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## Development of a multiplex immunoassay

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**FMD-DISCONVAC**  
**WP5: Improvement in FMD diagnostics**  
**Task 3:**  
**Development of a multiplex immunoassay**

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# Objectives

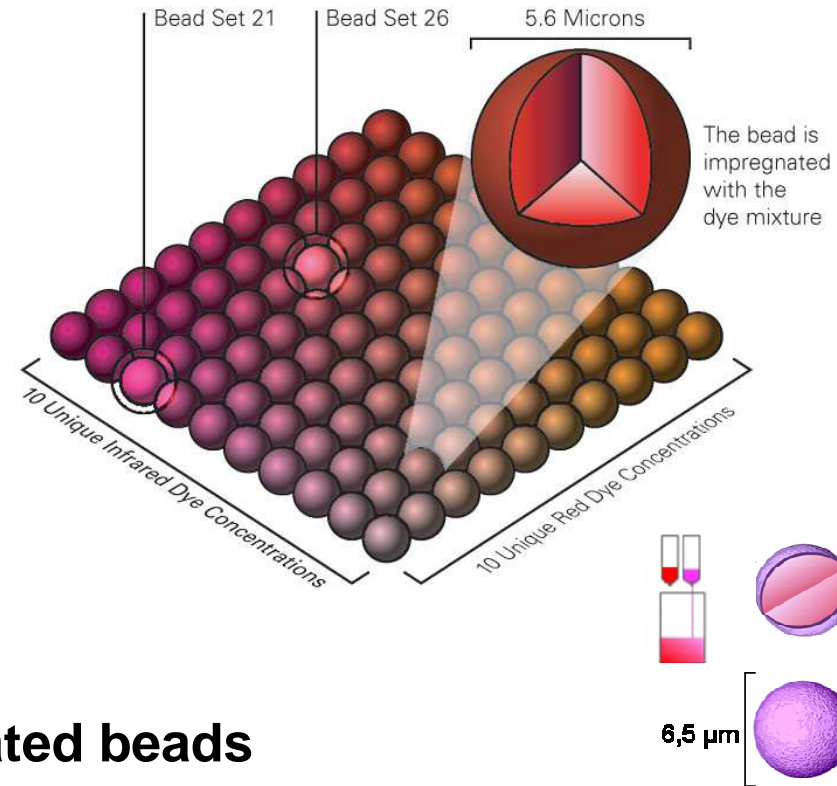
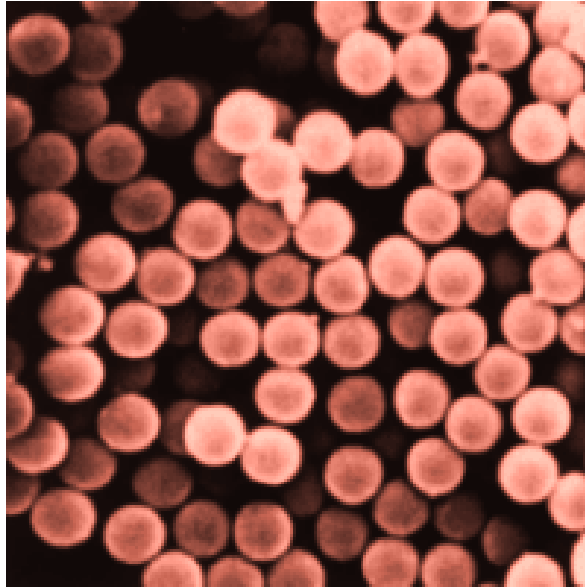
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Development of a multiplex immunoassay for FMD diagnosis by using the Luminex liquid array technology



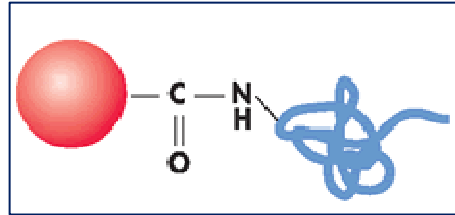
- ✓ Simultaneous detection of antibodies against vesicular disease viruses and FMDV structural and non structural proteins
- ✓ Serological discrimination between FMDV-infected and vaccinated animals in a single reaction

# Microspheres (I)



- Use of magnetic carboxylated beads
- $\emptyset$  6,5 µm
- each bead is impregnated with a unique infrared/ red dye ratio

# Microspheres (II)



## Magplex carboxylated beads

- ✓ Immobilization of 6–150 kDa proteins
- ✓ carbodiimide reaction (covalent)
- ✓ 5-10 $\mu$ g/ coupling reaction ( $1.25 \times 10^6$  beads)
- ✓ 2500 to 5000 beads/assay
- ✓ automatic magnetic bead washes

### **!!!Restrictions on use:**

The protein sample must be free of sodium azide, BSA, glycine, Tris or amine-containing additives and must be suspended in PBS, pH 7.4.

# Principles of the luminex technology

1. **100 Color Codes = 100 Simultaneous Tests**



Using a two-dye method, Luminex produces 100 distinct bead sets.

2. **Multiple Measurements With Color Separation**




Bio-Plex uses these uniquely color-coded beads to identify multiple assays in a single tube or well.

3. **Microspheres as Molecular Carriers**



To perform a test, thousands of probes are bound to the bead.

4. **Capturing the Target Molecule**



While suspended in a test sample, the bound probes collect molecules.

5. **Tagging the Reaction**




Fluorescently-labeled reporter tags bind to the sample molecule.

6. **Beads in a Fluid Stream**



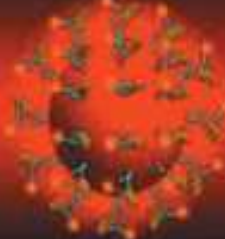
Precision fluidics align the beads in single file, and pass them through the lasers one at a time.

7. **One Laser Excites Molecular Tags**



Reactions are measured for fluorescence intensity and reported in real time.

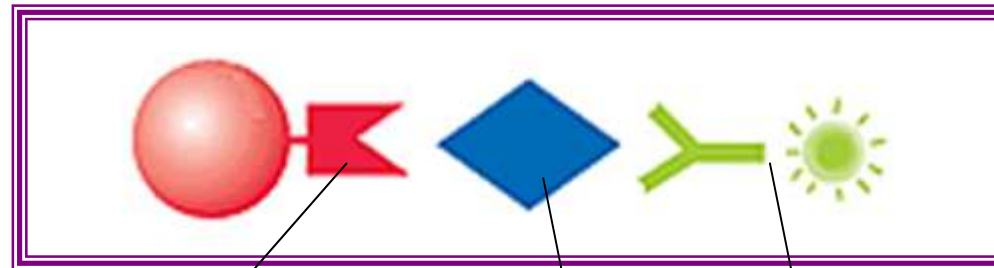
8. **Second Laser Excites Microsphere**



Fluorescence intensity of the bead identifies the reaction.



# Multiplex Immunoassay Principle (I)



## Capture molecule:

FMD SP : VP1 (of all serotypes)  
FMD NSP: 3ABC, 3A, 3B,3D and peptide 2B  
VSV SP  
SVDV SP

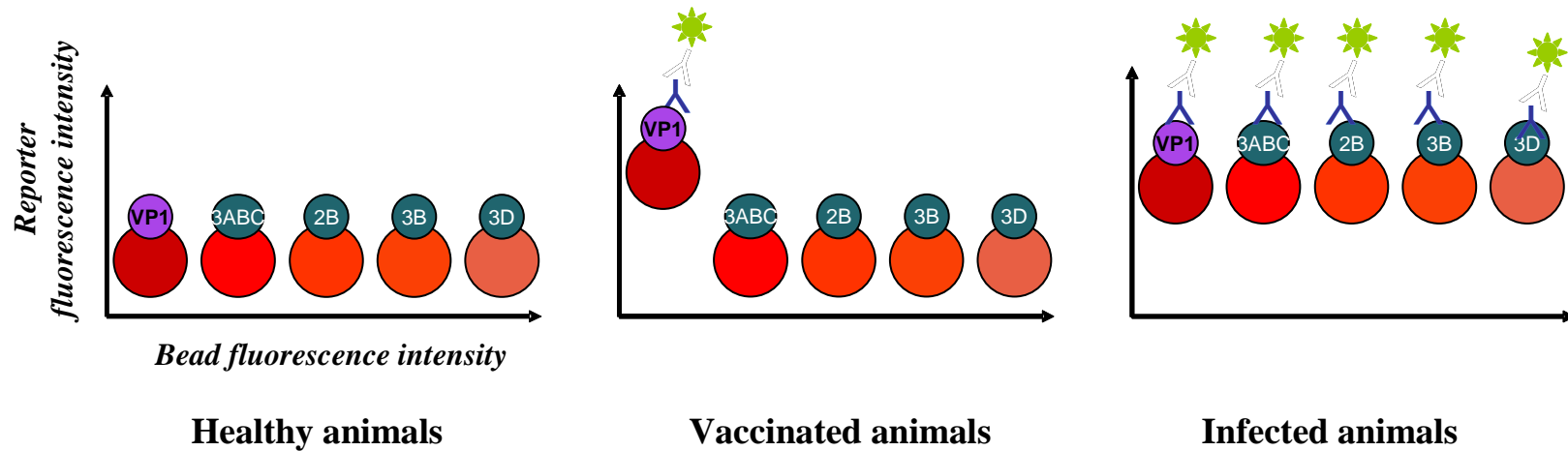
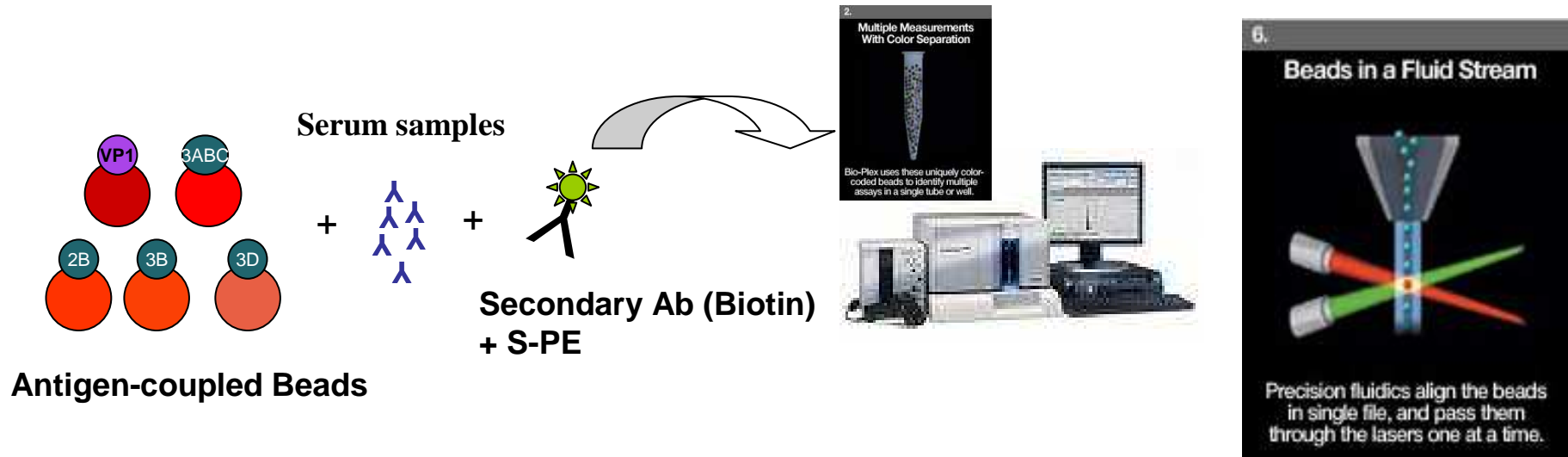
## Analyte:

Serum samples

## Reporter molecule:

Secondary anti-species antibody, biotin labeled.  
+ Streptavidine-Phyco-Erythrine (S-PE)

# Multiplex Immunoassay Principle (II)



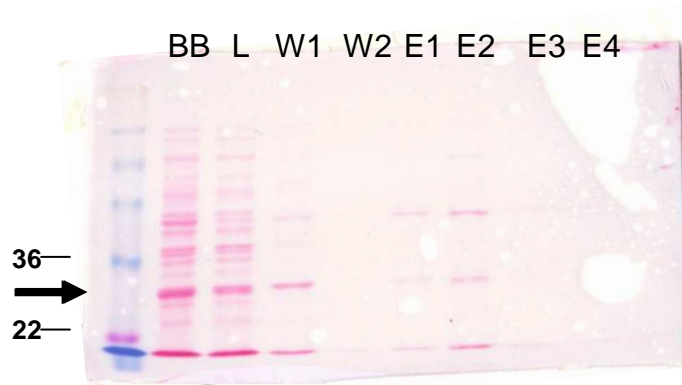


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# ANTIGENS PRODUCTION

# FMDV proteins expressed in E. Coli

## Capsid protein: VP1-6His

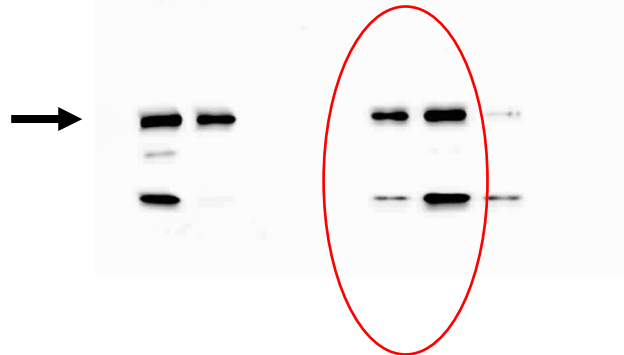


Purification on Ni-NTA column

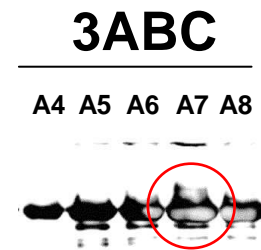
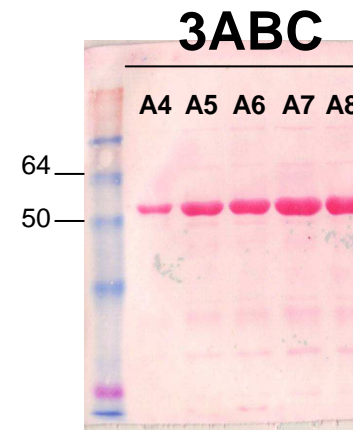


Dialysis and concentration

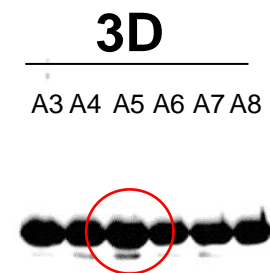
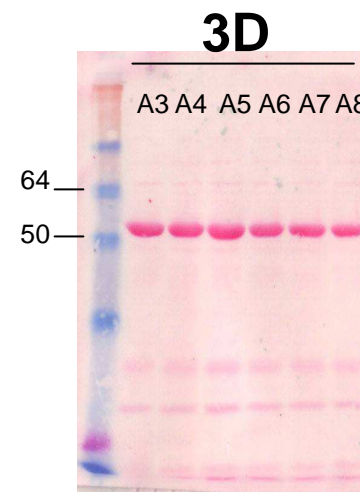
Anti-His



## Non structural proteins: 3ABC-6His, 3D-6His



Anti-His



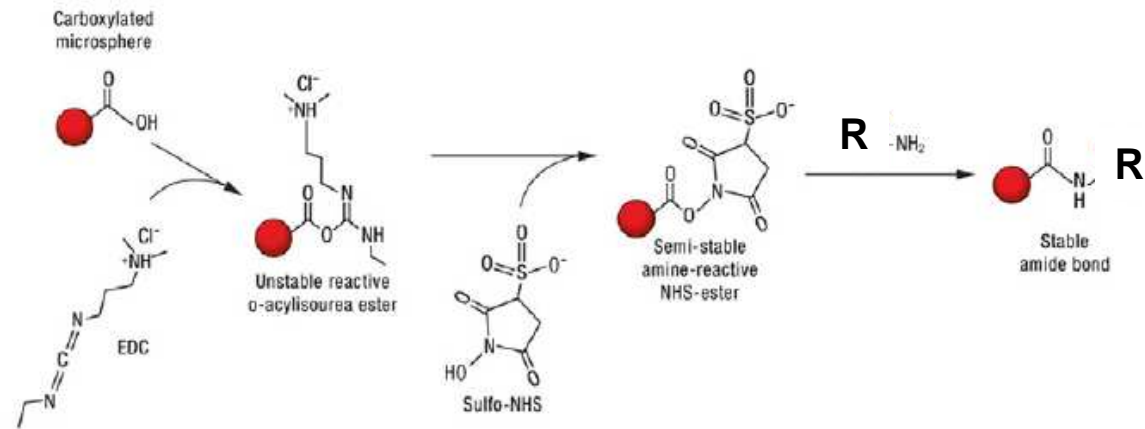
Anti-His



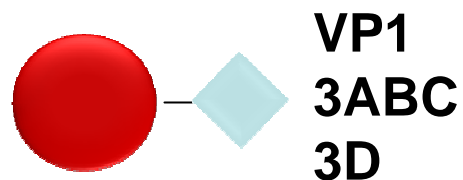
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# COUPLING CONDITIONS

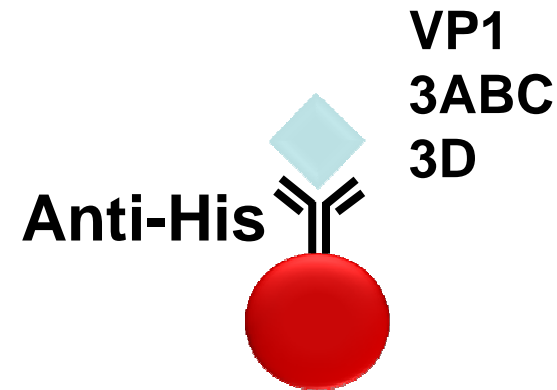
# Beads coupling : Two strategies



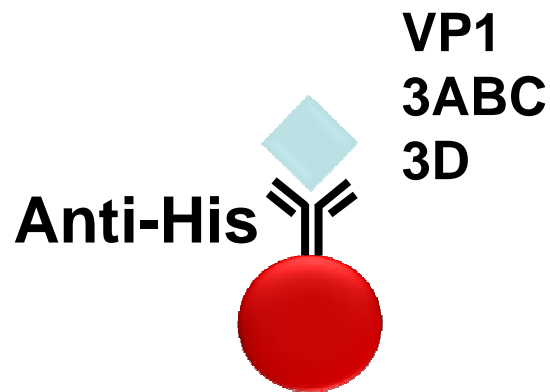
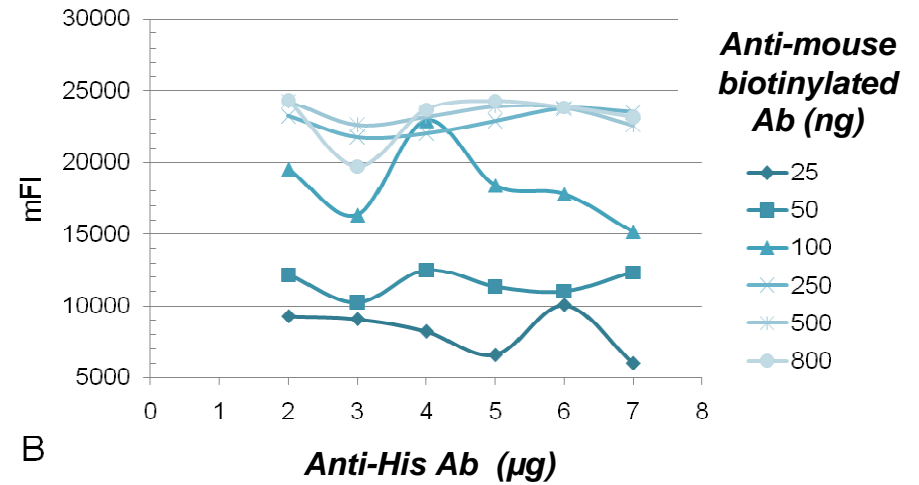
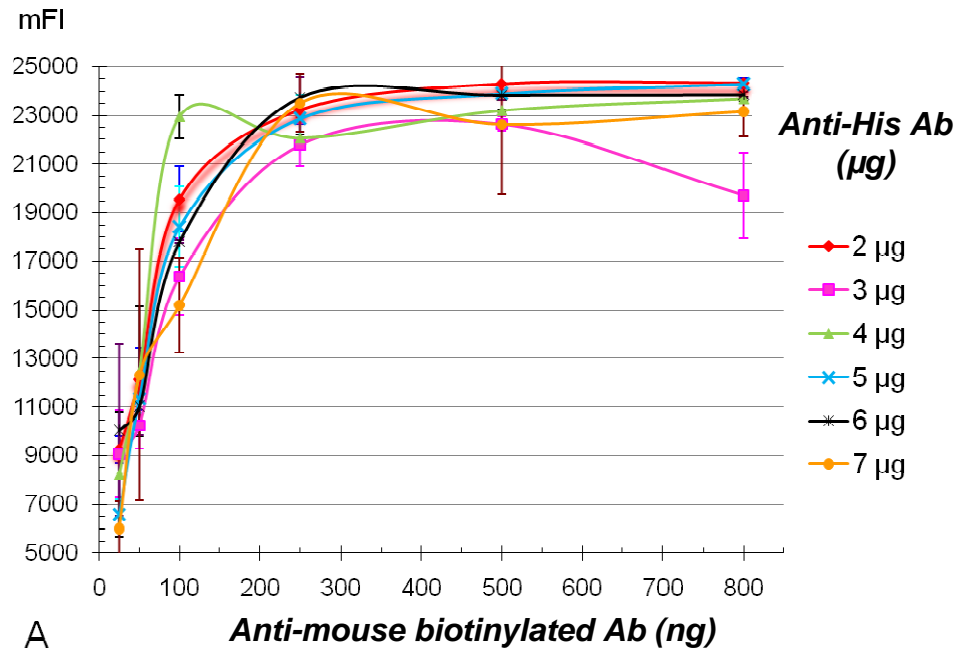
**Direct coupling:  
Bead/FMDVprotein**



**Indirect coupling:  
Bead/anti-His antibody**

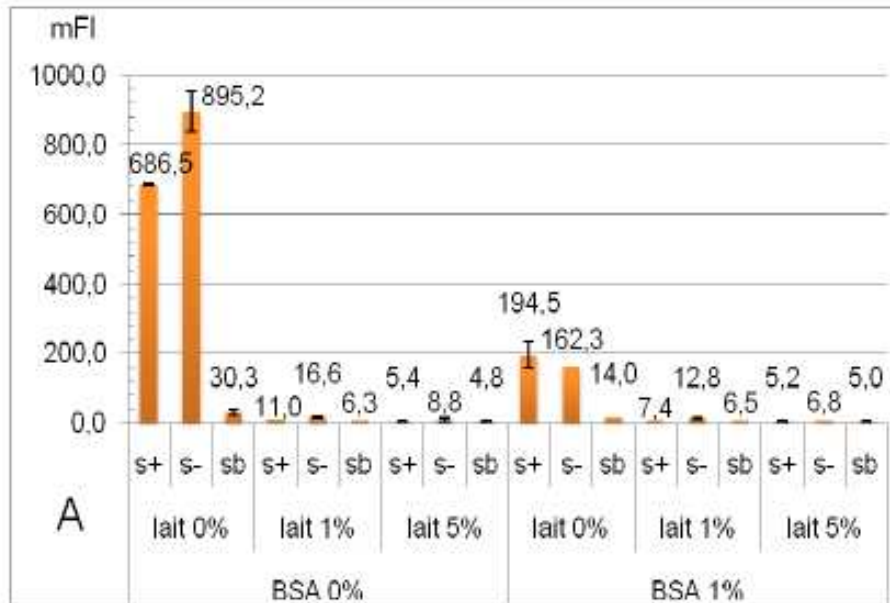


# Indirect coupling : optimization of the Anti-His Ab quantity

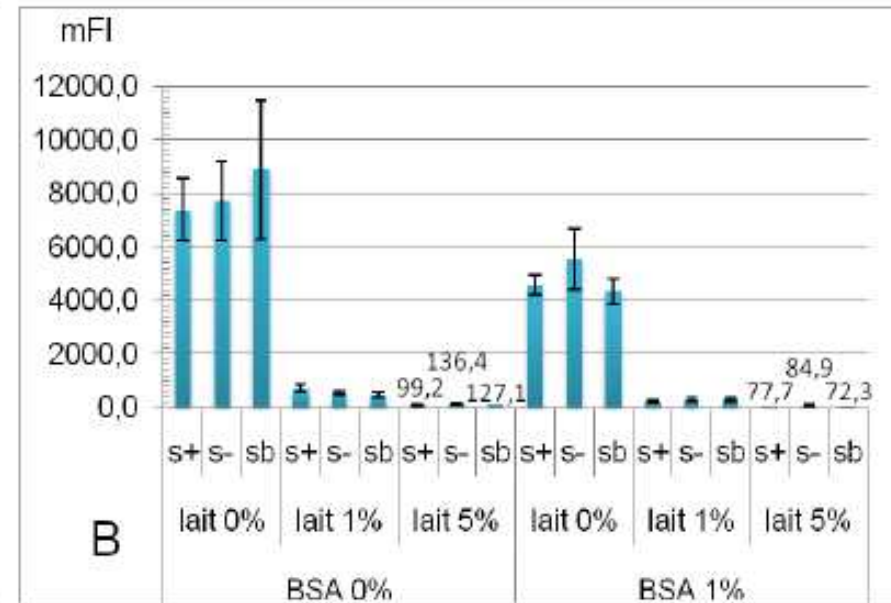


**Best results obtained with:**  
 2 µg Anti-His Ab  
 250-500 ng Biotinylated Ab

# Indirect coupling : optimization of the elisa blocking solution



*Uncoupled beads*



*Anti His (2µg) coupled beads*

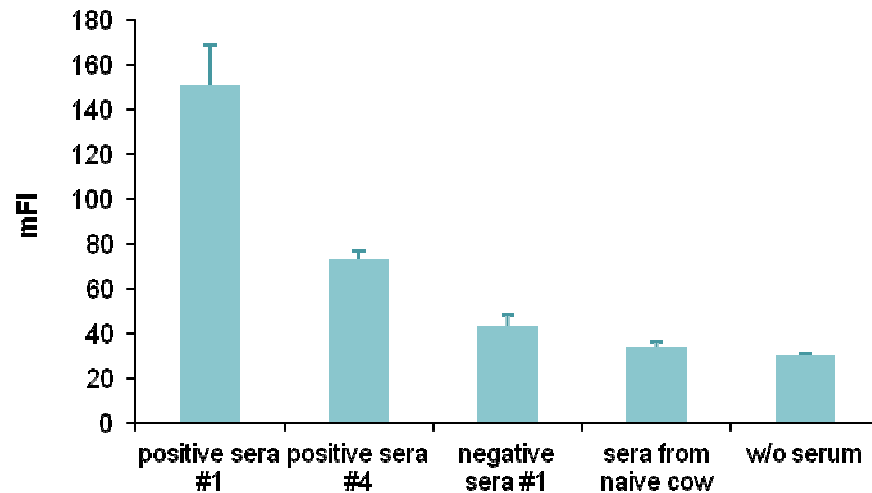
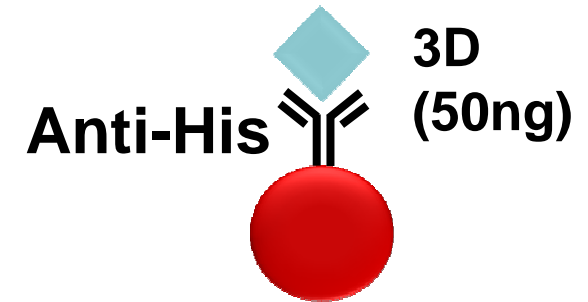
- Optimal elisa blocking solution (sb) formulation:  
 PBS (pH 7.2), Tween 20 (0.02%), milk (5%)
- Mfi for coupled beads = 10 x mfi uncoupled beads

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# Serological assay -preliminary results – (simplex)

# Indirect coupling

2500 beads/well (2  $\mu$ g Anti-His Ab)  
Serum dilution: 1/50  
Anti-cow biotin: 1/1000  
S-PE: 1/100



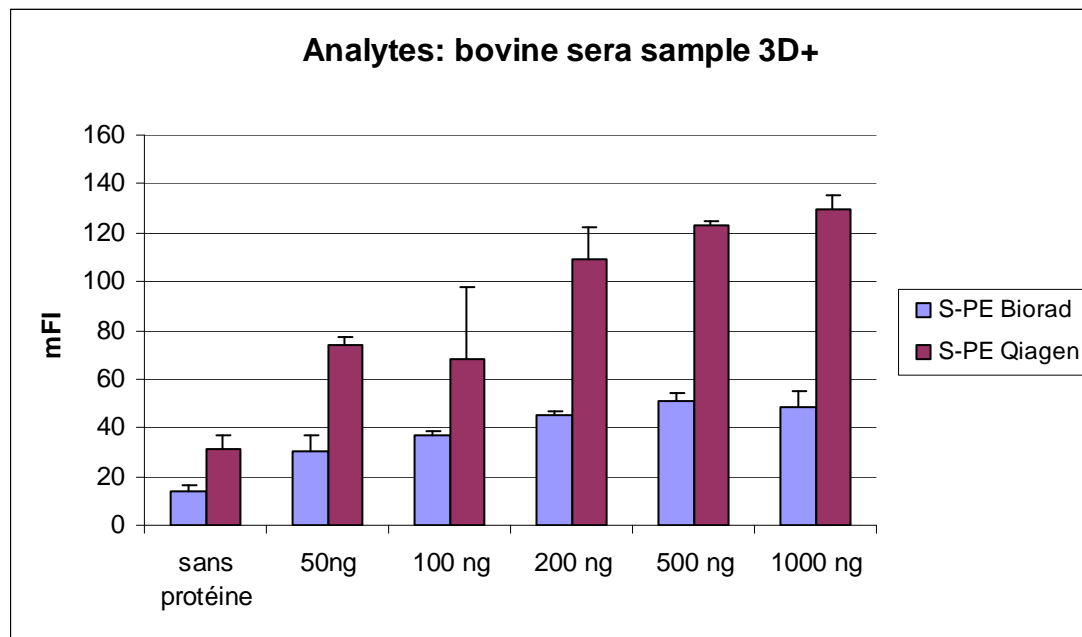
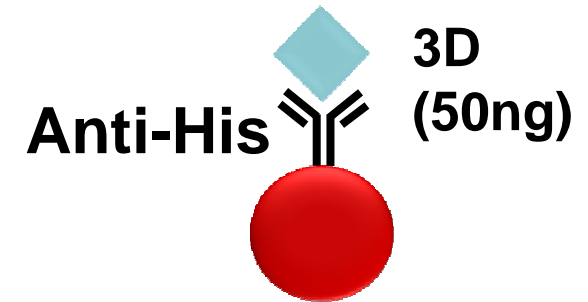
*Analytes: bovine sera samples*

Low mfi values  
signal-to-noise ratio < 4



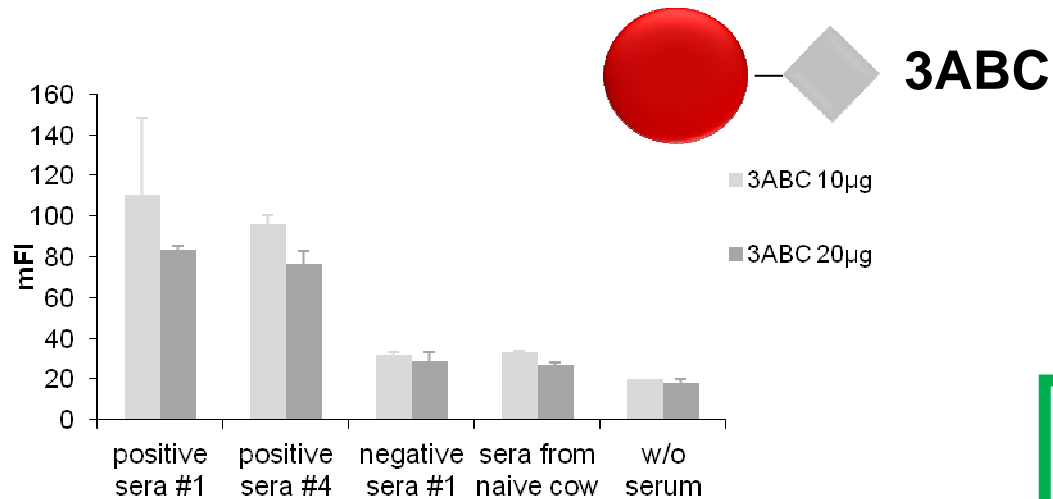
# Indirect coupling

2500 beads/well (2  $\mu$ g Anti-His Ab)  
3D his tag protein: 50 to 1000 ng/well  
Serum dilution: 1/50  
Anti-cow biotin: 1/1000  
S-PE: 1/100 (Biorad or qiagen)

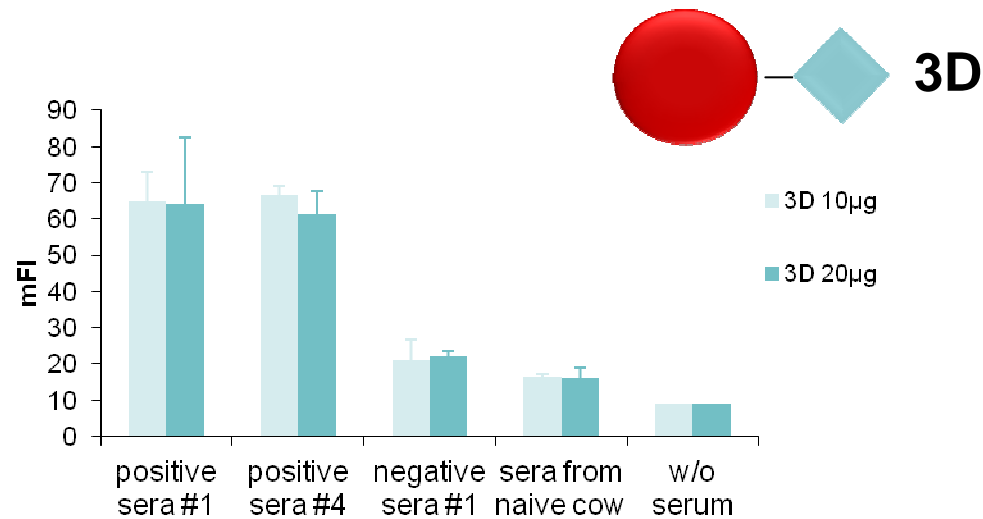


**Best results with SPE from qiagen**  
**Effect of 3D quantity on mfi values**

# Direct coupling

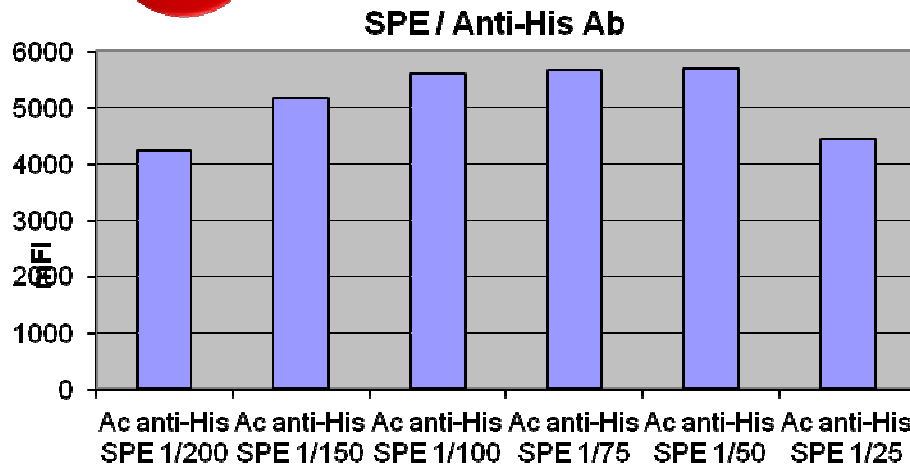
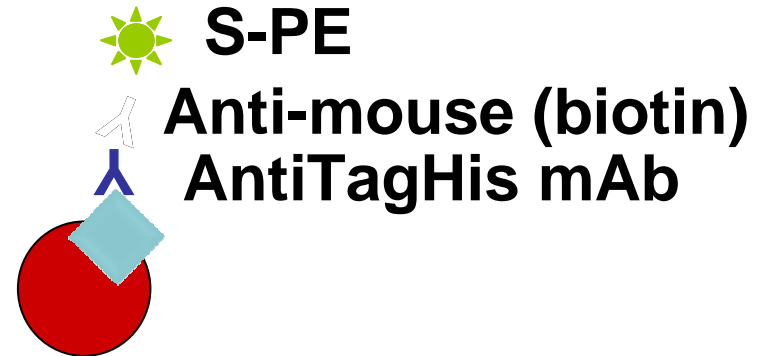
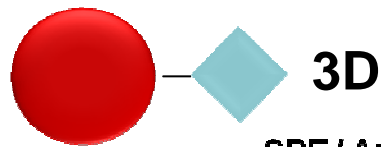


2500 beads/well  
10 or 20  $\mu$ g protein ( $1.25 \cdot 10^6$  beads)  
Serum dilution: 1/50  
Anti-cow biotin: 1/1000  
S-PE: 1/100

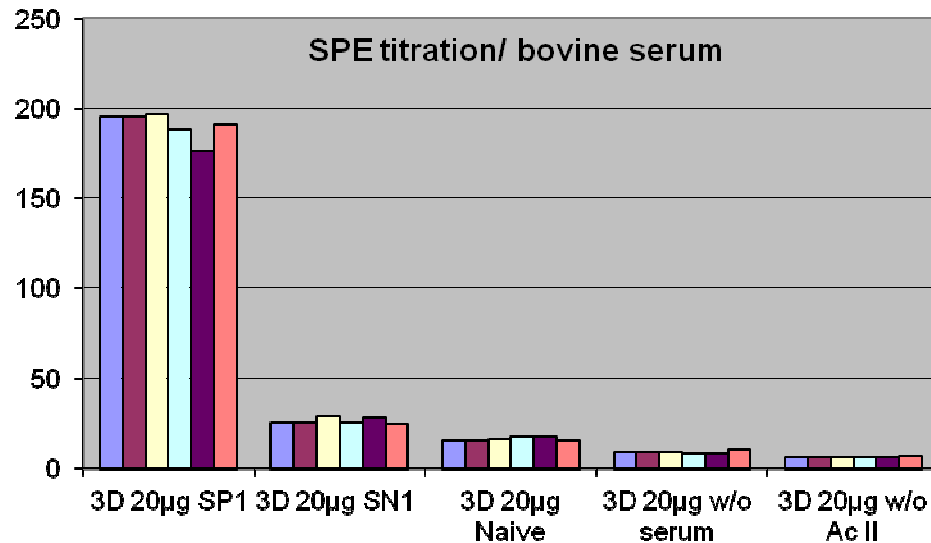


signal-to-noise ratio < 4

# Direct coupling (3D): streptavidine titration

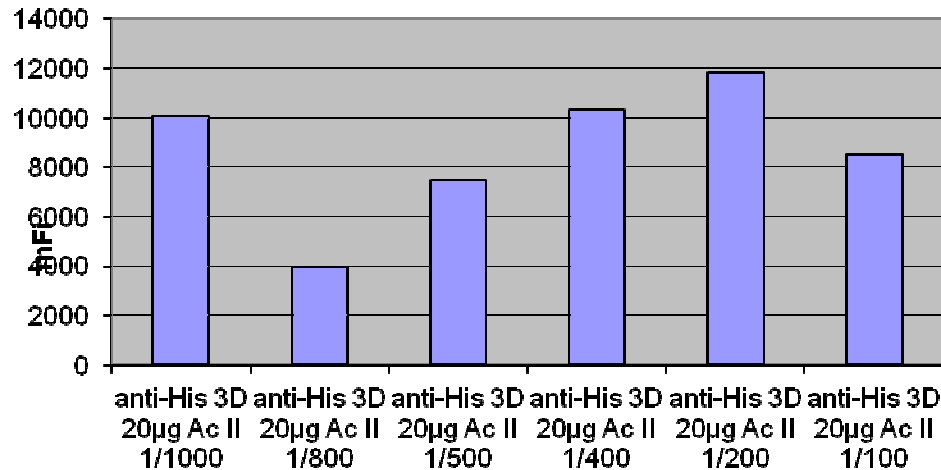
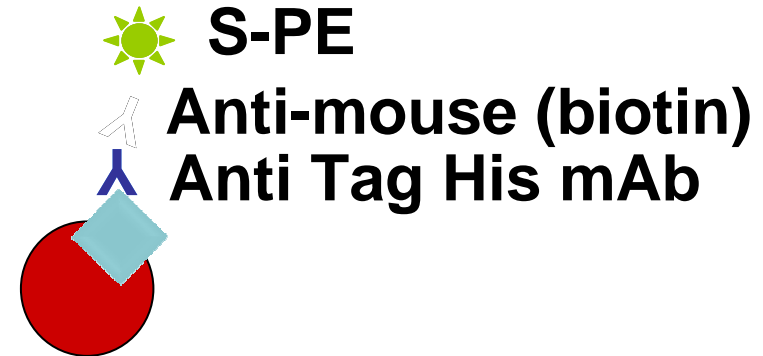
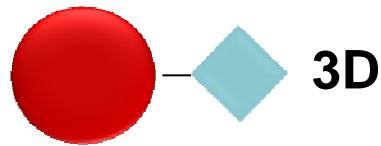


2500 beads/well  
 20  $\mu$ g protein ( $1.25 \cdot 10^6$  beads)  
 Serum dilution: 1/50  
 Anti-His Ab: 1/1000  
 Biotinylated Anti-cow or anti-mouse : 1/1000  
 S-PE: 1/25 to 1/200



**Optimal SPE dilution: 1/100**  
**Signal to noise ratio: 8**

# Direct coupling (3D): Biotinylated Ab titration

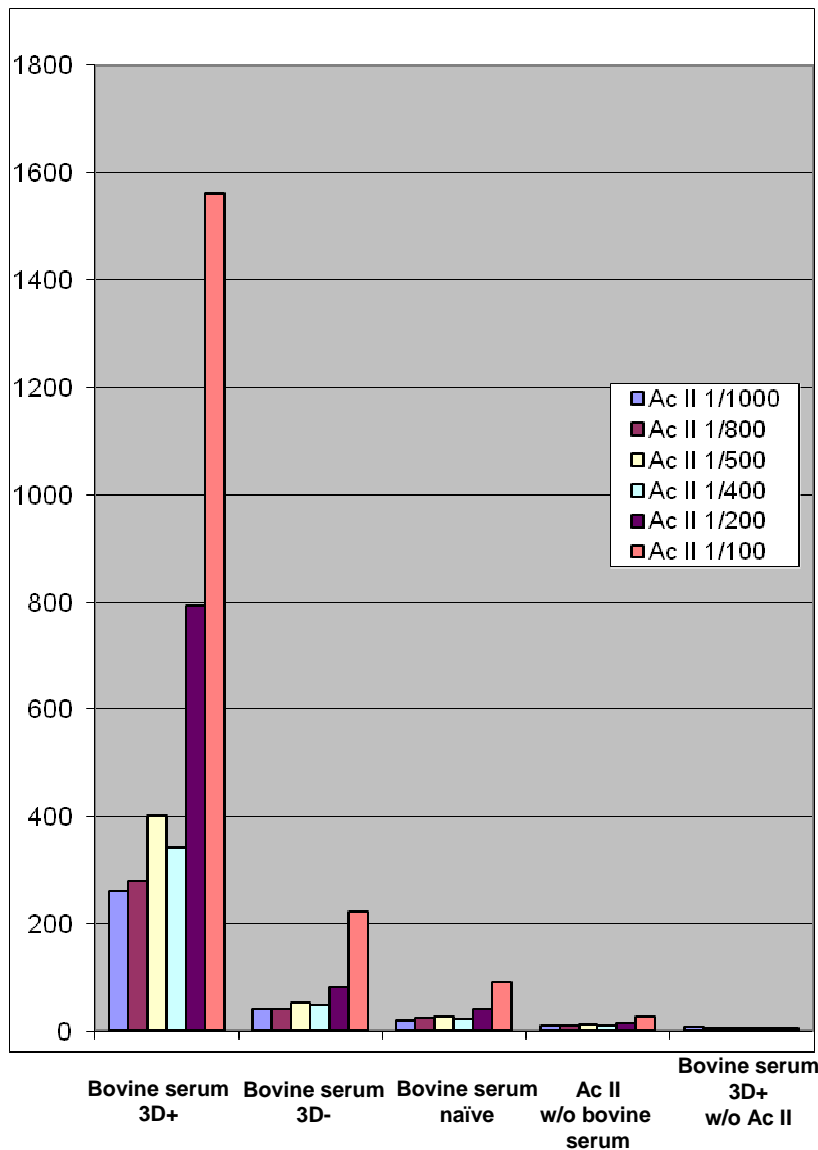


2500 beads/well  
20 µg protein ( $1.25 \cdot 10^6$  beads)  
Serum dilution: 1/50  
Anti-His Ab: 1/1000  
Biotinylated anti-mouse : 1/100 to 1/1000  
S-PE: 1/100

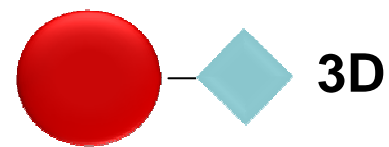
Optimal *Biotinylated anti-mouse* : 1/200  
Signal to noise ratio: 8

# Direct coupling (3D): Biotinylated Ab titration

mfi



2500 beads/well  
 20 µg protein (1.25.10<sup>6</sup> beads)  
 Serum dilution: 1/50  
 Anti-His Ab: 1/1000  
 Biotinylated Anti-cow : 1/100 to 1/1000  
 S-PE: 1/100



**Optimal SPE dilution: 1/100**  
**Anti-cow biotin: 1/200**  
**Signal-noise ratio: 10**

# Conclusions

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- ✓ **Two strategies to develop magnetic serological multiplex assay:**

**Indirect coupling**  
**Direct coupling**



- ✓ **Optimization of several critical parameters (simplex, 3D)**

**Elisa blocking solution**  
**quantity of Anti His Ab coupled**  
**SPE concentration**  
**biotinylated secondary Ab concentration**

# PERSPECTIVES

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- ✓ Producing proteins of interest in *E. coli*
  - FMD SP : VP1 (of all serotypes) *in progress...*
  - FMD NSP: 3ABC, 3A, 3B,3D and peptide 2B *in progress...*
  - VSV SP
  - SVDV SP
  
- ✓ Developing/ optimizing simplex serological immunoassay for each antigen coupled beads set and then developing multiplex assays
  
- ✓ Determining best conditions for storage of coupled beads

# Team « Biology of Picornaviruses »

## « BioPic »



Stéphane  
Zientara



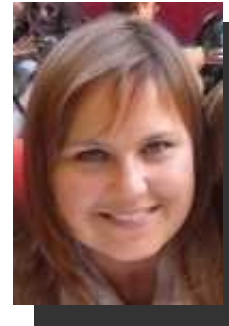
Labib  
Bakkali Kassimi



Sandra  
Blaise Boisseau



Anthony  
Relmy



Kamila  
Gorna



Aurore  
Romey



Margot Carocci



Aude Allemandou



Elisa Dominguez



Annabelle GARNIER



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*Thank you for your attention...*



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