LightMix® Kit *bcr-abl* t(9;22) M / m / µ and Abl1 Reference

Cat.-No. 40-0537-96

2014 Normalization against WHO International Standard
2016 Standard Row aligned to the ERM-AD623 Standard

Kit for relative quantification of *bcr-abl t(9;22) fusion transcript* versus Abl1 (cDNA); detection of exon a2 of Abl1 and optional discrimination of common *bcr-abl* breakpoints using:
Roche Diagnostics LightCycler® 1.x / 2.0 / 480 Instruments and cobas z 480 Analyzer.

Lyophilized mix of primers and probes for a total of 96 *bcr-abl* and Abl1 reactions each 20 µl volume. Store protected from light at 4-25℃. **Do NOT freeze lyophilized reagent !**

1. Introduction

The *bcr-abl* translocation t(9;22) can be found in 95% of all cases of chronic myeloid leukemia (*CML*), the most frequent leukemic disease in adults as well as in 25% of acute lymphoblastic leukemia (ALL) cases. The rearrangement generates a fusion transcript from the breakpoint cluster region (*bcr*) and the Abelson leukemia (Abl1) genes, resulting in a soluble and non-regulated tyrosine kinase, transforming normal cells to neoplastic CML-cells thus causing unlimited propagation of white blood cells. The fusion transcripts b3a2 and b2a2 (major breakpoint region, **M-bcr**) and e1a2 (minor breakpoint region, **m-bcr**) cover more than 95 % of all t(9;22) cases. The breakpoint region **µ-bcr** generates the fusion transcripts e19a2 (e19 is referred also as C3)[2] which is much more rare. The common fusion partner is exon **a2** of *Abl1* gene, while the fusion to exon **a3** is very rare.[1]

Besides FISH analysis, the most accepted diagnosis method is based on reverse transcription Real-Time-PCR detection of the fusion transcripts. The HybProbe based LightCycler® test from Emig et al. 1999[3] has been used in routine for more than one decade.

During therapy with tyrosine kinase inhibitors (eg. Imantinib) or a after bone marrow transplantation the expression of the fusion transcript is no longer detectable or reduced to very low levels (Minimal Residual Disease, MRD), requiring an extremely sensitive detection method in order to recognize a relapse immediately.

This kit provides an accurate system to quantify the *bcr-abl* fusion transcripts **b2a2**, **b2a3**, **b3a2**, **b3a3**, **e1a2**, **e1a3**, **e19a2**, **e19a3** against the reference gene Abl1 as recommended in Beillard et al., 2003 [4].

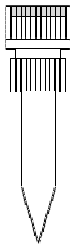
The standards included in the kit are aligned to the International Standard (IS) to allow a direct comparison with the results from other laboratories. The lot-specific correction factor is included in the Certificate of Analysis (CoA).

In the initial diagnosis of the disease the amount of the fusion transcript is very high and it is of clinical interest to discriminate which fusion transcript is present. An optional procedure allows to identify the specific transcripts **M-bcr**, **m-bcr**, or **µ-bcr**.; a melting curve analysis included in the protocol identifies the fusion transcript containing the exon **a2** of *Abl1* gene.

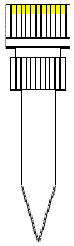
When necessary, differentiation **b2a2** versus **b3a2** and **b2a3** versus **b3a3** can be achieved by comparing on a gel the PCR products of the patients versus the included controls.

2. Set Contents

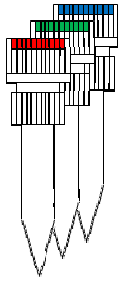
2.1 Parameter-Specific Reagents (PSR):



1x **Reference** Narrow vial with white cap containing:
premixed forward and reverse primers and probes
for **96 reactions** ***Abl1*** transcript (reference gene)

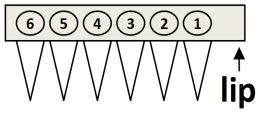


1x **Fusion** Narrow vial with yellow cap containing:
premixed probes and reverse (but no forward) primer
for **96 reactions** ***bcr-abl*** *t(9;22)* fusion transcript
(reagents common to all translocation variants)
and the *Abl1* exon ***a2*** / ***a3*** discrimination probe.



Three narrow vials containing **forward primer** for **96 reactions:**

1x **Red cap** exon 13 forward **primer** for *bcr-abl t(9;22)* **M-bcr**
1x **Green cap** exon 1 forward **primer** for *bcr-abl t(9;22)* **m-bcr**
1x **Blue cap** exon 19 forward **primer** for *bcr-abl t(9;22)* **µ-bcr**

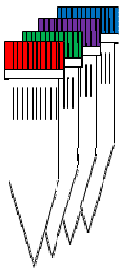


2.2 Controls and Standards :

1x Standard row with 6 lyophilized plasmid standards
bcr-abl t(9;22) fusion transcript *DNA* ***b3a2*** from 10^1 to 10^6 copies per reaction

1x Sealing foil for the standard row (dispose standard row after single use)

Four wide vials containing **controls:**



Cap		containing
1x	**Red**	**control** DNA *bcr-abl t(9;22)* ***b3a2*** fusion transcript *Abl1* plasmid representing a **1%** sample
1x	**Violet**	**control** DNA *bcr-abl t(9;22)* ***b3a3*** fusion transcript *Abl1* plasmid representing a **0.02%** sample
1x	**Green**	**control** DNA *bcr-abl t(9;22)* ***e1a2*** fusion transcript *Abl1* plasmid representing a **10%** sample
1x	**Blue**	**control** DNA *bcr-abl t(9;22)* ***e19a2*** fusion transcript *Abl1* plasmid representing a **0.1%** sample

Note: The above numbers are expressing 'target equivalents' per reaction (not copies per ml sample).

1x **Certificate of Analysis** (CoA)
with the Correction Factor (Normalization to International Standard)

2.3 Overview of Set Content and Dilution Instructions

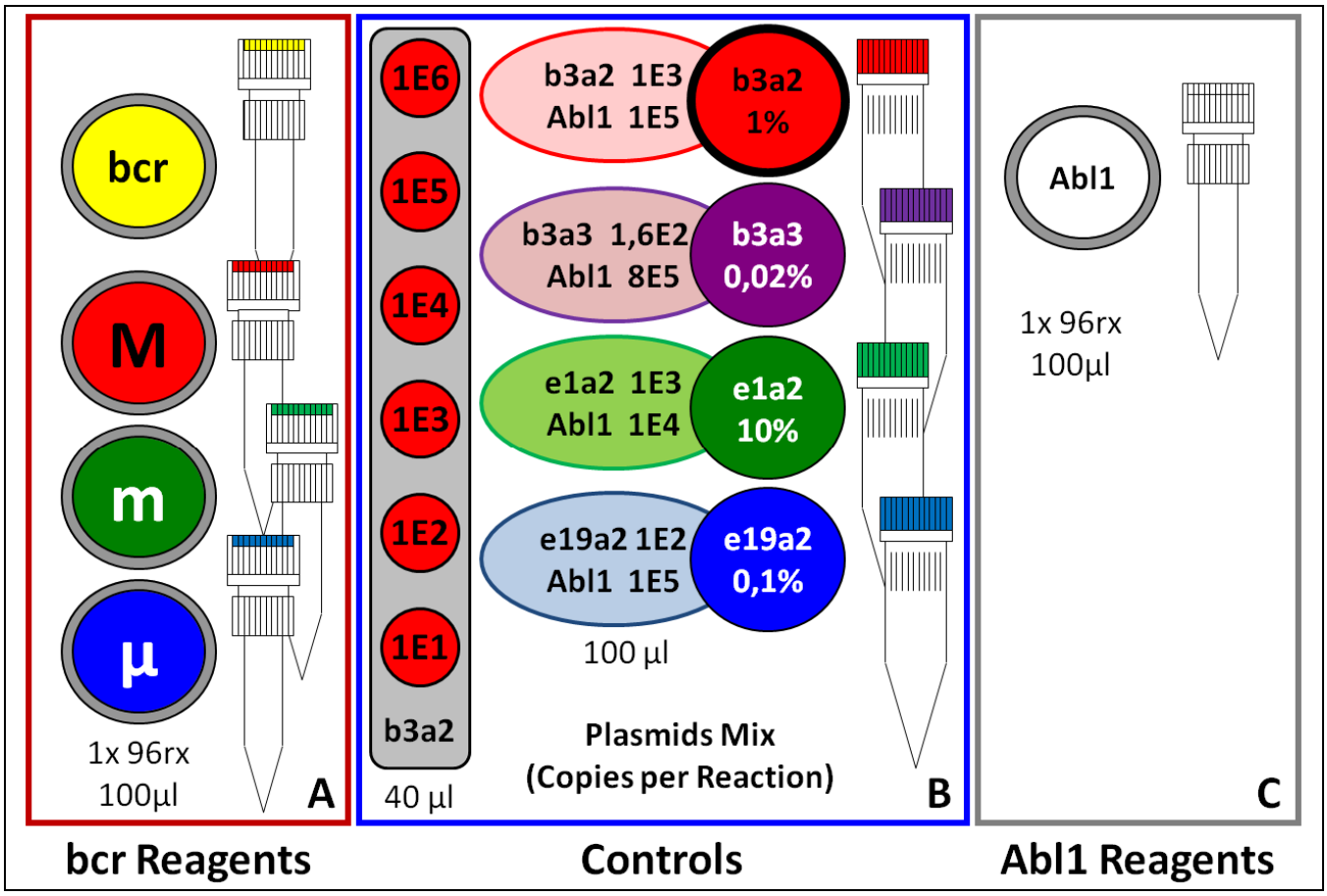


Fig. 1

3. Additional Reagents and Items

3.1 Required

Roche Diagnostics	Cat.-No.
LightCycler® FastStart DNA Master HybProbe	03 003 248 001
Transcriptor First Strand cDNA Synthesis Kit	04 896 866 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	04 929 292 001
LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	04 729 692 001

3.2 Optional

High Pure RNA Isolation Kit	11 828 665 001
TriPure Isolation Reagent	11 677 157 001

3.3 Instruments

Capillary based instruments:	
LightCycler® 1.x Instrument	discontinued
LightCycler® 2.0 Instrument	12 011 468 001
Multiwell based instruments:	
LightCycler® 480 Instrument	discontinued
LightCycler® 480 II Instrument	05 015 278 001
cobas z 480 Analyzer	05 200 881 001

4. Description

Fragments of different sizes (depending on the type of *bcr-abl t(9;22)* fusion transcript) are amplified from cDNA using primers located in *bcr* exons b2, e1, e19, and in *abl* exon a3 as depicted in figure 2 (A). The reference transcript Abl1 is amplified in a separate reaction (B).

The PCR products are detected with LightCycler® Red 640 labeled hybridization probes.

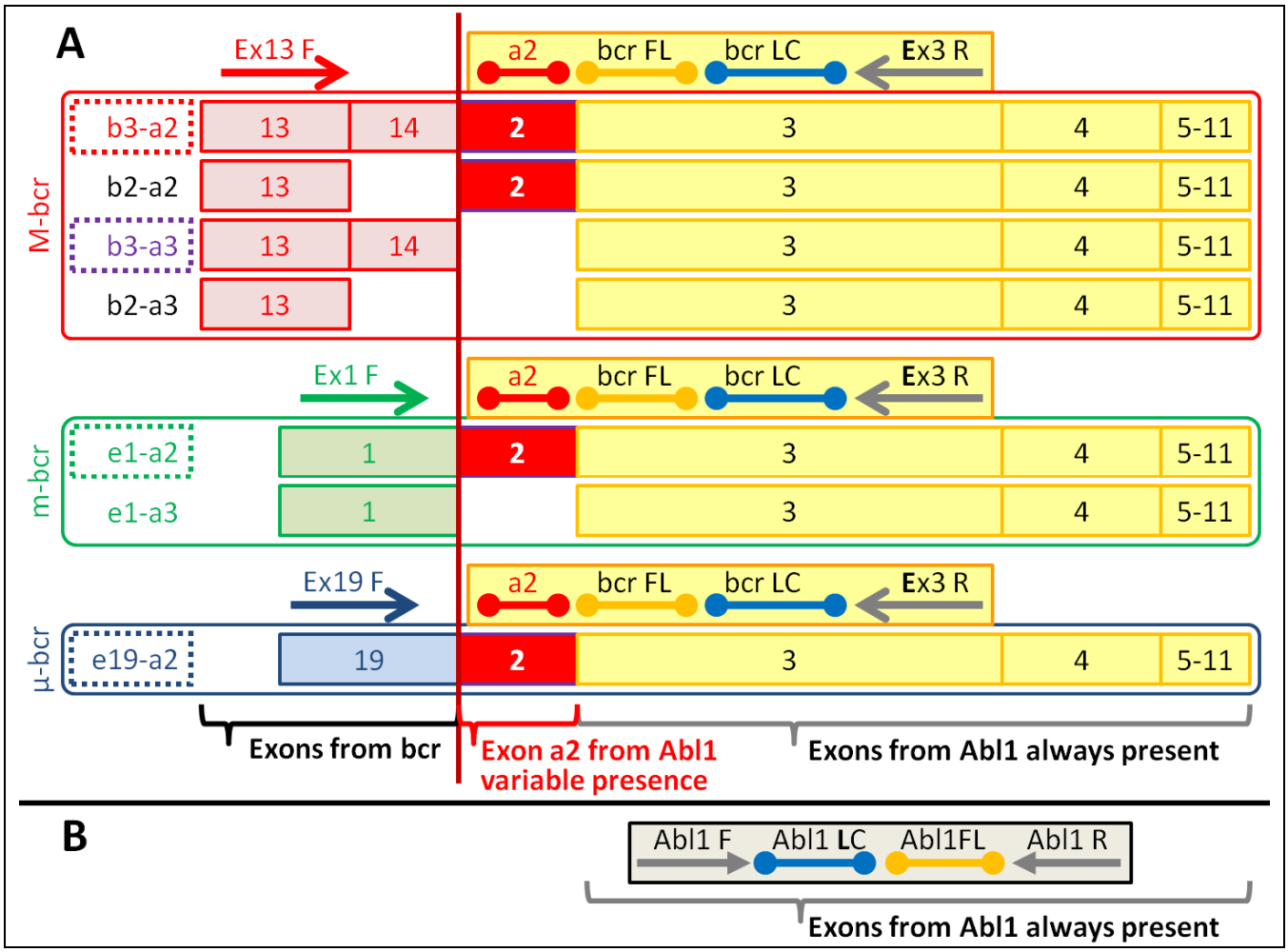
The different shape of the amplification curves helps to prevent to mix up *bcr-abl* and Abl1 results (see figures 5 or 8 for illustration).

The supplied standard row allows to determine the linear range of the reaction, to estimate the absolute quantity of *bcr-abl* and Abl1 in unknown samples, and to calculate the ratio between the fusion transcript and the Abl1 reference:

$$\frac{\textit{bcr-abl t(9;22)}\text{ fusion transcript}}{\text{Abl1}}$$

This assay provides the individual forward primers specific for the **M-bcr**, **m-bcr** and **µ-bcr** brake point transcripts in three distinct vials; for routine analysis all three primers must be combined for contemporaneous fusion transcript quantification. When the identification of the patient specific break point is requested, these primers can be used independently.

An Abl1 exon **a2** specific probe allows identification of fusion transcripts containing this exon, displaying an additional peak at Tm = 59°C in a melting curve analysis.



Schematic representation of primers position and possible fusion transcripts Fig. 2

5. Differential Utilization of the Kit Reagents

5.1 Quantification of M-m-μ Fusion Transcripts

Prepare the reaction mix as described in column **"M-m-μ"** of Table 1 (chapter 9.1.1). The reagents allow the contemporaneous detection and quantification of all fusion transcripts and of the total Abl1 transcript cDNA (see chapter 9.1.2) enabling to calculate the ratio.

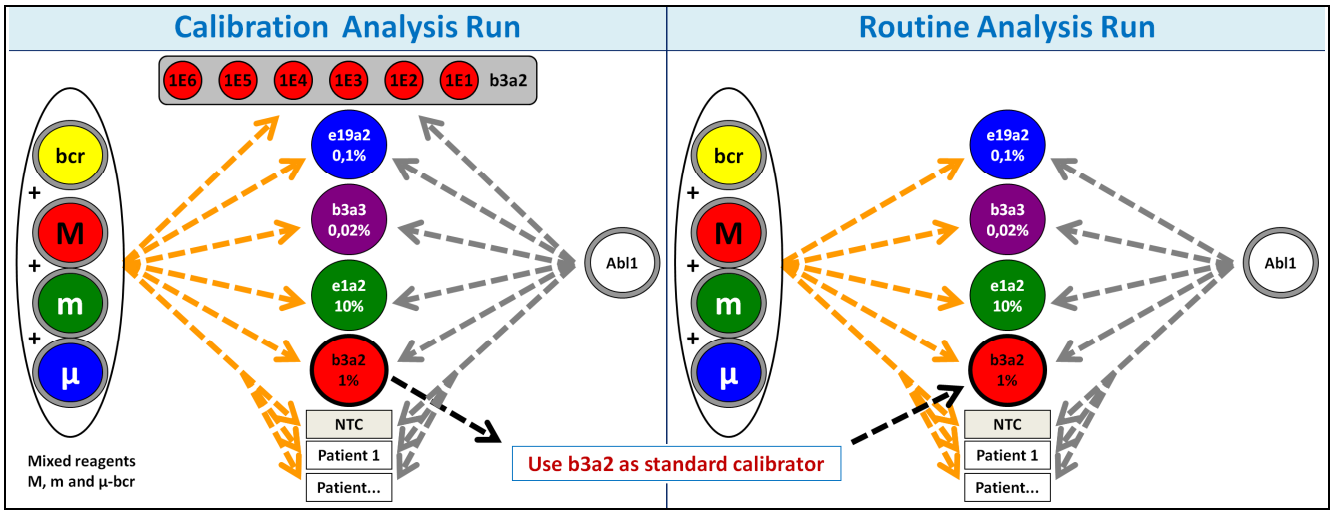


Fig. 3

The quantification of *bcr-abl* and Abl1 targets obtained in the Calibration Run (first run, left panel) from the **1% b3a2 vial** (red cap) will be utilized for the recall of the standard curves in the Analysis Run (successive runs, right panel); more reproducible results are obtained when standards and controls are analyzed in duplicate or triplicates.

5.2 Optional detection of Abl1 exon 2

Melting curve analysis, performed after amplification, allows to discriminate fusion transcripts containing the exon **a2** (peak at ~ 59°C) versus the exon **a3** of the Abl1 gene (no peak).

5.3 Optional discrimination of the Fusion Transcripts

Optionally samples can be tested with three independent reactions to discriminate amongst **M-bcr** or **m-bcr** or **μ-bcr** transcripts (see chapter 12 for details).

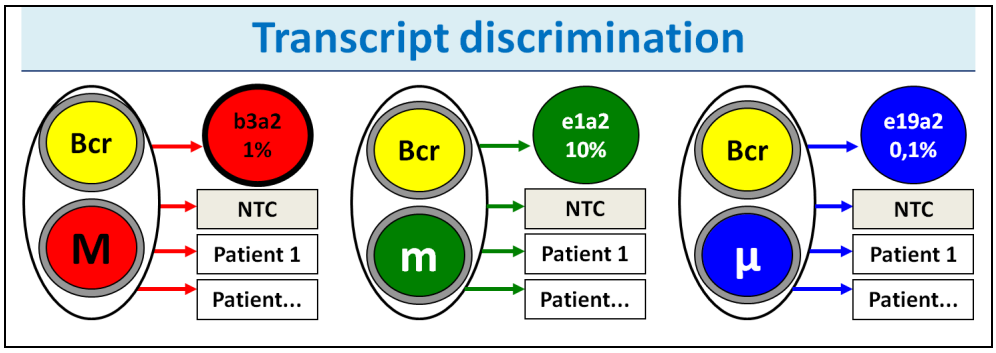


Fig. 4

Note: This utilization of the ***bcr-abl*** reagent will reduce the kit productivity accordingly.

6. Product Characteristics and Specifications

• Instruments:

Roche LightCycler® 1.x / 2.0 / 480 Instruments or cobas z 480 Analyzer
For use in **LightCycler® 1.x Instruments** we recommend upgrade to software version 4.1.
Use of **software version 3.5.3** is permitted: read channel F2 instead of channel 640.

• Color Compensation:

Not necessary.

• RetroTranscription:

Roche Diagnostics Transcriptor First Strand cDNA Synthesis Kit

• Mastermix:

Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'.

• Run time (PCR)

PCR results (50 cycles and melting curve) are obtained within 60 minutes with capillary based instruments and 85 minutes with multiwell based Instruments.

• Sensitivity

These reagents detect at least 10 copies/reaction for both *bcr-abl t(9;22) fusion transcript* and Abl1 (as tested in an exemplary system, using cloned targets as reference).

• Measuring range

The linear measuring range of the assay is 10^2 to 10^6 copies/reaction for both *bcr-abl t(9;22) fusion transcript* and Abl1.

• Storage and Stability

Store lyophilized reagents protected from light at room temperature (18-25℃).
Please see the expiry on the outer package label. **Do not freeze** lyophilized reagents.

Dissolved reagents are stable for at least 10 days when stored refrigerated (4℃) in the dark.

7. Sample Preparation

Prepare an aqueous solution of RNA (e.g. Roche Diagnostics High Pure RNA Isolation Kit or TriPure Isolation Reagent) from White Blood Cells (WBC) or bone marrow; whole blood is less suited for *bcr-abl* testing.

Prepare 20 µl of cDNA using Roche Diagnostics 'Transcriptor First Strand cDNA Synthesis Kit' using 1.0 µg of RNA following the instructions included in the kit.
Since PCR testing requires 20 µl cDNA (4 x 5 µl) add 5 µl water reserve volume. Mix gently!

Optionally, to simplify the procedure, use 1.2 µg RNA for reverse transcription and scale up all volumes and amounts to a final volume of 25 µl.

Note: The results depend not only on the quality but also on the total quantity of cDNA used. In order to reach the desired sensitivity of 0.001% *bcr-abl* fusion transcript the limiting factor of the test is the absolute quantity of the reference. In order to avoid stochastic variations we request a minimum amount of 5 copies *bcr-abl* per reaction thus the needed total quantity of the Abl1 reference transcript must be equal or more than 5 x 10^5 copies per reaction.

Samples with less than 10^4 copies of Abl1 potentially report false-negative results.

8. Reagent Preparation

Start programming before preparing the solutions. See the operator's manual for details.

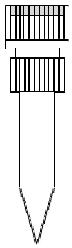
Negative Control: Run always at least one no-template control (NTC) - replace the template cDNA with water.

Positive Control: Include all four provided positive controls in every run - replace the template with the control DNA. For quantification (first run) use a fresh solution prepared from the provided standard row (single use only).

Warnings and Precautions:

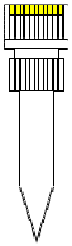
- Handle the positive controls at a different work place than the reagents.
- Use dedicated pipets.
- Use different lab coats (if possible).
- Change gloves after work steps.

8.1. Preparation of Parameter-Specific Reagents (PSR):



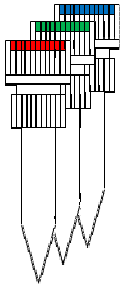
Add 100 µl PCR-grade water to:
Narrow vial with white cap, 96 reactions ***Abl1*** transcript
mix the solution (vortex) and spin down.

►**Use 1 µl** of reagent for a 20 µl PCR reaction to quantify *Abl1* transcript.



Add 100 µl PCR-grade water to:
Narrow vial with yellow cap, 96 reactions ***bcr-abl*** *t(9;22)* fusion transcript
mix the solution (vortex) and spin down.

►**Use 1 µl** of reagent for a 20 µl PCR reaction to quantify **M-m-µ** transcription variants.



Add 100 µl PCR-grade water to:
Narrow vial with **red cap** forward **primer** for *bcr-abl t(9;22)* **M-bcr**
Narrow vial with **green cap** forward **primer** for *bcr-abl t(9;22)* **m-bcr**
Narrow vial with **blue cap** forward **primer** for *bcr-abl t(9;22)* **µ-bcr**
mix each solution (vortex) and spin down.

►**Use 1 µl** of reagent for a 20 µl PCR reaction (see chapter 8 for details).

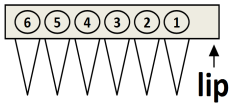
Dissolved reagents are stable for at least 10 days when stored refrigerated (4℃) in the dark.

8.2. Preparation of the Standard Row

Note: Use a different work place to avoid contamination of the reagents.

The plasmid standard is provided in 6 quantities of 10 to 10^6 target equivalents per 5 µl (once dissolved).

Start with the lowest concentrated standard (first tube from the extended lip).
Use a pipette tip to punch a hole in the sealing foil.



Add 40 µl PCR-grade water to each vial of the row.
Mix the target DNA by carefully pipetting ten times the solution up and down.

►**Use 5 µl** plasmid standard for a 20 µl PCR reaction.

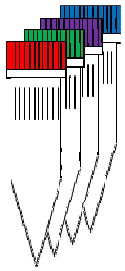
This standard solution is not long-term stable. Use only fresh prepared solutions as quantification reference. The standard row is intended for single use only.

After adding the plasmid standard to the reaction mix use the provided sealing foil to close the vials and dispose it. Reopening may cause contaminations of the work-space (aerosol).

8.3. Preparation of Control DNA

Add 100 µl PCR-grade water to each wide vial

Cap			containing
1x	**Red**	10^3	**control** DNA *bcr-abl t(9;22)* ***b3a2*** fusion transcript
		10^5	*Abl1* plasmid representing a **1%** sample
1x	**Violet**	$1.6x10^2$	**control** DNA *bcr-abl t(9;22)* ***b3a3*** fusion transcript
		$8x10^5$	*Abl1* plasmid representing a **0.02%** sample
1x	**Green**	10^3	**control** DNA *bcr-abl t(9;22)* ***e1a2*** fusion transcript
		10^4	*Abl1* plasmid representing a **10%** sample
1x	**Blue**	10^2	**control** DNA *bcr-abl t(9;22)* ***e19a2*** fusion transcript
		10^5	*Abl1* plasmid representing a **0.1%** sample



Mix the Control DNA by vortexing. Spin down to collect the solution.

This solution is stable at least ten days when stored refrigerated at 4℃, for long term storage freeze at -20℃. Avoid repeated freezing thawing cycles.

Remember that opening of the vials may cause contaminations of the work-space (aerosol).

►**Use 5 µl** Control DNA for a 20 µl PCR reaction.

Note: Pipetting Procedure.

The error for the final ratio can be grossly estimated as the sum of the errors of the two independent measurements for *bcr-abl* and Abl1.
Ensure to use calibrated pipettes and accurate procedures to achieve reliable results.

9. Preparation of Reaction Mixes

9.1. Quantification

It is possible to analyze *bcr-abl* and Abl1 in two independent runs and calculate the ratio outside the software; this procedure maximize productivity in capillary based instruments.

9.1.1. Reaction mix for Quantification of *bcr-abl* Fusion Transcripts.

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions and controls to be cycled, plus one additional reaction.

***bcr-abl* t(9;22) Fusion Transcript Quantification**	
M-m-µ	**Component**
6.6 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart kit)
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
1.0 µl	Narrow vials with yellow cap **bcr** Reverse Primer
1.0 µl	Narrow vials with **red cap vial M-bcr**
1.0 µl	Narrow vials with **green cap vial m-bcr**
1.0 µl	Narrow vials with **blue cap vial µ-bcr**
2.0 µl	Roche LightCycler® FastStart DNA Master HybProbe (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix. Mix gently (no vortex), spin down.

Note: A high percentage of imprecision is due to a non-homogeneous reaction mix ! Table 1

9.1.2. Reaction mix for Quantification of Abl1 Transcript.

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

***Abl1* Transcript Quantification**	
	Component
9.6 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart kit)
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
1.0 µl	Narrow vials with white cap ***Abl1*** transcript
2.0 µl	Roche LightCycler® FastStart DNA Master HybProbe (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix. Mix gently (no vortex), spin down.

Note: A high percentage of imprecision is due to a non-homogeneous reaction mix ! Table 2

9.1.3. Set up of the Reaction

Transfer 15 µl reaction mix to each capillary or each well and
add 5 µl of sample or control DNA/standard for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to seal the multiwell plate and spin down.

10. Capillary Based Instruments

10.1. Programming

The protocol consists of four program steps

1. **Denaturation**: sample denaturation and enzyme activation
2. **Cycling**: PCR-amplification of the target DNA
3. **Melting**: melting curve analysis for identification of the PCR product derived from the target DNA
4. **Cooling**: cooling the instrument

Program Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:20	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	**0.2**	20
Acquisition Mode	None	None	Single	None	None	None	**Continuous**	None

Table 3

10.2. Data Analysis

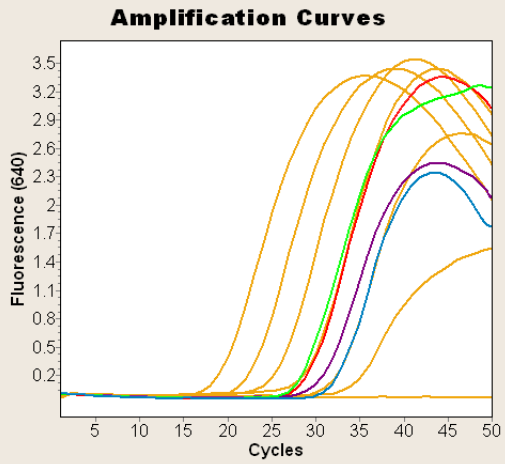
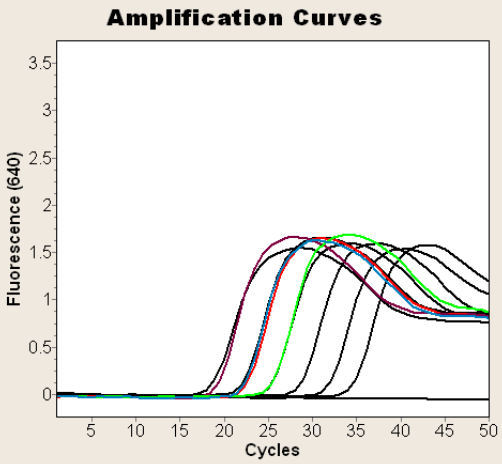
Perform data analysis, as described in the Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *bcr-abl t(9;22)* and *Abl1* data in channel 640, Quantification mode.

The negative controls (NTC) must show no signal.
Refer to **chapter 13** for data validation.

The provided standard row should yield Cp values between about 18 and 36 cycles (calculated with Second Derivative Maximum method).

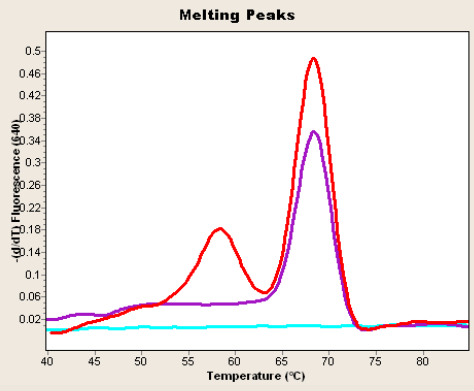
10.3. Sample Data – Typical Results for capillary based Instruments

***bcr-abl t(9;22)* Fusion Transcript**	***Abl1* Reference Transcript**	**LightCycler® 2.0 Instrument**
		Channel 640 (F2) Quantification mode (Second Derivative Maximum): Sample data for *bcr-abl* t(9;22) 1E01: Cp 34-36 1E02: Cp 31-33 1E03: Cp 28-30 1E04: Cp 25-27 1E05: Cp 21-24 1E06: Cp 18-20
Quantification for ***bcr-abl* t(9;22) fusion transcripts** (see chapters 8 and 9) Yellow Standard row **red b3a2 1%** control vial violet b3a3 0.02% control vial green e1a2 10% control vial blue e19a2 0.1% control vial	Quantification for **Abl1 Reference gene** (see chapters 8 and 9) Black Standard row **red b3a2 1%** control vial violet b3a3 0.02% control vial green e1a2 10% control vial blue e19a2 0.1% control vial	*bcr-abl* and Abl1 have different shape of the amplification curves. This prevents to mix up results.

LightCycler® 2.0 sample data *bcr-abl t(9;22)* and Abl1. For lot-specific Cp values see the CoA. Fig.5.

10.4. Detection of exon a2 of Abl1 gene in Fusion Transcripts

Only fusion transcript containing Abl1 exon **a2** display a melting peak at **~ 59°C** .

***bcr-abl t(9;22)* Fusion Transcript**	**LightCycler® 2.0 Instrument**
	Channel 640 (F2) **Negative Control** No melting curve **b3a3** 1 peak fusion transcript. **b3a2** 1 peak fusion transcript. 1 specific peak for exon a2 at Tm= 59°C

LightCycler® 2.0 melting curve analysis Fig. 6

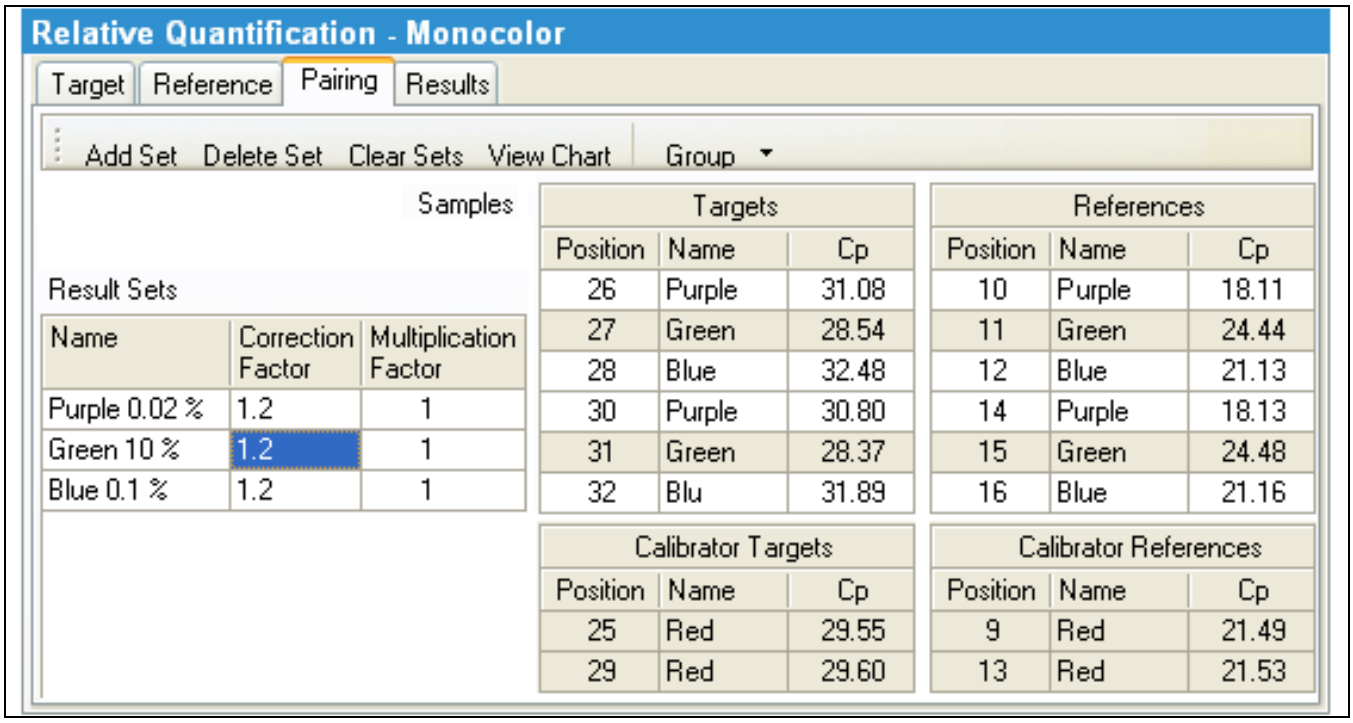
For identification of the **M-bcr**, **m-bcr**, **µ-bcr** type fusion transcripts, refer to **chapter 12.**

10.5. Relative Quantification - Use of the Correction Factor (software version 4.1)

The Positive Control ***b3a2 1%*** has been compared with the International Standard (IS) to be used as **calibrator** in “Relative Quantification – Monocolor” analysis as described below.

The **Correction Factor** is lot-specific and it is contained in the Certificate of Analysis (CoA).

For a correct alignment with the IS, insert the Correction Factor in the “Pairing” tab before calculating results. The normalized *Bcr-abl*/Abl1 ratio are displayed in the “Results” tab.



Correction Factor Fig. 7

In the 'Manual Pairing' section replace '1.2' with the respective lot-specific correction factor from the CoA.

11. Multiwell based Instruments

11.1. Programming

Reaction Volume: 20 µl

Detection Format:
LightCycler® 480 Instrument: 498-640
LightCycler® 480 II Instrument: 498-640
cobas z 480 Analyzer: 498-645

The protocol consists of four program steps

1. **Denaturation**: sample denaturation and enzyme activation
2. **Cycling**: PCR-amplification of the target DNA
3. **Melting**: melting curve analysis for identification of the PCR product derived from the target DNA
4. **Cooling**: cooling the instrument
5.

Program Step:	**1**	**2**			**3**			**4**
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:20	00:00:30	00:01:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Acquisition Mode	None	None	Single	None	None	None	**Continuous**	None
Acquisitions [per °C]	-	-	-	-	-	-	**3**	-

Table 4

11.2. Data Analysis

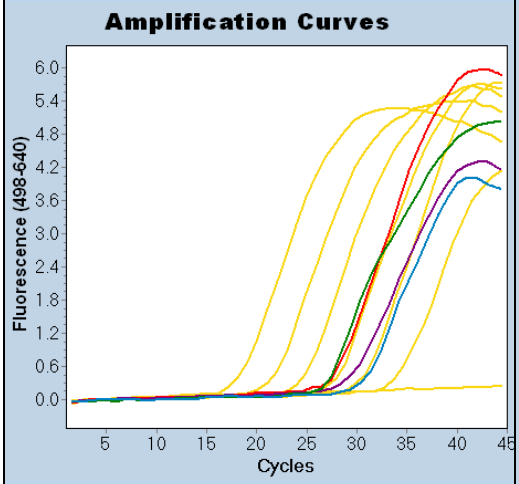
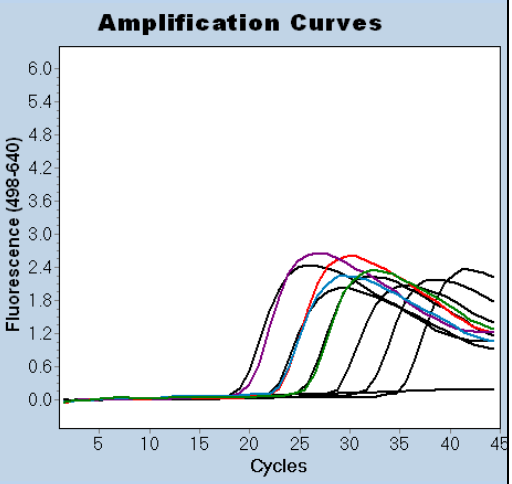
Perform data analysis, as described in the Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *bcr-abl t(9;22)* data in Quantification mode.

The negative control (NTC) must show no signal.
Refer to **chapter 13** for data validation.

The provided standard row should yield Cp values between about 18 and 36 cycles (calculated with Second Derivative Maximum method).

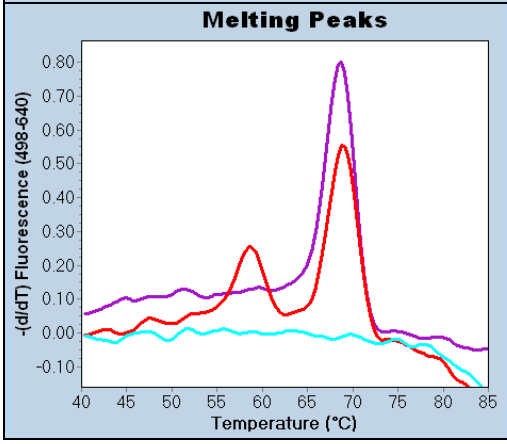
11.3. Sample Data – Typical Results for multiwell based Instruments

***bcr-abl t(9;22)* Fusion Transcript**	***Abl1* Reference Transcript**	**LightCycler® 480II Instrument**
		Filter Combination: 498-640 Quantification mode (Second Derivative Maximum): Sample data for *bcr-abl* t(9;22) 1E01: Cp 34-36 1E02: Cp 31-33 1E03: Cp 28-30 1E04: Cp 25-27 1E05: Cp 21-24 1E06: Cp 18-20
Quantification for ***bcr-abl* t(9;22) fusion transcripts** (see chapters 8 and 9) Yellow Standard row **red b3a2 1%** control vial violet b3a3 0.02% control vial green e1a2 10% control vial blue e19a2 0.1% control vial	Quantification for ***Abl1* Reference gene** (see chapters 8 and 9) Black Standard row **red b3a2 1%** control vial violet b3a3 0.02% control vial green e1a2 10% control vial blue e19a2 0.1% control vial	*bcr-abl* and Abl1 have different shape of the amplification curves. This prevents to mix up results.

LightCycler® 480 sample data *bcr-abl t(9;22)* and Abl1. For lot-specific Cp values see the CoA. Fig.8.

11.4. Detection of exon a2 of Abl1 gene in Fusion Transcripts

Only fusion transcript containing Abl1 exon **a2** display a melting peak at **~ 59°C** .

***bcr-abl t(9;22)* Fusion Transcript**	**LightCycler® 480 II Instrument**
	Filter Combin. 498-640 **Negative Control** No melting curve **b3a3** 1 peak fusion transcript. **b3a2** 1 peak fusion transcript. 1 specific peak for exon a2 at Tm= 59°C

LightCycler® 480 melting curve analysis Fig. 9

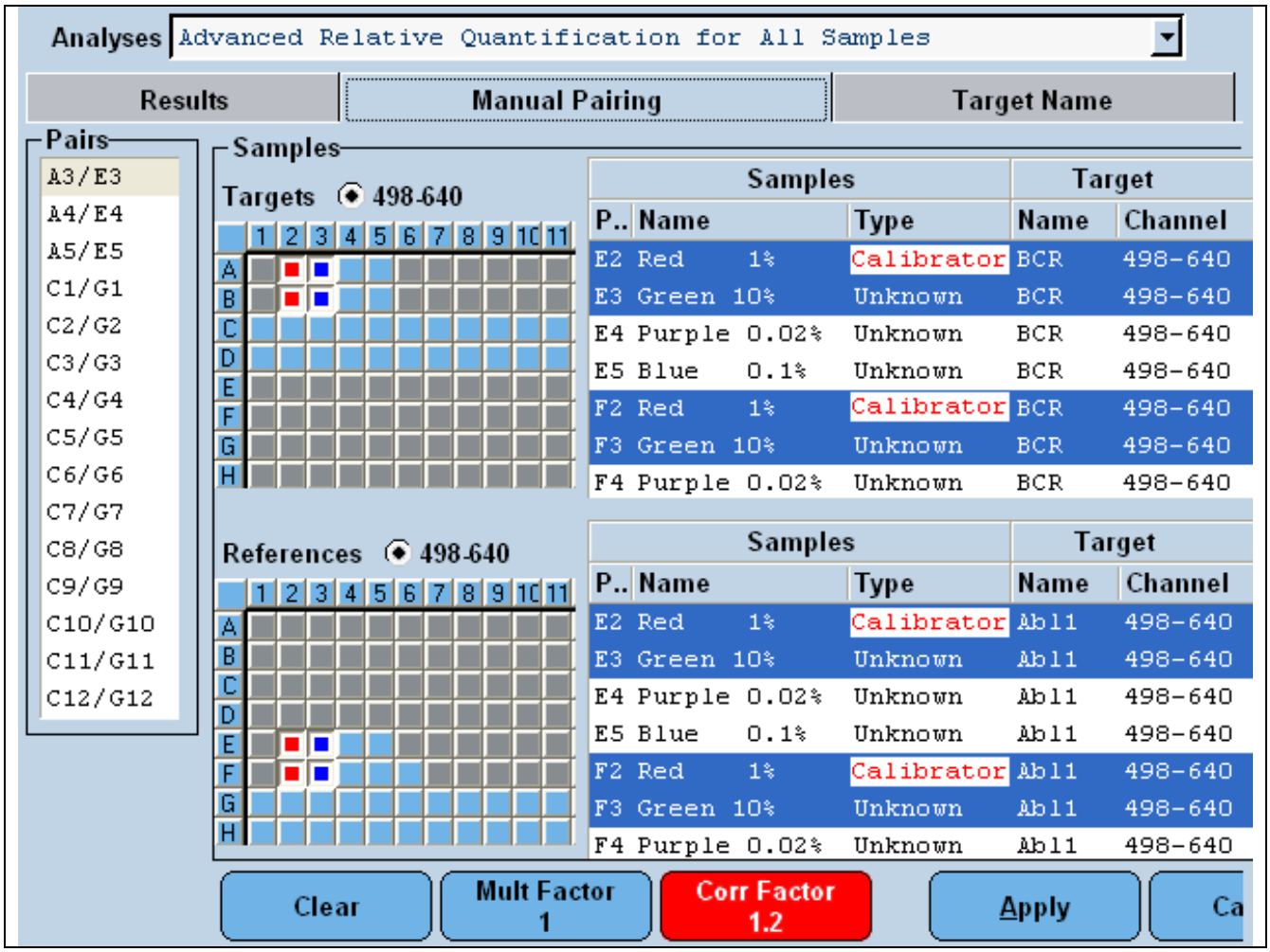
For identification of the **M-bcr**, **m-bcr**, **µ-bcr** type fusion transcripts, refer to **chapter 12.**

11.5. Relative Quantification - Use of the Correction Factor

The positive control ***b3a2 1%*** has been compared with the International Standard (IS) to be used as **calibrator** in “Relative Quantification – Monocolor” analysis as described below.

The correction factor is lot-specific and it is contained in the Certificate of Analysis (CoA).

Before calculating the results insert the Correction Factor in the “Manual Pairing” tab.
The right most column of the “Results” tab expresses the normalized *Bcr-abl*/Abl1 ratio.



Correction Factor Fig. 10

In the 'Manual Pairing' section replace '1.2' with the respective lot-specific correction factor from the CoA.

12. Discrimination of Fusion Transcripts

12.1 Rational

Each sample is tested in three separate reactions for **M-bcr**, **m-bcr** or **µ-bcr** transcripts.

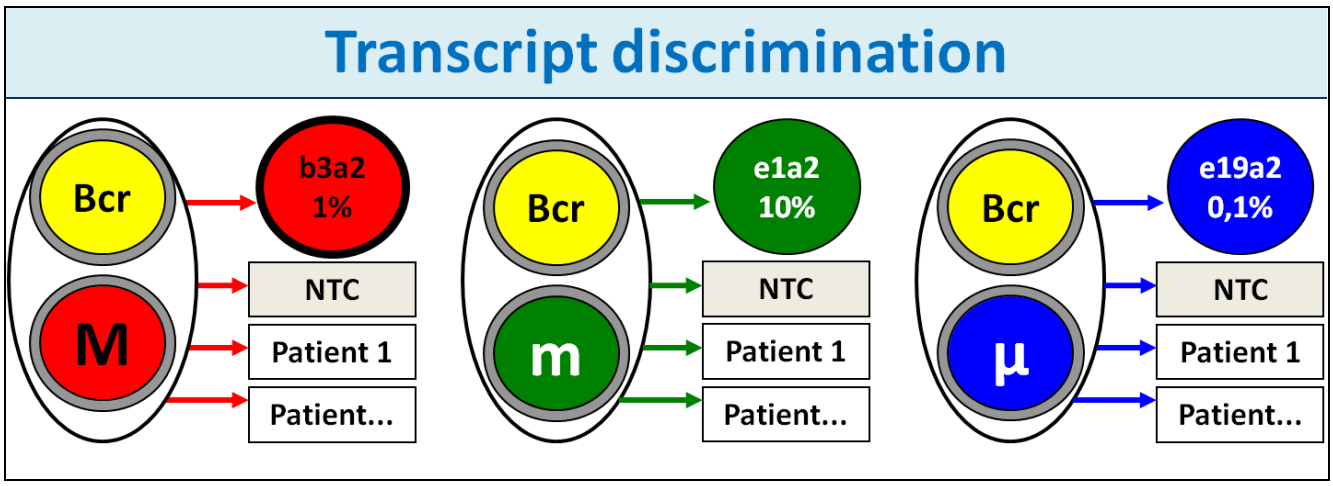


Fig. 11

Quantification of the total *Abl1* transcript cDNA usually is not required; if needed, follow instructions in chapter 9.1.2 and calculate the ratio.

12.2. Reaction mixes for *bcr-abl* fusion Transcript Discrimination

In three cooled reaction tubes, prepare the reaction mixes by multiplying each volume for a single reaction by the number of reactions to be cycled (sample(s), negative and positive controls), plus one additional reaction. It is not necessary to analyze samples in duplicate.

***bcr-abl* t(9;22) Fusion Transcript Discrimination**			
Component	**M-bcr**	**m-bcr**	**µ-bcr**
water, PCR-grade (colorless cap, provided with the Roche Master kit)	**8.6 µl**	**8.6 µl**	**8.6 µl**
Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	**2.4 µl**	**2.4 µl**	**2.4 µl**
Narrow vials with yellow cap **bcr** Reverse Primer	**1.0 µl**	**1.0 µl**	**1.0 µl**
Narrow vials with red cap vial **M-bcr**	**1.0 µl**	**------**	**------**
Narrow vials with green cap vial **m-bcr**	**------**	**1.0 µl**	**------**
Narrow vials with blue cap vial **µ-bcr**	**------**	**------**	**1.0 µl**
Roche LightCycler® FastStart DNA Master HybProbe (red cap, for preparation see Roche manual)	**2.0 µl**	**2.0 µl**	**2.0 µl**
Volume of reaction mix. Mix gently, spin down.	**15.0 µl**	**15.0 µl**	**15.0 µl**

Note: A high percentage of imprecision is due to a non-homogeneous reaction mix ! Table 5

Transfer 15 µl reaction mix to each capillary or each well and
add 5 µl of sample or control DNA/standard for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to seal the multiwell plateand spin down.

12.3. Sample Data – Typical Results for multiwell based Instruments

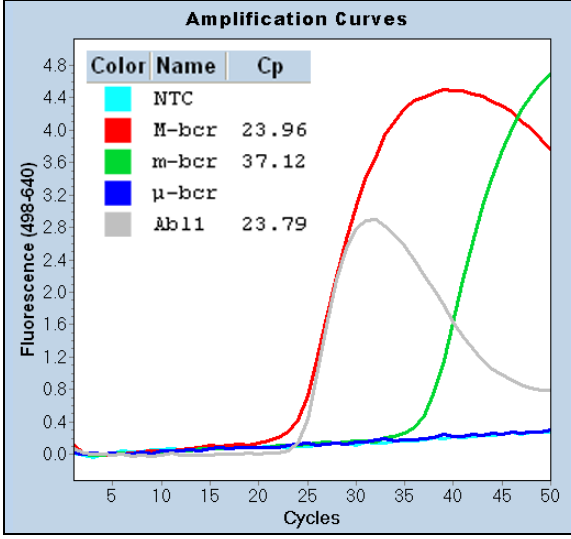
***bcr-abl t(9;22)* Fusion Transcript discrimination**	**LightCycler® 480 II Instrument**
	Filter Combination: 498-640 Patient cDNA independently tested for: **Red** line **M-bcr** **Green** line **m-bcr** **Blue** line **µ-bcr** **Gray** line **Abl1** **The patient fusion transcript is M-bcr** The **m-bcr** signal seen here some 13 cycles later than **M-bcr** is not unusual and, according to the literature, is due to the amplification from alternative spliced RNA.

Fig. 12

Note: Capillary based instruments generate similar results.

12.4. Fusion Type Identification

Clinical Sample(s)			**Control**	
Identification of fusion type			**NTC**	
M-bcr	**m-bcr**	**µ-bcr**		**Result**
no amplification	no amplification	no amplification	negative	Negative (not detectable)
amplification signal			negative	Positive for **M-bcr** fusion transcripts
	amplification signal		negative	Positive for **m-bcr** fusion transcripts
		amplification signal	negative	Positive for **µ-bcr** fusion transcripts
not relevant	not relevant	not relevant	positive	Contamination ! Repeat procedure.

Identification of *bcr-abl* fusion transcripts Table 6

Note 1: High positive M-bcr samples might also show also an amplification for m-bcr (see figure 12).
Note 2: PCR products can be run on a gel and compared for the specific length. Reference plasmids b3 and samples missing exon b3 (thus having 75 bases less) will generate a shorter band on the gel.

13. Run and Sample Validation

Positive Controls				NTC	
M-m-µ *bcr-abl* and Abl1					Result
b3a2 √	*b3a3* √	*e1a2* √	*e19a2* √	negative	Reagents working (passed)

Reagents validation. Table 7.

Clinical Sample(s)	Control	
Routine Analysis (chapter 9)	NTC	
ABL1		Result
Amplification signal **>** 5E5	negative	Sample is valid
Amplification signal **>** 1E4	negative	Sample is valid Detection limit reduced to 0.01%
Amplification signal **<** 1E4	negative	Sample outside range Detection limit reduced to 0.1%
not relevant	positive	Not valid - Contamination ! Repeat procedure.

Possible results for Abl1 The detection limit for *bcr-abl* depends on the Abl1 results. Table 8

Clinical Sample(s)		Control	
Routine Analysis (chapters 9)	Melting Curve (chapters 10.4 and 11.4)	NTC	
M-m-µ bcr-abl			Result
no amplification	No peak	negative	Negative (not detectable)
amplification signal	Peak (~ 59℃)	negative	Sample is valid. Positive for *bcr-abl* ABL1 exon 2 is **contained** in fusion transcript
amplification signal	No peak	negative	Sample is valid. Positive for *bcr-abl* ABL1 exon 2 is **absent** in fusion transcript
not relevant	not relevant	positive	Not valid - Contamination ! Repeat procedure.

Possible results for *bcr-abl t(9;22) fusion transcripts* Table 9

The detection limit for *bcr-abl* depends on the Abl1 results.

14 Index max

15.References

[1] BCR first exon sequences activate the bcr/abl tyrosine kinase oncogene of Philadelphia chromosome positive human leukemia. Muller AJ, Young JC, Pendergast AM. *Molecular Cell Biology* **11**:1785-1792(1991)

[2] Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction). Pane F. et al., Blood (1996) 88, 2410-14

[3] Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Emig M. et al., Leukemia (1999) 13, 1825-1832

[4] Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) – a Europe against cancer program. Beillard et al. Leukemia. 2003 Dec;17(12):2474-86

16. Material Safety Data (MSDS)

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or carcinogenic do not require a MSDS.

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

17. Version History

Notes in red mark events that require to change laboratory procedures
Notes in blue mark improvements and changes in composition

V121122	Combined kit version 40-0537-16 *bcr-abl* and 40-0357-16 Abl. New feature: Differentiation of three fusion transcripts M/m/µ. The performance for detection and quantification remained unchanged : No repeat of laboratory evaluations required.
V131208	Reference to the International Standard Included
V140115	Detection of Abl1 exon a2 in fusion transcript. Manual structure revised.
V160313	The Standard Row has been aligned with the European Standard ERM-AD623; amounts increased by nearly 30%, causing about 0.4 cycles earlier Cp values and virtually lower copy numbers for patient samples (change with lot 27361504).

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