DigitalTRACE™ Operator's Manual

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Introduction

Welcome to the DigitalTRACE™ Digital PCR Analysis System Operator's Manual and Help System. This document serves as both the DigitalTRACE™ Operator's Manual and the help system found within the TRACE Analysis™ Software package.

Navigate to your subject of interest and find the solution to your question. If your topic of interest is not listed or you would like to receive additional information, do not hesitate to contact us. The content of this help system is regularly updated. We encourage you to inform us of inaccuracies or suggestions. We do our utmost to implement your suggestions swiftly, such that you and other DigitalTRACE™ users may benefit from it.

JETA Molecular BV.
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Warning and Precautions

Product Use Limitations

This version of TRACE Analysis™ Software is for Research Use Only. It is not intended for use in diagnostic procedures.

No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the DigitalTRACE™ kits or components (collectively referred to as DigitalTRACE™ Kits herein) with the following terms:

The DigitalTRACE™ Kits may be used solely in accordance with the DigitalTRACE™ Kits manual and for use with components contained in the kit only. JETA Molecular grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the DigitalTRACE™ Kits manual and additional protocols available at www.jetabv.com.

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JETA Molecular assumes no responsibility for any inaccuracies that may be contained in this manual.

JETA Molecular reserves the right to make improvements to this manual and/or to the products described in this manual, at any time without notice.

If you find information in this manual that is incorrect, misleading, or incomplete, we would appreciate your comments and suggestions. Please send them to info@jetabv.com.

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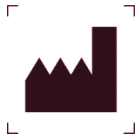
DigitalTRACE™ is a registered trademark of ElsworthMolecular Holding BV. in the U.S. and/or certain other countries. QTRACE® Products and Software are licensed exclusively to JETA Molecular BV. from ElsworthMolecular Holding BV.

MicroAmp® is a registered trademark of Thermo Fisher.

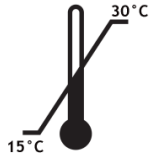
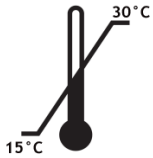
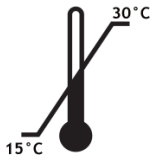
Materials

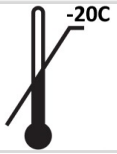
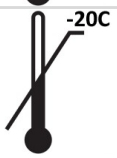
Keys to Symbols

The following symbols appear within the labeling of the DigitalTRACE™ Products:

Label	Name	Meaning
	Research Use Only	The RUO mark indicates that this product is for research use only, not clinical use.
	Lot number	The lot number identifies the reagent batch.
	Reference	The product reference code of the product.
	Manufacturer of the device and production date	The company name of the manufacturer of the product, JETA Molecular BV., and our address: Krommewetering 101C, 3543 AN Utrecht, The Netherlands, EU The date next to this label is the date of manufacture.
	Number of tests	The kit contains enough reagents for <N> tests. The number next to this symbol shows the total number of tests that you can perform
	Expiry date	The expiry date of the kit. The date next to this label is the expiration date of the item that expires the soonest in the kit.
	Storage temperature	The lowest and highest temperature the contents can be safely exposed to in storage.
 www.jetamolecular.com	Consult instructions for use	The user should read the instructions for use (IFU) to learn how to use the product in a safe and efficient way.

Materials Provided

REF	Name	Description	Storage Conditions	Unit
121045	DigitalTRACE™ QIAcuity Genotyping Plate	Two ABI MicroAmp® Optical 96-well plates pre-arrayed with INDEL dPCR Assays; individually sealed		Box
121056	DigitalTRACE™ EP QIAcuity Genotyping Plate	Two ABI MicroAmp® Optical 96-well plates pre-arrayed with INDEL dPCR Assays; individually sealed		Box
121227	MultiTRACE™ v3 Genotyping Plate	Four ABI MicroAmp® Optical 96-well plates pre-arrayed with INDEL Assays; individually sealed		Box

REF	Name	Description	Storage Conditions	Unit
711294	DigitalTRACE™ Universal Positive Control	360µl buffered solution containing synthetic DNA serving as positive control		Tube
	DigitalTRACE™ INDEL Assays	26µl buffered solution containing a mix of primers and probe for detecting the variant of interest in FAM and a reference assay in HEX		Tube
	DigitalTRACE™ HLA Assays	26µl buffered solution containing a mix of primers and probe for detecting the variant of interest in FAM and a reference assay in HEX		Tube

DigitalTRACE™ Monitoring Assay Reference Numbers:

REF	Name		REF	Name
811140	DigitalTRACE™ INDEL Assay 102		811030	DigitalTRACE™ INDEL Assay 748
811141	DigitalTRACE™ INDEL Assay 113		811053	DigitalTRACE™ INDEL Assay 755
811142	DigitalTRACE™ INDEL Assay 120		811167	DigitalTRACE™ INDEL Assay 777
811001	DigitalTRACE™ INDEL Assay 137		811032	DigitalTRACE™ INDEL Assay 784
811143	DigitalTRACE™ INDEL Assay 157		811168	DigitalTRACE™ INDEL Assay 795
811144	DigitalTRACE™ INDEL Assay 176		811034	DigitalTRACE™ INDEL Assay 824
811145	DigitalTRACE™ INDEL Assay 183		811036	DigitalTRACE™ INDEL Assay 840
811146	DigitalTRACE™ INDEL Assay 198		811169	DigitalTRACE™ INDEL Assay 874
811147	DigitalTRACE™ INDEL Assay 222		811170	DigitalTRACE™ INDEL Assay 884
811004	DigitalTRACE™ INDEL Assay 235		811171	DigitalTRACE™ INDEL Assay 892
811005	DigitalTRACE™ INDEL Assay 240		811040	DigitalTRACE™ INDEL Assay 916

811148	DigitalTRACE™ INDEL Assay 252		811172	DigitalTRACE™ INDEL Assay 923
811006	DigitalTRACE™ INDEL Assay 267		811173	DigitalTRACE™ INDEL Assay 936
811149	DigitalTRACE™ INDEL Assay 275		811041	DigitalTRACE™ INDEL Assay 948
811009	DigitalTRACE™ INDEL Assay 312		811042	DigitalTRACE™ INDEL Assay 954
811150	DigitalTRACE™ INDEL Assay 333		811174	DigitalTRACE™ INDEL Assay 962
811011	DigitalTRACE™ INDEL Assay 345		811175	DigitalTRACE™ INDEL Assay 971
811013	DigitalTRACE™ INDEL Assay 359		811176	DigitalTRACE™ INDEL Assay 987
811014	DigitalTRACE™ INDEL Assay 361		811177	DigitalTRACE™ INDEL Assay 990
811064	DigitalTRACE™ INDEL Assay 386			
811151	DigitalTRACE™ INDEL Assay 396		811078	DigitalTRACE™ HLA Assay H005
811015	DigitalTRACE™ INDEL Assay 408		811080	DigitalTRACE™ HLA Assay H007
811016	DigitalTRACE™ INDEL Assay 425		811083	DigitalTRACE™ HLA Assay H017
811017	DigitalTRACE™ INDEL Assay 434		811085	DigitalTRACE™ HLA Assay H022
811152	DigitalTRACE™ INDEL Assay 441		811087	DigitalTRACE™ HLA Assay H025
811153	DigitalTRACE™ INDEL Assay 450		811088	DigitalTRACE™ HLA Assay H028
811018	DigitalTRACE™ INDEL Assay 469		811091	DigitalTRACE™ HLA Assay H036
811154	DigitalTRACE™ INDEL Assay 472		811092	DigitalTRACE™ HLA Assay H038
811155	DigitalTRACE™ INDEL Assay 482		811093	DigitalTRACE™ HLA Assay H039
811156	DigitalTRACE™ INDEL Assay 493		811095	DigitalTRACE™ HLA Assay H043
811054	DigitalTRACE™ INDEL Assay 519		811096	DigitalTRACE™ HLA Assay H045
811021	DigitalTRACE™ INDEL Assay 531		811098	DigitalTRACE™ HLA Assay H051
811022	DigitalTRACE™ INDEL Assay 548		811099	DigitalTRACE™ HLA Assay H052
811157	DigitalTRACE™ INDEL Assay 555		811100	DigitalTRACE™ HLA Assay H053
811158	DigitalTRACE™ INDEL Assay 567		811101	DigitalTRACE™ HLA Assay H054
811159	DigitalTRACE™ INDEL Assay 574		811133	DigitalTRACE™ HLA Assay H102
811160	DigitalTRACE™ INDEL Assay 585		811134	DigitalTRACE™ HLA Assay H103
811161	DigitalTRACE™ INDEL Assay 597		811279	DigitalTRACE™ HLA Assay H104
811023	DigitalTRACE™ INDEL Assay 601		811280	DigitalTRACE™ HLA Assay H105
811024	DigitalTRACE™ INDEL Assay 615		811281	DigitalTRACE™ HLA Assay H106
811026	DigitalTRACE™ INDEL Assay 634		811282	DigitalTRACE™ HLA Assay H107
811027	DigitalTRACE™ INDEL Assay 650		811283	DigitalTRACE™ HLA Assay H108
811162	DigitalTRACE™ INDEL Assay 663		811290	DigitalTRACE™ HLA Assay H110
811163	DigitalTRACE™ INDEL Assay 670		811291	DigitalTRACE™ HLA Assay H111
811164	DigitalTRACE™ INDEL Assay 678		811292	DigitalTRACE™ HLA Assay H112
811165	DigitalTRACE™ INDEL Assay 681		811293	DigitalTRACE™ HLA Assay H113
811166	DigitalTRACE™ INDEL Assay 694		811296	DigitalTRACE™ HLA Assay H114
811028	DigitalTRACE™ INDEL Assay 706		811297	DigitalTRACE™ HLA Assay H115
811065	DigitalTRACE™ INDEL Assay 710		811298	DigitalTRACE™ HLA Assay H116
811051	DigitalTRACE™ INDEL Assay 721		811299	DigitalTRACE™ HLA Assay H117
811029	DigitalTRACE™ INDEL Assay 736		811300	DigitalTRACE™ HLA Assay H118

REF	Name	Description
341048	TRACE Analysis™ Software	Minimum System Requirements: Windows 7, 2 GB RAM, 250 MB free disk space, Network connection allowing TCP/IP traffic to and from port 3500, Microsoft .NET framework 4.5. Recommended System Requirements: Windows 7, 2 GB RAM, Internet connection for license validation and automatic updates, 500 MB free disk space, Microsoft .NET framework 4.5

REF	Name	Description
331307	DigitalTRACE™ System Operator's Manual	Operator's Manual for DigitalTRACE™ Analysis System

Materials Sold Separately

Additional Reagents Required but not Provided

Item name	Catalog number
Modified TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) (also called TE 0.1 Buffer or TE-4 Buffer)	Not applicable
Molecular grade water (DNase and RNase free)	Not applicable
QIAcuity Probe PCR Kit (Qiagen)	250102
ddPCR Supermix for Probes (Biorad)	1863023

Additional Equipment Required but not Provided

Item name	Catalog number
QIAcuity One, 5plex (Qiagen)	9245359
Biorad QX200 Droplet Digital PCR System (Biorad)	1864001
Adjustable single channel pipettes (0.5-1000 µL capacity)	Not applicable
Adjustable multi-channel, multi-dispensing pipettes (0.5-200 µL capacity)	Not applicable
Vortex mixer with flat rubber platform head	Not applicable
Centrifuge	Not applicable
Centrifuge with microtiter plate assembly	Not applicable
PC for the installation of TRACE Analysis™ Software	Not applicable
QIAcuity Analysis Suite Software (Qiagen)	Not applicable
QuantaSoft or QX Manager Software (Biorad)	Not applicable

Additional Consumables Required but not Provided

Item name	Catalog number
Pipette Tips, disposable, sterile, aerosol-resistant, filtered, capable of dispensing up to 20, 200, and 1000 µL.	Not applicable
1.5 mL microcentrifuge tubes	Not applicable

Lint-free tissue	Not applicable
Gloves, powder-free	Not applicable
96-well plates or strip tubes	Not applicable
Plate seals	Not applicable
QIAcuity Nanoplate 26k 24-well (Qiagen)	250001
QIAcuity Nanoplate 8.5k 96-well (Qiagen)	250021
DG8 Cartridges (Biorad)	1864008
DG8 Gaskets (Biorad)	1863009
ddPCR 96-well PCR Plates (Biorad)	12001925
PCR Plate Heat Seal, foil, pierceable (Biorad)	1814040
Droplet Generation Oil for Probes (Biorad)	1863005
ddPCR Droplet Reader Oil (Biorad)	1863004

Recommended DNA Isolation

Blood samples should be collected in ACD or EDTA anticoagulation tubes.

Purified DNA should have an A260/A280 ratio between 1.7 and 2.0.

We recommend using a fluorometric method to accurately quantify DNA

If necessary, DNA should be diluted in 10mM Tris, pH 8.0; 0.1mM EDTA (TE) or nuclease-free H_2O before use.

The optimal amount of template DNA to use in genotyping is 10 ng per well.

Safety Information

Read the “Safety Information” sections of any reagents or kits specified in “Materials” Section before starting.

When working with chemicals always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate Safety Data Sheets (SDSs) available from the specified product supplier.

Product reagents chemical component overview can be found in the SDSs of the DigitalTRACE™ Product and are available upon request.

Dispose product components as general medical waste.

TRACE Analysis™ Software does not provide a mechanism to edit data files obtained from the dPCR System or result files once they are created.

Warnings

Use good laboratory practices for sample handling and tracking.
Use only recommended materials, procedures, and equipment.
Use sterile disposable pipettes and filtered pipette tips.
Wear appropriate personal protective equipment (*e.g.,* safety glasses, disposable gloves, and protective clothing) when handling samples and reagents.
Clean and disinfect all work surfaces with a 10% bleach (0.525% sodium hypochlorite) solution and follow with 70% ethanol, ensuring that all bleach residue is removed.
Assays should be run by individuals experienced in good laboratory practices and who have been previously trained to use the equipment by the original equipment manufacturer (OEM).
Operate, calibrate and maintain all instruments and equipment according to procedures provided by the manufacturers.
To reduce the risk of contamination, the area where amplified DNA is handled must be physically isolated from the work areas for sample preparation and qPCR setup.
Do not use components past their expiration date.
Do not dilute reagents.

Visually inspect wells or tubes after pipetting steps to detect operator errors with pipetting, sample transfer, etc.
To prevent repeated freeze/thaw cycling of reagents during frozen storage, do not store reagents within freezers that use an automatic defrost function (*i.e.,* frost-free).
Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent tubes.
To prevent contamination, after aliquots are removed do not return the remaining volume to the original tube.
Comply with all local, state, or national laws and regulations related to chemical storage and disposal.

CHEMICAL HAZARD. Ethanol is a flammable liquid and vapor. Exposure can cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact can dry the skin.
Exposure can cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing and gloves.

CHEMICAL HAZARD. Bleach (sodium hypochlorite) is a corrosive liquid and vapor. Exposure can cause severe irritation or damage to eyes, skin and the respiratory system. Harmful if swallowed. Prolonged or repeated contact can lead to sensitization (*e.g.,* irritation) if skin damage occurs during exposure. Medical conditions that can be aggravated by exposure to high concentrations of vapor or mist include heart conditions or chronic respiratory problems such as asthma, emphysema, chronic bronchitis or obstructive lung disease. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Shipping and Storage

DigitalTRACE™ Genotyping Plates are shipped at ambient temperature. The DigitalTRACE™ INDEL Monitoring Assays are shipped frozen.

Examine the shipment upon receipt and if the integrity of the products has been compromised during shipment, immediately contact your local customer support representative.

Upon receipt, the individual components should be stored according to the temperatures listed on the labels.

Note: When storage recommendations are observed, both unopened and opened/recapped tubes are stable until the expiration date indicated on the label. Genotyping plates are stable when stored in their original packaging. Do not use any component after the expiration date.

Do not use any component that visibly shows signs of having been compromised (e.g., particulate matter, presence of foreign debris, cloudy appearance, discoloration).

Technical Assistance

For technical assistance and more information:

Please contact your local distributor

or JETA Molecular - info@jetabv.com

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Principle of the Method

Background Information

The DigitalTRACE™ and MultiTRACE™ Genotyping Plates, DigitalTRACE™ INDEL Assays and TRACE Analysis™ Software meet the needs of any research application that requires highly sensitive detection and quantification of the genome of one individual in the background of another individual or individuals. A genetic chimera is an organism with two or more genetically distinct cell populations, *i.e.,* cell populations with different genomes.

Chimerism can arise in humans through a variety of means, such as inheritance, maternal-fetal stem cell trafficking during gestation, blood vessel sharing in fraternal twin gestation, blood transfusions, bone marrow transplantation, cord blood transplantation, and solid organ transplantation. The presence of two distinct human genomes in a sample can also occur simply through the mixing of human cells from more than one individual, for example, when two cell lines are cross-contaminated, or in forensic tissue samples.

The DigitalTRACE™ INDEL assays are digital polymerase chain reaction (dPCR) assays based upon self-quenched, hydrolysis probe chemistry. In a dPCR reaction, a dye-labeled oligonucleotide probe enables the detection of a specific PCR product at the end PCR cycling. The high sensitivity is the result of the very large dynamic range of the real-time amplification method and is limited essentially by the input copy number of total DNA that can be added to the dPCR reaction. In the Monitoring test, the assays are formulated with the target in channel FAM and the reference assay (RNase P) in channel HEX.

The DigitalTRACE™ HLA Assays are digital polymerase chain reaction (dPCR) assays based upon self-quenched, hydrolysis probe chemistry. The assays are formulated with the target in channel FAM and the reference assay (RNase P) in channel HEX. These assays are designed to detect the loss of HLA heterozygosity after haploidentical HSCT.

TRACE Analysis™ Software was designed specifically for the DigitalTRACE™ INDEL Assay Set. The software provides a streamlined workflow for both the Genotyping and Monitoring tests. The software guides the user through assay setup, performs data analysis, generates results reports and stores the data collected for samples over time.

Product Overview

The DigitalTRACE™ System consists of DNA Genotyping plates, 70 individual DigitalTRACE™ INDEL assays, 31 DigitalTRACE™ HLA assays and TRACE Analysis™ Software. The DigitalTRACE™ INDEL Assays are a set of 70 genetic markers that are able to differentiate, and then quantify, the contributors to a human-mixed DNA sample. Each of the 70 assays is designed to a distinct bi-allelic insertion/deletion (INDEL) or copy number polymorphisms in the human genome. The DigitalTRACE™ HLA assays are designed to detect the loss of a particular HLA allele after haploidentical HSCT. The TRACE Analysis™ Software guides the user through reaction set-up for both screening and quantification, and analyzes the collected data. The procedure for determining the level of a genome of interest in a sample consists of two parts: a genotyping test and a quantification (monitoring) test.

Genotyping Test

In the initial genotyping test, the DNAs that comprise a mixed DNA sample are analyzed using a DigitalTRACE™ or MultiTRACE™ Genotyping Plate, to identify all of the informative assays for the samples. An informative assay is an assay for a marker allele that is present (positive) in one individual genome and absent (negative) in the other genome.

The DigitalTRACE™ Genotyping Plate contains a set of 43 quantification assays and the reference (RNase P) assay that serves as both a positive control and a No Template Control (NTC).

The DigitalTRACE™ EP QIAcuity Genotyping Plate represents an extended panel of dPCR markers that can be used for dPCR monitoring in the case of need for additional markers allowing to distinguish between donor and recipient DNA. This plate contains a set of 27 quantification assays and the reference (RNase P) assay that serves as both a positive control and a No Template Control (NTC).

The MultiTRACE™ Genotyping Plate contains a set of 45 quantification assays and the reference (RNase P) assay that serves as both a positive control and a No Template Control (NTC).

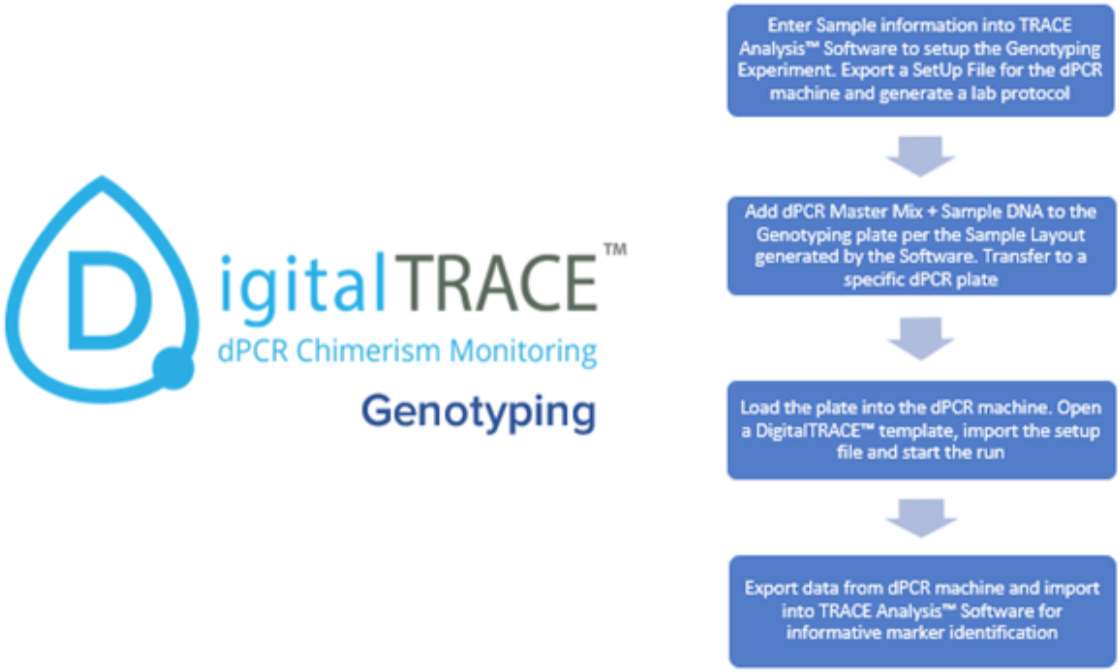
Monitoring Test

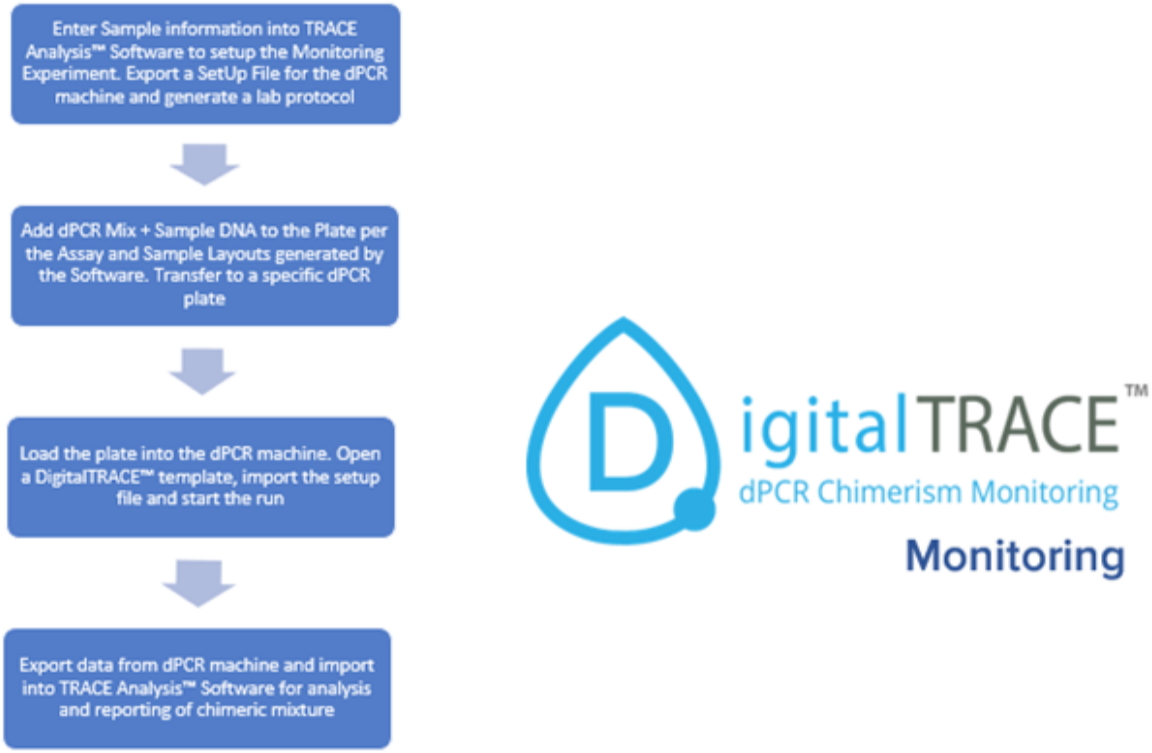
In the Monitoring (quantification) test, two or more of the informative assays identified in the genotyping test is used to quantify the DNA of interest in an unknown sample. Any of the informative assays identified in the genotyping test can be used to perform a quantification test. The amount positive for the informative allele in the unknown sample is determined relative to the amount of the reference gene, and the result is expressed as a percentage (ratio). For example, a result of 5% indicates that there is 5% of genome A in the unknown sample.

The informativeness of a multi-locus genotyping panel is a measure of the probability of finding at least one informative assay between two individual genomes (or DNA samples). Informativeness is calculated from the population frequency estimates of the alleles used to make up a multi-locus genotyping panel, and thus differs between ethnic populations. In addition, the informativeness of any panel of polymorphic loci is higher in unrelated individuals than in related individuals.

The performance of the DigitalTRACE™ System has been verified to a level of 0.1% minor component DNA in 150 ng total DNA.

DigitalTRACE Workflow





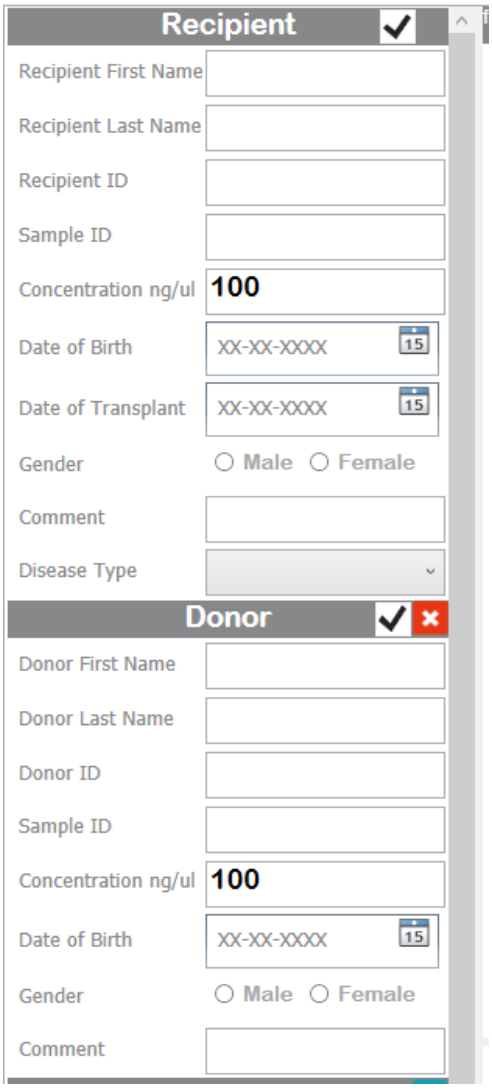
Genotyping Test

Genotyping Test Protocol

Genotyping Test Protocol - QIAcuity

Change the instrument type in the Preferences of the TRACE Analysis™ Software to QIAcuity and Plate type to Qiacuity, v1.

To generate a new record in TRACE Analysis™ Software, in the Section labeled “Recipient” enter the Recipient Name, Recipient Identifier and a unique Sample Identifier. While “Date of Birth” is an optional field for all samples, a “Date of Transplant” must be entered, if you ultimately want to have the data stored and reported in a temporal manner.



For a sample which should be genotyped against the Recipient sample, enter the Donor Name, Donor Identifier and unique Sample Identifier. You can genotype up to eight samples on a single plate using TRACE Analysis™ Software. TRACE Analysis™ Software also allows you to virtually compare multiple samples, independent of when they were genotyped. Based on the data from verification studies, JETA Molecular recommends the use of 10 ng DNA input per well for genotyping. (DNA inputs are customized in the software's Preferences for Concentrations). Enter

the concentrations of your samples to let the software calculate with.
After all samples to be comparatively genotyped are entered into the Sample window, press the "Screen" button to add the samples to the plate set up file. You will see the samples now added to the 96-well plate in the middle of the screen.

The colors in the small plate images (Sample View and Assay View) at the bottom of the window are enabled in the large plate image, by touching the colored plate image of interest. By touching the same image again, the large plate colors disappear. (For Genotyping, the assays are already in the wells and are not added by the operator).
Once the samples have been added virtually to your plate, and the experiment name have been entered, press the "Export Setup to PCR" button.
Browse to the location where you want the file saved and name it as you wish. This file can then be imported into your dPCR machine's DigitalTRACE template to execute the dPCR analysis.

Once the file is saved, TRACE Analysis™ Software generates a protocol, based on the experimental inputs and the settings in the preferences menu. Print out this protocol.

1. **Set up all reactions in a pre-PCR lab, under ambient conditions without ice.**
2. Collect all DNA samples to be screened, as well as QIAcuity typing plate together with 4x Probe PCR Master Mix and de-ionized H_2O.
3. Briefly vortex and centrifuge all tubes before opening.
4. For each sample to be genotyped, lable a tube and a Mix containing sample DNA, de-ionized H_2O and 4x Probe PCR Master Mix as suggested by TRACE Analysis™ Software in Table 1. A No Template Control (NTC) Mix is prepared with de-ionized H_2O and 4x Probe PCR Master Mix.
   * - Make a Ten-Fold Dilution (1:10) of Sample

Table 1. Master Mix Composition

Sample 1 dPCR Mix	1 x	13 x
4x Probe PCR Master Mix	3,0 µl	39,0 µl
001 DNA	0,5 µl	*6,5 µl
H_2O	8,5 µl	110,5 µl
	12,0 µl	156,0 µl

Sample 2 dPCR Mix	1 x	13 x
4x Probe PCR Master Mix	3,0 µl	39,0 µl
002 DNA	0,5 µl	*6,5 µl
H_2O	8,5 µl	110,5 µl
	12,0 µl	156,0 µl

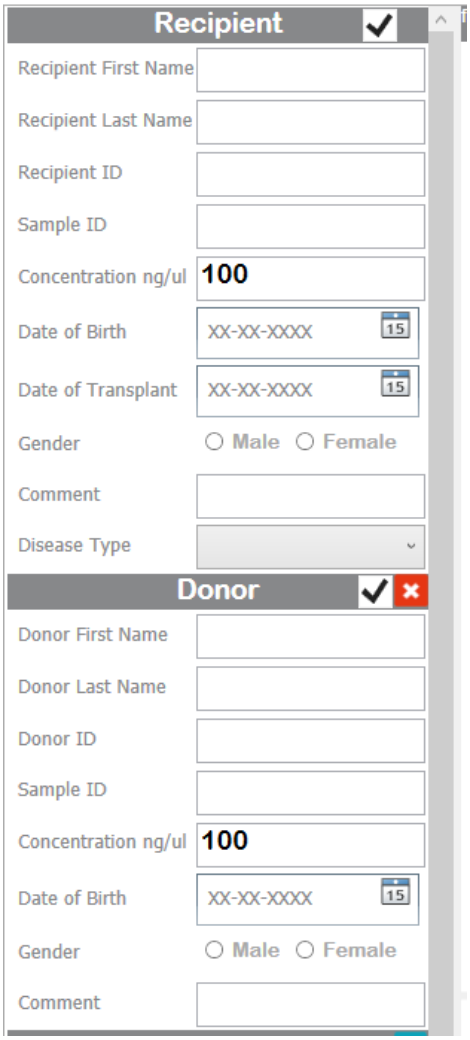
NTC dPCR Mix	1 x	10 x
4x Probe PCR Master Mix	3,0 µl	30,0 µl
H_2O	9,0 µl	90,0 µl
	12,0 µl	120,0 µl

5. **Vortex each tube to thoroughly mix the contents and centrifuge briefly to collect the reaction mix at the bottom of the tube.**
6. Remove the adhesive cover from the genotyping plate.
7. Deliver 13.2 µl of each Sample Mix and NTC mix to the Typing plate as defined in TRACE Analysis™ Software's Assay Layout view.
8. **An automated multichannel pipette is recommended in this step to minimize pipetting repetition and increase accuracy.**
9. Visually inspect plate wells from the sides and bottom to confirm consistent volume.
10. Seal the plate with an Adhesive Film.
11. **IMPORTANT! Vortex the plate to mix the contents of each well.** Centrifuge the plates briefly using a plate centrifuge to collect the contents at the bottom of the wells.
12. **Remove the Adhesive cover very carefully.**
13. Transfer 12 µl of each prepared reaction mix into a single column of a 96-well 8.5K Nanoplate. Seal the Nanoplate with the compatible plate sealer.
14. Load the Nanoplate into the QIAcuity digital PCR system.
15. Launch the QIAcuity Software Suite.
16. Open the DigitalTRACE typing template and import the Sample Setup sheet generated by TRACE Analysis™ Software.
17. Save the file and start the run.

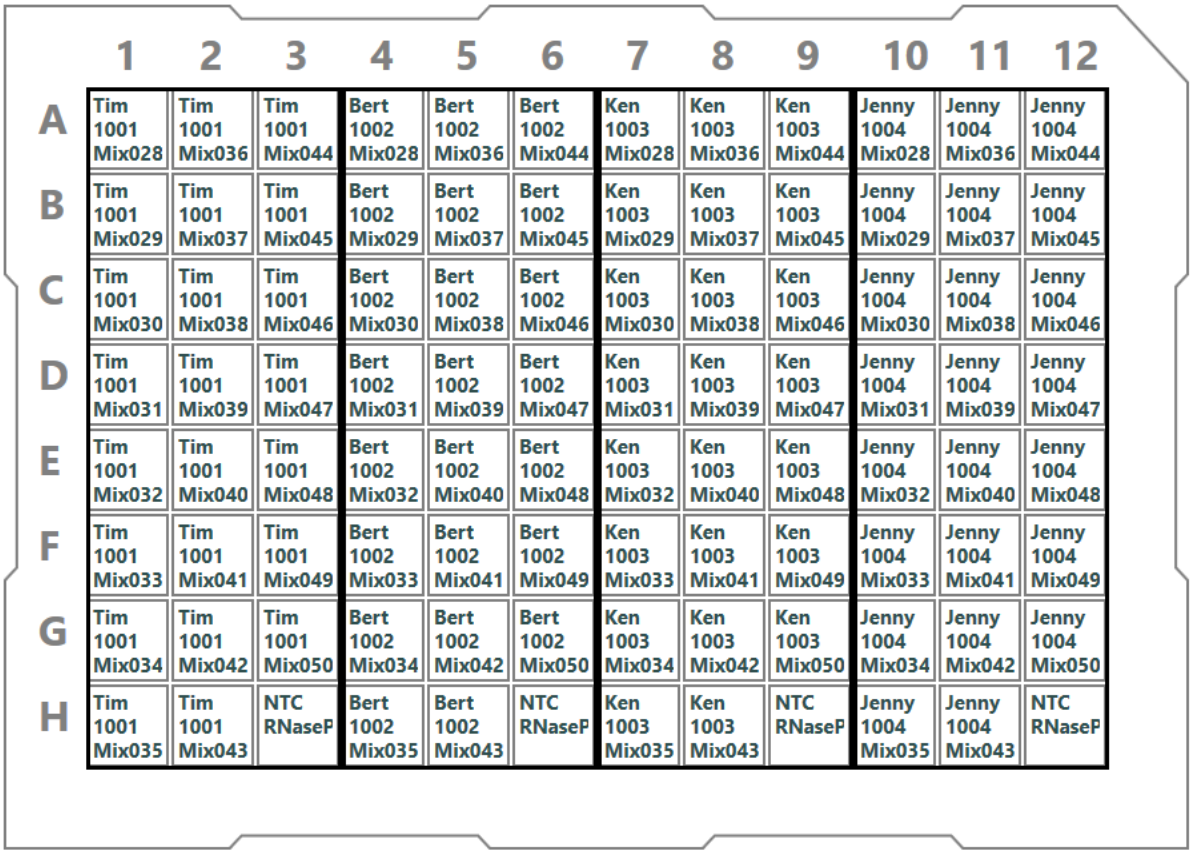
Genotyping Test Protocol - Biorad

Change the instrument type in the Preferences of the TRACE Analysis™ Software to Biorad QX-200 and Plate type to MultiTRACE, v3.

To generate a new record in TRACE Analysis™ Software, in the Section labeled “Recipient” enter the Recipient Name, Recipient Identifier and a unique Sample Identifier. While “Date of Birth” is an optional field for all samples, a “Date of Transplant” must be entered, if you ultimately want to have the data stored and reported in a temporal manner.



For a sample which should be genotyped against the Recipient sample, enter the Donor Name, Donor Identifier and unique Sample Identifier. You can comparatively genotype up to four samples on a single plate using TRACE Analysis™ Software. TRACE Analysis™ Software also allows you to virtually compare multiple samples, independent of when they were genotyped. Based on the data from verification studies, JETA Molecular recommends the use of 10 ng DNA input per well for genotyping. (DNA inputs are customized in the software's Preferences for Concentrations). Enter the concentrations of your samples to let the software calculate with.
Once all samples to be comparatively genotyped are entered into the Sample window, press the “Screen” button to add the samples to the plate set up file. You will see the samples now added to the 96-well plate in the middle of the screen.



	1	2	3	4	5	6	7	8	9	10	11	12
A	Tim 1001 Mix028	Tim 1001 Mix036	Tim 1001 Mix044	Bert 1002 Mix028	Bert 1002 Mix036	Bert 1002 Mix044	Ken 1003 Mix028	Ken 1003 Mix036	Ken 1003 Mix044	Jenny 1004 Mix028	Jenny 1004 Mix036	Jenny 1004 Mix044
B	Tim 1001 Mix029	Tim 1001 Mix037	Tim 1001 Mix045	Bert 1002 Mix029	Bert 1002 Mix037	Bert 1002 Mix045	Ken 1003 Mix029	Ken 1003 Mix037	Ken 1003 Mix045	Jenny 1004 Mix029	Jenny 1004 Mix037	Jenny 1004 Mix045
C	Tim 1001 Mix030	Tim 1001 Mix038	Tim 1001 Mix046	Bert 1002 Mix030	Bert 1002 Mix038	Bert 1002 Mix046	Ken 1003 Mix030	Ken 1003 Mix038	Ken 1003 Mix046	Jenny 1004 Mix030	Jenny 1004 Mix038	Jenny 1004 Mix046
D	Tim 1001 Mix031	Tim 1001 Mix039	Tim 1001 Mix047	Bert 1002 Mix031	Bert 1002 Mix039	Bert 1002 Mix047	Ken 1003 Mix031	Ken 1003 Mix039	Ken 1003 Mix047	Jenny 1004 Mix031	Jenny 1004 Mix039	Jenny 1004 Mix047
E	Tim 1001 Mix032	Tim 1001 Mix040	Tim 1001 Mix048	Bert 1002 Mix032	Bert 1002 Mix040	Bert 1002 Mix048	Ken 1003 Mix032	Ken 1003 Mix040	Ken 1003 Mix048	Jenny 1004 Mix032	Jenny 1004 Mix040	Jenny 1004 Mix048
F	Tim 1001 Mix033	Tim 1001 Mix041	Tim 1001 Mix049	Bert 1002 Mix033	Bert 1002 Mix041	Bert 1002 Mix049	Ken 1003 Mix033	Ken 1003 Mix041	Ken 1003 Mix049	Jenny 1004 Mix033	Jenny 1004 Mix041	Jenny 1004 Mix049
G	Tim 1001 Mix034	Tim 1001 Mix042	Tim 1001 Mix050	Bert 1002 Mix034	Bert 1002 Mix042	Bert 1002 Mix050	Ken 1003 Mix034	Ken 1003 Mix042	Ken 1003 Mix050	Jenny 1004 Mix034	Jenny 1004 Mix042	Jenny 1004 Mix050
H	Tim 1001 Mix035	Tim 1001 Mix043	NTC RNaseP	Bert 1002 Mix035	Bert 1002 Mix043	NTC RNaseP	Ken 1003 Mix035	Ken 1003 Mix043	NTC RNaseP	Jenny 1004 Mix035	Jenny 1004 Mix043	NTC RNaseP

Once the samples have been added virtually to your plate, and the experiment name have been entered, press the “Export Setup to PCR” button.

Browse to the location where you want the file saved and name it as you wish. This file can then be imported into your dPCR machine’s DigitalTRACE template to execute the dPCR analysis.
Once the file is saved, TRACE Analysis™ Software generates a protocol, based on the experimental inputs and the settings in the preferences menu. Print out this protocol.

1. **Set up all reactions in a pre-PCR lab, under ambient conditions without ice.**
2. Open a MultiTRACE® Genotyping Plate Pack and remove the genotyping plate.
3. Label the genotyping plate with the genotyping test name.
4. Collect the four DNA samples to be screened, as well as 2x ddPCR Supermix Master Mix and de-ionized H_2O. Briefly vortex and centrifuge all tubes before opening.
5. Label five 1.5 ml microcentrifuge tubes:
    a) Sample 1 Mix
    b) Sample 2 Mix
    c) Sample 3 Mix
    d) Sample 4 Mix
    e) NTC Mix
6. For each sample to be genotyped, prepare a ddPCR Mix containing sample DNA, de-ionized H_2O and 2x ddPCR Supermix as suggested by TRACE Analysis™ Software in Table 1. A No Template Control (NTC) Mix is prepared with de-ionized H_2O and 2x ddPCR Supermix.

Table 1. Master Mix Composition

Sample 1 dPCR Mix	1 x	27 x
2x ddPCR Supermix	11,0 µl	297,0 µl
1001 DNA	0,5 µl	13,5 µl
H_2O	10,5 µl	283,5 µl
	22,0 µl	594,0 µl

Sample 2 dPCR Mix	1 x	27 x
2x ddPCR Supermix	11,0 µl	297,0 µl
1002 DNA	0,5 µl	13,5 µl
H_2O	10,5 µl	283,5 µl
	22,0 µl	594,0 µl

Sample 3 dPCR Mix	1 x	27 x
2x ddPCR Supermix	11,0 µl	297,0 µl
1003 DNA	0,5 µl	13,5 µl
H_2O	10,5 µl	283,5 µl
	22,0 µl	594,0 µl

Sample 4 dPCR Mix	1 x	27 x
2x ddPCR Supermix	11,0 µl	297,0 µl
1004 DNA	0,5 µl	13,5 µl
H_2O	10,5 µl	283,5 µl
	22,0 µl	594,0 µl

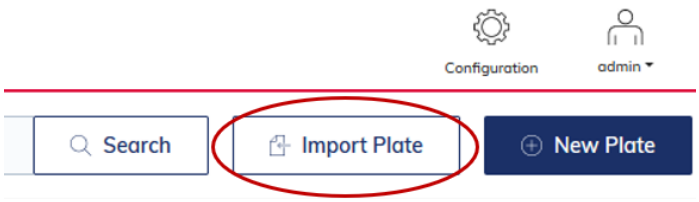
NTC dPCR Mix	1 x	5 x

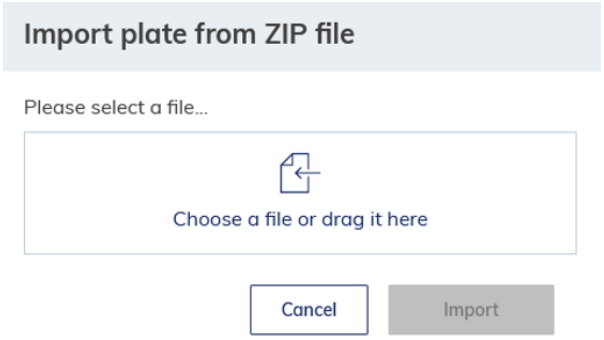
2x ddPCR Supermix	11,0 µl	55,0 µl
H_2O	11,0 µl	55,0 µl
	22,0 µl	110,0 µl

7. Vortex each tube to thoroughly mix the contents and centrifuge briefly to collect the reaction mix at the bottom of the tube.
8. Remove the adhesive cover from the genotyping plate.
9. Dispense 22 µl of the Sample 1 Mix into Wells A1-G3 by columns of the genotyping plate.
10. Dispense 22 µl of the Sample 2 Mix into Wells A4-G6 by columns of the genotyping plate.
11. Dispense 22 µl of the Sample 3 Mix into Wells A7-G9 by columns of the genotyping plate.
12. Dispense 22 µl of the Sample 4 Mix into Wells A10-G12 by columns of the genotyping plate.
13. Dispense 22 µl of the 5X PCR Master Mix/NTC mixture to wells H3, H6, H9, H12.
14. **A repeat pipettor is recommended to minimize pipetting repetition and increase accuracy.**
15. Refer to the DNA Sample Layout Plate Layout at the end of the protocol.
16. Visually inspect plate wells from the sides and bottom to confirm consistent volume.
17. Seal the plate completely with MicroAmp® Optical Adhesive Film using the MicroAmp® Adhesive Film Applicator.
18. **IMPORTANT! Vortex the plate to mix the contents of each well.**Centrifuge the plates briefly using a plate centrifuge to collect the contents at the bottom of the wells.
19. **Remove the Adhesive cover very carefully.**
20. **!!!The following steps from 21 to 26 are only for manual droplet generator users:**
21. Transfer 20 µl of each prepared sample to the sample wells (middle row) of the DG8 cartridge.
22. Add 70 µl of droplet generation oil to each oil well of the DG8 cartridge.
23. Hook the gasket over the cartridge holder using the holes on both sides.
24. Load the cartridge in the QX200 droplet generator.
25. When droplet generation is complete, remove the disposable gasket from the holder and discard it.
26. Pipet 40 µl of the contents of the droplets into a single column of a 96-well PCR plate.
27. Seal the PCR plate with foil plate seals that are compatible with the PX1 PCR plate sealer and the needles in the QX200 droplet reader.
28. Place the plate into the thermal cycler for PCR amplification.
29. Load the plate after amplification into QX200 droplet reader.
30. Import the Sample Setup sheet generated by TRACE Analysis™ Software.
31. Save the file and start the droplet reader.

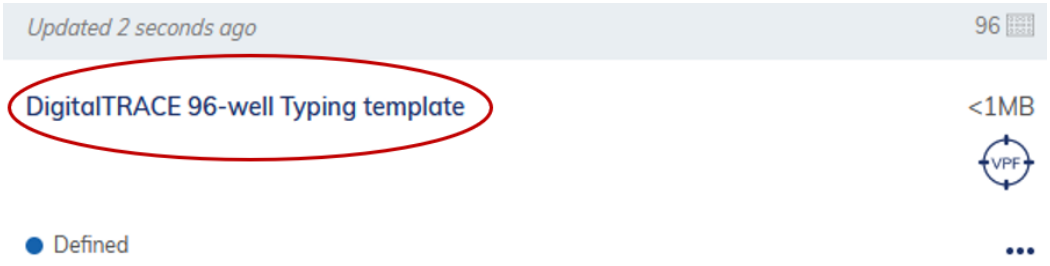
Experiment Setup in QIAcuity Suite Software

Create a new QIAcuity Plate by selecting New Plate.
Load a template by selecting Plate templates and import the appropriate DigitalTRACE Genotyping template



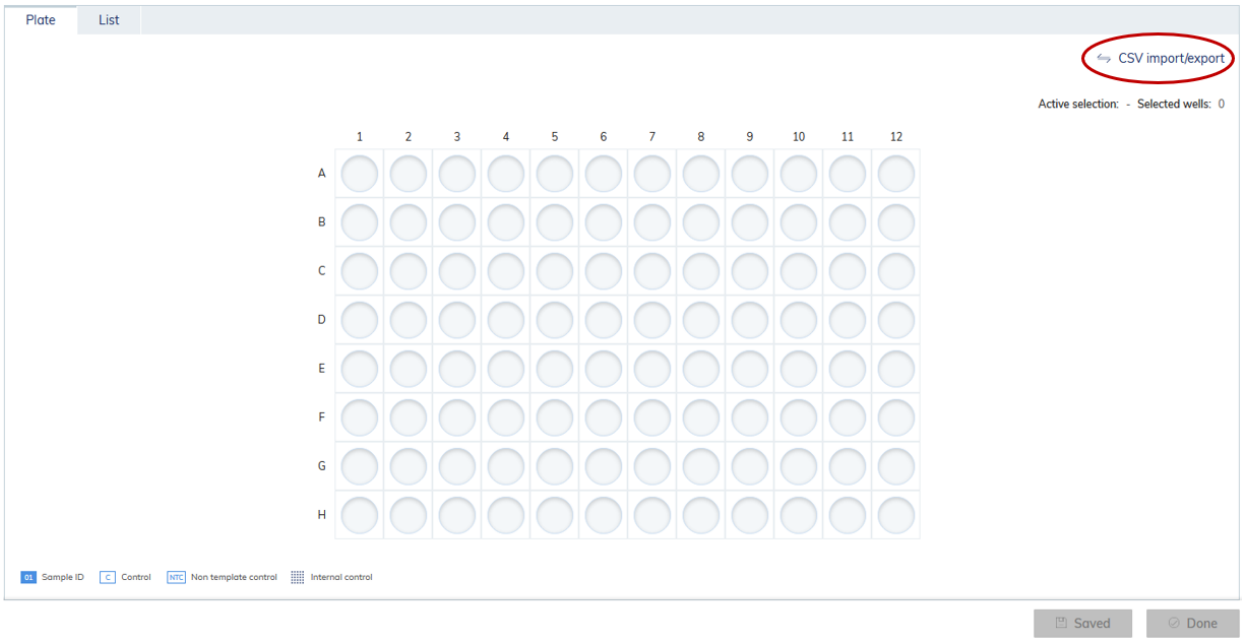


Press Save Plate. The new plate will appear in the main window of the QIAcuity Software Suite.

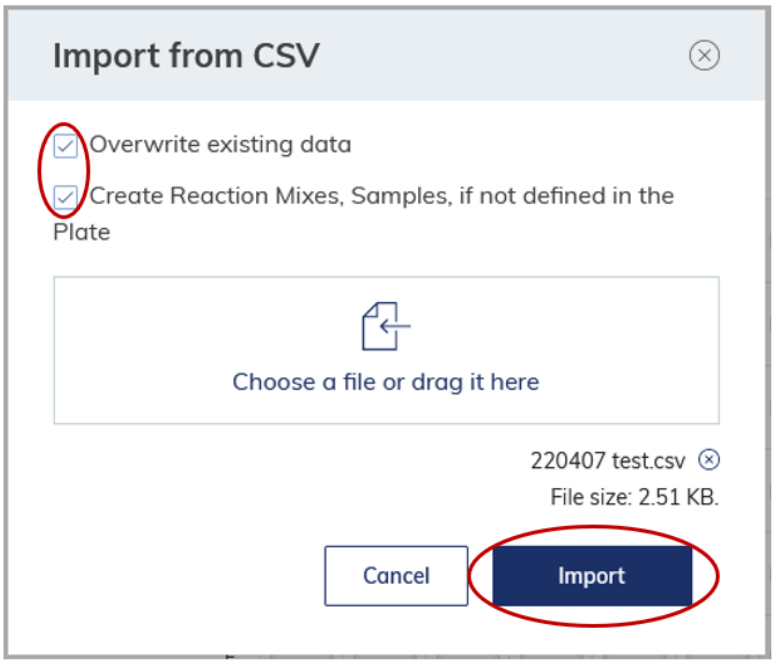


Click on the plate name to open the plate configuration procedure. Type in a new plate name.

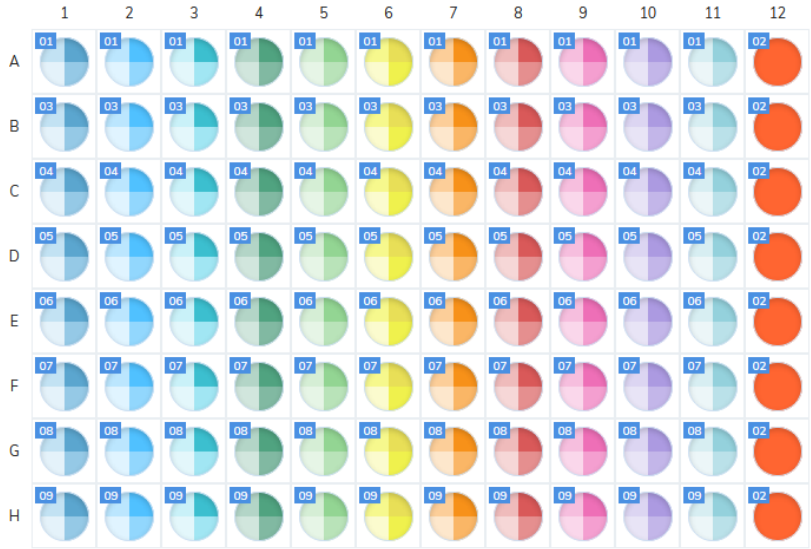
Import the Sample Setup sheet (.csv) generated by TRACE Analysis™ Software by selecting Plate layout tab and CSV import/export.



When importing the plate setup, select both options, "Overwrite existing data" and "Create Reaction Mixes, Samples if not defined in the Plate".



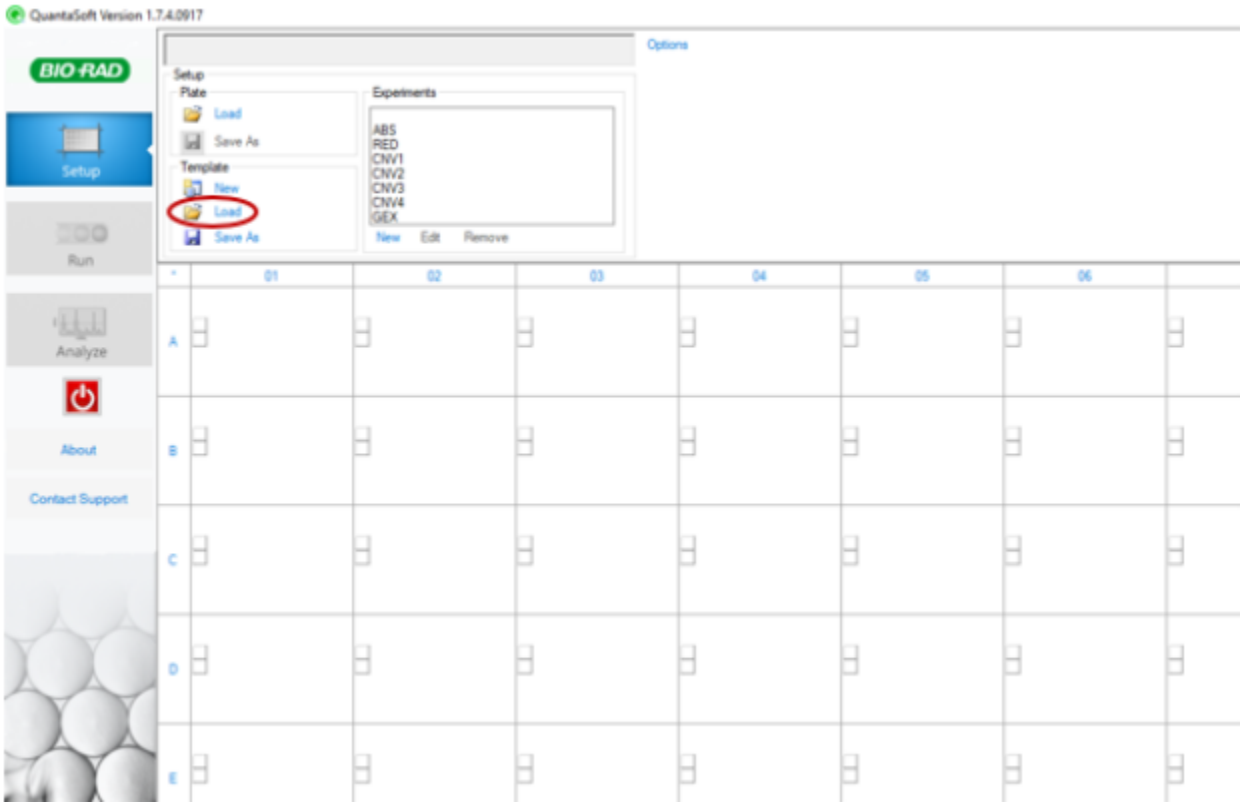
Inspect if all your selected samples are represented on the Plate figure.



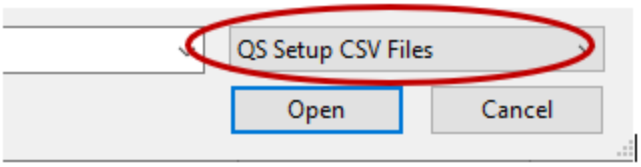
Select Done to finish the setup.
Start the run.

Biorad Droplet Reader Setup in QuantaSoft

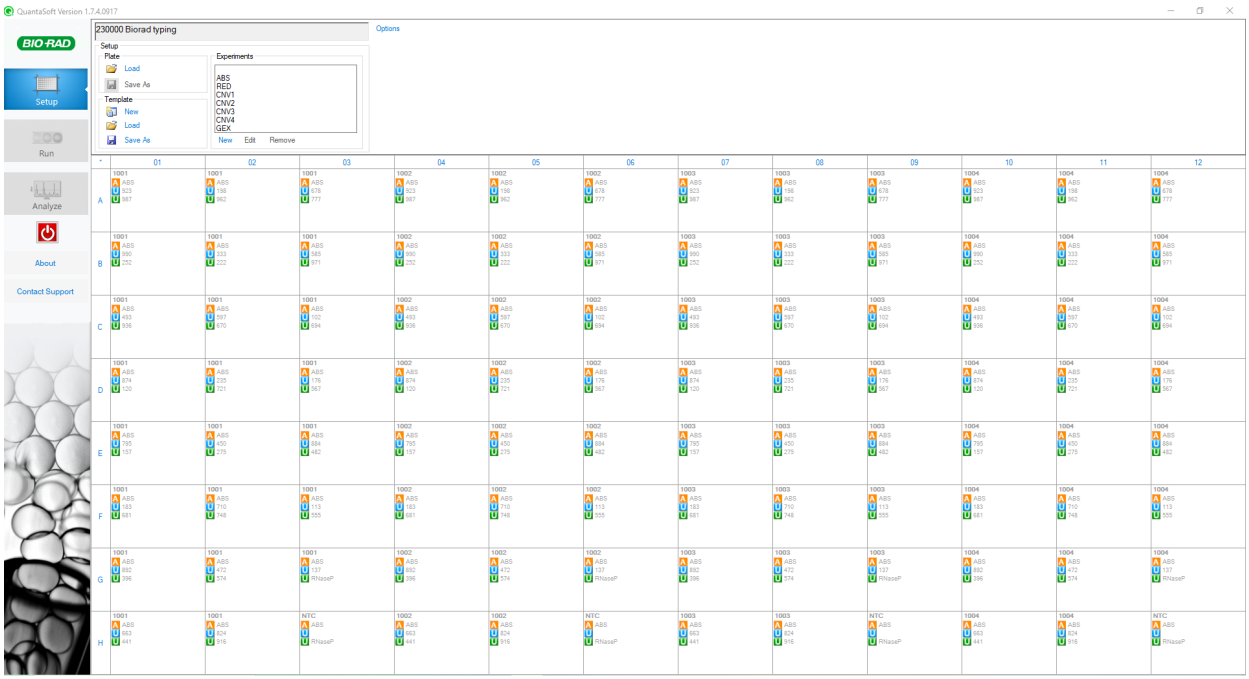
To read the signal after the PCR cycling was completed, setup an experiment in QuantaSoft.
Load a template by clicking on Load



Select QS Setup CSV Files as file format



Import the appropriate DigitalTRACE Genotyping template



Start the droplet reading run.

Thermal Cycling and imaging Protocol for DigitalTRACE™ Products

The DigitalTRACE™ System will deliver optimal results when the following thermal profile is used in dPCR.

Thermal cycling and imaging protocol - QIAcuity

Number of cycles	Temperature	Time
1	95 °C	3 min
40	95 °C	15 s
	60 °C	30 s

Use the following imaging parameters:

Channel	Exposure duration	Gain
Green	500 ms	6
Yellow	500 ms	6
Orange	Off	Off
Red	300 ms	4
Crimson	400 ms	4

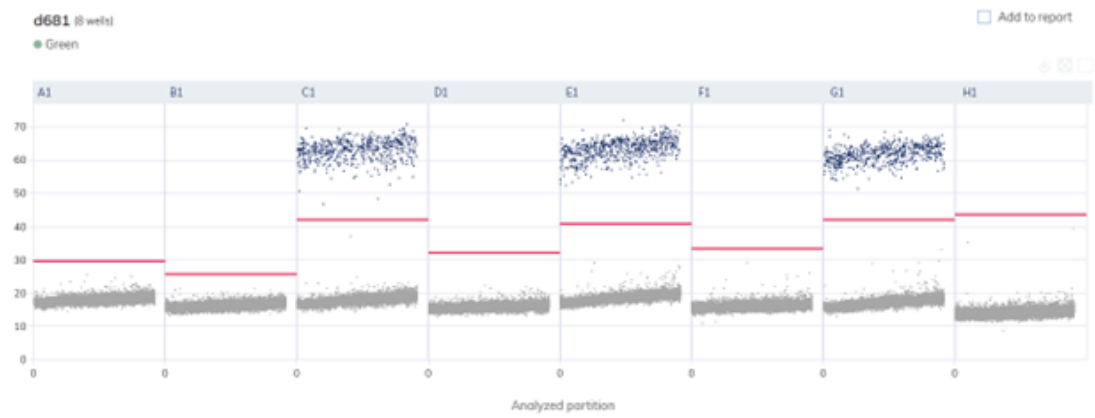
Thermal cycling protocol - Biorad

Number of cycles	Temperature	Time	F
1	95 °C	10 min	
40	94 °C	30 s	
	59 °C	60 s	
1	98 °C	10 min	

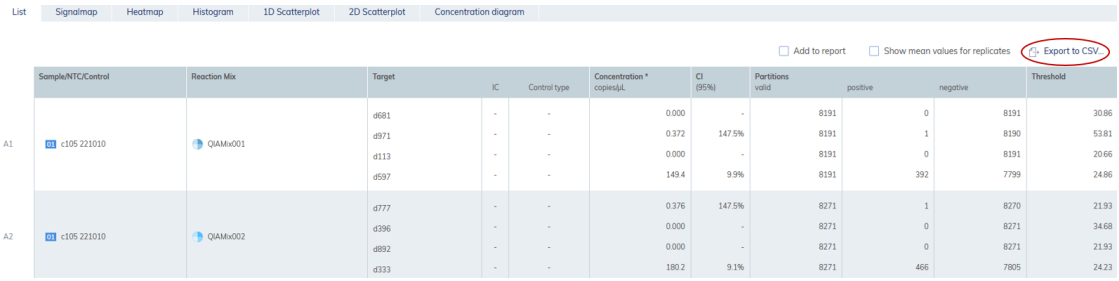
Genotyping Data Analysis and Report

Genotyping Data Analysis and Report - QIAcuity

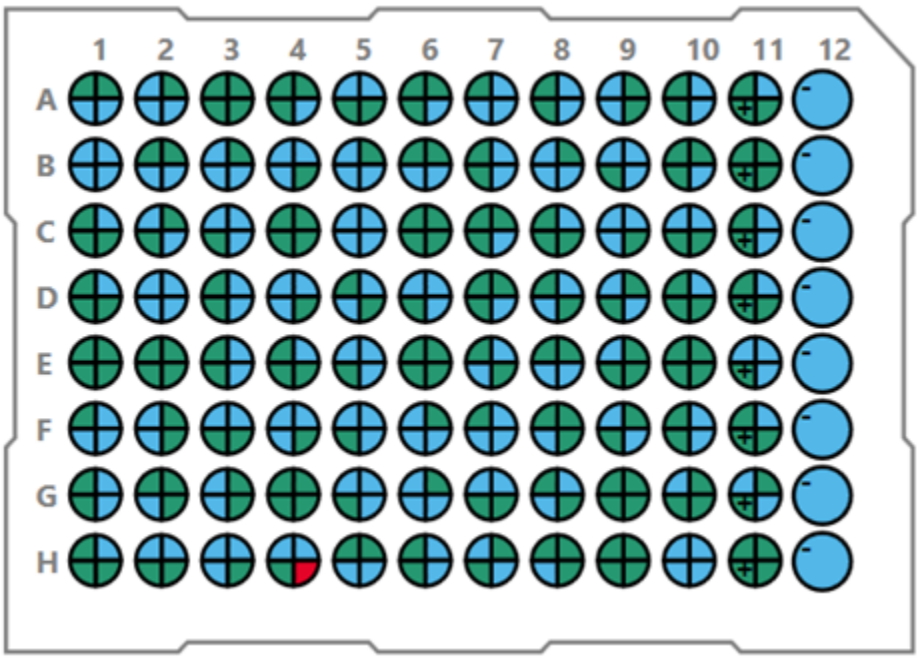
After the QIAcuity run has finished, check in the QIAcuity Software Suite if the automatic thresholds are correct.



Select all wells on the plate and analyze per target (not per channel), export data by selecting Export to CSV.



Import dPCR data to TRACE Analysis™ Software by clicking the “Import PCR Data”  button.
Browse to the location of your exported dPCR data file and select it.
TRACE Analysis™ Software will perform a quality analysis on the data and will present the data in the plate view.
There are three quality scores given to genotyping data: 1) positive (green), 2) negative (blue) and 3) atypical (red).
These values are represented accordingly in the plate image by three different colors:



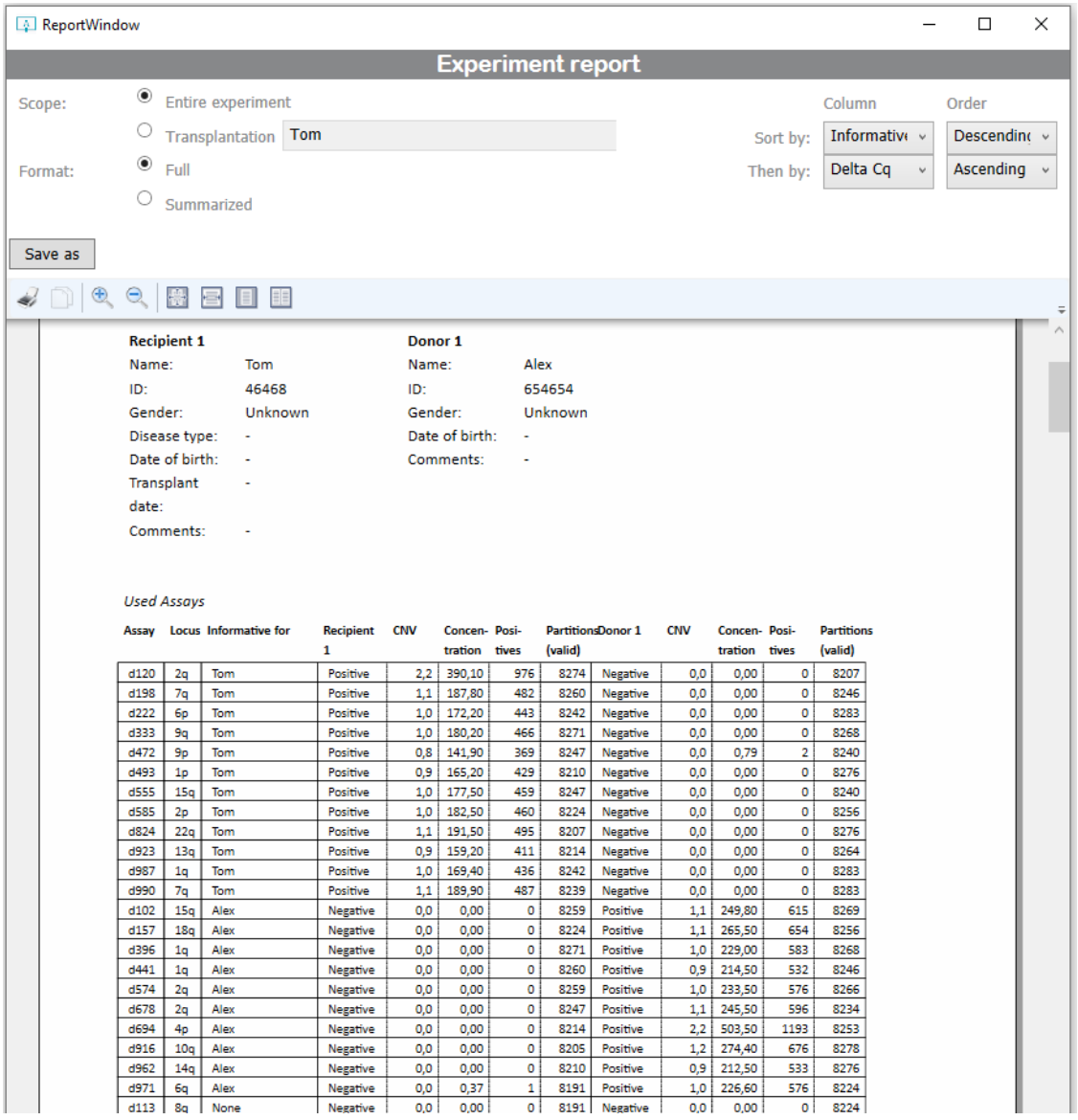
An atypical assay results will exclude the assay from consideration as a potentially informative assay for all samples grouped in the analysis.

After inspecting the quality of the data, pressing the “Calculate” Calculate button makes TRACE Analysis™ perform comparative genotyping analysis. It will determine and display markers which are informative for all samples in a group.

Press the "Report" Report... button to generate the Genotyping Report

TRACE Analysis™ Software displays each assay which was informative for a single sample in the group being compared, and it also displays the chromosomal location of the informative assays, as well as the positive or negative status of the assays for visual inspection.

The report generated from a TRACE Analysis™ Genotyping experiment may be sorted to provide a custom view of the data.



Recipient 1

Name:	Tom
ID:	46468
Gender:	Unknown
Disease type:	-
Date of birth:	-
Transplant date:	-
Comments:	-

Donor 1

Name:	Alex
ID:	654654
Gender:	Unknown
Date of birth:	-
Comments:	-

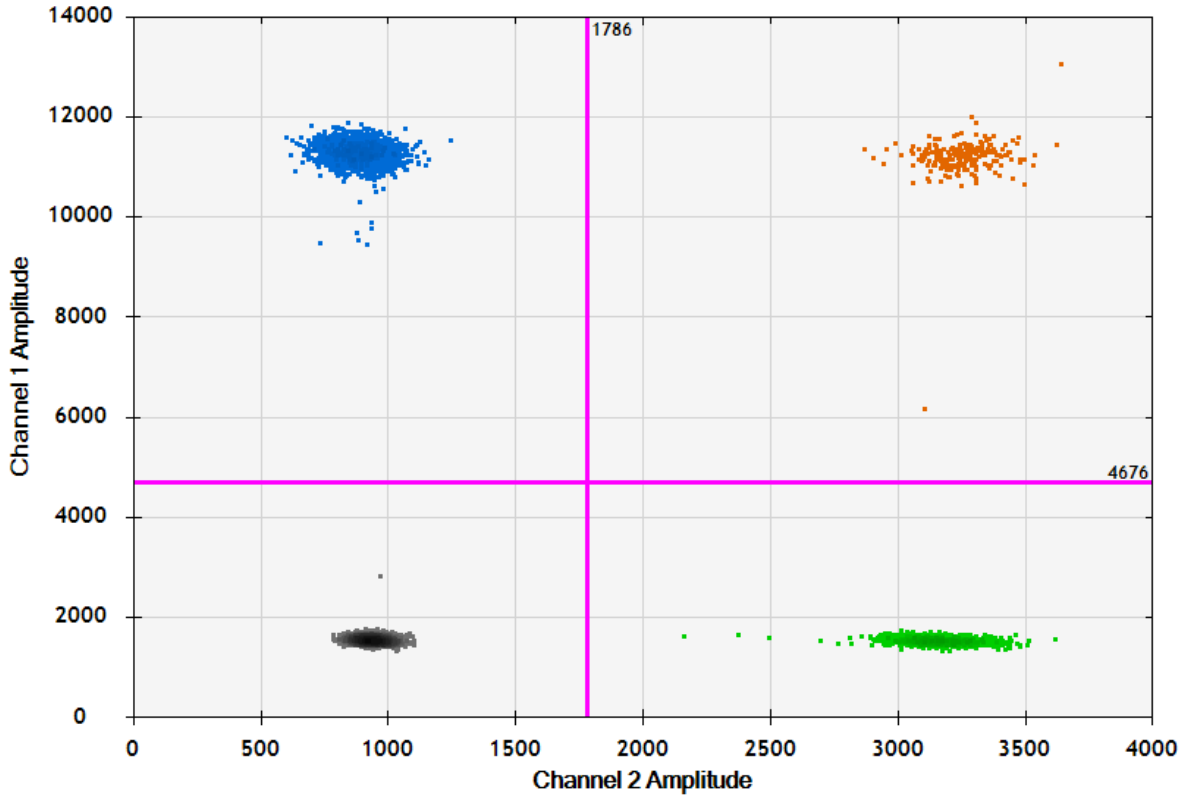
Used Assays

Assay	Locus	Informative for	Recipient 1	CNV	Concen-tration	Posi-tives	Partitions (valid)	Donor 1	CNV	Concen-tration	Posi-tives	Partitions (valid)
d120	2q	Tom	Positive	2,2	390,10	976	8274	Negative	0,0	0,00	0	8207
d198	7q	Tom	Positive	1,1	187,80	482	8260	Negative	0,0	0,00	0	8246
d222	6p	Tom	Positive	1,0	172,20	443	8242	Negative	0,0	0,00	0	8283
d333	9q	Tom	Positive	1,0	180,20	466	8271	Negative	0,0	0,00	0	8268
d472	9p	Tom	Positive	0,8	141,90	369	8247	Negative	0,0	0,79	2	8240
d493	1p	Tom	Positive	0,9	165,20	429	8210	Negative	0,0	0,00	0	8276
d555	15q	Tom	Positive	1,0	177,50	459	8247	Negative	0,0	0,00	0	8240
d585	2p	Tom	Positive	1,0	182,50	460	8224	Negative	0,0	0,00	0	8256
d824	22q	Tom	Positive	1,1	191,50	495	8207	Negative	0,0	0,00	0	8276
d923	13q	Tom	Positive	0,9	159,20	411	8214	Negative	0,0	0,00	0	8264
d987	1q	Tom	Positive	1,0	169,40	436	8242	Negative	0,0	0,00	0	8283
d990	7q	Tom	Positive	1,1	189,90	487	8239	Negative	0,0	0,00	0	8283
d102	15q	Alex	Negative	0,0	0,00	0	8259	Positive	1,1	249,80	615	8269
d157	18q	Alex	Negative	0,0	0,00	0	8224	Positive	1,1	265,50	654	8256
d396	1q	Alex	Negative	0,0	0,00	0	8271	Positive	1,0	229,00	583	8268
d441	1q	Alex	Negative	0,0	0,00	0	8260	Positive	0,9	214,50	532	8246
d574	2q	Alex	Negative	0,0	0,00	0	8259	Positive	1,0	233,50	576	8266
d678	2q	Alex	Negative	0,0	0,00	0	8247	Positive	1,1	245,50	596	8234
d694	4p	Alex	Negative	0,0	0,00	0	8214	Positive	2,2	503,50	1193	8253
d916	10q	Alex	Negative	0,0	0,00	0	8205	Positive	1,2	274,40	676	8278
d962	14q	Alex	Negative	0,0	0,00	0	8210	Positive	0,9	212,50	533	8276
d971	6q	Alex	Negative	0,0	0,37	1	8191	Positive	1,0	226,60	576	8224
d113	8q	None	Negative	0,0	0,00	0	8191	Negative	0,0	0,00	0	8224

To create an anonymized report, go to the Anonymized Reporting Section.

Genotyping Data Analysis and Report - Biorad

After the reading process has finished, check in the QuantaSoft Software if the automatic thresholds are correct.



Select all wells on the plate and export data by selecting Export CSV.

Import dPCR data to TRACE Analysis™ Software by clicking the “Import PCR Data” button.
Browse to the location of your exported dPCR data file and select it.
TRACE Analysis™ Software will perform a quality analysis on the data and will present the data in the plate view.
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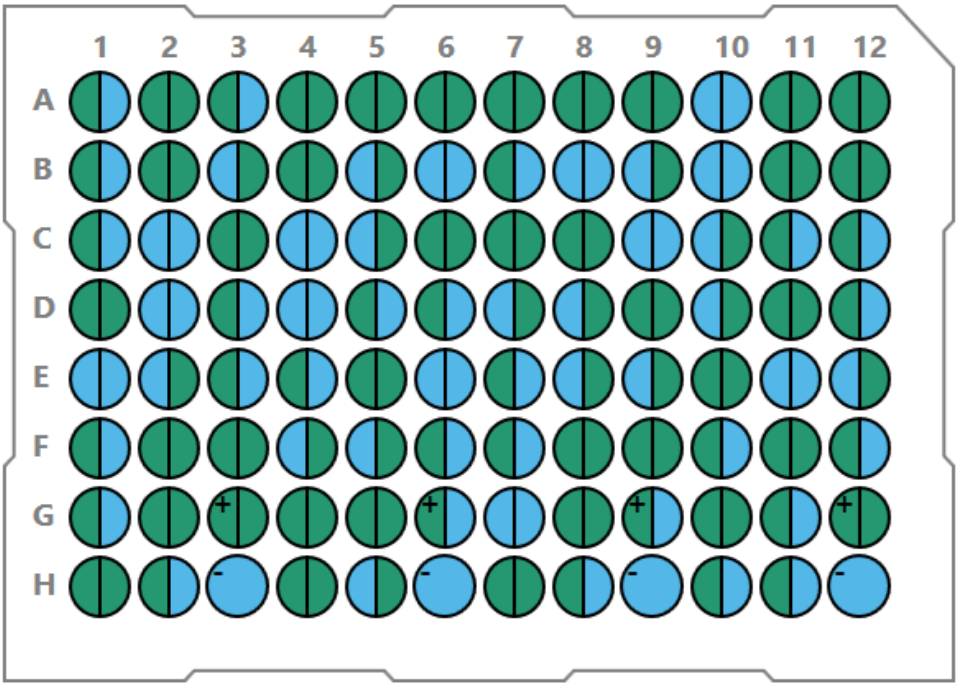
These values are represented accordingly in the plate image by three different colors:



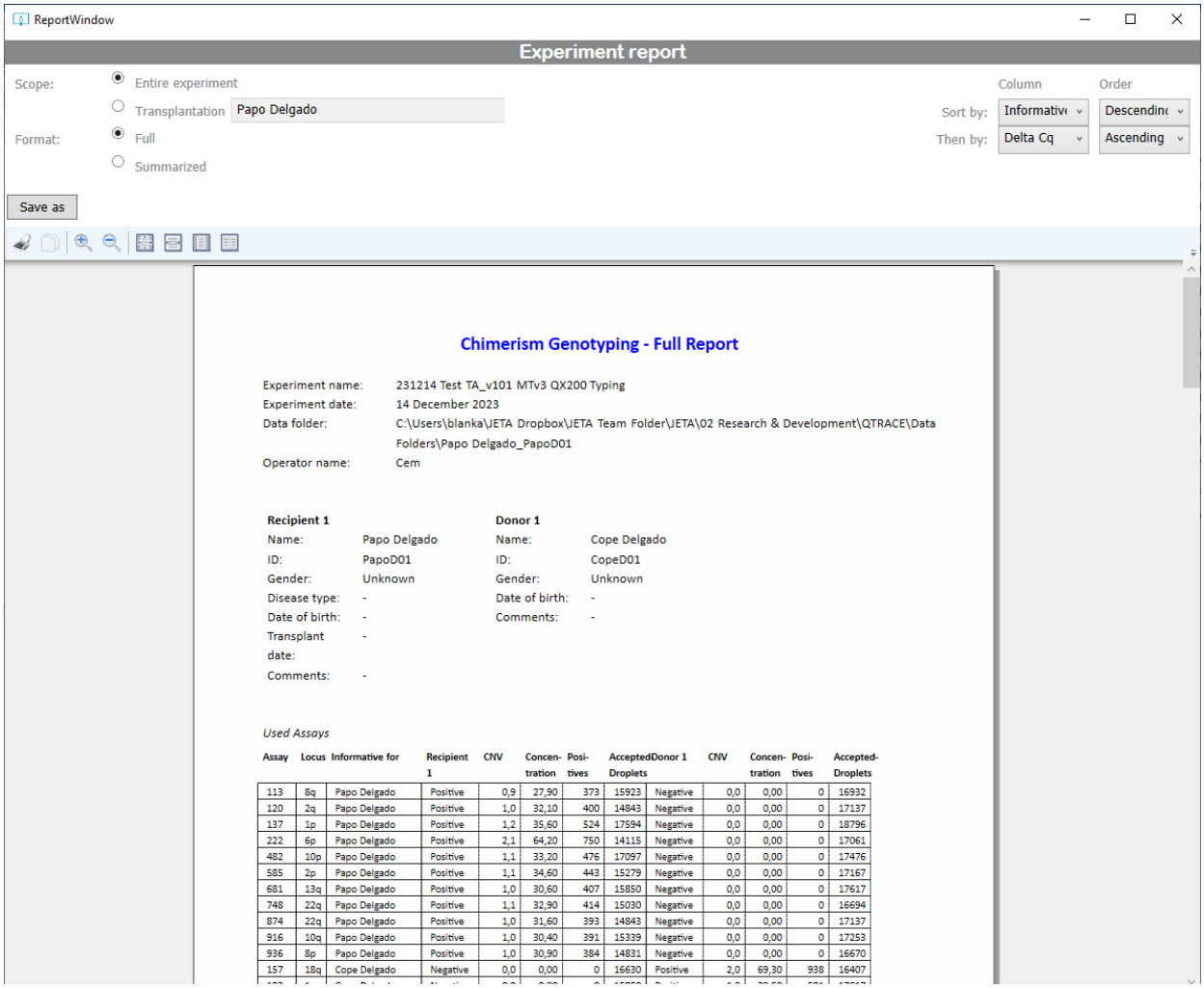
An atypical assay results will exclude the assay from consideration as a potentially informative assay for all samples grouped in the analysis.

After inspecting the quality of the data, pressing the “Calculate” Calculate button makes TRACE Analysis™ perform comparative genotyping analysis. It will determine and display markers which are informative for all samples in a group.

Press the "Report" Report... button to generate the Genotyping Report

TRACE Analysis™ Software displays each assay which was informative for a single sample in the group being compared, and it also displays the chromosomal location of the informative assays, as well as the positive or negative status of the assays for visual inspection.

The report generated from a TRACE Analysis™ Genotyping experiment may be sorted to provide a custom view of the data.



Assay	Locus	Informative for	Recipient 1	CNV	Concentration	Positives	Accepted Droplets	Donor 1	CNV	Concentration	Positives	Accepted Droplets
113	8q	Papo Delgado	Positive	0,9	27,90	373	15923	Negative	0,0	0,00	0	16932
120	2q	Papo Delgado	Positive	1,0	32,10	400	14843	Negative	0,0	0,00	0	17137
137	1p	Papo Delgado	Positive	1,2	35,60	524	17594	Negative	0,0	0,00	0	18796
222	6p	Papo Delgado	Positive	2,1	64,20	750	14115	Negative	0,0	0,00	0	17061
482	10p	Papo Delgado	Positive	1,1	33,20	476	17097	Negative	0,0	0,00	0	17476
585	2p	Papo Delgado	Positive	1,1	34,60	443	15279	Negative	0,0	0,00	0	17167
681	13q	Papo Delgado	Positive	1,0	30,60	407	15850	Negative	0,0	0,00	0	17617
748	22q	Papo Delgado	Positive	1,1	32,90	414	15030	Negative	0,0	0,00	0	16694
874	22q	Papo Delgado	Positive	1,0	31,60	393	14843	Negative	0,0	0,00	0	17137
916	10q	Papo Delgado	Positive	1,0	30,40	391	15339	Negative	0,0	0,00	0	17253
936	8p	Papo Delgado	Positive	1,0	30,90	384	14831	Negative	0,0	0,00	0	16670
157	18q	Cope Delgado	Negative	0,0	0,00	0	16630	Positive	2,0	69,30	938	16407

Monitoring Test

Once recipient specific markers have been found, quantification is performed for the monitoring of chimerism. In the Monitoring test, one or more of the informative assays is used to quantify the DNA of interest in an unknown sample. Any of the informative assays identified in the genotyping can be used to perform monitoring. The fraction of DNA positive for the informative marker in the unknown composition is measured using dPCR.

Monitoring Test Protocol

Monitoring Test Protocol - QIAcuity and Biorad

For Post samples wherein genotyping data was either determined using qPCR or not determined using TRACE Analysis™ Software, please see section on Assigning Informative Assays.

In order to perform a Monitoring experiment with TRACE Analysis™ Software using both the QIAcuity or Biorad dPCR instrument, select the name of your Recipient Sample in the Sample Entry window from the drop-down menu.

Once the Recipient Sample name is found and selected, choose from a pop-up window to use monitoring plate format which must be selected before in the Preferences. The selected sample's identifying information initially entered will appear.

Type in the Post sample information:

A unique Sample ID must be entered in the Sample ID field.

A sample Date must be entered.

Check the Sample type which is being tested - Sample Types can be defined by the User in the Preferences menu.

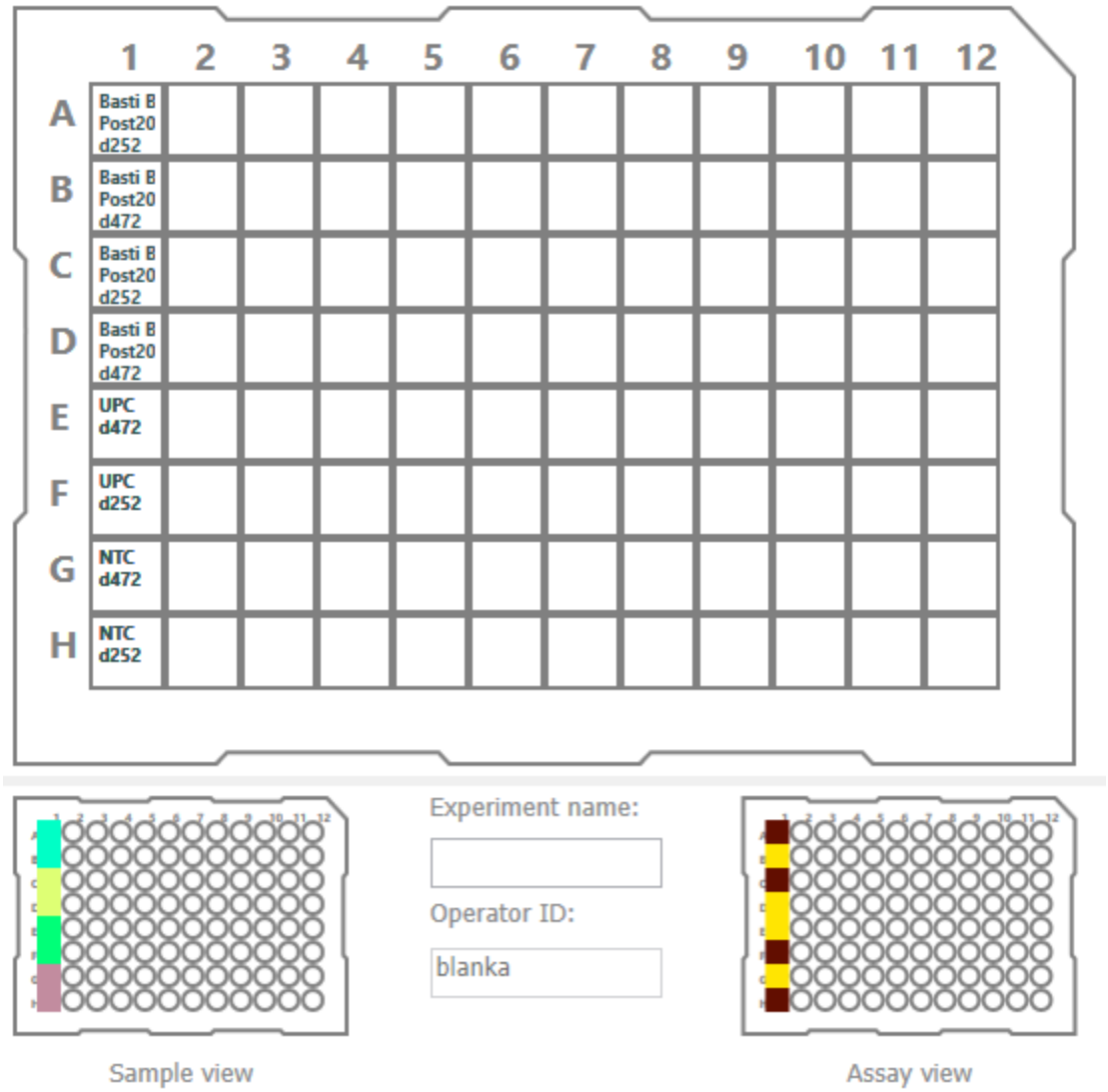
Click the plus sign next to the "Add Sample" tab

In the Informative Assays window, choose the loci to be tested by selecting assays from the list based on the screening test results.

A positive control (UPC) and a negative control (NTC) is automatically added to the plate layout.

You can toggle between coloring for the Sample View and the Assay View by pressing the appropriate small plate at the bottom of the window. One View shows the placement of monitoring and control samples. The other view shows the placement of the Assays.

Monitoring Samples are arranged into groups by TRACE Analysis™ Software, based on the Preferences set for the monitoring machine. You can drag the wells to rearrange the samples within the plate. In this case, both controls were placed to the first column.



Proceed to add more samples to fill the plate as per your needs.

Once all samples have been added to your plate, press the black “Export Setup to PCR” button.
Browse to the location where you want the file saved and name it as you wish. This file can then be imported into your dPCR machine’s template file to execute the dPCR analysis.

Once the file is saved, TRACE Analysis™ Software generates a protocol, based on the experimental inputs and the settings in the preferences menu.

The following protocol is an example output from TRACE Analysis™ Software for a Monitoring test using QIAcuity dPCR:

1. **Set up all reactions in a pre-PCR lab, under ambient conditions without ice.**
2. Briefly vortex and centrifuge all tubes before opening.
3. Prepare DNA dilutions as specified in Table 1:

Table 1. DNA dilutions

No.	Name	Sample ID	Concentration	Sample Volume	Water Volume	Total Volume	Amount per Reaction
1	Post Sample	Post_test	26 ng/µl	12,69 µl	48,91 µl	61,60 µl	150 ng
2	Post Sample	Post2_test	41 ng/µl	8,05 µl	53,55 µl	61,60 µl	150 ng

4. Prepare for chosen informative dPCR Assay the following mixture:

Table 2. Master Mix + dPCR Assay mixture

Assay Mix	QIAcuity 4x Master Mix	dPCR 20x assay mix [µl]	Total Volume
d359	46,00 µl	9,20 µl	55,20 µl
d626	46,00 µl	9,20 µl	55,20 µl

5. **IMPORTANT! Vortex at least 5 seconds and spin briefly each prepared Master Mix + dPCR Assay mixture.**
6. Deliver 13,2 µl of QIAcuity Probe Master Mix + dPCR Assay Mix to a 96-well plate as defined in TRACE Analysis™ Software's Assay Layout view.
7. Add 30,8 µl of each Sample DNA dilution as indicated by TRACE Analysis™ Software's Layout View. Where NTC and UPC are indicated add 30,8 µl water for NTC and 30,8 µl from Universal Positive Control for UPC.
8. **IMPORTANT! Vortex at least 15 seconds the 96-well plate to mix the contents of each reaction. Centrifuge the 96-well plate briefly using a microcentrifuge.**
9. Transfer 40 µl of each prepared reaction mix into a Nanoplate. Seal the Nanoplate with the compatible plate sealer.
10. Load the Nanoplate into the QIAcuity digital PCR system.
11. Launch the QIAcuity Software Suite.
12. Open the DigitalTRACE template and import the Sample Setup sheet generated by TRACE Analysis™ Software.
13. Save the file and start the run.

Set up the experiment in the QIAcuity Software Suite like a Genotyping test by using the same cycling parameters and use the following imaging parameters:

Number of cycles	Temperature	Time
1	95 °C	3 min
40	95 °C	15 s
	60 °C	30 s

Channel	Exposure duration	Gain
Green	500 ms	6
Yellow	500 ms	6
Orange	Off	Off
Red	Off	Off
Crimson	Off	Off

Then load a template by selecting Plate templates and import the appropriate DigitalTRACE Monitoring template. Press Save Plate.

Import the Sample Setup sheet (.csv) generated by TRACE Analysis™ Software by selecting Plate layout tab and CSV import/export.
Import plate setup and select both options, Overwrite existing data and Create Reaction Mixes, Samples if not defined in the Plate.
Inspect if all your selected samples are represented on the Plate figure. Select Done to finish the setup.
Start the run.

After the QIAcuity run has finished, check in the QIAcuity Software Suite if the automatic thresholds are correct.
Select all wells on the plate and analyze per target (not per channel), export data by selecting Export to CSV.

The following protocol is an example output from TRACE Analysis™ Software for a Monitoring test using Biorad ddPCR:

1. **Set up all reactions in a pre-PCR lab, under ambient conditions without ice.**
2. Briefly vortex and centrifuge all tubes before opening.
3. Prepare DNA dilutions as specified in Table 1:

Table 1. DNA dilutions

No.	Name	Sample ID	Concentration	Sample Volume	Water Volume	Total Volume	Amount per Reaction
1	Basti Bob	Post201	20 ng/µl	17,25 µl	5,52 µl	22,77 µl	150 ng
2	Basti Bob	Post202	20 ng/µl	17,25 µl	5,52 µl	22,77 µl	150 ng

4. Prepare for chosen informative dPCR Assay the following mixture:

Table 2. ddPCR Supermix + dPCR Assay mixture

Assay	ddPCR supermix for probes (no dUTPs) [uL]	dPCR assay [uL]	Total Volume
d252	46,00 µl	4,60 µl	50,60 µl
d472	46,00 µl	4,60 µl	50,60 µl

5. Deliver 12.1 µl of ddPCR Supermix + dPCR Assay mixture to a 8 strip PCR tube as defined in TRACE Analysis™ Software's Assay Layout view.
6. Add 9.9 µl of each Sample DNA dilution and water for NTC wells as indicated by TRACE Analysis™ Software's Layout View.
7. **IMPORTANT! Vortex the 8 strip tube to mix the contents of each reaction. Centrifuge the 8 strip tubes briefly using a microcentrifuge.**
8. **!!!The following steps from 9 to 14 are only for manual droplet generator users:**
9. Transfer 20 µl of each prepared sample to the sample wells (middle row) of the DG8 cartridge.
10. Add 70 µl of droplet generation oil to each oil well of the DG8 cartridge.
11. Hook the gasket over the cartridge holder using the holes on both sides.
12. Load the cartridge in the QX200 droplet generator.
13. When droplet generation is complete, remove the disposable gasket from the holder and discard it.
14. Pipet 40 µl of the contents of the droplets into a single column of a 96-well PCR plate.
15. Seal the PCR plate with foil plate seals that are compatible with the PX1 PCR plate sealer and the needles in the QX200 droplet reader.
16. Place the plate into the thermal cycler for PCR amplification.
17. Load the plate after amplification into QX200 droplet reader.
18. Import the Sample Setup sheet generated by TRACE Analysis™ Software.
19. Save the file and start the droplet reader.

Use the same cycling parameters as for a Genotyping test:

Number of cycles	Temperature	Time	F
1	95 °C	10 min	
40	94 °C	30 s	
	59 °C	60 s	
1	98 °C	10 min	

The droplet reading process is the same as for a Genotyping test.

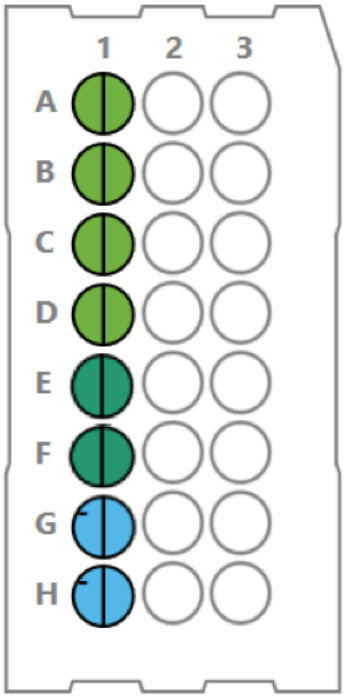
After the reading process has finished, check in the QuantaSoft Software if the automatic thresholds are correct.

Select all wells on the plate and export data by selecting Export CSV.

Monitoring Data Analysis and Report

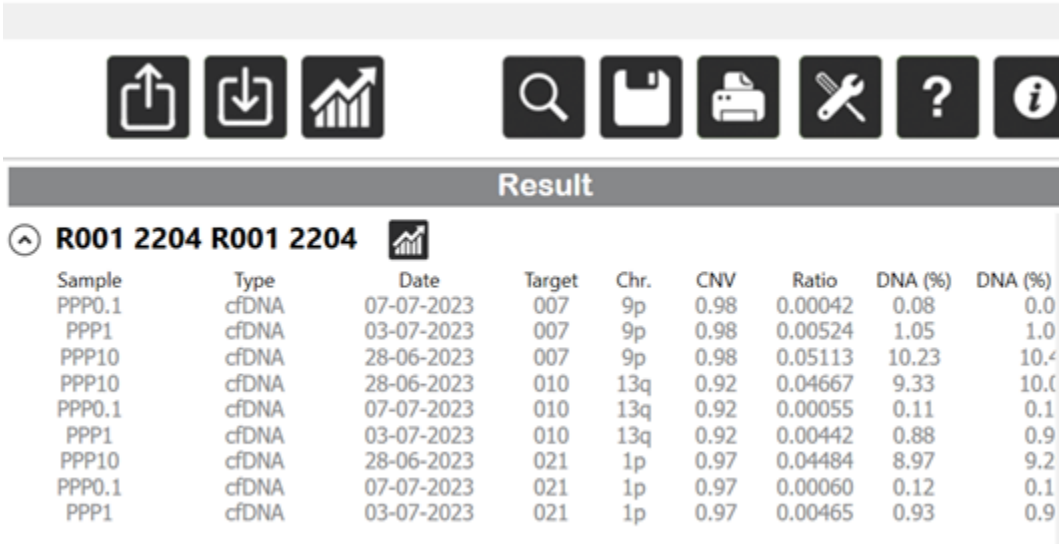
Import dPCR data by clicking the “Import PCR Data” button  , and browse to the location of your exported dPCR data file and select it.

TRACE Analysis™ Software uses a light green/dark green/light blue coding for well highlighting.



Light green wells represent post samples. Dark green wells represent the positive control DNA. Light blue wells represent non-template control (NTC)

The calculations for each sample are displayed in the right-hand Result window.



Result

R001 2204 R001 2204

Sample	Type	Date	Target	Chr.	CNV	Ratio	DNA (%)	DNA (%)
PPP0.1	cfDNA	07-07-2023	007	9p	0.98	0.00042	0.08	0.0
PPP1	cfDNA	03-07-2023	007	9p	0.98	0.00524	1.05	1.0
PPP10	cfDNA	28-06-2023	007	9p	0.98	0.05113	10.23	10.4
PPP10	cfDNA	28-06-2023	010	13q	0.92	0.04667	9.33	10.0
PPP0.1	cfDNA	07-07-2023	010	13q	0.92	0.00055	0.11	0.1
PPP1	cfDNA	03-07-2023	010	13q	0.92	0.00442	0.88	0.9
PPP10	cfDNA	28-06-2023	021	1p	0.97	0.04484	8.97	9.2
PPP0.1	cfDNA	07-07-2023	021	1p	0.97	0.00060	0.12	0.1
PPP1	cfDNA	03-07-2023	021	1p	0.97	0.00465	0.93	0.9

Click on Select assays and then on Report... to generate the Monitoring Report.

As more data is collected for a particular sample over time, TRACE Analysis™ Software provides

a longitudinal view.

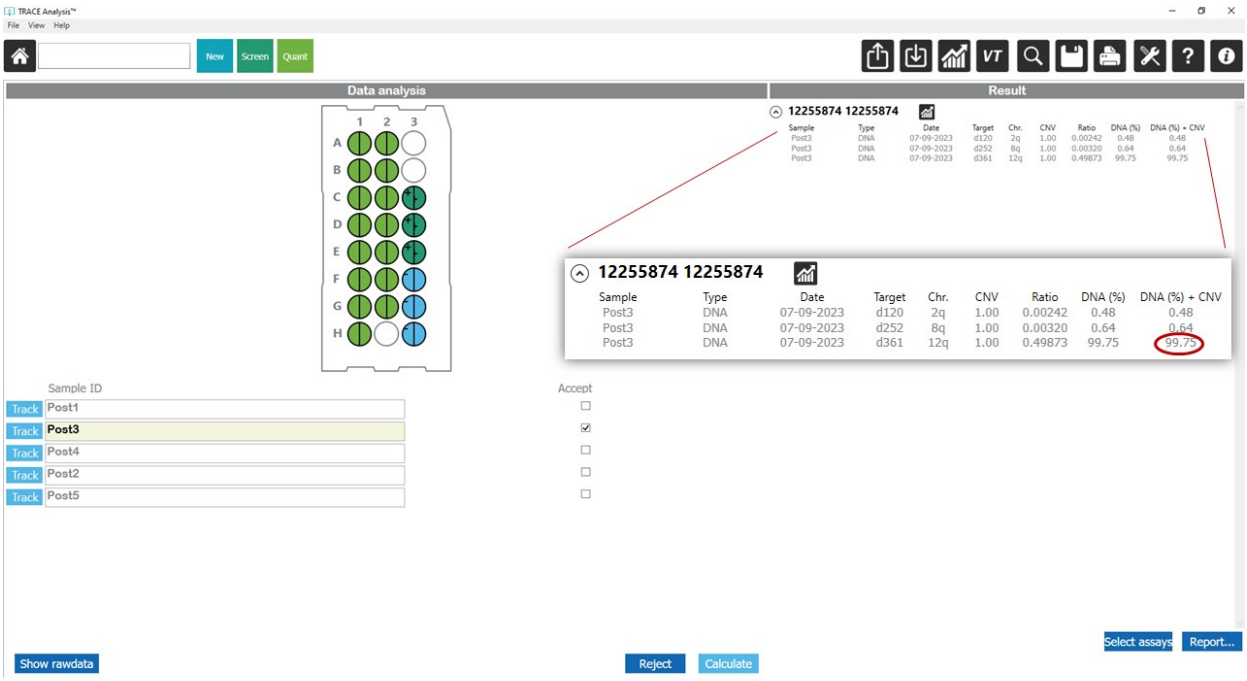
To view the composite set of data for an individual sample, press the “Overview” button 

Activating this action takes you to a screen showing all the information input for a particular group of samples as well as all of their genotyping and monitoring data.

Assay Filtering from Monitoring Reports

Assay Filtering is a feature which allows a user to selectively remove all data from a particular assay in the final report. This may be important, for example, if one pipette tip from a multi-channel pipette did not properly dispense the necessary reagents to a set of reactions. While the data may appear to have good precision - all negatives in this example - it may not be accurate.

In the following image, there is a failure of Assay d361 in the selected sample.



If the plate is approved, under the Results panel, there is 99.75% reported for Assay d361, while the other assays show a result under 0.7%.

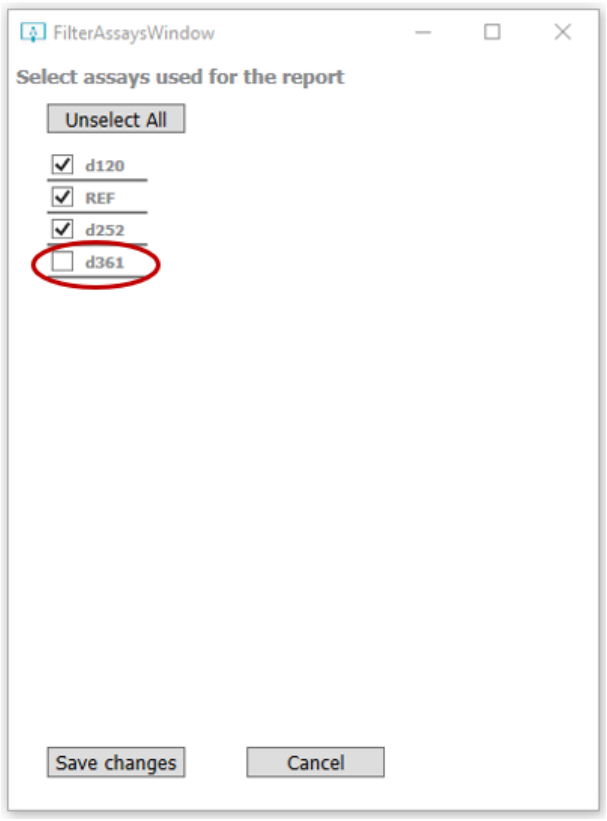
If a report is made at this point, the data from Assay d361 will be included in the calculations and the quantification result will be largely overestimated.

Cell fractions

Sample	Type	Date	Target	Chr.	CNV	DNA (%)	Informative for
Post3	DNA	07 September 2023	d120	2q	1,0	0.48	12255874 12255874
Post3	DNA	07 September 2023	d252	8q	1,0	0.64	12255874 12255874
Post3	DNA	07 September 2023	d361	12q	1,0	99.75	12255874 12255874
					Mean:	33.62	

In order to remove the data for Assay d361 from the Report, after approving the data, press the 'Select Assays' Select assays button.

The 'SelectAssaysWindow' will appear. In this window, users can choose to exclude an assay and its data from reporting.



In this example, once Assay d361 is de-selected and the changes saved, select the 'Report' button, the data of excluded assay is no longer present in the report and the average percentage reflect only the included data.

Cell fractions

Sample	Type	Date	Target	Chr.	CNV	DNA (%)	Informative for
Post3	DNA	07 September 2023	d120	2q	1,0	0.48	12255874 12255874
Post3	DNA	07 September 2023	d252	8q	1,0	0.64	12255874 12255874
					Mean	0.56	

Assigning Infromative Assays

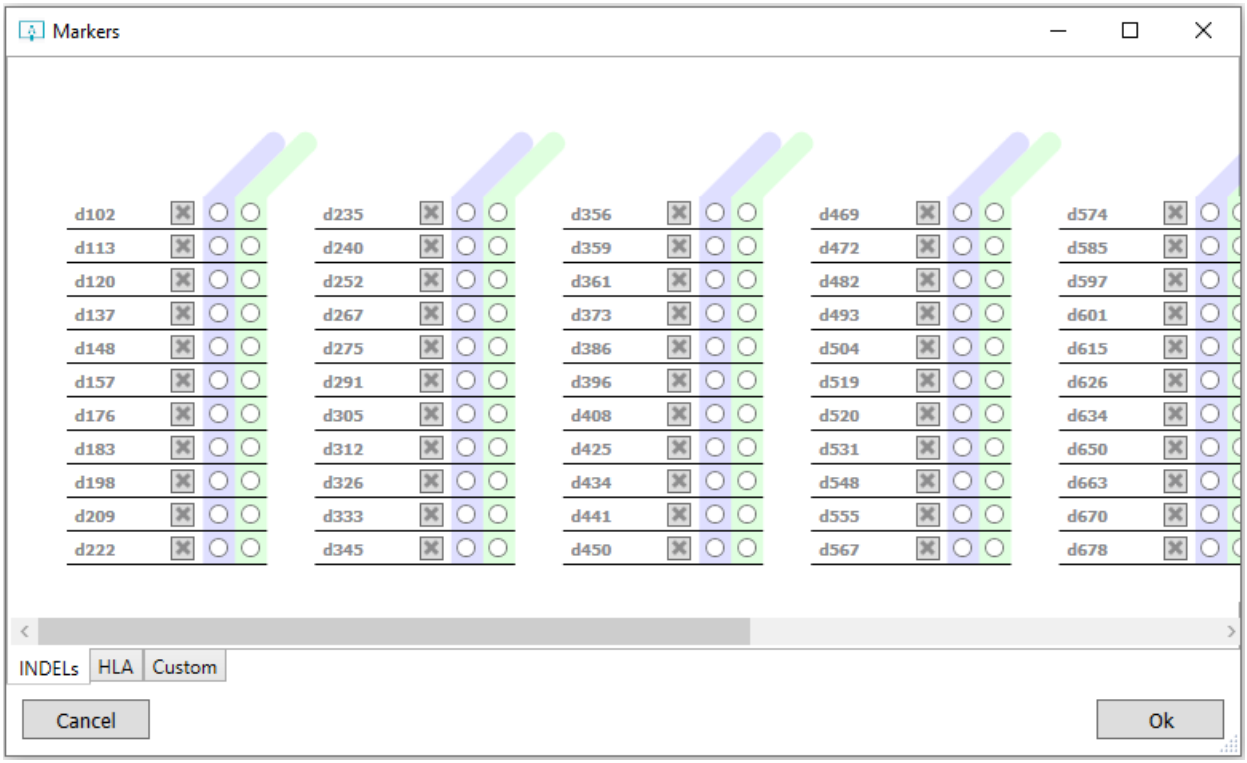
TRACE Analysis™ Software allows you to perform sample monitoring and leverage all the features of DigitalTRACE™, without the need to genotype the sample in advance. If you know that an assay is informative for your sample, you can designate its informative status and then use it as you normally would.

In order to use this feature of DigitalTRACE™ product:

Enter all the information necessary about the Recipient and the Donor samples.
Enter information related to the Post Sample(s) being tested

In the Informative Markers section, click the "Add Marker" button.

When this button is clicked, a window opens with all Assays available for assignment.



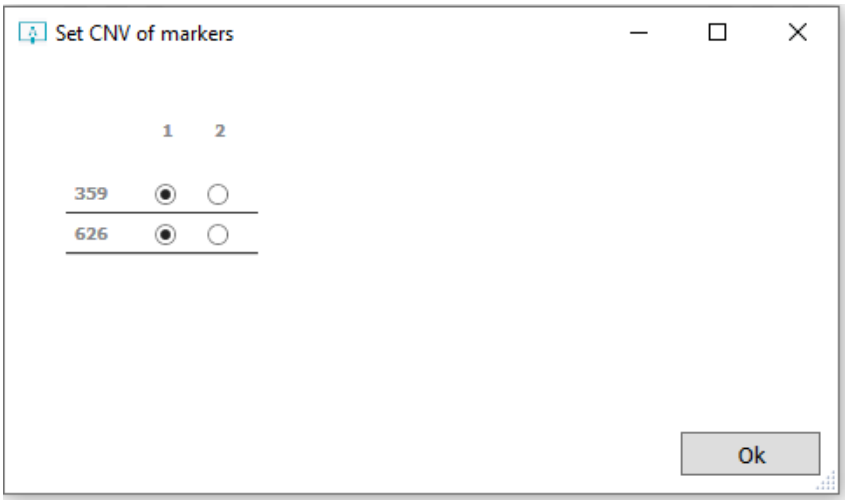
Assign the informative assays by clicking the circle and sample combination which is appropriate.

Press the 'x' to reset the choice for that assay.

Once the marker(s) have been assigned, click Ok.

Now the markers are selectable for the sample in the "Markers" window. TRACE Analysis™ Software will save this information, so it only must be entered once for a given sample.

A new window will ask to set the CNV of selected markers to proceed and the samples will be added to the plate.



Once all the information about the sample has been entered, click the “Quant” button

Software Overview

Use of TRACE Analysis™ Software facilitates setting up dPCR based tests for QIAcuity and Biorad platform, analyzes data, calculates, and displays analyzed data and stores sample-specific information for easy retrieval or exporting to a laboratory information management system. All data files generated by the software are stored in .xml format, for ease of data transfer.

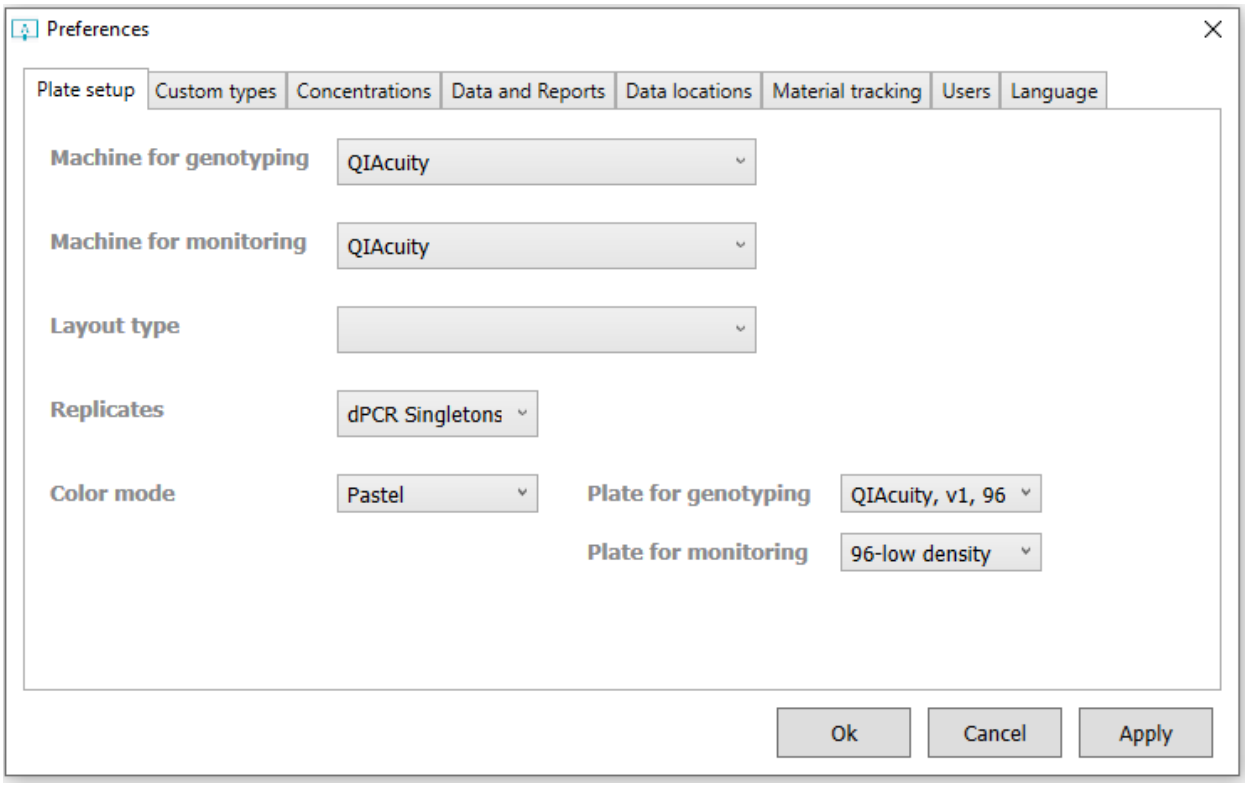
Preferences

The Preferences  Menu of TRACE Analysis™ Software allows for customization of many experimental parameters.
Through the preferences window, you can choose default settings for common variables, as well as enter information which may be unique to their testing regime or laboratory.

There are four main tabs within the window which enable changes to the experimental plate setup, manual entry of sample types, disease states and reference samples, experimental protocol variables and data storage customization. These customized parameters are found on the Plate Setup, Custom Types, Concentrations and Data Locations tabs.

There are also tabs enabling material tracking, user management and choice of language.

Plate Setup



Default machine:
Define the default dPCR analysis platform by clicking the radio button appropriate for the machine in use. TRACE Analysis™ Software will generate the appropriate sample setup .txt or .csv file for the machine, as well as will be able to analyze the results exported from that machine.

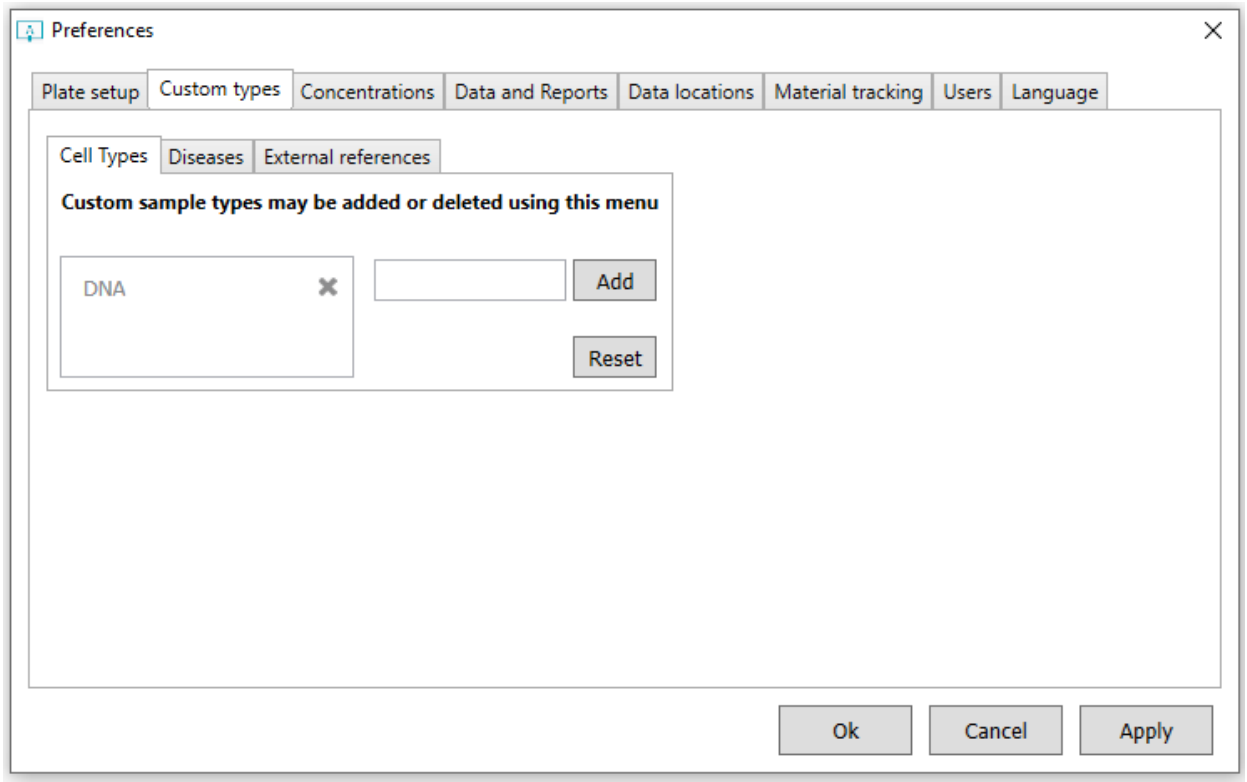
Replicates:
Define whether to perform quantitative analysis using duplicates or singletons.

Color mode:
The color highlights which appear on the software interface and the protocols can be modified to use a scheme which is preferred by the user.

Plate Configuration:
This preference selects the plate in use for genotyping and monitoring.

Custom Types

In the Custom types tab of the Preferences Menu, users may define the sample types being used, the diseases associated with the samples as well as any external reference materials which may be used.

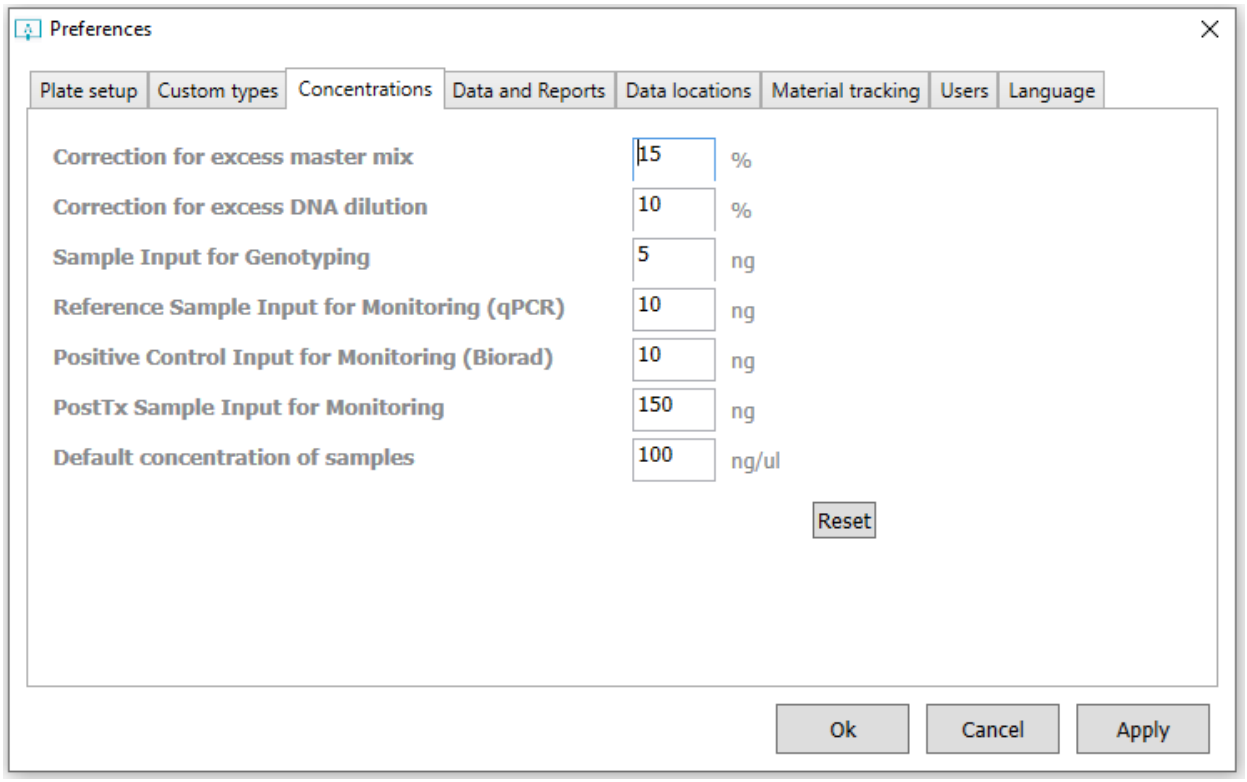


Cell Types tab:
Define the origin of the materials being tested. You can type a sample material in the empty box near the Add button, then press Add, and the sample type will now appear as an option in TRACE Analysis™. The small "x" on the same line as the sample type is used to remove that type from the software.

Diseases tab:
Define custom disease types

External references tab:
Define external reference samples (for qPCR only)

Concentrations



Define the variables used in protocol generation and experimental execution.

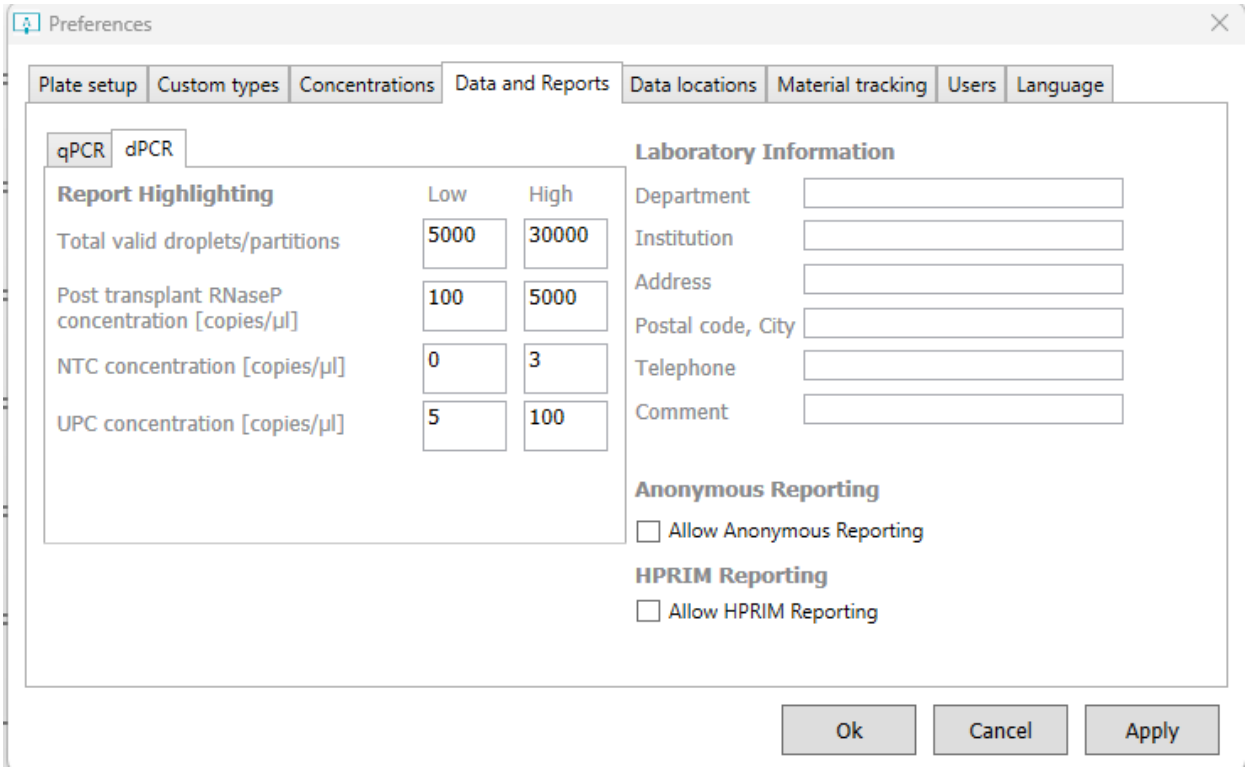
Define how much excess master mix and DNA dilution to use in the experimental protocol.

TRACE Analysis™ calculates the volumes needed, based on the experimental setup, and then adds these additional factors to provide more than enough of each solution to execution the experiment.

Define the sample input for genotyping. Based on the data from verification studies, JETA Molecular recommends the use of 10 ng DNA input per well for genotyping.

Define the default concentrations of samples. This number will appear for all samples and can be altered if necessary.

Data and Reports

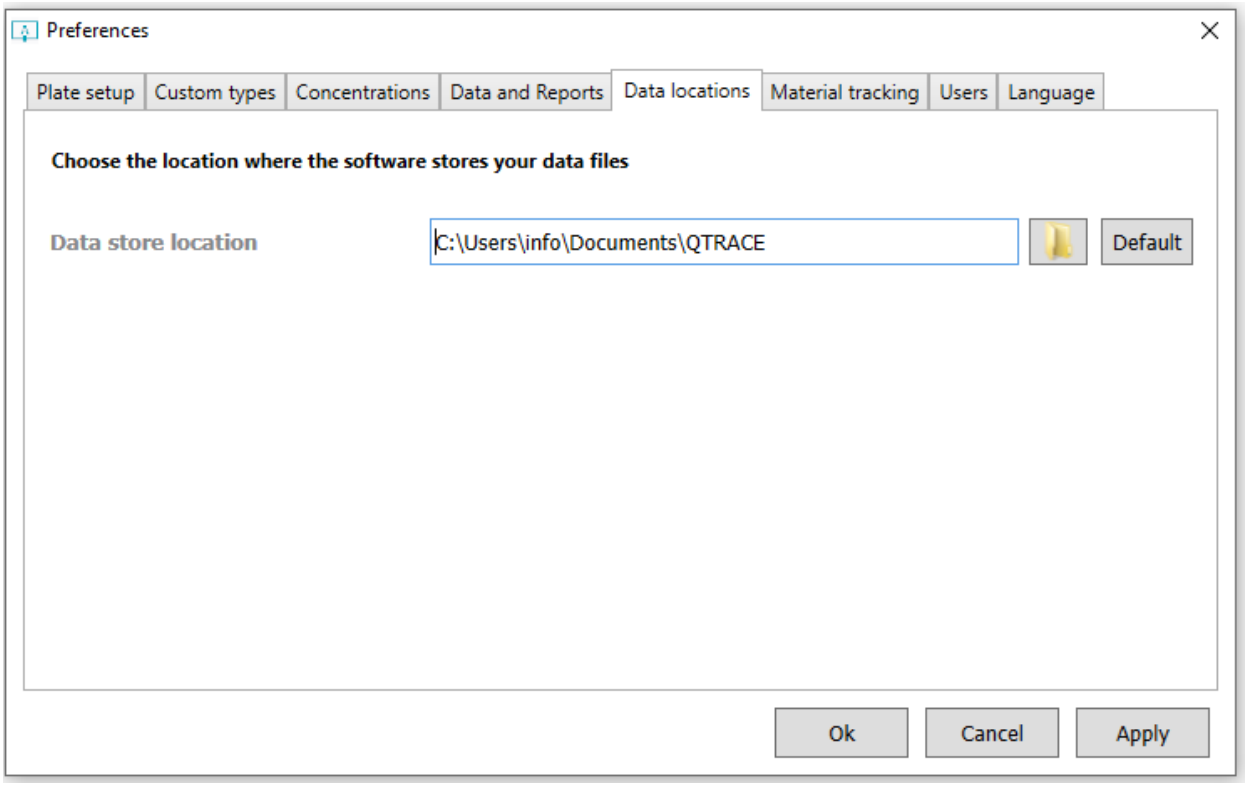


In order to achieve more customized data analysis, TRACE Analysis™ has a tab called Data and Reports in the Preferences section of the software.
In Report Highlighting, users could choose to have portions of their report highlighted, if values generated fall outside an expected range. The user can choose up to four different data quality inspections to be performed on monitoring data, with values outside of the input ranges highlighted automatically on the report. The report highlighting section enables us to define a range of values outside of which the data will be highlighted in the report.

Protocols and Reports can be customized by adding institutional information under the Laboratory Information section. Any information entered here will appear at the top of all protocols and reports generated by TRACE Analysis™ software.

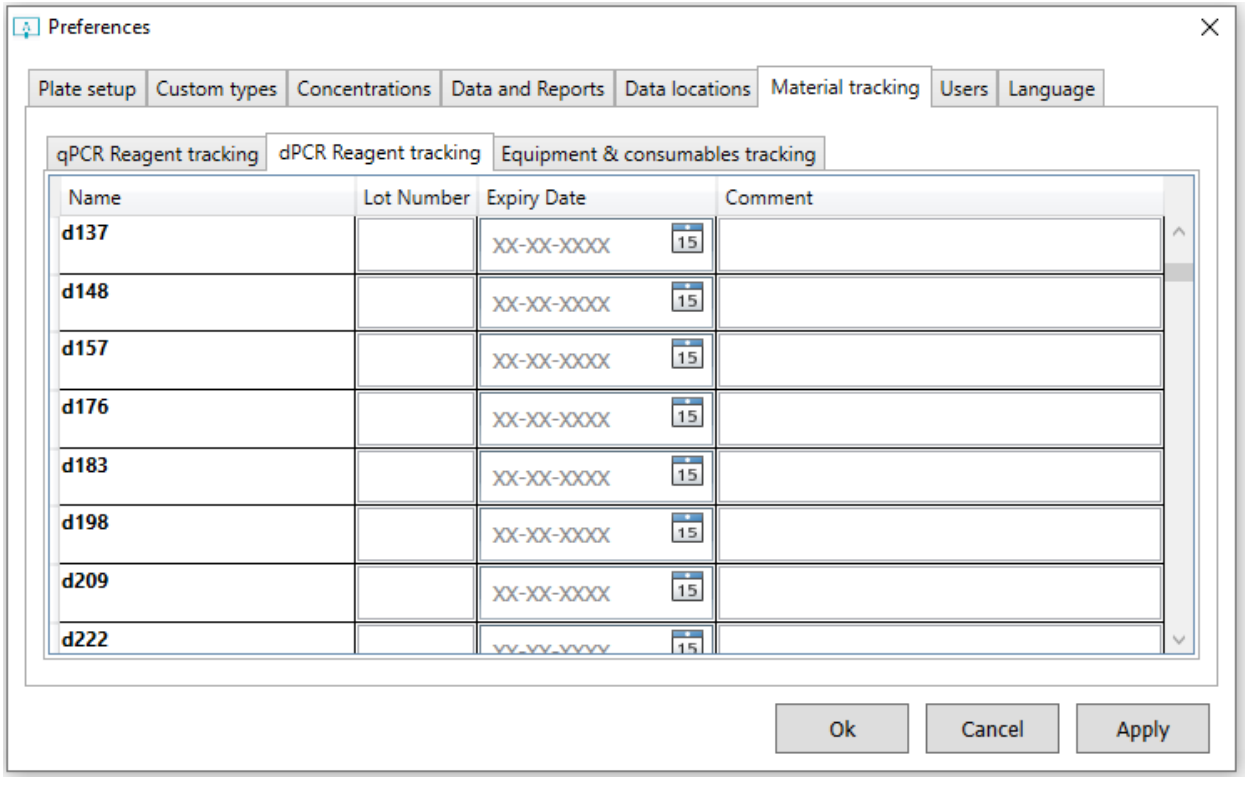
To create an anonymized report, go to the Anonymized Reporting Section.

Data Locations



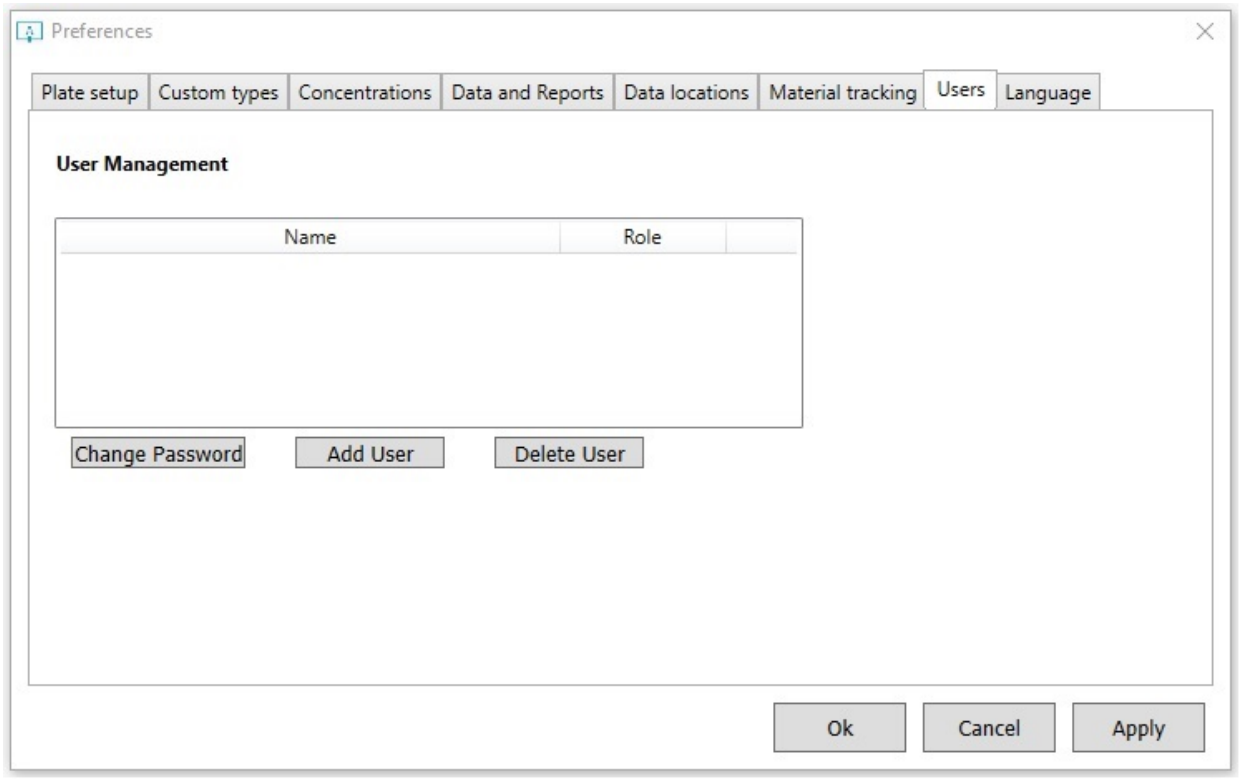
Define where TRACE Analysis™ Software stores the data it generates. For laboratories using multiple copies of TRACE Analysis™ Software, this location is likely best set as a shared location on a server. When all local copies of TRACE Analysis™ Software point to the same data storage location, all copies can read and write to the same data files, eliminating the need to transfer files between computers.

Materials Tracking



This tab is where reagent lot numbers and expiration dates may be entered into TRACE Analysis™ Software. This information will populate protocols and reports, eliminating the need for the operator to write the information each time.

Users



This tab is where User profiles are managed by an individual with 'Supervisor' rights in TRACE Analysis™ Software.

TRACE Analysis™ Software restricts access to the software as well as functions within the software. When TRACE Analysis™ launches for the first time, User Profiles need to be established. Once logged in with TRACE Analysis™ credentials, a Supervisor account needs to be created. In the Users tab, there is an option for creating New Users.

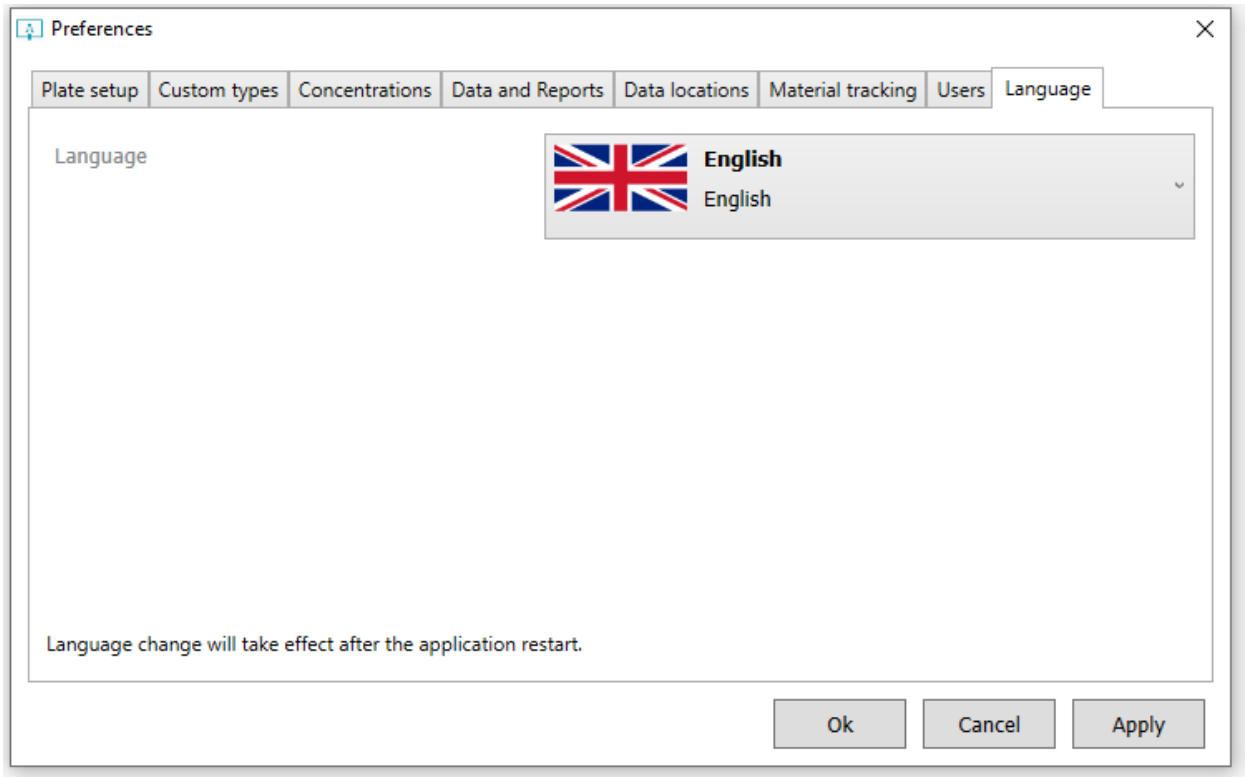
By clicking 'Add User' a dialog box opens wherein the details of the new user can be added.

After the Supervisor has created an account, multiple additional "Supervisor," "Advanced User," and "Analyst" accounts can be made.

Analysts and Advanced Users are allowed to change their passwords, and to edit the Reagent tracking Preferences information. All other Preferences settings are controlled by the Supervisor. Advanced Users can inspect and can validate a result.

User	Edit all tabs in Preferences	Results Validation	Add or Delete Users/Passwords	Edit Reagent Tracking Data
Supervisor	Yes	Yes	Modify All Users and Passwords	Yes
Advanced User	No	Yes	Modify Personal Password	Yes
Analyst	No	No	Modify Personal Password	Yes

Language



This tab enables to select a language for the TRACE Analysis™ Software. Language change will take effect after the application restart.

Software Buttons

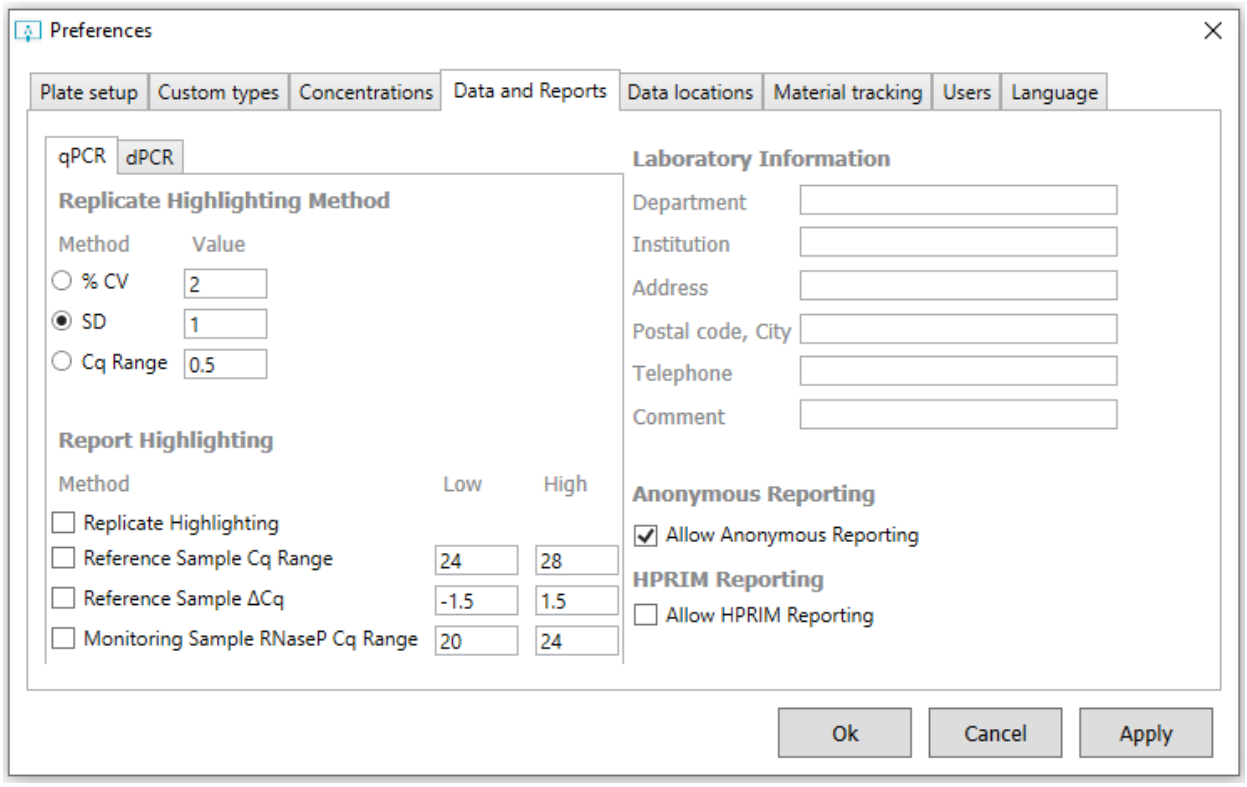
Icon	Name	Meaning
	Home	Return to the home screen of TRACE Analysis™ Software
	New	Add a new recipient record to the database. A record will always require a recipient name, unique recipient identifier and a sample identifier.
	Genotype	After entering recipient and donor specific information, choosing Screen will start creating a genotyping experiment by placing your samples on the plate.
	Quantify	After selecting a recipient to monitor and entering sample specific information, the Quant button adds your sample to a monitoring experiment.
	Export	Export an experiment sample setup file for use with a PCR instrument.
	Import	Import the .txt or .csv results file from your PCR instrument to review the data collected.
	Overview	Shows all available data for a transplantation: informative markers and quantitative analyses.

	Browse Experiment	Browse all experiments previously created in TRACE Analysis™ Software. By pressing the open button, you can re-open the imported data files.
	Save	Update information about the recipients or donors. Do not update information about the recipient or donor if there is PCR data waiting to be analyzed for them.
	Print	Print all typing and monitoring results from the currently selected recipient data.
	Preferences	Set your preferred instrument, sample types, number of replicates and disease types.
	Help	Review this manual directly via TRACE Analysis™ Software to search for useful tips, tricks, and troubleshooting.
	About	Technical information about TRACE Analysis™ Software, such as version, license, contact information.
	Remove	Remove a sample from comparative genotyping.
	Reset	Reset the experiment plate completely.
	Undo	Reset the last placement onto the experimental plate.
	Redo	Reset the previous "Undo" action onto the experimental plate.

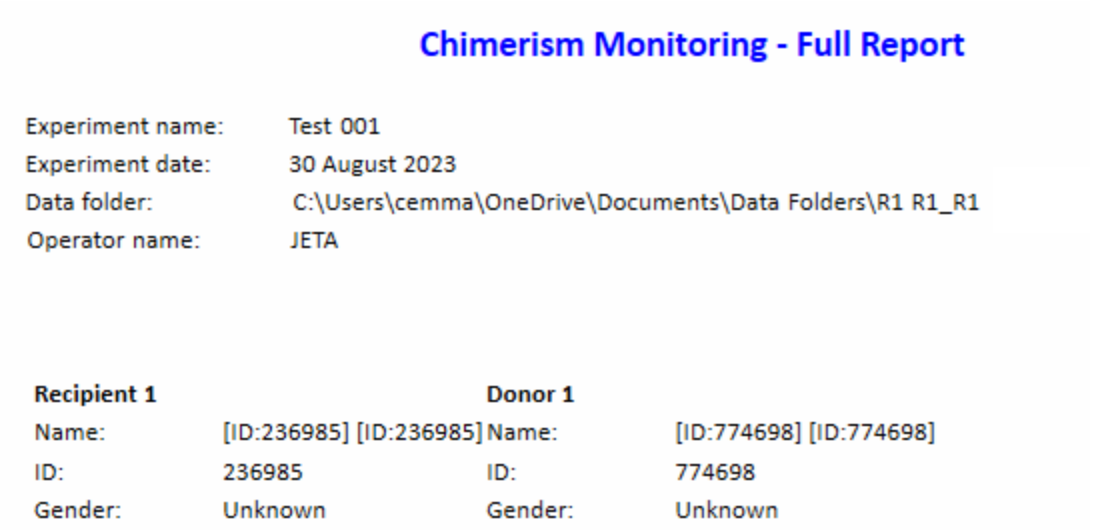
Anonymized Reporting

The ability to generate anonymized reports from TRACE Analysis™ Software is an option in the Preferences.

On the 'Data and Reports' tab of the Preferences, there is a check box which allows for reports to be generated without the names of the recipient and donor(s) appearing on them.



With the 'Allow Anonymous Reporting' option checked, Reports generated from TRACE Analysis™ Software will have the Recipient ID or the Donor ID replacing the name of the individuals.



Chimerism Monitoring - Full Report

Experiment name: Test 001
Experiment date: 30 August 2023
Data folder: C:\Users\cemma\OneDrive\Documents\Data Folders\R1 R1_R1
Operator name: JETA

Recipient 1		**Donor 1**	
Name:	[ID:236985] [ID:236985]	Name:	[ID:774698] [ID:774698]
ID:	236985	ID:	774698
Gender:	Unknown	Gender:	Unknown

Glossary and Definitions

Term	Definition
Bi-allelic	An allele which exists in two variant forms - a major and minor allele. Individuals may be homozygous for either variant or heterozygous
Experiment	A collection of genotyping and monitoring reactions that are carried out simultaneously (i.e., on a single plate)
INDEL	Abbreviation for insertion/deletion polymorphism; a class of DNA mutation characterized by the loss or gain of genetic material at a specific locus
Informative assay	An assay capable of distinguishing between genetic material from two or more sources; An informative assay is an assay for a marker allele that is present (positive) in one individual genome and absent (negative) in the other genome(s)
Monitoring assay	An assay designed for use in quantification of a specific marker, allele, or analyte. The assay must demonstrate high specificity for accurate quantification and high sensitivity to achieve a desirable limit of detection
Sample	A sample is a unique donor or recipient
Passive reference	A dye that produces fluorescence signal. The fluorescence signal in the reference channel is measured to determine the number of valid partitions in a well. Differences in the signal intensities between partitions are normalized and the fluorescence signals in the target channels are corrected accordingly.
Threshold	The intensity of fluorescence that must be exceeded for each reaction to be seen as positive
Partition	A compartment where the PCR reaction takes place
Nanoplate	QIAcuity dPCR plate with several single partitions
CNV	Copy Number Variation (CNV) refers to a type of genetic variation in which the number of copies of a particular segment of DNA differs between individuals

UPC	Universal Plasmid Control (UPC). A synthetic control sample possessing the targets for Assays in the DigitalTRACE™panel (typically 100% target DNA)