Journal of Visualized Experiments

Sequencing small non-coding RNA from formalin-fixed tissues and serum-derived exosomes from castration-resistant prostate cancer patients

--Manuscript Draft--

Editor   August 16, 2019

Journal of Visualized Experiments

Dear Editors

Thanks for the review of our invited original manuscript entitled 'Sequencing small non-coding RNA from formalin-fixed tissues and corresponding serum-derived exosomes from castration-resistant prostate cancer patients' for publication in your journal. We sincerely thank the reviewers and editorial board for their constructive comments. We have addressed the concerns that were raised in the previous version and incorporated the suggestions as detailed in the enclosed 'response to editor' letter. We sincerely thank the editors for their helpful suggestions. We hope that we have satisfactorily addressed all the raised concerns in the revision and hope for a favorable consideration. Please note that there is a change in institutional affiliation for the first author and corresponding author.

Thank you so much!

Sincerely,

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1 **TITLE:**
2 **Sequencing Small Non-coding RNA from Formalin-Fixed Tissues and Serum-Derived Exosomes**
3 **from Castration-Resistant Prostate Cancer Patients**
4
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23 **KEYWORDS:**
24 small RNA sequencing, exosomes, FFPE tissues, prostate cancer, miRNA, biomarker
25

26 **SUMMARY:**
27 Therapy resistance often develops in patients with advanced prostate cancer, and in some cases,
28 cancer progresses to a lethal subtype called neuroendocrine prostate cancer. Assessing the small
29 non-coding RNA-mediated molecular changes that facilitate this transition would allow better
30 disease stratification and identification of causal mechanisms that lead to development of
31 neuroendocrine prostate cancer.
32

33 **ABSTRACT:**
34 Ablation of androgen receptor (AR) signaling by androgen deprivation is the goal of the first line
35 of therapy for prostate cancer that initially results in cancer regression. However, in a significant
36 number of cases, the disease progresses to advanced, castration-resistant prostate cancer
37 (CRPC), which has limited therapeutic options and is often aggressive. Distant metastasis is
38 mostly observed at this stage of the aggressive disease. CRPC is treated by a second generation
39 of AR pathway inhibitors that improve survival initially, followed by the emergence of therapy
40 resistance. Neuroendocrine prostate cancer (NEPC) is a rare variant of prostate cancer (PCa) that
41 often develops as a result of therapy resistance via a transdifferentiation process known as
42 neuroendocrine differentiation (NED), wherein PCa cells undergo a lineage switch from
43 adenocarcinomas and show increased expression of neuroendocrine (NE) lineage markers. In
44 addition to the genomic alterations that drive the progression and transdifferentiation to NEPC,

45 epigenetic factors and microenvironmental cues are considered essential players in driving
46 disease progression. This manuscript provides a detailed protocol to identify the epigenetic
47 drivers (i.e., small non-coding RNAs) that are associated with advanced PCa. Using purified
48 microRNAs from formalin-fixed paraffin-embedded (FFPE) metastatic tissues and corresponding
49 serum-derived extracellular vesicles (EVs), the protocol describes how to prepare libraries with
50 appropriate quality control for sequencing microRNAs from these sample sources. Isolating RNA
51 from both FFPE and EVs is often challenging because most of it is either degraded or is limited in
52 quantity. This protocol will elaborate on different methods to optimize the RNA inputs and cDNA
53 libraries to yield most specific reads and high-quality data upon sequencing.
54
55 **INTRODUCTION**:
56 Prostate cancer is driven by androgens acting via AR signaling. Therefore, targeting the AR
57 pathway is the mainstay treatment for the disease[1]. However, resistance often ensues as a result
58 of targeted therapies and the disease progresses to an advanced metastatic CRPC[2]. CRPC is
59 treated by second generation of AR therapy that includes enzalutamide and abiraterone[2,3] which
60 improves survival initially. However, lethal variants such as NEPC often emerge in 25–30% of
61 treated CRPC cases that have limited treatment options, leading to increased mortality[3]. NEPC
62 arises via a reversible transdifferentiation process known as NED, wherein PCa cells undergo a
63 lineage switch from adenocarcinomas and show decreased expression of AR and increased
64 expression of NE lineage markers including enolase 2 (ENO2), chromogranin A (CHGA), and
65 synaptophysin (SYP)[4]. Given that these resistance variants arise as a result of therapeutic
66 interventions, it is essential to decipher pathways that lead to generation of this deadly, hard to
67 treat form of PCa.
68
69 The genomics of NEPC was recently deciphered in an exhaustive study done by Beltran et. al.
70 whereby copy number alterations, mutations, single nucleotide polymorphisms (SNPs), and DNA
71 methylation from patient-derived metastatic tissues were analyzed[5]. Despite significant
72 advancement in the understanding of the genomics of this aggressive form of PCa, little is known
73 about the epigenetic factors, including the small non-coding RNAs (microRNAs) that are involved
74 in the transition of CRPC-adenocarcinoma to NEPC. MicroRNAs (miRNAs) are 22bp long, double-
75 stranded RNAs that act primarily by suppressing gene expression post-transcriptionally by
76 sequence-specific interactions with the 3'-untranslated regions (UTRs) of cognate mRNA targets[6].
77 Several oncomiRs and tumor suppressors have now been identified, and their role in regulating
78 disease onset and metastasis has been well studied in different cancers[6,7]. These small non-
79 coding RNAs often serve as very important targets in controlling disease mortality[6,8,9]. More
80 recent research has been focused on understanding the paracrine effects of miRNAs in cancer
81 metastasis via their transport in EVs, such as exosomes, that flow in the bloodstream and allow
82 tumor cells to send these secondary messengers to metastatic locations in a nuclease-free
83 environment[10-12]. EVs carry miRNAs from the tumor cells to transfer the transforming effects
84 from the host cells[12]. Identification of EVs as secondary messengers of tumor cells can therefore
85 be useful in noninvasive detection of disease severity.
86
87 The miR-1246 is highly upregulated in EVs from aggressive PCa, and it indicates the disease
88 severity[13]. These EV-associated miRNAs not only serve as noninvasive biomarkers of the disease,

89 but also play functionally important roles in driving tumorigenesis. Thus, it essential to
90 understand the significance of the miRNA repertoire that is associated with resistant forms of
91 PCa to allow better identification of noninvasive biomarkers as well as their functional
92 significance.
93
94 The advent of next generation sequencing has offered the most comprehensive platform to study
95 the details of tumor landscape involving alterations in the genome such as mutations,
96 chromosomal relocations, chromothripsis, and methylation, all of which contribute significantly
97 to the form and nature of cancer[14-16]. Likewise, it is also an essential tool to understand the vast
98 epigenomic changes that occur in a tumor cell and that are often important players in disease
99 severity[17]. With the aim of understanding the miRNA repertoire associated with the generation
100 of NEPC, small RNA sequencing was performed on FFPE metastatic CRPC tissues and their
101 corresponding serum-derived EVs. RNA derived from either of these two sample sources is (1)
102 low in yield and (2) of bad quality due to the degradation that often happens due to formalin
103 fixation and EV isolation. Furthermore, generating cDNA libraries is a critical, but cumbersome,
104 step of a sequencing run. Thus, methods for isolating these RNAs and using them to generate
105 libraries for small RNA sequencing require optimization to generate accurate and reliable data.
106
107 There are several methods to profile miRNA expression in different samples, including RT-PCR,
108 microarrays, and in-situ hybridization (ISH). A protocol using FFPE tissue-derived RNA to evaluate
109 miRNA expression by RT-PCR and ISH was recently published[18]. More recent technologies offer
110 more exhaustive and comprehensive platforms for profiling miRNA expression in a sample.
111 NanoString nCounter offers a sensitive miRNA detection platform[19], but the detection is often
112 limited by the number of miRNAs that are available in the array (~2,000). In such a scenario, a
113 more sensitive and exhaustive platform such as next generation sequencing offers much wider
114 depth of miRNA identification and simultaneous profiling in different samples[20]. The method has
115 been used to determine the miRNA signatures in urine or plasma from PCa patients[21-23]. In the
116 current article, a protocol is presented to use a next generation sequencing platform to study
117 miRNA profiles associated with aggressive CRPC using FFPE tissues and serum-derived EV RNAs.
118
119 **PROTOCOL**:
120
121 This study was conducted in accordance with the ethical guidelines of US Common Rule and was
122 approved by the University of California San Francisco (UCSF) Committee on Human Research.
123
124 **1. Microdissection**
125
126 NOTE: FFPE metastatic CRPC tissues with adenocarcinoma features (CRPC-Adeno) or NE
127 differentiation (CRPC-NE) were obtained from the Prostate Cancer Biorepository Network
128 (PCBN). Ten-micron PCa sections on glass slides were prepared for manual microdissection as
129 discussed previously[18]. To briefly summarize:
130
131 1.1. Deparaffinize the tissue sections by soaking the slides in xylene (2x, 10 min each). Then
132 rehydrate the sections by incubating the slides for 5 min each in graded ethanol (100%, 95%,

133 90%, 80%, and 70%), followed by a distilled water wash and staining with hematoxylin (30 s soak).
134

135 1.2. Following hematoxylin staining, wash the tissue sections with water. Then dehydrate the
136 tissue sections by placing them in graded ethanol (70%, 80% 90%, 95%, 100%) (5 min each)
137 followed by xylene (5 min).
138

139 1.3. Analyze the corresponding hematoxylin and eosin (H&E) slides to identify the tumor areas
140 (i.e., adenocarcinomas or NED) and mark these tumor areas and normal adjacent areas with the
141 assistance of a board-certified pathologist. Using these H&E slides as roadmaps, mark the
142 hematoxylin-stained tissues obtained in the above step to distinguish between the tumor and
143 normal areas.
144

145 1.4. Dissect the marked tissue slide carefully under the microscope using a scalpel blade.
146

147 1.5. Collect the dissected tissue from normal and cancerous areas in separate tubes using
148 electrostatically charged gel loading pipette tips. The charged tip attracts the dissected tissue and
149 allows for maximum tissue recovery after dissection. Once the tissue sticks to the charged tip,
150 cut the tip into an empty 2 mL collection tube.
151

152 **2. Isolating serum-derived EVs**
153

154 NOTE: Frozen patient serum samples collected from patients with metastatic CRPC with
155 adenocarcinoma characteristics (CRPC-adeno) or NE differentiation (CRPC-NE) were procured
156 from PCBN using an approved IRB protocol and stored at -80 °C prior to their use. Serum samples
157 were collected from the same set of patients as those used for microdissected tissues to allow
158 comparison between the corresponding tissues and the serum-derived EVs. A commercially
159 available serum EV isolation reagent was used to collect the EVs.
160

161 2.1. Thaw frozen patient serum samples at 4 °C.
162

163 2.2. Use 110 µL of thawed serum sample and spin at 2,000 x g for 30 min.
164

165 2.3. Collect the supernatant for subsequent EV/exosome isolation and discard the debris pellet.
166 Add 30 µL of serum exosome isolation reagent to the supernatant and incubate at 4 °C for 30
167 min.
168

169 2.4. Spin at 10,000 x g for 10 min. Collect the EV-depleted serum carefully without disturbing the
170 pellet in a separate 1.5 mL tube and store at -80 °C for future analysis, if needed.
171

172 2.5. Resuspend the EV pellet in 70 µL of phosphate buffered saline (PBS) prepared in nuclease-
173 free water. Keep an aliquot for the particle tracking analyses system to assess the quality and
174 number of particles. Store the rest of the sample at -80 °C until further analysis.
175

176 **3. RNA isolation from microdissected prostate tissues and EVs**

177

178 NOTE: Total RNA was isolated from microdissected tissues and purified EVs using a commercially
179 available kit (**Table of Materials**) per the manufacturer's instructions with a few modifications.
180 The following sections briefly describe these procedures with special emphasis on the important
181 steps:

182

183 3.1. Isolate the RNA from the microdissected tissues.

184

185 3.1.1. Resuspend the microdissected FFPE tissue in 150 μL of proteinase K buffer. Mix by
186 vortexing and pipette out the residual tissue from the tip. Spin at 11,000 x g for 1 min.

187

188 3.1.2. Add 10 μL of proteinase K to the pellet. Wait for 2 min until the pellet turns white. Pipette
189 up and down a few times.

190

191 3.1.3. Incubate at 56 °C for 16 min. Vortex every 3 min.

192

193 3.1.4. Remove samples and let the heat block reach 80 °C. Incubate the samples on the block for
194 16 min. Vortex every 3 min.

195

196 3.1.5. Incubate on ice for 3 min.

197

198 3.1.6. Spin at 20,000 x g for 15 min. Transfer the supernatant to a new 2 mL tube.

199

200 3.1.7. Add 16 μL of DNase booster buffer followed by 10 μL of DNase I. Mix by inverting the tube
201 gently and incubate at room temperature (RT) for 15 min.

202

203 3.1.8. Add 320 μL of red blood cell (RBC) lysis buffer. Mix by inverting the tube and then add
204 1,120 μL of 100% ethanol. Immediately transfer on spin columns stored at 4 °C. Spin at 8,000 x g
205 for 30 s.

206

207 3.1.9. Wash the columns 2x with wash buffer and spin the columns at 20,000 x g for 5 min to
208 remove residual wash buffer.

209

210 3.1.10. Allow the spin column to air dry for 2–3 min, then elute with 19 μL of RNase-free water.
211 Quantitate the purified RNA on a spectrophotometer and normalize the sample to 40 ng/μL.

212

213 **3.2. RNA isolation from serum-derived EVs '**

214

215 3.2.1. Isolate the exosomal RNA using a kit. Add 75 μL of lysis buffer A and 9.3 μL of lysis buffer B
216 to 50 μL of the purified EV suspension.

217

218 NOTE: Keep mixing the sample by inverting the tube for about 10 min to allow complete lysis of
219 the EVs.

220

221 3.2.2. Add 130 µL of 100% ethanol and immediately mix by inverting the sample. Transfer on a
222 spin column and then spin at 3,300 x *g* for 1 min.

224 3.2.3. Wash the column 2x with the wash buffer. Spin the column at 1,300 x *g* for 3 min to
225 completely remove the wash buffer.

227 3.2.4. Allow the column to air dry for 2 min. Add 18 µL of elution buffer to the column and let it
228 sit for 2 min. Spin at 400 x *g* for 1 min followed by a second spin at 5,800 x *g* for 3 min.

230 3.2.5. Repeat step 3.2.4 with the eluted buffer included in the kit to increase the yield of the RNA
231 from the sample.

233 3.2.6. Quantify the sample on a spectrophotometer and normalize it to 40 ng/µL.

235 **4. Library preparation for small RNA sequencing**

237 NOTE: A small RNA library prep kit (**Table of Materials**) was used to generate cDNA libraries from
238 the isolated RNA samples. The steps to generate libraries, purify, and quality check them prior to
239 running on a sequencer are crucial to any sequencing protocol as they eventually determine the
240 quality of output data from a run. Therefore, proceed carefully with each step. The guidelines
241 from the manufacturer suggest using a minimum of 50 ng of RNA. Given the poor quality and low
242 amounts of total RNA usually obtained from these two sample sources, this protocol is optimized
243 for RNA concentrations as low as 100 ng. The protocol below is for one sample of a library.

245 4.1. Generate adapter-ligated cDNA.

247 4.1.1. Use 5 µL of a normalized (40 ng/µL) RNA sample. Add 1 µL of 3' adaptor. Preheat the
248 thermal cycler to 70 °C prior to incubating the samples. Incubate for 2 min. Immediately transfer
249 the samples on ice prior to moving onto the next step.

251 4.1.2. In a separate tube, mix 2 µL of ligation buffer, 1 µL of RNase inhibitor, and 1 µL of T4 RNA
252 Ligase 2, a deletion mutant. Mix by pipetting up and down and add 4 µL of this mix to the tube
253 with the RNA/3' adaptor.

255 4.1.3. Transfer to the thermal cycler that has been preheated to 28 °C and incubate for 1 h.

257 4.1.4. Add 1 µL of stop solution while keeping the samples on the thermal block. Incubate at 28
258 °C for another 15 min.

260 4.1.5. Remove the tubes and keep on ice immediately after this step.

262 4.1.6. In a separate tube, add 1 µL of 5' adaptor.

264 4.1.7. Transfer to a thermal cycler that has been preheated to 70 °C and incubate for 2 min.

265

266 4.1.8. Immediately place the tube on ice to prevent re-annealing of the adaptors.

267

268 4.1.9. Add 1 µL of 10 mM ATP to the tube of denatured 5' adaptor. Mix by pipetting and add 1 µL
269 of T4 RNA ligase to the mix. Mix by pipetting and add 3 µL of this mix to the tube containing the
270 3' adaptor-ligated RNA. Transfer on the thermal cycler that has been preheated to 28 °C and
271 incubate for 1 h.

272

273 4.1.10. Immediately transfer to ice and either proceed further with the samples or store at -20
274 °C for future use.

275

276 4.1.11. To perform RT-PCR, use 6 µL of each adaptor-ligated RNA and add 1 µL of RNA RT primer
277 to it. Transfer to the thermal cycler that has been preheated to 70 °C. Incubate for 2 min.

278

279 4.1.12. In a separate tube, mix 2 µL of 5x strand buffer, 0.5 µL of 12.5 mM dNTPs, 1 µL of 100 mM
280 DTT, 1 µL of RNase inhibitor, and 1 µL of reverse transcriptase. Add 5.5 µL of this mix to the
281 adaptor-ligated RNA and transfer to the thermal cycler that has been preheated to 50°C. Incubate
282 for 1 h.

283

284 4.1.13. Transfer the adaptor-ligated cDNA immediately to ice.

285

286 4.1.14. In a separate tube, mix 25 µL of PCR mix, 2 µL of RNA PCR primer, 2 µL of RNA PCR primer
287 index, and 8.5 µL of RNase-free water. Add 37.5 µL of this mix to 12.5 µL of adaptor-ligated cDNA.
288 Transfer to the thermal cycler that has been preheated to 98 °C. Program the thermal cycler as
289 suggested in the manufacturer's protocol with the cycle number modified to 15 instead of 11.

290

291 NOTE: Sequences of all the primers used are listed under **Table of Materials**.

292

293 4.1.15. Keep the samples at 4 °C after completion. Proceed with them or store at -80 °C for future
294 use.

295

296 4.2. Purify and perform quality control for the ligated cDNA.

297

298 NOTE: Amplified cDNA was gel-purified by following the manufacturer's protocol except for a few
299 modifications to improve the quality and yield of purified cDNA samples as explained below.

300

301 4.2.1. Use 6% TBE polyacrylamide gels to purify the amplified cDNA. Dilute the high-resolution
302 ladder (HRL) and custom RNA ladder (CRL) with equal volumes of DNA loading dye.

303

304 4.2.2. Add 10 µL of DNA loading dye to each cDNA sample. Run 30 µL of each sample in two
305 separate lanes adjacent to each other with CRL and HRL in the intermediate space between the
306 two different samples.

307

308 4.2.3. Run the gel in 1x TBE buffer at 145 V for approximately 30–40 min.

309

310   NOTE: Keep track of the lower blue front of the loading dye. Stop the electrophoresis when it
311   approaches the bottom of gel.

312

313   4.2.4. Transfer the gel in 1x TBE buffer with Ethidium Bromide (EtBr) at 0.5 μg EtBr/1 mL 1x TBE.

314

315   CAUTION: EtBr is a known carcinogen and each step from here until the excision from the gel
316   should be performed with extreme caution.

317

318   4.2.5. Visualize the gel in a transilluminator and cut the gel precisely between the 145 bp and 160
319   bp marker.

320

321   NOTE: The adaptor dimers run very close to the 145 bp marker. Therefore, cut the gel slightly
322   above the 145 bp marker to avoid contamination with adaptor dimer.

323

324   4.2.6. Transfer the gel to DNA breaking tubes kept in 2 mL collecting tubes. Spin at 20,000 x g for
325   2 min. Add 300 μL of RNase-free water and allow it to rotate gently on a rocker overnight at RT.

326

327   4.2.7. To perform the DNA precipitation, on the following day transfer the contents of the tube
328   to 5 μm filter tubes. Spin at 600 x g for 10 s.

329

330   NOTE: Do not let tubes spin longer than 10 s as the gel passes through the filter.

331

332   4.2.8. Add 2 μL of glycogen, 30 μL of 3 M NaOAc, 2 μL of 0.1x pellet paint, and 975 μL of 100%
333   ethanol to the eluted DNA. Spin at 17,000 x g at 4°C for 20 min.

334

335   4.2.9. Discard the supernatant and add 75% ethanol to wash the pellet. Spin at 17,000 x g at 4 °C
336   for 5 min. Discard the supernatant and incubate the tubes at 37 °C to completely evaporate the
337   ethanol.

338

339   4.2.10. Resuspend the pellet with 12 μL of 10 mM Tris-HCl pH 8.5.

340

341   4.2.11. Perform a library quality check by diluting the purified cDNA by 10x (1:10) and using 1 μL
342   of diluted cDNA to run on a bio-analyzer.

343

344   4.2.12. Make sure the cDNA product size corresponds to the range of small RNA (136 bp–160 bp)
345   in the electropherogram. Calculate the total molarity for each sample. Normalize each sample to
346   2 nM using Tris-HCl pH 8.5.

347

348   4.3. Denature and sequence the purified cDNA.

349

350   4.3.1. Pool the libraries by mixing equal volumes of each 2 nM sample in a single 200 μL PCR tube.
351   Add 10 μL of freshly prepared 0.2 N NaOH to 10 μL of pooled library.

352

353 4.3.2. Mix immediately by vortexing, and spin at 280 x *g* for 1 min. Incubate at RT for 5 min.
354

355 4.3.3. Add 10 µL of 200 mM Tris-HCl pH 7 to 20 µL of the denatured library. Mix by vortexing and
356 spin at 280 x *g* for 1 min.
357

358 4.3.4. Add 970 µL of prechilled hybridization buffer to the library and mix well by vortexing. The
359 library is at a concentration of 20 pM. Keep on ice until it is finally diluted.
360

361 NOTE: The final dilution is done right before running on sequencer.
362

363 4.3.5. Add 117 µL of the denatured library to 1,183 µL of prechilled hybridization buffer. Mix by
364 inverting the tube and pulse centrifuge. The final concentration of the library is now 1.8 pM.
365

366 4.3.6. Add 1.3 µL of PhiX to the 1.3 mL of final library. To initiate a run on a sequencer, follow the
367 on-screen steps on the software interface, review the run parameters, including read type (single
368 read), read length (number of cycles for each read), library ID, and select **Start**.
369

370 **5. Data analysis**
371

372 5.1. At the end of the sequencer run, obtain the resulting sequencing data as fastQ files. Use the
373 appropriate software to analyze the resulting sequencing data.
374

375 5.2. Open the fastQ toolkit application and press the **Launch** button.
376

377 5.3. Select the files from the list of samples in the software and select where the data will be
378 saved.
379

380 5.4. Add a string name that applies to each trimmed sequence. Keep the stringency to 0.9. Input
381 the adapter sequence to trim as "TGGAATTCTCGGGTGCCAAGG" from the 3' end of each
382 sequenced sample**.** Expand the **Read Filtering** tab and select a minimum read length of "**5**". Select
383 the **Acknowledge** box and press the **Continue** button to start trimming. The trimmed files will be
384 saved in the project folder upon the end of the analysis.
385

386 5.5. Open the **small RNA v.1.0** application on the software and press the **Launch** button.
387

388 5.6. Select the project where the files will be saved and sent after the analysis.
389

390 5.7. Select the **Novel miRs and Differential miRs** function in the application. Segregate the
391 groups as "**Control**" and "**Test**" for differential analysis and add the trimmed files to be analyzed
392 in their respective groups.
393

394 5.8. Select the **Launch** button for initiating the analysis. Following the analyses, download the
395 resulting files.
396

REPRESENTATIVE RESULTS:

The library was prepared after RNA isolation and a quality check was performed. Gel purification for the amplified cDNA library prepared from RNA isolated from microdissected tissues and serum-derived EVs is shown in **Figure 1** and **Figure 2**. The product size for adaptor-ligated miRNAs corresponded to about 136–160 bp for each sample. **Figures 1A**-**B** are marked to show the range of the gel that should be precisely excised for small RNA library preparation. **Figures 2A**-**B** show the gel electrophoresis and electropherograms for the microdissected tissue and serum-derived EVs. **Supplementary Figure 1** shows a representative result from the electrophoresis machine and the method to calculate molarity.

Steps to obtain the library metrics prior to running on a sequencer were performed. **Figure 3** shows the representative metrics from a small RNA sequencing run demonstrating the expected cluster density, QC score, cluster passing filter percentage, and estimated yield and error rates. **Figure 3A** and **Figure 3C** show the tables with all the run parameters and graph for QC score over different cycles. **Figures 3B,D** are graphical representations of the error rates for PhiX alignment over different cycles. The provided data are from the on-site analysis software.

A commercially available software application (**Table of Materials**) was used to analyze the sequenced samples from microdissected tissue and serum-derived EVs. The sequenced samples were trimmed off the adaptor sequences using the FASTq toolkit followed by analysis on the "**Small RNA v.1.0**" application. **Figure 3** shows the resulting data upon analysis. **Table 1** and **Table 2** show total reads from microdissected tissue and serum-derived EVs sequencing run. **Figure 3A** and **Figure 3B** show the small RNA length distribution for the two sample sources.

FIGURE AND TABLE LEGENDS:

Figure 1: Library preparation for sequencing small non-coding RNA. Polyacrylamide cDNA gel for the amplified libraries from (**A**) microdissected FFPE tissue RNA and (**B**) RNA from purified serum-derived EVs.

Figure 2: Quality check for sequencing small non-coding RNA. Representative image for gel electrophoresis and electropherogram for purified cDNA library for (**A**) microdissected FFPE tissue and (**B**) serum-derived EVs.

Figure 3: Run metrics for small RNA sequencing run. Tabular and graphical representation of run parameters % Q ≥30, cluster density, cluster passing filter %, estimated yield, and Q Score for sequencing run from (**A**) microdissected FFPE tissue and (**C**) serum-derived EVs. Graphical representation of error rates for PhiX alignment over different cycles for sequencing run from (**B**) microdissected FFPE tissue and (**D**) serum-derived EVs. Errors bars represent standard deviation for the error rates for each cycle.

Figure 4: Expected outcomes from a small non-coding RNA sequencing run. Small RNA read lengths for library from (**A**) Microdissected FFPE tissue and (**B**) serum-derived EVs.

441 **Table 1: Total sequencing reads for a small RNA sequencing run from microdissected FFPE**
442 **tissue.** Percentage of reads corresponding to small RNAs for sequenced FFPE samples that were
443 obtained after analysis.
444
445 **Table 2: Total sequencing reads for a small RNA sequencing run from purified serum-derived**
446 **EVs.** Percentage of reads corresponding to small RNAs for sequenced EV samples obtained after
447 analysis.
448
449 **Supplementary Figure 1: Quality check for sequencing small non-coding RNA.** Representative
450 results from an electrophoresis machine run showing the total molarity of the indicated sample.
451
452 **DISCUSSION:**
453 In this article, we describe a protocol to isolate RNA from FFPE tissues and serum-derived EVs
454 using kits that were optimized to increase the yield and quality of isolated RNAs. Further, the
455 purified RNAs were used to generate cDNA libraries for small RNA sequencing. Both of the listed
456 steps are essential in determining the quality and depth of the sequencing reads that are the final
457 result from the run[24]. Therefore, optimizing these crucial steps is critical to a successful
458 sequencing run.
459
460 An important step in the isolation of FFPE-derived RNA is the digestion with proteinase K that
461 allows for removal of cross-linked formalin from the tissue. This step has been optimized by
462 incubating the sample at 55 °C and 80 °C for about 16–18 min each. This resulted in an effective
463 increase in the quality of isolated RNA by an increase in the 260/280 ratio. Sequencing reads are
464 often variable for a run that includes multiple samples. Therefore, controlling the amount of RNA
465 inputs at the start of library preparation allows for uniformity of the library. Using normalized
466 RNA helps in leveling a sequencing run uniformly across different samples. The most critical step
467 in the library preparation is the purification of amplified adapter ligated cDNAs. It is very
468 important to carefully excise the gels above the 140 bp marker, because a slight deviation
469 towards the bottom of the marker leads to contamination of the adapter dimers. Lastly,
470 normalizing the cDNA library is the most important step of the sequencing protocol and should
471 be done accurately based on the calculated normality from the electrophoresis machine.
472
473 In case of an adapter dimer contamination, one can rerun the purified library on a TBE gel and
474 repurify the desired product. However, the yield of the cDNA library in such an extraction is
475 significantly decreased, which may affect the read length from the sample. For an estimate on
476 the concentration of the purified cDNA library, one can dilute the samples prior to running on
477 the electrophoresis machine based on the product intensity in the TBE gels and prepare different
478 dilutions (i.e., 5x, 10x, or 25x). To further improve the estimation of the purified cDNA libraries,
479 running a qPCR in addition to electrophoresis helps to evaluate template levels and allows more
480 accurate normalization.
481
482 Although ultracentrifugation is the gold standard for isolation of EVs from different sources, a
483 lower yield of the particles from such an extraction often limits the use of this method in purifying
484 EVs where the starting material is limited and a significant amount of input RNA is needed for

downstream applications. Thus, use of the total exosome isolation reagent provides a much quicker and higher yield method for EV isolation from limited sources like serum and plasma from clinical samples.

In all, this protocol details the methods for generating cDNA libraries in a more time effective manner while taking into consideration the yield and quality of the cDNA libraries to get accurate and comprehensive sequencing reads. Given the potential of exosomes as a source of cancer biomarkers, this method for next generation sequencing (NGS) analyses of exosomal miRNA content will be helpful for research in the field.

ACKNOWLEDGMENTS:

DISCLOSURES:

The authors have no conflict of interest to declare.

REFERENCES:

1. Heinlein, C. A., Chang, C. Androgen receptor in prostate cancer. *Endocrine Reviews*. **25** (2), 276-308 (2004).

2. Tilki, D., Schaeffer, E. M., Evans, C. P. Understanding Mechanisms of Resistance in Metastatic Castration-resistant Prostate Cancer: The Role of the Androgen Receptor. *European Urology Focus*. **2** (5), 499-505 (2016).

3. Vlachostergios, P. J., Puca, L., Beltran, H. Emerging Variants of Castration-Resistant Prostate Cancer. *Current Oncology Reports*. **19** (5), 32 (2017).

4. Aggarwal, R., Zhang, T., Small, E. J., Armstrong, A. J. Neuroendocrine prostate cancer: subtypes, biology, and clinical outcomes. *Journal of the National Comprehensive Cancer Network*. **12** (5), 719-726 (2014).

5. Beltran, H. et al. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. *Nature Medicine*. **22** (3), 298–305 (2016).

6. Calin, G. A., Croce, C. M. MicroRNA signatures in human cancers. *Nature Reviews Cancer*. **6** (11), 857–866 (2006).

7. Coppola, V., De Maria, R., Bonci, D. MicroRNAs and prostate cancer. *Endocrine-Related Cancer*. **17** (1), F1–17 (2010).

8. Ambs, S. et al. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. *Cancer Research*. **68** (15), 6162–6170 (2008).

9. Lu, J. et al. MicroRNA expression profiles classify human cancers. *Nature*. **435** (7043), 834–

529   838 (2005).

530   10.   Bhagirath, D., Yang, T. L., Dahiya, R., Saini, S. MicroRNAs as Regulators of Prostate Cancer
531   Metastasis. *Advances in Experimental Medicine and Biology*. **1095**, 83–100 (2018).

532   11.   Hoshino, A. et al. Tumour exosome integrins determine organotropic metastasis. *Nature*.
533   **527** (7578), 329–335 (2015).

534   12.   Colombo, M., Raposo, G., Thery, C. Biogenesis, secretion, and intercellular interactions of
535   exosomes and other extracellular vesicles. *Annual Review of Cell and Developmental Biology*. **30**
536   255–289 (2014).

537   13.   Bhagirath, D. et al. microRNA-1246 Is an Exosomal Biomarker for Aggressive Prostate
538   Cancer. *Cancer Research*. **78** (7), 1833–1844 (2018).

539   14.   Witte, J. S. Prostate cancer genomics: towards a new understanding. *Nature Reviews*
540   *Genetics*. **10** (2), 77–82 (2009).

541   15.   Kim, J. H. et al. Deep sequencing reveals distinct patterns of DNA methylation in prostate
542   cancer. *Genome Research*. **21** (7), 1028–1041 (2011).

543   16.   Robinson, D. et al. Integrative clinical genomics of advanced prostate cancer. *Cell*. **161** (5),
544   1215–1228 (2015).

545   17.   Kim, J., Yu, J. Interrogating genomic and epigenomic data to understand prostate cancer.
546   *Biochimica Et Biophysica Acta*. **1825** (2), 186–196 (2012).

547   18.   Bucay, N. et al. miRNA Expression Analyses in Prostate Cancer Clinical Tissues. *Journal of*
548   *Visualized Experiments*. 10.3791/53123 (103) (2015).

549   19.   Geiss, G. K. et al. Direct multiplexed measurement of gene expression with color-coded
550   probe pairs. *Nature Biotechnology*. **26** (3), 317–325 (2008).

551   20.   Tam, S., de Borja, R., Tsao, M. S., McPherson, J. D. Robust global microRNA expression
552   profiling using next-generation sequencing technologies. *Laboratory Investigation*. **94** (3), 350–
553   358 (2014).

554   21.   Rodriguez, M. et al. Identification of noninvasive miRNAs biomarkers for prostate cancer
555   by deep sequencing analysis of urinary exosomes. *Molecular Cancer*. **16** (1), 156 (2017).
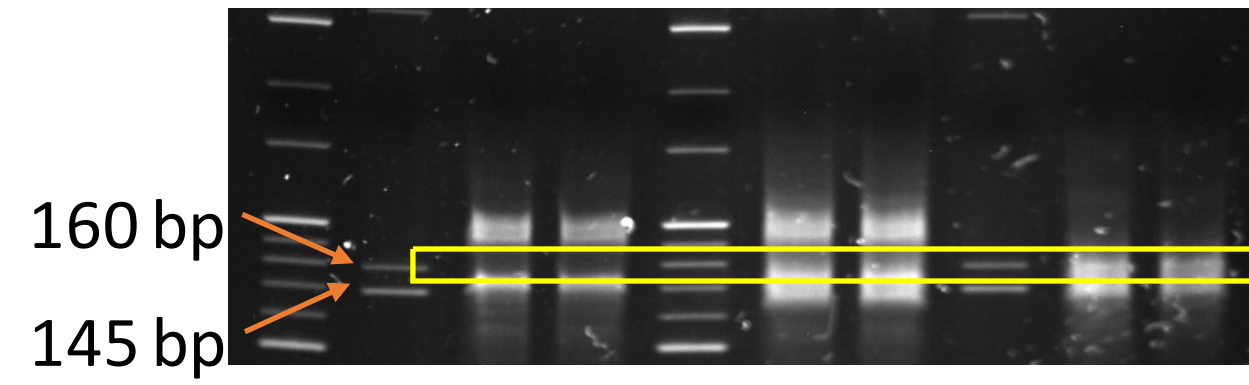
556   22.   Guelfi, G. et al. Next Generation Sequencing of urine exfoliated cells: an approach of
557   prostate cancer microRNAs research. *Scientific Reports*. **8** (1), 7111 (2018).

558   23.   Daniel, R. et al. A Panel of MicroRNAs as Diagnostic Biomarkers for the Identification of
559   Prostate Cancer. *International Journal of Molecular Sciences*. **18** (6), 1281 (2017).
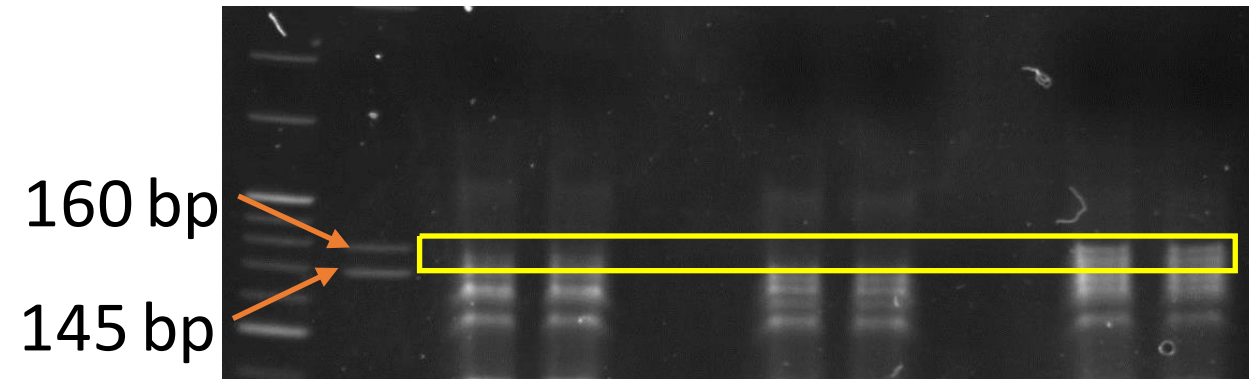
560   24.   Xuan, J., Yu, Y., Qing, T., Guo, L., Shi, L. Next-generation sequencing in the clinic: promises
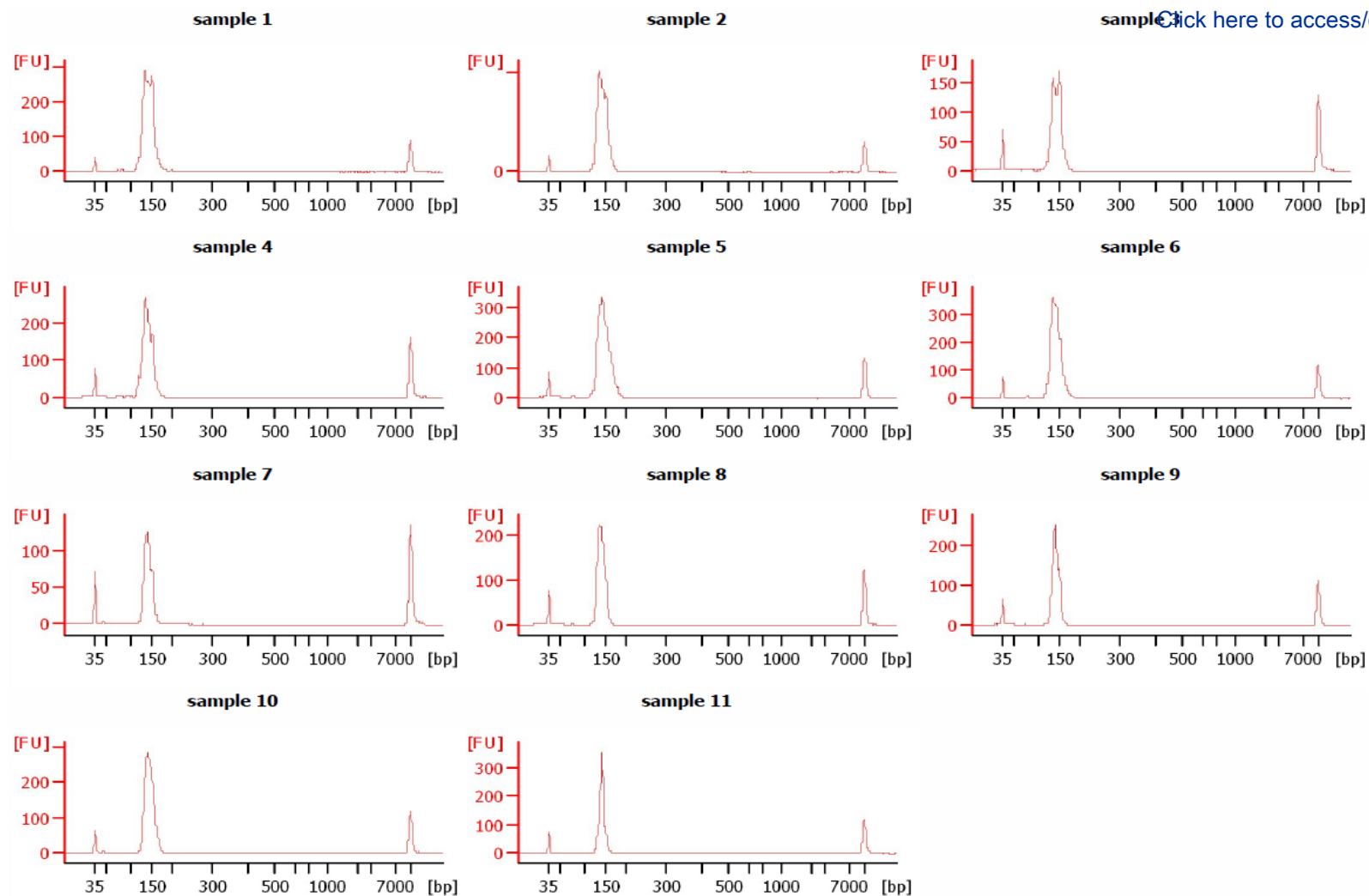561   and challenges. *Cancer Letters*. **340** (2), 284–295 (2013).

562

A

160 bp

145 bp

B

160 bp

145 bp

A

Parameters	Expected values
%Q >=30	94.4 %
Cluster Density	190 K/mm^2
Cluster passing filter	82 %
Estimated yield	7.3 Gb

B

C

Parameters	Expected values
%Q >=30	92.5 %
Cluster Density	240 K/mm^2
Cluster passing filter	80.5 %
Estimated yield	9 Gb

D

Table 1
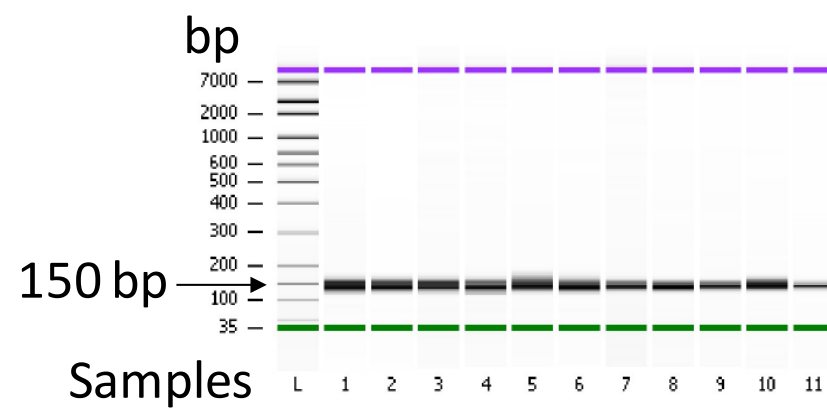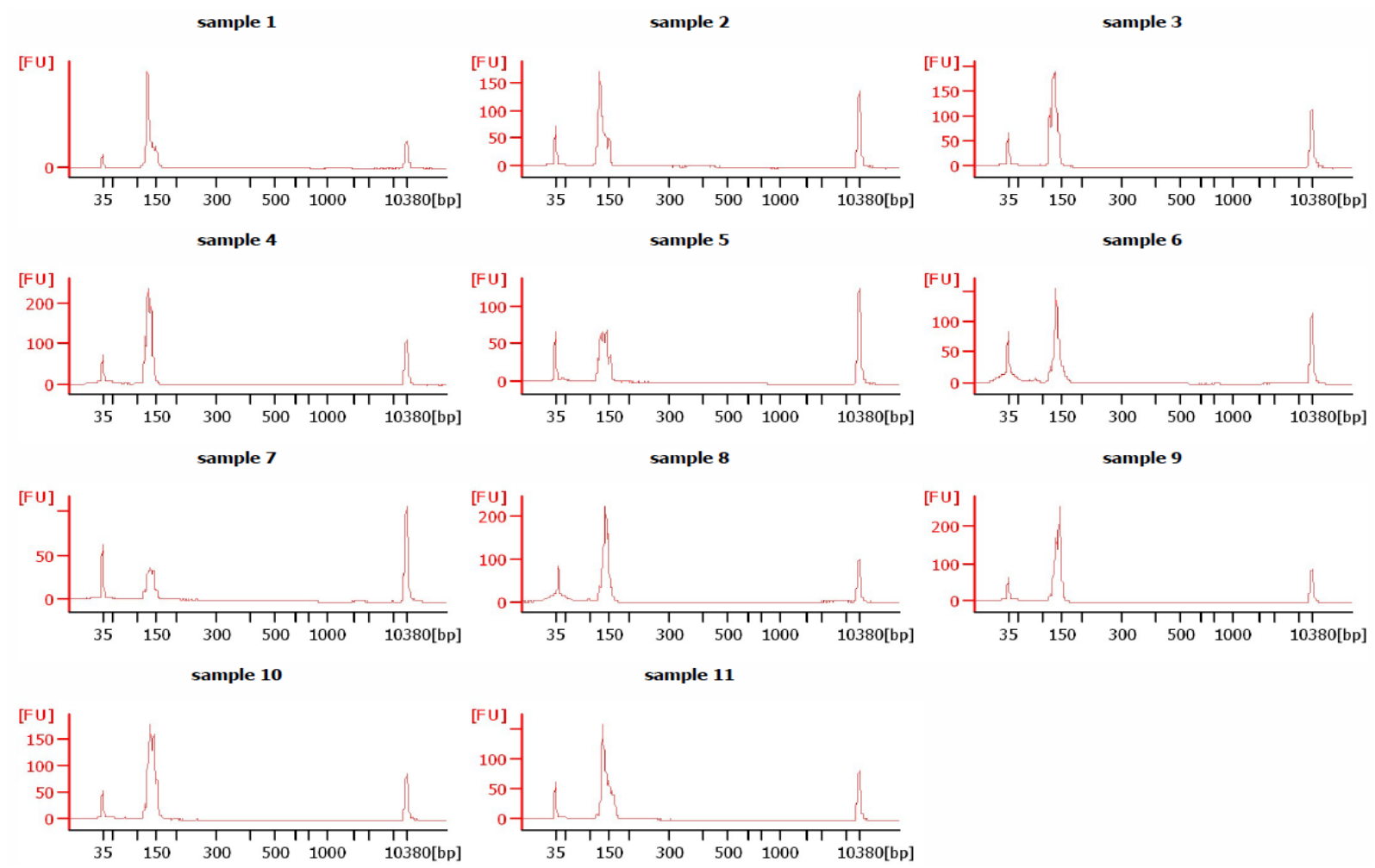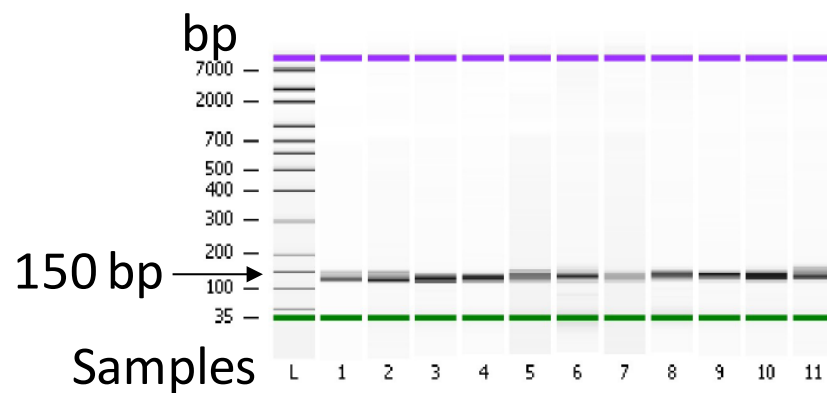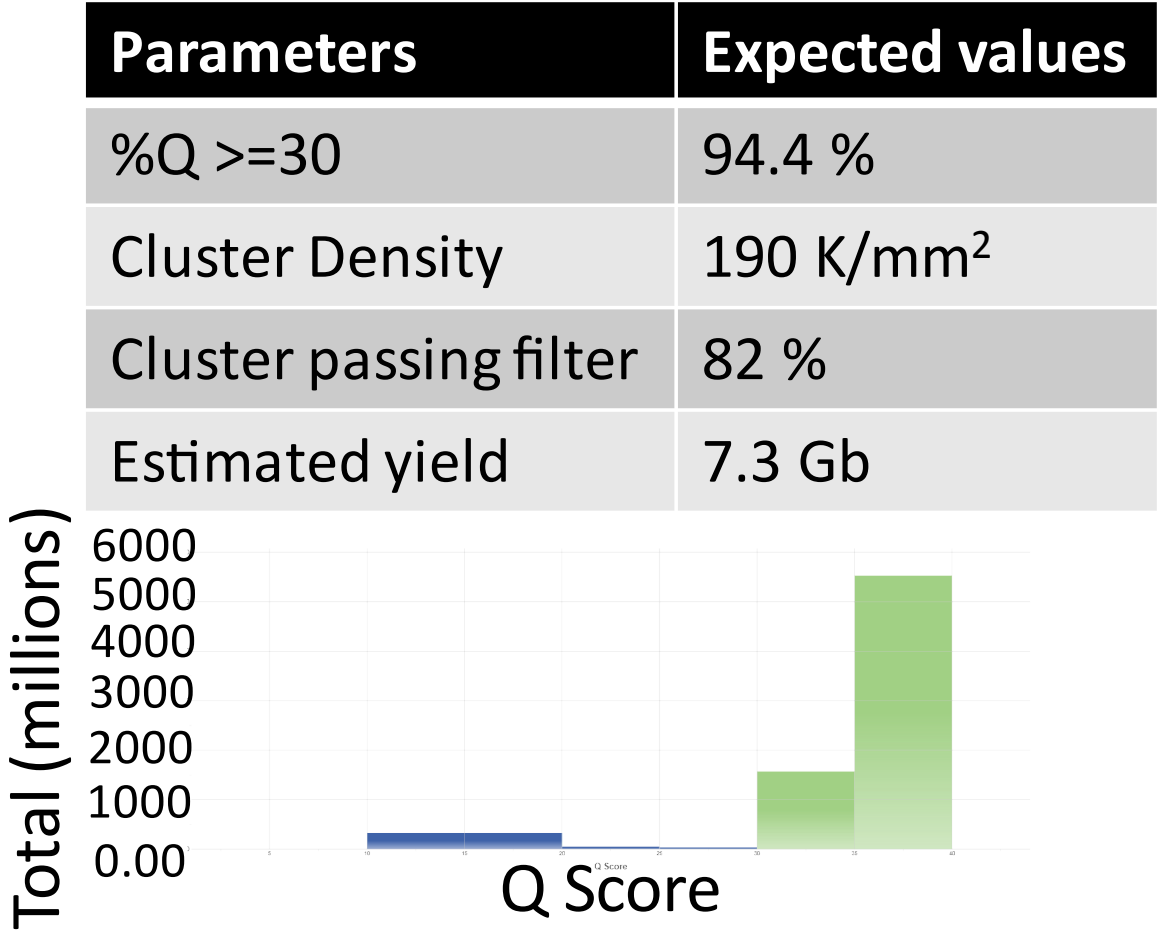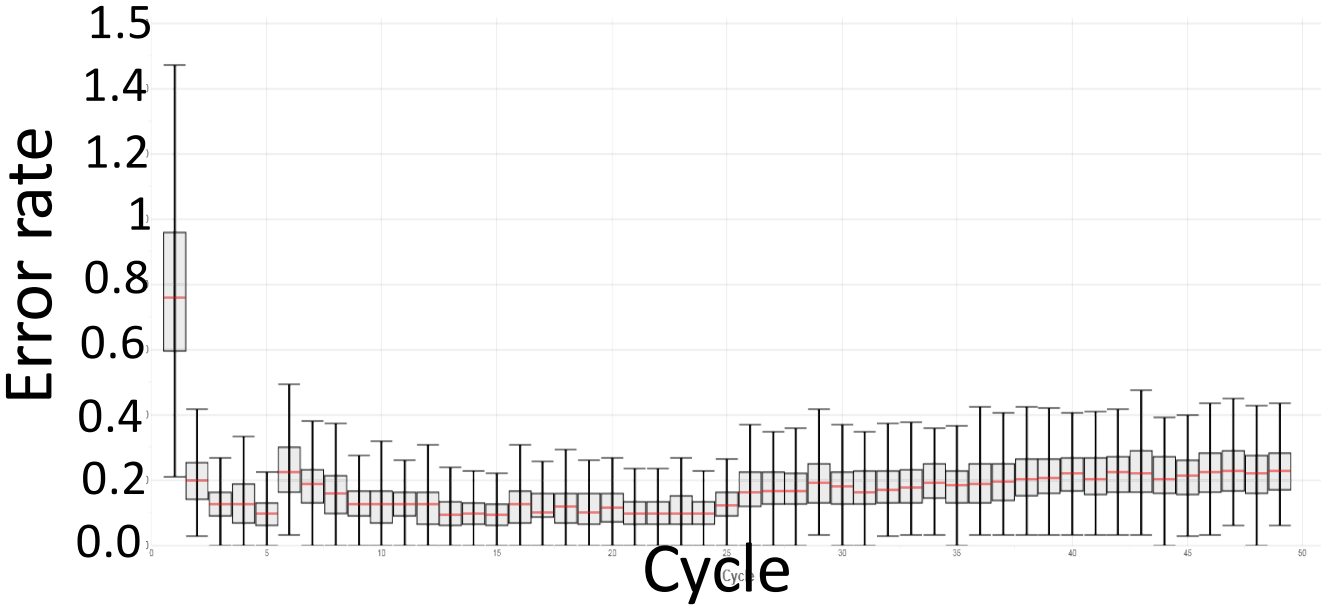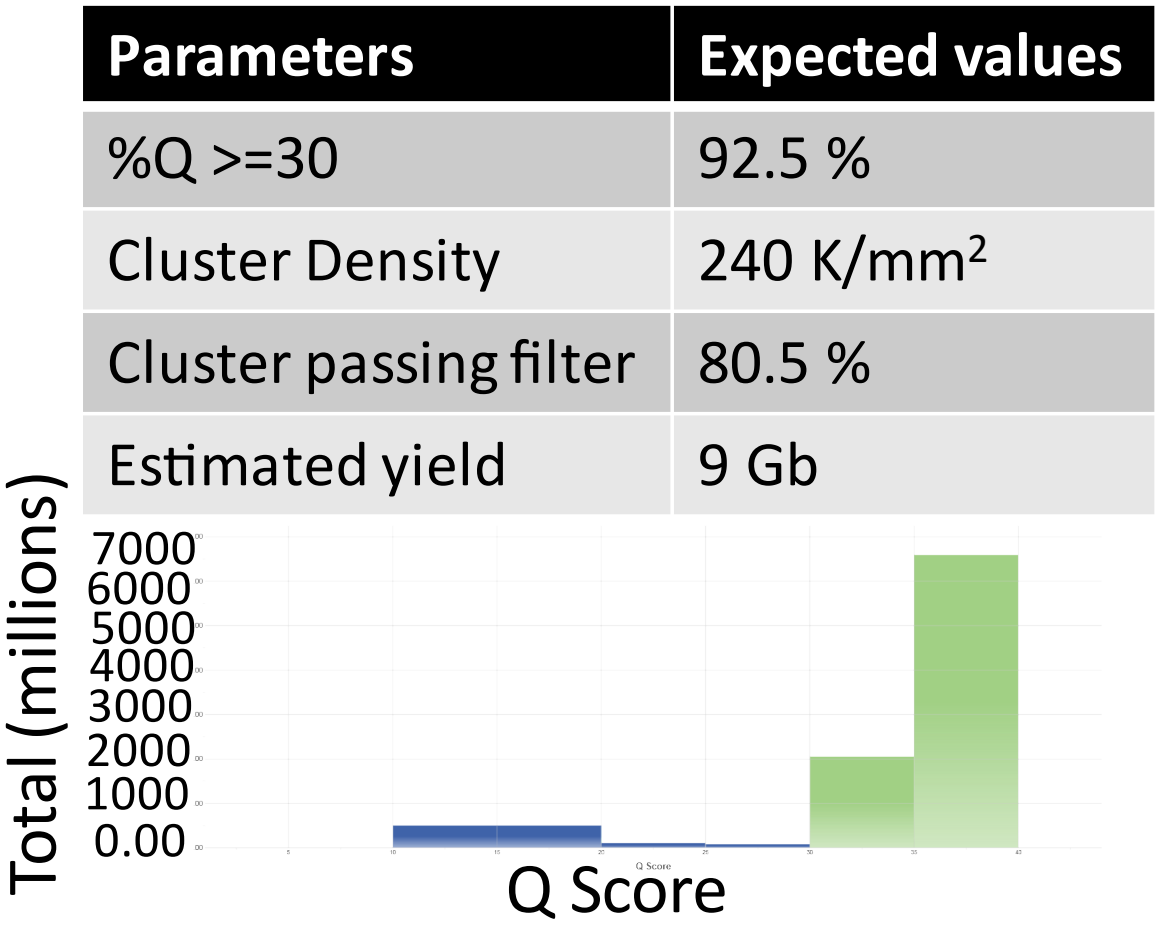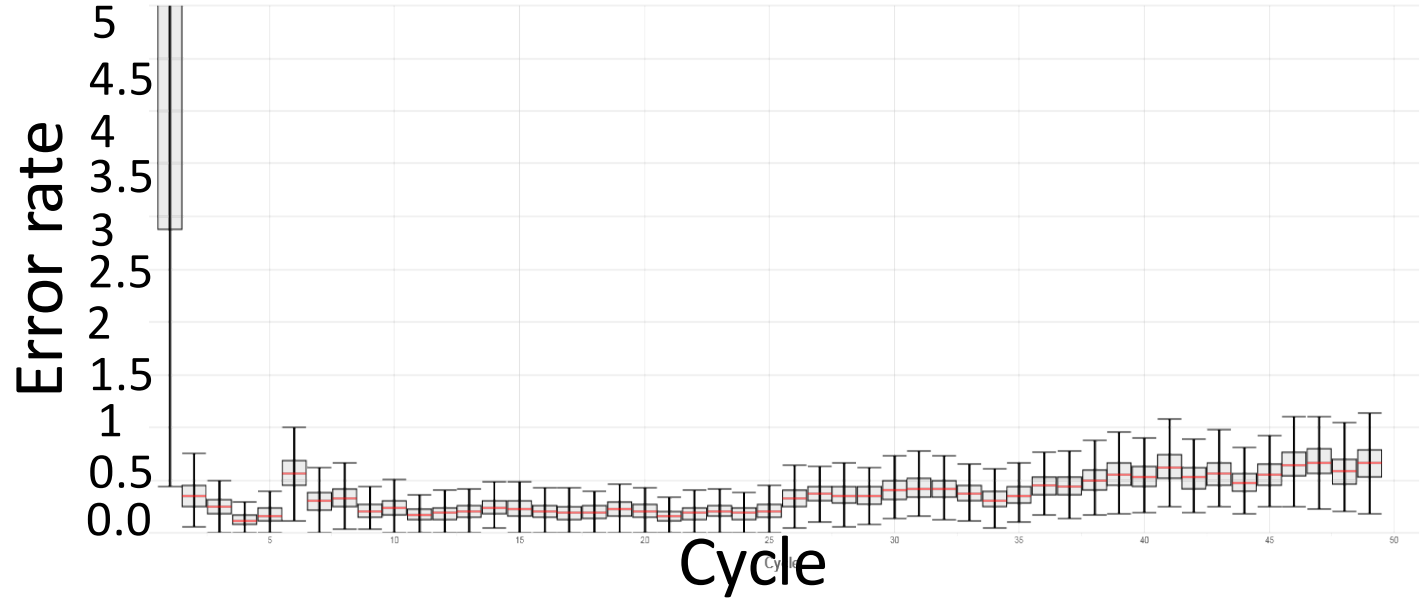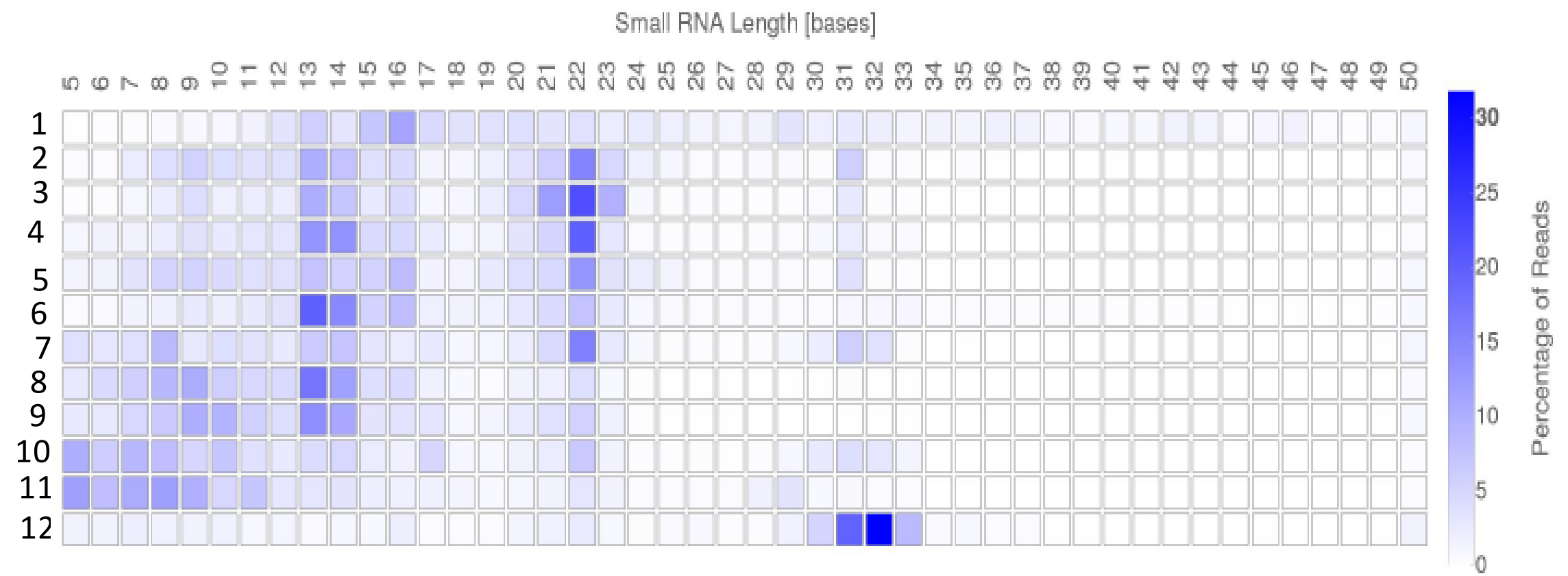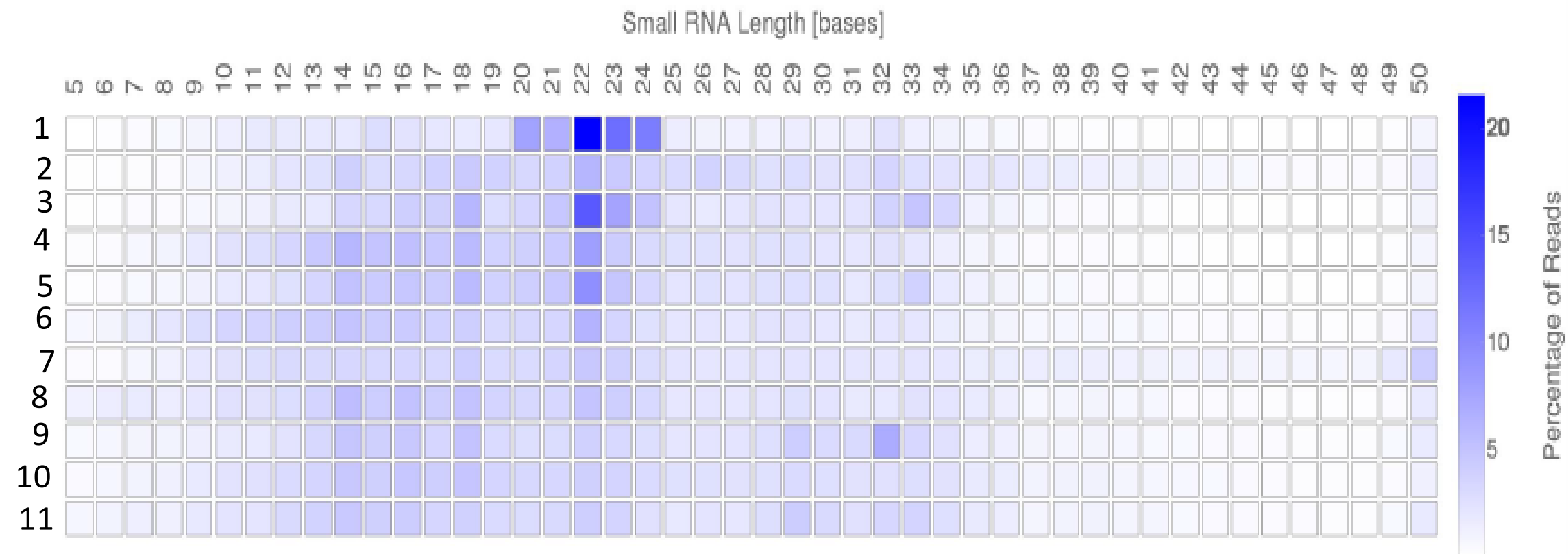
Sample	Total sequencing reads
1	9,268,658
2	7,000,551
3	15,641,204
4	15,046,681
5	14,282,756
6	12,738,970
7	15,721,318
8	8,851,578
9	13,216,879
10	11,466,162
11	19,114,932

Table 2

Sample	Total sequencing reads
1	23,088,960
2	17,769,806
3	9,037,884
4	8,726,243
5	7,323,571
6	28,937,388
7	7,652,149
8	13,007,122
9	5,434,386
10	9,305,941
11	9,453,760
12	8,279,773

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3M NaOAc pH 5.5	USB Corp.	75897 100 mL	
5 µm filter tubes	IST Engineering Inc.	5388-50	
6% TBE polyacrylamide gels	Novex	EC6265BOX	
BaseSpace	Illumina		Analysis software
Bio-analyzer	Agilent		
DNA loading dye	Novex	LC6678	
Eppendorf Thermostat plus	Eppendorf 1.5 mL		
EtBr	Pierce	17898	
Ethyl alcohol 200 proof	Pharmco	111000200	
Exosomal RNA isolation kit	Norgen	58000	
Gel breaking tubes	IST Engineering Inc.	3388-100	
Glycogen molecular biology grade	Thermo Scientifc	R0561	
Hematoxylin Select	Stat lab	SL401	
Microcentrifuge	Fisher Scientific	13-100-676	accuSpin Micro 17R
miRNeasy FFPE kit	Qiagen	217504	
Nanodrop	Thermo Scientifc	Nano Drop 1000	
Nanosight NTA	Malvern	LM14	
NextSeq 500 Sequencer	Illumina		
NextSeq 500/550 Mid Output Kit v2 (150 cycles)	Illumina	FC-404-2001	
Pellet paint	Millipore	70748-3	
Superscript II Reverse Transcriptase	Invitogen	18064014	
T4 RNA Ligase 2 Deletion Mutant	Lucigen	LR2D11310K	
TBE Running buffer (5X)	Novex	LC6675	
Thermal cycler	MJ Research	PTC100	
Total exosome isolation reagent (from serum)	Invitrogen	4478360	
TrueSeq small RNA library Prep kit-Set A (Indexes 1-12)	Illumina	RS-200-0012	

Xylene	Fisher Scientific	X3P- 1GAL	
RNA 3' Primer			GUUCAGAGUUCUACAGUCCG
RNA 5' Primer			TGGAATTCTCGGGTGCCAAGG
Stop Oligo			GAAUUCCACCACGUUCCCGUG
RNA RT Primer			GCCTTGGCACCCGAGAATTCCA
RNA PCR Primer			AATGATACGGCGACCACCGAG
RNA PCR Index Primer			CAAGCAGAAGACGGCATACGA
			6 bases in adapter
RNA PCR Index Primer 1			CGTGAT
RNA PCR Index Primer 2			ACATCG
RNA PCR Index Primer 3			GCCTAA
RNA PCR Index Primer 4			TGGTCA
RNA PCR Index Primer 5			CACTGT
RNA PCR Index Primer 6			ATTGGC
RNA PCR Index Primer 7			GATCTG
RNA PCR Index Primer 8			TCAAGT
RNA PCR Index Primer 9			CTGATC
RNA PCR Index Primer 10			AAGCTA
RNA PCR Index Primer 11			GTAGCC
RNA PCR Index Primer 12			TACAAG

ACGAUC

GG

A

ATCTACACGTTCAGAGTTCTACAGTCCGA

GAT[6 bases]GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

List of Primers

RNA 3' Primer	GUUCAGAGUUCUACAGUCCGACGAUC
RNA 5' Primer	TGGAATTCTCGGGTGCCAAGG
Stop Oligo	GAAUUCCACCACGUUCCCGUGG
RNA RT Primer	GCCTTGGCACCCGAGAATTCCA
RNA PCR Primer	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCG
RNA PCR Index Primer	CAAGCAGAAGACGGCATACGAGAT[6 bases]GTGACTGGAGTTCCTTG(
	6 bases in adapter
RNA PCR Index Primer 1	CGTGAT
RNA PCR Index Primer 2	ACATCG
RNA PCR Index Primer 3	GCCTAA
RNA PCR Index Primer 4	TGGTCA
RNA PCR Index Primer 5	CACTGT
RNA PCR Index Primer 6	ATTGGC
RNA PCR Index Primer 7	GATCTG
RNA PCR Index Primer 8	TCAAGT
RNA PCR Index Primer 9	CTGATC
RNA PCR Index Primer 10	AAGCTA
RNA PCR Index Primer 11	GTAGCC
RNA PCR Index Primer 12	TACAAG

GA
GCACCCGAGAATTCCA

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Dear Editors

We are sincerely thankful for thorough review and for suggesting modifications that would improve the quality of the article. The suggested modifications have now been included in the revised version. Please find below line by line response to each comment.

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
Response 1: Manuscript has been proofread for spelling or grammatical errors.

Protocol Language Comments:
Comment 2: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
Response 2: The language of the protocol has been checked for imperative voice.

Comment 3: Lines 142-145, 162-164, 207-214 etc should be NOTES.
Response 3: The above mentioned lines have been now included as NOTES in the manuscript.

Comment 4: Section 5 must be rewritten as steps in the imperative voice.
Response 4: As suggested, we have edited section 5.

Protocol Detail Comments:
Comment 5: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response 5: As suggested, we have added these details. Please refer to revised sections 4.3.11 and 5 of the revised manuscript.

Comment 6: 1.1: unclear what the "same ethanol gradient and xylene" is.
Response 6: We have now included details on these steps in revised version.

Comment 7: 1.4: Is any reagent used? Unclear why the tip is cut and how the collection of dry sample is performed? Is only cancerous tissue collected?
Response 7: To collect the tissues, no reagent is used. Rather, the electrostatically charged tip attracts the dissected tissue and allows for maximum tissue recovery after dissection. Once the tissue sticks to the charged tip, the tip is cut into an empty 2 mL collection tube. This description has been included in revised version. Cancerous tissue and/or normal adjacent areas are collected.

Comment 8: Section 2: What kind of serum is this?
Response 8: These are patient-derived serum samples isolated from metastatic CRPC cases with adenocarcinoma or NE differentiation. Serum samples were collected from same set of patients as those used for microdissected tissues to allow comparison between corresponding tissues and serum-derived EVs. The section has been modified in the manuscript for clarification.

Comment 9: 3.2.1: What are the compositions of lysis buffer A and B?

Response 9: Buffer A and B were part of exosomal RNA isolation kit. A line on using kit based method has been included for better understanding.

Comment 10: 3.2.4, What is the composition of elution buffer?
Response 10: Elution buffer was part of exosomal RNA isolation kit. A line on using kit based method has been included for better understanding.

Comment 11: 4.2.1, 4.2.4, 4.2.8, 4.2.10, 4.2.12: "Keep on pre-heated PCR" appears incomplete. Please check.
Response 11: The line has been modified in the manuscript as suggested.

Comment 12: 4.1: List all primers in the table of materials.
Response 12: We have added list of primers in 'Table of Materials'. Please refer to sheet 2 'list of primers' under the revised table.

Protocol Numbering Comment:

Comment 13: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.
Response 13: We have modified and numbered the protocols according to the required format as suggested by the Editor.

 Protocol Highlight comment:

Comment 14: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Response 14: The manuscript has been accordingly highlighted, taking in to consideration the above mentioned comments.

Discussion comment

Comment 15: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response 15: Discussion has been reviewed and checked to fit the format description as suggested by the Editor.

Figures comment

Comment 16: Fig 1: The numerous panels on the right hand side of this figure are too small to read. Please make this a separate figure.

Response 16: Figure 1 has been separated to Fig1, 2 and Fig. S1 for better clarity and ai files for each figure have been provided

Comment 17: Figure/Table Legends: 1) Fig 2: define error bars.

Response 17: The error bars are standard deviations for the error rates for PhiX alignment over different cycles. The line has been modified and included in the figure legend for the corresponding figure.

References comments:

Comment 18: Please edit your references to comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

Response 18: As suggested, we have edited references.

Comment 19: Please spell out journal names.

Response 19: We have spelled out full journal names for all the references.

Commercial Language comment:

Comment 20: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are nanosight, nanodrop, mini-spin column, Qubit, (Illumina, PCR block, geldoc, bioanalyzer 1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Response 20: As suggested, all the commercial sounding words as mentioned above have been replaced to more generic names in the manuscript.

Other comments:

Comment 21: Please define all abbreviations at first use.

Response 21: Abbreviations have been defined in the manuscript at first use.

Comment 22: If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
Response 22: All figures and tables have not been published previously.

Again, we sincerely thank the editor for helpful comments. We hope that we have satisfactorily addressed all the raised concerns in the revision and hope for a favorable consideration.

Thank you!

Sincerely

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Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]
1	◀	35	125.00	5,411.3
2		142	847.91	9,042.1
3		152	295.78	2,950.7
4	▶	10,380	75.00	10.9

11.992 nM (Total molarity)