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

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45 **Author contributions:** PB, VQ, ESE, AND, IU, MI, TO, AE, SD, AR, KK, PR performed the

46 experiments and raw data pre-processing. PB, VQ, MS and MI analyzed the data. EMF, DMC,

47 BC, DCD and DK designed the experiments. PB, DK and EMF wrote the manuscript with input

48 from all authors. DK and EMF conceived the study, which was supervised by EMF. DK, BC, AE,

49 DCD, DMC, KK and MS obtained funding for the study.

SUMMARY

52   Accurate profiling of T-cell receptor (TCR) repertoires is key to monitoring adaptive immunity.

53   We systematically compared TCR sequences obtained with 9 methods applied to aliquots of

54   the same T-cell sample. We observed marked differences in accuracy and intra- and inter-

55   method reproducibility for alpha (TRA) and beta (TRB) TCR chains. Most methods showed lower

56   ability to capture TRA than TRB diversity. Low RNA input generated non-representative

57   repertoires. Results from 5'RACE-PCR methods were consistent among themselves, while

58   differing from the RNA-based multiplex-PCR results. gDNA-based multiplex-PCR methods also

59   differed from each other. Using an in silico meta-repertoire generated from 108 replicates, we

60   found that one gDNA-based method and two non-UMI RNA-based methods were more

61   sensitive than UMI methods in detecting rare clonotypes, despite the better clonotype

62   quantification accuracy of the latter. This study delineates the advantages and limitations of

63   different TCR sequencing methods, which should help the study, diagnosis and treatment of

64   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

89   (UMI) to limit PCR amplification bias and sequencing errors[14,29–31]. Each method has potential

90   advantages and limitations[27,32–35]. Specifically, DNA-based methods are believed to be more

91   quantitative and can be used in situations where RNA quality may not be guaranteed. In

92   contrast, RNA-based methods are believed to be more sensitive because of the presence of

93   multiple mRNA copies per cell, and also are more amenable to UMI incorporation[36]. However,

94   the relative robustness and accuracy of the different approaches have not been systematically

95   compared. Here, we compared 9 different TCRseq library preparation protocols by analyzing

96   the TCR repertoire of aliquots of the same T-cell sample.

97

98   ## RESULTS

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

100   We set out to compare 9 different academic or commercial protocols for library preparation

101   and sequencing (**Supplementary material and methods**; **Supplementary Table 1**) based either

102   on RACE-PCR (RACE-1 to RACE-6) or on multiplex-PCR (mPCR-1 to 3). We sequenced nucleic

103   acids from $CD4^+CD25^-CD127^+$ effector T-cells (**Supplementary Fig.1a**) sorted from two healthy

104   donors (experiments A&B). In experiment A, we evaluated the accuracy and sensitivity of the

105   different methods by spiking donor A T-cell RNA (RACE-1 to RACE-6 and mPCR-3) or DNA

106   (mPCR-1 and mPCR-2) aliquots with different amounts of RNA or DNA from Jurkat cells

107   (**Supplementary Fig.1b**). In experiment B, we analyzed the impact of decreasing amounts of the

108   input material quantity by processing donor B RNA aliquots of 100 ng and 10 ng (**Supplementary**

109   **Fig.1c**). In both experiments, the $CD4^+CD25^-CD127^+$ T-cells were sorted, and the RNA and DNA

110   were extracted and aliquoted in a single laboratory. Triplicates of aliquots were distributed to

111   service providers and academic laboratories. Raw and/or pre-filtered sequences data were all

112   processed using MiXCR[37].

113   We obtained from 5.10^5 to 2.10^6 reads per aliquot depending on the method (**Supplementary**

114   **Fig.2a-b**). Numbers of unique V, J and VJ sequences as well as UMI distribution for RACE-1 and

115   RACE-2 (**Supplementary Fig.2a-c**) were comparable between all the methods. Numbers of TCR

116   sequences and clonotypes were correlated in a method-dependent manner, but not globally,

117   suggesting that the sequencing depth required for a given number of clonotypes is method-

118   dependent (**Supplementary Fig.2d**).

119

120   **Replicability and reproducibility differ among methods**

121   For each method, we first analyzed the proportion of reads that were identified as TCRs (**Fig.1a**

122   **and Supplementary Fig.2**). For 7/9 methods, we observed 20 to 60% of non-aligned reads,

123   which were mainly explained by no V and/or J sequence identification. TCR sequences had a

124   high-quality score (phred > 30, **Fig.1b**) and contained less than 1% PCR errors (**Fig.1c**), except

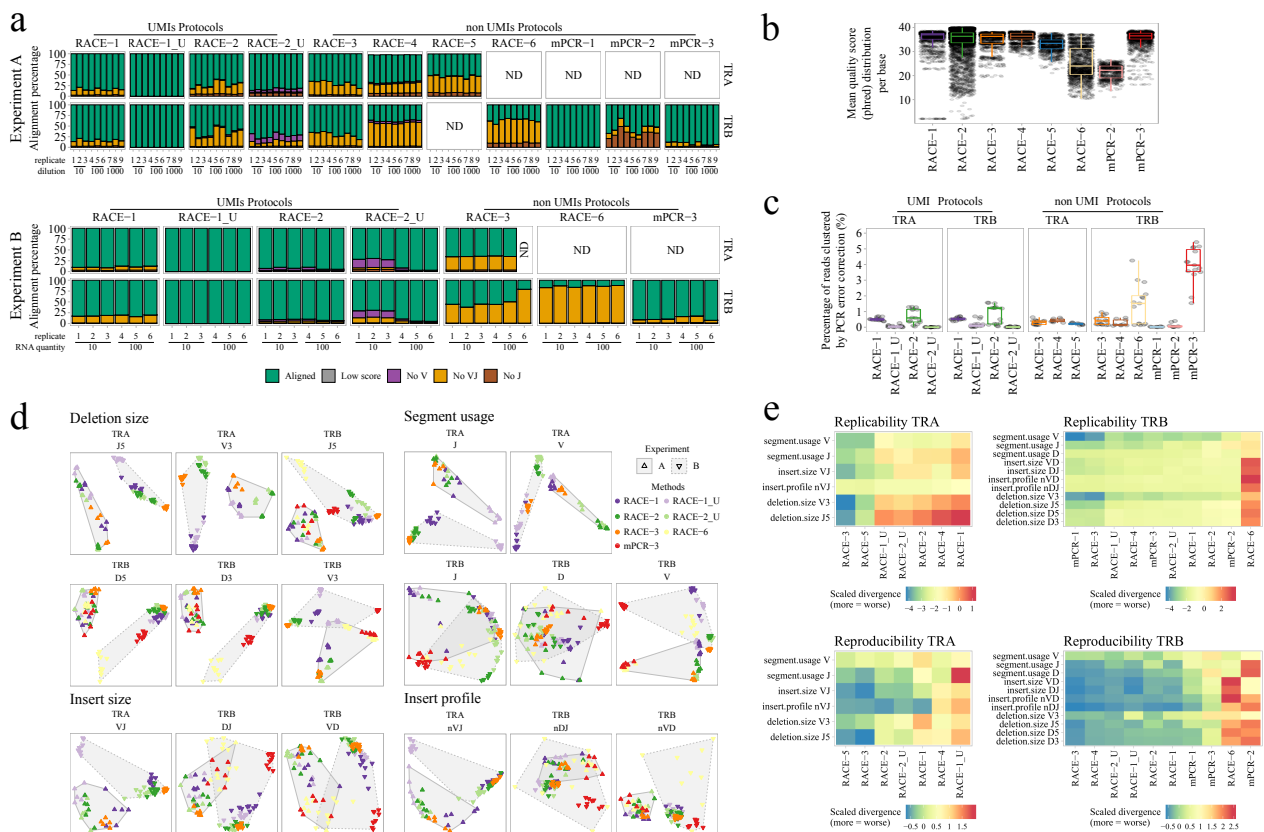125   for RACE-2, RACE-6, mPCR-2 and mPCR-3. Note that these parameters could not be assessed

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

126   for one of the commercialized mPCR-1 for which undisclosed proprietary pre-processing of the

127   data is performed.

128   Using a VDJ rearrangement model (Methods), we computed 17 rearrangement parameters for

129   TRA and TRB sequences from experiments A&B (**Supplementary Fig.3**) and calculated Jensen-

130   Shannon Divergence (JSD) distances between samples per parameter. Multi-Dimensional

131   Scaling (MDS, **Fig.1d**) showed that, within each experiment, samples obtained with the same

132   method clustered together, suggesting that each method imposed its methodological imprint

133   on the repertoire profile.

134   We further compared the different library methods' replicability (i.e. the similarity among data

135   obtained with the same method) and reproducibility (i.e. the similarity among data obtained

136   with different methods) using JSD as a measure of the distance between datasets[38]. **Figure 1e**

137   showed that for TRB, both the replicability and reproducibility of RACE-6 and mPCR-2 are lower

138   than for all the other methods tested. However, when considering TRA, replicability is higher

139   for RACE-3 and RACE-5 and reproducibility is higher for RACE-3, RACE-5 and RACE-2 (with and

140   without UMI). Since RACE-6 showed extremely low replicability for TRB samples and was not

141   reproduced by any other methods, we excluded it from further analysis. Altogether, our results

142   showed that many fundamental parameters of the TCR repertoire, as well as inter-sample

143   replicability and reproducibility, vary between the different methods tested.

144

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

146   We compared the TRBV usage obtained from the sequencing data with the percentage of TRBV

147   protein expression quantified by flow cytometry (FC) (**Fig.2a and Supplementary Figs.4a-b**).

148   mPCR-1 data were highly correlated with FC data (**Fig.2b**, $R^2>0.9$, $P < 5.10^{-12}$), which likely

149   reflects the undisclosed proprietary filtering by the provider. All other methods also showed a

150   significant R^2 Pearson correlation score ranging from 0.4 to 0.8, $P < 0.05$) with TRBV protein

151   expression (**Fig**.**2a-b**), except for mPCR-3 ($R^2<0.2$, $P > 0.05$). The Pearson correlation of TRBV

152   gene usage within replicates prepared with the same method (**Fig.2c**) was high ($R^2>0.9$).

153   However, clustering showed that mPCR-3 formed a distinct cluster with a low correlation score

154   ($R^2<0.5$) with other methods. The RACE methods data were highly correlated between each

155   other ($R^2>0.8$), except RACE-1 and RACE-1_U, which had a lower correlation ($0.6<R^2<0.7$).

156   mPCR-1 and mPCR-2 formed an independent "DNA cluster" with an $R^2>0.6$ when compared to

157   RACE replicates and a low correlation with mPCR-3 ($R^2<0.4$). This low correlation with mPCR-3

158   could in part be explained by a skewed TRBV9, TRBV29-1 and TRBV20-1 usage (**Supplementary**

159   **Fig.4c**). Spearman correlation scores were higher between FC data and mPCR-3 as well as RACE-

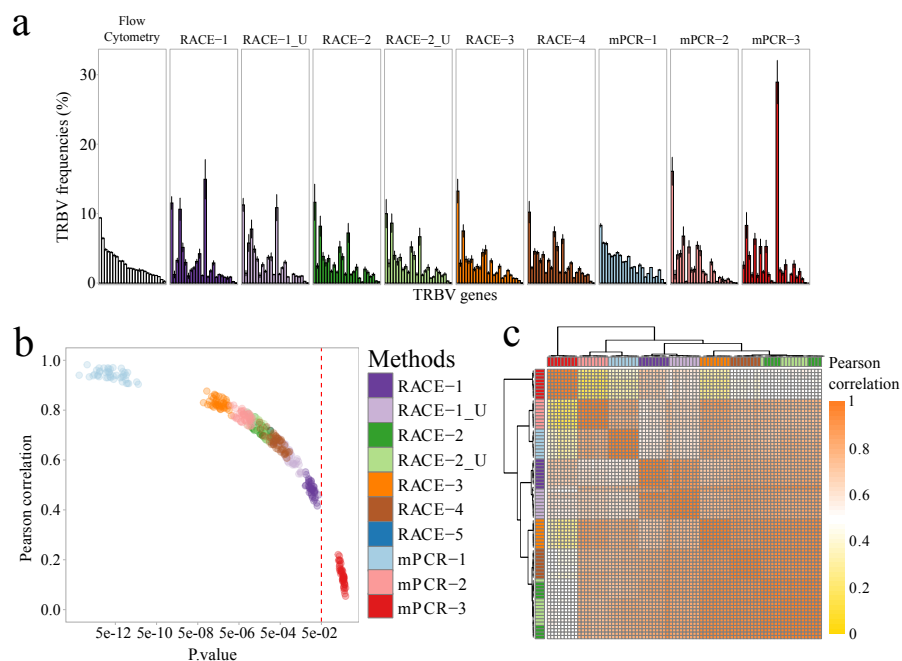160   1, and globally between the methods (**Supplementary Fig.4d-e**). In summary, RACE-PCR

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

161   methods and gDNA-based mPCR methods showed comparable TRBV usage results, in contrast

162   with the mPCR-3 RNA based method.

163

164   **Robustness of TRA and TRB detection is method-dependent**

165   We compared the similarity and composition of the 1% most predominant clonotypes

166   (1%_MPC) detected by each method. The Morisita-Horn similarity index (MH) was calculated

167   for each replicate across all the methods for both TRA (**Fig.3a-left**) and TRB sequences (**Fig**.**3a-**

168   **right**). TRA repertoires from RACE-3 and RACE-5 clustered together, inter- and intra-replicates

169   having a high degree of similarity (MH≈0.8). RACE-1, RACE-2 and RACE-4 have a lower inter-

170   and intra-method similarity (0.2<MH<0.5), but a higher similarity with RACE-3 and RACE-5.

171   Comparable clustering was obtained with the Jaccard similarity index (JSI), a measure

172   independent of clonotype frequency (**Supplementary Fig.5a**). For the TRB repertoires, MH

173   scores were low when comparing RACE and mPCR protocols (MH≈0.36), but high within the

174   RACE cluster (0.6>MH>0.9). There was poor similarity between the results of the three mPCR

175   methods, regardless of the template. Differences between RACE and mPCR methods

176   disappeared when calculating the JSI, suggesting a bias in clonotype frequency, as expected

177   when comparing RNA- with DNA-based methods, but less when comparing RNA-based

178   methods. Similar results were obtained by iteratively increasing the percentage of clonotypes

179   (**Supplementary Fig.5b**). Rényi diversity profiles (**Supplementary Fig.5c**) showed comparable

180   results for TRB with all the methods, but the diversity of TRA varied depending on the method.

181   However, the potential diversity estimated using Chao extrapolation was variable between

182   methods (**Supplementary Fig.5d**).

183   To test a possible bias in capturing the TRA diversity for some methods, we pooled and

184   compared the three spiking replicates per method from experiment A, as suggested by Greiff

185 et al.[24]. The MH similarity significantly increased for all the RACE-based methods for TRA

186 (**Fig.3b-top**) (except RACE-3) and for TRB (**Fig.3b-bottom**), with the TRA MH similarity remaining

187 lower than that of TRB. Similar observations were made for mPCR replicates. This suggests that

188 for a given depth of sequencing, the TRB diversity is better captured than that of TRA.
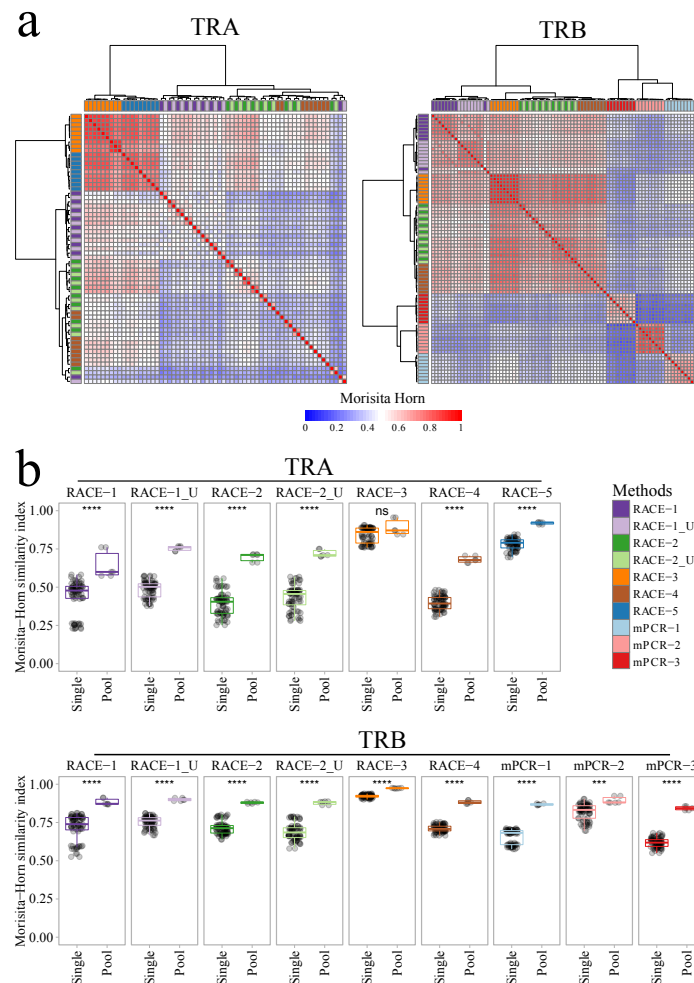
Fig. 3: The reproducibility of detection of major TCR clonotypes by different methods.

189

Detection sensitivity of rare TCRs depends on the method

191 To determine the accuracy of the different library amplifications for different clonotype

192 frequencies, we compared the observed frequencies of the TCR from the Jurkat spike-in to their

193 theoretical frequencies of 1/10, 1/100 and 1/1000. (**Supplementary Fig.1b**). TRA observed

194 frequencies were on average 3 times lower than expected (**Fig.4a-top**; Supplementary Table 2

195 and Supplementary Fig.6a). In contrast, TRB frequencies were on average 3 times higher than

196 the theoretical percentage, except for mPCR-1 (**Fig.4a-bottom**; **Supplementary Table 2 and**

197 **Supplementary Fig.6a**). For most of the methods, except RACE-1_U, RACE-4 and mPCR-3, the

198 ratio between the different dilutions was maintained, as shown by the mean slope values close

199 to 1 (**Fig.4b**).
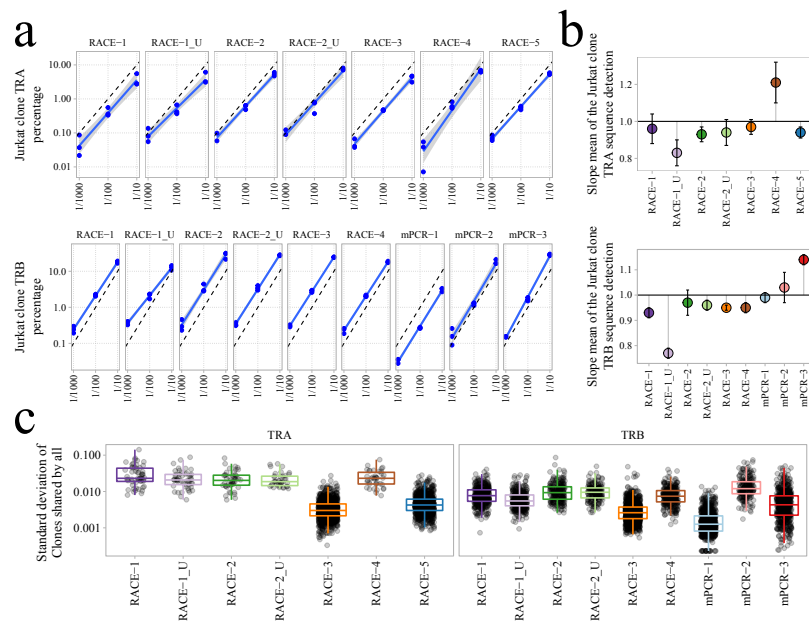
Fig. 4: Sensitivity of TCR sequence detection by different methods.

200 We then compared the inter-sample variation in clonal frequency for those TCR sequences

201 shared between all replicates of an individual method (excluding the Jurkat clone). **Figure 4c**

202 represents the standard deviation of the frequency of each shared clonotype (dots) per method

203 (see details in **Supplementary Fig.6b-d**). For TRA, RACE-3 and RACE-5 had the highest number

204 of clonotypes shared between the 9 replicates and the lowest standard deviation. For TRB, all

205 the methods captured a high number of shared clonotypes, and mPCR-1 and RACE-3 had the

206 lowest standard deviation. Finally, pooling all the clonotypes from all the replicates, we

207 identified 9 TRA and 31 TRB clonotypes shared by all the replicates of all methods,

208 corresponding to the most predominant clonotypes (**Supplementary Fig.7**). RACE-3, RACE-5

209 (both RNA-based) and mPCR-1 (DNA_based) showed the lowest inter-sample variability in TCR

210 frequency.

211

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

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

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

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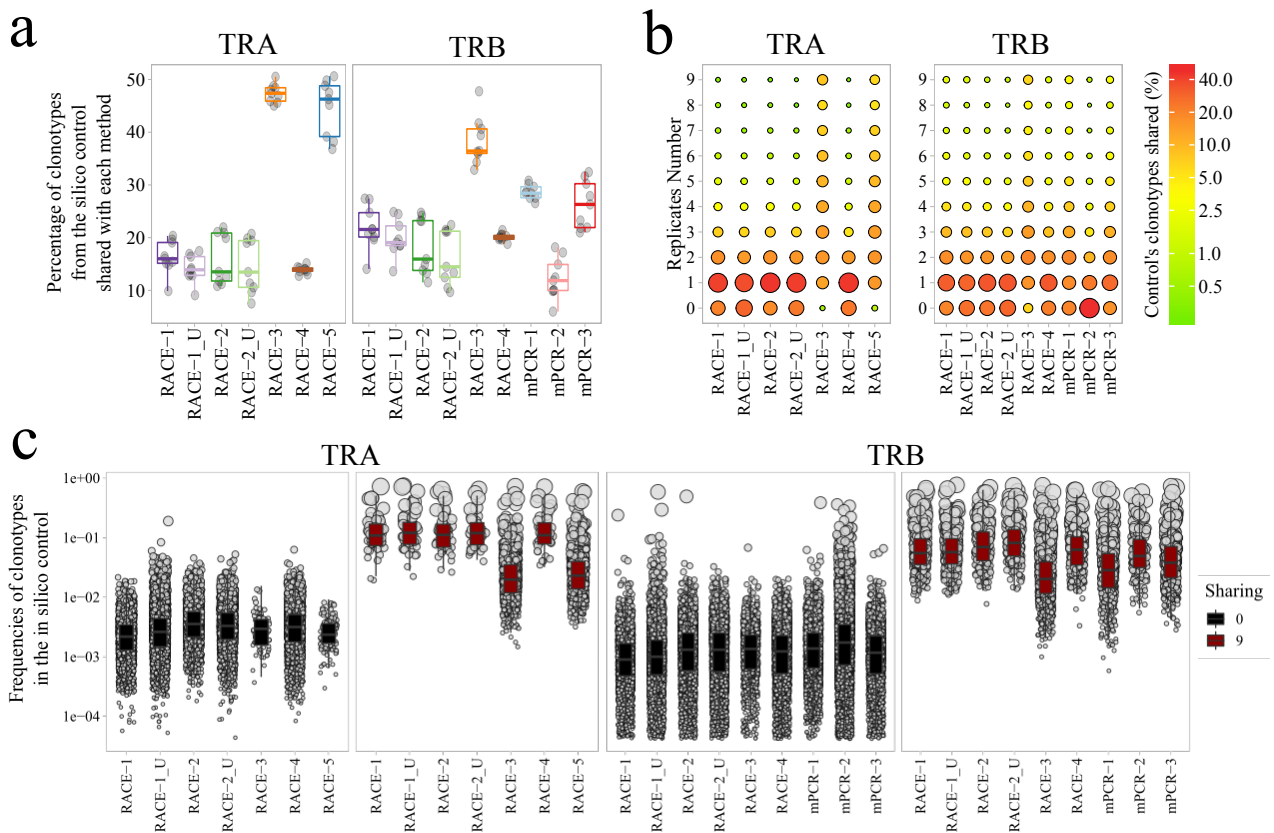
Fig. 5: Sharing with robust and representative meta-repertoire.

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).

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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. ~~Non-UMI RACE methods may thus offer some advances when it is~~

~~important to capture maximum repertoire diversity.~~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[42]~~, which~~

~~becomes critical with tighter cluster density on modern Illumina machines.[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[43,44]~~

~~as well as for the BCR, but not TCR, repertoire[45], still at a very low diversity level. The~~

~~construction of such gold standard repertoires is currently very costly and remains a major~~

333 ~~challenge that the Adaptive Immune Receptor Repertoire Community (AIRR-C)[46], engaged in~~

334 ~~AIRR-seq standardization[47–49], may tackle in the future.~~

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340 construction of such gold standard repertoires is currently very costly and remains a major

341 challenge that the Adaptive Immune Receptor Repertoire Community (AIRR-C)[48], engaged in

342 AIRR-seq standardization[49–51], may tackle in the future. Finally, in this study some data were

343 pre-processed using proprietary (mPCR-1, mPCR-3) or published[30,52] (RACE-1_U and RACE-2_U)

344 tools and then aligned and error-corrected using MiXCR (v2.1.10)[37]. To further optimize TCR

345 data accuracy, it would also be interesting to benchmark available software analysis tools,

346 especially regarding UMI analysis and sequence alignment. Our datasets generated using

347 different methods should be a valuable complement to using datasets generated purely in

348 vitro[53,54].

349 In conclusion, the take-home messages from this work are the following. Firstly, there are

350 satisfactory TCRseq methods based on either DNA or RNA input, and in both cases the amount

351 of material impacts both diversity and the detection of rare clones. Secondly, various methods

352 are optimal for detecting maximal diversity, while others most accurately quantify the

353 abundance of specific clonotypes. For the latter, UMI-based methods are potentially more

354 accurate, although they could miss relevant but rare clones. In contrast, non-UMI RACE

355 methods are more sensitive in capturing rare clones, especially for TRA. Thirdly, the availability

356 of raw data is crucial in allowing reliable and reproducible in-depth analyses of TCR repertoires;

18

357 the mPCR-1 service provider does not provide access to raw sequence data, while mPCR-1 and

358 mPCR-3 do not disclose the proprietary pre-processing filters. In contrast, the RACE-2 provider

359 provides raw data and all preprocessing algorithms. We summarized our results as well as

360 practical aspects in Table 1. Regarding the results, we calculated for each method a rank value

361 for Replicability, reliability and sensitivity based on various measures (**Table 1** and

362 **Supplementary file**). We also summarized cost per sample, presence of controls or standards,

363 format of the method and raw data availability. The Table 1 highlight the advantages and

364 disadvantages of the different methods which could serve as guidance for end-users. Improved

365 and more sophisticated data analyses are essential to extract the full power of TCR repertoire

366 data. We anticipate that now that TCR sequencing has come of age, the next key developments

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

368 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

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

370

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

395   measured on a NanoDrop1000 (Thermo Scientific™) and RNA integrity was determined on a

396   Bioanalyzer (Agilent®) with measurements higher than 8. RNA and DNA extraction and

397   validation were performed at the Sorbonne Université laboratory.

398

399   *Aliquot preparation for method comparison*

400   In experiment A, 100 ng of RNA or DNA from the CD4$^+$ effector T-cells sorted from donor A was

401   split into 3 aliquots that were spiked with different amounts of RNA or DNA from the Jurkat cell

402   line, at ratios of 1/10, 1/100 and 1/1000. Each spiked aliquot was further split into 3 and all

403   replicates were processed by all methods tested (7 for RNA and 2 for DNA; **Supplementary**

404   **Fig.1b**). With experiment B, we analyzed the impact of the input material quantity. RNA from

405   sorted CD4$^+$ effector T-cells of donor B was extracted, split into 15 aliquots of 100 ng each and

406   15 aliquots of 10 ng each and processed in triplicate using 5 of the RNA-based methods

407   (**Supplementary Fig.1c**). Aliquots were prepared at the Sorbonne Université laboratory and sent

408   to the partners.

409

410   *Flow Cytometry*

411   Vβ identification was performed on enriched CD4$^+$ effector T-cells from experiment A (see

412   *Blood effector T cell isolation* for enrichment procedure) stained with the IOTest Beta Mark TR

413   Repertoire Kit (Beckman Coulter®) according to the manufacturer's protocol as well as with

414   CD4-APC, CD127-BV421, CD25-PECy7. Data acquisition was performed on a Cytoflex®

415   (Beckman Coulter®) using CytExpert® software. FlowJo® was used for data analysis. Vb

416   frequencies were calculated on CD4$^+$CD25$^-$CD127$^+$ gated cells (**Supplementary Fig.4a-b**).

417   Staining was performed at the Sorbonne Université laboratory.

418

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. NineThe nine protocols for TCR library preparation compared in this study were selected based on AIRR-seq impact or involving (citationsaccording to at least one the following criteria: published using by other groups than the one who developed it (mPCR-1, mPCR-3, RACE-1, RACE-2, RACE-4 and associatedRACE-5), (ii) their association with recognizedwell-known analysis tools (RACE-1, RACE-2, mPCR-2) and sequencing(iii) commercially available (RACE-2, RACE-3, mPCR-1, mPCR-3). Sequencing protocols were used followingharmonized taking into account published recommendations or recommendations provided by the manufacturer of commercial kits or by the owner or userusers of the protocol. All protocols are detailed in Supplementary material and methods.

TCR deep sequencing data processing

FASTQ raw data file 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[29,30]. FASTQ and FASTA files were then processed for TRB and TRA sequence

FASTQ raw data files were obtained from each method, except for

448 Multiplex-1 & 2, for which we obtained, respectively, FASTA file and FASTQ files following

449 proprietary pre-processing. For RACE-1 and RACE-2, UMI pre-processing was performed

450 following protocols published elsewhere[30,31,52]. FASTQ and FASTA files were then processed for

451 TRB and TRA sequence annotation using the MiXCR software[37] (v2.1.10) with RNA-Seq default

452 parameters (-p rna-seq –s hsa) as available online. MiXCR extracts TRA and TRB repertoire

453 providing correction of PCR and sequencing errors.

454

455 *Data analysis*

456 Statistical comparisons and multivariate analyses were performed using R software version

457 3.5.0 (www.r-project.org). We used the ggplot2 package to generate figures[55], except

458 heatmaps. More complex analyses are detailed in the next section.

459

460 *Comparing VDJ rearrangement statistics*

461 An empirical VDJ rearrangement model for each method was built as follows. We analyzed

462 clonotype tables to obtain comprehensive statistics of VDJ rearrangements including the

463 frequencies of V/D/J segment usage, number of added N Bases (namely "insert profile", i.e. the

464 probability distribution of having A/T/G/C inserted in the N-region of CDR3 given that we

465　observe a certain base inserted before it) and V/J segment trimming bases, with the IGoR

466　package[56]. This model is built in a 'greedy' way in the sense that it uses best alignments provided

467　by MiXCR rather than running expectation maximization procedures as described in Murugan

468　et al.[57]. We utilized the Jensen-Shannon divergence (JSD) between distributions of VDJ usage

469　to define the following two statistics that we use for comparative analysis of different TCRseq

470　methods: 1) *replicability* measured as the distance between different samples produced by the

471　same protocol and 2) *reproducibility* measured as the distance between samples produced by

472　two different protocols. MDS used for sample mapping was performed on rank-transformed

473　distances to avoid the distorting effect of outliers. All the analyses involve VDJ usage inferred

474　from weighted data (TCR clonotype is weighted by its frequency in the sample) to account for

475　TCRseq method amplification biases.

476

477　*Similarity analysis*

478　Pearson and Spearman correlations, the Morisita-Horn index[58] (MH) and the Jaccard similarity

479　index[59] (JSI) were used to assess the similarity between samples. The MH index takes into

480　account the relative abundance of species in the sample, while the JSI is a measure of the

481　intersection between two populations relative to the size of their union, and is independent of

482　relative abundances. Both indices vary between 0 (no overlap) and 1 (perfect overlap). JSI and

483　MH were calculated using the DIVO package[60] on R. In order to discriminate indices represented

484　by a heatmap with the pheatmap package[61], we used a different set of colors. The Pearson and

485　Spearman correlations are presented as yellow/white/orange (**Fig.2c and Supplementary**

486　**Fig.4e**), MH is presented as blue/white/red (**Fig.3a**) and JSI is presented as purple/yellow/green

487　(**Supplementary Fig.5a**).

488

489 *Diversity profiling*

490 The diversity was analyzed using two indices. Rényi entropy[62] is a generalization of Shannon

491 entropy, which increases when both species richness and evenness are high. Rényi entropy is

492 a function of a parameter α spanning from (i) the species richness (α=0), which corresponds to

493 the number of clonotypes regardless of their abundance, to (ii) the clonal dominance (α→+∞),

494 corresponding to the frequency of the most predominant clonotype. For α=1, the Shannon

495 diversity index is computed. The exponential of the Rényi entropy corresponds to the actual

496 number of clonotypes in the datasets[63] and is used to build a diversity profile[64]. It was

497 computed using the entropy package[65] on R. ChaoE[66] index was calculated with the iNEXT

498 package[67] as a measure of extrapolation of the possible number of clonotypes based on the

499 observed clonotypes. Rarefaction curves were interpolated from 0 to the current sample size

500 and then extrapolated to the size of the largest of samples, allowing comparison of diversity

501 estimates. Interpolation and extrapolation were based on ChaoE multinomial models[68].

502

503 *Meta-repertoire construction*

504 We generated an in silico meta-repertoire from the sequences obtained from the 108

505 replicates (45 for TRA and 63 for TRB). This meta-repertoire, for each chain, was designed to

506 minimize biases by (i) pooling all clonotypes from the 9 datasets and removed singletons to

507 avoid introducing noise due to PCR errors, (ii) Selecting non-reprocessed datasets, meaning

508 before UMI, (iii) keeping only clonotypes found by at least 3 different methods to avoid bias

509 toward one particular method. The threshold was defined to reach a dataset size as close as

510 possible to the original datasets to avoid additional sampling, (iv) normalizing the size of each

511 dataset to the lowest dataset to ensure the same weighting for each method. Completion of

512   the representative meta-repertoire was achieved by pooling all the datasets. This generated a

513   pooled dataset of 14 458 TRA and 18 735 TRB clonotypes.

514

Data Availability

516   All the fastq data obtained in this study, including the Jurkat Clone E6-1 (ATCC®TIB-152™) cell

517   line TCR alpha and beta sequences, were deposited in the NCBI Sequence Read Archive

518   repository following MiAIRR standard recommendations[47] under the BioProject ID

519   PRJNA548335. Source data for TCRVb flow cytometry data are provided as **Supplementary**

520   **Fig.4a-b**. All other data are available from the corresponding author upon request.

521

Code Availability

523   All software packages and programs are publicly available and open source. Scripts used to

524   analyze the data with MiXCR are available from https://mixcr.milaboratory.com ; Decombinator

525   from https://github.com/innate2adaptive/Decombinator; MiGEC from

526   https://github.com/mikessh/migec; detailed VDJ rearrangement statistics scripts are available

527   from https://github.com/antigenomics/repseq-protocol-comparison. There is no restriction on

528   the use of the code or data.

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684

685

686 # FIGURE LEGENDS

687 **Fig. 1: Performance statistics and VDJ rearrangement model of each method for experiments**

688 **A and B.**

689 **a**, The proportion of sequence reads aligned for TRA or TRB genes for each TCRseq replicate

690 per experiment (Experiment A, top, Experiment B, bottom). The bars represent the percentage

691 of TRA and TRB alignment, and the reason for alignment failure is color coded. **b**, Distribution

692 of the reads quality control (QC) for each method over all datasets, computed with fastQC

693 software (www.bioinformatics.babraham.ac.uk/projects/fastqc). **c**, Percentage of reads

694   collapsed after PCR error correction for all samples in the study. For each method, the MiXCR

695   clustering strategy was applied to correct for PCR errors and collapse reads. Each box-plot

696   represents the percentage of clustered reads. **d**, Multi-dimensional scaling (MDS) of V(D)J

697   recombination parameters. MDS was performed based on the Jensen-Shannon Divergence

698   (JSD) calculated between replicates on weighted VDJ segment usage (Segment usage), non-

699   template nucleotide insert size distributions (Insert size), V/D/J segment trimming distributions

700   (Deletion size), and nucleotide frequencies in N-inserts (Insert profile). JSD values were

701   transformed to rank for better visualization. Solid and dotted polygons outline samples from

702   experiments A and B, respectively. Colors represents the different methods as in B (only

703   methods used in both experiments are presented). **e**, Replicability and reproducibility of the

704   TRA and TRB repertoires for each method. The average JSD calculated in D (rows) for TRA (left)

705   and TRB (right) measured between replicates produced by the same method (Replicability, top)

706   or replicates of a given method and all other protocols (Reproducibility, bottom) was used as

707   distance metric to compare different protocols (columns). Columns are sorted according to the

708   mean   scaled   distance   (averaged   over   all   rows)   from   the   lowest   (best

709   replicability/reproducibility) to the highest (worst replicability/reproducibility). Distance values

710   are shown using a color scale. Jurkat TCR sequences were removed from datasets for this

711   analysis.

712

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

714   **a**, Flow cytometry and TCRseq TRBV frequencies. Bar plots represent the TRBV frequencies

715   calculated from flow cytometry stained CD4$^+$ T effector cells for the 24 TRBV for which

716   antibodies are available and from the TCRseq data, considering only clonotypes annotated for

717   the same 24 TRBV (original TRBV frequencies were used accordingly). Histograms of the 24

718   TRBV frequencies are organized by decreasing order using frequencies obtained by flow

719   cytometry as a reference reference (TRBV20-1, TRBV19, TRBV12-3/4, TRBV28, TRBV2, TRBV3-

720   1, TRBV30, TRBV6-5/9, TRBV9, TRBV5-1, TRBV4-1/2, TRBV27, TRBV29-1, TRBV6-6, TRBV11-2,

721   TRBV10-3, TRBV25-1, TRBV6-2, TRBV18, TRBV5-5, TRBV14, TRBV5-6, TRBV13, TRBV4-3). **b,**

722   TRBV usage correlation between flow cytometry and TCRseq. Pearson's correlation of the TRBV

723   frequencies between the 5 flow cytometry datasets and the 9 TCRseq replicates was calculated

724   for each method. The plot is represented by the correlation score (y-axis) and the *P*-value (x-

725   axis) of the correlation, allowing the classification of the methods. **c,** Heatmap of the Pearson

726   correlations between each replicate for the distribution of TRBV gene usage (n=62). The

727   Euclidean distance was used for hierarchical clustering as a color-coded matrix ranging from 0

728   (yellow, maximum dissimilarity) to 1 (orange, maximum similarity). Jurkat TCR sequences were

729   removed from datasets for this analysis.

730

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

732   **a,** Heatmaps of the Morisita-Horn similarity index (MH). MH scores were calculated between

733   each replicate across all methods for the top 1% of most predominant clonotypes (MPC) for

734   TRA (left) and TRB (right). The Euclidean distance was used for hierarchical clustering as a color-

735   coded matrix ranging from 0 (blue, maximum dissimilarity) to 1 (red, maximum similarity). **b,**

736   Comparison between individual replicates (Single) and pooled replicates (Pool) by the MH

737   similarity index. Datasets from replicates of the same dilution were pooled for each method to

738   get 1 pooled sample per dilution. Singletons (count=1) were removed; MH similarity scores

739   were calculated for the top 1% of most predominant clonotypes for TRA (left) and TRB (right).

740   Jurkat TCR sequences were removed from datasets for this analysis.

741

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

743   **a**, Jurkat clone percentage. Jurkat TRA (top) and TRB (bottom) clonotype percentages were

744   calculated for each experiment per dilution (1/10, 1/100 and 1/1000 spike-in) and are

745   represented by the blue dots. The blue line represents linear regression and the black dashed

746   line represents the theoretically expected percentage. **b**, Slope of the Jurkat tracking linear

747   regression. Slope was computed between dilution with standard deviation by method for TRA

748   (top) and TRB (bottom). **c**, Standard deviation of the clonotypes shared among the 9 replicates

749   (except Jurkat clone) per method, for TRA (left) and TRB (right).

750

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

752   **a**, Replicate sharing fraction in meta-repertoire repertoire (focus on meta-repertoire

753   clonotypes) for TRA (left) and TRB (right). The values represented correspond to the percentage

754   of clonotypes from each replicate per method found in the meta-repertoire, median and the

755   1st and 3rd quartiles are shown. **b**, Replicability of replicate methods with meta-repertoire for

756   TRA (left) and TRB (right). By chain, heatmaps on the left represent the fraction, which

757   corresponds to the percentage of meta-repertoire clonotypes found in 1 to 9 replicates per

758   method (0: unseen in any of the replicates). **c**, Distribution of meta-repertoire clonotypes in the

759   replicates by methods for TRA (left) and TRB (right). Each dot represents a meta-repertoire

760   clonotype and the boxplot represents the average frequencies. Black boxplots with

761   corresponding gray dots represent the unseen clonotypes (0) and red boxplots with

762   corresponding gray dots represent the clonotypes found by the 9 replicates (9). Each method

763   is represented independently. Jurkat TCR sequences were removed from datasets for this

764   analysis.

765

766 Table 2 : Comparative performance of the nine TCRseq molecular methods. For each method,

767 an average rank score for TRA (top) and TRB (bottom) sequencing has been calculated for

768 Replicability, Reliability, and Sensitivity (three first column) and practical information have

769 been summarized (4 last columns). Ranks have been calculated as the average of the ranks for

770 results 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.

771 4*c*, 5*b* & Supplementary Fig. 2a, 5c for "Sensitivity". Rank values are comprised between 2

772 (best) and 7 (worst) and represented as bars with their values. Details are provided as

773 Supplementary information. Cost per sample" is expressed in USD as per current prices for a

774 depth of 1 million TCR sequences per sample on a 25 million reads sequencing format. The

775 costs cover reagents for library preparation to sequencing. *mPCR1 and mPCR3 price ranges

776 correspond to the cost for either purchasing kits (lowest price) or service up to sequencing

777 and basic data analyses from the provider.