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Research paper

A fast and robust method to clone and functionally validate T-cell receptors

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ABSTRACT

Sequencing, cloning and functional testing of T-cell-receptor (TCR) α- and β-chains from T-cell clones is often required in immunotherapy and in immunological research. However, the determination of the TCR chains by a simple PCR is not possible, since, in contrast to the 3′ constant domain and untranslated region (UTR), no conserved sequences are present in the 5′ region. Furthermore, subsequent functional testing of cloned TCRs requires laborious cell-culture experiments, often involving primary human material and time-consuming viral transduction strategies. Here we present a universal PCR-based protocol, adapted from the capswitch technology, that allows for amplification of the TCR α- and β-chain mRNAs without knowledge of the TCR variable domain subtype by attaching a designed sequence to the mRNA's 5′ end. Two different MelanA/HLA-A2-specific and one HIVgag/HLA-A2-specific TCR were cloned that way, and were functionally tested by a newly developed easy, fast, and low-cost method: we electroporated Jurkat T cells simultaneously with TCR-encoding RNA and an NFAT-reporter construct, and measured the activation status of the cells upon specific stimulation. The results of this assay correlated with the cytokine release, functional avidity, proliferative activity, and the ability to recognize MelanA/HLA-A2-presenting tumor cells of bulk T cells electroporated with RNA encoding the same TCR. Together these two protocols represent a rapid and low-cost tool for the identification and functional testing of TCRs of T-cell clones, which can then be applied in immunotherapy or immunological research.

1. Introduction

Sequencing, cloning, and characterization of T-cell receptors (TCRs) is often performed, as TCRs in a genetic format match several applications. Numerous groups already showed that T lymphocytes can be reprogrammed *in vitro* with a new (e.g. tumor) specificity by retroviral transduction with TCR-encoding vectors (Clay et al., 1999; Morgan et al., 2003; Roszkowski et al., 2005; Schaft et al., 2003b; Stanislawski et al., 2001; Willemsen et al., 2000; Zhao et al., 2005), or, more recently, by electroporation with TCR-encoding RNA (Cohen et al., 2005; Hofmann et al., 2008; Schaft et al., 2006; Zhao et al., 2005, 2006a,b). The clinical use of reprogrammed T cells in cancer immunotherapy has recently emerged, as clinical responses were observed in melanoma patients upon transfer of autologous T lymphocytes, retrovirally transduced with an anti-MelanA/HLA-A2 TCR (Morgan et al., 2006). To possess a TCR in a genetic format also allows for the introduction of directed mutations, which can improve stability and functionality (Cohen et al., 2007; Scholten et al., 2006; Kuball et al., 2007), or for affinity maturation in selective systems like the phage-display system (Zhao et al., 2007). Unhinging a TCR from its originating T cell is a prerequisite to characterize this TCR,

Abbreviations: UTR, untranslated region; TCR, T-cell receptor; PCR, polymerase chain reaction; wt, wild type; PMA, phorbol myristate acetate; NFAT, nuclear factor of activated T cells.

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avoiding inaccuracies arising from the differentiation state and other characteristics of the *in vitro* expanded T-cell clone.

Another application that should not be underestimated, is the use of TCR-transfected T cells as tool for detection and quantification of antigen presentation *in vitro*. In contrast to T-cell clones that often display signs of senescence and oscillate in their activity (Demotte et al., 2002), TCR-transfected T cells can be generated in sufficient numbers and of constant quality, resulting in informative and reproducible data.

Up to now, the identification and cloning of TCR α- and β-chains with unknown variable domains from specific T-cell clones were time-consuming and expensive (Kalams et al., 1994). Since each newly evolved human T cell generates its own individual TCR by somatic recombination, they display a high diversity. For the TCR α-chain, 44–46 functional variable gene segments exist, of which one is joined by a random process to one of 50 different joining regions, generating the variable and hypervariable domains of the α-chain (Lefranc and Lefranc, 2001). By RNA splicing, these are then fused to the sequence that encodes the TCR α-chain constant region. The TCR β-chain is assembled by a similar mechanism, but contains an additional diversity region (Fig. 1A) (Lefranc and Lefranc, 2001). Therefore, TCR α-chain or β-chain mRNAs can have 46 or 67 different 5′ ends, respectively. Consequently, to identify and clone an unknown full-length TCR by plain RT-PCR, even when using degenerated primers up to 29 PCRs for the α-chain and 48 PCRs for the β-chain were needed (Kalams et al., 1994). Alternative strategies involve attaching defined sequences to the 5′ end of the cDNA (corresponding to the 3′ end of the mRNA). These techniques include e.g. the RACE-technique (Sambrook and Russel, 2001) and the SMART technique (Zhu et al., 2001). Here we present a universal PCR protocol, based on the latter, which allows amplifying and cloning of the TCR α- and β-chain cDNAs from small amounts of mRNA without previous knowledge of the TCR variable domain subtype by attaching a designed sequence to the 5′ end.

TCRs can vary in their affinity and specificity, and the functionality of newly cloned and then transferred TCRs is not guaranteed. Informative examination of a cloned TCR, however, requires its expression in living cells, its binding to the corresponding antigen-peptide presented on MHC molecules,

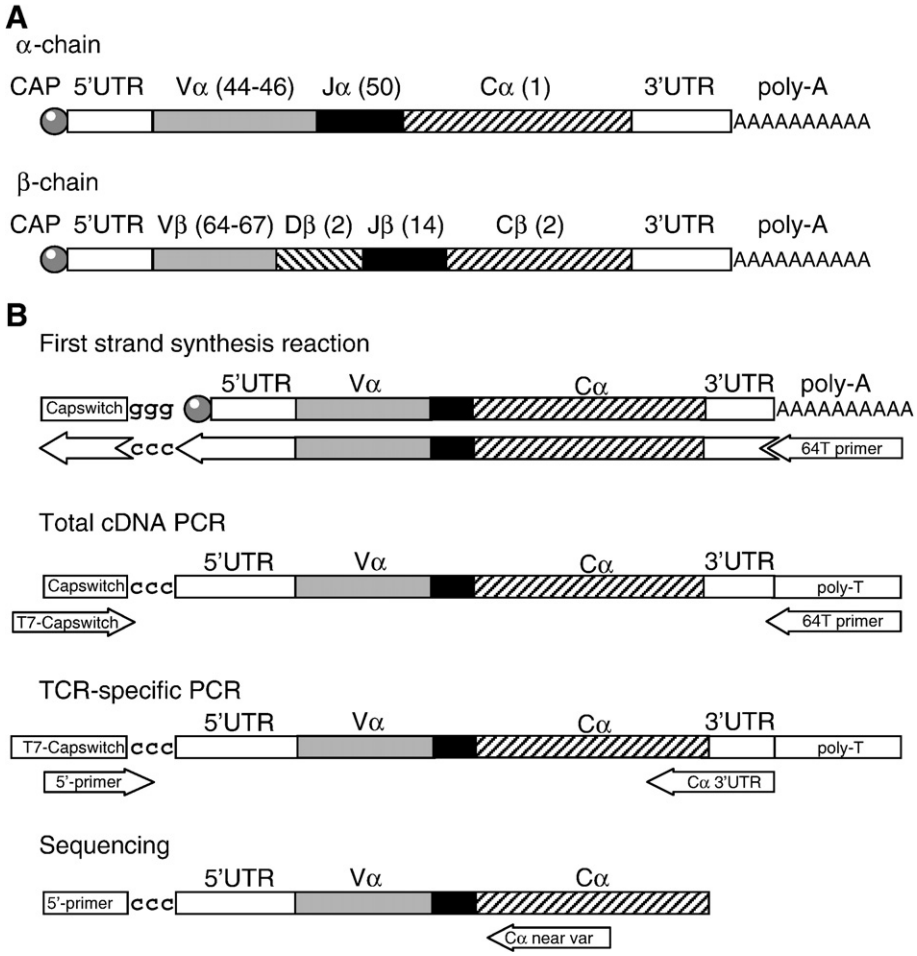

Fig. 1. T-cell receptor PCR strategy. (A) Structure of the TCR α- and β-chain mRNAs. The α-chain mRNA consists of a 5′ untranslated region (5′ UTR), a variable region (Vα), a joining region (Jα), and the constant region (Cα), followed by the 3′ untranslated region (3′UTR), and the poly-A tail. The β-chain is composed in a similar way, with an additional diversity region (Dβ) between Vβ and Jβ. The approximate number of different gene segments coding for each region is indicated in brackets. (B) Strategy to amplify the TCR α- and β-chain genes, (depicted is only the strategy for the α-chain). For detailed description see Results section.