RAS Lifesciences Pvt Ltd
Striving for better health

Amplisure® BCR-ABL PCR Kit

(Real Time Quantitative Kit)

IVD

Product Insert

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Introduction

BCR-ABL is an activated protein kinase resulting from the reciprocal translocation of the long arms of chromosomes 9 and 22 t (9;22), which is commonly referred to as the ***Philadelphia chromosome*** (Ph+). The Ph chromosome is present in more than 95 % of cases of Chronic Myeloid Leukemia (CML). It is also found in 5 % of Acute Lymphoblastic Leukemia (ALL) in children and 10 - 25 % of ALL cases in adults.

In majority of the CML cases, in 30 % to 50 % of Ph+ ALL cases in adults and in 20 % to 30 % of Ph+ ALL cases in children the breakpoint of the *BCR* gene occurs in the so-called "major breakpoint cluster region **M-BCR**. This region is located between exon 12 and 16 (also known as exon b1-b5). Most frequently breakpoints are found in exons b2 and b3 of the *BCR* gene and exons a2 and a3 of the *ABL* gene. Virtually all CML-affected patients carry the b3a2 (55 %) or b2a2 (40 %) translocation.

Depending on the precise location of the BCR-ABL fusion the molecular weight of the protein can range from 185 to 230 kDa. Three clinically important variants are the p190, p210 and p230 isoforms. p190 is generally associated with acute lymphoblastic leukemia (ALL), while p210 is generally associated with chronic myeloid leukemia but can also be associated with ALL. p230 is usually associated with chronic neutrophilic leukemia.

Detecting and identifying the translocation not only provides useful information for diagnosis and prognosis of the different types of leukemia, but also provides the means for monitoring the **Minimal Residual Disease (MRD)**. RT-PCR has been shown to be an accurate and highly sensitive method for detection of the BCR-ABL fusion gene, and is more sensitive than Fluorescence *In Situ* Hybridization (FISH) or cytogenetics.

Product Description

Amplisure® BCR-ABL PCR Kit (Real Time Quantitative Kit) is an *in-vitro* diagnostic kit for quantitation of Major BCR-ABL fusion transcripts (b2a2and b3a2) in blood/bone marrow samples of prediagnosed CML or ALL patients. It is used to monitor molecular response during treatment with TK (Tyrosine kinase) inhibitors. The kit contains the necessary reagents for performing BCR-ABL quantitation by Real-time PCR.

Fusion transcript detection by Real Time polymerase chain reaction (PCR) is based on the amplification of b2a2and b3a2 transcript region. The assay principle is based on Taqman probes which allow higher specificity and sensitivity. The internal control used in the kit is ABL gene. The kit is calibrated with WHO standards and IS calibrator is supplied to report the quantitation values in International Scale.

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 to reduce the risk of contamination in the laboratory should include 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 of 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 (QT-BCR-50)	100 rxns (QT-BCR-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 BCR-ABL PPM	BCR-ABL Primer-Probe Mix	1 x 100 µL	2 x 100 µL
Lavender	RAS BCQS1 (2×10^5 copies/µL)	BCR-ABL Quantitation Standards	1 x 60 µL	2 x 60 µL
Lavender	RAS BCQS2 (2×10^4 copies/µL)		1 x 60 µL	2 x 60 µL
Lavender	RAS BCQS3 (2×10^3 copies/µL)		1 x 60 µL	2 x 60 µL
Lavender	RAS BCQS4 (2×10^2 copies/µL)		1 x 60 µL	2 x 60 µL
Natural	RAS BCR-ABL PC	Positive RNA control expressing BCR-ABL fusion transcript (100%)	1 x 50 µL	2 x 50 µL
Natural	RAS IS Calibrator (0.1%)	Positive RNA control expressing BCR-ABL/ABL transcript (0.1%)	1 x 50 µL	2 x 50 µL
White	MBGW	Molecular Biology Grade water	1 x 1.0 mL	2 x 1.0 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. Real Time PCR machine
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® BCR-ABL PCR Kit (Real Time Quantitative Kit) is tested against predetermined specifications to ensure consistent product quality.

Sample Type/Collection/Storage/Transport

Sample Type

K_2EDTA-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 using Agilent Bioanalyzer; if available.

qPCR Protocol

Preparation of Reaction Mastermix

Quantitation procedure with Amplisure® BCR-ABL PCR Kit (Real Time Quantitative Kit) involves *1 step RT qPCR*. It is recommended that a minimum of three standards, IS calibrator 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 BCR-ABL PPM	2.0
RNA/BCQS/IS Calibrator /BCR-ABL PC/ MBGW	6.0

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

PCR Programming

The Amplisure® BCR-ABL PCR Kit (Real Time Quantitative 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/Standard/NTC) for each slot.
3. When Standard is selected as sample type, mention the quantitation value in copies/µL (2E4, 2E3 etc.) The quantitation value of standards for BCR/ABL and ABL would be same.
4. Select the channel for acquisition (FAM/Yakima Yellow)

Sl. No.	Name of channel	Source wavelength (nm)	Detection wavelength (nm)
1.	FAM (BCR-ABL)	470	510
2.	YAKIMA YELLOW (ABL)	530	555

5. 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 BCR-ABL fusion transcript copies in FAM channel and ABL gene copies 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/standards 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.

Setting the threshold for the qPCR Data analysis

The threshold should be set either automatically (by the machine itself)/ or manually just above the background signal of the negative controls and negative samples by referring to R_n/*Cycle* amplification plot. The mean threshold value calculated from these experiments will most likely work for the majority of future runs, but the user should nevertheless review the generated threshold value at regular intervals.

Result and Interpretation

The values for unknown samples would appear in the result column in *copies/µL* in FAM Channel and Yakima Yellow channel. Samples showing no amplification in FAM channel should show amplification in Yakima yellow channel (≥ 10,000 copies) to avoid false negative results due to the quality of RNA, and then only results should be considered. The negative control should not show any value in the result column for FAM and Yakima Yellow channel. Any amplification in the negative control indicates cross contamination.

As standards are tenfold dilution, the theoretical slope of the curve is –3.3. A slope between –3.0 and –3.7 is acceptable as long as R^2 is >0.95. However, a value for R^2 >0.98 is desirable for accurate results.

Normalized copy number (NCN)

The ABL copy numbers (ABL CN) and BCR-ABL copy numbers (BCR-ABL CN) obtained in the test results should be used to calculate the Normalized copy number for samples and IS calibrator.

The ratio of these CN values gives the normalized copy number (NCN):

$$\text{NCN (\%)} = \frac{\text{BCR-ABL CN}}{\text{ABL CN}} \times 100$$

The NCN result obtained for the RAS IS Calibrator must be within the interval 0.05–0.2. Otherwise, NCN values cannot be converted to the International Scale. Furthermore, the whole experiment must be rejected if the RAS positive RNA control is not detected.

International Scale Conversion of Results

Use the experimental RAS IS calibrator NCN result (NCN Cal), and its assigned value (RAS IS-Cal value) indicated in the contents table, to calculate the normalized copy number on the international scale for unknown samples (IS-NCN sample).

$$\text{IS-NCN sample} = \frac{\text{NCN Sample x RAS IS Cal value}}{\text{NCN RAS IS Cal}}$$

Troubleshoot

Observation	Possible cause	Solution
No amplification signal for Standards in FAM/Yakima Yellow Channel 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 standards (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/Standards/Work area.	1. Use fresh aliquots of Standards/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

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

Assay Characteristics

Analytical Sensitivity of qPCR assay

The analytical sensitivity is determined independent from the selected extraction method, using an international standard panel of known concentrations ie. 1st WHO International Genetic Reference Panel for quantitation of BCR-ABL translocation by RQ-PCR (NIBSC code: 09/138).

To determine the analytical sensitivity of the Amplisure® BCR-ABL PCR Kit (Real Time Quantitative Kit), a standard dilution series was set up from 100 copies /µL to 1 copy/µL and analyzed with Amplisure® BCR-ABL PCR Kit (Real Time Quantitative Kit).

Testing was carried out on 10 different days on 3 replicates. The results were analyzed by statistical analysis.

The analytical sensitivity of* Amplisure® BCR-ABL PCR Kit (Real Time Quantitative Kit) *is 5 copies /µL.

Linear Range

The linear range (analytical measurement) of the Amplisure® BCR-ABL PCR Kit (Real Time Quantitative Kit) was determined by analyzing a dilution series of BCR-ABL quantitation standards from $2x10^7$ copies/µL to 2 copies/µL. The dilution series has been calibrated against the 1st WHO International Genetic Reference Panel for quantitation of BCR-ABL translocation by RQ-PCR (NIBSC code: 09/138).
Each dilution was tested in replicates (n= 3) using the Amplisure® BCR-ABL Quantitative Kit.

The linear range of the Amplisure® BCR-ABL PCR Kit (Real Time Quantitative Kit) has been determined to cover concentrations from ***4 copies /µL to 2 x 10^7 copies/µL***.

Specificity

The specificity of the Amplisure® BCR-ABL PCR Kit (Real Time Quantitative 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.

Cross Reactivity Data

A potential cross-reactivity of the Amplisure® BCR-ABL PCR Kit (Real Time Quantitative Kit) was tested using the control group listed below. None of the tested pathogens has been reactive.

Pathogen Tested	Cross Reactivity with the BCR-ABL
Adenovirus	-ve
Herpes Simplex Virus-1	-ve
Herpes Simplex Virus-2	-ve
Epstein Barr virus	-ve
Human Immunodeficiency Virus	-ve
Cytomegalo virus	-ve
Hepatitis B virus	-ve
Hepatitis C virus	-ve
Enterovirus	-ve
BK virus	-ve
MTB complex	-ve
Plasmodium sps.	-ve

Abbreviations

Abbreviation	*Expansion*
RNA	Ribonucleic Acid
IC	Internal Control
RNases	Ribonucleases
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
qPCR Protocol	Quantitative PCR protocol
MBGW	Molecular Biology Grade Water
RT PCR	Real Time PCR
NTC	No Template Control
FAM	Carboxyfluorescein
ROX	Carboxy-X-rhodamine
NIBSC	National Institute for Biological Standards and Control
IU	International Units
WHO	World Health Organization
BLAST	Basic Local Alignment Search Tool
MTB complex	Mycobacterium tuberculosis complex
Sps	Species
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

QT-BCR-50 : 50 rxns
QT-BCR-100 : 100 rxns