1   # Systematic assessment of commercially available low-input miRNA library preparation

2   kits

3   Fatima Heinicke[1], Xiangfu Zhong[1], Manuela Zucknick[2], Johannes Breidenbach[3], Arvind

4   Sundaram[1], Siri T. Flåm[1], Magnus Leithaug[1], Marianne Dalland[1], Andrew Farmer[4], Jordana

5   M. Henderson[5], Melanie A. Hussong[6], Pamela Moll[7], Loan Nguyen[8], Amanda McNulty[4],

6   Jonathan M. Shaffer[6], Sabrina Shore[5,9], HoiChong Karen Yip[8], Jana Vitkovska[7], Simon

7   Rayner[1], Benedicte A Lie[1]*, Gregor D. Gilfillan[1]*

8   [1] Department of Medical Genetics, Oslo University Hospital and University of Oslo, Oslo,

9   0450 Norway

10   [2] Department of Biostatistics, Oslo Centre for Biostatistics and Epidemiology, University of

11   Oslo, Oslo, Norway

12   [3] Norwegian Institute for Bioeconomy Research, National Forest Inventory, Ås, 1431Norway

13   [4] Takara Bio USA, Inc., Mountain View, CA, USA

14   [5] TriLink Biotechnologies LLC, San Diego, CA, USA

15   [6] QIAGEN Sciences, Frederick, MD 21703, USA

16   [7] Lexogen GmbH, Vienna, Austria

17   [8] SeqMatic, LLC, Fremont, CA 94539, USA

18   [9] Current address: Singular Genomics, La Jolla, CA, USA

19   * contributed equally

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Abstract

High-throughput sequencing is increasingly favoured to assay the presence and abundance of micro RNAs (miRNAs) in biological samples, even from low RNA amounts, and a number of commercial vendors now offer kits that allow miRNA sequencing from sub-nanogram (ng) inputs. However, although biases introduced during library preparation have been documented, the relative performance of current reagent kits has not been investigated in detail. Here, six commercial kits capable of handling <100ng total RNA input were used for library preparation, performed by kit manufactures, on synthetic miRNAs of known quantities and human biological total RNA samples. We compared the performance of miRNA detection sensitivity, reliability, titration response and the ability to detect differentially expressed miRNAs. In addition, we assessed the use of unique molecular identifiers sequence (UMI) tags in one kit. We observed differences in detection sensitivity and ability to identify differentially expressed miRNAs between the kits, but none were able to detect the full repertoire of expected miRNAs. The reliability within the replicates of all kits was good, while larger differences were observed between the kits, although none could accurately quantify the majority of miRNAs. UMI tags, at least within the input ranges tested, offered little advantage to improve data utility. In conclusion, biases in miRNA abundance are heavily influenced by the kit used for library preparation, suggesting that comparisons of datasets prepared by different procedures should be made with caution. This article is intended to assist researchers select the most appropriate kit for their experimental conditions.

Keywords: microRNA, miRNA, small RNA-seq, library preparation, sequencing bias, low RNA input, NGS, Next Generation Sequencing, UMI

Introduction

46

47   Micro RNAs (miRNAs) are ~22 nucleotide long non-coding small RNAs that regulate gene

48   expression at a post-transcriptional level by binding to their mRNA targets to inhibit

49   translation. First discovered in the early 1990s [1,2], miRNAs have been shown to impact

50   biological processes such as cellular differentiation and development [3-6,7-9,10-12,13,14].

51   Alterations in miRNA expression have been observed in various diseases [15,16,17] and an

52   accurate method for detecting and measuring miRNA expression is therefore crucial. In

53   recent years, next generation sequencing (NGS) has evolved as the method of choice. The

54   main advantages of NGS, compared to qPCR and microarray techniques, are the possibility

55   to discover novel miRNAs and the ability to detect differences in miRNA sequences on a

56   single base level. Furthermore, NGS enables the study of low-abundance miRNAs, which is

57   especially useful when examining miRNAs in specific cell types or body fluids like serum

58   and plasma. Accordingly, the latest miRNA library preparation kits allow inputs as low as

59   100 picograms total RNA. The library preparation process typically consists of (i) addition of

60   adapter sequences onto the miRNA, (ii) reverse transcription and (iii) PCR amplification

61   prior to sequencing. The kits investigated in this study used both two adapter and single-

62   adapter circularization protocols which can broadly be divided into two classes: those

63   employing RNA ligases (e.g. T4 RNA ligase) and those employing polyadenylation (poly-A)

64   and template-switching oligonucleotides to attach adapter sequences to the single-stranded

65   miRNAs.

66

67   Despite the reported advantages of NGS, the miRNA abundance detected by sequencing and

68   that in the original sample have been shown to differ by up to four orders of magnitude [18]. In

69   particular, the addition of adapters onto the miRNA insert has been identified as a major

70   contributor to this bias [19,20]. For protocols utilizing T4 RNA ligase, adapter ligation is

3

71   influenced by the ligase used, the miRNA insert and adapter primary sequence, as well as the

72   GC content and the secondary structures of miRNA insert and adapter [19-23]. For poly-A

73   utilizing protocols, the enzyme poly (A) polymerase has also been reported to be influenced

74   by miRNA primary sequence and secondary structure [24]. Other reported possible sources of

75   bias during library preparation include the reverse transcription and PCR steps, with PCR in

76   particular able to introduce both amplification bias and duplicate reads, but results have been

77   contradictory [19-21]. A recent study recommended the use of unique molecular identifiers

78   (UMI) to mitigate the reverse-transcription and PCR biases in future experiments [25]. Previous

79   studies also reported that the incorporation of UMIs into sequence adapters resulted in

80   improved accuracy both in RNA-seq and smallRNA-seq analysis [26,27].

81

82   In this study we aimed to systematically assess the miRNA repertoire and frequency observed

83   in NGS data using six different low-input library preparation protocols (Table 1).

84   Commercial vendors marketing kits stating compatibility with total RNA amounts ≤100 ng

85   were invited to participate. The performance of the protocols was compared with regard to

86   their detection rate sensitivity, reliability and ability to identify differentially expressed

87   miRNAs. In addition, the relevance of UMIs was studied. All analyses were performed on

88   low-input well-defined synthetic miRNA and human-derived total RNA samples.

89

90   **Results**

91   **Experimental design and miRNA read yields**

92   Synthetic miRNA and biologically derived human total RNA samples (21 samples in total)

93   were distributed to participating companies for library preparation (Figure 1a). Upon return

94   of libraries, library yield and size were measured (Supplementary Figure 2). Libraries were

95   pooled based on their miRNA content with the aim of obtaining at least 5 million reads

96   apiece. Based on the presence of additional small RNAs in some libraries, particularly those

97   prepared with the CATS and SMARTer-beta reagents, which displayed a wider range of

98   insert sizes, we did not expect all libraries to produce similar read counts at this stage.

99   Nevertheless, a large number of reads from both CATS and SMARTer-beta kits failed to pass

100   QC threshold filters.

101   The number of sequencing reads obtained ranged from 400,000 to more than 33 million reads

102   for the individual libraries (Supplementary Table 4).

103

104   For all library preparation kits, the greatest proportion of reads were discarded during

105   mapping, most likely as a result of not allowing for any mismatches (Figure 1b and

106   Supplementary Figure 3). The absolute number of reads excluded differed between the kits.

107   As anticipated, a higher fraction of miRNA reads was obtained in the synthetic miRNA

108   samples compared to the human total RNA samples (Figure 1b), since the human total RNA

109   samples also contain additional classes of small RNA. SMARTer-beta and CATS returned

110   the lowest proportion of miRNA reads both in the synthetic miRNA and the human total

111   RNA samples compared to the other library preparation kits while TailorMix, followed by

112   QIAseq produced the highest proportions of miRNA reads (Figure 1b).

113

114   To comprehensively evaluate the sensitivity and reliability of the library preparation kits, the

115   synthetic miRNA samples were randomly down-sampled to 2.5 million and human total

116   RNA samples to 0.75 million miRNA reads. The libraries of SMARTer-beta and CATS did

117   not reach these thresholds and were therefore excluded from further analysis. The results

118   presented hereafter are based on calculations using the down-sampled data with the exception

119   of the differential expression and UMI analyses for which raw (not down-sampled) miRNA

120   reads were used.

121

122   **Detection rate sensitivity**

123   Applying a relaxed detection threshold where miRNAs were defined as detected if one or

124   more read counts were registered, the detection rate sensitivity for all kits ranged from 94.7%

125   to 99.1%, and all miRNAs could be detected in at least one kit and replicate. QIAseq

126   followed by TailorMix detected the highest numbers of miRNAs in all three replicates in all

127   the mixes (Figure 2a). QIAseq and TailorMix also missed the fewest miRNAs in either one,

128   two, or all three triplicates. When comparing the detection rate sensitivity of the 1.0ng

129   synthetic miRNA samples (mix A-D) with the 0.1ng synthetic miRNA samples (mix E), no

130   striking difference in the number of detected miRNAs could be observed for any of the kits

131   (Figure 2a).

132

133   Most of the miRNAs that were undetected in QIAseq and TailorMix were neither detected by

134   the other two kits (Supplementary Figures 4 and 5). TailorMix was the only reagent that

135   detected each of the 903 equimolar miRNAs in at least one sample (Supplementary Figure 5).

136   srLp followed by CleanTag showed the highest numbers of kit-specific undetectable

137   miRNAs.

138

139   Analysis of the 40 non-equimolar miRNAs revealed that miRNAs undetected in one or more

140   replicates belonged mostly to miRNAs present at low levels (Supplementary Figure 6), with

141   QIAseq showing the highest detection rate, again followed by TailorMix. Notably, CleanTag

142   and srLp failed to detect some miRNAs present at relatively high concentrations in all the

143   replicates (mix C and D, Supplementary Figure 6). However, even though the majority of the

6

144   non-equimolar miRNAs could be detected in all replicates, the analysis indicated that factors

145   in addition to miRNA abundance influence detection rate sensitivity.

146

147   We next compared the performance at different detection thresholds, i.e. 1, 10, 50, 100, 200

148   read counts per million (CPM) for synthetic miRNA samples in all mix triplicates for each kit

149   (Figure 2b). With the exception of some of the non-equimolar miRNA oligonucleotides

150   present at the lowest concentration, all synthetic miRNAs should in theory obtain CPM

151   values above 200 with the library size of 2.5 million mapped miRNA reads. However, a

152   sharp decline in detection was observed at increasing CPM thresholds. Nonetheless, QIAseq

153   followed by TailorMix consistently detected the highest number of miRNAs across all

154   thresholds.

155

156   **Intra-rater and Inter-rater reliability**

157   Rlog transformed miRNA count data were used for the for intra- and inter-rater reliability

158   calculations. Intra-rater reliability calculations (the concordance between miRNA read counts

159   within the replicates of the library preparation kit) revealed excellent reliability for the

160   synthetic miRNA and the human total RNA samples within all tested kits with ICC values

161   above 0.99 and 0.98, respectively (Supplementary Table 5). Similarly, very strong

162   correlations were found when Pearson correlation coefficients were calculated ($r > 0.97$,

163     $p < 0.05$) (Supplementary Figure 7 and 9a). Bland-Altman plots, which describe the

164   agreement between two replicates by presenting the difference of them against the mean, also

165   showed good agreement (Supplementary Figure 8 and 9b). For all Bland-Altman

166   comparisons the bias was close to 0. The line of equality (not presented in our Bland-Altman

167   plots) was always within the agreement limits, which indicates a lack of systematic error in

168   the measurements within the replicates. All in all, strong intra-rater reliabilities were

169   observed within the samples prepared by each kit.

170

171   For the inter-rater reliability calculations (concordance of read counts seen between the

172   different kits) the first replicate of each mix, RA or healthy control sample was randomly

173   chosen. The synthetic miRNA and the human total RNA samples revealed good and excellent

174   inter-rater reliability with ICC values above 0.83 and 0.95 respectively (Supplementary Table

175   6). The correlation between the different kits was above 0.76 ($p < 0.05$) for the synthetic

176   miRNA and above 0.92 for the human total RNA samples (Supplementary Figure 10).

177   However, differences in the correlations between the reagents were seen for the synthetic

178   miRNA samples. The kits with the highest correlations ($r > 0.94$, $p < 0.05$) were, independent

179   of whether mix A-E was considered, CleanTag and srLp while QIAseq showed the lowest

180   correlation to the other kits. The Bland-Altman plots revealed no systematic error when

181   comparing the different kits to each other (Supplementary Figures 11 and 12). The limits of

182   agreements were smallest for CleanTag and srLp across all tested mixes in the synthetic

183   miRNA samples indicating a high agreement between those two kits. In summary, a modest-

184   to-good inter-rater reliability was obtained when comparing the mix-specific replicates of the

185   four miRNA library preparation kits with each other, with QIAseq showing the greatest

186   differences from the other reagents.

187

188   The reliability measured against the theoretical miRNA concentration was only assessed for

189   the synthetic miRNA samples. For the 903 equimolar miRNAs, the fold deviation of the first

190   replicate of mix A from the median count for that sample was calculated as a rlog ratio

191   (Supplementary Figure 13). When the absolute value of the rlog fold deviation for a miRNA

192   was less than or equal to one, the miRNA was counted as equimolar. For the four kits this

193   was the case for 39.8 to 42.0% of the equimolar miRNAs. The remaining miRNAs showed a

194   bias towards over-representation (positive rlog fold change) rather than under-representation.

195   The coefficient of variation of the rlog counts across all replicates for the equimolar miRNAs

196   was lowest for QIAseq, followed by TailorMix, CleanTag and srLp, respectively

197   (Supplementary Table 7).

198

199   For the 40 non-equimolar miRNAs, the correlation between the rlog counts of each library

200   preparation kit and their theoretical concentration varied between the mixes for all kits.

201   Overall, mix A and mix E showed greater correlations ($0.41 < r > 0.61$, $p < 0.05$) than mix B

202   to mix D ($0.08 < r > 0.47$, $p < 0.05$) (Supplementary Table 8). QIAseq showed the highest

203   correlation coefficients across all samples. All in all, these results suggest that on one hand

204   the reliability between the reagents is good, but on the other hand that none of the reagents

205   are ideally suited for accurate miRNA quantification.

206

207   **Differential expression**

208   Most miRNA profiling studies aim to identify differentially expressed (DE) miRNAs

209   between samples of interest. When comparing mix A and mix B of the synthetic miRNA

210   samples, ideally all 40 non-equimolar miRNAs should be detected as DE with a log2 fold

211   change greater than or equal to one. All kits detected between 32 to 35 DE miRNAs (Figure

212   3a). However, some of those miRNAs (CleanTag, TailorMix and srLp=2 and QIAseq=1)

213   were from the pool of equimolar miRNAs. Of the 40 non-equimolar miRNAs, 26 were

214   detected to be DE by all kits, although they did not always agree on the log fold changes

215   (Figure 3b). The non-equimolar miRNAs hsa-miR-1199-5p, hsa-miR-22-5p and hsa-miR-

216   940, which were three of the ten miRNAs expected to show the lowest fold differences (fold

217   change of 2) between mix A and mix B, could not be detected as DE by any of the reagents.

9

218

219   In order to control as best possible that the levels of miRNA in mix A and mix B were as

220   expected, we performed quantitative reverse-transcriptase PCR assays on 16 selected non-

221   equimolar miRNAs (Supplementary Figure 14), which confirmed the intended ratios in the

222   starting material.

223

224   Differential expression analysis of the human total RNA samples revealed different numbers

225   of DE miRNAs detected by the kits. CleanTag detected 19 DE miRNAs, QIAseq and

226   TailorMix detected two DE miRNAs each, while srLp did not detect any (Figure 3c). With

227   the exception of hsa-miR-486-3p, no overlap between the DE miRNAs was seen amongst the

228   kits (Figure 3d).

229
230   **Titration response**

231   The titration response of the 40 non-equimolar miRNAs in mixes A - D (Figure 1a) was

232   compared by scoring a miRNA as titrating or non-titrating based on detection in the expected

233   concentration order in the four mixes. Since there were five miRNAs at each chosen

234   concentration, the fraction of titrating miRNAs (0, 0.2, 0.4, 0.6, 0.8 or 1) was calculated for

235   each reagent kit for each concentration group (Table 2). The highest fraction of titrating

236   miRNAs was seen for QIAseq, which correctly scored all miRNA concentrations with greater

237   than 2-fold differences in mix A through mix D.

238

239   **Effectiveness of QIAseq unique molecular identifier sequence tags**

240   QIAseq was the only kit included in this study that implements unique molecular identifiers

241   (UMIs) during library preparation, which are claimed to enable more accurate quantification

242   of miRNAs. For both synthetic miRNA and human total RNA samples, very strong Pearson

243 correlations were observed between the rlog transformed raw read and UMI counts

244 (Supplementary Figure 15). Comparison of the rlog sum of all UMI and ordinary read counts

245 revealed the sum of UMI counts to be negligibly smaller than the ordinary read counts for

246 both synthetic miRNA and human total RNA samples (Supplementary Figure 16a, b).

247

248 To further examine whether UMI read counts might reduce undesirable over-representation

249 of miRNAs that were favourably amplified or sequenced, we examined the abundance of the

250 ten miRNAs with the highest ordinary read counts for each sample and compared this to their

251 respective UMI counts (Supplementary Figure 16c,d). Amongst those miRNAs no

252 overestimation of the ordinary read counts was observed compared to the UMI counts.

253

254 **Discussion**

255 Several publications have revealed discrepancies between the frequencies of miRNAs present

256 in the original samples and those detected by sequencing approaches [18 20]. The adapter

257 ligation steps in the small library preparation procedure, in addition to miRNA sequence and

258 structure, have emerged as being most critical when trying to explain the discrepancy [18 19 21 43]

259 [44 20 22]. As an alternative to the ligase-dependent ligation step in library preparation, poly-

260 adenylation based procedures have been developed. Additional biases might be introduced

261 during reverse transcription and PCR steps, but in this case results have been contradictory

262 ([19-21], [25]). The use of UMI tags has therefore been suggested to remove this potential bias[25].

263 Here we performed a comprehensive comparison of six low input small RNA sequencing

264 reagents utilizing both ligase-depend, polyA-based and single-adapter methods, including one

265 kit that employed UMI tags. Note that we assessed here only the performance of the kits to

266 identify miRNAs; other small RNA species that may be captured were not assessed.

11

267

Sequencing yields and miRNA read proportions

269   Considerably different numbers of raw reads were obtained from the different kits. The kits

270   from TailorMix and QIAseq returned the highest miRNA read counts both in the synthetic

271   miRNA and the human total RNA samples. However, raw read outputs cannot be used to

272   judge the performance of a method. Furthermore, since the samples from SMARTer-beta

273   were sequenced alone in a single lane, we cannot exclude that technical issues affecting only

274   that lane were responsible for the low raw read numbers that passed filters. The input range

275   tested in this study was at or below the indicated range stated for the SMARTer-beta kit (100

276   ng -1 ug total RNA or 2 ng – 200 ng enriched small RNA); this may have resulted in the

277   observed poor performance. Since this study was performed, the kit has been re-optimized

278   and released with a new formulation and improved performance. Nonetheless, the low

279   proportion of reads mapping to miRNAs from both the CATS and SMARTer-beta was

280   clearly evident, which could be attributable to inefficient removal of other small RNA species

281   during library preparation. However, greater numbers of reads that were not counted as

282   miRNA (due to imperfect match in length to the database reference sequence) were

283   noticeable for CATS, which may indicate that polyadenylation-based methods are trickier to

284   process during data analysis, due to uncertainties on the length of the poly-A tail added. To

285   reduce the influence of technical aspects (e.g. different library size selection and purification

286   methods, as well as raw read yields) on the comparisons, all miRNA counts were down-

287   sampled to the same levels. CATS and SMARTer-beta did not reach the selected thresholds

288   and were therefore excluded from further analysis.

289

Detection rate sensitivity

When applying low detection rate sensitivity thresholds, most synthetic miRNAs could be detected by the four remaining kits, indicating that all of them may be suited to assess the overall miRNA repertoire. However, when applying more stringent detection thresholds ranging from 1cpm to 200cpm, greater differences in detection rates between the kits became evident, and QIAseq and TailorMix emerged as the most sensitive. It is worth noting that kit specific biases played a greater role in miRNA detection than input RNA amounts, at least within the ranges tested here (0.1-1.0 ng miRNA).

Reliability

Intra-rater reliability showed very high concordance between miRNA counts within the replicates of a miRNA library preparation kit, independent of the kit, for both synthetic miRNA and human total RNA inputs. Similar results have been reported by Giraldez, et al. [44] and Wright, et al. [25], although they refer to intra-rater reliability as reproducibility and consistency respectively. The intra-rater reliability was strong both for 0.1ng and 1.0ng synthetic miRNA samples for all kits in our study (data not shown) which is promising given current interest in using low RNA inputs derived from small biological specimens.

In concordance with the findings reported by Giraldez, et al. [44], Coenen-Stass, et al. [45] and Wright, et al. [25], inter-rater reliability (concordance of read counts seen between the different kits, also called reproducibility or consistency across replicates) was lower compared to the intra-rater reliability. In particular, QIAseq deviated from the other kits, but we stress that this does not indicate poorer performance. QIAseq employs a different 3´adapter sequence compared to the other three kits which may underlie the dissimilar preference for subset of miRNAs observed. These observations underscore the emerging conclusion that kit-specific

315   differences should be considered by any researchers comparing miRNA-seq datasets, as

316   supported by another recent study [46]. Notably, the concordance between the miRNA counts

317   measured and the expected concentration for the synthetic miRNA samples was low, and

318   revealed that none of the library preparation kits could accurately quantify the majority of

319   miRNAs.

320

321   **Differential expression**

322   Differential expression analysis of synthetic miRNA mix A versus mix B revealed that all

323   kits could detect at least 31 out of 40 non-equimolar miRNA correctly as DE (fold change

324   ≥ 2). MiRNAs hsa-miR-1199-5p, hsa-miR-22-5p and hsa-miR-940 were never detected as

325   DE by any of the kits. These miRNAs were present at two-fold concentration differences, the

326   lowest fold change tested, which can be challenging. In general, all reagents displayed greater

327   problems to detect small fold-change differences, reminiscent of results seen in the recent

328   study by Giraldez, et al. [44].

329

330   Our study offered the additional possibility to study levels of false positive DE miRNAs

331   detected from the 903 equimolar miRNAs. Equimolar miRNAs found to be DE were

332   characteristically detected as DE with low fold-changes and showed little agreement between

333   the kits, consistent with their being false positive calls. Taken together, QIAseq showed

334   slightly higher sensitivity (true positives) and slightly higher specificity (fewer false

335   positives) than the other reagents, although the false-positive calls did fall within the

336   expected rate set for the analysis (False discovery rate = 0.05). Reinforcing these conclusions,

337   the titration response analysis clearly demonstrated the superior performance of the QIAseq

338   reagents to most faithfully represent the levels of miRNAs in input material.

339

14

340   It nonetheless appears that the different reagents have differing preferences for particular

341   miRNAs. The primary sequence of terminal miRNA nucleotides [18], secondary structure

342   affecting ligation sites [47] and co-folding of the miRNA and ligated adapters [21] have all been

343   documented as sources of bias affecting miRNA detection. Interestingly, the 3' adapter

344   sequence in the QIAseq kit differs from the other three kits analysed. However, our attempts

345   to explain the differences observed between the kits based on primary sequence or secondary

346   structure analyses were inconclusive (data not shown).

347

348   Greater differences between kits were observed by examining DE miRNAs detected when

349   comparing the RA patient pool and healthy control pool of human CD8+ T cell RNA, where

350   the number of DE miRNA varied between none (srLp) to 19 (CleanTag). There are few

351   preceding studies of miRNAs from blood-isolated CD8+ cells in rheumatoid arthritis, but

352   some of the miRNAs found to be DE in this study have previously been associated with RA,

353   e.g. miR-221-3p [48], miR-223-3p [49-51], miR-374b-5p [52] and miR-486-3p [52], however further

354   confirmation is needed. Worryingly, in addition to the varying number of DE miRNA

355   detected by the different kits, there was almost no concordance between the miRNAs

356   identified. Taken together, it is advisable to interpret DE miRNA results from studies

357   employing different library preparation methods with caution.

358

359   **Re-analysis of QIAseq dataset utilising UMIs**

360   Reverse transcription and PCR-amplification may be potential sources of bias during library

361   preparation, and PCR can also introduce duplicate reads. QIAseq was the only kit tested to

362   address the issue of duplicate reads by the inclusion of UMIs, however, under the employed

363   conditions, no appreciable difference between UMI counts and the ordinary read counts were

364   detected, mirroring the findings of Wong, et al. [46]. Fu, et al. [27] observed that higher fractions

365   of PCR duplicates could be observed when reducing the starting material, but when

366   comparing the 1.0ng and 0.1ng synthetic miRNA samples, no difference in the proportion of

367   PCR duplicates was seen. Nonetheless, it remains possible that at lower concentrations than

368   tested here, UMIs may prove useful for the elimination of duplicates to improve dataset

369   quality.

370

371   In conclusion, the QIAseq kit from QIAGEN consistently demonstrated performance at, or

372   near, the top for all metrics examined. It should be mentioned that QIAGEN made an error

373   affecting samples 1-8 in their first attempt at library preparation and were supplied with

374   replacements. With the exception of performance in the titration response assay, the

375   TailorMix kit from SeqMatic closely followed. Lexogen´s srLp and Trilink´s CleanTag kit

376   also performed well, and the majority of differences we detected point to kit-specific biases.

377   However, whilst the experiments conducted here show that sequencing is a very sensitive

378   method for detecting miRNAs, even at low abundance, it is also clear that none of the kits

379   performed impressively with regard to accurately reflecting the relative input levels of all

380   miRNAs. There is clearly room for improvements in this regard for the development of

381   further enhanced reagents or methods to accurately quantitate miRNA levels.

382

383   **Material and Methods**

384   **Study material**

385   The performance of six miRNA library preparation kits was examined using low-input

386   material consisting of synthetic miRNA samples or human-derived total RNA samples. To

387   maximize the possibility that each procedure was performed under optimum conditions,

16

388   samples were distributed to the kit vendors for library construction. Sequencing libraries were

389   returned to the Norwegian Sequencing Centre for sequencing and data analysis.

390   **Synthetic miRNA samples**

391   The synthetic miRNA samples consisted of a mixture of equimolar and non-equimolar

392   miRNAs. The miRXplore Universal Reference (Miltenyi, California, United States),

393   comprising 962 HPLC purified, 5' phosphorylated, synthetic oligonucleotides of human,

394   mouse, rat and viral miRNA origin, was used as an equimolar miRNA pool. For the non-

395   equimolar pool, 40 additional HPLC purified, 5'phosphorylated, synthetic oligonucleotides

396   representing human miRNA were purchased from Eurofins MWG Synthesis GmbH (Bavaria,

397   Germany). Altogether five different miRNA mixes were created (denoted mix A to mix E,

398   Figure 1a). Mix A and Mix B consisted of the equimolar miRNA pool supplemented with the

399   non-equimolar pool present at eight different concentration ratios between the two mixes

400   spanning a 100-fold range (Supplementary Table S1). Mix C was a titration of 0.75 mix A

401   and 0.25 mix B, while mix D was a titration of 0.25 mix A and 0.75 mix B. In the case of

402   mixes A-D, the total miRNA concentration was 30 nM, with individual equimolar miRNAs

403   present at 30 pM and other miRNAs ranging from 3 – 300 pM. Mix E consisted of the same

404   miRNAs as mix A but at a 10-fold lower concentration. Due to the low concentrations in the

405   five synthetic miRNA mixes, the samples were blended with yeast (*Saccharomyces*

406   *cerevisiae)* total RNA, which does not contain known endogenous miRNAs [28], to minimise

407   degradation and loss of material due to adhesion to plasticware, and to mimic the more

408   complex total RNA mixtures encountered under typical usage. In each mix, the final RNA

409   content was 2 ng/µl, with miRNA representing approx. 10% (w/w) of the total amount (mixes

410   A-D) or 1% (mix E). The samples were distributed in triplicates to the participating vendors.

411   To each of the triplicates in mix A to mix E, one additional specific miRNA (miR-147a, miR-

412   212-3p or miR-412-3p) was added to check that the replicates were processed independently

413   throughout library preparation and were not combined into a single sample to increase

414   reproducibility.

415

416   To verify the intended ratios of the synthetic miRNA sample starting material, quantitative

417   reverse-transcriptase PCR was performed using 16 pre-designed TaqMan® Small RNA

418   assays (Thermo Fisher Scientific, Waltham, MA USA) according to manufacturer's

419   instructions. Assay details are provided in Supplementary Material and Methods. Relative

420   abundances of miRNAs in mixes A and B were measured by absolute quantification relative

421   to a standard curve.

422   **Human-derived total RNA samples**

423   Peripheral blood CD8+ T cells were magnetically sorted from newly diagnosed rheumatoid

424   arthritis (RA) patients (n=4) and healthy controls (n=4) using the EasySep cell isolation

425   system (Stemcell technologies, Vancouver, Canada). The RNA/DNA/Protein Purification Kit

426   (Norgen Biotek, Ontario, Canada) was used to isolate total RNA. Only RNA samples with

427   RNA integrity values above 8.5 were used for downstream analysis. To ensure the desired

428   amount of total RNA input for the miRNA library preparation, the four RA patients and the

429   four healthy controls were mixed together to obtain one pooled RA and one pooled healthy

430   control sample respectively. Triplicates of these different sample types were distributed to the

431   participants.

432   **miRNA library preparation**

433   Each participant was asked to prepare miRNA libraries from the 21 samples described above

434   using their specific miRNA library preparation kit. For optimization purposes the participants

435   received a further 20 ng of synthetic miRNA (blend of Mix A and Mix B) and 200 ng total

436   human RNA. All participants were requested to use the same Illumina i7 index sequence for

18

437   the same sample to avoid any possible effect of these sequences on the downstream library

438   preparation and sequencing process. Detailed sample and index information can be found in

439   Supplementary Table S2.

440

441   At the time of writing, four of the six kits were commercially available in the formats used

442   for this study (CATs, QIAseq, CleanTag and TailorMix). A fifth kit, srLp, was also

443   commercially available, but with different index primer sequences. For comparison purposes

444   and to avoid possible bias arising from the use of different indexes, this participant

445   synthesised custom index primers complying with the index sequences specified in this

446   article. The SMARTer kit used in the study had not been released for purchase, but a

447   modified version is now available. It should be noted that this study is not exhaustive, since

448   two library preparation suppliers meeting the input amount inclusion criteria (PerkinElmer,

449   formerly Bioo Scientific, and NEB) declined to participate. Detailed descriptions of the

450   library preparation conditions employed by the producers of the specific reagents are

451   supplied in the Supplementary Material and Methods.

452

453   **Sequencing**

454   All libraries were sequenced at the Norwegian Sequencing Center on the same single-read

455   flow cell of a HiSeq 2500 (Illumina, San Diego, CA) with 75 bp reads generated using v4

456   clustering and SBS reagents according to the manufacturer´s instructions. To avoid

457   sequencing lane bias, the libraries of srLp, QIAseq, TailorMix, CATS and CleanTag were

458   randomly distributed over five lanes of the flow cell, equivalent to sequencing 21 libraries per

459   lane (Supplementary Table S4). Due to concerns that the SMARTer beta libraries contained a

460   large proportion of non-miRNA inserts (higher molecular weight products than expected,

461   making it challenging to obtain equivalent numbers of reads per sample), these libraries were

462   sequenced independently from the other participants on a single lane (Supplementary Figure

463   1),

Bioinformatic analysis

Read mapping and reference sequences

466   Primary base calling and quality scoring was performed using RTA v1.18.66.4 (Illumina),

467   followed by demultiplexing and processing with Bcl2fastq v2.18.0.12 (Illumina).

468   For trimming of the 3' adapter, cutadapt v1.15 [29] with parameter –m 10 was used. Detailed

469   information about adapter sequences is provided in the Supplementary Material and Methods.

470   Read mapping was performed using bowtie v1.1.2 [30] with parameters –a and --norc. No

471   mismatch was allowed. As reference, the expected pools of synthetic miRNAs (962 synthetic

472   equimolar miRNAs originating from the miRXplore universal reference and 40 non-

473   equimolar miRNAs) were used for the synthetic miRNA samples, and the mature human

474   miRNA sequences specified in miRBase[31] v21 for the human total RNA samples. We

475   confirmed that all replicates had been processed separately by verifying the presence/absence

476   of spiked replicate-specific miRNAs in the datasets from each sample. Further analysis

477   revealed that 59 of the miRNA sequences included in the miRXplore Universal Reference

478   were identical to sequences in the *Saccharomyces cerevisae* (sacCer3) genome

479   (Supplementary Table S3). To avoid potential miscounting of yeast fragments in the

480   downstream analysis, these miRNA were excluded and only the remaining 903 miRNA of

481   the miRXplore Universal Reference were analysed further. Mapped reads (restricted to

482   miRNAs matching exactly to the reference sequence and length) were counted using a

483   custom python script (available upon request).

Read count modelling

484

485   With the exception of differential expression and UMI analysis, all further downstream

486   analyses were performed on down-sampled mapped miRNA reads to minimise confounding

487   factors arising from sources such as read numbers and proportions of adapter dimer reads,

488   which can be influenced by the purification method chosen and by pipetting errors. Random

489   down-sampling to 2.5 million reads was performed for the synthetic miRNA samples and to

490   0.75 million reads for the human total RNA samples. The seed number was set to 123.

491

492   In miRNA-seq count data, the average observed variance across samples increases with

493   higher average expression of the miRNA. If this heteroscedastic behaviour of the count data

494   is not taken into account, the results of most downstream analyses will be dominated by

495   highly expressed and highly variable miRNAs. We therefore transformed count data, where

496   indicated, with the rlog function of DeSeq2[32] (v1.20.0), which produces a superior

497   homoscedastic output than log2 transformation for low- and high-expressed genes [32].

Data analysis

498

Detection rate sensitivity and reliability

499

500   Data and statistical analyses were performed using R v3.5.2 [33] and Python v2.7.13. Unless

501   otherwise stated, ggplot2 [34] was used for data visualization. Synthetic miRNA and human

502   total RNA down-sampled read count data were used in the detection rate sensitivity analysis.

503   Upset plots were produced using the R package UpSetR[35] v1.4.0.

504

505   Rlog transformed synthetic miRNA and human total RNA count data were used for assessing

506   the reliability of the library preparation kits, on which intra-class correlation (ICC), Pearson

507   correlation and Bland-Altman agreements calculations were performed. For ICC, the two-

508   way mixed effects model, absolute agreement and single rater (ICC(3,1)) were applied using

21

509   the R package psych[36] v1.8.4. ICC values were interpreted according to the recommendations

510   of Koo and Li [37] where ICC values above 0.9, between 0.75 and 0.9, between 0.5 and 0.75

511   and below 0.5 indicate excellent, good, moderate and poor reliability respectively. Thresholds

512   described by Chan [38] were used for the Pearson correlation where correlations above 0.8,

513   between 0.6 and 0.8, between 0.3 and 0.6 and below 0.3 are described as very strong,

514   moderately strong, fair and poor respectively. The R corrplot package[39] v0.84 was utilized for

515   correlation plots and the R BlandAltmanLeh package[40] v0.31 for Bland Altman calculations.

Differential expression and titration response

517   Original read count data of mix A and mix B were used for the differential expression

518   analysis using the R package edgeR[41] v3.22.3. For the synthetic miRNA samples a read count

519   filtering of 3 counts per million (cpm) in at least two libraries was applied to the differential

520   expression analysis while a filter of 20cpm in at least two libraries was used for the human

521   total RNA samples. miRNAs were defined as significantly differentially expressed after

522   multiple testing adjustment with the methods of Benjamini and Hochberg controlling for a

523   false discovery rate of 0.05. In addition, only those miRNA with |log2 FC| > 1 between the

524   tested conditions were kept.

525

526   The titration response of the 40 non-equimolar miRNAs of the synthetic miRNA samples was

527   examined in mixes A to D according to the analyses published by Shippy, et al. [42]. Average

528   rlog expression values for each miRNA were calculated across the three replicates of each of

529   mixes A to D. If the average expression values for each miRNA followed the expected

530   concentration trend (across the four possible concentrations seen in each mix), it was scored

531   as titrating. Any deviations from the expected trend were scored as non-titrating.

532 **UMI analysis**

533 QIAGEN's analysis tool Geneglobe was used for assessing the effectiveness of QIAseq's

534 UMIs. For the synthetic miRNA samples the option "other" was chosen for mapping while

535 "human" was chosen for the human total RNA samples during the primary data analysis. The

536 resulting count table included UMI (after PCR duplicate removal) and raw (before PCR

537 duplicate removal) read counts for each miRNA in the samples. Before analysing the

538 correlation between UMI and raw read counts, the counts were rlog transformed.

539

540 **Acknowledgements**

543 **Funding statement**

546 **Data availability statement**

547 Raw sequencing fastq files and miRNA count tables will be made available in the Gene

548 Expression Omnibus database.

Disclosure of interest

549

550   AF and AM are employees of Takara Bio USA Inc., JMH is an employee of, and SS a

551   former employee, of TriLink Biotechnologies LLC. MAH and JMS are employees of

552   QIAGEN Sciences. PM and JV are employees of Lexogen GmbH. LN and HKY are

553   employees of SeqMatic LLC. FH, XZ, MZ, JB, AS, STF, ML, MD, SR, BAL and GDG

554   report no conflict of interest.

555

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682   miR-24 and miR-125a-5p as plasma biomarkers for rheumatoid arthritis. *PloS
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685   **Appendices**

686   **Supplementary Figures** (document: Supplementary_figures_Heinicke_etal2019.docx)

687   **Supplementary Tables** (document: Supplementary_tables_Heinicke_etal2019.xlsx)

688   **Supplementary Material and Methods** (document:

689   Supplementary_Material_and_Methods_Heinicke_etal2019.docx)

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705 **Table 1: Small RNA library preparation methods tested in this study.**

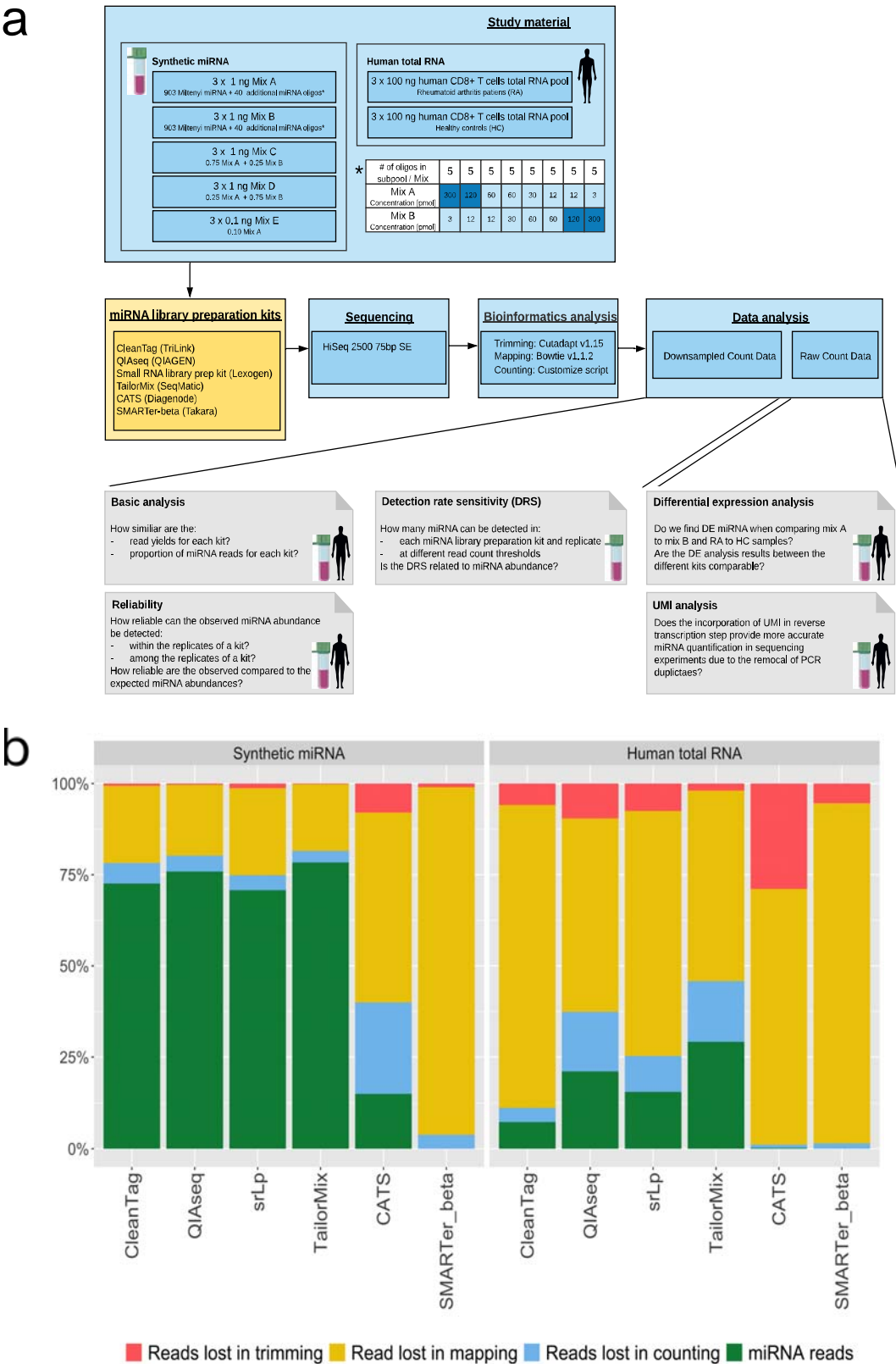| Method Name	Commercial supplier	Key points*	Max. input volume tolerated	Reported RNA input range (varies with type of input tested)	Maximum number of indexes available	Method types
CATS Small RNA-Seq Kit (CATS)	Diagenode s.a.	Single-tube, 4-step process of polynucleotide tailing, reverse transcription and PCR amplification. 1 purification step (2 if size selection required).	8 µl	0.1 – 100 ng	24	Poly-A based. 2-adapter procedure
Small RNA-Seq Library Prep Kit (srLp)	Lexogen GmbH [Cat. No. 052, 058]	4-step procedure of 3' adapter ligation, 5' adapter ligation, reverse transcription and PCR. 2 purification steps (3 if size selection required).	6 µl	0.05 – 1000 ng	96	Ligase based. 2-adapter procedure
QIAseq miRNA Library Kit (QIAseq)	QIAGEN [Cat. No. 331502 or 331505]	5-step procedure of 3' adapter ligation, 5' adapter ligation, reverse transcription and PCR. 2 purification steps (3 if size selection required).	5 µl	1 – 500 ng	96	Ligase based. UMI incorporated. 2-adapter procedure
TailorMix microRNA Sample Preparation Kit Version 3 (TailorMix)	SeqMatic LLC.	4-step procedure of 3' adapter ligation, 5' adapter ligation, reverse transcription and PCR. 2 purification steps, including a final PAGE gel excision.	6 µl	1– 1000 ng	96	Ligase based. 2-adapter procedure
SMARTer® miRNA-seq Kit (Beta version) (SMARTer)	Takara Bio USA Inc.	5-step procedure of 3' mono-adapter ligation, dephosphorylation, adapter dimer blocking, circularisation, reverse transcription and PCR. 2 purification steps, including a final PAGE gel excision.	4 µl	100 – 1000 ng total RNA or 2 – 200 ng enriched small RNA	48	Ligase based. Single adapter procedure

| CleanTag™ Small RNA Library Prep Kit (CleanTag) | TriLink BioTechnologies, LLC. | Single-tube, 4-step procedure of 3' adapter ligation, 5' adapter ligation, reverse transcription and PCR. 1 purification step. | 10µl | 1 – 1000 ng | 48 | Ligase based. 2-adapter procedure |

706   * A step is defined as a labwork period that culminates in an incubation longer than 5
707   minutes.
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729 **Table 2: Fraction of titrating miRNAs (n=5) in each of the eight concentration groups. Average rlog**

730 **expression values for the 40 non-equimolar miRNAs were calculated across the three replicates each of**

731 **mixes A to D. Each miRNA was scored as titrating if the average values followed the expected trend in**

732 **concentrations from high to low or vice versa across mixes A to D.**

Conc. Ratio	CleanTag	QIAseq	srLp	Tailor Mix
0.01	1	1	1	1
0.1	0.8	1	1	1
0.2	1	1	0.8	0.8
0.5	0.8	0.6	0.4	0.6
2	0.6	0.8	0.8	0.2
5	0.4	1	1	0.8
10	0.6	1	1	0.6
100	0.8	1	0.8	0.8

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745   **Figure 1**

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747 **Figure 2**

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749   **Figure 3**

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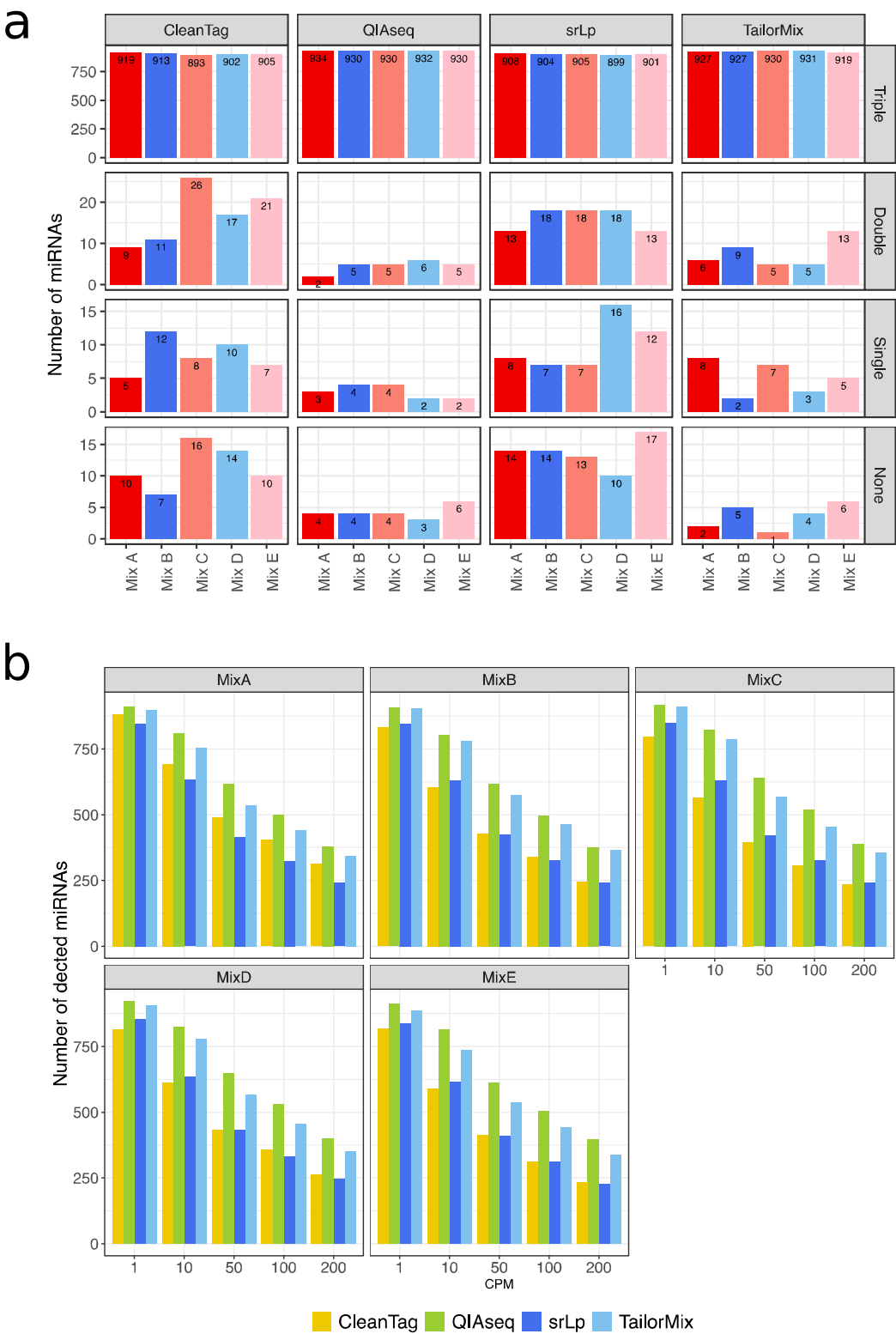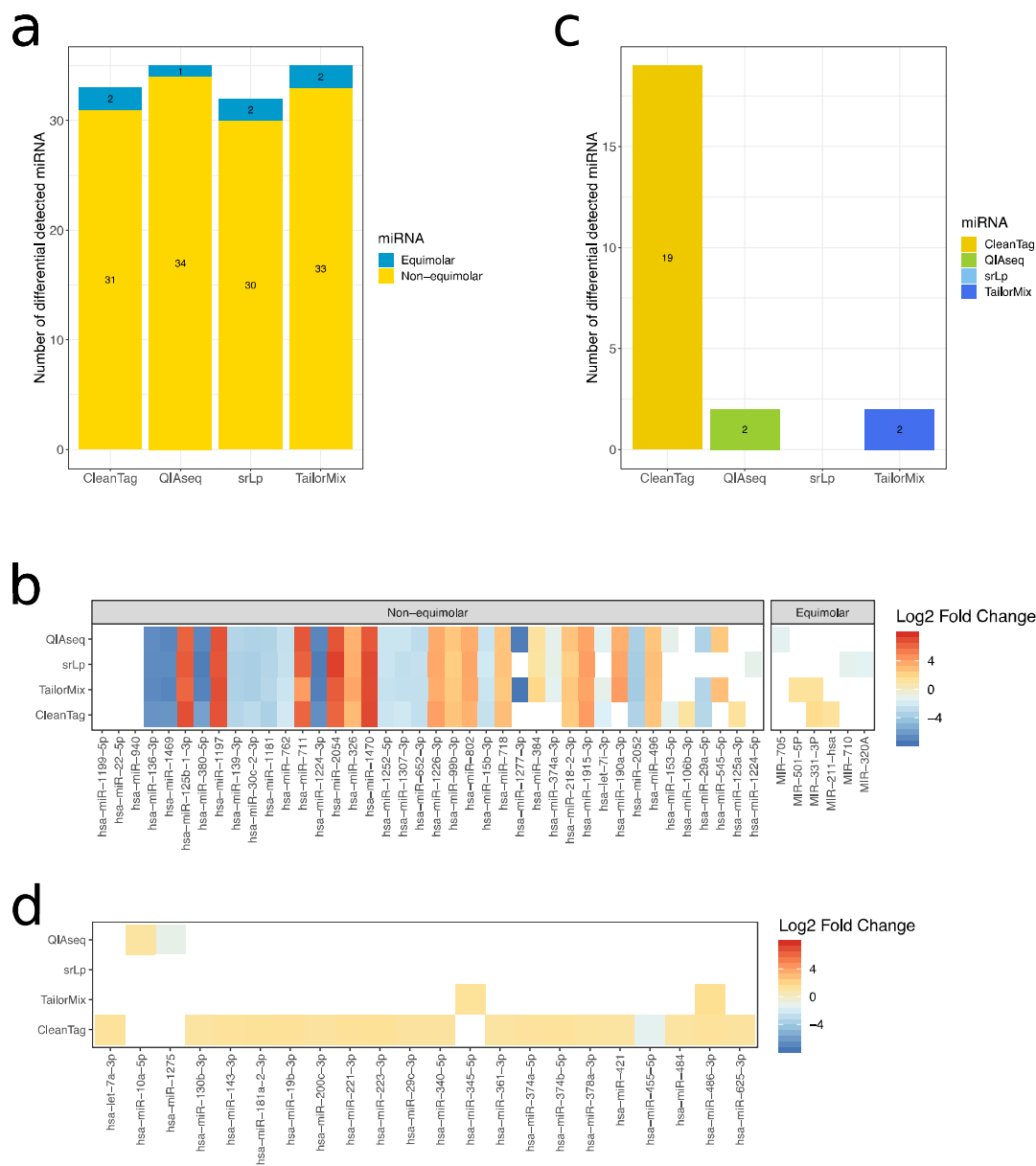| Figure	Caption
1	**Experimental design and sequencing read distribution. A: Overview of the study material, miRNA library preparation kits used, sequencing, bioinformatics and data analysis. Steps presented in blue boxes were performed in-house, while the step presented in the yellow box was executed by the indicated library preparation vendors. Grey boxes represent individual data analysis steps. B: Percentage of reads that were removed during the bioinformatic analysis and final miRNA proportion remaining (green). Trimming refers to removal of adapter sequences, mapping to miRNA reference alignment, and counting to filtering of aligned miRNAs that did not have the same length as the reference sequence. Results presented are the mean of 15 replicates in the synthetic miRNA (left) and the mean of six replicates in the human total RNA samples (right).** **Figure 1 A was created using images from Servier Medical Art (Servier. www.servier.com, licensed under a Creative Commons Attribution 3.0 Unported License).**
2	**Detection rate sensitivity. A: Bar charts presenting number of miRNAs detected in all replicates (Triple), in 2 out of 3 replicates (Double), in 1 out of 3 replicates (Single) or not detected in any replicate (None) across all synthetic miRNA mixes and all library preparation kits. The maximum number of detectable miRNAs is 943 (903 equimolar and 40 non-equimolar miRNA). B: Bar charts for various read count thresholds in the synthetic miRNA samples. A miRNA is defined as detected when it is (i) expressed in all three replicates of the mix and (ii) the read counts are greater or equal to the count per million (CPM) threshold displayed on the x-axis. The colours of the bars represent the reagents.**
3	**Differential expression analysis. Kit-specific number of differentially expressed miRNA detected for A: synthetic miRNA samples (mix A versus mix B) and C: human total RNA samples (RA versus healthy control). miRNA-specific log2 fold changes across the different kits for B: synthetic miRNA samples and D: human total RNA samples.**

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