RAS Lifesciences Pvt Ltd
Striving for better health

Amplisure® PML- RARA Qualitative PCR Kit
(Qualitative Mutation Analysis Kit)

IVD

Product Insert

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Introduction

The PML-RARA fusion gene transcripts are the result of translocations at t(15;17)(q22;q21). These translocations are associated with the majority of APL cases (> 90%), a distinct AML subset with M3 cytomorphology. APL accounts for 10–15% of all AML cases. The balanced reciprocal translocation t(15;17) leads to the fusion of the promyelocytic leukemia (PML) gene to the retinoic acid receptor alpha (RARA) to generate the PML-RARA fusion protein. The chimeric PML-RARA protein is a transcriptional repressor.
Diagnostic criteria for PML RARA mutation expression is associated with impaired myeloid differentiation. RARA breakpoints always occur in intron and exon. Depending on the location of breakpoints within the RARA site, intron 6, exon 6 and intron 3, the respective PML-RARa transcript subtypes referred to as long (L or bcr1), variant (V or bcr2) and short (S or bcr3), may be formed. They represent 55%, 5% and 40% of the cases respectively.

Product Description

Amplisure® PML-RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit) is an *in-vitro* nucleic acid amplification test for the qualitative detection of PML RARA for specific bcr1, bcr2, bcr3 fusion transcripts in human clinical samples. The kit contains all the reagents necessary for qualitative mutations analysis.

The assay is based on Real time PCR with Taqman probe chemistry. The bcr1, bcr2, bcr3 fusion transcripts are detected in the FAM channel, while the Internal Control (ABL gene) is detected in Yakima Yellow channel. The detection ABL in Yakima Yellow channel itself works as an internal control for the assay.

Recommended Work areas

Molecular Diagnostics work area includes:

a) Sample preparation area/room – for extraction of nucleic acids from clinical samples
b) Pre-PCR area/room - for setting up PCR reaction
c) PCR area/room – for performing PCR using the thermocyclers

As part of Good Laboratory Practices (GLP), it is recommended to have dedicated areas to avoid cross contamination.

General Precautions

Precautions while extracting Nucleic acid

Always wear proper attire (powder free gloves, facemask and Head cap) before starting the nucleic acid extraction procedure. During preparation of samples, compliance with good laboratory practices are essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of ribonucleases (RNases) into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with RNA.

The Sample Preparation Area is dedicated to processing samples. All reagents used in the Sample Preparation Area should remain in this dedicated area at all times. Laboratory coats, pipettes, pipette tips and vortex mixer used in the Sample Preparation Area must remain in this area and not be moved to the Pre-PCR/PCR area. Discard the gloves before leaving this area. Do not bring amplified product into the Sample Preparation Area. Usage of filter tips is recommended while sample preparation and should be performed in a Biosafety cabinet.

Precautions while setting up a PCR reaction

PCR assay is sensitive and any accidental introduction of product from previous amplification reactions leads to incorrect results. Hence, measures should be taken to reduce the risk of contamination in the laboratory which includes physically separating the activities involved in performing PCR and complying with good laboratory practices.
It is recommended to have proper cleaning procedures to minimize the risk of cross contamination and carry over contamination (e.g. DNA OUT™, RNase OUT™, 0.1% Sodium Hypochlorite, Fumigation etc.).
It is recommended that areas should be defined in Pre-PCR room for preparation of mastermix and addition of templates. Laboratory coats and equipment used in the Pre-PCR Area must remain in this area and should not be moved to the Sample Preparation Area.

Precautions for post PCR or equipment area/room

The Real time PCR instrument/s should be kept in a separate segregated area away from Sample preparation area and Pre-PCR area.

Precautions after completion of Real time PCR assay

The reaction tubes or strips should be properly discarded without opening the caps, after the completion of run to avoid carry over contamination.

Usage Limitations

1. The kit and all its components are for *in-vitro* diagnostics only.
2. The product is to be used by personnel specially trained in the *in-vitro* diagnostics procedures only.
3. Follow the product insert strictly for optimal PCR results.
4. Do not use the kit beyond the expiry date mentioned on the kit box.
5. Follow the guidelines provided in product insert for sample collection, storage and transport.
6. For ideal performance, store the kit under recommended conditions only.

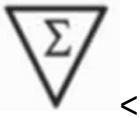
Safety Precautions

1. All patient specimens should be considered as potentially infectious and handled in a BSL2 biosafety hood with BSL3 practices.
2. Wear personal protective equipment, including gloves, head cap, face mask and lab coats when handling kit reagents/sample extraction. Wash hands thoroughly using detergents before and after performing the test.
3. Do not smoke, drink or eat in areas where kit reagents and/or human specimens are being used.
4. Dispose the unused kit reagents and human specimens as per regulatory guidelines.

Storage Conditions and Product Stability

1. All the kit reagents should be stored at -20°C. Replace all the kit components immediately at -20°C after usage.
2. Repeated thawing and freezing (more than 6 x) of all kit reagents should be avoided, as it reduces assay sensitivity. If needed, make aliquots of the kit reagents according to the volume used in the protocol prior to freezing.
3. Allow reagents to be thawed completely on Ice/4°C prior to use.
4. Kit reagents are stable through the end of the expiration date indicated on the box when stored at -20°C.

Symbols

Description of Symbol	Denotation
	*in-vitro* Diagnostic medical device
	Consult Instruction manual (Product Insert) for use
	Lot Number of the kit or Kit contents
	Catalogue number of Kit
 <N>	Contains sufficient for <N> reactions (Pack Size)
	Manufacturer
	Temperature limitation (Storage Condition)
	Use by MMM-YYYY (Expiry Date)
	Biological risk (handle carefully)
	Important Note

Kit Components

Color Coding (Caps)	Contents	Description	50 rxns (EP-PML-50)	100 rxns (EP-PML-100) 2 x 50 rxns
Yellow	RAS qRNA PCR Mix	DNA Amplification Reagent	1 x 750 µL	2 x 750 µL
Red	RAS RT Mix	cDNA synthesis Reagent	1 x 350 µL	2 x 350 µL
Brown	RAS PML- RARA PPM	Primer-Probe Mix	1 x 100 µL	2 x 100 µL
Natural	RAS PML- RARA PC	Positive control	1 x 50 µL	2 x 50 µL
White	MBGW	Molecular Biology Grade water	1 x 1.00 mL	2 x 1.00 mL

Materials required but not supplied

The materials which are required but not supplied are listed below:

1. RNA Extraction kit
2. Biosafety Cabinet
3. PCR Hood
4. Calibrated variable micropipettes
5. Sterile pipette filter tips (aerosol free)
6. Vortex mixer
7. Dry Bath
8. Benchtop centrifuge with rotor for 1.5 mL reaction tubes
9. Thermal cycler
10. Strip Tubes and Caps (0.2 mL) or PCR Tubes (0.2 mL) or 96 well plate
11. Cooling block (96 x 0.2 mL tubes)
12. 1.5 mL centrifuge tubes
13. 1.5 mL centrifuge tube stand

14. Cooling block (2 mL tubes)
15. Sterile powder free gloves
16. Facemask
17. Head cap
18. Lab coats

Quality Systems

In accordance with ISO-certified Quality Management System (9001:2008 and 13485: 2003) of RAS Lifesciences, each lot of Amplisure® PML-RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit) is tested against predetermined specifications to ensure consistent product quality.

Sample Type/Collection/Storage/Transport

Sample Type

K_2EDTA- Whole Blood, Bone marrow.
Heparinized Blood must not be used as they inhibit the PCR reaction

Sample Collection, Storage and Transport

3-5 mL of blood has to be drawn into a K_2EDTA vaccutainer. Cap and swirl the tubes for uniform mixing of blood and K_2EDTA. Collection and storage of unstabilized whole blood is not recommended for PCR analysis, because RNA degradation occurs in blood stored *ex vivo*. (Preferred to add RNA stabilizer at the time of collection)

The samples should be shipped at 2 to 8 °C and should be stored at 4°C. Ideally the sample should be processed within 24 hours of collection. If longer storage is required; remove the erythrocytes and store the cells at -70 °C.

Assay Procedure

RNA Extraction

RNA extraction from blood or bone marrow should be done following the standard procedure using TRIZOL® reagent (Invitrogen). Follow the manufacturer's instructions mentioned in the manual for RNA extraction. However the customer can also validate their own extraction process using other RNA extraction Kits.

The quality and sensitivity of the assay is largely dependent on the quality of sample RNA. We therefore recommend quantifying the purified RNA by Agarose gel electrophoresis and spectrophotometry prior to usage. Also check the quality of RNA by Agilent Bioanalyzer; if available.

PCR Protocol

Preparation of Reaction Mastermix

Detection procedure with Amplisure® PML-RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit) involves *1 step RT PCR*. A positive control and a negative control (MBGW should be used as negative control) are required to be included in a single run for acquiring proper results.

Set up a real time single step RTPCR reaction as follows:

Components	Volume per reaction (µL) (for final vol. of 30 µL)
RAS qRNA PCR Mix	15.0
RAS RT Mix	7.0
RAS PML- RARA PPM	2.0
RNA /PML-RARA PC/ MBGW	6.0

Place the PCR plate/tubes/strips in real time thermocycler.

PCR Programming

The Amplisure® PML -RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit) is validated on the following instruments:

- Rotor-Gene™ 6000
- Rotor-Gene™ Q 5plex
- ABI 7500 DX Real-Time PCR System
- ABI 7300 Real-Time PCR System
- Eppendorf Realplex 4
- Bio-Rad™ CFX 96

Plate setup

1. Program the plate setup by labeling the slots as per tube/strip/plate labels. The sequence of labeling of slots should be the same way as the tube/strip/plate is kept in the machine.
2. Select the type of sample (Unknown sample/positive control /NTC) for each slot.
3. Select the channel for acquisition (FAM/Yakima Yellow).

Sl. No.	Name of channel	Source wavelength (nm)	Detection wavelength (nm)
1.	FAM (PML-RARA)	470	510
2.	Yakima Yellow (ABL)	530	555

4. For background calibration in different instruments, follow the procedure described below:

Rotor-Gene™ 6000	- Perform 'Gain optimization'
Rotor-Gene™ Q 5plex	- Perform 'Gain optimization'
ABI 7500 DX Real-Time PCR System	- Select Passive Reference dye 'ROX'
ABI 7300 Real-Time PCR System	- Select Passive Reference dye 'ROX'
Eppendorf Realplex 4	- Select 'ROX' for background calibration
Bio-Rad™ CFX 96	- Select 'ROX' for background calibration

Preparation of reaction mastermix and cycling conditions are same for all the instruments listed in the product insert. For instrument specific protocols, please contact our technical support team at amplisure@raslifesciences.com

Cycling conditions

1. Configure the following program in the machine.

Steps	No. of cycles	Temperature (°C)	Time
1 (Reverse Transcription)	1	42	15 min.
2 (Initial denaturation)	1	95	10 min.
3 (PCR cycling)	40	95	15 sec.
		60*	60 sec.
* **Plate Read/Data Acquisition in FAM and Yakima Yellow channel**			

2. Set the reaction volume as 30 µL.
3. Plate read/Data Acquisition for FAM and Yakima Yellow channel should be incorporated in the second stage of step 3 (60°C/60 sec).
4. The ideal run time for the assay is 120 minutes. Note: *In case of Eppendorf Realplex 4, select RAMP rate as 35%.*

Preparation of reaction mastermix and cycling conditions are same for all the instruments listed in the product insert. For instrument specific protocols, please contact our technical support team at amplisure@raslifesciences.com

Data Analysis

1- Analyze the data after completion of the run.
2- Analyze the PML- RARA fusion transcript in FAM channel and ABL gene in Yakima Yellow channel.
3- Check the R_n/*Cycle* amplification plot and R_n/*Cycle* amplification plot to observe the amplification signal generated by different samples/Positive control in the run. Compare both the plots for data analysis.
4- Also look for noisy signals, if observed as it might not give you a proper result.

Result and Interpretation

The Ct value of samples in Yakima Yellow channel (ABL gene) should be before 30 cycles for the result to be considered valid.

Interpret the values for unknown samples based on the observations as described in the following table and there should be no amplification in negative control.

Observation	Interpretation	Conclusion
Amplification signal detected in PML RARA channel (FAM) and in ABL channel (Yakima Yellow)	PML RARA fusion transcript detected	Proceed for further Analysis
Amplification signal not detected in PML RARA channel (FAM) but detected in ABL channel (Yakima Yellow)	PML RARA fusion transcript not detected	
Amplification signal not detected in PML RARA channel (FAM) and in ABL channel (Yakima Yellow)	Possible inhibition of PCR	Repeat RNA extraction process and repeat the assay*

Troubleshoot

For any other technical query; please contact amplisure@raslifesciences.com

Observation	Possible cause	Solution
No amplification signal for Positive control in FAM/Yakima Yellow Channel	1. One of the components may not have been added. 2. Incorrect channel selection 3. Incorrect programming of the real time machine. 4. Instrument is not working properly	1. Repeat the assay by following the correct protocol and addition of reagents 2&3. Please recheck the PCR program 4. Contact manufacturer of thermocycler for technical support.
Weak amplification signal for positive control (Signal below threshold) in FAM /Yakima Yellow Channel	1. Improper PCR programming. 2. Inaccurate dispensing of reagents 3. Possible deterioration of kit components due to improper storage	1. Repeat the assay by following the correct protocol 2. Minimize Pipetting errors/Check for calibration status of pipettes
Identical/Similar Ct values observed in FAM/Yakima Yellow channel	1. Possible contamination of Kit reagents /Work area.	1. Use fresh aliquots of Positive control/Kit Reagents (if available) 2. Clean the PCR rack/Pipettes thoroughly as per GLP 3. Clean and Fumigate the work area overnight prior to use

Assay Characteristics

Analytical Sensitivity of PCR assay

Sensitivity of the assay was determined by serially diluting the positive control of PML RARA RNA from 100 to 1 copy/PCR. Sensitivity of the assay was determined as 10 copies /PCR.

To determine the analytical sensitivity for Amplisure® PML-RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit), positive control of PML-RARA RNA ranging from 1 copy/ reaction to 100 copies /reaction were tested using the Amplisure® PML- RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit).

The analytical sensitivity of the Amplisure® PML- RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit) is 10 copies /PCR.

Specificity

The specificity of the Amplisure® PML-RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit) is ensured by the selection of the primers and probes, as well as the selection of stringent reaction conditions. The primers and probes were checked for possible homologies to all published sequences (Genbank) by BLAST analysis to avoid any homology with other organisms.

Cross Reactivity Data

A potential cross-reactivity of the Amplisure® PML-RARA Qualitative PCR Kit (Qualitative Mutation Analysis PCR Kit) was tested using the control group listed below. None of the tested pathogens has been reactive. No cross-reactivity appeared with mixed infections.

Pathogen Tested	Cross reactivity with the PML RARA Primers/Probes
Herpes Simplex Virus-1	-
Herpes Simplex Virus-2	-
Epstein Barr virus	-
Human Immunodeficiency Virus	-
Hepatitis B virus	-
Hepatitis C virus	-
Parvovirus	-
Dengue virus	-
Chikungunya Virus	-
Cytomegalovirus	-
Janus kinase 2	-
BK Virus	-
E. coli	-

Abbreviations

Abbreviation	***Expansion***
PML RARA	Promyelogenous leukemia- Retinoic Acid Receptor Alpha
JAK	Janus Kinase
DNA	Deoxyribonucleic Acid
DNases	Deoxyribonucleases
PCR	Polymerase Chain Reaction
BSL2	Bio Safety Level 2
BSL3	Bio Safety Level 3
mL	Milli Liters
µL	Micro Liters
K_2EDTA	Potassium Ethylene Diamine Tetra Acetate
g	Relative Centrifugal Force
MBGW	Molecular Biology Grade Water
NTC	No Template Control
FAM	Carboxyfluorescein
ROX	Carboxy-X-rhodamine
BLAST	Basic Local Alignment Search Tool
Rxn	Reaction

References

1. Handbook "Sampling, Transportation, and Storage of Clinical Material for PCR Diagnostics", developed by Federal State Institution of Science Central Research Institute of Epidemiology of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being, Moscow, 2008.

Ordering Information

EP-PML -50 : 50 rxns
EP-PML -100 : 100 rxns