

## Supplementary Material

### Methods and Patients

#### *GvHD prophylaxis*

All Patients received GVHD prophylaxis with a calcineurin inhibitor, often combined with mycophenolate mofetil. The only exception were patients, who received T cell depleted transplants by means of the CliniMACS device. These patients did not receive any medication as GVHD prophylaxis. In case of an unrelated donor T cell depletion with either alemtuzmab, anti-thymocyte-globuline or CliniMACS device was provided.

#### *Donor characteristics*

39 Patients received a graft from an unrelated donor (34 matched, 5 mismatched unrelated donor) and 26 patients from a sibling donor (21 matched, 4 mismatched, 1 haploidentical donor) and one patient received a graft from a child (haploidentical). The mismatched patients had only one HLA antigen or allele discrepancy compared to the donor.

### Quantitative TREC-analysis

#### *Double Cloning of the Standards*

#### **Primer sequences and genomic DNA**

All primer, specific for either sjTREC, 10 D $\beta$ J $\beta$ TREC (D $\beta$ 1-J $\beta$ 1.1 –1J $\beta$ 1.6 or D $\beta$ 2-J $\beta$ 2.1-2.4) or the CD3 $\gamma$ -chain were taken from the human germline sequence as described by Dion et al.<sup>1</sup> and Poulin et al.<sup>2</sup> (Genbank Accession Number AE000661, U66061 and X06026). For D $\beta$ 1-J $\beta$ 1.1 –1J $\beta$ 1.6 and for D $\beta$ 2-J $\beta$ 2.1-2.4 only one standard has been generated which contains a consensus sequence for the respective target gene. The genomic DNA was taken from healthy volunteers or fetal thymic tissue (BioChain, Hayward, USA).

Primer to generate the cloning product were: (MWG, Ebersberg, Germany):

sj-out5            5'-CTCTCCTATCTCTGCTCTGAA-3'

sj-out3            5'-ACTCACTTTTCCGAGGCTGA-3'

Db1-out5 5'-ACAGTGCCTGGAGGTGAGAA-3'  
Db1-out3 5'-CTCATCTGGGCCTGTCCTTGT-3'  
Db2-out5 5'-CACTGTGACTTTCCCCAAGC-3'  
Db2-out3 5'-TATTCCTTGGAAGCCGAGT-3'  
CD3-out5 5'-ACTGACATGGAACAGGGGAAG-3'  
CD3-out3 5'-CCAGCTCTGAAGTAGGGAACATAT-3'

### Vector construction

The amplification of DNA fragments was performed with the Advantage 2 Kit (BD Biosciences, Palo Alto, USA), concerning the manufacturer's instructions. We used 150 ng of DNA, the primer concentration was adjusted to 10  $\mu$ M. The PCR was performed in a volume of 50  $\mu$ l. An initial denaturation step for 1 min with 95°C, was followed by 30 cycles at 95°C for 30 sec and 68°C for 1 min plus 10 min at 70°C. The product was isolated by 1.5% agarose gel separation with an subsequent extraction from the gel by means of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), concerning the instructions of the manufacturer. As a first step the product amplified from the CD3 $\gamma$ -gene was cloned into the pCR2.1-TOPO-vector using the TOPO TA Cloning Kit (Invitrogen, Grand Island, NY, USA) and was then transformed into TOP10 One Shot Chemically competent E.coli bacteria (Invitrogen). These bacteria were finally plated on agar plates containing ampicillin. Plasmid bearing colonies were picked and transferred to LB medium containing 5mg/ml ampicillin and incubated for at least 20 hours at 37°C with constant shaking. The recombinant plasmid bearing the CD3 $\gamma$ -gene was then isolated using the QIAprep Miniprep Kit (Qiagen, Hilden). The correct length of the cloned fragment was then confirmed by EcoR1 digestion (Boehringer, Germany). For the cloning of the second gene fragment we constructed a new T vector from CD3 $\gamma$ -bearing pCR2.1-TOPO-vector. The first step was a blunt end restriction using EcoRV, followed by a silicagel extraction using the QIAquick 8 PCR Purification Kit (Qiagen, Hilden). Next we established dTTP overhangs using 5  $\mu$ l PCR 10x buffer (Applied Biosystem, Heidelberg, Germany), 1  $\mu$ l dTTP (Amersham Biosciences) and 0,5  $\mu$ l 100mM Taq Polymerase (AmpliTaq, Applied Biosystem). The reaction was incubated for 2 hours at 72°C. After purification the new T vector was solved in 50  $\mu$ l of water. The three different target genes (sjTREC, consensus D $\beta$ 1-J $\beta$ 1.1 –I $\beta$ 1.6 or D $\beta$ 2-J $\beta$ 2.1-2.4) were ligated into the vector by means of the TOPO TA Cloning Kit (Invitrogen). The transformation, bacterial cultivation and plasmid preparation was performed as described above. The length of the fragments was then checked by BstXI (Boehringer,

Germany) digestion and the vectors were sequenced afterwards using the DNA sequencing kit (Applied Biosystem), the CD3 out Primer as well as a T7-Primer (Promega, Mannheim, Germany).

### ***Quantitative Real-time PCR analysis***

#### **Multiplex-PCR**

We performed a two round PCR: A multiplex first round was followed by a nested second round PCR. Primermix sjTREC: sj-out5, sj-out3, CD3-out5 und CD3-out3.

We used the following primer for the D $\beta$  first round-PCR.

Primermix D $\beta$ 1-J $\beta$ 1.1 –1J $\beta$ 1.6: D $\beta$ 1-out 5'-CTCATCTGGGCCTGTCCTTGT-3'  
1.1-out 5'-AACCTAGGACCCTGTGGATG-3'  
1.2.-out 5'-CTCTCTATGCCTTCAATGTG-3'  
1.3.-out 5'-AAGGGAACACAGAGTACTGGAA-3'  
1.4.-out 5'-GGATCACACGGGGCCTAATT-3'  
1.5.-out 5'-GAAACTGAGAACACAGCCAAGAA-3'  
1.6.-out 5'-ATCCTCCCTCTTATGTGCATGG-3'

Primermix D $\beta$ 1- Standard: D $\beta$ 1-out5, D $\beta$ 1-out3, CD3-out5 and CD3-out3.

Primermix D $\beta$ 2-J $\beta$ 2.1-2.4: D $\beta$ 2-out 5'-TATTCCTTGAAGCCGAGT-3'  
2.1.-out 5'-CTCCTCTGCAAATTGGTGGT-3'  
2.2.-out 5'-CACCGTGCTAGGTAAGAA-3'  
2.3.-out 5'-TACTGGGTAAGGAGGCGGTT-3'  
2.4.-out 5'-GGCTGACAGTGCTCGGTAA-3'  
CD3-out5 and CD3-out3

Primermix D $\beta$ 2- Standard: D $\beta$ 2-out5, D $\beta$ 2-out3, CD3-out5 and CD3-out3.

All primers were purchased from MWG, Ebersberg, Germany and were soluted in TE buffer at a final concentration of 100  $\mu$ M. The nucleotides were purchased from Amersham Bioscience, Taq buffer and Taq polymerase from Applied Biosystem, Heidelberg.

Peripheral blood mononuclear cells were isolated from peripheral blood of the patients by Ficoll-Hypaque density gradient centrifugation. The isolation of CD3+ positive cells was performed with magnetic beads from MiniMACS columns (Miltenyi, Bergisch Gladbach, Germany). The genomic DNA was isolated with the QIAamp Blood Kit (Qiagen, Hilden, Germany) as described by the manufacturer. The double cloned standards (see above) were diluted 10 fold in every dilution step

starting from  $10^6$  copies to generate the standard curves. All measurements of the sjTREC were done in duplicates, the  $\beta$ TREC however, in triplicates and the shown values represent the mean value of all measurements of the respective samples.

**Reaction mix: MasterMix 1**

Aqua dest.	15 $\mu$ l
Nucleotide(10mM)	1 $\mu$ l
Primermix 10x	5 $\mu$ l
DNA Template	4 $\mu$ l in H <sub>2</sub> O

**Reaction mix: Master Mix 2**

Aqua dest.	19 $\mu$ l
10xTaq Puffer	5 $\mu$ l
Taq Polym.	1 $\mu$ l

**PCR-Program:**

1. Denaturation	95°	5 minutes
2. Denaturation	95°	30 seconds
3. Annealing	60°	30 seconds
4. Elongation	72°	1 minute
step 2-4	22	cycles
5. 72°	10 min	
6. Final cooling	to 4°	

### ***Quantification via real-time PCR***

In order to quantify the patient samples by means of the Light cycler hybridization technology (Roche diagnostics, Basel, Switzerland) they were diluted after the first-round multiplex PCR. For the sjTREC the dilution step was 1:10 for D $\beta$ TREC it was 1:100. For every PCR product, sjTREC, D $\beta$ 1 and D $\beta$ 2, as well as the CD3 $\gamma$  products the second round of PCR was performed in the same run but in separated reaction tubes as independent experiments.

The quantification was performed using the Light Cycler Fast Start DNA Master<sup>PLUS</sup> HybProbe Kit (Roche). Since both 3' and 5' primers used for the second round PCR for the D $\beta$ 1 and D $\beta$ 2 quantifications are located near the end of the D $\beta$ 1 and D $\beta$ 2 segments, we introduced a control PCR to definitely exclude the coamplification of unarranged loci. For this purpose we paired a 5' primer located (immediately before the breakpoint) in a region which is not excised during TCR

rearrangement (D $\beta$ 1 control-5 or D $\beta$ 2-control5) with the 3' second round primer located in the respective D $\beta$  consensus sequence (D $\beta$ 1-in3 and D $\beta$ 2-in3). We could not detect any significant amplification or impact of the germline DNA, when using the identical hybridization probes.

The following primer were employed in the second round PCRs:

sj-in5	5'-GCTCTGAAAGGCAGAAAGAGG-3'
sj-in3	5'-ACATTTGCTCCGTGGTCTGTG-3'
CD3-in5	5'-TGGCTGTCCTCATCCTGG-3'
CD3-in3	5'-CTTGGCCTATGCCCTTTTGG-3'
D $\beta$ 1-in5	5'-TGATTCAACTCTACGGGAAACC-3'
D $\beta$ 1-control5	5'-GCTGTAACATTGTGGGGACAG-3'
D $\beta$ 1-in3	5'-ATCTGGGCCTGTCCTTGT-3'
D $\beta$ 2-in5	5'-TTCAGGTAGAGGAGGTGCTTTT-3'
D $\beta$ 2-control5	5'-TGGTGTAACATTGTGGGGACT-3'
D $\beta$ 2-in3	5'-GACAGGGAGATGGACAGGTG-3'

The following hybridization probes (HS) were employed in the analysis:

sj-HS1	5'-TCACAGCTATCCCAAGCCCCACGCTGACG--FL-3'
sj-HS2	5'-LCRed640-ATCACGGCCGAAAACACACTCTGATGCCA--PH
CD3-HS1	5'-GGCTGAAGGTTAGGGATACCAATATTCCTGTCTC--FL-3'
CD3-HS2	5'-LC705-CTAGTGATGGGCTCTTCCCTTGAGCCCTTC--PH
D $\beta$ 1-HS1	5'-GCAATTTGGTTCAGAATGCCTCTGCCTC--FL-3'
D $\beta$ 1-HS2	5'-LCRed640-CTCCTGATCACATGTCAGACCAAGACTGTG--PH
D $\beta$ 2-HS1	5'-TGGGAAGAGTTCTCTGGGGCTGGTCCCAG--FL-3'
D $\beta$ 2-HS2	5'-LCRed640-TGTGGTCTTGCAGGGTCCCCCAACCCAGCGA--PH

All primer were again purchased from MWG (Ebersberg, Germany) and soluted in TE buffer at a concentration of 100  $\mu$ M. For the real-time PCR the primer for sjTREC, D $\beta$ 1 and CD3 were diluted to 40  $\mu$ M, for D $\beta$ 2 at 20  $\mu$ M in TE. The hybridization probes were purchased from TIB-Molbiol (Berlin, Germany) and diluted in TE to a concentration of 8  $\mu$ M.

Reaction mix:	H <sub>2</sub> O.	9 $\mu$ l
	Primer 3'	0,5 $\mu$ l
	Primer 5'	0,5 $\mu$ l
	HS1	0,5 $\mu$ l

HS2	0,5 µl
H-Mix	4µl
Template	5µl

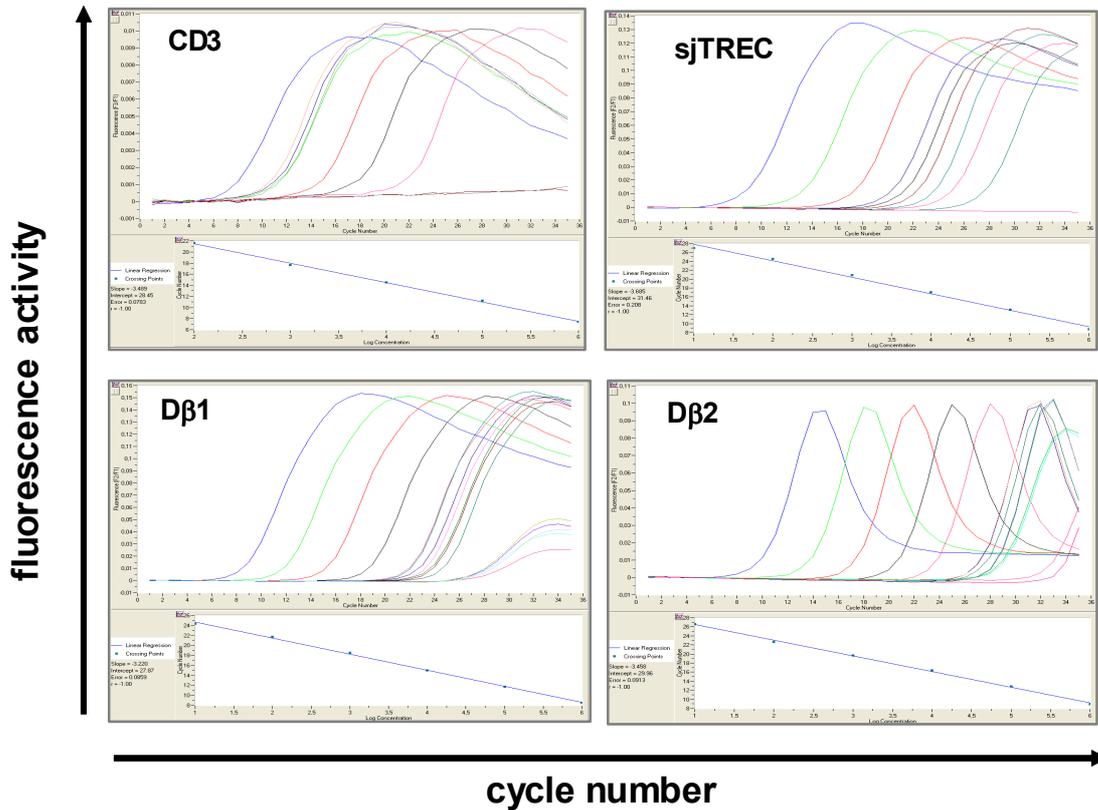
PCR-Program Light-cycler:	1. Denaturation	95°	10 minutes
	2. Denaturation	95°	10 seconds
	3. Annealing	60°	10 seconds
	4. Elongation	72°	25 seconds
	step 2-4		35 cycles

The number of the  $\beta$ TREC was calculated as 1.3 fold the sum of the two D $\beta$ TREC (D $\beta$ 1 + D $\beta$ 2), similar as described <sup>1</sup>. A serial dilution of this control vectors and subsequent amplification and quantification is shown in Figure 1 of the supplementary material. The slope varied between -3.685 and -3.220 corresponding to an overall reaction efficacy of 1.86 and 2.0 and the regression coefficient of the control vector dilution remained at least 0.99 without deleting any dilution step. Since we omitted the separate analysis of each single  $\beta$ TREC gene we lost the ability to screen for diversity, however it was possible to assess sjTREC and  $\beta$ TREC quantitatively with a reduced number of total PCR reactions. The reproducibility of our modified assay was checked by a crossing point analysis. The mean standard deviation in at least 5 independent measurements was 0.52% for CD3, 0.38% for sjTREC, 0.35% for D $\beta$ 1 and 0.31% for D $\beta$ 2 respectively, about a measurement range from 10<sup>5</sup> to 10<sup>1</sup> copies. Since we used D $\beta$ -consensus sequences our modified assay employes 8 PCR reactions less in the first preamplification round and 16 PCR reactions less in the second round of amplification.

## References for the Supplementary Material

1. Dion ML, Poulin JF, Bordi R, Sylvestre M, Corsini R, Kettaf N, et al. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. *Immunity*. 2004;21:757-768.
2. Poulin JF, Sylvestre M, Champagne P, Dion ML, Kettaf N, Dumont A, et al. Evidence for adequate thymic function but impaired naive T-cell survival following allogeneic hematopoietic stem cell transplantation in the absence of chronic graft-versus-host disease. *Blood*. 2003;102:4600-4607.

Figure 1



**Figure 1. Supplementary Material. Amplification characteristics of the four target genes.** Original light cycler plots for each of the four target genes. All genomic sequences were individually cloned into a pCR 2.1-TOPO vector together with the CD3 $\gamma$  amplification product. All plots show a serial dilution ranging from  $10^6$  or  $10^5$  to  $10^1$  or 1 respectively as well as 2-4 individual samples of healthy volunteers.