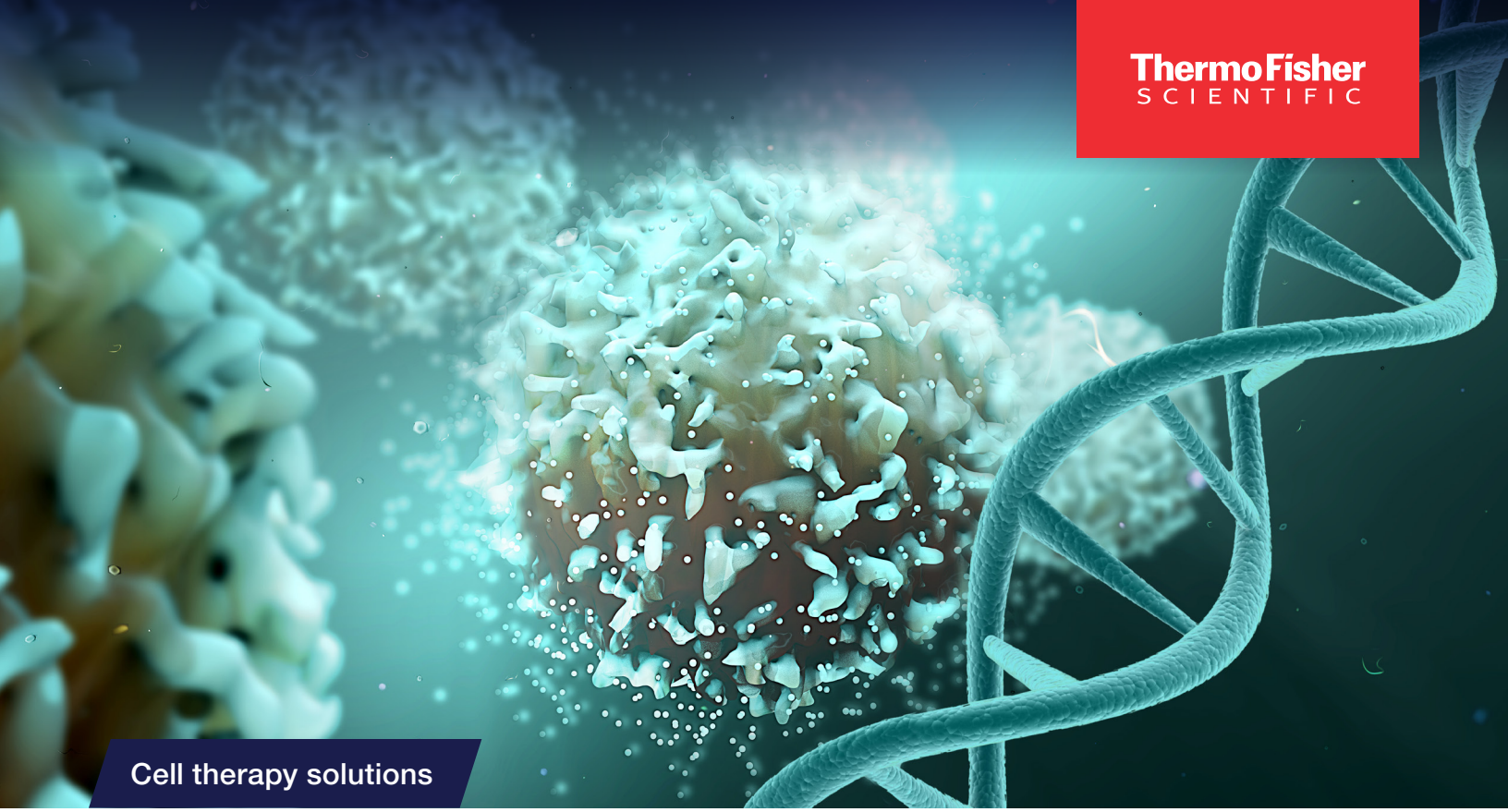

CAR-T cell release testing with qPCR and dPCR

In this application note, we show:

- Characterization and functionality assessment of CAR-T cells with qPCR and dPCR
- qPCR and dPCR methods are complementary in CAR-T cell QC workflows
- Thermo Fisher Scientific™ Kits facilitate analysis of CAR-T cell development and function

Chimeric Antigen Receptor (CAR) T-cell therapy is an innovative and highly promising approach in cancer treatment, particularly for hematologic malignancies. This therapy involves genetic engineering of patient's T cells to express a CAR, which enables them to recognize and thereby eliminate cancer cells. Quality control (QC) release testing for CAR-T cell products is a critical aspect of ensuring the safety, efficacy, and consistency of these advanced therapies. The testing process involves assessing various quality attributes, including safety, identity, purity, and potency. These quality attributes require various testing modalities, including flow cytometry and genetic testing. Each modality provides unique insights into different aspects of CAR-T cell characteristics and performance. In this application note, we highlight how qPCR and dPCR-based methods can be used to help with regulatory guidelines for CAR-T therapy development.

Both qPCR and dPCR methods can be used for safety, identity and potency testing of CAR-T cells. Each method has its own advantages and limitations as discussed extensively elsewhere [1,2]. Briefly, qPCR is a cost-effective method with faster turn-around time, higher throughput, easy scalable reaction volume, and is suitable for presence/absence tests and relative quantification. dPCR excels at applications requiring highly accurate, precise quantification. dPCR provides absolute quantification without the need for standard curves and exerts higher tolerance to PCR inhibitors.

One consideration to keep in mind is that the dynamic range of dPCR is limited, and thus, may require multiple dilutions of samples with unknown target concentrations to ensure falling into the measurable range.

Herein, we describe a potential workflow for CAR-T cell QC testing using qPCR and dPCR technologies (Figure 1). In particular, we highlight workflows that focus on aspects that determine successful construction and are notable for regulatory filings, including:

- **Safety:** analysis of replication competent virus, determining vector copy number in transduced cells, mycoplasma contamination
- **Identity:** Expression of the CAR transgene in transduced cells
- **Potency:** Gene expression patterns of immune-related genes indicating T cell function

And we present the advantages of dPCR and qPCR for the specified applications.

Source of CAR-T cells

Anti-CD19 CAR-T cells (Catalog #78171) and untransduced T cells (Catalog #78170) were obtained from BPS Bioscience or generated in house. In-house generated CAR-T cells were used for mycoplasma testing.

Automated nucleic acid purification on the KingFisher system

Nucleic acids and gDNA were prepared from CAR-T cells and non-transduced control cells using the Applied Biosytems™ PrepSEQ™ Sample Preparation Kits according to the kit instructions on the Thermo Scientific™ KingFisher™ Flex. Table 1 lists the kits used for the various assays. In brief, triplicate reactions of 0.5-1x106 cells were processed along with negative and positive extraction controls. DNA and RNA concentrations were measured using Invitrogen™ Qubit™ kits for the experiments requiring standardized DNA or RNA input amounts.

Table 1. Overview of sample preparation kits

Test	Sample prep protocol	Analyte
RCL	Applied Biosystems™ PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit	DNA
VCN	Applied Biosystems™ PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit	DNA
Mycoplasma	Applied Biosystems™ PrepSEQ™ 1-2-3 Mycoplasma Nucleic Acid Extraction Kit	DNA
CAR expression	Applied Biosystems™ PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit	RNA
Expression profile	Applied Biosystems™ PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit	RNA

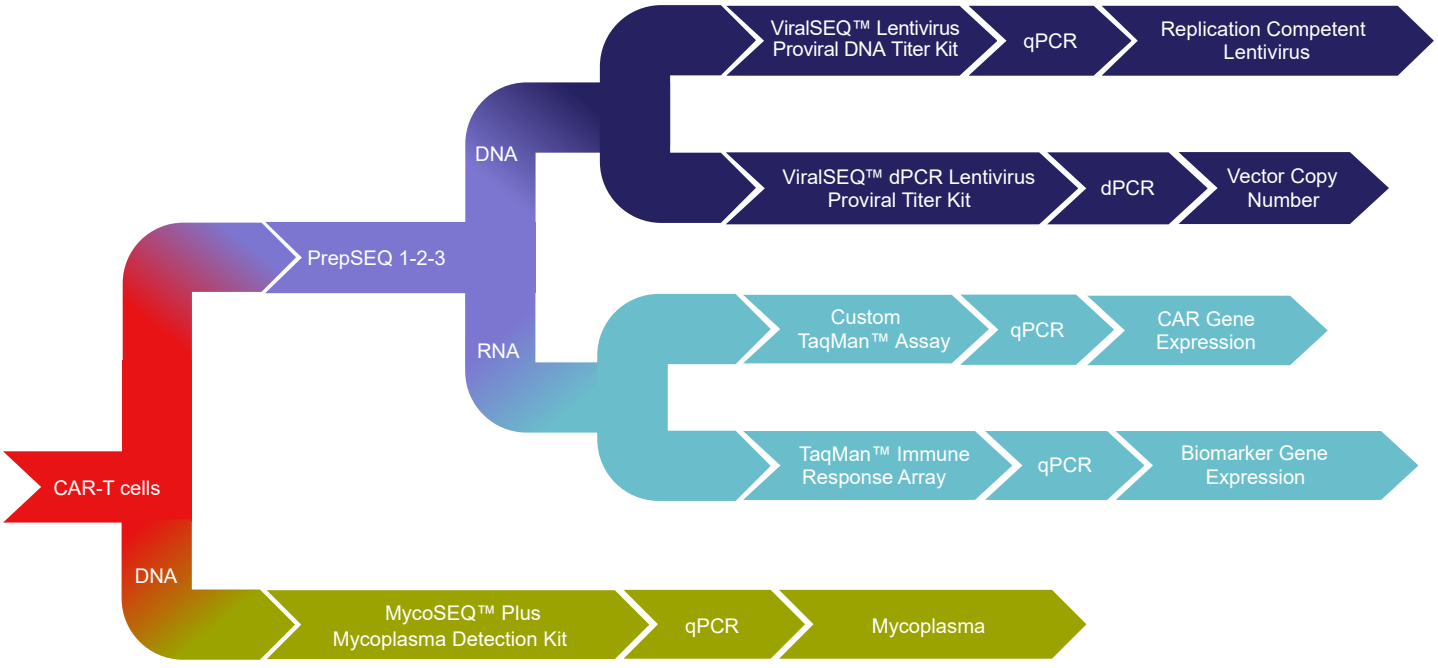

Figure 1. Workflows for QC tests described in this app note. Starting from CAR-T cells, the Applied Biosystems™ PrepSEQ 1-2-3 Kit purifies both DNA and RNA. The DNA can be used to quantify the replication competent lentivirus and the copy number of virus in the genome. The RNA can be used to quantify CAR gene expression and to verify important biomarkers are expressed. A separate kit, the Applied Biosystems™ MycoSEQ Plus Kit, is used to determine presence or absence of mycobacteria.

Quantifying replication competent lentivirus

Approach

Lentiviral vectors are commonly used to introduce CAR sequences into T-cells. There is a theoretical safety risk of generating a replication competent lentivirus (RCL) through recombination during the manufacturing of the virus. RCL could infect non-target cells in the patient and alter cellular function [3]. Therefore, the FDA recommends assessing the presence of replication competent viruses using a cell-based assay. However, PCR may be appropriate for lot release testing of ex vivo transduced cells when time constraints are present [4].

The vesicular stomatitis virus envelope glycoprotein (VSV-G) is commonly used for pseudo-typing of lentiviruses and the coding sequences of this envelope would be incorporated into a replication competent virus [5]. Therefore VSV-G is a suitable target for rapid and sensitive qPCR detection of replication competent lentivirus pseudo typed with the envelope [6]. The absence of RCL is demonstrated by a negative result in the VSV-G detection assay for genomic DNA from CAR-T cells. Since this is a simple presence/absence test and the amount of DNA to be screened requires a large sample input volume, qPCR is commonly used.

Methods

A qPCR assay for detection of replication competent virus was designed by targeting the vesicular stomatitis virus envelope glycoprotein (VSV-G). Briefly, a positive control was generated by digesting the Gibco™ LV-MAX™ Lentiviral Packaging Mix with Thermo Scientific™ FastDigest HindIII, purifying with the Thermo Scientific™ GeneJET Gel Extraction and DNA Cleanup Micro Kit and quantification by dPCR using a VSV-G assay (DFRWEKY). A triplex assay was assembled for detection of VSV-G consisting of the same VSV-G assay, an internal control assay (catalog number 4448484, assay ID: Ac00010014_a1) against a synthetic DNA sequence “Xeno” (A39175) and a reference assay for RNAse P gene detection (Primer limited, catalog number 4485715). qPCR reactions were set up with the Applied Biosystems™ TaqPath ProAmp™ master mix, assay oligos for VSV-G, Xeno and RNaseP, 1000 copies of the synthetic template and gDNA samples with up to 200ng gDNA per reaction. A standard thermocycling profile for the TaqPath ProAmp master mix was used on the Applied Biosystems™ QuantStudio™ 7 Pro and data analyzed by the design and Analysis software.

Results

A triplex assay for detection of VSV-G, an internal control and a reference gene (RNase P for quantification of genome copies in the sample) was assembled and linearity of the assay assessed in the presence of 200ng of genomic DNA. As shown in Figure 2A, the assay has excellent linearity (100.4% efficiency, R2> 0.99) from 10 to 1x106 copies per PCR reaction. At the lowest level (10 copies) of input, average Cq ranged from 35.91 to 36.38, averaging at 36.2. No signal was detected in negative controls.

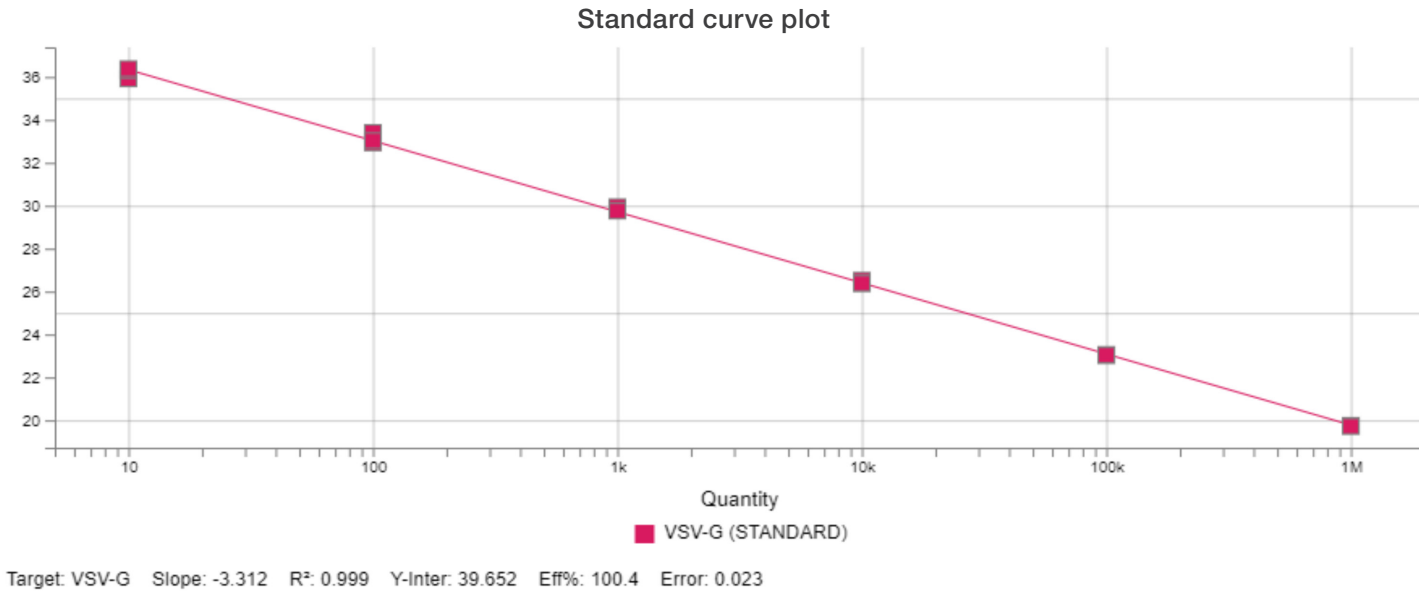

Figure 2A: Linearity of VSV-G assay for replication competent lentivirus detection, as obtained from the using reagents described in the text. Linearity over a wide dynamic range and high correlation (R2) is critical for accurate quantification.

A preliminary cut of was set at cycle 38 for VSV-G positivity.

The internal control assay showed stable Cq (Cq range from 29.21-29.71 in individual replicates, average at Cq 29.43) excluding the sample with highest VSV-G input, which had delayed Cq at 31.2 (data not shown). This is acceptable since the internal control can be exceptioned if the sample is strongly positive for VSV-G. It rather serves as a control for the samples negative for VSV-G to confirm that the negative result is not due to PCR inhibition in these samples.

Likewise, the genomic reference assay produces a stable Cq ranging from 22.08-22.48 in individual replicates (data not shown), confirming the presence and amplification of genomic DNA in the samples.

We used this assay to screen genomic DNA extracted from CAR-T cells and non-transduced control T-cells for the presence of the VSV-G. As shown in Figure 2B, the transduced CAR-T cells (Td) but not non-transduced cells (Ntd) were positive for VSV-G detection (Cq below cut off at cycle 38, dashed red line). The CAR-T cells produced a Cq from 33.6-34.4, which translates to 20-40 copies per reaction or 1 copy in 1500-3000 cells. The detected VSV-G DNA is most likely carry over plasmid DNA from the virus generation, and not replication competent virus, as has been observed by others [7], particularly if the virus was not produced and purified under GMP manufacturing conditions [5].

As shown in Figure 2B the internal control assay yielded very similar Cq for all samples, indicating good PCR performance and no effects of PCR inhibition were observed. Acceptance range limits for sufficient PCR performance need to be determined based on the verification/validation data sets.

The reference gene assay serves as an internal extraction and sample input control for the T-cell samples. This assay yielded similar Cq for T-cells compared to the PC samples, which contained 200ng of DNA, confirming the theoretical gDNA input amount in the T-cells was amplified in PCR. The Cq acceptance range for this reference gene assay need to be established during process and assay development.

The data, taken collectively indicates that qPCR is a suitable method to analyze VSV-G in CAR-T cell products the due to its large dynamic range, ability to multiplex, and robust bench workflow. The proposed triplex assay controls for extraction efficiency, sample input amount and potential PCR inhibition. In addition to release testing of manufactured CAR-T cell products, this VSV-G assay may be used for process development [5], as well as for research into post infusion RCL patient monitoring [7].

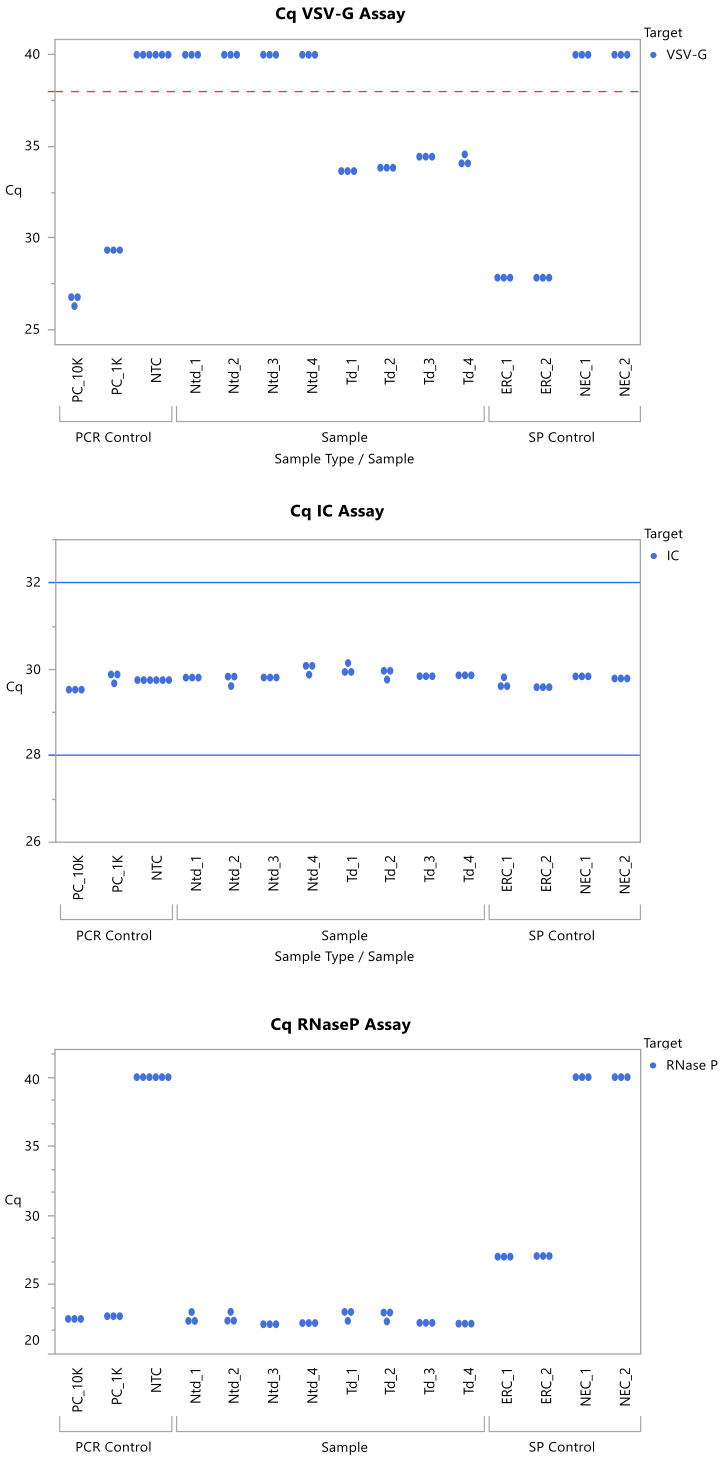

Figure 2B. VSV-G detection in CAR-T cells. A triplex PCR assay was designed that included a probe for VSV glycoprotein gene (FAM), an internal amplification probe (VIC), and a human genomic DNA probe (ABY). Cq was set to 40 if amplification was undetectable. PCR positive controls (PC_10K and PC_1K) were positive for all targets. No template control (NTC) showed no amplification for RNase P or VSV-G, but was positive for internal amplification. Four non-transduced cell samples (NTC_1-4) amplified RNaseP and internal control, but did not have VSV-G amplification. In contrast, four transduced cell samples (Td_1-4) had amplification with all probes. Finally, the extraction controls (ERC) were positive for all probes, while the negative extraction controls (NEC) were negative for all but internal amplification control. The Cq values of VSV-G were used to quantify vector using the above standard curve as described in the text.

Determining vector copy number

Approach

Regulatory guidelines mandate that the average vector copy number (VCN) in CAR-T cell products must be assessed and maintained at less than 5 copies per cell [8]. This assessment is a critical step prior to patient infusion, as it helps ensure the safety and efficacy of the therapy. By keeping the VCN below this threshold, the risks of cytokine release syndrome, insertional mutagenesis, genotoxicity and subsequent cancer development are minimized [9,10]. Additionally, maintaining an optimal VCN allows proper CAR expression, ultimately enhancing patient safety and treatment efficacy [11].

Average VCN of CAR-T cells is calculated by the average number of transgenes per cell, normalized by the transduction efficiency [12]. Previous studies employed the use of both qPCR assays including standard curves [10,13] as well as dPCR assays [11,12,14,15,16] for quantification of transgene and reference genes.

Methods

For dPCR testing, a duplex assay mix from the Applied Biosystems™ ViralSEQ™ dPCR Lentivirus Proviral Titer Kit was combined with an ABY™ dye-labeled QSY™ human RNase P reference assay (catalog number 4485714) and the universal dPCR master mix. Restriction enzyme EcoRI was added to the dPCR reaction to ensure fragmentation of the genomic DNA for accurate quantification of the genomic DNA, as proposed by others [12]. Five nanograms DNA per dPCR reaction was tested for each of the sample prep replicates in duplicates. The pLenti6.2 control vector was used as positive control at 1000 copies per reaction. The plasmid was linearized by digestion with Thermo Scientific™ FastDigest EcoRI, purified with the GeneJET Gel Extraction and DNA Cleanup Micro Kit and DNA copies quantified by dPCR using the same ViralSEQ dPCR Lentivirus Proviral Titer assay. The dPCR reactions were loaded onto the MAP16 plates per manufacturer's recommendation. A thermocycling protocol recommended for gDNA applications was used including an initial 10-minute incubation at 37C for the restriction digest.

For qPCR quantitation, the ViralSEQ Lentivirus Proviral DNA Titer Kit was used with slight modifications. To quantify cell DNA, a triplex assay was created by adding ABY-labelled RNase P reference assay to the duplex assay provided in the kit, and EcoR1 was added to the reaction as described above. A 15 minute incubation step was included before initiating PCR to insure fragmentation; otherwise all reactions and PCR conditions were followed according to the kit. Results were analyzed using the Applied Biosystems™ AccuSeq Real-Time PCR software version 3.2. This SAE-capable solution performs QC analysis of all reactions and calculates the quantity of viral DNA in

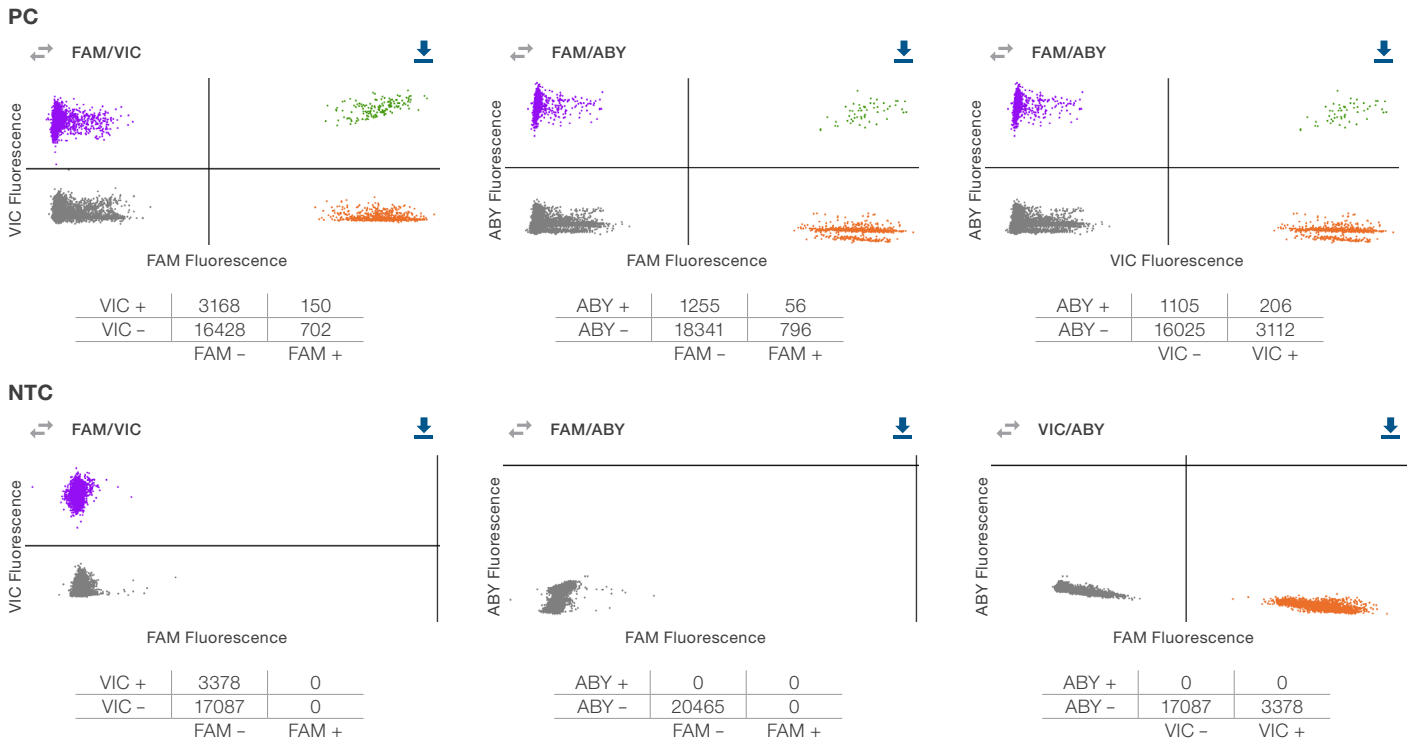

Figure 3: Verification of dPCR assay for copy number testing. A well-designed multiplex reaction should show good separation of positive clusters under all comparisons. The clustering for a positive control (PC) and no template control (NTC) is shown for all pairwise comparisons. Note that the positive clusters for FAM (LTR-3), VIC (Internal Amplification Control) and ABY (RNaseP) are clear and distinct and have no background.

each sample. Genomic DNA was quantified by comparing RNase P levels to a standard curve of known amounts of CEPH DNA run concurrently. Note, when developing and producing plasmids and viral vectors in house rather than outsourcing, the Applied Biosystems™ resDNASEQ™ system provides accurate, rapid, and reproducible quantitation of residual DNA, critical to the quality and safety of many biotherapeutic reagents. For more information about the products discussed above, please visit thermofisher.com/celltherapy-pharma-analytics.

Average VCN per cell is obtained by dividing the transgene concentration by the cell number, the latter equivalent to half of the reference gene concentration. Average CAR-T cell VCN is obtained by normalizing the VCN per cell with the percentage of transduced cells.

Results

For detection by dPCR, a triplex assay composed of an assay targeting the integrated lentiviral vector, an internal control assay and a reference gene were assembled. The VCN triplex dPCR assay was tested with the positive and negative control to ensure good cluster separation and accurate quantification. As shown in Figure 3, positive and negative controls produced expected clusters with good separation. The quantification was close to expected input values (data not shown).

The VCN triplex dPCR assay was used to assess VCN in transduced CAR-T cells and control cells. As shown in Table 2, the assay detected no viral vector in non-transduced control cells nor in negative controls. The IC showed stable levels. The reference assay amplified the genomic DNA in cells and positive controls. The average VCN per cells in the sample was around 0.7. The average VCN per CAR-T cells was calculated at around 1.4 copies per CART-cell, based on minimum 50% transduction efficiency determined by flow cytometry.

A similar triplex assay was used for qPCR detection. The internal PCR control was detected in all wells, demonstrating successful amplification (average Cq = 25.93, stdev = 0.26). As seen with dPCR, there was no detectable viral vector in non-transduced cells nor in negative controls (Table 3). The average VCN per CAR-T cell was 1.38, agreeing with the value calculated by dPCR.

Table 2. Overview of sample preparation kits

Sample	Reps	Proviral average of conc.	IC average of conc.	RNAseP average of conc.	VCN_{Cells}	Max VCN_{CAR}
PC	4	106.82	401.13	158.79		
NTC	4	0.14	406.57	0.00		
ERC_1	2	96.76	409.05	80.55		
ERC_2	2	96.59	420.70	80.88		
NEC_1	2	0.12	406.97	0.00		
NEC_2	2	0.00	410.21	0.00		
Td_1	2	76.53	417.10	237.19	0.65	1.29
Td_2	2	87.20	416.44	241.12	0.73	1.45
Td_3	2	87.09	410.17	256.85	0.68	1.36
Td_4	2	86.38	416.40	248.82	0.70	1.39
Ntd_1	2	0.00	404.08	231.97		
Ntd_2	2	0.06	414.20	239.61		
Ntd_3	2	0.06	421.74	243.59		
Ntd_4	2	0.06	402.24	226.19		

Table 3. Overview of sample preparation kits

Sample	Reps	Proviral average copies	RNAseP average copies	VCN_{Cells}	Max VCN_{CAR}
NTC	3	No Amp	No Amp		
ERC_1	3	4666	740.9		
ERC_2	3	4558	1000.9		
NEC_1	3	No Amp	No Amp		
NEC_2	3	No Amp	No Amp		
Td_1	3	30956.4	44927.4	0.69	1.38
Td_2	3	29717.8	49404.3	0.60	1.20
Td_3	3	34374.8	48326.2	0.71	1.42
Td_4	3	31718.3	42232.2	0.75	1.50
Ntd_1	3	No Amp	34063.6		
Ntd_2	3	No Amp	35949.8		
Ntd_3	3	No Amp	54808.7		
Ntd_4	3	No Amp	35949.8		

Taken together, we demonstrated robust workflows for both dPCR and qPCR to determine the average VCN in CAR-T cells preparations. These assays may be used for CAR-T cell release testing and assessing VCN in patient samples. Importantly, these methods need to be combined with flow cytometry or other methods (for the % CAR-T cells in the sample) to determine the VCN per CAR-T cell.

Mycoplasma detection

Approach

Mycoplasma testing is a critical quality control measure for CAR-T cell therapies. Regulatory bodies such as the U.S. Food and Drug Administration (FDA) have specific guidelines for mycoplasma testing in cell and gene therapies to ensure that the CAR-T cell drug product is not contaminated [17,18]. Mycoplasmas are a group of bacteria that can contaminate cell cultures and adversely affect the CAR-T cell manufacturing process and potentially patient safety. Rapid mycoplasma tests (including PCR-based tests) are acceptable for live cells as an alternative to compendial culture-based methods.

qPCR was chosen as method for mycoplasma detection since it is a simple presence/absence readout. The Applied Biosystems™ MycoSEQ™ Plus Mycoplasma Detection Kit was designed for detection of mycoplasma in complex bioproduction samples to meet or exceed guidance on sensitivity and specificity expectations as described in European Pharmacopoeia (E.P. 2.6.7, 2007), US Pharmacopoeia (US63) and Japanese Pharmacopoeia. When used with a suitable sample preparation method, the MycoSEQ Plus Kit can detect less than 10 CFU/mL or the genomic equivalent of 10 GC/mL.

Methods

Mycoplasma detection was performed according to the instructions in the user guide for the MycoSEQ Plus Mycoplasma Detection Kit. Briefly, 10µL of sample was tested along with positive and negative PCR controls on the Applied Biosystems™ QuantStudio™ 5 instrument. The run was set up and data were analyzed with AccuSeq Real-Time PCR software version 3.2.

Results

The results of Mycoplasma testing using the MycoSEQ kit and AccuSeq software are easily interpreted. A screenshot of a successful test is shown in in Figure 4. Each assay included controls that verify the performance, and an interpretation of these controls and unknown samples is provided by the software. In this experiment, the CAR-T cell transduced cell extract analyzed had no mycoplasma contamination.

Call summary

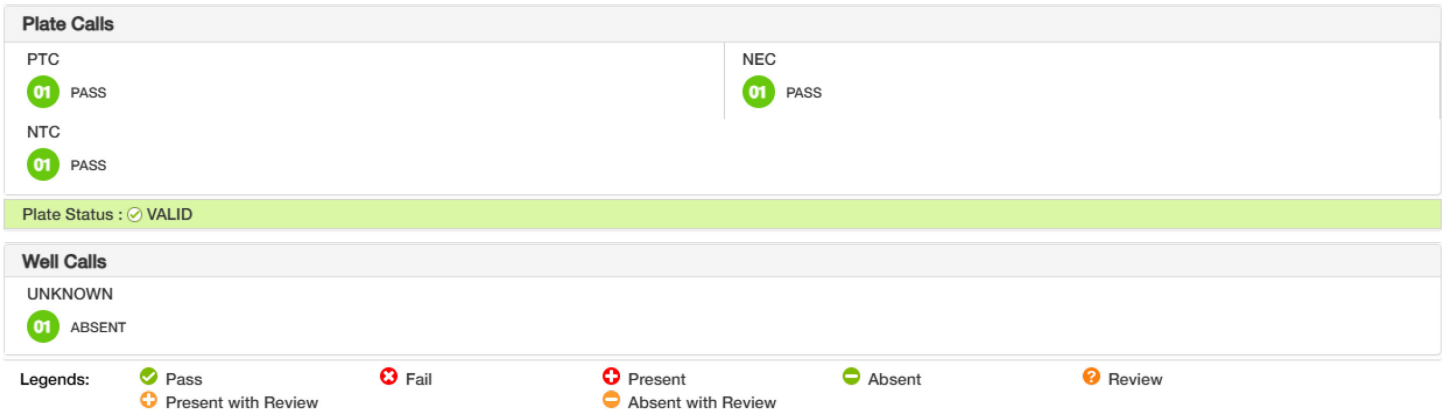

Figure 4: Mycoplasma test results. Screenshot of an output from AccuSeq Mycoplasma Testing module. The software examines controls and makes a call for unknowns based on control PCR reactions. Note that in this case the CAR-T sample (unknown) did not contain detectable mycoplasma. PTC: positive test control, NTC: negative test control, NEC: negative extraction control.

The MycoSEQ Plus Mycoplasma Detection Kits provides results in less than five hours and can be used for routine in process testing as well as for lot release testing. This test may be used as an alternative to the standard 28-day culture test or in conjunction with culture-based methods by providing preliminary results while waiting for results from a compendial method.

Quantification of CAR gene expression

Approach

In order for the engineered CAR-T cell to be effective, the chimeric antigen receptor gene must be expressed. For this, RNA from the cells would be reverse transcribed and the CAR expression levels determined either by qPCR or dPCR. However, because there could be a large range of expression and precise quantification may not be necessary, qPCR is the most practical option for verifying CAR expression. Since many CAR genes may be unique, it might be necessary to design a custom TaqMan assay that will recognize the unique gene. To determine relative expression, however, any of the off-the-shelf reference gene controls, for example B2M, can be used to analyze CAR expression relative to the housekeeping gene.

Methods

CAR expression was assessed by 1-Step RT-qPCR. An assay for detection of the CAR mRNA was designed using the Applied Biosystems™ TaqMan® Gene Expression Assays option in the Custom TaqMan® Assay Design Tool on the Thermo Fisher website (Assay ID: APEP3XX). DNase treatment of total nucleic acids eluates from the Applied Biosytems™ PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit protocol (5ng of RNA) was performed with TURBO DNA-free™ Kit to remove the genomic DNA per instructions in the product information sheet. CAR expression was detected using a 1-Step-RTqPCR duplex assay targeting the transgene mRNA (FAM-labelled) along with a B2M reference gene assay (Cat no 4326319E). The Applied Biosystems™ TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix was used for the duplex assay. For each sample prep, 0.5ng of treated RNA was processed in triplicate PCR reactions on the Quantstudio 7 Pro instrument with standard conditions and data analyzed in the Design and Analysis software.

Results

Identity of CAR-T cells was confirmed by detecting the CAR transgene expression. Since high expression levels are expected, qPCR was chosen for detection of the CAR transgene mRNA. A 1-Step-RT-qPCR duplex assay was designed to detect the transgene transcript along with a reference gene as internal control. As shown in Table 3, high levels of transgene were detected in CAR-T cells but not non-transduced control cells. Acceptance limits for the transgene levels need to be determined during process and analytical development.

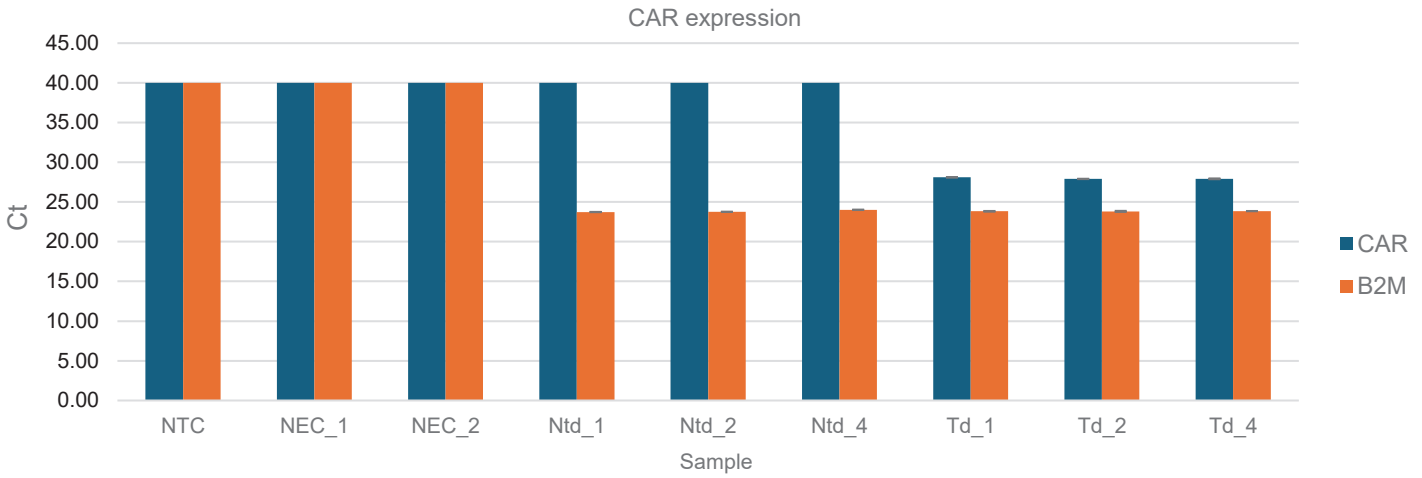

Figure 5. qPCR analysis of CAR gene expression in cells. A custom TaqMan assay was designed that queries the CAR sequence (CAR) in the transduced T cells. An off-the shelf b2-macroglobulin control (B2M) was also included for a duplex reaction. Expression was measured in transduced (Td) and untransduced (Ntd) cells. Note these are Cq values; lower Cq values mean higher expression. A Cq value of 40 means no expression was detected. B2M was not detected in the negative controls (NTC and NEC), but was detected in cell lysates. The CAR gene was only detectable in transduced cells. The average and STD of triplicate measurements is shown.

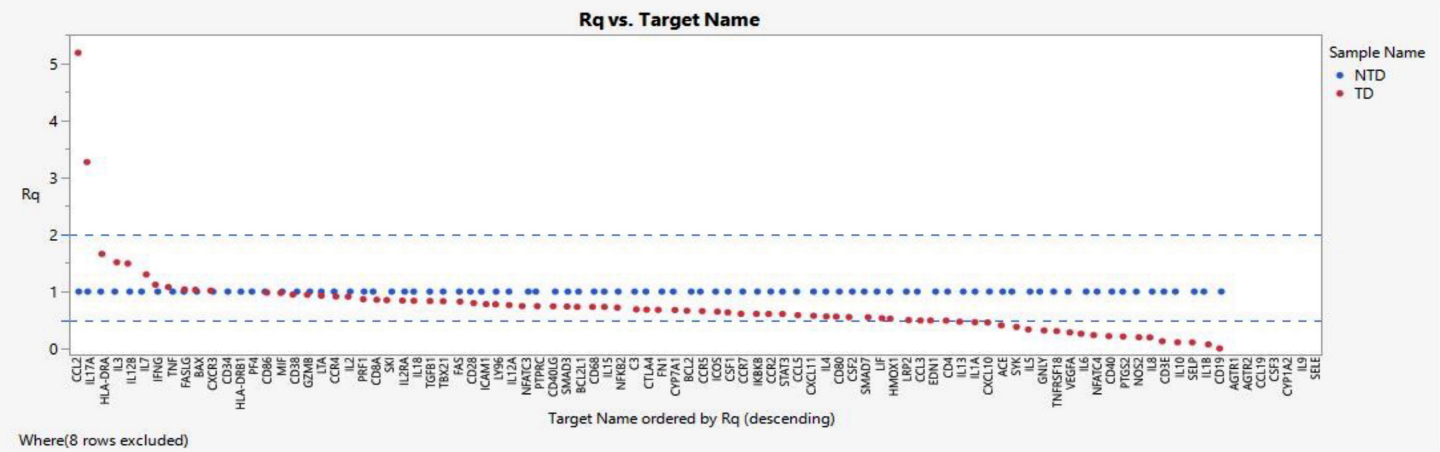

Figure 6: Immune response gene expression analysis. Two replicates from transduced (TD) and untransduced (NTD) were analyzed using the TaqMan Human Response assay panel. Expression levels were normalized to four internal control genes (*18S, GUSB, GAPDH and HPRT*), then normalized to NTD expression levels; fold-differences are plotted as Rq values on y-axis. Genes that are upregulatated or downregulated by more than twofold fall above (Rq > 2) or below (Rq < 0.5) the dotted lines.

Immune response gene expression

Approach

The expression profiling of cytokines and other immune response genes can potentially act as biomarkers for CAR-T cell activity. qPCR was chosen for gene expression analysis, since it allows high throughput analysis of many targets. Relative expression levels of immune response markers in CAR-T cells and non-transduced control cells were evaluated using a TaqMan assay qPCR array for immune response genes. The array consists of 92 Taqman assays for gene expression analysis, encompassing cell surface receptors, stress response elements, oxidoreductases, proteases, transcription factors, signal transduction proteins, cytokines, cytokine receptors, chemokines, chemokine receptors, cell cycle and protein kinases.

Methods

Expression of Immune Response genes in CAR-T cells and non-transduced control cells was analyzed using the Applied Biosystems™ TaqMan™ Array Human Immune Response assays. Two sample preparation replicates were processed for transduced and not transduced T-cells. First, eluates from the PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit (528ng RNA) were treated with DNase (ezDNase) to remove genomic DNA. Treated RNA was reverse transcribed with the Invitrogen™ SuperScript™ IV VILO™ Master Mix according to kit instructions. cDNA was then mixed with TaqPath™ qPCR Master Mix, CG and 5ng cDNA per well added to the Taqman array plate. PCR was performed on Quantstudio 7 Pro with standard thermocycling conditions for the respective TaqPath master mix. Data were analyzed using the RQ cloud app. Relative expression was calculated using the 4 reference genes in the panel *(18S, GUSB, GAPDH, HPRT)*, relative to the non-transduced cells.

Results

Upregulation of gene expression (>2 fold) in CAR-T cells vs non-transduced cells was observed for *CCL2* and *IL-17A* (see Figure 5). *CCL2* is one of the cytokines known to be involved in T cell chemotactic trafficking and antitumor Th1 responses [19]. IL-17A was reported to be expressed in CAR-T cells with killing activity [20,21]. Several genes were expressed at lower levels (<0.5 fold) in CAR-T cells or below detection limit, including *CD3E, IL-1B, and IL-10*.

Interestingly, *CD19*, a marker for B-cells was detected at much lower levels (0.4%) in CAR-T cells vs non-transduced control cells. *CD40*, another marker found on B-cells but not exclusively associated with this cell type was also at lower levels in CAR-T cells. Presence of residual B cells during the first days of PBMC cultivation is expected, and their reduction or absence in the transduced samples may confirm the killing activity of the generated CAR-T cells [22].

Markers for macrophages, endothelial cells, platelets and other cell types (*IL-1B, IL-8, NOS2, PTGS2, SELP, VEGFA*) were also detected at lower levels in the transduced cells, indicating that their cell numbers are decreasing. The remaining genes showed no significant difference between CAR-T cells and not transduced control cells, e. g. *IFNG* and *TNF*, as reported previously [22]. Other genes, including *AGTR1, AGTR2, CD34, CCL19, CSF3, CYP1A2, PF4, SELE*, expressed in smooth muscle cells, hematopoietic stem cells, dendritic cells, macrophages, platelets and endothelial cells, respectively, were below detection limit in either cell type as expected.

The expression stimulated by CARs is influenced by the specific intracellular signaling domain incorporated in the construct [23], and it will require characterization for each individual CAR construct.

The array can be utilized to profile gene expression levels throughout the process development phase. This characterization helps in understanding the expression patterns and identifying critical genes. Once these gene expression levels are thoroughly characterized, specific acceptance ranges can be established. These predefined ranges can then be used as benchmarks for QC during later stages, including release testing, to support consistency with established standards.

Summary

In this application note, we showed a workflow for testing the nucleic acid components of CAR-T cells using qPCR and dPCR platforms, including automated nucleic acid extraction using the KingFisher liquid handling instruments. Furthermore, we described the various kits available from the Applied Biosystems portfolio for quantifying replication-competent virus, vector copy number, testing presence of mycoplasma, expression of the CAR, and verifying expression of immune response genes. Together, these results demonstrate the strengths of qPCR and dPCR technologies and how each of the methods contribute to a robust CAR-T QC workflow.

> To learn how Thermo Fisher can partner with you and streamline your therapy development workflow, contact us.

Ordering information

Description	Catalog number
Sample preparation, Nucleic Acid quantification	
KingFisher Flex Purification System	24074431
KingFisher Apex Purification System	5400930
KingFisher Presto Purification System	5400830
Qubit 4 Fluorometer	Q33238
Applied Biosystems™ PrepSEQ™ Nucleic Acid Sample Preparation Kit	A50485
Applied Biosystems™ PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit	4452222
Applied Biosystems™ PrepSEQ™ 1-2-3 Mycoplasma Nucleic Acid Extraction Kit	4443789
Qubit dsDNA BR	Q33265
Qubit RNA HS	Q32852
Quality control instruments	
Applied Biosystems™ QuantStudio™ Absolute Q Digital PCR System	A52864
Applied Biosystems™ QuantStudio™ 7 Pro Real-Time PCR System, 96-well, 0.1 mL	A43163
Applied Biosystems™ QuantStudio™ 7 Pro Real-Time PCR System, 96-well, 0.2 mL	A43162
Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 96-well, 0.1 mL	A28138
Software	
Applied Biosystems™ QuantStudio™ Absolute Q Digital PCR Software Release Version: 6.3.3	
Design & Analysis Software Release Version:2.8.0	
Applied Biosystems™ AccuSEQ™ Real-Time PCR Detection Software v3.2	A58643
RQ cloud App	

Ordering information (continued)

Description	Catalog number
Quality control reagents	
Applied Biosystems™ TaqPath™ ProAmp™ Master Mix	A30866
Applied Biosystems™ TaqMan™ RNase P Assay, ABY™ dye/QSY™ quencher, primer-limited	4485715
Applied Biosystems™ TaqMan™ Gene Expression Assay, VIC primer-limited, ID: Ac00010014_a1	4448484
Applied Biosystems™ TaqMan™ Universal DNA Spike-In Control	A39175
Applied Biosystems™ Absolute Q™ Universal DNA Digital PCR Master Mix (5X)	A72710
Applied Biosystems™ Absolute Q™ Custom FAM Copy Number dPCR assay, ID: DFRWEKY	A55325
Applied Biosystems™ TaqMan™ Gene Expression Assay, VIC, ID: Ac00010014_a1	4448489
Human B2M (Beta-2-Microglobulin) Endogenous Control (VIC™/MGB probe, primer limited)	4326319E
Custom TaqMan™ Gene Expression Assay, FAM, ID: APEP3XX	4331348
Applied Biosystems™ ViralSEQ™ dPCR Lentivirus Proviral Titer Kit	A59318
Applied Biosystems™ QuantStudio™ Absolute Q MAP16 Plate Kit	A52865
Applied Biosystems™ TaqMan™ RNase P Assay, ABY™ dye/QSY™ quencher	4485714
Gibco™ LV-MAX™ Lentiviral Packaging Mix	A43237
Invitrogen™ pLenti6.2-GW/EmGFP Expression Control Vector	V36920
Thermo Scientific™ FastDigest HindIII	FD0504
Thermo Scientific™ FastDigest EcoRI	FD0274
Thermo Scientific™ GeneJET Gel Extraction and DNA Cleanup Micro Kit	K0831
Applied Biosystems™ DNA Dilution Buffer	4405587C
Invitrogen™ TURBO DNA-free™ Kit	AM1907
Applied Biosystems™ TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix	A58666
Invitrogen™ SuperScript™ IV VILO™ Master Mix Kit with ezDNase	11766050
Applied Biosystems™ Taqman™ control dilution buffer	A49889
Applied Biosystems™ MycoSEQ™ Plus Mycoplasma Detection Kit	A55124
Applied Biosystems™ ViralSEQ™ Lentivirus Proviral DNA Titer Kit	A53561
Applied Biosystems™ ViralSEQ™ dPCR Lentivirus Proviral Titer Kit	A59318
Applied Biosystems™ ViralSEQ™ Lentivirus Physical Titer Kit	A52597
Applied Biosystems™ ViralSEQ™ dPCR Lentivirus Physical Titer Kit	A59317
Applied Biosystems™ TaqMan™ Array Human Immune Response	4414073
Applied Biosystems™ TaqPath™ qPCR Master Mix, CG	A16245
CEPH Control DNA	403062

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