Mentype® **DIPquant**

Instructions for Use

The qPCR Application for the Allele-Specific Quantification of the Chimerism Status

For in vitro diagnostic use

CE IVD

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REF
45-015xx*-0025
45-015xx*-0050
45-01591-0100

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xxx* - defines the locus-specific order number

Made in Germany

Biotype GmbH develops, produces, and distributes PCR-based applications for medical diagnostics.

Our Mentype® test kits guarantee highest quality standards.

We are at your disposal for further information and suggestions.
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Content

Mentype® **DIPquant**

1. Intended Use

Mentype® **DIPquant** applications are in-vitro diagnostic medical devices based on the real-time PCR technology (qPCR) for the allele-specific and quantitative analysis of molecular chimerism after allogenic bone marrow and blood stem cell transplantation by using deletion/insertion polymorphisms (DIPs, also referred to as INDELs: see Chapter 14, "References").

Mentype® **DIPquant** applications are intended exclusively for professional use in specialized laboratories. Personnel should be trained in the techniques of qPCR and in the use of in-vitro diagnostic medical devices (IVDD).

2. Background Information

It is crucial to analyze of molecular chimerism after allogeneic bone marrow and blood stem cell transplantation to monitor the transplant engraftment or to detect an imminent rejection reaction of the graft at an early stage. 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 patient and donor informative DIP loci using the Mentype® **DIPscreen** application, 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 advisable to analyze samples with mixed chimerism by using the Mentype® **DIPscreen** kit (Guideline on Allogeneic Stem Cell Transplantation of the German Association for Bone Marrow and Blood Cell Transplantation, Bader et al., 2016).

3. Product Description of Mentype® DIPquant

With 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 patient samples can be performed in parallel in one qPCR run.

The calculation of chimerism is based on the **ΔΔ**Cp 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 recipient DNA isolated before the transplantation (**pre-HSCT calibrator**) must be analyzed, together with the reference assay (β-globin gene) and the respective recipient-specific qPCR assay (see Chapter 9.1).

3.1 qPCR Instruments

Mentype® **DIPquant** was verified and validated with the Roche Lightcycler® 480 instrument II real-time PCR system (Roche Diagnostics International AG, Rotkreuz, CH).

The use of Mentype® **DIPquant** assays with other qPCR instruments must be verified and validated by the user.

3.2 Sample Type

Mentype® **DIPquant** assays were validated with the 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.

3.3 Sensitivity/Specificity

3.3.1 Measuring Range of Chimerism Samples

Owing to qPCR technology, the optimal measuring range of chimerism samples with Mentype® **DIPquant** assays is between 0 % and 12.5 % of recipient or donor DNA fractions in the patient sample (mixed sample). In this area, the qPCR setting can be applied as described in Chapter 9.2. For samples > 12.5 % of the recipient or donor DNA, as well as for mixed chimerism samples, it is recommended to increase the number of replicates or use the Mentype® **DIPscreen** kit.

3.3.2 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 submerged DNA 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/PCR [%]	0,013		0,025		0,05		0,2	
Mentype® **DIPquant** Assay 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						
	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						

4. Warnings and Safety Instructions

The following potentially hazardous substances are contained in this test kit:

Table 2. Potentially hazardous substances contained in Mentype® **DIPquant** assays

Kit component	Reagent	Danger
Reaction Mix **D**	Sodium azide NaN	Toxic if swallowed, toxic upon contact with acids

Please refer to the material safety data sheet (SDS) of Biotype® products that are sent on request by writing to support@biotype.de. For SDS of reagents not included in the test kit, please contact the respective manufacturer.

Read the instructions carefully before using the product.

Please check upon arrival of the product and its components for number, type and filling (see Chapter 5.1 Kit Content), correct labelling, frozen reagent status, and integrity of reagent packs.

When using the assays, wear gloves, a lab coat, and, if necessary, eye protection.

Avoid nuclease (DNase/RNase) contamination of the samples by using DNase/RNase-free disposable pipette tips with aerosol-tight filters.

Use separate workspaces and equipments for sample preparation (pre-PCR), master mix preparation, and sample post-processing and analysis (post-PCR). Store the positive controls spatially separated from the kit components.

Additional controls may be necessary in keeping with the guidelines or requirements of country-specific medical associations, authorities, or accreditation organizations.

Do not use components of the kit that have exceeded their expiration date. Do not mix any batches.

Discard sample and test waste in keeping with local safety regulations.

4.1 Quality Assurance

All contents of the test kit are subjected to intensive quality assurance by Biotype GmbH. The quality of the test kits is continuously checked to ensure unrestricted usability. Please contact us at info@biotype.de for questions regarding quality assurance.

5. Material Provided

5.1 Kit Content

The Mentype® **DIPquant** kit contains the following components that can be used to perform up to 100 reactions:

Table 3. Packaging sizes and included components of Mentype® **DIPquant** kit; *only available as Mentype® **DIPquant** Reference assays

Reagent	Volume per Packaging Size		
	25 reactions	**50 reactions**	**100 reactions***
Nuclease-free Water	1.5 ml	2 x 1.5 ml	2 x 1.5 ml
Reaction Mix **D**	125 µl	2 x 125 µl	1 x 500 µl
Mentype® **DIPquant HLDxxx-D/-I** Primer Mix	63 µl	2 x 63 µl	1 x 250 µl
MultiTaq2 DNA Polymerase	10 µl	2 x 10 µl	1 x 40 µl

5.2 Order Information

Please order Mentype® **DIPquant** kits via mail to sales@biotype.de, including the order numbers from Table 4 and Table 14 (page 21).

Table 4. General form of ordering numbers for Mentype® **DIPquant** assays; *xx defines locus-specific Mentype® **DIPquant** order number

Product Name	Packaging Size	Cat. No.
Mentype® **DIPquant**	25 reactions	45-015xx* -0025 (*see Table 14)
Mentype® **DIPquant**	50 reactions	45-015xx* -0050 (*see Table 14)
Mentype® **DIPquant** Reference Assay	100 reactions	45-01591-0100

5.3 Additionally Required Reagents and Equipment not included in the Kit

The following reagent is available for the selection of informative donor- and patient-specific Mentype® **DIPquant** assays in a single multiplex-PCR:

Table 5. Ordering information for genotyping kit Mentype® **DIPscreen;** *yyy defines the packaging size

Reagent	Provider	Order Number
Mentype® **DIPscreen**	Biotype GmbH	45-45410-0yyy*

Note: Primer binding sites of Mentype® **DIPquant** assays differ from those of Mentype® **DIPscreen**. In rare cases, mutations occurring in the primer binding sites can

generate allelic dropouts. Owing this, genotyping differences between Mentype® **DIPquant** assays and Mentype® **DIPscreen** may occur. So, Mentype® **DIPscreen** results should be always verified by pre-selected informative Mentype® **DIPquant** assays before using the latter in chimerism monitoring (see also 9.1, Quantification before Transplantation (pre-HSCT).

The following materials and instruments are required for qPCR amplification:

- Suitable real-time PCR instruments (see Chapter 3.1, qPCR Instruments)
- Suitable DNA purification kits (see Chapter 7.2.1, DNA Isolation)
- Suitable instrument for quantitative measurement of the DNA concentration after purification (see Chapter 7.2.1, DNA Isolation)
- A table centrifuge with a rotor for 2 ml reaction tubes
- 96-well reaction plates or reaction tubes for use with 96-well reaction plates, appropriate lids or sealing folia, and a centrifuge with a rotor for microliter plates
- A laboratory shaker (e. g. Vortex), suitable for 96-well reaction plates or reaction tubes
- Pipettes and disposable aerosol-tight filter pipette tips
- Disposable powder-free gloves

Note: Make sure that all the devices are installed, maintained, and calibrated in keeping with the manufacturers' instructions. Make sure that all the reagents for operating the respective qPCR device are present (for use, see instructions of the respective device manufacturer).

6. Storage

Mentype® **DIPquant** assays are delivered on dry ice. The ingredients of the assays are frozen. If one or more components are not frozen after reception or if the tubes are damaged during transportation, please contact Biotype GmbH for further assistance (support@biotype.de).

The components must be stored at -25 °C to -15 °C. Frequent thawing and freezing should be avoided. A maximum of 8 freeze-thaw cycles should not be exceeded.

The Mentype® **DIPquant** assays must be kept protected against light.

The shelf-life of the test kit is indicated on the packaging label.

7. Working Procedures

7.1 Overview of Chimerism Analysis with Mentype® DIP-Products

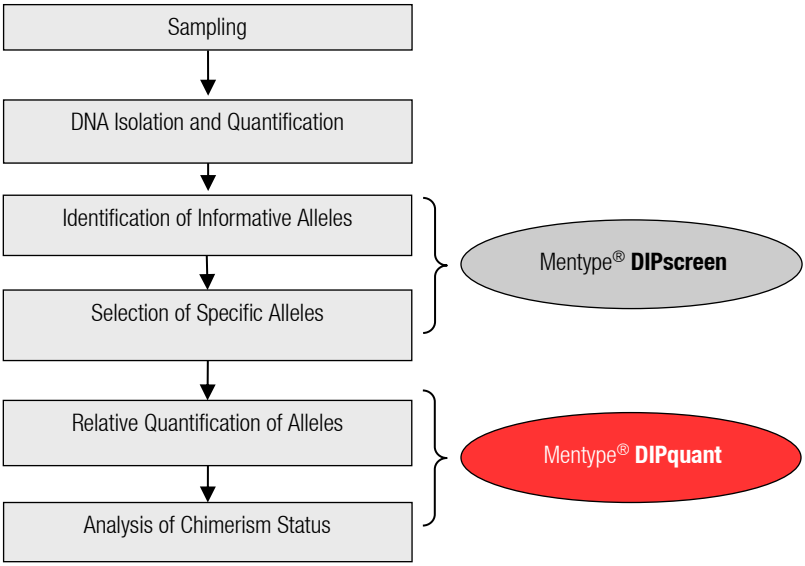

Figure 1. From sampling to analysis: chimerism analysis with Mentype® **DIPscreen** and Mentype® **DIPquant**

7.2 Sample Preparation and DNA Insert Volume

7.2.1 DNA Isolation

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.

Note: For accurate results, DNA quantification is required (e. g., DNA quantification by UV/VIS-spectroscopy at A260 nm and quality determination by the A260/A280 ratio, which should be between 1.7 and 2.0).

7.2.2 Template-DNA

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.

7.3 Applying the Master Mix

All reagents should be well-mixed (vortex) and centrifuged briefly (approx. 10 s) before applying the master mix. The volume of the DNA to be used depends on its concentration. For optimal results, the DNA concentration should be adjusted to 250 ng per reaction. The total volume of the PCR mixture should always be 25 µl.

Consider the positive and negative controls for the number of qPCR reactions to be applied. Add 1-2 additional reactions to the total number to compensate for pipetting errors.

Table 6 shows the volumes of kit components used with a sample volume of 5.0 µl (template DNA) and a reaction volume of 25 µl.

Table 6. Master mix set-up for one reaction Mentype® **DIPquant** using 5 µl DNA

Component	Volume per qPCR Reaction
Nuclease-free Water	12.1 µl
Reaction Mix **D***	5.0 µl
Mentype® **DIPquant** **HLDxxx-D/-I** Primer Mix	2.5 µl
Multi Taq2 DNA Polymerase (hot start, 2.5 U/µL)	0.4 µl
Total volume of master mix	**20.0 µl**
Template DNA (50 ng/µl)	5.0 µl

* contains Mg^{2+}, dNTPs, BSA

Note: Store your DNA samples in nuclease free water or dilute in TE buffer (10 mM Tris HCl, pH 8.0, and 1 mM EDTA), e. g. 0.1 x TE buffer.

7.3.1 Positive Control

For positive control, use 5 µl of a pre-typed, allele-specific positive control DNA (5 ng/µl) instead of the template DNA. Pipette the control DNA, instead of the template DNA, into the tubes containing the qPCR master mix.

Table 7 shows the volumes of the kit components used with 5.0 µl of a control DNA and a reaction volume of 25 µl.

Table 7. Master mix set-up for Mentype® **DIPquant** assays using a 5 ng/µl Positive Control Sample

Component	Volume per qPCR Reaction
Nuclease-free Water	12.1 µl
Reaction Mix **D***	5.0 µl
Mentype® **DIPquant** **HLDxxx-D/-I** Primer Mix	2.5 µl
Multi Taq2 DNA Polymerase (hot start, 2.5 U/µL)	0.4 µl
Total volume of master mix	**20.0 µl**
Control DNA (5 ng/µl)	5.0 µl

* contains Mg^{2+}, dNTPs, BSA

7.3.2 Negative Control

For negative control, pipette 5 µl of nuclease-free water instead of the template-DNA into the reaction vessels containing the qPCR master mix.

Table 8 shows the volumes of the kit components used with 5.0 µl of nuclease-free water and a reaction volume of 25 µl.

Table 8. Master mix set-up for Negative Control of Mentype® **DIPquant** assays using 5 µl Nuclease-free Water

Component	Volume per qPCR Reaction
Nuclease-free Water	12.1 µl
Reaction Mix **D***	5.0 µl
Mentype® **DIPquant** **HLDxxx-D/-I** Primer Mix	2.5 µl
Multi Taq2 DNA Polymerase (hot start, 2.5 U/µL)	0.4 µl
Total volume of master mix	**20.0 µl**
Nuclease-free Water	5.0 µl

* contains Mg^{2+}, dNTPs, BSA

7.4 Reaction Volume

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 Chapter 9.1 and 9.2) or 5 µl of positive or negative Control.

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

The reaction vessels 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.

8. qPCR Program and Amplification

8.1 Instrument Settings and Amplification Parameters

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.

8.2 Detection Parameters

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.

8.3 qPCR Amplification Parameters*

Multi Taq2 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 9. qPCR amplification parameter to be used for Mentype® **DIPquant** assays

Temperature	Time	
94 °C	4 min (hot start to activate Multi Taq2 DNA Polymerase)	
94 °C	30 s	45 Cycles
62 °C	**45 s**	

* Validated with Roche Light Cycler® LC480 (Standard Heating-Rates of 4.4 °C/s and Cooling-Rates of 2.2 °C/s).

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

Create a sample list with the selected settings.

9. Recommended Set-up for Analysis

The recipient's portion in the mixed sample should be measured to address the optimal measuring range of qPCR (see Chapter 3.3.1)

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 10), **or**
- **2** different **recipient-specific alleles** (Allele of Interest, AOI) in **triplicates** (see Table 11)
- Per DNA and time-point, the **active reference** (REF, ß-Globin) must be measured at least in **duplicates** (better in triplicates)
- in each assay, a **negative (NTC)** and **positive Control (PC)** should be run additionally

Table 10. Set-up 1: use of 3 specific Mentype® **DIPquant** in duplicates and the Mentype® **DIPquant** Reference Assays in triplicates

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

Table 11. Set-up 2: use of 2 specific Mentype® **DIPquant** in triplicates and the Mentype® **DIPquant** Reference Assays in duplicates

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

9.1 Quantification before Transplantation (pre-HSCT)

The recipient DNA before transplantation (pre-HSCT calibrator) must be analyzed together with the reference assay (β-globin gene) and the respective recipient-specific qPCR assays to calibrate the analysis. The value of this quantification is set at the 100 % recipient level.

To ensure the specificity of the recipient-specific qPCR assays, an analysis of the donor DNA should be carried out. This corresponds to the 0 % recipient level.

Table 12. Example for the set-up of a multi-well plate before transplant (pre-HSCT calibrator)

	1	2	3	4
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
E		AOI-1 Donor	AOI-2 Donor	AOI-3 Donor
F		AOI-1 Donor	AOI-2 Donor	AOI-3 Donor

REF: active reference assay; AOI 1-3: recipient-specific assays; preTx: recipient DNA before transplantation as calibrator; Donor: donor sample to test on specificity; NTC: No Template Control; PC: positive control

9.2 Quantification after Transplantation/Monitoring (post-HSCT)

Chimerism monitoring should be done with the patient DNA being freshly isolated at the respective monitoring times. For safe analysis, the active reference, three recipient-specific alleles and positive and negative controls should be carried out (see Table 13).

Table 13. Example for the set-up of a multi-well plate after transplantation (monitoring)

	1	2	3	4
A	Ref Monitoring 1	AOI-1 Monitoring 1	AOI-2 Monitoring 1	AOI-3 Monitoring 1
B	Ref Monitoring 1	AOI-1 Monitoring 1	AOI-2 Monitoring 1	AOI-3 Monitoring 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

REF: active reference assay; AOI 1-3: recipient-specific assays; Monitoring 1: first monitoring sample after transplantation; NTC: No Template Control; PC: positive control

10. Analysis

10.1 Data Analysis

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 **ΔΔ**Cp 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.

10.2 Verification of Results

The qPCR run is valid if the Cp values of positive control corresponds 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.

11. 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 **ΔΔ**Cp 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.

Calculation

11.1 Quantification of Pre-HSCT Samples, Calibrator

1. Calculate individual Cp values for the Reference (REF) and the informative alleles “Alleles of Interest” (AOI) for the recipient DNA
2. Calculate the **Δ**Cp for each AOI to the REF gene (**Δ**Cp C = Cp AOI – Cp REF)
3. The **Δ**Cp value equals the calibrator (**Δ**Cp C) in the post-HSCT calculation (100 % recipient)

11.2 Quantification of Post-HSCT Samples

1. Calculate the individual Cp values for the Reference (REF) and the informative alleles “Alleles of Interest” (AOI) for the recipient DNA
2. Calculate the **Δ**Cp for each AOI to the REF gene (**Δ**Cp C = Cp AOI – Cp REF)
3. The resulting **Δ**Cp value will be used to calculate the unknown status **Δ**Cp U
4. Calculate **ΔΔ**Cp to quantify the chimerism (**ΔΔ**Cp = **Δ**Cp U – **Δ**Cp C)
5. Calculate the percentage % of the recipient component that is dependent on the efficiency of the qPCR; % recipient = $((1+E)^{-(\Delta\Delta Cp)})$ x 100. In case of the qPCR efficiency of 100%, use the reduced formula $(2^{-(\Delta\Delta Cp)})$ x 100.

12. Interpretation of Unexpected Results

12.1 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 Taq2 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 the 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 in the correct fluorescence channel. Check the settings of your qPCR instrument for the fluorescence color (6-FAM and ROX) 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 1x or 0.1x TE. Use a 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.

12.2 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 the 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.

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.

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 Chapter 7.2.1).

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.

12.3 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 also Chapter 5.3, 9.1). 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 monitoring.

13. Order Information

Table 14. Detailed ordering information for allele-specific Mentype® **DIPquant** assay

DIPquant Assay	25 Reactions	50 reactions
Reference*	45-01591-0025	45-01591-0050
SRY	45-01590-0025	45-01590-0050
SMCY	45-01589-0025	45-01589-0050
HLD23-I	45-01538-0025	45-01538-0050
HLD38-I	45-01558-0025	45-01558-0050
HLD48-I	45-01560-0025	45-01560-0050
HLD53-D	45-01561-0025	45-01561-0050
HLD53-I	45-01562-0025	45-01562-0050
HLD67-D	45-01567-0025	45-01567-0050
HLD67-I	45-01568-0025	45-01568-0050
HLD70-D	45-01569-0025	45-01569-0050
HLD70-I	45-01570-0025	45-01570-0050
HLD79-I	45-01576-0025	45-01576-0050
HLD82-D	45-01577-0025	45-01577-0050
HLD82-I	45-01578-0025	45-01578-0050
HLD84-D	45-01579-0025	45-01579-0050
HLD84-I	45-01580-0025	45-01580-0050
HLD88-D	45-01581-0025	45-01581-0050
HLD88-I	45-01582-0025	45-01582-0050
HLD91-D	45-01585-0025	45-01585-0050
HLD91-I	45-01586-0025	45-01586-0050
HLD97-I	45-01588-0025	45-01588-0050
HLD101-D	45-01501-0025	45-01501-0050
HLD101-I	45-01502-0025	45-01502-0050
HLD103-D	45-01505-0025	45-01505-0050
HLD103-I	45-01506-0025	45-01506-0050
HLD104-D	45-01507-0025	45-01507-0050
HLD104-I	45-01508-0025	45-01508-0050
HLD105-D	45-01509-0025	45-01509-0050
HLD105-I	45-01510-0025	45-01510-0050
HLD106-D	45-01511-0025	45-01511-0050
HLD106-I	45-01512-0025	45-01512-0050
HLD110-I	45-01514-0025	45-01514-0050
HLD112-I	45-01516-0025	45-01516-0050

DIPquant Assay	25 Reactions	50 reactions
HLD114-D	45-01517-0025	45-01517-0050
HLD114-I	45-01518-0025	45-01518-0050
HLD116-D	45-01519-0025	45-01519-0050
HLD116-I	45-01520-0025	45-01520-0050
HLD128-D	45-01523-0025	45-01523-0050
HLD128-I	45-01524-0025	45-01524-0050
HLD131-D	45-01525-0025	45-01525-0050
HLD131-I	45-01526-0025	45-01526-0050
HLD133-I	45-01528-0025	45-01528-0050
HLD134-D	45-01529-0025	45-01529-0050
HLD134-I	45-01530-0025	45-01530-0050
HLD140-I	45-01532-0025	45-01532-0050
HLD152-D	45-01533-0025	45-01533-0050
HLD163-D	45-01535-0025	45-01535-0050
HLD163-I	45-01536-0025	45-01536-0050
HLD301-D	45-01539-0025	45-01539-0050
HLD301-I	45-01540-0025	45-01540-0050
HLD304-D	45-01541-0025	45-01541-0050
HLD305-D	45-01543-0025	45-01543-0050
HLD305-I	45-01544-0025	45-01544-0050
HLD307-D	45-01545-0025	45-01545-0050
HLD307-I	45-01546-0025	45-01546-0050
HLD310-D	45-01549-0025	45-01549-0050

* also available as packaging size 100 reactions (45-01591-0100)

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15. Trademarks and Disclaimers

Registered names, trademarks, etc. used in this document, even if not specifically marked as such, are not to be considered unprotected by law: Biotype®, Mentype® (Biotype GmbH); LightCycler® (Roche Diagnostics International AG); QIAamp® (QIAGEN GmbH); NucleoSpin® (Macherey-Nagel GmbH & Co. KG); FAM™, ROX™ (Life Technologies Ltd.).

Mentype® **DIPscreen** and Mentype® **DIPquant** assays are CE-marked kits according to the European In-vitro Diagnostic Device Directive 98/79/EC. The kits are not available as in-vitro diagnostic devices outside this regulatory area.

16. Symbols

Manufacturer

Consult instructions for use

Catalogue number

In-Vitro-Diagnostic Medical Device

Verification and Validation of Mentype® DIPquant qPCR-Amplifications Kits

A Analytical Performance Data (Verification)

A 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. For preanalytics, DNA isolation and DNA quantification, see Chapter B b).

A b) Analytical Specificity and Limit of Blank (LoB)

Aim: The product consists of 57 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).

A 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 $LoB - (LOD + 2 \times \delta) \geq 2$, where LoB is the Cp value determined non-parametrically according to A 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.

A d) Measuring Range of the Assays

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

Method: The experiments included all data from A b) and A 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 ≤ Cp ≤ 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.

A e) Batch Variation and Test Performance at LoD

Aim: The concentration of the ingredients of Reaction Mix D and the Multi Taq2 DNA Polymerase are 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 MultiTaq2 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 15 and Table 16.

Table 15. 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 16. 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

A 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 MultiTaq2 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.

A 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 was 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 has 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.

B Clinical Performance Data

B a) Ethical and Regulatory Aspects

A clinical performance evaluation according to §§ 20-24 of the German Medical Devices Act was conducted with a waiver from the approval for medical devices with low-safety risk granted by the German National Competent Authority. The protocol was approved by the local ethics committee of a clinical study center. All the participants were adult, sui juris, and gave written informed consent.

B b) Preanalytics, DNA Isolation and DNA Quantification

Venous ETDA blood samples were used (e. g., S-Monovette® K2E, Sarstedt AG & Co. KG, Nuembrecht, DE). DNA isolation from whole blood was carried out with the QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, DE) according to the manufacturer's instruction. DNA concentrations were determined via ultraviolet–visible absorption spectroscopy at 260 nm.

B c) Concordance Analysis

All patients were sex mismatched after allogeneic hematopoietic stem cell transplantation to allow genotyping by fluorescence in situ hybridization (FISH) by using the gonosome specific CE-IVD CEP® X SpectrumOrange™/Y SpectrumGreen™ Direct Labeled Fluorescent DNA Probe Kit (Abbott GmbH & Co KG, Wiesbaden, DE). A PCR-based chimerism analysis was performed with Mentype® **DIPquant** (qPCR), Mentype® **DIPscreen** (Biotype GmbH), multiplex-PCR combined with capillary electrophoresis using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, US-CA), and Mentype® **Chimera**® (Biotype GmbH), multiplex-PCR combined with capillary electrophoresis using an ABI Prism® 3100 Genetic Analyzer. Using Mentype® **DIPquant,** 250 ng of DNA per reaction were applied. All the kits were used in keeping with the manufacturers' instructions for use.

B d) Results and Discussions

At first, all informative STR and DIPsystems of the donor-recipient pairs were determined. In addition, sex was confirmed by genotyping with the amelogenin marker, which is also part of Mentype® **DIPscreen** and Mentype® **Chimera**®. A total of 54 ETDA blood samples were collected from 6 patients on different days after blood stem cell transplantation. The mean of all informative STR and DIP biomarkers (2-7) was used for chimerism analysis in case of multiplex-PCR genotyping combined with capillary electrophoresis. In case of Mentype® **DIPquant,** three informative qPCR assays and the reference-gene were selected, as described in Chapter 10.2, according to the instruction for use, and then, analyzed in duplicates. In general, the minor allele was determined.

The results of the comparison tests are depicted in Figure 2 to Figure 4.

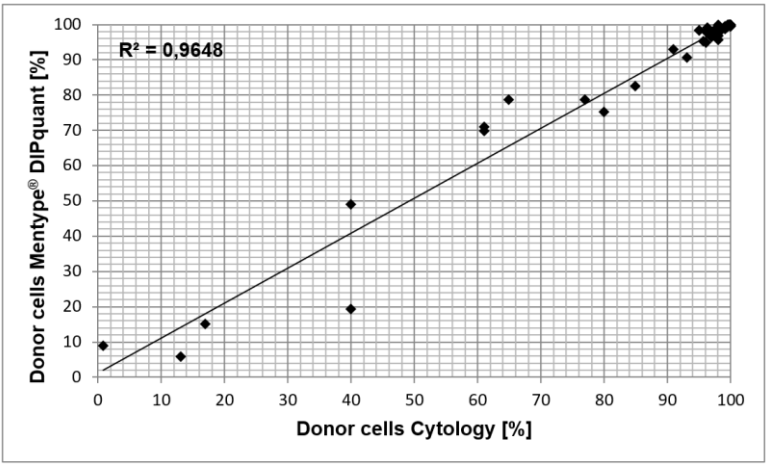

Figure 2. Comparison of Mentype® **DIPquant** and cytology for chimerism analysis

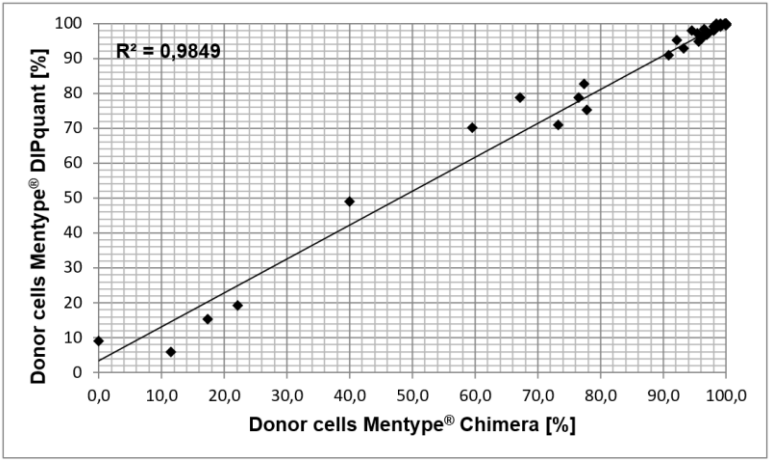

Figure 3. Comparison of Mentype® **DIPquant** and Mentype® **Chimera**® for chimerism analysis

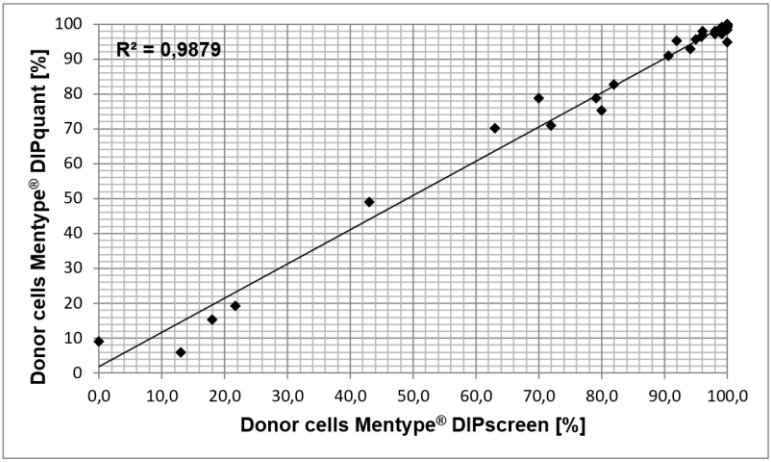

Figure 4. Comparison of Mentype® **DIPquant** and Mentype® **DIPscreen** for chimerism analysis

The coefficient of determination (R squared) of Mentype® **DIPquant** in comparison with FISH, Mentype® **Chimera**®, and Mentype® **DIPscreen** was 0.9648, 0.9849, and 0.9879 respectively. The best concordance was achieved with Mentype® **DIPscreen,** which possesses the same biomarkers: lesser concordance of FISH reflects technical differences. At least 200 cells should be counted according to the manufacturer's instruction for use. However, better results are achieved with more than 500 cell counts (Buño et al. 2005) - this was not obtained with all the samples.

The scientific validity of all tested biomarkers for chimerism analysis has been frequently shown in the literature (Thiede et al. 2001; Thiede and Lion 2001; Wilhelm et al. 2002; Buño et al. 2005), and PCR based tests are already accepted in clinical guidelines (Bader et al. 2016). At first, FISH was used as a comparative test. This technique still possesses a local value in sex mismatched transplantations. However, it shows only a sensitivity level of 1% for the minor cell population. Multiplex-PCR based on short tandem repeats (STR), such as Mentype® **Chimera**®, is often referred to as the golden standard of chimerism analysis (Bader et al. 2016). Biallelic markers like DIPs offer technical advantages like no stutter peaks in capillary electrophoresis or their suitability for qPCR, which can reach sensitivity levels of less than 0.1% (Wilhelm et al. 2002; Bader et al. 2016).

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Notes

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