

INSTRUCTIONS FOR USE

PlentiPlex™ assay for sensitive detection of the *KIT* D816V mutation



#Cat. No.: 7030-7033



Version: 2.3

Date of Revision: June 2021

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Δ IMPORTANT:

Please read these instructions carefully before using the PlentiPlex™ Mastocytosis CE IVD assay. It is recommended to save the “Instructions for use” for future use. Purchasers of PlentiPlex™ Mastocytosis CE IVD assay are only granted the right of use, but no general licensing or patent rights.

1. INTENDED USE

PlentiPlex™ Mastocytosis CE IVD is intended for in vitro diagnosis of the aspartic acid to valine mutation in codon 816 of the KIT proto-oncogene receptor tyrosine kinase (KIT D816V, c.2447A>T) in human genomic DNA (gDNA) purified from blood samples. The test will provide an assessment of the presence of the examined mutation constituting down to 0.01 % of a human gDNA sample.

PlentiPlex™ Mastocytosis CE IVD is to be used by trained laboratory personnel in a professional laboratory environment to assist the diagnosis of mastocytosis. The test is provided with all necessary reagents for amplification of the human *KIT* D816V allele by real-time PCR.

1.1 INDICATIONS FOR USE

The obtained results of PlentiPlex™ Mastocytosis CE IVD are intended for identification of the presence of the *KIT* D816V mutation as part of the World Health Organization (WHO) diagnostic criteria to assist the diagnosis of Mastocytosis (Valent et al. 2016).

2. SUMMARY AND EXPLANATION OF THE ASSAYS

Mastocytosis is a disorder characterized 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 three 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) (Valent et al. 2016). 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.

PlentiPlex™ Mastocytosis CE IVD is based on PentaBase’s highly sensitive DNA technology and can, if provided with sufficient input of DNA of sufficient quality, detect 0.01% of *KIT* D816V mutated gDNA in a background of wild type gDNA.

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

Table 1. List of investigated mutations

3. TECHNOLOGY AND REAGENTS

PlentiPlex™ Mastocytosis CE IVD combines allele-specific PCR with PentaBase's novel and selective technologies comprising: 1) HydrolEasy™ probes, 2) SuPrimers™ for specific and sensitive amplification, and 3) BaseBlockers™. The technology is applicable on standard real-time PCR instruments using standard procedures. Pentabases are synthetic DNA analogues comprising a flat heteroaromatic, hydrophobic molecule and a linker. They are inserted into the oligonucleotides at fixed positions during synthesis. PlentiPlex™ Mastocytosis CE IVD contains both standard oligonucleotides and pentabase-modified oligonucleotides (HydrolEasy™ probes, SuPrimers™, and BaseBlockers™). Using PlentiPlex™ Mastocytosis CE IVD, somatic mutations can be detected quickly (in less than one and a half hour), sensitively (5-200 ng gDNA input per well/vial) and selectively (0.01% limit of detection of *KIT* D816V mutation in a wild type gDNA background), by real-time PCR analysis.

3.1 HYDROLASY™ PROBES

A **HydrolEasy™** probe is similar to a standard hydrolysis probe (also referred to as a TaqMan™ probe) labeled with a fluorophore at the 5' end, a quencher at the 3' end, but with the addition of pentabases giving the probe a significantly improved signal-to-noise ratio, higher specificity and higher sensitivity compared to conventional hydrolysis probes. HydrolEasy™ probes in PlentiPlex™ Mastocytosis CE IVD assays are labeled with PentaGreen™ (λ_{abs} . 495 nm and λ_{Em} . 516 nm, detected on the same channel as FAM™) in combination with Green Quencher™, or as PentaYellow™ (λ_{abs} . 533 nm and λ_{Em} . 557 nm, detected on the same channel as VIC™, VIC™, TET™) in combination with Yellow Quencher™.

3.2 SUPRIMERS™

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

3.3 BASEBLOCKERS™

BaseBlockers™ are DNA sequences modified with several pentabases, allowing for the specific and strong binding to a target sequence. In PlentiPlex™ Mastocytosis CE IVD, the BaseBlockers™ are designed to bind to wild type gDNA targets, suppressing false positive signals from the wild type templates and ensuring high specificity and robustness of the assay. Along with SuPrimers™, the BaseBlockers™ minimize or eliminate the risk of false positive signals. The BaseBlocker™ principle is illustrated below.

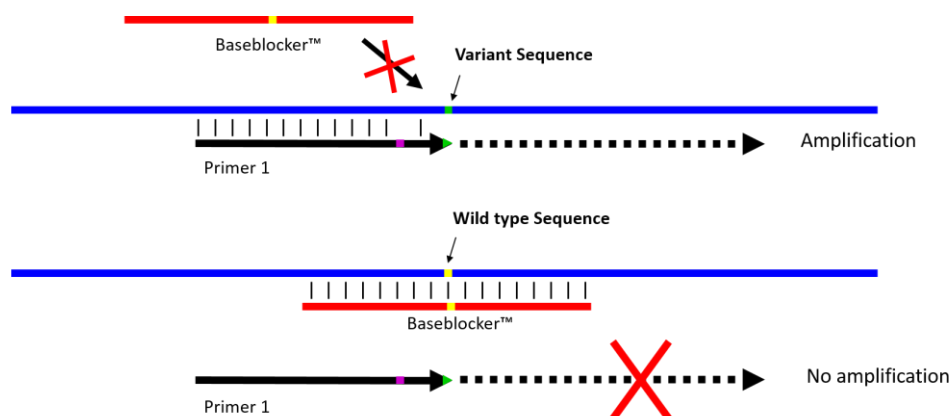


Figure 1: Illustration of how BaseBlockers™ function in PlentiPlex™ assays. A BaseBlocker™ binds to and blocks the wild type template from being amplified. In contrast, the BaseBlocker™ does not inhibit amplification of a template with a single nucleotide mutation and the result is a selective amplification of mutated gDNA in a wild type background.

4. ASSAY FORMAT AND DESIGN

4.1 FORMAT

PlentiPlex™ Mastocytosis CE IVD is supplied in either a "Ready-To-Use" or "Dispense Ready" version. PlentiPlex™ Mastocytosis CE IVD Ready-to-use assays are provided in either 12 or 60 reactions in pre-aliquoted PCR strips (Table 2) while Dispense Ready assays are provided in 20 or 50 reactions (Table 3).

PlentiPlex™ Mastocytosis contains the following reagents:

Reference assay

- Reference assay primer/probe mix (labeled with PentaGreen™, for detection on green (FAM™) channel)
- Internal control primer/probe mix (labeled with PentaYellow™, for detection on yellow (VIC™) channel)
- AmpliQueen™ Master mix

Mutation assay

- Mutation assay primer/probe/BaseBlocker™ mix (labeled with PentaGreen™, for detection on green (FAM™) channel)
- Internal control primer/probe mix (labeled with PentaYellow™, for detection on yellow (VIC™) channel)
- AmpliQueen™ Master mix

Mastocytosis KIT D816V Assay					
Strip #	Gene	Tube #	Content	Mutation	Corresponding reference
IM1	KIT	A	KIT Reference 1		
		B	KIT Simplex 1	D816V	KIT Reference 1
		C	KIT simplex 1	D816V	KIT Reference 1
		D	KIT simplex 1	D816V	KIT Reference 1

Table 2. PlentiPlex™ Mastocytosis CE IVD Ready-to-use assay components. Each tube contains 20 µL in total (7,5 µL primer/probe-mix and 12,5 µL master mix).

Mastocytosis KIT D816V Assay						
Gene	Tube #	Content	Mutations	Corresponding reference	Volume 20x	Volume 50x
KIT	1	KIT Reference 1			150 µL	375 µL
	2	KIT Simplex 1	D816V	KIT Reference 1	450 µL	1125 µL
	3	Master mix			1000 µL	2500 µL

Table 3. PlentiPlex™ Mastocytosis CE IVD Dispense Ready assay components. Each tube contains reagents for either 20 or 50 reactions.

4.2 INTERNAL CONTROL

An internal control assay is included in the primer-probe mixes of the assay and comprise a HydrolEasy™ probe labeled with PentaYellow™ (measured on the same fluorescence channel as VIC™, VIC® or TET™) and a primer set. The internal control assay is used to assess whether an amplification has taken place in reactions with negative signal from the PentaGreen™ labeled 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. See section 8 "Data analysis" for more details.

4.3 REFERENCE ASSAY

The reference assay targets 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 HydrolEasy™ probe labeled with PentaGreen™ (measured on the FAM™ channel), 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.

4.4 MUTATION ASSAY

The mutation assay (Table 2-3) 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 (see section 8 “Data Analysis” for more details). The mutation assay contains a HydrolEasy™ 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 mutation-assay is optimized to the conditions specified in section 7 and it is therefore important that these are followed to avoid misleading results. 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. See section 8 “Data Analysis” for more details.

4.5 EQUIPMENT AND REAGENTS NOT SUPPLIED WITH PLENTIplex™ MASTOCYTOSIS CE IVD

The use of PlentiPlex™ Mastocytosis CE IVD will require the following equipment and consumables:

- Template DNA (extracted mutant gDNA)
- Applied Biosystems QuantStudio™ Real-Time PCR instrument
- Plastic products (tubes/plates) that are compatible with the instrument^
- Dedicated pipettes and tips for preparing PCR mixes
- Dedicated pipettes and tips for addition of DNA sample
- Centrifuge for spinning tubes/plates
- Nuclease-free H₂O (sterile)

^ PlentiPlex™ Mastocytosis CE IVD ready-to-use assays include PCR strips for use in analysis (can be provided in either 0.1 mL or 0.2 mL strips)

5. SAFETY, SHIPMENT AND STORAGE

General laboratory precautions should be taken. PlentiPlex™ Mastocytosis CE IVD should only be used by personnel who has been trained in the appropriate techniques. All chemicals and biological material should be considered as potentially hazardous. When working with the assay suitable personal protective equipment (lab-coat, disposable gloves and safety glasses) should be used. It is recommended that all work is carried out in appropriate facilities. All waste should be disposed as clinical waste.

5.1 PRECAUTIONS

The following precautions should be taken when working with PlentiPlex™ Mastocytosis CE IVD:

- The assay is only intended for *in vitro* diagnostics
- PlentiPlex™ Mastocytosis CE IVD is not intended for sole diagnosing of mastocytosis, but only as a supplement to other diagnostic factors
- The mutational status determined by PlentiPlex™ Mastocytosis CE IVD should always be considered alongside other disease factors when making treatment decisions
- Avoid several freeze/thaw cycles of the reagents as this might impair the performance of PlentiPlex™ Mastocytosis CE IVD assays. Limit to a maximum of eight times

- Verify eligibility of the DNA samples as DNA samples can be non-homogeneous and of varying quality, which might affect the analysis
- The delivered reagents should not be diluted further. Further dilution can cause loss of performance and increase the risk of false negative and false positive results
- Use the specified volumes. It is not recommended to reduce the specified volumes as the results can be affected
- No reagents should be substituted by others if the optimal performance should be maintained
- It is recommended to use one of the platforms, validated to ensure full PlentiPlex™ Mastocytosis CE IVD performance. For more information, see section 4.5 “Equipment and Reagents not supplied with PlentiPlex™ Mastocytosis CE IVD”
- Due to the presence of HydrolEasy™ probes, assays should be protected from light
- Use extreme caution not to contaminate reagents and samples. It is recommended to separate preparation of PCR mixes and gDNA addition. Dedicated pipettes should be used, and it is recommended to have separate areas for sample preparation and PCR running
- PCR tubes should not be opened after completing the PCR program
- All used instruments and equipment should be calibrated and meet their original specifications

5.2 SHIPMENT

PlentiPlex™ Mastocytosis CE IVD assays are shipped on either dry ice or blue ice cooled to -80°C. If the PlentiPlex™ Mastocytosis CE IVD packaging has been opened during transport or if the products are not frozen upon arrival, please contact your local distributor or PentaBase A/S (see section 11 “Manufacturer and Distributors”). Please also contact your local distributor or PentaBase A/S if the shipment is missing a certificate of analysis, reagents or a “Quick Guide”.

5.3 STORAGE

PlentiPlex™ Mastocytosis CE IVD assays should after arrival immediately be stored at maximum -18°C. Repeated freeze/thaw cycles should be avoided. If the assays are stored under the recommended conditions, they should be stable until the date stated.

6. SPECIMENS

Specimens should be human genomic DNA extracted from whole blood samples or similar. The samples should be collected and stored after standard pathology methodologies to ensure optimal quality. Extracted gDNA should be stored at maximum -18°C until use.

6.1 RECOMMENDED PROCEDURE FOR EXTRACTION OF gDNA FROM WHOLE BLOOD

DNA isolation should be performed using a kit designed, approved and quality controlled for extraction of genomic DNA from whole blood according to the manufacturer’s protocol.

7. PLENTIPLEX™ MASTOCYTOSIS CE IVD PROTOCOL

Before using the assay, it is recommended to carefully read this instructions for use. PlentiPlex™ Mastocytosis CE IVD is supplied in either a "Ready-to-use" or "Dispense Ready" version. When using PlentiPlex™ Mastocytosis CE IVD Dispense Ready assays, it is recommended to collect samples in larger batches for most effective use of reagents and to avoid repeated freeze/thaw cycles and waste. For each sample, a reference assay must be included in the mutation analysis (Table 2-3). These should be analyzed in the same PCR run to ensure minimal variation.

7.1 READY-TO-USE

1. Thaw the reaction mixtures and spin down
2. Add 5 µL extracted gDNA (1-40 ng/µL) to the reference and mutation assays. It is recommended to include a no template control (NTC) in each run. Add sterile water instead of gDNA.
3. Close lids and spin down
4. Place the strips into the instrument and run the protocol described in Table 4
5. Analyze the samples in accordance with the analysis rules. For more information, see section 8 “Data analysis”

7.2 DISPENSE READY

1. Run the mutation specific assay in triplicates
2. Thaw the reaction mixtures, mix and spin down
3. Add 12.5 µL master mix to all tubes/wells
4. Add 7.5 µL of reference mix to one tube/well per patient and mix carefully by pipetting
5. Add 7.5 µL of mutant mix to three tubes/wells per patient and mix carefully by pipetting
6. Add 5 µL extracted gDNA (1-40 ng/µL) from each sample to the mutation assays and the corresponding reference. Mix carefully by pipetting. It is recommended to include a NTC in each run. Add sterile water to the NTC instead of gDNA.
7. Seal all tubes/wells and spin down. Make sure that there are no bobbles in the solutions
8. Place tubes or plate in the instrument and run the protocol as described in Table 4
9. Analyze the samples in accordance with the analysis rules. For more information, see section 8 “Data analysis”

7.3 PCR RUN PROTOCOL

PCR amplification using PlentiPlex™ Mastocytosis CE IVD assay should be performed according to the PCR protocol described in table 4.

Protocol	Temperature (°C)	Time (s)	Ramping (°C/s)	Number of cycles	Dye
Hold	95	120	-	-	-
Cycling	94	15	1.6	45	FAM™ (Primary assay) VIC™ (Internal control) ROX™ (Passive reference) Measure fluorescence intensity at the end of each cycle
	60	40	1.6		

Table 4. PlentiPlex™ Mastocytosis CE IVD Real-time PCR protocol

To set up a new PlentiPlex™ Mastocytosis CE IVD PCR run using the QuantStudio™ software:

1. Click *Create New Experiment* and select *Experiment Setup* or *From Template* if the experiment setup has been stored previously as a template.
2. When creating a new experiment, in the *Properties tab*, select the applicable *Instrument type* and *Block type* if required and choose *Standard Curve* as the Experiment type, *TaqMan® Reagents* as the Chemistry and *Standard* as the Run mode.
3. In the *Method tab*, set the reaction volume to 25 µl, remove Step2 in the Hold stage and set the temperature of Hold Stage step1 to 95°C for 2 min. In the PCR stage, set the step1 temperature to 94°C for 15 sec and the step2 temperature to 60°C for 40 sec. Check that step2 data collection (camera icon) is on. Set the cycle number to 45.
4. In the *Plate tab*, choose ROX and the Passive Reference.
5. Click *Advanced Setup* and enter the name of the target, (e.g. Mastocytosis KIT D816V) and select FAM as the *Reporter* and NFQ-MGB as the *Quencher*.

6. In the *Targets* window, click the *Add* button to add a second target. Enter the name of the target (Internal Control) and select VIC as the *Reporter* and NFQ-MGB as the *Quencher*.
7. In the *Samples* window, enter the name of the samples and mark the wells used for each sample.
8. In the *Run* tab, click the START RUN button.

8. DATA ANALYSIS

When using PlentiPlex™ Mastocytosis CE IVD real-time PCR assays, determining the cycle threshold (Ct) is a central part of the data analysis procedure. Ct is defined as the cycle in which the fluorescence signal of a given assay exceeds the threshold value. The threshold is set to 10% of the reference fluorescence signal at cycle 45 (Figure 2).

8.1 SETTING QUANTSTUDIO™ SOFTWARE ANALYSIS PARAMETERS

1. After the run has finished, click the *Results* tab.
2. In the *Amplification Plot* window, click the eye icon to configure the plot. Set the Plot Type to ΔRN vs Cycle and the Graph Type to *Linear*.
3. Uncheck the Threshold: Auto check box. The threshold can now be moved manually using the pointer or the threshold values can be entered in the field.
4. Click the *Plot properties* icon and select the Y Axis tab. Check the *Auto adjust range* check box in the *Range* window.
5. Select the target with the FAM reporter in the *Amplification Plot* window and select the wells to be analyzed in the *View* window.

8.2 DETERMINING THE MUTATIONAL STATUS

Use the following protocol to determine the mutational status:

1. Analyze the mutation assay replications against the corresponding reference for one sample at a time
2. Set the threshold at 10% of the reference signal strength at cycle 45 (Figure 2). The threshold line can be used to read the normalized fluorescence value of the reference assay at cycle 45 by moving it manually to align with the reference amplification curve.

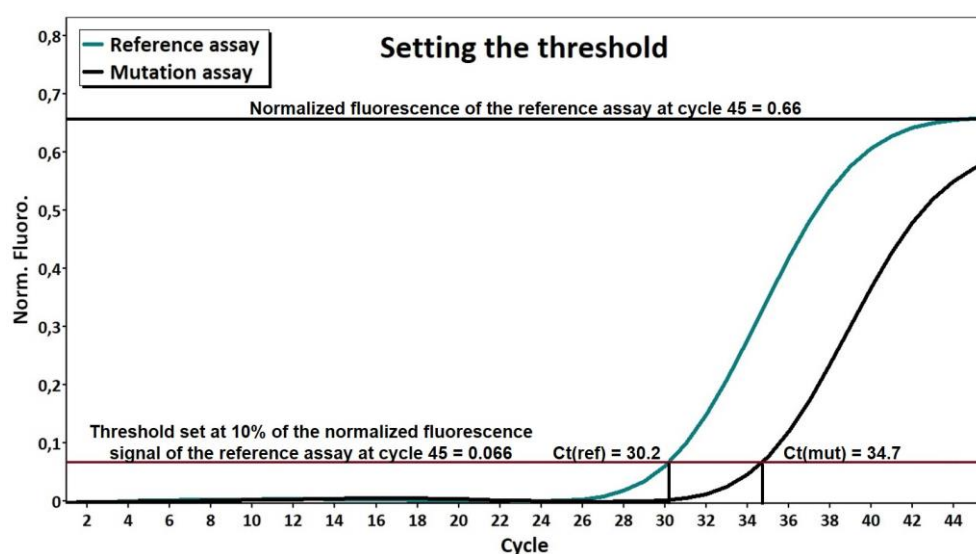


Figure 2. Setting the threshold. Read the fluorescence value (ΔRn) for the reference assay at cycle 45 and set the threshold at 10% of this value. This threshold is used for determination of assay Ct values.

3. If NTC samples have been included in the run, verify that no signal is seen before Ct = 38 for the reference and Ct = 44 for mutation analyses. A positive signal in the NTC before these limits indicates contamination, which may influence the results. Data should not be used if the NTC control is positive

4. Read the Ct value for the reference assay and validate that the reference sample is suitable for analysis, cf. Table 6.

Ct for reference	Quality	Comments
Ct, reference < 23	Not valid	The amount of input DNA is too high which might affect the assay. The analysis should be repeated with lower input of DNA
23 ≤ Ct, reference ≤ 32	Optimal	The amount of input DNA is valid for mutation analysis. Please note that the closer the reference Ct is to 23, the higher the number of potential <i>KIT</i> D816V gDNA templates are analyzed.
Ct, reference > 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.

Table 6. Acceptable Ct values for the reference assay.

5. Calculate delta Ct (dCt)-values as the mean of the two lowest Ct-values produced by the mutation-specific assay subtracted the Ct-value of the reference assay. A Ct-value of 45 should be used for the dCt-value calculations in reactions producing no Ct-value in 45 cycles.
6. The following criteria for *KIT* D816V mutation positivity should be applied; 2 or 3 of the 3 mutation-specific replicates produce a Ct-value below 44.00 and the mean dCt-value of the two lowest Ct-values produced by the mutation-specific assay is below 15.00 (Table 7).

Ct and ΔCt for mutation-specific assay	Conclusion	Comments
2 or 3 of 3 mutation-specific replicates with a Ct-value < 44.00 and the mean dCt-value of the two lowest Ct-values produced by the mutation-specific assay < 15.00.	<i>KIT</i> D816V mutation Positive	The sample fulfils the criteria for <i>KIT</i> D816V positivity when the reference Ct is valid and when 2 or 3 of the 3 mutation-specific replicates produce a Ct-value below 44.00 and the mean dCt-value of the two lowest Ct-values produced by the mutation-specific assay is below 15.00.
0 or 1 of 3 mutation-specific replicates with a Ct-value < 44.00 or the mean dCt-value of the two lowest Ct-values produced by the mutation-specific assay ≥ 15.00.	Sample does not fulfill criteria for <i>KIT</i> D816V mutation positivity	The sample does not fulfill the criteria for <i>KIT</i> D816V mutation positivity if there are 0 or 1 of 3 mutation-specific replicates with a Ct-value lower than 44.00 or the mean dCt-value of the two lowest Ct-values produced by the mutation-specific assay are equal to or higher than 15.00.

Table 7. Determination of *KIT* D816V mutational status based on Ct and mean dCt values.

Examples of PlentiPlex™ Mastocytosis CE IVD *KIT* D816V mutational status calculations are shown in Table 8.

Sample	Ct Reference	Mutation-specific assay						Mean dCt (based on the two lowest mutation-specific assay replication Ct values)	Conclusion
		Replication 1		Replication 2		Replication 3			
		Ct	ΔCt	Ct	ΔCt	Ct	ΔCt		
1	27.00	41.00	<u>14.00</u>	40.00	<u>13.00</u>	43.00	16.00	(14.00+13.00)/2 = 13.50	<i>KIT</i> D816V mutation positive
2	26.00	42.00	<u>16.00</u>	41.00	<u>15.00</u>	43.00	17.00	(16.00+15.00)/2 = 15.50	Sample does not fulfill criteria for <i>KIT</i> D816V mutation positivity

Table 8. Mastocytosis *Kit* D816V mutational status calculation examples.

8.3 INTERNAL CONTROL ANALYSIS

In reactions with no or late amplification by the PentaGreen™ labeled assays (FAM™ green channel), it should be validated that template has been added and/or amplification has taken place by examining the fluorescence from the internal control assay (VIC™ yellow channel). This is done by selecting the target with the VIC™ reporter (Internal control). All wells with added DNA should show a clear PCR amplification curve. **Note:** The internal control (VIC) assay will typically display a lower ΔRn value than the primary mutation assays (FAM) and should be displayed separately. Furthermore, the internal control assay includes suboptimal primer concentrations to reduce the risk of interference with the mutation assays. Therefore, the

internal control assay is only intended for verification of the addition of DNA but not for precise quantitation of the amount added.

9. NON-CLINICAL PERFORMANCE

9.1 ANALYTICAL SENSITIVITY – LIMIT OF BLANK

The specificity and performance of PlentiPlex™ Mastocytosis CE IVD 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.

9.2 ANALYTICAL SENSITIVITY – LIMIT OF DETECTION

The analytical sensitivity of PlentiPlex™ Mastocytosis CE IVD 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. 2011, Kristensen et al. 2014, Kristensen et al. 2017, Kristensen et al. 2019, section 12 “References”). 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 characterized 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., *manuscript submitted for publication*).

9.3. STRESS TOLERANCE

Freeze-thaw cycle stability

Assay performance has been evaluated during repeated freeze-thaw cycles in a 24 hour and 48 hour time period. The PlentiPlex™ Mastocytosis CE IVD assay was subjected to 8 freeze-thaw cycles. At each cycle, 6 reactions were moved into new tubes and stored at 5 degrees until analysis. The stock mix was frozen in 2 h intervals or overnight. Assay performance was found to be unchanged after 8 freeze-thaw cycles during both time periods.

In-use stability

In-use stability of PlentiPlex™ Mastocytosis was evaluated by placing PlentiPlex™ Mastocytosis dispense-ready tubes at room temperature (20°C) and exposed to light (without receiving direct sunlight) for 8 hours followed by storage at -20°C for 7 and 14 days, respectively. Assay performance was found not to be affected. The effect of exposure to direct sunlight has not been tested and should be avoided.

Stability at elevated temperature

Examination of stability after prolonged exposure to high temperature and subsequent long term storage at correct temperature (-20°C) has not been tested. Therefore, the contents of any shipment received that is no longer cold (temperature is clearly below room temperature) should be regarded as damaged and returned to PentaBase.

Long term stability

Assay performance was evaluated after storage at -20°C for 15 months. Assay performance was found to be within specifications. Current shelf life of Ready-to-use assays is however limited to 9 months and further studies are ongoing to investigate further shelf life extension.

10. CLINICAL PERFORMANCE

Validation of the PlentiPlex™ Mastocytosis CE IVD assay has been performed at Mastocytosis Centre Odense University Hospital (MastOUH). MastOUH represents the National Danish Mastocytosis Centre and is a European Competence Network on Mastocytosis (ECNM) Center of Excellence and ECNM Reference Center within the fields of allergology, dermatology and molecular medicine/KIT mutation analysis (www.ecnm.net). The Department of Pathology, Odense University Hospital, which is a partner in MastOUH has performed more than 5000 clinical *KIT* D816V mutation analysis using the MastOUH method (MastOUH-assay) that has been invented at the department and published in a series of articles (Kristensen et al. 2011, Kristensen et al. 2014, Kristensen et al. 2017, Kristensen et al. 2019, section 12 “References”). The MastOUH-assay is currently used in at least 30 different laboratories according to the most recent UK-NEQAS KIT D816V trial (trial 192001).

10.1 CLINICAL PERFORMANCE OF PLENTIPLX™ MASTOCYTOSIS CE IVD

The clinical specificity and sensitivity of the PlentiPlex™ Mastocytosis CE IVD assay has been compared to the MastOUH-assay standard protocol (without re-analysis) by analyzing 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). 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., *manuscript submitted for publication*).

PlentiPlex™ Mastocytosis CE IVD vs. MastOUH-assay standard protocol:

Overall agreement = 110/115 = 96%

Wilson confidence interval (confidence level 0.95) = 0.90 - 0.98

Agreement wild type = 58/58 = 100%

Wilson confidence interval (confidence level 0.95) = 0.94 - 1.00

Agreement mutation = 84/89 = 94%

Wilson confidence interval (confidence level 0.95) = 0.88 - 0.98

The calculated *KIT* D816V allele burden of PlentiPlex™ Mastocytosis 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., *manuscript submitted for publication*).

11. TROUBLESHOOTING

The troubleshooting guide shown in Table 10 below covers some of the most frequent questions and problems that can occur with the use of PlentiPlex™ Mastocytosis CE IVD assay and how they might be solved.

Problem	Solution
NTC signal	The assay is contaminated. Find the cause of contamination by checking all sources such as water, pipettes or facilities. If the contamination can't be located contact Pentabase A/S. For contact details, see section 11 "Manufacturer and Distributors"
No internal control signal (PentaYellow™)	There is no lower threshold for the Yellow channel. No internal control signal is only a problem if there is no signal on the green channel either. This indicates that no amplification has occurred. This might be due to low amount or low quality of gDNA or the presence of PCR inhibitors. Repeat the PCR with higher gDNA quality. If there is a signal on the reference assay (in the green channel) with Ct<30 but no signal in the internal control of the mutation assays, then try to dilute the gDNA

	five times and repeat the PCR.
No reference signal (PentaGreen™)	No reference signal indicates that a low amount or low quality gDNA has been used. If there are no signal (before Ct = 44) in the mutation-specific assays either, the purification of gDNA should be re-done. If a signal is observed in some of the mutation-specific assays or in other reference assays with the specific sample, the analysis could be re-run using present extraction of gDNA
No signal from mutation specific assay (PentaGreen™)	Check that there is signal for the internal control (yellow channel). If there is a signal this sample does not comprise the specific mutation
Ct(reference) < 23	The amount of input gDNA is too high. This can affect the performance of PlentiPlex™ Mastocytosis CE IVD. Repeat the PCR with lower input of gDNA if possible
Ct(reference) > 32	The amount of input gDNA is too low. If possible, repeat the PCR with higher input of gDNA. If the mutation-specific analysis is positive, the sample is most likely mutated

Table 10. Troubleshooting

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13. MANUFACTURER AND DISTRIBUTORS

11.1 MANUFACTURER

	<p>PentaBase A/S Petersmindevej 1A 5000 Odense C, Denmark</p>
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11.1 TECHNICAL ASSISTANCE

For technical assistance in Denmark, contact PentaBase A/S:

www.pentabase.com

support@pentabase.com

Phone: +45 3696 9496

For technical assistance in all other countries, contact your local distributor. A complete list of distributors is available at www.pentabase.com.

11.2 DATE OF REVISION

PlentiPlex™ Mastocytosis CE IVD protocol was revised in November 2019.

Change history

Version No.	Effective Date	Significant Changes	Previous version
1.02	08.05.2018	New Procedure	N/A
1.10	15.04.2019	Changed the maximum gDNA concentration from 80 to 40 ng/μl and corrected table 6 Ct cut off value for the reference assay from Ct≥21 to Ct≥23	1.02
2.0	01.11.2019	Updated product from research-use-only to CE IVD. Updated description of intended use and summary and explanation of assays (section 1 and 2). Updated required equipment (section 4.5), PCR run protocol and analysis (section 7 and 8). Added performance data (section 9+10) and updated reference list (section 11).	1.10

14. PLENTIPLEx™ MASTOCYTOSIS CE IVD PRODUCT OVERVIEW

Assay	PlentiPlex™ Ready-to-use	Assay #	Catalogue # 12; 60 reactions	PlentiPlex™ Dispense Ready	Catalogue # 20; 50 reactions
Mastocytosis	KIT D816V Ready-to-use (CE IVD)	IM3	7030-7031	KIT D816V Dispense Ready (CE IVD)	7032-7033

15. ADDITIONAL INFORMATION

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16. NOTES
