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## **Flow cytometry on the move. Extending feasibility of the Cytomics FC500: from homologous blood transfusion detection to soluble molecules multiassay.**

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### **Abstract**

Flow cytometry is commonly used in research and clinical diagnostic for the analysis of properties of cells. Recently, antidoping laboratories worldwide introduced flow cytometry techniques and instrumentation for homologous blood transfusion detection. Although this unique flow cytometry application is cell based, we explored the feasibility of extending the field of application of our instrument Cytomics FC500 (Beckman Coulter) by using it as a platform for soluble molecules, bead-based multiassay detection.

Most of the techniques for the measurement of soluble analytes employ ELISA-based technology. Flow cytometry instrumentation also may be used as a readout platform for sandwich immunoassay by using microsphere of different sizes and color for multiplexed immunoassay.

This work shows how to broaden the application of an instrumentation that requires a significant initial investment by WADA laboratories and that is presently used only within the restricted field of application of the identification of doping by detecting homologous blood transfusion.

### **Introduction**

Antidoping laboratories worldwide introduced flow cytometry techniques and instrumentation for homologous blood transfusion detection. Although the majority of the applications are cell-based, recently flow cytometry has been used also for the multiparametric analysis of soluble molecules.

In this work, we coated microspheres with primary capture antibodies specifically reacting with each of the analytes to be detected by the multiplex system. We developed a basic panel to detect molecules related to Growth Hormone/IGF1 axis. Once we developed the multiassay system on our FC500 beckman coulter flow cytometer, we performed an application study aimed to explore the potential of cytokines expression in "ex vivo" models of blood transfusion.

### **Experimental**

Carboxyl modified microspheres (5 micron diameter) are purchased from Bangs Laboratories Inc (Fishers IN, USA). All antibody pairs for the target molecules were purchased from R&D systems: hGH, IGF1, IGFBP3, Prolactin, Interleukin-6, Interleukin-8. Covalent attachments of antibodies to microspheres is achieved by activating the carboxyl groups with water-soluble carbodiimide. Protocol for covalent coupling has been taken from [www.bangslabs.com](http://www.bangslabs.com), with slight modifications.

A multiplex assay protocol has been then developed. Firstly, a mixture of beads populations has been made considering 3000 beads for each target in a total volume of 75  $\mu$ L. 25  $\mu$ L of samples has been added to the mixture. 50  $\mu$ L of biotin-conjugated secondary antibody mixture (each secondary antibody concentration previously titered for optimal conditions) has been added. After incubation of 2 hours at room temperature, beads were washed two times with PBS. Supernatant was discarded after centrifugation. 50  $\mu$ L of Streptavidin-PE was added to all tubes and an incubation step of 45 minutes followed. Then beads were washed again two times with PBS. Supernatant was discarded after centrifugation.

500  $\mu$ L PBS were added to all tubes, then beads were ready to be analyzed by the flow cytometer. Flow cytometric analysis: each set composed by several subpopulations of beads is stained internally with different amount of dye, so that each subset can be clearly identified on a 2-D plot and not overlapping each other. Y-axis detects target fluorochrome and X-axis detects the reporter. Subset of beads shown in Figure 1 represents a panel created for the simultaneous detection of Human Growth Hormone (isoform 22KDa) and molecules directly or indirectly related to hGH administration. MFI values obtained for the standard samples were used to create the calibration curves. Standard curves were created with LBIS software.

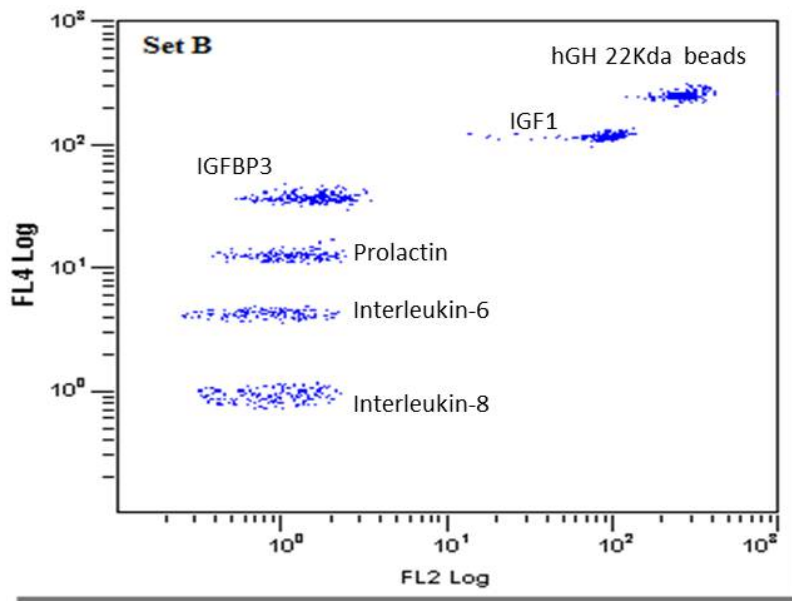


Figure 1: A panel of beads created for the flow cytometric multiassay of soluble molecules related to growth hormone. Flow cytometer clearly separates all different population of beads so no carryover is achieved at the detection level.

## Results and Discussion

FC500 flow cytometer clearly identifies subsets of microspheres coated with different capture antigens without carryover among beads subpopulations.

We also validated the performances for each of the populations of soluble molecules (Figure 2).

A correlation study in which 15 human serum samples were analyzed for hGH 22KDa using both the implemented flow cytometric multiassay and WADA standard reference method for hGH isoforms has been made. Results (Figure 3) show high correlation between the two assays (R-square= 0,883).

In the perspective of extend the field of application of this approach we performed an analysis of cytokines' pattern of activation on an "ex vivo" model of blood transfusion. Cytokines are a large family of small glycoproteins that act mainly as immunomodulating agents in the up and down-regulation of several genes related to erythropoiesis and cellular immune

response. Cytokines were here analyzed on a total of 130 blood plasma samples (80 males and 50 females) coming from blood routine samples received by our laboratory.

We determined normal reference range for males and females and the production of cytokines in blood samples after a storage period (up to 40 days) has been monitored. A blood transfusion has been accomplished ex-vivo. Stored samples were mixed with fresh samples (donor at 10%). Mixed samples were incubated a 37°C in a 5% carbon dioxide atmosphere. We tested cytokines production of cytokines at regular intervals up to 24 hours. Differences between males and females in the reference value of Interleukin-6 and Interleukin-8 were observed. Secondly, after 30 days of storage blood samples showed high levels of two cytokines (IL-2 and IL-8) compared to basal levels. Finally, ex-vivo transfused samples showed increased levels of IL-8 compared to basal non transfused samples. The activation of IL-8 as a consequence of transfusion has a magnitude higher than the one observed after the storage period.

Analyte target	Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	cv% intra-day	cv% inter-day	Carryover
hGH 22KDa	0 - 50	0.100	0.200	4.5	7.1	Undetected
IGF-1	0 - 50	2.000	5.000	5.3	8.2	Undetected
IGFBP3	0 - 10	0.250	0.500	5.8	7.5	Undetected
Prolactin	0 - 20	0.010	0.150	3.6	6.7	Undetected
Interleukine-6	0 - 20	0.002	0.005	2.5	7.6	Undetected
Interleukine-8	0 - 20	0.002	0.050	2.6	7.5	Undetected

Figure 2: Performance validation results of the flow cytometric multiassay. This subset of beads has been developed to detect soluble molecules related directly or indirectly to the GH/IGF1 axis.

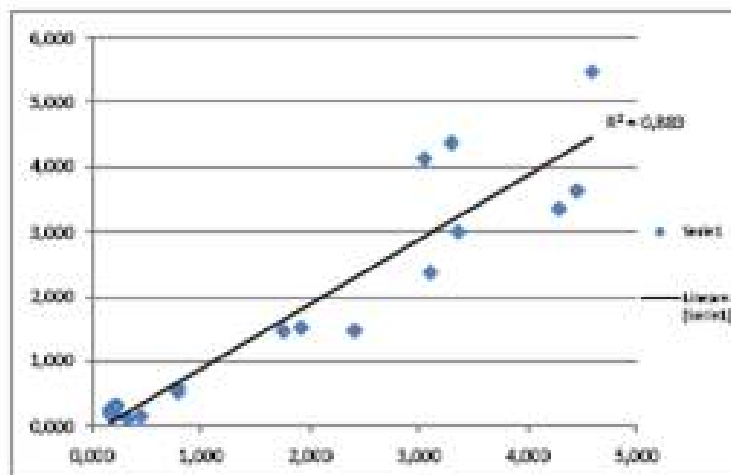


Figure 3: Correlation analysis of 15 human serum samples analyzed for hGH 22Kda isoform both with flow cytometry multiassay developed in this work and Wada reference isoform method (CMZ).

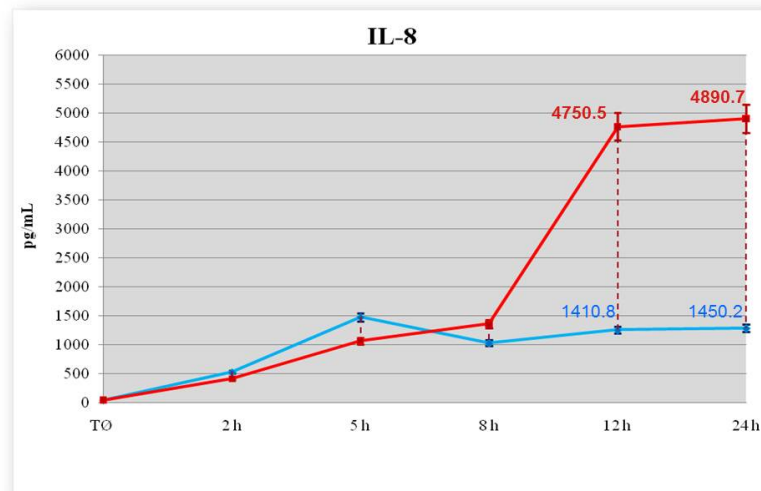


Figure 4: The ex-vivo experiment on a transfused sample (red line) and non-transfused (blue line) shows a marked increase of the concentration of Interleukin-8 in the transfused sample. Interleukin-8 can be a suitable marker for a study on blood transfusion strategies of detection.

## Conclusions

This study shows essentially how to broaden the application, within the anti-doping field, of a common flow cytometer, an instrumentation that requires a significant initial investment by the WADA laboratories and that is presently used for reduced workload that is the identification of doping by homologous blood transfusions. Here we have shown the potentiality of flow cytometry for the multiassay analysis of soluble molecules in human serum. All reagents used in this work (microspheres, antibodies and buffers) are commercially available so this opens up the possibility for a laboratory to develop "home-made" multiassays. It is also possible to expand the panel of target molecules to be analyzed according to the combination of different populations of beads. Moreover, we believe that the proposed application study on cytokines represents a promising first step in the aim to find new strategies and target markers for the future development of a method to detect autologous blood transfusion in doping control.

## References

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