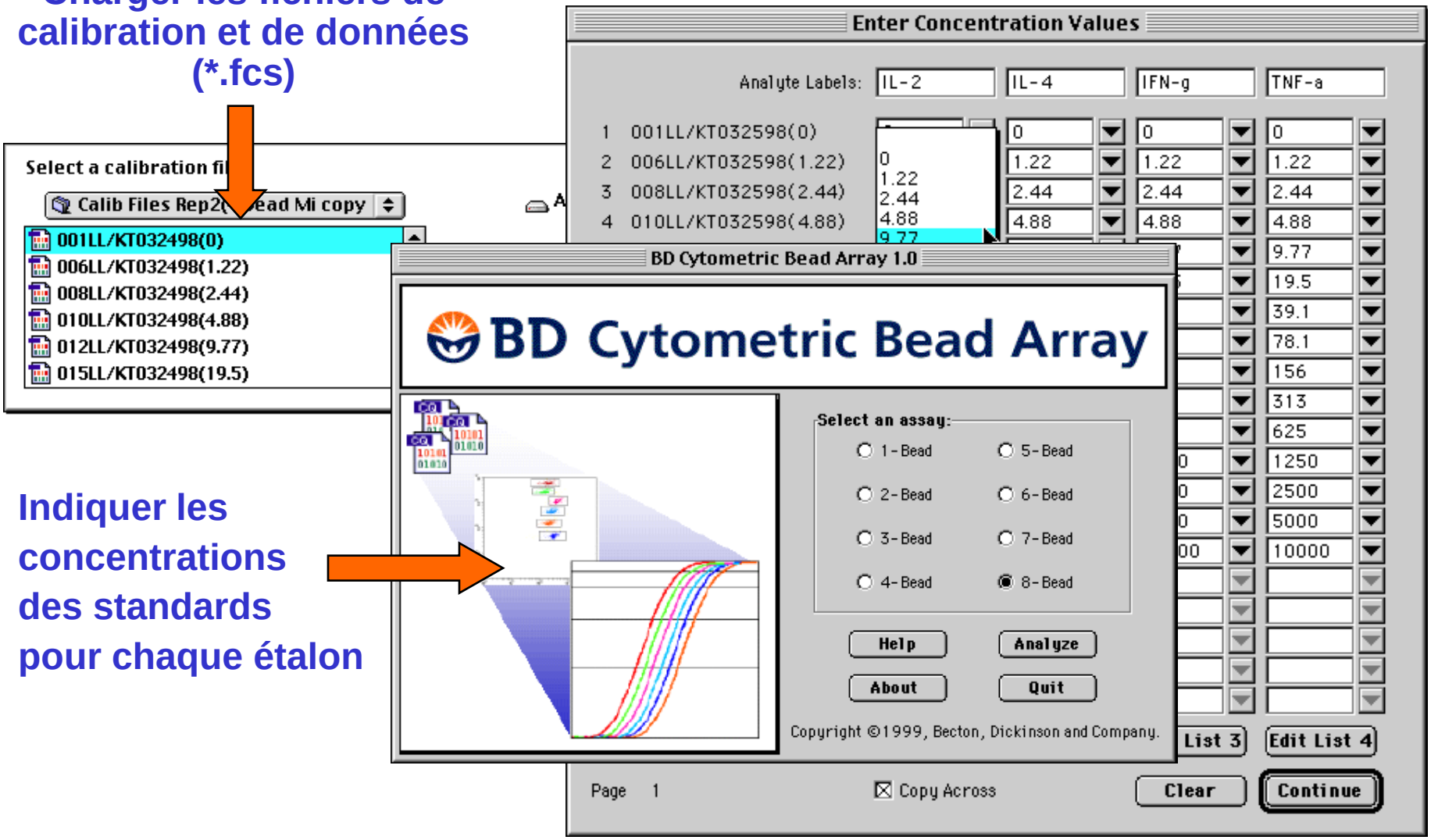
Gammes étalon - calcul - CBA

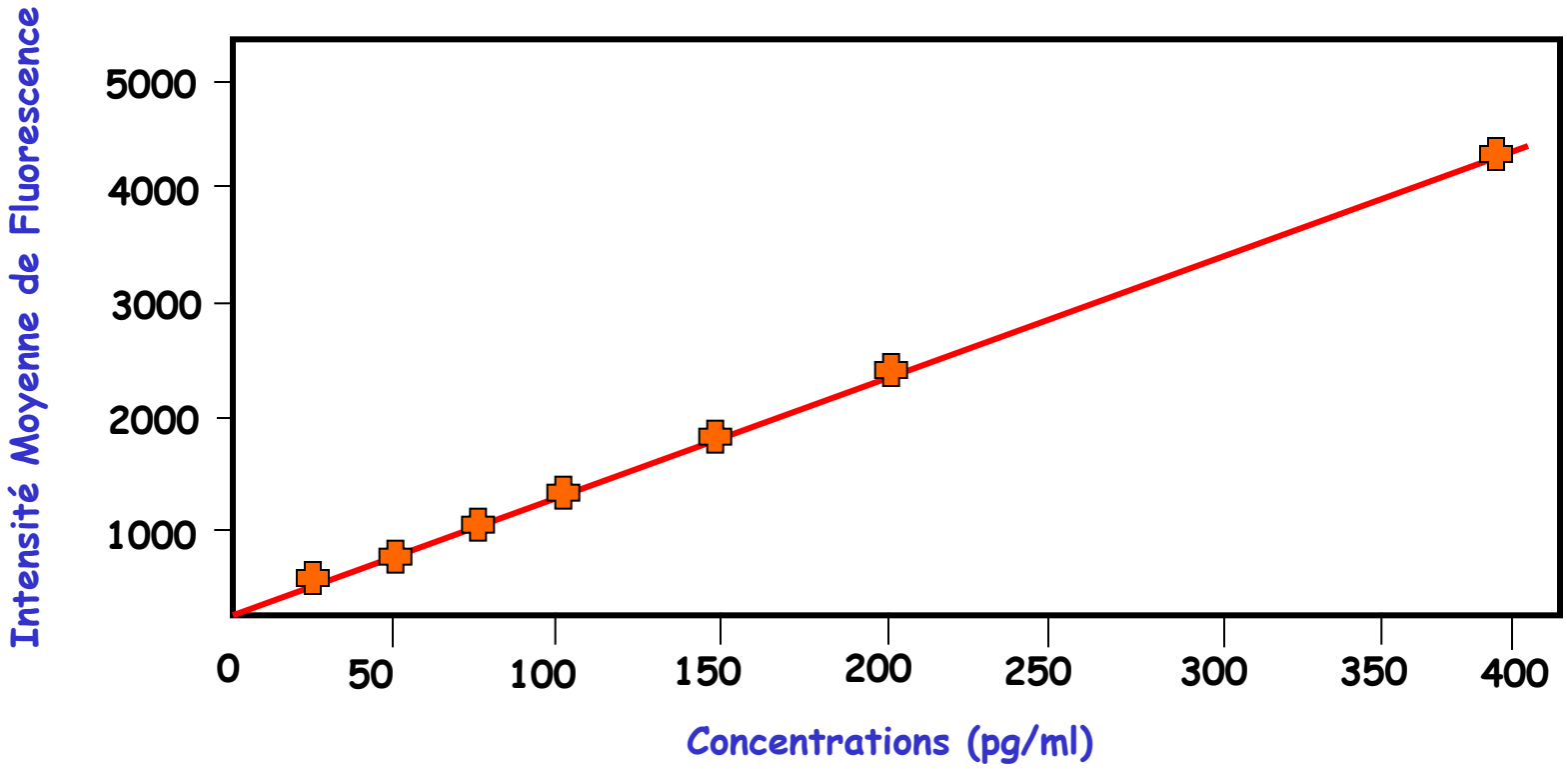
Charger les fichiers de calibration et de données (*.fcs)

Indiquer les concentrations des standards pour chaque étalon



Gamme étalon : ELISA

- Régression linéaire



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

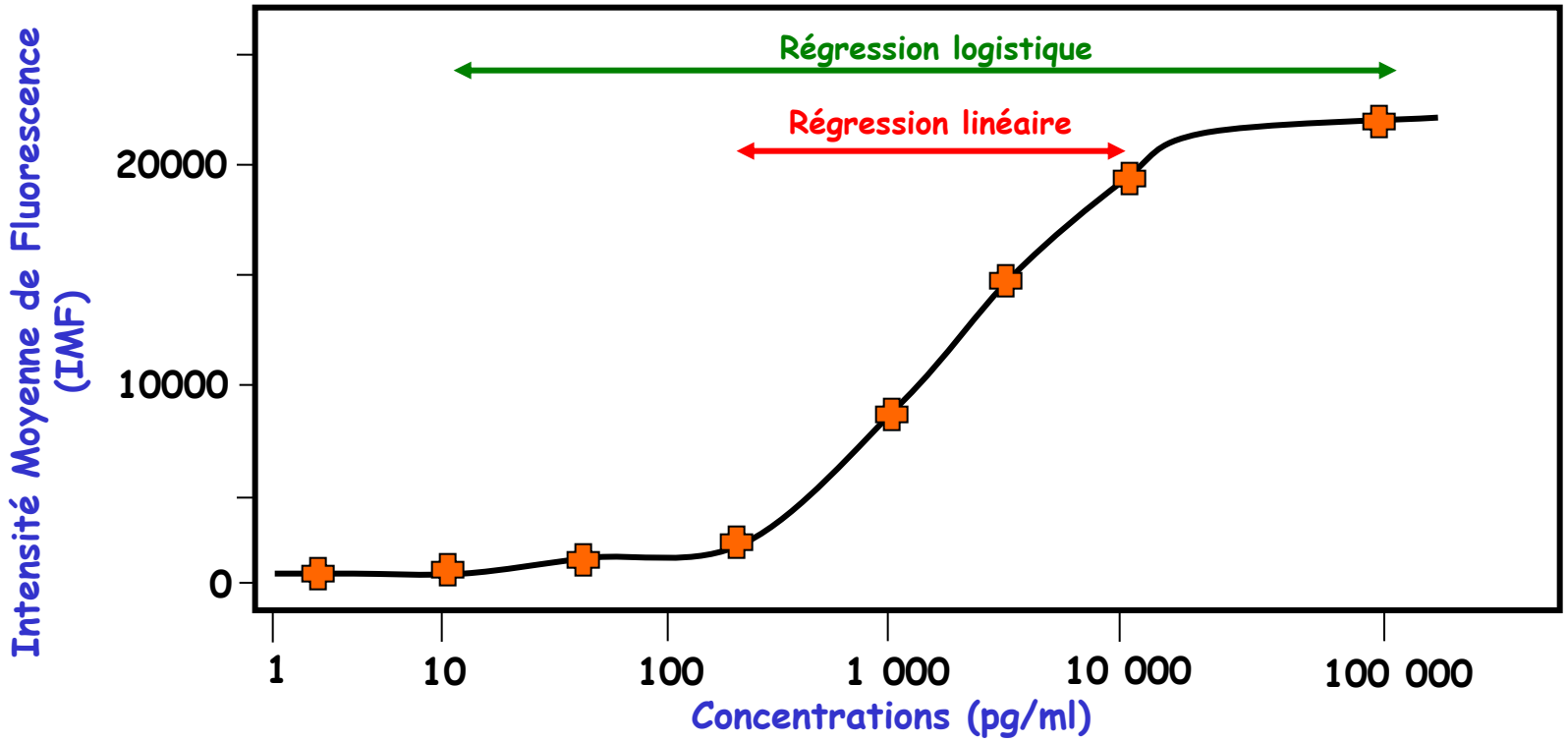
Gamme étalon : microbilles/analyses multiplexes

-Régression logistique

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

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

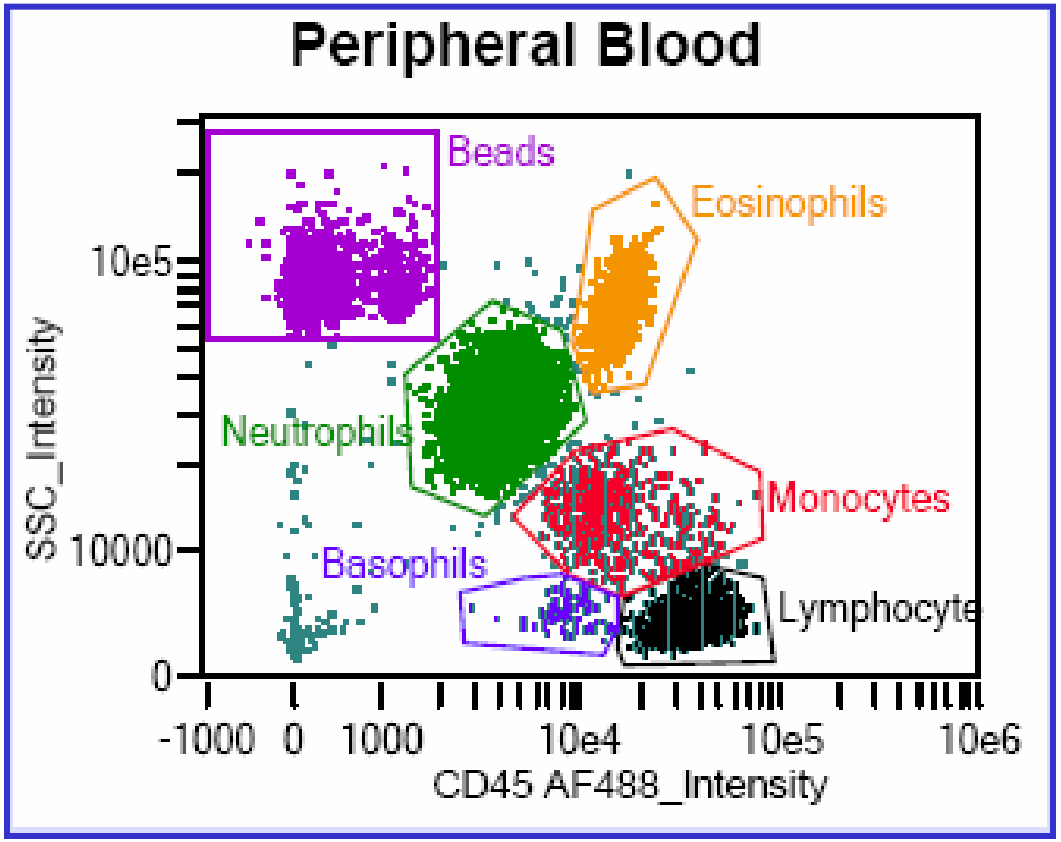
Avec le logiciel BD les valeurs de a, b, c et d sont définies automatiquement pour chaque courbe.



° 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

Phénotypage et analyses moléculaires



Multiplexed cell classification and cytometric bead array analysis using the ImageStream100 imaging cytometer.

Philip J. Morrissey, Thaddeus George, Brian Hall, and David Basiji.
Amnis Corporation, 2505 3rd Ave, Suite 210, Seattle, WA 98121-1480 www.amnis.com

Analyses cytokiniques multiplexes sur extraits cellulaires et tissulaires

revue générale

abc

Ann Biol Clin 2009 ; 67 (4) : 381-93

Méthodes d'analyses moléculaires multiplexes sur supports solides ou en milieu liquide pour l'identification de biomarqueurs protéiques dans les fluides biologiques et les extraits cellulaires ou tissulaires

Multiplexed analysis for identification and evaluation of novel biomarkers in biological fluids, tissue and cell extracts

S. Lehmann[1]
A. Dupuy[2]
J.-L. Beaudeux[3]
G. Lizard[4]

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[2] Laboratoire de biochimie, Hôpital Lapeyronie, Montpellier
[3] Laboratoire de biochimie, Hôpital Charles Foix, AP-HP, Ivry-sur-Seine
[4] Centre de Recherche Inserm 866 (Lipides-Nutrition-Cancer ; équipe Biochimie Métabolique et Nutritionnelle), Université de Bourgogne,

Résumé. Cet article de synthèse a été réalisé dans le cadre du groupe de travail « Groupe préanalytique et analyses multiplexes en protéomique » de la SFBC 2007-2008. Il fait le point sur les méthodes d'analyses multiplexes sur supports solides ou en milieu liquide utilisant alors les principes de la cytométrie en flux. Ces approches s'inscrivent dans une démarche d'analyse du protéome mais elles peuvent également avoir de l'intérêt en analyses biomédicales. Elles peuvent en effet donner des informations diagnostiques, pronostiques ou thérapeutiques dans diverses pathologies humaines. Il s'agit donc ici de faire un état des lieux décrivant les techniques d'analyses protéiques multiplexes déjà accessibles dans les laboratoires d'analyses biologiques ainsi que les outils disponibles en recherche clinique.

Mots clés : *analyses moléculaires multiplexes, biopuces, système Randox, technologie Luminex, cytométrie en flux, protéomique clinique*

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

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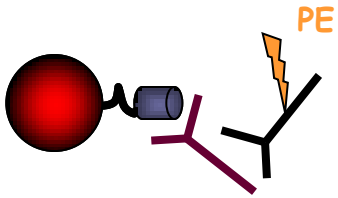
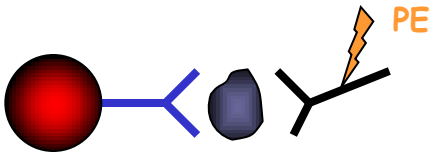
Analyses réalisables, caractéristiques analytiques

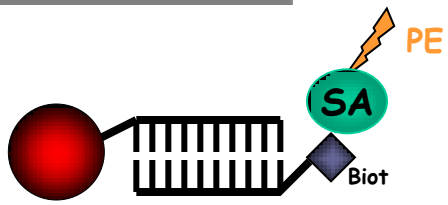
- Immunoessai



ou



- PCR



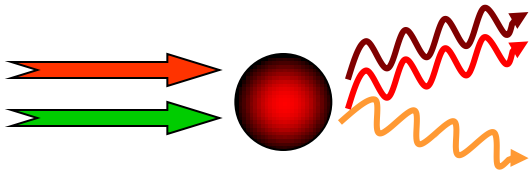
Anticorps

Antigène

Séquence nucléotidique

- Détection

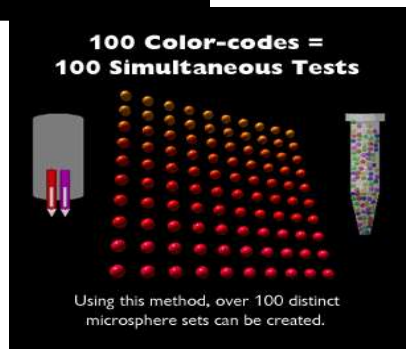
Laser rouge 635 nm
Laser vert 532 nm

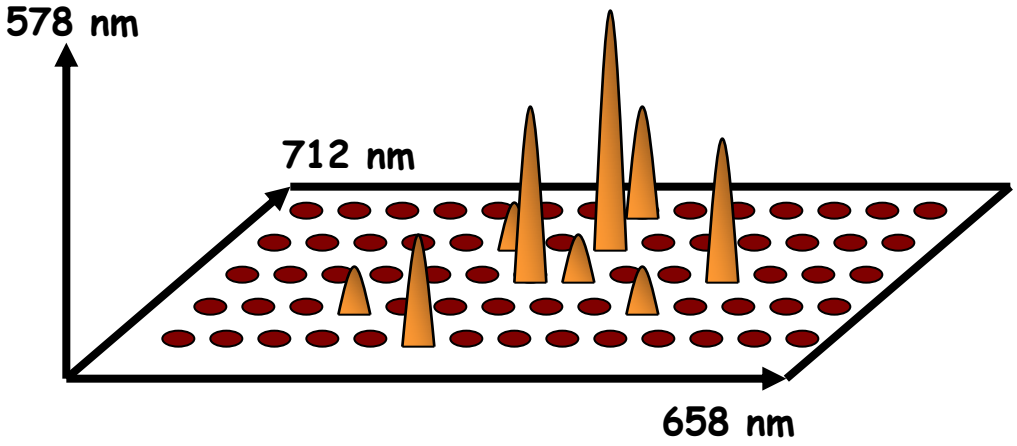


Microsphères: émission 658 et 712 nm

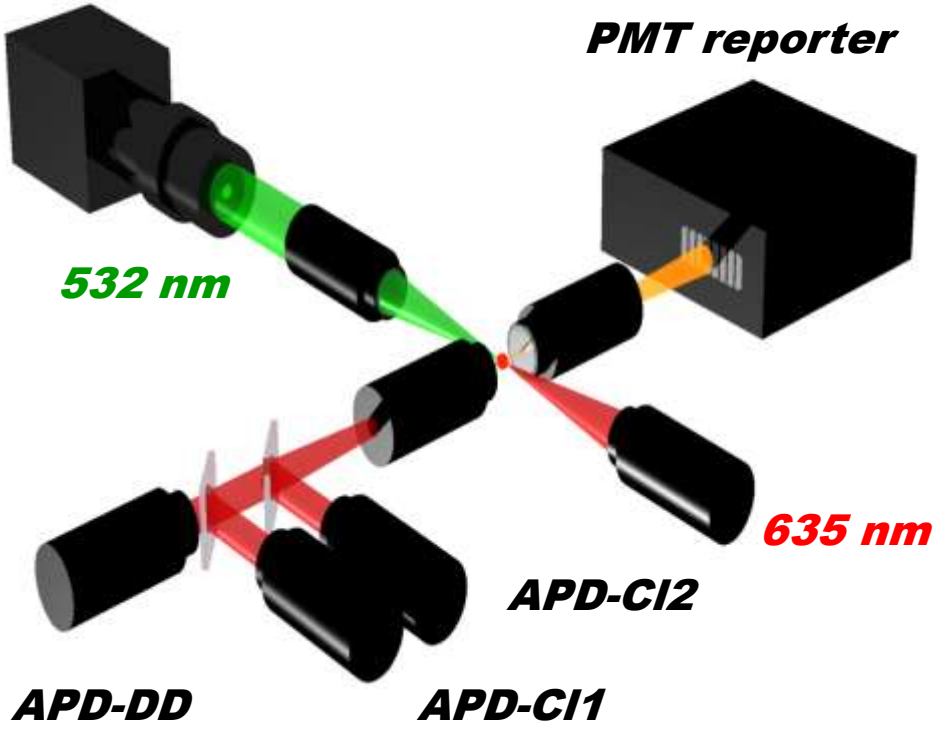
PE: émission à 578 nm

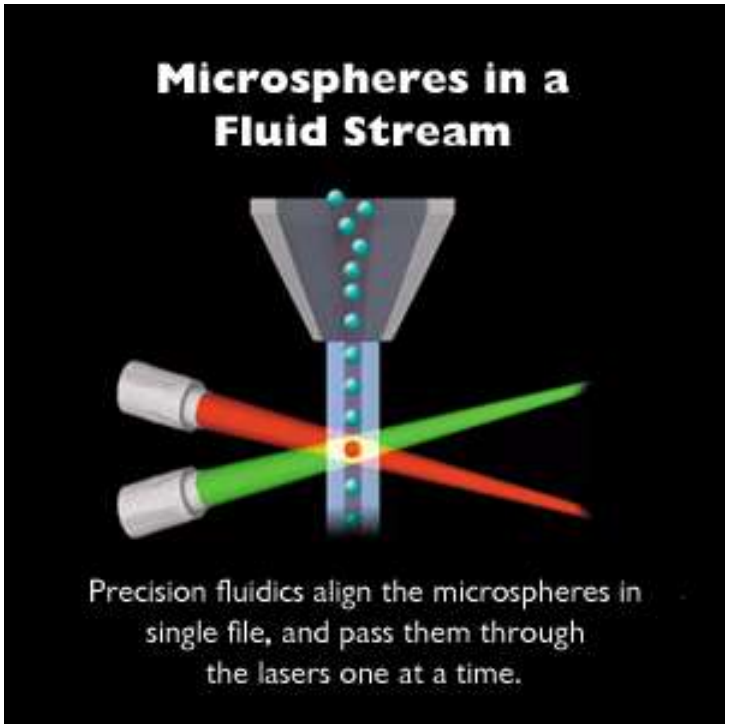
- Acquisition et exploitation des résultats





Technologie LUMINEX
Lasers et excitation

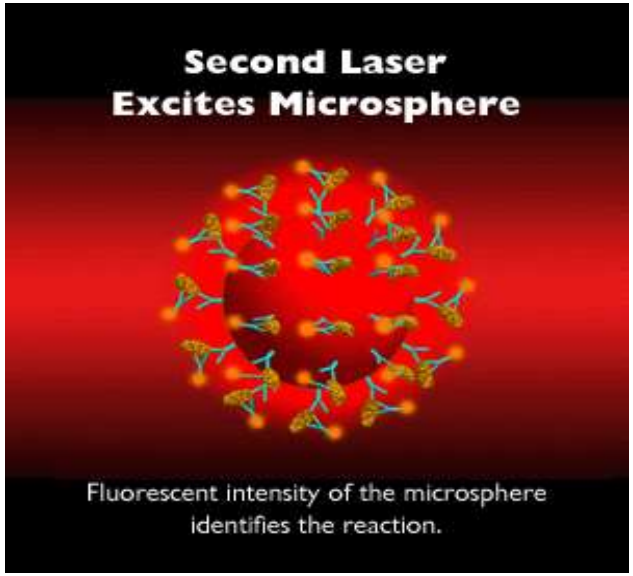


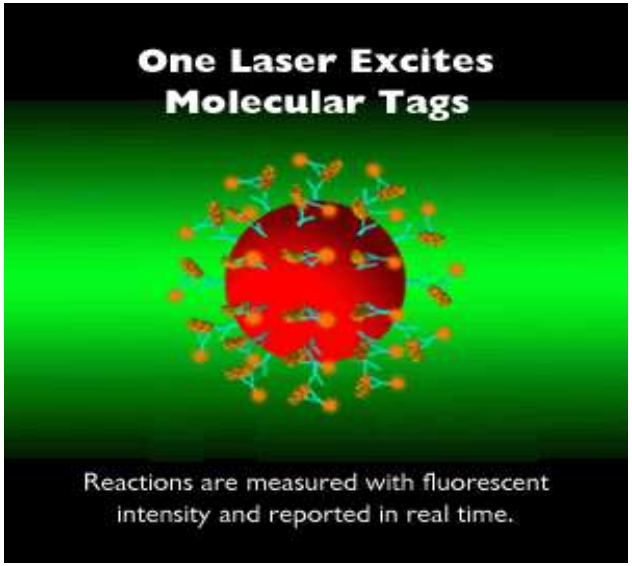


- ***1ére excitation***
  - ° laser rouge
- ***2ème excitation***
  - ° laser vert

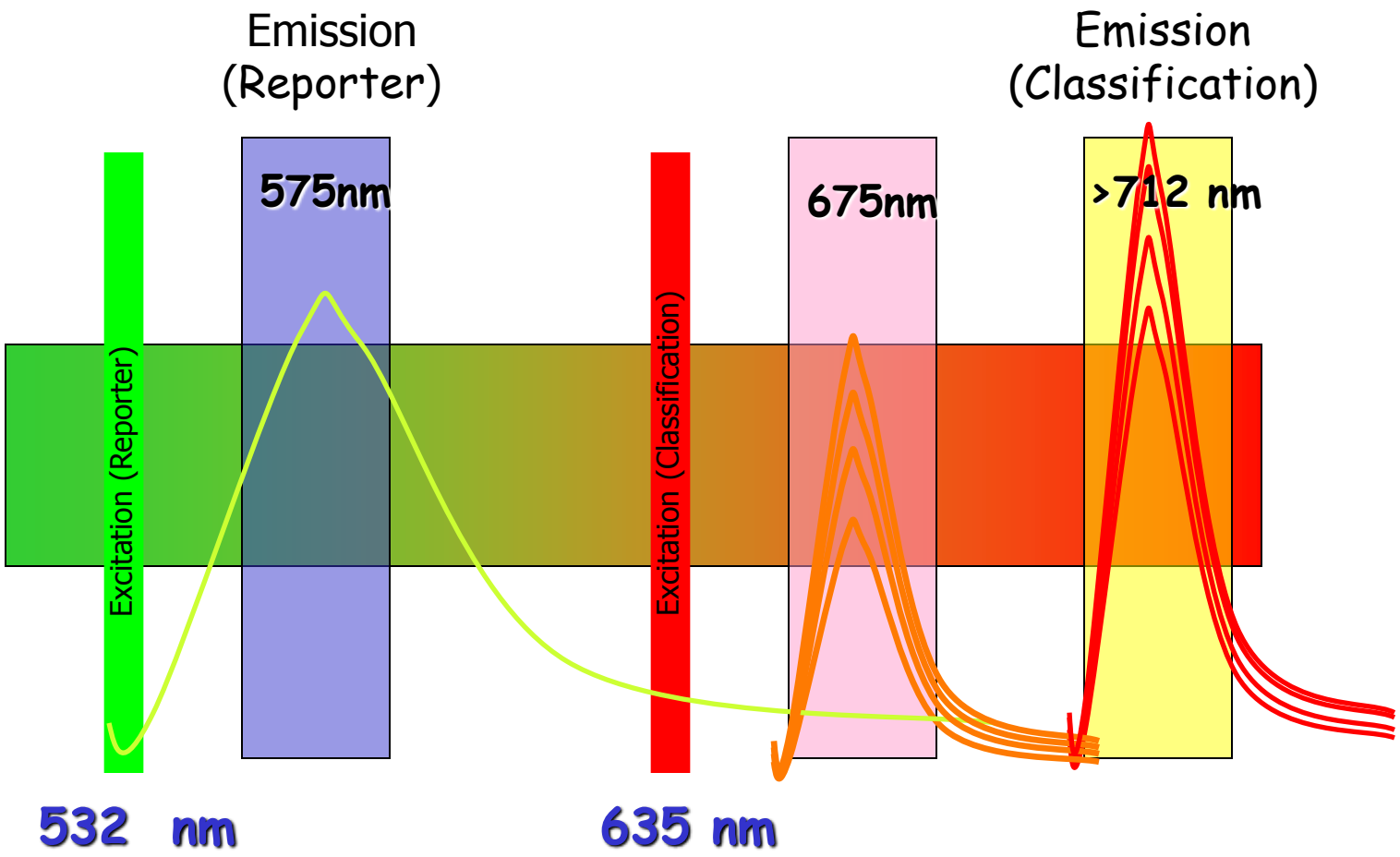
Technologie LUMINEX

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

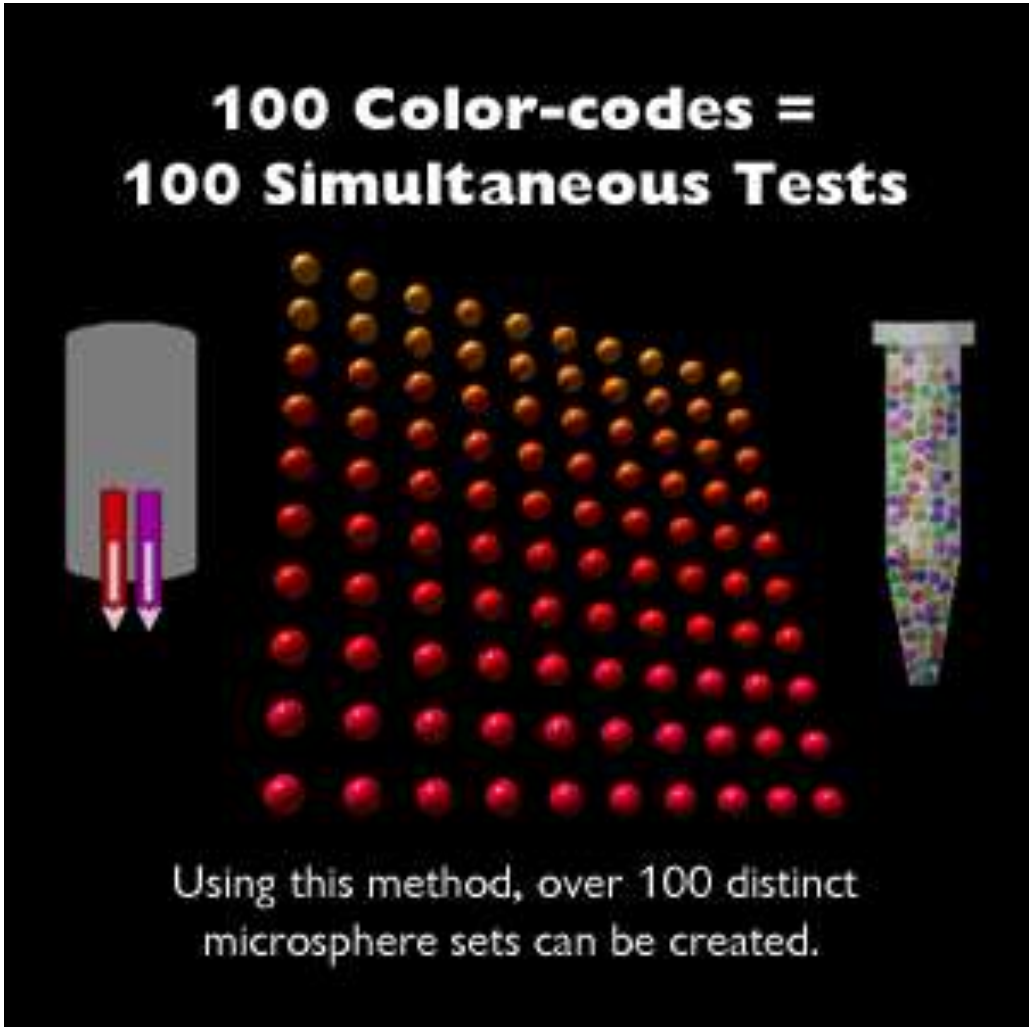




Technologie LUMINEX
Banc optique



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



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- **Lower-cost platform**
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MAGPIX INSTRUMENT SPECIFICATIONS

Accuracy and Precision

- Sample uptake volume ± 5%
- Classification of microspheres > 80%
- Misclassification of microspheres ≤ 2%
- Distinguishes 1 to 50 unique xMAP® microspheres in a single sample
- Temperature control ± 1°C of target
- Sample carryover ≤ 4%
- Soluble background fluorescence emissions at 590 nm ± 24 nm automatically subtracted from fluorescence intensity values

Sensitivity

- Detect ≤ 700 fluorochromes phycoerythrin (PE) per xMAP microsphere

Optics

- Reporter channel, LED excitation: 511 nm ± 27 nm
- Reporter channel dynamic range: ≥ 3 decades of detection
- Reporter / Classification detector: CCD
- Reporter / Classification A/D resolution 16 bits
- Focus Lens: 5x magnification

Fluidics

- 96-well plate capability with additional off-plate reagent area
- Piercing probe capability
- Auto adjusting probe capability
- Sample uptake volume 20 µL – 200 µL.
- Internal / removable drive bottle and waste bottle
- Disposable Drive bottle volume: 650 mL (8 plates)
- Waste bottle volume: 850 mL

Electronics:

- USB 2.0 communications
- Input voltage range: 100 – 120 or 200 – 240 VAC and 50 – 60Hz

Setup

- Installation ≤ 2 hours
- System calibration < 10 minutes

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- Analyze multiple 96-well plates per batch
- Analyze multiple assay templates per plate
- Automatic sampling from a 96-well plate. The following microtiter plates are compatible with the MAGPIX plate holder: flat bottom, conical, round, filter bottom, [overall height no more than 19 mm (0.75 inches)], any color
- Drive and waste bottles hold enough volume to run up to eight 96-well plates between refills
- Detect and distinguish surface reporter fluorescence emissions at 590 nm ± 24 nm on the surface of 1-50 unique xMAP microsphere sets in a single sample

General

- Physical dimensions: 16.5 cm (6.5 inches) W x 60 cm (23.5 inches) D x 43 cm (17 inches) H. Additional space required for the monitor/PC stand, keyboard, mouse and barcode scanner does not exceed 64.8 cm (25.5 inches) W x 61 cm (24 inches)
- Weight: up to 18 kg (40lbs)
- Temperature control: Maintains samples at a constant temperature from 35 °C - 60 °C (95 °F - 131 °F)
- Warm up time: 15 minutes
- Plate throughput time: ≤ 60 minutes

Operating Environment:

- Temperature: 15 °C – 35 °C (59 °F – 95 °F)
- Humidity: 20% - 80%RH, non-condensing
- Altitude: up to 2400 m (7874 ft) above mean sea level

Monitor Specifications

- Screen resolution and number of colors: SXGA 1280 x 1024 with 32-bit color
- Screen size: 43 cm (17")

PC Specifications

- Processor: 2.8 GHz Intel® Core 2
- Main memory: 4 G RAM
- Hard disk drive: 160 GB
- Communication/Ports: four USB version 2.0 compatible high speed ports
- DVD-RW drive
- Operating system: Microsoft® Windows® XP Professional, SP3
- CE marked and UL listed

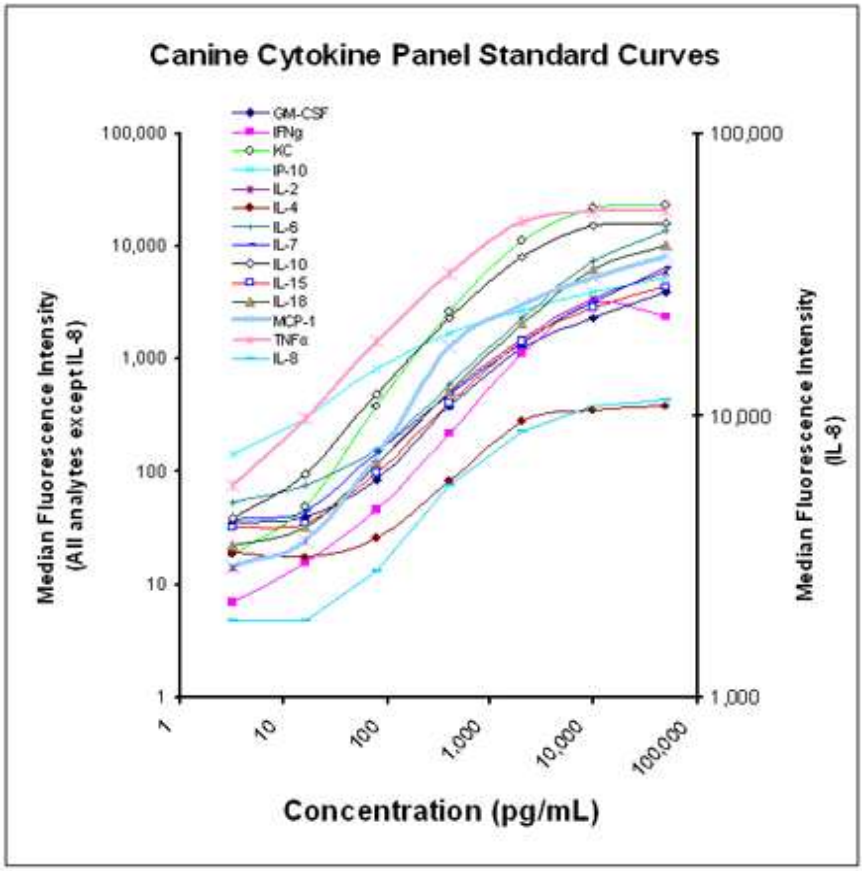
Lit. No. PF3545EN00 07/10 BP-GEN-10-03329 Printed in U.S.A.

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.

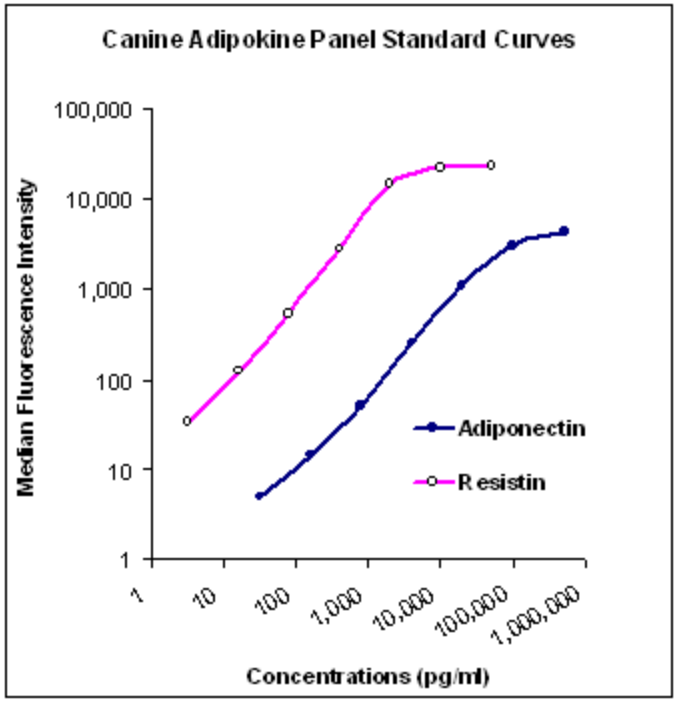
Maladies métaboliques : obésité

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

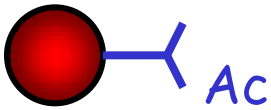
Mouse (CVD) Biomarker Panel 1

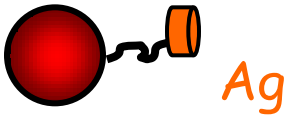
sE-Selectin, sVCAM-1, sICAM-1, MMP-9, tPAI-1

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

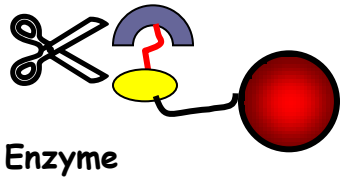
Techniques multiplexes complémentaires

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





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



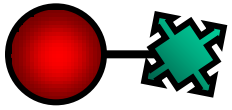
Agent 'quenchant'

Séquence peptidique

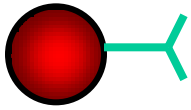
fluorochrome

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

Streptavidine



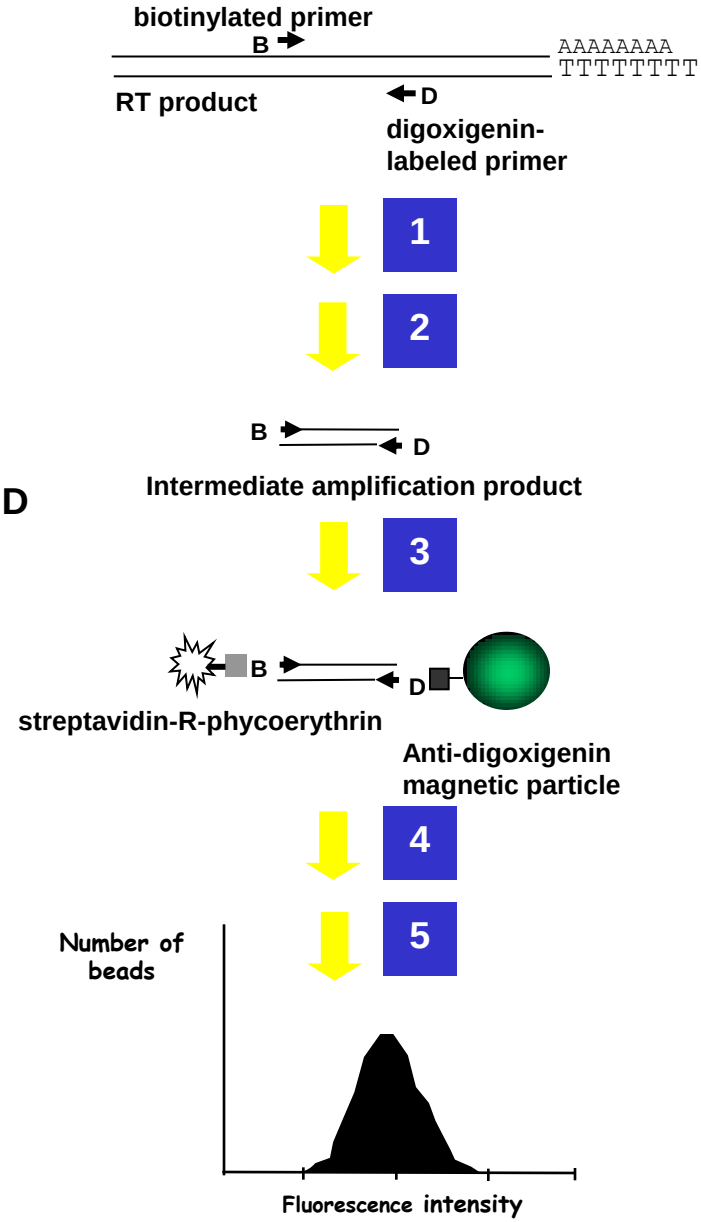
Ac (Dig, DNP)



° Interactions moléculaires

° Identification de phosphoprotéines: voies métaboliques

Microbilles et PCR en flux

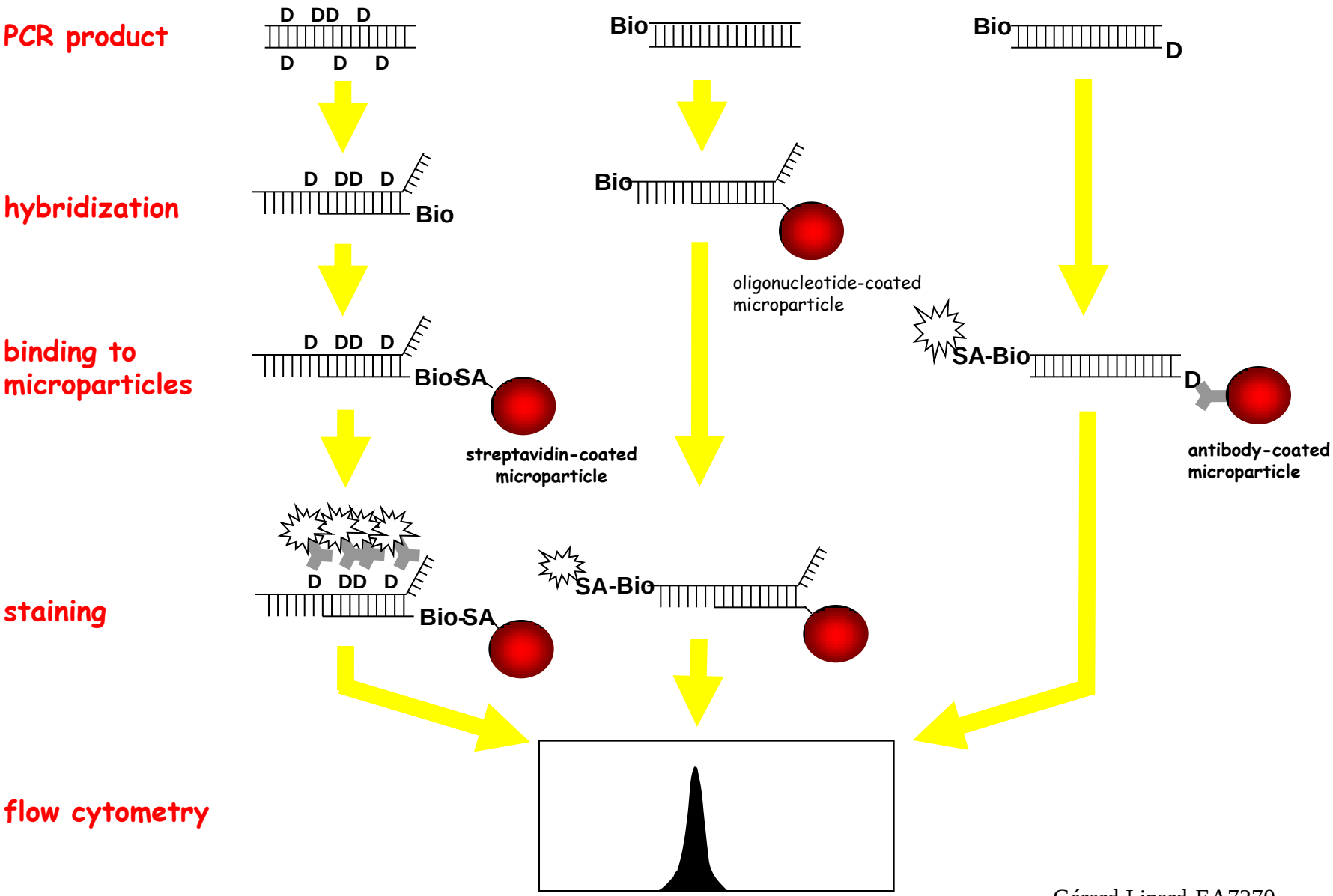


1. PCR reaction
   - using primers labeled with digoxigenin and biotin
   - For quantification PCR reaction is stopped in the exponential phase
   - 60 min
2. Removal of unincorporated primers
   - using silica particles
   - 5 min
3. Binding and staining of PCR products
   - using microparticles coated 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

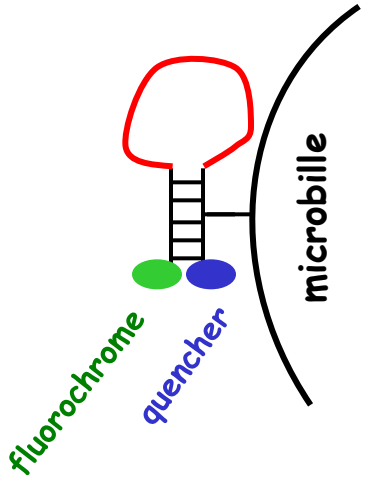
Total: 90 min

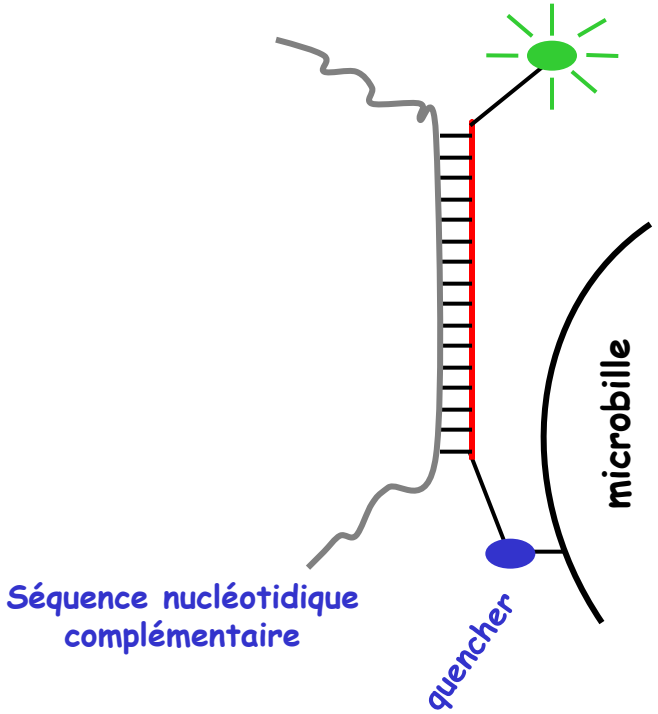
Wedemeyer *et al*. Clin Chem 2002, 48 : 1398-1405.

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



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





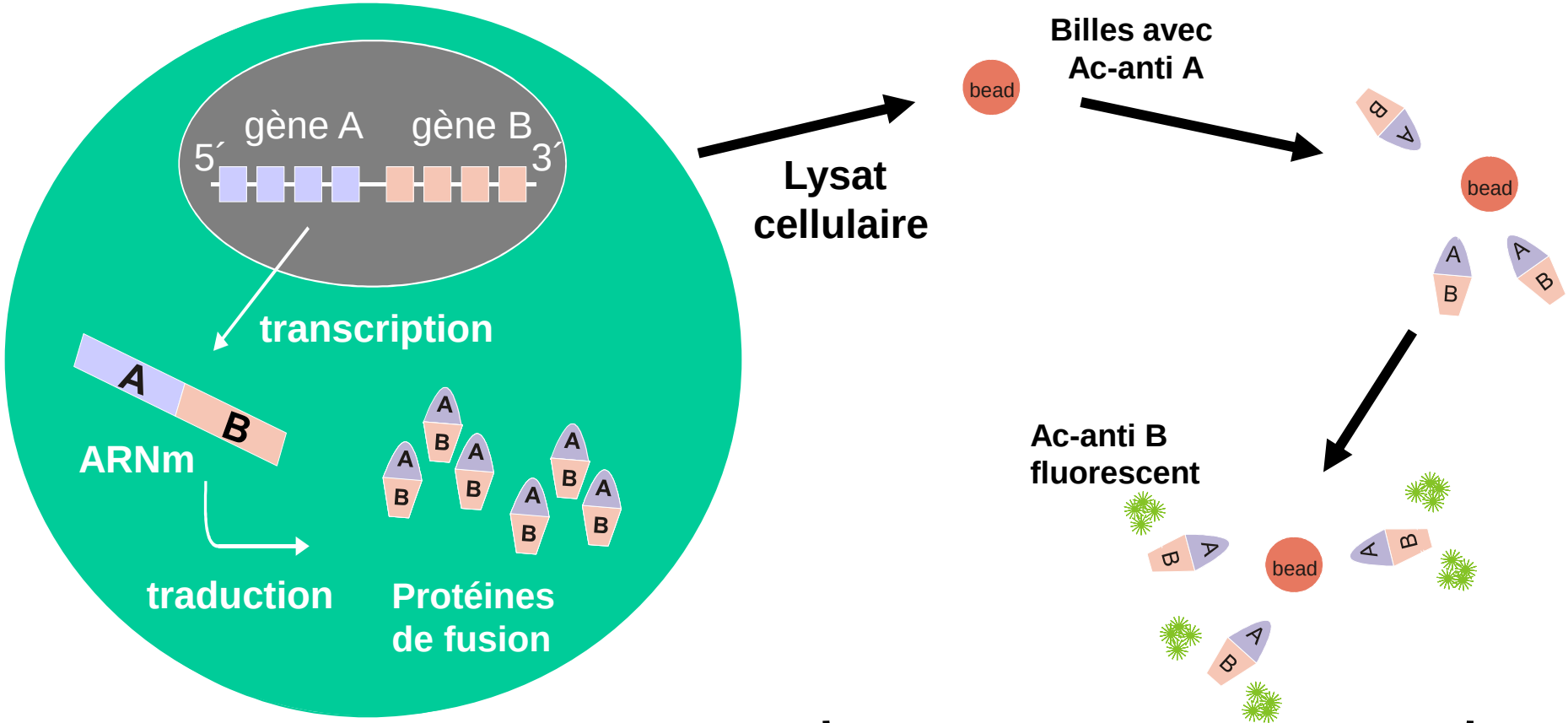
- Pas de séquence nucléotidique complémentaire, seule la microbille émet de la fluorescence,

- 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

Identification de protéines de fusion



Synthèse d'une protéine de fusion

Détection d'une protéine de fusion par analyse multiplexe

- Zhang QY *et al*. 2002; 16: 144-149
- Chan HE *et al*. Methods Cell Biol 2007; 378: 167-174
- Jilani I *et al*. Leuk Res 2008; 32: 936-943

Gérard Lizard-EA7270

Combinaison d'analyses multiplexes envisageables pour l'analyse cytokiniques par cytométrie en flux

Analyse des ARNm de cytokines

↓

Analyse des cytokines intracellulaires

↓

Analyse des cytokines sécrétées

↓

Voies métaboliques impliquées
- Utilisation d'inhibiteurs
- Etude des protéines phosphorylées (JNK, p38, ERK1/2……)

↓

Identification des Facteurs de transcription impliqués

Automatisation

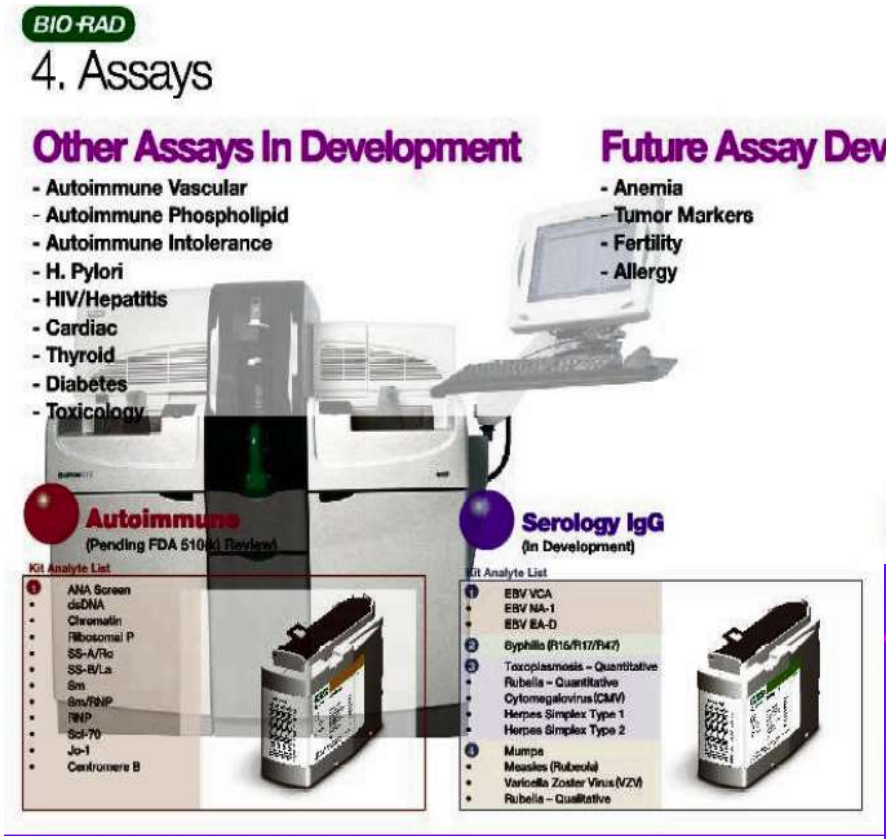
Automatisation des Réactions

Répartion des échantillons,
Répartition des microbilles
Répartition des réactifs
Lavages

Acquisition et Exploitation Automatiques des Résultats

Feuilles de Résultats

Technologie LUMINEX : automatisation



Microbilles, analyses multiplexes, cytométrie et… environnement

•**Optimisation des performances (sensibilité, rapidité, capacité de stockage) des cytomètres en flux**

*** Standardisation des résultats**

*** Préservation de l'environnement**

- Appareil de petites tailles (faible consommation d'énergie),

- Microméthodes d'analyses (réduction de l'utilisation de matières premières et diminution du rejet de déchets : préservation de l'environnement).

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