Gene expression analysis

Importance of data reproducibility for development of safe gene therapies

In this application note we:

- Introduce the importance of reproducible quantitation of gene expression to investigate potential sources of vector-induced immunotoxicity in gene therapy
- Demonstrate reproducibility of measuring gene expression across multiple Applied Biosystems™ QuantStudio™ 7 Pro Real-Time Systems using the Applied Biosystems™ TaqMan® Array Human Inflammation Panel
- Demonstrate reproducibility of measuring gene expression from well-to-well and across multiple 96-well plates and across multiple 384-well Applied Biosystems™ TaqMan® Array Cards

Introduction

After decades of research, viral-based vectors have now become an integral part of clinically approved gene therapies. Delivering and inserting genes into the host genome are natural behaviors of viruses and are cornerstones of gene therapy. However, viral infection can also provoke deleterious immune responses. In gene therapy, a virus-induced immune reaction can interfere with gene transfer, destroy the functional transgene product, eliminate transduced cells, or even threaten the life of the gene therapy recipient. [1,2,3] Consequently, evading unintended immune responses is a primary obstacle in ensuring the safety as well as the efficacy of gene therapies. Understanding the interplay among gene therapy vector components and deleterious immune responses may be especially important in developing safer gene therapies.

Immune response in gene therapy

Several studies have revealed that many components of gene therapy can cause or influence both innate and adaptive immune responses. [1,4] Immunogenic factors may include the route of vector administration, the target tissue, specific vector components, the viral strain, capsid structure, viral DNA or RNA, vector dose, viral-encoded proteins, impurities, the transgene product itself, or downstream effects.

The first immune response, occurring within a few hours following vector transfer, is the innate immune secretion of inflammatory cytokines around the therapeutic administration site. If the innate reaction is too aggressive, adverse effects can occur ranging from mild flu-like symptoms to multiple-organ failure. Within a few days after vector administration, the innate immune response gives way to the adaptive immune response. [4] The adaptive immune response leads to long-term immunity, which can present multiple safety challenges for gene therapy.

Recombinant adeno-associated virus (rAAV) vectors comprising viral capsid proteins, the vector DNA expression cassette, and products generated by it can themselves be immunogenic due to their interaction with toll-like receptors (TLRs), which recognize specific conserved signatures (pathogen-associated molecular pattens, PAMPs). [4] Different TLRs recognize specific PAMPs. For example, TLR2 recognizes ligands on rAAV capsids at the cell membrane, and TLR3, TLR7, TLR8, and TLR9 sense the AAV genome at different stages of transcription. An immune response launched by TLRs can block expression of the therapeutic transgene and present risks of adverse immune reactions. [5] Engineering rAAV vectors with altered genetic sequences to modify TLR PAMPs may offer approaches to mitigate TLR-initiated immune response and advance understanding of gene therapy safety.

Gene expression analysis to investigate immune response

Due to the paramount importance of ensuring safety across multiple populations and the complexity of developing a new gene therapy product, gene therapy studies can grow into large collaborations among multiple research teams in different geographic areas. For example, in October 2021, the US FDA, US NIH, ten pharmaceutical companies, and five non-profit organizations partnered to accelerate development of gene therapies for rare diseases [6] When even gathering enough data for a comprehensive study requires such extraordinary efforts, experimental reproducibility across all sites is essential for the researchers to trust the results. Robust and reliable technologies must be used to generate study data that provides researchers with confidence to draw conclusions.

Here, using quantitative real-time PCR (qPCR) to evaluate the expression of 90 inflammation-associated genes and six reference genes, we show how this genetic technology can be used to investigate immune responses. Gene expression analysis using qPCR is a reliable, sensitive, and reproducible approach to quantify transgene expression and investigate the potential sources of immunotoxicity in gene therapy such as viral genes, adventitious contaminants, therapy-induced tissue damage, and off-target activity. The data presented here show excellent well-to-well and instrument-to-instrument data concordance in the QuantStudio 7 Pro Real-Time PCR System, demonstrating the robustness of the platform and the assays.

Methods

We tested the reproducibility of measuring gene expression on the QuantStudio 7 Pro Real-Time PCR System using the TaqMan Array Human Inflammation Panel. This panel consists of 96 TaqMan Assays that encompass the 90 inflammation-associated and six reference (housekeeping) genes pre-spotted and dried down into either 96-well plates or 384-well TaqMan Array Cards. In separate preparations, we synthesized cDNA from 10 ng and 100 ng of universal human reference (UHR) RNA using Invitrogen™ SuperScript™ VILO™ Master Mix.

384-well TaqMan Array Cards were spotted in quadruplicate so that four samples could be run simultaneously. PCR was done using Applied Biosystems™ TaqMan® Fast Advanced™ Master Mix and cycled according to standard protocols. Analysis of resulting C_q values was performed using Applied Biosystems™ QuantStudio™ Design and Analysis Software using default baseline calling values. Further analysis was performed in Microsoft™ Excel™ or SAS JMP™ software.

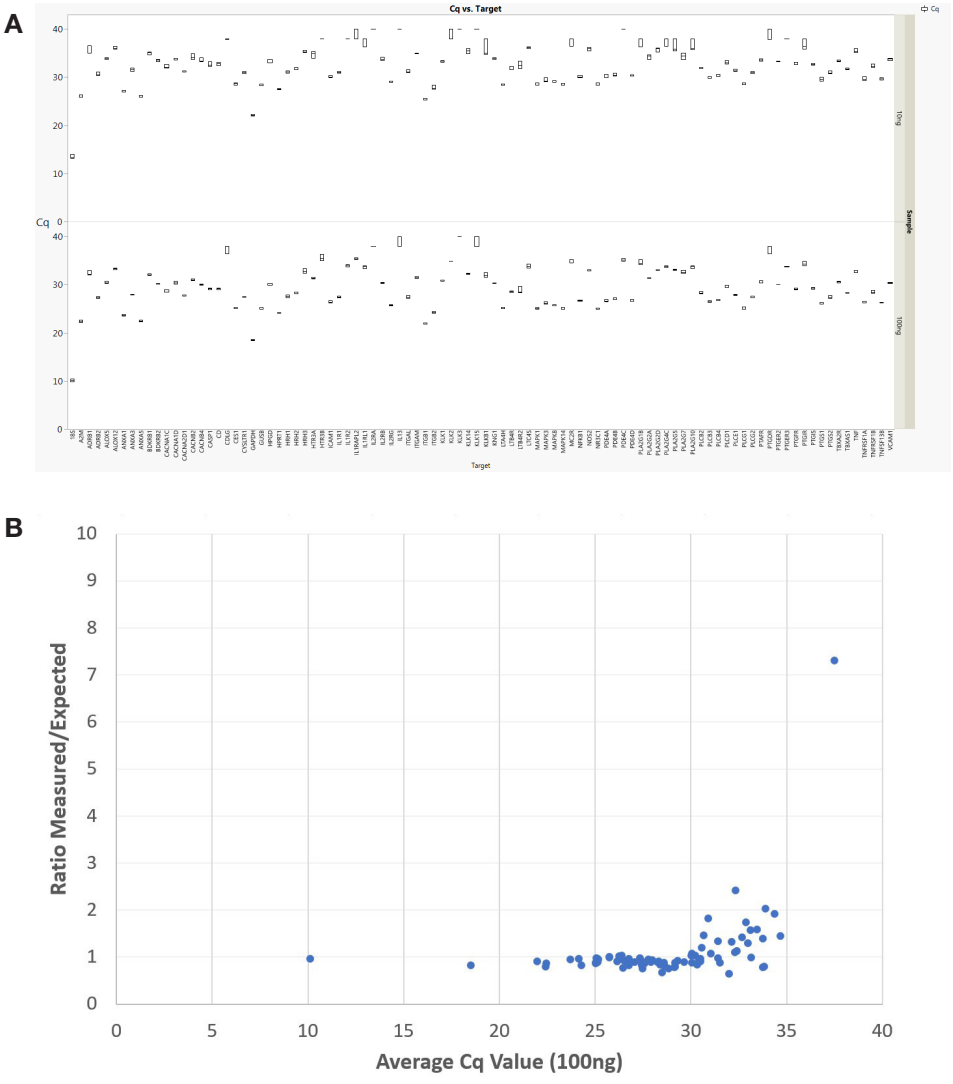


Figure 1. Expression range of gene expression assays. We analyzed the reproducibility of gene expression measurements using a TaqMan® Array 96-well Human Immune Response Plate with Fast Advanced Master Mix and UHR RNA. (A) The expression levels and variability in the transcript levels from 10 ng input RNA (top) and 100 ng input RNA (bottom) are shown. (B) The accuracy of the measurements was evaluated by determining how close to a 10-fold difference was measured. An exact 10-fold difference would give a ratio of 1.0 (y-axis). As the C_q increases (decreasing transcript abundance, C_q x-axis), the measurements become more variable and the ratio increases.

Results

We first determined the range of inflammation-related transcript levels detected in the UHR RNA sample. Using 10 ng of RNA for cDNA synthesis, the C_q ranged from about 13.5 for the 18S gene to greater than 38 (undetectable) for 13 other genes (Figure 1A, upper panel). For 100 ng UHR RNA, the C_q values ranged from 10.1 C_q for the 18S gene to greater than 38 (undetectable) for six other genes. Although seven of the genes (*HTR3B, IL1R2, IL1RAPL2, KLK2, MC2R, PDE4C, PTGER3*) were too low to be detected in the 10 ng sample, these genes were detected in the 100 ng sample, but at low levels (Figure 1A, lower panel). Five other genes (*CD40LG, IL13, IL2RA, KLK3, KLK15*) were not detected in either sample. Note that these expression levels were the result of measurements made on three different combinations of instruments and blocks. (See Reproducibility for additional data.)

To measure the accuracy of the measured C_q values, we calculated the C_q relative ratio as measured in 100 ng vs. 10 ng samples. We subtracted the average C_q for 100 ng from the average C_q for 10 ng, raised the result to the power of 2, then divided that number by 10. Using that formula, a perfectly accurate set of measurements would produce a value of 1.00. Overall, for C_q values less than about 30, this accuracy measurement was about 1.1. As expected, as the transcript levels decreased, the spread between the 10 ng and 100 ng C_q measurements increased (Figure 1B). Nevertheless, even for those transcripts for which C_q was greater than 30, the C_q average accuracy measurement was 1.4. This means that accurate transcript level measurements are possible even at very low transcript levels.

Reproducibility

To show the reproducibility of the measurements on different instruments, we performed RT-qPCR on three different QuantStudio 7 Pro Real-Time PCR Systems. Each of these instruments had a different 96-well Fast PCR interchangeable heat block installed. One plate each of 10 ng and 100 ng cDNA input amounts was run on each instrument configuration. The C_q values measured were extremely reproducible. When pairwise comparisons were made between the 10 ng plates, the correlation coefficient (R2) averaged 0.985. Similarly, pairwise correlations between the 100 ng plates averaged 0.992. Even comparing the 10 ng measurements to 100 ng measurements, the correlation was extremely high (R2 = 0.971). These results demonstrate that using the 96-well Fast plate format of the TaqMan Array Human Inflammation Panel, the inter-instrument reproducibility is extremely high (Figure 2).

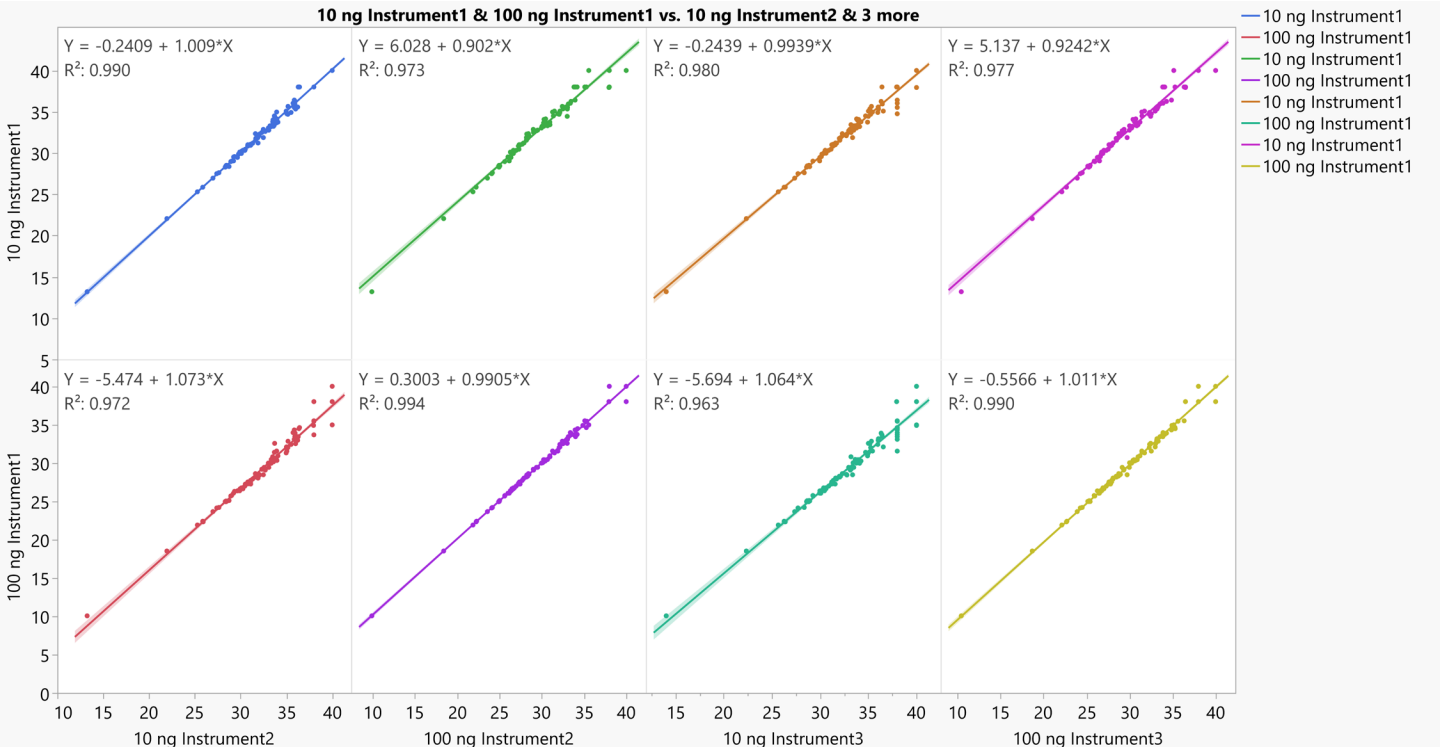
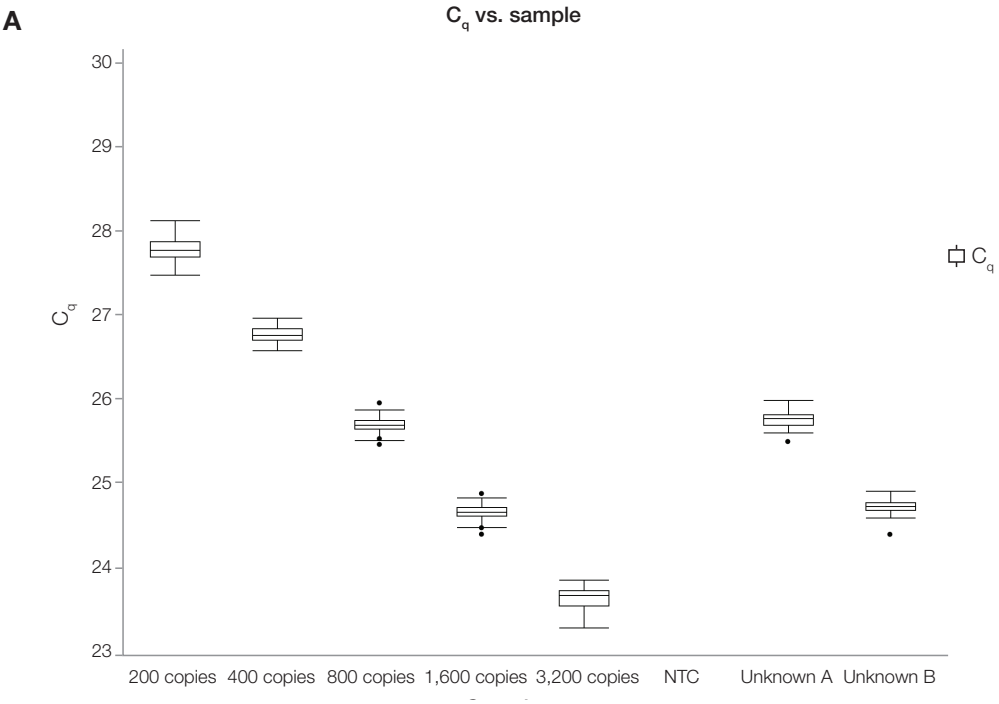


Figure 2. Reproducibility of gene expression measurements using 96-well Fast plates on different instruments. The cDNA from 10 ng and 100 ng UHR RNA was analyzed on three different instruments. Pairwise comparisons are shown. The correlations from instrument to instrument were very high.

We next measured the reproducibility of TaqMan Array Cards. We first examined the well-to-well reproducibility of TaqMan Array Cards on the QuantStudio 7 Pro Real-Time PCR System by plating standards containing known copy numbers of RNase P transcript onto a single card pre-spotted with RNase P assays. For this experiment, each concentration was measured in replicates in 48 separate chambers (Figure 3). The resulting C_q measurements were extremely close at all concentrations, with an average coefficient of variation (standard deviation/average C_q; CV) of <0.4% for each set, and linear correlation of C_q to copy number of about 0.9945 C_q.



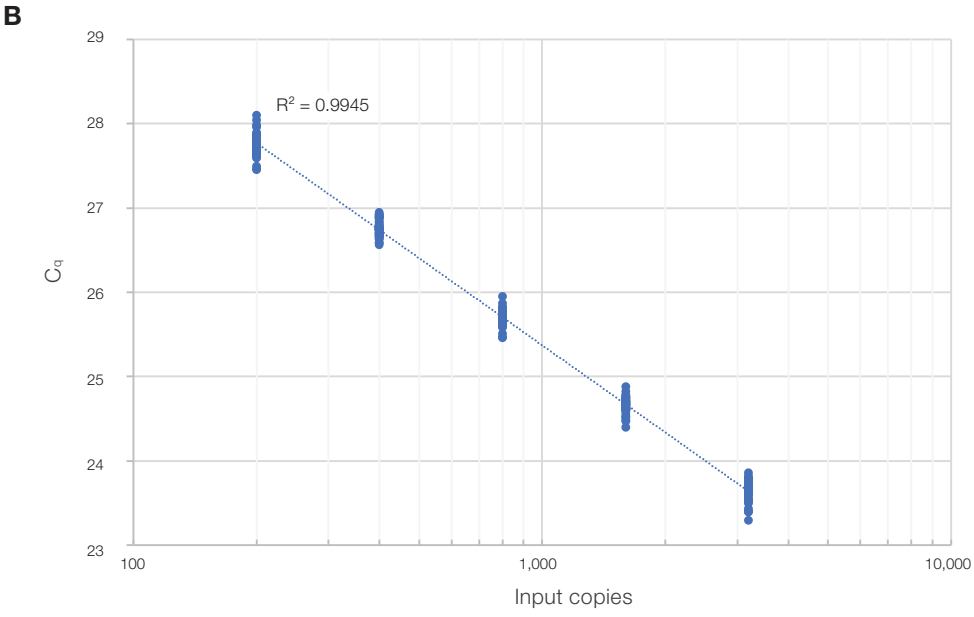


Figure 3. Well-to-well consistency of transcript measurements on a 384-well TaqMan Array Card. Each sample on the x-axis was measured in 48 different wells. **(A)** The range of measurements is shown. **(B)** The linear correlation and range of the C_q values for known input amounts are shown.

Next, we analyzed the reproducibility of measurements on different TaqMan Array Cards. For this experiment, we used the TaqMan Array Card format of the TaqMan Array Human Inflammation Panel (96 genes). As described previously, we also used cDNA synthesized from 10 ng and 100 ng of UHR RNA. These cards were analyzed on a single instrument with a single removable 384-well TaqMan Array Card heat block. Each sample was analyzed in at least four different runs on three different cards. The pairwise correlations observed between the four different data sets obtained with 10 ng input RNA on three different cards averaged 0.982 (Figure 4A). Similarly, the pairwise correlations between the five different data sets collected with 100 ng input RNA on three different cards averaged 0.984 (Figure 4B). These data indicate that TaqMan Array Cards produce highly reproducible measurements of transcript levels.

Finally, we determined the reproducibility of the 384-well TaqMan Array Card measurements on three different instruments. As described previously, we used three different QuantStudio 7 Pro Real-Time PCR Systems, each installed with its own interchangeable heat block. The pairwise correlations of the three different data sets collected using 10 ng input RNA averaged 0.954, and the pairwise correlations of the three different data sets collected using 100 ng input RNA averaged 0.978 (Figure 5). Even comparing 10 ng to 100 ng data sets, the average correlation for detectable genes was 0.955. Again, these results demonstrate that even when different instruments are used, the combination of 384-well TaqMan Array Cards and the QuantStudio 7 Pro Real-Time PCR System produces highly reproducible results.

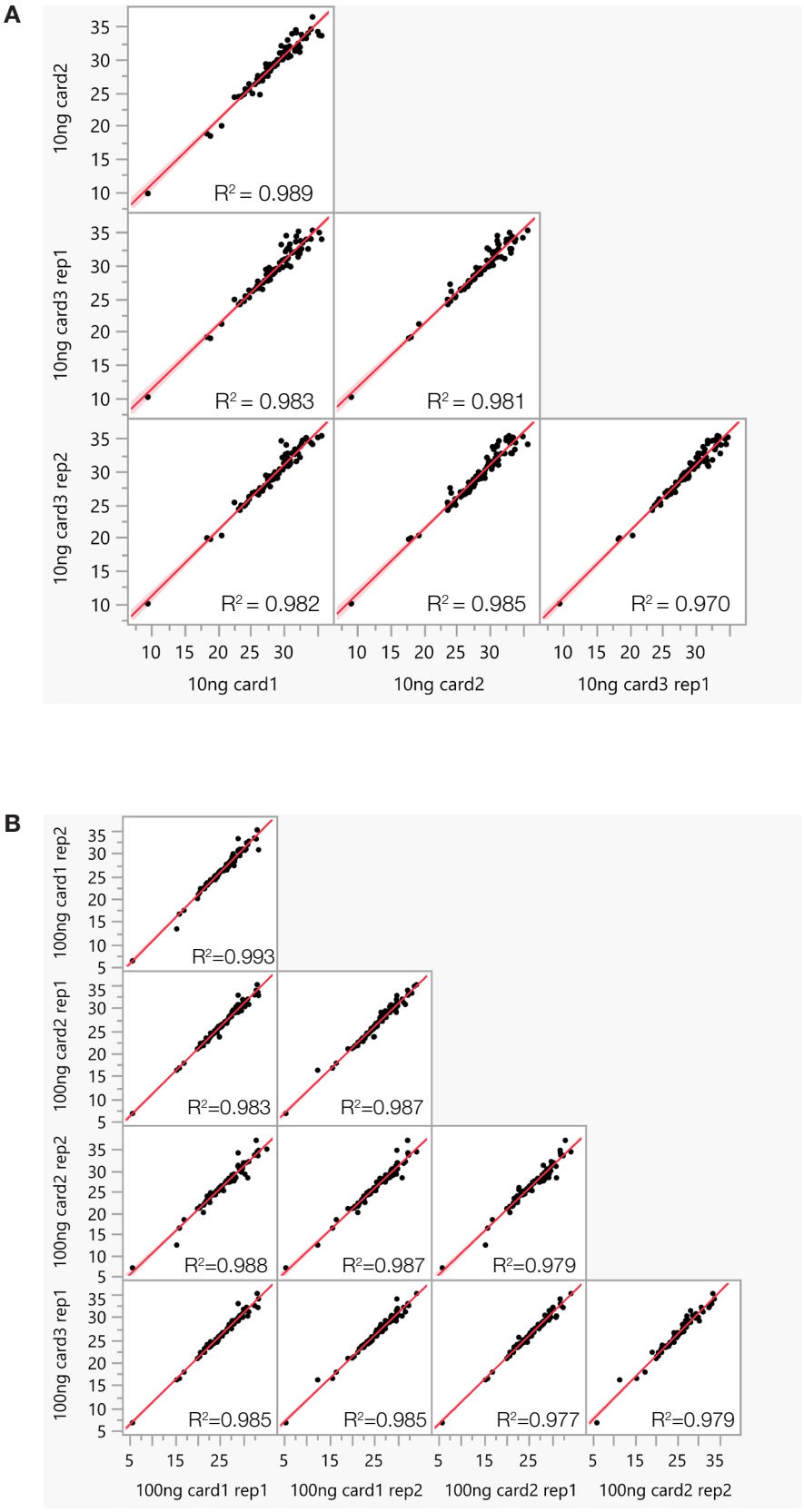


Figure 4. Reproducibility of gene expression measurements on different 384-well TaqMan Array Cards. The cDNA from 10 ng and 100 ng UHR RNA was analyzed using three different cards. Pairwise comparisons are shown for the **(A)** 10 ng measurements and **(B)** 100 ng measurements. Card-to-card correlations were very high.

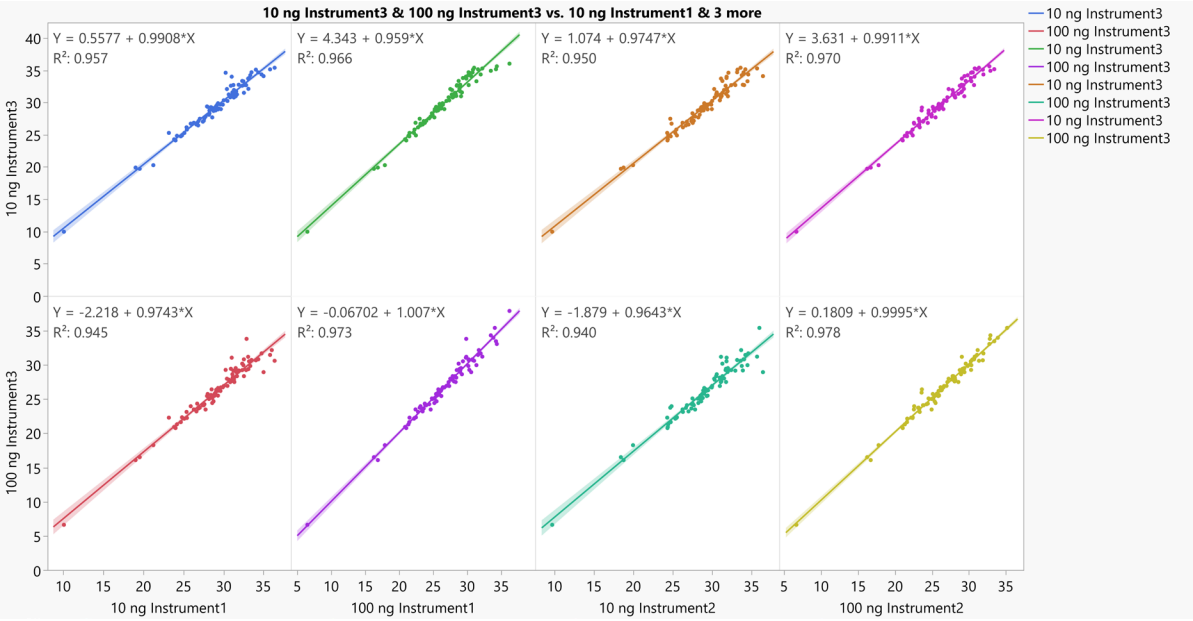


Figure 5. Reproducibility of gene expression measurements using 384-well TaqMan Array Cards on different instruments. The cDNA from 10 ng and 100 ng UHR RNA was analyzed on different TaqMan Array Cards on three different instruments. The correlations from instrument to instrument were very high.

Conclusion

Ongoing optimization of AAV vector efficiency and specificity, as well as discovery of new adenovirus strains in nature, offer opportunities for new AAVs to be explored as potential therapeutic constructs. New vector strains and designs have potential to introduce genetic elements that elicit an immune response, so they must be investigated for both efficacy and safety. When the therapeutic product, viral capsid, or other vector elements may be culprits in gene therapy-induced toxicity, exploring activation or silencing of gene expression may help to guide vector design and construction to balance therapeutic efficacy against potential toxicity. qPCR is a reliable, sensitive, and reproducible approach to quantify gene expression and investigate the potential genetic sources and vector-design mitigations of immunotoxicity in gene therapy.

The scale of research into potential gene therapies, particularly for rare diseases, often requires that a study be distributed across multiple locations. Investigators must have confidence that the results obtained at one site are consistent with results obtained at other sites participating in the study. The results presented in this application note demonstrate excellent well-to-well and instrument-to-instrument data concordance in the Applied Biosystems™ QuantStudio™ 7 Pro Real-Time PCR System in quantifying gene expression using the TaqMan® Array Human Inflammation Panel, highlighting its utility in gene therapy research.

References

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TaqMan qPCR gene expression assays can be used to detect and measure upregulation or downregulation of both the gene delivery vehicle and host-associated genes. TaqMan Assays provide the flexibility to select from more than 20 million predesigned assays, as well as custom designs and individual primers and probes. The QuantStudio 7 Pro Real-Time PCR System simplifies qPCR experiments by deploying motorized interchangeable blocks, intuitive software, and a large touchscreen interface. Facial authentication at log-in, voice commands, and remote monitoring facilitate hands-free operation. Dye multiplexing and automation capabilities provide a platform for efficient expansion and scale-up.

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