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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Staphylococcal enterotoxins (SEs) are potential biological threat agents. Exposure to SEs can result in systemic shock and death of military personnel. Understanding the molecular mechanism of SEs function is a fundamental prerequisite for development of effective therapeutics and regimens for protection against the biological effects of SEs exposure. The enterotoxins, such as SEB, bind as folded proteins to both major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells and germline-encoded variable domain sequences of specific T cell receptor (TCR) beta chain on T lymphocytes. The resulting activation of T cells contributes to several serious diseases and lethal toxic shock syndrome. In this proposal, the molecular bases for human TCR-SE-human MHC class II interaction will be addressed. A major advancement to the field will be in construction and preparation of a soluble form of human TCR protein in large quantities. Interaction of TCR with soluble human HLA-DR1 in complex with antigenic peptides and SEA and SEB will be examined. The interaction kinetics will be determined by the use of multiple methods that include real time detection of interactions by surface plasmon resonance.				
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Introduction

Staphylococcal enterotoxins (SEs) are potential biological threat agents. Exposure to SEs can result in systemic shock and death of military personnel. The enterotoxins, such as SEB, bind as folded proteins to both major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells and germline-encoded variable domain sequences of specific T cell receptor (TCR) beta chain on T lymphocytes. The resulting activation of T cells contributes to several serious diseases and lethal toxic shock syndrome. Rational design of strategies for prevention (vaccines) or treatments of such diseases may only be possible if we have insights into the mechanisms of T cell activation by enterotoxins. Thus, our efforts have been to investigate such mechanisms. Specifically, we are set up to study the molecular interactions of isolated TCR with their natural ligands, peptide/MHC and enterotoxins.

Strategies and procedures

Production of TCR protein has been a long challenge over the past decade. For some unknown reasons, production of soluble TCR has successfully been possible only for very few clonotypes. Therefore, we attempted undertaking our efforts towards this goal bearing in mind that we might have to tackle multiple strategies. In the first effort, we tried a bacterial expression system, because of the possibility of getting large quantities of folded protein once all the technical issues were successfully addressed.

Two individual constructs for bacterial expression of α and β TCR as leucine-zipper fusion proteins were designed. The leucine zipper used here is based on the modified sequence of the naturally occurring leucine-zipper region of Fos and Jun oncoproteins (O'Shea et al., Current Biology, 1993). The α construct, α -ALZ, consist of the genes encoding the α -chain variable and constant domains of Clone1 TCR fused via a linker to the acidic peptide of the leucine zipper. The β -construct, β -BLZ, is made up of the genes encoding the β -chain variable and constant domains of Clone1 TCR fused, through a linker, to the basic peptide of the leucine zipper. In addition the β -construct also has a free unpaired cysteine after the last residue of the basic leucine zipper peptide to facilitate specific immobilization (for BIAcore studies) and fluorescent labeling. The TCR sequences were included up to the cysteine residues involved in the interchain disulfide bridge at the C-terminus of constant domains. A cysteine residue was added to the c-terminus of β chain to facilitate immobilization of TCR in proper orientation for biophysical studies.

The cDNA of α and β T-Cell receptor from Clone 1 T-Cells were constructed using RT-PCR. From the cDNA, the individual constructs for α and β TCR for bacterial expression were made using PCR amplification as follows. The 5' primers were designed to introduce an initiating methionine codon, as part of a NcoI site, just before the first residue of the mature α and β subunits as well as to introduce a XbaI site upstream of NcoI site for cloning purposes. The 3' primers were designed to exclude all the residues following the interchain disulfide as well as to introduce a BamHI site immediately following this cysteine residue. Both such engineered α and β -TCR were cloned into Pbluescript II KS vector as XbaI-BamHI fragments.

The DNA coding for the acid/basic leucine zipper and the linker (GSTTAPS) connecting the zipper to the TCR subunits were made using six synthetic oligonucleotides. Briefly, six primers (3 each for the sense and the anti-sense strands), designed to make cohesive BamHI and HindIII sites on either side, were melted and annealed. These annealed double-stranded dna, coding for either the acid-LZ peptide linker or the basic-LZ peptide linker, were then ligated following the BamHI end of pBluescript construct carrying either the α - or β -TCR. Codon usage in *E. coli* was used as a basis for constructing the dna sequence coding for the leucine-zipper. Furthermore, the unpaired cysteine in the constant domain of β -TCR was mutated to Alanine, to enable more efficient refolding, using Quik Change Mutagenesis technique (Stratagene). The α -ALZ and β -BLZ clones were entirely sequenced to ensure the coding regions had the intended sequence. The NcoI –HindIII fragments of α -ALZ and β -BLZ constructs were then cloned in to pET21-d and pET23-d (Novagen) expression vectors bearing the T7 polymerase promoter. pET21-d has a T7lac promoter contains a lac operator sequence downstream of T7 and offers a more stringent control over uninduced basal protein expression and is typically used to express proteins that may be toxic to bacteria. pET23-d has a plain T7 promoter and works very well with not-so toxic proteins.

Protein expression was attempted in 2 different bacterial strains – BL21(DE3) pLys S and BL21(DE3)-CodonPlus-RIL (Stratagene). BL21(DE3)-CodonPlus-RIL (cells contain extra copies of the genes that code for t-RNAs (Arginine, Isoleucine and Leucine), which most frequently limit translation of heterologous proteins in *E. coli*).

4 possible combinations in terms of expression vectors and hosts were tried. They were:

- 1) pET21d- based plasmid in BL21(DE3) pLys S cells
- 2) pET23d- based plasmid in BL21(DE3) pLys S cells
- 3) pET21d- based plasmid in BL21(DE3)-CodonPlus-RIL cells.
- 4) pET23- based plasmid in BL21(DE3)-CodonPlus-RIL cells.

As for α goes, there was very little expression observed with BL21(DE3) pLys S cells both with pET21d and pET23d vectors. But noticeable expression was observed with BL21(DE3)-CodonPlus-RIL cells both with pET21d and pET23d vectors. The combination that worked the best so far, in terms of the size of the inclusion-body like pellets, is pET21d- α -ALZ plasmid in BL21(DE3)-CodonPlus-RIL cells. As for the β chain goes, both cell lines and both vectors worked very well. Out of the possible combinations, pET21d-mut β -BLZ plasmid in BL21 (DE3) pLys S and BL21(DE3)-CodonPlus-RIL cells work very well.

The proteins were expressed individually as inclusion bodies in the bacterial strain, BL21(DE3)-CodonPlus-RIL (Stratagene). Inclusion bodies were purified and solubilized in either urea or guanidine. The α and β chains were renatured together by dilution refolding of the denatured protein to form heterodimeric TCR. We followed the refolding procedure described by Garboczi et al first. Later on, we modified his procedure for optimization of refolding.

Beta chain was expressed in inclusion bodies at or > 90% purity, as evaluated by SDS-PAGE and Coomassie staining. However, alpha chain expression was poor and in mixture with many other proteins. Changing the strain of expressing *E. coli* helped to increase the proportion of the α chain relative to other proteins but did not eliminate expression of contaminating proteins significantly.

Small scale refolding tests were set up to evaluate efficiency of the refolding conditions. We tested different denaturants, changes in pH, presence or absence of glycerol at varying concentrations (0-30 %), and different ratios of the denatured individual proteins. We continue our efforts in evaluating refolding conditions to find the optimal conditions for TCR folding and large scale purification.

Parallel with our protein purification and refolding efforts, we have been working towards establishing the technology needed for accomplishing specific Aim 2. A novel membrane immobilization strategy for investigating interactions of TCR inserted in cell membrane with the ligand in real time was attempted. Membrane fragments of T cell clones bearing TCR and other proteins involved in T cell activation, such as CD4, were isolated and used for immobilization on the surface of L1 chip of BIACore. This technique can be utilized for measuring interaction of TCR inserted in membranes with superantigens or peptide/MHC ligands in natural environment. Purified TCR immobilized on the surface of BIACore chip will be compared to the membrane anchored native TCR for accurate determination of the mechanism of interaction. Possibilities of TCR clustering in interaction with MHC/peptide and different superantigens will be evaluated.

We found that we can properly fold TCR from bacteria, as confirmed by ELISA, but with a low yield. We attempted to optimize refolding of TCR by; **a)** mixing a larger proportion of a chain protein with the beta chain, **b)** try several protein folding methods, and **c)** use other strains of bacteria expressing the gene to increase compatibility with the a chain. In addition, we tried adding different concentrations of glycerol (known to improve proper folding of denatured proteins) to the folding mixture. Five percent glycerol helped refolding slightly, whereas other concentrations of glycerol up to 50% did not lead to improved folding over the glycerol free folding mixture.

Baculovirus expression system:

Unfortunately, our tedious efforts in obtaining high yields of correctly folded bacterial produced TCR $\alpha\beta$ did not lead to successful production. Although we detected a small amount of TCR that were identifiable by the anti TCR antibodies specific for the native protein, much of the folded protein was incorrectly folded.

In face of difficulties in developing an effective refolding protocol for TCR produced by *E. coli*, we have decided to return to utilizing the baculovirus-mediated insect cell expression system. The major advantage of the baculovirus system over a bacterial expression system is that it typically produces the recombinant protein with proper folding and disulfide bond formation. As such, it obviates the need for developing

a refolding procedure for the expressed protein. However, the drawback is its low yield at production level of ~1 mg /L supernatant.

Because we have had positive results from our previous utilization of the baculovirus expression system offered by BD Pharmingen (Natarajan et al., 1999), we have decided to keep on using their system. We are currently in the process of producing a baculovirus transfer vector containing the coding regions for the extracellular portions of both the alpha and beta chains of the Clone 1 TCR.

Our subcloning procedure is as follows:

1. PCR-amplify the alpha- and beta-chain coding regions from the bacterial expression vectors containing them. Capture each PCR product in a TA-cloning vector (pCR2.1, Invitrogen), and check for the absence of PCR-introduced point mutations through sequence analysis (Appendix Figure 1).
2. Separately subclone each of the PCR-amplified alpha- and beta-chain coding regions into pAcGP67C, a vector from Pharmingen containing the coding region for an insect cell-optimized signal sequence immediately upstream of the cloning site (Appendix Figure 2).
3. PCR-amplify the chimeric signal sequence-TCR alpha chain cassette (and the analogous construct for the beta chain) from the vectors constructed in step 2. Capture the PCR products in the pCR2.1 TA cloning vector, and check for the absence of PCR-introduced point mutations through sequence analysis (Appendix Figure 3).
4. Sequentially subclone the signal sequence-TCR alpha chain cassette and the signal sequence-TCR beta chain cassette into pAcUW51, a dual promoter baculovirus transfer vector from Pharmingen. The two cassettes are thus placed under transcriptional control of two separate promoters on the same vector. At this point, this transfer vector is ready to be cotransfected with the baculovirus genomic DNA into insect cells for virus production (Appendix Figure 4-5).

Accomplishments:

A- Bacterial expression system;

1. Beta chain of TCR was produced in inclusion bodies of the expressing bacteria to over 90 % purity
2. Alpha chain of TCR was produced in inclusion bodies but mixed with many other proteins
3. At present, we can properly fold TCR from bacteria, as confirmed by ELISA, but with a low yield. We attempted to optimize refolding of TCR by; a) mixing a larger proportion of a chain protein with the beta chain, b) try several protein folding methods, and c) use other strains of bacteria expressing the gene to increase compatibility with the alpha chain. Adding 5% glycerol helped refolding.

B- Baculovirus expression system;

We have completed steps 1-4 (above and appendix 1-5) for cloning of modified TCR alpha chain into baculovirus double promoter shuttle vector. In the next few days, the beta chain will be cloned in the same recombinant vector as well. Then, the vector will be used for transfection of baculovirus. The recombinant virus will be passed in SF9 cells to increase the virus titer and ultimately will be used for infecting Hy-5 cells for production of correctly folded protein. The produced protein will be concentrated and affinity purified

Reference

Erin K. O'Shea, Kevin J. Lumb and Peter S. Kim. Current Biology Peptide 'Velcro': Design of a heterodimeric coiled coil (1993) 3: 658-667.

Natarajan, S. K., Stern, L. J., and Sadegh-Nasseri, S. (1999). Sodium dodecyl sulfate stability of HLA-DR1 complexes correlates with burial of hydrophobic residues in pocket 1, *J Immunol* 162, 3463-70.

Appendices

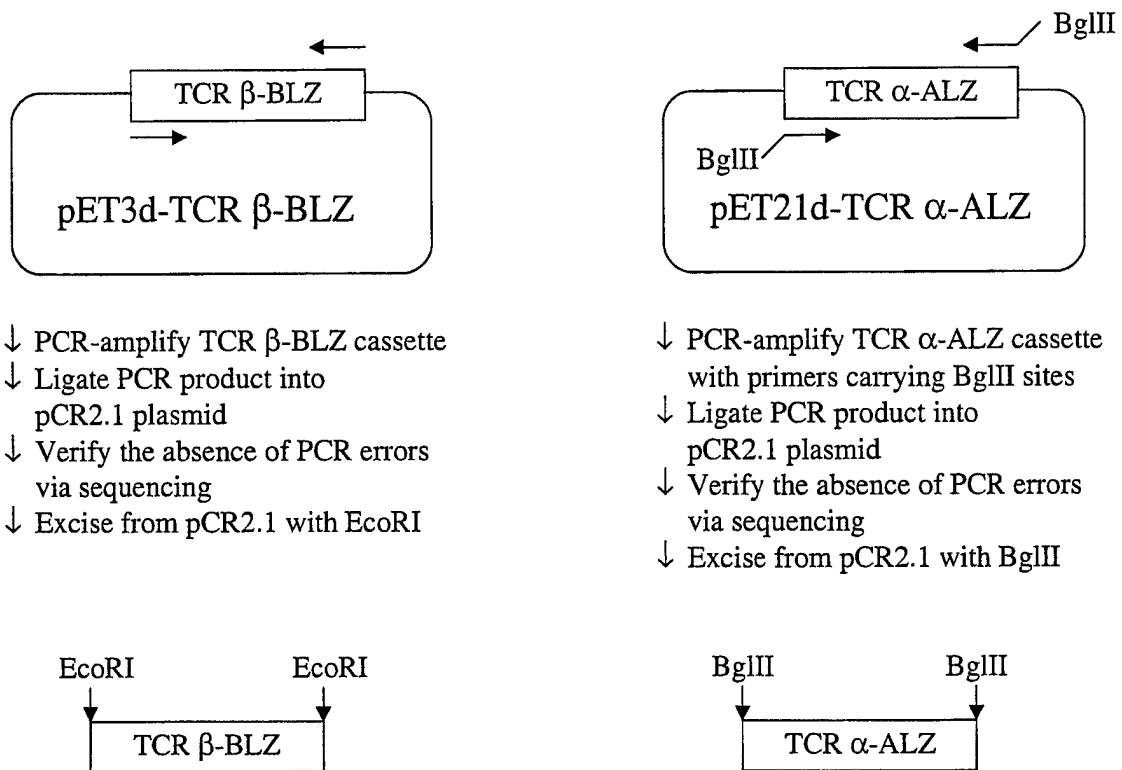


Figure 1. PCR cloning of TCR a and b chain sequences into pCR2.1 plasmid

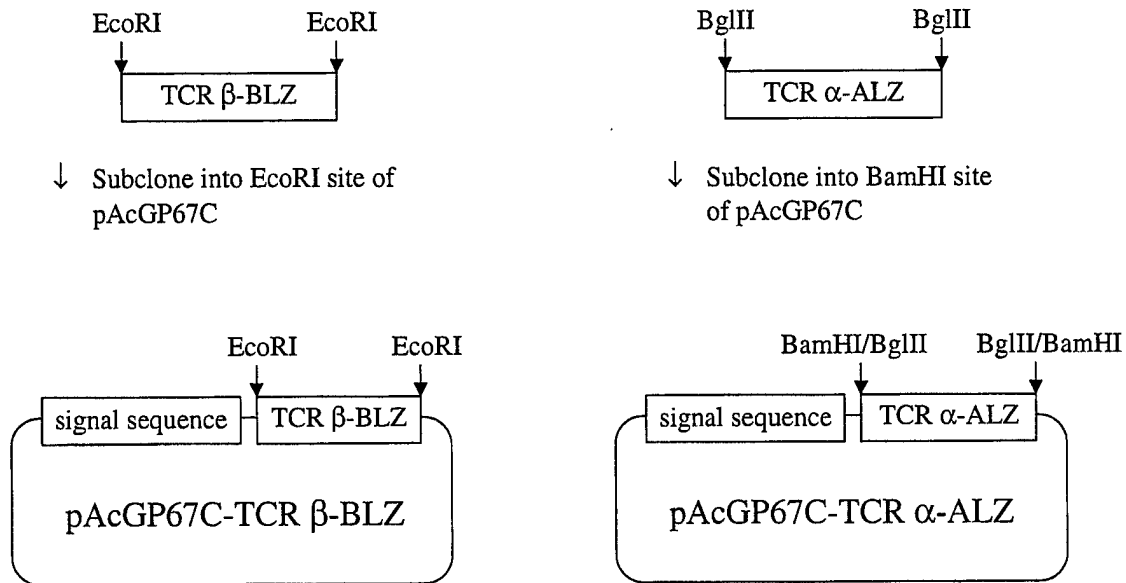
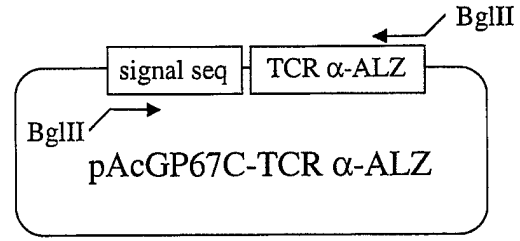
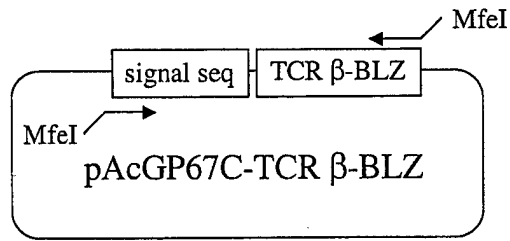


Figure 2. Subcloning of TCR a and b fragments into pAcGP67C plasmid containing signal sequence.



- ↓ PCR-amplify signal seq-TCR β -BLZ cassette with primers containing MfeI sites
- ↓ Ligate PCR product into pCR2.1 plasmid
- ↓ Verify the absence of PCR errors via sequencing
- ↓ Excise from pCR2.1 with MfeI

- ↓ PCR-amplify signal seq-TCR α -ALZ cassette with primers containing BglII sites
- ↓ Ligate PCR product into pCR2.1 plasmid
- ↓ Verify the absence of PCR errors via sequencing
- ↓ Excise from pCR2.1 with BglII

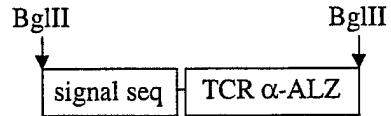
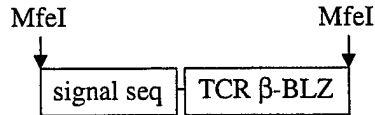


Figure 3. PCR amplification of signal sequence-TCR α and β Leucine zipper cassettes.

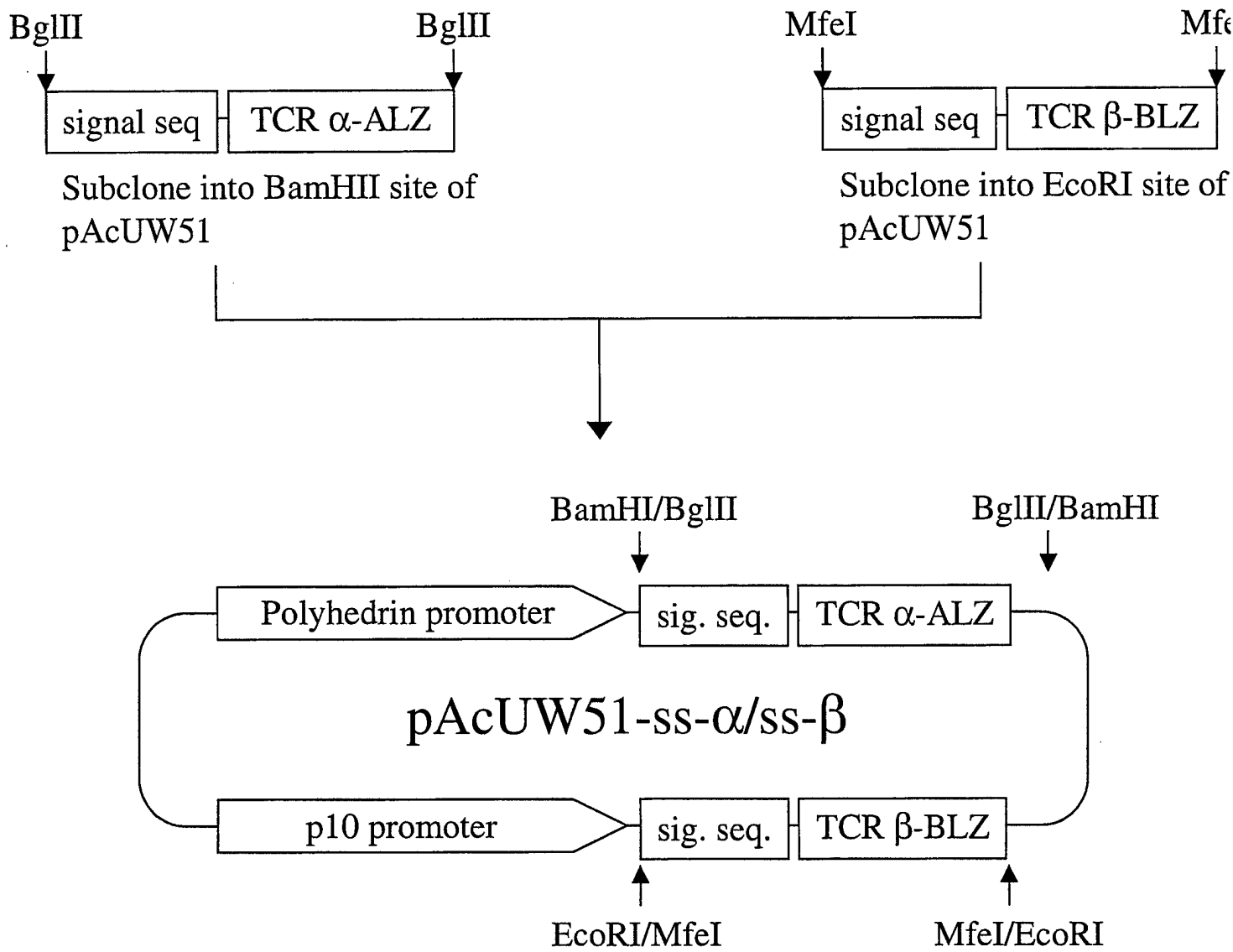


Figure 4. Subcloning of signal seq-TCR α -LZ, and signal seq-TCR β -LZ into pAcUW51 double vector

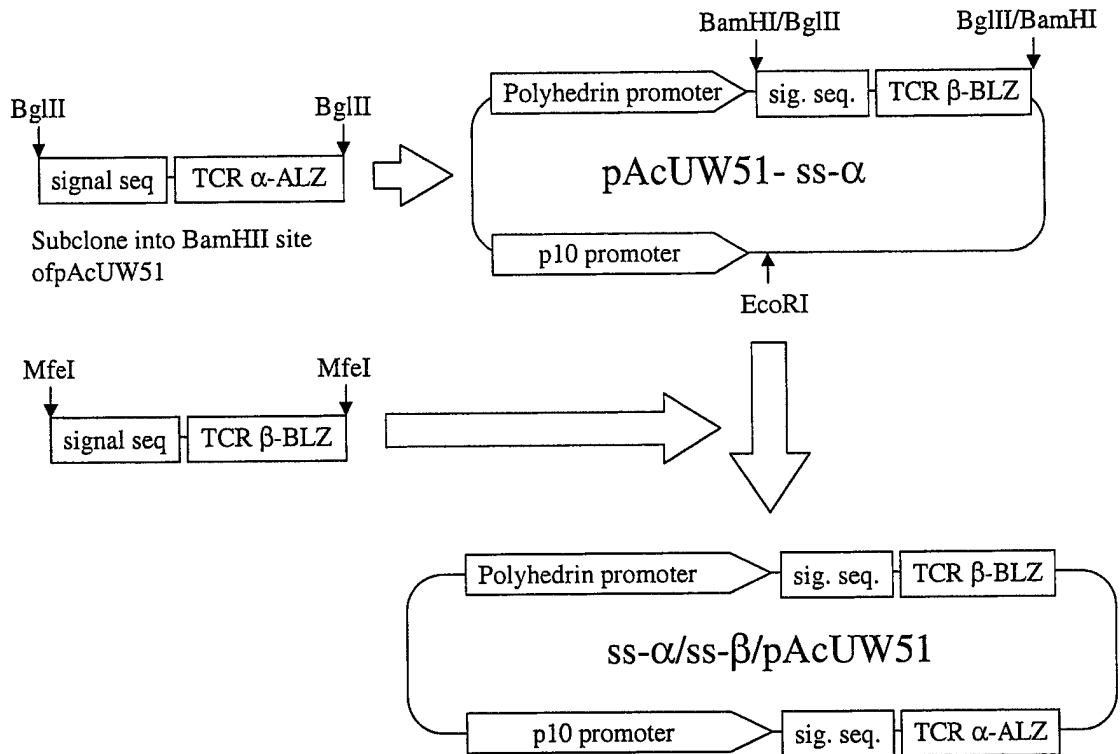


Figure 5 Figure 5. Generation of the final recombinant pAcUW51 vector containing both a and b chains of clone 1 TCR.