PlentiPlex™ MYD88

Waldenström Lymphoma qPCR Assay

In Vitro Diagnostic Assay for Detection of MYD88 L265P

INSTRUCTIONS FOR USE

REFERENCE NUMBERS

Dispense Ready (DR)
1500 (20 reactions)

Ready-to-Use (RTU)
1849 (12 reactions)
1850 (24 reactions)

Version 4.0
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CE IVD

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1 Intended purpose

PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay is a semi-quantitative real-time Polymerase Chain Reaction (PCR) assay intended for *in vitro* diagnosis of the genomic DNA (gDNA) change giving rise to leucine to proline mutation in codon 265 (L265P) of the Myeloid differentiation primary response 88 (MYD88) protein. Samples shall be obtained from Formalin-Fixed Paraffin-Embedded (FFPE) tissue or blood samples. The assay is used with real-time PCR systems and samples can be prepared using automated platforms or in manual workflows. The obtained results of PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay are intended for identification of the presence of the MYD88 L265P, facilitating discrimination between Lymphoplasmacytic lymphoma/Waldenström macroglobulinemia (LPL/WM) and non-Hodgkin lymphoma.

1.1 Intended user

PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay is intended for use by healthcare professionals or qualified laboratory personnel instructed and trained in the techniques of real-time qPCR as well as proficient in handling biological samples. Medical interventions based on results from this product requires medical authorisation.

2 Test principle

2.1 Explanation of the assay

PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay combines allele-specific PCR with PentaBase's novel and selective technologies comprising both standard and modified synthetic oligonucleotides such as HydrolEasy® probes and BaseBlockers™ for specific and sensitive amplification. The technology is applicable on standard real-time PCR equipment including PentaBase's own portfolio of instruments using standard procedures. Modified oligonucleotides contain at least one synthetic DNA analogue (called pentabases) comprising a flat heteroaromatic, hydrophobic molecule and a linker. These modifications are inserted into the oligonucleotides at fixed positions during synthesis. Using PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay, MYD88 L265P somatic mutations can be detected quickly (in less than two hours), sensitively (5-50 ng gDNA input per well/vial) and selectively (0.6% and 0.75% MYD88 L265P mutation detection limit in a wild-type gDNA background from whole blood and FFPE samples, respectively), by real-time qPCR analysis.

2.1.1 HydrolEasy® probes

A HydrolEasy® probe is similar to a standard hydrolysis probe (also referred to as a TaqMan™ probe[1]) labelled with a fluorophore at the 5' end, a quencher at the 3'end, but with the addition of pentabases. HydrolEasy® probes are based on oligos 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 PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay are labelled with PentaGreen™ (λ_{ex}. 495 nm and λ_{em}. 516 nm, detected on the same channel as FAM) in combination with a Green Quencher™, and with PentaYellow™ (λ_{ex}. 533 nm and λ_{em}. 557 nm, detected on the same channel as HEX/VIC®[2]/TET) in combination with a Yellow Quencher™.

2.1.2 BaseBlockers™

BaseBlockers™ are oligonucleotides modified with several pentabases, allowing for specific and strong binding to a target sequence (**Figure 1**). In PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay, the BaseBlocker™ technology is designed to bind to wild-type gDNA targets, suppressing wild-type DNA amplification by hindering the binding of the mutation-specific primer to the wild-type template. The presence of BaseBlockers™ ensures high specificity and robustness of the assay and eliminates the risk of false positive signals.

[1]Taqman is a registered tradename of Roche Molecular Systems, Inc.

[2]VIC is a registered tradename of Applied Biosystems, Inc.

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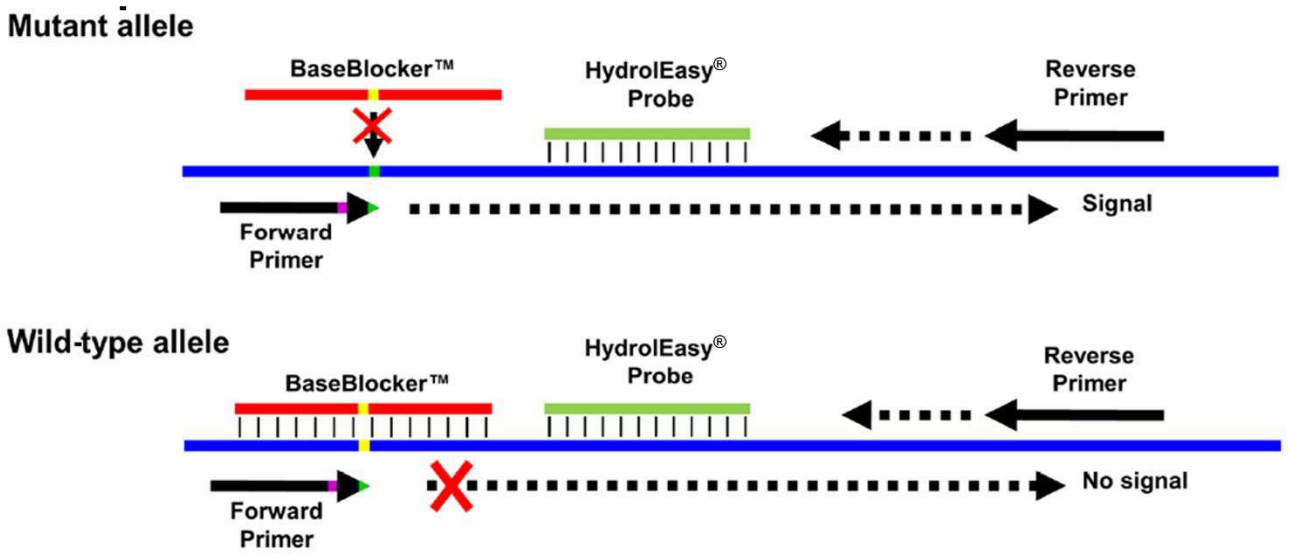

Figure 1. Illustration of how BaseBlockers™ function in PlentiPlex™ assays. A BaseBlocker™ binds to and blocks the wild-type template from being amplified (modified from Riva et al. 2017[3]). In contrast, the BaseBlocker™ does not inhibit amplification of a template with a single nucleotide mutation and the result is a selective amplification of mutated gDNA in a wild-type background.

2.2 Product variants

PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay is supplied as either Dispense Ready (DR) or 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.

2.3 Principle of the procedure

2.3.1 Internal control

An internal control assay is included in both the reference and mutation-specific assay. It is comprised of a HydrolEasy® probe labelled with PentaYellow™ (measured on the same fluorescence channel as HEX/VIC®/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 assay as possible. The signal from the internal control assay may be affected by positive amplification in the reference and mutation-specific assay. See section 7 for more details.

2.3.2 Reference assay

The reference assay targets a genomic region within the MYD88 gene 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 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. See section 7 for more details.

2.3.3 Mutation-specific assay

The mutation-specific assay targets the genomic region containing the MYD88 L265P mutation (c.794T>C) and is used to determine the presence of the mutation in a sample (**Table 1**). The mutation-specific assay contains a HydrolEasy® probe labelled with PentaGreen™ (measured at the FAM channel), a BaseBlocker™ (to reduce or eliminate non-specific amplification of wild-type template), a mutation-specific primer set, and an internal control assay. The mutation-specific assay is optimised to the conditions specified in section 6 and it is therefore important that these are followed to avoid misleading results. The Ct values of the mutation-specific assay and the corresponding reference are used to determine whether a sample is positive or negative for a given mutation. See section 7 for more details.

Table 1. The MYD88 mutation detected with PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay.

Assay	CDS mutation	Amino acid substitution	Cosmic ID
MYD88 L265P	c.794T>C	p.Leu265Pro	COSM85940

[3]Riva et al., 2017; PMID: 28636636

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3 Reagents and materials

The materials provided with the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay can be found in **Table 2**. Materials and instruments required, but not provided can be found in **Table 3**.

3.1 Storage and stability

Refer to the label for expiry date and storing conditions. Repeated thawing and freezing should be kept to a minimum and should not exceed 12 freeze-thaw cycles.

3.1.1 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.
Used Ready-to-Use PCR tubes and dispensed Primer-Probe and Master Mix should be disposed following your local guidelines on disposal of biological waste. The reagents included in PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay are not for reuse.

3.2 Materials provided

Table 2. List of materials provided with the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay as either Dispense Ready (DR) or Ready-to-Use (RTU).

Product variant	Description	Tube no.		Kit components	Mutation	Contents
		DR	RTU			
Y1	**PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay**	1 2 3 4	A B - -*	MYD88 Reference 1 MYD88 L265P Simplex AmpliQueen™ Master Mix MYD88 Positive Control	- L265P - L265P	Synthetic DNA Synthetic DNA PCR Master Mix Synthetic DNA

*Delivered separately in a tube.

3.3 Materials and Instruments required but not provided

Materials and instruments required but not provided are listed in **Table 3**. PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay is designed to run on open platforms with at least two-channels (green and yellow) and has been validated using samples purified with the BasePurifier™ Nucleic Acid Extraction System (BasePurifier™, PentaBase ref. no. 715 and analysed with the BaseTyper™ 48 (PentaBase, ref. no 754), CFX96 Touch (Bio-Rad, ref. no. 18450974), QuantStudio™ 5 (Applied Biosystems™ ref. no. A28574), Mic qPCR Cycler (Bio Molecular Systems), or LightCycler® 480 II (Roche, ref. no. 05 015 278 001) real-time PCR instruments. 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, to verify cycle thresholds and cut-offs. Contact PentaBase A/S or your local distributor for support.

Table 3. Materials required but not provided for use with PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay.

Materials
Pipettes (1-10 µL, 10-100 µL)
Sterile pipette tips
Centrifuge for spinning PCR tubes, strips or plates
Nuclease-free water for No Template Control (NTC)
DNA extraction method or instrument
DNA extraction method or instrument (e.g., BasePurifier™ 32, PentaBase A/S)
DNA extraction kit for FFPE or blood 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 instruments
Real-time PCR instrument (e.g., BaseTyper™, Pentabase)

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4 Warnings and precautions

- For *in vitro* diagnostic use.
- The mutational status determined by PlentiPlex™ MYD88 Waldenström Lymphoma 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 eye protection when handling biological samples and reagents. Laboratory gloves must be changed between handling different biological samples. Furthermore, work in clean environments to avoid contaminations.
- Remove gloves and wash hands thoroughly after handling samples and reagents.
- Do not use reagents that have expired.
- Do not use damaged PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay tubes.
- Do not use a PlentiPlex™ MYD88 Waldenström Lymphoma 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.
- 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. Refer to section 7 for more information.
- Consult relevant real-time qPCR Instrument User Guides for additional warnings, precautions, and procedures to reduce the risk of contamination.
- Dispose used PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay tubes, pipette tips, and specimen tubes according to local, state, and federal regulations for hazardous biological material.
- Minimise the exposure of PlentiPlex™ MYD88 Waldenström Lymphoma 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.
- Use caution not to contaminate reagents and samples. Dedicated pipettes should be used, and it is recommended to have separate areas for sample preparation and PCR running. Change pipette tip between loading of sample to the reference and the mutation specific tube to avoid contamination of the reagents. Furthermore, use caution not to contaminate patient sample PCR tubes by adding PlentiPlex™ MYD88 L265P Positive Control or several patient samples into the same PCR tube.

5 Sample handling

5.1 Sample collection, transport, and storage

Specimens shall be human genomic DNA extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tissue or whole blood. It is recommended that FFPE and blood samples are collected, transported, processed, and stored according to ISO 20166-3:2018[4] and ISO 20186-2:2019[5], respectively, to ensure optimal DNA quality.

5.2 Purification

Extraction of genomic DNA from FFPE and blood samples should be performed using genomic DNA extraction kits and/or procedures specially designed for handling of FFPE and whole blood samples according to the manufacturer´s instructions including steps for deparaffinisation and sample digestion to remove PCR inhibitors embedded in the sample. It is recommended to evaluate DNA integrity and amplifiability by PCR-based methods according to ISO 20166-3:2018.

5.3 Positive Control and No Template Control

It is recommended to include at least one positive control (provided) and one No Template Control (NTC) (e.g., nuclease-free water, not provided) in each PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay run. The provided positive control consists of approximately 120 MYD88 L265P synthetic DNA copies and 1.2 ng human wild type gDNA per microliter.

NOTE: The positive control (and the NTC) should be added to both a reference and a mutation-specific assay.

[4]Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for formalin-fixed and paraffin-embedded (FFPE) tissue — Part 3: Isolated DNA

[5]Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood — Part 2: Isolated genomic DNA

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6 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.

6.1 Dispense Ready

1. Add 12.5 µL AmpliQueen™ RT-qPCR Master Mix to each PCR tube (vial, strip, plate).
2. Add 7.5 µL of Reference Mix or Mutant Mix to the PCR tubes.
3. Add 5 µL of extracted DNA (1-10 ng/µL) (sample or positive control) to both the mutation-specific assay and the corresponding reference. It is recommended to include an NTC in each run. Add sterile water to the NTC instead of gDNA.
4. Seal all tubes.
   'Optional step: Briefly vortex strips (2-3 sec.) to enhance elimination of air bubbles.'
5. 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.
6. Place the PCR tubes in the Real-Time PCR instrument and run the RT-qPCR program (**Table 4**).

6.2 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 (1-10 ng/µL) (sample or positive control) to both the mutation-specific assay and the corresponding reference. It is recommended to include an NTC in each run. Add sterile water to the NTC instead of gDNA.
3. Seal all tubes.
   'Optional 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.
   Place the PCR tubes in the Real-Time PCR instrument and run the RT-qPCR program (**Table 4**).

6.3 Real-time qPCR program

Table 4. RT-qPCR program for running PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay. Fluorescence is acquired on the green and yellow channel at the second step in the 3-step amplification (at 57°C).

Protocol	Temperature [°C]	Time [sec]	Cycles	Channel
Stage 1				
Hold	95	120	1	
Stage 2 (1-45)				
3-step amplification	94	15	45	
	57	10		FAM/SYBR (green) HEX/VIC®/TET (yellow)
	72	15		

7 Data Analysis

7.1 Baseline and threshold settings

The data analysis procedure for the PlentiPlex™ MYD88 Waldenström Lymphoma 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**).

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.

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**).

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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.

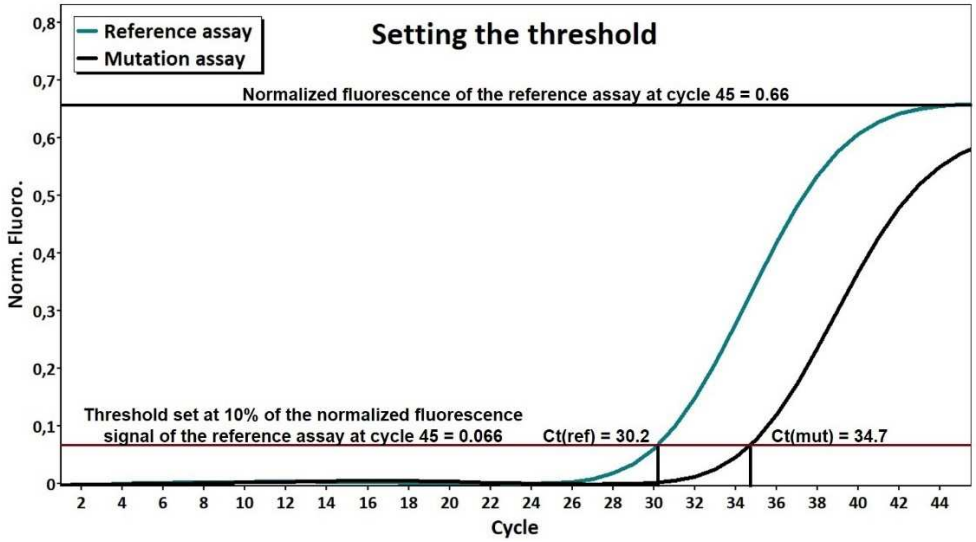

Figure 2. Setting the threshold. Read the fluorescence value for the reference assay at cycle 45 and set the threshold at 10% of this value. This threshold is now used to determine Ct values of the reference assay and the corresponding mutation-specific assay. In the example shown, Ct(reference) = 30.2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

7.1.1 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

7.2 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 and positive controls are either valid or borderline according to **Table 6**.
    a. If some of the samples do not meet the requirements for valid or borderline reference assay Ct values as described in **Table 6**, rerun the samples with either more ($Ct_{(reference)}$ > 36) or less ($Ct_{(reference)}$ < 25) DNA.
    b. If the reference assay (Tube A) green channel Ct values for the positive control sample(s) are invalid, the whole run is invalid, and the results should not be used (See **Table 6**). Make sure that the qPCR program has been defined correctly and that the instrument is acquiring on FAM (green) and HEX/VIC®/TET (yellow) channels (**Table 4**). If the qPCR program is correct, the positive control reagents might be degraded or the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay might not be functional. If you cannot locate the root cause of the problem, please contact PentaBase A/S or your local distributor for support.
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) 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.

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

Reference assay	Interpretation	Comments
Ct < 25	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.
25 ≤ Ct ≤ 30	Valid	The amount of input DNA is optimal for mutation analysis.
30 < Ct ≤ 36	Borderline	The amount of input DNA is lower than recommended. The sensitivity is affected hereby. If the MYD88 L265P mutation is not detected, the analysis should if possible be repeated with a higher amount of input DNA (see **Table 7**).
Ct > 36	Not valid	The amount of input DNA is too low. The analysis should be repeated with a higher amount of input DNA.

7.2.1 Calculating ΔCt

To determine if the MYD88 L265P mutation is detected or not, a ΔCt value is calculated for the green channel assays for each sample and positive control. The ΔCt value is defined as the difference between the green channel Ct value of the mutation-specific assay (Tube B) subtracted the green channel Ct value of the corresponding reference assay (Tube A):

$$\Delta Ct = Ct_{(mutation-specific\ assay)} - Ct_{(reference\ assay)}$$

1. To verify that the PCR run has been performed correctly, for each positive control with a valid or borderline $Ct_{(reference\ assay)}$ value (**Table 6**), calculate the green channel ΔCt for the mutation-specific assay (Tube B) against the corresponding reference assay (Tube A).
2. Verify that the ΔCt of included positive control(s) < 4.
    a. If the green channel ΔCt of any included positive control(s) is ≥ 4, the whole run is invalid, and the results should not be used. Make sure that the qPCR program has been defined correctly and that the instrument is acquiring on FAM (green) (**Table 4**). If the qPCR program is correct, the positive control reagents might be degraded or the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay might not be functional. If you cannot locate the root cause of the problem, please contact PentaBase A/S or your local distributor for support
3. Calculate ΔCt for each sample with a valid or borderline $Ct_{(reference\ assay)}$ value (**Table 6**).

7.3 Interpretation of results

Determination of the MYD88 L265P mutational status is based on the green channel Ct and calculated ΔCt values as described in **Table 7**.

1. If the green channel $Ct_{(mutation-specific\ assay)}$ is equal to or lower than 39 and the ΔCt is equal to or below 9, the MYD88 L265P mutation is detected (**Table 7**).
2. If the $Ct_{(mutation-specific\ assay)}$ is equal to or lower than 39 and the ΔCt is above 9, or if the $Ct_{(mutation-specific\ assay)}$ is above 39 the MYD88 L265P mutation is not detected.

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Table 7. Interpretation of the MYD88 L265P mutational status based on the green channel Ct and ΔCt values of PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay.

Ct		ΔCt	Interpretation	Comments
Reference assay (Tube A)	Mutation-specific assay (Tube B)			
Ct < 25	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.
25 ≤ Ct ≤ 30	≤ 39	≤ 9	MYD88 L265P mutation detected	The amount of input DNA is optimal for mutation analysis.
		> 9	MYD88 L265P mutation not detected	
	> 39	NA	MYD88 L265P mutation not detected	
30 < Ct ≤ 36	≤ 39	≤ 9	MYD88 L265P mutation detected	The amount of input DNA is lower than recommended. The sensitivity is affected hereby. *If the MYD88 L265P mutation is not detected, the analysis should if possible be repeated with a higher amount of input DNA.
	> 39	> 9	MYD88 L265P mutation not detected*	
Ct > 36	Any	Any	Invalid	The amount of input DNA is too low. The analysis should be repeated with a higher amount of input DNA.

7.3.1 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 7.1.

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 7.3 for details regarding lack of internal control signal.

7.3.2 Invalid reference and internal control results

7.3.2.1 No reference signals

If there is no signal from the reference assay in neither the green nor the yellow channel, it indicates that a low amount of gDNA or a low quality gDNA has been used. If the signal also is absent (or Ct is higher than 39) in the mutation-specific assay, the purification of gDNA and/or the PCR analysis should be performed again.

7.3.2.2 No internal control signals

If no signal occurs from the internal control assay, make sure that the PCR program has been defined correctly and that the emitted light is acquired on the 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 gDNA or the presence of PCR inhibitors. 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. 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-specific assay, a PCR inhibitor may be present in the sample. Try to dilute the gDNA five times and repeat the PCR.

7.3.3 Estimation of mutation frequency

PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay is a semi-quantitative test that can be used to estimate the mutation frequency of the indicated MYD88 L265P mutation. The % mutation frequency is described as:

$$Estimated\ mutation\ frequency = \frac{1}{2^{(\Delta Ct)}} \cdot 100\%$$

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An example is shown below:

1. Calculation of ΔCt when $Ct_{(reference\ assay)} = 31.3$ and $Ct_{(mutation-specific\ assay)} = 34.7$:

$$\Delta Ct = Ct_{(mutation-specific\ assay)} - Ct_{(reference\ assay)} = 34.7 - 31.3 = 3.4$$

2. Calculation of estimated mutation frequency:

$$Estimated\ mutation\ frequency = \frac{1}{2^{(\Delta Ct)}} \cdot 100\% = \frac{1}{2^{(3.4)}} \cdot 100\% = 9.5\%$$

8 Performance evaluation

8.1 Analytical sensitivity

Evaluation of analytical sensitivity of both whole blood and FFPE samples was performed on the BaseTyper™ Instrument (48 well block with 4 channel detection). In addition, the analytical sensitivity of whole blood samples was performed on the CFX96 Touch (Bio-Rad), QuantStudio™ 5 (Applied Biosystems™), Mic qPCR Cycler (Bio Molecular Systems), and LightCycler® 480 II (Roche) real-time PCR instruments.

8.1.1 Limit of Blank

The limit of blank (LoB) of the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay was determined to be ΔCt = 13.8 and ΔCt = 12.4 in whole blood and FFPE samples, respectively, using the BaseTyper™ Instrument (**Figure 3**). The LoB was determined using extracted gDNA from 40 and 20 replicates of human whole blood and FFPE wild type tissue samples, respectively, at a total amount of 50 ng per reaction. In addition, the LoB of the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay was determined to be ΔCt = 13.2, ΔCt = 16.8, ΔCt = 13.8, and ΔCt = 16.0 on the CFX96 Touch, QuantStudio™ 5, Mic qPCR Cycler and LightCycler® 480 II real-time PCR instruments, respectively, using 20 replicates of 50 ng whole blood wild type gDNA (**Table 8**).
The whole blood replicates were based on two whole blood samples, and the FFPE replicates were based on three FFPE samples.

8.1.2 Limit of Detection

The limit of detection (LoD) was determined to be 0.60% in whole blood with a 95% confidence level using the BaseTyper™ Instrument. The LoD was evaluated using 20 replicates consisting of synthetic MYD88 L265P (Twist Bioscience) spiked into a 50 ng wild-type human gDNA background extracted from whole blood. This was also determined to be the limit of quantification for whole blood. 19 out of 20 replicates of extracted gDNA from whole blood were detected by PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay. The LoD of PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay when using gDNA from FFPE tissue was determined to be 0.75% with a 95% confidence level by using 20 replicates consisting of MYD88 L265P spiked into a 40 ng wild-type human gDNA background extracted from FFPE (**Figure 3**) analysed on the BaseTyper™ Real-Time PCR Instrument. This was also determined to be the limit of quantification for FFPE.
Furthermore, the LoD of the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay was 0.85% with a 95% confidence level on the CFX96 Touch, QuantStudio™ 5, Mic qPCR Cycler, and LightCycler® 480 II real-time PCR instruments using 20 replicates of 50 ng whole blood wild type gDNA (**Table 8**).
The whole blood replicates were based on two whole blood samples, and the FFPE replicates were based on three FFPE samples.

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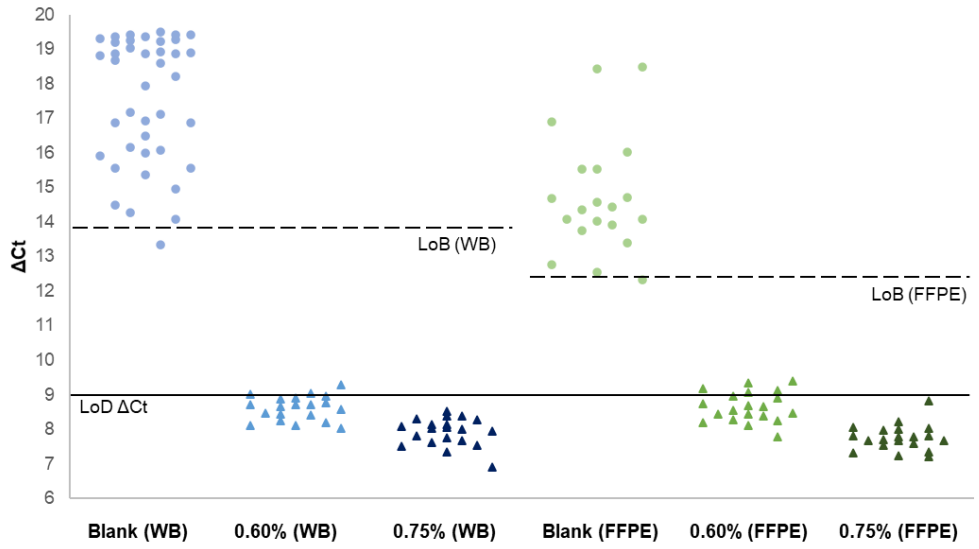

Figure 3. Left side: ΔCt values for 40 replicates of the blank sample (blue dots) and 20 replicates of both the 0.60% and 0.75% MYD88 L265P mutated samples (light blue and dark blue triangles) for human gDNA extracted from whole blood (WB). The LoD was determined as 0.60% for a ΔCt ≤ 9.0 and the LoB ΔCt was found to be 13.8, illustrated by the dotted black line. 19 out of 20 replicates of the 0.60% samples have a ΔCt ≤ 9. The LoD ΔCt ≤ 9 threshold is illustrated by the black full line. *Right side:* ΔCt values for 20 replicates of the blank sample (green dots) and 20 replicates of both the 0.60% and 0.75% MYD88 L265P mutated samples (light and dark green triangles) for human gDNA extracted from FFPE tissue. The LoD was determined to be 0.75% with a ΔCt ≤ 9.0 and the LoB ΔCt was 12.4 illustrated by the dotted black line. 20 out of 20 replicates of the 0.75% samples have a ΔCt ≤ 9 in FFPE background, whereas only 15 out of 20 replicates of the 0.60% samples have a ΔCt ≤ 9. The data illustrated are analysed using the BaseTyper™ Real-Time PCR Instrument.

Table 8. Analytical sensitivity of PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay for whole blood samples using the BaseTyper™ real-time PCR instruments.

Analytical sensitivity of the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay												
	MYD88 L265P concentration (%) spiked into wild-type Whole Blood gDNA											
	Blank			0.50%			0.60%			0.75%		
	Replicates ΔCt>9 / Total	LoB ΔCt	Mean $Ct_{(mut)}$ (PentaGreen™)	Replicates ΔCt≤9 / Total	Mean ΔCt	Mean $Ct_{(mut)}$ (PentaGreen™)	Replicates ΔCt≤9 / Total	Mean ΔCt	Mean $Ct_{(mut)}$ (PentaGreen™)	Replicates ΔCt≤9 / Total	Mean ΔCt	Mean $Ct_{(mut)}$ (PentaGreen™)
BaseTyper™	40 / 40	13.8	40.3	14 / 20	8.9	35.1	19 / 20	8.6	34.5	20 / 20	7.9	34.4

Table 9. Analytical sensitivity of PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay for FFPE samples using the BaseTyper™ real-time PCR instruments.

Analytical sensitivity of the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay									
	MYD88 L265P concentration (%) spiked into wild-type FFPE gDNA								
	Blank			0.60%			0.75%		
	Replicates ΔCt>9 / Total	LoB ΔCt	Mean $Ct_{(mut)}$ (PentaGreen™)	Replicates ΔCt≤9 / Total	Mean ΔCt	Mean $Ct_{(mut)}$ (PentaGreen™)	Replicates ΔCt≤9 / Total	Mean ΔCt	Mean $Ct_{(mut)}$ (PentaGreen™)
BaseTyper™	20 / 20	12.4	41.4	15 / 20	8.6	35.7	20 / 20	7.8	34.5

8.1.3 Linearity

The assay shows linearity from 0.3% to 30% MYD88 L265P DNA spiked in a 50 ng wild-type human genomic DNA background extracted from whole blood with a R^2 of 0.997. The PCR efficiency were 114% and 103% for the green channel on the reference assay and the mutation-specific assay, respectively. Furthermore, the PCR efficiency were 99% and 92% for the yellow channel on the reference assay and the mutation-specific assay, respectively.

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8.2 Analytical precision

8.2.1 Within-Laboratory Repeatability and Reproducibility

The analytical precision was validated by within-laboratory repeatability and reproducibility (**Table 10**). The validation was performed by two different persons at four different days spread over two different weeks using four different BaseTyper™ qPCR instruments and three different LOTs of PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay. The templates tested were 0%, 2% and 10% MYD88 L265P mutated DNA spiked into 50 ng wild type gDNA extracted from whole blood as well as a positive control. Within-laboratory repeatability was determined as the 3 replicate ΔCt variance (standard deviation (SD)) within each run performed in the same laboratory. The 3 replicate ΔCt variance was averaged across 4 days, 4 instruments and 3 lots. Within-laboratory reproducibility was determined as the total within-laboratory variance when tests were performed across 4 days, 4 instruments, 3 lots and 3 replicates. Both within-laboratory repeatability and reproducibility showed low variance within the acceptance range listed in **Table 10** for all four templates. However, the reproducibility SD for the 0% mutated DNA template was just within the acceptance range. The higher SD is caused by varying ΔC_t values for the blank samples just as seen in the LoB study illustrated in **Figure 3**.

Table 10. Repeatability and reproducibility for PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay. Standard deviation (SD) is listed.

Dye	Targets	Mean (ΔCt)	Repeatability	Reproducibility
			SD	SD
PentaGreen™	0% MYD88 L265P mutated DNA in gDNA background	14.2	1.33	2.5
	2% MYD88 L265P mutated DNA in gDNA background	6.7	0.26	0.26
	10% MYD88 L265P mutated DNA in gDNA background	4.3	0.21	0.35
	MYD88 L265P Positive Control	2.2	0.51	0.55
Dye	**Targets**	**Mean (Ct)**	**Repeatability**	**Reproducibility**
			SD	**SD**
PentaYellow™	0% MYD88 L265P mutated DNA in gDNA background	26.6	0.21	0.48
	2% MYD88 L265P mutated DNA in gDNA background	26.6	0.13	0.34
	10% MYD88 L265P mutated DNA in gDNA background	26.7	0.23	0.38
	MYD88 L265P Positive Control	29.1	0.38	0.41
Acceptance range			≤2	≤2.5

8.3 Analytical specificity

A single genetic variation was found in the primer binding region of the MYD88 gene by analysis in UCSC database[6], however the SNP is very rare (frequency of 0.005%). There is no reason to believe that the assay is not specific for the MYD88 L265P mutation. In addition, a common SNP was found in the probe binding region of the internal control. It was concluded by wet lab testing that the SNP do not affect the performance of the internal control. The primers were found to be 100% specific towards their target regions by blasting in NCBI Primer-BLAST[7] and no cross reactivity was found between the two primer sets.

8.4 Clinical evaluation

For the clinical evaluation, a blinded study using 21 whole blood (WB) and 21 FFPE matrix replicates spiked with MYD88 L265P DNA was set up (**Table 11**). The samples were handled by Person 1 who spiked the replicates to a concentration of 0% (wild-type), 1%, 5% and 10% MYD88 L265P in a background of 6-10 ng/µL human gDNA. The samples were handed over to Person 2 who performed the qPCR. Afterwards, the data was analysed by Person 3. Neither Person 2 nor Person 3 had any knowledge about which replicates were spiked with MYD88 L265P DNA. 42 out of 42 replicates were identified correctly by the PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay, where 18 of the replicates were wild-type samples (6 WB and 12 FFPE replicates), 12 of the replicates were 1% MYD88 L265P (6 WB and 6 FFPE replicates), 12 of the replicates were 5% MYD88 L265P (6 WB and 6 FFPE replicates), and finally, there were three 10% FFPE replicates. The whole blood background was based on one whole blood samples, and the FFPE background was based on two FFPE samples.

Table 11. Analysed sample data versus actual sample status from a blinded clinical study involving 0% (wt, wildtype), 1%, 5%, and 10% MYD88 L265P spiked whole blood (WB).

Clinical performance: Whole blood samples		**PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay**		
		Mutated	**Wild-type**	**Total**
Sample	**Mutated**	6	0	6
	Wild-type	0	15	15
	Total	6	15	21
Overall agreement was 21/21 or 100% (CI95%: 85.5-100%)				
Wild-type agreement was 15/15 or 100% (CI95%: 79.6-100%)				
Mutation agreement was 6/6 or 100% (CI95%: 61.0-100%)				

[6] https://genome.ucsc.edu/
[7] https://blast.ncbi.nlm.nih.gov/Blast.cgi

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Table 12. Analysed sample data versus actual sample status from a blinded clinical study involving 0% (wt, wildtype), 1%, 5%, and 10% MYD88 L265P spiked FFPE samples.

Clinical performance: FFPE samples		PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay		
		Mutated	**Wild-type**	**Total**
Sample	**Mutated**	12	0	12
	Wild-type	0	9	9
	Total	12	9	21
Overall agreement was 21/21 or 100% (CI95%: 85.5-100%)				
Wild-type agreement was 9/9 or 100% (CI95%: 70.1-100%)				
Mutation agreement was 12/12 or 100% (CI95%: 75.8-100%)				

Table 13. Analysed sample data versus actual sample status from a blinded clinical study involving 0% (wt, wildtype), 1%, 5%, and 10% MYD88 L265P spiked whole blood (WB) and FFPE samples.

Clinical performance: FFPE and Whole blood (total)		PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay		
		Mutated	**Wild-type**	**Total**
Sample	**Mutated**	24	0	24
	Wild-type	0	18	18
	Total	24	18	42
Overall agreement was 42/42 or 100% (CI95%: 91.6-100%)				
Wild-type agreement was 18/18 or 100% (CI95%: 82.4-100%)				
Mutation agreement was 24/24 or 100% (CI95%: 86.2-100%)				

9 Limitations

- PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay has been validated for use with MYD88 L265P spiked whole blood samples and MYD88 L265P spiked colorectal FFPE 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 gDNA or the presence of PCR inhibitors in the sample.
- PlentiPlex™ MYD88 Waldenström Lymphoma qPCR Assay is validated for use with 5-50 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.
- PlentiPlex™ MYD88 Waldenström Lymphoma 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 internal control assay contains suboptimal primer concentrations and amplification may be inhibited by amplification by the PentaGreen™-labelled assay in the same reaction.

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10 Symbols

The following symbols are used in labelling of PlentiPlex™ MYD88 Waldenström Lymphoma 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 electronically available instructions for use

11 Manufacturer and distributors

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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.