Microsphere-based Immunofluorescence Assay for the Concurrent Detection of West Nile and St. Louis Encephalitis Virus IgM Antibodies

SAFER • HEALTHIER • PEOPLE

Objective

To replace the WN and SLE MAC-ELISAs with a single, equally sensitive, faster test.

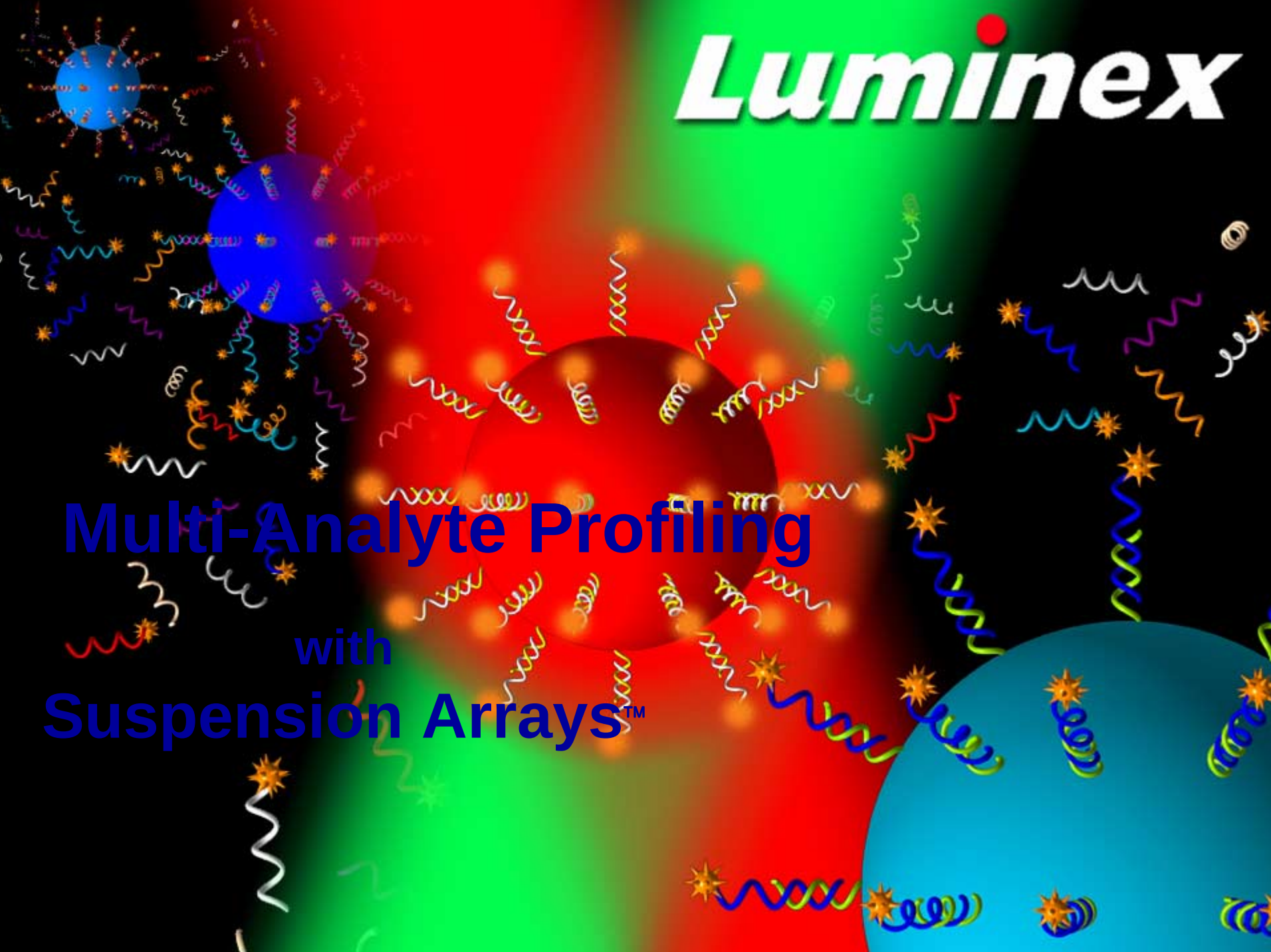
WN and SLE viruses co-circulate in parts of the US and are routinely tested for concurrently.

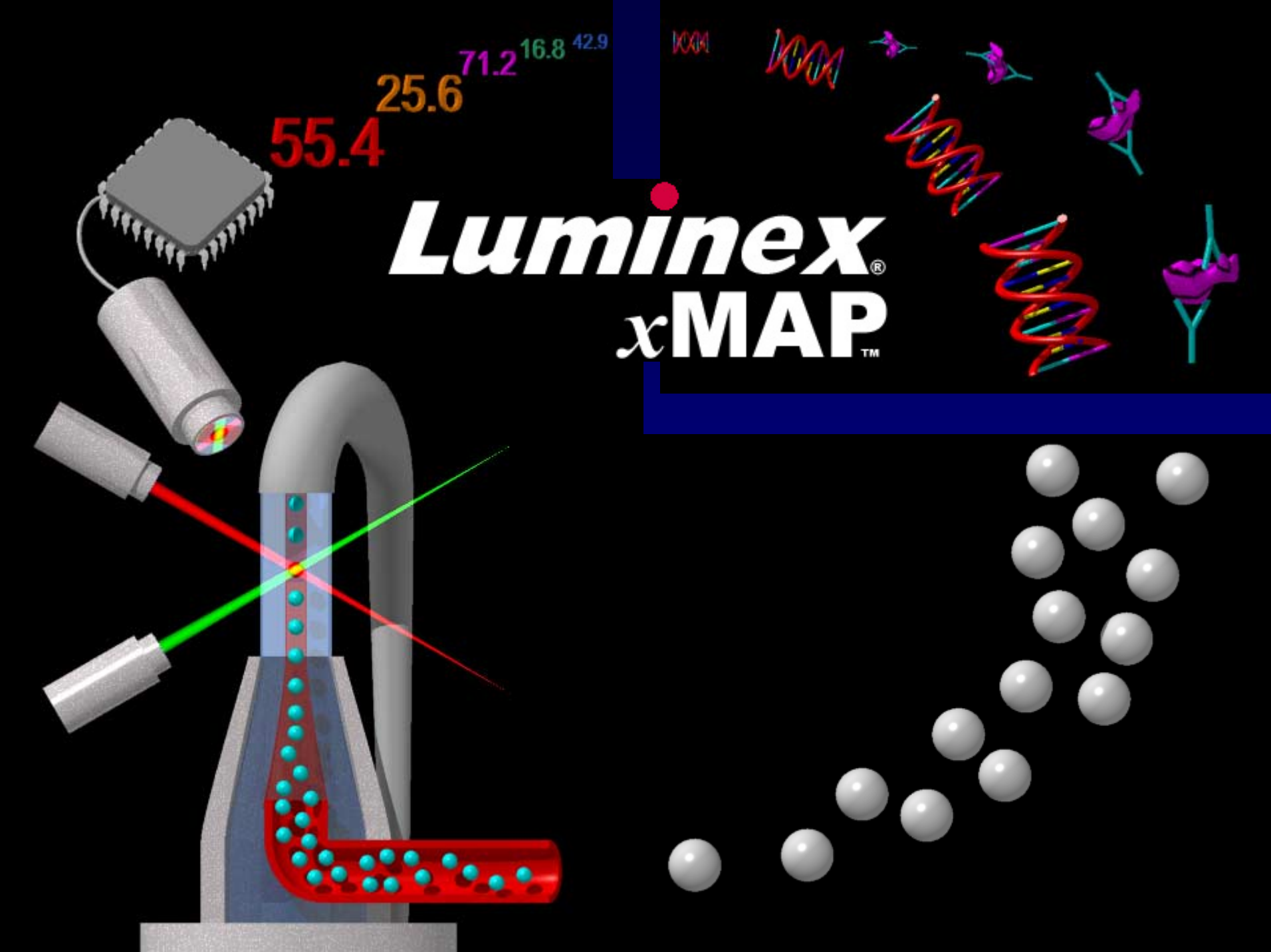
Principle

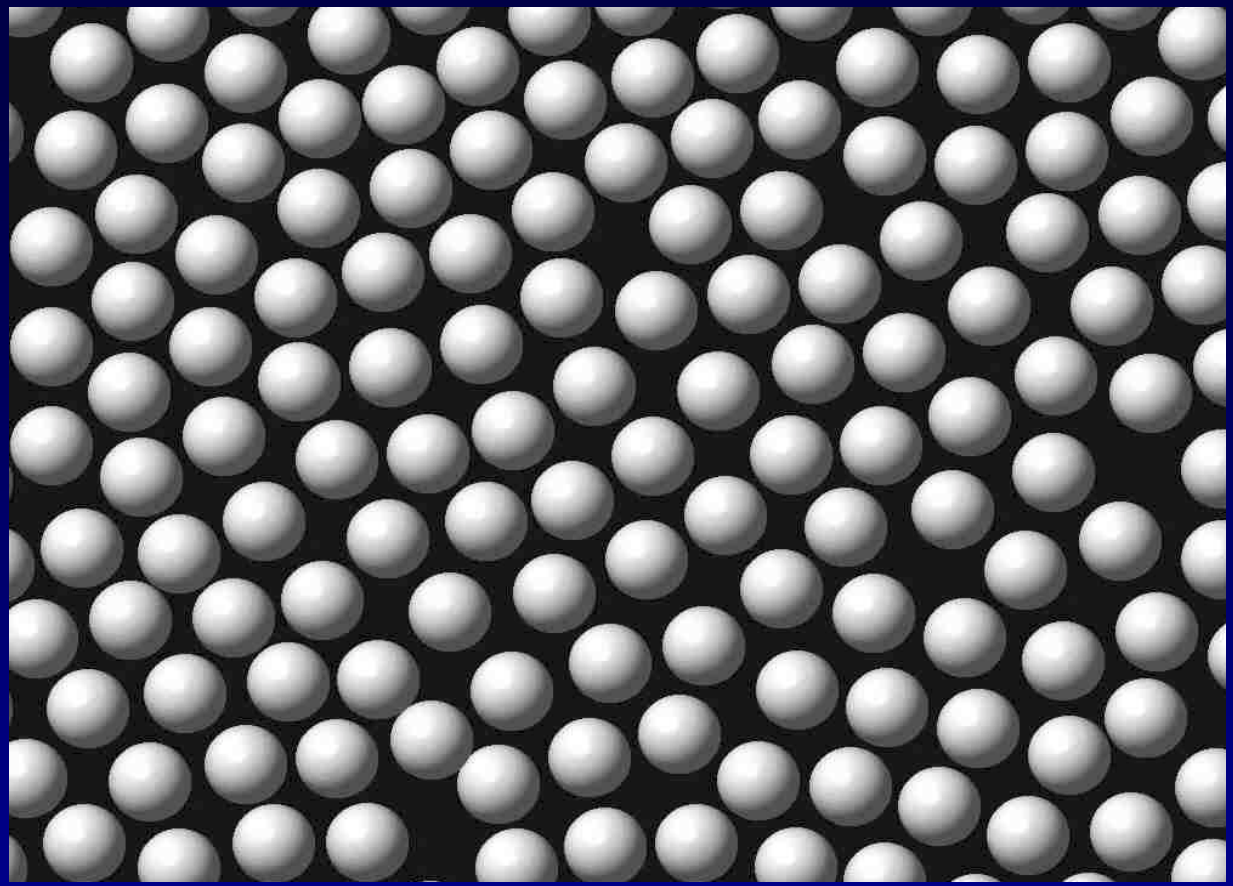
Microsphere-based immunologic assays (MIA's) are similar to ELISAs, except instead of being attached to a plate, the assay components are attached to microspheres, and results are read using a modified flow cytometer.

Similar problems to the ELISA are likely to arise.

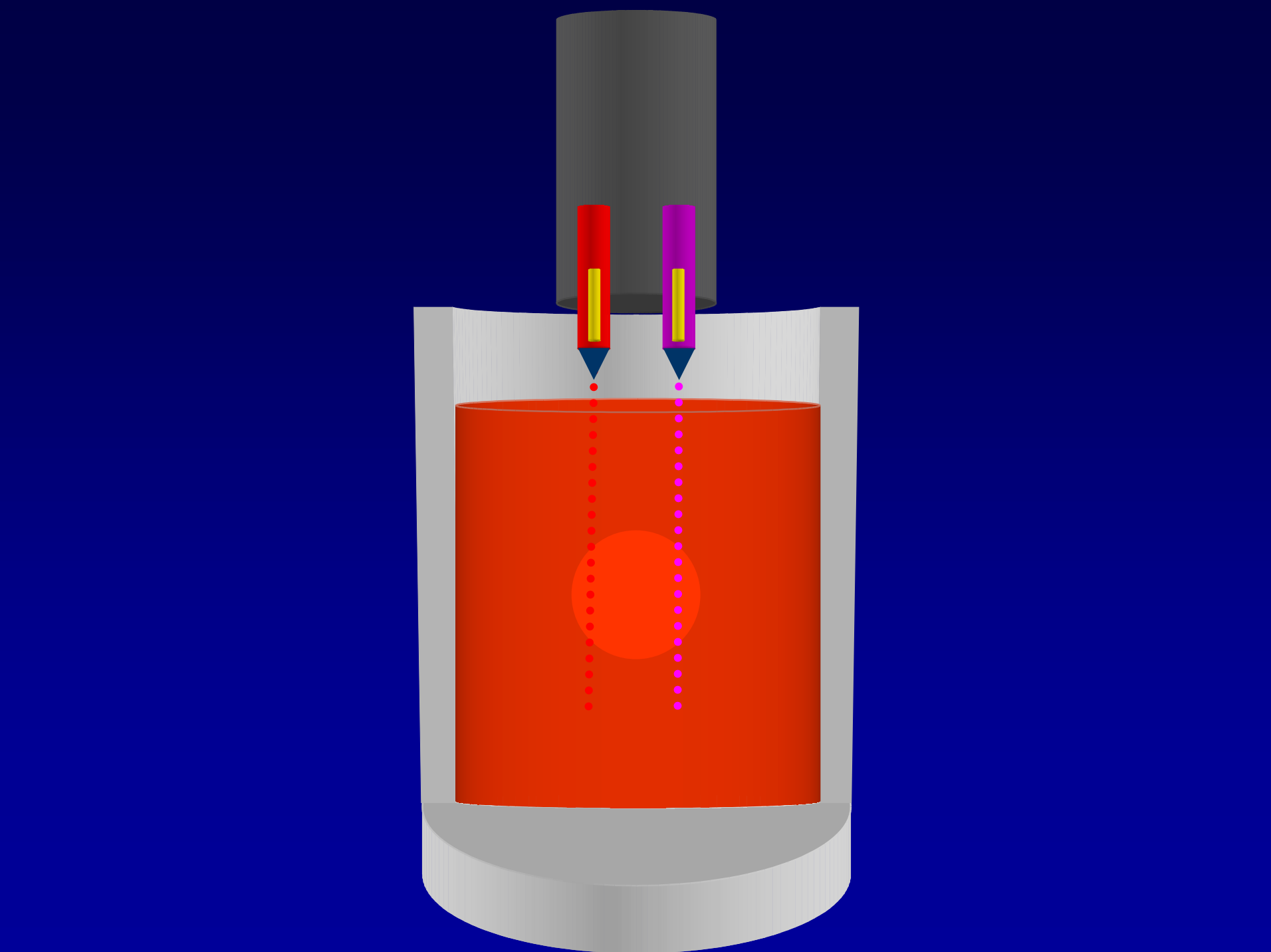
How the technology works

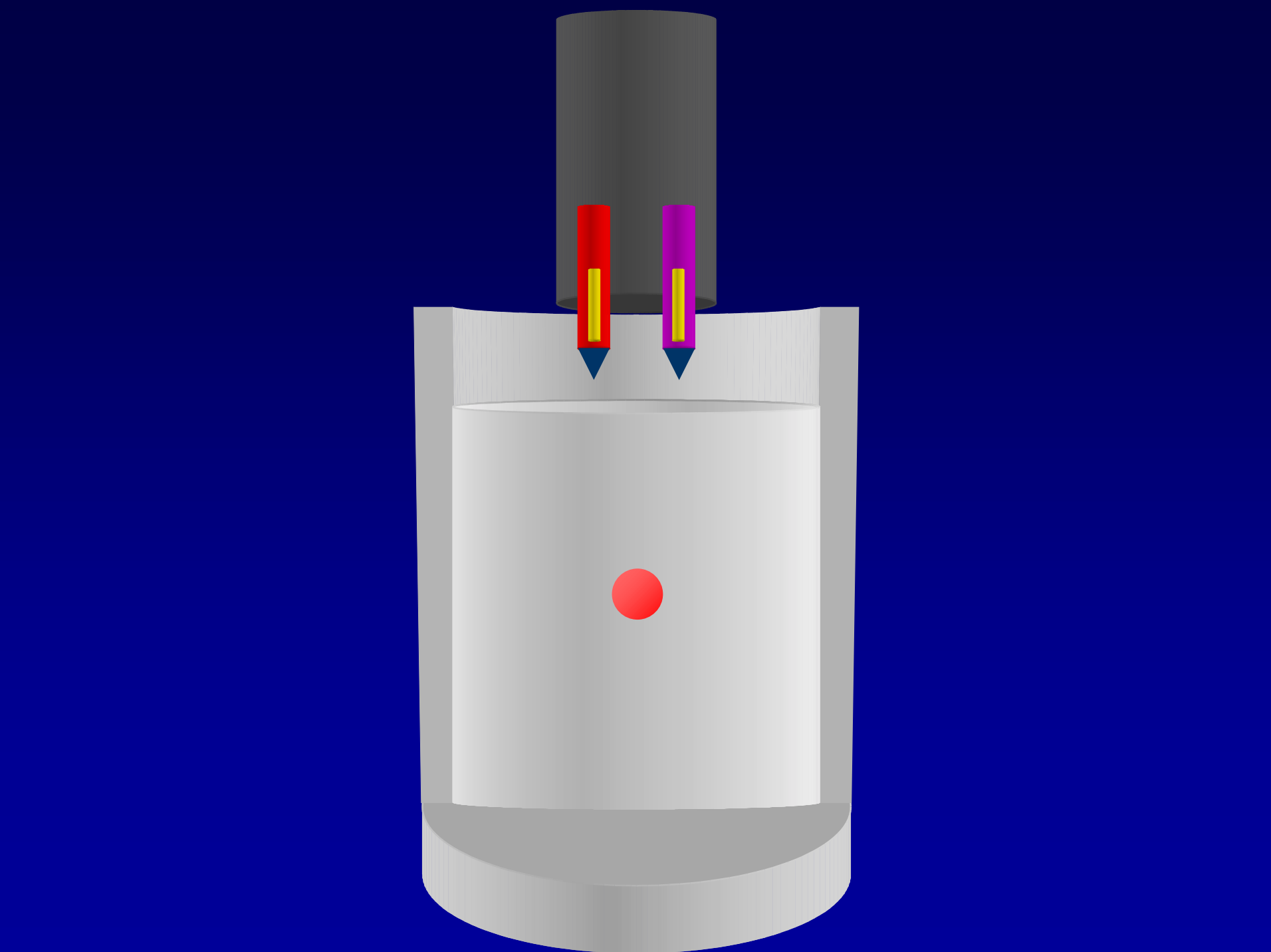

Luminex
Multi-Analyte Profiling
with
Suspension Arrays™

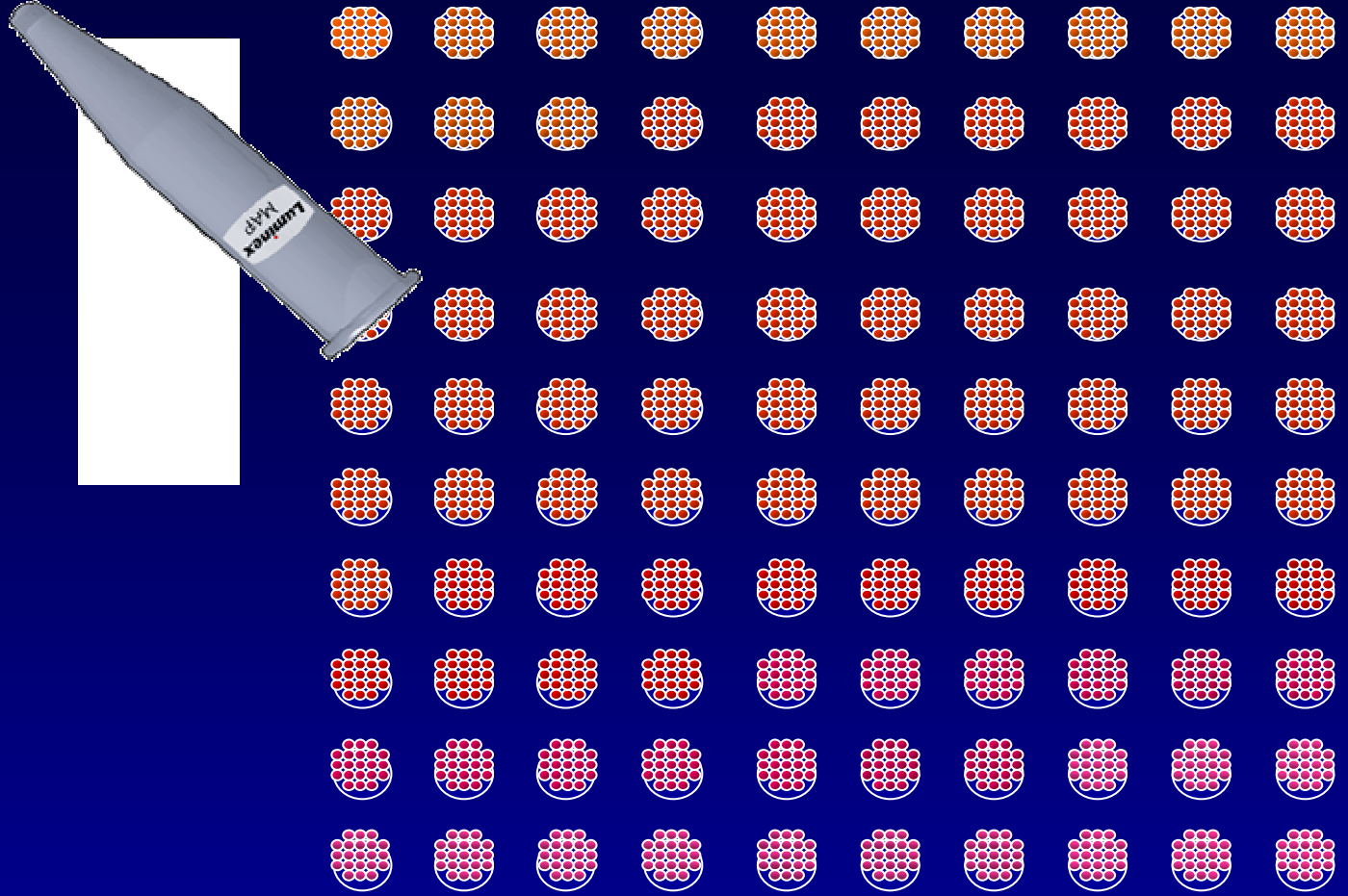

55.4
25.6
71.2
16.8
42.9
Luminex®
xMAP™



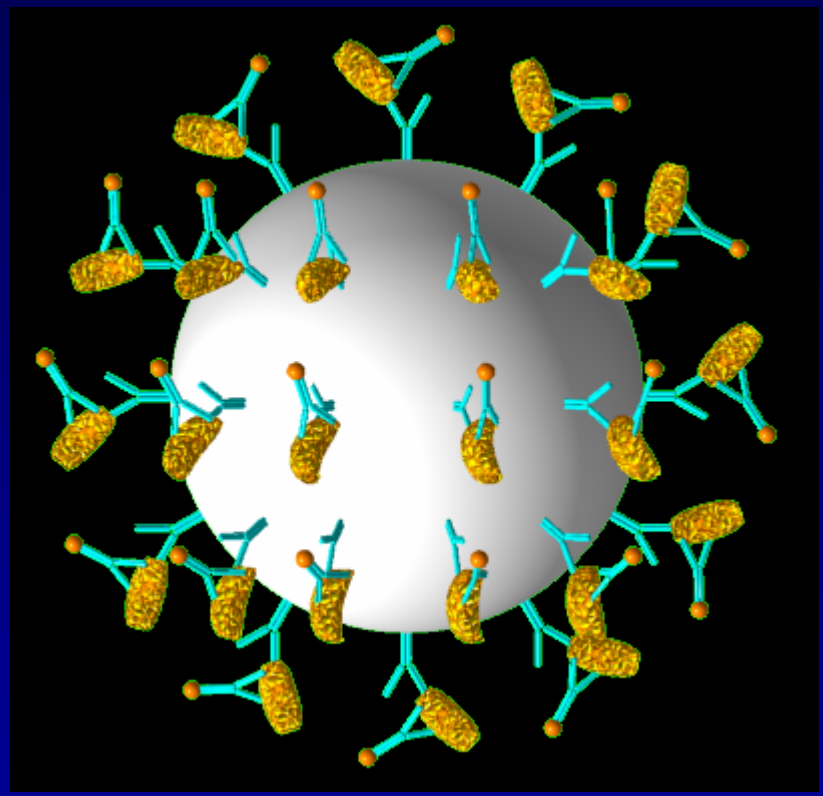
The bioassays are performed on microspheres (or beads). The beads are all the same size but different colors. Different colored beads can carry different biological tests. The software identifies which test is being identified based on the individual bead.

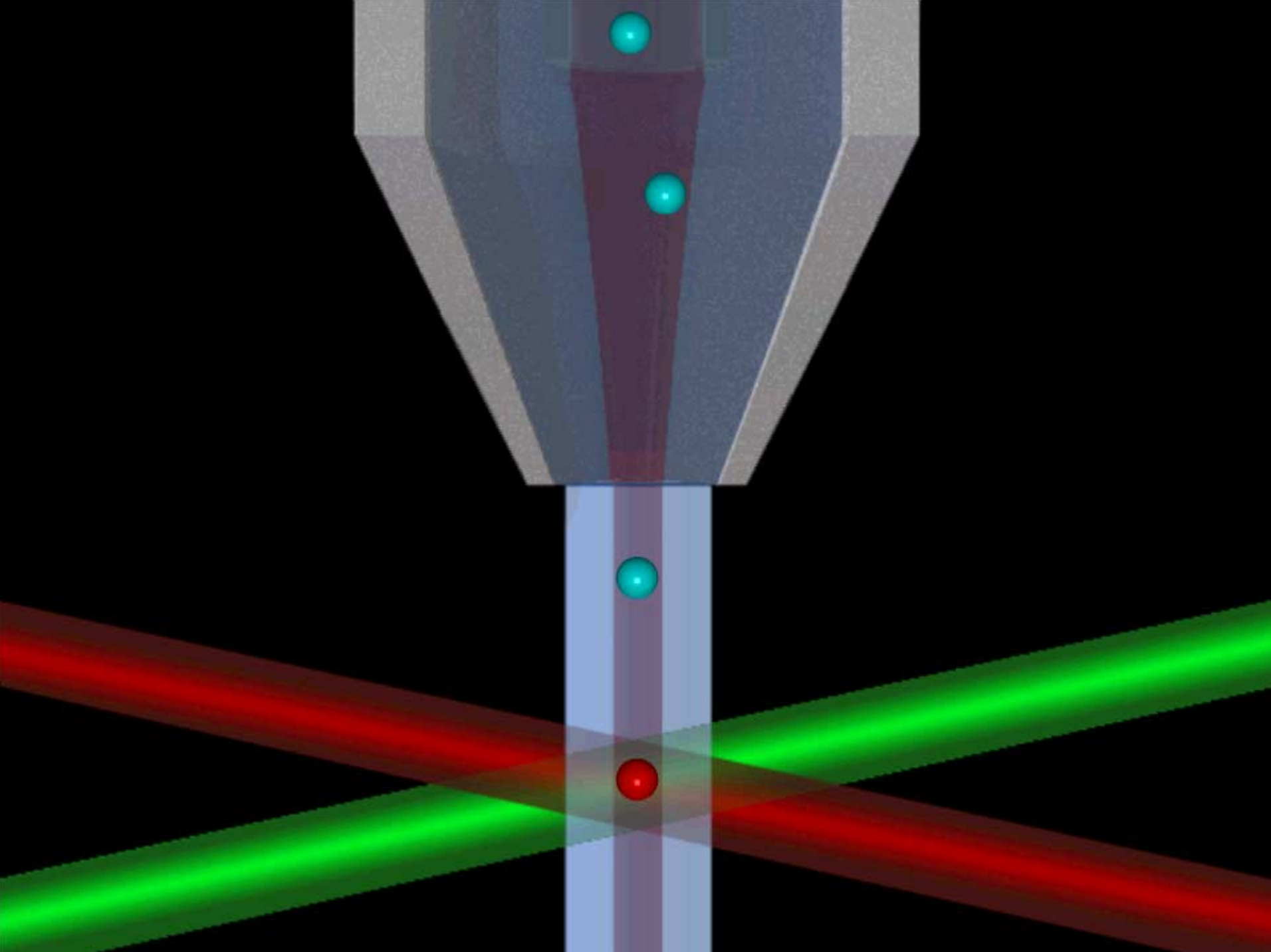


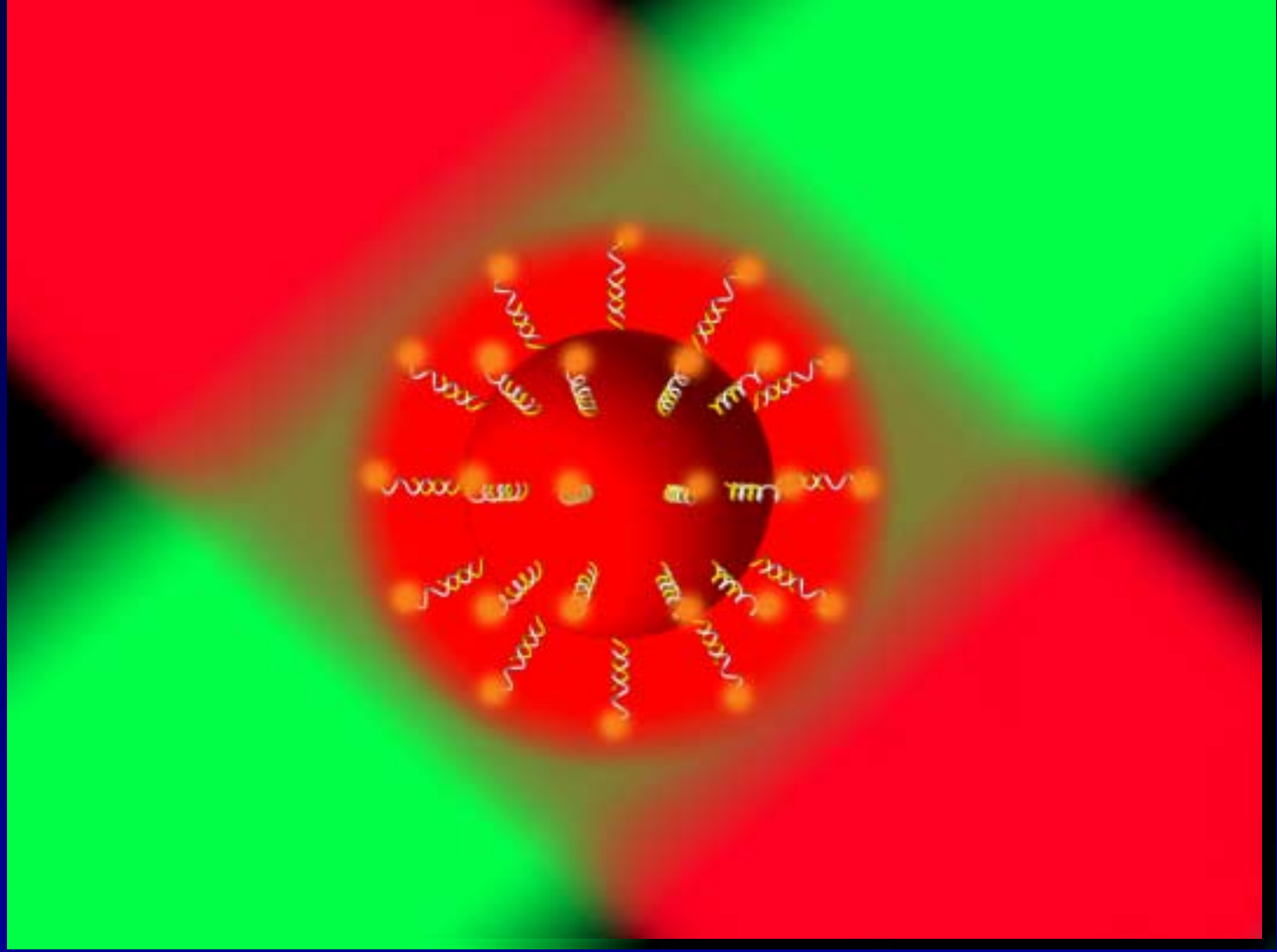




All the bead sets can be mixed in a well or a tube. The instrument sorts the data based on the unique color ratios of the beads. The individual bead sets are tagged with biological tests where the instrument identifies the set number, and measures the binding results for the test.







A green laser quantifies the surface fluorescence, which represents the biological reaction. Simultaneously, the bead sets are classified by a red laser.


DELL
Luminex 100
Luminex 100

The beads used in the test , and the detecting instrument, are manufactured and sold by Luminex Corp, Austin, TX.

The Luminex 100IS instrument is also sold through corporate partners such as Bio-Rad, Qiagen, Marligen, Miraibio, Upstate, LINCO and others, sometimes under different product names.

Approaches for arboviral diagnostics

1. Bead-antigen…IgM…anti-IgM PE
2. Bead-anti-IgM…IgM…antigen…MAbPE
3. Bead-MAb…antigen…IgM…anti-IgM PE

Which approach to take?

1. Bead-antigen….problem with antigen purity

2. Bead-anti IgM…coupling good but test failed

3. Bead-MAb….this approach works well and allows different types of antigen preparation to be used.

Preparation of stock reagents prior to the test

- **Couple beadsets 32 and 57 to purified MAb 6B6C-1. Stable for > 1yr. The process takes about 1 day. Uses standard luminex protocol.**

- **Add WN antigen to set 32 and SLE antigen to set 57 at a predetermined concentration. Can be stored at 4 deg. C for 1 month. The process takes 1 hour.**

- **Add negative cos-1 antigen to set 32 and negative mouse brain antigen to set 57 in the same amounts as the viral antigens. Can be stored at 4 deg. C for up to 1 month.**

Protein G treatment of serum

- **The preponderance of IgG in serum will often overwhelm the IgM signal. Protein G (a bacterial protein) coupled to sepharose matrix binds all subclasses of mammalian IgG but does not bind IgM, so IgG can be cleaned from a serum sample.**

- **Success of this shown by comparative IgM and IgG ELISAs (maintenance of IgM activity, decrease in IgG activity).**

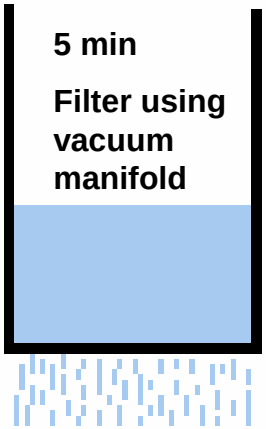
- **For single samples, use Mini-Rapi-Sep units; for multiple samples use 5 ul protein G in a filter plate format**

Method

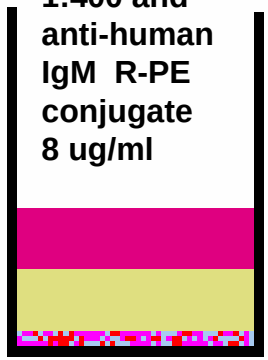
1 Presoak

5 min

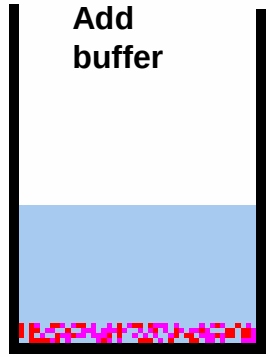
Filter using vacuum manifold



3 Add serum 1:400 and anti-human IgM R-PE conjugate 8 ug/ml



5 Add buffer

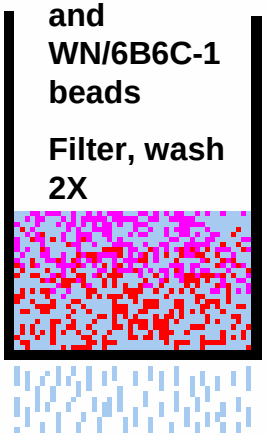


Buffer= 1% BSA in PBS All steps performed at room temp

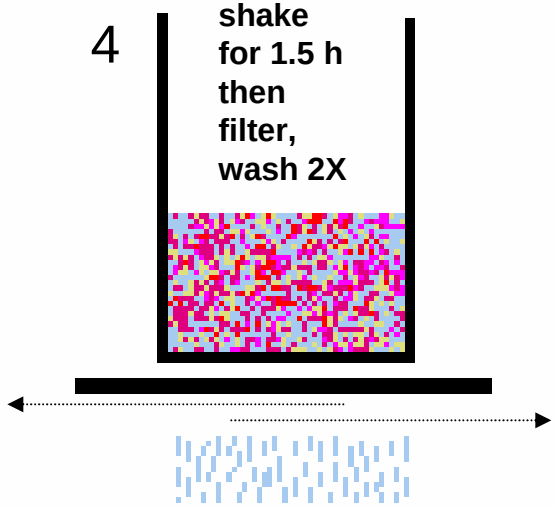
2500 beads per antigen per well are used.

2 Add SLE/6B6C-1 and WN/6B6C-1 beads

Filter, wash 2X

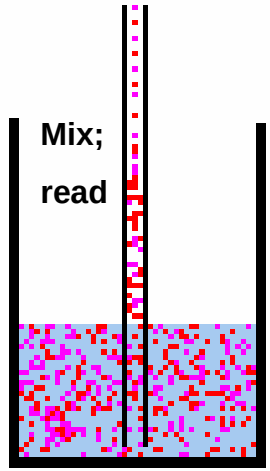


4 Mix; shake for 1.5 h then filter, wash 2X



6 Mix;

read



During method development the following were determined:

1. All reagent parameters were titrated to gain optimal results

2. The quantity of IgM in the positive controls were similar

3. The duplex arrangement did not compromise the results

4. The co-addition of serum and detector did not reduce the signal

5. Plates could be read in series when washed and kept in the dark for at least 4 h without signal reduction

6. Co-addition of beads in their antigen solutions did not produce cross-contamination when washed immediately

7. A longer reaction time yielded improved results

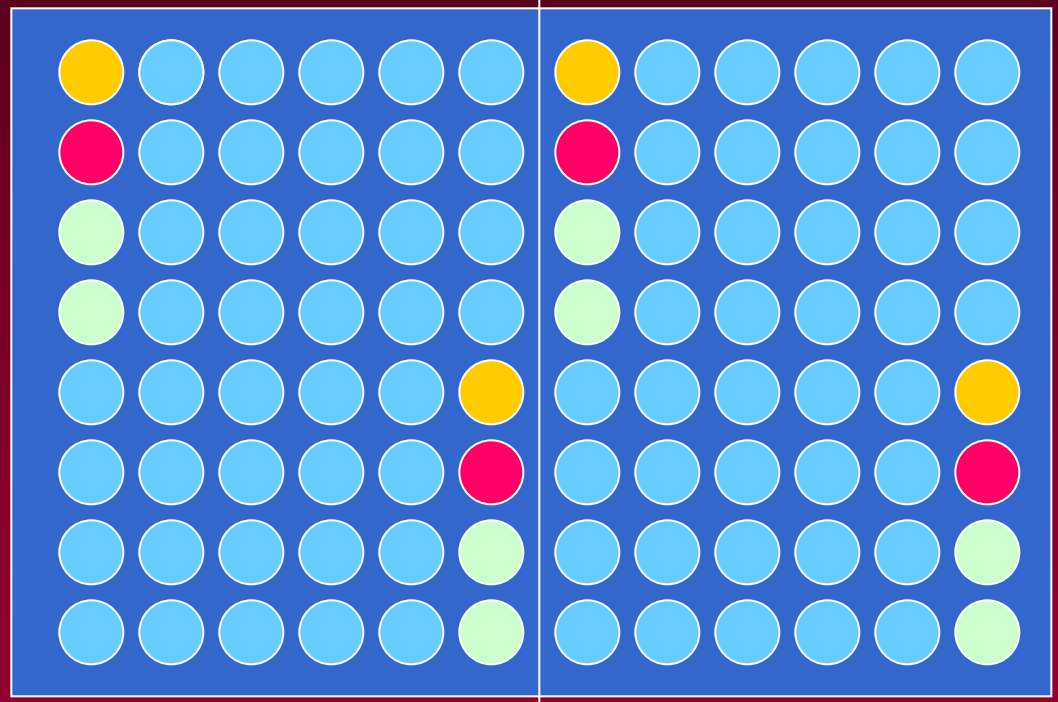
Typical plate format

WN pos control serum

SLE pos control serum

Neg control serum

PG-treated test serum



Bead set 32/WN Ag

+

Bead set 57/SLE Ag

Bead set 32/N Cos Ag

+

Bead set 57/N SMB Ag

How long does the duplex assay take?

Pretreatment and dilution of sample(s) with protein G: 1 min for a single sample; about 1 hour for a whole plate of 40 samples.

Assay setup: 15 min for a single sample; 30 min for a whole plate.

Incubation time: 1.5 hours

Read time: 5 min for a single sample and controls;
1.5 hours for a whole plate.

Total time: Approx. 2 hours for a single sample; 4.5 hours for a whole plate.

e Tools Help

Lumine

Single Step

Home | Run Batch | Maintenance | Diagnostics | Acq. Detail

Luminex100 Integrated System 2

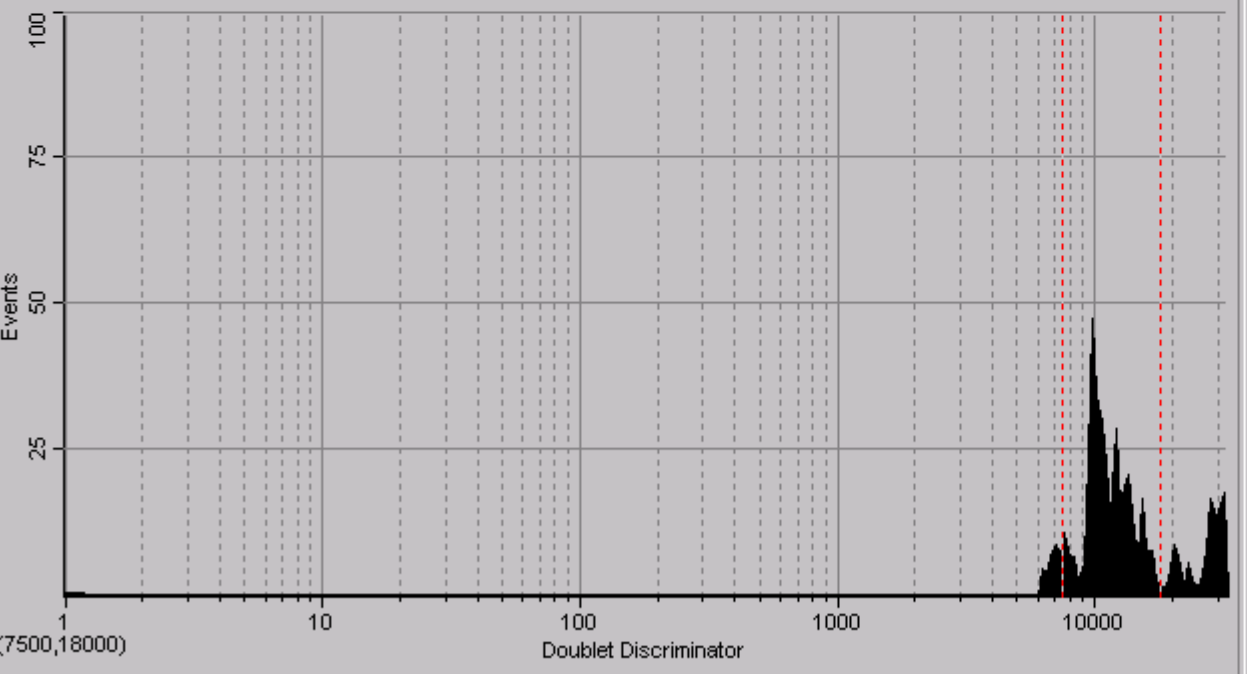
Batch Name: Batch,1/22/04,9:55_FILEMODE
Batch Description: <None>

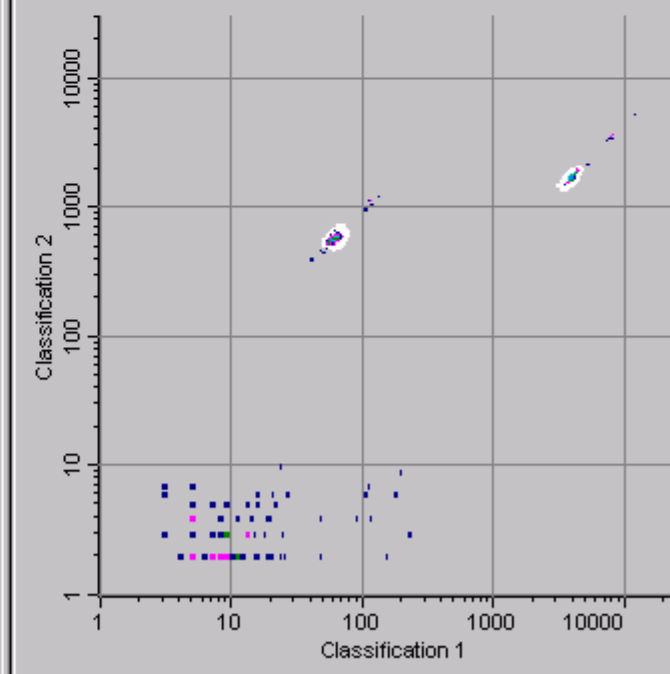
Help

Autosize | Statistic: Median

Loc	Sample	WN IgM (32)	SLE IgM (57)
G5, Plate1	Acquire Pa39	3736.00	109.50
H5, Plate1	Acquire Pa40	1566.50	45.00
A6, Plate1	Acquire Pa41	2662.00	54.00
B6, Plate1	Acquire Pa42	2155.00	77.00
C6, Plate1	Acquire Pa43	31.00	71.50
D6, Plate1	Acquire Pa44	3174.00	154.00

Show Bead: <all>





Command State	Device Status	Device Activity	Laser Status	Total Events/Sec	Region Events/Sec
Paused	Standby, FILE	Idling	Lasers Off	141	66

Example of median fluoresecent indices (MFIs) for WN, SLE, and negative specimens

	WN Ag	NRec Ag	SLE Ag	NSMB Ag
WN	3720	77	97	58
SLE	109	43	2398	73
NEG	45	38	58	64

Standardization

For results to be comparable to one another (antigen to antigen, plate to plate), the raw MFIs are adjusted as follows:

1. WN & SLE MFIs divided by appropriate negative control antigen MFIs

2. SLE and WN positive controls compared to account for differences in antigen reactivity, and test results normalized according to the ratio.

3. Plates standardized
   - WN, SLE & Neg control samples on plate calibrated to other plates
   - Some hint of residual bead/antigen lot dependence

- Alternative, within-plate standardization not as successful

Classification Methods (how the final result is arrived at)

- **Mean ± 3×SD**
  - Does not determine classification by optimizing group sensitivities
  - Does not consider WN & SLE results simultaneously
- **3-Way ROC Analysis**
  - Does optimize group sensitivities
  - WN & SLE results considered simultaneously
  - Too inflexible to account for WN & SLE relationship
- **Linear discriminant analysis**
  - Does optimize group sensitivities
  - WN & SLE results considered simultaneously
  - Assumes common correlation for the groups
- **Quadratic discriminant analysis (QDA)**
  - Does optimize group sensitivities
  - WN & SLE results considered simultaneously
  - Admits different correlation for the groups

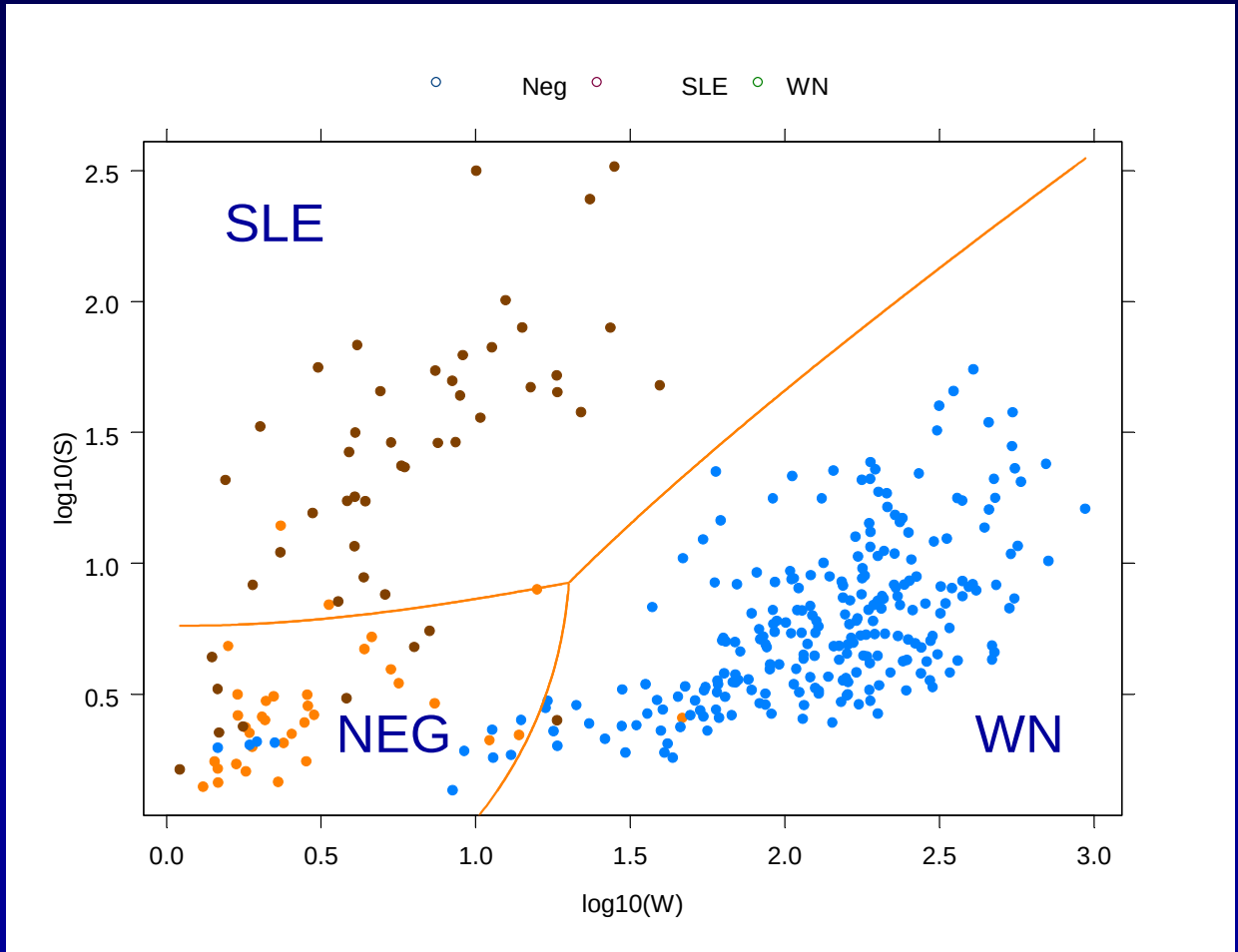
Classification Algorithm

- **Quadratic Discriminant Analysis (QDA)**
- **Evaluate *predictive accuracy* using cross-validation**

The end result is an algorithm that allows one to say a sample is IgM positive to WN or SLE or is negative for both. This is different from the MAC-ELISA.

The result can be arrived at by embedding the algorithm in the Luminex method (Excel).

MIA Standardized Data & QDA Classification Rule



N=334

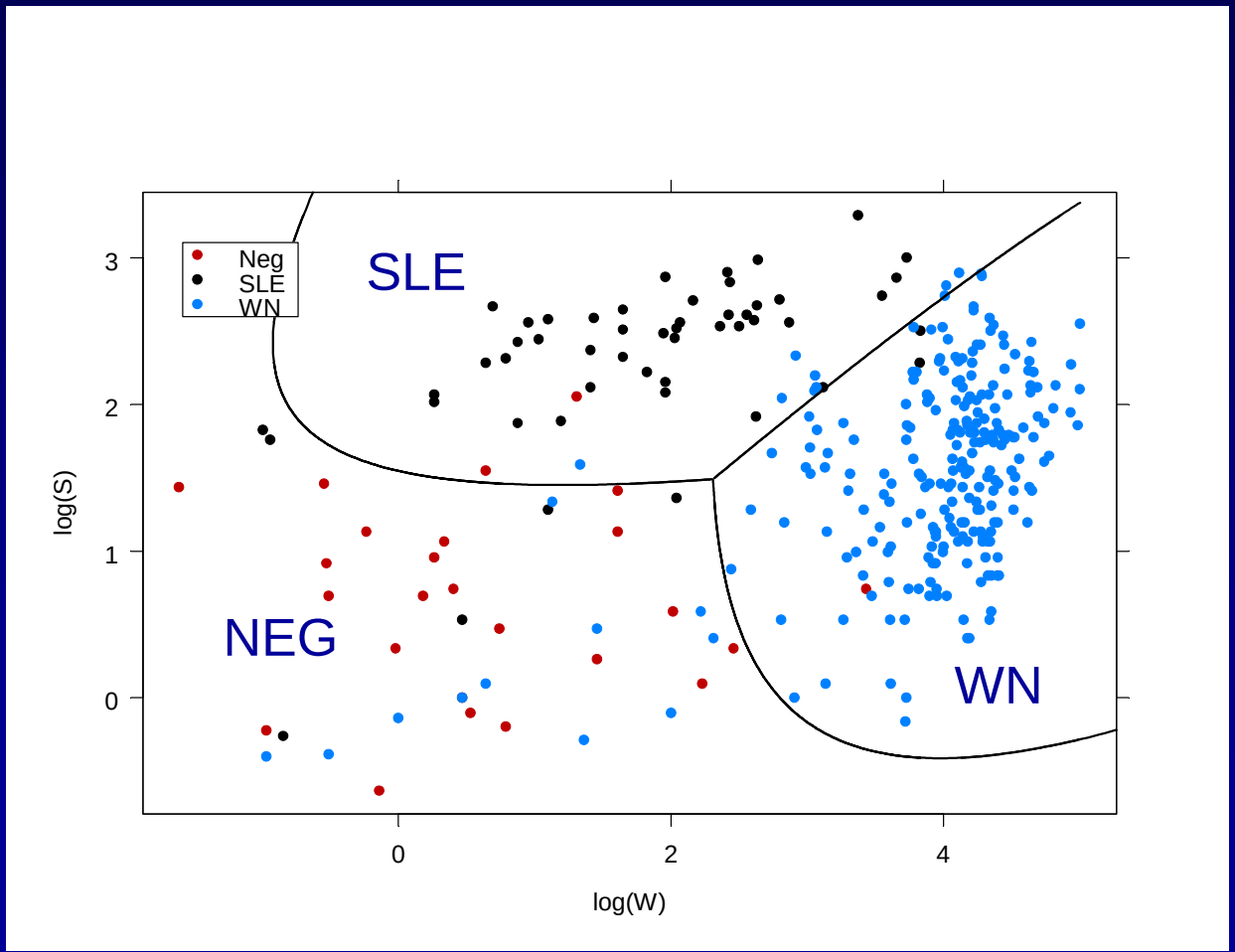
Colored dots represent the PRNT (truth) result. Serum only.

MIA QDA Cross-validation Results

		PRNT			Overall
		WN	SLE	Neg	
QDA	WN	241	1	2	
	SLE	0	39	3	
	Neg	10	8	30	
n		251	48	35	334
%Correct classification		96	81	86	87

*

ELISA QDA Classification



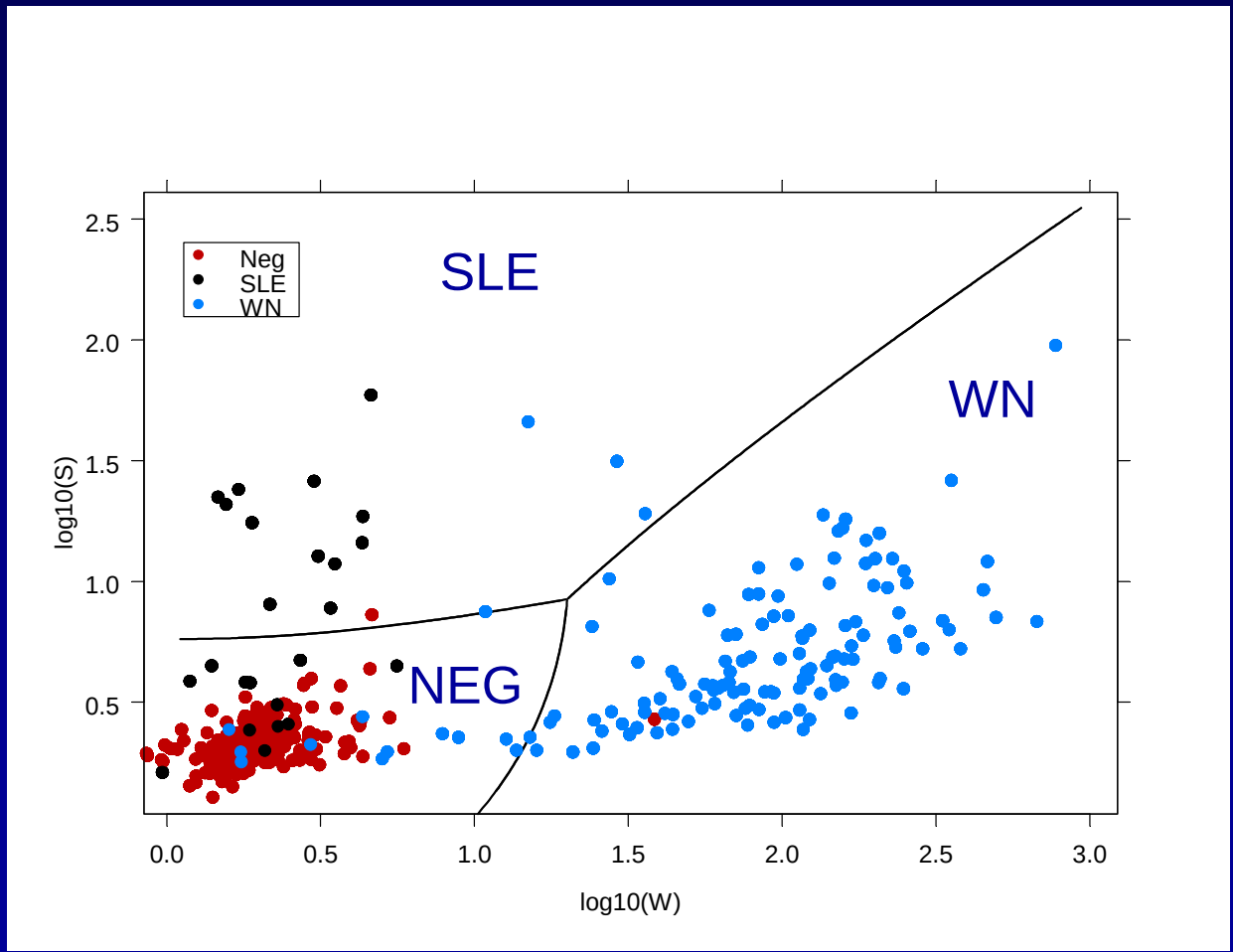
N=326

ELISA Cross-validation Results

		PRNT			Overall
		WN	SLE	Neg	
QDA	WN	230	3	1	
	SLE	9	42	2	
	Neg	11	6	22	
n		250	51	25	326
ELISA % correct classification		92	82	88	87
MIA % correct classification		96	81	86	87

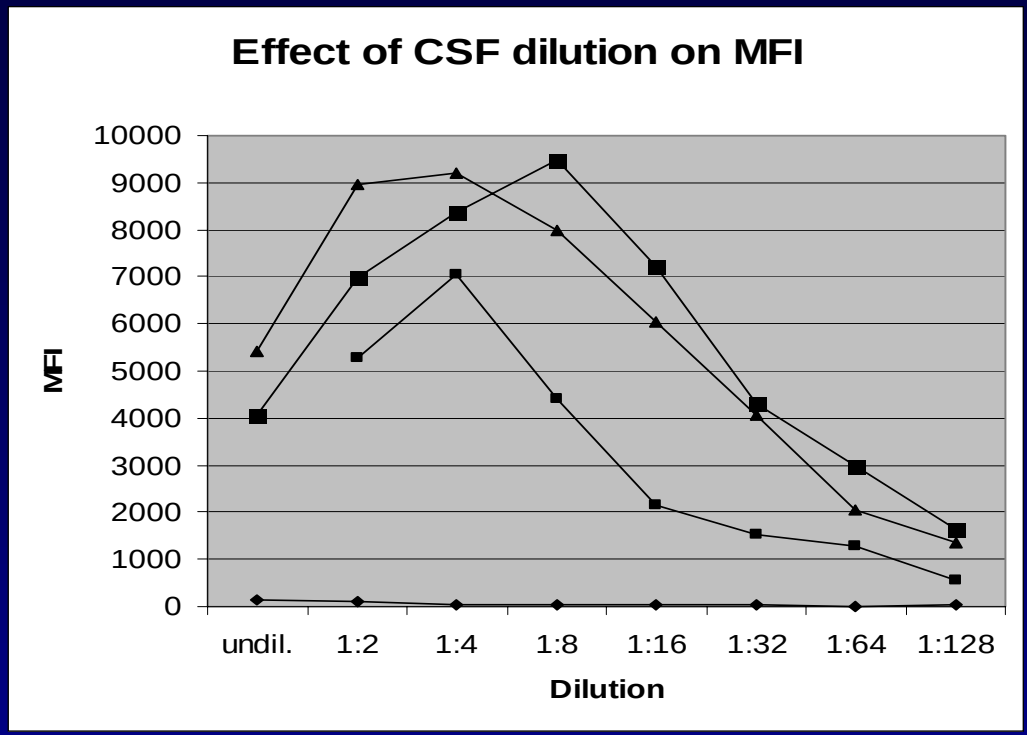
*

ELISA Classifications against MIA QDA for data not included in the cut-off analysis



N=527

Cerebrospinal fluid



- Optimal MFI readings for CSF are produced at a 1:5 dilution. Samples were not treated with protein G.
- Only 25 ul of CSF is required for the SLE/WN MIA as opposed to 200 ul for the MAC-ELISA.
- 29/30 CSF results correlated between the MIA and MAC-ELISA.
- The highest MFI reading out of 80 CSF samples was 9270.

Cost comparison based on 40 samples:

WN/SLE MIA		WN + SLE ELISA	
Plates	$13.20	Plates	$20.00
Coupled beads (lab)	$4.17	Coating antibody	$2.20
Coupled beads (com.)	$31.87	Test reagents	$1.00
Protein G	$4.50	Wash buffer	$8.00
R-PE anti-hu IgM	$13.20	TMB susbstrate	$23.00
6B6C-1 (pure)	$0.75	Antigen	$14.00
System fluid	$1.10	Labware	$10.00
Antigen	$14.39		
Labware	$10.00		
Hands-on tech time:		Hands-on tech time:	
4.5h (lab bead prep)	$85.00	3.25 h	$65.00
1.25 (com bead prep)	$25.00		
Mean cost/pt sample w/ lab bead prep	$3.65	Mean cost/pt sample for WN + SLE	$3.58
Mean cost/pt sample w/ com. bead prep	$2.85		

Advantages of the WN/SLE duplex MIA compared to the ELISA

- Decreased turnaround time
- A single result: WN or SLE or negative
- Overall slightly better accuracy
- Less hands-on technician time
- Slightly less costly
- Technically easier
- Numeric manipulations (standardizations) can be performed using the software
- Integratable software
- Statistically sound basis for cut-off values and no equivocal results
- More flexible calculation algorithm for more accurate IgM comparisons
- Volume of CSF needed is less

Future directions

- Refine the cut-off values
- Introduce the MIA test into the ADB laboratory as a routine test
- Use the software to perform all calculations and eliminate as much paperwork as possible
- Contract out the coupling of microspheres
- Add other arboviruses to make a multiplex assay
- Add a category that defines an uninterpretable result due to background reactions on the normal antigens
- Modify the test to detect IgG

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