

Biotech Health Sci. 2014 November; 1(3): e26375.

Published online 2014 November 28.

Research Article

# Novel Real Time Polymerase Chain Reaction Approach for Rapid Detection of the Residual *Escherichia coli* Genomic DNA in Biopharmaceutical Products Establishment of Real Time Polymerase Chain Reaction to Detect Residual gDNA

Taghi Naserpour Farivar<sup>1,2</sup>; Babak Mamnoon<sup>1</sup>; Mohsen Karimi Arzenani<sup>3</sup>; Dariush Ilghari<sup>2,\*</sup><sup>1</sup>Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, IR Iran<sup>2</sup>Department of Clinical Biochemistry and Genetics, Qazvin University of Medical Sciences, Qazvin, IR Iran<sup>3</sup>Department of Molecular Medicine, Pasteur Institute of Iran, Tehran, IR Iran

\*Corresponding author: Dariush Ilghari, Department of Clinical Biochemistry and Genetics, Qazvin University of Medical Sciences, Qazvin, IR Iran. Fax: +98-283331007, E-mail: d.ilghari@gmail.com

Received: October 24, 2014; Accepted: October 24, 2014

**Background:** Contamination of therapeutic recombinant proteins with residual host cell DNA must be controlled under the regulatory standards.

**Objectives:** The current study established a new rapid, sensitive real time polymerase chain reaction (PCR) approach to measure the reliably of the residual *Escherichia coli* (*E. coli*) host cell genomic DNA in the recombinant streptokinase and alfa interferon preparations.

**Materials and Methods:** In this assay, a specific primer pair was utilized to amplify a 115 base pair sequence inside the *E. coli* 16S rRNA using SYBR Green Chemistry. This method enabled the authors to detect a very small quantity of the residual genomic DNA, as low as 0.8 pg, in the protein-based drugs. This method can, therefore, offer a dependable way to quantitatively analyze the major contaminant of biopharmaceutical products, the host cell DNA, during the manufacturing process.

**Results:** SYBR Green PCR master mix may contain a source of DNA contamination during its manufacturing process.

**Conclusions:** The current study data showed that *E. coli* host cell DNA contamination in streptokinase and alfa interferon manufactured in the Pasteur institute of Iran is much lower than the safety limits suggested by the FDA.

**Keywords:** Streptokinase; Alfa interferon; Real Time PCR

## 1. Background

Recombinant therapeutic proteins are extensively used as biopharmaceutical products for human therapies. They can be expressed in bacteria, yeasts, mammalian cells, or in transgenic plants and animals (1, 2). *Escherichia coli* is one of the most commonly used host cells to produce biopharmaceuticals due to its well-characterized genetic map, and rapid growth on inexpensive substrates (3-5). Despite the fact that employment of *E. coli* as an expression system has substantial advantages to manufacture therapeutic proteins, contamination of the bioproducts with the bacterial DNA is a major concern during the manufacturing process. Residual *E. coli* genomic DNA in biopharmaceuticals is considered as a potential risk factor (6). It is suggested that the DNA-based contaminants could act as antigens or immunogenic agents in the patients receiving the protein-based drugs. It is also hypothesized that the DNA impurities could be integrated into the genome of the recipient cells and express a new foreign gene, or alter the level of gene expression (7). It is, therefore, cru-

cial for the pharmaceutical industries to quantitatively monitor the DNA impurities (8) and keep the amount of the DNA contaminants in the bioproduct preparations as low as the safety limit suggested by the regulatory authorities such the food and drug administration (FDA) (9). Recently, real time polymerase chain reaction (PCR) assay is greatly aimed as a new method to detect and quantify PCR products simultaneously (10). Real time PCR is already employed to detect bacterial, fungal and viral loads in recombinant therapeutics (11-15). The current study established a new highly sensitive real time PCR approach based on SYBR Green Chemistry to detect a very small quantity of the residual *E. coli* host cell DNA in the recombinant streptokinase and alfa interferon samples produced in the Pasteur Institute of Iran. Streptokinase and interferon are recombinant protein-based drugs produced in *E. coli* host cells (16, 17). Streptokinase is clinically used as an intravenous thrombolytic agent to treat acute myocardial infarction (18). Interferons are a family of cytokines, acting as integral parts of the in-

nate immune response. As secreted ligands, interferons target specific cell surface receptors (19), eliciting the interferon-mediated antiviral response through four main pathways (20). Collectively, the obtained data showed that the innovative real time PCR method exploited, offers a precise reliable way to detect the *E. coli* DNA contaminants as low as 0.8 pg in the bioproduct preparations.

## 2. Objectives

The current study established a new rapid, sensitive real time Polymerase Chain Reaction (PCR) approach to measure the reliability of the residual *Escherichia coli* (*E. coli*) host cell genomic DNA in the recombinant streptokinase and alfa interferon preparations.

## 3. Materials and Methods

### 3.1. Experimental

#### 3.1.1. Cell Culture

*Escherichia coli* W3110 strain was provided by the Pasteur Institute, Iran. A single colony of this strain was inoculated into 5 mL of Luria-Bertani (LB) broth, and incubated overnight at 37°C, shaking at 250 rpm.

#### 3.1.2. Preparation of *Escherichia coli* Genomic DNA

The cells were recovered by centrifugation at 12 rpm for two minutes. Genomic DNA was purified using DNeasy Blood & Tissue kit (Qiagen Co., Iran) according to the manufacturer's protocol. DNA concentration was measured by ND-1000 spectrophotometric analysis at 260/280 nm (Biorad Co., USA).

#### 3.1.3. Primer Design and PCR Test

Four sets of oligonucleotide forward and reverse primers targeting the conserved region of the eubacterial 16S rRNA gene were designed using Primer Express software of the Applied Biosystems (Foster city Co., USA), and the sequence information was found in the NCBI GeneBank database (Accession Number: J01859). The primers were synthesized by Bioneer Corporation (Korea). PCR reactions were carried out in a master cycler personal (Eppendorf Co., Germany) using Ex Taq polymerase (TaKaRa Co., Japan) according to the manufacturer's instruction. The cycle parameters were as follows: initial heat denaturation at 94°C for one minute, followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. To ensure complete extension, the reaction mixture was incubated for five minutes at 72°C. The amplified DNA products were analyzed by gel electrophoresis on a 1.5% agarose gel (Sigma Corp Co., USA).

#### 3.1.4. Master Mix Preparation

Reaction mixture containing nuclease free water and SYBR Premix Ex Taq II (TaKaRa Co., Japan) was incubated with 0.1 U/rxn DNase I (Amersham Co., USA) and 0.5 mM MgCl<sub>2</sub>, for 60 minutes at 37°C. In order to inactivate DNase I, reaction mixture was heated at 65°C for 10 minutes.

#### 3.1.5. Optimization of the Real Time PCR

To determine the efficiency of the primers, *E. coli* genomic DNA was serially diluted five-fold. PCR was performed for each set of primer pairs using the serially diluted genomic DNA templates in a Perkin-Elmer 7300 sequence detection system (Applied Biosystem Co., USA). Optimum reaction conditions in a total volume of 20 µL were obtained with 10 pM forward primer (0.5 µL), 10pM reverse primer (0.5 µL), 2x SYBR Green PCR Master mix (TaKaRa Co., Japan) containing Taq DNA polymerase, SYBR Green I, Tris-HCl, MgCl<sub>2</sub>, KCl, and deoxynucleotide triphosphate (total volume of 10 µL), 0.4 µL ROX (6-carboxy-x-rhodamine), nuclease free water (7.6 µL), and 1 µL template genomic DNA. Amplification was performed with an initial heat denaturation at 95°C for one minute, followed by 40 cycles of denaturation at 95°C for 20 seconds, and combined annealing/extension at 60°C for 35 seconds in the 96-well optical plates (Applied Biosystems Co., USA). A mixture of nuclease free water and PCR master mix was used as non template control (NTC) in each experiment. All reactions were run in duplicate.

#### 3.1.6. DNA Extraction From Recombinant Therapeutic Proteins

Due to the presence of inhibitory agents in the final products, DNA was extracted from Active Pharmaceutical Ingredients (API) of streptokinase and alfa interferon. To achieve this purpose, five batches of each drug, produced in five successive weeks, were selected for DNA purification. After Phenol/Chloroform extraction, supernatant was transferred to a microcentrifuge tube, and DNA was precipitated using 400 mL/L isopropanol. After washing twice with 700 mL ethanol, DNA pellets were resuspended in nuclease free water.

#### 3.1.7. Detection of DNA Contamination

PCR was carried out using seven serially diluted *E. coli* genomic DNA templates, along with the DNA extracted from each streptokinase and alfa interferon. One microliter of each serially diluted *E. coli* genomic DNA, 1µL of the DNA extracted from streptokinase, and 1 µL of the DNA extracted from alfa interferon, in each reaction, were amplified separately in a total volume of 20 mixture containing 10 pM forward primer (0.5 µL), 10 pM reverse primer (0.5 µL), 2x SYBR Green PCR Master mix (10 µL), 0.4 µL ROX, and nuclease free water (7.6 µL). All reactions were run in duplicate. Non template controls were added in the experiment. Cycling conditions were one cycle at 95°C for

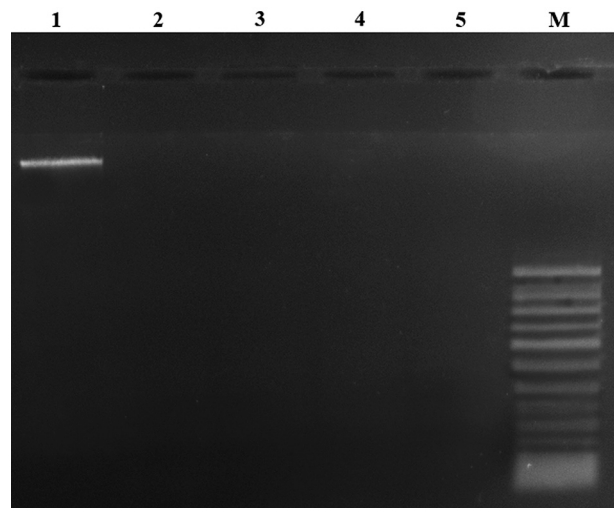
one minute, followed by 40 cycles at 95 °C for 20 seconds, and 60 °C for 35 seconds.

### 4. Results

#### 4.1. DNase I Treatment

SYBR Green PCR master mix may contain a source of DNA contamination during its manufacturing process. In order to find the best duration of DNase treatment to eliminate the initial contaminants, 0.1 U/rxn of the DNase I was incubated with 10 ng genomic DNA samples in 15, 30, 60, and 120 minutes, separately. In addition, 10 ng of the untreated genomic DNA was used as the negative control. Gel electrophoresis data showed that, in contrast to the negative control, all DNA samples were completely digested upon incubation with the enzyme during all different periods (Figure 1). To ensure the complete digestion, master mix was treated with DNase I (0.1 U/rxn) for 60 minutes, prior to each PCR performance.

**Figure 1.** Agarose Gel Electrophoresis of the PCR Products

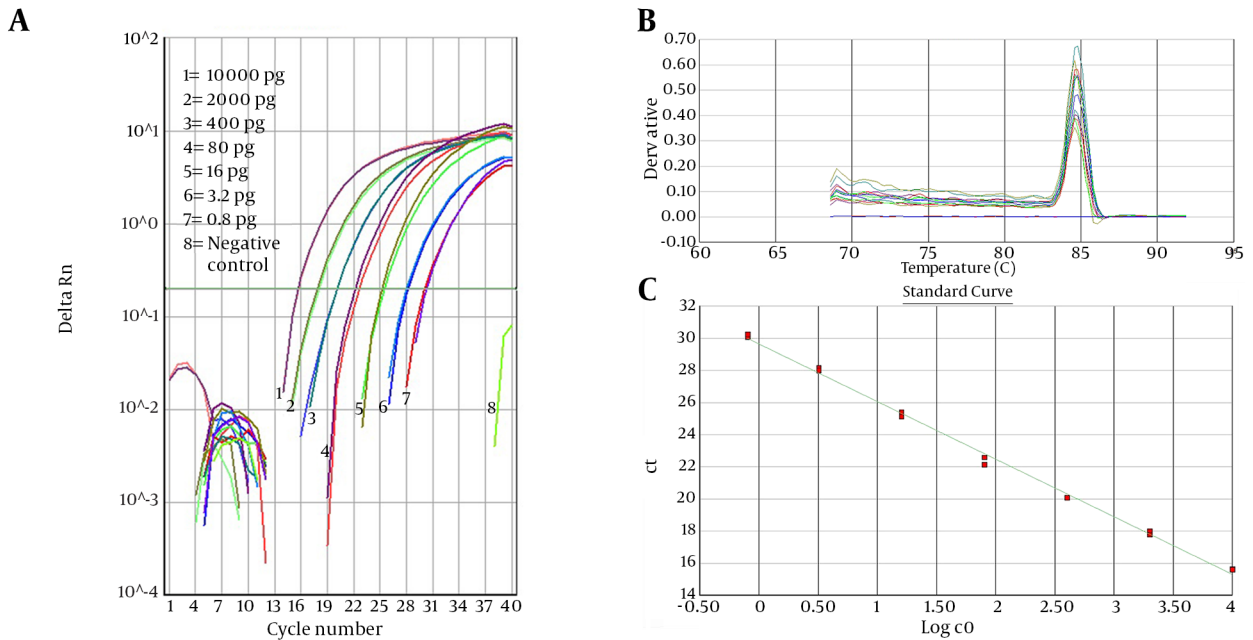


Lane 1, undigested sample; lane 2, digestion after 15 minutes; lane 3, digestion after 30 minutes; lane 4, digestion after 60 minutes; lane 5, digestion after 120 minutes; M, 100 bp ladder.

**Table 1.** Sequences of the Oligonucleotide Primer Pairs

Forward Primer	Reverse Primer	Nucleotide Position	PCR Product Size
<b>16S-F1</b> AGAAGCTTGCTCTTTGCTGA	16S-R1 CTTGGTCTTGCGACGTAT	78-197	120
<b>16S-F2</b> AAAGGAGACTGCCAGTGATA	16S-R2 AGGTCGCTTCTCTTTGTATG	1149-1263	115
<b>16S-F3</b> CATTGACGTTACCCGAGAA	16S-R3 CGCTTACGCCAGTAATCC	476-576	101
<b>16S-F4</b> CCATGAAGTCGGAATCGCTAG	16S-R4 ACTCCATGGTGTGACGG	1326-1419	94

**Figure 2.** Optimization of the Real Time PCR Approach to Measure Residual Host Cell DNA



A, amplification curve; B, melting curve analysis; C, standard curve.

### 4.2. Primer Selection

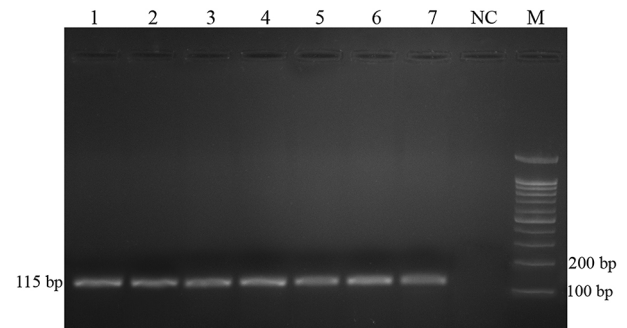
The oligonucleotide primers specific for the conserved region of the *E. coli* 16S rRNA gene were designed (Table 1.). Although, all primer pairs were amplified with the specific target regions, the primer pair 16S2 displayed a greater specificity and efficiency with minimal primer pair formation (data not shown).

### 4.3. Optimization and Sensitivity of Real Time PCR

Optimization of the real time PCR depends on several parameters such as primer and cation concentrations, annealing/extension temperature and time, and the length and sequence of template in the reaction mixture. In the current study, the optimal primer and Magnesium concentrations were 10 pM and 4 mM, respectively. The optimal annealing/extension temperature was 60 °C, and the annealing time was 35 seconds. With short amplified sequences (Amplicons), it may be sufficient to touch 95 °C and immediately start cooling (10). The sequence length selected in the current experiment was 115 bp. Seven serially 5-fold dilutions of *E. coli* genomic DNA from 10,000 pg to 0.8 pg were amplified, and the amplification curve was plotted (Figure 2 A). The sensitivity of this method was 0.8 pg. Melting curve analysis of the PCR products displayed the specific identification of the amplicons without any primer dimer formation (Figure 2 B). In order to quantify

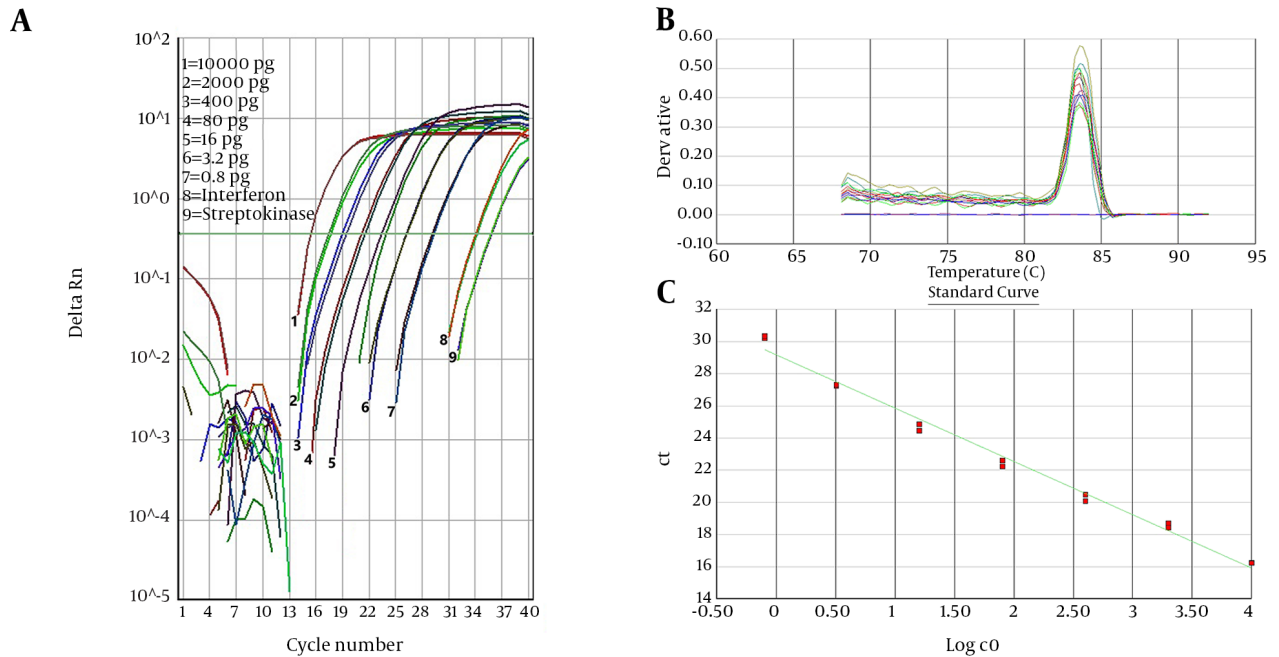
the products, standard curve was generated by plotting the logarithm of genomic DNA concentration versus cycle threshold (Ct) values (Figure 2 C). Agarose gel electrophoresis of the amplified products showed a 115 bp amplicon (Figure 3). The standard curve presented Ct values between  $15.62 \pm 0.028$  and  $30.06 \pm 0.126$ . Ct values were used to calculate PCR efficiency from the given slope in SDS 1.2.3 software according to the equation:  $Y = -3.58X + 29.65$  ( $R^2 = 0.996$ ). PCR efficiency =  $(10^{[-1/\text{slope}] - 1}) \times 100$ . The PCR efficiency was 94%.

**Figure 3.** Agarose Gel Electrophoresis of the Amplified Products



Lane 1, 10'000 pg; lane 2, 2000 pg; lane 3'400 pg; lane 4, 80 pg; lane 5, 16 pg; lane 6, 3.2 pg; lane 7, 0.8 pg; lane 8, negative control.

**Figure 4.** Accuracy of the Real Time PCR Approach to Measure DNA Contamination in Biopharmaceuticals



A, amplification curve; B, melting curve analysis; C, standard curve.

**Table 2.** DNA Content of Recombinant Protein-Based Drugs

Recombinant Protein	Drug Concentration, mg/mL	DNA Content, pg/mL of Product
Streptokinase	1.2	0.005
Alfa interferon	1.1	0.015

#### 4.4. Amplification of the DNA Extracted from Streptokinase and Interferon

Serially diluted *E. coli* genomic DNA preparations, one pair of negative controls, and the DNA extracted from streptokinase and alfa interferon preparations were run in duplicate (Figure 4 A). Melting curve analysis of the PCR products did not show any primer pair formation (Figure 4 B). The standard curve presented Ct values between  $16.23 \pm 0.000$  and  $30.22 \pm 0.101$  (Figure 4 C). PCR efficiency was calculated from the given slope in SDS 1.2.3 software according to the equation:  $Y = -3.31X + 29.16$  ( $R^2 = 0.99$ ). The PCR efficiency was 100%.

#### 4.5. Quantitative Detection of Residual Host Cell DNA

The mean of the two Ct values (mCt) obtained from the duplicate amplification of DNA extracted from each streptokinase and alfa interferon, were calculated separately. The mCt values (36.89 and 35.14 from streptokinase and alfa interferon, respectively), were put instead of Y values in the standard curve equation ( $Y = -3.31X + 29.16$ ), to achieve X values. After getting the common logarithm of X values, the estimated amount of residual *E. coli* host cell DNA was achieved from the recombinant therapeutic proteins (Table 2). The values calculated from the experiments, were much lower than those of the permitted by the FDA (9).

## 5. Discussion

Recombinant therapeutic proteins are extensively used for the human treatment purposes. Host cell genomic DNA contamination in biopharmaceuticals is a great concern. Hence, the FDA and other regulatory agencies have provided specific quality control and safety criteria, which require the manufacturers to quantify DNA impurities in all recombinant products at intermediate stages in the process, as well as on the final products (21, 22). The method involved to measure contaminations must be accurate and sensitive enough to determine the minimum level of impurities. For years, expensive radioactive-based methods, including the species-specific DNA hybridization assay were used to quantify the amount of residual host cell DNA in biopharmaceuticals (23). Ji et al. reported a safer nonradioactive slot-blot hybridization assay to quantify residual *E. coli* DNA levels in purified protein drugs with the sensitivity to detect 2.5 pg *E. coli* DNA (24). Subsequently, Gregory et al. described a safer and less ex-

pensive alternative approach, i.e. PCR amplification and dioxygenin labeling of the genes encoding 5S rRNA followed by an affinity-based collection to detect 1pg per ml of the extracted *E. coli* genomic DNA (25). Although the Gregory's detection method seemed very safe and sensitive, it was very labor-intensive to be applied routinely in the biopharmaceutical companies. This made investigators to search for a more inexpensive and feasible approach that could be applied readily in the companies. Real time PCR is recently reported as an alternative to detect and quantify DNA sequence in a manufacturing product simultaneously. This method, however, was not employed routinely in the pharmaceutical industries mainly because the real time PCR reactions were inhibited by various inhibitors present in the biopharmaceutical products (26). The current study established a novel and rapid detection approach for a precise quantitative measurement of residual *E. coli* host cell DNA in the two widely used recombinant protein-based drugs, i.e. streptokinase and alfa interferon. This method, which was based on real time PCR technique and SYBR Green Chemistry, enabled the authors to reliably detect very small quantities of the genomic DNA, as low as 0.8 pg, in one milliliter of the recombinant protein preparations (Figure 2 D). The high sensitivity and specificity of this approach are most probably due to the fact that 16S rRNA gene is present in multiple copies in the genome of all known bacteria, belonging to the eubacterial kingdom (27). Although probes, including those labeled with fluorescent dyes or scorpion ones could have been applied to significantly enhance the sensitivity of real time PCR (28, 29), the SYBR Green technology was exploited to establish a reasonably sensitive inexpensive detection approach. It is known that primer dimer formation is one of the major problems with the SYBR Green use. To avoid this problem, the real time PCR reaction condition was optimized by designing accurate primers, finding optimal cation (4 mM) and primer concentration (10 pM), and optimal annealing temperature and time (60 °C for 35 seconds). It is also noteworthy that when the real time PCR reactions were initially utilized to quantify the DNA contaminants, the reactions were restricted by inhibitor(s). To eliminate the inhibitors, the residual host *E. coli* DNA was first extracted from the Active Pharmaceutical Ingredients (API) of biopharmaceuticals and then subjected to the PCR reactions. Moreover, to preclude any DNA cross-contamination, i.e. to remove any possible DNA contaminants other than the host genomic *E. coli* DNA, the master mixtures used for the real time PCR reactions were

treated with DNase I prior to the reactions. In summary, the current study succeeded to establish a new real time PCR approach based on SYBR Green Chemistry to amplify highly conserved sequences of the *E. coli* 16S rRNA gene with a detection accuracy of 0.8pg genomic DNA per ml of the recombinant protein-based drugs. The sensitivity of the current detection method is three times more than that of offered by the slot blot hybridization assay (24). The current study data showed that *E. coli* host cell DNA contamination in streptokinase and alfa interferon manufactured in the Pasteur institute of Iran is much lower than the safety limits suggested by the FDA.

## Acknowledgements

This project was supported by the Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Iran. Authors would like to offer their special thanks to Dr. Ali Khamesipour helped to improve the final version of the manuscript. Authors are also grateful to all the staff members of the Molecular Medicine Department of the Pasteur institute of Iran, Tehran.

## Funding/Support

This project was supported by the Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Iran.

## References

- Rasala BA, Muto M, Lee PA, Jager M, Cardoso RM, Behnke CA, et al. Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. *Plant Biotechnol J*. 2010;**8**(6):719-33.
- Swiech K, Picanco-Castro V, Covas DT. Human cells: new platform for recombinant therapeutic protein production. *Protein Expr Purif*. 2012;**84**(1):147-53.
- Baneyx F. Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotechnol*. 1999;**10**(5):411-21.
- Jana S, Deb JK. Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Appl Microbiol Biotechnol*. 2005;**67**(3):289-98.
- Swartz JR. Advances in *Escherichia coli* production of therapeutic proteins. *Curr Opin Biotechnol*. 2001;**12**(2):195-201.
- Lahijani R, Duhon M, Lusby E, Betita H, Marquet M. Quantitation of host cell DNA contaminate in pharmaceutical-grade plasmid DNA using competitive polymerase chain reaction and enzyme-linked immunosorbent assay. *Hum Gene Ther*. 1998;**9**(8):1173-80.
- Walsh G. *Proteins: Biochemistry and Biotechnology*. West Sussex: John Wiley & Sons, Inc; 2002.
- Rathore AS, Sobacke SE, Kocot TJ, Morgan DR, Dufield RL, Mozier NM. Analysis for residual host cell proteins and DNA in process streams of a recombinant protein product expressed in *Escherichia coli* cells. *J Pharm Biomed Anal*. 2003;**32**(6):1199-211.
- Riggin A, Luu VT, Lobdell JK, Wind MK. A non-isotopic probe-hybridization assay for residual DNA in biopharmaceuticals. *J Pharm Biomed Anal*. 1997;**16**(4):561-72.
- Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, et al. The real-time polymerase chain reaction. *Mol Aspects Med*. 2006;**27**(2-3):95-125.
- de Wit C, Fautz C, Xu Y. Real-time quantitative PCR for retrovirus-like particle quantification in CHO cell culture. *Biologicals*. 2000;**28**(3):137-48.
- Wolf JJ, Wang L, Wang F. Application of PCR technology in vaccine product development. *Expert Rev Vaccines*. 2007;**6**(4):547-58.
- Jeong HS, Shin JH, Park YN, Choi JY, Kim YL, Kim BG, et al. Development of real-time RT-PCR for evaluation of JEV clearance during purification of HPV type 16 L1 virus-like particles. *Biologicals*. 2003;**31**(3):223-9.
- Martins SA, Prazeres DM, Cabral JM, Monteiro GA. Comparison of real-time polymerase chain reaction and hybridization assays for the detection of *Escherichia coli* genomic DNA in process samples and pharmaceutical-grade plasmid DNA products. *Anal Biochem*. 2003;**322**(1):127-9.
- Mohammadi T, Savelkoul PH, Pietersz RN, Reesink HW. Applications of real-time PCR in the screening of platelet concentrates for bacterial contamination. *Expert Rev Mol Diagn*. 2006;**6**(6):865-72.
- Mahmoudi S, Abtahi H, Bahador A, Mosayebi G, Salmanian AH. Production of recombinant streptokinase in *E. coli* and reactivity with immunized mice. *Pak J Biol Sci*. 2010;**13**(8):380-4.
- Babaeipour V, Shojaosadati SA, Maghsoudi N. Maximizing Production of Human Interferon-gamma in HCDC of Recombinant *E. coli*. *Iran J Pharm Res*. 2013;**12**(3):563-72.
- Karimi Z, Babashamsi M, Asgarani E, Niakan M, Salimi A. Fermentation, fractionation and purification of streptokinase by chemical reduction method. *Iran J Microbiol*. 2011;**3**(1):42-6.
- Fensterl V, Sen GC. Interferons and viral infections. *Biofactors*. 2009;**35**(1):14-20.
- Sadler AJ, Williams BR. Interferon-inducible antiviral effectors. *Nat Rev Immunol*. 2008;**8**(7):559-68.
- Wang KY, Guo YJ, Sun SH, Shi K, Zhang S, Wang KH, et al. 16S rRNA gene probe quantitates residual host cell DNA in pharmaceutical-grade plasmid DNA. *Vaccine*. 2006;**24**(14):2656-61.
- Tatalick LM, Gerard CJ, Takeya R, Price DN, Thorne BA, Wyatt LM, et al. Safety characterization of HeLa-based cell substrates used in the manufacture of a recombinant adeno-associated virus-HIV vaccine. *Vaccine*. 2005;**23**(20):2628-38.
- Wolter T, Richter A. Assays for controlling host-cell impurities in biopharmaceuticals. *Bioprocess Int*. 2005;**3**(2):40-6.
- Ji X, Lee K, DiPaolo B. High-sensitivity hybridization assay for quantitation of residual *E. coli* DNA. *Biotechniques*. 2002;**32**(5):1162-7.
- Gregory CA, Rigg GP, Illidge CM, Matthews RC. Quantification of *Escherichia coli* genomic DNA contamination in recombinant protein preparations by polymerase chain reaction and affinity-based collection. *Anal Biochem*. 2001;**296**(1):114-21.
- Wilson IG. Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol*. 1997;**63**(10):3741-51.
- Brosius J, Dull TJ, Sleeter DD, Noller HF. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J Mol Biol*. 1981;**148**(2):107-27.
- Singh J, Batish VK, Grover S. A scorpion probe-based real-time PCR assay for detection of *E. coli* O157:H7 in dairy products. *Foodborne Pathog Dis*. 2009;**6**(3):395-400.
- Wang Y, Li Y, Yang C, Hui L, Han Q, Ma L, et al. Development and application of a universal Taqman real-time PCR for quantitation of duck hepatitis B virus DNA. *J Virol Methods*. 2013;**191**(1):41-7.