Instructions for use

Imegen® PML-RARA

Ref. IMG-111

RUO

Fabricated by:

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All Health in Code S.L. products undergo strict quality control. Imegen® PLM-RARA has passed all internal validation tests, thus guaranteeing the reliability and reproducibility of each assay.

If you have any questions about the use of this product or its protocols, feel free to contact our Technical Department:

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Modifications to the Instructions for Use (IFU)		
Version 05	AUG 2023	Enzyme reagent renaming in sections 5, 6 and 9.
Version 04	MAR 2023	Enzyme reagent naming change in sections 5 and 6.
Version 03	SEP 2022	Change of manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain. Change of manufacturer's identification from Imegen to HEALTH IN CODE, S.L.
Version 02	NOV 2018	Content review.

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01 General information

PML–RARA transcripts, which are the molecular result of the t(15;17)(q22;q21) translocation, are associated with the majority of acute progranulocytic leukemia (APL) cases. The genes that are fused in the t(15;17) translocation are *PML*, located on chromosome 15, and *RARA* (the gene that codes for retinoic acid receptor alpha), located on chromosome 17. This fusion produces an oncogene encoding a protein that acts as a transcriptional silencer.

The fusion breakpoint of *RARA* is always in intron 2, but for *PML* there are three possibilities: intron 6, exon 6, or intron 3, which in turn result in three possible *PML–RARA* fusion variants, being bcr1 (representing 55% of *PML–RARA* translocations), bcr2 (5% of cases), and bcr3 (40%), respectively.

References

- *Leukemia. 2003; Volume 17: 2318–2357. doi:10.1038/sj.leu.2403135 2318–2357. doi:10.1038/sj.leu.2403135*
- *Int J Clin Exp Pathol. 2015; 8(11): 15294–15300. Epub 2015 Nov 1.*

02 Intended use

The Imegen® PML–RARA kit uses a combination of oligonucleotides and hydrolysis probes in real-time PCR to amplify and quantify the *PML–RARA*:bcr1 rearrangement. This kit allows calculating the number of copies of the rearrangement and the endogenous gene *GUS*, by comparing it with a single plasmid that contains a copy of the two amplification targets. Moreover, this genetic analysis allows the user to detect minimal residual disease (MRD). The theoretical sensitivity level that can be reached is MR4 (0.01% of *PML–RARA*).

Limit of quantification (LoQ) is the minimum quantifiable value and matches the greatest dilution included in the standard curve. Therefore, the LoQ is established at 125 total copies both for the reference gene *GUS* and for the *PML–RARA* rearrangement.

This test requires 10 µL of cDNA synthesized by reverse transcription of total RNA extracted from peripheral blood samples.

Imegen® PML–RARA is intended solely for research use and is aimed at professionals working in molecular biology.

03 Safety warnings and precautions

- It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
- Do not mouth-pipette.
- Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- Any cuts, abrasions, and other skin injuries must be properly protected.
- Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the case of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with a large amount of water.
- Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
- This product requires the manipulation of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and manipulate them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
- This kit has been validated with specific equipment and under specific conditions that may vary widely among laboratories. Therefore, it is recommended that each laboratory conduct an internal validation when the kit is to be used for the first time.
- The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code S.L.
- The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code S.L. but are considered by the user equivalent to those provided in the kit.

04 Content and storage conditions of the kit

This kit contains sufficient reagents to perform 48 real-time PCR reactions.

The reagents included in this kit are as follows:

- ***PML-RARA Master Mix***: Oligonucleotides and hydrolysis probes (FAM™) specifically designed to detect the *PML-RARA:bcr1* rearrangement.
- ***GUS Master Mix***: Oligonucleotides and hydrolysis probes (FAM™) specifically designed to detect the reference gene *GUS*.
- ***PML-RARA Standard:*** Plasmid at a concentration of 25 x 10^4 copies/µL of *PML-RARA* and *GUS* in a 1:1 ratio.

Reagents	Color	Vials	Storage
PML-RARA Master Mix	Blue cap	2 x 24 reactions	4 °C
GUS Master Mix	Yellow disc	2 x 24 reactions	4 °C
PML-RARA Standard.	Blue cap	4 vials	4 °C

Table 1. Componentes of the Imegen® PML-RARA kit

**The reagents in this kit are freeze-dried. Once rehydrated, the reagents must be stored at -20 °C*

05 Equipment, reagents and materials not included in the kit

Equipment:

- Real-time PCR thermal cycler (FAM channel)
- Micropipettes (10 µL, 20 µL, and 200 µL)
- Vortex mixer
- Centrifuge

Reagents:

- Hot Start PCR Master Mix (TaqMan™ Environmental Master Mix 2.0, ThermoFisher Scientific)
- Nuclease-free water

NOTE: This kit does not include the necessary reagents for RNA reverse transcription to cDNA.

Materials:

- Filter pipette tips (10 µL, 20 µL, and 200 µL)
- Sterile 1.5-mL tubes
- Fungible optical material compatible with the real-time PCR thermal cycler
- Latex gloves

Related kits

Health in Code S.L. offers a real-time PCR screening kit that can detect and distinguish between the the bcr1, bcr2, and bcr3 *PML-RARA* oncogene variants: Imegen® PML-RARA Screening (Ref: IMG-130).

06 Assay protocol

06.1 | Preparation of the PCR reagents

All the reagents included in this kit are freeze-dried. Before using the kit, the first step is to rehydrate the reagents by adding the amounts of nuclease-free water indicated in the table below. To enable resuspension of each component, it is recommended to shake and spin the tubes containing the reagents and store them at 4 °C for one hour before use.

Reagents	Rehydration
PML-RARA Master Mix	130 µL water/vial*
GUS Master Mix	130 µL water/vial*
PML-RARA Standard.	50 µL water/vial*

Table 2. Rehydration volume for the components of the kit

(*) If these reagents are not to be used immediately after rehydration, storage at -20 °C is recommended.

06.2 | Preparation of standard curves

Once *PML-RARA Standard* has been rehydrated, serial 1:10 dilutions must be prepared to generate the standard curves. These curves allow quantifying the number of copies of both the endogenous gene (*GUS*) and the bcr1 variant *PML-RARA* rearrangement. The number of copies of the positive control is $25x10^4$. It is recommended to prepare the control dilutions immediately before performing the assay.

01 Thaw *PML-RARA Standard*. Vortex and spin.

02 Perform four serial 1:10 dilutions by adding 5 µL of standard and 45 µL of water until a control with a concentration of 25 copies has been obtained.

03 Vortex and spin the tube after preparing each dilution.

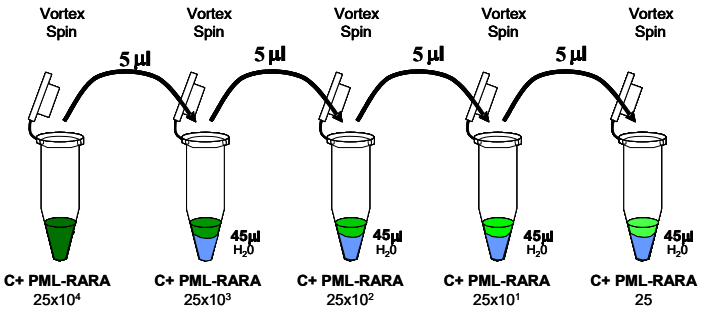

Figure 1. Protocol to prepare the standard curve using PML-RARA Standard

06.3 | Preparation of amplification reactions

To perform the test using the Imegen® PML-RARA kit, two independent PCR master mixes must be prepared.

01 Thaw the following reagents:

- *PML-RARA Master Mix*
- *GUS Master Mix*
- cDNA from test samples
- *Hot Start PCR Master Mix (TaqMan™ Environmental Master Mix 2.0,* ThermoFisher Scientific) (not included)

02 Vortex each reagent to mix thoroughly and keep on ice.

To 1.5 mL tubes, add the necessary amounts of the reagents specified below. To perform the calculations, it is recommended either to add a sufficient amount of reagents to perform one extra reaction or to add an extra 10% of each reagent.

NOTE: It is important to calculate the required volumes for each mix taking into account the number of samples to be tested, as well as the necessary reactions to build the standard curve and to analyze a negative PCR control (no-template control, NTC).

	Volume per reaction	
Reagents	**PML-RARA Master Mix**	**GUS Master Mix**
PML-RARA Master Mix	5 µL	-
GUS Master Mix	-	5 µL
*TaqMan™ Environmental Master Mix 2.0 **	10 µL	10 µL

(*) See section 5. Equipment, reagents, and materials not included in the kit

03 Vortex the tubes containing the PCR master mixes and dispense 15 µL to each well of the optical consumable.

04 Moreover, the following reagents must be added to the corresponding wells:

- 5 µL of sample cDNA (in duplicate)
- 5 µL of each *PML-RARA Standard* dilution
- 5 µL of nuclease-free water (negative control, NTC)

PML-RARA Master Mix	
Standard 25 x 10^4 copies/µl	cDNA 1_R1
Standard 25 x 10^3 copies/µl	cDNA 1_R2
Standard 25 x 10^2 copies/µl	cDNA 2_R1
Standard 25 x 10^1 copies/µl	cDNA 2_R2
Standard 25 copies/µl	NTC

GUS Master Mix	
Standard 25 x 10^4 copies/µl	cDNA 1_R1
Standard 25 x 10^3 copies/µl	cDNA 1_R2
Standard 25 x 10^2 copies/µl	cDNA 2_R1
Standard 25 x 10^1 copies/µl	cDNA 2_R2
Standard 25 copies/µl	NTC

Figure 2. Example of a PCR template. R, replicates; NTC, no-template control; Standard, PML-RARA Standard

05 Place the tubes or plates into the real-time PCR thermal cycler and configure settings for the amplification program as indicated in the next section.

06.4 | Settings for the real-time PCR program

- **Type of experiment**: Quantitation – Standard curve
- **Ramp rate**: Standard
- **Reaction volume**: 20 µl
- **ROX™ baseline reference**: Included
- **Fluorophores of TaqMan® probes**:

Probe	Fluorophore	*Quencher*
PML-RARA (bcr1)	FAM™	TAMRA*
GUS	FAM™	TAMRA*

Table 3. Information about probes

(*) In a StepOne PCR System (ThermoFisher Scientific), this field must be indicated as "None"

- **Optimal program**:

Fields	Phase 1 Enzyme activation		Phase 2 PCR	
No. of cycles	1 initial cycle	1 initial cycle	50 cycles	
			Denaturation	Annealing / Extension
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 minutes	10 minutes	15 seconds	1 minute*

Table 4. Optimal PCR program for the 7500 FAST or StepOne PCR Systems

(*) Fluorescence detection

07 Analysis of results

The following recommendations should be followed to ensure an adequate analysis of results:

NEGATIVE CONTROLS

- Verify the lack of amplification in **negative controls (NTC)**. If amplification is detected, it is recommended to repeat the test to rule out accidental contamination.

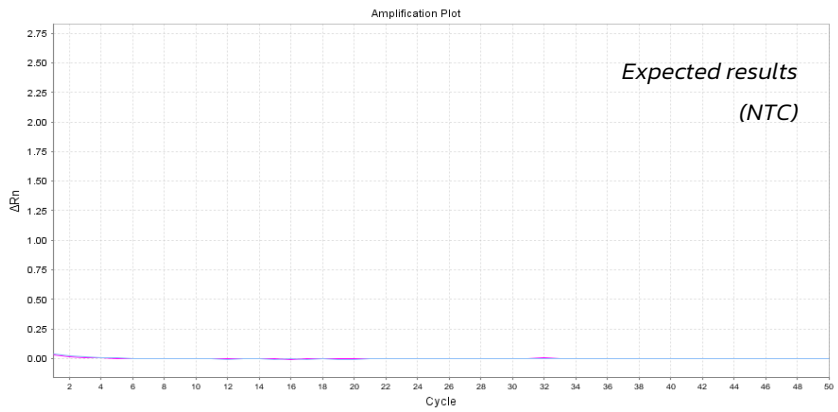

Figure 3. Expected result for the negative control (NTC)

STANDARD CURVE (*PML-RARA STANDARD*)

- Confirm that serial dilutions prepared using the PML-RARA Standard produce suitable standard curves for both PML-RARA and GUS when linear regression is adjusted to logarithmic copy numbers:
  - Slope: Range from -3.1 to -3.7
  - Coefficient of determination: R^2 >0.980.

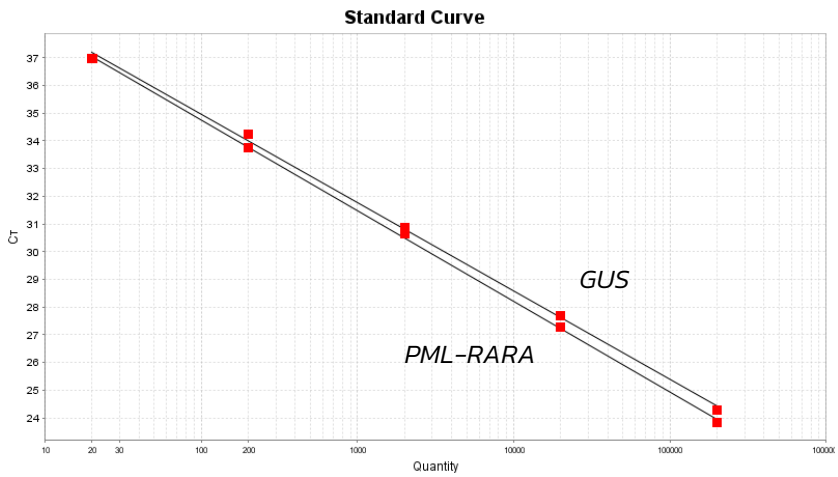

Figure 4. Linear regression of standard curves for GUS and PML-RARA

If no amplification is detected in *PML-RARA Standard*, see Section 8 (Troubleshooting). The highest concentration of the standard is 25 x 10^4 copies per µL (1,250,000 total copies) and the lowest is 25 copies per µL (125 total copies).

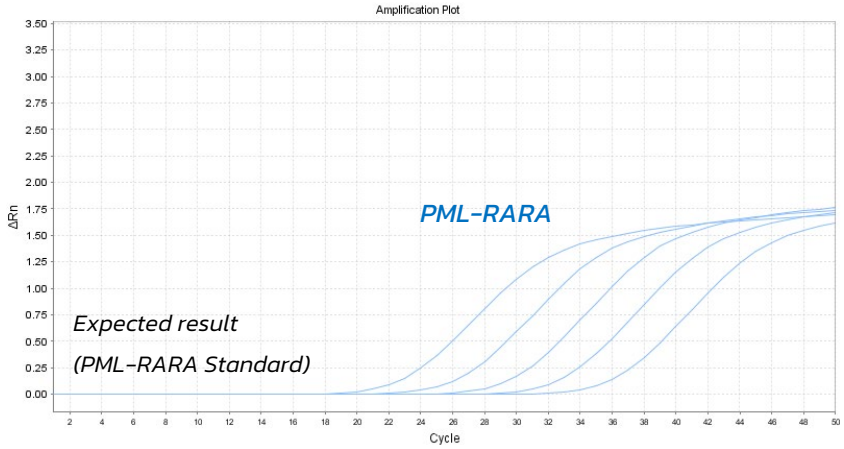

Figure 5. Serial dilutions used to build the standard curve for the PCR PML-RARA system

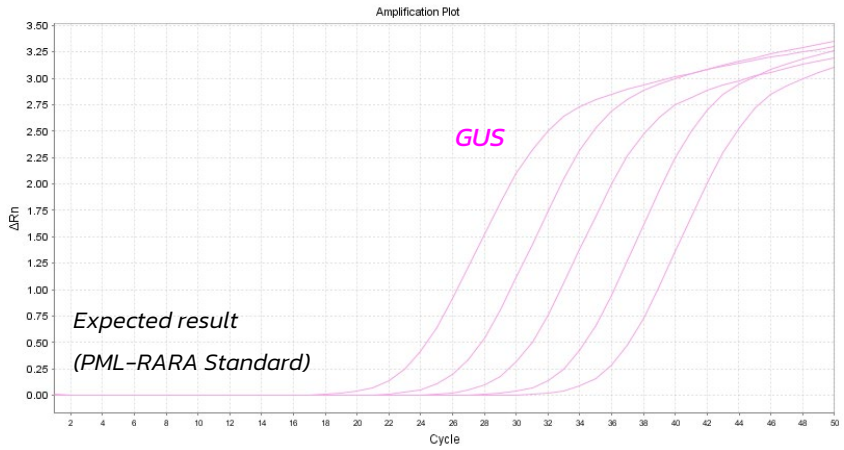

Figure 6. Serial dilutions used to build the standard curve for the PCR GUS system

cDNA SAMPLES

GUS Master Mix

- Verify that the reference gene (*GUS*) is detected in all samples in reactions using ***GUS Master Mix***. *GUS* is a constitutively expressed gene; therefore, amplification of the endogenous gene allows verifying that the sample contains enough DNA of sufficient quality.

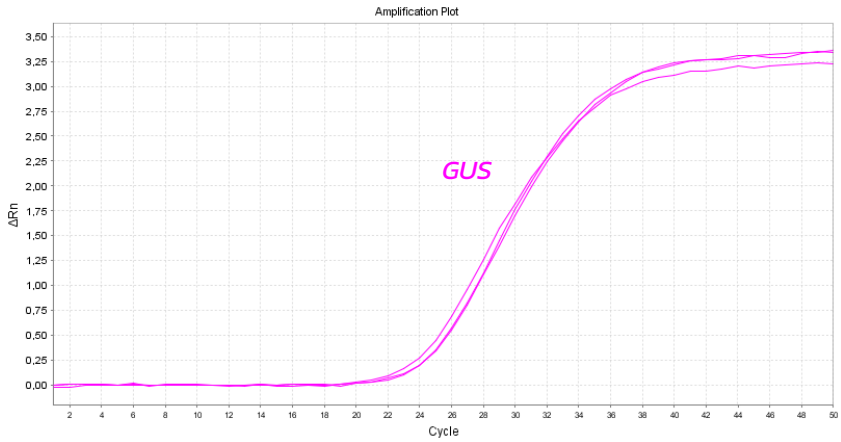

Figure 7. Expected result for a good-quality cDNA sample with the GUS system.

PML-RARA Master Mix & GUS Master Mix

- After verifying all controls, cDNA samples are analyzed. The analyzed sample shows translocation of PML-RARA bcr1 if amplification is detected in reactions using the P***PML-RARA Master Mix***, as shown below:
  - Negative sample:

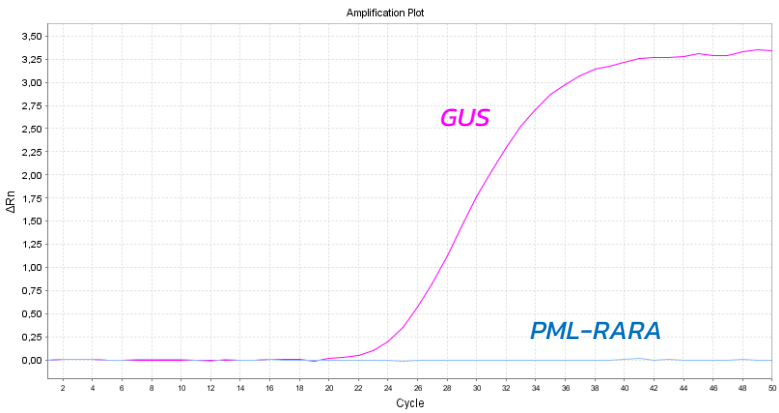

Figure 8. Expected result in non-pathogenic cDNA samples. The GUS system amplifies said gene, but the PML-RARA system does not amplify said oncogene.

- Positive sample:

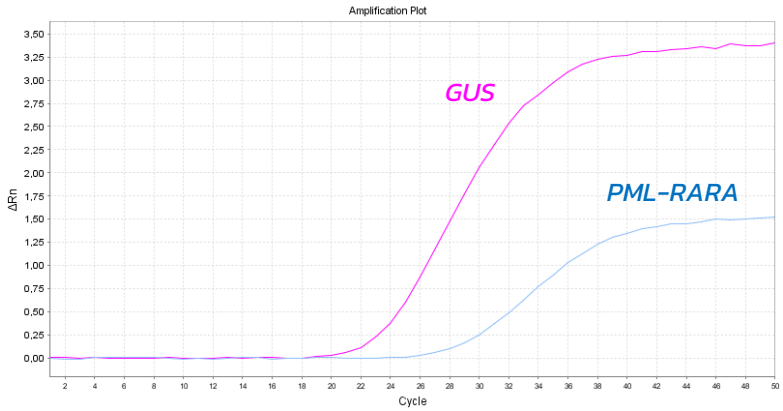

Figure 9. Expected result in pathogenic cDNA samples. Both systems amplify their targets: the GUS gene and the PML-RARA oncogene.

- The normalized copy number (NCN) will be calculated from the number of copies of the reference gene (GUS) and of the PML-RARA (bcr1) rearrangement. The rearrangement is quantified (NCN) by the following formula:

$$NCN = \frac{PML - RARA_{CN}}{GUS_{CN}}$$

NCN = Normalized copy number

08 Troubleshooting

The following table shows results that may be obtained while using positive controls (*PML-RARA Standard*), negative controls, and cDNA samples. If an unexpected result is obtained, the interpretation of the result and the most likely reason for the result are given in the following table:

Control	PML-RARA	GUS	Result / Interpretation
(PML-RARA Standard)	+	+	Expected result
	–	–	Incorrect PCR settings [1]
	+	–	
	–	+	
cDNA sample	–	+	Expected result
	+	+	
	+	–	Incorrect PCR settings [1]
	–	–	cDNA samples failed to amplify [2]
Negative control (NTC)	–	–	Expected result
	+	+	Contamination with human cDNA or the standard [3]

Table 5. Interpretation of possible results from Imegen® PML-RARA

(1) Incorrect PCR settings: An amplification error may be due to a technical issue during PCR configuration. Make sure the amplification program and fluorescence detection settings are correct.

(2) cDNA sample failed to amplify: Failure to amplify the reference gene in the cDNA sample might suggest that the quantity or quality of the cDNA sample is compromised. In this case, it is advised to perform a new test, RNA extraction, or synthesis of new cDNA samples before interpreting the results.

(3) Contamination with human cDNA or the positive control (standard): PCR contamination could be caused by improper sample handling, the use of contaminated reagents, or environmental contamination. To fix this issue, deep cleaning of the laboratory where PCRs are prepared is advised, including the equipment and material used. If necessary, use new aliquots from PCR reagents and finally prepare the PCR reactions containing the positive controls to avoid any cross-contamination.

09 Limitations

09.1 | Equipment

Imegen® PML-RARA has been validated for use with the following real-time PCR thermal cyclers:

- *StepOnePlus™ Real-Time PCR System* (ThermoFisher Scientific)
- *7500 FAST Real-Time PCR System* (ThermoFisher Scientific)

Technically, the kit is compatible with any real-time PCR equipment that allows detecting fluorescence emitted by the fluorophore FAM™.

If a different brand or model of thermal cycler is used, the amplification program may need to be adjusted. Should you need further information or advice, please contact our technical support service.

09.2 | Reagents

Imegen® PML-RARA has been validated using the reagents included in the kit and the DNA polymerases recommended by the manufacturer of the real-time PCR thermal cyclers used for validation:

- *M-MLV RT* (Moloney murine leukemia virus reverse transcriptase).
- *Hot Start PCR Master Mix (TaqMan™ Environmental Master Mix 2.0,* ThermoFisher Scientific)

If a different PCR enzyme other than DNA polymerase is to be used for test validation, it is advised to perform a previous validation step using the new reagents. Please contact our Technical Support Team for additional information.

Moreover, this kit does not include the necessary reagents for RNA extraction or reverse transcription of RNA to cDNA. For optimal results, it is recommended to follow local guidelines for *PML-RARA* testing.

09.3 | Product stability

Optimal performance of this product is achieved provided that the specified recommended storage conditions are applied as specified in section 4 (Content and storage conditions of the kit) of this manual.

For any questions about the applications of this product or its protocols, please contact our Technical Department:

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