Alien Reference RNA QRT-PCR Detection Kit for Monitoring the Overall Performance of QRT-PCR Assays

Bahram Arezi, Melissa McCarthy & Holly Hogrefe
Agilent Technologies, Stratagene Products Division, La Jolla, CA 92037

Introduction

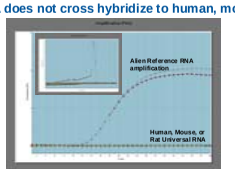
Exogenous RNA controls are increasingly used in QRT-PCR experiments to normalize variations that arise due to the presence of co-purified inhibitors (1,2). In addition, RNA controls allow assay standardization from experiment-to-experiment, across platforms, and between laboratories (3). We previously described the use of a highly sensitive exogenous Alien RNA transcript (Alien QRT-PCR Inhibitor Alert, cat. no. 300600) for monitoring the presence of common QRT-PCR inhibitors in RNA samples. When inhibitors are present, the Alien QRT-PCR Inhibitor Alert kit detects a delay in threshold cycle (Ct value) between Alien RNA amplified in the presence of sample RNA versus Alien RNA alone. The Inhibitor Alert kit employs SYBR Green chemistry, which provides high sensitivity, but does not allow multiplex amplification of Alien RNA alongside a transcript of interest.

We've recently expanded our line of exogenous controls to include TaqMan® primers and probe (FAM-and VIC-labeled) sets for detection of exogenous Alien RNA transcript in 1-step and 2-step QRT-PCR assays. As we will demonstrate, the Alien probe systems generate standard curves showing broad dynamic range (9-orders of magnitude) and single-copy detection of Alien RNA in 2-step QRT-PCR. In addition, the Alien probe system is sensitive to a variety of QRT-PCR inhibitors, and allow multiplex detection of Alien and sample RNA in the same tube.

Results

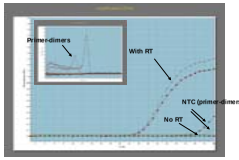
Alien Reference RNA template is a 500 bp *in vitro* transcript made from a computer generated sequence that possesses 50% GC-content and is non-homologous to sequences in GenBank.

Part 1:Alien Reference RNA does not cross hybridize to human, mouse, or rat sequences



Amplification reactions contained 100nM of Alien RNA primers and either 10^7 copies of Alien RNA target or 50ng of Human, Mouse, or Rat Universal Reference RNA (Stratagene) in separate tubes. Amplifications were performed with the Brilliant® SYBR® Green QRT-PCR Master Mix (1-step). No amplification products were detected using Human, Mouse, or Rat Universal Reference RNA.

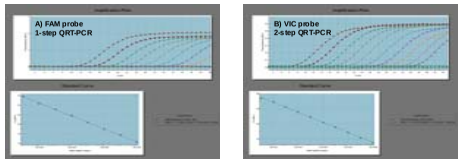
Part 2: Alien Reference RNA is devoid of contaminating DNA



Amplification reactions were performed using Brilliant II SYBR Green QRT-PCR Master Mix (1-step), 10^5 copies of Alien Reference RNA, and 100nM of Alien RNA primers with and without the reverse transcriptase as indicated. No amplification is detected in the no-RT control.

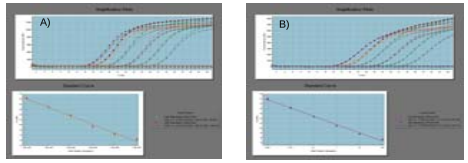
Part 3: Alien Reference RNA primer/probe sets exhibit high amplification efficiency, sensitivity, and dynamic range

a) 1-step and 2-step singleplex QRT-PCR



In panel A, Brilliant II QRT-PCR Master Mix (1-step) was used to generate a standard curve with the Alien Reference RNA FAM primer/probe set from 10^8 to 10 copies (7 orders of magnitude). In panel B, Brilliant II QRT-PCR Master Mix (2-step) was used to generate a standard curve with the Alien Reference RNA VIC primer/probe set from 10^9 to 1 copy (9 orders of magnitude). One-step QRT-PCR resulted in amplification efficiency of 97%, Rsq of 1.000, and detection limit of 10 copies. Two-step QRT-PCR generated amplification efficiency of 100%, Rsq of 0.999, and detection limit of 1 copy.

b) 2-step multiplex QRT-PCR



Results-continued

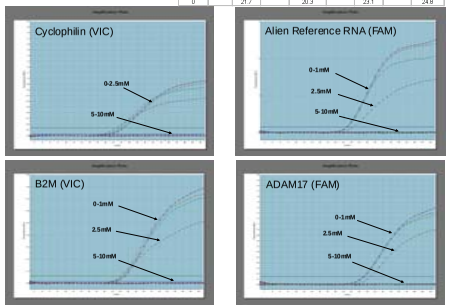
Brilliant Multiplex QPCR Master Mix was used to generate standard curves for cyclophilin (VIC) and Alien Reference RNA (FAM) individually or in multiplex. Amplifications were performed using Human QPCR Reference RNA ranging from 100 ng to 0.001 ng, and Alien Reference RNA ranging from 10^7 to 10^2 copies. Panel A shows cyclophilin standard curves in the absence (SAF = Single reaction) or presence (DAF = Duplex reaction) of Alien Reference RNA amplification. Panel B shows Alien Reference RNA standard curves in the absence (SAF = Single reaction) or presence (DAF = Duplex reaction) of cyclophilin amplification.

Part 4: Alien Reference RNA amplification is sensitive to the presence of common QRT-PCR inhibitors

In order to detect inhibiton, amplification of Alien Reference RNA alone (singleplex) is compared to the amplification of Alien Reference plus sample RNA (in singleplex or multiplex format). A delay in Ct of Alien Reference plus sample RNA suggests that the RNA sample contains inhibitors. To mimic this situation, we added two common RT-PCR inhibitors (EDTA and ethanol) to Alien Reference plus sample RNA reactions. For each set of inhibitor reactions, an HPLC water control was used to determine the Ct for "no inhibition" reactions. We demonstrate that Alien Reference RNA is sensitive to these known inhibitors in singleplex and multiplex probe formats, and hence should be able to detect a wide range of "unknown" inhibitors in RNA samples.

a) EDTA in one-step singleplex QRT-PCR:

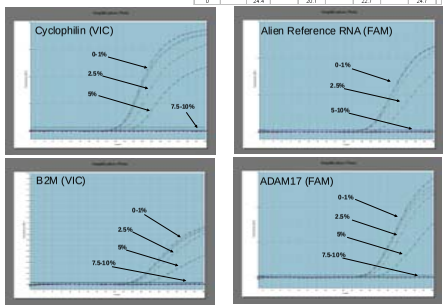
	Alien	Cyclophilin	B2M	ADAM17
EDTA mM	Ct (dRn)	Ct (dRn)	Ct (dRn)	Ct (dRn)
10	No Ct	No Ct	No Ct	No Ct
7.5	No Ct	No Ct	No Ct	No Ct
5	No Ct	No Ct	No Ct	No Ct
2.5	23.0	19.8	22.1	26.4
1	21.7	20.6	22.3	24.8
0.5	21.7	19.8	23.0	24.7
0	21.7	20.3	23.1	24.8



Amplifications were performed using Brilliant II one-step QRT-PCR kit. 10^6 copies of Alien Reference RNA and 10ng Human Reference RNA were added to the reactions. EDTA was titrated into the reactions at the concentrations indicated above. Inhibition of amplification of Alien Reference RNA and ADAM17 (low abundance target) was detected at EDTA concentrations of ≥ 2.5mM with Ct delays of 1.7 and 1.6, respectively. Amplifications of cyclophilin and B2M (medium to high abundance) were, however, only marginally affected (a slight reduction in final fluorescence but no Ct delay) at 2.5mM EDTA. Concentrations of ≥ 5mM EDTA inhibited all target amplifications. Therefore, Alien Reference RNA and ADAM17 are more sensitive to the inhibitory effects of EDTA compared to B2M and cyclophilin.

b) Ethanol in one-step singleplex QRT-PCR:

	Alien	Cyclophilin	B2M	ADAM17
EtOH%	Ct (dRn)	Ct (dRn)	Ct (dRn)	Ct (dRn)
10	No Ct	No Ct	No Ct	No Ct
7.5	No Ct	No Ct	No Ct	No Ct
5	No Ct	24.3	26.8	26.4
2.5	27.3	21.3	23.8	25.3
1	24.8	20.6	23.1	25.0
0.5	24.0	20.3	23.2	24.5
0	24.4	20.1	22.7	24.7

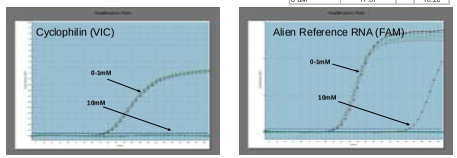


Amplifications were performed using Brilliant II one-step QRT-PCR kit. 10^6 copies of Alien Reference RNA and 10ng Human Reference RNA were added to the reactions. Ethanol was titrated into the reactions at the concentrations indicated above. When 2.5% ethanol was added into the reaction, amplifications of Alien Reference RNA, cyclophilin, B2M, and ADAM17 targets were delayed by 2.5, 1.3, 0.7, and 0.9 Cts, respectively. At 5% ethanol, Alien Reference RNA failed to amplify whereas all other targets produced delayed Cts, suggesting that Alien Reference RNA is more sensitive to the inhibitory effects of ethanol compared to all the targets tested here.

Results-continued

c) EDTA in two-step singleplex QRT-PCR:

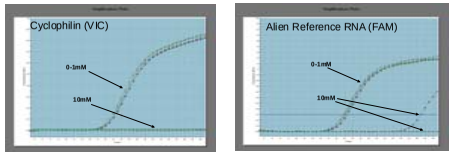
	Cyclophilin	Alien
EDTA	Ct (dRn)	Ct (dRn)
10 mM	No Ct	36.74
1 mM	18.24	18.67
100 uM	17.37	17.82
10 uM	17.46	18.26
1 uM	17.45	17.95
0 uM	17.87	18.20



Amplifications were performed using Brilliant II two-step QRT-PCR kit. 10^7 copies of Alien Reference RNA and 10ng Human Reference RNA were added to the cDNA synthesis reactions. EDTA was titrated into the reactions at the concentrations indicated above. Inhibition of amplification of both Alien Reference RNA and cyclophilin was observed at ≥ 10mM EDTA.

d) EDTA in two-step multiplex QRT-PCR:

	Cyclophilin	Alien
EDTA mM	Ct (dRn)	Ct (dRn)
10	No Ct	41.0
1	22.7	22.6
0.5	21.9	21.8
0.05	22.1	21.9
0.005	21.9	21.9
0	22.6	22.3



Amplifications were performed using Brilliant Multiplex QPCR kit. 10^7 copies of Alien Reference RNA and 10ng Human Reference RNA were added to the cDNA synthesis reactions. EDTA was titrated into the reactions at the concentrations indicated above. Both Alien Reference RNA and cyclophilin amplifications are completely inhibited at 10mM EDTA similar to the results obtained above (part 4, section c).

Conclusions

- Alien Reference RNA QRT-PCR detection kit is used 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.

- Alien Reference 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 kit includes an exogenous RNA transcript, two primers and a TaqMan probe. The TaqMan probe is provided in with FAM (#300602) or VIC (#300604) dye for multiplexing flexibility.

- When multiplexing Alien Reference RNA with a gene-of-interest (GOI), general multiplexing guidelines should be followed to minimize competition. These guidelines include using spectrally distinct fluorophores, a multiplex QPCR kit (e.g., Brilliant Multiplex kit), and limiting primer/probe concentration for higher abundance targets.

- If the presence of an inhibitor in RNA samples is suspected, researchers are encouraged to re-purify or dilute the RNA sample (if target abundance allows it) and repeat the amplification. Furthermore, a two-step QRT-PCR format may be more appropriate to use due to increased tolerance of QRT-PCR inhibitors.

- Alien Reference RNA QRT-PCR detection kit will be commercially available on 11/26/08.

References

1. Smith, R.D. et al, *Exogenous Reference RNA for Normalization of Real-time Quantitative PCR*, Biotechniques, 2003. 34:p 88-91.
2. Nolan, T. et al., *SPUD: A quantitative PCR assay for the detection of inhibitors in nucleic acid preparations*, 2006, 351: p 308-310.
3. Hartman, L.J. et al, *Development of a novel internal positive control for TaqMan based assays* , Molecular and Cellular Probes, 2005. 19: p 54-59.

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