User Bulletin #5

ABI PRISM® 7700 Sequence Detection System

August 10, 1998 (updated 01/2001)

SUBJECT: Multiplex PCR with TaqMan® VIC Probes

Overview

Applied Biosystems now offers probes constructed with the new TaqMan® VIC reporter dye. The characteristics of the VIC dye make it an excellent candidate to replace existing TaqMan® JOE and HEX reporter dyes. The increased signal strength and improved spectral resolution also make VIC-labeled probes the ideal second probe for a multiplex PCR system.

This user bulletin describes the characteristics of VIC probes in relation to the existing JOE probes. It also contains guidelines for defining limiting primer concentrations in a one- or two-step multiplex reverse transcription-polymerase chain reaction (RT-PCR) system using VIC probes.

The following topics are covered in this user bulletin:

IMPORTANT To use VIC probes on the ABI PRISM® 7700 Sequence Detection System (SDS), you must first calibrate the instrument with the Sequence Detection Systems Spectral Calibration Kit (P/N 4305822). This kit contains the new SYBR® Green and VIC fluorescent dye standards used to update the spectra components file in the SDS software. See *User Bulletin #4: Generating New Spectra Components* (P/N 4306234).

Note All documents referred to in this user bulletin are available though the internet at the Applied Biosystems technical support documentation library or through Fax-on-Demand (see "To Obtain Documents on Demand" on page 16 for information).

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Applied Biosystems

Characteristics of TaqMan VIC Probes

Introduction

Applied Biosystems designed the VIC dye as a replacement for the TaqMan® JOE and HEX dyes currently in use with ABI PRISM 7700 instruments. The VIC dye features improvements in fluorescent signal strength, spectral resolution, and production cost. This section describes these characteristics of the VIC reporter dye in comparison to the existing reporters.

Increased Signal Strength

The fluorescent signal from VIC dye is nearly four times stronger than JOE. Figure 1 illustrates the difference between the emissions of the two dyes. In combination with the improved spectral resolution, the increased signal strength of the VIC dye is extremely important in multiplex systems.

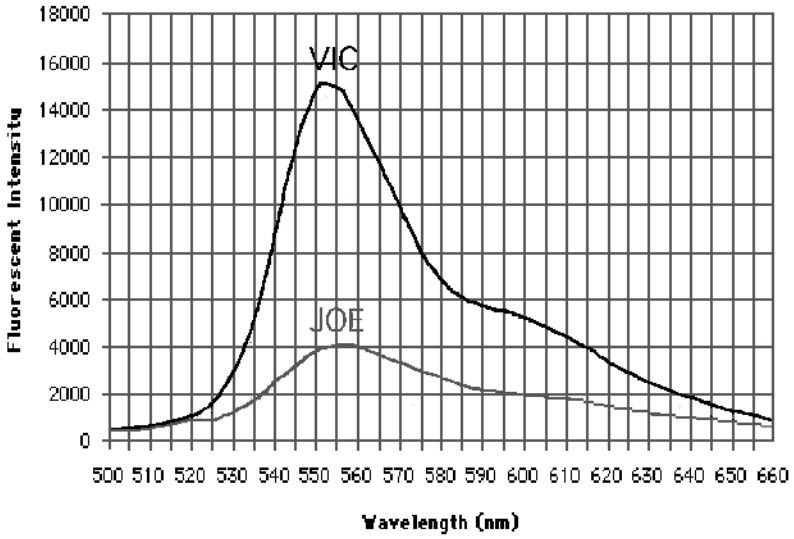

Figure 1. Emission spectra of VIC and JOE dye standards (200nM)

Improved Spectral Resolution

Spectral resolution refers to the degree of separation between the spectra of dyes in a set. Figure 2 illustrates the spectral resolution of four dyes used in TaqMan assays.

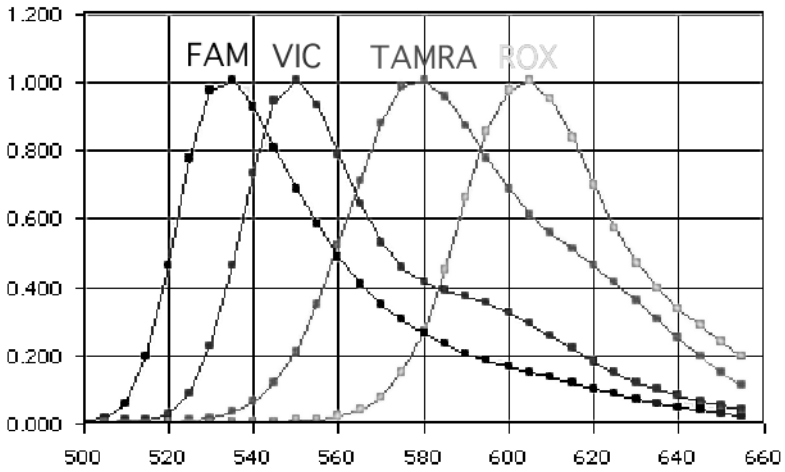

Figure 2. Dual probe (VIC and FAM) system pure dye view

Because of experimental variation in measuring multiple dye spectra, multicomponenting introduces some error into the determination of each dye's contribution. The degree of error depends on how well the various dyes are spectrally resolved. The less the spectral overlap between the dyes, the less the error. Therefore, reporter dyes with the largest difference in emission maxima provide the most accurate quantitation with two probes in one tube (i.e. 6-FAM and VIC).

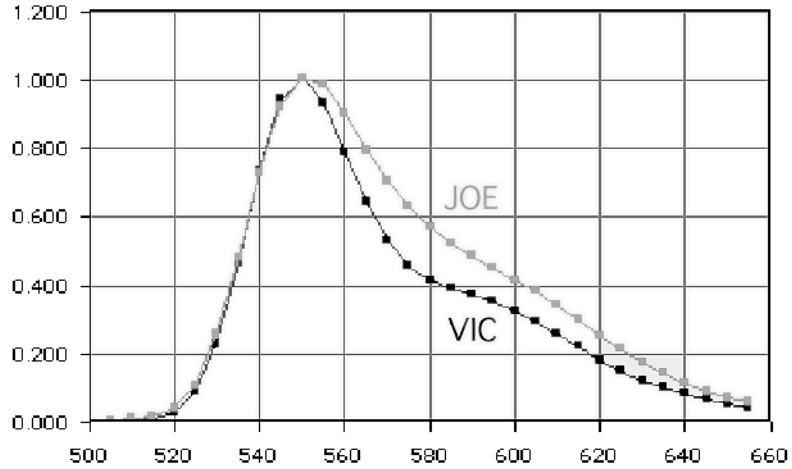

Figure 3. VIC and JOE pure dye view

Figure 3 illustrates the improved spectral resolution of the VIC dye. The VIC spectrum consists of a narrow peak with a half band width 15 percent less than that of JOE. Consequently, the VIC spectrum overlaps less with surrounding dyes and introduces less error into the system.

Probe Availability and Production Cost

Unlike the JOE and HEX reporters, probes labeled with the VIC dye are available at three synthesis scales. Probes labeled with HEX and JOE are only available in the 50,000–100,000 pmol scale because they are difficult to synthesize and must undergo additional purification. VIC dye synthesis chemistry however, is similar to that of TaqMan® FAM and TET dyes. Consequently, Applied Biosystems can offer VIC probes in the three quantities listed below at prices lower than current HEX and JOE labeled probes.

Part #	Description	Quantity (pmol.)
450025	TaqMan Probe, HPLC purified 5´-Fluorescent label: FAM, VIC or TET 3´-Fluorescent label: TAMRA + HPLC Purification	5,000–6,000
450024	TaqMan Probe, HPLC purified 5´-Fluorescent label: FAM, VIC or TET 3´-Fluorescent label: TAMRA + HPLC Purification	15,000–25,000
450003	TaqMan Probe, HPLC purified 5´-Fluorescent label: FAM, VIC or TET 3´-Fluorescent label: TAMRA + HPLC Purification	50,000–100,000

Multiplex RT-PCR

Introduction

The increased signal strength and improved spectral resolution of VIC probes make them ideal second probes for a multiplex system. This section contains brief instructions for defining limiting primer concentrations using one- and two-step RT-PCR.

Multiplex PCR is the use of more than one primer pair in the same tube. You can use this method for relative quantitation where one primer pair amplifies the target and another primer pair amplifies the endogenous reference. You can perform a multiplex reaction using either the Standard Curve Method or the Comparative C_T Method. See "Multiplex PCR (Same Tube)" on page 16 of the *ABI PRISM 7700 Sequence Detection System User Bulletin #2* (P/N 4303859) for a detailed description of multiplex PCR.

Multiplex Strategy

The strategy for performing two independent reactions in the same tube is to adjust primer concentrations such that accurate threshold cycles (C_T) are obtained for both targets. In order to do this, it is necessary to limit the primer concentrations for the majority species. By doing so, the amplification of the majority species can be stopped before it can limit the common reactants available for amplification of the minority species.

Limiting Primer Protocol

To define limiting primer concentrations, perform the following experiments:

Step	Action	See Page
1	Evaluate the Relative Abundance of Target and Reference Species	6
2	Determine the Optimal Primer Concentrations	6
3	Determine the Linear Dynamic Range	7
4	Define Limiting Primer Concentrations	8
5	Verify the Limiting Primer Concentrations	11

Limitations of the Limiting Primer Protocol

The following limiting primer protocol does not guarantee successful determination of limiting primer concentrations for all assays. If you are unable to define limiting primer concentrations after completing the limiting primer protocol, use one of the following alternative methods for quantitation:

- Run the reactions in separate tubes. By separating the reactions you permit each reaction to proceed unaffected by the other, thereby eliminating the competition for common reagents.
- Redesign and retest the primers to find limiting concentrations. If either primer used in the reaction has an actual melting temperature (T_m) lower than expected, it may preclude determination of limiting primer concentrations. In this case, Applied Biosystems generally recommends that you lengthen the primer by one or two nucleotides to increase its T_m. By increasing the T_m of the primer, you are able to achieve robust amplification at lower primer concentrations.

Evaluate the Relative Abundance of Target and Reference Species

Understanding Relative Abundance

By understanding the relative abundance of target and reference species, it is possible to control the competition between the reactions. Once the relative abundance of the competing species are known, preventing domination by the abundant species becomes a simple matter of limiting the primers. Thus, it is important that you understand the relative abundance of your target and reference species before conducting the limiting primer protocol.

If...	Then...
the concentration of one species is always greater than the other **Note** For example, the concentration of rRNA is always greater than mRNA for total RNA systems.	limit the concentrations of the abundant species primers.
the abundance of one RNA species is unknown	limit the primer concentrations for both amplicons

Determine the Optimal Primer Concentrations

Optimal Primer Concentrations

Applied Biosystems suggests that you establish the optimal probe and primer concentrations for your reaction before attempting to determine limiting primer concentrations. The optimal primer concentrations are those that yield the maximum ΔR_n and the lowest C_T values for your assay. These values are used to verify the results from the "Define Limiting Primer Concentrations" experiment on page 8.

Determining the Optimal Primer Concentrations

To determine the optimal primer concentrations for your RT-PCR system, follow the "Optimize Primer Concentrations" procedure on page 18 of the *TaqMan® Universal PCR Master Mix Protocol* Revision A (P/N 4304449) with the following changes.

Step 4 of the Optimal Primer procedure now reads:

Step	Action
4	At the end of runs, tabulate the results for ΔR_n **and C_T**. Choose the minimum forward- and reverse- primer concentrations that yield **the lowest C_T** and the maximum ΔR_n.

(Changes to the procedure are shown in bold text.)

Determining the Optimum Probe Concentration

Optimal probe concentration should be determined for each quantitative assay with the procedure outlined on pages 20–21 of the *TaqMan® Universal PCR Master Mix* Protocol (P/N 4304449).

Determine the Linear Dynamic Range

Linear Dynamic Range and RT-PCR

The linear dynamic range refers to the range of initial template concentrations over which accurate C_T values are obtained. By defining the linear dynamic range for your system, you can identify the possible starting nucleic acid concentrations for your sample.

Defining the Linear Dynamic Range

To determine the linear dynamic range of your multiplex system, perform the template titration experiment on pages 16–29 of the TaqMan®*Gold RT-PCR Kit Protocol* Revision A (P/N 402876). This experiment will define the upper and lower boundaries for the dynamic range of your system.

Selecting an Initial Template Concentration

The minimum template concentration for your system can be defined by constructing a standard curve from the results of the defining the linear dynamic range experiment (see page 7 of the *ABI PRISM 7700 Sequence Detection System User Bulletin #2* for instructions on how to construct the plot).

Although any initial template concentration within the dynamic range can be used to determine limiting primer concentrations, lower initial template concentrations will yield greater sensitivity for your assay. Therefore, select an initial template concentration within the dynamic range of your assay that represents the lowest concentration of input nucleic acid that you can expect for any sample.

Example 1: Linear Dynamic Range Analysis

Figure 4 illustrates an example of the linear dynamic range for a one-step RT-PCR run using a 1–10^5 pg range of initial template concentrations.

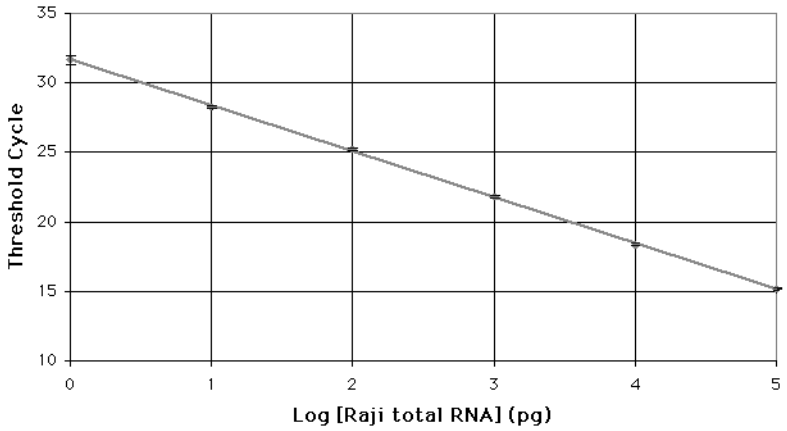

Figure 4. Standard Curve (Human GAPDH mRNA System). Each data point represents the average of triplicate reactions.

All initial template concentrations plotted on the graph appear to be in the linear dynamic range for the system. Therefore, any total RNA concentration within the 1–10^5 pg range can be used. However, the greatest sensitivity will be achieved if the limiting primer experiment is run at 1 pg initial total RNA.

Define Limiting Primer Concentrations

Description

Once you establish the initial template concentration for your system, defining limiting primer concentrations becomes a matter of varying the primer concentrations and observing the trends in ΔR_n and C_T. Because initial template concentration is held constant for all wells in the matrix, the forward and reverse primers will be directly responsible for any variation in the data.

Limiting Primer Matrix

To define limiting primer concentrations, run a matrix of forward and reverse primer concentrations using the value of the minimum initial template found in the previous section. Figure 5 illustrates a recommended matrix of forward and reverse primers varying in concentration from 20–100 nM.

For 100 nM Rev 100 nM	For 100 nM Rev 80 nM	For 100 nM Rev 60 nM	For 100 nM Rev 40 nM	For 100 nM Rev 20 nM
For 80 nM Rev 100 nM	For 80 nM Rev 80 nM	For 80 nM Rev 60 nM	For 80 nM Rev 40 nM	For 80 nM Rev 20 nM
For 60 nM Rev 100 nM	For 60 nM Rev 80 nM	For 60 nM Rev 60 nM	For 60 nM Rev 40 nM	For 60 nM Rev 20 nM
For 40 nM Rev 100 nM	For 40 nM Rev 80 nM	For 40 nM Rev 60 nM	For 40 nM Rev 40 nM	For 40 nM Rev 20 nM
For 20 nM Rev 100 nM	For 20 nM Rev 80 nM	For 20 nM Rev 60 nM	For 20 nM Rev 40 nM	For 20 nM Rev 20 nM

Figure 5. Limiting primer matrix: A matrix of varying concentrations of forward and reverse primers (20–100 nM).

Perform triplicate reactions for each primer combination within the matrix. As in the previous step, follow the procedure outlined in pages 16–29 of the TaqMan® *Gold RT-PCR Kit Protocol*.

Interpreting Results

Ideally, limiting primer concentrations will have negligible effects on the C_T while decreasing product yield. Therefore, desirable primer concentrations are those that show a reduction in ΔR_n but no effect on C_T.

Graphically, the primer concentrations of interest will be those combinations that:

- fall within the plateau region of the forward and reverse primer concentration vs. C_T graph
- achieve a ΔR_n lower than the maximum value for the assay

IMPORTANT If the graph of forward and reverse primer concentration vs. C_T does not plateau, then limiting primer concentrations are unobtainable. See “Limitations of the Limiting Primer Protocol” on page 5 for alternative methods for quantitation.

Example 2: One-Step Multiplex GAPDH RT-PCR Amplification

Figures 6 and 7 illustrate the ΔR_n and C_T trends found by varying concentrations of forward and reverse primers (20–100 nM). Figure 6 demonstrates that the C_T is affected by variations in the concentrations of both primers. By decreasing the forward primer concentration below 40 nM or the reverse primer concentration below 60 nM, the C_T increased by at least one cycle.

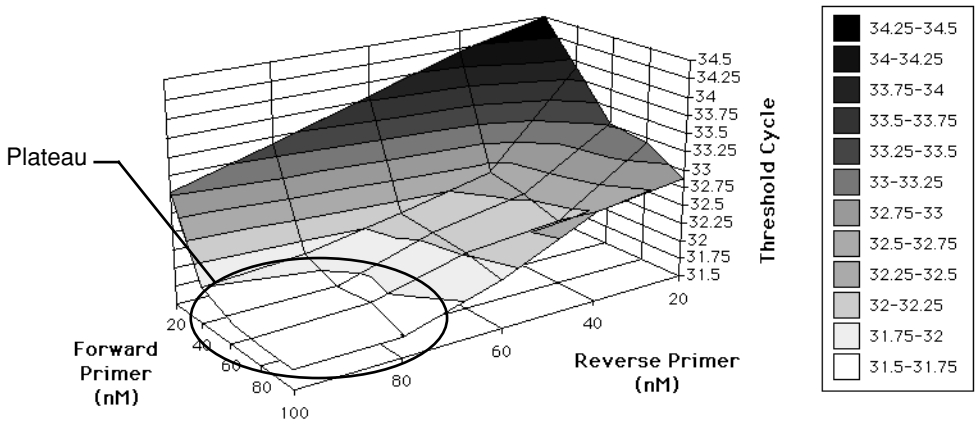

Figure 6. Comparison of primer concentrations and C_T from one-step Human GAPDH mRNA RT-PCR amplification using combinations of forward and reverse primers (20–100 nM).

Figure 7 demonstrates that lower ΔR_ns are achieved by decreasing both primer concentrations. Therefore, final product yields can be regulated by limiting the primer concentrations.

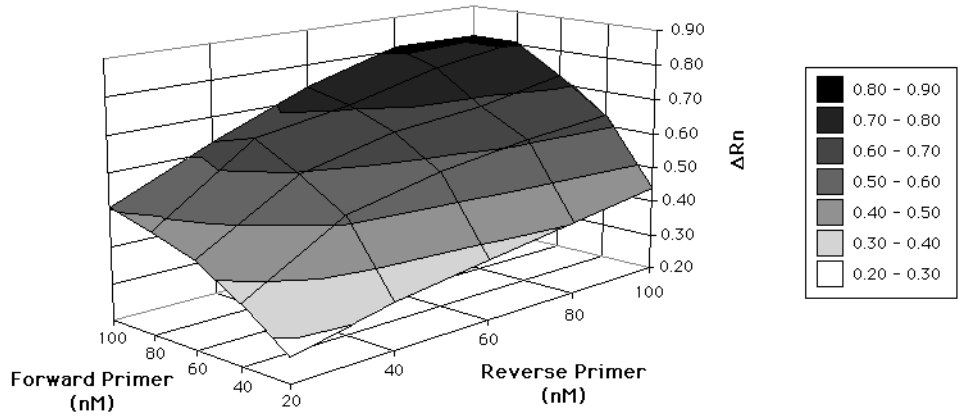

Figure 7. Comparison of primer concentrations and ΔR_n from one-step Human GAPDH mRNA RT-PCR amplification using combinations of forward and reverse primers (20–100 nM).

Note For the example above, 50 nM forward primer and 80 nM reverse primer were selected as limiting primer concentrations in order to provide an experimental margin for error.

Example 3: Two-Step Multiplex GAPDH RT-PCR Amplification

Figures 8 and 9 illustrate the ΔR_n and C_T trends found in two-step RT-PCR by varying the concentrations of forward and reverse primers (20–100 nM). Unlike the one-step RT-PCR in Example 2, the concentrations of forward and reverse primers can be reduced to 50 nM for the corresponding two-step system. Typically, the reverse primer concentration is higher for one-step RT-PCR than for the corresponding two-step system. This is because the reverse primer is responsible for both reverse transcription and PCR priming in one-step RT-PCR.

Figure 8 shows that although the C_T was unchanged by the decrease in reverse primer concentration, the change in forward primer concentration had a significant effect. For example, the C_T rose nearly two cycles when the forward primer concentration fell below 40 nM.

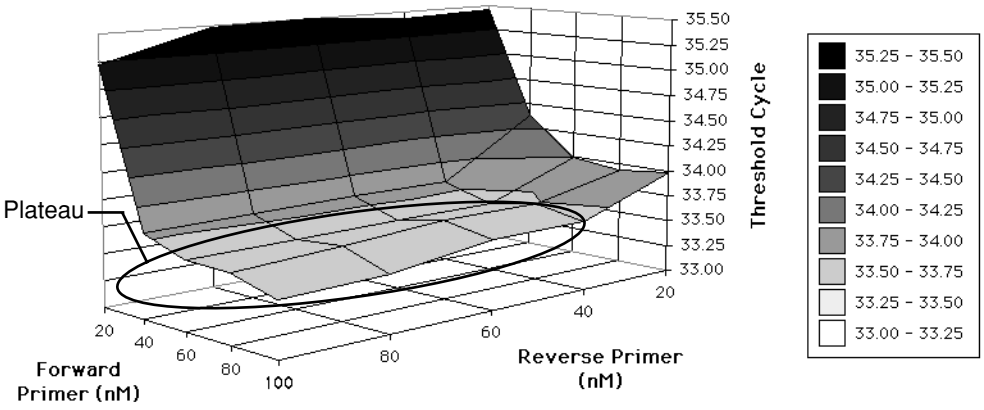

Figure 8. Comparison of primer concentrations and C_T from two-step Human GAPDH mRNA RT-PCR amplification using combinations of forward and reverse primers (20–100 nM).

Figure 9 shows the corresponding relationship between primer concentrations and ΔR_n. It demonstrates that lower product yields can be achieved by decreasing forward and reverse primer concentrations.

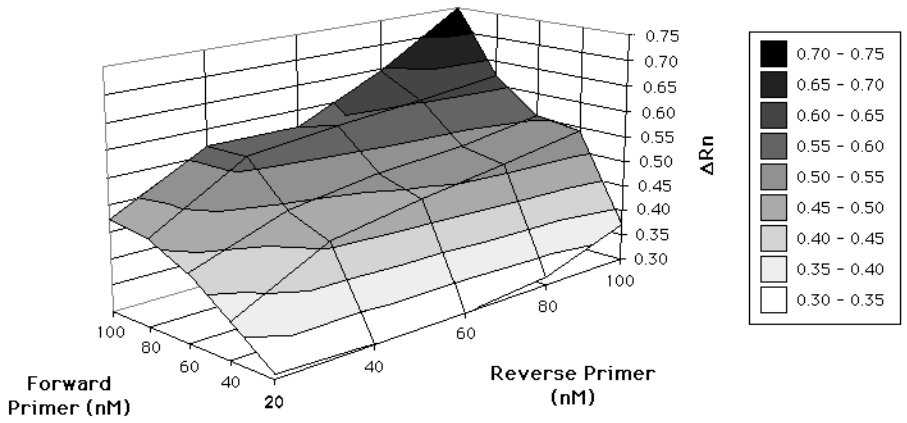

Figure 9. Comparison of primer concentrations and ΔR_n from two-step Human GAPDH mRNA RT-PCR amplification using combinations of forward and reverse primers (20–100 nM).

Note For the example above, 50 nM forward primer and 50 nM reverse primer were selected as limiting primer concentrations in order to provide an experimental margin for error.

Verify the Limiting Primer Concentrations

Confirming Results

After defining limiting primer concentrations in the previous step, they should be verified experimentally. This is accomplished by running primer limited multiplex RT-PCR in parallel with non-primer limited RT-PCR in separate tubes. Because limiting primer concentrations should not affect the C_T, the values obtained from both the multiplex and separate tube experiments should match. As in the previous step, follow the procedure outlined in pages 16–29 of the TaqMan® *Gold RT-PCR Kit Protocol*.

Example 4: Primer Verification

Figure 10 illustrates the comparison of the standard curves for primer limited (multiplex) and non-primer limited (separate tubes) RT-PCR. The results verify that the C_T values determined for this target were not affected by limiting the primer concentrations.

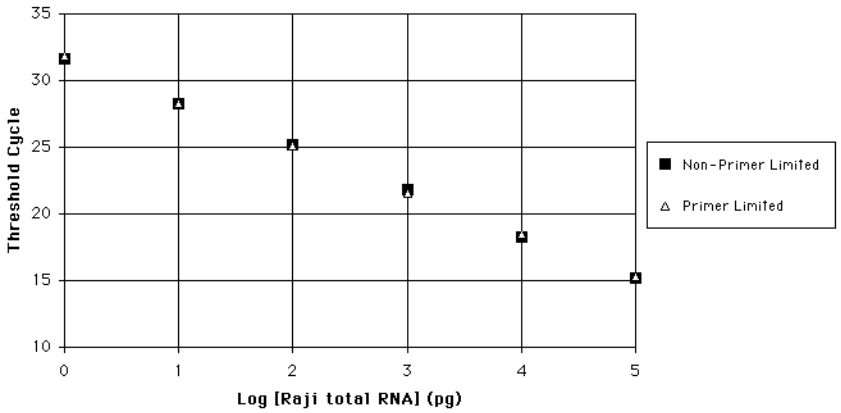

Figure 10. Threshold cycle vs. initial template concentration (Human GAPDH mRNA System). Each data point represents the average of triplicate reactions.

RT-PCR With Known Limiting Primers

Once you define the limiting primers in your system, perform the RT-PCR assay as described on pages 16–29 of the TaqMan®*Gold RT-PCR Kit Protocol* with the modifications to the reaction mix shown in Tables 1 and 2 (changes shown in bold print).

Table 1. One-step Multiplex RT-PCR Reaction Mix Components

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	—
10X TaqMan Buffer A	5.0	1X
25mM $MgCl_2$	11	5.5 mM
10mM deoxy ATP	1.5	300 µM
10mM deoxy CTP	1.5	300 µM
10mM deoxy GTP	1.5	300 µM
20mM deoxy UTP	1.5	600 µM
Target Primers and Probe	**x**	**(see below[b])**
Endogenous Reference Primers and Probe	**y**	**(see below[b])**
AmpliTaq Gold DNA Polymerase (5.0 U/µL)	**0.5**	**0.05 U/µL**
MultiScribe Reverse Transcriptase (MuLV) (50 U/µL)	0.25	0.25 U/µL
RNase Inhibitor	1.0	0.4 U/µL

a. Volume of RNase-free water (µL) = 26.25–(RNA Sample Volume+x+y)

b. Depending on the relative abundance of the target and reference (see table on page 6), use either the optimal or limiting primer concentrations as determined on page 6 or page 8.

Table 2. Two-step Multiplex PCR Mix Reaction Components

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	—
TaqMan Universal PCR Master Mix (2X)	25.0	1X
Target Primers and Probe	**v**	**(see below[b])**
Endogenous Reference Primers and Probe	**w**	**(see below[b])**

a. Volume of RNase-free water (µL) = 25.0–(cDNA Sample Volume+v+w)

b. Depending on the relative abundance of the target and reference (see table on page 6), use either the optimal or limiting primer concentrations as determined on page 6 or page 8.

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