

## Construction of CTLA-4-Ig Fusion Gene in pBudCE4.1 Expression Vector

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## Abstract

**Background:** CTLA-4 inhibitory signals prevent cell cycle progression and IL-2 production, leading to a halt on an ongoing immune response. CTLA4-Ig fusion proteins contain the extracellular domain of CTLA-4 and Fc fragment of human IgG antibody. In this study we aimed to fuse the *ctla-4* gene encoding the extracellular domain of CTLA-4 molecule with *igg1* gene encoding Fc region of human IgG.

**Methods:** After primer design, PCR reaction was performed using *pfu* polymerase enzyme and specific primers. PCR amplified fragment was ligated into the vector containing the human *igg1* gene. The resulting fusion fragment of *ctla-4* and human *igg1* genes was ligated to pBudCE4.1 expression vector.

**Results:** Extracellular domain of *ctla-4* gene was ligated to *igg1* gene and then *ctla-4-ig* fragment was cloned into pBudCE4.1 vector. Construction of the expression vector was confirmed by restriction pattern analysis and sequencing.

**Conclusion:** By confirming the construct, in the next step, the recombinant DNA will be used to produce CTLA4-Ig recombinant protein for the clinical uses.

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**Keywords:** Abatacept, CTLA-4 antigen, CTLA4-Ig, Recombinant DNA

## Introduction

Following the T cell activation, cytotoxic T lymphocyte antigen-4 (CTLA-4), a negative regulatory molecule, will be expressed on T cells<sup>1,2</sup>. CTLA-4 is a homolog of CD28 but binds B-7 molecules with greater affinity. Inhibitory signals of this molecule inhibit cell cycle progression and IL-2 production, leading to a halt on an ongoing immune response<sup>3-6</sup>.

Regarding the central role of CTLA-4 in down-regulation of the immune responses, co-stimulatory receptors became an important target for drug development. The best example is the CTLA4-Ig fusion protein, containing the extracellular domain of CTLA-4 and the constant region of human IgG antibody. This fusion protein can inhibit T cells dependent immune responses<sup>5,7,8</sup>.

In this study we aimed to fuse extracellular domain of *ctla-4* gene to Fc region of human *igg1* gene. This recombinant DNA could be used to produce CTLA4-Ig protein and studying its function in future studies.

## Materials and Methods

## Enzymes and chemicals

All chemicals and antibiotics were purchased from Sigma, Merck (Germany) and Invitrogen (France), unless stated otherwise. DNA-modifying enzymes and

restriction enzymes were obtained from Fermentas.

## Vectors, microorganisms and growth conditions

*Escherichia coli* DH5 $\alpha$  (CinnaGen, Iran) as a host and pBudCE4.1 as an expression vector were used. IgG1 containing vector was provided kindly by Dr. Rabbani (Avicenna Research Institute, Iran). *Escherichia coli* (*E. coli*) were cultured in LB medium at appropriate temperature (37°C) with shaking (150 rpm).

## PCR amplification and CTLA4-Ig fragment construction

Extracellular domain of *ctla-4* gene was amplified using specific primers (CTLA4-FOR/CTLA4-fuse) and pUCCTLA4 vector as template. For subsequent cloning of the PCR-derived fragments, *SalI* and *BamHI* restriction sites were added to the 5'-end of these primers, respectively (Table 1). pUCCTLA-4 (synthetic construct) was used to amplify the *ctla-4* gene with *pfu* polymerase enzyme. PCR products were purified by High Pure PCR Product Purification Kit (Roche, Germany). The purified fragment was digested simultaneously with vector containing human *igg1* gene using *SalI/BamHI* enzymes and then were ligated. *E. coli* DH5 $\alpha$  cells were transformed using CaCl<sub>2</sub> method<sup>9</sup>. Recombinant colonies were confirmed by PCR using

## Construction of *ctla4-Ig* Fusion Gene in pBudCE4.1 Expression Vector

Table 1. Oligonucleotides (primers) used in present study (restriction sites were showed in bold)

Primers	Sequence 5'to 3'	Orientation	5' cloning site
<b>A) Primers used for amplification of <i>ctla4-Ig</i></b>			
CTLA-4 FOR	5'-TT <b>GT</b> CGACAGCCACCATGGCTTGCCTT-3	Sense	<i>SalI</i>
CTLA-4 fuse	5'-TTGGAT <b>CC</b> GTGAGAATCTGGGCA-3'	Anti-sense	<i>Bam</i> HI
<b>B) Universal primers</b>			
T7f	5' GTAAAACGACGGCCAGT	Sense	--
pBudCE4.1r	5' CAGGAAACAGCTATGAC	Anti-sense	--
M13f	5'-GTAAAACGACGGCCAGT-3'	Sense	--
M13r	5'-AACAGCTATGACCATG-3'	Anti-sense	--

specific primers. Plasmid DNA preparation was done using QIAGEN Mini Prep Kit (Germany).

### Construction of CTLA4-Ig expression vector

Vector containing the *ctla4-ig* fragment digested by *SalI/XbaI* enzymes and subcloned into pBudCE4.1 *SalI/XbaI* cloning sites with the methods mentioned before. Recombinant colonies were confirmed by digestion with cloning enzymes and PCR pattern.

### Sequence and computer analysis

Cloned DNA fragment in pBudCE4.1 (50-200 ng/ $\mu$ l) was sequenced by a Commercial Service (Bio-ner, South Korea).

## Results

*ctla-4* fragment was amplified using specific primers and *pfu* polymerase. A specific band about 483 bp showed the expected size (Figure 1). Extracellular domain of *ctla-4* gene was inserted into the *SalI* and *Bam*HI pGEMIgG vector and designated as pGEMCTLA4-Ig. The new construct was confirmed by restriction pattern using *SalI/Bam*HI and *SalI/XbaI* enzymes and PCR product pattern. According to the size of *ctla-4* gene external domain (483 bp) and *igg1* gene (993 bp), the resulting fragment (1476 bp) confirmed the fusion of extracellular domain of *ctla-4* to human *igg1* gene (Figure 2).

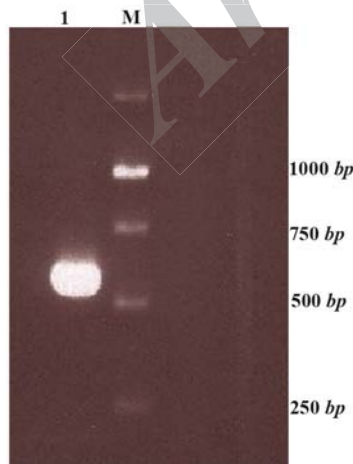


Figure 1. *ctla-4* gene PCR product with *Pfu* polymerase enzyme  
M: 1 kb ladder  
1: PCR product using specific primers (CTLA4- FOR / CTLA4-fuse).

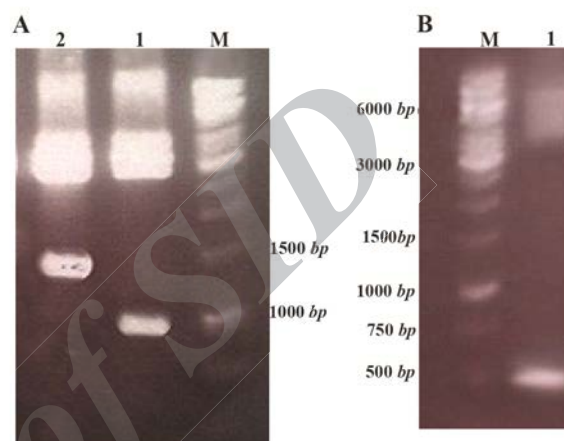


Figure 2. Confirmation of new construct with digestion pattern  
A) 1: Digestion of vector containing *igg1* gene with *SalI/XbaI* enzymes  
2: Digestion of new construct with *SalI/XbaI* enzymes  
B) 1: Digestion of new construct with *SalI/Bam*HI enzymes.

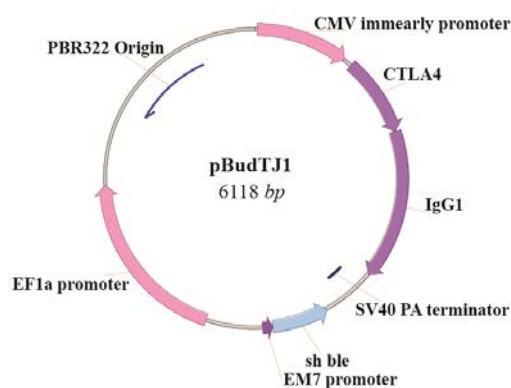


Figure 3. pBudTJ1 schematic view.

To clone *ctla4-ig* fragment in pBudCE4.1 expression vector, pGEMCTLA4-Ig construct was digested and gel purified fragment was cloned into pBudCE4.1 vector. Construction of the expression vector was confirmed by restriction pattern analysis using *SalI* and *XbaI*. The cloned fragment was sequenced by T7f/pBudCE4.1r universal primers. DNA sequencing showed an open reading frame, 1476 bp in length, encoding a 492 amino acid polypeptide. The new construct designated pBudTJ1 is shown in figure 3.

### Discussion

Co-stimulatory molecules play a critical role in controlling the immune response. The central role of CD28 family, especially the CTLA-4, makes it a useful tool for immunotherapy in autoimmune disease and transplant rejection<sup>6,10</sup>.

Two approaches have been selected in respect to the potential clinical applications of CTLA-4 in immunotherapy, anti CTLA-4 antibody and CTLA4-Ig, respectively<sup>7,11</sup>. CTLA4-Ig is a fusion protein containing the extracellular domain of CTLA-4 and the Fc portion of human IgG1. This protein is capable of preventing the stimulatory effect of CD28 through competing and binding to B-7s on APCs<sup>12-14</sup>.

In this study, we have fused the extracellular domain of CTLA-4 to the Fc fragment of the human IgG1 antibody. The resulting construct was ligated to pBud-CE4.1 expression vector and confirmed by sequencing. Analysis of the fusion sequence revealed an open reading frame encoding a protein of 445 amino acids with predicted molecular mass of about 50 *kDa* without glycosylation (ExPASy). In subsequent study, this recombinant DNA can be used to produce CTLA4-Ig recombinant protein.

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