# DEVELOPMENT OF STANDARDIZED FLOW CYTOMETRY TOOLS TO ASSESS IMMUNOPHENOTYPING IN IMMUNOTOXICOLOGY STUDIES


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## Abstract

Immunotoxicology evaluations in animal models are mandatory pre-clinical studies of the drug discovery process, as they determine potential adverse health effects that a new drug may have on the immune system of patients. For this reason, the testing strategies need to be reliable and reproducible to accurately assess safety. Among Different Immunotoxicology tests recommended by the ICH Harmonized Tripartite Guideline, Flow Cytometry-based Lymphocytes phenotyping is a useful assay.

To address this, we have developed standardized and automated tools to determine rapidly lymphocyte phenotype in a rat model.

By using a cocktail of pre-calibrated labeled antibodies that give minimum emission spectral overlap (FITC/PC7/APC), we enable the addition of a wide variety of PE-labeled antibodies as well as the use of the dead cell exclusion 7-AAD marker. In addition to this flexible 5-color Lymphocyte Immunophenotyping assay, a no wash Red Blood Cell lysing system was optimized for enhanced accuracy. Furthermore, immunostaining and lysing steps can be processed on the Biomek Laboratory Automation Workstation and analysed on the FC 500 cytometer, with auto-setup panels and gating protocols provided.

Various examples are shown to demonstrate the usefulness of our methodology. For example, the described technique and analysis were applied for the detection of activated T cells following mitogenic activation with Concanavalin A, or for the exclusion of dead cells in Bone Marrow samples. We have established and compared the results obtained on Rat Peripheral Blood samples processed both manually and on the automation platform, and shown no significant difference. In addition, the sensitivity and specificity of our method to known immuno-suppressive drugs and/or immunogenic agents has been evaluated.


## Introduction

All new human pharmaceuticals should be evaluated for the potential to produce immunotoxicity, which means immunosuppression, immunoenhancement, allergenicity or drug-specific autoimmunity. Nonclinical / preclinical testing can help to reveal the first two of these adverse effects, except that, apart from the so-called "standard toxicity studies", no standardized practical approaches are currently available; methods to raise data in practice are not described! Here we take the flow cytometry-based lymphocyte subset phenotyping of rat biological samples as one example of tests recommended [by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)] in the "Immunotoxicity Studies for Human Pharmaceuticals" ICH S8 harmonized tripartite guideline. Usually, these are performed by home brewing tests mixing reagent components and analysis instruments from various origins that may prove difficult to reconcile one to the other, and to validate properly. We describe a set of reagents and adapted pre analysis tools that have been designed and developed to fit with the Biomek NX Liquid Handling system and the FC 500 MPL Flow Cytometer from Beckman Coulter. By design, this methodology aims at streamlining the preparation steps, with easy procedures, whatever the sample to be used, and whatever the lymphocyte sub-family level we want to reach. It is also aimed at opening the process to automation, while giving the freedom to manually perform small testing series with the same level of confidence in the results. We performed different evaluations of this methodology to demonstrate its flexibility linked to high robustness.

## Materials and Methods

### Animals and Study Protocol

Tests were performed mainly with Wistar rats and more rarely with Sprague Dawley or Brown Norway strains. Female (F) and Male (M) rats were assigned to a control group and 3 experimental groups as follow: Group 1: 10 F & 10 M treated with PBS; Group 2: 10 F & 10 M treated with $10^8$ SRBCs per animal by i.v. on day 2; Group 3: 10 F & 10 M treated with Cyclophosphamide (CY 40 mg/kg by i.p on day 1) + SRBCs ($10^8$ per animal by i.v on day 2); Group 4: 10 F & 10 M treated with Cyclophosphamide (CY 80 mg/kg by i.p on day 1) + SRBCs ($10^8$ per animal by i.v on day 2). Hematological investigations were performed on blood and organ samples were taken at terminal sacrifice on day 7. Lymphocyte enumeration following rat treatment with CY is known to be reflective of immunosuppression.

### Monoclonal Antibodies (mAbs)

The following mouse mAbs were used to develop the three color combinations.

- IOTest® Anti-Rat CD3-FITC / CD4-PC7 / CD8-APC (PN A32909): anti-rat CD3, clone 1F4, IgM; anti-rat CD4, clone OX38, IgG2a; anti-rat CD8, clone OX8, IgG1.
- IOTest Anti-Rat CD3-FITC / CD45RA-PC7 / CD161-APC (PN A32910): anti-rat CD3, clone 1F4, IgM; anti-rat CD45RA, clone OX33, IgG1; anti-rat CD8, clone 10/78, IgG1.

### Sample Preparation (manual)

The cell population of interest is stained with monoclonal antibodies and the red blood cells in each sample are lysed in a No wash "Fix-and-Lyse" procedure based on the combination of VersaLyse™ Lysing Solution (PN IM3648) and IOTest 3 Fixative Solution (PN IM3515).

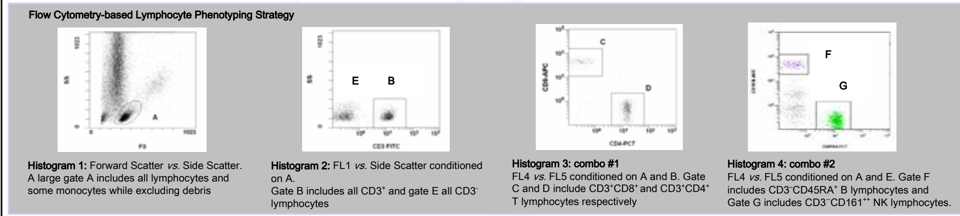
Briefly, 25 µL of biological sample (whole blood, or spleen cell preparation, or lymph node preparation, or broncho-alveolar lavage preparation) are incubated with 25 µL of mAbs combination for 20 minutes at room temperature (RT), protect from light. The Fix & Lyse mixture is then added to the samples and incubated for 10 minutes at RT, protect from light. The stained samples will be keep at 2-8°C until use (≤ 2hrs following the lysis).

### Flow Cytometry

The flow cytometer must be equipped to detect Forward Scatter, Side Scatter and three fluorescence channels allowing the analysis of FITC-, PC7-, and APC-conjugated antibodies (respectively 504 – 541 / 750 – 810 / 650 – 680 nm maximal peak emission). APC conjugates require an exciting source of 633 nm (He-Ne laser) or 635 nm (Red diode laser).

1- Prepared samples are analyzed on FC 500 / Multi Carousel Loader (MCL) or FC 500 / Multi Plate Loader (MPL) using respectively Beckman Coulter Cytomics™ CXP™ and MXP™ analysis software. In all experiments, a mixture of Flow-Set™ Fluorospheres (PN 6607007) and Flow-Set 675 Fluorospheres (PN 6607120) were used to set up the PMT values while compensations were set by using Cyto-Comp Cells (PN 6607023) stained separately with either CD45-FITC (PN IM0782), CD45-PC7 (PN IM3548) or CD45-APC (PN IM2473).

2- Analysis: a large gate is drawn around lymphocytes on an FS/SS histogram and the $CD3^+$ and $CD3^-$ subpopulations were analyzed as described below.




**Histogram 1:** Forward Scatter *vs.* Side Scatter. A large gate A includes all lymphocytes and some monocytes while excluding debris

**Histogram 2:** FL1 *vs.* Side Scatter conditioned on A. Gate B includes all $CD3^+$ and gate E all $CD3^-$ lymphocytes

**Histogram 3: combo #1** FL4 *vs.* FL5 conditioned on A and B. Gate C and D include $CD3^+CD8^+$ and $CD3^+CD4^+$ T lymphocytes respectively

**Histogram 4: combo #2** FL4 *vs.* FL5 conditioned on A and E. Gate F includes $CD3^-CD45RA^+$ B lymphocytes and Gate G includes $CD3^-CD161^{++}$ NK lymphocytes.

### Sample Preparation using the Biomek® NX Span-8

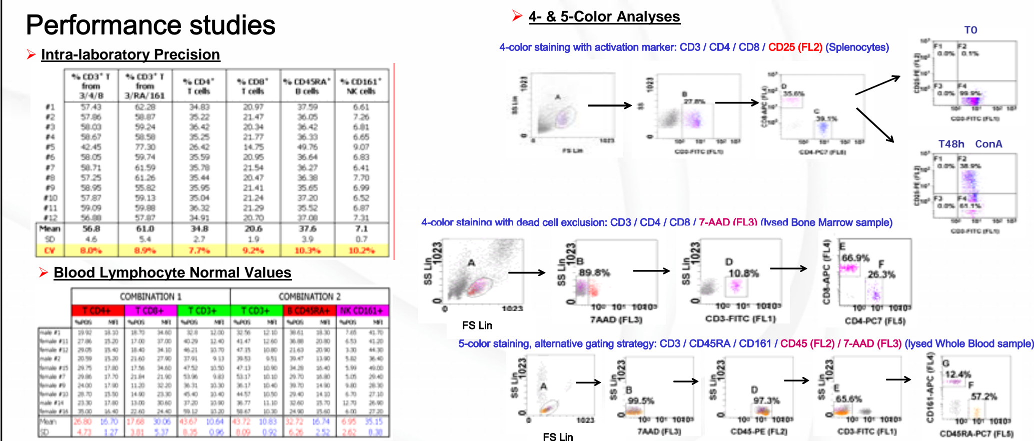
In some experiments, the sample preparations (blood or cell suspensions + antibodies mixing in view of flow cytometric immunophenotyping) have been performed with a Beckman Coulter Biomek NX Span-8 Laboratory Automated Workstation, using a series of eight 1000-µL teflon® needles to distribute biologic samples and reagents. The Span-8 option allows for liquid level sensing. We have optimized the method to provide a walk-away preparation system (follow-up of reagent and sample levels, mixing step optimized to avoid sedimentation of blood cells, specific aspiration process to eliminate risk of contaminations using blowout air gap and leading air gap, etc.).

Mixing has been performed by multi aspiration / dispense of fluids in microtubes (Micronic, Ref. M32022) arranged on a 96-socket carrier rack. After preparation, the rack has been directly transferred on the plate loader of an FC 500 MPL.

A fully integrated installation of the FC 500 MPL plate loader can be proposed as optional feature, with a direct link with the Biomek NX Span-8 working area.

Span-8 Device

## Performance studies




### Intra-laboratory Precision

| | % $CD3^+$ T from 3/4/8 | % $CD3^+$ T from 3/RA/161 | % $CD4^+$ T cells | % $CD8^+$ T cells | % $CD45RA^+$ B cells | % $CD161^+$ NK cells |
|---|---|---|---|---|---|---|
| #1 | 57.43 | 62.28 | 34.83 | 20.97 | 37.59 | 6.61 |
| #2 | 57.86 | 58.87 | 35.22 | 21.47 | 36.05 | 7.26 |
| #3 | 58.03 | 59.24 | 36.42 | 20.34 | 36.42 | 6.81 |
| #4 | 58.67 | 58.58 | 35.25 | 21.77 | 36.33 | 6.65 |
| #5 | 42.45 | 77.30 | 26.42 | 14.75 | 49.76 | 9.07 |
| #6 | 58.05 | 59.74 | 35.59 | 20.95 | 36.64 | 6.83 |
| #7 | 58.71 | 61.59 | 35.78 | 21.54 | 36.27 | 6.41 |
| #8 | 57.25 | 61.26 | 35.44 | 20.47 | 36.38 | 7.70 |
| #9 | 58.95 | 55.82 | 35.95 | 21.41 | 35.65 | 6.99 |
| #10 | 57.87 | 59.13 | 35.04 | 21.24 | 37.20 | 6.52 |
| #11 | 59.09 | 59.88 | 36.32 | 21.29 | 35.52 | 6.87 |
| #12 | 56.88 | 57.87 | 34.91 | 20.70 | 37.08 | 7.31 |
| **Mean** | **56.8** | **61.0** | **34.8** | **20.6** | **37.6** | **7.1** |
| SD | 4.6 | 5.4 | 2.7 | 1.9 | 3.9 | 0.7 |
| CV | 8.0% | 8.9% | 7.7% | 9.2% | 10.3% | 10.2% |

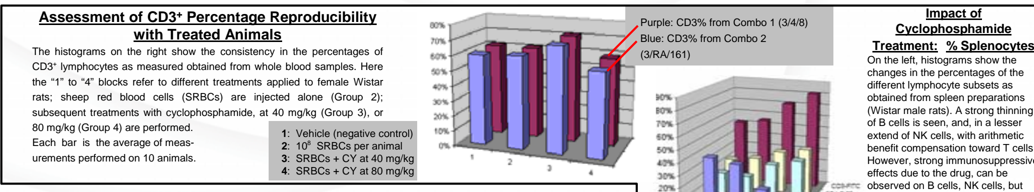
### Blood Lymphocyte Normal Values

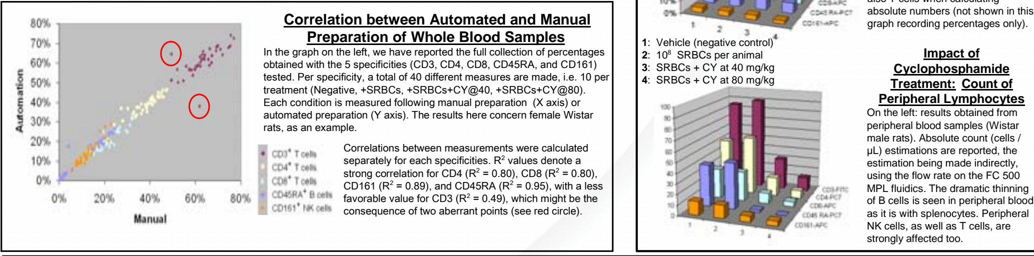
| | COMBINATION 1 | | | | | | COMBINATION 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T CD4+ | | T CD8+ | | T CD3+ | | T CD3+ | | B CD45RA+ | | NK CD161+ | |
| | %POS | MFI | %POS | MFI | %POS | MFI | %POS | MFI | %POS | MFI | %POS | MFI |
| male #1 | 19.92 | 18.10 | 18.70 | 34.60 | 32.8 | 12.00 | 32.56 | 12.10 | 38.61 | 18.30 | 7.65 | 41.70 |
| female #11 | 27.86 | 15.20 | 17.00 | 37.00 | 40.29 | 12.40 | 41.47 | 12.60 | 36.88 | 20.80 | 6.53 | 41.20 |
| female #12 | 29.05 | 15.40 | 18.40 | 34.10 | 46.21 | 10.70 | 47.15 | 10.80 | 21.63 | 20.90 | 3.30 | 44.30 |
| male #2 | 20.59 | 15.20 | 21.60 | 27.90 | 37.91 | 9.13 | 39.53 | 9.51 | 39.47 | 13.90 | 5.82 | 36.40 |
| female #15 | 29.75 | 17.80 | 17.56 | 34.60 | 47.52 | 10.50 | 47.13 | 10.90 | 34.28 | 16.40 | 5.99 | 49.00 |
| female #7 | 29.86 | 17.70 | 21.84 | 21.90 | 53.96 | 9.83 | 53.17 | 10.10 | 29.70 | 16.80 | 5.05 | 29.40 |
| female #9 | 24.00 | 17.90 | 11.20 | 32.20 | 36.31 | 10.30 | 36.17 | 10.40 | 39.70 | 14.90 | 9.80 | 28.30 |
| female #10 | 28.70 | 15.50 | 14.90 | 23.30 | 45.40 | 10.40 | 44.57 | 10.50 | 29.40 | 14.10 | 6.70 | 27.10 |
| male #14 | 23.30 | 17.80 | 13.00 | 30.60 | 37.20 | 10.90 | 36.77 | 11.10 | 32.60 | 15.70 | 12.70 | 26.90 |
| female #16 | 35.00 | 16.40 | 22.60 | 24.40 | 59.12 | 10.20 | 58.67 | 10.30 | 24.90 | 15.60 | 6.00 | 27.20 |
| Mean | 26.80 | 16.70 | 17.68 | 30.06 | 43.67 | 10.64 | 43.72 | 10.83 | 32.72 | 16.74 | 6.95 | 35.15 |
| SD | 4.73 | 1.27 | 3.81 | 5.37 | 8.35 | 0.96 | 8.09 | 0.92 | 6.26 | 2.52 | 2.62 | 8.38 |

### Assessment of $CD3^+$ Percentage Reproducibility with Treated Animals

The histograms on the right show the consistency in the percentages of $CD3^+$ lymphocytes as measured obtained from whole blood samples. Here the "1" to "4" blocks refer to different treatments applied to female Wistar rats; sheep red blood cells (SRBCs) are injected alone (Group 2); subsequent treatments with cyclophosphamide, at 40 mg/kg (Group 3), or 80 mg/kg (Group 4) are performed.

Each bar is the average of measurements performed on 10 animals.




### Correlation between Automated and Manual Preparation of Whole Blood Samples

In the graph on the left, we have reported the full collection of percentages obtained with the 5 specificities (CD3, CD4, CD8, CD45RA, and CD161) tested. Per specificity, a total of 40 different measures are made, i.e. 10 per treatment (Negative, +SRBCs, +SRBCs+CY@40, +SRBCs+CY@80). Each condition is measured following manual preparation (X axis) or automated preparation (Y axis). The results here concern female Wistar rats, as an example.

Correlations between measurements were calculated separately for each specificities. $R^2$ values denote a strong correlation for CD4 ($R^2 = 0.80$), CD8 ($R^2 = 0.80$), CD161 ($R^2 = 0.89$), and CD45RA ($R^2 = 0.95$), with a less favorable value for CD3 ($R^2 = 0.49$), which might be the consequence of two aberrant points (see red circle).

### Impact of Cyclophosphamide Treatment: % Splenocytes

On the left, histograms show the changes in the percentages of the different lymphocyte subsets as obtained from spleen preparations (Wistar male rats). A strong thinning of B cells is seen, and, in a lesser extend of NK cells, with arithmetic benefit compensation toward T cells. However, strong immunosuppressive effects due to the drug, can be observed on B cells, NK cells, but also T cells when calculating absolute numbers (not shown in this graph recording percentages only).

### Impact of Cyclophosphamide Treatment: Count of Peripheral Lymphocytes

On the left: results obtained from peripheral blood samples (Wistar male rats). Absolute count (cells / µL) estimations are reported, the estimation being made indirectly, using the flow rate on the FC 500 MPL fluidics. The dramatic thinning of B cells is seen in peripheral blood, as it is with splenocytes. Peripheral NK cells, as well as T cells, are strongly affected too.

## Discussion

The data presented here demonstrates that the methodology is suitable for the immunophenotyping of T-, B- and NK-cell populations in various rat biological samples. The main features of this methodology are:

1- Robustness, since straightforward results can be obtained with whole blood samples, spleen or lymph node preparations, and bone marrow samples. 2- Flexibility, since you can perform the sample preparation into regular lysing tubes and into small-sized tube that fit into rack with microplate format. Also, from an analytical point of view, you can add to the three color combinations a choice of viability dye (7-AAD), gating tool (CD45-PE), or cell-status / -activation markers (e.g. CD25-PE). You might even combine drop ins, would you need to perform at the same time dead cell exclusion and CD45-gating. 3- Easiness since a no wash lysis procedure is involved and an automated preparation process is possible using the Biomek NX S-8, and can be fully synchronized with the flow cytometric acquisition on the FC 500 MPL.

Further evaluations are still to be performed, one of which being to explore absolute counting of the lymphocyte subsets using Flow-Count™ Fluorospheres, a feature that would allow a direct determination of absolute counts, together with the full standardization of this methodology.

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