TRUPCR™

TRUPCR® BCR-ABL1 Kit

Version 2.0

FOR MEASURING DEEP MOLECULAR RESPONSE

1. **Calibrated to report on International Scale using First WHO International Genetic Reference Panel for quantitation of BCR-ABL1 translocation by RQ-PCR (NIBSC code: 09/138).**

2. **Measuring standards are calibrated to European reference material ERM-AD623a-f, produced and certified under the responsibility of the Institute for Reference Materials and measurements of the European Commission's Joint Research Centre.**

3. **Deep molecular response reporting based on European Treatment and Outcome Study (EUTOS) guidelines.**

4. **Guidelines for assessment and monitoring the response to first and second line tyrosine kinase inhibitors (TKIs) based on European LeukemiaNet (ELN) recommendations.**

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CE IVD

TRUPCR® BCR-ABL1 Kit

Version 2.0

FOR MEASURING DEEP MOLECULAR RESPONSE

CE IVD

REF Product No.: 3B1267/1268

48 & 96 tests

Temperature limitation

MAY 2016

Manufactured By-

3B BlackBio Biotech India Ltd.

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INTENDED USE

The **TRUPCR® BCR-ABL1 Kit** is intended for the quantitative detection and differentiation of BCR-ABL1 fusion gene transcript Major (M), minor (m), and micro (µ) in bone marrow or peripheral blood samples of chronic myeloid leukemia (CML) or acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML) using real time PCR system. This kit can also be used to evaluate the level of molecular response up to 5-log reduction (deep molecular response); results can be used for minimal residual disease follow-up.

LEUKEMIA AND BCR-ABL1

Leukemia is defined as neoplastic proliferation of abnormal white blood cells (WBCs). These abnormal white blood cells accumulate and interrupt the production of normal and functional WBC s as well as the synthesis of erythrocytes and platelets resulting in anemia and thrombocytopenia. Myelogenous leukemia is present when myeloid cell lineages such as granulocytes or monocytes are affected but when lymphocytes are affected then lymphocytic leukemia is present[1]. The difference between the acute and chronic leukemias is that the acute leukemias are rapidly progressing immature cells (immature cell proliferation which is blast or blast equivalent) while the chronic leukemias are slowly progressing mature cells. Broadly, leukemias are classified into four subtypes: acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML) [2]. Chronic myeloid leukaemia (CML) is a clonal stem cell disorder characterized by increased proliferation of myeloid lineage. Annual incidence of CML varies from non western countries to western countries, the age-adjusted incidence vary considerably 0.7-1.8/100000 between different parts of world[3]. CML is the commonest adult leukaemia in India and the annual incidence ranges from 0.8–2.2/100,000 population in males and 0.6–1.6/100,000 population in females in India[4]. It is slightly more common in males than in females. In general, the male predominance has been estimated to be 1.3-1.4 to 1[5].

The crucial genetic events in CML is an acquired genetic abnormality, which is a result of reciprocal chromosomal translocation between chromosome 22 and chromosome 9 -

t(9;22)(q34;q11). This translocation between the long arms of chromosome 9 and 22 results in a shortened chromosome 22, commonly known as the Philadelphia chromosome (Ph)[6] and It is found in 95% of CML patients, and in 5% of children and 15-30% of adults with acute lymphoid leukaemia (ALL)[7]. This translocation results when the Tyrosine Kinase human homologue of the Abelson Murine leukaemia Virus (ABL 1) gene from chromosome 9 merges with the breakpoint cluster region (BCR) gene on chromosome 22, resulting in a BCR-ABL1 fusion gene on 22q11 that encodes for uncontrolled tyrosine kinase activity[8].

In most cases, ABL1 gene exons 2 to 11 are translocated to the major break point cluster region (M-BCR) of the BCR gene on chromosome 22, forming a hybrid BCR-ABL1 gene. It is then transcribed into an 8.5 kb chimeric messenger ribonucleic acid (mRNA) with an e14a2 or e13a2 (also known as b3a2 or b2a2) junction, and then mRNA is translated into a p210 BCR-ABL1 fusion protein[9]. In rare cases of CML and in common cases of Ph positive ALL, the breakpoint of chromosome 22 occurs in a minor breakpoint cluster region (m-BCR), and results in a e1a2 junction mRNA and a 190 kDa BCR-ABL1 fusion protein, which is termed as p190 BCR-ABL1[10]. In very rare cases of CML and in AML, a third breakpoint is detected downstream of the M-BCR between exons e19 and e20 of BCR gene, it is termed μ-BCR. Its transcription product is a mRNA with e19a2 junction and its final product is a 230 kDa BCR-ABL1 protein which is termed p230 BCR-ABL1 (Fig. 1).

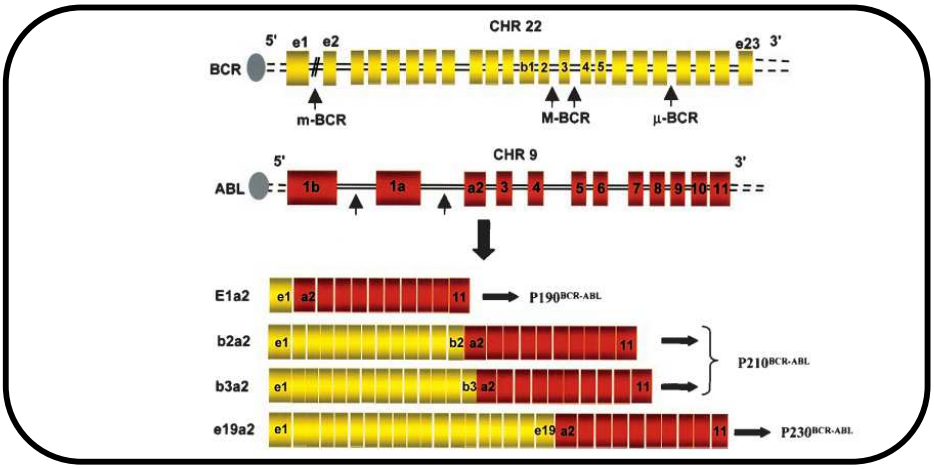

Fig. 1 Locations of the breakpoints in the ABL1 and BCR genes and structure of the chimeric BCR/ABL1 mRNA transcripts derived from the various breaks (adapted from Salesse and Verfaillie, 2002[6]).

MONITORING DISEASE RESPONSE TO TYROSINE KINASE INHIBITOR (TKIS) THERAPY IN CML

Diagnostic testing plays a key role in the management of CML. Diagnosis of CML has been rapidly changing over the years with sensitive diagnostic tests. Earlier, diagnosis only included complete blood count (CBC), comprising differential and platelet count, marrow aspiration, and marrow biopsy. Recent development in diagnosis for CML involves more sensitive tests such as standard cytogenetics, fluorescent in-situhybridization (FISH) and quantitative polymerase chain reaction (QPCR).

The therapeutic progress of CML patients receiving first-line TKI therapy is measured by reaching a continuum of milestones; assessed using techniques of increasing sensitivity[11]. Gils FJ, 2011[11] defined different response Levels in Chronic Myeloid Leukemia Therapy as following

Hematological Response

A complete hematological response (CHR) is defined as the complete normalization of peripheral blood counts and spleen size. Blood counts and differentials are required bi-weekly until a CHR has been achieved and confirmed and then at least every three months thereafter. The treatment goal is to achieve a CHR within one to three months after the start of treatment.

Cytogenetic Response

Cytogenetic monitoring is the most widely used technique to monitor treatment response in patients with CML. Cytogenetic response is determined by bone marrow metaphase chromosome analysis and is based on the number of Ph+ metaphases. A complete cytogenetic response (CCyR) indicates that a patient has no Ph+ metaphases.

Major molecular response (MMR)

Molecular response is defined by the magnitude of reduction in peripheral blood BCR-ABL1 transcript levels. The first level of response evaluated on the molecular scale, a major molecular response (MMR) and its definition originates from the International Randomized Study of Interferon Versus STI571[12] which corresponds to a 3-log reduction in BCR–ABL1 transcript levels from a standardized baseline (≤ 0.1% BCR–ABL1 on the International Scale [13]).

Deep molecular response (MR)

According to laboratory recommendations, developed as part of the European Treatment and Outcome Study for CML (EUTOS)[14], deeper levels of molecular response may also be defined according to the International Scale as MR^4, $MR^{4.5}$ and MR^5 (Fig. 2).

Deep molecular response (MR^4) corresponds to a 4-log reduction in BCR–ABL1 transcript levels from a standardized baseline (≤ 0.01% BCR–ABL1 on the International Scale).

Deep molecular response ($MR^{4.5}$) corresponds to a 4.5-log reduction in BCR–ABL1 transcript levels from a standardized baseline (≤ 0.0032% BCR–ABL1 on the International Scale).

Deep molecular response (MR^5) corresponds to a 5-log reduction in BCR–ABL1 transcript levels from a standardized baseline (≤ 0.001% BCR–ABL1 on the International Scale).

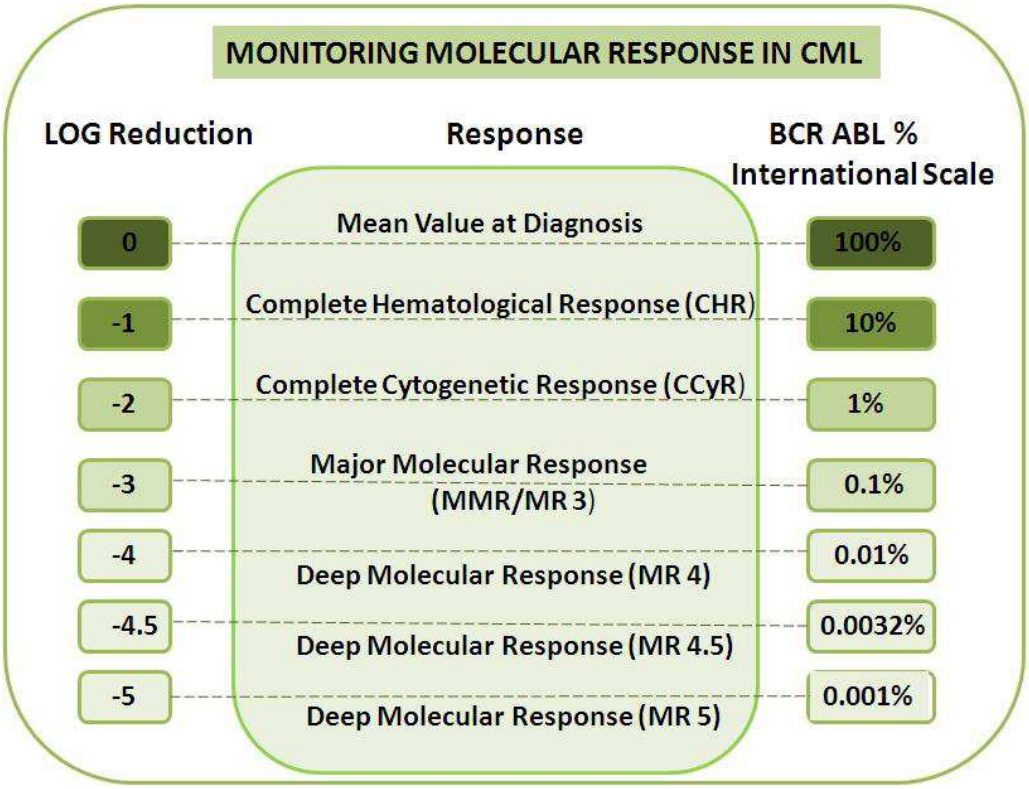

Fig. 2 Disease Response to Tyrosine Kinase Inhibitor Therapy in CML

ASSESSING AND MONITORING THE TKIS RESPONSE

The European LeukemiaNet (ELN) recommends[15] to assess and monitor the response using both conventional cytogenetics chromosome banding analysis (CBA) of at least 20 marrow cells metaphases) and real-time quantitative polymerase chain reaction (RT-Q-PCR). Cytogenetics should be performed at 3, 6, and 12 months, until a complete cytogenetic response (CCyR) is achieved. CBA of marrow cell metaphases can be substituted by fluorescence-in-situ-hybridization (FISH) of at least 200 blood cells nuclei, only once a CCyR has been achieved. RT-Q-PCR should be performed on buffy coat blood cells every 3 months. ELN acknowledged that if RT-Q-PCR methodology is standardized and the results are expressed according to the International Scale (IS) as BCR-ABL1%, the response can be assessed using only RT-Q-PCR, not only after a CCyR has been achieved but also from the beginning because RT-Q-PCR is more sensitive and does not require marrow sampling. Once a major molecular response (BCRABL1≤0.1% IS) has been achieved, RT-Q-PCR can be performed every 3 to 6 months, depending on baseline risk (warnings), transcripts level, and transcripts level fluctuations. In the patients who discontinue the treatment after a stable and deep molecular response, molecular monitoring must be ensured monthly for 1 year and at least every 3 months thereafter.

ELN distinguishes three grades of response. These definitions are clinically important because they provide a guide to therapy. “Optimal” means that the treatment should be continued because the response predicts for an excellent outcome and an almost normal survival length. “Failure” means that the treatment should be changed because the response is such that the patient is at a significant risk of progression and death.”Warning” defines an intermediate category of responses: on one hand, it is acknowledged that the response and the outcome could be better, but on the other hand, it is also acknowledged that there are no solid data to make a specific treatment recommendation on how the treatment should be changed to improve the response or the outcome. The definitions of the responses to first-line treatment (Tables 1) were based on solid data. The definitions of the responses to second-line treatment (Table 2) were provisional and were mainly based on dasatinib and nilotinib data.

Table 1. ELN definition of the response to TKIs, first-line

Time	Optimal	Warning	Failure
Baseline	NA	High risk or CCA/Ph +	NA
3 months	BCR/ABL1≤10 % or Ph + ≤35 %	BCR-ABL1 > 10 % or Ph + 36–95 %	Non CHR or Ph+ >95 %
6 months	BCR-ABL1≤1 % or Ph + 0 (CCyR)	BCR-ABL1 1–10 % or Ph + 1–35 % (PCyR)	BCR-ABL1 > 10 % or Ph + >35 %
12 months	BCR-ABL 1≤0.1 % (MMR)	BCR-ABL1 0.1–1 %	BCR-ABL1 > 1 % or Ph + ≥1%

Table 2. ELN definitions of the response to TKIs, second line

Time	Optimal	Warning	Failure
Baseline	NA	High risk, CHR never achieved, Loss of CHR, CyR never achieved	NA
3 months	BCR-ABL1≤10 % or Ph + <65 %	Ph + 65–95 %	No CHR BCR-ABL1 > 10 % Ph + >95 % New mutations
6 months	BCR-ABL1≤10 % or Ph + ≤35 % (MCyR)	Ph + 35–65 %	BCR-ABL1 > 10 % Ph + >65 % New mutations
12 months	BCR-ABL≤1 % or Ph + 0 (CCyR)	BCR-ABL1 1–10 % or Ph + 1–35 % (PCyR)	BCR-ABL1 > 10 % Ph + >35 % New mutations

NA - not applicable, CCyR - complete cytogenetic response, PCyR- partial cytogenetic response, MCyR- Major cytogenetic response, CHR- complete hematological response

PRINCIPLE AND PROCEDURE OF TRUPCR® BCR-ABL1 Kit

TRUPCR® BCR-ABL1 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® BCR-ABL1 detection is a Real-Time amplification test for the quantitative detection of BCR-ABL1 b2a2, b3a2, e1a2 and e19a2 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 BCR-ABL1 (Major, Minor and Micro) 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.

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

TRUPCR® BCR-ABL1 KIT REAGENTS

Reagent	Description	Volume in µL 48 reactions	Volume in µL 96 reactions
2X High Master Mix	Mix for Real time PCR	480 µL x 4	480 µL x 8
Major BCR-ABL1 Primer probe mix	Primer and probe mix for Major-BCR-ABL1 detection	24 µL x 2	24 µL x 4
Minor BCR-ABL1 Primer probe mix	Primer and probe mix for Minor-BCR-ABL1 detection	24 µL x 2	24 µL x 4
Micro BCR-ABL1 Primer Probe Mix	Primer and probe mix for Micro-BCR-ABL1 detection	24 µL x 2	24 µL x 4
ABL1 Primer Probe Mix	Primer and probe mix for ABL1 detection	24 µL x 2	24 µL x 4
Standards BCR-ABL1 and ABL1	STD 1 (1.08 X 10^6 copies/µl) STD 2 (1.08 X 10^5 copies/µl) STD 3 (1.08 X 10^4 copies/µl) STD 4 (1.08 X 10^3 copies/µl) STD 5 (1.08 X 10^2 copies/µl) STD 6 (1.08 X 10^1 copies/µl)	30 µL each	60 µL each
RNase free Water	RNase free Water	1000 µL	1500 µL

RECOMMENDATIONS FOR SAMPLE PROCESSING AND 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[13], although ideally samples should be processed within 24-36 hours for most sensitive measurement of Minimum Residual Disease[15].

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

For most sensitive measurement of Minimum Residual Disease the extracted RNA should immediately be converted in to cDNA and cDNA should then be used in PCR reaction immediately.

The extracted RNA can be store at --80°C for future use[20].

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 FOR SAMPLES

NOTE: TRUPCR® BCR-ABL1 kit is a multi-tube format kit. Hence, each sample will be split in separate tubes for separate transcript detection. For all three transcripts four separate tubes has to be run for Major, Minor, Micro BCR-ABL1 and ABL1

Prepare the PCR Mix as follows: following 4 columns represents four tubes for each sample

Name of the Reagent	MBCR-ABL1 (Major)	mBCR-ABL1 (Minor)	µBCR-ABL1 (Micro)	ABL1
Multiplex Master Mix	10 µl	10 µl	10 µl	10 µl
Major BCR-ABL1 Primer probe mix	1 µl	-	-	-
Minor BCR-ABL1 Primer probe mix	-	1 µl	-	-
Micro BCR-ABL1 Primer probe mix	-	-	1 µl	-
ABL1 Primer probe mix	-	-	-	1 µl
Sample cDNA	5 µl	5 µl	5 µl	5 µl
Nuclease free Water	4 µl	4 µl	4 µl	4 µl
Total reaction volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

BCR-ABL1 AND ABL1 QUANTITATIVE STANDARD

The standards in TRUPCR® BCR-ABL1 kit are plasmid-based standards and the kit uses single plasmid technology containing BCR-ABL1 and ABL1 targets to limit variability. The value of the copy number concentration of the plasmid is calibrated and traceable to "ERM®-AD623' Certified Reference Material for the Quantification of BCR-ABL1 Transcripts produced by European Commission, Institute for Reference Materials and Measurements. The kit is provided with 6 standards ranging from 10^6 copies to 10 copies of BCR-ABL1 and ABL1. In order to ensure accurate standard curves, 3 standard dilutions (10^6, 10^5 & 10^4) for ABL1, and 6 standard dilutions (10^6-10^1) for BCR- ABL1 should be included in each run.

2. REACTION PREPARATION FOR STANDARDS

Prepare six reactions for BCR-ABL1 Standards

Name of the Reagent	STD 1 (10^6 copies)	STD 2 (10^5 copies)	STD 3 (10^4 copies)	STD 4 (10^3 copies)	STD 5 (10^2 copies)	STD 6 (10^1 copies)
Multiplex Master Mix	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
Major BCR-ABL1 Primer probe mix	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Standards	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
Nuclease free Water	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl
Total reaction volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

Prepare three reactions for ABL1 standards

Name of the Reagent	STD 1 (10^6 copies)	STD 2 (10^5 copies)	STD 3 (10^4 copies)
Multiplex Master Mix	10 µl	10 µl	10 µl
ABL1 Primer probe mix	1 µl	1 µl	1 µl
Standards	5 µl	5 µl	5 µl
Nuclease free Water	4 µl	4 µl	4 µl
Total reaction volume	**20 µl**	**20 µl**	**20 µl**

3. 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	

Passive Reference Dye - ROX

4. CHANNEL SELECTION

Define the following setting for channel selection

Detection	Detector channel	Reporter	Quencher	Gain Setup
BCR - ABL1	Green	FAM	None	Auto
ABL1	Green	FAM	None	Auto

QUALITATIVE RESULT ANALYSIS

To check the BCR ABL1 positive or negative status of samples or type of transcript present in sample following Table should be refer

Case	Amplification Signals in ABL1	Amplification Signals in BCR-ABL1			Interpretation
		Major	Minor	Micro	
1	Present	Present	Absent	Absent	Sample is positive for Major BCR-ABL1 translocation
2	Present	Absent	Present	Absent	Sample is positive for Minor BCR-ABL1 translocation
3	Present	Absent	Absent	Present	Sample is positive for Micro BCR-ABL1 translocation
4	Present (But less than 10,000)	Absent	Absent	Absent	Test Sample has been degraded and re-sampling should be done
5	Absent	Absent	Absent	Absent	PCR inhibition, retest the sample

***There is a possibility of late amplification of minor transcript in the high load major transcript positive samples due to the presence of exon 1 in major BCR ABL1 fusion transcript positive sample. (For Details refer page No. 23)**

QUANTITATIVE RESULT ANALYSIS

The Quantitative result analysis must be done with standard curve method and Standard Curve should be run for both ABL1 and BCR-ABL1. Standard Curve must be derived from a minimum of three dilution points, although 6 for BCR –ABL1 and 3 for ABL1 are recommended. As standards are tenfold dilution, the theoretical slope of the curve is –3.3. Guidelines defines slope range to –3.2 and –3.6, however 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.

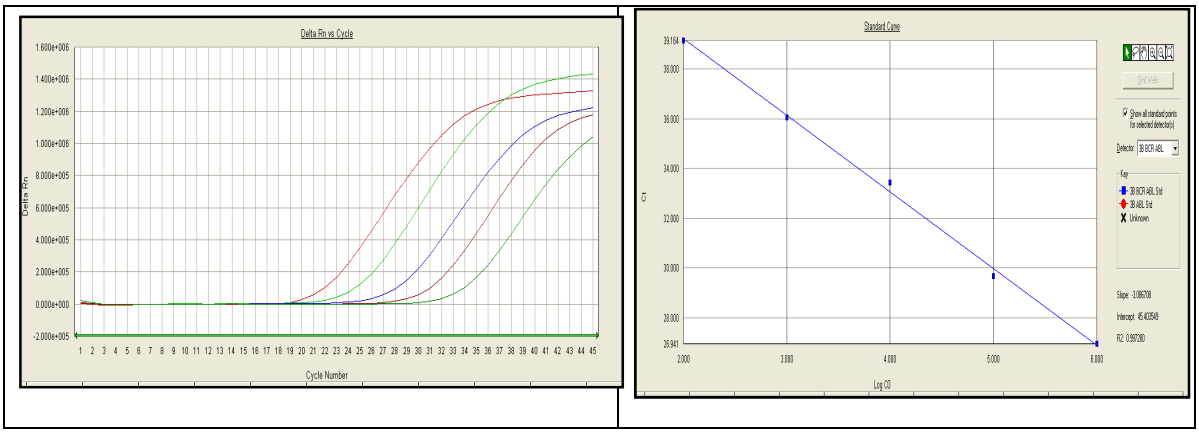

Fig.3 BCR-ABL1 Standards and Standard Curve

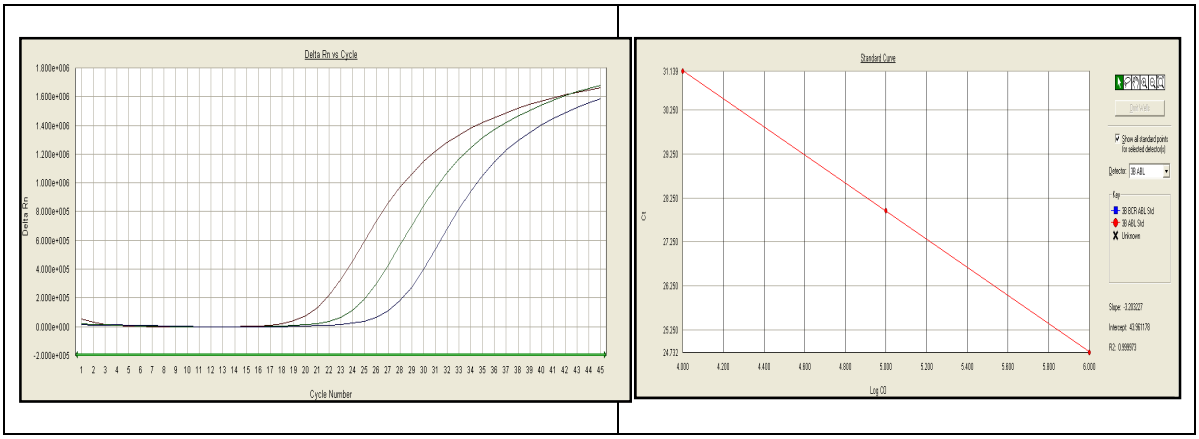

Fig.4 ABL1 Standards and Standard Curve

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 BCR-ABL1 and ABL1. Samples showing no amplification in BCR-ABL1 should show amplification in ABL1 (Absolute copy number ≥ 10,000 copies)[16] 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 BCR-ABL1 and ABL1. Any amplification in the negative control indicates cross contamination.

Absolute Copy number (aCN)

The standards allow the generation of standard curves for the BCR-ABL1 fusion transcript and the transcripts of the housekeeping genes ABL1. The standard curves permit determination of the copy number of the BCR-ABL1 fusion transcript and the transcripts of the housekeeping genes ABL1.

For a correct absolute quantification, make sure the copy number obtained using the standard curves are multiplied by 10.

[This step is necessary due to the fact the 5µl of double stranded plasmid (contains two copies of each target) is used to generate standard curve whereas the cDNA fragments from the clinical sample are single stranded (contain one copy of a target)] [18]

Absolute copy number (aCN) = Copy number X 10

Normalized copy number (NCN)

The ABL1 absolute copy numbers (ABL1 aCN) and BCR-ABL1 absolute copy numbers (BCR-ABL1 aCN) 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):

$$\text{NCN (\%)} = \frac{\text{BCR-ABL1 aCN}}{\text{ABL1 aCN}} \times 100$$

INTERNATIONAL SCALE CONVERSION OF RESULTS

First WHO International Genetic Reference Panel for quantitation of BCR-ABL1 translocation by RQ-PCR (NIBSC code: 09/138)[17] has been assigned different defined value for % BCR-ABL1/ABL1 in 2009 by the Expert Committee on Biological Standardization (ECBS) of the World Health Organization (WHO). These Primary standards were used to calibrate Secondary reference standards. A lot specific Correction Factor (CF) will be determined using secondary standards calibrated against First WHO International Genetic Reference Panel for quantitation of BCR-ABL1 translocation by RQ-PCR (NIBSC code: 09/138) and will be provided with each kit. To convert result in international scale (IS) Normalized copy number (NCN) % following formula should be used

IS NCN % = NCN % X Lot specific CF

MEASURING MOLECULAR RESPONSE[14]

MAJOR MOLECULAR RESPONSE (MMR)

Case	IS-NCN % of Sample	ABL Copies	MMR status	Remarks
1	> 0.1 %	< 10000	Inconclusive	Poor Quality Sample
2	> 0.1 %	> 10000	No MMR	Major Molecular Response not achieved
3	≤ 0.1 %	< 10000	Inconclusive	Poor Quality Sample
4	≤ 0.1 %	> 10000	**MMR (MR 3)**	Major Molecular Response achieved with ≥3-log reduction from IRIS baseline[12]

DEEP MOLECULAR RESPONSE (DMR) IN CASE OF DETECTABLE DISEASE

Case	IS-NCN % of Sample	ABL Copies	DMR status	Remarks
1	≤ 0.01 %	< 10000	Inconclusive	Poor Quality Sample
2	> 0.01 %	> 10000	No DMR	Deep Molecular Response not achieved
3	≤ 0.01 %	10000–31999	**DMR-4**	Deep Molecular Response achieved with ≥4-log reduction from IRIS baseline[12]
4	≤ 0.0032 %	32000–99999	**DMR-4.5**	Deep Molecular Response achieved with ≥4.5-log reduction from IRIS baseline[12]
5	≤ 0.001 %	≥100000	**DMR-5**	Deep Molecular Response achieved with ≥5-log reduction from IRIS baseline[12]

DEEP MOLECULAR RESPONSE (DMR) IN CASE OF UNDETECTABLE DISEASE

Case	BCR ABL Copies	ABL Copies	DMR status	Remarks
1	Not Detected	< 10000	Inconclusive	Poor Quality Sample
2	Not Detected	10000–31999	**DMR-4**	Deep Molecular Response achieved with ≥4-log reduction from IRIS baseline[12]
3	Not Detected	32000–99999	**DMR-4.5**	Deep Molecular Response achieved with ≥4.5-log reduction from IRIS baseline[12]
4	Not Detected	≥100000	**DMR-5**	Deep Molecular Response achieved with ≥5-log reduction from IRIS baseline[12]

PERFORMANCE CHARACTERISTIC

LIMIT OF BLANK (LOB):

Limit of blank (LOB) was determined following CLSI/NCCLS EP17-A2 documents[19] on healthy whole blood samples, 10 samples, over a seven-day period, three replicates of each concentration were tested with kits of three different lots. The LOB was found to be equal to 0.94 copies of BCR-ABL1 transcript.

LIMIT OF DETECTION (LOD):

The limit of detection (LoD or analytical sensitivity) was determined following CLSI/NCCLS EP17-A2 documents[19] by analyzing dilution series of ERM®-AD623' Certified Reference Material for the Quantification of BCR-ABL1 Transcripts produced by European Commission, Institute for Reference Materials and Measurements. The ERM®-AD623 control was diluted and processed to give concentrations of 5 copies, 3 copies and 1 copy per amplification reaction and over a seven-day period, three replicates of each concentration were tested with kits of three different lots. The LOD was found to be equal to 1.76 copies of BCR-ABL1 transcript. However according to guidelines[15] any positive sample with estimated copy numbers of < 3 should be scored as 3. Beyond this, laboratory at its own discretion can report copy numbers less than 3.

LINEARITY:

The linear range (analytical measurement) of the TRUPCR® BCR-ABL1 Kit was determined by mixture of Leukemia cells in different ratio to get range of BCR-ABL1 % in different logs. The study was performed on RNA extracted from mixture of positive and negative BCR-ABL1 cell lines. Five different levels of Major, Minor and Micro were tested in triplicates. The results for Major BCR-ABL1 linearity ranges from 0.0008 to 98 Mbcr NCN, for Minor it ranges from 0.002 to 82 mbcr NCN and Micro BCR-ABL1 linearity range was 0.005 to 76 µbcr NCN.

PRECISION:

Precision data of the TRUPCR® BCR-ABL1 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 Major, Minor and Micro BCR-ABL1. For intra-assay variability assay 20 replicates per sample (Mbcr, mbcr and µbcr) were analyzed. Inter-assay experiment was performed on 3 alternate days with 5 replicates per samples per run were analyzed.

Major BCR-ABL 1	Average (NCN %)	Standard Deviation	Coefficient of Variation (%)
Intra- Assay Variability	12.7	1.37	10.81
Inter- Assay Variability	12.5	0.38	3.11

Precision data for Major BCR-ABL1 of the TRUPCR® BCR-ABL1 Kit

Minor BCR-ABL 1	Average (NCN %)	Standard Deviation	Coefficient of Variation (%)
Intra- Assay Variability	12.16	1.82	15.02
Inter- Assay Variability	13.59	2.32	17.07

Precision data for Minor BCR-ABL1 of the TRUPCR® BCR-ABL1 Kit

Micro BCR-ABL 1	Average (NCN %)	Standard Deviation	Coefficient of Variation (%)
Intra- Assay Variability	17.13	3.04	17.74
Inter- Assay Variability	14.36	2.70	18.84

Precision data for Micro BCR-ABL1 of the TRUPCR® BCR-ABL1 Kit

INTERPRETATION OF HIGH LOAD MAJOR FUSION TRANSCRIPT

As the major transcript is a fusion of exon 13 or exon 14 of BCR gene with ABL gene the transcript also have exon 1 of BCR gene therefore when sample positive for high load of major will also have primer binding sites for minor transcript (exon 1) and will amplify with minor primers however due to bigger product size late Ct can be observed. In such case only major transcript has to be considered.

Below are the few examples.

Case	Major BCR ABL1 Ct	Minor BCR ABL1 Ct	Major BCR ABL1 NCN%	Minor BCR ABL1 NCN%	Interpretation
1	21.349	36.375	44.891	0.0013	Major Only
2	22.855	39.000	72.491	0.0010	Major Only
3	27.078	Undetermined	29.871	Undetected (With Sufficient ABL1)	Major Only
4	21.501	37.259	57.807	0.00107	Major Only
5	Undetermined	23.090	Undetected (With Sufficient ABL1)	6.761	Minor Only

TRACEABILITY:

The TRUPCR® BCR-ABL1 Kit is traceable to following international reference material

No.	Traceable component of kit	Traceable to
1.	International scale (IS) reporting	First WHO International Genetic Reference Panel for quantitation of BCR-ABL 1 translocation by RQ-PCR (NIBSC code: 09/138)[17]
2	Quantitative standards for BCR ABL 1 and ABL 1	European reference material ERM-AD623a-f, produced and certified under the responsibility of the Institute for Reference Materials and measurements of the European Commission's Joint Research Centre[18]

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.
5. The kit has been designed according to the "Europe Against Cancer" (EAC) and Guidelines for the measurement of BCR-ABL1 transcripts in chronic myeloid leukaemia[13] with the updated international recommendations.
6. A lot specific Correction Factor (CF) provided with kit is determined using secondary standards calibrated against First WHO International Genetic Reference Panel for quantitation of BCR-ABL1 translocation by RQ-PCR (NIBSC code: 09/138)[17].
7. The BCR-ABL1 standards provided with this kit are plasmids with MBCR-ABL1 target gene; however they are calibrated with standards of mBCR-ABL1 & µBCR-ABL1 for efficiency, sensitivity and specificity.
8. The kit is calibrated to report on international scale (IS) using First WHO International Genetic Reference Panel for quantitation of BCR-ABL1 translocation by RQ-PCR (NIBSC code: 09/138) only for MBCR-ABL1 target therefore sample positive for mBCR-ABL1 & µBCR-ABL1 should be reported in NCN % only.

STORAGE AND HANDLING

All the components of TRUPCR® BCR-ABL1 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.

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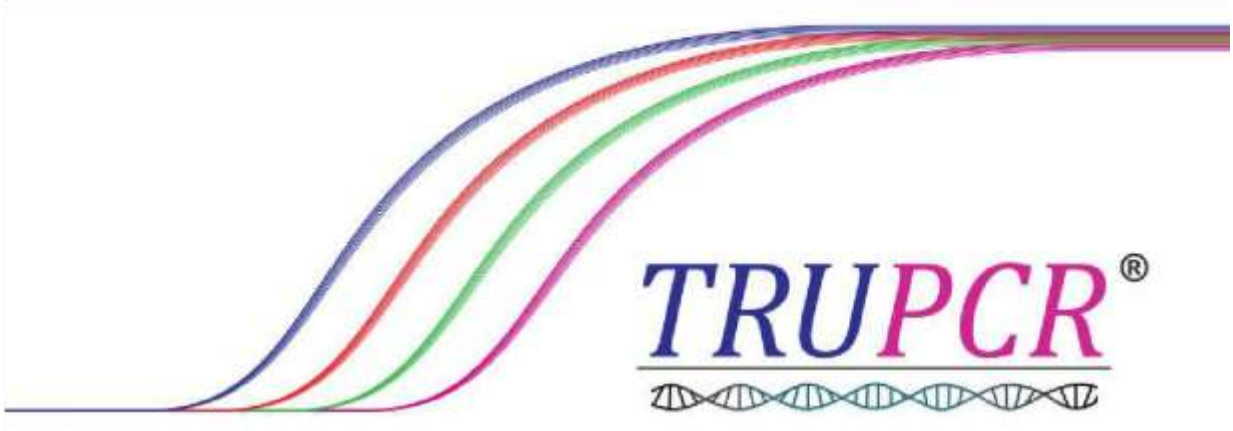

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