Benchmarks

Production of retroviral constructs for effective transfer and expression of T-cell receptor genes using Golden Gate cloning

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Here we present an improved strategy for producing T-cell receptor (TCR)-expressing retroviral vectors using a Golden Gate cloning strategy. This method takes advantage of the modular nature of TCR genes by directly amplifying TCR α and β variable regions from RNA or cDNA, then cloning and fusing them with their respective constant region genes resident in a retroviral TCR expression vector. Our one-step approach greatly streamlines the TCR vector production process in comparison to the traditional three-step procedure that typically involves cloning whole TCR genes, producing a TCR expression cassette, and constructing a retroviral construct. To date, we have generated TCR vectors that transferred seven functional human/rhesus macaque TCRs into primary T cells. The approach also holds promise for the assembly of other genes with defined variable regions, such as immunoglobulins.

One powerful approach to studying T-cell receptor (TCR) properties and T-cell function is to clone TCR genes into retroviral vectors and transfer antigen specificity into other cells (1–3). A drawback to this approach, however, is that the cloning of the TCR genes into retroviral vectors is a somewhat complicated and time-consuming process. Full gene synthesis of a TCR gene cassette is an alternative, but this approach is relatively expensive, entails significant production time, and still requires cloning into a retroviral vector.

Here we describe a Golden Gate method that combines the conventional steps for cloning TCR genes into a single process (Figure 1A), expediting TCR vector

production. In a normal workflow, TCR vectors can be produced in 1 work week using this Golden Gate method: (i) Day 1—PCR amplification and PCR product gel analysis; (ii) Day 2—Golden Gate reaction and bacterial transformation; (iii) Day 3—pick bacterial colonies and liquid culture transformants for screening; (iv) Day 4—plasmid preparation and restriction enzyme screening for recombinants, and liquid culture candidate clones in 50-mL mid-scale cultures; (v) Day 5—plasmid preparation for additional testing by transduction. In contrast, the conventional three-step protocol involving TCR cloning, expression cassette construction, and retroviral vector construction requires ~3 weeks. Even gene

synthesis, given our experience, takes ~3 weeks.

To develop this system, we modified our previously published MSGV-based retroviral vector system that expresses both TCR α and β chains (1,2) to allow for one-step Golden Gate cloning of both V α and V β (variable) regions by fusing them to their respective C-regions residing in a retroviral vector (Figure 1A). Thus, V-region Golden Gate cloning (4–6) allows for precise and efficient fusion of DNA through directional use of type II restriction sites and iterative cycles of restriction/ligation. Both V regions of codon-optimized TCR chains in either human or rhesus macaque TCR vectors were replaced with two pairs of *BbsI* type II restriction sites oriented to produce four base overhangs in the vector at human/rhesus-conserved coding sites to enable V-region fusion with the respective constant regions. The *BbsI* sites in the vector, which are separated by a short unstable stuffer fragment that provides spacing for efficient cleavage, cannot themselves reclone into the vector (Figure 1A).

Producing a TCR vector using our methodology entails (Figure 1A): (i) producing *BbsI*/V-region sense and antisense primers that introduce complementary sites for V-region fusion; (ii) PCR amplification of the unique V-region sequences from cDNA/RNA and adding *BbsI* sites that produce Site 1 and 2 cohesive ends to the V α -region flanks and Site 3 and 4 ends to that of the V β region; and (iii) carrying out the Golden Gate reaction with both V-region PCR products and the V-region acceptor vector to produce a MSGV TCR retroviral vector. There are two versions of the vectors: For studies of TCRs that have tetramers available for detection (1), the acceptor vector is ideal. For TCRs that have no reagents for specific detection, the acceptor-tNGFR vector should be used to be able to identify transduced T cells via NGFR expression.

A detailed protocol is provided in the Supplementary Material. We assume that the 5' sequences of the TCR chain open reading frames have been determined by sequencing. The sequencing of TCRs has been described by others (7–9). Using the template provided in Figure 1B, V-region cloning primers are designed: Site 1, 5' V α

METHODS SUMMARY

We present a method to simultaneously clone and assemble TCR sequences directly from RNA/cDNA into a retroviral vector using Golden Gate cloning of PCR products.

B Primer Design Template

$V\alpha$ Sense Site 1
 TTTTGAAGACTTCACC + add 5' $V\alpha$ S Seq
 Tm ~72

$V\alpha$ Antisense Site 2
 TTTTGAAGACTTTCTG GAT ATT TGG + add 3' $V\alpha$ AS Seq
 Tm ~72

$V\beta$ 5' Sense Site 3
 TTTTGAAGACTTC CCT GGC CCT + add 5' $V\beta$ S Seq
 Tm ~72

$V\beta$ 3'Antisense Site 4
 TTTTGAAGACTTG ATC CTC + add 3' $V\beta$ AS Seq
 Tm ~72

C $V\alpha$ Cloning

$V\beta$ Cloning

Figure 1. Golden Gate TCR cloning process. (A) Overview of the cloning process is presented in three steps (i–iii). Golden Gate–specific sequences in primers appear as thin lines labeled as Site 1, 2, 3, or 4. (B) Primer design template with the cohesive ends used for joining underlined. Care should be taken to use the correct sense and orientation. S Seq: sense primer sequence; AS Seq: antisense primer sequence. (C) Molecular details of the Golden Gate reaction for both the $V\alpha$ and $V\beta$ insertions into the vector. Sequence features are displayed above, and translations are shown below each sequence. P2A is a self-cleaving peptide cassette (1,2) used to produce both TCR chains from a single open reading frame.

region sense sequence starting with the methionine codon; Site 2, 3' V α antisense sequence starting just upstream of the C α proline; Site 3, 5' V β region sense sequence starting with the methionine codon; and Site 4, 3' V β antisense sequence starting just upstream of the C β glutamate codon. The TCR V-region-specific sequence in each primer should have a T_m of ~72°C. These primers can be used to amplify either RNA or cDNA to produce the V α and V β insert PCR fragments. For RNA, we amplify 0.1–1 μ g of RNA with the SuperScript III/One-Step RT-PCR System (Life Technologies, Carlsbad, CA) using an RT-PCR profile of 50°C/45 min, followed by 95°C/2 min, then 35 cycles at 95°C/30 s, 68°C/30 s, 72°C/60 s, and then 72°C/3 min. For cDNA, we amplify 0.5–0.05 μ g of cDNA with the Platinum Taq SuperMix system (Life Technologies) with an amplification profile of 95°C/60 s, then 35 cycles at 95°C/30 s, 68°C/30 s, 72°C/60 s, and 72°C/3 min.

Golden Gate cloning of the amplified V-regions into the acceptor vector (Figure 1, A and C) is accomplished through iterative restriction/ligation in a 20- μ L reaction containing 100 ng of acceptor TCR vector and 50 ng of each V-region PCR product in ligase buffer with 10 U of *Bbs*I (New England Biolabs, Ipswich, MA), and 400 cohesive end units of T4 ligase (New England Biolabs), carried out for 5 cycles at 37°C/10 min and 16°C/15 min followed by a 80°C/5 min inactivation step. Linear DNAs are digested with 10 U of Plasmid Safe Exonuclease (Epicenter, Madison, WI) and 1 mM ATP, incubating for 1 h at 37°C then enzyme inactivation at 70°C/30 min.

Of the 22 μ L Golden Gate reaction, 5 μ L are transformed into One Shot TOP10 Chemically Competent *E. coli* (Life Technologies) following the provider's recommendations, and 10% of the transfor-

Figure 2. Functional properties of transduced CM9-11 TCR in primary rhesus macaque T cells. Flow cytometry analysis is presented of cells transduced with the CM9-11 TCR vector produced by Golden Gate cloning after surface staining with CD8 antibody and MHC/CM9 tetramer or intracellular staining for Mip1 β and interferon- γ (IFN γ) after stimulation with CM9 peptide.

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mation is plated onto 100 µg/mL ampicillin Luria Broth agar. Typical transformations yielded 6736 total colonies per transformation, on average. Screening colonies of human TCR transformants for recombinant plasmids with the *Bsr*GI restriction enzyme yields diagnostic bands (unique fragments in bold) for the following TCR constructs: HuTCR recombinant, **6936/1045** bp versus **6033/666** bp for the acceptor vector and HuTCR-NGFR recombinant, **5842/1883/1042** bp versus **5942/1547/665** bp. For rhesus constructs, *Kpn*I enzyme digestion yields diagnostic bands for RhTCR recombinants at 3686/**2103**/1209/828/466 bp versus 3686/**1343**/1209/466 bp and for pRhTCR-NGFR, 3686/**3460**/1209/466 bp versus 3686/**2701**/1209/466 bp. It should be noted that because the V regions can harbor additional restriction sites, a diagnostic band might be cleaved into smaller bands. Overall, 56% of the colonies screened were recombinants with appropriate gene fusions as confirmed by DNA sequencing. However, in some cases, typically with large numbers of transformants, there is a high background of empty vector clones. In this event, less acceptor plasmid should be used, a new PCR product generated, or more reaction

cycles performed. Also, it is important to use PCR products yielding a single band on an agarose gel. Otherwise, purification of the ~400 bp V-region PCR products should be done.

Candidate TCR vector DNA constructs were examined for TCR expression by CD3 rescue (10) of the TCR-deficient Jurkat RT3.5-T3.5-clone 12 cells (11). The retroviral vector was produced by transfecting the TCR constructs into a murine retroviral packaging line, in our case, the Phoenix-RD114 packaging line (12). Vector was transduced into the indicator cells and observed by flow cytometry for gain of CD3 expression. Successful vectors were then used for studies (1). Representative data from an SIV CM9 epitope-specific TCR vector, MSGV CM9–11, are presented in Figure 2. The CM9–11 TCR was isolated from cDNA obtained from a rhesus macaque T-cell clone that responded to the CM9 immunodominant SIV Gag peptide (13). The CM9–11TCR vector was used to transduce CD8⁺ primary rhesus T cells, which were then analyzed by flow cytometry. Transduced cells exhibited robust CM9 tetramer staining and possessed strong and specific induction of interferon γ and Mip1 β expression upon CM9 peptide

stimulation when compared with untransduced cells (Figure 2).

This method greatly reduces the time required to construct a TCR-expressing vector, from several weeks using 3 steps to just 1 week using a single step. Because the V-regions are directly cloned as individual PCR products, each recombinant represents a unique sampling of the V-region amplification. Therefore, in contrast to current methods that are restricted to only one pair of sequences that may contain PCR errors, screening several Golden Gate clones increases the likelihood of isolating a functional TCR without PCR-induced mutations as the cloned V-regions are independent events. Indeed, some of our prior defective conventional TCR constructs were successfully obtained using our new procedure, including the CM9–11 vector (Figure 2). Vectors can be produced even when *Bbs*I sites are present in the V-regions; the CM9–11 V β contains a *Bbs*I site (Figure 2), yet it still produced a vector, albeit with 5-fold fewer recombinants, likely due to the requirement for ligation at the retained *Bbs*I site. While no TCR has yet had *Bbs*I sites in both V α and V β regions, theoretically, those regions should be able to be cloned because Golden Gate cloning can assemble multiple DNA pieces in one reaction (4–6). However, the presence of two *Bbs*I sites would certainly reduce the efficiency of the cloning.

Overall, our new method provides for a rapid and efficient way to produce expression-ready retroviral TCR vector constructs directly from T-cell RNA/cDNA. Because TCRs are heterodimers requiring two matching chains, isolating functional TCRs from nonclonal sources where there could be two or more TCRs present is problematic, as there are 2ⁿ combinations for mixtures of *n* clones. Although it would be very labor- and time-intensive to apply the conventional approach in this case, the ability to produce a library of TCRs from a mixture is possible with the Golden Gate method. This would be especially useful for nonclonal samples containing limited numbers of cells that cannot be feasibly cultured, such as a biopsy or tumor sample, because of its efficiency and independent sampling of sequences. Finally, our approach mimicking the domain-swapping strategy that generates TCR genes in vivo could streamline the production of viral vectors expressing other variable proteins, especially immunoglobulins.

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Author contributions

L.V.C. performed all of the DNA manipulations and sequence analysis; M.T.T. performed cell culture and flow cytometry; S.J. performed flow cytometry; C.O. provided assistance in manuscript presentation and experimental design; D.E.O. conceived the concept, designed the vectors, performed cell culture and retroviral vector manipulations, and wrote the manuscript.

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Competing interests

The authors declare no competing interests.

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