Protocol for Multiplex RT-qPCR Analysis Using PrimePCR™ Assays

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 provides an off-the-shelf solution for increasing throughput and reducing reagent costs through the adoption of multiplex assays.

Materials

- iQ Multiplex Powermix, catalog #1725849
- PrimePCR Probe Assays for Gene Expression, **bio-rad.com/primepcr**
- Hard-Shell® Thin-Wall 96- or 384-Well Skirted PCR Plates, catalog #HSP9655 or HSP3805, respectively
- Microseal® 'B' Adhesive Seals, catalog #MSB1001

Equipment

- CFX96 Touch™ or CFX384 Touch™ Real-Time PCR Detection System, catalog #1855196 or 1855484, respectively

Note: The CFX384 Touch System has four fluorescent channels and is capable of four-plex assays.

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

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

Optional: Preamplification

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

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 CFX96 Touch Systems.

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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 CFX96 Touch or CFX384 Touch Real-Time PCR Detection 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 Manager™ 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 results generated 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, please contact our technical support team for assistance

Visit **bio-rad.com/web/multiplex** for more information.

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Web site *bio-rad.com* ***USA*** *1 800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *43 1 877 89 01 177* ***Belgium*** *32 (0)3 710 53 00* ***Brazil*** *55 11 3065 7550* ***Canada*** *1 905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *420 241 430 532* ***Denmark*** *45 44 52 10 00* ***Finland*** *358 09 804 22 00* ***France*** *33 01 47 95 69 65* ***Germany*** *49 89 31 884 0* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 459 6100* ***India*** *91 124 4029300* ***Israel*** *972 03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *81 3 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *31 (0)318 540 666* ***New Zealand*** *64 9 415 2280* ***Norway*** *47 23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 (0) 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *46 08 555 12700* ***Switzerland*** *41 026 674 55 05* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *66 662 651 8311* ***United Arab Emirates*** *971 4 8187300* ***United Kingdom*** *44 020 8328 2000*

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