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RESEARCH ARTICLE

Overcoming Biopharmaceutical Interferents for Quantitation of Host Cell DNA Using an Automated, High-Throughput Methodology

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Abstract

The rapid development of biologics and vaccines in response to the current pandemic has highlighted the need for robust platform assays to characterize diverse biopharmaceuticals. A critical aspect of biopharmaceutical development is achieving a highly pure product, especially with respect to residual host cell material. Specifically, two important host cell impurities of focus within biopharmaceuticals are residual DNA and protein. In this work, a novel high-throughput host cell DNA quantitation assay was developed for rapid screening of complex vaccine drug substance samples. The developed assay utilizes the commercially available, fluorescent-sensitive Picogreen dye within a 96-well plate configuration to allow for a cost effective and rapid analysis. The assay was applied to in-process biopharmaceutical samples with known interferences to the dye, including RNA and protein. An enzymatic digestion pre-treatment was found to overcome these interferences and thus allow this method to be applied to wide-ranging, diverse analyses. In addition, the use of deoxycholate in the digestion treatment allowed for disruption of interactions in a given sample matrix in order to more accurately and selectively quantitate DNA. Critical analytical figures of merit for assay performance, such as precision and spike recovery, were evaluated and successfully demonstrated. This new analytical method can thus be successfully applied to both upstream and downstream process analysis for biologics and vaccines using an innovative and automated high-throughput approach.

Keywords High-throughput experimentation · Fluorescence · Vaccine development · Biological development · Process research and development · Automated assay development · DNA quantification · Protein interference · RNA interference · Enzymatic digestion

Introduction

Recent world crises, including the Ebola outbreak and COVID-19 pandemic, have brought to light the demand for accelerated timelines for the development of biologics and vaccines (1–4). In turn, analytical methodologies need to increase their throughput to meet these rapid timelines without compromising data integrity. To accomplish this feat, the development of platform assays that can be applied in a modality agnostic manner is highly beneficial for the robust assessment of both biologics and vaccines.

The manufacture of biopharmaceuticals, including both biologics and vaccines, often requires the use of a cell substrate to efficiently produce the desired drug substance (5–7). The resulting drug substance must in turn be carefully scrutinized for the presence of process related impurities such as residual host cell protein and deoxyribonucleic acid (DNA) (8–10) to maintain the quality, safety, and efficacy of the product. In particular, residual host cell DNA is defined as the sum of DNA and fragments present in biological samples derived from recombinant host cells during expression (11). The World Health Organization and U.S. Food and Drug Administration have strict guidelines as to the accepy limits of both the concentration of host cell DNA (10 ng/dose) and the size of the residual DNA (200 base pairs) in the final drug product (12, 13).

A variety of methodologies exist that can be applied for precise host cell DNA quantification. The most widely used technology is quantitative polymerase chain reaction (qPCR) (14–16). This approach uses highly specific DNA primers to detect the host cell DNA (17). For this reason, a number of

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qPCR assays have been commercialized for popular cell substrates, such as *Escherichia coli*, *Pichia pastoris*, Vero, and Chinese Hamster Ovary cells. Highly specific primers allow for minimal interference; however, depending on the commercial assay being utilized, varying coverages may exist. Additionally, DNA extraction may be needed in complex matrices, adding to the time and complexity of the assay. While qPCR allows for a highly specific method, the early development of biopharmaceuticals would benefit from a platform method to be universal to all cell substrates, measure smaller DNA fragments, and rapidly assess samples.

In addition to qPCR, fluorescence-based probe methods are frequently used to quantify host cell DNA. These methods rely on a fluorogenic dye, like SYBR Green (18) or Picogreen (19), that has specific interactions with the DNA. Fluorescence-based methods are highly advantageous, as their application can be generally applied across all biopharmaceuticals, have lower associated costs than qPCR, and are easily transferrable (20). However, a variety of common pharmaceutically relevant process impurities and formulation components, such as protein, RNA, and detergents (21, 22), can interfere with DNA quantitation using these fluorescent dyes. Therefore, to enable a robust fluorescence-based method, a thorough evaluation of the potential interference effects and their subsequent removal needs to be performed.

In this work, the commercially available, fluorescent sensitive Picogreen dye was selected as the basis for a quantitative, high-throughput host cell DNA assay. Picogreen is able to bind to double-stranded DNA through specific charge interactions (19). Picogreen has been applied in several formats to successfully quantify host cell DNA (21, 23, 24). However, these studies fail to overcome the common, pharmaceutically relevant interferences associated with nonspecific binding to the reagent, including protein, RNA, and detergents (21, 22). This renders the current methodologies incompatible with many prominent vaccines such as mRNA vaccines and others utilizing RNA-based viruses as they contain high levels of RNA (2, 3, 25). Here, we assess the application of our proposed fluorescence-based assay to measure residual DNA in complex live-virus vaccine (LVV) drug substance. This enveloped RNA virus contained significant concentrations of RNA encapsulated in a protein and lipid shell as well as process related impurities including extracellular vesicles and large aggregates of host cell DNA and protein. Systems of a similar nature have been utilized in the development of vaccines targeting HIV, Chikungunya, West Nile Virus, and many other prominent pathogens (26–28). Interactions of the process related impurities in the drug substance were shown to have shielding effects, preventing access to the nucleotides within the drug substance. We herein demonstrate that these shielding-based interference effects can be overcome via addition of the detergent deoxycholate (DOC). Additionally, DOC has a known virucidal effect on various viruses such as influenza, HIV-1 viruses, and Rauscher leukemia by causing partial or complete disruption of the virion lipid membrane (29–31). This is a critical function of DOC to disrupt the viral components as well. DOC addition was further optimized to overcome this interference most effectively. Next, we demonstrate successful removal of RNA and protein interferences by enzymatic digestion with RNase A followed by Proteinase K addition. Finally, detailed evaluation and subsequent optimization of interferences of our proposed assay, resulting from addition of these reagents, was accomplished. To the best of our knowledge, this is the *first* report of a high-throughput, fluorescence-based analytical method utilizing the commercial Picogreen dye to quantitate host cell DNA in the presence of significant interferents commonly encountered within biopharmaceuticals, including RNA, protein, and detergents. Our proposed analytical method can thus be broadly applied to complex biopharmaceuticals with high specificity, precision, and accuracy for host cell DNA quantitation.

Materials and Methods

Reagents and Materials Components of the Quant-iT™ Picogreen™ dsDNA Assay Kit, including the Picogreen reagent and 100 µg/mL λDNA standard stock (Thermo Fisher Scientific), were utilized. A calf thymus DNA (Sigma-Aldrich) standard and ribosomal RNA standard — 16S and 23S rRNA from *E. coli* (Thermo Fisher Scientific) — were diluted in phosphate-buffered saline (PBS) (Cytiva). Buffering components such as PS-80 (Thermo Fisher Scientific), Proteinase K (Thermo Fisher Scientific), Tris–EDTA 100× buffer (Sigma-Aldrich), and 5 M sodium chloride solution (Invitrogen) were also utilized. The process-related samples of a live-virus vaccine drug substance were obtained from our Vaccine Process Development colleagues.

Consumable materials equipped on the Freedom EVO 200 Liquid Handling system (Tecan) include the following: 180 µL MCA 96 filtered, sterile tips (Corning-Axygen), 300 mL Nalgene Flat Bottom Reservoir (Thermo Fisher Scientific), Costar 96-well 2 mL polypropylene assay block (Thermo Fisher Scientific), 2 mL cryogenic vials (Nalgene), and Costar opaque 96-well microplates (Thermo Fisher Scientific). Additionally, a reusable 1000 mL Reagent Trough (Tecan) was equipped on the deck.

DNA Quantitation Assay on an Automated Liquid Handler Our proposed analytical method is based on the commercially available Picogreen Assay. In short, this is a fluorogenic technique that results in increased fluorescence of the Picogreen dye upon binding to DNA. A Freedom EVO 200 Liquid Handling system (Tecan) was utilized to automate our proposed analytical method and applied throughout

all experiments described in this work. The liquid handling system is equipped with an 8-channel pipetting arm, a multichannel arm (96 channels), and a microtiter-plate gripping arm. The system also has an integrated Infinite F500 microplate reader (Tecan). Microcide SQ (Hamilton) (3.05%) and 1 N NaOH (Fisher) were used as solvents to clean the fixed tips on the 8-channel arm in between sample handling steps. Samples are prepared in a Costar 96-well 2-mL deep well polypropylene plate (Fisher Scientific). The following steps were performed by the Tecan workstation. First, a standard curve was prepared in duplicate from a 100-µg/mL λDNA standard stock (Thermo Fisher Scientific) by dilution with a diluent consisting of 10 mM Tris, 1 mM EDTA, 100 mM NaCl, 200 µg/mL Proteinase K, and 0.01% Polysorbate-80, pH 8.0. Concentrations of 1, 2.5, 5, 10, 25, 50, 100, 150, 250, 400, and 500 ng/mL λDNA were prepared. Drug substance samples were diluted in 10 mM Tris, 1 mM EDTA, 100 mM NaCl, 200 µg/mL Proteinase K, and 0.01% Polysorbate-80, pH 8.0. Multiple sample dilutions were prepared to target the quantitative range of the assay, and drug substance samples were analyzed in triplicate. Standards and samples were both incubated for 10 min. A minimum dilution of 1:1 with 10 mM Tris, 1 mM EDTA, 100 mM NaCl, 200 µg/mL Proteinase K, and 0.01% Polysorbate-80, pH 8.0, was necessary to ensure proper digestion of residual protein within the samples via Proteinase K. Next, 100 µL of each prepared standard and sample was transferred to a Costar opaque 96-well plate (Fisher Scientific). An SBS reservoir containing Picogreen reagent (Thermo Fisher Scientific) diluted 1:200 in 10 mM Tris and 1 mM EDTA was placed on the automated liquid sample handler. To each standard and sample, 100 µL of the pre-diluted Picogreen reagent was added using the multichannel arm. The plate is then transferred using the robotic gripper arm to the Infinite F500 plate reader (Tecan), and the fluorescence of each well is measured at an excitation of 485 nm and emission of 535 nm. Ten exposures were performed per well and the gain is optimized based on the well with the highest intensity. The standard curve was generated by log–log linear regression of the mean of the given replicates. The DNA concentration of each sample was then interpolated from this standard curve.

Method to Overcome Interference Effects via Enzymatic Pre-treatment of Drug Substance Pre-treatment buffers were prepared containing 25 U of Benzonase (Millipore Sigma) and/or 0.4% DOC (Sigma-Aldrich) in PBS (Cytiva). Benzonase was prepared in a solution of PBS containing 100 mM $MgCl_2$ (Sigma-Aldrich), as Mg^{2+} is a necessary co-factor for Benzonase activity (32). The pretreatment of the drug substance was performed using the automated Freedom EVO 200 Liquid Handling system (Tecan). The drug substance was then diluted 1:1 into the pre-treatment buffer within a 96-well 2 mL deep well plate. For untreated samples, the drug substance was diluted 1:1 in PBS within the 96-well 2 mL deep well plate. The samples were incubated at room temperature for 2 h. Samples were subsequently diluted 1:1 with 10 mM Tris, 1 mM EDTA, 100 mM NaCl, 200 µg/mL Proteinase K, and 0.01% Polysorbate-80, pH 8.0. The 96-well plate containing both Benzonase-treated and untreated samples was then transferred to the Liquid Handler (Tecan) and the automated, high-throughput Picogreen Assay proceeded as described above. As needed, further dilution of the samples into the linear range of the assay was performed. Measurements of each sample were taken in triplicate, with the mean result reported.

Pre-treatment buffers were prepared containing 5 µg/mL RNase A (Invitrogen) and/or DOC (Sigma-Aldrich) at varied concentrations (0.0125 to 0.4%) in PBS (Cytiva). The drug substance was then diluted 1:1 into the pre-treatment buffer or diluted 1:1 in PBS for untreated samples in a 96-well 2-mL deep well plate. The samples were incubated at room temperature for 2 h before being diluted 1:1 with 10 mM Tris, 1 mM EDTA, 100 mM NaCl, 200 µg/mL Proteinase K, and 0.01% Polysorbate-80, pH 8.0. The 96-well plate was then transferred to the Liquid Handler (Tecan), and the automated, high-throughout Picogreen Assay proceeded as described above.

Results and Discussions

Fully Automated, High-Throughput Picogreen DNA Quantitation Assay A fully automated Picogreen-based assay has been developed using the Tecan Evo Liquid Handling system to allow for high throughput preparation and analysis of samples. In brief, an 11-point standard curve is generated in duplicate through serial dilution of a controlled DNA reference material from 1 to 500 ng/mL. Unknown samples are diluted into this linear range, in which multiple dilutions can be prepared of the same sample to ensure accuracy and precision of the resulting host cell DNA quantitation. After the designated dilution, the samples and standards are incubated with the assay diluent for 10 min to allow the Proteinase K, which is present in the diluent, to digest residual protein and remove this interferent (21). The samples and standards are transferred to an opaque, black 96-well plate suitable for fluorescence measurements. The Quant-iT™ Picogreen® double stranded DNA reagent is added and mixed with both the samples and references through a controlled aspiration and dispense process. The plate is transferred to a plate reader directly connected to the liquid handler. For analysis, the plate is exposed to 485 nm excitation, and the fluorescence emission is measured at 535 nm.

A standard curve is generated for each independent analysis of our proposed platform Picogreen-based assay (Fig. 1). Specifically, duplicate measurements of the given standards are averaged, and the log of the mean result is reported as the *y*-axis of the calibration curve. The *x*-axis is the log of the prepared DNA concentration of the standards, ranging from 1 to 500 ng/mL DNA. The lower limit of the reference curve is set by the limit of quantification. The acceptance criteria set by the FDA and WHO guidance is to have less than 10 ng/dose, making the 1 ng/mL detection limit reasonable. The upper limit of the reference curve was determined based upon the expected range of hcDNA concentrations observed through process development. Concentrations of hcDNA are not expected to exceed 5000 ng/mL, and with the ability of the automated system to dilute samples in the linear range, the upper limit of 500 ng/mL lends itself to realistic dilutions. The resulting calibration curve (Fig. 1) demonstrates extremely high linearity, in which an R2 value of 0.99 is observed. Furthermore, the dynamic range of this method is observed to be high, in which the 11 total standards analyzed across 2.5 orders of magnitude of DNA concentration all shows high linearity. Given this method is able to assess both low and high concentrations of DNA (i.e., 1 to 500 ng/mL DNA), the analysis of diverse biopharmaceuticals with wide-spanning DNA concentrations can be accomplished in a broad, straightforward manner. In order to be applicable as a platform methodology, the large range in the calibration curve and ability to prepare multiple dilutions through an automated approach is critical to the feasibility to implement appropriately.

Additional analytical figures of merit for analysis of DNA reference materials were elucidated (Table I). In brief, the percent relative standard deviation (%RSD) confirms the high immediate precision of the automated assay, in which values range from 0.6 to 6.3%. The average %RSD across all concentration ranges analyzed is 2.6%, further highlighting the precision of the method. In addition, the percent error observed when correlating the expected DNA concentration of a given standard to that of the measured DNA concentration was also very low, with values ranging from 0.2 to 7%. Moreover, the average error across all concentration ranges analyzed is 3.9%, highlighting the low error observed for this method.

Table I Results of Quantitation of DNA Reference Standards for Evaluation of Analytical Method. Expected DNA Concentration (ng/mL) Determined by Preparation of Reference Standard at Varying Concentrations. Measured DNA Concentration (mg/mL) Is the Mean Value of Duplicate Measurements. Statistical Analysis of Measured vs. Expected DNA Concentrations Are Provided as Relative Standard Deviation (RSD, %) and Error (%)

Expected DNA concentration (ng/mL)	Measured DNA concentration (ng/mL)	RSD (%)	Error (%)
1.0×10^{0}	1.0×10^{0}	1.4	0.2
2.5×10^{0}	2.4×10^{0}	1.2	3.2
5.0×10^{0}	5.2×10^{0}	6.3	4.8
1.0×10^{1}	9.6×10^{0}	0.6	4.3
2.5×10^{1}	2.3×10^{1}	4.0	4.8
5.0×10^{1}	5.2×10^{1}	2.6	4.3
1.0×10^{2}	1.1×10^{2}	2.4	7.3
1.5×10^{2}	1.6×10^{2}	5.4	4.2
2.5×10^{2}	2.5×10^{2}	0.9	0.9
4.0×10^{2}	3.9×10^{2}	2.3	2.1
5.0×10^{2}	4.7×10^{2}	1.0	6.3

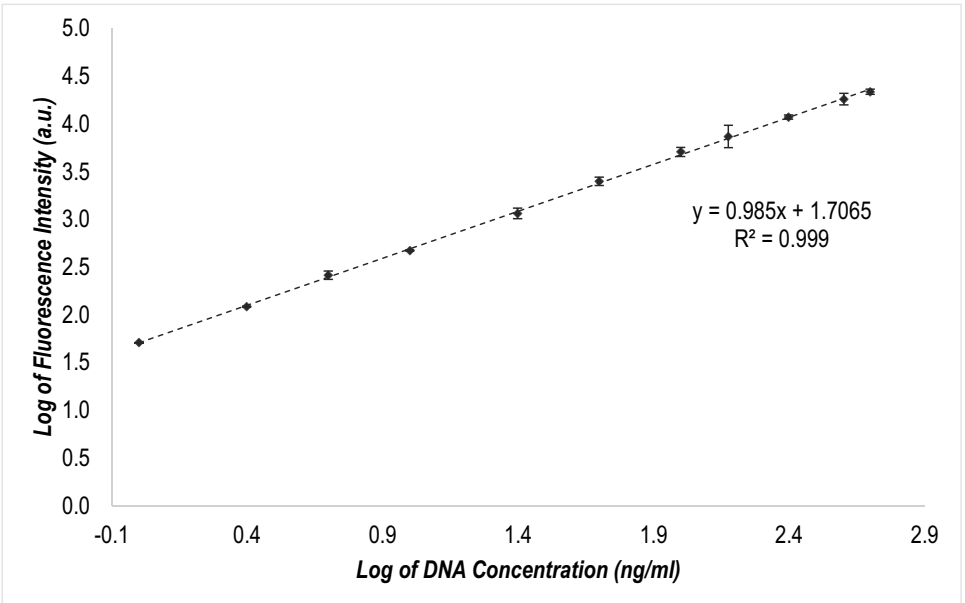

Fig. 1 Results from analysis of DNA reference material using the fluorescence-based analytical method. DNA standards were analyzed at concentrations of 1, 2.5, 5, 10, 25, 50, 100, 150, 250, 400, and 500 ng/mL in duplicate. The resulting standard curve displays the log of the average fluorescence signal of duplicate measurements of all prepared DNA reference materials

Using this automated, high-throughput platform assay, drug substance samples can undergo multiple dilutions along the given linear range, a key aspect for reliable quantitation. Moreover, using this fully automated platform, it is possible to quantify DNA in 384 samples in less than 4 h. With the basic analytical figures of merit now established for our proposed method, a detailed evaluation can now be performed to determine how process-related impurities in biopharmaceuticals may interfere with the assay and subsequent strategies to overcome these interferences.

Matrix Interactions Shield Host Cell DNA and RNA from Digestion Several common, pharmaceutically relevant species are known to interfere with Picogreen-based DNA quantitation, including protein, RNA, and detergents (21, 22). In order to have a platform technology applicable to a wide range of biopharmaceuticals, a robust method for mitigating these interferences needed to be developed. As described here, Proteinase K is incorporated for digestion of protein, and thus, protein interference effects are overcome and accounted for. This work now focuses on understanding RNA and detergent interferences.

A complex LVV drug substance containing an enveloped RNA genome was selected for evaluation using the Picogreen DNA quantitation assay to assess methods for overcoming interferences. The sample had known complexities, such as high residual protein and genomic RNA. Due to the intricacies of the LVV drug substance, there was a high probability of interactions between components of the sample (i.e., host cell proteins, vesicles, process impurities) with the host cell DNA and genomic RNA. Such interactions with DNA could lead to a shielding effect — a prevention of the dye reaching the DNA (33) — that would result in inaccurate quantitation, as the Picogreen dye would be unable to interact appropriately with the host cell DNA (19). Additionally, sample matrix interactions with RNA may make it more difficult to remove this interference (34).

To investigate the potential shielding effects of the sample matrix, an evaluation was first done to determine the accessibility of the host cell DNA and genomic RNA by digestion via Benzonase (35, 36). Benzonase is an endonuclease that is commonly used in purification processes to remove residual DNA and RNA by digestion into small nucleotides (37–39). As the Picogreen Assay signal is a result of the presence of DNA and RNA interference, the addition of Benzonase should result in a very large decrease in the observed DNA concentration. If a large decrease in signal is not observed, this would be indicative that Benzonase activity is being decreased. Our hypothesis is that if signal does not largely decrease, it is because Benzonase is unable to access the DNA and RNA. Interactions the nucleic acids have with matrix interferants could be inadvertently blocking it from interacting with Benzonase. Benzonase has very robust activity and is widely used in the pharmaceutical industry; activity would not be compromised by the pH, ionic strength, or temperature of this system (32).

Analysis of the drug substance was performed in triplicate using this Picogreen-based assay. The resulting DNA concentration was 2720 ± 82 ng/mL (Fig. 2). A pre-treatment with 25 U of Benzonase was applied to the same sample, and the resulting concentration was 1460 ± 97 ng/mL, a decrease of only 48%. The decrease in concentration was substantially less than expected, indicating that the DNA and RNA

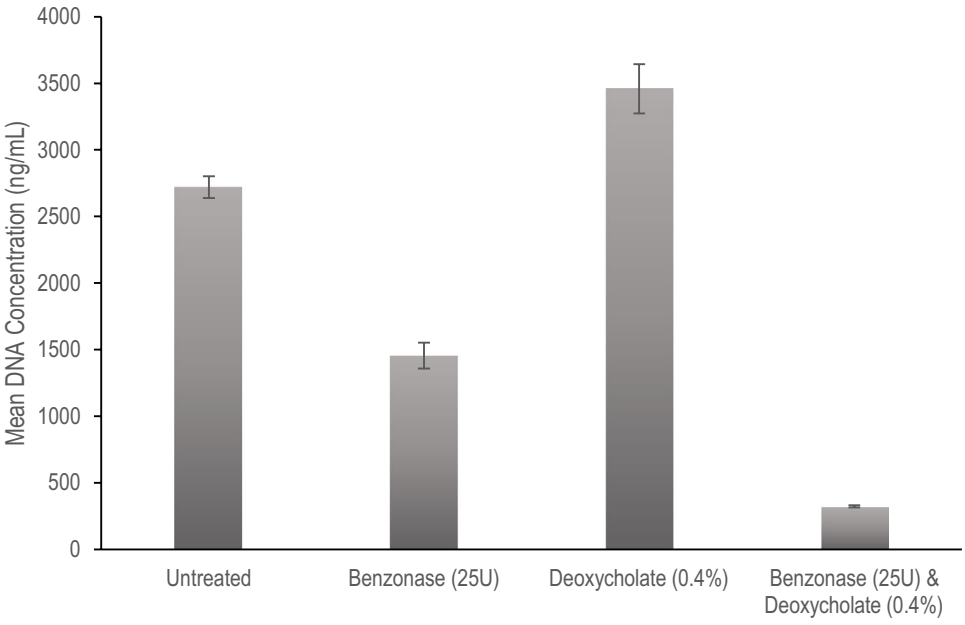

Fig. 2 Benzonase treatments reveal inaccessibility of DNA/RNA in matrix. The LVV drug substance was treated with 25U of Benzonase which resulted in only a 48% decrease in apparent DNA concentration. In order to disrupt the sample matrix interactions and viral components, a pre-treatment with 0.4% deoxycholate (DOC) was applied. In pre-treatments containing 0.4% DOC, Benzonase digestion now led to a 90% decrease in DNA and RNA content. Reported values are the mean of triplicate measures and the standard deviation is represented by error bars

in the system were shielded from digestion by Benzonase. To disrupt potential interactions, overcome shielding, and allow the enveloped nucleic acids to be more susceptible to digestion, DOC was selected as a pre-treatment for the drug substance because of its ability to break up interactions and viral envelopes (31). DOC has demonstrated success in disrupting lipids, improving the accessibility of hydrophobic proteins to digestion, as well as increasing the activity of the protease trypsin (40, 41). Applying a pre-treatment buffer containing DOC therefore could disrupt shielding interactions and increase the activity of Benzonase and Proteinase K in the diluent as well.

The addition of 0.4% DOC to the drug substance was evaluated in the Picogreen-based method. The resulting apparent DNA concentration was 3460 ± 185 ng/mL (Fig. 2). An increase of the apparent DNA concentration was observed, indicating that a portion of the nucleotides was previously being shielded from interacting with the Picogreen reagent. The drug substance was then pre-treated with a solution containing 25 U of Benzonase and 0.4% DOC, and the resulting apparent DNA concentration was 323 ± 9 ng/mL. This 90% decrease indicates that DOC was largely successful in breaking up matrix interactions and viral components to make the DNA and RNA more accessible and increasing the Benzonase activity. The positive shift of the DOC is also reflected in this measurement, as the resulting measurement is non-zero, and as such, a matrix matching of all reagents can further assist in platform, robust analysis. These results confirm that the biopharmaceutical drug substance benefits from the presence of a surfactant, such as DOC, to break up matrix interactions and fully expose DNA and process impurities.

RNase A Selectively Removes RNA Interferences The addition of deoxycholate to the LVV drug substance allowed for full accessibility of the host cell DNA and genomic RNA in the sample matrix for subsequent Benzonase digestion. To quantify solely the host cell DNA, next RNA needed to be selectively removed (21, 22), and given the success of the Benzonase treatments, an enzymatic digestion was performed. Enzymatic pre-treatments have the benefit of high specificity to their substrate as well as low costs (42). Additionally, all enzymes added as a pre-treatment will eventually themselves be digested by Proteinase K in the designed platform assay, removing any potential interference they may cause. RNase A was selected as the most specific means to digest RNA in the sample (43).

Several controls were evaluated to ensure RNA could be removed as an interferant and allow for selective quantitation of DNA in the Picogreen-based assay (Table II). A control of calf thymus DNA was quantified using the Picogreen-based assay, and the resulting mean DNA concentration of triplicate measurements was 89 ± 4 ng/mL. A control of 500 ng of RNA resulted in a measurement of 459 ± 26 ng/mL, and the large positive shift was noted, confirming RNA is a strong interferant. The linearity of the RNA response was not explored, as the intention was to only determine the signal interference and not quantify RNA in this assay. The DNA control was then treated with 5 µg/mL of RNase A. The resulting DNA quantification was 88 ± 3 ng/mL, a recovery of 99% of the control DNA. As we observed full recovery of the control DNA, this serves as confirmation that RNase A at this concentration will not non-specifically digest DNA, a challenge that has been noted in the literature at high concentrations of RNase A (44). In addition, the negligible differences between the DNA control with and without the presence of RNase A further confirm RNase A is not itself causing any interference, as the Proteinase K in the assay diluent is able to eliminate all potential protein interference. When evaluating 500 ng of RNA in the

Table II RNase A Selectively Degrades RNA and Is Unaffected by DOC. A Control Study Was Evaluated to Determine the Specificity of the Picogreen-Based DNA Quantitation Assay After Pre-treatment with RNase A. A Control of DNA Was Evaluated with and without the Presence of RNA. RNA Led to a Large Interference That Was Removed in Conditions with RNase A. RNase A Remained Effective in the Presence of DOC. Reported Values Are the Mean of Triplicate Values Unless Otherwise Indicated

Sample description	Mean DNA concentration (ng/ml)	Standard deviation (ng/mL)	Recovery of DNA (%)
DNA control	89	4	100
500 ng RNA	459	26	-
0.4% DOC	23	N/A*	-
DNA; 5 µg/mL RNase A	88	3	99
500 ng RNA; 5 µg/mL RNase A	3.8	N/A*	-
DNA; 500 ng RNA; 5 µg/mL RNase A	97	6	109
DNA; 500 ng RNA; 0.4% DOC	516	16	580
DNA; 500 ng RNA; 5 µg/mL RNase A; 0.4% DOC	101	4	113

*Further dilutions resulted in quantitation below LOD; one replicate performed

presence of RNase A, robust digestion of the RNA is noted, with signals nearing the limit of quantitation. A baseline of the total interferences proposed to arise in the system was evaluated by measuring the DNA concentration of the control DNA, 500 ng RNA, and 0.4% DOC. The resulting DNA quantification was determined to be 516 ± 16 ng/mL. The percent recovery of the control DNA was 580%, indicating the severity of the interferences from RNA and DOC. Next, a control containing DNA and RNA with an RNase A pre-treatment was evaluated. The resulting DNA concentration was determined to be 97 ± 6 ng/mL, a percent recover of 109%, suggesting that the RNA interference was greatly diminished, but the RNA was not fully digested. Lastly, the ability for RNase A to function in the presence of DOC was evaluated, as DOC is a critical component of our proposed method's pre-treatment process for complex biological samples. The control confirms that RNase A is still active in the presence of DOC as the resulting DNA concentration was determined to be 101 ± 4 ng/mL DNA, a recovery of 113% of the control DNA.

With established controls in place, a sample of the same LVV drug substance used in the previous studies described herein (Fig. 2) was applied again to the Picogreen-based platform assay. The resulting DNA concentration, reported as the mean of three replicates, was 2650 ± 56 ng/mL (Table III). The resulting DNA quantification confirms the high precision of the assay with a %RSD of 2% across the two replicate runs. The sample was again subjected to pre-treatment with 0.4% DOC, resulting in a DNA concentration of 3130 ± 185 ng/mL. The increase of 18% in DNA concentration again suggesting that the host cell DNA is now fully exposed. An increase of this magnitude is well above the observed variability of the assay and is thus considered significant. A concentration of 5 µg/mL of RNase A was incorporated into a pre-treatment with the drug substance, and a significant decrease in DNA concentration was noted to a value of 848 ± 49 ng/mL. This finding suggests that RNA was indeed causing interference and RNase A is successfully able to overcome this interference. Finally, the sample was pre-treated with a mixture of 0.4% DOC and 5 µg/mL RNase A. The pre-treatment was successful in digesting a larger portion of RNA, thus indicating that a portion of RNA was inaccessible to RNase A. This also suggests that the concentration of DOC selected was sufficient for disrupting the viral components as previous precent indicating that only 500 µg/mL was necessary to disrupt influenza-A virus (31). The final DNA concentration was reported as 535 ± 20 ng/mL, a decrease of 80% of the initial untreated condition. In order to fully elaborate on the criticality of DOC in providing the most accurate result, a *t*-test was performed comparing the resulting DNA concentration for "drug substance; 5 µg/mL RNase A" and "drug substance; 0.4% deoxycholate; 5 µg/mL RNase A." The resulting *p* value was 0.001, well below 0.05, indicating the statistical significance of DOC in increasing the efficiency of RNase A in our treatments. Through a combination of disrupting matrix interactions and digesting RNA interferences, the Picogreen-based platform method is now applicable to complex biological samples. The studies presented in this work demonstrate that RNase A selectively removes RNA as an interferant in complex biological samples and that DOC continues to provide a means of disrupting matrix interactions.

Deoxycholate Optimization Achieves Disruption of Interactions in the Matrix and Viral Components While Maintaining the Lowest Possible Concentration to be Amenable to Liquid Handling DOC was found to be a critical reagent in disrupting interactions within a complex biopharmaceutical sample. However, DOC was also found to produce a small positive shift in the assay (Table II). An evaluation of potential DOC concentrations was conducted to identify the lowest DOC concentration that still provides matrix disruptions, to decrease any potential effects of the observed DOC-based positive shift. The host cell DNA was quantified in the LVV drug substance against concentrations of DOC ranging from 0.4 to 0.0125% with 5 µg/mL of RNase A also present (Fig. 3). The critical micelle concentration (CMC) of DOC varies from 2 to 6 mM (0.08 to 0.25% w/v) depending on the ionic strength of the solution; therefore, the full range of the CMC was evaluated as well as points above and below the range.

Table III RNase A Treatment Removes RNA Interference. The Function of RNase A in the Complex LVV Drug Substance Sample Matrix Was Evaluated. RNase A Digestion Led to a Decrease in the Interference of RNA and the Addition of Deoxycholate Enhanced the RNA Digestion. The Reported Mean DNA Concentration Is the Result of Triplicate Measurements

Sample description	Mean DNA concentration (ng/mL)	Standard deviation (ng/mL)	Comparison to untreated (%)
Untreated drug substance	2650	56	100
Drug substance; 0.4% deoxycholate	3130	185	118
Drug substance; 5 µg/mL RNase A	848	49	31
Drug substance; 0.4% deoxycholate; 5 µg/mL RNase A	554	20	20

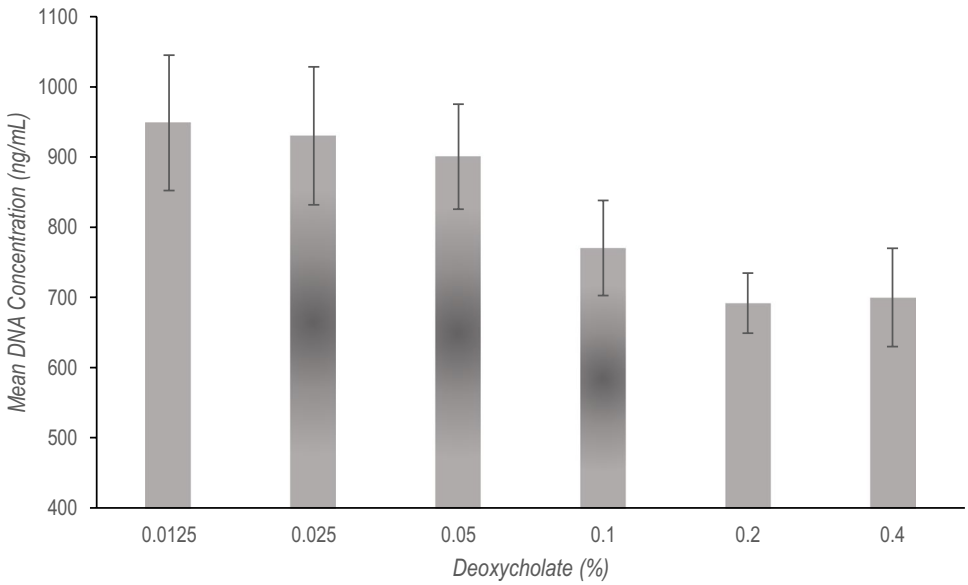

Fig. 3 Optimization of deoxycholate (DOC) concentrations in pre-treatment conditions. Varied concentrations of DOC were evaluated for their ability to disrupt interactions within the LVV drug substance matrix and allow for interferences, such as RNA, to be fully exposed to digestion via RNase A. The selected concentrations range from 0.0125 to 0.4%. The lowest apparent DNA concentration was noted at 0.2% DOC. Reported values are the mean of triplicate measures and the standard deviation is represented by error bars

At lower concentrations of DOC, a positive shift in the apparent DNA concentration was noted. Our findings suggest that at low concentrations of DOC, full disruption of the matrix interactions does not occur. The genomic RNA is not fully exposed to digestion and is likely leading to the increased apparent DNA concentration. As the concentration of DOC increases, the apparent DNA quantification decreases again indicating that a minimal level of DOC is required to fully expose interferent RNA. A plateau of the DNA concentration readout begins occurring from 0.1 to 0.4% DOC, until there is an observed percent difference of 1.2% between DOC concentrations of 0.2 and 0.4%. As the expected CMC concentration has a maximum value of 0.25%, the combination of the small percent difference and this known value drew the conclusion that we had accomplished reaching the CMC. As such, further increases to the DOC concentration would provide no benefit or further disruption to the matrix and virus components.

Selecting the lowest DOC concentration is advantageous to minimize interference and be more amenable to the automated instrumentation, so 0.2% DOC was determined to be the optimal concentration. The final selected concentration of DOC was also at the high end of the proposed concentration to achieve CMC. This allows the flexibility in this platform method to assess a range of ionic strengths in the sample buffers. For samples with extraordinarily high ionic strengths like the elution from ion exchange chromatography, the samples can be greatly diluted and still fall within the linear range of the technique to ensure varying ionic strengths do not interfere with the function of DOC.

The final optimal conditions for pre-treatment of the drug substance were selected as 0.2% DOC and 5 µg/L RNase A. In order to fully account for all matrix interferences of the pre-treatment buffer, equal proportions of DOC and RNase A were added to the DNA standard and assay diluent in the Picogreen-based platform method. A summary of the entirety of the proposed analytical method, including pre-treatment, dilution, and analysis, is displayed (Fig. 4).

The final optimized method, including pre-treatments and matrix-matched reagents, was applied to an upstream, complex sample of the biopharmaceutical drug substance for a final evaluation with a spiking recovery study (Table IV). The sample was specifically chosen as a rigorous test for the proposed method, in which the upstream, complex biopharmaceutical sample represents a historically difficult-to-analyze scenario. A successful recovery was noted ranging from 78 to 83% in various spiking amounts. These values align well with reported spike recoveries for host cell DNA in live-attenuated vaccines drug substance, which are far less complex matrices. Reported values for rabies, dengue, and inactivated poliovirus vaccine have spike recoveries that range from 77 to 128% using a highly specific qPCR methodology (9). This demonstrates both the precision and the robustness of this assay to be applied to not only purified drug substance, but also in-process, unpurified, or upstream samples — enabling rapid process and formulation development efforts across biologic and vaccine production. Furthermore, all samples, from early-stage purification through to the final drug substance, demonstrated improved accuracy, specificity, and precision of the reported DNA concentration after the applied pre-treatment method outlined herein was adopted. This method thus provides a direct means of quantifying host cell DNA in complex biopharmaceutical in a straightforward, high-throughput manner.

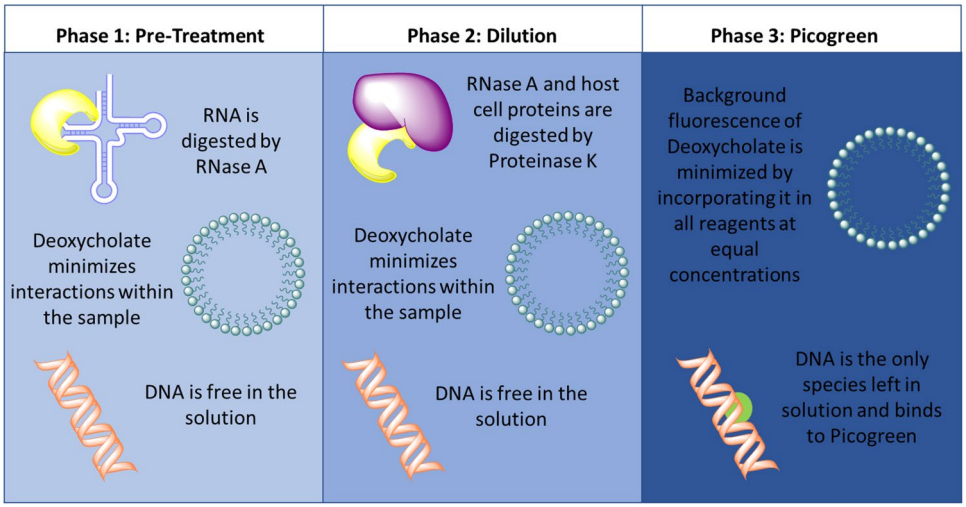

Fig. 4 Summary of the proposed analytical method, including pre-treatment, dilution, and Picogreen-based analysis, in which elimination of common biopharmaceutical interferences was accomplished. During the first phase of the method, the samples are pre-treated with RNase A and deoxycholate (DOC). This eliminates RNA contaminants and minimizes interactions within the sample. In the second phase, the samples are diluted into the linear range of the assay. The dilution buffer contains Proteinase K to eliminate the RNase A and any residual host cell proteins. Finally, in the last phase, DNA is the only species left to interact with the Picogreen dye. Background fluorescence of DOC is eliminated by keeping its concentration constant in all buffers, reagents, and reference material

Table IV Results of Spike Recovery Studies Using Upstream LVV Sample. The DNA Spike Solution Had a Concentration of 30 ng/mL and the Drug Substance Sample Used Had an Initial Concentration of 58 ng/mL. Measured DNA Concentration (mg/mL) Is the Mean Value of Triplicate Measurements, and the Expected DNA Concentration Is Based Upon the Concentration of the Amount of Spiking Solution Used

Sample description	Expected DNA concentration (ng/mL)	Measured DNA concentration (ng/mL)	Spike recovery (%)
Low DNA spike in sample	87	69	80
Medium DNA spike in sample	132	103	78
High DNA spike in sample	207	173	83

Conclusions

A novel method was developed to provide an efficient, high-throughput assay for host cell DNA quantitation in complex biopharmaceuticals. This work was able to overcome common, pharmaceutically relevant interferences, such as protein, RNA, and detergent, with a Picogreen fluorescence-based approach. RNA was determined to be an interferent that produced a large positive increase in the apparent DNA concentration. Through a strategically designed method, RNase A was able to digest the RNA and then itself be digested by Proteinase K to remove residual protein interferences. Additionally, deoxycholate (DOC) was shown to have a profound effect on disrupting matrix interactions and allowing for full removal of the RNA interference through disruption of the viral components. Matrix-matching efforts in the reference material and assay diluent allow for the interference of this detergent to be easily overcome, as well. This robust design allows for rapid analysis of diverse, complex samples to accelerate process development of biopharmaceuticals. This methodology is the first, fluorescence-based host cell DNA quantification that is appliable to vaccines with high concentrations of RNA like the mRNA vaccines as well as biologics and vaccines from all cell substrates. In a time of accelerated needs for the development of biologics and vaccines, the method outlined herein is readily automated and can help accelerate research and development efforts with low costs and high output.

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Declarations

Conflict of Interest The authors declare no competing interests.

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