

780 Dubuque Avenue
So. San Francisco, CA 94080, U.S.A.
Tel: (800) 989-6296 / Fax:(650)871-2857
<http://www.maximbio.com>
E-mail: mbi@maximbio.com

Custom Real Time PCR Kit for Mouse sFLT1 Gene Expression
Cat No. TP-10045: Probe Set

**INSTRUCTION
MANUAL**

ID-M10204
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The use of Taqman[®] fluorogenic probes in 5' nuclease assays is covered by U.S. Patent No. 6,214,979, 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent No. 5,538,848, owned by the Perkin-Elmer Corporation. Purchase of the Custom Real Time PCR Kit does not provide a license to use this patented technology. A license to practice this technology must be obtained from Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404 or from Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, CA 94501.

The Polymerase Chain Reaction (PCR) process is covered by patents owned by Hoffman-LaRoche. Use of the PCR process requires a license. A license for diagnostic purposes may be obtained from Roche Molecular System. A license for research may be obtained by the purchase and the use of authorized reagents and DNA thermocyclers from the Perkin-Elmer Corporation or by negotiating a license with Perkin-Elmer.

This product is intended for research use only and not for diagnostic purposes.

INTRODUCTION

Analysis of the temporal and spatial distribution of RNA expression provides researchers with important clues about the genes' function in their own systems. Northern Blot and RNase Protection Assay are the most widely used procedures for determining the abundance of a specific mRNA in a total or poly(A) RNA sample. RT-PCR, especially the homogenous Real Time RT-PCR, provides an easy and accurate method to detect gene expression^{1,2,3}.

The Real Time PCR Gene Expression Kit was developed as a research tool for real-time *in vitro* quantitative evaluation of gene expression. Real Time PCR Kit provides all the components necessary to perform a 5' nuclease assay. Primers and FRET probes for Human IL-8

(Genbank Accession: Y00787) were designed to generate a 182 bp PCR product and an optimal fluorescent signal under the recommended conditions.

The purpose of Real Time PCR technology is to detect known sequences of genomic, plasmid, or complementary DNA (cDNA). In RNA quantitation assays, the Real Time PCR Gene Expression Kit is used in a single step RT-PCR or the second step of a two-step reverse transcription reaction (RT-PCR) protocol. The template is cDNA generated from a reverse transcription reaction. The Real Time PCR Gene Expression Kit may be used for Real Time or Plate Read (end-point) detection of DNA and cDNA. See Instrument instruction for details.

GENERAL CONSIDERATIONS

Please follow the recommended procedures below:

1. Wear a clean lab coat not previously worn while handling amplified PCR products or used during sample preparation, and clean gloves when preparing samples for PCR amplification.
2. Change gloves whenever you suspect that they have been contaminated.
3. Maintain separate areas and dedicate equipment and supplies for:
 - Sample preparation
 - PCR setup
 - PCR amplification
 - Analysis of PCR products
4. Never bring amplified PCR products into the PCR setup area.
5. Open and close all sample tube carefully. Try not to splash or spray PCR samples.
6. Keep reactions and components capped as much as possible.
7. Use a positive-displacement pipet or aerosol-resistant pipet tips.
8. Clean lab benches and equipment periodically with a 10% bleach solution.

Fluorescent Contaminants

As fluorescent contaminants may interfere with the assay and give false-positive results, it may be necessary to include a No-Amplification Control tube that contains sample, but no enzyme. If the absolute fluorescence of the No-Amplification Control is greater than that of the No-Template Control after PCR, fluorescent contaminants may be present in the sample or in the heat block of the thermal cycler.

Performing Routine Analysis

For routine assays, perform analysis using optimum primer and probe concentrations and specified thermal cycling conditions as described in this protocol.

Use optimized conditions to amplify DNA or cDNA obtained from RT. The following ranges of DNA and RNA can be used:

- Genomic DNA: 0.1ng - 1 µg
- RNA: 10 pg - 100 ng

TAQMAN OVERVIEW

The PCR reaction acts on the 5'-nuclease activity of *Taq* DNA Polymerase to cleave a FRET probe during PCR. The FRET probe contains a reporter dye at its 5'-end and a quencher dye at its 3'-end.

When the probe has not been cleaved, the proximity of the reporter dye to the quencher dye effectively suppresses the reporter fluorescence primarily by Forster-type energy transfer (Forster, 1948; Lakowicz, 1983).

During the course of the Real Time PCR reaction, cleavage of the probe separates the reporter dye from the quencher dye and results in increased fluorescence of the reporter dye. The increase in PCR products can thereby be detected by observing the increase in fluorescence of the reporter dye, as illustrated below (Figure 1).

If the target gene of interest is present during PCR, the probe will specifically anneal between the forward and reverse primer sites. The *Taq* Polymerase will cleave the probe between the reporter and quencher sites in every cycle only if the probe has hybridized to the target gene.

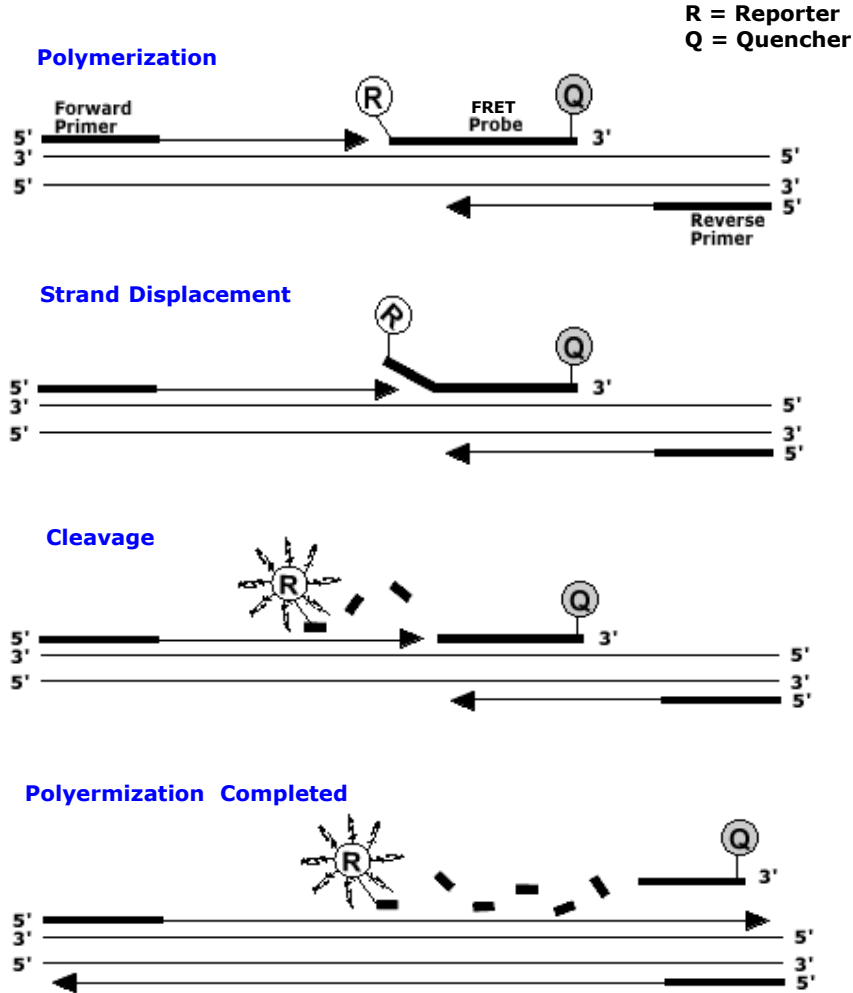


Figure 1 The forklike-structure-dependent, polymerization-associated 5'-3' nuclease activity of *Taq* DNA Polymerase during PCR.

PROBE KIT MATERIALS AND REAGENTS

TP-10045
FRET probe reaction kit
Store all reagents at -20°C

| Product Code | Kit Component | Amount |
|--------------|--|--------|
| mFLT1-PB1 | 10X FRET PCR Probes* for Mouse sFLT1 Instruction Manual | 500 µl |

Materials & Equipment Not Included:

- ABI PRISM 7700 System or its equivalent
- Taq DNA Polymerase: Perkin-Elmer Taq or its equivalent
- Maxim RT Kit: Cat. No. RT-40001 or RT-40002
- Maxim Single Step RT-PCR Kit Cat No.: RT-40003
- Pipettes: P1000, P200, and P20
- Pipette tips
- Fluorescent Plate Reader
- Mouse sFLT1 primer Cat No.: TM-60045

REAL TIME PCR FRET PROBE

Catalog No.: TP-10045

Gene ID: Mouse sFLT1

Size/Form: One vial of 500 µL 10X FRET Probe Solution (~1 nmole in TE buffer)

Probe T_m: 69°C

cDNA reference: GenBank Accession#: D88690

Related Products: Mouse sFLT1 primer with control Cat No.: TM-60045

Storage:

The FRET Probe Solution should be stored at -20°C. If used within one week, Primer Pair Solution and Positive Control Solution may also be stored at 2-8°C. The FAM fluorophore and Black Hole Quencher are light-sensitive and should be protected from light whenever possible.

Suggested Reaction Conditions:

The FRET Probe Solution is intended for use in the detection of the DNA fragment by PCR. The FRET probe Solution should be briefly centrifuged to concentrate the contents in the bottom of the tube before use. For each 50µL PCR reaction, use 2-5 µL of the FRET Probe Solution. Sufficient reagent is supplied for 100 individual detection reactions.

INTERPRETING RESULTS

Black Hole Quencher

The hybridization probes are custom-designed to spectrally pair fluorophores and quenchers; each are linked covalently so as not to interfere with probe-target hybridization. The Black Hole Quencher (BHQ) dyes are a new class of dark quenchers that have no fluorescence of their own, essentially removing background problems that other quenchers have. BHQ dyes also prevent fluorescence until hybridization has taken place. Probes that incorporate BHQ dyes provide a powerful and specific means to identify and quantify a diversity of biomolecules.

Multi-componenting

Multicomponenting is the term used to distinguish the contribution each individual dye makes to the fluorescent spectra. The overlapping spectra from the pure dye components generate the composite spectrum. This spectrum represents one-fluorescent reading from one well. Current pure dye menus available for Multicomponent Analysis are:

| | |
|------------|--------------------|
| Reporters: | FAM, TET, JOE, HEX |
| Quenchers: | BHQ |

R_n and ΔR_n Values

R_n⁺ is the R_n value of a reaction containing all components including the template.

R_n⁻ is the R_n value of an unreacted sample. This value may be obtained from the early cycles of a Real Time run, those cycles prior to a detectable increase in fluorescence. This value may also be obtained from a reaction not containing template.

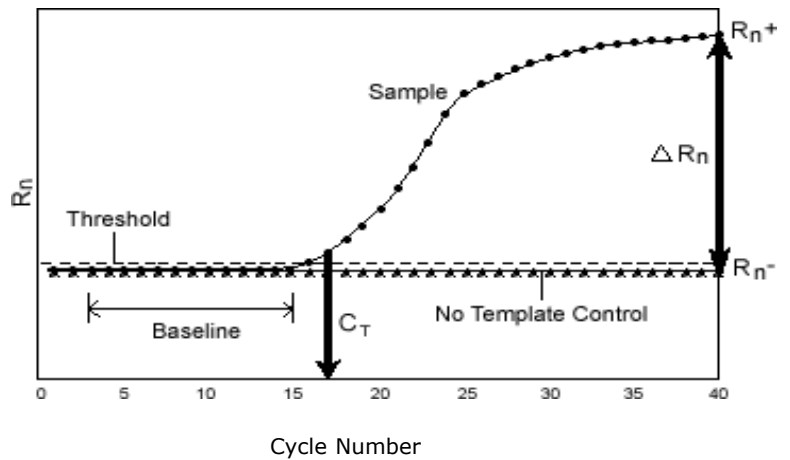
ΔR_n is the difference between R_n⁺ value and the R_n⁻ value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions.

The following equation expresses the relationship of these terms:

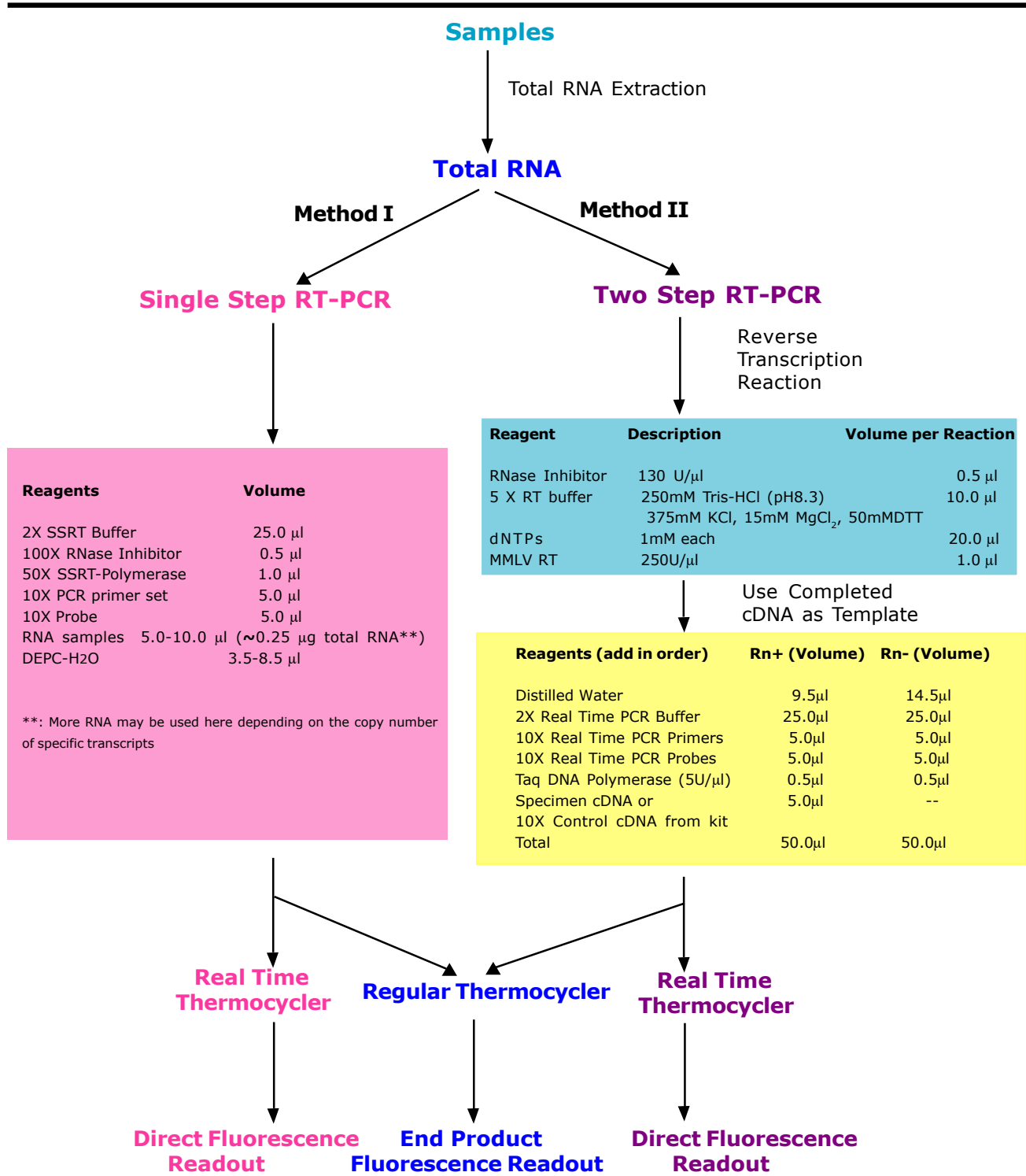
| | | | |
|----------------------------------|-----------|---|--|
| $\Delta R_n = (R_n^+) - (R_n^-)$ | $R_n^+ =$ | Emission Intensity of Reporter Emission Intensity of Passive Reference | PCR with template |
| | $R_n^- =$ | Emission Intensity of Reporter Emission Intensity of Passive Reference | PCR without template or early cycles of a Real Time reaction |

Real Time Detection

The threshold cycle or C_T value, is the cycle at which a statistically significant increase in ΔR_n is first detected. Threshold is defined as the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor. On the graph to the right, the threshold cycle occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.



TWO ALTERNATIVE METHODS OF REAL TIME PCR



SINGLE STEP RT-PCR PROCEDURE

Method I:

Single Step RT Kit available separated from Maxim: Cat.No. RT-40003, 50 reactions

A. Preparing a RT-PCR Master Mix

1. Quickly thaw each tube; place on ice.

Note: Thaw RT Buffer at room temperature before use and vortex to resuspend crystals. If residual precipitate fails to dissolve, spin and use supernatant.

2. Spin each tube briefly in a microcentrifuge and return to ice.

3. Prepare a Master Mix as described below. Prepare sufficient Master Mix for all of your reactions plus one additional reaction to ensure adequate volume. You should plan to perform one positive control reaction in addition to your experimental reactions. An optional negative control can be performed as well.

B. Suggested Single Step RT-PCR Real Time Protocol:

| Reagents | Volume |
|-----------------------|---|
| 2X SSRT Buffer | 25.0 μ l |
| 100X RNase Inhibitor | 0.5 μ l |
| 50X SSRT-Polymerase | 1.0 μ l |
| 10X PCR primer set | 5.0 μ l |
| 10X Probe | 5.0 μ l |
| RNA samples | 5.0-10.0 μ l (\sim 0.25 μ g total RNA**) |
| DEPC-H ₂ O | 3.5-8.5 μ l |

** : More RNA may be used here depending on the copy number of specific transcripts

C. PCR Thermocycle Profile:

A typical thermo-profile is given below using a Real Time PCR machine or by reading fluorescence after cycles have been completed.

| Temperature | Time | Cycles |
|--------------|------------------|--------|
| 50°C | 30 min | 1X |
| 95°C | 5 min | 1X |
| 95°C 60°C | 15 sec 60 sec | 40-50X |

D. Launch the Proper Real Time Software, or Read Fluorescence after Cycles.

TWO STEP RT-PCR PROCEDURE Method II:

RT Kit available separated from Maxim: Cat.No. RT-40001, 10 reaction, or RT-40002, 50 reactions

A. RT Protocol:

The isolation of undegraded, intact RNA is an essential prerequisite for successful first strand synthesis and PCR amplification. Care should be taken to avoid RNase contamination of buffers and containers used for RNA work by pretreating with DEPC, autoclaving, and baking. Always wear sterile gloves when handling reagents. Use cDNA derived from 10^{5-4} cells (0.01-0.1 μ g cDNA) and apply them to each Taqman reaction.

1. Prepare total RNA, mRNA or use the control GAPDH RNA which is provided in Maxim's RT kit.
2. Equilibrate 3 water baths: 37°C, 70°C and 95°C.
3. **On ice**, pipet 1-2 μ g mRNA or 10 μ g total RNA (from 10^6 cells) dissolved in pure water or 2 μ l control GAPDH RNA into a RNAase free reaction vial. We strongly recommend including a positive control reaction when setting up a RT-PCR reaction for the first time.
4. Add sterile water to a final volume of 14.5 μ l.
5. Add 4 μ l random hexamer (50 μ M) or Oligo(dT) (50 μ M).
NOTE: The hexamer and Oligo(dT) RT reactions may be run simultaneously.
6. Incubate tube(s) at 70°C for 5 minutes and quickly chill on ice.
7. Begin your RT reaction by adding the following reagents to your hexamer or Oligo mixture:

| Reagent | Description | Volume per Reaction |
|-----------------|---|---------------------|
| RNase Inhibitor | 130 U/ μ l | 0.5 μ l |
| 5 X RT buffer | 250mM Tris-HCl (pH8.3) 375mM KCl, 15mM MgCl ₂ , 50mMDTT | 10.0 μ l |
| dNTPs | 1mM each | 20.0 μ l |
| MMLV RT | 250U/ μ l | 1.0 μ l |

8. Incubate the RT mixture at 37°C for 60 minutes.
9. Then, heat RT mixture at 95°C for 10 minutes and quickly chill on ice.
10. Add another **50 μ l** water or 0.1X TE buffer.
11. 1-5 μ l of above cDNA is sufficient for most genes in a standard Real Time reaction. However, more or less DNA may be needed depending on the copy number of the specific gene.

TWO STEP RT-PCR PROCEDURE Method II:

B. Suggested Two Step Real Time PCR Protocol:

1. Taq DNA polymerase from Perkin-Elmer or its derivatives are recommended.
2. Reaction Mixture Preparation:
 - A. Set up the Real Time PCR reactions with the test samples, and Real Time PCR Primers, FRET Probes and Buffers provided in the kit according to the table below:

| Reagents (add in order) | Rn+ (Volume) | Rn- (Volume) |
|----------------------------------|-------------------------------|-------------------------------|
| Distilled Water | 9.5 μ l | 14.5 μ l |
| 2X Real Time PCR Buffer | 25.0 μ l | 25.0 μ l |
| 10X Real Time PCR Primers | 5.0 μ l | 5.0 μ l |
| 10X Real Time PCR Probes | 5.0 μ l | 5.0 μ l |
| Taq DNA Polymerase (5U/ μ l) | 0.5 μ l | 0.5 μ l |
| Specimen cDNA or | 5.0 μ l | -- |
| 10X Control cDNA from kit | | |
| Total | 50.0 μl | 50.0 μl |

- B. EDTA concentration in the test sample must not exceed 0.5 mM because Mg⁺⁺ concentration in PCR Buffers is limited to certain ranges. Additional Mg⁺⁺ may be added to the PCR mixture to compensate for EDTA. We strongly recommend running a PCR reaction with the provided positive control. Since the PCR DNA polymerase needed in each reaction is in a very small volume, it is recommended that all of the PCR components be premixed in a sufficient quantity for daily needs and then dispensed into individual reaction vials. This will help you to achieve more accurate measurements. Hot Start PCR is highly recommended ^{7,10}.

C. PCR Thermocycle Profile:

Reaction profiles will need to be optimized according to the machine type and needs of user. Please take note that temperature variations occur between different thermocyclers, therefore, the annealing temperature in the sample profile below is given as a range. It will be necessary to determine the optimal temperature for your individual thermocycler. An example of a time-temperature profile for the positive control PCR reaction optimized for Perkin Elmer machine type 9600 or ABI PRISM 7700 is provided below:

| Temperature | Time | Cycles |
|--------------|------------------|--------|
| 95°C | 5 min | 1X |
| 95°C 60°C | 15 sec 60 sec | 40-50X |

D. Launch the Proper Real Time Software, or Read Fluorescence after Cycles.

TROUBLESHOOTING

1. REAL TIME AMPLIFICATION

| Observation | Possible Cause | Recommended Action |
|---|--|--|
| 1.1. $\Delta R_n <$ No template Control ΔR_n , and no amplification plot | 1.1a. Inappropriate reaction conditions 1.1b. Incorrect dye components chosen 1.1c. Reaction component omitted 1.1d. Degraded template or no template 1.1e. Reaction inhibitor present | 1.1a. Re-optimize RT-PCR 1.1b. Check dye component 1.1c. Check all reagents added 1.1d. Repeat with fresh template 1.1e. Repeat with purified template |
| 1.2. $\Delta R_n <$ No template Control ΔR_n , and both reactions show an amplification plot. | 1.2a. Amplicon contamination of reagents 1.2b. Template contamination of reagents | 1.2a. Check for contamination 1.2b. Use fresh reagents |
| 1.3. Shifting R_n value during the early stages of PCR (cycles 0-5) | 1.3. Fluorescent emissions have not stabilized to buffer conditions of reaction mixture. This does not affect PCR, or the final results. | 1.3a. Reset lower value of baseline range. 1.3b. Pre-formulate the probe, primer, and PCR reaction mixture to allow the reaction mixture to equilibrate. |
| 1.4. Abnormal amplification | 1.4. Ct value $<$ 15, amplification signal detected in early cycles | 1.4a. Dilute the sample to increase Ct. 1.4b. Reset lower value of baseline range. |
| 1.5. Ct value is higher than expected | 1.5a. Less template added than expected. 1.5b. Sample has degraded. | 1.5a. Increase sample amount. 1.5b. Evaluate sample integrity. |
| 1.6. Small ΔR_n . | 1.6a. PCR efficiency is poor. 1.6b. Low copy number of target. | 1.6a. Recheck the optimization 1.6b. Increase starting copy number |
| 1.7. Ct value is lower than expected | 1.7a. More sample added than expected. 1.7b. Contamination. | 1.7a. Reduce sample amount 1.7b. Review "contamination" section |
| 1.8. Standard deviation of Ct value $>$ 0.16. | 1.8. Inaccurate pipetting. | 1.8. Calibrate pipettors. |

PRECAUTIONS AND STORAGE

Precautions

The National Institute of Occupational Safety and Health (NIOSH) has issued a bulletin citing the potential explosion hazard due to the reaction of sodium azide with copper, lead, brass, or solder in plumbing systems. Although sodium azide is added at minimal concentration, it is recommended that a copious amount of water be used to flush the drain pipeline after disposal of these reagents in the plumbing system.

Storage

1. Store all Real Time PCR Kit components at -20°C. Under these conditions components of the kit are stable for 6 months.
2. Isolate the kits from any sources of contaminating DNA, especially amplified PCR product.
3. Do not mix Real Time PCR Kit components that are from different lots. Each lot is optimized individually.

REFERENCES

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