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DNA spike studies for demonstrating improved clearance on chromatographic media

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ABSTRACT

DNA spike clearance methods were used to demonstrate improved clearance factors on anion exchange and hydrophobic interaction columns used in the production of human therapeutic proteins. DNA clearance at large-scale was first measured for a monoclonal antibody expressed in Chinese Hamster Ovary (CHO) cells and an antibody fragment expressed in *Escherichia coli*. Small-scale spike experiments were then performed on individual chromatographic steps using host-specific DNA paired with TaqMan PCR assay methods. This approach has advantages of improved specificity, sensitivity, cost and throughput compared to other types of spike clearance methods. The anion exchange column used in the monoclonal antibody process was shown to have very high capacity for CHO DNA, resulting in greater than 7.1 log reduction. The anion exchange and hydrophobic interaction columns used in the antibody fragment process were shown to have high *E. coli* DNA clearance capability, with greater than 5.1 and 5.3 logs clearance, respectively. Compared to the large-scale process, higher log reduction values were achieved in small-scale spike clearance studies by challenging the chromatographic steps with load DNA levels 2–5 logs higher than the large-scale process levels. Using highly specific and sensitive spike clearance methods, we demonstrated consistently high DNA clearance factors for each of the production processes that meet industry and regulatory standards for human therapeutics. The method is applicable to a broad range of industrial scale processes where demonstration of the robustness of DNA clearance is necessary to support development or licensure of biopharmaceutical products.

1. Introduction

The manufacture of human therapeutic proteins often utilizes mammalian Chinese Hamster Ovary (CHO) or bacterial *E. coli* production systems. Constituents of the production process such as deoxyribonucleic acid (DNA), host cell proteins and endotoxin must subsequently be removed or reduced by the downstream purification process to acceptable levels to ensure patient safety [1,2]. The World Health Organization (WHO) has recommended that DNA levels be consistently reduced to less than 10 ng DNA per dose for proteins intended for human therapeutics that are produced by continuous cell line culture such as CHO [3]. The DNA limit recommended by the WHO has been widely adopted by the biotechnology industry. This DNA limit demands a robust purification process and an assay with high sensitivity for the detection of DNA.

Since DNA has a net negative charge due to its phosphate groups, anion exchange chromatography has been widely utilized for purifying plasmid [4] and chromosomal DNA [5,6]. Anion exchange membranes [7] and DEAE monolithic columns [8] have also been used. In addition to ion exchange methods, various other methods have also been used to purify DNA. Affinity chromatography methods such as Protein A [6,9], lysine [6], hydroxyapatite [10], and hydrophobic interaction [5] have all been used to remove DNA.

Production processes for proteins often consist of a combination of various purification methods in order to achieve the desired DNA clearance and reduction of other process-related impurities and product variants. It is prudent to demonstrate the robustness of individual steps and the overall process capability to clear challenge levels of process-related impurities such as DNA, host cell proteins, virus and resin leachables. This approach gives added assurance that the process is capable of consistent performance even with natural variability in the starting harvested cell culture fluid from mammalian or bacterial production systems. One method for performing challenge studies is to evaluate spike clearance. This method has been previously recommended by the European Agency for the Evaluation of Medicinal Products [11] and the Food and Drug Administration [12] as a method for validating or demonstrating DNA clearance. DNA spike clearance has been performed using radio labeled DNA [13]. Radio labeling can improve the sensitivity of detection of DNA; however, it requires the use of hazardous chemicals, specialized equipment, and segregated laboratory areas. Fluorimetry has also been utilized for measuring the

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level of DNA in spike clearance studies [5]. Another alternative is to perform spike clearance studies using DNA that is representative of the host cell DNA and to pair this with a sensitive and specific DNA assay method such as polymerase chain reaction (PCR). This approach ensures that DNA clearance achieved during spike clearance studies is representative of the DNA clearance expected in the large-scale production process. Robust and improved clearance factors can also be demonstrated through the use of efficient, high throughput, and sensitive DNA analysis methods such as TaqMan PCR.

Prior published work with spike clearance studies have used non-host-specific DNA for load spiking which can then preclude the use of specific and sensitive DNA assays such as TaqMan PCR [5,13]. Previous DNA spike clearance methods which used non-specific DNA analysis methods (Threshold® Total DNA, fluorimetry, radio labeling, and others) have several disadvantages [5,13]. First these non-specific DNA assay methods lack specificity due to detection of human or other contaminating DNA. Cross-contamination of DNA often leads to high assay failure rates and decreases the sensitivity of the assay due to false positive results in the chromatographic pools used to assess log clearance values. Secondly, these methods have decreased sensitivity due to quantification limits which reduces the apparent log clearance values and limits the assessment of actual process robustness. In cases where high therapeutic product doses are required, these assays are frequently not sensitive enough to establish that a process meets the WHO recommended DNA limit of less than 10 ng/dose. Last, these assay methods have limitations in throughput and cost compared to TaqMan PCR methods. This manuscript is the first to describe using host-specific DNA for the load spiking in combination with TaqMan PCR assay methods to improve the measurement of DNA clearance. Using these methods we demonstrated higher log DNA clearance compared to more conventional spike clearance methods thereby establishing the robustness of the tested chromatographic steps.

This method was applied to two types of production processes to demonstrate the application and advantages over conventional methods. Robust DNA removal from a therapeutic monoclonal antibody derived from a CHO production system and an antibody fragment derived from an *E. coli* production system that meet the recommended WHO limit of less than 10 ng DNA per dose are described. Five large-scale runs were first used to validate the DNA clearance level in each production process. Individual steps in each process were then identified for further clearance evaluation using spike clearance studies performed at small-scale. For the CHO monoclonal antibody process, herring DNA was initially used for the spike, and DNA was quantified with a conventional non-specific Threshold® Total DNA assay. In later work, CHO DNA spikes were used and DNA was quantified with a CHO DNA specific TaqMan PCR assay to demonstrate improved DNA clearance over conventional methods. To evaluate the DNA clearance in the *E. coli* antibody fragment process, *E. coli* DNA spikes were used and DNA was quantified with an *E. coli* TaqMan PCR assay to show applicability across a broad range of production processes.

2. Materials and methods

2.1. Preparation of DNA solutions for spike experiments

Herring DNA Type XIV was from Sigma–Aldrich (St. Louis, MO). Herring DNA was sheared to an average size of less than 2 kbp (Fig. 1) by passing the DNA through a 26-gauge needle 20 times. The herring DNA stock solution was in a buffer solution of 25 mM Tris, 50 mM NaCl at pH 8.0. CHO DNA was purified from DP12 CHO cells (Genentech Inc., South San Francisco, CA). The Qiagen Blood and Cell culture DNA Maxi Kit (Valencia, CA) or the Stratagene DNA extraction kit (La Jolla, CA, no. 200600) was used to purify the CHO DNA. The CHO DNA stock solution was in a buffer solution of 10 mM Tris, 1 mM EDTA at pH 8.0. DNA was sonicated twice to an average size of less than 2 kbp as measured by agarose gel (Fig. 1). *E. coli* DNA was type VIII sodium salt *E. coli* strain B from Sigma–Aldrich (St. Louis, MO). *E. coli* DNA was sonicated for 10 s six times to achieve an average size of less than 2 kbp as measured by agarose gel (Fig. 3). The *E. coli* DNA stock solution was in a buffer solution of 1 mM Tris, 0.1 mM EDTA at pH 7.5. The concentration of DNA in the stock solutions was measured by UV absorbance at 260 nm (A_{260} = 1 corresponds to 50 μg DNA/mL). The size of both the herring, CHO, and *E. coli* DNA in the stock solutions and the DNA contained in the monoclonal antibody harvested cell culture solution were measured by 0.8% or 1.2% Agarose E-gels containing ethidium bromide (Invitrogen, Carlsbad, CA). Images were taken using the Fluor S UV imaging system (Biorad, Hercules, CA). 1 kbp DNA ladder was included as a molecular weight marker (New England Biolabs, Ipswich, MA). For DNA spike runs, the stock solution was spiked into the column load and mixed for a minimum of 30 min prior to loading the column.

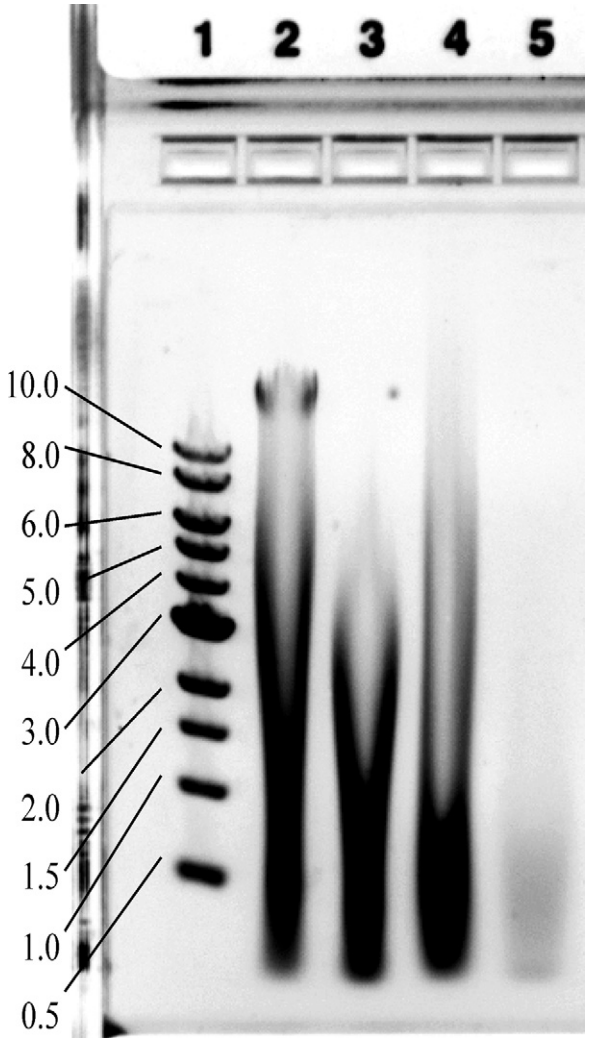

Fig. 1. Size analysis of DNA from spike stock solutions and monoclonal antibody HCCF by 0.8% agarose gel. The size of the CHO DNA in the stock solution after sonication was comparable to the size of CHO DNA entering the monoclonal antibody process (<2 kbp). Lane 1: 1 kbp DNA ladder, 2: CHO DNA after one sonication-5 μg, 3: CHO DNA after second sonication-5 μg, 4: Herring DNA after shearing using a needle-5 μg, 5: monoclonal antibody HCCF-0.3 ng DNA.

2.2. Proteins, chromatographic media and systems

Protein molecules (monoclonal antibody and antibody fragment) were from Genentech Inc. (South San Francisco, CA). Anion exchange media was Q-Sepharose FF from GE Healthcare, Uppsala, Sweden. Hydrophobic interaction media was Hi-Propyl from Mallinckrodt Baker Inc., Phillipsburg, NJ. Small-scale chromatography experiments were conducted on an ÄKTAexplorer (GE Healthcare, Uppsala, Sweden) and protein retention was monitored by UV absorbance at 280 nm.

2.3. Monoclonal antibody chromatographic methods

For anion exchange experiments, a 0.66 or 1.6 cm i.d. glass Omnifit column (Sigma–Aldrich, St. Louis, MO) was packed to a bed height of 19 cm, total bed volume V_t = 6.5 or 38 mL. Anion exchange experiments were run under isocratic chromatographic conditions with a flow rate of 100 cm/h and an equilibration and wash buffer composition of 25 mM Tris, 50 mM NaCl, pH 8.0. The column was pre-equilibrated with 200 mM Tris, pH 8.0 for three column volumes (CV) followed by 3 CV of equilibration buffer. The anion exchange load was adjusted to pH 8.0 using 1.5 M Tris base. The column was loaded at a density of 100 mg protein per mL resin. Since the column was operated in flow through mode, the load density represents the level of antibody applied to the resin and not the amount bound to the resin. Pooling was initiated at 0.2 OD during the load phase and concluded at 0.5 OD during the wash phase.

2.4. Antibody fragment chromatographic methods

For hydrophobic interaction (HIC) experiments, a 0.66 cm i.d. glass Omnifit column (Sigma–Aldrich, St. Louis, MO) was packed to a bed height of 20 cm, total bed volume V_t = 6.8 mL. HIC experiments were performed at a flow rate of 200 cm/h. The column was pre-equilibrated with 50 mM MES pH 6.0 for 4 CV. The column was equilibrated for 3 CV using 0.37 M sodium citrate pH 6.0. The HIC load was adjusted by adding 0.54 volume of 1 M sodium citrate pH 6.0. The column was loaded at a density of 9 mg antibody fragment per mL resin. After loading, the column was washed for 4 CV using 0.26 M sodium citrate pH 6.2. The product was then eluted using 50 mM MES pH 6.0 and the pool was collected from 1.0 OD to 0.5 OD.

For anion exchange experiments, a 0.66 cm i.d. glass Omnifit (Sigma–Aldrich, St. Louis, MO) column was packed to a bed height of 20 cm, total bed volume V_t = 6.8 mL. Experiments were run at a flow rate of 100 cm/h. The column was pre-equilibrated with 25 mM Tris, 100 mM NaCl pH 9.0 for 3 CV. The column was equilibrated for 4 CV using 25 mM Tris, 9 mM NaCl pH 9.1. The anion exchange load was adjusted to pH 9.1 by the addition of 1.5 M Tris and then diluted with water to reach a conductivity of 1.4 mS/cm. The column was loaded at a density of 10 mg protein per mL resin. After loading, the column was washed for 3 CV using equilibration buffer. Gradient buffer A was 25 mM Tris pH 9.0 and gradient B buffer was 25 mM Tris, 100 mM NaCl pH 9.0. The product was eluted using a linear gradient from 20% to 65% gradient B over 15 CV and the pooling was collected from 2.0 OD to 1.0 OD.

2.5. Protein concentration and protein yield

Protein concentration in the monoclonal antibody anion exchange load and pool was determined by measuring the UV absorbance at 280 and 320 nm and using equation: mg/mL = $((A_{280} - A_{320})/1.7) \times$ dilution factor. Protein concentration in the antibody fragment HIC load was measured by analytical Protein G affinity chromatography (Applied Biosystems, Poros G/20, 4.6 mm i.d. × 100 mm, total bed volume V_t = 1.7 mL, 20 μm particle diameter, Foster City, CA). Samples were loaded onto the column with a loading buffer of 100 mM sodium phosphate, 250 mM sodium chloride pH 6.3 and then eluted with 0.2% TFA, 2 M urea, 250 mM sodium chloride pH 2.5 solvent at a flow rate of 2 mL/min. The detector wavelength was set at 280 nm. The sample injection volume was 50 μL and the range of the assay was 0.05–1.0 mg/mL. Protein concentrations in the antibody fragment HIC pool, anion exchange load, and anion exchange pool were determined by measuring the UV absorbance at 280 nm and 320 nm and using equation: mg/mL = $((A_{280} - A_{320})/1.9) \times$ dilution factor. The total protein in the load and pool was determined using equation: total protein (mg) = protein concentration (mg/mL) × pool volume (mL). The product yield was determined by equation: % yield = [total protein in pool (mg)/total protein in load (mg)] × 100.

2.6. DNA assays

Herring DNA was measured using the Threshold® Total DNA assay (Molecular Devices, Sunnyvale, CA) following the vendor's protocol. The Threshold® assay is specific for single-stranded DNA. Samples were diluted so that the DNA content was within the standard curve range of 10–400 pg/mL. CHO and *E. coli* DNA were quantified using TaqMan PCR assays. Extraction of CHO and *E. coli* DNA from test samples was performed using Qiagen QIAamp Viral RNA kit (Valencia, CA). DNA TaqMan PCR reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) containing specific forward primer, reverse primer, and probe. The *E. coli* DNA TaqMan PCR assay was performed following the procedure described in BioTechniques [14]. The CHO DNA TaqMan PCR assay was initially developed by BioReliance (Rockville, MD) and was introduced to Genentech under a licensing agreement. The CHO DNA primer set targets a repetitive sequence of DNA in the *Cricetulus griseus* genome. Amplification was performed using the ABI PRISM™ 7900 HT Sequence Detection System (PerkinElmer, Waltham, MA). Samples were quantified by comparison to DNA standards. The standard curve range was 10–100,000 pg/mL for *E. coli* DNA and 1–10,000 pg/mL for CHO DNA. For all the DNA assays, the following calculation was used to obtain the reported results: ng DNA/mg product = corrected DNA value (ng/mL, converted from pg/mL)/product concentration (mg/mL).

3. Results and discussion

3.1. DNA clearance in a monoclonal antibody process

The monoclonal antibody was produced by Chinese Hamster Ovary (CHO) mammalian cell culture which is considered a continuous cell line. For this type of cell line, strict guidelines apply in demonstrating clearance of cellular DNA [3,11]. The World Health Organization (WHO) has recommended that DNA levels in biological products produced by recombinant DNA technology in continuous cell lines be consistently reduced to levels of less than 10 ng/dose [3]. To demonstrate DNA reduction to acceptable levels, we first measured the clearance in the large-scale purification process. Harvested cell culture fluid was purified through three chromatographic steps consisting of Protein A affinity, anion exchange and cation exchange chromatography followed by a tangential flow filtration (TFF) formulation step. The DNA level in each of the in-process pools as measured by the CHO DNA TaqMan PCR assay method is shown in Table 1. The CHO DNA TaqMan PCR assay method is specific for detecting CHO DNA. The DNA clearance steps in the process were shown to be the Protein A and anion exchange chromatographic steps, with 2.9–3.0 and greater than 4.7–5.1 log clearance, respectively. Log reduction is the level of DNA

Table 1
DNA clearance in a monoclonal antibody process from five large-scale runs. DNA was measured by CHO DNA TaqMan PCR and results were rounded to two significant figures.

Process pool	DNA level (ng/mg)	DNA clearance (log)
HCCF	7,900–15,000	na
Protein A	7.4–19	2.9–3.0
Anion exchange	<0.00014 to <0.00015	>4.7 to >5.1
Cation exchange	<0.00012 to <0.00013	na
TFF (UFDF)	<0.000031 to <0.000057	na
Overall clearance	na	8.3 to >8.7

clearance from the chromatographic load to the pool expressed as $\log_{10}$. After the anion exchange step, the DNA level in all five runs was less than the detection limit of the assay (1 pg/mL), so clearance factors for other process steps were not calculated. Overall, at least 8.3 log DNA clearance was demonstrated. For this particular molecule, the maximum dose is 1770 mg of antibody. Therefore, based on the highest DNA level in the TFF pool, the purification process consistently reduced DNA levels to less than 0.10 ng of DNA per dose, which is 100-fold lower than the limit recommended by the WHO. Since this value is based on the performance of five large-scale runs, we wanted to further evaluate the capability of the process to clear more challenging levels of DNA. The performance of the anion exchange step showed that 7.4–19 ng DNA per mg antibody could be reduced to less than the assay detection limit, indicating that the anion exchange step may be capable of clearing more challenging levels of DNA. Therefore, this step was chosen for further evaluation using spike clearance studies.

Two different stock solutions were chosen for the DNA spike clearance studies on the anion exchange column. Experiments were initially performed with herring DNA since it is easily obtained from a commercial vendor. However, detection of herring DNA required use of the less sensitive and less specific Threshold® Total DNA assay, which had a detection limit of 10 pg/mL. Experiments were also performed with CHO DNA which was prepared in house. The use of CHO DNA was advantageous since it could be detected with a very specific and sensitive (1 pg/mL) CHO DNA TaqMan PCR assay. The specificity and sensitivity of this assay method allowed for demonstration of greater DNA log reduction values.

Prior work testing DNA clearance has suggested that larger fragments of DNA bind more strongly to anion exchange resins due to the presence of more negatively charged phosphate residues compared to smaller DNA fragments which can therefore influence the separation [13]. In preparing the DNA stock solutions, we verified that the final size of the DNA in the stock spike solution was representative of the size of the DNA in the actual production process so that the spike study results would be comparable to those expected in the large-scale production process. The agarose gel in Fig. 1 shows that the CHO DNA, after sonication, and the herring DNA, after shearing using a 26-gauge needle, are comparable in size to the DNA in the monoclonal antibody harvested cell culture fluid (HCCF). The agarose gel in Fig. 2 shows the size of the CHO DNA stock solution before and after sonication. CHO DNA was larger in size before sonication (>10 kbp) than after sonication (<2 kbp). The reduction in the size of the CHO DNA observed in the harvested cell culture fluid is likely due to the shear force encountered during the continuous feed centrifugation operation used to harvest the CHO cells during the large-scale process.

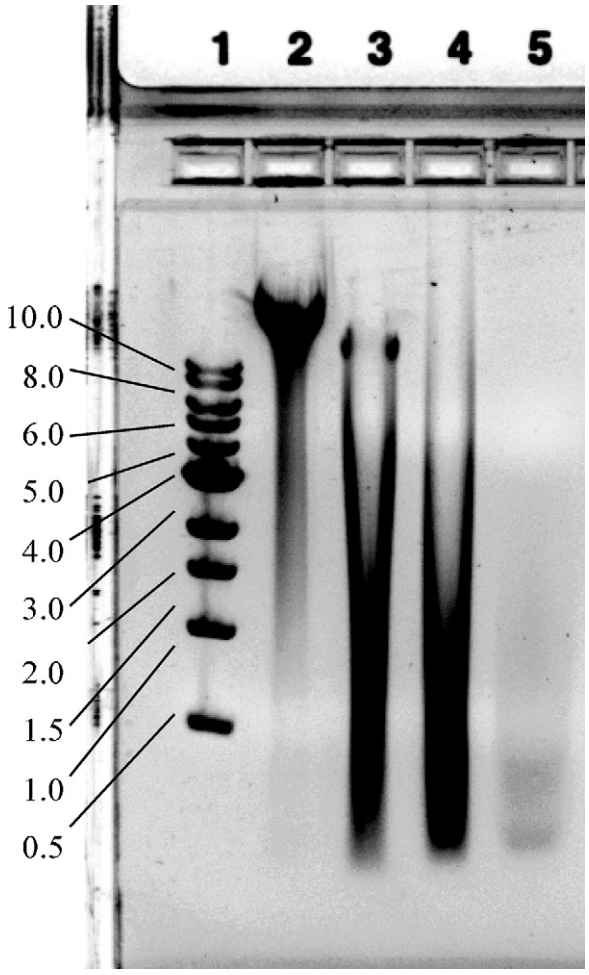

Fig. 2. Size analysis of CHO DNA stock solution by 1.2% agarose gel before and after sonication. Prior to sonication the average size of the CHO DNA stock solution was >10 kbp and after sonication the average size was reduced to <2 kbp. Lane 1: 1 kbp DNA ladder, 2: CHO DNA no sonication-5 μg 3: CHO DNA after one sonication-5 μg, 3: Herring DNA after shearing using a needle-5 μg, 4: monoclonal antibody HCCF-0.3 ng DNA.

Table 2
DNA spike clearance on scaled-down anion exchange column using monoclonal antibody feedstock. DNA results were rounded to two significant figures.

Type of DNA used for spike	DNA assay method	Antibody yield (%)	Load DNA (ng/mg)	Pool DNA (ng/mg)	Resin DNA capacity (ng/mL)	DNA clearance (log)
Control, no spike	Threshold	101	0.81	0.032	78	1.4
Herring	Threshold	97	50	0.018	5,000	3.4
Herring	Threshold	100	120	0.046	12,000	3.4
Herring	Threshold	101	200	0.060	20,000	3.5
Herring	Threshold	101	660	0.033	66,000	4.3
Herring	Threshold	94	24,00	0.053	240,000	4.7
Control, no spike	TaqMan PCR	98	7.4	<0.00013	>740	>4.8
CHO	TaqMan PCR	101	1,000	<0.00013	>100,000	>6.9
CHO	TaqMan PCR	100	1,000	<0.00013	>100,000	>6.9
CHO	TaqMan PCR	99	1,000	<0.00013	>100,000	>6.9
CHO	TaqMan PCR	93	1,800	<0.00014	>180,000	>7.1
CHO	TaqMan PCR	95	1,800	<0.00013	>180,000	>7.1

Table 3
Overall DNA clearance capability in the monoclonal antibody process. Analysis performed using the CHO DNA TaqMan PCR assay method.

Process step	Clearance (log)	Test method
Protein A	2.9–3.0	No spike/endogenous
Anion exchange	>7.1	Spike run
Overall process	>10.0 to >10.1	Combined

The herring and CHO DNA spike experiments on the anion exchange column are shown in Table 2. Since the positively charged resin is operated in isocratic mode, it was expected that the negatively charged DNA would remain bound to the resin while the monoclonal antibody flowed through the column. For the herring DNA experiments, five different levels of DNA spikes were used, with the highest level at 2400 ng/mg. The herring DNA level in the pools ranged from 0.018 to 0.060 ng/mg, which demonstrated the capacity of the process to clear 3.4–4.7 logs of DNA, compared to 1.4 logs observed with normal process levels in the control or non-spiked small-scale run. Using the Threshold® Total DNA assay, DNA was detected at very low levels in all the pools, including a small-scale control run. This was unexpected since the DNA level was approximately 10-fold lower in the load to the small-scale control run compared to the levels detected in the large-scale process by the more specific CHO DNA TaqMan PCR assay. Since the Threshold® Total DNA assay method is not specific for CHO DNA, the level of detectable DNA observed in the anion exchange pools may be due to contaminating non-process-related DNA. Using the Threshold® Total DNA assay method, we were only able to demonstrate that the process cleared DNA to 32–106 ng/dose after the anion exchange step, which does not meet the WHO recommended DNA limit of less than 10 ng/dose. Since the large-scale anion exchange step (Table 1) had demonstrated complete clearance of up to 19 ng CHO DNA per mg of antibody using the CHO DNA TaqMan PCR assay method, we performed additional spike clearance studies using CHO DNA to take advantage of this more specific and sensitive assay method.

Two different spike levels were used for the CHO DNA spike experiments and the runs were performed in triplicate or duplicate (Table 2). The results from all the CHO DNA anion exchange runs confirm that the resin has a high capacity for binding DNA, since the DNA levels in the pools from all the CHO DNA spike experiments were less than the detection limit of the assay. All of the pools from these runs contained less than 0.00014 ng/mg, which resulted in acceptable levels of less than 0.2 ng DNA per dose. CHO DNA spike experiments where complete DNA clearance was achieved showed the resin capacity to be greater than 180,000 ng DNA per mL resin. The resulting clearance of DNA at challenge levels was greater than 7.1 logs, which was 2.0 logs higher than demonstrated with normal process DNA levels on the anion exchange step. High clearance levels for DNA on the anion exchange column were expected given the likely strong association between the negatively charged DNA phosphate groups and the quaternary amine ligand on the Q-Sepharose FF resin. The yields for all DNA spike clearance runs were also comparable to those of the control runs, indicating normal column performance (Table 2). Combining the 2.9 log DNA clearance on the Protein A step with the greater than 7.1 log clearance achieved on the anion exchange step using spike clearance studies, the overall process clearance capability is greater than 10 logs (Table 3). Spike clearance studies, therefore, were valuable in demonstrating additional process capability. These data provide confidence that the purification process is capable of reducing higher DNA levels that may enter the process due to feedstream variability, while still achieving acceptable levels in the final process pool intended for human therapeutic use.

Table 4
DNA clearance in an antibody fragment process from five large-scale runs. DNA was measured by *E. coli* DNA TaqMan PCR and results were rounded to two significant figures.

Process pool	DNA levels (ng/mg)	DNA clearance (log)
Centrate	<0.050–0.090[a]	na
Cation exchange	<0.0067 to <0.0091	>1.0
Hydrophobic interaction	<0.0035 to <0.0038	na
Mixed mode	<0.0051 to <0.0055	na
Anion exchange	<0.0054 to <0.0057	na
TFF (UFDF)	<0.00020 to <0.00021	na

[a] Only one run showed detectable level of DNA in the centrate.

3.2. DNA clearance in an antibody fragment process

The antibody fragment was produced using an *E. coli* microbial production process. To demonstrate DNA reduction to acceptable levels, we first measured DNA clearance in five large-scale runs. The level of DNA entering the process was measured using the *E. coli* DNA TaqMan PCR assay method (Table 4). The centrate from four of the five runs showed DNA levels that were less than the *E. coli* TaqMan PCR assay detection limit (<10 pg/mL or <0.063 ng/mg). The very low level of DNA (0.090 ng/mg) detected in the centrate from one of the runs was easily reduced to below the limit of detection on the subsequent cation exchange chromatographic step. This demonstrated that the cation exchange step is capable of greater than 1.0 log clearance of DNA ($n = 1$). The very low or undetectable level of DNA entering the purification process can be attributed to conditioning steps during the upstream harvest operation used to generate the centrate (data not shown). The highest therapeutic dose for the antibody fragment was set at 0.5 mg. The level of DNA measured in the final TFF pool was <0.00021 ng/mg, which corresponds to a human therapeutic exposure level of <0.0001 ng DNA per dose that meets, and is 5 logs lower than, the WHO recommended limit for DNA derived from continuous cell lines. Although we demonstrated that the antibody fragment process had consistent and robust DNA clearance, the 10 ng/dose recommended DNA limit for continuous cell lines does not apply to microbial derived products (WHO [3]). Regardless, we used this limit to help us evaluate the overall process performance. Since the level of DNA entering the process was either very low or undetectable, even using the very sensitive *E. coli* TaqMan PCR assay method, we were unable to evaluate the clearance capability of the downstream purification steps (HIC, mixed mode, anion exchange). Since only five runs were used to evaluate the level of DNA entering the process, it is possible that, with a higher number of runs, the DNA level could exceed 0.090 ng/mg in the starting feedstream. To ensure that the process had sufficient capacity to clear DNA, we performed small-scale spike clearance studies on the HIC and anion exchange steps to evaluate the process robustness and capacity to clear higher levels of DNA.

E. coli DNA is commercially available, so no additional in house purification was required. In addition, the *E. coli* DNA TaqMan PCR assay was also compatible with the commercially available *E. coli* DNA, so we could utilize the sensitivity and specificity of this assay in performing the spike experiments. *E. coli* DNA was prepared for the antibody fragment spike studies, and the size of the DNA after shearing by syringe was measured to be 0.5–2 kbp by agarose gel (Fig. 3). We were unable to compare the size of the process DNA to the prepared DNA spike solution since the levels of DNA entering the purification process were either undetectable or too low to measure by agarose gel. Five different spike levels were evaluated for the *E. coli* DNA spike experiments on the HIC step (Table 5). At the lower spike levels, DNA was cleared to below the detection limit of the assay (<0.0033 ng/mg), which demonstrated that the HIC column is capable of completely clearing at least 600 ng

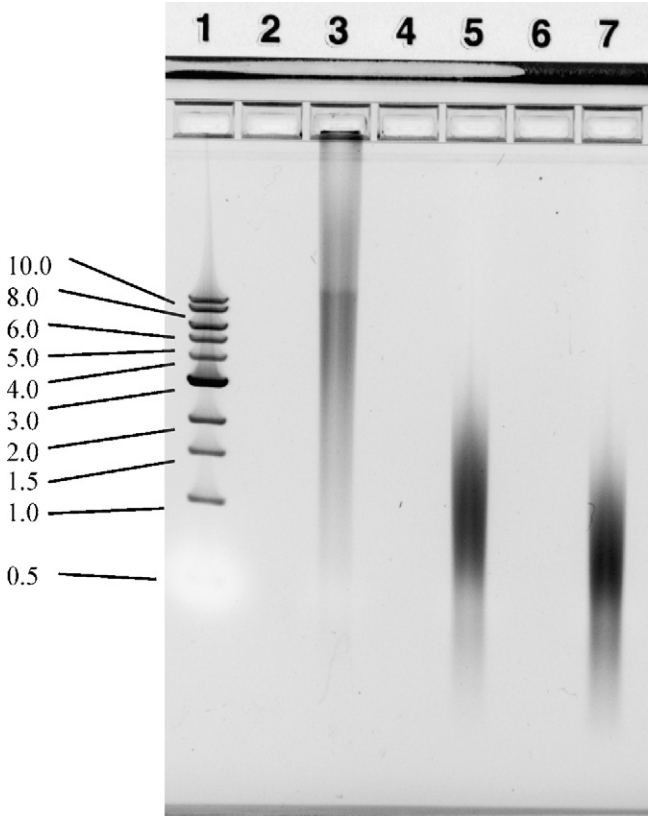

Fig. 3. Size analysis of *E. coli* DNA stock solution by 1.2% agarose gel before and after sonication. After sonication the size of the *E. coli* DNA stock solution was reduced to an average size of <2 kbp. Lane 1: 1 kbp ladder, 2: blank, 3: *E. coli* DNA stock solution, 4: blank, 5: *E. coli* DNA stock solution sonicated 2× 10 s, 6: blank, 7: *E. coli* DNA stock solution sonicated 6× 10 s.

DNA/mg antibody fragment, resulting in greater than 5.3 log clearance. At the higher spike levels of 1400 and 2500 ng/mg, DNA was detectable in the pool which demonstrated the upper limit of the column's DNA clearance capability. The yields for all spike runs were comparable to the control run (Table 5).

The mechanism of binding and retention of genomic DNA on HIC resins is less understood than that on anion exchange resins. Prior work in this field [15] has shown two possible mechanisms for separating genomic DNA by HIC chromatography. The interaction of genomic DNA binding to HIC supports depends primarily on the level of exposed hydrophobic aromatic bases. Single-stranded genomic DNA was found to bind to the HIC support due to exposed hydrophobic base groups while double-stranded DNA did not interact with the HIC support. In addition, in the absence of factors which promote hydrophobic interactions, the mechanism of separation between double- and single-stranded genomic DNA was shown to be based on a size exclusion mechanism. Size exclusion based separation showed that single-stranded genomic DNA (MW < 0.7 kbp) had some retention while double-stranded genomic DNA (MW > 20 kbp) was not retained and eluted in the column void volume.

To determine where DNA resolved on the HIC column used to purify the antibody fragment, we used the run with the highest spike level of 2500 ng/mg and measured the DNA levels in different fractions throughout the HIC step. Each of the fractions showed detectable DNA (Table 6), with the highest levels found prior to product elution in the load flow through (40%), and wash (36%). It is expected that the majority of DNA contained in the spike solution is double-stranded DNA since it was prepared using non-denaturing methods, consequently the majority of DNA would not be expected to bind to the HIC resin. Lower levels of DNA were found in the pre-pool (0.01%), elution pool (0.24%) and in the regeneration (0.19%) and storage (0.04%) phases. These data indicated that, compared to the antibody fragment, the majority of the DNA is less hydrophobic in its behavior on the HIC column. It was interesting that DNA was observed in all the HIC fractions for the high spike run, which could indicate variability in the DNA that is being separated, loss of resolution during the wash and pre-elution phases, or more DNA binding to the antibody fragment. Based on prior work into the mechanism of DNA retention on HIC resins [15], it is possible that a small amount of the DNA contained in the spike solution either has some exposed hydrophobic groups allowing retention on the resin or that the size after shearing allows for retention by the size exclusion mechanism. Since DNA was only detected in the elution pools from the runs with the two highest spike levels, it is unknown if this same distribution of DNA, in particular low levels of DNA in the post-elution phases, would have been observed in the lower spike level runs since they were not analyzed.

Table 5
E. coli DNA spike clearance on scaled-down HIC column in an antibody fragment process. DNA was measured by *E. coli* TaqMan PCR and results were rounded to two significant figures.

Run	Product yield (%)	Load DNA (ng/mg)	Pool DNA (ng/mg)	DNA clearance (log)
1, control (no spike)	99	0.0046	<0.0032	>0.16
2	96	56	<0.0033	>4.2
3	96	310	<0.0031	>5.0
4	96	600	<0.0031	>5.3
5	94	1,400	0.0041	5.5
6	89	2,500	6.5	2.6
Spike run range	89–96	56–2,500	<0.0031–6.5	2.6–5.5

Table 6
E. coli DNA TaqMan PCR analysis of HIC fractions in an antibody fragment process during 2500 ng/mg spike clearance run.

Phase	DNA (ng/mL)	Phase volume (mL)	DNA (ng)	DNA recovered (%)
Load	14,322	10.5	150,381	na
Load flow through	5,770	10.5	60,585	40
Wash	1,970	27.2	53,584	36
Pre-pool	2.98	6.7	20.0	0.01
Pool	19.85	18.0	357.3	0.24
Regeneration	14.00	20.4	285.6	0.19
Storage	1.81	34.0	61.54	0.04
Total	na	na	114,893	76

Table 7
E. coli DNA spike clearance on scaled-down anion exchange column in an antibody fragment process. DNA was measured by *E. coli* DNA TaqMan PCR and results were rounded to two significant figures.

Run	Product yield (%)	Load DNA (ng/mg)	Pool DNA (ng/mg)	DNA clearance (log)
1, control (no spike)	89	<0.028	<0.0049	na
2	88	270	<0.0049	>4.7
3	90	270	<0.0048	>4.8
4	87	670	<0.0049	>5.1
5	87	670	<0.0048	>5.1
Spike run range	87–90	270–670	<0.000013 to <0.00014	>4.7 to >5.1

Table 8
Overall DNA clearance capability in the antibody fragment process. Analysis performed using the *E. coli* DNA TaqMan PCR assay method.

Process step	Clearance (log)	Test method
Cation exchange	>1.0	No spike/endogenous
HIC	>5.3	Spike run
Anion exchange	>5.1	Spike run
Overall process	>11.4	Combined

Two different spike levels were used for the *E. coli* DNA spike experiments on the anion exchange column and the runs were performed in duplicate (Table 7). The anion exchange step for the antibody fragment process was run in a bind and gradient elution mode with a different pH and conductivity compared to the flow through step used for the monoclonal antibody process. These differences could impact the removal of DNA from the product pool. DNA was undetectable in the product pools from all of the spike runs. This demonstrated that the anion exchange column, when operated in either flow through or bind and gradient elution mode (under the conditions specified), was robust in removing DNA from the two types of production processes. These data support the expectation that anion exchange chromatography is a generally robust operation for DNA removal. The level of DNA in the pools from the spike runs on the anion exchange step for the antibody fragment process were all less than 0.0049 ng/mg, demonstrating greater than 5.1 log DNA clearance. The yields for all spike runs were also comparable to the control run. Three DNA clearance steps were identified for the antibody fragment process. The cation exchange step showed 1.0 log clearance ($n = 1$), and the HIC and anion exchange steps showed greater than 5.3 and 5.1 logs clearance, respectively, through the use of spike studies. Thus the overall purification process, not including the harvest step, was capable of 11.4 log clearance (Table 8).

4. Conclusions

Spike removal studies can demonstrate improved DNA log reduction values because clearance of very challenging levels of DNA in the load feedstocks for chromatographic steps is possible. Individual process steps can be challenged with many fold higher DNA levels than the large-scale process would ever see, thereby allowing the true maximum DNA clearance limits of a process step to be quantified. These studies demonstrate consistent clearance of DNA in the large-scale purification processes for a monoclonal antibody and an antibody fragment intended for human therapeutic use. Small-scale DNA spike clearance studies demonstrated robust and high DNA clearance capability of specific steps in each of the purification processes. Small-scale spike studies, using host-specific DNA and specific and sensitive TaqMan PCR assay methods in tandem were successfully used to demonstrate that the monoclonal antibody and antibody fragment processes were capable of 2.0 and 10.4 log higher DNA clearance, respectively compared to the clearance measured in the large-scale process. These studies demonstrate that the purification processes for each molecule are robust in clearing DNA to acceptable levels, even when challenged with levels higher than expected under normal process conditions. Protein A, anion exchange and hydrophobic interaction chromatography were shown to be reliable DNA clearance steps. Using high CHO DNA spike levels and the sensitive and specific CHO TaqMan PCR assay method, we demonstrated higher DNA clearance (>7.1 log) on the anion exchange resin than previously reported [5,6,13]. The methods presented have improved specificity and sensitivity over previous methods. Using these methods we demonstrated higher log DNA clearance values thus better characterizing the robustness of the chromatographic steps. These methods can be applied to other production processes to test robustness and to demonstrate process capability beyond levels achievable under normal operating conditions. The spike method described here could also be combined with new Quality by Design process characterization approaches to rapidly define a broad operating space for DNA clearance. These methods can be used to demonstrate that production processes intended for human therapeutics achieve consistent and robust DNA clearance that meets industry and regulatory standards.

Nomenclature

CHO	Chinese Hamster Ovary
CV	column volume
HCCF	harvested cell culture fluid
HIC	hydrophobic interaction chromatography
PCR	polymerase chain reaction
TFF	tangential flow filtration
UFDF	ultrafiltration, diafiltration

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