BIO·RAD

QXDx™ BCR-ABL %IS Kit

Instructions For Use

$\sum$/96

CE

IVD

US: Rx Only

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Translations

Product documents are provided in additional languages on electronic media.

Symbols Lexicon

CE European Conformity	Manufacturer	EC REP Authorized Representative in the European Union
LOT Lot Number	Use by	IVD For In Vitro Diagnostic Use
Temperature Limit	REF Catalog Number	Consult Instructions for Use
Σ Number of Tests	USE For use with	SN Serial Number
Rx Only Prescription Use Only	LATEX Contains Latex	

BIO-RAD TECHNICAL SUPPORT

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SAFETY AND REGULATORY COMPLIANCE

The QX200™ System has been tested and found to be in compliance with all applicable requirements of the following safety and electromagnetic standards:

1. IEC 61010-1:2010 (3rd ed.), EN61010-1:2010 (3rd ed). Electrical Equipment for Measurement, Control, and Laboratory Use — Part 1: General requirements

2. EN 61326-1:2006 (Class A). Electrical equipment for measurement, control, and laboratory use. EMC requirements, Part 1: General requirements

3. UL 61010-1:2004, Laboratory equipment, Test & Measurement Equipment and Industrial Process Controls

4. CAN/CSA 22.2 No 61010-1-04, Safety Requirements for Electrical. Equipment for Measurement, Control, and Laboratory Use, Part I: General. Requirements

5. This equipment generates, uses, and can radiate radiofrequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference, in which case the user will be required to correct the interference at their own expense.

CE The CE mark indicates that the manufacturer ensures the product conforms with the essential requirements of the European Directive for in-vitro diagnostic medical devices 98/79/EC.

SA The CSA mark indicates that a product has been tested to Canadian and U.S. standards, and it meets the requirements of those applicable standards.

This equipment has been tested and found to comply with the limits for a Class A digital device pursuant to Part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment.

The Waste Electrical and Electronic Equipment Directive symbol indicates that when the end- user wishes to discard this product, it must be sent to separate collection facilities for recovery and recycling.

This instrument is for use only by trained personnel.

Do not position the equipment so that it is difficult to operate the plug of the power supply. The plug of the power supply is the disconnect device.

No serviceable parts inside.

QXDX BCR-ABL %IS KIT FOR QX200 SYSTEM WARNINGS AND PRECAUTIONS

For in vitro diagnostic use. For healthcare professional use.

This test kit should be handled only by qualified personnel trained in laboratory procedures and familiar with their potential hazards. Wear appropriate protective clothing, gloves and eye/face protection and handle appropriately with the requisite Good Laboratory Practices.

PPE (PERSONAL PROTECTIVE EQUIPMENT) TRAINING

Proper use of gloves is recommended with use of oils and sample plates. OSHA requirements for PPE are set forth in the Code of Federal Regulations (CFR) at 29 CFR 1910.132 (General requirements); 29 CFR 1910.138 (Hand protection); 29 CFR 1926.95 (Criteria for standard personal protective equipment). Any gloves with impaired protective ability should be discarded and replaced. Consider the toxicity of the chemicals and factors such as duration of exposure, storage, and temperature when deciding to reuse chemically exposed gloves. Features to aid glove selection for handling of machines, assays, oils, and cleaning solvents:

- Butyl gloves are made of a synthetic rubber and protect against peroxide, hydrofluoric acid, strong bases, alcohols, aldehydes, and ketones.

- Natural (latex) rubber gloves are comfortable to wear and feature outstanding tensile strength, elasticity, and temperature resistance.

- Neoprene gloves are made of synthetic rubber and offer good pliability, finger dexterity, high density, and tear resistance; they protect against alcohols, organic acids, and alkalis.

- Nitrile gloves are made of copolymer and provide protection from chlorinated solvents such as trichloroethylene and tetrachloroethene; they offer protection when working with oils, greases, acids, and caustic substances.

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INTENDED USE

The QXDx BCR-ABL %IS test, performed on Bio-Rad's QXDx™ Droplet Digital PCR System, is an in vitro nucleic acid amplification test for the quantitation of BCR-ABL1 and ABL1 chromosomal transcripts in total RNA from whole blood of diagnosed, t(9;22)-positive, Chronic Myeloid Leukemia (CML) patients expressing BCR-ABL1 fusion transcripts type e13a2 and/or e14a2. The test measures the e13a2 and/or e14a2 transcripts of BCR-ABL1, normalized to the ABL1 endogenous control. Results are reported as percent reduction from a baseline of 100% on the International Scale (%IS) and on a log molecular reduction (MR) scale.

The test does not differentiate between e13a2 and e14a2 transcripts and does not monitor other rare fusion transcripts resulting from t(9;22). This test is not intended for diagnosis of CML.

PRINCIPLE OF THE PROCEDURE

The QXDx BCR-ABL %IS test uses random primed reverse transcription in combination with Droplet Digital PCR (ddPCR) technology. BCR-ABL translocations e13a2 (b2a2) and e14a2(b3a2) are simultaneously amplified, detected and quantified. The QXDx BCR-ABL %IS test quantitates copies of the two BCR-ABL fusion transcripts e13a2 (b2a2) and/or e14a2 (b3a2) in the FAM channel and the ABL transcript as an endogenous control in the HEX channel, using total RNA extracted from human blood[1-4]. The QXDx BCR-ABL %IS test quantifies the amount of BCR-ABL transcript as a ratio of BCR-ABL/ABL, then returns the value on the International Scale.

The QXDx BCR-ABL %IS Kit is intended for use on Bio-Rad QXDx Droplet Digital PCR (ddPCR) System. The QXDx AutoDG™ ddPCR system consists of the Automated Droplet Generator (AutoDG) and Droplet Reader (DR). The QXDx BCR-ABL %IS Kit includes reagents sufficient for 96 samples including calibrator-checks and controls. Each kit contains two lot-matched IS calibrator-checks. The IS calibrator-checks are formulated at approximately 0.1% BCR-ABL/ABL, which is at the point of major molecular response (MMR or MR3), and at approximately 10% BCR-ABL/ABL (MR1). The IS calibrator-checks are traceable to the First WHO international Genetic Reference Panel (09/138)[5]. The calibrator-checks are used to ensure that the assay is traceable to the First WHO international genetic reference standards.

Besides an endogenous control (ABL), the QXDx BCR-ABL %IS kit also includes 3 external controls: high positive control, low positive control and BCR-ABL Negative/ABL Positive control (negative control).

Reporting Format: %IS and MR

Two measures are commonly used for monitoring CML.

1. International Scale percent ratio (%IS), calculated by dividing the number of BCR-ABL copies by the reference ABL copies times 100 then multiplied by a laboratory-specific correction factor (CF) (Baccarani, *et al.* 2006)[1].

2. Molecular log Reduction level (MR) (Hughes *et al.* 2006, Branford *et al*, 2006, 2008)[4], which is a log10 transform of %IS.

$$\%IS = \frac{BCR - ABL\ copies}{ABL\ copies} * 100 * CF$$

$$MR = \log_{10}(100\%IS) - \log_{10}(\%IS) = 2 - \log_{10}(\%IS)$$

The %IS ratio is reported in linear scale, while MR is the %IS ratio converted to log10-scale and subtracted from baseline, MR0.0 is 100%IS. We will use the notation of MRx.y to indicate MR level, where x.y is the numeric value of the measure. For example, MR3.0 refers to a thousand fold (10^3) reduction from nominal MR0.0. It also means a ratio of 0.1% between detected BCR-ABL copies over ABL copies, corrected to IS.

QXDx™ BCR-ABL %IS Kit

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The QXDx BCR-ABL %IS test reports both %IS and MR values. As intermediate results, detected copies of BCR-ABL and ABL transcripts are reportable and can be used to assess the certainty or power of the measurement. The %IS values are traceable to the WHO primary reference materials ("standards") (White et al 2010)[5], which target four %IS levels; 10%IS, 1%IS, 0.1%IS, 0.01%IS or (MR1, MR2, MR3, and MR4) respectively.

Statistical entities like a mean or a standard deviation in this document are expressed in either MR or %IS units. Table 1 below illustrates the relationship between %IS values and their associated MR values for selected levels.

%IS	MR
10	1.0
1	2.0
0.32	2.5
0.1	3.0 (MMR)
0.032	3.5
0.01	4.0
0.0032	4.5
0.002	4.7
0.001	5.0

Table 1: *%IS Values and Associated MR Values*

Overview of the QXDx BCR-ABL %IS Test Workflow

The standard test workflow and the time required per step are depicted in the table 2 below.

Step	Description	Uses	Estimated Hands-on Time	Estimated Instrument Time
1	Extract RNA from whole blood in EDTA tubes	Variable	Variable	Variable
2	RT reaction		<30 min	~60 min
3	Plate setup and droplet generation		<30 min	~1 min/test ~48 min/plate
4	PCR amplification		<5 min	~120 min.
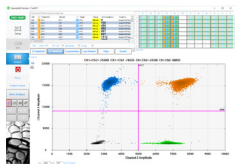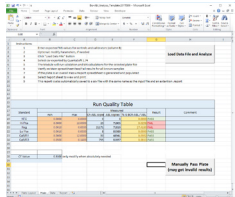
Step	Description	Uses	Estimated Hands-on Time	Estimated Instrument Time
5	Droplet reading		<5 min	~2.5 min/test ~120 min/plate
6	Manual thresholding, export csv		10-20 min/plate	N/A
7	Aggregation of data and reporting		~15 min/plate or Variable with 3rd party tools	N/A

Table 2: *QXDx BCR-ABL %IS Test – CE-IVD Workflow*

RNA extraction, step 1, can be performed by a variety of standard methods, including Maxwell® CSC RNA Blood Kit, Qiagen RNeasy® Mini and Trizol extraction. Steps 2-5 are performed with reagents and instrumentation provided by Bio-Rad, see below. Manual thresholding and export is done with QuantaSoft v 1.7.4. The final step of data aggregation and reporting can be done either by lab developed or with 3rd party tools.

3rd party tool instructions for download and use can be found on the BCR-ABL product page. The tool takes raw data generated by QS 1.7.4 software and provides an output into International Scale or MR value.

REAGENTS AND INSTRUMENTS

Materials Provided

Σ	The QXDx BCR-ABL %IS Kit contains sufficient reagents to process 96 samples or quality control specimens when run in 2 wells per sample. The kit contains the following items:

Catalog #	Name	QTY (Tubes)	Volume	Storage Conditions
12006134	QXDx BCR-ABL %IS Kit	1	---	-25°C to -15°C
12004581	QXDx BCR-ABL Primers & Probes	1	250 µL	-25°C to -15°C
12004586	QXDx Nuclease Free Water	2	1500 µL	-25°C to -15°C
12004569	QXDx 5x iScript Select Reaction Mix	1	500 µL	-25°C to -15°C
12004572	QXDX 2X Supermix	3	1000 µL	-25°C to -15°C

QXDx™ BCR-ABL %IS Kit

Catalog #	Name	QTY (Tubes)	Volume	Storage Conditions
12004571	QXDx iScript Advanced Reverse Transcriptase	1	125 µL	-25°C to -15°C
12004582	QXDx RT Primers	1	500 µL	-25°C to -15°C
12004585	QXDx BCR-ABL 0.1%IS	2	50 µL	-25°C to -15°C
12004588	QXDx BCR-ABL 10%IS	2	50 µL	-25°C to -15°C
12004584	QXDx BCR-ABL H-CTRL	2	50 µL	-25°C to -15°C
12004583	QXDx BCR-ABL L-CTL	2	50 µL	-25°C to -15°C
12004573	QXDx BCR-ABL Neg-CTRL	2	50 µL	-25°C to -15°C
12006672	QXDx BCR-ABL %IS Kit IFU, CE-IVD	1		

NOTE: *Safety Data Sheets (SDS) are available at www.bio-rad.com*

Storage and Handling

- Store all cDNA and ddPCR components at -25°C to -15°C in a constant temperature freezer.
- Store all RNA Controls at -80°C.
- Do not vortex the reverse transcriptase (RT).
- Keep RT frozen until use and promptly place back in freezer after use.
- Avoid extended exposure to light for the BCR-ABL1/ABL1 primer/probe mix at room temperature.
- Mix BCR-ABL1/ABL1 primer/probe mix thoroughly to ensure homogeneity while avoiding bubbles.
- Gently mix by vortexing and centrifuge all other vials before opening.
- Do not vortex calibrator or control vials.
- Do not freeze/thaw QXDx Supermix more than 5 times.
- Expiration dates for each of the reagents are indicated on the individual component labels.

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents and Consumables

For QX200 AutoDG ddPCR Dx System Use:

Catalog #	Name	QTY (Tubes)	Volume	Storage Conditions
12001922	**QXDx AutoDG Consumable Pack**	**1**		**15°C to 30°C**
	ddPCR Pierceable Foil Seals	50		15°C to 30°C
	ddPCR 96 Well Plates	15		15°C to 30°C
	DG32™ Cartridges w/ Gaskets	15		15°C to 30°C
	Pipet Tip for AutoDG (Racks)	10		15°C to 30°C
	AutoDG Oil for Probes	1		15°C to 30°C

Catalog #	Name	QTY (Tubes)	Volume	Storage Conditions
12002526	**QXDx Droplet Reader Oil Pack**	**1**		
12002526	QXDx Droplet Reader Oil Pack	1	1 L	15°C to 30°C

For QX200 Droplet Generator Use (manual droplet generator):

Catalog #	Name	QTY (Tubes)	Volume	Storage Conditions
12001921	**QXDx Consumable Pack**			**15°C to 30°C**
	ddPCR 96-Well Plates	5		15°C to 30°C
	ddPCR Pierceable Foil Heat Seal	50		15°C to 30°C
	Droplet Generation Oil for Probes	2	7 mL	15°C to 30°C
	DG8™ Cartridges	24		15°C to 30°C
	DG8 Gaskets	24		15°C to 30°C
	Instructions Manual	1		

Catalog #	Name	QTY (Tubes)	Volume	Storage Conditions
12002526	**QXDx Droplet Reader Oil Pack**	**1**		
12002526	QXDx Droplet Reader Oil Pack	1	1L	15°C to 30°C

Instruments

Description	Catalog #
QX200 AutoDG Droplet Digital PCR Dx System	**17002229**
QX200 Droplet Reader, CE-IVD	12001045
QX200 Automated Droplet Generator, CE-IVD	12001630

or

Description	Catalog #
QX200 AutoDG Droplet Digital PCR System	**1864100**
QX200 Droplet Reader, IVD	1864003
QX200 Automated Droplet Generator, IVD	1864101

or

Description	Catalog #
QX200 Droplet Digital PCR Dx System	**17000034**
QX200 Droplet Reader, IVD	12001045
QX200 Droplet Generator, IVD	12001049

or

Description	Catalog #
QX200 Droplet Digital PCR System	**1864101**
QX200 Droplet Reader	1864003
QX200 Droplet Generator	1864002

Description
Thermal Cycler
Required: Thermal Cyclers with Specifications equivalent to the following: • Accuracy: +/- 0.2°C • Uniformity: +/- 0.4°C well-to-well within 10 sec • Adjustable ramp capability with required ramp rate: up to 2°C/sec • Temperature range: 0-100°C

Description	Catalog #
PX1™ PCR Plate Sealer	**1814000**

Description
General Laboratory Equipment

• Pipets (Rainin or Eppendorf)
   ○ 1-10 µL, 10-100 µL, 20-200 µL, and 100-1000 µL Plate Centrifuge or Plate Spinner
• Vortex Pipets (Rainin or Eppendorf)
   ○ 1-10 µL, 10-100 µL, 20-200 µL, and 100-1000 µL

Other Reagents and Consumable

Description
Reagents for RNA Purification
Any commercially available, lab-validated sample preparation methodology is acceptable. Example: Qiagen, Trizol, Promega Maxwell RNA Blood Kit.

Description	Catalog #
ddPCR 2x Control Buffer	**1863052**
For use in blank wells with Droplet Generation Oil	

GENERAL PRECAUTIONS AND WARNINGS

- For In Vitro Diagnostic (IVD) use only
- For professional use only
- All biological specimens should be treated as if they are capable of transmitting infectious agents. All human specimens should be treated with standard precautions. Guidelines for specimen handling are available from the World Health Organization or U.S. Centers for Disease Control and Prevention.
- Use proper laboratory safety procedures for working with chemicals and handling specimens.
- Change gloves while transporting and working with different reagents.
- Use 10% bleach followed by 70% alcohol to wipe the surface areas of workspace.
- Ensure that Droplet Generator and Droplet Reader have a dedicated space in separate areas to avoid amplicon contamination.
- Failure to obey procedures and conditions described in this document and use of reagents other than those provided in this kit can cause incorrect results and adverse effects.
- Do not substitute QXDx BCR-ABL %IS Kit reagents with other reagents.
- Do not mix reagents from different lots of QXDx BCR-ABL %IS Kit.
- If using the same thermal cycler for RT and PCR, place the thermal cycler alongside Droplet Generator or in a separate location than the reader.
  NOTE: *The plate will be foil sealed during PCR amplification.*
- Perform test setup and template addition in different locations, with dedicated pipets and pipettors.
- Due to the extreme sensitivity of RNA and ddPCR, the entire test must be performed under RNAse/DNAse-free conditions.
- Optimize workflow and space to eliminate carryover contamination.
- Ensure that regular maintenance and calibration are performed on all equipment according to manufacturer's recommendations.
- Use nuclease-free tips and reagents, and routinely clean pipets.
- Ensure that only a recommended thermal cycling protocol is used for optimal results.
- Do not use DEPC-treated water for PCR amplification
- Some substances may interfere with the test: certain drugs (e.g., heparin), highly lipemic samples, hemolyzed samples, icteric samples, or samples with high levels of protein.
- Consult your institution's environmental waste personnel on proper disposal of used plates, consumables, and reagents. This material may exhibit characteristics of federal EPA Resource Conservation and Recovery Act (RCRA) hazardous waste requiring specific disposal requirements.
- Check state and local regulations as they differ from federal disposal regulations. Institutions should check their country hazardous waste disposal requirements.
- The quality of the test result is largely dependent on the amount and quality of the sample being analyzed. It is recommended that the quality and quantity of the RNA sample be measured as specified in this manual.

SPECIMEN COLLECTION, TRANSPORT AND STORAGE

- Collect a minimum of 5 mL of whole blood in tubes containing EDTA as anticoagulant.
- When not in use, store blood specimens at 2-8°C for up to 72 hours.

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QXDX BCR-ABL %IS TEST PROTOCOL

Overview

The kit contains enough reagents to run a total of 192 ddPCR wells. When all samples, controls and calibrators are run in two-well format, the provided reagents are sufficient for 84 samples. When higher sensitivity is required, unknown samples can be run in more than 2 wells, for example 4. In this case, kit reagents will be sufficient for fewer samples.

Plate Layouts

Each plate must be run with 6 known samples: No Template Control (NTC), QXDx High Positive Control (Hi Pos), QXDx Negative Control (Neg), QXDx Low Positive Control (Lo Pos), Calibrator-check QXDX ~10%IS Cal, and Calibrator-check QXDx ~0.1%IS Cal. All of these known samples are always run in two wells each. Run quality is scored on the data produced from these controls and calibrator-checks, see below. The unknown samples must be run in at least two wells. When higher sensitivity is required, the unknown samples can be run in 4-well format. Data from all wells with the same sample are aggregated in a digital way to produce the total counts of molecular targets and %IS and MR values for the sample. A special type of sample, called 2x Control Buffer can be run, but will not be analyzed or reported. 2x Control Buffer must be used for buffer wells in all empty wells of each column with samples (see Figures 1-3).

	Knowns		Unknowns									
	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	Sample 3	Sample 3	Sample 11	Sample 11	Sample 19	Sample 19	Sample 27	Sample 27	Sample 35	Sample 35
B	Hi Pos	Hi Pos	Sample 4	Sample 4	Sample 12	Sample 12	Sample 20	Sample 20	Sample 28	Sample 28	Sample 36	Sample 36
C	Neg	Neg	Sample 5	Sample 5	Sample 13	Sample 13	Sample 21	Sample 21	Sample 29	Sample 29	Sample 37	Sample 37
D	Lo Pos	Lo Pos	Sample 6	Sample 6	Sample 14	Sample 14	Sample 22	Sample 22	Sample 30	Sample 30	Sample 38	Sample 38
E	~0.1% IS	~0.1% IS	Sample 7	Sample 7	Sample 15	Sample 15	Sample 23	Sample 23	Sample 31	Sample 31	Sample 39	Sample 39
F	~10% IS	~10% IS	Sample 8	Sample 8	Sample 16	Sample 16	Sample 24	Sample 24	Sample 32	Sample 32	Sample 40	Sample 40
G	Sample 1	Sample 1	Sample 9	Sample 9	Sample 17	Sample 17	Sample 25	Sample 25	Sample 33	Sample 33	Sample 41	Sample 41
H	Sample 2	Sample 2	Sample 10	Sample 10	Sample 18	Sample 18	Sample 26	Sample 26	Sample 34	Sample 34	Sample 42	Sample 42

Figure 1: *A plate layout when all known and unknown samples are run in two-well format. There are 42 unknown samples on one plate.*

	Knowns		Unknowns									
	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	Sample 3	Sample 3	Sample 11	Sample 11						
B	Hi Pos	Hi Pos	Sample 4	Sample 4	Sample 12	Sample 12						
C	Neg	Neg	Sample 5	Sample 5	Sample 13	Sample 13						
D	Lo Pos	Lo Pos	Sample 6	Sample 6	Sample 14	Sample 14						
E	~0.1% IS	~0.1% IS	Sample 7	Sample 7	buffer	buffer						
F	~10% IS	~10% IS	Sample 8	Sample 8	buffer	buffer						
G	Sample 1	Sample 1	Sample 9	Sample 9	buffer	buffer						
H	Sample 2	Sample 2	Sample 10	Sample 10	buffer	buffer						

Figure 2: *A plate layout with less than 42 unknown samples. The last 8 wells are filled with buffer (not read) to complete 8 samples per column. Remaining empty columns are not filled or read.*

	Knowns		Unknowns									
	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3	Sample 3		
B	Hi Pos	Hi Pos	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4	Sample 4		
C	Neg	Neg	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5	Sample 5		
D	Lo Pos	Lo Pos	Sample 6	Sample 6	Sample 6	Sample 6	buffer	buffer	buffer	buffer		
E	~0.1% IS	~0.1% IS	Sample 7	Sample 7	Sample 7	Sample 7	buffer	buffer	buffer	buffer		
F	~10% IS	~10% IS	Sample 8	Sample 8	Sample 8	Sample 8	buffer	buffer	buffer	buffer		
G	Sample 1	Sample 1	Sample 9	Sample 9	buffer	buffer	buffer	buffer	buffer	buffer		
H	Sample 2	Sample 2	Sample 10	Sample 10	buffer	buffer	buffer	buffer	buffer	buffer		

Figure 3: *A plate layout where some unknown samples are run in 2, 4-well formats. Buffer is used to complete 8 samples per column. Remaining empty columns are not filled or read.*

It is recommended that samples are spread between wells in a horizontal direction. This way, if there is a failure at a droplet chip level (column), the other wells may provide sufficient information for a sample to be scored. Use 2x control buffer diluted 1:1 with water in all empty wells of each column.

NOTE: *As with any PCR-based molecular test, nucleic acid contamination must be protected against by routine decontamination of bench space and pipets, separation of RNA/DNA handling from assay preparation areas, and proper PPE. We recommend that template-free areas and template-plus areas are spatially separated, have separate pipets and tools, and be routinely decontaminated.*

PRE-ANALYTICAL STEPS

Total RNA extracted via common sample preparation methodologies from whole blood or buffy coat is compatible with Droplet Digital PCR methods. The most common methodologies for RNA extraction are Maxwell® CSC RNA Blood Kit, Qiagen RNeasy® Mini and Trizol extraction. For optimal results, at least 10 million (1 x 107) nucleated cells should be collected and extracted into RNA. Since RNA is known to be subject to degradation by ubiquitous RNases present in human specimens, it is recommended to minimize sample handling steps and to process the blood samples within 24 hours of collection for the most sensitive measurements of BCR-ABL quantities (Hughes et. al, 2006)[4]. RNA quality, purity and quantity can greatly affect the results. Therefore it is recommended, but not required, that purified total RNA be evaluated for quality and purity by standard spectrophotometric methods. The corresponding OD260 concentration should be ~100 ng/μL; purity as estimated by OD_{260}/OD_{280} ratio should be >1.6, and OD_{260}/OD_{230} ratio should be >1.2.

Test sensitivity is dependent on the RNA input. It is recommended to validate an RNA Isolation method yielding greater than 100 ng/μL and to target 750-1250 ng total RNA input per test. Purified total RNA should be adjusted to target concentration of ~100 ng/μL for efficient test setup and should be tested immediately after extraction or stored frozen at -80°C until ready for testing.

Catergory	Target Specification
Blood Volume	1.5-10 mL
Cell Count	≥1E+07
RNA Concentration	75-125 ng/ μL
RNA Purity	OD_{260}/OD_{280} ratio > 1.6 OD_{260}/OD_{230} ratio > 1.2

RT REACTION PREPARATION

1. Thaw purified RNA at room temperature for up to 10 minutes, then place on ice or cold block.

   NOTE: *Do not vortex; you may spin the tubes.*

2. Adjust RNA samples to ~100 ng/µL, if not already done.

3. Place iScript Advanced Reverse Transcriptase on ice or cold block removed from freezer <u>and</u> when ready to use.

   NOTE: *Do not vortex Advanced Reverse Transcriptase.*

4. Thaw the following components to room temperature for up to 15 minutes.

   a. QXDx 5x iScript Select Reaction Mix

   b. QXDx RT Primers

   c. QXDx Nuclease-free water

5. Mix or vortex each component tube in step 4 thoroughly with lid closed, and briefly centrifuge to collect contents to the bottom of the tube.

6. Place each of the components on ice or cold block.

7. RT Master Mix Preparation and loading RT plate:

   a. Prepare RT Master Mix according to the number of samples, controls and calibrators being processed.

   b. See Table 3 for RT Master Mix preparation volumes.

   c. **NOTE:** *The RT plate can be placed on ice while loading the plate.*

RT Master Mix Volume		# of Samples* (samples + overage) Volume				
# of Samples		1	16	32	48	96
Plates			1/3 Plate	2/3 Plate	1 Plate	2 Plates
Component	Volume	1x	(16+2)x	(32+3)x	(48+4)x	(96+4)x
QXDx Nuclease-Free Water	µL	~3.75	~67.50	~131.25	~195	~375
QXDx 5x iScript Select Reaction Mix	µL	5	90	175	260	500
QXDx RT Primers (Nonamers)	µL	5	90	175	260	500
QXDx iScript Advanced Reverse Transcriptase	µL	1.25	22.5	43.75	65	125
RNA Sample* (750-1250 ng)	µL	10				
Total	µL	25	450	875	1300	2500

Table 3: *Master Mix Volume based on # of Samples.*

NOTE: ** Sample(s) include patients samples, controls, and calibrators to be run on the plate.*

8. For multiple samples, it is recommended to prepare the RT Master Mix with the above components, (excluding RNA) and then dispense into each reaction well.

   a. See Figure 4 for required plate layout

	Knowns		Unknowns									
	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC		Sample 3		Sample 11							
B	Hi Pos		Sample 4		Sample 12							
C	Neg		Sample 5		Sample 13							
D	Lo Pos		Sample 6		Sample 14							
E	~0.1% IS		Sample 7		buffer							
F	~10% IS		Sample 8		buffer							
G	Sample 1		Sample 9		buffer							
H	Sample 2		Sample 10		buffer							

Figure 4: *Required RT plate layout*

b. Mix all of the RT Master Mix components (excluding RNA) well and aliquot 15 µL per sample into every other column of the 96-well PCR plate.

   NOTE: *An electronic repeater pipet may be used for efficiency.*

c. Add 10 µL of Calibrators and Controls in the appropriate RT wells.

d. Add 10 µL of extacted RNA sample volume in the appropriate RT wells.

e. Mix the reaction wells gently by pipetting (10 times), carefully avoiding forming bubbles.

f. Cover the plate with Foil Seal using a PX1 PCR Plate Sealer.

   i. Set the PX-1 plate sealer to 180 degrees for 5 seconds (not default conditions).)

   ii. Place RT plate on the room temperature aluminum block.

   iii. Cover the plate with one sheet of pierceable foil seal, where the red stripe should be visible when the foil is placed on the plate.

   iv. Touch the **Seal** button to seal the plate; (this will close the door, and initiate heat sealing).

      NOTE: *Do not use the metal frame.*

   v. Remove the plate and block from PX-1 plate sealer.

   vi. Ensure all of the wells on the plate are sealed by checking that the depressions of the wells are visible on the foil.

      NOTE: *For more detailed instruction refer to PX1 Plate Sealer Instruction Manual.*

g. Spin plate for 1 minute @ 1000 rcf to remove any bubbles. If bubbles remain, spin the plate again.

h. Once sealed and spun, the plate is ready for thermal cycling.

NOTE: *Remember to remove the metal block from PX1 PCR plate sealer in order to avoid overheating the block.*

9. Transfer the plate to the Thermal Cycler and run the protocol shown in Table 4.

Primer Binding	10 min. @ 25°C
Reverse Transcription	45 min. @ 42°C
RT Inactivation	5 min at 85 degrees
Hold	Up to 24 hours at 4° C

Table 4: *Thermal Cycler Protocol*

*NOTE: *4°C hold allows the user to store RT reaction up to 24 hours before processing.*

NOTE: *if using C1000 Thermal Cycler, set the sample volume at 25µL and lid temperature to 105 °C.*

10. Upon completion of RT preparation (cDNA), spin the plate for 1 minute @ 1000 rcf to collect condensate.

11. Store cDNA on ice and use immediately; alternatively, store at 4°C for up to 24 hours if not used immediately.

ddPCR SETUP AND DROPLET GENERATION

WARNING: *Prior to removing all components, ensure that the bench space has been properly cleaned with 10% bleach and 70% alcohol.*

1. Thaw the following components to room temperature for approximately 15 minutes.

   a. QXDx 2x Supermix

   b. QXDx BCR-ABL Primers & Probes,

   c. cDNA sample(s)* plate (~640 ng/test)

2. Mix individual component tubes thoroughly using a vortex to ensure homogeneity, and carefully check for precipitates. If precipitate exists, mix the tube content thoroughly, and then centrifuge.

   NOTE: *Supermix material is more viscous than other components.*

3. Centrifuge all component tubes for 30 seconds @ 1000 rcf, to collect contents at the bottom of the tubes.

4. Prepare the ddPCR Master Mix for the number of reactions according to Table 5.

ddPCR Volume	# of Samples* (samples + overage) Volume					
Component	**Volume**	**1x**	**(16+2)x**	**(32+3)x**	**(48+4)x**	**(96+4)x**
QXDx Nuclease-Free Water	µL	6.5	117	227.5	338	650
QXDx ddPCR Supermix	µL	25	450	875	1300	2500
QXDx 20X BCR-ABL Primers/Probes	µL	2.5	45	87.5	130	250
cDNA Sample(s)*	µL	16				
Total	µL	50	900	1750	2600**	5000*

Table 5: *ddPCR Plate Volumes based on # of samples (established for RT plate).*

NOTE: ** *Total volume of 2600 µL for 48 samples (1 plate) and 5000 µL for 96 samples (2 plates) is more than 2 mL tube can hold. Recommend 5 mL tube for those samples.*

NOTE: * *Sample(s) include patient samples, controls, and calibrators to be run on the plate.*

5. For multiple samples, it is recommended to prepare a ddPCR Master Mix with the above components, (excluding cDNA sample), and then dispense cDNA into each reaction well of ddPCR plate.

   a. See Figure 5 for required plate layout.

   b. Mix all of the ddPCR Master Mix components thoroughly by using a vortexer.

   c. Aliquot 34 µL/well of ddPCR Master Mix (without cDNA sample) into the plate according to Figure 5.

   d. Using a 50, 100, or 200 µL 8-channel pipettor with filter tips, puncture foil seal of the cDNA plate.

   NOTE: *Normal practice has found that P20 pipet tips do not break the foil seal. Avoid using P20 pipetter for this step.*

Best Practices for recovering an adequate volume of cDNA:

   i. Set a pipette to the volume being pipetted and, without engaging the plunger, puncture the foil seal on on the plate. The pipette tips should extend no more than 2 mm below the seal.

   ii. Once the holes are formed, raise the pipette tips above the seal and engage the plunger.

iii. With plunger engaged lower the tips to the bottom of the wells and widen the holes by gently rocking the tips back and forth. Widening the foil seal holes allows the pipette tips to reach the bottom of the wells. This is important for recovering the full volume of cDNA required for optimal performance.

iv. With the pipette tips still in the cDNA, very slowly aspirate the required volume. After aspiration stops, leave the pipette tips in the wells for an additional 5 sec to allow pressure to equilibrate.

e. Add 16 µL of cDNA sample (patient, control, calibrator or buffer) into each well of ddPCR plate.

f. Carefully pipet up and down ~5 times to mix with ddPCR Master Mix, then fully expel the sample.

g. Repeat step f for all samples.

h. After all cDNA samples are added to the plate, use a 50 or 100 µL pipettor (can be multichannel) and set at 35µl.

i. Pipet each reaction up and down at least 15 times at 50% (25 µL) of total volume (50 µL) , carefully avoiding bubbles.

WARNING: *It is critical that you pipet 15 times to ensure viscous material is well-mixed.*

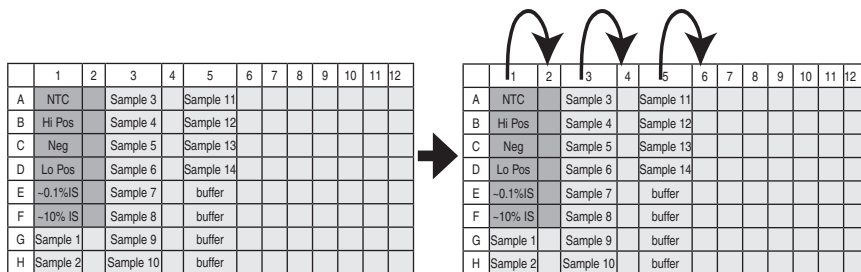j. Transfer 25 µL of each sample into neighboring well to create duplicate wells as shown in **Figure 5**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC		Sample 3		Sample 11							
B	Hi Pos		Sample 4		Sample 12							
C	Neg		Sample 5		Sample 13							
D	Lo Pos		Sample 6		Sample 14							
E	–0.1%IS		Sample 7		buffer							
F	~10% IS		Sample 8		buffer							
G	Sample 1		Sample 9		buffer							
H	Sample 2		Sample 10		buffer							

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC		Sample 3		Sample 11							
B	Hi Pos		Sample 4		Sample 12							
C	Neg		Sample 5		Sample 13							
D	Lo Pos		Sample 6		Sample 14							
E	–0.1%IS		Sample 7		buffer							
F	~10% IS		Sample 8		buffer							
G	Sample 1		Sample 9		buffer							
H	Sample 2		Sample 10		buffer							

Figure 5: ddPCR plate layout

NOTE: *if the total number of samples and controls is less than a multiple of 8 (i.e., 8,16,24, etc.) then fill the remaining wells with 25 µL ddPCR control buffer or excess reagents.* **Droplets will not form if the wells are left empty.**

6. Seal plate with foil seal using the PX-1 plate sealer.

   i. Set the PX-1 plate sealer to 180 degrees for 5 seconds (not default conditions).

   ii. Place RT plate on the room temperature block.

   iii. Cover the plate with one sheet of pierceable foil seal; the red stripe should be visible when the foil is placed on the plate.

   iv. Touch the Seal button to seal the plate; this will close the door, and initiate heat sealing.

   NOTE: *Do not use the metal frame.*

   v. Remove the plate and block from PX-1 plate sealer.

7. Briefly centrifuge the plate for 30 seconds @ 1000 rcf to collect contents at the bottom of the plate and remove bubbles.
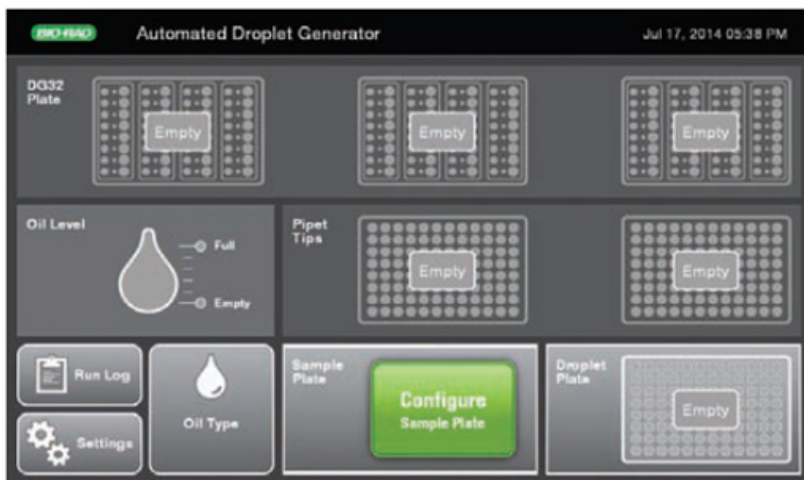
Droplet Generation

Figure 6: *Automated Droplet Generator System Layout for items*

NOTE: *Refer to the QX200 Automated Droplet Generator manual for detailed and complete instructions for use.*

WARNING: *Prior to setting up, ensure that the bench space and instrument surface area have been properly cleaned with 10% bleach and 70% alcohol.*

1. Gather all of the consumable materials needed to set up the Droplet Generator.

    •   DG32 Cartridges, Pipet Tips, Droplet Generation Oil, Sample Plate, Droplet Plate (where generated droplets appear at run completion).

    NOTE: *Ensure that the Trash container is sitting in the appropriate location on the AutoDG.*

2. Open the AutoDG door and load 96-well Sample ddPCR plate onto the QX200 Automated Droplet Generator in the "Sample Plate" position. The indicator light should turn green.

3. Touch the **Configure Sample Plate** button on the AutoDG interface and select appropriate number of columns in which your samples are located. (Plate name and plate notes are optional.) Click **OK**.

NOTE: *If the total number of samples and controls is less than a multiple of 8 in a single column of the plate, fill the additional column wells with 25 μL ddPCR 1X control buffer or excess 1X Master Mix to ensure droplet formation.*

NOTE: *Only full columns can be selected on the AutoDG Configure Screen, (see figure 7).*
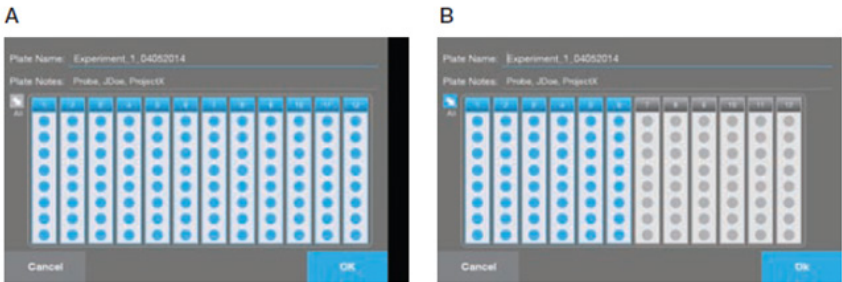
Figure 7: *Plate A has 96 wells selected for 48 samples; Plate B has 48 wells selected for 24 samples.*

4. Based on the number of columns selected on the sample plate, yellow indicator lights identify consumables that need to be loaded on the instrument.

5. Open the AutoDG door and load appropriate consumables until associated indicator lights are green.

   a. Load the DG32 cartridges (with green gaskets to the right) into the plate holders at the back of the system. The holders are keyed for proper orientation, and when placed correctly, the light will turn green.

   b. Load Pipet Tips along the center row of the instrument after removing the plastic wrap and box lid.
   WARNING: *only full tip boxes should be loaded on the system.*

   c. Load empty 96-well droplet plate with a blue cooling block in the Droplet Plate location; the light should turn green.

   d. Load the AutoDG Oil on the left side by removing the cap, and twisting the bottle into the tower. Select the type of AutoDG Oil on the Instrument User Interface "Droplets for Probes" the oil type selected will turn blue. The Oil level icon on the screen will turn blue and display the current oil level in the bottle.
   NOTE: *this step is required only if the oil level is not sufficient for the run.*

6. Once all of the indicators on the AutoDG are green, press the blue Start Droplet Generation button.
   WARNING: *Ensure that you back away from the instrument, as this will cause the door to close and the run to initialize.*

7. AutoDG user interface will ask the user to confirm "Start Run". The run will not start until the user presses the **Start Run** button.
   NOTE: *The screen will indicate the time remaining until droplets are generated and ready. If the droplet formation stops for any reason, "Run Terminated" message will appear on AutoDG screen. If the run is terminated determine the cause of the failure and follow the instructions in the troubleshooting section.*

8. Upon successful completion of the droplet generation run, remove and seal the "droplet plate" with a pierceable foil seal within 30 minutes of run completion.
   WARNING: *Upon completion of the AutoDG run, it is important to foil seal and start thermal cycling within 30 minutes.*

   i. Set the PX-1 plate sealer to 180 degrees for 5 seconds (not default conditions).

   ii. Place RT plate on the room temperature block.

   iii. Cover the plate with one sheet of pierceable foil seal; the red stripe should be visible when the foil is placed on the plate.

   iv. Touch the Seal button to seal the plate; this will close the door, and initiate heat sealing.
   NOTE: *Do not use the metal frame.*

   v. Remove the plate and block from PX-1 plate sealer.

9. Now the droplet plate is ready for thermal cycling.

PCR AMPLIFICATION (THERMAL CYCLING)

Once the 96-well plate containing the droplets is sealed, place it into the thermal cycler for PCR amplification.

WARNING: *Prior to setting up, ensure that the bench space and instrument surface area have been properly cleaned with 10% bleach and 70% alcohol.*

Follow the Thermal Cycling Protocol in Table 6 for optimal QXDx BCR-ABL results.

Cycling Step	Number of Cycles	Temperature (°C)	Time	Ramp Rate
Enzyme Activation	1	95	10 min	2°C/sec
Denaturation	5	94	30 sec	
Annealing/Extension		60	1 min	
Denaturation	35	94	30 sec	
Annealing/Extension		64	1 min	
Enzyme Deactivation	1	95	10 min	
Droplet Stabilization	1	4	30 min	
Hold (optional)	1	4	24 hours	

Table 6: BCR-ABL/ABL1 Thermal Cycling Protocol

WARNING: *It is critical to set the Ramp Rate @ 2°C/sec, as default ramp rates differ for different cyclers.*

NOTE: *if you use C1000 Touch Thermal Cycler, use a heated lid set to 105°C and set the sample volume to 40 μL (default sample volume is 30 μL). Additionally, refer to C1000 Touch Thermal Cycler manual for protocol configuration.*

For other thermal cyclers, see Appendix A for suggested settings.

DROPLET READING

Preparing for Data Acquisition

1. Before reading the plate, set up a template file for the plate. This can be done either on the instrument PC or on a workstation running QuantaSoft 1.7.4. The plate settings are as follows:
   - Experiment: RED
   - Supermix: ddPCR Supermix for Probes(no dUTP)
   - Target1: Name: BCR-ABL
   - Target1: Type: Ch1 Unknown
   - Target2: Name: ABL
   - Target2: Type: Ch2 Unknown

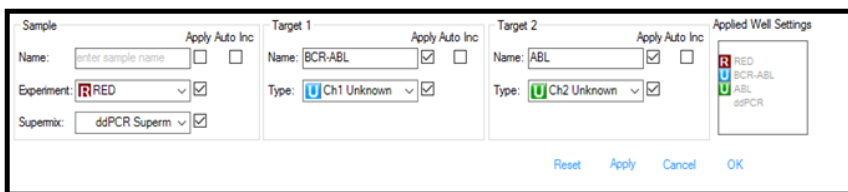Figure 8 shows a view of the QuantaSoft panel and its settings.

Figure 8: *Droplet Reader Setup Parameters*

2. Each sample in the used wells should be named. The standard names for the known samples are:

    •   NTC – no template control
    •   Hi Pos – high positive control
    •   Neg – negative control
    •   Lo Pos – low positive control
    •   ~0.1% IS – calibrator-check at approximately 0.1% IS
    •   ~10% IS– calibrator-check at approximately 10% IS
    •   Buffer – samples that will not be read; these are added to complete partially used columns

3. Name the unknown samples according to your lab procedures. Refer to the Software Manual for more details.

WARNING: *Ensure all columns to be read have all 8 wells filled. Use buffer solution as needed to fill empty well.*

Data Acquisition

1. On the instrument PC, start QuantaSoft and load the template file for the plate to be run. Make sure all information is complete and accurate.

2. Press the **Run** button on the left panel. The Reader will start reading droplets from the requested wells and will do preliminary automated analysis. You can view progress and the quality of the data during the run.

    WARNING: *Do not interfere with the acquisition process: do not use the PC for any other tasks and minimize vibrations around the system.*

3. After run completion, a "*.qlp" file is saved on the instrument PC hard drive. This file can be analyzed on the same PC or at a workstation running QuantaSoft 1.7.4.

4. Dispose the plate in accordance with your local, state, or federal waste disposal laws and regulations.

ANALYSIS

Load the .qlp file generated by the reader and select Analyze on the left panel.

1. Check accepted events.

    a. Select all wells.
    b. Click Events button and Total checkbox.

   c.   Any wells with less than 10000 accepted events should be excluded from further analysis.

   d.   Record all wells to be excluded.

2.   Prepare for thresholding.

   a.   Click the **2D Amplitude** button

   b.   Click **Options** and select **fixed** to lock axis.
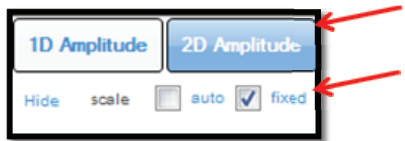
3.   Verify positive controls and thresholds.

   a.   Select the wells with the Hi Pos sample.

   b.   Set thresholds ~ 1/3 between the negative and the positive clusters in each channel. The 2-D plot should look like Figure 9.
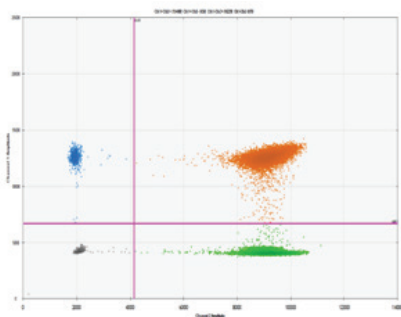

Figure 9: *2-D for Positive Control*

   c.   Record threshold values.

4.   Set thresholds for the whole plate.

   a.   Select all wells.

   b.   Apply plate-level common thresholds for the selection by setting them at the levels of the Hi Pos thresholds.

5.   Check other control and calibrator thresholding.

   a.   NTCs – If more than 10 ABL copies or 1 BCR-ABL copy is detected, fail the run.

   b.   All other known samples – verify thresholds do not cut through any clusters.

6.   Inspect thresholding on unknown samples.

   a.   Verify clusters are not cut in the middle.

   b.   Check for any other unusual phenotypes.

       ○   For examples of unusual phenotypes, see Appendix.

   c.   Exclude compromised wells from further analysis or adjust thresholds, if necessary.

Data Export

1. Select all wells and then deselect excluded wells by "Ctrl+ click" on each one.

2. Select **Both** in the dropdown box and click **Export CSV** to save data in merged and single-well format to a .csv file.
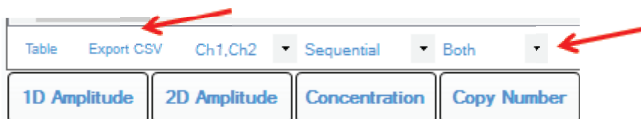
All samples with more than 1 passing well will be merged at a digital, droplet level. Any samples with only 1 passing well will be reported at a single-well level. Digital merging is the most accurate method for data aggregation and will provide the most sensitive and robust measurements.

Results

The CSV file contains data in a tabular format. Each row is an individual sample (merged or unmerged). When merging has been applied, the well notation changes to Mxx, to indicate merged wells. When wells have not been merged, original naming is preserved (i.e. A01, A02, etc.). The column with a header "Ratio" contains the ratio of BCR-ABL/ABL copies. To compute %IS ratio and MR level, use these formulas.

$$\%IS = Ratio \times 100 \times CF$$

$$MR = \log_{10}(100\%IS) - \log_{10}(\%IS) = 2 - \log_{10}(\%IS)$$

CF is the lot-specific conversion factor and can be found in the kit insert.

If you have excluded wells, some samples may only have a single well reporting. Results (BCR-ABL and ABL) from these singletons are in the CSV file below the results from the merged wells.

QUALITY CONTROL

Controls

The kit ships with three controls. In order to report on the unknown samples, the following criteria must be met:

Control name	Function	Expected result
Hi Pos	High positive control	MR0.3-MR1 (10%-50%IS)
Neg	Negative control	Neg. (less than MR5.3 / 0.0007%IS)
Lo Pos	Low positive control	MR3-MR5 (0.001-0.1%IS)

Calibrator-Checks

Two calibrator-checks are provided. They are used to ensure valid quantification capability of the kit. The values obtained for each calibrator-check are not used to modify reported values of the unknown samples. Make sure that reported %IS values are as expected:

Calibrator-check name	Expected result
~0.1% IS	Between min and max value provided in the insert
~10% IS	Between min and max value provided in the insert

Depiction fo the Traceability of Calibrator-Checks to the WHO Reference Standard Panel
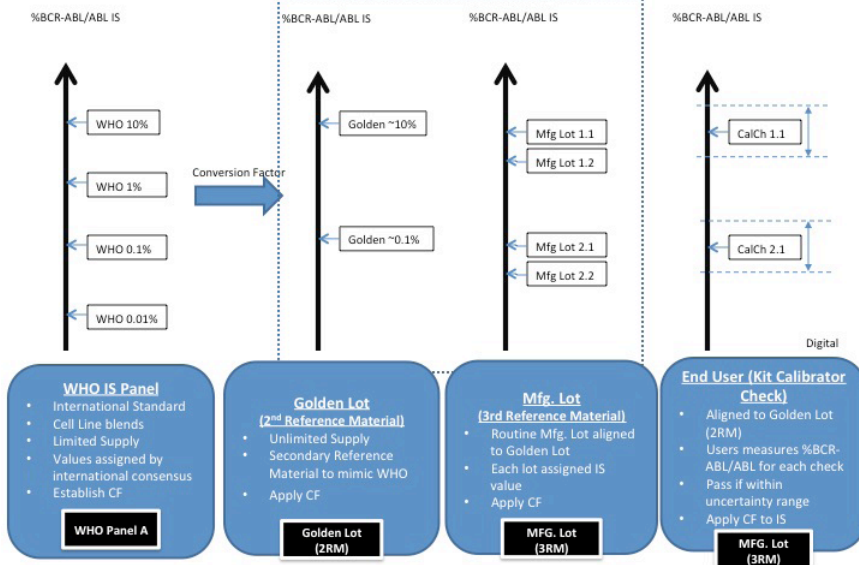
The calibrator-checks are used to ensure that the assay is still traceable to the First WHO international genetic reference panel.

Unknown Samples

The test allows for 2 or more wells per unknown sample to be used. Each well should report at least 10000 ABL copies **per Cross recommendations**[6] You may choose to use wells with less than 10000 ABL copies, but this may lead to lower sensitivity for this sample. Digital merging aggregates all copies of ABL and BCR-ABL transcripts between all wells used to provide a total ratio of BCR-ABL/ABL copies, expressed as %IS and MR level. Additional checks for concordance between the wells may be performed as needed.

WARNING: *In cases where only one well per sample is valid, the number of ABL copies may not be sufficient to score high MR levels. Check ABL copies per well in the CSV file to verify sensitivity.*

INTERPRETATION OF RESULTS

When all control and calibrator-check tests pass, results from unknown samples may be reported. In general, using more wells per sample allows for higher sensitivity.

Current European LeukemiaNet (ELN) Recommendations are summarized as follows:

BCR-ABL (IS)	3 Months	6 Months	12 Months	>12 Months
>10%				
1%-10%				
0.1% - <1%				
<0.1%				

Code	Clinical Considerations
RED	Evaluate patient compliance and drug interactions Mutational Analysis
YELLOW	Evaluate patient compliance and drug interactions Mutational Analysis
GREEN	Monitor response and side effects

TROUBLESHOOTING

ddPCR is a highly sensitive technology that allows detection and characterization of problems with the sample, reaction or instrumentation in a robust and reliable fashion. The following lists the most common failure modes with their phenotypes, descriptions and suggested resolution.

Shredding – some droplets have been shredded, usually at read time (see Figure 10).

Resolution: Do not use the data; exclude the well. If problem persists, check for particle contamination in the lab.
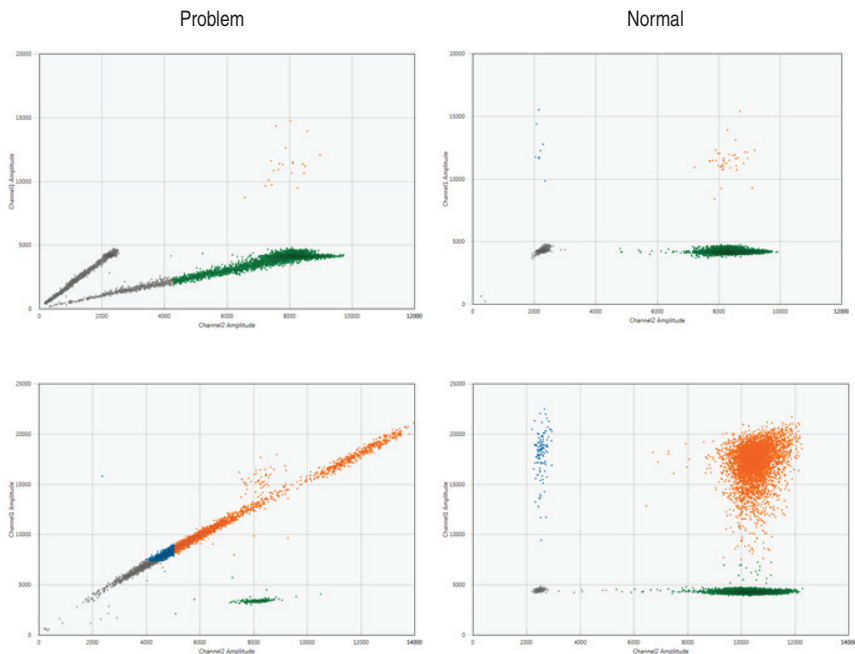
Problem   Normal

Figure 10: *Shredding – Problem vs. Normal*

Split clusters – droplet populations are not clearly separated, usually due to problems with the RT reaction (see Figure 11).

Resolution: If possible, adjust well thresholds; frequently the counts do not change significantly. Otherwise, exclude the well.
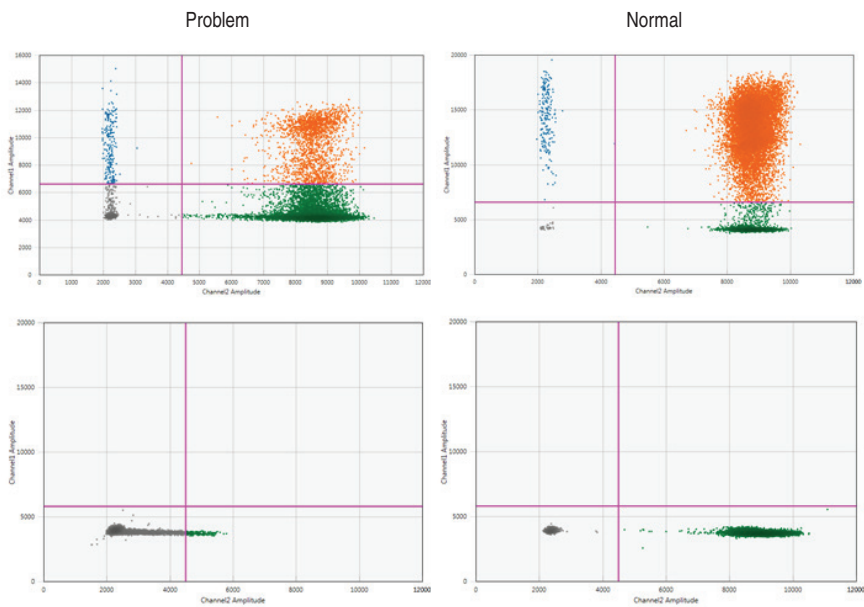
Problem   Normal

Figure 11: Split Clusters - Problem vs. Normal

Mirroring – droplet with two distinct sizes (see Figure 12).

Resolution: Exclude well from analysis.
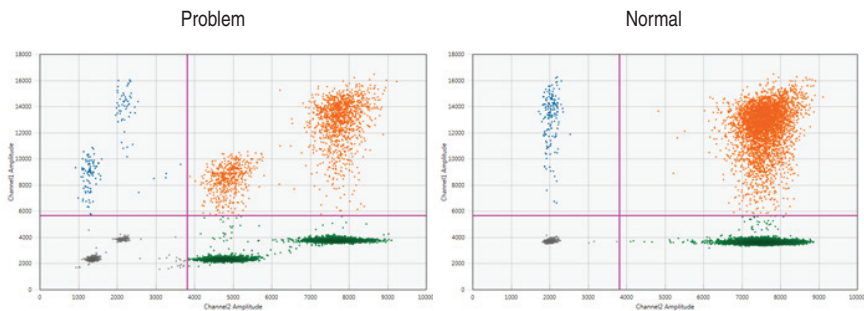
Problem   Normal

Figure 12: Mirroring – Problem vs. Normal

Thermocycler failure – temperature profile aberrations lead to poor PCR (see Figure 13).

Resolution: Exclude well from analysis. Check performance of thermocycler.
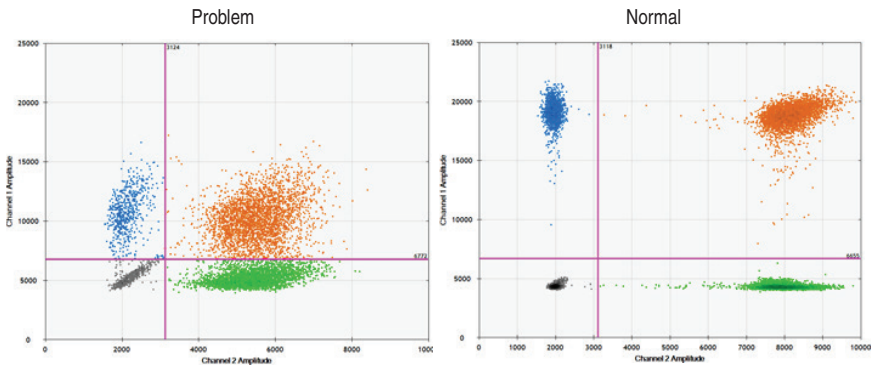
Figure 13: *Thermal Cycling – Problem vs. Normal*

Plate Seal Failure – plate seal failed, evaporation has led to variable droplet volumes (see Figure 14)

Resolution: Exclude well from analysis. Check performance of plate sealer.

Figure 14: *Plate Seal Failure – Problem vs. Normal*

ANALYTICAL PERFORMANCE CHARACTERISTICS

Limit of Blank (LoB)

This study followed CSLl EP17-A2, Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures. 36 samples from different non-leukemic human RNA specimens that were presumed negative for BCR-ABL were used. Each negative sample was tested in 2-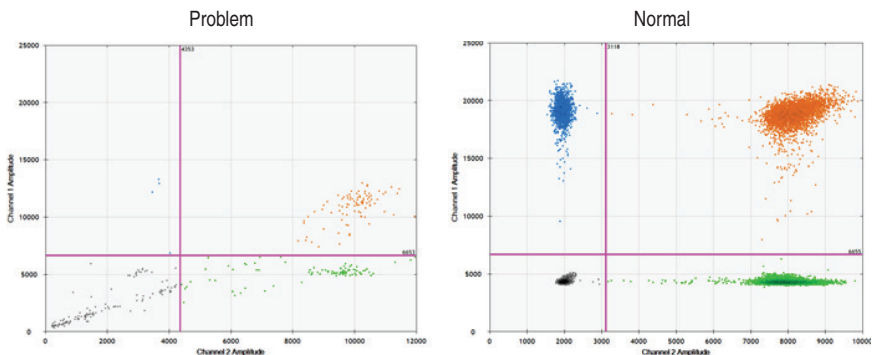well sets with 1 instrument, 1 operator, 2 lots and over 3 days, for a total of 72 independent measurements. All results were rank-ordered by total detected BCR-ABL copies. The 95% quantile was between sample ranks 68 and 69, both of which had 0 BCR-ABL copies. Thus the LoB at 95% is 0 BCR-ABL copies in 2 well.

Additionally, 48 no-template-control (NTC) samples were run in 2 wells with 1 operator, 1 instrument, 3 runs and 2 lots. The samples were rank-ordered by total BCR-ABL copies detected, with a 95% quantile between ranks 46 and 47. For both lots the LoB was 0 BCR-ABL copies.

Limit of Detection (LoD)

This study followed CLSI EP17-A2, Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures. Three positive BCR-ABL RNA patient sample pools were prepared to achieve approximately MR4.0 values. Pool 1 used a mix of 15 patients positive for the E13a2 and/or E14a2 variants. Pool 2 contained 5 patients positive for the E13a2 variant. Pool 3 contained 5 patients positive for the E14a2 variant. One negative BCR-ABL RNA sample pool was prepared and used in the dilution of the positive patient sample pools. Each positive pool was serially diluted with the negative pool. Resulting MR levels spanned from MR4.0 to MR6.0. Dilutions were tested in 20 replicates by 1 operator on 1 system with 4 independent lots, in 2 wells per test. Analyses by the Probit method yielded a LoD at 95% confidence level at MR4.7 (0.002%IS).

Additionally, a sample from the College of American Pathologists (CAP) proficiency testing program at CAP-assigned MR4.7 was analyzed in 60 single-wells (20 independent RT reactions, each with 3 ddPCR independent reactions, 1 operator, 1 lot, 1 instrument). In-silico combination of individual wells into 1-well test, 2-well tests and 4-well tests yielded same LoD at MR 4.4 in 1-well, LoD at MR4.7 in 2-wells and LoD at MR5.0 in 4-well tests. Single well and four well analysis of the same probit data yielded LoDs of MR4.4 and MR5.0, respectively for 1 and 4 well test.

	1-Well	2-Well	4-Well
%IS Value	0.004	0.002	0.001
MR	4.4	4.7	5.0

Limit of Quantitation (LoQ)

This study followed CLSI EP17-A2, Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures. The same set of samples and experiments as in the LOD study was used. Dilutions spanned from MR4.0 to MR6.0. The measured %CV was less than 76% down to MR4.7 level (0.002%IS). The Limit of Quantitation is MR 4.7 (0.002%IS) in 2 wells. Additionally, (0.0045%IS) or MR4.34 in single well, and 0.0008%IS or MR5.0 in quadruplicate well format.

Precision

The precision was verified as SD ≤0.25 for MR values ≤4.6. This study followed CSLI EP5-A3, Evaluation of Precision of Quantitative Measurement Procedures. The study was run on 3 days, with 2 instruments and 3 reagent lots, by 2 operators and 3 replicate runs. Samples at 8 different %IS levels – MR0.7, MR1.4, MR2.4, MR2.8, MR 3.3, MR 3.6, MR 4.1 and MR 4.6 – were tested. Table 7 summarizes the measured total imprecision in MR and %IS units.

Sample ID	N	Mean MR Level	MR Total Precision			Mean %IS Level	% BCR-ABL	Total Precision
			SD	%CV			SD	%CV
MR1	108	1.40	0.031	2.214		3.98	0.277	6.977
MR2	108	2.47	0.046	1.862		0.34	0.035	10.346
MR2.5	108	2.80	0.049	1.750		0.16	0.018	11.132
MR3	108	3.31	0.081	2.447		0.05	0.009	18.000
MR3.5	108	3.63	0.103	2.837		0.02	0.005	22.314
MR4	108	4.13	0.165	3.995		0.0079	0.003	36.709
Cell Line Control 1	108	0.73	0.017	2.329		18.73	0.714	3.815
Cell Line Control 1	108	4.66	0.251	5.386		0.0023	0.002	69.565

Table 7: Precision Summary

Linearity

The Linearity was demonstrated from at least MR 0.3 (50%IS) to MR 4.7 (0.002%IS). This study followed CSLI EP6-A2, Evaluation of the Linearity of Quantitative Measurement Procedures. 10 CML-positive human samples with the two transcripts e13 and e14 (5 each) were blended and diluted into a CML-negative human blood sample. The dilution levels spanned from MR0.3 (50 %IS) to MR4.7 (0.002%IS). For the e14 blend, additional RNA, extracted from a non-reactive positive 3t3 cell line, was added to normalize to 100ng/μL. The negative human sample was boosted by a negative HeLa cell line to increase ABL level. Testing was performed with two thermocyclers and two readers over four days by a single operator. Both e13 and e14 blends exhibited linearity with R^2 of 0.996 for both (see Figure 15 and 16). Measured slopes were 1.03 and 1.04, respectively. In addition, 2nd- and 3rd-order polynomial fits were assessed. From the 2nd-order fit, the absolute deviations from 1st-order linearity were ≤0.09 MR units for all levels.
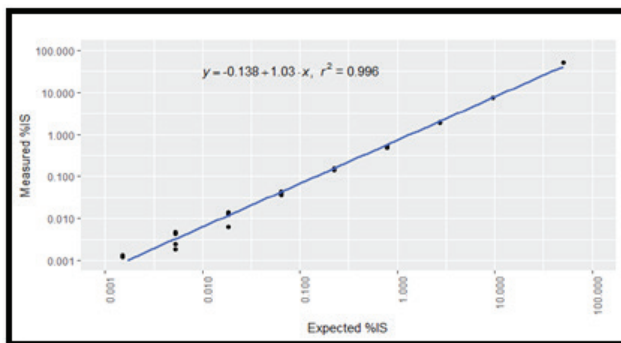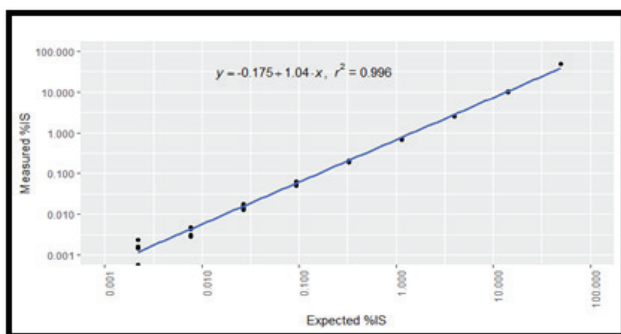
Figure 15: e13a2 linearity

Figure 16: e14a2 linearity

REPORTABLE RANGE

The reportable range spans from the upper limit of linearity at MR0.3 (50%IS) to the LoQ MR4.7 (0.002%IS) for 2 wells.

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Traceability

Traceability to the WHO Primary Reference Materials was verified with slope of 1.0 and intercept parameter of 0.0-0.1. Traceability to the 1st WHO International Genetic Reference Panel for quantitation of BCR-ABL translocation by RQ-PCR (NIBSC code: 09/138) was demonstrated by measuring the WHO Reference Panel with 3 lots of calibrator-checks and 5 lots of independent reagent lots and comparing the measured values to the values published in the Reference Panel's Instructions for Use (NIBSC, 2012). Each of the 4 WHO Reference Panel members was tested with 5 reagent lots in 4 replicates across 5 runs (1 run per lot).

The measured MR values for each level of the WHO Primary panel were adjusted by a common derived correction factor, CF=0.93. The measured MR values were compared to the published MR values through an additional regression analysis to determine slope and intercept values. The analysis showed excellent correlation with R^2 values of 1.0. The slope of the lines was between values 0.98 to 1.04 and the intercepts were calculated between -0.06 to 0.07.

Analytical Specificity

Analytical Specificity supports detection of BCR-ABL1 transcripts e13a2 and e14a2 exclusively, no cross-reactivity with transcripts e1a2 (p190) and e19a2 (p230) to at least MR5.0 level. Two (2) samples were prepared by blending in-vitro transcribed p190 or p230 with RNA extracted from normal human blood from 2 donors. Four (4) dilutions of each sample were prepared by varying the amount of negative RNA used to achieve levels between 0.005-30% ratios. All samples were tested on 1 instrument with 1 lot by 1 operator in at least 4 replicates. All measurements reported 0.000%IS for both e13a2 and e14a2. The kit is specific to at least MR5.0 level.

Interfering Substances

Endogenous

The effect of structurally unrelated substances such as endogenous blood components (proteins and lipids) and exogenous molecules such as anticoagulants on the performance of the QXDx™ BCR-ABL %IS Kit was determined by extracting RNA from whole blood specimens in the presence and absence of the potential interferents. The potential interferents were added to the whole blood samples prior to extraction. For each interferent, a control sample without interferent was tested.

For both the control and test samples, five replicate extractions were performed using the Promega Maxwell CSC RNA Blood Kit. Following extraction, the absorbance at 260nm was measured and replicates adjusted to 100ng/µL by adding DNA suspension buffer as needed. Each extracted sample was tested in duplicate for a total of ten tests per interferent. No interference was observed with any of the tested substances.

Endogenous substance testing

Test	N	%IS Within Run Precision %CV	Mean %IS	%IS 95% Confidence Interval	Does the Test Range Fall in the Control Precision Range?
Cholesterol	10	36.5	0.005%	0.002% - 0.007%	Yes - Pass
Conjugated Bilirubin	10	36.5	0.007%	0.004% - 0.01%	Yes - Pass
EDTA	10	10.7	0.094%	0.075% - 0.113%	Yes - Pass
Hemoglobin	10	17.9	0.045%	0.041% - 0.049%	Yes - Pass
Heparin	10	10.7	0.100%	0.088% - 0.112%	Yes - Pass
Triglycerides	10	36.5	0.004%	0.002% - 0.006%	Yes - Pass
Unconjugated Bilirubin	10	37	0.005%	0.002% - 0.007%	Yes - Pass

Exogenous substance testing

Test	Measured Mean MR	MR 95% Confidence Interval	MR Acceptable Range (Control CI ± 0.5 Log)	Result (Does the Test Sample MR CI fall within the Control Sample CI ± 0.5 Log?)
Guanidinium-containing lysis buffer	3.57	3.49 - 3.68	3 - 4.09	PASS
Ethanol	3.5	3.45 - 3.56	3 - 4.09	PASS
Phenol	3.53	3.46 - 3.63	3 - 4.09	PASS
Final Wash Buffer	3.53	3.47 - 3.59	3 - 4.1	PASS
Genomic DNA	3.58	3.53 - 3.63	3 - 4.1	PASS

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RNA Input

In an effort to determine RNA Input requirements, four (4) RNA samples were created by mixing RNA from one (1) negative donor blood with four (4) RNA pools, consisting of two (2) patients each, extracted from CML+ donor blood. Samples were diluted to 200ng/µL and varying volumes were tested targeting RNA inputs from 125ng to 1500ng. All samples yielded equivalent results down to 125ng/µL per test, however decreased precision occurs with lower inputs.

MRgroup	input (ng)	+/n tests	meanIS	ISCV	meanMR	MRSD
MR1	125.0	4/4	17.844%	0.84	0.75	0
MR1	250.0	4/4	18.056%	1.46	0.74	0.01
MR1	500.0	4/4	18.698%	0.91	0.73	0
MR1	1000.0	4/4	17.138%	0.72	0.77	0
MR1	1500.0	4/4	14.840%	1.47	0.83	0.01
MR2	125.0	4/4	0.879%	9.75	2.06	0.04
MR2	250.0	4/4	0.969%	6.44	2.01	0.03
MR2	500.0	4/4	1.016%	0.51	1.99	0
MR2	1000.0	4/4	0.972%	3.95	2.01	0.02
MR2	1500.0	4/4	0.966%	3.27	2.02	0.01
MR3	125.0	8/8	0.087%	24.76	3.06	0.12
MR3	250.0	8/8	0.085%	13.09	3.07	0.05
MR3	500.0	8/8	0.096%	9.06	3.02	0.04
MR3	1000.0	8/8	0.092%	12.58	3.04	0.06
MR3	1500.0	8/8	0.084%	16.09	3.08	0.07
MR4	125.0	8/8	0.017%	55.73	3.77	0.28
MR4	250.0	8/8	0.012%	27.54	3.92	0.12
MR4	500.0	8/8	0.018%	28.08	3.74	0.15
MR4	1000.0	8/8	0.016%	32.7	3.81	0.17
MR4	1500.0	8/8	0.014%	20.96	3.87	0.09

CLINICAL PERFORMANCE CHARACTERISTICS

Thirty-four (34) clinical specimens were collected and processed at an independent clinical laboratory in Europe. Standard lab procedures were followed for sample processing and analysis by the lab developed qPCR test, which was calibrated to the WHO IS standard. The QXDx BCR-ABL %IS test was performed in 4 wells. Analysis of the data was performed both in 4-well and in 6 2-well pairs (format is shown in Figure 17). Reproducibility assessed from the 6 2-well replicates was excellent, with maximum %CV of 46% (in %IS space) for the specimen near MR4.0, comparable with the values from the analytical reproducibility studies.

Concordance between the lab-developed clinical qPCR test and the QXDx BCR-ABL %IS test was also excellent, R^2 of 0.976 and slope of 1.03. Concordance in MMR (MR3 or 0.1%IS) status was excellent at 98%. A single discordant sample was off by 0.35 log.
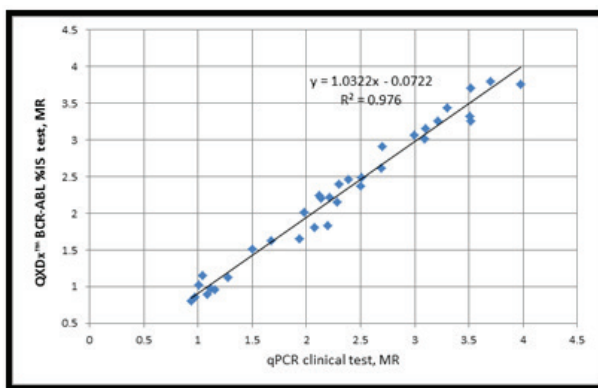

Figure 17: *Clinical Performance*

LIMITATIONS

The Test is designed only to detect the e13a2 and e14a2 fusion transcripts and not e1a2, e19a2 or other rare transcripts resulting from t(9;22).

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APPENDIX A: THERMAL CYCLERS

Required Specifications: Thermal Cyclers with Specifications equivalent to the following:

- ○ Accuracy: +/- 0.2°C
- ○ Uniformity: +/- 0.4°C well-to-well within 10 sec
- ○ Adjustable ramp capability with required ramp rate: up to 2°C/sec
- ○ Temperature range: 0-100°C

Thermal Cyclers: A study was performed to demonstrate equivalency of thermal cyclers instruments for use with QXDx BCR-ABL %IS Kit. The thermal cycler below meet the specifications requirement provided above. This study evaluates following 4 thermal cyclers:

Thermal Cycler	Catalog Number	Instrument Type	Ramp Setting	95% CI w/ ± 0.5 log of reference
Bio-Rad C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module	185-1197	Reference	2°C/s	
Bio-Rad C1000 Touch Thermal Cycler with 96-Well Fast Reaction Module	185-1196	Test	2°C/s	Pass
Life Technologies Veriti Dx 96-well Thermal Cycler, 0.2m	4452300	Test	2°C/s (93% Ramp Rate)	Pass
Eppendorf Mastercycler® Pro, with Control Panel, 230 V/50 – 60 Hz	6321000515	Test	20% Ramp Rate	Pass

For each samples, the allowable range was calculated as the mean MR value obtained on the reference thermal cycler (Bio-Rad C1000 Touch Thermal Cycler with 96 Deep well reaction module) plus or minus 0.5 log. All samples tested met 95% confidence interval (CI).

APPENDIX B: NAME CHANGE

June 9, 2017

Subject: ddPCR diagnostics product Brand Name change

Dear Valued Customer,

Since the initial launch of Bio-Rad Droplet Digital PCR platform, Bio-Rad has been using QX200 Droplet Digital PCR to identify its systems brand.

As part of Bio-Rad's transition of the technology from RUO tools to a diagnostic solution, we at Bio-Rad have decided to identify the diagnostic product line with a new product family brand called: **QXDx™**. Hence, we will be working to update any current and future diagnostics product with a brand name prefix **QXDx™**.

The product brand and resulting name change does not affect the form, fit, or function, or claims of the specific products.

Starting June 15th, 2017, commercial documentation will be updated in a phased in process over a period of time to reflect the QXDx™ brand to identify diagnostics product line for digital PCR.

During this phased in approach we will commonly refer to the product with dual names: The Registered name and a Proprietary/Common name until we are able to complete our registration of the common name as the registered name.

Sample documentation will identify the Proprietary and Registered name. Then quickly transition to using the Proprietary name throughout the rest of its uses. The products that will have a proprietary name is as follows:

Instrumentation System:

Catalog #	Registered Name	Proprietary/Common Name (New)
17002229	QX200 AutoDG™ Droplet Digital PCR Dx System	QXDx AutoDG ddPCR System
12001045	QX200 Droplet Reader, IVD	QXDx Droplet Reader
12001630	QX200 Automated Droplet Generator, IVD	QXDx Automated Droplet Generator
17000034	QX200 Droplet Digital PCR Dx System	QXDx ddPCR System
12001045	QX200 Droplet Reader, IVD	QXDx Droplet Reader
12001049	QX200 Droplet Generator, IVD	QXDx Droplet Generator

Reagents & Consumables:

Catalog #	Registered Name	Proprietary/Common Name (New)
17001378	ddPCR Dx Universal Kit for AutoDG	QXDx Universal Kit for AutoDG ddPCR System
12001922	ddPCR Dx AutoDG Consumable Pack	QXDx AutoDG Consumable Pack
12003031	ddPCR Dx AutoDG Supermix Pack	QXDx AutoDG Supermix Pack
12002526	ddPCR Dx Droplet Reader Oil Pack	QXDx Droplet Reader Oil Pack
17001379	ddPCR Dx Universal Kit for QX200 ddPCR System	QXDx Universal Kit for ddPCR System
12001921	ddPCR Dx Consumable Pack	QXDx Consumable Pack
12002544	ddPCR Dx Supermix Pack	QXDx Supermix Pack
12002526	ddPCR Dx Droplet Reader Oil Pack	QXDx Droplet Reader Oil Pack

Sincerely,

Marketing Manager,

Bio-Rad, Digital Biology Center

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