MOLECULAR DIAGNOSTICS: immunetechniques and molecular biology methods in routine laboratory

- Multiplex immunological methods

Timea Berki MD, PhD

Multiplex immunserological methods

1. Microbead based methods: - Flow cytometry
   - Becton Dickinson
   - Bender MedSystem
   - Luminex platform

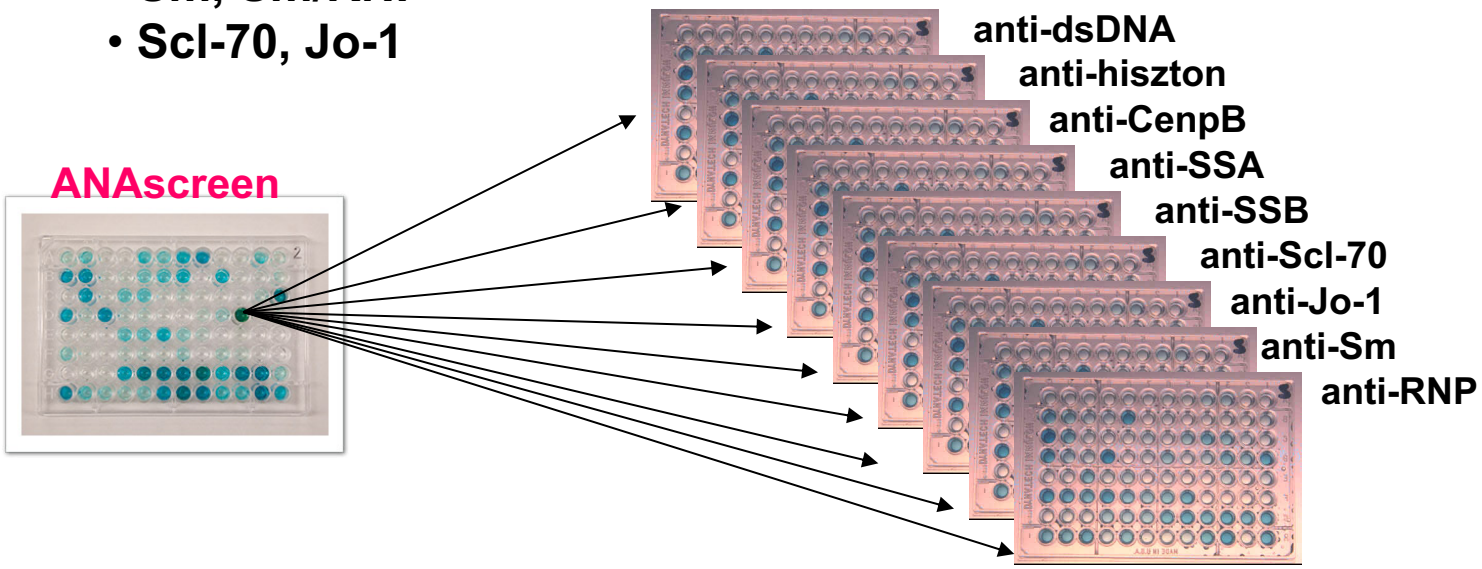
2. Protein chip methods: „microarray”: - chip reader
   - glass
   - plastic
   - silica
   - nano-well

ANAcreen on microplate ELISA

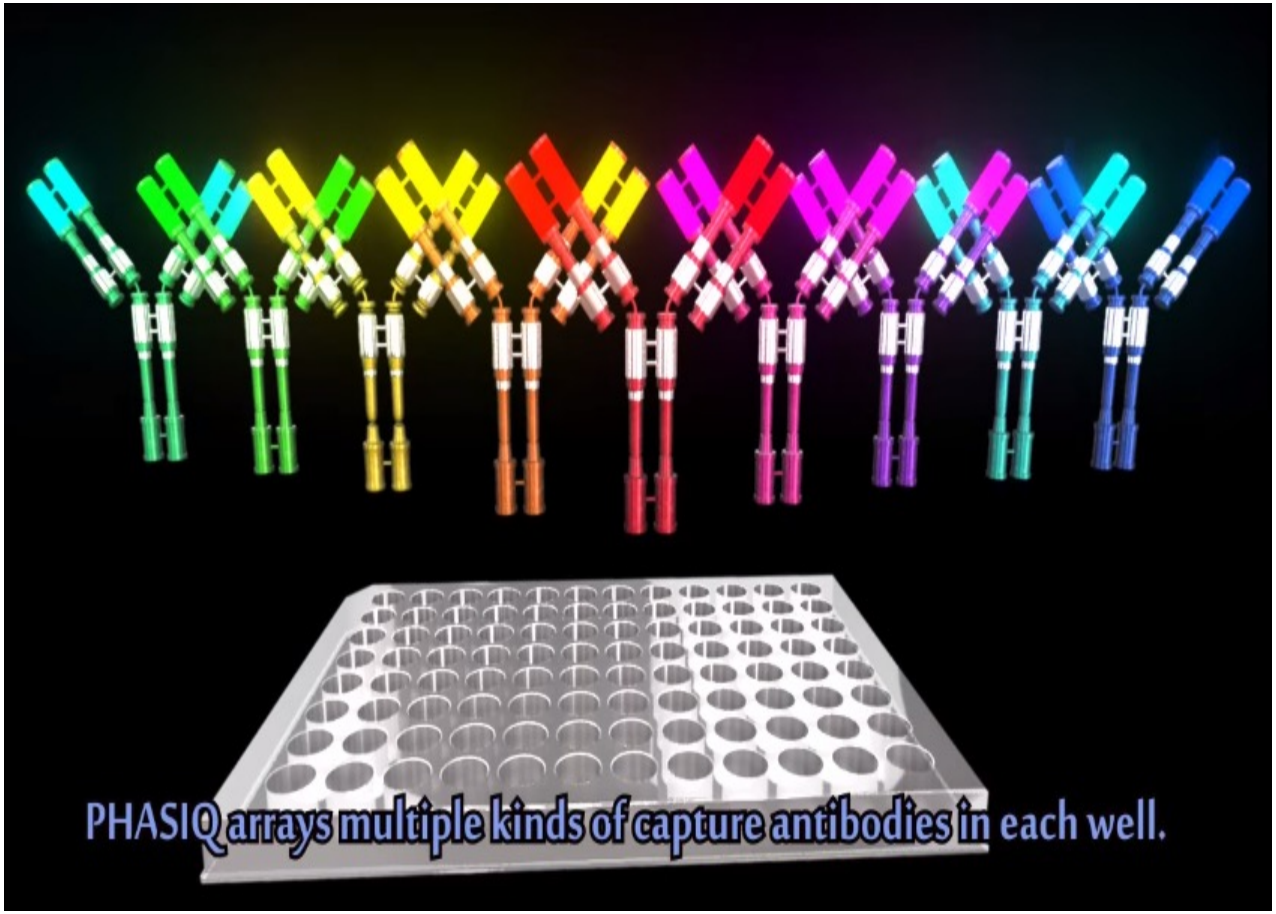
Az ELISA lemezt Hep2 sejtek magjából kivont antigének keverékével érzékenyítik:

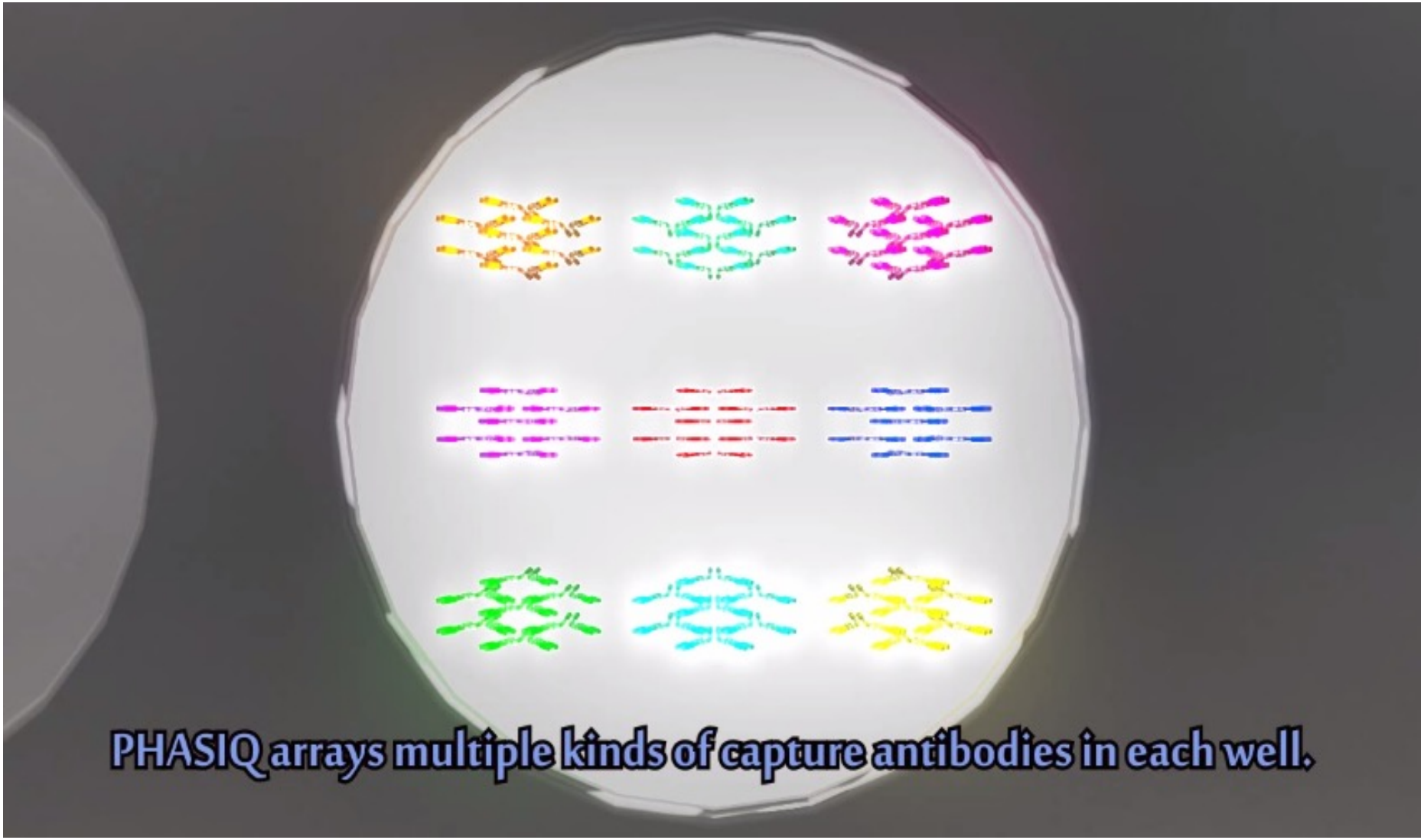
- **dsDNA**
- **hiszton**
- **centromer**
- **SSA/Ro, SSB/La**
- **Sm, Sm/RNP**
- **Scl-70, Jo-1**

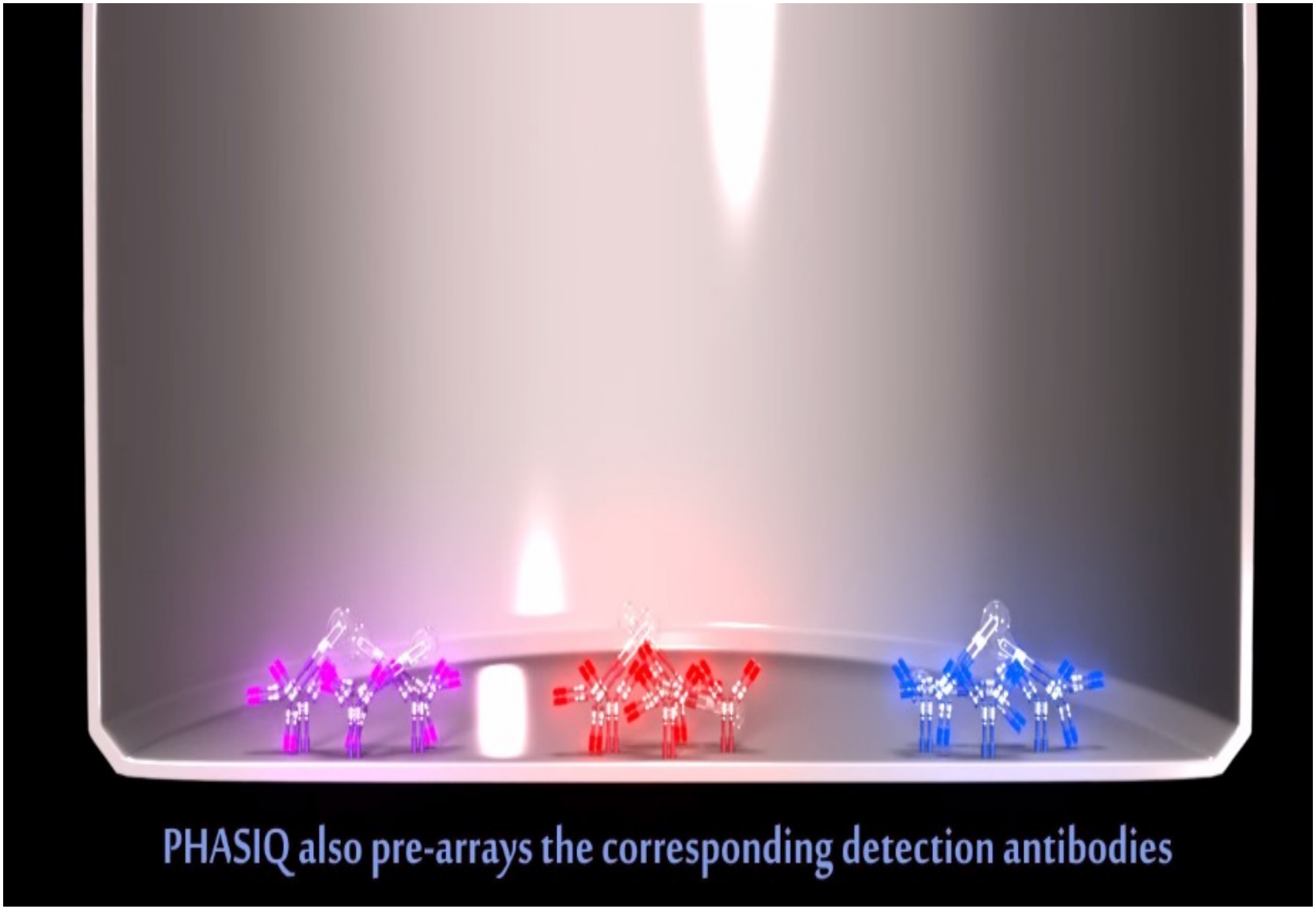
Pozitivitás esetén egy-egy antigénnel fedett külön ELISA lemezeken un. „kifejtő” vizsgálatok következnek

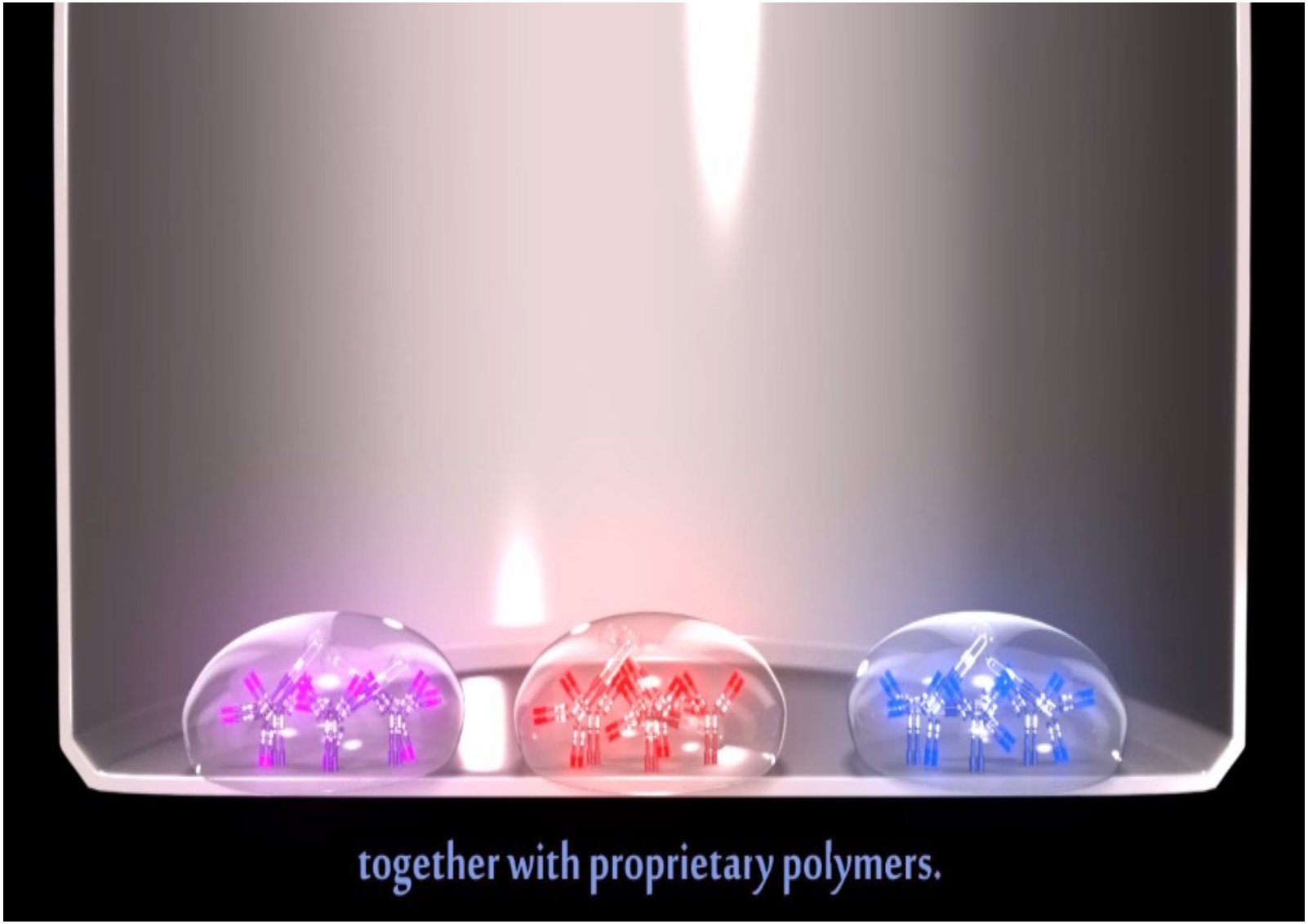


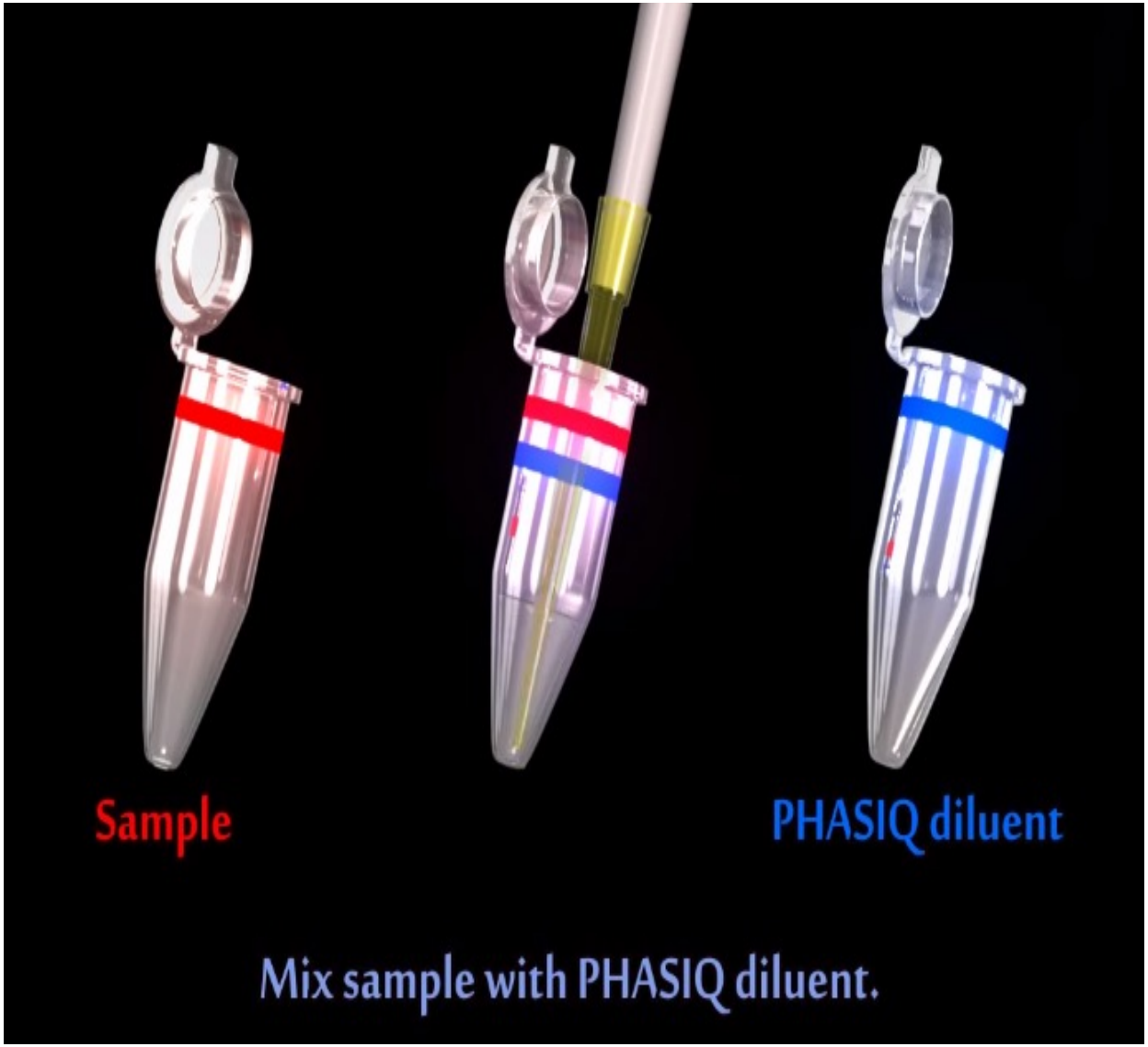
Multiplex sandwich ELISA

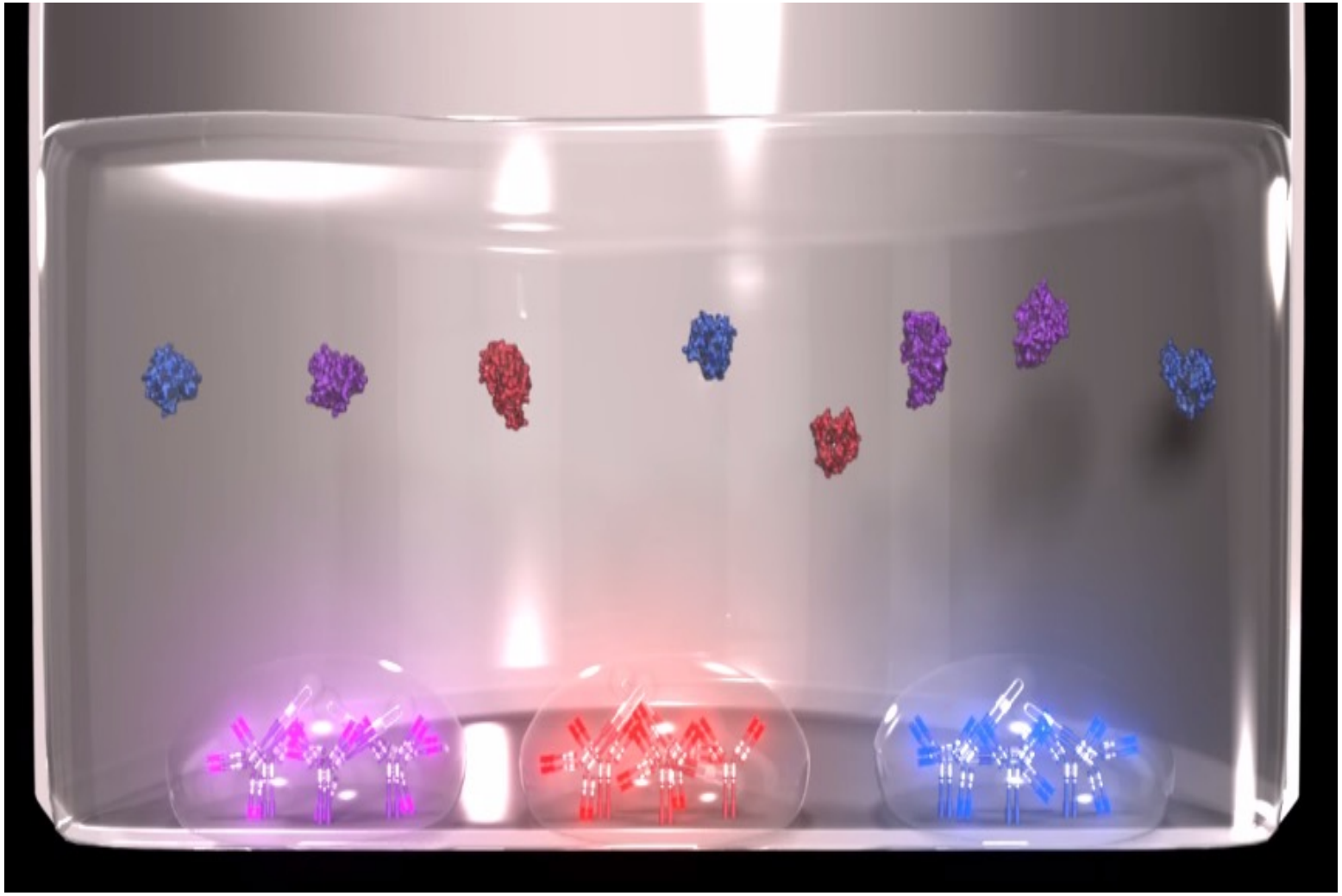


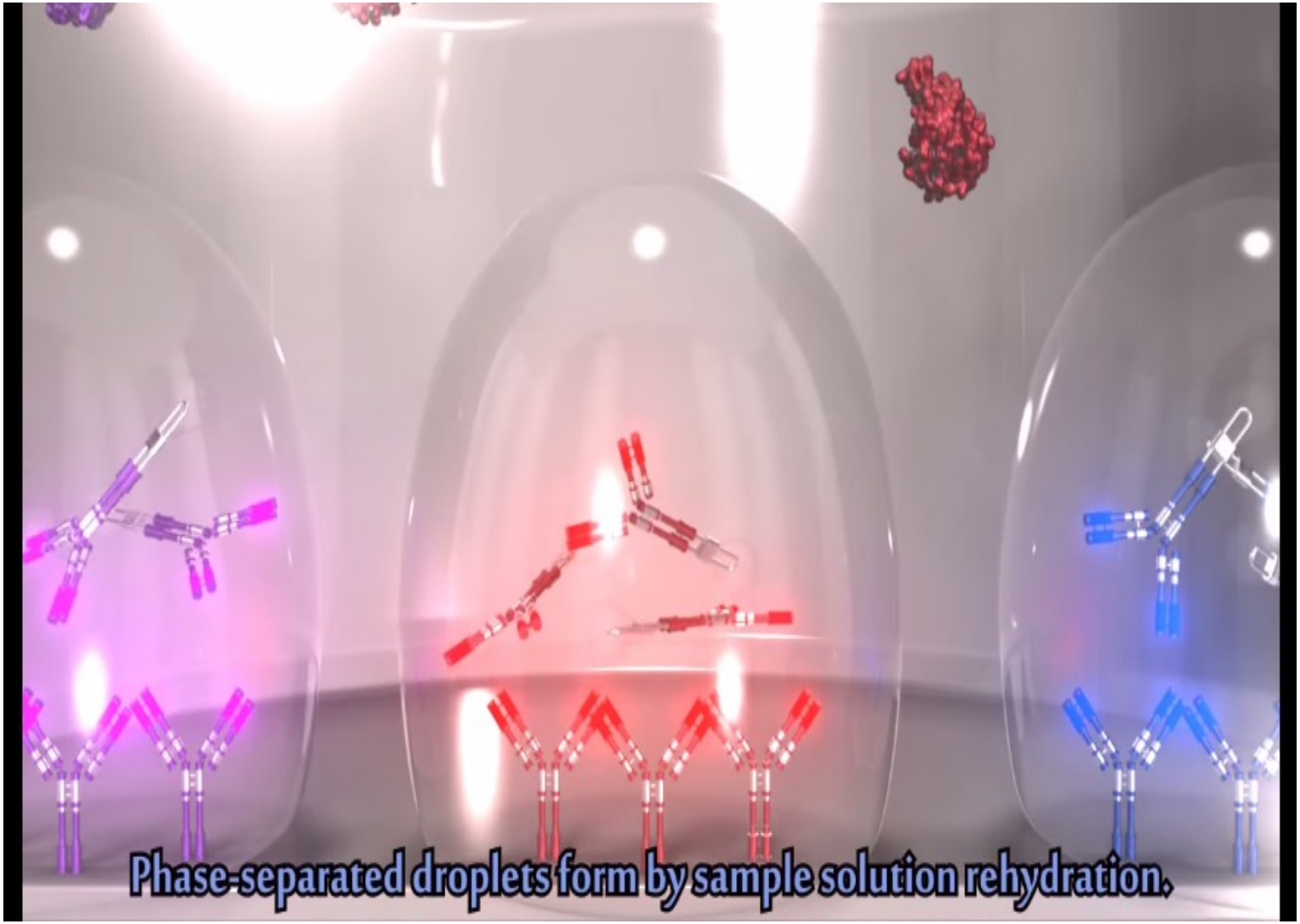

PHASIQ arrays multiple kinds of capture antibodies in each well.

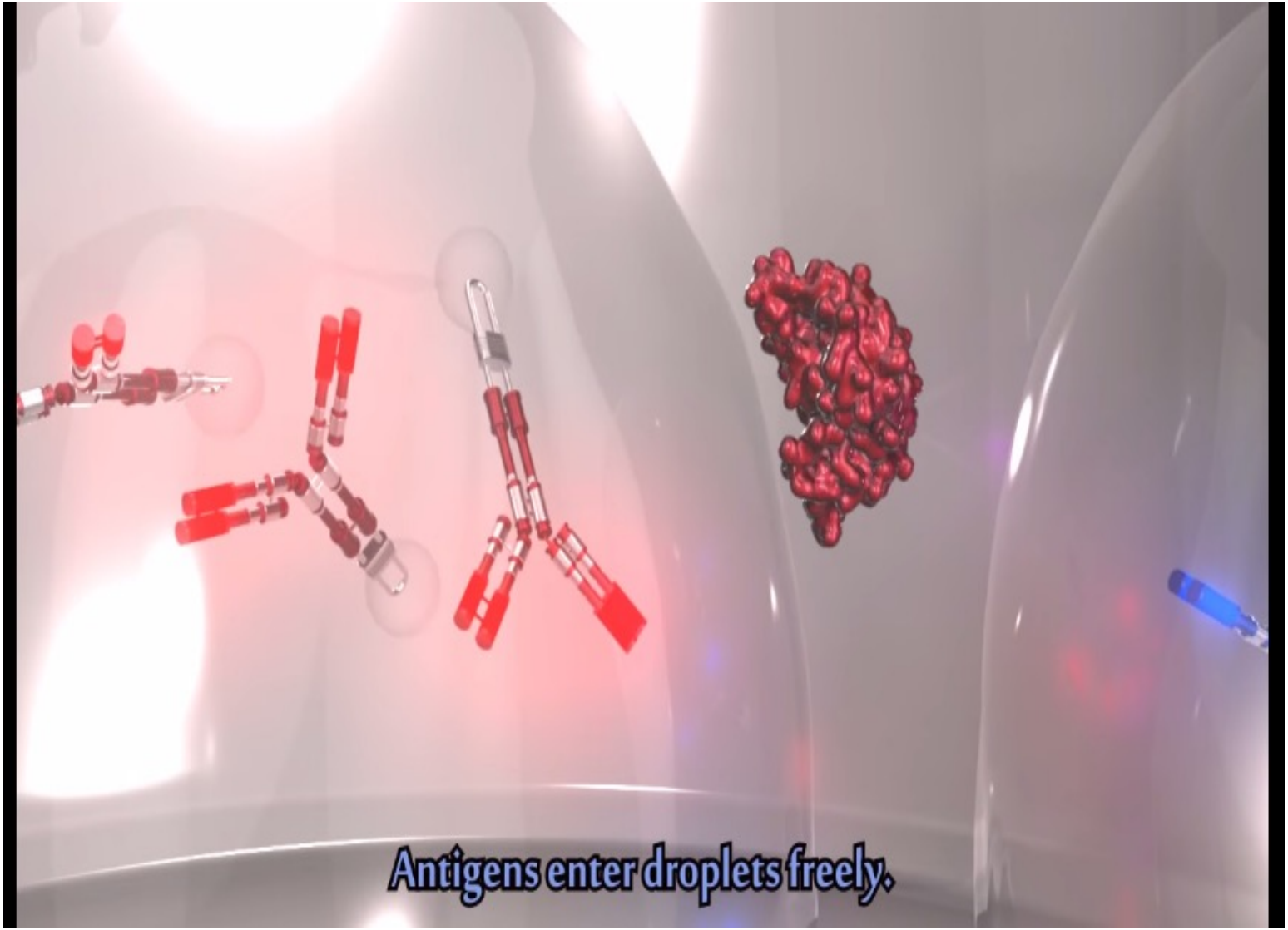

PHASIQ also pre-arrays the corresponding detection antibodies

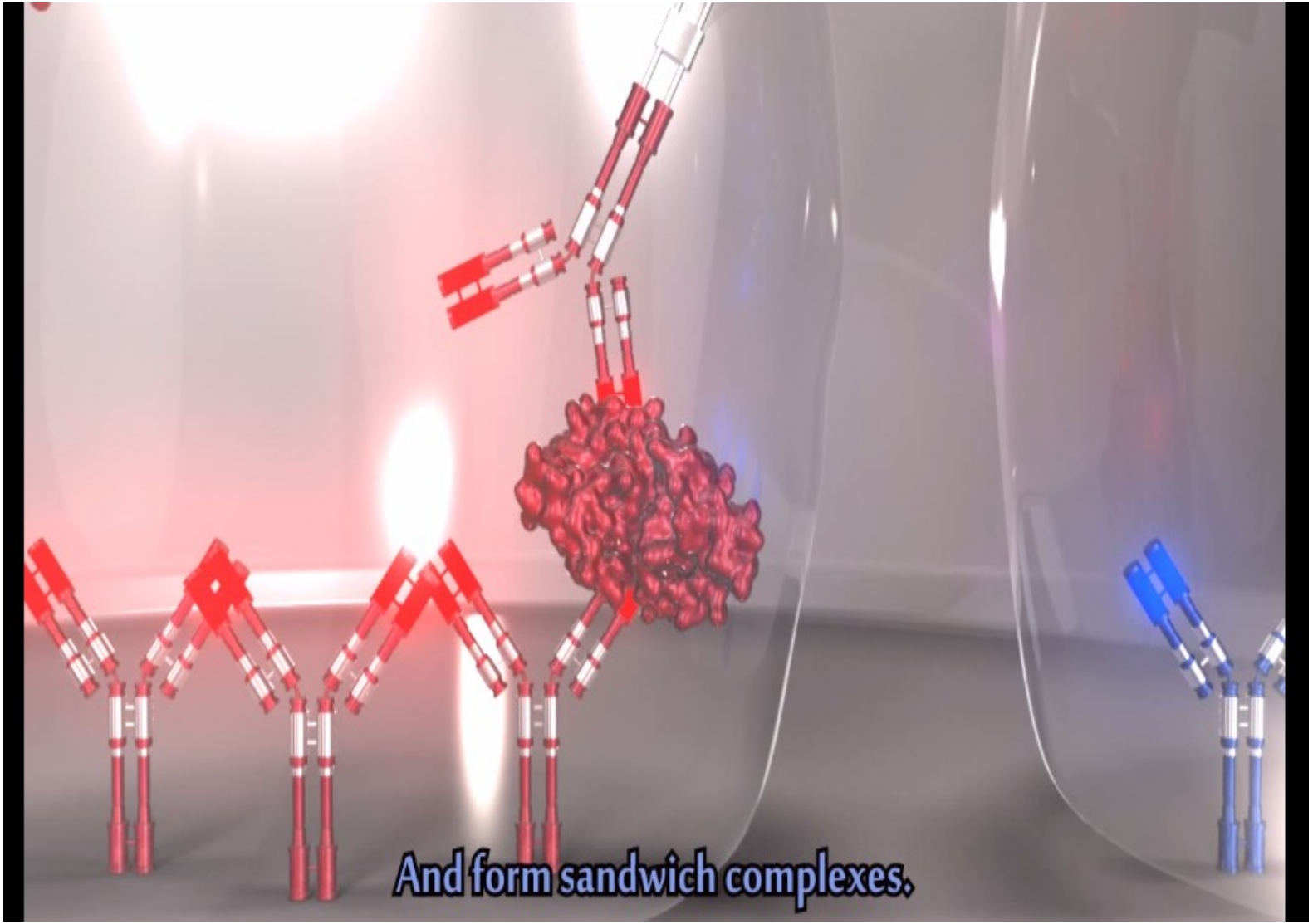

together with proprietary polymers.

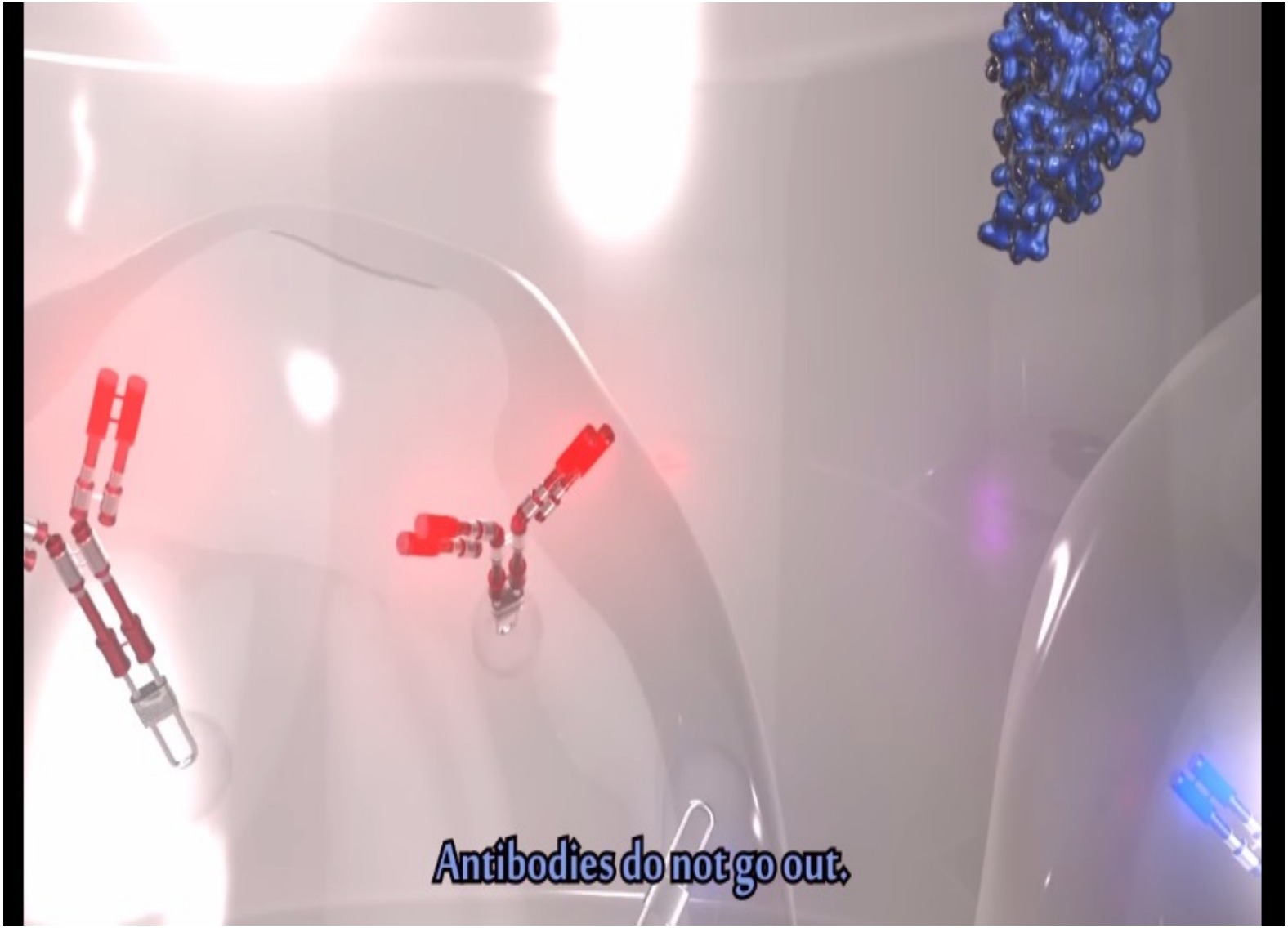

Sample
PHASIQ diluent
Mix sample with PHASIQ diluent.

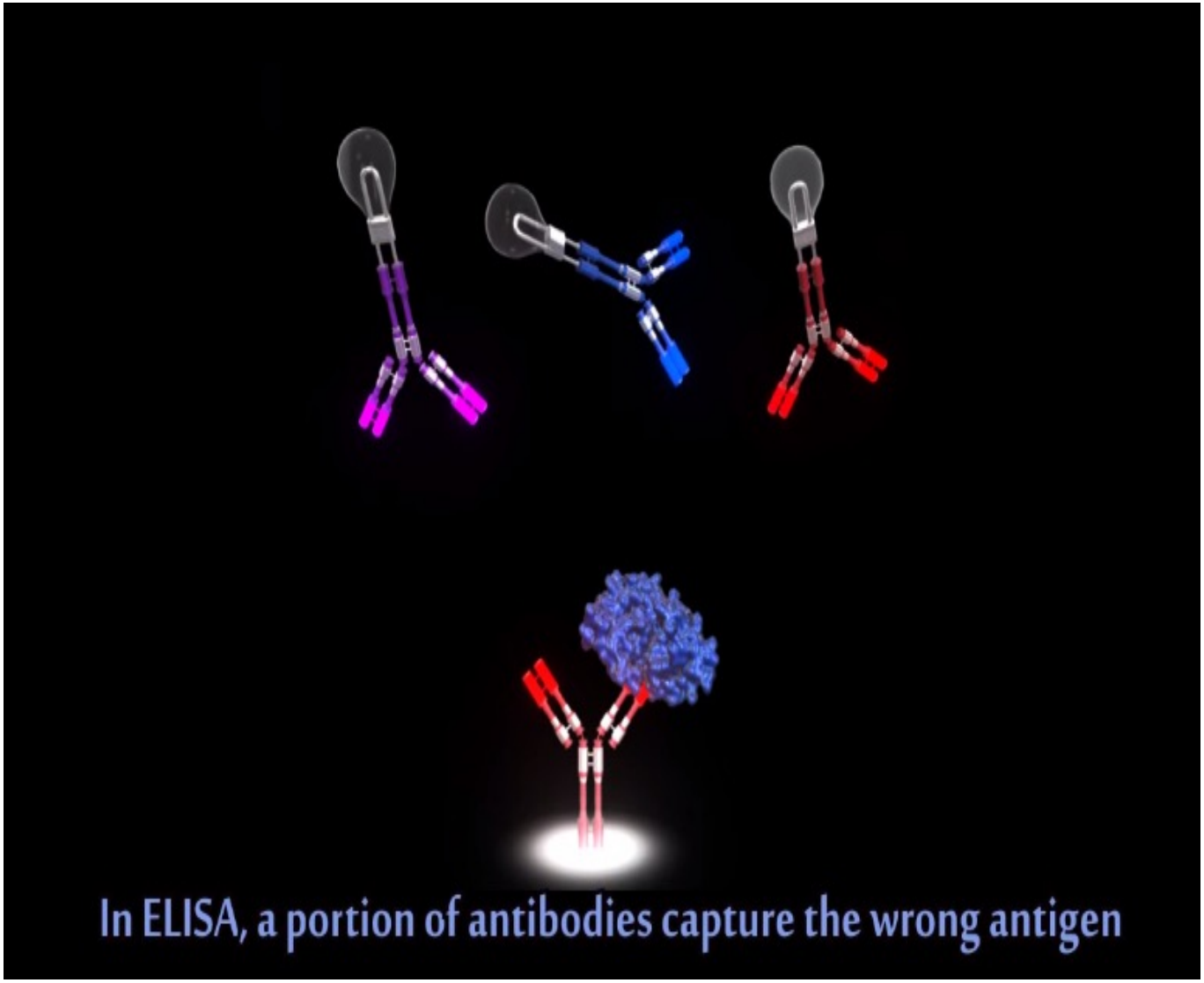


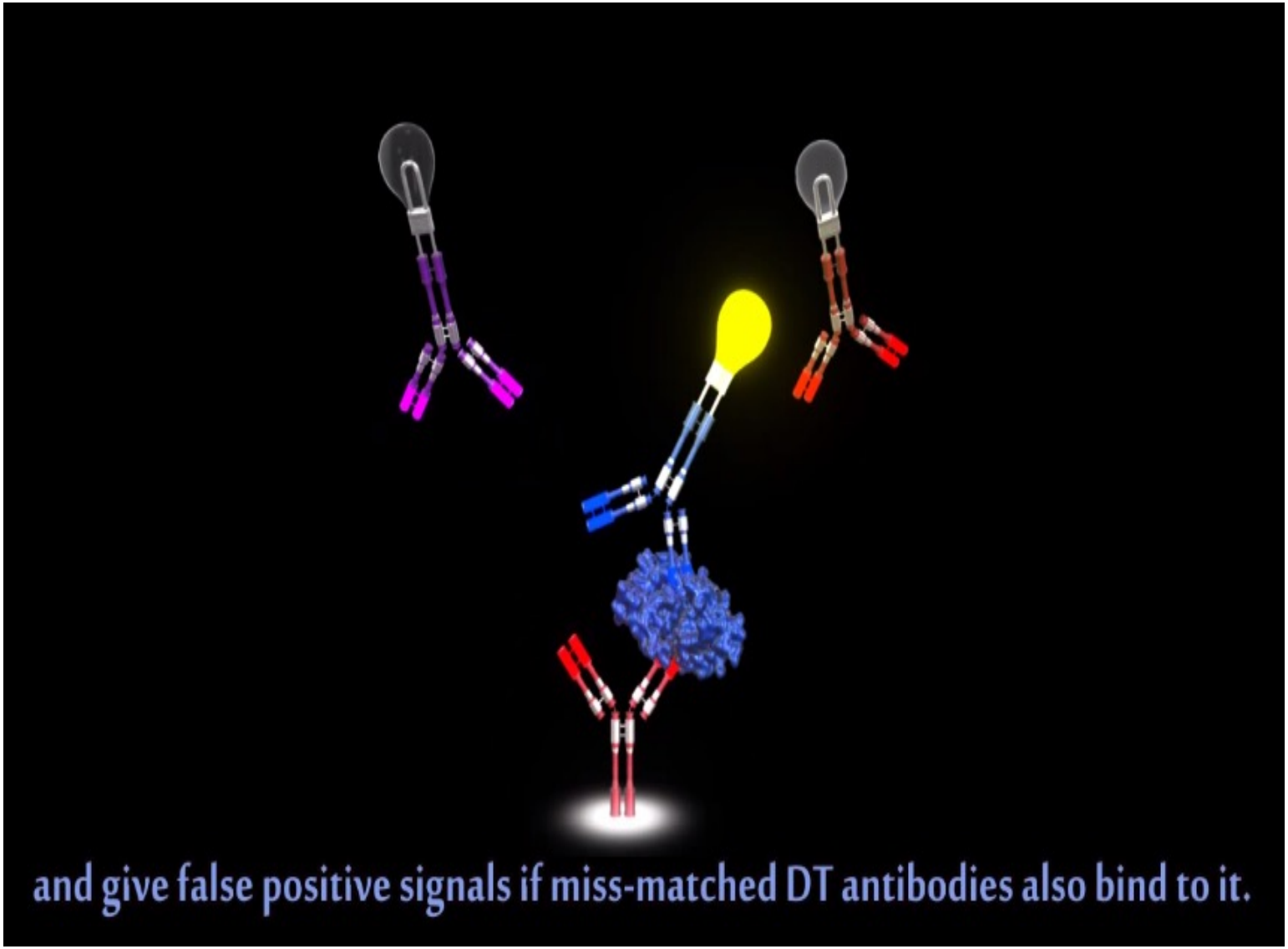

Phase-separated droplets form by sample solution rehydration.

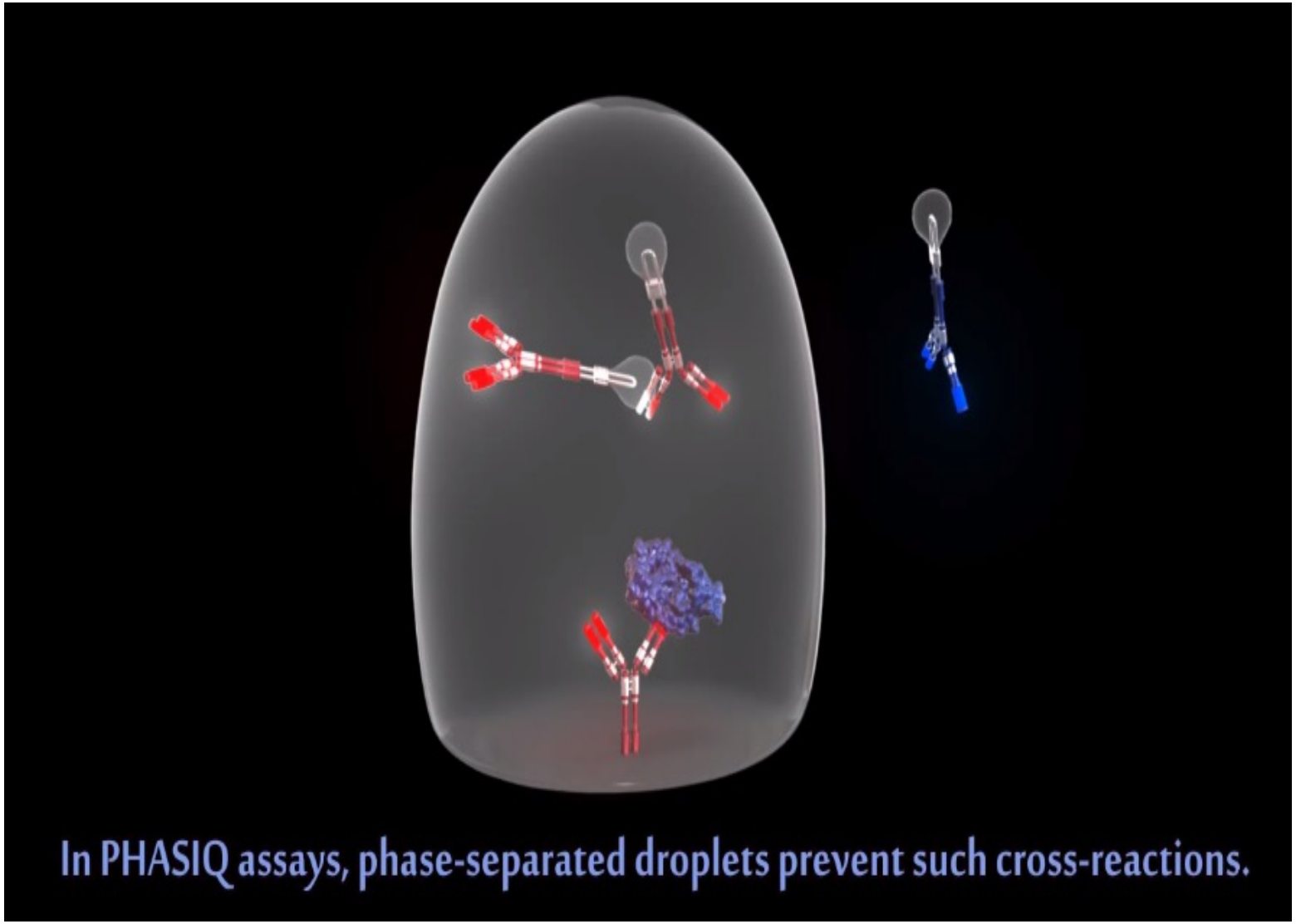

Antigens enter droplets freely.

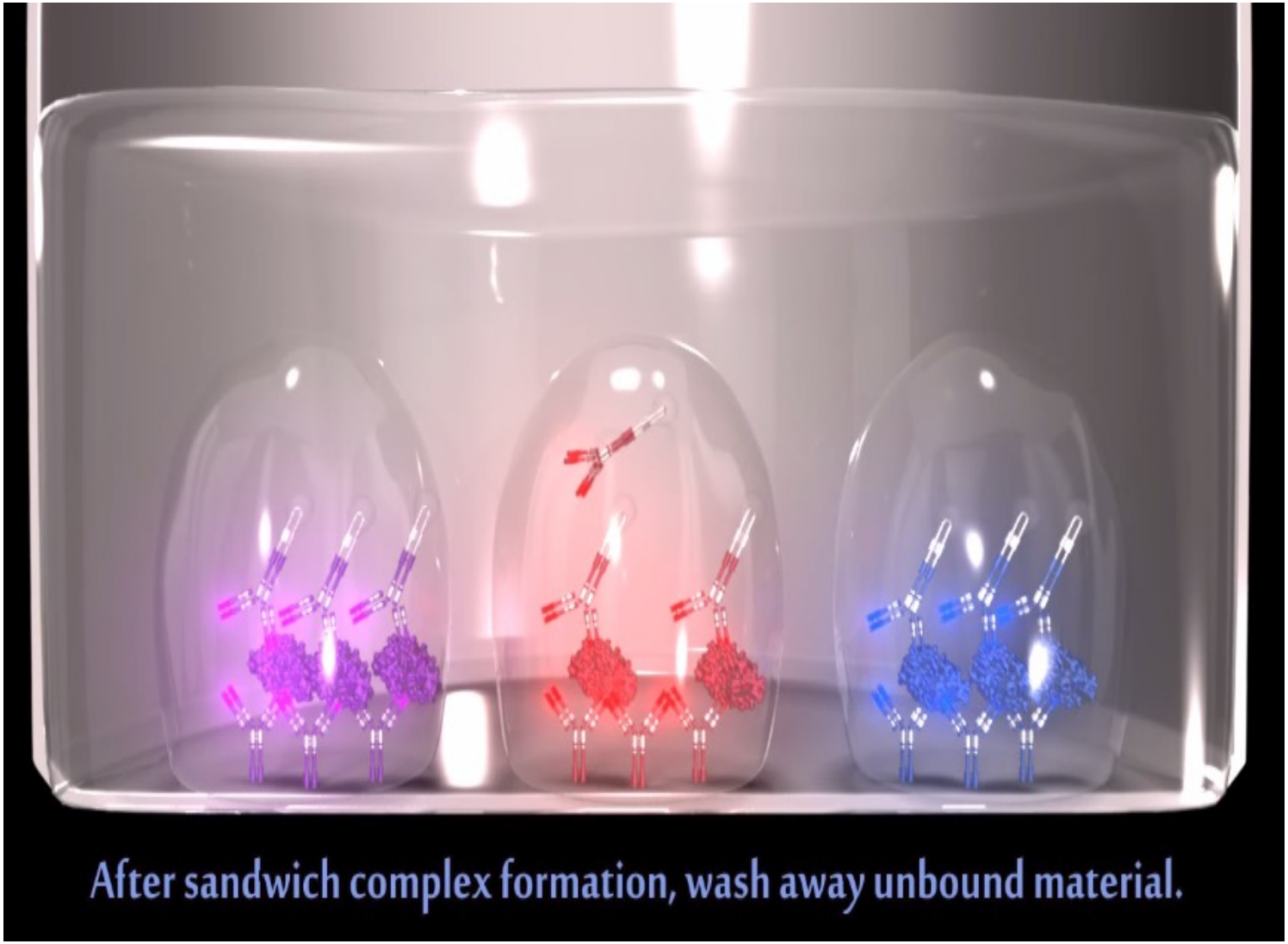

And form sandwich complexes.

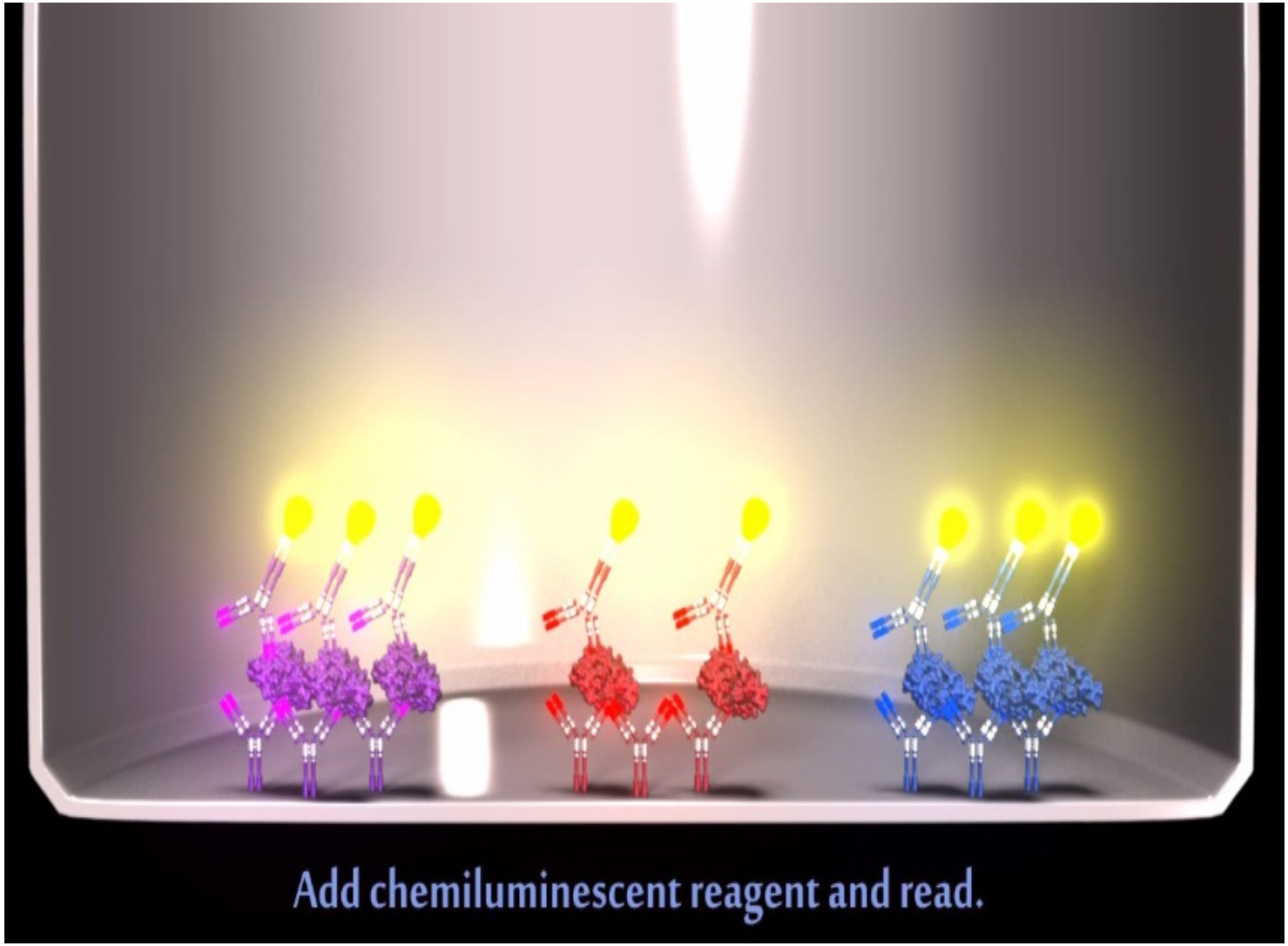

Antibodies do not go out.

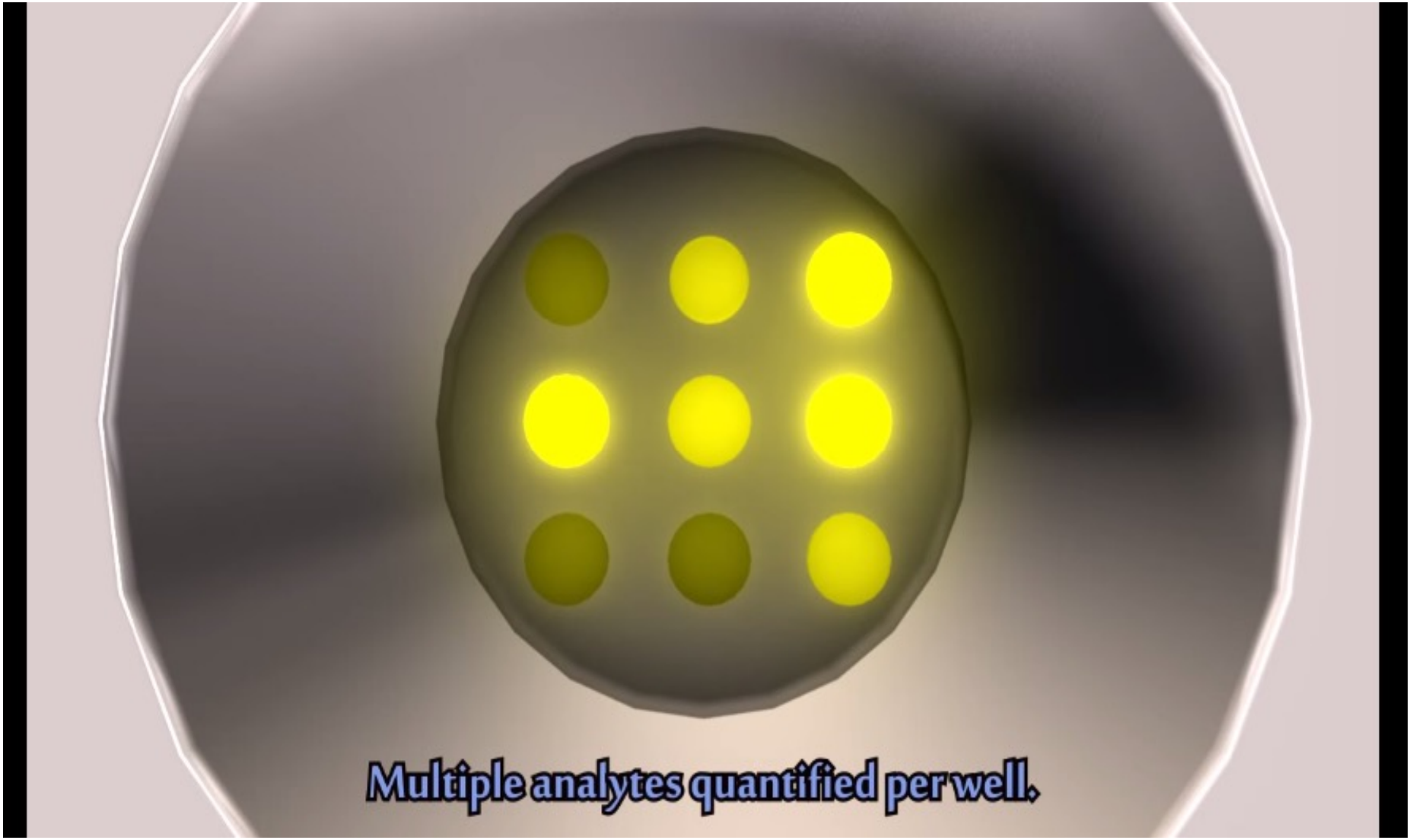

In ELISA, a portion of antibodies capture the wrong antigen


and give false positive signals if miss-matched DT antibodies also bind to it.


In PHASIQ assays, phase-separated droplets prevent such cross-reactions.

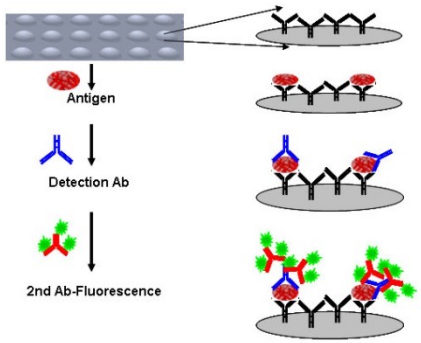

After sandwich complex formation, wash away unbound material.


Add chemiluminescent reagent and read.


Multiple analytes quantified per well.

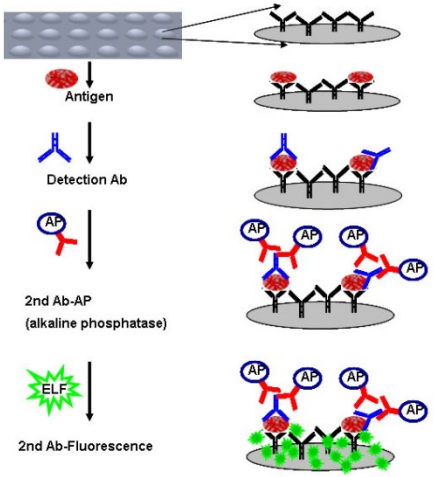
Antibody microarray

Fluorescence-linked Imminosorbent Assay

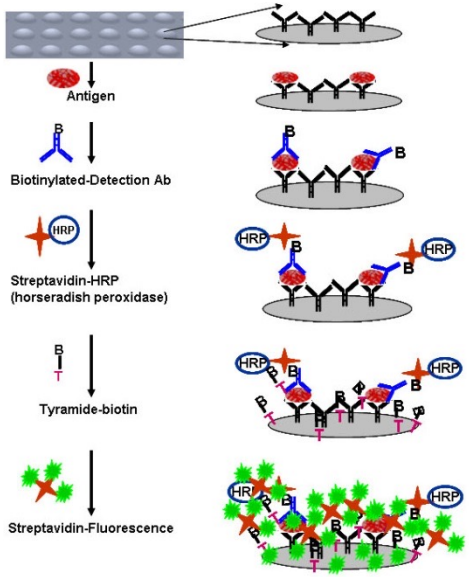


A few ways to create and detect antibody microarrays

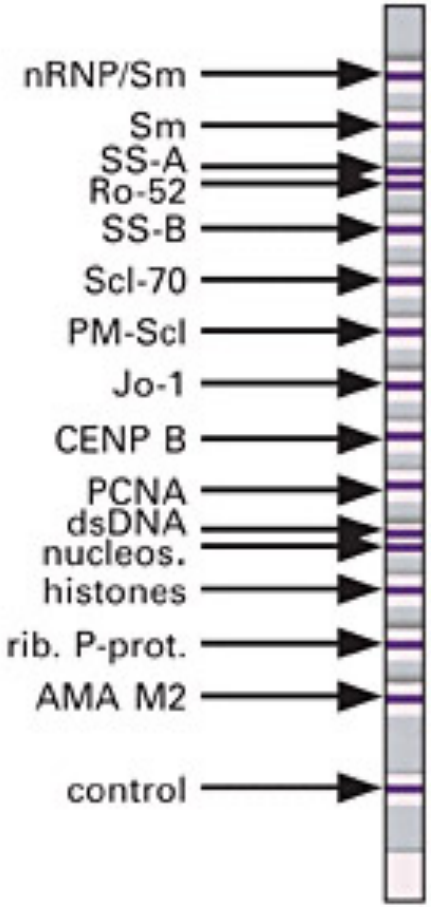
Enzyme-linked Immunosorbent Assay (ELISA)
Using insoluable fluorescence dye



Enzyme-linked Immunosorbent Assay (ELISA)
Using Tyramide Signal Amplification (TSA)
technology



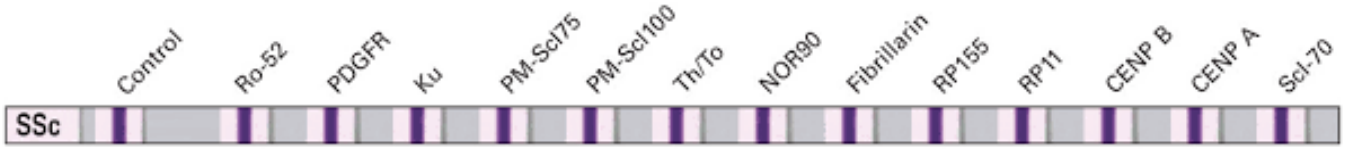
Multiplex ANA-Profile Immunoblot



its advantage: - 1 sample 1 run
- fast

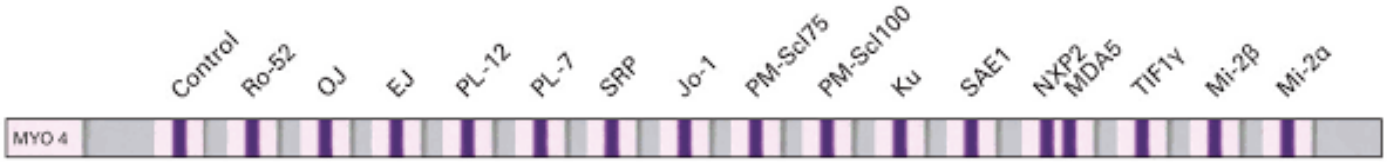
disadvantage:
- antigen is denatured, the conformation is different than that in ELISA
- false-negative reactions
- some antigens cannot be investigated

Systemic sclerosis profile IgG – EUROLINE



- **Scl-70** – DNA-topoisomerase I – purified protein, 65% sensitivity, specific for dcSSc
- **CENP A és CENP B** - centromer A an B recombinant protein. 11-13 % sensitivity 80-95% specificity in lcSSc.
- **RNS polimeráz III:** RP11 and RP155 recombinant proteins in dcSSc 5-22 %
- **Fibrillarin -U3-RNP**. In dcSSc with internal organ involvement (pulmonary hypertonia, myositis and kidney).
- **NOR90** - nucleolus-organising region 90kDA <5% + in SSc.
- **Th/To** - *7-2-RNP/7-2-RNA protein complex*. >5% + in SSc
- **PM-Scl komplex** PM-Scl100 és PM-Scl75: 10-12% + in SSc indicates pulmonary and joint involvement.
- **KU**: <5% when overlapping polymyositis exists with primer pulmonaris hypertonia.
- **PDGFR** – platelet derived growth factor receptor rekombinant.
- **Ro-52** - 52 KDa-os component of Ro rekombináns. Nem SSc specifikus, más autoimmun betegségekben pl. Sjögren, SLE, MCTD AIHepatitisben és infekciókban is pozitív lehet

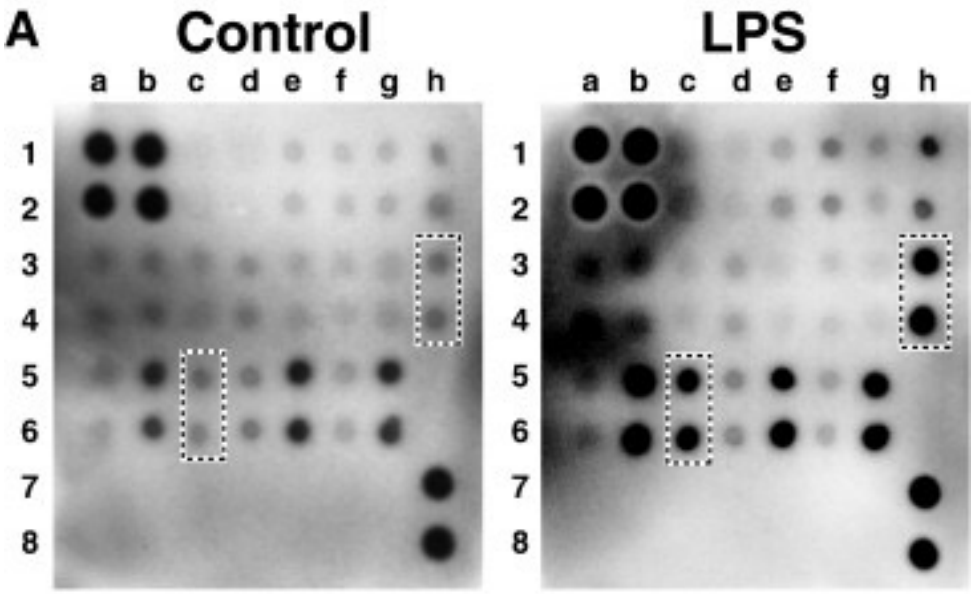
Autoimmune myositis diagnostics - EUROLINE



- Dermato- és polymyositis
- Idiopathic myositis
- Anti-synthase syndrome
- Inclusion body myositis
- Overlap syndrome
- Some can associate with lung, breast or colon cc

Multiplex citokine protein array

A



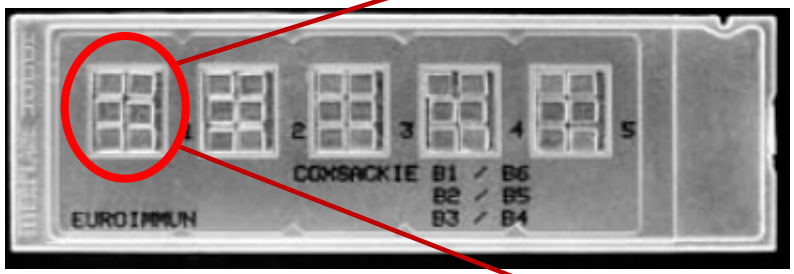
B

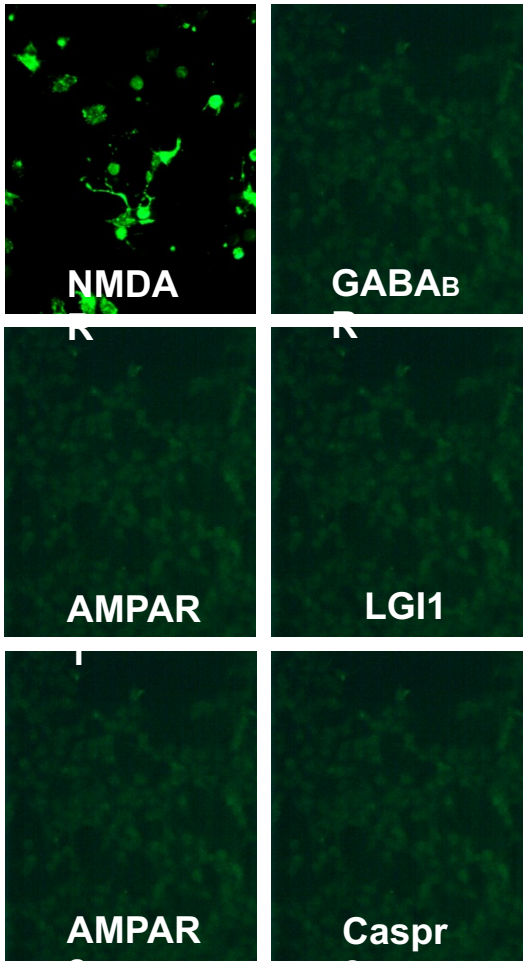
	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fraktalkine
2	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fraktalkine
3	CM-CSF	INF-γ	IL-1α	IL-1β	IL-4	IL-6	IL-10	LIX
4	CM-CSF	INF-γ	IL-1α	IL-1β	IL-4	IL-6	IL-10	LIX
5	Leptin	MCP-1	MIP-3α	B-NGF	TIMP-1	TNF-α	VEGF	Blank
6	Leptin	MCP-1	MIP-3α	B-NGF	TIMP-1	TNF-α	VEGF	Blank
7	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Pos
8	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Pos

MIP-3 – CCL20
LIX- CXCL-5

Diagnostics of autoimmune encephalitis:
Cell-base indirect immunfluorescent BIOCHIP assy

1. Incubation of petients sera (1:10x) ior liquor (1:1) on a biochip surface
2. anti-human IgG-FITC secondary antibody
3. Microscopic evaluation

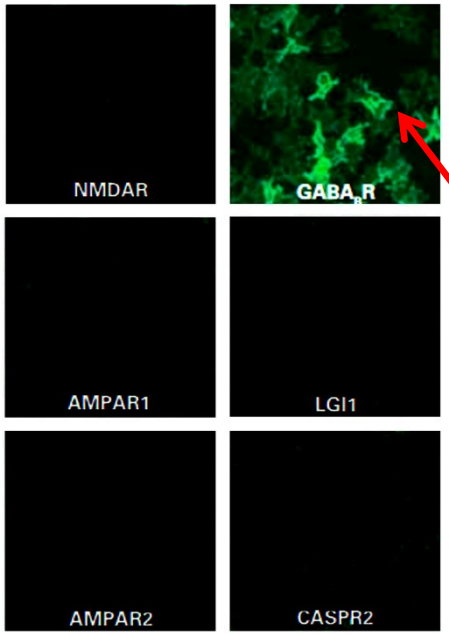


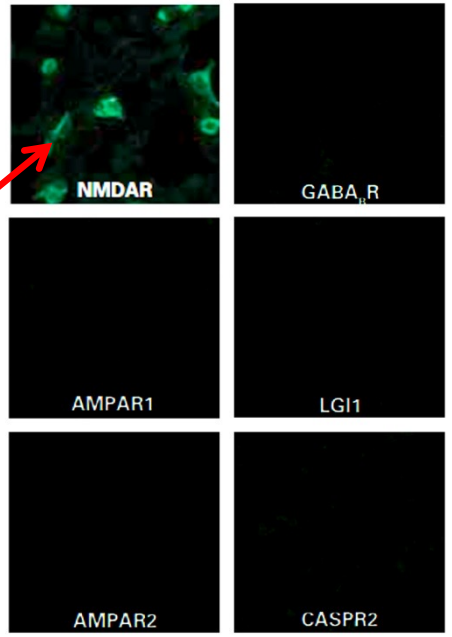


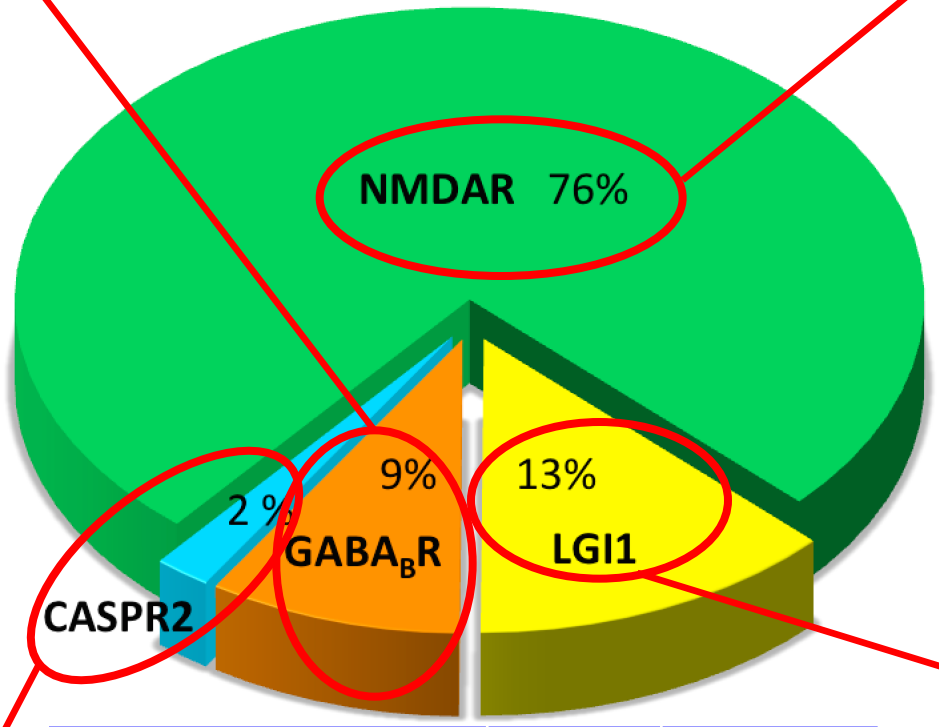
On the biochip on each place HEK293 cells are immobilized each transfected with the gene of e different CNS receptor

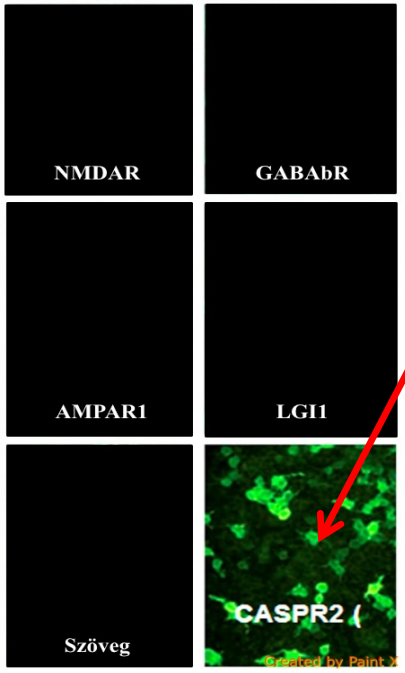
Csépány T, Illés Zs. Klinikai neuroimmunológia. Mátyus-Benten Kiadó, Debrecen-

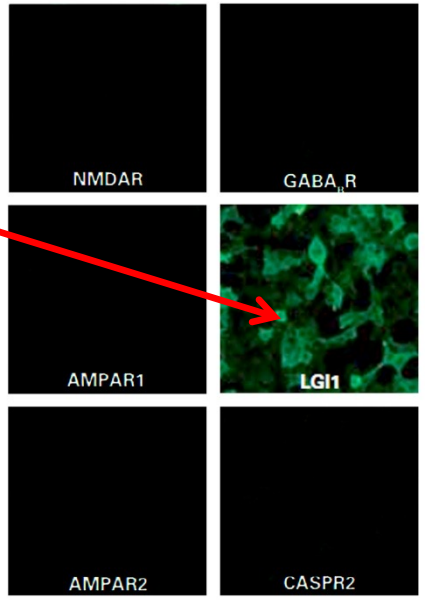
AIE Biochip









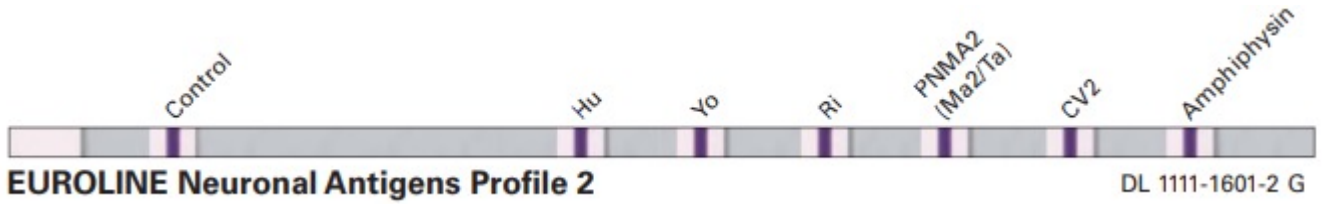


Total demands (2011.06.30.-2017.08.08.)	836	
Petiens number	717	100%
Positivity (n=)	54	7,53%

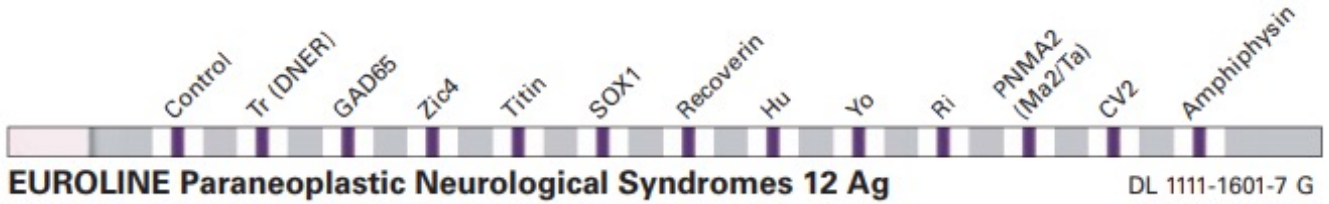
A többi receptor ellen nem találtunk pozitív mintát.

Line-immunoblot for detection of onconeural antibodies

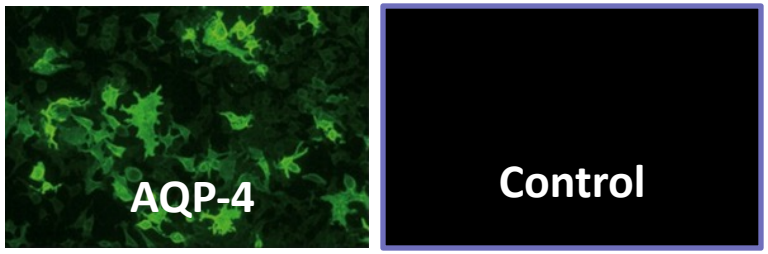
6 antigén
→



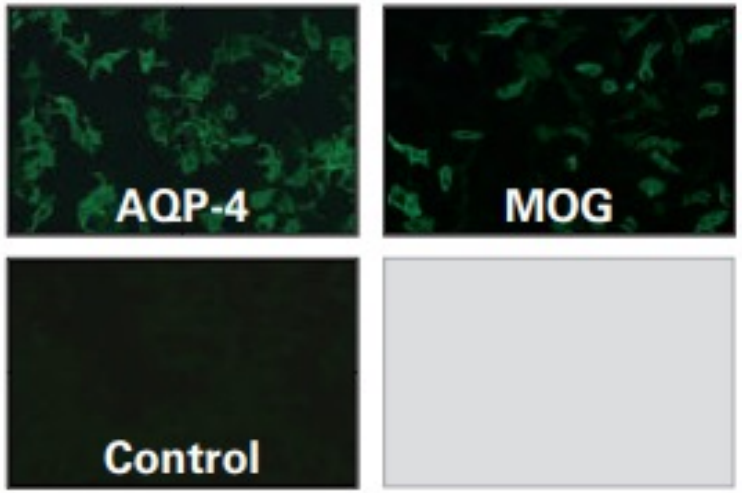
12 antigén
→



Neuromyelitis optica spectrum disease (NMOSD)



ANTI-AQP4 MOSAIC IIF TEST



NMOSD SCREEN 1

CombiChip Autoimmune 1.0

FOR DIAGNOSIS OF THE AUTOIMMUNE DISEASES VASCULITIS & COLLAGENOSIS

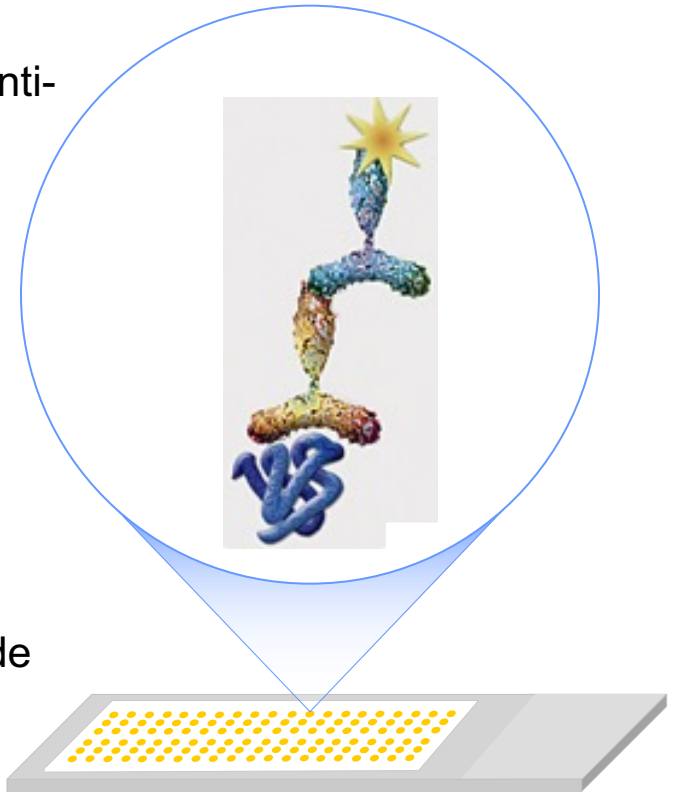
- Simultaneous quantitative analysis of 14 autoantibodies
- Easy as an ELISA test
- Up to 64 samples in one working cycle

Antigen Array

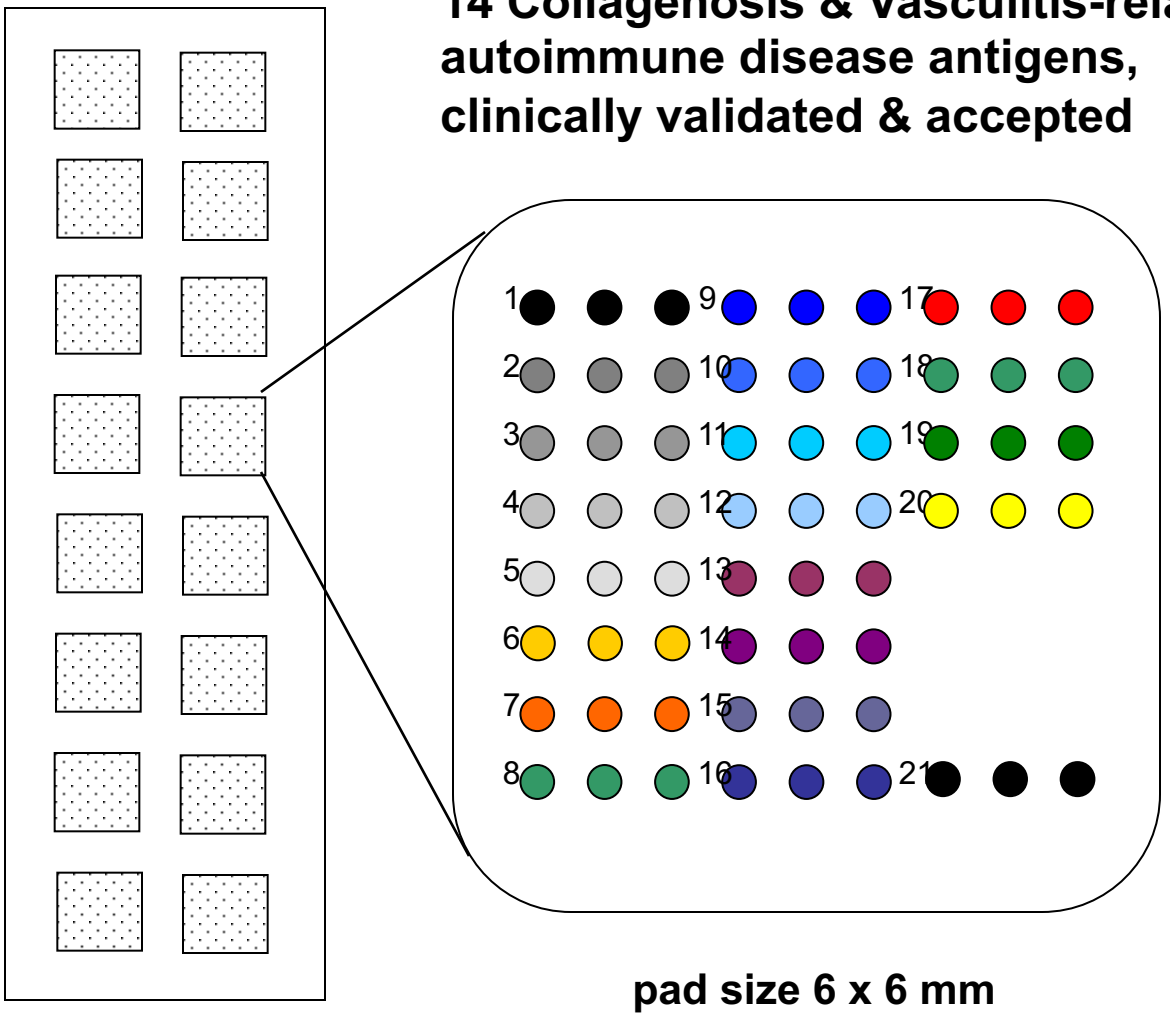
Detection
fluorophor-labelled anti-IgG antibody binds

Serum-Incubation
auto-antibody binds to antigen

Printing
antigen on FAST Slide



CombiChip Autoimmune 1.0 Layout



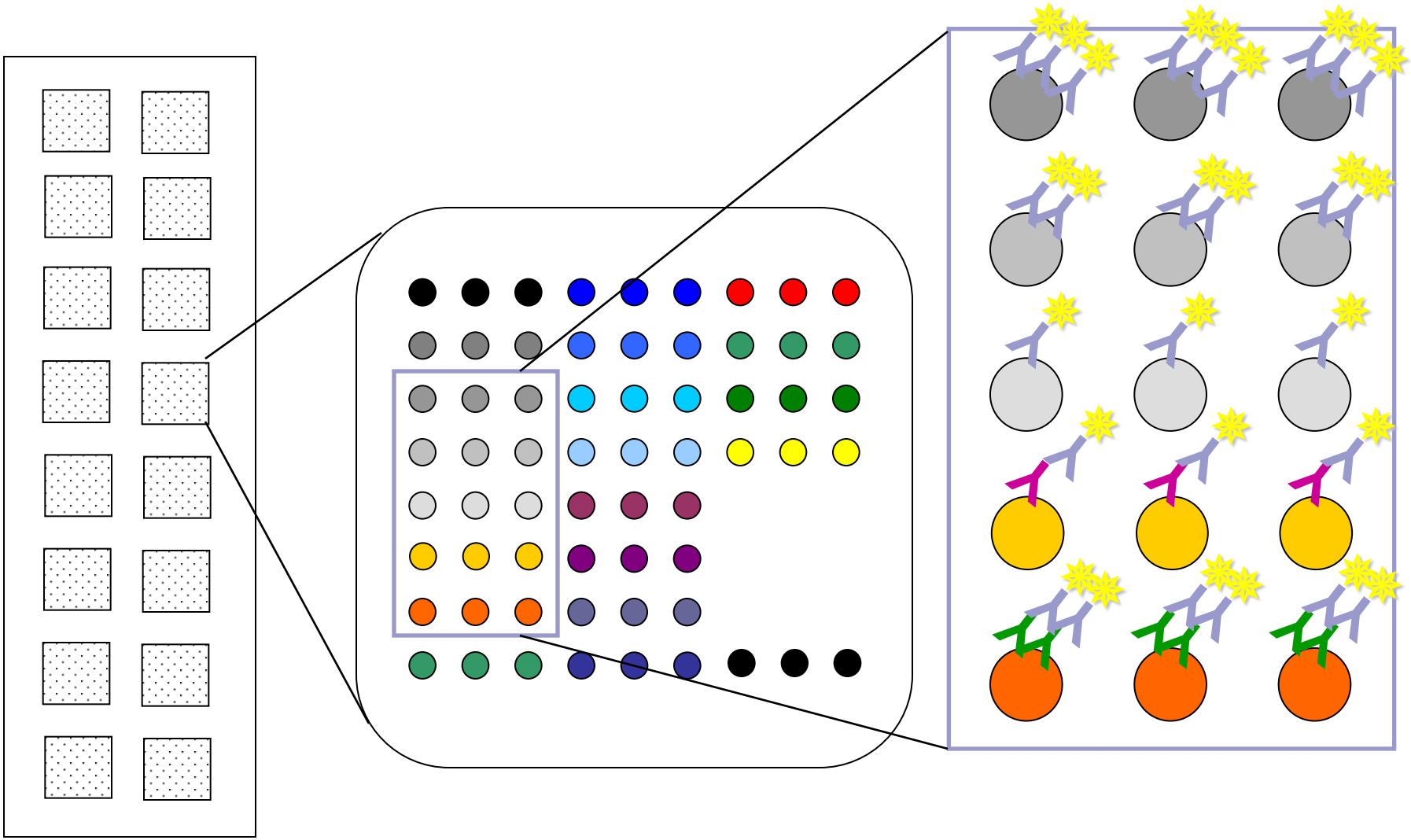
14 Collagenosis & Vasculitis-related autoimmune disease antigens, clinically validated & accepted

pad size 6 x 6 mm

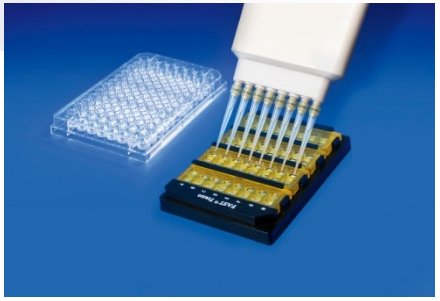
1-5 = calibrator*
6 = CENP-B
7 = U1-70k
8 = Sm
9 = SSA/Ro52
10 = SSA/Ro60
11 = SSB/La
12 = Mi-2
13 = PM/Scl-75
14 = PM/Scl-100
15 = Jo-1
16 = Scl-70
17 = Pr3
18 = MPO
19 = dsDNA
20 = buffer
21 = “Landing Lights”
(= positive assay control)

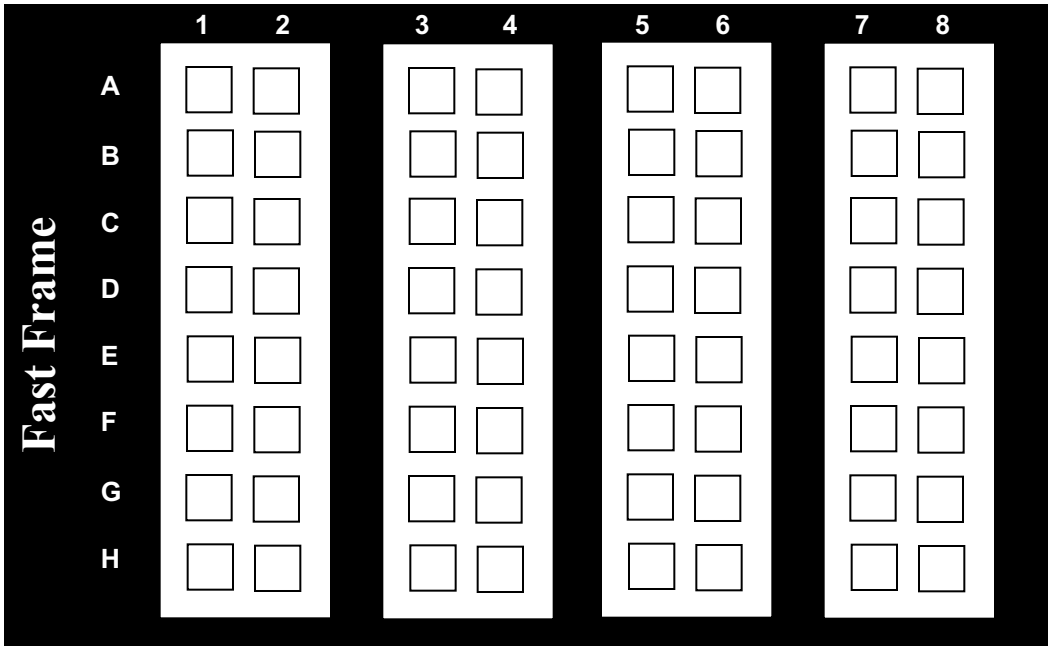
* patent pending

CombiChip Autoimmune Assay



Microplate-like Liquid Handling





Process up to 4 slides with 64 identical arrays

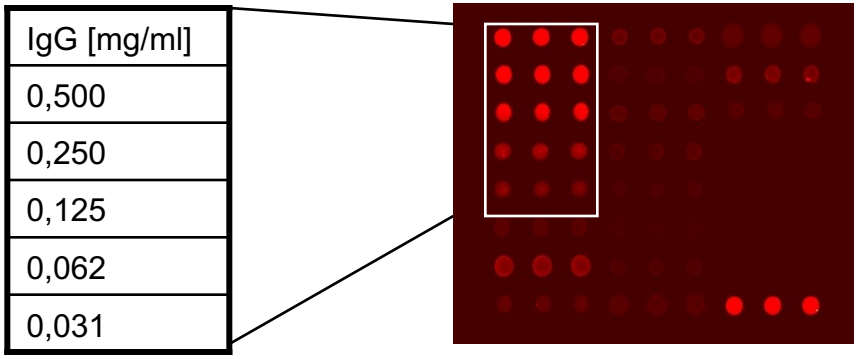
To process up to 64 different patient samples at once

CombiChip Autoimmune 1.0 Scan

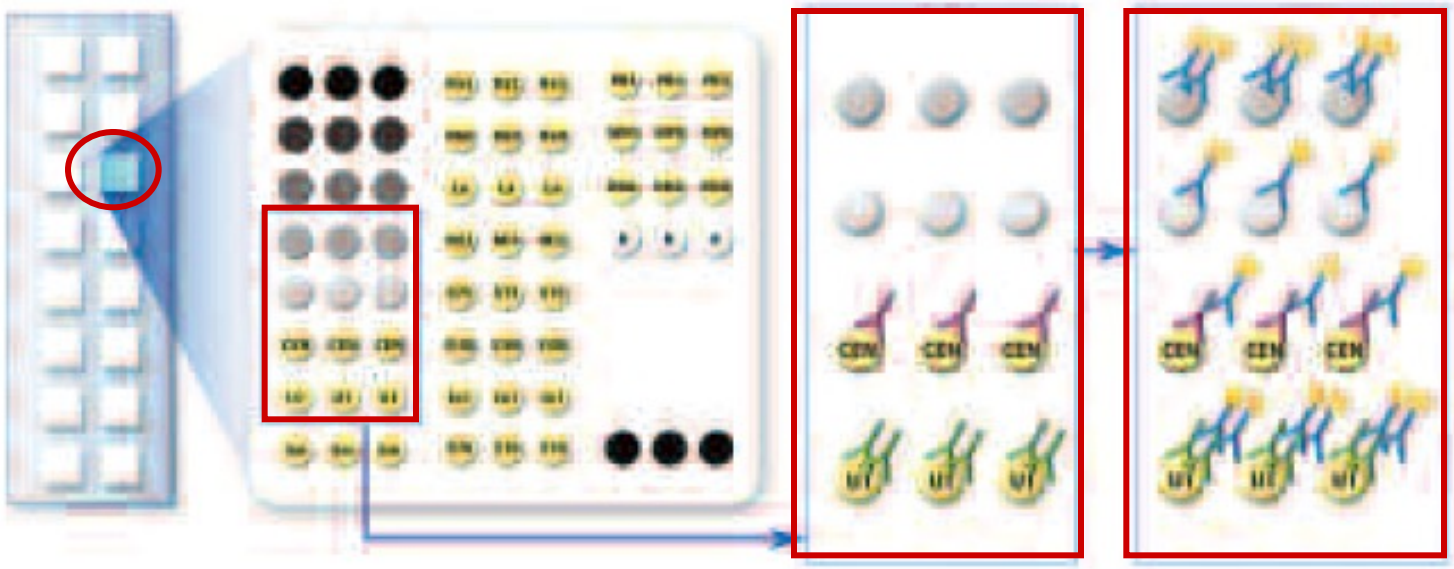
Eredmény:

16-bit Greyscale Digital Image (tiff-Format)

Kalibráció:



Fluoreszcent CombiChip



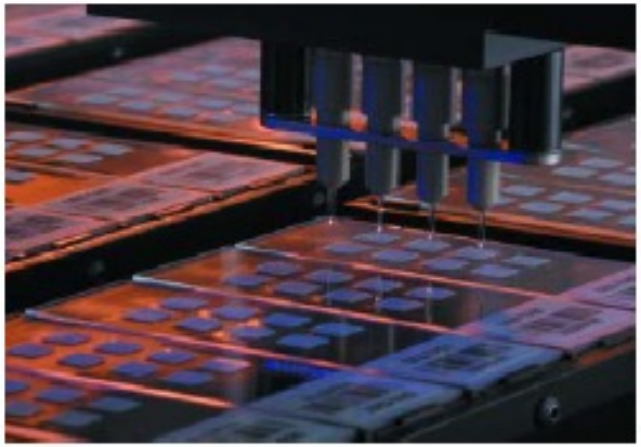
70 µl of diluted sample (1:100) is incubated on each array pad.

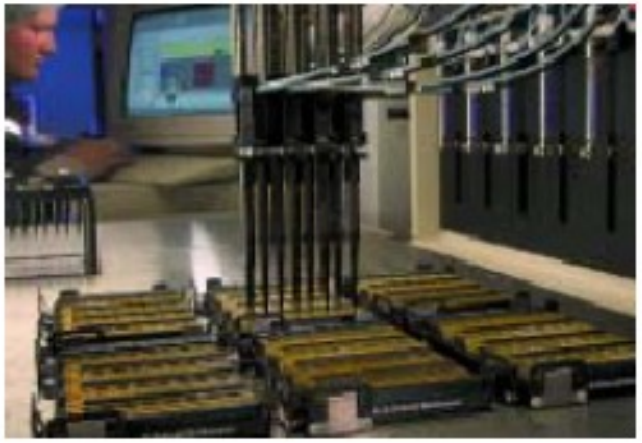
Step A	The reactive autoantibodies bind to their specific antigen
Step B	Anti–human IgG conjugate binds to human IgG antibodies, including the calibrator curve consisting of human IgG

CombiChip is then scanned in a fluorescent imager, and data are analyzed.

Whatman®
Schleicher & Schuell

... weltweit führend für Protein Biochips



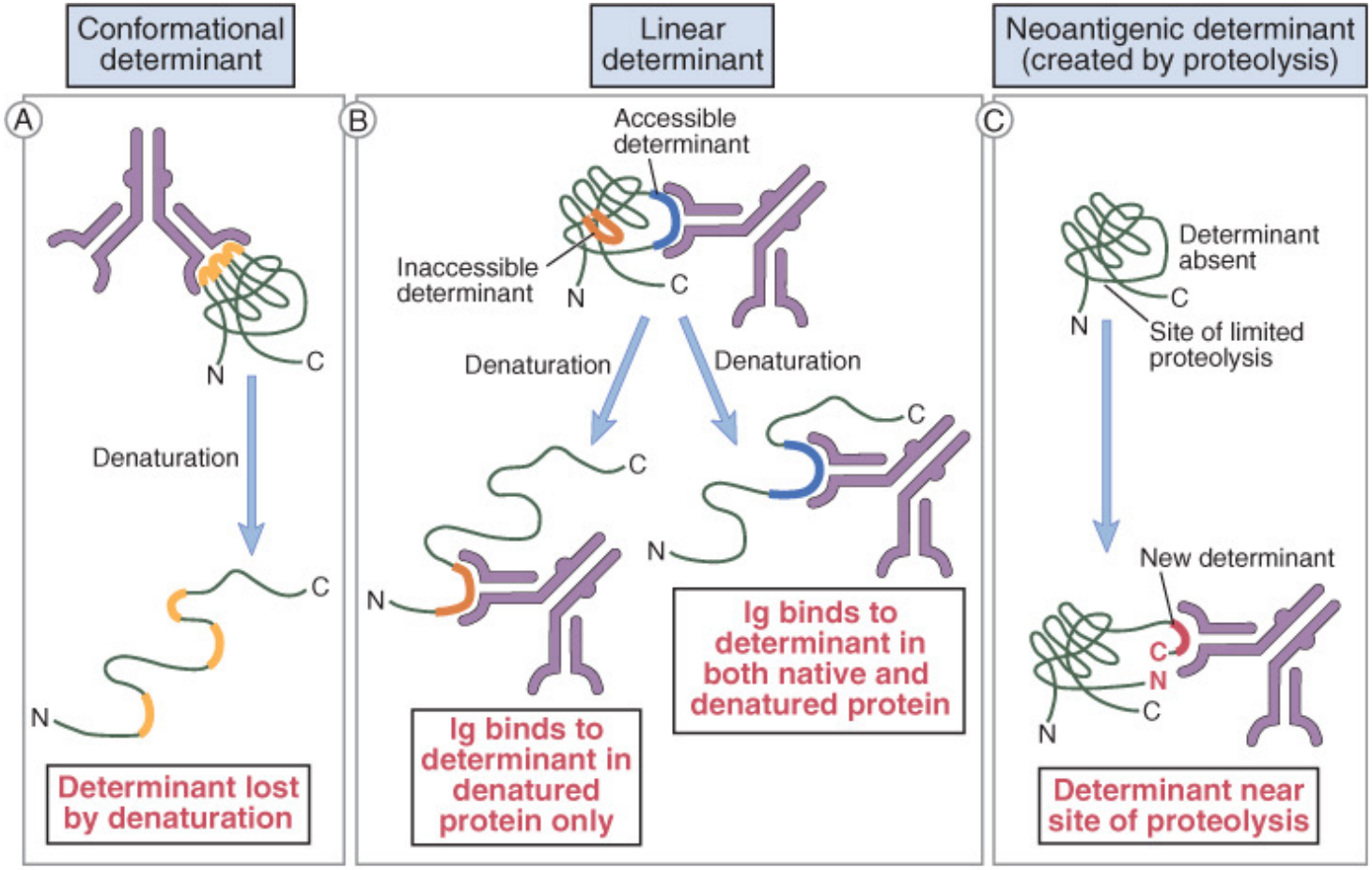


Protein microarray attachment methods

Requirements: Strong binding, retention of function, high signal:noise, low background

Diffusion, e.g polyacrylamide gel (Hydrogel), agarose	No protein modification, high capacity, low background, random orientation
Surface adsorption e.g. PVDF, Nitrocellulose Poly-L-lysine	Protein denaturation, high capacity, variable background, random orientation
Covalent e.g, Silanes (various) aldehyde	Protein modified, strong attachment, high sensitivity, random or uniform orientation
Affinity Biotin/Streptavidin, His/Ni-NTA	Protein modified, strong attachment, low background, random or uniform orientation

Antigen determinants



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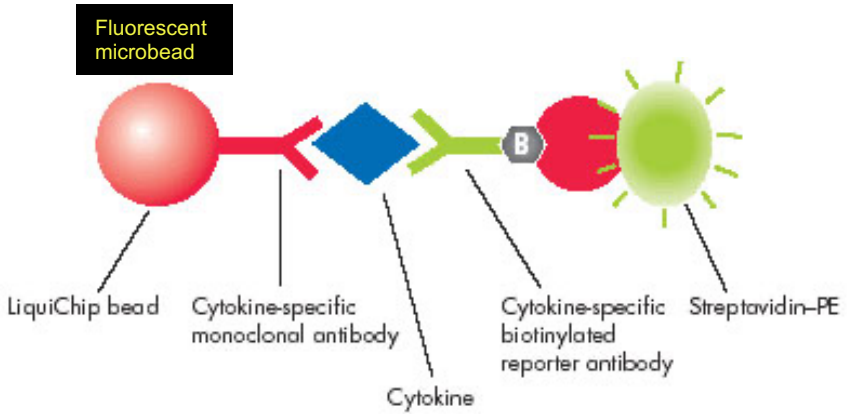
Multiplex microbead array (MMA)



1. Capture antibody bound to the microbead

2. Reporter antibody

Step 1

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Step 2

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Step 3

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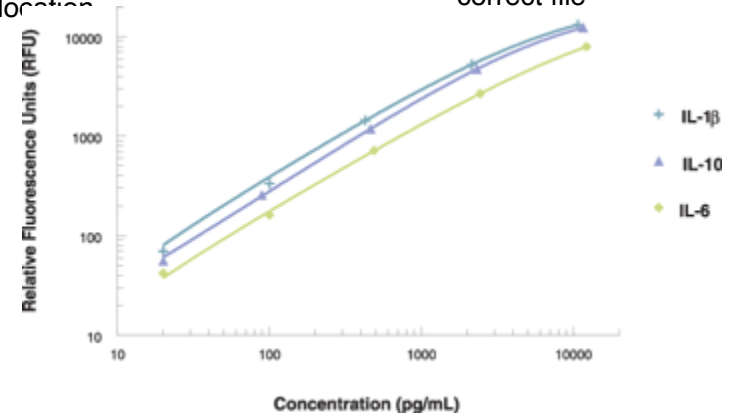
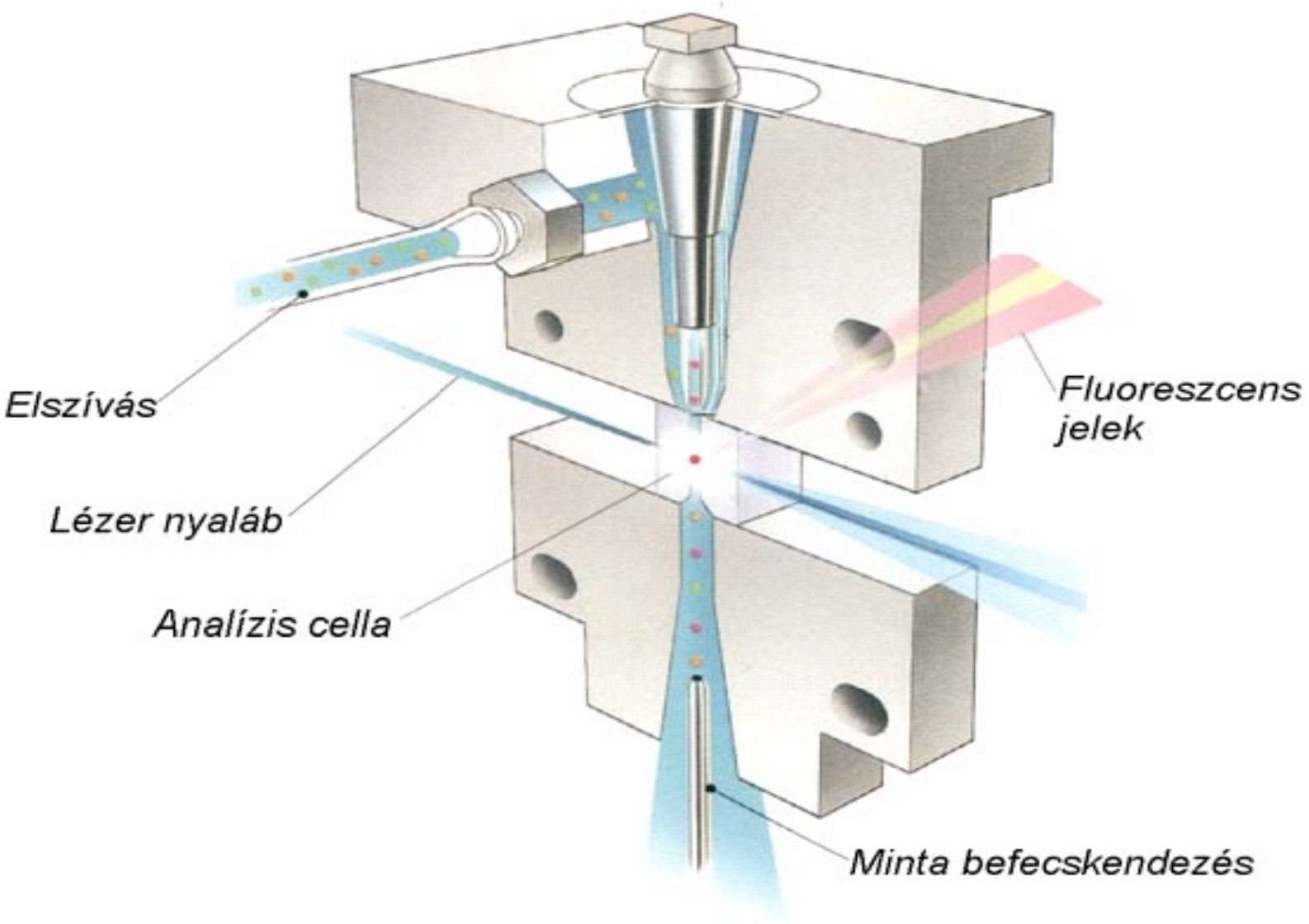
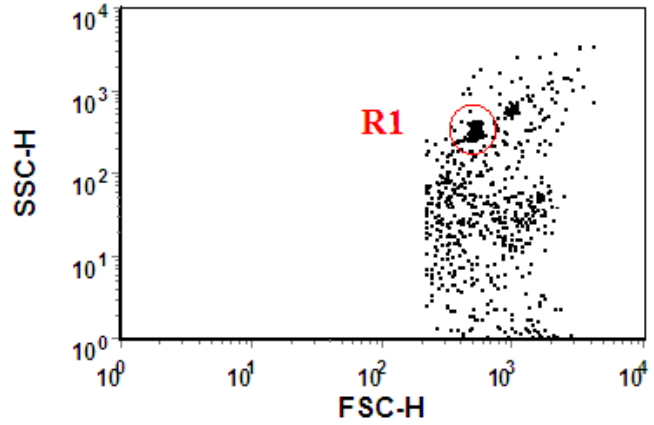
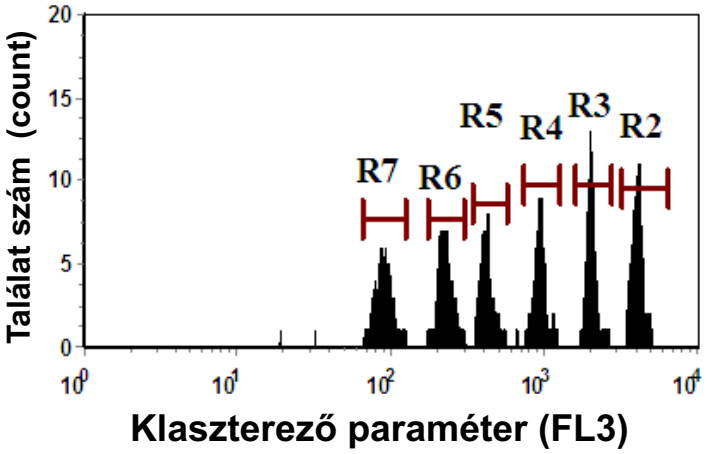


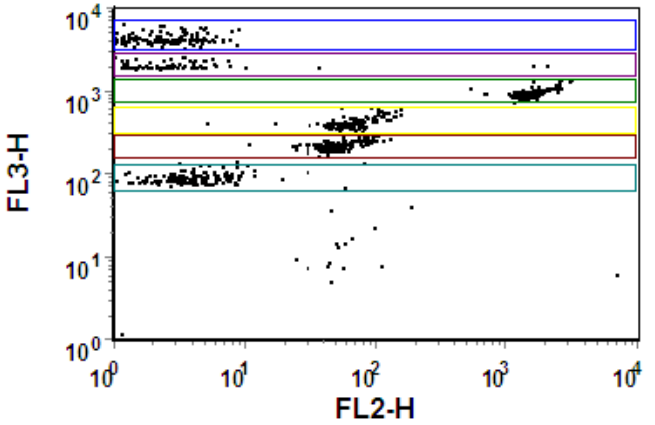
Typical standard curves for IL-1β, IL-10 and IL-6 run simultaneously.


Elszívás
Lézer nyaláb
Analízis cella
Fluoreszcens jelek
Minta befecskendezés

Human inflammatory cytokine bead array - CBA kit



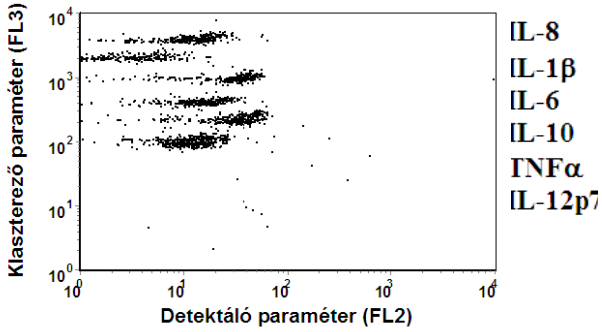




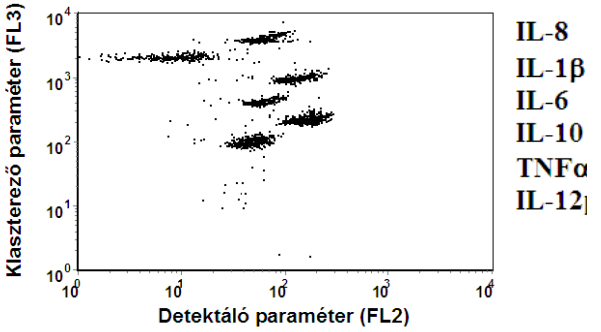
IL-8
IL-1β
IL-6
IL-10
TNFα
IL-12p70

Region #	# of Events	X Geometric Mean	X Arithmetic Mean
None	1201	26.83	322.22
1	1201	26.83	322.22
2	227	1.96	2.38
3	123	2.4	33.11
4	216	1536.98	1596.05
5	201	65.7	69.66
6	236	53.82	56.57
7	175	3.81	5.46

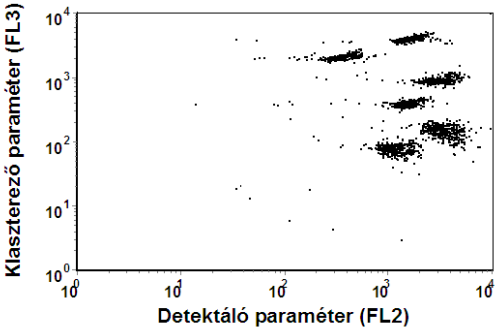
Calculation of standard curve



20 pg/ml

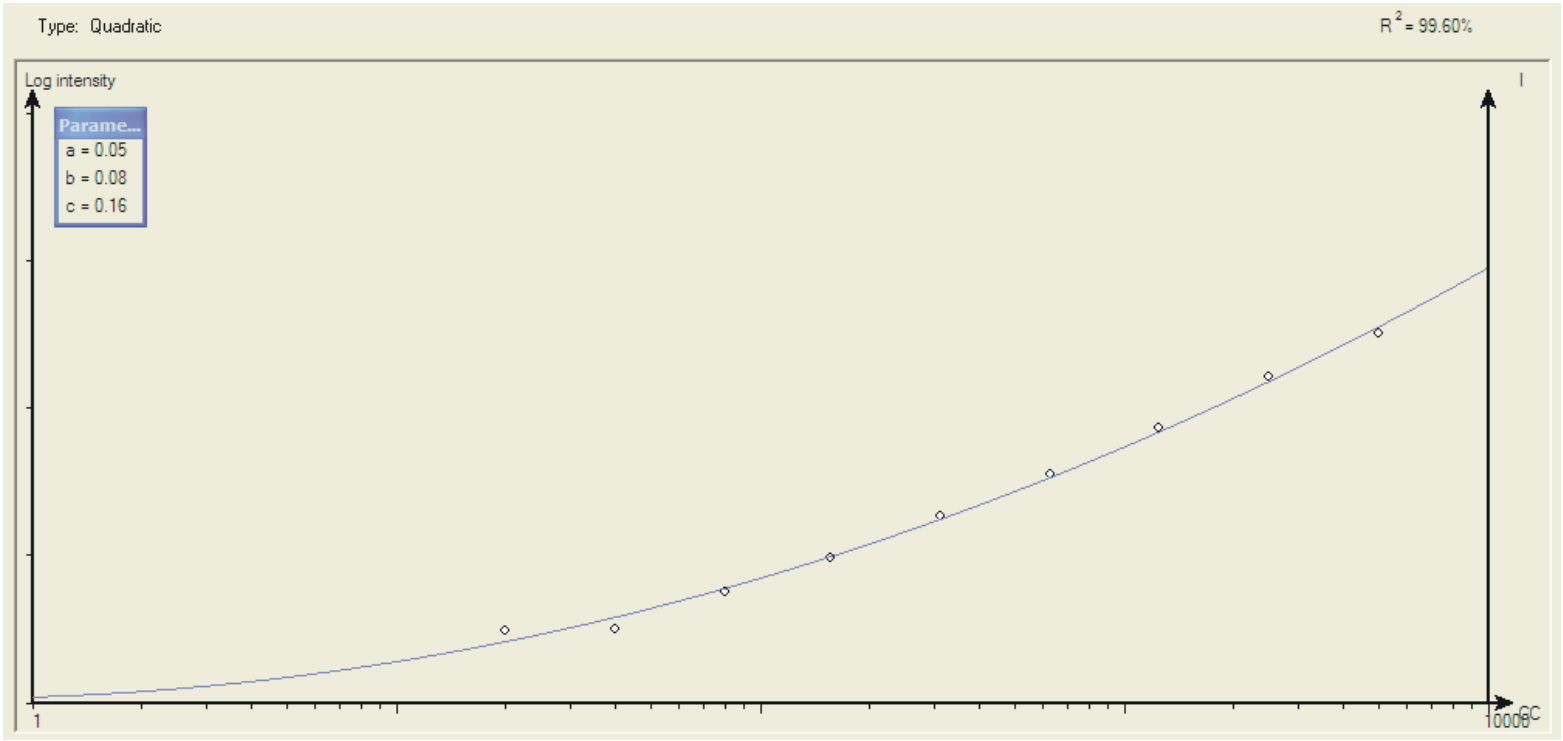


625 pg/ml



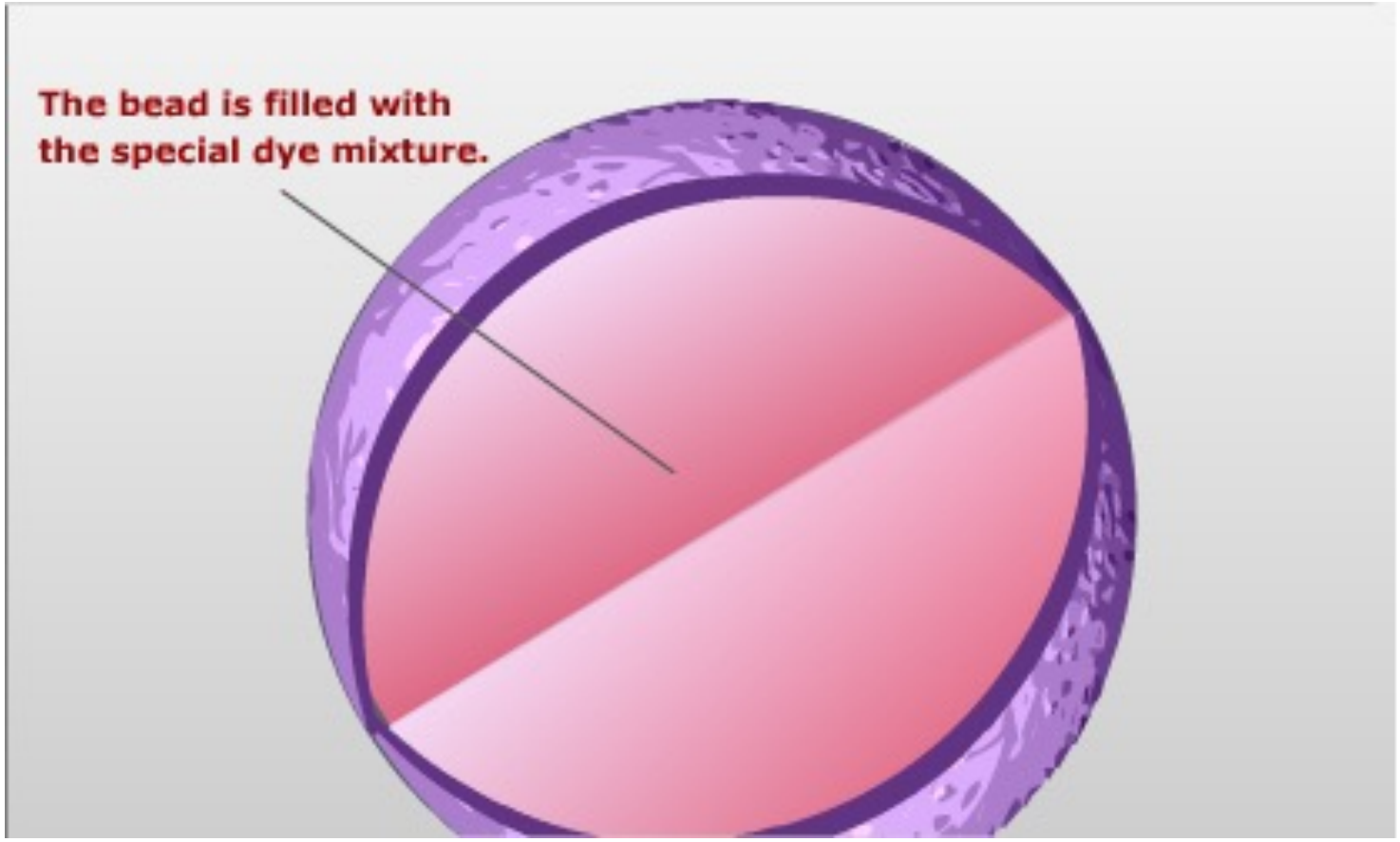
5000 pg/ml

FL2 MFI



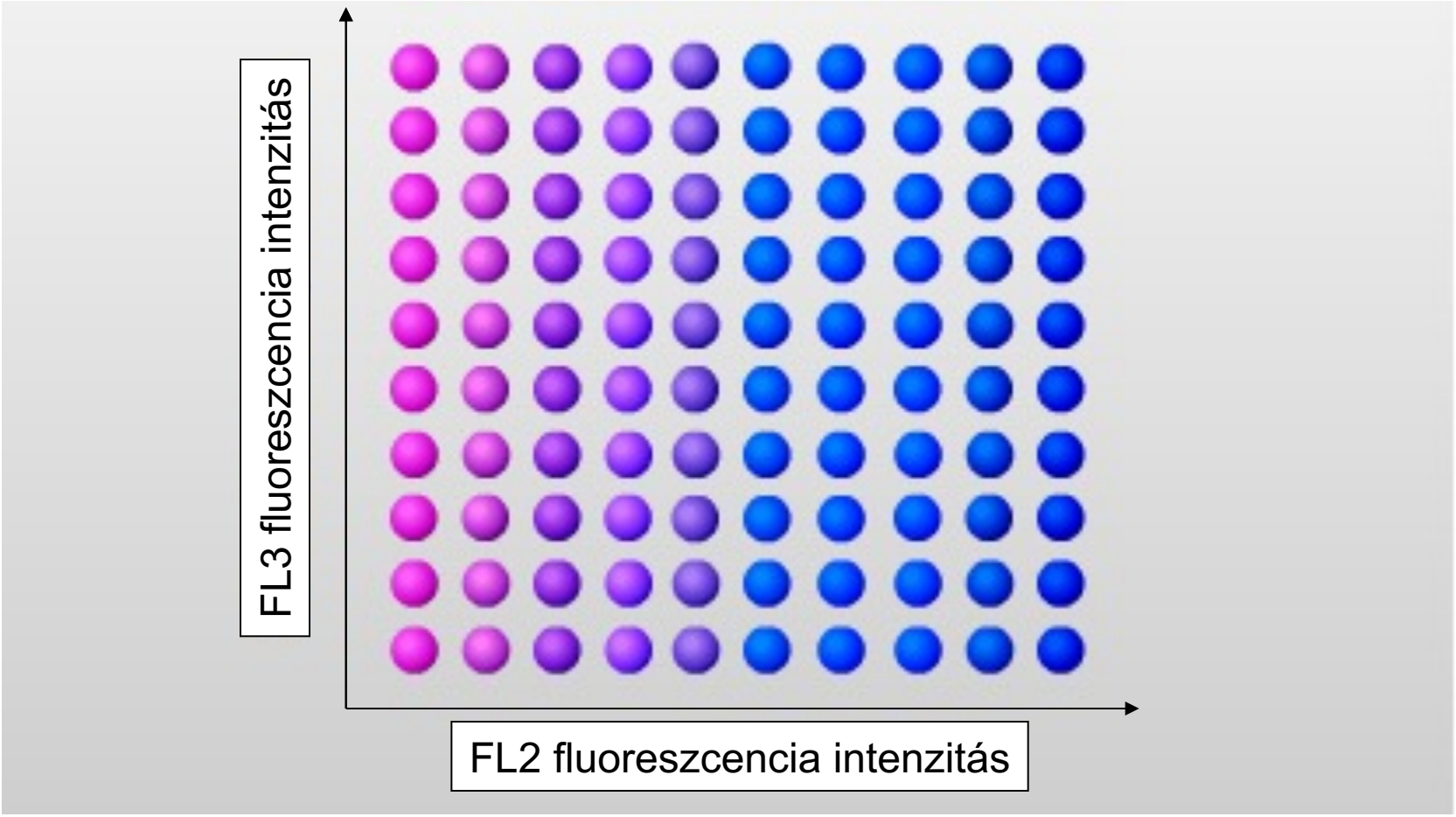
CC 10.000

Luminex xMAP technology

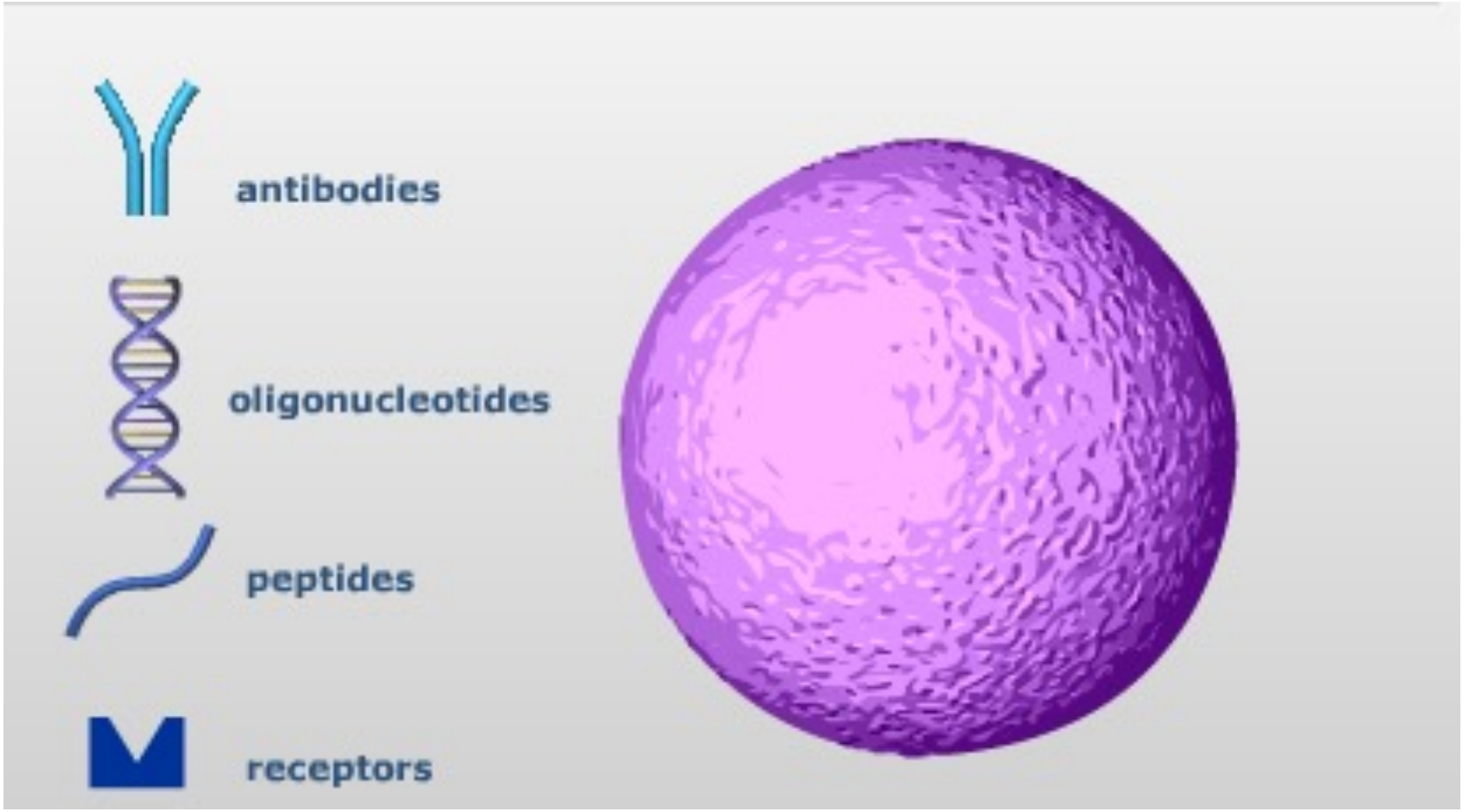


Luminex xMAP technológia

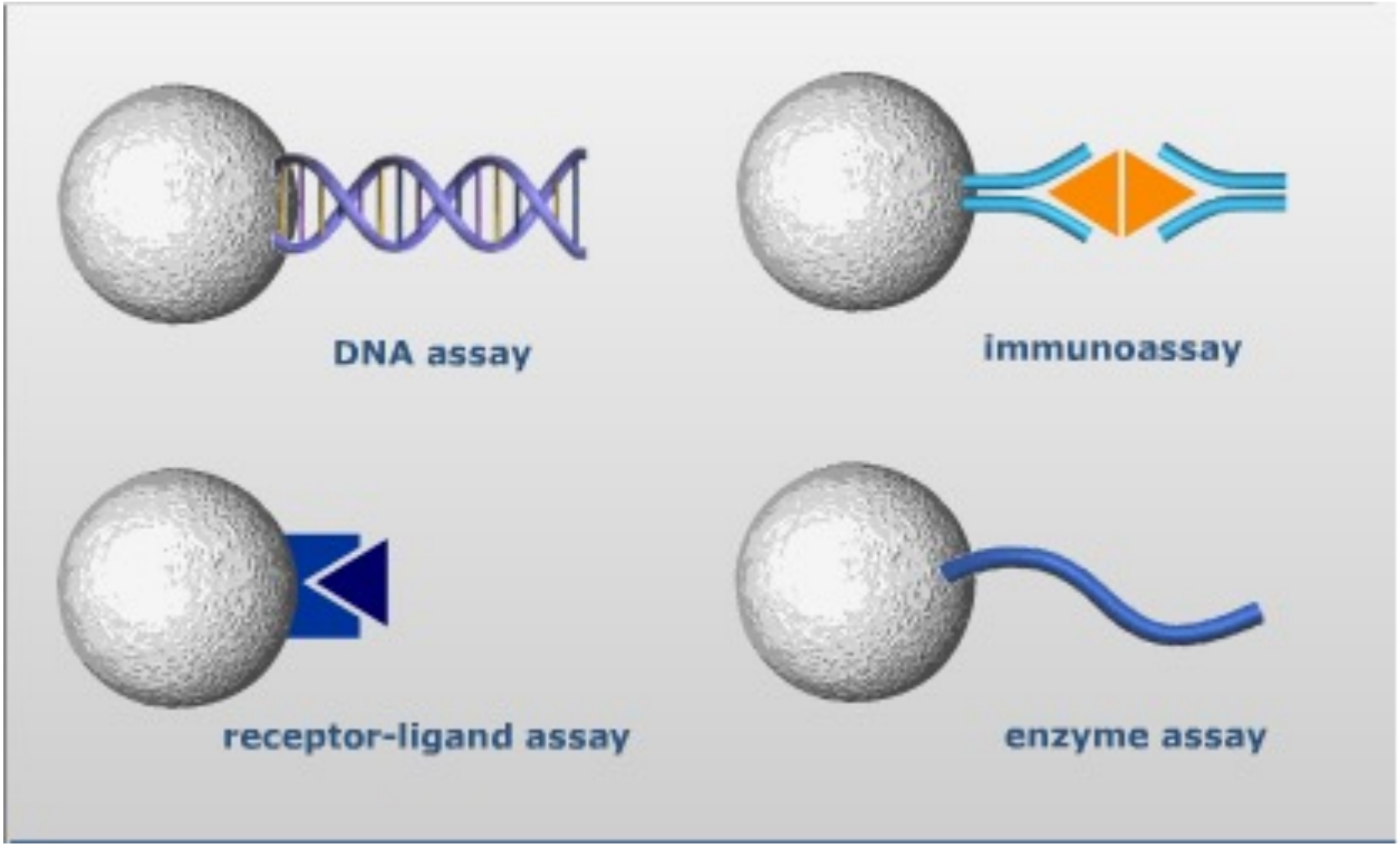
A különböző gyöngy klasztereket össze lehet keverni. Mivel minden egyes gyöngy egyéni felismerési jelet hordoz, az xMAP detekciós rendszer detektálni tudja, hogy melyik gyöngy melyik klaszterhez tartozik. Elméletileg 100 különböző mérést lehet így egyetlen mintában egyszerre elvégezni.



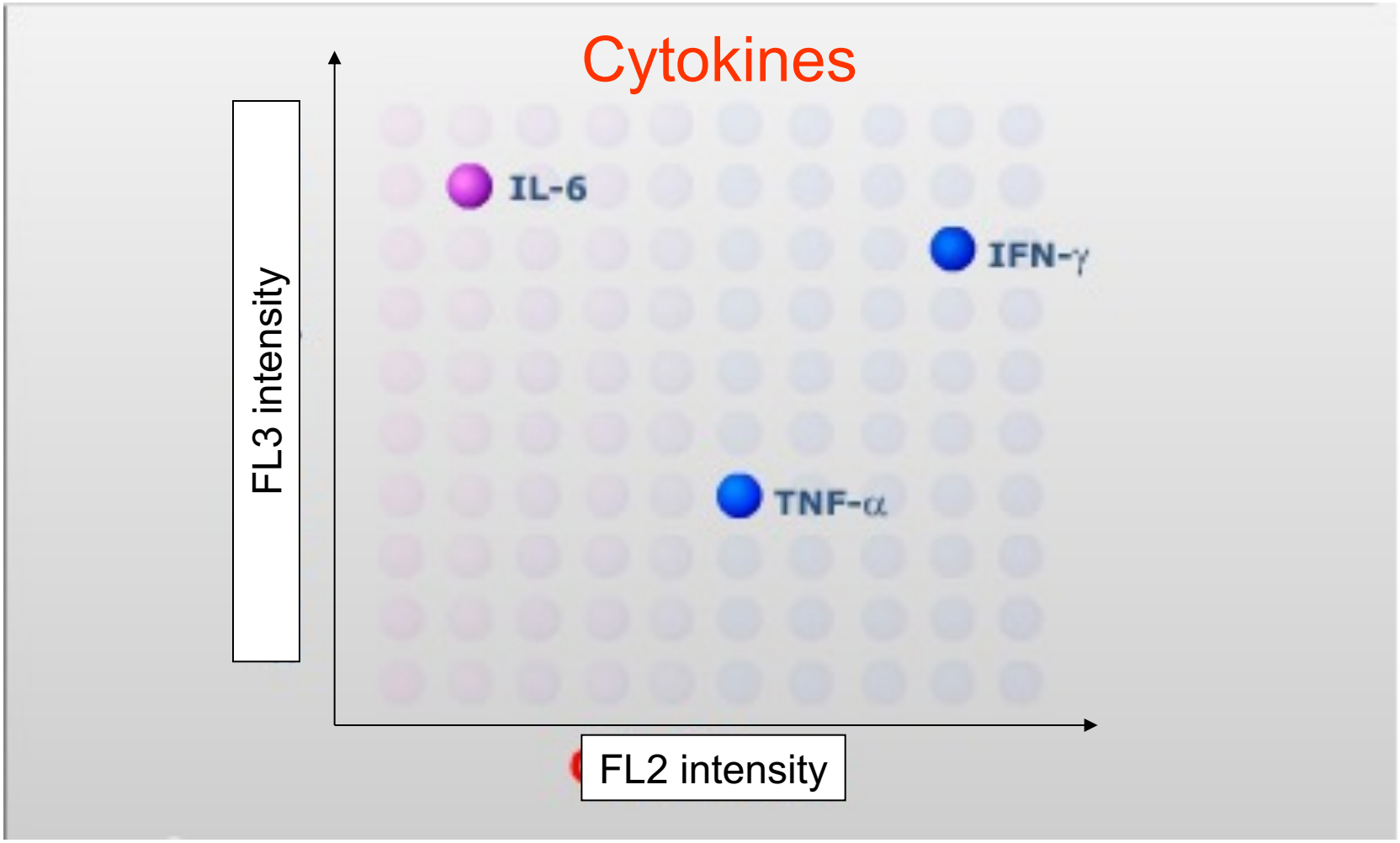
Immobilization of molecules on microbead surface



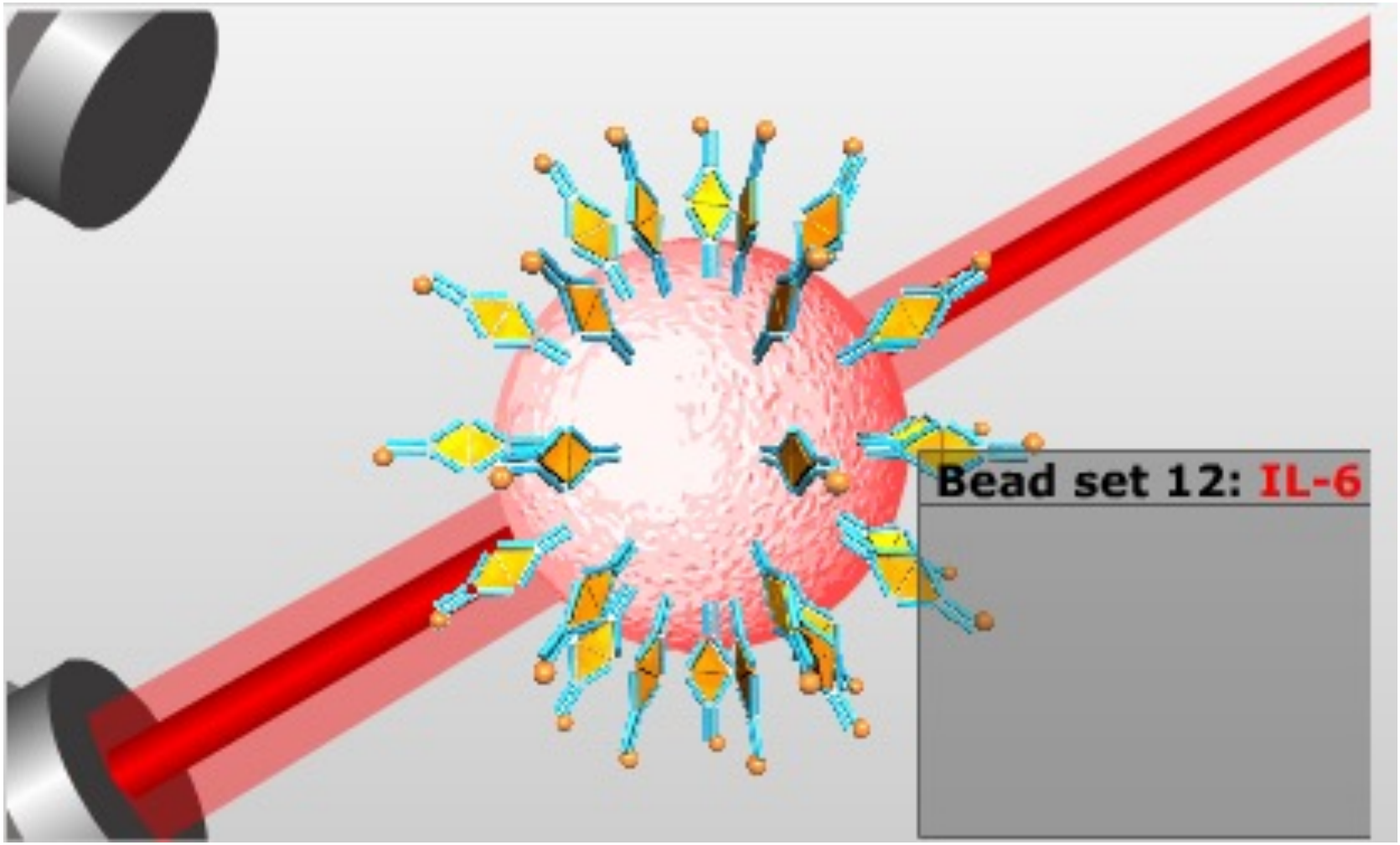
Covalent bindig of different molecules alow measurement of different reactions.


DNA assay
immunoassay
receptor-ligand assay
enzyme assay

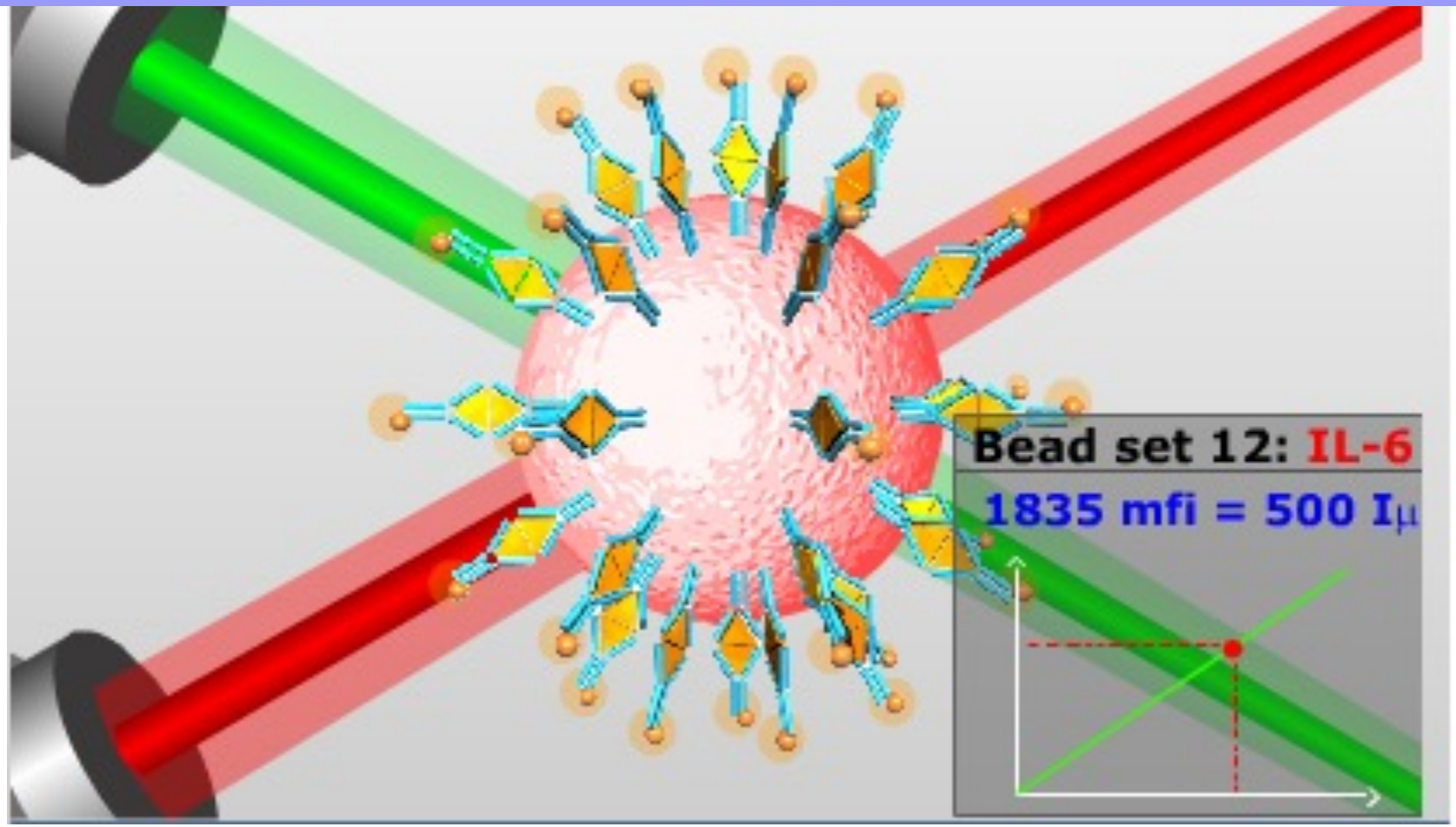
Luminex xMAP technology



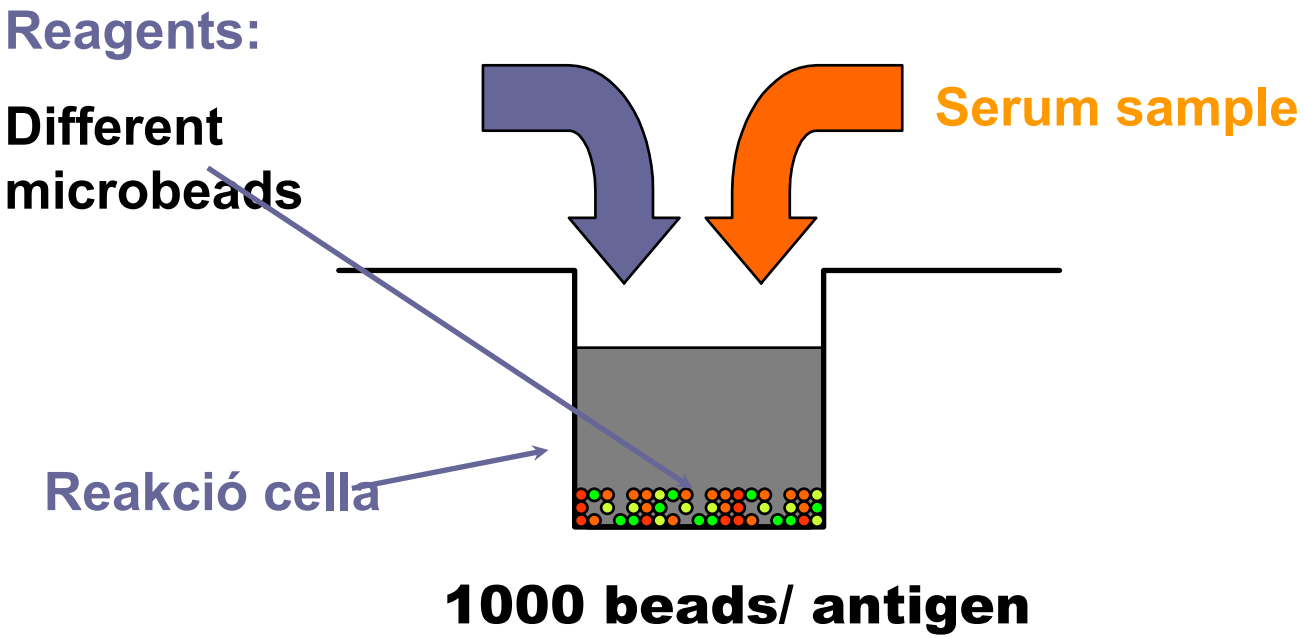
Detection fo mikrobead clusters



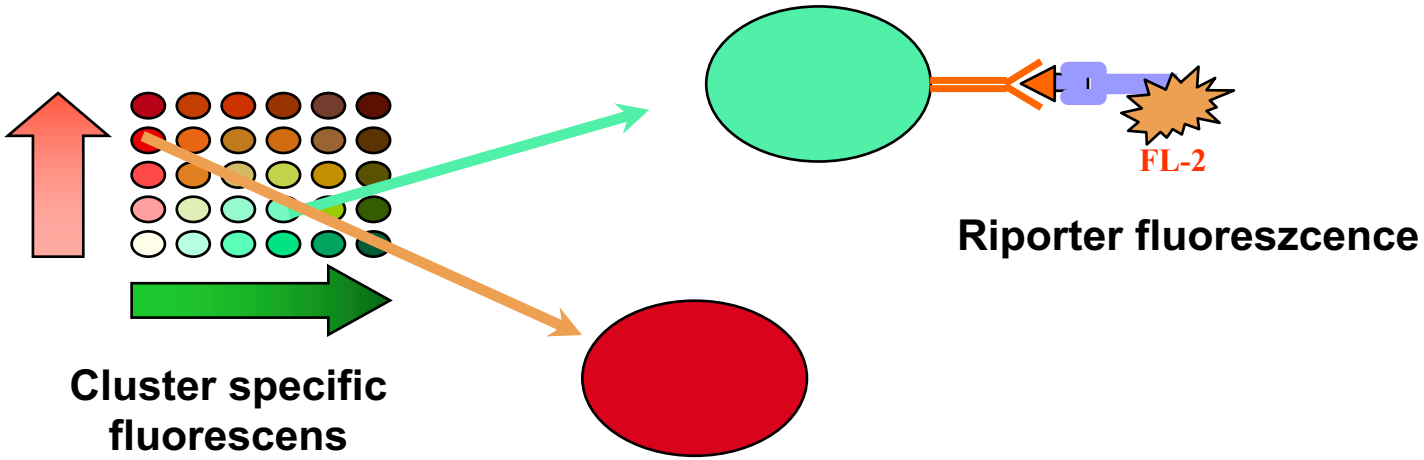
Detection of reporter fluorescenc



Multiplex Microbead Ass(rr)ay (MMA)



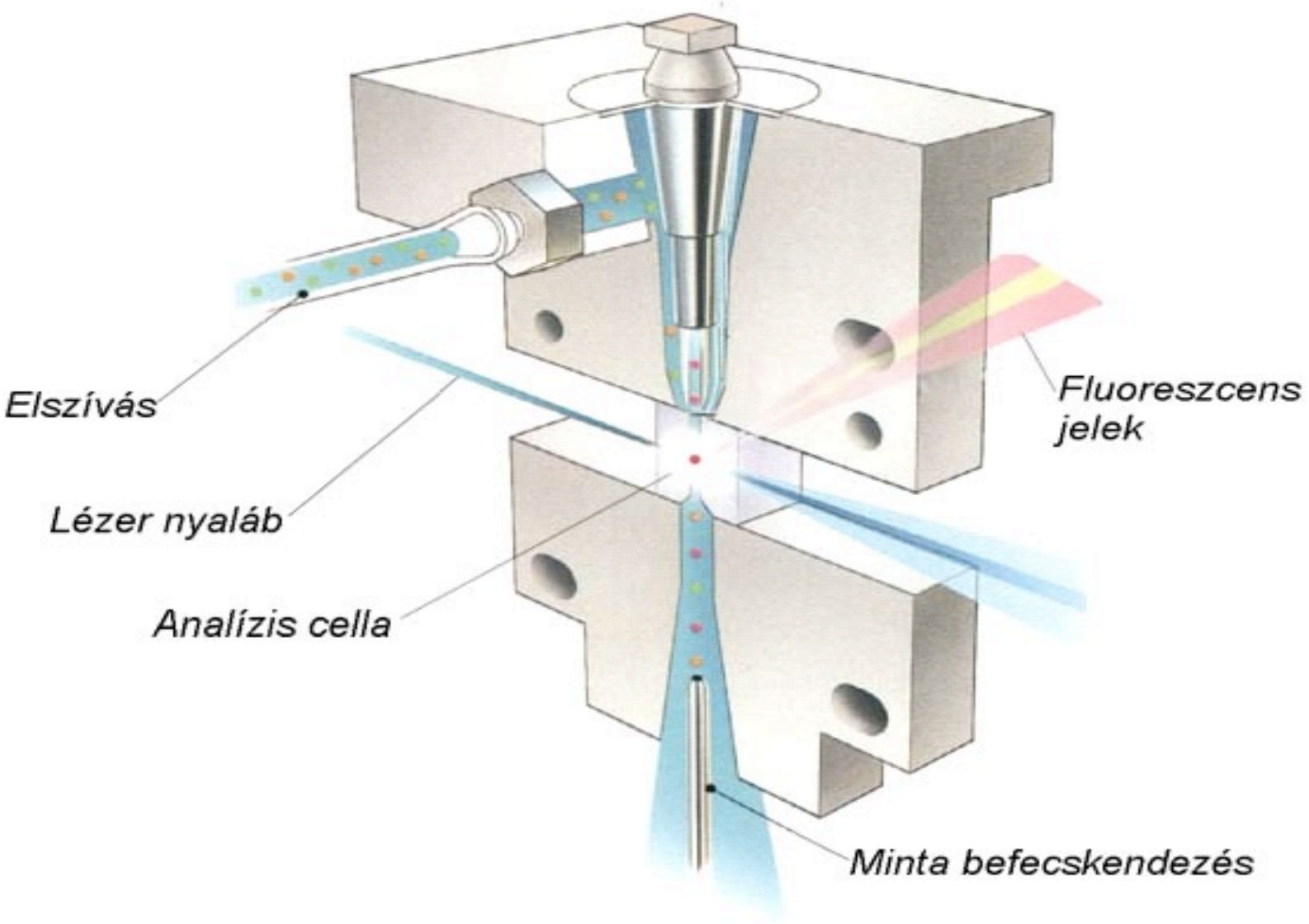
Multiplex Microbead Assay



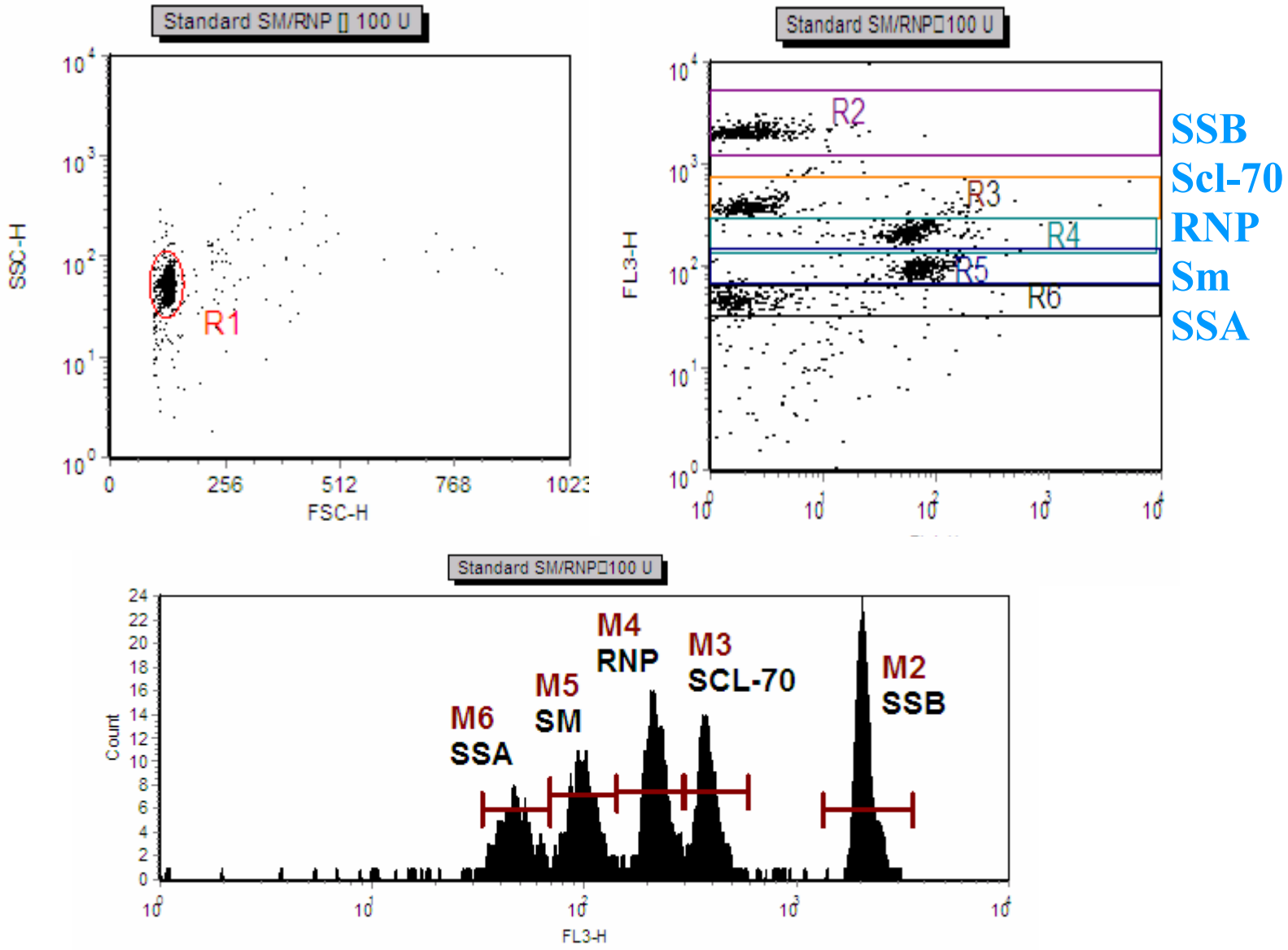
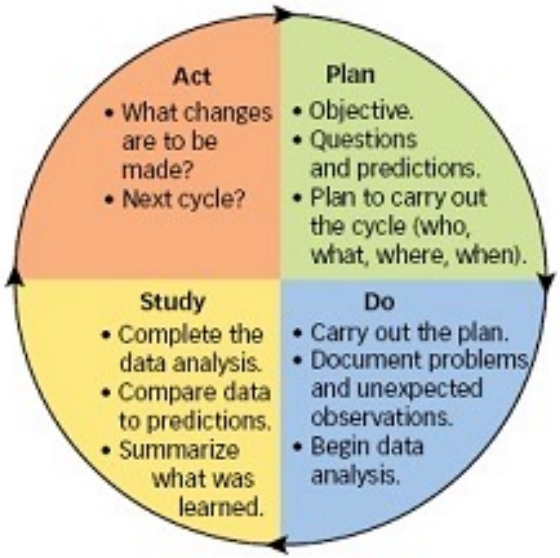
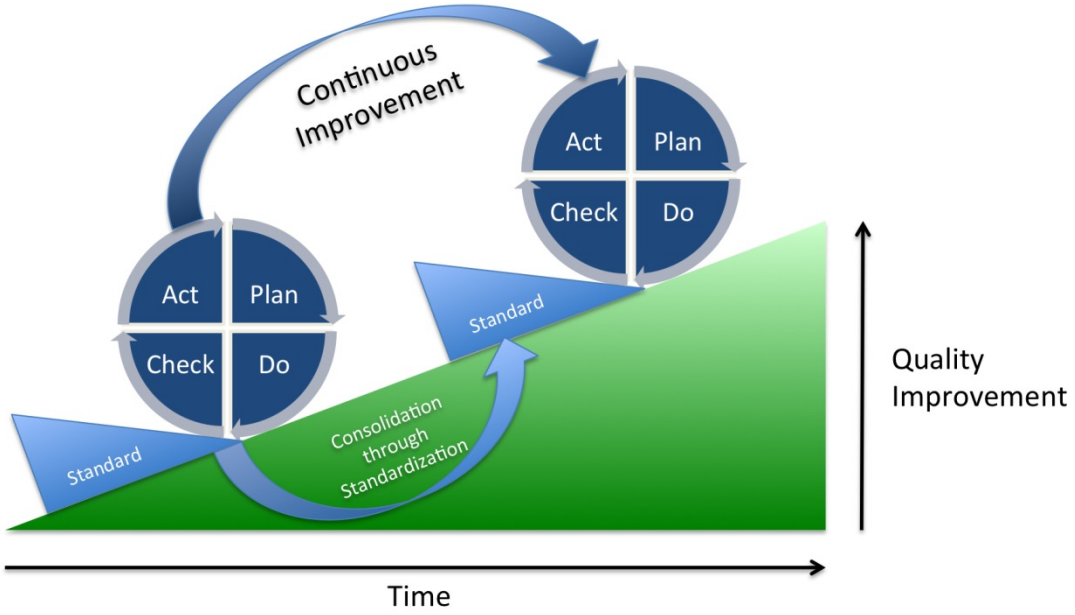
- Each microbead cluster has its own fluorescence

- Each cluster is specific for a different Aab or cytokine

- Concentration dependent riporter fluorescenece is determined


Elszívás
Lézer nyaláb
Analízis cella
Fluoreszcens jelek
Minta befecskendezés

MMA flow cytometry



PDCA: plan–do–check–act/adjust = Deming cycle