MLL-AF4 REAL TIME PCR DETECTION KIT

ONKOTEST R2020-20

onkotest

Keep the kit at -15°C to -25°C

Rev. 1.4

www.onkotest.com

Product Information

Chromosomal region 11q23 is frequently rearranged in acute leukemias. The nature of rearrangements is mostly balanced translocations, but also includes unbalanced translocations, inversions, insertions, and tandem duplications. The MLL gene - a transcriptional regulator - resides in this region, and is frequently involved in reciprocal exchanges with various partner genes. One of the most common rearrangements is t(4;11)(q21;q23), in which a chimeric oncogene is formed between MLL and its translocation partner AF4. The t(4;11)(q21;q23) translocation is present in approximately 10% of acute lymphoblastic leukemia (ALL) patients; most frequently in infant leukemia where it is observed over 80%. The t(4;11)(q21;q23) translocation has also been associated with treatment related leukemia (secondary to epipodophyllotoxins). Different breakpoints in the genes result in multiple MLL-AF4 mRNA products with different sizes. Different clustering patterns of breakpoints are observed in infant and non-infant patients (Table 1). It is important to perform molecular diagnostic screening for the presence of t(4;11), along with t(9;22), t(12;21) and t(1;19) in pediatric ALL to determine prognosis and therapeutic approaches.

Table 1: MLL-AF4 fusions

Fusion type(MLL-AF4)	Infant	Non-infant
Exon9-exon5	Less than 10%	16%
Exon9-exon4	rare	25%
Exon10-exon5	Less than 10%	Less than 5%
Exon10-exon4	18%	39%
Exon11-exon5	Less than 10%	rare
Exon11-exon4	55%	Less than 5%

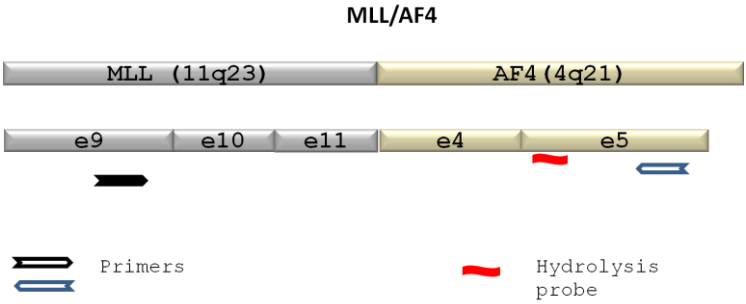


Figure 1: Annealing locations of the PCR primers and hydrolysis probe

The onkotest R2020-20 kit, uses florescent labeled probes to detect the MLL-AF4 chimeric gene product. The kit is designed to detect the presence of all chimeric mRNA's given in Table 1. This kit will not detect mRNA breakpoint products that are outside the sequence confined by the PCR primers provided within the kit.

Kit Contents

Tube	Labeling	Volume& Content
1	Real Time Master Mix (2X)	565 µl (2X) - enough for 56 reactions. Ready to use real time PCR reaction mix.
2	MLL-AF4 Primer Mix	40 µl- enough for 20 patient samples, 8 positive control and 8 negative control reactions.
3	MLL-AF4 probe	40 µl- enough for 20 patient samples, 8 positive control and 8 negative control reactions.
4	Positive Control	40 µl- contains a positive control template enough for 8 reactions.
5	ABL Primer Mix	20 µl- enough for real time reactions of 20 patient samples.
6	ABL Probe	20 µl- enough for real time reactions of 20 patient samples.
7	H_2O (PCR grade)	500 µl- Nuclease free. PCR grade. Use for reaction mixture preparation and as template for negative control reactions.
8	ROX (6µM)	40 µl

The onkotest R2020-20 kit is designed to work with all real time thermal cycler instruments (the onkotest R2020-20 kit has been optimized to work without ROX dye, it can easily be used with ABI real time instruments without ROX. Nevertheless if ROX is to be used, add 1 µl to each reaction tube (the final concentration will be 300nM after the addition of 1 µl ROX). If ROX is added, do not forget to also reduce dH_2O by 1 µl to keep the total reaction volume at 20 µL)

Kit Description

The **onkotest R2020-20** kit uses cDNA of patients (converted from total RNA by reverse transcription) as template for PCR reactions. Real time PCR will amplify target the MLL-AF4 and ABL regions by using highly specific primers provided with the kit. The hydrolysis probe will specifically hybridize to its own complementary target sequence within the amplified PCR product. It is a dual labeled probe in which the fluorophore FAM (6-carboxyfluorescein) is covalently attached to the 5'-end; while a quencher, BHQ (Black hole quencher) is attached at the 3'-end. The quencher molecule quenches the fluorescence emitted by the fluorophore, so as long as the fluorophore and the quencher are in proximity, fluorescence signals are inhibited. When the probe hybridizes to its specific sequence, the 5´–>3´ exonuclease activity of the polymerase degrades the probe and breaks the close proximity between the fluorophore and the quencher. As a result the fluorophore is relieved of the quenching effect and is able to emit fluorescence which can be detected (Figure 2). The fluorescence detected is directly proportional to the amount of fluorophore released, therefore also directly proportional to the amount of DNA template present in the PCR. The hydrolysis probe is both highly sensitive and highly specific; for it requires the presence and amplification of its complementary sequence by the specific primers provided within **onkotest R2020-20** kit.

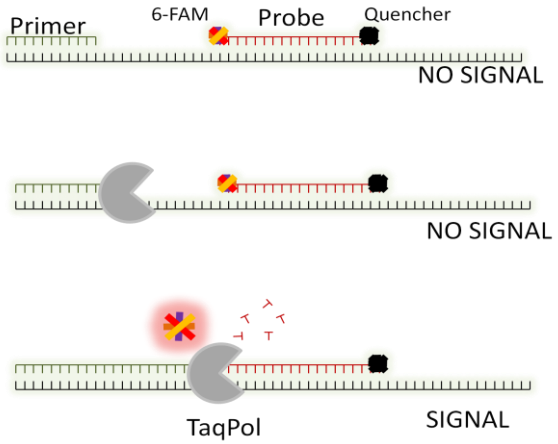


Figure 2: Hydrolysis probe

The positive and internal controls provided with the kit are aimed to prevent false negative results that may be the outcome of failed reactions or poor template quality. Using H_2O as a negative control aims to prevent false positive results by the detection of cross contamination.

Sample Material:

The **onkotest R2020-20** kit is intended for the accurate detection of MLL-AF4 transcripts in bone marrow or peripheral blood samples (EDTA - ethylenediaminetetraacetic acid is the preferred as anticoagulant, heparin may inhibit the PCR reaction) of patients. The kit uses cDNA (converted from total RNA by reverse transcription) as template for the PCR reactions. Using patient RNA directly as template will not yield results.

Number of Tests:

The **onkotest R2020-20** kit contains the reaction mix, primers and probes enough for MLL-AF4 and internal control ABL reactions of 20 patient samples. The kit also provides the reaction mix, primers and probes enough for 8 positive control and 8 negative control reactions.

Handling & Storage

The components of kit should be stored at -20^0 C. Protect fluorescent probes (tubes 3 and 6) from light. While setting up PCR reactions, kit components should be placed on ice. Multiple freeze-thaw cycles may result in the degradation of protein reagents.
IMPORTANT: In order to prevent cross contamination, the positive control tube inside the kit should be stored separately from test samples and added to the reaction mix in a separate area during assay preparation.

Materials Required But Not Provided

Equipment

Real-time thermal cycler
Laminar flow hood/biological safety cabinet
Vortex
Spin-down microcentrifuge
Micropipettes

Consumables

Sterile filtered pipette tips
Serological pipettes
0,2 ml PCR tubes/Capillary tubes/strip tubes/plates (depending on the real-time instrument)

Important Notes & Precautions:

- Separate areas dedicated for RNA isolation, cDNA/PCR reaction preparation and agarose gel electrophoresis is strongly recommended.
- Lab coats and safety equipment (goggles ect.) should be specifically designated to each area
- Separate micropipette sets for each area is strongly recommended.
- Micropipette tips should be DNAse - RNAse free and preferably filtered.
- In order to prevent cross contamination between patient samples, total RNA isolation of samples should be performed separately.
- Avoid biohazard exposure by observing universal precautions when handling all biological materials.

Before You Begin

Before starting be sure the tube contents are fully dissolved. Mix tube contents briefly by vortexing, followed by a spin-down centrifugation to bring down tube contents. Keep all test samples and kit contents on ice when in use.

Procedure

Negative control (NC) - (dH_2O): Tube number 7 is the "no template negative control". This control replaces template cDNA with water in the PCR reactions. It aims for the detection of any cross contamination. The negative control should not give any positive signal. In cases where a positive reaction signal is detected, **ALL** reactions must be repeated. The kit provides MLL-AF4 primers and probes enough for 8 reactions.

Positive control (PC): Tube number 4 contains positive control template. MLL-AF4 detection **MUST BE** positive when this control is used. Otherwise all reactions for all samples must be repeated. It gives a band 113 bp in size on agarose gel electrophoresis. To avoid false positive results resulting from cross contamination we strongly suggest the addition of positive control template in to its reaction mix in a separate area. The kit provides positive control template enough for 8 reactions. This positive control template should not be diluted when used. We recommend the positive control to be evaluated after the 13th cycle. Signal observed at lower cycle numbers should be evaluated as false positive.

Patient Sample Internal Control (IC)-(ABL): The ABL gene product is used as internal control. cDNA from patient samples are used as template for this reaction. The PCR product size is 123 bp. Patient sample PCRs that are negative for ABL, designate poor template quality (RNA and/or cDNA unsuitable for analyses). In case of a negative internal control (negative result for ABL expression), a new cDNA conversion from total RNA should be performed and both MLL-AF4 and ABL PCR reactions should be repeated. New RNA extraction from patient bone marrow/peripheral blood should be performed in cases where a second negative result for the internal control is obtained.

Preparation of the Real-time PCR Mix

Real-time PCR Reaction Panel for One Patient *:

	Negative Control **	Positive Control	Patient ABL	Patient MLL-AF4
dH_2O (tube 7)	8 µl	3 µl	3 µl	3 µl
Real time master mix (2X)(tube 1)	10 µl	10 µl	10 µl	10 µl
MLL-AF4 primer mix (tube 2)	1 µl	1 µl	-	1 µl
MLL-AF4 probe (tube 3)	1 µl	1 µl	-	1 µl
Positive Control (tube 4)	-	5 µl	-	-
ABL primer (tube 5)	-	-	1 µl	-
ABL probe (tube 6)	-	-	1 µl	-
Patient cDNA template	-	-	5 µl	5 µl

Total reaction volume 20 µL

* When more than one patient sample is being tested in a single reaction panel, using one positive and one negative reaction control will be sufficient.
**dH_2O replaces the template

Thermal Profile for Real-Time PCR Instrument

Denaturation	95˚C	10 minutes	1 cycle
Reaction	95˚C	10 seconds	35 cycle
	58˚C	40 seconds (signal acquisition step)*	
Cooling	40 °C	1 minute	1 cycle

When creating your thermal profile if your instrument requires you to define parameters for the 5' and 3' ends: select 6-FAM for the 5 prime end, and "quencher dye" for the 3 prime end.

Evaluation:

The instrument you use will evaluate the real-time PCR reactions using its own software. The onkotest R2020-20 kit has been optimized to perform MLL-AF4 gene product analyses with very high sensitivity. PCR primers and probes have been design to prevent non-specific reactions. Nevertheless we recommend running the PCR products on 2% agarose gel electrophoresis and visualizing the expected specific band. The positive control is expected to be a 113 bp size product. Expected PCR product sizes for different chimeric genes are given in table 2 .The expected band size for ABL is 123 bp (Figure 3).

Table 2: Expected PCR product sizes for different chimeric genes

Fusion type(MLL-AF4)	PCR size
Exon9-exon5	113 bp
Exon9-exon4	158 bp
Exon10-exon5	244 bp
Exon10-exon4	289 bp
Exon11-exon5	357 bp
Exon11-exon4	403 bp

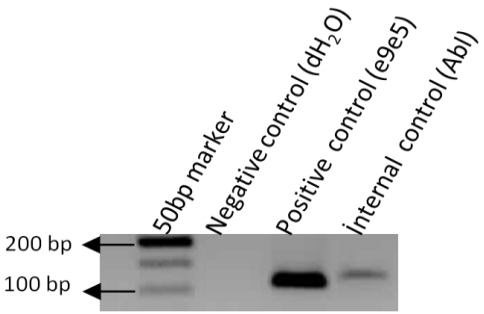


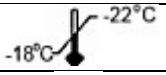
Figure 3: Agarose gel electrophoresis of PCR control reactions.

Product Specifications

Kit Capacity	20 samples
Control Gene	ABL
Reported Values	MLL-AF4 Transcript
Components	MLL-AF4 primer mix/probe ABL primer mix/probe DNA Plasmid Control (MLL-AF4)
Tested Platforms	Roche® LC480, LightCycler® 1.5, 2.0; ABI® StepOnePlus™; Corbett® Rotor-Gene® 6000
Product Order No.	R2020-20

Symbols

The following symbols may appear on the packaging and labeling:

CE	CE sign
	Manufacturer
	Consult instructions for use
IVD	In vitro diagnostic
	Manufacturing date
REF	Catalog number
LOT	Lot number
	Temperature limitation
	Expiration date

CONTACT:

ONKO TEST GENETİK ARAŞTIRMA PROJELENDİRME
SANAYİ VE TİCARET LTD.ŞTİ.

Dokuz Eylul University
DEPARK -(Technology Development Zone) Office: 202
Balcova, İZMİR-TURKEY
Tel: 90-232-4642088
90-216-5732591
info@onkotest.com