Protocol for One-Step Multiplex RT-qPCR Analysis Using PrimePCR Probe Assays and Reliance One-Step Multiplex Supermix

Protocol

Abstract

Perform one-step multiplex RT-qPCR with minimal cross-reactivity using Reliance One-Step Multiplex Supermix and PrimePCR Probe Assays expertly designed for gene expression. This protocol provides an off-the-shelf solution for sensitive, reproducible detection of up to five targets directly from RNA while increasing throughput and reducing reagent costs through the use of multiplex assays.

Materials

- Reliance One-Step Multiplex Supermix (catalog #12010176, #12010220, #12010221)
- 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

- CFX96 Touch or CFX384 Touch Real-Time PCR Detection Systems (catalog #1855196 or #1855484, respectively)
- To enable walk-away high-throughput qPCR operation, CFX Automation System II (catalog #1845075)

Note: The CFX384 Touch System has four fluorescent channels and is capable of 4-plex assays.

Before You Begin

You will first need to prepare your RNA sample for use in one-step multiplex RT-qPCR. You can either lyse your sample or isolate total RNA. We recommend the following options:

Lysate Preparation

- Generation of lysate from cell cultures
  - SingleShot Cell Lysis RT-qPCR Kits (catalog #1725080, #1725081)

RNA Isolation

- Total RNA isolation from most samples
  - Aurum Total RNA Mini Kit (catalog #7326820)
  - PureZOL RNA Isolation Reagent (catalog #7326880)

Procedure

The Reliance One-Step Multiplex Supermix is delivered in a 4x ready-to-use format. To use the mix, thaw the vial on ice to 4°C. Thoroughly mix the vial and briefly centrifuge to ensure all components are at the bottom of the tube. Store on ice protected from light until ready to use.

If using automated liquid handling, let sit at ambient temperature for 10 min to further reduce the viscosity of the supermix.

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 1x concentration in this protocol.

Table 1. Preparation of one-step multiplex assay pool(s).

Component	Per 20 µl Reaction	Per 10 µl Reaction
PrimePCR Assay-FAM	1.0 µl	0.5 µl
PrimePCR Assay-HEX	1.0 µl	0.5 µl
PrimePCR Assay-TEX	1.0 µl	0.5 µl
PrimePCR Assay-Cy5	1.0 µl	0.5 µl
PrimePCR Assay-Cy5.5*	1.0 µl	0.5 µl
Total	**5.0 µl**	**2.5 µl**

* This channel is available only on CFX96 Touch Systems.

2. Prepare a one-step multiplex RT-qPCR master mix according to Table 2 that contains all of the components listed (except the RNA sample). An excess of 10% should be prepared to account for pipetting variation.

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

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

Table 2. Preparation of one-step multiplex RT-qPCR master mix.

Component	Per 20 µl Reaction	Per 10 µl Reaction
4x Reliance Supermix	5 µl	2.5 µl
Multiplex assay pool	5 µl	2.5 µl
RNA template*	Varies	Varies
Nuclease-free water	To 20 µl	To 10 µl
Total	**20 µl**	**10 µl**

* 1 pg–1 µg of purified RNA or up to 10 µl of lysed sample can be used.

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.
5. Program the thermal cycling protocol on a CFX96 Touch or CFX384 Touch Real-Time PCR Detection System according to Table 3.

Table 3. Thermal cycling protocol.*

Step		Temperature, °C	Time	Number of Cycles
Reverse transcription		50	10 min	1
DNA polymerase activation and template denaturation		95	10 min	1
Amplification	Template denaturation	95	10 sec	35–40
	Annealing/extension and plate read	60	30 sec	

* Fast cycling protocols can be used on fast thermal cyclers with additional optimization recommended for best performance.

6. Load the PCR plate onto the CFX System and start the RT-qPCR run program.
7. When thermal cycling is complete, perform data analysis in CFX Manager or 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 results generated side by side with the individual assays in the same qPCR experiment. In addition, keep the following in mind:

- Pair the brighter fluorophores with low-expressing targets and the dimmer fluorophores with high-expressing targets
- Reactions for the individual assays should be set up using the same amount of Reliance One-Step Multiplex Supermix and RNA 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/RelianceOneStep** for more information.

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

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