

Designing T-Cell Receptor Constructs Using Golden Gate Cloning

Worcester Polytechnic Institute



University of Massachusetts Medical School



Advisor: Jill Rulfs PhD (BBT)
Author: Maxwell Studley (BBT)

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Abstract:

Latent TB infections are a result of the disease's ability to evade host immune defenses and remain in a quiescent asymptomatic state. Clonal expansion of T-cells within granulomas of individuals with active and latent TB provides an opportunity to determine the antigens capable of promoting an adaptive immune response within a human subject. Using sequence information from T-cells of patients with Latent Tuberculosis, T cell receptor (TCR) variable region inserts were designed. The objective of this work is to utilize Golden Gate cloning of the compiled TCR sequences to create a repertoire of specific TCRs for eventual expression using retroviral vectors.

Introduction:

Epidemiology:

Mycobacterium tuberculosis is the infectious organism responsible for causing the majority of Tuberculosis (TB) disease outbreaks and infections (34). The World Health Organization reported in 2018 that TB was responsible for 1.3 million deaths in HIV-negative, and 300,000 in HIV-positive individuals, and that 10.0 million people developed the disease globally (45). Active or symptomatic TB is transmitted as an aerosol through the respiratory tract and targets alveolar macrophages as the site of primary infection (11). Early clearance of *M. tuberculosis* infection is attributed to the innate immune response and alveolar macrophage elimination of the infection without initiation of the adaptive immune system (42, 26). Due to *M. tuberculosis*' close evolution with the human host immune system, disease progression increases when components of the adaptive immune response are recruited. Analysis of *Mycobacterium tuberculosis* Complex (MTBC) phylogeography suggests that MTBC has been undergoing co-evolution with humans for tens of thousands of years (7). This co-evolutionary relationship is demonstrated predominantly during persistence of latent TB infections (LTBI) in the diseases' ability to evade host immune defenses and remain in a quiescent asymptomatic state (3). It is hypothesized that this alternative disease progression allowed the human-obligate pathogen to remain dormant until the individual passes reproductive age and then progress to the clinically active disease in older age. This mechanism would have allowed ancient *Mycobacterium tuberculosis* to retain its human host pool while avoiding self-extinction in small groups of hunter gatherers (10, 34).

Immunology:

Current research investigations into TB are directed toward pursuing the means to treat or promote the immune system's defense and clearance of LTBI. Targeting LTBI would reduce the reservoir of potentially infectious TB within the population and reduce overall transmission and disease prevalence (13). The asymptomatic stage of infection is mediated via the *M. tuberculosis*: macrophage interaction (39). Initiated by alveolar macrophage phagocytosis of *M. tuberculosis* and promoted by either mannose or surfactant protein A (38, 16, 39) the bacteria is sequestered to an intracellular vacuole where virulence factors such as lipoarabinomannan (LAM) inhibit phagosomal maturation and elimination of the pathogen (43, 22). Persistence of the *Mycobacterium tuberculosis* (Mtb) is achieved through the development of granulomas that function to contain and localize the adaptive immune response. These accumulations of infected alveolar macrophages, interstitial macrophages, lung myeloid dendritic cells, monocytes, and neutrophils create a balance of pro/anti-inflammatory responses surrounding a necrotic center (17, 18). Utilizing genes that inhibit apoptosis of the infected cells *M. tuberculosis* maintains a favorable replicative environment until determinants cause necrosis of the infected host cell and release of internally replicated bacterium (4, 19). Stimulated apoptosis of the infected macrophages and dendritic cells are promoted via Mtb translocation from the phagosome into the cytosol (4, 41). T-cell mediated immunity is dually manipulated by Mtb targeting antigen presentation on both MHC class I and II. CD4 T-cells are unable to identify Mtb infected antigen presenting cells within the lung as a result of the 19-kDa lipoprotein antigen (LpqH) which reduces cellular presentation of MHC II in infected macrophages (4, 32, 14). Cytotoxic CD8 T-cell response mediated by the MHC I complex, an important pathway for immunity against intracellular pathogens, provides one potential pathway to identify effective adaptive immune system response toward infected antigen presenting cells (APCs) (4). This pathway is reliant upon Mtb antigen presentation on MHC I during translocation into the cytosol or via cross-presentation from the phagosome to the recycled MHC I from the ER (36, 4, 21, 19). An additional process for presenting Mtb antigens on MHC I is possible as uninfected APCs can uptake cellular debris from infected cells that have undergone apoptosis (4, 37).

T-Cell Mediated Immunity:

Naive CD8 T-cell priming post recognition of pathogen-activated MHC class I APCs propagates clonal expansion and differentiation of effector T-cells (29, 33). Differentiated effector T-cells, specifically cytotoxic T-cells, are capable of identifying infected APCs presenting Mtb antigens on MHC I receptors and facilitating lysis or apoptosis of these infected cells (44).

Clonal expansion of T-cells within granulomas of individuals with LTBI provides an opportunity to determine Mtb antigens capable of promoting an adaptive immune response within a human subject (33). Sequencing of these granulomas reveals the frequencies of clonally expanded T-cell receptors (TCRs) that are potentially specific to Mtb antigens. Through collaboration with a South African research center, human granulomas from patients with Tb were sequenced and frequencies of TCR alpha and beta variable chains and hypervariable regions were compiled. The objective of this study is to utilize Golden Gate cloning of the compiled TCR sequences

to create a repertoire of specific TCRs for expression in Jurkat cells using retroviral vectors and analysis via flow cytometry of the antigens the TCRs are responding to.

T-Cell Receptor Complex:

T-Cell Receptors (TCRs) are transmembrane heterodimers composed of complementary determining regions (CDRs) present on T-lymphocytes of the adaptive immune system (5, 29). The two amino acid chains of interest, dimerized via a disulfide bond are the Alpha and Beta chains (5, 29). Alternatively, these chains can be composed of Gamma and Delta sequences (30). Studies have demonstrated Gamma: Delta TCR complexes operate via dissimilar mechanisms of antigen presentation, recognition, and signaling in comparison to Alpha: Beta complexes (25, 1). Due to limited research in elucidating these dissimilar mechanisms, Gamma: Delta TCRs are not considered in this study (12). The analysis of identifying Human TCRs responding to Mtb antigens in this research is focused specifically to Alpha: Beta complex TCRs. The fundamental structure of TCR Alpha: Beta complexes is illustrated in *Image 1*.

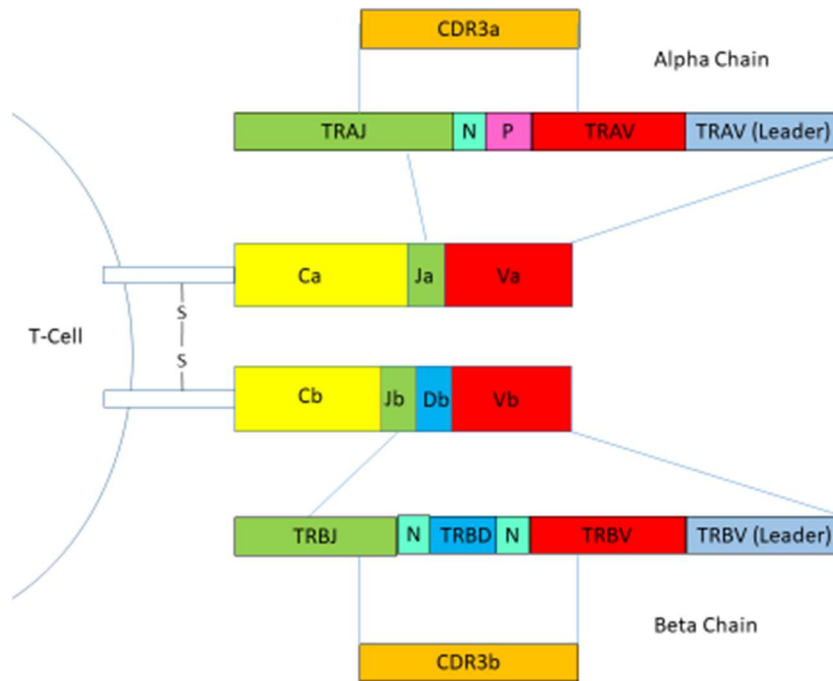


Image 1, Basic $\alpha:\beta$ TCR Complex structure, illustrates junction diversity and localization of CDR3 hypervariable region

Both Alpha and Beta chains can be further divided into variable ($V\alpha/V\beta$) and constant regions ($C\alpha/C\beta$) (29). Regions of the variable alpha ($V\alpha$) chain are composed of the $V\alpha$ and $J\alpha$ subunits (31). Variable beta ($V\beta$) chains are comprised of the $V\beta$, $D\beta$, and $J\beta$ subunits (40, 5, 44). Subunits are classified as variable (V), diversity (D), and Joining (J) (5, 44). These classifications correspond to components of the TCR “hypervariable region” (CDR3) (8, 1, 10). The $V\alpha/V\beta$ regions are further divided into three complementary determining regions (CDRs) classified as CDR1, CDR2, and CDR3 (6, 15, 40, 31). TCR functionality via the MHC: peptide: TCR ternary complex relies on these CDR sites in which each alpha and beta chain forms three loops (2, 44, 20, 15). CDR1 and CDR2 relate to the “germline” variable gene segments and form the peripheral loops of the TCR that engage with the MHC complex, mediating both MHC recognition and specificity (28, 2). The CDR3 selectively contacts the specific complementary peptide fragment presented on the MHC complex binding groove (40, 2, 6). Through these complementary determining regions, MHC recognition and specificity is created by CDR1 and CDR2 while CDR3 conveys specificity to the bound peptide fragment (40, 2, 6, 31). The diversity of CDR3 regions, which allows the T-cell to recognize the variety of intracellularly digested peptide fragments, is due to junctional sequence diversity (40, 20). During gene rearrangement of the V, D, and J TCR regions, both the alpha and beta chain modifications are implemented in the form of both N- and P- nucleotides (20, 40). N- nucleotides are inserted between the junctions of the V, D, and J segments during gene segment arrangement (20). Similarly P- nucleotides are found at these gene segment junction and are reciprocal repeats of the sequences located at the terminal ends of the gene segments (20).

Golden Gate Reaction Scheme:

Golden Gate Cloning is an effective method for taking advantage of the modular nature of TCR genes and preparing them in constructs for retroviral expression (8). This cloning strategy utilizes the Type IIS restriction enzyme to implement restriction cut sites upstream of Type IIS recognition sites (8, 9, 35). Cut sites located downstream of recognition sequence and residual overhangs, respectively promote removal of restriction enzyme responsiveness to final expression constructs and directionality during ligation resulting in a highly efficient single step reaction (8). Removal of recognition sequences from the final ligated construct allows overhangs to be specified to the intended sequence without residual scarring of the sequence of interest as seen in alternative restriction enzyme practices. *Image 2* illustrates the design of the acceptor vector and shows the directionality of the *BbsI* cut sites and residual overhangs.

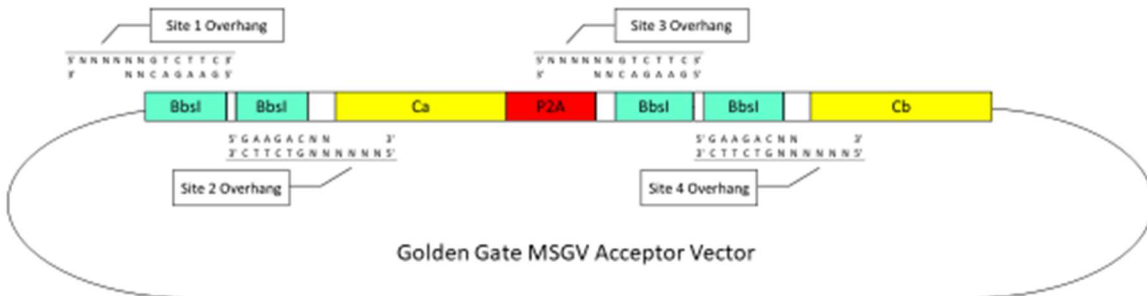


Image 2, Golden Gate MSGV Acceptor Vector, reveals the location of the TCR constant regions in addition to presence of P2A self-cleavage site

Vector design was based off of previous work from David Ott's lab, MSGV Hu-Acceptor and MSGV Hu-Acceptor PGK-NFGR plasmids available through Addgene (8, 9, Addgene plasmid # 64270 & 64269). These predesigned Acceptor Plasmids are unique in that they include MSGV retroviral components, both TCR constant alpha and beta regions joined by a P2A multicistronic self-cleavage site, and *BbsI* Type IIS restriction enzyme recognition sites to facilitate insertion of TCR alpha and beta variable regions (8, 9, Addgene). The P2A (porcine teschovirus-1 2A) has been noted to possess the highest "self-cleavage" efficiency of the 2A peptides, resulting in co-expression of adjacent genes (27, 23).

Methodology:

Designing TCR Variable Inserts:

TCR alpha and beta chain variable regions were designed by combining the sequenced V, D, and J gene segments from the human subjects and identifying the junctional N- and P- nucleotides to create the specific amino acid sequence of the CDR3. Each subject's TCR alleles were determined via sequencing. The V α and V β chains are comprised of the TRAV, TRAJ, TRBV, TRBD, and TRBJ alleles (24). Provided with the amino acid sequence of the TCR specific CDR3, these allele sequences were combined using ApE and SnapGene software while the allele sequences were obtained from the online IMGT database. Using the Expansy software to translate the DNA sequence to the amino acid sequence, the junctional insertions sites of the N- and P- nucleotides were determined. Alpha and Beta chain variable sequences were then flanked by *BbsI* type IIs restriction enzyme sites. Presence of these sites would then allow these variable inserts to be used in Golden Gate reaction. The reading frame of the variable insert was confirmed within the Acceptor Vectors from David Ott using the NEB Golden Gate software. The variable acceptor constructs were designed in the pUC57 plasmid with ampicillin resistance and created by Addgene.

Plasmid Transformation:

TCR variable chain entry pUC57 plasmids from Addgene were transformed into NEB 5-alpha competent *E. coli* HF (New England Biolabs) and cultured in SOC outgrowth media. These transformants were then grown on 100ug/mL Amp LB agar plate and colonies were selected for plasmid preparation. The Qiagen: Miniprep (Qiagen) kit was used for both Entry plasmids and Acceptor plasmids. Products of the miniprep kit were analyzed via Nanodrop for purity and DNA concentration.

Confirming Acceptor and Entry Plasmid Sequences:

The Acceptor Plasmid MSGV Hu-Acceptor and MSGV Hu-Acceptor PGK-NGFR (8, Addgene plasmid # 64270 & 64269) were digested using 1uL of *BsrGI* (New England Biolabs), for 30 min, at 37C to confirm intended plasmid size through restriction digest and gel electrophoresis on a 0.5% gel for 60 min at 100V loading 15 uL of product and 2 uL of loading dye. Each gel completed all utilized the 1Kb Plus DNA Ladder (Invitrogen). The identified bands from the digest revealed the anticipated results of Hu-Acceptor **667**, **6072** and Hu-NGFR **665**, **1735**, **5669** (Fig 7).

In order to confirm sequences of the *BbsI* recognition sites and overhangs in the Acceptor plasmids the following primers were designed by imputing the Acceptor plasmid sequences into Benchling, and ordered through IDT (Integrated DNA Technologies). Primers were designed with complementarity to portions of the Acceptor Plasmid sequence. The primers for the V α entry sequence site are upstream of the V α site and inward while the downstream primer anneals in the upstream portion of the C α region for the reverse sequence of the V α insert. Primer designations for the V β region follow a similar design where the upstream primer is specific to the terminal C β sequence and the downstream primers is complementary to the start of the C β region. Using this format for primer specificity these primers can be used across V α /V β insert variations allowing a more streamlined procedure for completing sequencing of the TCR regions of interest. *Figure 1* presents the designed primers.

V α -Forward (T_m = 58.2°C)
5' CACGAAGTCTGGAGACCTCTGG 3'

C α -Reverse (T_m = 58.5°C)
5' CTTTTCCACCAGCTTCACGTCG 3'

C α /V β -Forward (T_m = 57.7°C)
5' CGAGGACACATTCTTCCCAAGC 3'

C β -Reverse (T_m = 57.9°C)
5' TCTTTCAGAGGCTGGGGATCG 3'

Figure 1, Primers Designed for specificity with the Golden Gate Acceptor Plasmids

Eton Bioscience Inc. provided sequencing services to provide sequence data used to confirm the correct Acceptor sequence. Restriction digest using the *EcoRI* and *HindIII* restriction enzyme (New England Biolabs) was used to confirm the correct anticipated size of the pUC57 plasmid containing the TCR variable insert. 0.5 uL of both

EcoRI and *HindIII*, using 1 ug of DNA were digested at 37C for 2 hours and inactivated at 80C for 10 min. Digestion products were run on a 1% EtBr gel at 110V for 60 min. The pUC57 digest with *EcoRI* and *HindIII* produced the anticipated bands V α insert **497, 2635** and V β insert **515, 2635** (Fig 8).

PCR Preparation of Variable Inserts:

Primers specific for the pUC57 plasmid variable alpha and beta inserts were designed and used to perform PCR amplification of the variable insert for use in the Golden Gate Reaction (Figure 2). The primers were designed to recognize outside of variable insert of the entry plasmid allowing the primers to be used across the pUC57 entry vectors.

pUC57 Forward (T_m = 57.5°C)
5' CAGTGAATTCGAGCTCGGTACC 3'

puC57 Reverse (T_m = 58.2°C)
5' TGTTGTGTGGAATTGTGAGCGG 3'

Figure 2, Primers Designed for specificity with the pUC57 Plasmid

PCR was conducted using 10X Taq Buffer and Taq polymerase (Invitrogen #18067017), 1 uL of primer, and 2 uL of pUC57 entry vector template. Thermocycler conditions were run at 94°C/5 min, followed by 30 cycles of 94°C/30 s, 54°C/15 s, 68°C/45 s, and then 68°C/5 min, and a 4°C hold. 4 uL of PCR products was subsequently run on a 1% EtBr gel at 110V for 60 min (Fig 9,10). The expected amplicons of V α and V β at 572 bp and 590 bp respectively were purified from the gel using Qiagen PCR Cleanup Kit (Qiagen) and concentration and purity determined by Nanodrop. These purified amplicons were used as the variable inserts in the Golden Gate Reaction scheme.

Golden Gate Reaction:

The Golden Gate reaction was completed using 50 ng of both variable entry plasmids in addition to 100 ng of acceptor sequence. Per 50 ng Entry and 100 ng Acceptor DNA, 2 uL of T4 DNA ligase buffer, 2 uL *BbsI*, and 1uL *T4* ligase were added. Restriction and ligation was completed in the same reaction as both *BbsI*-HF and *T4* ligase (New England Biolabs) were added and run in the thermocycler programs presented (Figure 3 & 4).

First Thermocycler Program	
Temperature	Time
37°C	10 min
16°C	5 min
repeat x5 cycles	
80°C	5 min
total time	80 min

Figure 3, First Thermocycler Program for Golden Gate Reaction

After the initial restriction ligation was completed, 1uL of both ATP buffer and Plasmid safe exonuclease (Epicenter) was added to digest linear DNA fragments, followed by the thermocycler program in table 2.

Second Thermocycler Program	
Temperature	Time
37°C	60 min
70°C	30 min

Figure 4, Second Thermocycler Program for Golden Gate Reaction

The design of both reagent volumes and thermocycler settings are based off of the protocol followed by David Ott (8, 9). Confirmation of the Golden Gate constructs were completed through transformation of the Golden Gate Plasmids into NEB 5-alpha competent *E. coli* HF (New England Biolabs). Transformation was completed by adding 50 uL of thawed DH5 alpha competent *E. coli* and incubated on ice for 30 min prior to being heat shocked in a 42C water bath for exactly 30 seconds. Heat shock was followed by placing the tube of transformants on ice for 5

min before adding 905 mL of SOC outgrowth media and incubated for 1 hr at 37C on shaker at 225 rpm. The transformants were plated on LB ampicillin (100mg/mL) plates. Digestion via *BsrGI* (New England Biolabs) was completed using the same procedure as the empty acceptor plasmids. The primers initially designed for confirming the Acceptor plasmid sequence also served as the primers to confirm appropriate variable insert positioning in the final Golden Gate constructs by sequencing services through Eton Bioscience Inc.

Results:

Initial Golden Gate Reaction:

The original design of the Golden Gate reaction scheme intended to use both the Acceptor and Entry plasmids in the one step reaction. Variable regions used in this initial reaction design were $V\alpha$ -2.88 and $V\beta$ -3.31. Both Acceptor Plasmid MSGV Hu-Acceptor and MSGV Hu-Acceptor PGK-NGFR (8, 9, Addgene plasmid # 64270 & 64269) were used, these constructs were designated as Hu.1A & NGFR.1A. Concentrations and thermocycler protocol previously completed by David Ott's lab was attempted and the Golden Gate constructs were transformed into DH5 alpha competent *E. coli* (New England Biolabs). Construct plasmids were isolated using the Qiagen: Miniprep Kit (Qiagen). To confirm presence and expected plasmid size these constructs were digested using the *BsrGI* restriction enzyme (New England Biolabs). These digest products are presented in *Figure 5 & 6*.

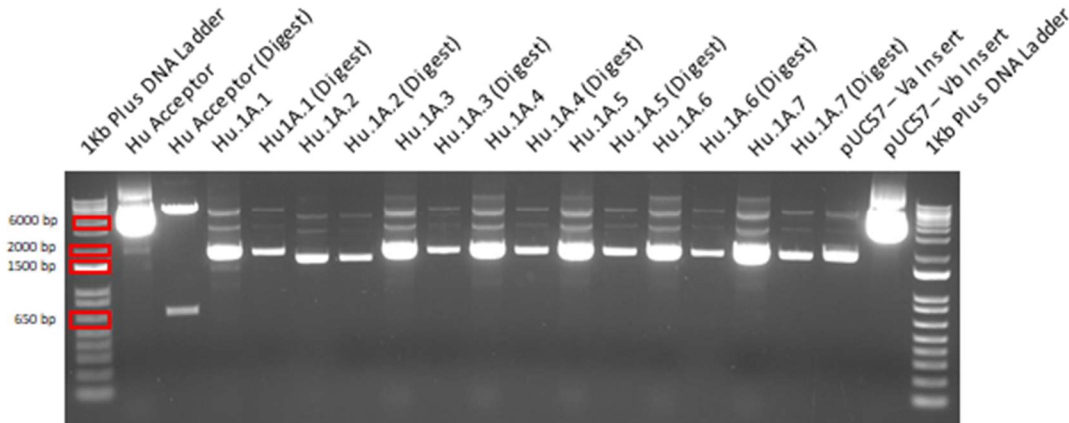


Figure 5, Hu.1A (1-7) Golden Gate *BsrGI* Digest, 1% Agarose gel for 60 min at 100V

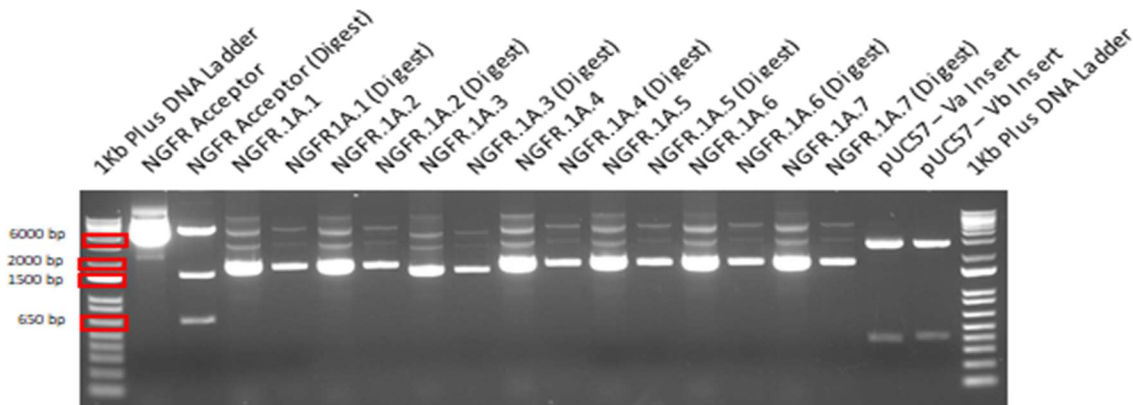


Figure 6, NGFR.1A (1-7) Golden Gate *BsrGI* Digest, 1% Agarose gel for 60 min at 100V, variable insert plasmids pUC57 Va and Vb are both digests of *EcoRI* and *HindIII*.

The digest present in *Figures 5 & 6* was expected to produce bands corresponding to: Hu.1A (1-7) **1033, 6420** and NGFR.1A (1-7) **1033, 1949, 5842**. Instead digested Golden Gate constructs produced bands similar to undigested plasmid constructs. Noted in *Figure 5* the pUC57 Va insert fragment aligns with digests of the Golden Gate Constructs. From these results it was proposed that during the Golden Gate reaction scheme, undigested pUC57 entry plasmids remained in the mixture and were subsequently not removed during the cleanup utilizing the plasmid safe exonuclease. This would have allowed the pUC57 entry plasmids to be transformed into the DH5 alpha *E. coli* (New England Biolabs) due to the pUC57 plasmid sharing the ampicillin resistance as the Golden Gate Acceptor plasmid.

Troubleshooting the Reaction Design:

To establish that both the entry and acceptor plasmids had not been altered due to transformation, restriction digests were completed of each to identify the expected band size. The Acceptor Plasmids MSGV Hu-

Acceptor and MSGV Hu-Acceptor PGK-NGFR (8, 9, Addgene plasmid # 64270 & 64269) were digested using the *BsrGI* restriction enzyme (New England Biolabs) and the gel digest is presented in *Figure 7*.

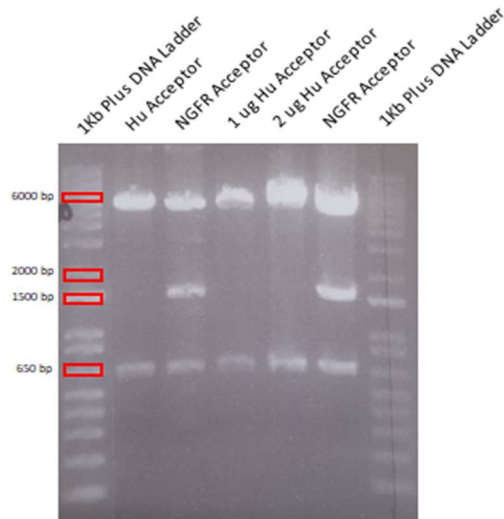


Figure 7, Hu and NGFR Acceptor BsrGI Digest, run on 0.5% gel for 60 min at 100V

The identified bands from the digest (*Fig. 7*) revealed the anticipated results of Hu-Acceptor **667**, **6072** and Hu-NGFR **665**, **1735**, **5669**. This digest provided evidence to support that the plasmid is the expected size. In order to confirm the sequences of the *BbsI* type IIS restriction sites and the specific directional overhangs the primers designed for sequencing of the Golden Gate constructs (*Fig. 1*) to confirm variable insert reading frame was used for the empty Acceptor. The sequence alignments completed from the sequence data provided from Eton Bioscience confirmed the correct and anticipated *BbsI* restriction sites and overhangs.

Confirmation of the Entry pUC57 size was completed using the *EcoRI* and *HindIII* restriction enzyme (New England Biolabs). The results of the digest are present in *Figure 8*.

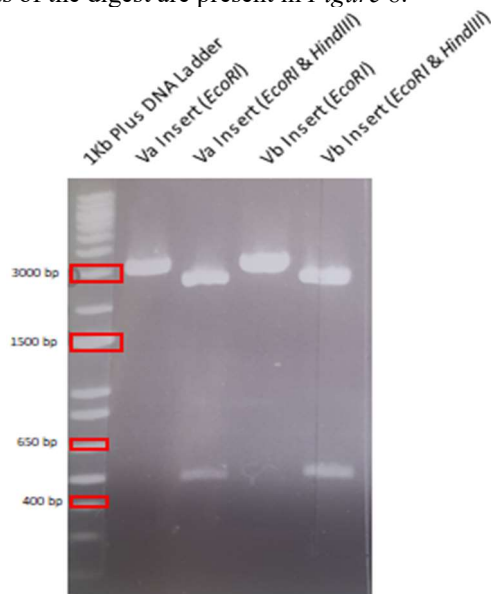


Figure 8, pUC57 Entry plasmid EcoRI and HindIII digest, using Va-2.88 and Vb-3.31, run on 1% gel at 110V for 60 min

The pUC57 digest with *EcoRI* and *HindIII* produced the anticipated bands Va-2.88 insert **497**, **2635** and Vb-3.31 insert **515**, **2635** (*Fig 8*). Plasmid size and sequence confirmation indicated that difficulties in producing the expected Golden Gate constructs was likely due to the presence of the ampicillin resistant pUC57 plasmid in the reaction scheme and subsequent transformation.

PCR Production of Variable Inserts:

Removal of the pUC57 plasmid from the Golden Gate reaction was achieved by using PCR to amplify the $V\alpha$ and $V\beta$ inserts of the entry plasmids. Using these PCR products instead of the entry plasmids in the Golden Gate reaction allowed the subsequent transformation to be uncontaminated with additional plasmids containing ampicillin resistance. Using the primers specific to the pUC57 (Fig. 2) allowed the multiple variants of variable entry sites to be amplified and prepared. Figure 9 & 10 provide images of the gels that correspond to the PCR reaction products.

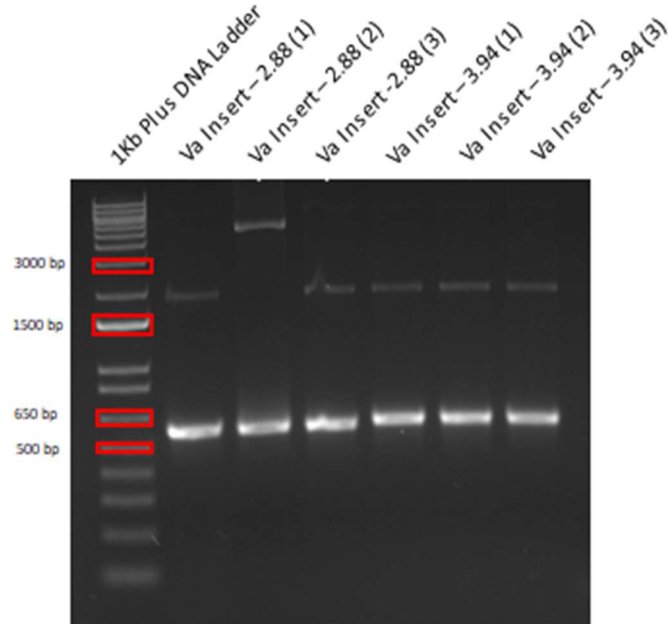


Figure 9, PCR amplification of the $V\alpha$ -2.88 and $V\alpha$ -3.94 variable regions, run on 1% gel at 110V for 60 min

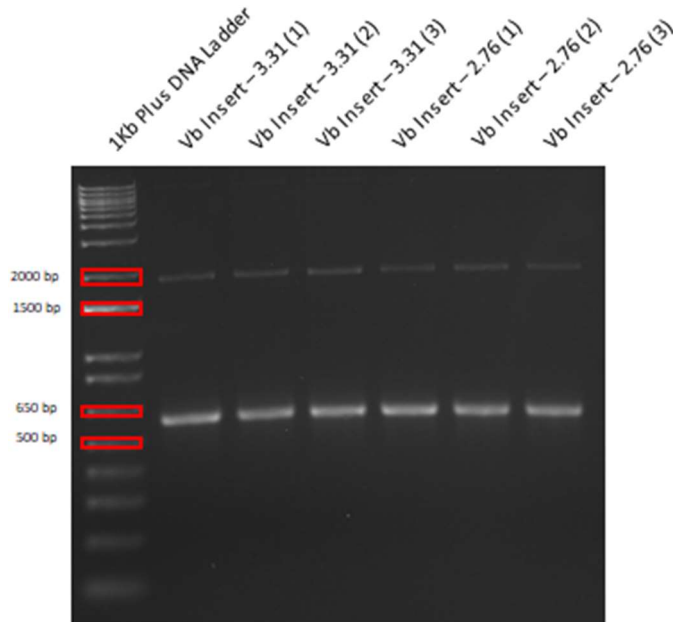


Figure 10, PCR amplification of $V\beta$ -3.31 and $V\beta$ -2.76 variable regions, run on 1% gel at 110V for 60 min

The PCR amplicons for the 1A TCR construct had anticipated bands of: $V\alpha$ -2.88, **572** and $V\beta$ -3.31, **596** represented on the gel (Fig 9 & 10). The variable inserts in addition to the inserts for the 2C TCR construct were isolated using the Qiagen PCR Cleanup Kit (Qiagen). Re-attempting the Golden Gate Reaction involved using the isolated PCR amplicons instead of the entry pUC57 plasmid. These PCR products still possessed the *BbsI* enzyme

recognition sites in addition to the variable inserts since the primers designed for the PCR reaction annealed outside of these regions of interest.

Golden Gate Construct Digestion:

After troubleshooting the Golden Gate Reaction scheme and replacing the entry plasmids with the PCR products the same reaction procedure as previously attempted was implemented (Fig 3 & 4). The Golden Gate reaction was followed by transformation into DH5 alpha Competent *E. coli* (New England Biolabs) and the plasmids were isolated using the Qiagen Plasmid Miniprep Kit.

Digestion of the Golden Gate Constructs using the *BsrGI* restriction enzyme (New England Biolabs) produced bands that indicate the presence of the V α and V β variable inserts present within the Acceptor plasmid. The Golden Gate constructs are of the variable insert pairs 1A: V α -2.88/ V β -331 and 2C: V α -3.94 /V β -2.76. Both construct designs were digested in triplicate (1A.1-3 and 2C.1-3). The expected band sizes for Hu1A are **1033, 6420** and Hu2C is **525, 6420**. The expected band sizes for NGFR1A are **1033, 1949, 5842** and NGFR2C is **538, 1949, 5842**.

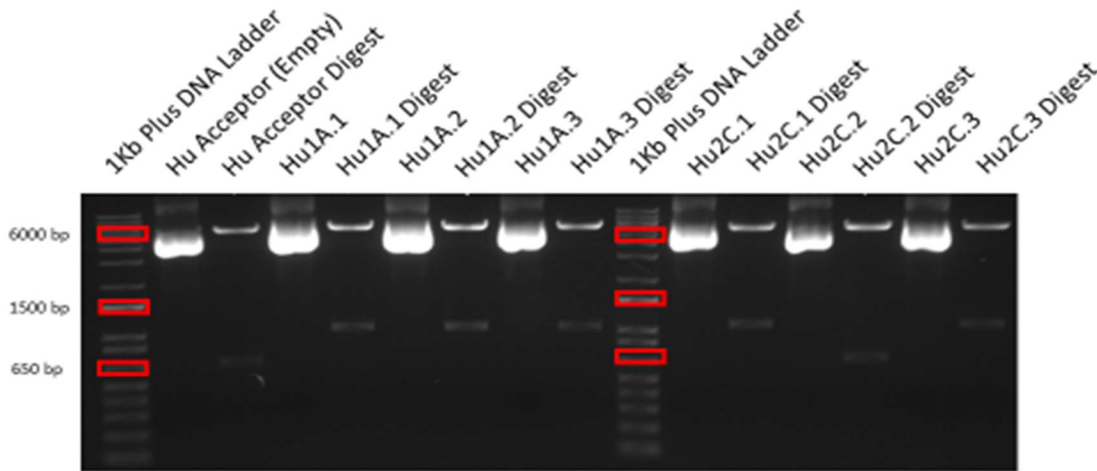


Figure 11, Hu Golden Gate Digest with *BsrGI*, run on 1% gel at 110V for 60 min,

The bands in Fig 11 corresponding to Hu.1A are present at the expected bp throughout Hu.1A 1-3. Hu.2C only has the expected bands for the construct of Hu.2C.2. Hu.2C.1 and Hu.2C.3 do not have bands at the anticipated 525 length.

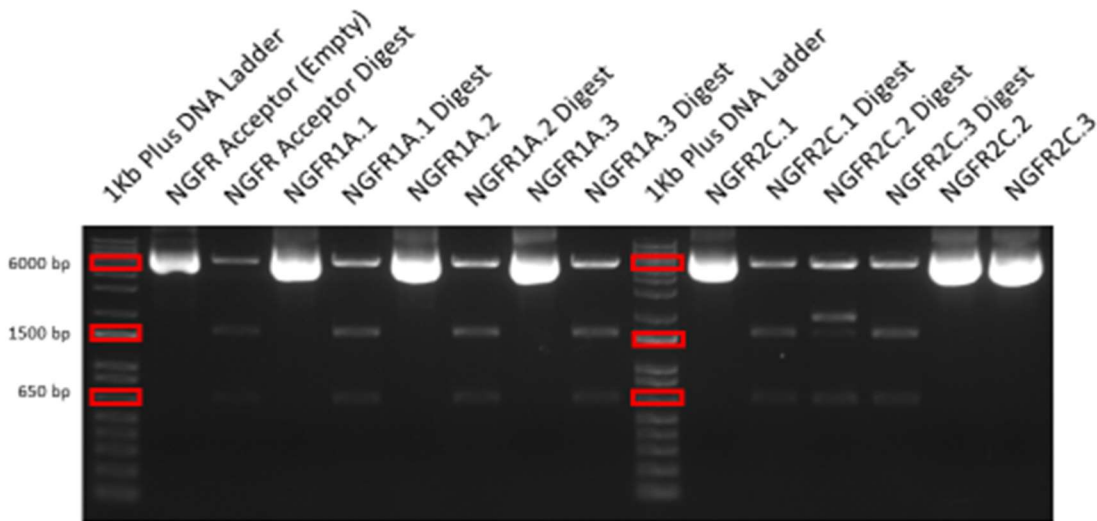


Figure 12, NGFR Golden Gate Digest with *BsrGI*, run on 1% gel at 110V for 60 min.

The bands in Fig 12 corresponding to NGFR2C 1-3 all have approximate correspondence to what was anticipated for the digestion. The 1A construct with did not produce the bands at the anticipated length.

Discussion:

Interpreting Golden Gate Plasmid Digestion with BsrGI:

The digestion products based on band length presented in *Figure 11* and *12* suggest that the Golden Gate reaction was successful in inserting the variable $V\alpha$ and $V\beta$ fragments specifically in the Hu acceptor in constructs Hu1A.1 – Hu1A.3 (*Fig 11*) and NGFR construct in NGFR2C.1 – NGFR2C.3 (*Fig 12*). Throughout these Acceptor vectors, and unlike the previous digest of Golden Gate constructs (*Fig. 5 & 6*) additional unexplainable bands that previously been present are not identified. Sequencing of these Golden Gate constructs using the designed primers (*Fig 1*) could potentially provide further insight into determining if the constructs reveal the presence of the variable inserts. This would help identify if these constructs were simply ill represented on the gel digestion. Otherwise, unpredicted sequence data could suggest problems in the specific overhangs causing interruptions in the variable insert.

Correcting the Golden Gate Reaction Scheme:

The Golden Gate reaction was able to produce bands of the anticipated size in certain constructs when the pUC57 plasmid with ampicillin resistance was excluded from the reaction and subsequent procedures. Initially, the motivation for being able to use the entry plasmid instead of relying on PCR amplification was that the plasmids could easily be transformed and cultured to increase the amount of plasmid DNA containing variable inserts available for Golden Gate reactions. Avoiding the requirement for PCR was also pursued to reduce the cost of the Golden Gate scheme and remove the potential for changes in the sequence during amplification. The presence of the ampicillin resistance in the pUC57 suggests that due to the entry vector having a smaller size than the Golden Gate constructs the DH5 alpha competent *E. coli* were preferentially transformed by the smaller pUC57 plasmid as opposed to the larger Golden Gate plasmids. Replacing the pUC57 entry plasmid with a plasmid that contains an alternative antibiotic is predicted to alleviate the problems experienced in this Golden Gate scheme. Changing the antibiotic resistance in the entry plasmid is also predicted to allow the experimenter to use the Golden Gate scheme using the Acceptor and Entry plasmids and avoid PCR. Changing the antibiotic resistance in the entry plasmid would allow only the Acceptor plasmid to be specifically selected for in the growth on LB ampicillin following the Golden Gate reaction and transformation into NEB DH5 alpha competent *E. coli* (New England Biolabs). Colonies would not be afforded the potential to preferentially transform the smaller entry plasmid.

Next Steps and Future Direction:

After sequencing the Golden Gate constructs using the primers in *Figure 1* the next steps involve transduction of the Golden Gate plasmid into a murine retroviral packaging cell line. The murine retrovirus produced will subsequently be used to transfect the TCR construct into a Jurkat cell line. Using flow cytometry, the Jurkat cells will be analyzed to identify the presence of the transmembrane TCR complex. The murine NGFR will serve as a control to track the effectiveness of the transfection/transduction process through identification of its presence in the steps of analysis via flow cytometry. Using the modular design of the Golden Gate reaction scheme allows future researchers to create a repertoire of TCRs by interchanging $V\alpha$ and $V\beta$ sequences among TCR constructs. This functionality allows a variety of potential TCR constructs to be identified with a specific patient and investigated in terms of their capacity to recognize and respond to Mtb antigens.

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