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AFP mRNA Expression in Peripheral Blood Real Time RT-PCR Kit
User Manual

LT029200RT

For use with ABIPrism® 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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1. Intended Use
AFP mRNA real time RT-PCR Kit is used for the detection of AFP mRNA in mononuclear cell 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
α-Fetoprotein (AFP), a serum protein produced in large amounts during fetal life, rapidly reduces from late fetal life and is essentially scarce in normal adults. The synthesis of AFP is often associated with the development of HCC and yolk sac tumors. The detection of serum AFP provides a useful marker for diagnosis and prognosis of these tumors. However, the serum AFP level does not always correspond to the clinical stage of HCC. Recent molecular biological techniques have provided a method for detecting malignant cells in the peripheral blood by amplification of messenger RNA (mRNA) of various genes specific to a particular cell type from peripheral blood mononuclear cells. AFP mRNA has been demonstrated to be one of the candidate molecules for detecting HCC cells in the blood. AFP mRNA in the peripheral blood of patients with hepatocellular carcinoma (HCC) may indicate hematogenous spread of HCC. AFP mRNA real time RT-PCR kit contains a specific ready-to-use system for the detection of the AFP mRNA using RT-PCR (Reverse Transcription Polymerase Chain Reaction) in the real-time PCR system. The master contains three Super Mixes for the specific amplification of M-BCR,m-BCR and μ-BCR. The reaction is done in one step real time RT-PCR. The first step is a reverse transcription (RT), during which the AFP mRNA 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 BCR-ABL fragment is performed in fluorimeter **channel FAM** with the fluorescent quencher BHQ1. An external positive control (1×10^7copies/ml) supplied, allows the determination of the gene load. For further information, please refer to section 9.3 Quantitation.

4. Kit Contents

Ref.	Type of reagent	Presentation 25rxns
1	AFP Super Mix	1 vial, 480μl
2	RT-PCR Enzyme Mix	1 vial, 28μl
3	Molecular Grade Water	1 vial, 400μl
4	AFP Positive Control (1×10^7copies/ml)	1 vial, 30μl

Analysis sensitivity:1×10^3copies/ml ； LOQ：2×10^3～1×10^8 copies/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°C 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
- Vortex mixer
- Cryo-container
- Sterile filter tips for micro pipets
- Disposable gloves, powderless
- Refrigerator and Freezer
- Real time PCR system
- Real time PCR reaction tubes/plates
- Pipets (0.5μl – 1000μl)
- Sterile microtubes
- Biohazard waste container
- Tube racks
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)

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.

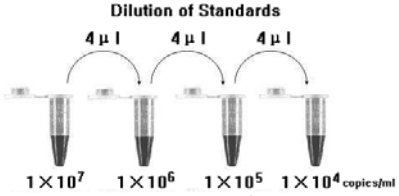
8. Sample Collection, Storage and transport
- Collected samples in sterile tubes.
- Specimens can be extracted immediately or frozen at -20°C to -80°C.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents.

9. Procedure

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

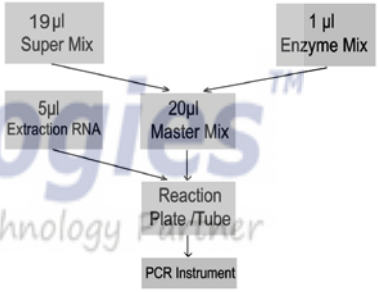
RNA Extraction Kit GEN 52-904 LT

9.2 Quantitation
The kit can be used for **quantitative** or **qualitative** real-time RT-PCR. A positive control defined as 1×10^7copies/ml is supplied in the kit.
For performance of quantitative real-time PCR, Standard dilutions must prepare first as follows. Molecular Grade Water is used for dilution.
Dilution is not needed for performance of qualitative real-time PCR.
Take positive control (1×10^7copies/ml) as the starting high standard in the first tube. Respectively pipette **36ul** of Molecular Grade Water into next three tubes. Do three dilutions as the following figures:



To generate a standard curve on the real-time system, all four dilution standards should be used and defined as standard with specification of the corresponding concentrations.
Attention:
A. Mix thoroughly before next transfer.
B. The positive control contains high concentration of the target DNA. Therefore, be careful during the dilution in order to avoid contamination.

9.3 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, standards, 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.
2) Pipet **20μl** AFP 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 10min	1cycle
95°C for 15min	1cycle
95°C for 15sec, 60°C for 1min (Fluorescence measured at 60°C)	40cycles

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. Calibration for quantitative detection: Input each concentration of standard controls at the end of run, and a standard curve will be automatically formed.

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

Channel / Control	Ct value FAM
Molecular Grade Water	UNDET
Positive Control(qualitative assay)	≤35
QS（quantitative detection）	Correlation coefficient of QS curve≤—0.98

13. Data Analysis and Interpretation
The following results are possible:

	Ct value FAM	Result Analysis
1#	UNDET	Below the detection limit or negative
2#	≤38	Positive; and the software displays the quantitative value
3#	38～40	Re-test; if it is still 38~40, report as 1#