Alien Reference RNA QRT-PCR Detection Kit

INSTRUCTION MANUAL

Catalog #300602 (FAM detection)
#300604 (VIC detection)
Revision A

For In Vitro Use Only
300602-12

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Alien Reference RNA QRT-PCR Detection Kit

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MATERIALS PROVIDED

Catalog #300602, 300604

Materials Provided	Concentration	Quantity	
		Catalog #300602	Catalog #300604
Alien RNA Transcript	3×10^{10} copies/µl in 0.1 mM EDTA/RNase-free H_2O	20 µl	20 µl
Alien Probe Forward Primer	2.5 µM	100 µl[a]	100 µl[a]
Alien Probe Reverse Primer	2.5 µM	100 µl[a]	100 µl[a]
Alien FAM Probe	4 µM	100 µl[a]	—
Alien VIC probe	4 µM	—	100 µl[a]

[a]Sufficient reagents are provided for 100 reactions.

STORAGE CONDITIONS

All Components: –80°C

Notes *The Alien probe and primer solutions may also be stored at –20°C.*

Protect the Alien probe solution from light during storage (e.g., store the probe solution inside the provided box or a similar container).

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler (e.g. Stratagene Mx3005P and Mx3000P QPCR Systems)
QRT-PCR reagents [e.g. Brilliant II QRT-PCR Master Mix, 1-step (Catalog #600809) or Brilliant II QRT-PCR, AffinityScript Two-Step Master Mix (Catalog #600827) or Brilliant Multiplex QPCR Master Mix (Catalog #600553)]
Nuclease-free PCR-grade water

Revision A

INTRODUCTION

Real-time QRT-PCR is a powerful tool for mRNA quantitation in biological samples. The Alien Reference RNA QRT-PCR Detection Kit is a high-quality external reference RNA control system for QRT-PCR experiments using probe-based detection. The Alien reference RNA is useful for validating the quality of experimental RNA samples, interpreting the quality of QRT-PCR data, and monitoring the overall performance of QRT-PCR assay reagents and instrumentation. The Alien RNA transcript is an *in vitro*-transcribed RNA molecule that is nonhomologous to any known nucleic acids.

When used for sample or assay validation, the Alien reference RNA is added to experimental RNA samples and amplification of the Alien target is monitored to evaluate the quality of the sample. Reduced Alien target amplification in the test sample, relative to a reference sample of Alien RNA alone, reveals the presence of a PCR inhibitor in the test sample, or other biologically irrelevant sources of sample-to-sample variation in the assay. Figure 1 shows an example of detection of inhibition by ethanol in an RNA sample using the Alien Reference RNA QRT-PCR Detection Kit.

The Alien Reference RNA QRT-PCR Detection Kit is also well suited for assay standardization applications. Using the Alien Reference RNA as a reference control to generate standard curves allows data comparisons from multiple experiments, across platforms, and between laboratories. The Alien RNA is produced in large lots and subjected to stringent quality-control measures to ensure the availability of consistent reference RNA material over long-term experimental studies.

The Alien Reference RNA QRT-PCR Detection Kit can be used in either 1-Step (single-tube) or 2-Step (two-tube) QRT-PCR assays that employ probe-based detection. Separate protocols are provided for 1-Step and 2-Step QRT-PCR applications. QRT-PCR assays for the Gene of Interest (GOI) and Alien reference targets may be run in the same tube using multiplex detection (see *Preprotocol Considerations* for multiplexing guidelines).

Properties of the Alien RNA Transcript

The Alien RNA transcript is a ~500-nt, polyadenylated RNA molecule that is synthesized by *in vitro* transcription. The Alien RNA transcript is nonhomologous to all known nucleic acid sequences currently in public databases, as determined by BLAST comparisons against NIH sequence databases. In addition to its unique sequence, the Alien RNA transcript was designed with a GC content of approximately 50% and is predicted to have little secondary structure. The RNA has been extensively tested in QRT-PCR assays, in combination with the optimized primers and probe provided, to ensure optimal QRT-PCR amplification efficiency, thus allowing maximum sensitivity and specificity. Using the reaction conditions specified in the *Protocols* section, amplification is highly specific for the expected amplicon. The Alien RNA transcript is free of contaminating DNA.

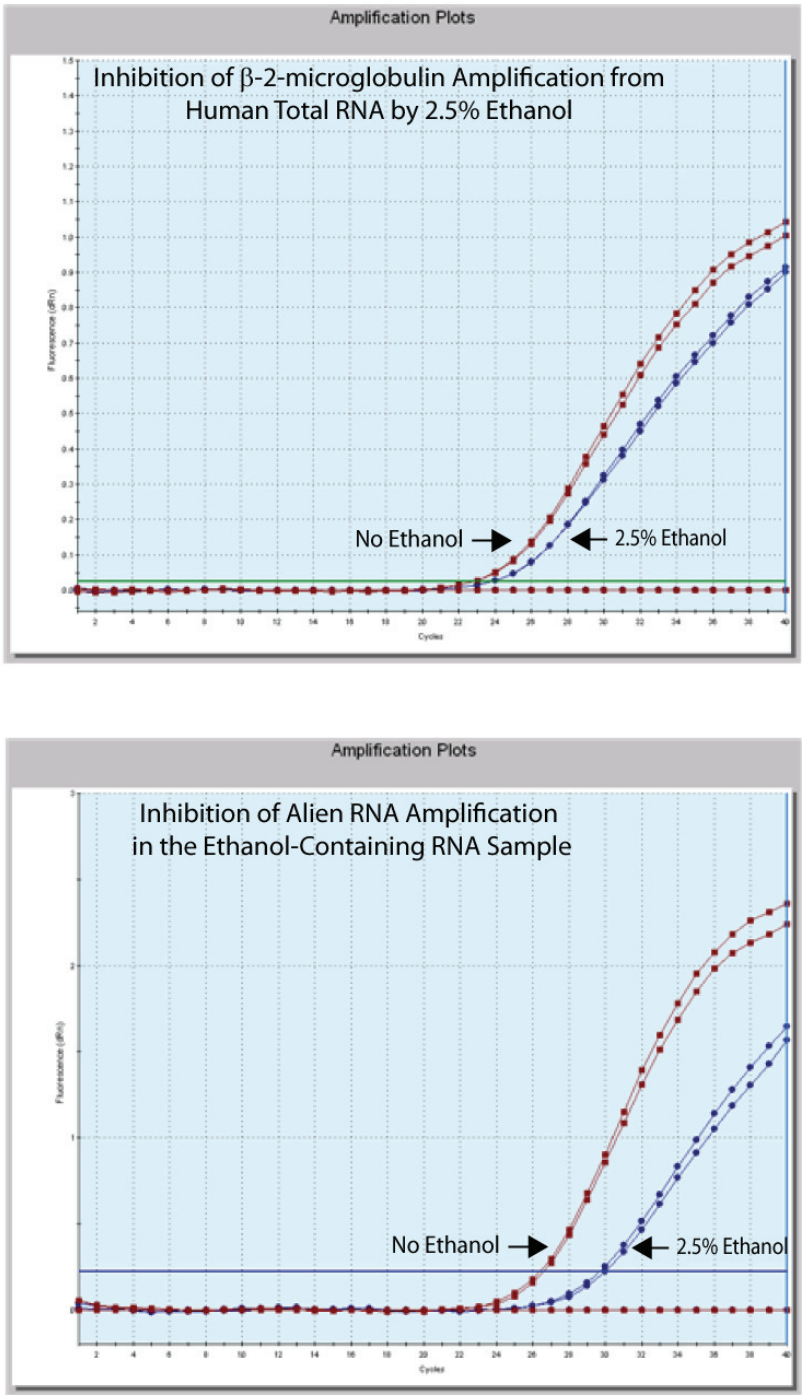

Figure 1 Detection of inhibition by 2.5% ethanol using the Alien Reference RNA QRT-PCR Detection Kit. The upper panel shows amplification of the β-2-microglobulin (B2M) target from 10 ng human total RNA (Stratagene QPCR Reference Total RNA, Human) in the absence and presence of 2.5% ethanol (final concentration in the QRT-PCR reaction). The lower panel shows amplification of the Alien RNA target from reactions in which 10^5 copies of Alien RNA transcript was added to 10 ng human total RNA samples, in the absence and presence of 2.5% ethanol (final concentration in the QRT-PCR reaction). In the presence of 2.5% ethanol, a delay of 1 Ct value was observed for the B2M target, and a delay of 3 Ct values was observed for the Alien RNA target. Experiments were performed using the Brilliant II 1-Step QRT-PCR Master Mix. Fluorescence data was collected and analyzed using the Mx3005P QPCR System.

PREPROTOCOL CONSIDERATIONS

Aliquoting and Storage of the Alien RNA Transcript

Like other purified RNA molecules, the Alien RNA transcript is sensitive to multiple freeze-thaw cycles and to storage at low concentration. The first time the RNA is used, the stock tube should be thawed and aliquoted (typically, a scheme of 20 × 1 µl aliquots is optimal), with the aliquots stored at –80°C. For each experiment, thaw a fresh aliquot and prepare serial dilutions, using the dilution scheme specified in the *Protocols* section. Storage and re-use of diluted RNA transcript solutions is not recommended.

Alien FAM Probe and Alien VIC Probe Handling Guidelines

Minimize exposure of the Alien FAM Probe or Alien VIC Probe solution to light during storage and use.

Avoid subjecting the Alien FAM Probe or Alien VIC Probe solution to an excessive number of freeze-thaw cycles. If your experimental design involves using small quantities of Alien probe solution in each experiment, consider dispensing the probe solution into several aliquots prior to use.

Multiplex Detection of GOI and Alien Targets

Multiplex QPCR allows two or more targets to be amplified and analyzed in the same reaction vessel by using spectrally distinct fluorophores for the detection of each amplicon. Multiplex QRT-PCR has been validated for the Alien transcript with several GOI targets using the Brilliant Multiplex QPCR Master Mix (Stratagene Catalog #600553) in conjunction with the AffinityScript QPCR cDNA Synthesis Kit (Stratagene Catalog #600559). For best results, multiplex assays should be performed using reagent systems, like the Brilliant Multiplex QPCR Master Mix, that have been optimized for multiplex applications.

Dye Compatibility

All probes used in a given multiplex experiment must be labeled with spectrally distinct fluorophores that have minimal overlap in excitation and emission spectra. See the following table for Alien FAM Probe and Alien VIC Probe dye compatibility for multiplex experiments run on the Stratagene Mx3000P or Mx3005P QPCR instrument. If you are using another QPCR platform, consult the instrument manufacturer's literature for dye compatibility information.

Alien Probe Dye	FAM	TET	HEX	JOE	VIC	Cy3	TAMRA	ROX	Texas Red	Cy5
FAM	—	NO	YES	YES	YES	YES	YES	YES	YES	YES
VIC	YES	NO	NO	NO	—	NO	NO	YES	YES	YES

Assay Compatibility

When designing multiplex QRT-PCR assays, it is important to verify that there is no interference between reaction components that prevents quantitative amplification of each target. The best way to test for interference is to run each assay in separate tubes as well as both assays multiplexed in the same tube and verify equivalent Ct results for each target run in singleplex vs. multiplex. If interference is detected, the multiplex assay requires reoptimization. (As a general guideline, interference is indicated by ΔCt values >1 for singleplex vs. multiplex conditions.) Use the reaction conditions given for the Alien target in *Protocols* as the starting point for optimization for this target. Refer to the following guidelines for additional multiplex QPCR optimization suggestions:

- Amounts of Alien target added to the test reactions may require optimization according to the abundance of the GOI target. In general, multiplexing with high-abundance targets may require increasing the amount of Alien target per reaction, while multiplexing with low abundance targets may require reducing the amount of Alien transcript.

- The optimal concentration of the upstream and downstream primers for each target may differ from optimal concentrations for singleplex assays. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate endpoint fluorescence for a given target concentration. The best concentrations of the upstream and downstream primers are not always of equal molarity. Alien upstream and downstream primers are provided separately to allow for independent optimization of each primer when necessary.

- When the GOI is present at high abundance, the limiting primer concentrations should be determined for the GOI target, to avoid competition among QPCR targets. The limiting primer concentration is the primer concentration that results in the lowest endpoint fluorescence intensity without affecting the Ct. Limiting primer concentrations are determined by titrating the forward and reverse primer concentrations while maintaining constant probe and target concentrations.

- Reoptimize probe concentrations by titrating the concentration of probe to determine the concentration that results in the lowest Ct and an adequate endpoint fluorescence for a given target concentration.

Note *To conserve precious RNA samples, optimization experiments may be carried out using an abundant, high quality total RNA preparation, such as the Stratagene QPCR Human Reference RNA (Catalog #750500)or the Stratagene QPCR Mouse Reference RNA (Catalog #750600), as template.*

Using a Passive Reference Dye

Many QRT-PCR assay systems include a passive reference dye that is added to each reaction to monitor non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized, and compensates for changes in fluorescence between wells caused by minor volume differences in reaction tubes. A passive reference dye (with excitation and emission wavelengths of 584 nm and 612 nm, respectively) is included with each of the Brilliant QRT-PCR reagent kits, which are employed in the specific protocols provided. When using this passive reference dye, it is important to use a dilution appropriate to the specific QPCR platform used in the experiment. Although addition of the reference dye is optional when using the Mx3005P, Mx3000P or Mx4000 system, with other real-time PCR instruments (including the ABI 7900HT and ABI PRISM® 7700) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If using Stratagene's Mx3000P, Mx3005P, or Mx4000 instruments, use the reference dye at a final concentration of 30 nM. If using the ABI PRISM 7700 instrument, use the reference dye at a final concentration of 300 nM. For other instruments, use the following guidelines for passive reference dye optimization. For instruments that allow excitation at ~584 nm (including most tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED), begin optimization using the reference dye at a final concentration of 30 nM. For instruments that do not allow excitation near 584 nm, (including most laser-based instruments) begin optimization using the reference dye at a final concentration of 300 nM.

PROTOCOLS

Preparing Dilutions of the Alien RNA Transcript

The first time the Alien RNA transcript is used, the stock tube (containing 3×10^{10} copies/µl) should be thawed and aliquoted, with the aliquots stored at –80°C. For each experiment, thaw a fresh aliquot and prepare serial dilutions according to the needs of the experiment.

The optimized conditions provided below include 10^5 copies of Alien transcript per QRT-PCR reaction, provided by adding 2 µl of a dilution containing 5×10^4 copies/µl. To prepare this RNA dilution, thaw an aliquot of the Alien RNA transcript stock on ice. Dilute a 1-µl aliquot with 59 µl of RNase-free H_2O, to a final concentration of 5×10^8 copies/µl. From this initial 1:60 dilution, prepare 10-fold serial dilutions in RNase-free H_2O.

For standardization applications, prepare serial dilutions of the Alien RNA transcript spanning the desired copy number range. The optimized assay conditions listed below generate high quality standard curves for the range of 10^8 to 10^1 copies Alien RNA per reaction.

One-Step QRT-PCR: Optimized Conditions for Alien Target QRT-PCR

The protocol provided below details optimized conditions for analysis of the Alien reference target in one-step QRT-PCR. Depending on the experimental design, reactions may include other components, such as test RNA samples. The amount of H_2O in the reaction should be adjusted for the volumes of these reaction-specific components.

Conditions were optimized using the Brilliant II QRT-PCR Master Mix Kit, 1-Step (Stratagene Catalog #600809). This kit includes an optional passive reference dye, which must be diluted according to the QPCR platform used (see *Preprotocol Considerations* for more information). The protocol may be adapted to other single-tube QRT-PCR reagent systems (please consult the manufacturer's instructions for reaction mixture specifications).

Optimized QRT-PCR Reaction Mixture (25 µl Reaction)

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl
- 12.5 µl 2× Brilliant II QRT-PCR master mix
- 1 µl Alien FAM probe or Alien VIC probe (4 µM)
- 1 µl Alien probe forward primer (2.5 µM)
- 1 µl Alien probe reverse primer (2.5 µM)
- 0.375 µl **diluted** reference dye (optional)
- 1 µl RT/RNase block enzyme mixture
- 2.0 µl **diluted** Alien RNA transcript (10^5 copies total, or appropriate copy number for the specific experiment)

1-Step QRT-PCR Thermal Profile

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[a]	95°C
40[b]	15 seconds	95°C
	1 minute[c]	60°C

[a] Initial 10 minute incubation is required to fully activate the DNA polymerase.

[b] Increase the number of cycles to 45 when performing standard curves that include $<10^3$ copies of the Alien target per reaction.

[c] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

Protocol Modifications for Multiplex One-Step QRT-PCR

When analyzing GOI and Alien targets in multiplex, make the following modifications to the protocol above:

- Use a reagent system that has been optimized for multiplex QRT-PCR to limit competition between targets.
- Verify that amplification of the Alien target and the GOI target are not affecting each other. If interference between the two targets is detected, reoptimize the assay for primer, probe, and template amounts (see *Multiplex Detection of GOI and Alien Targets* in *Preprotocol Considerations* for optimization guidelines).

Two-Step QRT-PCR: Optimized Conditions for Alien Target QRT-PCR

The protocol provided below details the optimized conditions for analysis of the Alien reference target in two-step QRT-PCR. Depending on the experimental design, reactions may include other components, such as test RNA samples. The amount of H_2O in the reaction should be adjusted for the volumes of these reaction-specific components.

Conditions were optimized using the Brilliant II QRT-PCR, AffinityScript Two-Step Master Mix Kit (Stratagene Catalog #600827). This kit includes an optional passive reference dye, which must be diluted according to the QPCR platform used (see *Preprotocol Considerations* for more information.) The protocol may be adapted to other two-tube QRT-PCR reagent systems (please consult the manufacturer's instructions for reaction mixture specifications).

Optimized cDNA Synthesis Reaction (20 µl Reaction)

Nuclease-free PCR-grade H_2O to adjust the final volume to 20 µl
10.0 µl cDNA synthesis master mix (2×)
3.0 µl oligo(dT) primer (0.1 µg/µl)
1.0 µl AffinityScript RT-RNase Block enzyme mixture
2.0 µl **diluted** Alien RNA transcript (10^5 copies total, or appropriate copy number for the specific experiment)

cDNA Synthesis Thermal Profile

Cycles	Duration of cycle	Temperature	Purpose
1	5 minutes	25°C	Primer annealing
1	15 minutes[a]	42°C	cDNA synthesis
1	5 minutes	95°C	Termination

Optimized QPCR Reaction Mixture (25 µl Reaction)

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl
12.5 µl Brilliant II QPCR master mix (2×)
1 µl Alien FAM probe or Alien VIC probe (4 µM)
1 µl Alien probe forward primer (2.5 µM)
1 µl Alien probe reverse primer (2.5 µM)
0.375 µl **diluted** reference dye (optional)
2 µl cDNA (from synthesis reaction detailed above)

QPCR Thermal Profile

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40[b]	15 seconds	95°C
	1 minute[c]	60°C

[a] Initial 10 minute incubation is required to fully activate the DNA polymerase.
[b] Increase the number of cycles to 45 when performing standard curves that include $<10^3$ copies of the Alien target per reaction.
[c] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

Protocol Modifications for Multiplex Two-Step QRT-PCR

When analyzing GOI and Alien targets in multiplex, make the following modifications to the protocol above:

- Use the Brilliant Multiplex QPCR Master Mix (Stratagene Catalog #600553), in conjunction with the AffinityScript QPCR cDNA Synthesis Kit (Stratagene Catalog #600559).
- Verify that amplification of the Alien target and the GOI target are not affecting each other. If interference between the two targets is detected, reoptimize the assay for primer, probe, and template amounts (see *Multiplex Detection of GOI and Alien Targets* in *Preprotocol Considerations* for optimization guidelines.)

Interpretation of Results

Examine the Ct values obtained for reference reactions (Alien RNA transcript alone) and for each of the test reactions (Alien RNA transcript added to experimental RNA). Calculate the ΔCt value for each of the test reactions relative to the reference reactions. Compare the magnitude of the ΔCt values to the variation of Ct values among replicates to determine whether any of the test samples show a significant increase in the Ct for Alien RNA amplification.

Recommendations for Removing QRT-PCR Inhibitors

If QRT-PCR inhibition is detected in an experimental RNA sample, a variety of approaches may be considered for reducing the inhibitor concentration to acceptable (non-inhibitory) levels. First, when all of the targets used in the experiment (gene of interest and any normalizer targets) are present in sufficiently high abundance, it may be possible to simply dilute the experimental RNA sample prior to running the QRT-PCR assay. For some target/inhibitor combinations, dilution in the 10–100 fold range may result in a reduction of the inhibitor concentration to acceptable levels, while the concentration of the targets remains in the linear range of the QRT-PCR assay.

A second approach, which may be suitable for experiments using lower abundance targets, is purification of the total RNA sample either by ethanol precipitation or by using spin column-based RNA purification methods (e.g. Stratagene Absolutely RNA Purification Kits). Either purification method may, however, result in some loss of RNA, so these methods should only be considered if the sample contains a sufficiently large quantity of RNA.

TROUBLESHOOTING

Observation	Suggestion
The Ct obtained for the Alien RNA transcript increases over time	Avoid subjecting the Alien RNA transcript to multiple freeze-thaw cycles. Use fresh aliquots of the concentrated stock, stored at –80°C, to prepare dilutions for each experiment.
Ct values obtained for the Alien RNA target are lower in inhibitor test samples compared to reference samples	Verify that the Alien primer mix does not amplify sequences within the experimental RNA samples. Perform amplification reactions lacking the Alien RNA transcript but including the experimental RNA sample and the Alien RNA primer mix.
Increased fluorescence with cycling in no template control reactions	The reagents are contaminated. Repeat the experiment using a fresh set of reagents.
Little or no increase in fluorescence with cycling	Ensure that the cycling program includes the 10 minute incubation at 95°C in order to activate SureStart *Taq* DNA polymerase.
Ct values for Alien target are consistently higher in test reactions compared to reference reactions	Verify that the amount of total RNA added does not exceed the RNA capacity of the assay. Excess amounts of nucleic acids are inhibitory to the PCR amplification reaction. The RNA capacity of the assay is determined by the format of the assay (1-step vs. 2-step) as well as the specific reagent system used. When using Stratagene Brilliant QRT-PCR Master Mix kits, the capacity is 5 µg when performing 2-step QRT-PCR and 4 µg when performing 1-step QRT-PCR. A titration should be performed, using pure total RNA (e.g. Stratagene QPCR Human Reference Total RNA, catalog #750500) to determine the total RNA capacity of other reagent systems.
	For multiplexed assays, amplification of GOI may interfere with amplification of the Alien target. Reoptimize the multiplex assay according to the parameters outlined in *Preprotocol Considerations*. Alternatively, redesign the experiment for singleplex detection of each target.
Inhibition of Alien RNA amplification that is not addressed by sample purification or dilution	Verify that the amount of total RNA added does not exceed the RNA capacity of the assay. Adding excess RNA can lead to false-positive indications of inhibitors in RNA samples.
	The purification or dilution step may not have reduced the inhibitor concentration enough to remove the inhibition. Where possible, try further dilution of the experimental RNA sample or complete an additional round of purification. Some inhibitors may not be removed by the purification method chosen.

ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.