base composition and backbone effects on hybridization*. *Nucleic Acids Res.* 25:3718–3723.
- Kwok, S. and Higuchi, R. 1989. *Avoiding false positives with PCR*. *Nature* 339:237-238.
- Liew S-N., Lazaruk K., Wong, L, Stevens, J., et al. 2005. *Determining the Copy Number of Genes Using Real-Time Quantitative PCR*. [poster] At American Society for Human Genetics Annual Meeting, Oct. 25-29, Salt Lake City, Utah.

- Luo H.-R., Gaedigk, A., Aloumanis, V., Wan, Y.J. 2005. *Identification of CYP2D6 impaired functional alleles in Mexican Americans*. Eur J. Clin. Pharmacol. 61:797-802.
- Maaroufi, Y., Ahariz N., Husson M., and Crokaert, F. 2004. *Comparison of different methods of isolation of dna of commonly encountered candida species and its quantitation by using a real-time PCR-based assay*. J. Clin. Microbiology 42:3159-3163.
- Mullis, K.B. and Faloona, F.A. 1987. *Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction*. Methods Enzymol. 155:335-350.
- Ouahchi, K., Lindeman, N., and Lee, C., 2006. *Copy number variants and pharmacogenomics*. Pharmacogenomics 7:25-29.
- Oscarson, M. 2001. *Genetic polymorphisms in the cytochrome P450 2A6 (CYP2A6) gene: implications for interindividual differences in nicotine metabolism*. Drug Metabolism and Disposition 29:91-95.
- Rao Y., Hoffmann E., Zia M., Bodin L., et al. 2000. *Duplications and defects in the CYP2A6 gene: identification, genotyping, and in vivo effects on smoking*. Mol. Pharmacol. 58(4):747-55.
- Rebeck T.R., 1997. *Molecular epidemiology of the human glutathione S-transferase genotypes GSTM1 and GSTT1 in cancer susceptibility*. Cancer Epidemiol. Biomarkers Prev. 6:733-43.
- Roses, A.D. 2004. *Pharmacogenetics and Drug Development: The Path to Safer and More Effective Drugs*. Nature Reviews Genetics 5:645-56.
- Saiki, R.K., Scharf, S., Faloona, F.A., et al. 1985. *Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia*. Science 230:1350-1354.
- Shastry, B.S. 2006. *Pharmacogenetics and the concept of personalized medicine*. Pharmacogenomics Journal 6:16-21.
- Smits K. M., Gaspari L., Weijenberg M. P., et al. 2003. *Interaction between smoking, GSTM1 deletion and colorectal cancer: results from the GSEC study*. Biomarkers 8:299-310.
- Szakács, G., Annereau, J.P., Lababidi, S., et al. 2004. *Predicting drug sensitivity and resistance: Profiling ABC transporter genes in cancer cells*. Cancer Cell 6:129-137.
- Thier, R., Brüning, T., Roos, P.H., et al. 2006. *Markers of genetic susceptibility in human environmental hygiene and toxicology: The role of selected CYP, NAT and GST genes*. International Journal of Hygiene and Environmental Health 206:149-171.
- Topcul, Z., Chiba, I., Fujieda, M., et al. 2002. *CYP2A6 gene deletion reduces oral cancer risk in betel quid chewers in Sri Lanka*. Carcinogenesis 23:595-598.
- Wilkinson, G.R., 2005. *Drug metabolism and variability among patients in drug response*. N. Engl. J. Med. 352:2211-21.
- Wong, G.K., Yang, Z., Passey, D.A., et al. 2003. *A population threshold for functional polymorphisms*. [letter] Genome Research 13:1873-1879.
- Xie H-G., Kim R.B., Wood A.J.J., Stein C.M. 2002. *Molecular basis of ethnic differences in drug disposition and response*. Ann. Rev. Pharmacologic Toxicity. 41:815-850.
- Xu, C., Goodz, S., Sellers, E.M., Tyndale, R.F., 2002. *CYP2A6 genetic variation and potential consequences*. Adv. Drug Deliv. Rev. 54:1245-56.

Zanger, U.M., Raimundo, S., and Eichelbaum, M. 2004. *Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry*. Naunyn Schmiedebergs Arch. Pharmacol. 369:23-37.

Haque, K.A., Pfeiffer, R.M., Beerman, M.B., Struewing, J.P., Chanock, S.J., and Bergen, A.W. 2003. *Performance of high-throughput DNA quantification methods*. BMC Biotechnol. Oct 28;3-20.

Index

A

Absolute quantitation 48
Allele
 Frequency 9
 Nomenclature 9
Allelic discrimination
 Plate read 27
 Plot 27, 30, 31
Assay database 14
Assay Information File (AIF)
 Assay ID 81
 Columns 81
 Well Location 81
Autocalling 27, 59

B

biohazard safety 94

C

Chromosome
 Location 9
 X 38
 Y 38
Classification scheme 27
Cluster
 Diffuse 54, 58
 Trailing 54, 58
Context sequence 9
Coriell cell line 8
Custom Assay Design Tool (CADT) 8, 14

D

DME Index 14
DNA
 Degradation 48
 Quantitation 48
DNA delivery
 Dried-down DNA 20, 21
 Wet DNA 20, 21
documentation, related 95
Drug Metabolism Enzyme (DME)

 Phase I
 Phase II
 Transmembrane transporters
Drug Metabolism Index 9

F

FAM dye 27, 65
FAM dye-labeled probe 9
Fluorometric analysis 48

G

Genotyper Software 27

H

Haplotypes maps 8
HapMap 8
Hardy-Weinberg
 Equilibrium equation 59

I

Indels 8

L

limited product warranty 97
LIMS 81

M

Material Data Safety Sheets (MSDSs). *See* Safety Data Sheets (SDSs)
Minor groove binding (MGB) 9
Minor groove-binding (MGB) 8
Minor-groove binding (MGB)
 Probe 65
Multi-nucleotides polymorphism (MNP) 8

N

Non-fluorescent quencher (NFQ) 9

O

Outliers 54, 59

P

PCR inhibition 48
Polymorphism 9
Probe hybridization 65

R

related documentation 95
Reporter dye information 9
Rescaling 59

S

safety
 biohazard 94
Safety Data Sheets (SDSs), obtaining 96
Supplementary Sample Information (SSI) 27

support, obtaining 97

T

Tag SNP 8
terms and conditions 97
training, information on 97

U

UV spectroscopy 48

V

VIC dye 27, 65
VIC dye-labeled probe 9

W

warranty 97

Headquarters

5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288

For support visit lifetechnologies.com/support or email techsupport@lifetech.com

lifetechnologies.com

