



DxFLEX Standardization study

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IN THIS PAPER YOU WILL

Learn how DxFLEX provides simplified standardization setup workflow between different instruments

Know DxFLEX can use one single standardized setting, reducing complexity in instrument setup calibration, simplifying the workflow and consequently saving laboratory costs and time.

Discover how DxFLEX delivers intra and inter lab consistent results after standardization, enabling experimental reproducibility and result reliability.

Introduction

In flow cytometry immunophenotyping, it is essential that results are comparable among laboratories, distinguishing true biological changes from technical artifacts ^{1,2}.

Flow cytometry standardization is a complex procedure widely reviewed in the literature ⁴⁻⁵, which includes many other concepts like panel marker design, sample processing, system setup and instrument performance monitoring. From a user point of view, the most difficult aspects to control are: instrument setup, performance monitoring, compensation matrix calculation and the setting of voltage/gain applied to each fluorescence detector.

Daily instrument performance monitoring ensures reproducibility of the analysis and also standardizes the positions of cell clusters in histograms obtained from different instruments ³. By setting voltage/gain to place the control particles at a particular target channel, a relatively similar instrument setup is achieved ^{4,5}. If one or more parameters are out of range from the target MFI (Mean Fluorescence Intensity), the system performance could be compromised. In case a readjustment is needed, the voltages/gains modification and the application of these changes to the panel's settings must be done manually. This makes standardization among different instruments difficult and time consuming.

The new DxFLEX includes a QC/Standardization module that automatically enables instrument quality control and standardization procedures. Quality control checks the adequate signal strength and precision of the instrument. The module monitors the MFI, tracks gain changes for any specific assay and directly links them to the panel experiment settings for automatic modifications.

In this paper we will review how to perform a complete standardization process and we will discuss the results obtained in a multicenter standardization study.

Material and Methods

Three different DxFLEX have been standardized in the study. They were located at: 1) University of Leipzig, German. 2) Hospital Dr Peset, Valencia, Spain. 3) Beckman Coulter FR, Paris, France.

In order to minimize the experimental variability due to samples and antibody handling, additional Beckman Coulter solutions have been adopted:

- **Samples:** ClearLLab Control Cells Normal, a liquid preparation of stabilized human erythrocytes and leukocytes to verify the accuracy/reproducibility of the steps involved in immunophenotyping. (Beckman Coulter PN. B90002)
- **MoAb Panel combination:** DURAClone IM Phenotyping Basic Tube kit*, expert-designed dry antibody panel for clinical research studies, which eliminates error-prone antibody pipetting and extensive reagent inventory management. (Beckman Coulter PN. B53309)

488 nm					635 nm			405 nm	
FITC	PE	ECD	PC5.5	PC7	APC	A700	APC-AF750	PB	KrO
CD16	CD56	CD19	-	CD14	CD4	CD8	CD3	-	CD45

Fig. 1. DURAClone IM Phenotyping Basic Tube is for Research Use Only, not for use in diagnostic procedure.

Additional material:

- Plastic test tubes 12 x 75 mm
- PBS Buffer (Beckman Coulter PN. 6603369)
- Flow-Set Pro beads (Beckman Coulter PN. A63492*)
- Versalyse lysing solution (Beckman Coulter PN. B53309)
- IOTest3 Fixative Solution (Beckman Coulter PN. A07800)
- Centrifuge accepting the sample tubes (typically 12x75 mm)
- Equipment to handle blood and antibodies (pipettes, tips, gloves, etc.)

Staining protocol and experiment workflow

Every lab prepared 5 samples as follows:

- Add 100 μ L of ClearLLab Control Cells Normal to an IM Basic reagent DURAClone tube.
- Vortex at high speed for 15 second.
- Incubate for 15 minutes at room temperature (RT), protected from light.
- Add 2 mL of VersaLyse.
- Add 50 μ L IOTest3 Fixative Solution.
- Vortex at high speed for 5 seconds.
- Incubate for 20 minutes at room temperature (RT), protected from light.

* DURAClone IM Phenotyping Basic Tube is for Research Use Only, not for use in diagnostic procedures.

FlowSet Pro: Research Use only. Not for use in diagnostic procedures.

FlowSet Pro has not been validated as part of the DxFLEX CE system and is only meant to serve as an example to demonstrate the general standardization process. DxFLEX Daily QC Fluorospheres or any other reference material that is relevant for your applications may be used as the standardization samples.

Each sample was acquired 3 times

- Centrifuge at 200 x g for 5 minutes.
- Aspirate or decant the supernatant and discard.
- Gently tap the cell pellet.
- Re-suspend cell pellet in 0.5 mL PBS.

Each sample was acquired 3 times

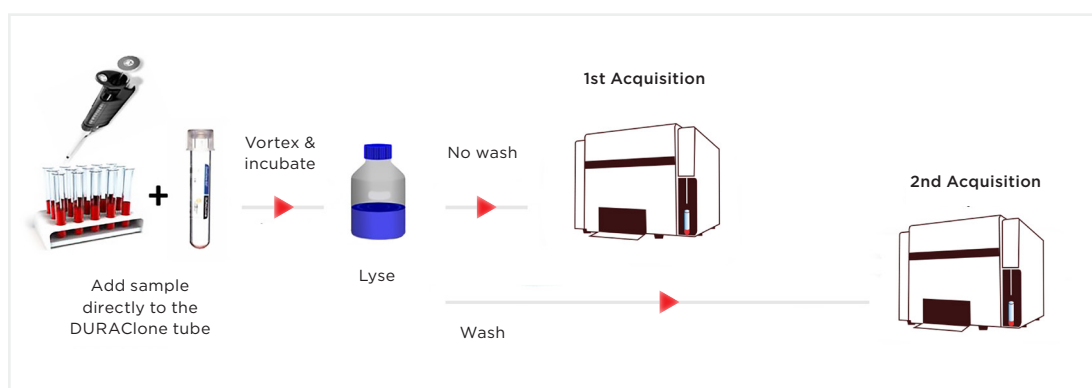


Fig. 2. Experiment workflow.

Instrument setup

(CHAPTER 5 Instrument Quality Control and Standardization, CHAPTER 6 Data Acquisition and Sample Analysis, CHAPTER 7 Compensation from the DxFLEX IFU PN. C44966.AA)

Standardized Target values

In order to run all samples under the same conditions, optimized settings were obtained following a gaintration protocol described on the Beckman Technical Note “**Characterization of Gains on the CytoFLEX**” (document FLOW-656502.20).

FlowSet Pro (Beckman Coulter PN. A63492*) was run with the obtained settings. The resulting median fluorescence intensities were added into the standardization tool and the .tgt file generated and shared across the participants in the multicenter study.

Standardization target file generation

1. Go to the “QC/Standardization” menu and open “Start QC/Standardization”.
2. Go to the “Settings” menu and open “Standardization Target Library”.
3. Click “ADD”.

* FlowSet Pro: Research Use only. Not for use in diagnostic procedures.

FlowSet Pro has not been validated as part of the DxFLEX CE system and is only meant to serve as an example to demonstrate the general standardization process. DxFLEX Daily QC Fluorospheres or any other reference material that is relevant for your applications may be used as the standardization samples.

- Complete the standardization target library. See Fig. 3

Item	Lot No.	Expires
FSpro	71	2020-05-12

Lot No.: 71
Expires: 2020-05-12

Threshold

Type	Channel	Mode	Value
Standardization Test Setting	FSC(Height)	Manual	100000
Acquisition Setting	FSC(Height)	Manual	200000

Signal

Channel	Median	Median Tolerance(%)
FSC	150000.00	5.00
SSC	502963.80	5.00
FITC	510572.60	2.50
PE	1661614.00	2.50
ECD	885173.50	2.50
PC5.5	425027.80	2.50
PC7	104847.50	2.50
APC	16149.30	2.50
APC-A700	354213.90	2.50
APC-A750	206050.50	2.50
PB450	274898.90	2.50
KO525	169785.80	2.50
Violet610	225232.50	2.50
Violet660	68851.30	2.50
Violet780	30227.50	2.50

Add... Delete Edit... Duplicate... Import... Export... Close

Fig. 3. DXFLEX targets obtained with Flow Set Pro Lot 71 after gain optimization. Data for illustrative purposes only.

- Select OK to save the target value.
- Once all the values are introduced, "Close". A "standardization target file" is automatically generated (FSpro-71.tgt). The file was then exported and shared with other participants in the study, who imported it into their standardization library.

Running a standardization experiment

Once the tgt file was imported, the standardization can be performed.

- Open the QC/Standardization module of the sw **QC/Standardization**
- Select Standardization.
Check the configuration of the system is the Default one.
Check the Carrier settings to fix the position of the tube on the carousel.
- Select the Flow-Set Pro lot number (In this case, 71).
- Dispense 15-20 drops (about 0.5 mL) of Flow Set Pro Beads in a tube and run the standardization tool.
- The obtained gains are labelled "Standard settings". They are automatically saved in the library as "S" settings.
- These settings will be automatically updated each time the standardization protocol is run.

Compensation matrix generation

Compensation matrix was generated using the automatic compensation application from DxFlex (Please refer to DxFLEX IFU).

ClearLLab control Cells, stained with IM Basic compensation kit tubes have been acquired with “Standard settings”.

The resulting “IM Basic compensation” was saved into the DxFLEX Compensation Library.

Panel experiment creation and set up

- Select ‘File → New Panel Experiment’.
- Select location and a name, e.g. ‘IM Basic standardization experiment’.
- Add New Empty sample, and on the generated Sample 1 right click and select “new tube”.
- Select ‘Settings → Set Channel’: select the corresponding channels and enter the label description. “Close”.

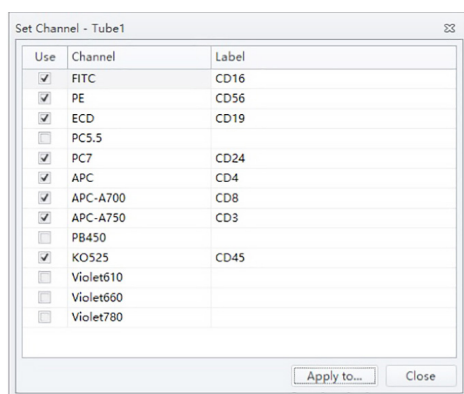


Fig. 5. Set label window.

- Right click on the “Tube 1” and “Link to Settings from Catalog”. Link to the standardized “Standard settings”.
- Import the compensation matrix by selecting  ‘Compensation Matrix: Import from Library’.
- Select the “IM Basic compensation” compensation matrix and import compensation matrix only.

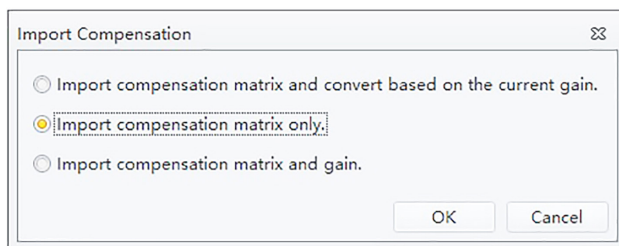


Fig. 6. Import compensation window.

- Generate the acquisition worksheet.

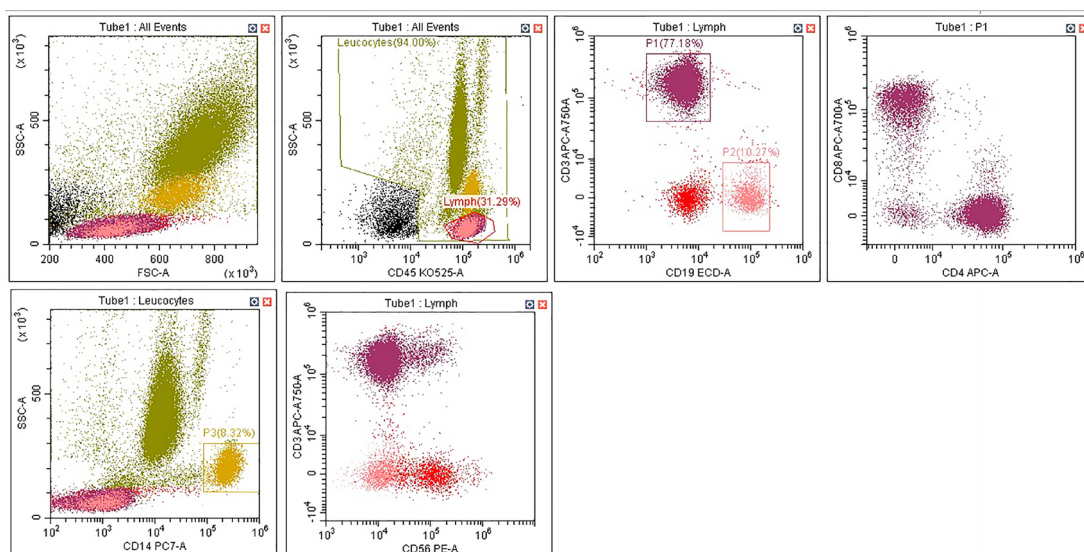


Fig. 7. Example of Analysis in CytoExpert for DxFLEx software. Plots are for illustrative purposes only.

- Rename “Tube 1” as “1 run” and “Duplicate without data” two times to generate the triplicate runs for the sample.

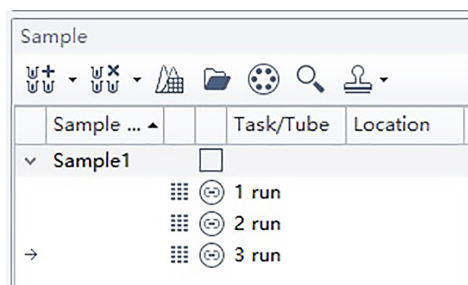


Fig. 8. Panel experiment screen shot.

- Right click on Sample 1 and “Duplicate without data” five times in order to create the complete acquisition worklist.
NB: This worklist will be run twice under different sample procedures: before wash and after 1 wash cycle

Sample acquisition

Every sample was acquired 3 times after lysis, before washing, and 3 times after washing, using the compensation and settings previously generated. Acquisition was performed at fast speed and stop condition was set at 50,000 on the “Leucocytes” gate.

Data analysis: Gating Strategy and off line compensation

Data from the 3 evaluation instruments were collected and analyzed in Kaluza C, where the same gating strategy and compensation have been applied.

Analyzed data have been exported for Excel statistical analysis.

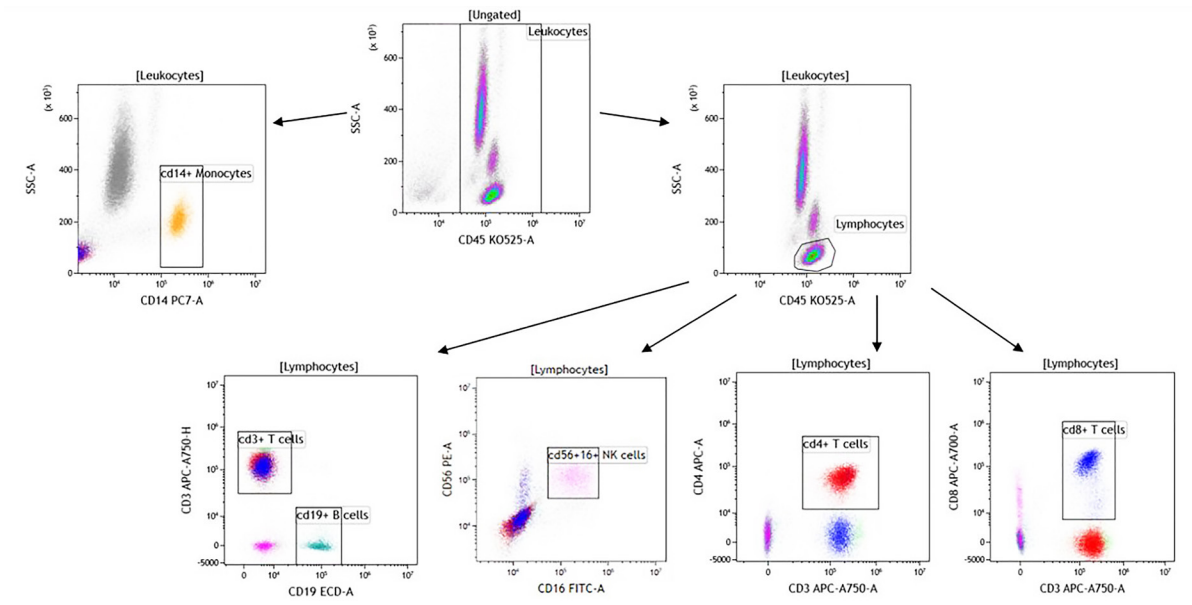


Fig. 9. example of gating strategy in Kaluza C software.

Compensation									
<Linked>									
Spillover (%)									
	FL1	FL2	FL3	FL5	FL6	FL7	FL8	FL10	
FL1		0.81	1.17	1.43	0.27	0.38	0.04	1.04	
FL2	28.39		28.50	5.50	0.27	0.45	0.06	1.15	
FL3	6.34	26.96		2.29	0.35	0.25	0.05	0.55	
FL5	0.14	0.61	4.49		0.39	1.54	1.75	0.20	
FL6	0.00	0.00	0.56	0.48		0.57	4.59	0.25	
FL7	0.00	0.00	0.33	0.35	35.15		2.14	0.17	
FL8	0.00	0.00	0.70	10.41	18.05	52.05		0.45	
FL10	2.10	0.04	0.40	0.63	0.17	0.17	0.03		

Fig. 10. compensation matrix applied for analysis. Data are for illustrative purposes only.

Results & Discussion

Sample procedure impact on the result

Paired t test data was performed, as shown in Fig. 11, and the data shows no significant difference between washed and no washed procedure.

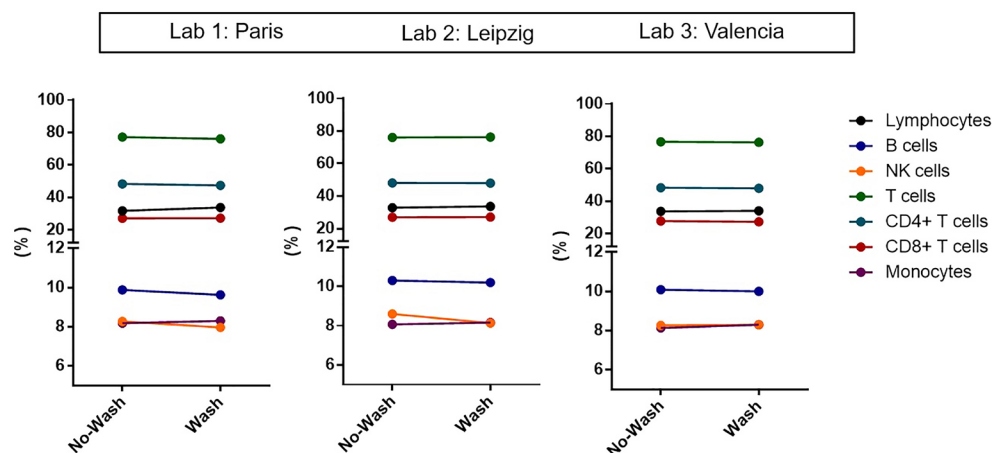


Fig. 11. Paired t test datas, each point is the median of 5 samples. Lymphocytes and Monocytes percentages have been calculated over total leucocytes, all the other populations over the total amount of lymphocytes.

Additional statistic evaluation was performed, as shown in chart 1, average values between washed and no washed sample procedure are similar. Furthermore, standard deviation and coefficient variation have been calculated and all average CVs for different sites are less than 2.5%.

Villepinte, Paris						Leipzig, Germany						Valencia, Spain								
NO WASHED			WASHED			NO WASHED			WASHED			NO WASH			WASHED					
AVG	ST DEV	CV	AVG	ST DEV	CV	AVG	ST DEV	CV	AVG	ST DEV	CV	AVG	ST DEV	CV	AVG	ST DEV	CV			
9.87	0.38	2.83%	9.63	0.21	2.18%	cd19+	10.29	0.20	1.91%	10.20	0.24	2.34%	cd19+	10.08	0.16	1.60%	10.00	0.24	2.39%	cd19
77.15	0.49	0.64%	76.02	0.41	0.54%	cd3+	75.99	0.42	0.55%	76.21	0.39	0.51%	cd3+	76.60	0.28	0.36%	76.22	0.41	0.53%	cd3
8.27	0.24	2.96%	7.97	0.19	2.43%	NK	8.59	0.27	3.20%	8.13	0.27	3.27%	NK	8.26	0.15	1.82%	8.30	0.23	2.78%	NK
48.23	1.00	2.07%	47.42	0.50	1.06%	cd3+cd4+	48.00	0.37	0.77%	47.88	0.44	0.92%	cd3+cd4+	48.33	0.20	0.42%	47.92	0.40	0.83%	cd3+cd4
27.02	0.31	1.14%	27.14	0.30	1.11%	cd3+cd8+	26.99	0.45	1.68%	27.02	0.38	1.42%	cd3+cd8+	27.74	0.21	0.77%	27.32	0.20	0.73%	cd3+cd8
8.18	0.14	1.68%	8.30	0.13	1.55%	Mono	8.06	0.11	1.35%	8.16	0.07	0.92%	Mono	8.14	0.10	1.18%	8.23	0.14	1.69%	Mono
31.74	1.73	5.44%	33.69	0.33	0.99%	Lymphs	32.85	0.29	0.88%	33.62	0.29	0.87%	Lymphs	33.66	0.25	0.75%	34.06	0.26	0.77%	Lymphs
average CV 2.40%			average CV 1.41%				average CV 1.48%			average CV 1.46%				average CV 0.99%			average CV 1.39%			

Intra lab result: accuracy within the same instrument

Data resulting after ANOVA analysis in Fig. 12 show no statistical differences between samples for each population in both sample procedures.

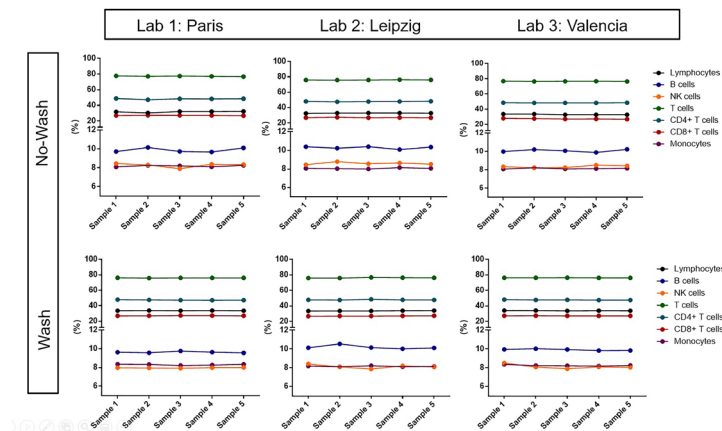


Fig. 12. Repeated measures one-way ANOVA datas, each point is the median of 3 repetitions. Lymphocytes and Monocytes percentages have been calculated over total leucocytes, all the other populations over the total amount of lymphocytes.

Since we demonstrated there is no significant difference in sample procedure, all data have been pooled and analyzed together. Resulting CVs reflect intra lab variation and, as shown in chart 2, all average CVs for different sites are less than 2.5%.

Villepinte, Paris				
NO WASHED + WASHED				
AVG	ST DEV	CV		
9.75	0.28	2.83%	cd19+	
76.59	0.73	0.95%	cd3+	
8.12	0.27	3.30%	NK	
47.82	0.89	1.86%	cd3+cd4+	
27.08	0.31	1.15%	cd3+cd8+	
8.24	0.15	1.77%	Mono	
32.72	1.58	4.84%	Limphs	
average CV		2.38%		

Leipzig, Germany				
ALL DATA (NO WASHED + WASHED)				
AVG	ST DEV	CV		
10.25	0.22	2.19%	cd19+	
76.10	0.42	0.55%	cd3+	
8.36	0.35	4.24%	NK	
47.94	0.41	0.86%	cd3+cd4+	
27.00	0.42	1.56%	cd3+cd8+	
8.11	0.10	1.28%	Mono	
33.24	0.48	1.46%	Limphs	
average CV		1.73%		

Valencia, Spain				
NO WASH + WASH				
AVG	ST DEV	CV		
10.04	0.21	2.10%	cd19	
76.39	0.40	0.53%	cd3	
8.28	0.20	2.40%	NK	
48.10	0.38	0.80%	cd3+cd4+	
27.51	0.30	1.08%	cd3+cd8+	
8.23	0.14	1.65%	Mono	
33.88	0.33	0.96%	Linfo	
average CV		1.36%		

Chart 2. Averages, Standard Deviations and Coefficient Variations results when pooling washed and no washed data.

Having a low coefficient of variability between sample replicates is crucial in demonstrating an assay was well-run and the resultant data is precise and accurate. All the centers show high consistency data in each detected population among the different acquisitions, either in no washed or washed sample preparations. It shows high intra lab reliability and accuracy of the method.

Intra lab results: Accuracy across multiple sites

Data from different centers have been analyzed in order to evaluate inter lab variability. ANOVA analysis in Fig. 13 shows no statistical differences were found between laboratories for each population.

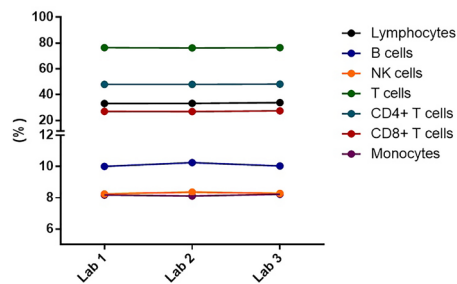


Fig. 13. Lab 1: Paris, Lab 2: Leipzig, Lab 3: Valencia.

Repeated measures one-way ANOVA datas, each point is the median of both procedures in 5 samples. Lymphocytes and Monocytes percentages have been calculated over total leucocytes, all the other populations over the total amount of lymphocytes.

All the data have been analysed together and average CV is less than 2.5%, as shown in chart 3.

All Samples Paris+Leipzig+Valencia			
ALL DATA (NO WASHED + WASHED)			
AVG	ST DEV	CV	
10.00	0.34	3.35%	cd19+
76.35	0.61	0.80%	cd3+
8.25	0.32	3.89%	NK
47.92	0.66	1.38%	cd3+cd4+
27.11	0.40	1.47%	cd3+cd8+
8.18	0.14	1.73%	Mono
33.12	1.16	3.49%	Limphs
average CV		2.30%	

Chart 3: Averages, Standard Deviations and Coefficient Variations results when considering all data (washed and no washed data) from different centers.

In addition, images have been also evaluated in the study. In particular, histogram overlays have been performed and positive population MFI (Mean Fluorescence Intensities) variability between centers have been analyzed.

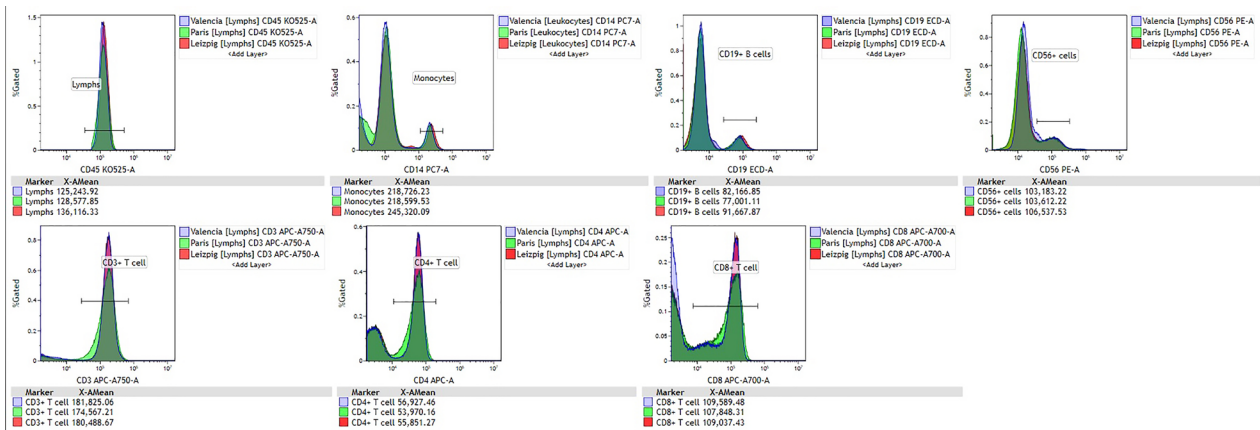


Fig. 14. Histograms overlays of the detected populations between different centers. MFI used for statistical analysis are displayed.

MFI have been analyzed and average CV is less than 3.5%, as shown in chart 4.

MFI comparison			
AVG	ST DEV	CV	
130185.87	5088.33	3.91%	Lymphs
225181.18	10391.03	4.61%	Monocytes
84475.76	6337.43	7.50%	CD19+
104267.17	972.82	0.93%	CD56+
178437.47	3826.95	2.14%	CD3+
54550.62	1783.06	3.27%	CD3+4+
108715.38	1594.52	1.47%	CD3+8+
average cv		3.41%	

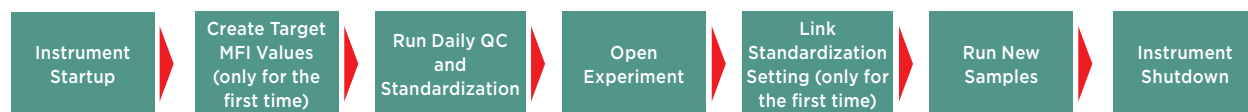
Chart 4. Histogram MFI Averages, Standard Deviations and Coefficient Variations results from different centers.

Data regarding % and MFI showed no statistical difference across centers has been detected. Inter lab consistency enables experimental reproducibility and result reliability.

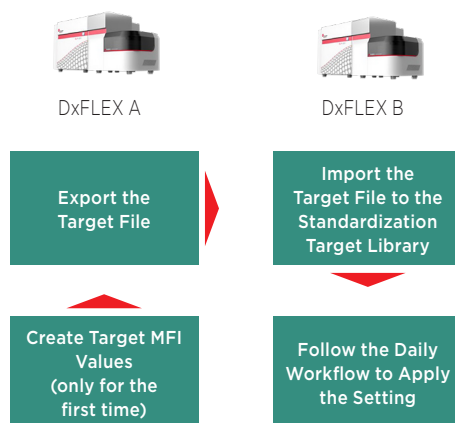
Conclusion

Simplified standardization setup workflow on one or between DxFLEX platforms.

A streamlined application standardization setup workflow will improve efficiency in clinical research studies. The DxFLEX platform provides a built-in standardization function in the CytExpert for DxFLEX software, which enables daily auto-calibration of gain settings under user defined target values and implements the same target values on different DxFLEX platforms for the same application.



In order to reduce variation in study outcome between laboratories and improve consistency between clinical research sites, in this white paper we demonstrate that Beckman Coulter DxFLEX system provides a simplified and reliable standardization workflow, while delivering consistent results on cell samples across instruments.



References

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DxFLEX is an IVD instrument that is available only in countries where the regulatory approval is obtained from the local regulatory agencies. Please check with your local sales representatives before placing your orders.



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