Title: Tandem Dyes: Stability in cocktails and compensation considerations

Running Title: Tandem spill-over variation & stability in cocktails

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Abstract:

Background: The stability and performance of tandem conjugated antibodies can be impaired when stored in antisera cocktails (1,2). This, and the need for frequent re-compensation due to the possible spectral spill over variation between tandem lots, reduces the robustness of clinical flow cytometry panels that include tandems. Since tandems are required for standard 8-10 colour screens, further studies of the stability of tandems in cocktails and their spill over variability are warranted.

Methods: The performance of PE- and APC-tandems stored in cocktails was tested on fresh bone marrow, preserved blood and lyophilised cell samples over 1-, 6- or 8-week periods respectively, and their spill over matrices were compared. The observed correction factor differences were used as the basis for analysing how the application of an incorrect compensation matrix could influence data interpretation.

Results: Signal intensities and background fluorescence remained constant for all fluorochromes in the cocktails tested. Spill over correction factors for different PE-Cy7 mAbs did not exceed or were only marginally higher than those for non-tandem organic dye conjugated mAb. By applying the correction factor differences observed between tandem mAb lots to clinical data, it was found that the over and under compensation would not alter the clinical interpretation.

Conclusions: Tandems can be safely stored and used in cocktails. However, each cocktail should be tested on relevant material prior to use. Exact compensation settings are a requirement for accurate data. Provided that careful evaluation of tandem compensation requirements is carried out, certain tandems may use a generic compensation matrix.

Key Terms: tandem conjugates, monoclonal antibody cocktails, spectral spill-over, compensation matrix.

INTRODUCTION

Tandem conjugates ('tandems') for use in multi-colour flow cytometry were first introduced more than 20 years ago (1, 2). Tandems comprise two fluorochromes: one donor and one receiver. The fluorescence resonance energy transfer (FRET) efficiency between the donor and the receiver may vary between batches of a particular tandem. Hence, the spectral spill-over characteristics can differ from lot-to-lot (3-5). Therefore it is generally recommended that each tandem lot is re-compensated (4,6-8). It is important to not expose tandems to light, or other conditions that can break the coupling between the two dyes, since a de-coupled donor component will emit at its usual wavelength range instead of the longer wavelengths expected for the tandem (7). Storage in dark bottles increases the durability of tandems (3). On the other hand, the performance of tandem mAbs that are stored in antisera cocktails has been questioned. One study showed that the fluorescence signals of two q-dot-conjugated mAbs and that of a tandem-conjugated mAb were significantly reduced after storage for 3 days in a cocktail (1).

These issues pose concerns about the use of tandem conjugates in clinical laboratories, where the antibody turn-over is high. If tandems are selected for commonly used antibodies they may be used several times daily: This could potentially accelerate light-induced degradation. The use of antibody cocktails is often favoured for six-ten colour screens, as these help speed

up the work and prevent the risk of adding the wrong antisera or volume. The inability to use tandems in cocktails would hamper the work-flow in the lab. In addition, due to the high antibody turnover, compensation of new lots would be required very frequently. Although this is a straightforward procedure, most diagnostics laboratories are very busy and use several tandems that each requires individual compensation. In addition, the number of appropriately cytometry trained staff can be low. Consequently, it is highly likely that new lots are introduced without prior compensation. Subsequent off-line compensation could be applied, but this strategy may cause an unacceptable delay in obtaining diagnostic results, and may also add to the quality control work load.

For these reasons, the use of tandem conjugates would reduce the overall robustness of diagnostic multicolour flow cytometry (MFC). Nonetheless, the use of MFC for haematology-oncology diagnostics has proven very useful and, until suitable alternative fluorochromes are available, tandem conjugates are needed to achieve MFC. In addition, the last few years have seen an increased in the use of 405nm ('violet') lasers in clinical laboratories. Several different violet excited organic fluorochromes are commercially available. Most have emission maxima around 420-455nm ('blue channel') or 500-530nm ('yellow channel'). Violet laser excited tandems are commercially available. However, the majority of mAb excited by the violet laser that are in clinical use today are not tandems and therefore not expected to cause particular concerns with regard to lot-to-lot variations.

Here, we examine the use of tandem conjugated antibodies from a haematology-oncology diagnostics laboratory perspective. We analysed the performance of tandems stored in cocktails, their lot-to-lot spectral spill-over variation, and how incorrect compensation matrices may influence data interpretation. Since violet laser exited fluorochromes are relatively new in clinical laboratories, we also looked at the spectral spill over characteristics of different organic fluorochromes exited by violet lasers.

MATERIALS AND METHODS

Stability of tandems in antibody cocktails

Used to label preserved blood and fresh bone marrow

Reagents: Cytometer Setup & Tracking (CS&T) Beads, CompBeads, and Multi-Check Control whole blood ('Multi-Check Control') were obtained from BD Biosciences. CytoComp lyophilised cells were from Beckman Coulter. Monoclonal antibodies for cocktail 1: CD4-fluorescein isothiocyanate (FITC) (SK3), CD8-Phycoerythrin (PE) (SK1), CD19-peridinin-chlorophyll α complex – cyanine5.5 (PerCP-Cy5.5) (J25C1), CD2-PE-cyanine7 (PECy7) (S5.2), CD20-allophyocyanine (APC) (L27), CD3-APC-Cy7 (SK7), CD56-V450 (B159) and CD45-V500 (2D1), all from BD Biosciences. Kappa-FITC (Rabbit polyclonal Ab) and Lambda-PE (Rabbit polyclonal Ab) were from Alere.

Method: Preserved blood and Lyophilised cells: The cocktail was stored in a dark glass bottle at 4°C. The Multi-Check Control was stored and handled according to the manufacturer's instructions, and aliquots were removed weekly for labelling. CytoComp cells were reconstituted according to the manufacturer's instructions. A new vial of cells was reconstituted each week prior to use, and the same batch of cells was used throughout the study. The antibody cocktail was used to label cells on the day of preparation and subsequently on a weekly basis for 6 (Multi-Check) or 8 (CytoComp cells) weeks. To mimic daily use and light exposure, the cocktails were placed on the bench, on 4°C cool-blocks, with the cap removed for 10 minutes each day for 5 days/week throughout the test period. The time periods were deemed to reflect the combined time that cocktail bottles are opened when used several times a day.

Fresh bone marrow: A second cocktail consisting of CD16-FITC (3G8), CD117-PC7 (95C3), HLA-DR-Pacific Blue (PB, Immu-357, all from Beckman Coulter), CD13-PE (L138), CD34-PerCp-Cy5.5 (8G12), CD33-APC (P67.6), CD11b-APC-Cy7 (ICRF44), and CD45-V500 (2D1), all from BD Biosciences) was made and stored as above. A fresh (2-5 hours old)

bone marrow sample was stained with this cocktail, or the same antibodies but individually pipetted, for five consecutive days. Daily light exposure was as above but for 20 minutes each day.

Flow cytometry: A Canto II flow cytometer with standard laser and filter sets and FACSDiva software version 6.1.3 was used. The instrument was calibrated daily with CS&T beads and the analysis protocol used the daily corrected CS&T bead PMT voltages to ensure consistency of fluorescence signals over the eight week period. Compensation was set using CompBeads and the Diva automated compensation programme according to the manufacturer's instructions.

Analysis: Preserved blood and Lyophilised cells: Cells were identified on a CD45-V500 (x-axis, logarithmic scale) and side scatter (y-axis, linear scale) plot. $CD45^+$ cells were displayed on a forward scatter (FSC) (x-axis, linear scale) and side scatter (SSC) (y-axis, linear scale) plot. For the first cocktail, lymphocytes were gated and displayed on plots as follows: CD2-PE-Cy7 (x-axis) and CD3-APC-Cy7 (y-axis); and CD19-PerCP-Cy5.5 (x-axis) and CD20-APC (x-axis). A CD4-FITC (x-axis) and CD8-PE (y-axis) plot was gated on $CD3^+$ cells. A Kappa-FITC (x-axis) and Lambda-PE (y-axis) plot was gated on $CD20^+$ cells. $CD56^+$ cells were identified in a plot of CD56-V450 (x-axis) and CD2-PE-Cy7 (y-axis) gated on all lymphocytes.

Fresh bone marrow. Data was analysed using Kaluza software. Neutrophils and monocytes were gated in a SSC (y-axis) CD33 (x-axis) plot. Neutrophils were then displayed on CD13 (y-axis) CD11b (x-axis) and CD13 (y-axis) CD16 (x-axis) plots and gates set around the most mature (Step 4) and immature (Step 1 or Step 2) populations (9-11). Monocytes were displayed in a DR (y-axis) 11b (x-axis) plot, and immature cells gated as $CD11b^{dim/negative}$ $HLA\text{-}DR^{bright}$ (9-12). A gate around the bone marrow progenitor cell compartment (13) extended to include erythroid cells was set in a SSC-A (y-axis) CD45 (x-axis) plot. From this, $CD34^+CD117^+$ myeloid progenitors were gated in a CD117 (y-axis) CD34 (x-axis) plot and $CD117^+HLA\text{-}DR^-$ erythroid and myeloid progenitors from a CD117 (y-axis) HLA-DR (x-

axis) plot. All fluorochrome plots used logarithmic scales. The percentage positive and median fluorescence intensity for each fluorochrome was then recorded.

Comparison of compensation matrices for mAbs conjugated to the same fluorochrome

Monoclonal antibodies: FMC7-FITC (FMC7), CD2-FITC (S5.2), CD38-FITC (HB7), CD57-FITC (HNK-1), CD81-FITC (JS-81),CD103-FITC (Ber-ACT8), CD8-PE (SK1), CD13-PE (L138), CD14-PE (MΦP9), CD56-PE, (NCAM16.2), CD59-PE (p282H19), CD79a-PE (HM47), CD123-PE (9F5), CD4-PerCP (SK3), CD19-PerCP (4G7), CD34-PerCP (8G12),CD-5PE-Cy7 (L17F12), CD10-PE-Cy7 (HI10a), CD14-PE-Cy7 (M5E2), CD19-PE-Cy7 (SJ25C1), CD25-PE-Cy7 (2A3), CD38-PE-Cy7 (HB7), CD117-PE-Cy7 (104D2), CD5-APC (L17F12), CD8-APC (RPA-T8), CD11c-APC (S-HCL-3), CD23-APC (EBVCS-5), CD33-APC (P67.6), CD55-APC (IA10), CD138-APC (MI15), CD45-V450 (H130), CD3-APC-Cy7 (SK7), CD20-APC-Cy7 (L27), CD45-APC-Cy7 (2D1), and HLA-DR-APC-H7 (L243), were from BD Biosciences. CD11b-FITC (CDIS1/18) was from Caltag Medsystems. MPO-FITC (CLB-MPO-1) was from Alere / Dako and CD160-PE (By55) was from Beckman Coulter.

Method: Seven combinations of seven antibodies were selected, each containing antibodies conjugated to FITC, PE, PerCP, PECy7, APC, APCCy7 and V450 (Supplementary Table 1). The combinations were then used to label CompBeads according to the manufacturer's instructions.

Flow cytometry and compensation: The compensation matrices for each combination of fluorochrome conjugated antibodies were performed as above compared.

Compensation matrices for different instruments

Monoclonal antibodies: CD3-PE (UCHT1), CD3-PC5.5 (UCHT1), CD3-APC-Alexa750 (UCHT1), CD3-PB (UCHT1), CD5-PC7 (SFCI12T4D11), CD5-APC (SFCI12T4D11), CD5-APC-Alexa750 (SFCI12T4D11), CD8-PC5.5 (B9.11), CD8-KO (B9.11), CD16-PB (3G8),

CD19-PC5.5 (J3 119), CD19-APC (J3 119), CD20-APCAlexa-750 (89B), CD36-FITC (FA6.152), CD41-PC7 (P2), CD42b-FITC (SZ2), CD45-FITC (J.33), CD45-PE (J.33), CD45-PC5.5 (J.33), CD45-PC7 (J.33), CD45-APC (J.33), CD45-APC (J.33), CD45-APC-Alexa750 (J33), CD45-PB (J.33), CD45-KO (J.33), CD56-PE ((N901), CD79a-APC (HM47), CD117-PC7 (95C3), CD160-PE (BY55) and FMC7-FITC (FMC7) were from Beckman Coulter.

Method. Four combinations of eight antibodies were selected, each containing antibodies conjugated to FITC, PE, PC5.5, PC7, APC, APC-Alexa Fluor750 (APC-AF750), Pacific Blue (PB) and Krome Orange (KO) (**Supplementary Table 2**). The combinations were then used to label CompBeads according to the manufacturer's instructions.

Flow cytometry. The compensation matrices for each combination of antibodies were determined using software available on the two instruments: A Canto II with standard laser and filter configuration and FACSDiva software version 6.1.3 and a Navios (Beckman Coulter) with a standard three colour configuration and software. Both instruments were optimised for use and the optimised PMT voltages were used for the compensation protocol. The compensation matrices for each combination of fluorochrome conjugated antibodies were compared on the two instruments.

Spectral spill-over of commonly used tandems from different manufactures and Spectral spill-over of violet-excited fluorochromes

Monoclonal antibodies: CD3-APC-H7 (SK7), CD3-PB (UCHT1), CD4-V500 (RPAT4), CD5-PE-Cy7 (LI7F12), CD4-BV421 (RPA-T4), CD45-V500 (2D1) and CD56-V450 (B159) were from BD Biosciences. CD3-APC-AF700 (UCHT1), CD5-PC7 (BL1a), CD20-PB (B9E9) and CD45-KO (J.33) was from Beckman Coulter. CD3-APC-eFluor750 (UCHT1) and CD5-PE-Cy7 (UCHT2) was from eBioscience.

Method: Compensation matrices for tandems and violet laser excited fluorochromes from three or two different manufacturers, respectively, were performed on the same day, and in the same FACSDiva software version 6.1.3 compensation set-up experiment.

Flow cytometry and compensation set-up. As described above.

Lot-to-lot variation of spectral spill-over

Monoclonal antibodies: CD5-PE-Cy7 (LI7F12), CD3-APC-H7 (SK7), CD117-PE-Cy7 (104D2) and HLA-DR-APC-H7 (L243), all from BD Biosciences.

Method: Lot-to-lot comparisons were performed on separate days in two laboratories. New lots were compared with old lots by over-acquiring the old tandem conjugate data in the FACSDiva software version 6.1.3 compensation set-up experiment. Since the same compensation set-up experiment was used for all lots tested, the PMT voltages used were constant over time: PMT voltage adjustments for day-to-day drift were not applied. The average CS&T delta voltage adjustment requirement during the time period concerned did not exceed 20V for any channel.

Flow cytometry and compensation set-up: As described above.

Interpretation of incorrectly compensated data

Monoclonal antibodies:

CD38-PE (HB7), CD26-PE (L272), CD5-PerCP-Cy5.5 (L17F12), and CD3-APC-H7 (SK7) were from BD Biosciences; CD5-PC7 (BL1a), CD10-PC7 (ALB1), and CD19-PC7 (J3-119) were from Beckman Coulter, CD7-APC (eBio124-1D1) was from eBioscience.

Method: FCS data files derived from routine diagnostic 7 or 8 colour screens were analysed in FACSDiva software version 6.1.3. Using the correct compensation matrix as a starting point, the matrix related to tandem spill-over was increased or decreased in a step-wise manner using off-line compensation. The visual interpretation of antigen expression patterns, median fluorescence intensity (MFI) and population size (% positive cells) of gated populations were measured for each compensation setting.

Routine diagnostic specimens were labelled with antibodies for 15 minutes at room temperature in the dark. After red cell lysis (Pharmlyse, BD Biosciences, used according to manufacturer's instructions) samples are washed in PBS and centrifuged at 600g for four minutes. Samples were acquired within 30 minutes.

Flow Cytometry: As described above. The cytometer was set at medium flow rate; acquisition rate did not exceed 8,000 events/second. The window extension was 7.0, forward and side scatter voltages and forward scatter threshold were set to include all leukocyte populations, including a complete lymphocyte cloud and, in some cases, the complete nuclear red cell cloud.

Statistical Analysis

For the stability of cocktails and the variation in compensation matrices, the mean, standard deviation (SD) and coefficient of variation (CV) were calculated using Microsoft XL software.

RESULTS

Stability of tandems in antibody cocktails used to label preserved blood and fresh bone marrow

Preserved blood. On initial analysis the CytoComp cells bound all cocktail antibodies except CD56-V450, Kappa-FITC and Lambda-PE. The Multi-Check Control bound all antibodies. **Table 1** shows the mean values for the percentage positive cells and median fluorescence intensities of the eight analyses performed on CytoComp cells. There was very little variation in the percentage positive values, with CVs of five or less for the six fluorochromes analysed. The CVs for the MFI's were higher, though all were below 10. Similarly, the results for the six analyses of the Multi-Check Control showed very little variation in the percentage positive values, with CVs of five or less. The exception was CD56-V450, which had a CV of 12.3. There was more variability in MFI's with CV < 10 for all antibodies tested except CD56-V450, which had a MFI CV of 23.7 (**Table 1**). The only consistent change over time was a reduction in CD8-PE MFI on the Multi-Check control (**Supplementary Table 3**). This however, was not seen during the eight weeks testing of the Cytocomp cells (**Supplementary Table 4**). Visual interpretation of expression patterns did not give cause for any concerns; and

neither cell type had any evident increase in background fluorescence over time for any of the fluorochromes used (**Figure 1**).

Fresh bone marrow. MFIs and population sizes for all antigens and populations investigated remained very similar between cells labelled with cocktail or daily individually aliquoted mAbs, over the five day test period. A slight decrease in HLA-DR-PB on monocytes labelled with the cocktail, compared to daily individually aliquoted mAbs, was found. This difference did not influence the proportions of the HLA-DR$^{dim\text{-}mod}$ CD11b^{dim} mature /HLA-DR^{bright} CD11b^{dim} immature monocytes as determined by the HLA-DR/CD11b gate. The CD16 MFI on mature neutrophils was marginally higher for samples labelled with the cocktail (**Supplementary Table 5**). Again, this did not affect the maturation patterns studied, which all rely on the range of CD11b, CD13 and CD16 expression intensities on the maturing cells. There was no obvious difference in background fluorescence between cocktail and individually aliquoted mAbs at any time point tested. In particular, looking at signals from the tandem donor fluorochromes, we found no increase in CD33-APC signals of either APC positive populations (CD33$^+$ Monocytes), APC dim populations (CD33$^+$ Neutrophils) or fully negative cells (CD33$^-$ lymphocytes) (**Supplementary Table 5** and data not shown). Similarly, cocktail generated PE signals from CD13-PE dim immature neutrophils or CD13$^-$ lymphocytes did not increase over time (**Supplementary Table 5** and data not shown).

Finally, data were examined for artefacts which were defined as additional populations seen in the cocktail labelled samples but not detected in the individually aliquoted tests. No artefacts were identified in any of the cocktail labelled samples upon close examination systematically plotting each fluorochrome against all others (data not shown).

Comparison of compensation matrices for mAbs conjugated to the same fluorochrome

Most clinical laboratories use a generic compensation matrix for all antisera conjugated to non-tandem fluorochromes. For example, all FITC-conjugated mAbs would use the same matrix. To explore whether a generic compensation matrix could be used also for tandem conjugated mAbs, the matrices for seven separate FITC, PE, APC and PE-Cy7 conjugated

mAbs and three different APC-Cy7 mAbs were compared. The largest variation within a group was for the APC-Cy7 conjugated mAbs: Their APC-Cy7 spill-over into the APC channel varied from 20.26% to 31.07% (**Table 2**). The correction factors for all PE-Cy7 conjugated mAbs were quite similar, and did not markedly differ more than those for non-tandem conjugated mAbs (**Table 2**).

Compensation matrices for different instruments

The four antibody cocktail combinations are shown in **Supplementary Table 2**. The Mean and SD for the compensation matrices are shown in **Supplementary Figure 1**. The compensation matrices were similar on the two instruments.

Spectral overlap between tandems from different manufacturers

Compensation matrices for PE-Cy7 / PC7 conjugated mAbs from three separate manufacturers are shown in **Table 3**. The greatest difference was for spill over into the PE channel: The difference between the maximum and minimum values in absolute terms was 0.72%. APC-tandems that use different receiver fluorochromes were also tested. Their correction factors for APC varied greatly: The maximum difference was 38.4%. Their PC7 correction factors varied from 5.24% to 6.38% (**Table 4)**.

Spectral overlap for violet excited fluorochromes

Table 5 and 6 show compensation matrices for violet excited fluorochromes from two different companies. The 'blue' emitting fluorochromes PB, V450 and BV421 mainly spill-over into the 'yellow' 510 +/- 25 nm channel. Notably, this was less for BV421 than for either PB or V450 (**Table 5)**. KO and V500 mainly spill over into the 'blue' 450+/- 25nm channel and to a lesser extent, into the FITC channel (**Table 6**).

Correction factor lot-to-lot variation for tandem mAbs

Compensation matrices for five different lots of the same CD5-PE-Cy7 or CD3-APC-H7 mAb, recorded on the same cytometer in laboratory 1 are shown in **Figure 2 (A-B)**. Similarly the compensation matrices for five different lots of the same CD117-PE-Cy7 or HLA-DR-APC-H7 mAb, recorded on the same cytometer in laboratory 2 are shown in **Figure 2 (C-D).** The two laboratories produced very similar compensation matrices for the conjugates even though different monoclonal antibodies were studied. The variation in the % compensation was less than 2% for all conjugates investigated. Several lots of other tandem conjugated mAbs, including PC7 and PerCp-Cy5.5 conjugates, were tested in the same way by laboratory 1 and 2 and were found to have less variation than CD5-PE-Cy7 or CD3-APC-H7 (data not shown).

Interpretation of incorrectly compensated data

Please see discussion below.

DISCUSSION

Tandem conjugate stability in antibody cocktails

The main reason for false results in multicolour flow cytometry is thought to be incorrect compensation. This is not disputed here; yet, the flow cytometry-related non-conformities that have occurred since the introduction of MCF in our laboratories are chiefly related to the addition of incorrect antisera to sample tubes or the omission of an antibody. Notably, these incidences occur more frequently for panels that do not have pre-made cocktails. The use of cocktails is, in our experience, helpful as it saves time and avoids human error. However, to date, few publications have reported the stability of antisera, including tandem mAbs, used in cocktails. Biancotto et al (1) tested a cocktail containing 14 mAbs over a 4-day period, and found that the qdot 605-, qdot 655-, and PE-Cy5-conjugated mAbs did not perform well after 3 days. Interestingly, the authors did not report a problem with the other tandem mAbs used in the cocktail: APC-Cy7, PE-Cy7, PerCP-Cy5.5, PE-TR and PE-Cy5.5 conjugated mAbs. Rawstron et al (2) investigated two separate cocktails, each containing six mAbs (including APC-H7- and PE-Cy7-conjugated mAbs), over a 28-day period. They observed no loss of the

PE-Cy7 or APC-H7 signals during this time, but did detect an increase in background APC-H7 intensity on APC positive cells. This effect was limited to one of the cocktails, and to cells with strong expression of the antigen binding the APC-conjugated mAb.

The present study tested two cocktails. Both cocktails contained antibodies from different manufacturers and therefore possibly a mixture of different buffers. The first cocktail was used to examine lymphocyte subsets and was tested on commercially available preserved and lyophilised cell controls over a 6 and 8-week period, respectively. A consistent decrease in CD8-PE MFI was observed for the preserved cell control but not for the lyophilised cell control. This would suggest that the cocktail used performed equally well at all time points, while the preserved cells had reduced capacity for binding the CD8 antibody over time. Possibly, freshly reconstituted lyophilised cells offer a more stable vehicle for testing antibody cocktails. The second cocktail targeted myeloid antigens and was tested on fresh bone marrow samples over a 5-day period. For both the cocktails, none of the observed changes would have altered the clinical outcome: Visual interpretation of expression patterns did not reveal differences, nor did the minor numerical differences influence data interpretation. The data presented here, along with that reported by Biancotto et al (1) and Rawstron et al (2), suggest that the performance of each separate cocktail needs to be evaluated, and that performance may differ depending on the test material. Here, we used three substrates for testing the cocktails: preserved or lyophilised control cells and fresh bone marrow samples. Preserved and lyophilised cells have the advantage of consistency: the population size and fluorescence intensity should remain the same throughout the test period. Fresh samples include all normal cell populations and therefore allow for monitoring non-specific binding of several cell subsets as well as performance of antibodies specific for antigens with different expression levels on the same, or different, cell subsets. The possibility of tube-to-tube variation, rather than cocktail shelf-life, causing different results may be greater when using fresh samples. It should also be noted that none of these choices offers the possibility of testing cocktails on neoplastic populations. LeRoy et al (14) showed that APC-

tandems may be degraded depending on cell type present in the sample. The effect was relatively immediate (within 30 minutes) and also occurred in single mAb-stained samples. It is, therefore, difficult to see how results influenced by cell type in the manner described by LeRoy et al (14) would be different should the APC tandem be stored singly or in a cocktail.

PE-tandem mAbs, particularly PE-Cy7, are photosensitive (7). Both cocktails tested here were stored in light protective bottles, and were subjected to a single daily light exposure of 10 minutes (when used to label preserved cells) or 20 minutes (when used to label fresh bone marrow). The different exposure levels were selected because they reflect the average time spent on the bench for the respective cocktails in our two laboratories. This level of light exposure did not appear to cause tandem degradation, at least not at a level that affected the data analysed here.

Comparison of compensation matrices for mAbs conjugated to the same fluorochrome

Clinical labs in the UK often use a generic antibody when setting compensation matrices for non-tandem fluorochromes. Analysis of groups of 7 different FITC, PE, APC and PE-Cy7 conjugated mAbs showed that the PE-Cy7 spill over into the PE channel did not vary over that of, for example, APC spill over into the APC-Cy7 channel. Most variation in the required compensation occurred for the APC-Cy7 conjugated mAbs, between APC and APC-Cy7. This may be due to the variation in protein:fluorochrome ratio for these tandems or the efficiency of the resonance energy transfer between the two fluorochromes in that particular tandem conjugate. It is however difficult to compare spill over values for the different fluorochromes. The degree of spill over is diverse, ranging from an approximate 15 % for FITC into PE to below 0.1% for other combinations. Moreover, the effect of correction factor change on the cell population MFIs for the different fluorochromes may not be the same across all combinations.

Compensation matrices for different instruments

Comparison of compensation matrices on Canto II and Navios showed that the greatest amount of compensation was required for PE – FITC, PC7 – PC5.5, APC – APC-Alexa750 and KO – PB on both instruments. The variations observed between the two cytometer's matrices reflect their different filter set up, and would not prevent standardisation and harmonisation on different types of instruments.

Violet excited fluorochromes

It is generally accepted that non-tandem fluorochromes have very similar spill-over characteristics regardless of production line and most clinical laboratories would use them interchangeably in compensation matrices. Newer organic fluorochromes that are excited by violet lasers are now used extensively by many laboratories. These include dyes such as PB, V450, brilliant blue and brilliant violet dyes (15), normally detected using 450 +/- 25nm filters. Also KO and V500 that are normally detected using 510+/- 25 nm filters. So far, our experience is that PB, V450, V500 and KO do not vary more than other organic dyes from lot-to-lot. However, more extensive data sets should be recorded to confirm this observation. Despite fairly similar emission spectra, the spill over corrections required for these fluorochromes are different, as shown in **Tables 5 and 6**. Off-line compensation checks showed that their compensation matrices cannot be used interchangeably in our set-up (data not shown). To the contrary, the two main different 'green' emitting fluorochromes used in clinical laboratories, FITC and Alexa 480, have very similar matrices in our systems and may be used interchangeably (data not shown). Our data highlights that individual matrices are required for the different violet laser exited fluorochromes.

Correction factor variation between lot-to-lot and different manufacturers

Due to the lot-to-lot variation of tandem conjugates we investigated whether different compensation values may be required for each lot. Different antibodies conjugated to PE-Cy7 or APC-H7 were found to have relatively similar compensation matrices for different lots. In

addition the compensation matrices for the tandems were found to be very similar even when analysed on different instrument from the same manufacturer in different laboratories. The difference in compensation between lots was always less than 2% and in most cases less than 1%. Some of the differences between the matrices shown in **Figure 2** are likely due to daily drift, since PMT voltages were not corrected for day-to-day variations in cytometer performance (see materials and methods). The matrices for PE-Cy7 conjugated mAbs from three different companies did not vary over those for the different PE-Cy7 lots from one single company. It should be noted that some of the companies may use the same production line. In contrast, the APC-tandems tested varied greatly, particularly for spill over into the APC channel. This was expected, since many companies use different receiver fluorochromes for their APC-tandems, for example, H7, Cy7 and Alexa Fluor 700/750 which was the case for the mAbs tested here.

Interpretation of incorrectly compensated data

We have shown that the spectral spill over for different lots of tandem antibodies may vary. This raise the question whether the variation observed would affect data interpretation? The extent of the effect of incorrect compensation on MCF data depends not only on the magnitude of the change in the correction factor, but also on the particular antibody/fluorochrome combinations used, and on the expression patterns studied. We therefore investigated several fcs files derived from various antibody and cell combinations, using correct and incorrect off-line compensation. Importantly, numerical evaluation of results is not always the unique way to check changes since visual interpretation based on pattern comparison plays a large role in diagnostic procedures. Therefore, expression patterns, as well as percentage positive cells and MFIs for gated populations were evaluated.

The greatest effect tends to be observed for antigens that are weakly expressed where the population of interest ranges from a negative to weakly/moderate pattern of antigen expression (**Supplementary Figures 2b** and **c**) when compared to more highly expressed antigen (**Supplementary Figure 2a**). Where the population of interest is relatively well

separated from the remaining cells, as is the case for the CD7 negative T cells shown in **Supplementary Figure 2d**, even larger deviations from correct compensation settings (up to 10%) would not cause a false result. However, should a population be defined as $CD5^{+}CD26^{-/dim}$ (as in **Supplementary Figure 2b**), the reported population size would be affected by compensation changes of less than 2%. That said, a population is rarely defined by one antigen alone but, rather, by a series of gates (sequential Boolean gating). For the example shown in **Supplementary Figure 2b**, the Sezary cells were also identified by the complete loss of CD7, and the population size was gated on according to $CD3^{+}/CD4^{+}/CD7^{-}/CD26^{-/dim}$ expression. Similarly, the $CD5^{+}$ B cells shown in **Supplementary Figure 2c** would be investigated for expression of several other antigens before reported as "abnormal". In addition, in many cases the analyst would notice that the data was either over or under compensated.

Perhaps of most concern is the effect of incorrect compensation on small population sizes that are defined by altered antigen expression, rather than by a clear positive or negative population. An example is MRD analysis. Using data files for B-ALL and AML MRD-positive and -negative cases, it was not possible to create a false positive or false negative result by changing one or several correction factors within 2%, including channels used by non-tandem antibodies (data not shown). Similarly it was not possible to alter the interpretation of MDS-related monocyte and neutrophil maturation patterns (16). All these tests were based on populations defined by four antigens, and the analysis was always interpreted manually. Current and, particularly, future computer based analysis may take smaller changes in fluorescence intensity into account than are currently used for manual analysis.

What is a clinically safe compensation strategy for busy laboratories?

The safest strategy for obtaining accurate data must surely be to check the spill over characteristics for each new lot of tandem antibody. If the lot is different from the previous lot, then compensation must be performed. It is not possible to advocate the creation of an

inexact system or approach to diagnostic MCF. At the same time, it is recognised that many clinical laboratories have time pressures and, sometimes, a lack of staff with adequate training in cytometry. Thus it is realistic to assume that, at least on occasion, re-compensation of new tandem antibody lots has not taken place prior to data reporting, and/or that compensation settings for one PE-Cy7-conjugated mAb are used for a different PE-Cy7-conjugated mAb.

Knowing the spill over variation between tandem lots and between tandem mAbs from different companies is helpful for evaluating how the final result might be influenced by using a wrong compensation matrix. We have shown here that for at least certain tandem mAbs, compensation for each new lot is not needed, and it could also be safe with a generic compensation matrix for the PE-Cy7 mAbs used in this study. It must be emphasised that each tandem and the combinations it is used in require validation prior to using such protocols. Further strategies that guard against false results derived from incorrect compensation would include choosing the appropriate hardware, such as lasers, mirrors and filters, as well as careful selection of fluorochrome conjugates. If possible, the same configuration and voltage settings could be used for all panels and tubes, since the application of a wrong compensation matrix would then cause less erroneous analysis. It would also be useful to identify antibody combinations that are more susceptible to smaller (up to 2%) compensation deviations.

This study focussed on a few commonly used tandems. Several tandem fluorochromes are currently available, and more are likely to be developed. It is hoped that these, and other fluorochromes, will enable further expansion of clinical MCF. The tests carried out in this study would be useful for selecting which fluorochromes and dyes to include in assays that require a robust performance.

Looking to the future, manufacturers may provide lyophilised cocktails. These could be made from large batches of mAb and validated by the manufacturer such that lot to lot variation is reduced. This would alleviate some of the consideration commented upon here.

ACKNOWLEDGMENTS

The authors would like to thank Nan Jiang and Kelly Lundsten at Biolegend for useful discussions and Nicki Senior for help with the compensation matrix analysis performed on the Navios.

Table 1.

Analysis of the percentage positive cells and the median fluorescence intensity (MFI) of six fluorochromes in a cocktail used to label CytoComp cells over an eight week period and Multi-Check Control cells over a six week period.

	CytoComp cells				Multi-Check Control cells			
	% positive		MFI		% positive		MFI	
mAb	**Mean (SD)**	**CV**	**Mean (SD)**	**CV**	**Mean (SD)**	**CV**	**Mean (SD)**	**CV**
CD2-PE-Cy7	81.2 (1.2)	1.5	3110 (258)	8.3	78.0 (1.5)	2.0	4836 (445.1)	9.2
CD3-APC-Cy7	76.2 (0.8)	1.0	9543 (537)	5.6	74.2 (1.6)	2.2	7006 (503)	7.2
CD4-FITC	60.5 (0.8)	1.4	2434 (166)	6.8	57.1 (1.0)	1.8	6248 (501)	8.1
CD8-PE	30.9 (0.6)	1.9	21024 (1408)	6.7	30.3 (0.6)	1.8	33522 (2626)	7.8
CD19-PerCP-Cy5.5	9.5 (0.5)	5.0	845 (55)	6.5	13.9 (0.6)	4.2	2571 (240)	9.3
CD20-APC	9.4 (0.4)	3.8	15158 (1471)	9.7	14.1 (0.7)	4.9	17309 (1288)	7.4
CD56-V450	n.a		n.a		1.1 (0.1)	12.3	4077 (968)	23.7
Kappa-FITC	n.a		n.a		55.6 (1.7)	3.0	12723 (561)	4.4
Lambda-PE	n.a		n.a		39.1 (21.6)	4.1	30446 (2181)	7.2

Table 2. Compensation matrices for tandem and non-tandem mAbs. The spill-over correction factors for mAbs within a given non-tandem group of mAbs are very similar. This was the case also for the PE-Cy7 conjugated mAbs tested.

			Percent spill-over into:						
		mAb	**FITC**	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**APC**	**APC-Cy7**	**V500**
Monoclonal antibodies conjugated to:	FITC	CD57		15.26	2.38	0.25	0.00	0.00	3.45
		CD11b		15.36	2.49	0.31	0.00	0.00	3.79
		MPO		15.18	2.43	0.33	0.02	0.02	3.88
		CD2		14.64	2.25	0.29	0.00	0.01	3.80
		CD103		15.14	2.42	0.28	0.00	0.00	3.72
		CD81		15.27	2.50	0.30	0.00	0.01	3.98
		CD38		15.85	2.57	0.28	0.06	0.00	4.00
	PE	CD13	1.10		18.02	2.00	0.02	0.00	0.01
		CD56	1.10		18.17	2.02	0.01	0.00	0.02
		CD79a	1.15		18.31	2.01	0.04	0.01	0.02
		CD160	1.15		18.23	2.07	0.03	0.00	0.05
		CD123	1.11		18.14	2.03	0.02	0.00	0.01
		CD14	1.11		18.04	2.02	0.02	0.00	0.02
		CD8	1.05		17.66	2.28	0.04	0.00	0.07
	PE-Cy7	CD117	0.10	1.71	3.25		0.03	1.48	0.00
		CD5	0.10	1.73	3.28		0.03	1.49	0.03
		CD10	0.09	1.25	3.01		0.02	1.55	0.03
		CD25	0.11	1.76	3.39		0.06	1.41	0.02
		CD19	0.09	1.50	3.04		0.02	1.69	0.01
		CD38	0.10	1.50	3.09		0.02	1.51	0.00
		CD14	0.10	1.65	3.00		0.04	1.53	0.04
	APC	CD33	0.01	0.00	1.09	0.17		2.84	0.00
		CD22	0.00	0.00	1.09	0.16		3.50	0.05
		CD23	0.01	0.00	1.10	0.17		2.85	0.02
		CD5	0.02	0.01	1.08	0.16		2.87	0.04
		CD11c	0.01	0.00	1.06	0.18		2.87	0.01
		CD138	0.01	0.00	1.09	0.18		2.88	0.02
		CD55	0.00	0.00	1.09	0.19		3.10	0.05
	APC-Cy7	CD3	0.07	0.00	0.85	7.73		31.07	0.13
		CD45	0.09	0.02	0.61	8.00		30.57	0.05
		CD20	0.15	0.05	0.67	8.64		20.26	0.20

Table 3. Comparison of spectral overlaps for PE-Cy7 conjugates from different manufactures. The mAbs tested were different CD5-specific clones.

	PE-Cy7 / PC7		
Percent	eBioscience (UCHT2)	Beckton Dickinson (LI7F12)	Beckman Coulter (BL1a)
FITC	0.07	0.05	0.07
PE	1.24	1.65	0.93
PerCP-Cy5.5	3.46	3.68	3.24
APC	0.02	0.05	0.04
APC-H7	2.63	2.42	2.91
Pacific Blue	0.00	0.00	0.00
V500	0.00	0.00	0.00

Table 4. Variation in spectral overlap between different APC-tandem conjugates. The antibodies were all specific for CD3, the clone and tandem conjugates are indicated in the table.

	APC-Tandem		
Percent	APC-eFluor750 (UCHT1)	APC-H7 (SK7)	APC-AF700 (UCHT1)
FITC	0.00	0.00	0.00
PE	0.00	0.00	0.00
PerCP-Cy5.5	0.56	0.23	0.48
PC7	5.54	6.38	5.24
APC	49.80	11.40	40.68
Pacific Blue	0.00	0.00	0.00
V500	0.00	0.00	0.00

Table 5. Spectral overlap for antibodies conjugated to 420-455nm emitting fluorochromes.

Percent	CD20-PB	CD3-PB	CD19-V450	CD56-V450	CD4-BV421
FITC	1.79	1.87	1.55	1.54	1.18
PE	0.16	0.23	0.15	0.19	0.12
PerCP-Cy5.5	0.00	0.01	0.03	0.03	0.00
PC7	0.00	0.00	0.00	0.00	0.00
APC	0.02	0.00	0.05	0.00	0.01
APC-H7	0.00	0.01	0.00	0.00	0.00
KO	45.55	45.83	38.76	38.31	16.54

Table 6. Spectral overlap for antibodies conjugated to 500-530nm emitting fluorochromes.

	V500 / KO		
Percent	CD45-V500	CD4-V500	CD45-KO
FITC	1.34	1.45	0.63
PE	0.03	0.34	0.06
PerCP-Cy5.5	0.02	0.05	0.00
PC7	0.02	0.00	0.10
APC	0.00	0.00	0.00
APC-H7	0.02	0.00	0.03
Pacific Blue	7.77	7.37	1.22

LITERATURE CITED

1. Biancotto A, Fuchs JC, Williams A, Dagur PK, McCoy JP, Jr. High dimensional flow cytometry for comprehensive leukocyte immunophenotyping (CLIP) in translational research. J Immunol Methods 2011;363:245-61.
2. Rawstron AC, Bottcher S, Letestu R, Villamor N, Fazi C, Kartsios H, de Tute RM, Shingles J, Ritgen M, Moreno C and others. Improving efficiency and sensitivity: European Research Initiative in CLL (ERIC) update on the international harmonised approach for flow cytometric residual disease monitoring in CLL. Leukemia 2013;27:142-9.
3. Forman MA, Gupta RK. Tandem dyes for flow cytometry: can we overcome quality concerns? MLO Med Lab Obs 2007;39:24, 26.
4. Stelzer GT, Marti G, Hurley A, McCoy P, Jr., Lovett EJ, Schwartz A. U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: standardization and validation of laboratory procedures. Cytometry 1997;30:214-30.
5. Stewart CC, Stewart SJ. Four color compensation. Cytometry 1999;38:161-75.
6. Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Methods 2000;243:77-97.
7. Hulspas R, Dombkowski D, Preffer F, Douglas D, Kildew-Shah B, Gilbert J. Flow cytometry and the stability of phycoerythrin-tandem dye conjugates. Cytometry A 2009;75:966-72.
8. Wood B. 9-color and 10-color flow cytometry in the clinical laboratory. Arch Pathol Lab Med 2006;130:680-90.
9. Matarraz S, Lopez A, Barrena S, Fernandez C, Jensen E, Flores-Montero J, Rasillo A, Sayagues JM, Sanchez ML, Barcena P and others. Bone marrow cells from myelodysplastic syndromes show altered immunophenotypic profiles that may contribute to the diagnosis and prognostic stratification of the disease: a pilot study on a series of 56 patients. Cytometry B Clin Cytom 2010;78:154-68.

10. van Lochem EG, van der Velden VH, Wind HK, te Marvelde JG, Westerdaal NA, van Dongen JJ. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. Cytometry B Clin Cytom 2004;60:1-13.

11. Wood BL. Flow cytometric diagnosis of myelodysplasia and myeloproliferative disorders. J Biol Regul Homeost Agents 2004;18:141-5.

12. Wells DA, Benesch M, Loken MR, Vallejo C, Myerson D, Leisenring WM, Deeg HJ. Myeloid and monocytic dyspoiesis as determined by flow cytometric scoring in myelodysplastic syndrome correlates with the IPSS and with outcome after hematopoietic stem cell transplantation. Blood 2003;102:394-403.

13. Arnoulet C, Bene MC, Durrieu F, Feuillard J, Fossat C, Husson B, Jouault H, Maynadie M, Lacombe F. Four- and five-color flow cytometry analysis of leukocyte differentiation pathways in normal bone marrow: a reference document based on a systematic approach by the GTLLF and GEIL. Cytometry B Clin Cytom 2010;78:4-10.

14. Le Roy C, Varin-Blank N, Ajchenbaum-Cymbalista F, Letestu R. Flow cytometry APC-tandem dyes are degraded through a cell-dependent mechanism. Cytometry A 2009;75:882-90.

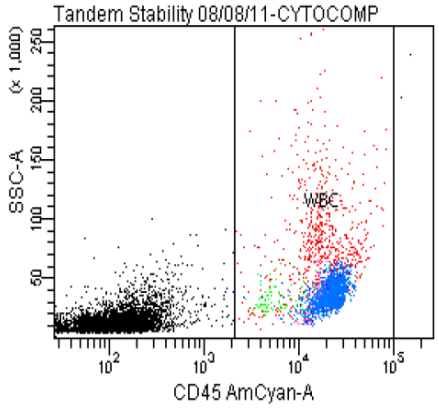
15. Chattopadhyay PK, Gaylord B, Palmer A, Jiang N, Raven MA, Lewis G, Reuter MA, Nur-ur Rahman AK, Price DA, Betts MR and others. Brilliant violet fluorophores: a new class of ultrabright fluorescent compounds for immunofluorescence experiments. Cytometry A 2012;81:456-66.

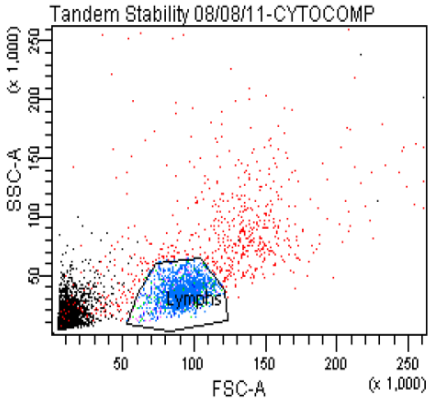
16. Westers TM, Ireland R, Kern W, Alhan C, Balleisen JS, Bettelheim P, Burbury K, Cullen M, Cutler JA, Della Porta MG and others. Standardization of flow cytometry in myelodysplastic syndromes: a report from an international consortium and the European LeukemiaNet Working Group. Leukemia 2012;26:1730-41.

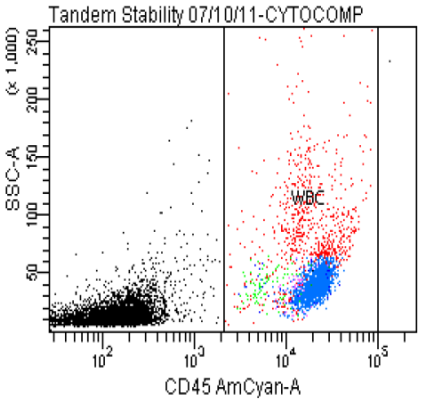
Figure Legends.

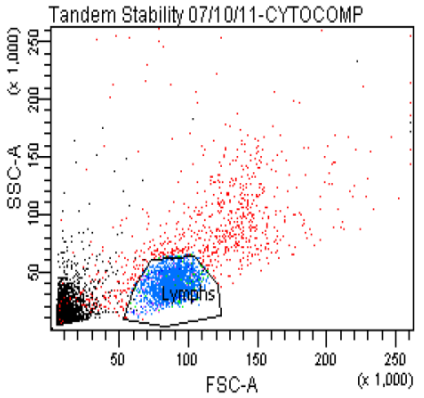
Figure 1. CytoCheck cells labeled with the cocktail of antibodies. **A**. Cells labeled with cocktail at beginning of stability study. **B**. Cells labeled with the cocktail after eight weeks of storage. Very little change in expression patterns or background fluorescence was observed.

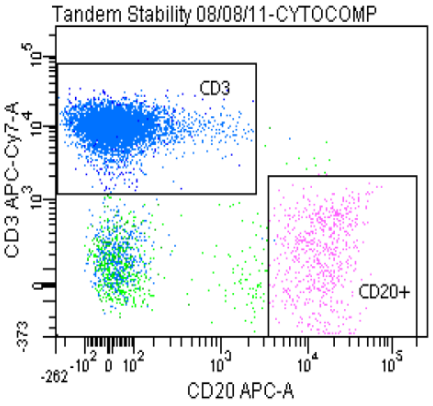
Figure 2. Lot-to-lot spill-over variation for tandem mAbs. The spectral overlap for 5 different lots each of different tandem mAbs were collated in two separate laboratories. **A** CD5-PE-Cy7, laboratory 1; **B** CD3-APC-H7, laboratory 1; **C** CD117-PE-Cy7, laboratory 2 and **D** HLA-DR-APC-H7, laboratory 2. The largest correction factor difference between the APC-H7 mAbs lots was 1.75%, for spill over into APC. For the PE-Cy7 mAbs lots, the largest variation was 1.84%, for spill over into APC-H7.

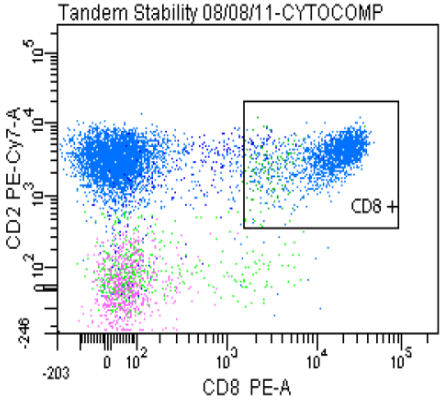


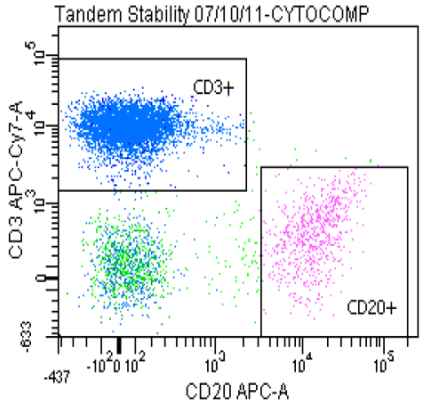


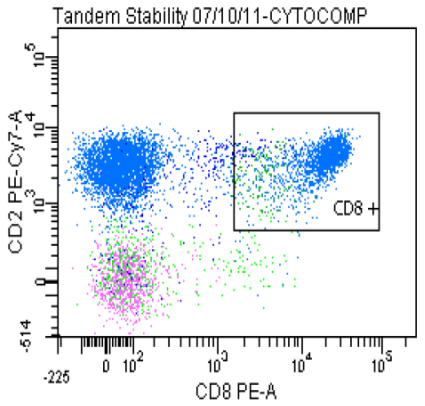


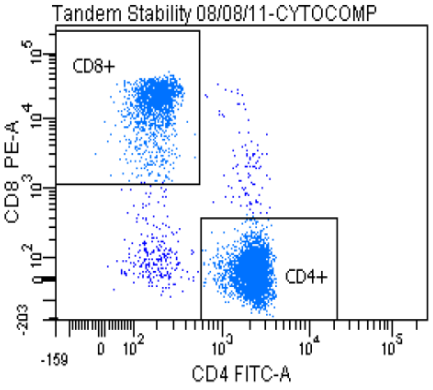












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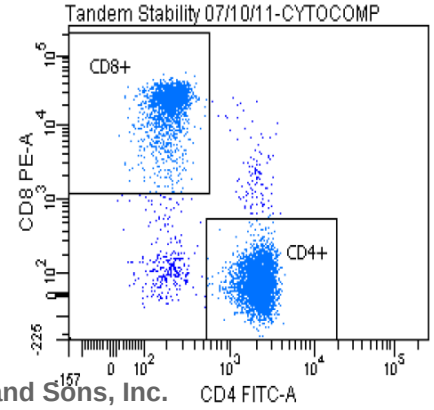
WBC	9,695	32.3	32.3
Lymphs	7,057	72.8	23.5
CD20+	668	9.5	2.2
CD2	5,757	81.6	19.2
CD8 +	1,817	31.6	6.1
CD3	5,312	75.3	17.7
CD4+	3,287	61.9	11.0
CD8+	1,669	31.4	5.6



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CD20+	731	9.6	2.4
CD2+	6,167	81.4	20.6
CD8 +	1,985	32.2	6.6
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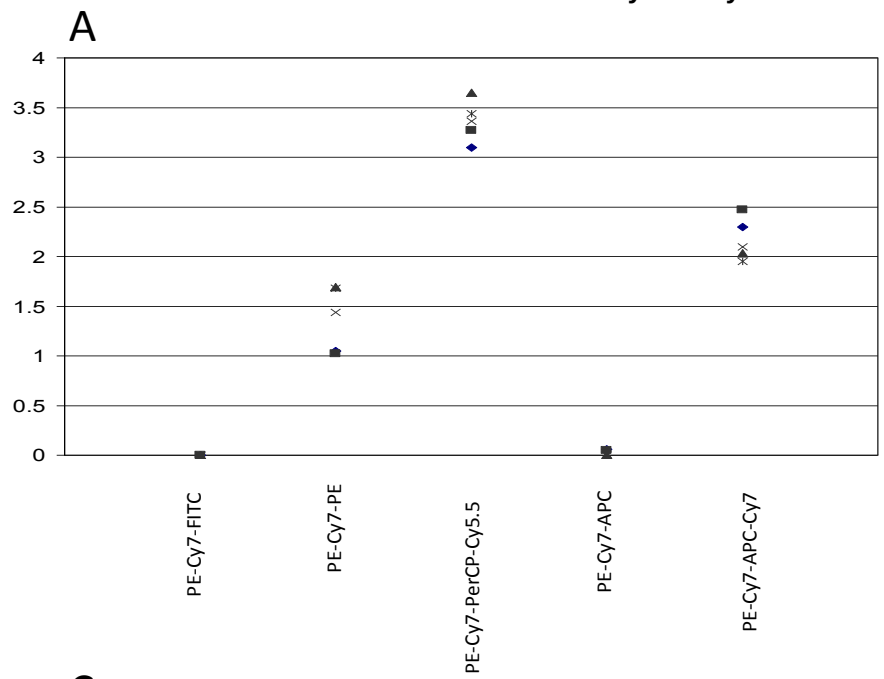
CD8+	1,839	32.1	6.1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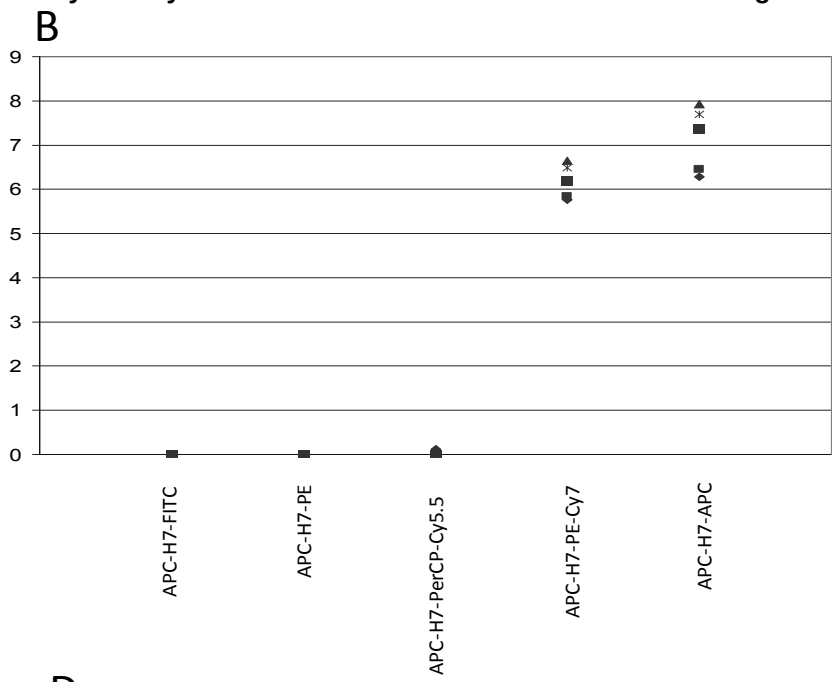
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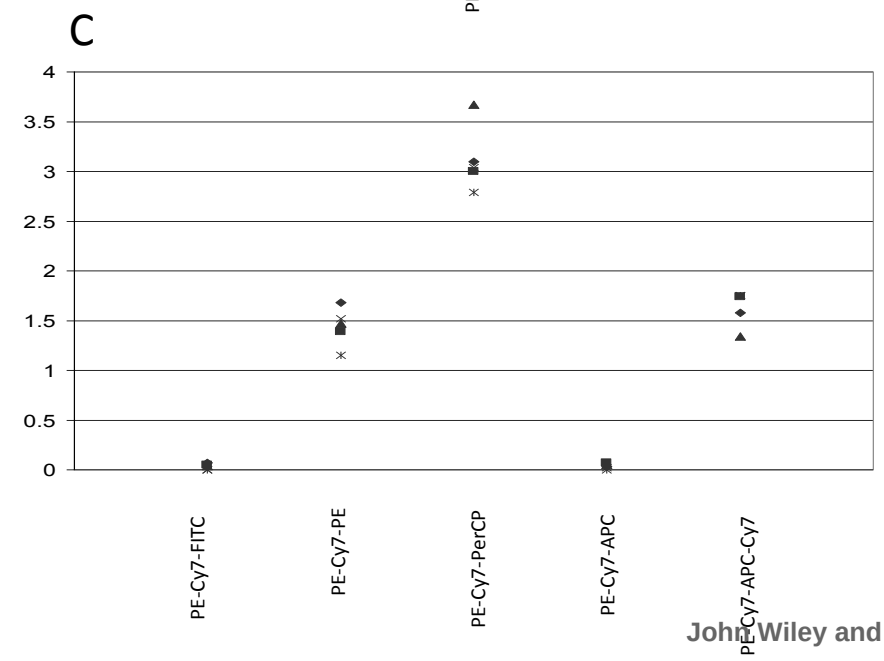
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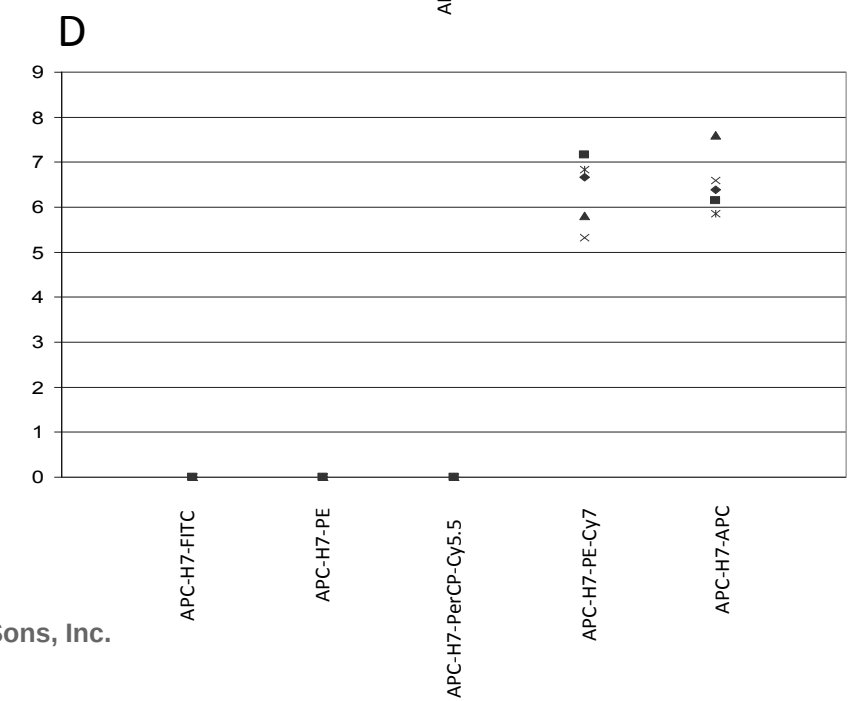
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Supplementary Data: Tables

Supplementary Table 1. Combinations of antibodies used to label preserved and lyophilised cells over a six and eight week period respectively.

	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4	Cocktail 5	Cocktail 6	Cocktail 7
FITC	CD57	CD11b	MPO	FMC7	CD2	CD103	CD81
	CD13	CD56	CD79a	CD79a	CD160	CD123	CD14
PerCP-Cy5.5	CD34	CD4	CD19	CD34	CD34	CD34	CD34
PECy7	CD117	CD5	CD10	CD38	CD25	CD19	CD38
	CD33	CD33	CD23	CD8	CD5	CD11c	CD138
APCCy7/H7	CD3(Cy7)	CD3 (Cy7)	HLA-DR (H7)	HLA-DR (H7)	CD20	CD20	CD3
	CD45	CD45	CD45	CD45	CD45	CD45	CD45

Supplementary Table 2. Cocktail combinations analysed a Canto II and a Navios.

Fluorochrome	Cocktail 1	Cocktail 2	Cocktail 3	Cocktail 4
FITC	CD36	CD45	FMC7	CD42b
PE	CD160	CD3	CD45	CD56
PC5.5	CD19	CD45	CD8	CD3
PC7	CD5	CD117	CD45	CD41
APC	CD79b	CD45	CD19	CD5
APC-Alexa750	CD20	CD5	CD3	CD45
Pacific Blue	CD16	CD3	CD45	CD16
Krome Orange	CD45	CD8	CD45	CD8

Supplementary Table 3. Analysis of percentage positive and median fluorescence intensity of six fluorochromes in a cocktail used to label Multi-Check Control whole blood over a six week period. The mean, SD and CV for six analyses are also shown.

Week	CD2-PE-Cy7		CD3-APC-Cy7		CD4-FITC		CD8-PE		CD19-PerCP-Cy5.5		CD20-APC		CD56-PB		Kappa-FITC		Lambda-PE	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
1	76.5	5160	75.5	7188	56.7	6686	30.4	36352	14.6	2372	15.3	18214	1.3	4119	56.7	11778	36.7	30154
2	76.8	4948	74.6	7713	59.0	6945	30.0	37196	14.5	2398	14.1	18717	1.0	3272	57.6	12637	37.5	31677
3	78.2	4042	75.3	6456	56.3	5642	29.7	32549	13.3	2378	13.3	15750	1.2	2805	56.8	12900	39.2	26400
4	78.1	5231	75.0	7301	57.4	6272	30.9	32766	14.2	2825	14.4	18352	1.0	4483	55.0	13489	40.7	32050
5	77.7	4609	73.7	6425	57.1	6139	31.0	31271	13.4	2538	13.7	16838	1.0	5572	53.7	12927	40.0	32223
6	80.8	5023	71.2	6955	56.3	5805	29.8	30997	13.5	2912	13.8	15985	1.0	4209	53.8	12606	40.2	30172
Mean	78.0	4836	74.2	7006	57.1	6248	30.3	33522	13.9	2571	14.1	17309	1.1	4077	55.6	12723	39.1	30446
SD	1.5	445.1	1.6	503	1.0	501	0.6	2626	0.6	240	0.7	1288	0.1	968	1.7	561	21.6	2181
CV	2.0	9.2	2.2	7.2	1.8	8.0	1.8	7.8	4.2	9.3	4.9	7.4	12.3	23.7	3.0	4.4	4.1	7.2

Supplementary Table 4. Analysis of percentage positive cells and median fluorescence intensity of six fluorochromes in a cocktail used to label CytoComp cells over an eight week period. The mean, SD and CV for eight analyses are also shown. The positive populations were all gated from CD45/FSC/SSC gated lymphocytes, except for $CD4^+$ and $CD8^+$ cells, these values are derived from the $CD3^+$ gated lymphocytes.

Week	CD2-PE-Cy7		CD3APC-Cy7		CD4-FITC		CD8-PE		CD19-PerCP-Cy5.5		CD20-APC	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
1	81	3057	75	9226	60	2325	31	19532	9.9	902	9.5	15107
2	79.9	3035	75.6	9549	59.9	2486	30.7	21984	9.7	856	9.5	15739
3	80.9	3268	77	10580	60.7	2695	31	22623	10	897	10	16623
4	82.9	3166	76.4	9566	62.4	2548	31.2	21364	9.2	840	9.2	16059
5	79.4	2676	75.7	8683	59.7	2259	29.8	18732	9	724	8.8	13548
6	82.1	3591	76.8	9835	60.4	2586	31.1	22585	9.3	857	9.4	16993
7	82.4	3091	77	9424	60.5	2318	30.8	20417	8.8	836	9.1	12887
8	81.4	2999	75.7	9487	60.4	2259	31.9	20952	10	848	9.6	14308
Mean	81.2	3110	76.2	9543	60.5	2434	30.9	21024	9.5	845	9.4	15158
SD	1.2	258	0.8	537	0.8	166	0.6	1408	0.5	55	0.4	1471
CV	1.5	8.3	1.0	5.6	1.4	6.8	1.9	6.7	5.0	6.5	3.8	9.7

Supplementary Table 5: Comparison of MFIs and population sizes for bone marrow samples stained in parallel with freshly aliquoted mAbs and the same mAbs stored in a cocktail. The cocktail was kept and used for five consecutive days, and tested each day on a different, fresh sample. The positive populations were gated as described in materials and methods. P=progenitor cells, N=neutrophils, M=monocytes, pos=positive, C=cocktail, IA=individually aliquoted.

		Day 1		Day 2		Day 3		Day 4		Day 5	
Gate	**Parameter**	**C**	**IA**	**C**	**IA**	**C**	**IA**	**C**	**IA**	**C**	**IA**
CD45+	CD45 MFI	56	47	41	40	44	44	55	52	31	30
P	CD45 MFI	15	11	3	4	3	3	5	4	4	4
N	CD33 MFI	6	6	25	26	13	14	25	27	12	12
M	CD33 MFI	18	18	88	91	54	58	104	109	65	68
N	% pos of total $CD45^+$	48	53	65	66	52	53	40	40	61	61
M	% pos of total $CD45^+$	8	7	7	7	9	9	13	13	5	5
N	CD11b MFI on immature cells	0	0	0	0	0	0	0	0	1	0
N	CD11b MFI on mature cells	19	19	40	41	25	26	31	32	19	19
N	CD13 MFI on immature cells	31	31	65	63	37	37	27	27	33	32
N	CD13 MFI on mature cells	82	81	71	72	65	63	75	74	76	74
N	% immature in 13/11b plot	8	9	8	8	8	8	21	21	4	4
N	% mature in 13/11b plot	59	57	60	60	63	64	17	17	71	72
N	CD16 MFI on mature cells	289	284	342	337	339	332	192	189	234	224
N	CD16 MFI on immature cells	5	5	4	4	4	4	4	4	3	3
M	DR MFI	61	57	33	34	64	72	32	35	61	63
M	% immature in DR/11b plot	5	6	17	17	15	16	9	10	12	12
P	% $CD34^+CD117^+$	10	9	9	10	5	5	5	5	8	8
P	% $CD117^+$ DR^-	9	9	16	16	7	7	25	22	7	6
$CD34^+$ $CD117^+$	CD34 MFI	22	19	19	19	13	13	13	13	16	16
$CD34^+$ $CD117^+$	CD117 MFI	14	14	20	21	19	18	16	16	16	15

Supplementary Figures: Legends

Supplementary Figure 1. Compensation matrices for different instruments. Matrices for four antibody cocktail combinations were generated on a Canto II and a Navios platform. The figure shows the mean compensation correction factor for each fluorochrome used (see supplementary Table 2). Error bars indicate standard deviation.

Supplementary Figure 2. Analysis of incorrectly compensated data. The plots show four different examples of off line compensation using step-wise increased or decreased correction factors for tandem mAb spill over into the donor fluorochrome channel or a neighbouring channel. All gates were kept constant. As expected, a more prominent effect on MFIs and population sizes was observed for weakly expressed antigens (**2B** and **2C**) compared to strongly expressed antigens (**2A**) or cell subsets with a near loss of antigen expression (**2D**).

A Changes in CD38-PE expression in B-ALL caused by overcompensation for PC7 spill over into PE. A diagnostic B-ALL bone marrow sample was stained with CD45-V500, CD19-APC, CD10-PC7, CD34-PerCP-Cy5.5, CD38-PE and CD20-FITC. Mononuclear cells were gated in a FSC-A/SSC-A plot. $CD19^+$ mononuclear cells were gated in a CD19/SSC-A plot. $CD19^+CD45^-$ blasts were gated from $CD19^+$ cells in a CD45/SSC-A plot. The plots in the figure show mononuclear cells in green, and B-ALL blasts in magenta. The correction factor for PC7 spill-over into the PE channel is indicated in the lower left corner of each plot. The resulting CD38 MFI for the total blast population is indicated in the upper right corner of respective plot. The CD38 dim gate is set below the normal hematogone CD38 expression level in regenerating bone marrows. The percentage CD38 dim B-ALL blasts is written inside this gate, in the lower right corner.

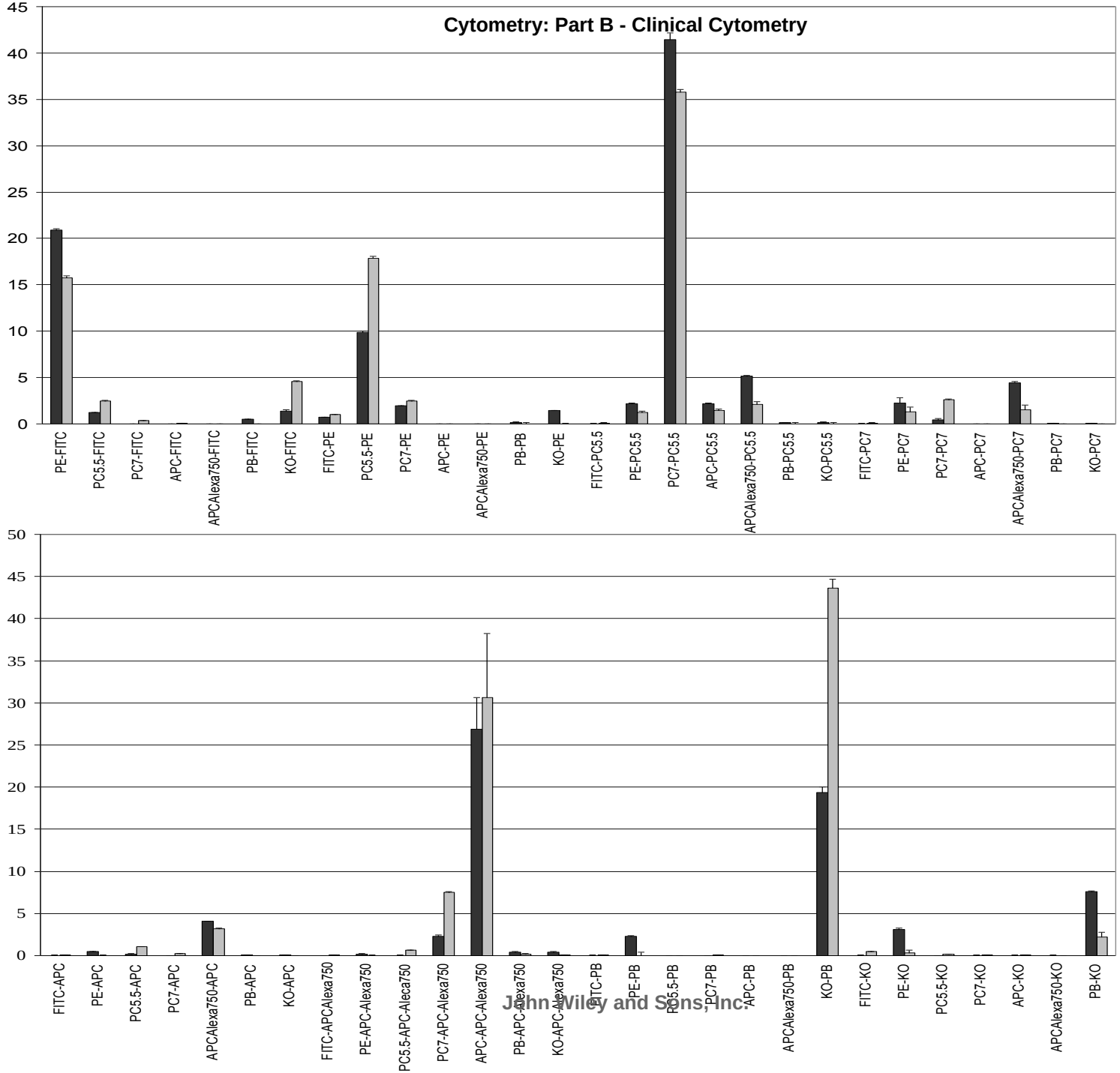
B Effect on under compensation for PC7 spill over into PE on CD26-PE expression on Sezary cells. A peripheral blood sample from a known Sezary Syndrome patient was stained with CD45-V500, CD4-BV421, CD3-APC-H7, CD7-APC, CD5-PC7, CD8-PerCP, CD26-PE and CD2-FITC.$CD3^+$ and $CD3^+CD4^+$ T cells were gated in a CD3/CD4 plot from CD45/FSC-A/SSC-A gated lymphocytes. The plots in the figure show lymphocytes (green), $CD3^+$ cells (light blue) and $CD3^+CD4^+$ cells (darker blue). The correction factor change is shown in the lower left corner, percentage CD26 weak/negative cells of $CD3^+CD4^+$ cells on middle left side and the CD26 MFI on $CD3^+CD4^+$ cells in the top right corner. The arrow indicates a potential point where visual interpretation may cause suspicion of incorrect compensation.

C Effect on CD5-PerCp-Cy5.5 expression on mature B cells by under compensation of PC7 spill over into PerCp-Cy5.5. A peripheral blood sample with normal leukocyte populations was stained with CD45-V500, CD20-PB, CD23-APC, CD19-PC7, CD5-PerCp-Cy5.5, Lambda-PE and Kappa-FITC. Mononuclear cells were gated in FSC-A/SSC-A and CD45/SSC-A plots. $CD19^+$ cells were gated from mononuclear cells in a CD19/SSC-A plot. $CD19^+CD5^+$ cells were gated from $CD19^+$ cells in the CD5/CD19 plot shown. The plots show mononuclear cells (green), $CD19^+$ cells (blue) and $CD19^+CD5^+$ cells (red). The correction factor change is shown in the lower left corner, the percentage $CD5^+$ cells of total $CD19^+$ cells in the $CD5^+$ gate in each plot, and the CD5-PerCp-Cy5.5 MFI for the $CD19^+$ cells in the top left corner.

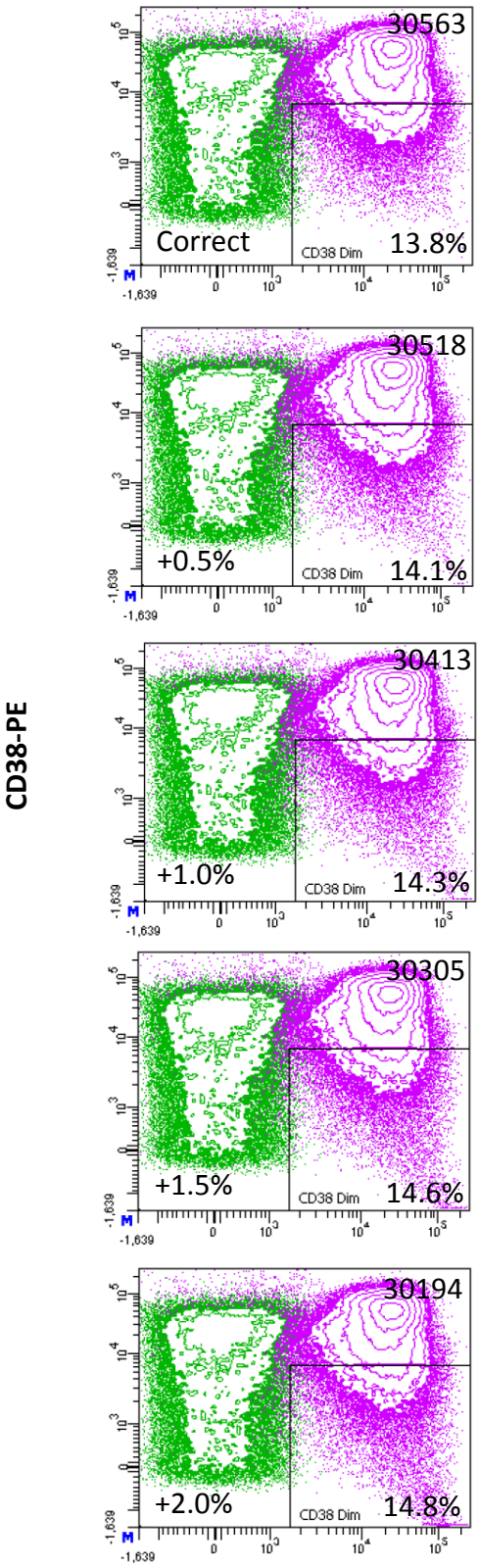
The arrow indicates a potential point where visual interpretation may cause suspicion of incorrect compensation.

D Altered correction factor for APC7 Spill-over into APC: Effect on CD7 expression on normal peripheral blood $CD3^+$ T cells. The sample was stained with CD45-V500, CD4-BV421, CD3-APC-H7, CD7-APC, CD5-PC7, CD8-PerCP, CD26-PE and CD2-FITC. Lymphocytes were gated in FSC-A/SSC-A and CD45/SSC-A plots. $CD3^+$ lymphocytes were gated in a CD3/CD2 plot and, from these, CD7 wkr $CD3^+$ cells in the plots shown. These display lymphocytes (green), $CD3^+$ cells (blue) and CD7 dim cells (red). The correction factor change is indicated in the lower left corner, the percentage CD7 dim $CD3^+$ cells on middle right, and the CD7 MFI for $CD3^+$ cells in the top right corner. The arrow indicates a potential point where visual interpretation may cause suspicion of incorrect compensation.

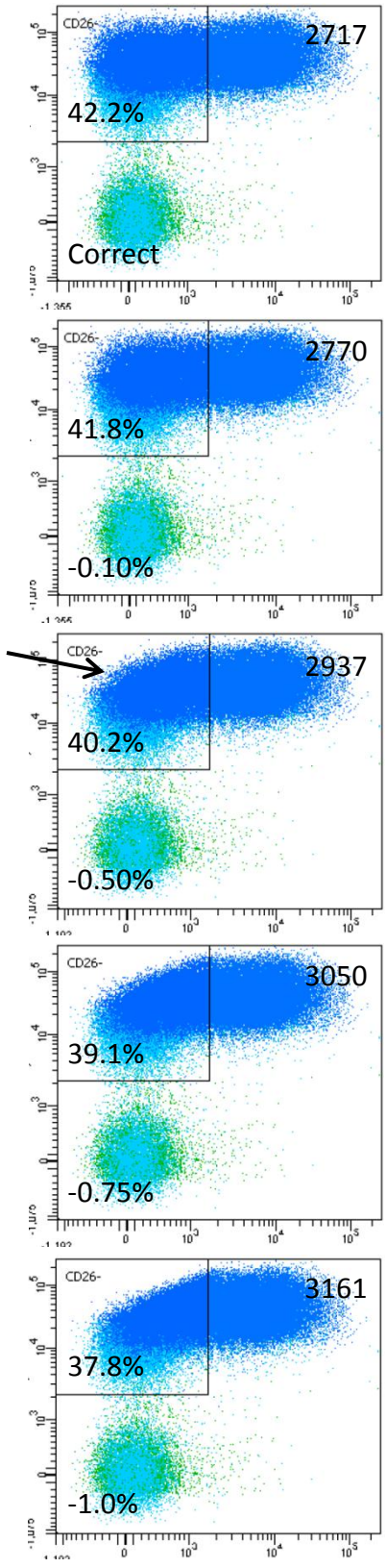
Supplementary Figure 1



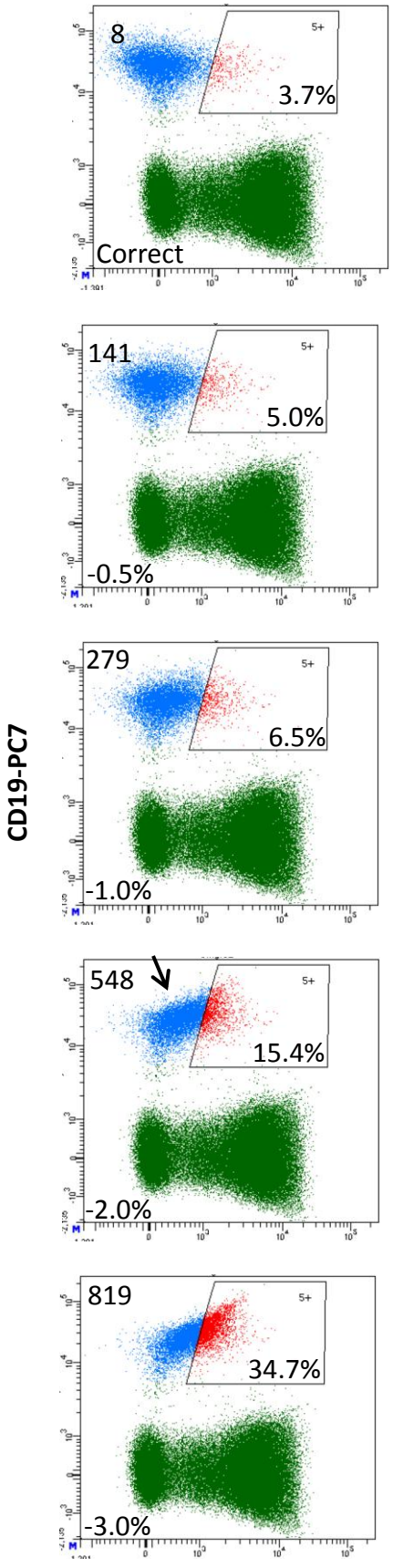
Supplementary Figure 2a



Supplementary Figure 2b



Supplementary Figure 2c



Supplementary Figure 2d