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QUALITY-CONTROLLED SPUTUM ANALYSIS BY FLOW CYTOMETRY
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1 **TITLE:**
2 Quality-Controlled Sputum Analysis By Flow Cytometry
3
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26
27 **SUMMARY:**
28 This protocol describes an efficient method for dissociating sputum into a single cell suspension
29 and the subsequent characterization of cellular subsets on standard flow cytometric platforms.
30
31 **ABSTRACT:**
32 Sputum, widely used to study the cellular content and other microenvironmental features to
33 understand the health of the lung, is traditionally analyzed using cytology-based methodologies.
34 Its utility is limited because reading the slides is time-consuming and requires highly specialized
35 personnel. Moreover, extensive debris and the presence of too many squamous epithelial cells
36 (SECs), or cheek cells, often renders a sample inadequate for diagnosis. In contrast, flow
37 cytometry allows for high-throughput phenotyping of cellular populations while simultaneously
38 excluding debris and SECs.
39
40 The protocol presented here describes an efficient method to dissociate sputum into a single cell
41 suspension, antibody stain and fix cellular populations, and acquire samples on a flow cytometric
42 platform. A gating strategy that describes the exclusion of debris, dead cells (including SECs) and
43 cell doublets is presented here. Further, this work also explains how to analyze viable, single
44 sputum cells based on a cluster of differentiation (CD)45 positive and negative populations to

45   characterize hematopoietic and epithelial lineage subsets. A quality control measure is also
46   provided by identifying lung-specific macrophages as evidence that a sample is derived from the
47   lung and is not saliva. Finally, it has been demonstrated that this method can be applied to
48   different cytometric platforms by providing sputum profiles from the same patient analyzed on
49   three flow cytometers; Navios EX, LSR II, and Lyric. Furthermore, this protocol can be modified
50   to include additional cellular markers of interest. A method to analyze an entire sputum sample
51   on a flow cytometric platform is presented here that makes sputum amenable for developing
52   high-throughput diagnostics of lung disease.
53
54   **INTRODUCTION:**
55   Technical advancements in the hardware and software of flow cytometers have made it possible
56   to identify many distinct cell populations simultaneously[1–4]. The utilization of the flow cytometer
57   in hematopoietic cell research, for example, has led to a much better understanding of the
58   immune system[2] and the cellular hierarchy of the hematopoietic system[5], as well as the
59   diagnostic distinction of a multitude of different blood cancers[6–8]. Although most sputum cells
60   are of hematopoietic origin[9–11], flow cytometry has not been widely applied to sputum analysis
61   for diagnostic purposes. However, several studies suggest that the evaluation of immune cell
62   populations in sputum (the most significant subset of cells) may be of great help in diagnosing
63   and/or monitoring diseases such as asthma and chronic obstructive pulmonary disease (COPD)[12–
64   15]. Moreover, the existence of epithelial-specific markers that can be used in flow cytometry
65   allows the interrogation of the following most significant subset of cells in sputum, lung epithelial
66   cells.
67
68   In addition to the ability to analyze many distinct cell populations of different tissue origins, a
69   flow cytometer can evaluate large numbers of cells in a relatively short period. In comparison,
70   slide-based, cytological types of analyses often require highly specialized personnel and/or
71   equipment. These analyses can be labor-intensive, which leads to only a proportion of the
72   sputum sample being analyzed[16].
73
74   Three critical issues limit the widespread use of sputum in flow cytometry. The first issue relates
75   to the collection of sputum. Sputum is collected through a huff cough that expels mucus from
76   the lungs into the oral cavity, subsequently spitting into a collection cup. Since the mucus travels
77   through the oral cavity, there is a high chance of SEC contamination. This contamination
78   complicates the specimen analysis, but the problem is easily rectified on a flow cytometric
79   platform, as shown in this study.
80
81   Not everyone can produce sputum spontaneously; therefore, several devices have been
82   developed to assist with the sputum collection in a non-invasive manner[17]. The nebulizer is one
83   such device and has been shown to produce reliable sputum samples[18–20]. Although the nebulizer
84   is a very effective way of non-invasively collecting sputum, its use still requires a setting at a
85   medical facility with specialized personnel[21]. In contrast, handheld devices such as the lung
86   flute[22–24] and the acapella[16,25] can be used at home since they are very user-friendly. These assist
87   devices are both safe and cost-effective.
88

89   For us, the acapella gave consistently better results than the lung flute[16], and therefore, the
90   acapella device has been chosen for sputum collections. A 3-day collection sample was decided
91   because the primary purpose for using sputum is to develop a lung cancer detection test[16]. It has
92   been shown that a 3-day sample increases the likelihood of lung cancer detection compared to a
93   1- or 2-day sample[26–28]. However, other methods of sputum collection may be preferable for
94   different purposes. If a different sputum collection method is used than the one described here,
95   it is recommended to carefully titrate each antibody or dye used for flow cytometric analysis;
96   very little data is available on how different sputum collection methods affect the targeted
97   proteins for flow cytometry.
98

99   The second issue dampening the enthusiasm for using sputum for diagnostics, primarily related
100   to flow cytometry, is cell number. The problem is the collection of sufficient viable cells for a
101   reliable analysis. Two studies demonstrated that sputum samples collected by non-invasive
102   methods, with the help of an assist device, contain enough viable cells that can be utilized in
103   clinical diagnosis or research studies[16,24]. However, neither of these studies addressed the issue
104   of cell numbers concerning flow cytometry.
105

106   For the studies that form the basis for this protocol, sputum samples were collected from
107   participants at high risk for developing lung cancer following approved institutional guidelines for
108   each study site. High-risk participants were defined as between 55–75 years, having smoked 30
109   pack-years and having not quit smoking within the past 15 years. Patients were shown how to
110   use the acapella device according to the manufacturer's instructions[29] and collected sputum for
111   three consecutive days at home. The sample was kept in the refrigerator until the last collection.
112   On the final collection day, the sample was shipped to the laboratory overnight with a frozen cold
113   pack. The samples were processed into a single cell suspension on the day they were received.
114   With this method of sputum collection, more than enough viable cells are obtained for a reliable
115   flow cytometric analysis.
116

117   Lastly, and related to the previous cell number issue, is the question of how to release the sputum
118   cells from its mucinous environment. How can the cells be kept viable and create a single cell
119   suspension that does not clog the flow cytometer? Based on initial work by Pizzichini et al.[30] and
120   Miller et al.[31], this protocol describes an easy and reliable method for sputum processing into a
121   single cell suspension that is suitable for flow cytometric analysis. This method has used well-
122   established guidelines in flow cytometry[32–34] to develop an efficient antibody labeling strategy to
123   identify hematopoietic and epithelial cells in sputum and provide instrument settings, quality
124   control measures, and analysis guidelines standardizing sputum analysis on a flow cytometric
125   platform.
126

127   **PROTOCOL:**
128   Sputum samples were collected from participants between 55–75 years, having smoked 30 pack-
129   years and having not quit smoking within the past 15 years. These participants were at high risk
130   of developing lung cancer. The samples were collected following approved institutional
131   guidelines for each study site. All steps of the sputum processing are performed in a biological
132   safety cabinet with appropriate personal protective equipment.

1.   Reagent preparation before starting sputum dissociation

1.1   Thaw 1% Paraformaldehyde (PFA), 25 mL per sample on ice, and keep cold until use.

CAUTION: PFA is toxic by inhalation and skin contact. Prepare a fixative according to the manufacturer's instructions and freeze at -20 °C in 25 mL aliquots until use.

1.2   Approximate the weight of the sample and thaw enough 0.1% Dithiothreitol (DTT) for step 2.2 and bring it to 37 °C. (Aliquots of DDT should be stored at -20 °C before use.)

1.3   Bring enough 0.5% N-acetyl- L-cysteine (NAC) up to 37 °C for step 2.2. (NAC should be made fresh weekly and stored at 4 °C before use.)

2.   Sputum dissociation

2.1   Weigh the sputum sample to determine the volumes of dissociation reagents.

NOTE: A sample is considered to be small if the initial weight is ≤3 g, medium if >3 g but ≤8 g, large if >8 but ≤16 g, and extra-large if a sample weighs >16 g. The indications small, medium, large, and extra-large will be used throughout the protocol. The quantity of reagents required for dissociation and labeling differ according to the size of the sputum sample.

2.2   Transfer a small sample to a clean 50 mL conical tube, a medium sample to a clean 250 mL plastic disposable bottle, or a large and extra-large sample to a clean 500 mL plastic disposable bottle. Add 1 mL/g sample weight of 0.5% NAC and 4 mL/g sample weight of 0.1% DTT.

2.3   Vortex at maximum speed (for 15 s), and then rock at room temperature (at maximum speed) for 15 min.

2.4   Dilute the sample with four volumes of Hank's Balanced Salt Solution (HBSS) (based on the total volume of sample + reagents) to neutralize the NAC and DDT; vortex quickly at maximum speed and rock at room temperature for 5 min at maximum speed.

2.5   Filter the cell suspension through 100 µm nylon mesh cell strainer(s) into one or more 50 mL conical centrifuge tube(s) to create a single-cell suspension.

2.6   Centrifuge the cells at 800 x g for 10 min at 4 °C. Aspirate the supernatant, combine all pellets in one 15 mL conical tube, and then wash the pellets with HBSS using the same conditions.

2.7   Resuspend the cell pellet in a volume of buffer determined by the initial weight of the sputum sample.

176　NOTE: A small sample is resuspended in 250 µL of HBSS. A medium sample is resuspended in 760
177　µL of HBSS. Large and extra-large samples are resuspended in 1460 µL of HBSS.

178

179　2.8　　Take an aliquot of the cell suspension for a live/dead cell count using Trypan Blue.

180

181　NOTE: For a small sample, use 5 µL. For a medium, large or extra-large sample, use 10 µL. Dilute
182　1:10 with HBSS.

183

184　2.8.1　Mix 10 µL of the sputum dilution with 30 µL of 0.4% Trypan Blue to achieve a final sample
185　dilution of 1:40. Load into the counting chambers of a hemocytometer for a cell count.

186

187　NOTE: It may be necessary to adjust the final dilution if the cell numbers are too low or too high
188　to achieve an accurate count. Consulting Guiot et al.[20] for correctly distinguishing sputum cells
189　from SECs and debris is strongly recommended here. This is essential for an accurate cell count.

190

191　2.9　　From the extra-large sample, remove 50 x 10^6 cells from the total and add to a new tube
192　with enough added HBSS to create a total volume of 1700 µL.

193

194　NOTE: Consider this a large sample for the remainder of the protocol. Left-oversamples can be
195　discarded or used for other purposes.

196

197　**3.　　Antibody and viability dye staining**

198

199　3.1　　Choice of antibody and staining dye

200

201　NOTE: **Table 1** shows the antibodies and viability dye that are used in this protocol and the cell
202　populations they identify.

203

204　3.1.1　Label the tubes containing the sputum cells (see **Table 2** for labels).

205

206　3.1.2　Use 5 mL flow cytometry tubes (compatible with the flow cytometer used) for the sample
207　tube with the unstained cells and the tube with the isotype control. Use 15 mL conical tubes for
208　the blood and epithelial tube samples.

209

210　NOTE: These samples will be transferred to flow cytometry tubes following antibody staining and
211　fixation.

212

213　3.2　　Label the compensation tubes (**Table 3**).

214

215　NOTE: Use 5 mL flow cytometry tubes compatible with the flow cytometer being used.

216

217　3.3　　Add the amount of HBSS, antibody and/or dye to each of the sputum cell tubes and
218　compensation tubes as indicated in **Table 2** and **Table 3**, respectively.

219

NOTE: Add buffer (HBBS), antibodies, and dye to all the tubes before adding cells or compensation beads to ensure that all tubes' staining time is consistent.

3.4   Add the amounts of sputum cell volume listed in **Table 2** to the assay tubes.

3.5   Add compensation beads to the compensation tubes as listed in **Table 3**.

3.6   Incubate all the tubes (assay and compensation tubes) on ice, protected from light, for 35 min. Then, fill the tubes with ice-cold HBSS and centrifuge at 4 °C for 10 min at 800 x g.

3.7   For the compensation tubes, aspirate the supernatant as close to the pellets as possible and flick the pellets to loosen.

3.8   Add 0.5 mL of cold HBSS to the compensation tubes, store them on the ice at 4 °C and protect them from light until needed for flow cytometry analysis.

3.9   Aspirate the supernatant from the unstained isotype, blood, and epithelial tubes after centrifugation (step 3.6 from the antibody staining section) and loosen the pellets by flicking the tubes.

4.   Fixation with 1% Paraformaldehyde (PFA)

4.1   Add cold 1% PFA (which should be thawed by now) to the unstained, isotype, blood, and epithelial tubes; 2 mL to the unstained and isotype tubes, and 10 mL to the blood and epithelial tubes.

4.2   Incubate the tubes on ice, protected from light for 1 h. Vortex quickly at maximum speed after 30 min.

4.3   Fill the tubes with ice-cold HBSS. Then, centrifuge tubes at 4 °C for 10 min at 1600 x g.

4.4   Aspirate as much supernatant as possible without disturbing the cell pellet and flick the tube with the fingers to loosen the cells.

4.5   Add 200 μL of cold HBSS to the unstained and isotype tubes.

4.6   Calculate the volume of HBSS for resuspension of the blood and epithelial tube according to the total cell count.

NOTE: Resuspension volume = 0.15 x [total cell count (step 8 of sputum dissociation) / 10^6]. Use 50 x 10^6 as the cell count for an extra-large sample.

4.7   Store all sample and compensation tubes, protected from light on the ice at 4 °C until flow cytometry analysis is performed.

264

265 NOTE: This protocol has not been tested for storage for more than 24 h.

266

267 **5.   Data acquisition on the flow cytometer**

268

269 5.1   Apply appropriate startup procedures for the flow cytometer being used.

270

271 NOTE: This section of the protocol assumes that the person operating the flow cytometer is
272 trained in the use of the instrument available to them, especially concerning daily procedures
273 including checking the stability of the optics and fluidics systems, techniques for standardizing
274 light scatter and fluorescence intensity, as well as calculating and applying the correct
275 compensation matrix.

276

277 5.2   Use the mixture of National Institute of Standards and Technology (NIST) beads to ensure
278 that forward scatter and side scatter voltages are set to place the NIST beads to span the entire
279 plot without placing the beads too close to the axes.

280

281 NOTE: This step is crucial to ensure that debris smaller than 5 µm can be eliminated by gating
282 post-acquisition analysis. Depending on the flow cytometer used, be mindful that the smallest
283 beads are not being excluded with the threshold (when using the LSR II or the Lyric) or with the
284 high discriminator (using the Navios EX flow cytometer). For the Navios EX, a gain of 2 was used
285 for the forward and side scatter, a voltage of 236 for forward scatter, and 250 for side scatter.
286 For the LSR II, a forward scatter voltage of 165 and a side scatter voltage of 190 was used.

287

288 5.3   Set the flow rate to medium (LSR II) or high (Navios EX).

289

290 NOTE: The medium or high flow rate for most instruments can be used to acquire the sputum
291 tubes. It is important to note that using too slow of a flow rate or too dilute of the sample may
292 result in the cells settling, resulting in increased vortexing which is not desired. Therefore,
293 adhering to the calculated resuspension volume in step 4.6 should result in cellular density that
294 can be acquired quickly but not clog the machine.

295

296 5.4   Adjust the voltages for each parameter used for the scatter and fluorescent parameters
297 to place the cell populations on the scale. Use the figures with the gating strategy as guidance on
298 how to adjust the voltages accordingly.

299

300 NOTE: Ensure that all the parameters needed are selected in the parameter selection window
301 before the acquisition or that data will not be acquired.

302

303 5.5   Acquire data for the unstained sputum sample first, followed by the isotype-stained
304 sample, and then the blood tube and the epithelial tube.

305

306 NOTE: If the cell suspension is too concentrated to allow the flow cytometer to run the samples
307 without clogging, the samples may be further diluted with HBSS.

308
309 **REPRESENTATIVE RESULTS:**
310 This protocol was developed with a clinical laboratory setting in mind. The focus during the
311 development of the protocol was on simplicity, efficiency, and reproducibility. It was found that
312 the most time-consuming step in the processing of sputum was counting the cells. Therefore, the
313 protocol is set up in such a way that sputum processing and cell labeling can be performed
314 independently from cell counting without loss of time. An accurate cell count, which is still
315 necessary to dilute the sample appropriately for an unobstructed run, can then be obtained
316 during the antibody labeling incubation period.
317
318 This protocol uses a sputum weight measure instead of a cell count as an indication of how much
319 antibody/dye to use for optimal cell labeling. However, there is a great degree of variation in the
320 weight of the sputum samples; of the 126 samples that were analyzed, the weight ranged from
321 0.57 g to 38.30 g. **Figure 1A** shows that the correlation between sputum weight and cell yield is
322 not strong. Therefore, the samples were divided into four categories; the median weights of the
323 samples were 2.1 g for small samples, 5.0 g for medium samples, 11.2 g for large samples, and
324 22.0 g for extra-large samples (**Figure 1B**). However, there were still samples that yielded far
325 more cells than expected based on their weight (**Figure 1C**), the majority of the samples clustered
326 nicely. For small samples, the median cell yield was 8.0×10^6 cells, for medium samples $13.0 \times$
327 10^6, for large samples 35.4×10^6, and extra-large samples 93.0×10^6.
328
329 Each antibody used in this protocol was titrated. A concentration on the plateau phase (**Figure**
330 **2A**) with the highest staining index (**Figure 2B**) of the titration curve was chosen as the working
331 concentration. Variations in cell numbers will not dramatically alter the staining intensity. This
332 antibody titration and testing is essential and should be set up with care for each new antibody
333 or dye used in this protocol[34,35]. When an antibody is titrated with care, it should stain 10 to 50-
334 fold more cells than the number of cells on which the antibody was titrated[34,35]. An example of
335 this principle is provided in **Table 4**. The anti-human CD45-PE antibody was titrated on 1×10^6
336 cells in a total volume of 400 µL. If this antibody to staining volume is extrapolated to the protocol
337 (see **Table 2**, which shows the CD45-PE quantity and staining volumes for each sample size), for
338 a small sample, 0.625×10^6 cells will be stained, 1.25×10^6 cells will be stained for a medium
339 sample, and 2.5×10^6 cells for a large sample (column A in **Table 4**). A 50-fold excess in cell number
340 for each category is calculated to be 31.25×10^6, 62.5×10^6, and 125×10^6 cells, respectively
341 (column B). As shown in columns C and D, the median cell number of each size category and the
342 largest sample in each category are well within the 50-fold range.
343
344 Despite the apparent differences in size and cell yield between the various sizes of the sputum
345 samples, the median percent SEC contamination in each category is very similar. **Figure 3A** shows
346 the percentage of SECs found in individual samples, stratified according to their sputum weight
347 category. The main concern was with SECs since these cells are not representative of lung tissue
348 but of the oral cavity. However, the average SEC contamination is less than 20% for all categories.
349 **Figure 3B** shows the percentage of viable cells found in these samples. Excluding the SECs, the
350 average viability was approximately 72% for the small, medium, and large sample size categories.
351 It was slightly higher (79%) for the extra-large category.

352

353 **Figure 4** shows a typical gating strategy to separate the sputum cells of interest from debris, dead
354 cells (which also includes the contaminating SECs[36]), and cell clumps. **Figure 4A** shows the use of
355 NIST beads to set gates to eliminate debris smaller than 5 µm or larger than 30 µm, while **Figure**
356 **4B** applies this gate on sputum cells. **Figure 4C** shows a typical sputum profile when viewed as
357 side scatter width (SSC-W) against forward scatter width (FSC-W), the width gate. This profile is
358 used to eliminate small debris that presents itself along the SSC-W and FSC-W axes. The
359 elimination of dead cells is shown in **Figures 4D** and **Figure 4E**. The unstained control (**Figure 4D**)
360 is used to determine the cut-off for FVS510 positivity; cells that stain positive for FVS510 above
361 the negative control are considered dead and will not be used in the sputum cell analysis (**Figure**
362 **4E**). Lastly, a singlet gate is applied to remove cell doublets from the analysis, which is shown in
363 **Figure 4F**. Thus, the cells that have made it through the selection gates shown in **Figures 4B**,
364 **Figure 4C**, **Figure 4E,** and **Figure 4F** represent live, single sputum cells ready for subsequent
365 analysis with the antibodies used in this protocol.

366

367 The use of the anti-CD45 antibody in this labeling protocol allows the separation of live, single
368 sputum cells into a blood (CD45$^+$) cell compartment and a non-blood (CD45$^-$) cell compartment.
369 The latter category includes epithelial cells and other non-blood cells. The top profile in **Figure**
370 **5A** shows the use of an unstained sputum sample to set the cut-off for CD45 positivity, rendering
371 the gates to capture CD45$^+$ cells and CD45$^-$ cells. The bottom profile of **Figure 5A** shows both
372 these gates applied to a sputum sample stained with the anti-CD45 antibody. Staining with
373 additional antibodies is then used to identify blood cell-specific populations within the CD45$^+$
374 gate (**Figure 5B**) and other non-blood cell populations, including epithelial populations in the
375 CD45$^-$ gate (**Figure 5C**). In both the figures, the top profiles show how isotype-stained sputum is
376 used to set the double negative populations, whereas the bottom profiles show the antibody-
377 stained sputum cells. **Figure 5B** (bottom) shows six different CD45$^+$ cell populations created with
378 the cocktail of antibodies detectable in the fluorescence (FL) 1 channel (anti-CD66b, CD3, CD19
379 antibodies) and the anti-CD206 detectable in the FL3 channel. Sorting experiments have revealed
380 that lung-specific macrophages reside in the gates identified with M. The presence of cells in
381 these gates thus indicates that the sputum sample was derived from the lung and was not saliva
382 (Bederka et al., manuscript in preparation). **Figure 5C** (bottom) shows the gating quadrants
383 created with the anti-pan-cytokeratin (panCK) detectable in the FL1 channel and anti-epithelial
384 cell adhesion molecule (EpCAM) antibodies detectable in the FL3 channel among the CD45$^-$
385 sputum cells.

386

387 This labeling protocol has been extensively tested on the Navios EX and the LSR II. Some
388 preliminary experiments were performed on the Lyric, but without the extensive instrument
389 optimization that was done for the other two machines. Therefore, detailed instrument settings
390 are provided for the Navios EX and the LSR II in the Materials sheet, but not for the Lyric. For
391 understanding the similarities and variations between these three flow cytometric platforms that
392 can be used to analyze sputum cells, profiles obtained from the various machines can be
393 compared in **Figure 6** and **Figure 7**. Two large sputum samples were processed, pooled, labeled,
394 separated into three equal portions, and subsequently acquired on each flow cytometer
395 mentioned above. **Figure 6**, which is similar to **Figure 4**, compares the gating strategy for

396 eliminating debris, dead cells, and cell clumps. **Figure 7**, which is identical to **Figure 5**, compares
397 the blood and non-blood profiles from the data acquired on the various flow cytometers.
398
399 Comparing the various profiles obtained from the three different flow cytometers shows that the
400 same basic profiles can be observed with each instrument. The differences to pay attention to
401 are the SSC-W/FSC-W (width gate) plots (**Figure 6**, second row) and the linear scales of the scatter
402 plots (**Figures 6** and **7**). The scatter width plot generated on the Navios EX shows an inclusion
403 gate (red box), meaning that all cells included in the gate are further analyzed; the events along
404 the axes in the left-bottom corner are excluded. The scatter width plots on the LSR II, and Lyric
405 do not show a similar deposit of events along these same axes. This discrepancy is likely due to
406 the very sensitive threshold used on the Navios EX, leading to some small debris in the previous
407 size-exclusion gate (top row of **Figure 6**). On occasion, debris is seen along the axes in the scatter
408 width profile generated on the LSRII, but it is in the right-bottom corner. In those cases, an
409 exclusion gate (as indicated by the dashed red box in the middle profile in row 2 of **Figure 6**) is
410 used to eliminate these events from further analysis.
411
412 While the log scales appear slightly different on the plots between the Navios EX and the Lyric
413 and LSR II cytometers, the Navios EX does have the option to acquire data using more decades.
414 Implementing more decades depends on the context of the experiment and the desired
415 sensitivity to visualize the populations of interest.
416
417 An additional notable difference between the Navios EX and LSR II and Lyric cytometers is the
418 appearance of the profile of the singlet gate. The Navios EX cytometer is equipped with a
419 rectangular flow cell with a different collection angle than the LSR II. The Navios EX contains three
420 software-controlled collection angles to optimize the forward angle light scatter to achieve the
421 sensitivity appropriate for particle size to analyze. The polygon gate for the Navios EX cytometer
422 will need to be adjusted to a slightly lower angle than the singlet gate for the LSRII. The singlet
423 gate for the Lyric can be optimized to get a profile similar to that seen with the LSR II. Using the
424 sputum sample, the area scaling factor would need to be optimized for each laser on the Lyric to
425 achieve a singlet gate similar to the LSR II.
426
427 All the profiles shown in **Figure 4** to **Figure 7** were analyzed with FlowJo software, version 10.6.
428 The .fcs files can be found in the FlowRepository (https://flowrepository.org), under IDs FR-FCM-
429 Z3LX, FR-FCM-Z3MJ, and FR-FCM-Z3MM and can be used to practice the sputum analysis as
430 illustrated in these figures.
431
432 **FIGURE AND TABLE LEGENDS:**
433
434 **Figure 1: Sputum weights and cell yield.** (**A**) Depicted is the relationship between sputum weight,
435 determined before processing, and total cell yield, determined after processing using a
436 hemocytometer. Each bullet represents an individual sample, 126 in total. (**B**) Sputum samples
437 were stratified into four categories based on their weight: small for samples weighing up to 3 g,
438 medium for samples weighing more than 3 g up to 8 g, large for samples weighing more than 8 g
439 up to 16 g, and extra-large samples for those weighing more than 16 g. (**C**) Shown is the

440 distribution of cell yield for the samples in each category. The red bars in (B) and (C) represent
441 the median values in each category.

443 **Figure 2: Antibody titration for anti-human CD45-PE.** (**A**) The CD45-PE (IgG1) titration curve with
444 the mean fluorescence intensity (MFI) of the positive population plotted versus the
445 concentration of the CD45-PE antibody plateaus at 1 µg/mL. (**B**) Titration curve depicting the
446 staining index versus the antibody concentration shows the highest staining index is at 1 µg/mL.
447 Staining index was calculated as: [CD45 MFI positive population - CD45 MFI negative population]
448 / [2 * Standard Deviation]. Based on **Figure 2A** and **Figure 2B**, 1 µg/mL was chosen as the optimal
449 concentration for the CD45-PE antibody.

451 **Figure 3: Proportion of SECs and dead cells in sputum samples is consistent among the four**
452 **weight categories.** (**A**) The proportion of SECs in sputum samples is classified according to their
453 weight category. The percent SEC contamination was determined by hemocytometer as part of
454 the total cell count. (**B**) Cell viability of sputum samples excluding SECS, determined by
455 hemocytometer and the trypan blue exclusion method. For each graph, the red lines represent
456 the median values.

458 **Figure 4: Gating strategy for excluding debris, dead cells, and doublets in a sputum sample.** (**A**)
459 NIST beads of sizes 5 µm, 20 µm, and 30 µm were used to set the gate indicated by the red box
460 to exclude debris smaller than 5 µm and larger debris above 30 µm and close to the axis. (**B**) A
461 sputum sample was acquired using the same voltages for the forward and side scatter as that of
462 the NIST beads. The gate created in (A) was applied to exclude debris. (**C**) Small debris close to
463 the axis was excluded in this scatter width plot. (**D**) An unstained sputum sample was used to set
464 the cut-off (red line) for the negative, unstained population for the viability dye. (**E**) The cut-off
465 created in D was applied to the stained sputum sample to form a gate (red box) to include live,
466 viable cells. (**F**) The singlet gate indicated by the red polygon excludes cells that do not fall on the
467 diagonal, eliminating doublets and/or clumps.

469 **Figure 5: Gating strategy of sputum cells into blood and non-blood cell compartments.** (**A**)
470 Unstained sputum cells derived from the singlet gate are used to set the cut-off (red line) on the
471 negative population for CD45 (top). The cut-off from the top profile is applied to the stained
472 sputum sample (bottom) to differentiate CD45 positive (CD45$^+$) and negative populations (CD45$^-$
473). (**B**) CD45$^+$ sputum cells stained with isotype antibodies for FITC/AF488 are used to set the gates
474 on the negative population (top). The same gates are applied to the CD45$^+$ sputum cells stained
475 with blood cell markers CD3, CD19, CD66b, and CD206 (bottom). (**C**) Quadrant gates are placed
476 on the CD45$^-$ sputum cells stained with isotype controls (top) and applied to the CD45$^-$ cells from
477 the sputum sample stained with the epithelial markers pan-cytokeratin (panCK) and EpCAM
478 (bottom).

480 **Figure 6: Gating strategy to exclude debris, clumps, and dead cells of stained sputum cells on**
481 **three flow cytometric platforms.** Two large sputum samples were processed, pooled, labeled,
482 and divided into three equal portions for acquisition on the Navios EX (**A**), LSR II (**B**), and the Lyric
483 (**C**) flow cytometers. Top row: Sputum samples were gated (red box) to exclude very small and

484 large debris. Second row: The large red box in the Navios EX plot represents an inclusion gate
485 that includes the cells but excludes debris. The dashed small red box seen in the LSR II plot
486 represents an exclusion gate to eliminate debris from further analysis. Further optimization with
487 the Lyric is needed to determine where debris exclusion gating is needed. Therefore, no gate is
488 present in that plot. Third row: red rectangular gates include live cells for further analysis. Bottom
489 row: polygon gate has single cells by excluding doublets that fall outside the diagonal of the gate.
490
491 **Figure 7: Gating strategy for sputum stained for blood and epithelial markers on three flow**
492 **cytometric platforms.** The analysis shown in **Figure 6** is continued in **Figure 7**; all viable, single
493 cells (bottom row, **Figure 6**) were divided into CD45$^+$ and CD45$^-$ populations (first row, **Figure 7**),
494 so that blood cell-specific markers and epithelial cell-specific markers could further delineate
495 these populations. Second row: profile of blood cell markers CD3/CD19/CD66b and CD206 from
496 CD45$^+$ cells. Third row: epithelial cell markers panCK and EpCAM from CD45$^-$ cells.
497
498 **Table 1: Antibodies and viability stains used.** List of antibodies and viability dye used in this
499 protocol. Indicated are the cellular subsets they each identify.
500
501 **Table 2: Content of tubes with sputum cells.** Use this table as referenced in the protocol to add
502 the specified volumes of antibodies and viability stain for the appropriate sample size.
503
504 **Table 3: Content of compensation tubes.** Use this table as referenced in the protocol to add the
505 specified volumes of antibodies to the compensation beads.
506
507 **Table 4: Anti-human CD45-PE concentration for all samples in each sample size category.**
508
509 **DISCUSSION:**
510 The cellular content of sputum includes a large variety of wide-ranging cells, often accompanied
511 by a lot of debris[37]. In addition, sputum analysis requires a quality control that confirms the
512 sample is collected from the lung instead of the oral cavity[38]. Therefore, it is not as simple to
513 analyze sputum by flow cytometry as it is for blood, for example, which releases a much cleaner
514 and homogeneous cell suspension. This protocol has addressed all these issues: providing
515 instrument settings using specific size beads to ensure that both the smallest and largest cell
516 populations can be detected, a gating strategy to eliminate debris, cell clumps, contaminating
517 SECs and other dead cells, and lastly, a quality control measure for ensuring a sputum sample is
518 from the lung instead of being mostly saliva.
519
520 There are critical steps in the protocol in working with sputum that is worth pointing out. First,
521 cell yield can be drastically impacted by the number of nylon cell strainers used in step 2.5 of the
522 sputum dissociation part of the protocol. Multiple strainers may be required to avoid losing too
523 many cells due to clogging of the strainer. When the flow through the strainers has slowed down
524 noticeably, a new strainer should be used. Second, the cell pellet of sputum samples can be very
525 loose, especially if there is high contamination of SECs. Therefore, it is essential not to aspirate
526 too close to the pellet when removing the supernatant after centrifuging the samples. Aspirating
527 too close to the pellet may result in cell loss, if not loss of the entire pellet. Third, this protocol

528 requires a fixation step. This preserves the cells and their staining profile and serves as a safety
529 measure to protect the flow cytometer operator. Running samples on certain flow cytometers
530 may present increased biological hazards due to the potential for aerosol production[39]. The PFA
531 fixation helps to protect the operator from potential pathogens in the sputum sample. Fourth,
532 and perhaps the most challenging aspect of preparing sputum samples for flow cytometric
533 analysis, is cell counting. Cell counting is complicated because of the large variety of cell types
534 present in the sputum. Counting machines are often limited by the cell size range they can
535 capture and are therefore less reliable than a hemocytometer. However, cell counting of sputum
536 samples by hemocytometer, which is excellently described by Guiot et al.[20], is tedious and
537 requires practice to become proficient. An accurate cell number is vital in determining the
538 sample's final resuspension volume to allow for a reasonable flow rate and prevent clogs in the
539 flow cytometer. The presence of the very large SECs in the sputum and the many smaller cell
540 clumps that are not broken up by the dissociation buffer, nor caught by the filters, increase the
541 likelihood of clogging the flow cytometer. Therefore, it is recommended to spend some time
542 determining the best cell concentration/flow rate for data acquisition using the available flow
543 cytometer. Additionally, ensure that the flow cytometer has the appropriate flow cell and nozzle
544 size (or probe) capable of measuring the bigger cell populations present in the sputum.
545
546 Even when proficiency in cell counting has been achieved, it is still a time-consuming process.
547 Therefore, the reagents' determination for cell labeling in this protocol is based on the sample
548 size (as judged by weight) rather than cell number. This allows for more efficient use of time since
549 the manual cell count can be completed during the time needed for the antibody and viability
550 dye staining. However, there are three notable exceptions to this. If a sample is >16 g (the so-
551 called extra-large samples), the manual cell count should occur before staining so that expensive
552 reagents can be conserved by only staining 25 x 10^6 cells each for the blood and epithelial tubes.
553 This cell number gives very reliable profiles in the settings described in this protocol. Another
554 potential exception is the case of a very small sample. The estimation is that a minimum of 1–2 x
555 10^6 cells is needed for the flow cytometry analysis as presented in this protocol to generate
556 reliable profiles (data not shown). Therefore, if a sample contains fewer than 1–2 x 10^6 cells, it
557 may not be worth continuing with the procedure since the final result will likely be unacceptable.
558
559 Specific labeling reagents that work well on peripheral blood cells or cell lines may work very
560 differently on blood cells contained in sputum (unpublished observations). Therefore, it is
561 recommended to titrate each antibody or other labeling reagents according to well-established
562 protocols[34,35] before they are used in flow cytometry experiments. Most antibody titrations
563 should give comparable results when staining up to 10 to 50-fold; however, it is advised to do
564 additional titrations with cell numbers higher than that range[34]. When a reagent concentration
565 has been determined, it is essential to keep the incubation time of that reagent the same as in
566 the actual experiment. For that reason, the protocol stresses adding all the buffer and reagents
567 to the tubes before the cells or compensation beads are added. This order of adding reagents
568 and cells/beads together allows for higher consistency in labeling times. If, for some reason, the
569 experimental incubation time cannot be kept similar to that used for antibody/dye titration, the
570 latter should be repeated with an incubation time that is feasible during experiments.
571

Sputum samples have been widely used as a diagnostic specimen to study the pathophysiology of various illnesses affecting the lung. Tuberculosis[40,41], COPD[13,42], asthma[43], cystic fibrosis[44,45], lung cancer[46–48], and rheumatoid arthritis[49] are among the many illnesses where sputum has been studied due to the advantage of it being a non-invasively obtained specimen. More recently, during the coronavirus pandemic, sputum has been used to detect prolonged viral shedding in patients hospitalized with coronavirus disease 2019 (COVID-19)[50].

Various technologies, including reverse transcription-polymerase chain reaction (RT-PCR), protein analysis, microscopy, and flow cytometry, have been used to identify disease-specific markers in sputum. Using a flow cytometric platform to analyze sputum samples is not widely used, but its use increases. With recent advancements in the technology of flow cytometers[3,51,52], as well as advancements in the chemistries of fluorophores, generation of new antibody clones, and development of various dyes to identify dead cell populations[51,53,54], the possibility of designing antibody panels aimed at diagnosing human diseases has dramatically increased. Moreover, the recent push for automating the analysis of flow cytometric data[55,56] will eliminate the potential bias in manually reading and interpreting flow data. These new developments in flow cytometry will significantly facilitate the exploration of sputum as a clinical diagnostic.

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All the authors are past or current employees of bioAffinity Technologies.

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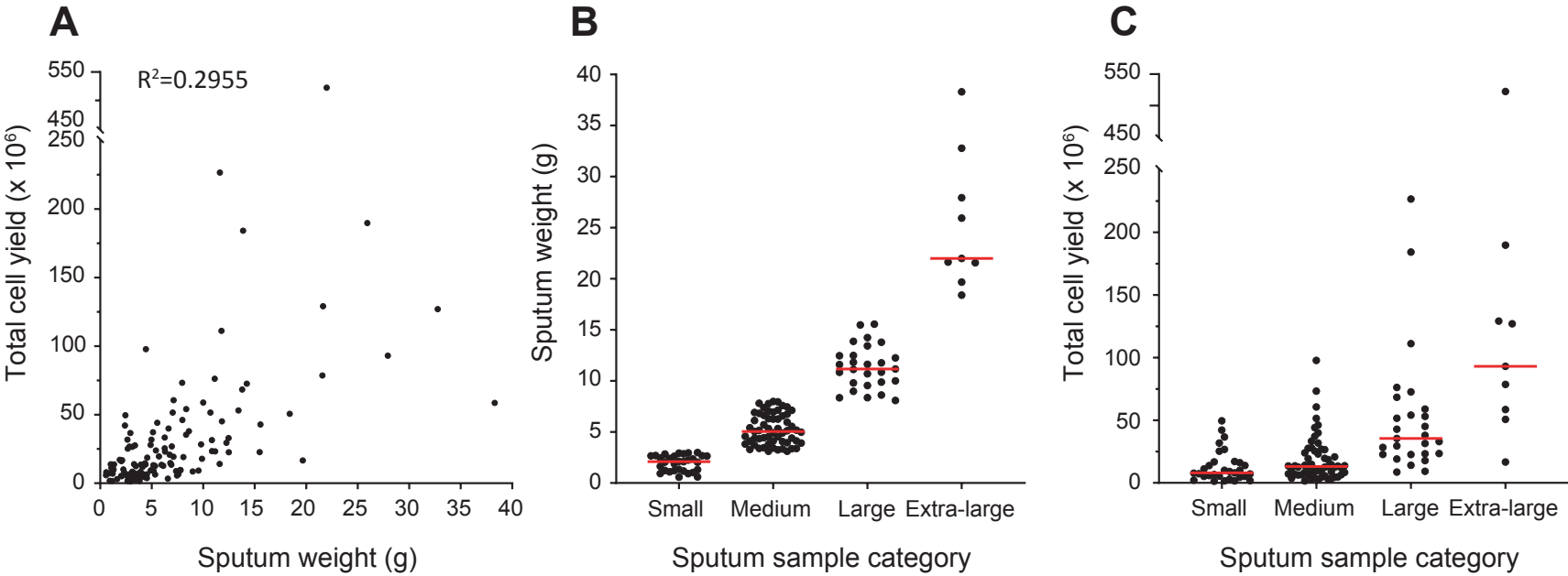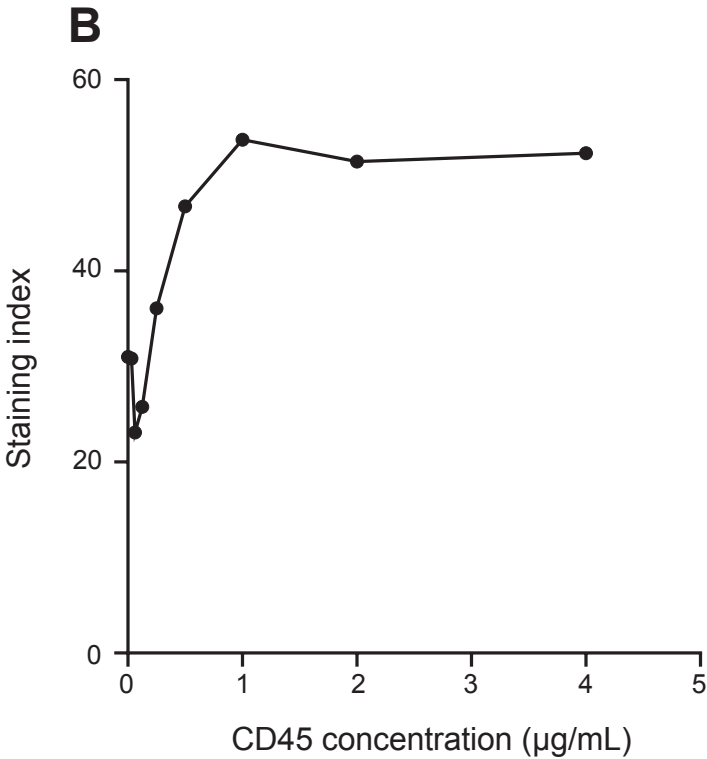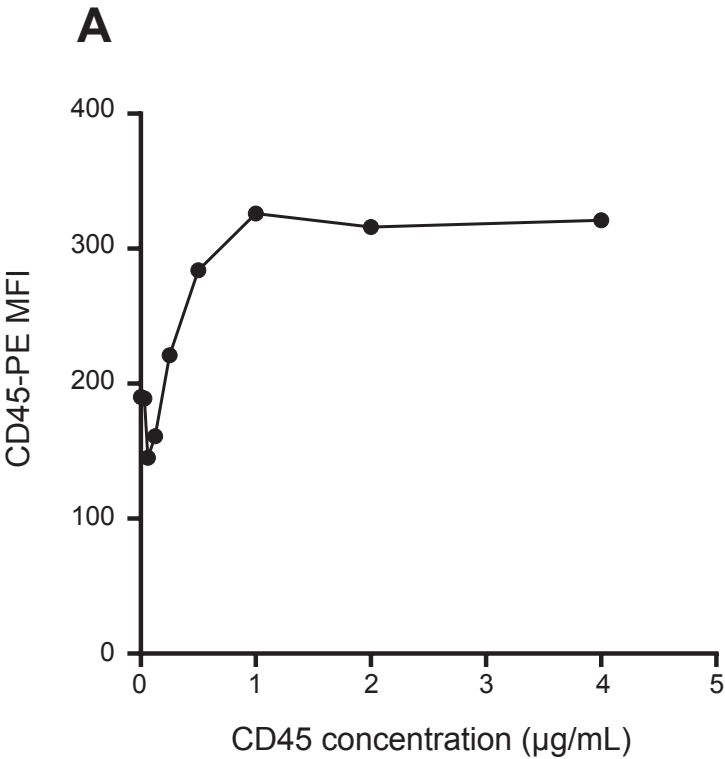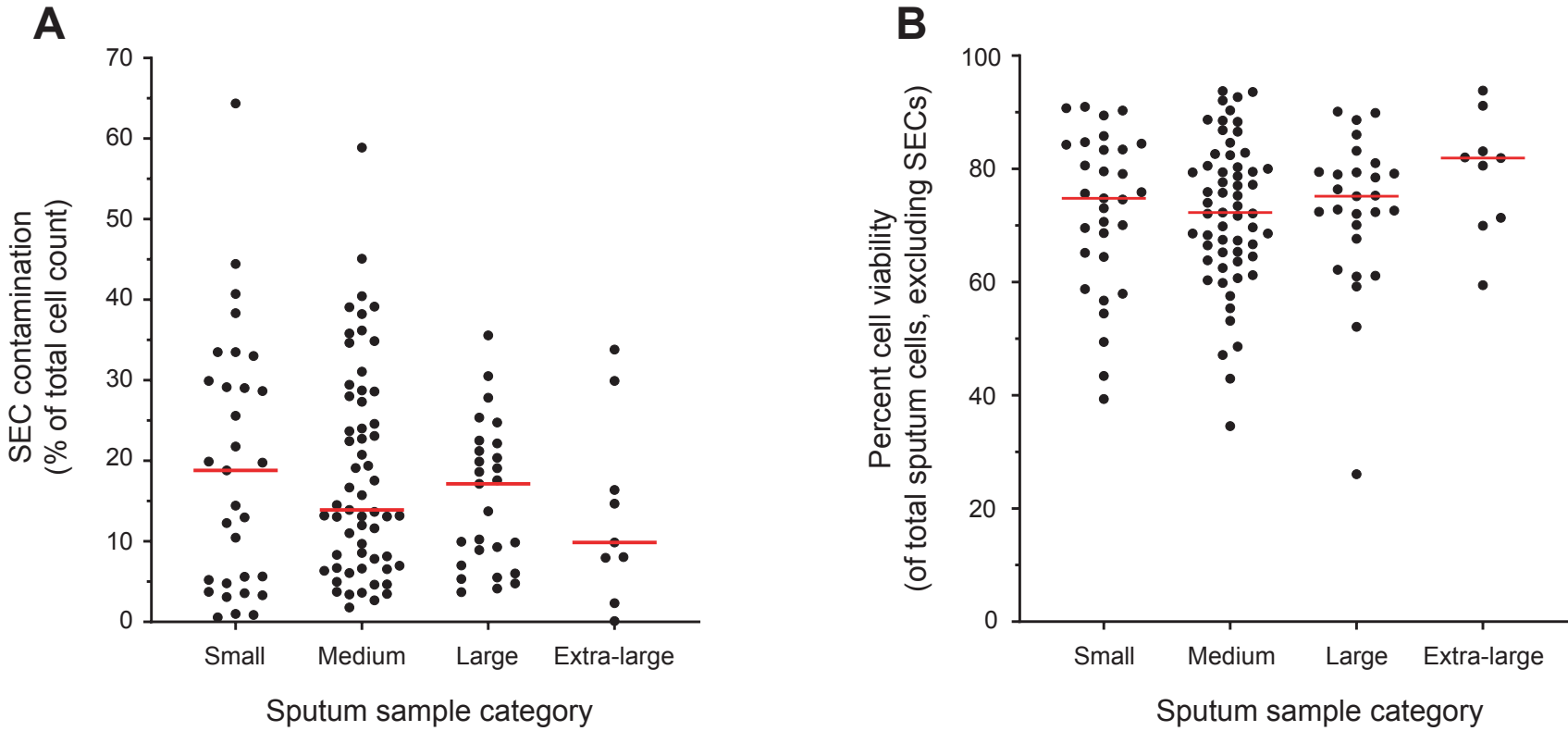743   (2016).
744

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Exclusion of debris

Exclusion of dead cells

Exclusion of doublets

Figure 5

Figure 5

A Single cells

B **CD45⁺** cells

C **CD45⁻** cells

Figure 6
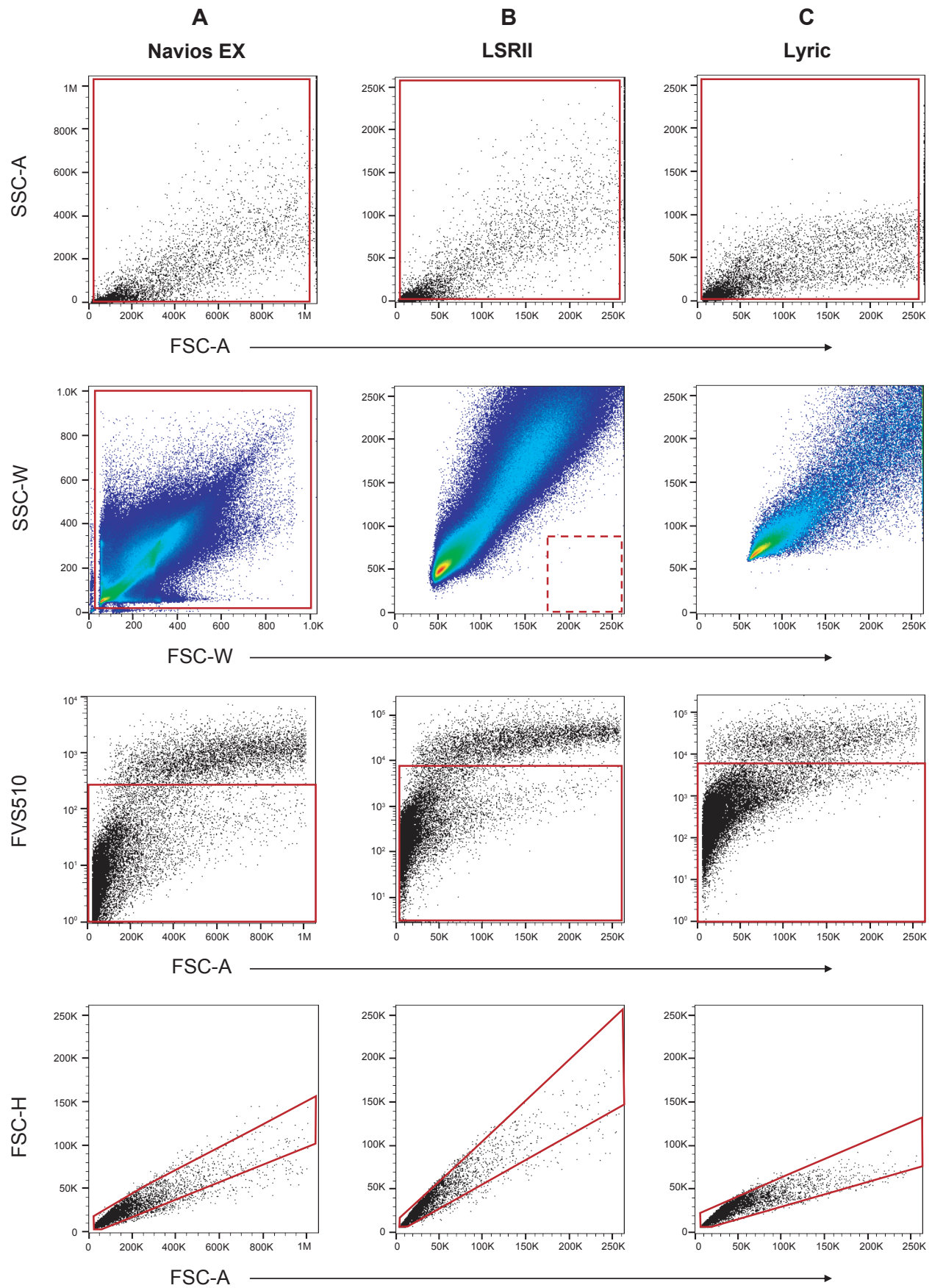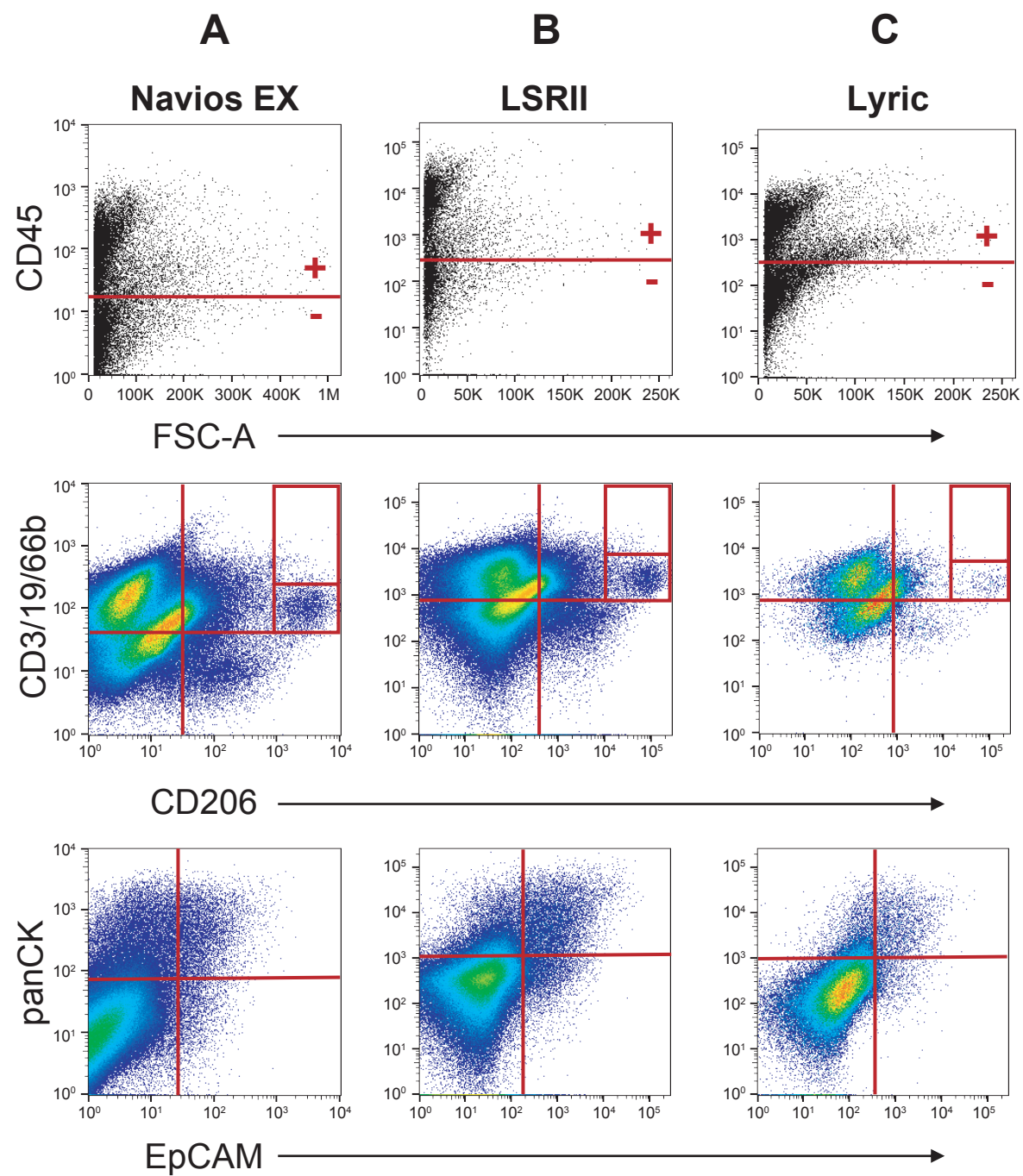
Figure 6

Figure 7

Table 1
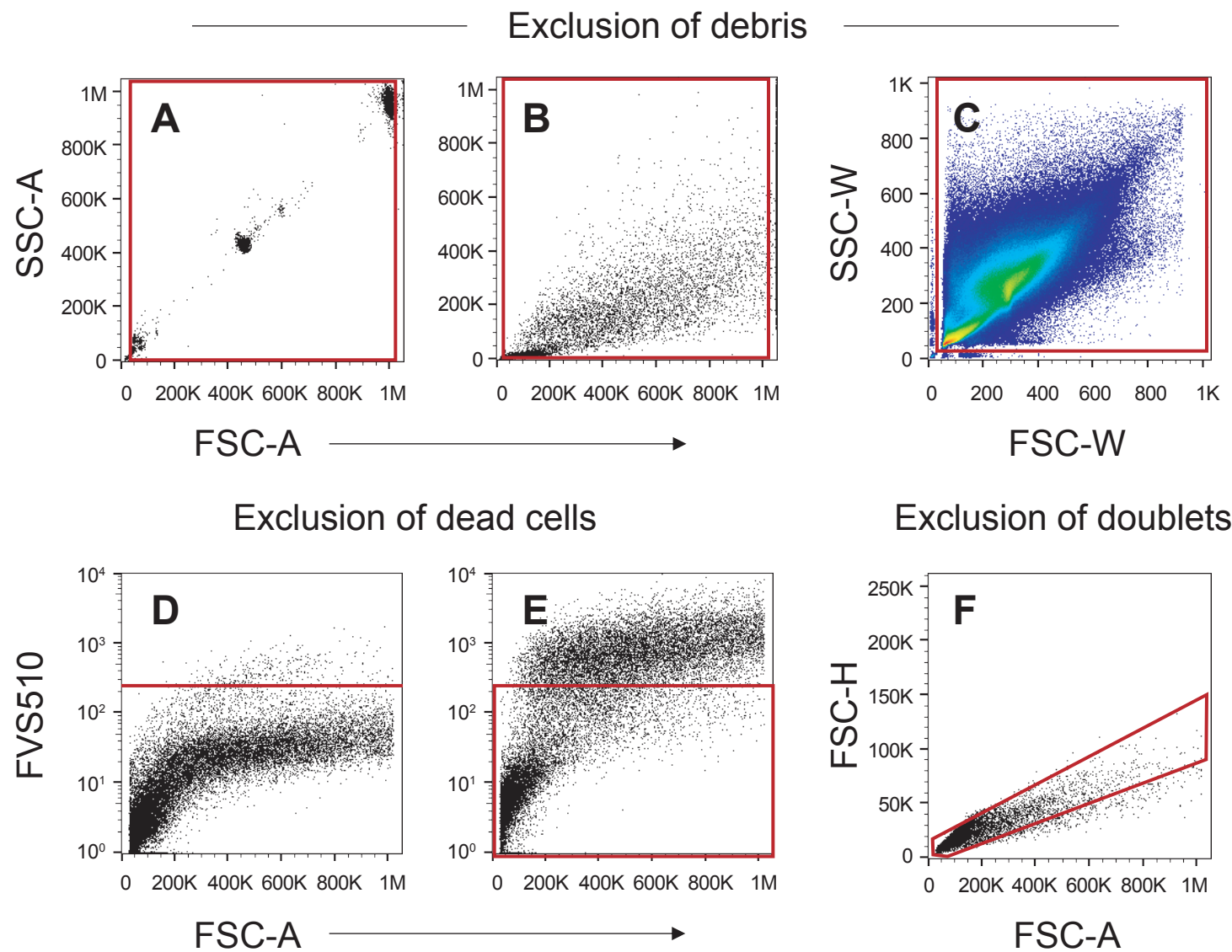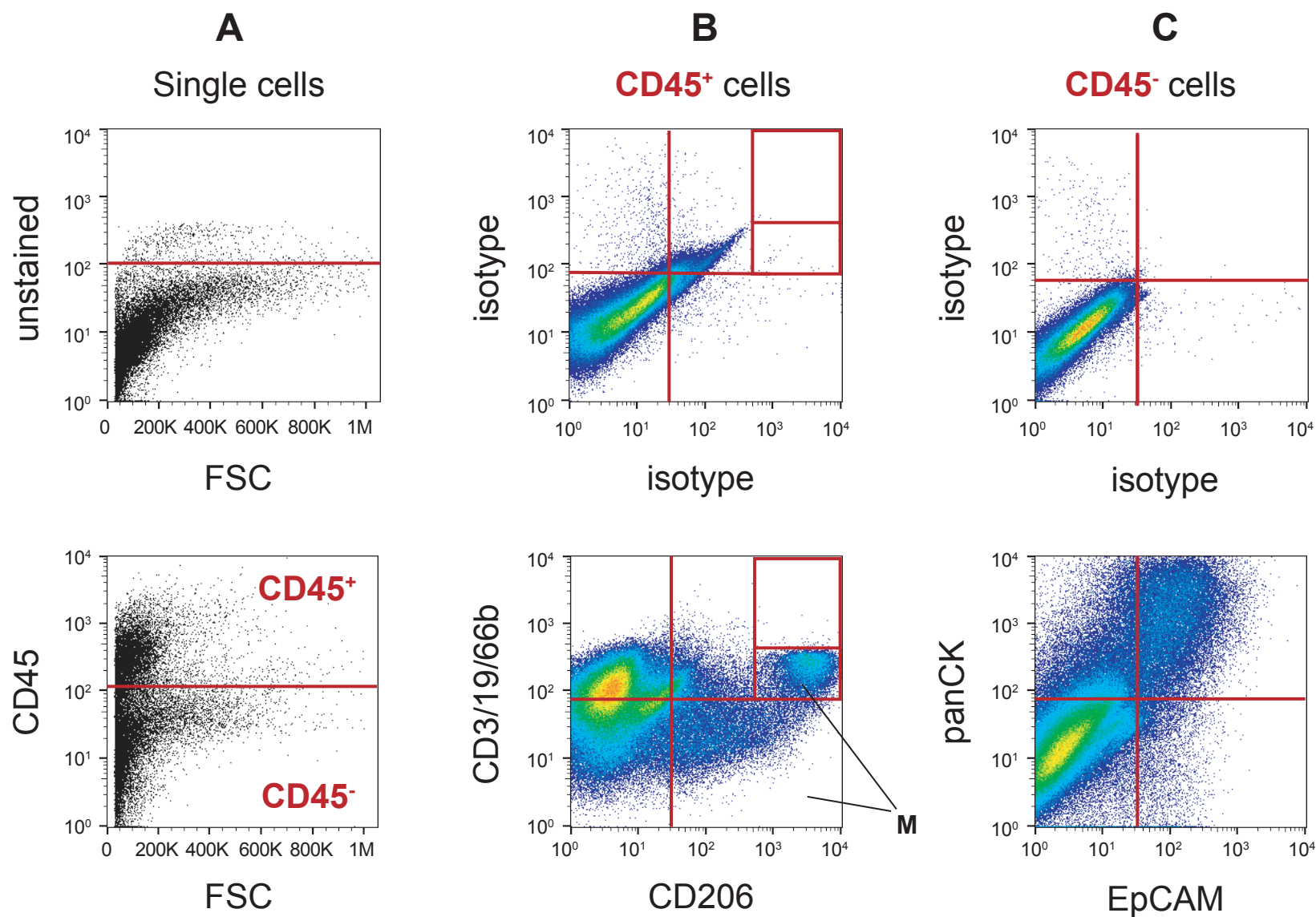
Antibody/stain
FVS510
Anti-human CD45 – PE
Anti-human CD66b – FITC
Anti-human CD3 – Alexa488
Anti-human CD19 – Alexa488
Anti-human CD206 – PE-CF594
Anti-human EpCAM – PE-CF594
Anti-human pan-cytokeratin (panCK) – Alexa488
IgG1κ – Alexa488
IgG1κ – FITC
IgG1κ – PE-CF594
Anti-human CD45 – BV510

Purpose
Viability stain
Pan-leukocyte marker; PE compensation
Granulocyte marker
T cell marker
B cell marker; FITC/Alexa488 compensation
Lung macrophage marker; PE-CF594 compensation
Epithelial cell marker; PE-CF594 compensation
Epithelial cell marker
CD3/CD19/panCK isotype control
CD66b isotype control
CD206/EpCAM isotype control
FVS510 compensation

Table 2   Click here to access/download;Table;Grayson_Table2.xlsx ↓

Tube (Label)	Sample size*	HBSS (µL)	FVS510 (µL)	CD45 (µL)	
Unstained	small	80			
	medium	50			
	large	50			
					IgG1κ - Alexa488
Isotype control	small	60.65	0.6	10	2
	medium	30.65	0.6	10	2
	large	30.25	1	10	2
					CD206
Blood	small	92.875	1.5	25	1.875
	medium	65.75	3	50	3.75
	large	102.5	10	100	7.5
					EpCAM
Epithelial	small	96.5	1.5	25	10
	medium	73	3	50	20
	large	117	10	100	40

 * Samples are considered small, medium or large based on weight determined in step 1 of the

§ Cells should be added *after* all reagents have been distributed, including those in the compen

Other antibodies (µL)			Cells [§] (µL)
			20
			50
			50
IgG1κ- FITC	IgG1κ - PE-CF594		
6	0.75		20
6	0.75		50
6	0.75		50
CD3	CD19	CD66b	
5	1.25	7.5	115
10	2.5	15	350
20	5	30	725
PanCK			
2			115
4			350
8			725

sputum dissociation section of the protocol.

sation tubes (Table 3).

Table 3   Click here to access/download;Table;Grayson_Table3.xlsx ⬇

Tube (Label)	Reagents added (µL)				
	HBSS	CD45-PE	CD206-PE-CF594	EpCAM-PE-CF594	CD19-Alexa488
PE comp.	76	4	x	x	x
PE-CF594 comp.	72	x	4	4	x
Alexa488/FITC comp.	60	x	x	x	20
BV510 comp.	60	x	x	x	x

* The positive (+) and negative (-) beads should be added *after* all reagents have been distributed a
prepared.

comp. = compensation

CD45-BV510	Reagents added (drop)	
	CompBead (+)	CompBead * ( )
x	1	1
x	1	1
x	1	1
20	1	1

nd the tubes in Table 2 have been

Table 4

Sample size	Cell number (x 10^6)			
	A In staining volume *	**B** 50-fold access [¶]	**C** Median sample (fold access) [#]	**D** Largest sample (fold-access) [#]
Small	0.625	31.25	8.0 (12.8)	24.77 (39.6)
Medium	1.25	62.5	13.0 (10.4)	48.87 (39.1)
Large	2.5	125	35.4 (14.2)	113.5 (45.4)

* Staining volumes used in the protocol (Table 2). Cell number is extrapolated from how the titration curve was performed; 1 µg/mL of CD45-PE and 1 x 10^6 cells per 200 µL.

[¶] 50-fold access of cell numbers in Column A.

[#] fold access in columns C and D are calculated by dividing the number presented in the cells of columns C or D by the number in the corresponding cell in column A.

Click here to access/download

Table of Materials

62785_R2_Table of Materials.xlsx

Nilanjana Saha, Ph.D.

Review Editor

JoVE

San Antonio, July 12, 2021

Dear Dr. Saha:

We hereby submit the second revision of our manuscript (JoVE62785R1), "***Quality-controlled sputum analysis by flow cytometry***" by Grayson *et al.,* for publishing consideration*.* We would like to thank the editorial office for their time and effort dedicated to reviewing our manuscript.

We have addressed all the editorial concerns by responding in the comment bubble. There were no questions regarding the figures or tables. Therefore, we left these unchanged.

We have proofread the entire manuscript and found two mistakes that we corrected. These corrections involved lines 255 and 256, where we changed 106 to 10^6.

We added 1 reference per request (reference # 29).

We confirm that this manuscript has not been published elsewhere, nor is it under consideration for publication at another journal. Thank you for your time and consideration. We look forward to hearing from you.

Sincerely,

Vivienne I. Rebel, M.D., Ph.D.