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A non-isotopic probe-hybridization assay for residual DNA in biopharmaceuticals [1]

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Abstract

Although most biopharmaceuticals are highly purified, there is a theoretical concern that such recombinant products could be contaminated with oncogenic or bacterial DNA. A crucial part of the control of such biologicals is to ensure they do not contain more residual DNA than a safety limit suggested by the regulatory agency. Currently, the FDA has suggested a 100 pg per dose limit for residual DNA. DNA probes labeled with a radioisotope such as ^{32}P have been commonly used in hybridization tests. Because of the radiation safety concern, we chose to develop a procedure for assessing DNA levels by either a dot or slot blot hybridization technique using a nonisotopic DNA probe and immuno-enzymatic detection. A minimum detectable limit (MDL) of < 10 pg DNA mg^{-1} protein can be achieved. Method validation data demonstrated that the precision, reproducibility, and robustness of this approach are appropriate for quality control. © 1997 Elsevier Science B.V.

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1. Introduction

DNA assays are needed [1–5] in rDNA biopharmaceutical processes to demonstrate genetic stability, characterize the cell banks, validate the effectiveness of the purification process in DNA removal, and ensure the quality of the final bulk. Recently, a number of new analytical techniques, including several non-isotopic methods, have been reported for DNA determination [6–15]. Although in some cases these techniques can achieve single-copy DNA sensitivity, most of these detection techniques are designed for research purposes only and are not currency applicable to the development of assays for quality control or regulatory compliance. The literature about the analysis of nucleic acids in related biopharmaceuticals is very limited [16,17]. The analytical challenges of DNA assays in biopharmaceuticals are: (1) to achieve the sensitivity necessary for quantitating residual DNA at or near the method minimum detectable concentration (MDC); and (2) to have the robustness required for quality control.

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We have successfully developed methods to assay residual source DNA in therapeutic proteins utilizing a commercially available, non-isotopic DNA detection kit (SulfoPROBE kit, Sigma, St. Louis, MO). This kit provides mouse monoclonal antibody to sulfonated cytosine for immunochemical detection of sulfonated DNA. This approach was previously used to estimate the DNA content of nuclei in cultured rat lens epithelial cells [18] and to measure hepatitis B virus DNA in human serum [19]. In this paper, we demonstrate that this detection scheme can be tailored and optimized to assay residual DNA in biopharmaceuticals, and that the sensitivity and robustness demanded for ensuring product quality can be achieved using this approach. Briefly, following denaturation, the DNA probe is labeled by chemical modification, whereby cytosine moieties of DNAs are sulfonated using sodium bisulfite in the presence of methoxyamine [20]. The residual DNA extracted from the samples was denatured to single-stranded DNA and blotted on the nylon membrane. The DNA, cross-linked to the nylon membrane, was hybridized with the sulfonated DNA probe, which was then recognized using an antibody specific for sulfonated cytosines. A second antibody conjugated with alkaline phosphatase (AP) is used with a chromogenic substrate for the colorimetric reaction.

This paper discusses the parameters we used for method validation to ensure the robustness of the assays. A dot-blot format was used to determine residual DNA of *Escherichia. coli* origin in the Lispro insulin analog. A slot-blot format showed that better MDC can be achieved with a higher sample load when assaying residual DNA of mammalian cell line origin in activated protein C (aPC). We also demonstrated that this non-isotopic probe-hybridization approach can be used to determine DNA levels in the Lispro process samples. The assay data of the in-process samples, when combined with the corresponding final bulk assay data, can be used to effectively validate the DNA removal by the manufacturing process purification steps.

2. Experimental

2.1. Apparatus

The Bio-Dot SF apparatus was obtained from Bio-Rad (Melville, NY). Nylon membrane (0.45 μ) was purchased from Pall Biodyne (Glen Cove, NY). Heat sealable hybridization bags were from BRL (Gaithersburg, MD). The Stratalinker/UV crosslinker, Model 1800, was from Stratagene (La Jolla, CA). The SpeedVac concentrator was from Savant Instruments, (Farmingdale, NY).

2.2. Reagents

Heparin, salmon testes DNA, and the SulfoPROBE Kit, containing the following reagents—modification solutions A and B, anti-modified DNA (developed in the mouse, clarified ascites containing monoclonal antibodies), anti-mouse IgG alkaline phosphatase conjugate (antibody developed in goat), nitro blue tetrazolium (NBT), 5-bromo-4 chloro-3-indolyl phosphate (BCIP) in dimethylformamide (50 mg ml^{-1}), bovine serum albumin (BSA) and dextransulfate—were purchased from Sigma (St. Louis, MO). SDS, Tween-20, proteinase K, t-RNA, and DNase 1 were from Boehringer Mannheim (Indianapolis, IN). Chloroform and NaCl were from Mallinckrodt (Chesterfield, MO). Phenol–chloroform premixed with isoamyl alcohol was from Ameresco (Solon, OH). Glycerol, formamide, NaOH, $MgCl_2$, and EDTA tetrasodium salt were from EM Science (Gibbstown, NJ). Tris–HCl was from Fisher Biotech (Pittsburgh, PA). Reagent water was obtained from a Millipore Milli-Q water-purification system (Bedford, MA). All other chemicals were analytical reagent grade unless otherwise indicated.

2.3. Working solutions

All working solutions were sterile filtered by utilizing a sterile, low extractable membrane, 0.22 μm, mounted on a disposable polystyrene bottle from Corning (Corning, NY). These solutions were stored in the refrigerator and were stable for at least 1 month unless indicated otherwise.

Neutralization solution was 1.5 M NaCl and 0.5 M Tris–HCl, adjusted to pH 7.2. The denaturing solution was 1.5 M NaCl and 0.6 M NaOH. The hybridization solution was prepared by mixing 0.5 ml 2 M Tris–HCl, 2.8 ml 5M NaCl, 2.0 ml 50% dextran sulfate, 4.0 ml 5% SDS, 0.70 ml water and 10.0 ml deionized formamide. (To deionize formamide, mix 50 ml formamide with 5 g of mixed-bed resin, stir gently for 30 min at 4°C, and filter twice through Whatman No. 1 filter paper. Store in 10-ml aliquots at 20°C.) Blocking buffer was 50 mM Tris–HCl, 25 mM NaCl, 1 mM EDTA, tetrasodium salt and 0.3% (v/v) Tween 20; adjusted to pH 7.5. Blocking solution A was 28.6 mg heparin and 3.0 g Carnation natural nonfat dry milk dissolved in 10 ml blocking buffer, freshly prepared. Conjugation solution was 3 g Carnation natural nonfat dry milk dissolved in 10 ml blocking buffer, freshly prepared. 20X SSC was 3 M NaCl and 0.3 M sodium citrate, adjusted to pH 7.0. Washing solution No. 1 was 2X SSC containing 0.1% SDS. Washing solution No. 2 was 0.1X SSC containing 0.1% SDS. Washing solution No. 3 was 1000 ml water containing 3.0 ml Tween 20 and 30.0 g NaCl. Substrate buffer was O.1 M Tris–HCl, O.1 M NaCl, and 5mM $MgCl_2$, adjusted to pH 9.5. Chromogenic substrate solution was 3 mg nitroblue tetrazolium (NBT) and 40 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 10 ml substrate buffer. This solution was freshly prepared prior to use. The 0.5 M EDTA was adjusted to pH 8.0. TE buffer was 10 mM Tris–HCl and 1 mM EDTA. The 5 mg ml^{-1} t-RNA was prepared in sterile filtered water. The 5000 U ml^{-1} DNase 1 solution was prepared in 0.15 M NaCl, 50% glycerol, and 25 mM Tris buffer, adjusted to pH 7.0–8.0. The 70% and 95% ethanol were chilled prior to use.

2.4. Preparation of reference DNA

The reference DNA for Lispro experiments was prepared from cultures of proprietary *E. coli* strains. Single colonies were purified from the cell banks, phenotypically tested to verify identity, and grown in shake flask cultures. Whole cell DNA was isolated from the strain containing the Lispro expression plasmid. Host cell DNA was isolated from the host strain that did not contain the Lispro expression plasmid. Covalently closed circular Lispro expression plasmid DNA was purified by cesium chloride density gradient ultra centrifugation in the presence of propidium iodide [21]. Reference cell bank DNA for the aPC experiment was obtained from the manufacturer's working cell bank and purified in Dr B.W. Grinnell's laboratory [22,23] at the Lilly Research Laboratories. The concentration of reference DNA in the stock solution was determined by UV absorbance at 260 nm [21].

2.5. Preparation of sulfonated DNA probe

Host cell DNA, whole cell/cell bank DNA, and plasmid DNA were sulfonated separately as follows: On day 1, reference DNA (host cell DNA, plasmid DNA, or whole cell/cell bank DNA) was diluted in sterile filtered, purified water to a final concentration in the range of 0.1–0.5 μg μl^{-1}. The DNA was denatured by boiling for 5 min, and immediately chilled on ice for about 10 min. One volume (e.g. 1 ml) of modification solution A from the Sigma SulfoPROBE kit was added to one volume (e.g. 1 ml) of the above denatured DNA solution and it was mixed well by vortexing; then 0.5 volume (e.g. 0.5 ml) of modification solution B from the Sigma SulfoPROBE kit was added. This mixture was vortexed to mix it well and left at room temperature overnight. On day 2, 100 ng of t-RNA (5 mg ml^{-1}) per μl of modification mixture was added, followed by adding 2.5 volumes (e.g. 6.25 ml) of cold 100% ethanol, and this solution was kept at -20°C for about 2 h. The mixture was centrifuged at $15\,000 \times g$ at 4°C for 10 min, the supernatant was decanted, then the pellet was rinsed with cold 70% ethanol and again rinsed with cold 95% ethanol by gently rotating the tube. This pellet was evaporated in a SpeedVac Concentrator (Savant Instruments, Farmingdale, NY, or equivalent) until it was dry (for approximately 3 h). The resulting sulfonated DNA was dissolved in TE buffer (10 mM Tris HC1, pH 7.4 and 1 mM EDTA, pH 8.0) to achieve a final concentration of 100 μg sulfonated DNA ml^{-1}. (Note that the final concentration of

sulfonated DNA was based on the assumption of 50% DNA recovery. Because the sulfonated DNA probe was used in large excess to effectively probe a minute quantity of residual DNA, the precise concentration assignment of the probe solution is therefore not critical.) This sulfonated DNA solution was aliquoted in 100 μl per vial and stored at −20°C until use (stable at least 3 months).

2.6. Preparation of reference DNA calibration solutions

Three types of the reference DNA (whole cell or cell bank DNA) working standard solutions were used for assay evaluation: (1) absolute reference DNA working standard (labeled as A): These working standards were generated directly from the stock reference DNA solution by serial dilution in 50 mM Tris–HCl, pH 9.5. This set of standards had not been treated with proteins, enzymes, or any other reagents used in routine sample preparation procedure; (2) reference DNA working standards without protein compensation (labeled as NC): This set of standards should be free from protein matrix interference and served as a procedure control; (3) protein compensated reference DNA working solutions (labeled as C).

For the Lispro experiment, the reference DNA calibration solutions, NC and C, were used for assay evaluation. Stock solutions with DNA levels of 20, 10, 5, 2.5, 1, and 0 pg μl^{-1} in 50 mM Tris pH 7.4 were prepared; 5% SDS solution and proteinase K was added to give 0.1% SDS and a ratio of 1:25 (proteinase K:Lispro) by weight in the final solution. A sufficient amount of Tris buffer (50 mM, pH 9.5) was added to give a 1:5 dilution. DNA levels for the six non-compensated standards are 20, 10, 5, 2.5, 1, and 0 pg 5 μl^{-1} blot, respectively.

For the aPC experiment, protein-compensated, non-compensated, and absolute reference standards were used. The DNA levels were 100, 40, 20, 10, 5, 2, and 0 pg DNA per blot, corresponding to 25, 10, 5, 2.5, 1.25, 0.5, and 0 ppb DNA in protein.

2.7. Preparation of sample solutions

For the Lispro experiment, sample solutions were prepared at 25 mg ml^{-1} in 50 mM Tris pH 9.5 for assays. For aPC, a sample solution of 50 mg ml^{-1} in sterile filtered water was prepared. Proteinase K solution and 5% SDS solution were added to give 0.1% SDS and a ratio of 1:25 (proteinase K:protein) by weight in the final solution. The spiked sample ('S') of the Lispro experiment was prepared by adding 50 μl of the 5 pg μl^{-1} stock reference DNA to 200 μl aliquot of the 25 mg ml^{-1} Lispro solution (i.e. a spiking level of 50 pg mg^{-1} or 50 ppb DNA in Lispro). The nonspiked sample ('NS') was prepared by adding 50 μl of the 50 mM Tris pH 7.4 buffer to 200 μl aliquot of the 25 mg ml^{-1} Lispro solution. For aPC experiment, the reference DNA sample was spiked at 5 ppb.

2.8. Removal of protein interference

All sample and calibration solutions were digested with proteinase K overnight at 55°C. Then enzymes and any undigested proteins were extracted with phenol–chloroform, and finally, any remaining trace phenol was extracted with chloroform.

2.9. Assay design

Three types of reference DNA working standard solutions were used to evaluate the assay: absolute reference DNA (labeled as A), reference DNA working standards without protein compensation (labeled as NC), and protein-compensated reference DNA (labeled as C). Sample solution and sample solution spiked with reference cell bank DNA at the decision limit were blotted side-by-side on the membrane for comparison.

For the Lispro experiment, the dot-blot format was used. A 5 μl aliquot of sample (containing about 100 μg protein) or standard solution was blotted onto the membrane with a 20 μl size Pipetman (see Fig. 1 for the suggested pattern for reference standard and sample application). For the aPC experiment, the slot-blot format was used. A commercially available Bio-Dot SF ap-

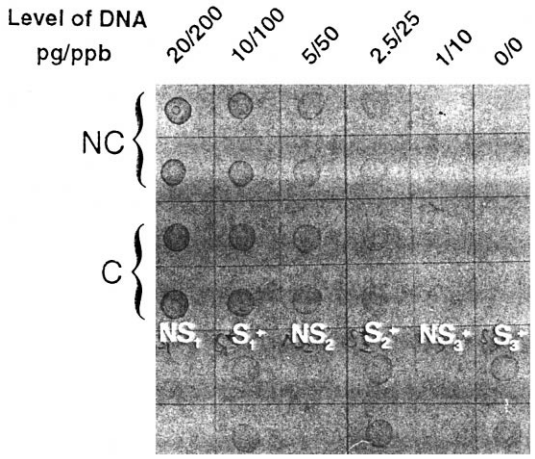

Fig. 1. Dot-blot residual DNA assay of LysPro samples NC (non compensated) is reference DNA with no protein added; C is reference DNA, compensated with 100 μg protein control; NS is non-spiked sample; S+ is sample spiked with 50 ppb DNA; S1, S2 and S3 are samples from bulk lots 1, 2, and 3.

paratus was used to prepare slot blots. A 500 μl aliquot of each DNA sample or standard solution was applied to each slot. The protocol suggested by the Bio-Dot manufacturer's package insert was followed for the sample application (see Fig. 2 for the suggested pattern for reference standard and sample application).

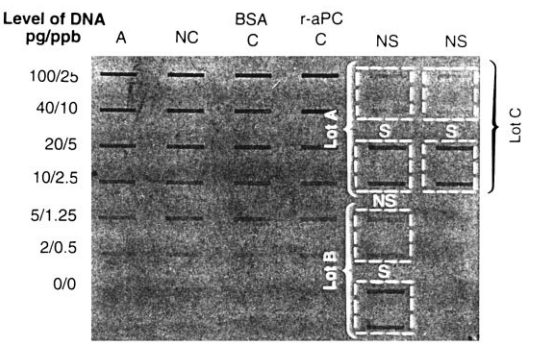

Fig. 2. Slot-blot residual DNA assay of r-aPC. A (absolute reference standard): DNA standard directly blotted on the membrane; NC: DNA reference standard, no protein added, which was used as a procedure control; BSA/C: DNA reference standard, compensated with BSA (each 500 μl blot containing 4 mg BSA); r-aPC/C: DNA reference standard, compensated with r-aPC (each 500 μl blot containing 4 mg r-aPC, control); NS: non-spiked r-aPC samples, each blot containing 4 mg of protein. As shown, the DNA level in non-spiked samples is <5 ppb. S: r-aPC samples spiked with 5 ppb reference DNA. Lots A, B, and C are r-aPC samples.

2.10. Denaturating and fixation of DNA onto the membrane

After the membrane was blotted with standard and sample solutions, it was air-dried. Then it was briefly soaked in the denaturing solution for about 1 min. After the membrane was removed from the denaturing solution and the excess solution was drained off, it was dipped in the neutralizing solution for about 1 min. After the membrane was removed from the neutralizing solution and the excess solution was drained off, it was air-dried (about 10 min).

The DNA on the membrane was further treated with 120 000 μ joules of UV irradiation (about 20–5 s) using a UV Crosslinker, Autolink mode. Finally, the membrane was placed on a glass plate and baked in an oven for 2 h at 80°C.

The above treatment ensured that the maximum amount of DNA blotted on the membrane was denatured into ssDNA and tightly cross-linked on the membrane.

2.11. Hybridization

After the membrane was treated with UV irradiation and baked at 80°C, it was placed in a plastic hybridization bag, and 75 μl of the hybridization solution and 0.75 μl of freshly denatured (about 20 min before use) salmon testes DNA per cm^2 of membrane were added. (The DNA was boiled about 5 min and then chilled quickly on ice for about 10 min to denature it.)

The bag was sealed, free of air bubbles, and incubated in a 40–45°C water bath for 1 h. The bag was then removed from the water bath and the previously denatured probe solutions were added. (Note the denaturing process is the same as described above.) A probe mixture of 1:1 (w:w) plasmid-DNA probe:reference cell bank DNA probe at the concentration range of 1.3–2.5 μg ml^{-1} was used for the Lispro experiments. A probe concentration range of 12–17 μg ml^{-1} of the hybridization solution was used in the aPC experiments. The incubation was continued in a water bath at 40–45°C overnight.

2.12. Post-hybridization immunochemical reaction and colorimetric visualization

The membrane was washed three times with approximately 250 ml of washing solution I, for about 10 min each time, on a shaker plate with gentle agitation. It was again washed three times with approximately 250 ml of washing solution II for about 10 min on a shaker plate. The membrane was then placed in a new plastic bag and treated with blocking solution A for 30 min at room temperature by adding into the bag 150 μl of blocking solution A per cm^{-1} of membrane. This blocking step was done on a shaker plate with gentle agitation. Then anti-modified DNA solution was added. The anti-modified DNA solution was prepared in the range 1/250–1/500 (v/v) anti-modified DNA in blocking solution A. The bag was resealed and incubated on the shaker plate for 1 h at room temperature. The membrane was washed three times with approximately 250 ml washing solution III for 10 min each time in a plastic box. This was done with gentle agitation on a shaker plate at room temperature. The membrane was then put in a fresh incubation bag and 150 μl conjugation solution per cm^2 of membrane was added. Then the anti-mouse IgG alkaline phosphatase conjugate solution was added. The anti-mouse IgG alkaline phosphatase conjugate solution was prepared in the range 1/750–1/1000 (v/v) enzyme conjugated antibody solution in conjugation solution. The bag was sealed and incubated on the shaker plate for 2.5–3 h at room temperature. The membrane was washed again following the same procedure as described above, but this step was repeated four times. In a new incubation bag, 150 μl of the chromogenic substrate solution was added per cm^2 of membrane. The bag, free of air bubbles, was placed on a glass plate, and incubated in a dark oven at 37°C until the spots at the lowest level (1 pg 10 ppb^{-1}) become well visualized. (The color developed in 5–15 min). This color development was critical; therefore, the membrane was checked frequently to avoid the color being overdeveloped. The bag was removed from the oven and the membrane was removed from the bag. The membrane was placed in a glass evaporation dish and the color reaction was stopped by rinsing the membrane for 30 s in ice-cold 95% ethanol. The ethanol bath was swirled frequently to ensure proper rinsing. After the membrane was removed from the ethanol solution, it was rinsed in cold water and air dried on a glass plate. The membrane was stored dry in the dark in a sealed plastic bag.

2.13. DNase treatment protocol

A research Lispro sample that contained trace residual DNA was used for this experiment. Protein samples (Lispro) with or without 50 ppb DNA added to the sample were incubated with DNase. The hybridization assay results of the DNase treated samples were compared to the assay results of samples without DNase treatment.

On day 1, approximately 25 mg Lispro was accurately weighed into a 2.0 ml sterile tube. An appropriate amount of 5% SDS solution and 50 mM Tris pH 9.5 was added to give 0.1% SDS and a Lispro concentration of 25 μg μl^{-1} in the final volume.

A 200 μl aliquot of the 25 μg μl^{-1} Lispro solution was transferred to each of four 2.0 ml sterile tubes, labeling them as tube 1, non-spiked sample (NS); tube 2, spiked sample (S*); tube 3, non-spiked sample treated with DNase (S + D); and Tube 4, spiked sample and treated with DNase (S* + D).

To tube 1, a 40 μl aliquot of 50 mM Tris pH 7.4 was added. To tube 2, a 30 μl aliquot of 50 mM Tris pH 7.4 and a 10 μl aliquot of the 25 μg μl^{-1} stock reference cell bank DNA were added. To tube 3, a 10 μl aliquot of 0.1 M $MgCl_2$, a 20 μl aliquot of DNase 1 (5000 U ml^{-1}), and a 10 μl aliquot of 50 mM Tris pH 7.4 were added. This tube was incubated in a 37°C water bath for 3 h. To tube 4, a aliquot of 10 μl 1.1 M $MgCl_2$, a 20 μl aliquot of DNase 1 (5000 U ml^{-1}), and a 10 μl aliquot of dhe 25 pg μl^{-1} stock reference cell bank DNA were added. This tube was incubated in a 37°C water bath for 3 h. After 3 h incubation of tube 3 and tube 4, proteinase K (20 mg ml^{-1}) was added to all the four tubes. The amount of proteinase K to be added should result in ratio of 1:25 by weight (proteinase K:protein).

Fig. 3. Effect of nuclease treatment. S is a protein sample (100 μg) containing trace residual DNA; S* is a sample spiked with 5 pg (50 ppb) DNA; S + D is a sample treated with DNAse; S* + D is a sample spiked with 5 pg (50 ppb) DNA and treated with DNAse.

All four tubes were incubated overnight in a 55°C water bath. The next day, samples were extracted by phenol–chloroform premixed with isoamyl alcohol and then with chloroform only. The extraction procedure was the same as the extraction described previously for standard and sample preparation. Fig. 3 shows the arrangement of the standards and samples on the membrane.

2.14. Selectivity of DNA probes

The three kinds of DNA, reference or whole cell DNA, plasmid DNA, and chromosomal DNA of *E. coli* host cell, with or without protein (Lispro) compensation, were blotted on three membranes for study of selectivity of various DNA probes.

The first membrane was hybridized with denatured-sulfonated host cell chromosomal DNA. The second membrane was hybridized with denatured-sulfonated plasmid DNA, and the third membrane was hybridized with a mixture of denatured-sulfonated reference whole cell DNA and plasmid DNA.

2.15. Size of DNA fragment

Southern blot technique [24] was performed to separate fragments of reference DNA by size. Reference DNA was digested by two enzymes (*Hae*III and *HpA*II) for 1–2 h. The Southern blot pattern was then transferred onto the nylon membrane and analyzed following the sample analysis section (Sections 2.10, 2.11 and 2.12) of this method.

2.16. Range study

A Plackett-Burman experimental design was performed to study the applicable range (upper range (+) and lower range (−)) of nine experimental parameters used in the Lispro study. Nine factors in the assay were chosen for the study (see Table 1 for the description of the setups).

The experiment was carried out utilizing 12 pieces of membrane. Samples were blotted on all 12 membranes and each membrane was treated with conditions that are various combinations of the selected nine factors.

2.17. Study of intermediate precision

Three lots of bulk substance were assayed by three analysts independently. Each analyst prepared one piece of membrane that was blotted with NC and C reference standard curves, as well as the unspiked and the 50 ppb spiked spots of the three lots of samples. The intermediate precision was evaluated by visually comparing the color intensity of both standard curves and of the unspiked and the 50 ppb spiked spots of the three lots of samples on the three membranes.

2.18. Assay of in-process samples

Sample volumes equivalent to 5 mg of protein were taken, and the samples were dried utilizing a SpeedVac (Savant) to remove organic solvent in the matrix. After reconstitution with 50 mM Tris buffer, the samples were centrifuged using Centricon-10 tubes to remove salts. Samples were then subjected to the same protein removal procedure as described in Section 2.8, then the aqueous portion was blotted on the nylon membrane. The blotted membrane was treated with alkaline solution and then, baked for 2 h at 80°C to denature the DNA. Finally, the probe mixture, sulfonated whole cell DNA plus plasmid DNA, was added for hybridization.

3. Results and discussion

3.1. Data acceptance and evaluation

A limit test was used to evaluate whether a sample had indeed passed a decision limit. A

Table 1
Plackett-Burman design for range study of nine experimental factors

Factors	Experimental parameters	Lower range (−)	Upper range (+)
*X*1	Baking time for membrane (h)	1.5	2.5
*X*2	Baking temperature for membrane (°C)	75	85
*X*3	Hybridization temperature (°C)	40	45
*X*4	Concentration of anti-sulfonated DNA (v/v)	1:500	1:250
*X*5	Concentration of anti-mouse IgG alkaline phosphatase conjugate (v/v)	1:1000	1:750
*X*6	Concentration of DNA probe (μg ml^{-1})	1.0	2.5
*X*7	pH of substrate solution	9.0	10.0
*X*8	Incubation time for complexation of anti-mouse IgG alkaline phosphatase conjugate (h)	2.5	3.0
*X*9	Incubation time of substrate solution (min)	10	15

Membrane	*X*1	*X*2	*X*3	*X*4	*X*5	*X*6	*X*7	*X*8	*X*9
1	+	−	+	+	+	−	−	−	+
2	−	−	+	−	−	+	−	+	+
3	−	+	−	−	+	−	+	+	+
4	−	+	−	+	+	+	−	−	−
5	+	−	−	+	−	+	+	+	−
6	+	+	+	−	−	−	+	−	−
7	−	+	+	+	−	−	−	+	−
8	+	+	−	−	−	+	−	−	+
9	−	−	+	−	+	+	+	−	−
10	−	−	−	+	−	−	+	−	+
11	+	−	−	−	+	−	−	+	−
12	+	+	+	+	+	+	+	+	+

sample was spiked with reference whole cell/cell bank DNA at the decision limit. Sample blots, with or without spiked reference DNA, were compared. For a sample to pass the test, the color intensity of the unspiked sample blot must be negligible when compared to the 50 ppb spiked sample blot. In addition, the color intensity of the 50 ppb spiked sample blot must be equivalent to the color intensity of the 50 ppb blot on the compensated standard curve. DNA levels in the samples can be estimated by comparing unspiked sample blots to the blots of the protein-compensated calibration standards. When the sample blot fell between two levels, the higher level was always reported as the worst-case result.

The following criteria must be met for assay data to be acceptable:

1. For both standard curves (non-compensated and compensated), each level must be visible and distinguishable from the next level.
2. Both zero-control spots on non-compensated and compensated standard curves must have no visually detectable signals.

3.2. Matrix effect and controls

The phenol–chloroform extraction efficiency can be evaluated by comparing the signals of absolute standard curve and non-compensated curve. The matrix effect can be evaluated by comparing the signals of protein-compensated and non-compensated curves. The matrix effect between these two standard curves is minimized by the extensive effort for removal of protein from both standard curves. First, the protein is

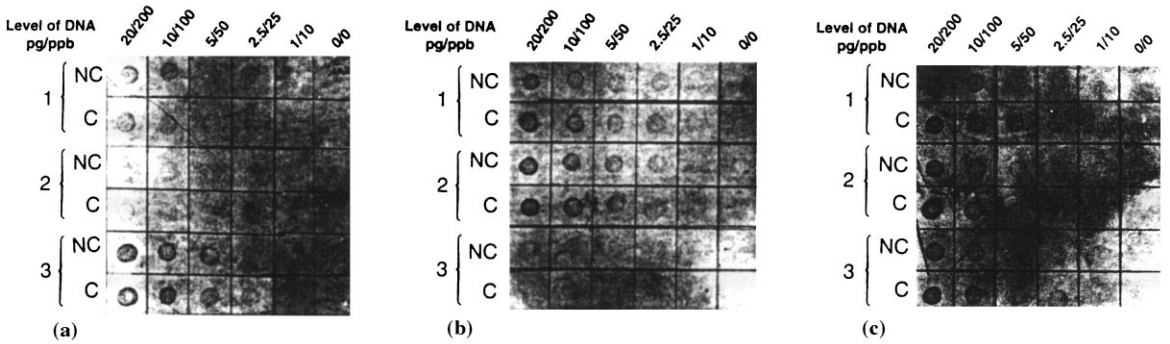

Fig. 4. Selectivity of (a) host cell DNA probes, (b) plasmid DNA probes, (c) a mixture of plasmid and whole cell DNA probes. NC (non-compensated) is reference DNA, no protein added; C is reference DNA, compensated with 100 μg protein control: (1) reference whole cell DNA; (2) plasmid DNA; (3) host cell chromosomal DNA.

removed by digestion with proteinase K overnight, then enzymes and any undigested proteins are removed using phenol–chloroform extraction, and finally, chloroform extraction is used to remove any remaining trace phenol. Therefore, there is no significant difference between the signals of protein compensated and non-compensated standard curves (Figs. 1 and 2). The aPC samples are treated the same way as the two standard curves for removal of protein and then are compared directly to the two standard curves. This treatment ensures that the results of the samples will account for any matrix effect.

3.3. Spike/recovery

The color intensity of the duplicate spots of the reference DNA spiked samples of three bulk lots on each membrane was nearly equivalent to the color intensity of the 50 ppb and 5 ppb spots of the compensated spots for Lispro and aPC, respectively. This result demonstrated that the spike/recovery of this method is excellent (Figs. 1 and 2).

3.4. Selectivity and detection of all specific source-DNA

The results showed that the host cell chromosomal DNA probe selectively hybridized the *E. coli* host cell DNA, as well as whole cell DNA which also contained the chromosomal DNA of *E. coli* host cell (Fig. 4A). The plasmid DNA showed only very faint color, which may be caused by trace host-cell chromosomal DNA co-purified during the preparation of plasmid DNA.

The results (Fig. 4B) showed that plasmid DNA probe selectively hybridized the plasmid DNA as well as whole cell DNA, which also contained plasmid DNA. The host cell chromosomal DNA showed only a very faint color (Fig. 4B). The plasmid DNA probe might contain trace amounts of host cell chromosomal DNA probe because the trace chromosomal DNA could be co-purified during the preparation of plasmid DNA.

In Fig. 4C the signal intensities of NC and C spots of three sources of DNA were the same. The results demonstrated that when whole cell DNA was used as reference DNA and a mixture of denatured-sulfonated whole cell DNA and plasmid DNA was used as probe as it was done in the routine protocol, both specific sources of DNA (plasmid DNA and chromosomal DNA of the host cell) can be detected.

All replicates of the membranes gave consistent results. The results of the third membrane (Fig. 4C) generated by both analysts demonstrated that this method would detect both plasmid and chromosomal DNA.

DNA probe used in this method is a mixture of sulfonated and denatured reference and plasmid DNA (1:1 mixture). Because DNA probes were used in excess (1.3–2.5 μg ml^{-1}), any trace level

of DNA specific to these probes should be detected.

3.5. Detection limit and the decision limit of the limit test

For the Lispro experiment, the detection limit was determined by spiking four different levels of source DNA (100, 50, 25 and 10 ppb) into three lots of Lispro bulk. The spiked samples were then assayed following the assay protocol (Sections 2.10, 2.11 and 2.12). This experiment was carried out by three analysts independently and each analyst produced an assay membrane. The detection limit of this method was determined to be 10 ppb after the evaluation of assay results of three lots of bulk material on all three membranes (data not shown).

The decision limit was set at 50 ppb of DNA in Lispro. This level is five times higher than the expected limit of detection (10 ppb) and less than the current regulatory limit (100 pg DNA per 0.5 mg of Lispro or 200 ppb. This amount of Lispro is equivalent to 14 U day^{-1}).

For the aPC experiment, the lowest DNA level that can be visualized on both the absolute standard curve and the no-protein-compensated standard curves is 2 pg DNA. The lowest DNA level that can be visualized on both aPC and BSA compensated standard curves is 0.5 pg DNA per mg protein (or 0.5 ppb) (Fig. 2).

3.6. The detectable size of reference DNA fragment

The assay results showed that reference DNA fragments as small as 300 base pairs was hybridized with a mixture of excess denatured-sulfonated reference DNA and plasmid DNA (data not shown).

3.7. Range study

There were no significant differences between the upper and lower range of six ($X3$, $X4$, $X5$, $X7$, $X8$, $X9$) out of nine factors studied. There are three factors ($X1$, $X6$, $X2$) in which significant differences were noted. Based on the results of this study and the previous method validation experience, the following range is suggested for the assay: The baking temperature for membrane should be between 80 and 85°C. The baking time should be in the range of 1.5–2.0 h. The probe concentration used for hybridization should be higher than 1 μg ml^{-1} (1.3–2.5 μg ml^{-1} was used for the assay).

3.8. Robustness

The independent assay of three lots of Lispro bulk substances performed by three analysts on three pieces of membranes gave consistent results. The robustness of the method was demonstrated.

3.9. Confirmation by DNase treatment

Four types of the samples were spotted on the membrane (Fig. 3): non-spiked samples (S), sample treated with DNase (S + D), 50 ppb DNA spiked sample (S*), and spiked samples plus DNase (S* + D). The results show that after DNase digestion, the DNA-spiked samples no longer exhibited the characteristic blue color that corresponds to the presence of DNA. This confirms that the blue signal is indeed due to the presence of DNA.

3.10. Process removal of DNA

The color intensity of the blots of the absolute calibration standards and the procedure calibration standards is comparable. We believe that no appreciable amount of DNA was lost during the extensive sample preparation/clean-up procedure. Therefore, the DNA levels in all the samples were estimated by directly comparing sample blots to the blots of the absolute calibration standards. To avoid the possibility that any residual component in the sample matrix would affect the assay, both spiked (spiked at the estimated detection limit) and nonspiked samples were analyzed and the resulting signals were compared. The signal intensity in several samples was inhibited. When we compared absolute calibration standards and spiked samples, an inhibited signal intensity for the spiked sample indicated a matrix interference.

Table 2
DNA levels in Lispro samples determined by dot-blot probe hybridization

Purification step	Trial 1 (ppb)	Trial 2 (ppb)	Trial 3 (ppb)	Trial 4 (ppb)
Composite solubilization	4 864 000	5 470 000	4 762 000	4 129 000
Step I	1000	5000	100	500
	500	1000	500	500
Step II	500	2500	500	100
	1000	1000	100	100
Step IIIA	100	100	100	100
	100	100	100	100
Step IIIB	100	100	100	100
	100	100	100	100
Step IV	<100	<100	<100	<100
	<100	<100	<100	<100
Bulk drug substance	<50	<50	<50	<50

IIIA and IIIB are identical purification steps of parallel process runs.

We did not use assay results where there was evidence of matrix interference.

In purification steps I, II, III(A), III(B), and IV, the spiked samples demonstrated excellent recovery, and there was a distinct difference in the signal intensity of spiked and nonspiked blots. The DNA levels of the samples were estimated by comparing them to the calibration curve. When the sample blot fell between two levels, the higher level (worst-case scenario) was always reported as the assay result. Samples from composite solubilization lots were diluted 10 000-fold for dot-blot DNA analysis. Therefore, the results estimated by visual comparison were multiplied by the dilution-fold to obtain the DNA levels in the original sample solutions. Assay results of the in-process samples as well as the final bulk substance of several campaigns are given in Table 2. These data demonstrated that DNA was effectively and consistently removed from Lispro bulk campaigns. The fact that all bulk lots made from the same manufacturing process passed the limit test—they contained less than 50 ppb of source DNA—further supported the process DNA removal validation.

4. Conclusions

We have demonstrated that utilizing sulfonated DNA probes in conjunction with immunochemical detection, trace residual source DNA in protein pharmaceuticals as low as 0.5 ppb can be detected. These methods can be optimized to have the specificity and robustness demanded for quality control. However, the hybridization procedure usually involves overnight incubation and multiple manipulations. In addition, extensive time and effort is also needed for removing interfering matrix protein prior to the hybridization step for a robust assay. The entire assay procedure normally took an experienced technician three days. To assay residual DNA in bulk protein for batch release is labor intensive, time consuming, and costly. An alternative strategy to effectively and meaningfully evaluate the removal of DNA in protein products is highly desirable [25]. Because DNA can be readily removed by modern production processes, a well-designed process removal validation protocol, with confirmation of a residual DNA assay for a select number (3–5) of representative production batches, should be ap-

propriate. The assays should be of appropriate quality and should use proper procedure controls to address the issues related to DNA removal. The laboriousness of the analysis technique and the frequency required for the assay should not be the primary emphasis. The frequency of residual DNA assay to confirm the removal of DNA should depend on faith in the process instead of faith in the assay. The batch release assays for control of residual DNA should not be necessary.

We have shown that this immunochemical detection following the hybridization of sulfonated DNA probes can be used to detect residual DNA in both bulk drug substances and in-process samples from key purification steps, demonstrating the effectiveness of DNA process removal.

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