Endpoint or Real-time Protocol

Assay Optimization

Build the standard curve using the conditions described in Assay Setup to initially test sensitivity and dynamic range of the Amplifluor™ Universal Detection System on the specific instrument being used (ie. thermocycler and detection instrument). The primers and target supplied with the kit are design to act as controls in assay development for the target of interest. The following parameters should be considered during assay characterization:

1. Thermocycler: Amplifluor™ PCR conditions must be optimized on the thermocycler using primers and control template for the target of interest.

2. Detection instrument sensitivity: With a sensitive fluorescence plate reader, the Amplifluor™ Universal Detection System detects a minimum of 1000 copies of the provided Control Template.

3. Dynamic Range: With sensitive detection instruments, up to 3 logs is observed. Once optimized, PCR cycling parameters should be kept constant and experimental samples that fall above the linear portion of the standard curve should be diluted and re-assayed.

4. Reproducibility: To control for normal assay setup errors, a minimum of sample duplicates are required for all fluorescence measurements.

Assay Setup

Assay setup and the required controls for each target of interest are as follows:

Table 1: Recommended Assay Setup

PCR reactions	Sample Type	Performed in
1-2	No Enzyme Control	Duplicate, optional
3-5	No Target Control	Triplicate
6-17	Control Template Dilutions	Duplicate
"n" number of reactions	Experimental Sample Dilutions	Duplicate

A. No Enzyme Control

Used to assess the level of background nonspecific amplification that may occur in the No Target Controls (reactions 3-5). The observed fluorescence of the No Enzyme

Control will be generally lower than that obtained for the No Target Control (see B below). This control is optional once assay optimization and characterization is complete.

B. No Target Control

Determines the limits of PCR sensitivity and tests reactions for possible background that may occur during amplification. The statistical significance of lowest detectable signal is determined by employing the z*M test using the average relative fluorescence units (M) and standard deviation (S) from the triplicate reactions (see Appendix). This control is also utilized to assess generation of primer-dimer PCR artifact or amplicon contamination of a kit reagent (*Troubleshooting*).

C. Control Template Dilutions

Generation of a standard curve is required with each experiment and for each target of interest. Serial 10 fold dilutions of the Control Template (10^2 to 10^7 copies per reaction) are amplified. It is recommended that target dilutions are run in duplicate for increased accuracy.

D. Experimental Sample Dilutions

In some instances, experimental samples may require dilution to quantitate target levels from the standard curve using the Amplifluor™ System. Amplify the experimental and Control Template dilutions. Run at least duplicates of all experimental samples.

> **Note**: Test several dilutions of experimental samples during assay characterization to determine which dilution provides a fluorescent signal that falls within the linear range of the standard curve. Table 2 is a recommended guideline.

Table 2: Diluting Experimental Samples

Final Dilutions	Stock Solution	TE Buffer
1. Stock cDNA sample	---------	---------
2. 1:10 dilution	*5 µl of #1*	*45 µl*
3. 1:100 dilution	*5 µl of #2*	*45 µl*

PCR Amplification Setup

1. Remove all reagents needed for amplification except *Taq* Polymerase from -20°C storage and thaw at room temperature. Place experimental samples and PCR reagents in separate racks in an area designated for PCR. Place the Control Template and subsequent dilutions in a rack in an area designated for DNA template work.

2. Program the thermocycler. To minimize potential background, **preheat the block to 95°C.** The cycling parameters presented in Table 5 were optimized using the Control Primers and Template provided with the kit.

3. Determine the number of Amplifluor™ reactions that will be run and place the required number of PCR tubes in a rack. (See Table 1: *Recommended Assay Setup*)

4. Add 2 µl of TE to tubes designated as No Enzyme Controls and No Target Controls (reactions 1-5).

5. Using pipettes designated only for aliquoting DNA, prepare an initial **working stock solution** of 5×10^6 copies/µl. Then, prepare 1:10 serial dilutions of the initial stock (5×10^6 copies/µl) with TE to obtain Control Template concentrations of 5×10^5, 5×10^4, 5×10^3, 5×10^2 and 5×10^1 copies/µl. (See Table 3: *Preparing Control Template Dilutions*)

Table 3: Preparing Control Template Dilutions

Final Concentration	Stock Solution	TE
1. 5×10^6 copies per µl	working stock	---------
2. 5×10^5 copies per µl	5 µl of #1	45 µl
3. 5×10^4 copies per µl	5 µl lot #2	45 µl
4. 5×10^3 copies per µl	5 µl of #3	45 µl
5. 5×10^2 copies per µl	5 µl of #4	45 µl
6. 5×10^1 copies per µl	5 µl of #5	45 µl

Notes:

1. Storage of the dilutions at lower concentration may result in a loss of target template.
2. The Control Template provided with the kit is at a concentration of 1 ng/µl (2×10^8 copies/µl). To prepare a stock solution of 5×10^6 copies/µl, add 2 µl of Control Template (1ng/µl) to 78 µl TE.

6. Add 2 µl of the diluted Control Template to duplicate tubes 6-17 in order of increasing concentration.

7. Add 2 µl of experimental samples to appropriately labeled tubes (n) using pipettes designated for aliquoting DNA only. *If necessary, dilute experimental samples.*

8. Prepare the PCR "Master Mix" without Taq Polymerase using the reagents listed in Table 4 (equilibrated to room temperature). Multiply each component volume by the total number of reactions being run to create the PCR "Master Mix". The volume of PCR "Master Mix" must be sufficient to include controls and all experimental samples as determined in step 3. Include extra reactions to compensate for pipetting errors.

Table 4: Preparation of PCR "Master Mix"

Components of Master Mix	Volume per reaction
dH_2O	10.25 µl
10× Reaction Mix A	2.5 µl
2.5 mM dNTP Mix	2.5 µl
10X Tailed Specific Primer (0.5 µM)	2.5 µl
10X Untailed Specific Primer (5.0 µM)	2.5 µl
Amplifluor™ UniPrimer™ (5.0 µM)	2.5 µl
Volume	**22.75 µl**

9. Aliquot 22.75 µl of the PCR Master Mix without Taq Polymerase to the No Enzyme Controls.

10. **Add Taq Polymerase** (0.25 µl x number of remaining reactions) to the PCR "Master Mix" and mix. Aliquot 23µl of the mixture into tubes containing experimental samples and Control Template dilutions. Change pipette tips with each addition.

11. Cap the tubes, spin briefly and place in preheated thermocycler.

12. Start PCR.

PCR Amplification

The cycling parameters in Table 5 provide a dynamic range of 10^3 to 10^6 copies at 40 cycles for amplification of the provided Control Template with the Amplifluor™ Universal Detection System.

Notes:

1. Amplification conditions for the target of interest require optimization with the Amplifluor™ Universal System. The conditions described below were optimized using the provided Control Primers. See Troubleshooting section if changes in cycling condition are necessary.
2. If experimental sample dilutions fall above the linear portion of the standard curve, dilute the sample further and retest.

Table 5: PCR Program for Endpoint Detection
(* Refer to Instrumentation for Real-time set-up)

Cycling Parameter	Time	Temperature
Preheat		95 °C
Denaturation:	4 minutes	95 °C
40 cycles*	15 seconds	95 °C
	20 seconds	55 °C
	40 seconds	72 °C
Final Extension	4 minutes	72 °C
Hold		4 °C

For optimal performance of your experiment, the PCR program and number of PCR cycles require optimization.