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Cellular Calibrators to Quantitate T Cell Receptor Excision Circles (TRECs) in Clinical Samples

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Abstract

T cell receptor excision circles (TRECs) are circular DNA molecules formed during rearrangement of the T cell receptor (TCR) genes during lymphocyte development. Copy number of the junctional portion of the δRec-ψJα TREC, assessed by quantitative PCR (qPCR) using DNA from dried blood spots (DBS), is a biomarker for newly formed T cells and absent or low numbers of TRECs indicate SCID (severe combined immunodeficiency) or T lymphocytopenia. No quantitation standard for TRECs exists. To permit comparison of TREC qPCR results with a reliable method for counting TRECs across different laboratories, we sought to construct a stable cell line containing a normal human chromosomal constitution and a single copy of the TREC junction sequence. A human EBV (Epstein Barr virus) transformed B cell line was transduced with a lentivirus encoding mCherry fluorescence, puromycin resistance and the δRec-ψJα TREC sequence. A TREC-EBV cell line, with each cell carrying a single lentiviral insertion was established, expanded and shown to have one TREC copy per diploid genome. Graded numbers of TREC-EBV cells added to aliquots of T lymphocyte depleted blood showed TREC copy number proportional to TREC-EBV cell number. TREC-EBV cells, therefore, constitute a reproducible cellular calibrator for TREC assays, useful for both population-based screening for severe combined immunodeficiency and evaluation of naïve T cell production in clinical settings.

Keywords

TREC; SCID; newborn screening; calibrator; test standardization; primary immunodeficiency

1. Introduction

T cell receptor excision circles (TRECs) are circular molecules formed from DNA excised during the T cell receptor (TCR) gene rearrangement process that generates a repertoire of T cells with diverse antigen specificities [1–3]. An intermediate rearrangement event in most developing T cells destined to express the αβ TCR results in the deletion of the TCRδ gene

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that lies 5′ to the TCR Jα region, generating a δRec-ψJα TREC (signal joint TREC), thereby ensuring that α and δ TCR protein chains are never co-expressed on the same T cell [3]. TRECs are stable, but are not replicated in mitosis as T cells undergo peripheral expansion. Thus the TREC number is a direct reflection of the emergence of newly formed T cells from the thymus. TRECs are most abundant in the peripheral blood of healthy newborns and become less so in older individuals. TRECs are deficient in individuals with T lymphocytopenia [4], which means that a quantitative PCR (qPCR) reaction across the joined ends of the δRec-ψJα TREC sequence, as described by Douek, *et al.* [5] can be used to distinguish healthy individuals from those with pathologic T lymphocytopenia. In particular, DNA from infant dried blood spots (DBS) that is deficient in TRECs may indicate clinically significant T lymphocyte compromise [6].

Severe combined immunodeficiency (SCID) comprises a collection of profound genetic defects of both cellular and humoral immunity [7–9]. Infants with SCID develop fatal infections unless immune function is restored [10] by bone marrow transplantation, enzyme replacement therapy or gene therapy ([11–14], reviewed in [15]). Because all infants with typical SCID fail to produce a diverse repertoire of peripheral T cells from their thymus, their blood is lacking in TRECs. SCID can therefore be detected in newborns by screening routinely collected DBS for TREC copy number. In addition, other newborn conditions with low numbers of T cells are also revealed by TREC screening. Furthermore, later in life, serial measurement of TRECs by qPCR can be used to document the status of thymic production of T cells in a variety of patients, such as those who have undergone hematopoietic cell transplantation (HCT) or other interventions such as treatments for malignancy or HIV infection ([1, 5, 16] and reviewed by [17, 18]).

TREC assays have become the method of choice for high throughput inexpensive screening for SCID [19–22]. In 2010, SCID was judged to meet the criteria for inclusion in the nationally recommended uniform newborn screening panel in the U.S. [23]. With compelling evidence from pilot studies and population-based data from states such as Wisconsin, Massachusetts and the Navajo Nation [21, 24–26, J. Puck, unpublished data], several states have initiated TREC screening of all newborns. TREC quantitation for each DBS DNA sample is arrived at by comparing its qPCR measurement to those of a calibration curve that comprises dilutions of a DNA plasmid containing the δRec-ψJα TREC sequence. As a control to avoid false positive results from unsatisfactory samples, DBS DNA is also used to amplify a genomic DNA segment, such as from the β-actin or *RNaseP* genes.

Each laboratory has had to adapt and optimize the PCR detection of TRECs in DNA prepared from DBS samples. However, the laboratory developed tests currently in place in newborn screening programs show significant variation in reported TREC copy number, despite consistent accurate characterization of proficiency test samples from the U. S. Center for Disease Control and Prevention (CDC) by these laboratories. Thus standardized interpretation of the TREC test is problematic. For example, while the California and Massachusetts newborn screening programs have each identified several infants with SCID and consistently characterize the “SCID-like”, “not SCID” and “SCID unsatisfactory” CDC proficiency samples accurately, California reports TREC copy numbers per μl of whole blood that are about 6-fold lower than those reported by Massachusetts (R. Vogt, CDC, unpublished data). Therefore, a calibration technique that can be validated by a method that is independent of qPCR is required; a stable cell line that can be used to make cellular calibrators could be suitable to standardize TREC testing. Good calibrators would not only allow consistent measurement of TREC copy number but would also be amenable to high volume production for distribution to all laboratories carrying out the TREC qPCR.

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An immortalized cell line with a normal complement of human genomic DNA and single copy of the δRec-ψJα TREC junction sequence could be suitable to generate reliable calibrators to establish uniformity in TREC testing.

2. Materials and methods

2.1 Transduction of an EBV transformed B cell line from a healthy donor

The δRec-ψJα TREC sequence [6] was released from a TOPO-blunt plasmid by digestion with *NotI* and *BamH1* restriction enzymes and then ligated into the lentiviral vector MP-283: pSicoR-BstXI-EF1a-puro-T2A-mCherry, (kindly provided by Michael McManus through the Lentiviral RNAi Core, UCSF, San Francisco, CA). MP-283-TREC lentiviral supernatant was harvested after transient transfection of 293 cells in DMEM medium with 10% fetal calf serum (FCS); lentivirus dilutions transduced into NIH3T3 cells were titered by flow cytometry to detect mCherry fluorescence and used to transduce an EBV B cell line from a healthy donor at m.o.i of 5 in a 6-well plate. The EBV cells and supernatant with 8 μg/ml of polybrene were centrifuged at 1800 rpm for 90 m at 37°C (adapted from [27, 28]). The co-culture was incubated overnight at 37°C in 5% CO_2, following which the EBV cells were washed and resuspended in RPMI 1640 medium with GlutaMax (Invitrogen), 20% FCS, penicillin, streptomycin, and 5 μg/ml puromycin to select transduced cells. A sham control was prepared as above, but with no lentivirus. Ten days post transduction; genomic DNA was prepared and tested for incorporation of the δRec-ψJα TREC sequence by PCR as described [5].

2.2 Isolation of a clone with a single copy of the δRec-ψJα TREC sequence

The transduced EBV cells were assessed for mCherry fluorescence, and positive cells were sorted using a BD FACS Aria II (BD Biosciences). Flow data were analyzed with FlowJo software (Tree Star Inc Version 9.4.1). The sorted cells were expanded and metaphase preparations were subjected to karyotype confirmation and fluorescence in situ hybridization (FISH) with the MP-283-TREC plasmid labeled with rodamine (red) as a probe (as described previously [29]). The bulk culture was further sorted based on mCherry fluorescence intensity to obtain a clonally pure cell population.

2.3 Ratio of genomic DNA to TREC insertion copy number in the TREC-EBV cell line

2.3.1 DNA extraction—Genomic DNA was prepared from 1×10^5 to 6.25×10^3 TREC-EBV cells (counted using a Vi-Cell automated cell viability analyzer, Beckman Coulter, Inc.) using the Gentra Puregene DNA isolation kit (Qiagen). A 16 h incubation with Proteinase K (20 mg/ml) at 65°C was used to maximize DNA yield from small cell numbers. The resulting DNA pellets were resuspended in 50 μl of hydration buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0, Qiagen). Quantitative PCR was carried out with 1/10 of the extracted material.

2.3.2 Relative quantification by qPCR for ratio determination—The TREC transgene was detected using primers and probe as reported by Harris *et al* [30], on an ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City, USA). The reaction mixture contained 10 μl of 1X Taqman Universal Master Mix (Applied Biosystems, Inc.), 0.5 μM of each TREC primer, 150 μM FAM-TAMRA probe, 0.04% BSA (New England Biolabs), 5 μl of template DNA and nuclease free water to 20 μl. PCR thermal profile was: 50°C for 2 m, 95°C for 5 m, followed by 40 cycles (95°C for 30 s, 60°C for 60 s). Reactions were run in duplicate in 384-well plates. TREC copy number per cell was determined by parallel amplification of the *RNaseP* gene, using the TaqMan *RNaseP* detection reagent (FAM) (Applied Biosystems, Inc.).

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The ratio of the copy numbers of the TREC transgene was compared to that of *RNaseP* by the comparative Ct method for relative quantification, using the modified Livak method [31] and the following equation:

$$\text{Ratio (target:reference)} = 2^{-\Delta Ct} = 2^{-(Ct\ \text{target} - Ct\ \text{reference})}$$

Equivalent amplicon sizes (≈80 bp), linearity (r >0.99) and slopes between 3.3–3.6 were obtained in serial dilutions of DNA from the TREC-EBV cell line, thereby ensuring similar amplification efficiencies for both, *RNaseP* and TREC.

2.4 Preparation of TREC-EBV reconstituted T cell depleted blood (TREC-EBV blood-based calibrators)

ACD anticoagulated blood from a healthy donor was centrifuged at 1500 rpm for 15 m, and the platelet-rich plasma was reserved. The original blood volume was restored with PBS with 0.1% BSA, and the cells were spun over a Ficoll gradient (MP Biomedicals). The granulocyte/erythrocyte rich pellet was retained; while the mononuclear fraction was incubated with anti-CD3 coated microbeads and passed twice over an automatic magnetic separation system (AutoMACS, Miltenyi Biotech) to deplete T cells. The T-depleted mononuclear fraction was added back to the granulocyte/erythrocyte pellet and platelet-rich plasma from the original centrifugation. The resulting blood was stained with antibodies against CD45, CD3, CD19 and CD16/56 labeled with PerCP, FITC, APC and PE respectively (BD Biosciences) and analyzed by flow cytometry to confirm T cell depletion. The T-depleted blood contained < 0.5% T cells (Suppl fig 1).

TREC-EBV cells in two-fold gradations were added to aliquots of T depleted blood, yielding TREC-EBV reconstituted T depleted blood (TREC-EBV blood-based calibrators). Absolute numbers of TREC-EBV cells added to each aliquot were determined using Trucount tubes (BD Biosciences), using the formula:

$$\text{Cell count}/\mu 1 = \frac{\#\ \text{events in cell population}}{\#\ \text{events in absolute count bead region}} \times \frac{\#\ \text{beads/test}}{\text{volume}}$$

where, # events in cell population was the number of mCherry positive cells, # events in absolute count bead region was the number of PE^+, $FITC^+$ Trucount beads acquired, # beads/test was the value from the BD Trucount tube pouch label, and volume was 50 μl. Untreated EBV cells (6000 per μl) were also added to the T depleted blood in amounts required to equalize the total genomic DNA in all samples. The samples were mixed thoroughly and 50 μl was spotted onto standard filters (Whatman source number 903), which were dried and stored at −20°C. Table 1 shows the constitution of the TREC-EBV blood-based calibrators.

2.5 DNA extraction from dried blood spots of the TREC-EBV blood-based calibrators

Disks, 3.2 mm in diameter, corresponding to ~3 μl of blood, were punched from each DBS into 96-well deep plates. Automated DNA extraction was performed (Autogen 965), using an overnight 0.5 mg/ml proteinase K digestion, with constant shaking at 65°C, phenol-chloroform extraction, and precipitation at high salt concentration with isopropanol. DNA was washed twice with 70% ethanol, resuspended in 50 μl of elution buffer (10mM Tris, 0.1mM EDTA, pH 8.0) and stored in adhesive sealed plates at 4°C.

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2.6 Quantitative PCR measuring the δRec-ψJα TREC sequence in dried blood spots of TREC-EBV blood-based calibrators

Quantitative PCR for the δRec-ψJα TREC sequence used primer, probe and control plasmid sequences of Douek *et al* [5]. Each 20 μl duplicate reaction contained 5 μl of calibrator DNA extract (~20 ng) TaqMan 2X PCR Master mix (Applied Biosystems), 40X BSA, 500 nmol/l forward and reverse primers and 150 nmol/l probe described previously [1, 6]. PCR conditions were 95°C for 5 m, 40 cycles (95°C for 30 s, 60°C for 1 m) (7900HT Prism Sequence detection system, Applied Biosystems). An amplification control targeting the β-actin gene segment was used as previously described [6], with the modification wherein each reaction contained 5 μl of template DNA. TREC copy numbers were calculated relative to calibration curves generated from serially diluted plasmids included in each run. TREC plasmid dilutions were 10^6, 10^5, 10^4, 10^3, 10^2, 50 and 25 TREC copies per reaction.

3. Results

3.1 Generation of a clone with a single copy of the δRec-ψJα TREC insert

Ten days post transduction of the EBV cell line with MP-283-TREC lentivirus, mCherry fluorescent transduced cells were bulk sorted (Figure 1A) and expanded. PCR analysis of genomic DNA from these cells revealed the δRec-ψJα TREC sequence, absent in sham transduced EBV cells (Figure 1B). Sanger sequencing of the PCR product confirmed the presence of the TREC construct (data not shown). The expanded cells were karyotyped and analyzed by FISH to determine the number of copies of the δRec-ψJα TREC sequence inserted (Figure 1C). Two observed patterns of lentivirus insertion included one with a single TREC signal in chromosome 4 and the other with multiple signals of varying intensity on chromosomes 4 and 15. To isolate the transduced cells with a single insertion, the $mCherry^{lo}$ cells were sorted from the broad positive fluorescent peak (Figure 2A), expanded and analyzed by FISH. Only a single locus of insertion was seen in each of > 20 metaphases and 400 interphases (Figure 2B, and data not shown). Re-analysis by flow cytometry of this population, termed E1, revealed a narrow peak of mCherry fluorescence equal to that of the cells of low fluorescence from the original mixed population (Figure 2C). These were expanded under puromycin selection to generate the TREC-EBV cell line. After more than 20 passages, analysis by flow cytometry still showed the presence of mCherry positive TREC-EBV cells and no contaminating, mCherry negative, non-transduced EBV cells (Suppl fig 2). TREC copy number verification by qPCR also remained unaffected following multiple passages (data not shown). Therefore, the TREC insertion was stable and did not affect growth or viability of the cell line, making it amenable to high volume production for distribution.

To confirm a single TREC insert per cell, qPCR reactions for the TREC lentiviral insertion and for *RNaseP* were carried out using genomic DNA from measured cell numbers of the TREC-EBV line. A TREC/*RNaseP* ratio of 0.41 ± 0.05 (Table 2) was observed, which is consistent with hemizygosity for the TREC insert as compared to the two copies of *RNaseP* per diploid genome.

3.2 Comparison of TREC-EBV blood-based calibrators with plasmid calibrators

The TREC-EBV blood-based calibrators (prepared from known graded numbers of TREC-EBV cells in T depleted blood and spotted onto filter paper) were assayed for the δRec-ψJα TREC insert copy number by qPCR. In the same run, a calibration curve comprising a set of the commonly used plasmid calibrators showed TREC copy numbers proportional to TREC-EBV cell numbers (Figure 3). Thus the TREC-EBV cells could be used to prepare a series of cellular calibrators with known numbers of TREC copies to undergo the same purification and analysis steps as DBS samples.

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4. Discussion

This work describes the development of a stable cell line that can be used to generate cell-based calibrators to determine absolute copy numbers of TRECs in DNA extracted from complex cellular mixtures, including DNA extracted from dried blood spots. An EBV B cell line was chosen as the host for transduction because these cells generally maintain a normal complement of human chromosomes. These B cells are unlikely to have rearranged their TCRs to form TRECs, and in any case after several passages would have lost through dilution any excision circles arising from receptor rearrangement events. Selection of transduced cells by mCherry fluorescence, verification of a clone with a single locus of insertion by FISH and karyotyping, and maintenance in puromycin selection assured that our cellular calibrator retained the characteristics of a normal diploid chromosomal constitution and a single δRec-ψJα TREC insertion locus per cell. The mean TREC:*RNaseP* ratio of 0.41 supports the premise that not only is there a single site of insertion, but that each cell has only one copy of the TREC sequence for the for every two copies of the diploid gene *RNaseP*.

Assays for the copy number of TRECs in peripheral blood are now conducted in clinical and research laboratories to monitor naïve T cells emigrating from the thymus (reviewed by [18, 22]). Detecting absence of TRECs in newborns by population-based screening of their routinely collected DBS permits early diagnosis of SCID from otherwise unsuspected and asymptomatic newborns, making possible prompt intervention to prevent severe infections and provide definitive treatment with optimal outcome [32].

One of the challenges for clinicians and researchers working with newborn screening programs for SCID has been to minimize the variation in absolute TREC copy numbers reported and establish consistent results in different laboratories across the country. Preparation of the calibration curve for the TREC qPCR using a plasmid requires calculation of copy numbers from the concentration of DNA in the plasmid preparation and molecular weight of the plasmid. However, sources of plasmid DNA vary and concentration of the plasmid may be overestimated due to contaminating *E. coli* genomic DNA. Furthermore, other impurities, present in variable amounts based on the methods of preparation of the plasmid, may also alter the efficiency of plasmid TREC amplification in the qPCR reaction and the dilution technique must be precise for every calibration curve preparation. Therefore the calibration curve is likely to vary between laboratories and between different batches of the control plasmid, resulting in different TREC copy number readouts for the same DBS sample. Given the expected inherent variability of the DBS sample itself, minimizing other sources of variability is important.

Use of the TREC-EBV cells will permit a more consistent and replicable calibration curve. TREC-EBV blood-based calibrators permit copy numbers to be measured by an independent method (cell counting) in addition to qPCR. They can be applied to filter paper and processed alongside unknown clinical samples, providing standardized DBS that very closely resemble the patient DBS samples being analyzed. Such TREC-EBV blood-based calibrators reflect the various steps from application of liquid blood to filter paper up to and including the qPCR assay. Storage by cryopreservation and propagation of the TREC-EBV line are straightforward, with puromycin selection ensuring that every cell retains its copy of the TREC sequence. Reconstitution of T depleted blood with graded numbers of TREC EBV cells is also straightforward and makes feasible the generation of large stocks of standardized calibrators that can be provided as dried blood spots or as DNA. Therefore, the obstacle in establishing reproducible results between laboratories, or within laboratories over time can be overcome with the availability of this cell line for cellular calibrators. Importantly, because the SCID newborn screening methodology depends on identifying an

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absence or low copy number of TRECs in an affected infant's sample, calibration curves made of cellular calibrators afford the high throughput newborn screening laboratories a significant level of consistency and increase the specificity of the test as an indicator of immune function. Finally, potential contamination of DBS samples by plasmid DNA, which could generate a false negative result in the DBS of an infant with SCID, can be avoided by use of cellular calibrators rather than a plasmid calibration curve.

Improved test performance will increase the utility of measuring TRECs, not just to screen DBS for T lymphocytopenia, but also to assess status of T cell production in other clinical settings. Monitoring TRECs as a biomarker for T cell reconstitution following hematopoietic cell transplantation from bone marrow or cord blood donors will assist in development of better transplantation protocols. Similarly, TRECs can guide T cell ablation during immunosuppressive chemotherapy and demonstrate T cell recovery after anti-retroviral therapy for HIV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

TREC

TCR

DBS

SCID

HCT

EBV

TREC-EBV

FISH

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Highlights

- T cell receptor excision circle (TREC) count by PCR shows thymic output of T cells.
- An EBV B cell line was made with a single copy of the TREC sequence.
- Graded TREC-EBV cell numbers added to T-depleted blood constituted calibrators.
- TREC copy number was proportional to the number of TREC-EBV cells.
- TREC-EBV cells are as a universal cellular calibrator for TREC assays.

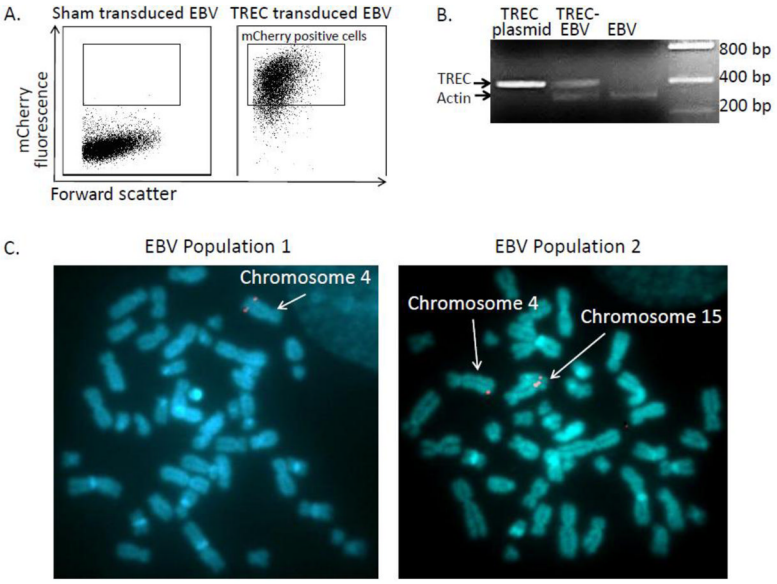

Figure 1.

A, Bulk sorting of MP-283-TREC transduced EBV cells for mCherry expression. Sham transduced cells showed no mCherry fluorescence.

B, Duplex PCR analysis for δRec-ψJα TREC and genomic actin sequences using the following templates: left, TREC plasmid; center, genomic DNA of TREC-transduced and sham-transduced EBV cells; right, size standards.

C, FISH analysis of MP-283-TREC transduced, bulk sorted cells, using the MP-283-TREC plasmid labeled in red. Two patterns of lentiviral insertion were detected: EBV population E1 with one insertion signal in chromosome 4 (white arrow) and E2 with one signal in chromosome 4 and multiple signals in chromosome 15 (white arrows).

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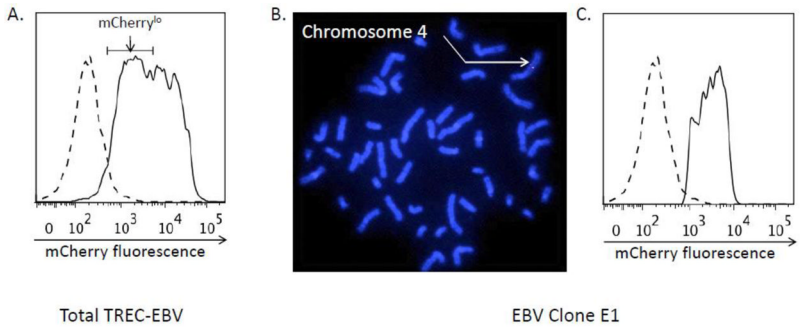

Figure 2.

A, Sorting of mCherry^{lo} cells to isolate cells derived from the population E1, with a single MP-283-TREC insertion site. Cells were stained with DAPI to exclude dead cells.

B, Representative FISH of mCherry^{lo} cells, probed with MP-283-TREC plasmid labeled in red, showing a single MP-283-TREC insert on chromosome 4 (white arrow) and a normal chromosomal constitution.

C, Analysis by flow cytometry of sorted mCherry^{lo} cells, confirming mCherry fluorescence at the lower range of the brightness of the original mixed population.

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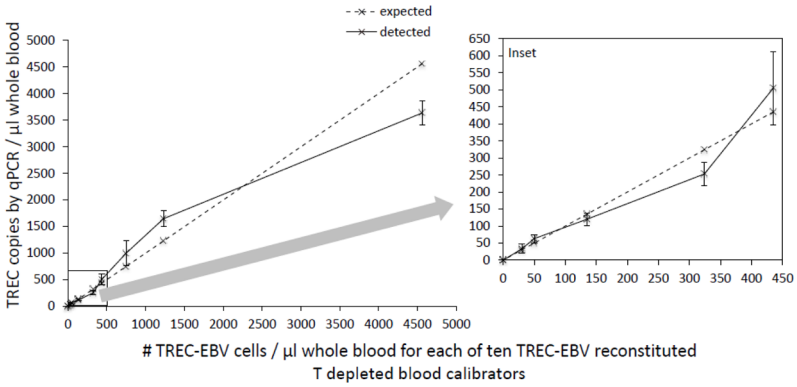

Figure 3.

Proportionality between the TREC-EBV cell numbers, obtained using Trucount beads, added to TREC-EBV reconstituted T-cell depleted blood, applied to filter paper and measured copy number of the δRec-ψJα TREC sequence as detected by quantitative PCR that used plasmid calibrators. Dashed line: expected copy numbers of the TREC sequence; solid line: measured copy numbers. Mean ± SD of 3 DNA extractions and duplicate quantitative PCR reactions for each of the ten calibrators.

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Table 1

Constitution of TREC-EBV blood-based calibrators and projected copy numbers

Estimated number of TREC-EBV cells per μl of T depleted blood	Number of TREC-EBV cells per μl obtained from TruCount tubes	Number of TREC sequences in each 3 μl dried blood spot punch, corresponding to 50 μl DNA	Expected TREC copy number per reaction
4000	4560	13680	1368
2000	1230	3690	369
1000	748	2244	224
500	435	1305	131
250	324	972	97
125	135	405	41
63	50	150	15
32	30	90	10
0	0	0	0

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Table 2

Ratio of expression levels of TREC and *RNaseP* calculated by the comparative Ct methods, obtained by quantitative PCR carried out using genomic DNA extracted from fixed numbers of TREC-EBV cells.

TREC-EBV cell number	qPCR determined Ct		TREC/*RNaseP* ratio
	TREC	*RNaseP*	
100,000	28.25	27.19	0.48
75,000	28.37	27.24	0.46
50,000	28.74	27.41	0.40
25,000	29.67	28.37	0.41
12,500	30.58	29.30	0.42
6,250	32.35	30.74	0.33
Average			0.41
Standard deviation			0.05