LightMix® Kit *PML-RAR t(15;17)*

Cat.-No. 40-0135-16

Kit with reagents for the detection of the translocation *PML-RAR t(15;17)-specifc* transcript (cDNA) using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 / 480 II or Cobas® Z480 Instruments.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25℃), do N OT freeze!**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 / Z480 Instruments see pages 6-7

1. Introduction

The translocation t(15;17) (q22;q21) causes a fusion of the promyelocytic leukemia gene (PML) to the retinoic acid receptor alpha gene (RARa) generating a chimeric PML-RARa fusion protein which acts as transcriptional repressor. This tranlocation is in indication for Acute Promyelocytic Leukemia (APL). The fuison products are distinguished by means of their breakpoint and occur in different frequencies :

Name	Type	Variant		Breakpoint	Frequency
PML/RARa bcr1	A	L	long	Intron 6	55%
PML/RARa bcr2	A	V	variant	Exon 6	5%
PML/RARa bcr3	B	S	short	Intron 3	40%

Tab. 1: Variants of PML-RAR

The detection of the PML-RAR fusion transcript has been accomplished with Real-Time PCR methods using TaqMan or hybridization probes.[1]

The PML-RAR specific detection assay is verified against the amplification of a control or reference transcript (housekeeping gene); for quantification purposes the Cp values are normalized against the reference gene, comparing delta Cp values. The commonly used glucose-6-phophate-dehydrogenase G6PDH [2] (available as LightMix® Kit 40-0137-16) has been meanwhile replaced by Abl1 (40-0357-16), following the recommendations of the EU consortium [3].

The LightMix® Kit *PML-RAR t(15;17)* provides a fast, easy and accurate system to identify and quantify this fusion transcripts in a nucleic acid extract.

This LightMix® Kit is tested on the LightCycler® 1.x / 2.0 / 480 and 480 II Instruments with Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe'. A 1-step RT PCR procedure was not tested.

[1] New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFBMYH11+ acute myeloid leukemia based on quantification of fusion transcripts . Schnittger et al., Blood (2003)

[2] 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

[3] 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

2. Description

Fragments of different sizes depending on the type of the *PML-RAR* fusion transcript are amplified from cDNA with specific primers. The resulting PCR products are detected with LightCycler® Red 640 labeled hybridization probes (detected in channel 640).

The supplied standard row allows to determine the linear range of the reaction and to estimate the quantity of the target sequence in unknown samples.
For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of channel 640, channel F3 instead of channel 705 and channel F1 instead of channel 530 for detection. We recommend upgrading LightCycler® 1.x Instruments to software version 4.1.

3. Set contents

6 Vials with red caps containing lyophilized primers and probes for each 16 reactions *PML-RAR*
1 Standard row with 6 lyophilized plasmid standards *PML-RAR DNA* 10^1 to 10^6 target equivalents / rxn
1 Sealing foil for the standard row

4. Additional reagents and items required

Roche Diagnostics:

LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
High Pure RNA Isolation Kit	Cat.-No. 11 828 665 001
Transcriptor First Strand cDNA Synthesis Kit	Cat.-No. 04 379 012 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 692 001

TIB MOLBIOL - Reference Genes (optional)

LightMix® Kit GAPHD 40-0137-16	Cat.-No. 05 945 305 001
LightMix® Kit Abl1 40-0357-16	Cat.-No. 05 945 674 001

5. Product characteristics

PCR results are obtained within 50 minutes (50 cycles and melting curve) with the LightCycler® 1.x / 2.0 Instruments and within 80 minutes (50 cycles and melting curve) with the LightCycler® 480 Instruments.

Sensitivity
These reagents detect 10 copies of *PML-RAR t(15;17)* cDNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 Instruments (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 of *PML-RAR t(15;17)* cDNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' with the LightCycler® 1.x / 2.0 / 480 Instruments.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25°C). See expiry on the outer label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4°C).

6. Experimental protocol

The following procedure was developed for use with the LightCycler® 1.x / 2.0 / 480 Instruments. Start programming before preparing the solutions. See the Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. Roche Diagnostics 'High Pure RNA Isolation Kit' combined with Roche Diagnostics 'Transcriptor First Strand cDNA Synthesis Kit').

Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA. For quantification always use a fresh solution prepared from the provided standard row (single use only).

6.1. Preparation of parameter-specific reagents (16 reactions):

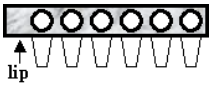
One reagent vial with a red cap contains primers and probes to run 16 PCR reactions for *PML-RAR*

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►**Use 4 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C. Avoid prolonged exposure t o light.

6.2. Preparation of the standard row



The target *DNA* is provided in 6 different quantities to yield from 10^1 to 10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. **Add 40 µl** PCR-grade water to each vial of the row. Mix the target DNA by pipetting the solution up and down 10 times.

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

This standard solution is not long-term stable and will lose sensitivity under prolonged storage. Use only fresh prepared solutions as quantification references. The standard row is intended for single use only.
After adding the target DNA to the LightCycler® reaction mix use the provided sealing foil to close the vials in order to avoid changes in the concentration due to evaporation.
Please note that opening of these vials may cause contaminations of the work-space (aerosol).

6.3. Preparation of the LightCycler® reaction mix

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.

For use with the Roche FastStart Master	
Single reaction	**Component**
6.6 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix

Table 2

Mix gently, spin down and **transfer 15 µl** each of the reaction mix to a LightCycler® capillary (LightCycler® 1.x / 2.0 Instrument) or to a multiwell plate (LightCycler® 480 Instrument).

Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.

Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1. 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:	Denaturation	Cycling			Melting			Cooling
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

(Melting not relevant for detection) Table 3

***Note**: Unified run conditions for hematology parameters. No changes in reagents made. The former cycling program yields equivalent results.*

7.2. Data Analysis

For use in LightCycler® 1.x Instruments select channel F2 instead of channel 640 for detection.

Perform data analysis, as described in the LightCycler® 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 *PML-RAR t(15;17)* data in channel 640, Quantification mode. The negative control (NTC) must show no signal.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *PML-RAR t(15;17)* should have Cp values between cycles 18 and 35 (Cp values calculated with Second Derivative Maximum method).

7.3. Sample Data – typical results

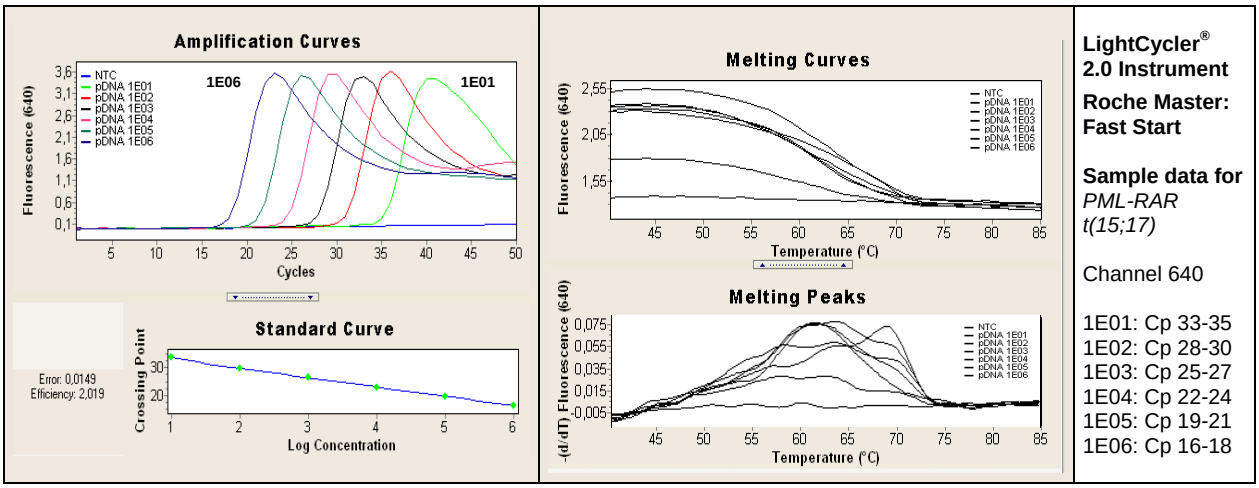


Fig.1. Sample data for the *PML-RAR t(15;17)* detection system.

Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with standard curve for *PML-RAR t(15;17)*. Right panel channel 640 melting analysis for *PML-RAR t(15;17)* (not relevant for detection).

Note: Fluorescence levels depend on instrument settings and can be may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

7.4. Interpretation of data

PML-RAR **(Sample)**	***PML-RAR*** **(Positive Control)**	**Reference Gene (Sample) ***	**No-target Control (NTC)**	**Result**
no amplification	amplification	amplification	negative	***PML-RAR translocation*** not detectable
amplification Cp < 36	amplification	not relevant	negative	Positive for ***PML-RAR***
no amplification	amplification	no amplification	not relevant	Sample problems: Repeat sample preparation
no amplification	no amplification	not relevant	not relevant	Inhibition: Repeat PCR
amplification signal	not relevant	not relevant	positive	Contamination Repeat experiment

* not provided with this kit

Tab. 4. Typical analysis results

8. LightCycler® 480 / Cobas® Z480 Instruments

8.1. 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

Detection Format:
LightCycler® 480 Instrument: 483-640
LightCycler® 480 II Instrument: 498-640
Cobas® Z480 Instrument: 498-645

Program Step:	Denaturation	Cycling			Melting			Cooling
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	00:00:30	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	-

(Melting not relevant for detection) Table 5

8.2. Data Analysis

Perform data analysis, as described in the LightCycler® 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 *PML-RAR t(15;17)* data with Filter Combination 483-640 Quantification mode. The negative control (NTC) must show no signal.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *PML-RAR t(15;17)* should have Cp values between cycles 18 and 35 (Cp values calculated with Second Derivative Maximum method).

8.3. Sample Data – typical results

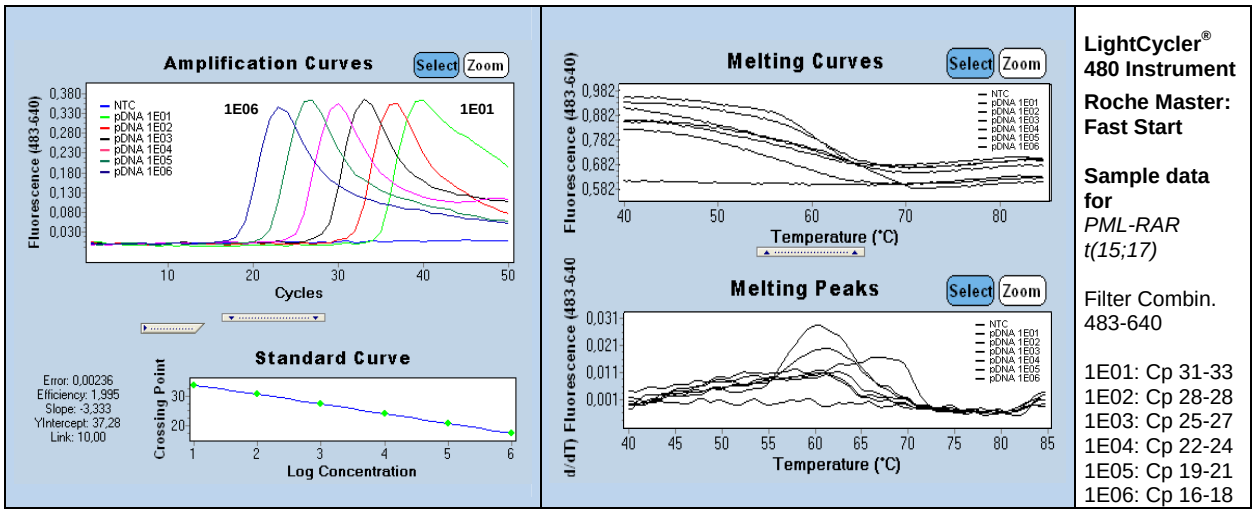


Fig.1. Sample data for the *PML-RAR t(15;17)* detection system.

Data from LightCycler® 480 Instrument. Left panel filter combination 483-640 quantification mode (Second Derivative Maximum) with standard curve for *PML-RAR t(15;17)*. Right panel filter combination 483-640 melting analysis for *PML-RAR t(15;17) (*not relevant for detection).

Note: Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

8.4. Interpretation of data

PML-RAR **(Sample)**	***PML-RAR*** **(Positive Control)**	**Reference Gene (Sample) ***	**No-target Control (NTC)**	**Result**
no amplification	amplification	amplification	negative	***PML-RAR translocation*** not detectable
amplification Cp < 35	amplification	not relevant	negative	Positive for ***PML-RAR***
no amplification	amplification	no amplification	not relevant	Sample problems: Repeat sample preparation
no amplification	no amplification	not relevant	not relevant	Inhibition: Repeat PCR
amplification signal	not relevant	not relevant	positive	Contamination Repeat experiment

* not provided with this kit

Tab. 6 Typical analysis results

9. Material Safety Data

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

10. Version history

V060307	Release version
V100826	Unified PCR protocol hematology
V130628	Cobase Z480 included, editorial changes, cut-off values

Roche SAP order n° 05947189001

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LightCycler® hybridization probes produced under license from Roche Diagnostics GmbH.

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