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Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV infected cells

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The human immunodeficiency virus type 1 (HIV-1) uses cell surface CD4 as a receptor to infect susceptible cells. Therefore, different forms of soluble CD4 (sCD4) molecules have been developed recently for potential therapeutic purposes. Here we describe a novel design of sCD4 molecules which exploit cytotoxic T cells as their effector function. The principle of bispecific antibodies was exploited and further developed to create new bispecific reagents which could retarget cytotoxic T cells of any specificity and thus, induce killing of HIV-1 infected cells. The most advanced molecules, Janusins, contain in one polypeptide chain the first two N-terminal CD4 domains and single chain combining site against the human CD3 complex (FvCD3).

Key words: bispecific antibodies/HIV-I/soluble CD4

Introduction

Since the realization that the immunodeficiency virus type I (HIV-I) exploits the cell surface CD4 molecule as its receptor (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985; Maddon *et al.*, 1986), soluble forms of CD4 (sCD4) have been designed to combat the virus. The first generation of sCD4 molecules contained basically only the extracellular part of the protein or truncated versions of it and, thus were only able to neutralize the free virus (Smith *et al.*, 1987; Deen *et al.*, 1988; Fisher *et al.*, 1988; Hussey *et al.*, 1988; Traunecker *et al.*, 1988). Further development led to second generation molecules like CD4−toxin (Chaudhary *et al.*, 1988) and CD4−Igs (Capon *et al.*, 1989; Traunecker *et al.*, 1989), which were also thought to destroy the infected cells by direct action of the toxin or by Fc mediated immune effector functions. Here we describe a third generation of sCD4 molecules which are based on the principle of bispecific antibodies and which exploit cytotoxic T cell activity. By using these antibodies, which contain one combining site against the T cell receptor (TcR) complex and the other against selected cell surface structure, cytotoxic T cells of any specificity can be directed onto and induced efficiently to kill the defined target (Perez *et al.*, 1985; Staerz *et al.*, 1985; Lanzavecchia and Scheidegger, 1987). We have generated two kinds of bispecific molecules: one is based on Ig and consists of modified human IgG3 whose Fab regions were replaced with anti-CD3 single chain combining site (FvCD3) (Huston *et al.*, 1988) and the first two N-terminal domains of CD4 (Traunecker *et al.*, 1988; Traunecker *et al.*, 1989). The other is a further developed molecule made wholly of a single polypeptide chain containing the FvCD3 and CD4 with no Ig Fc sequences. Here we show that these single chain molecules efficiently mediate the killing of HIV-I infected cells.

The usual approach to generate bispecific antibodies is to fuse two hydridoma cell lines producing the desired antibody specificities (Milstein and Cuello, 1983). Because of the random assortment of Ig heavy (H) and light (L) chains, these hybridomas produce a potential mixture of 10 different antibody molecules. Therefore, the bispecific antibodies have to be purified with cumbersome procedures which can considerably decrease the yield of the desired product. The way we have designed our bispecific molecules reduces the original heterogeneity of the mixture into three, and with a second construct finally into only one species.

Results and discussion

To produce bispecific molecules that can bridge T cells of any specificity to the HIV-I infected, gp120 expressing cells, we took advantage of previously produced CD4−human IgG3 (CD4−Hγ3) molecules, which were secreted by transfected myeloma cells as typical dimeric Ig-like molecules. We then wanted to introduce into these cells the specificity of anti-CD3 (the invariant part of TcR complex) to create a bispecific molecule. Since hγ H chains in CD4−Hγ proteins were modified by the deletion of the first region (CHl) domain, ensuring the L chain independent secretion, we produced the anti-CD3−Hγ3 in a way that obviates the need of independently assorting Ig L chains. To do this we used the established PCR methodology to clone V_H and V_L sequences (Orlandi *et al.*, 1989) from anti-CD3 hybridoma TR66 (Lanzavecchia and Scheidegger, 1987). The V_H and V_L clones were then fused together to generate FvCD3 construct, following basic procedures used to produce single chain antibody Fv fragments in bacteria with modifications (Huston *et al.*, 1988), which allow the expression in mammalian cells (see Materials and methods; Figure 1). Finally, complete expression vector pFvCD3−Hγ3 analogous to pCD4−Hγ3 were introduced into myeloma cells J558L alone or together with CD4 partners. Single transformants secreted typical Ig-like dimeric molecules (Figure 2) with the same specificity as the parent antibody TR66 (data not shown), and double transformants produced all the predictable three dimeric species, CD4−Hγ3, FvCD3−Hγ3 and the hybrid CD4−Hγ3/FvCD3−Hγ3 (Figure 2).

Although the molecules retained the binding specificities of both parent molecules (see below, Figure 3), they still have two obvious major drawbacks. Firstly, as mentioned above and also shown in Figure 2, correct heterospecific species is often a minor component and needs to be purified from the bivalent monospecific forms (Milstein and Cuello, 1983). Secondly, the Fc part of the molecule can mediate illegitimate targeting, i.e. T cells can be directed on any Fc receptor positive cells, a situation that is certainly not desirable.

To avoid these drawbacks, we created a prototype construct which represents a single mammalian expression unit encoding all CD4, FvCD3 and C$\varkappa$ regions in a single polypeptide chain (Figure 1). Myeloma cells transfected with this construct secreted the expected protein (Figure 2), which we named Janusin (CD4−FvCD3), after the Roman God Janus, often pictured as having two faces. Janusin could be purified easily from cultured supernatants, (~10 mg/l) in this case, simply by using monoclonal anti-$\varkappa$ (187.1) affinity columns (Yetton *et al.*, 1981).

First we tested if recombinant molecules had folded correctly and maintained both CD4 and anti-CD3 binding sites. This was indeed the case, since $CD3^+$ $CD4^-$ T cells incubated with Janusin or CD4−Hγ3/FvCD3−Hγ3 (still containing all three species) could be stained by anti-CD4 monoclonal antibody (Figure 3). We then tested whether these molecules could be able to retarget cytotoxic T cell clones against HIV-I infected cells. To study this possibility we used several human random $CD8^+$ cytotoxic T-cell clones and scored their ability to kill Jurkat cells either infected with HIV-I-HAN (Traunecker *et al.*, 1989) or uninfected in the presence of varying amounts of (CD4−FvCD3) Janusin (Figure 4). As shown for two different cytotoxic clones, only nanogram amounts of Janusins were sufficient for efficient killing. At higher concentrations (~1 μg/ml), non-infected cells were also killed. Therefore, we analyzed the preparation of Janusins in the FPLC system to detect potential multimers or aggregates, which could, in fact, bridge the CD3 molecules of cytotoxic clones to the CD3 molecules of Jurkat cells and induce killing of uninfected cells. FPLC analysis (Figure 2C) revealed the presence of about 5% of Janusins in multimeric forms, which could have been formed by denaturation during low pH (~3.0) elution step. To avoid aggregate formation, alternative purification methods, including perhaps sizing columns, have to be developed before potential *in vivo* application. This would also minimize the possibility that HIV-infected T cells would be activated to virus production by Janusins, since it has been shown that monovalent ligation of CD3 by a bispecific antibody does not deliver a triggering signal in the absence of a specific target (Roosnek and Lanzavecchia, 1989).

We believe that Janusins described here (or simple modifications of them, like omission of $\varkappa$ region), which combine the specificity of CD4 and effector functions of cytotoxic T cells could offer significant further development for sCD4-based AIDS therapy. Janusins exploit potent cytotoxic T cell responses, i.e. different compartment of the immune system is triggered compared with potential complement or ADDC activation by previous forms of soluble CD4 molecules, like CD4−Igs. In addition, activated T cells may provide efficient transport of these molecules as cell bound entities to the sites of infection and, thus, also the functional half lives of these molecules can be increased compared to free soluble molecules. Interestingly, Romeo and Seed (1991) have taken another approach to exploit

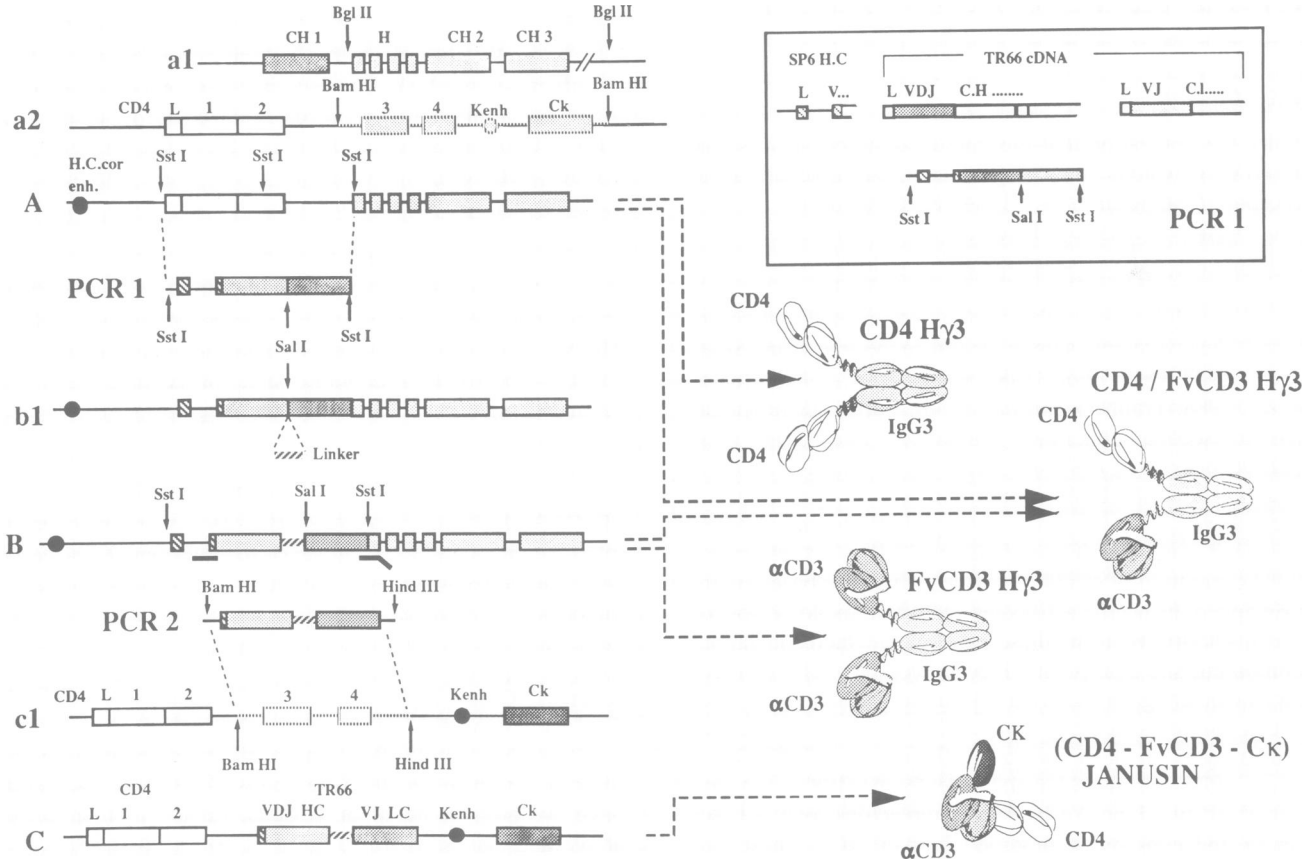

Fig. 1. Bispecific CD4/anti-CD3 constructs. The flow chart shows the engineering steps needed for the final constructs **A**. pCD4-Hγ3, **B**. pFvCD3−Hγ3 and **C**. Janusin i.e. pCD4−FvCD3-C$\varkappa$. The lower part of the figure shows the schematic representations of the corresponding proteins. Vectors to express the constructs in myeloma cells have been described previously (Traunecker *et al.*, 1988; Traunecker *et al.*, 1989). Exons are indicated by various boxes and introns by single lines. Filled circles represent $\varkappa$ or heavy chain (HC) enhancers as indicated. Only restriction sites used for constructions are shown. H = hinge, CHl, 2, 3 = constant region exons of human γ3 HC gene.

cytotoxic T-cell responses against gp120 expressing cells. Instead of using 'adaptor' molecules like us, they constructed novel T-cell receptors with the specificity of CD4 (Romeo and Seed, 1991). We also want to stress that we have used mammalian expression systems to produce Janusins as the recovery of biologically active molecules in bacterial expression systems can dramatically decrease when the number of independently folding protein domains increases. Although the Janusin structure is the simplest solution, we would like to point out that our approach to produce dimeric Ig-based bispecific molecules is a general one and allows molecular designs which can easily accommodate one or even two non-Ig binding moieties.

We also think that our approach to design single chain molecules can be applied more generally, e.g. Janusins with two different Fv domains could be exploited for retargeting T cells against tumors or to target deficient retroviruses to desired cells in the context of somatic gene therapy. Finally, it will be interesting to see whether Janusins of FcCD3/antigen designs have the potential to concentrate T cell help into relevant antigen specific B-cells and therefore, behave as 'super immunogens'.

Materials and methods

Construction of expression vectors

pCD4−Hγ3. Obtained by simply replacing the *Bam*HI fragment of pHT4-YK12 (Traunecker *et al.*, 1989) with the *Bgl*II fragment containing H, CH2 and CH3 exons of human γ3 HC genes from plasmid pSH2-γ3 (Huck *et al.*, 1986) (a gift from Dr M.-P.Lefranc) as indicated. Finally, biochemical selection markers were changed from *gpt* to *his* (Hartman and Mulligan, 1988).

FvCD3−Hγ3. First the V_L and V_H sequences of the anti-CD3 hybridoma TR66[15] without their leader (L) sequences were amplified by using the established PCR methodology (Orlandi *et al.*, 1989) with few modifications. RNA from TR66 cells was extracted by using the guanidine thiocyanate method (Chomcynski and Sacchi, 1987), and the first strand cDNA synthesis was made with MuMLV reverse transciptase and oligo(dT)primers by using standard conditions. The second strand synthesis was performed by T4 DNA polymerase and 5′ oligonucleotides 1570 for V_H and 1569 for V_L (Orlandi *et al.*, 1989) sequences in conditions described for site directed mutagenesis (Muta-Gene, Bio-Rad). Final amplification was then achieved by using the above mentioned oligonucleotides and 3′ oligonucleotides 1354 and 1329 complementary to mouse γHC and Cκ genes, respectively at standard PCR conditions (30 cycles) recommended by the manufacturer (GeneAmp DNA amplification kit, Perkin-Elmer Cetus). The L region of Sp6 HC gene (Köhler, 1984) containing the small intron was amplified by using 5′ oligonucleotide 1609 and 3′ oligonucleotide 1610 which is also complementary (20 bp) to oligonucleotide 1570 at its 5′ end (i.e. to the beginning of the amplified V_H and V_L fragment). Partial DNA sequencing of the amplified V_H and V_L fragments showed that they contained JH2

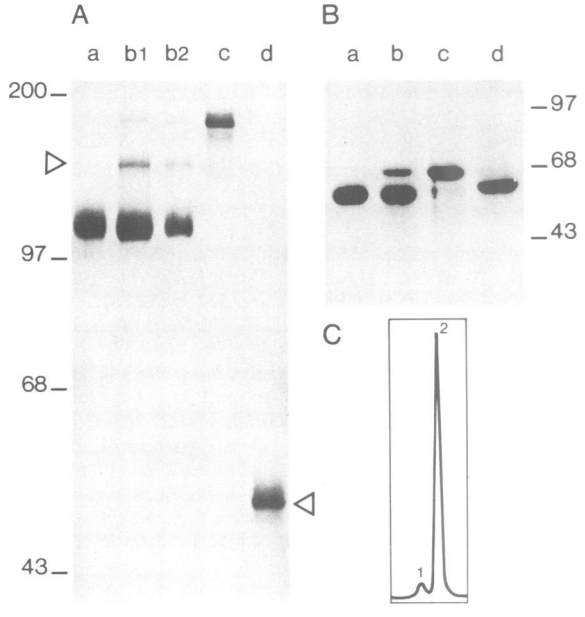

Fig. 2. Characterization of the bispecific molecules. Stable transformants were obtained by transfecting the constructs into J558L myeloma by protoplast fusion. **A** and **B**, analyses of purified proteins by 7% PAGE run under non-reducing (A) or under reducing (B) conditions. a, CD4−Hγ3; b, CD4−Hγ3/FvCD3−Hγ3; c, FvCD3−Hγ3; d, Janusin. Secreted proteins were purified in a single step, either by protein G (a−c), or by anti-κ monoclonal antibody (d) affinity columns. Approximately 5 μg of purified material were loaded and stained afterwards with Coomassie blue, except on lane bl, where 10 μg were analyzed. Arrowheads show the positions of Janusin (~52 kDa) and hybrid CD4−Hγ3/FvCD3−Hγ3 (~120 kDa) molecules in the non-reducing gel (A). Standard molecular weight markers are shown on the left (in thousands). **C**, gel filtration profile of Janusin preparation. FPLC was done by using Superose 12HR 10/60 column in PBS. The numbers indicate the positions of monomers and multimers.

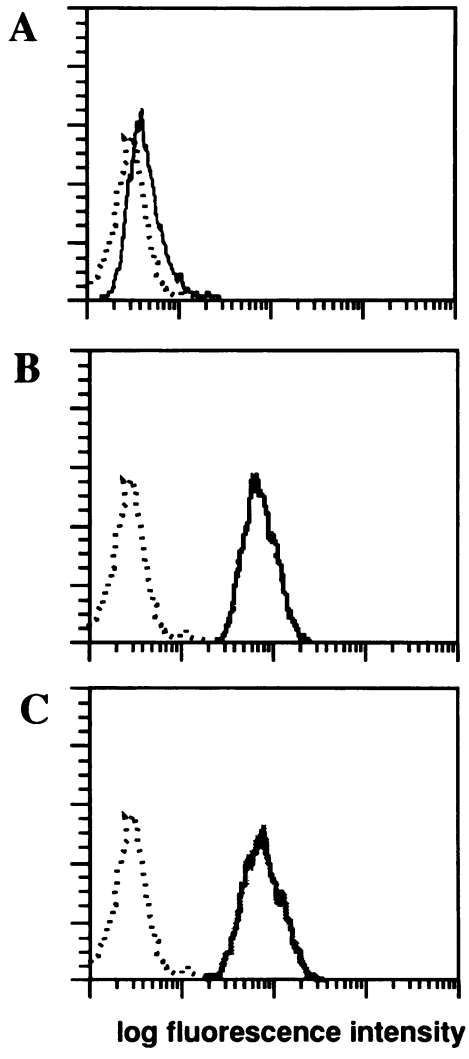

Fig. 3. Recombinant molecules express functional anti-CD3 and CD4 domains. A $CD3^+$, $CD4^-$ T cell clone was incubated with CD4-Hγ3 (**A**, 200 μg/ml), $CD4^-$ Hγ3/FvCD3−Hγ3 (**B**, 200 μg/ml containing all three species), Janusin (**C**, 20 μg/ml) or medium alone, washed and stained with an IgG2a anti-CD4 antibody (10A12), followed by FITC conjugated goat anti-mouse IgG2a. Cells were analyzed on a FACScan (Becton-Dickenson). Dotted line represents the second and third step reagents alone.

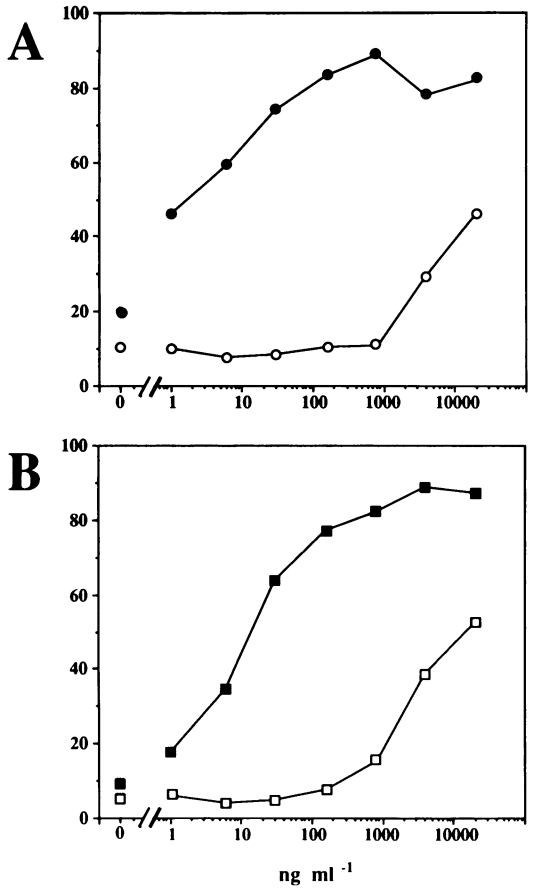

Fig. 4. (CD4−FvCD3) Janusins effectively retarget cytotoxic T cells on HIV-I infected cells. ^{51}Cr-labelled, HIV-I infected (Traunecker *et al.*, 1989) (●, ■) or uninfected (○, □) Jurkat cells were incubated with two independent CD8$^+$ cytotoxic T cell clones (**A,B**) in the presence of different concentrations of Janusin. Random CD8$^+$ clones were isolated from the peripheral blood of a healthy individual using PHA as previously described (Lanzavecchia and Scheidegger, 1987). A 5 h ^{51}Cr release assay was performed as described (Lanzavecchia and Scheideggar, 1987). E:T = 10. Percentage of ^{51}Cr release is plotted as a function of Janusin concentration (ng/ml).

and Jκ5 regions, respectively. This information allowed us to plan appropriate junctional and flanking oligonucleotides which were used to link Sp6L, V_H and V_L fragments together in one 35 cycle PCR reaction (Horton *et al.*, 1989): the flanking 5′ and 3′ oligonucleotides were 1609 and 2768, respectively, the latter having complementary (24 bp) to the end of the mouse Jκ2 sequence. The oligonucleotide 2139 which links V_H to V_L fragments had 20 bp complementarities to the end of the JH2 sequence and to oligonucleotide 1569, i.e. to the 5′ end of the V_L sequence. In addition, the *Sal*I restriction site was designed between these regions of complementarity. All the amplified fragments (Sp6L, V_H, V_L) were mixed with equimolar ratios while the flanking oligonucleotides were in 50-fold excess to the linking oligonucleotide during the PCR reaction. Note that no linking oligonucleotide was necessary between Sp6L and V_H fragments because their primary amplification resulted already to complementary ends. The full length product (PCRl) was then isolated and digested with *Sst*I restriction enzyme (flanking oligonucleotides contained terminal *Sst*I sites) and cloned into similarly digested pCD4−Hγ3. Finally, a synthetic piece of DNA with *Sal*I compatible ends (oligonucleotides 2119 and 2120), corresponding to the linking peptide between V_H and V_L domains, was cloned into a *Sal*I site of this intermediate plasmid (bl) to give the final pFvCD3−Hγ3 construct. The extra 18 amino acids thus introduced between V_H and V_L domains in the final protein have the sequence JH2-VEGGSGGSGGSGGSGGVD-V_L. *pCD4-FvCD3-Cκ (Janusin).* The FvCD3 region was amplified (PCR2) out of the FvCD3−Hγ3 by using oligonucleotide pair 2733 and 2767 which brought in the compatible *Bam*HI and *Hin*dIII restriction sites in addition to the splice donor site. This fragment was then cloned into pHT4−YK12 (Traunecker *et al.*, 1989) as indicated to give the final construct corresponding to Janusin.

Oligonucleotides used:

Oligo 1570: AGGTCAAACTGCAGCAGTCAGG 3′
GCG G T

Oligo 1569: GACATTCAGCTGACCCAGTCTCCA 3′

Oligo 1345: AAATAGCCCTTGACCAGGCATCC 3′

Oligo 1329: GTTAACTGCTCACTGGATCC 3′

Oligo 1609: AGTGTTCCTCTCTACA**GAGCTC**CTGACAACAC-
TGACTC 3′ ***Sst*I**

Oligo 1610: CCTGACTGCTGCAGTTTGACCTGGCAAT-
A C C GC
CGACACCTAT 3′
***Sal*I**

Oligo 2139: TGGAGACTGGGTCAGCTGAATGTC**GTCGAC**-
TGAGGAGACTGTGAGAGTGGTGCC 3′

Oligo 2768: AAGTGGGGIITT**GAGCTC**TTTCAGCTCCAGCTTG-
GTCCCAGC 3′ ***Sst*I**

Oligo 2119: TCGAAGGTGGAAGTGGAGGTTCTGGTGGAAGTG-
GAGGTTCAGGTGGAG 3′

Oligo 2120: TCGACTCCACCTGAACCTCCACTTCCACCAGAACC-
TCCACCTTCCACCT 3′

Oligo 2733: AATCTGAATT**GGATCC**AGGGCCTGAGGTGACA 3′
***Bam*HI**

Oligo 2767: TGTACTTAC/GTTTCAGCTCCAGCTTGGTCCCAGC 3′
SD

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