PlentiPlex™ MYD88

Made by PentaBase

INSTRUCTIONS FOR USE

PlentiPlex™ Assay for Sensitive Detection of the MYD88 L265P Mutation

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Δ IMPORTANT:

Please read these instructions carefully before using the PlentiPlex™ MYD88 L265P assay. It is recommended to save the "Instructions for use" for future use. Purchasers of PlentiPlex™ MYD88 L265P assay are only granted the right of use, but no general licensing or patent rights.

1. INTENDED USE

PlentiPlex™ MYD88 L265P assay is intended for in vitro diagnosis of the leucine to proline mutation in codon 265 of the Myeloid differentiation primary response 88 protein (MYD88 L265P) in genomic DNA (gDNA) samples. The test will provide an assessment of the presence of the examined mutation constituting down to 0.25% of a human genomic DNA (gDNA) sample (from formalin fixed paraffin-embedded tumor biopsies).

PlentiPlex™ MYD88 L265P assays are to be used by trained laboratory personnel in a professional laboratory environment with human gDNA samples (e.g. gDNA extracted from formalin fixed paraffin-embedded tissues from cancer). PlentiPlex™ MYD88 L265P assays **are not intended for diagnosing of cancer** but only as an aid to assist the oncologist's treatment planning.

The tests are provided in one or more boxes containing all necessary components for use including an "Instructions for Use" and a "Quick guide".

1.1 INDICATIONS FOR USE

The obtained results of PlentiPlex™ MYD88 L265P are intended for identification of the presence of the MYD88 L265P, facilitating discrimination between Lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia (LPL/WM) and non-Hodgkin lymphoma. Importantly, mutational status should always be considered alongside other disease factors when making treatment decisions.

2. SUMMARY AND EXPLANATION OF THE ASSAYS

MYD88 mutation detected with PlentiPlex™ MYD88 L265P			
Assay	**CDS mutation**	**Amino acid substitution**	**Cosmic ID**
MYD88 L265P	c.794T>C	p.Leu265Pro	COSM85940

Table 1. List of investigated mutations

3. TECHNOLOGY AND REAGENTS

PlentiPlex™ MYD88 L265P assay 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 equipment 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™ MYD88 L265P assay contains both standard oligonucleotides and pentabase-modified oligonucleotides (HydrolEasy™ probes, SuPrimers™, and BaseBlockers™). Using PlentiPlex™ MYD88 L265P, somatic mutations can be detected quickly (in less than one hour), sensitively (1-50 ng gDNA input per well/vial) and selectively (0.25% mutation in a wild type background of gDNA), by real-time PCR analysis.

3.1 HydrolEasy™ 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™ MYD88 L265P 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 HEX™, 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™ MYD88 L265P assay, 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.

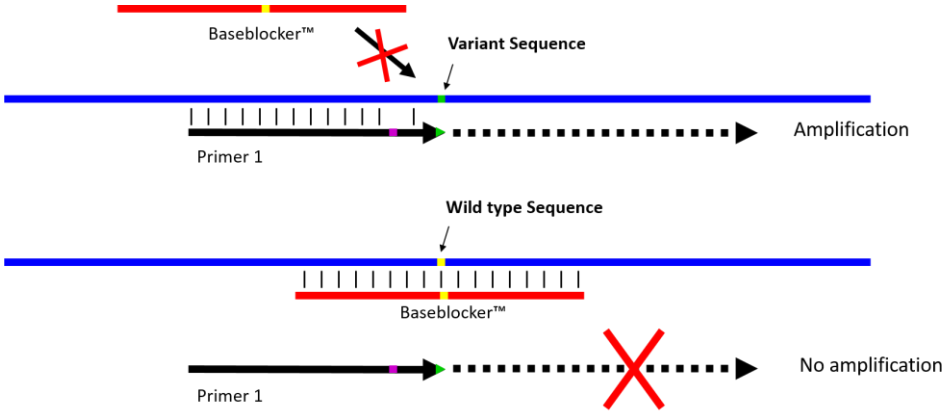

Figure 1: Illustration of how BaseBlockers™ function in PlentiPlex™ assays. A BaseBlocker™ binds to and blocks the wild type template from being amplified. In contrast, the BaseBlocker™ does not inhibit amplification of a template with a single nucleotide mutation and the result is a selective amplification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

4. Assay Format and Design

4.1 Format

PlentiPlex™ MYD88 L265P assay is supplied in either a "Ready-To-Use" or "Dispense Ready" version. PlentiPlex™ MYD88 L265P Ready-to-use assays are provided in either 1, 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™ MYD88 L265P assay 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 (HEX™) channel)
- Master mix

Mutation assay

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

MYD88 L265P Assay					
Strip #	**Gene**	**Tube #**	**Content**	**Mutations**	**Corresponding reference**
Y1*	**MYD88**	**A** **B**	**MYD88 Reference 1** MYD88 Simplex 1	L265P	MYD88 Reference 1

Table 2. PlentiPlex™ MYD88 L265P Ready-to-use assay components. Each tube contains 20 µL in total (7,5 µL primer/probe-mix and 12,5 µL master mix). *Research use only.

MYD88 L265P Assay						
Gene	**Tube #**	**Content**	**Mutations**	**Corresponding reference**	**Volume 20x**	**Volume 50x**
MYD88*	1 2 3	**MYD88 Reference 1** MYD88 Simplex 1 Master mix	L265P	MYD88 Reference 1	150 µL 150 µL 500 µL	375 µL 375 µL 1250 µL

Table 3. PlentiPlex™ MYD88 L265P Dispense Ready assay components. Each tube contains reagents for either 20 or 50 reactions. *Research use only.

4.2 Internal control

An internal control assay is included in all the primer-probe mixes of the different assays and comprise a HydrolEasy™ probe labeled 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 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 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.

4.4 Mutation assay

The mutation assay (Table 2-3) 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 (see section 8 “Data Analysis” for more details). The mutation 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), 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™ MYD88 L265P

The use of PlentiPlex™ MYD88 L265P will require the following equipment and consumables:

- Template DNA (extracted mutant gDNA)
- 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 H_2O (sterile)

* PlentiPlex™ MYD88 L265P has been validated on the following real-time PCR instruments: Rotorgene (Qiagen) and MyGo Pro (IT-IS Life). We recommend that one of these systems are used, but other instruments are likely applicable.

^ PlentiPlex™ MYD88 L265P 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™ MYD88 L265P 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™ MYD88 L265P assay:

- The assay is only for *in vitro* diagnostics
- The mutational status determined by PlentiPlex™ MYD88 L265P 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™ MYD88 L265P assay. 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™ MYD88 L265P performance. For more information, see section 4.5 “Equipment and Reagents not supplied with PlentiPlex™ MYD88 L265P”
- 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™ MYD88 L265P Ready-to-use assays are shipped on dry ice while Dispense Ready assays are shipped on either dry ice or blue ice. If the PlentiPlex™ MYD88 L265P 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™ MYD88 L265P assays should after arrival immediately be stored at maximum -15°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 fresh, frozen or formalin fixed paraffin-embedded (FFPE) tumor sections or similar. The samples should be collected and stored after standard pathology methodologies to ensure optimal quality. Extracted gDNA should be stored at maximum -15˚C until use.

6.1 Recommended procedure for extraction of gDNA

Several methods to extract gDNA can be used. Different methods have been validated under the development and validation of PlentiPlex™ MYD88 L265P, among other the Maxwell™ 16 FFPE Purification Kit (Promega, Cat. #AS1010). Regardless of method, it is recommended to follow the manufacturers protocol for gDNA extraction.

7. PlentiPlex™ MYD88 L265P protocol

Before using the assay, it is recommended to carefully read the full “Instructions for use”. When using PlentiPlex™ MYD88 L265P 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

- Thaw the reaction mixtures and spin down
- Add 5 µL extracted gDNA (1-10 ng/µL) to the reference and mutation assay. It is recommended to include a no template control (NTC) in each run. Add sterile water instead of gDNA.
- Close lids and spin down
- Place the strips into the instrument and run the protocol described in Table 4
- Analyze the samples in accordance with the analysis rules. For more information, see section 8 “Data analysis”

Protocol	Temperature	Time	Number of cycles	Data (channel)
Hold	95˚C	2 min	1	-
Cycling	94˚C	15 sec.	45	FAM™/SYBR™ (470 nm/510 nm) HEX™/VIC™/TET™ (538 nm/551 nm) Measure fluorescence intensity during annealing (57°C)
	57˚C	10 sec.		
	72˚C	15 sec.		

Table 4. PlentiPlex™ MYD88 L265P Real-time PCR protocol

7.2 Dispense Ready

- Thaw the reaction mixtures, mix and spin down
- Add 12.5 µL master mix to all tubes/wells
- Add 7.5 µL of reference mix or mutant mix to the tube/well and mix carefully by pipetting
- Add 5 µL extracted gDNA (1-10 ng/µL) from each sample to the mutation assay and the corresponding reference. Mix carefully by pipetting. It is recommended to include an NTC in each run. Add sterile water to the NTC instead of gDNA.
- Seal all tubes/wells and spin down. Make sure that there are no bobbles in the solutions
- Place tubes or plate in the instrument and run the protocol as described in Table 4
- Analyze the samples in accordance with the analysis rules. For more information, see section 8 “Data analysis”

8. Data analysis

In PlentiPlex™ MYD88 L265P 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). The Ct value is reflecting the DNA amount and any PCR inhibitors present in a sample.

8.1 Adjusting the baseline

Before setting the threshold value and calculating the Ct values, it is important that any baseline “drift” or fluctuation is corrected so that the baseline or background fluorescence is as close to zero as possible. 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 the instrument-specific guidelines for specific instructions when available.

IMPORTANT! In cases where it is not possible to adjust the baseline fluorescence to zero, the value of baseline fluorescence at cycle 20 should be added to the threshold value calculated by taking 10 % of the reference signal at cycle 45. An example of this is shown in Table 5.

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

Table 5. Setting the threshold.

8.2 Determining the mutational status

Use the following protocol to determine the mutational status:

1. Analyze the mutation assay against the corresponding reference for one sample at a time
2. Correct for "baseline drift" before setting the threshold:
   - Use slope correction/curve fitting when possible and/or define the baseline or background cycle interval to be between cycle 15 and cycle 20
3. Set the threshold at 10% of the reference signal strength at cycle 45 (Figure 2). Add any significant baseline fluorescence at cycle 20 to the threshold value (Table 5)

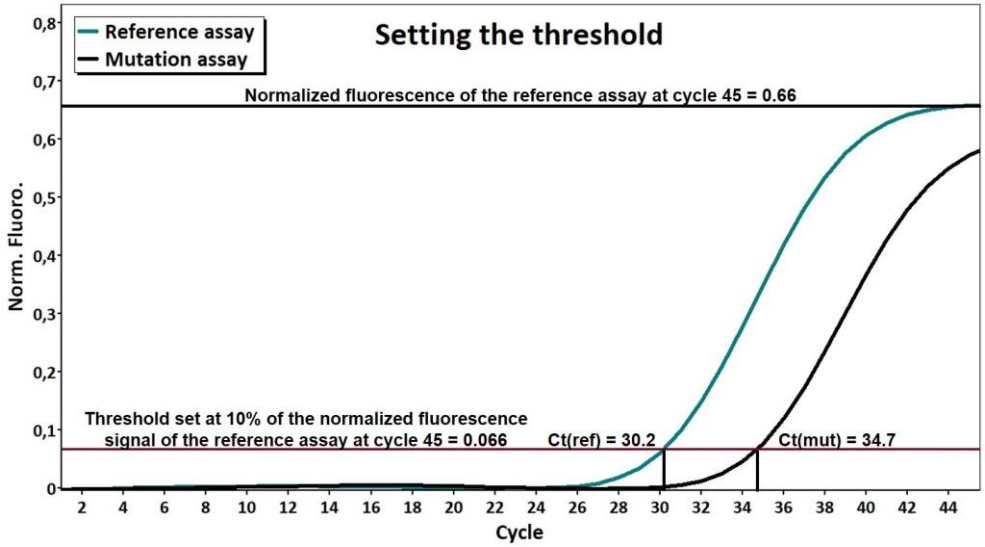

Figure 2: Setting the threshold. Read the fluorescence value for the reference assay at cycle 45 and set the threshold at 10% of this value. This setting for the threshold is now used for the analysis of the corresponding mutation assay. In the shown example, Ct(reference)=30.2 and Ct(mutation)=34.7. Thus, the sample is positive for the mutation analyzed.

4. If NTC samples have been included in the run, verify that no signal is seen before Ct = 38 for the reference and Ct = 45 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
5. Read the Ct value for the corresponding reference assay and validate that the reference sample is suitable for analysis cf. Table 6.

Ct for reference	Quality	Comments
Ct, reference <25	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
25≤ Ct, reference ≤31	Optimal	The amount of input DNA is optimal for mutation analysis
31< Ct, reference ≤36	Borderline	The amount of input DNA is lower than recommended. The sensitivity is affected hereby. The analysis should if negative be repeated with higher amount of input DNA if possible
Ct, reference >36	Not valid	The amount of input DNA is too low. The analysis should be repeated with higher amount of input DNA

Table 6. Acceptable Ct values for the reference assay.

6. Calculate ΔCt for each of the mutations having a Ct value equal to or lower than 39. A sample is positive for the mutation(s) of interest if the ΔCt is equal to or below 9 and negative if the ΔCt is above 9 cf. Table 7.

ΔCt for assay	Conclusion	Comments
ΔCt ≤9	Positive	The sample is mutation positive if ΔCt ≤9 and Ct ≤39
ΔCt >9	Negative	The sample is mutation negative if ΔCt ≥9 or Ct ≤39

Table 7. Mutation analysis

8.3 Internal control analysis

In reactions with no or late amplification by the PentaGreen™ labeled assay, it should be validated that template has been added and/or amplification has taken place by examining the fluorescence from the internal control assay (yellow channel). To set the threshold for the internal control assay, select the yellow channel and repeat steps 1-3 in section 8.3. **Note:** The internal control assay contains suboptimal primer concentrations and amplification may be inhibited by amplification by the PentaGreen™ labeled 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.

9. Troubleshooting

The troubleshooting guide shown in Table 8 below covers some of the most frequent questions and problems that can occur with the use of PlentiPlex™ MYD88 L265P 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 is no signal (before Ct = 39) in the mutation-specific assay 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
$Ct_{reference}$< 24	The amount of input gDNA is too high. This can affect the performance of PlentiPlex™ MYD88 L265P. Repeat the PCR with lower input of gDNA if possible
$Ct_{reference}$ > 36	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 8. Troubleshooting

10. References

11. Manufacturer and Distributors

11.1 Manufacturer

PentaBase A/S
Petersmindevej 1A
5000 Odense C, Denmark

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™ MYD88 L265P protocol was revised March 2020.

Change history

Version No.	Effective Date	Significant Changes	Previous version
1.00	08.05.2018	New Procedure	N/A
1.10	19.08.2018	Corrected table 6 Ct cutoff values for the reference assay	1.00
1.20	10.12.2018	Corrected table 3 Gene name	1.10
1.30	20.05.2019	Clarified description of specimens for intended use in section 6	1.20
1.31	31.03.2020	Removed incorrect instruction to use triplicate samples in section 7	1.30

12. Additional information

TaqMan™ is a trademark of Roche. 5-FAM™, VIC™, TET™ and HEX™ are trademarks and registered trademarks of Applera Corporation or its subsidiaries in the U.S. and certain other countries. PlentiPlex™, HydrolEasy™, SuPrimers™ and BaseBlockers™ are all trademarks belonging to PentaBase A/S. Products or parts of it must not be resold or transferred without PentaBases acceptance. PentaBase A/S takes certain reservation for changes. PentaBase A/S disclaim all responsibility for any errors that may appear in this Instructions for use. Furthermore, PentaBase A/S disclaim all responsibility for misinterpretation that can occur by using this product.

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13. NOTES