Mentype® DIPquant

The qPCR application for allele-specific quantification of the chimerism status

Handbook (HB)

RUO For research use only. Not for use in diagnostic procedures.

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A printed version of this handbook can be provided free of charge within 7 days.

For any further questions, please contact us:

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Product Description

The Mentype® DIPquant applications are research kits based on real-time PCR (qPCR) technology, which is used for allele-specific and quantitative analysis of molecular chimerism.

With these allele-specific Mentype® DIPquant singleplex assays, 55 DIP alleles and two Y-chromosomal regions can be individually addressed (see Table 1). The reference (REF) for the relative quantification is the β-globin gene. The qPCR parameters are universally set so that the analysis of different recipient-specific Mentype® DIPquant assays and multiple samples can be performed in parallel in one qPCR run.

The calculation of chimerism is based on the ΔΔCp qPCR method [Cp (crossing point) of Roche Lightcycler® qPCR instruments corresponding to the Ct value (cycle threshold) of other qPCR systems]. Therefore, for the relative quantification of chimerism, parallel measurement of the reference gene β-globin to the specific recipient locus is required.

To calibrate the analysis, the pure, unmixed DNA (preTx/recipient or donor calibrator) must be analyzed, together with the reference assay (β-globin gene) and the respective recipient-specific qPCR assay (see Quantification before mixture (preTx)).

Measuring Range of Chimerism Samples

Owing to qPCR technology, the optimal measuring range of chimerism samples with Mentype® DIPquant assays is between 0.05 % and 12.5 % of recipient or donor DNA fractions in the mixed samples. In this area, the qPCR setting can be applied as described in Quantification of mixed samples (postTx). For samples > 12.5 % of the recipient or donor DNA, as well as for mixed chimerism samples, it is recommended to use the multiplex PCR assay Mentype® DIPscreen (see Table 4).

Sensitivity and Specificity

The detection limit and sensitivity of Mentype® DIPquant assays depend on the quality and quantity of the used template DNA. Table 1 shows the sensitivity of the allele-specific Mentype® DIPquant markers in DNA mixtures. The mixtures were used with a DNA amount of 250 ng; the DNA utilized was homozygous for the allele-specific Mentype® DIPquant marker.

The maximum Cp value listed in Table 1 shows the range up to which signals from the corresponding Mentype® DIPquant assay can be specifically evaluated.

Table 1 Specific detection limits of Mentype® DIPquant assays

Sensitivity and specifity of Mentype® DIPquant assays								
Cell equivalent DIP allele/PCR	5	Max. Cp value	10	Max. Cp value	20	Max. Cp value	80	Max. Cp value
Concentration [pg/PCR]	31.5		63		126		500	
Ratio in 250 ng [%]	0.013		0.025		0.05		0.2	
Mentype® DIPquant HLD	23-I	36.7	67-I	37.7	82-I	33.5	79-I	31.1
	38-I	36.3	82-D	33.9	105-D	33.7	152-D	35.5
	48-I	36.5	84-D	38.0	140-I	35.0		
	53-D	37.5	101-I	37.4	301-D	32.5		
	53-I	35.0	103-D	37.2				
	67-D	36.2	104-D	34.1				
	70-D	32.5	131-D	33.6				
	70-I	35.6	131-I	34.2				
	84-I	37.3	305-D	33.9				
	88-D	35.6						
	88-I	31.6						
	91-D	34.1						
	91-I	35.2						
	97-I	36.4						
	101-D	35.7						
	103-I	36.6						
	104-I	35.5						
	105-I	34.5						
	106-D	36.9						
	106-I	34.2						
	110-I	35.6						
	112-I	34.8						
	114-D	35.5						
	114-I	37.4						
	116-D	35.9						
	116-I	34.6						
	128-D	35.4						
	128-I	32.7						
	133-I	35.9						
	134-D	35.3						

Sensitivity and specifity of Mentype® DIPquant assays		
	134-I	35.6
	163-D	35.2
	163-I	35.0
	301-I	35.9
	304-D	36.0
	305-I	35.6
	307-D	35.9
	307-I	37.5
	310-D	36.3
	REF	36.0
	SMCY	36.3
	SRY	36.6

Scientific Background

The term 'chimerism' describes a state in which a single organism harbors two or more genetically distinct cell populations. Human chimerism is defined as the presence of genetically distinct cell populations within a single individual. This condition can arise naturally, for example through tetragametic fusion, twin-twin cell exchange, or maternal-fetal microchimerism, or artificially, through medical procedures such as bone marrow or organ transplantation. Chimerism is fundamentally a biological state, that is not inherently pathological, and plays an important role in our understanding of human developmental biology. In evolutionary and developmental research, chimerism offers insights into cell lineage tracing, developmental plasticity, and immune tolerance. For example, microchimerism demonstrates how foreign cells can persist for decades, providing a natural model for long-term cell engraftment and inter-individual cell transfer.

Molecular chimerism analysis is carried out by the detection of deletion/insertion polymorphisms, which are extremely suitable for analysis by allele-specific qPCR technology as compared to other DNA-sequence motifs.

Following the identification of recipient and donor informative DIP loci using an application like Mentype® DIPscreen, quantitative chimerism analysis can be performed by using the corresponding Mentype® DIPquant singleplex assays. The flexible assay format allows the analysis of individual samples as well as large sample quantities with minimal material expenditure. Since the high sensitivity of the qPCR method is associated with limited accuracy in the field of mixed chimerism, it is recommended to analyze samples with mixed chimerism by using the Mentype® DIPscreen kit.

Materials provided

Kit content

The Mentype® DIPquant kits contains reagents that can be used to perform up to 25 or 100 reactions. They include the following components:

Table 2 content of the Mentype® DIPquant, * only available as Mentype® DIPquant Reference assay, # defines the specific locus

Reagent	Cap color		Volume per kit	
			25 reactions	100 reactions*
Nuclease-Free Water	Light blue	🟦	1.5 mL	2 x 1.5 mL
Reaction Mix D	Black	🟪	125 µL	500 µL
Mentype® DIPquant -HLDxxx#-D/-I Primer Mix -SRY Primer Mix -SMCY Primer Mix -Reference Primer Mix	Red	🟥	63 µL	250 µL
Multi Taq 2 DNA Polymerase	White	⬜	10 µL	40 µL

NOTE

Please note that the packaging size describes the number of testings **without** taking into account the number of required controls or the required excess for pipetting.

Reagent storage and handling

The kit is shipped on dry ice. The components of the kit should arrive frozen, except the Multi Taq 2 DNA Polymerase, which is stored in a buffer preventing freezing of the reagent.

Please check the completeness of the kit upon receipt. Immediately contact BIOTYPE GmbH if one or more components are not frozen, or if tubes, labeling or the packaging have been compromised during the shipment. Do not use kits that have been thawed upon arrival.

Store all components at -25 °C to -15 °C without light exposure. Especially the Mentype® DIPquant Primer Mix must be stored protected from light.

The expiry date is indicated on the kit box label. Do not exceed a maximum of 8 freeze-thaw cycles.

Material and devices required but not provided

General laboratory equipment

- Desktop centrifuge with rotor for 2 mL reaction tubes
- Centrifuge with a rotor for microtiter plates, if using 96 well reaction plates
- Vortex mixer
- Calibrated adjustable pipettes with disposal aerosol tight filter tips
- Suitable racks for 2 mL reaction tubes and 200 µL microtiter plates
- Cooling rack suitable for 2 mL tubes
- Disposable powder-free gloves
- NanoDrop™ One Spectrophotometer

NOTE

All materials used for PCR should have appropriate quality (DNA free and for molecular biology). Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturers' instructions and recommendations.

Reagents, kits and consumables

Table 3 Reagents required, but not provided

Reagent	Supplier	Order number
Mentype® DIPscreen PCR Amplification Kit, 25 reactions	BIOTYPE GmbH	45-12300-0025
Mentype® DIPscreen PCR Amplification Kit, 100 reactions	BIOTYPE GmbH	45-12300-0100
DIP Positive Control (5 ng/µL)	BIOTYPE GmbH	27-13201-0100
ChimerisMonitor RUO	BIOTYPE GmbH	46-14801-0000
NucleoSpin® Blood L Kit, 20 preps	Macherey Nagel GmbH	740954.20
QIAamp® DNA Blood Midi Kit, 20 preparations	QIAGEN GmbH	51183

Instruments and Software

Mentype® DIPquant was verified and validated with the Roche Lightcycler® 480 instrument II real-time PCR system (cat. no. 05015278001, Roche Diagnostics International AG, Rotkreuz, CH).

The use of Mentype® DIPquant assays with other qPCR instruments must be verified by the user. Make sure that the fluorescent dye FAM can be analyzed by the instrument.

To make data analysis easier and more intuitive, we recommend performing chimerism calculations using the ChimerisMonitor RUO software (version 3.0.7 or higher).

NOTE

Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturer´s instructions and recommendations.

Samples and Specimen

Mentype® DIPquant assays were validated with DNA isolated from citrated whole blood.

The product Mentype® DIPquant is validated for a DNA input of 250 ng per reaction. The use of larger amounts of DNA must be validated by the user.

Warning and Precautions

- Read the handbook carefully before using the product.
- Read the safety data sheets (SDS) and Non-Hazardous Statements (NHS) for all BIOTYPE products, which are available on request via support@biotype.de. For products that do not require a SDS as they do not contain an SVHC or are subject to other restrictions of Regulation 1272/2008 (CLP), BIOTYPE provides the SDS upon request.
- Please contact the respective manufacturers for copies of the SDS for any additionally needed reagents.
- Kit components of different kit lots must not be mixed.
- Aliquoting the kit components into other reaction vessels is not permitted.
- The use of this product is limited to personnel specially instructed and trained on molecular-genetic techniques and in (q)PCR techniques.
- Before the first use, check the product and its components for:
    - Integrity
    - Completeness with respect to number, type and filling (see chapter Materials provided)
    - Correct labelling
    - Frozenness upon arrival (except the Multi Taq 2 DNA Polymerase).
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Do not use a kit that has passed its expiration date.
- Discard samples and assay waste according to your local safety regulations.
- All instruments used must have been installed, calibrated, checked and maintained according to the manufacturer´s instructions and recommendations.

Procedure

Overview of the experimental workflow

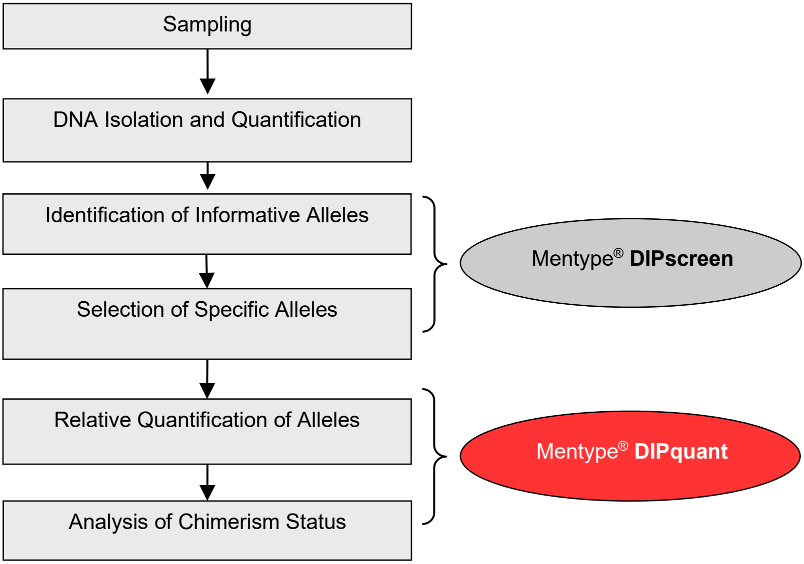

Figure 1 From sampling to analysis: chimerism analysis with Mentype® DIPscreen and Mentype® DIPquant

Selection of specific Mentype® DIPquant assays

The selection of suitable loci for the chimerism analysis with Mentype® DIPquant is dependent on the expected chimerism value. Please follow these recommendations for assay selection (see Table 4).

Table 4 Recommendation for the selection of suitable Mentype® DIPquant loci

Expected chimerism [% recipient]	Expected chimerism [% recipient]	Calibrator sample	Recommended loci
0.05 – 12.5	87.5 – 99.5	Recipient sample	3 recipient specific loci
87.5 – 99.5	0.05 – 12.5	Donor sample	3 donor specific loci
12.5 – 87.5	87.5 – 12.5	Use Mentype® DIPscreen for quantification	

Sample preparation

Raw sample requirements

Take at least a 200 µL sample of peripheral venous whole blood for the following procedure.

The handling of the raw sample material (peripheral venous whole blood) should follow the recommendation of the Clinical and Laboratory Standards Institute (CLSI) guideline MM05–A2 (2nd edition), where they state that whole blood can be stored at room temperature (22 °C to 25 °C) for up to 24 hours, or at 2 °C to 6 °C for 72 hours or more. Additionally, it is recommended that the anticoagulants used for whole blood collection are EDTA, citrate or heparin.

DNA extraction

The quality of the isolated DNA has an important influence on the performance and quality of the entire test system. Nucleic acid isolation methods must be employed, or the kits must be compatible with qPCR technology.

The following kits were tested and are suitable for nucleic acid isolation:

- NucleoSpin® Blood L Kit (Macherey Nagel GmbH, Düren, DE)
- QIAamp® DNA Blood MidiKit (Qiagen GmbH, Hilden, DE)

The use of alternative DNA isolation kits must be validated by the user.

DNA quantification and dilution

Quantify the DNA concentration by UV/VIS spectroscopy at 260 nm using the NanoDrop spectrophotometer.

When using spectrophotometry, use the elution buffer from the DNA extraction kit to measure the blank. The A260/A280 ratio should be in the range of 1.7 – 2.0, whereas the A260/A230 ratio should be in the range of 1.8 – 2.3, or according to the recommendations of the DNA extraction kit manufacturer.

Prepare the dilution freshly before usage. Use nuclease-free water as diluent.

NOTE

The allele-specific primer mix is **optimized for the use of 250 ng** of purified DNA, which corresponds to 41,666 cells (6 pg/cell). For optimal results the use of 250 ng DNA is recommended.

DNA storage

DNA should be stored undiluted at -25 °C to -15 °C for long term storage or according to the DNA isolation kit's manufacturer's information.

Control preparation

Positive Control PC

NOTE

DIP Positive Control (DPC, cat. no. 27-13201-0100, BIOTYPE GmbH) is a DNA mixture positive for all deletion and insertion polymorphisms (DIP) analyzed with Mentype® DIPquant, plus the loci SRY and SMCY. Therefore, it is the ideal universal qualitative external positive control for this application.

For positive control, use 5 µL of the DIP Positive Control or a pre-typed, allele-specific positive control DNA (5 ng/µL) instead of the template DNA.

Instead of the template DNA, pipette the control DNA into the wells containing the qPCR master mix.

No template control NTC

For negative control, pipette 5 µL of Nuclease-Free Water instead of the template-DNA into the reaction wells containing the qPCR master mix.

Master mix setup

Remove all components from the Mentype® DIPquant kit for the master mix setup:

- Nuclease-Free Water (light blue cap)
- Reaction Mix D (black cap)
- Mentype® DIPquant Primer Mix (red cap)
- Multi Taq 2 DNA Polymerase (white cap)

All frozen components should be thawed at room temperature (22 °C to 25 °C, ca. 30 min, protected from light) and homogenized by inverting the tubes or gentle vortexing. After this, briefly centrifuge the reagents (approx. 10 s). To uphold the principles of good laboratory practice, it is advisable to keep the Multi Taq 2 DNA Polymerase in a cooled environment as long as possible (e. g. cooling rack) prior to the master mix setup.

NOTE

Mix the Multi Taq 2 DNA Polymerase by flicking for longer stability – **do not vortex the enzyme**.

Prepare the PCR master mix according to Table 5 in an appropriately sized microcentrifuge tube for the total number of samples to be tested in a dedicated clean area. Include at least one PC and one NTC into your calculation.

Table 5 PCR master mix reaction setup, * The volume depends on the DNA concentration. If a higher volume of DNA template is used make sure to adjust the volume of Nuclease-Free Water. The total reaction (rxn.) volume per reaction must be 25.0 µL at all times.

	Volume		
Component	**1 rxn.**	**5 rxn.**	**10 rxn.**
Nuclease-Free Water*	12.1 µL	60.5 µL	121.0 µL
Reaction Mix D	5.0 µL	25.0 µL	50.0 µL
Mentype® DIPquant Primer Mix	2.5 µL	12.5 µL	25.0 µL
Multi Taq 2 DNA Polymerase	0.4 µL	2.0 µL	4.0 µL
DNA template or control sample	5.0 µL*	5 x 5.0 µL*	10 x 5.0 µL*
Total volume	**25.0 µL**	**125.0 µL**	**250.0 µL**

Pipette 20 µL of the qPCR mix (without the template DNA) into the reaction tubes (optical tubes) or the multi-well plate (optical multi-well). Next, add 5 µL of the specific DNA (see Pipetting Scheme in Quantification before mixture (preTx), Quantification of mixed samples (postTx)) or 5 µL of PC or NTC.

NOTE

First, prepare the NTC to avoid contamination of the control. Prepare the PC last to avoid cross contamination of the samples.

If possible, white PCR plates or reaction tubes should be used for qPCR instruments. This will minimize well-to-well fluorescence overexposure and thus improve the sensitivity of the assays.

The reaction tubes or the multi-well plates should be tightly sealed (optical caps, optical sealing) after pipetting.

Centrifuge the reaction batches briefly and place them in the instrument for analysis.

PCR amplification

Use the parameters listed below to create the protocol for qPCR amplification and detection. For instrument-specific settings, please refer to the instructions of the respective manufacturer or contact our technical support at support@biotype.de.

NOTE

6-FAM serves as a reporter fluorescent dye for all assays. Ensure that the correct filter set is selected in the software of the real-time PCR device.

Multi Taq 2 DNA Polymerase is reversibly inactive at a lower temperature to suppress the formation of unspecific amplification products. A "hot start" should be carried out before thermocycling to activate the enzyme.

Table 6 qPCR amplification parameter to be used for Mentype® DIPquant assays, validated with Roche Light Cycler® LC480 (Standard Heating-Rates of 4.4 °C/s and Cooling-Rates of 2.2 °C/s

Temperature	Time	
94 °C	4 min (hot start to activate Multi Taq 2 DNA Polymerase)	
94 °C	30 s	45 cycles
62 °C	**45 s**	

Data should be recorded during the annealing and elongation phase at 62 °C.

Create a sample list with the selected settings.

Recommended Setup for Analysis

The recipient’s portion in the mixed sample should be measured to address the optimal measuring range of qPCR (see Measuring Range of Chimerism Samples)

For the relative quantification of chimerism, the preparation of qPCR assays is recommended according to the following scheme:

- 3 different recipient-specific alleles (Allele of Interest, AOI) in duplicates (see Table 7, Table 8, Table 9)
- Per DNA and measurement, the active reference (REF, ß-Globin) must be measured at least in duplicates

- in each assay, a negative (NTC) and positive control (PC) should be run additionally

Table 7 Setup using 3 specific Mentype® DIPquant and the Mentype® DIPquant Reference Assays in duplicates

Assay	Replicates	Number investigated Loci
Specific DIPquant Assay	2	3
Reference-Assay (ß-Globin)	2	-

Quantification before mixture (preTx)

To calibrate the analysis, the recipient DNA before mixture (preTx calibrator) must be analyzed alongside the reference assay (β-globin gene) and the respective recipient-specific qPCR assays. This quantification is set at 100% recipient level.

To ensure the specificity of the recipient-specific qPCR assays, an analysis of the donor DNA is recommended. This corresponds to the 0 % recipient level.

Table 8 Example for the set-up of a multi-well plate of neate, unmixed samples (preTx calibrator), REF: active reference assay; AOI 1-3: recipient-specific assays; preTx: pure unmixed recipient DNA as calibrator; NTC: No Template Control; PC: positive control

	1 REF	2 AOI-1	3 AOI-2	4 AOI-3
A	REF preTx	AOI-1 preTx	AOI-2 preTx	AOI-3 preTx
B	REF preTx	AOI-1 preTX	AOI-2 preTX	AOI-3 preTX
C	REF NTC	AOI-1 NTC	AOI-2 NTC	AOI-3 NTC
D	REF PC	AOI-1 PC	AOI-2 PC	AOI-3 PC

Quantification of mixed samples (postTx)

Chimerism analysis should be performed using freshly isolated recipient DNA at the respective analysis times. To ensure a safe analysis, the active reference and three recipient-specific alleles, as well as the PC and NTC, should be analyzed (see Table 9).

Table 9 Example for the set-up of a multi-well plate with mixed samples, REF: active reference assay; AOI 1-3: recipient-specific assays; Quantification 1: first quantification sample; NTC: No Template Control; PC: positive control

	1 REF	2 AOI-1	3 AOI-2	4 AOI-3
A	REF Quantification 1	AOI-1 Quantification 1	AOI-2 Quantification 1	AOI-3 Quantification 1
B	REF Quantification 1	AOI-1 Quantification 1	AOI-2 Quantification 1	AOI-3 Quantification 1
C	REF NTC	AOI-1 NTC	AOI-2 NTC	AOI-3 NTC
D	REF PC	AOI-1 PC	AOI-2 PC	AOI-3 PC

Data Analysis

General

View the amplification plots for the entire qPCR run. A detailed analysis of raw data depends on the real-time PCR instrument used.

The threshold "baseline noise levels" should either be automatically defined or predefined for specific cycles (e. g. 3 - 15). Use the NTC to determine the respective threshold.

Since the ΔΔCp method is used for quantification, individually set values for the threshold have no effect on the results as long as all the assays of a sample are analyzed with the same threshold.

Information for data export and data processing can be found in the manual of your real-time device manufacturer. Export the sample name "Sample name" and the Cp values for subsequent calculations.

Verification of Results

The qPCR run is valid if the Cp values of positive control correspond to the values shown in Table 1, and the Negative Control shows no amplification < 45 cycles.

Using the donor DNA to control assay specificity, no signals should be detectable below the Cp values shown in Table 1.

Data Analysis using ChimerisMonitor RUO

The software ChimerisMonitor provides an intuitive and fast workflow for automated chimerism calculation. For a comprehensive guide on how to analyse DIPquant samples, please refer to the CSM RUO Handbook.

Raw data input

The pure, unmixed sample serves a calibrator for the following ΔΔCp calculation (see Table 4).

If preTx specific loci were used for chimerism analysis, the preTx sample must be analyzed as calibrator.

If donor specific loci were used for chimerism analysis, the donor sample must be analyzed as calibrator.

If both preTx and donor specific loci were used for chimerism analysis, both preTx and donor samples must be analyzed as calibrators. Please note that calculated chimerism values for postTx samples may differ between preTx and donor loci.

preTx sample: after verification of the Cp values, make sure to import the values for DIPquant Reference and the preTx specific DIPquant loci. If the preTx sample was tested with donor specific DIPquant loci for specificity, do not import these values in ChimerisMonitor to prevent incorrect calculations.

Donor sample: if donor specific loci are used for the chimerism analysis, import the Cp values for the donor sample obtained with DIPquant Reference and the donor specific DIPquant loci. If the donor sample was tested with preTx specific DIPquant loci for specificity, do not import these values in ChimerisMonitor RUO to prevent incorrect calculations.

Quantification

Manual quantification of qPCR data should be performed with the relative quantification method. Individually set thresholds during raw data analysis do not affect quantification by the ΔΔCp method as long as all the assays of a sample are analyzed with the same threshold.

Please use the NTC to set an appropriate threshold.

Quantification of calibrator samples (neat, unmixed samples)

1. Calculate individual Cp values for the Reference (REF) and the informative alleles “Alleles of Interest” (AOI) for the calibrator DNA (C)

2. Calculate the ΔCp for each AOI to the REF gene:

$$\Delta\text{Cp C} = \text{Cp AOI} - \text{Cp REF}$$

3. The ΔCp value equals the calibrator (ΔCp C) in the postTx calculation (100 % recipient)

Quantification of postTx samples

1. Calculate the individual Cp values for the Reference (REF) and the informative alleles “Alleles of Interest” (AOI) for the postTx DNA (U)
2. Calculate the ΔCp for each AOI to the REF gene:

$$\Delta\text{Cp U} = \text{Cp AOI} - \text{Cp REF}$$

3. The resulting ΔCp value will be used to calculate the unknown status ΔCp U
4. Calculate ΔΔCp to quantify the chimerism:

$$\Delta\Delta\text{Cp} = \Delta\text{Cp U} - \Delta\text{Cp C}$$

5. Calculate the percentage % of the calibrator that is dependent on the efficiency E of the qPCR (see Table 10):

$$\%\ \text{calibrator (preTx or donor)} = ((1+E)^{-(\Delta\Delta\text{Cp})}) \times 100$$

In case of the qPCR efficiency E of 100 %, use the reduced formula:

$$(2^{-(\Delta\Delta\text{Cp})}) \times 100$$

Table 10 qPCR efficiency E of the Mentype® DIPquant loci * Experimentally determined by serial dilution series of homozygote DNA, calculated values > 1.00 were set to 1.00. Values are embedded in the software and are applied when the calculation is performed with setting *Respect PCR efficiency in calculation*

DIPquant assay	qPCR Efficiency (E)*	DIPquant assay	qPCR Efficiency (E)*
Reference	0.95	HLD105-D	1.00
SRY	1.00	HLD105-I	0.95
SMCY	1.00	HLD106-D	1.00
HLD23-I	1.00	HLD106-I	0.92
HLD38-I	1.00	HLD110-I	0.97
HLD48-I	1.00	HLD112-I	1.00
HLD53-D	1.00	HLD114-D	0.90
HLD53-I	1.00	HLD114-I	1.00
HLD67-D	0.98	HLD116-D	0.93
HLD67-I	0.98	HLD116-I	1.00
HLD70-D	1.00	HLD128-D	0.92
HLD70-I	0.97	HLD128-I	1.00
HLD79-I	1.00	HLD131-D	0.95
HLD82-D	1.00	HLD131-I	0.90
HLD82-I	1.00	HLD133-I	1.00
HLD84-D	0.93	HLD134-D	0.95
HLD84-I	0.95	HLD140-I	1.00
HLD88-D	1.00	HLD152-D	1.00
HLD88-I	1.00	HLD163-D	1.00
HLD91-D	0.92	HLD163-I	1.00
HLD91-I	0.99	HLD301-D	1.00
HLD97-I	1.00	HLD301-I	1.00
HLD101-D	0.97	HLD304-D	1.00
HLD101-I	1.00	HLD305-D	1.00
HLD103-D	0.99	HLD305-I	0.98
HLD103-I	0.91	HLD307-D	1.00
HLD104-D	1.00	HLD307-I	0.92
HLD104-I	1.00	HLD310-D	0.90

Troubleshooting

Poor Signal or no Signal Detected

One or more components were not added to the reaction: Check the positive control amplification and repeat the qPCR if necessary.

The wrong assay was used for the analysis: Make sure that the allele-specific assays are compatible with the recipient-specific alleles.

Suboptimal qPCR conditions: Check the qPCR settings. Ensure that Multi Taq 2 DNA Polymerase activation is at 94 °C for 4 min. Check the annealing and elongation temperature. Make sure that the heating rate of the device is set to 4 °C/s and the cooling rate of the device to 2 °C/s.

The qPCR was inhibited: PCR inhibitors were not completely removed during DNA isolation. Make sure that DNA purification is done carefully and in keeping with the instructions for use of the kit manufacturer. Clean the DNA again or dilute the template. Repeat the qPCR with the purified or diluted DNA.

The data collection failed: Make sure that the fluorescence data collection was carried out at the right time on the correct fluorescence channel. Check the settings of your qPCR instrument for the fluorescence color (6-FAM) used in the assay.

Baseline or threshold issues: Set the threshold above the nonspecific background to get accurate Cp values. Use the procedure in the instruction for use of your qPCR instrument manufacturer. If possible, make the baseline and threshold settings manually.

Degradation of the template DNA: The degradation can take place during the preparation of the sample and during storage. Store the DNA in 1 x or 0.1 x TE. Use control DNA to check the integrity of the assay.

Degradation of the qPCR components: Check the durability of the components used, as well as the storage conditions. Avoid frequent freezing and thawing of the primer mix for more than 8 cycles. Ensure that the components are stored at -25 °C to -15 °C.

Fluctuations in the Signal Strength within the Replicas

Pipetting errors: Check and calibrate your pipettes regularly to avoid pipetting errors.

Variations in the master mix: Add 1 - 2 additional reaction volumes to compensate for pipetting errors during the setting up of the master mix. Mix the components carefully by brief vortexing and brief centrifugation (10 s). Pipette at least 5 µL of the template DNA.

qPCR was inhibited: PCR inhibitors were not completely removed during DNA isolation. Make sure that DNA purification is done carefully and in compliance with the instructions for use of the kit manufacturer. Clean the DNA again or dilute the template. Repeat the qPCR with the purified or diluted DNA.

Baseline or threshold issues: Set the threshold above the nonspecific background to get accurate Cp values. Use the procedure in the instruction for use of your qPCR instrument manufacturer. If possible, set the baseline and threshold settings manually.

Low sensitivity: The use of small amounts of DNA (optimal is 250 ng) can reduce sensitivity and reproducibility within the replicates. Please quantify the applied DNA amount and measure its quality with appropriate methods (see Sample preparation).

Signals in negative controls: To prevent contamination, use disposable pipette tips with aerosol-tight filters. Run a new qPCR with the nuclease-free water used. Store pre- and post-PCR reagents separately. Pipette the reaction mixture and the DNA, if possible, in different rooms.

Signals of Recipient-Specific Assays in Donor DNA

Use of large amounts (> 250 ng) of the template DNA: Reduce the template DNA amount to 250 ng. Before preparing the qPCR reaction, the DNA concentration must be determined.

Occurrence of false negative signals: In rare cases, mutations occurring in the primer binding sites can lead to allelic dropouts. Genotyping with the Mentype® DIPscreen could result in false negative results (see Material and devices required but not provided, Quantification before mixture (preTx)). Since the primer binding sites of the Mentype® DIPquant assays differ from those of the Mentype® DIPscreen, allele-specific signals can nevertheless be amplified in the qPCR. But this must be verified before the application of pre-selected Mentype® DIPquant assays in chimerism analysis.

Analytical Performance Evaluation

a) Human DNA

A biobank of more than 100 human DNA samples prepared from venous ETDA blood was used for all verification experiments. The samples were derived from unrelated healthy volunteers who gave their written informed consent.

b) Analytical Specificity and Limit of Blank (LoB)

Aim: The product consists of 54 assays for autosomal biallelic markers, two Y-chromosomal specific markers, and the reference gene. The specificity of the allele and Y-chromosomal specific Mentype® DIPquant assays must be ensured in the presence of an excess of the template DNA of the alternative allele or the X-chromosome, respectively.

Method: Real-time qPCR data of no template controls (NTC, $n \geq 12$) and controls with 250 ng homozygous DNA for the alternative allele or 250 ng female DNA (in case of Y-specific markers) ($n \geq 9$), which should not be detected, were collected for each Mentype® DIPquant qPCR assay.

Results: All NTC showed no false positive signals before 45 cycles. In case of 250 ng homogenous DNA for alternative allele or female DNA 26, Mentype® DIPquant qPCR assays showed no signals before 45 cycles. The other tests showed unspecific signals before 45 cycles. However, a stochastic distribution between 1 and 6 false positives was observed within 9 parallel measurements. So, the non-parametric analysis approach was used to calculate the LoB (CSLI 2012, data not shown).

c) Analytical Sensitivity and Limit of Detection (LoD)

Aim: Experiments were performed to establish the analytical Limit of Detection (LoD) for all qPCR tests.

Method: The calculation on whether the LoD meets the quality criterion to exclude false positive results was $\text{LoB} - (\text{LOD} + 2 \times \delta) \geq 2$, where LoB is the Cp value determined non-parametrically according to a), LoD is the mean Cp value of positive measurements, and δ is the standard deviation of LoD.

DNA mixtures were generated for all allele-specific tests by using 250 ng homozygous DNA per PCR for the alternative allele or 250 ng female DNA (in case of Y-specific markers) and different amounts of homozygous DNA of the Allele of Interest. The number of replicates was 6 (3 at two different days).

Results: At first, 31.5 pg DNA of the Allele of Interest was spiked corresponding to 0.01 % of the minor allele. Additional experiments with 63 pg (0.025 % minor allele), 126 pg (0.05 % minor allele), and 500 pg (0.2 % minor allele) respectively were conducted in cases where the quality acceptance criterion was not achieved. The results for all qPCR tests are depicted in Table 1.

d) Measuring Range of the Assays

Aim: The linear measurement range of the assays was determined.

Method: The experiments included all data from b) and c). In addition, serial dilutions of recombinant plasmids encoding DNA regions of the Alleles of Interest were measured in the range between 5 and 5 120 copies per reaction. In total, 11 dilutions, including non-template controls (NTC), were prepared and the number of replicates was 6 (3 at two different days).

Results: A linear measuring range of $24 \le Cp \le LOD$ was defined for all assays. A Cp value of 24 with 5 120 copies of the target allele corresponds to 12.5 % of the minor DNA in a mixture with a total of 250 ng DNA per reaction.

e) Batch Variation and Test Performance at LoD

Aim: The concentration of the ingredients of Reaction Mix D and the Multi Taq 2 DNA Polymerase is crucial for the performance of qPCR tests. Hence, the influence of batch variations of these kit components were tested.

Method: Four batches of Reaction Mix D and three batches of Multi Taq 2 DNA Polymerase were tested. The assays Mentype® DIPquant HLD53-I, Mentype® DIPquant HLD84-I, Mentype® DIPquant HLD101-I, Mentype® DIPquant HLD70-D, and Mentype® DIPquant HLD88-D were used for measurement. The qPCR was performed under standard conditions with the control DNA (General Positive Control) of two different DNA concentrations (50 pg per PCR reaction and 5 ng per PCR reaction). For each concentration, three parallel samples were performed. In addition, three blank values (NTCs) were carried for each DIPquant assay and each batch.

Results: The results are depicted in Table 11 and Table 12.

Table 11 Variations between four batches of Reaction Mix D

Mentype® DIPquant	5 ng Template		50 pg Template	
	Mean Cp	δ	Mean Cp	δ
HLD53-I	28.66	0.06	34.36	2.19
HLD84-I	28.31	0.44	36.75	2.95
HLD101-I	29.59	0.04	36.59	2.48
HLD70-D	27.83	0.05	34.46	1.26
HLD88-D	28.92	0.07	35.96	0.65

Table 12 Variations between three batches of Multi Taq2 DNA Polymerase

Mentype® DIPquant	5 ng Template		50 pg Template	
	Mean Cp	δ	Mean Cp	δ
HLD53-I	28.54	0.11	32.21	0.85
HLD84-I	28.71	0.69	37.82	2.69
HLD101-I	29.56	0.18	34.99	0.51
HLD70-D	27.92	0.05	34.99	0.51
HLD88-D	29.01	0.09	35.79	0.52

f) Measurement on two different days

Aim: The measurements were performed on two different days to show the influence of pipetting two independent master mixes and the instrument on the performance of the assay.

Method: For the simulation of possible pipetting errors by the user, ± 10 % volume fluctuations of the PCR buffer and Multi Taq 2 DNA Polymerase were compared with the standard reaction on 3 performance strong and 3 performance weak qPCR assays. The qPCR was performed under standard conditions with the control DNA (General Positive Control) of two different DNA concentrations (50 pg per PCR reaction and 5 ng per PCR reaction). For each concentration, three parallel samples were performed. In addition, three blank values (NTCs) were carried for each DIPquant assay and each batch.

Results: Possible pipetting errors with a volume fluctuation of ± 10 % have no influence on the performance of the selected Mentype® DIPquant assays by using 5 ng GPC. The acceptance criterion is achieved for all assays and for each simulated pipetting error. There are no failures and no non-specific by-products have been detected.

Using 50 pg GPC per reaction, broader variations >2 Cp are possible. Therefore, the usage of calibrated equipment like pipettes is mandatory.

g) In-use Stability

Aim: The stability of the reagents of the qPCR kit was tested after repeated freezing and thawing. This simulates the actual routine use of the product in a simulated (accelerated) process.

Method: Four Mentype® DIPquant assays were selected as examples. The primer probe mixtures were subjected to an 8-fold freezing and thawing cycle. The freezing was carried out for at least 30 minutes at -20 °C. The mixture thawed at room temperature, and the reagents were homogenized by shaking before use. A standard reaction was then carried out. To avoid any additional influence from the use of different DNAs, the GPC was used as a template. Two different DNA concentrations were selected for the assay (50 pg per PCR reaction and 5 ng per PCR reaction). For each concentration, three parallel samples were performed. In addition, three NTCs were carried for each Mentype® DIPquant assay.

Results: Frequent freezing and thawing have no negative effect on the performance of the Mentype® DIPquant assays. Detection using the primer probe mixture is also possible after an 8-fold freezing and thawing cycle. The Cp values vary minimally, and deviation is within the range of the qPCR thermocycler oscillation.

Explanation of Symbols

🏭	Manufacturer
LOT	Batch code
Σ <N>	Contains sufficient for <N> tests
ℹ	Consult electronic instructions for use
⌛	Use-by date
-25°C -15°C	Temperature limit
REF	Catalogue number
☀	Keep away from sunlight
☂	Keep dry

Further markings used in this handbook:

ℹ

⚠	Attention, be sure to follow this notice!
blue underlined text	Links leading to external content like homepages, e-mail addresses
black underlined text	Cross-links in the document for easy navigation
Black, bold, cursive text	Fields which are to be clicked in a software

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For technical advice, please contact our Customer Support Team:

e-mail: support@biotype.de

phone: +49 351 8838 400

References

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Ordering information

Direct your orders via email to sales@biotype.de.

NOTE

Individual components of the kits cannot be ordered separately.

Product	Packaging size	Order number	Order number
Mentype® DIPquant Reference	25 reactions	BT00067	45-01591-0025
Mentype® DIPquant Reference	100 reactions	BT00068	45-01591-0100
Mentype® DIPquant SRY	25 reactions	BT00066	45-01590-0025
Mentype® DIPquant SMCY	25 reactions	BT00065	45-01589-0025
Mentype® DIPquant HLD23-I	25 reactions	BT00038	45-01538-0025
Mentype® DIPquant HLD38-I	25 reactions	BT00047	45-01558-0025
Mentype® DIPquant HLD48-I	25 reactions	BT00048	45-01560-0025
Mentype® DIPquant HLD53-D	25 reactions	BT00049	45-01561-0025
Mentype® DIPquant HLD53-I	25 reactions	BT00050	45-01562-0025
Mentype® DIPquant HLD67-D	25 reactions	BT00051	45-01567-0025
Mentype® DIPquant HLD67-I	25 reactions	BT00052	45-01568-0025
Mentype® DIPquant HLD70-D	25 reactions	BT00053	45-01569-0025
Mentype® DIPquant HLD70-I	25 reactions	BT00054	45-01570-0025
Mentype® DIPquant HLD79-I	25 reactions	BT00055	45-01576-0025
Mentype® DIPquant HLD82-D	25 reactions	BT00056	45-01577-0025
Mentype® DIPquant HLD82-I	25 reactions	BT00057	45-01578-0025
Mentype® DIPquant HLD84-D	25 reactions	BT00058	45-01579-0025
Mentype® DIPquant HLD84-I	25 reactions	BT00059	45-01580-0025
Mentype® DIPquant HLD88-D	25 reactions	BT00060	45-01581-0025
Mentype® DIPquant HLD88-I	25 reactions	BT00061	45-01582-0025
Mentype® DIPquant HLD91-D	25 reactions	BT00062	45-01585-0025
Mentype® DIPquant HLD91-I	25 reactions	BT00063	45-01586-0025
Mentype® DIPquant HLD97-I	25 reactions	BT00064	45-01588-0025
Mentype® DIPquant HLD101-D	25 reactions	BT00011	45-01501-0025

Product	Packaging size	Order number	Order number
Mentype® DIPquant HLD101-I	25 reactions	BT00012	45-01502-0025
Mentype® DIPquant HLD103-D	25 reactions	BT00013	45-01505-0025
Mentype® DIPquant HLD103-I	25 reactions	BT00014	45-01506-0025
Mentype® DIPquant HLD104-D	25 reactions	BT00015	45-01507-0025
Mentype® DIPquant HLD104-I	25 reactions	BT00016	45-01508-0025
Mentype® DIPquant HLD105-D	25 reactions	BT00017	45-01509-0025
Mentype® DIPquant HLD105-I	25 reactions	BT00018	45-01510-0025
Mentype® DIPquant HLD106-D	25 reactions	BT00019	45-01511-0025
Mentype® DIPquant HLD106-I	25 reactions	BT00020	45-01512-0025
Mentype® DIPquant HLD110-I	25 reactions	BT00021	45-01514-0025
Mentype® DIPquant HLD112-I	25 reactions	BT00022	45-01516-0025
Mentype® DIPquant HLD114-D	25 reactions	BT00023	45-01517-0025
Mentype® DIPquant HLD114-I	25 reactions	BT00024	45-01518-0025
Mentype® DIPquant HLD116-D	25 reactions	BT00025	45-01519-0025
Mentype® DIPquant HLD116-I	25 reactions	BT00026	45-01520-0025
Mentype® DIPquant HLD128-D	25 reactions	BT00027	45-01523-0025
Mentype® DIPquant HLD128-I	25 reactions	BT00028	45-01524-0025
Mentype® DIPquant HLD131-D	25 reactions	BT00029	45-01525-0025
Mentype® DIPquant HLD131-I	25 reactions	BT00030	45-01526-0025
Mentype® DIPquant HLD133-I	25 reactions	BT00031	45-01528-0025
Mentype® DIPquant HLD134-D	25 reactions	BT00032	45-01529-0025
Mentype® DIPquant HLD134-I	25 reactions	BT00033	45-01530-0025
Mentype® DIPquant HLD140-I	25 reactions	BT00034	45-01532-0025

Product	Packaging size	Order number	Order number
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Mentype® DIPquant HLD163-D	25 reactions	BT00036	45-01535-0025
Mentype® DIPquant HLD163-I	25 reactions	BT00037	45-01536-0025
Mentype® DIPquant HLD301-D	25 reactions	BT00039	45-01539-0025
Mentype® DIPquant HLD301-I	25 reactions	BT00040	45-01540-0025
Mentype® DIPquant HLD304-D	25 reactions	BT00041	45-01541-0025
Mentype® DIPquant HLD305-D	25 reactions	BT00042	45-01543-0025
Mentype® DIPquant HLD305-I	25 reactions	BT00044	45-01544-0025
Mentype® DIPquant HLD307-D	25 reactions	BT00043	45-01545-0025
Mentype® DIPquant HLD307-I	25 reactions	BT00045	45-01546-0025
Mentype® DIPquant HLD310-D	25 reactions	BT00046	45-01549-0025
Mentype® DIPscreen PCR Amplification Kit	25 reactions	45-12300-0025	
Mentype® DIPscreen PCR Amplification Kit	100 reactions	45-12300-0100	
DIP Positive Control (5 ng/µL)	20 reactions (100 µL)	27-13201-0100	
ChimerisMonitor RUO	Demo / 1-year / 3-years license	46-14801-0000	

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