Quantitative Detection of Residual *E. coli* Host Cell DNA by Real-Time PCR

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**E. coli** has long been widely used as a host system for the manufacture of recombinant proteins intended for human therapeutic use. When considering the impurities to be eliminated during the downstream process, residual host cell DNA is a major safety concern. The presence of residual *E. coli* host cell DNA in the final products is typically determined using a conventional slot blot hybridization assay or total DNA Threshold assay. However, both the former and latter methods are time consuming, expensive, and relatively insensitive. This study thus attempted to develop a more sensitive real-time PCR assay for the specific detection of residual *E. coli* DNA. This novel method was then compared with the slot blot hybridization assay and total DNA Threshold assay in order to determine its effectiveness and overall capabilities. The novel approach involved the selection of a specific primer pair for amplification of the *E. coli* 16S rRNA gene in an effort to improve sensitivity, whereas the *E. coli* host cell DNA quantification took place through the use of SYBR Green I. The detection limit of the real-time PCR assay, under these optimized conditions, was calculated to be 0.042 pg genomic DNA, which was much higher than those of both the slot blot hybridization assay and total DNA Threshold assay, where the detection limits were 2.42 and 3.73 pg genomic DNA, respectively. Hence, the real-time PCR assay can be said to be more reproducible, more accurate, and more precise than either the slot blot hybridization assay or total DNA Threshold assay. The real-time PCR assay may thus be a promising new tool for the quantitative detection and clearance validation of residual *E. coli* host cell DNA during the manufacturing process for recombinant therapeutics.

**Keywords:** *E. coli* host cell DNA, real-time PCR, slot blot hybridization assay, total DNA Threshold assay

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The objective of this study is to develop a highly sensitive and specific detection method of E. coli host cell DNA using real-time PCR as an alternative means to the conventional slot blot hybridization assay and total DNA Threshold assay. In order to develop a convenient, rapid, and sensitive way of measuring the residual E. coli host cell DNA, a real-time PCR assay based on SYBR chemistry is proposed and will then be compared with the slot blot hybridization assay and the total DNA Threshold assay so as to validate the overall capability of these varying methods.

MATERIALS AND METHODS

Bacterial Strain and Culture Medium

The strain used in this study was E. coli KCTC 1102 harboring a plasmid pET 21b carrying the gene for the granulocyte colony-stimulating factor (Novogen Ltd., U.S.A.). The E. coli strain was grown in LB medium containing 50 µg/ml of ampicillin at 37°C.

Preparation of Genomic DNA

Genomic DNA was extracted from E. coli using an SV mini kit (General Bio System Inc., Korea) in accordance with the manufacturer’s instructions. DNA integrity and concentration were determined by spectrophotometric analysis at 260 nm and 280 nm (UV-1650 PC; Schimadzu Corp., Japan).

Primer Design and PCR Specificity Test

Oligonucleotide primers against the 16S rRNA gene (GenBank Accession No. J01859.1) were designed for the detection of E. coli DNA by real-time PCR using Primer3 [19]. The primers were synthesized by Bioneer Corp. (Korea). To determine the efficiency of the primers, genomic DNA extracted from E. coli was serially diluted 10-fold from 42,000 pg to 0.042 pg. A PCR reaction was then carried out with each primer pair using the templates of serially diluted genomic DNA. The PCR was performed in a Palm-Cycler (Corbett Research Ltd., Australia) using the following conditions: initial heat denaturation at 95°C for 2 min, followed by 40 cycles each of 95°C for 30 s, 54°C for 30 s, and 72°C for 35 s. Two µl of genomic DNA was amplified in a total volume of 25 µl mixture of 10 µM forward primer (1 µl), 10 µM reverse primer (1 µl), 2× GoTaq Green Master Mix (Promega Corp., U.S.A.) (12.5 µl), and nuclease free water (8.5 µl). To ensure complete extension, the reaction mixture was further incubated for 5 min at 72°C. Amplified DNA was analyzed by gel electrophoresis using a 1.5% (w/v) agarose gel (Sigma Corp., U.S.A.).

Optimization of Quantitative Real-Time PCR Assay

Real-time PCR was performed with a Rotor-Gene 3000 (Corbett Research Ltd., Australia) using the following conditions: an initial heat denaturation at 95°C for 15 min, followed by 40 cycles each of denaturation at 95°C for 10 s, annealing at different temperatures of 52, 54, 56, 58, or 60°C for 20 s, and an extension at 72°C for 30 s. Two µl of genomic DNA was amplified in a total volume of 20 µl mixture of a 10 µM forward primer (0.5 µl), a 10 µM reverse primer (0.5 µl), 4× AccuPower Greenstar PCR Premix containing Hot-Start Taq DNA polymerase, SYBR Green I, and deoxynucleotide triphosphate mix in a 5 µl quantity (manufactured by Bioneer Corp., Korea), and nuclease free water (12 µl). To ensure complete extension, the reaction mixture was further incubated for 10 min at 72°C. Immediately following PCR, a melting curve analysis was performed by raising the incubation temperature from 72 to 95°C in 0.2°C increments with a hold of 1 s at each increment. Real-time PCR conditions in relation to the primer concentration, annealing temperature and time, and MgCl₂ concentrations were optimized. Negative controls were run with each experiment. All reactions were run in duplicate.

Determination of Sensitivity and Reproducibility of Real-Time PCR Assay

To obtain a standard curve and to verify the sensitivity of the real-time PCR assay, serial 10-fold dilutions from 42,000 pg to 0.042 pg of E. coli genomic DNA, were amplified using the optimized conditions. A standard curve for quantification was generated by plotting the log of the DNA concentration of the known standard against the threshold cycle (Ct) value.