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"SensiQuant MASTER MIX"

SensiQuant e1a3 Master Mix

Detection and Measurement of the mRNA of BCR/ABL variants e1a3

(Transcript m-bcr)

REF SQT-041 For Research Use Only -25°C -15°C

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INTENDED USE

The **SensiQuant e1a3 MasterMix** product is a device for:

- the **detection** of BCR/ABL transcripts, t(9;22), Philadelphia chromosome, variant e1a3 (m-bcr), in total RNA extracted from peripheral blood samples and/or bone marrow samples;
- the **quantification** of BCR/ABL transcripts e1a3, normalized with transcript number of the control gene ABL, in total RNA extracted from peripheral blood samples and/or bone marrow samples.

The product **SensiQuant e1a3 MasterMix** has been designed for the detection and/or monitoring of BCR/ABL transcript, variant e1a3 in Chronic Myeloid Leukaemia (CML), Acute Myeloid Leukaemia (AML) and Acute Lymphoblastic Leukaemia (ALL) cases. The product could be used as support to evaluate the complete responses and to monitor the efficiency of therapies and the follow-up of Minimal Residual Disease (MRD).

The **SensiQuant e1a3 MasterMix** kit has been designed according to "Europe Against Cancer" studies (Gabert et al., Leukemia 2003) and is compliant with the updated international recommendations (Branford et al., Leukemia 2006; Hugues et al., Blood 2006).

To perform a QUANTITATIVE analysis, the SensiQuant e1a3 MasterMix kit must be exclusively used in association with the SensiQuant e1a3 Standard product.

To perform a QUALITATIVE analysis, the SensiQuant e1a3 MasterMix kit may be used in association with one of the four dilution points of the SensiQuant e1a3 Standard kit, as positive control.

The product should be used following the instructions given this manual, in combination with validated instruments and reagents. Any off-label use of this product, and/or modification of the components will void Bioclarma responsibility.

PRODUCT DESCRIPTION

The **SensiQuant e1a3 MasterMix** product is based on One-Step RT-PCR technology, which allows highly reproducible first-strand cDNA synthesis and real-time PCR within one tube continuously. Based on this, the **SensiQuant e1a3 MasterMix** kit makes possible maximum RT-PCR efficiency, sensitivity and specificity, while minimizing time and cost of the analysis.

The **SensiQuant e1a3 MasterMix** product provides:

- ***SensiQuant ABL Primers & Probe MIx:*** a ready to use 20X reaction mixture containing primers and probe specific for the amplification of the housekeeping gene ABL
- ***SensiQuant e1a3 Primers & Probe Mix:*** a ready to use 20X reaction mixture containing primers and probe specific for the amplification of the translocated gene BCR/ABL e1a3
- ***SensiQuant ABL Master Mix:*** a ready to use reaction mixture for real time amplification of the housekeeping gene ABL. The combination contains: reaction buffer, magnesium ions, dNTPs, Taq polymerase and water.
- ***SensiQuant e1a3 Master Mix:*** a ready to use reaction mixture for real time amplification of the translocated gene BCR/ABL e1a3. The combination contains: reaction buffer, magnesium ions, dNTPs, Taq polymerase and water.
- ***SensiQuant RT***: optimized and ready to use solution of MMLV Reverse Transcriptase. It must be added to amplification mixtures before each analysis. This reagent has been optimized to work with One step protocol, in which the first-strand cDNA synthesis and subsequent real-time PCR are done in a single tube

The assay provides two reactions in microplate with programmable heater equipped with optical fluorescence detection system (thermal cycler for real time):

- retro-transcription and real-time amplification reaction for the target gene BCR/ABL e1a3;
- retro-transcription and real-time amplification reaction for the housekeeping gene ABL (internal control and reference gene).

The specific probe, labelled with FAM for e1a3 and with HEX for ABL, is active when binds to the specific amplicon. Fluorescence increases with the increasing amount of amplicon and is measured and registered by the instrument.

The processing of the data obtained determines the presence and the titre of BCR/ABL e1a3 cDNA in the starting sample, compared with the reference ABL cDNA.

Performance evaluation was carried out on:

- Bio-Rad Instruments: CFX96, CFX Connect
- Applied Biosystems Instrument 7900.

Note: reagents *"SensiQuant e1a3 Primers & Probe Mix" and "SensiQuant ABL Primers & Probe Mix" could be used for the detection and quantitative measurement of the BCR/ABL e1a3 transcript and ABL transcript by* "Droplet Digital PCR" technology (ddPCR™ - Bio-Rad). To perform this kind of analysis, please refer to the Bio-Rad instruction manual.

TECHNOLOGICAL SPECIFICATION

The product **SensiQuant e1a3 MasterMix** must be used in combination with the **SensiQuant e1a3 Standard** kit.

The product provides 24 duplicate determinations for the mRNA of e1a3 (48 determinations in total) and 24 duplicate determinations for the mRNA of ABL (48 determinations in total), including standards and controls. The number of patients analysable in one session is 19.

The **test sensitivity** allows revealing the presence of about 5 DNA molecules target in 5 ul of sample added to the amplification reaction.

The **test linear measuring range** enables determination of a titre ranging between 1,000,000 and 10 target copies.

REAGENTS PROVIDED

	REAGENT	QUANTITY	COMPOSITION
	SensiQuant ABL Primers & Probe Mix	2 x 24 µl	ready to use 20X mixture of primers and probe specific for ABL
	SensiQuant e1a3 Primers & Probe Mix	2 x 24 µl	ready to use 20X mixture of primers and probe specific for BCR/ABL, e1a3 variants
	SensiQuant ABL Master Mix	2 x 350 µl	ready to use reaction mixture for ABL
	SensiQuant e1a3 Master Mix	2 x 350 µl	ready to use reaction mixture for BCR/ABL, e1a3 variants
	SensiQuant RT	1 x 30 µl	stabilized and ready to use solution of MMLV Reverse Trascriptase
	Ultrapure water	1 x 500 µl	ultrapure water for molecular biology

ADDITIONAL EQUIPMENT/REAGENT REQUIRED

- Laminar airflow hood.
- Disposable latex powder-free gloves or similar material.
- Vortex mixer.
- Microcentrifuge (12.000/14.000 RPM).
- Sterile micropipettes and tips with aerosol filter or positive displacement (0,5-10 µl, 2-20 µl, 5-50 µl, 50-200 µl, 200-1000 µl).
- RNase- and DNase free microplates adhesive seals.
- Real-time PCR instrumentation.

ACCESSORY PRODUCTS

The **known-quantity DNA standards are not** included in this kit. To perform these analytical steps the following accessory product, manufactured by Bioclarma, is recommended:

- **SensiQuant e1a3 Standard (cod. STD-041)**: known-quantity plasmid DNA to obtain the standard curves

WARNINGS AND PRECAUTIONS

This product is exclusively for research use.
Reagents and instructions supplied in this kit have been validated for optimal performance. Further dilution of the reagents or alteration of incubation temperatures may result in erroneous or discordant data.
All kit reagents are specifically formulated to be used in combination with **SensiQuant e1a3 Standard (code STD-041)** Bioclarma product. We advise against any mixing with other manufacturer's product to ensure optimal performance of the test.

Specific warnings for handling:

Molecular biology procedures, such as extraction, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results.
In use extreme caution to prevent:

- RNAse/DNAse contaminations, that might cause degradation of the template mRNA and the generated cDNA;
- mRNA or PCR carry-over contamination resulting in false positive signal.

We therefore **recommend** the following:

- Dispose separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce amplification products in the area designed for extraction/preparation of amplification reactions;
- Use lab coats, gloves and tools which are exclusively employed in the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification/detection of amplification products to the area designed for the extraction/preparation of the amplification reactions;
- Handle the samples under a laminar flow hood. The samples must be exclusively employed for this type of analysis;
- Avoid simultaneous opening of tubes containing different samples;
- Use fresh aerosol-resistant pipette tips for all pipetting steps and pipettes with positive displacement system. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA. Pipettes used to handle samples must be exclusively employed for this specific purpose;
- Handle the reagents under a laminar flow hood. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes employed to handle the reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips

employed must be sterile, free from DNases and RNases, free from DNA and RNA;
- Handle amplification products in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be employed exclusively for this specific purpose.

Warnings and general precautions:

Human tissues must be handled as if capable of transmitting infections and disposed of with proper precautions and in compliance with OSHA and/or CAP (or EU equivalent) guidelines.

- Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with biological samples must be treated with 3% sodium hypochlorite for at least 30 minutes or autoclaved at 121°C for one hour before disposal;
- Handle and dispose of all reagents and all assay materials as if they were potentially infective. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be treated and disposed of in compliance with the appropriate safety standards. Disposable combustible materials must be incinerated. Liquid waste containing acids or bases must be neutralized before disposal;
- Wear suitable protective clothing and gloves and protect eyes / face. Refer to the Material Safety Data Sheet (MSDS) for additional information.
- Never pipette solutions by mouth and avoid contact with skin and mucous membranes.
- Do not eat, drink, smoke or apply cosmetic products in the work areas.
- Wash hands carefully after handling samples and reagents.
- Dispose leftover reagents and waste in compliance with regulations in force.
- Read all the instructions provided with the product before running the assay.
- Follow the instructions provided with the product while running the assay.
- Do not use the product after the expiry date.
- Only use the reagents provided in the product and those recommended by the manufacturer.
- Do not mix reagents from different batches.
- Do not use reagents from other manufacturers' products.

Warnings and precautions specific to components:

The **SensiQuant e1a3 MasterMix** product carries the following precautionary statements (P):
P261. Avoid breathing dust/fume/gas/mist/vapours/spray.
P262. Do not get in eyes, on skin, or on clothing

Storage:

Store **SensiQuant RT, SensiQuant e1a3 Master Mix, SensiQuant ABL Master Mix, SensiQuant e1a3 Primers & Probe Mix and SensiQuant ABL Primers & Probe Mix components at -15°C to -25°C in a constant-temperature freezer.** Only one freezing and thawing cycle is allowed. Avoid further cycles of freezing and thawing, which can reduce the performance of the product.
Minimize exposure to light of the various components of the kit.

Store all kit components in original containers.

Mix gently by inversion and briefly centrifuge the tubes before opening.

These storage conditions apply to open components and those not yet open. The components stored under conditions other than those specified on the label may not provide correct performance negatively affecting the results of the tests.

Kit stability:

The kit will remain stable until the expiration date printed on the label under correct storage conditions. The product will maintain performance through the expiry date printed on the label.

SAMPLES AND CONTROLS

Samples:

This product is designed to be used with RNA extracted from:

- peripheral blood collected in EDTA or citrate,
- bone marrow collected in EDTA or citrate,
- lympho-monocyte or lymphocyte suspension.

To the RNA preparation, the following protocols/reagents are recommended:

- Manual Procedure (TRIzol Reagent – Invitrogen, Purezol – Biorad and similar);
- RNA extraction kit with columns (RNeasy Mini Kit – Qiagen);
- Semi-automatic extraction Procedure (Maxwell 16 – Promega).

Peripheral blood and Bone marrow collected in EDTA or citrate.
Peripheral blood samples and bone marrow samples must be collected according to laboratory guidelines, transported at +2° / +8°C and stored at +2° / +8°C for a maximum of four hours. When the starting material is peripheral blood it is advisable to separate lympho-monocytes on Ficoll™ according to laboratory guidelines. The optimum quantity of leukocytes for total RNA extraction is approximately 10,000,000 cells. Do not freeze peripheral blood in order to prevent degradation of RNA.

Lympho-monocyte or lymphocyte suspension.
The lympho-monocyte or lymphocyte suspension must be prepared accordingly to the laboratory guidelines, resuspended in sterile isotonic buffer and stored at +2° / +8°C for a maximum of four hours. The optimum quantity of lympho-monocytes and leukocytes for total RNA extraction is approximately 10.000.000 cells. Do not freeze cell suspension in order to prevent degradation of RNA.

Interfering substances:
The total RNA sample must not contain heparin, haemoglobin or Ficoll™ to prevent inhibitory effects on amplification reaction. There are no data available concerning possible inhibitory effects of antibiotics, antiviral drugs, chemotherapeutic drugs or immunosuppressant.

Preparation of RNA from patient specimens must be carried out with an approved procedure (TriZol, Invitrogen; PureZol, Bio-Rad). **The quality of the assay depends to a large extent on the quality of starting RNA.** We recommend verifying the quality of purified RNA by agarose gel electrophoresis or by Agilent Bioanalyzer prior to downstream analysis.

Sample concentration:

For each sample, the test requires:

- a retro-transcription/amplification reaction for the BCR/ABL e1a3 transcript (**in duplicate**);
- a retro-transcription/amplification reaction for ABL transcript (**in duplicate**).

The amount of RNA in reaction is:

1.5 µg of total RNA per well: **the complete analysis of a clinical specimen requires 6 µg of total RNA**. Dilute each RNA sample at a concentration of 0.3 µg/µl, so that 5 µl contains 1.5 µg of RNA.

OR

0.5 µg of total RNA per well: **the complete analysis of a clinical specimen requires 2 µg of total RNA**. Dilute each RNA sample at a concentration of 0.1 µg/µl, so that 5 µl contains 0.5 µg of RNA.

OR

0.25 µg of total RNA per well: **the complete analysis of a clinical specimen requires 1 µg of total RNA**. Dilute each RNA sample at a concentration of 0.05 µg/µl, so that 5 µl contains 0.25 µg of RNA.

It is recommended to prepare several sample aliquots in order to avoid cycles of freezing and thawing, which can reduce the quality of RNA.

Amplification controls:
It is absolutely mandatory to validate each amplification session with **negative and positive control reactions**.

As negative control use sterile nuclease free water, **Ultrapure water**, NEUTRAL cap tube, supplied with the product **SensiQuant e1a3 MasterMix**.

As positive control use the **SensiQuant e1a3 Standard product** manufactured by Bioclarma.

INSTRUCTIONS FOR USE

Before starting the session, it is important to do the following:

- referring to the instrument documentation, verify that the thermal cycler for Real Time is able to excite the fluorophores used and to measure the emission;
- referring to the instrument documentation, set the "detector" (fluorescence measured) and the type of reaction (sample, negative control, known quantity standard DNA) for each well used in the microplate. Add this information to the **Work Sheet** enclosed at the end of this document. We recommend to carefully follow the **Work Sheet** while dispensing reaction mixture and samples into the wells.

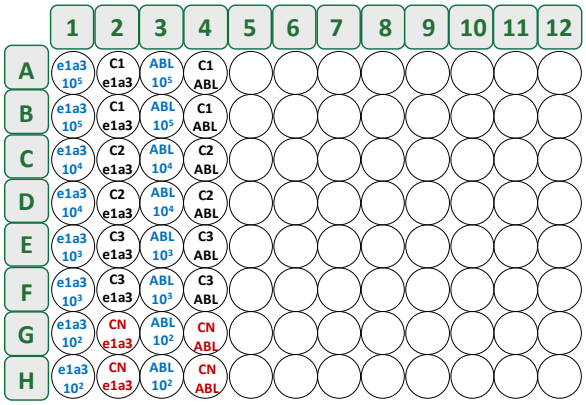
  Note: to titre target cDNA in the samples, set up two standard curves, one for e1a3 and one for ABL with the **SensiQuant e1a3 Standard** (10^5-10^4-10^3-10^2 copies). Each point of the curve is made at least as a technical duplicate.

  The point with 10 copies/reaction can be used as qualitative control to verify the LOD of the reaction

  OR

  Can be included in the standard curve e1a3 to obtain a quantitation curve with five points for the translocate BCR/ABL e1a3. If the 5 points curve is used, the total number of analysable samples in one session is **18**.

  The following is an example of how the analysis of 3 samples can be organized.



e1a3 10^5- e1a3 10^2: Standard for BCR/ABL e1a3; ABL 10^5-ABL 10^2: Standard for ABL; NC: Negative Control; C1-C3: Samples.

- referring to the instrument documentation set the cycling condition described below, indicating 50 cycles and a reaction volume of **20 µl**.

Bio-Rad CFX96 and CFX Connect Instruments

Amplification thermal cycle		
Temperature	**Timing**	**Phase**
50°C	10 min.	**cDNA synthesis**
95°C	5 min.	**RT enzyme inactivation**
95°C	10 sec.	**PCR cycling and detection (50 cycles)**
60°C (*)	30 sec.	

(*) Fluorescence Signal Acquisition

Fluorescence:

- FAM for the detection of BCR/ABL e1a3 transcript
- HEX for the detection of ABL transcript.

Analysis mode: single threshold with the option "baseline subtracted curve fit". Set the threshold value of 100 for all fluorescence channels.

Set the amplification diagram with the following colour code:

	Fluorescence	Target	Colour
	FAM	**BCR/ABL e1a3 probe**	**GREEN**
	HEX	**ABL probe**	**BLUE**

Applied Biosystems Instruments 7900

Amplification thermal cycle		
Temperature	**Timing**	**Phase**
50°C	10 min.	**cDNA synthesis**
95°C	5 min.	**RT enzyme inactivation**
95°C	20 sec.	**PCR cycling and detection (50 cycles)**
60°C (*)	1 min.	

(*) Fluorescence Signal Acquisition

Fluorescence:

- FAM for the detection of BCR/ABL e1a3 transcript
- HEX for the detection of ABL transcript.

Set the correct fluorescence channels, referring to instrument manual, and set the amplification diagram with the following colour code:

	Fluorescence	Target	Colour
	FAM	**BCR/ABL e1a3 probe**	**GREEN**
	HEX	**ABL probe**	**BLUE**

Passive reference: **ROX**

Analysis mode: standard curve with absolute quantification, standard protocol.
Set threshold set at 0.15 and the calculation range for the baseline from cycle 3 to cycle 15.

Amplification Set-Up:
Before starting the session, it is important to do the following:

- remove and thaw the test tubes containing the samples to be analysed. Mix gently and centrifuge the tubes to bring the contents to the bottom and keep in ice;
- remove and thaw the necessary **SensiQuant e1a3 Master Mix (GREEN cap tube), SensiQuant ABL Master Mix (BLUE cap tube), SensiQuant e1a3 Primers & Probe mix (YELLOW cap tube) and SensiQuant ABL Primers & Probe mix (WHITE cap tube),** remembering that each tube is enough for **24 reactions**. Mix gently by inversion and briefly centrifuge the tubes to bring the contents to the bottom and keep on ice;
- remove and place on ice the **SensiQuant RT (PURPLE cap tube)** tube containing the stabilized retro-transcriptase enzyme;
- remove and thaw the necessary **SensiQuant e1a3 STANDARD** tubes for the BCR/ABL e1a3 transcript and ABL standard curves. Mix gently and centrifuge for 5 seconds the tubes to bring the contents to the bottom and keep on ice;

1) Prepare the following "**reaction mixture e1a3**" containing **SensiQuant e1a3 Master Mix, SensiQuant e1a3 Primers & Probe mix** and **SensiQuant RT,** according to the number of samples being processed:

Reaction mixture e1a3	Cap tube	Volume for 1 reaction (uL)
SensiQuant e1a3 Master mix	**GREEN**	13,76
SensiQuant e1a3 Primers & Probe Mix	**YELLOW**	1
SensiQuant RT	**PURPLE**	0,24
	Total volume	**15**

Mix by inversion the tube and avoiding the creation of foam. Briefly centrifuge (max 5 sec) to bring the contents to the bottom and keep on ice.

2) Prepare the following "**reaction mixture ABL**" containing **SensiQuant ABL Master Mix, SensiQuant ABL Primers & Probe mix** and **SensiQuant RT** according to number of samples being processed:

reaction mixture ABL	Cap tube	Volume for 1 reaction (uL)
SensiQuant ABL Master mix	**BLUE**	13,76
SensiQuant ABL Primers & Probe Mix	**WHITE**	1
SensiQuant RT	**PURPLE**	0,24
	Total Volume	**15**

Mix by inversion the tube and avoiding the creation of foam. Briefly centrifuge (max 5 sec) to bring the contents to the bottom and keep on ice.

3) Dispense 15 µl of the "**reaction mixture e1a3**" on the bottom of the wells of the amplification microplate, as established on the **work sheet**.

4) Dispense 15 µl of the "**reaction mixture ABL**" on the bottom of the wells of the amplification microplate, as established on the **work sheet**.

5) Gently deposit 5 µl of **Ultrapure Water** (NEUTRAL cap tube) as amplification negative control in each of two e1a3 and ABL wells of the amplification microplate, as established on the **work sheet**.

6) Gently deposit 5 µl (corresponding to 1.5 µg or 0.5 µg or 0.25 ug) of the first **RNA** sample in each of two e1a3 and ABL wells. Similarly proceed with the remaining RNA samples, as established on the **work sheet**.

7) Gently deposit 5 µl of **SensiQuant e1a3 Standard** 10^2 copies in the two e1a3 wells and in the two ABL wells of the amplification microplate as established on the **work sheet.** Similarly proceed for the other **SensiQuant e1a3 Standard** (10^3, 10^4, 10^5 copies).

8) Carefully seal the amplification microplate using the amplification adhesive sheet and briefly centrifuge to bring the contents to the bottom of the wells.

9) Place the microplate in thermal cycler and run the defined program.

Test Interpretation:

- General validation:

The fluorescence values of the four standards obtained with the specific BCR/ABL e1a3 and ABL probes are used to calculate the **Standard Curves** of the amplification session and to validate the amplification and detection reactions.

For each Standard Curve (ABL and BCR/ABL e1a3), the linear regression analysis ($y = ax + b$) is applied. The a value correspond to the **slope** of the line and the b value correspond to the y-**intercept**. From the data is extrapolated the **Linear Correlation Coefficient** (R2) that defines the limits of acceptability for the standard curves, as described in the following table:

Linear correlation coefficient (R2)	Acceptability range	Amplification/ detection
BCR/ABL e1a3	0,990 ≤ R2 ≤ 1,000	VALID
ABL Standard Curve	0,990 ≤ R2 ≤ 1,000	VALID

If the R2 value is not included within the limits indicated, a problem have occurred during the amplification or detection phase (incorrect preparation of the reaction mixture, incorrect dispensing of reaction mixture or standards, degradation of probe or standard, incorrect standard position setting, incorrect thermal cycle setting) which may cause incorrect results.
The session is invalid and must be repeated. See the Troubleshooting Guide at the end of the document to identify a solution.

Since standards are scalar dilution with a factor 10, the theoretical slope of the curve is **-3,3**. Van der Velden et al., Leukemia 2003, said that a slope between -3,0 and -3,9 is acceptable if R2 is > 0,95. However, a R2 value greater than 0,99 is desirable to have precise results (Branford et al., Leukemia 2006).

The fluorescence values obtained with the specific BCR/ABL e1a3 and ABL probes in the amplification reactions of **Negative control** are used to validate amplification and detection reactions, as shown in the following table:

Threshold Cycle of Negative Control		Acceptability range	Amplification/ Detection
FAM, e1a3 probe	Undeterminated	NEGATIVE	CORRECT
HEX, ABL probe	Undeterminated	NEGATIVE	CORRECT

If the result of the Negative control amplification reaction is different from "Ct undetermined", it means that target DNA has been detected in the amplification reaction. Problems have occurred during the amplification phase (contamination), which may cause incorrect results and false positives.
The session is invalid and must be repeated. See the Troubleshooting Guide at the end of the document to identify a solution.

- Identification of BCR/ABL e1a3:

The **values of fluorescence** emitted by the specific probes for e1a3 and ABL in the amplification reactions of each sample and the **Threshold value** of fluorescence are used to detect the presence of target DNA through the determination of the **Threshold Cycle (Ct)**.

Note: Verify by using the instruments software tools that the Ct is determined by a rapid and regular increase of fluorescence value and not by isolated peaks or background signal increases.

When this kit is used to search for BCR/ABL e1a3, the results for each sample are used as described in the table below:

Sample Threshold Cycle		Sample suitability	Assay result	BCR/ABL e1a3 mRNA
FAM	HEX			
e1a3	ABL			
Ct Undetermined	Copies < 10.000 or undetermined	NOT SUITABLE	INVALID	/
	Copies > 10.000	SUITABLE	VALID, NEGATIVE	NOT DETECTED
Ct Determined	Copies < 10.000 or	SUITABLE	VALID, POSITIVE	PRESENT
	Copies > 10.000	SUITABLE	VALID, POSITIVE	PRESENT

If the sample amplification reaction indicates "Ct Undetermined" for e1a3 and "Copies < 10.000 or Ct Undetermined" for ABL, the target gene ABL was not detected efficiently. Problems have occurred during the amplification phase (inefficient or invalid amplification) or in the reverse transcription phase (inefficient or invalid reverse transcription) or in the extraction phase (absence of DNA or presence of inhibitors), which may cause incorrect results and false negatives.
The sample is not suitable, the assay is invalid and must be repeated beginning with extraction of a new sample

If the sample amplification reaction indicates "Ct Undetermined" for the e1a3 and "Copies > 10.000**"** for the ABL, the target gene ABL was detected efficiently, while the target gene BCR/ABL e1a3 was not detected. However, it is not possible to exclude the presence of e1a3 cDNA at a titre lower than the detection limit of

the product. In this case the result would be a false negative.
The results obtained with this assay must be interpreted in consideration of all the clinical data and the other laboratory tests done on the patient.

Please Note: If e1a3 is detected while the ABL amplification shows "Copies < 10.000 or Ct Undetermined", this means that the sample is still suitable in terms of quality and the positive result of the assay is valid. In this case, however, it is not possible to use the data for the quantitative analysis of the results (see following pages).

- Quantitative analysis of BCR/ABL e1a3:

The Ct values obtained with e1a3 specific probe and the e1a3 Standard Curve are used to calculate the **Quantity** of target DNA present in the amplification reactions of each sample.

The **SensiQuant e1a3 MasterMix** is able to measure from 1,000,000 to 10 copies of e1a3 cDNA *per* amplification reaction, as shown in the following table

Result of the sample (FAM e1a3)	BCR/ABL e1a3 cDNA per reaction
Quantity > 1 x 10^6	GREATER THAN 1.000.000 COPIES
10 ≤ Quantity ≤ 1 x 10^6	= quantity
Quantity < 10	FEWER THAN 10 COPIES

The Ct values obtained with ABL specific probe and the ABL Standard Curve are used to calculate the **Quantity** of target DNA present in the amplification reactions of each sample.

This kit is able to measure from 1,000,000 to 10 copies for ABL cDNA per amplification reaction. However, for the **quantitative analysis**, the useful interval is from 1.000.000 to 10.000 copies.

When this product is used to monitor the rate of e1a3, the **Quantity** results are used to calculate the total number of e1a3 cDNA copies (**sum of the copies in each replicate**) normalized to the total number of ABL cDNA copies (**sum of the copies in each replicate**), according to this formula:

$$\text{e1a3\%} = \frac{\text{Quantity of BCR/ABL e1a3 (sum of copies)}}{\text{Quantity of ABL (sum of copies)}} \times 100$$

Before calculating e1a3 % it is necessary to analyse the data obtained in the two repeat samples. The following table shows the different cases that might occur in one amplification session and the recommended approach to assess the data:

Sample	sample suitability	cDNA of e1a3	Quantity of e1a3 cDNA	Quantity of ABL cDNA
1st repeat	suitable	PRESENT	Sum quantity of e1a3	Sum quantity of ABL
2nd repeat	suitable	PRESENT		
1st repeat	suitable	NOT DETECTED	0	Quantity of ABL cDNA
2nd repeat	suitable	NOT DETECTED		
1st repeat	suitable	PRESENT (< 10 copies)	Sum quantity of e1a3	Quantity of ABL cDNA
2nd repeat	suitable	NOT DETECTED		
1st repeat	suitable	PRESENT (> 10 copies)	Sum quantity of e1a3	Quantity of ABL cDNA
2nd repeat	suitable	NOT DETECTED		
1st repeat	not suitable	PRESENT/NOT DETECTED	Retest the sample	
2nd repeat	suitable	PRESENT/NOT DETECTED		
1st repeat	not suitable	PRESENT/NOT DETECTED	Retest the sample	
2nd repeat	not suitable	PRESENT/NOT DETECTED		

Note: the results of this test should be interpreted in light of all clinical data and other laboratory tests done on the patient.

PROCEDURE LIMITATIONS

With this product:

- Only use total RNA extracted from peripheral and/or medullar blood samples, collected in EDTA or citrate, and lympho-monocytes and/or leukocytes cell suspensions.
- Do not use total RNA extracted from heparinised samples: heparin inhibits the reverse transcription and amplification reactions of nucleic acids and causes invalid results.
- Do not use total RNA contaminated with haemoglobin or Ficoll™: these substances may inhibit the reverse transcription reaction and amplification reactions of the nucleic acids and cause invalid results.

There are no data available concerning inhibition caused by antibiotics, antiviral drugs, chemotherapeutic drugs or immunosuppressant.

The results obtained with this product are subject to the correct collection, transport, storage and preparation of samples. To avoid incorrect results, take particular care during these pre-analytical phases.

Owing to its high analytical sensitivity, the nucleic acids amplification assay used in this product is subject to contamination from clinical samples that are positive for e1a3, from positive controls and from the amplification reaction products themselves. Contamination leads to false positive results. The product has been designed in such a way as to reduce contamination; nevertheless, this phenomenon can only be prevented by following good laboratory practices and by complying scrupulously with the instructions provided in this manual.

This product requires:

- personnel trained in the processing of potentially infective biological samples and chemical preparations classified as dangerous to prevent accidents with potentially serious consequences for the user and other people.

- the use of work clothes and premises that are suitable for the processing of potentially infective biological samples and chemical preparations classified as dangerous to prevent accidents with potentially serious consequences for the user and other people.

- personnel trained in molecular biology techniques, such as extraction, amplification and detection of nucleic acids, to avoid incorrect results.

- the presence of separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products to prevent false positive results.

- the use of special clothing and instruments for extraction/preparation of amplification reactions and for amplification/detection of amplification products to avoid false positive results.

A negative result obtained with this product suggests that the e1a3 cDNA has not been detected in the product of the reverse transcription obtained from the RNA extracted from the sample, but it may also contain the e1a3 cDNA at a lower titre than the detection limit for the product (detection limit for the product, see paragraph on Performance Characteristics on page 11); in this case the result would be a false negative.

As in the case of any diagnostic device:

- the results obtained with this product must be interpreted in consideration of all the clinical data and other laboratory tests done on the patient.
- there is a residual risk of obtaining invalid results, false positives and false negatives with this product. This residual risk cannot be eliminated or reduced any further. In particular situations such as emergency diagnoses, this residual risk can contribute to incorrect decisions with potentially grave consequences for the patient.

TROUBLESHOOTING GUIDE

Negative result for the control gene (ABL) and for BCR/ABL in all the samples, Standard OK	
Probable cause(s)	**Suggested Corrective Action(s)**
• Poor RNA sample quality	•Always check the RNA quality and concentration before starting
• RT step failure	• Check pipetting scheme and the set-up of the reaction
• Pipetting error or reagent exclusion	• Check pipetting scheme and the set-up of the reaction
• Inhibitory effects of the sample material, caused by insufficient purification	• Repeat RNA purification
Negative result for the control gene (ABL) in the samples, Standard OK	
Probable cause(s)	**Suggested Corrective Action(s)**
• Poor RNA sample quality	•Always check the RNA quality and concentration before starting
• RT step failure	• Check pipetting scheme and the set-up of the reaction
• Pipetting error or reagent exclusion	• Check pipetting scheme and the set-up of the reaction
Negative Standard Signal	
Probable cause(s)	**Suggested Corrective Action(s)**
• Pipetting error	• Check pipetting scheme and the set-up of the reaction
	• Repeat PCR run
• Inappropriate component storage	• Aliquot reagent
• Standard degradation	• Store the SensiQuant MasterMix kit at -15°C to -25°C protected from light
	• Avoid repeated freezing and thawing
	• Use new aliquots of SensiQuant Standard
Non linear standard Curve (R^2 < 0,99)	
Probable cause(s)	**Suggested Corrective Action(s)**
• Pipetting error	• Check pipetting scheme and the set-up of the reaction
	• Repeat PCR run
• Cross contamination or Aspecific Contamination	Replace all critical reagents
	• Avoid repeated freezing and thawing
	Repeat the experiment with new aliquots of all reagents
	Clean surfaces and instruments, wash lab coat, replace test tube s and tips in use.
• Partial standard degradation	• Store the SensiQuant MasterMix kit at -15°C to -25°C protected from light
	• Avoid repeated freezing and thawing
Passive Reference Incorrect Setting	• Check passive reference setting. Set ROX

Negative control (H2O) is positive	
Probable cause(s)	**Suggested Corrective Action(s)**
• Cross contamination	• Replace all critical reagents
	• Repeat the experiment with new aliquots of all reagents
	• Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination
	• Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use
• Microplate badly sealed	• Take care when sealing the microplate
• Probe degradation	• Store the SensiQuant MasterMix kit at -15°C to -25°C protected from light
No signal, even in standard controls	
Probable cause(s)	**Suggested Corrective Action(s)**
• Incorrect detection channel has been chosen	• Set correct channel
• Pipetting error or reagent exclusion	• Check pipetting scheme and the set-up of the reaction
	• Repeat PCR run
• Probe degradation	• Use new aliquots of SensiQuant MasterMix
• Standard degradation	• Use new aliquots of SensiQuant Standard
• Inhibitory effects of the sample material, caused by insufficient purification	• Repeat RNA purification
Fluorescence intensity too low	
Probable cause(s)	**Suggested Corrective Action(s)**
• Inappropriate storage of kit components	• Aliquot reagents
	• Store the SensiQuant MasterMix kit at -15°C to -25°C protected from light
	• Avoid repeated freezing and thawing
• Very low initial amount of target RNA	•Always check the RNA concentration before starting

REFERENCES AND SYMBOLS

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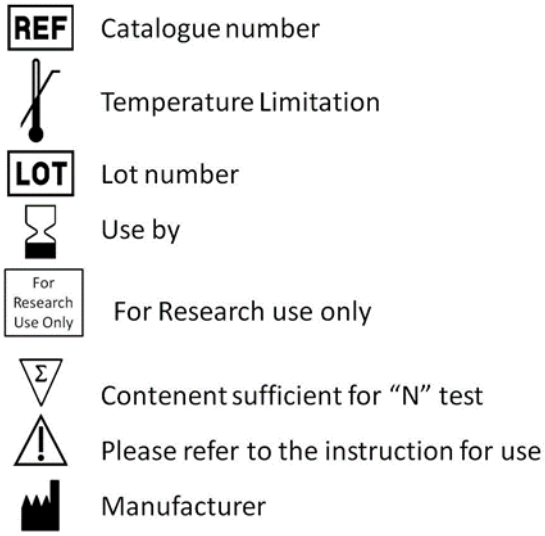

WORK PLAN

Date:

Operator: