

## 4plex, real-time one-step RT-PCR without optimization

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Multiplex, real-time PCR allows the simultaneous quantification of multiple targets in the same reaction, and is becoming increasingly common with the introduction of cyclers that can detect different fluorophores. However, use of PCR master mixes intended for singleplex analysis often requires time-consuming optimization of primer-probe sets before satisfactory results can be achieved. In contrast, QuantiTect® Multiplex Kits are specially designed for multiplex analysis, requiring no optimization of reaction and cycling conditions. We describe here the successful application of multiplex analysis to our vaccine research.

In our studies of new vaccine vectors, we used real-time RT-PCR assays to monitor the outcome of animal vaccination. This included analysis of the distribution and persistence of vaccine vectors in small animal models. In our development of a vesicular stomatitis virus (VSV) vector, we performed assays to detect vector nucleic acid and a vector-encoded gene (i.e., 2 viral RNA targets), an animal housekeeping gene (to normalize viral RNA levels), and exogenous RNA (to monitor the efficiency of RNA purification). We investigated the feasibility of analyzing these 4 RNA targets by multiplex, real-time RT-PCR. As the targets were not analyzed in separate wells, potential problems with well-to-well variation in starting template amount and reaction conditions were avoided.

First of all, we performed an experiment using RNA standards (synthetic oligonucleotides) with the QuantiTect Multiplex RT-PCR Kit. The 4 targets were the viral nucleocapsid gene (N); the HIV-1 gene, gag (G); the mouse housekeeping gene, RPL0 (R); and a spiked exogenous control, Armored-RNA (A). Multiplex, real-time one-step RT-PCR was carried out using 10-fold serial dilutions (from  $10^7$  to  $10^1$  copies) of the viral and mouse RNA targets (N, G, and R) and  $10^5$  copies of the exogenous RNA (A). Reactions were run on the Applied Biosystems® 7500 according to the instructions in the *QuantiTect Multiplex RT-PCR Handbook*. The probes were labeled with the dyes 6-FAM, VIC®, NED, and Cy®5 for the targets N, G, R, and A, respectively. The concentration of each primer or probe was 0.2  $\mu$ M.

### Inside

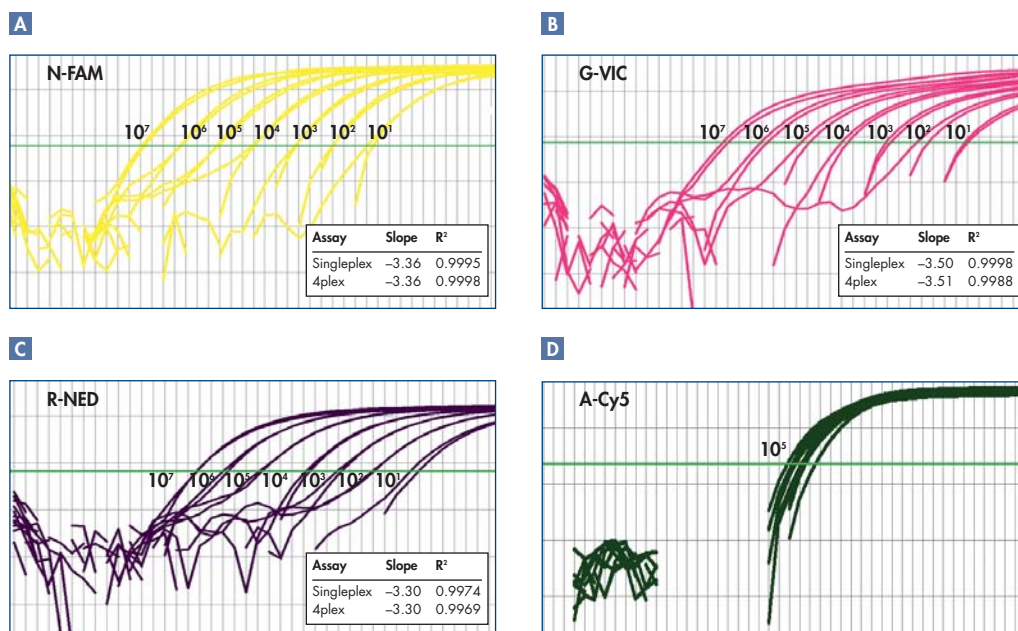
- Universal conditions for duplex real-time RT-PCR assays
- miRNA supplement
- Tips and tricks



For comparison, the targets were also analyzed by singleplex, real-time one-step RT-PCR: for these reactions, primer concentration was increased to 0.4  $\mu\text{M}$ , while probe concentration was kept at 0.2  $\mu\text{M}$ .

The amplification plots for the 4plex assays overlapped with those for the singleplex assays (Figure 1), showing equivalent  $C_T$  values at each dilution of the targets N, G, R, and A. The sensitivity of the 4plex and singleplex assays was also comparable, with detection of as little as 10 copies of the targets N, G, and R. These results demonstrate the reliability of the QuantiTect Multiplex RT-PCR Kit in multiplex, real-time RT-PCR.

Further experiments were carried out to analyze viral/mouse RNA purified from nasal turbinate homogenates by real-time one-step or two-step RT-PCR. Equivalent  $C_T$  values in 4plex and singleplex assays were also observed (see reference below).



**Figure 1. Comparable  $C_T$  values in 4plex and singleplex assays.** The QuantiTect Multiplex RT-PCR Kit was used with the Applied Biosystems 7500 to carry out 4plex, real-time one-step RT-PCR and, for comparison, singleplex, real-time one-step RT-PCR. The synthetic RNA templates were 10-fold serial dilutions (from  $10^7$  to  $10^1$  copies) of targets N, G, and R, and  $10^5$  copies of target A. The amplification plots for the 4plex and singleplex assays overlapped, demonstrating the reliability of the QuantiTect Multiplex RT-PCR Kit in multiplex analysis.

QuantiTect Multiplex Kits provided specific and sensitive amplification without the need for PCR optimization, even in multiplex assays where both high-abundance and low-abundance genes were analyzed. The kits not only helped us to increase the throughput of our real-time RT-PCR assays in our vaccine research with animal models, but also reduced concerns about false-negatives. In addition, we no longer have to perform time-consuming optimization of primer–probe sets, such as limiting primer concentrations.

Visit [www.qiagen.com/multiplex](http://www.qiagen.com/multiplex) to find out more about QuantiTect Multiplex Kits.

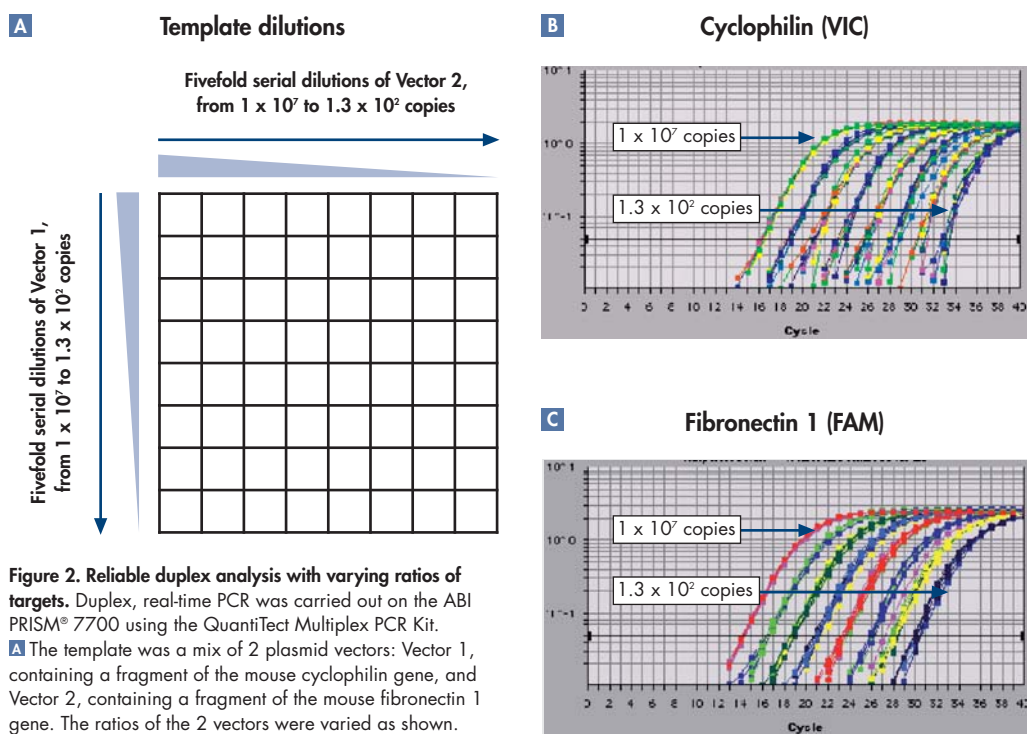
Data excerpted from Coleman, J.W. et al. (2007) Simultaneous Quantification of Four RNA Targets by Multiplex, Real-Time RT-PCR without Optimization. *BioTechniques* **43**, 369.

# Universal conditions for duplex real-time RT-PCR assays

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Duplex, real-time RT-PCR enables simultaneous quantification of 2 targets in the same reaction, which saves time, reduces costs, and conserves samples. However, universal reaction conditions that can be applied to a wide range of different duplex assays have not been validated until now. In this study, the performance of the QuantiTect Multiplex PCR Kit with varying ratios of targets and with different combinations of targets was evaluated. First of all, an 8 x 8 checkerboard validation procedure was set up, where a duplex assay was carried out with 64 different ratios of 2 targets (Figure 2A). For each dilution of one target, the  $C_T$  value was not affected when the dilution of the other target in the assay was varied from  $10^2$  to  $10^7$  copies (Figures 2B and 2C). In another experiment, 38 different duplex assays were carried out. Each duplex assay provided similar  $C_T$  values as control assays in which the targets were analyzed in separate reactions (see the reference at the end of this page).



**Figure 2. Reliable duplex analysis with varying ratios of targets.** Duplex, real-time PCR was carried out on the ABI PRISM® 7700 using the QuantiTect Multiplex PCR Kit.

**A** The template was a mix of 2 plasmid vectors: Vector 1, containing a fragment of the mouse cyclophilin gene, and Vector 2, containing a fragment of the mouse fibronectin 1 gene. The ratios of the 2 vectors were varied as shown.

**B** Amplification plots for cyclophilin (detection with VIC labeled probe). **C** Amplification plots for fibronectin 1 (detection with FAM labeled probe).

To read a longer version of this article, visit [www.qiagen.com/goto/GNL13](http://www.qiagen.com/goto/GNL13).

Data excerpted from Ishii, T. et al. (2007) Validation of universal conditions for duplex quantitative reverse transcription polymerase chain reaction assays. *Anal. Biochem.* **362**, 201.

## Efficient miRNA purification from FFPE sections

Effective purification of small RNAs is an essential step in miRNA research. However, most commercial RNA purification kits do not recover RNA molecules smaller than ~200 nucleotides (nt). The miRNeasy FFPE Kit is the latest addition to QIAGEN's range of RNA purification kits for miRNA studies. This kit enables purification of total RNA, including RNA from approximately 18 nt upwards, from formalin-fixed, paraffin embedded (FFPE) tissue sections and laser capture microscopy (LCM) samples from FFPE sections.

### Benefits of the miRNeasy FFPE Kit:

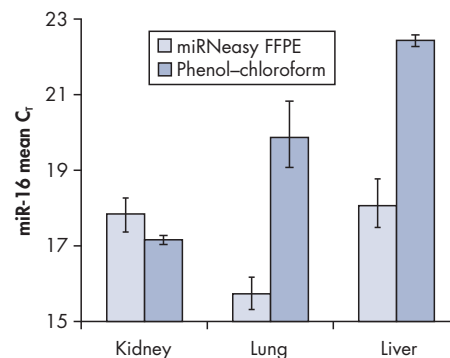
- Effective purification of miRNA and total RNA
- Novel method to overcome formalin crosslinking
- Efficient release of RNA without compromising integrity
- Optimal performance in downstream applications

### Overcoming crosslinking yields high-quality RNA

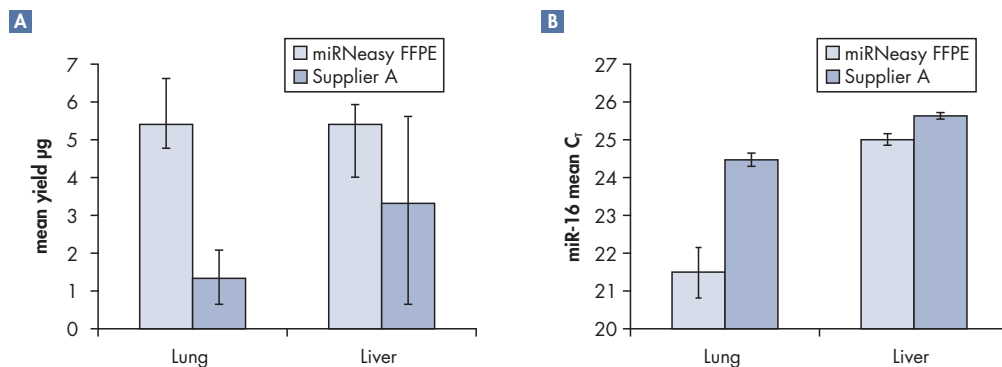
Fixing tissues with formalin leads to RNA–RNA and RNA–protein crosslinking which impairs RNA performance in downstream applications. The miRNeasy FFPE Kit provides special lysis and incubation conditions to reverse formalin crosslinking of RNA. This enables the purified RNA to deliver maximal performance in a range of applications, including real-time RT-PCR. A specially developed lysis buffer enables release of RNA from tissue sections while avoiding further RNA degradation. The kit also uses gDNA Eliminator spin columns for removal of genomic DNA contamination. Optimized binding conditions allow purification of all usable RNA from approximately 18 nt upwards. RNA yields and performance are superior to alternative methods of miRNA purification, such as using phenol-chloroform extraction (Figures 3 and 4).

### Purified RNA highly suitable for downstream miRNA quantification

The miRNeasy FFPE Kit allows purification of miRNA with total RNA for use in a variety of applications, such as quantitative, real-time RT-PCR with the miScript System (Figures 3 and 4B). The miScript System enables detection of multiple miRNAs and mRNA from a single cDNA synthesis reaction by SYBR® Green-based real-time PCR.



**Figure 3. Efficient RNA purification from FFPE tissues.** Total RNA including miRNA was purified from the indicated rat tissues using either the miRNeasy FFPE Kit or phenol-chloroform extraction. Purified RNA was used as a template in quantitative, real-time RT-PCR assays for the miRNA miR-16 using the miScript System. Results showed that for lung and liver tissues,  $C_T$  values were lower after purification using the miRNeasy Kit, indicating that higher amounts of miRNA were purified than when using phenol-chloroform extraction. For kidney tissue,  $C_T$  values were similar for both methods.  $C_T$  values were similar or lower after purification using the miRNeasy Kit for all tissue types tested.



**Figure 4. Superior yields and performance.** RNA was purified from FFPE sections of the indicated rat tissues using either the miRNeasy FFPE Kit or a similar kit from Supplier A. **A** RNA yields were determined by measuring absorbance at 260 nm. **B** Purified RNA was used as a template in quantitative, real-time RT-PCR assays for the miRNA miR-16 using the miScript System. Results showed that  $C_t$  values were lower after purification using the miRNeasy FFPE Kit, indicating that higher amounts of miRNA were purified than when using the kit from Supplier A.

### Ordering Information

Product	Contents	Cat. no.
miRNeasy FFPE Kit (50)	For 50 preps: 50 RNeasy® MinElute® Spin Columns, 50 gDNA Eliminator Spin Columns, Collection Tubes, Proteinase K, RNase-Free Reagents and Buffers	217404
miRNeasy Mini Kit (50)	For 50 preps: 50 RNeasy Mini Spin Columns, Collection Tubes, QIAzol® Lysis Reagent, RNase-Free Reagents and Buffers	217004
miRNeasy 96 Kit (4)	For 4 x 96 preps: 4 RNeasy 96 plates, Collection Microtubes (racked), Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217061
miScript System	The miScript Reverse Transcription Kit, miScript SYBR Green PCR Kit, and miScript Primer Assay are the 3 components of the system	Varies

Discover more about miRNeasy and miScript technologies at [www.qiagen.com/miRNA](http://www.qiagen.com/miRNA) !

# Tips and tricks

## Disruption and homogenization in Buffer RLT Plus

When disrupting and homogenizing tissues in Buffer RLT Plus (supplied with RNeasy Plus Kits and certain AllPrep® Kits), excessive foaming may occur. Excessive foaming can be prevented by adding Reagent DX (cat. no. 19088) to Buffer RLT Plus at a final concentration of 0.5% (v/v) before starting disruption and homogenization.

## Ordering Information

Product	Contents	Cat. no.
QuantiTect Multiplex PCR Kit (200)*†	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (with ROX dye), 2 x 2 ml RNase-Free Water	204543
QuantiTect Multiplex PCR NoROX Kit (200)†‡	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (without ROX dye), 2 x 2 ml RNase-Free Water	204743
QuantiTect Multiplex RT-PCR Kit (200)*†	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (with ROX dye), 100 µl RT Mix, 2 x 2 ml RNase-Free Water	204643
QuantiTect Multiplex RT-PCR NR Kit (200)†‡	For 200 x 50 µl reactions: 3 x 1.7 ml 2x Master Mix (without ROX dye), 100 µl RT Mix, 2 x 2 ml RNase-Free Water	204843

\* Recommended for cyclers from Applied Biosystems.

† Larger kit size available; please inquire.

‡ Recommended for cyclers from other suppliers.

To order trial-size QuantiTect Kits, visit [www.qiagen.com/goto/TrialKits](http://www.qiagen.com/goto/TrialKits)

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