Analyses Cytokiniques par Cytométrie en Flux

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St Etienne - 2018

Cytokines : domaines d'intérêt

- Pathologies
    * Maladies auto-immunes

    * Maladies liées à l'âge (maladies neurodégénératives, athérosclérose….)

    * Cancers

    * Traumatismes (post-chirurgie oculaire…)

- Pharmacologie

- Toxicologie (industries cosmétiques, alimentaires…)

Dosages des Cytokines : méthodologie

- **ELISA**

- **ELISPOT**

- **Cytométrie en Flux:**

  *** Cytokines intracellulaires**

  *** Sécrétion de Cytokines**

Quantification de Cytokines Intracellulaire

- Immunomarquages directs ou indirects en présence de monensine pour bloquer l'efflux de cytokines (amplification du signal),

- Informations fournies:

    * % de cellules produisant des cytokines,

    * Quantité de cytokines par cellules:
        - Valeurs souvent arbitraires (moyenne de fluorescence)
        - Valeurs absolues : (envisageable)

Présentation et exploitation des données

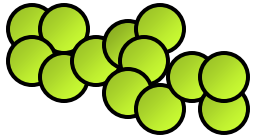
- Histogrammes : monoparamétriques, biparamétriques…

- Tableaux de données : % et/ou moyenne de fluorescence

- Analyses statistiques

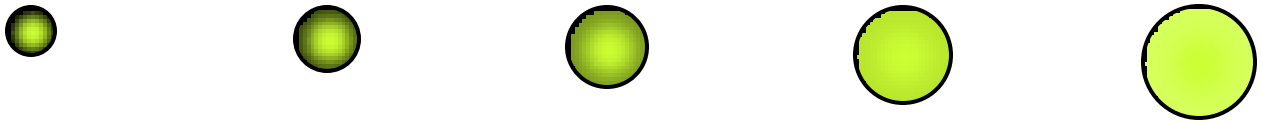
Microbilles: le début !

° Réglage et optimisation du cytomètre en flux

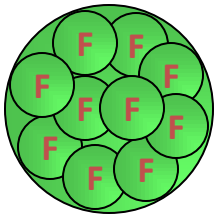


° Calibration en taille
et

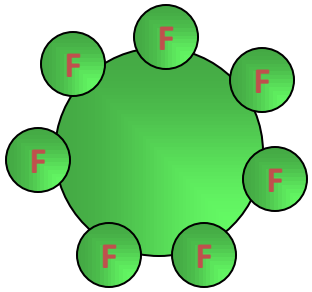
° Calibration en fluorescence
(équivalent de molécules fluorescentes par microbilles)



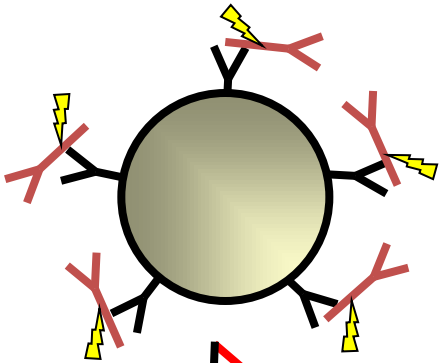
Différents types de microbilles



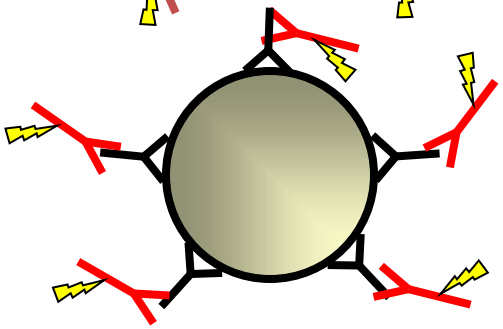
- microbille de calibration avec fluorochrome (F) interne



- microbille de calibration avec fluorochrome externe



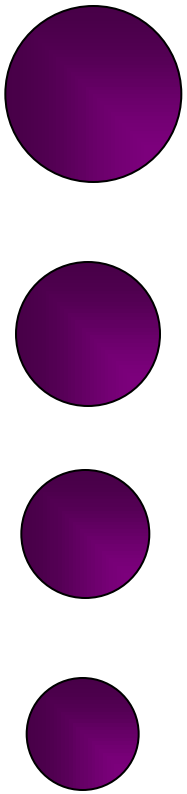
- microbille de calibration non fluorescente recouverte d'anticorps anti-IgG pour la quantification antigènique en immunofluorescence directe



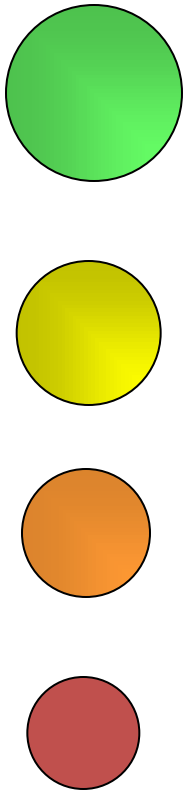
- microbille de calibration non fluorescente, mimant la cellule ayant fixé l'anticorps primaire, et sur laquelle se fixe le conjugué pour la quantification antigènique en immunofluorescence indirecte

Distinction de microbilles par cytométrie en flux

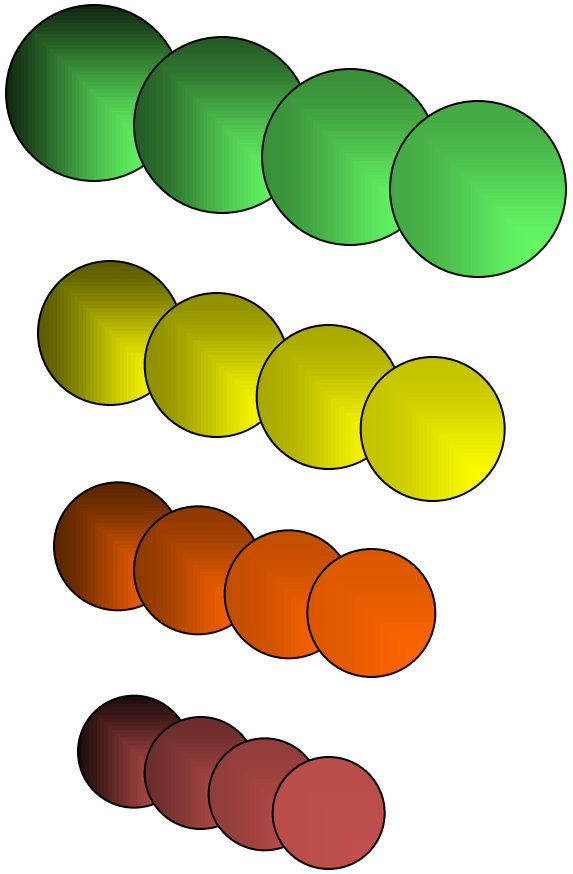
Tailles différentes



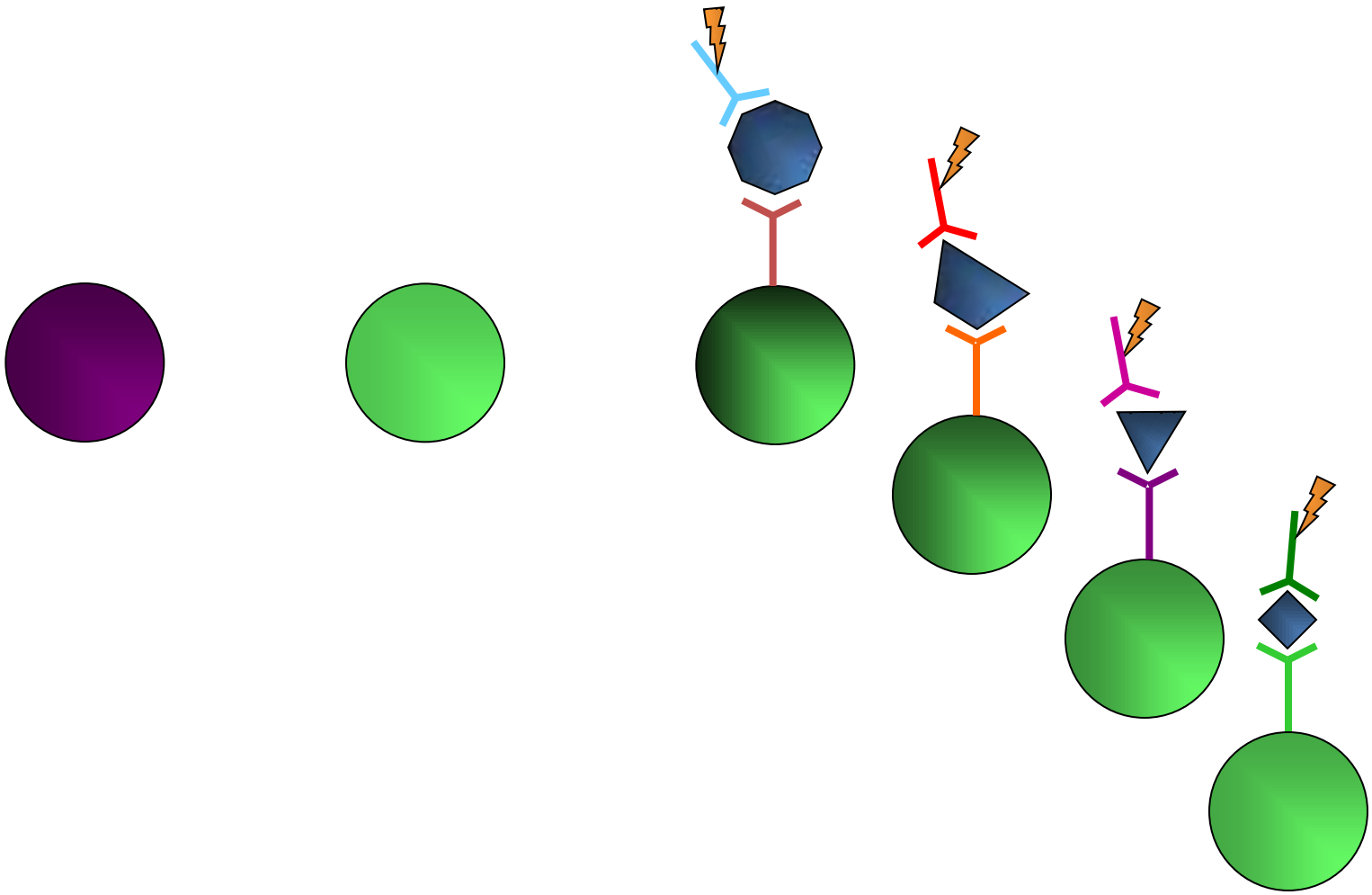
Fluorescences différentes



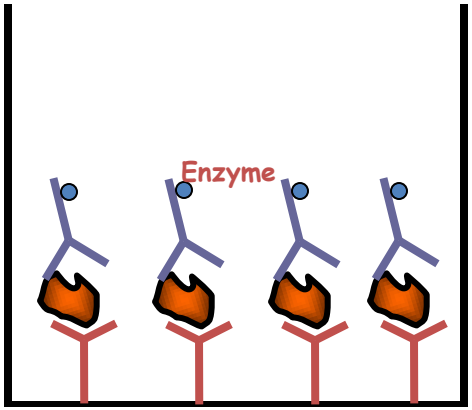
Intensités de fluorescences différentes



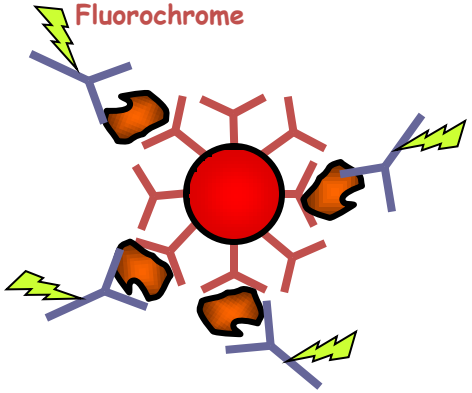
Microbilles et détection d'antigènes



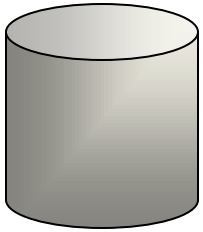
ELISA versus SAT (Suspension Array Technology)



ELISA



SAT



micropuits

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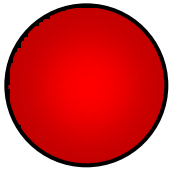
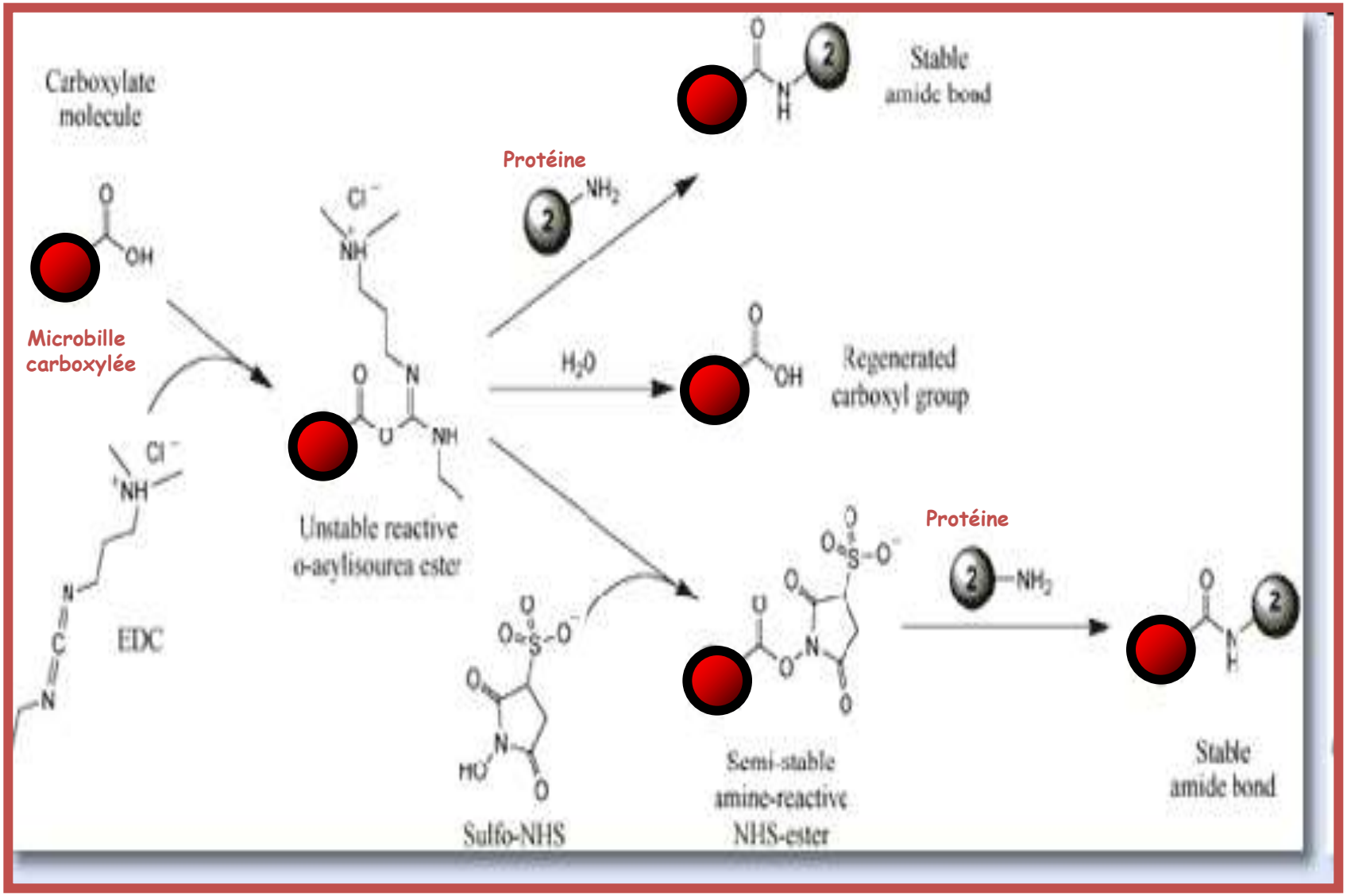


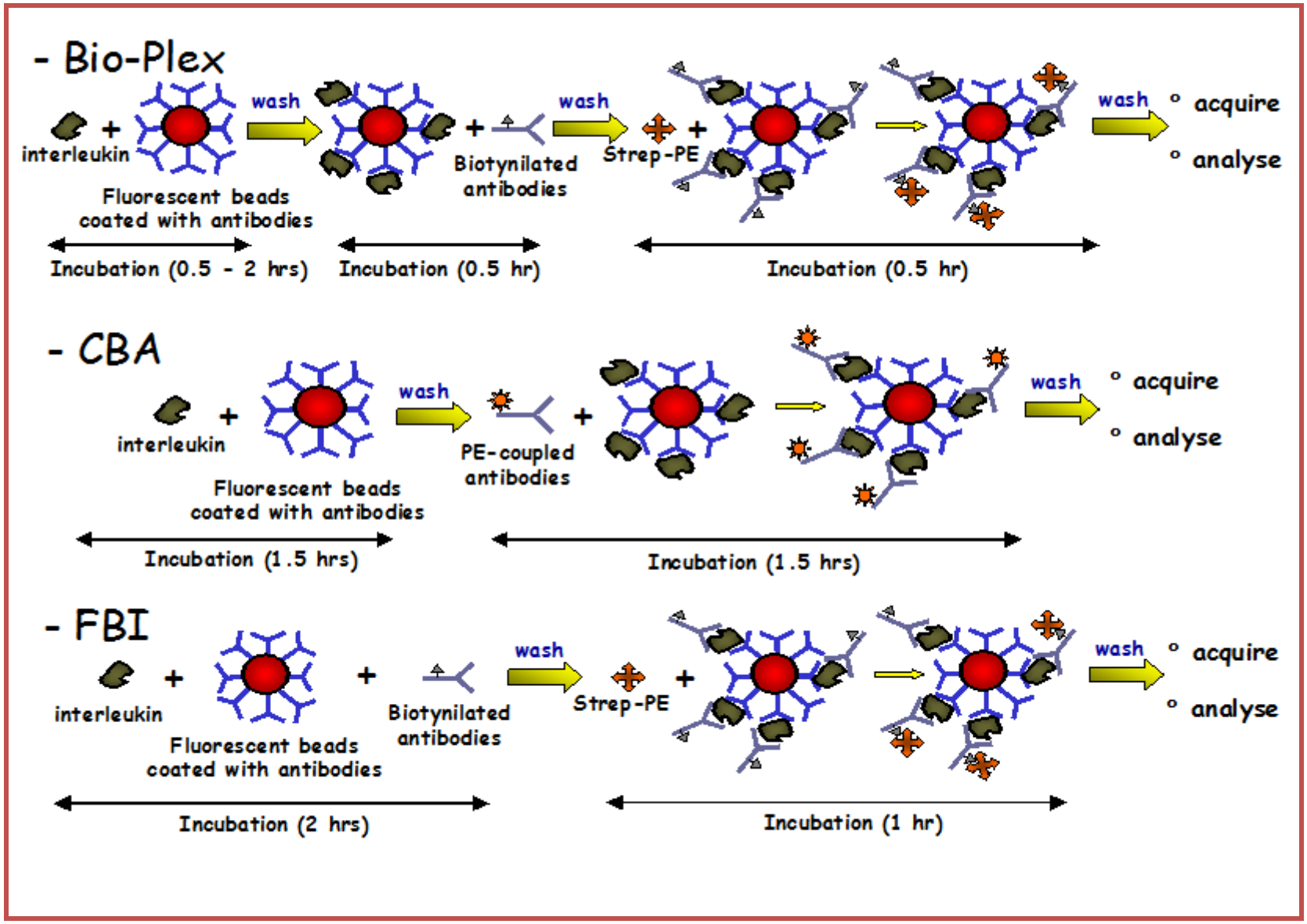
microbille

Couplage de protéines à des microbilles

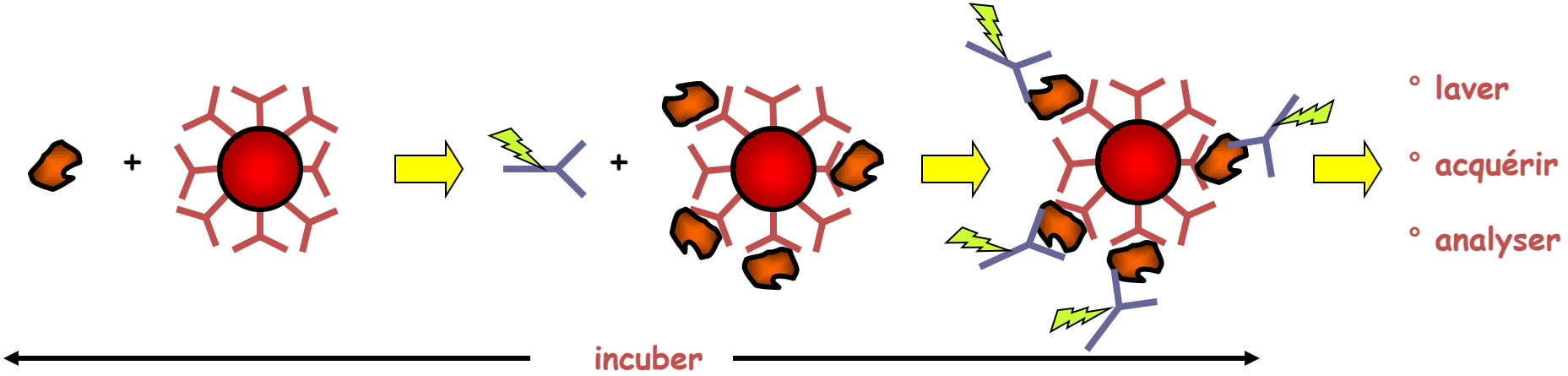


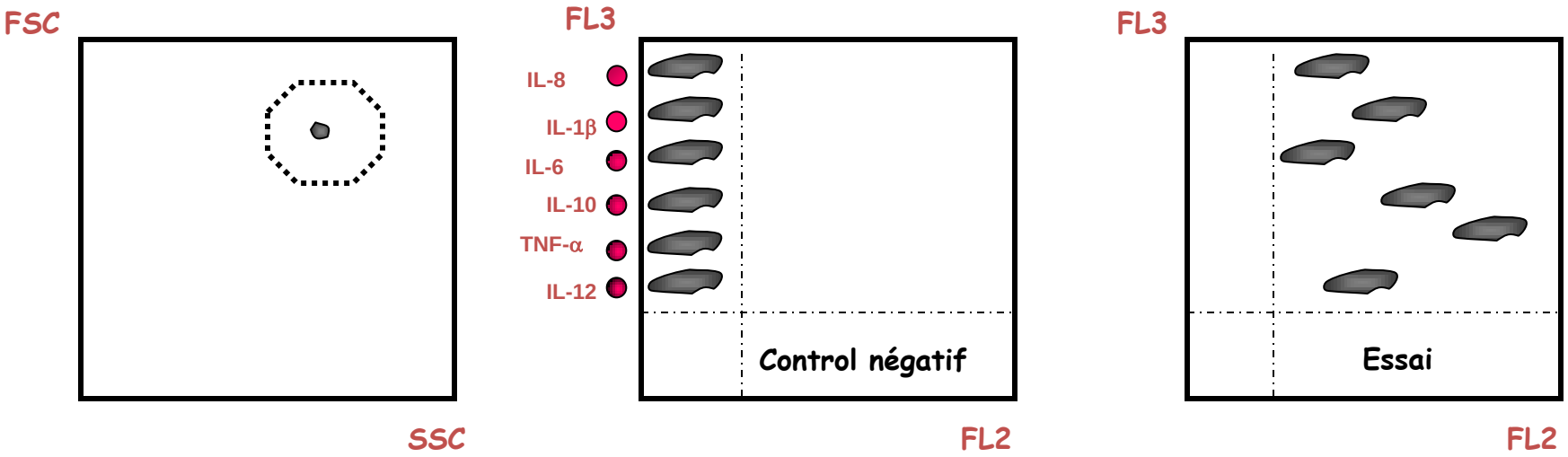
EDC = (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)

Techniques multiplexes et quantification de cytokines



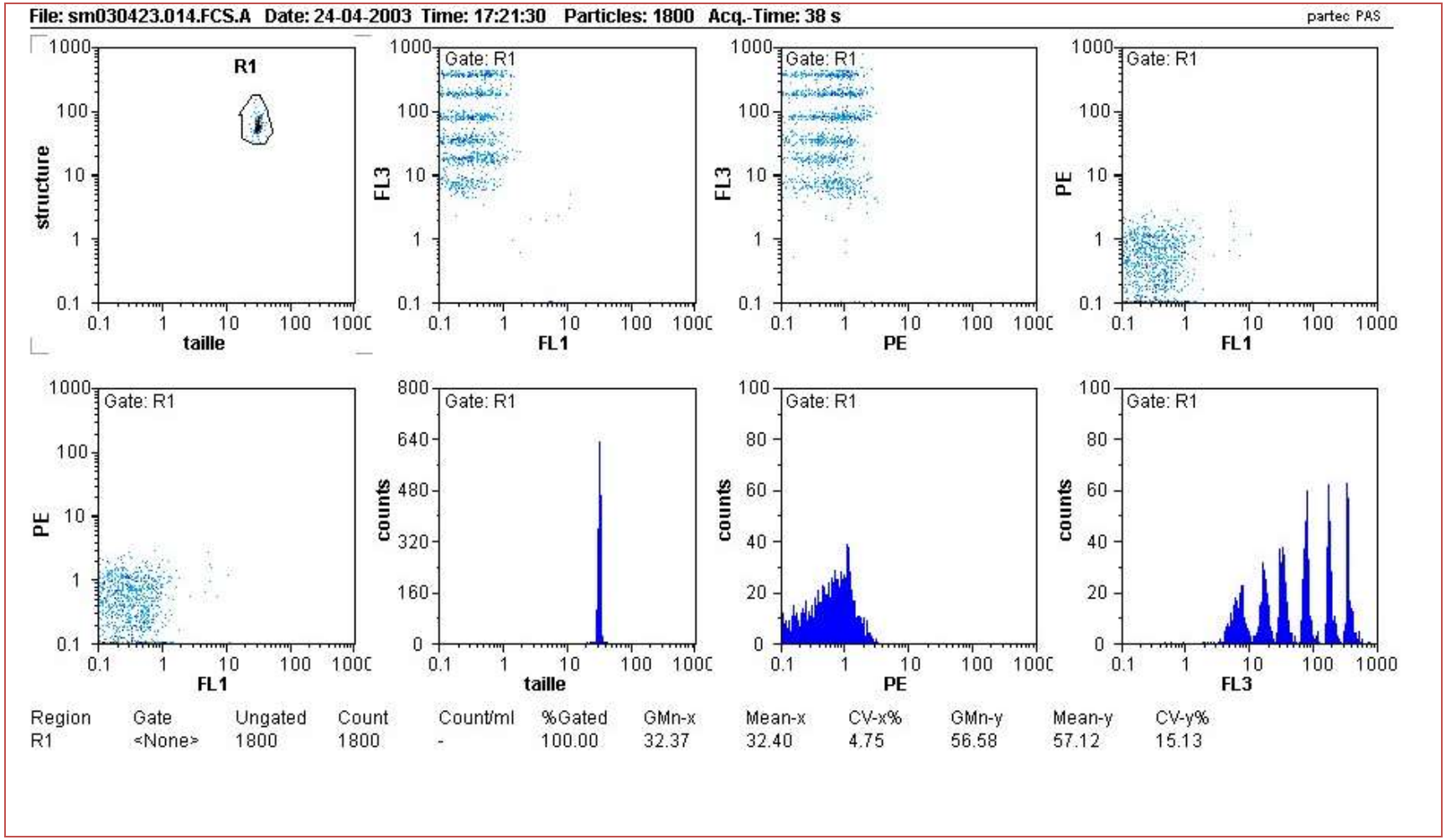
Cytometric Bead Array (CBA)





Analyse d'interleukines chez des prématurés: diagnostic d'infections materno-fœtales précoces

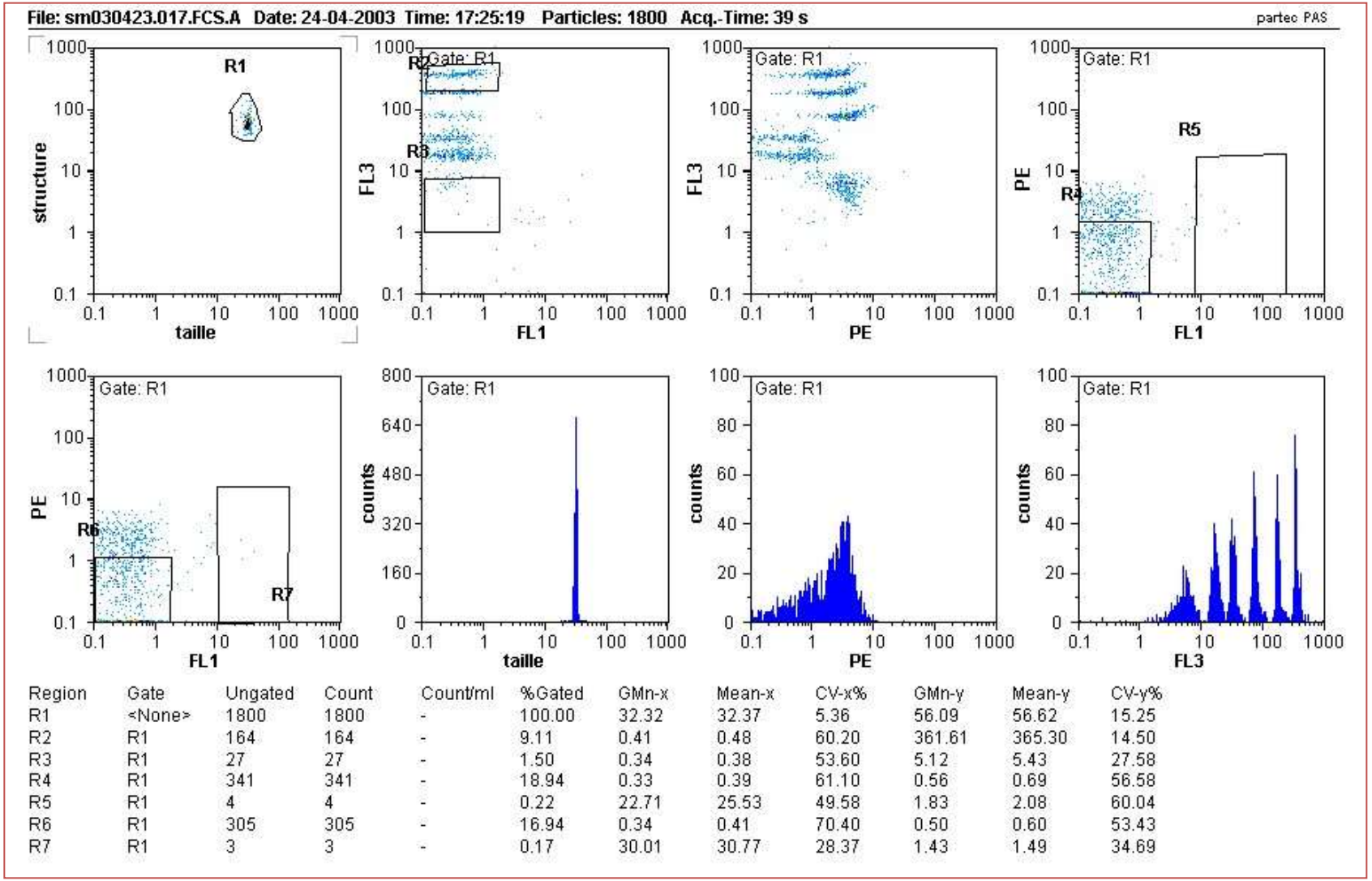
Profil cytokinique normal (5-10 µl de sérum)



Region	Gate	Ungated	Count	Count/ml	%Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	1800	1800	-	100.00	32.37	32.40	4.75	56.58	57.12	15.13

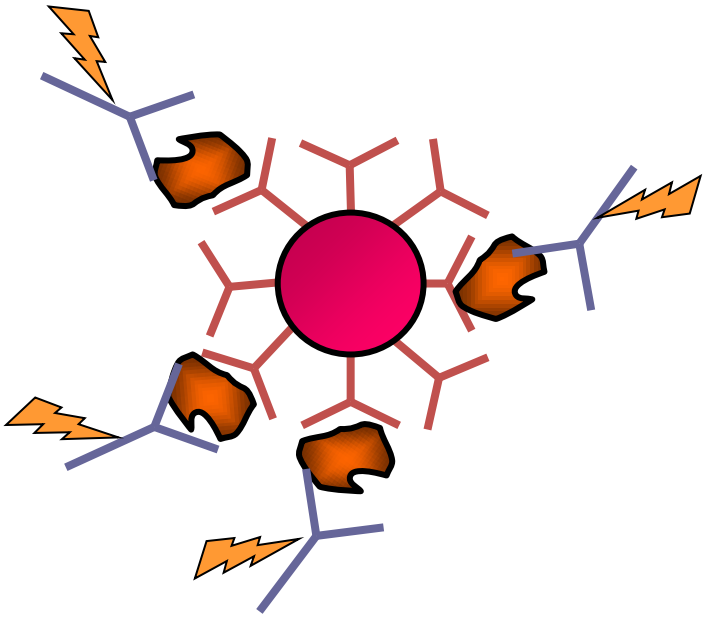
Analyse d'interleukines chez des prématurés: diagnostic d'infections materno-fœtales précoces

Profil cytokinique anormal (5-10 µl de sérum)



Region	Gate	Ungated	Count	Count/ml	%Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	1800	1800	-	100.00	32.32	32.37	5.36	56.09	56.62	15.25
R2	R1	164	164	-	9.11	0.41	0.48	60.20	361.61	365.30	14.50
R3	R1	27	27	-	1.50	0.34	0.38	53.60	5.12	5.43	27.58
R4	R1	341	341	-	18.94	0.33	0.39	61.10	0.56	0.69	56.58
R5	R1	4	4	-	0.22	22.71	25.53	49.58	1.83	2.08	60.04
R6	R1	305	305	-	16.94	0.34	0.41	70.40	0.50	0.60	53.43
R7	R1	3	3	-	0.17	30.01	30.77	28.37	1.43	1.49	34.69

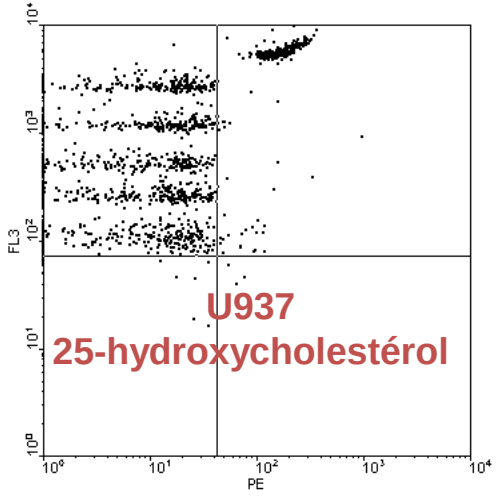
Dosages de cytokines en milieu de culture

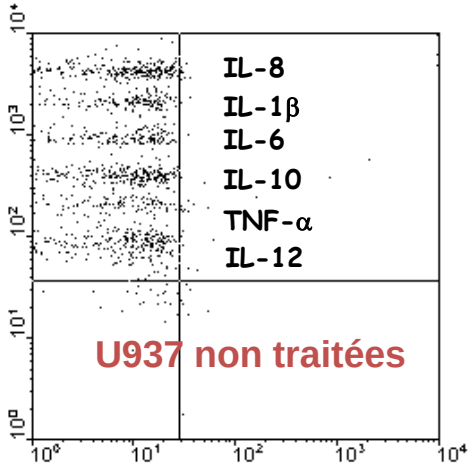


Laser bleu 488 nm

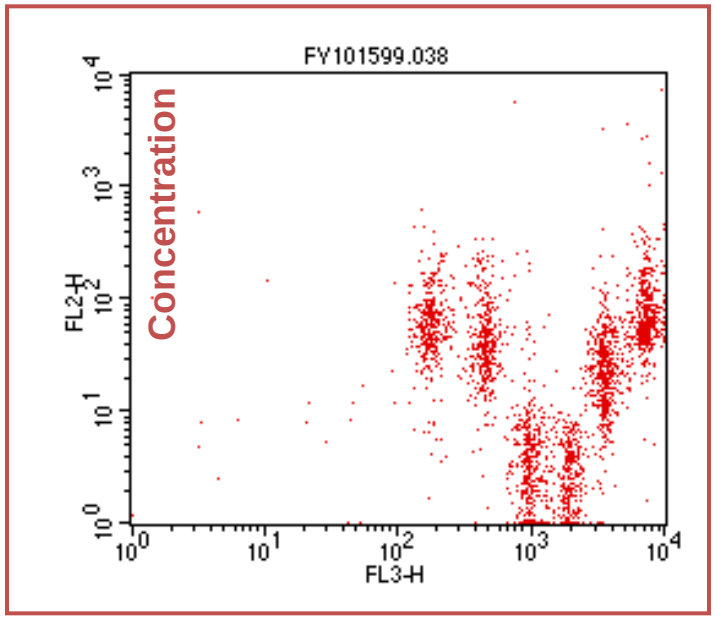
50 µl d'échantillon

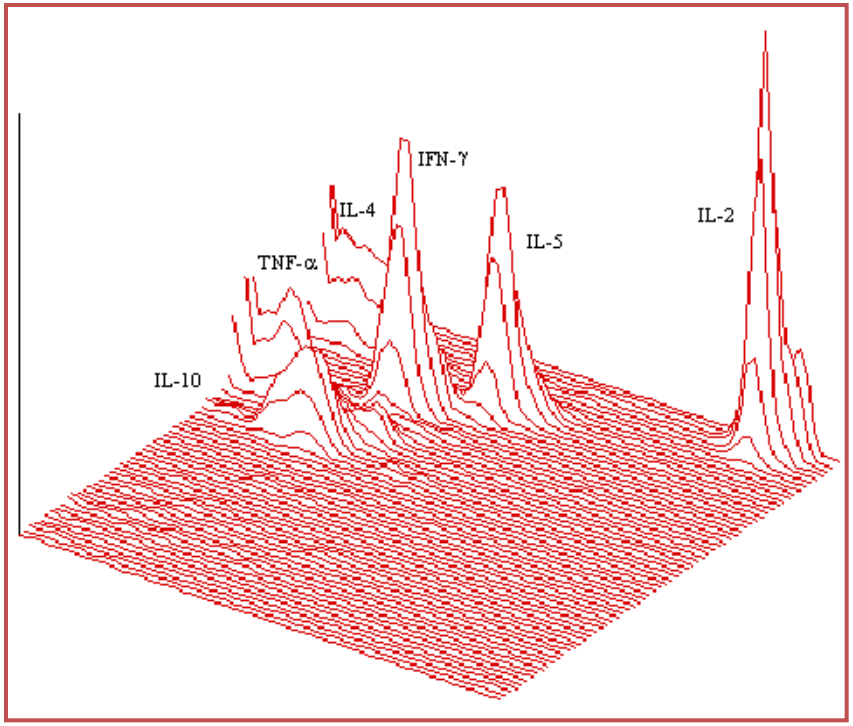
IL-8
IL-1β
IL-6
IL-10
TNF-α
IL-12





Dosages de cytokines dans les larmes



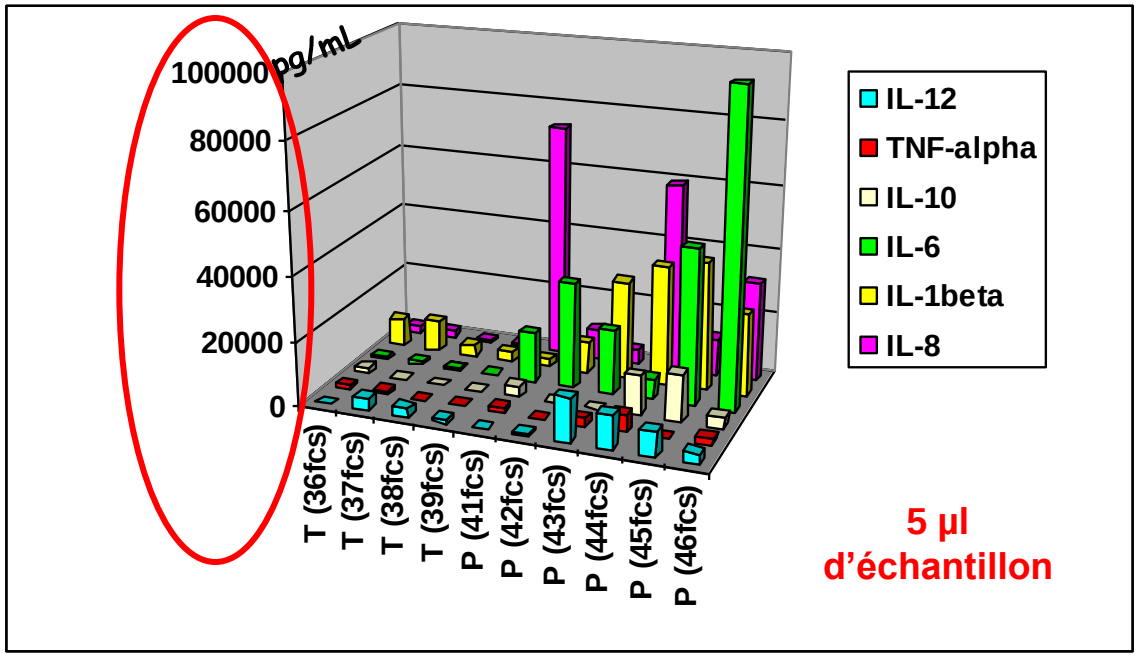


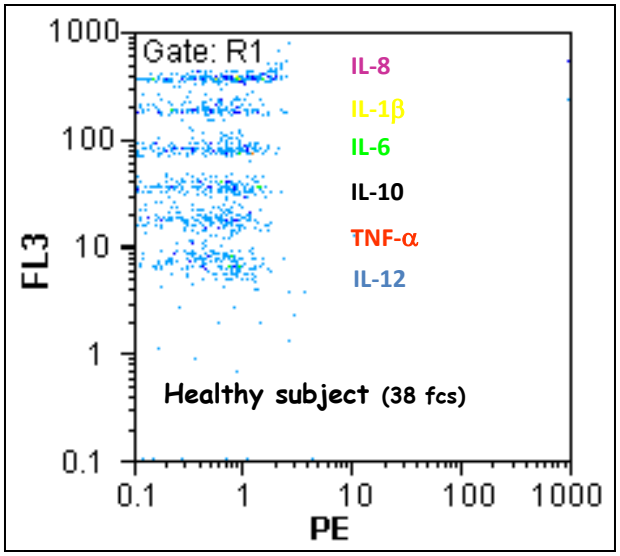
Cytokines Analysées

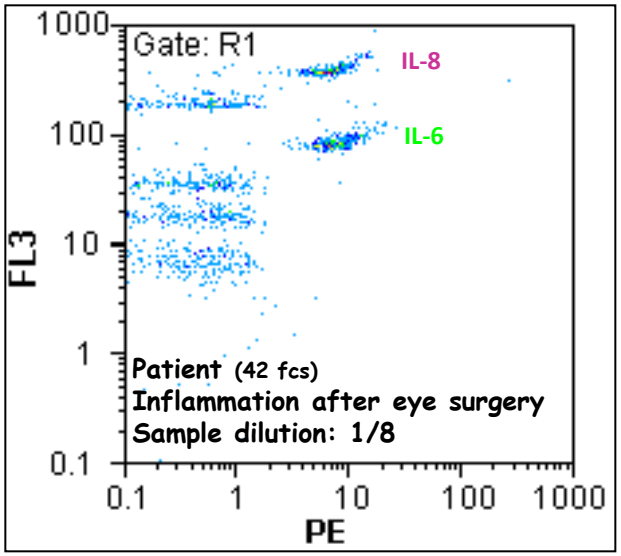
IFN-γ, TNF-α , IL-10, 5, 4, 2

Journal of Immunological Methods 254 (2001) 109–118

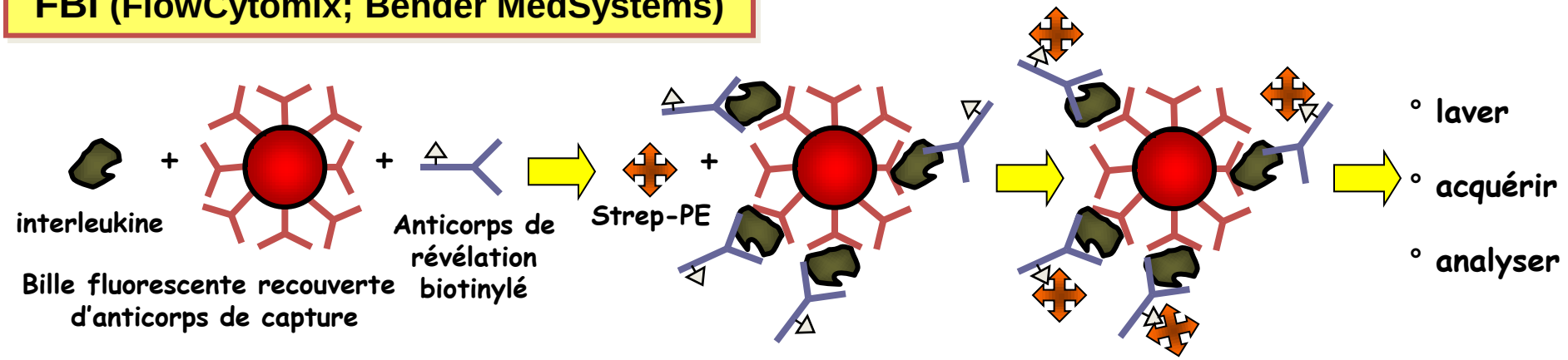
Dosages de cytokines dans les larmes : concentrations de cytokines mesurées



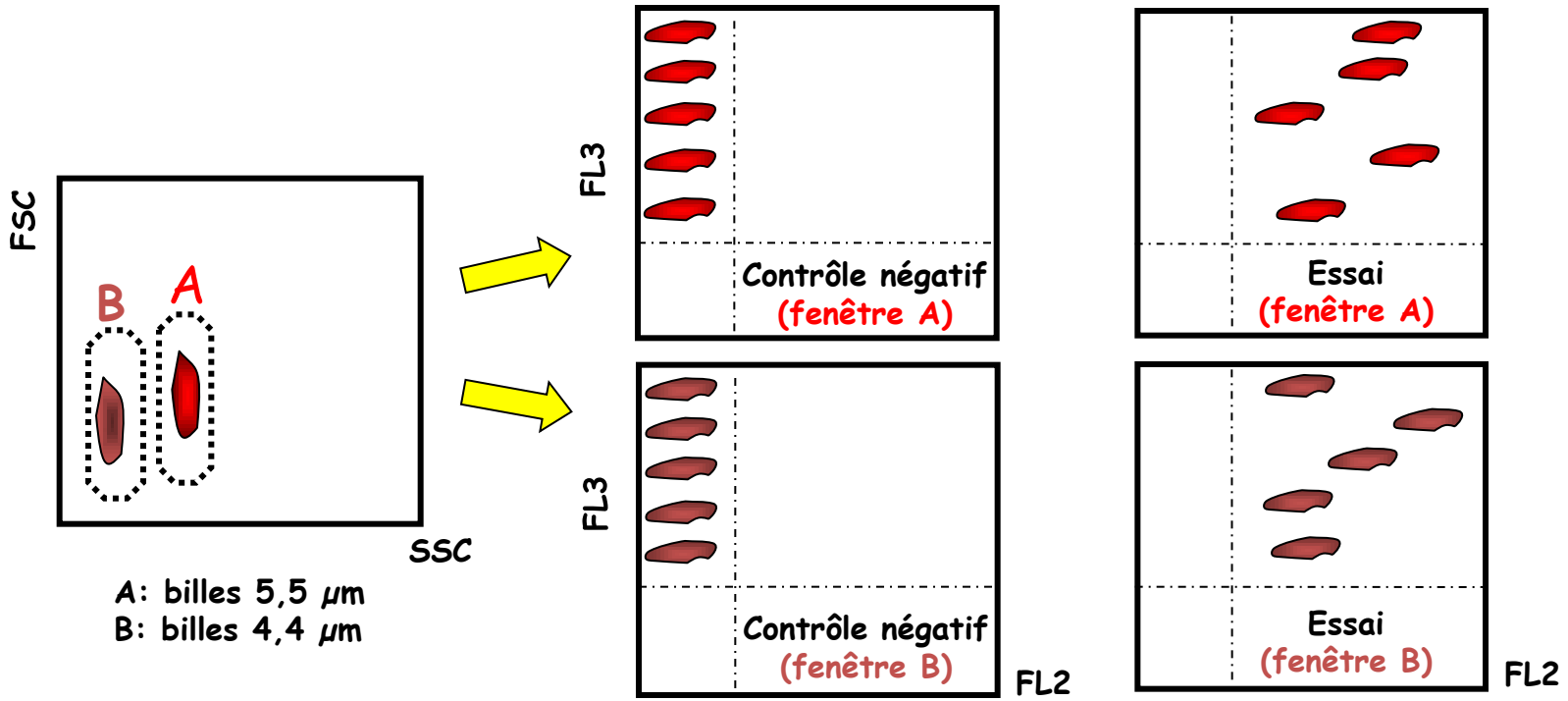




FBI (FlowCytomix; Bender MedSystems)

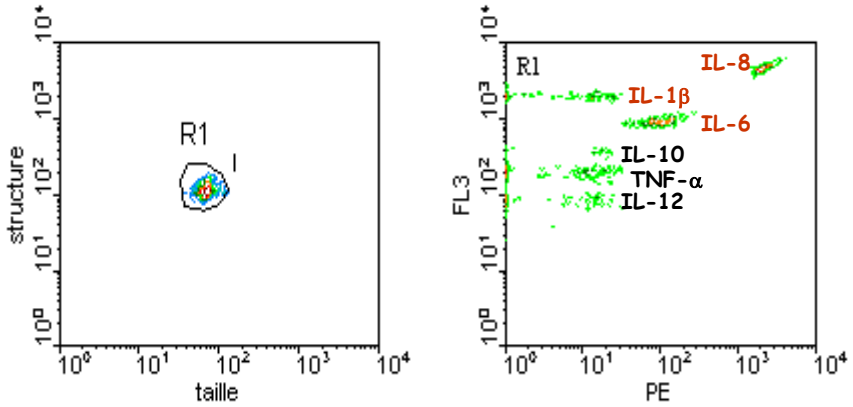


incuber

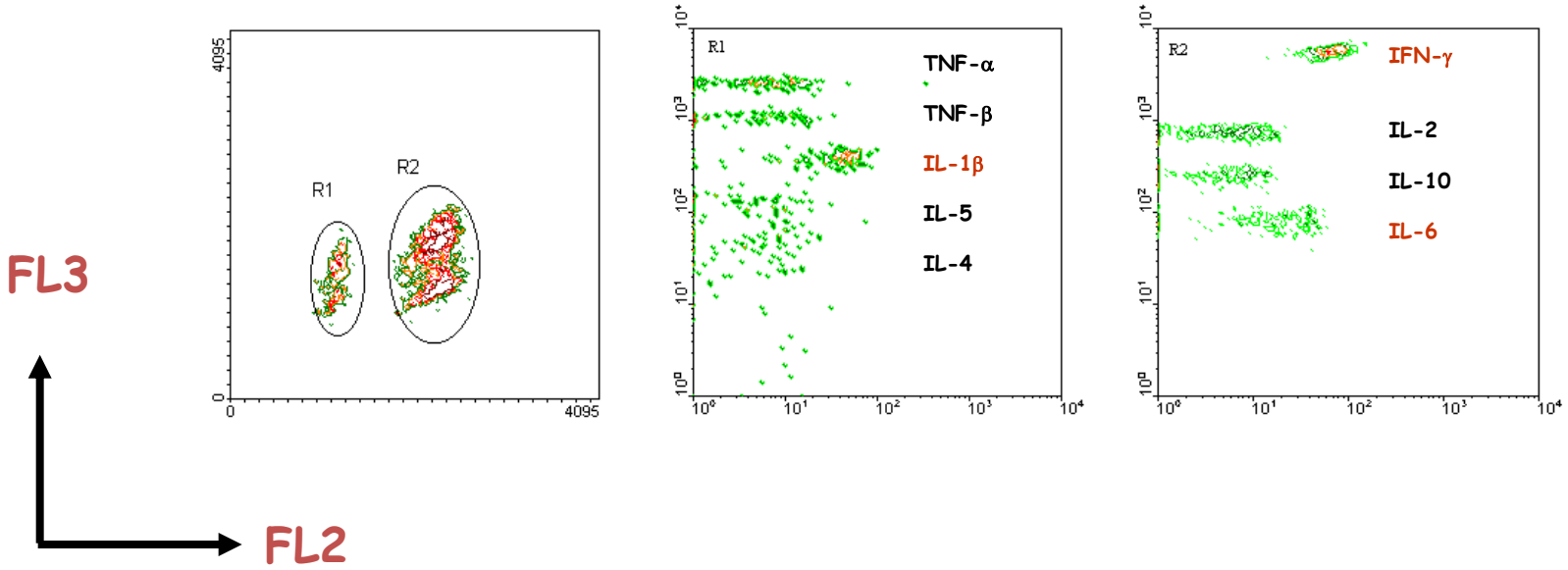


Dosages de cytokines : polyarthrite rhumatoïde

CBA



FACS-Fluorescent Bead Immunoassay



Volume d'échantillon : microbilles versus ELISA

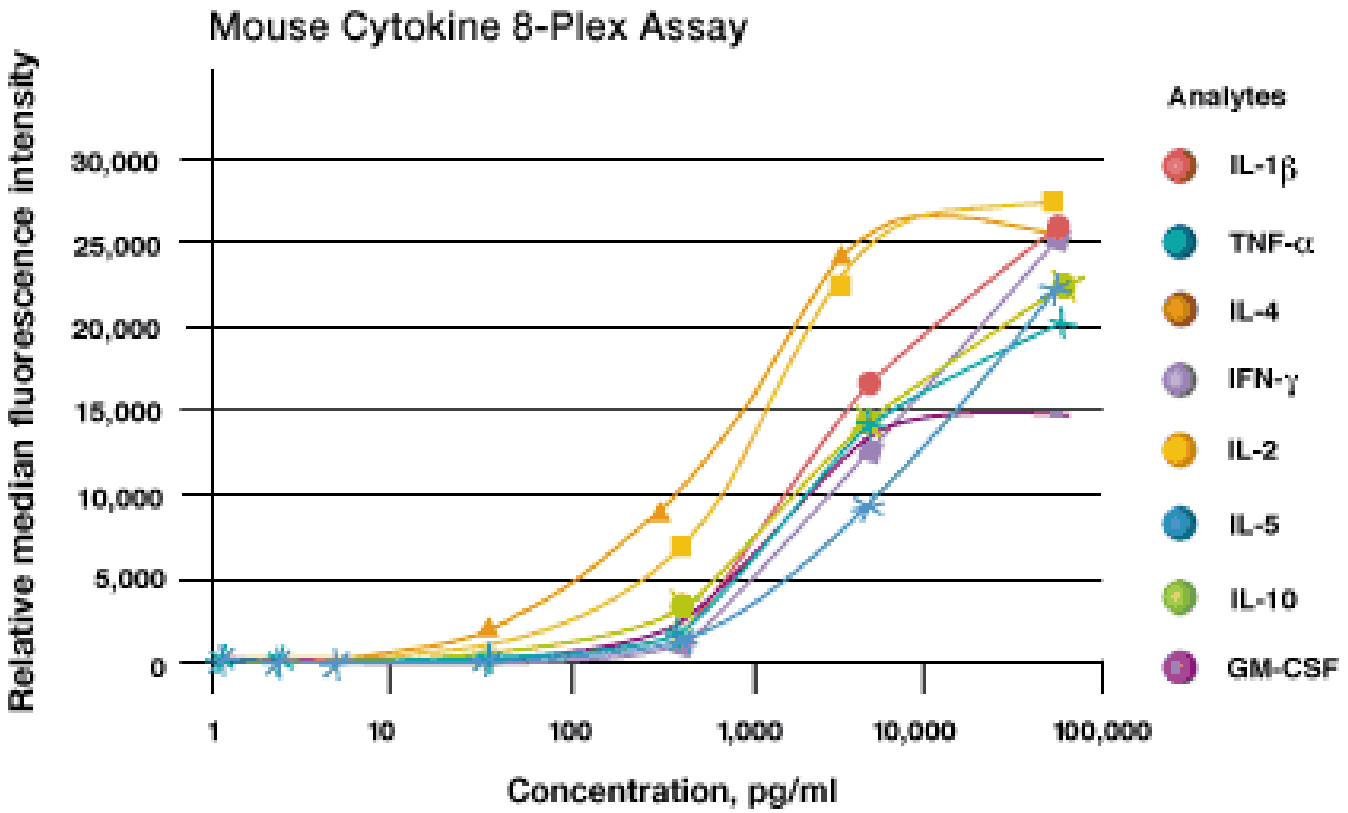
FlowCytomix and Conventional ELISA – Sample Volume

tebu-bio

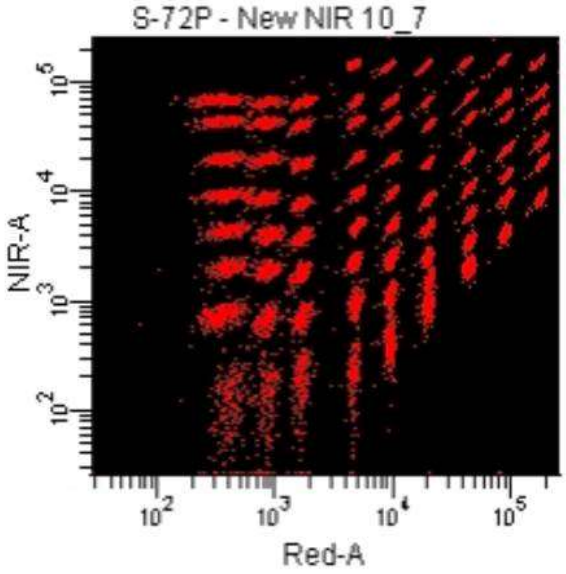
human	FlowCytomix	ELISAs
IL-1β	25 µl	50 µl
IL-2	25 µl	50 µl
IL-4	25 µl	50 µl
IL-5	25 µl	50 µl
IL-6	25 µl	50 µl
IL-8	25 µl	50 µl
IL-10	25 µl	50 µl
IFN-γ	25 µl	50 µl
TNF-α	25 µl	50 µl
TNF-β	25 µl	100 µl
total	**25 µl**	**550 µl**

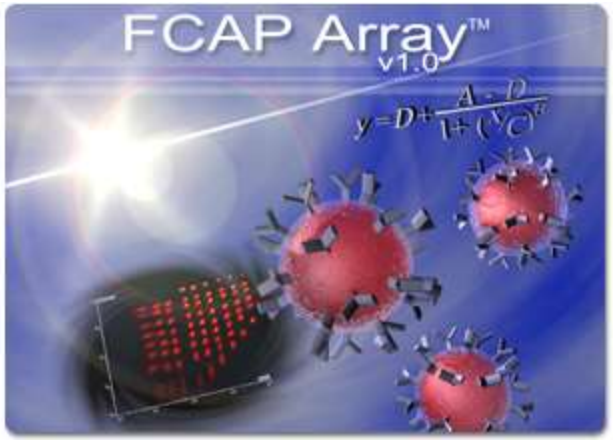
www.tebu-bio.com

Gammes étalon



Cytométrie et analyses moléculaires multiplexes : perspectives





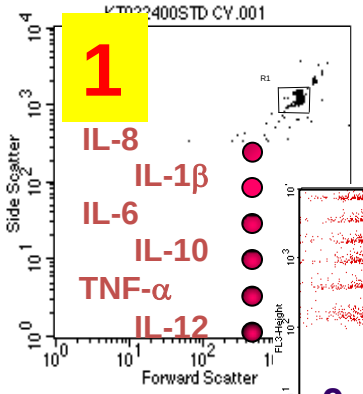
BD FACSCalibur™
BD™ LSR
BD FACSAria™
BD FACSCanto™
BD FACScan™
BD FACSVantage™

Coulter® EPICS XL™
Coulter® EPICS XL-MCL™
Cytomics FC 500
Cytomics FC 500 MPL

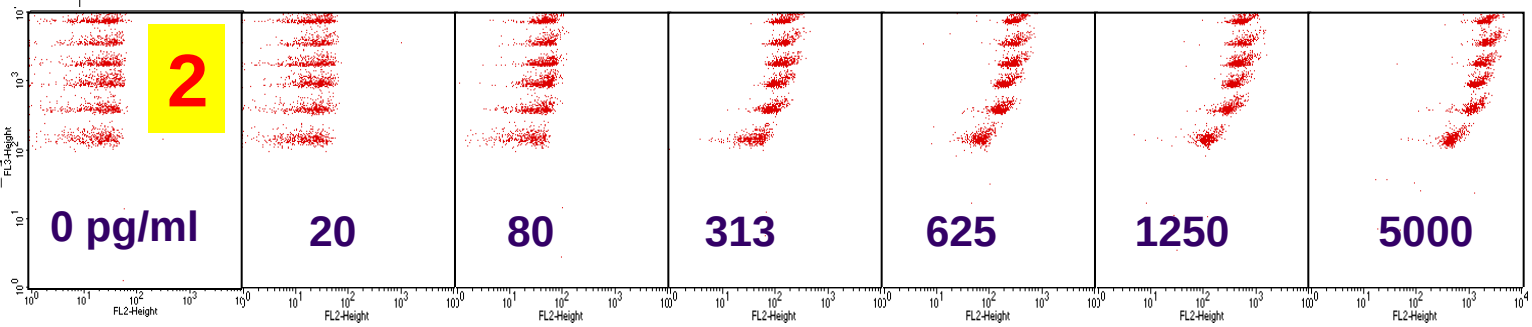
Luminex 100™

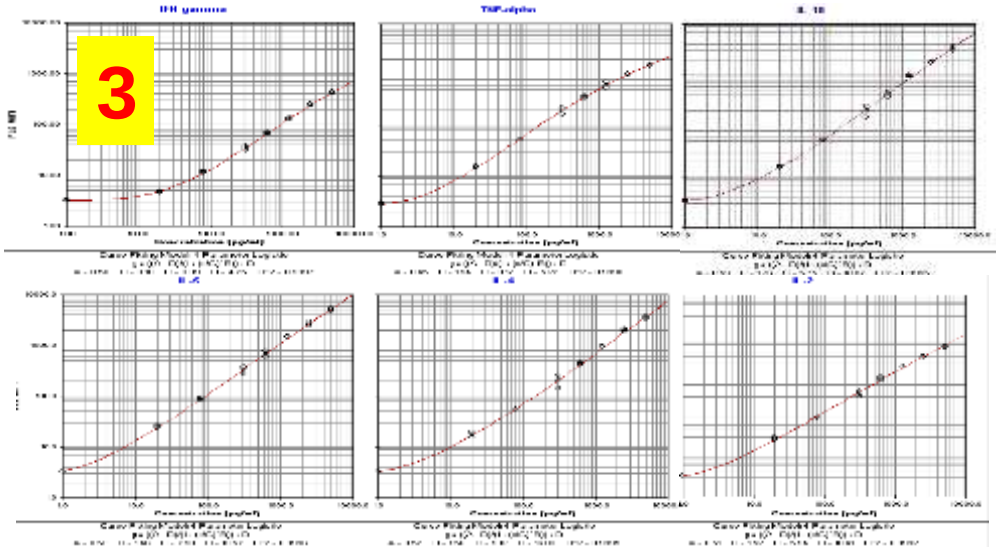
* Soft Flow Inc; www://softflow.com
* BD-Biosciences

Gammes étalon : obtention



Région sur les singulets

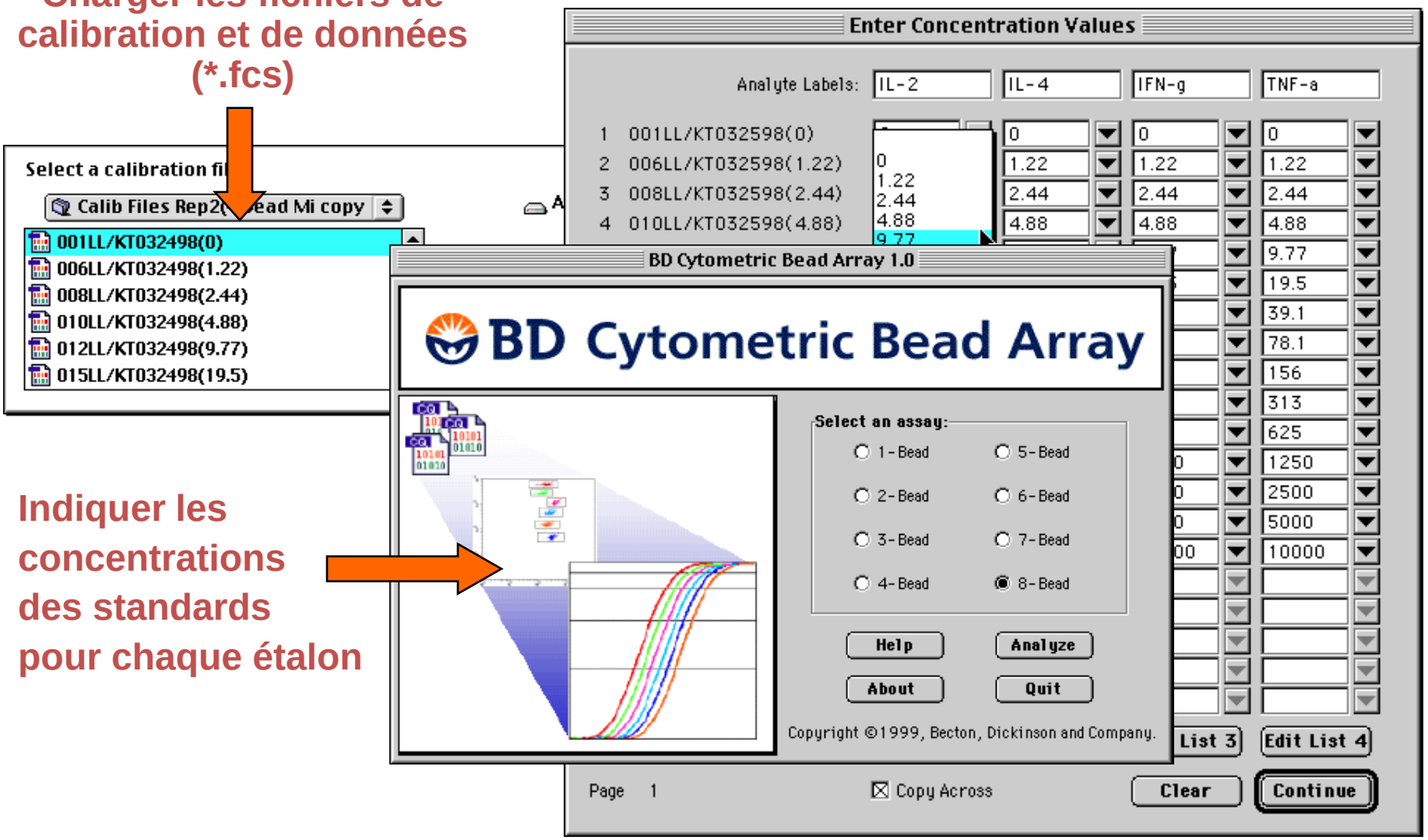




6 courbes standards automatiquement générées par logiciel

Gammes étalon - calcul - CBA

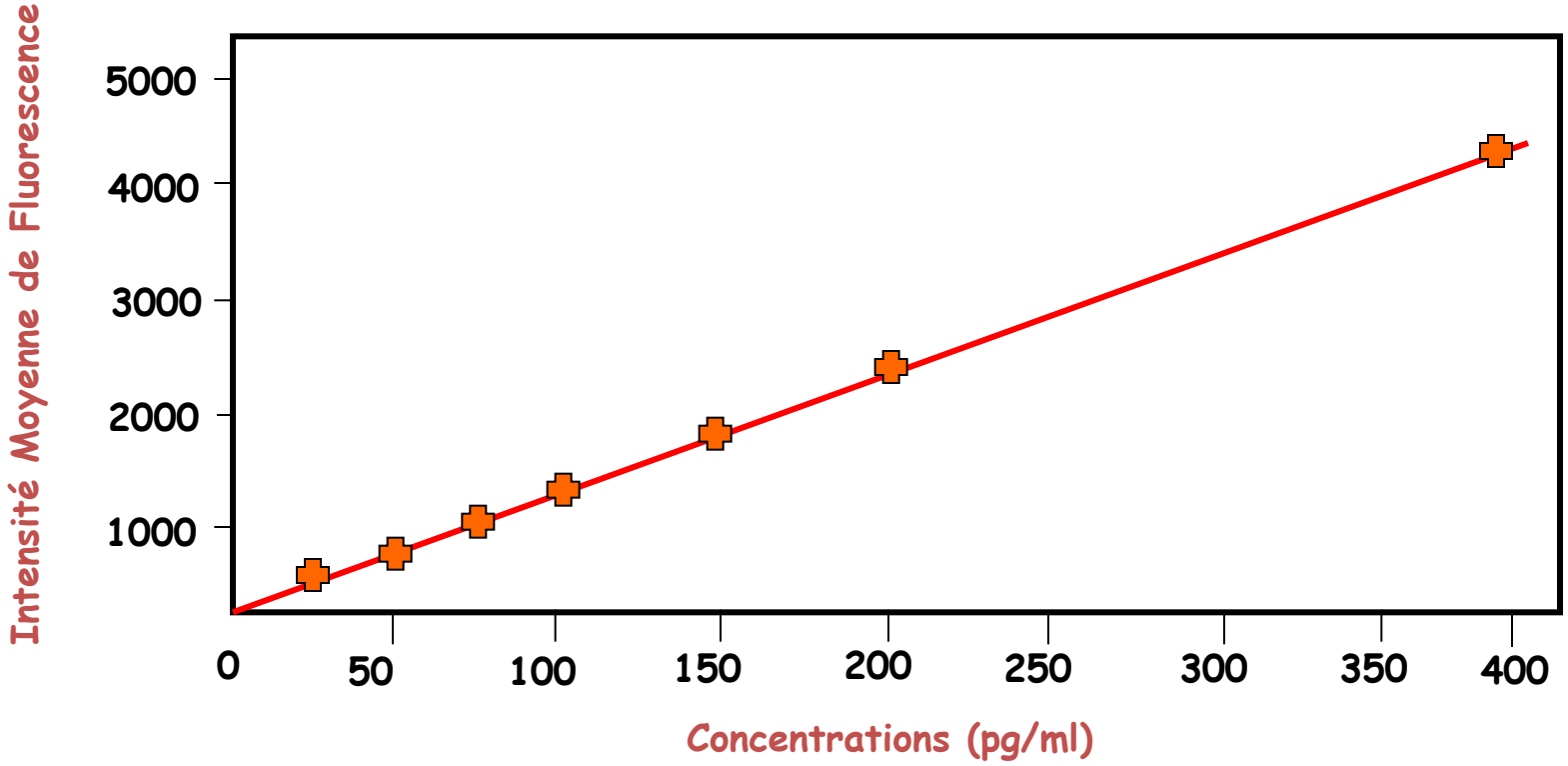
Charger les fichiers de calibration et de données (*.fcs)



Indiquer les concentrations des standards pour chaque étalon

Gamme étalon : ELISA

- Régression linéaire



Exemple: GM-CSF Bio-Plex cytokine assay, Bio-Rad

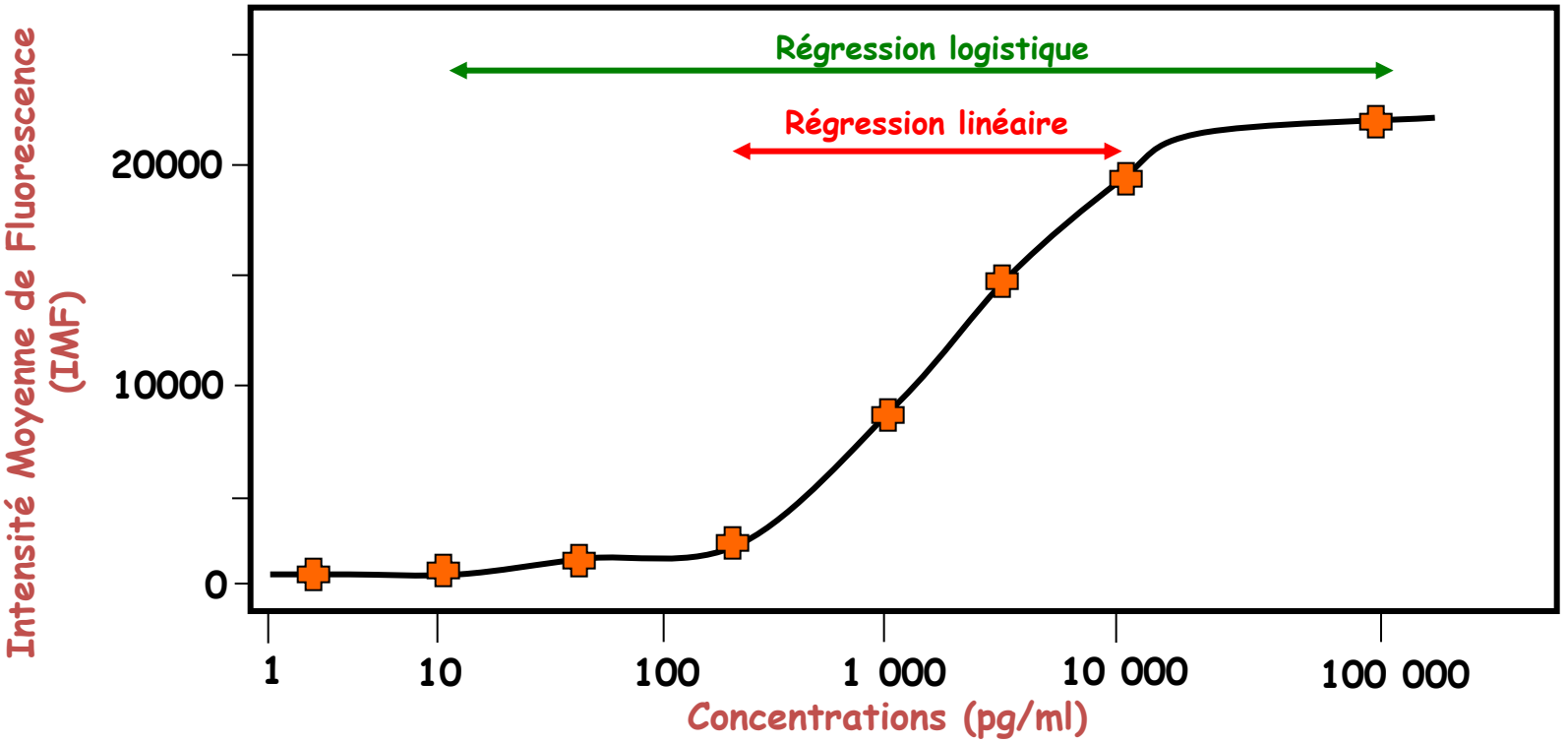
Gamme étalon : microbilles/analyses multiplexes

-Régression logistique

$y=((a-d)/(1+(x/c)^b) + d$ (dont l'inverse est: $x=c((a-y)/(y-d))^{1/b}$

a=IMF à concentration 0; b=pente partie linéaire; c=C50; d=IMF à concentration infinie

Avec le logiciel BD les valeurs de a, b, c et d sont définies automatiquement pour chaque courbe.



° Gammes étalon :

- BioRad : **1-10 000; 10 – 10 000; 1-100 000 pg/ml**
- BD-Biosciences : **1-5 000 pg/ml**
- Bender-Medsystems : **1-10 000 pg/ml**

Précautions pré-analytiques

- Stockage des échantillons à – 80°C

- Utiliser du plasma plutôt que du sérum (éviter l'exudation de cytokines)

- Sur milieux de cultures, extraits cellulaires et tissulaires (voire liquides biologiques): collecter en présence d'un **cocktail d'inhibiteurs de protéases**

Améliorations à apporter à la technologie

- Nécessité d'établir des valeurs de référence pour les molécules dosées par les méthodes multiplexes **(valeurs de référence à définir dans différents liquides biologiques)**

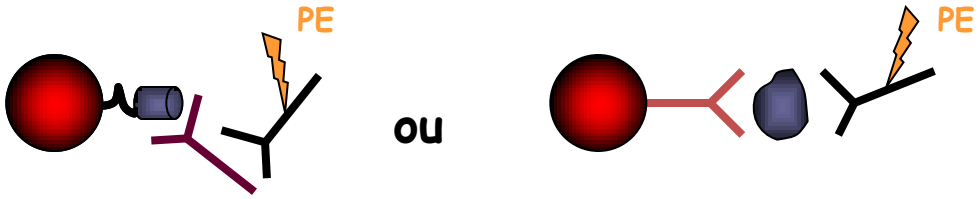
- **Comparer ces valeurs de référence par rapport à d'autres méthodes de quantification** (Radio-immunoessais, ELISA)

- Nécessité de **standards internationaux** au moins pour les cytokines et les facteurs de croissance présentant un intérêt clinique (et qui restent à définir en fonction des pathologies)

Technologie LUMINEX

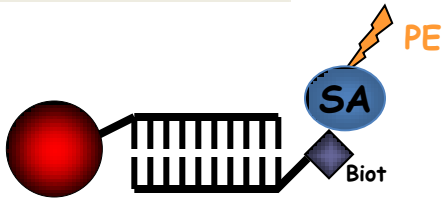
Technologie LUMINEX
Analyses réalisables, caractéristiques analytiques

- Immunoessai



Anticorps Antigène

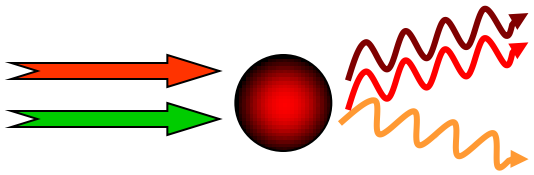
- PCR



Séquence nucléotidique

- Détection

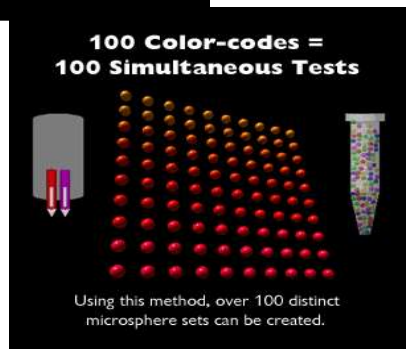
Laser rouge 635 nm
Laser vert 532 nm

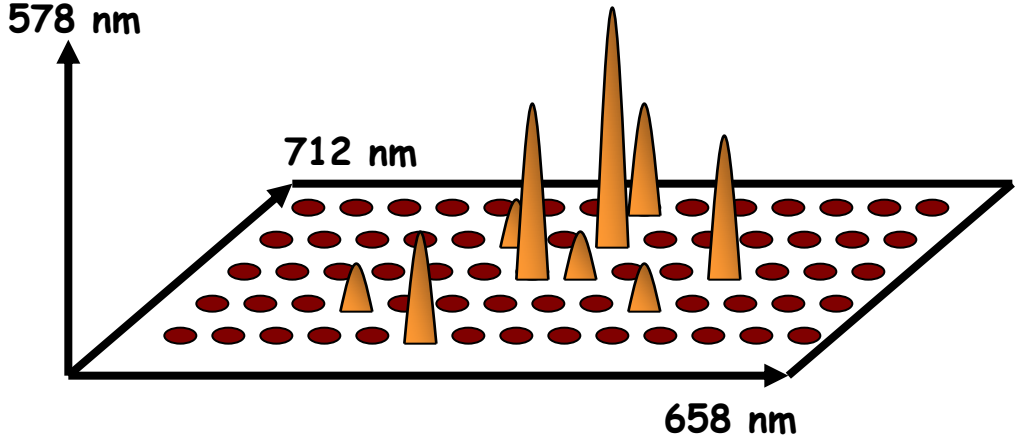


Microsphères: émission 658 et 712 nm

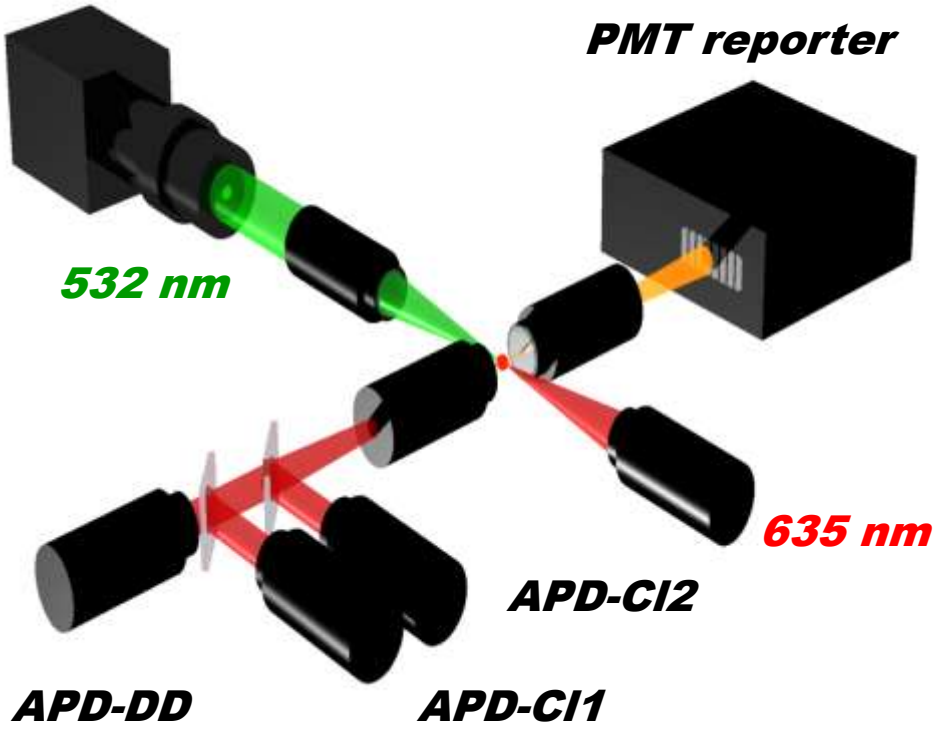
PE: émission à 578 nm

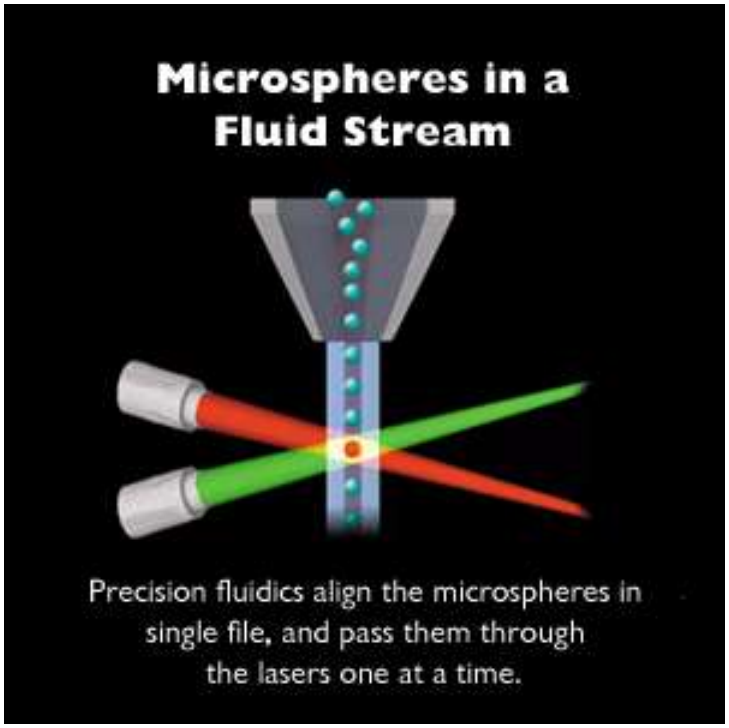
- Acquisition et exploitation des résultats





Technologie LUMINEX
Lasers et excitation

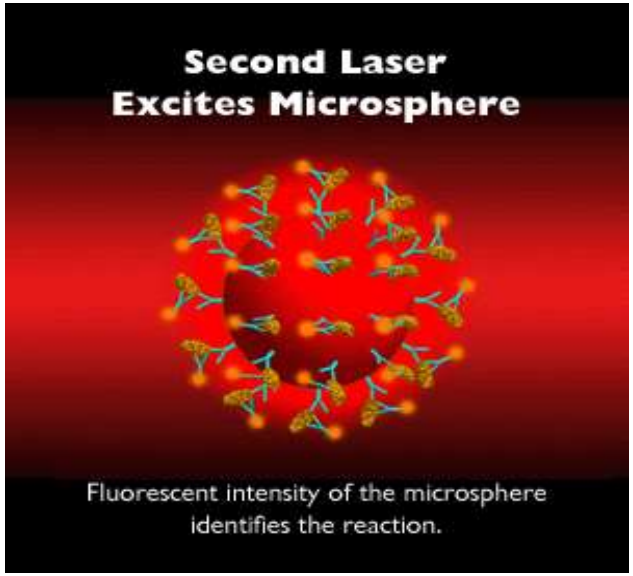


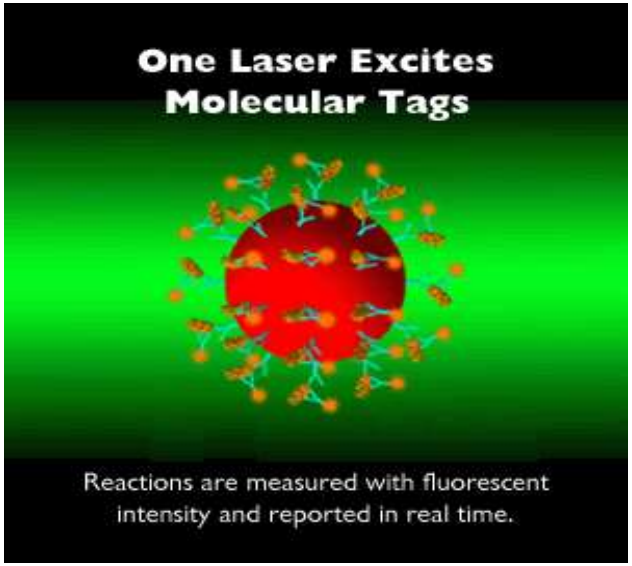


- ***1ére excitation***
  - ° laser rouge
- ***2ème excitation***
  - ° laser vert

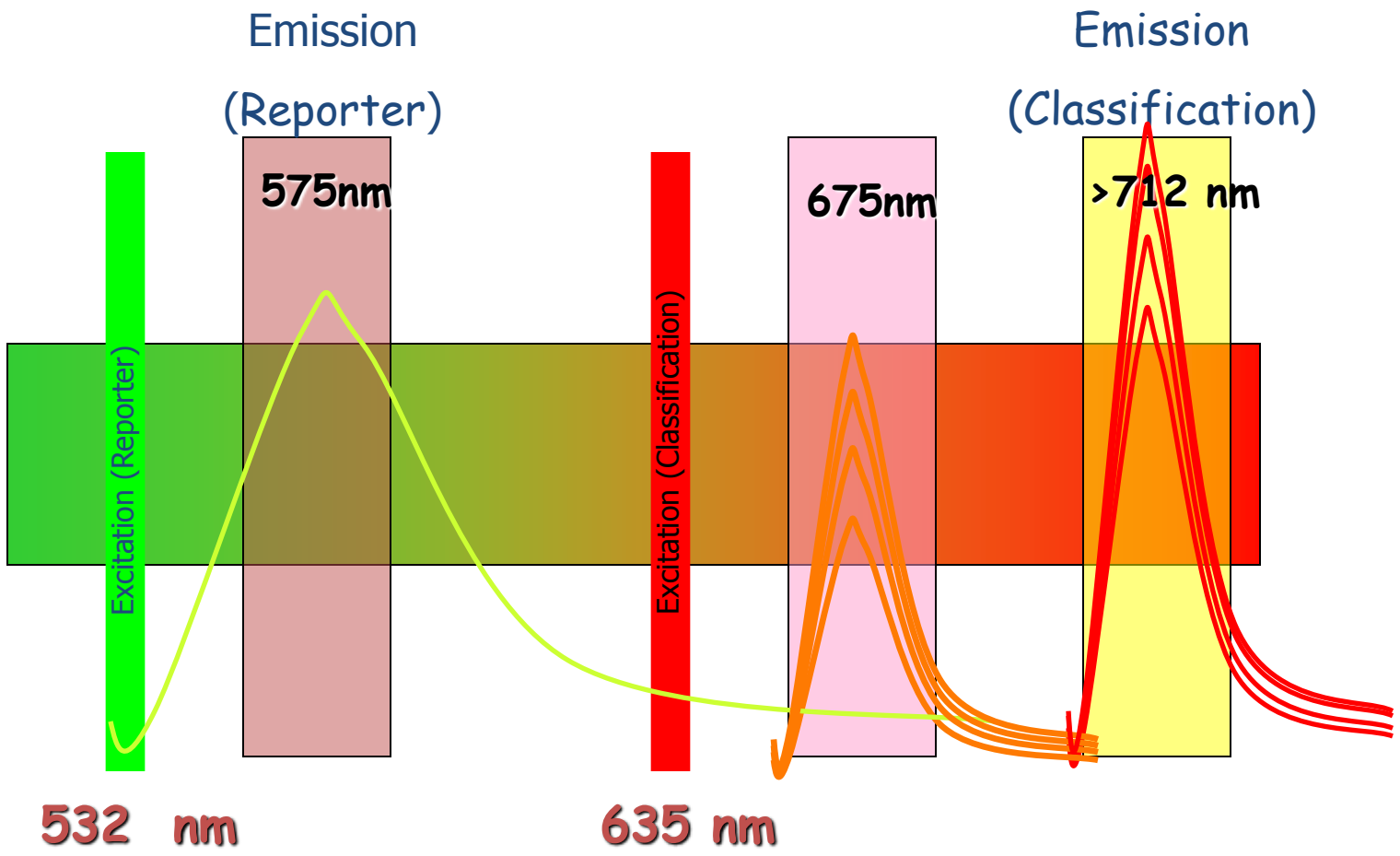
Technologie LUMINEX

Laser rouge : excitation des fluorochromes des microbilles (adressage)
Laser vert : excitation de la phycoérythrine (quantification des analytes)

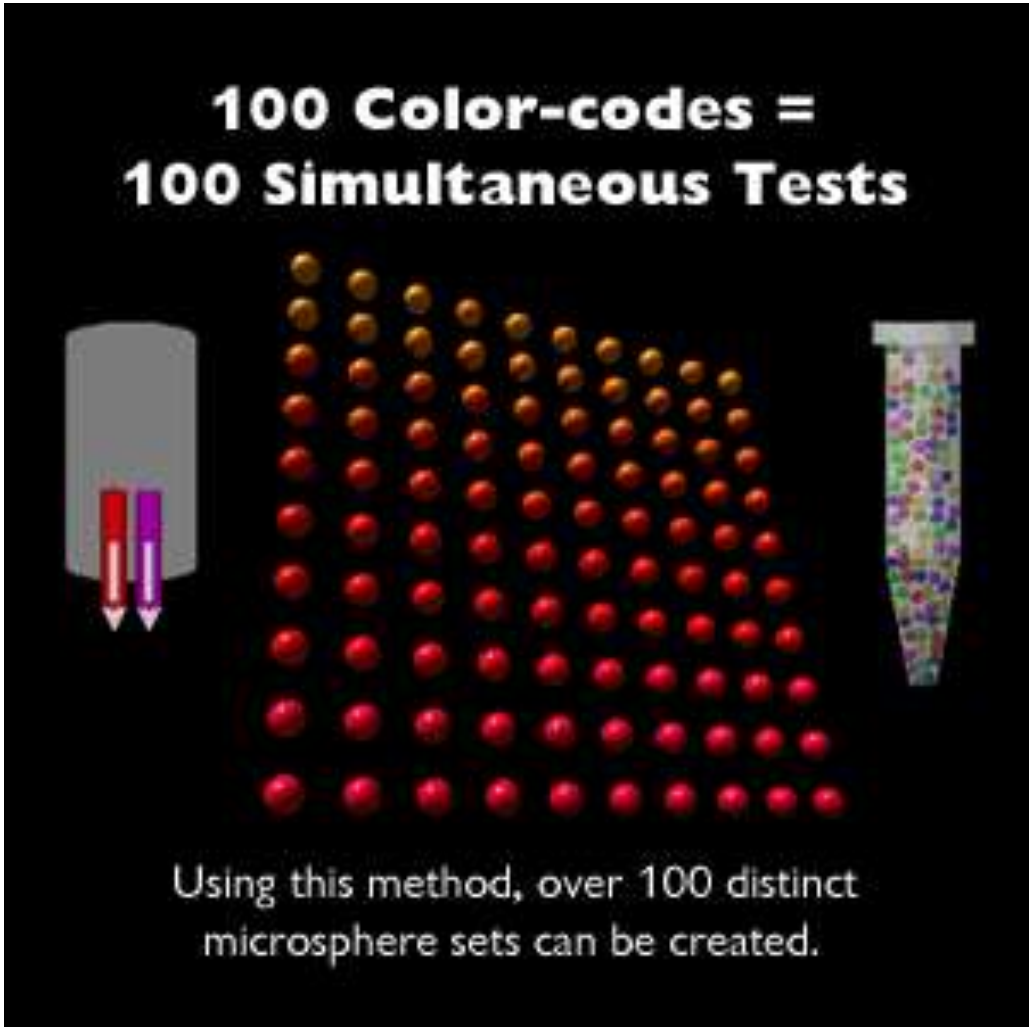




Technologie LUMINEX
Banc optique



Technologie LUMINEX
100 codes de couleurs
soit 100 adressages, soit 100 analytes quantifiés simultanément

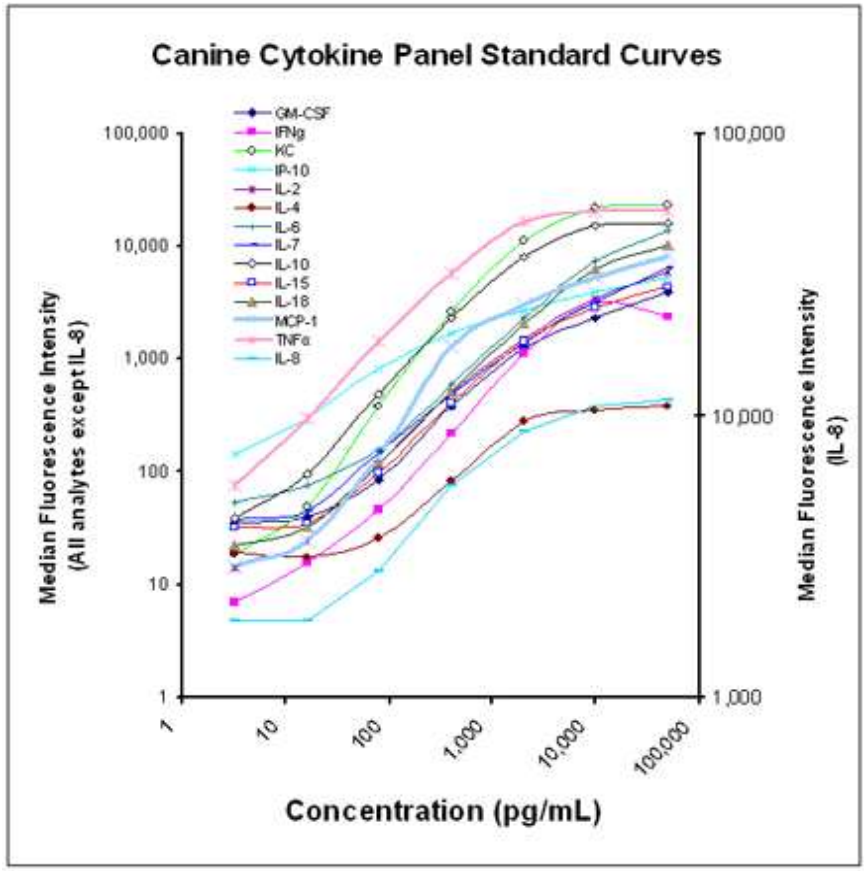


Cytokines

-Human, mouse, rat,monkey
(BD Biosciences, Biosource (Invitrogen))

-Human, mouse, rat
(Bender MedSystems)

-Human, dog, mouse, rat
(Linco (Millipore))



Canine (Dog) is a popular companion animal. It is also an important animal model for human biomedical research and drug development.
Because of limited availability of commercial canine-specific reagents, understanding of the pathogenesis of canine diseases and drug development has been limited.
The customizable **LINCOplex** canine cytokine/chemokine immunoassay panel is a useful tool for studying many canine diseases, vaccine/drug development and drug toxicities for both veterinary and biomedical research communities.

This kit may be used for the analysis of above cytokines and chemokines **in tissue/cell lysates, culture supernates, serum, plasma, other body fluids, and/or tissue extract samples**.

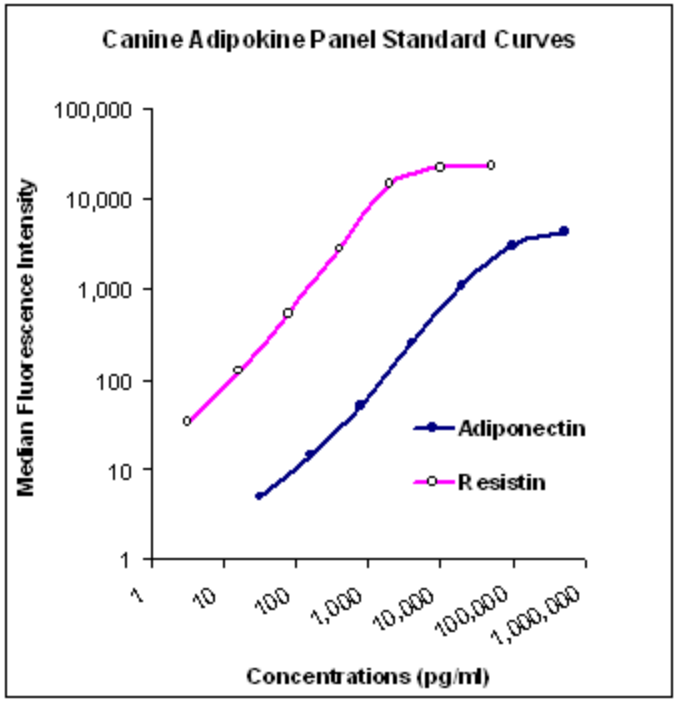
Maladies métaboliques : obésité

Adiponectines

-Human, mouse, rat,monkey
(BD Biosciences)

-Human, mouse, rat
(Bender Medsystems)

- Human (Adiponectin, IL-1β, IL-6, IL-8, HGF, NGF, Leptin, MCP-1, TNF-α, Resistin, PAI-1 (Active)/PAI-1 (Total)), Dog, mouse, rat *(Linco (Millipore))*



Obesity and diabetes are urgent health issues for canine (Dog) species. Since there is limited availability of canine specific immunoassay products, **LINCO** has developed new **LINCOplex** canine panels for use in studies to understand the pathogenesis of diabetes/obesity in canine models. LINCOplex canine endocrine hormones and adipokine biomarker assay panels are developed to provide research tools for veterinary and biomedical researchers who use canine models.
This multiplex assay kit manufactured by LINCO, Inc. is to be used for the simultaneous quantification of Adiponectin and Resistin **in canine serum, plasma, tissue extract, cell lysate, and culture supernatant samples of canine origin.**

Maladies cardiovasculaires

Cardiovascular Disease

Human (CVD) Biomarker Panel 1

sE-Selectin, sVCAM-1, sICAM-1, MMP-9, MPO, Adiponectin, PAI-1 (total)

Human (CVD) Biomarker Panel 2

CRP, SAA, SAP

Human Fibrinogen:

Fibrinogen

Human Haptoglobin:

Haptoglobin

Apolipoproteins

Human Apolipoprotein Panels

AI, AII, B, CII, CIII, E

Human (CVD) Biomarker Panel 3

IL-1β, IL-6, IL-8, IL-10, IFN-g, TNF-a, MCP-1, NT-proBNP, VEGF

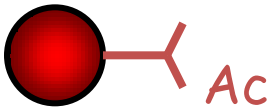
Mouse (CVD) Biomarker Panel 1

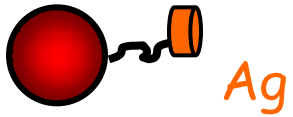
sE-Selectin, sVCAM-1, sICAM-1, MMP-9, tPAI-1

Human Cardiovascular Markers FlowCytomix (CD40L, P-Selectin, tPA, VCAM-1, IL-6, IL-8, MCP-1)

Techniques multiplexes complémentaires

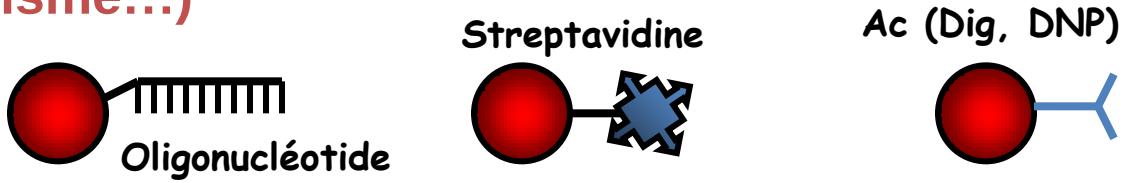
° Identification et quantification de protéines (cytokines, glucagon, insuline, leptines, anticorps,…)





° Mise en évidence d'activités enzymatiques (caspase-3)

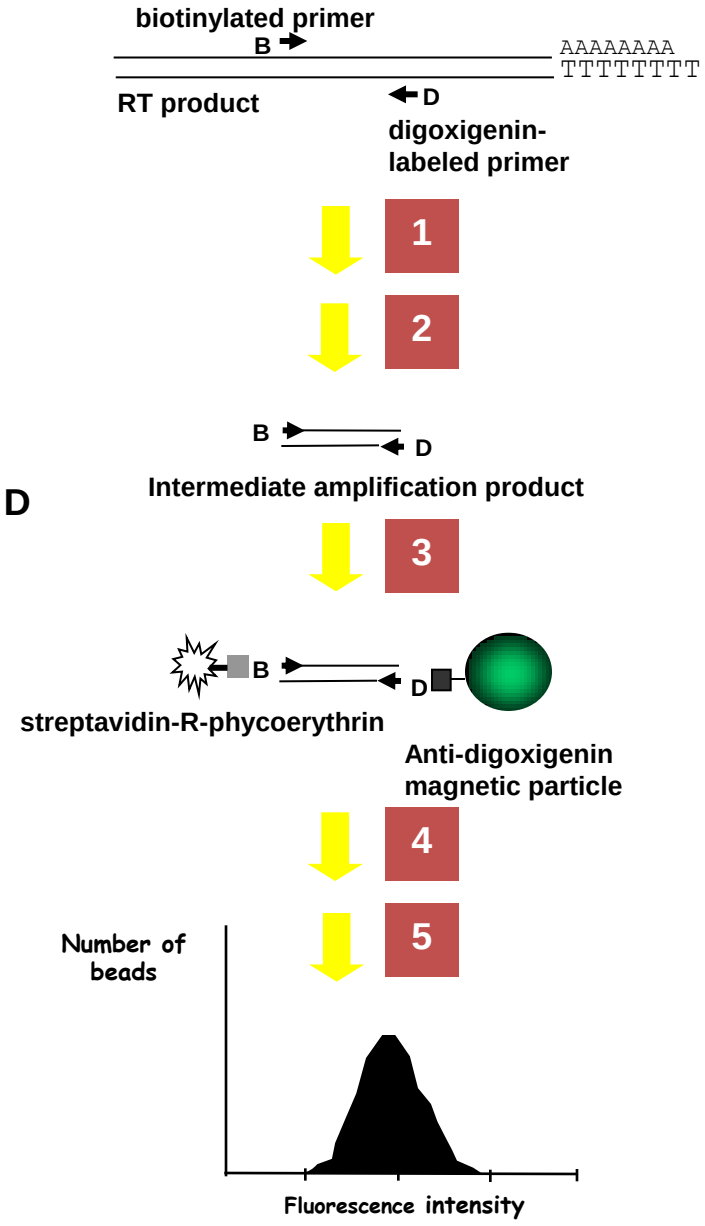
° Identification et quantification de séquences nucléotidiques (ADN ou ARN viral, bactérien ou parasitaire; analyse de polymorphisme…)



° Interactions moléculaires

° Identification de phosphoprotéines: voies métaboliques

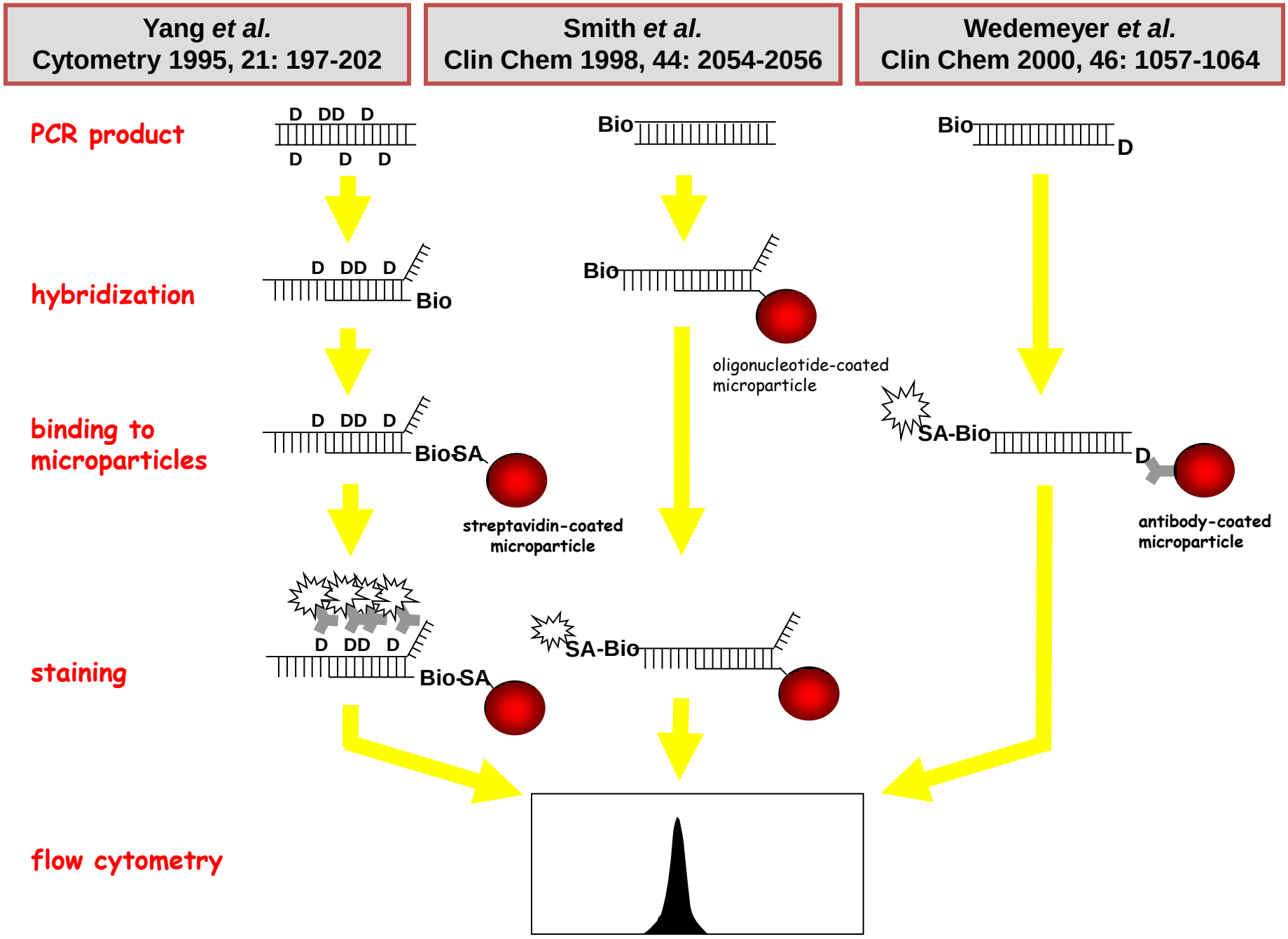
Microbilles et PCR en flux



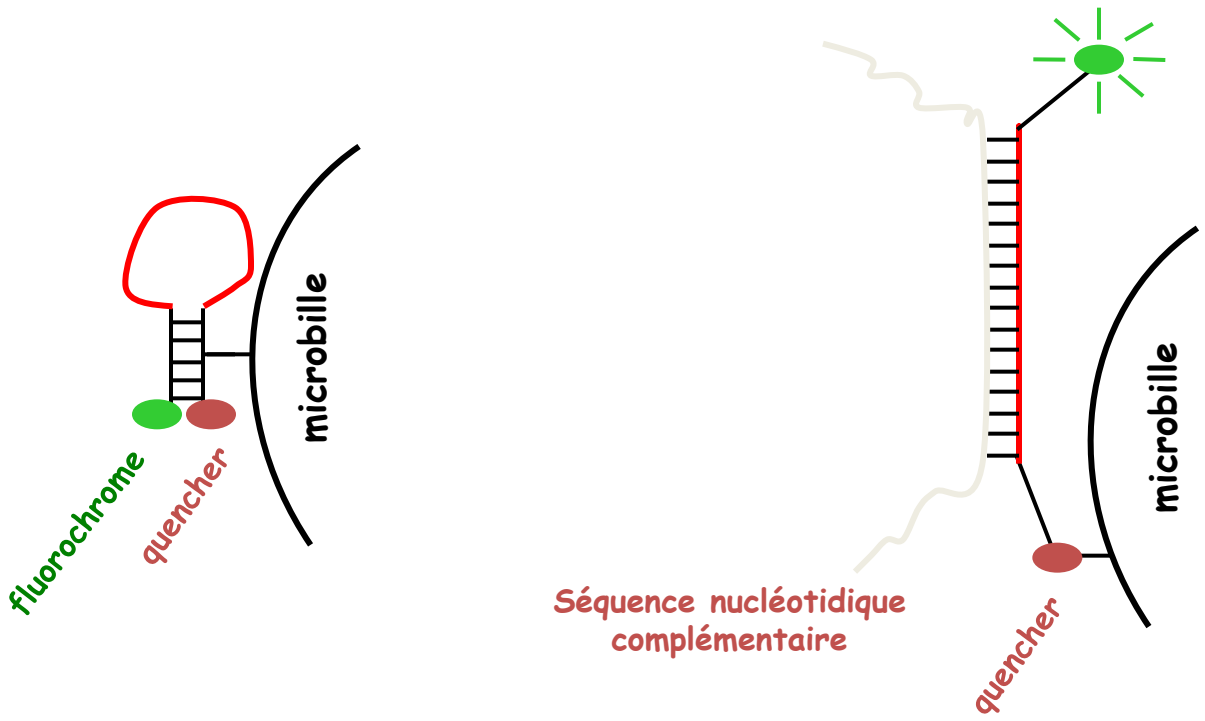
1. PCR reaction
   - using primers labeled with digoxigenin and biotin
   - For quantification PCR reaction is stopped in the exponential phase
   - 60 min
2. Removal of unincorporated primers
   - using silica particles
   - 5 min
3. Binding and staining of PCR products
   - using microparticles coated with anti digoxigenin antibody and streptavidin-R-phycorerythrin
   - 15 min
4. washing step
   - removing of unspecifically bound fluorescent dye
   - 5 min
5. flow cytometric measurement
   - 5 min

Total: 90 min

Wedemeyer *et al*. Clin Chem 2002, 48 : 1398-1405.


Yang *et al.*
Cytometry 1995, 21: 197-202
Smith *et al.*
Clin Chem 1998, 44: 2054-2056
Wedemeyer *et al.*
Clin Chem 2000, 46: 1057-1064
PCR product
hybridization
binding to microparticles
staining
flow cytometry
D DD D
D D D
Bio
BioSA
Bio-SA
SA-Bio
streptavidin-coated microparticle
oligonucleotide-coated microparticle
antibody-coated microparticle

Application de la technique 'Molecular beacon' à la détection d'ADN viraux à l'aide de microbilles



- Pas de séquence nucléotidique complémentaire, seule la microbille émet de la fluorescence,

- Avec séquence nucléotidique complémentaire, la microbille fluorescente libère la fluorescence du 'quencher'; L'intensité de fluorescence du 'quencher' est proportionnelle à la quantité de nucléotide complémentaire fixé.

- Pas de PCR préalable.

Horesjh D et *al.*, Nucleic Acid Res 2005, 33 (2): e13

Automatisation

Automatisation des Réactions

Répartion des échantillons,
Répartition des microbilles
Répartition des réactifs
Lavages

Acquisition et Exploitation Automatiques des Résultats

Feuilles de Résultats