Short Technical Report

High-Sensitivity Hybridization Assay for Quantitation of Residual *E. coli* DNA

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ABSTRACT

*Impurity assays for recombinant protein therapeutics are essential to ensure batch-to-batch consistency and to meet the FDA's criteria for a well-characterized biopharmaceutical. For determination of residual host cell DNA, membrane hybridization assays utilizing radiolabeled DNA probes prepared from the host cell's genomic DNA have traditionally been used for products derived from bacterial expression systems to obtain the required low picogram sensitivity. Nonradioactive methods, while desirable to eliminate radioactive waste disposal and safety issues, typically suffer from poor sensitivity and high backgrounds. We report the development of a suitably sensitive, nonradioactive assay to quantitate residual *E. coli* DNA levels in purified protein drugs by means of a slot-blot hybridization method. The assay utilizes digoxigenin-labeled *E. coli* DNA probes and SuperSignal® chemiluminescent substrate. The optimized chemiluminescent hybridization assay has both low background and high sensitivity, allowing routine detection of 2.5 pg *E. coli* DNA. The method can be tailored for detection/quantitation of DNA contamination in recombinant protein products expressed in *E. coli* or other bacterial expression systems.*

INTRODUCTION

The Food and Drug Administration (FDA) requires testing for residual DNA for impurity analysis of recombinant biotherapeutics. The Center for Biologics Evaluation and Research (CBER) has recommended that drug products contain less than 100 pg/dose of residual DNA, as determined with an assay having a detection limit of at least 10 pg (2). Membrane hybridization (dot or slot-blot) utilizing radiolabeled genomic DNA probes has traditionally been used to determine host-cell-specific DNA (4,5,7,8); sensitivity equal to that obtained with total DNA assays (e.g., Molecular Devices Threshold) can typically be achieved. Higher-sensitivity methods such as quantitative PCR require dedicated instrumentation and synthesis of custom primers. While membrane hybridization remains the "gold standard", quantitation of picogram levels of host cell DNA using nonradioactive methodology has remained a challenge. Chemiluminescent, colorimetric, and fluorescent DNA labeling and detection systems currently provide several alternatives to ³²P labeling for hybridization analysis (9). However, high backgrounds are typically encountered with many of the substrates, thus limiting the sensitivity of the host cell DNA detection. In particular, it is very difficult to detect host cell DNA at picogram levels in prokaryotic expression systems via hybridization because of the lower number of repetitive sequences in the bacterial genome.

Here, we have developed and optimized a sufficiently sensitive chemiluminescent hybridization assay for quantitation of residual *E. coli* DNA in a protein therapeutic. The assay utilizes

E. coli DNA probes labeled with digoxigenin (DIG), an anti-DIG HRP conjugate, and a high-sensitivity chemiluminescent substrate. Various parameters including probe concentration, probe labeling, hybridization buffer, stringency washing, blocking, conjugate concentration, and detection reagents have been optimized as a system for high sensitivity and low background. This improved nonradioactive assay can be used as an alternative to ³²P-based methods for quantitation of residual DNA in recombinant protein drugs expressed in *E. coli* and may be adapted for use with other prokaryotic or mammalian expression systems.

MATERIALS AND METHODS

DIG-High Prime and DIG Easy Hyb were purchased from Roche Applied Science (Indianapolis, IN, USA). DigDETEK® HP HRP Membrane Detection System was purchased from Enzo Diagnostics (Farmingdale, NY, USA). SuperSignal® nucleic acid chemiluminescent substrate was purchased from Pierce Chemical (Rockford, IL, USA). Hybond™ N⁺ nylon membrane, Hyperfilm ECL® X-ray film for chemiluminescence, and ECL Direct Labeling and Detection kit were purchased from Amersham Biosciences (Piscataway, NJ, USA). Saline sodium citrate (SSC) buffer, Tris-buffered saline (TBS), TBS with 3% nonfat milk, TBS with 0.05% Tween® 20, Tris-EDTA buffer, and SDS were purchased from Sigma (St. Louis, MO, USA). The blood and cell culture DNA extraction and purification kit was purchased from Qiagen (Valencia, CA, USA). Slot-blot manifold and Bio Dot® SF filter paper

were purchased from Bio-Rad Laboratories (Hercules, CA, USA). PS-3 chemiluminescent substrate was purchased from Lumigen (Southfield, MI, USA). The Ultra Lum UVC-508 UV Crosslinker was obtained from Marsh Biomedical Products (Rochester, NY, USA). Hybridization ovens were from Boekel Industries (Feasterville, PA, USA). The Personal Densitometer SI was obtained from Molecular Devices (Sunnyvale, CA, USA).

ECL Direct DNA Labeling and Detection

E. coli genomic and plasmid DNA were co-purified from *E. coli* cells using a QIAGEN® genomic DNA purification kit and sheared to 300–2000 bp using a micro-tip sonicator. DNA was denatured by incubating at 100°C for 10 min, and serial dilutions were applied to a positively charged nylon membrane using a slot-blot manifold. DNA was fixed to the membrane using UV transillumination (120 000 microjoules). DNA hybridization, washing, and detection were performed as described previously (3).

General Method Using DIG-Labeled Probes

Purified *E. coli* DNA was denatured and slot-blotted to a positively charged nylon membrane as described above and then fixed by UV cross-linking. Probes were prepared by labeling DNA with DIG using a random-primed DNA labeling procedure (DIG-High Prime). DIG-labeled DNA probes bind to DNA on the membrane via overnight hybridization. The membrane was washed using high stringency conditions (defined below) to remove the unbound probe. The DNA hybrid was detected using an HRP-conjugated anti-DIG antibody and a chemiluminescent substrate. Emitted light was exposed to X-ray film, and the autoradiographic signal was quantitated using densitometry (volume integration).

Probe Concentrations for the Detection of *E. coli* DNA

E. coli DNA standards (200, 100, 50, 25, 10, 5, and 2.5 pg) were immobilized to the membrane according to the gener-

al method described above. Each third of the membrane was incubated with DIG-labeled probe at three concentrations (50, 500, and 1000 ng/mL, respectively) in DIG Easy Hyb. After overnight incubation, the membranes were washed twice for 5 min with 2× SSC, 0.1% SDS at room temperature, twice for 30 min with 0.1× SSC, 0.1% SDS at 42°C, and twice for 5 min with 2× SSC at room temperature. After washing, the DNA hybrid was detected using a DigDETEK HP HRP Membrane Detection System at 1:10 000 dilution and an ECL chemiluminescent substrate.

Effects of the Hybridization Buffer

A membrane containing immobilized *E. coli* DNA standards was divided into two sections, each being prehybridized and then hybridized in DIG Easy Hyb buffer and ECL Gold hybridization buffer containing 5% blocking agent (supplied with the ECL kit), respectively, using a 1 µg/mL probe concentration. Stringency washes and detection were performed as described above.

Effects of Different Blocking Reagents after Stringency Washings

A membrane containing the *E. coli* DNA standards was incubated with DIG-labeled probe at 1 µg/mL concentration in ECL Gold hybridization buffer containing 5% blocking reagent. The hybridization and washing were performed as described above. After stringency washing, each third of the membrane was incubated at room temperature with TBS containing BSA (2%), casein (3%), or nonfat dry milk (3%), respectively. The DNA hybrid was then detected using the DigDETEK HP HRP Membrane Detection System at 1:10 000 dilution and an ECL chemiluminescent substrate.

Effects of Detection Reagent Washing Buffers

The *E. coli* DNA immobilized membrane was incubated with DIG-labeled probe at 1 µg/mL concentration in ECL Gold hybridization buffer containing 5% blocking reagent. Hybridization and washing were performed as described above. The membrane was then

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blocked with nonfat dry milk (3%). Following a 1-h incubation in HRP-conjugated anti-DIG antibody (1:10 000), each third of the membrane was washed with Tween 20 (0.05%), SDS (0.5%), or the washing buffer from the DigDE-TEK Hp HRP Membrane Detection System, respectively, for 3×15 min and then 2×5 min in $2\times$ SSC buffer at room temperature. The DNA hybrid was detected using an ECL chemiluminescent substrate.

Comparison of Different Chemiluminescent Substrates

The *E. coli* DNA immobilized membrane was incubated with DIG-labeled probe at 1 $\mu\text{g/mL}$ concentration in ECL Gold hybridization buffer containing 5% blocking reagent. The membrane was hybridized and washed using the conditions described above. The membrane was incubated with nonfat dry milk (3%) for 1 h, followed by an HRP-conjugated anti-DIG antibody (1:10 000) for 1 h. After washing with detection reagent washing buffer, one third of the membrane was detected using ECL chemiluminescent substrate, SuperSignal NA chemiluminescent substrate, and Lumigen PS-3 chemiluminescent substrate, respectively.

Chemiluminescent Hybridization Assay for Quantitative Determination of *E. coli* Residual DNA in Recombinant Protein Drugs

The denatured *E. coli* DNA standards (100, 75, 50, 25, 10, 5, and 2.5 pg) in assay buffer ($6\times$ SSC) were immobilized, in triplicate, to the membrane using a slot-blot manifold. Protein samples for analysis included spiked samples containing 10 and 50 pg *E. coli* DNA. One milligram of each spiked and unspiked sample (in triplicate) was then extracted with a commercially available kit (DNA Extractor kit; WAKO Chemicals USA, Richmond, VA, USA) to remove the protein from the DNA. Extracted samples were denatured and applied in the last three slots, respectively. The membrane was cross-linked under UV light and pre-hybridized in 10 mL ECL Gold hybridization buffer with 5% blocking agent for 1 h at 42°C. Denatured DIG-

labeled probe was added to 10 mL hybridization buffer to obtain 1 $\mu\text{g/mL}$ final concentration, and the membrane was incubated in a shaking hybridization oven overnight at 42°C. Post-hybridization stringency washes were performed using the conditions described above. The membrane was blocked with nonfat dry milk (3%) for 1 h and incubated in HRP-conjugated anti-DIG antibody (1:10 000) for 1 h at room temperature. After washing three times with detection reagent washing buffer (TBS, pH 8.0, 0.05% Tween 20), SuperSignal chemiluminescent substrate was applied and the membrane incubated for 5 min at room temperature. The membrane was wrapped in clear plastic wrap and exposed to X-ray film designed for chemiluminescence. Typical film exposures were 1–5 min.

RESULTS AND DISCUSSION

Since the ECL Direct nucleic acid labeling method has been used and validated previously in our laboratory for detection of residual Chinese hamster ovary (CHO) DNA (3), this method was initially applied to the detection of *E. coli* DNA. It was noted that the sensitivity was extremely poor (>200 pg), though it was effective in detecting CHO cell DNA quite well [limit of detection (LOD) = 6.1 pg]. The marked difference in the detection limit between *E. coli* and CHO DNAs may reflect the nature of the DNA sequences between prokaryotic and mammalian species; high copy numbers of repetitive sequences may affect hybridization kinetics and sensitivity. Therefore, increasing the probe concentration may compensate for the slower kinetics of bacterial DNA hybridizations. To test this possibility, various *E. coli* DNA probe concentrations (10, 20, and 50 ng/mL) were used, followed by the ECL detection procedure. These probes were directly labeled with HRP. As expected, very faint bands were detected for 100 and 200 pg *E. coli* DNA when 10 ng/mL probe was used. At a higher probe concentration (20 ng/mL), the signal intensity was sharply increased for 100 and 200 pg DNA, and faint bands were observed for 25 and 50 pg *E. coli* DNA. However, when the probe concentration

increased to 50 ng/mL, the background was too high to see the hybridization signal. At this concentration, nonspecific binding may be occurring between the HRP probe and the membrane. These data suggested that a high probe concentration is able to “drive” the hybridization; however, the higher background associated with the high probe masks any resulting sensitivity increase. Using the direct labeling ECL system, we could not overcome the background associated with these high probe concentrations. Thus, to detect low levels of *E. coli* DNA maintaining a chemiluminescent system, a new method needed to be developed and optimized to overcome the high backgrounds associated with high probe concentrations.

Effect of Probe Concentration on *E. coli* DNA Detection Using a DIG-Labeled Probe

In an effort to reduce the background and increase the sensitivity, we developed an alternative nonradioactive hybridization method for *E. coli* DNA using an indirect system that employs DIG-labeled probes. *E. coli* DNA was labeled with DIG (as described in detail in the Materials and Methods section), and high concentrations of probe (50, 500, and 1000 ng/mL) were used to hybridize immobilized *E. coli* DNA ranging from 0 to 200 pg/slot. Less than 25

Figure 1. Comparison of DIG Easy Hyb and ECL Gold hybridization buffers using DIG labeled probe. (A) DIG Easy Hyb buffer. (B) ECL Gold buffer. Probe concentration used in both instances is 1 $\mu\text{g/mL}$. Each panel contains duplicate loadings.

pg/spot of *E. coli* DNA could be detected with a higher probe concentration (500 and 1000 ng/mL) labeled with DIG. Remarkably, the 1000 ng/mL probe allowed us to detect as low as 5 pg DNA. These data clearly demonstrated that probe concentration is one of the most critical factors to increase the sensitivity for the detection of *E. coli* DNA. However, high backgrounds remained an issue when using the standard DIG hybridization buffer (DIG Easy Hyb). Though we tried to increase the stringency wash temperature or de-

tergent (SDS) concentration, the background was still higher than desired (data not shown).

Effects of the Hybridization Buffer

To reduce the background when using high concentrations of probe, we investigated the effect of changing the hybridization buffer. Parallel experiments were carried out using DIG Easy Hyb buffer and ECL Gold hybridization buffer with 5% blocking agent using a 1 µg/mL DIG-labeled probe.

Figure 2. Comparison of post-stringency blocking agents. (A) Membrane blocked with 2% BSA. (B) Membrane blocked with 3% casein. (C) Membrane blocked with 3% nonfat dry milk. Each panel contains duplicate loadings.

Figure 3. Chemiluminescent hybridization assay for quantitative determination of residual *E. coli* DNA in recombinant protein drugs. Columns A, B, and C represent *E. coli* DNA standards; columns D, E, and F: row 1, extracted blank; row 2, 1 mg extracted protein drug (unspiked); row 3, 10 pg *E. coli* DNA spiked into 1 mg protein drug; row 4, 50 pg *E. coli* DNA spiked into 1 mg protein drug. Rows 5–7 represent a repeat of rows 2–4 using a different batch of protein drug.

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Table 1. *E. coli* DNA Spike Recovery from Two Batches of Recombinant Protein Drugs

Sample	Mean (<i>n</i> = 3)	%RSD	% Recovery
Batch 1 + 10 pg Spike	10.57	6.8	105.7
Batch 1 + 50 pg Spike	50.38	10.6	100.8
Batch 2 + 10 pg Spike	11.52	6.1	115.2
Batch 2 + 50 pg Spike	47.34	5.9	94.7

Spiked DNA was extracted from the protein drug before slot blotting and hybridization using the DNA Extractor kit. Hybridization with DIG-labeled *E. coli* DNA probes was carried out in ECL Gold hybridization buffer overnight at 42°C using a 1 µg/mL probe concentration. Following stringency washes and incubation in secondary antibody (HRP-labeled anti-DIG), SuperSignal chemiluminescent substrate was applied, and the membrane was exposed to film. Quantitation was achieved from the film response using a Molecular Devices Personal Densitometer using volume integration. %RSD, percent relative standard deviation.

Figure 4. Standard curve of the assay. The chemiluminescent response of the DNA standards on film was quantitated with a Molecular Dynamics Personal Densitometer using volume integration. Each data point represents the mean of triplicate determinations.

While a similar detection limit was observed under both conditions, the hybridization background was remarkably reduced with the ECL Gold buffer (Figure 1). It should be noted that this hybridization buffer failed to reduce background when using high concentrations of direct HRP-labeled probe as generated in the ECL Direct kit. In addition, because of the reduction of the background in the presence of the blocking reagent, the hybridization signal intensity was increased at all the DNA concentrations tested. These data

demonstrated that the choice of hybridization buffer could considerably improve the signal-to-noise ratio for *E. coli* DNA hybridization.

Comparison of Different Post-Stringency Blocking Reagents

The effects of various blocking reagents following post stringency washings were evaluated. Among these reagents tested, 3% nonfat dry milk produced the best hybridization quality for its low hybridization background

and improved signal (Figure 2). Though 3% casein produced a very low background, the signal was reduced as well. The presence of 2% BSA was unable to effectively block the nonspecific antibody binding, possibly because of poor binding of the BSA to the positively charged nylon membrane. Therefore, our results indicate that to improve the quality of *E. coli* DNA hybridization, an appropriate post-stringency blocking reagent must be chosen to optimize the hybridization and detection.

Optimization of Anti-DIG Antibody Concentration

The optimal dilution of anti-DIG Antibody for use in the assay was found to be 1:10000. This dilution provided appropriate sensitivity and low background; lesser dilutions resulted in higher backgrounds.

Comparison of Different Detection Reagent Wash Buffer

Various detection reagent washing buffers (containing 0.05% Tween 20, 0.5% SDS, or the buffer from the DigDETEK Hp HRP Membrane Detection System) were compared for the *E. coli* DNA hybridization and detection. Similar detection background and signals were observed using these different detection reagent wash buffers.

Comparison of Different Chemiluminescent Substrates

To optimize the assay, we compared three commercially available HRP chemiluminescent substrates (ECL, SuperSignal, and PS-3). While all three substrates generated a low hybridization background, the detection sensitivity was in the order of SuperSignal > PS-3 > ECL.

Chemiluminescent Hybridization Assay for Quantitative Determination of *E. coli* Residual DNA in Recombinant Protein Drugs

Figure 3 shows a representative exposure of our hybridization assay for residual DNA in recombinant protein drugs expressed in *E. coli*. Quantitation of the film by densitometry revealed the

standard curve to have a four-parameter fit with $R^2 = 0.999$ (Figure 4). The estimated LOD was 9.6 pg based on a four-parameter logistic model calculation (6), although visually the 2.5 pg standard is clearly discernable. This compares to an LOD of 5 pg for a similar *E. coli* DNA assay utilizing radiolabeled probes (5); however, the methods used to calculate the LOD are different. DNA spike recovery using 10- and 50-pg spikes was 95%–115% (Table 1). Other imaging and quantitation methods (such as a CCD camera) may be used (1), which may provide greater dynamic range than film and thus could change the curve fitting and LOD calculations.

We conclude that the most important parameter in the development of this nonradioactive hybridization assay was the use of a relatively high (1 $\mu\text{g/mL}$) probe concentration. In addition, the optimal hybridization results were obtained by applying the following factors, including the selection of the hybridization buffer: the use of 3% non-fat dry milk as the post-stringency blocking agent and the use of an appropriate anti-DIG HRP conjugate dilution (1:10 000). Many of the reagents come as part of chemiluminescent DNA labeling and detection kits supplied by a single manufacturer; our results suggest that best performance may not be achieved from a single kit, but rather by mixing and matching components from several different kits and optimizing all conditions for the probe/target system being used. Therefore, the described assay may not be best suited for mammalian DNA hybridizations, where the desired sensitivity can probably be achieved using lower probe concentrations. Overall, this non-isotopic hybridization assay for bacterial DNA is very sensitive, efficient, and convenient. It can be used as an excellent alternative to isotopic detection and is suitable for quantitation of residual DNA in recombinant protein drugs when the use of a hybridization assay is desired.

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