Alien QRT-PCR Inhibitor Alert

INSTRUCTION MANUAL

Catalog #300600

Revision C.0

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300600-12

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Alien QRT-PCR Inhibitor Alert

MATERIALS PROVIDED

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Materials provided	Quantity	Concentration
Alien RNA Transcript	20 µl	3×10^{10} copies/µl in 0.1 mM EDTA/RNase-free H_2O
Alien Primer Mix	400 µl[a]	2.5 µM (1.25 µM each forward and reverse primers)

[a]Sufficient reagents are provided for 400 inhibitor test reactions.

STORAGE CONDITIONS

All Components: –80°C

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
QRT-PCR reagents [e.g. Brilliant II SYBR® Green QRT-PCR Master Mix, 1-step (Stratagene catalog #600825) or Brilliant SYBR® Green QRT-PCR, AffinityScript Two-Step Master Mix (Stratagene catalog #600585)]
Nuclease-free PCR-grade water

Revision C.0

INTRODUCTION

Real-time QRT-PCR is a powerful tool for mRNA quantitation in biological samples. Alien QRT-PCR Inhibitor Alert* is a high-quality external control for detecting inhibitors in RNA samples for quantitative RT-PCR experiments. The Alien RNA transcript is an *in vitro*-transcribed RNA molecule that has no significant homology to any known nucleic acids.

The inhibitor alert test is performed by comparing amplification of a known amount of Alien RNA in the absence and presence of the experimental RNA samples of interest. Specifically, the threshold cycle, or Ct, for amplification of the Alien RNA is measured in reference reactions containing the Alien RNA transcript alone. In separate inhibitor test reactions, each of the RNA samples of interest are added to an Alien RNA amplification reaction. An increased Ct for the Alien RNA in a specific test reaction, relative to the reference reactions, indicates the potential presence of one or more inhibitors of QRT-PCR. Figure 1 shows an example of detection of the inhibitor guanidine in a total RNA sample using the Alien QRT-PCR inhibitor alert system.

The Alien QRT-PCR inhibitor alert can be used to detect inhibitors in either 1-Step (single-tube) or 2-Step (two-tube) QRT-PCR assays that employ SYBR® Green dye for detection. Separate protocols are provided for 1-Step and 2-Step QRT-PCR applications.

The Alien QRT-PCR inhibitor alert is also well suited for assay standardization applications. Using the Alien QRT-PCR inhibitor alert 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.

Properties of the Alien RNA Transcript and Optimized Primers

The Alien RNA transcript is a ~500-nt, polyadenylated RNA molecule that is synthesized by *in vitro* transcription. The Alien RNA transcript does not have significant homology to any 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 provided, to ensure optimal QRT-PCR amplification efficiency, thus allowing maximum sensitivity for inhibitor detection. The Alien RNA transcript is free of contaminating DNA.

An optimized primer pair for amplification of the Alien RNA target is provided. The primer pair has been optimized for QRT-PCR efficiency, sensitivity, and specificity. Using the reaction conditions specified in the *Protocols* section, amplification is highly specific for the expected 239-bp amplicon, with very little primer-dimer formation.

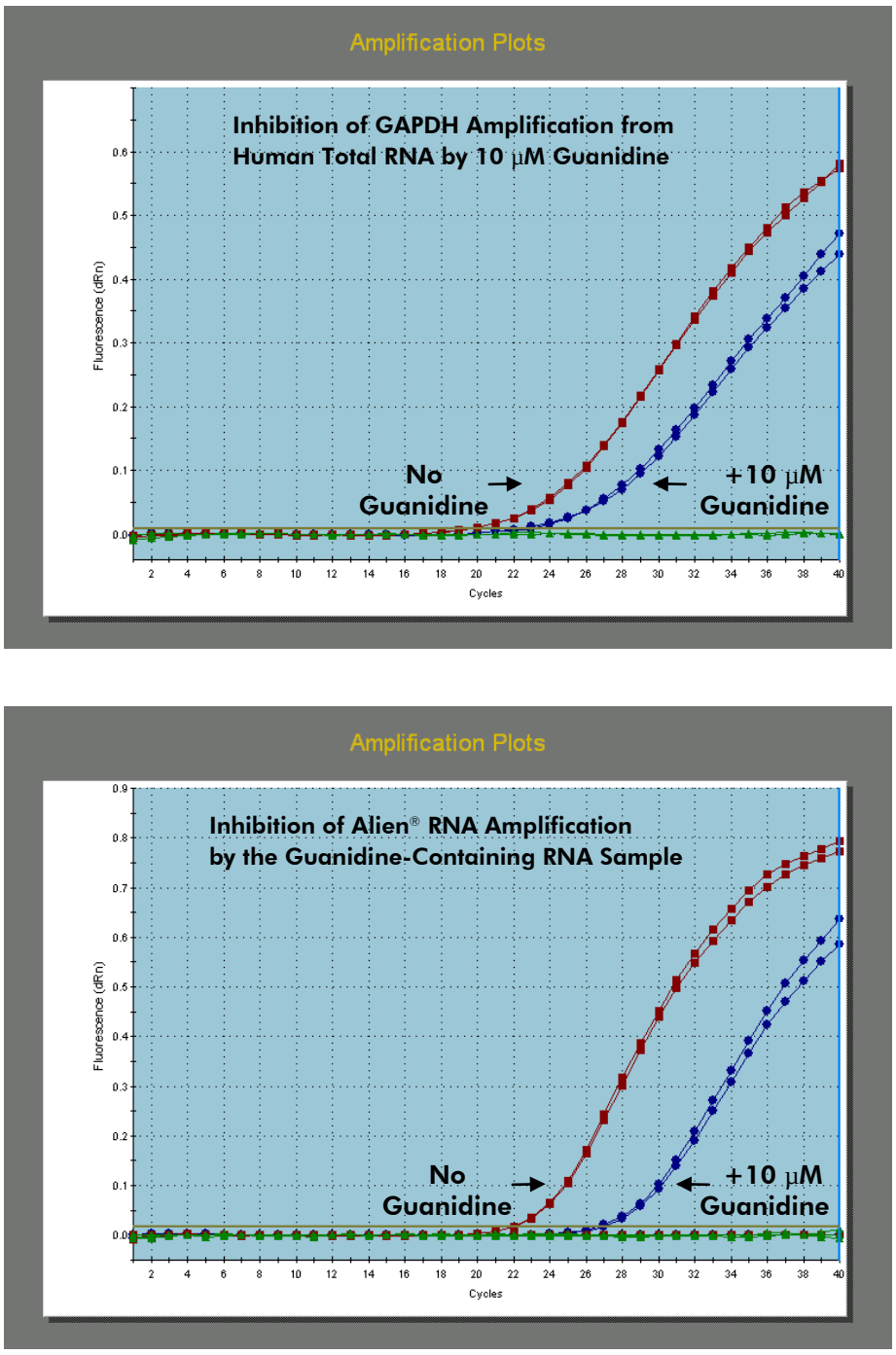

Figure 1 Detection of inhibition by 10 µM guanidine using the Alien QRT-PCR Inhibitor Alert. The upper panel shows amplification of the GAPDH target from human total RNA (50 ng Stratagene QPCR Reference Total RNA, Human) in the absence and presence of 10 µM guanidine (final concentration in the QRT-PCR reaction). The lower panel shows amplification of the Alien RNA target from samples containing a mixture of 10^5 copies of Alien RNA transcript and 50 ng human total RNA, in the absence and presence of 10 µM guanidine (final concentration in the QRT-PCR reaction). In the presence of 10 µM guanidine, a delay of 3 Ct values was observed for the GAPDH target, and a delay of 5 Ct values was observed for the Alien RNA target. Experiments were performed using the Brilliant SYBR® Green 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.

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 SYBR Green 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 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.

Total RNA Capacity of the Inhibition Assay

It has been shown that excess amounts of nucleic acids are inhibitory to the PCR amplification reaction. For this reason, there is an upper-limit to the amount of total RNA that may be added to the Alien QRT-PCR inhibitor alert assay, which 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 SYBR Green QRT-PCR Master Mix kits, the capacity is 1000 ng when performing 2-step QRT-PCR and 100 ng when performing 1-step QRT-PCR. A titration should be performed, using pure total RNA (e.g. Stratagene QPCR Reference Total RNA, catalog #750500) to determine the total RNA capacity of other reagent systems.

PROTOCOLS

Inhibitor Detection for 1-Step QRT-PCR Assays

The following protocol uses Brilliant II SYBR Green QRT-PCR master mix kit, 1-step (Stratagene catalog #600825), with analysis performed on Stratagene Mx3000P or Mx3005P instruments or the ABI PRISM 7700 instrument. This protocol may be adapted to other single-tube QRT-PCR reagent systems (please follow the reagent manufacturer's instructions for preparing the reaction mixture). Other QPCR platforms may also be used (please consult the instrument manufacturer's instructions for reference dye preparation and QRT-PCR program recommendations).

Preparing Dilutions of the Alien RNA Transcript

Thaw an aliquot of the Alien RNA transcript (stored at –80°C at 3×10^{10} copies/µl) on ice. Dilute a 1-µl aliquot of the stock 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 four 10-fold serial dilutions in RNase-free H_2O, to a final concentration of 5×10^4 copies/µl. The protocol provided below requires 2 µl of the final dilution (5×10^4 copies/µl) per QRT-PCR reaction.

Preparing 1-Step QRT-PCR Reactions

Note *It is prudent to set up a no-template control reaction to screen for contamination of reagents or false amplification.*

1. If the reference dye (provided with the Brilliant II SYBR Green QRT-PCR master mix kit, 1-step) will be included in the reaction, dilute the dye solution **1:500 for the Mx3000P and Mx3005P instruments,** or **1:50 for the ABI PRISM 7700 instrument** using nuclease-free PCR-grade H_2O. For other instruments, use the guidelines in *Preprotocol Considerations*.

   Note *If using a system other than the Mx3000P, Mx3005P or Mx4000 instruments, the use of the reference dye may be required for optimal results.*

2. Thaw the 2× SYBR Green QRT-PCR master mix and store on ice. Mix the solution well by gentle inversion prior to pipetting.

   Note *Once the master mix is thawed, store it on ice while setting up the reactions. Following initial thawing, store the unused portion at 4°C.* ***SYBR Green I dye (present in the master mix) is light-sensitive, solutions containing the master mix should be protected from light whenever possible.***

3. Prepare the experimental reactions by combining the following components *in order*. Prepare a single reagent mixture for all of the reactions in the run, plus at least one reaction volume excess, using multiples of each component listed below. Runs should include duplicate inhibitor test reactions (Alien RNA transcript plus experimental RNA) and duplicate reference (Alien RNA transcript only) reactions.

Reagent Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including experimental RNA, or dH_2O, added in step 5)
12.5 µl of 2× SYBR QRT-PCR master mix
1 µl of Alien Primer Mix (final concentration 50 nM each primer)
0.375 µl of the **diluted** reference dye (optional)
1 µl of RT/RNase block enzyme mixture
2.0 µl of **diluted** Alien RNA transcript (10^5 copies)

Note *A total reaction volume of 50 µl may also be used.*

4. Gently mix the reactions without creating bubbles (do not vortex), then distribute the mixture to the inhibitor test and reference reaction tubes.

5. Add *x* µl of each experimental RNA sample (up to 100 ng total RNA) to the appropriate inhibitor test reactions. Add an equivalent volume of QPCR-grade dH_2O to the reference (Alien RNA only) reactions.

Notes *The volume of RNA sample should match the volume to be used in the QRT-PCR assays that detect the target of interest, such that the two assays contain equivalent concentrations of any potential inhibitors.*

The RNA contributed by the experimental sample should not exceed 100 ng. Greater amounts of RNA are inhibitory to the RT-PCR reaction, and may generate false-positive results for the presence of inhibitors.

Reagent systems vary in the amount of RNA that inhibits the QRT-PCR reaction. See Preprotocol Considerations *for more information when using other QRT-PCR assay reagents.*

6. Gently mix the reactions without creating bubbles (do not vortex).

Note *Bubbles interfere with fluorescence detection.*

7. Centrifuge the reactions briefly.

Cycling Program for1-Step QRT-PCR

8. Place the reactions in the QPCR instrument and run the QRT-PCR program below.

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

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

[b] Set the instrument to detect and report fluorescence during both the annealing and extension steps of each cycle.

Dissociation Program

9. Following the QRT-PCR cycling program, perform a dissociation curve to verify that the fluorescence signal obtained is due to true amplification of the Alien RNA target. Follow the QPCR instrument manufacturer's guidelines for generating dissociation curves. The dissociation curve obtained is expected to appear similar to the sample data shown in Figure 2, where the fluorescence signal obtained is due to specific amplification of the Alien RNA target, with little or no primer-dimer formation.

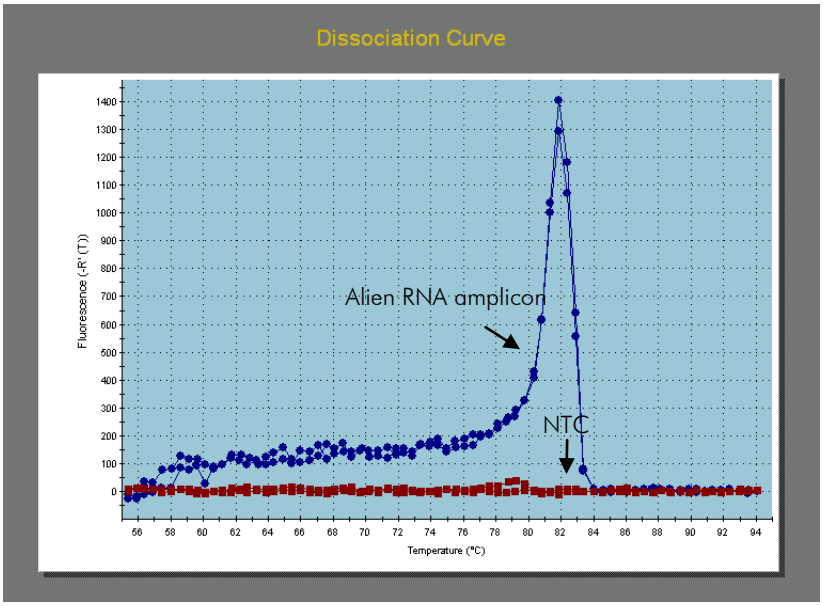

Figure 2 Expected dissociation curve profile for products of amplification of the Alien RNA target using the Alien primer mix. Primer-dimers (not present in this plot) are indicated by the presence of a dissociation peak centered at ~76°C for experiments analyzed using the Mx3005P QPCR system.

Inhibitor Detection for 2-Step QRT-PCR Assays

The following protocol uses Brilliant SYBR Green QRT-PCR, AffinityScript two-step master mix (Stratagene catalog #600585), with analysis performed on Stratagene Mx3000P or Mx3005P instruments or the ABI PRISM 7700 instrument. This protocol may be adapted to other two-tube QRT-PCR reagent systems (please follow the reagent manufacturer's instructions for preparing the reaction mixture). Other QPCR platforms may also be used (please consult the instrument manufacturer's instructions for reference dye preparation and RT-PCR program recommendations).

Preparing Dilutions of the Alien RNA Transcript

Thaw an aliquot of the Alien RNA transcript (stored at –80°C at 3×10^{10} copies/µl) on ice. Dilute a 1-µl aliquot of the stock 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 four 10-fold serial dilutions in RNase-free H_2O, to a final concentration of 5×10^4 copies/µl. The protocol provided below requires 2 µl of the final dilution (5×10^4 copies/µl) per QRT-PCR reaction.

Synthesis of First-Strand cDNA

1. Prepare the experimental reactions by combining the following components *in order*. Prepare a single reagent mixture for duplicate inhibitor test reactions and duplicate reference (Alien RNA transcript only) reactions, plus at least one reaction volume excess, using multiples of each component listed below.

cDNA Synthesis Reagent Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 20 µl (including experimental RNA, or dH_2O, added in step 3)
10.0 µl of cDNA synthesis master mix (2×)
3.0 µl of oligo(dT) primer (0.1 µg/µl)
1.0 µl of AffinityScript RT-RNase Block enzyme mixture
2.0 µl of **diluted** Alien RNA transcript (10^5 copies)

2. Gently mix the reagents without creating bubbles (do not vortex), then distribute the mixture to each of the inhibitor test and reference reaction tubes.

3. Add *x* µl of each experimental RNA sample (up to 1000 ng total RNA) to the appropriate inhibitor test reactions. Add an equivalent volume of QPCR-grade dH_2O to the reference (Alien RNA only) reactions.

   Notes *The volume of RNA sample should match the volume to be used in the QRT-PCR assays that detect the target of interest, such that the two assays contain equivalent concentrations of any potential inhibitors.*

   The RNA contributed by the experimental sample should not exceed 1000 ng. Greater amounts of RNA are inhibitory to the RT-PCR reaction, and may generate false-positive results for the presence of inhibitors.

   Reagent systems vary in the amount of RNA that inhibits the QRT-PCR reaction. See Preprotocol Considerations *for more information when using other QRT-PCR assay reagents.*

4. Incubate the reactions at 25°C for 5 minutes to allow primer annealing.

5. Incubate the reactions at 42°C for 15 minutes to allow cDNA synthesis.

6. Incubate the tubes at 95ºC for 5 minutes to terminate the cDNA synthesis reaction.

7. Place the completed first-strand cDNA synthesis reactions on ice for immediate use in QPCR.

QPCR Amplification of cDNA

Note *It is prudent to set up a no-template control reaction to screen for contamination of reagents or false amplification.*

Setting Up the QPCR Reactions

8. If the reference dye (provided with the Brilliant SYBR Green QRT-PCR master mix kit, 2-step) will be included in the reaction, dilute the dye solution **1:500 for the Mx3000P and Mx3005P instruments,** or **1:50 for the ABI PRISM 7700 instrument** using nuclease-free PCR-grade H_2O. For other instruments, use the guidelines in *Preprotocol Considerations*.

   Note *If using a system other than the Mx3000P, Mx3005P or Mx4000 instruments, the use of the reference dye may be required for optimal results.*

9. Thaw the 2× SYBR Green QPCR master mix and store on ice. Mix the solution well by gentle inversion prior to pipetting.

Note *Once the Brilliant SYBR Green QPCR master mix is thawed, store it on ice while setting up the reactions. Following initial thawing, store the unused portion at 4°C.* ***SYBR Green I dye (present in the master mix) is light-sensitive, solutions containing the master mix should be protected from light whenever possible.***

10. Prepare the QPCR reactions by combining the following components in order. Prepare a single reagent mixture for all of the test and reference samples, using multiples of each component listed below.

    QPCR Reagent Mixture

    9.125 µl of nuclease-free PCR-grade H_2O
    12.5 µl of Brilliant SYBR Green QPCR master mix (2×)
    1.0 µl of Alien Primer Mix (final concentration 50 nM each primer)
    0.375 µl of diluted reference dye (optional)

    Note *Total reaction volumes of 50 µl may also be used.*

11. Gently mix the reagents without creating bubbles (do not vortex) then distribute the mixture to the inhibitor test and reference reaction tubes.

12. Add 2 µl of each cDNA synthesis reaction to the appropriate QPCR reaction.

13. Gently mix the reactions without creating bubbles (do not vortex) and then centrifuge briefly.

    Note *Bubbles interfere with fluorescence detection.*

QPCR Cycling Program

14. Place the reactions in the QPCR instrument and run the program below.

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1.0 minute	55–60°C[b]
	30 seconds[c]	72°C

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

[b] The Alien inhibitor alert assay has been optimized using a range of annealing temperatures (55–60°C), allowing the assay to be run in tandem with typical QRT-PCR assays for other targets.

[c] Set the instrument to detect and report fluorescence during both the annealing and extension steps of each cycle.

Dissociation Program

15. Following the QRT-PCR cycling program, perform a dissociation curve to verify that the fluorescence signal obtained is due to true amplification of the Alien RNA target. Follow the QPCR instrument manufacturer's guidelines for generating dissociation curves. The dissociation curve obtained is expected to appear similar to the sample data shown in Figure 3, where the fluorescence signal obtained is due to specific amplification of the Alien RNA target, with little or no primer-dimer formation.

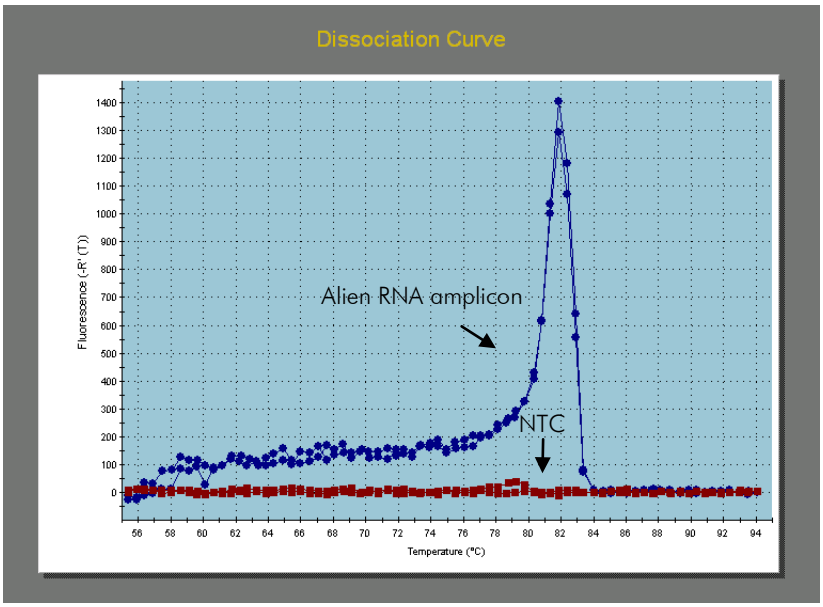

Figure 3 Expected dissociation curve profile for products of amplification of the Alien RNA target using the Alien primer mix. Primer-dimers (not present in this plot) are indicated by the presence of a dissociation peak centered at ~76°C for experiments analyzed using the Mx3005P QPCR system.

Interpretation of Results

Examine the Ct values obtained for the reference reactions (Alien RNA transcript alone) and for each of the inhibitor test reactions (Alien RNA transcript plus experimental RNA). Calculate the ΔCt value for each of the inhibitor test reactions relative to the reference reactions. Compare the magnitude of the ΔCt values to the variation of Ct values among duplicates to determine whether any of the inhibitor 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 an RNA purification spin column. 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
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.
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.
Inhibition of Alien RNA amplification that is not addressed by sample purification or dilution	Verify that the amount of experimental RNA added to the inhibition test reactions does not exceed 100 ng for 1-step RT-PCR or 1000 ng for 2-Step RT-PCR when using Brilliant SYBR Green QRT-PCR master mixes. The amounts of experimental RNA that may be accommodated using other reagent systems must be determined empirically.
	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.
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.
Primer-dimers observed in the melting curve	Verify that the Alien RNA dilutions were made correctly. While primer-dimers are not expected in amplification reactions containing 10^5 copies of the Alien RNA transcript, primer-dimers may be detected in reactions containing ≤10 copies and in no-template controls (NTC's).
Increased fluorescence with cycling in no template control reactions	The reagents are contaminated. Repeat the experiment using a fresh set of reagents.

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.