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Understanding the contribution of operator measurement variability within flow cytometry data analysis for quality control of cell and gene therapy manufacturing

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27 **Abstract – 150 words**

28 Flow Cytometry is a measurement technique used in Quality Control and in-process
29 measurements of biomanufactured Cell and Gene Therapy products. However, it contains
30 a number of sources of measurement variation at; sample preparation, instrument setup,
31 analysis, and post-analytical data analysis stages. The latter sees variation introduced
32 from operator subjectivity, which is investigated here to understand what effects the
33 interpretation of diagrammatical protocols have on inter-operator analysis.

34

35 36 operators from different sites were given a series of histograms to analyse, gating a
36 shifting peak. This was repeated with diagrammatical protocols to apply gates which
37 reduced inter-operator variation by up to 92%. Various control limits include and exclude
38 different results and when adjusted with a log transform differences in outlier discrimination
39 have been found. This research supports the use of Flow Cytometry diagrammatical
40 protocols to reduce the contribution of inter-operator variation and measurement
41 uncertainty associated within Cell and Gene Therapy manufacturing scenarios.

42 **Keywords**

43 Operator Variation, Cell Therapy Manufacture, Quality Control, Data Analysis, Flow
44 Cytometry, Biometrology

45 **Highlights**

- 46 • There is up to a 92% reduction in variation when operators follow prescribed
47 protocols for analysis.
- 48 • Different control limits can be used to define in or out of specification results, but the
49 effect of these limits must be carefully chosen to ensure values falling out of bounds
50 are understood.
- 51 • Log transforms, when applied to 'normalise' data, can change the outliers defined
52 by control limits.

53 **1.0 Introduction**

54 High value products require significant attention during their manufacture, to ensure
55 consistency of product to a detailed, quality release specification. High-value manufacture
56 covers a number of different industries, with Cell and Gene Therapy (CGT) manufacturing
57 [1] being a rapidly growing sector within the UK. The market for CGT products has grown
58 significantly in potential in recent years, and with licensed products such as Yescarta™ [2]
59 and Kymriah™[3] becoming available, an ever-increasing focus must be paid to Quality
60 Control (QC) of products, to maintain high standards now, and when scale up or out
61 across distributed biomanufacturing platforms becomes a possibility.

62
63 Any deviation in the biomanufacturing, and uncertainty in the measurement, can waste
64 critical time and costly materials, thereby impacting the safety of the patient as well as
65 increasing the cost of goods. Improved control over biomanufacture and release provides
66 greater certainty in decision-making, allowing better success rates of the expensive
67 therapeutics through the biomanufacturing route, thus better delivery of high-quality
68 therapeutics to the patient, although it is noted that measurement uncertainty may well
69 persist and requires management.

70
71 Flow Cytometry (FC) is a significant Quality Control (QC ¹) release measurement
72 technique used to test metrics such as cell count, purity and product characteristics
73 attributed to cellular phenotype and product definition [4]. FC is a laser-based analytical
74 method often using up to 18 exclusive analysis channels, that can interrogate multiple
75 combinations of fluorescent biomarkers (used to uniquely tag cells) at the single cell level
76 through the use of specific antibodies conjugated to distinct fluorophores. Cells can be

¹ QC = Quality Control

77 labelled with specific antigen markers which help to identify specific cellular states with
78 positive and negative controls. A preparation of labelled cells are hydrodynamically
79 focused through a laser pathway, with fluorescence emission spectra from each
80 fluorophore being recorded, using narrow line width optical separation mirrors and filters to
81 allow sensors and detectors to pick up specific bandwidths of light emitted from the
82 fluorophore markers attached to the cell surface.

83
84 Sample preparation time is lengthy and the irreversible nature of tagging the cells for this
85 technique currently limits its use in-process for Cell Therapy Product (CTP)² development,
86 where regulations stipulate that a sample cannot be administered to a patient post-
87 analysis. FC instruments can be integrated into Fluorescence Activated Cell Sorting
88 (FACS)³ platforms to sort the required cells on the basis of electrical impedance, but the
89 principle remains the same and the irreversible nature of fluorescence tagging means
90 these samples still cannot be administered post-analysis. Variation in staining reagents
91 and the sample preparation itself is a well-known problem in the industry, with many
92 international efforts made to reduce variation from these sources [6–11].

93
94 To set up the FC system, voltages relating to sensor gain and amplification of fluorescence
95 signals are set for the relevant laser channel being used for excitation of specific markers.
96 This can be optimised with fluorescent calibration beads and antigen marker titration, to
97 ensure cellular populations can be fully visualised and are not close to limits of sensitivity
98 of the instrument. The data relating to the measured fluorescence is visualised using a
99 series of scatter plots, with each point representing a single measured event or cell [5].
100 Typical FC analysis of cells requires a minimum of 30,000 cell events to be acquired.

² CTP = Cell Therapy Product

³ FACS = Fluorescence Activated Cell Sorting

101 These cell events can be visualised as histograms (for just one marker), or scatter plots to
102 visualise two or more markers at the same time. Different positive and negative
103 fluorescence areas can help to identify positive and negative markers on the cell surface
104 which relate to different cellular states to identify.

105

106 Assay or test determination and discrimination is a function of interpretation of the FC
107 output data, by an operator often applying a series of subjective software gates within
108 multi-dimensional scatter plots to determine positive and negative thresholds. This
109 requires correct discrimination of data sub-populations that are characterised by noisy
110 clusters of data, which introduces inherent subjectivity and increased measurement
111 uncertainty in the data analysis and assay conclusions.

112

113 FC is an example of complex metrology whereby whole instrument uncertainty statements
114 have yet to be fully defined. There are many sources of variation which can contribute to
115 the overall uncertainty of FC measurements, with the Ishikawa diagram in Figure 1
116 illustrating many of these components. The focus of the research reported here is, for the
117 first time, to understand the impact of operator subjectivity within Flow Cytometry data
118 analysis for a QC Cell Therapy exemplar. With respect to Figure 1, this is the Operator
119 branch of the Ishikawa diagram and encompasses all elements apart from the parallax
120 term. In addition, for the first time this research starts to define the variation magnitude that
121 would help to quantify an operator uncertainty term. In addition, common statistical tools
122 are mapped to determine the consequence on outlier definition and exclusion. Whilst the
123 work reported is not intended to develop or deliver a whole instrument uncertainty
124 statement, it is fully anticipated that the results of the operator variation analysis will
125 eventually inform and contribute to a detailed uncertainty analysis.

126

129 Figure 1: Ishikawa diagram listing multiple sources of uncertainty

130 2 column width

132 This paper has been organised to describe the protocols and standards used to generate
133 files for subjectivity studies (Section 2), with a definition of the statistical tools and outlier
134 discriminators used for data processing defined in Section 3, whilst Sections 4 and 5
135 demonstrate how these studies were conducted with participants. The study results have
136 been discussed in Section 6, with greater emphasis paid to outlier boundary estimators in
137 Section 7, to identify differences in control limits used within manufacturing contexts.

138

139 **2.0 Flow Cytometry Protocols and Standards**

140 **2.1 Standards and regulation**

141 In any manufacturing context, it is important to know and quantify the sources of variation
142 in a process or measurement, to better comprehend the allowable fluctuation within a
143 system. Within biomanufacturing and its associated regulatory capacity, all clinical testing
144 or pathology laboratories complying to ISO 15189 guidelines need to be able to quantify
145 uncertainty within a process or measurement [12] and are supported by the International
146 Committee for Harmonization (ICH)⁴ Guidelines for Good Manufacturing Practices
147 (GGMP) [13]. Within FC, there are an inherent number of variation sources as shown in
148 Figure 1, from upstream sample preparation through to data analysis, as well as the
149 choice of statistics used. Hence operator perception and decision making can increase the
150 variability of data being gated and analysed at a simplistic, univariate level. When faced
151 with analysing highly dimensional, compounding data, the variability in output may
152 potentially correlate with the complexity and difficulty of the data in question. However, ISO
153 15189 and the ICH GGMP currently do not recognise or accommodate for inter operator
154 variability.

155

156 **2.2 Software processing**

157 Software exists to assist with the subjectivity of gate application by human operators [14],
158 with many automated analysis platforms accessible for use applying initial gates based on
159 a variety of statistical clustering and Principal Components Analysis (PCA)⁵ methods.
160 Gating-ML (extensible Markup Language) facilitates inter-site comparison of data by
161 creating a file type that transfers pre-applied gates to data [15]. Various platforms and
162 open source code software such as SPADE (1, 2 or 3), RMeans and FlowClust, are

⁴ ICH = International Committee for Harmonization

⁵ PCA = Principal Components Analysis

163 available to group FC data to identify subsets based upon location of raw data values [16].
164 Whilst these partially replace the operator when identifying subsets, they can be difficult to
165 implement when the data is not easy to separate, often requiring manual checks post-
166 processing. There will also potentially be variability in the end results produced from
167 different analysis software, due to differences in algorithms, coding methods and statistical
168 analysis applied.

169

170 **2.3 Analysis standards**

171 The International Society of Analytical Cytology (ISAC)⁶ champions standards to aid gating
172 in the Flow Cytometry field; known as Optimised Multicolour Immunofluorescence Panels
173 (OMIPS)⁷ [17]. These Panels contain significant guideline information relating to the setup,
174 staining and analysis of the cells for immunophenotyping and characterisation of specific
175 immunological markers within certain disease states. As such they are a useful model
176 guideline series, even if the content may not be specific to the analysis being conducted.
177 However, they do contain information relating to antibody clone and suggested fluorophore
178 combinations for targets so do contribute to standardisation through both documentary
179 and physical means. However, even following the gating protocols in these standards, the
180 authors have observed that there still remain elements of subjective interpretation.

181

182 **2.4 External Quality Assessment**

183 Various clinical External Quality Assessment Schemes (EQAS)⁸ exist globally to monitor
184 variability and performance of multiple clinical and pathology centres analysing blood
185 samples using Flow Cytometry [18]. UK NEQAS runs multiple schemes for different
186 enumeration and classification assays and defines performance boundaries based upon

⁶ ISAC = International Society of Analytical Cytology

⁷ OMIPS = Optimised Multicolour Immunofluorescence Panels

⁸ EQAS = External Quality Assessment Schemes

187 the observed distribution and variation of data across centres when analysing standard
188 samples. Historically, 95% Confidence Intervals or 10% trimmed mean $\pm$ 2 Standard
189 Deviations (SD)⁹ have been used to specify data falling outside control limits, and so
190 identify centres for retraining. This facilitates continuous improvement and lowers overall
191 variation over time [18,19], but importantly EQA schemes do not identify sources of
192 uncertainty or evaluate uncertainty budgets. Through discussion with UK NEQAS, it has
193 been further reinforced that subjective interpretation is prevalent within FC manual gating.

194

195 However, when applying similar control limits or error estimators to patient data or a cell
196 therapy product, 'outliers' will be common within the probability distribution of the data
197 (parametric or non-parametric) and cannot be excluded because they represent a specific
198 patient or patient data upon which decisions must be based. Previous work has shown
199 that patient data for cell counts for cell therapy starting materials can vary by up to 4
200 orders of magnitude with positively skewed distributions, which potentially creates
201 significant difficulty when manufacturing a product that has to cope with this incoming
202 variation [20].

203

204 **2.5 Control limits for manufacture**

205 When defining control limits for product manufacture and release, validation is usually
206 based upon historical data sets and company specifications. Differences in these bounds
207 rely on correct analysis of population spread, to infer the correct location and spread
208 parameters. Methods can be broken down into three statistical approaches; Normal
209 statistics outlier estimators, Robust statistics outlier estimators, Multivariate statistics
210 outlier estimators.

211

⁹ SD = Standard Deviations

212 Normal distribution statistics are most commonly used to describe data, but this causes
213 problems when distributions are heavily skewed and non-parametric in shape. Log
214 transformation of this data is a traditionally common method employed to negate analysis
215 issues with differing data spreads, but when critical Quality Control Indicators (QCI)
216 decisions rely on data output, as well as the outcome of patient data regarding treatment,
217 it is best to focus on the raw data to ensure that resolution and discrimination are accurate.
218

219 3.0 Statistical Analysis Methods

220 Determining outliers is completed by calculation of location and spread values of a data
221 set in question. Location parameters such as the mean, median or trimmed means are
222 points used to represent a population and spread parameters such as Standard Deviation
223 or Absolute Deviation represent the amount of fluctuation around the defined location
224 parameter. Historically, boundaries such as $\text{Mean} \pm 2 \text{ SD}$ have been used, because they
225 approximately align to a 95% Confidence Interval, assuming normally distributed data.

226

227 3.1 Normal statistics outlier estimators

228 These parameters can be calculated with any substantial data set, as long as their
229 distribution represents that of the raw data in question. Often, normal distribution statistics
230 are used to process and represent data that may be non-parametric in nature, potentially
231 leading to misinterpreted information and a skewed judgement, conclusion or pass/fail
232 decisions. Robust statistics are an alternative, opting to use medians instead of means, for
233 example when data has a fat tail and mean values are often positioned further along the
234 tail than the highest frequency point. Using median location values negates this problem.

235

236 Table 1 identifies various outline boundaries that are based upon normal distribution and
237 robust statistics, found to be used across many examples of FC analysis and within other
238 industries. Whilst this is not an exhaustive list, the majority use univariate data to
239 understand distributions. Regression analysis requires two input variables to determine a
240 95% Confidence Interval, whereas $\text{Mean} \pm 3\text{SD}$ are traditional bounds, originally
241 championed in the use of control charts to monitor process quality over time [21]. These
242 have been amended for use in analytical chemistry QC [22] but were refined to $\text{Mean} \pm$
243 2SD in the 1990's FC EQAS [6,7,23], alongside use of 95% Confidence Intervals, defined
244 by Standard Error of the data set.

245

246

[Insert Table 1 Here]

Error Boundary Estimator	Normal/Robust Statistical Methods	Data Dimensionality	Outlier Limits/ Accommodates Data
Shewhart’s Control Charts Mean $\pm$ 3SD	Normal	1 parameter	Outlier limits
EWMA charts	Robust	1 parameter	Outlier limits
Mean $\pm$ 2SD Calculation from Type I errors	Normal Normal	1 parameter 1 parameter	Outlier limits Outlier limits
10% trimmed Mean $\pm$ 2SD	Normal	1 parameter	Outlier limits
Median $\pm$ 2MAD	Robust	1 parameter	Outlier limits
M-Estimators	Robust	1 parameter	Accommodates data
Paxton’s Criterion	Robust	1 parameter	Outlier limits
Linear regression models	Normal	Minimum 2 parameters	Outlier limits
Robust Multivariate regression	Robust, but transforms data to normal	Minimum 2 parameters	Outlier limits
Longitudinal mixed effects models	Robust	Minimum 2 parameters	Outlier limits
Bootstrapping	Normal, or log transformation of non-normal data	1 parameter	Outlier limits
Euroflow Personalised outlier estimator	Robust, but transforms data to normal	1 parameter	Outlier limits

247

248

249

250

Table 1: Common outlier estimator methods used within Flow Cytometry and other industries.

1.5 column width

251 3.2 Robust statistics outlier estimators

252 When considering robust statistics, the Exponentially-Weighted Moving Average
253 (EWMA)¹⁰ Chart (Table 1) is an alternative to the Mean $\pm$ 3SD method, that adjusts to the
254 data set and is often preferred when detecting small shifts in components [24]. The use of
255 EWMA charts to track data has been recommended more as an internal assessment [25],
256 because it can be easily understood in a similar manner to a Levy-Jennings plot, and for
257 this reason has not been used for analysis or plotted in subsequent figures. Trimmed
258 means are another alternative location parameter, involving recalculation of new spread
259 metrics from a refined data set removing a certain percentile of data from the extremes,
260 making the distribution more centralised [25]. This has been popular in the UK NEQAS FC
261 Schemes [26,27], allowing pathology laboratories identified as extremes to improve over
262 time. However, if used incorrectly it can remove extremes that require investigation into
263 causality, and still represent probable events occurring.

264

265 A robust alternative to the Mean $\pm$ 2SD, is the Median $\pm$ 2 Median Absolute Deviations
266 (MAD)¹¹ [28]. This uses the Median value of residuals from the location median to estimate
267 spread, which is more aligned to non-parametric distributions. An additional traditional
268 robust method was described for an early FC EQAS (Paxton's Criterion), that uses
269 parameters calculated to also define Box and Whisker plots, from 25th and 75th percentiles,
270 and subtraction and addition of the Interquartile Range respectively [29]. Bootstrapping is
271 a resampling method used as an alternative technique for validation [30,31] that derives
272 confidence intervals, making assumptions on the data probability distribution, and
273 assumes a normal distribution or uses a log-transform of non-normal data. This is a
274 suitable way of defining bounds if it fits the raw data or if the transform sufficiently equates.

¹⁰ EWMA = Exponentially-Weighted Moving Average

¹¹ MAD = Median Absolute Deviation

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3.3 Multivariate statistics outlier estimators

As previously mentioned, regression analysis is a common method for specifying data-fitness bounds. Linear regression analysis (Table 1) minimises the residual sum of squares and uses residual plots to detect outliers. Linear regression has been used to compare FC EQAS approaches [32], with further developments building upon this by using robust multivariate regression [33], which is less sensitive to outliers due to transformation of non-normal data.

Longitudinal mixed effects models have been employed more recently by UK NEQAS [34,35]. This uses a mixture of pass criteria based upon different residual and deviate values, allowing for multiple stratification levels of the data, based upon robust statistics. An alternative method uses M-estimators, that accounts for all of the data, but weights extreme values to shift them to an optimal boundary, also known as Winsorizing means. This does not technically exclude any data points as previously described methods do, but still uses normal statistics to define initial boundary points.

292 4.0 Histogram File Generation for this Study

293 A series of .fcs 3.0 files were generated using a BD Bioscience FACSCanto II Flow
294 Cytometer with 4-2-2 optical configuration, by running a suspension of BD Bioscience
295 Cytometer Setup and Tracking (CS&T)¹² beads [36]. The CS&T beads were a mix of dim,
296 medium and brightly fluorescing polystyrene beads and are used to monitor baseline and
297 daily performance of FC instruments. The beads were selected for use due to their lesser
298 variation compared to biological variance. The beads were run through the allophycocyanin
299 (APC)¹³ channel, excited by the red laser (633 nm). The APC channel was used, because
300 it is a common channel across a wide variety of FC instruments.

301
302 Voltages, and therefore fluorescence peaks, are adjusted to determine the optimal analysis
303 parameters in line with instrument sensitivity. The gain can be changed by voltage setting
304 to alter the position of the fluorescence peaks seen through the APC channel on a univariate
305 scale. Many methods have been developed to determine Flow Cytometry analysis
306 parameters, but the most popular, referred to as the 'Peak 2 method' involves running a
307 control sample at different voltage intervals and calculating the stain index [37].

308
309 The .fcs files generated for this work (Figure 2) were gathered at ten different voltages from
310 250 V to 700 V, in 50 V intervals, allowing for resolution differences to be seen whilst not
311 over-exerting the concentration of operators involved in the study. The .fcs figure elements
312 in Figure 2 show the progression of the three data peaks across the univariate scale, in
313 relation to the gain increase of the APC channel of the Flow Cytometer. This figure also
314 shows the range gates (red circle) applied to the histograms, which participants were asked
315 to copy.

¹² CS&T = Cytometer Setup & Tracking

¹³ APC = Allophycocyanin

[Insert Figure 2 Here]

318 Figure 2 Images of histograms provided for participant gating studies (250 V to 700 V)

319 2 column width.

320 **5.0 Operator Study Methodology**

321 36 participants were enrolled in this study to analyse the histogram files; 10 from an
322 academic institution, 19 from an industrial cell therapy process development team and 7
323 as part of a data gathering exercise at the 2017 FIRM Symposium [38]. Ethical and GDPR
324 approval and was obtained for this study by local University Human Participant Ethics Sub-
325 Committee. Participants completed the study individually and were asked to complete two
326 phases of analysis. The two phases aimed to monitor the effects of personal data
327 interpretation, in comparison to following a pre-defined protocol, or interpretation thereof,
328 with the hypothesis of reduced inter-operator variation for FC analysis when following the
329 latter.

330

331 **5.1 Phase 1 Histogram gating – Operator judgement**

332 During Phase 1, participants were asked to apply range gates to the area of the plots
333 where they estimated the medium fluorescing peak lay. Five of the plots were considered
334 to have poor peak separation (Figure 2 a-e), where there was difficulty in discerning
335 peaks. The remaining five files were considered to have good peak separation (Figure 2 f-
336 j) and to be more representative of good Flow Cytometry data. Participants used the third-
337 party software FlowJo Version 10.0.8r1 to examine the .fcs files and apply histogram
338 gates, using default bi-exponential scaling and maintaining the histogram view setting.

339

340 **5.2 Phase 2 Histogram gating – Protocol driven**

341 For Phase 2, participants were provided with printed images of range gates (highlighted by
342 the red circles in Figure 2) that had been pre-applied to the series of files and were asked
343 to adjust their gates to try and match these 'diagrammatical protocols'. These gates were
344 applied to aid precision of application, not accuracy, because accuracy can be translated
345 into different subject contexts. The poor gated files were included to demonstrate the

346 potential impact and worth of using diagrammatical protocols in difficult analysis
347 conditions.

348

349 **5.3 Analysis metrics**

350 Data was statistically and graphically processed in IBM SPSS for Statistics Version 24 and
351 Matlab R2018. Range gates applied to histograms of one fluorescence channel show
352 variation in spread and location parameters, chosen by perception of the operator. The
353 Robust Coefficient of Variation (rCV)¹⁴ (as defined in Eq. 1) is a commonly used metric
354 within FC data analysis to compare variation amounts between operators and
355 measurement phases. It helps the operator to understand and monitor variation within a
356 system, combining both location and spread parameters into one measure.

357

$$rCV (\%) = \frac{rSD}{Median} \times 100 \quad \text{Eq. (1)}$$

358

359 These rCV measures have been used to calculate a total range of variation between
360 operators (inter-operator), per histogram file, per phase. Range was used to monitor
361 reduction and therefore improvement in inter-operator variation attributed to the results,
362 because it is an easily understood measure of spread and monitors the total range rather
363 than an arbitrary measure of spread (such as standard deviation). The difference in range
364 between operators can be used to observe the effectiveness of protocol instigation, with
365 percentage of variation reduction monitored alongside this. Normality tests were
366 completed in IBM SPSS using the Shapiro-Wilk test along with the generation of Standard
367 Deviation (SD) data.

¹⁴ rCV = robust coefficient of variation

368 **6.0 Analysis of Results**

369 The *rCV* range between operators measures as defined in Table 2, is higher in Phase 1 –
370 Operator judgment (Figure 3 – left image – blue line), compared to the lower inter-operator
371 range in Phase 2 – Protocol driven (Figure 3 - left image – orange line), apart from the 250
372 V file. This shows better separation between the stages at all file values, and the different
373 inter-operator ranges for all files. The inter-operator ranges were calculated as the
374 difference between the minimum and maximum *rCV* values of all the operators gating
375 each peak within a certain file. The percentage reduction in range between operator
376 variation from Phase 1 to Phase 2 is shown by the bar chart in Figure 3 (right).

377

378

379

[Insert Figure 3 Here]

380

381

382

383

384

385

386

Figure 3: Comparison of inter-operator ranges of personal interpretation of histogram data (left) and percentage reduction from Stage 1 to Stage 2 results (right)

2 column width

[Insert Table 2 Here]

Voltage (V)	250	300	350	400	450	500	550	600	650	700
Figure 2	a	b	c	d	e	f	g	h	i	j
Phase 1 Personal interpretation inter-operator range (FI)	2150	400.0	142.0	104	6	7.6	10.3	8.2	10.3	10.6
Phase 2 Gating standard inter- operator range (FI)	2100	251.5	95.4	60.6	5.3	0.8	0.8	1.2	1.0	1.5
Reduction (%)	2	37	33	42	12	90	92	85	90	86

Table 2: Inter-operator ranges

1.5 column width

It can be seen that the notionally ‘good separation’ files (Figure 3, 500 V to 700 V) have a much greater reduction in variation between operators when they follow the diagrammatical protocol, reducing the amount of contributory variation to the end result.

In addition, when speaking to operators as they completed these studies, it became apparent that less informed or experienced users sometimes applied gating knowledge from other measurement techniques that generate peak spectra as measurement output (e.g. Mass, Raman and Infrared Spectrometry). This practice may or may not have been beneficial.

403 **6.1 Optimum voltage for this Flow Cytometry data**

404 The optimum voltage for this set of data would be 500 V. This method has been
405 demonstrated in Figure 4 [37], where the median operator *rCV* values from each voltage
406 file in Phase 1 were plotted to create a Stain Index *rCV* curve. Median values were plotted
407 for each file due to non-parametric distributions of operator results for files across Phase 1
408 and Phase 2 analysis. The inflection point that changes the curve to a plateau indicates
409 the optimum voltage, minimising the effect of background noise on the data. This voltage
410 file was selected to further investigate the distribution of an example data set, and the
411 effects of different statistical outlier methods (Section 3) for determining outliers and
412 control limits.

413

414 **[Insert Figure 4 Here]**

415
416 Figure 4: Stain Index *rCV* curve.

417 *1 column width.*

418

419

420 The variation for the 500 V file is reduced by 90% when operators used the
421 diagrammatical protocol. As the voltage files increase from 500 V, and therefore the
422 separation between the peaks increases, the reduction in variation is still very high, but the
423 trend decreases slightly from this optimum analysis point. This could be due to the gain
424 increase amplifying background noise (as well as the fluorescence peaks), creating
425 additional shoulders on the peaks, which operators also gated on. This demonstrates the
426 importance of determining optimal settings to ensure that no other variation sources affect
427 interpretation of a final result and understanding where larger sources of uncertainty come
428 into play.

429

430 Lower voltages reduce sensitivity, which can be seen in the files by the merging peaks.
431 Consequently, it becomes harder to discern the peaks from each other, and from
432 background noise. The 'poor separation' files have reduced variation between operators
433 when they follow the diagrammatical protocol, but this is not as extensive as the clearly
434 separated files. This further supports the requirement for proper setup when analysing
435 data through a complex metrology instrument.

436

437

438 7.0 Understanding the impact of outlier discrimination

439 Outlier discrimination has been explored using the 500 V Phase 1 analysis data as an
440 exemplar data set. Typically, within manufacturing analysis outliers or extreme values are
441 either removed from analysis or the outliers are addressed to facilitate future improvement
442 in process control. Table 1 summarizes different outlier estimator methods (boundary
443 specifications) used within a variety of industries including biomedical and cell therapy
444 biomanufacture. There is no one correct choice for outlier determination, with the choice
445 being based on commercial guidelines, quality standards, and/or method validation
446 exercises.

447
448 To demonstrate the effect that different estimators have on data, the Phase 1 500 V data
449 set was used to calculate different outlier discrimination intervals. This data set was initially
450 tested to describe the distribution, using IBM SPSS to calculate the descriptive statistics.
451 The Shapiro-Wilk test for departure of normality confirmed that the data set was non-
452 parametric ($p < 0.05$), alongside a strong negative Skew (-2.21) and high Kurtosis (5.27),
453 making the results more affected by the presence of 'outliers'.

454
455 To apply different error boundaries to the data set, the outlier discrimination methods were
456 screened to include those that could be applied to a univariate data set for data that is not
457 manufactured-part specific, and those that act as a set boundary and do not transform the
458 extreme values. Both normal distribution and robust statistics have been included. The
459 effect of logarithmic transformation on the data has also been investigated, because this is
460 a common method used to transform data to make better approximate to a normal
461 distribution. This allows non-parametric data to be more applicable to a wider range of
462 Gaussian-based statistical analyses and conforming to more traditional manufacturing
463 statistical process control paradigms.

464

465 The calculations used for both the raw and transformed data can be seen in Table 3.

466 Bootstrapping relies upon a 95% Confidence Interval calculation, so these have been

467 accounted for together, and Mean $\pm$ 3SD, 2SD, and 10% trimmed mean $\pm$ 2SD all use the

468 same SD calculation to generate control limits.

469

470

[Insert Table 3 Here]

Outlier estimator	Location Calculation	Spread calculation	Location value	Spread value	Lower Control Limit	Upper Control Limit	Control Limit Distance	Log transformed Lower Control Limit	Log transformed Upper Control Limit	Log transformed Control Limit Distance
Shewhart Control Charts	$\bar{x} = \frac{\sum x}{n}$ For trimmed data, 10% of the ordered data are removed before calculation	$s = \pm \sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}}$	15.59	5.16	10.43	20.75	10.32	1.02	1.36	0.34
Mean +/- 3SD										
Mean +/- 2SD				3.44	12.15	19.03	6.88	1.08	1.30	0.23
10% trimmed mean +/- 2SD			15.88	3.44	12.44	19.32	6.88	1.09	1.32	0.23
95% Confidence Intervals		$CI = \pm 1.96 \frac{s}{\sqrt{n}}$	15.59	0.55	15.04	16.14	1.10	1.17	1.21	0.04
Bootstrapping										
Med +/-2MAD	$Median = \left(\frac{n+1}{2}\right)$	$MAD = med(x_i - med(x))$	16.30	0.80	15.50	17.10	1.60	1.19	1.23	0.04
IQR Metrics		$IQR = 75th \%ile - 25th \%ile$ $LCL = 25th \%ile - IQR$ $UCL = 75th \%ile + IQR$			14.20	17.80	3.60	1.16	1.25	0.10

Table 3: Summary of outlier estimator methods used to distinguish outliers from the 500 V data set

2 column width

473

474 Figures 5 and 6 show plots representing these non-parametric and transformed results.

475 Figure 5 shows two histograms of operator rCV values for the 500 V file, where operators

476 were asked to apply gates based upon personal interpretation (upper histogram), and then

477 asked to follow protocols (lower histogram). The grey stepped bands represent the

478 calculated outlier discrimination boundaries derived using the information in Table 3. Any

479 histogram column falling within these boundaries is determined as being in specification.

480 Those falling outside of a boundary would then be classified as an outlier by that method.

481 The majority of participants fall within all bounds, but this shows the importance of correct

482 selection of discrimination for the process. If chosen control limits are too small, then

483 allowable variation could be cut out, causing a high defect rate and false negatives. Too

484 large, and the process is allowed to head towards a more out-of-control state and false

485 positives could be included in analysis, which is undesirable and makes location of the

486 source of the variation more difficult.

488

489 Figure 5: Application of different control limits applied to raw data

490 *2 column width.*

491

492 To show the impact of operators following use of a gating standard to obtain results, the
493 Phase 2 data was overlaid onto discriminatory bounds, as seen in Figure 5 (lower
494 histogram). All participants fall within the tightest boundary, specified by the Median $\pm$
495 2MAD. This confirms that causing operators to follow a visual protocol not only reduces
496 inter-operator variation but also reduces the chance of ‘outlier’ results, regardless of how

497 outliers are defined in this context. In the context of cell therapies and their manufacture,
498 these outliers will represent key patient data metrics and cannot be excluded.

499 **[Insert Figure 6 here]**

500
501 Figure 6: Application of different control limits applied to log transformed data

502 *2 column width*

503

504 As seen in Figure 5, all boundaries scale inwards and outwards, so overlaps can be seen
505 on both sides. The mean, 10% trimmed mean and median have all been plotted on top, to
506 show shift due to the distribution. The median value of the data fits best to represent the
507 skewed distribution because it sits within the histogram maxima.

508

509 The boundaries in Figure 6 were recalculated when the skewed data was normalised with
510 a log transform (noting the change of x-axis scaling). The outer boundaries in Figure 6
511 (upper histogram) all fall within the same gradient pattern towards the centre, however the
512 95% confidence interval and the Median ± 2 MAD bounds sit astride one another,
513 discounting various operators as outliers. Application of log transformed Phase 2 data to
514 these defined boundaries is shown again in Figure 6 (lower histogram), with operators
515 falling within the tightest boundaries when following a diagrammatical protocol to apply
516 their gates.

517

518 To monitor equality of outlier discrimination between the bounds specified for raw and
519 transformed data, Table 4 shows the different bounds used, and what outliers are
520 specifically discriminated against using this method (outliers identified using anonymous
521 coding). Log transformation is deemed appropriate in the context here where the outliers
522 are identical for each context and therefore labelled as 'Equal'.

523

524 All outlier boundaries calculated up to and including Paxton's Criterion are equal in the
525 number of outliers specified when using either the raw data or log transformed data. As
526 these boundaries become more refined, outliers are included due to the area of the peak
527 gated by the specific operators. Mean ± 3 SD has one outlier quantified by the gate applied
528 covering a small section of the peak. This does not include all events collected within the
529 peak and therefore has a skewed *rCV* value as a result. Mean ± 2 SD, 10% Trimmed Mean

530 $\pm 2SD$ and Paxton's Criterion have all specified outliers due to gates not covering the
 531 entirety of the peak, and the gradual inclusion of these outliers through more refined
 532 control limits sees these outlier gates become wider to include more of the peak,
 533 questioning the outliers have been defined due to differences in spread definition.

534 **[Insert Table 4 Here]**

	Outlier frequency	Outlier code	Log transformed outlier frequency	Outlier Code	Equal Outliers	Reason for outlier
Mean $\pm 3SD$	1	C4	1	C4	EQUAL	Range gate applied over smaller % of peak
Mean $\pm 2SD$	2	C1, C4	2	C1, C4	EQUAL	Range gate applied over smaller % of peak
10% trimmed mean $\pm 2SD$	2	C1, C4	2	C1, C4	EQUAL	Range gate applied over smaller % of peak
Paxton's Criterion	5	A3, B11, C1, C4, C7	5	A3, B11, C1, C4, C7	EQUAL	Range gate applied over smaller % of peak
95% Confidence Interval	29	A1-A10, B1-B5, B7,B8, B10-B13, B16, B17, C1, C3-C7	28	A1- A5, A7-A10, B1-B5, B7, B8, B10-B13, B16, B17, C1, C3,-C7	UNEQUAL	Additional outlier in raw data (A6)
Median $\pm 2MAD$	9	A3, A5, A6, B4, B11, B15, C1, C4, C7	11	A3, A5, A6, B4, B9, B11, B14, B15, C1, C4, C7	UNEQUAL	Additional outliers in log transform (B9, B14)

Table 4: Control limits used to specify outliers

2 column width

Both highly refined control limits (95% Confidence Interval and Median $\pm$ 2MAD) have an unequal number of outliers when boundaries are calculated with raw and log transformed data. When calculating the 95% Confidence Interval the raw data contained one extra outlier, which may be expected due to this being a statistical test using normal metrics applied to a non-parametric data set. Both the raw and log-transformed control limits for the 95% Confidence Interval contain a high number of outliers (29 and 28 respectively), leaving only 8 and 9 respective 'inliers' to represent the distribution. Due to the distribution having a high Kurtosis and Skew, this could affect the calculation of this confidence interval, due to its dependence on the standard deviation that is not located at the peak maxima.

The robust Median $\pm$ 2MAD control limits are unequal due to two extra outliers being present in the log transformed data. One could assume that this is because a robust method has been applied to 'normalized' data. However, this should still be effective in analysis despite of distribution shape.

555 8.0 Conclusion

556 Flow Cytometry is a core biometrology technique within clinical, research and the newly
557 defining Cell Therapy biomanufacturing industry. Subjective manual interpretation of FC
558 data is commonly practiced, however this subjective manual interpretation is a key
559 potential source of process control input variation and hence measurement uncertainty in
560 Cell Therapy manufacture. Reducing variability within Cell Therapy manufacture will help
561 to optimise the process and to better understand with greater certainty where variation
562 comes from, if the process fluctuates and exceeds control limits.

563

564 When considering manual FC data analysis, this univariate gating study results shows for
565 the first time that significant inter-operator reduction occurs when operators follow a
566 diagrammatical protocol, with up to 90% decreases in rCV values if operators are
567 presented with clearly separated peaks. If peak separation is reduced then benefit gain
568 from diagrammatical protocols is likewise reduced due to poorer peak discrimination by the
569 operators, even given a guiding protocol. Sensitivity thresholds require correct definition,
570 but over-amplifying to try and aid data definition can cause amplification and spill-over of
571 noise into identified measured regions.

572

573 As is the case in other industrial sectors, this supports the implementation and use of
574 standardised gating protocols in the field such as OMIPS for inter-site comparison. This
575 demonstrates one manner in which clinical but most importantly biomanufacturing centres,
576 can reduce biometrology variability within a Cell Therapy manufacturing context from data
577 analysis within Quality Control, although context for scale-up would require investigation.

578

579 Furthermore, this work has considered the importance of correct definition of control
580 and/or specification limits, in the context of the assumed shape of data distribution within a

581 specific process, either normalised or non-parametric. Formal method validation
582 traditionally defines these control limits, but in Cell Therapy manufacturing this can be
583 difficult on a patient-by-patient basis, due to lack of material available to complete multiple
584 replicates, along with the key point that notional 'outliers' cannot be discarded due to this
585 being true patient data.

586

587 The analysis of the 500 V exemplar data set has shown how selection of different control
588 limits (normalised and non-parametric based) leads to different definitions of outliers, with
589 some outliers being common to several control limits, whilst other outliers being unique to
590 a particular method. Given that there is minimal consensus as to selection and application
591 of the most appropriate control limit, this again exemplifies the potential for further variation
592 input within the biometrology feedback into a manufacturing process control scenario. In
593 addition, the patient specific nature of outliers in cell therapy statistical datasets is further
594 hindered by control limit selection and application.

595

596 Previous data from both healthy and diseased patients would be used to define Cell
597 Therapy manufacturing control limits, but there cannot be complete confidence as to
598 whether new patient data meets historical data sets and disease states are often much
599 more variable. This makes definition of initial control limits difficult. Recent EU guidelines
600 state a minimum of 3 validation runs are required to define control limits before a therapy
601 receives Market Authorization Approval (MAA)¹⁵ [39], with manufacturers taking a Risk-
602 based approach to validation. This number of validation runs is lower than other more
603 traditional industrial validation scenarios, but MAA would ensure manufacturers are aware
604 of additional variation sources in the Cell Therapy manufacturing process and the
605 uncertainty quantification of these. The better the control of variation of the biometrology

¹⁵ MAA = Market Authorization Approval

606 technique, the more assurance that any observed variation more probably originates from
607 the biological material and biomanufacturing process. This allows decisions to be made
608 with more confidence within Quality Control Processes.

609

610 This study has provided initial awareness of operator behaviour and the potential for
611 variation within subjective manual Flow Cytometry data processing. This will directly inform
612 the definition of operator analysis studies using simple to highly complex cell models and
613 datasets (in terms of significantly increasing dimensionality), with different gating scenarios
614 designed to quantify the variation present when gating clearly separated and poorly
615 separated cellular populations. However, it is important to recognise that software-based
616 auto-gating techniques may also be used for Flow Cytometry data analysis. Whilst not fully
617 automated in the true sense of the word, auto-gating techniques may aid the operator
618 during the data analysis. As yet, auto-gating techniques have not been assessed in a
619 comparable manner to these studies of operator manual data analysis.

620

621 In addition, the work reported here is not intended to develop or deliver a whole FC
622 instrument uncertainty statement. It is fully anticipated that the results of the operator
623 variation analysis will eventually inform a detailed operator uncertainty analysis, and
624 subsequently contribute to a whole FC instrument uncertainty statement.

625

626

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631

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637

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