TaqMan® Assays Shipped at Ambient Temperature Reduce Environmental Impact and Retain Their Quality and Stability

ABSTRACT

In order to minimize the adverse environmental impact of packaging and shipping products on dry ice, Life Technologies investigated the feasibility of shipping its TaqMan® Assay products at ambient temperature. This report describes stability and performance testing of three classes of assays (TaqMan® Gene Expression Assays, TaqMan® MicroRNA Assays, and TaqMan® SNP Genotyping Assays) after subjecting them to simulated summer ambient shipping conditions. Functional and stability testing demonstrated that TaqMan® Assay products that underwent simulated summer ambient shipping conditions performed as well as assays shipped on dry ice. By shipping at ambient conditions, the need for expanded polystyrene (EPS) coolers and dry ice is eliminated and the fuel consumption and greenhouse gas emissions from transporting the product are significantly reduced.

INTRODUCTION

The adverse environmental impact of shipping refrigerated or frozen products is tremendous. The annual carbon footprint to manufacture the EPS and convert it into coolers for our TaqMan® Assay products is approximately 100 tons (CO_2-equivalents). It takes nearly 300 barrels of crude oil equivalents and 400 MWhr of power annually to make the EPS coolers that are used to ship Life Technologies genomic assay products [1]. Additionally, 75,000 liters of water are consumed in the manufacture of the EPS coolers [1]. An average of 7 pounds of dry ice is added to each cooler to ensure the product is delivered frozen to our customers, further increasing the mass and dimensions of each package. Factoring in the number of shipments and average distance traveled per package and the fact that most packages are shipped via air, the annual total carbon footprint for transporting frozen oligonucleotides is in excess of 100 tons (CO_2-equivalents) [2].

Life Technologies has been systematically evaluating novel ways to minimize the impact of shipping products on dry ice, and the CO_2 footprint left by these products during distribution. One way to achieve this is to ship products at a temperature consistent with its demonstrated stability. By avoiding the cooler and refrigerant, products could be shipped in smaller boxes, which improves the carrier's freight density (less fuel and emissions per box) and reduces the amount of packaging materials requiring disposal or recycling. This enables Life Technologies to eliminate an annual total of nearly 26,000 kg (70,000 ft^3) of EPS from landfills and incinerators and replace it with recyclable corrugated paper packaging. By combining the elimination of EPS and reduction in impact attributed to transporting the added weight of the dry ice, we reduce the annual total carbon footprint from product delivery by over 200 tons (CO_2-equivalents) [1,2].

For many years, TaqMan® Assays have been shipped as preformulated liquid products on dry ice [3]. Extensive accelerated stability studies (4 weeks at 37°C) followed by real-time functional testing showed that TaqMan® Assay products are stable for at least 5 years when stored at –20°C. For this reason, the products were shipped under conditions aligned with their long-term stability temperature. Additional freeze/thaw stability data have shown that TaqMan® Assay products remain stable for multiple freeze/thaw cycles without compromising their functional performance. This paper describes the results from functional and stability studies carried out after TaqMan® Assay products were exposed to established summer shipment profiles. These experiments demonstrate that by shipping our TaqMan® Assay products under ambient conditions, not only can we supply researchers with the same superior quality product they are used to receiving, but we can reduce our environmental footprint in the process. This is a win for our company (eliminating the need for managing cold chain transport), a win for our customers (minimizing packaging waste), and a win for our planet (reducing resource consumption and total carbon footprint).

MATERIALS AND METHODS

Products Tested. TaqMan® Assays comprise a preformulated set of unlabeled gene-specific oligonucleotide primers and fluorescent dye–labeled minor groove binding (MGB) probe(s) provided in liquid form in approved Matrix storage tubes. Assays were selected to represent the widest range of performance as well as chemical, sequence, and structural motifs. In this paper a total of 42 different TaqMan® Assays were subjected to simulated summer ambient shipping conditions and subsequently measured for physical integrity and functional performance. These assays were selected from five product lines that fell into three representative TaqMan® Assay products, based on their formulations (Table 1).

Note: TaqMan® Copy Number Assays, Custom TaqMan® Copy Number Assays, ncRNA Assays, and pri-miRNA assays were not included in this testing as the tested assay products were considered to be similar enough to be representative of all product lines. TaqMan® CNV Reference Gene and Endogenous Control kits were not included in this study, but are being investigated to "go ambient" in the near future.

Creating Replicates. To help eliminate manufacturing lot variability when creating the replicates, individual tubes representing the various assays were taken from inventory stock, pooled, and aliquoted into Matrix tubes at the same fill volume as specified for the manufactured product. Six replicate tubes and six replicate control tubes were prepared for each assay, for a total of 252 test sample tubes and 252 control sample tubes (matched replicate sets). The control tubes were kept at –20°C for the duration of the testing.

Simulated Shipping

Temperature Exposure. To simulate temperatures incurred during shipping, samples were placed in a cycling environmental chamber (temperature and humidity controlled) programmed to reproduce a "worst-case" 144 hr (6 day) summer temperature profile (sequentially ran two times for a total of 288 hr) (Figure 1), previously established from testing profiles established at Amgen [4]. This profile mimics temperature extremes encountered from over 2,500 shipments during summer months between the latitudes of 59.9° north and 37.8° south. Due to a failure in the chamber control system, an additional 3-day room temperature (~25°C) incubation was added to the cycle as indicated in the graph (Figure 1) [4]. Samples were exposed to the 144 hr temperature cycle twice to account for inter-company shipments before being delivered to our customers. Testing of winter ambient conditions was not considered due to the extensive, positive historical data on freeze/thaw and low-temperature stability of TaqMan® Gene Expression and SNP Assays (data not shown).

Handling. In addition to the summer ambient temperature cycle, the products and packaging were subjected to reduced pressures and drop and vibrational tests as detailed under International Safe Transit Association (ISTA 3A) simulated performance protocols [5]. To ensure ambient/liquid product will arrive intact, we redesigned the packaging for orders of 16 tubes or less. To simulate the damage-producing pressure, motions, and forces typically encountered in the shipping and distribution environment, the package was tested according to the ISTA 3A Test Protocol [5].

Analytical Testing

Volume and Concentration. Volume and concentration for all test sample tubes were measured before and after the ambient shipping simulation. Volume was measured by gravimetric analysis and concentration was measured spectroscopically at OD_{260}.

Stability/Integrity. Structural integrity changes in test samples compared to controls was measured by reverse phase HPLC (RP-HPLC). Samples were analyzed using an Agilent HPLC1100 System with a quaternary pump system. HPLC columns used were Transgenomic OligoSep HC (5.5–6.0 micron, porous, alkylated polymer (DVB), 4.6 x 50 mm, P/N NUC-99-3560)). Mobile phases used were 0.1 M TEAA (triethylamine acetate) in water and 0.1 M TEAA in 75% acetonitrile/25% water.

Functional Performance

TaqMan® Gene Expression Assays. Sixty matched test and control tubes from 10 TaqMan® Gene Expression Assays (20X formulation) and 36 matched test and control tubes from 6 TaqMan® Gene Expression Assays (60X formulation) were functionally tested. Reactions were set up following the standard product protocol for a 20 µL reaction volume with 4 replicates for each tube. Ten nanograms of cDNA synthesized from Universal Human RNA (Stratagene) was used as the template for all reactions. PCR reactions were conducted with TaqMan® Universal PCR Master Mix (P/N 4304437) and run on a 7900HT Real-Time PCR System using universal cycling conditions (95°C, 10 min; 95°C, 15 sec; 60°C, 1 min for 40 cycles). SDS 2.3 software was used to generate the amplification plots and determine C_t values (autobaseline; threshold set at 0.2). C_t variability and no-template control (NTC) C_t values were calculated using JMP 8 statistical software.

TaqMan® MicroRNA Assays. Thirty-six matched test and control sample tubes from 6 TaqMan® MicroRNA Assays were processed. The RT step was performed using a TaqMan® MicroRNA Reverse Transcription Kit (P/N 4366596). TaqMan® Universal PCR Master Mix, No UNG was used for qPCR (P/N 4304437). A mixture of 5 ng each Human Brain and Human Lung Total RNA (Ambion®) was used as the template in 15 µL RT reactions. The RT reaction products were used in 10 µL PCR reactions at a final dilution of 1:15. qPCR reactions were run, with 4 replicates for each assay tube, on a 7900HT Real-Time PCR System using universal cycling conditions (95°C, 10 min; 95°C, 15 sec; 60°C, 1 min for 40 cycles). SDS 2.3 software was used to generate the amplification plots and determine C_t values (autobaseline; threshold set at 0.2). C_t and NTC variability was calculated using JMP 8 statistical software.

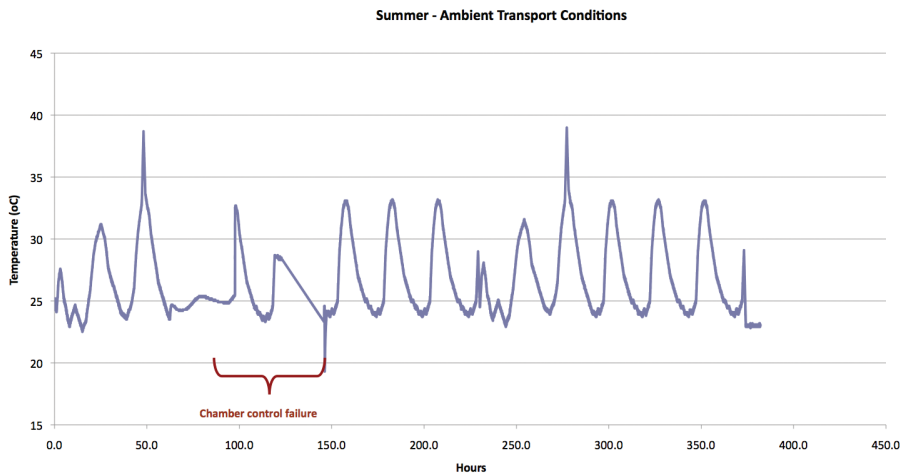


Figure 1. 144-Hr Summer Temperature Profile Used to Simulate Shipping Temperatures. The summer temperature profile was used to mimic average high temperature extremes between the latitudes of 59.9° north and 37.8° south (profile derived from the Amgen protocol as described). A controller failure induced a 3-day increase in exposure to normal room temperatures, further increasing the stringency of the challenge.

TaqMan® DME and SNP Genotyping Assays. Matched test and control samples were processed for 10 TaqMan® Drug Metabolism (DME) Genotyping Assays (20X formulation) and 10 TaqMan® SNP Genotyping Assays (80X formulation). The SNP assays were diluted to 20X working stock solutions with TE before use. TaqMan® DME and SNP Genotyping Assays were performed following the manufacturer's recommended protocol. Templates for these tests consisted of 20 distinct genomic DNA samples (Coriell Cell Repositories, www.coriell.org/ccr/ccrsumm.html), containing homozygous and heterozygous targets for all assays tested. The DNAs were normalized, dispensed onto 384-well plates, and dried down. NTC reactions were included. Reaction mixes were prepared for 10 µL reactions using TaqMan® Universal PCR Master Mix, No UNG. PCR was performed using the GeneAmp® PCR System 9700. SNP PCR was run using universal cycling conditions while DME PCR was run using more PCR cycles and longer extension time (50 cycles x 90 seconds). Endpoint data were collected using the Applied Biosystems® 7900HT Real-Time PCR System. FAM™ dye, VIC® dye, and FAM™:VIC® dye Rn ratios and allelic discrimination (cluster) plots were generated using SDS 2.3 software. JMP 8 software was used to calculate variability for both Rn-NTC and FAM™ and VIC® Rn ratios.

Statistical Analysis. The number of replicate tubes for each assay (test and control) was predetermined by the sample size calculator of JMP 8 software using prior functional performance data with α = 0.01 and power = 0.99. For each of the selected assays, 12 tubes were combined and split back into 12 tubes (6 control and 6 test samples). Each assay was run with 6 replicates for control and 6 replicates for test (12 tubes for each assay) as predetermined by JMP 8 software. For the qPCR run, each tube was run with 4 technical replicates, thus each control assay and test assay had 24 data points. We used the SAS JMP statistical package to test for significant differences between test and control assays. For TaqMan® Gene Expression and MicroRNA Assays, C_t variability and NTC C_t values were analyzed. Variability for both Rn-NTC and FAM™:VIC® Rn ratios were calculated for TaqMan® DME and SNP Genotyping Assays.

Table 1. TaqMan® Assays Tested.

Product	Description	Part Number	Concentration	Number of Assays
1. TaqMan® Gene Expression Assays				
TaqMan® Gene Expression Assays, inventoried	2 oligonucleotide primers 1 MGB probe with FAM™ dye*	4331182	20X	10
Custom TaqMan® Gene Expression Assays, large scale	2 oligonucleotide primers 1 MGB probe with FAM™ dye*	4332079	60X	6
2. TaqMan® MicroRNA Assays				
TaqMan® MicroRNA Assays	1 oligonucleotide primer† 2 oligonucleotide primers 1 MGB probe with FAM™ dye*	4427975	5X 20X	6
3. TaqMan® SNP Genotyping Assays				
TaqMan® Drug Metabolism Genotyping Assays	2 oligonucleotide primers 1 MGB probe with FAM™ dye‡ 1 MGB probe with VIC® dye‡	4362691	20X	10
TaqMan® SNP Genotyping Assays, large scale	2 oligonucleotide primers 1 MGB probe with FAM™ dye‡ 1 MGB probe with VIC® dye‡	4351374	80X	10

* TaqMan® minor groove binding (MGB) probe with FAM™ dye.

† TaqMan® MicroRNA Assays consist of a reverse transcription (RT) primer and TaqMan® Assay. This is the oligonucleotide primer for priming the RT step.

‡ Allele-specific TaqMan® MGB probes with distinct dyes for detecting the alleles for the specific polymorphism of interest; VIC® dye is linked to the 5´ end of the Allele 1 probe, and FAM™ dye is linked to the 5 end of Allele 2 probe.

RESULTS

Analytical Testing

Volume and Concentration. Negligible differences were observed for both volume and concentration (data not shown).

Stability/Integrity. RP-HPLC was used to create peak profiles of the oligonucleotide TaqMan® Assay components (FAM™ and VIC® dye-labeled MGB probe oligonucleotides and unlabeled oligonucleotide primers) using UV/VIS absorbance detection. Forty-two matched test and control tubes, representing samples from all assays, were analyzed. An example of the data is shown in Figure 2. Test and control peak profiles were compared. Test samples were judged as identical to matched controls (no degradation). For this study, the comparisons of all 42 test samples analyzed were shown to be identical to their matched controls, confirming that the simulated summer ambient shipping protocol did not affect product integrity.

Functional Performance

TaqMan® Gene Expression Assays. Functional performance for both types of

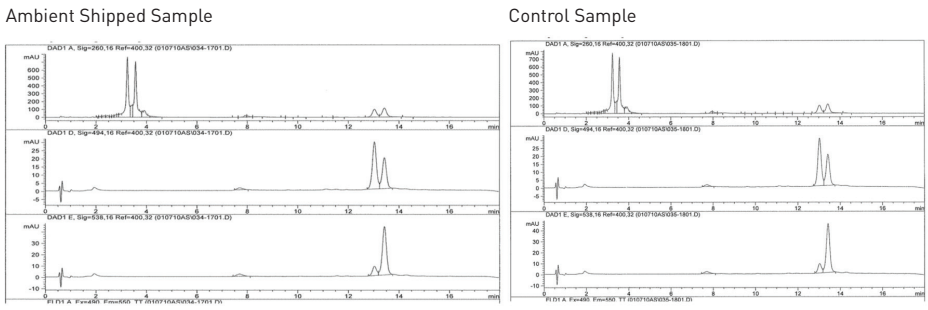
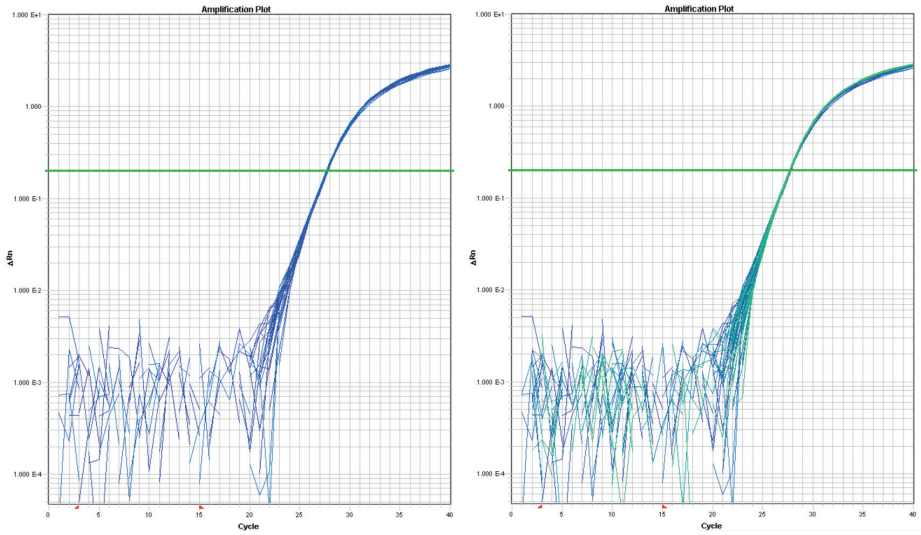


Figure 2. Simulated Summer Ambient Shipping Does Not Affect Oligonucleotide Stability—Representative Data. The effect of simulated summer ambient shipping on oligonulceotide integrity was measured by comparing RP-HPLC profiles of paired test and control samples. The HPLC chromatogram profiles of the testing samples are comparable to the profiles of the control samples. There was no indication of probe or primer degradation in the ambient-shipped SNP assay C___1305119_10 (**A**) compared to its matched control (**B**).



Panel A. Panel B.

Assay ID	Mean Difference (C_t)	p Value	Pass/Fail
Hs01374556_m1	–0.01	0.2357	Pass

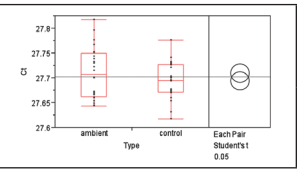
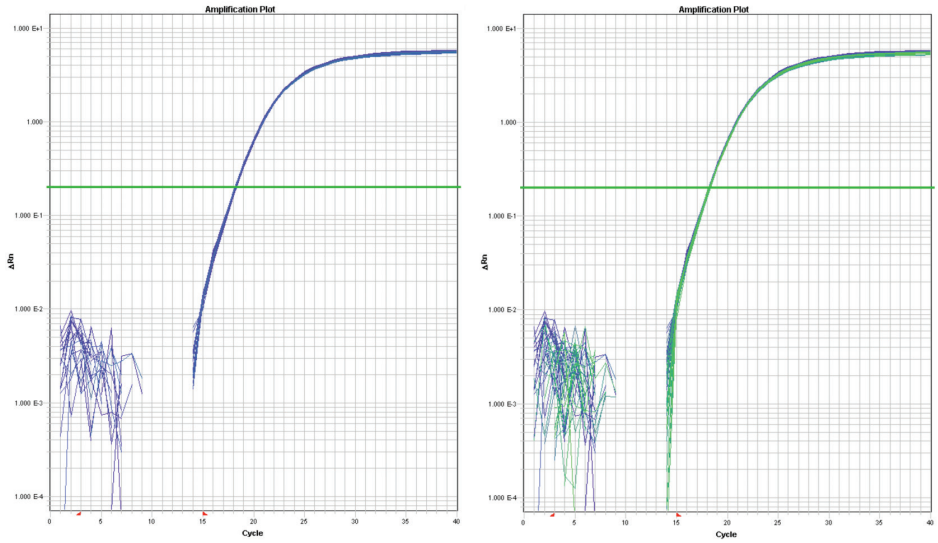


Figure 3. TaqMan® Gene Expression Assay (60X). The effect of simulated summer ambient shipping on assay functional performance was evaluated by performing real-time qPCR of paired test and control samples. Assay ID: Hs01374556_m1. (**A**) Amplification plot for control sample stored at –20°C (blue, n = 24). (**B**) Amplification plots for ambient test sample (green, n = 24) overlaid with control sample (blue, n = 24). The table below the plot shows the mean difference between the average C_t of 24 samples (6 control and test tubes, 4 qPCR replicates of each tube). The box plot and circle plot show the distribution of the C_t values and a visual display of the results of the variance analysis (student's t-test).



Panel A. Panel B.

Assay ID	Mean Difference (C_t)	p Value	Pass/Fail
391	0.04	<.0070	Pass

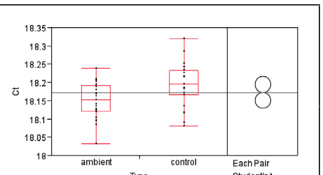


Figure 4. TaqMan® MicroRNA Assay. The effect of simulated summer ambient shipping on assay functional performance was evaluated by performing real-time qPCR followed by RT-PCR of paired test and control samples. Assay ID: 391. (**A**) Amplification plot for control sample stored at –20°C (blue, n = 24). (**B**) Amplification plots for ambient test sample (green, n = 24) overlaid with control sample (blue, n = 24). The table below the plot shows the mean difference between the average C_t of 24 samples (6 control and test tubes, 4 qPCR replicates of each tube). The box plot and circle plot show the distribution of the C_t values and a visual display of the results of the variance analysis (student's t-test).

TaqMan® Gene Expression Assays, at 20X and 60X concentration, was assessed by functional analysis of qPCR amplification results. A statistical evaluation of C_t variability and C_t value for the NTC test was performed between the test and control assays. All 60 matched test and control sample tubes from the 10 TaqMan® Gene Expression Assays and all 36 matched test and control sets from the 6 TaqMan® Gene Expression Assays were tested. Representative results for matched pairs (test and control) are shown in Figure 3, with results for all assays provided in Appendix 1 and 2. The data showed that the functional performance as measured by C_t value is equivalent between ambient-shipped assays and control assays for both TaqMan® Gene Expression products tested. All 20X formulated assays showed no statistical difference ($p > 0.05$) (Figure 3). In addition, the mean difference in C_t between the test and control was <0.1 for all assays tested. Two of the 60X formulated assays had $p < 0.05$; however, the mean difference between the test and control tubes was <0.15. The NTC testing showed that all assay tubes, control and test, showed C_t >38.

TaqMan ® MicroRNA Assays. Functional performance for TaqMan® MicroRNA Assays was assessed by qPCR followed by calculation of C_t variability and C_t value for the NTC. These tests also included an RT step prior to PCR amplification (to test the RT primer included in these assay products). Representative results (matched test and control pairs) are shown in Figure 4, with results for all assays provided in Appendix 3. The data showed that the functional performance is equivalent between ambient-shipped samples and control samples for MicroRNA Assays as measured by the C_t value. Two assays showed statistical differences ($p < 0.05$); however, the mean differences between test and control C_t are less than 0.2. NTC for all tubes tested gave C_t >38.

Amplification plots from test and control assay tubes for TaqMan® Gene Expression Assays and TaqMan® MicroRNA Assays displayed identical profiles, and when overlayed were indistinguishable.

TaqMan® SNP and TaqMan® DME Genotyping Assays. Functional performance for TaqMan® Genotyping Assays was assessed by measuring the difference in the end point ΔRn (Rn-NTC) value between the test

versus control sample tubes for FAM™- and VIC®-labeled probes averaged across the 20 gDNA samples. The FAM™:VIC® Rn ratio was also calculated. All 60 matched pairs of test and control samples for both products were assayed. Representative results for 4 assays from each (matched test and control pairs) are shown in Figures 5 and 6, with results for all assays provided in Appendix 4 and 5. Comparison of ΔRn and FAM™:Vic® Rn ratios showed that most assays had no statistical difference between the test and control for these measurements. Only a few assays showed a difference ($p < 0.05$) for ΔRn; however, the mean difference between control and test for DME assays was less than 5% and for SNP Genotyping Assays was less than 3%.

Cluster plots for the test and control assays for DME and SNP assays were plotted together. The profiles were very similar, showing good clusters, and the test assays were indistinguishable from the control. FAM™, VIC®, and FAM™:VIC® clusters were well separated and clearly defined for both test and control samples (Figure 6). In addition, there was no difference in the genotyping calls.

CONCLUSIONS

The data described in this paper demonstrate that ambient shipping of TaqMan® Gene Expression Assay, TaqMan® MicroRNA Assay, TaqMan® DME Genotyping Assay, and TaqMan® SNP Genotyping Assay products has no effect on their quality, integrity, and functional performance.

After eliminating factors such as manufacturing lot variability by pooling and re-splitting samples, analytical testing (including measurements of volume, concentration, and purity) showed that the samples were not impacted when shipped under simulated summer ambient shipping conditions.

For the TaqMan® Gene Expression Assay and TaqMan® MicroRNA Assay products, functional assays compared test and control amplification plots, C_t variability, and NTC C_t values. All of the test samples were essentially identical to the control samples and fell within specifications for these parameters. Likewise, functional data consisting of cluster plots, Rn variability, and FAM™:VIC® Rn ratios for the test sample tubes compared to control sample tubes from TaqMan® DME and SNP Genotyping Assay products also fell well within performance specifications for these parameters.

We will no longer be shipping in EPS (also known as Styrofoam®) coolers, and dry ice is no longer needed. Thus, external packaging will be reduced in size by over 92%. For orders comprising 16 tubes or less we will be using a recyclable plastic holder; orders 17 and larger will continue to be shipped in Matrix racks. The ambient packaging, from the corrugate outer box to the plastic and paper containers, are fully recyclable.

The results presented here validate the change to ambient shipping, and provide the researcher confidence that when shipped ambient, TaqMan® Assays will exhibit no statistically significant difference in function or stability compared to dry ice–shipped products. In addition to ensuring our customers will continue to receive the highest quality possible, this study enables us to significantly reduce the environmental impact of transport of these products. Life Technologies' consumption of non-renewable raw materials will decrease by over 300 barrel equivalents of oil every year and reduce our water utilization by over 75,000 liters. Our customers will see a reduction of 26,000 kg (76,000 ft^3) of EPS waste. Our planet will see a reduction of CO_2 emissions by over 200 tons every year. Finally, the packaging used to deliver the products, from the corrugate outer box to the plastic and paper containers, are fully recyclable. Please reuse the containers when possible and, when you cannot reuse, please recycle.

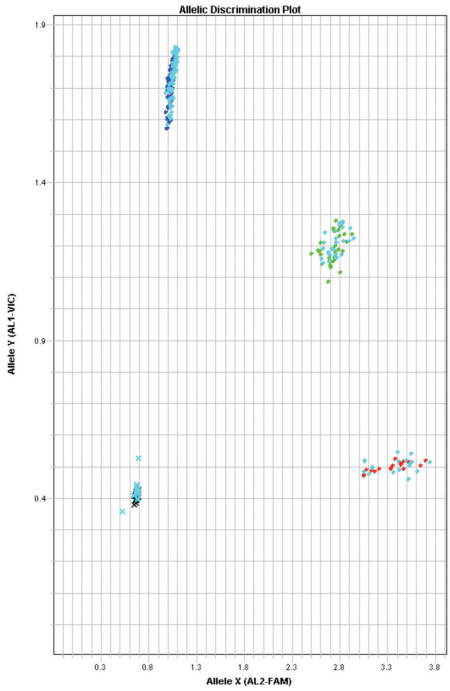


Figure 5. TaqMan® DME Assay (20X). The effect of simulated summer ambient shipping on assay functional performance was evaluated by performing PCR on paired test and control samples. Assay ID: C___7817764_10. The red, dark blue, and green dots in this overlaid cluster plot represent data points from the control samples for Allele X, Allele Y, and heterozygote XY genotypes, respectively. The test samples are shown as the turquoise dots for corresponding Allele X, Allele Y, and heterozygote XY genotype clusters.

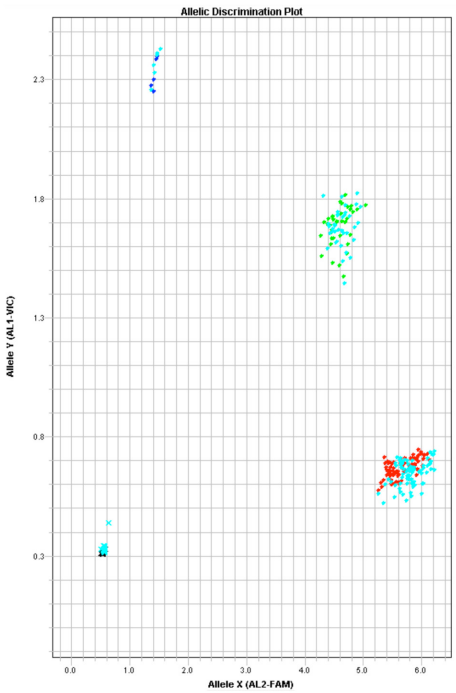
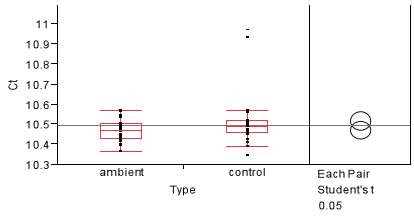
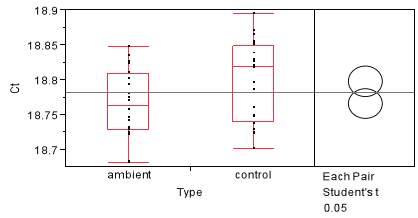
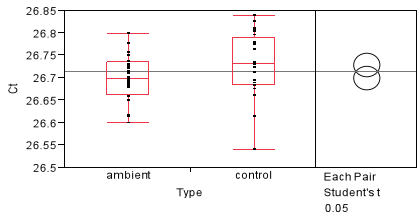
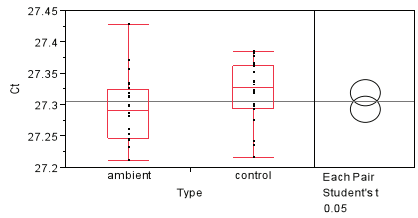
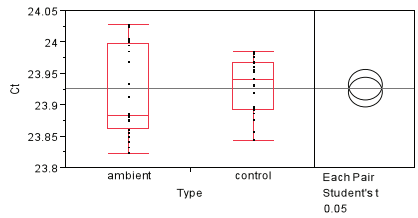
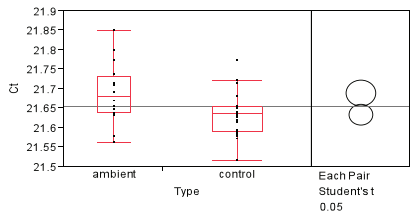
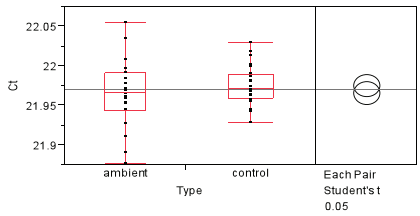
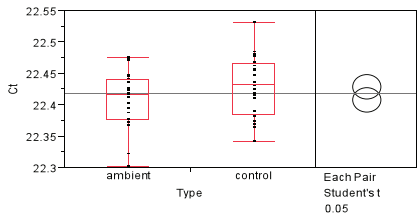
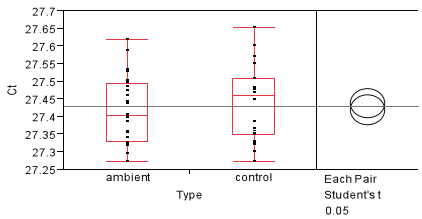
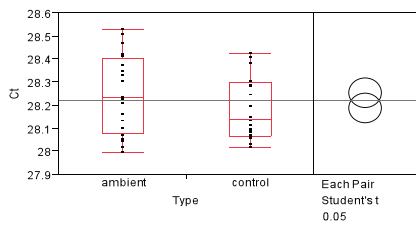


Figure 6. TaqMan® SNP Assay (80X). The effect of simulated summer ambient shipping on assay functional performance was evaluated by performing PCR on paired test and control samples. Assay ID: C___3084793_20. The red, dark blue, and green dots in this overlaid cluster plot represent data points from the control samples for Allele X, Allele Y, and heterozygote XY genotypes, respectively. The test samples are shown as the turquoise dots for corresponding Allele X, Allele Y, and heterozygote XY genotype clusters.

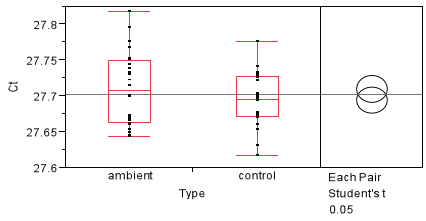
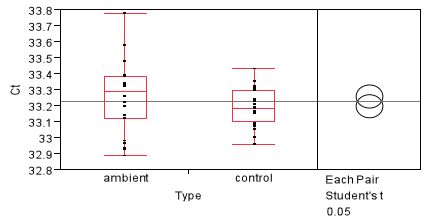
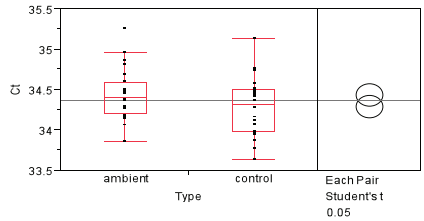
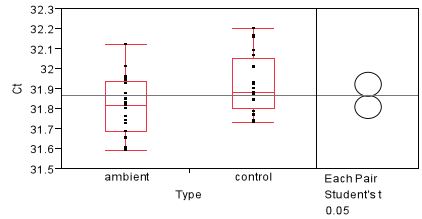
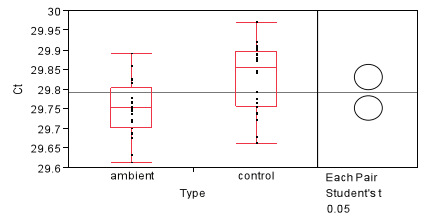
Appendix 1: TaqMan® Gene Expression Assays (20X). Statistical comparison of the functional performance.

TaqMan® Gene Expression Assay (20X)	Mean Difference (C_t)	p Value	Variance Analysis
Hs99999901_m1	0.05	0.157	
Hs99999905_m1	0.03	0.040	
Hs00609297_m1	0.03	0.114	
Hs99999910_m1	0.03	0.068	
Hs99999908_m1	0.01	0.480	

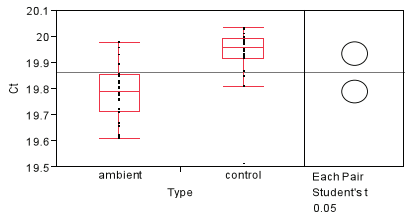
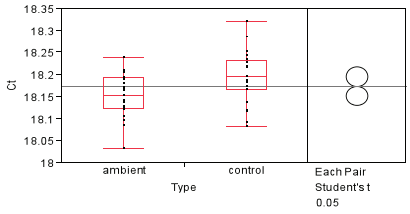
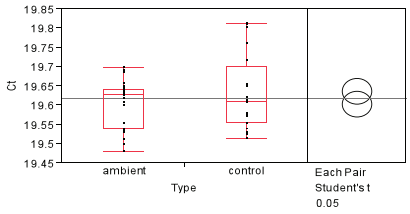
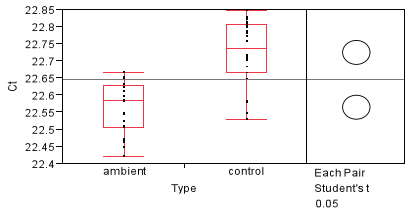
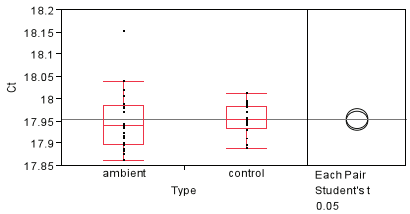
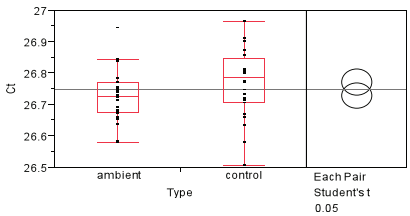
Appendix 1: continued

TaqMan® Gene Expression Assay (20X)	Mean Difference (C_t)	p Value	Variance Analysis
Hs99999904_m1	–0.06	0.02	
Hs00157244_m1	0.01	0.374	
Hs00192574_m1	0.02	0.135	
Hs00237047_m1	0.02	0.518	
Hs00156385_m1	–0.07	0.153	

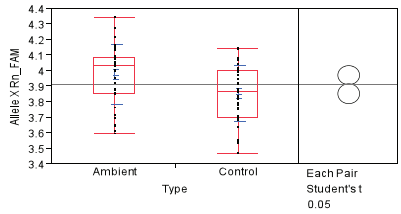
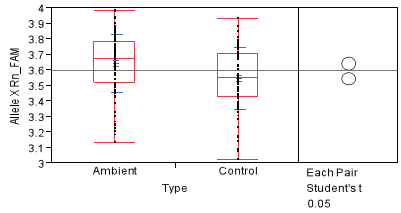
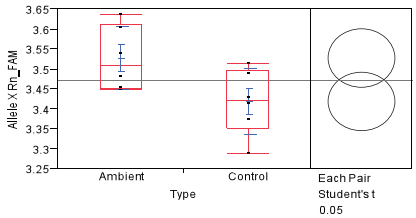
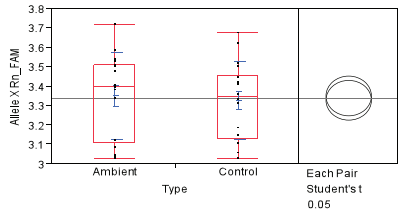
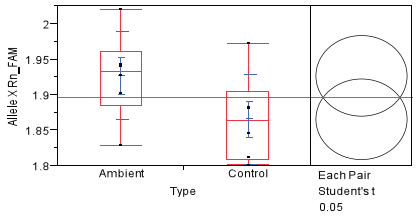
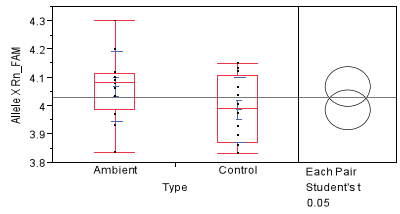
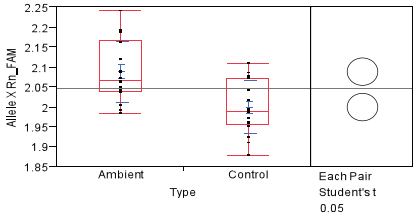
Appendix 2: TaqMan® Gene Expression Assays (60X). Statistical comparison of functional performance.

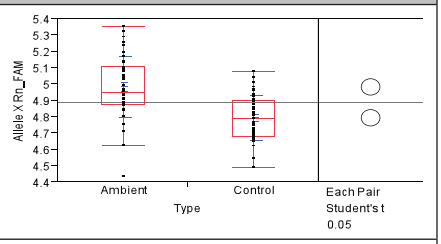
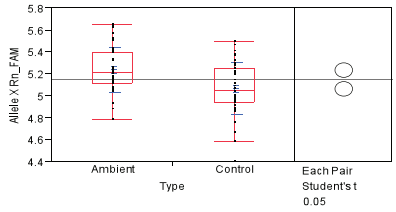
TaqMan® Gene Expression Assay (60X)	Mean Difference (C_t)	p Value	Variance Analysis
Hs01374556_m1	-0.01	0.236	
Hs03454268_m1	–0.06	0.229	
Hs03454539_m1	–0.15	0.147	
Hs03454556_s1	0.11	0.009	
Hs00940773_s1	0.08	0.001	

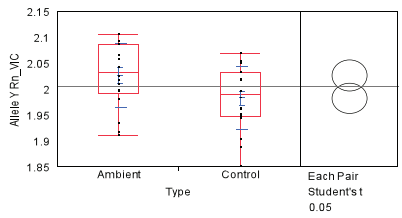
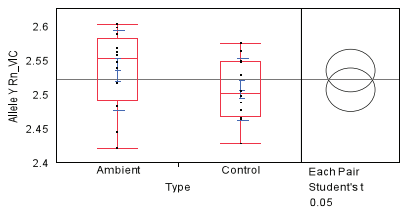
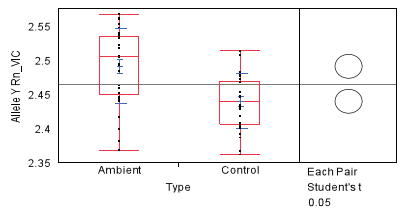
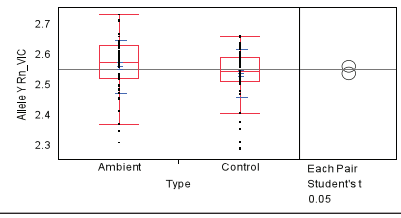
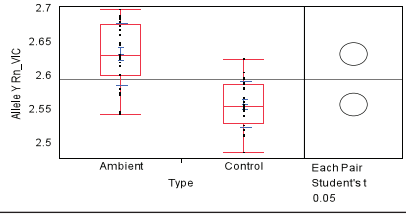
Appendix 3: TaqMan® MicroRNA Assays. Statistical comparison of functional performance.

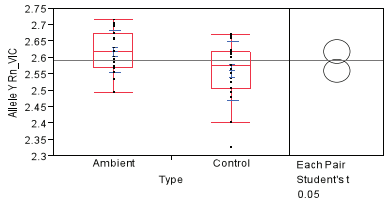
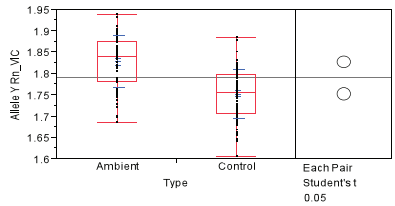
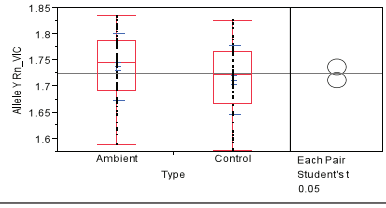
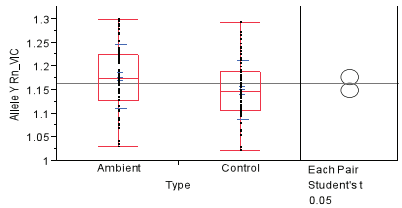
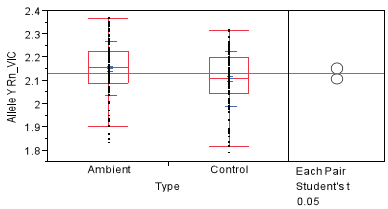
TaqMan® Micro RNA Assay	Mean Difference (C_t)	p value	Variance Analysis
377	0.14	<.0001	
391	0.04	<.0070	
397	0.03	0.168	
403	0.16	<.0001	
450	0.00	0.758	
491	0.04	0.150	

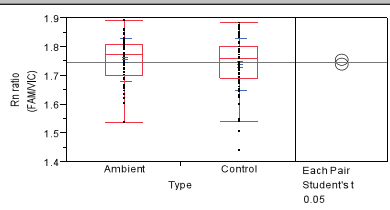
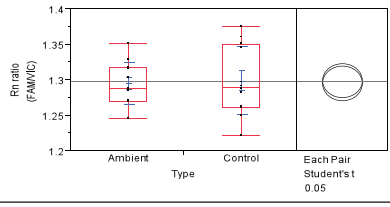
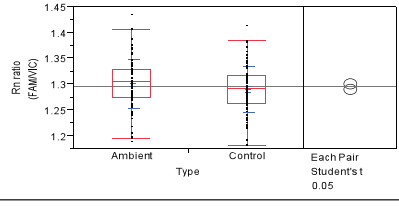
Appendix 4: TaqMan® DME Genotyping Assays. Statistical comparison of Rn values.

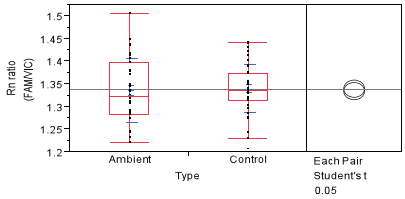
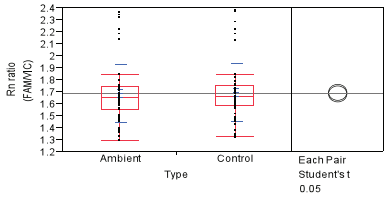
DME FAM Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C____287260_10	36	3.97	0.19	3.85	0.18	−0.12	0.007	
C___1823316_20	96	3.64	0.19	3.55	0.20	−0.09	0.001	
C___2461184_20	6	3.53	0.08	3.42	0.08	−0.11	0.041	
C___7817764_10	18	3.35	0.22	3.33	0.20	−0.02	0.745	
C___7817765_60	6	1.93	0.06	1.87	0.06	−0.06	0.119	
C__26236594_10	12	4.07	0.12	3.99	0.12	−0.08	0.104	
C__26823969_10	18	2.09	0.08	2.00	0.07	−0.09	0.001	

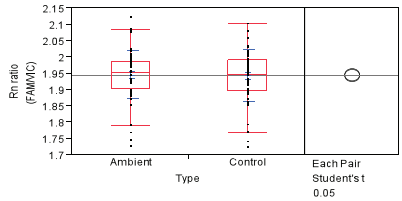
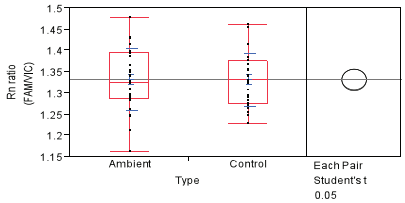
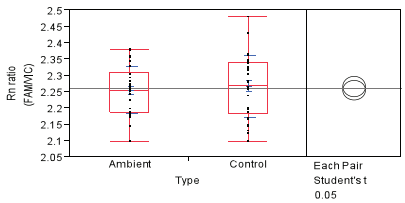
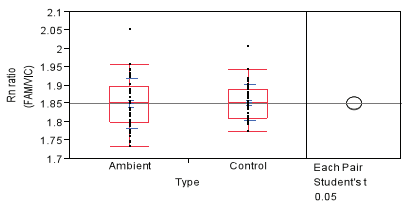
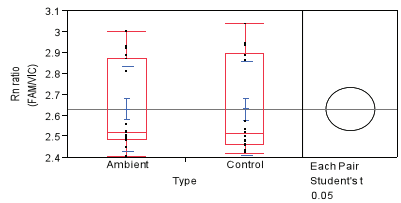
DME FAM Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C__27102414_10	42	4.98	0.19	4.79	0.14	−0.19	0.000	
C__32449742_20	42	5.24	0.21	5.07	0.23	−0.17	0.001	

DME VIC Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C____287260_10	18	2.03	0.06	1.98	0.06	−0.05	0.038	
C___1823316_20	12	2.54	0.06	2.51	0.05	−0.03	0.200	
C___2461184_20	30	2.49	0.05	2.44	0.04	−0.05	0.000	
C__26236594_10	66	2.56	0.09	2.53	0.08	−0.03	0.101	
C__27102414_10	24	2.63	0.05	2.56	0.03	−0.07	0.000	

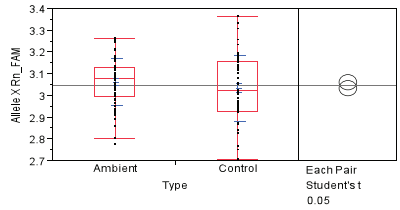
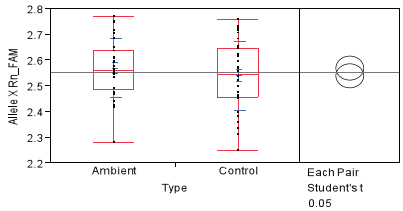
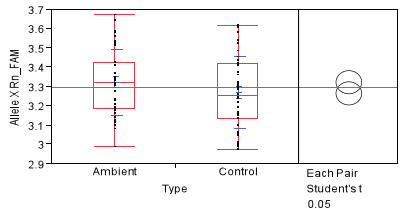
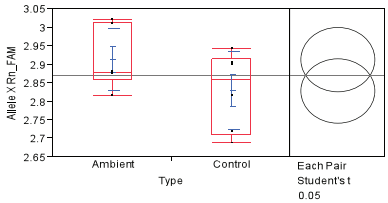
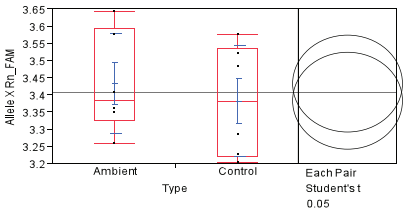
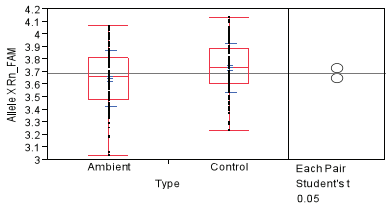
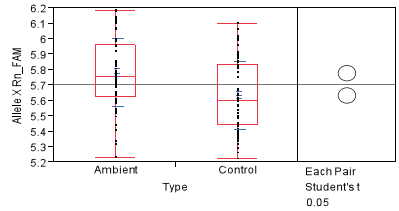
DME VIC Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C__32449742_20	19	2.62	0.06	2.56	0.09	−0.06	0.027	
C__26823969_10	72	1.83	0.06	1.75	0.06	−0.08	0.000	
C___7817764_10	72	1.74	0.06	1.71	0.07	−0.03	0.022	
C___7817765_60	66	1.18	0.07	1.15	0.06	−0.03	0.013	
C__27102431_D0	101	2.15	0.11	2.11	0.12	−0.04	0.007	

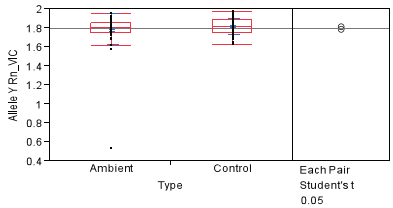
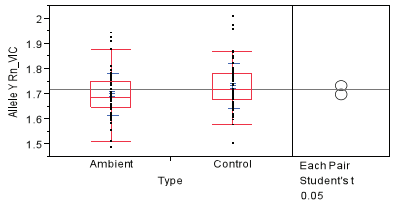
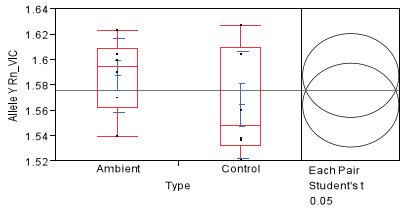
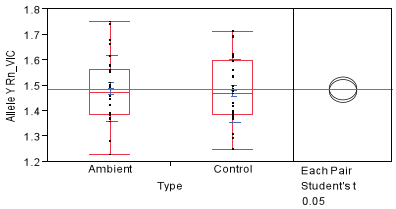
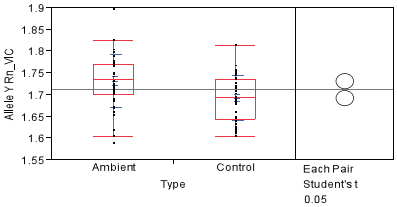
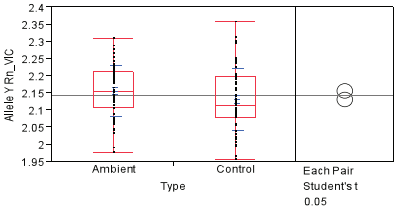
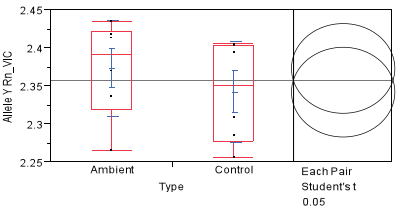
DME FAM/VIC Rn Ratio								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C____287260_10	66	1.76	0.07	1.74	0.09	−0.02	0.274	
C___1823316_20	12	1.30	0.03	1.30	0.05	0.00	0.801	
C___2461184_20	84	1.30	0.05	1.29	0.04	−0.01	0.122	

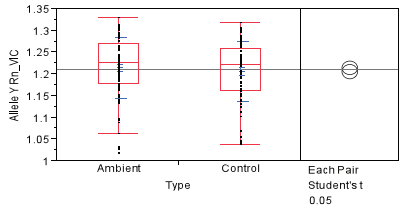
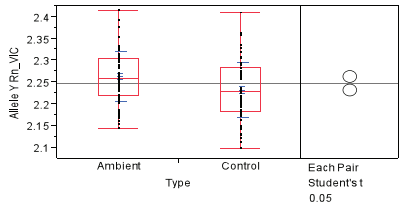
DME FAM/VIC Rn Ratio								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C__26236594_10	42	1.34	0.07	1.34	0.05	0.00	0.727	
C__27102414_10	54	1.68	0.24	1.69	0.24	0.01	0.818	

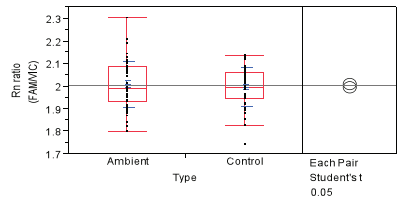
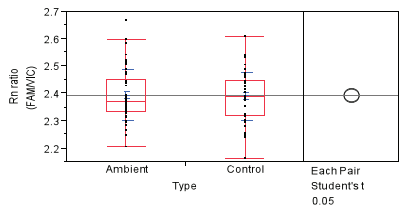
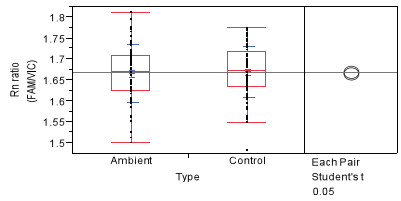
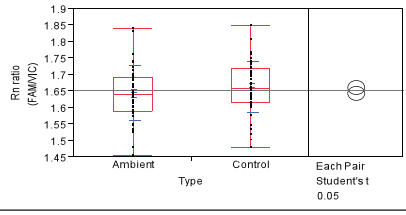
DME FAM/VIC Rn Ratio								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C__32449742_20	60	1.95	0.07	1.94	0.08	–0.01	0.880	
C__26823969_10	30	1.33	0.07	1.33	0.06	0.00	0.929	
C___7817764_10	30	2.25	0.07	2.27	0.10	0.02	0.586	
C___7817765_60	48	1.85	0.07	1.85	0.05	0.00	0.882	
C__27102431_D0	18	2.63	0.20	2.63	0.22	0.00	0.990	

Appendix 5: TaqMan® SNP Genotyping Assays. Statistical comparison of Rn values.

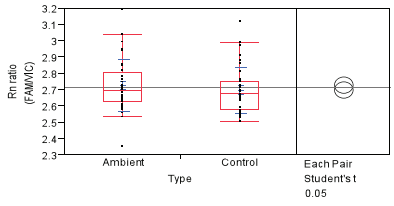
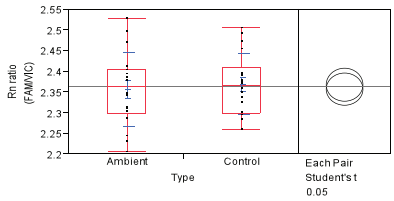
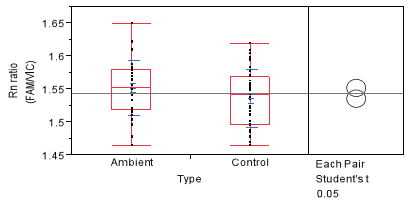
VA SNP FAM Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C___1305119_10	60	3.06	0.11	3.03	0.15	−0.03	0.266	
C___1202883_20	30	2.57	0.11	2.54	0.13	−0.03	0.355	
C___1305007_10	36	3.32	0.17	3.27	0.19	−0.05	0.194	
C__27004199_10	6	2.91	0.08	2.83	0.11	−0.08	0.155	
VA SNP FAM Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	P-Value	Assay Variance
C__11738050_10	6	3.43	0.15	3.38	0.16	−0.05	0.583	
C___8726802_20	120	3.65	0.23	3.73	0.20	0.08	0.003	
C___3084793_20	78	5.78	0.22	5.63	0.22	−0.15	0.000	

VA SNP VIC Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C___2292799_10	72	1.78	0.17	1.81	0.08	0.03	0.196	
C___2292797_20	72	1.70	0.09	1.73	0.09	0.03	0.022	
C___1305119_10	6	1.59	0.03	1.56	0.04	−0.03	0.296	
C___1305007_10	30	1.49	0.13	1.48	0.12	−0.01	0.772	
C__27004199_10	42	1.73	0.06	1.69	0.05	−0.04	0.002	
VA SNP VIC Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C__11738050_10	60	2.16	0.07	2.13	0.09	-0.03	0.120	
C___3084793_20	6	2.37	0.06	2.34	0.07	-0.03	0.429	

VA SNP VIC Rn								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C___1202883_20	72	1.21	0.07	1.21	0.07	0.00	0.458	
C___29347861_10	78	2.26	0.06	2.23	0.06	−0.03	0.002	

VA SNP FAM/VIC Rn Ratio								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C___1305119_10	54	2.01	0.10	2.00	0.08	−0.01	0.488	
C___1305007_10	54	2.39	0.09	2.39	0.09	0.00	0.824	
C__27004199_10	72	1.66	0.07	1.67	0.06	0.01	0.700	
C__11738050_10	54	1.64	0.08	1.66	0.08	0.02	0.262	

Appendix 5: *continued*

VA SNP FAM/VIC Rn Ratio								
Assay	n	Ambient Mean	Ambient SD	Control Mean	Control SD	Mean Difference	p Value	Assay Variance
C___3084793_20	36	2.73	0.16	2.70	0.14	−0.03	0.399	
C___1202883_20	18	2.36	0.09	2.37	0.07	0.01	0.703	
C___29347861_10	42	1.55	0.04	1.54	0.04	−0.01	0.097	

NOTES

NOTES

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