# 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 a 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

52 LNA miRNA PCR assay, yielded results comparable to control samples, indicating that the membrane
53 material does not interfere with the assay.
54
55 **Conclusions.** Using PExA, we identified several miRNAs that are known to be dysregulated in various
56 pulmonary disorders. The enrichment of extracellular secretory components in the core protein list
57 from our proteomic analysis, along with the elevated RNAse activity in the respiratory tract, suggests
58 that the detected miRNAs may be encapsulated within extracellular vesicles. These miRNAs are
59 particularly intriguing due to their potential role in intercellular communication during both normal
60 and pathological lung processes. Our findings suggest that PExA holds significant potential as a non-
61 invasive tool for studying extracellular vesicle-mediated miRNA cargo in the small airways.

62 INTRODUCTION

63

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

87

88 MicroRNAs (miRNAs) are small, conserved non-coding RNAs that regulate gene expression post-
89 transcriptionally (8). They play a crucial role in various biological processes, and their dysregulation is
90 linked to many pulmonary diseases (9-11). miRNAs are found in numerous body fluids and
91 extracellular spaces, including RTLF, either associated with other biomolecules or encapsulated in
92 extracellular vesicles (EVs) (12-14). Acting as functional vehicles of intercellular communication, EVs
93 transport miRNAs during pathological processes, protecting them from degradation and thereby
94 enhancing their stability (15). Given their importance in diseases processes (9, 10), as well as relative
95 stability, miRNAs hold great potential for various clinical applications, including longitudinal

96 monitoring. Non-invasive molecular tools for studying lung-derived miRNAs are emerging, though
97 not yet rigorously evaluated or in clinical use (16). Existing publications on non-invasive approaches
98 focus on exhaled miRNAs, primarily using exhaled breath condensate (EBC) for sampling (16-18).
99 However, it is still not fully understood where EBC originates from, and there are uncertainties on its
100 formation. Radioactive markers studies have revealed that EBC predominantly captures central
101 airways, rather than providing a clear picture of the RLTF in the smaller airways (19).
102
103 Given the pivotal roles of extracellular vesicles (EVs) and miRNAs in intercellular communication and
104 regulatory processes in lung diseases, in this publication we describe the development of a miRNA
105 analysis approach for non-invasive interrogation of the distal lung using the PExA technology in
106 healthy subjects. To our knowledge we were the first to report detection of miRNA in PExA samples.
107 By exploring the feasibility of detecting miRNAs in PExA samples, our study contributes to the
108 advancement of respiratory research and disease diagnostics.

109 MATERIALS AND METHODS

110

111 **Sample collection**

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

130

131 **Protein workflow**

132 Two distinct PEx proteome analyses were designed to evaluate both the technical reproducibility,
133 the detection limit, and the reproducibility of repeated sampling the same individual on different
134 days. In Experiment 1, we collected 240 ng PEx sample from one healthy subject and divided it into
135 three replicates composed of three PEx dots (3 replicates of 72 ng PEx). In Experiment 2, we
136 collected another sample from the same subject on a different day, maintaining the same time of
137 the day, dividing it into five different replicates, each composed of a single PEx sPOT (5 replicates of
138 24 ng PEx). Subsequently, we proceeded with the protein analysis workflow.

139

140 **Non-targeted proteome analysis**

141 Excised PEx membranes spots were wetted with a total volume of 20 µL lysis buffer composed of
142 0.2% n-dodecyl-β-D-maltoside (DDM; Sigma-Aldrich) in 100 mM triethylammonium bicarbonate

143 (TEAB; Fluka) and sonicated in water bath for 10 min. Suspensions were vortexed and centrifuged at
144 low speed for 5 min at room temperature to sediment PEx sPOTs. Subsequently, samples were
145 digested by adding 10 µL of 20 ng/uL sequencing grade trypsin (Promega) into the lysis buffer, with a
146 final trypsin concentration of 6.7 ng/µL. Following a short vortexing samples were incubated for 4 h
147 at 37˚C and centrifuged at 10,000 x *g* for 5 min. The supernatants were transferred to sample vials
148 and dried in a vacuum concentrator (Eppendorf) until dry.
149
150 Peptides were reconstituted in solvent A (0.1% formic acid, FA in 2% acetonitrile, ACN) and 2 µL of
151 each sample was injected using an Ultimate 3000 nano-flow UHPLC system (Thermo Fisher
152 Scientific). Peptides were captured on a 2-cm Acclaim PepMap trap column (Thermo Fisher
153 Scientific) and separated on a heated 25-cm long EASY-Spray C18 column (Thermo Fisher
154 Scientific) applying a 90 min long gradient: 4-26% of solvent B (0.1% FA in 98% ACN) in 90 min, 26-
155 95% in 5 min, and 95% of solvent B for 5 min at a flow rate of 300 nL/min.
156
157 Mass spectra were acquired on an Orbitrap Eclipse tribrid mass spectrometer (Thermo Fisher
158 Scientific) equipped with high-field asymmetric ion mobility spectrometry (FAIMS) in *m/z* 300 to
159 1500 at resolution of R=120,000 (at *m/z* 200) for full mass, targeting $8x10^5$ ions in maximum 100 ms,
160 followed by data-dependent higher-energy collisional dissociation (HCD) fragmentations of
161 precursor ions with a charge state 2+ to 6+, using 20 s dynamic exclusion. The tandem mass spectra
162 of the top precursor ions were acquired in 1 s cycle time by the linear ion trap in rapid mode,
163 targeting $3x10^4$ ions for maximum injection time of 20 ms, setting quadrupole isolation width to 1 Th
164 and normalized collision energy (HCD) to 30%. FAIMS was operated at three different compensation
165 voltages in each cycle using either -40, -60 or -80 V.
166
167 **Proteome data analysis**
168 The raw files were searched against the human UniProt database using MS Amanda v2.0 search
169 engine (24) loaded into Proteome Discoverer 2.5 software (Thermo Fisher Scientific). MS1 precursor
170 mass tolerance was set at 10 ppm, and MS2 tolerance was set at 0.6 Da. Search criteria included
171 variable modifications of oxidation (+15.9949 Da) on methionine residues, deamidation (+0.984 Da)
172 asparagine and glutamine residues. Search was performed with full trypsin/P digestion and allowed
173 a maximum of two missed cleavages on the peptides analyzed from the sequence database. The
174 false-discovery rates of proteins and peptides were set at 0.05. All data were quantified using the
175 label-free quantitation node of Precursor Ions Quantifier through the Proteome
176 Discoverer calculating protein abundances based on precursor abundances. The abundance levels of

177 all proteins were normalized using the total peptide amount normalization node in the Proteome
178 Discoverer.
179
180 **Protein-protein interaction (PPI) Network and Pathway analysis**
181 Pathway enrichment analysis and PPI network visualization was performed using STRING database v.
182 12.0 (25). All 69 proteins identified in both examinations were analysed. Keratins were eliminated
183 due to possible contamination concerns, and a few protein IDs were not found in the STRING
184 database. The final pathway analysis and visualization network included 62 proteins. For the PPI
185 network, the minimum required interaction score was set to medium confidence (cutoff 0.4). The
186 obtained network was then transferred to Cytoscape v. 3.9.1 (26) for graphical processing.
187
188 **miRNA workflow**
189 Two PExA samples were collected from two healthy subjects. We extracted all available PEx sPOTs
190 (10) from the first membrane and then, we separately processed two and one PEx sPOTs, from the
191 second membrane. Subsequently, we proceeded with the RNA extraction workflow. Details of the
192 protocol are reported in **Figure 2A**.
193
194 **RNA extraction**
195 Vials containing PEx sPOT were transferred directly from -80°C storage to dry ice to prevent
196 premature thawing, and 260 µL of Buffer RLT (Qiagen) was added while still in a frozen state.
197 Samples were transferred to room temperature and incubated for 10 min with intermittent
198 vortexing, then mixed through pipetting until PEx sPOT were disintegrated, thereby ensuring the
199 creation of a pseudo-uniform solution. Total RNA, including the miRNA fraction, was extracted using
200 the miRNeasy Tissue/Cells Advanced Micro Kit (Qiagen) according to manufacturer's instruction. The
201 gDNA Eliminator Spin Column step is essential as it allows to separate any paper-based particles
202 from the liquid, that could interfere with any downstream steps. The RNA fraction was eluted in 15
203 µL nuclease-free water, and the process of elution was performed twice with an incubation time of 5
204 min each to increase the elution efficiency. For samples destined for the qRT-PCR-based miRNA
205 assays, 1µl of RNA spike-in mix (Qiagen) was added to the lysis buffer during RNA isolation to
206 monitor RNA extraction efficacy.
207
208 **Library Preparation and RNA sequencing**
209 The QIAseq miRNA Library Kit (Qiagen, Hilden, Germany) was used to prepare the library, which has
210 integrated Unique Molecular Indices (UMIs) to facilitate measurement of pre-amplification miRNA

211 levels. The libraries were sequenced on the NextSeq 2000 instrument (Illumina, San Diego, US) with
212 a 100 nt (Read1) 8 nt (Index1) setup using 'P2' flowcell. The fastq data were uploaded to the RNA-
213 seq Analysis Portal (RAP) 5.0 powered by QIAGEN CLC Genomics Workbench to obtain the UMI raw
214 counts (reference: miRBase_v22, Homo sapiens; analysis workflow version: 1.2).
215
216 **cDNA Synthesis and qRT-PCR based miRNA assays**
217 For validation experiments using qRT-PCR based miRNA assays, a set of samples were collected in an
218 identical manner as for the RNAseq analysis described above. Following extraction, RNA was reverse
219 transcribed using miRCURY Locked Nucleic Acid (LNA™) Reverse Transcription (RT) kit (Qiagen)
220 according to the manufacturer's instruction. For all samples, the reaction mix included the maximum
221 allowable amount of RNA (6.5µL) in a final volume of 10 µL. The cDNA synthesis control (UniSp6) and
222 the PCR amplification Ctrl (cel-miR-39-3p, called UniSp3) coming from the Spike-in mix (Qiagen) were
223 introduced to the reverse transcription reaction to assess its efficacy. A miRCURY LNA miRNA
224 Custom PCR Panel (Qiagen) was used and the set of primers for the miRNAs selected for the custom
225 plate were all validated for qPCR. cDNA samples were amplified using miRCURY LNA™ SYBR Green
226 PCR kit (Qiagen) and the real-time reaction was performed using a QuantStudio™ 7 Flex Real-Time
227 PCR System (Applied Biosystem Foster City, CA, USA), with the following protocol: 95°C for 2 min,
228 followed by 40 cycles consisting of denaturation (10 s at 95°C) and combined annealing/extension
229 (60 s at 56°C). Melting curve analysis from 60–95°C was performed post-amplification to evaluate
230 product specificity.
231
232 **RNAse activity test**
233 The presence of RNase activity in bronchoalveolar lavage fluid (BALF) and PEx samples was assayed
234 using the RNaseAlert® Lab Test Kit (Thermo Fisher Scientific). BALF samples were kept undiluted and
235 thawed on ice when frozen. The assays were conducted following the manufacturer's protocol, with
236 fluorescence measured using the Varioskan™ LUX multimode microplate reader at an excitation
237 wavelength of 490 nm. Additional DBS membranes were pre-treated with RNAse inactivating
238 reagents prior to sample collection, including RNaseZap™ (ThermoFisher Scientific) and lysis buffer
239 from the miRNeasy Tissue/Cells Advanced Micro kit (Qiagen).
240
241 **Evaluation of the potential interference of the membrane in the extraction of the RNA**
242 To test if the membrane matrix materials interfere with the RNA extraction procedure and workflow,
243 5 PEx sPOTs each of DBS an FTA membrane were wetted with 0.2 µL of RNA spike-in mix (Qiagen),
244 making a total of 1 µL across 5 punches. RNA extraction was then carried out as described above. To

245 control for adsorption effects, a positive control was performed by adding 1 µL of the RNA spike-in
246 mix directly to lysis buffer, without including any PEx sPOTs membrane.
247
248 **Ethical Statement**
249 Analysis of miRNA and proteins collected by the PExA method has been approved by the Swedish
250 Ethical Authorities (Case no. 2022-03768-01). For the purpose of methods optimizations, PEx
251 material was stripped of all personal identifiers at the time of collection, and all downstream
252 handling and analysis was performed in a de-identified manner.

253 RESULTS

254

255 **PEx protein content**

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

271

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

273 Subsequently, we sequenced the PEx material to analyze the miRNA composition. Samples were
274 processed in accordance with the instructions provided in the material and methods section. A total
275 181 miRNAs were detected with at least 1 UMI count in at least 1 of the samples. A set of 39 miRNAs
276 were detected with at least 10 UMI counts in at least one of the samples (**Figure 2B**). The amount of
277 UMI counts is higher in the samples processed with 10 PEx sPOTs and then they gradually decrease
278 in the samples processed with 2 and 1 PEx sPOTs for both the subjects employed for this analysis.
279 The number of counts in the sample with only 1 PEx sPOTs are extremely low or absent for most of
280 the miRNAs. Most PExA-identified miRNAs are present in respiratory tract miRNA atlases (lungs,
281 bronchus, and saliva (27, 28)), as reported in the right part of the **Figure 2B.** The highest number of
282 UMI counts was registered by miR-125b-5p in the 10 PEx sPOTs of subject 1, and then the number
283 gradually decreased in the 2 and 1 PEx sPOT settings. Subject 2 exhibits the same trend, but with
284 lower counts. We detected many members of the let-7 miRNA family (let-7a-5p, let-7b-5p, let-7c-5p,
285 let-7e-5p, let-7f-5p, let-7i-5p) with high UMI counts. They exhibit the same reduction trend as miR-
286 125b-5p, transitioning from 10 to 2 to 1 PEx sPOTs.

287

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

289 During the QC analysis of miRNA-seq data (data not shown), a significant proportion of reads that
290 were shorter than expected mapped to RNAs other than miRNA, indicating potential RNA
291 degradation. We then performed a fluorimetric assay to evaluate the presence of RNAse activity in
292 the airways and exclude any contamination coming from the PEx tools and workflow. First, we
293 measured RNase activity in PExA samples collected on a DBS membrane and in fresh and frozen
294 BALF samples to get another biological sample coming from the airways to compare (**Figure 3A**). All
295 samples have significantly higher fluorescence signal than the negative control, indicating strong
296 RNase presence. We then measured the RNAse activity on the membrane only and applying some
297 RNAse inactivation strategies (**Figure 3B**). The fluorescence level of the DBS membrane alone was
298 comparable to the negative control. Samples collected on DBS without any protective reagents
299 resulted in being contaminated by RNAses, even if the activity was a bit less in the samples that were
300 immediately frozen at -80°C after the collection. In samples that were collected on DBS that had
301 been pre-treated with lysis buffer from the RNA extraction kit, or in samples that were collected on
302 an FTA membrane the RNAse contamination is minimal and comparable to the negative control.

303

304 **Validation of different miRNA using real-time PCR technology**

305 From the qRT-PCR based miRNA assays few miRNAs were identified with CT values less than 35
306 (**Figure 4A**). Although we currently lack controls for the regular quantification, miR-125b-5p appears
307 to be the most abundant based on the clear melting curve plot (**Figure 4B**), as reported in the
308 RNAseq experiment. However, some miRNAs have not been identified using this technique. The
309 internal controls for RNA extraction (UniSp2 and UniSp4), cDNA synthesis (UniSp6), and PCR
310 amplification (UniSp3) appear to be reliable and uniform across the different samples, ensuring a
311 high level of reproducibility in the overall workflow (**Table S4**).

312

313 **The membranes for PExA collection do not interfere with the extraction of the RNA and the**
314 **downstream steps**

315 To evaluate if the membranes, DBS or FTA, interfere with the workflow we used the spike-in mix
316 used during the main experiment. We decided to use this mix instead of a real PEx sample
317 considering extremely low amount of RNA in PEx samples and the potential variability of the
318 collection procedures that we are trying to understand. The spike-in mix was added directly to pre-
319 cut PEx sPOTs and processed following the PExA RNA extraction process performed in the main
320 experiment and miRCURY LNA miRNA PCR assay steps. CT values for five synthetic miRNAs of the

321 spike-in kit (UniSp5, UniSp4, UniSp2 as RNA extraction controls, UniSp6 for cDNA synthesis, and
322 UniSp3 for RT-PCR) were comparable to controls where the mix was processed without membranes
323 (**Figure 5**). Thus, the DBS and FTA membranes do not interfere with RNA extraction nor affect the
324 enzymes used in cDNA synthesis and RT-PCR.

## DISCUSSION

In this study, we report for the first time the presence of miRNAs in material collected using the PExA methodology, and we provided novel insights into the existence of EV-miRNAs in these types of samples. A total of 181 miRNAs were detected in PEx spots using RNA-seq analysis, with 39 identified with high confidence, and a subset further verified with qPCR. In parallel, 163 proteins were detected using non-targeted proteomics, with 69 proteins identified reproducibly. Pathway enrichment analyses revealed that many of these proteins are associated with extracellular vesicles and secretory pathways, indicating the presence of EVs. Moreover, RNase activity measurements in PEx samples as well as BALF samples indicating that the sequenced miRNAs are of intravesicular origin.

PExA has recently gained interest as a non-invasive method for collecting material from the distal lung compartment to study molecular changes in health and disease. Commonly used sampling techniques such as bronchoscopy and induced sputum may exert discomfort for research subjects, and their invasive nature makes repeated sampling and longitudinal follow-up challenging. PEx particles have been shown to originate from respiratory tract lining fluid (RTLF) of the distal airway and alveolar region (2, 4) with the strongest evidence coming from the detection of surfactant proteins predominantly produced in the distal airways (21, 29). It has also been shown that the protein content of PEx particles is rather stable, at least in healthy individuals, and thus can be used as a source to identify biomarkers of lung pathologies (4) and a number of previous publications (2, 4, 19-21) have reported the presence of numerous proteins in PEx samples. In this study, we performed non-targeted proteome analyses with the aim of investigating the minimal quantity of PEx material required for analysis without compromising the reproducibility of the assay. The results of the proteome analyses of PEx samples demonstrated a significant enrichment of GO terms related to extracellular vesicles (EVs), including exosomes, terms already reported to be enriched in PEx (2). Our findings thus further strengthen the notion that the protein content of PEx can be identified with mass spectrometry-based methods and that PEx samples contain EVs, including EVs of exosomal origin. Furthermore, we showed that this information can be captured even when using a single PEx sPOT for the analysis. Since a single PEx membrane has 10 PEx sPOTs that can be retrieved for the analysis, this observation paves the way for the analysis of multiple classes of biomolecules (e.g., proteins, lipids, RNAs) from a single sampling occasion, which previously have required several sample sessions from each subject.

358 It has previously been reported that EVs can function as a delivery system for miRNAs and facilitate
359 intercellular communication in the respiratory tract (9, 13), and numerous fundamental physiological
360 processes in health and diseased states are regulated by miRNAs (7-9). Our group, as well as others,
361 have detected exosomes and their miRNA cargo in various clinical lung samples, including BALF and
362 sputum supernatants in patients with asthma as well as in healthy subjects (14, 30). Cells are known
363 to adjust the production and composition of EVs in response to various stimuli, and quantitative
364 analysis of their cargo may thus provide useful insights into pulmonary disorders (30). The
365 observation that a significant portion of the PEx proteome is associated with EVs suggests that intact
366 miRNAs could be present in such samples, as their miRNA cargo would be protected from
367 degradation (11, 31). Shi et al. provided a comprehensive report on exhaled miRNAs in lung cancer
368 research utilizing the EBC sampling techniques (16). Specifically, they examine the efficacy of a
369 selected panel of 24 miRNAs in differentiating individuals with non-small cell lung cancer from
370 control subjects. Although further refinements of their exhaled miRNA interrogation technique are
371 required, the researchers have shown that the detection of miRNAs in EBC using qPCR can lead to a
372 modest enhancement in differentiating between cases and controls, beyond what can be achieved
373 with clinical factor models alone (16). Nevertheless, the composition of biomolecules in EBC is
374 significantly affected by their physical characteristics as well as their relative concentrations in the
375 RTLF (32). Moreover, dilution poses uncertainty in EBC, as well as in BAL, where external fluid
376 addition complicates concentration calculations and normalization. Conversely, PEx samples are dry
377 upon collection, thus enhancing the reliability of concentration measurements (4). For these
378 reasons, we decided to perform a miRNA-seq analysis of PEx samples in an attempt to explore the
379 feasibility of analyzing exhaled microRNA through this non-invasive method and optimize its
380 application for studying lung diseases.
381
382 The miRNAs identified through RNAseq in PEx sample have previously been reported in multiple
383 studies investigating miRNA profiles of the respiratory tract, including samples from lungs and
384 bronchi reported in the Lung Atlas project (27, 28). In particular, the highly conserved let-7 miRNA
385 family was detected in relatively high counts in both subjects, with let-7b-5p and let-7c-5p being the
386 most abundant (33, 34). The significant level of preservation observed in let-7 family
387 members across several animal species implies that they likely have crucial and maybe comparable
388 functions in the biological processes of different organisms (33, 34). As summarized by Lee et al. in
389 their comprehensive review of the let-7 family (35), it appears that they exert two primary biological
390 functions: crucial regulator of terminal differentiation and fundamental tumour suppressor.
391 Furthermore, let-7 family members in different respiratory diseases, including chronic obstructive

392 pulmonary disease, idiopathic pulmonary fibrosis, and asthma (36), providing a good rationale for
393 detecting them as biomarkers at different stages of diseases. For example, based on the miRNA
394 profile of exosomes derived from BALF, let-7 family members were included in a subset of miRNAs
395 that distinguishes asthmatics from healthy participants at baseline (30). The most common let-7
396 member found in PEx, let-7b-5p, was extensively studied by Koeppen et al. who demonstrated that
397 EV-derived let-7b-5p reduced biofilm formation and increased antibiotic sensitivity in *P. aeruginosa*
398 (36). Moreover, PEx samples contained four of the five most common miRNAs detected in airway
399 epithelial cells-EVs by Koeppen and colleagues (36), which account for more than half of all miRNA
400 sequence reads. Identification of miRNAs that are directly produced and encapsulated by airway
401 epithelial cells provides further support for the concept that PEx samples can yield insights into intra-
402 vesicular communication within the small airways. Other abundant miRNAs detected in PEx samples
403 from both patients were miR-125b-5p and miR-125a-5p. miR-125 family is found to regulate a set of
404 genes that mediate cellular responses against pathogens through modulating, for example, IL6 and
405 type I IFN pathways (37). In addition, the miR-125 family has been shown to be implicated in lung
406 carcinogenesis; however, the reports seem to find both oncogenic and tumor-suppressive functions
407 of these miRNAs (37). To address the potential problem of saliva contamination of the PEx samples,
408 an issue that has been raised in the past for PExA, we also compared our results with the miRNA of
409 the salivary glands (4). miR-4508, miR-1246, and miR-4492 were the only three miRNAs identified in
410 PEx that, based on the miRNA tissue atlas, are expressed in the bronchus and/or lung but not in the
411 salivary glands, but they are not the most expressed. Thus, based on these results, we cannot
412 completely exclude the possibility that our identified miRNAs originate from saliva; however, our
413 proteomic data did not detect amylase, the most prevalent protein in saliva, which has previously
414 been cited as evidence to deny the presence of saliva in PEx samples (4).
415
416 In addition to the existing literature about the RNA degradation occurring in biological matrices (11,
417 31), we demonstrated that PEx membranes following sample collection, as well as BALF, display a
418 very high RNase activity. These findings confirm that the airway microenvironment, specifically
419 samples originating from the RTLF, is rich in RNases, thereby the miRNAs detected in the PEx
420 samples are derived from EVs, where they are protected from RNase activity; otherwise, they would
421 have been rapidly degraded.
422
423 The presence of a selection of miRNAs detected by miRNA sequencing was validated using custom-
424 designed miRCURY LNA™ SYBR Green PCR assay. Despite the low abundance of PEx material
425 approaching the assay's detection limit, the most highly abundant miRNA species based on UMI

426 reads—including miR-125b-5p and certain let-7 family members relevant to lung function were
427 detectable even in a single PEx spot. Furthermore, our dilution series experiments showed that
428 increasing the number of PEx sPOTs used for RNA extraction resulted in an increased miRNA UMI
429 count for both individuals tested (Figure 2). However, in contrast to the corresponding proteome
430 analysis, using more than one PEx sPOT for RNA extraction is recommended. While several mature
431 miRNAs were detectable in a single PEx spot, 2 or more PEx spots considerably increased the
432 number of different miRNA species detected, as well as the qualitative information of the sampling.
433 However, the dilution series experiments indicated that using the full panel of 10 spots produced in
434 a single sampling session may not be beneficial. Given these findings, we propose that designating
435 five PEx sPOTs for protein analysis and five for miRNA analysis represents the optimal method
436 moving forward. This method guarantees adequate data from an individual patient in a single PExA
437 sampling session, facilitating the integration of protein and miRNA insights. Moreover, protein
438 content may function as a prospective normalizing factor for miRNA data in the future, especially as
439 additional research is required to identify universally stable miRNAs appropriate for quantitative
440 normalization.
441
442 CONCLUSION
443 This proof-of-concept study demonstrates that miRNAs can be detected in PEx samples and that the
444 miRNAs identified can be relevant to use in studying and monitoring different lung diseases and
445 conditions. Furthermore, the enrichment of proteins from the EV- and exosome compartments
446 along with the high level of RNAse activity detected both in BALF and PExA membranes suggests that
447 the detected miRNAs are derived from intact vesicles, which are known to offer protection via
448 encapsulation. Taken together, we demonstrate the feasibility of the PExA method as a tool for the
449 collection of non-invasive liquid biopsies to study EV-derived miRNAs from the small airways.

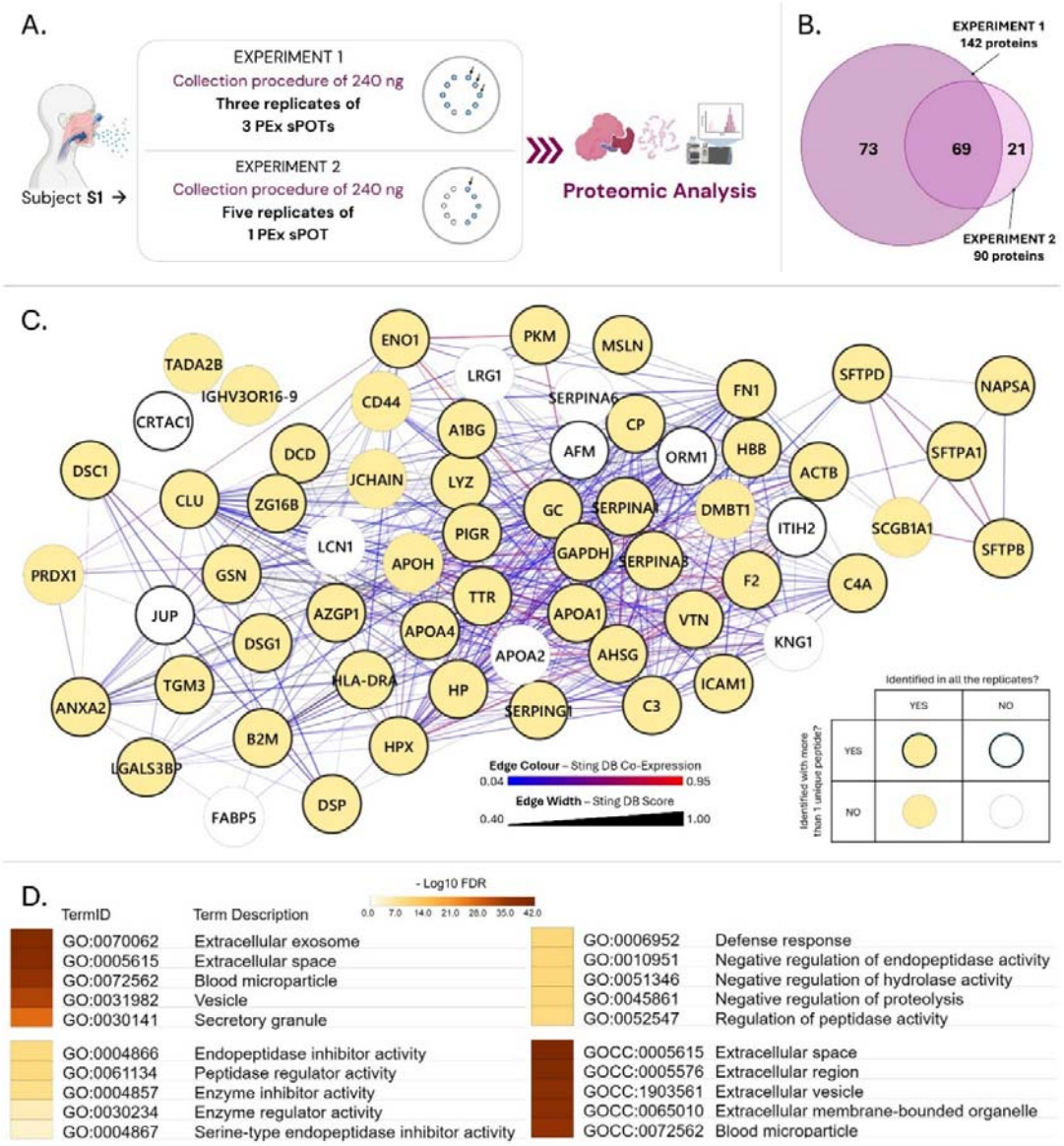



***Figure 1.*** *Proteins detected in PEx and their associated Gene Ontology (GO) categories.* **A.** Graphical outline of the experimental workflow. **B.** Pie chart with the number of proteins identified in both experiments. **C.** The protein-protein interaction (PPI) network of proteins identified in both experiments. The legend describes the features of the network's elements. Edges represent the StringDB score, describing 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 (Light grey: no interaction). The yellow nodes with black border represent proteins with the highest level of confidence for identification (> 1 unique peptide/protein). **D.** Pathway enrichment analysis based of proteins identified in both experiments. The table are reported the top five enriched terms for all three GO categories and subcellular

461 compartments. Color codes display the significance scores (FDR -Log10). All data were obtained from
462 stringDB (25).

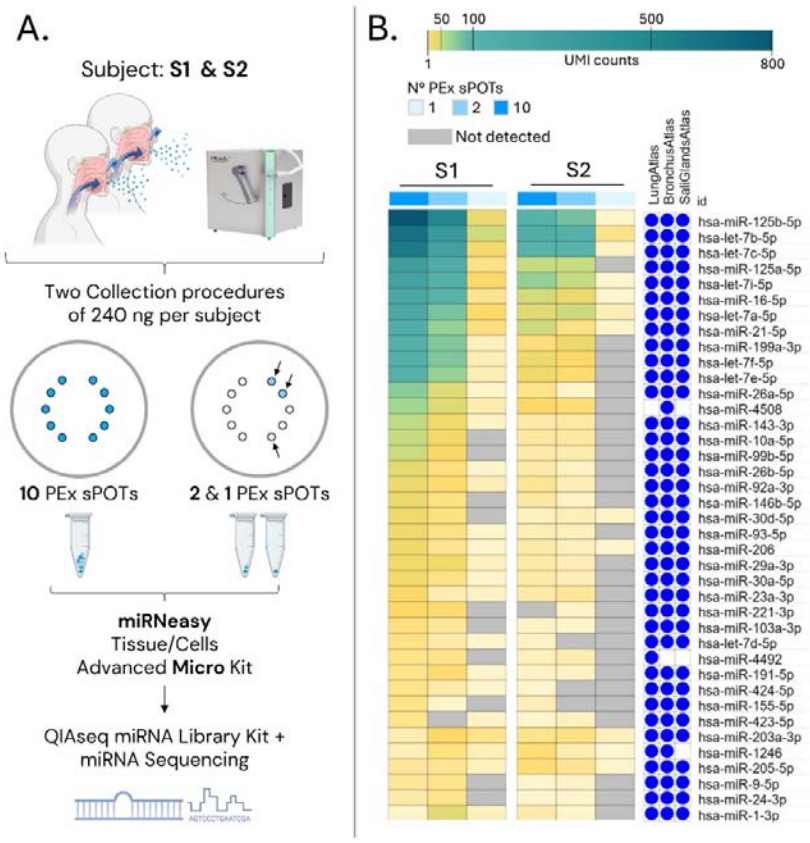
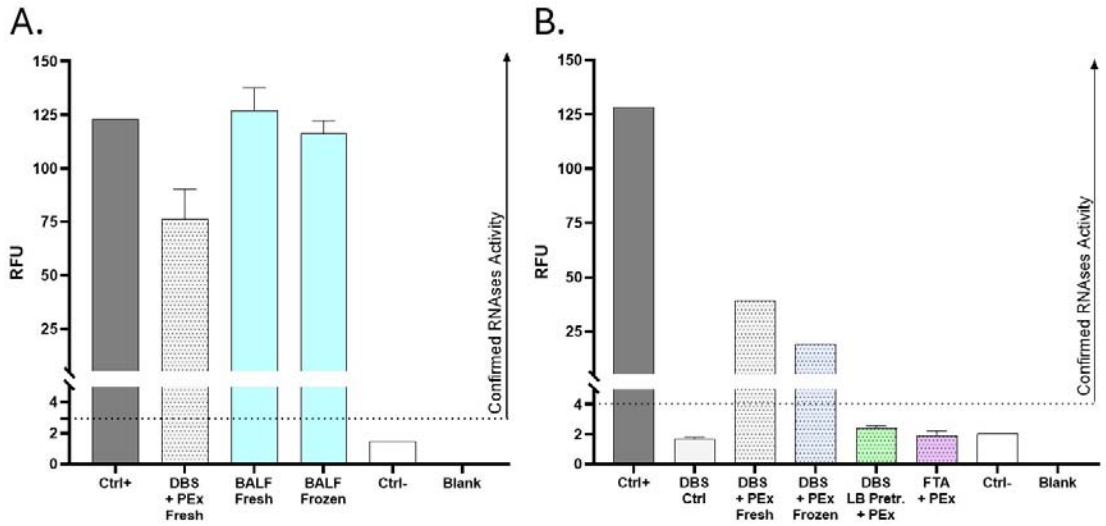



***Figure 2.*** *miRNA identified in PEx samples on DBS membrane using RNAseq.* **A.** Graphical outline of the experimental workflow. **B.** List of detected miRNAs. Data are presented as UMI counts color coded from dark green (UMI=800) to light yellow (UMI=1). Each of the two subjects are represented by 3 columns, related to 10, 2 and 1 PEx sPOTs of starting material respectively. The right columns display gene lists sourced from or miRNATissueAtlas2 (with expression mean > 10 as cut-off (27, 28)). A blue dot indicates the presence of the PExA identified miRNA in the corresponding list.




470

471 ***Figure 3.*** *Evaluation of RNAse activity presence in the airways.* **A.** Bar graph showing the level of
472 RNAse activity in different sample types collected from the airways, including PEx samples collected
473 on DBS membrane, fresh BALF, and frozen BALF. **B.** Bar graph showing the level of RNAse activity in
474 PEx samples using different condition and membrane matrices (Ctrl +: positive control provided by
475 the kit, with a known concentration of RNAse; DBS Ctrl: DBS membrane without sample; LB Pretr. =
476 DBS membrane pretreated with lysis buffer from the RNA extraction kit; Ctrl -: negative control,
477 reaction containing RNase-free water). The dotted line represents fluorescence levels exceedingly
478 twofold the corresponding negative control, which is defined as the threshold for determining
479 whether samples are affected by RNase activity. Data are reported as mean with range when more
480 than one replicate. The dotted texture highlights samples with PEx material.

481

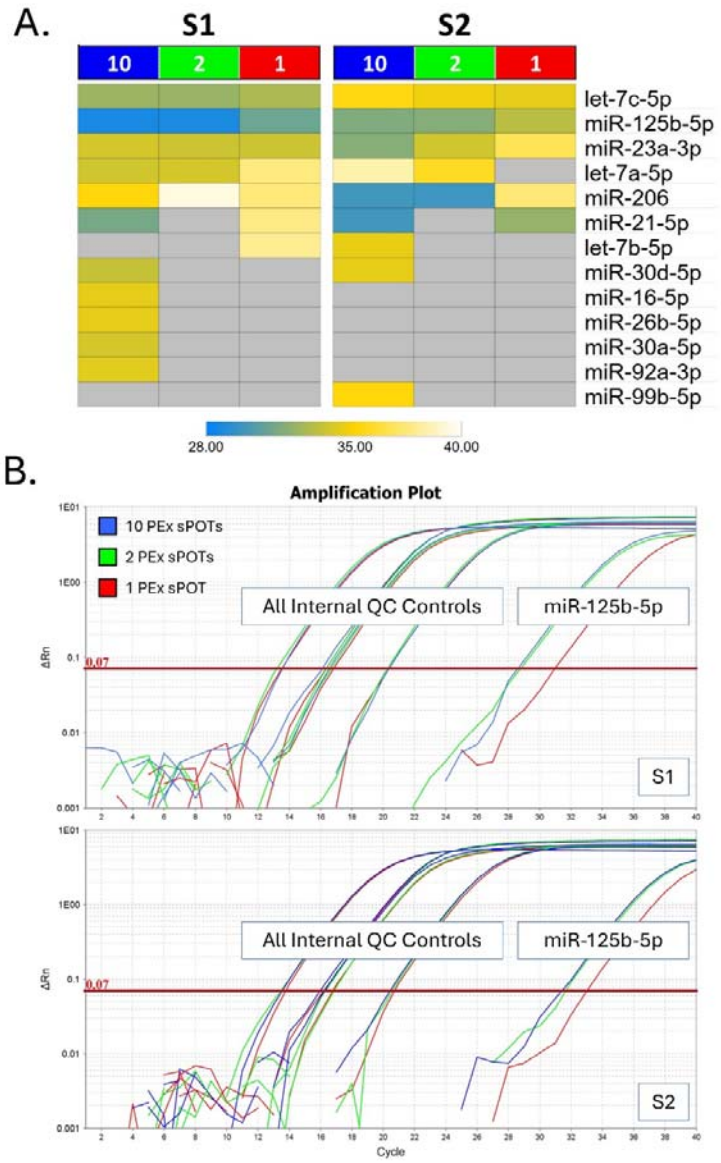
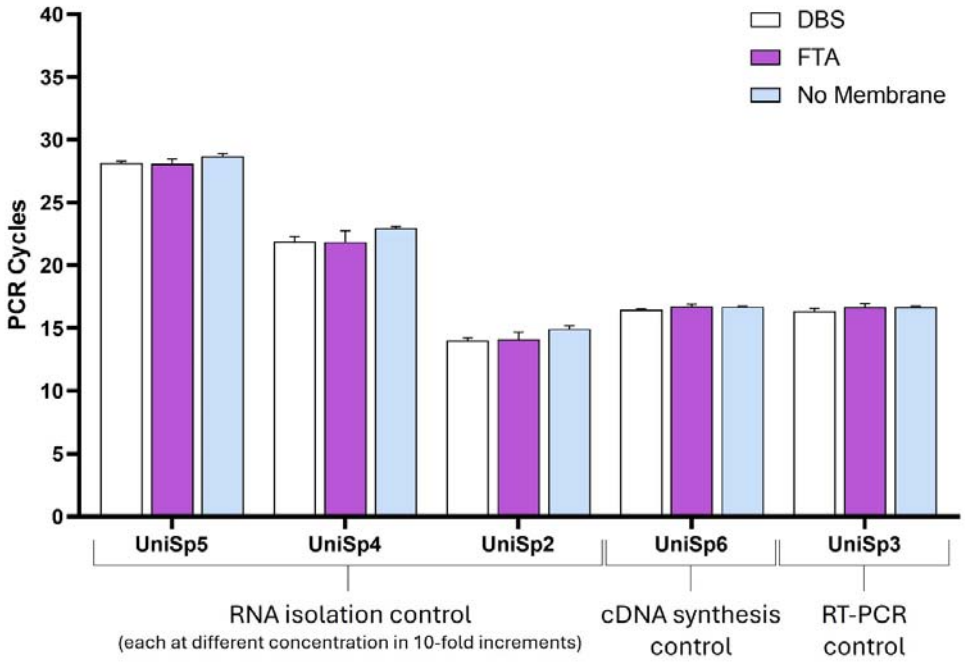



***Figure 4.*** *miRCURY LNA™ SYBR Green PCR assay in PEx samples.* Results of PCR array performed on the same set of samples utilized in the RNAseq analysis. **A.** Visualization of miRNA presence across diverse samples. Grey cells represent undetected miRNAs in the respective sample. **B.** Amplification plot of the internal controls of the array and the most abundant miRNA in S1 and S2 (miR125b-5p).




487

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

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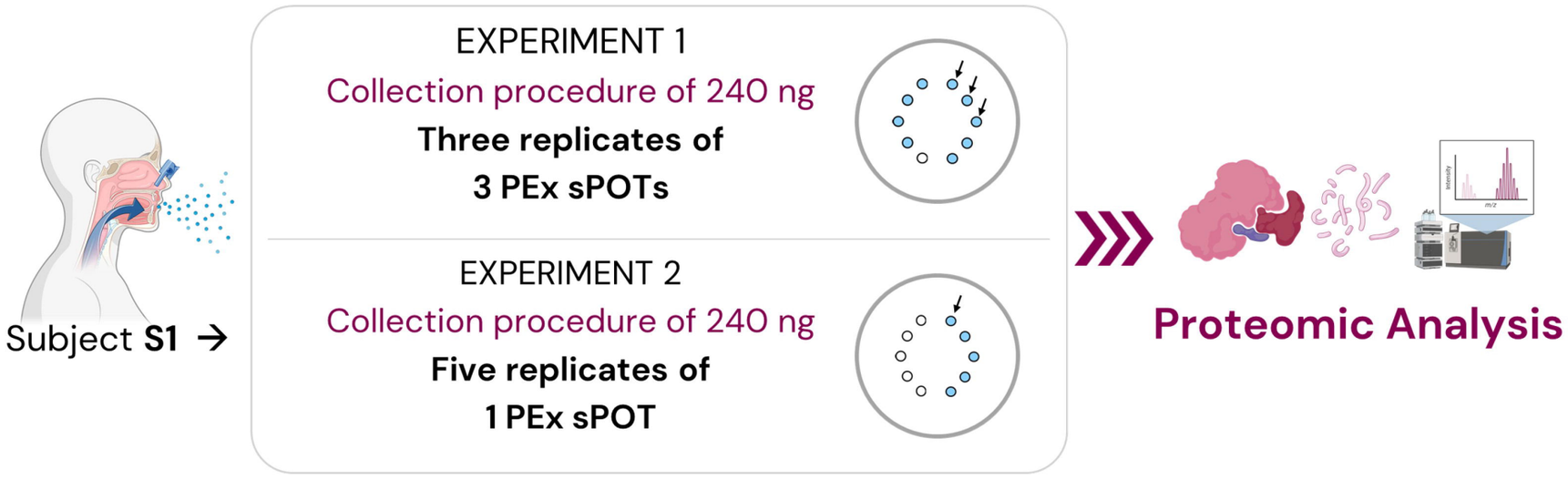
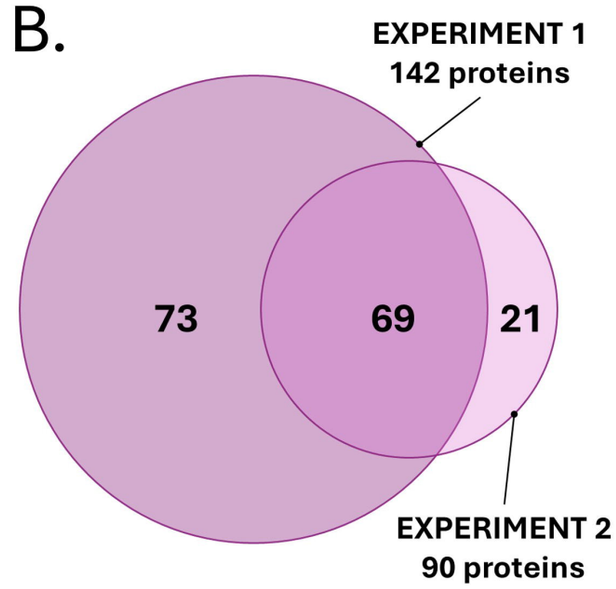
598

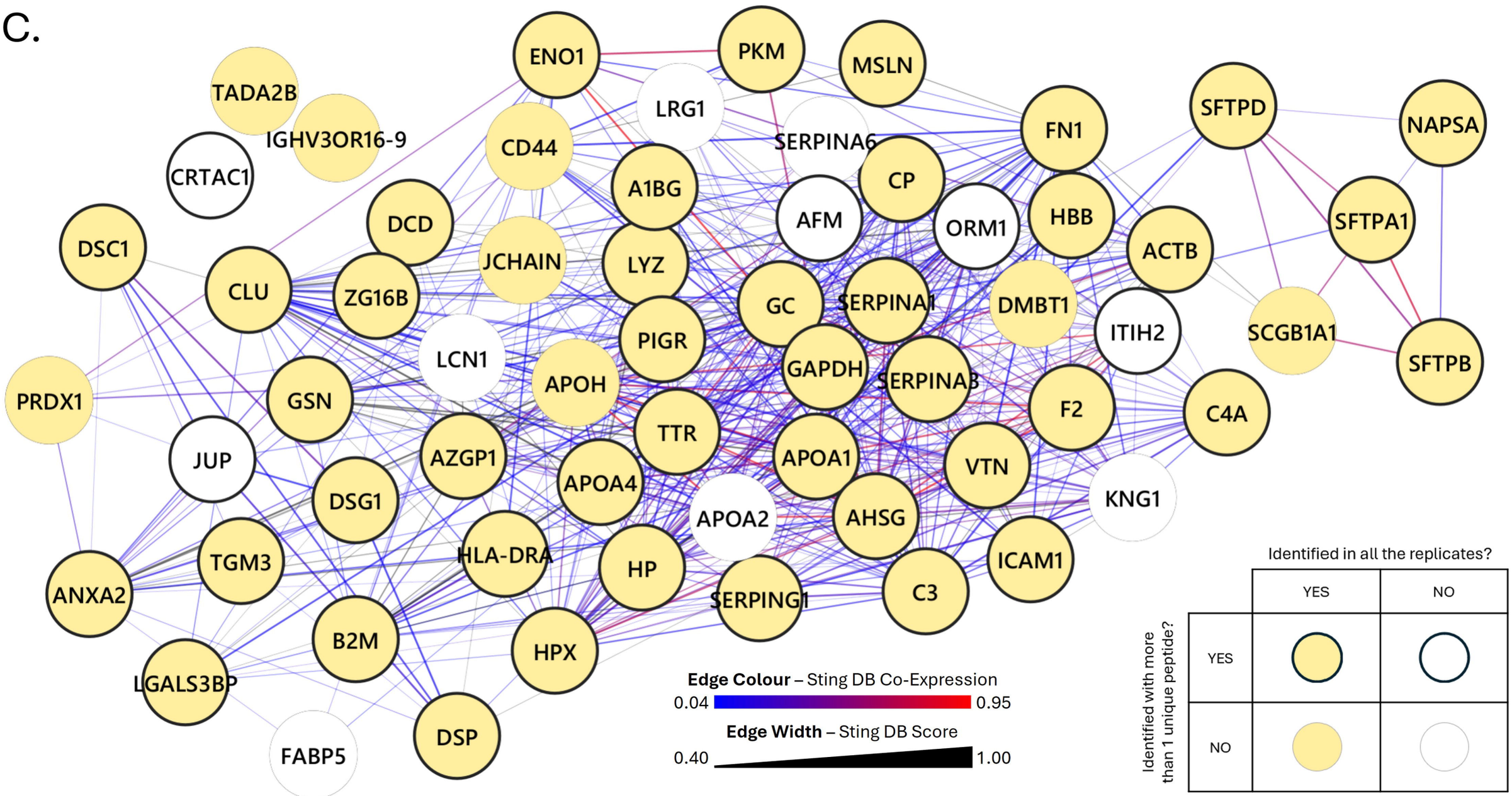
A.


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.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IGHV3OR16-9
CRTAC1
DSC1
CLU
ZG16B
DCD
CD44
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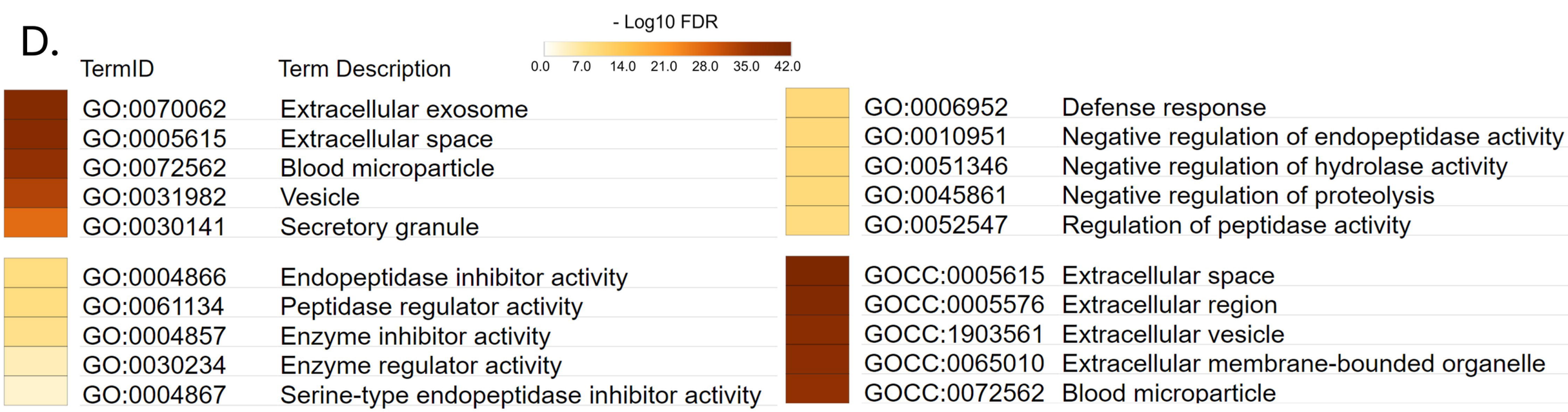
LRG1
ENO1
PKM
MSLN
SERPINA6
FN1
SFTPD
NAPSA
CP
AFM
ORM1
HBB
ACTB
SFTPA1
GC
SERPINA1
DMBT1
ITIH2
SCGB1A1
SFTPB
PIGR
GAPDH
SERPINA3
F2
C4A
PRDX1
GSN
LCN1
APOH
TTR
APOA1
VTN
JUP
AZGP1
APOA4
APOA2
AHSG
KNG1
DSG1
HLA-DRA
HP
ICAM1
TGM3
ANXA2
SERPING1
C3
B2M
HPX
LGALS3BP
FABP5
DSP
Edge Colour – Sting DB Co-Expression
0.04
0.95
Edge Width – Sting DB Score
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
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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
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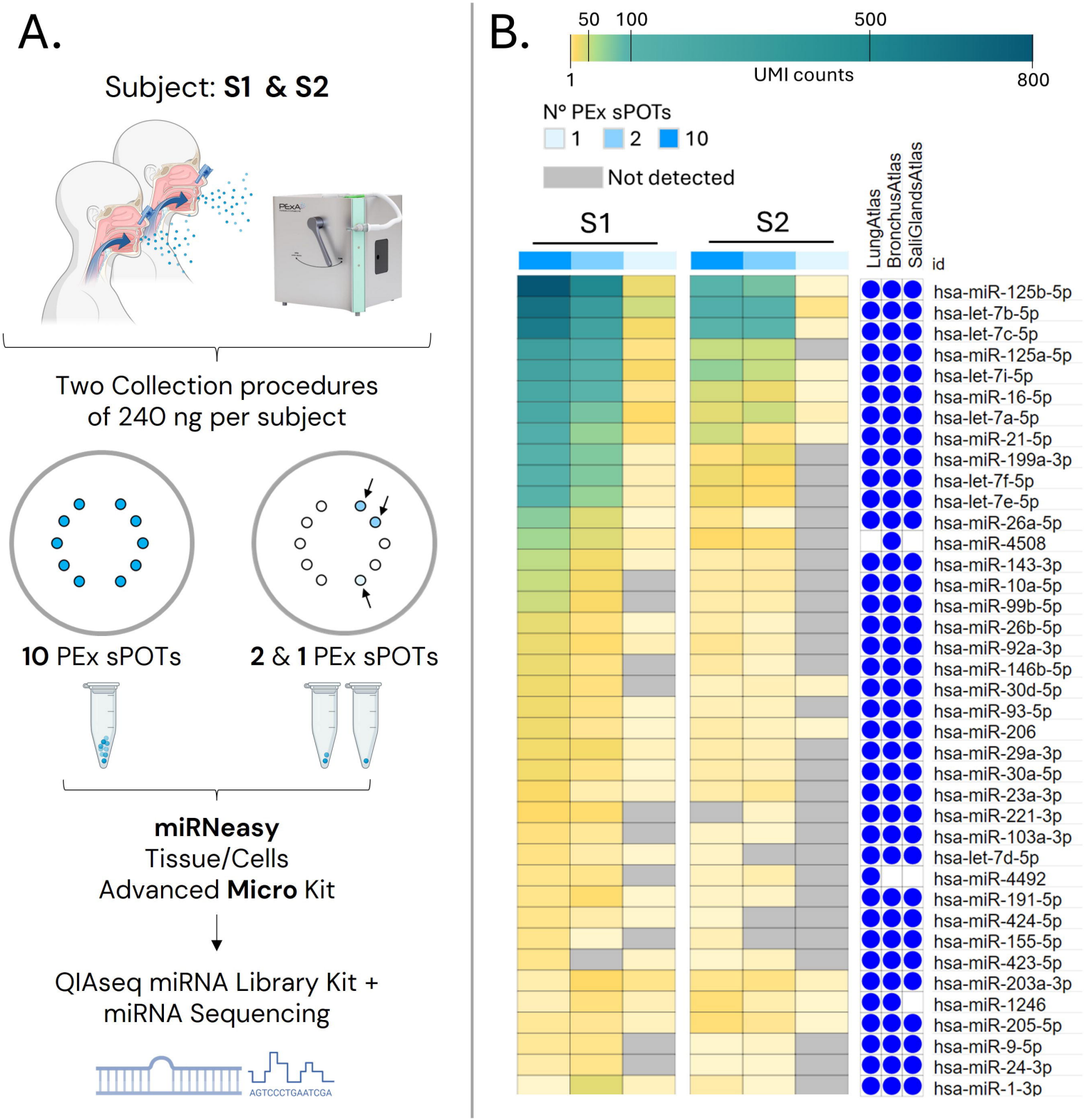
GOCC:0072562 Blood microparticle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Subject: S1 & S2
Two Collection procedures
of 240 ng per subject
10 PEx sPOTs
2 & 1 PEx sPOTs
miRNeasy
Tissue/Cells
Advanced Micro Kit
QIAseq miRNA Library Kit +
miRNA Sequencing
AGTCCCTGAATCGA
B.
50
100
500
1
UMI counts
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
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hsa-miR-146b-5p
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hsa-miR-93-5p
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hsa-miR-29a-3p
hsa-miR-30a-5p
hsa-miR-23a-3p
hsa-miR-221-3p
hsa-miR-103a-3p
hsa-let-7d-5p
hsa-miR-4492
hsa-miR-191-5p
hsa-miR-424-5p
hsa-miR-155-5p
hsa-miR-423-5p
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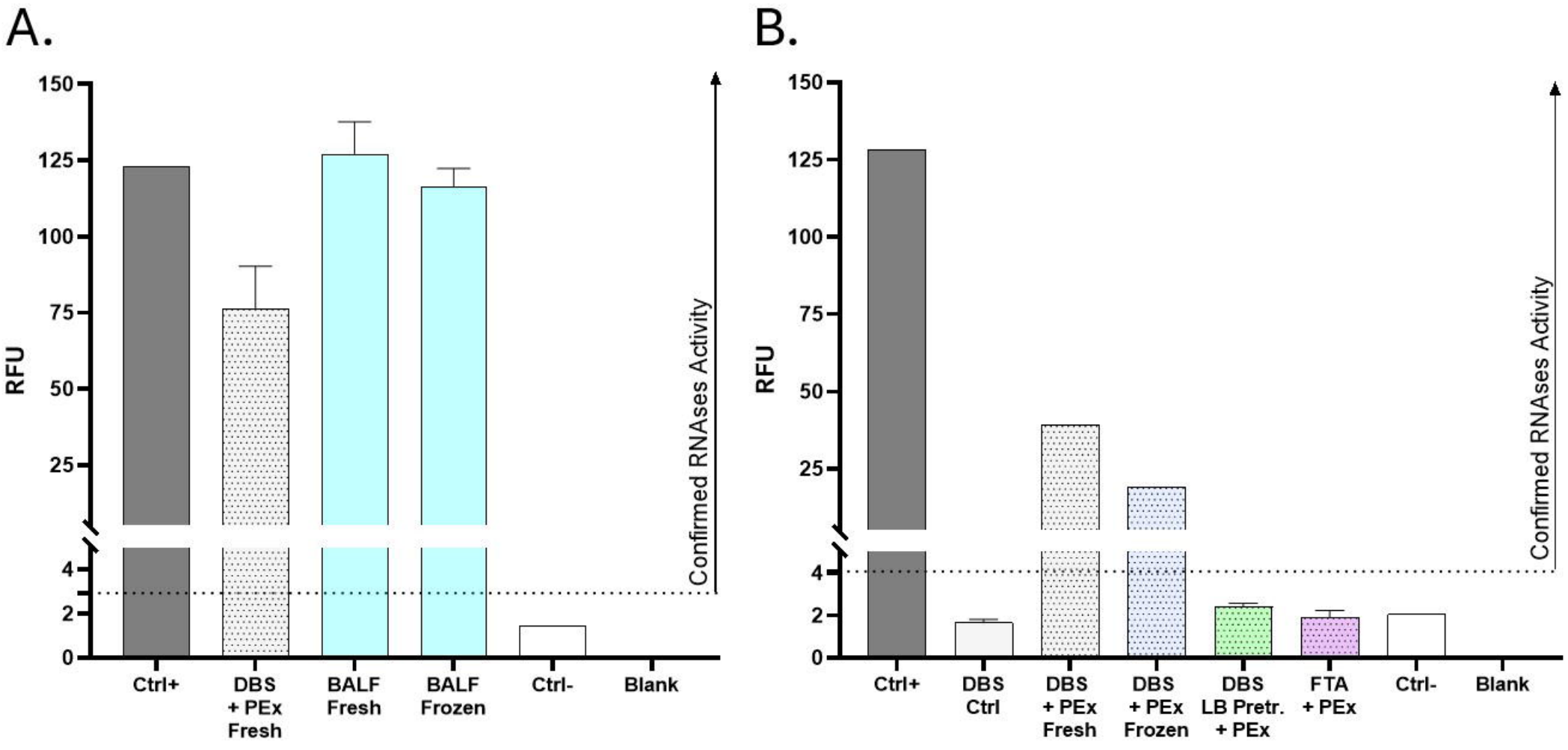
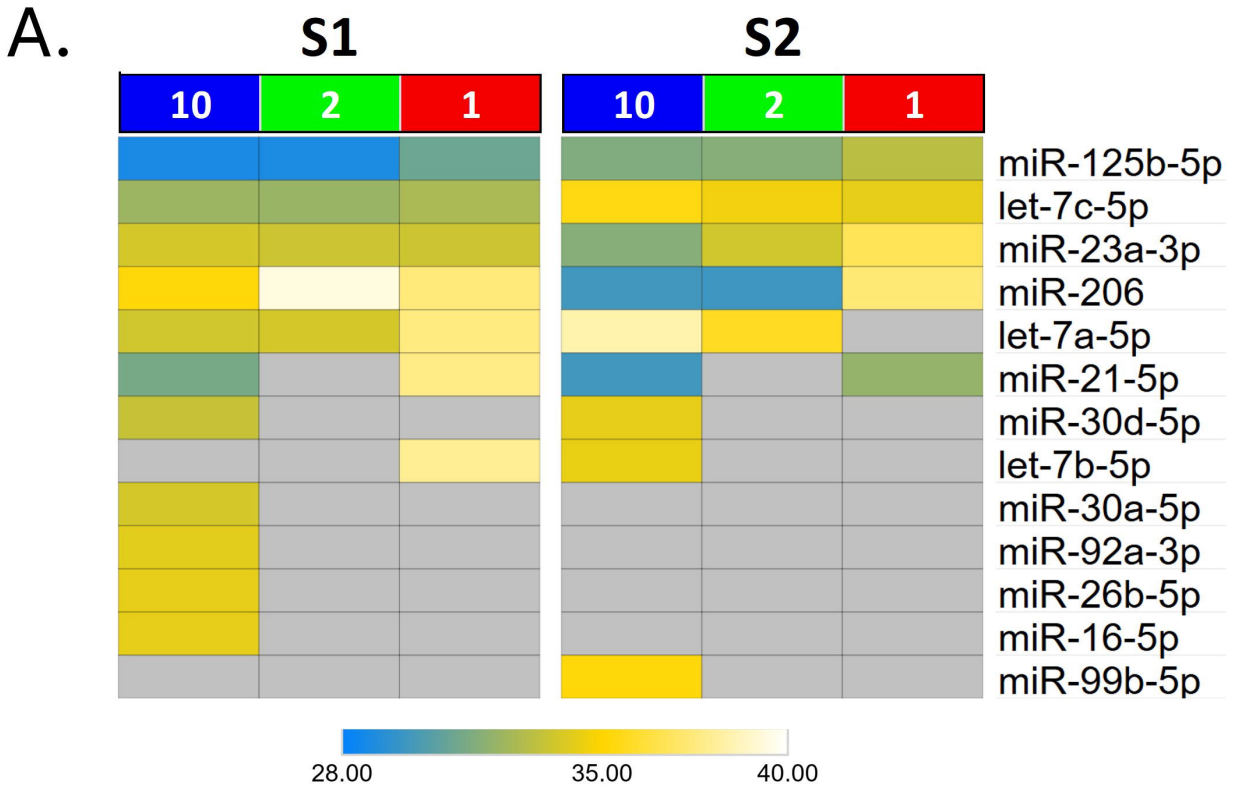

A.
RFU
150
125
100
75
50
25
4
2
0
Confirmed RNAses Activity
Ctrl+
DBS + PEx Fresh
BALF Fresh
BALF Frozen
Ctrl-
Blank
B.
RFU
150
125
100
75
50
25
4
2
0
Confirmed RNAses Activity
Ctrl+
DBS Ctrl
DBS + PEx Fresh
DBS + PEx Frozen
DBS LB Pretr. + PEx
FTA + PEx
Ctrl-
Blank


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
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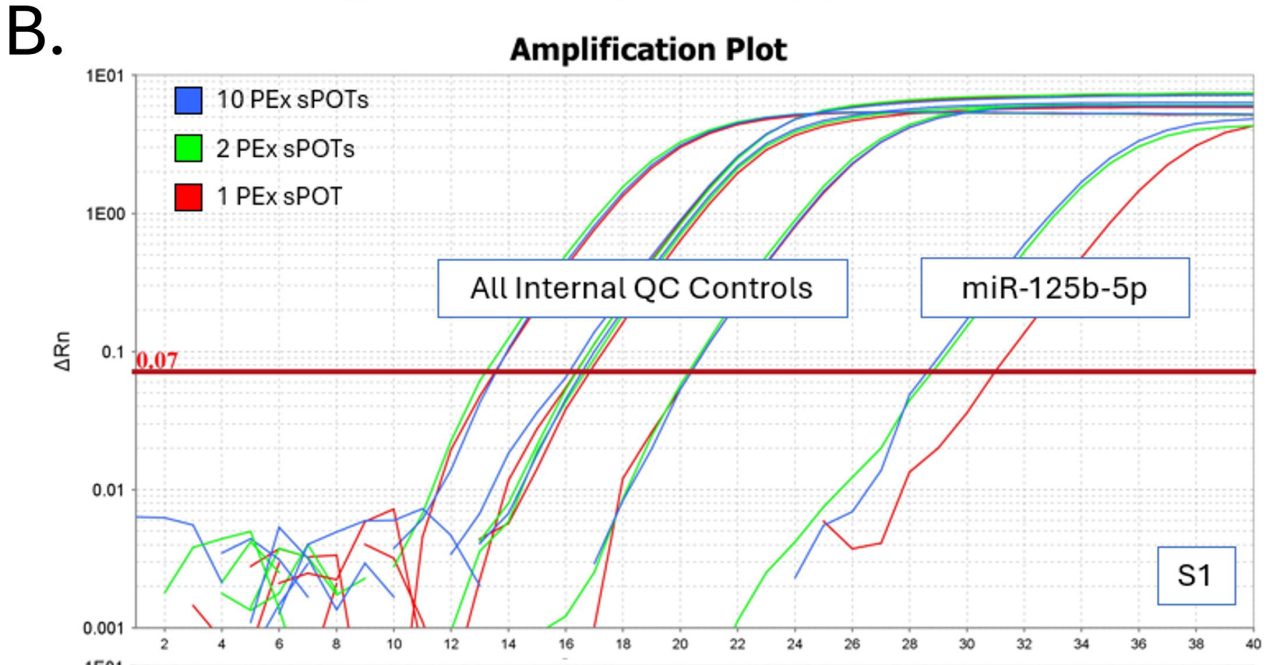
28.00
35.00
40.00


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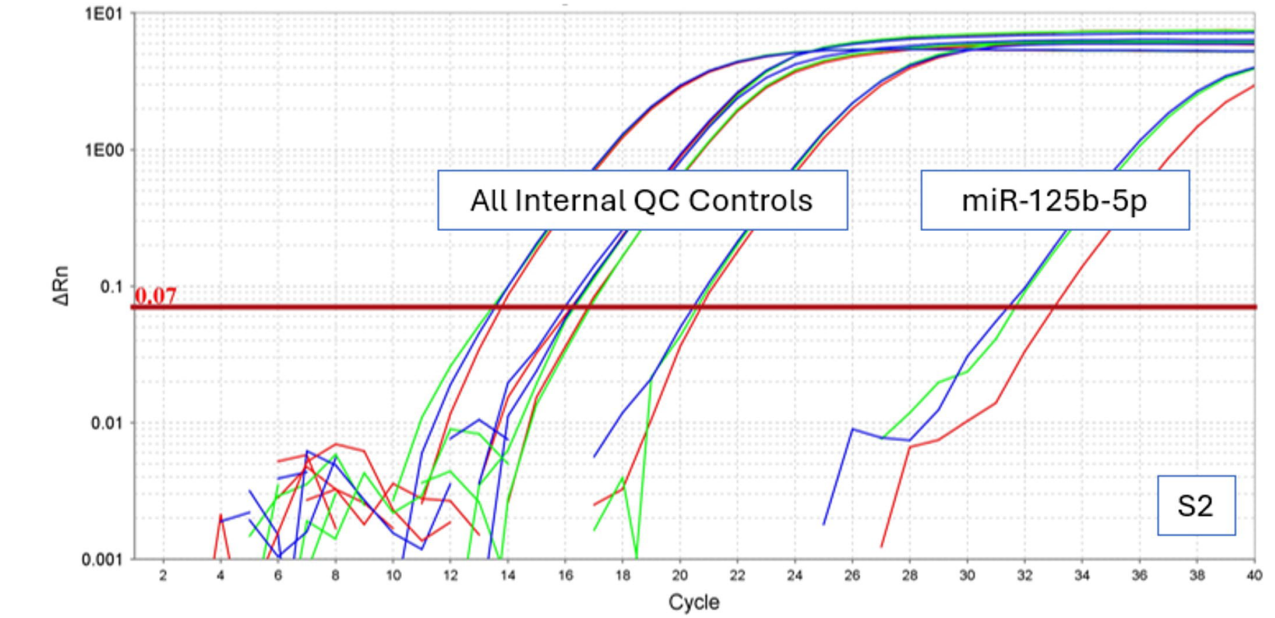
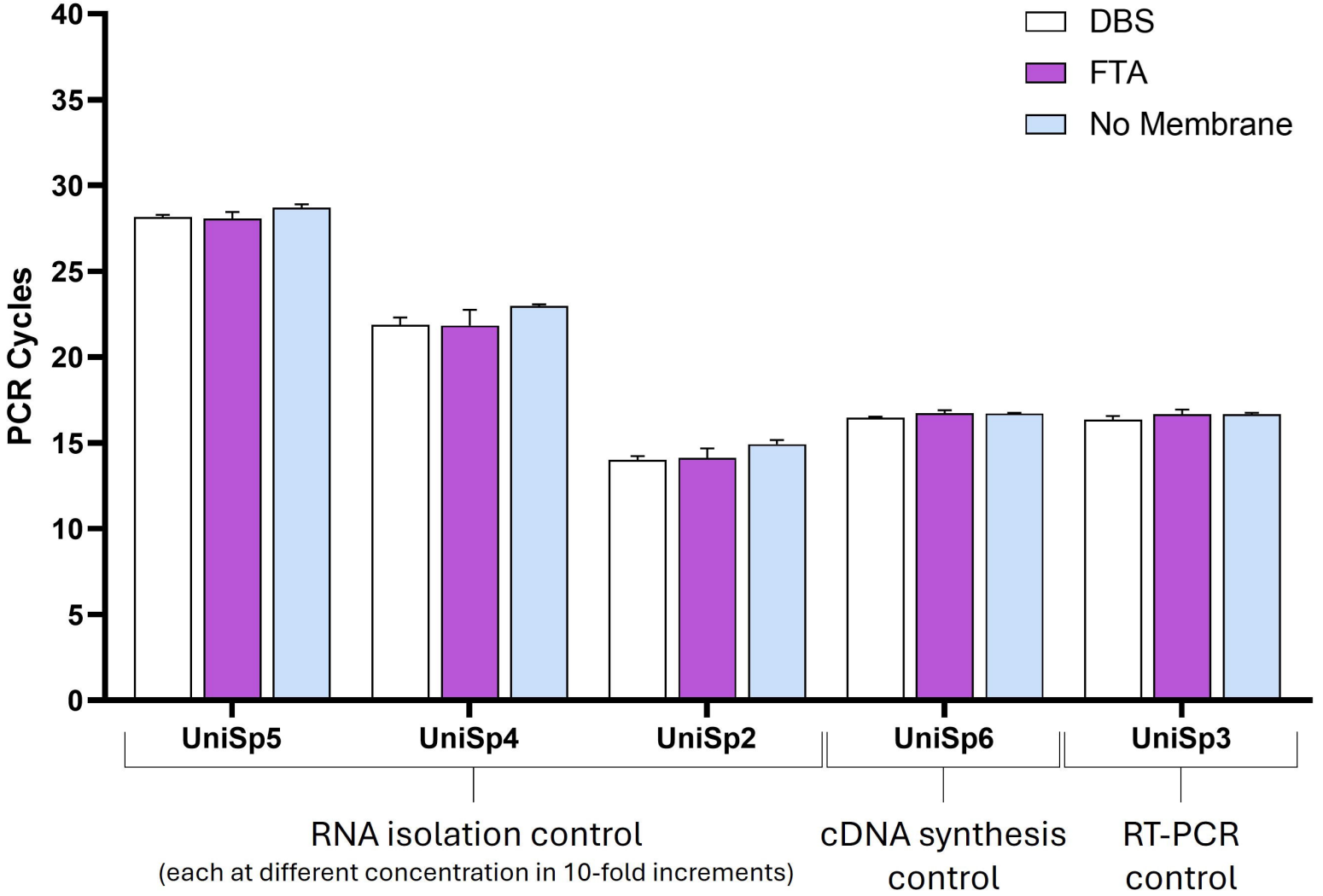
2 PEx sPOTs
1 PEx sPOT
All Internal QC Controls
miR-125b-5p
0.07
ΔRn
S1


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