Benchmarking of T cell receptor repertoire profiling methods reveals large systematic biases

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Systematic study of T-cell receptor repertoire profiling reveals large methodological biases: lessons from a multicenter study

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SUMMARY

Accurate profiling of T-cell receptor (TCR) repertoires is key to monitoring adaptive immunity. We systematically compared TCR sequences obtained with 9 methods applied to aliquots of the same T-cell sample. We observed marked differences in accuracy and intra- and inter-method reproducibility for alpha (TRA) and beta (TRB) TCR chains. Most methods showed lower ability to capture TRA than TRB diversity. Low RNA input generated non-representative repertoires. Results from 5'RACE-PCR methods were consistent among themselves, while differing from the RNA-based multiplex-PCR results. gDNA-based multiplex-PCR methods also differed from each other. Using an in silico meta-repertoire generated from 108 replicates, we found that one gDNA-based method and two non-UMI RNA-based methods were more sensitive than UMI methods in detecting rare clonotypes, despite the better clonotype quantification accuracy of the latter. This study delineates the advantages and limitations of different TCR sequencing methods, which should help the study, diagnosis and treatment of human diseases.

INTRODUCTION

T-cell receptors (TCR), which drive T-cell activation by antigenic peptide recognition, are heterodimers formed by an α and a β chain[1] produced by somatic V(D)J rearrangements during thymopoiesis[2] of 47V and 61J functional TRA genes and 48V, 2D, 12J functional TRB genes[3]. The stochastic V(D)J recombination generates a combinatorial diversity that is further increased by random nucleotide excision and addition at the V(D)J junctions. The independent recombination and subsequent pairing of TRA and TRB chains add an additional level of combinatorial diversity. Recently, computational chain pairing experiments suggested that the potential diversity of the paired repertoire is $\sim 2 \times 10^{19}$ TCRs[4], while the number of different TRB clonotypes in an individual has been estimated to range from 10^6 to 10^8 [5–7]. The TCR repertoire is dynamic, as lymphocytes are continuously generated, die and expand in response to stimulation, and reflects both an individual's immune potential and history.

Analysis of the TCR repertoire by deep sequencing (TCRseq) is increasingly used to measure lymphocyte dynamics in health, in pathological contexts such as autoimmune disease, infections and cancer[8–14], and following interventions such as vaccination[11,15–18]and immunotherapy[19–22], with the goal of identifying TCR biomarkers of disease or of clinical response to treatment and to stratify patients for precision medicine[23]. These diverse applications have different requirements in terms of sensitivity, specificity and depth. Accurately capturing the TCR repertoire therefore presents great challenges. A large number of TCRseq methods have been developed. They are all complex multistep protocols, and each step may have a profound impact on the TCRseq data and hence on their interpretation[24]. Methods can be broadly classified as DNA- or RNA-based, and the latter can be categorized as using multiplex PCR (mPCR) with panels of V and J primers[5,25,26] or using rapid amplification of cDNA-ends by PCR (RACE-PCR)[14,27–29] optionally incorporating unique molecular identifiers

(UMI) to limit PCR amplification bias and sequencing errors[14,29–31]. Each method has potential advantages and limitations[27,32–35]. Specifically, DNA-based methods are believed to be more quantitative and can be used in situations where RNA quality may not be guaranteed. In contrast, RNA-based methods are believed to be more sensitive because of the presence of multiple mRNA copies per cell, and also are more amenable to UMI incorporation[36]. However, the relative robustness and accuracy of the different approaches have not been systematically compared. Here, we compared 9 different TCRseq library preparation protocols by analyzing the TCR repertoire of aliquots of the same T-cell sample.

RESULTS

Experimental design to evaluate the robustness of human T-cell receptor repertoire analysis

We set out to compare 9 different academic or commercial protocols for library preparation and sequencing (**Supplementary material and methods**; **Supplementary Table 1**) based either on RACE-PCR (RACE-1 to RACE-6) or on multiplex-PCR (mPCR-1 to 3). We sequenced nucleic acids from $CD4^+CD25^-CD127^+$ effector T-cells (**Supplementary Fig.1a**) sorted from two healthy donors (experiments A&B). In experiment A, we evaluated the accuracy and sensitivity of the different methods by spiking donor A T-cell RNA (RACE-1 to RACE-6 and mPCR-3) or DNA (mPCR-1 and mPCR-2) aliquots with different amounts of RNA or DNA from Jurkat cells (**Supplementary Fig.1b**). In experiment B, we analyzed the impact of decreasing amounts of the input material quantity by processing donor B RNA aliquots of 100 ng and 10 ng (**Supplementary Fig.1c**). In both experiments, the $CD4^+CD25^-CD127^+$ T-cells were sorted, and the RNA and DNA were extracted and aliquoted in a single laboratory. Triplicates of aliquots were distributed to service providers and academic laboratories. Raw and/or pre-filtered sequences data were all processed using MiXCR[37].

We obtained from 5.10^5 to 2.10^6 reads per aliquot depending on the method (**Supplementary Fig.2a-b**). Numbers of unique V, J and VJ sequences as well as UMI distribution for RACE-1 and RACE-2 (**Supplementary Fig.2a-c**) were comparable between all the methods. Numbers of TCR sequences and clonotypes were correlated in a method-dependent manner, but not globally, suggesting that the sequencing depth required for a given number of clonotypes is method-dependent (**Supplementary Fig.2d**).

Replicability and reproducibility differ among methods

For each method, we first analyzed the proportion of reads that were identified as TCRs (**Fig.1a and Supplementary Fig.2**). For 7/9 methods, we observed 20 to 60% of non-aligned reads, which were mainly explained by no V and/or J sequence identification. TCR sequences had a high-quality score (phred > 30, **Fig.1b**) and contained less than 1% PCR errors (**Fig.1c**), except for RACE-2, RACE-6, mPCR-2 and mPCR-3. Note that these parameters could not be assessed

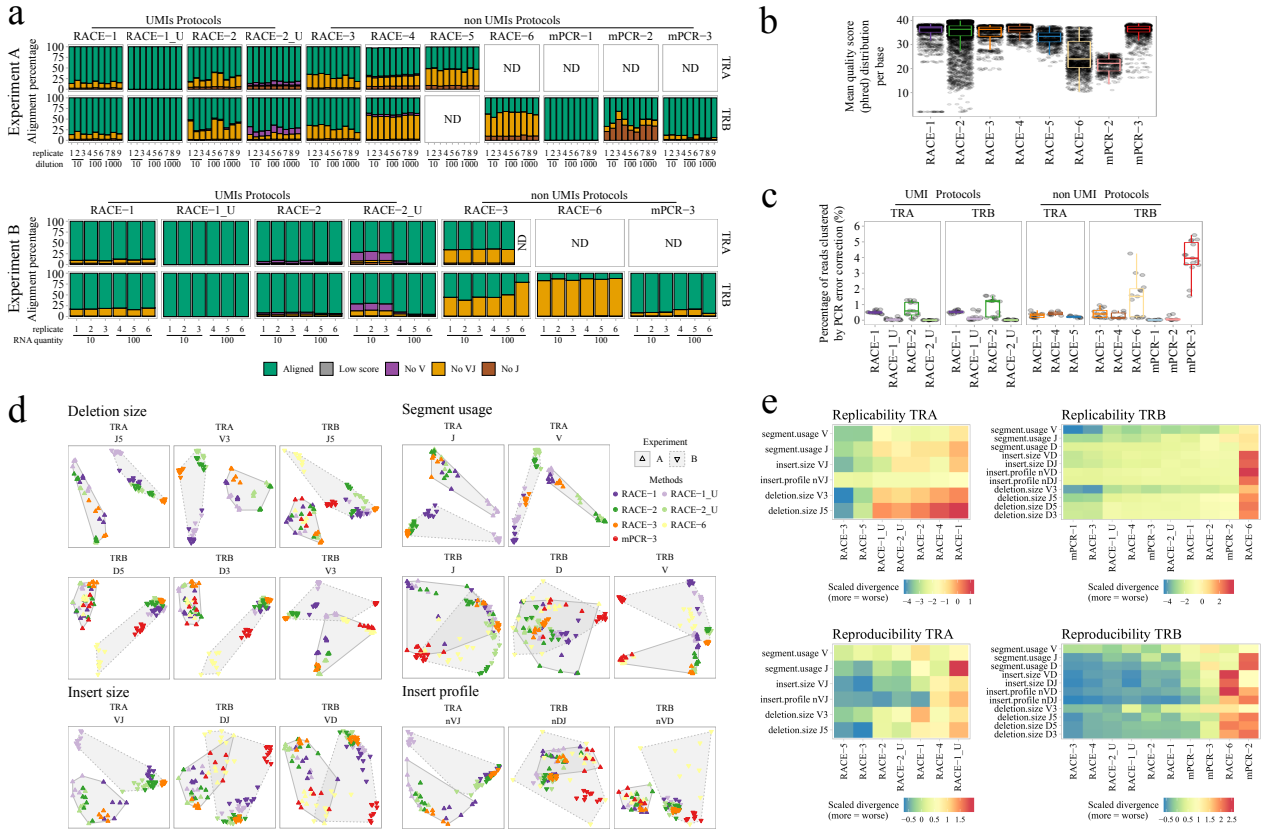

Fig. 1: Performance statistics and VDJ rearrangement model of each method for experiments A and B.

for one of the commercialized mPCR-1 for which undisclosed proprietary pre-processing of the data is performed.

Using a VDJ rearrangement model (Methods), we computed 17 rearrangement parameters for TRA and TRB sequences from experiments A&B (**Supplementary Fig.3**) and calculated Jensen-Shannon Divergence (JSD) distances between samples per parameter. Multi-Dimensional Scaling (MDS, **Fig.1d**) showed that, within each experiment, samples obtained with the same method clustered together, suggesting that each method imposed its methodological imprint on the repertoire profile.

We further compared the different library methods' replicability (i.e. the similarity among data obtained with the same method) and reproducibility (i.e. the similarity among data obtained with different methods) using JSD as a measure of the distance between datasets[38]. **Figure 1e** showed that for TRB, both the replicability and reproducibility of RACE-6 and mPCR-2 are lower than for all the other methods tested. However, when considering TRA, replicability is higher for RACE-3 and RACE-5 and reproducibility is higher for RACE-3, RACE-5 and RACE-2 (with and without UMI). Since RACE-6 showed extremely low replicability for TRB samples and was not reproduced by any other methods, we excluded it from further analysis. Altogether, our results showed that many fundamental parameters of the TCR repertoire, as well as inter-sample replicability and reproducibility, vary between the different methods tested.

The observed TRBV gene usage varies between RACE- and multiplex-PCR RNA-based methods.

We compared the TRBV usage obtained from the sequencing data with the percentage of TRBV protein expression quantified by flow cytometry (FC) (**Fig.2a and Supplementary Figs.4a-b**). mPCR-1 data were highly correlated with FC data (**Fig.2b**, R^2>0.9, $P < 5.10^{-12}$), which likely reflects the undisclosed proprietary filtering by the provider. All other methods also showed a significant R^2 Pearson correlation score ranging from 0.4 to 0.8, $P < 0.05$) with TRBV protein expression (**Fig.2a-b)**, except for mPCR-3 (R^2<0.2, $P > 0.05$). The Pearson correlation of TRBV gene usage within replicates prepared with the same method (**Fig.2c**) was high (R^2>0.9). However, clustering showed that mPCR-3 formed a distinct cluster with a low correlation score (R^2<0.5) with other methods. The RACE methods data were highly correlated between each other (R^2>0.8), except RACE-1 and RACE-1_U, which had a lower correlation (0.6<R^2<0.7). mPCR-1 and mPCR-2 formed an independent "DNA cluster" with an R^2>0.6 when compared to RACE replicates and a low correlation with mPCR-3 (R^2<0.4). This low correlation with mPCR-3 could in part be explained by a skewed TRBV9, TRBV29-1 and TRBV20-1 usage (**Supplementary Fig.4c**). Spearman correlation scores were higher between FC data and mPCR-3 as well as RACE-1, and globally between the methods (**Supplementary Fig.4d-e**). In summary, RACE-PCR

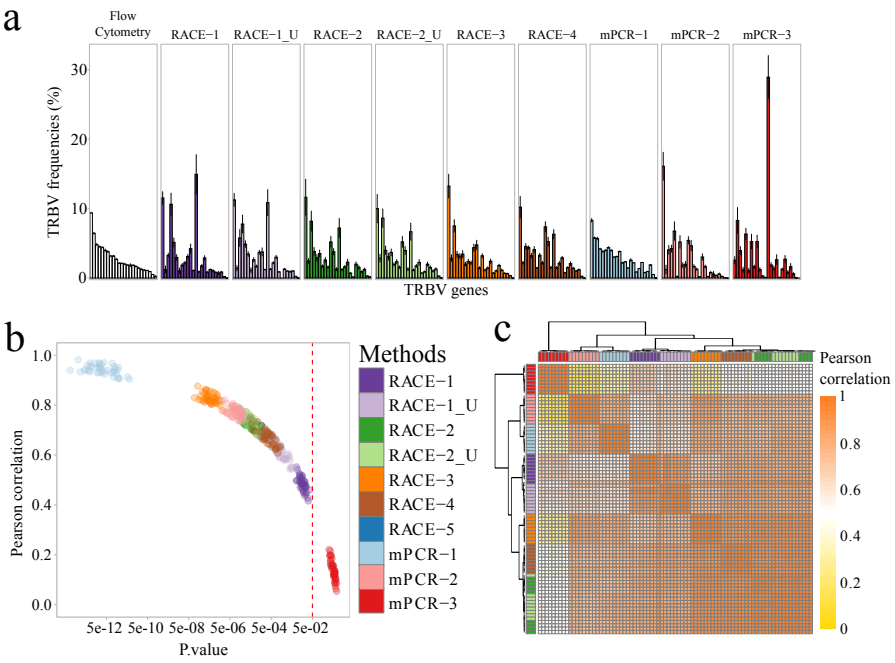

Fig. 2: TRBV usage comparison between flow cytometry and TCRseq.

methods and gDNA-based mPCR methods showed comparable TRBV usage results, in contrast with the mPCR-3 RNA based method.

Robustness of TRA and TRB detection is method-dependent

We compared the similarity and composition of the 1% most predominant clonotypes (1%_MPC) detected by each method. The Morisita-Horn similarity index (MH) was calculated for each replicate across all the methods for both TRA (Fig.3a-left) and TRB sequences (Fig.3a-right). TRA repertoires from RACE-3 and RACE-5 clustered together, inter- and intra-replicates having a high degree of similarity (MH≈0.8). RACE-1, RACE-2 and RACE-4 have a lower inter- and intra-method similarity (0.2<MH<0.5), but a higher similarity with RACE-3 and RACE-5. Comparable clustering was obtained with the Jaccard similarity index (JSI), a measure independent of clonotype frequency (Supplementary Fig.5a). For the TRB repertoires, MH scores were low when comparing RACE and mPCR protocols (MH≈0.36), but high within the RACE cluster (0.6>MH>0.9). There was poor similarity between the results of the three mPCR methods, regardless of the template. Differences between RACE and mPCR methods disappeared when calculating the JSI, suggesting a bias in clonotype frequency, as expected when comparing RNA- with DNA-based methods, but less when comparing RNA-based methods. Similar results were obtained by iteratively increasing the percentage of clonotypes (Supplementary Fig.5b). Rényi diversity profiles (Supplementary Fig.5c) showed comparable results for TRB with all the methods, but the diversity of TRA varied depending on the method. However, the potential diversity estimated using Chao extrapolation was variable between methods (Supplementary Fig.5d).

To test a possible bias in capturing the TRA diversity for some methods, we pooled and compared the three spiking replicates per method from experiment A, as suggested by Greiff

et al.[24]. The MH similarity significantly increased for all the RACE-based methods for TRA (**Fig.3b-top**) (except RACE-3) and for TRB (**Fig.3b-bottom**), with the TRA MH similarity remaining lower than that of TRB. Similar observations were made for mPCR replicates. This suggests that for a given depth of sequencing, the TRB diversity is better captured than that of TRA.

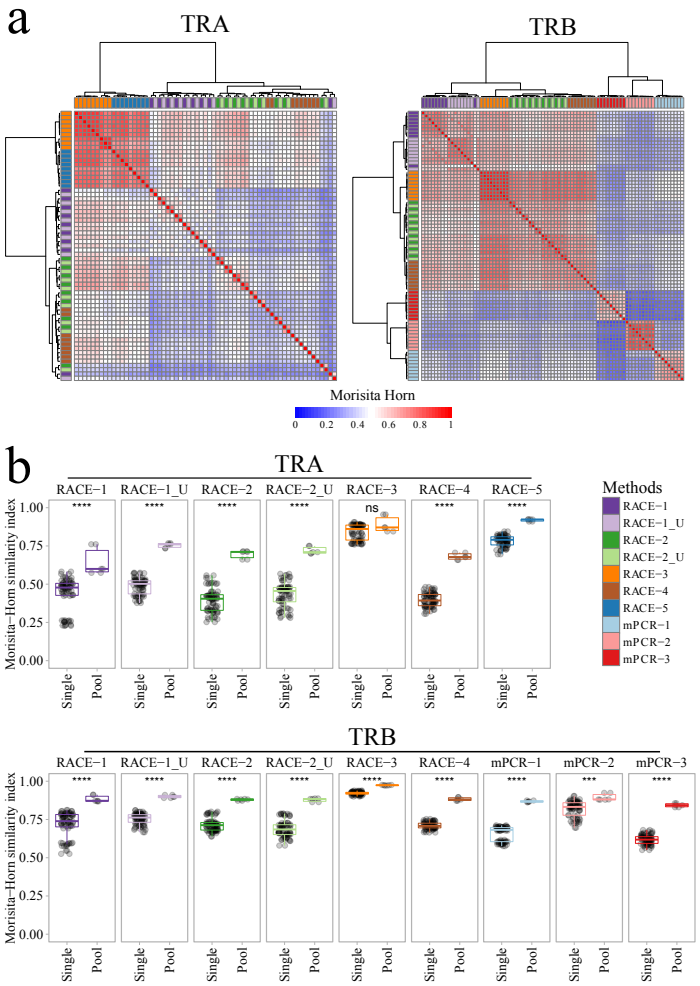

Fig. 3: The reproducibility of detection of major TCR clonotypes by different methods.

Detection sensitivity of rare TCRs depends on the method

To determine the accuracy of the different library amplifications for different clonotype frequencies, we compared the observed frequencies of the TCR from the Jurkat spike-in to their theoretical frequencies of 1/10, 1/100 and 1/1000. (**Supplementary Fig.1b**). TRA observed frequencies were on average 3 times lower than expected (**Fig.4a-top**; **Supplementary Table 2**

and Supplementary Fig.6a). In contrast, TRB frequencies were on average 3 times higher than the theoretical percentage, except for mPCR-1 (Fig.4a-bottom; Supplementary Table 2 and Supplementary Fig.6a). For most of the methods, except RACE-1_U, RACE-4 and mPCR-3, the ratio between the different dilutions was maintained, as shown by the mean slope values close to 1 (Fig.4b).

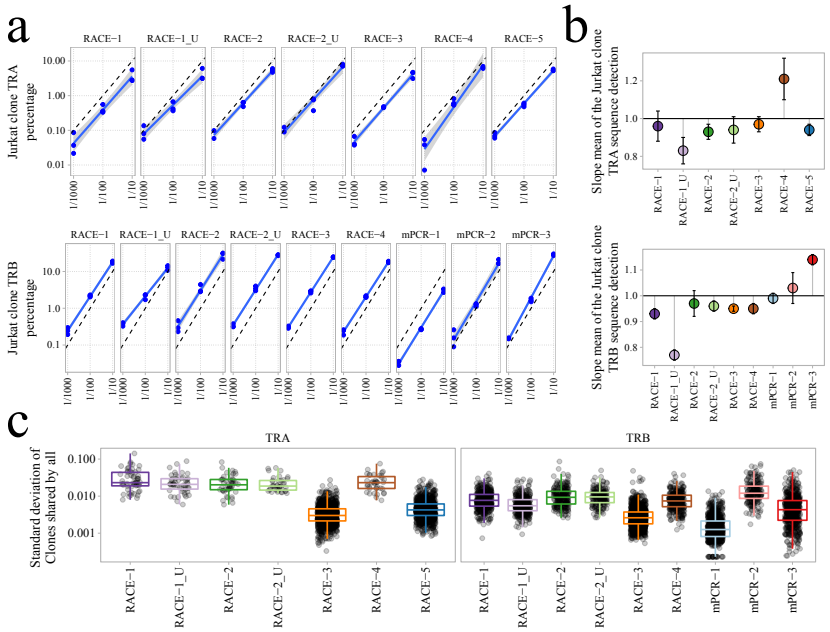

Fig. 4: Sensitivity of TCR sequence detection by different methods.

We then compared the inter-sample variation in clonal frequency for those TCR sequences shared between all replicates of an individual method (excluding the Jurkat clone). Figure 4c represents the standard deviation of the frequency of each shared clonotype (dots) per method (see details in Supplementary Fig.6b-d). For TRA, RACE-3 and RACE-5 had the highest number of clonotypes shared between the 9 replicates and the lowest standard deviation. For TRB, all the methods captured a high number of shared clonotypes, and mPCR-1 and RACE-3 had the lowest standard deviation. Finally, pooling all the clonotypes from all the replicates, we identified 9 TRA and 31 TRB clonotypes shared by all the replicates of all methods, corresponding to the most predominant clonotypes (Supplementary Fig.7). RACE-3, RACE-5 (both RNA-based) and mPCR-1 (DNA_based) showed the lowest inter-sample variability in TCR frequency.

211

212 The quantity of starting material impacts TCR diversity capture

213 One major limitation when analyzing TCR repertoire is the number of T-cells that can be
214 analyzed. Focusing on 4 RNA-based methods, we analyzed the influence of input RNA quantity
215 on TRA and TRB repertoires (Supplementary Fig.1c). We compared two sets of samples, one
216 containing 10 ng or 100 ng (corresponding to 10^4 and 10^5 cells, respectively). For all the
217 methods, the richness was higher with large (100 ng) than small (10 ng) samples
218 (Supplementary Fig.8a). Rényi diversity profiles (Supplementary Fig.8b) showed that when
219 alpha < 2 (i.e. when the diversity metric is influenced by rare clones), the diversity of small
220 samples is less than that of larger ones. In contrast, at alpha = 2 (Simpson index) or above,
221 diversity profiles of both samples overlap. Thus, a low RNA input influences the number of rare
222 TCR sequences detected, but not the distribution of the more abundant TCRs.
223 Finally, we evaluated the inter-sample similarity as a function of RNA input quantity by
224 calculating the MH index with either the TRVJ combination usage (VJ_usage), all clonotype
225 frequencies (Overall), or with the frequencies of the 1% most predominant clonotype
226 (1%_MPC) (Supplementary Fig.8c-middle). For TRA, the similarity between 10 ng replicates was
227 lower at the level of VJ usage and of all clonotypes compared with that between 100 ng
228 replicates (Supplementary Fig.8c-top&bottom). For TRB, the results were comparable
229 regardless of the quantity (MH>0.5). When focusing on the 1% MPC, the similarity was
230 comparable regardless of the quantity for both TRA and TRB. These results indicated that RNA
231 quantity impacts rare clonotype detection.
232

233 Reliability and sensitivity of each method highlighted using an in silico meta-repertoire

234 One unavoidable issue when aiming at capturing the diversity of a repertoire is sampling, i.e.
235 only a fraction of the cells are analyzed and then a fraction of their nucleic acids[24]. To better
236 assess the ability of each method robustly to capture rare and frequent clonotypes, we took
237 advantage of the fact that altogether we generated 45 TRA and 63 TRB replicates of the same
238 cell sample. We aggregated these results to generate an in silico meta-repertoire. To ensure
239 the accuracy of the TCR sequences composing this meta-repertoire, we removed singletons
240 and kept clonotypes found by at least 3 methods.

241 We first analyzed how many of the clonotypes present in this meta-repertoire were detected
242 by each method. For TRA (**Fig.5a-left**), RACE-3 and RACE-5 datasets included up to 50% of the
243 meta-repertoire clonotypes (MRC) compared to 10 to 20% for the other RACE method datasets.
244 Similar results were found for TRB (**Fig.5a-right**). We then computed for each method the
245 fraction of MRC found in 0, 1, 2, 3 etc. up to 9 replicates. The dot-heatmaps (**Fig.5b**) showed
246 that for TRA, RACE-3 and RACE-5 clearly outperformed the other methods, capturing up to 40%
247 of the MRC in all 9 replicates (**Fig.5b-left**; Replicate number=9) and missing (i.e. never captured
248 in any of the 9 replicates) less than 1% of the MRC (**Fig.5b-left**; Replicate number=0). The other
249 RACE protocols detected only 1% of MRC in all 9 replicates and missed 15 to 20% of the MRC

(Fig.5b-left). In contrast, there was much less difference between the methods for TRB (Fig.5b-right).

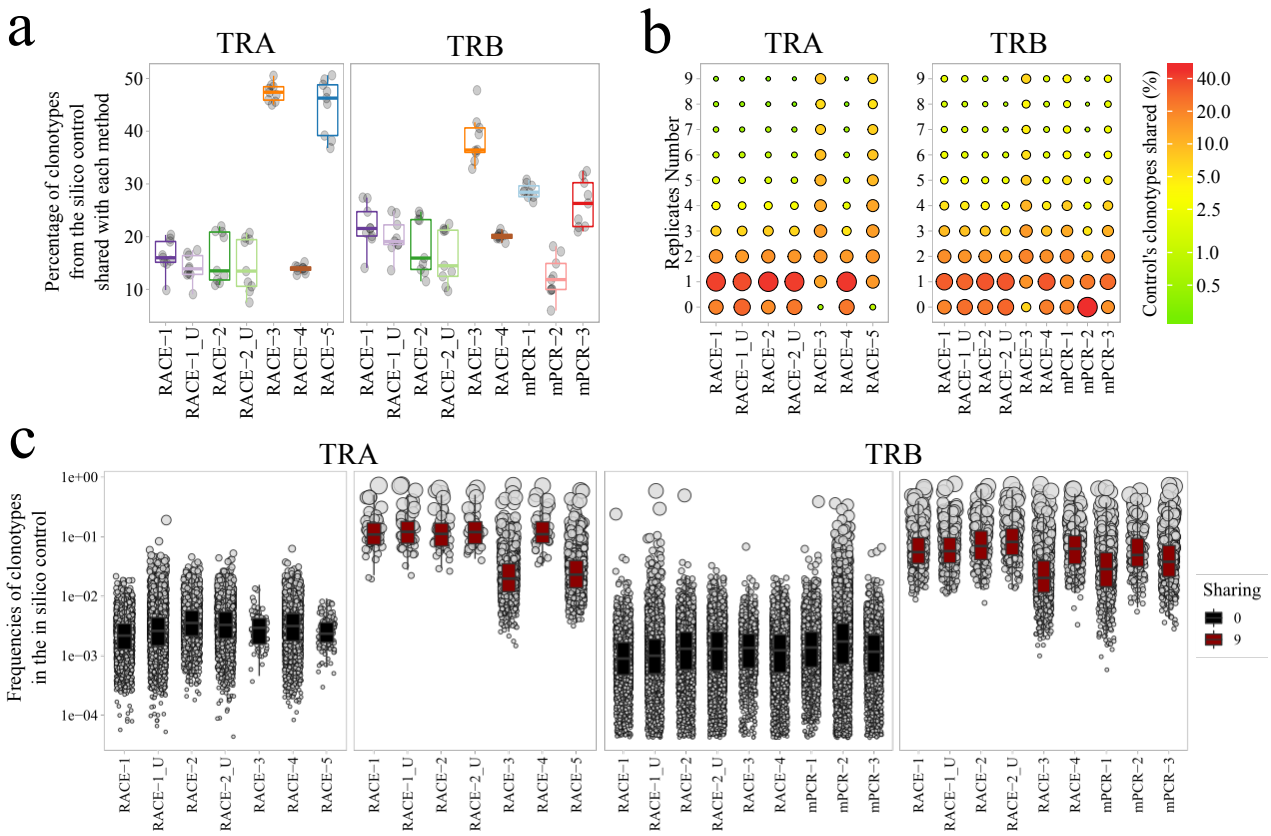

Fig. 5: Sharing with robust and representative meta-repertoire.

Finally, we analyzed the frequency of MRC TCRs that were detected or not by each method (Fig.5c and Supplementary Fig.9). For TRA (Fig.5c-left), the frequency of MRC found in 9 replicates (red boxplots) ranged from 1% to 0.001% for RACE-3 and RACE-5 and from 1% to 0.05% for the other methods. In contrast, clonotypes not detected in any replicates (black boxplots) were present at 10- to 100-fold lower abundance. A similar overall pattern was seen for TRB, although the frequencies were shifted to a lower range. This analysis suggested that RACE-3 and RACE-5 had increased sensitivity, and hence were able to detect a larger proportion of clonotypes at lower abundances. These differences were more evident for TRA than for TRB (Fig.5c-right). The other methods compared behaved very similarly to each other. Importantly, those results were independent of sample size (Supplementary Fig.10).

DISCUSSION

Interpreting the TCR repertoire is an increasingly important tool in understanding the underlying causes of immune-mediated diseases and in assisting the development of new immunotherapeutic strategies. However, despite hundreds of TCRseq studies in the last decade using a variety of different methodologies, there has been no systematic study comparing them.

In this work, we compared methods developed by academics, at a time when there was little or no reliable commercial service provision, with some currently available commercial methods. Both RNA- and gDNA-based methods were included. To avoid mis-implementation of protocols, each method (including appropriate pre-processing of sequence data) was performed by the laboratory or commercial provider (except for kit providers) that developed them.

Unexpectedly, some consistent differences were observed in TRBV usage when compared to FC measurement of TRBV-encoded proteins, especially for RNA-based profiling. This might reflect bias in amplification of RNA transcripts according to their expression levels, more efficient transcription of some V genes, or differences in nonsense-mediated decay[39]. Further studies, using single-cell RNAseq may shed light on this phenomenon.

Working with human samples often imposes limits on the number of available T-cells. Notably, lymphopenia is a common feature in people undergoing treatment (transplantation, immunosuppressive therapy) or with autoimmune disease[40] and infections. Additionally, T-cell subsets of interest, as well as available counts of tumor-infiltrating T-cells, may be limited. Therefore, it is important to identify which methods provide reliable TCRseq profiles for small numbers of T-cells. In this context, we observed that, regardless of the method, starting from

286 a highly polyclonal population, the initial amount of material is critical to obtaining
287 representative results, notably in terms of diversity and rare clone detection.
288 Although our study focused on polyclonal CD4 T-cells from healthy repertoires, we analyzed a
289 wide range of global and sequence-specific repertoire parameters, including V(D)J gene usage,
290 junctional diversity, repertoire diversity and sequence sharing. These parameters are all
291 relevant to any other alpha/beta T-cell populations, as indeed are all parameters routinely used
292 to analyze repertoires of samples from pathological and clinical human samples[41].
293 Because our study incorporated multiple replicates tested with each method, we were able to
294 explore method replicability, i.e. the ability of each method to reproduce the same repertoire
295 from different sub-samples from the same individual. Our results showed that, except mPCR-
296 3, all the methods provided consistent results among replicates. We also evaluated the
297 reproducibility, i.e. the extent to which different methods record the same results when applied
298 to the same sample. We observed a low degree of TRB clonotype overlap between repertoires
299 amplified from gDNA and RNA (cDNA), perhaps reflecting differences in gDNA and RNA copy
300 numbers. The four RACE methods produced relatively similar repertoires as revealed by the
301 Morisita-Horn index. The mPCR on gDNA showed low reproducibility between methods,
302 suggesting that the choice of multiplexing primers might bias the amplification of some
303 clonotypes, as suggested previously[34]. However, most RACE methods (not tested for mPCR)
304 had a lower efficiency in capturing TRA rather than TRB diversity, which may reflect the 2- to 3-
305 fold lower number of TRA transcripts than TRB transcripts[31].
306 Finally, sensitivity is important for the study of circulating blood T-cells, especially when the
307 goal is to track a few expanded clones associated with infection or autoimmunity, or in
308 response to treatment. However, assessing sensitivity based on sample overlap is a complex
309 performance metric, since it is impacted by experimental variability, but also by sampling. In

order to tackle this problem directly, we generated an in silico meta-repertoire which provided a more robust platform with which to directly compare the sensitivity performance of the different methods. Interestingly, using this standard, we found that two non-UMI methods (RACE-3 and RACE-5) had greater sensitivity than UMI-based methods (RACE-1 and RACE-2) and were able to detect clonotypes at a 10-fold lower frequency. In part, this results from the reads-per-UMI cutoff, which may lead to a decrease in observed TCR diversity if sequencing coverage is not sufficient. For example, introducing a hard cutoff which discards all UMIs with less than 5 reads leads to a decrease in observed TCR diversity. UMI-based methods may be more accurate for assessing clonotype frequency, in line with their use to quantify and correct for PCR errors and bias[42,43]. Furthermore, a threshold of 2-4 reads per UMI efficiently protects against artefacts and cross-sample contamination[44], which becomes critical with tighter cluster density on modern Illumina machines. UMI-based methods may require several replicates or higher sequencing coverage to consistently and unambiguously identify rare TCR sequence clonotypes. Noteworthy, both RACE-1 and RACE-2 methods performed better after UMI correction (see Table 1).

Such in silico standards may be of value in further comparative TCRseq method evaluation, although ideally synthetic repertoires recapitulating at least the extent of the TRAVJ and TRBVJ combinations and distributions may provide an even more robust alternative. Two such approaches have been proposed for specific clone detection in Minimal Residual Diseases[45,46] as well as for the BCR, but not TCR, repertoire[47], still at a very low diversity level. The construction of such gold standard repertoires is currently very costly and remains a major challenge that the Adaptive Immune Receptor Repertoire Community (AIRR-C)[48], engaged in AIRR-seq standardization[49–51], may tackle in the future. Finally, in this study some data were pre-processed using proprietary (mPCR-1, mPCR-3) or published[30,52] (RACE-1_U and RACE-2_U)

tools and then aligned and error-corrected using MiXCR (v2.1.10)[37]. To further optimize TCR data accuracy, it would also be interesting to benchmark available software analysis tools, especially regarding UMI analysis and sequence alignment. Our datasets generated using different methods should be a valuable complement to using datasets generated purely in vitro[53,54].

In conclusion, the take-home messages from this work are the following. Firstly, there are satisfactory TCRseq methods based on either DNA or RNA input, and in both cases the amount of material impacts both diversity and the detection of rare clones. Secondly, various methods are optimal for detecting maximal diversity, while others most accurately quantify the abundance of specific clonotypes. For the latter, UMI-based methods are potentially more accurate, although they could miss relevant but rare clones. In contrast, non-UMI RACE methods are more sensitive in capturing rare clones, especially for TRA. Thirdly, the availability of raw data is crucial in allowing reliable and reproducible in-depth analyses of TCR repertoires; the mPCR-1 service provider does not provide access to raw sequence data, while mPCR-1 and mPCR-3 do not disclose the proprietary pre-processing filters. In contrast, the RACE-2 provider provides raw data and all preprocessing algorithms. We summarized our results as well as practical aspects in Table 1. Regarding the results, we calculated for each method a rank value for Replicability, reliability and sensitivity based on various measures (Table 1 and Supplementary file). We also summarized cost per sample, presence of controls or standards, format of the method and raw data availability. The Table 1 highlight the advantages and disadvantages of the different methods which could serve as guidance for end-users. Improved and more sophisticated data analyses are essential to extract the full power of TCR repertoire data. We anticipate that now that TCR sequencing has come of age, the next key developments

in the field will come from novel methods of data analysis, as has been the case in the related field of global transcriptomics.

TR chain	Method	Replicability	Reliability	Sensitivity	Cost per sample	Controls & standards	Format type	fastq data availability
TRA	**RACE-1**	7	4	4	~230	-	lab protocol	YES
	RACE-1_U	4	5	4	~230	UMI	lab protocol	YES
	RACE-2	5	4	5	230-280	-	service or kit	YES
	RACE-2_U	4	5	5	230-280	UMI	service or kit	YES
	RACE-3	3	2	3	~150	-	kit	YES
	RACE-4	5	6	4	~150	-	lab protocol	YES
	RACE-5	2	3	3	~300	-	lab protocol	YES
TRB	**mPCR-1**	3	3	3	~350-550*	synthetic TCRs	service or kit	NO
	mPCR-2	6	7	7	~230	-	lab protocol	YES
	mPCR-3	5	5	3	~350-550*	-	service or kit	YES
	RACE-1	6	5	4	~230	-	lab protocol	YES
	RACE-1_U	4	6	5	~230	UMI	lab protocol	YES
	RACE-2	6	6	6	230-280	-	service or kit	YES
	RACE-2_U	6	6	7	230-280	UMI	service or kit	YES
	RACE-3	2	2	3	~150	-	kit	YES
	RACE-4	3	5	4	~150	-	lab protocol	YES

Table 1: Comparative performance of the nine TCRseq molecular methods.

MATERIAL AND METHODS

Blood effector T cell isolation

Peripheral blood mononuclear cells (PBMC) from two healthy blood donors (Etablissement Français du sang; French Blood Center) were obtained with written informed consent for biomedical research. The experiments carried out were in conformity with the Helsinki Declaration on Biomedical Research. Donors A (experiment A) and B (experiment B) were both men, 36 and 54 years old, respectively. $CD3^+CD4^+CD127^+CD25^-$ cells ($CD4^+$ T effector cells) were sorted at the Sorbonne Université laboratory as follows: $CD4^+$ cells were isolated by Lymphoprep (Stemcell®) density gradient and positive selection using the Dynabeads™ CD4 Positive Isolation Kit (Invitrogen®). Enriched $CD4^+$ T-cells were then labeled with anti-$CD3^+$, $CD4^+$, $CD127^+$ and $CD25^+$ antibodies and effector T-cells were sorted on a FACS ARIA II with a purity > 95% (Supplementary Fig.1a).

Jurkat cell culture

The Jurkat cell line with a known TCR (TRAV8-4-CAVSDLEPNSSASKIIF-TRAJ3; TRBV12-3-CASSFSTCSANYGYTF-TRBJ1-2) (clone E6-1), from ATCC, was grown in 5% CO_2, in RPMI 1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin at the Sorbonne Université laboratory.

RNA and DNA extraction

In experiment A, DNA and RNA were both extracted using TRIzol Reagent (Invitrogen®) from 5 million Jurkat cells and 20 million $CD4^+$ T effector cells and, in experiment B, only RNA was extracted using the RNAqueous-Kit (Invitrogen®) from 7.2 million $CD4^+$ T effector cells following the manufacturer's recommendations. DNA concentration and RNA concentration were

measured on a NanoDrop1000 (Thermo Scientific™) and RNA integrity was determined on a Bioanalyzer (Agilent®) with measurements higher than 8. RNA and DNA extraction and validation were performed at the Sorbonne Université laboratory.

Aliquot preparation for method comparison

In experiment A, 100 ng of RNA or DNA from the $CD4^+$ effector T-cells sorted from donor A was split into 3 aliquots that were spiked with different amounts of RNA or DNA from the Jurkat cell line, at ratios of 1/10, 1/100 and 1/1000. Each spiked aliquot was further split into 3 and all replicates were processed by all methods tested (7 for RNA and 2 for DNA; Supplementary Fig.1b). With experiment B, we analyzed the impact of the input material quantity. RNA from sorted $CD4^+$ effector T-cells of donor B was extracted, split into 15 aliquots of 100 ng each and 15 aliquots of 10 ng each and processed in triplicate using 5 of the RNA-based methods (Supplementary Fig.1c). Aliquots were prepared at the Sorbonne Université laboratory and sent to the partners.

Flow Cytometry

Vβ identification was performed on enriched $CD4^+$ effector T-cells from experiment A (see *Blood effector T cell isolation* for enrichment procedure) stained with the IOTest Beta Mark TR Repertoire Kit (Beckman Coulter®) according to the manufacturer's protocol as well as with CD4-APC, CD127-BV421, CD25-PECy7. Data acquisition was performed on a Cytoflex® (Beckman Coulter®) using CytExpert® software. FlowJo® was used for data analysis. Vb frequencies were calculated on $CD4^+CD25^-CD127^+$ gated cells (Supplementary Fig.4a-b). Staining was performed at the Sorbonne Université laboratory.

TCR library preparation and sequencing

The nine protocols for TCR library preparation compared in this study were selected according to at least one the following criteria: published use by groups other than the one who developed it (mPCR-1, mPCR-3, RACE-1, RACE-2, RACE-4 and RACE-5), (ii) their association with well-known analysis tools (RACE-1, RACE-2, mPCR-2) and (iii) commercially available (RACE-2, RACE-3, mPCR-1, mPCR-3). Sequencing protocols were harmonized taking into account published recommendations or recommendations provided by the manufacturer of commercial kits or by the owner or users of the protocol. All protocols are detailed in Supplementary material and methods.

TCR deep sequencing data processing

FASTQ raw data files were obtained from each method, except for Multiplex-1 & 2, for which we obtained, respectively, FASTA file and FASTQ files following proprietary pre-processing. For RACE-1 and RACE-2, UMI pre-processing was performed following protocols published elsewhere[30,31,52]. FASTQ and FASTA files were then processed for TRB and TRA sequence annotation using the MiXCR software[37] (v2.1.10) with RNA-Seq default parameters (*-p rna-seq –s hsa*) as available online.MiXCR extracts TRA and TRB repertoire providing correction of PCR and sequencing errors.

Data analysis

Statistical comparisons and multivariate analyses were performed using R software version 3.5.0 (www.r-project.org). We used the ggplot2 package to generate figures[55], except heatmaps. More complex analyses are detailed in the next section.

Comparing VDJ rearrangement statistics

An empirical VDJ rearrangement model for each method was built as follows. We analyzed clonotype tables to obtain comprehensive statistics of VDJ rearrangements including the frequencies of V/D/J segment usage, number of added N Bases (namely "insert profile", i.e. the probability distribution of having A/T/G/C inserted in the N-region of CDR3 given that we observe a certain base inserted before it) and V/J segment trimming bases, with the IGoR package[56]. This model is built in a 'greedy' way in the sense that it uses best alignments provided by MiXCR rather than running expectation maximization procedures as described in Murugan et al.[57]. We utilized the Jensen-Shannon divergence (JSD) between distributions of VDJ usage to define the following two statistics that we use for comparative analysis of different TCRseq methods: 1) *replicability* measured as the distance between different samples produced by the same protocol and 2) *reproducibility* measured as the distance between samples produced by two different protocols. MDS used for sample mapping was performed on rank-transformed distances to avoid the distorting effect of outliers. All the analyses involve VDJ usage inferred from weighted data (TCR clonotype is weighted by its frequency in the sample) to account for TCRseq method amplification biases.

Similarity analysis

Pearson and Spearman correlations, the Morisita-Horn index[58] (MH) and the Jaccard similarity index[59] (JSI) were used to assess the similarity between samples. The MH index takes into account the relative abundance of species in the sample, while the JSI is a measure of the intersection between two populations relative to the size of their union, and is independent of relative abundances. Both indices vary between 0 (no overlap) and 1 (perfect overlap). JSI and MH were calculated using the DIVO package[60] on R. In order to discriminate indices represented

by a heatmap with the pheatmap package[61], we used a different set of colors. The Pearson and Spearman correlations are presented as yellow/white/orange (**Fig.2c and Supplementary Fig.4e**), MH is presented as blue/white/red (**Fig.3a**) and JSI is presented as purple/yellow/green (**Supplementary Fig.5a**).

Diversity profiling

The diversity was analyzed using two indices. Rényi entropy[62] is a generalization of Shannon entropy, which increases when both species richness and evenness are high. Rényi entropy is a function of a parameter α spanning from (i) the species richness ($\alpha=0$), which corresponds to the number of clonotypes regardless of their abundance, to (ii) the clonal dominance ($\alpha \to +\infty$), corresponding to the frequency of the most predominant clonotype. For $\alpha=1$, the Shannon diversity index is computed. The exponential of the Rényi entropy corresponds to the actual number of clonotypes in the datasets[63] and is used to build a diversity profile[64]. It was computed using the entropy package[65] on R. ChaoE[66] index was calculated with the iNEXT package[67] as a measure of extrapolation of the possible number of clonotypes based on the observed clonotypes. Rarefaction curves were interpolated from 0 to the current sample size and then extrapolated to the size of the largest of samples, allowing comparison of diversity estimates. Interpolation and extrapolation were based on ChaoE multinomial models[68].

Meta-repertoire construction

We generated an in silico meta-repertoire from the sequences obtained from the 108 replicates (45 for TRA and 63 for TRB). This meta-repertoire, for each chain, was designed to minimize biases by (i) pooling all clonotypes from the 9 datasets and removed singletons to avoid introducing noise due to PCR errors, (ii) Selecting non-reprocessed datasets, meaning

before UMI, (iii) keeping only clonotypes found by at least 3 different methods to avoid bias toward one particular method. The threshold was defined to reach a dataset size as close as possible to the original datasets to avoid additional sampling, (iv) normalizing the size of each dataset to the lowest dataset to ensure the same weighting for each method. Completion of the representative meta-repertoire was achieved by pooling all the datasets. This generated a pooled dataset of 14 458 TRA and 18 735 TRB clonotypes.

Data Availability

All the fastq data obtained in this study, including the Jurkat Clone E6-1 (ATCC®TIB-152™) cell line TCR alpha and beta sequences, were deposited in the NCBI Sequence Read Archive repository following MiAIRR standard recommendations[47] under the BioProject ID PRJNA548335. Source data for TCRVb flow cytometry data are provided as **Supplementary Fig.4a-b**. All other data are available from the corresponding author upon request.

Code Availability

All software packages and programs are publicly available and open source. Scripts used to analyze the data with MiXCR are available from https://mixcr.milaboratory.com ; Decombinator from https://github.com/innate2adaptive/Decombinator; MiGEC from https://github.com/mikessh/migec; detailed VDJ rearrangement statistics scripts are available from https://github.com/antigenomics/repseq-protocol-comparison. There is no restriction on the use of the code or data.

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FIGURE LEGENDS

Fig. 1: Performance statistics and VDJ rearrangement model of each method for experiments A and B.

a, The proportion of sequence reads aligned for TRA or TRB genes for each TCRseq replicate per experiment (Experiment A, top, Experiment B, bottom). The bars represent the percentage of TRA and TRB alignment, and the reason for alignment failure is color coded. **b**, Distribution of the reads quality control (QC) for each method over all datasets, computed with fastQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc). **c**, Percentage of reads

665 collapsed after PCR error correction for all samples in the study. For each method, the MiXCR
666 clustering strategy was applied to correct for PCR errors and collapse reads. Each box-plot
667 represents the percentage of clustered reads. **d**, Multi-dimensional scaling (MDS) of V(D)J
668 recombination parameters. MDS was performed based on the Jensen-Shannon Divergence
669 (JSD) calculated between replicates on weighted VDJ segment usage (Segment usage), non-
670 template nucleotide insert size distributions (Insert size), V/D/J segment trimming distributions
671 (Deletion size), and nucleotide frequencies in N-inserts (Insert profile). JSD values were
672 transformed to rank for better visualization. Solid and dotted polygons outline samples from
673 experiments A and B, respectively. Colors represents the different methods as in B (only
674 methods used in both experiments are presented). **e**, Replicability and reproducibility of the
675 TRA and TRB repertoires for each method. The average JSD calculated in D (rows) for TRA (left)
676 and TRB (right) measured between replicates produced by the same method (Replicability, top)
677 or replicates of a given method and all other protocols (Reproducibility, bottom) was used as
678 distance metric to compare different protocols (columns). Columns are sorted according to the
679 mean scaled distance (averaged over all rows) from the lowest (best
680 replicability/reproducibility) to the highest (worst replicability/reproducibility). Distance values
681 are shown using a color scale. Jurkat TCR sequences were removed from datasets for this
682 analysis.
683
684 **Fig. 2: TRBV usage comparison between flow cytometry and TCRseq.**
685 **a**, Flow cytometry and TCRseq TRBV frequencies. Bar plots represent the TRBV frequencies
686 calculated from flow cytometry stained $CD4^+$ T effector cells for the 24 TRBV for which
687 antibodies are available and from the TCRseq data, considering only clonotypes annotated for
688 the same 24 TRBV (original TRBV frequencies were used accordingly). Histograms of the 24

TRBV frequencies are organized by decreasing order using frequencies obtained by flow cytometry as a reference reference (TRBV20-1, TRBV19, TRBV12-3/4, TRBV28, TRBV2, TRBV3-1, TRBV30, TRBV6-5/9, TRBV9, TRBV5-1, TRBV4-1/2, TRBV27, TRBV29-1, TRBV6-6, TRBV11-2, TRBV10-3, TRBV25-1, TRBV6-2, TRBV18, TRBV5-5, TRBV14, TRBV5-6, TRBV13, TRBV4-3). **b**, TRBV usage correlation between flow cytometry and TCRseq. Pearson's correlation of the TRBV frequencies between the 5 flow cytometry datasets and the 9 TCRseq replicates was calculated for each method. The plot is represented by the correlation score (y-axis) and the *P*-value (x-axis) of the correlation, allowing the classification of the methods. **c**, Heatmap of the Pearson correlations between each replicate for the distribution of TRBV gene usage (n=62). The Euclidean distance was used for hierarchical clustering as a color-coded matrix ranging from 0 (yellow, maximum dissimilarity) to 1 (orange, maximum similarity). Jurkat TCR sequences were removed from datasets for this analysis.

Fig. 3: The reproducibility of detection of major TCR clonotypes by different methods.

a, Heatmaps of the Morisita-Horn similarity index (MH). MH scores were calculated between each replicate across all methods for the top 1% of most predominant clonotypes (MPC) for TRA (left) and TRB (right). The Euclidean distance was used for hierarchical clustering as a color-coded matrix ranging from 0 (blue, maximum dissimilarity) to 1 (red, maximum similarity). **b**, Comparison between individual replicates (Single) and pooled replicates (Pool) by the MH similarity index. Datasets from replicates of the same dilution were pooled for each method to get 1 pooled sample per dilution. Singletons (count=1) were removed; MH similarity scores were calculated for the top 1% of most predominant clonotypes for TRA (left) and TRB (right). Jurkat TCR sequences were removed from datasets for this analysis.

713 Fig. 4: Sensitivity of TCR sequence detection by different methods.

714 **a**, Jurkat clone percentage. Jurkat TRA (top) and TRB (bottom) clonotype percentages were
715 calculated for each experiment per dilution (1/10, 1/100 and 1/1000 spike-in) and are
716 represented by the blue dots. The blue line represents linear regression and the black dashed
717 line represents the theoretically expected percentage. **b**, Slope of the Jurkat tracking linear
718 regression. Slope was computed between dilution with standard deviation by method for TRA
719 (top) and TRB (bottom). **c**, Standard deviation of the clonotypes shared among the 9 replicates
720 (except Jurkat clone) per method, for TRA (left) and TRB (right).

721

722 Fig. 5: Sharing with robust and representative meta-repertoire.

723 **a**, Replicate sharing fraction in meta-repertoire repertoire (focus on meta-repertoire
724 clonotypes) for TRA (left) and TRB (right). The values represented correspond to the percentage
725 of clonotypes from each replicate per method found in the meta-repertoire, median and the
726 1st and 3rd quartiles are shown. **b**, Replicability of replicate methods with meta-repertoire for
727 TRA (left) and TRB (right). By chain, heatmaps on the left represent the fraction, which
728 corresponds to the percentage of meta-repertoire clonotypes found in 1 to 9 replicates per
729 method (0: unseen in any of the replicates). **c**, Distribution of meta-repertoire clonotypes in the
730 replicates by methods for TRA (left) and TRB (right). Each dot represents a meta-repertoire
731 clonotype and the boxplot represents the average frequencies. Black boxplots with
732 corresponding gray dots represent the unseen clonotypes (0) and red boxplots with
733 corresponding gray dots represent the clonotypes found by the 9 replicates (9). Each method
734 is represented independently. Jurkat TCR sequences were removed from datasets for this
735 analysis.

736

737 **Table 1: Comparative performance of the nine TCRseq molecular methods.** For each method,
738 an average rank score for TRA (top) and TRB (bottom) sequencing has been calculated for
739 Replicability, Reliability, and Sensitivity (three first column) and practical information have been
740 summarized (4 last columns). Ranks have been calculated as the average of the ranks for results
741 from Fig. 1*e*, 2*c*, 3*b*, 4*c* for "Replicability"; Fig. 1*e*, 2*b*, 4*b*, 5*a*, 5*b* for "Reliability"; Fig. 4*c,* 5*b* &
742 Supplementary Fig. 2a, 5c for "Sensitivity". Rank values are comprised between 2 (best) and 7
743 (worst) and represented as bars with their values. Details are provided as Supplementary
744 information. Cost per sample" is expressed in USD as per current prices for a depth of 1 million
745 TCR sequences per sample on a 25 million reads sequencing format. The costs cover reagents
746 for library preparation to sequencing. *mPCR1 and mPCR3 price ranges correspond to the cost
747 for either purchasing kits (lowest price) or service up to sequencing and basic data analyses
748 from the provider.