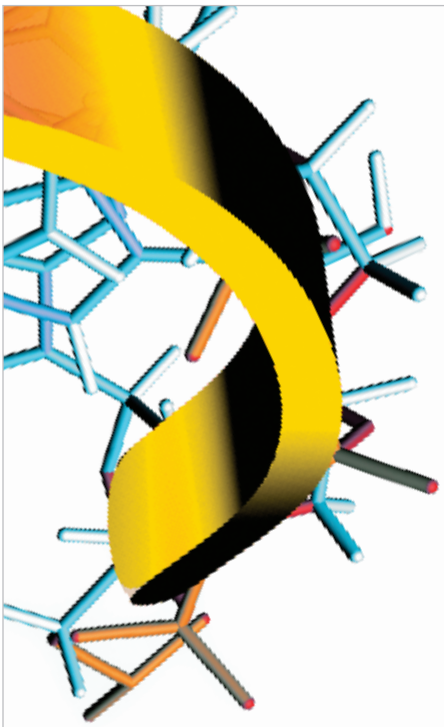

Product Stability Study

TaqMan® Gene Expression Assays

Introduction

Applied Biosystems has designed and manufactured over 40,000 TaqMan® Gene Expression Assays for quantitative gene expression analysis of human, mouse, and rat genes. These assays are inventoried at Applied Biosystems manufacturing facility, and are available for immediate sale as off-the-shelf products. Each gene-specific assay consists of a singe tube containing a fluorescently labeled TaqMan® MGB probe, a forward primer, and a reverse primer. The shelf life of these assays has recently been extended to five years—three years beyond the original control date. To establish assay stability, functional testing based on QC specifications was done to evaluate assay performance over real and accelerated time. A description of these tests and results of the temperature stability study are described in this white paper.

Objective

The objective of the temperature stability study was to determine the maximum storage time and temperature possible for TaqMan Gene Expression Assays in 20X concentration while meeting required performance specifications.

Method

Eight randomly selected TaqMan Gene Expression Assays were used for stability testing (Table 1). The probes and primers were manufactured using current validated manufacturing processes, and only those that passed manufacturing QC criteria were included in the experiment (Table 2). The sequence-specific TaqMan MGB probe and primers for each target were pooled together to create a single lot at 20X final concentration—the standard concentration of a TaqMan Gene Expression Assay. The 20X mix contains a 5 µM probe and 18 µM of each primer in TE. After pooling, the pH of each assay was 7.8. Each pooled assay was aliquoted into approved plasma-treated Matrix tubes with Matrix caps in a volume sufficient for one test. The tubes were stored at four different temperatures: -20°C, 4°C, 23°C, and 37°C. The higher temperatures were used for the accelerated studies, which allowed for rapid stability predictions. Tubes were removed weekly for nine weeks to perform functional testing.

Accelerated Testing

Accelerated testing is commonly used to make stability predictions. Since chemical reaction rates (for first order reactions) generally double with each 10°C temperature increase, accelerated stability predictions can be made according to the following formula, derived from the Arrhenius Equation[1]:

$$\text{Predicted Stability} = \text{Accelerated Stability} \times 2^{\Delta T/10}$$

Where ΔT is the difference between the normal storage temperature and the sample storage temperature. The greater this difference is, however, the less reliable the prediction. Therefore, the predictions were modified (reduced) based on previous testing of probes and primers done by Applied Biosystems. The predicted stabilities are shown in Table 3.

Functional Testing

Three templates were used for functional testing: an artificial template (AT), genomic DNA (gDNA), and a no template control (NTC). A single-stranded artificial template consisting of the forward primer, probe, and reverse primer (antisense strand) sequences was synthesized, purified, and quantitated spectrophotometrically at OD260. An aliquot of a 10^{-8} dilution of the AT was used for each reaction. Next, 10 ng genomic DNA isolated from a Raji cell line was used to test for cDNA specificity (exon/exon junctions).

For each time point, the assay stored at -20°C served as the control and was tested in parallel with the assay stored at higher temperatures. Four different assays stored at four different temperatures were tested in duplicate on the same 96-well plate, and each plate was prepared in duplicate. Thus, each assay temperature (treatment) and time point was replicated four times. The final reaction volume was 25 µL per well (reaction components are shown in Table 4). The robotic Biomek was used to set up the assay plates.

Plates were thermal-cycled on an ABI PRISM® 7700 Sequence Detection System using standard thermal cycling conditions recommended for TaqMan Gene Expression assays: 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension for 1 minute at 60°C.

Data Analysis

Analysis was performed with SDS software v1.9.1 using a manual baseline set at 3–15 and a threshold setting at 0.05. C_T values were determined for each assay at all time points and treatments, and were compared to the -20°C control value within treatments. Statistical analysis was performed to determine Analysis of Variance (ANOVA) for C_T for each assay treatment and time point. The maximum ΔRn was determined and compared to the ΔRn of the -20°C control point.

Table 1. TaqMan Gene Expression Assays

Assay ID	Gene ID
Hs00180993_m1	PMAIP1
Hs00181772_m1	BAI1
Hs00181271_m1	CHRNB4
Hs00181613_m1	ANGPT1
Hs00181633_m1	CDS1
Hs00181777_m1	BAI1
Hs00181992_m1	ITPR3
Hs00182215_m1	PLRG1

Table 2. Manufacturing QC Criteria

Test	Criteria
Mass Spectrometry	Measured mass-calculated mass = < 0.3%
Yield	Each probe = > 4,000 pmols Each primer = > 80,000 pmols
No Template Control	C_T > 38

Table 3. Predicted Stability of TaqMan Gene Expression Assays

Week to test	4°C (Months)	24°C (Months)	37°C (Months)	45°C (Months)
1			3	4
2	2	4	5	8
3	3	6	8	12
4	4	8	11	16
6	6	12	16	24
8	8	16	21	32
10	10	20	26	40
18	18	36	47	72
25	25	50	66	100
30	30	60	79	120

Table 4. Reaction Components

Template	5 µL
TaqMan® Universal PCR Master Mix (No UNG)	12.5 µL
20X TaqMan® Assay	1.25 µL
RNAse Free Water	6.25 µL
Final Volume	25 µL

Table 5. Pass Criteria for TaqMan Gene Expression Assays

Template	C_T Range	ΔRn	+/- C_T from Control
Artificial Template	14–23	ΔRn ≥ 0.95	0.5
Genomic DNA	≥ 35	N/A	N/A
No Template Control	38–40	N/A	N/A

Table 6. Variance Components (Weeks 1–5)

Component	Var. Comp.	% Total	Sqrt. (Var. Comp.)
Assay (Set ID)	2.111	50.0	1.453
Week	1.861	44.1	1.364
Treatment	0.000	0.0	0.000
Within	0.250	5.9	0.500
Total	4.224	100.0	2.055

Table 7. Variance Components (Weeks 7–72)

Component	Var. Comp.	% Total	Sqrt. (Var. Comp.)
Assay (Set ID)	6.359	92.1	2.521
Week	0.428	6.2	0.654
Treatment	0.000	0.0	0.000
Within	0.115	1.7	0.339
Total	6.902	100.0	2.627

Results

Established QC specifications were used as criteria to evaluate the performance of the assays on the three different templates (Table 5). All assays passed NTC and genomic testing for each treatment and time. C_T values for NTC on all assays and treatment times were > 38, which indicates that contamination did not occur during the testing. Likewise, amplification of each assay and treatment time with genomic DNA gave C_T values greater than 35, as expected for TaqMan Gene Expression Assays which are designed to span transcript exon-exon junctions.

Artificial template testing had to meet the following criteria. First, the AT had to be diluted so that the final template concentration gave C_T values of 14–23. The C_T range takes into consideration variations in template preparation and final concentration as well as differences attributed to plate-to-plate and run-to-run variations.

Secondly, the ΔRn for each time and temperature had to be greater than or equal to 0.95. The last requirement allows for +/- 0.5 C_T between the control (-20ºC) and each of the test treatments at each time point. When 10^{-8} dilution for each AT was tested, the C_T value for the eight assays for all treatments and time points fell within the acceptable range for all three conditions. All ΔRn values were > 0.95 and the C_T difference between the assay kept at the control temperature and each of the test temperatures were within the specified C_T.

A shift in C_T values was seen for all assays between week five and seven, and was attributed to a systematic change in the instrument (preventive maintenance and spectral dye recalibration). Assay performance under different treatments remained the same since no difference in C_T and ΔRn could be detected among the treatments.

Analysis of variance allows comparison of independent samples (assays) exposed to different treatments (storage temperature), and identifies which components contribute to any variability. To avoid introducing the system-related bias mentioned above, the first five time points were analyzed separately from the later time points. The variance components and the results of the analysis are shown in Table 6 (weeks 1 through 5) and Table 7 (weeks 7 through 72). The components listed in these tables are the Set ID (the assay), the Week (run-to-run variation, sample preparation), Treatment (temperature) and Within (accounts for imbedded procedural effects within the experiment). The major components for the first five weeks of testing (Table 6) were the Set ID and Week, contributing 50% and 44.1%, respectively. For the later weeks (Table 7), the assay alone was the only component contributing

to variability (92.1%). The variation due to different temperature treatments is negligible for the first five weeks of testing and for the following seven weeks. For the duration of the study, the temperature treatments accounted for 0% of total variation.

Conclusion

We have looked at the stability of TaqMan Gene Expression Assays over an extended period of time at real and accelerated temperatures. These studies demonstrate that TaqMan Gene Expression Assays are stable for longer than 30 weeks when stored at -20ºC, 4ºC, 23ºC, and 37ºC in Matrix tubes and assayed under the conditions described here. This corresponds to a five-year shelf life when stored at -20ºC (Table 3). These ongoing studies allow us to estimate the shelf life for TaqMan Gene Expression Assays stored at -20ºC in Matrix tubes to be five years. As stated in our TaqMan Gene Expression Assays product insert, freeze thaw cycles should be kept to a minimum (a maximum of 10 freeze-thaw cycles is recommended). Since accelerated testing is a model and should be supported by real-time testing, we plan to continue to accumulate real-time data on a regular schedule.

Reference

[1] Arrhenius equation: **http://www.shodor.org/UNChem/advanced/kin/arrhenius.html**

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