TRUPCR™

TRUPCR® PML-RARA QUANTITATIVE KIT

Version 1.1

Detection and Quantitation of PML-RARA Fusion Transcript
(BCR1, BCR 2 and BCR3)

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CE IVD

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Version 1.1

Detection and Quantitation of PML-RARA Fusion Transcript (BCR1, BCR2 and BCR3)

CE IVD

REF Product No.: 3B1259/3B1260

48 & 96 tests

Temperature limitation

DEC 2016

Manufactured by-

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EC	REP

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INTENDED USE

The **TRUPCR® PML-RARA QUANTITATIVE KIT** is intended for the quantitative detection and differentiation of PML-RARA fusion long (BCR1), variant (BCR2) and short (BCR3) transcript in bone marrow or peripheral blood samples of Acute promyelocytic (M3) leukemia (APL) using real time PCR system. This kit also differentiates between BCR 2 variant with 5' break point or 3' break point.

ACUTE PROMYELOCYTIC (M3) LEUKEMIA AND PML-RARA

Acute promyelocytic leukemia (APL or AML-M3) is a subtype of acute myeloid leukemia with distinct clinical and histopathologic features. Genetically, APL is characterized by a unique chromosomal anomaly. APL accounts for 10–15% of all AML cases. Investigations suggest that 99% of APL patients harbor a translocation between chromosomes 15 and 17, which fuses the retinoic acid receptor alpha (RaRa) gene on chromosome 17 with the PML gene on chromosome 15. Approximately 30% to 40% of t(15;17)-positive cases demonstrate a breakpoint within intron 3 of the PML gene. This breakpoint region is referred to as bcr3 and results in the fusion of PML exon 3 with RAR alpha exon 3. The bcr1 is detected in approximately 50% to 55% of positive cases. It occurs within intron 6 of the PML gene and results in the fusion of PML exon 6 and RAR alpha exon 3. The most uncommon breakpoint region, bcr2, is involved in roughly 5% to 10% of t(15;17)-positive cases. Unlike bcr1 and bcr3, the breakpoint of bcr2 occurs within an exon. The bcr2 breakpoint occurs at inconsistent sites within exon 6 of the PML gene resulting in the fusion of a variable portion of PML exon 6 with exon 3 of the RAR alpha gene. The location of bcr1, bcr2, and bcr3 produces fusion transcripts of varying lengths that, as a result, also are referred to as the long, variant, and short forms, respectively [1-6] (Fig. 1).

A number of researchers have reported on the clinical relevance associated with the specific type of PML-RAR alpha fusion transcript expressed in an individual. Although a subject of some debate, it has been reported that the location of the breakpoint within the PML gene may influence prognosis. Some reports suggest that APL-positive individuals who express the short-form fusion transcript tend to have decreased periods of clinical remission compared with

patients with the long form.[7-9] In addition; individuals who express the variant form may have a decreased response to treatment with all-trans retinoic acid (ATRA), depending on where the break occurs within PML exon 6.[5] Fusion transcript sequence analysis has suggested that individuals expressing the bcr2 transcript with a breakpoint 5' to nucleotide 1709 of the PML gene have reduced sensitivity to ATRA. Furthermore, individuals expressing the variant transcript with a breakpoint 3' to nucleotide 1709 may demonstrate high sensitivity to ATRA indistinguishable from individuals expressing the short or long forms.[5] However at present, the clinical significance of each transcript remains uncertain.

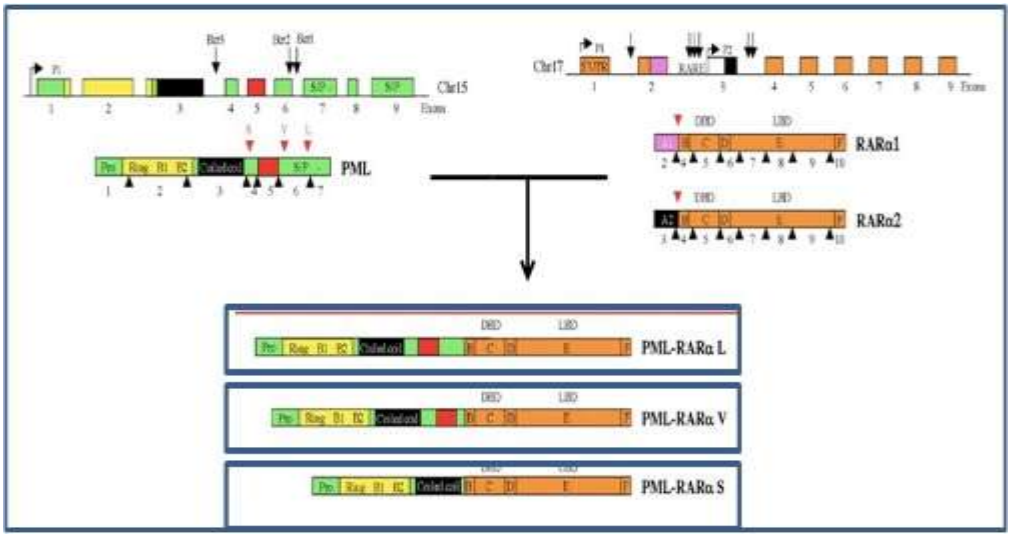


Fig. 1 Locations of the breakpoints in the PML and RARA genes and structure of the chimeric PML-RARA mRNA transcripts derived from the various breaks

PRINCIPLE

TRUPCR® PML-RARA QUANTITATIVE KIT is an RT-qPCR assay based on oligonucleotide hydrolysis principle which allows higher specificity and sensitivity. In real-time PCR, the fluorescent signal is generated from the presence of an oligonucleotide probe specific for target DNA sequence. The probe contains a fluorescent dye molecule on its 5’ end and a quencher molecule on its 3’ end. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Fluorescence resonance energy transfer (FRET).The probe hybridizes with one of the chains of the amplified fragment. During synthesis of a complementary chain, Taq DNA polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. As a result, the fluorescent dye and quencher dye are separated, and the total fluorescence of reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The fluorescent signal is measured in each cycle of reaction, and the threshold cycle value is determined from the obtained curve. The threshold cycle is proportional to the initial number of DNA copies in a sample and its value allows qualitative comparisons of analyzed and control samples.

TRUPCR PML-RARA detection is a Real-Time amplification test for the quantitative detection of PML-RARA BCR1, BCR2 & BCR3 fusion transcripts in bone marrow or peripheral blood samples. It has two-step protocol in which total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe of PML-RARA and ABL1. The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* extends from the primer bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5'-3' exonuclease activity of the *Taq* DNA polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them and leading to an increase in fluorescence from the reporter and a decrease in fluorescence from the quencher.

TRUPCR PML-RARA kit differentiates between BCR1 and BCR3 using different tube format, whereas differentiation between BCR1 and BCR2 is based on three different dye labelled probes (FAM, HEX and Texas Red).

REAGENTS

The Kit contains amplification reagents for performance of 48/96 amplification reactions. Thaw and handle reagents on ice. Do not freeze/thaw Kit vials repeatedly. In case of frequent use, we recommend to aliquot the contents of the vials into 10 reactions each. This will also rule-out kit/ reagent contamination.

REAGENTS FOR REVERSE TRANSCRIPTION

Reagent	Description	Volume in µL 48 reactions	Volume in µL 96 reactions
RRT 1	RT mix	384 µL	768 µL
RRT 2	Enzyme mix	72 µL	144 µL
RRT 3	Primer mix	240 µL	480 µL
RRT 4	Enhancer mix	24 µL	48 µL

REAGENTS FOR PCR

Reagent	Description	Volume in µL 48 reactions	Volume in µL 96 reactions
Multiplex Master Mix	Hot-start DNA polymerase Reaction Buffer dNTPs (dATP, dCTP, dGTP, dTTP) $MgCl_2$ and stabilizers	360 µL x 4	360 µL x 8
BCR1/BCR2 PML-RARA Primer probe mix	Primer and probe mix for BCR1 and BCR2 PML-RARA detection	24 µL x 2	24 µL x 4
BCR3 PML-RARA Primer probe mix	Primer and probe mix for BCR3 PML-RARA detection	24 µL x 2	24 µL x 4
ABL1 Primer Probe Mix	Primer, probe mix for ABL1 detection	24 µL x 2	24 µL x 4
Standards BCR1/BCR2	BCR1/BCR2 STD 1 (10^6 copies/5 µl) BCR1/BCR2 STD 2 (10^5 copies/5 µl) BCR1/BCR2 STD 3 (10^4 copies/5 µl) BCR1/BCR2 STD 4 (10^3 copies/5 µl) BCR1/BCR2 STD 5 (10^2 copies/5 µl)	30 µL each	60 µL each
Standards BCR3	BCR3 STD 1 (10^6 copies/5 µl) BCR3 STD 2 (10^5 copies/5 µl) BCR3 STD 3 (10^4 copies/5 µl) BCR3 STD 4 (10^3 copies/5 µl)	30 µL each	60 µL each

	BCR3 STD 5 (10^2 copies/5 µl)		
Standards ABL1	ABL1 STD 1 (10^5 copies/5 µl) ABL1 STD 2 (10^4 copies/5 µl) ABL1 STD 3 (10^3 copies/5 µl)	30 µL each	60 µL each
RNase free water	Sterilized water	1500 µL	1500 µL

RNA EXTRACTION

The samples should be shipped at 2 to 8 °C and should be stored at 4°C. To prevent significant degradation of transcripts, samples should be processed within 72 hours of collection, although ideally samples should be processed within 24-36 hours for most sensitive measurement of Minimum Residual Disease.

RNA Extraction from EDTA-Blood or Bone marrow can be performed with a recommended procedure using any of the following kits:

Sample Material	Nucleic Acid Isolation Kit	Cat No.
EDTA Blood/Bone Marrow	3B SpeedTools RNA Blood Kit	350
	Qiagen RNeasy Mini Kit	74104
	QIAamp RNA Blood Mini Kit	52304

The extracted RNA should be store at -20°C for shorter duration and at -80°C for longer duration.

REVERSE TRANSCRIPTION PCR PROTOCOL

1. REACTION PREPARATION

Name of the Reagent	Quantity per reaction
RRT 1	8 µl
RRT 2	1.5 µl
RRT 3	5 µl
RRT 4	0.5 µl
Sample RNA	1 µg
Total reaction volume	**25 µl**

NOTE:

1. Add up to 10 µl sample RNA (1 µg/rxn) and the OD 260/280 of the RNA should be measured spectrophotometrically and should be between 1.7 and 2.0.
2. The standards provided are DNA standards so no need to run reverse transcription step for standards and can be directly included in PCR run.

2. PROGRAM SET UP

Define the following setting for Temperature Profile

Step	Temperature, °C	Time	Cycles
1	25	10 min	1
2	47	60 min	1
3	70	05 min	1

REAL TIME PCR PROTOCOL

1. REACTION PREPARATION

NOTE:

1. Three separate tubes has to be run for BCR1/BCR2, BCR3 and ABL1
2. BCR1/BCR2: Prepare five reactions for standards and 1 reaction for Negative control.
   BCR3: Prepare five reactions for standards and 1 reaction for Negative control.
   ABL 1: Prepare three reactions for standards and 1 reaction for Negative control.

Prepare the PCR Mix as follows:

Name of the Reagent	BCR1/ BCR2 PML-RARA	BCR3 PML-RARA	ABL1
Multiplex Master Mix	10 µl	10 µl	10 µl
BCR1/BCR2 PML-RARA Primer probe mix	1 µl	-	-
BCR3 PML-RARA Primer probe mix	-	1 µl	-
ABL1 Primer probe mix	-	-	1 µl
Sample cDNA	5 µl	5 µl	5 µl
Nuclease free Water	4 µl	4 µl	4 µl
Total reaction volume	**20 µl**	**20 µl**	**20 µl**

2. PROGRAM SET UP

Define the following setting for Temperature Profile and Dye Acquisition

Step	Temperature, °C	Time	Dye Acquisition	Cycles
1	94	10 min	-	1
2	94	15 sec	-	45
	60	01 min	Yes	

Reference Dye - NONE

3. CHANNEL SELECTION

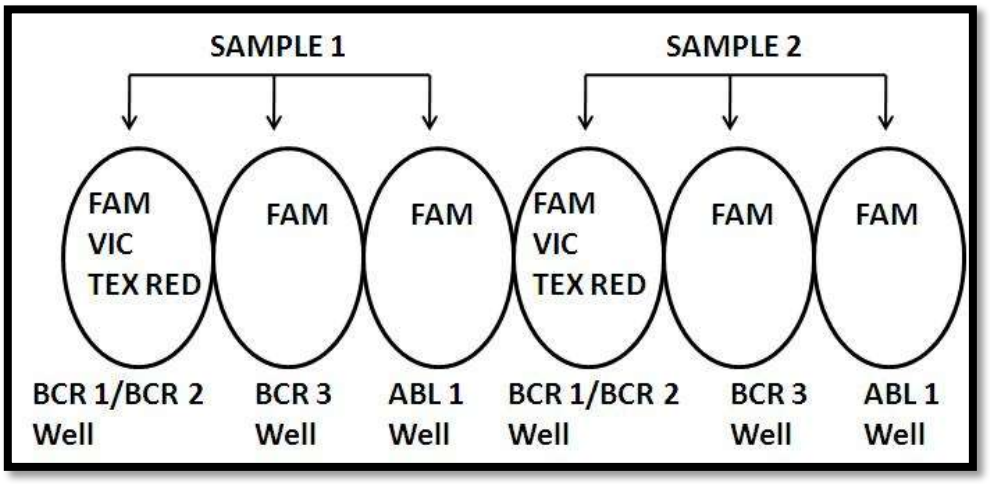
3.1 Define the following setting for channel selection for *Rotor-Gene Q Series*

Detection		Detector channel	Reporter	Gain Setup
PML-RARA	BCR3 tube	Green	FAM	Auto
	BCR1/BCR2 tube	Green, Yellow and Orange	FAM, HEX and Tex red	Auto
ABL1	ABL1 tube	Green	FAM	Auto

3.2 Define the following Targets for channel selection for *Applied Biosystems® 7500, 7500 fast, Quantstudio* series

No.	Target	Reporter	Quencher	Select in wells with
1	BCR 1	FAM	None	BCR 1/ 2 PPM
2	BCR 2 – 5' BP	VIC/HEX	None	BCR 1/ 2 PPM
3	BCR 2 – 3' BP	TEXAS RED	None	BCR 1/ 2 PPM
4	BCR 3	FAM	None	BCR 3 PPM
5	ABL 1	FAM	None	ABL 1 PPM

3.3 Selection of targets in wells



QUALITATIVE RESULT ANALYSIS

Case	Amplification Signals in ABL1	Amplification Signals in PML-RARA				Interpretation
		BCR3	BCR1/ BCR2			
			FAM	HEX	TEX RED	
1	Present	Present/Absent	Present	Present	Present	Sample is positive for BCR1 PML-RARA translocation
2	Present	Present	Absent	Absent	Absent	Sample is positive for BCR3 PML-RARA translocation
3	Present	Present/Absent	Present	Present	Absent	Sample is positive for BCR 2 3' break point PML-RARA translocation
4	Present	Present/Absent	Present	Absent	Absent	Sample is positive for BCR 2 5' break point PML-RARA translocation
5	Present	Absent	Absent	Absent	Absent	Sample is negative for any PML-RARA translocation
6	Absent or less than 10000 copies	Absent	Absent	Absent	Absent	Retest the sample

STANDARD CURVES AND QUALITY CRITERIA

Standard curve can be established using standards with a known number of copies and precise amount of target present in the test sample can be determined. The standard curves are plasmid-based. In order to ensure accurate standard curves, 3 standard dilutions for ABL1, 5 standard dilutions for BCR1 and 5 standard dilutions for BCR3 are provided with this kit.

As standards are tenfold dilution, the theoretical slope of the curve is –3.3. A slope between –3.0 and –3.9 is acceptable as long as R^2 is >0.95. However, a value for R^2 >0.98 is desirable for precise results.

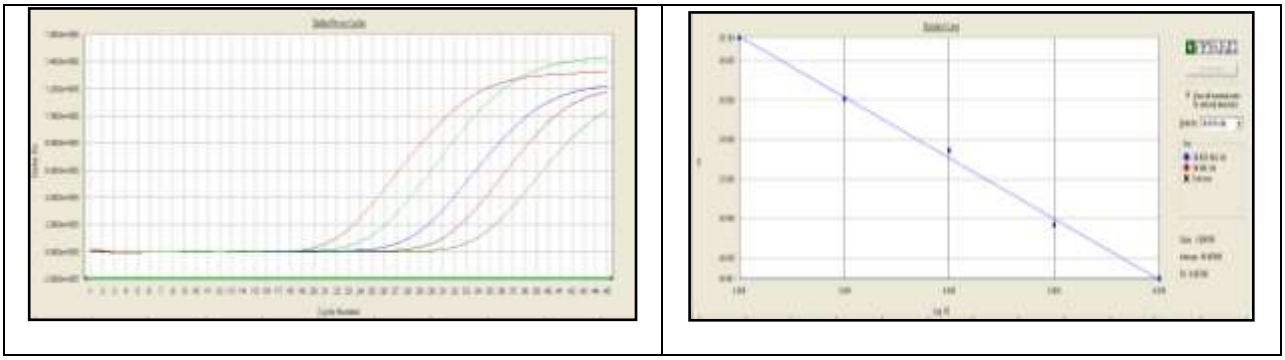


Fig.3 PML-RARA Standards and Standard Curve

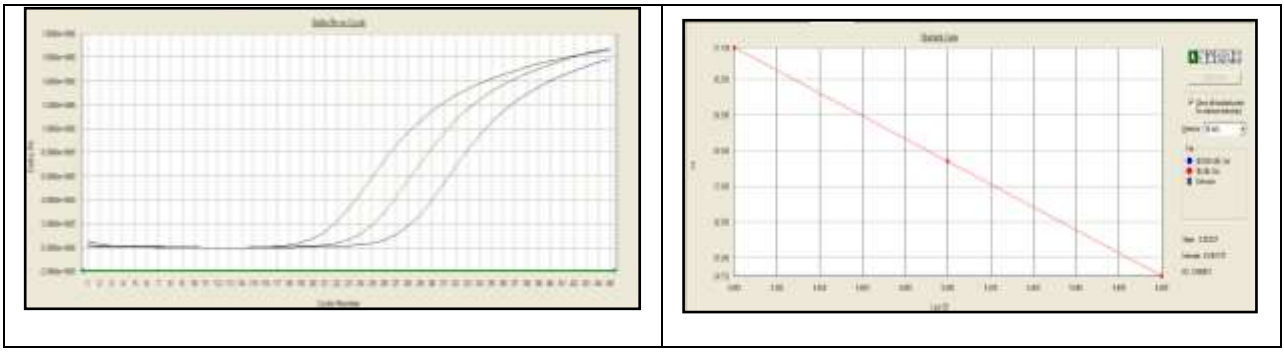


Fig.4 ABL1 Standards and Standard Curve

QUANTITATIVE RESULT ANALYSIS

Analysis of the results should be performed by trained personnel who have received the required training for analysing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration. The values for unknown samples would appear in the result column in copies/µL in FAM Channel for BCR1, BCR3 and ABL1. Samples showing no amplification in PML-RARA should show amplification in ABL1 (≥ 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 PML-RARA and ABL1. Any amplification in the negative control indicates cross contamination.

Normalized copy number (NCN)

The ABL1 copy numbers (ABL1 CN) and PML-RARA copy numbers (PML-RARA CN) obtained in the test results should be used to calculate the Normalized copy number for samples.

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

$$\text{NCN FOR BCR1 (\%)} = \frac{\text{BCR1 CN}}{\text{ABL1 CN}} \times 100$$

$$\text{NCN FOR BCR3 (\%)} = \frac{\text{BCR3 CN}}{\text{ABL1 CN}} \times 100$$

PERFORMANCE CHARACTERISTIC

LINEARITY:

The linear range (analytical measurement) of the TRUPCR® PML-RARA QUANTITATIVE KIT was determined by mixture of cells in different ratio to get range of PML-RARA % in different logs. The study was performed on RNA extracted from mixture of positive and negative PML-RARA cell lines and samples. Five different levels of BCR1, BCR 2 & BCR3 were tested in triplicates. The results for BCR3 linearity range from 0.01 to 98 BCR3 NCN, for BCR1 it ranges from 0.04 to 88 BCR1 NCN and for BCR 2 range from 0.07 to 82 BCR 2 NCN.

PRECISION:

Precision data of the TRUPCR® PML-RARA QUANTITATIVE KIT was determined as intra-assay variability and inter-assay variability. Variability data are expressed in terms of standard deviation and coefficient of variation. The study was performed on positive and negative samples of BCR1, BCR2 and BCR3 PML-RaRa. For intra-assay variability assay 20 replicates per sample were analyzed. Inter-assay experiment was performed on 3 alternate days with 5 replicates per samples per run were analyzed.

BCR1 PML-RARA	Average (NCN %)	Standard Deviation	Coefficient of Variation (%)
Intra- Assay Variability	19.8	2.39	12.07
Inter- Assay Variability	19.25	1.97	10.23

Precision data for BCR1 of the TRUPCR® PML-RARA Kit

BCR3 PML-RARA	Average (NCN %)	Standard Deviation	Coefficient of Variation (%)
Intra- Assay Variability	18.89	1.52SS	8.04
Inter- Assay Variability	19.06	1.93	10.12

Precision data for BCR3 of the TRUPCR® PML-RARA Kit

BCR 2 PML-RARA	Average (NCN %)	Standard Deviation	Coefficient of Variation (%)
Intra- Assay Variability	21.8	1.99	9.12
Inter- Assay Variability	21.71	1.67	7.69

Precision data for BCR2 of the TRUPCR® PML-RARA Kit

NOTE:

1. The users must be trained and familiar with real time PCR technology prior to the use of this kit.
2. Any diagnostic results generated must be interpreted in conjunction with other clinical or laboratory findings.
3. It is the user's responsibility to validate system performance for any procedures used in their laboratory which are not covered by the TRUPCR performance studies.
4. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

STORAGE AND HANDLING

All the components of TRUPCR® PML-RARA QUANTITATIVE KIT should be stored at -20˚C and stable until the date of expiry stated. The reagents can be aliquoted and stored at -20˚C in-order to maintain the stability and sensitivity.

MATERIAL AND DEVICES REQUIRED BUT NOT PROVIDED

- Adjustable pipettes with sterile filter or positive displacement tips
- Disposable powder-free gloves
- Sterile bidistilled water

- Sterile 1.5 ml and 2 ml microcentrifuge tubes
- 50 ml conical tubes
- Vortex mixer
- Heating-block for incubation at 70^{o}C
- Water Bath
- Desktop centrifuge
- Real time PCR
- Laminar airflow cabinet
- PCR vials (0.2 ml, thin-walled)
- 96 – 100% ethanol
- Personal protection equipment (lab coat, gloves, goggles)

KIT IS COMPATIBLE TO USE WITH FOLLOWING REAL TIME PCR INSTRUMENTS

- Applied Biosystems™ 7500
- StepOne and StepOnePlus
- QuantStudio® 3, 5 and 12
- Rotor-Gene Q
- Bio-Rad CFX96, CFX384
- AriaMx Real-Time PCR
- Roche - LightCycler® 480 -II
- Line gene K real time PCR

GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.

- Use disposable gloves, laboratory coats, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all samples and unused reagents in compliance with local authorities' requirements.
- Clean and disinfect all sample or reagent spills using a disinfectant, such as 0.5 % sodium hypochlorite or other suitable disinfectant.
- Avoid contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact, immediately flush with water, seek medical attention.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of PCR.
- The laboratory process must be uni-directional; it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

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TRUPCR®

TRUPCR® Molecular Diagnostic Kits

Oncology	**TRUPCR® BCR-ABL Quantitative Kit - M m µ**	*Detection, differentiation and quantitation of BCR-ABL major (M), minor (m) and micro (µ) transcripts. Reporting of Major transcripts ratios on WHO IS.*
	TRUPCR® JAK 2 QT Kit	*Detection and quantitation of Jak2 V617F allele burden on real-time PCR*
	TRUPCR® PML/RARA Quantitative Kit	*Differentiation and quantitation of BCR1, BCR2 and BCR3 transcripts*
	TRUPCR® KRAS Qualitative Kit	*Detection of 22 mutations across codons 12, 13, 59, 61, 117 & 146 of exons 2, 3 & 4*
	TRUPCR® EGFR Mutation Kit	*Detection of 32 different mutations in a single run*
	TRUPCR® AML Panel Kit*	*Qualitative detection of diagnostic markers (AML1-ETO, CBFB MYH11, BCR ABL1 and PML RARA) and prognostic markers (FLT3 ITD/TKD, C KIT and NPM1) of acute myelogenous leukaemia (AML) in peripheral blood samples using real time and conventional PCR system.*
	TRUPCR® ALL Panel Kit*	*Detection and differentiation of fusion genes (E2A/PBX1,TEL/AML1, MLL-AF4, MLL-ENL, MLL AF9 and BCR ABL1) associated with acut lymphoblastic leukaemia.*
	TRUPCR® Leukemia Panel Kit*	*Detection of E2A-PBX1, TEL-AML1, MLL-AF4, BCR-ABL1, CBFB MYH11, AML1-ETO, PML-RARA & ABL1 in single panel kit on real-time PCR*
	TRUPCR® MPN Mutation Panel Kit*	*Detection of BCR-ABL1, JAK-2, CALR & MPL in single panel kit on real-time PCR*
Genetics	**TRUPCR® HLA B27 Kit**	*Detection of highest number of HLA B27 allelic subtypes*
Infectious Disease	**TRUPCR® MTB/NTM Nested Kit**	*Detection of Mycobacterium DNA from any sample type on real-time PCR*
	TRUPCR® H1N1 Detection Kit	*Based on CDC certified primers and probes for the detection of type A influenza virus, pandemic influenza A virus and pandemic H1N1 influenza virus*
Drug Resistance	**TRUPCR® Rifampicin Resistant MTB Detection Kit**	*Detection of MTBC & Rifampicin resistance from any sample type*
Coagulation Factor	**TRUPCR® Thrombophilia Panel Kit**	*Detection of 3 Markers: Factor V, Factor II, MTHFR in single panel kit on real-time PCR*
Virology	**TRUPCR® CMV QT Kit**	*Detection and quantitation of Cytomegalovirus on real-time PCR*
	TRUPCR® HSV 1/2 Kit	*Detection of Herpes Simplex virus 1 & 2 on real-time PCR*
Tropical Diseases	**TRUPCR® Dengue/Chikungunya/ Malaria Kit**	*Simultaneous detection of Dengue & Chikungunya and P.falciparum, P.Vivax & Mixed infection on real-time PCR*
Women's Health	**TRUPCR® HPV 16/18 Kit**	*Detection & differentiation of HPV 16 and HPV 18 genotypes on real-time PCR*
	TRUPCR® HPV HR-16/18 Kit	*Detection of 14 High risk HPV genotypes & differentiation of HPV 16 and HPV 18 on real-time PCR*

* All Markers are also available as individual test.

To know more about complete product range & technical details please visit our website

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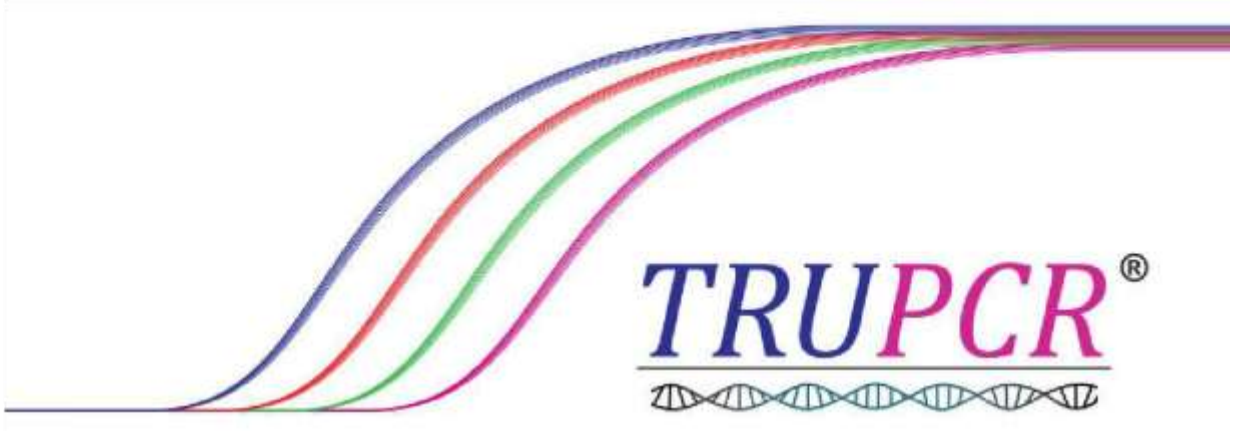

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