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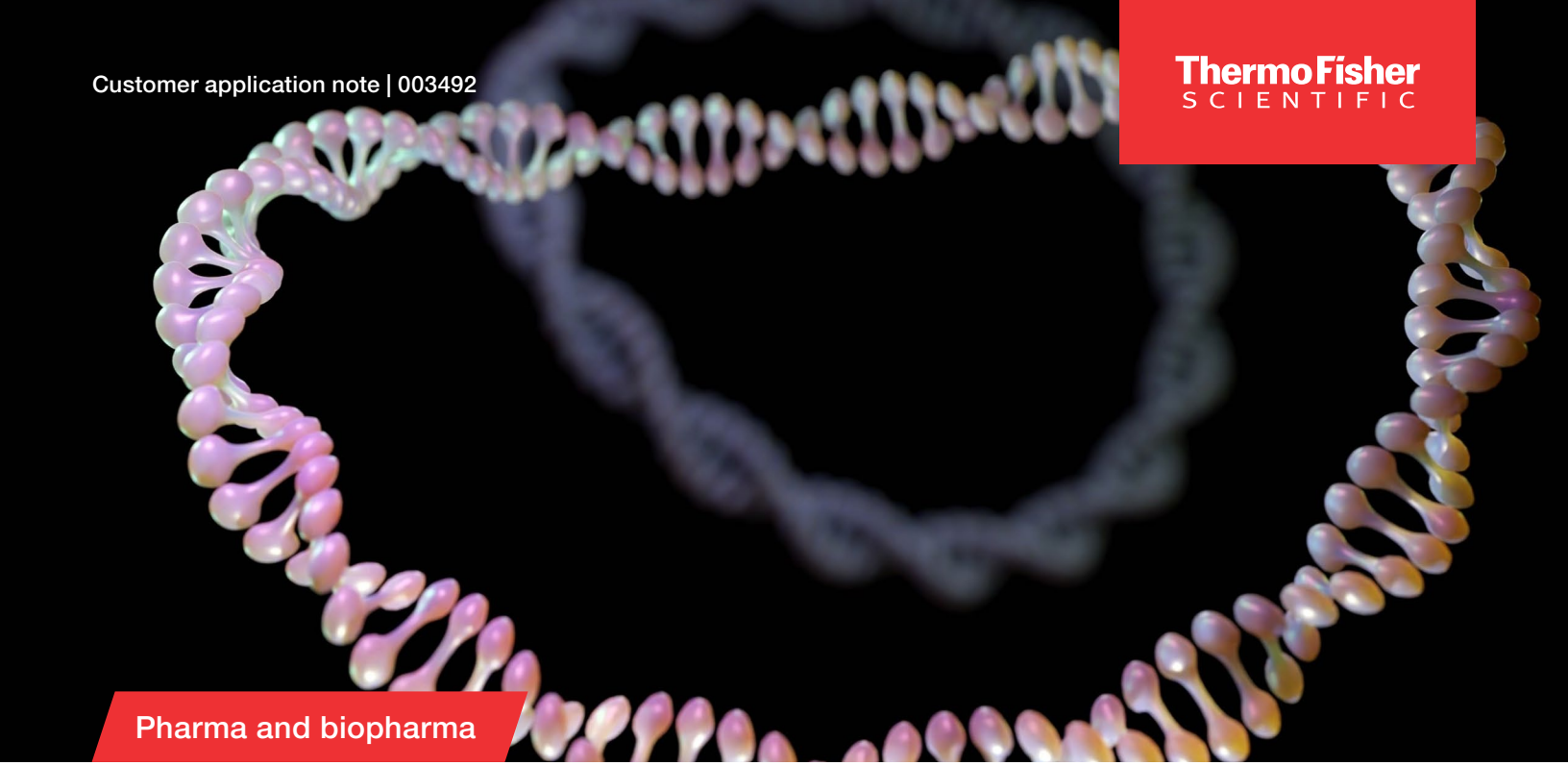

Pharma and biopharma

Quantification of residual hcRNA in plasmid DNA samples using IP-RP-UHPLC

Authors

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Keywords

Residual, hcRNA, quantification, plasmid, vector, DNA, *E. coli*, adeno-associated virus, AAV, impurity

Application benefits

- Quantification of residual hcRNA between 1 and 10 µg/mL
- Short run time for high-throughput analysis

Goal

To develop a method for the quantification of residual host cell RNA (hcRNA) during the manufacture and release of plasmid vectors produced in *E. coli*.

Introduction

Plasmid vectors are an essential component in the manufacturing of cell and gene therapy products such as adeno-associated viruses (AAV). Plasmids are commonly produced using recombinant *E. coli* strains and released through bacterial cell lysis. This can result in the introduction of host cell impurities, such as RNA, which can elicit an undesired immune response.[1] It is therefore essential to monitor the clearance of hcRNA within the production process.

Residual hcRNA levels in plasmid samples for clinical use should be <0.2 µg/mg of plasmid DNA (0.02% RNA) as per USP <1047>.[2] As starting materials in cell and gene therapy manufacturing, release specifications of <1–5% residual hcRNA have been proposed.[3,4] Quantification assays include agarose gel electrophoresis, reverse transcription quantitative polymerase chain reaction (RT-qPCR) and high-performance liquid chromatography (HPLC).

In agarose gel electrophoresis, Invitrogen™ SYBR™ Gold stain selectively binds to double-stranded DNA (dsDNA), single-stranded DNA (ssDNA) as well as RNA, showing the highest affinity for dsDNA. This can result in specificity issues for RNA detection, especially in the presence of plasmid DNA. A minimum of ≈1–2 ng RNA can be detected in the absence of DNA.[5,6] BioPhorum attendees reported a detection limit of 20 ng RNA in 5 µg plasmid DNA (0.4% RNA).[3]

RT-qPCR assays require sequence-specific primers for hcRNA, therefore not all hcRNA present may be detected. Alternatively, using random primers, RNA cannot be amplified selectively, resulting in specificity issues due to plasmid DNA in the sample.

As an alternative to agarose gel electrophoresis and RT-qPCR, HPLC-based assays provide high accuracy, precision, sensitivity, and throughput. In this article, we present an ultra-high performance liquid chromatography (UHPLC)-based assay for the quantification of hcRNA with a range response of 1–10 µg/mL, and quantification limit (QL) of 1 µg/mL, using UV detection. The presented assay was developed on the Thermo Scientific™ Vanquish™ Horizon UHPLC in conjunction with a Design of Experiments approach using a connected solution between Chromeleon CDS and S-Matrix™ Fusion QbD™ software (Figure 1).

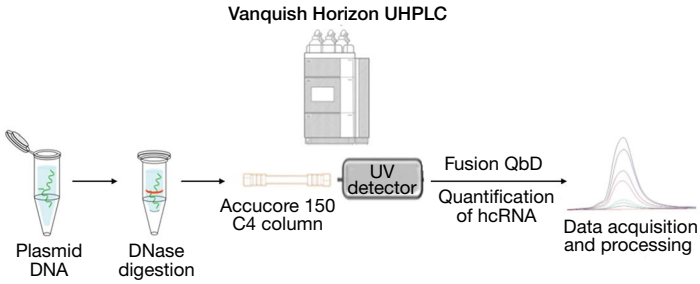

Figure 1. Workflow overview.

Experimental

Invitrogen™ TURBO™ DNase (Cat. No. AM2238), Invitrogen™ RT-PCR grade water (Cat. No. AM9935), and Invitrogen™ *E. coli* Total RNA (Cat. No. AM7940; 1 mg/mL) were used in this work. A Thermo Scientific™ Accucore™ 150-C4 column (2.1 mm × 30 mm, 2.6 µm, Cat. No. 16526-032130) provided separation. 1 M Triethylammonium acetate (TEAA) solution (HPLC grade) and acetonitrile (LC/MS grade) were obtained from reputable sources.

A 7-point calibration curve (0.5–10 µg/mL RNA) and control standard were prepared by combining master mix (10x TURBO DNase buffer and TURBO DNase) with a stock of each standard containing plasmid DNA. All samples, standards, and controls were incubated according to the manufacturers' instructions.

Using Fusion QbD software, a 3-level response surface Design of Experiments was created [flow rate (0.25, 0.50, 0.75 mL/min), final % organic (50, 62.5, 85), column temperature (20, 40, 60 °C)]. Each parameter combination was analyzed for peak height and USP signal to noise (S/N). Twenty parameter combinations were assessed, including replicates of reference/center points, using two different columns (total of 40 runs). Additionally, the Robustness Simulator™ of Fusion QbD was utilized to predict method performance. The method operable design region was based on the obtained models.

Instrumentation

Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of:

- System Base Horizon/Flex (Cat. No. VF-S01-A)
- Vanquish Binary Pump H (Cat. No. VH-P10-A-02)
- Split sampler FT (Cat. No. VF-A10-A)
- Column compartment H (Cat. No. VH-C10-A)
- Variable wavelength detector F (Cat. No. VF-D40-A) with 11 µL standard bio flow cell (Cat. No. 6077.0200)

The instrument and final method details are listed in Table 1.

Table 1. Instrument and method details.

Wavelength	260 nm
Column	Accucore 150-C4, 2.1 x 30 mm, 2.6 µm, (Cat. No. 16526-032130)
Mobile phase A	100 mM TEAA
Mobile phase B	100 mM TEAA in 50% acetonitrile (aq)
Flow rate	0.5 mL/min
Injection volume	5 µL
Column temperature	60°C
Autosampler temperature	5°C
Gradient	Time (min) % B 0.00 15.0 2.00 15.0 3.50 81.5 5.00 81.5 5.75 100.0 6.75 100.0 6.85 15.0 9.00 15.0

Software

- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software, version 7.3.1
- Fusion QbD software, version 9.9.2

Results and discussion

The Fusion QbD Robustness Simulator with a target process capability (Cpk) ≥ 1.33 (6 sigma) showed high robustness of the model. The model provided by Fusion QbD was then used to determine the optimum method by Best Answer Search and was assessed for range response (Figures 2 and 3).

The method demonstrated good range response, as shown in Figure 3 (R^2 > 0.99). The control standard showed good accuracy and precision, with an RSD of 3.5% and recovery of 108%. As shown in Figure 2, despite the heterogenous nature of the analyte, the method allowed for the elution of all RNA species as a single peak. Less abundant RNA sizes were observed as a shoulder at higher concentrations of the standard.

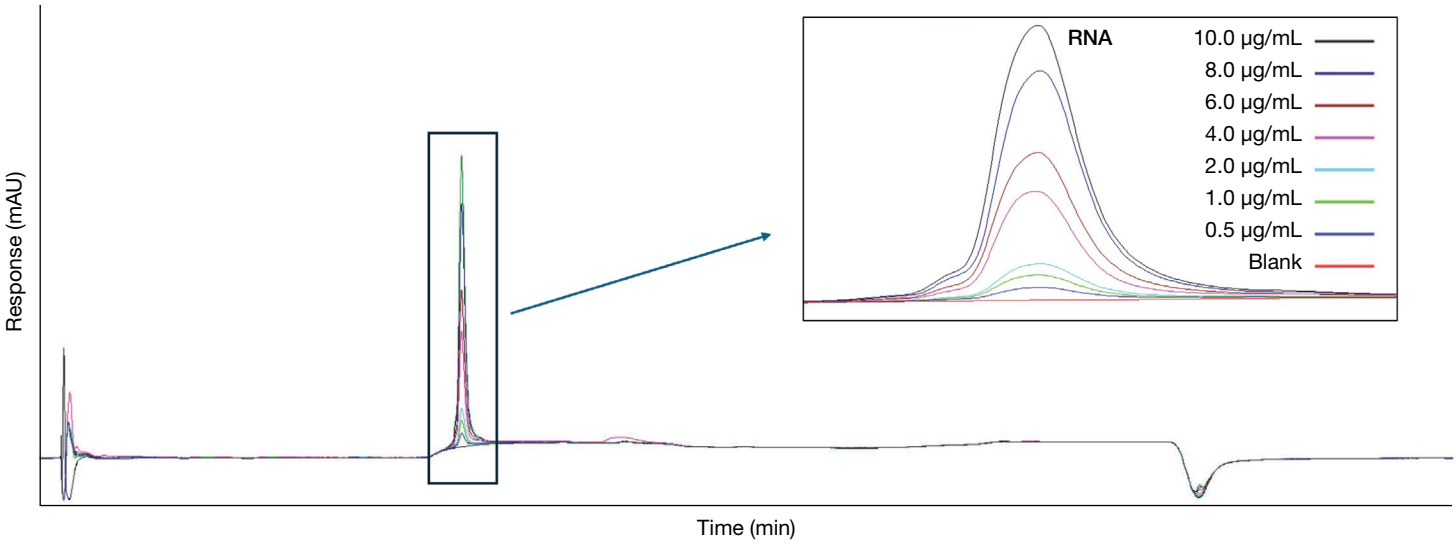

Figure 2. Overlaid chromatograms of the RNA calibration curve (see Table 1 for method details).

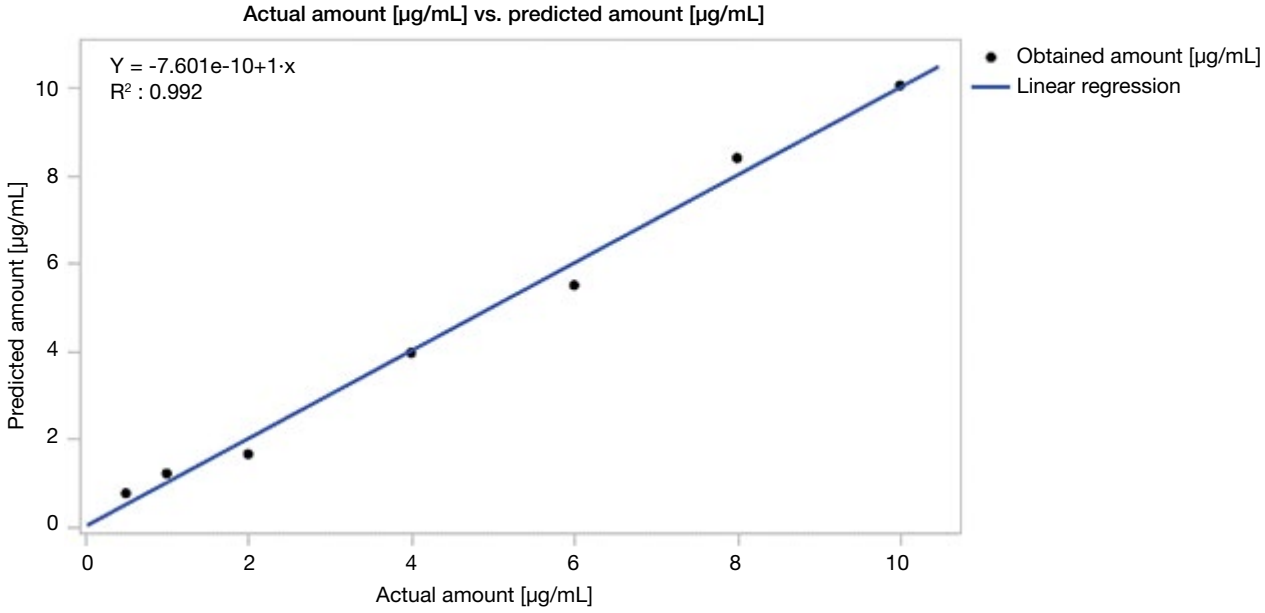

Figure 3. Actual vs. predicted RNA concentration.

Despite the lowest standard (0.5 µg/mL) having a USP S/N of 51, the accuracy was outside the acceptance criteria. Therefore, the QL was increased to 1.0 µg/mL, for a final 6-point calibration curve (1–10 µg/mL). To demonstrate the specificity of the assay, plasmid DNA samples were spiked to 1 µg/mL RNA and treated with TURBO DNase. As shown in Figure 4, the mean RNA concentration of triplicate preparations was 1.11 µg/mL, indicating selective digestion of plasmid DNA in the presence of RNA.

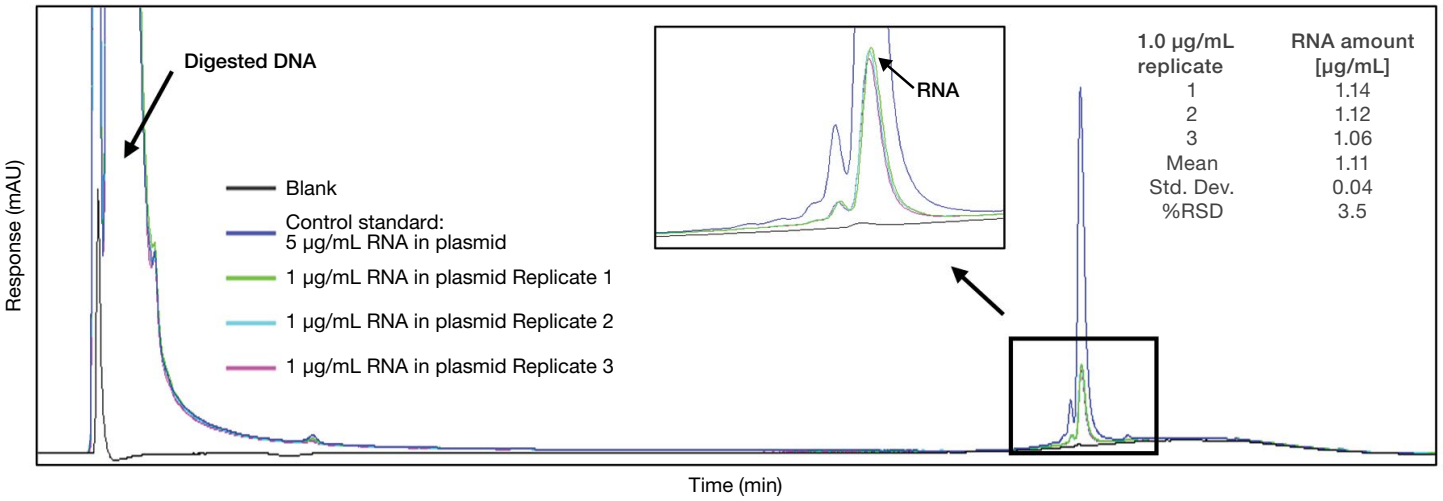

Figure 4. Selective plasmid DNA digestion in the presence of RNA with TURBO DNase.

Conclusion

The aim of this application was to develop a method for the quantification of residual hcRNA, for the purpose of in-process and release testing of plasmids manufactured in *E. coli.* The analytical target profile outlined the following requirements:

- Detection limit: ≤1 µg/mL
- Quantification limit: 1.0–5.0 µg/mL (1–5% RNA per mg DNA)
- Range response: 1–10 µg/mL
- Specific for RNA

Using Fusion QbD, a robust and sensitive method for the quantification of residual hcRNA was successfully developed, which met the analytical target profile. The method demonstrated high precision with a quantification limit of 1 µg/mL and range response of 1–10 µg/mL. Selective digestion of DNA with TURBO DNase allowed for the quantification of hcRNA in preparations containing high levels of plasmid DNA. Furthermore, a run time of 9 minutes was achieved, enabling high-throughput analysis.

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