INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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Inovace studijních programů AF a ZF MENDELU směřující k vytvoření mezioborové integrace CZ.1.07/2.2.00/28.0302

Tato prezentace je spolufinancovaná z Evropského sociálního fondu a státního rozpočtu České republiky

Uplatnění ELISA a nových imunologických postupů (Luminex) při sériové diagnóze virů

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evropský sociální fond v ČR
EVROPSKÁ UNIE
MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY
OP Vzdělávání pro konkurenceschopnost

Nové molekulárně-genetické metody detekce rostlinných virů a jejich využití v biotechnologiích
ÚEB AV ČR Olomouc

Obsah

- **Trochu historie ze sérologické diagnózy rostlinných virů**
  - příprava diagnostických protilátek
  - testy precipitace, aglutinace, immunodifuze,
  - RIDT, latex
- **ELISA**
  - první popisy a aplikace v rostlinné virologii
  - protilátky a komerční dostupnost, sériové testy
  - varianty a modifikace : DAS, PT, TAS,(Fab fragment), koktejl (two-steps), APAb, Dot-blot (NCM, nylon)
- **LUMINEX xMAP (xTAG)**
- **Další vývoj - ? Molekulární metody (Real time RT-PCR, Isothermal amplifikace)**

Kdy a kde to u nás začalo ?
Přes milníky ale i suché vývojové větve až k ELISA ?

: Precipitační a aglutinační testy
1939: Jermoljev a Hruška - 3 publikace o sérologii virových chorob u brambor

1955: Jermoljev a Průša - 3 publikace o zdokonalení výroby antisér proti rostlinným virům (řepa)

1955: Pozděna, Čech, - (řepa, chmel, brambory)

1960: Nohejl, Červenka ……
(Fliegl, Kameníková)

1963: Chod, Albrechtová (řepa, mrkev …)

1968: Polák (tulipány, řepa, hrách, fazol)
……
: Immunodifúze - RIDT (v 80. letech NDR)

: Latexová aglutinace (v 80. letech též u nás)

……..

ELISA 1977: **Clark a Adams**. …. Richter (1978), Casper, Gugerli, deBokx, Tamada….

……..

- *ULV ELISA s 4-MUP (Ashersleben)*
- *Peroxidázové konjugáty (NDR, Maďarsko, ČR)*

……

Výrobna antisér při VŠÚO v Olomouci

Vývoj MAbs (ÚMB, ÚEB, VŠÚO, VÚB, Virol. Ústav SAV) a rekombinantní protilátky

Komerční dodavatelé diagnostických kitů (Boehringer, Bioreba, Sanofi, Lowe, Agdia, Adgen, IPO Primediagnostics)

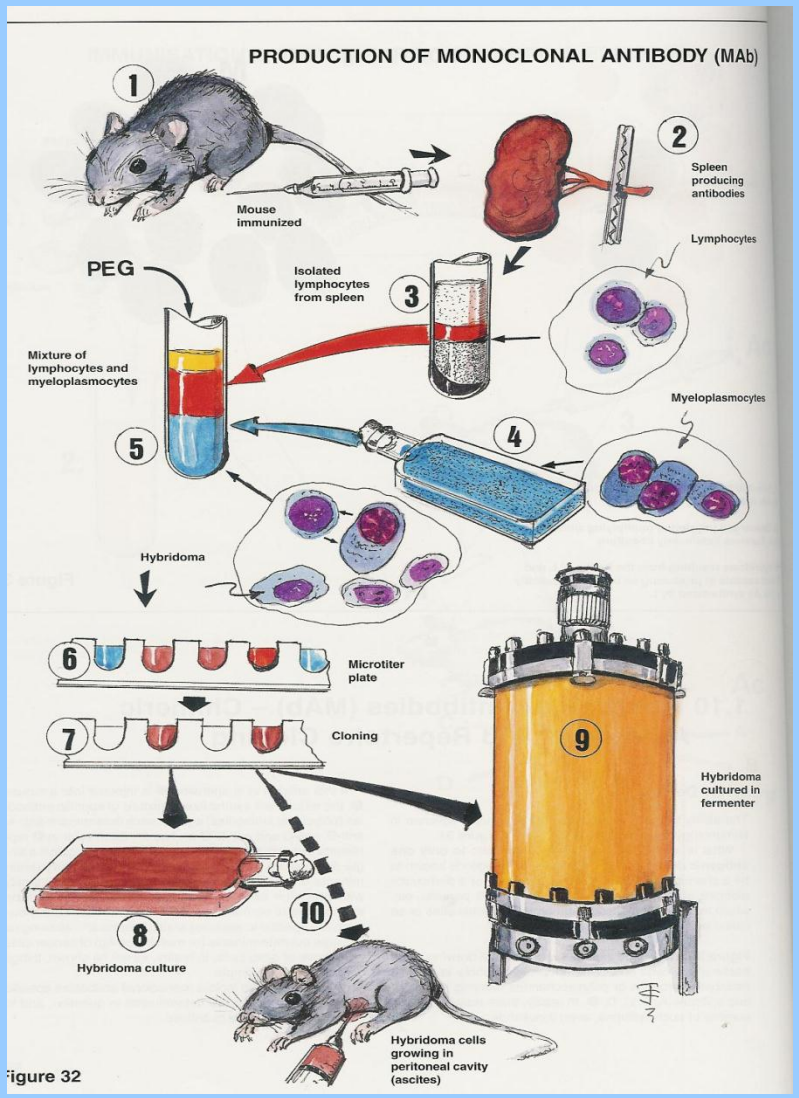
……

Zavedení sériových testů ELISA ve šlechtění a certifikaci sadby bramboru (od 80. let v Holandsku, Německu, ČR)

Pozděna, Špak, Filigarová, Čeřovská
Polák, Žák, Křístek
Gallo, Matisová, Franěk
Navrátil, Havránek

Příprava diagnostických protilátek

Masová produkce diagnostických antisér
– králíci, ovce, koně

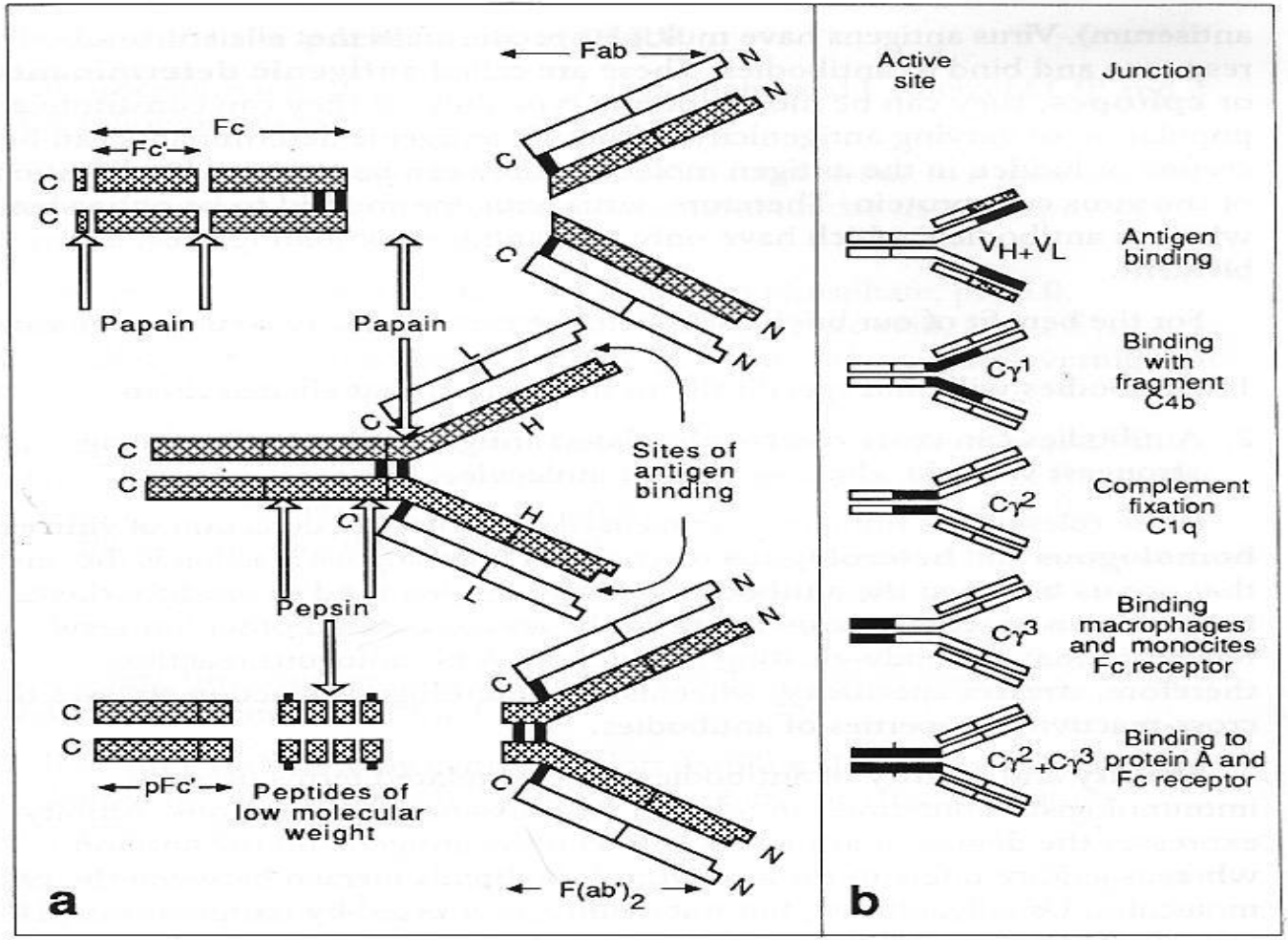


Strategie přípravy rekombinantních protilátek

Struktura IgG

a) produkty digesce pepsinem a papainem

b) funkce různých částí struktury

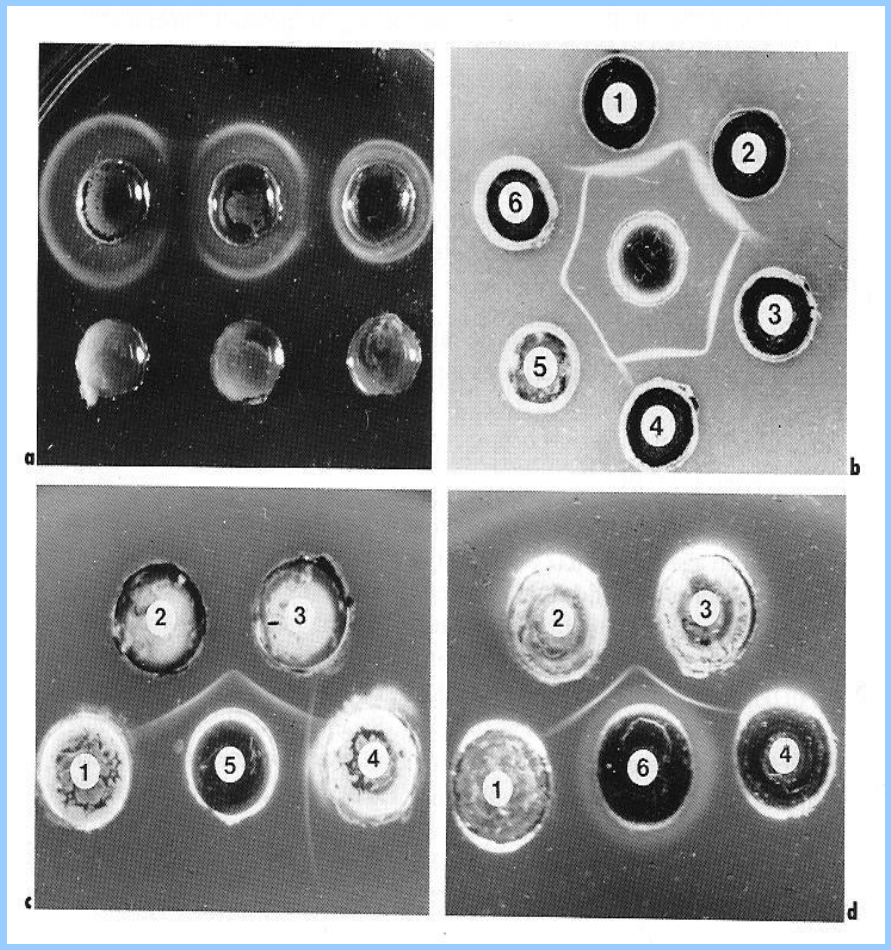


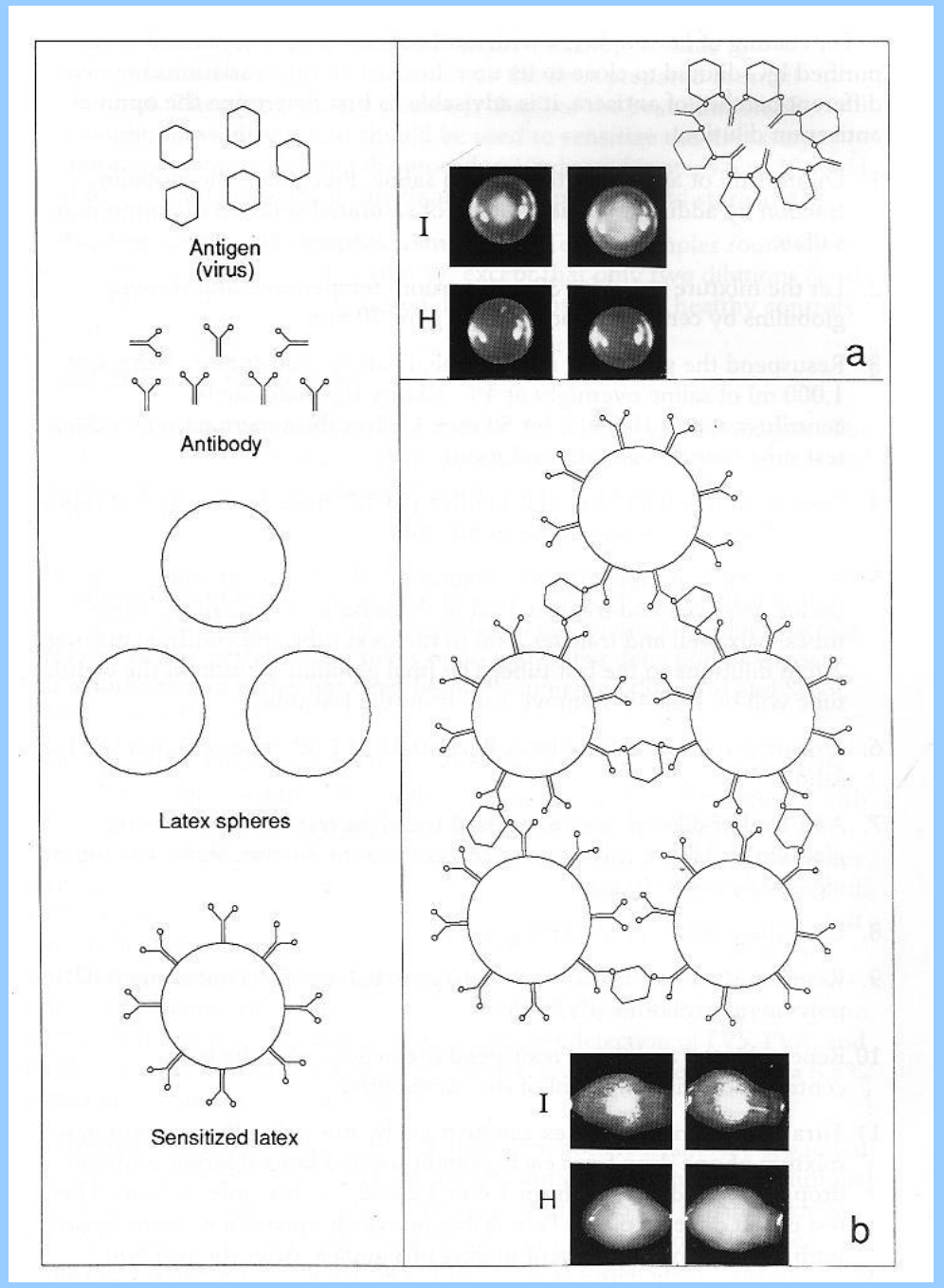
Sérologická diagnóza

mikroprecipitace

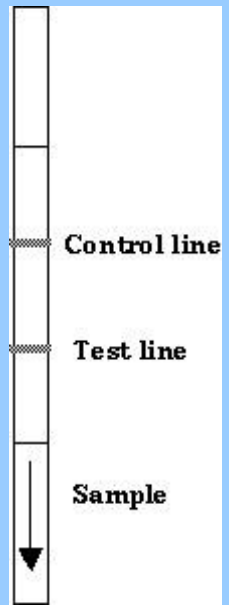
latexová aglutinace

immunodifúze



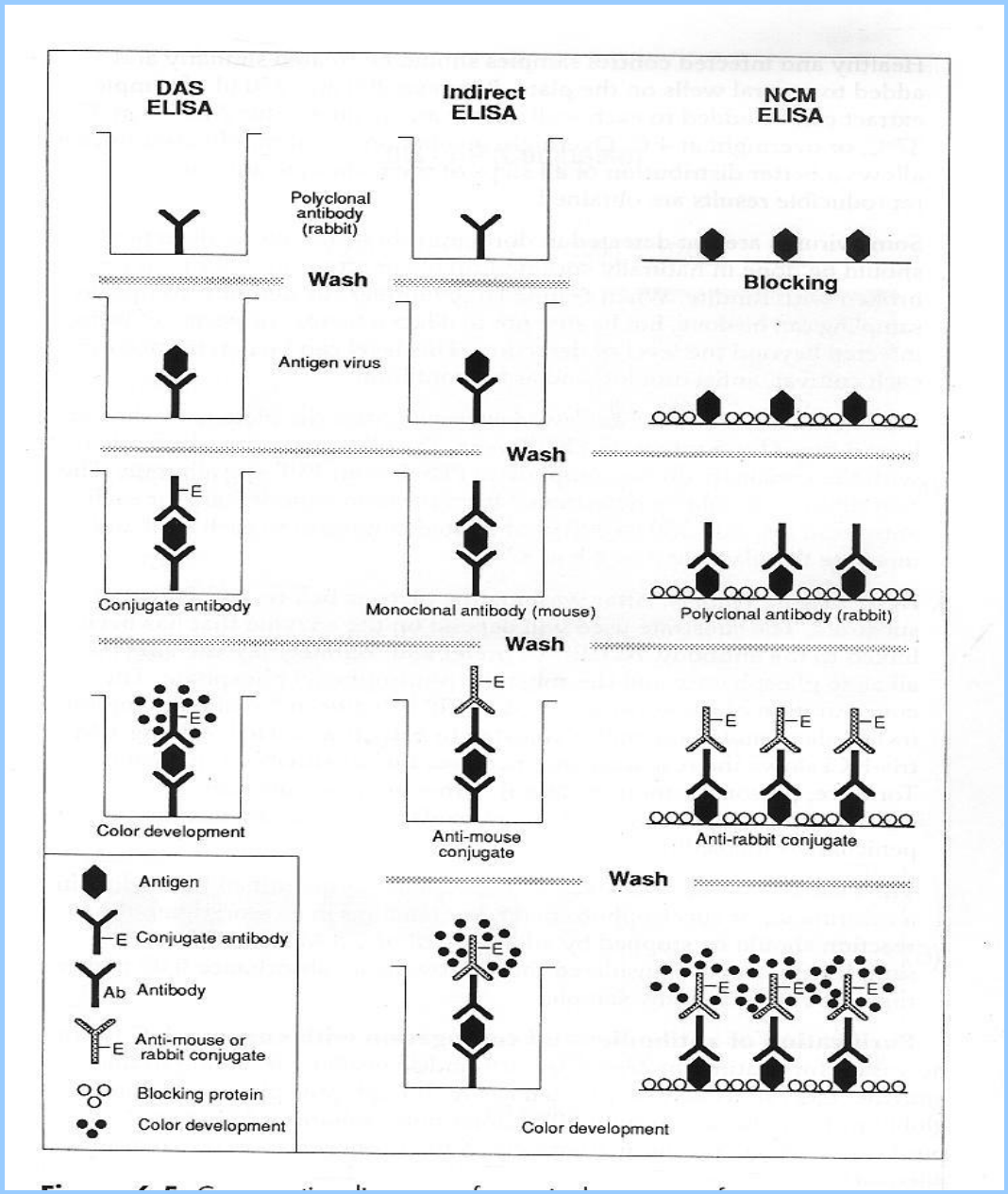


Možnost instantní diagnózy (např. Immunostrip test Agdia, Bioreba, ….)



Modifikace ELISA

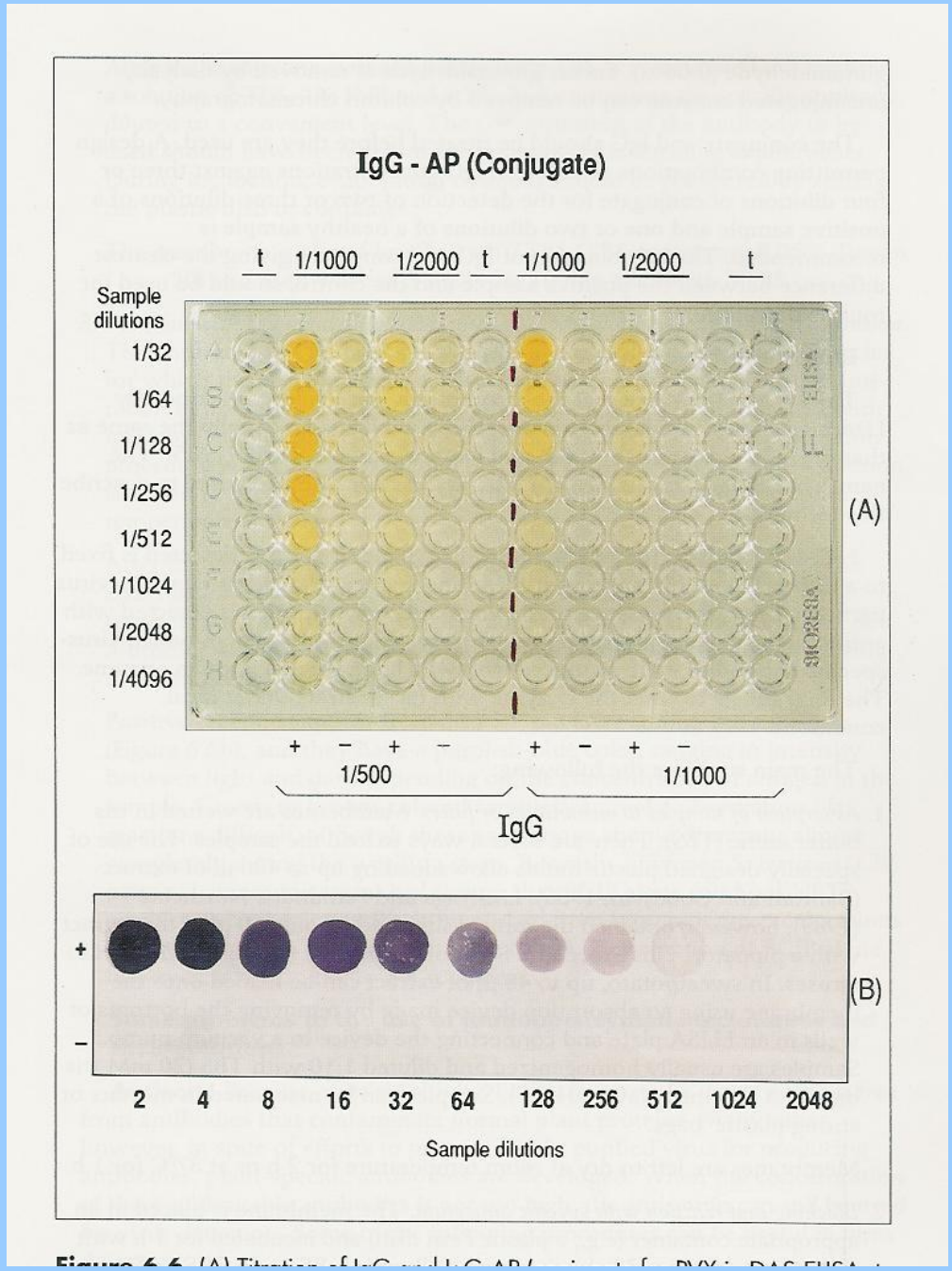
- DAS ELISA
- Indirect ELISA
- NCM ELISA



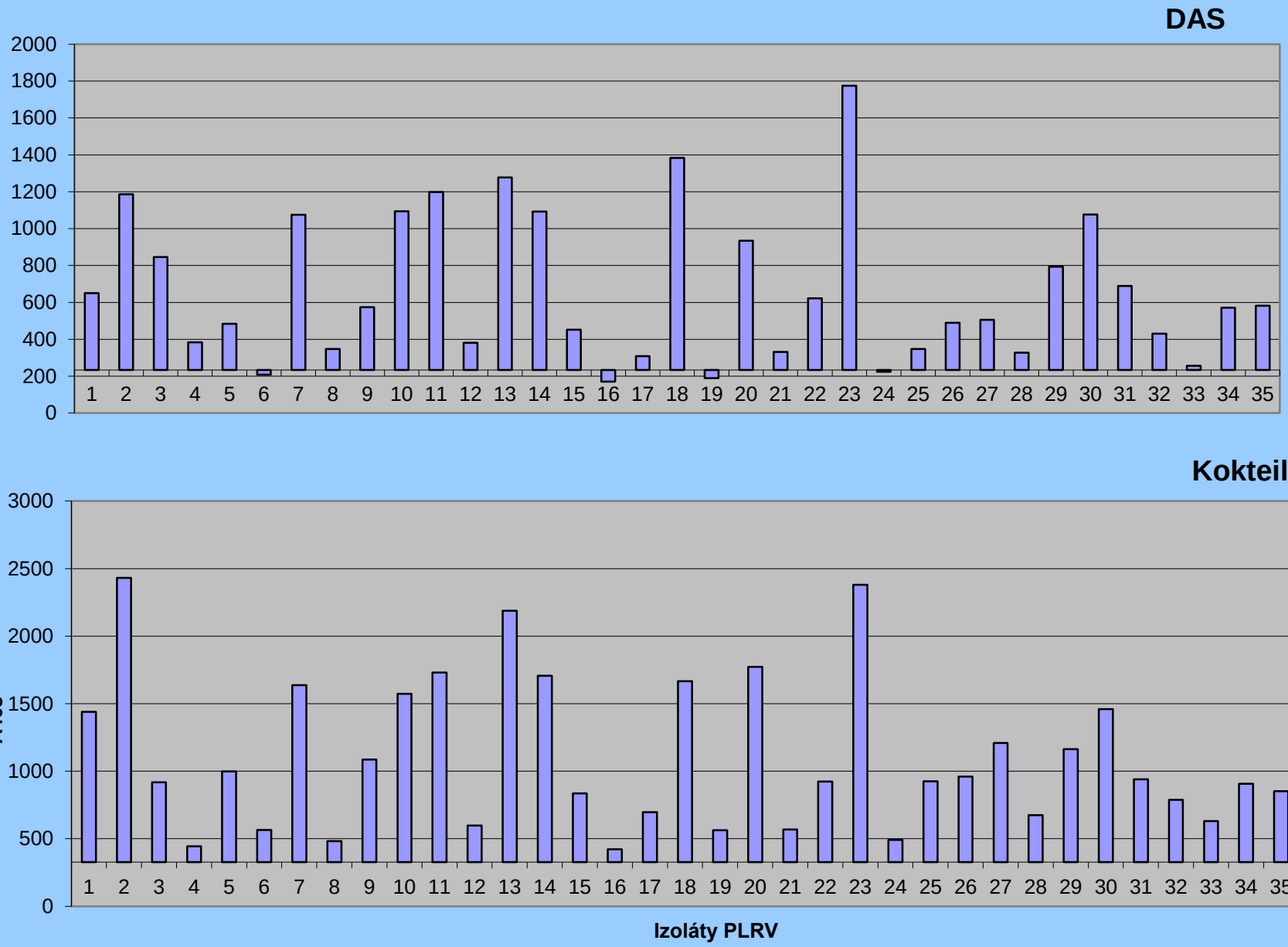
Hodnocení výsledku diagnózy

DAS ELISA

NCM ELISA



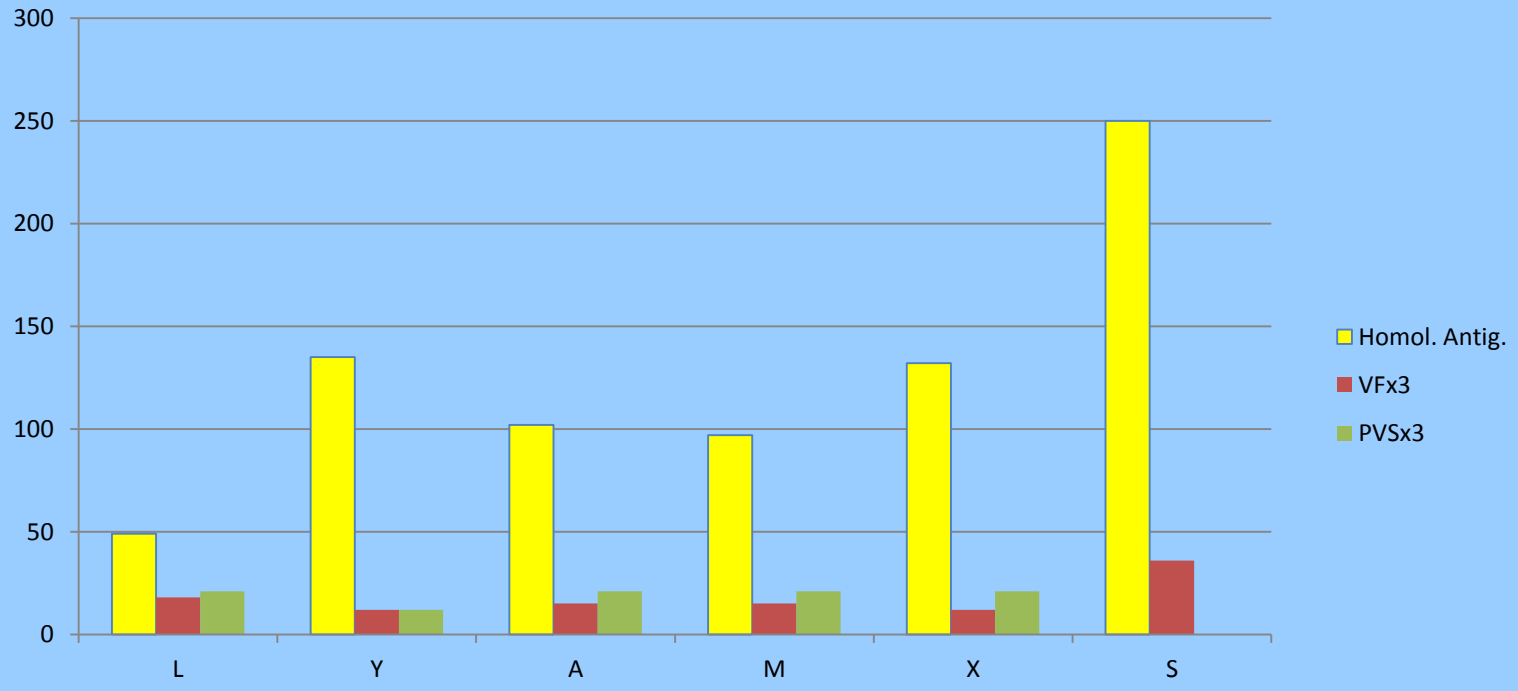
DAS ELISA vs Koktejl ELISA izoláty PLRV, kit Primediagnostics



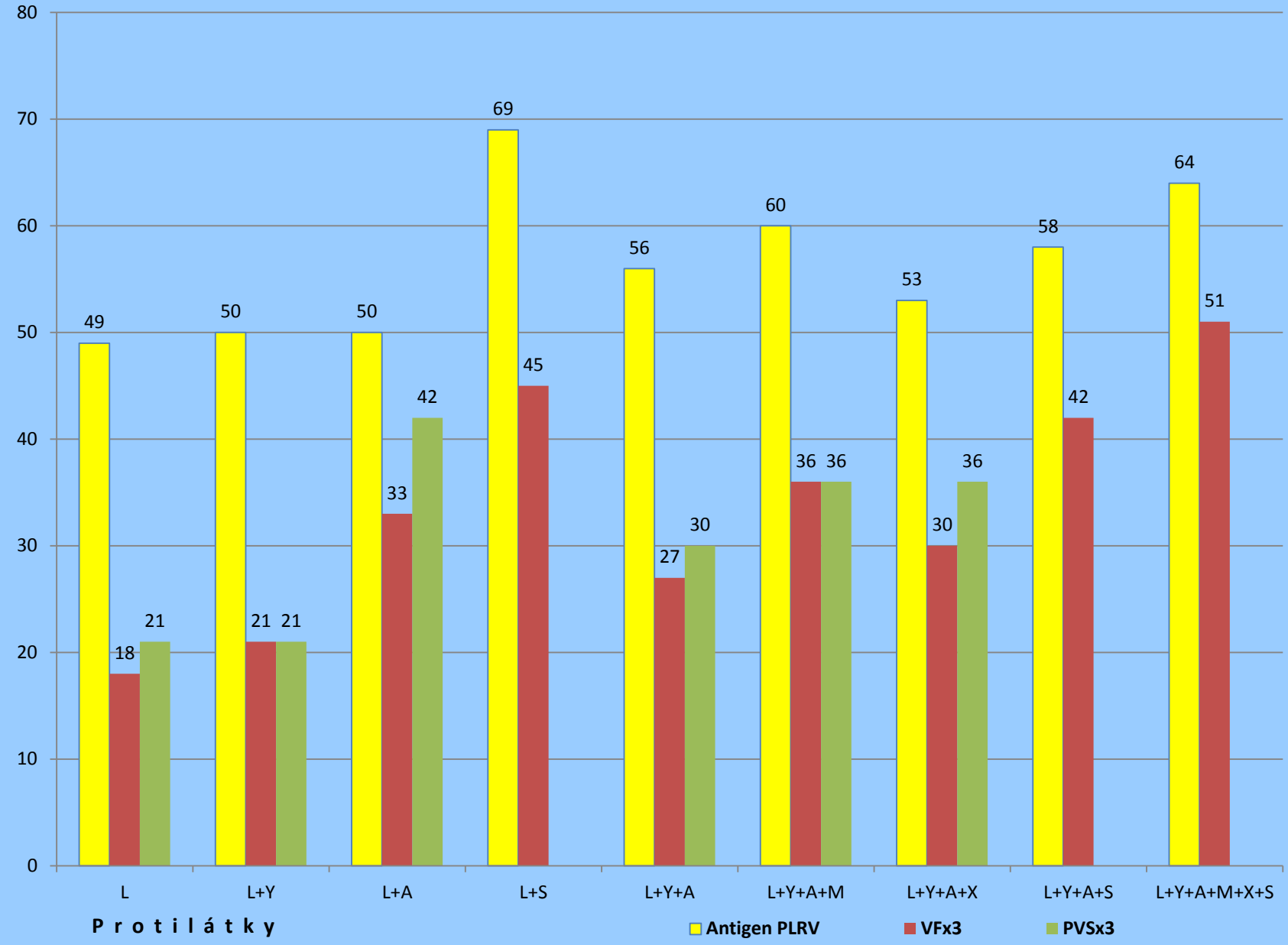
APAb

Hodnoty absorbance A405 u homologních antigenů a kontrol x 3

	L	Y	A	M	X	S
Homol. Antig.	490	1350	1020	970	1320	2500
VFx3	180	120	150	150	120	360
PVSx3	210	120	210	210	210	



Absorbance uměle polyvalentních protilátek s homologním antigenem PLRV, šťávou z bezvirových a PVS infikovaných rostlin bramboru po 60 min. inkubaci

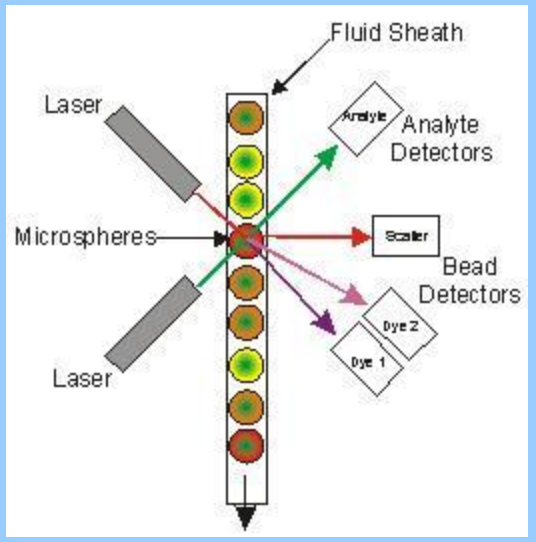


Shrnutí APAb:

Pro racionalizaci sériových testů lze s protilátkami vytvářet a účelově používat zejména bivalentní kombinace PLRV+PVY a PLRV+PVA,
případně trivalentní kombinace PLRV+PVY +PVA, zahrnující všechny "těžké" viry bramboru, ovšem bez odlišení jednotlivých druhů.

Kombinace uměle vícevalentních protilátek zahrnujících PVS je nezbytné dodatečně ověřit z hlediska jejich specifičnosti, případně interference s protilátkami dalších virů.

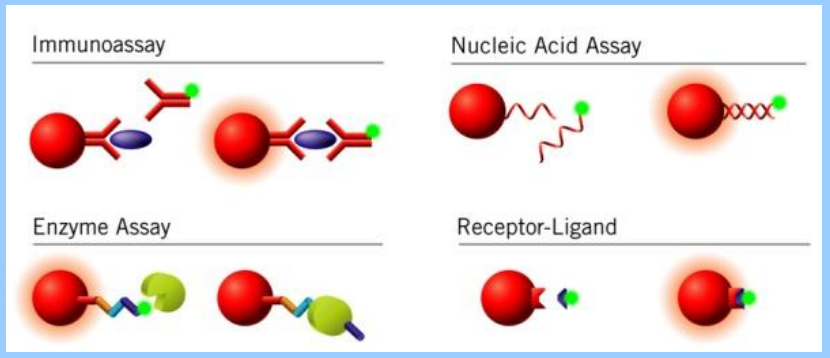
Luminex xMAP technology

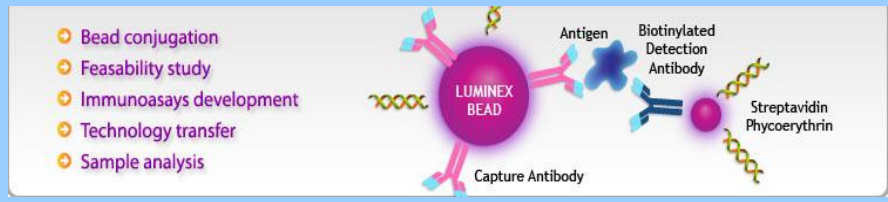


Similarities Luminex *x*MAP and ELISA

- Principle
- Sample preparation
- Antibodies

- Based on a multiple and simultaneous analysis, in the same well
- Fluorescent detection using a flow cytometer, microbeads dyed with fluorescent colours and lasers detection
- Different constructions (e.g. microbeads, 1st antibody; 2nd biotiniled antibody, streptavidine-phytoerytrine conjugate.





Serological reagents

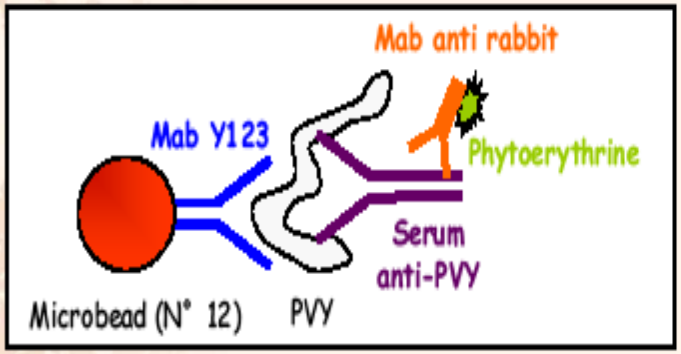
- Examples

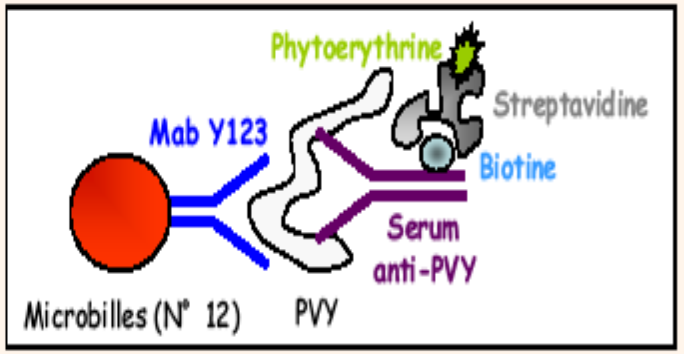
- Monoclonal antibodies (INRA/FN3PT): - Mab Y123 (polyvalent) - Mab Y^N
- Polyclonal antibody (INRA/FN3PT): - Serum anti-PVY

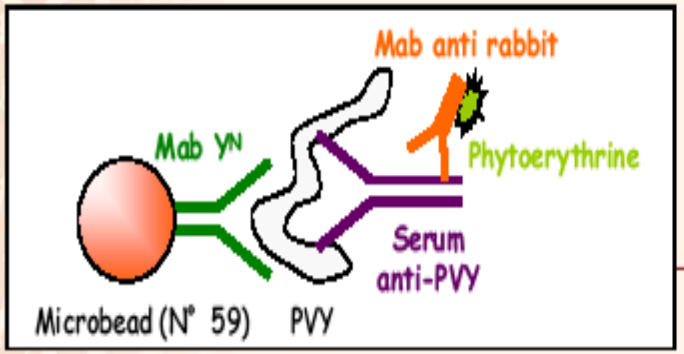
Purified virus

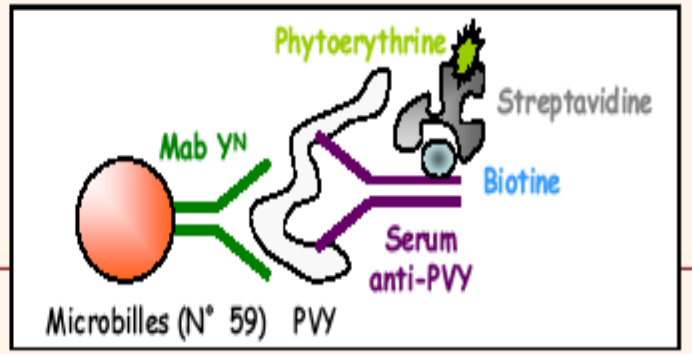
- $PVY^{NTN}NZ$

Different constructions



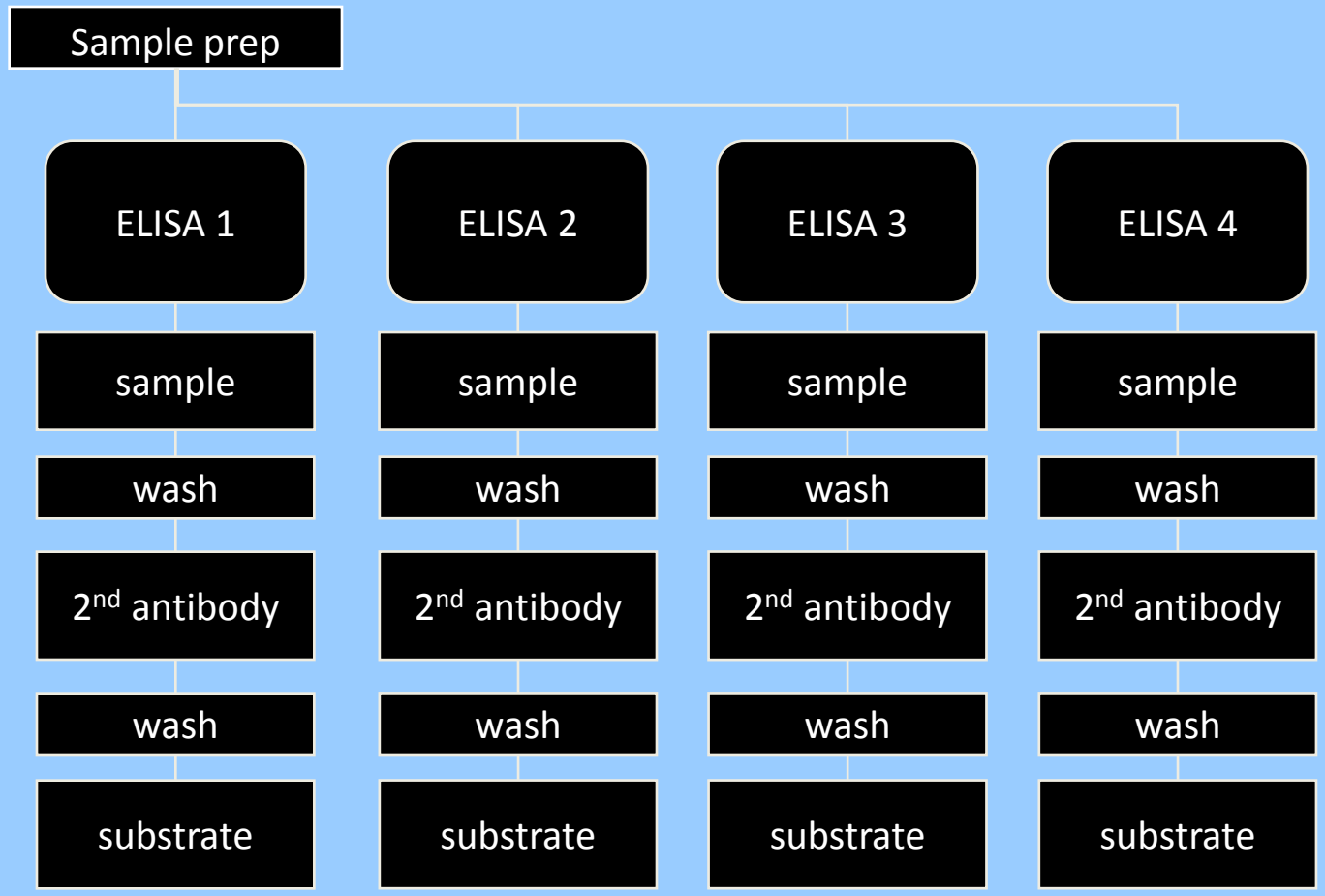




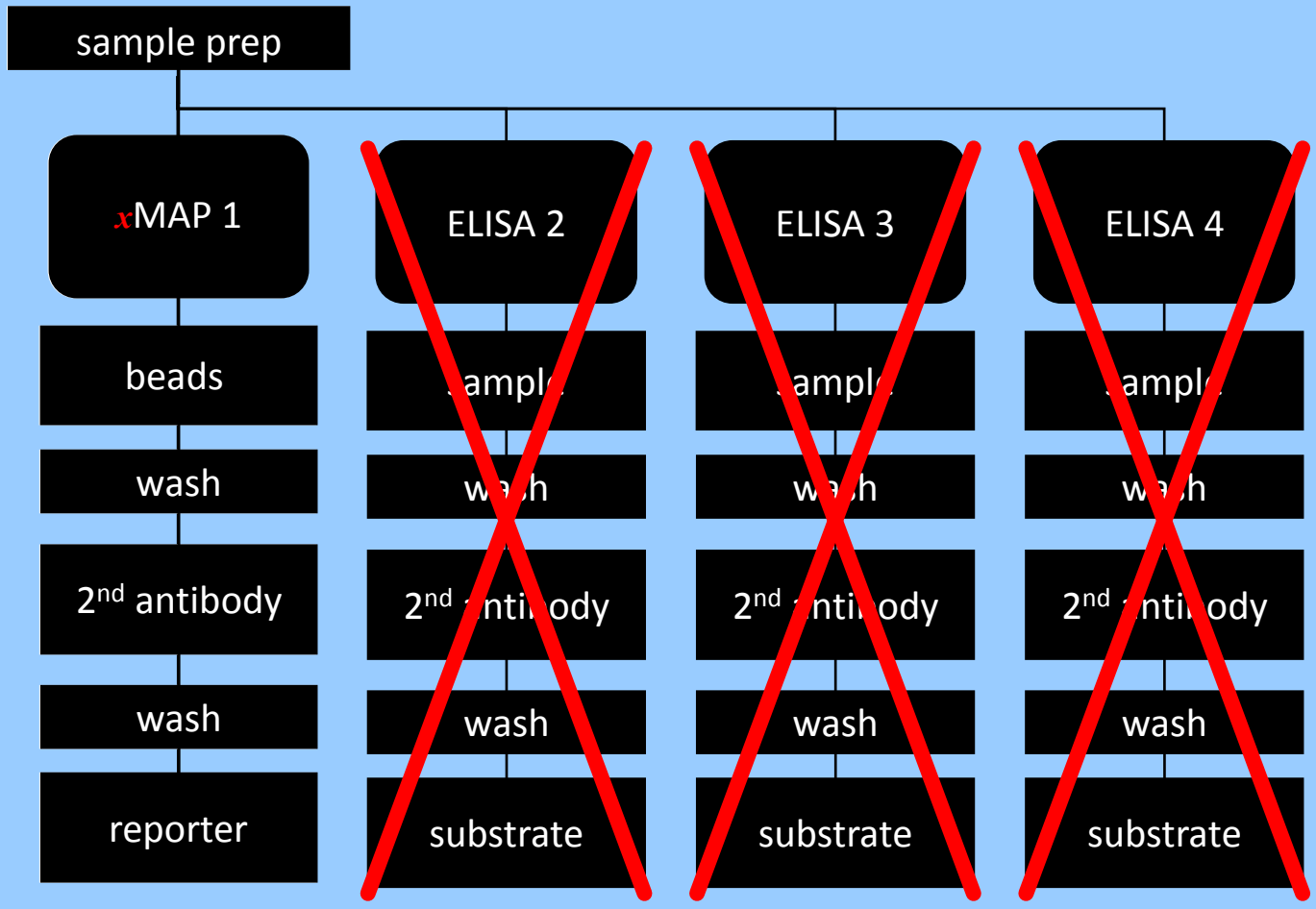


Glais, 2012

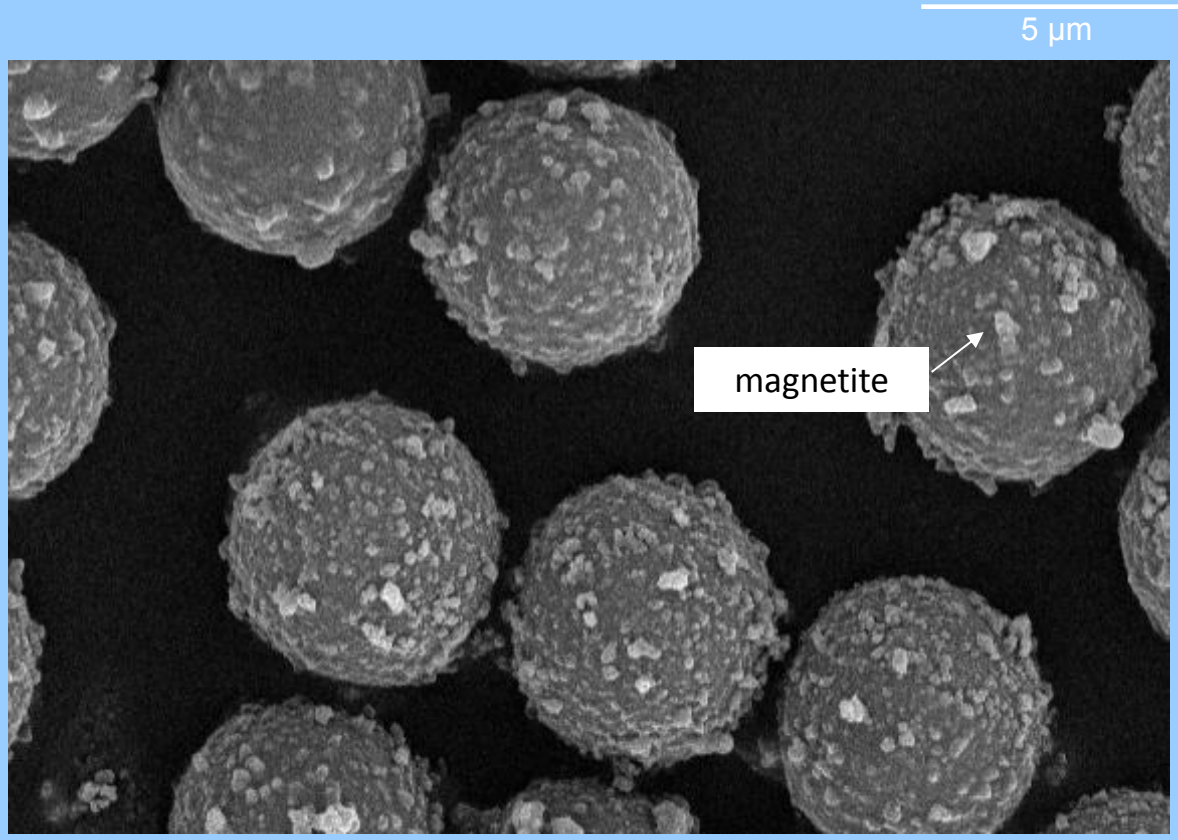
ELISA vs Luminex xMAP



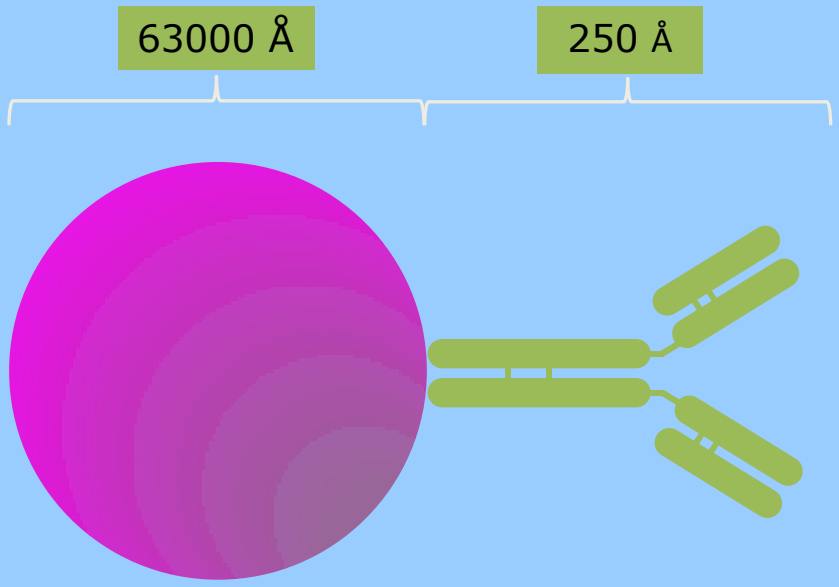
ELISA vs Luminex xMAP



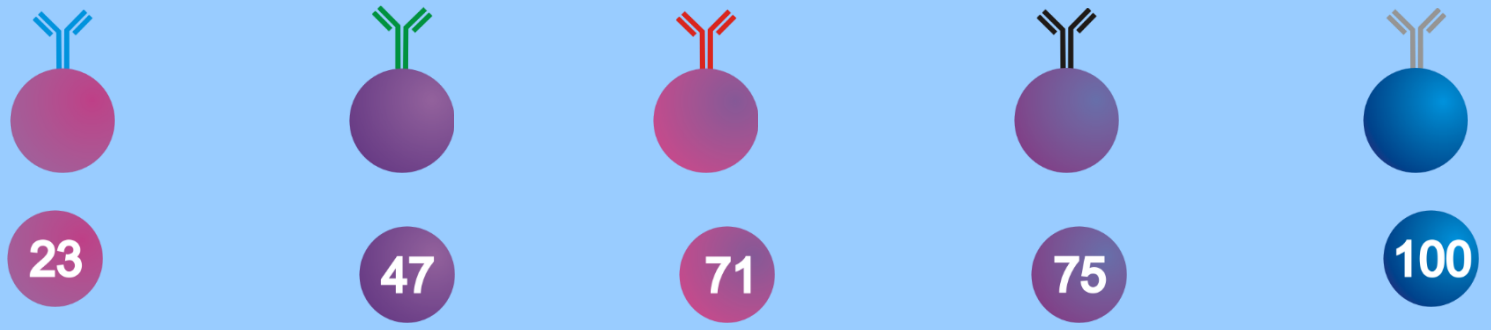
SEM, MagPlex-C Microsféry



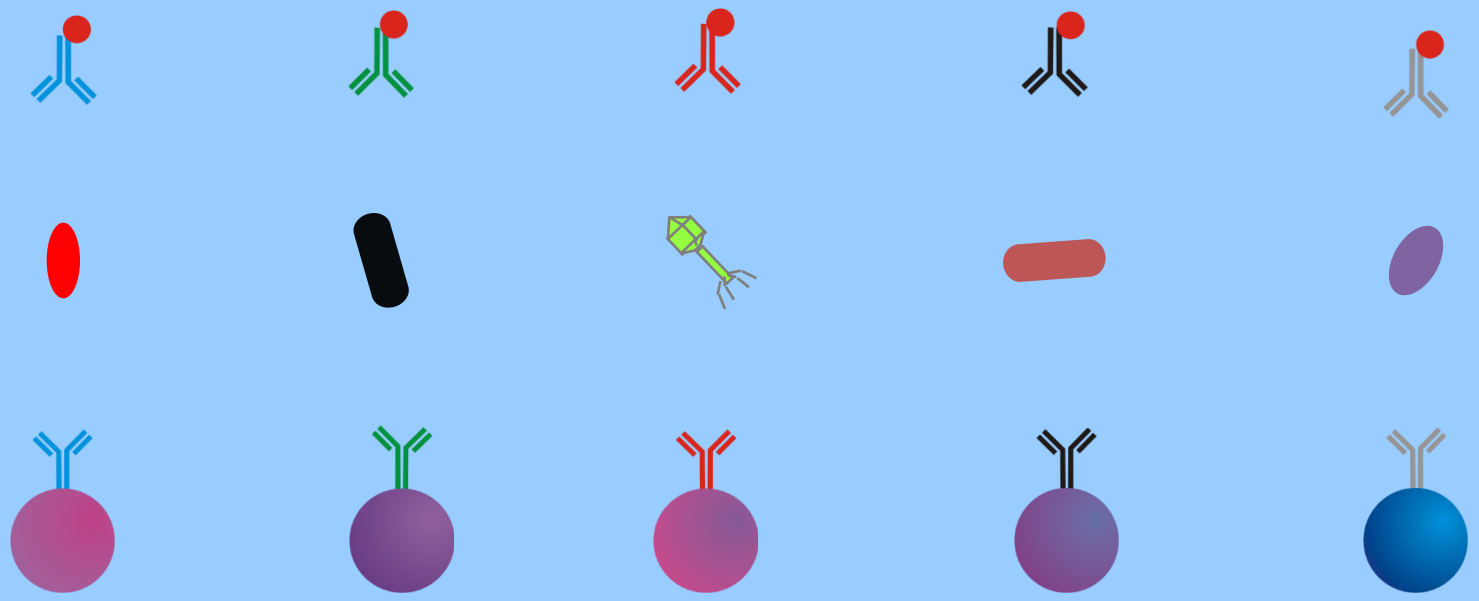
Technologie: mikrosféry



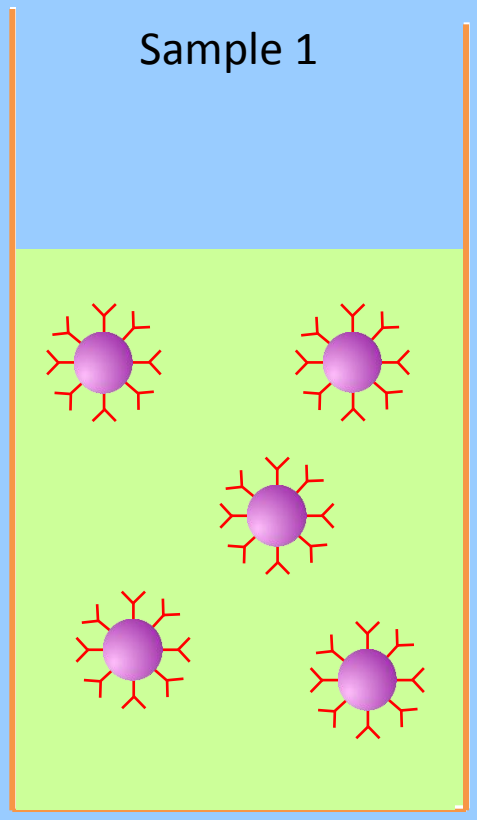
Technologie mikrosfér



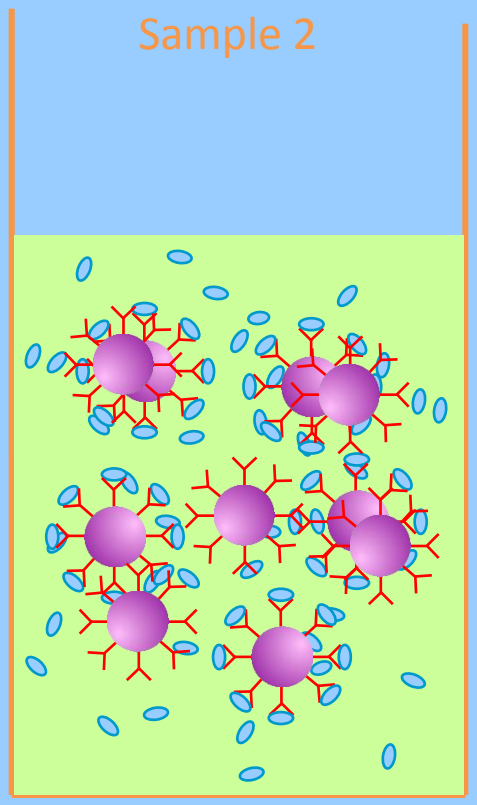
Technologie mikrosfér



Technologie: *x*MAP

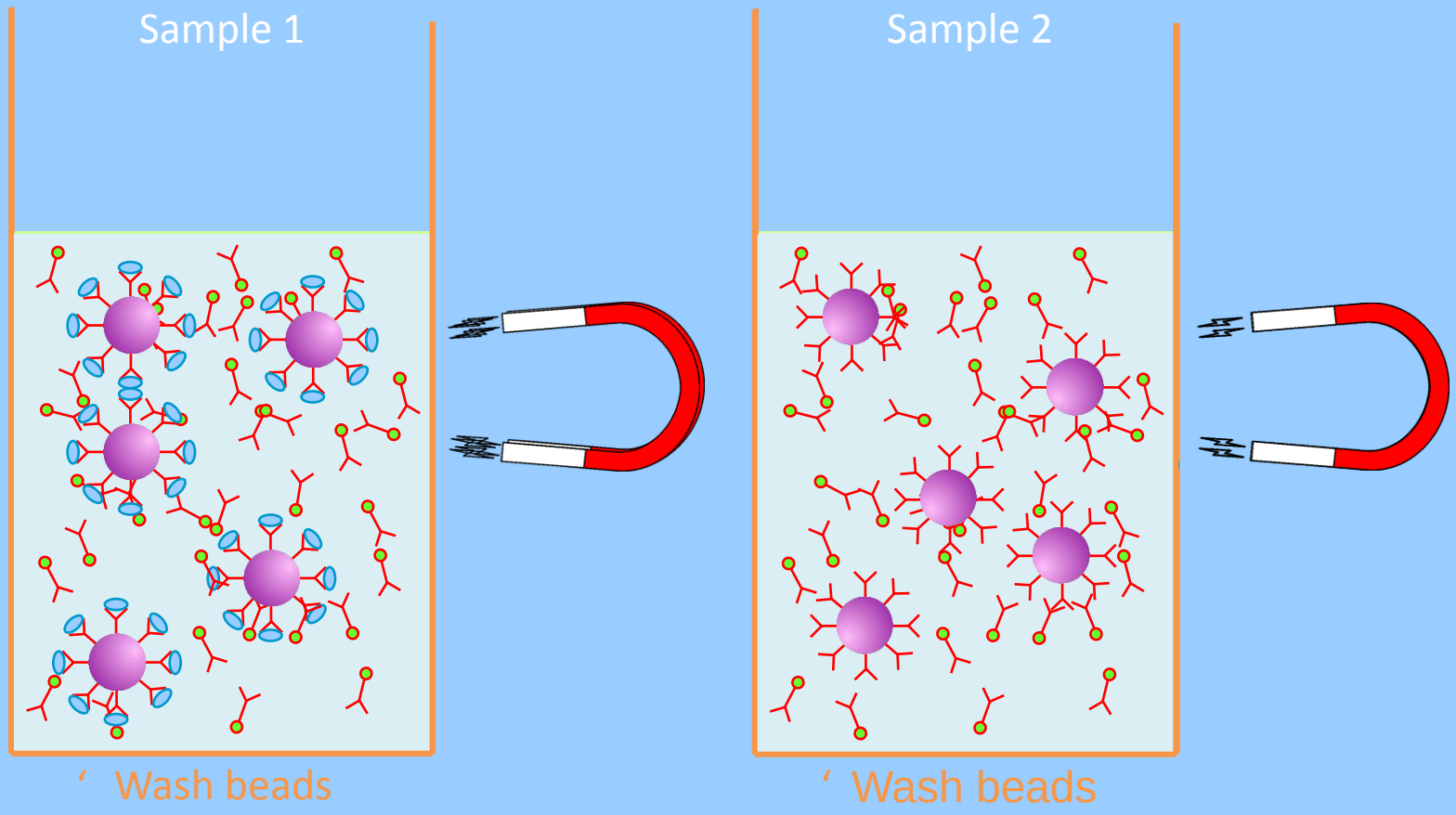


Add beads

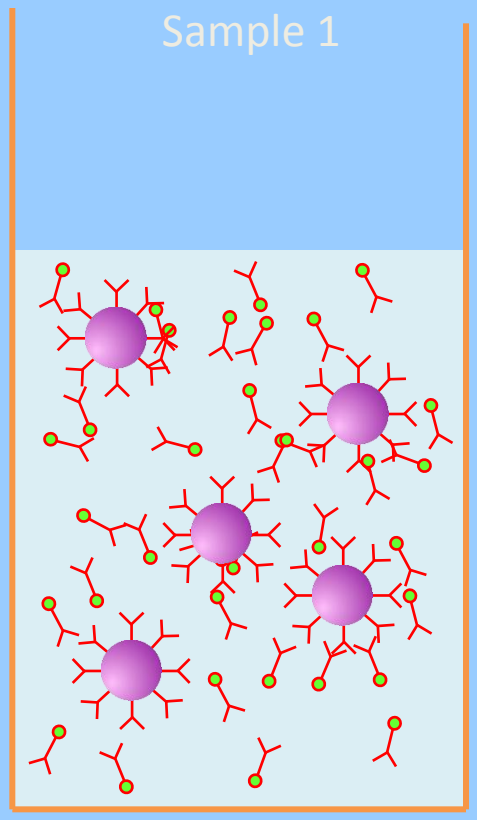


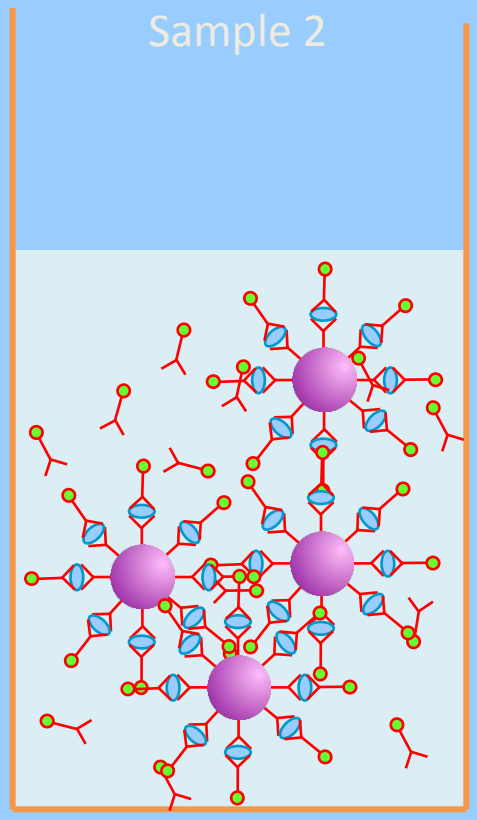
Add sample

Technologie: *x*MAP

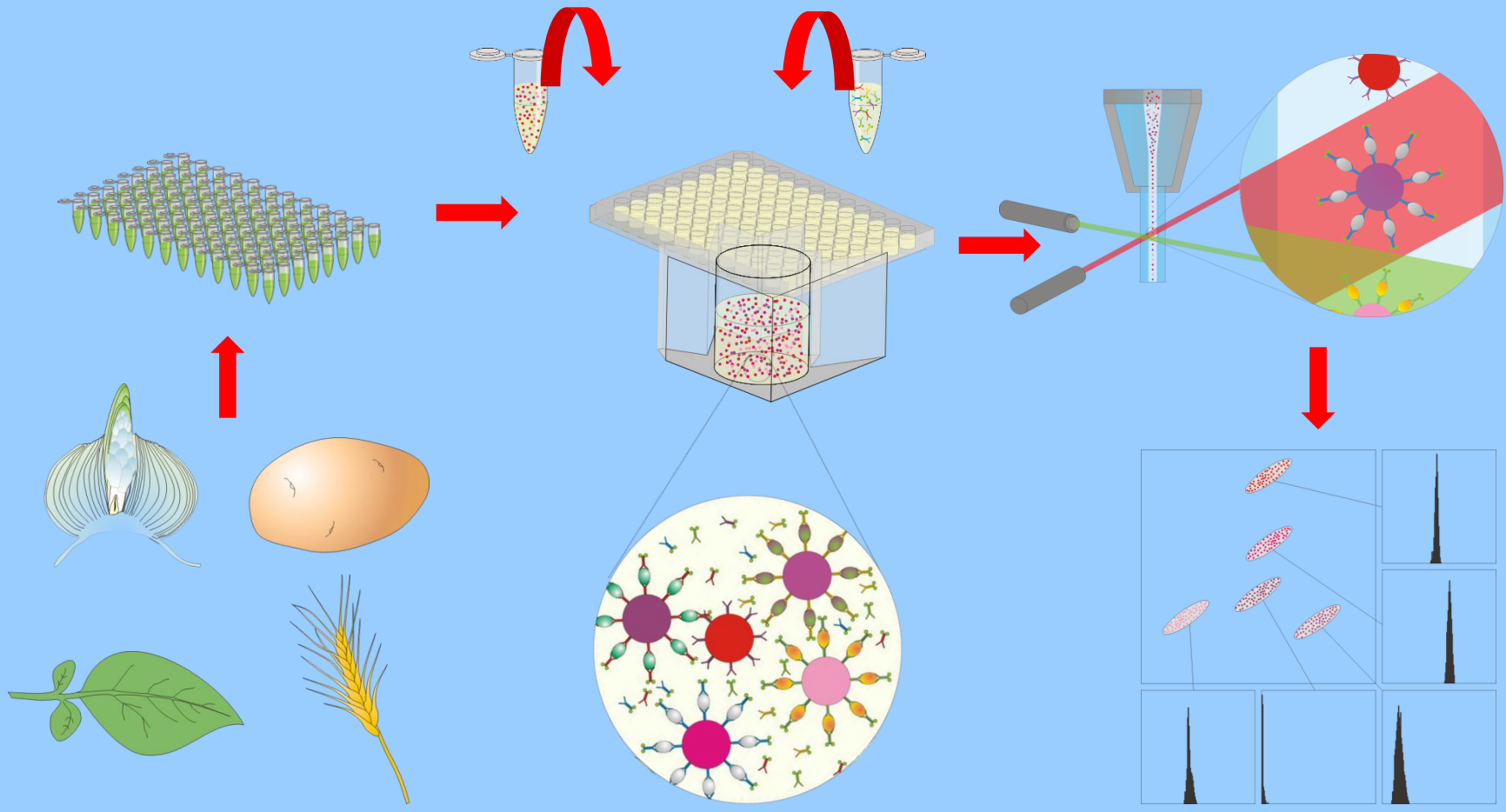


Technologie: *x*MAP





Procedura *x*MAP



Výsledky *x*MAP: Karafiát 6-plex

Sample	CarMV	CERV	CLV	CNFV	CRSV	CVMV
CarMV	268	11	11	13	11	18
CERV	13	166	12	16	11	19
CIRV	21	16	21	20.5	18	25
CLV	15	13	886.5	17	14	22
CNFV	17	14	15	58	13	22
CRSV	17	13	15	16	2404	21
CVMV	17	14	14	18	15	68
INSV	16	10	14	16	12	23.5
TSVW	15	12	13	14.5	13	20
KMV	17	14	14	16	13	20
mix PC	168	136	562	37.5	1976	42
Neg control	16	12	14	15.5	13.5	21
Treshold	22.6	18.1	22.3	22.3	19.5	27.4
background	16.4	12.9	14.3	16.25	13.35	21.15
SD	2.069591	1.72884	2.668749	2.003469	2.05548	2.082333
gain	3	3	3	3	3	3

CarMV – Carnation mottle virus
CERV - Carnation etched ring virus
CLV - Carnation latent virus
CNFV - Carnation necrotic fleck virus
CRSV - Carnation ringspot virus
CVMV - Carnation vein mottle virus

Výsledky *x*MAP: Karafiát 6-plex

Sample	CERV	CNFV	CRSV	CVMV	CarMV	CLV	CIRV
NC	57.0	428.0	46.0	57.1	53.0	67.0	44.0
NC	60.0	518.0	42.5	55.5	42.0	57.0	87.0
CRSV	71.0	3110.0	15845.0	99.0	83.0	6449.0	49.5
CVMV	48.0	2499.5	75.0	4593.0	91.0	11126.0	61.5
CRSV	68.0	3385.0	16910.0	101.0	78.0	6326.0	46.0
CNFV	134.5	3720.0	3356.0	1570.0	11688.0	14405.5	66.0
CNFV	233.0	3770.5	163.5	3657.0	15551.0	22675.0	35.0
CarMV	68.5	6202.5	70.5	87.0	10210.5	10243.5	64.0
NC	51.0	631.0	61.0	49.0	62.5	86.0	50.0
NC	70.0	469.0	56.0	64.5	50.0	72.0	64.0
CRSV	174.0	1335.0	17444.0	112.0	137.0	6666.0	46.0
CVMV	81.0	2245.0	105.0	5543.0	73.0	9829.0	90.0
CRSV	73.0	4211.0	183.0	86.0	59.0	4133.0	53.0
CNFV	114.0	2349.5	117.0	1044.0	14422.0	14868.5	44.0
CNFV	335.0	2599.0	75.0	2036.0	11690.5	1111.0	64.0
CarMV	71.0	6334.0	119.5	104.0	26046.0	11675.0	66.0
CVMV	90.5	4212.0	72.0	2074.0	15037.0	4379.0	40.5
CarMV	116.0	2589.5	101.5	113.0	17662.0	13196.0	53.0

Sample	CERV	CNFV	CRSV	CVMV	CarMV	CLV	CIRV
CLV	74.0	1659.5	10533.5	100.0	17231.0	9296.5	66.0
CLV	126.0	4410.5	11166.0	151.0	16880.0	13187.0	93.0
CVMV	80.0	4602.0	5838.5	4368.0	12318.0	8399.5	54.0
CarMV	48.0	111.0	48.0	52.0	6604.0	59.0	46.0
CERV	3232.0	165.0	57.0	73.0	100.0	62.0	59.0
CLV	73.5	78.5	55.0	72.0	83.0	11105.0	70.0
CVMV	73.0	4651.0	60.5	1913.0	16567.5	16162.5	72.0
CarMV	54.0	1262.0	56.8	87.5	17607.5	14421.0	46.5
CLV	68.0	1369.0	10093.0	86.5	14560.0	14263.5	51.0
CLV	86.0	4104.5	11926.0	106.0	15751.0	9217.0	83.0
CVMV	67.0	6630.5	75.0	5570.0	14442.5	9072.5	76.0
CNFV	38.5	356.8	47.0	78.0	135.5	29.0	80.0
CRSV	53.0	70.0	15667.0	104.0	49.0	56.0	34.0
CVMV	55.0	118.0	99.0	474.5	77.0	82.0	63.0
Gezond	53.5	791.0	51.0	68.0	44.0	65.5	50.5
CIRV	267.5	372.0	224.0	312.0	352.0	281.0	277.0

Výsledky *x*MAP: Kalanchoë

Sample	KMV		TSWV		INSV	
NC	✓	46.0	✓	54.0	✓	57.0
NC	✓	65.5	✓	67.0	✓	77.0
KMV pos	✗	175.0	✓	58.5	✓	62.0
KMV pos	✗	281.0	✓	66.0	✓	87.5
KMV pos	✗	489.0	✓	56.0	✓	57.0
TSWV pos	✓	62.0	✗	2512.5	✓	77.0
TSWV pos	✓	67.0	✗	2553.0	✓	87.0
TSWV pos	✓	70.5	✗	3956.0	✓	96.0
NC	✓	65.0	✓	69.0	✓	73.0
NC	✓	73.0	✓	71.0	✓	70.0
KMV pos	✗	217.0	✓	55.0	✓	54.0
KMV pos	✗	324.0	✓	54.0	✓	56.0
KMV pos	✗	375.0	✓	60.0	✓	61.5
TSWV pos	✓	88.0	✗	1980.0	✓	79.0
TSWV pos	✓	69.5	✗	2611.5	✓	82.5
TSWV pos	✓	65.0	✗	4494.0	✓	127.0
Plant neg	✓	60.0	✓	48.0	✓	50.0
KMV pos	✗	433.0	✓	94.0	✓	74.0
TSWV pos	✓	66.0	✗	1437.0	✓	78.0
INSV	✓	54.5	✓	115.0	✗	9589.5
Treshold		159		154		164

KMV – Kalanchoe mosaic virus
TSWV – Tomato spotted wilt virus
INSV – Impatiens necrotic spot virus

Výsledky vlastních experimentů od 2011 ve VÚB

Procedure

- 50 µl extract + 50 µl buffer/beads
- Incubate 15 minutes at room temperature
- Wash
- Buffer + add 2nd antibody
- Incubate 30 minutes at room temperature
- Wash
- Add SA-PE for 15 minutes
- Measure and analyse

Originál Primediagnostics

Beads
(043,044,046,053,054,056)
Y L A X M S

2nd biotinylated antibodies

Positive controls

Streptavidin – R- Phycoerythrin

Buffers
SEB (PBS, ovalbumin, PVP, Tween)
Microsphere working solution
(PBS, milk powder, Tween)
Wash Buffer
(PBS, milk powder, Tween)

know how a akomodace (optimalizace) postupu.

Magnetická podložka

- **Optimální ředění jednotlivých komponentů**
- **Možnost záměny pufrů**
- **Inkubace – třepačka Biosan (RPM, čas, teplota)**

- **Vymývání (počet, opakování)**

Izoláty jednotlivých virů (rostlinky in vitro, skleníkové rostliny)

Porovnání dvou extrakčních pufrů

pufr	virus	protilátky PVY	PLRV	PVA	PVX	PVM	PVS
SEB	1	120	**854**	237	39	17	25
	2	2470	35	350	48	63	51
	3	330	36	**327**	59	25	54
	4	122	38	135	66	**15055**	84
	5	122	26	157	**21854**	20	18
	6	66	20	166	221	63	**13125**
	VF	78	20	105	35	18	19
	p	70	19	147	17	8	12

pufr	virus	protilátky PVY	PLRV	PVA	PVX	PVM	PVS
Tris	1	55	741	96	18	10	11
	2	2501	20	210	25	22	38
	3	54	29	758	24	20	19
	4	85	40	139	124	15037	29
	5	78	23	82	18047	19	28
	6	79	26	121	250	49	11326
	VF	54	18	84	28	17	25
	p	81	12	103	22	6	15

Manuál : Postup přípravy vzorků pro Luminex xMAP s manuální separací

•Beads – šejkr TK3S; ředění v BWB pufru dle optimálních parametrů;
nanesení na paletu po 50 ul/jamka

•Antigen (ředěný cca 1:10 v/v v extrakčním pufru SEB nebo Tris)
nanesení na paletu po 50 ul/jamka, promísení pipetou

•Inkubace – šejkr Biosan 600 ot/min, 60 min., pokojová teplota

•Vymývání – Přenesení palet na magnetický separátor, prodleva 1 min. Odcáknutí; aplikace 100 ul pufru WB / opakovat 2x

•Biotinil. protilátky – ředění v WB pufru dle optimálních parametrů; nanesení na paletu po 100 ul/jamka

•Inkubace (60 min.) a vymývání (1x)

•SAPE – ředění v WB pufru dle optimálních parametrů;
nanesení na paletu po 50 ul/jamka

•Inkubace (20 min.)

•Ředění produktu ve WB pufru – 75ul/jamka –šejkr 5 min

•**Zpracování v kalibrovaném přístroji Luminex 100/200**

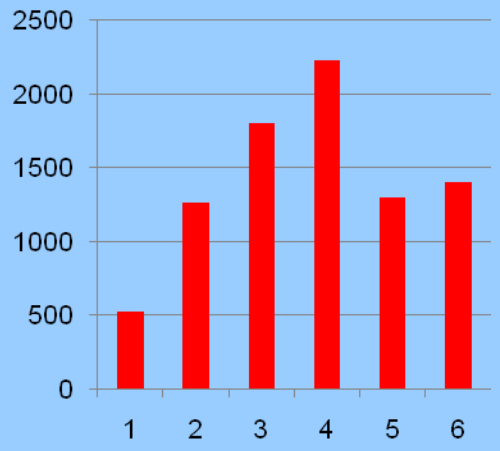
Kompletní přístrojové vybavení pro diagnózu LUMINEX


Luminex
User:
Log Off
Help
XPONENT
SOFTWARE SOLUTIONS
Home
Samples
Batches
Results
Protocols
Maintenance
Admin
Current Batch
Saved Batches
LIS Results
Reports
Now displaying batch "New Batch 10".
Instructions
View current run statistics and analyte progress per well. For more analysis and result options for this batch, select Details or go to Saved Batches when batch acquisition completes.
Results
Statistic: Median
Analyte: Select an Analyte
Current Well
1,D5
Single Step
Well Sample Run Sta... PVY PLRV PVA PVX PVM PVS
1,A5 Unknown25 Ok 142 185 198 18330.5 280 175.5
1,B5 Unknown26 Ok 171 199 216 23426 317 323.5
1,C5 Unknown27 Warning 94 86 161 16766 3478.5 24329
1,D5 Unknown28
Plate
1
Count
Doublet Discriminator
(7500,15500)
Classification 2
Classification 1
Date Message Code
3/10/2011 1:06:45 AM Sample Preparation started. 0
3/10/2011 1:06:51 AM Sample Preparation completed with status SUCCEEDED. 0
3/10/2011 1:06:51 AM Detect started. 0
Save Image
Progress
Well Report
System Status
Connection: Connected
Command: Detect
System State: Active
Thursday 3/10/2011 1:07 AM
Batch Running
Pause
Stop
Eject
Delta Cal Temp: -1.1°C
Sheath Pressure: 7.8 psi
XY Status: D5, 24.5°C
Laser: Ready, 3 hr 59 min remain
Region Events: 39/sec
Total Events: 48/sec

Reactivity of diffrent potato virus isolates in multiplex Luminex xMAP

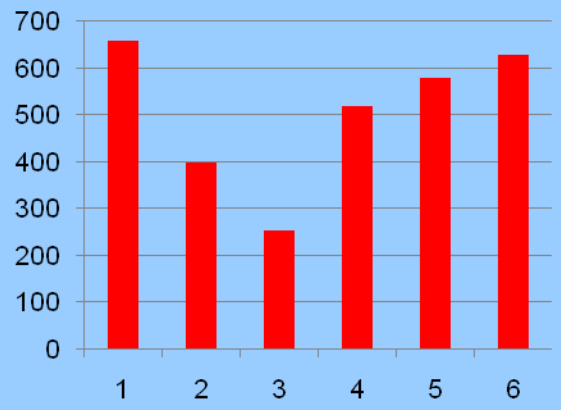
PVY

Treshold : **162**



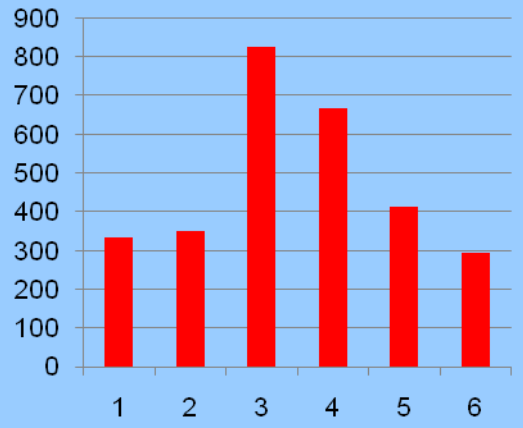
PLRV

140

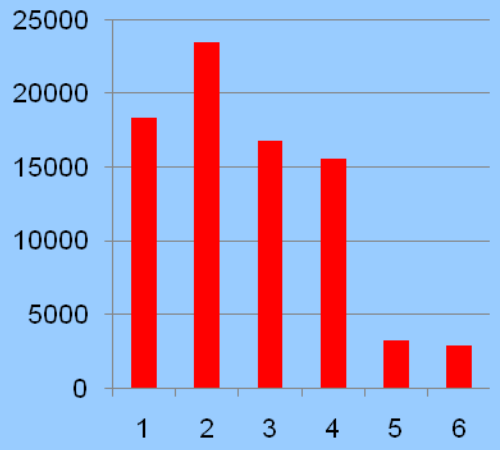


PVA

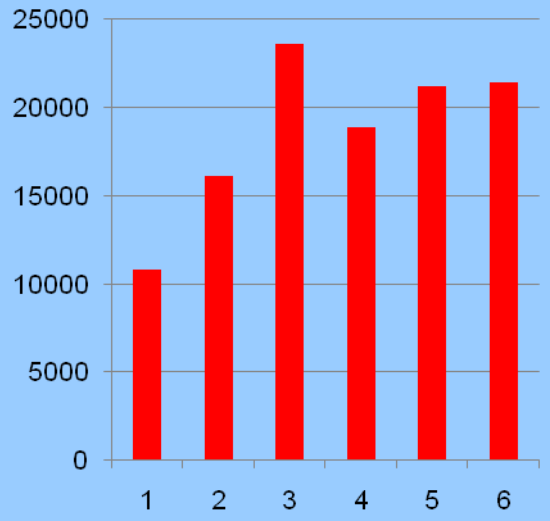
298



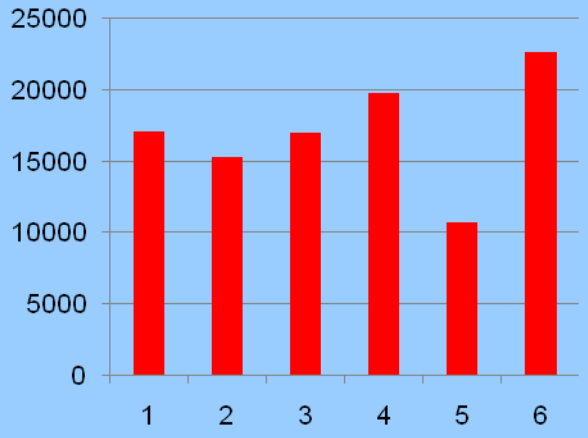
PVX



PVM



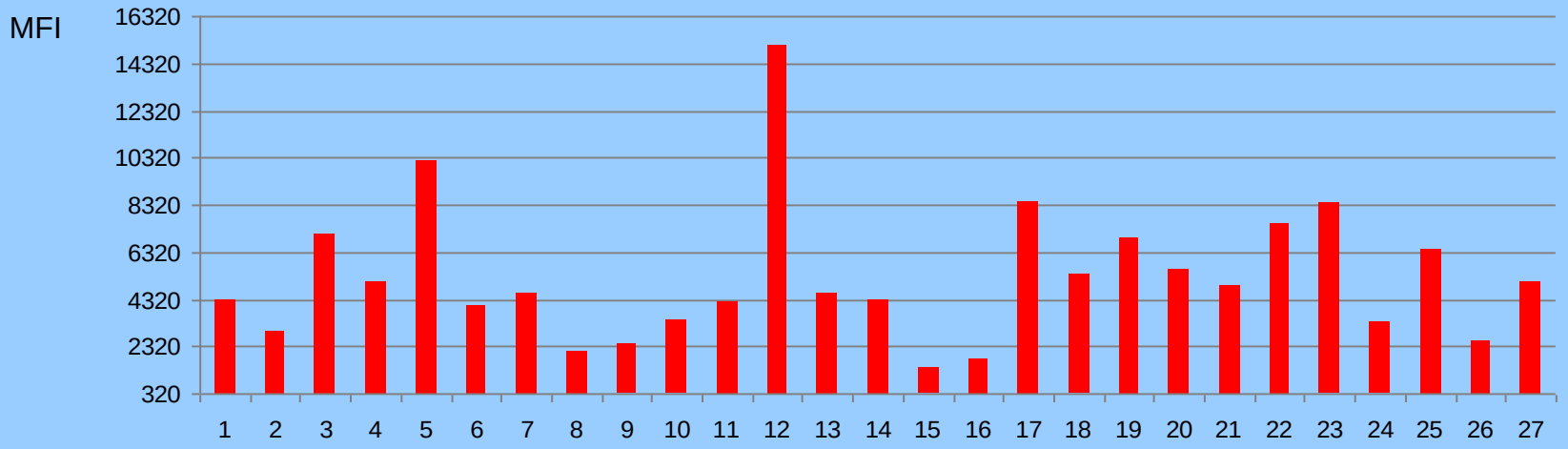
PVS

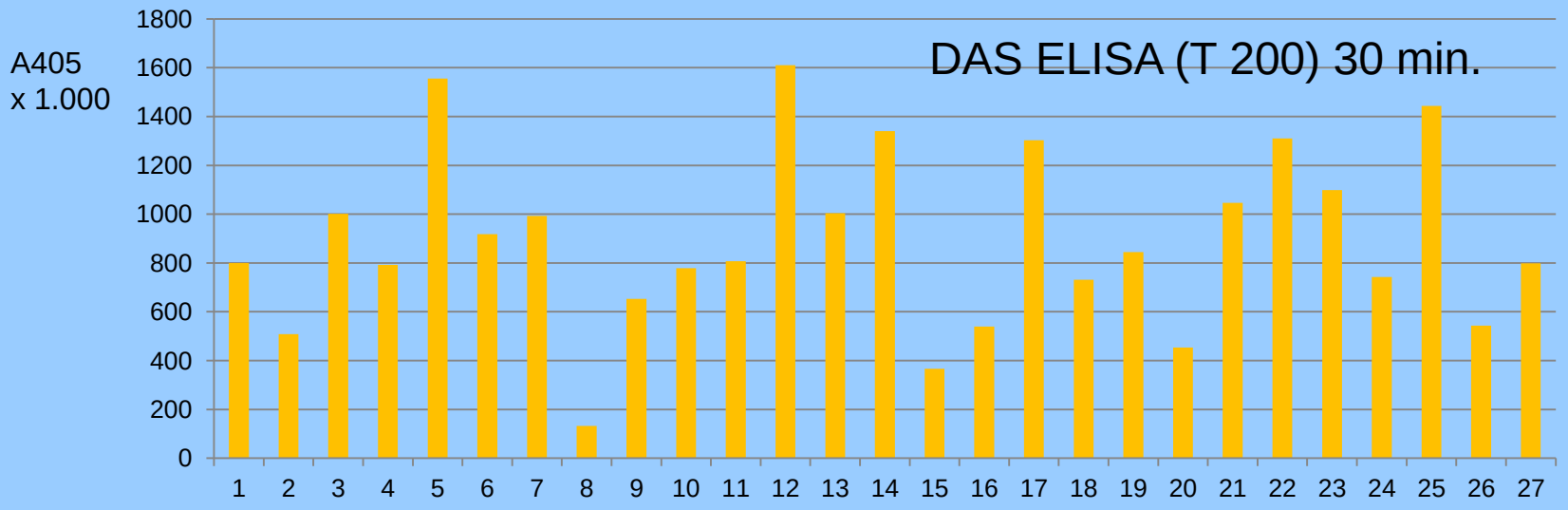


Treshold: **320** **488** **230**

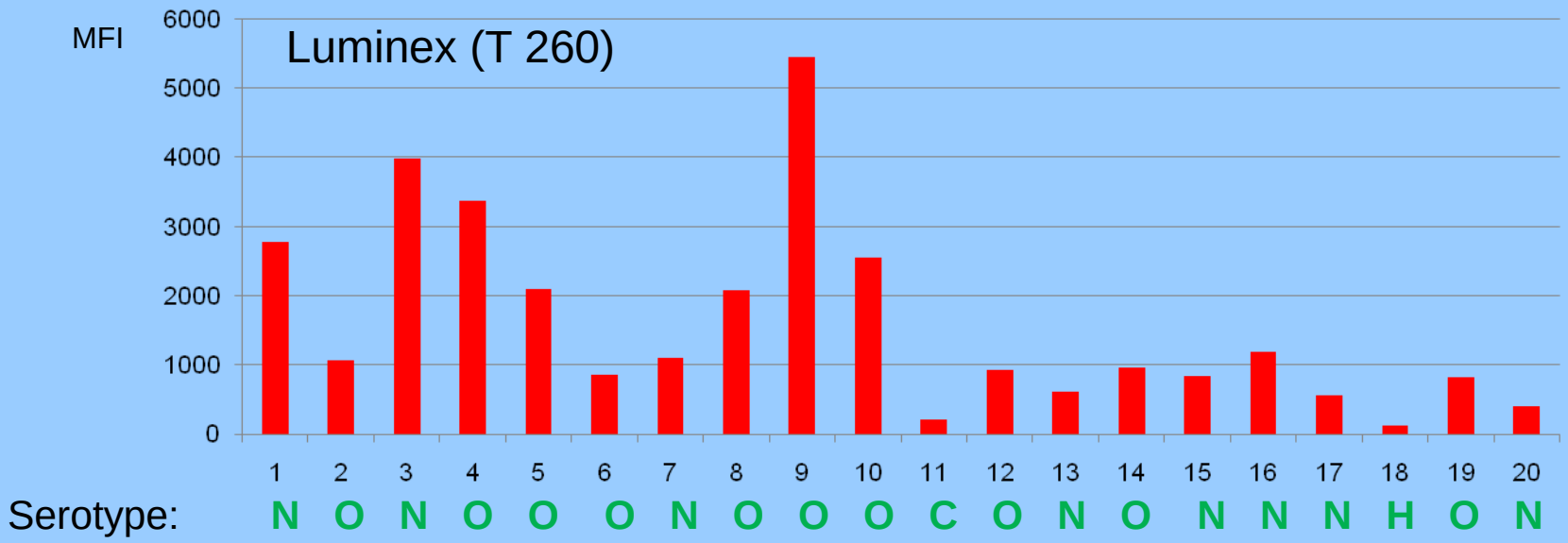
Reactivity of different PVX isolates in Luminex xMAP and DAS ELISA

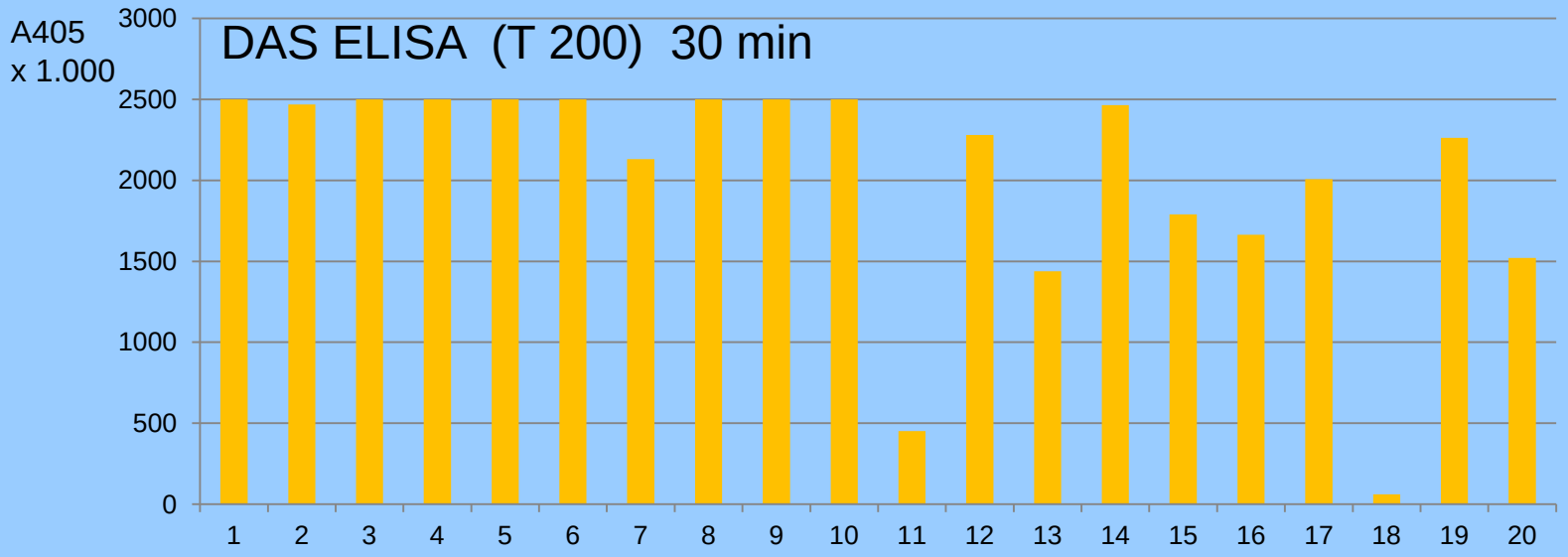
PVX isolates Luminex (T 320)

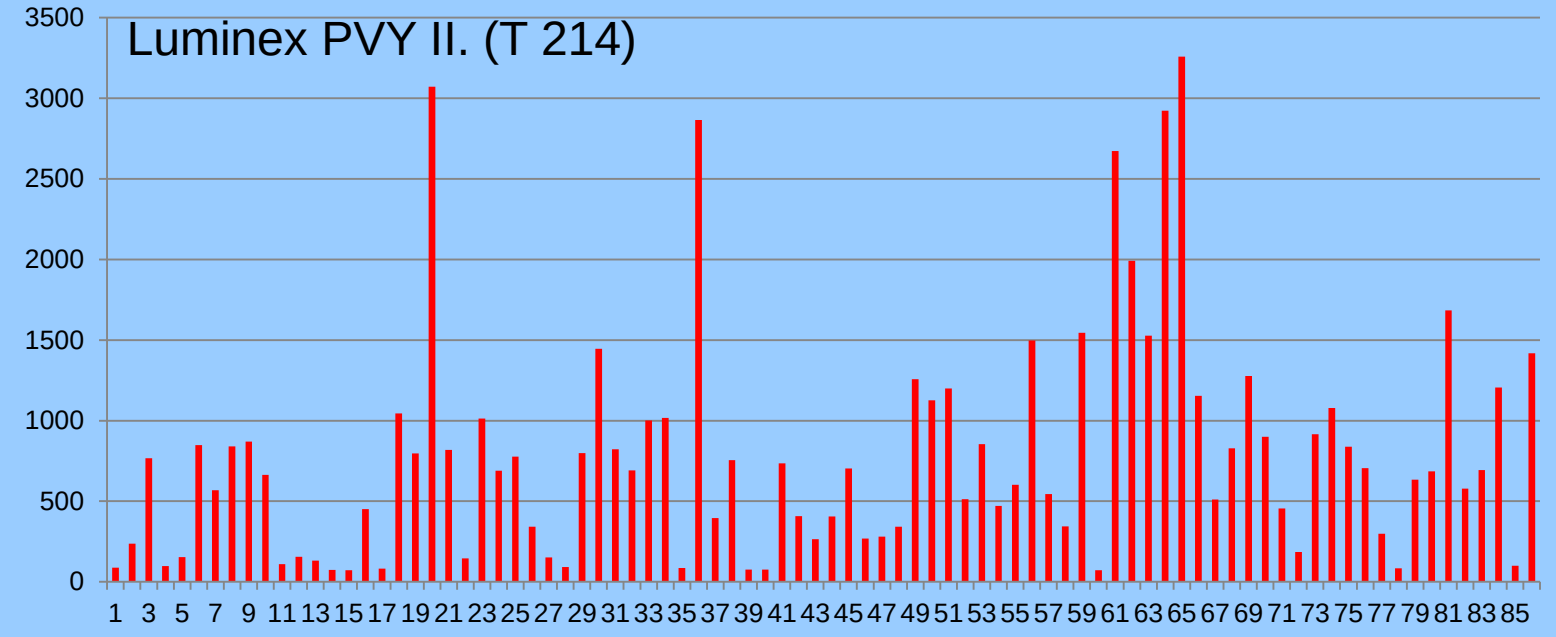


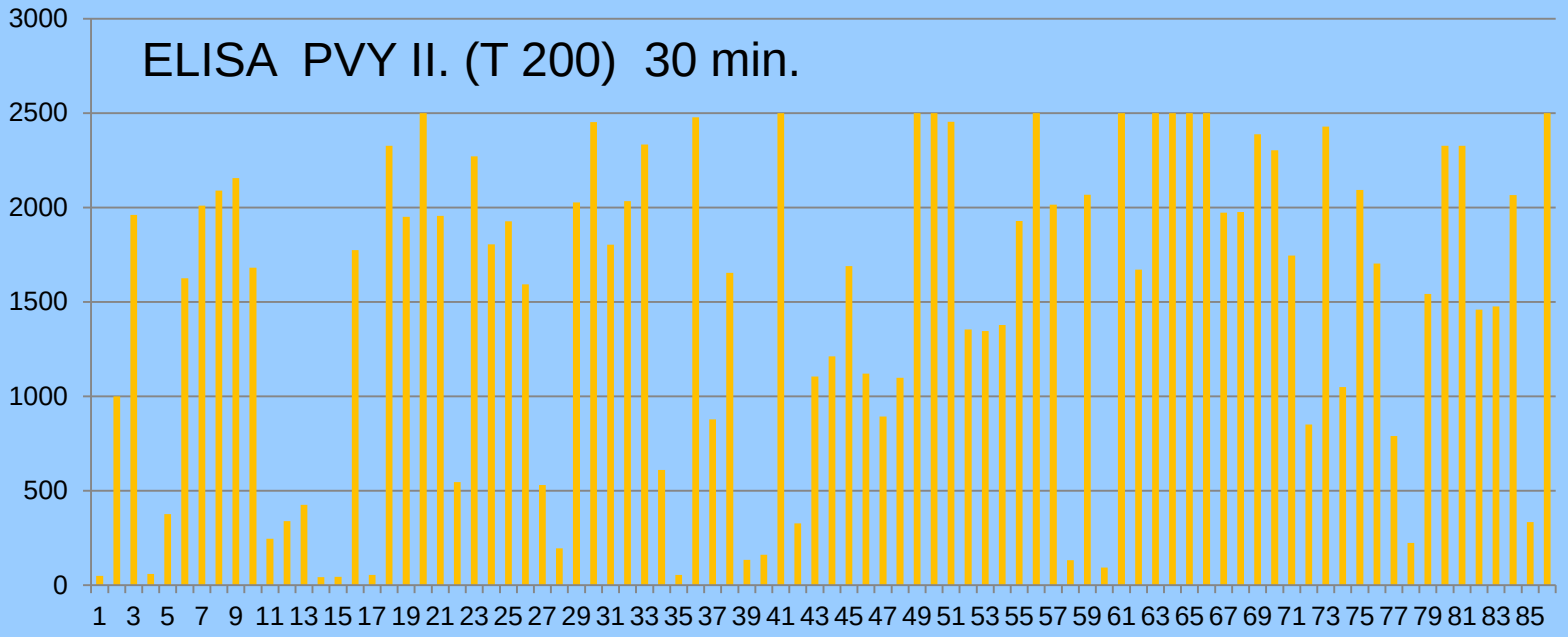


Reactivity of different PVY isolates in Luminex xMAP and DAS ELISA

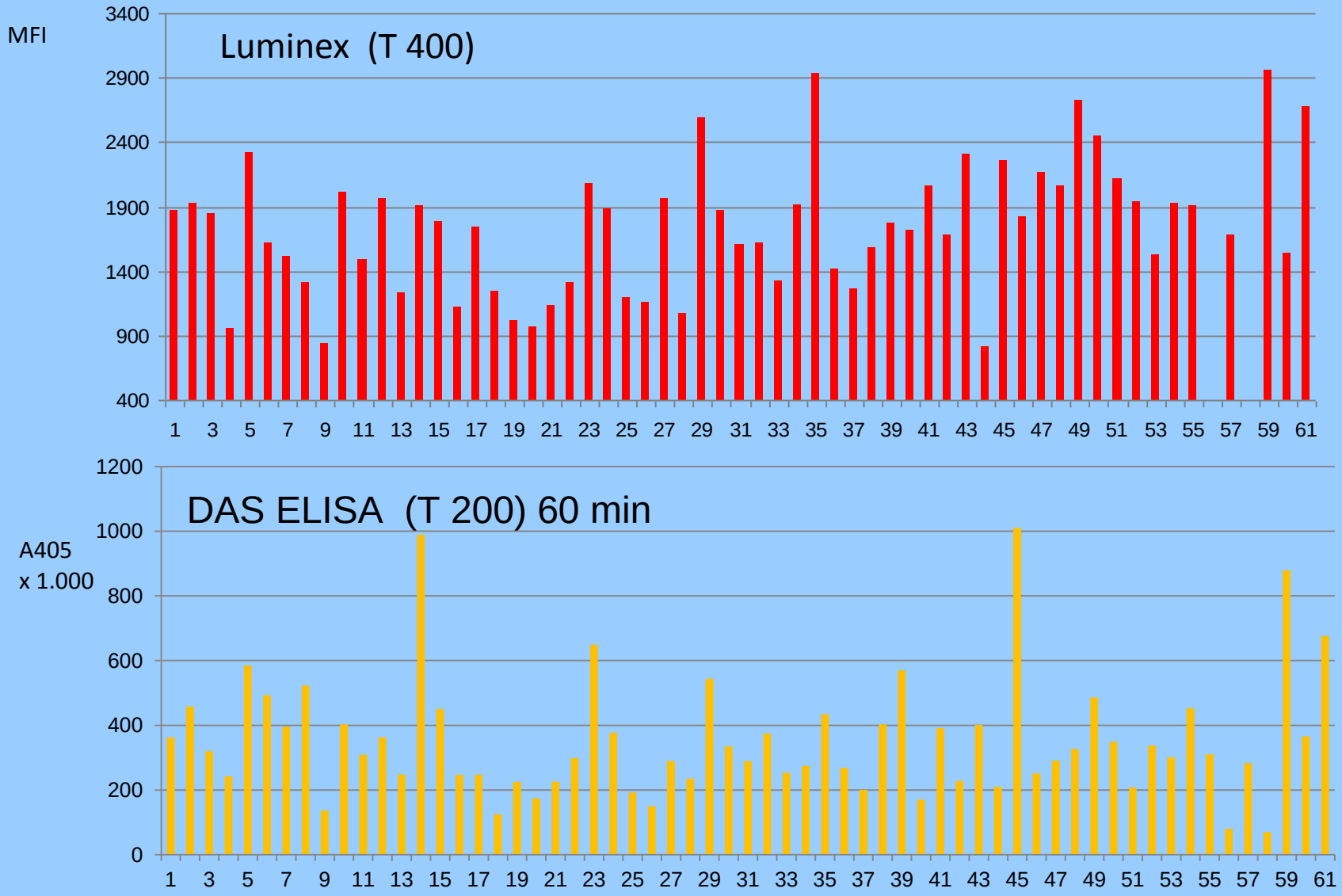




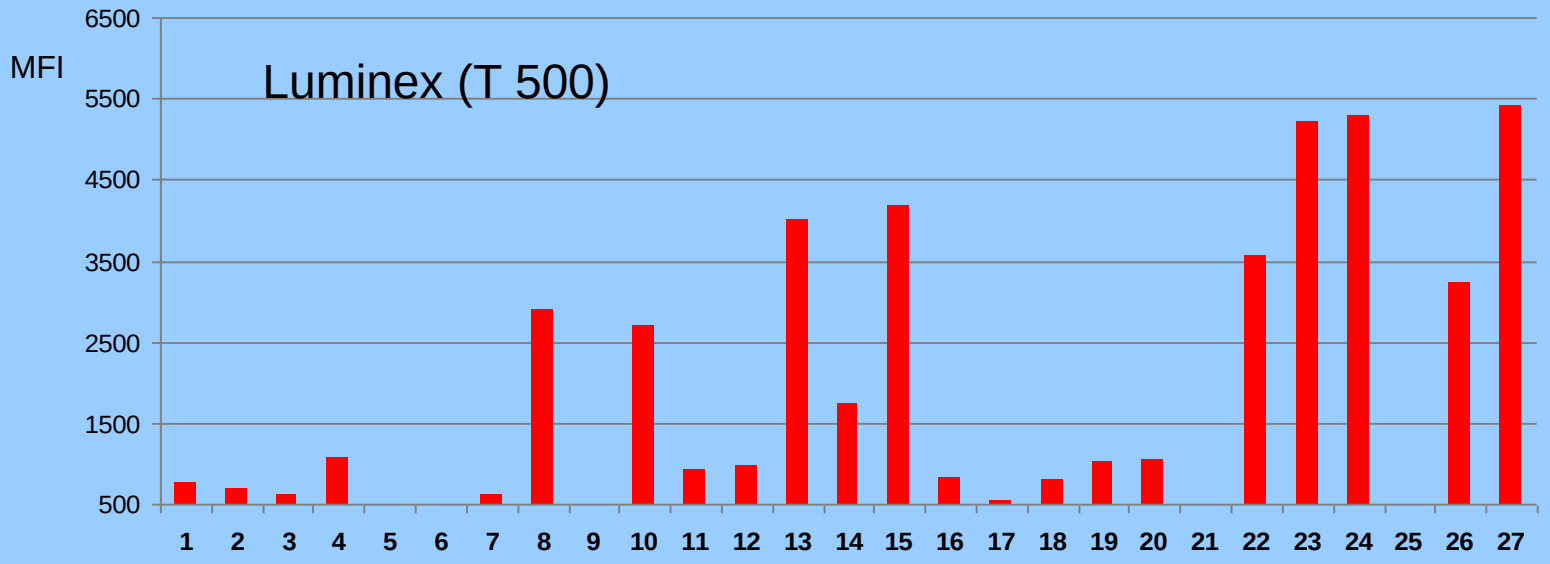

Luminex PVY II. (T 214)
3500
3000
2500
2000
1500
1000
500
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77 79 81 83 85

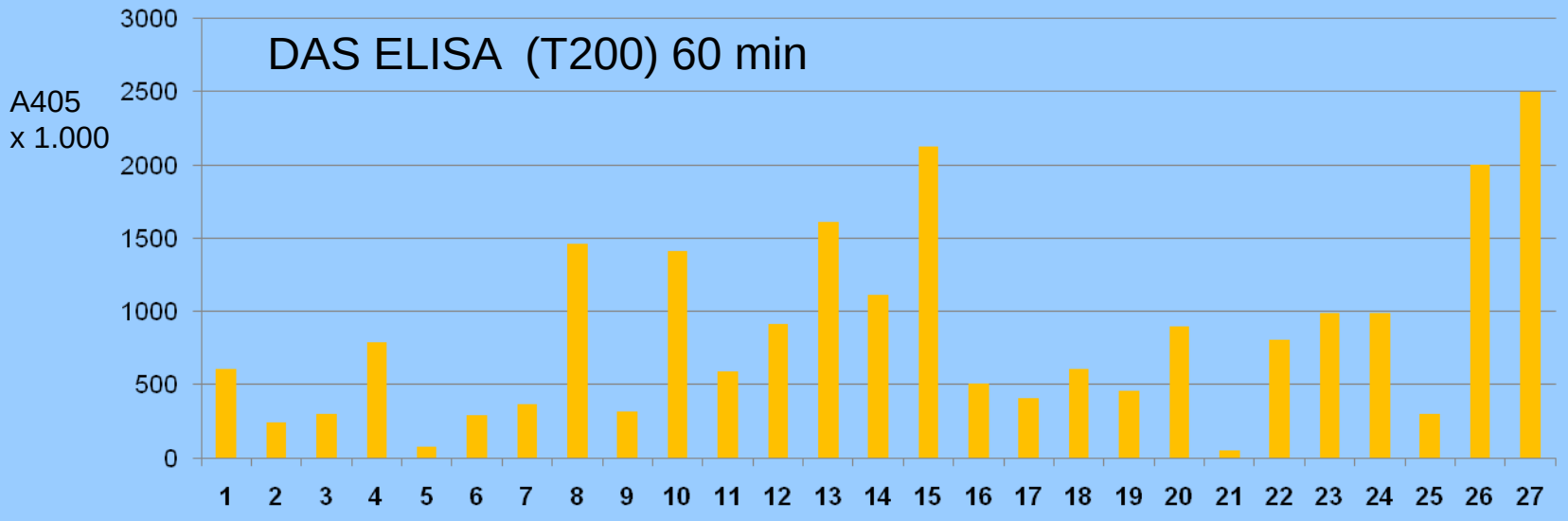

ELISA PVY II. (T 200) 30 min.
3000
2500
2000
1500
1000
500
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77 79 81 83 85

Reactivity of different PLRV isolates in Luminex xMAP and DAS ELISA

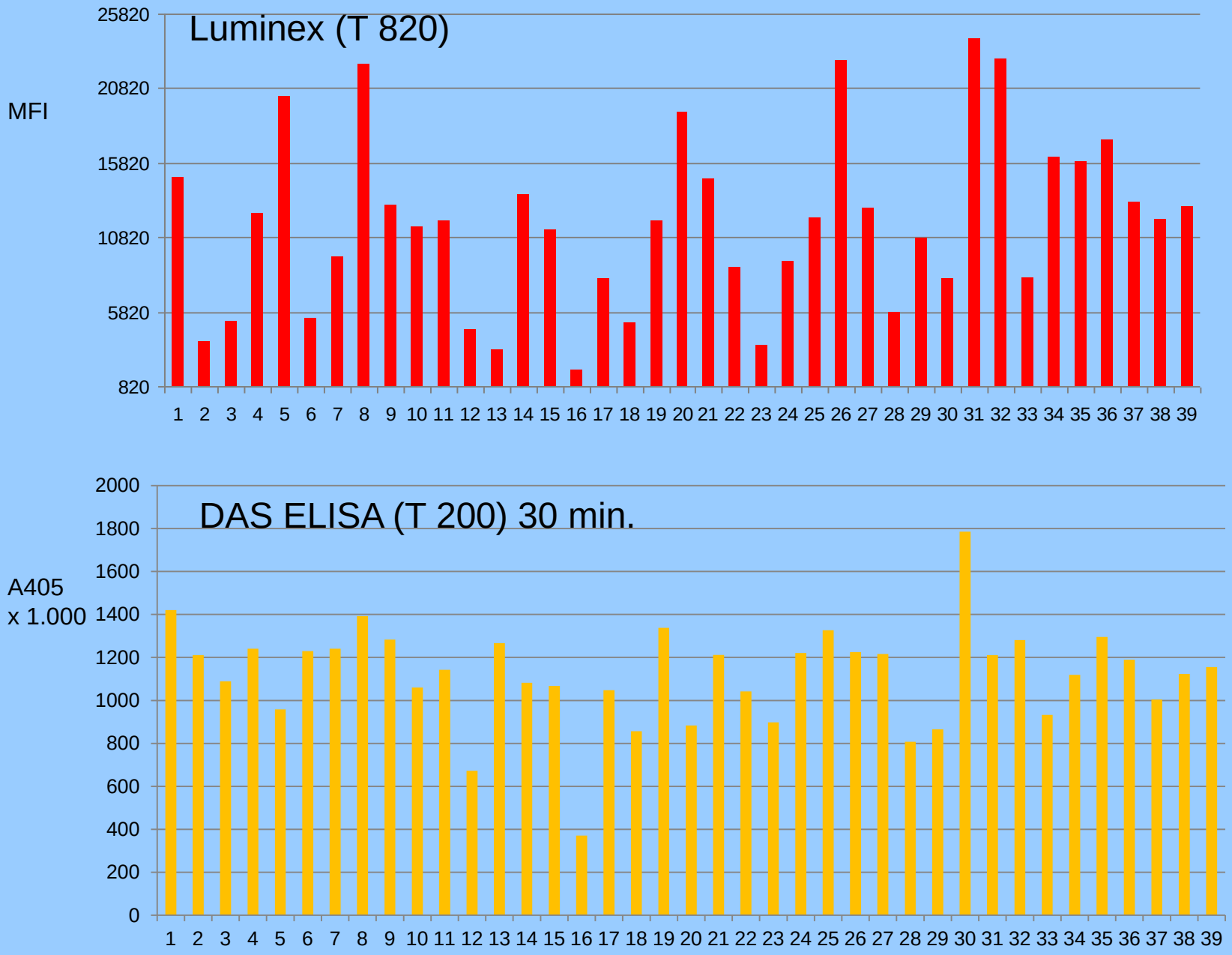


Reactivity of different PVA isolates in Luminex xMAP and DAS ELISA





Reactivity of different PVM isolates in Luminex xMAP and DAS ELISA



Influence of two incubation modes on fluorescence intensity in Multiplex x-MAP of six potato viruses

		PVY		PLRV		PVA	
Mode	sample	average	range	average	range	average	range
a)	virus - inf.	**304**	**143-478**	**114**	**88-143**	**140**	**128-172**
	virus-free	**66**		**61**		**60**	
b)	virus - inf.	**3416**	**1867-4871**	**703**	**459-1187**	**257**	**186-288**
	virus-free	**118**		**55**		**64**	

		PVM		PVX		PVS	
Mode	sample	average	range	average	range	average	range
a)	virus - inf.	**2760**	**2480-3145**	**6000**	**2514-12693**	**2600**	**1148-4285**
	virus-free	**61**		**73**		**56**	
b)	virus - inf.	**1364**	**1266-1482**	**5473**	**1675-12858**	**1670**	**715-2382**
	virus-free	**21**		**27**		**44**	

a) Incubation time: 40 + 60 + 30 min.

b) Incubation time : 80 + 90 + 60 min.

PVM Izolát	PVY	PLRV	PVA	PVX	PVM	PVS	Elisa PVM
1	144	201	252	265	14811	16444	1420
2	131	202	245	250	3809	204	1211
3	152	234	266	285	5222	180	1089
4	148	200	252	282	12429	210	1241
5	150	239	267	267	20252	998	958
6	143	197	249	279	5431	177	1229
7	141	202	239	292	9490	189	1241
8	152	242	245	281	22409	187	1393
9	133	187	237	271	12953	4536	1283
10	139	191	248	263	11527	5626	1060
11	133	199	249	254	11930	174	1142
12	136	194	250	280	4661	163	672
13	142	201	248	278	3277	166	1266
14	170	373	280	307	13631	210	1082
15	126	192	226	247	11368	5616	1068
16	122	180	227	246	1906	3212	371
17	129	192	238	256	8061	7952	1048
18	140	192	249	281	5117	178	856
19	137	203	244	251	11955	1930	1338
20	144	193	251	266	19234	9939	883
21	134	190	248	265	14696	193	1212
22	138	196	254	276	8840	5735	1042
23	132	182	221	260	3581	1616	898
24	132	199	235	256	9195	15294	1221
25	139	212	237	266	12108	267	1327
26	139	208	245	263	22709	8881	1225
27	167	307	271	276	12752	12656	1216
28	130	183	240	268	5761	7575	807
29	212	300	325	325	10756	6538	865
30	132	206	236	261	8039	17417	1785
31	120	183	217	239	24133	229	1210
32	131	178	235	270	22831	15149	1281
33	133	193	231	246	8074	5042	932
34	147	208.5	247	297	16235	7633	1119
35	138	1073	234	239	15899	13044	1295
36	136	186	243	283	17389	231	1189
37	134	745	242	265	13202	159	1004
38	134	197	243	269	11972	169	1123
39	124	177	236	267	12922	165	1155

Barbora E3 – Luminex xMAP multiplex

č.	PVY	PLRV	PVA	PVX	PVM	PVS
1	98.5	49	262	31.5	15	32
2	158	32	266	37	14	26.5
3	114	54	339	25	14	22
4	6604	35.5	285.5	28	16	28
5	137	23.5	273	41	10	13.5
6	85.5	65	263.5	27	7.5	23
7	4728	42	264	33	132.5	25746.5
8	181.5	36	255	28	8	29
9	148	11	213.5	29	13	24
10	153.5	18	243.5	36	7.5	13.5
11	144.5	31	327.5	26	14.5	31
12	299	NaN	277	45	12	19
13	226	27	173	19	14	40
14	780.5	17.5	237	28	14	16.5
15	331	33	277.5	44	4.5	283
16	127	28	256	27.5	11	14
17	122	33	210.5	34	7	11
18	142	35	231.5	17	3	197
19	114.5	21.5	237	21.5	18.5	15
20	92	NaN	229.5	21	11	50.5
21	114.5	21.5	221.5	22	24.5	19
22	126	14	224.5	23.5	6	12
23	114	27	255.5	28	9	99
24	131	13	259	24	9	23.5
25	90.5	27	196.5	17	133	25407
26	91	38.5	191	23	11	52
27	142	32	181	23.5	10	14
28	110.5	39	228	28	5	12.5
29	94.5	18	201	21.5	7	14
30	119	30	226.5	28.5	84	17761
31	210.5	19.5	229	44.5	19	14.5
32	110	26	201.5	26.5	12	18.5
33	60	43	237	27.5	11	19
34	96.5	22	232	32.5	177.5	21437
35	2378	34	219	32	28	3786
36	1491.5	14	250.5	18	162	28118
37	111	25	231	19.5	9	40
38	72	60	222.5	26	11.5	9
39	73	25	163	31	12	19
40	103	23.5	226	15	37	18389.5
41	38	NaN	131	19.5	8.5	6
42	4569	28	200	28	18	77
43	207	36	254	15.5	13	9
44	111.5	34.5	256	32	19	479
45	124	25.5	243.5	26	7	18
46	131	90.5	297.5	30	9	20
47	79.5	37.5	214	18.5	17	13
48	138	14	262	18	14	77
č.						
49	115	29	252	24	11	15
50	106	16	216.5	21	14	13
51	89	6	203.5	18	9.5	12
52	131	28	202	22	7	22.5
53	131	28	267	22	22	5955
54	102.5	22.5	249.5	42	21	29
55	6415	40	189	18	14	17.5
56	105	19.5	205	39	10	15
57	114	24.5	145	26.5	10	28
58	153.5	17	207.5	19	7	13
59	144	33	219	23	21	42
60	177.5	31.5	206.5	26	48	23
61	87	23	206	30	6	33.5
62	110	38	243	33.5	11	20.5
63	125	20	183.5	30	10	23.5
64	102	12	198	37.5	54	19087
65	109	21	216	14	10	35
66	96	32.5	245.5	18.5	15	14
67	4558.5	26	179	30	19	10
68	115	46	184	11	11	21
69	69	27	207	20	54	22626.5
70	6659	18	281	33	28	11697
71	110.5	30	273.5	29	96.5	23446
72	314	24	252.5	31	8	247
73	53	17	125.5	9	6	6
74	99.5	40	219	29	16	13
75	159	45.5	237	42	8	15.5
76	127	13	201	28	9	14
77	60	29	152.5	30	57.5	18583
78	1263	18	140	17	15	32.5
79	4754	24.5	262.5	43.5	50	24388
80	89	15.5	147	17	13	12
81	84.5	16	158	14	11	39
82	91	11	168.5	24	11	167
83	114	13	173	25	8	13
84	92.5	12	132.5	22	8	240
85	87	35	164	22	9	13
86	82	12	170.5	27.5	12	13.5
87	82.5	24	185	27	17	14
88	107	13.5	241	21	8	13
89	48	11	139	19.5	30	20630.5
90	131	17.5	165	14	9	22.5
91	86	25	188	39	183	26249.5
92	2113	44	262.5	36	18	42
93	231	1679.5	275.5	63	31.5	50
94	65	35.5	1228	26	6.5	13.5
95	72.5	119	254	215	16362	136
96	125	48	153	23859	82.5	58.5
(3xMFI)	370	201	682	303	*200*	*300*

Comparison of ELISA and LUMINEX
(greenhouse plants, postharvest 2012)

Virus	Sum.	pozit. pozit.	neg. neg.	pozit. neg.	neg. pozit.
∑	2209	353	1846	5	5
PLRV	374	33	336	3	2
PVY	421	88	330	0	3
PVA	304	5	298	1	0
PVM	304	20	284	0	0
PVX	351	19	331	1	0
PVS	455	188	267	0	0

Criterium (new method vs. reference method)

[1)]Accuracy – probability of finding exact results (++ and --/∑x100)
(353+1846/2209) **99,55%**

Specificity – true negative rate (--/∑neg.x100)
(1846/1856) **99,46%**

Sensitivity – true positive rate (++ /∑pos.x100)
(353/363) **97,25%**

Summary Multiplex Luminex xMAP vs ELISA
Post Harvest Testing 2012 – greenhouse potato plants survey

Total number of samples tested		**9**	
Total cases (negative or positive for six potato viruses)		**2.209**	
Number of positive detections matching for both methods		**353**	**(16.0%)**
Number of negative detections matching for both methods	**1.846**		
Number of detections Luminex negative – ELISA positive		**5**	
Number of detections Luminex positive – ELISA negative		**5**	
Total non-matching cases		**10**	
Percentage of non-matching cases		**0,45%**	
Total matching cases		**2.199**	
Percentage of matching cases[1)]		**99,55%**	

Shrnutí *x*MAP

- Redukce pracovních a materiálových nákladů
- Citlivost srovnatelná s ELISA a IF
- Rychlost : 3 hodiny oproti 2 dnům uELISA
- Snadné použití
- Dostupné pro řadu objektů

Technologie *x*TAG : TSPE

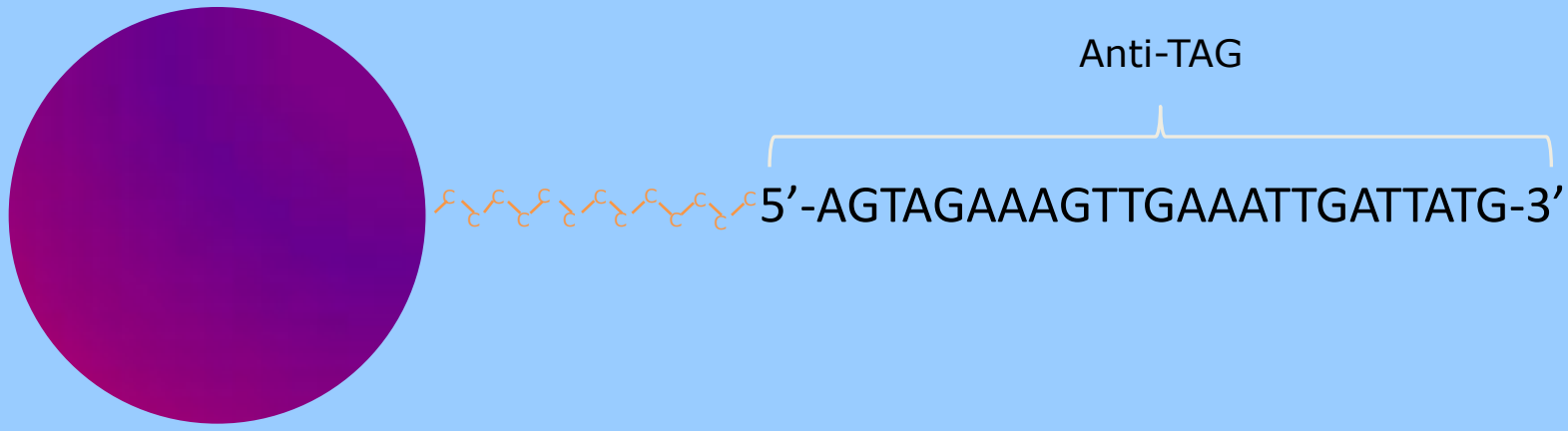
- Workflow
  - RNA extraction
  - cDNA synthesis
  - Multiplexed PCR
  - Remove excess primers and dNTPs
  - Use purified amplicons as template for TSPE
  - Linear TSPE reactions (incorporation of biotin-dCTP)
  - Microsphere hybridization
  - Add streptavidin-*R*-phycoerythrin
  - Analysis

*x*TAG technology

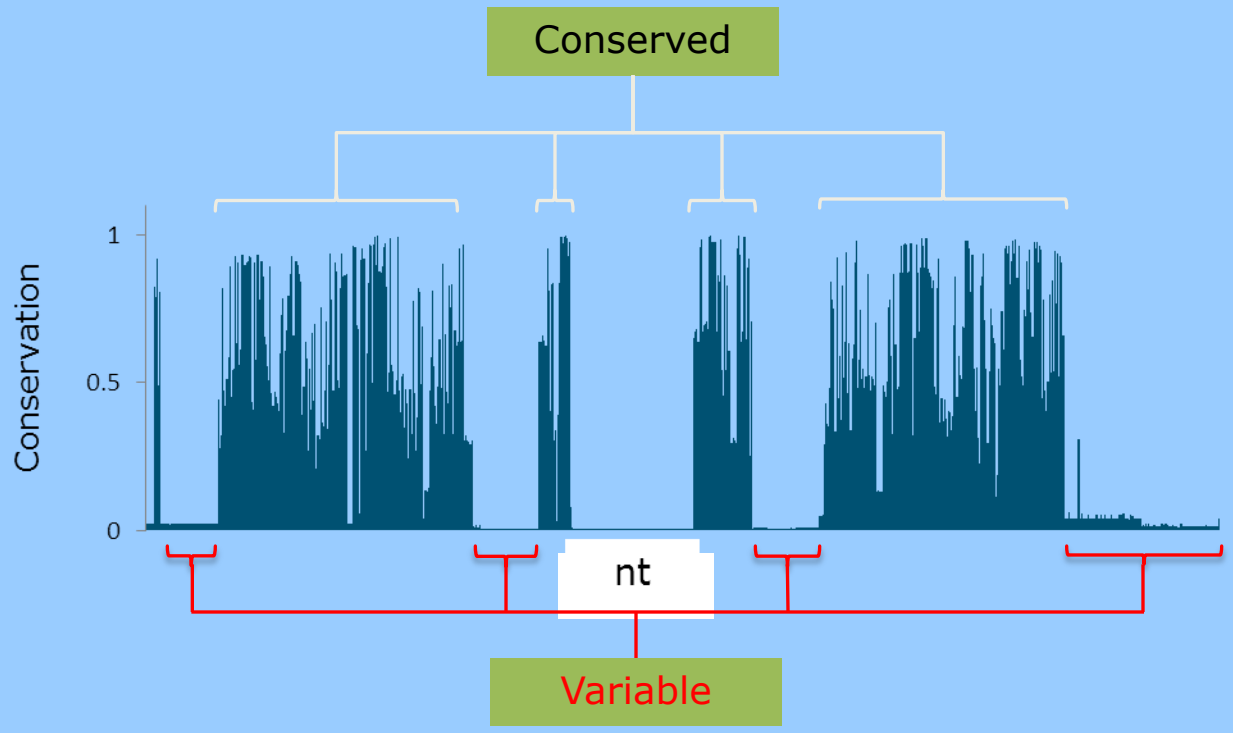
- Design ‘outer’ primers
  - Multiplex amplification
  - Conserved region
- Design TSPE primers
  - Linear amplification
  - Variable region

*x*TAG technology

- *x*TAG
  - 12 C spacer + 24-mer nucleotides (A, T and G)

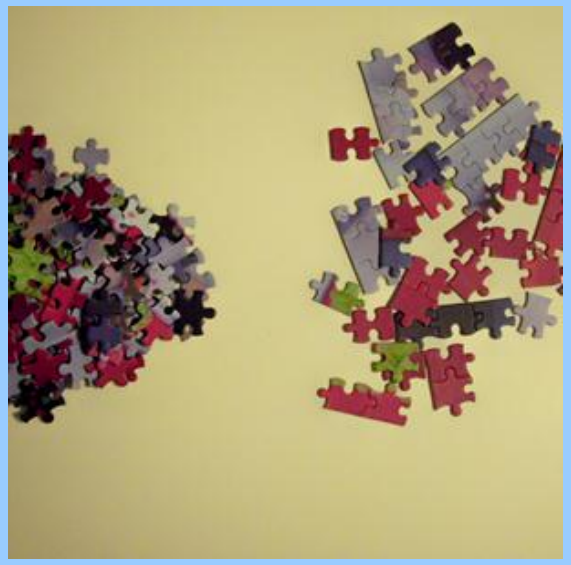


*x*TAG technology



*x*TAG technology

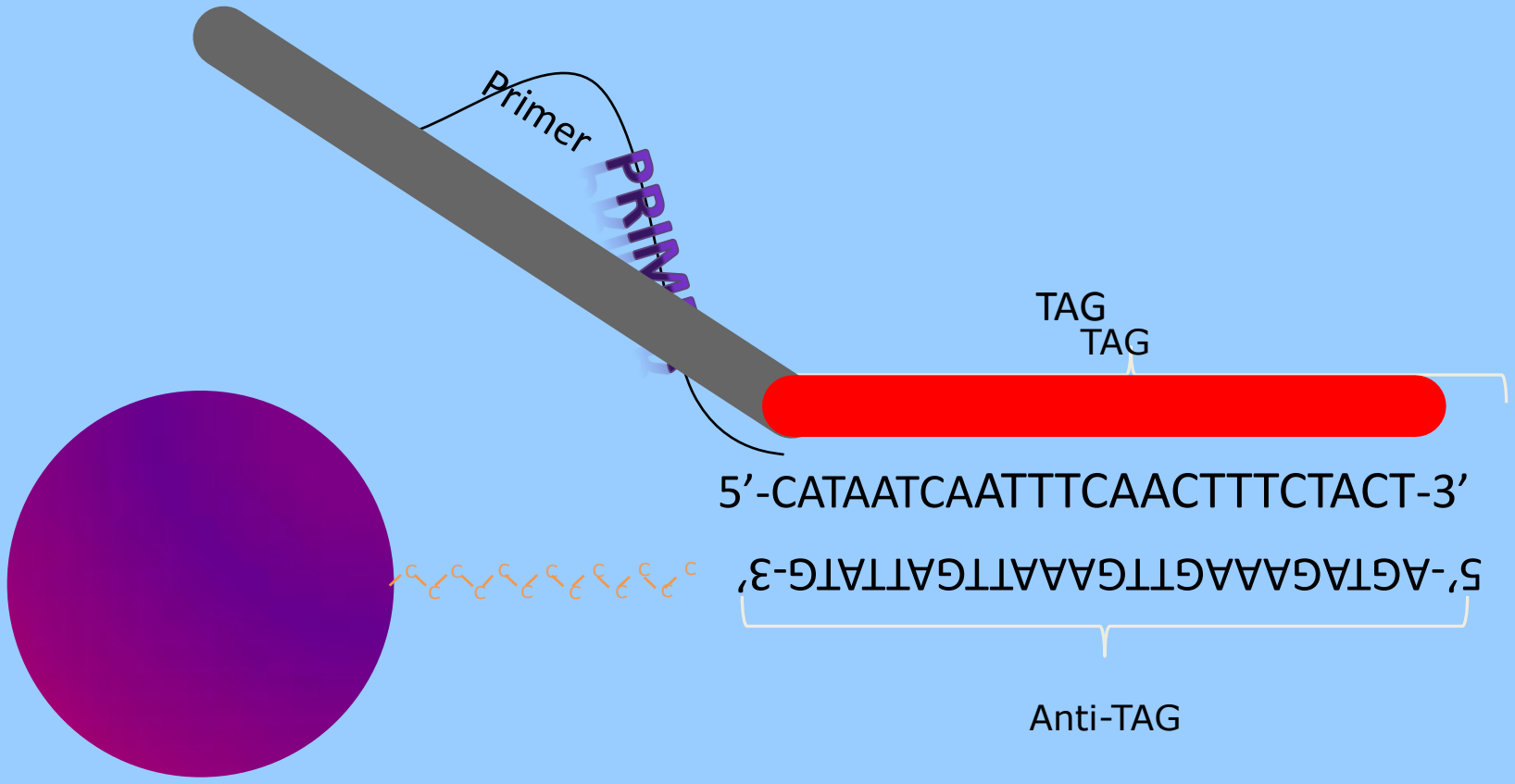
Raw ‘sequences’



Pre-amplification

TSPE primers

*x*TAG technology: TSPE



*x*TAG technology: TSPE

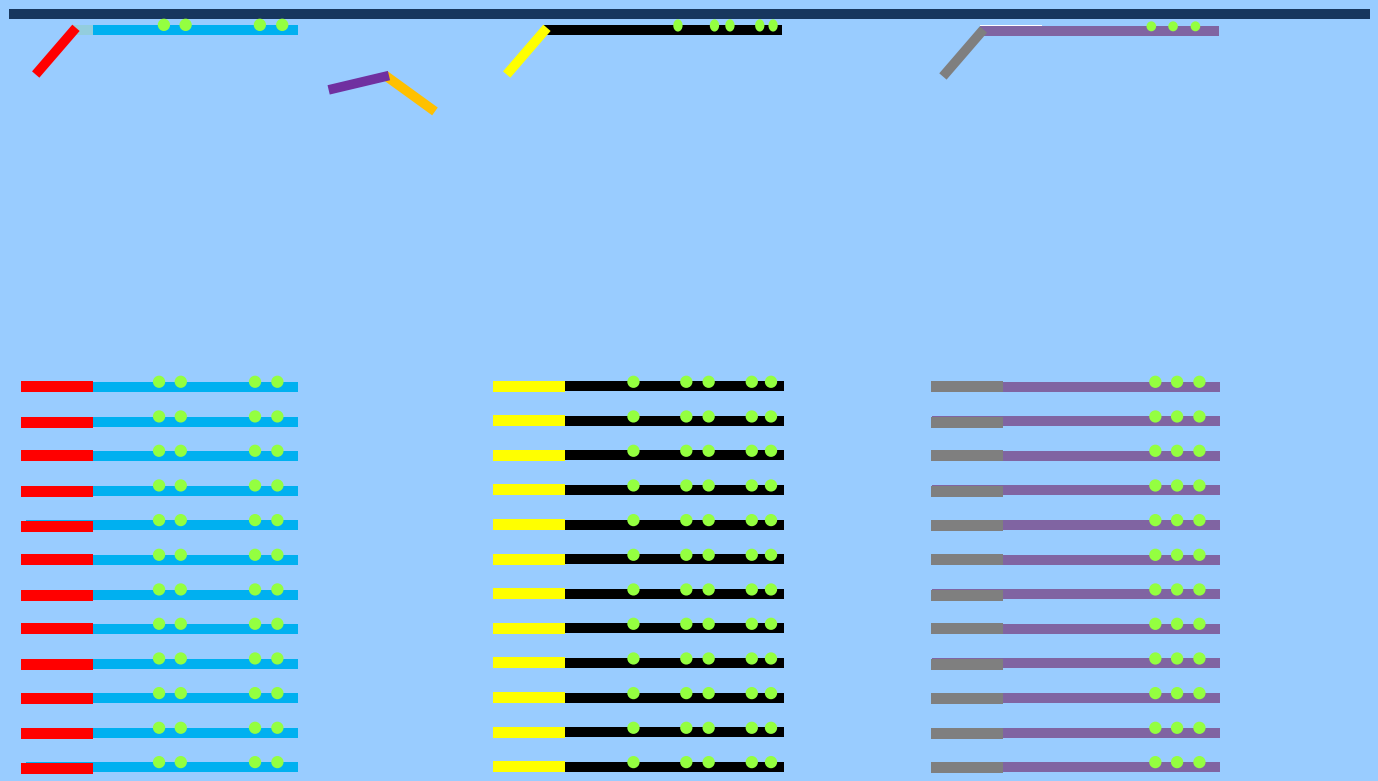
Outer primer spacing: 500-2000 nucleotides

Pre amplification of targets

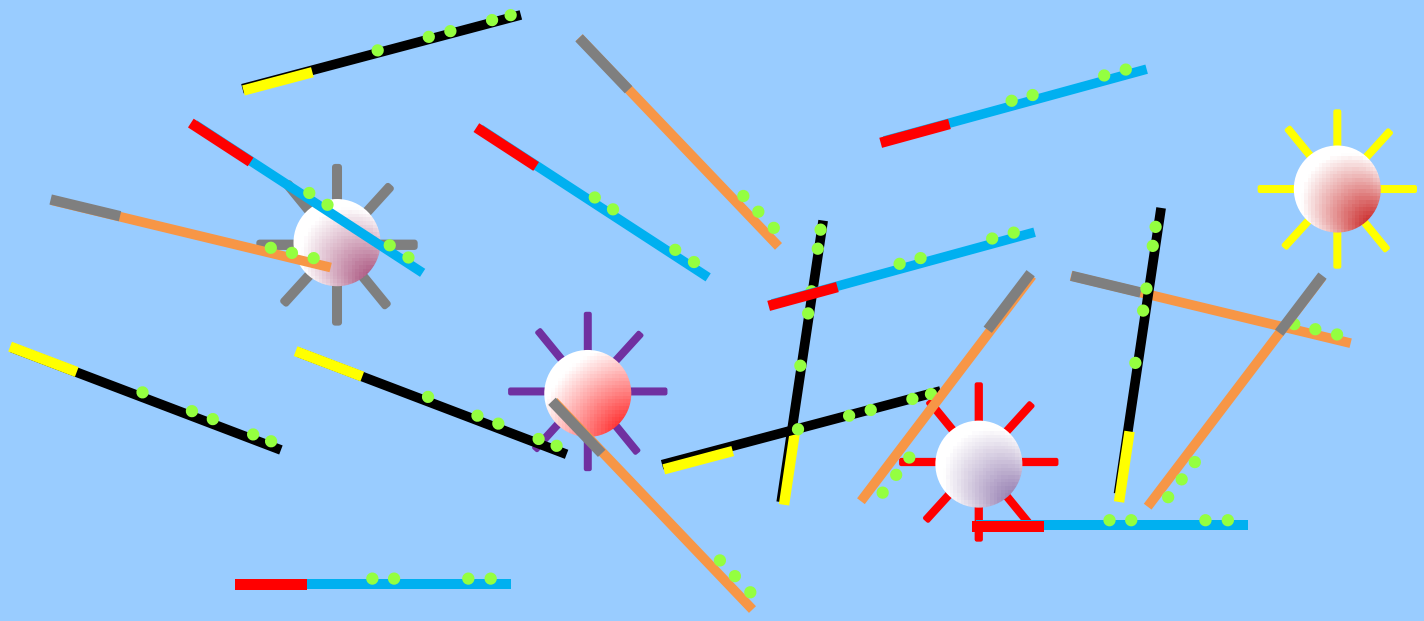
*x*TAG technology: TSPE

Pre amplification of targets

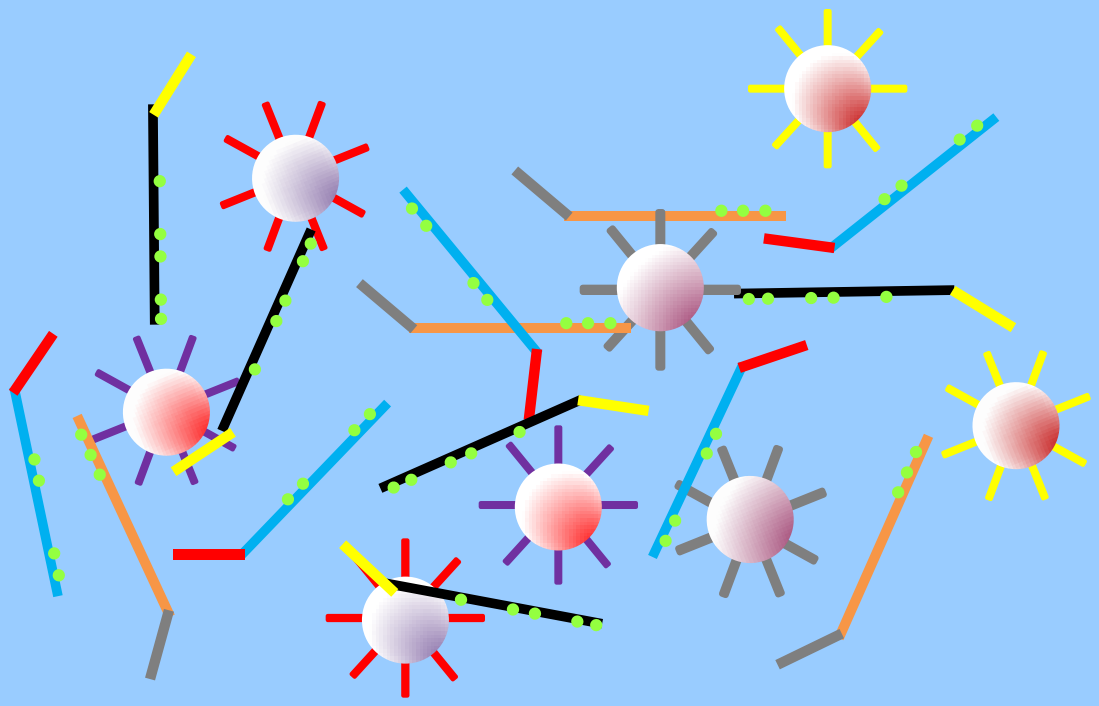
*x*TAG technology: TSPE



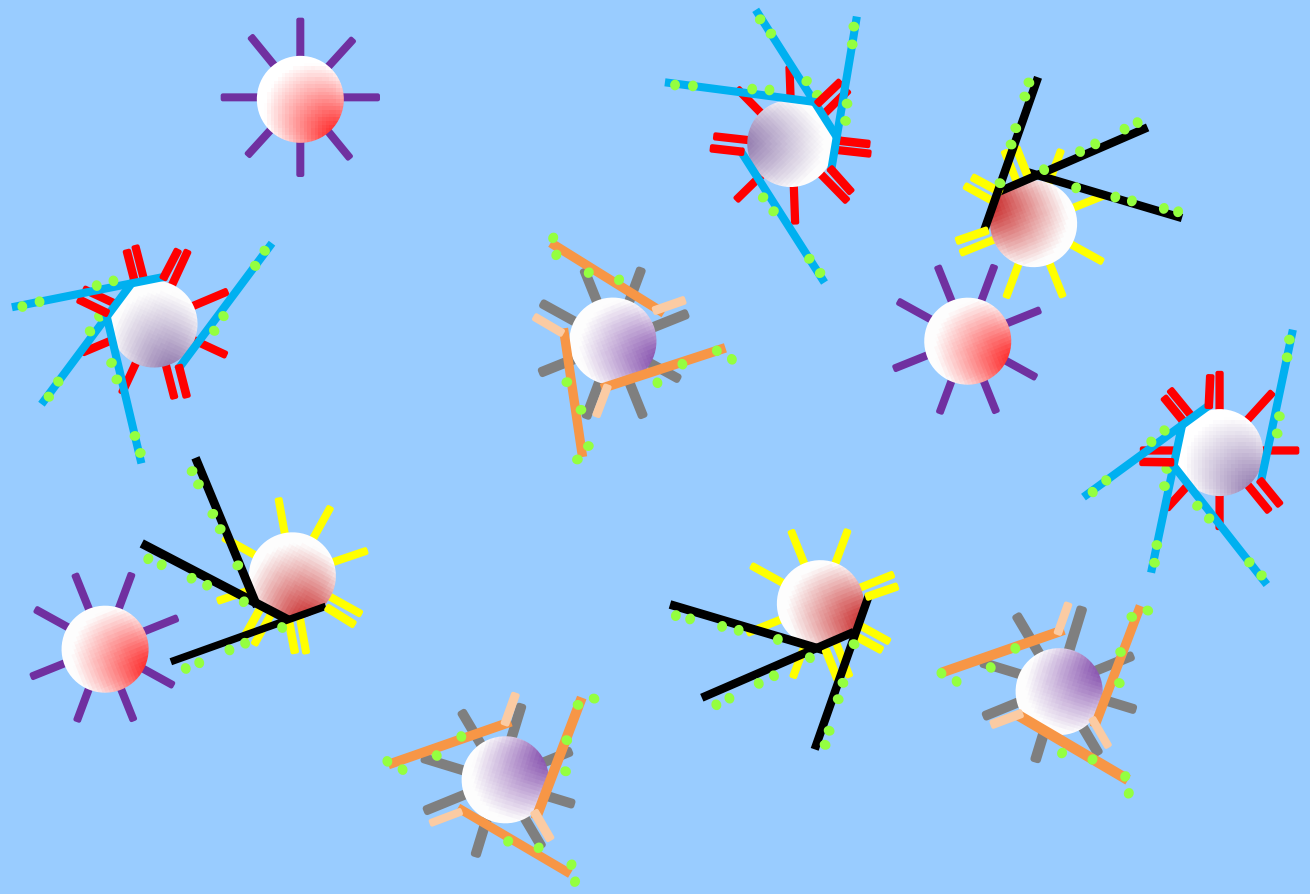
*x*TAG technology: TSPE



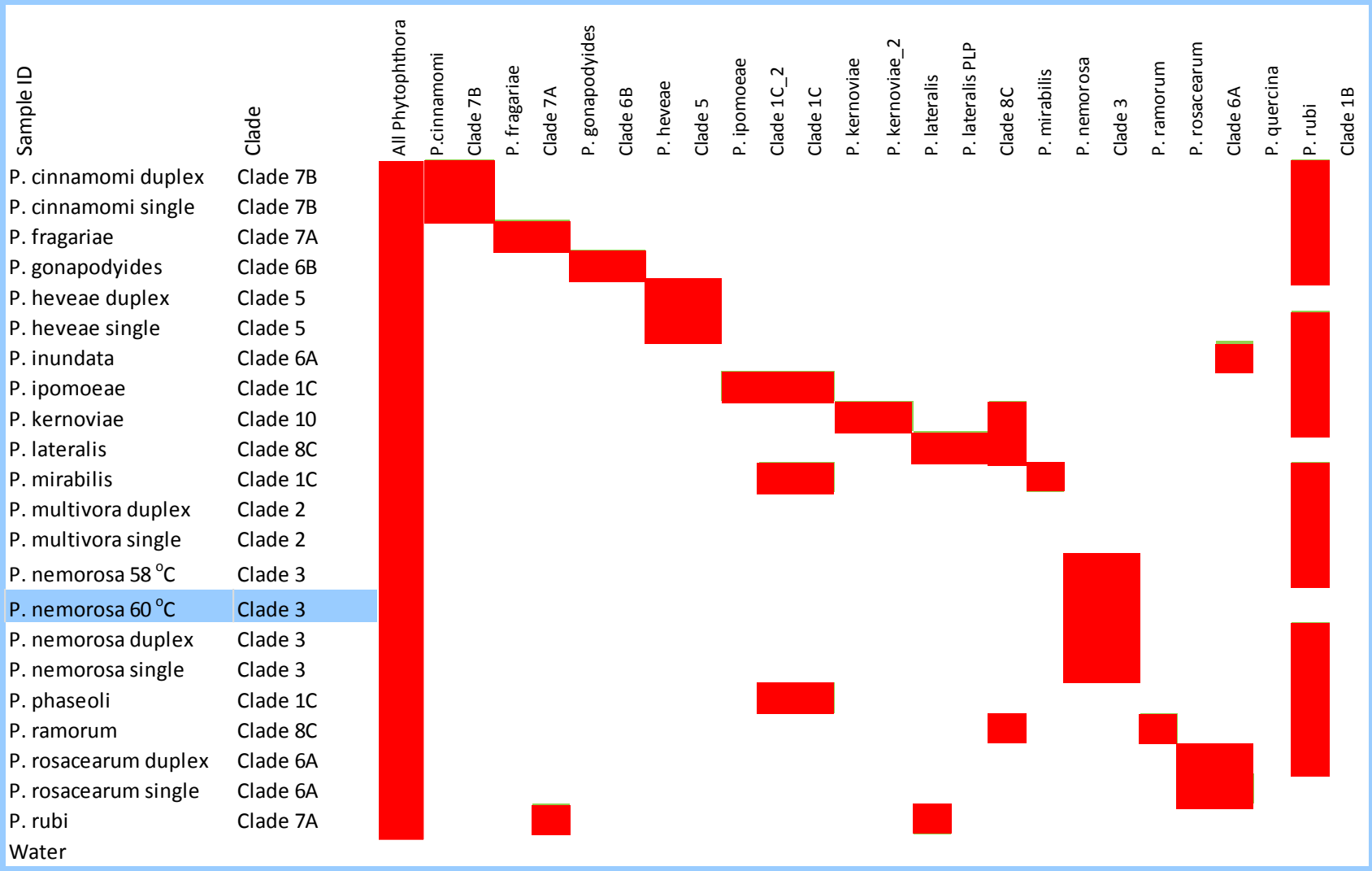
*x*TAG technology: TSPE method



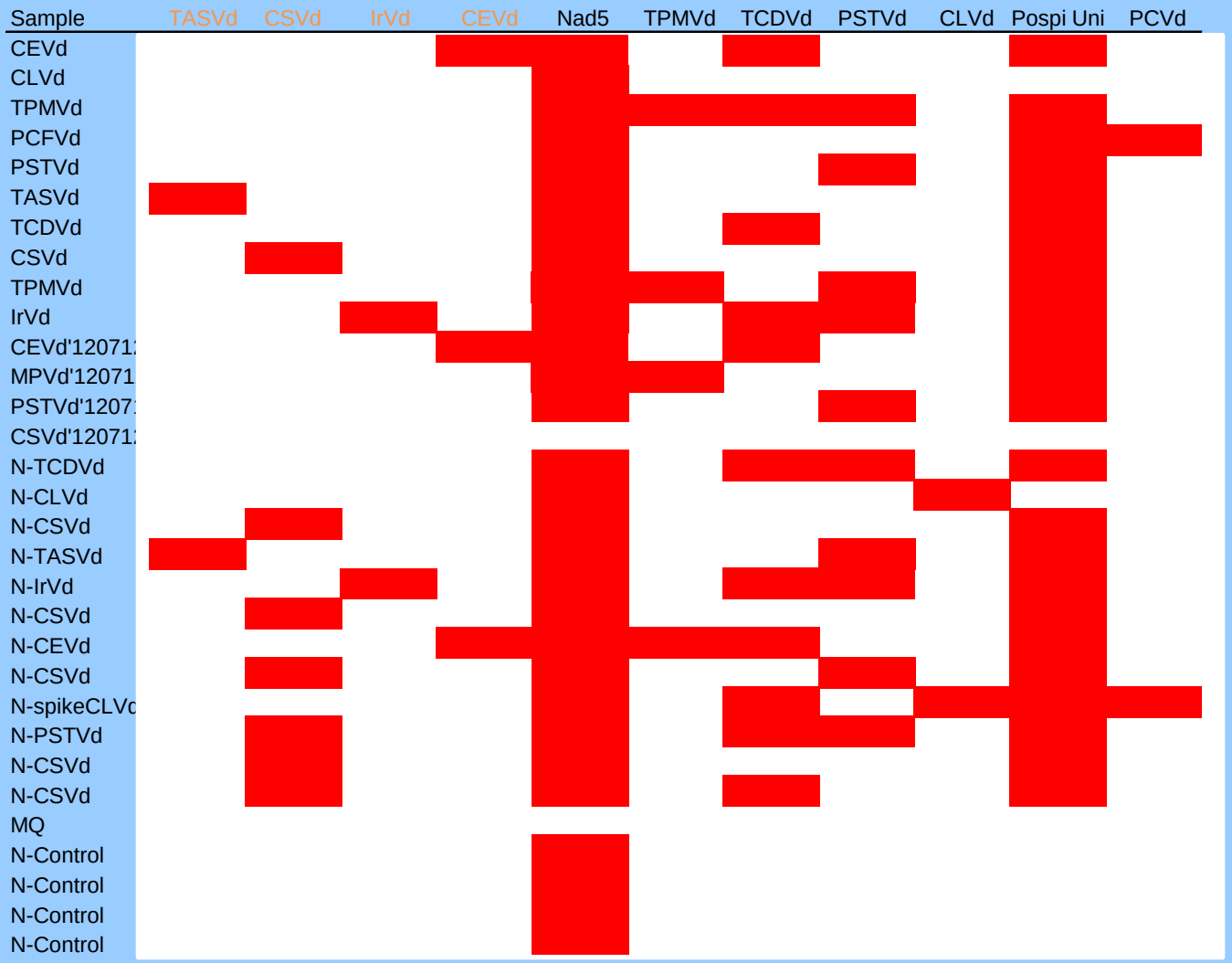
*x*TAG technology: TSPE method



*x*TAG technology: TSPE Phytophthora



*x*TAG technology: TSPE Pospiviroid test



Conclusions *x*TAG

- Wide application in pathogen detection
  - Fungi
  - Viroïds
  - Viruses
- Multi loci detection improves reliability
- Cost effective and fast (6-8 hours)
- One tube reaction published

Conclusions *x*TAG continued

- *x*TAG pospiviroids
  - One assay for 11 different targets
  - Multiple loci for each determinant possible
    - Increased reliability
  - Qualitative
  - Robust
  - Sensitivity equal to TaqMan
  - True multiplex assay

Methods for detection of plant viruses
Past – present – future ?

BIOREBA

Biological (Bioassays)

- Inoculation to herbacious plants
- Indexing (grafting to indicator plants)

Molecular

- PCR 25 years old
- Real time PCR
- Arrays (Gene-Chip)
- Isothermal amplification (e.g. LAMP and others)

Potential for future application in routine post harvest tests

Serological

- Immuno electron microscopy
- Western
- Labeled antibody techniques
- Arrays (PepChip)
- Luminex

→ Enzyme label (ELISA)

Today most widely used technique in routine potato virus detection (Certification) 30 years old

Many isothermal amplification methods:

BIOREBA

LAMP

Loop Mediated Isothermal Amplification 65° C

Eiken Chemicals Co.

RPA

Recombinase Polymerase Amplification 37° C

TwistDx's

NEAR

Nicking Enzyme Amplification Reaction 60° C

Ionian Technologies

NASBA

Nucleic acid sequence based amplification 41° C

Premier Biosoft

Post-harvest laboratory tests

(for routine virus detection → potato certification)

Europe: Mostly ELISA, PCR (often for early deliveries)
Belgium,France, Germany (*6), Luxembourg, The Netherlands, Scotland, Switzerland, Czech Republic, Poland (*7), Russia (*3)

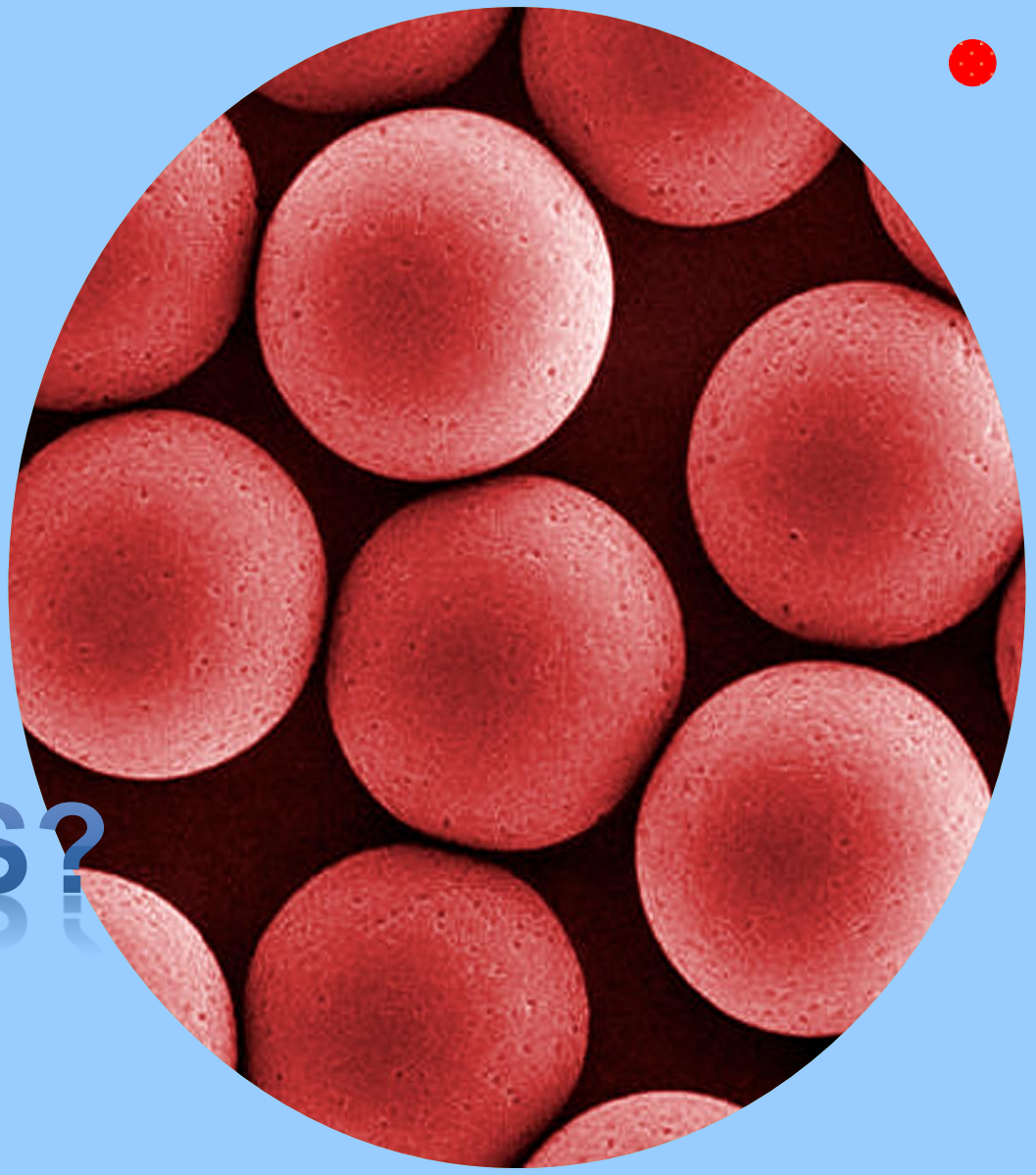
South Africa: ELISA

North America: ELISA (if post-harvest laboratory tests are done)
Canada: ELISA, some PCR
USA (*5) : only Idaho, Alaska and Montana are doing post-harvest laboratory tests (ELISA)

South America: ELISA
Argentina, Paraguay, Brazil

*Ranking in world production year 2005 (weight):
1 China, 2 India, 3 Ruusia, 4 Ukraine, 5 USA, 6 Germany, 7 Poland

Díky



QUESTIONS?