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Leukemia FLT3-TKD Mutation Real Time RT-PCR Kit User Manual

LT021920RT50

For use with ABI Prism®7000/7300/7500/7900/Step One Plus; iCycler iQ™4/iQ™5; Smart Cycler II;Bio-Rad CFX 96;Rotor Gene™6000; Mx3000P/3005P;MJ-Option2/Chromo4; LightCycler®480 Instrument

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50 −20 °C CE

1. Intended Use

Leukemia FLT3-TKD Mutation Real Time RT-PCR Kit is used for the detection of Leukemia FLT3-TKD Mutation in leukocyte by using real time PCR systems.

2. Principle of Real-Time PCR

The principle of the real-time detection is based on the fluorogenic 5'nuclease assay. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities in real-time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

3. Product Description

FLT3-TKD mutations are small mutations in the activation loop of FLT3, mostly representing point mutations in codon D835 or deletions of codon I836. They induce constitutive tyrosine phosphorylation leading to activation of the receptor tyrosine kinase and are supposed to represent gain-offunction mutations. The FLT3-TKD mutations occurred in approximately 7% in adults AML.

Leukemia FLT3-TKD Mutation Real Time RT-PCR Kit contains a specific ready-to-use system for the detection of the leukemia FLT3-TKD Mutation using RT-PCR (Reverse Transcription Polymerase Chain Reaction) in the real-time PCR system. The reaction is done in one step real time RT-PCR. The first step is a reverse transcription (RT), during which the mutant gene is transcribed into cDNA. Afterwards, a thermostable DNA polymerase is used to amplify the specific gene fragments by means of PCR (polymerase chain reaction). Fluorescence is emitted and measured by the real time systems optical unit during the PCR. The detection of amplified fragment is performed in fluorimeter **channel FAM** with the fluorescent quencher BHQ1. An external positive control is supplied.

4. Kit Contents

Ref.	Type of reagent	Presentation	50rxns
1	FLT3-TKD Super Mix	1 vial, 480µl	x2
2	RT-PCR Enzyme Mix	1 vial, 28µl	x2
3	Molecular Grade Water	1 vial, 400µl	x2
4	FLT3-TKD Positive Control	1 vial, 30µl	x2

Analysis sensitivity: 1×10^3copies/ml;

Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors .If you use the RNA extraction kits recommended, the analysis sensitivity is the same as it declares. However, when the sample volume is dozens or even hundreds of times greater than elution volume by some concentrating method, it can be much higher.

5. Storage

- All reagents should be stored at -20 °C. Storage at +4 ℃ is not recommended.
- All reagents can be used until the expiration date indicated on the kit label.
- Repeated thawing and freezing (> 3x) should be avoided, as this may reduce the sensitivity of the assay.
- Cool all reagents during the working steps.
- Super Mix should be stored in the dark.

6. Additionally Required Materials and Devices

- Biological cabinet
- Real time PCR system
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)
- Vortex mixer
- RNA extraction kit
- Real time PCR reaction tubes/plates
- Cryo-container
- Pipets (0.5 µl – 1000 µl)
- Sterile filter tips for micro pipets
- Sterile microtubes
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator and freezer
- Tube racks

7. ⚠ Warnings and Precaution

- Carefully read this instruction before starting the procedure.
- For in vitro diagnostic use only.
- This assay needs to be carried out by skilled personnel.
- Clinical samples should be regarded as potentially infectious materials and should be prepared in a laminar flow hood.
- This assay needs to be run according to Good Laboratory Practice.
- Do not use the kit after its expiration date.
- Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
- Once the reagents have been thawed, vortex and centrifuge briefly the tubes before use.
- Prepare quickly the Reaction mix on ice or in the cooling block.
- Set up two separate working areas: 1) Isolation of the RNA/ DNA and 2) Amplification/ detection of amplification products.
- Pipets, vials and other working materials should not circulate among working units.
- Use always sterile pipette tips with filters.
- Wear separate coats and gloves in each area.
- Do not pipette by mouth. Do not eat, drink, smoke in laboratory.
- Avoid aerosols.

8. Sample Collection, Storage and transport

- Collected samples in sterile tubes.
- Specimens can be extracted immediately or frozen at -20°C to -80°C.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents

9. Procedure

9.1.1 Leukocytes separation

You can use commercial erythrocytes lysis buffer to remove the erythrocytes from blood samples. Please refer to the specific instructions for erythrocytes lysis buffer.

Attention: Do not use the lymphocytes separation media to obtain leukocytes.

The leukocytes precipitate obtained can be directly used for RNA extraction, and it can also be dissolved in RNA extraction reagents (such as Trizol, RLT buffer) for long-time storage at -80℃. It's strongly recommended not to store the leukocytes precipitate without any RNA extraction reagent.

9.1.2 RNA extraction from leukocytes

RNA extraction kits are available from various manufacturers. You may use your own extraction systems or the commercial kits based on the yield. For the RNA extraction, please comply with the manufacturer's instructions. The recommended extraction kit is as follows:

Nucleic Acid Isolation Kit	Cat. Number	Manufacturer
Qiagen RNeasy Mini Kit	74106	QIAGEN

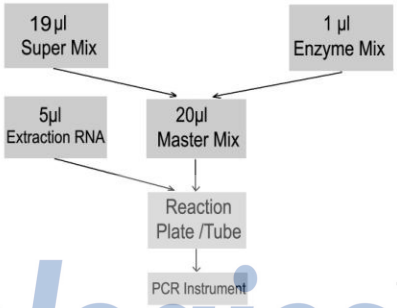
e.g. RNA extraction with Trizol

1)Add 1 ml Trizol into the leukocytes, and pipet up and down several times to make cells fully dissolved;(Increase the volume of Trizol proportionately if leukocytes are more than 5×10^6 cells).
2) Add 0.2 ml chloroform and shake the tube by vortex for 15 sec at least;
3) incubate the tube at room temperature for 2-3 min
4) Centrifuge the sample at 13,000 rpm for 15 min at 4 ℃.
The following procedures should be operated on ice box;
5) Transfer the aqueous phase above (approximately 0.4-0.6 ml) into a new 1.5 ml centrifuge tube, avoiding disturbing any of the white interphase.
6) Add pre-cooled isopropanol into the aqueous phase, and mix by pipetting up and down for 10 times.
7) Incubate for 1 hour at -20 ℃;
8) Centrifuge at 13,000 rpm for 15 min at 4 ℃, and carefully remove the supernatant from the tube;
9) Add pre-cooled 75% ethanol and gently pipet RNA pellet;
10) Centrifuge the sample at 13,000 rpm for 15 min at 4 ℃, and carefully remove the supernatant from the tube avoiding disturbing the RNA pellet;
11) Add 40 µl DEPC-H_2O to the RNA pellet after Air drying for 5-10 min, then shake gently.
12) Centrifuge instantaneously, and incubate for 10 min at room temperature to make RNA fully dissolved.

Extracted RNA can be used for following PCR reactions immediately, or stored at -80 ℃ for long time.

9.2 RT-PCR Protocol

The Master Mix volume for each reaction should be pipetted as follows:



1) The volumes of Super Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of controls and sample prepared. Molecular Grade Water is used as the negative control. For reasons of unprecise pipetting, always add an extra virtual sample. Mix completely then spin down briefly in a centrifuge. Mix completely then spin down briefly in a centrifuge.
2) Pipet **20µl** Master Mix with micropipets of sterile filter tips to each of the real time PCR reaction plate/tubes. Separately add **5µl** RNA sample, positive and negative controls to different plate/tubes. Immediately close the plate/tubes to avoid contamination.
3) Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.
4) Perform the following protocol in the instrument:

45°C for 20min	1cycle
95°C for 5min	1cycle
95°C for 15sec, 58°C for 30 sec, 72°C for 45 sec (Fluorescence measured at 58°C)	45cycles

Selection of fluorescence channels	
FAM	Target Nucleic Acid

5) ⚠ If you use ABI Prism® system, please choose **"none"** as **passive reference** and **quencher.**

10. Threshold setting: just above the maximum level of molecular grade water.

11. Quality control: Negative control and positive control must be performed correctly, otherwise the sample results is invalid.

Channel / Control	Ct value
	FAM
Molecular Grade Water	UNDET
Positive Control(qualitative assay)	≤35

12. Data Analysis and Interpretation

The following results are possible:

	Ct value	Result Analysis
1	UNDET	Below the detection limit or negative
2	≤43	Positive; the sample contains FLT3-TKD mutation;
3	43~45	Re-test; If it is still 43~45, report as I#