Journal of Immunological Methods xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Measurement of absolute T cell receptor rearrangement diversity

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ARTICLE INFO

Article history:
Received 6 November 2010
Received in revised form 21 January 2011
Accepted 1 March 2011
Available online xxxx

Keywords:
T cell receptor
Diversity
Rearrangement
Sequence
Repertoire
AmpliCot

ABSTRACT

T cell receptor (TCR) diversity is critical for adaptive immunity. Existing methods for measuring such diversity are qualitative, expensive, and/or of uncertain accuracy. Here, we describe a method and associated reagents for estimating the absolute number of unique TCR Vβ rearrangements present in a given number of cells or volume of blood. Compared to next generation sequencing, this method is rapid, reproducible, and affordable. Diversity of a sample is calculated based on three independent measurements of one Vβ–Jβ family of TCR rearrangements at a time. The percentage of receptors using the given Vβ gene is determined by flow cytometric analysis of T cells stained with anti-Vβ family antibodies. The percentage of receptors using the Vβ gene in combination with the chosen Jβ gene is determined by quantitative PCR. Finally, the absolute clonal diversity of the Vβ–Jβ family is determined with the AmpliCot method of DNA hybridization kinetics, by interpolation relative to PCR standards of known sequence diversity. These three component measurements are reproducible and linear. Using titrations of known numbers of input cells, we show that the TCR diversity estimates obtained by this approach approximate expected values within a two-fold error, have a coefficient of variation of 20%, and yield similar results when different Vβ–Jβ pairs are chosen. The ability to obtain accurate measurements of the total number of different TCR gene rearrangements in a cell sample should be useful for basic studies of the adaptive immune system as well as in clinical studies of conditions such as HIV disease, transplantation, aging, and congenital immunodeficiencies.

1. Introduction

The repertoire of millions of different T cell receptors (TCRs) is thought to play an important role in protecting humans from infections and cancers, but the large size of the repertoire has made it difficult to study. An inexpensive and rapid method for the quantitative measurement of the absolute number of different TCRβ sequences found in a cell sample would be useful for investigations in clinical and basic immunology. The method would enable a more comprehensive analysis of the development, maintenance, and impact of the TCR repertoire in the setting of clinical conditions such as HIV disease, transplantation, aging, and congenital immunodeficiencies. Absolute diversity measurements would also facilitate mathematical modeling of the development and health of the immune repertoire. At a technical level, a robust method for the measurement of absolute TCR diversity would also inform key aspects of experimental design, e.g., of the number and type of cells that must be sampled so that the full amount of diversity present in given population can be determined.

We present here an approach towards analysis of TCR diversity that is patterned after that of Arstila et al. (2000) who multiplied several parameters to estimate the total repertoire size. In like manner, we combine and validate three independent measurements–of V segment usage, J segment usage, and VJ family sequence diversity–to calculate the diversity of the TCRβ chain repertoire. Instead of direct sequencing of TCRβ chain rearrangements, we use AmpliCot,

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doi:10.1016/j.jim.2011.03.001

a method that uses Cot analysis (DNA hybridization kinetics) to measure the sequence diversity of a PCR amplicon (Baum and McCune, 2006). Using a biological standard of known diversity, we show that the method is accurate, sensitive, and specific. Given this method, it will now be possible to deduce absolute TCR diversity in a manner that is both reproducible and affordable. The absolute measurements shown in this paper provide additional evidence validating the accuracy of AmpliCot measurements of TCR diversity, either for relative or for absolute diversity measurements.

2. Materials and methods

2.1. Cell samples

Whole blood samples were obtained from healthy individuals after obtaining informed consent. Naïve T cell samples were obtained from buffy coats from healthy blood donors (Stanford Blood Bank) enriched for T cells with a negative-selection rosetting kit (Rosette-Sep, Stem Cell). $CD3^+CD4^+CD45RA^+CCR7^+CD57^-$ T cells were then sorted to >99% purity on an Aria cell sorter (BD Biosciences). The number of sorted events agreed well with manual counting of the cells with a hemocytometer. The cell viability exceeded 95% as assessed with trypan blue exclusion. Vβ gene usage in this naïve cell population was determined by further FACS analysis using anti-Vβ antibodies (see Table 1).

2.2. RNA preparation and reverse transcription

Cells were centrifuged at 3000 rpm × 10 min at 4 °C. RNA was prepared using the RNeasy Plus kit (Qiagen). The cell pellets were resuspended in buffer RLT plus, vortexed for 2 min, and then frozen at −80 °C until RNA preparation was performed. RNA yields were measured with a Nanodrop spectrophotometer, and quality was analyzed with the Agilent Bioanalyzer, yielding RNA Integrity Numbers >8.0, and minimal genomic DNA contamination peaks. RNA was reverse transcribed with the Omniscript enzyme (Qiagen) and an oligo dT_{15} primer (Promega), according to manufacturer's directions.

2.3. Quantitative PCR

Plasmid standards for quantitative PCR were made by amplifying TCR cDNAs with Vβ and Cβ primers (Integrated DNA Technologies; see Table 2 for sequences) and then cloning the PCR products into the T vector pCR2.1 (Invitrogen). Specific V–J–C rearrangements were identified through sequencing. Plasmid templates of known mass (determined through spectrophotometry) were digested with EcoRI and then serially diluted in the presence of sheared salmon sperm DNA carrier. The V–J–C plasmids were used as absolute PCR standards to determine yields of TCR cDNA. The plasmid clones were also used as calibrators for determining the proportional use of a particular V–J pair among all rearrangements bearing a selected Vβ gene, since by definition they contained a 1:1 ratio of V–C to V–J templates. Serial dilutions of the plasmid template were run with every experiment to provide actual amplification efficiency measurements for use in relative abundance calculations with a modification of the Pfaffl equation (Pfaffl, 2001). All real time PCR reactions used SYBR Green hot start mastermix (MC Lab) and were incubated at 95 °C × 10 min, followed by 35 cycles of 95 °C × 15 s, 60 °C × 30 s, and 72 °C × 30 s in an MJ Opticon 1 (Bio-Rad).

2.4. AmpliCot

AmpliCot measurements were performed as previously described (Baum and McCune, 2006), except that an extra round of sample PCR was added to increase the concentration of DNA in the annealing reaction and to obviate the need for real-time monitoring of the V–J amplification process. The first round V–J amplification was performed in a 20 μL reaction using 2 μL of V–C amplification product template for 12 cycles. This product was diluted 1:10 into a second round V–J amplification reaction for 3 cycles. The second round PCR reaction usually had a volume of 100 μL seeded with 10 μL first round PCR product; 25 μL concentrated annealing buffer was then added. In the experiment shown in Fig. 4B, the second round reaction had a volume of 50 uL seeded with 5 μL first round PCR product; 75 μL dilute annealing buffer was then added. (This was done to lower the DNA concentration in the annealing reaction to permit more precise measurement of samples of low diversity). In all cases the volumes of the sample and reference wells during the annealing reaction were 50 μL each, and the final concentrations of buffer components were 250 mM NaCl, 5× SYBR, 20 mM MOPS, pH 7.5, 0.03% Brij-700 and 10 mM EDTA.

Table 1
Overview of parameters. Listed are the measured parameters discussed in this paper (**v**, **j** and **a**) as well as the total TCR β chain diversity (**d**) calculated from these parameters. For each of these, the method used for measurement, the calibration standard used for these measurements (if applicable), the validation "gold standard" used to assess accuracy, and the measured precision (interassay coefficient of variation) are listed.

Parameter	Definition	Method	Calibration standard	Validation "gold standard"	Precision (Coefficient of variation)
v	Fraction of cells using a selected Vβ family	Flow cytometry with anti-Vβ antibodies	None	Quantitative PCR using flow-sorted lymphocytes	NA
j	Fraction of cells using a specific Vβ family that use a selected Jβ family	Quantitative PCR	Plasmid clone containing a single VJC β gene rearrangement	Summation of all j values measured for a single Vβ family	12%
a	Number of different sequences in a selected VβJβ family	AmpliCot (DNA hybridization kinetics)	PCR templates containing known amount of diversity	Titration of a diverse pool of cDNA templates	16%
d	Number of different TCR β chain sequences in a sample	Calculated from a/(v j)	None	Known number of naïve cells entered into assay	20%

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Table 2
TCRβ PCR primers.

Vβ protein	Antibody clone	Vβ gene [a]	Vβ primer sequences
1	BL37.2	TRBV9	out: CTGACTTGCACTCTGAACTAAACC in: CTCAGCTTTGTATTTCTGTGCCAG
2	MPB2D5	TRBV20-1	GCAGCTTCTACATCTGCAGT
3	CH92	TRBV28	out: TTCTGGAGTCCGCCAGCACC in: ATCTATGTACCTCTGTGCCAG

Primer	Sequence
Cβ	CGACCTCGGGTGGGAACAC
Jβ2S1	TGGCCCGAAGAACTGCTCA
Jβ2S2	CTTCTCCAAAAAACAGCTCCC
Jβ2S3	CCTGGGCCAAAATACTGCGT

[a] The correspondence between human Vβ gene and protein nomenclature is according to Rowen et al. (1996).

2.5. AmpliCot sequence diversity standards

Artificial PCR templates of known diversity, as diagrammed in Fig. 1C, were used to calibrate the AmpliCot assay to allow measurement of absolute sequence diversity (Baum et al., 2010). Briefly, oligonucleotide templates containing between 1 and 25,000 sequences were generated by the bulk ligation of different numbers of 6-nucleotide-long "words" at each of three positions. The words were designed to have optimal hybridization characteristics: they had identical GC content and they differed from each other at a minimum of 3 positions, to permit resolution of the various word combinations with stringent hybridization. The ends of the standard library contained Type IIs restriction enzyme sites, allowing ends matching any VβJβ primer pair to be substituted. The resulting standards had melting temperatures within 1 °C of true TCR sequences, permitting them to be amplified and run in the AmpliCot assay alongside lymphocyte cDNA samples.

3. Calculation

AmpliCot can only measure the diversity of a single VβJβ family (approximately 1/200th of the repertoire) at a time. This is because different VβJβ families may require different annealing temperatures to achieve adequate stringency and because measurement of a larger fraction of the repertoire would require higher Cot values than can be conveniently achieved. The number of unique TCR β chain rearrangements in a sample is estimated on the basis of several measured parameters, using the equation:

$$\mathbf{d} = \mathbf{a} / (\mathbf{vj}), \quad (1)$$

where:

d is diversity, or the number of unique TCR β chain rearrangements in the sample. This measurement must be defined in terms of a denominator describing the sample size, such as volume of blood or tissue, or number of cells;

a is the number of unique TCR β chain rearrangements in a specified VβJβ family

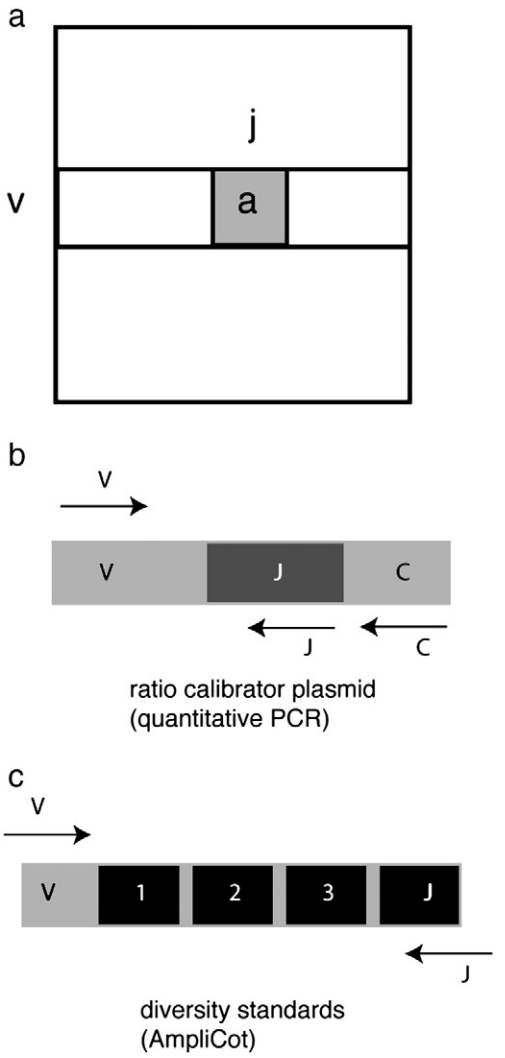

Fig. 1. Overview of method. A. Schematic of method. The area of the large square represents all TCRβ rearrangements in the sample. The small square represents a selected VβJβ family of TCR β chains. **v** represents the proportion of receptors using a single Vβ family and **j** represents the proportion of receptors using the chosen Vβ that also use a selected J. The sequence diversity of the selected VβJβ family, measured with AmpliCot, is represented as **a**. This schematic shows that the method relies on the VβJβ family chosen to be representative of the sequence diversity of the whole sample. If the VβJβ family contains an atypical clonal expansion, then the total TCRβ diversity will be underestimated. B. Quantitative PCR standards for measuring J usage. For each VβJβ family studied, an actual TCRβ sequence was cloned. Because this clone contains a 1:1 ratio of VβJβ to V–C templates, it can be used as a calibrator for determining the ratio of V–J to V–C templates (the parameter **j**) in an unknown sample. C. AmpliCot standards for measuring the sequence diversity of a VβJβ family. PCR-amplified oligonucleotides served as sequence diversity standards made through ligation of "word" oligonucleotides at three positions. The number of words used at each position creates combinatorial diversity of the sequence library. The ends of the library can be replaced with Vβ and J sequences to match any VβJβ pair.

v is the fraction of cells in the sample using the chosen Vβ;

j is the fraction of cells using the specified Vβ that use the chosen Jβ.

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This calculation is represented schematically in Fig. 1 and more details on the parameters measured are provided in Table 1.

This calculation has two implications. First, since the estimate of total diversity is derived from three estimated parameters, the measurement error is a product of that of the component measurements. We discuss the methods of measurement and their accuracy and precision in the Results section 4. Second, this calculation makes the assumption that the repertoire has a fixed relationship between the number of receptors and the diversity of receptors. To the degree that the repertoire contains small numbers of expanded clones in particular VβJβ families, this assumption is not true, and multiple VβJβ pairs must be measured to achieve a reasonable estimate.

4. Results

4.1. Correspondence of antibody staining and primer specificity

We used flow cytometry with Vβ family specific antibodies to measure the percentage of T cells using a particular Vβ family member. To make this approach useful, we first validated that the anti-Vβ staining corresponded precisely with Vβ primer sequences used to amplify members of the same family. We selected three frequently used Vβ families—ones for which a commercially available antibody stained the entire family and for which a PCR strategy could be designed that could amplify all the family members without also amplifying any other Vβ families. The antibody clones and corresponding PCR primers are shown in Table 2. For several Vβ families, nested primers were required to achieve specific amplification of the desired family alone. In these cases, the first-round primer is labeled "out" and the second-round primer is labeled "in." To test the specificity of our primers, $CD3^+$ T cells were stained with several anti-Vβ antibodies and sorted into $Vβ^+$ and $Vβ^-$ fractions. Quantitative PCR was then performed with primers corresponding to that Vβ family and, as shown in Fig. 2, the signal (transcripts/cell) was log orders higher in the $Vβ^+$ compared to the $Vβ^-$ fraction, as expected. It is more challenging to demonstrate that the primers are also sensitive, i.e., to show that they are able to amplify TCR genes from all of the cells staining with a given Vβ antibody. Certainly, some rare TCR gene rearrangements that have extensive deletions of the Vβ genomic sequences will lack the sequences to match our primers. We did find, however, that all of our primers detected equivalent TCR gene copy numbers per $Vβ^+$ cell, which suggests that they all had a similar degree of sensitivity. While this method can be used to measure the frequency of several representative Vβ families, it cannot be used to measure all of them: some Vβ families have antibodies that cannot be precisely matched with specific PCR primers and many Vβ families lack antibodies altogether.

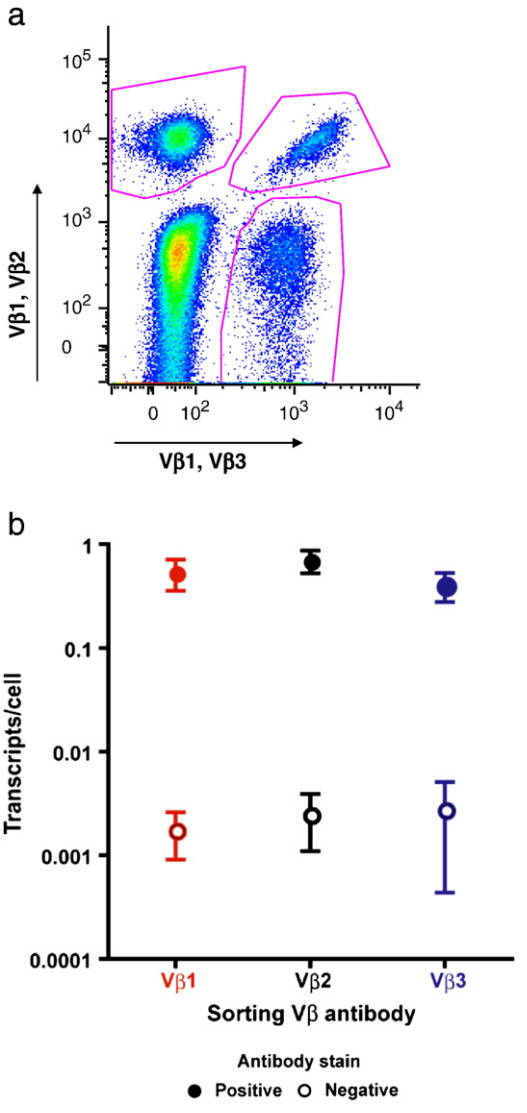

Fig. 2. Correspondence of Vβ antibody specificity and PCR primer specificity. A. Vβ antibody staining. $CD4^+$ naïve T lymphocytes were defined by gating on forward and side scatter, singlet events, and the phenotype $CD3^+CD4^+CD45RA^+CCR7^+CD57^-$. This flow plot shows the staining results for Vβ 1–FITC and PE, Vβ2–FITC, and Vβ3–PE (using the Vβ antibody nomenclature described in Table 2). Cells positive for FITC only are $Vβ2^+$; those positive for PE only are $Vβ3^+$; those positive for both FITC and PE are $Vβ1^+$; those not staining with either color presumably use one of the other Vβ families. B. Proportion of Vβ cDNAs in $Vβ^+$ vs. $Vβ^-$ fractions. Cells staining positive or negative for Vβ1, Vβ2, and Vβ3 antibodies, as shown in panel A, were flow sorted to 99% purity from three donors. Each sample was collected and processed in duplicate. The number of transcripts amplifying with the corresponding Vβ primers listed in Table 2 was measured using quantitative PCR relative to plasmid clone standards. Results were normalized to cell number. Error bars show standard deviations.

4.2. Quantitative PCR assay for Jβ usage

Since Jβ-specific antibodies are not commercially available, quantitative PCR was used to measure the fraction of cells with a specified Vβ that used a chosen Jβ. The relative abundance of VβJβ and VβCβ templates was measured with VβJβ and VβCβ primer pairs. Because these amplifications used different primer pairs and had variable fluorescence thresholds, a known standard was required to permit a comparison. The abundance ratio of these templates was determined with reference to a calibrator (a plasmid containing a VβJβCβ region clone, therefore with a 1:1 ratio of VβJβ to VβCβ). A modified Pfaffl equation (Pfaffl, 2001) was used

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to measure ***j***, the fraction of cells using a specified Vβ that also use a specified Jβ:

$$j = \frac{\left[\text{Efficiency}_{VJ\text{primerpair}}\right]^{\left[\text{Ct}_{VJ\text{standard}} - \text{Ct}_{VJ\text{sample}}\right]}}{\left[\text{Efficiency}_{VC\text{primerpair}}\right]^{\left[\text{Ct}_{VC\text{standard}} - \text{Ct}_{VC\text{sample}}\right]}}. \quad (2)$$

Serial dilutions of the calibrator plasmid were used to calculate the primer pair amplification efficiencies (always between 1.8 and 2.1 copies per cycle, data not shown). A high temperature extension step was required to obtain relatively uniform amplification efficiencies for all of the primer pairs, likely because of the high GC content of some of the J subunits (data not shown). Ct values for standards and samples were measured on the same plate with the same threshold. The $\text{Ct}_{VJ\text{standard}}$ and $\text{Ct}_{VC\text{standard}}$ values were measured using the same dilution of plasmid template.

To test the precision of these measurements, we made independent replicate measurements of Jβ usage for samples from several donors (Fig. 3A). These measurements were indeed reproducible with a coefficient of variation of 12%. The accuracy of these percentage measurements was determined by calculating the percentage use for all 13 Jβs and the same Vβ. As expected, these values summed to approximate 100% in the case of three donors (Fig. 3B).

4.3. AmpliCot assay for VβJβ family sequence diversity

Most TCR β gene diversity is due to the sequence diversity of the CDR3 region, created through nucleotide deletion and addition in the recombination process. The AmpliCot assay (Baum and McCune, 2006), based on the principles of DNA hybridization kinetics, was used to measure this sequence diversity for individual VβJβ families. Artificial oligonucleotide standards of known diversity (Baum et al., 2010) were amplified and annealed in parallel with unknown samples so that it would be possible to measure absolute, rather than relative, sequence diversity. Replicate samples from several donors were measured, yielding reproducible results with a coefficient of variation of 16% (Fig. 4A). Validating the accuracy of these AmpliCot measurements with naturally occurring TCR sequences is difficult because it is not trivial to construct lymphocyte samples of precisely known TCR diversity that contain more than a few cloned sequences. To create samples where the expected diversity could be measured, cDNA was prepared from millions of naïve $CD4^+$ T cells and then diluted so that each sample would contain 10^5 or fewer cDNAs using any particular VβJβ pair. Since these cDNAs were prepared from a large pool of naïve $CD4^+$ T cells, the expectation was that each would likely carry a unique sequence. The exact number of cDNAs using a given VβJβ pair in each sample was measured using quantitative PCR with a VβJβ primer pair, with a plasmid clone serving as an absolute abundance standard. The results of this experiment, shown in Fig. 4B, show an excellent agreement between the number of transcripts in the sample and the measured diversity of the sample when several different VβJβ combinations were tested. At low numbers of transcripts, the measured diversity slightly exceeded the number of transcripts detected, likely due to two factors. First, our quantitative PCR assay had imperfect sensitivity for small numbers of transcripts. Second, AmpliCot experiments using standard salt and DNA concentrations tend to overestimate the diversity of low-diversity samples due to difficulties in measuring rapid annealing times.

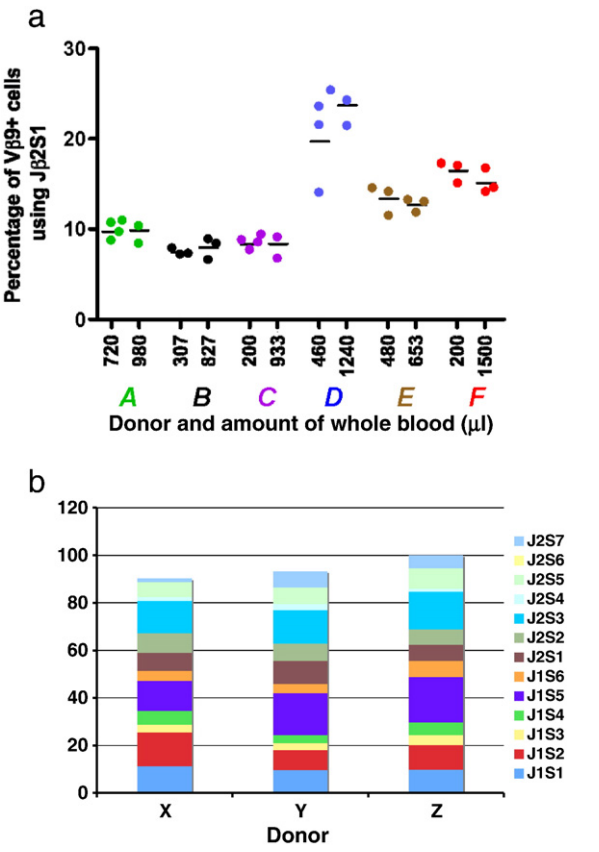

Fig. 3. Quantitative PCR measurement of J usage. A. Interassay variability. Samples of two different volumes of whole blood (and therefore containing different numbers of T cells) were prepared in triplicate from six donors (36 samples altogether). The percentage of Vβ9 transcripts using J2S1 was determined with quantitative PCR in three independent runs for the triplicate samples. The results are independent of the size of the cell samples, as expected, and indicate low interassay variability. B. Validation by addition. Buffy coat samples were obtained from three donors (different from those of Fig. 3A). Percentages of Vβ9 transcripts using each of the 13 possible Js were measured using quantitative PCR and then added together. The sums of these measurements were within 10% of the expected 100% total for three donors.

4.4. Total TCR β chain repertoire diversity

Finally, to validate our measurements of total TCR β chain repertoire diversity, samples were prepared from several donors with small, known numbers of $CD4^+$ naïve T cells. The number of different TCR β sequences in each sample (d) was calculated from measurements of a, v and j, and these measurements were repeated for several independent VβJβ pairs. Results from replicate samples indicated that this measurement of the total sample diversity based on a given VβJβ pair was quite reproducible with a CV of 20% (Fig. 5). The precision of this calculated value is lower than those previously presented because it reflects a compounding of error from the three component measurements.

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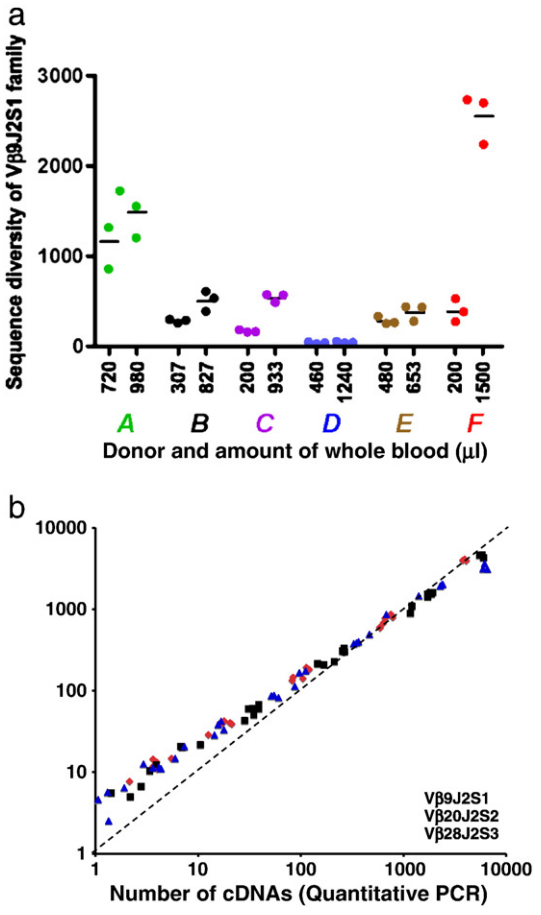

Fig. 4. Quantitative AmpliCot assay. A. Interassay variability of AmpliCot assay. Samples described in Fig. 3A had their diversity of the V9J2S1 family determined with AmpliCot, again in triplicate runs. As expected, in contrast to Fig. 3A, the numbers of cells in the samples now affect the results. The interassay variability remains low. B. Validation by titration. A cDNA preparation from millions of sorted naïve CD4$^+$ T cells was diluted into samples from which three VβJβ pairs (Vβ9J2S1, Vβ20J2S2, and Vβ28J2S3) were amplified. The number of template cDNA molecules containing a given VβJβ pair in each sample was measured with quantitative PCR, shown on the x-axis. The sequence diversity of these molecules was measured with AmpliCot and is shown on the y-axis. The expected linear relationship between these variables is shown as a dashed line.

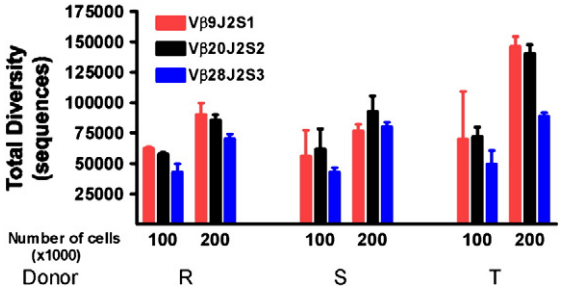

Fig. 5. Validation of overall diversity measurement. Samples containing 1×10^5 or 2×10^5 naïve CD4$^+$ T cells were prepared in triplicate from three donors. The diversity of each of these cell samples was calculated based on **v**, **j**, and **a** measured for three different VβJβ pairs. Error bars show the standard deviation.

Given the relatively small number of CD4$^+$ naïve T cells used, we expected that the actual TCR β chain diversity in each sample should be equal to the number of cells in that sample (Fig. 5). This expectation may not have been fully correct: multiple cells in the samples may have shared identical TCRβ rearrangements due to homeostatic proliferation, phenotype reversion of atypical memory cells, and the fact that some TCR rearrangements are far more likely to occur than others (Quigley et al., 2010). In fact, the measured values were generally within a factor of two of the expected value. While the absolute values were not fully accurate, the calculated values for the three different VβJβ pairs were well correlated, and when the number of cells in a sample was doubled, the measured diversity also increased (although it did not double, as might be expected). We also note that this test, using TCR rearrangements from naïve T cells where the frequency distribution of clones was expected to be relatively even, provides ideal circumstances for the assay. The assay would likely be less accurate for samples with significant inequality of the clone frequencies.

Low cDNA yields may have limited the TCR diversity present in the cell-based samples, contributing to the discrepancy between expected and measured TCR diversity in this experiment. Our TCRβ cDNA yields averaged two molecules per cell, with lower yields per cell for samples containing larger numbers of cells. Therefore, the full complement of receptor genes in the cell sample may not have been represented in the fraction of the cDNA product used in each V–C PCR reaction. Further, despite our best efforts, decreases in cell viability and RNA integrity due to sorting may have also limited the TCR diversity present in our samples. The cDNA per cell yield and the measured TCR diversity per cell were consistent for a given sample across all Vβ families, consonant with day-to-day variations in the quality of our sort preparations. This may also explain why the cDNA measurement experiment (Fig. 3B) demonstrated better accuracy and reproducibility than AmpliCot measurements of diversity of these cell-based samples.

5. Discussion

5.1. Reproducible, accurate measurement of absolute TCR diversity

Here, we provide methods and standards to estimate the absolute sequence diversity of a VβJβ family of TCR β genes, using independent measurements of Vβ gene usage by flow cytometry, of J gene usage by quantitative PCR, and of VβJβ sequence diversity using AmpliCot. Each of these component measurements has 90% accuracy and inter-assay coefficients of variation of 16% or less. When combined, these measurements can be used to calculate the absolute number of expressed TCR β chain rearrangements in a sample with an inter-assay coefficient of variation of 20% and accuracy within a factor of two. If the diversity of naïve T cell populations lacking significant clonal expansions is measured, such measurements are largely in agreement, independent of which VβJβ family is chosen as a basis for measurement. Other types of samples (e.g., those containing substantial numbers of non-naïve T cells) will require multiple VβJβ

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family measurements to be made to avoid estimation errors due to the presence of clonal expansions within the repertoire.

5.2. Comparison with other methods

A number of creative methods have been proposed in the past for assessing the diversity of the immune repertoire. In general, these methods have not been suitable for use in clinical studies because they are qualitative, costly, and/or time consuming. With the advent of next generation sequencing technologies, it is now possible to simply sequence large portions of the repertoire, but brute force sequencing remains too expensive and time-consuming to apply to more than a handful of samples at a time. Because cost limits the number of replicate measurements that can be made, these methods are vulnerable to sample preparation artifacts. Further, large data sets are obtained and their analysis can be challenging: it is difficult to differentiate true unique sequences from RT-PCR or sequencing errors; and statistical methods that use the discovery rate of new sequences to estimate the total number of sequences can be affected by the frequency distribution of sequences in the sample. Although sequencing methods are improving, it is concerning that the early set of publications using high throughput sequencing on a handful of samples has made estimates of repertoire diversity differing by more than an order of magnitude (Robins et al., 2009; Wang et al., 2010; Boyd et al., 2009; Freeman et al., 2009). The lower cost of our method permits more replicate measurements, permitting more samples to be studied and better characterization of the reproducibility of the sampling and measurement procedures. Finally, many sequencing approaches attempt to amplify the entire TCR repertoire at once in multiplex reactions, which is difficult to do with high sensitivity and low bias. Indeed, some early sequencing papers have reported Vβ usage frequencies at odds with those measured by flow cytometry (Melenhorst et al., 2008). Compared to these methods, our approach of amplifying a single Vβ family at a time, using individual specific primers, is more robust.

The most widely used method to date, called spectratyping (or immunoscope), requires multiple electrophoretic measurements of the sizes of PCR amplified TCR genes (Pannetier et al., 1995; Gorski et al., 1994). While spectratyping/immunoscope is useful for following the presence or absence of specific expanded TCR clones, it provides little information about the diversity of the nonexpanded pool of TCR clones. When tested on known numbers of naïve T cells, spectratyping/immunoscope turns out to be relatively insensitive for changes in diversity (Baum and McCune, 2006). Finally, the data produced by this method are inherently qualitative. While statistical approaches for deriving semi-quantitative diversity measurements from spectratyping/immunoscope data have been proposed, their sensitivity and reproducibility are unclear because no single method has been adopted (Hori et al., 2002; Long et al., 2006; Kepler et al., 2005; Killian et al., 2002).

5.3. Limitations

Most molecular approaches to TCR diversity, including our own, have reduced the problem to the more tractable measurement of TCRβ chain nucleotide sequence diversity, a measurement that is likely correlated with but not identical to the clonotypic and functional diversity of the repertoire. Short of sorting subpopulations of cells using particular Vα families (Arstila et al., 2000), no existing method is able to provide information about the diversity of TCRα and TCRβ heterodimers; accordingly, the full genetic diversity of a naïve T cell sample, where cells expressing the same β chain may pair with different α chains, cannot be routinely measured. However, in memory/effector populations, where each β chain appears to pair exclusively with a single α chain (Arstila et al., 2000), this is not a limitation. Because TCR–peptide–MHC interactions are of relatively low affinity and are often promiscuous, it will be a great challenge to extrapolate the diversity of binding specificities from the diversity of TCR chain sequences. Finally, measuring the number of genetic rearrangements can be useful in studies of repertoire ontogeny or dynamics, but the measurement does not give information about the functional status of the cells carrying the receptors. All these caveats aside, it is still a reasonable hypothesis that individuals with an order of magnitude reduction in TCRβ sequence diversity will probably have limitations in cellular immunity. Our measurement method has the precision to test this hypothesis.

All methods for measuring TCR diversity are affected, to varying degrees, by the fact that TCR clones are distributed at unequal frequencies in the repertoire. Immunoscope/spectratyping and limiting dilution methods are the most severely affected. Sequencing-based methods are also affected, but the extent depends on the size of the sampling performed and the statistical methods used to estimate the total diversity. The AmpliCot-based method presented here is affected to an intermediate degree, in two ways. First, changes in the distribution of sequences in a VβJβ pair will affect the measured sequence diversity with AmpliCot: The more unequal the distribution of sequences, the more AmpliCot will underestimate the total number of sequences. Despite this inaccuracy in the raw count of sequences, this abundance-dependent bias may actually be a useful feature of a diversity measurement. An example is Simpson's Diversity Index, commonly used in ecology and proposed for use in immunology (Venturi et al., 2007). Second, the strategy put forward here, like any that calculates diversity from measurements of a subset of the repertoire, assumes that the diversity of a VβJβ family relative to the diversity of the entire TCR repertoire is proportional to the abundance of cells using that VβJβ family relative to all of the cells in the sample. This assumption is more likely to be true in T cell subpopulations that contain a large number of rearrangements in a relatively even distribution. In the case of an effector memory population, VβJβ-family based sampling is likely to be misleading. If a VβJβ family bearing a large expansion is chosen, the size of the repertoire is likely to be underestimated (because this family will have a small **a** and large **v** and **j**). (Subject D in Figs. 3A and 4A provides an example of this.) Conversely, if a family without an expansion is chosen, the size of the repertoire will be overestimated (because this family will have a relatively large **a** and a small **v** and **j**). The solution to this second problem will be to make AmpliCot measurements of multiple VβJβ pairs. For cell samples containing very small numbers of expanded clones, such as antigen-specific populations, amplification and sequencing

Please cite this article as: Baum, P.D., et al., Measurement of absolute T cell receptor rearrangement diversity, J. Immunol. Methods (2011), doi:10.1016/j.jim.2011.03.001

using a universal C region primer will clearly be a better approach for measuring diversity (Douek et al., 2002).

5.4. Importance of sample preparation and replicate measurements

To date, most attempts to measure T cell diversity via PCR amplification have used cDNA rather than genomic DNA as starting material. This is largely due to the difficulty of amplifying TCR genes efficiently from a complex DNA template. However, using RNA is problematic because results are very dependent on cell recovery and viability (particularly if flow sorting is performed), RNA yields, the degree to which the RNA is degraded, the proportion of RNA used in the assay, and the efficiency of the reverse transcription reaction. With TCR cDNA yields under five copies per cell, it is easy for a bottleneck to occur at this step in the protocol, particularly if the cDNA product is split into several PCR reactions. Indeed, low cDNA copies per cell may partly explain why our calculated TCR diversity in Fig. 5 was less than the expected one sequence per cell. Unfortunately, because diversity is a property of the sample size while abundance is not, it is impossible to normalize for differences in RNA input in a diversity assay, the way it would be for a quantitative PCR assay, because one cannot tell whether a given yield of cDNA represents many messages from a few cells or a few messages from many cells. Our method has relatively low cost and rapid turnaround relative to current high throughput sequencing platforms. These important features will permit the study of larger numbers of samples than it has been possible to study via sequencing, providing increased statistical power that will be critical given the large amount of inter-subject variability and vagaries of sample collection in clinical studies.

5.5. Choice of denominator for TCR diversity measurements

In addition to the importance of sample integrity, quantitative measurements of TCR diversity are also dependent on the size of the sample. Quantitative diversity measurements of naive lymphocytes will usually be limited by the number of cells in the sample, and are therefore only interpretable if the nature of the sample is specified. While it is logistically difficult to measure the TCR diversity of all lymphocytes in the body, it is also likely that the most functionally relevant denominator is a much smaller volume or cell number. An important goal for the future will be to determine the ideal sample size for measuring TCR diversity that best correlates with immunocompetence for a given clinical or biological function.

5.6. Potential applications of this method

This method will be useful for basic studies measuring the diversity of T lymphocytes or lymphocyte subpopulations in studies analyzing large numbers of samples. For example, we are applying this method to study the effects of HIV infection on immune repertoire diversity, and also to study the effects of antiviral and immune stimulatory treatments. The method could also be used to study other patients expected to manifest changes in TCR diversity, such as congenital immunodeficiency patients, bone marrow transplant recipients, and the elderly. Moreover, the methods described here are easily adapted to mice and may be useful for experiments studying the creation and maintenance of the TCR repertoire.

6. Conclusion

We have described a low-cost and robust method for quantitative measurement of the diversity of the entire set of TCR rearrangements in a T cell sample. Despite the relatively low measurement error of our method, preliminary data indicate that TCR diversity measurements are noisy due to variability in sample preparation and significant individual variation in TCR diversity. This method makes it possible to perform the large numbers of replicate quantitative measurements required for clinical studies of repertoire diversity. Because the calculation method presented here is modular, investigators may choose to adopt components of it and adapt others. First, as sequencing costs continue to decrease, the method could potentially be adapted to use sequencing rather than AmpliCot for the determination of sequence diversity of a VβCβ or VβJβ pair. Compared to sequencing the entire repertoire, this would be less expensive and enable broader coverage of the sequences in the sample. Moreover, while it is difficult to amplify the full TCR repertoire without either bias or compromised sensitivity, it is relatively straightforward to amplify specific VβCβ or VβJβ gene families with defined primer pairs with high sensitivity and minimal bias. Quantitative PCR methods could potentially be substituted to estimate Vβ usage (Sriram et al., 2007) or VβJβ usage (Wettstein et al., 2008) if live cells or flow cytometry equipment were not available. Finally, it can be very costly to prepare samples that contain equal, large numbers of well-purified T cell subpopulations that are required for fully measuring absolute repertoire diversity. More rearrangements may exist, particularly in the naïve $CD4^+$ T cell repertoire, than can be sampled in a peripheral blood sample of a reasonable size. In many clinical and experimental situations, the relative diversity of samples is more important than the absolute diversity. We anticipate that in many such cases, measurements will be made with AmpliCot alone, greatly reducing the time and cost involved.

Acknowledgments

We thank McCune lab members for discussions and comments on the manuscript. We thank Saunak Sen for statistical consultation.

This work was funded by grants from the National Institutes of Health (K23 AI 073100 to P.D.B., and R37 AI40312, and UO1 AI43864 to J.M.M.) and from the California HIV/AIDS Research Program (an IDEA Grant to P.D.B.). J.M.M. is a recipient of the NIH Director's Pioneer Award Program, part of the NIH Roadmap for Medical Research, through grant DPI OD00329.

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Please cite this article as: Baum, P.D., et al., Measurement of absolute T cell receptor rearrangement diversity, J. Immunol. Methods (2011), doi:10.1016/j.jim.2011.03.001