microRNA and non-targeted proteome analysis of liquid biopsies from the distal lung collected by Particles in Exhaled Air (PExA) reveals presence from extracellular vesicles

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ABSTRACT

Background. Early detection is essential to ensure prompt treatment selection for many lung disorders, which are becoming more prevalent. Unfortunately, invasive methods are often used to examine the distal parts of the lungs for diagnosis and to establish disease biomarkers. The growing need to explore the bio-molecular mechanisms in various lung diseases highlights the importance of non-invasive methods, particularly in longitudinal studies. Thus, the use of particles in exhaled air (PExA), a non-invasive technique for sampling epithelial lining fluid from distal airways, is gaining attention. MicroRNAs (miRNAs) are crucial in modulating protein expression both intracellularly and intercellularly, often transported via extracellular vesicles. Dysregulated miRNAs have been linked to many pulmonary diseases, and their relative stability, especially when encapsulated in EVs, makes them promising biomarkers. Although underreported, exhaled miRNA identified with other non-invasive techniques shows potential as a molecular tool. Therefore, here we report the initial steps in the miRNA detection in PExA, offering opportunities to study miRNA roles in both normal and diseased states in a non-invasive manner.

Methods. Exhaled particles were collected from healthy subjects using the PExA 2.0 instrument following prescribed guidelines. Different samples were processed for non-targeted proteome analysis using mass spectrometry and RNA extraction followed by miRNAseq workflow for low input starting material. The detection of some miRNAs was confirmed with the miRCURY LNA miRNA PCR assays. The quality of the whole workflow was evaluated with a RNAses activity test and with synthetic controls miRNAs (spike-in kit).

Results. Proteomic analysis identified over 50 proteins across experiments and samples, with a notable enrichment of proteins associated with extracellular vesicles, including blood microparticles, and secretory granules. miRNA-seq revealed 39 mature miRNAs, the majority of which have been previously reported to be detected in the airways and respiratory disorders. Some were also reported to be secreted by primary human airway epithelial cells via extracellular vesicles. Notably, miRNA-125b and members of the let-7 family were among the most abundant. Fluorometric assays showed significant RNase activity in both PExA and other lung-related samples, such as bronchoalveolar lavage fluid, suggesting that this activity originates from the airways and is independent of the sampling techniques used. The workflow for extraction and processing of the PExA collection membrane, tested with abundant synthetic miRNAs and analyzed using the miRCURY LNA miRNA PCR assay, yielded

results comparable to control samples, indicating that the membrane material does not interfere with the assay.

Conclusions. Using PExA, we identified several miRNAs that are known to be dysregulated in various pulmonary disorders. The enrichment of extracellular secretory components in the core protein list from our proteomic analysis, along with the elevated RNAse activity in the respiratory tract, suggests that the detected miRNAs may be encapsulated within extracellular vesicles. These miRNAs are particularly intriguing due to their potential role in intercellular communication during both normal and pathological lung processes. Our findings suggest that PExA holds significant potential as a non-invasive tool for studying extracellular vesicle-mediated miRNA signalling in the small airways.

INTRODUCTION

Early detection of lung diseases is important to avoid loss of lung function and holds the potential to significantly enhance the quality of life for affected individuals. Timely intervention has the capacity to slow down or even halt the progression of the disease, leading to superior long-term outcomes. Today, we rely on invasive techniques such as lung biopsies or bronchoalveolar lavage (BAL) for biomarker discovery, and in some cases for diagnosis. While invasive methods provide valuable insights, their resource intensiveness may be prohibitive, may be inappropriate or impractical for longitudinal studies, and not be well tolerated for some patient groups. It is thus essential to balance the benefits with the potential risks and patient discomfort associated with these procedures [1]. As such, the development of non-invasive sampling techniques of the distal lung is pivotal to facilitate biomarker discovery, longitudinal monitoring or large-scale screening of respiratory disease. Particles in exhaled air (PExA) represents a unique sampling method in that it permits non-invasive sampling of the epithelial lining fluid and airway exudates from the small airways and alveoli [2], a.k.a. liquid biopsies from the distal airways. The approach involves impaction of exhaled aerosols onto comprising ultrafine droplets of respiratory tract lining fluid (RTLF) onto a membrane[3]. These droplets are generated and exhaled after a breathing manoeuvre that facilitates the closure and reopening of the small airways. PEx samples have been shown to contain several types of biomolecules, including proteins [2, 4] and lipids [5] known to be present in the RTLF in the distal airways and alveolar region. Accordingly, this type of sampling method can be especially relevant for disorders involving the small airways, such as asthma and COPD [2, 6, 7]. Despite a number of recent advancements of the sampling method and associated publications, our understanding of the material collected and its relevance to disease diagnosis and monitoring remains limited. As such, further benchmarking and analysis is needs to unlock the full potential of this non-invasive technique.

MicroRNAs (miRNAs) are small, conserved non-coding RNAs that regulate gene expression post-transcriptionally [8]. They play a crucial role in various biological processes, and their dysregulation is linked to many pulmonary diseases [9, 10]. miRNAs are found in numerous body fluids and extracellular spaces, including RTLF, either associated with other biomolecules or encapsulated in extracellular vesicles (EVs) [11-13]. Acting as functional vehicles of intercellular communication, EVs transport miRNAs during pathological processes, protecting them from degradation and thereby enhancing their stability [14]. Given their importance in diseases processes [9, 10], as well as relative stability, miRNAs hold great potential for various clinical applications, including longitudinal monitoring. Non-invasive molecular tools for studying lung-derived miRNAs are emerging, though not

yet rigorously evaluated or in clinical use [15]. Existing publications on non-invasive approaches focus on exhaled miRNAs, primarily using exhaled breath condensate (EBC) for sampling [15-17]. However, it is still not fully understood where EBC originates from, and there are uncertainties on its formation. Radioactive markers studies have revealed that EBC predominantly captures central airways, rather than providing a clear picture of the RLTF in the smaller airways [18].

Given the pivotal roles of extracellular vesicles (EVs) and miRNAs in intercellular communication and regulatory processes in lung diseases, in this publication we describe the development of a miRNA analysis approach for non-invasive interrogation of the distal lung using the PExA technology in healthy subjects. To our knowledge we were the first to report detection of miRNA in PExA samples [19]. By exploring the feasibility of detecting miRNAs in PExA samples, our study contributes to the advancement of respiratory research and disease diagnostics.

105 MATERIALS AND METHODS

106

107 **Sample collection**

108 PEx were collected using the PExA 2.0 instrument (PExA AB, Gothenburg, Sweden), according to
109 previously described methods [20, 21]. The collection was performed by impaction on dried blood
110 spot (DBS) membranes (Whatman 903 Protein saver, specially tailored for PExA, PExA AB, Gothenburg,
111 Sweden) or on Flinders Technology Associates (FTA) membranes (QIAcard FTA Elute Indicating Micro,
112 Qiagen, specially tailored for PExA, PExA AB), a chemically coated cellulose membrane type that lyse
113 cells on contact, denature proteins and protect nucleic acids from degradation. All subjects performed
114 a standardised breathing manoeuvre [22, 23] with a mouthpiece and a two-way, non-rebreathing
115 valve (Series 1420; Hans Rudolph, Shawnee, KS, USA), wearing a nasal clip during all process. Prior to
116 the sampling, the subjects took a minimum of three tidal breaths of HEPA-filtered air via the PExA
117 instrument to eliminate particle contamination from the surrounding ambient air. From each subject,
118 240 ng of exhaled particles were collected. Following the collection process, the sample holder
119 containing the impact membrane was directly placed in a dust- and RNAse-free container, and the
120 area of the membrane impacted by the particles (PEx sPOT) were excised using a puncher specifically
121 designed for this purpose. Subsequently, the PEx sPOTs were transferred to 1.5 ml Eppendorf® DNA
122 LoBind tubes for miRNA analysis, or Protein LoBind tubes for proteomic analyses, and stored at -80°C
123 until use. All the procedure to prepare the sample holder and to collect the PEx sPOTs were performed
124 under a laminar flow hood under sterile conditions to prevent sample contamination.

125

126 **Protein workflow**

127 Two distinct PEx proteome analyses were design to evaluate both the technical reproducibility, the
128 detection limit, and the reproducibility of repeated sampling the same individual on different days. In
129 Experiment 1, we collected 240 ng PEx sample from one healthy subject and divided it into three
130 replicates composed of three PEx dots (3 replicates of 72 ng PEx). In Experiment 2, we collected
131 another sample from the same subject on a different day, maintaining the same time of the day,
132 dividing it into five different replicates, each composed of a single PEx sPOT (5 replicates of 24 ng PEx).
133 Subsequently, we proceeded with the protein analysis workflow.

134

135 **Non-targeted proteome analysis**

136 Excised PEx membranes spots were wetted with a total volume of 20 µL lysis buffer composed of 0.2%
137 n-dodecyl-β-D-maltoside (DDM; Sigma-Aldrich) in 100 mM triethylammonium bicarbonate (TEAB;
138 Fluka) and sonicated in water bath for 10 min. Suspensions were vortexed and centrifuged at low

139 speed for 5 min at room temperature to sediment PEx sPOTs and lysed proteins. Subsequently,
140 samples were digested by adding 10 µL of 20 ng/uL sequencing grade trypsin (Promega) into the lysis
141 buffer, with a final trypsin concentration of 6.7 ng/µL. Following a short vortexing samples were
142 incubated for 4 h at 37˚C and centrifuged at 10,000x *g* for 5 min. The supernatants were transferred
143 to sample vials and dried in a vacuum concentrator (Eppendorf) until dry.
144 Peptides were reconstituted in solvent A (0.1% formic acid, FA in 2% acetonitrile, ACN) and 2 µL of
145 each sample was injected using an Ultimate 3000 nano-flow UHPLC system (Thermo Fisher Scientific).
146 Peptides were captured on a 2-cm Acclaim PepMap trap column (Thermo Fisher Scientific) and
147 separated on a heated 25-cm long EASY-Spray C18 column (Thermo Fisher Scientific) applying a 90
148 min long gradient: 4-26% of solvent B (0.1% FA in 98% ACN) in 90 min, 26-95% in 5 min, and 95% of
149 solvent B for 5 min at a flow rate of 300 nL/min.
150 Mass spectra were acquired on an Orbitrap Eclipse tribrid mass spectrometer (Thermo Fisher
151 Scientific) equipped with high-field asymmetric ion mobility spectrometry (FAIMS) in *m/z* 300 to 1500
152 at resolution of R=120,000 (at *m/z* 200) for full mass, targeting $8x10^5$ ions in maximum 100 ms,
153 followed by data-dependent higher-energy collisional dissociation (HCD) fragmentations of precursor
154 ions with a charge state 2+ to 6+, using 20 s dynamic exclusion. The tandem mass spectra of the top
155 precursor ions were acquired in 1 s cycle time by the linear ion trap in rapid mode, targeting $3x10^4$ ions
156 for maximum injection time of 20 ms, setting quadrupole isolation width to 1 Th and normalized
157 collision energy (HCD) to 30%. FAIMS was operated at three different compensation voltages in each
158 cycle using either -40, -60 or -80 V.
159
160 **Proteome data analysis**
161 The raw files were searched against a human UniProt database using MS Amanda v2.0 search engine
162 [Ref: https://doi.org/10.1002/rcm.9088] loaded into Proteome Discoverer 2.5 software (Thermo
163 Fisher Scientific). MS1 precursor mass tolerance was set at 10 ppm, and MS2 tolerance was set
164 at 0.6 Da. Search criteria included variable modifications of oxidation (+15.9949 Da) on methionine
165 residues, deamidation (+0.984 Da) asparagine and glutamine residues. Search was performed with full
166 trypsin/P digestion and allowed a maximum of two missed cleavages on the peptides analyzed from
167 the sequence database. The false-discovery rates of proteins and peptides were set at 0.05. All data
168 were quantified using the label-free quantitation node of Precursor Ions Quantifier through the
169 Proteome Discoverer calculating protein abundances based on precursor abundances. The abundance
170 levels of all proteins were normalized using the total peptide amount normalization node in the
171 Proteome Discoverer.
172

173 **Protein-protein interaction Network (PPI) and Pathway analysis**

174 Pathway enrichment analysis and PPI network visualization was performed using STRING database v.
175 12.0 [24]. All 69 proteins identified in both examinations were analysed. Keratins were eliminated due
176 to possible contamination concerns, and a few protein IDs were not found in the STRING database.
177 The final pathway analysis and visualization network included 62 proteins. For the PPI network, the
178 minimum required interaction score was set to medium confidence (cutoff 0.4). The obtained network
179 was then transferred to Cytoscape v. 3.9.1[25] for graphical processing.

180

181 **miRNA workflow**

182 Two PExA samples were collected from two healthy subjects. We extracted all available PEx sPOTs
183 (10) from the first membrane and then, we separately processed two and one PEx sPOTs, from the
184 second membrane. Subsequently, we proceeded with the RNA extraction workflow. Details of the
185 protocol are reported in **Figure 2A**.

186

187 **RNA extraction**

188 The vials containing PEx sPOT were transferred from the storage state to dry ice to prevent premature
189 thawing. 260 µL of Buffer RLT (Qiagen) were added to each sample to disrupt it while it was in a frozen
190 state and allowed to incubate at room temperature for 10 min. The samples were periodically
191 vortexed, and pipette mixed through pipetting until PEx sPOT were disintegrated, thereby ensuring
192 the creation of a pseudo-uniform solution. Total RNA, including the miRNA fraction, was extracted
193 using the miRNeasy Tissue/Cells Advanced Micro Kit (Qiagen) according to manufacturer's instruction.
194 The gDNA Eliminator Spin Column step is essential as it allows to separate any paper-based particles
195 from the liquid, that could interfere with any downstream steps. The RNA fraction was eluted in 15 µL
196 nuclease-free water, and the process of elution was performed twice with an incubation time of 5 min
197 each to increase the elution efficiency. For samples destined for the qRT-PCR-based miRNA assays, 1µl
198 of RNA spike-in mix (Qiagen) was added to the lysis buffer during RNA isolation to monitor RNA
199 extraction efficacy.

200

201 **Library Preparation and RNA sequencing**

202 The QIAseq miRNA Library Kit (Qiagen, Hilden, Germany) was used to prepare the library, which has
203 integrated Unique Molecular Indices (UMIs) to facilitate measurement of pre-amplification miRNA
204 levels. The libraries were sequenced on the NextSeq 2000 instrument (Illumina, San Diego, US) with a
205 100 nt (Read1)- 8 nt (Index1) setup using 'P2' flowcell. The fastq data were uploaded to the RNA-seq

206 Analysis Portal (RAP) 5.0 powered by QIAGEN CLC Genomics Workbench to obtain the UMI raw counts
207 (reference: miRBase_v22, Homo sapiens; analysis workflow version: 1.2).
208
209 **cDNA Synthesis and qRT-PCR based miRNA assays**
210 For validation experiments using qRT-PCR based miRNA assays, a set of samples were collected in an
211 identical manner as for the RNAseq analysis described above. After extraction, RNA was reverse
212 transcribed using miRCURY Locked Nucleic Acid (LNA™) Reverse Transcription (RT) kit (Qiagen)
213 according to manufacturer's instruction. For all samples, the reaction mix included the maximum
214 allowable amount of RNA (6.5µL) in a final volume of 10 µL. The cDNA synthesis control (UniSp6) and
215 the PCR amplification Ctrl (cel-miR-39-3p, called UniSp3) coming from the Spike-in mix (Qiagen) were
216 introduced to the reverse transcription reaction to assess its efficacy. A miRCURY LNA miRNA Custom
217 PCR Panel (Qiagen) was used and the set of primers for the miRNAs selected for the custom plate were
218 all wet-lab validated for qPCR. cDNA samples were amplified using miRCURY LNA™ SYBR Green PCR
219 kit (Qiagen) and the real-time reaction was performed using a QuantStudio™ 7 Flex Real-Time PCR
220 System (Applied Biosystem Foster City, CA, USA), with the following protocol: 95°C for 2 min, followed
221 by 40 cycles consisting of denaturation (10 s at 95°C) and combined annealing/extension (60 s at 56°C).
222 Melting curve analysis from 60–95°C was performed post-amplification to evaluate product
223 specificity.
224
225 **RNAse activity test**
226 The presence of RNase activity in BALF and PExA samples was assayed using the RNaseAlert® Lab Test
227 Kit (Thermo Fisher Scientific). BALF samples were kept undiluted and thawed on ice when frozen. The
228 assays were conducted following the manufacturer's protocol, with fluorescence measured using the
229 Varioskan™ LUX multimode microplate reader at an excitation wavelength of 490 nm. As part of this
230 test some DBS membrane were pre-treated, before collecting the samples, with the reagents that
231 destroy RNases, like RNaseZap™ (ThermoFisher Scientific) and lysis buffer from the miRNeasy
232 Tissue/Cells Advanced Micro kit (Qiagen).
233
234 **Evaluation of the potential interference of the membrane in the extraction of the RNA**
235 To test if the membrane matrix materials interfere with the RNA extraction procedure and workflow,
236 5 PEx sPOTs each of DBS an FTA membrane were wetted with 0.2 µL of RNA spike-in mix (Qiagen),
237 making a total of 1 µL across 5 punches. RNA extraction was then carried out as described above. To
238 control for adsorption effects, a positive control was performed by adding 1 µL of the RNA spike-in
239 mix directly to lysis buffer, without including any PEx sPOTs membrane.

240

241 **Ethical Statement**

242 Analysis of miRNA and proteins collected by the PExA method has been approved by the Swedish
243 Ethical Authorities (Case no. 2022-03768-01). For the purpose of methods optimizations, PEx material
244 was stripped of all personal identifiers at the time of collection, and all downstream handling and
245 analysis was performed in a de-identified manner.

246 RESULTS

247

248 **PEx protein content**

249 Non-targeted proteome analysis of all replicates of the two experiments revealed a total of 163
250 proteins detectable in DBS PExA membranes, with 69 proteins in common between the two
251 experiments (**Figure 1A**). We identified 142 proteins in the first experiment (72 with more than 1
252 unique peptide) and 90 in the second (44 with more than 1 unique peptide). Of the 69 common
253 proteins, 58 were identified in all replicates of both experiments. All the proteins in this list that are
254 present in the STRING database are shown in **Figure 1B**, relevant information and detailed gene names
255 are reported in supplementary material (**Table S1**). The provided list exhibited strong, statistically
256 significant enrichment (false discovery rate, FDR ≤ 0.05) for various terms associated with extracellular
257 secretion-related components, such as extracellular exosome, blood micro-particle, vesicle and
258 secretory granule (**Figure 1C**). Complete list and further details of the pathway analysis are reported
259 in the supplementary materials (**Table S2**). On one PEx sPOTs, 70 proteins were identified in all the 5
260 replicates, of which 43 were identified with more than one unique peptide, that give strong
261 statistically significant enrichment for terms associated with extracellular release processes (data
262 showed in supplementary material, **Table S3**), providing a good confidence of the analysis performed
263 even in one single PEx sPOT.

264

265 **miRNA content evaluation on DBS membrane using miRNA NGS sequencing**

266 A total 181 miRNAs were detected with at least 1 UMI count in at least 1 of the samples. A set of 39
267 miRNAs were detected with at least 10 UMI counts in at least one of the samples (**Figure 2B**). The
268 amount of UMI counts is higher in the samples processed with 10 PEx sPOTs and then they gradually
269 decrease in the samples processed with 2 and 1 PEx sPOTs for both the subjects employed for this
270 analysis. The number of counts in the sample with only 1 PEx sPOTs are extremely low or absent for
271 most of the miRNAs. Most PExA-identified miRNAs are present in respiratory tract miRNA atlases
272 (lungs, bronchus, and saliva[26, 27]), as reported in the right part of the **Figure 2B.** The highest number
273 of UMI counts was registered by miR-125b-5p in the 10 PEx sPOTs of subject 1, and then the number
274 gradually decreased in the 2 and 1 PEx sPOT settings. Subject 2 exhibits the same trend, but with lower
275 counts. We detected many members of the let-7 miRNA family (let-7a-5p, let-7b-5p, let-7c-5p, let-7e-
276 5p, let-7f-5p, let-7i-5p) with high UMI counts. They exhibit the same reduction trend as miR-125b-5p,
277 transitioning from 10 to 2 to 1 PEx sPOTs.

278

279 **RNA degradation evaluation in lung related samples**

280 During the QC analysis of miRNA-seq data (data not shown), a significant proportion of reads that
281 were shorter than expected mapped to RNAs other than miRNA, indicating potential RNA degradation.
282 We then performed a fluorimetric assay to evaluate the presence of RNAse activity in the airways and
283 exclude any contamination coming from the PEx tools and workflow. First, we measured RNase
284 activity in PExA samples collected on a DBS membrane and in fresh and frozen BALF samples to get
285 another biological sample coming from the airways to compare (**Figure 3A**). For the BALF we tested
286 the supernatant only and the complete version with cells. All samples have significantly greater
287 fluorescence than the negative control, indicating strong RNase contamination. We then measured
288 the RNAse activity on the membrane only and applying some RNAse inactivation strategies (**Figure**
289 **3B**). The fluorescence level of the DBS membrane alone was comparable to the negative control.
290 Samples collected on DBS without any protective reagents resulted in being contaminated by RNAses,
291 even if the activity was a bit less in the samples that were immediately freeze at -80°C after the
292 collection. In samples that were collected on DBS that had been pre-treated with lysis buffer from the
293 RNA extraction kit, or in samples that were collected on an FTA membrane the RNAse contamination
294 is minimal and comparable to the negative control.
295
296 **Validation of different miRNA using real-time PCR technology**
297 From the qRT-PCR based miRNA assays few miRNAs were identified with CT values less than 35 (**Figure**
298 **4A**). Although we currently lack controls for the regular quantification, miR-125b-5p appears to be the
299 most abundant, as reported in the RNAseq experiment. However, some miRNAs have not been
300 identified using this technique. The internal controls for RNA extraction (UniSp2 and UniSp4), cDNA
301 synthesis (UniSp6), and PCR amplification (UniSp3) appear to be reliable and uniform across the
302 different samples, ensuring a high level of reproducibility in the overall workflow (**Table S4**).
303
304 **The membranes for PExA collection do not interfere with the extraction of the RNA and the**
305 **downstream steps**
306 To evaluate if the membranes, DBS or FTA, interfere with the workflow we used the spike-in mix used
307 during the main experiment. We decided to use this mix instead of a real PEx sample considering
308 extremely low amount of RNA in PEx samples and the potential variability of the collection procedures
309 that we are trying to understand. The spike-in mix was added directly to pre-cut PEx sPOTs and
310 processed following the PExA RNA extraction process performed in the main experiment and miRCURY
311 LNA miRNA PCR assay steps. CT values for five synthetic miRNAs of the spike-in kit (UniSp5, UniSp4,
312 UniSp2 as RNA extraction controls, UniSp6 for cDNA synthesis, and UniSp3 for RT-PCR) were
313 comparable to controls where the mix was processed without membranes (**Figure 5**). Thus, the DBS

314 and FTA membranes do not interfere with RNA extraction or affect the enzymes used in cDNA
315 synthesis and RT-PCR.

316 DISCUSSION

317 This pilot study discusses the initial setup and optimization processes for miRNA analysis in PEx
318 samples. It is the first exploration of miRNAs in PEx to date, and it was performed on healthy people
319 to assess the approach's feasibility. As previously pointed out in other publications, the composition
320 of PEx is similar to that of respiratory tract lining fluid (RTLF), as exhalation after breath-holding at
321 residual volume results in the release of a large number of minute particles generated from the
322 fluid coating the small airways [2, 4]. The analysis previously reported of the PEx proteome highlighted
323 a significant enrichment of extracellular proteins associated with exosomes [2], and mass
324 spectrometry-based identification showed stable protein content in healthy individuals, affirming its
325 reliability [4]. Although a wealth of information regarding the protein content of PExA samples is
326 currently available, we wanted to go further with our own analysis to establish which proteins could
327 be used in the future as internal controls to normalize RNA or other types of data. In details, we wish
328 to investigate both the minimum material quantity required for analysis, as well as the reliability of
329 the assay. Our proteomic analysis performed on PEx samples correlates precisely with the findings
330 already reported, showing extremely significant enrichments for terms related to extracellular
331 secretory-related components, such as extracellular exosome and vesicle. Furthermore, we show that
332 this information can be captured even when analysing a single PEx sPOT, paving the way for
333 combinatory analysis of various biomolecules (e.g., proteins and miRNAs) using a single membrane,
334 considering the 10 PEx sPOTs available for each of them. All these results point to the highly likely
335 existence of diverse types of EVs in PEx samples. Numerous physiological and diseased states are
336 known to be regulated by miRNAs contained in EVs that facilitate intercellular communication, and
337 this has also been reported for the lungs[9, 12]. In detail, exosomes and their enclosed miRNAs have
338 been detected in various clinical lung samples, including bronchoalveolar lavage fluid (BALF) and
339 sputum supernatants, during the investigation of respiratory diseases, like asthma[28]. Furthermore,
340 cells could adjust the production and composition of these EVs in response to various stimuli;
341 therefore, examining them provides useful insights into pulmonary disorders[29]. The observation
342 that a significant portion of the PEx proteome exhibits a statistically significant association with
343 extracellular vesicles and granules provided us with persuasive evidence that intact miRNAs
344 could exist in such samples, as they are protected when they enter the vesicles. Given the limitations
345 provided by the small amount of starting material, we used a combination of kits and resources to
346 extract and analyse miRNAs from small amounts of starting material. The methods appeared effective,
347 as we were able to discover several mature miRNAs. The kit's incorporation of the UMI that we
348 used enabled us to obtain a realistic panoramic view of the miRNA that could be identified in the
349 samples, unaffected by the PCR duplicate bias, which is a frequent issue with degraded or low-

350 abundance RNA samples. As reported in the results, for both subjects, the number of counts
351 decreased with the reduction of the number of PEx sPOTs employed for the RNA extraction. In
352 particular, it seems that using only one PEx sPOT is not enough to get miRNA information, as it seems
353 to be for protein analysis. A substantial presence of RNAse activity was observed in the PEx sample
354 after the collection, as well as in BALF, but not in the membrane before the collection that we prepared
355 in a sterile and dust-free environment. Moreover, the RNAse activity is comparable to the negative
356 controls if the membranes contain chemicals able to protect the RNA. These findings indicate that the
357 membrane is RNAse-free, whereas the airways and related samples are highly contaminated with
358 RNAses. This supports the hypothesis that extracellular miRNAs found in the respiratory tract that are
359 used for intercellular communication require protection in order to reach their target, and so the
360 miRNAs we discovered are most likely from EVs, where they are protected from RNAse activity, since
361 we don't expect to collect cells in PExA samples due to the size of the particles collected (0.5–7 µm).
362 To confirm the presence of some of the miRNAs discovered in miRNA sequencing, and to start testing
363 a reproducible and fast technique to use in clinical practice, we selected the most abundant ones and
364 designed a custom-made miRCURY LNA™ SYBR Green PCR assay from Qiagen. This product employs
365 LNA-enhanced and Tm-normalised primers, which allow for accurate and reliable quantification of
366 individual miRNAs with exceptional sensitivity and an extremely low background. With this easy-to-
367 assay and commercially available tool we were able to confirm the existence of some of the selected
368 miRNA in PEx. Even though we can't consider this system quantitative yet, since we haven't identified
369 stable miRNAs in all samples that we can use in normalization, the most abundant miRNA from the
370 sequencing data is the one with the lowest CTs (miR-125b).
371 The identified miRNAs are also reported in other publications that analyse miRNA in the lung. In
372 particular, the let-7 miRNA family, which is well represented in PEx samples, showed relatively high
373 counts for both subjects, with let-7b-5p and let-7c-5p having the highest expression. The significant
374 level of preservation observed in let-7 family members across several animal species implies that they
375 likely have crucial and maybe comparable functions in the biological processes of different organisms
376 [30, 31]. As summarized by Lee and colleagues in their comprehensive review of the let-7 family [32],
377 it appears that they exert two primary biological functions: crucial regulator of terminal differentiation
378 and fundamental tumour suppressor. Furthermore, let-7 family members, like numerous EV-secreted
379 eukaryotic miRNAs, are implicated in different respiratory diseases, including chronic obstructive
380 pulmonary fibrosis, idiopathic pulmonary fibrosis, and asthma [33], providing a good rationale for
381 detecting them as biomarkers at different stages of clinical studies. For example, based on a miRNA
382 profile of exosomes performed on BALF, some of these family members were included in a small set
383 of miRNAs able to distinguish asthmatics from healthy participants at baseline [28]. Regarding the

384 most common let-7 member found in PEx, let-7b-5p, Koeppen and colleagues discovered that this
385 miRNA could control genes involved in the development of biofilms in a bacterial species such as *P.*
386 *aeruginosa*. Specifically, they demonstrated that let-7b-5p delivered by EVs from airway epithelial cells
387 reduces biofilm formation and increases antibiotic sensitivity in *P. aeruginosa* [33]. Moreover, PEx
388 samples contained four of the five most common miRNAs detected in airway epithelial cells-EVs by
389 Koeppen and colleagues [33], which account for more than half of all miRNA sequence reads.
390 Identification of miRNAs that are directly produced and encapsulated by airway epithelial cells
391 provides further support for the concept that PEx samples can yield insights into intra-vesicular
392 communication within the small airways, given that PEx particles theoretically form in the lower
393 respiratory tract via the mechanism of rupture of the RTLF film during airway reopening after airway
394 closure. Other miRNAs that were detected in PEx samples from both patients with a high number of
395 counts were miR-125b-5p and miR-125a-5p. Evidence from many articles suggests that the miR-125
396 family plays a controvert role in diseases, acting as both tumour suppressors and promoters across
397 different carcinomas [34]. What is clearer is that miR-125 influences the fate of immune cells by
398 targeting and regulating a set of target genes to enhance the effect against intra- or extracellular
399 pathogens or by responding to the regulation of specific signal stimulation like IL and IFN [34], key
400 mediators in pulmonary diseases like asthma. However, efforts to use miR-125 as a target or
401 biomarker are ongoing and require further research.
402 Looking at the miRNA atlas, the majority of the miRNAs detected in PEx are reported to be present in
403 the lungs and bronchi, the only two elements of the respiratory system available in the miRNA tissue
404 atlas. We then checked if they were also present in the respiratory glands, since some concerns had
405 arisen in the past given the risk of saliva contamination of the samples since PEx are exhaled through
406 the oral cavity [4]. MiR-4508, miR-1246, and miR-4492 are the only three miRNAs identified in PEx
407 that, based on the miRNA tissue atlas, exhibit expression in the bronchus and/or lung but not in the
408 salivary glands, but they are not the most expressed. Thus, based on these results, we cannot
409 completely exclude the possibility that our identified miRNAs originate from saliva; however, our
410 proteomic data did not detect amylase, the most prevalent protein in saliva, which has previously
411 been cited as evidence to deny the presence of saliva in PEx samples [4].
412 The investigation of miRNA detection in non-invasive samples is gaining attention since techniques
413 such as BALF and induced sputum have varying degrees of impact on patients and cannot be repeated
414 multiple times, especially in diseased patients. Shi and colleagues provided a comprehensive report on
415 exhaled miRNAs in lung cancer research utilizing the EBC sampling technique [15]. Specifically, they
416 examine the efficacy of a selected panel of 24 miRNAs in differentiating individuals with non-small cell
417 lung cancer from control subjects. Although further refinement of their exhaled miRNA interrogation

418 technique is required, the researchers have shown that the detection of miRNAs in EBC using qPCR
419 can lead to a modest enhancement in differentiating between cases and controls, beyond what can
420 be achieved with clinical factor models alone [15]. Nevertheless, the composition of biomolecules in
421 EBC is significantly affected by their physical characteristics as well as their relative concentrations in
422 the RTLF [35]. Moreover, dilution poses uncertainty in EBC, as well as in BAL, where external fluid
423 addition complicates concentration calculations and normalization. Conversely, PEx samples are dry
424 upon collection, thus enhancing the reliability of concentration measurements [4]. Therefore, this
425 underscores the importance and value of our findings, highlighting the efficacy of the method (PEx)
426 we employed for miRNA identification as a non-invasive tool of significant interest.

427 CONCLUSION

428 This proof-of-concept study aims to verify that miRNAs can be detected in PExA samples, thereby
429 showing its utility as a non-invasive method for collecting small airway samples for biomarker analysis
430 in lung disorders. Using PExA, we identified a series of miRNAs previously shown to be of relevance
431 for pulmonary disorders using two complementary techniques. Moreover, we strongly believe that
432 the identified miRNAs were detected intact due to their protection via encapsulation within EVs. This
433 is supported by the enrichment of extracellular secretory-related components in the core protein list
434 from PExA samples, along with the demonstrated high RNAse activity in the respiratory tract, which
435 would have otherwise rapidly degraded the miRNAs. This opens the possibility of studying EV-
436 mediated intracellular communication in distal small airways using PExA as a non-invasive technique.
437 However, this is only the first step; we are still in the process of understanding the feasibility of miRNA
438 detection in this type of sample. Further investigations and efforts must be made to establish internal
439 controls for moving forward with a quantitative evaluation, as well as to understand intra- and inter-
440 subject variability. Our efforts to understand the core of proteins that can always be detected in PEx
441 sPOTs can help in this purpose of identifying internal controls to use in the process of normalizing the
442 miRNAs until we will not find stable miRNAs that can be detected in the same amount in all samples.

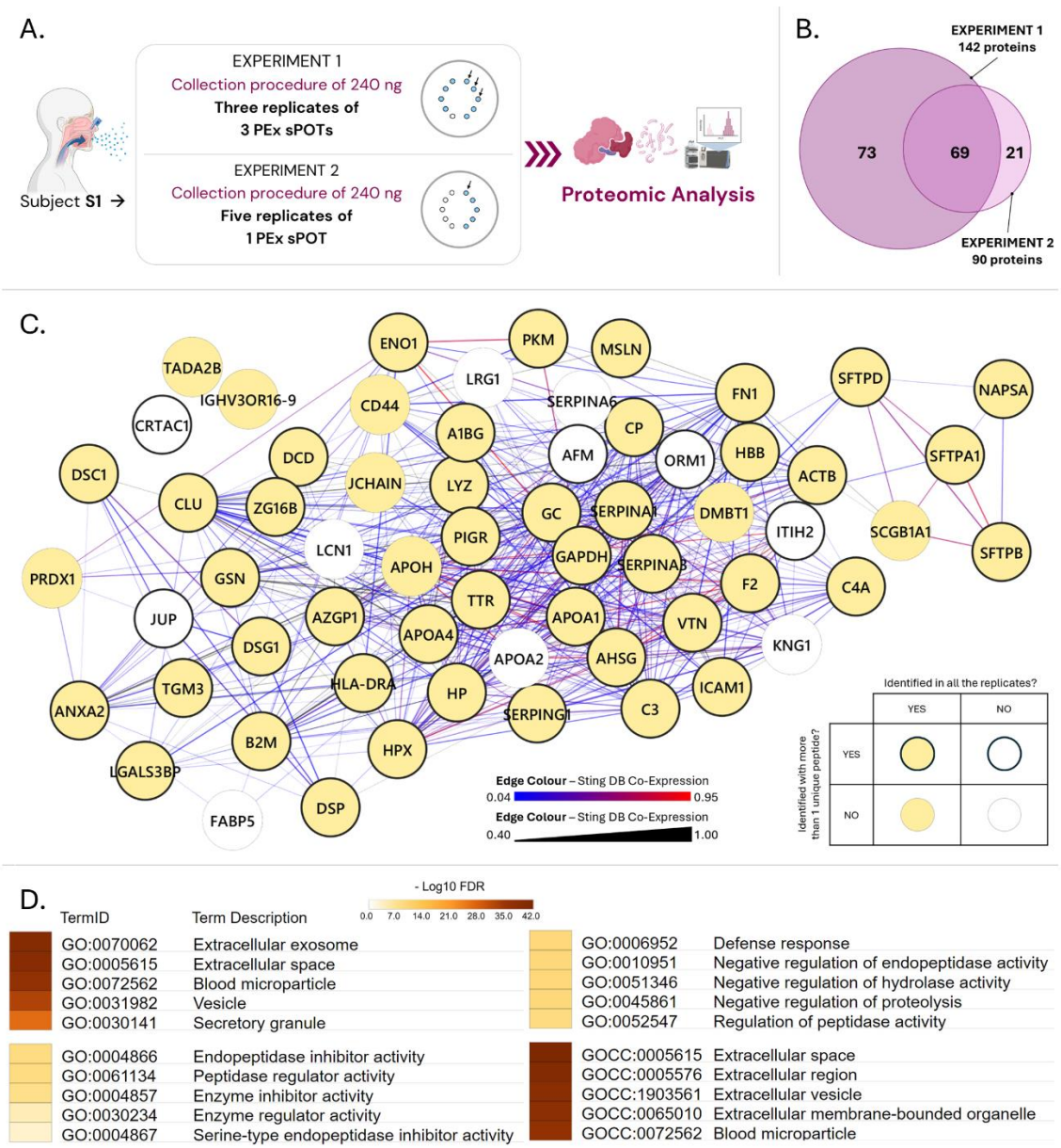

Figure 1. *Proteins observed in PEx with their biological function and location.* **A.** Graphical outline of the experimental workflow. **B.** Pie chart with the number of proteins identified in both experiments. **C.** The protein-protein interaction (PPI) network of the proteins identified in both experiments; the legend describes the features of the network's elements. The edges represent the StringDB score, i.e., how likely STRING is to judge the confidence of the correspondent PPI. For the network realization, the minimum required interaction score was set at 0.4 (medium confidence). The edges are colored based on the co-expression interaction score (with no interaction of this type, the edge is grey). The yellow nodes with black border represent the proteins with the highest level of confidence for identification PExA samples, since they were identified in all the replicates with more than 1 unique peptide. **D.** Pathway analysis of the proteins identified in both experiments. In the table are reported the top five enriched terms for all three GO categories and subcellular compartments. For each

455 enriched term, the -Log10 false discovery rate (FDR) is provided. All the data was obtained from
456 stringDB[24].

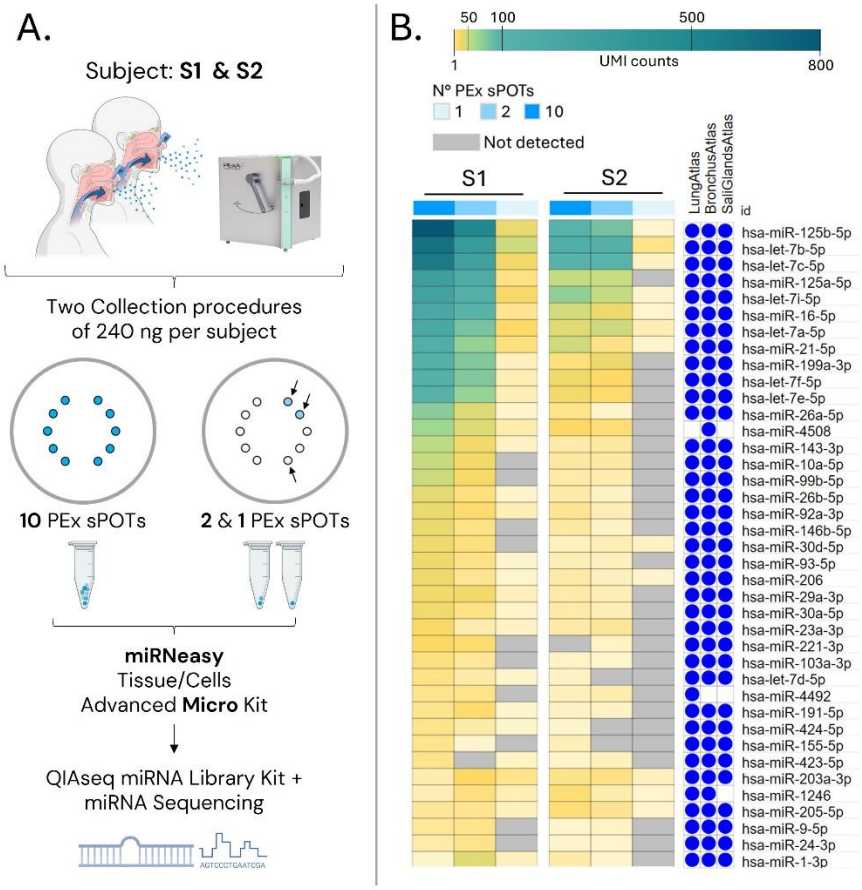

Figure 2. *miRNA identified in PEx samples on DBS membrane using RNAseq.* **A.** Graphical outline of the experimental workflow. **B.** List of the miRNA identified. Data are presented as UMI counts in a colour scale from dark red to green. Each left column represents a sample, related to two subjects with varying amounts of PEx sPOTs. The right columns display gene lists sourced from or miRNATissueAtlas2 (with expression levels exceeding 10 as cut-off[26, 27]). A blue dot indicates the presence of the PExA identified miRNA in the corresponding list.

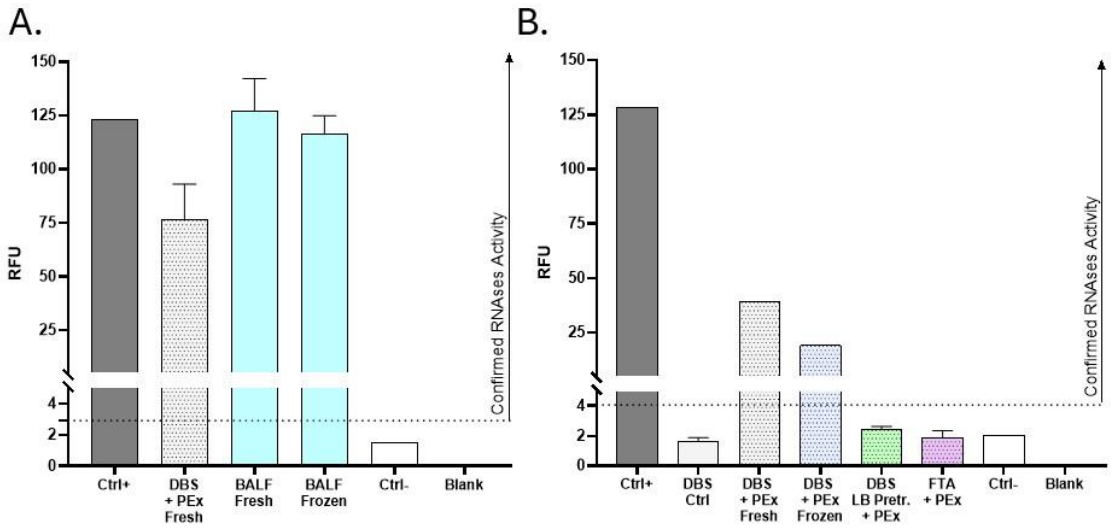

Figure 3. *Evaluation of RNAse activity presence in the airways.* **A.** The barplot shows the level of RNAse activity in different sample types coming from the airways, like PEx samples collected on DBS membrane, as well as in fresh and frozen BALF. **B.** The barplot shows the level of RNAse activity in the PEx samples using different condition and membranes (DBS Ctrl is the membrane alone, without any sample on it; LB Pretr. = the DBS membrane was pretreated with the lysis buffer from the RNA extraction kit). In both plots, according to the manufacturer, samples with fluorescence levels exceeding 2-fold of the corresponding negative control (dotted line) are considered affected by the presence of RNase activity. All the graph-bars in the two plots are reported as mean ± SD of two different replicates (except for the controls and the blank). The dotter texture of bar plots highlights samples with PExA.

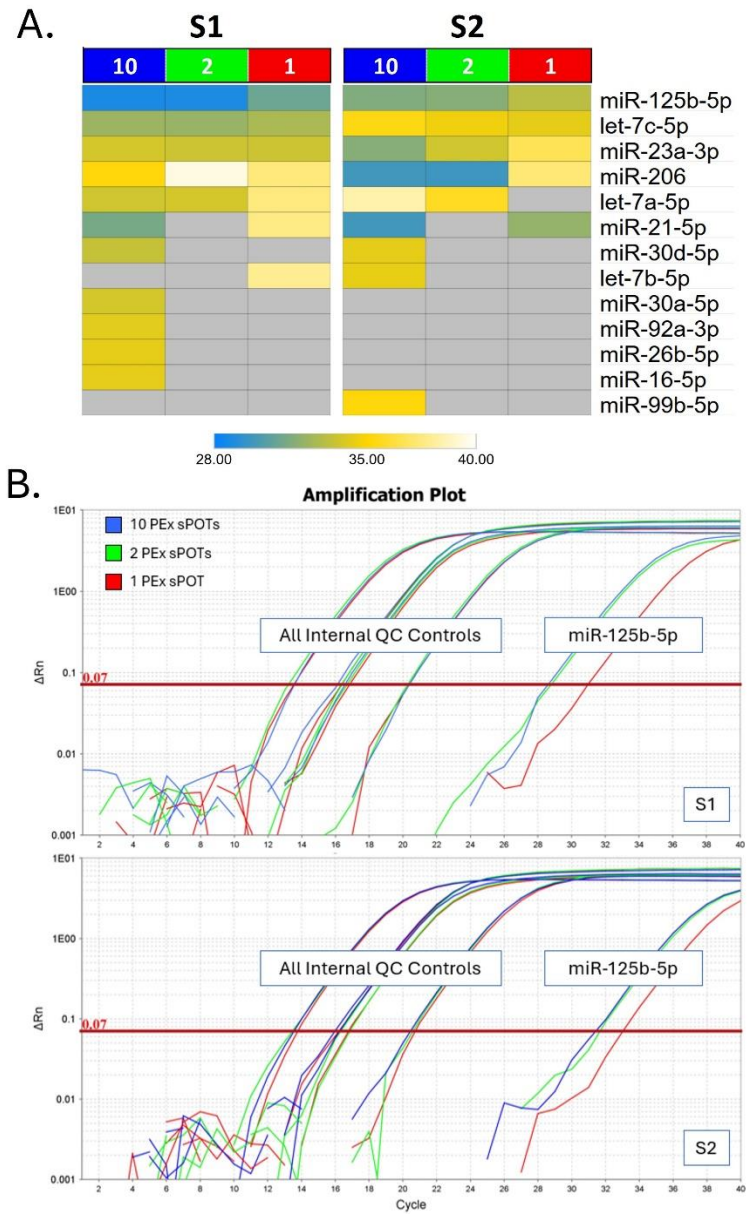

Figure 4. *miRCURY LNA™ SYBR Green PCR assay in PEXA samples.* Results of the PCR array performed on the same set of samples as in the RNAseq analysis. **A.** Visualization of miRNA presence across diverse samples. The heatmap is constructed using a color scale that represents the detected Ct values, with a threshold set at 0.07. Grey cells represent undetected miRNAs in the respective sample. **B.** Amplification plot of the internal controls of the array and the most abundant miRNA in S1 and S2 (miR125b-5p).

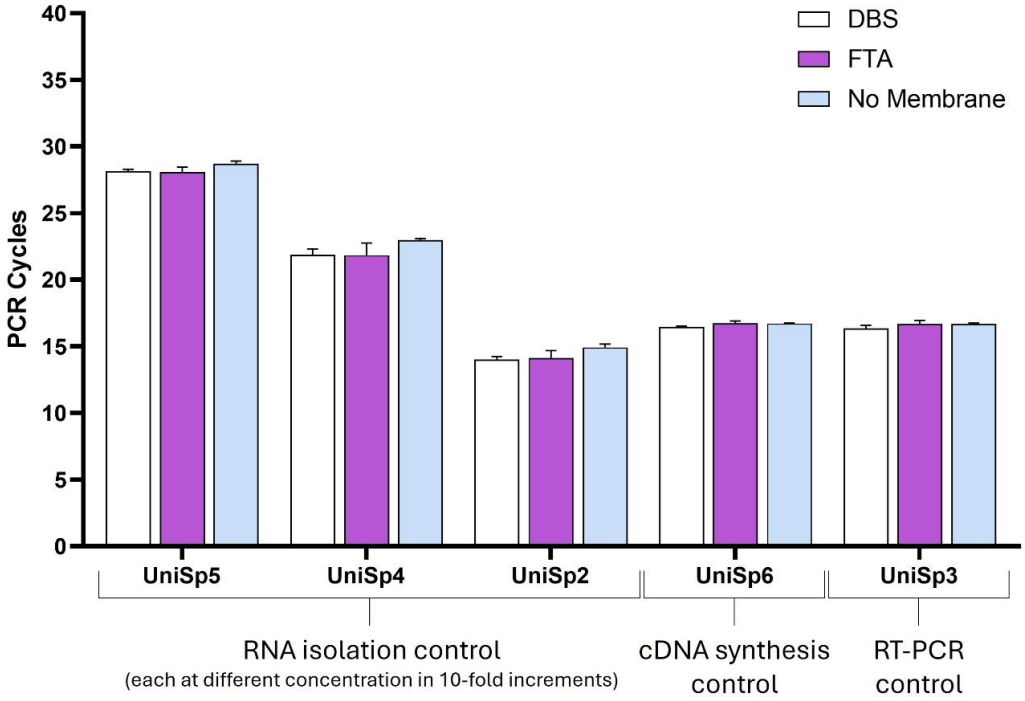

Figure 5. *Evaluation of potential membrane interference during RNA extraction.* PCR cycle thresholds for RNA spike-in controls (UniSp5, UniSp4, UniSp2) for RNA isolation, cDNA synthesis (UniSp6), and RT-PCR (UniSp3) show comparable results between DBS (white), FTA (purple), and no membrane (blue) conditions, with no statistically significant differences among the conditions (Kruskal-Wallis test). All graph bars are reported as mean ± SD of three different replicates.

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489

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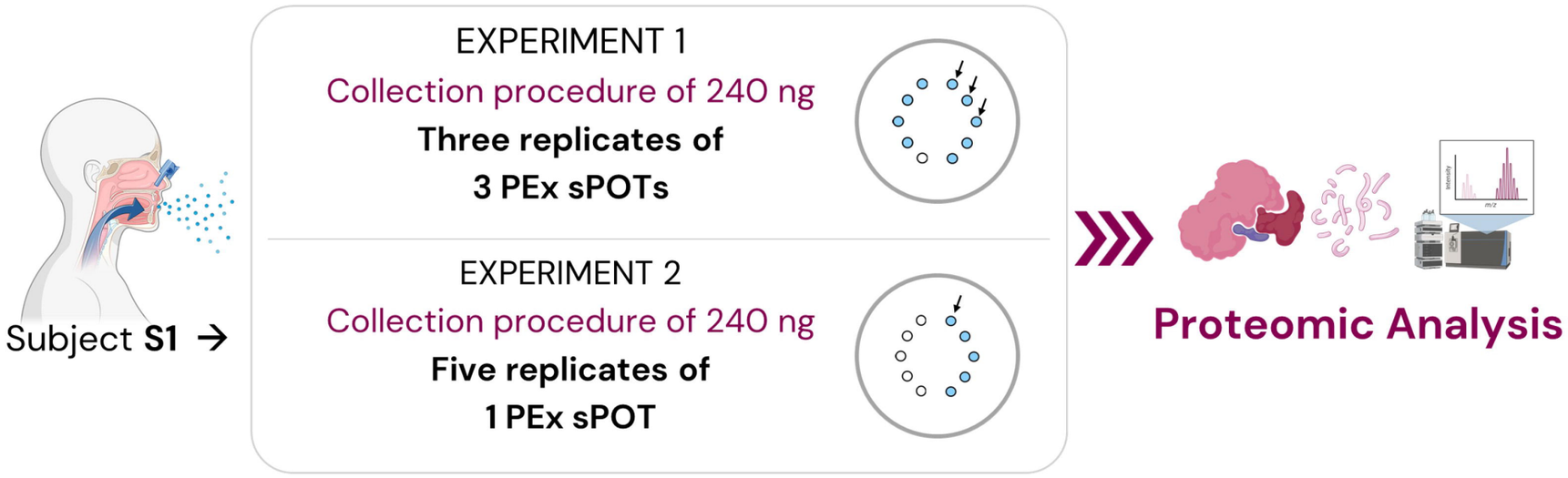
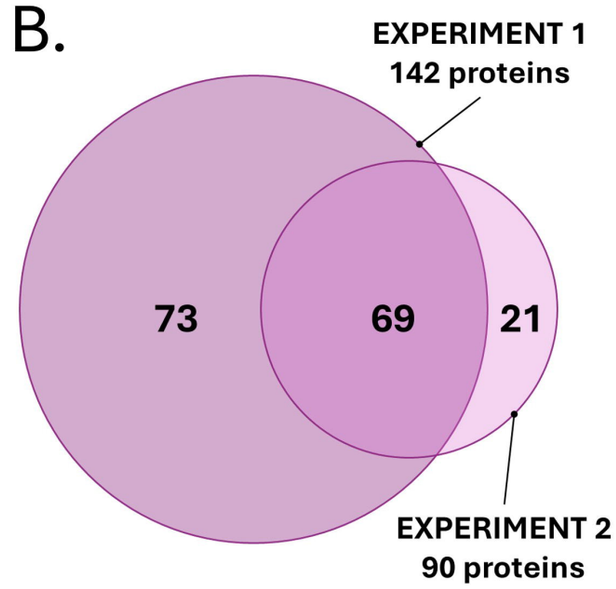
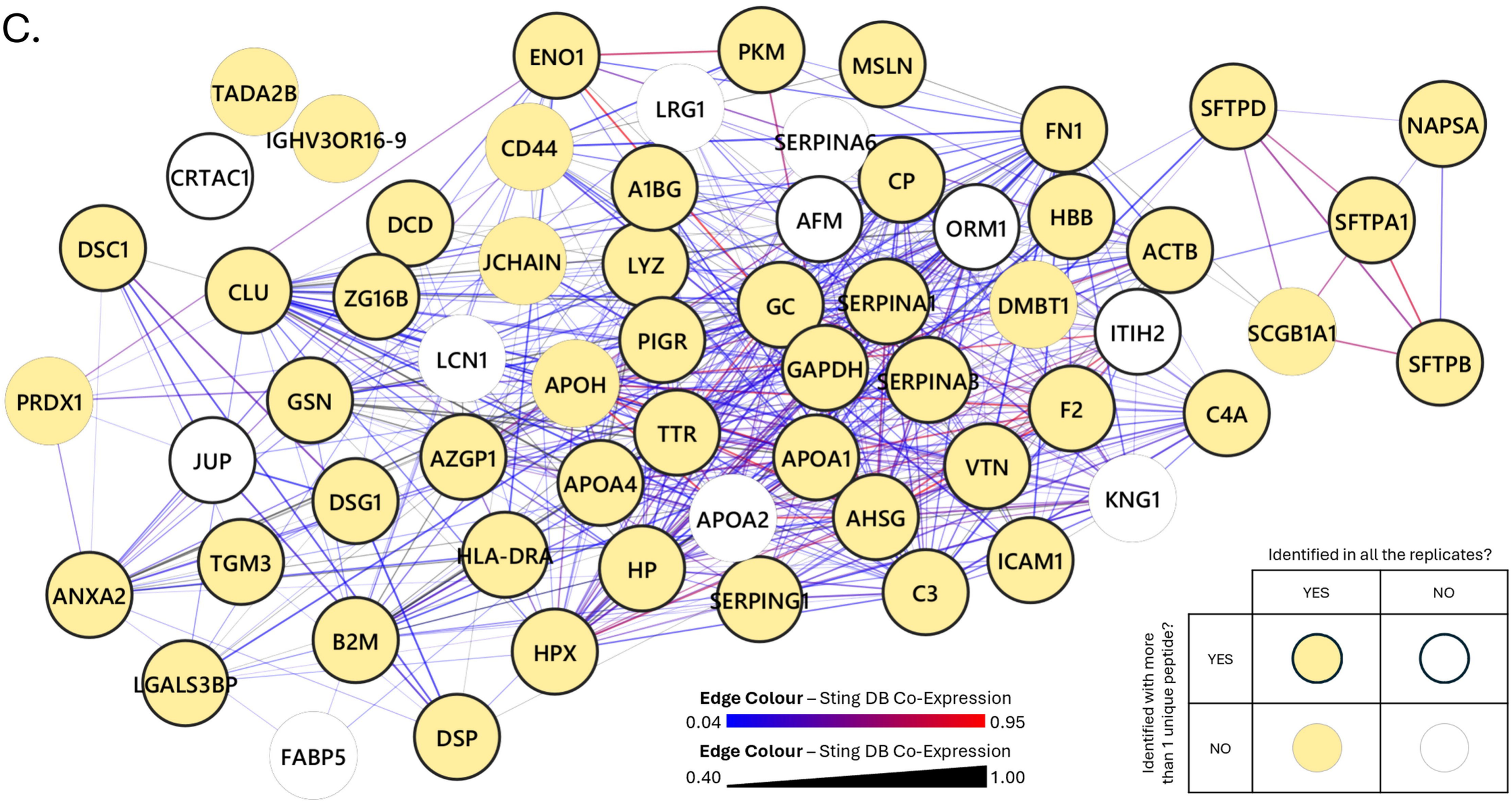
A.


Subject S1 →
EXPERIMENT 1
Collection procedure of 240 ng
Three replicates of
3 PEx sPOTs
EXPERIMENT 2
Collection procedure of 240 ng
Five replicates of
1 PEx sPOT
Proteomic Analysis

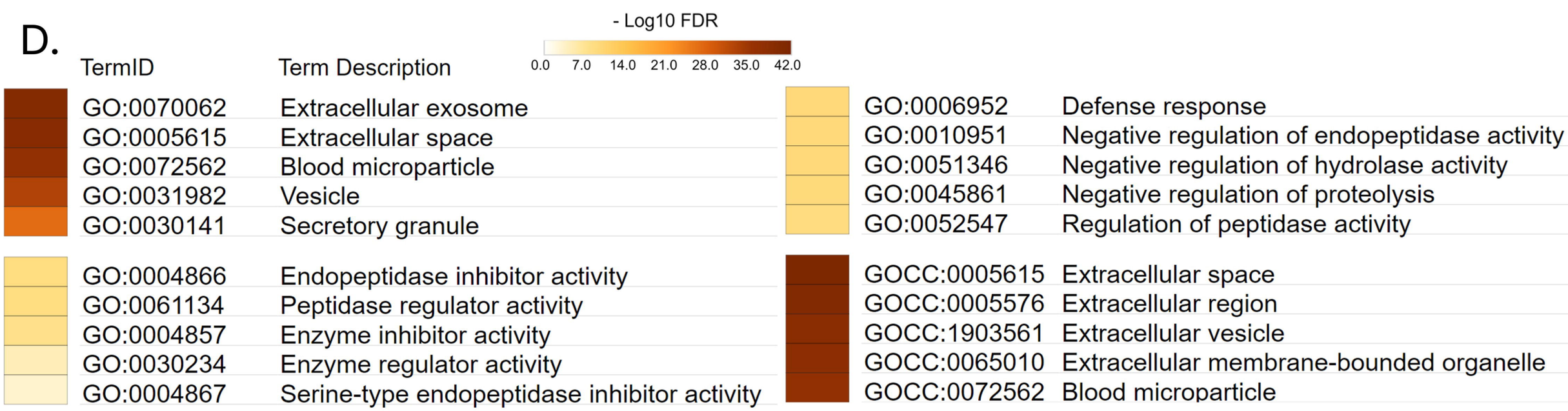
B.

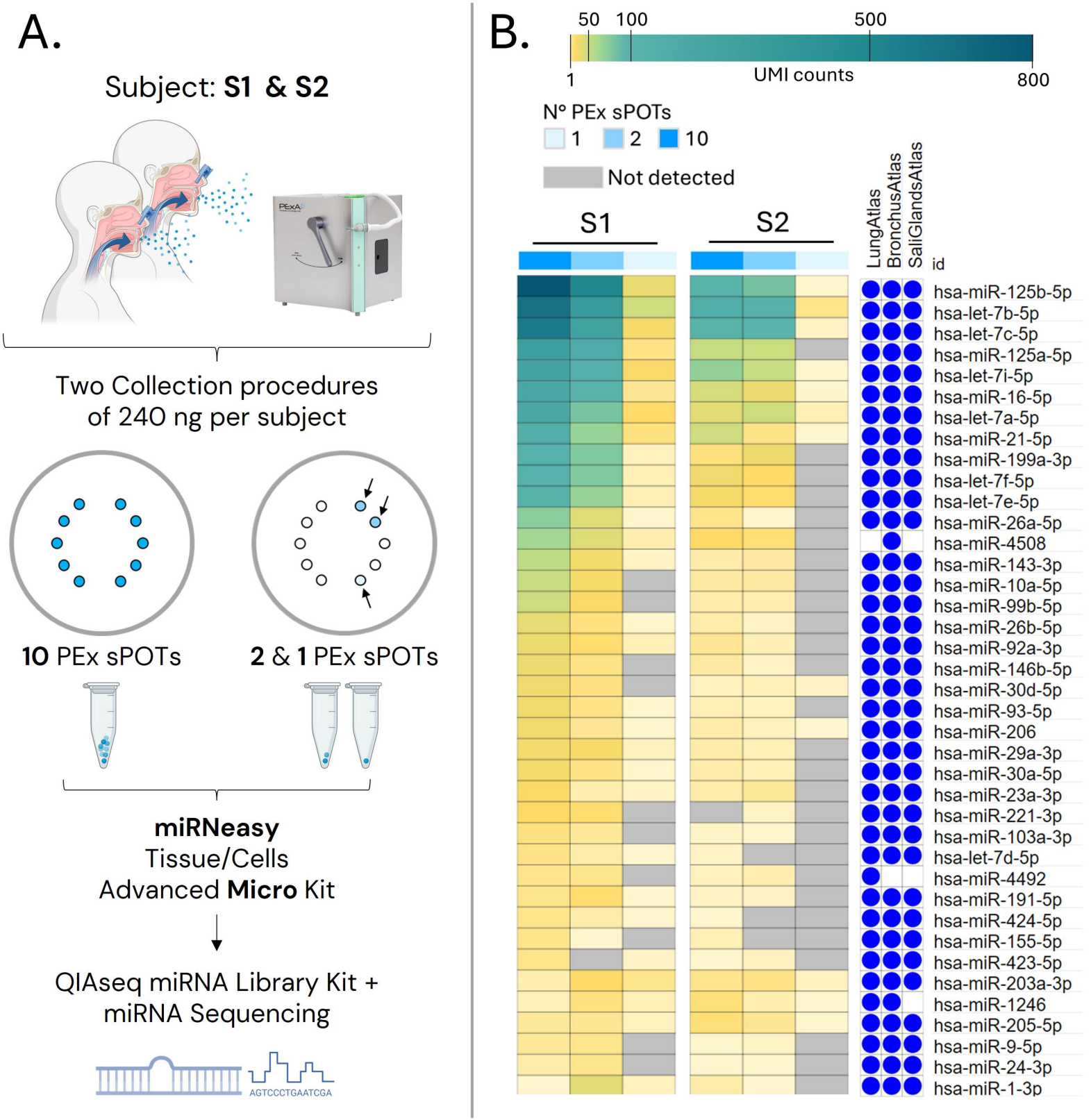

EXPERIMENT 1
142 proteins
73
69
21
EXPERIMENT 2
90 proteins

C.


ENO1
PKM
MSLN
TADA2B
IGHV3OR16-9
LRG1
SERPINA6
FN1
SFTPD
NAPSA
CRTAC1
CD44
A1BG
CP
AFM
ORM1
HBB
SFTPA1
DSC1
DCD
JCHAIN
LYZ
ACTB
CLU
ZG16B
GC
SERPINA1
DMBT1
ITIH2
SCGB1A1
LCN1
PIGR
GAPDH
SERPINA3
SFTPB
PRDX1
GSN
APOH
F2
C4A
TTR
JUP
AZGP1
APOA1
VTN
APOA4
DSG1
APOA2
AHSG
KNG1
TGM3
HLA-DRA
HP
ICAM1
ANXA2
SERPING1
C3
B2M
HPX
LGALS3BP
DSP
FABP5
Edge Colour – Sting DB Co-Expression
0.04
0.95
Edge Colour – Sting DB Co-Expression
0.40
1.00
Identified in all the replicates?
YES
NO
Identified with more than 1 unique peptide?
YES
NO

D.


- Log10 FDR
0.0
7.0
14.0
21.0
28.0
35.0
42.0
TermID
Term Description
GO:0070062 Extracellular exosome
GO:0005615 Extracellular space
GO:0072562 Blood microparticle
GO:0031982 Vesicle
GO:0030141 Secretory granule
GO:0006952 Defense response
GO:0010951 Negative regulation of endopeptidase activity
GO:0051346 Negative regulation of hydrolase activity
GO:0045861 Negative regulation of proteolysis
GO:0052547 Regulation of peptidase activity
GO:0004866 Endopeptidase inhibitor activity
GO:0061134 Peptidase regulator activity
GO:0004857 Enzyme inhibitor activity
GO:0030234 Enzyme regulator activity
GO:0004867 Serine-type endopeptidase inhibitor activity
GOCC:0005615 Extracellular space
GOCC:0005576 Extracellular region
GOCC:1903561 Extracellular vesicle
GOCC:0065010 Extracellular membrane-bounded organelle
GOCC:0072562 Blood microparticle


A.
Subject: S1 & S2
Two Collection procedures
of 240 ng per subject
10 PEx sPOTs
2 & 1 PEx sPOTs
miRNeasy
Tissue/Cells
Advanced Micro Kit
QIAseq miRNA Library Kit +
miRNA Sequencing
AGTCCCTGAATCGA
B.
50
100
500
1
UMI counts
800
Nº PEx sPOTs
1
2
10
Not detected
S1
S2
LungAtlas
BronchusAtlas
SaliGlandsAtlas
id
hsa-miR-125b-5p
hsa-let-7b-5p
hsa-let-7c-5p
hsa-miR-125a-5p
hsa-let-7i-5p
hsa-miR-16-5p
hsa-let-7a-5p
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hsa-let-7e-5p
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hsa-miR-206
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hsa-miR-30a-5p
hsa-miR-23a-3p
hsa-miR-221-3p
hsa-miR-103a-3p
hsa-let-7d-5p
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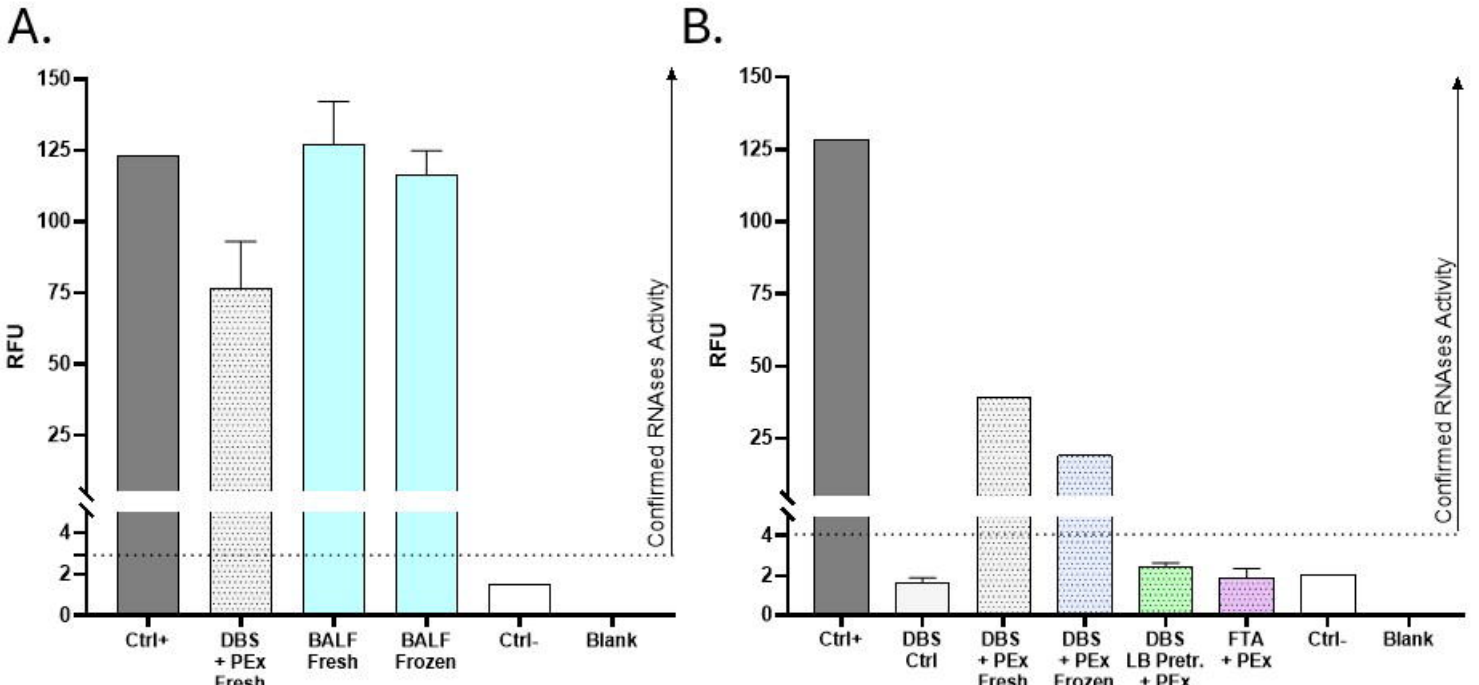
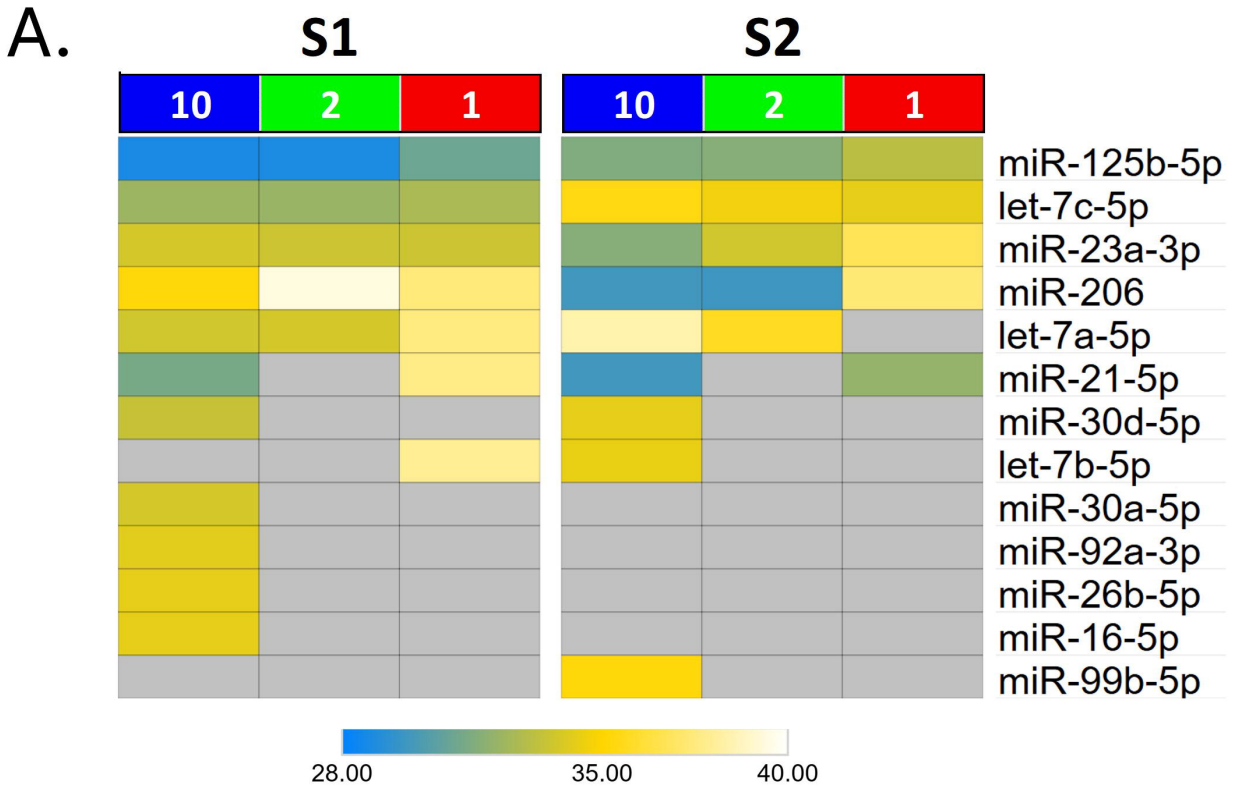
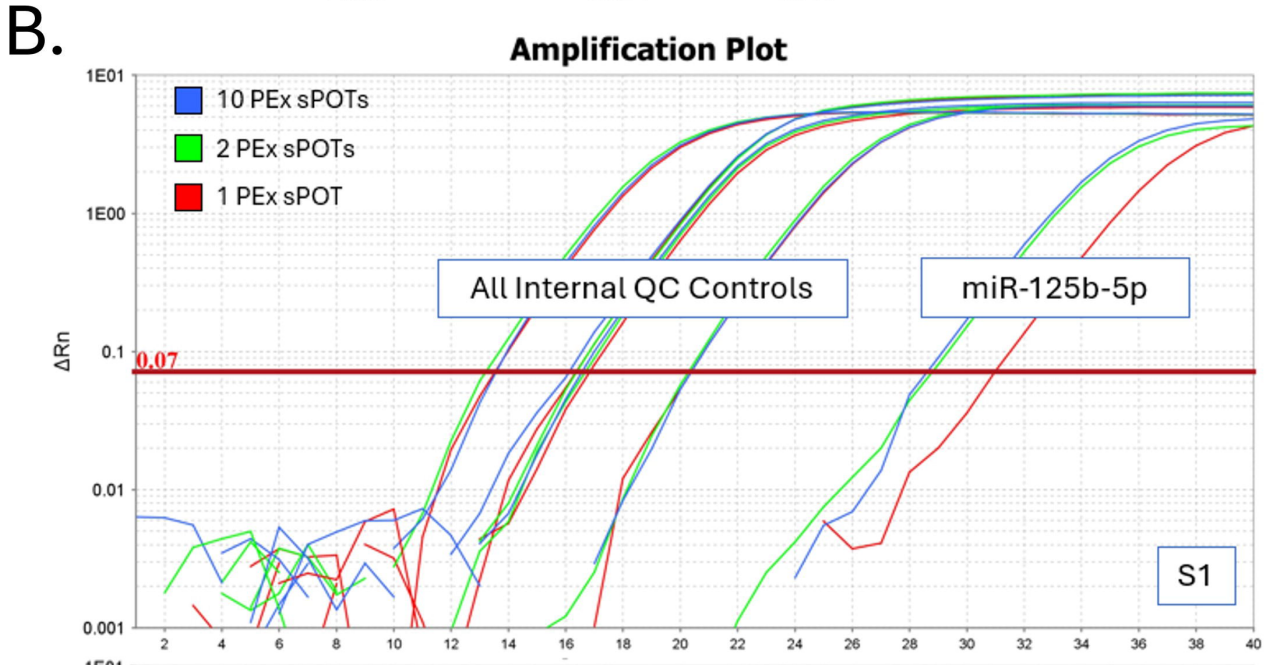
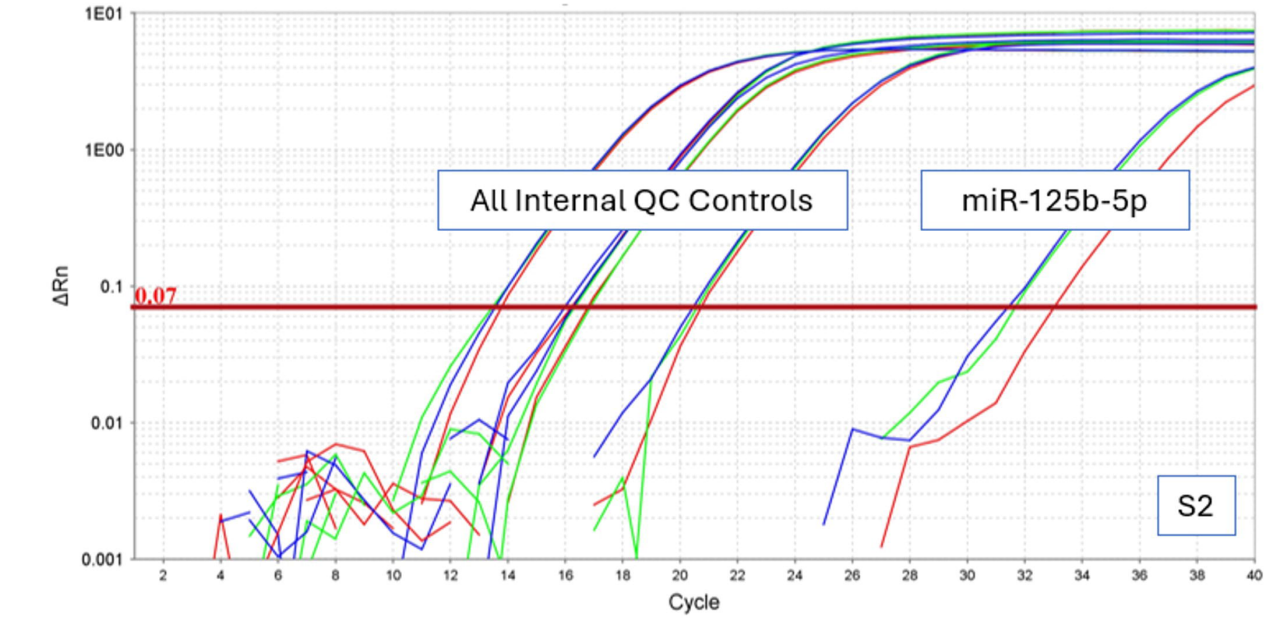
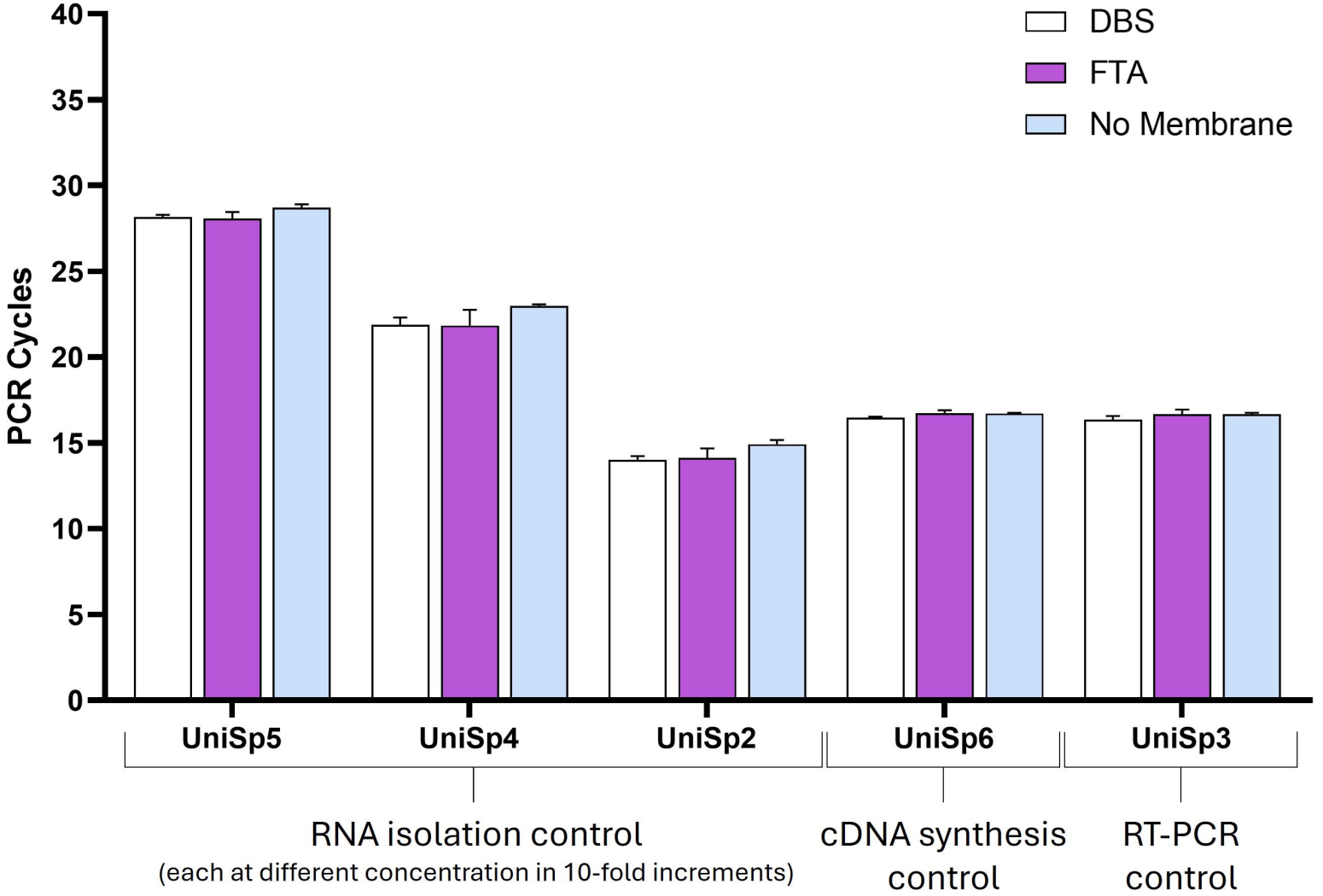
hsa-miR-1-3p


A.
B.
RFU
150
125
100
75
50
25
4
2
0
Confirmed RNAses Activity
Ctrl+
DBS + PEx Fresh
BALF Fresh
BALF Frozen
Ctrl-
Blank
DBS Ctrl
DBS + PEx Frozen
DBS LB Pretr. + PEx
FTA + PEx


A.
S1
S2
10
2
1
10
2
1
miR-125b-5p
let-7c-5p
miR-23a-3p
miR-206
let-7a-5p
miR-21-5p
miR-30d-5p
let-7b-5p
miR-30a-5p
miR-92a-3p
miR-26b-5p
miR-16-5p
miR-99b-5p
28.00
35.00
40.00


B.
Amplification Plot
10 PEx sPOTs
2 PEx sPOTs
1 PEx sPOT
All Internal QC Controls
miR-125b-5p
0.07
ΔRn
S1


All Internal QC Controls
miR-125b-5p
0.07
ΔRn
S2
Cycle


DBS
FTA
No Membrane
PCR Cycles
40
35
30
25
20
15
10
5
0
UniSp5
UniSp4
UniSp2
UniSp6
UniSp3
RNA isolation control
(each at different concentration in 10-fold increments)
cDNA synthesis control
RT-PCR control