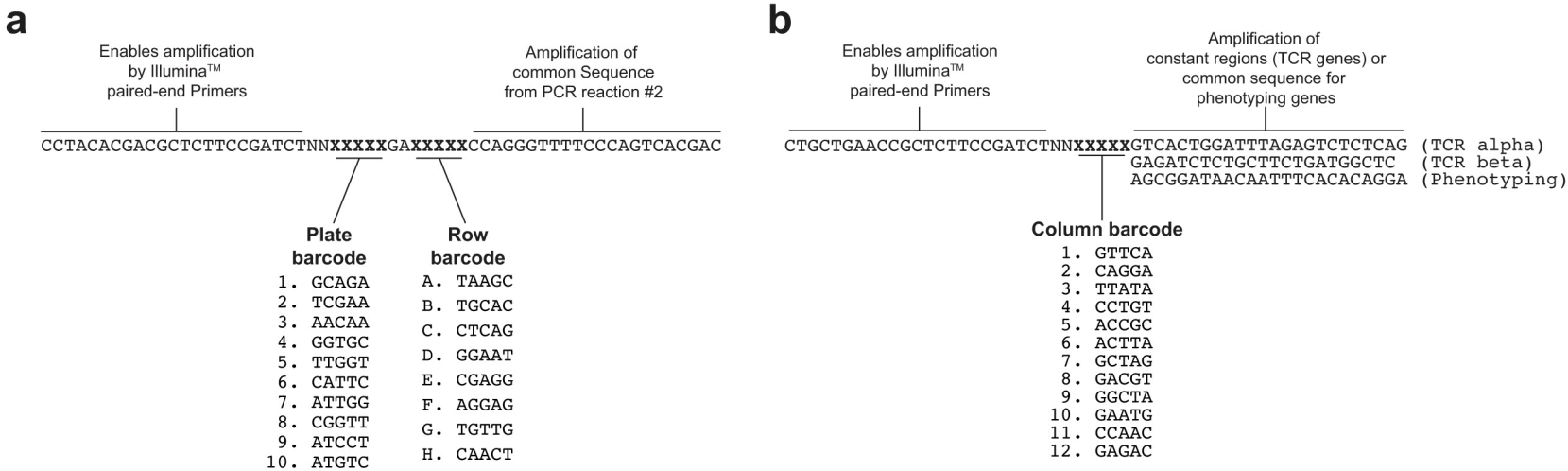
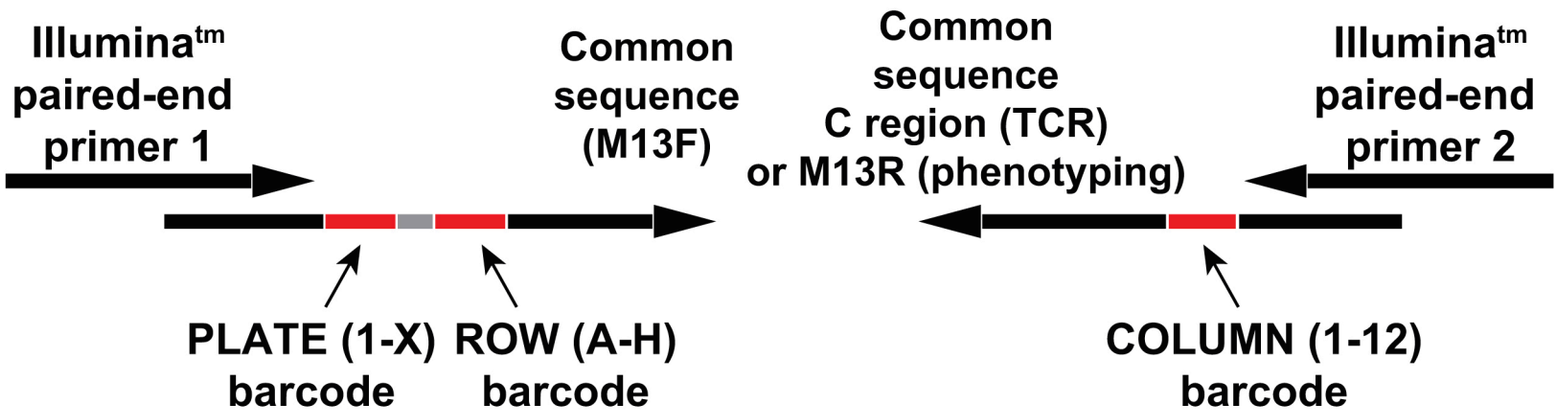
Supplementary Figure 1

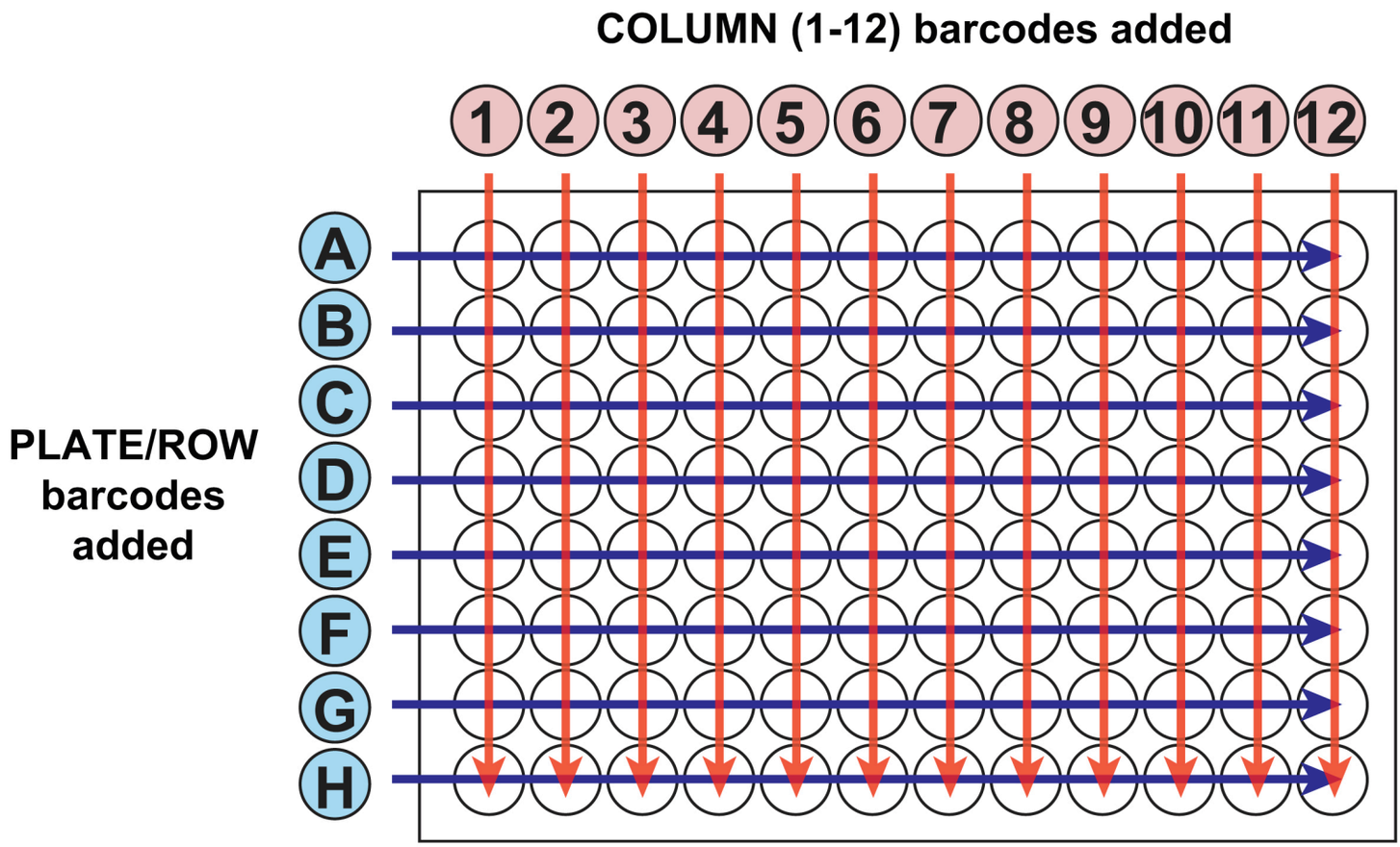


Supplementary Figure 1. **Barcoding primer design.** (**a**) 5' primers incorporate barcodes that specify plate and row and bind and amplify a common sequence that is incorporated into all 5' primers from PCR reaction #2. The outside sequence allows for amplification using IlluminaTM Paired-End primers. (**b**) 3' primers incorporate barcodes that specify column. The primers amplify nested constant region sequences for TCRα/β or a common sequence incorporated into all 3' cytokine primers. The outside sequence allows for amplification using IlluminaTM paired-end primers.

Nature Biotechnology: doi:10.1038/nbt.2938

Supplementary Figure 2

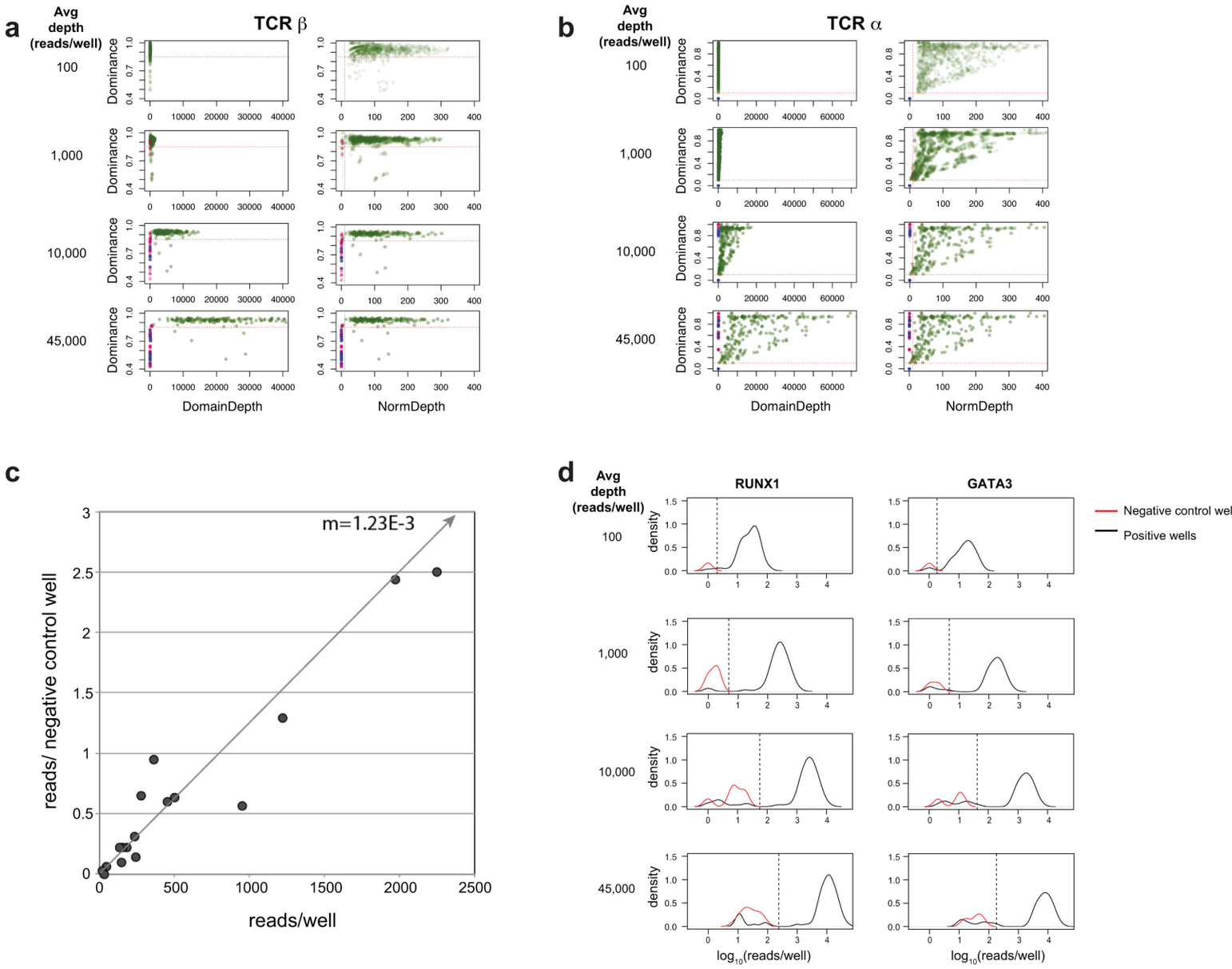




Supplementary Figure 2. Schematic for barcoding (third) PCR reaction. An aliquot from the second PCR reaction product is used as a template for this reaction. To each well within a particular row within a given plate, a distinct 5' primer is added by multichannel pipette that specifies row. To each well within a column, a distinct 3' primer is added by multichannel pipette that specifies column. The reaction is performed with Illumina™ paired-end primers in all wells, which enable sequencing on the Illumina™ MiSeq™ platform.

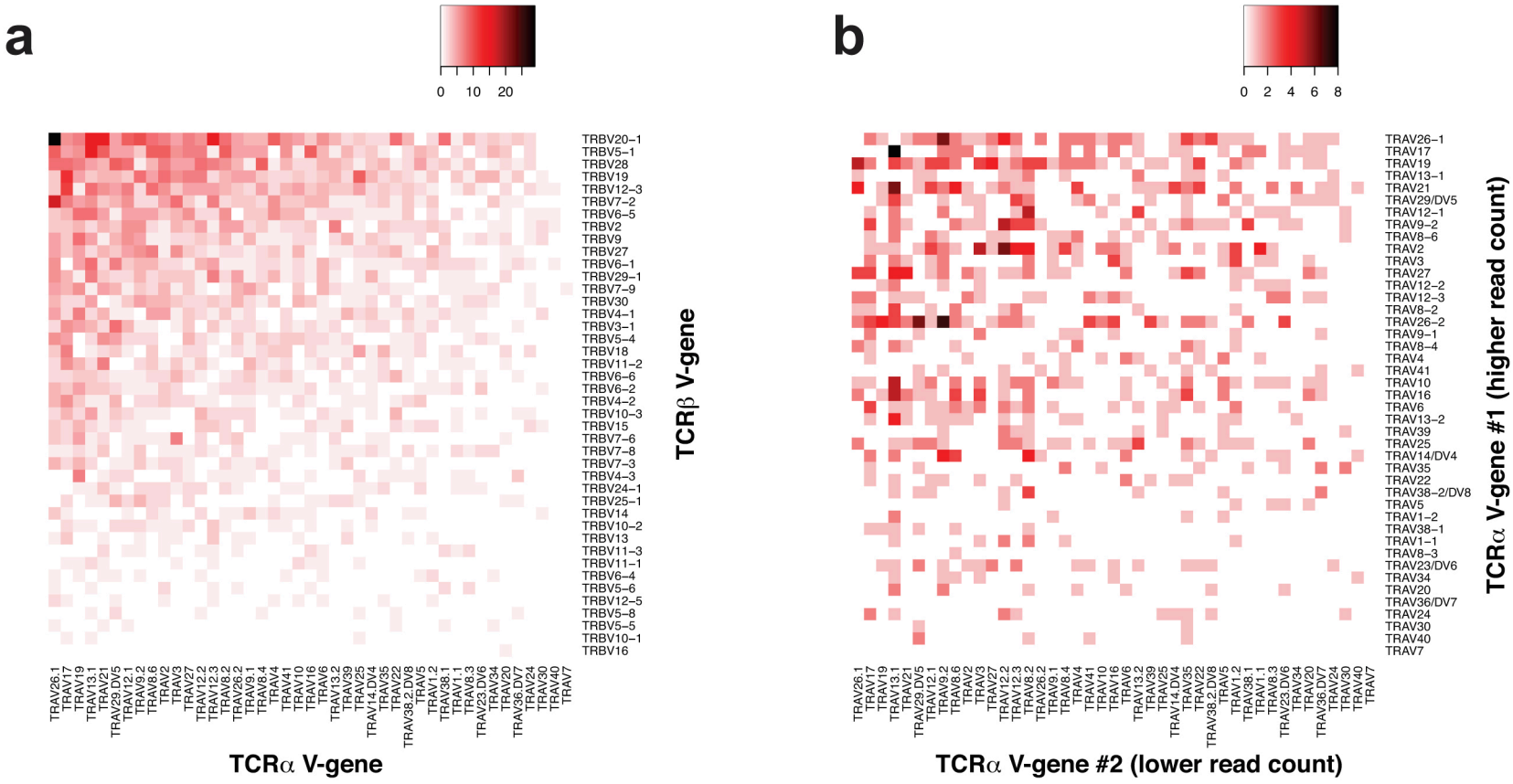
Nature Biotechnology: doi:10.1038/nbt.2938

Supplementary Figure 3



Supplementary Figure 3. Validation of true-positive cutoff criteria by very deep sequencing (see Methods). (**a-b**) TCR cutoff criteria. Two plates containing single cells and reagents (green dots), reagents but no cells (red), and neither reagents nor cells (blue), were subjected to TCRβ (a) or TCRα (b) sequencing to an average depth of over 45,000 reads per well. The plates were then randomly subsampled to depths ranging from 100 to 45,000 average reads per well (left column). Depths of 100, 1,000, 10,000 and 45,000 are shown. Left columns show raw number of TCR domain-specific reads per well (DomainDepth, x-axis) as a function of domain dominance of that TCR clone within the well (y-axis). Right columns shows normalized read number of TCR clones (NormDepth, x-axis) as a function of domain dominance of that TCR clone within the well (y-axis). NormDepth (Normalized Depth) is the ratio of raw domain-specific reads over the average number of domain-specific reads per well present in the sequencing run, and is used to normalize read number to exclude background regardless of number of sequencing depth. A NormDepth cutoff of 10% reliably excludes 100% of negative control wells across the dynamic range of 100 to 45,000 reads per well. For TCR analysis, samples were also evaluated based on Domain dominance, a measure of dominance of a single TCR sequence in all reads of that domain type (TCRβ, TCRα) in the well. Domain dominance further excludes negative control wells and wells containing more than one cell or cross contaminating sequences. A Domain Dominance cutoff of > 85% for TCRβ was established to exclude negative control wells, as well as wells potentially containing more than one sorted cell (a). The DomainDominance cutoff for TCR alpha was set at > 10% to account for the possibility of multiple alpha chain expression (b). We apply both Domain Dominance cutoff and Normalized-Depth cutoffs in our analysis, to eliminate all negative control wells within a dynamic range of 100 to 45,000 reads per well. In all positive control wells, sequencing depth does not impact successful classification of the dominant clone's identity. (**c**) Background of phenotypic parameters as a function of total number of reads of that parameter on a given plate. Two plates, containing a combination of single stimulated T cells and negative control wells were analyzed for expression of phenotypic parameters. For each individual parameter, background reads (y-axis, reads per negative control well) is plotted in relation to total reads (x-axis, reads per well). The ratio of background reads/well to reads/well in all wells is ~1.23x10-3. (**d**). A single plate containing 80 wells with single T cells and 16 negative control wells was analyzed across the dynamic range of 100 to 45,000 reads per well. RUNX1 and GATA3, the two parameters containing the highest background, were assessed in 80 wells containing T cells and 16 negative control wells. Histogram depicts average read count per well (x-axis) and relative density (y-axis) for wells containing T cells (black) and negative control wells (red). Only wells containing at least 1 read for RUNX1 (left) or GATA3 (right) are shown. Dotted line represents 1 SD below the mean of log read counts per well of all wells containing reads, which is the cutoff established to exclude background.

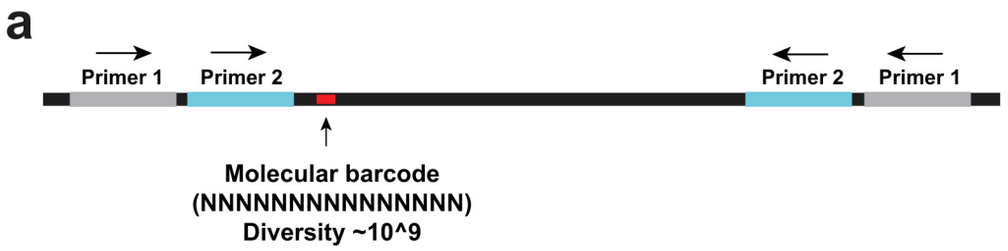
Supplementary Figure 4

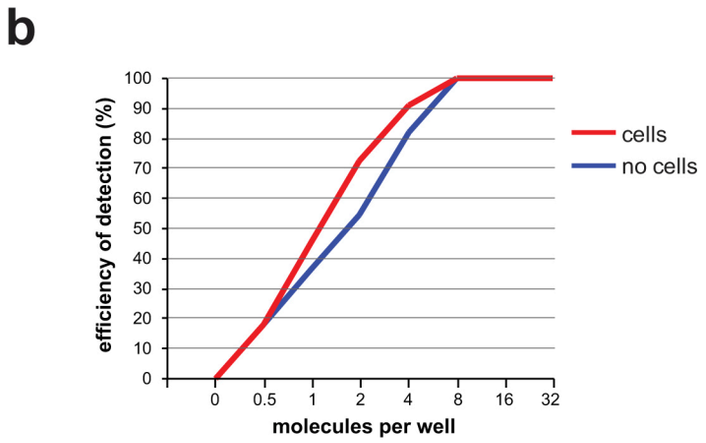


Supplementary Figure 4. Human TCR V-gene usage in single T cells. (**a**) Observed frequency of all possible TCR α/β combinations observed in 2,721 non-redundant TCRs where a productive TCRβ gene and a single productive TCRα gene were obtained. For both TCRα and TCRβ, some V-genes are used at a higher frequency than others, and their combinations appear largely in proportion to independent abundance. (**b**) Observed frequency of all possible double TCRα combinations observed in 999 non-redundant TCRs where two TCRα chains were identified, one or more being productive. The dominantly detected gene is plotted on the y-axis, while the gene with lower read counts is plotted on the y-axis. Dual TCRα cells appear to select chains as a function of TCRα frequency and there is no systemic bias observed with respect to TCRα chain co-expression within a particular cell.

Nature Biotechnology: doi:10.1038/nbt.2938

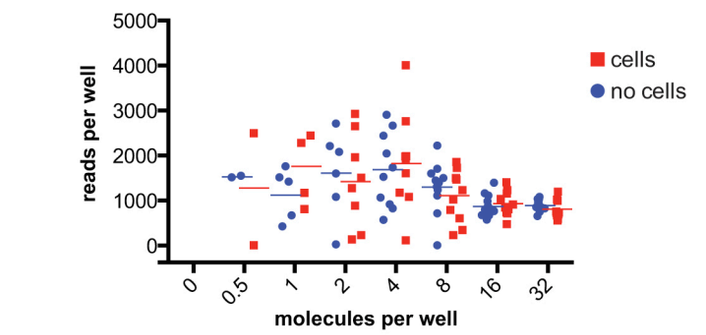
Supplementary Figure 5

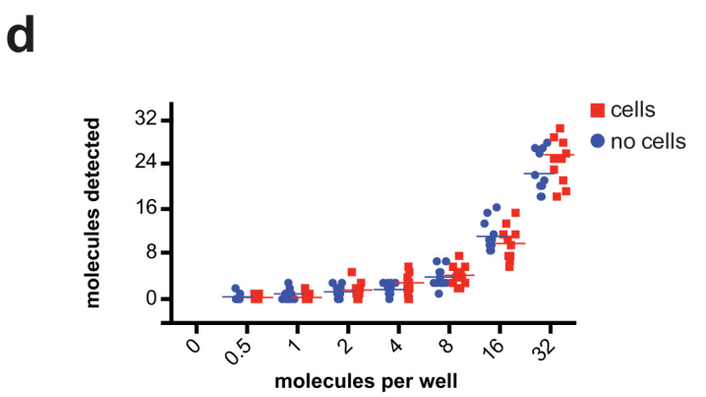




e

GENE	READ/WELL
FOXP3	550
GATA3	478
GZMB	353
IFNG	605
IL17A	442
PRF1	283
RORC	974
TBET	389
TGFB1	156
TNF	524
BCL6	584
RUNX1	517
RUNX3	493





Supplementary Figure 5. Analysis of the affect of transcript abundance on assay sensitivity and read count. A synthetic IL-17 template (a) was spiked into 2 plates at various dilutions from 0 to 32 molecules per well (indicated on x-axis of plots in (b-d)). Into 1 of these 2 plates, a single stimulated T cell was also sorted into each well. One well from each row was left without template as a negative control. Subsequent RT-PCR reactions and analysis were performed per protocol. (**a**) Design of the synthetic IL-17 template, which contains primer sequences for amplification and a molecular barcode containing 15 random nucleotides. (**b**) Sensitivity of detection of exogenous IL-17 template. Sensitivity is scored as a percentage of wells (out of 11 total wells per dilution) where the template was detected as present. (**c**) Total reads of exogenous IL-17 template in wells where exogenous IL-17 template was detected above background. Two wells also containing endogenous IL-17 (from cells added to the wells) were excluded from the analysis. (**d**) Number of uniquely barcoded molecules detected per well. To account for the presence of sequencing and PCR error, a similarity threshold was set above which molecular barcodes were scored as being equivalent. No molecules sharing the same barcode were repeated throughout the plates. (**e**) Mean read counts per well of phenotypic parameters present in at least 50 cells within our tumor and colon T cell set.

Supplementary Figure 6

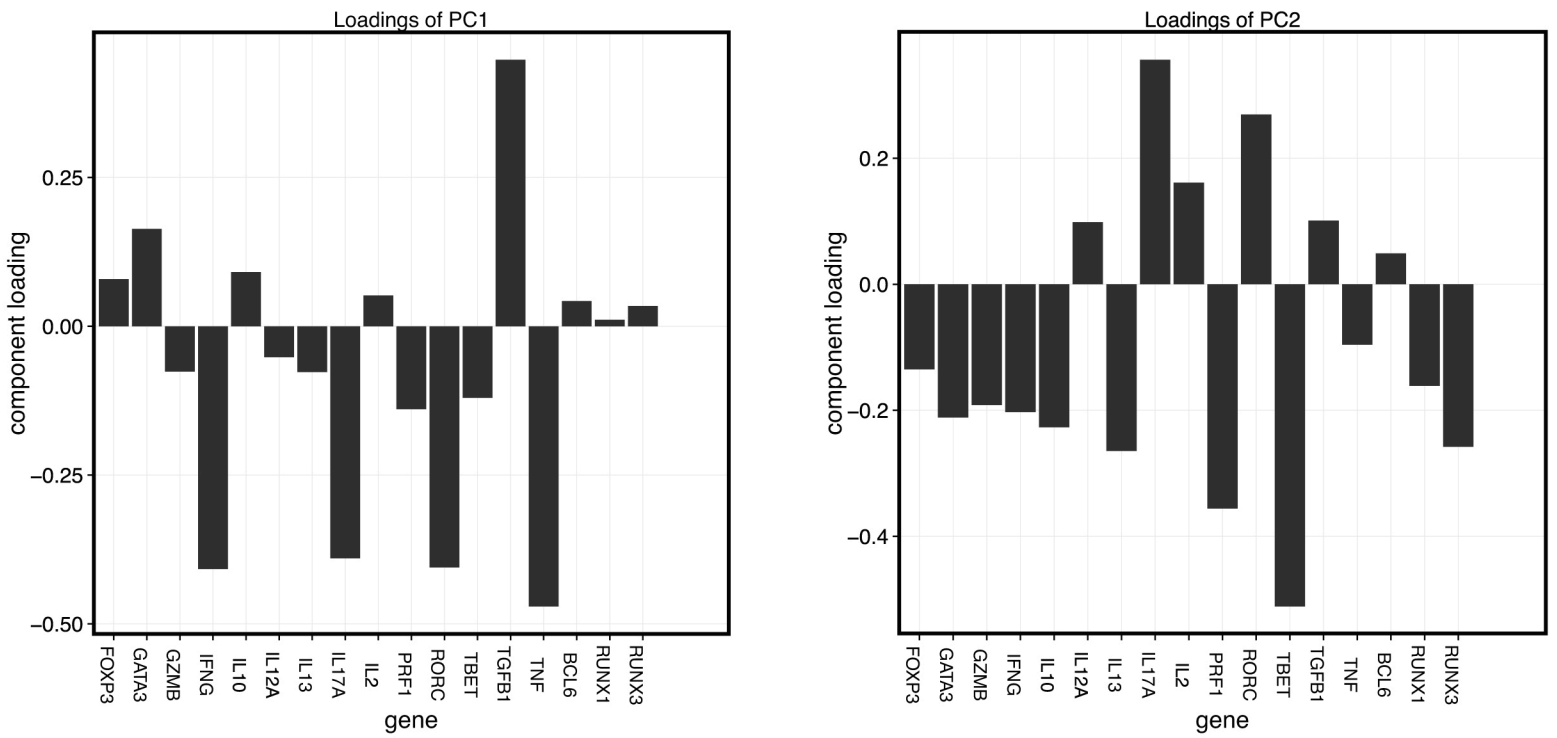
Clone	TRBV	CDR3	TRBJ
A(52) B(8)	TRBV 13	C A S S **L** A S **M** G V G E L F F tgt gcc agc agc **cta** gc**g** ag**t** at**g** gg**t** gtc ggg gag ctg ttt ttt C A S S **S** A S **G** G V G E L F F tgt gcc agc agc t**cg** gca ag**c** g**gg** gg**a** gtc ggg gag ctg ttt ttt	TRBJ 2-2

Clone	TRAV	CDR3	TRAJ
A(52) B(8)	TRAV 38-2	C A Y R P N Y G G A T N K L I F tgt gct tat agg **cca** aat tat ggt ggt gct aca aac aag ctc atc ttt C A Y R P N Y G G A T N K L I F tgt gct tat agg **ccg** aat tat ggt ggt gct aca aac aag ctc atc ttt	TRAJ 32

Supplementary Figure 6. Nucleic acid sequence of two expanded TIL T cell clones sharing an identical TCR alpha chain and similar TCR beta. N-nucleotide additions are indicated in yellow. D-region sequence is indicated in grey.

Nature Biotechnology: doi:10.1038/nbt.2938

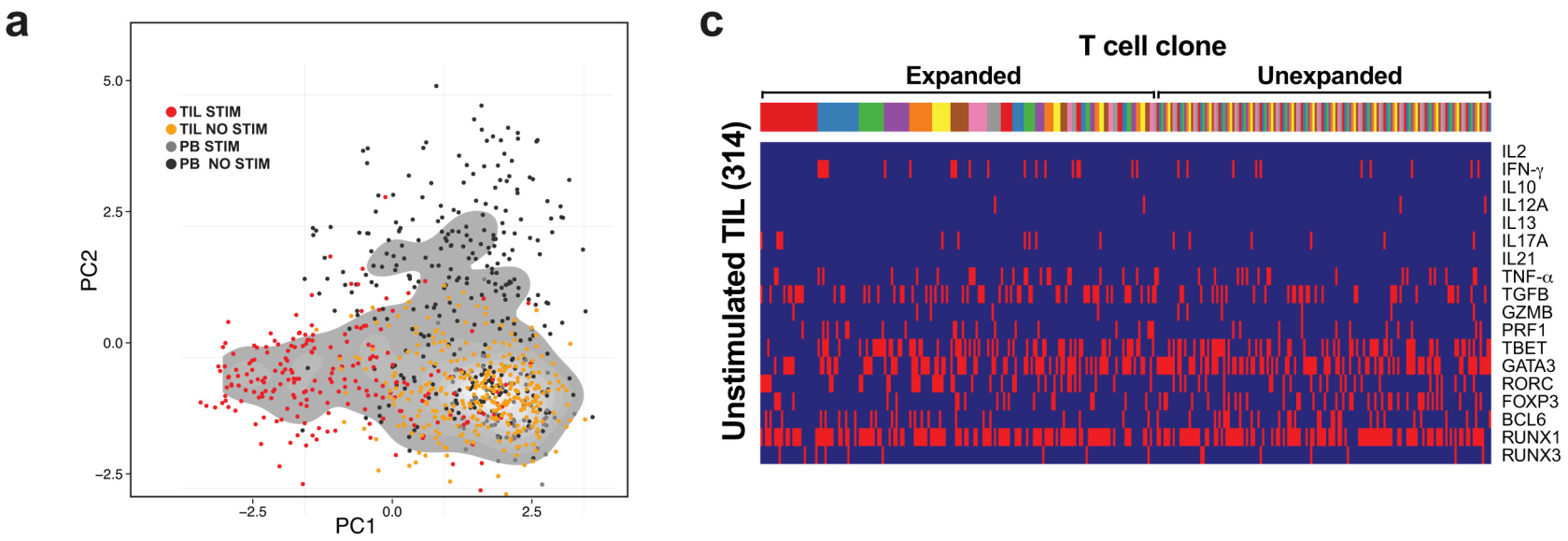
Supplementary Figure 7



Supplementary Figure 7. Principle component analysis parameter loadings for the PC1 and PC2, depicted in Fig. 3a.

Nature Biotechnology: doi:10.1038/nbt.2938

Supplementary Figure 8



Supplementary Figure 8. Analysis of stimulated vs. unstimulated CD4+ T cells from tumor and peripheral blood (**a**) Principle component analysis of unstimulated vs PMA/inomycin stimulated CD4+ T cells from tumor and peripheral blood. (**b**) Principle component analysis parameter loadings for the PC1 and PC2, depicted in (a) are shown. (**c**) Heat map showing multi-parametric phenotypic analysis from unstimulated CD4+ T cells from tumor. Individual T cells are grouped by TCR sequence. Each color on bar represents a distinct TCR sequence.

Nature Biotechnology: doi:10.1038/nbt.2938

Supplementary Table 1

TRAV1, Reaction 1	CTGCACGTACCAGACATCTGGGTT
TRAV2, Reaction 1	GGCTCAAAGCCTTCTCAGCAGG
TRAV3, Reaction 1	GGATAACCTGGTTAAAGGCAGCTA
TRAV4, Reaction 1	GGATACAAGACAAAAGTTACAAACGA
TRAV5, Reaction 1	GCTGACGTATATTTTTTCAAATATGGA
TRAV6, Reaction 1	GGAAGAGGCCCTGTTTTCTTGCT
TRAV7, Reaction 1	GCTGGATATGAGAAGCAGAAAGGA
TRAV8, Reaction 1	AGGACTCCAGCTTCTCCTGAAGTA
TRAV9, Reaction 1	GTATGTCCAATATCCTGGAGAAGGT
TRAV10, Reaction 1	CAGTGAGAACACAAAGTCGAACGG
TRAV12.1, Reaction 1	CCTAAGTTGCTGATGTCCGTATAC
TRAV12.2, Reaction 1	GGGAAAAGCCCTGAGTTGATAATGT
TRAV12.3, Reaction 1	GCTGATGTACACATACTCCAGTGG
TRAV13.1, Reaction 1	CCCTTGGTATAAGCAAGAACTTGG
TRAV13.2, Reaction 1	CCTCAATTCATTATAGACATTCGTTC
TRAV14, Reaction 1	GCAAAATGCAACAGAAGGTCGCTA
TRAV16, Reaction 1	TAGAGAGAGCATCAAAGGCTTCAC
TRAV17, Reaction 1	CGTTCAAATGAAAGAGAGAAACACAG
TRAV18, Reaction 1	CCTGAAAAGTTCAGAAAACCAGGAG
TRAV19, Reaction 1	GGTCGGTATTCTTGGAACTTCCAG
TRAV20, Reaction 1	GCTGGGGAAGAAAAGGAGAAAGAAA
TRAV21, Reaction 1	GTCAGAGAGAGCAAACAAGTGGAA
TRAV22, Reaction 1	GGACAAAACAGAATGGAAGATTAAGC
TRAV23, Reaction 1	CCAGATGTGAGTGAAAAGAAAGAAG
TRAV24, Reaction 1	GACTTTAAATGGGGATGAAAAGAAGA
TRAV25, Reaction 1	GGAGAAGTGAAGAAGCAGAAAAGAC
TRAV26.1, Reaction 1	CCAATGAAATGGCCTCTCTGATCA
TRAV26.2, Reaction 1	GCAATGTGAACAACAGAATGGCCT
TRAV27, Reaction 1	GGTGGAGAAGTGAAGAAGCTGAAG
TRAV29, Reaction 1	GGATAAAAATGAAGATGGAAGATTCAC
TRAV30, Reaction 1	CCTGATGATATTACTGAAGGGTGGA
TRAV34, Reaction 1	GGTGGGGAAGAGAAAAGTCATGAA
TRAV35, Reaction 1	GGTGAATTGACCTCAAATGGAAGAC
TRAV36, Reaction 1	GCTAACTTCAAGTGGAATTGAAAAGA
TRAV38, Reaction 1	GAAGCTTATAAGCAACAGAATGCAAC
TRAV39, Reaction 1	GGAGCAGTGAAGCAGGAGGGAC
TRAV40, Reaction 1	GAGAGACAATGGAAAACAGCAAAAAC
TRAV41, Reaction 1	GCTGAGCTCAGGGAAGAAGAAGC
TRBV2, Reaction 1	CTGAAATATTCGATGATCAATTCTCAG
TRBV3-1, Reaction 1	TCATTATAAATGAAACAGTTCCAAATCG
TRBV4, Reaction 1	AGTGTGCCAAGTCGCTTCTCAC
TRBV5-4,8, Reaction 1	CAGAGGAAACTYCCCTCCTAGATT
TRBV5-1, Reaction 1	GAGACACAGAGAAACAAAGGAAACTTC
TRBV6-1, Reaction 1	GGTACCACTGACAAAGGAGAAGTCC
TRBV6-2,3, Reaction 1	GAGGGTACAACTGCCAAAGGAGAGGT
TRBV6-4, Reaction 1	GGCAAAGGAGAAGTCCCTGATGGTT
TRBV6-5,6, Reaction 1	AAGGAGAAGTCCCSAATGGCTACAA
TRBV6-8, Reaction 1	CTGACAAAGAAGTCCCCAATGGCTAC
TRBV6-9, Reaction 1	CACTGACAAAGGAGAAGTCCCCGAT
TRBV7-2, Reaction 1	AGACAAATCAGGGCTGCCCAGTGA
TRBV7-3, Reaction 1	GACTCAGGGCTGCCCAACGAT
TRBV7-8, Reaction 1	CCAGAATGAAGCTCAACTAGACAA
TRBV7-4,6, Reaction 1	GGTTCTCTGCAGAGAGGCCTGAG
TRBV7-7, Reaction 1	GGCTGCCCAGTGATCGGTTCTC
TRBV7-9, Reaction 1	GACTTACTTCCAGAATGAAGCTCAACT
TRBV9, Reaction 1	GAGCAAAAGGAAACATTCTTGAACGATT
TRBV10-1,3, Reaction	GGCTRATCCATTACTCATATGGTGTT
TRBV10-2, Reaction 1	GATAAAGGAGAAGTCCCCGATGGCT
TRBV11, Reaction 1	GATTCACAGTTGCCTAAGGATCGAT
TRBV12-3,4, Reaction	GATTCAGGGATGCCCGAGGATCG
TRBV12-5, Reaction 1	GATTCGGGGATGCCGAAGGATCG
TRBV13, Reaction 1	GCAGAGCGATAAAGGAAGCATCCCT
TRBV14, Reaction 1	TCCGGTATGCCCAACAATCGATTCT
TRBV15, Reaction 1	GATTTTAACAATGAAGCAGACACCCCT
TRBV16, Reaction 1	GATGAAACAGGTATGCCCAAGGAAAG
TRBV18, Reaction 1	TATCATAGATGAGTCAGGAATGCCAAAG
TRBV19, Reaction 1	GACTTTCAGAAAGGAGATATAGCTGAA
TRBV20-1, Reaction 1	CAAGGCCACATACGAGCAAGGCGTC
TRBV24-1, Reaction 1	CAAAGATATAAACAAAGGAGAGATCTCT
TRBV25-1, Reaction 1	AGAGAAGGGAGATCTTTCCTCTGAGT
TRBV27-1, Reaction 1	GACTGATAAGGGAGATGTTCCTGAAG
TRBV28, Reaction 1	GGCTGATCTATTTCTCATATGATGTTAA
TRBV29, Reaction 1	GCCACATATGAGAGTGGATTTGTCATT
TRBV30, Reaction 1	GGTGCCCCAGAATCTCTCAGCCT
TRAC, Reaction 1	CGGTGAATAGGCAGACAGACTTGT
TRBC, Reaction 1	ACCAGTGTGGCCTTTTGGGTGTG

TRAV1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**AGGTCGTTTTTCTTCATTCCTTAGTC
TRAV2, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**ACGATACAACATGACCTATGAACGG
TRAV3.1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTTTGAAGCTGAATTTAACAAGAGCC
TRAV4.1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTCCCTGTTTATCCCTGCCGAC
TRAV5.1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**AAACAAGACCAAAGACTCACTGTTC
TRAV6, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**AAGACTGAAGGTCACCTTTGATACC
TRAV7, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**ACTAAATGCTACATTACTGAAGAATGG
TRAV8, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCATCAACGGTTTTGAGGCTGAATTTAA
TRAV9, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GAAACCACTTCTTTCCACTTGGAGAA
TRAV10, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TACAGCAACTCTGGATGCAGACAC
TRAV12, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GAAGATGGAAGGTTTACAGCACA
TRAV13.1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GACATTCGTTCAAATGTGGGCGAA
TRAV13.2, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GGCAAGGCCAAAGAGTCACCGT
TRAV14, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TCCAGAAGGCAAGAAAATCCGCCA
TRAV16, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCTGACCTTAACAAAGGCGAGACA
TRAV17, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TTAAGAGTCACGCTTGACACTTCCA
TRAV18, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCAGAGGTTTTCAGGCCAGTCCT
TRAV19, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TCCACCAGTTCCTTCAACTTCACC
TRAV20, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCCACATTAACAAAGAAGGAAAGCT
TRAV21, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCCTCGCTGGATAAATCATCAGGA
TRAV22, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**ACGACTGTCGCTACGGAACGCTA
TRAV23, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CACAATCTCCTTCAATAAAAGTGCCA
TRAV24, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**ACGAATAAGTGCCACTCTTAATACCA
TRAV25, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GTTTGGAGAAGCAAAAAAGAACAGCT
TRAV26.1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CAGAAGACAGAAAGTCCAGCACCT
TRAV26.2, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**ATCGCTGAAGACAGAAAGTCCAGT
TRAV27, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**ACTAACCTTTCAGTTTGGTGATGCAA
TRAV29, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTTAAACAAAAGTGCCAAGCACCTC
TRAV30, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**AATATCTGCTTCATTTAATGAAAAAAAGC
TRAV34, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CCAAGTTGGATGAGAAAAAGCAGCA
TRAV35, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTCAGTTTGGTATAACCAGAAAGGA
TRAV36, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GGAAGACTAAGTAGCATATTAGATAAG
TRAV38, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTGTGAACTTCCAGAAAGCAGCCA
TRAV39, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CCTCACTTGATACCAAAGCCCGT
TRAV40, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**AGGCGGAAATATTAAAGACAAAAACTC
TRAV41, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GATTAATTGCCACAATAAACATACAGG
TRBV2, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCCTGATGGATCAAATTTCACTCTG
TRBV3-1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TCTCACCTAAATCTCCAGACAAAGCT
TRBV4, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CCTGAATGCCCCAACAGCTCTC
TRBV5-4,8, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTCTGAGCTGAATGTGAACGCCT
TRBV5-1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CGATTCTCAGGGCGCCAGTTCTCT
TRBV6-1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TGGCTACAATGTCTCCAGATTAAACAA
TRBV6-2,3, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CCCTGATGGCTACAATGTCTCCAGA
TRBV6-4, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GTGTCTCCAGAGCAAACACAGATGATT
TRBV6-5,6, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GTCTCCAGATCAACCACAGAGGAT
TRBV6-8, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GTCTCTAGATTAAACACAGAGGATTTC
TRBV6-9, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GGCTACAATGTATCCAGATCAAACA
TRBV7-2, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TCGCTTCTCTGCAGAGAGGACTGG
TRBV7-3, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CGGTTCTTTGCAGTCAGGCCTGA
TRBV7-8, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CCAGTGATCGCTTCTTTGCAGAAA
TRBV7-4,6, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TCTCCACTCTGAMGATCCAGCGCA
TRBV7-7, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCAGAGAGGCCTGAGGGATCCAT
TRBV7-9, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTGCAGAGAGGCCTAAGGGATCT
TRBV9, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTCCGCACAACAGTTCCCTGACTT
TRBV10-1,3, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CAGATGGCTAYAGTGTCTCTAGATCAAA
TRBV10-2, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GTTGTCTCCAGATCCAAGACAGAGAA
TRBV11, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCAGAGAGGCTCAAAGGAGTAGACT
TRBV12-3,4, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCTAAGATGCCTAATGCATCATTCTC
TRBV12-5, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CTCAGCAGAGATGCCTGATGCAACT
TRBV13, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TCTCAGCTCAACAGTTCAGTGACTA
TRBV14, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCTGAAAGGACTGGAGGGACGTAT
TRBV15, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GATAACTTCCAATCCAGGAGGCCG
TRBV16, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GCTAAGTGCCTCCCAAATTCACCC
TRBV18, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GGAACGATTTTCTGCTGAATTTCCCA
TRBV19, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GGTACAGCGTCTCTCGGGAGAAGA
TRBV20-1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GGACAAGTTTCTCATCAACCATGCAA
TRBV24-1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TGGATACAGTGTCTCTCGACAGGC
TRBV25-1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CAACAGTCTCCAGAATAAGGACGGA
TRBV27-1, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**TACAAAGTCTCTCGAAAAGAGAAGAGGA
TRBV28, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GGGGTACAGTGTCTCTAGAGAGA
TRBV29, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**GTTTCCCATCAGCCGCCCAAACCTA
TRBV30, Reaction 2	**CCAGGGTTTTCCCAGTCACGAC**CAGACCCCAGGACCGGCAGTTCAT
TRAC, Reaction 2	CAGACAGACTTGTCACTGGATTTAG
TRBC, Reaction 2	CTTTTGGGTGTGGGAGATCTCTG

Supplementary Table 1. TCR sequencing primers for first two PCR reactions. Common sequences are indicated in bold.

Supplementary Table 3

AlphaBC1	CTGCTGAACCGCTCTTCCGATCTNN**GTTCA**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC2	CTGCTGAACCGCTCTTCCGATCTNN**CAGGA**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC3	CTGCTGAACCGCTCTTCCGATCTNN**TTATA**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC4	CTGCTGAACCGCTCTTCCGATCTNN**CCTGT**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC5	CTGCTGAACCGCTCTTCCGATCTNN**ACCGC**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC6	CTGCTGAACCGCTCTTCCGATCTNN**ACTTA**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC7	CTGCTGAACCGCTCTTCCGATCTNN**GCTAG**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC8	CTGCTGAACCGCTCTTCCGATCTNN**GACGT**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC9	CTGCTGAACCGCTCTTCCGATCTNN**GGCTA**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC10	CTGCTGAACCGCTCTTCCGATCTNN**GAATG**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC11	CTGCTGAACCGCTCTTCCGATCTNN**CCAAC**GTCACTGGATTTAGAGTCTCTCAG
AlphaBC12	CTGCTGAACCGCTCTTCCGATCTNN**GAGAC**GTCACTGGATTTAGAGTCTCTCAG
BetaBC1	CTGCTGAACCGCTCTTCCGATCTNN**GTTCA**GAGATCTCTGCTTCTGATGGCTC
BetaBC2	CTGCTGAACCGCTCTTCCGATCTNN**CAGGA**GAGATCTCTGCTTCTGATGGCTC
BetaBC3	CTGCTGAACCGCTCTTCCGATCTNN**TTATA**GAGATCTCTGCTTCTGATGGCTC
BetaBC4	CTGCTGAACCGCTCTTCCGATCTNN**CCTGT**GAGATCTCTGCTTCTGATGGCTC
BetaBC5	CTGCTGAACCGCTCTTCCGATCTNN**ACCGC**GAGATCTCTGCTTCTGATGGCTC
BetaBC6	CTGCTGAACCGCTCTTCCGATCTNN**ACTTA**GAGATCTCTGCTTCTGATGGCTC
BetaBC7	CTGCTGAACCGCTCTTCCGATCTNN**GCTAG**GAGATCTCTGCTTCTGATGGCTC
BetaBC8	CTGCTGAACCGCTCTTCCGATCTNN**GACGT**GAGATCTCTGCTTCTGATGGCTC
BetaBC9	CTGCTGAACCGCTCTTCCGATCTNN**GGCTA**GAGATCTCTGCTTCTGATGGCTC
BetaBC10	CTGCTGAACCGCTCTTCCGATCTNN**GAATG**GAGATCTCTGCTTCTGATGGCTC
BetaBC11	CTGCTGAACCGCTCTTCCGATCTNN**CCAAC**GAGATCTCTGCTTCTGATGGCTC
BetaBC12	CTGCTGAACCGCTCTTCCGATCTNN**GAGAC**GAGATCTCTGCTTCTGATGGCTC
PhenotypeBC1	CTGCTGAACCGCTCTTCCGATCTNN**GTTCA**AGCGGATAACAATTTCACACAGGA
PhenotypeBC2	CTGCTGAACCGCTCTTCCGATCTNN**CAGGA**AGCGGATAACAATTTCACACAGGA
PhenotypeBC3	CTGCTGAACCGCTCTTCCGATCTNN**TTATA**AGCGGATAACAATTTCACACAGGA
PhenotypeBC4	CTGCTGAACCGCTCTTCCGATCTNN**CCTGT**AGCGGATAACAATTTCACACAGGA
PhenotypeBC5	CTGCTGAACCGCTCTTCCGATCTNN**ACCGC**AGCGGATAACAATTTCACACAGGA
PhenotypeBC6	CTGCTGAACCGCTCTTCCGATCTNN**ACTTA**AGCGGATAACAATTTCACACAGGA
PhenotypeBC7	CTGCTGAACCGCTCTTCCGATCTNN**GCTAG**AGCGGATAACAATTTCACACAGGA
PhenotypeBC8	CTGCTGAACCGCTCTTCCGATCTNN**GACGT**AGCGGATAACAATTTCACACAGGA
PhenotypeBC9	CTGCTGAACCGCTCTTCCGATCTNN**GGCTA**AGCGGATAACAATTTCACACAGGA
PhenotypeBC10	CTGCTGAACCGCTCTTCCGATCTNN**GAATG**AGCGGATAACAATTTCACACAGGA
PhenotypeBC11	CTGCTGAACCGCTCTTCCGATCTNN**CCAAC**AGCGGATAACAATTTCACACAGGA
PhenotypeBC12	CTGCTGAACCGCTCTTCCGATCTNN**GAGAC**AGCGGATAACAATTTCACACAGGA
PEprimer1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PEprimer2	AAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Supplementary Table 3. Column barcoding primers used for the third PCR reaction and Illumina™ paired-end primers.

Supplementary Table 5

	TCR beta				TCR alpha (primary)				TCR alpha (secondary)			
Well	**V-gene**	**J-gene**	**CDR3**	**#**	**V-gene**	**J-gene**	**CDR3**	**#**	**V-gene**	**J-gene**	**CDR3**	**#**
E12	TRBV9	TRBJ1-5	CASSAGPKNQPQHF	4781	TRAV12-2	TRAJ23	CAVRNQGGKLIF	2058				
F8	TRBV9	TRBJ1-5	CASSAGPKNQPQHF	1773	TRAV12-2	TRAJ23	CAVRNQGGKLIF	1062				
A6	TRBV4-2	TRBJ2-7	CASSPGAIEGISYEQYF	3832	TRAV12-3	TRAJ5	CAMSLRSRTGRRALTF	895				
E9	TRBV4-2	TRBJ2-7	CASSPGAIEGISYEQYF	7550	TRAV12-3	TRAJ5	CAMSLRSRTGRRALTF	474				
B2	TRBV28	TRBJ2-2	CASSYGDPGGLDGELFF	2157	TRAV27	TRAJ39	non-productive	878	TRAV8-4	TRAJ48	CAVSLISNFGNEKLTF	866
E1	TRBV28	TRBJ2-2	CASSYGDPGGLDGELFF	3222	TRAV8-4	TRAJ48	CAVSLISNFGNEKLTF	1286				
H3	TRBV28	TRBJ2-2	CASSYGDPGGLDGELFF	97	TRAV8-4	TRAJ48	CAVSLISNFGNEKLTF	59	TRAV27	TRAJ39	non-productive	16
E5	TRBV30	TRBJ2-1	CAWTLGGNEQFF	4272	TRAV13-1	TRAJ45	CAASRSTAGGGADGLTF	563	TRAV22	TRAJ9	CAGRAGGFKTIF	276
E7	TRBV30	TRBJ2-1	CAWTLGGNEQFF	7412	TRAV13-1	TRAJ45	CAASRSTAGGGADGLTF	78	TRAV22	TRAJ9	CAGRAGGFKTIF	33
G1	TRBV30	TRBJ2-1	CAWTLGGNEQFF	258	TRAV13-1	TRAJ45	CAASRSTAGGGADGLTF	42	TRAV22	TRAJ9	CAGRAGGFKTIF	38
H10	TRBV30	TRBJ2-1	CAWTLGGNEQFF	204	TRAV13-1	TRAJ45	CAASRSTAGGGADGLTF	31	TRAV22	TRAJ9	CAGRAGGFKTIF	16

Supplementary Table 5. Determination of two TCRα genes from single T cells. Four T cell clones were clonally expanded and repeated within the TCR validation set. Multiple TCRα chains were detected in two of these clones. Well location, V and J gene usage, CDR3 sequence, and number of reads are indicated.

Nature Biotechnology: doi:10.1038/nbt.2938

Supplementary Table 7

	# positive /60 positive	Sensitivity	# positive /36 negative	Specificity	Prevalence (% positive CD45RO/total CD45RO)	Postive predictive value	Negative predictive value
IFNγ	55	91.67%	0	100.00%	6.25%	100.00%	99.40%
TNFα	46	76.67%	2	94.44%	35.48%	88.30%	88.00%
IL2	58	96.67%	1	97.22%	65.70%	98.50%	93.89%
IL10	50	83.33%	1	97.22%	2.46%	43.10%	99.60%
IL13	56	93.33%	0	100.00%	26.03%	100.00%	97.70%
IL17	49	81.67%	0	100.00%	4.26%	100.00%	99.20%
FOXP3	54	90.00%	0	100.00%	3.80%	100.00%	99.62%

Supplementary Table 7. Sensitivity, specificity, positive and negative predictive value of single-cell phenotypic detection compared to cytokine capture assays and CD25 expression in the case of FOXP3.

Supplementary Information. Explanation of software for analysis

Paired end fastq files are unpacked and joined by shared regions of overlap. The resulting joined reads are converted to fasta. Paired end fasta files are demultiplexed as barcode-demultiplex.pl --dnafile=<joinedfile.fa>

DNA sequence file in fasta format to produce individual files for each plate/well.
Usage: ./barcode-demultiplex.pl --dnafile

Each well is then analyzed with well-vdjfasta.pl --file=<joinedfile-<plate><well>.fa
Usage: ./well-vdjfasta.pl

```
 --file=seqs.fa     input dna file in fasta
 --dna=1            output a header-annotated dna fasta file
 --aa=1             output a header-annotated aa fasta file
 --c2m=1            output a header-annotated c2m fasta file
 --verbose=1        produce verbose messages while running
 --vdb="alt.fa"   alternative variable segment blast database
 --ddb="alt.fa"   alternative D segment blast database
 --jdb="alt.fa"   alternative J segment blast database
 --cdb="alt.fa"   alternative constant segment blast database
```

Each well is then profiled using
cell-profiler.pl --file=<joinedfile-<plate><well>.dnaH3.fa

Usage: ./cell-profiler.pl

```
 --file          dna sequence file in fasta format
 --do_cytokines  optionally analyze cytokines
 --min_depth     minimum depth for reporting a cytokine or chain
 --min_percenth  minimum percent dominance for asserting beta chain
 --min_percentl1 minimum percent dominance for asserting first alpha chain
 --min_percentl2 minimum percent dominance for asserting possible second
alpha chain
```