Protocol for Multiplex RT-qPCR Analysis Using PrimePCR Assays

Abstract

Multiplex real-time PCR using Bio-Rad's PrimePCR Assays and iQ Multiplex Powermix enables quantitative relative gene expression analysis of up to five targets in qPCR. Traditionally, multiplex qPCR experiments have been challenging to design. This protocol describes an off-the-shelf solution for increasing throughput and reducing reagent costs through the adoption of multiplex assays. For one-step multiplex RT-qPCR using Reliance One-Step Multiplex Supermix and PrimePCR Probe Assays, refer to bulletin 7197.

Materials

- iQ Multiplex Powermix, catalog #1725849
- PrimePCR Probe Assays for Gene Expression
- Hard-Shell™ Thin-Wall 96- or 384-Well Skirted PCR Plates, catalog #HSP9655 or HSP3805, respectively
- Microseal™ 'B' Adhesive Seals, catalog #MSB1001

Equipment

- CFX Opus 96 Real-Time PCR System, catalog #12011319
- CFX Opus 384 Real-Time PCR System, catalog #12011452

Software

- CFX Maestro Software, catalog #12013758

Note: Compatible with Windows 7 and 10. Includes USB installation drive, video quick guides, and PDF instruction manual.

Before You Begin

You will first need to isolate total RNA and prepare first-strand cDNA for use in multiplex qPCR. We recommend the following options:

RNA Isolation

- Total RNA isolation from most samples
  - Aurum Total RNA Mini Kit, catalog #7326820
  - PureZOL RNA Isolation Reagent, catalog #7326880
- Rapid isolation of total RNA from tissue culture samples
  - SingleShot Cell Lysis RT-qPCR Kits, catalog #1725080

cDNA Synthesis

- First-strand cDNA synthesis
  - iScript Advanced cDNA Synthesis Kit, catalog #1725037
- First-strand cDNA synthesis with genomic DNA elimination
  - iScript gDNA Clear cDNA Synthesis Kit, catalog #1725034
- First-strand cDNA synthesis for processing samples with inhibitors and degraded RNA (e.g., FFPE samples)
  - Reliance Select cDNA Synthesis Kit, catalog #12012802

Optional: Preamplification

- Preamplification for analysis of targets from a limited sample
  - SsoAdvanced PreAmp Supermix, catalog #1725160
  - PrimePCR PreAmp Assays

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Procedure

Protocol

1.1 Prepare the multiplex PrimePCR assay pool(s) according to Table 1. Scale the size of the pool(s) to the total number of reactions that will be performed. Prepare an excess of at least 10% to account for pipetting variation.

Note: If multiplexing fewer than five targets, use water or TE buffer with low EDTA in place of unused PrimePCR Assays.

Note: PrimePCR Assays are used at a 0.5x concentration in this protocol.

Table 1. Preparation of multiplex assay pool(s).

Component	Per 20 µl Reaction	Per 10 µl Reaction
PrimePCR Assay-FAM	0.5 µl	0.25 µl
PrimePCR Assay-HEX	0.5 µl	0.25 µl
PrimePCR Assay-TEX 615	0.5 µl	0.25 µl
PrimePCR Assay-Cy5	0.5 µl	0.25 µl
PrimePCR Assay-Cy5.5*	0.5 µl	0.25 µl
Total	**2.5 µl**	**1.25 µl**

* This channel is available only on CFX Opus 96 Systems.

1.2 Prepare a multiplex qPCR master mix according to Table 2 that contains all of the components listed except the cDNA sample. An excess of 10% should be prepared to account for pipetting variation.

1.3 Aliquot the appropriate volume of the multiplex qPCR master mix to a Hard-Shell Thin-Wall 96- or 384-Well Skirted PCR Plate and add the cDNA sample to complete the reaction mix.

Note: If using technical (reaction) replicates, prepare the reaction mix at a larger scale using PCR tube strips or iQ PCR Plates. Mix well and then dispense replicate reactions to a Hard-Shell Plate.

Table 2. Preparation of multiplex qPCR reaction mix.

Component	Per 20 µl Reaction	Per 10 µl Reaction
iQ Multiplex Powermix	10 µl	5 µl
Multiplex assay pool	2.5 µl	1.25 µl
cDNA sample*	Varies	Varies
Nuclease-free water	Varies	Varies
Total	**20 µl**	**10 µl**

* The qPCR reactions should not contain more than 20% of the undiluted cDNA reaction.

1.4 Seal the plate with a Microseal 'B' Adhesive Seal and vortex to mix reagents. Centrifuge briefly to collect all reaction components at the bottom of the plate.

1.5 Analyze the plate using a CFX Opus 96 or CFX Opus 384 Real-Time PCR System. Select **User-defined** as the run type and use the following cycling protocol: 95°C for 2 min followed by 40 cycles of 95°C for 10 sec and 60°C for 45 sec. Ensure that the scan mode is set to **All Channels**.

1.6 Analyze the relative gene expression data using CFX Maestro Software.

Important Considerations for Multiplex Validation

- Interactions between assays can potentially compromise target quantification in a multiplex qPCR assay. To validate a multiplex qPCR assay, compare generated results side by side with the individual assays in the same qPCR experiment. In addition, keep the following in mind:
  - Reactions for the individual assays should be set up using the same amount of iQ Multiplex Powermix and cDNA sample as in the multiplex reaction, but the individual PrimePCR Assay should be used at a 1x concentration
  - A representative set of samples spanning expression ranges should be used
  - The difference in Cq value between singleplex and multiplex reactions should be less than one cycle
  - Caution should be used when comparing results for targets with lower expression levels where qPCR results can exhibit stochastic variability

Tips

If your CFX instrument is not calibrated for the fluorophores in the PrimePCR Probe Assays, contact our technical support team for assistance.

Visit **bio-rad.com/primepcr** for expertly predesigned probe assays for gene expression.

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