A computational solution to improve biomarker reproducibility during long-term projects

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Abstract

Biomarkers are fundamental to basic and clinical research outcomes by reporting host responses and providing insight into disease pathophysiology. Measuring biomarkers with research-use ELISA kits is universal, yet lack of kit standardization and unexpected lot-to-lot variability presents analytic challenges for long-term projects. During an ongoing two-year project measuring plasma biomarkers in cancer patients, control concentrations for one biomarker (PF) decreased significantly after changes in ELISA kit lots. A comprehensive operations review pointed to standard curve shifts with the new kits, an analytic variable that jeopardized data already collected on hundreds of patient samples. After excluding other reasonable contributors to data variability, a computational solution was developed to provide a uniform platform for data analysis across multiple ELISA kit lots. The solution (*ELISAtools*) was developed within open-access R software in which variability between kits is treated as a batch effect. A defined best-fit Reference standard curve is modelled, a unique Shift factor "S" is calculated for every standard curve and data adjusted accordingly. The averaged S factors for PF ELISA kit lots #1-5 ranged from -0.086 to 0.735, and reduced control inter-assay variability from 62.4% to <9%, within quality control limits. S factors calculated for four other biomarkers provided a quantitative metric to monitor ELISAs over the 10 month study period for quality control purposes. Reproducible biomarker measurements are essential, particularly for long-term projects with valuable patient samples. Use of research-use ELISA kits is ubiquitous and judicious use of this computational solution maximizes biomarker reproducibility.

Introduction

In virtually every research project with real or potential clinical application, biomarkers provide valuable data to monitor presence or progression of disease, as well as therapeutic susceptibility or efficacy. Biomarker data monitor defined outcomes and there is considerable discussion about whether investigators should disclose incidental research findings to study participants [1-3]. Intrinsic to this discussion is the need for reproducible study data and this presents challenges, particularly with long-term studies. Data rigor and reproducibility is a systemic problem [4] and a priority issue for the NIH. It is an analytic challenge for long-term studies because research laboratories often do not have standardized operations nor validated biomarker assay reagents that adhere to the quality assurance and quality control standards for diagnostic use as required by the Clinical Laboratory Improvement Act (CLIA; www.cdc.gov/clia).

Protein biomarkers are measured frequently in plasma, serum or other matrices by solid phase Enzyme-Linked Immunosorbent Assay (ELISA) methods in which the antigen of interest in the sample is bound by antibodies and the amount of bound antigen is proportional to the signal strength that develops in the assay. There have been only 136 *in vitro* diagnostic ELISA kits or kit components cleared or approved by the FDA since 2000, but there are hundreds of commercially available ELISA kits labeled for “research purposes only” from dozens of vendors. Typically these are vetted by the manufacturer for sensitivity, selectivity, intra/inter-assay variability, stability and storage needs, but they are not required to adhere to federal CLIA guidelines. Lot-to-lot variability between ELISA kits is either not relevant or is manageable for short-term projects, but challenges arise for quality assurance when multiple lots of research ELISA kits are used in long-term

projects. A research laboratory may operate under NIH biomarker recommendations [5] and Good Clinical Laboratory Practice guidelines with appropriate training, auditing, assay validation and proficiency testing [6], but it does not have jurisdiction over kit reagents controlled by the manufacturer. In general practice, expected inter-assay coefficients of variation (CV) for ELISA standard curves will be within 10~20%, but if a commercial ELISA standard curve suddenly shifts significantly with a new kit lot and validated control data shifts outside limits, then the current patient biomarker concentrations cannot be compared with those quantified months earlier. An experienced laboratory can rescue data from one ELISA with a failed standard curve [7], but long-term quality assurance poses other challenges. Either all the samples have to be re-assayed, which introduces variables such as freeze/thaw issues, or the data discarded, all of which wastes precious patient samples and resources.

This was encountered by our research group with one commercial ELISA kit during an on-going two year project to evaluate effects of pre-analytic variables on plasma thrombosis biomarkers in patients. The project developed thirty-five standard operating procedures (SOPs) that define and document operations from blood acquisition to transport, processing, assay and storage. Nine biomarkers are quantified by research-use ELISA kits. For one biomarker, biomarker “PF”, after months of reproducible assays, we observed a significant shift of our standard curves and internal control results when assayed with new kits lots. The scope of the problem was revealed by review of data from 5 kit lots over 10 months and 65 ELISA plates. Rigorous review by the quality assurance team did not identify laboratory or operational pre-analytic contributions and similar changes were not observed with the other ELISAs. The project had encountered an unexpected analytic variable and the data from 420 plasma samples collected over 10

93 months could not be compared. The manufacturer was responsive but ultimately unable
94 to resolve the problem.

95 To rescue our patient data, we developed a computational solution with a
96 sufficiently generalized approach such that it may be used by others facing a similar
97 situation. In the solution, the lot-to-lot variability in ELISA kits is treated as a batch effect,
98 and a defined Reference standard curve is modelled with either a four- or five-parameter
99 logistic function. Based on this Reference curve, a Shift factor ("S") can be calculated and
100 applied retrospectively to every standard curve from every ELISA plate over many
101 months, and the biomarker concentrations for that plate are adjusted accordingly. In this
102 way, the data collected from many ELISA plates over many months can be compared on
103 a uniform platform. Once instituted, calculating the Shift factor for each ELISA standard
104 curve or each kit lot provides an expedient way to rapidly monitor standard curves as a
105 quality assurance metric and to facilitate data management.

106 Materials and methods
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108 ELISA kits
109

110 Biomarker ELISAs with at least two kit lots were analyzed for the current study.
111 ELISAs for human P-selectin/CD62P, human myeloperoxidase and human plasminogen
112 activator inhibitor-1/serpin E1 were provided by R&D Systems (Minneapolis, MN, USA).
113 The ELISA kit vendor for the biomarker of focus for the current study (biomarker "PF") is
114 not provided for discretionary reasons. Five lots of the biomarker PF ELISA kits were
115 received over a 10 month time period. All ELISA kits were a standard 96-well format,
116 sandwich antibody-based ELISA designated "for research purposes".

Assays and Equipment

ELISA kits were stored at 4 ± 2°C in a cooler (Helmer Scientific, Noblesville, IN, USA) equipped with alarmed wireless external temperature monitoring (SensoScientific, Inc., Simi Valley, CA). Temperature logs were reviewed and constant temperatures without drift were verified. All kits were used within the manufacturer's expiration date. Plasma samples stored at -80°C were thawed just before assay in a 37°C water bath for <10 minutes, gently mixed, and kept on ice. Plasma samples were assayed according to a detailed standard operating procedure (SOP) for each biomarker ELISA that includes the manufacturer's procedural steps. The SOP also included required documentation for every ELISA plate for operator, date, plate ID, critical reagents (date received, lot number, dilution/concentration, expiration date), incubation times (date, start/stop times, temperature), equipment (manufacturer, model, serial number) and a section to document any deviations from the SOP. Every plate included kit standards prepared according to the manufacturer's instructions. The standards were added to triplicate wells in the first three columns of the provided 96 well plate (columns A-C, rows 1-8 using standard plate designations).

For every biomarker and every plate, an internal spiked plasma-based control sample (BMC Control, see below) was included in triplicate wells. Samples were added to the plates in with calibrated pipettes, washing steps were performed with an automated plate washer (Biotek model Elx50, Winooski, VT) and developed color was quantified by measuring optical density (O.D.) at the appropriate wavelength with a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA). The acceptable coefficient of variation (CV) of triplicate wells for each standard, control or unknown was ≤15%. For biomarker

141 PF, incubations with samples and detection antibody were done at 37°C in a dry incubator
142 per the manufacturer's instructions and perimeter wells were not used for patient samples
143 to prevent possible evaporation complications due to the elevated incubation
144 temperature. Other ELISAs were performed at room temperature. Initial data analyses
145 were done with SoftMax Pro version 7 (Molecular Devices).

146 BMC Control Preparation and Storage

147

148 Every ELISA plate included a human pooled plasma control sample that had been
149 spiked with supplemental biomarker and designated the BMC Control. For each
150 biomarker, the BMC Control was made in bulk volume according to the respective ELISA
151 SOP and stored at -80°C in small aliquots for single use. For biomarker PF, lyophilized
152 human pooled citrated plasma (Sigma Aldrich, catalogue P9523-5ML) was reconstituted
153 with deionized water at room temperature for at least 15 minutes with gentle mixing,
154 diluted with appropriate buffer to the same ratio as the patient samples and then spiked
155 with reconstituted biomarker PF standard prepared from the same manufacturer's kit but
156 purchased expressly for this purpose. On the assay day, a BMC Control aliquot was
157 thawed just before assay in a 37°C water bath for <10 minutes, mixed gently and added
158 to each plate in triplicate wells. Two preparations of BMC Controls were made and
159 aliquoted for storage: one in August 2017 (C1) and one in October 2017 (C2). Both
160 preparations were made with PF standard from kit lot #1. The mean O.D. ± S.D. for C1=
161 0.759 ± 0.095 (CV=13%, n=26 plates) and 0.672 ± 0.062 (CV=9%, n=16 plates) for C2.

162 Data Analysis and Derivation of Shift Factor "S"

163

164 The approach was implemented in the statistical package R, an open software
165 environment for statistical computing and graphics that accepts ELISA optical density

166 data and standard concentration data for calculation of a best-fit Reference standard
167 curve. The lot-to-lot variability is modelled as a fixed batch effect calculated as the
168 difference between each plate's standard curve and the Reference curve. This difference
169 is designated the Shift factor "S". An adjusted plate standard curve is derived using the
170 S factor and used to adjust the biomarker concentrations.

171 The Reference and standard curves are fitted with four- or five-parameter logistic
172 functions (4pl, 5pl). These functions are well established models to relate analyte
173 concentrations to their response signal intensities in immunoassays [8-10]. The 5pl has
174 the form of:

175 $$Y = a + \frac{d - a}{\left(1 + e^{\frac{xmid - x}{scal}}\right)^g} \quad (1)$$

176 where Y is the signal intensity of measurement (OD in ELISA assays); x is the log-
177 transformed concentration of analytes; a and d are the lower and upper asymptotes of
178 signal intensity, respectively; $xmid$ is the x value of the curve's inflexion point; $scal$ is
179 the scale parameter or the inverse of the slope of the curve at the inflexion point ($x = xmid$
180); g is the factor controlling the curve asymmetry. When g takes a value of 1, the 5pl
181 becomes the 4pl function.

182 The 5pl could also be written equivalently as a non-logarithm or exponential form,

183 $$Y = A + \frac{D - A}{\left(1 + \left(\frac{x'}{C}\right)^B\right)^g} \quad (2)$$

184 where Y and g are identical to the parameters in eq. (1); x' is non-log transformed
185 concentration and equal to e^x; A and D are identical to its equivalent lower-case letter

parameters in eq.(1); B and C are equivalent to $xmid$ and $scal$, but have an exponential value of them. In this study, the log-form equation is used for implementation.

To analyze data from multiple plates or lots of ELISA kits, batch effects have to be modelled and corrected for data comparison. Many biological or technical factors could impact immunoassay reproducibility and lead to batch effects [11-15]. Lot-to-lot variability between PF ELISA kits is among such factors. We proposed to model and correct it as a fixed batch effect. It first assumes the lot-to-lot differences mainly result from the variable quantitation of standard analyte concentrations, which can expressed as:

$$\begin{aligned} c &= c_0 \times N \\ \log(c) &= \log(c_0) + \log(N) \end{aligned} \tag{3}$$

where c_0 and c are the real and provided standard analyte concentrations from the manufacturer, respectively, and N is the fold difference between them. We can use x, x_0 and S to replace $\log(c)$, $\log(c_0)$, and $\log(N)$ and rewrite the above log-transformed equation as:

$$x_0 = x - S \tag{4}$$

Therefore the statistical model of the standard curves can be written as:

$$\begin{gathered} Y_{ijk} = a + \frac{d-a}{\left(1+e^{\frac{xmid - x_{ij} + S_i}{scal}}\right)^g} + \varepsilon_{ijk} \\ i = 1,2....n;\ j = 1,2...m;\ k = 1,2..l \end{gathered} \tag{5}$$

where a, d, $xmid$, $scal$ and g are the parameters for the 5pl as in eq.(1); Y_{ijk} is the observed signal intensity (measured OD in ELISA); x_{ij} is the log-transformed

concentration of the jth standard analytes in the ith batch; S_i, the Shifting factor, is the log fold difference between the known concentration and the true one; i and n are the ith and total batch number, respectively; j and m are the jth and total number of standards, respectively; k and l are the kth and total number of measurements; ε_{ijk} is the random error for each measurements. This equation can be further rewritten into:

$$Y_{ijk} = a + \frac{d-a}{\left(1+e^{\frac{xmid_i - x_{ij}}{scal}}\right)^g} + \varepsilon_{ijk} \qquad (6)$$

where $xmid_i = xmid + S_i$ and all other parameters are the same as in eq.(5). As a result, the model indicates that the standard curves of the same batch differ from each other as a result of random errors of measurements, while differences between curves from different batches is ascribed to inaccurate quantitation of analyte concentrations. Furthermore, the standard curves from different batches follow the 5pl functions (or 4pl) with the identical parameters of a, d, $scal$ and g, but different $xmid$. The differences are defined by the Shift factor, S_i, which is estimated through the non-linear regression together with other 5pl parameters.

To do the batch normalization/correction, the analyte concentrations in unknown samples are first estimated based on unadjusted standard curves and then the Shift factor S of the batch is applied to obtain the final quantities:

$$\left(x_{ij}\right)_{adj} = \hat{x}_{ij} - S_i \qquad (7)$$

223 where x is the log-transformed analyte concentrations in the unknown sample, i and j
224 are the batch and standard number as in eq.(5), respectively.

225 It might not be possible to know the precise concentrations of analyte in the
226 standard samples (c_0 in eq.(3)) without other validation methods, such as proteomics [16].
227 Therefore, we designated one batch as the Reference batch, in which the analyte
228 concentrations of the standard samples were treated as accurate, and from that we
229 estimated the Shift factor S of all other batches relative to it. The data from 28 standard
230 curves from PF ELISA kit lot #1 (batch #1) was used to calculate a 4pl reference curve
231 for biomarker PF. For other biomarkers, at least four representative standard curves from
232 at least two lots and three operators were chosen to model the best-fit 4pl Reference
233 curve.

234 Statistical Analysis
235

236 Student's t-tests were performed for analysis of inter-assay differences. Analysis of
237 variance between groups was performed with Bonferroni post-test. $P<0.05$ was
238 considered significant.

239 Software Availability
240

241 An ELISA data analysis tool (*ELISAtools*) with the ability to correct batch effects
242 has been implemented in the statistical R software (version 3.5.1) and is available freely
243 for academic use at https://github.com/BULQI/ELISAtools. Instructions for calculation of
244 S factors is provided in Supplemental Methods.
245

Results

Procedurally, the ELISA manufacturer's directions were followed for reagents, buffers, assay temperature, reagent incubations and wash times. Operationally, these instructions were supplemented with documentation for each plate that included operator, kit reagent lot numbers and clock times for reagent additions and incubation periods. Including a BMC control sample on each plate permitted comparison of data over months (Fig 1). Control optical density (O.D.) readings were consistent with time even with different ELISA kit lots (Fig 1A,B). Myeloperoxidase data (lots #1,2) is shown as a comparison with biomarker PF (lots #1-5). Observations were similar for the other seven biomarkers (data not shown). Myeloperoxidase O.D.s were slightly higher for lot #2, but calculated antigen concentrations were stable (Fig 1C). In contrast, calculated PF concentrations in the BMC controls (preparations C1 and C2) decreased by an average 62.4% between ELISA kit lot #1 and lot #5 over the time (Fig 1D), exceeding our quality control limits. This disconnect between O.D. readings and calculated PF biomarker concentrations over time raised problems for the 420 patient samples already analyzed.

Fig 1. Biomarker Controls with Time

ELISAs were completed over ~10 months as described in Methods for biomarkers myeloperoxidase and PF. (A,B) Optical density (O.D. at 450nm) readings and biomarker concentrations calculated from each plate's standard curve (C,D) for the BMC internal control samples are shown *versus* time. Two ELISA kit lots were used for myeloperoxidase, and five kit lots for PF. Two BMC control preparations were used for PF (C1 and C2), and one BMC control preparation for myeloperoxidase. OD readings for PF controls are reasonably constant with time, but unlike myeloperoxidase, PF concentrations decreased by 62.4% over the study period.

271 Loss of PF antigenicity in the BMC controls during freezer storage could be a
272 contributor. BMC control preparation C1 was prepared in August 2017 and C2 in October
273 2017, and stored aliquots were used over the study period. The averaged optical density
274 values for C1 were higher than C2 ($P<0.01$; C1 O.D.= 0.759 ± 0.095, CV=9%;n=26 plates;
275 C2 O.D. = 0.672 ± 0.062, CV=13%; n=16 plates). However, O.D. values over time for
276 each preparation were reasonably stable (Fig 1B), suggesting PF antigenicity did not
277 change significantly during storage. Despite similar O.D. values, a plate with control C1
278 had a calculated PF concentration of 723.9 pg/mL (mean, triplicate wells) in October 2017
279 with kit lot #1, but the same control calculated as 238.5 pg/mL in June 2018 with kit lot #5
280 (Table 1). A similar change was observed for BMC control preparation C2 samples.
281 Other than the kit itself, a detailed operations review did not identify significant changes
282 in PF antigenicity, environment, equipment or operator contributions that could explain
283 the large change in calculated PF concentrations observed in the BMC controls (data not
284 shown). Notably, no similar changes were observed with the other eight biomarker kits
285 (1-6 kit lots) over the same period.

Table 1. BMC Controls and ELISA Kit Lot number (#)

Assay Date	BMC Control C1	BMC Control C2	Kit Lot #	O.D. (450nm)*	PF Conc. (pg/mL)
10/11/2017	x		1	0.686	723.9
06/19/2018	x		5	0.638	238.5
02/19/2018		x	1	0.717	605.4
06/04/2018		x	5	0.684	225.1

* Mean O.D. ± S.D. is 0.759 ± 0.095 for C1 (CV=13%, n=26) and 0.672 ± 0.062 (CV=9%, n=16)

286 In contrast, comparison of PF standard curves from kit lot #1-5 showed a trend
287 with time (Fig 2A) that paralleled changes observed with the BMC control concentrations.
288 Expected variability between standard curves within the lot was observed (Fig 2B), but
289 lot-to-lot variability showed a left-shift trend. The averaged standard curves for lots #1
290 and #2 (September, November 2017) were similar, but lots #3-5 (April, May 2018) curves
291 had increasing O.D. at each standard concentration. The expected consequence of a left-
292 shift in standard curves will be lower biomarker PF concentrations, which was observed
293 (Fig 1, Table 1).
294
295 **Fig 2. Lot-to-Lot Variability in PF ELISA.**
296 (A) The averaged standard curves for each lot of PF ELISA as the mean optical density
297 O.D. ± S.D. at each standard concentration. N= 28, 19, 8, 4, 9 curves for lots #1-5,
298 respectively. (B) Variability of standard curve optical density at 450nm within and
299 between PF ELISA kit lots #1 and #5.
300
301 To address this analytic variable and rescue our patient data, a strategy was
302 developed such that each PF standard curve is compared to a best-fit Reference curve
303 (O.D. *versus* PF concentration) and a Shift factor ("S") calculated that quantifies the
304 difference between each plate's standard curve and the Reference curve. An adjusted
305 plate standard curve equation is derived and the biomarker concentrations are re-
306 calculated. Thus, every plate has a unique adjustment, based on the calculated S factor
307 for that plate, and results are generated on a uniform platform. A small S factor value
308 indicates the original standard curve on that plate is similar to the best-fit Reference curve.
309 Conversely, a large S factor indicates a larger left- or right-shift relative to the best-fit

310 Reference curve. The PF Reference curve was fitted with a 4-parameter logistics (4pl)
311 equation common to many ELISA analyses, but a 5-parameter logistics (5pl) curve fit
312 option is also available (Supplemental Methods).

313 Assignment of biomarker PF data to calculate the Reference curve was based on
314 our available data, the manufacturer's screening data, and judgment. We used data from
315 28 standard curves (PF kit lot #1) consistent over six months and three operators to
316 calculate the (4pl) Reference curve. Fig 3 shows the Reference curve with unadjusted
317 averaged standard curves for lots #2 and #5. Lot #2 curves had an average S factor of
318 0.0690 (n=19) indicating small differences from the Reference curve. In contrast, lot #5
319 curves had an average S factor of 0.6994 (n=9), indicating a substantial shift relative to
320 the Reference curve.

321

322 **Fig 3. Reference Curve for calculation of S factor.**

323 The best-fit, 4-parameter logistic (4pl) Reference curve (closed circle, R, solid line) from
324 twenty-eight PF ELISA kit lot #1 standard curves is shown as derived from the indicated
325 equation and D= -0.01; A= 3.20; C= 1300.00; B= -1.30. The averaged standard curves
326 for kit lot #2 has an S factor = 0.0690 (open square; n=19 curves) and S= 0.6994 (open
327 triangle; n=9) for curves from kit lot #5.

328

329 S factors for each PF standard curve were calculated (Fig 4). Data from lot #1 was
330 used to fit the Reference Curve, so the average S factor is close to 0 as expected. A shift
331 from the Reference curve for lots #3-5 is shown by their higher average S factors. One
332 lot #2 plate had an S = 0.7032, which is 20.8-fold higher than the average S factor from
333 the remaining plates (mean S=0.0338 ± 0.158; n=18). Data from this plate is under

334 review. Average S factors for lots #3-5 are similar, but those from lot #3 have greater
335 inter-assay variability. The CV for lot #3 S factors is 44.2% (n=8), compared to 16.7%
336 (n=4) for lot #4 and 11.5% (n=9) for lot #5. Whether this is due to manufacturer's
337 differences, sample size or laboratory-based variables is not known.
338
339 **Fig 4. Calculated Shift Factors for PF ELISA Standard Curves.**
340 A 4pl Reference curve was derived from PF kit lot #1 standard curves data and a Shift
341 factor "S" was calculated to quantify the difference between each plate's standard curve
342 and the best-fit reference curve. The S factors for each curve in lots #1-5 is shown with
343 the mean (horizontal line) for each lot. S factors for lots #1 and #2 curves are not different,
344 but those for lots #3-5 differ significantly from lot #1 (***P<0.0001, ANOVA with Bonferroni
345 post-test).
346
347 The BMC control PF concentrations for preparations C1 and C2 were re-calculated
348 using the S factor for each plate's standard curve. The reference curve was based on kit
349 lot #1 data and C1 was assayed primarily with kit lots #1 and #2. Thus, the average PF
350 concentration did not change significantly (P=0.843), but the variance was reduced (P=
351 0.002), as expected (Fig 5). C2 controls were included primarily on plates from kit lots
352 #3-5, so the difference after correction with S factors is significant (P<0.0001).
353
354 **Figure 5. BMC Control PF Concentrations.**
355 A BMC control sample from preparation C1 or C2 was included on each ELISA plate for
356 the 10 month study period and assayed with kit lots #1-5. C1 was used primarily on plates
357 from kit lots #1 and #2. C2 was used primarily on plates from kit lots #3-5. Standard

358 curves from each plate were adjusted according to their calculated S factor and the control
359 PF concentrations were re-calculated. The data shows the PF concentrations before and
360 after correction with the S factors. ***P<0.0001; ns, not significant (Student's t test).
361
362 S factors for myeloperoxidase, soluble P-selectin and plasminogen activator
363 inhibitor-1 were calculated (Table 2). Data for 4pl Reference curves was chosen from
364 four standard curves performed by 3 different operators over at least 6 months to
365 represent the composite data and inter-assay variability. One myeloperoxidase curve
366 had an S = 1.099, and this data is under review. All BMC controls were within quality
367 control limits. Overall, their calculated S factors agree with consistent BMC Control
368 values over time and between kit lots.
369

Table 2. Average S Factors (min, max; N) for Biomarker Standard Curves from Kit Lots

Biomarker	Lot 1	Lot 2	Lot 3
Myeloperoxidase	0.041 (-0.352, 0.347; 10)	0.505 (0.085, 1.10; 35)	0.289 (0.231, 0.382; 3)
Soluble P-selectin	-0.032 (-0.294,0.156; 42)	0.105 (-0.153, 0.393; 4)	----
Plasminogen activator inhibitor-1	-0.226 (-0.220,0.107; 22)	-0.056 (-0.373,0.047; 22)	-0.008 (-0.043,0.039; 2)

370
371

Discussion

Acquiring ELISA data using commercial research kits over a prolonged period presents challenges for quality control needs. There is potential impact of multiple operators, antigen stability, environmental and equipment drift, lot-to-lot reagent variability, and lack of validated controls [15, 17-20]. It is virtually impossible to manufacture a new reagent lot that is identical in all respects to prior lots, and reagent variability occurs even with commercial diagnostic assay reagents [21]. Yet minimizing these influences is necessary so patient or other experimental data can be compared with confidence over the study period. In this study, changing BMC control concentrations for biomarker PF first raised the potential of excessive lot-to-lot variability in the ELISA kits. The optical density data did not change significantly, but calculated PF concentrations greatly decreased, and this disconnect triggered the comprehensive data review. After excluding other reasonable contributors, the S factor strategy was developed to provide a uniform platform for comparison of patient PF data collected over many months.

Calculation of S factors allowed retrospective adjustment of each plate's standard curve. The averaged S factors for PF kit lots #1-5 ranged from -0.086 to 0.735, and reduced the BMC control inter-assay variability to within our quality control limits (<9%). Review of the S factors for each standard curve was useful in that we could rapidly identify possible outliers. One curve each from PF ELISA lot #1 (high) and lot #3 (low), and one for myeloperoxidase (high) were sufficiently different and those plates were tagged for review. Unanticipated variability occurred even with SOPs, required documentation, equipment calibration and heightened operator awareness of pre-analytic variables. Controlling pre-analytic variables for quality assurance and method validation is difficult

396 [22] and identifying trends on a day-to-day basis is largely subjective. Once a Reference
397 curve is established, calculating the S factor for each curve provides rapid and
398 quantitative comparative data.

399 Few research-use ELISA kits include validated controls, so the early decision to
400 include spiked plasma controls on every plate was advantageous. One limitation of
401 laboratory-made controls is that each preparation will be slightly different and those
402 differences may not be apparent until enough plates have been run to set the quality
403 control ranges. Access to independently standardized and validated controls in a variety
404 of matrices (plasma, serum, urine, etc) for established and emerging biomarkers would
405 be a valuable resource for investigators and facilitate more consistent biomarker results
406 between laboratories. This in turn supports the goals of improved data rigor and
407 reproducibility, and the more ethical discussion regarding disclosure of validated data to
408 study participants [23-25].

409 The problem of inter-assay variability is not new [26] and various approaches are
410 proposed to quantify data that are acquired in batches using conversion of a signal from
411 known samples into a meaningful value for unknown samples [15, 27]. For *in vitro*
412 diagnostic ELISA kits used in a clinical laboratories, quality assurance is provided by a
413 validated control sample(s) with defined value limits. Research-only ELISA kits do not
414 have this foundation, yet are used universally. Calculation of S factors and retrospective
415 re-analysis with adjusted standard curves is useful when quality control values exceed
416 limits and environmental, procedural, equipment or operator contributions are ruled out.

417 This strategy should be used judiciously primarily because data choices for the
418 Reference curve is partially subjective. We chose data from PF ELISA lot #1 based on
419 volume of data, relative consistency over many months and similarity to the

420 manufacturer's data for that lot. We could have chosen standard curve data from lot #5
421 plates instead, but there were fewer curves over a shorter time period and our collective
422 experience judged this to be a less favorable choice. We do not anticipate using this
423 strategy for the other biomarkers. Their inter-assay ELISA variability is acceptable, their
424 BMC controls remain within limits, and there is no justifiable need to adjust the curves.
425 That said, we have found the S factors to be a useful monitor of ELISA outcomes to
426 rapidly identify pre-analytic factors, such as operator differences, that otherwise may be
427 difficult to identify by visual inspection of the data, particularly for long-term projects.

428 There are other approaches to adjust for batch effects in immunoassays similar to our
429 current work. More complicated statistical approaches are employed, such as the mix-
430 effect model [15] and the iterative maximum likelihood method [27]. They assume a linear
431 relationship between the measured signals and the analyte concentrations, which is only
432 an approximation and has its own limitations. Our implementation takes the form of a non-
433 linear logistic function. Our proposed statistical model is simple and the assumption is
434 appropriate for the observed lot-to-lot variability (Supplementary Figure 2).

435 We focused on developing an accessible and generalizable strategy to solve similar
436 issues as long as the assumptions are met. The software is implemented in R and the
437 data input uses a format familiar to those who use standard 96 well plates for ELISAs. It
438 is written for either Mac or PC and a choice of 4pl or 5pl curve fitting is provided. The
439 software is open source and in the public domain, with instructions for data input in the
440 Supplemental Methods.

441

442

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551

552 # Supporting Information
553

554 Supplemental Methods provides instructions on how to load and use ELISAtools for
555 calculation of S factor(s).
556

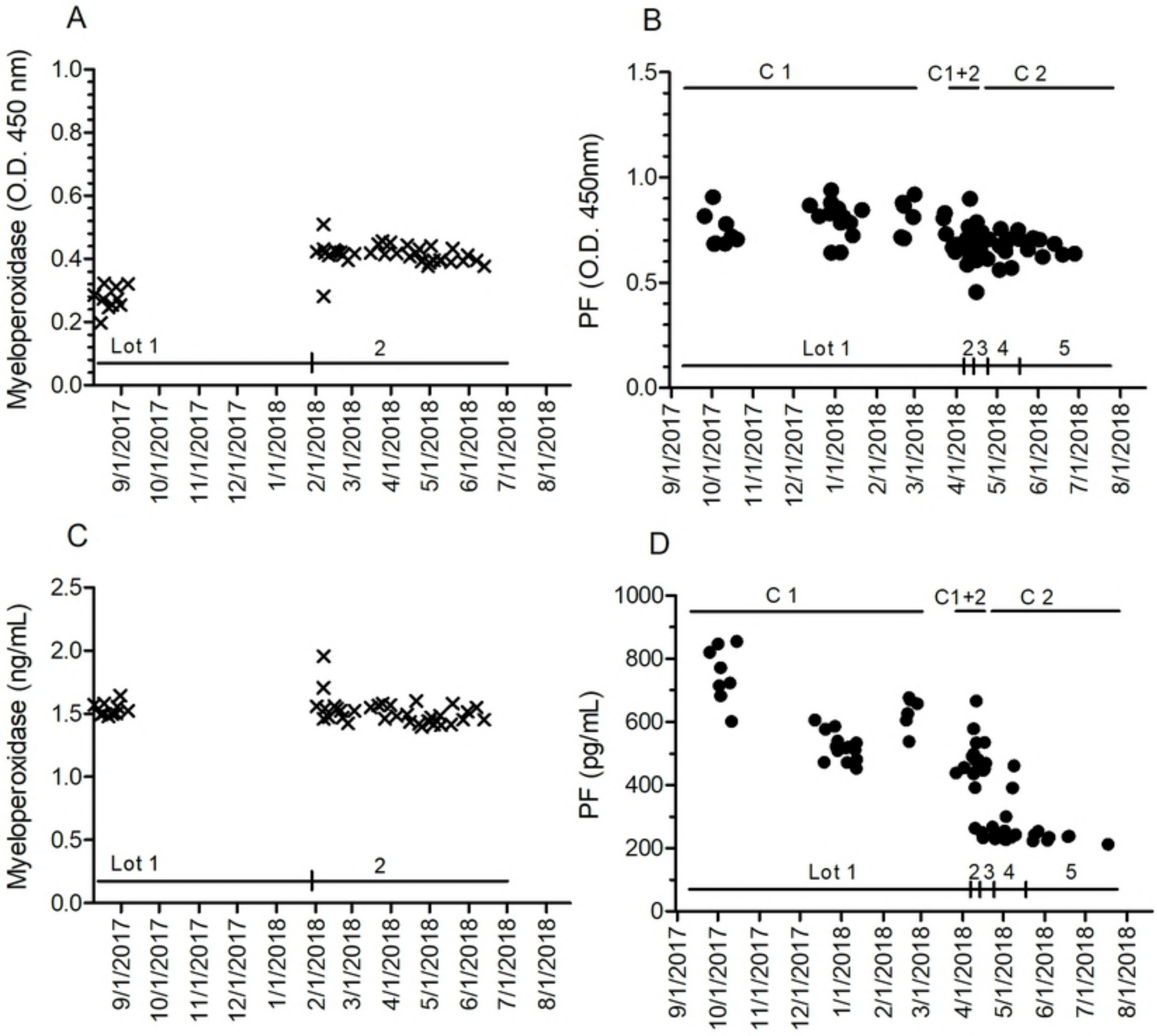
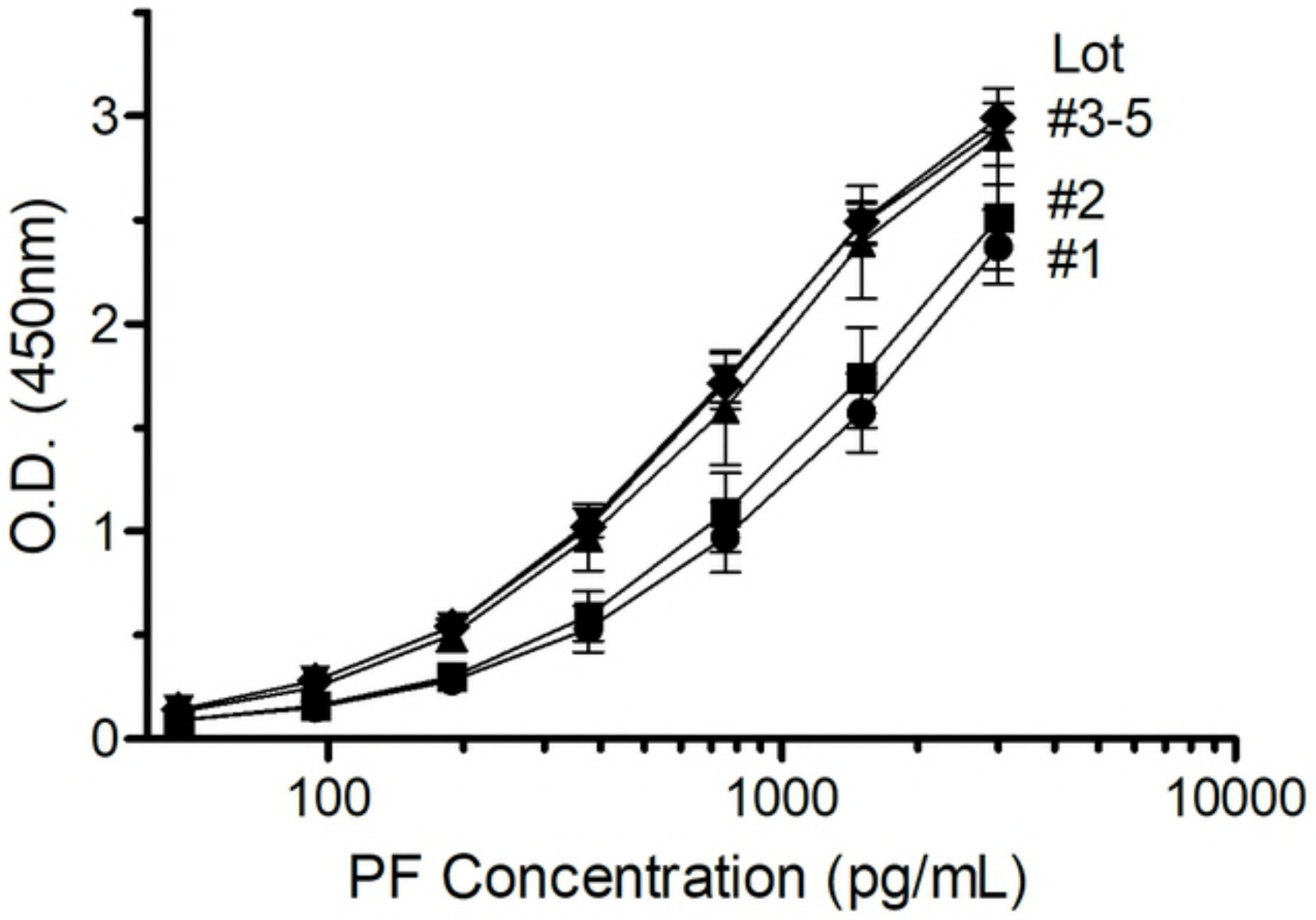

A
Myeloperoxidase (O.D. 450 nm)
1.0
0.8
0.6
0.4
0.2
0.0
Lot 1
2
9/1/2017
10/1/2017
11/1/2017
12/1/2017
1/1/2018
2/1/2018
3/1/2018
4/1/2018
5/1/2018
6/1/2018
7/1/2018
8/1/2018
B
PF (O.D. 450nm)
1.5
1.0
0.5
0.0
C 1
C1+2
C 2
Lot 1
2 3 4
5
C
Myeloperoxidase (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
D
PF (pg/mL)
1000
800
600
400
200
0

Figure 1

A


Lot
#3-5
#2
#1
O.D. (450nm)
0
1
2
3
100
1000
10000
PF Concentration (pg/mL)

B

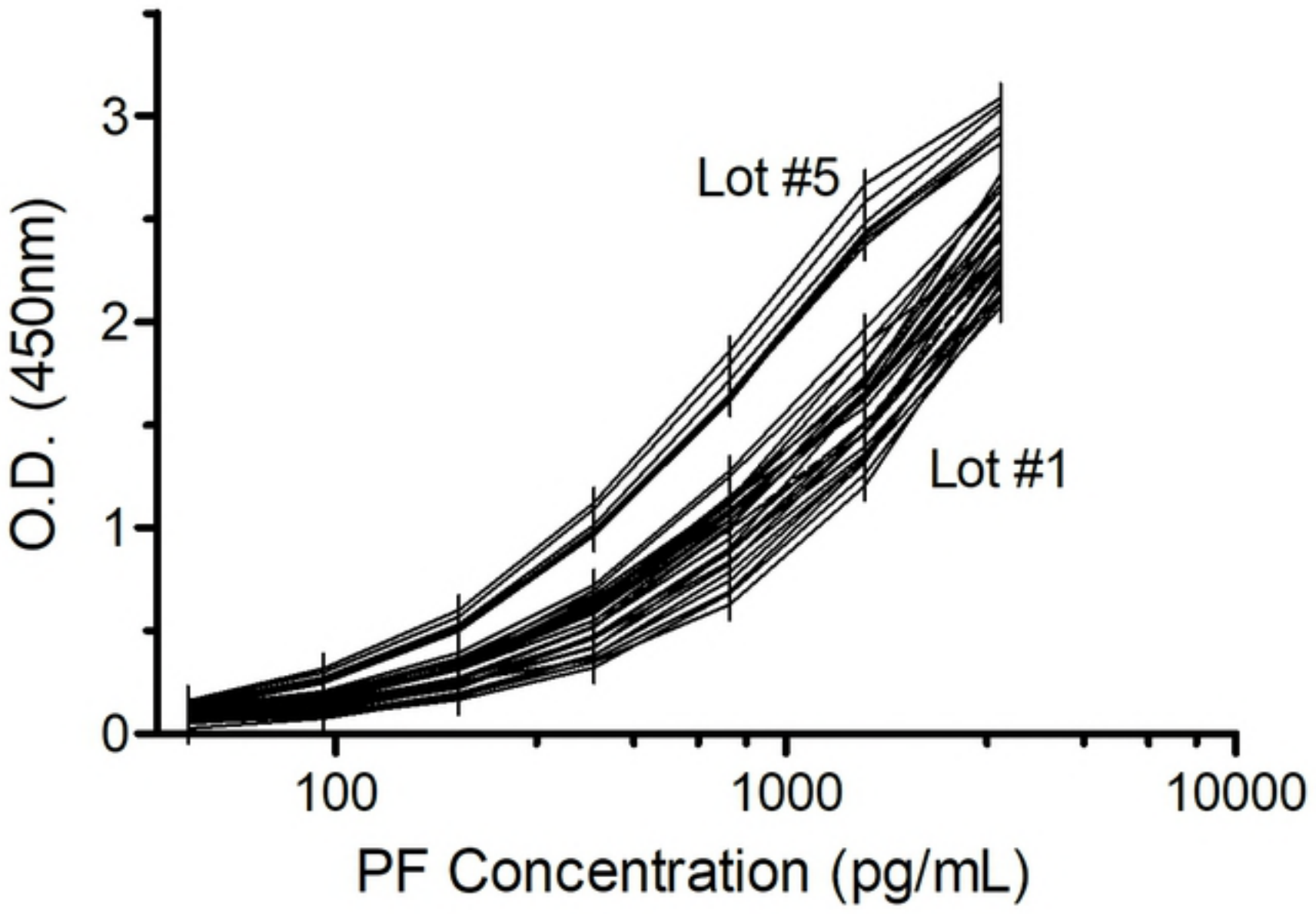

Lot #5
Lot #1
O.D. (450nm)
0
1
2
3
100
1000
10000
PF Concentration (pg/mL)

Figure 2

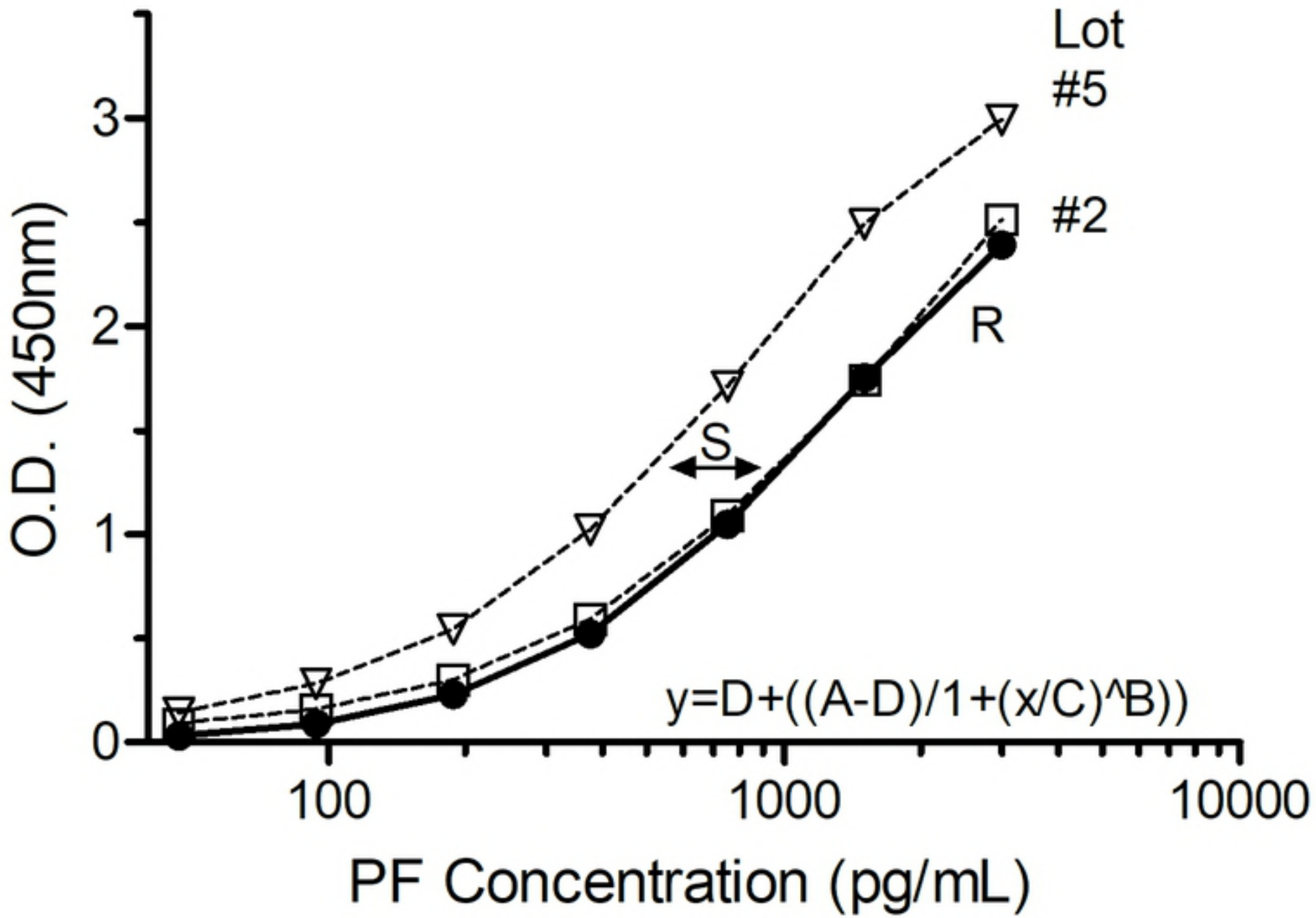

Lot
#5
#2
R
S
y=D+((A-D)/1+(x/C)^B))
O.D. (450nm)
0
1
2
3
100
1000
10000
PF Concentration (pg/mL)

Figure 3

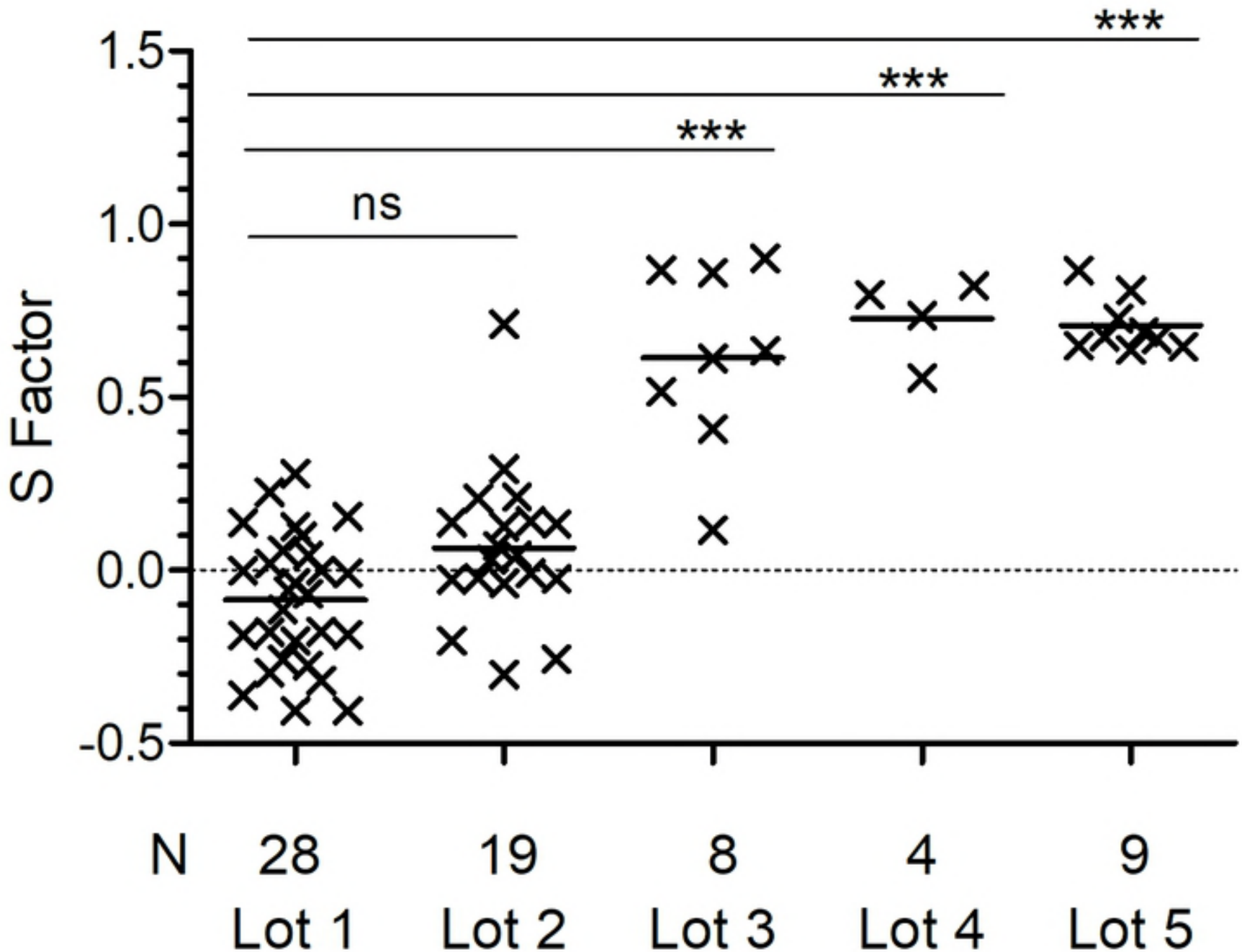


Figure 4

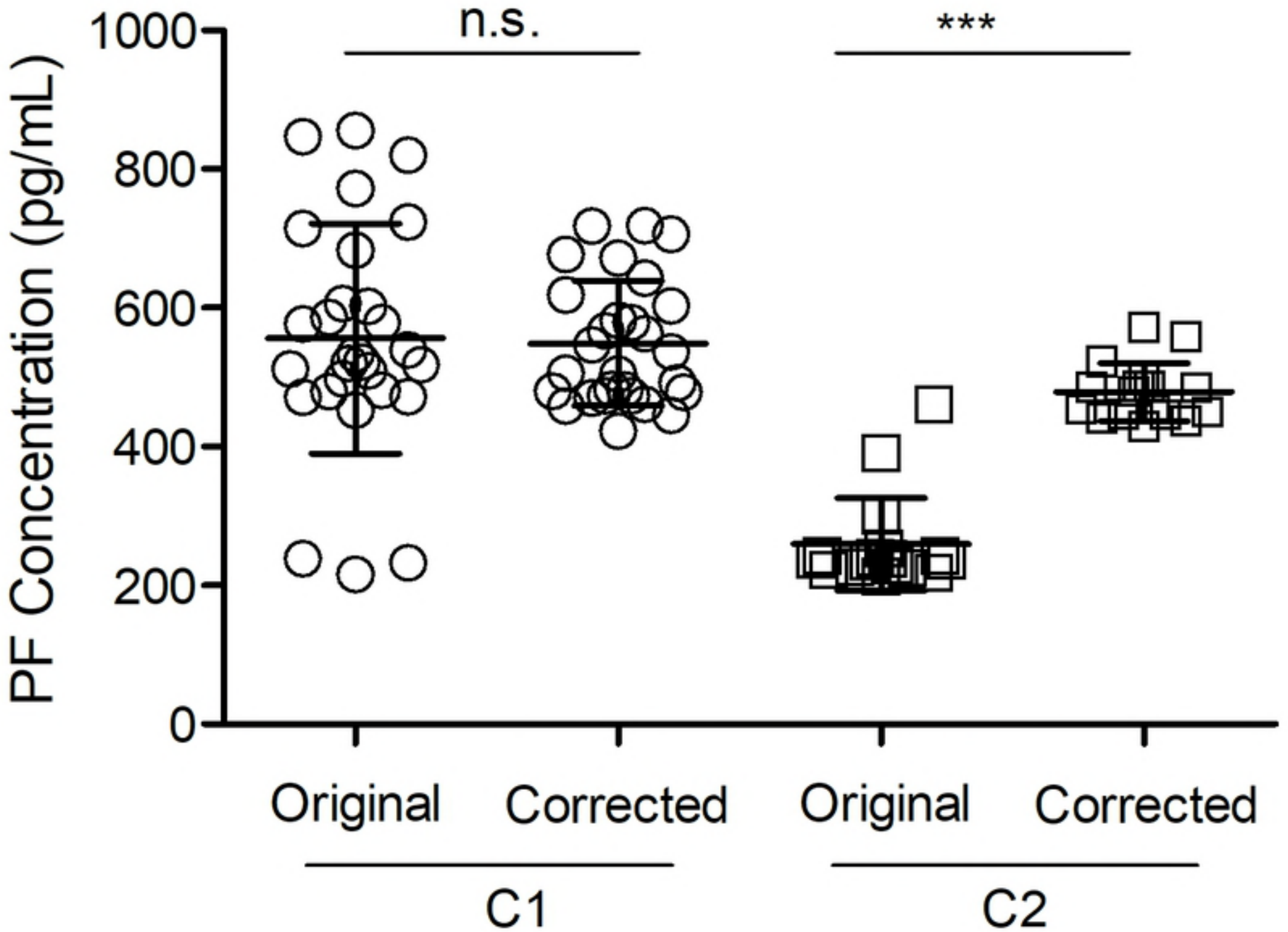

n.s.
***
PF Concentration (pg/mL)
1000
800
600
400
200
0
Original
Corrected
Original
Corrected
C1
C2

Figure 5