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Short Communication

Quantitation of residual *Escherichia coli* DNA in recombinant biopharmaceutical proteins by hybridization analysis

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Introduction

Recombinant bovine somatotropin (rbSt) is a biopharmaceutical protein which is intended for parental administration to lactating dairy cattle, in order to increase milk yield and feed efficiency [1]. Since rbSt is expressed by a runaway plasmid which has been inserted into the bacterium *Escherichia coli*, potential rbSt purification process contaminants include host cell proteins, and nucleic acids. Currently, 10–100 pg of contaminating deoxyribonucleic acid (DNA) of host cell origin, per dose of a human parental biopharmaceutical, is considered to be an acceptably safe level [2]. It may not be necessary for parenterally administered veterinary recombinant biopharmaceuticals to achieve the level of purity which is implied by these guidelines.

Generic spectrophotometric, wet-chemical, chromatographic or electrophoretic/staining methods for characterizing nucleic acids lack appropriate sensitivity for quantitating process contaminant DNA, at the suggested levels, in bulk purified recombinant proteins. This is due to either an inherent lack of sensitivity, or to the one million to one billion-fold excess of interfering recombinant protein in the analytical sample. However, DNA hybridization analysis is an accepted method by which suitable quantitation of DNA at the one part-per-billion (ppb) level has been achieved [15]. The literature related to the analysis of nucleic acids in related biopharmaceutical samples is extremely limited [16], and not devoted to the development of quantitative assay methods. For analytical, as well as regulatory reasons, we have developed a slot-blot hybridization assay which is capable of detecting and quantitating 10 pg of residual *E. coli* DNA in rbSt. This assay requires the use of a specific plasmid probe which has been ^{32}P-labelled to high specific activity, and a characterized process-dependent mixed chromosomal–plasmid DNA standard.

Experimental

Apparatus

Slot-blots for hybridization were prepared using a standard Minifold II slot blotter (Schleicher and Schuell, Inc., Keene, NH, USA). Scintillation counts of ^{32}P-labelled DNA were obtained with an LS3801 scintillation counter (Beckman Instruments, Palo Alto, CA, USA). Hybridizations, and post-hybridization blot washes, were performed using a heating, rotating SciEra Hot Shaker waterbath (Bellco Biotechnology, Bellco Glass, Inc., Vineland, NJ, USA). Centrifugation of small volumes was performed with a Microfuge 12 (Beckman Instruments, Palo Alto, CA, USA) which was placed inside a refrigerator and equilibrated at 4°C. Autoradiograms [3] were obtained with a laser densitometer (LKB Produkter AB, Bromma, Sweden). Scan data were obtained and analysed by PEAKFIND and LINFIT programs with a VAX computer (Digital Equipment Corporation, Maynard, MA, USA).

Reagents and materials

Ammonium acetate, sodium acetate, sodium chloride, sodium hydroxide, magnesium chloride and high purity water were purchased from Mallinkrodt Chemical Co. (St. Louis, MO, USA). Ethanol and chloroform were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI, USA). Isoamyl alcohol was purchased from A. T. Baker Chemical Co. (Phillipsburg, NJ, USA). Sodium citrate, sodium dextran sulphate, sodium dodecyl sulphate (SDS), tris(hydroxymethylaminomethane) (Tris) and salmon sperm DNA (sspDNA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The sspDNA was repurified three times by phenol–chloroform extraction and cold ethanol precipitation [4]. Molecular biology grade phenol was purchased from International Biotechnologies, Inc. (New Haven, CT, USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Transfer ribonucleic acid (tRNA) was purchased from Boehringer-Mannheim, GmbH (Germany). The tRNA was repurified three times by phenol–chloroform extraction and cold ethanol precipitation before use [4]. Carnation instant non-fat dry milk was used for blot background suppression [9].

Mixed chromosomal–expression plasmid standard DNA (mcpDNA) was extracted [4] from proprietary rbSt expression plasmid-containing *E. coli* cells obtained from T. W. Evans, Bioprocess Engineering, The Upjohn Co. (Kalamazoo, MI, USA). The cells were harvested under full scale fermentation conditions, to assure the appropriate ratio of process-dependent plasmid DNA to chromosome DNA which would be expected for the random process contaminant DNA fragments found in rbSt bulk protein samples, as a result of a given expression and purification process. The DNA was sheared to an average of <2000 base pairs in length [5]. Plasmid-free chromosomal standard DNA (chrDNA) was extracted [4] from proprietary *E. coli* "parental" strain cells which were obtained from R. J. Kirschner, Bioprocess Research and Development, The Upjohn Co. (Kalamazoo, MI, USA). This chrDNA was sheared in the same manner [5]. Proprietary expression plasmid DNA (plsDNA) was obtained in a restriction-enzyme (single cut) linearized form from C. C. Tomich, Molecular Biology Research, The Upjohn Co. (Kalamazoo, MI, USA) and purified by cesium chloride gradient.

Nylon-66 filters (0.45 μm) were purchased from Rainin Instrument Co. (Woburn, MA, USA) and were used to filter all buffer solutions. Pre-cut Nytran blot-hybridization membranes (0.45 μm) were purchased from Schleicher and Schuell, Inc. (Keene, NH,

USA). Heat sealable bags for hybridization were obtained from Sears, Roebuck and Co. (Kalamazoo, MI, USA).

Alpha-^{32}P-dCTP and alpha-^{32}P-dGTP (each 3000 Ci mmol^{-1}) were purchased from DuPont-NEN (Boston, MA, USA). Nick translation kits were purchased from Bethesda Research Laboratories (Gaithersburg, MD, USA). Autoradiography cassettes, enhancement screens and X-O-MAT AR film were purchased from Kalamazoo X-Ray Sales (Kalamazoo, MI, USA).

Assay protocol

Samples (1 mg) of bulk lyophilized rbSt powder were each reconstituted with 500 μl of 0.01 M Tris, pH 8.0, in 1.5 ml polyethylene snap-cap microcentrifuge tubes. Carrier tRNA (500 ng) was added to each sample, followed by thorough vortex mixing. Each sample was extracted [4] with an equivalent volume of phenol (saturated with 0.01 M Tris, pH 8.0), accompanied by vigorous shaking for 10 min, at 25°C. Phase separation was achieved by centrifugation at 5000 *g* for 15 min, at 4°C. The aqueous phase of this separation was extracted with an equivalent volume of chloroform/isoamyl alcohol (20:1, v/v) accompanied by vigorous shaking for 10 min, at 25°C. Phase separation was achieved by centrifugation at 5000 *g* for 15 min, at 4°C. The aqueous phase (about 500 μl), which contained the extracted nucleic acids, was adjusted with 100 mcl of a buffer consisting of 2.5 M sodium acetate, 0.25 M sodium chloride, 0.05 M magnesium chloride, 0.25 M Tris (pH 7.5) [6]. Following the addition of 2–3 vol of 95% ethanol and thorough mixing, this sample was precipitated at −20°C for at least 6 h, and then centrifuged at 12,000 *g* for 45–60 min. (By this extraction method, in the absence of carrier tRNA, the recovery of nanogram quantities of standard mcpDNA which had been spiked into *E. coli*-DNA-free purified pituitary bovine somatotropin (pbSt) was <65%. However, with the addition of an excess of carrier tRNA, the recovery of various quantities of spiked standard mcpDNA, ranging from 10 pg to 5 ng, was virtually 100%.) The supernatant which resulted from the post-precipitation centrifugation was removed with a long-tipped Pasteur pipette and discarded, and each pellet was resuspended with 100 μl of 0.01 M Tris, pH 8.0, with vigorous vortex mixing.

Prior to blotting [7], the resuspended DNA samples and the mcpDNA standards which had been generated by serial dilution in 0.01 M Tris, pH 8.0 each received 10 μl of 3 M NaOH, followed by incubation at 65°C for 60 min. This treatment effected denaturation of double-stranded DNA to single strands. The samples were cooled to 25°C, and received 110 μl of 2 M ammonium acetate, pH 7.0. Each sample was applied to a well of the slot-blot device and filtered very slowly under vacuum, through a NYTRAN membrane which had been saturated previously with 1 M ammonium acetate (pH 7.0). After removing the excess moisture from the edge of the blot with filter paper, it was dried at 37°C for 60–90 min.

The dried blot was pre-hybridized in a sealed plastic bag at 65°C, in a heating–rotating waterbath, for 16 h. The pre-hybridization buffer consisted of 10–20 ml of the following: 10% sodium dextran sulphate, 1% SDS, 1 M sodium chloride [8], and 5 mg ml^{-1} of non-fat dry milk [9]. After that, 1–2 μg of probe plsDNA, which had been nick-translated to 2.0–2.5 × 10^8 cpm μg^{-1} and which was <24 h old [10], was mixed with 100 μg of re-purified sspDNA "carrier" in 1 ml of purified water. The probe mix was denatured by boiling for 10 min and added to the pre-hybridization buffer in the bag. The resealed bag was returned to the waterbath and hybridization was allowed to proceed for 16–18 h, at 65°C.

The blot was removed from the bag and washed [8] twice, for 5 min each wash, at 25°C, with 100 ml of 2 × SSC. (Stock 20 × SSC is: 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0 [11].) The blot was washed three times, for 30 min each wash, at 65°C, with 200 ml of 2 × SSC, 1% SDS. The blot received two final washes, for 30 min each wash, at 25°C, with 100 ml of 0.1 × SSC. It was dried at 25°C on a clean filter paper, and analysed by autoradiography at −70°C for 16–24 h [3]. The resulting autoradiogram was scanned by transmittance densitometry.

Results and Discussion

Certain practical considerations have influenced the choice of default parameters for the hybridization assays [12]. Firstly, the efficiency of hybridization of DNA strands which are in solution phase, with homologous DNA strands which have been adsorbed onto a solid phase, is only about 70%. Secondly, the approximate quantity of DNA extracted from any random rbSt sample may range from picograms to nanograms, and there may be many individual test samples per blot. Consequently, for quantitation purposes, the labelled probe must be in excess to insure saturation of all the target sequences. Thirdly, the many copies of an induced runaway expression plasmid may comprise more than 50% of the DNA in the host *E. coli* cell. Under a given set of hybridization parameters these few plasmid DNA gene sequences (each in many copies) will exhibit more favourable hybridization kinetics for themselves, than will the many chromosomal DNA gene sequences (each in relatively few copies) for themselves [12]. Therefore, relatively stringent default conditions (65°C) were chosen to maximize the plasmid sequence hybridization, and to minimize the already relatively low chromosomal sequence hybridization. Thus, sensitivity is enhanced by maximizing the specific activity of a labelled pure plasmid probe, instead of labelling a mixed plasmid–chromosomal DNA probe. True quantitation of total contaminant DNA is assured by rigorous comparison of the signal which is obtained from rbSt DNA extracts with an appropriate mixed chromosome–plasmid DNA standard curve. This assay standard should represent that DNA composition which would be found in production rbSt sample extracts, as a result of a given bulk protein purification protocol. The range of standard concentrations employed in this study was suitable for the quantitation of residual DNA in production lots of bulk rbSt.

Figure 1 is an autoradiogram of an example blot which has been probed with 1 μg (2×10^8 cpm) of ^{32}P-labelled expression plasmid B (plsB) DNA. Hybridization of this probe with the purified plsB DNA standards which were immobilized on the right side of the blot is excellent, with clear detection of the 10 pg standard. Depending upon exposure time the dynamic range of the X-ray film may be limited at either end of a given curve. However, a plot of log DNA concentration versus log densitometer peak scan area, for the 10 pg–1 ng linear region of this standard curve, had a slope of 1.02, an intercept of 4.2 and a correlation coefficient of 0.998. Triplicate determinations, from similar curves, at the 1 ng level, gave a relative standard deviation of 21–28%. In contrast, the mcpDNA curve (mixed chromosomal DNA and plsB DNA, left side of the blot, Fig. 1) was depressed by an average of 40%. The 10 pg standard was still detectable, but was outside the linear region for this exposure. This could be easily corrected by increasing the autoradiography exposure time. The linear region for this curve (50 pg–5 ng) had a slope of 1.28, an intercept of 3.76 and a correlation coefficient of 0.991. The depression in this response was attributed to the lack of hybridization of

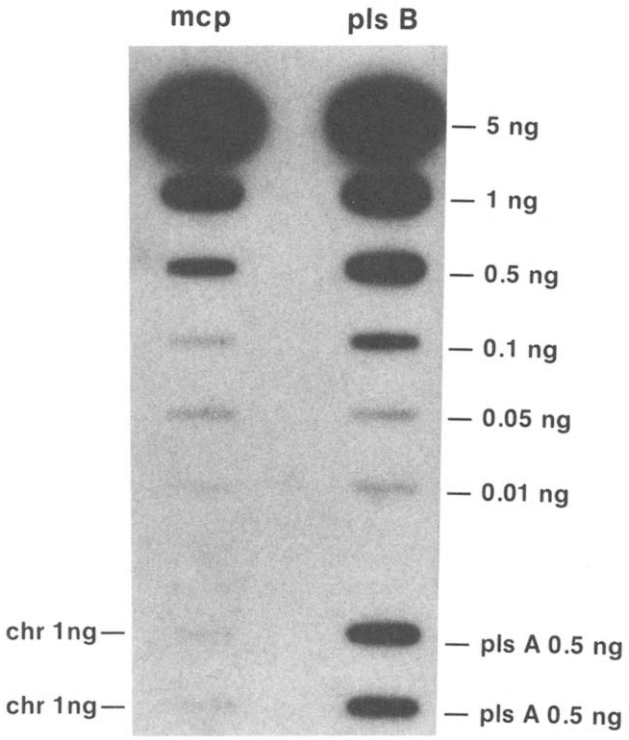

Figure 1
Autoradiogram obtained from a 24-h X-ray film exposure, of a blot which had been probed with 2×10^8 cpm (1 mcg) of ^{32}P-labelled expression plasmid B (plsB) DNA. Key to blotted test samples: plsB, rbSt expression plasmid B DNA dilutions; mcp, mixed *E. coli* chromosomal–plsB standard DNA dilutions; plsA, rbSt expression plasmid A DNA (which is partially homologous in sequence with plsB), duplicate samples; chr, purified *E. coli* chromosomal DNA, duplicate samples.

plsB probe with chromosomal DNA, which made up 40% of the total DNA in the standard. As one might predict, the cross hybridization of plsB with 1 ng chrDNA (lower left) was poor (≤0.6%). Cross hybridization of the probe with carrier tRNA was ≤0.0026% (not shown on this blot). The cross hybridization of plsB with plsA, a different, but partially sequence-related rbSt expressed plasmid (lower right), was only 36%. Under these conditions the sensitivity of the assay was strongly dependent upon the homology (sequence relatedness) of the plasmid probe for the appropriate plasmid component of the analyte DNA, and was minimally affected by the presence of chromosomal DNA or carrier tRNA. Quantitation of the extracted test samples for "total" DNA, through the use of an appropriate labelled plasmid of very high specific activity, was then dependent only upon comparison with a well characterized process-dependent plasmid–chromosome DNA standard. The standard must necessarily be obtained from a production scale batch of *E. coli* expressing the protein of interest (e.g. rbSt), from the appropriate plasmid. While suitably sensitive, and appropriately quantitative, this assay is not truly generic for total DNA quantitation, due principally to the specific nature of the probe. For each biopharmaceutical protein product, a separate plasmid probe and process-dependent DNA standard must be characterized. This is not a trivial undertaking when dealing with multiple plasmid expression systems, for multiple

biopharmaceuticals. The labour intensive nature of this type of methodology may be significantly improved, however, by betascanner data acquisition directly from the blots. A very recent standard curve (10 pg–5 ng, mean values of triplicate determinations), obtained in 3 h by direct betascan from a blot like the one used to obtain the autoradiogram in Fig. 1, had a slope of 1.17, an intercept of 4.77 and a correlation coefficient of 0.997 [J. E. Stanchfield, personal communication; 13, 14].

Very recent developments in the generic quantitation of DNA suggest that immunospecific detection and quantitation by biosensor [17], or by chemical modification and immunodetection [18], may soon become acceptably sensitive alternatives to hybridization analysis. The principal advantages which might make these desirable pharmaceutical analysis techniques, would be: the avoidance of radioactive isotopes, and decreased turn-around time for analytical samples, possibly as a result of automation of the method.

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