



PlentiPlex™ KIT

Mastocytosis qPCR Assay

In vitro Diagnostic Assay for Sensitive Detection of KIT D816V

INSTRUCTIONS FOR USE

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REFERENCE NUMBERS

Dispense Ready (DR)
7032 (20 reactions)
Ready-to-Use (RTU)
7030 (12 reactions)

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1 Intended purpose

PlentiPlex™ KIT Mastocytosis qPCR Assay is a semi-quantitative real-time Polymerase Chain Reaction (PCR) assay intended for *in vitro* diagnosis of the genomic change giving rise to the aspartic acid to valine mutation in codon 816 of the human KIT proto-oncogene receptor tyrosine kinase (KIT D816V). Samples are genomic DNA (gDNA) purified from blood samples. The obtained results of PlentiPlex™ KIT Mastocytosis qPCR Assay are intended for the identification of the presence and/or quantity of the KIT D816V mutation as part of the World Health Organization (WHO) diagnostic criteria to assist the diagnosis of Mastocytosis.

1.1 Intended user

PlentiPlex™ KIT Mastocytosis qPCR Assay is intended for use by healthcare professionals or qualified laboratory personnel instructed and trained in the techniques of real-time PCR as well as proficient in handling biological samples. Medical interventions based on results from this product requires medical authorisation.

2 Background

Mastocytosis is a disorder characterised by growth and accumulation of neoplastic mast cells within various organs and most commonly the skin. The WHO criteria for diagnosis of systemic mastocytosis include identification of at least one major criterion as well as one minor criterion or identification of at least three minor criteria. The major systemic mastocytosis criterion is multifocal dense infiltrates of mast cells (≥ 15 mast cells in aggregates) in bone marrow biopsies and/or in sections of other extracutaneous organ(s). The minor criteria are: 1) more than 25% of all mast cells are atypical cells (type I or type II) on bone marrow smears or are spindle-shaped in mast cell infiltrates detected on sections of visceral organs, 2) *KIT* point mutation at codon 816 in the bone marrow or another extracutaneous organ, 3) mast cells in bone marrow or blood or another extracutaneous organ exhibit CD2 and/or CD25, and 4) baseline serum tryptase level >20 ng/mL (in case of an unrelated myeloid neoplasm, item 4 is not valid as a systemic mastocytosis criterion)¹. Although the fraction of cells carrying the *KIT* D816V mutation is often very low ($<0.1\%$ mutated allele), presence of the mutated allele is well correlated with mastocytosis.

3 Test principle

3.1 Explanation of the assay

PlentiPlex™ KIT Mastocytosis qPCR Assay CE IVD combines allele-specific PCR with PentaBase's novel and selective technologies comprising HydrolEasy® probes, SuPrimers™ and BaseBlockers™ for specific and sensitive target amplification. The PlentiPlex™ KIT Mastocytosis qPCR Assay is based on PentaBase's unique DNA technologies (referred to as pentabases) which are synthetic DNA analogues comprising a flat heteroaromatic, hydrophobic molecule and a linker. They are inserted into the oligonucleotides at fixed positions during synthesis of the oligonucleotides. The PlentiPlex™ KIT Mastocytosis qPCR Assay contain both standard oligonucleotides and oligonucleotides modified with pentabases.

3.1.1 HydrolEasy® probe

A HydrolEasy® probe is similar to a standard hydrolysis probe (also referred to as a TaqMan® probe²) labelled with a fluorophore at the 5' end and a quencher at the 3' end, but with the addition of pentabases. HydrolEasy® probes are based on oligonucleotides modified with pentabases, giving the probe a significantly improved signal-to-noise ratio, higher specificity, and higher sensitivity compared to conventional hydrolysis probes. HydrolEasy® probes in the PlentiPlex™ KIT Mastocytosis qPCR Assay are labelled with PentaGreen™ (detected on the same channel as FAM) in combination with Green Quencher™, or as PentaYellow™ (detected on the same channel as HEX, VIC^{®3}, TET) in combination with Yellow Quencher™.

3.1.2 SuPrimers™

SuPrimers™ are standard DNA primers modified with one or more pentabases. Pentabases in primers may provide increased specificity, sensitivity, and reduce primer-dimer formation.

3.1.3 BaseBlockers™

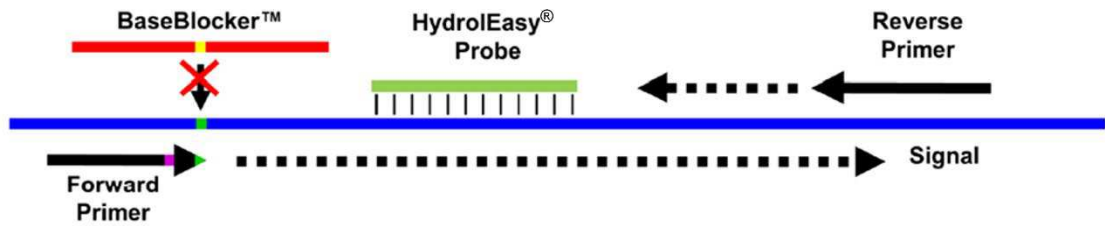
BaseBlockers™ are oligonucleotides modified with several pentabases, allowing for specific and strong binding to a target sequence (**Figure 1**). In the PlentiPlex™ KIT Mastocytosis qPCR Assay, the BaseBlockers™ are designed to bind to wild-type DNA targets, suppressing wild-type DNA amplification by hindering the binding of the mutation specific primers to the wild-type template. The presence of BaseBlockers™ ensures high specificity and robustness of the assay. Along with SuPrimers™, the BaseBlockers™ minimise the risk of false positive signals.

¹ Valent P, Akin C, Metcalfe DD. (2017). Mastocytosis: 2016 updated WHO classification and novel emerging treatment concepts. *Blood* 2017 Mar 16;129(11):1420-1427. DOI: [10.1182/blood-2016-09-731893](https://doi.org/10.1182/blood-2016-09-731893)

² Taqman is a registered trademark of Roche Molecular Systems, Inc

³ VIC is a registered trademark of Applied Biosystems, Inc.

Mutant allele



Wild-type allele

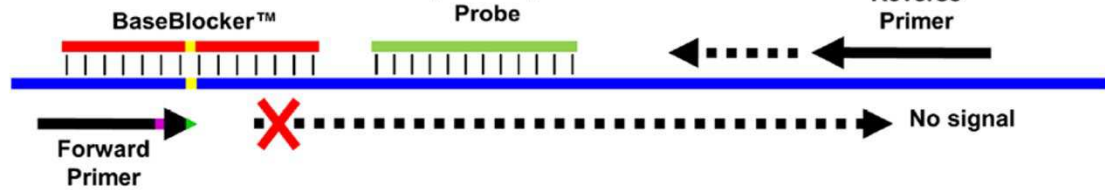


Figure 1. Illustration of how BaseBlockers™ function in PlentiPlex™ assays. A BaseBlocker™ binds to and blocks the wild-type template from being amplified (modified from Riva et al. 2017⁴). In contrast, the BaseBlocker™ does not inhibit amplification of a template with a single nucleotide mutation and the result is a selective amplification of mutated DNA in a wild-type background.

3.2 Product variants

PlentiPlex™ KIT Mastocytosis qPCR Assay is supplied as either Dispense Ready (DR) and Ready-to-Use (RTU). The DR version includes Primer-Probe Mix and Master Mix in separate tubes, which need to be dispensed into suitable plasticware before addition of template. The RTU version is pre-dispensed and only need the addition of template before real-time qPCR.

3.3 Principle of the procedure

3.3.1 Internal control

An internal control assay is included in both the reference and mutation-specific assays. It is comprised of a HydrolyEasy® probe labelled with PentaYellow™ (measured on the same fluorescence channel as HEX, VIC® and TET) and a primer set. The internal control assay is used to assess whether template has been added and that amplification has taken place in reactions with negative signal from the PentaGreen™ labelled assay in the same reaction. The primers in the control assay are designed to be inefficient and are located outside the area of all frequently known mutations. In this way, the internal control assay will have as little impact on the effectiveness of the reference and the mutation-specific assays as possible. The signal from the internal control assay may be affected by positive amplification in the reference and mutation-specific assays.

3.3.2 Reference assay

The reference assay primers target a genomic region with no known sequence variations and is used to assess the amount of amplifiable DNA in the sample. The reference assay contains a HydrolyEasy® probe labelled with PentaGreen™ (measured on the same channel as FAM), a mutation-independent primer set, and an internal control assay. The reference assay runs in its own tube or well. The fluorescence signal of the reference assay is used for calculating the threshold value which is again used to determine the cycle threshold (Ct) of the assay of interest. Cycle threshold for the reference assay is a measure of amount of amplifiable DNA in the evaluated sample and used for calculation of the mutation ΔCt.

3.3.3 Mutation assay

The mutation-specific assay targets the genomic region containing the KIT D816V mutation (c.2447A>T) and is used to determine the presence of the mutation in a sample (**Table 1**) The mutation assay contains a HydrolyEasy® probe labelled with PentaGreen™ (measured on the FAM channel), a BaseBlocker™ (to reduce or eliminate non-specific amplification of wild type), a mutation-specific primer set, and an internal control assay. The Ct values of the mutation assay and the corresponding reference are used to determine whether a sample is positive or negative for a given mutation.

Table 1. The KIT mutation detected with PlentiPlex™ KIT Mastocytosis qPCR Assay.

Assay	CDS mutation	Amino acid substitution	Cosmic ID
KIT D816V	c.2447A>T	p.Asp816Val (D816V)	COSM1314

⁴Riva et al., 2017; PMID: 28636636

4 Reagents and materials

The materials provided with the PlentiPlex™ KIT Mastocytosis qPCR Assay can be found in **Table 2**. Materials and instruments required, but not provided can be found in **Table 3**.

4.1 Storage

Refer to the label for expiry date. Repeated thawing and freezing should be kept to a minimum and should not exceed 10 freeze-thaw cycles.

4.1.1 In-use stability

When in use, the assay components should be returned to the freezer promptly after use to minimise the time at room temperature and exposure to light.

Please disposed the component following your local guidelines on disposal of plastic waste including non-hazardous PCR components. The reagents are not for reuse.

4.2 Materials provided

Table 2. List of materials provided with the PlentiPlex™ KIT Mastocytosis qPCR Assay and the mutation detected as either Dispense Ready (DR) or Ready-to-Use (RTU).

Product variant	Description	Tube no.		Kit components	Mutation	Contents
		DR	RTU			
Y1	PlentiPlex™ KIT Mastocytosis qPCR Assay	1	A	KIT Reference 1	-	Synthetic DNA
		2	B	KIT Simplex 1	D816V	Synthetic DNA
		-	C	KIT Simplex 1	D816V	Synthetic DNA
		-	D	KIT Simplex 1	D816V	Synthetic DNA
		3	-	AmpliQueen™ qPCR Master Mix	-	qPCR Master Mix

4.3 Materials and instruments required but not provided

Materials and instruments required but not provided are listed in **Table 3**. PlentiPlex™ KIT Mastocytosis qPCR Assay is designed to run on open platforms with at least two-channels (green and yellow). Other real-time PCR platforms are likely applicable, but it is highly recommended that a specific validation is performed using clinical samples and reference controls when running on other instruments than in **Table 3** to verify cycle thresholds and cut-offs.

Table 3. Materials and instruments required but not provided.

Materials
Pipettes (1-10 µL, 10-100 µL)
Sterile pipette Tips
Centrifuge for spinning PCR tubes, strips or plates
Sterile water for No Template Control (NTC)
DNA extraction method or instrument
DNA extraction method or instrument (e.g., BasePurifier™, PentaBase A/S, Ref.No. 715)
DNA Extraction kit for FFPE samples (e.g., Nucleic Acid Extraction Kit for FFPE DNA Extraction, Xi'an TianLong Science and Technology Co., Ltd., distributed by PentaBase A/S, Ref. No. T165H)
Real-time qPCR instrument
Real-time PCR instruments including: QuantStudio™ (Applied Biosystems), BaseTyper (PentaBase A/S), MyGo Pro (IT-IS Life Science), MIC (Bio Molecular Systems)

5 Warnings and precautions

- For *in vitro* diagnostics use.
- The mutational status determined by the PlentiPlex™ KIT Mastocytosis qPCR Assay should always be considered alongside other clinical symptoms and diagnosis when making treatment decisions.
- Good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary. Wear laboratory coats, laboratory gloves, and hair protection to avoid contamination. Work in clean environments to avoid contaminations.
- Do not use reagents that have expired.
- Do not use damaged PlentiPlex™ KIT Mastocytosis qPCR Assay tubes.
- Do not use a PlentiPlex™ KIT Mastocytosis qPCR Assay pre-dispensed in a Ready-to-Use PCR tube that has been dropped while open.
- Do not open the tubes or unseal wells during or after amplification following completing the PCR program.
- Verify eligibility of the DNA samples as DNA samples can be non-homogeneous and of varying quality, which might affect the analysis. In cases of suspected DNA degradation, it is recommended to verify DNA integrity and the amount of amplifiable DNA by a PCR-based method.
- The internal control assay contains suboptimal primer concentrations and amplification may be inhibited by amplification by the PentaGreen™ labelled assay in the same reaction. Thus, the result is valid in cases where there is only amplification in the green (FAM/SYBR) channel.
- Be aware of the placement and orientation of the PCR tubes in the PCR machine in relation to how the samples are named in the PCR software.
- Baseline drift, a slowly rising signal in the amplification plot with no or late exponential phase, may lead to false positive results if not corrected.
- Consult relevant real-time qPCR Instrument User Guides for additional warnings, precautions, and procedures to reduce the risk of contamination.
- Dispose of used PlentiPlex™ KIT Mastocytosis qPCR Assay tubes, pipette tips, and specimen tubes according to local, state, and federal regulations for laboratory and plastic waste. The device does not pose any biohazard risks.
- Minimise the exposure of PlentiPlex™ KIT Mastocytosis qPCR Assay to light due to the presence of light sensitive HydrolEasy® probes.
- The reagents should not be diluted to a lower concentration than stated in the protocol. This may affect the performance of the assay.
- Do not substitute the reagents with others, as it may affect the performance of the assay.
- Inadequate or inappropriate sample collection, storage, and transport may yield incorrect or invalid results.

6 Sample handling

6.1 Sample collection, transport, and storage

Specimens should be human genomic DNA extracted from whole blood samples or similar. It is recommended that whole blood samples are collected, transported, processed, and stored according to ISO 20186-2:2019⁵ to ensure optimal DNA quality.

6.2 Sample purification

Extraction of genomic DNA from whole blood samples should be performed using genomic DNA extraction kits and/or procedures specially designed for handling of whole blood samples according to the manufacturer's instructions. It is recommended to evaluate DNA integrity and amplifiability by PCR-based methods according to ISO 20186-2:2019.

⁵ ISO 20186-2:2019 Molecular in vitro diagnostic examinations — Specifications for venous whole blood - Part 2: Isolated genomic DNA

7 Procedure

Analyse each sample with both the reference assay and the mutation-specific assay. These should be analysed in the same PCR run to ensure minimal variation. The mutation-specific assay should be run in triplicates.

7.1 Dispense Ready

1. Add 12.5 µL AmpliQueen™ qPCR Master Mix to each PCR tube (vial, strip or plate).
2. Add 7.5 µL of Reference Mix to one PCR tubes per patient.
3. Add 7.5 µL of Mutant Mix to three PCR tubes per patient.
4. Add 5 µL of extracted DNA (1-40 ng/µL) from each sample to the mutation assay and the corresponding reference. It is recommended to include an NTC in each run. Add sterile water to the NTC instead of DNA.
5. Seal all tubes.
'Recommended step: Briefly vortex strips (2-3 sec.) to enhance elimination of air bubbles'
6. Spin down the PCR tubes (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the tubes to eliminate air bubbles.
7. Place the PCR tubes in the real-time PCR instrument and run the specified real-time qPCR program (**Table 4**).

7.2 Ready-to-Use

1. Spin down the PCR strips (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the tubes to eliminate air bubbles.
2. Add 5 µL of extracted DNA (1-40 ng/µL) from each sample to the reference well and to the three wells with mutation assay. It is recommended to include an NTC in each run. Add sterile water to the NTC instead of DNA.
3. Seal all tubes.
'Recommended step: Briefly vortex strips (2-3 sec.) to enhance elimination of air bubbles'
4. Spin down the PCR tubes (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the tubes to eliminate air bubbles.
5. Place the PCR tubes in the real-time PCR instrument and run the real-time qPCR program (**Table 4**).

7.3 Real-time qPCR program

Table 4. Real-time qPCR program for running PlentiPlex™ KIT Mastocytosis qPCR Assay.

Protocol	Temperature [°C]	Time [sec]	Ramping (°C/s)	Cycles	Channel
Stage 1					
Hold	95	120	-	-	
Stage 2 (1-45)					
Amplification	94	15	1.6	45	FAM/SYBR (green) HEX/VIC®/TET (yellow)
	60	40	1.6		

8 Data Analysis

8.1 Baseline and threshold settings

The data analysis procedure for the PlentiPlex™ KIT Mastocytosis qPCR Assay involves determining the cycle threshold (Ct). The Ct is defined as the cycle in which the fluorescence signal of a given assay exceeds the threshold value which is set as part of the analysis procedure. The Ct value reflects the amount of amplifiable target DNA present in the sample. The threshold should be set to 10% of the reference assay (Tube A) fluorescence signal at cycle 45 for each sample (**Figure 2**). The threshold should be set for each channel individually. The mutation analysis is performed in the green (FAM) channel and the internal control analysis is performed in the yellow (HEX/VIC) channel.

Before setting the threshold and calculating the Ct values, it is important that any baseline drift or fluctuation is corrected so that the normalised baseline or background fluorescence is as close to zero as possible. Baseline drift is a slowly rising signal in the amplification plot with no or late exponential phase and can occur when baselining has not been done properly. Different instrument manufacturers use different approaches to adjust the baseline. These include slope correction, curve fitting, setting a baseline cycling interval, and ignoring the first cycles in the run. Please refer to instrument-specific guidelines for specific instructions when available or contact PentaBase A/S or your local distributor for technical assistance.

If baseline drift cannot be corrected and/or there is any doubt about the quality of the amplification curve, the sample should be rerun.

In cases where it is not possible to adjust the normalised background fluorescence to zero, the value of background fluorescence at cycle 20 should be added to the threshold value calculated by taking 10 % of the reference signal at cycle 45 (**Table 5**).

NOTE: If baseline drift cannot be corrected and/or there is any doubt about the quality of the amplification curve, the sample should be rerun.

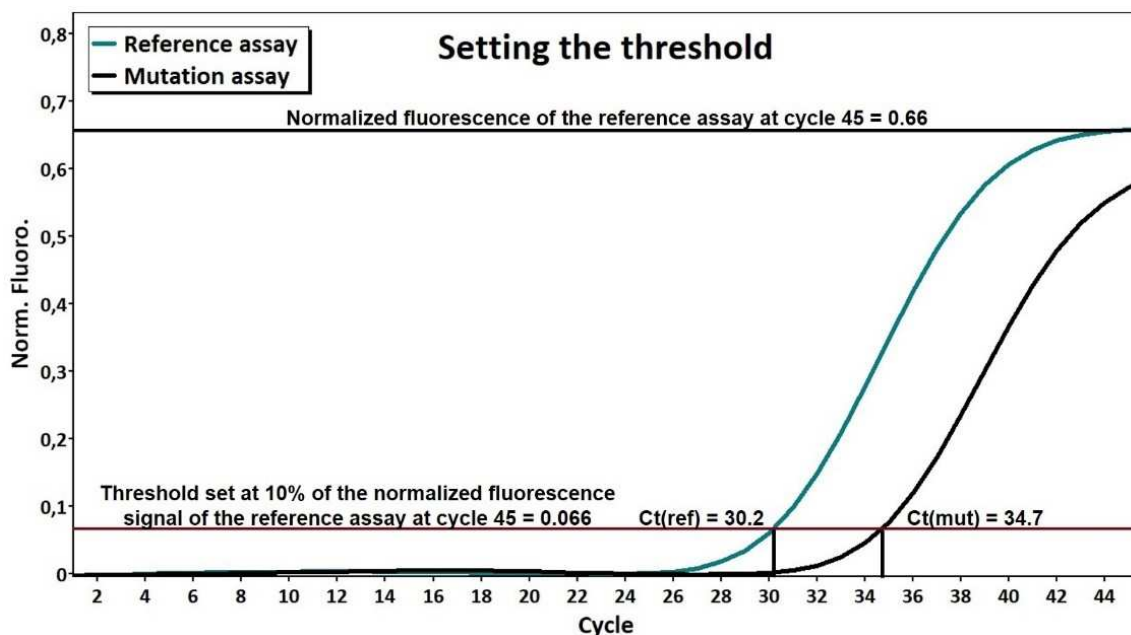


Figure 2. Setting the threshold. Read the fluorescence value for the reference assay at cycle 45 and set the threshold at 10% of this value. This threshold is now used to determine Ct values of the reference assay and the corresponding mutation-specific assay. In the example shown, Ct(reference) = 30.2 and Ct(mutation) = 34.7. Thus, in accordance with **Table 7.**, the sample is positive for the mutation analysed.

8.1.1 Correcting for baseline drift and setting the threshold

1. Use slope correction/curve fitting when possible and/or define the baseline or background cycle interval to be between cycle 15 and cycle 20.
2. Set the threshold to 10% of the signal in the reference assay at cycle 45 (**Figure 2**). Add any significant baseline fluorescence at cycle 20 to the threshold value (**Table 5**).

Table 5. Setting the threshold in cases where the background fluorescence is above 0.

Reference fluorescence at cycle 45	10% of reference fluorescence at cycle 45	Assay baseline/background fluorescence at cycle 20	Threshold value
3	0.3	0	0.3
3	0.3	0.2	0.5

8.1.2 Verifying sample input and validity of the run

Before determining the mutational status of the examined samples, the sample input and the validity of the run should be verified.

1. Verify that the reference assay (Tube A) green channel Ct values for all samples are valid according to **Table 6**.
2. If NTC samples have been included in the run, verify that the green and yellow channel reference assay (Tube A) Ct values > 38 and that the green and yellow channel mutation-specific assay (Tube B-D) Ct values > 45 for included NTC(s). Runs with NTC Ct values at or below these limits indicate contamination and the results of the whole run should be deemed invalid. Find the cause of contamination by checking or replacing all potential sources of the contamination such as pipettes and instruments. If the contamination cannot be located, contact PentaBase A/S or your local distributor.

Table 6. Acceptable green channel Ct values for the reference assay.

Reference assay Ct	Interpretation	Comments
Ct < 23	Not valid	The amount of input of DNA is too high which might affect the performance of the assay. The analysis should be repeated with lower input of DNA.
23 ≤ Ct ≤ 32	Valid	The amount of input DNA is optimal for mutation analysis.
Ct > 32	Not optimal	The amount of input DNA is low. If the sample is negative for the mutation, the analysis should be repeated with higher amount of input DNA if possible since a reduced number of copies of gDNA are analyzed leading to a substantial risk of false negatives.

8.1.3 Determining the mutational status

Determination of the mutational status is based on a combination of mutation assay replicate Ct values and the mean of calculated ΔCt values as described below:

1. Analyze the mutation assay replications (green channel) against the corresponding reference for one sample at a time.
2. Set the threshold at 10% of the reference signal strength at cycle 45 as described in section 8.1.2 (Figure 2).
3. Read the Ct value for each of the mutation assay replicates (Tubes B-D).
4. For replicates with Ct < 44, calculate the mean ΔCt (see **Table 7** for examples) of the two replicates with the lowest Ct values:

$$\Delta Ct_{\text{Replicate 1 with Ct} < 44} = Ct_{(\text{Replicate 1 with Ct} < 44)} - Ct_{(\text{Reference assay})}$$

$$\Delta Ct_{\text{Replicate 2 with Ct} < 44} = Ct_{(\text{Replicate 2 with Ct} < 44)} - Ct_{(\text{Reference assay})}$$

$$\text{Mean } \Delta Ct = \frac{\Delta Ct_{(\text{Replicate 1})} + \Delta Ct_{(\text{Replicate 2})}}{2}$$

Thus, if all 3 replicates have Ct values < 44, only the two replicates with the lowest Ct values should be used for calculating the mean ΔCt value. Thus for:

$$Ct_{(\text{Replicate 1 with Ct} < 44)} < Ct_{(\text{Replicate 2 with Ct} < 44)} < Ct_{(\text{Replicate 3 with Ct} < 44)},$$

only replicate 1 and replicate 2 Ct values are used for calculating the mean ΔCt value.

Table 7. PlentiPlex™ KIT Mastocytosis qPCR Assay mutational status calculation.

Sample	Reference Ct	Mutation-specific assay						Replicates with Ct < 44	Mean ΔCt	Conclusion
		Replicate								
		1		2		3				
Ct	ΔCt	Ct	ΔCt	Ct	ΔCt					
1	27.00	41	14	40	13	43	16	3	$\frac{14+13}{2} = 13.50$	KIT D816V mutation positive
2	26.00	42	16	41	15	43	17	3	$\frac{16+15}{2} = 15.50$	Sample does not fulfil criteria for KIT D816V mutation (Mean $\Delta Ct > 15$)
3	31.00	41	10	45	14	ND	NA	1	NA	Sample does not fulfil criteria for KIT D816V mutation (only 1 replicate with Ct < 44)
4	31.00	45	14	43	12	39	8	2	$\frac{12+8}{2} = 10$	KIT D816V mutation positive

8.1.3.1 Interpretation of mutation status

Samples are positive for the KIT D816V mutation when the reference assay Ct value is valid, the Ct values of at least two mutation assay replicates are < 44, and the mean Δ Ct of the replicates with two lowest mutation assay Ct values is < 15 (**Table 8** and examples in **Table 7**).

Table 8. Determination of the KIT D816V mutational status based on the green channel Ct and Δ Ct values of PlentiPlex™ KIT Mastocytosis qPCR Assay. * Δ Ct values refer to the mean of two replicates values of the mutation-specific assay subtracted the Ct value of the corresponding reference assay.

Ct		Mean Δ Ct	Interpretation	Comments
Reference assay (Tube A)	Mutation-specific assay (Tubes B-D)			
< 23	Any	Any	Invalid	The amount of input of DNA is too high which might affect the performance of the assay. The analysis should be repeated with lower input of DNA.
23 ≤ Ct ≤ 32	< 44 (minimum two replicates)	< 15*	KIT D816V mutation detected	The amount of input DNA is optimal for mutation analysis.
		≥ 15*	KIT D816V mutation not detected	
	≥ 44 (minimum two replicates)	Any	KIT D816V mutation not detected	
Ct > 32	< 44 (minimum two replicates)	< 15*	KIT D816V mutation detected	The amount of input DNA is lower than recommended. The sensitivity is affected hereby. ^If the KIT D816V mutation is not detected, the analysis should if possible be repeated with a higher amount of input DNA.
	≥ 44 (minimum two replicates)	≥ 15*	KIT D816V mutation not detected [^]	

8.1.3.2 Internal control analysis

In reactions with no or late amplification by the PentaGreen™-labelled assay (reference or mutation-specific assay, green channel), it should be validated that template has been added and/or amplification has taken place by examining the fluorescence from the PentaYellow™-labelled internal control assay (yellow channel). To set the threshold for the internal control assay, select the yellow channel and repeat the steps in Section 8.1.1.

NOTE: The internal control assay contains suboptimal primer concentrations and amplification may be inhibited by amplification by the PentaGreen™ labelled assay in the same reaction. Thus, the Ct value of the internal control assay is only indicative of the amount of template added to the reaction and cannot be used for precise quantification of DNA. See Section 8.1.4.2 for details regarding lack of internal control signal.

8.1.4 Invalid samples

8.1.4.1 No reference signal

Cases where there is no signal from the reference assay in neither the green nor the yellow channel indicate that a low amount or low quality of DNA has been used, that PCR inhibitors are present in the sample, or that the PCR program is incorrect. Make sure that the qPCR program has been defined correctly and that the instrument is acquiring on FAM/SYBR (green) and HEX/VIC®/TET (yellow) channels (**Table 4**). If there is no signal in the mutation-specific assay either, the purification of DNA should be re-done. If a signal is observed in some of the mutation-specific assays the sample should be re-run using the same extraction of DNA.

8.1.4.2 No internal control signal

If no signal occurs from the internal control assay, make sure that the qPCR program has been defined correctly and that the instrument is acquiring on FAM/SYBR (green) and HEX/VIC®/TET (yellow) channels (**Table 4**). No internal control signal indicates that no amplification has occurred. This might be due to low amount or low quality of DNA or the presence of PCR inhibitors. **NOTE:** No internal control signal is only a problem if there is also no signal on the green channel either (reference and/or mutation-specific assay) in the specific PCR reaction tube. Thus, if there is a signal in the green channel in the specific PCR tube lacking internal control signal, the result is valid. If there is also no signal in the green channel the purification of DNA should be re-done and the analysis re-performed.

8.1.4.3 Signal in NTC

If NTC samples have been included in the run, verify that the green and yellow channel reference assay (Tube A) Ct values > 38 and that the green and yellow channel mutation-specific assay (Tubes B-D) Ct values > 45 for included NTC(s). Runs

with NTC Ct values below these limits indicate contamination and the results of the whole run should be deemed invalid. Find the cause of contamination by checking or replacing all potential sources of the contamination such as pipettes and instruments. If the contamination cannot be located, contact PentaBase A/S or your local distributor.

9 Performance evaluation

9.1 Analytical sensitivity

9.1.1 Limit of Blank

The specificity and performance of PlentiPlex™ KIT Mastocytosis qPCR Assay in the absence of template has been established and evaluated during assay validation and is evaluated during quality control of produced lots using 200 ng of wild type (WT) human genomic DNA and PCR grade water, respectively. The criteria for approval of assays are $Ct_{(200\text{ ng WT})} > 45$ and $\Delta Ct_{(200\text{ ng WT})} > 20$ in 4 out of 6 replications.

9.1.2 Limit of Detection

The analytical sensitivity of PlentiPlex™ KIT Mastocytosis qPCR Assay has been evaluated by comparison to a method (MastOUH-assay) developed by the Mastocytosis Centre Odense University Hospital (MastOUH) (MastOUH-assay) and published in a series of articles (Kristensen et al. 2011⁶, Kristensen et al. 2014⁷, Kristensen et al. 2017⁸, Kristensen et al. 2019⁹). The PCR-efficiency of PlentiPlex™ Mastocytosis CE IVD assay was found to be identical to the MastOUH assay PCR-efficiency of 94% for the mutation-specific assay and 100% for the reference assay as previously characterised using fourfold dilutions of *KIT* D816V mutation-positive DNA in wild type DNA (Kristensen et al. 2011). The resulting non-clinical limit of detection is 0.01% *KIT* D816V-positive alleles when using 15 as the ΔCt cutoff (Kristensen et al. 2020¹⁰).

9.2 Clinical evaluation

The clinical specificity and sensitivity of the PlentiPlex™ KIT Mastocytosis qPCR Assay have been compared to the MastOUH-assay standard protocol (without re-analysis) by analysing DNA from 58 control samples from healthy blood donors as well as DNA from 115 clinical follow-up blood samples from known *KIT* D816V mutation-positive MastOUH patients (D816V previously detected in BM, skin, and/or blood) (Table 9). The analysis was performed using the same DNA extractions and according to the manufacturer's recommendations (PentaBase-assay technology and protocol described in detail in manufacturer's 'instructions for use') using the QuantStudio™ 12K instrument (Applied Biosystems) (Kristensen et al. 2020¹⁰).

The calculated *KIT* D816V allele burden of PlentiPlex™ KIT Mastocytosis qPCR Assay was found to be highly similar to the MastOUH assay (N=84, P<0.001, R = 98) and thus showing a similar clinical PCR-efficiency as the MastOUH assay and a clinical limit of detection of 0.01% (Kristensen et al. 2020¹⁰).

Table 9. Clinical performance of PlentiPlex™ KIT Mastocytosis qPCR Assay.

Clinical performance: Whole blood samples		PlentiPlex™ KIT Mastocytosis qPCR Assay		
		KIT D816V Positive	KIT D816V Negative	Total
MastOUH Assay Standard protocol	KIT D816V Positive	84	5	89
	KIT D816V Negative	0	58	58
	Total	84	63	147
Overall agreement was 142/147 or 92.3% (CI95%: 92.3-98.5%)				
Wild-type agreement was 58/63 or 92.1% (CI95%: 82.7-95.6%)				
Mutation agreement was 84/89 or 94.4% (CI95%: 87.5-97.6%)				

⁶ Kristensen T, Vestergaard H, Møller MB. Improved detection of the *KIT* D816V mutation in patients with systemic mastocytosis using a quantitative and highly sensitive real-time qPCR assay. *J Mol Diagn* 2011 13:180-8. DOI: [10.1016/j.jmoldx.2010.10.004](https://doi.org/10.1016/j.jmoldx.2010.10.004)

⁷ Kristensen T, Vestergaard H, Bindslev-Jensen C, Møller MB, Broesby-Olsen S. Sensitive *KIT* D816V mutation analysis of blood as a diagnostic test in mastocytosis. *Am J Hematol* 2014 89:493-8. DOI: [10.1002/ajh.23672](https://doi.org/10.1002/ajh.23672)

⁸ Kristensen T, Vestergaard H, Bindslev-Jensen C, Mortz CG, Kjaer HF, Ollert M, Møller MB, Broesby-Olsen S. Prospective evaluation of the diagnostic value of sensitive *KIT* D816V mutation analysis of blood in adults with suspected systemic mastocytosis. *Allergy* 2017 72:1737-43. DOI: [10.1111/all.13187](https://doi.org/10.1111/all.13187)

⁹ Kristensen T, Broesby-Olsen S, Vestergaard H, Bindslev-Jensen C, Mortz CG, Kjaer HF, Møller MB. Mastocytosis Centre Odense University Hospital (MastOUH). Towards rational diagnostics in mastocytosis: clinical validation of sensitive *KIT* D816V mutation analysis of unfractionated whole-blood. *Leuk Lymphoma* 2019 60:268-70. □ DOI: [10.1080/10428194.2018.1460475](https://doi.org/10.1080/10428194.2018.1460475)

¹⁰ Kristensen T, Vestergaard H, Bindslev-Jensen C, Møller MB, Broesby-Olsen S; Mastocytosis Centre Odense University Hospital (MastOUH). Clinical validation of a new commercial highly sensitive *KIT* D816V mutation analysis in mastocytosis. *Allergy*. 2020 Jun;75(6):1489-1491. DOI: [10.1111/all.14165](https://doi.org/10.1111/all.14165)

10 Limitations

- PlentiPlex™ KIT Mastocytosis qPCR Assay has only been validated for use with whole blood specimens.
- Incorrect collection, storage, DNA extraction, transportation or handling of the sample could cause false test results due to low amount or poor quality of DNA or the presence of PCR inhibitors in the sample.
- PlentiPlex™ KIT Mastocytosis qPCR Assay is validated for use with 5-200 ng of DNA per reaction. Using DNA input amounts lower or higher than this may lead to incorrect test results. A negative test result does not exclude the presence of mutated DNA at levels below the detection limit of the assay.
- Rare mutations within the genomic DNA regions of the KIT gene covered by the oligonucleotides used in the PlentiPlex™ KIT Mastocytosis qPCR Assay may impair the detection of the indicated KIT D816V mutation.
- PlentiPlex™ KIT Mastocytosis qPCR Assay is a semi-quantitative test that will only provide an estimate of the mutation frequency. The test is not for accurate detection of mutation frequency.
- The Ct value of the internal control assay is only indicative of the amount of template added to the reaction and cannot be used for precise quantification of DNA.
- PlentiPlex™ KIT Mastocytosis qPCR Assay is not compatible with the LightCycler® 96 System (Roche).

11 Symbols

The following symbols are used in labelling of PlentiPlex™ KIT Mastocytosis qPCR Assay.



Date/country of manufacture



In vitro diagnostic medical device



Use-by date



Do not reuse



Contains sufficient for <n>



Manufacturer



Temperature limit



CE marking of conformity; this device is in conformity with the applicable requirements for CR of an *in vitro* diagnostic medical device



Consult electronic available instructions for use

12 Manufacturer

PentaBase A/S
Petersmindevej 1A
DK-5000 Odense C

Telephone: +45 36 96 94 96
Email: info@pentabase.com
Webpage: www.pentabase.com

For technical assistance please contact your local distributor or PentaBase A/S. A complete list of distributors is available at www.pentabase.com.

NOTICE TO USERS: any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.