applied biosystems

resDNASEQ™ HEK293 and E1A Fragment Length Quantitative Assays: Comprehensive Solution for GMP Lot Release in Gene Therapy and Vaccine Manufacturing

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ABSTRACT

Purpose Biopharmaceutical products must limit host cell residual DNA contaminants to prevent genotoxicity and immunotoxicity risks to patients. Existing regulatory guidelines for residual host cell DNA require ≤10ng per dose, with a DNA size of 200bp or lower. In viral vector production, the amount, size, and oncogenic sequences of encapsidated DNA is of additional concern. To address this need Thermo Fisher Scientific has developed two complementary assays designed to meet regulatory guidance. The Applied Biosystems™ resDNASEQ™ Quantitative HEK293 DNA kit quantifies total residual host cell DNA and the The Applied Biosystems™ resDNASEQ™ E1A DNA Fragment Length Kit performs sizing analysis of short (86bp), medium (200bp) and long (476bp) fragments targeting the E1A oncogene.

Method To demonstrate performance, DNA in both kits were diluted to create a 5-point linear standard curve for interpolation of unknown sample concentrations. DNA spiked in complex matrix formulations, representing various stages in the bioproduction workflow, were extracted manually and using the KingFisher™ Flex Extraction System using the PrepSEQ™ Residual DNA Sample Preparation kit. Real-time PCR was performed on both QuantStudio™ 5 and 7500 Fast real-time PCR instrument and data analyzed on AccuSEQ™ analysis software to verify performance specifications such as sensitivity, specificity, accuracy, linearity and robustness.

Results The limit of quantitation (LOQ) for both HEK293 and E1A Fragment assays were 0.3pg and 30 copies respectively. All standard curve points conformed to a linear curve fit with R^2 > 0.99 and PCR efficiency of 100% +/- 10%. The assays showed high accuracy in the various bioprocessing sample matrices and high specificity when spiked with cross-reactants, including other cell line DNA, viral and bacterial gDNA. Spiked sample recoveries measured on both the QuantStudio 5 and 7500 Fast were within the 70-130% range.

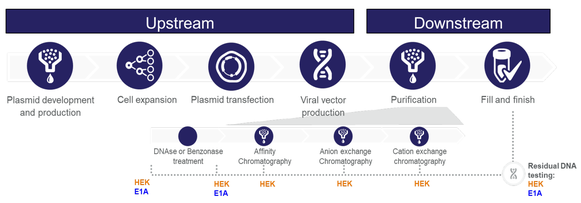

Figure 1: resDNASEQ HEK293 and E1A Fragment Quantitative Assay testing points for process development and QC

INTRODUCTION

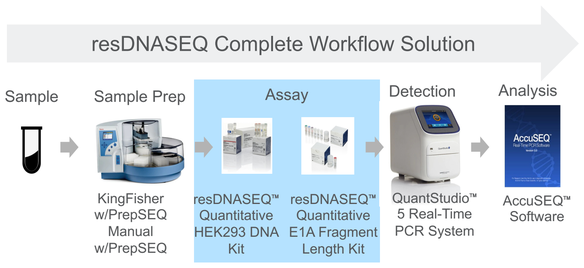

Figure 2: resDNASEQ workflow

MATERIALS AND METHODS

Performance was validated across multiple conditions:

- 2 Manufactured Lots
- 2-4 Days x 3 Operators x 2 Instruments x 2 Sites
- Applied Biosystems™ 7500 Fast Real-Time PCR Instrument + AccuSEQ™ 2.1 Software, Applied Biosystems™ QuantStudio™ 5 Real-Time PCR Instrument
  + AccuSEQ™ 3.0 Software
- All measurements were run in triplicate

RESULTS

resDNASEQ HEK293 and E1A Fragment Standard Curve: In order to perform accurate quantitation of residual genomic HEK293 DNA and E1A DNA for gene therapy and vaccine manufacturing, a standard curve was generated from the standard provided in the kit. Both standard curves are generated using serial dilutions to generate 5 standards which were run in triplicate using qPCR (Quantstudio 5 and Fast 7500) and is shown in Figure 1. The Ct CV% were also calculated for each standard level and met the criteria (each less than 10% CV%).

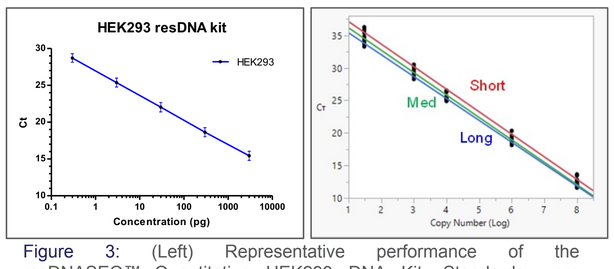

Figure 3: (Left) Representative performance of the resDNASEQ™ Quantitative HEK293 DNA Kit. Standards were diluted to 3,000 pg, 300 pg, 30 pg, 3 pg, and 0.3 pg per PCR well. (Right) Representative performance of the resDNASEQ™ Quantitative HEK293 DNA Kit. Standards for short, medium and long assays were diluted to 10^8, 10^6, 10^4, 1000, 30 and 10 copies per PCR well.

Standard curve fit parameters were calculated for resDNASEQ HEK293 and the slope of -3.28, R^2 = 0.997 and Efficiency of 101.7 and fell within acceptable limits of R^2>0.99 and Efficiency within 90 – 110%.
Standard curve fit parameters were calculated for E1A fragments assays – short / medium / long and the slopes were -3.34 / -3.36 / -3.44 , R2 = 0.997 and Efficiencies were 99% / 98.6% / 95.6%. All results fall within acceptable limits of R^2 >0.99 and Efficiency within 90 – 110%. Reproducibility was investigated by running multiple standard curves in two different laboratories. The results are shown in Figures 4 and 5 and show that results at both sites fall into acceptable ranges.

Figure 4. Summary of Quantstudio 5 standard curve parameters for resDNASEQ HEK293 Assay kits

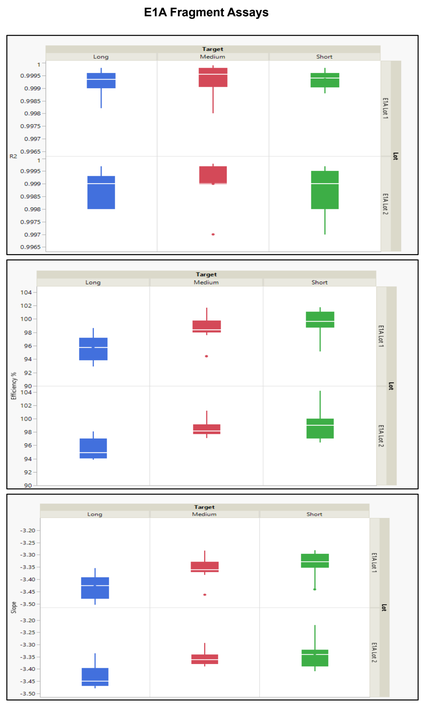

Figure 5. Summary of Quantstudio 5 standard curve parameters for resDNASEQ E1A Fragment Assay kits

Effect of Inhibitors: A robust assay must give consistent and accurate results in the presence of inhibitors in samples. The results of our assay where three different inhibitors typically found in the manufacturing process (benzonase, Tween and LV-MAX) were used as shown in Figure 5. The inhibitors had no effect on PCR detection and sensitivity. The results from Internal Positive Control (FAM channel) in presence of inhibitor E, F and G presented the same Ct detection. This indicates that the assays are robust enough to quantitate samples present complex manufacturing matrices.

resDNASEQ HEK293

PR ID	Metric	Passing Criteria	Plate 11	Plate 12	Plate 13	Plate 14
PR-2.1	The product shall perform in the presence of benzonase	Inhibitor E samples 1-3; average VIC Ct between 27-35	P	P	P	P
PR-2.1	The product shall perform in the presence of excess DNA of other species	Inhibitor F samples 1-2; average VIC Ct between 27-35	P	P	P	P
PR-2.1	The product shall perform in the presence of detergent	Inhibitor H samples 1-3; average VIC Ct between 27-35	P	P	P	P
PR-2.1	The product shall perform in the presence of cell culture media	Inhibitor I sample 1; average VIC Ct between 27-35	P	P	P	P

Inhibitor ID	Formulation
Inhibitor E	DNA Dilution Buffer with 25U/ml Benzonase.
Inhibitor F	DNA Dilution Buffer with Tween20_0.5%
Inhibitor G	DNA Dilution Buffer with 1 X LV-MAX
Inhibitor H	Tween-20 0.1 - 0.5%
Inhibitor I	1x EXPI293 Expression Medium

Standard Curve Point	Formulation
SD1	10^8 copies
SD2	10^6 copies
SD3	10.,000 copies
SD4	1000 copies
SD5	30 copies
SD6	10 copies

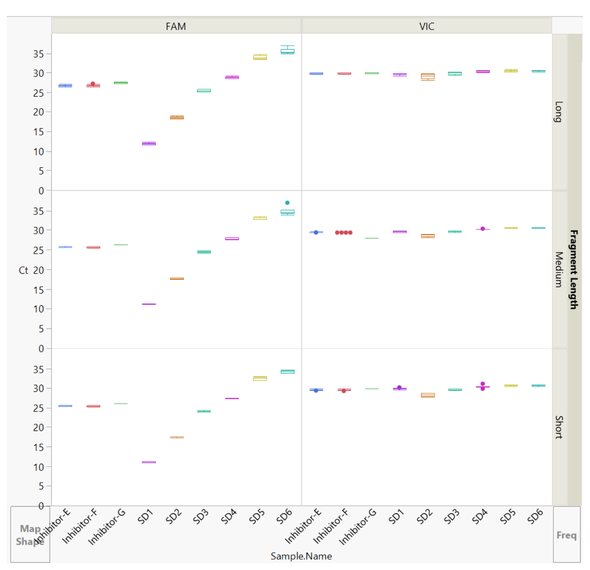

Figure 6. Ct values of Inhibitors E, F and G in both the IPC and HEK293/E1A FAM channels from Quantstudio 5 instrument

Assay Specificity: The resDNAseq HEK293 and E1A Fragment Assay kits demonstrated high specificity (Figures 7 and 8) in the presence of other cross-reactants from other sources (3ng non-specific DNA spikes).

resDNASEQ HEK293

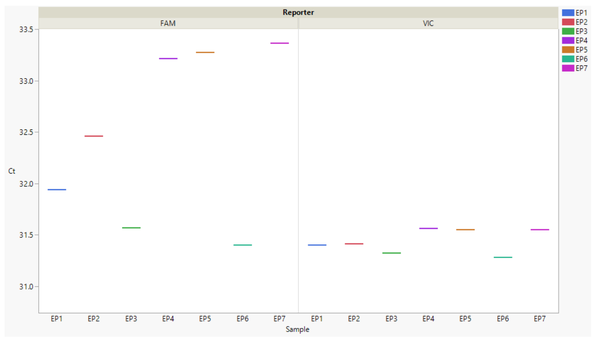

Figure 7: Specificity study showed all cross reactants yield negative result (high Ct) when tested with resDNASEQ HEK293 assay; where Ct > Ct of LOD for HEK293 assay..

Cross Reactant List	
No. of Item	3ng per Reaction
EP1	E.coli
EP2	MulV2
EP3	Bovine
EP4	pAV1
EP5	MDCK
EP6	CHO
EP7	NS0

resDNASEQ E1A Fragment Assays

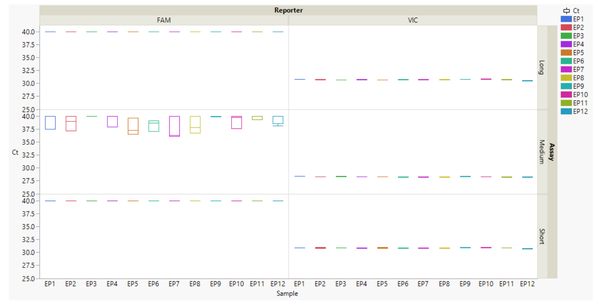

Figure 8: Specificity study showed all cross reactants yield negative result (high Ct) when tested with resDNASEQ E1A Fragment assays; where Ct > Ct of LOQ for E1A assay.

Cross Reactant List	
No. of Item	3ng per Reaction
EP1	Human DNA
EP2	Rabbit
EP3	Bovine
EP4	Chicken
EP5	MDCK
EP6	CHO
EP7	Yeast
EP8	Mouse (BALB/c)
EP9	SF9
EP10	Baculovirus
EP11	E.coli
EP12	Vero

CONCLUSIONS

Robust, highly sensitive and reliable real time PCR assays for quantitation of residual DNA impurities in HEK293 cell lines were developed to support gene therapeutics. The kits can quantitate genomic HEK293 presence as well as different fragment sizes of E1A oncogene to help enable manufacturers of gene therapies and vaccines meet regulatory requirements. Both assays met the following key metrics:

- The limit of quantitation (LOQ) was consistently achieved at 30 copies. The limit of detection (LOD) was 10 copies.
- Robustness was demonstrated by R-squared (R2) values greater than 0.99 and PCR efficiency of 100 ± 10% from the standard curves based on PCR performed using multiple reagent lots, multiple days, and multiple operators. Spike recoveries observed in common gene therapy workflow matrices were within 70-130%, which is tighter than the USP <509> guidance (50-150%).
- The assay also demonstrated no negative effect in presence of inhibitors and showed high specificity in the presence of off-target DNA.

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TRADEMARKS/LICENSING

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