INTRODUCTION

Researchers carrying out quantitative, multiplex, real-time PCR and RT-PCR require master mixes that provide accurate, simultaneous quantification of multiple targets in the same reaction without time-consuming optimization. Our results at Wyeth Research show that QuantiTect® Multiplex Kits from Qiagen are well suited for multiplex analysis of up to four RNA targets in two-step and one-step real-time RT-PCR, requiring no PCR optimization and providing the same efficiency as single-target amplification (singleplex) reactions.

Multiplex, real-time PCR refers to the use of multiple fluorescently-labeled oligonucleotide probes for the quantification of amplicons produced by different primer pairs in the same reaction. This method is becoming more common with the introduction of cyclers capable of spectrally resolving different fluorophores (e.g., Applied Biosystems® 7500, Mx3000P®, LightCycler® 480, and iCycler iQ®), and with the development of master mixes that facilitate the quantification of multiple targets in the same reaction tube. Some master mixes intended for singleplex analysis have been adapted for multiplex analysis1,2, but require time-consuming optimization of primer–probe sets. Therefore, evaluating and selecting a suitable master mix is critical when establishing multiplex assays.

APPLICATION OF MULTIPLEX, REAL-TIME PCR TO VACCINE RESEARCH

Multiplex analysis can be applied to many different fields, such as vaccine research. To advance studies of new vaccine vectors, we are developing sensitive real-time RT-PCR assays to monitor the outcome of vaccination of animals3. An important aspect of characterizing new viral vectors is to analyze their distribution and persistence in small animal models. For vesicular stomatitis virus (VSV) vector development, a sensitive method to detect vector nucleic acid and a vector-encoded gene (i.e., viral RNA targets) is required in order to monitor the extent and duration of vector replication. Since we are interested in quantifying these viral RNA targets as well as an animal housekeeping gene (to normalize viral RNA levels) and exogenous RNA (to monitor RNA purification efficiency), we investigated the feasibility of 4plex, real-time RT-PCR. Analyzing targets in the same well, instead of in separate wells, would avoid potential problems with well-to-well variation in starting template amount and reaction conditions.

OPTIMIZED MASTER MIXES FOR MULTIPLEX, REAL-TIME PCR

Qiagen offers two kits for multiplex analysis: the QuantiTect Multiplex PCR Kit (for PCR and two-step RT-PCR procedures) and the QuantiTect Multiplex RT-PCR Kit (for one-step RT-PCR procedures). The optimized PCR master mix in the kits ensures that amplicons are amplified with equal efficiency and sensitivity in both multiplex and singleplex reactions. This is achieved through various reaction components in the master mix that create the conditions for high specificity in PCR. Firstly, a balanced combination of K+ and NH4+ ions and a novel PCR additive called synthetic factor MP work together to promote stable and efficient annealing of primers to the nucleic acid template. Secondly, a stringent hot-start Taq DNA polymerase becomes active only after incubation at 95°C, preventing the formation of nonspecific products and primer–dimers in every PCR cycle. The QuantiTect Multiplex RT-PCR Kit is also supplied with an RT mix for highly efficient and sensitive reverse transcription over a wide range of RNA template amounts.

EVALUATION OF QUANTITECT MULTIPLEX KITS

To determine whether the QuantiTect Multiplex RT-PCR Kit could amplify four targets simultaneously in multiplex, real-time, one-step RT-PCR, we analyzed RNA standards (synthetic oligonucleotides) or RNA purified from nasal turbinate homogenates from mice inoculated intranasally with a VSV vector encoding an HIV-1 gene. The four targets were the viral nucleocapsid gene (N), the HIV-1 gene, gag (G), a
mouse housekeeping gene, RPLO (R), and a spiked exogenous control, Armored-RNA (A). We also analyzed these four targets by multiplex, real-time, two-step RT-PCR using the QuantiTect Multiplex PCR Kit. The sensitivity and specificity of the 4plex assays were determined by comparison with the corresponding singleplex assays.

MULTIPLEX, REAL-TIME, ONE-STEP RT-PCR USING STANDARDS

Multiplex, real-time, one-step RT-PCR was carried out using 10-fold serial dilutions (from $10^7$ to $10^1$ copies) of synthetic RNA oligonucleotides of three of the amplicons (N, G, R) and $10^5$ copies of the spiked control (A). Reaction setup and cycling were carried out according to the QIAGEN protocol for 4plex analysis with the QuantiTect Multiplex RT-PCR Kit on the Applied Biosystems 7500. Probes were labeled with 6-FAM (N gene), VIC® (G gene), NED (R gene), and Cy®5 (A gene), and primers and probes were each used at 0.2 μM. For the singleplex reactions, the probe concentration was set at 0.2 μM, while the primer concentrations were increased to 0.4 μM. The amplification plots achieved with the 4plex assay overlapped with those from the singleplex assays, indicating equivalent threshold cycle numbers ($C_T$) at each dilution for gene N (Fig. 1A), gene G (Fig. 1B), gene R (Fig. 1C), and gene A (Fig. 1D). The sensitivity of the 4plex and singleplex assays was comparable, with detection of 10–100 copies for each gene. This sensitivity was maintained when mixed targets were done with genes N, R and A fixed at $10^5$ copies and gene G fixed at 100 copies or genes G, R and A fixed at $10^5$ copies and gene N fixed at 100 copies (data not shown). The specificity of the four primer–probe sets was confirmed in control reactions in which the template molecules were absent/modified (data not shown).

MULTIPLEX, REAL-TIME, ONE-STEP RT-PCR USING VIRAL/MOUSE RNA

Viral/mouse RNA purified from nasal turbinate homogenates was analyzed by real-time, one-step RT-PCR in both 4plex and singleplex assays. Reactions were run using the QuantiTect Multiplex RT-PCR Kit and the recommended primer–probe concentrations and cycling conditions. A comparison of the 4plex and singleplex assays showed equivalent $C_T$ values for each gene (Table 1).

MULTIPLEX, REAL-TIME, TWO-STEP RT-PCR USING VIRAL/MOUSE RNA

To determine whether the QuantiTect Multiplex PCR Kit could be used in real-time, two-step RT-PCR, viral/mouse RNA purified from nasal turbinate homogenates was analyzed by real-time, two-step RT-PCR in both 4plex and singleplex assays. Reactions were run using the QuantiTect Multiplex RT-PCR Kit and the recommended primer–probe concentrations and cycling conditions. A comparison of the 4plex and singleplex assays showed equivalent $C_T$ values for each gene (Table 1).
RNA from nasal turbinate homogenates was analyzed in 4plex and singleplex assays. Equivalent \( C_T \) values were achieved for each gene regardless of whether a 4plex or a singleplex assay was performed (Table 2).

**CONCLUSION**

QuantiTect Multiplex Kits accurately quantified multiple targets simultaneously in the same reaction tube when tested with RNA standards or with total/viral RNA from mouse tissues. This helped us at Wyeth Research to increase the throughput of our animal studies and to reduce reagent costs and concerns about false-negatives. Since our target genes may be expressed at different levels after animal inoculation, lengthy optimization of primer–probe sets and limiting primer experiments were often necessary in order to prevent abundant genes from dominating the amplification reaction and affecting detection of less abundant genes. With QuantiTect Multiplex Kits, this time-consuming optimization was not needed. Whether these kits were used in two-step or one-step RT-PCR, they provided sensitive and specific detection of target genes, giving equivalent \( C_T \) values in both 4plex and singleplex assays. Since optimization of primer–probe sets and cycling conditions are not necessary, QuantiTect Multiplex Kits represent an advanced tool for quantitative, real-time PCR and RT-PCR.

**REFERENCES**


**TRADEMARKS**

Trademarks: QuantiTect® (QIAGEN Group); Applied Biosystems®; VIC® (Applera Corporation or its subsidiaries); Cy® (GE Healthcare); iCycler iQ® (Bio-Rad Laboratories, Inc.); LightCycler® (Roche Group); Mx3000P® (Stratagene).