QIAGEN Supplementary Protocol

Pre-amplification of gDNA and cDNA using the QIAGEN® Multiplex PCR *Plus* Kit

This protocol has been developed for use with the QIAGEN Multiplex PCR *Plus* Kit (cat. nos. 206151 and 206152) to enable simultaneous amplification of up to 80 targets, up to 300 bp in length (ideally 80–150 bp) using cDNA or gDNA as a template. This protocol describes pre-amplification of cDNA for applications such as two-step RT-PCR for gene expression analysis of multiple targets. It also describes gDNA pre-amplification for SNP/mutation detection from small amounts of gDNA for subsequent qPCR, end-point PCR, and Pyrosequencing®.

IMPORTANT: Please read the *QIAGEN Multiplex PCR* Plus *Handbook*, paying careful attention to the "Safety Information" and "Important Notes" sections, before beginning this procedure. The QIAGEN Multiplex PCR *Plus* Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

Equipment and reagents

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- PCR tubes/strips/plates
- Pipets and pipet tips (aerosol resistant)
- Thermal cycler
- Primers
- Primers should be purchased from an established oligonucleotide manufacturer. Lyophilized primers should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry. Primer stock solutions should be stored in aliquots at –20°C.

Important points before starting

- **Always use the cycling conditions specified in this protocol.**
- **Use equal concentrations (0.1 µM) of all primers.**
- For optimal results, we recommend using primer pairs with a T_m of ≥68°C. For multiplex PCR primer design, see Appendix A, page 38 of the *QIAGEN Multiplex PCR* Plus *Handbook*.
- Prepare a 10x primer mix as described in page 13 of the *QIAGEN Multiplex PCR* Plus *Handbook*.
- **Do not use Q-Solution®** reagent that is provided with the kit.
- **Do not use CoralLoad® Dye** provided with the kits when performing qPCR or capillary sequencing subsequently.
- **PCR must start with an activation step of 5 minutes at 95°C** to activate HotStarTaq® *Plus* DNA Polymerase (see step 5 of this protocol).

Procedure

1. Thaw the 2x Multiplex PCR Master Mix (if stored at –20°C), template cDNA or gDNA, RNase-free water, and primer mix. Mix the solutions completely before use.

It is important to mix the solutions completely before use to avoid localized concentrations of salts. Preparing a mixture of all primers avoids pipetting of individual primers for each experiment, reducing pipetting time and increasing reproducibility of results.

2. Prepare a reaction mix according to Table 1.

The reaction mix typically contains all the components required for multiplex PCR, except the template DNA. Prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. Primer concentration should be 0.1 µM per primer (up to 80 primer pairs/reaction).

Table 1. Reaction composition using 2x Multiplex PCR Master Mix

Component	Volume/reaction	Final concentration
Reaction mix		
2x Multiplex PCR Master Mix*	12.5 µl	1x
10x primer mix, 1 µM each primer	2.5 µl	0.1 µM†
RNase-free water	Variable	–
Template DNA		
Template cDNA or gDNA, added at step 4	Variable	1–50 ng DNA
Total volume	25 µl	–

* Provides a final concentration of 3 mM $MgCl_2$.

† A final primer concentration of 0.1 µM is optimal for most primer–template systems. However, in some cases, using other primer concentrations (i.e., 0.02–0.1 µM) may further improve amplification performance.

3. **Mix the reaction mixture thoroughly and dispense appropriate volumes into PCR tubes or wells.**

   Mix gently, for example, by pipetting the reaction mix up and down a few times. Due to the hot start, it is not necessary to keep samples on ice during reaction setup.

4. **Add template DNA (1–50 ng/reaction) to the individual PCR tubes or wells containing the reaction mix. See Table 1.**

5. **a) Program the thermal cycler according to the manufacturer's instructions.**

   b) Place the PCR tubes/plates/strips in the thermal cycler and start the cycling program as outlined in Table 2 or 3.

   Each PCR program must start with an initial heat-activation step at 95°C for 5 min to activate HotStarTaq *Plus* DNA Polymerase.

6. **After amplification, samples can be stored overnight at 2–8°C or at –20°C for long-term storage or subjected to a second round of PCR or qPCR amplification.**

   Note: Use 0.2–2 µl for subsequent PCR amplification (25 µl final reaction volume). Additional purification of samples prior to subsequent amplification is not required.

Table 2. Thermal cycler conditions — using cDNA as template

Step	Time	Temperature	Additional comments
Initial activation step	5 min	95°C	HotStarTaq *Plus* DNA Polymerase is activated by this step.
2-step cycling:			
Denaturation:	15 s	95°C	
Annealing/extension:	120 s	60°C	Do not change annealing/extension conditions.
Number of cycles:	8–14		Do not use more than 14 cycles as the PCR must still be in a linear range to avoid uneven amplification of different amplicons.

Table 3. Thermal cycler conditions — using gDNA as template

Step	Time	Temperature	Additional comments
Initial activation step	5 min	95°C	HotStarTaq *Plus* DNA Polymerase is activated by this step.
3-step cycling:			
Denaturation:	30 s	95°C	
Annealing:	30 s	60°C	The annealing temperature of 60°C is suitable for most PCR systems when using gDNA as a template. For bisulfite-converted DNA, use 55°C.
Extension:	120 s	72°C	
Number of cycles:	8–14		Do not use more than 14 cycles as the PCR must still be in a linear range to avoid uneven amplification of different amplicons.

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