

Quality Control of Widely Used Therapeutic Recombinant Proteins by a Novel Real-Time PCR Approach

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ABSTRACT

Background: Existence of bacterial host-cell DNA contamination in biopharmaceutical products is a potential risk factor for patients receiving these drugs. Hence, the quantity of contamination must be controlled under the regulatory standards. Although different methods such as hybridization assays have been employed to determine DNA impurities, these methods are labor intensive and rather expensive. In this study, a rapid real-time PCR test was served as a method of choice to quantify the *E. coli* host-cell DNA contamination in widely used recombinant streptokinase (rSK), and alpha interferon (IFN- α) preparations. **Methods:** A specific primer pair was designed to amplify a sequence inside the *E. coli* 16S rRNA gene. Serial dilutions of DNA extracted from *E. coli* host cells, along with DNA extracted from Active Pharmaceutical Ingredients of rSK, and IFN- α samples were subjected to an optimized real-time PCR assay based on SYBR Green chemistry. **Results:** The test enabled us to detect a small quantity of genomic DNA contamination as low as 0.0002 pg in recombinant protein-based drugs. For the first time, this study showed that DNA contamination in rSK and IFN- α preparation manufactured in Pasteur Institute of Iran is much lower than the safety limit suggested by the US FDA. **Conclusion:** Real-time PCR is a reliable test for rapid detection of host-cell DNA contamination, which is a major impurity of therapeutic recombinant proteins to keep manufacturers' minds on refining drugs, and provides consumers with safer biopharmaceuticals. **DOI: 10.7508/ibj.2016.01.008**

Keywords: DNA contamination, Real-time PCR, Streptokinase, Interferon-alpha (IFN- α)

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INTRODUCTION

Therapeutic recombinant products are extensively used for human medical purposes. Recombinant proteins can be expressed in various types of living organisms^[1,2]. *Escherichia coli* is one of the most commonly used host cells for the manufacture of biopharmaceuticals due to its well-characterized genetic map^[3-5]. Although using *E. coli* for the production of therapeutics has many advantages, contamination of biopharmaceuticals with bacterial DNA has become a major concern for manufacturers.

Genomic DNA contamination in biopharmaceuticals has been considered as a possible risk factor^[6]. It has been supposed that DNA contaminants may be integrated into the genome of patient's recipient cells and express a new foreign gene or alter the level of gene expression^[7].

It is, therefore, crucial for biopharmaceutical industries to monitor DNA contamination^[8] and keep the quantity of impurities in bioproducts as low as a safety limit suggested by the regulatory authorities such as FDA, which must be lower than 100 pg of DNA per milligram of protein^[9]. Generally, the

hybridization assay and total DNA threshold assay have been used for the quantitative detection of host cell DNA in pharmaceuticals; however, these methods are time-consuming and expensive^[10].

In recent years, great interests have been focused on real-time PCR assay as a new method for simultaneous detection and quantification of PCR products^[11]. Real-time PCR has been employed for detection of bacterial, and viral loads in therapeutic proteins^[12-16]. In this study, we established a novel sensitive real-time PCR test based on SYBR Green chemistry to detect a small quantity of *E. coli* host-cell DNA contamination in rSK and IFN- α samples produced at Pasteur Institute of Iran. Streptokinase and interferon are protein-based drugs produced in *E. coli* host cells^[17,18]. Streptokinase is used as an intravenous thrombolytic agent for treatment of acute myocardial infarction^[19]. Interferon acts as an integral part of the innate immune response and targets specific cell-surface receptors^[20], which are conducive to the interferon-mediated antiviral response^[21].

MATERIALS AND METHODS

Cell culture

E. coli W3110 strain was provided from Pasteur Institute of Iran. As the first step of normalization, a single colony of the cells was used to inoculate into 5 ml of Luria-Bertani broth. The colony was incubated at 37°C overnight with shaking at 250 rpm until the optical density at 600 nm reached 3.0, which was approximately equivalent to 2.4×10^9 cells/ml (Perkin-Elmer Lambda UV/visible Spectrophotometer, Applied Biosystems, UK).

Preparation of *E. coli* genomic DNA

Cells were precipitated by centrifugation at $17226 \times g$ for 2 min. Genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. At the second step of normalization, Optical density (260/280 nm and 260/230 nm) and concentration were measured using a NanoPhotometer 2000c (Thermo Science, USA).

Primer design and PCR test

In this study, four sets of forward and reverse primers were designed for the conserved region of the eubacteria 16S rRNA gene using Primer Express software (Foster City, USA). The sequence information is found in the NCBI GenBank database (Accession Number: J01859). The specific oligonucleotide primers were designed for the conserved region of 16S rRNA gene (Table 1), and primer pairs were synthesized by Bioneer Corporation

(Korea). All primer pairs were used in different PCR runs from the viewpoint of primer dimer formation. Among the designed primers, the primer pair 16S₂ displayed higher specificity and efficiency with minimal primer pair formation.

PCR reaction was carried out in a Mastercycler Personal (Eppendorf, Germany). The cycle parameters were as follows: initial heat denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. To ensure the complete extension, reaction mixture was incubated at 72°C for 5 min. Genomic DNA (10 ng) was amplified in a total volume of 20 μ l mixture containing 10 pM each of forward and reverse primer (0.5 μ l), 10 μ l Ex Taq Master Mix 2 \times (Takara, Japan, and 8 μ l nuclease free water. PCR products were analyzed by electrophoresis on 1.5% agarose gel (Sigma, USA).

Optimization of real-time PCR

In order to normalize and calculate primer efficiency, different concentrations of primer pairs were assessed. In this assay, SYBR Green I was used as a fluorescent dye. Real-time PCR reactions were carried out via an ABI 7300 real-time PCR instrument (Applied Biosystems, USA).

Amplification efficiency was validated using five-fold serial dilutions of DNA template as 2000, 400, 80, 16, 3.2, 0.64, 0.128, 0.025, 0.005, 0.001, and 0.0002 pg. Standard curve was drawn by plotting the logarithmic input DNA concentration versus mean cycle of threshold (mCt). Subsequently, the slope of standard curve was determined and PCR efficiency was calculated using the formula; $E = (10^{[-1/\text{slope}]} - 1) \times 100$.

Optimum reaction condition in a total volume of 20 μ l was obtained with 10 pM each forward and reverse primer (0.5 μ l), 10 μ l SYBR Green PCR Master Mix 2 \times (Takara, Japan) containing Taq DNA polymerase, SYBR Green I, Tris-HCl, MgCl₂, KCl, and deoxynucleotide triphosphate, 0.4 μ l ROX dye (6-Carboxy-x-rhodamine), 7.6 μ l nuclease-free water, and 1 μ l template genomic DNA. Thermal cycling program was performed with an initial heat denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 20 s, and combined annealing/extension at 60°C for 35 s and 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. In the melting stage, temperatures were raised 0.5°C for each step. Dissociation stage was performed after the amplification stage to verify the specificity of the PCR products. Each amplicon was identified by its specific melting curve T_m. A reaction including PCR Master Mix and nuclease-free water was used as non-template control in each experiment. All reactions were run in duplicate.

Table 1. Sequences of oligonucleotide primer pairs

Forward primer	Reverse primer	Nucleotide position	product size (bp)
16S-F1 AGAAGCTTGCTCTTTGCTGA	16S-R1 CTTTGGTCTTGCGACGTTAT	78-197	120
16S-F2 AAAGGAGACTGCCAGTGATA	16S-R2 AGGTCGCTTCTCTTTGTATG	1149-1263	115
16S-F3 CATTGACGTTACCCGCAGAA	16S-R3 CGCTTTACGCCAGTAATTCC	476-576	101
16S-F4 CCATGAAGTCGGAATCGCTAG	16S-R4 ACTCCCATGGTGTGACGG	1326-1419	94

DNA extraction from therapeutic proteins

Due to the presence of inhibitory agents in final products, DNA was extracted from Active Pharmaceutical Ingredients of rSK and IFN- α samples. To achieve this purpose, five batches of each drug, which were produced for five successive weeks, were selected for DNA purification. After phenol/chloroform extraction, the supernatant was transferred into a microcentrifuge tube. Then, DNA was precipitated using 400 μ l isopropanol. After washing twice with 700 μ l ethanol, DNA pellets were suspended in nuclease-free water.

Detection of DNA contamination

DNA templates, including 1 μ l each serially diluted *E. coli* genomic DNA, 1 μ l DNA extracted from rSK, and 1 μ l DNA extracted from IFN- α were subjected to real-time PCR test. The total volume of each reaction mixture was 20 μ l containing 10 pM each forward and reverse primer (0.5 μ l), SYBR Green PCR Master Mix 2 \times (10 μ l), ROX dye (0.4 μ l), nuclease-free water (7.6 μ l), and 1 μ l DNA template. All reactions were run in duplicate, and non-template controls were added to the experiment. Cycling conditions were hold at 95°C for 1 min, followed by 40 cycles at 95°C for 20 s, and 60°C for 35s. PCR products were visualized on 1.5% agarose gel.

Validation of real-time PCR

According to the FDA guidelines for industry, namely bioanalytical method, validation, the reproducibility, precision, linearity, and detection limit of the real-time PCR assay were validated. *E. coli* genomic DNA (100 μ g) was fragmented using *Hind*III and *Eco*RI enzymes, and fragmented DNA was used as the standard DNA. The reproducibility and precision were measured using 50 and 100 pg of *E. coli* genomic DNA (gDNA), and the concentration of samples were measured three times on different days. The mCt values, and average of standard deviations (SD) were also calculated.

For detection of linearity, standard samples containing 2000, 400, 80, 16, 3.2, 0.64, 0.128, 0.025, 0.005, 0.001, and 0.0002 pg of *E. coli* gDNA, along with negative samples, were prepared. The

concentrations of samples were obtained three times on different days. Standard curves were plotted for each experiment, and the correlation coefficient was calculated using least square method.

The detection limit was evaluated using minimum level of concentration at which the samples can be detected. Standard samples containing 2000 to 0.0002 pg of genomic DNA, along with negative solutions, were used for the validation of real-time PCR assay.

RESULTS

Amplification of DNA extracted from rSK and IFN- α

Serially five-fold dilution of *E. coli* genomic DNA from 2000 to 0.0002 pg, one pair of negative controls, and DNA extracted from rSK and IFN- α preparations were run in duplicate (Fig. 1A). Melting curve analysis of PCR products did not show any primer-dimer formation (Fig. 1B). Moreover, this graph demonstrated melting of amplification fragment at 83.9°C. The post-PCR analysis confirmed and showed the target fragment (115 bp) without non-specific products (Fig. 2).

The slope of standard curve, y-intercept, determination of coefficient (R^2), and PCR efficiency were obtained as -2.63, 25.64, 0.99, and 100%, respectively. Standard curve presented Ct values (mean \pm SD) between 15.58 ± 0.163 and 34.03 ± 0.037 (Fig. 1C).

Quantitative detection of DNA contamination

The mean of two Ct values (mCt) obtained from duplicate amplification of DNA extracted from each rSK and IFN- α was calculated separately. The mCt values (32.89 and 33.28 for rSK and IFN- α , respectively) were put instead of "Y" values in the standard curve equation ($Y = -2.63X + 24.72$) to calculate "X" values. After getting the common logarithm of "X" values, the estimated amounts of *E. coli* host-cell DNA contamination in rSK and IFN- α preparation were 0.0012 and 0.0017 pg per mg of products, respectively (Table 2). The values calculated from the experiments were much lower than the limit permitted by the FDA^[9].

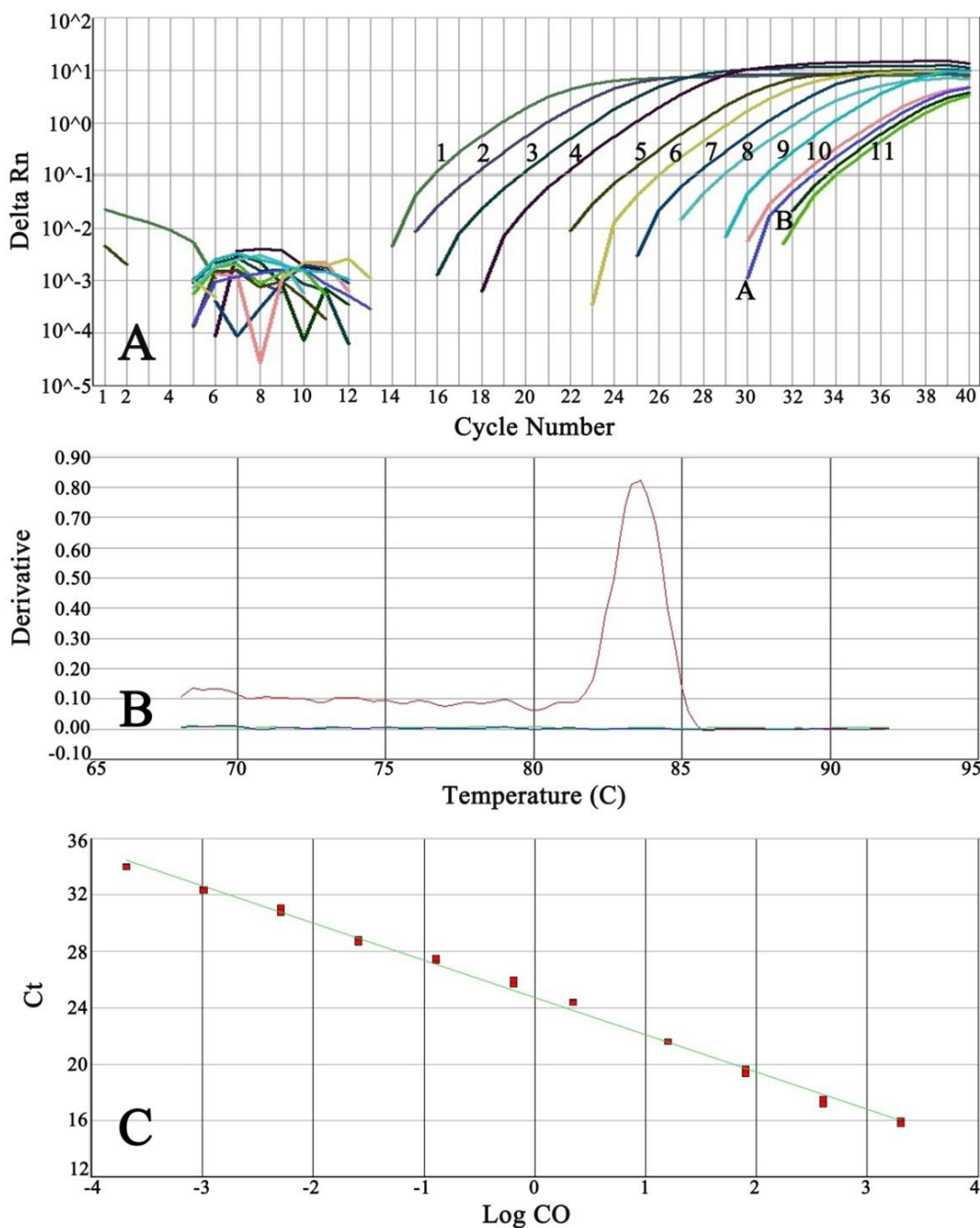


Fig. 1. Specificity of real-time PCR test for the quantity of DNA contamination in biopharmaceuticals; (A) Amplification plot of 5-fold serial diluted *E. coli* genomic DNA concentrations along with the amplification plot of DNA extracted from therapeutic proteins; 1, 2000 pg; 2, 400 pg; 3, 80 pg; 4, 16 pg; 5, 3.2 pg; 6, 0.64 pg; 0.0128 pg; 8, 0.025 pg; 9, 0.005 pg; 10, 0.001 pg; 11, 0.0002 pg; A, Alpha interferon; B, Streptokinase; (B) Melting curve analysis of PCR products; (C) Standard curve.

Validation of real-time PCR

In the present study, some quality control procedures, including the reproducibility, precision, detection limit, and linearity of real-time PCR method were performed. The reproducibility was determined by calculating the mCt values as 19.17, 19.64, and 18.88 for 50 pg of standard DNA, and 20.33, 20.44,

and 20.12 for 100 pg of standard DNA on different days (Table 3). The precision was evaluated as 50.67 ± 1.28 and 101.12 ± 1.44 for 50 and 100 pg of standard DNA, respectively (Table 4). The linearity was calculated by a standard curve using the least square method. The mean value of the correlation coefficient obtained from three experiments was 0.99.

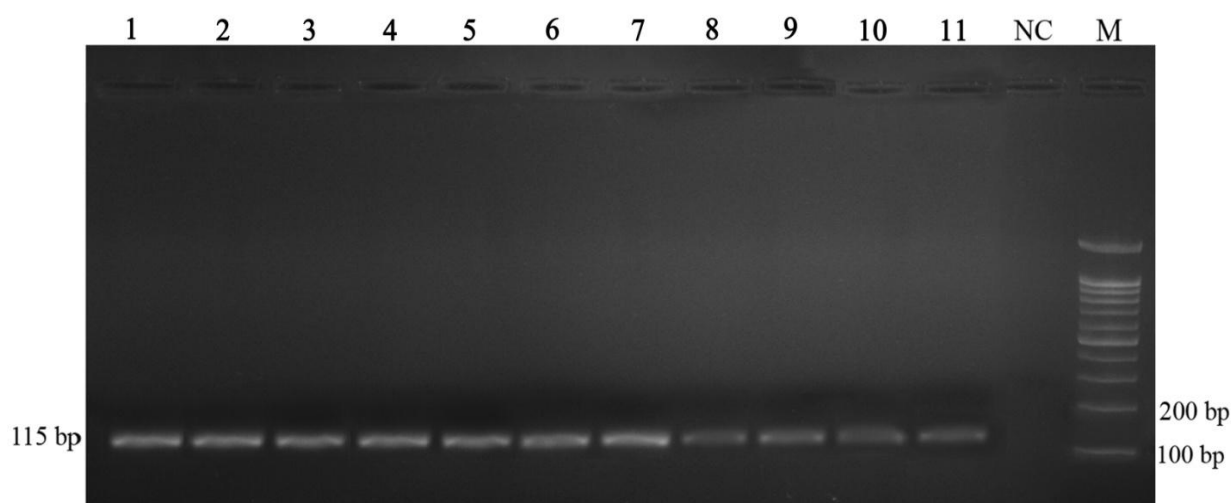


Fig. 2. Gel electrophoresis of PCR products. Lane 1, 2000 pg; Lane 2, 400 pg; Lane 3, 80 pg; Lane 4, 16 pg; Lane 5, 3.2 pg; Lane 6, 0.64 pg; Lane 7, 0.128 pg; Lane 8, 0.025 pg; Lane 9, 0.005 pg; Lane 10, 0.001 pg; Lane 11, 0.0002 pg, NC; negative control, M; marker.

Table 2. DNA content of recombinant protein-based drugs

Recombinant protein	Drug concentration (mg/ml)	DNA content (pg/mg of product)
rSK	1.2	0.0012
IFN- α	1.1	0.0017

The detection limit of the assay was 0.0002 pg of DNA. The range of the standard curve was from 0.0002 to 2000 pg of DNA.

DISCUSSION

Therapeutic recombinant proteins are widely used for human therapies. Host-cell genomic DNA contamination in biopharmaceuticals is a potential risk factor for consumers. Therefore, FDA and other regulatory agencies have defined specific quality control and safety criteria, which manufacturers are required to quantify DNA impurities in recombinant therapeutics at different stages of production^[22,23]. To detect the minimum amount of impurities, the method involved in the quantitative detection of DNA contamination must be accurate and sensitive enough.

For a few years, radioactive-based expensive

methods, including the species-specific DNA hybridization assay, have been used to quantify bacterial host-cell DNA contamination in biopharmaceuticals [10]. Ji *et al.*^[24], reported a safe non-radioactive slot-blot hybridization assay to quantify *E. coli* DNA contamination levels in purified protein-based drugs with a sensitivity to detect 2.5pg *E.coli* DNA. Afterwards, Gregory *et al.*^[25] described a safer and less-expensive alternative method, i.e. PCR amplification and dioxigenin labeling of the genes encoding 5S rRNA followed by an affinity-based collection, for detection of 1 pg/mL of the extracted *E.coli* genomic DNA^[25]. Although the Gregory's method seemed sensitive, it was capital-intensive to be applied routinely in the biopharmaceutical industries. This made researchers to search for even more inexpensive assays that might be applied readily by manufacturers. Real-time PCR has recently been reported as an alternative method for simultaneous detection and quantification of DNA sequence in manufactured products. In this study, a novel real-time PCR test, based on SYBR Green chemistry, was established for the precise measurement of *E. coli* host-cell DNA contamination in two widely used recombinant protein-based drugs, i.e. rSK and IFN- α . Despite the fact that real-time PCR has many

Table 3. The reproducibility of assay

Concentration of standard DNA (pg)	mCt		
	Day 1	Day 2	Day 3
50	19.17	19.64	18.88
100	20.33	20.44	20.12

Table 4. The precision of assay

Concentration of standard DNA (pg)	Concentration of calculated DNA (mean \pm SD)
50	50.67 \pm 1.28
100	101.12 \pm 1.44

privileges, it has not been employed routinely in the pharmaceutical industries, mainly because real-time PCR reactions may be inhibited by various agents that are present in biopharmaceuticals^[26]. In this experiment, to eliminate the inhibitory effect of the agents, *E. coli* genomic DNA was first extracted from the Active Pharmaceutical Ingredients of biopharmaceuticals and then was subjected to real-time PCR test. Using this method, we are able to detect reliably a very small amount of bacterial genomic DNA. The gene copy number was calculated according to the molecular mass of the *E. coli* genome, namely 2.86×10^9 Da or 4.7491×10^{-3} pg. There are seven genomic copies of the 16S rRNA gene in each bacterium^[27], and the molecular mass of each copy is 6.79×10^{-4} pg. Thus, the sensitivity of our assay was 0.0002 pg equivalent to 1 copy of the 16S rRNA gene (equivalent to one *E. coli* bacterium). Although some kinds of probes, including those labeled with fluorescent dyes, can significantly enhance the specificity of real-time PCR^[28,29], we exploited SYBR Green chemistry to establish an inexpensive detection assay. It is known that primer-dimer formation is one of the major problems using SYBR Green dye. To avoid this problem, we optimized the real-time PCR reaction condition by designing accurate primers as well as by finding the most effective salt concentration (4 mM), primer concentration (10 pM), optimal annealing temperature and time (60°C for 35 s).

In conclusion, we have succeeded to establish a new real-time PCR approach based on SYBR Green chemistry for measurement of *E. coli* genomic DNA impurities in biopharmaceuticals, with a detection limit of 0.0002 pg DNA per mg of therapeutic recombinant proteins. The present study show that *E. coli* host-cell DNA contamination in rSK and IFN- α manufactured at Pasteur Institute of Iran is much lower than the safety limit suggested by FDA.

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CONFLICT OF INTEREST. None declared.

REFERENCES

- Rasala BA, Muto M, Lee PA, Jager M, Cardoso RM, Behnke CA, Kirk P, Hokanson CA, Crea R, Mendez M, Mayfield SP. Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. *Plant biotechnology journal* 2010; **8**(6): 719-733.
- Swiech K, Picanco-Castro V, Covas DT. Human cells: new platform for recombinant therapeutic protein production. *Protein expression and purification* 2012; **84**(1): 147-153.
- Baneyx F. Recombinant protein expression in *Escherichia coli*. *Current opinion in biotechnology* 1999; **10**(5): 411-421.
- Jana S, Deb JK. Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Applied microbiology and biotechnology* 2005; **67**(3): 289-298.
- Swartz JR. Advances in *Escherichia coli* production of therapeutic proteins. *Current opinion in biotechnology* 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. *Humane gene therapy* 1998; **9**(8): 1173-1180.
- Walsh G. *Proteins: Biochemistry and Biotechnology*. 2nd edition. West Sussex: John Wiley & Sons Ltd. 2002; pp: 205-206.
- Rathor 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. *Journal of pharmaceutical and biomedical analysis* 2003; **32**(6): 1199-1211.
- Riggin A, Luu VT, Lobdell JK, Wind MK. A non-isotopic probe-hybridization assay for residual DNA in biopharmaceuticals. *Journal of pharmaceutical and biomedical analysis* 1997; **16**(4): 561-572.
- Wolter T, Richter A. Assay for controlling host-cell impurities in biopharmaceuticals. *BioProcess International*. 2005; **3**: 40-46.
- Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, Sindelka R, Sjogreen B, Strombom L, Stahlberg A, Zoric N. The real-time polymerase chain reaction. *Molecular aspects of medicine* 2006; **27**(2-3): 95-125.
- deWit C, Fautz C, Xu Y. Real-time quantitative PCR for retrovirus-like particle quantification in CHO cell culture. *Biologicals* 2000; **28**(3): 137-148.
- Wolf JJ, Wang L, Wang F. Application of PCR technology in vaccine product development. *Expert review of vaccines* 2007; **6**(4): 547-558.
- Jeong HS, Shin JH, Park YN, Choi JY, Kim YL, Kim BG, Ryu SR, Baek SY, Lee SH, Park SN. 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): 223-229.
- 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. *Analytical biochemistry* 2003; **322**(1): 127-129.
16. Mohammadi T, Savelkoul PH, Pietersz RN, Reesink HW. Applications of real-time PCR in the screening of platelet concentrates for bacterial contamination. *Expert review of molecular diagnosis* 2006; **6**(6): 865-872.
 17. Mahmoudi S, Abtahi H, Bahador A, Mosayebi G, Salmanian AH. Production of recombinant streptokinase in *E. coli* and reactivity with immunized mice. *Pakistan journal of biological sciences* 2010; **13**(8): 380-384.
 18. Babaeipour V, Shojaosadati SA, Maghsoudi N. Maximizing production of human interferon- γ in HCDC of recombinant *E. coli*. *Iranian journal of pharmaceutical research* 2013; **12**(3): 563-572.
 19. Karimi Z, Babashamsi M, Asgarani E, Niakan M, Salimi A. Fermentation, fractionation and purification of streptokinase by chemical reduction method. *Iranian journal of microbiology* 2011; **3**(1): 42-46.
 20. Fensterl, V and Sen GC. Interferons and viral infections. *Biofactors* 2009; **35**: 14-20.
 21. Sadler AJ, Williams BR. Interferon-inducible antiviral effectors. *Nature reviews immunology* 2008; **8**(7): 559-568.
 22. Wang KY, Guo YJ, Sun SH, Shi K, Zhang S, Wang KH, Yi-Zhang Chen, ZH. 16S rRNA gene probes quantitates residual host cell DNA in pharmaceutical-grade plasmid DNA. *Vaccine* 2006; **24**(14): 2656-2661.
 23. Tatalick LM, Gerard CJ, Takeya R, Price DN, Thorne BA, Wyatt LM, Anklesaria, P. Safety characterization of HeLa-based cell substrates used in the manufacture of a recombinant adeno-associated virus-HIV vaccine. *Vaccine* 2005; **23**(20): 2628-2638.
 24. Ji X, Lee K, DiPaolo B. High-sensitivity hybridization assay for quantitation of residual *E. coli* DNA. *Biotechniques* 2002; **32**(5): 1162-1167.
 25. 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. *Analytical Biochemistry* 2001; **296**(1): 114-121.
 26. Wilson IG. Inhibition and facilitation of nucleic acid amplification. *Applied and environmental microbiology* 1997; **63**(10): 3741-3751.
 27. Brosius J, Dull TJ, Sleeter DD, Noller HF. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *Journal of molecular biology* 1981; **148**(2): 107-127.
 28. Singh J, Batish VK, Grover S. A scorpion probe-based real time PCR assay for detection of *E. coli* 0157: H7 in dairy products. *Foodborne pathogens and diseases* 2009; **6**(3): 395-400.
 29. Wan Y, Li Y, Yang C, Hui L, Han Q, Ma L, Wang Q, Yang G, Liu Z. Development and application of a universal TaqMan real-time PCR for quantitation of duck hepatitis B virus DNA. *Journal of virological methods* 2013; **191**(1): 41-47.