Construction of TNF-α and IL-6 Fusion Protein Gene Clone

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Abstract: TNF-α (tumor necrosis factor-α) and IL-6 (interleukin-6) are key cytokines secreted by macrophages that trigger acute-phase responses. TNF-α primarily regulates immune cells, induces fever, and promotes apoptosis. By stimulating the production of IL-6 and IL-1, it can lead to cachexia, a state of systemic wasting observed in the late stages of cancer, tuberculosis, hemophilia, and other diseases. This condition reduces the time available for treatment and accelerates patient mortality. In this project, the TNF-α and IL-6 genes were fused using PCR amplification, followed by large-scale purification and recovery of the products. The resulting fusion gene was cloned into a high-expression plasmid vector for enhanced expression, transfected into E. coli, and subsequently screened and sequenced to obtain the desired fusion protein. The aim of the TNF-α-IL-6 fusion protein is to form a receptor trimer after interacting with its ligand. By occupying specific receptor binding sites, the fusion protein disrupts the correct signaling pathway of TNF-α, reducing the likelihood of TNF-α binding to cells, thereby decreasing IL-6 production, alleviating cachexia symptoms, and prolonging the survival of cancer patients to provide more time for further treatment.

Keywords: Tumor; Cachexia; Fusion protein

Introduction

Fusion proteins are protein products generated by connecting the coding regions of two distinct cytokines through DNA recombination technology, controlled by a single regulatory sequence. Under artificial conditions, these fusion genes can express chimeric proteins. Detailed discussions on fusion proteins have been presented by Wang Jianhua [1], and Huang Shuqi et al. investigated optimal expression conditions for specific fusion proteins [2]. Currently, some recombinant fusion proteins with both specificity and efficacy have entered clinical trials, demonstrating therapeutic potential in fields such as anti-tumor, anti-rheumatoid arthritis, and anti-HIV treatment, providing new therapeutic strategies for human diseases.

Fusion proteins play a unique role in immune regulation. Moutih Rafei et al. discovered that a granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-15 fusion protein can induce regulatory B cells with immunosuppressive properties [3]. Jian Hui Wu et al. found that a GM-CSF and IL-15 fusion protein can transmit asymmetric signals through IL-15 receptor complexes, leading to paradoxical immune suppression [4]. Additionally, Jacques Galipeau et al. have filed patents related to the application of fusion proteins in modulating immune responses [5].

Given that TNF-α can act on macrophages to induce the production of IL-6, which also triggers acute-phase responses, and that biologically active TNF-α exists as a tightly bound trimer, this study proposes the fusion of IL-6 and TNF-α genes. Upon injection into the body, the fusion protein is expected to transmit incorrect signals intracellularly, thereby reducing IL-6 production and alleviating the symptoms of cachexia, ultimately alleviating the suffering of cancer patients.

1 Materials and Methods

1.1 Experimental Design

1.1.1 Workflow

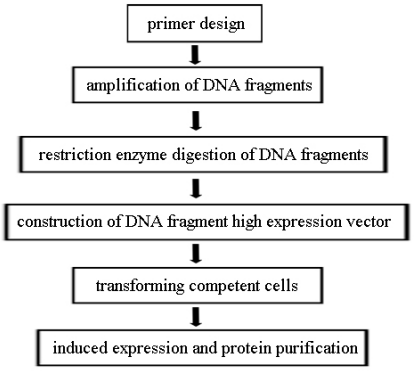


Fig. 1 Workflow Diagram for TNF–α and IL–6 Fusion Protein Construction

1.1.2 Experimental Design for Optimization of PCR Conditions

Table. 1 Experimental Design for PCR Optimization Control

Enzyme / Annealing Temperature	2×Tag Mix	2×Pfu Mix	TransStart FastPfu DNA Polymerase	DNA Polymerase
40° C				
45° C				
50° C				
55° C				
60° C				

1.2 Experimental Materials

1.2.1 Experimental Materials and Reagents

(1) Human TNF-α template gene

(2) Human IL-6 template gene

(3) Mouse TNF-α template gene

(4) Mouse IL-6 template gene

(5) PCR primers (designed in-house and synthesized by the laboratory)

(6) DNA polymerases for PCR (2xTag Mix, 2xPfu Mix, TransStart FastPfu DNA Polymerase, high-fidelity DNA polymerase)

(7) TAE buffer

(8) Agarose gel

(9) Agarose gel recovery kit

(10) Plasmid mini-prep kit

(11) Trans10 chemically competent cells

(12) EGFP-N1-Tie2 plasmid vector

(13) T4 DNA ligase

(14) Restriction endonucleases (BamH1, EcoR1)

(15) D2000 DNA Marker

1.2.2 Experimental Instruments

(1) PCR thermocycler

(2) Microwave oven

(3) Power supply for electrophoresis and gel combs

(4) Gel imaging system

(5) Nanodrop 2000 spectrophotometer

(6) Centrifuge

(7) Constant-temperature water bath

(8) Constant-temperature shaker

1.3 Methods

1.3.1 Primer Design

Primers were designed using Primer Premier 5 software by incorporating BamH1 (GGATCC) and EcoR1 (GAATTC) restriction enzyme recognition sites. After adding the linker sequence between the TNF-α and IL-6 gene fragments, a total of eight primers were designed for the human and mouse TNF-α and IL-6 genes.

TNF-α (Human)

- Forward: 5'-GCGGATCCATGAGCACTGAAAGCATGA-3'
- Reverse: 3'-GCGAATTCTCACAGGGCAATGATCCCAA-5'

IL-6 (Human)

- Forward: 5'-GCGGATCCATGAACTCCTTCTCCACAAG-3'
- Reverse: 3'-GCGAATTCCTACATTTGCCGAAGAGCCC-5'

TNF-α (Mouse)

- Forward: 5'-GCGGATCCATGAGCACAGAAAGCATGA-3'
- Reverse: 3'-GCGAATTCTCACAGAGCAATGACTCCAA-5'

IL-6 (Mouse)

- Forward: 5'-GCGGATCCATGAAGTTCCTCTCTGCAAG-3'
- Reverse: 3'-GCGAATTCCTAGGTTTGCCGAGTAGATC-5'

1.3.2 Incorporation of Linker Sequence into TNF-α and IL-6 Genes

The TNF-α and IL-6 gene fragments were fused with the linker sequence using PCR amplification. During the amplification process, the primers annealed to the template DNA, and the linker sequence was introduced into the final fusion product.

1.3.3 Preparation of Agarose Gel

A 25 mL agarose gel was prepared by dissolving 2.5 g of agarose in 25 mL of TAE buffer and heating for 2 minutes.

After adding 2.5 μL of loading dye, the solution was allowed to cool and solidify to form the gel.

1.3.4 DNA Fragment Recovery

Under UV illumination, the portion of the gel containing the target DNA fragment was excised. The DNA was purified from the gel using an agarose gel recovery kit and stored at 4°C.

1.3.5 Restriction Enzyme Digestion of DNA Fragments

In a sterile EP tube, 12 μL of double-distilled water, 2 μL of 10X buffer, and 1 μL of restriction enzymes (0.5 μL BamH1 and 0.5 μL EcoR1) were added along with 5 μL of plasmid DNA. After brief centrifugation, the reaction was incubated at 37°C for 1–2 hours.

1.3.6 Cloning into High-Expression Plasmid Vector

Linearized DNA fragments were ligated into the plasmid vector using T4 DNA ligase during the PCR reaction to form recombinant plasmids.

1.3.7 Transformation of Competent Cells

The recombinant plasmids were transformed into Trans10 chemically competent E. coli cells. Since the plasmid contained a kanamycin resistance gene, the cells were cultured in a shaking incubator at 37°C overnight in the presence of kanamycin.

2 Results and Analysis

2.1 Human TNF-α and IL-6 Genes with Linker Sequence Insertion Experiment

2.1.1 Optimization of Annealing Temperature for Linker Incorporation in Human TNF-α and IL-6 Genes

Following the method described above for inserting the linker sequence into the human TNF-α and IL-6 genes, an annealing temperature gradient was set during PCR. The PCR products corresponding to each temperature were loaded into the wells of an agarose gel. The electrophoresis results are shown in Figure 3.

Given that the length of the human TNF-α gene is 702 bp and that of the IL-6 gene is 639 bp, the electrophoresis results confirmed successful incorporation of the linker sequence into both genes. The data indicated that the optimal annealing temperature for the PCR amplification of the TNF-α gene was 50°C, while for the IL-6 gene, the optimal annealing temperature was 55°C.

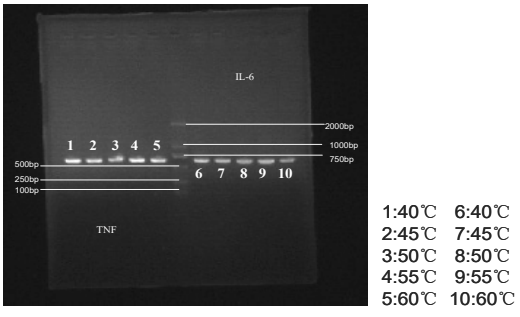


Fig. 2 Electrophoresis Results for Optimal Annealing Temperature Exploration of Human TNF-α and IL-6 Genes with Linker Sequence (The PCR products corresponding to different annealing temperatures were loaded into the wells as indicated in the legend. The central lane contains the D2000 DNA marker bands. The specificity of the PCR products is reflected by the intensity of the bands.)

2.1.2 Incorporation of Linker Sequence into Human TNF-α Gene

Following the method described above for incorporating the linker sequence into the human TNF-α and IL-6 genes, and based on the results of experiment 2.1.1, the annealing temperature during PCR was set to 50°C. The PCR products were then loaded into the wells of the agarose gel. The electrophoresis results are shown in Figure 4.

Upon analysis of the electrophoresis results, the effective length of the TNF-α gene was confirmed to be 702 bp. By comparing with the D2000 DNA marker on the left side of the gel, the bright band corresponding to the target gene was identified. This section of the gel was excised and the DNA was recovered for further use.

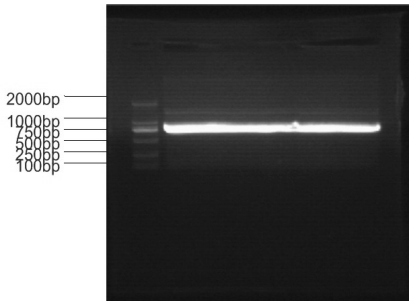


Fig. 3 Electrophoresis Results of Human TNF–α Gene with Linker Sequence

2.1.3 Incorporation of Linker Sequence into Human IL-6 Gene

Following the method described above for incorporating the linker sequence into the human TNF-α and IL-6 genes, and based on the results of experiment 2.1.1, the annealing temperature during PCR was set to 55°C. The PCR products were then loaded into the wells of the agarose gel. The electrophoresis results are shown in Figure 5.

Upon analysis of the electrophoresis results, the effective length of the IL-6 gene was confirmed to be 639 bp. By comparing with the D2000 DNA marker on the right side of the gel, the bright band was identified as the target gene. This section of the gel was excised, and the DNA was recovered for subsequent experiments.

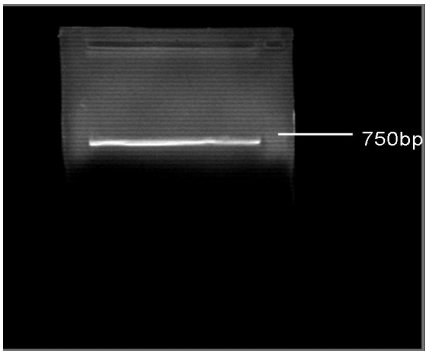


Fig. 4 Electrophoresis Results of Human IL–6 Gene with Linker Sequence

2.2 Fusion of Human TNF-α and IL-6 Genes into a Fusion Protein Gene

2.2.1 Exploration of the Optimal DNA Polymerase for Fusion Gene Construction

Taq polymerase has an error rate of 1 in 1,000 base pairs, making it unsuitable for PCR preparation of fusion genes due to potential mutations. Therefore, Pfu polymerase, known for its high fidelity, was used. However, experimental observations indicated that Pfu polymerase exhibited low efficiency due to the high GC content of the template genes. Thus, an experiment was designed to identify the most suitable polymerase for linking the two genes.

Four PCR reaction systems were set up, each containing a different polymerase (2xTag Mix, 2xPfu Mix, TransStart FastPfu DNA Polymerase, and high-fidelity DNA Polymerase). After the PCR reactions, the products were loaded into the wells of an agarose gel. The electrophoresis results are shown in Figure 6.

Upon analysis of the electrophoresis results, the PCR product bands obtained with high-fidelity DNA Polymerase were the brightest. This indicates that high-fidelity DNA Polymerase has the highest efficiency and specificity. Therefore, the fusion of the two genes (human TNF-α and IL-6) should use high-fidelity DNA Polymerase for optimal PCR performance.

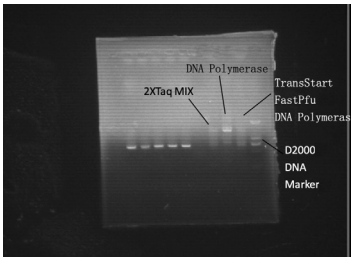


Fig. 5 Electrophoresis Results for Optimal DNA Polymerase Selection for Fusion of Human TNF–α and IL–6 Genes

2.2.2 Exploration of the Optimal Annealing Temperature for Fusion of Human TNF-α and IL-6 Genes

High-fidelity DNA polymerase was used in the PCR system. An annealing temperature gradient was applied, and the PCR products of the fused human TNF-α and IL-6 genes at different annealing temperatures were loaded into the wells of an agarose gel. The electrophoresis results are shown in Figure 7.

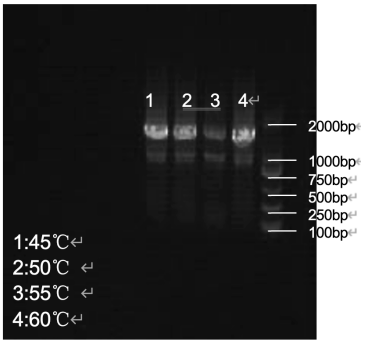


Fig. 6 Electrophoresis Results for Optimal Annealing Temperature for Fusion of Human TNF–α and IL–6 Genes

Analysis of Electrophoresis Results:The length of the fused human TNF-α and IL-6 gene band was confirmed to be 1356 bp, indicating the successful formation of the target fusion band. The agarose gel electrophoresis results showed that the brightest band was obtained at an annealing temperature of 60°C, demonstrating the highest PCR efficiency for the fusion of the two genes at this temperature. Therefore, 60°C was determined to be the optimal annealing temperature for the PCR reaction of the human TNF-α and IL-6 gene fusion.

2.2.3 Fusion of Human TNF-α and IL-6 Genes into a Fusion Protein Gene

Based on the results of experiments 2.2.1 and 2.2.2, the optimal DNA polymerase and annealing temperature for the fusion of the two genes were identified. To further enhance the efficiency due to the high GC content of the genes, a PCR system was developed using a GC content enhancer. The components of the PCR system were as follows:

- Template DNA: 1.7 μL
- High-fidelity DNA polymerase: 0.5 μL

- dNTPs: 8 μL
- Q5 enhancer: 10 μL
- Forward primer: 1 μL
- Reverse primer: 1 μL
- Double-distilled water: 18.3 μL
- Buffer: 10 μL

The PCR product obtained using this system at an annealing temperature of 60°C was loaded into the wells of an agarose gel. The electrophoresis results are shown in Figure 8.

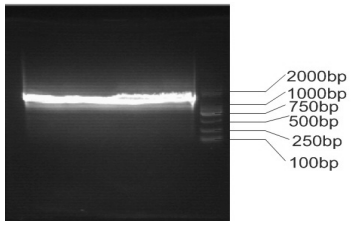
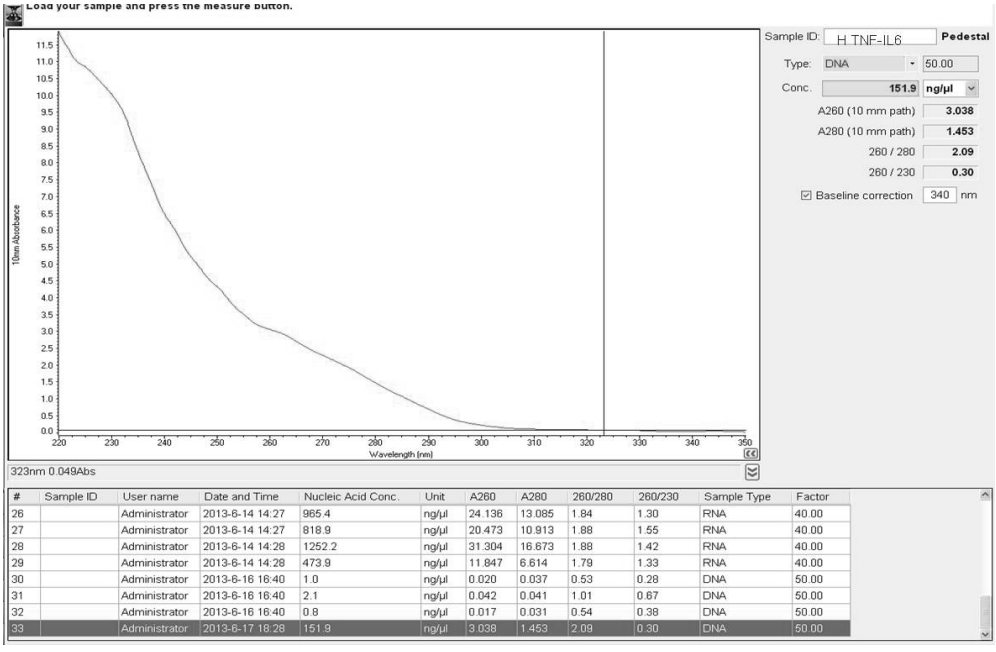


Fig. 7 Electrophoresis Results of Human TNF–α and IL–6 Gene Fusion into a Fusion Protein Gene

The effective length of the human TNF-α gene is 702 bp, and that of the human IL-6 gene is 639 bp. Therefore, the expected length of the fused gene is 1341 bp, and 1356 bp after including the linker sequence. By comparing with the D2000 DNA marker bands on the right side of the gel, it was confirmed that the human TNF-α-IL-6 fusion protein gene was successfully obtained. The bright band was excised from the gel and recovered. The concentration was measured using a NanoDrop 2000 spectrophotometer, and the result was 151.9 ng/μL, as shown in Figure 9.



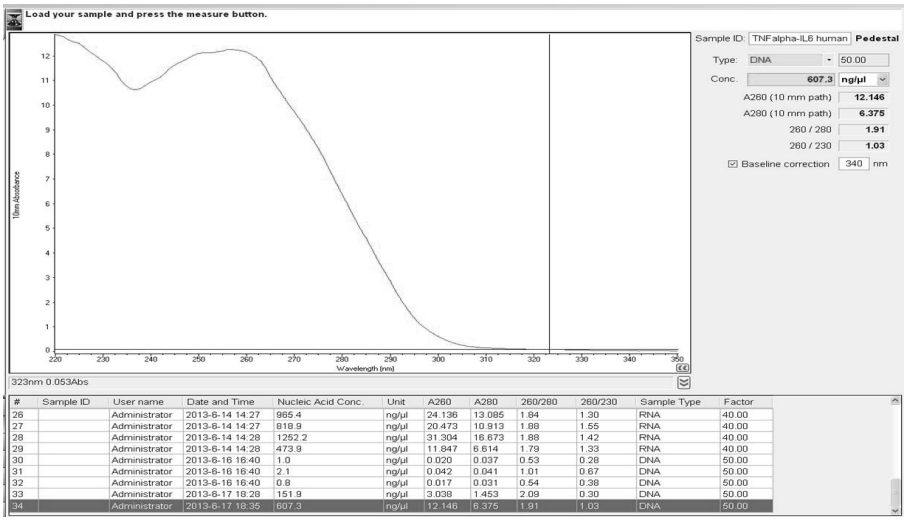
#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
26		Administrator	2013-6-14 14:27	965.4	ng/μl	24.136	13.085	1.84	1.30	RNA	40.00
27		Administrator	2013-6-14 14:27	818.9	ng/μl	20.473	10.913	1.88	1.55	RNA	40.00
28		Administrator	2013-6-14 14:28	1252.2	ng/μl	31.304	16.673	1.88	1.42	RNA	40.00
29		Administrator	2013-6-14 14:28	473.9	ng/μl	11.847	6.614	1.79	1.33	RNA	40.00
30		Administrator	2013-6-16 16:40	1.0	ng/μl	0.020	0.037	0.53	0.28	DNA	50.00
31		Administrator	2013-6-16 16:40	2.1	ng/μl	0.042	0.041	1.01	0.67	DNA	50.00
32		Administrator	2013-6-16 16:40	0.8	ng/μl	0.017	0.031	0.54	0.38	DNA	50.00
33		Administrator	2013-6-17 18:28	151.9	ng/μl	3.038	1.453	2.09	0.30	DNA	50.00

Fig. 8 Concentration of Human TNF–α–IL–6 Fusion Protein Gene

2.3 Transformation into Competent Cells and Plasmid Extraction

Following the methods described above for restriction enzyme digestion, cloning into a high-expression plasmid vector,

and transformation into competent cells, the fusion protein gene was subjected to the respective procedures. The amplified plasmids were extracted and verified by electrophoresis to confirm that the plasmid contained the fusion protein gene. Subsequently, the concentration of the plasmid was measured using a NanoDrop 2000 spectrophotometer, and the results are shown in Figure 10.



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33		Administrator	2013-6-17 18:28	151.9	ng/µl	3.038	1.453	2.09	0.30	DNA	50.00
34		Administrator	2013-6-17 18:35	607.3	ng/µl	12.146	6.375	1.91	1.03	DNA	50.00

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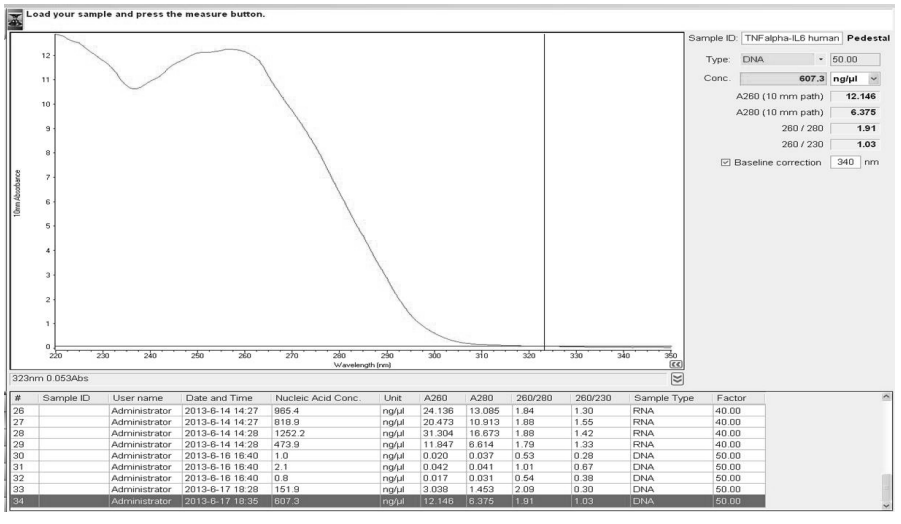

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Fig. 9 DNA Concentration Extracted from Competent Cells

As shown in Figure 10, a DNA absorption peak was observed at 260 nm, indicating a high level of DNA content. The

measured DNA concentration at this point was 607.3 ng/μL.

3 Discussion

3.1 Optimal Annealing Temperature for Inserting the Linker Sequence into Human TNF-α and IL-6 Genes

Due to differences in the number of base pairs and nucleotide sequences, different annealing temperatures are required during PCR to ensure efficient and precise primer-template binding. The annealing temperature is a critical factor in PCR amplification, making temperature optimization an essential step. After setting a gradient and comparing the product concentrations at different temperatures, the optimal annealing temperature for inserting the linker into the human TNF-α gene was determined to be 50°C, and for the IL-6 gene, 55°C.

3.2 Optimal DNA Polymerase for the Fusion of Human TNF-α and IL-6 Genes

The human TNF-α-IL-6 fusion gene sequence has a high GC content, which directly reduces PCR efficiency. Taq and Pfu polymerases are commonly used in laboratory PCR. Taq polymerase has a higher amplification efficiency than Pfu polymerase but a higher error rate, which may cause issues during subsequent protein expression. Pfu polymerase has a lower error rate but lower catalytic efficiency. Neither polymerase proved ideal for amplifying the fusion gene. Therefore, a comparison experiment was conducted to identify the most suitable polymerase for this study. The results indicated that high-fidelity DNA polymerase exhibited high catalytic efficiency and strong specificity during the PCR amplification of the fusion gene, as shown by the bright, low-background bands in the electrophoresis results. Thus, high-fidelity DNA polymerase was identified as the optimal choice for fusing the human TNF-α and IL-6 genes.

3.3 Optimal PCR System for the Fusion of Human TNF-α and IL-6 Genes

To address the challenge of high GC content in the fusion gene, a GC enhancer was added to the reaction system, and high-fidelity DNA polymerase was used. The optimized PCR system for fusing the human TNF-α and IL-6 genes is as follows:

- Template DNA: 1.7 μL
- High-fidelity DNA polymerase: 0.5 μL
- dNTPs: 8 μL
- Q5 enhancer: 10 μL
- Forward primer: 1 μL
- Reverse primer: 1 μL
- Double-distilled water: 18.3 μL
- Buffer: 10 μL

3.4 Optimal Annealing Temperature for Fusion of Human TNF-α and IL-6 Genes

The efficiency of the PCR reaction is highest at the optimal annealing temperature. By comparing the band intensity after electrophoresis at different annealing temperatures, the highest reaction efficiency was observed at 60°C. Therefore, 60°C was determined to be the optimal annealing temperature for fusing the human TNF-α and IL-6 genes.

4 Conclusion

During the construction of the human TNF-α-IL-6 fusion protein, key parameters for each step were identified, yielding

significant intermediate results:

1.The optimal annealing temperature for inserting the linker into the human TNF-α gene was 50°C, while for the IL-6 gene, it was 55°C.

2.High-fidelity DNA polymerase was identified as the most suitable polymerase for the PCR amplification of the fusion gene.

3.The optimized PCR system for the fusion of the human TNF-α and IL-6 genes included:

- Template DNA: 1.7 μL
- High-fidelity DNA polymerase: 0.5 μL
- dNTPs: 8 μL
- Q5 enhancer: 10 μL
- Forward primer: 1 μL
- Reverse primer: 1 μL
- Double-distilled water: 18.3 μL
- Buffer: 10 μL

4.The optimal annealing temperature for the fusion of the human TNF-α and IL-6 genes was determined to be 60°C.

5.The human TNF-α-IL-6 fusion gene was successfully obtained with a concentration of 151.9 ng/μL.

6.The fusion gene was successfully cloned into the plasmid vector and transformed into E. coli. The plasmid was re-extracted, yielding a concentration of 607.3 ng/μL.

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