

In Silico Analysis, Cloning and Expression of Recombinant CD166 in *E. coli* BL21 (DE3) as a Marker for Detection and Treatment of Colorectal Cancer

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Abstract

Introduction: Colorectal cancer is the third most common type of tumors, with more than 1.2 million new cases resulted in 600 thousand deaths annually and ranks fourth in terms of mortality worldwide. The activated leukocyte cell adhesion molecule (ALCAM) also called CD166 is a marker of colorectal cancer (CRC) stem cells. The expression of CD166 increase in colorectal cancer. Also with advancement of illness in different stages of cancer, this expression increased. So, it could be a reasonable marker for Detection and Treatment of Colorectal cancer. The purpose of this study is to produce recombinant protein CD166 for cancer therapy or early detection of colorectal cancer cells.

Methods: In this study, the sequence of CD166 was optimized for expression in *E. coli* using online tools and cloned into pET28a as an expression vector. The recombinant pET28a was transformed into the *E. coli* BL21DE3 using heat shock method and expression of recombinant CD166 was examined using SDS-PAGE.

Results: The synthetic gene of CD166 was located between NcoI/BamHI and XhoI restriction sites and cloned into pBSK (+) vector. The presence of this gene in pET28a was determined by colony and confirmed by restriction digestion. Gene of CD166 were expressed in *E. coli* BL21 DE3. The results of the SDS-PAGE technique confirmed the expression of recombinant 53 kDa CD166 in a bacterial expression system.

Conclusion: A portion of the CD166 gene was expressed as a recombinant in *E. coli*. This could be a good candidate to produce a vaccine for cancer therapy or colorectal cancer diagnostic test.

Keywords: Colorectal cancer; Cloning and Expression; Recombinant Protein; CD166/ALCAM

Introduction

Cancer is one the oldest diseases occurring among humans and animals with a history longer than the prehistoric era. Developing countries account for at least two-thirds of cancer patients in the world [1]. Colorectal cancer is fourth common type of cancer with an estimated of 1/2 million new diagnosed cases annually. This disease is the third cause of death among the cancers in the world [2-4].

Factors which increase the risk of colorectal cancer may be considered as age over 50, colorectal polyps, family history of colorectal cancer, genetic disorders, history of being diagnosed with cancer, Krone or inflammatory bowel disease, diet, smoking, etc. [5].

Molecular or genetic changes can also be involved in colorectal cancer but some genetic changes can increase the risk of colorectal cancer such as changes in *HNPCC* or *APC2* genes [6]. Symptoms of colorectal cancer are diarrhoea, constipation, blood in the stool, a completely empty bowel feeling, narrowing of the stool and losing weight without reason.

Colorectal cancer may be treated using various methods depending on the location of the tumor, in the rectum and colon, and the stage of the disease, such as surgery, radiotherapy, chemotherapy or a combination of all these methods, in which surgery is the most common treatment method [6].

CD166, also known as ALCAM (Activated Leukocyte Cell Adhesion Molecule), is a marker of colorectal cancer stem cells, which emerges by aggressive tumors. Adhesion molecules like the CD166 are important

for cell survival, cell growth and motility, and for invasion during tumor progression and metastases [7-12].

The presence of CD166 at the tumor surface indicates the shortening of survival [13]. When the cells become cancerous, CD166, which is located on the outer surface of colorectal cancer cells, is noticeably overexpressed at the surface [14].

CD166 is a member of the immunoglobulin superfamily and was identified by expression cloning based on its ability to bind to CD6 making use of COS cells transfected with cDNA libraries. CD166 consists of five extracellular immunoglobulin domains (two NH2-terminal, membrane-distal variable-[V]-type folds and three membrane-proximal constant-[C2]-type immunoglobulin folds), a transmembrane region, and a short cytoplasmic tail. ALCAM is able to mediate homophilic as well as heterophilic (CD6) interactions. The gene

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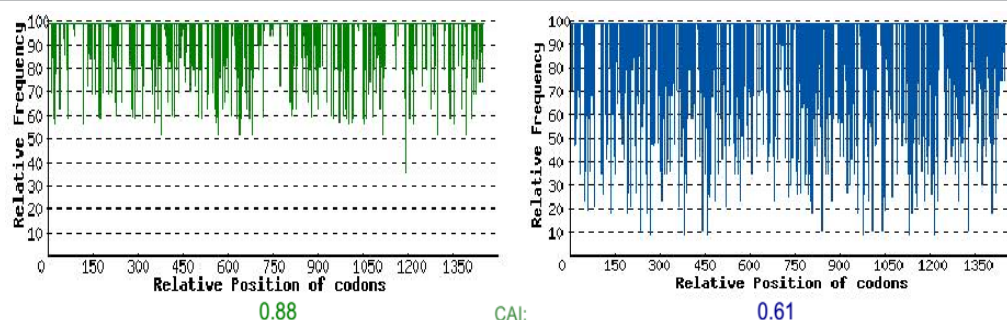


Figure 1: Codon adaptation index (CAI); Right: before Optimization, Left: After optimization.

encoding *CD166* is located on the long arm of human chromosome 3 (3q13.1-q13.2). It is organized into 16 exons that span nearly 150 kb of DNA [15-17].

In addition, this protein is considered to be a recognizable anti-gene that may lead to the production of antibody in an immunized mouse with human intestine cancerous cells [18]. The *CD166* protein is also considered as a well-known molecular marker for targeted treatment used for patients suffering colorectal cancer [19,20]. In this research bioinformatics analysis, cloning and surface expression of *CD166* were applied to study and to analyze the potential of optimization of diagnosis methods such as generating diagnostic kits to diagnose the colorectal cancer cells before becoming malignant and the possibility producing a vaccine for colorectal cancer.

CD166 plays a crucial role in the invasion and the development of tumor in colorectal cancer and is assumed as a cancer stem cells marker. Besides, the extracellular of this protein has a region with appropriate size. it performs an important role in the reactions and interactions of the protein, for either hemophilic or heterophilic reactions. *CD166* is composed of two components of 93 and 110-amino acids and three components of 84, 77 and 86 amino acids, given that, this experiment is aimed to clone and express *CD166* to be used in practical, diagnostic and remedial plans.

Materials and Methods

Materials

Luria Bertani (LB) media was prepared according to Sambrook and Russel. Antibiotic screening was performed on LB agar plates using ampicillin at 100 µg/ml and Kanamycin at 20 µg/ml which was obtained from Sigma, USA. Concentrations of IPTG (100 µg.mL⁻¹) were used for the induction of *CD166* gene. This reagent was purchased from Merck, Germany. The restriction enzymes were purchased from Fermentas (Lithuania).

Bioinformatics analysis and optimization and chemical synthesis of gene *CD166*

The sequence of the specified gene was estimated via uniprot KB/ Swiss-Port website and the national center of biotechnology information (NCBI). Afterwards, after 3' sequences, 6 histidine amine acids (His-Tag) were inserted followed by TAA stop codon. In the end, NcoI and BamHI enzymes restriction sites were embedded at 5' terminal and XhoI enzyme restriction site at 3' terminal. In the following, codon optimization was performed using American GenScript Company to gain access to the most correct expression in prokaryotic host. In the following, the gene was chemically synthesized and delivered as a clone in pBSK (+) vector (Biomatik Corporation, Canada).

Amplify the *CD166* gene

In order to amplify the *CD166* gene, the *E. coli* TOP10 was transformed with plasmid containing *CD166* gene using heat shock method. Then, plasmid extraction, a double digestion was done with NcoI and XhoI.

Gene *CD166* sub cloning

E. coli BL21 (DE3) was used as an expression host cell. Digested fragments were analyzed by agarose gel electrophoresis and pET28a expression vector and *CD166* fragments were purified. Finally, *CD166* sequence was ligated to the pET28a using T4 DNA ligase. After an overnight incubation at 37°C, the plasmid extraction from appeared colonies were performed.

Transformation of recombinant vector (pet-28a-*CD166*)

In this research, the competent *E. coli* BL21 (DE3) host cells were prepared with calcium chloride. The accuracy of transformation was verified by double digestion of plasmid with NcoI and XhoI enzymes.

Expression of *CD166* gene

Protein expression was analyzed using SDS-PAGE analysis technique. *E. coli* BL21 (DE3) harboring the *CD166*_pet-28a (+) positive colony was grown in 100 ml LB broth at 37°C including 20 µg/ml Kanamycin to achieve an optical density (OD) of 0.8 and at this time, 20 µL IPTG (100 µg.mL⁻¹) was added to 50 ml LB broth. At 37°C, incubation was continued. The samples from induced culture for 6 h at 150 rpm and 37°C after induction. Samples were accumulated, centrifuged at 5000 rpm for 5 min and 4°C. Supernatant was discarded and 80 µL Urea (8 M) was added to the precipitate. To run the samples, the mixture (precipitated sample and Urea) was dissolved in 1 × SDS-PAGE sample buffer. The prepared samples and protein molecular weight marker was heated for 7 min at 95°C. Then each sample and marker was loaded on the 12% SDS-PAGE gel and run on the constant voltage of 100 V.

Results

Sequence optimization and gene synthesis

In the native nucleotide sequence, there were parameters that could decrease the efficiency of expression system in the *E. coli* host cell. Thus, these parameters were substituted to optimize clones to a higher efficiency system. The parameters changed through optimization were Codon Adaptation Index (CAI) and frequency of optimal codons (FOP) and the GC content adjustment.

CAI is variable between 0 and 1, in which the closer index to 1 shows better expressional conditions. In the native sequence the CAI was 0.61 which was changed to 0.88 By changing the nucleotides (Figure 1).

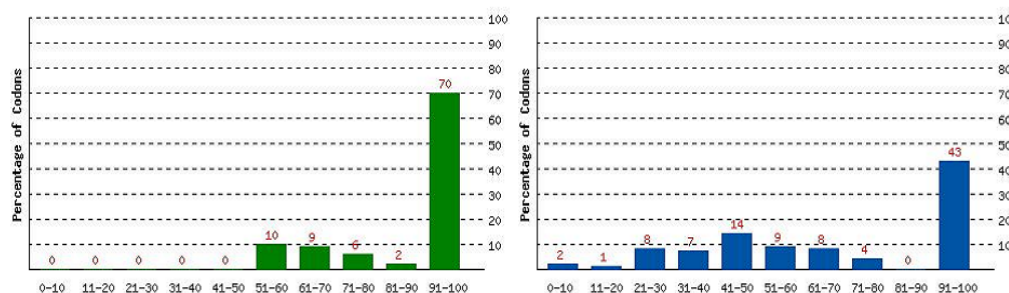


Figure 2: Frequency of optimal codons (FOP); Right: before Optimization, Left; After optimization.

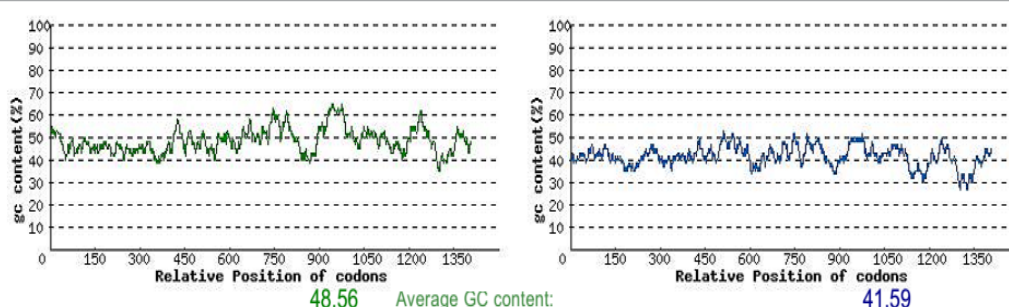


Figure 3: GC content adjustment; Right: before Optimization, Left; After optimization.

The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. In the native sequence the FOP was 43 which was changed to 70 (Figure 2).

A suitable sequence has a G-C Content Adjustment of 30% to 70% (50% on average). Through codon optimization, the G-C content adjustment was increased from 41.59 to 48.56, which results in an increase in the half-life of mRNA of the gene (Figure 3). Considering the modified parameters, nucleotide sequence of original and optimized sequences was compared for Peer to Peer (Figures 4 and 5).

Amplify the *CD166*

To amplify the recombinant pBSK plasmid containing *CD166* DNA, transformation process was carried out for *E. coli* top 10 competent cell and the colonies grown on medium containing ampicillin antibiotic were selected (Figure 6).

Followed by confirming pBSK plasmid containing the *CD166* gene using the electrophoresis method, double digestion technique was applied to confirm the presence of *CD166* gene in the plasmid, considering *NcoI* and *XhoI* enzyme restriction sites embedded at the two ends of synthesized gene (Figure 7).

Sub-cloning of *CD166* in pET28a

CD166 DNA was digested and it was ligated into similarly digested pET-28a expression plasmid. In the following, *E. coli* BL21 (DE3) competent cells were transformed by recombinant construct. Afterwards, the transformed bacteria were inoculated in a medium containing kanamycin and incubated for an overnight. Plasmid extraction was performed and was confirmed on gel electrophoresis. After confirming the presence of recombinant pET-28a plasmid, enzyme double digestion technique was performed. Observing a 1463 bp band related to the target gene and a 5369 bp band to the pET-28a

expression plasmid indicated the cloning of target gene into the pET-28a plasmid (Figures 8 and 9).

Discussion

With nearly 135,000 new cases diagnosed annually, CRC is the third most prevalent cancer in the United States [21]. Concentrate on cancer initiation and development has dominated the effort to better understand disease pathology and guide therapeutic approaches. Hence, the cancer stem cell (CSC) hypothesis, which suggests that cancer is driven by cells harboring stem cell-like qualities, offers one explanation for why many current therapeutic approaches ultimately result in relapse of disease. In this hypothesis, some CSCs may be quiescent and, thus, evade eradication by standard cytotoxic therapies designed to target proliferating cells. These surviving cells can then proceed to support tumor growth and have the potential to initiate metastatic or recurrent disease [22-32].

ALCAM has been reported to be involved in tumorigenesis of CRC and to function as a CSC marker. Correlation of the *CD166* expression pattern with aggressive disease has led to efforts for targeting this molecule as a cancer therapeutic.

Bioinformatics is gathering, implementation and analysis of a large quantity of biological data, which its most essential application is sequence analysis. Another practical application of bioinformatics is the optimization of newly composed gene considering the host cell, which was done in this research. Codon optimization leads to a better expression, precision, and performance of the target gene in the host cell.

Monoclonal antibodies (MAbs) are a relatively new innovation in cancer treatment. At present, some monoclonal antibodies have increased the efficacy of the treatment of certain tumors with acceptable safety profiles. *CD166* is a cell-surface antigen that is proposed as the antigen of cancer stem cell in CRC [33]. Recently, the recognition

Optimized	9	ATGGCGTGGTACACCGTCAACTCGGCCCTATGGCGATACGATTATTATTCCGTGTCTCTC
Original	9	ATGGCATGGTATACGTAAATTCAGCATATGGAGATACCATTCATACCTTGCCGACTI
Optimized	69	GATGTCCCGCAGAACCTGATGTTTGGTAAATGGAAATATGAAAACCGGATGGCTCGCCG
Original	69	GACGTACCTCAGAATCTCATGTTTGGCAAATGGAAATATGAAAAGCCGATGGCTCCCCA
Optimized	129	GTGTTTATTGCGTTCCTAGCTCTACCAAAAAATCTGTGCAGTATGATGACGTCCCGGAA
Original	129	GTATTTATTGCCCTTCAGATCCTCTACAAAGAAAAGTGTGCAGTACGACGATGTACCGAA
Optimized	189	TACAAAGATCGCCTGAACCTGTCTGAAATTACACCCTGAGTATCTCCAACGCCCGTATT
Original	189	TACAAAGACAGATTGAACCTCTCAGAAAACCTACACTTTGTCTATCAGTAATGCAAGGATC
Optimized	249	AGTGATGAAAACCGCTTTGTGTGCATGCTGGTTACGGGAAGACAAATGTCTTTGAAGCACCG
Original	249	AGTGATGAAAAGAGATTGTGTGCATGCTAGTAACTGAGGACAACGTGTTTGAGGCACCT
Optimized	309	ACCATGTTTAAAGTCTTCAACAGCCGAGCAAAACCGGAAATCGTGTCTAAAGCTCTGTT
Original	309	ACAATAGTCAAGGTGTTCAAAGCAACCATCTAAACCTGAAATTTGAAGCAAGCACTGTT
Optimized	369	CTGGAACCGGAACAACCTGAAAAAACTGGGTGATTGTATTAGTGAAGATTCTTATCCGAC
Original	369	CTCGAAACAGAGCAGCTAAAAAAGTTGGGTGACTGCATTTCAGAAAGACAGTTATCCAGA
Optimized	429	GGCAACATCACCTGGTACCGTAATGGTAAAGTTCTGCACCCGCTGGAAGGCGCAGTGGT
Original	429	GGCAATATCACATGGTACAGGAATGGAAAAAGTGTACATCCCTTGAAGGAGCGGTGGT
Optimized	489	ATCATCTTCAAAAAAGAAATGGACCCGGTCACCCAGCTGTATACCATGACGTCTACCCCT
Original	489	ATAATTTTAAAAAGGAAATGGACCCAGTGTACCTGCTATACCATGACTTCCACCCCT
Optimized	549	GAATACAAACACGAAAGCTGACATTCAAATGCCGTTTACGTGACGTGTACCTATTAC
Original	549	GAGTACAAGACAACCAAGTGTGACATTACAAATGCCATTACCTGCTCGGTGACATATT
Optimized	609	GGCCCGTCCGGTCAGAAAACCATTCATTCAGAACAAAGCGGTCTTCGATATTATTACCC
Original	609	GGACCATCTGGCCAGAAAACCAATTCATCTGAACAGGCGAGTATTGTATATTACTATCC
Optimized	669	ACGGAAACAGGTCACCATCCAAGTGCTCCGCCGAAAAACGCCATCAAAGAAAGGCGACAA
Original	669	ACAGAGCAGGTGACAAATACAGGTGCTGCCACCAAAAAATGCCATCAAAGAGCGGATTA
Optimized	729	ATTACCTGAAATGCTGGGTAAACGGTAACCCGCCCGCGGAAGAAATTTCTGTTCTATCT
Original	729	ATCACTCTTAAATGCTTAGGGAATGGCAACCCCTCCCCAGAGGAATTTTGTGTTTACTT
Optimized	789	CCGGGCCAGCCGGAAAGGTATTTCGAGTTTCAACACGATATACCCTGACGGATGTGCGT
Original	789	CCAGGACAGCCCGAAGGAATGAAGAGCTCAAACTACTTACACAGTGTGAGGAGTGTG
Optimized	849	AATGCAACCGGTGATTACAAATGTAGTCTGATCGACAAAAATCTATGATCGCGCTTACC
Original	849	AATGCAACAGGAGACTACAAGTGTTCCTGTAGACAAAAAAGCATGATGTCTTCAAC
Optimized	909	GCCATTACCGTTTCTATCTGATCTGAGTCTGAACCCGTCGGCGGAAGTCAACCCGTAC
Original	909	GCTATCAGGTTTCACTATTGGAATTTGTCTTAAACCAAGTGGAGAAGTGTACATGACAC
Optimized	969	ATTGGTGACGCTCTGCCGGTGTCTCTGACGATCTCAGCATCGCGCAACGCTACCGTCGT
Original	969	ATTGGTGATGCCCTACCCGTGTCTGACACAATATCTGTCTAGCAGGAATGCAACTGTGGT
Optimized	1029	TGGATGAAAGATAAATATTCTGCTGCGCTCATCGCCGTCATTTAGCTCTCTGCACTATCA
Original	1029	TGGATGAAAGATAACATCAGGCTTCGATCTAGCCCGTCATTTTCTAGTCTTCATTATCA
Optimized	1089	GACGCGGGTAATTACGTGTGTGAAACGGCCCTGCAAGAAAGTTGAAGGCCTGAAAAACG
Original	1089	GATGCTGGAAACTATGTCTGCCGAAAGTCTCTGCAGGAGGTTGAAGGACTAAAGAAAAAG
Optimized	1149	GAAAGCCTGACCCTGATCGTGAAGGTAAACCGCAGATTAAATGACCAAGAAAAACCGA
Original	1149	GAGTCATTGACTCTCATTTGGAAGGCAAACTCAAAATAAAATGACAAAGAAAACTGA
Optimized	1209	CCGTCAGGTCTGTGAAAACCAATTATCTGCCATGTGGAAGGCTTCCCGAAACCGGCCAT
Original	1209	CCCAGTGGACTATCTAAAACAAATAATCTGCCATGTGGAAGGTTTTCCAAAGCCAGCCAT
Optimized	1269	CAGTGGACCAATTACGGGCGAGCGGTTCTGTTATTAACCAAACCGAAGAAAGCCCGTATAT
Original	1269	CAATGGACAATTACTGGCAGTGGAGCGTCATAAACCAACAGAGGAATCTCCTTATAT
Optimized	1329	AATGGCCGCTATTACTCAAAAATTATCATTTCGCCGGAAGAAAACGTTACCCGTGACGTG
Original	1329	AATGGCAGGTATTATAGTAAAAATTATCATTTCCTCCTGAAGAGAAATGTTACATTAACCTG
Optimized	1389	ACCGCGGAAATCAACTGGAACGCAACCGTCAACTCTCTGAATGTGCTCTCATCATCATCA
Original	1389	ACAGCAGAAAACCAACTGGAGAGAACAGTAAACTCCTTGAATGTCTCTCATCACCATCA
Optimized	1449	CATCACTAA
Original	1449	CATCACTAA

Figure 4: DNA alignment; Comparing nucleotide for peer to peer in the primary and optimized sequences.

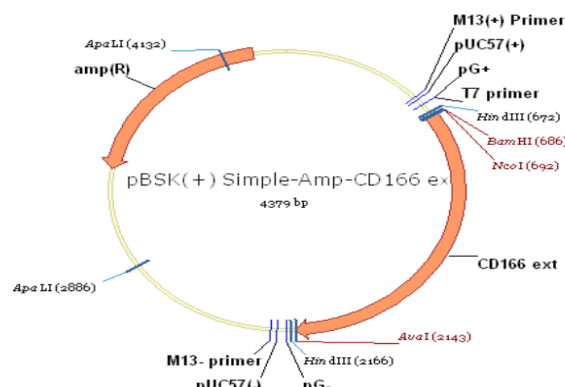


Figure 5: The map of pBSK plasmid containing CD166 gene.

of the tumor related antigen has introduced a new foundation in the immunotherapy of special antigens. The major aim of vaccine experiments initiated from the last decade was to induce a specific immune response against the cancer antigens [34-37].

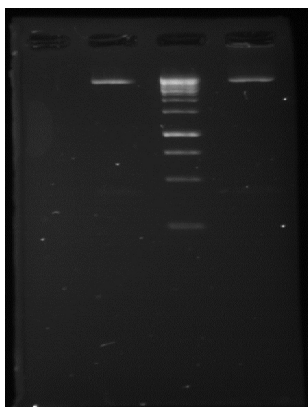


Figure 6: Extraction of transformed plasmid (pBSK plasmid containing *CD166* gene); M, GeneRuler™ 1 kb ladder; Lane 1 and 2: Extraction of transformed plasmid.

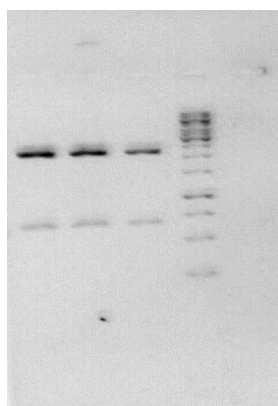


Figure 7: Double digestion of pBSK (+) vector (with the *NcoI* and the *XhoI*); M, GeneRuler™ 1 kb ladder (Fermentas, Lithuania) and Lane 1, 2 and 3 double digested plasmid.

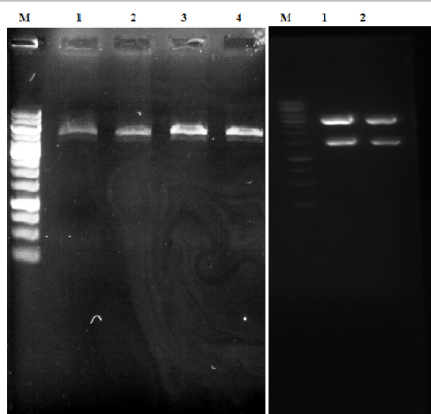


Figure 8: (A) Extraction of transformed plasmid (pET-28a Recombinant Vector); M, GeneRuler™ 1 kb ladder; Lane 1, 2, 3 and 4: Extraction of transformed plasmid; (B) The double digestion of pET-28a recombinant vector (with the *NcoI* and the *XhoI*); M, GeneRuler™ 1 kb ladder; Lane 1 and 2 double digestion.

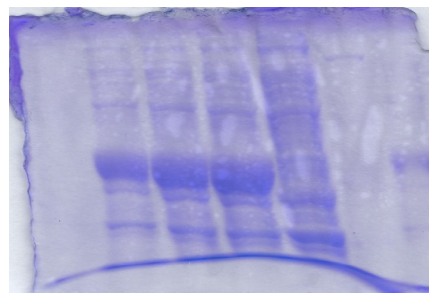


Figure 9: Expression analysis of recombinant CD166 produced in *E. coli* BL21 (DE3) by SDS-PAGE; line M, protein marker (CMG, Iran); line 1, 2 and 3: Induction of *E. coli* BL21 (DE3) with IPTG; lane 4: Non-induced *E. coli* BL21 (DE3).

In this study, with the induction of *CD166* protein in *E. coli* BL21 (DE3), to take initial steps to purify and separate, we made an effort to take initial steps to purify and separate them in a way to be used for *in vitro* induction of immune cells. The results of this study can be used to improve the diagnostic approaches and cancer remedial techniques.

Conclusion

By analyzing the expression of *CD166* protein in normal and tumor tissues, the excess expression of *CD166* protein was demonstrated in the colorectal cancer, as result this protein is considered to be the exclusive marker of colorectal cancer. Considering the correlation between *CD166* protein and tumor growth, this protein is suggested to be a marker for fighting methods against colorectal cancer. In this research, underlying studies have been provided using bioinformatics analysis, cloning and expression of the *CD166* protein, in order to employ this protein as a vaccine and to produce colorectal cancer detection kits.

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