applied biosystems

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Genotyping Experiments

APPLICATION GUIDE

for use with:

OpenArray™ Genotyping Plates

QuantStudio™ 12K Flex instrument with OpenArray™ block (AccuFill™ System)

TaqMan™ Genotyper Software

Publication Number MAN0014405

Revision A.0

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

DISCLAIMER

TO THE EXTENT ALLOWED BY LAW, LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

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.

Revision history

Table 1 Revision history of Pub. no. MAN0014405

Revision	Date	Description
A.0	25 February 2016	New document

Corporate entity

Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

TRADEMARKS

Contents

1 Introduction and workflow overview

CAUTION! ABBREVIATED SAFETY ALERTS. Hazard symbols and hazard types specified in procedures may be abbreviated in this document. For the complete safety information, see the "Safety" appendix in this document.

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

This guide describes the OpenArray™ plate high-throughput, sample-to-result workflow for genotyping CFTR mutations in buccal and leukocyte samples. The workflow uses:

- TaqMan™ SNP Genotyping Assays in OpenArray™ plates
- QuantStudio™ 12K Flex instrument with OpenArray™ block (AccuFill™ System)
- *(Optional)* Thermo Fisher Cloud GT Analysis module at **www.thermofisher.com/us/en/home/cloud.html**
- TaqMan™ Genotyper Software

Qualified TaqMan™ SNP Genotyping Assays for CFTR mutations

We offer a collection of ~200 qualified TaqMan™ SNP Genotyping Assays that have been optimized for genotyping key CFTR mutations. Target coding and regulatory mutations were selected from the Cystic Fibrosis Mutation Database (CFTR1) Database (**www.genet.sickkids.on.ca**) and the Clinical and Functional Translation of CFTR (CFTR2) Database (**www.cftr2.org**). Additional TaqMan™ SNP Genotyping Assays for CFTR mutations are available from our extensive predesigned SNP assay collection or can be ordered through our custom TaqMan™ SNP assay design service.

Qualified CFTR mutation assays demonstrate accurate, reproducible performance in multiple rounds of testing and perform well with DNAs isolated from blood leukocytes and buccal cell samples isolated using optimized MagMAX™ DNA Multi-Sample Ultra Kit protocols (see "Isolate DNA from buccal swabs" on page 22 and "Isolate DNA from blood leukocytes" on page 26).

The diverse nature and the rarity of key CFTR mutations presented challenges in developing TaqMan™ assays. We designed the assays using the proprietary Applied Biosystems™ TaqMan™ SNP Genotyping Assays design pipeline:

- Nontarget SNP positions in mutation context sequences were stringently masked to avoid designing assays over known SNPs that could interfere with assay performance.
- The target uniqueness of assay designs was checked by alignment to genomic sequences.
- All assay designs were qualified to meet stringent specificity and sensitivity criteria:
  - Assays were initially run in real-time PCR on both 384-well plates and OpenArray™ plates with 47 Coriell gDNA samples that include 44 total characterized CFTR mutations (**www.coriell.org**) .
  - Because most CFTR mutations are rare and control gDNAs are not available for all targets, assays were also tested with synthetic DNA controls representing three genotypes for each CFTR variant, to ensure robust discrimination of alleles and genotypes .

Workflow: OpenArray™ plate CFTR experiments

Chapter 3, "Isolate DNA using the MagMAX™ DNA Multi-Sample Ultra Kit"

▼

Chapter 4, "Prepare and run OpenArray™ CFTR SNP genotyping experiments"

▼

Chapter 5, "Analyze CFTR genotyping data"

Review assay guidelines for non-standard assays.

IMPORTANT! If you are interrogating mutations that require two assays, review the data for both assays together.

▼

Chapter 6, "Confirm positive results"

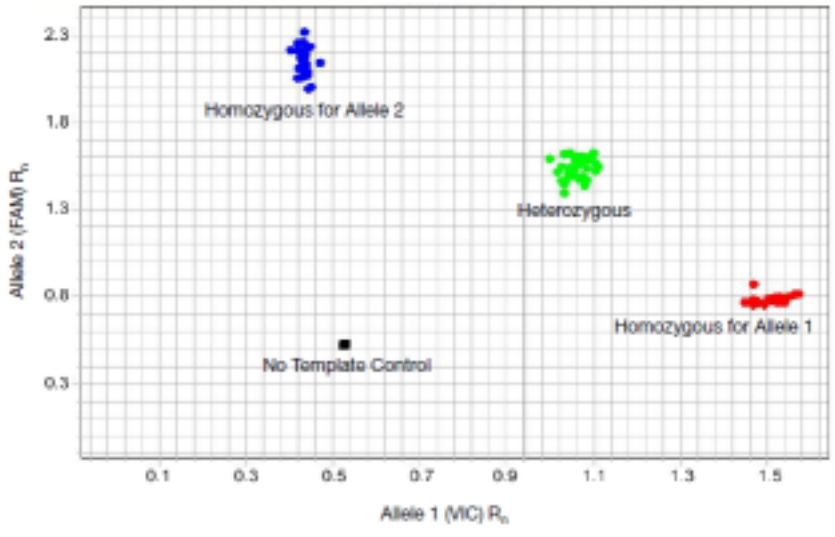
2 Background and tools for assay content selection

TaqMan™ SNP Genotyping Assays

TaqMan™ SNP Genotyping Assays contain sequence-specific forward and reverse primers to amplify the genomic region of interest and two TaqMan™ MGB probes with Non-fluorescent quencher (NFQ):

- One probe labeled with VIC™ dye detects the Allele 1 sequence.
- One probe labeled with FAM™ dye detects the Allele 2 sequence.

Data is analyzed by cluster plot analysis: FAM™ dye signal is plotted on the Y-axis and VIC™ dye signal is plotted on the X-axis. Samples homozygous for the FAM™ or VIC™ dye-labeled alleles form clusters along the Y- or X-axis. Heterozygous samples contain both FAM™ and VIC™ dye signals and are grouped in clusters roughly along the diagonal position between the homozygous clusters.



CFTR nomenclature

CFTR researchers often use descriptive legacy nomenclature to identify mutation targets. Many legacy names are imprecise; for example, amino acid change names are often used instead of the specific nucleotide change.

In an effort to standardize CFTR mutation names, the Cystic Fibrosis Mutation Database (CFTR1 database **www.genet.sickkids.on.ca**) converted legacy CFTR nomenclature to Human Genome Variation Society (HGVS) nomenclature, which uses standardized rules for naming variants mapped to reference genome, transcript, and protein sequences. The new CFTR nucleotide and protein names and the associated legacy names are provided on the CFTR1 database website (**www.genet.sickkids.on.ca**), and a subset of these are also listed on the Clinical and Functional Translation of CFTR website (CFTR2 database **www.cftr2.org**).

Assay context sequences

The context sequence is the nucleotide sequence surrounding the SNP site. It 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: [Allele 1 = VIC™ dye / Allele 2 = FAM™ dye].

The reporter dye information is represented in the assay context sequence, which is posted on **thermofisher.com**. It is also provided in the Assay Information File:

- OpenArray™ plates – Download from **www.thermofisher.com/OA-platefiles**
- TaqMan™ Array 384-well plates – Provided with your order
- Single-tube – Download from **www.thermofisher.com/taqmanfiles**

Note that for many, but not all, of the qualified CFTR mutation assays, the wild type allele is identified by the VIC™ dye-labeled probe and the mutant allele by the FAM™ dye-labeled probe.

For example, in Assay ID: C_151693869_10, which targets F508del, c.1521_1523delCTT (SNP ID: rs113993960), has the following context sequence: The VIC™ dye-labeled probe is associated with the deletion allele and the FAM™ dye-labeled probe is associated with the wild type allele.

```
5'-CTGGCACCATTAAAGAAAATATCAT[-/CTT]TGGTGTTTCCTATGA
TGAATATAGA-3'
```

Plate products and formats for CFTR assays

TaqMan™ OpenArray™ Genotyping plate products and formats

OpenArray™ plates contain pre-plated, dried down TaqMan™ SNP Genotyping Assays.

Array format	Number of assays[1]	Number of samples	Cat. no.
32	26	96	4471114 (4470171[2])
64	60	48	4471115 (4470172[2])
128	120	24	4471116 (4470173[2])
192	180	16	4471117 (4470174[2])

[1] Number of assays recommended for optimal performance in this format.

[2] Cat. no. if ordered through a sales representative

TaqMan™ SNP Genotyping Assay single-tube products and formats

TaqMan™ single-tube assays can be used to confirm positive results.

Product Scale	Number of reactions		Assay mix formulation	Cat. no. (Human)
	384-well	96-well		
Small	1500	300	40x	4351379
Medium	5000	1000	40x	4351376
Large	12000	2400	80x	4351374

Optional CFTR controls

The CFTR TaqMan™ Assay Controls (Cat. no. A30421) can optionally be used in CFTR experiments to verify results and to assist with troubleshooting.

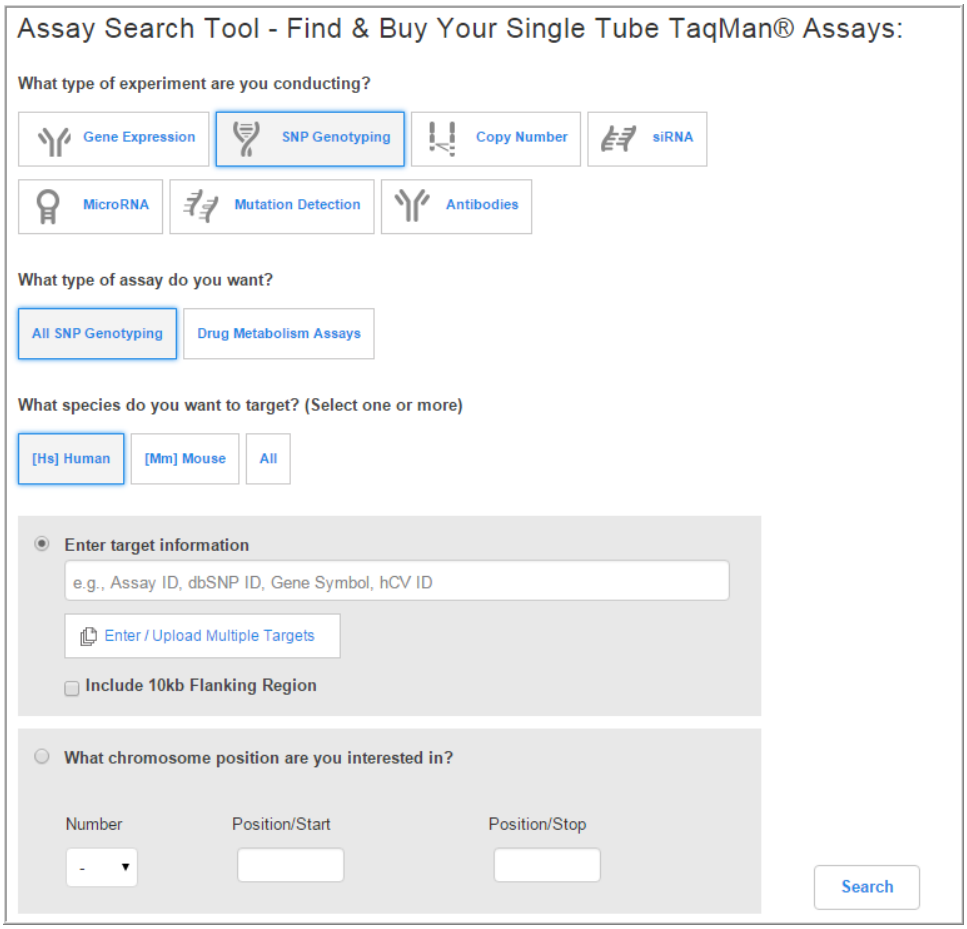
For information on the controls kit, refer to *CFTR TaqMan™ Assay Controls Quick Reference* (Pub. no. MAN0014633).

Tools for finding qualified TaqMan™ CFTR mutation assays

- Assay search tool available at **www.thermofisher.com/ordertaqman**
- CFTR TaqMan™ SNP Genotyping Assays Index available at **www.thermofisher.com/cftr**

Search for TaqMan™ CFTR assays

1. Go to **www.thermofisher.com/ordertaqman**.



2. Select **SNP Genotyping** ▸ **All SNP Genotyping** ▸ **[Hs] Human**.

3. Under target information, enter **CFTR**, then click **Search** to search for the complete mutation assay set.

   Optionally, specify other search terms (including rsSNP ID and assay ID) or specify chromosome position. You can use the Enter/Upload Multiple Targets option to specify multiple search terms.

4. Under Narrow Your Results (to the left of the results), select **CFTR** Gene and **Qualified** Assay Type.

5. Review the information for each assay: click **View Assay on Map**, **View Details**, and **View Allele Frequency** (if available).

   Note the following:

   - Read any Important Information notes associated with the assay (click the Important Information box next to an Assay ID). Notes provide information required to make ordering decisions and analyze data. For example, two assays may be required to interrogate one target mutation (for example, triallelic SNPs or CFTRdele2,3).
   - The annotation for triallelic SNP assays and large deletion assays is tied to the SNP ID and not to specific alleles. Review the Important Information or the assay context sequence to determine the target alleles of each assay.
   - View Details provides allele nomenclature (mutation names: legacy, nucleotide, and protein), which links to the CFTR1 database website

6. If you need a list of assays for ordering, select the assays of interest, then click **Export** (at the top of the page).

   The exported results contain the assay ID and annotations (catalog number, SNP, gene, context sequence, genomic location, and allele nomenclature).

CFTR TaqMan™ SNP Genotyping Assays Index

Download from **www.thermofisher.com/cftr**.

This file contains a comprehensive list of the qualified TaqMan™ CFTR mutation assays with the following annotations:

- Assay ID
- Gene symbol
- Mutation common name
- Mutation cDNA name
- Mutation protein name
- Mutation legacy name
- SNP ID
- NCBI cds annotation
- NCBI protein annotation
- NCBI assembly build
- NCBI assembly location
- Context sequence

Non-standard CFTR TaqMan™ SNP genotyping assays

CFTR TaqMan™ SNP genotyping assays to triallelic SNPs

CFTR triallelic SNPs have three bases that occur at the same genomic location. Triallelic SNP alleles are interrogated using two TaqMan™ assays. Each assay contains a probe for the wild type allele and a probe for one of the mutant alleles.

To generate accurate sample genotypes, run the two assays independently on the same samples, then analyze the allelic discrimination results together and compare the expected cluster positions from both assays. For information, see "Analysis guidelines: SNP genotyping assays to triallelic SNPs" on page 47.

Table 2 TaqMan™ CFTR assays to triallelic SNPs

SNP ID	Assay ID[1]	Mutation legacy name	Mutation HGVS nucleotide name	Mutation HGVS protein name	Assay alleles labeled with VIC™/FAM™ dye
rs121908751	C_32545261C_10	E92K	c.274G>A	p.Glu92Lys	G/A
rs121908751	C_32545261D_20[2]	E92X	c.274G>T	p.Glu92Ter	G/T
rs121908761	C_ 26083714C_10[2]	Y1092X(C->A)	c.3276C>A	p.Tyr1092X	C/A
rs121908761	C_ 26083714D_20	Y1092X(C->G)	c.3276C>G	p.Tyr1092X	C/G
rs121908799	C172767555C_30	2183AA->G	c.2051_2052delAAinsG	p.Lys684SerfsX38	AA/G
rs121908799	C172767555D_20	2183delAA	c.2051_2052delAA	p.Lys684ThrfsX4	AA/-
rs121908805	C_64676233C_10	S466X(TAA)	c.1397C>A	p.Ser466X	C/A
rs121908805	C_64676233D_20	S466X(TAG)	c.1397C>G	p.Ser466X	C/G
rs77932196	C___656878C_30[2]	R347P	c.1040G>C	p.Arg347Pro	C/G
rs77932196	C___656878D_20	R347H	c.1040G>A	p.Arg347His	A/G
rs80055610	C_27861436C_30	R560T	c.1679G>C	p.Arg560Thr	G/C
rs80055610	C_27861436D_20[2]	R560K	c.1679G>A	p.Arg560Lys	G/A

[1] Assay IDs for an assay pair contain the same core number with a C or D.

[2] This assay has nonspecific activity for the nontarget allele. You must run and analyze both triallelic SNP assays to ensure accurate results.

CFTR TaqMan™ SNP genotyping assays to regions with multiple mutations

Some qualified CFTR mutation assays cover probe-binding sites that contain one or more mutations that are close to the target mutation. These nearby nontarget mutations do not typically interfere with genotype analysis of the target mutation in heterozygous samples because these rare mutations are not found together in cis on the same chromosome.

Table 3 TaqMan™ SNP Genotyping Assays to the CFTR F508del region mutations

SNP ID	Assay ID	Mutation Legacy name	Mutation HGVS nucleotide name	Mutation HGVS protein name	Assay alleles labeled with VIC™/FAM™ dye
rs1800091	C__59055679_20	I506V	c.1516A>G	p.Ile506Val	A/G
rs121908745	C_151693868_10	I507del	c.1519_1521delATC	p.Ile507del	ATC/–
rs1801178	C___8853252_20	I507V	c.1519A>G	p.Ile507Val	A/G
rs113993960	C_151693869_10	F508del	c.1521_1523delCTT	p.Phe508del	–/CTT
rs74571530	C_100964862_10	F508C	c.1523T>G	p.Phe508Cys	G/T

Table 4 TaqMan™ SNP Genotyping Assays to the CFTR 2184delA region mutations

SNP ID	Assay ID	Mutation Legacy name	Mutation HGVS nucleotide name	Mutation HGVS protein name	Assay alleles labeled with VIC™/FAM™ dye
rs121908799	C172767555D_20	2183delAA	c.2051_2052delAA	p.Lys684ThrfsX4	AA/–
rs121908799	C172767555C_30	2183AA->G	c.2051_2052delAAinsG	p.Lys684SerfsX38	AA/G
rs121908786	C_172767550_10[1]	2184insA	c.2052_2053insA	p.Gln685ThrfsX4	–/A
rs121908746	C_172767547_20	2184delA	c.2052delA	p.Lys684AsnfsX38	A/–

[1] Assay C_172767550_10 to the rare 2184insA homopolymer mutation is not recommended in the OpenArray™ plate format . This assay distinguishes 7 from 8 A bases. The separation between heterozygous and wild type clusters in allelic discrimination plots is very low. Review real-time PCR trace data to confirm suspected heterozygous results.

CFTR TaqMan™ SNP genotyping assays to the 5T/7T/9T polymorphism

The CFTR 5T/7T/9T polymorphism, or triallelic polyT variant, is a component of the intron 8 polyAG/polyT mutation. The 5T mutation affects CFTR RNA splicing, resulting in low levels of CFTR protein. The 5T mutation is incompletely penetrant.

Two assays are required to genotype the polyT mutation:

- The 5T assay contains a probe for the wild type 7T allele (VIC™ dye) and a probe for the 5T mutant allele (FAM™ dye).
- The 9T assay contains a probe for the wild type 7T allele (FAM™ dye) and a probe for the 9T variant allele (VIC™ dye).

To generate accurate sample genotypes, run the two assays independently on the same samples, then analyze the allelic discrimination results together and compare the expected cluster positions from both assays. For information, see "Analysis guidelines: SNP genotyping assays to the 5T/7T/9T polymorphism" on page 53.

Table 5 TaqMan™ SNP Genotyping Assays to the CFTR 5T/7T/9T (polyT) mutation

SNP ID	Assay ID	Mutation Legacy name	Mutation HGVS nucleotide name	Mutation HGVS protein name	Assay alleles labeled with VIC™/FAM™ dye
rs387906372	C203006801C_10	5T	c.1210-12T[5_9]	No protein name	TT/– (7T/5T)
rs387906372	C203006801D_20	9T	c.1210-12T[5_9]	No protein name	TT/– (9T/7T)

CFTR TaqMan™ SNP genotyping assays to large CFTR mutations

CFTRdele2,3 and CFTRdele22,23 are large deletion mutations that are each interrogated by a pair of modified TaqMan™ copy number assays. Each assay contains one functional probe:

- Wild type allele assay – One functional probe (VIC™ dye) and one non-functional probe (FAM™ dye)
- Mutant allele assay – One functional probe (FAM™ dye) and one non-functional probe (VIC™ dye)

The non-functional probe allows these copy number assays to be run with SNP assay sets.

To generate accurate sample genotypes, run the two assays independently on the same samples, then analyze the allelic discrimination results together and compare the expected cluster positions from both assays. For information, see "Analysis guidelines: SNP genotyping assays to large CFTR mutations" on page 54.

Table 6 TaqMan™ SNP Genotyping Assays to large deletion mutations

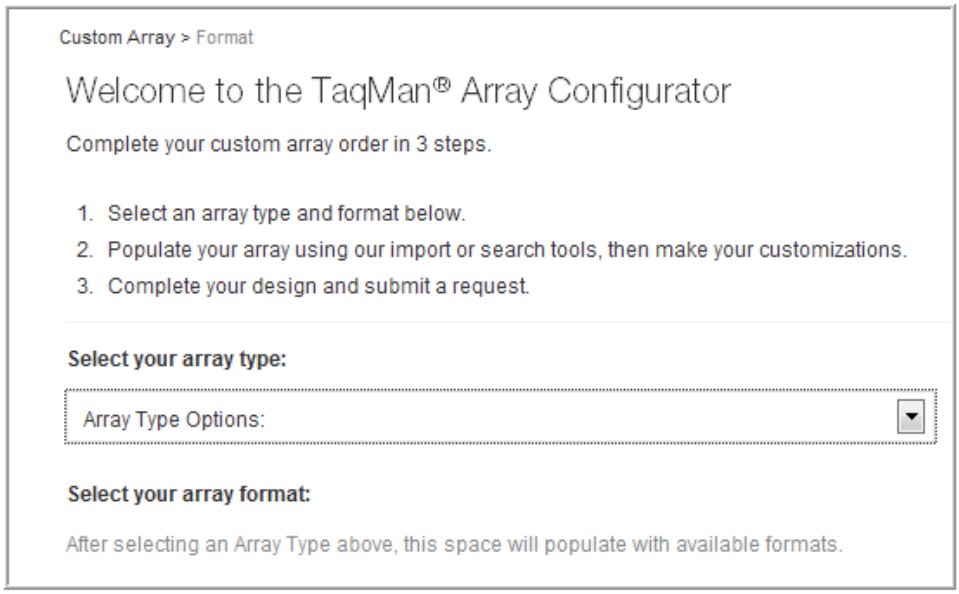
Assay ID[1]	Mutation legacy name	Mutation HGVS nucleotide name	Mutation HGVS protein name	Assay alleles labeled with VIC™/FAM™ dye
C990000002A_10	CFTRdele2,3	c.54-5940_273+10250del21kb	p.Ser18ArgfsX16	–/mu
C990000002B_20	CFTRdele2,3	c.54-5940_273+10250del21kb	p.Ser18ArgfsX16	wt/–
C990000003A_10	CFTRdele22,23	c.3964-78_4242+577del1.5kb	No protein name	–/mu
C990000003B_20	CFTRdele22,23	c.3964-78_4242+577del1.5kb	No protein name	wt/–

[1] Assay IDs for an assay pair contain the same core number with A or B.

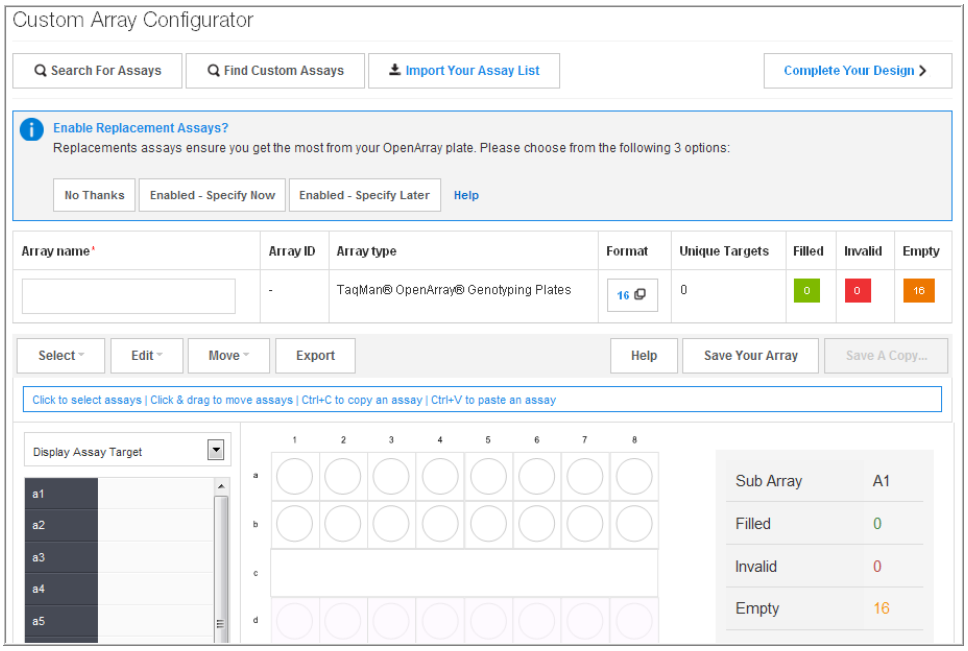
Order assays

Order custom OpenArray™ plates

1. Go to **www.thermofisher.com/order/custom-array/**.



2. For array type, select **TaqMan™ OpenArray™ Genotyping Plates**.
3. Click **View Layout** to display the assay format in a plate.
4. Click **Select** to configure a plate.



5. Click **Import Your Assay List**, then select a tab-delimited text file (.txt) containing Assay IDs or paste the IDs in the Enter a list of Assays IDs field, then click **Import Entered List**.

6. When the plate is configured, click **Complete Your Design**, then follow the screen instructions to complete the order.

Order single tube TaqMan™ assays

You can order single-tube assays in two ways:

- "Search for TaqMan™ CFTR assays" on page 11, add assays to the shopping cart, then complete the order.
- If you have already obtained catalog numbers and IDs (Assay IDs), click **Quick Order** at the top of any page at **thermofisher.com**.

3 Isolate DNA using the MagMAX™ DNA Multi-Sample Ultra Kit

Kit contents and storage

Component	Cat. no. A25597[1] (500 rxns)	Cat. no. A25598[2] (2500 rxns)	Storage
Proteinase K[3]	4 mL	5 × 4 mL	–25°C to –15°C
PK Buffer	96 mL	5 × 96 mL	15°C to 30°C
Multi-Sample DNA Lysis Buffer	100 mL	5 × 100 mL	
RNase A[4]	2 × 1.25 mL	10 × 1.25 mL	–25°C to –15°C
DNA Binding Beads[3]	8 mL	5 × 8 mL	2°C to 8°C
Nuclease-free Water	100 mL	5 × 100 mL	15°C to 30°C
Wash Solution 1 Concentrate	80 mL[5]	5 × 80 mL[5]	
Wash Solution 2 Concentrate	162 mL[5]	5 × 162 mL[5]	

Component	Cat. no. A25597[1] (500 rxns)	Cat. no. A25598[2] (2500 rxns)	Storage
DNA Elution Buffer 1	25 mL	5 × 25 mL	15°C to 30°C
DNA Elution Buffer 2	25 mL	5 × 25 mL	

[1] Also available as Cat. no. A25919, containing Cat. no. A25597 with **one additional tube** each of Proteinase K (4 mL) and DNA Binding Beads (8 mL).

[2] Also available as Cat. no. A25920, containing Cat. no. A25598 with **5 additional tubes** of Proteinase K (4 mL each) and **5 additional tubes** of DNA Binding Beads (8 mL each).

[3] Proteinase K is also available as Cat. no. A25561 and DNA Binding Beads are also available as Cat. no. A25562.

[4] Not used for DNA isolation from buccal swabs.

[5] Final volume; see "Before first use of the kit: prepare Wash Solutions" on page 23.

Required materials not supplied

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

Table 7 Required materials not included with the kit

Item	Source
One of the following instruments	
(Recommended) KingFisher™ Flex Magnetic Particle Processor	Cat. No. 5400630
MagMAX™ Express-96 Magnetic Particle Processor	—[1]
Equipment	
Plate shaker capable of shaking plates at a minimum of 900 rpm	MLS
Analog Vortex Mixer	Fisher Scientific 02-215-365
Adjustable micropipettors	MLS
Multi-channel micropipettors	MLS
(*Optional but recommended*) Magnetic Stand-96	Cat. No. AM10027
Plates and combs	
Deep Well Plates, one of the following:	
KingFisher™ Flex Microtiter Deepwell 96 Plate, Sterile	Cat. No. 95040460
MagMAX™ Express-96 Deep Well Plates	Cat. No. 4388476
Standard Well Plates, one of the following:	
KingFisher™ 96 KF Microplate	Cat. No. 97002540

Item	Source
MagMAX™ Express-96 Standard Plates	Cat. No. 4388475
Tip Combs, one of the following:	
KingFisher™ 96 Tip Comb for DW Magnets	Cat. No. 97002534
MagMAX™ Express-96 Deep Well Tip Combs	Cat. No. 4388487
Other consumables	
MicroAmp™ Clear Adhesive Film	Cat. No. 4306311
RNase-free Microfuge Tubes (2.0 mL)	Cat. No. AM12425
Conical tubes (15 mL)	Cat. No. AM12500
Conical tubes (50 mL)	Cat. No. AM12502
Aerosol-resistant pipette tips	MLS
Reagent reservoirs	MLS
(*Optional*) Paraffin film	MLS
Reagents	
Ethanol, 200 proof (absolute)	MLS
Isopropanol, 100% (molecular grade or higher)	MLS

[1] Not available for sale.

Table 8 Additional materials required for buccal swabs

Item	Source
Laboratory incubator with slatted shelves capable of reaching 65°C	MLS
One of the following types of buccal swabs, or equivalent buccal swabs with foam tips:	
Puritan™ PurFlock™ Ultra Flocked Swabs	Fisher Scientific 22-025-192
Puritan™ HydraFlock™ Swabs, standard tip	Puritan 25–3306–H
Sterile Foam Tipped Swabs	Puritan 25-1506 1PF
4N6FLOQSwabs™, regular tip	Cat. No. 4473979
(*Optional*) Proteinase K, 500 reactions (4 mL)	Cat. No. A25561
(*Optional*) DNA Binding Beads, 500 reactions (8 mL)	Cat. No. A25562

Table 9 Additional materials required for blood leukocytes

Item	Source
Centrifuge capable of spinning deep well plates at 1700 × g	MLS
Laboratory incubator with slatted shelves capable of reaching 65°C	MLS
Ammonium Chloride (A.C.S. grade or higher)	MLS
EDTA (0.5M), pH 8.0	Cat. No. AM9260G

Download the KingFisher™ Flex program

The programs required for these protocols are not pre-installed on the KingFisher™ Flex Magnetic Particle Processor.

1. On the MagMAX™ DNA Multi-Sample Ultra Kit web page, scroll down to the Product Literature section.

2. Click the appropriate file for your sample type to download the program to your computer.

Sample type	Program
Buccal swabs	A25597_Blood_Buccal
Leukocytes	A25597_Leukocytes

3. See *Thermo Scientific™ KingFisher™ Flex User Manual* (Cat. No. N07669) and *BindIt™ Software User Manual* (Cat. No. N07974) for instructions for installing the program on the instrument.

Set up the sample layout

Set up the sample plate layout using either of the following tools, which provide sample tracking from the 96-well plate used for DNA isolation to the 96-well sample plate .csv file used for import into OpenArray™ Sample Tracker Software.

Include at least 2 no template controls (NTCs) per plate.

Tool	Obtain from	Description
OA_Genotyping_CalcSheet	**thermofisher.com/oaqrc**	Contains a sample layout tab, additional sample tracking tabs, and instructions for using an adjustable pipettor to transfer samples from the 96-well plate to the 384-well sample plate.
96-well Sample Plate 1.csv template	On the computer on which the Sample Tracker Software is installed: C:\Program Files\Applied Biosytems\Sample Tracking Utility\examples	Contains a sample layout tab.

Isolate DNA from buccal swabs

This section contains brief procedures. For detailed information, refer to *MagMAX™ DNA Multi-Sample Ultra Kit (human buccal swabs) User Guide* (Pub. no. MAN0010293).

Refer to *Best Practices for Collection of Buccal Swabs Quick Reference (Genotyping Experiments)* (Pub. no. MAN0014348) for sample collection instructions.

Guidelines for buccal swab preparation

- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- Equilibrate buccal swabs to room temperature to maximize DNA recovery.
- Remove the buccal swabs from the lysate by:
  - Option 1: Transfer the lysate to a new plate.
    This option eliminates contamination risks and saves time. To transfer lysates, set a multi-channel micropipettor to ~300 µL and transfer one row at the time. Each well should contain 200–250 µL after transfer.
  - Option 2: Remove the swabs from the plate using forceps.
    Rinse the forceps in 70% ethanol between samples, to prevent cross-contamination. Press the swabs against the side of the well when removing them, to prevent sample loss.
- Cover the plate during the incubation and shaking steps to prevent spill-over and cross-contamination. The same MicroAmp™ Clear Adhesive Film can be used throughout the procedure, unless it becomes contaminated.

- If you use a plate shaker other than the Thermo Scientific™ Compact Digital Microplate Shaker, verify that:
  - The plate fits securely on your plate shaker.
  - The recommended speeds are compatible with your plate shaker. Ideal shaker speeds allow for thorough mixing without splashing.
- Per-plate volumes for reagent mixes are sufficient for one plate plus overage. To calculate volumes for other sample numbers, refer to the per-well volume and add 5% overage.
- (*Optional*) To prevent evaporation and contamination, cover the prepared processing plates with paraffin film until they are loaded into the instrument.
- This procedure is optimized for processing of one swab per well. It is possible to process two swabs in one well when a higher concentration of DNA is required.
- If the DNA yield is lower than expected, extend the Proteinase K digestion time to 45 minutes.

Before first use of the kit: prepare Wash Solutions

Prepare the Wash Solutions from the concentrates:

- Add 25 mL of isopropanol to Wash Solution 1 Concentrate, mix, and store at room temperature.
- Add 132 mL of ethanol to Wash Solution 2 Concentrate, mix, and store at room temperature.

Before each use of the kit: prepare DNA Binding Bead Mix

Vortex DNA Binding Beads thoroughly, then combine with Nuclease-free Water according to the following table.

Component	Volume per well	Volume per plate
DNA Binding Beads	16 µL	1.6 mL
Nuclease-free Water	24 µL	2.4 mL
Total DNA Binding Bead Mix	40 µL	4 mL

Store DNA Binding Bead Mix at room temperature for up to 24 hours.

Digest the samples with Proteinase K

Ensure that the incubator is preheated to 65°C.

1. Place the swab, swab-head down, in a well of a MagMAX™ Express-96 Deep Well Plate (one per well).

   If two swabs were collected, store the second swab as a backup sample.

2. Break enough of the stick off the swabs so that the swabs sit in the wells without protruding.

3. Prepare sufficient PK Mix according to the following table. Invert PK Mix several times to thoroughly mix components.

IMPORTANT! Prepare the PK Mix just before use. Do not place PK Buffer or PK Mix on ice, to avoid precipitation.

Component	Volume per well	Volume per plate
Proteinase K	8 µL	800 µL
PK Buffer	192 µL	19.2 mL
Total PK Mix	200 µL	20 mL

4. Add 200 µL of PK Mix to each sample well of a MagMAX™ Express-96 Deep Well Plate (PK Plate).

IMPORTANT! Do not touch any part of the swab with the pipet tip when pipetting the PK Mix in the sample wells.

5. Seal the plate with a MicroAmp™ Clear Adhesive Film and shake the sealed plate for 5 minutes at 900–950 rpm.

6. Incubate for 20–45 minutes at 65°C.

IMPORTANT! Arrange plates in the incubator to allow adequate flow around the plate wells, to ensure that samples quickly reach and maintain the incubation temperature.

Set up the processing plates

1. While the samples are incubating, set up the Wash, Elution, and Tip Comb Plates outside the instrument as described in the following table.

Plate ID	Plate position[1]	Plate type	Reagent	Volume per well
Wash Plate 1	2	Deep Well	Wash Solution 1	150 µL
Wash Plate 2	3	Deep Well	Wash Solution 2	150 µL
Wash Plate 3	4	Deep Well	Wash Solution 2	150 µL
Elution Plate[2]	5	Standard	DNA Elution Buffer 1	50 µL
Tip Comb	6	Deep Well	Place a MagMAX™ Express-96 Deep Well Tip Comb in a MagMAX™ Express-96 Deep Well Plate.	

[1] Position on the instrument.

[2] The instrument prompts the user to add DNA Elution Buffer 2 to the Elution Plate, after incubation with DNA Elution Buffer 1.

2. (*Optional*) To prevent evaporation and contamination, cover the prepared processing plates with paraffin film until they are loaded into the instrument.

Add Multi-Sample DNA Lysis Buffer, isopropanol, and DNA Binding Bead Mix

1. (*Optional*) If condensation is present at the end of the 65°C incubation, briefly centrifuge the plate for 1–2 minutes at 1500 × *g*.
2. Add 200 µL of Multi-Sample DNA Lysis Buffer to each sample.
3. Seal the plate with a MicroAmp™ Clear Adhesive Film and shake for 5 minutes at speed 8 (900–950 rpm).
4. Transfer lysates to the wells of a new MagMAX™ Express-96 Deep Well Plate and discard the plate containing the buccal swabs.
5. Add 240 µL of isopropanol to each sample, seal the plate, and shake for 5 minutes at speed 8 (900–950 rpm).
6. Vortex the DNA Binding Bead Mix at moderate speed to ensure thorough mixing, add 40 µL to each sample, then proceed immediately to DNA isolation (next section).

   If you see that the beads in the DNA Binding Bead Mix are settling, vortex the mix again briefly before continuing to pipette.

Process samples on the instrument

1. Select the program on the instrument.
   - MagMAX™ Express-96 Magnetic Particle Processor: **4413021 DW blood**
   - KingFisher™ Flex Magnetic Particle Processor: **A25597_Blood_Buccal**
2. Start the run, remove the temporary paraffin plate seals (if present), and load the prepared processing plates in their positions when prompted by the instrument (see "Set up the processing plates" on page 24).
3. Load the PK plate (containing lysate, isopropanol, and DNA Binding Bead Mix) at position 1 when prompted by the instrument.
4. When prompted by the instrument (approximately 28–30 minutes after initial start):
   a. Remove the Elution Plate from the instrument.
   b. Add 50 µL of DNA Elution Buffer 2 to each sample well.

      IMPORTANT! Add DNA Elution Buffer 2 immediately after the prompt, to prevent excessive drying of any beads that are still captured on the Tip Comb.

   c. Load the Elution Plate back onto the instrument, and press **Start**.

5. At the end of the run (approximately 30–35 minutes after initial start), remove the Elution Plate from the instrument and seal immediately with a new MicroAmp™ Clear Adhesive Film.
   - *(Optional)* Eluates can be transferred to a new storage plate after collection.
   - If precipitated DNA is visible, pipet up and down 5–10 times before sealing the plate, to ensure complete resuspension.
   - If excess bead residue is seen in the wells, place the Elution Plate on the Magnetic Stand-96 to capture any residue prior to downstream use of the DNA.

IMPORTANT! Do not allow the purified samples to sit uncovered at room temperature for more than 10 minutes, to prevent evaporation and contamination.

The purified samples are ready for immediate use. Alternatively, store the covered Elution Plate:

- At 2–6°C for up to 24 hours.
- At –20°C or –80°C for long-term storage.

Isolate DNA from blood leukocytes

This section contains brief procedures. For detailed information, refer to *MagMAX™ DNA Multi-Sample Ultra Kit (blood leukocytes) User Guide* (Pub. no. MAN0010818).

Guidelines for blood leukocyte preparation

- If the whole blood is frozen prior to use, thaw the sample at 25–37°C in a water bath until it is completely liquid, and place on ice until needed.
- Perform all steps at room temperature (20–25°C) unless otherwise noted.
- When mixing samples by pipetting up and down, avoid creating bubbles.
- Cover the plate during the incubation and shaking steps to prevent spill-over and cross-contamination. The same MicroAmp™ Clear Adhesive Film can be used throughout the procedure, unless it becomes contaminated.
- Perform incubation in ice by covering the plate in ice up to the sides without covering the top of the wells.
- Verify that:
  - The plate fits securely on your plate shaker.
  - The recommended speeds are compatible with your plate shaker. Ideal shaker speeds allow for thorough mixing without splashing.
- Per-plate volumes for reagent mixes are sufficient for one plate plus overage. To calculate volumes for other sample numbers, refer to the per-well volume and add 5% overage.

Before first use of the kit

- Prepare the Wash Solutions from the concentrates:
  - Add 25 mL of isopropanol to Wash Solution 1 Concentrate, mix, and store at room temperature.
  - Add 132 mL of ethanol to Wash Solution 2 Concentrate, mix, and store at room temperature.
- Prepare 10X Red Blood Cell (RBC) Buffer as indicated in the following table, mix, and store at 2–8°C.

Component	Quantity	Final concentration
Ammonium chloride	9 g	1.68 M
0.5 M EDTA, pH 8.0	400 µL	2 mM
Water	up to 100 mL	—
Total 10X RBC Buffer	100 mL	—

Before each use of the kit

- Preheat the incubator to 65°C.
- Prepare fresh 1X RBC Buffer as indicated in the following table, mix, and store on ice until use.

Component	Volume per well	Volume per plate
10X RBC Buffer	60 µL	6 mL
Water	540 µL	54 mL
Total 1X RBC Buffer	600 µL	60 mL

Lyse the red blood cells and pellet the leukocytes

1. Invert the collection tube 5 times to ensure thorough mixing of the sample.
2. Transfer 200 µL of whole blood to each sample well.
3. Add 600 µL of ice-cold 1X RBC Buffer to each sample and mix by pipetting up and down 10 times with a multi-channel micropipettor.
4. Seal the plate with a MicroAmp™ Clear Adhesive Film.
5. Submerge the plate in ice that is halfway up the side of the plate, then incubate 15 minutes. Mix briefly twice during the incubation by shaking the plate for 15 seconds at 900–950 rpm.

   Note: The cloudy suspension becomes translucent during incubation, indicating lysis of the erythrocytes.
6. Centrifuge at ≥1700 × *g* for 15 minutes.

7. Carefully remove and discard the supernatant (~700 µL) without disturbing the leukocyte pellet.

   Note: Up to 100 µL of supernatant can be left in the well to avoid loss of sample. When removing the supernatant, aspirate from the side of the well, not the center.

8. Seal the plate with the MicroAmp™ Clear Adhesive Film, then place on ice.

Digest the samples with Proteinase K

Ensure that the incubator is preheated to 65°C.

1. Prepare sufficient PK Mix according to the following table. Invert PK Mix several times to thoroughly mix components.

   IMPORTANT! Prepare the PK Mix just before use. Do not place PK Buffer or PK Mix on ice, to avoid precipitation.

Component	Volume per well	Volume per plate
Proteinase K	0.8 µL	80 µL
PK Buffer	4.2 µL	420 µL
Total PK Mix	5.0 µL	500 µL

2. Add 5 µL of PK Mix to each sample well and mix by pipetting up and down 5 times with a multi-channel micropipettor.

3. Seal the plate with a MicroAmp™ Clear Adhesive Film and shake the sealed plate for 5 minutes at 900–950 rpm.

4. Incubate for 15 minutes at 65°C.

   IMPORTANT! Arrange plates in the incubator to allow adequate flow around the plate wells, to ensure that samples quickly reach and maintain the incubation temperature.

   During the incubation at 65°C, follow "Set up the processing plates" on page 29.

Proceed immediately to "Add Multi-Sample DNA Lysis Buffer, Bead/RNase A Mix, and isopropanol" on page 29.

Set up the processing plates

1. During the incubation at 65°C, set up the Wash, Elution, and Tip Comb Plates outside the instrument as described in the following table.

Table 10 Processing plates

Plate ID	Plate position[1]	Plate type	Reagent	Volume per well
Wash Plate 1	2	Deep Well	Wash Solution 1	150 µL
Wash Plate 2	3	Deep Well	Wash Solution 2	150 µL
Wash Plate 3	4	Deep Well	Wash Solution 2	150 µL
Elution Plate[2]	5	Standard	DNA Elution Buffer 1	50 µL
Tip Comb	6	Deep Well	Place a MagMAX™ Express-96 Deep Well Tip Comb in a MagMAX™ Express-96 Deep Well Plate.	

[1] Position on the instrument

[2] The instrument prompts the user to add DNA Elution Buffer 2 to the Elution Plate, after incubation with DNA Elution Buffer 1.

2. (*Optional*) To prevent evaporation and contamination, cover the prepared processing plates with paraffin film until they are loaded into the instrument.

Add Multi-Sample DNA Lysis Buffer, Bead/RNase A Mix, and isopropanol

1. Add 400 µL of Multi-Sample DNA Lysis Buffer to each sample and mix by pipetting up and down 5 times with a multi-channel micropipettor.
2. Seal the plate with a MicroAmp™ Clear Adhesive Film, then shake for 5 minutes at 900–950 rpm.
3. Prepare Bead/RNase A Mix according to the following table.

IMPORTANT! Prepare the Bead/RNase A Mix up to 20 minutes before use. Prolonged storage at room temperature can reduce its efficiency.

Vortex the DNA Binding Beads at moderate speed to form a uniform suspension before pipetting.

Component	Volume per well	Volume per plate
DNA Binding Beads	16 µL	1.6 mL
RNase A	5 µL	500 µL
Nuclease-free Water	19 µL	1.9 mL
Total Bead/RNase A Mix	40 µL	4 mL

4. Vortex the Bead/RNase A Mix at moderate speed to ensure thorough mixing, then add 40 µL to each sample, then use a multi-channel micropipettor to mix by pipetting up and down 5 times.

   If you see that the beads in the Bead/RNase A Mix are settling, vortex the mix again briefly before continuing to pipette.

5. Seal the plate with the MicroAmp™ Clear Adhesive Film and shake for 5 minutes at 900–950 rpm.

6. Add 240 µL of isopropanol to each sample, briefly shake for 30 seconds at 900–950 rpm, then proceed immediately to process the samples on the instrument (next step).

Process samples on the instrument

1. Select the program **A25597_Leukocytes** on the instrument.
   If you are running your samples on the MagMAX™ Express-96 Magnetic Particle Processor, use the program **4413021_DW_blood**

2. Start the run, remove the temporary paraffin plate seals (if present), and load the prepared processing plates to their positions when prompted by the instrument (see Table 10).

3. Load the sample plate (containing lysate, isopropanol, and Bead/RNase A Mix) at position 1 when prompted by the instrument.

4. When prompted by the instrument (approximately 23 minutes after initial run):
   a. Remove the Elution Plate from the instrument.
   b. Add 50 µL of DNA Elution Buffer 2 to each sample well.

      IMPORTANT! Add DNA Elution Buffer 2 immediately after the prompt, to prevent excessive drying of any beads that are still captured on the Tip Comb.

   c. Load the Elution Plate back onto the instrument, and press **Start**.

5. At the end of the run (approximately 27 minutes after initial start), remove the Elution Plate from the instrument and seal immediately with a new MicroAmp™ Clear Adhesive Film.
   - If precipitated DNA is visible, pipet up and down 5–10 times before sealing the plate, to ensure complete resuspension.
   - *(Optional)* Eluates can be transferred to a storage plate after collection.
   - If excess bead residue is seen in the wells, place the Elution Plate on the Magnetic Stand-96 to capture any residue prior to downstream use of the DNA.

   IMPORTANT! Do not allow the purified samples to sit uncovered at room temperature for more than 10 minutes, to prevent evaporation and contamination.

The purified samples are ready for immediate use. Alternatively, store the covered Elution Plate:

- At 2–6°C for up to 24 hours.
- At –20°C or –80°C for long-term storage.

DNA concentration and quantification

We recommend that you prepare 50 ng/µL DNA stock solutions for 825 pg DNA/33 nL SNP genotyping reaction.

Note: Samples with low concentration (<10 ng/µL) or PCR inhibitors may fail to amplify and/or cluster properly, and can yield inaccurate results.

Quantify DNA by any of the following methods. Before quantifying, mix the samples well to ensure sample homogeneity, especially if samples have been stored.

- **RNase P Ct value method** (recommended) – See Appendix A, "RNase P Quantification for genotyping experiments"
- **UV absorbance measurements –** Use a NanoDrop™ or other comparable instrument. Pure gDNA should have an A_{260}/A_{280} ratio of approximately 1.8–2.0.
- **Fluorometric analysis** – Use a Qubit™ dsDNA BR or HS Assay Kit.

Prepare and run OpenArray™ CFTR SNP genotyping experiments

This chapter contains brief procedures. For detailed procedures, refer to:

Title	Pub. no.
QuantStudio™ 12K Flex Real-Time PCR System: OpenArray™ Experiments User Guide	4470935
OpenArray™ Sample Tracker Software Quick Reference	4460657
OpenArray™ AccuFill™ System User Guide	4456986
TaqMan™ OpenArray™ Genotyping Troubleshooting Guide	MAN0011115

Workflow

"Generate 384-well sample plate layouts in OpenArray™ Sample Tracker Software" on page 34

▼

"Set up the PCR reactions in the 384-well plate for AccuFill™ system transfer" on page 35

▼

"Set up the AccuFill™ instrument" on page 36

▼

"Transfer reactions to the OpenArray™ plate in the AccuFill™ instrument" on page 37

▼

"Seal the OpenArray™ plate" on page 38

▼

"Run the OpenArray™ plate(s) on the QuantStudio™ 12K Flex instrument" on page 39

▼

"Check the QC Images" on page 40

▼

"Analyze results in TaqMan™ Genotyper Software" on page 44

Required materials: OpenArray™ plate workflow

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

Item	Source
Genomic DNA	We recommend that you prepare 50 ng/µL DNA stock solutions for 825 pg DNA/33 nL SNP genotyping reaction. From Chapter 3, "Isolate DNA using the MagMAX™ DNA Multi-Sample Ultra Kit"
(Optional) CFTR TaqMan™ Assay Controls	A30421
OpenArray™ 384-Well Sample Plates (microplates), black	4482221
OpenArray™ plates with CFTR assays	Custom ordered
TaqMan™ OpenArray™ Genotyping Master Mix (5 mL)	4404846
Biomek Seal and Sample Foil Lids (for pre-plating step)	Beckman Coulter 538619

Item	Source
OpenArray™ AccuFill™ System Tips	4458107
QuantStudio™ 12K Flex OpenArray™ Accessories Kit (contains the items needed to assemble up to 10 plates: 12 lids and plugs, 12 immersion fluid syringes, and 2 carriers)	4469576

Instruments and software required:

- OpenArray™ Sample Tracker Software
- QuantStudio™ 12K Flex OpenArray™ Plate Press 2.0.
- QuantStudio™ 12K Flex instrument with OpenArray™ block (AccuFill™ System)

Generate 384-well sample plate layouts in OpenArray™ Sample Tracker Software

The software maps samples from the 96-well layout used for DNA isolation to a 384-well sample plate layout as .csv files that are used by OpenArray™ AccuFill™ software.

See "One-time procedures" on page 41 to:

- Set up optimized folder locations and software preferences before performing this procedure for the first time.
- Download the SPF file(s) for the OpenArray™ plate(s) before starting.

1. Generate the 96-well sample plate .csv file using the OA_Genotyping_CalcSheet or 96-Well Sample Plate 1.csv template, then save it to the Sample Tracker 96-well Input folder.

   The 96-Well Sample Plate 1.csv file is provided in the AccuFill™ software installation. Enter or copy the sample names in 96-Well Sample Plate 1.csv, then **Save as** a new .csv-format file.

   The OA_Genotyping_CalcSheet automatically generates the sample .csv file.

2. In the Sample Tracker Software Properties page, select **Genotyping** for Experiment Type, then select the appropriate settings for OpenArray™ Plate and Pipettor.

3. In the Samples page, click  **Import**, then select and import the sample .csv file.

4. In the Sample Mapping page, confirm that the samples for a single OpenArray™ plate are assigned to one color.

   If necessary, correct the OpenArray™ Plate and Pipettor settings in the Properties page.

5. In the Sample Mapping page, click the **384-Well Plate** tab, then click **Export** ▸ **Export *.csv**.

6. Select **384-Well Plate (for AccuFill)**, then save the exported file.

Plate layouts for the 384-well sample plates are saved to individual .csv files in the Sample Tracker 384-well CSV Files folder.

Set up the PCR reactions in the 384-well plate for AccuFill™ system transfer

For best results, dilute DNA samples to 50 ng/µL before setting up the PCR reactions.

IMPORTANT! The 4 × 12 area(s) of the 384-well plate being filled must match the area(s) designated in Sample Tracker Software for that set of samples.

1. Remove the OpenArray™ plate from the freezer and allow it to thaw in its sleeve, unopened, at room temperature (~15 minutes).

   The OpenArray™ plate must be completely thawed before transferring reactions to it from the 384-well plate.

2. Mix the 2X Master Mix gently. Do not invert the bottle.

3. Vortex the Assay Working Stock, then centrifuge briefly.

4. Following the plate layout determined in Sample Tracker Software, add master mix, then UltraPure™ DNase/RNase-Free Distilled Water (NTCs) or DNA samples, to a 384-well microplate (OpenArray™ 384-Well Sample Plate). Include 2 NTCs on each plate.

Component	OpenArray™ Genotyping Plate Format		
	16	32	64 +
TaqMan™ OpenArray™ Genotyping Master Mix	1.5 µL	2.0 µL	2.5 µL
One of the following: • UltraPure™ DNase/RNase-Free Distilled Water (NTCs) • DNA sample	1.5 µL	2.0 µL	2.5 µL
Total reaction volume	3.0 µL	4.0 µL	5.0 µL

5. Seal the plate with an aluminum foil seal, remove the foil flap, mark the edges of the filled 4 × 12 area with a pen, then score the foil along those lines.

   Do not remove the foil from the scored area at this time.

6. Vortex the sealed plate for 5 seconds, then centrifuge the plate for 1 minute at 1,000 rpm.

   Vortex gently to avoid bubble formation.

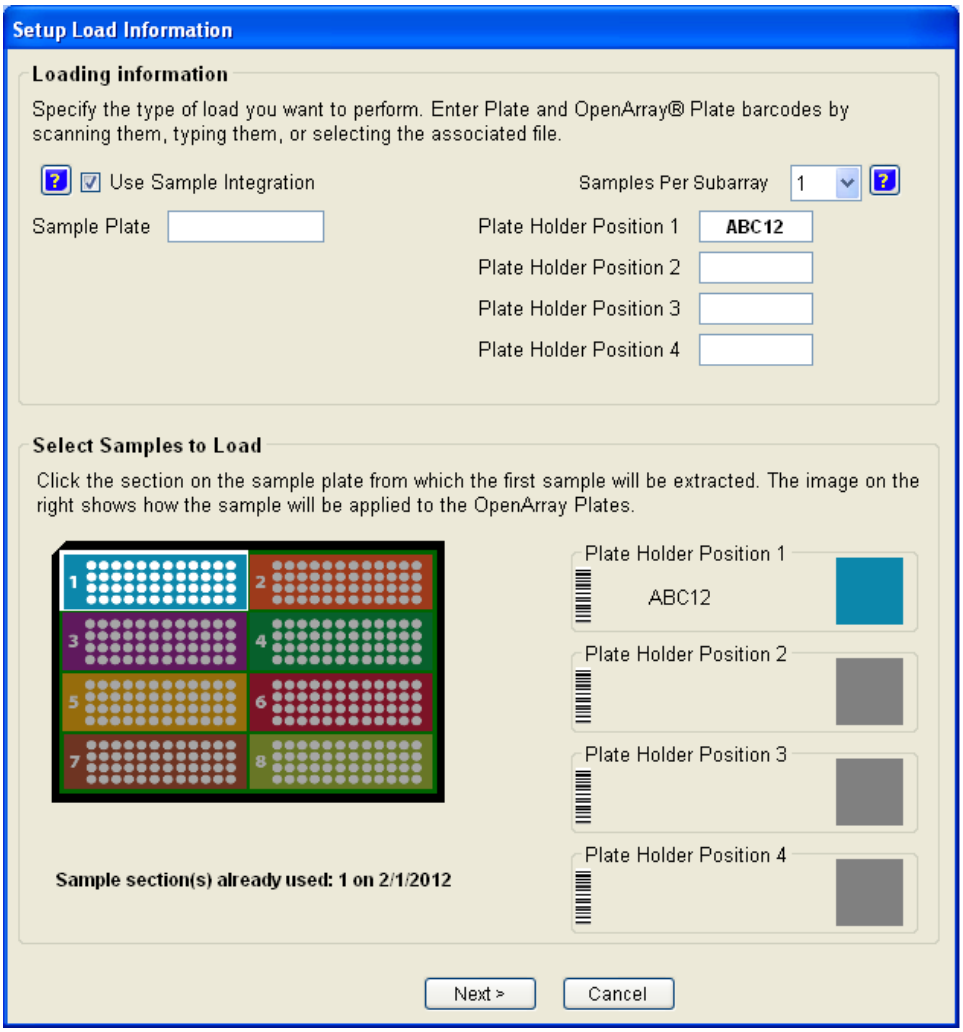
If you make a sample layout error:

- Before the AccuFill™ procedure – Repeat "Generate 384-well sample plate layouts in OpenArray™ Sample Tracker Software" on page 34 with a corrected sample.csv file.
- After the AccuFill™ procedure – See "To recover from layout errors in the 384-well sample plate" on page 40.

Set up the AccuFill™ instrument

IMPORTANT! Before proceeding, check the tip expiration date (shown on the outer box that contains the trays of tips). Do not use tips that exceed the expiration date.

1. In the OpenArray™ AccuFill™ software, click **Setup and Load**.
2. In the Setup Load Information window, verify that the Use Sample Integration checkbox is selected.



3. Click **Browse** to the right of Sample Plate, then select the 384-well sample plate .csv file that was generated with Sample Tracker Software.

4. Click **Browse** to the right of the plate holder position corresponding to the OpenArray™ of interest, then select the .spf file corresponding to the desired OpenArray™ plate.

5. Click the corresponding 4 × 12 area of the 384-well plate, then click **Next** to open the Setup Deck window.

6. Ensure that:
   - Tip boxes are loaded in the AccuFill™ instrument in the displayed configuration.
   - Lids are removed from the tip boxes.
   - The waste bin in the instrument is emptied.

7. In the Setup Deck window:
   a. Click the **The tips are configured as shown above** checkbox.

   b. Click the **The Waste Bin is empty** checkbox.

Transfer reactions to the OpenArray™ plate in the AccuFill™ instrument

Ensure that the OpenArray™ plate is thoroughly thawed before starting this procedure.

1. Prepare the items needed to seal the OpenArray™ plates (next section), because the OpenArray™ plate must be sealed promptly after being loaded with the reactions (this section).
   a. Ensure that the QuantStudio™ OpenArray™ Plate Press is ready.

   b. Gather and remove from its packaging an OpenArray™ Lid, plug, syringe with OpenArray™ Immersion Fluid, and syringe tip.

   c. Attach the syringe tip to the syringe and carefully push some of the fluid through the tip to remove air bubbles, then lay the syringe aside.

2. Remove the OpenArray™ plate from its sleeve and place it in the Plate Holder of the AccuFill™ instrument.

   Ensure that the bar code on the OpenArray™ plate is facing left and the serial number is facing right.

3. Using forceps, peel off the foil over the 384-well plate area being used.

4. Close the instrument door.

5. In the AccuFill™ software Setup Deck window:
   a. Click the **The OpenArray Plate is in the Plate Holder** checkbox.

b. Click the **Remove foil from the highlighted section of the Sample Plate** checkbox.

c. Click **Load**.

6. As soon as the Remove OpenArray Plate window appears (after ~90 seconds), open the instrument door, and remove the loaded OpenArray™ plate.

7. Proceed immediately to seal the OpenArray™ plate (next section).

   Note: For best results, seal the OpenArray™ plate within 90 seconds of completion of loading, to prevent evaporation.

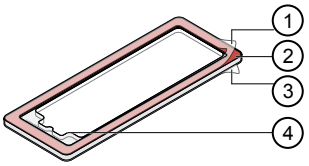
Seal the OpenArray™ plate

IMPORTANT! Handle the OpenArray™ plate and case only by the edges throughout this procedure.

1. Place the filled OpenArray™ plate in the QuantStudio™ 12K Flex OpenArray™ Plate Press 2.0.

   Ensure that the bar code is facing left and the serial number is facing right.

2. Remove the clear plastic sheets from the top and the bottom of the lid, remove the red protective film around the edge of the OpenArray™ lid, then seat the lid on the OpenArray™ case in the plate press.

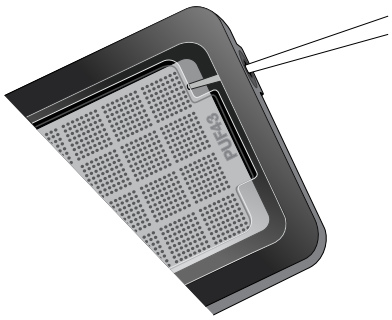


① Protective film (remove)
② Adhesive
③ Protective film (remove)
④ Notched end (align with serial number)

3. Engage the press mechanism until the green flashing light changes to a steady green light (~20 seconds).

4. Disengage the press, then remove the OpenArray™ case.

5. While holding the OpenArray™ case by the edges, insert the prepared syringe tip into the port in the case and carefully inject immersion fluid until the case is filled.



It is important that the syringe tip is in front of the array when filling the case with immersion fluid.

Minimize creation of air bubbles when you dispense the fluid; one small air bubble in the case is acceptable.

6. Continuing to hold the case vertically, remove the syringe tip, insert the screw end of the OpenArray™ Plug into the port and rotate clockwise until the black handle breaks off.

7. Clean the case with a laboratory wipe that has been thoroughly sprayed with ethanol, then dry the case with a clean laboratory wipe.

Run the OpenArray™ plate(s) on the QuantStudio™ 12K Flex instrument

1. On the instrument touchscreen, touch to extend the loading arm, and place the OpenArray™ plate(s) on the plate adapter.

   Ensure that the plate barcode and serial number are facing the front of the instrument.

2. Touch to retract the loading arm.

3. On the Home screen of the QuantStudio™ 12K Flex Software, select **Run ▸ OpenArray**.

4. In the Select Instrument pane, select the QuantStudio™ instrument.

5. Click **Get Plate IDs** to import the barcode(s) of the OpenArray™ plate(s).
   Once the OpenArray™ serial numbers appear, the loaded .spf files corresponding to each plate should appear in the Setup File field.
   If not, click **Browse**, then select the correct loaded .spf file from the Loaded SPF folder.

6. (*Optional*) If desired, click **Browse** to change the QuantStudio™ Experiment File Location.

7. (*Optional*) If desired, change the software-determined Experiment File Name.
8. On the instrument touchscreen, touch **Start Run**.
   The instrument pauses at 41 or 42 seconds prior to the end of the run. Wait for the system to complete the run before opening the .eds file.
9. Transfer the .eds file from the instrument to an accessible location for analysis in Genotyper Software.
10. Check the QC images for loading issues or leaks; see "Check the QC Images" on page 40.

Check the QC Images

Check the QC images before analysis in Genotyper Software. For additional information, see the *TaqMan™ OpenArray™ Genotyping Troubleshooting Guide* (Pub. no. MAN0011115).

1. In the instrument software Export tab, set a uniquely named folder for the OpenArray™ plate of interest, then click **Export QC Images**.
2. View POST-READ_CHANNEL_4.tiff (ROX™ image), to check for loading quality issues.
3. View s03_c001_t02_p0001_m2_x3_e1_cp#_spotfind.tiff for leaks or other displaced sample issues.
   cp# identifies in which position in the instrument an array was run.
   If a problem is found, view s01_c001_t02_p0001_m2_x3_e1_cp#_spotfind.tiff to see if the issue existed even before cycling (this is useful for troubleshooting).
4. View STAGE3_CYCLE1_CHANNEL_1.tiff and STAGE3_CYCLE1_CHANNEL_2.tiff to check for any fluorescent abnormalities, and to confirm any problem seen in the spotfind image.
5. Note any abnormalities found, as well as all other potentially relevant information related to the setup of the run.

To recover from layout errors in the 384-well sample plate

After the AccuFill™ procedure, you can recover from plate layout errors that were made during setup of the reactions in the 384-well sample plate. Refer to the *OpenArray™ Sample Tracker Software Quick Reference* (Pub. no. 4460657) for additional information.

1. Create a corrected sample .csv file.
2. Repeat "Generate 384-well sample plate layouts in OpenArray™ Sample Tracker Software" on page 34, but select **OpenArray Plate X (for QuantStudio)** when exporting from Sample Tracker Software.
3. Import the corrected .csv file into the QuantStudio™ 12K Flex Software.
   You can import either before starting the run or after the run is complete.

One-time procedures

Set up default folders and software preferences

This procedure simplifies the file locations used in the OpenArray™ AccuFill™ instrument software.

Set up the default file locations and preferences before using the OpenArray™ AccuFill™ system for the first time. You must be logged in as an administrator.

1. Create four folders in a convenient location on the same computer drive as the AccuFill™ software:
   - SPF Files
   - Sample Tracker 96-well Input
   - Sample Tracker 384-well CSV Files
   - Loaded SPF Files

2. (*Optional*; not required if using the OA_Genotyping_CalcSheet.) Navigate to *<drive>*:`\Program files(x86)\AppliedBiosystems\OpenArray Sample Tracker\Examples`, copy 96-Well Sample Plate 1.csv, then paste it in the Sample Tracker 96-well Input folder.

3. In OpenArray™ Sample Tracker Software, select **View ▸ Preferences**, and enter preferences as indicated.

Field	Selection
Experiment type	**Genotyping**
OpenArray Plate	Select the OpenArray™ format that will be run most often, for example, **Genotyping – 64**
Pipettor	**Adjustable** or **Fixed**
Import Data Directory	Sample Tracker 96-well Input folder
Export Data Directory	Sample Tracker 384-well CSV Files folder

4. In the AccuFill™ software, select **Instrument ▸ Edit Preferences**, then:
   a. Click the **Require Sample Integration** checkbox.
   b. Select the indicated folders.

For this AccuFill™ folder...	Select this folder...	Folder contents
OpenArray™ Plate File Input Folder	SPF Files	.spf files for your OpenArray™ plates; contain assay name and location
Sample Plate File Folder	Sample Tracker 384-well CSV Files	.csv 384-well sample plate layout files
Loaded OpenArray™ Plate File Folder	Loaded SPF Files	Integrated .spf files that are generated during processing with AccuFill™ Software.

5. In the QuantStudio™ 12K Flex instrument software, select **Tools ▸ Preferences ▸ OpenArray**, then select the Loaded SPF Files folder for the software Setup Folder.

   Note: If the instrument software is not on same computer as the AccuFill™ software, you will need to transfer the loaded SPF files to the computer running the instrument software.

Download SPF files

Set up the optimized folder locations and software preferences before downloading SPF files. See "Set up default folders and software preferences" on page 41.

For custom OpenArray™ plates, have the sales order number, lot number, and serial number for each plate ready.

- The sales order number is located on the packing slip and order confirmation email.
- The lot number and serial number are found on the pouch of each OpenArray™ plate.

1. At **www.thermofisher.com/OA-platefiles**, select **TaqMan™ OpenArray™ Custom Genotyping Plates QuantStudio 12K Flex** in the Select Your Product dropdown list.

   Note: Do not choose from the bottom half of the dropdown list, under OpenArray™ Real Time PCR Platform.

2. Select **I want to download all available SPF & AIF files** or **I want to download a specific SPF file**, enter the sales order number, lot number, and serial number, as required, then click **Submit**.

3. Save the SPF file(s) to the desktop SPF Files folder.

5 Analyze CFTR genotyping data

This chapter contains brief procedures. For detailed information, refer to *TaqMan™ Genotyper Software Getting Started Guide* (Pub. no. 4448637).

Analysis options for CFTR genotyping data

Analyze allelic discrimination plots for CFTR genotyping data using any of these tools:

Software	Features
TaqMan™ Genotyper software	• Desktop software • Create studies • Overlay data from multiple plates
QuantStudio™ 12K Flex software	• Desktop software • View real-time trace data that is needed for positive result confirmation
Thermo Fisher Cloud GT Analysis module	• Cloud software • Create studies • Overlay data from multiple plates • View real-time trace data that is needed for positive result confirmation

Analyze results in TaqMan™ Genotyper Software

For non-standard assays, review the data for both assays together to ensure accurate genotyping results.

Confirm all positive results as described in the next chapter.

1. In Genotyper Software, click **Create Study from Template**, then select and open the desired template (.lat).

   Note: If you have not created a template, see "Create a study template" on page 45.

2. In the Workflow Menu pane, select **Setup** ▸ **Experiments**.

3. Click **Import**, then select and import one or more OpenArray™ experiment files (.eds).

4. Ensure that the Use Hardy-Weinberg for Analysis option is selected in the Analysis Settings.

5. Click **Analyze**.

6. Inspect the call data for the SNP assay in the scatter plot.

7. (*Optional*) If desired, manually assign the genotype: select the sample(s), right-click, then select the genotype.

8. Select the **Inspected** checkbox next to the assay, then click **Save**.

9. Repeat the inspection for each SNP assay.

10. In the Workflow Menu pane, select **Export** ▸ **Analysis Data**.

11. In the Export Study Properties pane:
    - Select **Analysis Results** ▸ **Advanced**.
    - Select **One File**.
    - Enter the filename and location, and select **(*.txt)**.

12. Click **Export preview**, then click **Start Export** in the new window.

One-time procedures

Create a study template

If you will use a Reference Panel, generate the Reference Panel before creating the study template, so that the Reference Panel can be imported into the template.

If necessary, first download the Assay Information File (AIF) from **www.thermofisher.com/OA-platefiles**.

1. In the TaqMan™ Genotyper Software Home screen, click **Create Study**.
2. In the Workflow Menu pane, select **Setup ▸ Properties**, then enter the study properties.
   - For Instrument Type, select **QuantStudio™ 12K Flex Real-Time PCR System**.
   - For Experiment Type, select **Real-time**.
3. In the Workflow Menu pane, select **Setup ▸ Assays**.
4. Click **Import**, then select and import the Assay Information File (AIF).
5. (*Optional*) Import the Reference Panel:
   a. In the Workflow Menu pane, select **Setup ▸ References**.
   b. Click **Import**, then select and import the Reference Panel file of interest.
6. Select **File ▸ Generate Template**, enter a template name (.edt), then save the template in the desired location.

(*Optional*) Generate a Reference Panel

A reference panel consists of high-quality data for all the assays in the study. The data can be chosen from multiple OpenArray™ plates.

For best results, generate a new Reference Panel for each lot of OpenArray™ plates.

If necessary, first create a study containing data that will be used as reference samples. Set **QuantStudio™ 12K Flex Real-Time PCR System** for Instrument Type, and **Real-time** for Experiment Type in the study properties.

1. In TaqMan™ Genotyper Software, open the study that contains data points to be used as reference samples.
2. In the Workflow Menu pane, select **Analysis ▸ Results**.
3. Click-drag in the scatter plot to select one or more data points.
   Select data points for each homozygous genotype (FAM™ dye-labeled or VIC™ dye-labeled) and the heterozygous genotype.
   The non-selected data points will fade.
4. Right-click in the scatter plot, then select **Tag for Ref Panel**.
5. Repeat the selection and tag steps for additional data points.

6. Confirm that the correct samples have been tagged as reference samples in the Results table.

   In the Results tab, **Reference Sample** must be checked in the **View** dropdown list to view the tags.

7. Repeat the data point selection, tag, and confirmation steps for each SNP assay of interest.

8. Select **File ▸ Save**.

9. Select **File ▸ Generate Reference Panel**, enter a name for the panel and save the file (.lap) to the desired location.

(*Optional*) Add samples to an existing Reference Panel

During analysis in Genotyper Software, you can add samples to an existing Reference Panel.

The Reference Panel must already be imported into the study containing samples that will be added to the Reference Panel.

1. In the scatter plot of the study, select data points for the samples of interest, right-click in the plot, then select **Tag for Ref Panel**.

2. Select **File ▸ Save**.

3. Select **File ▸ Generate Reference Panel**, enter a name for the panel and save the file (.lap) to the desired location.

Analyze data in QuantStudio™ 12K Flex or Thermo Fisher Cloud Cloud software

For non-standard assays, review the data for both assays together to ensure accurate genotyping results.

Confirm all positive results as described in the next chapter.

To use the QuantStudio™ 12K Flex software or related instrument software:

- In analysis settings, select **Analyze Real-Time Rn – Median (Rna to Rnb)**. This setting will normalize for any run and system noise to improve data accuracy.
- Select **Analysis ▸ Allelic Discrimination Plot**.

To use the Thermo Fisher Cloud GT Analysis module:

- Go to **www.thermofisher.com/us/en/home/cloud.html**
- Create a project and import one or more OpenArray™ experiment files (.eds).
- Access the Genotyping (GT) module.
- Analyze data.

Analysis guidelines: SNP genotyping assays to triallelic SNPs

Several important CFTR mutations are triallelic SNPs (three bases occur at the same genomic location). Triallelic SNP alleles are interrogated using two TaqMan™ assays.

Example

- Assay 1: Wild type T, Mutant A [non-target mutant allele for Assay 2]
- Assay 2: Wild type T, Mutant G [non-target mutant allele for Assay 1]

Expected results

Always examine data for both assays to ensure correct genotyping results.

- Samples that are heterozygous for a mutation run as heterozygous with the assay designed to that mutant allele and typically run with the wild type samples in the second assay (because only the wild type allele is detected in the second assay).
- Some assays exhibit nonspecific probe activity for the nontarget mutant allele, and heterozygous samples containing the nontarget mutant allele can be incorrectly called as heterozygous for the target mutant allele.

Example data

The following figures show example OpenArray™ data generated using wild type genomic DNAs and synthetic heterozygous mutation controls for four pairs of triallelic SNP assays. In each pair, one assay shows nonspecific probe activity for the nontarget mutant allele. The result for the heterozygous control sample containing the nontarget mutant allele is circled in each data plot.

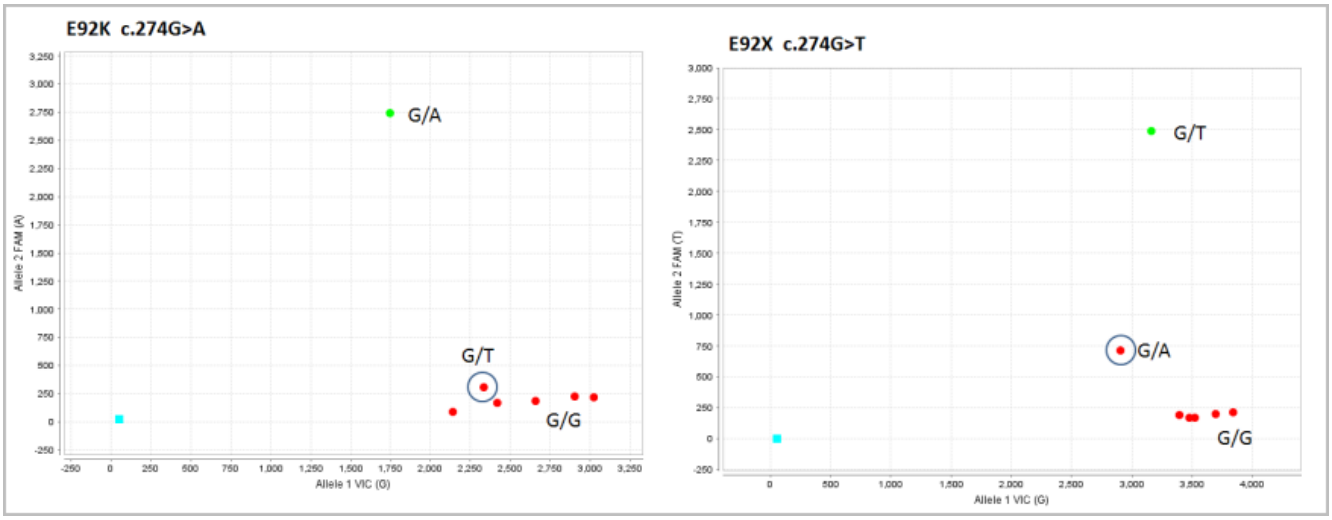


Figure 1 Triallelic SNP rs121908751 assay results. Left: E92K c.274G>A assay (C_32545261C_10) to the G wild type and A mutant allele; the mutant allele probe is specific and the heterozygous sample containing the nontarget T allele runs with wild type samples. Right: E92X c.274G>T assay (C_32545261D_20) to the G wild type and T mutant allele; nonspecific probe activity is observed for the nontarget A allele. Without evaluation of data for both assays together, the G/A sample could be incorrectly called as a G/T heterozygote.

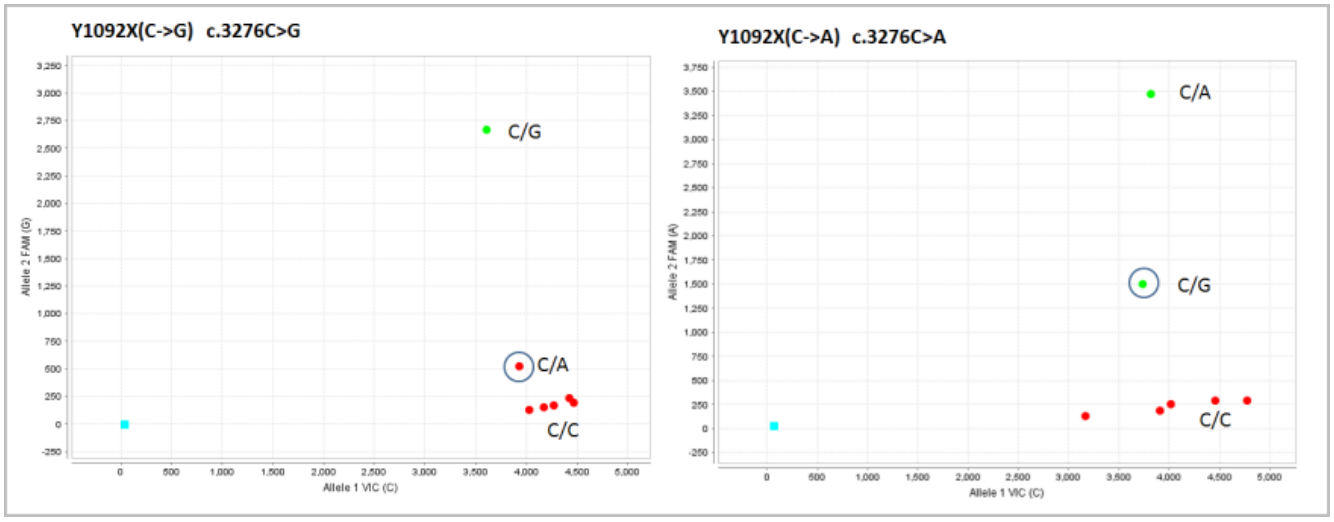


Figure 2 Triallelic SNP rs121908761 assay results. Left: Y1092X(C->G) c.3276C>G assay (C_26083714D_20) to the C wild type and G mutant allele; the mutant allele probe is specific and the heterozygous sample containing the nontarget A allele runs with wild type samples. Right: Y1092X(C->A) c.3276C>A assay (C_26083714C_10) to the C wild type and A mutant allele; nonspecific probe activity is observed for the nontarget G allele. Without evaluation of data for both assays together, the C/G sample could be incorrectly called as a C/A heterozygote.

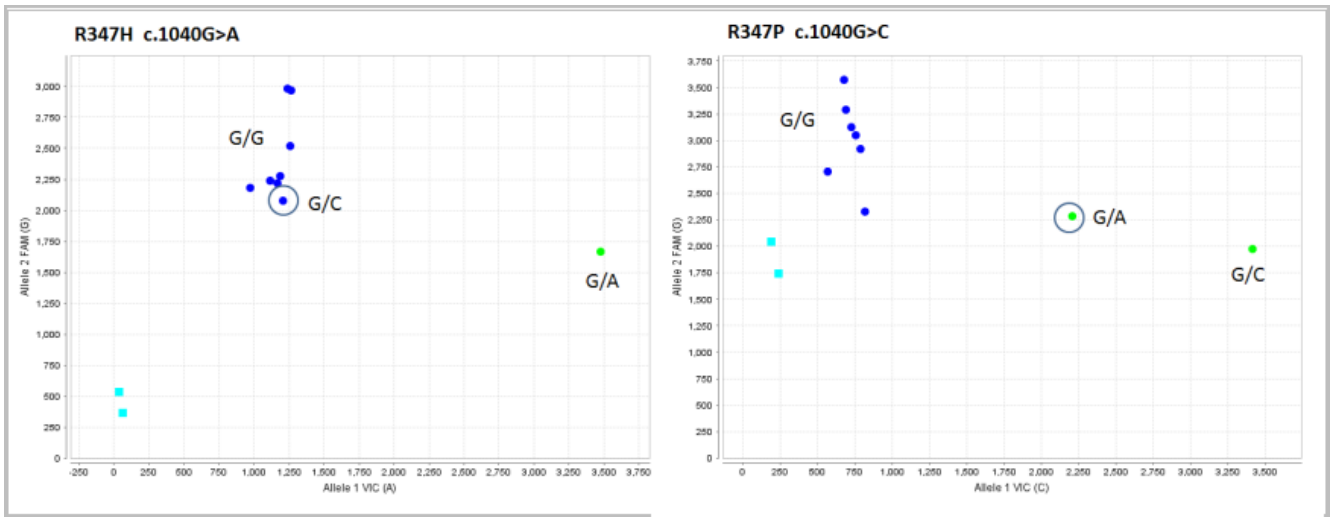


Figure 3 Triallelic SNP rs77932196 assay results. Left: R347H c.1040G>A assay (C___656878D_20) to the G wild type and A mutant allele; the mutant allele probe is specific and the heterozygous sample containing the nontarget C allele runs with wild type samples. Right: R347P c.1040G>C assay (C___656878C_30) to the G wild type and C mutant allele; nonspecific probe activity is observed for the nontarget A allele. Without evaluation of data for both assays together, the G/A sample could be incorrectly called as a G/C heterozygote.

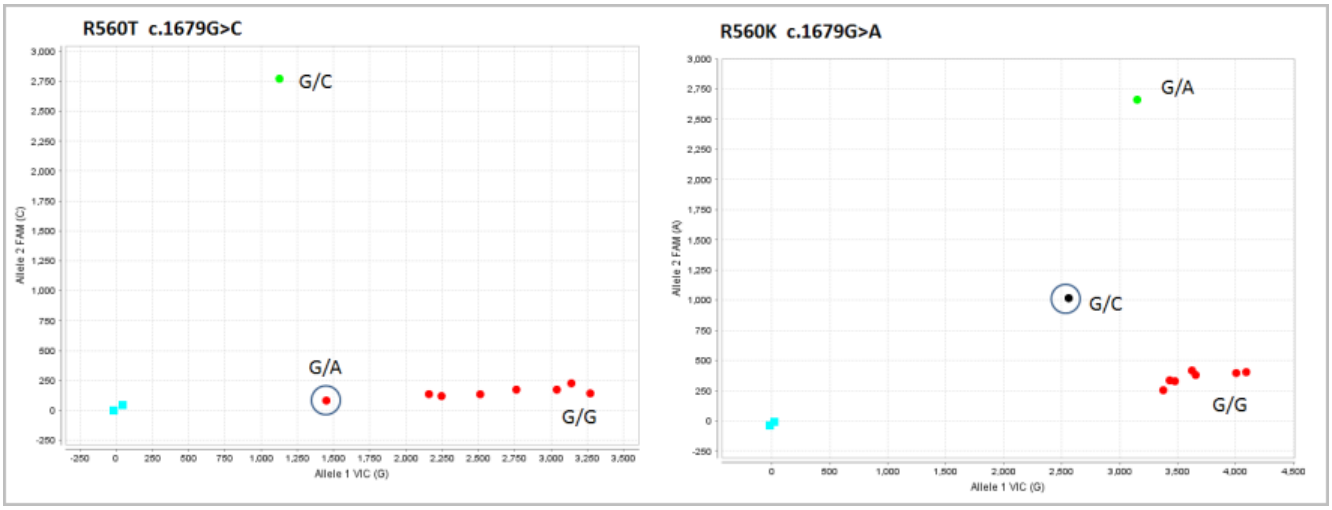


Figure 4 Triallelic SNP rs80055610 assay results. Left: R560T c.1679G>C assay (C_27861436C_30) to the G wild type and C mutant allele; the mutant allele probe is specific and the heterozygous sample containing the nontarget A allele runs with wild type samples. Right: R560K c.1679G>A assay (C_27861436D_20) to the G wild type and A mutant allele; nonspecific probe activity is observed for the nontarget C allele. Without evaluation of data for both assays together, the G/C sample could be incorrectly called as a G/A heterozygote.

Analysis guidelines: SNP genotyping assays to regions with multiple mutations

Some qualified CFTR mutation assays cover probe-binding sites that contain one or more mutations that are close to the target mutation. These nearby nontarget mutations do not typically interfere with genotype analysis of the target mutation in heterozygous samples because these rare mutations are not found together in cis on the same chromosome. For example, the F508del assay does not detect the I507del mutation.

Expected results

- A sample that is heterozygous for a target mutation:
  - Runs as a heterozygous sample with the assay to the target mutation.
  - Runs with wild type samples with an assay to a nearby mutation. The signal for the heterozygous mutation sample may be lower than the signal for wild type samples because only one chromosome (wild type) is detected.
- A sample that is homozygous for a target mutation:
  - Runs as a homozygous mutation sample with the assay to the target mutation.
  - Is not detected by assays to nearby mutations and runs with or near the NTCs.
- A sample that contains two nearby mutations on different chromosomes (for example, a compound heterozygote):
  - Runs as a homozygous mutant with the assays that target each mutation (wild type probes cannot bind).
  - Is not detected by assays to other nearby mutations.

Always examine data for all assays to ensure correct genotyping results.

Example data F508del region

Figure 5 and Figure 6 show OpenArray™ data for the F508del region, which contains five mutations that are covered by qualified TaqMan™ SNP genotyping assays:

- Samples that contain the F508del, I507del, and F508C mutations are detected by their respective assays (Figure 5 A, B, and C).
- Samples that are homozygous for the F508del mutation do not amplify with assays designed to the nearby mutations; they run close to the NTCs (Figure 5 A through D and Figure 6).
- Heterozygous samples tested with assays to nearby mutations may show lower signal than other samples in the wild type cluster (Figure 6)

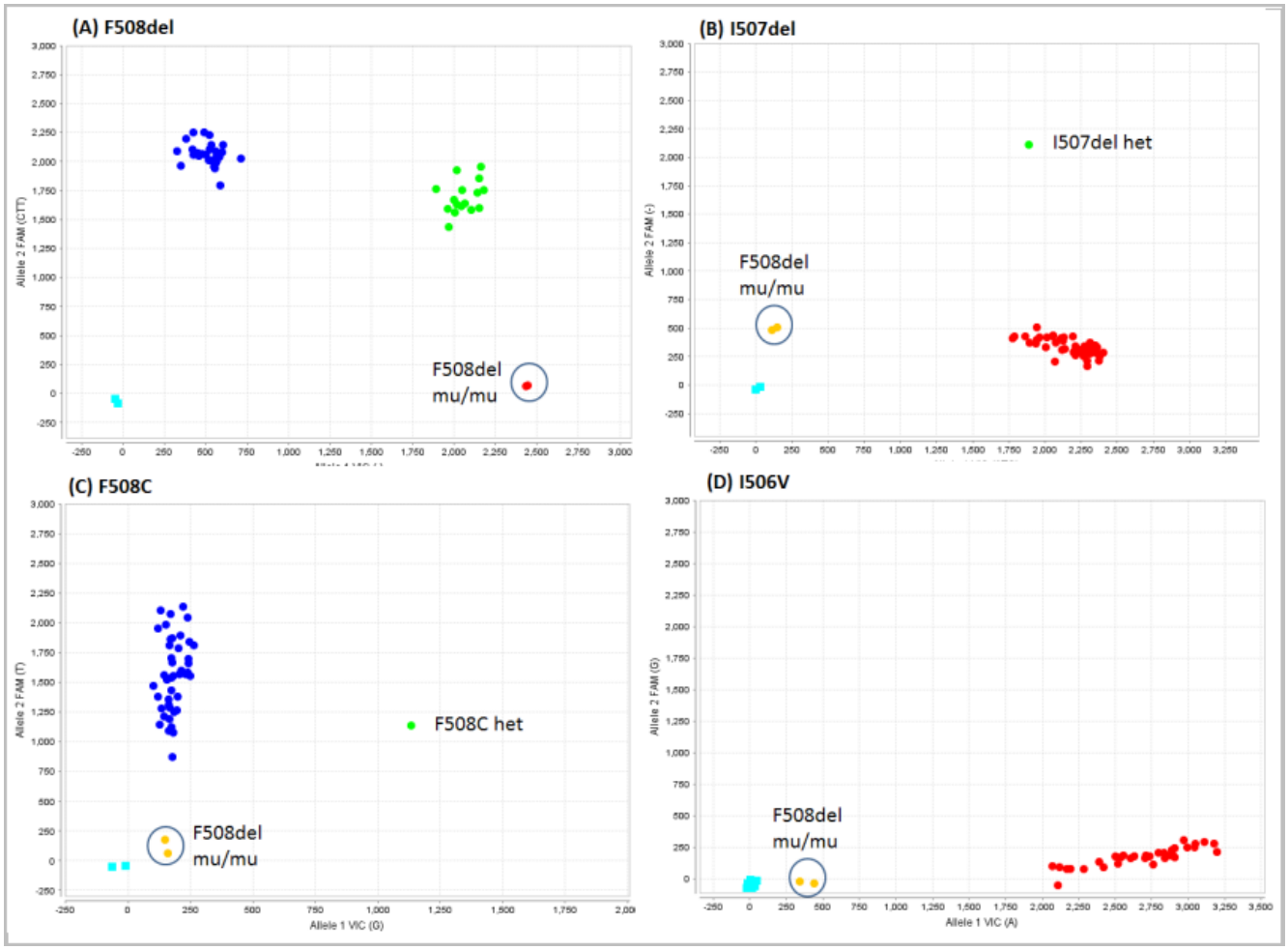


Figure 5 F508del region assay results: (A) F508del (C_151693869_10) (B) I507del (C_151693868_10), (C) F508C (C_100964862_10), (D) I506V (C__59055679_20). Two F508del mu/mu homozygous mutation samples are circled in each data plot. Heterozygous I507del and F508C samples are shown in (B) and (C). [Samples: Coriell cell line gDNA (not available for I506V)]

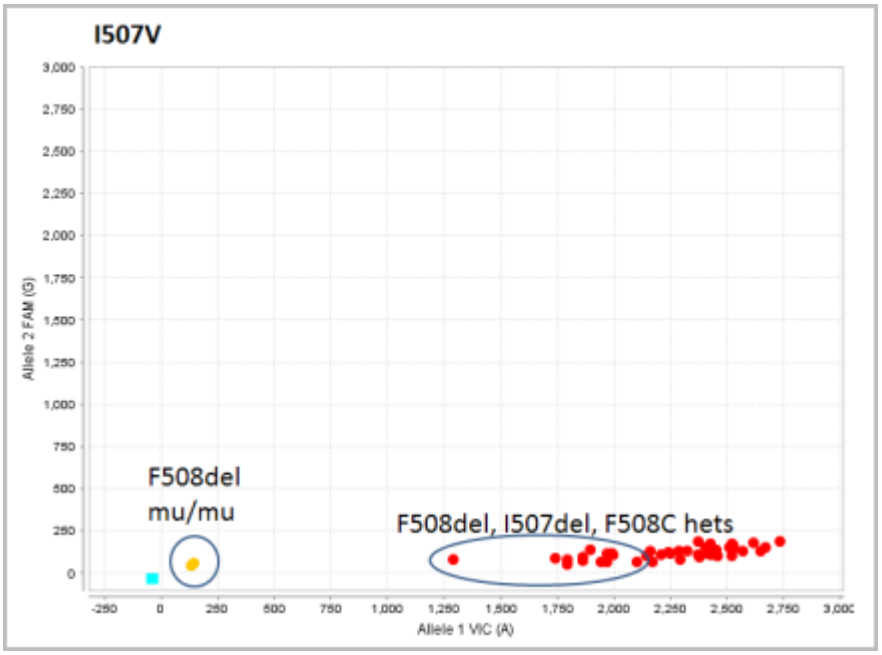


Figure 6 F508del region assay results: (E) I507V (C___8853252_20). Two F508del mu/mu homozygous mutation samples are circled. The approximate positions of the F508del, I507del, and F508C heterozygous samples in the I507V assay wild type cluster are shown in (E). [Samples: Coriell cell line gDNA (not available for I507V)]

Example data 2184delA region

Figure 7 shows OpenArray™ data for the 2184delA region, which contains four mutations that are covered by qualified TaqMan™ SNP genotyping assays:

- Samples that contain the 2184delA and 2183AA>G mutations are specifically detected by their respective assays (Figure 7 A, B).
- A small amount of nonspecific probe activity for the 2184delA heterozygous samples may be observed with the 2184delA and 2183delAA assays, and signal may be lower than signal for other samples in the wild type cluster (Figure 7 B and C).

  Note: Data is not shown for the 2184insA assay, which is not recommended for OpenArray™ plate format.

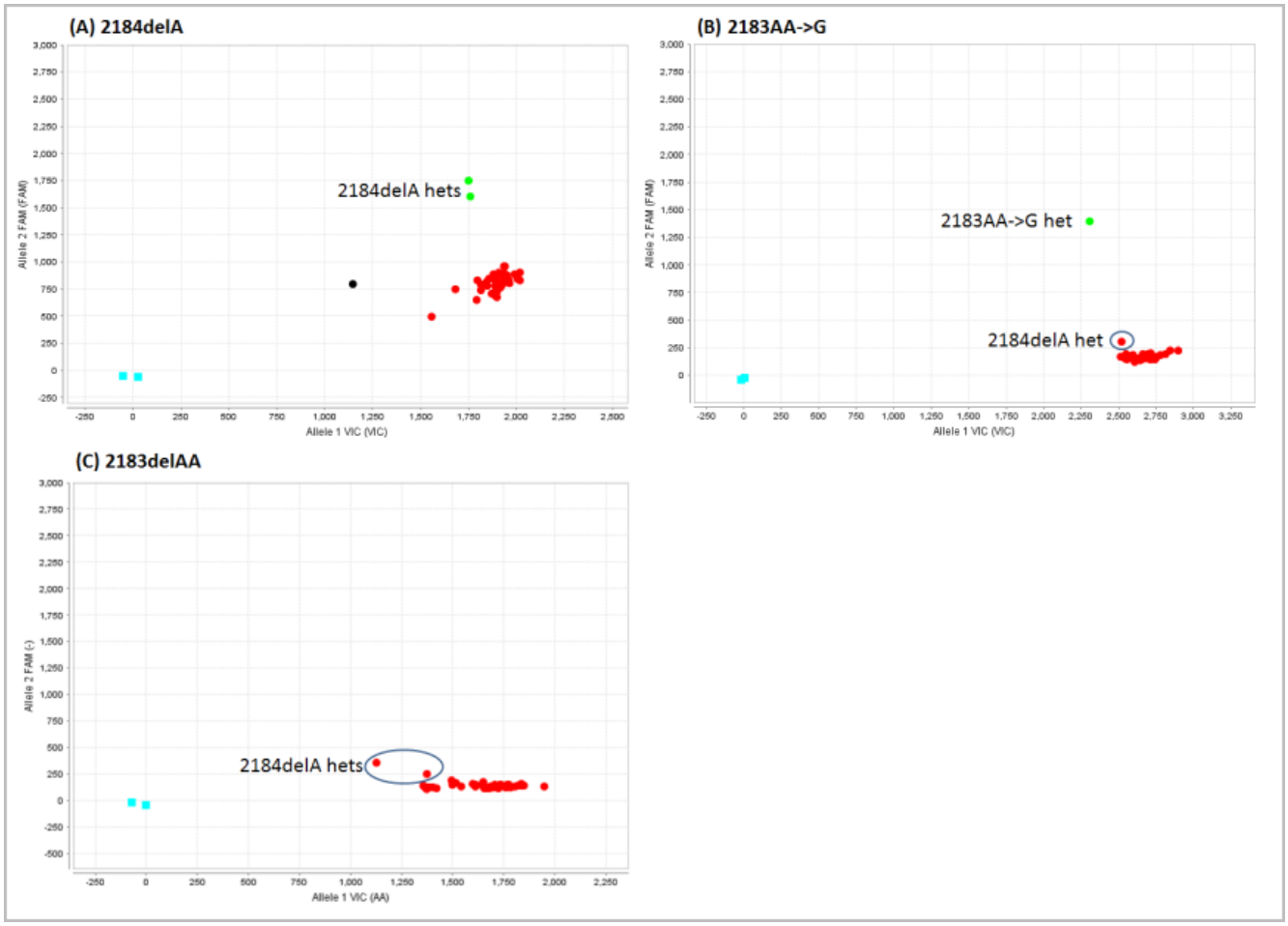


Figure 7 2184delA region assay results. (A) 2184delA (C_172767547_20), (B) 2183AA->G (C172767555C_30), (C) 2183delAA (C172767555D_20). Heterozygous 2184delA and 2183AA->G samples identified by specific assays are indicated in (A) and (B); 2184delA heterozygous samples in the wild type cluster of the 2183AA->G and 2183delAA assays are shown in (B) and (C), respectively. [Samples: Coriell cell line gDNA (not available for 2184delAA)]

Analysis guidelines: SNP genotyping assays to the 5T/7T/9T polymorphism

Each of the two assays used to interrogate the triallelic polyT variant contain one probe to the wild type 7T allele and one probe to one of the mutant alleles (5T or 9T). Because of nonspecific activity, the 7T probe in the 5T assay cannot distinguish 7T and 9T alleles, but this does not interfere with genotype analysis.

Expected results

Always examine data for both assays to ensure correct genotyping results.

The following translation table lists possible assay results and the corresponding genotype.

Table 11 polyT genotype and expected results with the 5T and 9T assays

Genotype	7T/5T assay result 7T (TT) = VIC™ dye 5T (-) =FAM™ dye 9T=unreported	9T/7T assay result 9T (TT) =VIC™ dye 7T (-) = FAM™ dye 5T=unreported
5T/5T	-/-	-/- , und, or noamp[1]
5T/7T	TT/-	-/-
5T/9T	TT/-	TT/TT, und[2]
7T/7T	TT/TT	-/-
7T/9T	TT/TT	TT/-
9T/9T	TT/TT	TT/TT

[1] 5T/5T samples may be weakly detected by the 7T probe and run at the lower end of the 7T (FAM™ dye) homozygous cluster (OpenArray™ plates), or may run closer to the NTCs (384-well plates).

[2] 5T/9T samples may be weakly detected by the 7T probe and run between the heterozygous and 9T (VIC™ dye) homozygous cluster.

Data analysis workflow

1. Examine the 5T assay results to identify samples that contain 5T alleles and note the following:
   - 5T homozygous samples run in the -/- (FAM™ dye homozygous) cluster.
   - 5T/7T or 5T/9T heterozygous samples run as heterozygotes.
   - Samples that do not contain a 5T allele and that have 7T/7T, 7T/9T, or 9T/9T genotypes run in the TT/TT (VIC™ dye homozygous) cluster.
2. Examine the 9T assay results to determine the 7T and 9T allele component of the samples:
   - 7T homozygous samples run in the -/- (FAM™ dye homozygous) cluster.
   - 9T homozygous samples run in the TT/TT (VIC™ dye homozygous) cluster.
   - 7T/9T heterozygous samples run as heterozygotes.

- For samples that contain the unreported 5T allele:
  - 5T/5T samples may be weakly detected by the 7T probe and run at the lower end of the 7T (FAM™ dye) homozygous cluster (OpenArray™ plates), or may run closer to the NTCs (384-well plates).
  - 5T/7T samples run in the 7T homozygous cluster.
  - 5T/9T samples typically run in the 9T homozygous cluster but may also run between clusters.

Example data

Figure 8 shows OpenArray™ data for 5T and 9T assays.

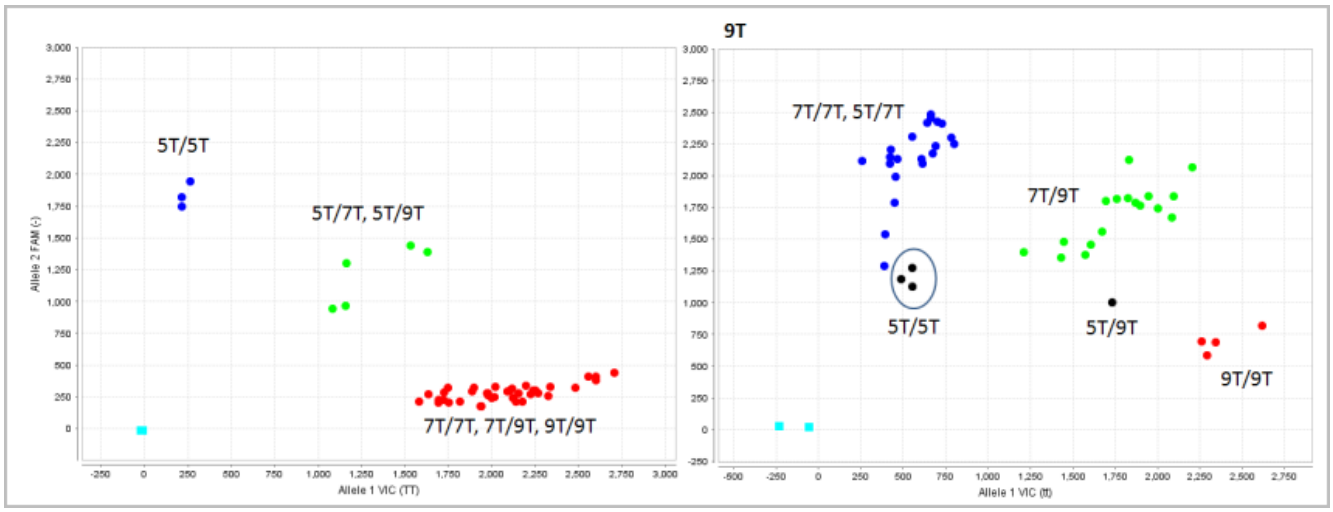


Figure 8 PolyT region 5T/7T/9T assay results. Left: 5T (C203006801C_10) assay contains 5T and 7T allele probes; the 7T allele probe also detects the 9T allele, so 5T/7T and 5T/9T samples run as 5T heterozygous. Right: 9T (C203006801D_20) assay contains 7T and 9T allele probes; 5T/5T samples run near the bottom of the 7T homozygous cluster. In this experiment, the 5T/9T sample ran between clusters instead of with the 9T homozygous cluster. [Samples: gDNAs isolated from blood leukocytes]

Analysis guidelines: SNP genotyping assays to large CFTR mutations

CFTRdele2,3 and CFTRdele22,23 are large CFTR mutations that are each interrogated by a pair of modified TaqMan™ copy number assays. Each assay contains one functional probe:

- Wild type allele assay – One functional probe (VIC™ dye) and one non-functional probe (FAM™ dye)
- Mutant allele assay – One functional probe (FAM™ dye) and one non-functional probe (VIC™ dye)

The non-functional probe allows these copy number assays to be run with SNP assay sets.

Expected results

- Heterozygous samples amplify with both assays and run in the VIC™ dye homozygous cluster with the wild type assay and in the FAM™ dye homozygous cluster with the deletion mutation assay.
- Homozygous wild type samples amplify only with the wild type assay and run in the VIC™ dye homozygous cluster.
- Homozygous wild type samples do not amplify with the mutation assay, but the software sometimes assigns genotypes to wild type samples. Change these calls to noamp.

Genotype	Wild type assay result VIC™ dye	Mutation assay result FAM™ dye
wt/wt	wt/wt	No amp
mu/wt	wt/wt	mu/mu

Example data

Figure 9 shows OpenArray™ data for CFTRdele2,3 assays.

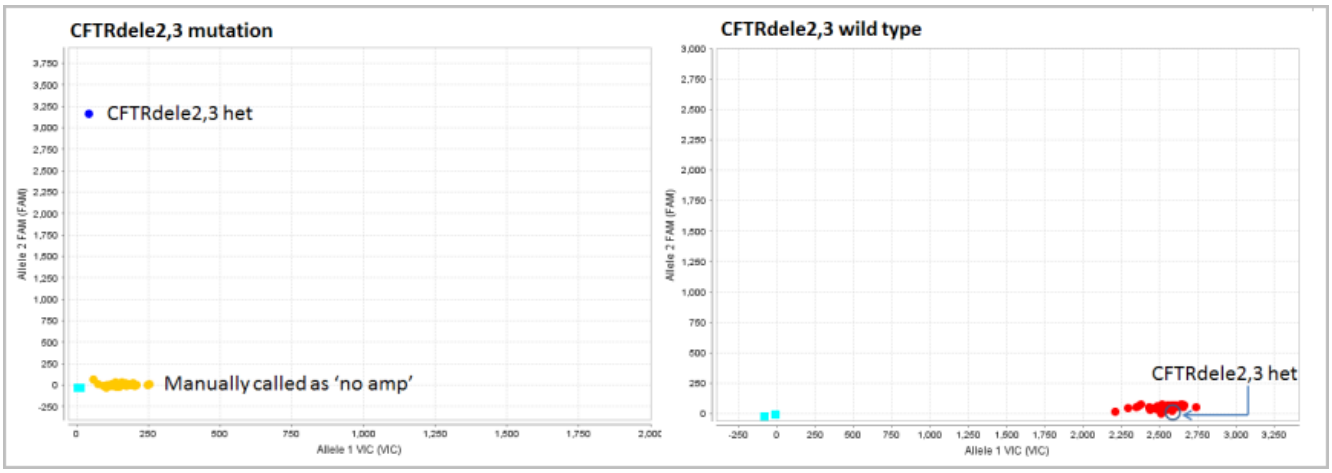


Figure 9 CFTRdele2,3 mutant and wild type assay results. Left: CFTRdele2,3 (C990000002A_10) mutation assay; heterozygous samples run in the FAM™ homozygous cluster; genotype calls were manually changed to "no amp". Right: CFTRdele2,3 wild type assay (C990000002B_20); wild type and heterozygous samples run in the VIC™ homozygous cluster. [Samples: Coriell cell line gDNAs]

6 Confirm positive results

Guidelines for confirmation of positive results

Examine real-time trace data in the allelic discrimination plot to confirm that results are accurate and are not caused by a rare system error. You can examine real-time trace data with the QuantStudio™ 12K Flex Real-Time PCR System software or the Thermo Fisher Scientific Cloud GT module.

Confirm all ambiguous or homozygous results using any of the following methods:

- Repeat the original experiment.
- Run the sample with single-tube assays on 96- or 384-well plates.
- Run the sample on an additional technology such as capillary electrophoresis sequence analysis.

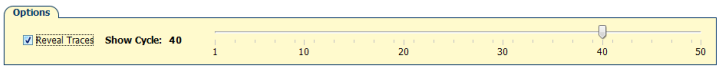
Review real-time trace data to confirm positive results

For assays with positive results, review the real-time trace data to ensure the genotyping results are valid.

Real-time sample traces for true positive results are:

- Linear or curved as appropriate for the sample genotype (samples of the same genotype should have traces with similar trajectories)
- Readily distinguished from the homozygous wild type sample traces

Review real-time trace data:

Software	Procedure
QuantStudio™ 12K Flex	1. In analysis settings, select **Analyze Real-Time Rn – Median (Rna to Rnb)**. This setting will normalize for any run and system noise to improve data accuracy. 2. Select **Analysis** ▸ **Allelic Discrimination Plot**, then click  in the toolbar.  3. Select **Reveal traces**. 
Thermo Fisher Cloud Cloud	1. Go to **www.thermofisher.com/us/en/home/cloud.html**. 2. Create a project and import one or more OpenArray™ experiment files (.eds). 3. Open the GT module. 4. Analyze data. Real-time trace data is displayed by default in the allelic discrimination plots.

Real-time trace data examples

The traces in this section were generated using the QuantStudio™ 12K Flex software and are for example purposes only.

Figure 10 is an example of a true positive result that the software can accurately genotype.

Figure 11 is an example of a result (marked with an x) that the software may inaccurately genotype.

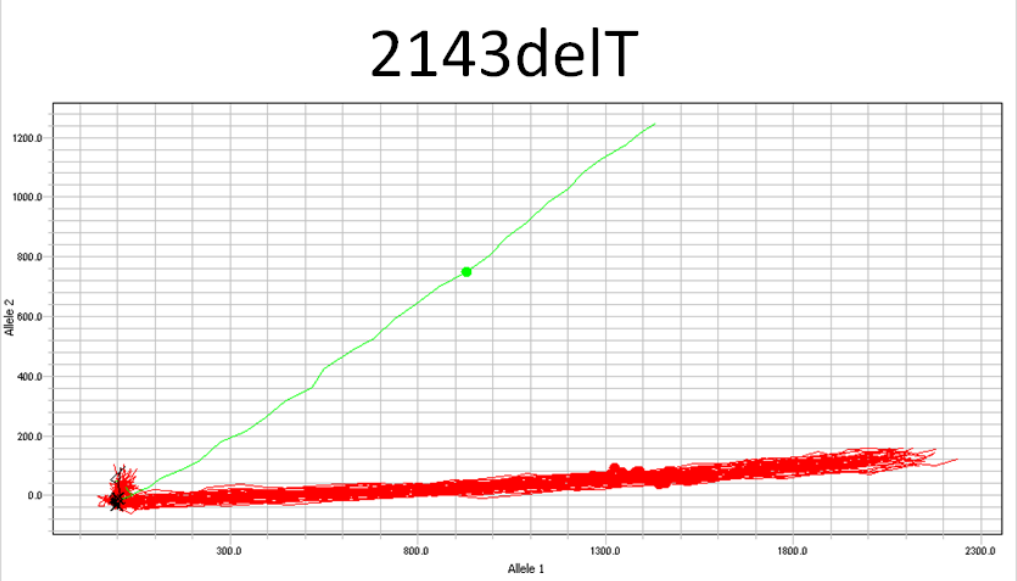


Figure 10 Heterogygote (green) and homozygous (red) real-time data traces are clearly distinguished.

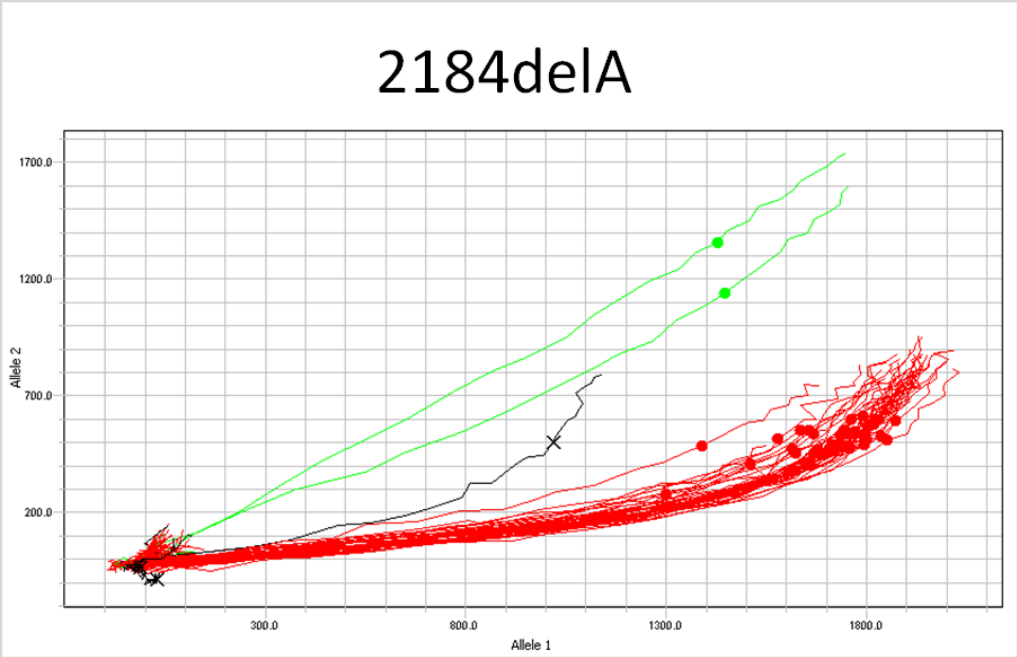


Figure 11 Wild type sample real-time data trace trajectory (black) varies significantly from the trajectories for true heterozygotes (green) and for other homozygous (red) samples. In addition, nonspecific probe activity can cause homozygous samples (red) to cluster closer to heterozygous samples at later PCR cycles, but this clustering does not typically interfere with genotype calls. If necessary, data points from an earlier PCR cycle can be used to resolve data points that merge together at later cyles (for example, use 40 cycles instead of 50 cycles for analysis).

Prepare and run single-tube experiments

Workflow: single-tube CFTR experiments

"Prepare the DNA sample plate: wet DNA" on page 61 or

"Prepare the DNA sample plate: dry DNA" on page 61

▼

"Perform PCR (single-tube assays)" on page 62

▼

Chapter 5, "Analyze CFTR genotyping data"

If you are interrogating mutations that require two assays, review the data for both assays together.

Required materials: single-tube workflow

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

Item	Source
Genomic DNA	5 to 20 ng (diluted in nuclease-free water or 1X TE buffer as needed) From Chapter 3, "Isolate DNA using the MagMAX™ DNA Multi-Sample Ultra Kit"
TaqMan™ Genotyping Master Mix (1 × 1 mL)	4371353
TaqMan™ Genotyping Master Mix (1 × 10 mL)	4371355
TaqMan™ Genotyping Master Mix (1 × 50 mL)	4371357
TaqMan™ Genotyping Master Mix (2 × 10 mL)	4381656
TaqMan™ Genotyping Master Mix (2 × 50 mL)	4381657
CFTR assays, 20X Assay Working Stock	See "Order single tube TaqMan™ assays" on page 17.
(Optional) CFTR TaqMan™ Assay Controls	A30421
MicroAmp™ Optical Reaction Plates, any of the following:	
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	4346906
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	4306737
MicroAmp™ Optical 384-Well Reaction Plate with Barcode	4309849
UltraPure™ DNase/RNase-Free Distilled Water *or* TE, pH 8.0 (1X TE buffer)	10977-015 AM9849

Instruments and software required:

- QuantStudio™ 12K Flex instrument or thermal cycler

Prepare the DNA sample plate: wet DNA

All wells being tested with a given assay must contain similar amounts of sample DNA for best results. Include at least 10 samples and 2 NTCs for each assay.

1. (Recommended for gDNA) Quantify the DNA (see "DNA concentration and quantification" on page 31), then dilute in nuclease-free water or 1X TE buffer to 5 to 20 ng per well.
2. Mix the 2X Master Mix gently. Do not invert the bottle.
3. Vortex the Assay Working Stock, then centrifuge briefly.
4. Calculate the number of reactions needed for each assay.
5. Calculate the volume of each component needed. Include extra reactions to compensate for the volume loss that occurs during pipetting.

Component	384-Well Plate	96-Well Fast Plate	96-Well Plate
TaqMan™ Genotyping Master Mix	2.50 µL	5.00 µL	12.50 µL
CFTR TaqMan™ SNP Genotyping Assay	0.25 µL	0.50 µL	1.25 µL
UltraPure™ DNase/RNase-Free Distilled Water	Varies based on DNA concentration		
One of the following: • UltraPure™ DNase/RNase-Free Distilled Water (NTCs) • DNA sample			
Total reaction volume	5.00 µL	10.00 µL	25.00 µL

Prepare the DNA sample plate: dry DNA

All wells being tested with a given assay must contain similar amounts of sample DNA for best results. Include at least 10 samples and 2 NTCs for each assay.

1. Add 5 to 20 ng of DNA to each well. Use 2 to 5 µL sample volumes to minimize drying time.
2. Cover the plate and allow the samples to dry at room temperature in a dark, amplicon-free location.

   If you are using gDNA, do not heat the plate to dry, because it can affect gDNA recovery.
3. Mix the 2X Master Mix gently. Do not invert the bottle.
4. Vortex the Assay Working Stock, then centrifuge briefly.
5. Calculate the number of reactions needed for each assay.

6. Calculate the volume of each component needed. Include extra reactions to compensate for the volume loss that occurs during pipetting.

Component	384-Well Plate	96-Well Fast Plate	96-Well Plate
TaqMan™ Genotyping Master Mix	2.50 µL	5.00 µL	12.50 µL
CFTR TaqMan™ SNP Genotyping Assay	0.25 µL	0.50 µL	1.25 µL
UltraPure™ DNase/RNase-Free Distilled Water	2.25 µL	4.50 µL	11.25 µL
Total reaction volume	5.00 µL	10.00 µL	25.00 µL

Perform PCR (single-tube assays)

Follow the instructions in the instrument user guide for this procedure.

1. Set the following thermal cycling parameters:

Step	Temperature	Duration	Cycles
Activate	95°C	10 minutes	HOLD
Denature	95°C	15 seconds	40
Anneal/ Extend	60°C	1 minute	40

If you are using a real-time PCR instrument, select **Standard** mode in thermal cycler settings.

IMPORTANT! Do not use Fast Mode thermal cycling conditions with TaqMan™ Genotyping Master Mix, even when using Fast plates, blocks, or thermal cyclers.

2. Set the following reaction volumes:

MicroAmp™ Optical Reaction Plate	Reaction volume
384-well	5.00 µL
Fast 96-well	10.00 µL
96-well	25.00 µL

3. Load the plate and start the run.

A

RNase P Quantification for genotyping experiments

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

In this procedure, the quantification method uses TaqMan™ Control Genomic DNA (human) as a calibrator instead of a standard curve. The OA_Genotyping_CalcSheet calculates the concentration of test DNA samples with this calibrator method.

Note: The OA_Genotyping_CalcSheet assumes duplicate RNase P reactions in a 384-well plate layout.

Download the OA_Genotyping_CalcSheet from **thermofisher.com/oaqrc**.

Required materials

Item	Source[1]
TaqMan™ Copy Number Reference Assay, human, RNase P (20X)	Cat. nos. 4403326 (750 rx), 4403328 (3000 rx)
TaqMan™ Control Genomic DNA (human; ready for use)	Cat. no. 4312660
TaqMan™ Genotyping Master Mix (2X)[2]	Cat. no. 4371357[3]

[1] thermofisher.com

[2] Any TaqMan™ Master Mix can be used, but ensure that the run mode on the instrument is appropriate for the master mix.

[3] 50 mL; other sizes available

Before you begin

- For best results, dilute test DNA samples 1:10 in Nuclease-Free Water.
- Determine the total number of reactions required. Include:
  - Diluted test DNA samples.
  - Calibrator DNA sample.
  - No-template control (NTC) reactions; use Nuclease-Free Water in place of DNA sample.

  Note: For best results, include a minimum of two reactions for each sample. The OA_Genotyping_CalcSheet is set up for duplicate reactions.
- Generate the RNase P Sample .txt file from the OA_Genotyping_CalcSheet.

Set up and run the PCR, then quantify the DNA

1. Prepare a reaction mix for the required number of reactions plus 10% overage.

Component	Volume per reaction[1]
TaqMan™ Genotyping Master Mix (2X)	5 µL
TaqMan™ RNase P Assay (20X)	0.5 µL
Nuclease-Free Water	2.5 µL
Total	8 µL

[1] Refer to the OA_Genotyping_CalcSheet to easily calculate volumes for multiple reactions.

2. Transfer 8 µL of the reaction mix to the wells of a 384-well PCR reaction plate.
3. Add 2 µL of sample DNA, control DNA, or Nuclease-Free Water to the appropriate wells.
4. Seal the plate with optical film.
5. Set up the real-time instrument with the following settings:
   - Experiment type: **Standard Curve**
   - Mode: **Standard**
   - Reporter: **VIC™**
   - Quencher: **TAMRA™**
   - Passive reference: **included**
6. Assign the sample names: import the RNase P Sample .txt file generated from the OA_Genotyping_CalcSheet.
7. Load the plate and start the run.
8. After the run is complete, deselect all but the Results tab, select a file location, then export the results.

9. Remove duplicate sample IDs from the exported data by opening the exported data file in Excel™, then:
   a. Copy and paste sample names and their corresponding C_T mean values into a new sheet.
   b. Select both columns and use the **Remove Duplicates** function.
10. Calculate the concentration of test DNA samples: copy and paste the Sample Names and Mean C_T values into the OA_Genotyping_CalcSheet, and enter the dilution factor of the test DNA samples into the OA_Genotyping_CalcSheet.

B

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/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Related documentation

Document	Publication number
CFTR TaqMan™ Assay Controls Quick Reference	MAN0014633
OpenArray™ Sample Tracker Software Quick Reference	4460657
QuantStudio™ 12K Flex Real-Time PCR System: OpenArray™ Experiments User Guide	4470935
OpenArray™ AccuFill™ System User Guide	4456986
TaqMan™ OpenArray™ Genotyping Troubleshooting Guide	MAN0011115
TaqMan™ Genotyper Software Getting Started Guide	4448637

Customer and technical support

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

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

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

Index

T

W