Construction of plasmids expressing recombinant B cell epitopes of PD1

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Abstrak

Latar Belakang: *Pengobatan kanker di Indonesia umumnya menggunakan pengobatan dengan kemoterapi atau dengan operasi. Efek samping dari pengobatan ini antara lain adalah kerontokan rambut, mual dan penurunan berat badan. Saat ini sedang berkembang alternatif terapi kanker dengan menggunakan immunoterapi. Kemampuan sel kanker untuk menghindar dari sistem imun disebabkan adanya protein PD-1 pada sel T yang berikatan dengan ligannya PD-L1.*

Metode: *Penelitian ini merupakan penelitian awal yaitu pembuatan rekombinan PQE PD-1 dan menggunakan bagian soluble dari PD-1 yang disebut dengan EP2PD1 yang akan digunakan untuk pembuatan antibodi monoklonal dan sistem pendeteksi antibodi monoklonal. Metode pembuatan rekombinan PD-1 dan EP2PD1 dengan cara penentuan sekuens epitop sel B yang paling imunogenik dilanjutkan dengan amplifikasi sekuen tersebut dengan PCR dan diligasi ke vektor pengekspresi PQE80.*

Hasil: *Telah terbentuk konstruksi rekombinan PQE80 PD-1 dan PQEEP2PD1 yang diverifikasi menggunakan PCR koloni, pemotongan enzimatik dan sekuensing. Hasil penelitian menunjukkan bahwa epitop PD1 telah terklona ke PQE 80 dan tidak ditemukan mutasi dalam urutan asam amino.*

Kesimpulan: *Konstruksi yang dibuat tidak mempunya mutasi dan dapat dilanjutkan untuk pembuatan antibodi monoklonal.* ***(Health Science Journal of Indonesia 2019;10(1):1-7)***

Kata Kunci: *PD1, Epitop, Kanker, Immunotherapy*

Abstract

Background: Medications on cancer to date in Indonesia is mostly by surgical or chemotherapy, this type of medications is not always curing the patients. The side effect of the chemotherapy drugs sometimes more challenging such as hair loss, nausea and lost weight. One of the promising targets for cancer is using immune therapy. Cancer cells can avoid immune response by surprising immunity through activation of specific inhibitory signalling pathways, referred to as immune checkpoints. Immune check points like PD-1, PD-L1 are breakthrough therapies in oncology and this monoclonal antibody have been approved by the FDA for treatment. In this research we develop full PD-1 and part of PD1 sequence as an insert then we construct with plasmid PQE80L. This recombinant called PQE PD-1 and PQEEP2PD1. The aim of this study is to make recombinant which would be used to detect PD1 full clone monoclonal antibodies.

Methods: In this study, we designed our recombinants using Indonesian HLA and others using in silico models, this prototype will not only cover Indonesian patients but also other country.

Result: The result showed that the epitope sequence of PD1 has been clone to PQE 80 wt and verified using colony PCR, Enzyme Digestion and Sanger Sequencing. The Clone than will be expressed and injected to animal model to produce antibody.

Conclusion: Construction of recombinant PQE PD-1 and PQE EP2PD1 are constructed without any mutation in the sequence, this recombinant can be used in the next study for protein expression of PQE PD-1 and PQE EP2PD1. ***(Health Science Journal of Indonesia 2019;10(1):1-7)***

Keywords: PD1, Epitope , Cancer, Immunotherapy

Current treatment of cervical dysplasia is limited to excisional or ablative procedures that remove or destroy cervical tissue. These procedures have efficacy rates of approximately 90% but are associated with morbidity and expense. Additionally, surgical treatments remove only the dysplastic tissue, leaving normal-appearing HPV-infected tissue untreated.[1]

There are five main therapeutic modalities for cancer: surgery, radiation, chemotherapy, targeted therapy, and immunotherapy. With a few exceptions, the first four modalities are focused squarely on cancer itself. Immunotherapy represents conceptually a unique way of dealing with cancer which is to focus on eliminating cancer indirectly by harnessing the power of the host's immune system.[1] The presence of tumour – associated inflammatory cells in tumours raises an important question, which is one of the most important challenges in oncology. During the early stage of tumour development, cytotoxic immune cells such as NK cells, and CD8+ recognize and eliminate the more immunogenic cancer cells.[2]

In 2011 the immune check point inhibitor (ipilimumab) that used CTLA-4 antibodies was approved by the FDA for melanoma therapy. There are currently 2 types of immune FDA approved therapy as treatment cancer (1) inhibitors of either the programmed death receptor 1 (PD-1) or its ligand (PD-L1), or (2) cytotoxic T cell lymphocyte-associated protein 4 (CTLA-4).[3,4] A good example of the advanced checkpoint molecules that mediates tumour -induced immune suppression is programmed death-1 (PD-1). Physiologically, the PD-1/PD-L1 pathway had emerged as a result of the need to control the degree of inflammation at locations expressing the antigen, in order to protect normal tissue from damage. There was a remarkable expression of the PD-1 protein on the surface of all activated T cells. When a T cell recognizes the antigen expressed by the MHC complex on the target cell, inflammatory cytokines are produced, initiating the inflammatory process.[5,6]

These cytokines result in PD-L1 expression in the tissue, activating the PD-1 protein on the T cell leading to immune tolerance, a phenomenon where the immune system lose the control to mount an inflammatory response, even in the presence of actionable antigens.[5]

The evolution of immune checkpoint inhibitors as anticancer treatment options represents one of the most successful approaches in cancer drug discovery in the past few years. Someday might be possible to make cancer an easy disease to cure.

In this study, we constructed recombinant PQE80 with PD1 full sequences and recombinant of part of epitopes from PD1. The purpose of obtaining a full PD1 sequence construction was to obtain PD1 protein to be injected into mice and to obtain PD1 monoclonal antibody after going through the stages of making monoclonal antibodies. Whereas the making of part of PD1 epitope which we call PQEEP2PD1 is intended as a detection and selection of monoclonal antibody of full PD1 sequence. We designed sequence of Full PD1 and EP2PD1 with unique epitope for single B-cell. Because monoclonal antibodies generated from a single B cell, it is important to get sequence epitope with high affinity. To get this we analyze our full sequence PD1 using in silico method. We use www.IEDB .org epitope B linear prediction with Kolaskar and Tongaonkar antigenicity method, with threshold number 1,007. From the result we find that locations 145-217 of protein have threshold above 1,007. We amplified this sequence and become EP2PD1 sequence. This study gives a preliminary data in DNA level that is important for the future research in expression of PD1 protein and production of PD 1-anti monoclonal antibodies as therapies of cancerous diseases.

MATERIAL AND METHODS

Analysis of PD-1 Gene

The PD1 gene was made by conducting alignment genes from the genebank. After the alignment, codon optimization was done so that the gene that is ordered is easy to express. Codon optimization was performed using in silico. The optimized genes were added with B*amHI* and S*acI*. The gene were synthesised by *Macrogene.*

Analysis in-Silico of PD-1 Protein

In silico analysis (*www.IEDB.org*) was carried out to determine epitopes of B cells that play a role in the immunology process. The analysed PD-1 DNA sequence was applied to websites and its results some epitopes for targeting PD-1 anti monoclonal antibodies.

Construction of Recombinant PQE80−PD-1and PQE EP2PD-1

The Synthetic PD-1 DNA fragment was restricted using *Bam*HI and *Sac*I enzymes .The restricted PD-1 DNA fragment was inserted to vector PQE 80 that had been restricted by the same enzymes. The EP2PD1

were amplified using PCR with synthetic PD-1 as a template. Locations of EP2PD1 was at 147-217 as mentions above and amplified using primers and TaqHifi enzyme from *Invitrogen.* After amplified, the amplicon then purified using PCR purification from Qiagen and digested using *Bam*HI and *Sac*I enzymes. The ligation was performed by using T4 enzyme and T4 DNA ligase. The ratio of vector and insert DNAs for ligation is 1: 3. Transformation was conducted by chemical induction method using heat shock. The transformant value was 7.664 x 103 cfu efficiency is 1.15 x 105 cfu / µgIn the results of the negative control transformation, no growing colonies were found so that there was no bacterial contamination when making competent TOP 10 E. *coli* cells. Selection of transformants was performed by PCR colonies. PCR. Primers PQE F: CCCGAAAAGTGCCACCTG and PQE R: GTTCTGAGGTCATTACTGG were used for PCR. The PCR was performed by the following compositions: 95^0C for 2 minutes for early denaturation, For denaturation we at 95^0C for 10 seconds, annealing at 95^0C for 30 seconds extended time at 68^0C for 1 minute and final extension at 68^0C for 2 minutes the PCR cycle is 35 cycle. The thermal cycler (BioRad) was performed by the following condition. The PCR results were visualized at 0.8% agarose gel.

Sequencing

DNA sequencing was conducted to determine the correctness of DNA sequences and in-frame between DNA insert and plasmid DNA backbone. Sequencing were using sanger method and for analysis sequencing was analysed with Bio Edit software. Based on the sequencing results, the DNA insert was successfully inserted in the frame on the PQE80 vector. The sequencing results were then aligned with the reference sequence and initial DNA PD1 gene to known any mutations that were caused by cloning process.

Ethical Clearence

The study is laboratory experiment which does not involve any human and animal as an object.

RESULTS

B Cell Epitop Prediction

A study was performed to determined which epitope might elicit cellular or humoral immune response. B-cell epitopes can be defined as a surface accessible clusters of amino acids, which are recognized by secreted antibodies or B-cell receptors. Figure 1, analysis of linear epitope prediction was using with Kolaskar and Tongaonkar antigencity method, with threshold number 1,007 at www.IEDB.org. Briefly we input the PD-1 sequence to find which position have the highest rank. The threshold of peptide prediction is 1,007, epitope give score above the threshold is the epitope were amplified.

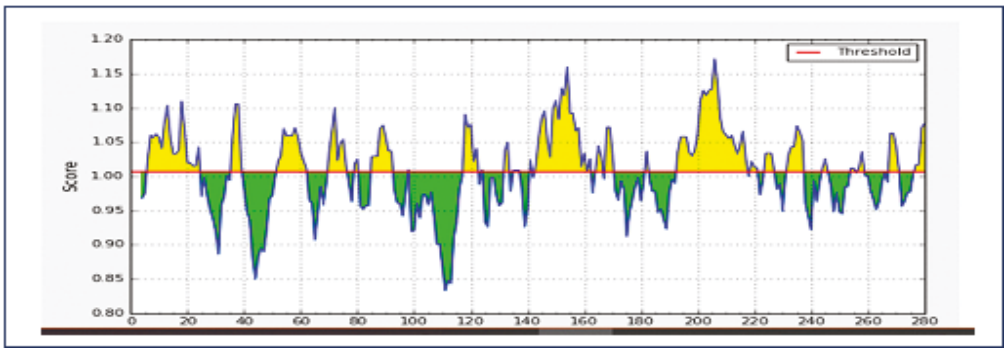

Figure 1. Result of linear epitop B cell Prediction using Kolaskar and Tongaonkar antigencity method

Table 1. The score of prediction PD-1 epitop to B cell

No	Start	End	Peptide	Lenght	Score
1	6	24	TQRLCATRTICATTTCRAA	19	0,998
2	52	62	FTPLKIQFTII	11	1,011
3	70	76	ARIFFQR	7	0,963
4	85	92	GTTCCTAT	8	0,961
5	143	160	FCTQAFFNLCFRCQANCT	18	1,026
6	193	217	LTRLATIFRKCCQFIGLIARAHTIP	25	1,022
7	232	237	KGCIIT	6	1,052

From the result we choose peptide position at 145- 217 for amplification of EP2PD1 insert. As a template we use PD-1 full length (Table 1).

Amplification of EP2PD1

Full-length PD-1 sequence it was used to amplify EP2PD1 insert. Primer used in this amplification was purchase from IDT with forward sequence : 5'-ccggaagcttatgcagattccg-3' and reverse sequence : 5'-ggccaagcttgctcgggctcat-3'.

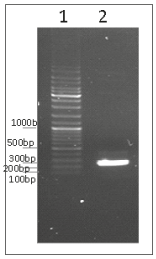

Figure 2. Amplification of EP2PD1 using PCR Taq Hifi Invitrogen.

Next, the amplification of EP2PD1 using PCR with Taq Hifi Polymerase, the amplicon had a molecular base pair 220 bp (figure 2, lane 2) which is consistent with the design of epitope prediction .

Detection of Insert PD-1 By PCR Colony

Verification of recombinant was using PCR colony. This PCR performed as early step to identified cloned that have PD1 insert. The colony use for this analysis is the colony from replica plating at ligation and transformation procedure which is 12 colony (Figure 3).

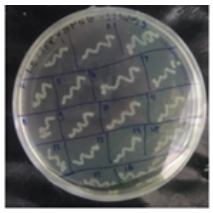

Figure 3. Replica plate of PQE80 PD-1

The Primer were use are PQE-*forward* and PQE-*reverse*. Those primer amplified Multiple Cloning Site (MCS) of PQE 80 which about ± 175 bp. To confirm the colony has insert of PD-1, length of amplified DNA from PCR colony is ± 1056 bp (175 bp + 881 bp). Electrophoresis visualisation showed that colony number 2,3,4,6,and 8 are predicted carrying PD-1 insert, showed with band at ± 1056 bp.

PCR colony also conducted to PQEEP2PD1 using primer PQE-*forward* and PQE-*reverse* confirm, to confirm the colony has insert of EP2PD-1, length of amplified DNA from PCR colony is ± 395 bp (175 bp + 220 bp). Electrophoresis visualisation at Figure 5 showed that colony number 1,2,3,4, are predicted carrying EP2PD-11 insert.

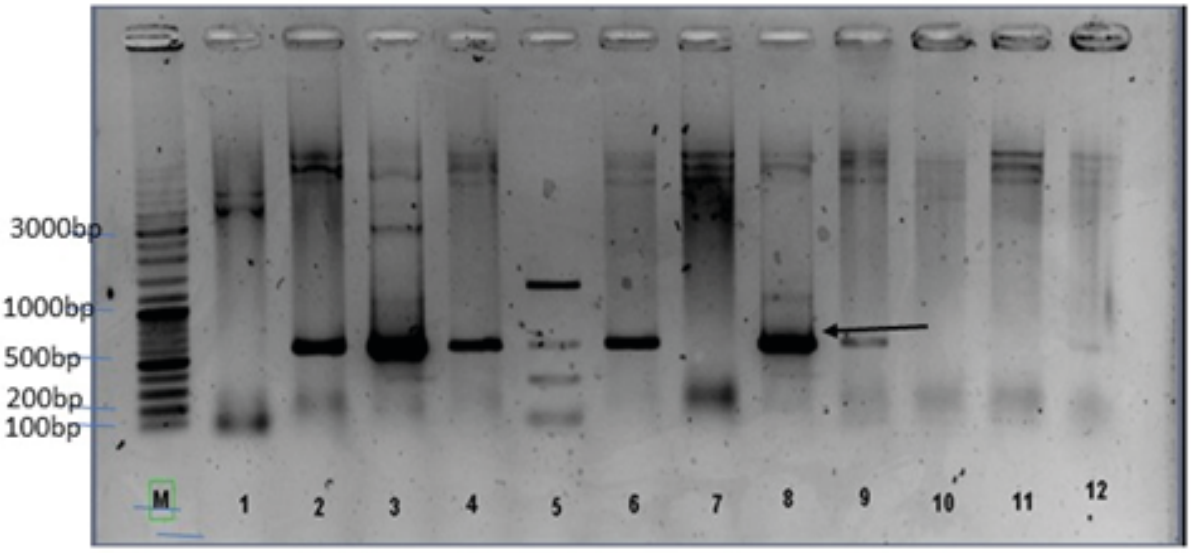

Figure 4. PCR Colony result, lane M: Gene ruler marker from Invitrogen, Lane 1: colony PQE PD-1 No:1, Lane 2: colony PQE PD-1 No:2, Lane 3,: colony PQE PD-1 No:3 Lane 4 : colony PQE PD-1 No:4, Lane 5 : colony PQE PD-1 No:5, Lane 6: colony PQE PD-1 No:6, Lane 7: colony PQE PD-1 No:7, Lane 8: colony PQE PD-1 No:8, Lane 9: colony PQE PD-1 No:9 Lane 10 : colony PQE PD-1 No:10, Lane 11 ; colony PQE PD-1 No:11, Lane 12 : colony PQE PD-1 No:1

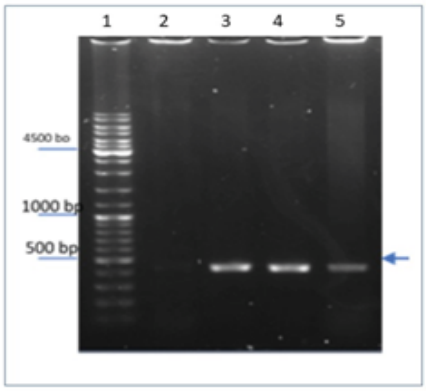

Figure 5. PCR Colony result, lane 1: Gene ruler marker from Invitrogen, Lane 2: colony PQE EP2PD1 No:1, Lane 3: colony PQE EP2PD1 No:2, Lane 4,: PQE EP2PD1 No:3 Lane 5 : PQE EP2PD1 No:4

Verification of the insert PD-1 and EP2 PD-1 by restriction enzymes

Colony that predicted have insert of PD-1, then inoculate to Luria Berthani medium with volume of culture 3 ml overnight. The next day culture were performed plasmid isolation using QIAprep Spin Miniprep Kit. The concentration of DNA plasmid was 111,6 ng/µL with total volume of 41 µL and the ratio of A260/280 value is 1,92. After plasmid isolation we performed verification of insert using enzyme digestion with B*am*HI and S*ac*I. The purpose of this method is to excrete PD-1 insert. Colonies containing insert will showed 4068 bp and 818 bp band (Figure 6).

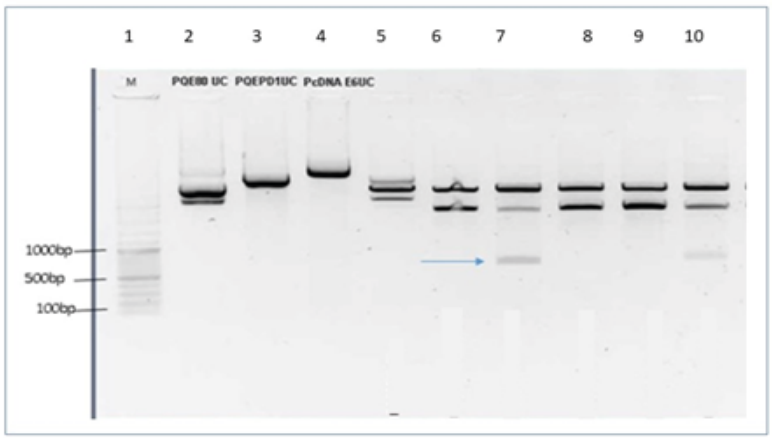

Figure 6. Verification of insert PQE PD-1 colony number 2,3,4,6,and 8 using BamHI and SacI. lane 1: Gene ruler marker from Invitrogen, Lane 2: PQE80 Uncut colony, Lane 3: PQE PD-1 Uncut, Lane 4,:PcDNA E6 Lane 5 : PQE 80 cut BamHI-SacI, Lane 6 : PQE PD-1 (2) cut BamHI-SacI , Lane 7: PQE PD-1 (3) cut BamHI-SacI, Lane 8: PQE PD-1 (4) cut BamHI-SacI, Lane 9: PQE PD-1 (6) cut BamHI-SacI, Lane 10: PQE PD-1 (8) cut BamHI-SacI

From the digestion result using agarose 0,8% tells that colony with DNA fragment expected (818 bp) are colony PQE PD-1(3) and PQE PD-1(8).

The same protocol also conducted to colony of PQE EP2PD1, for this colony the insert were excrete using B*am*HI and H*ind*III. All the digest verification protocol was performed single digest only, B*am*HI then followed by H*ind*III using NEB buffer 3 and incubate at 37^0C for 4 hours. After incubation period the plasmid was ran at agarose gel 0,8% for visualisation.

DNA Sequencing

DNA sequencing was conducted to determine nucleotide bases of inserted DNA. DNA sequencing was using Sanger method, and for analysis was using BioEdit. To find out if there is any mutation analysis was compare to reference gene from gene bank for PQE PD-1 (8) and fpr PQE EP2PD1 (1) was compare with full length of PQE PD-1(8). From data sequencing result (Figure 8) we can find out that there is no mutation of PQE EP2PD1 (1) in the recombinant, also with the PQE PD-1 (8).

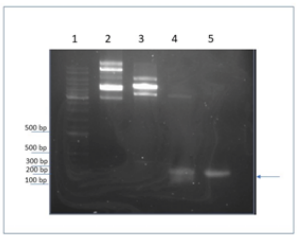

Figure 7. Verification of insert PQE EP2 PD-1 colony number 1,2, using BamHI and HindIII lane 1: Gene ruler marker from Invitrogen, Lane 2: PQE80 Uncut, Lane 3: PQE80 cut with BamHI and HindIII , Lane 4,: PQE EP1PD-1 (1) cut BamHI and HindIII, Lane 5 : PQE EP1PD-1 (2) cut BamHI and HindIII

From Visualisation of digested plasmid was using agarose 0,8% we can find that expected band (220 bp) was inserted to PQE 80 vector (Figure 7).

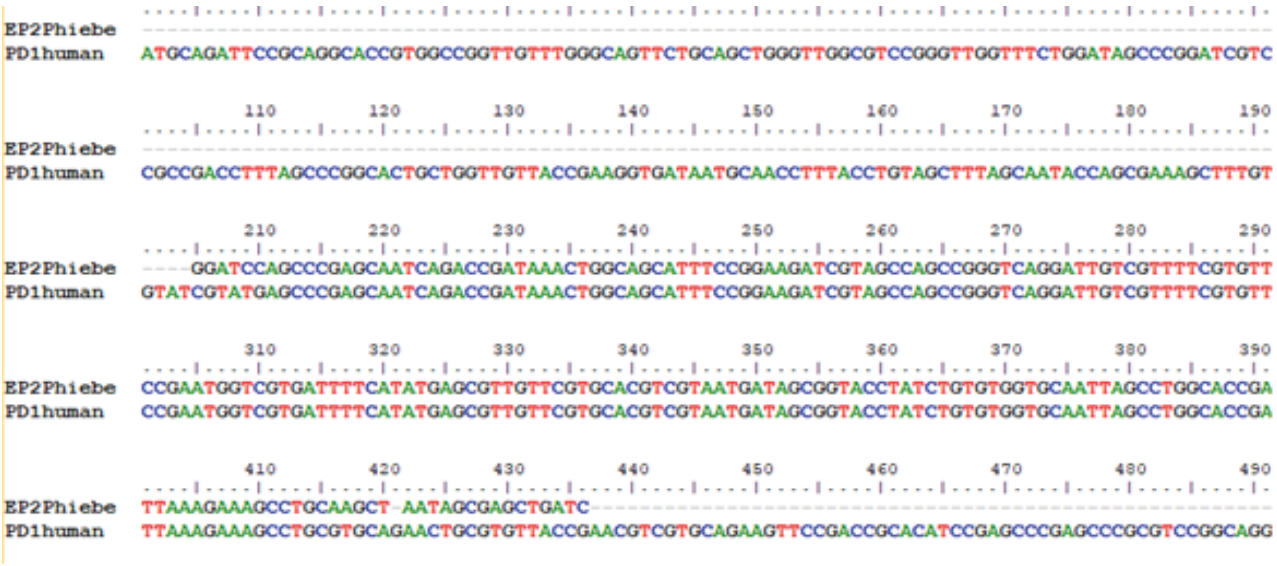

Figure 8. Sequencing result of PQE EP2PD1 (1) using Sanger method, comparation of sequence was using PD-1 full gene.

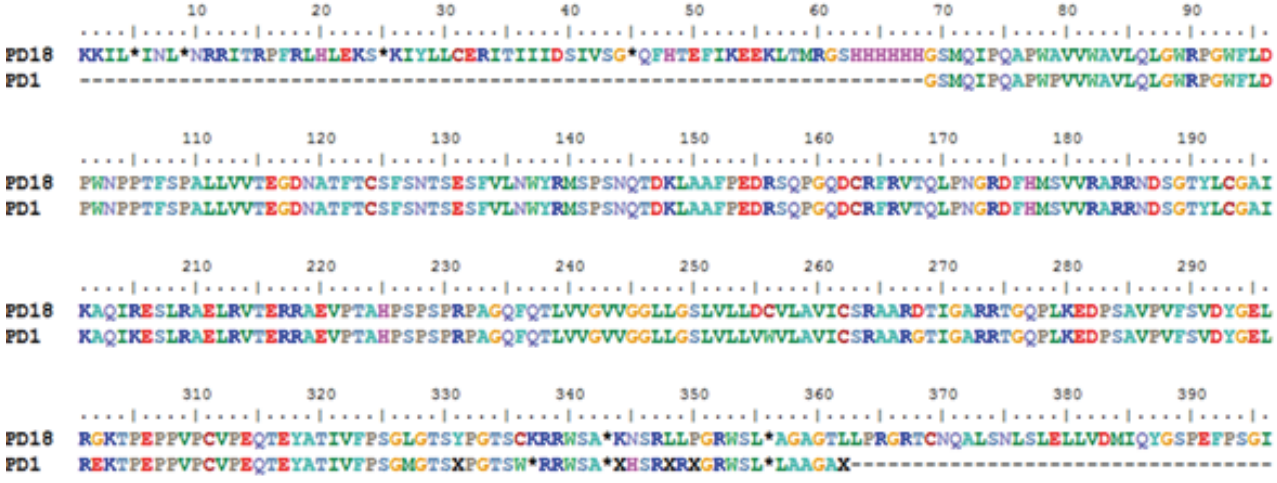

Figure 9. Sequencing result of PQE PD-1 (8) using Sanger method, comparation of sequence was using PD-1 full gene.

From sequencing result in Figure 9, we found that there is no mutation at recombinant PQE PD-1 (8). It means that this two clone a can be a candidate for antibody monoclonal development.

DISCUSSION

In this study, we have proved to be successful in constructing PD-1 human sequence and EP2PD1 sequence into prokaryote expression system PQE80. The purpose of his study is to make recombinant of PQE PD-1 which is going to be use for development of antibody monoclonal PD1. According to Xu research.[6] not all part of PD1 are easily to express specially domain region but other studies showed that the extracellular domain of PD-1 could be express. One study was carried out to express a human PD-1-GST fusion protein and it was also found that the fusion protein was completely in inclusion bodies.[6] EP2PD1 is located at 145-217 amino acid which is an extra cellular domain according to software https://protein-sol.manchester.ac.uk (data not showed). Since this epitope is protrude it is possible to make this epitope as tools for detect efficiency of antibody monoclonal PD-1 or as DNA vaccine which combine with other immunogenic factor.

This sequence were optimized to B cell because B cells expressing high-affinity antibodies differentiate into antibody-secreting plasma cells and memory B cells that mediate humoral immunity against pathogens[7] but in spite of advance in B- cell epitope mapping , it is important to note that antibodies raise against peptides often lack the ability to bind native proteins due to unstructured nature of the peptide.[9] In

this study we check the antigenicity of peptide using Kolaskar and Tongaankar method with threshold value 1,007. At the score value not all epitopes have the highest score only four of them the rest is below the threshold. For constructing EP2PD1 we choose epitope position from 145- 217 which have threshold value higher than 1,007. This might be the meaning that this epitope doesn't have proper activity to interact with circulating tumour reactive CD8+, these finding suggest that there is other factor to be added to modulate activity of epitope EP2PD1 like IL 12.[10]

E. *coli* was select in this study as expression system because this system have many advantages, it has doubling time is about 20 min[11], it means that a culture with a 1/100 dilution of starter culture may reach stationary phase in a few hour, this make E.coli a robust system to amplified DNA and highly density cultures are easily to achieved.[12] In this study we use PQE 80 (Qiagen) as vector plasmid because this vector have properties of 6x His tag at the N-Terminal of the protein, make it the insert if ligated in framed easily to purified[13] and also the PQE series from Qiagen was provide with termination codons it means that the 3' end of the insert does not need to determined accurately.

In conclusion, construction of recombinant PQE PD-1 and PQE EP2PD1 are constructed without any mutation in the sequence. It is means that this recombinant can be used in the next study for protein expression of PQE PD-1 and PQE EP2PD1, which will be proceed to development of monoclonal antibodies.

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