# Development and Validation of Multiplexed and Quantitative SARS-CoV-2 Serological Assays

**NIST MML/BBD**: Linhua Tian, Elzafir Elsheikh, Sarah Inwood, Sheng Lin-Gibson, Tara Eskandari, Kenneth Cole and Lili Wang*

**NIST ITL/SED:** Paul Patrone, Anthony Kearsley

**NIH/NCI:** Dominic Esposito, Ligia Pinto, and Jim Cherry

**NIH/VRC:** Kizzmekia Corbett and Barney Graham

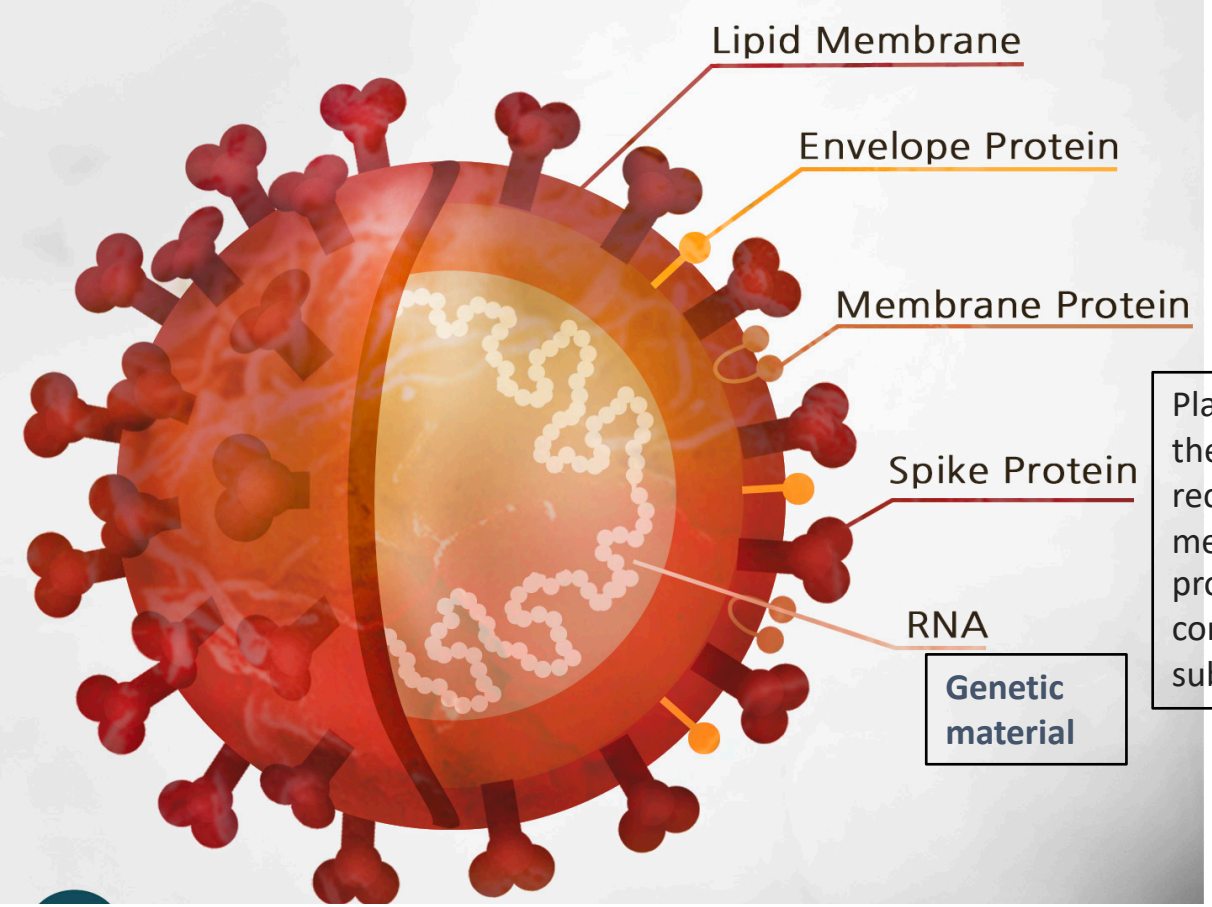
**NIH/Clinical Center:** Roa Harb and Alan Remaley

**BARDA/ASPR/OS:** Rosemary Humes, BARDA/ASPR/OS

**UK NIBSC:** Giada Mattiuzzo and Mark Page

**LGC SeraCare:** Russell Garlick

# SARS-CoV-2: The virus




https://bpsbioscience.com/sars-cov-2-coronavirus-covid-19

- alpha and beta coronaviruses are known to infect humans by spreading through the air and are responsible for about 10-30% of colds worldwide.
- Seven human coronaviruses (HCoVs) have now been identified:
  - HCoV-229E,
  - HCoV-OC43,
  - HCoV-NL63,
  - HCoV-HKU1,
  - SARS-CoV (which causes severe acute respiratory syndrome)
  - MERS-CoV (Middle East respiratory syndrome)
  - SARS-CoV-2

Cross reactivity

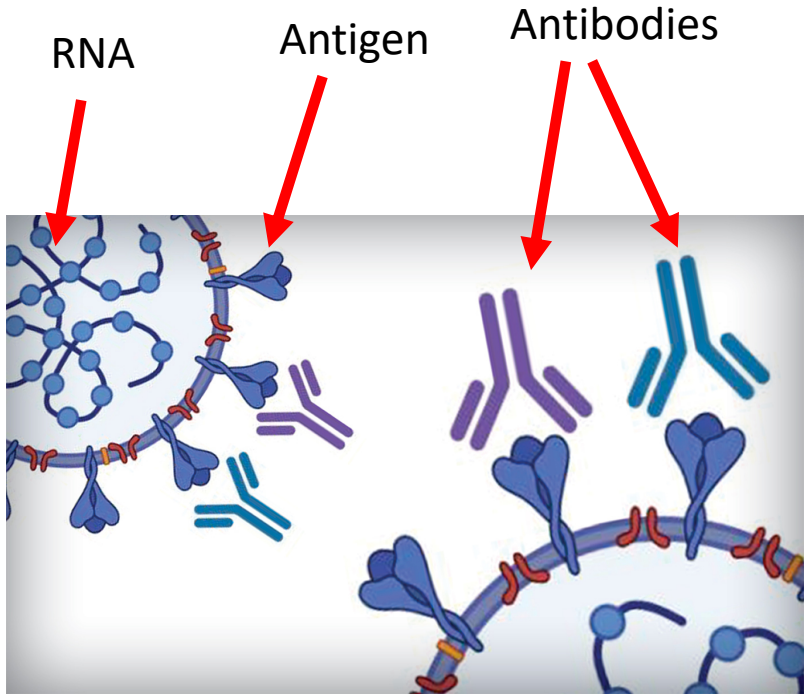
# COVID-19: The disease

- Non-symptomatic – no obvious *sign* of the disease
- Symptomatic – fevers, chills, vomiting, loss of taste, muscle aches, etc.
- Severity – mild to severe

- Infection – invasion by the virus
- Reinfection – invasion by the virus again
- Immune response – defense/response to the infection
- Immunity – the capability of multicellular organisms to resist the infection

# SARS-CoV-2: Diagnostics

- Current practice – Detect the presence of viral **RNA** via molecular testing (PCR)
  - NIST develop a RGRM (P. Vallone et al)
- Current practice – Detect the presence of **antibodies (protein produced by the patient)** via ELISA
  - NIST developed multiplexed  immunoassays
- Emerging methods:
  - Isothermal amplification technologies: RT-LAMP
  - Detection of **antigen** via mAb
  - CRISPR-based detection methods




https://news.utexas.edu/2020/09/11/antibody-test-developed-for-covid-19-that-is-sensitive-specific-and-scalable/

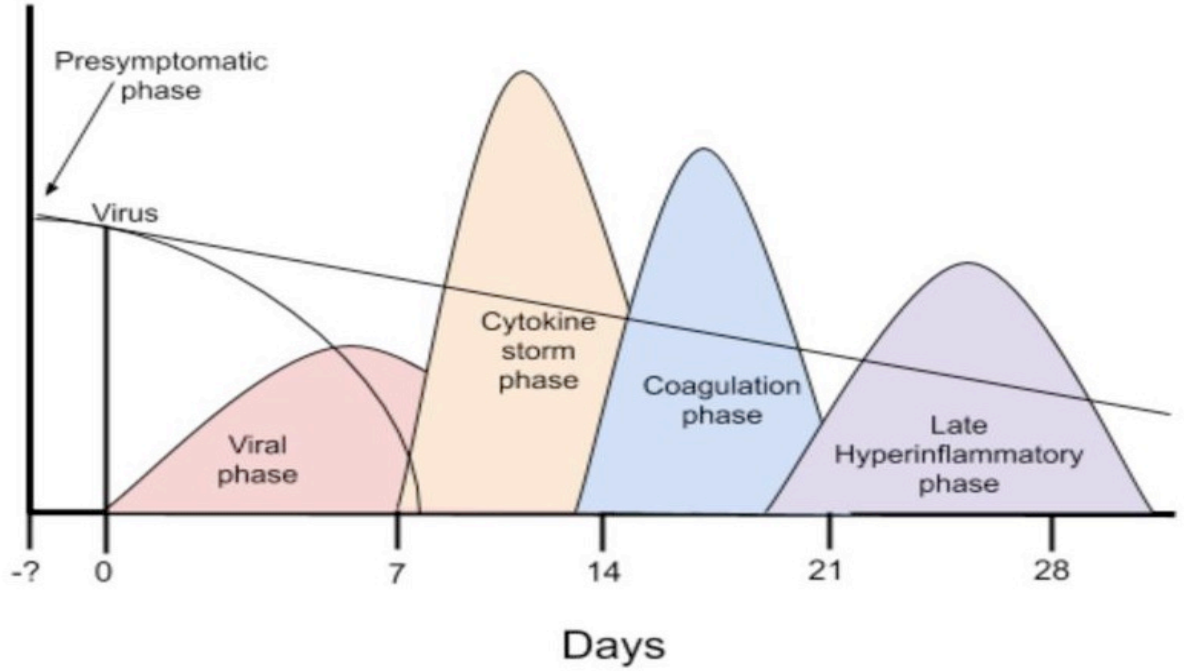
# Phases of COVID-19

NOTE:

Disease progression is highly varied

Effective treatments depend on the phases of the disease

Knowledge is needed for vaccine development




## 01

Infection phase: virus multiplies inside the body and is likely to cause mild symptoms (antiviral, convalescent plasma, mAb, etc.)

## 02

Pulmonary phase: immune system becomes strongly affected by infection, leads to primarily respiratory symptoms (steroids, anticoagulant, etc.)

## 03

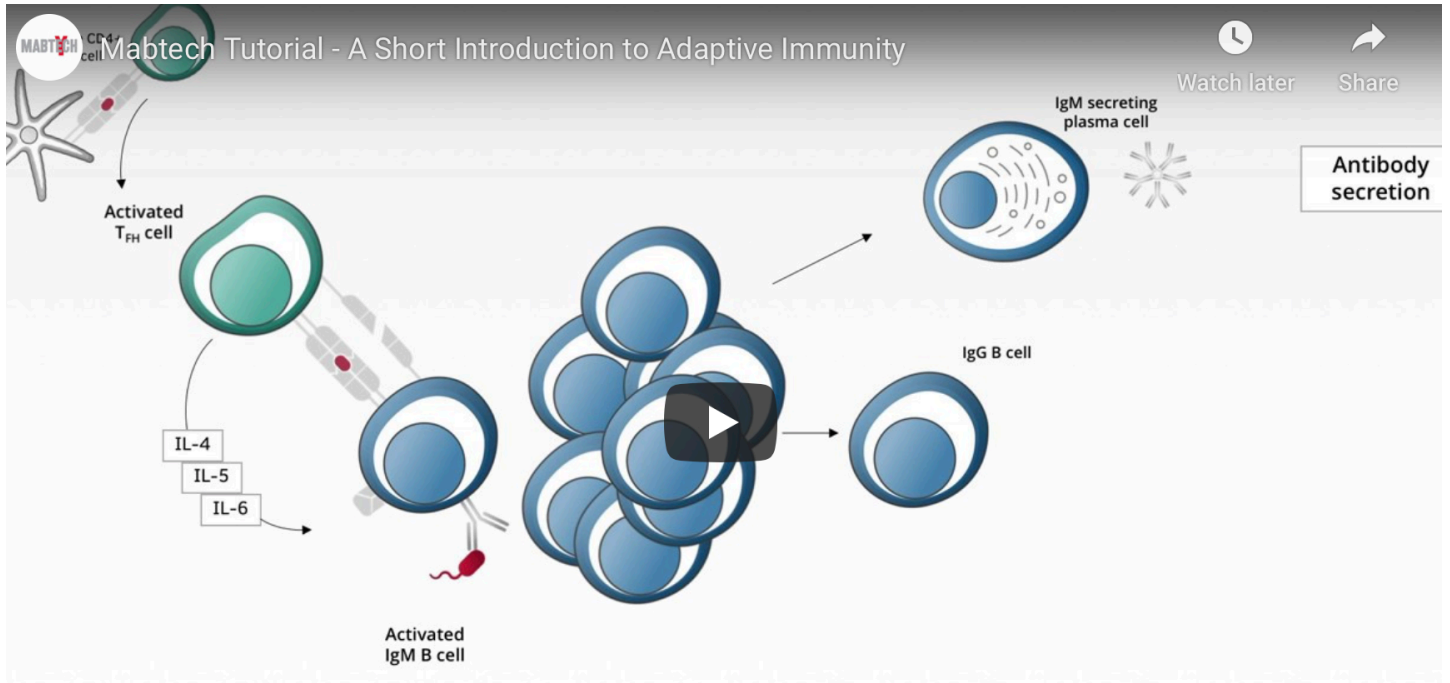
Hyperinflammatory phase: occurs when a hyperactivated immune system may cause injury to the heart, kidneys, and other organs

https://www.forbes.com/sites/williamhaseltine/2020/07/24/the-complex-pathogenesis-of-covid-19/#2c69b9a52c33

## Immunity

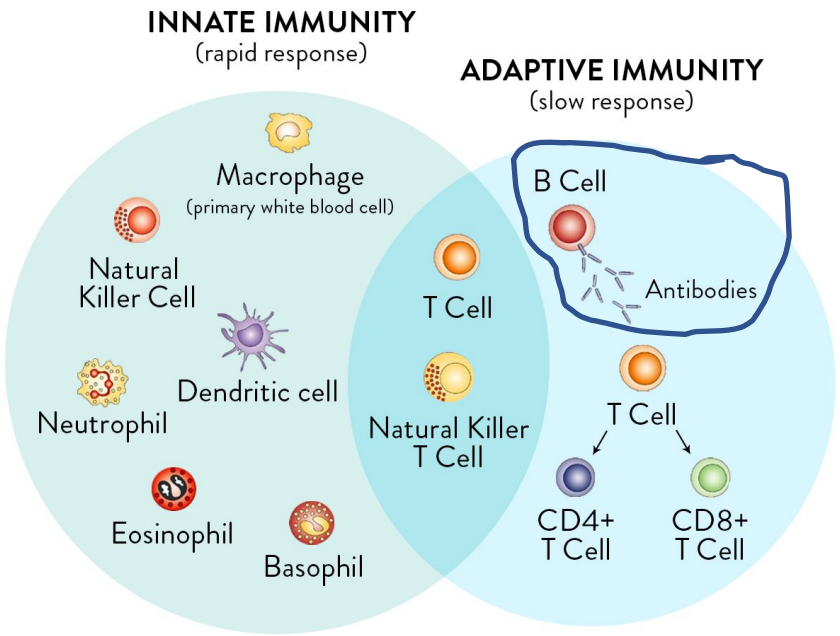
- Important to understand the body's response to infection and reinfection
- Immune system is highly complex, includes many cell types working together to recognize and destroy foreign pathogens
- Provides perspectives for needed **measurands** to advance diagnostic, vaccine and therapeutic development, and biosurveillance

## 15-minute crash course on adaptive immunity

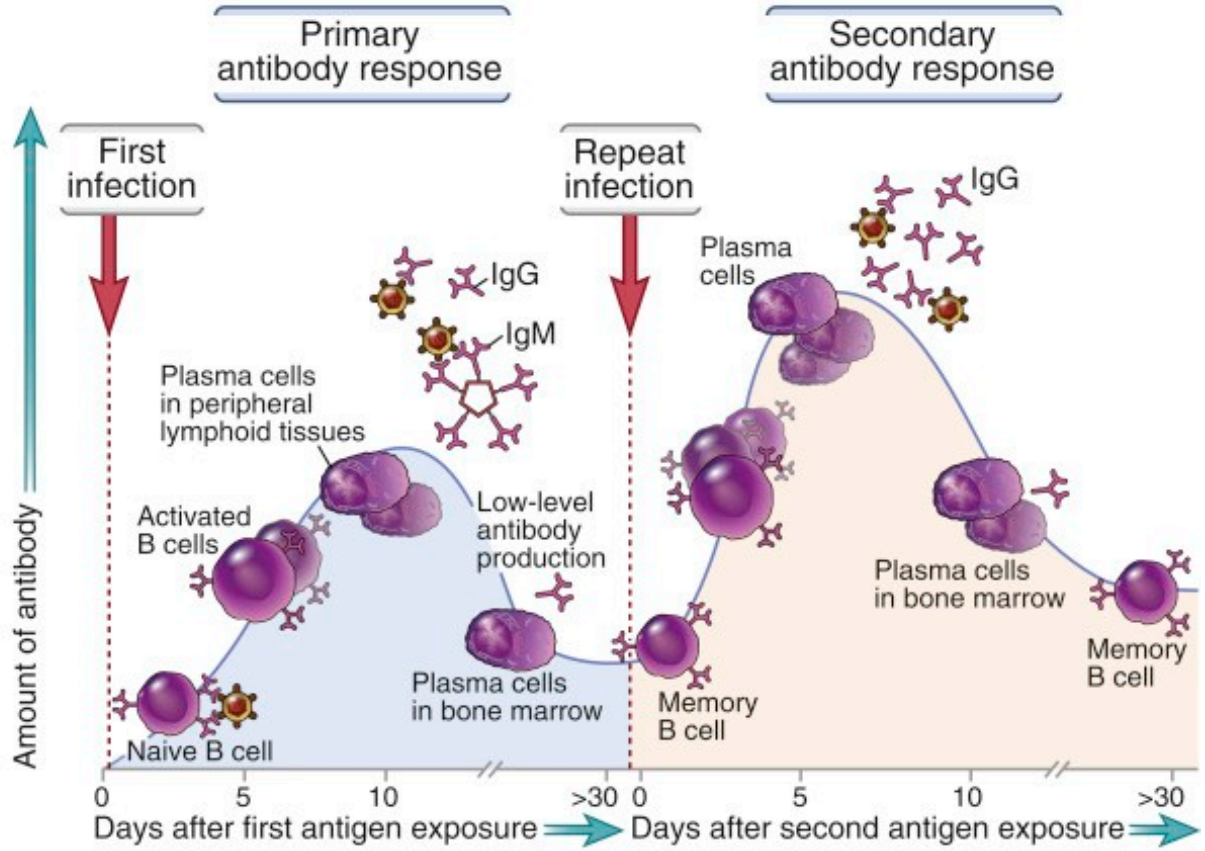



https://www.mabtech.com/knowledge-center/videos?gclid=EAIaIQobChMI34n_7YXS6wIVD9vACh00cQ1cEAAYASABEgLNTfD_BwE

# Measurement of Antibodies: Serology



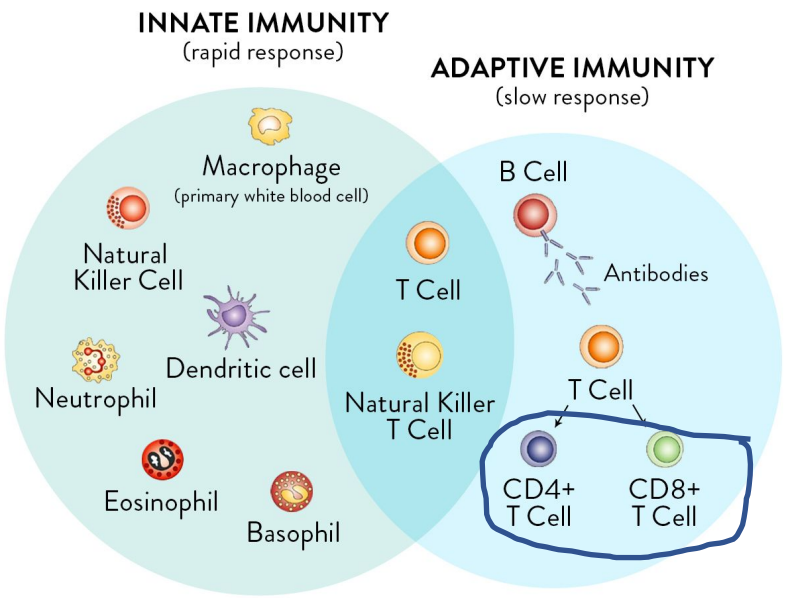

A

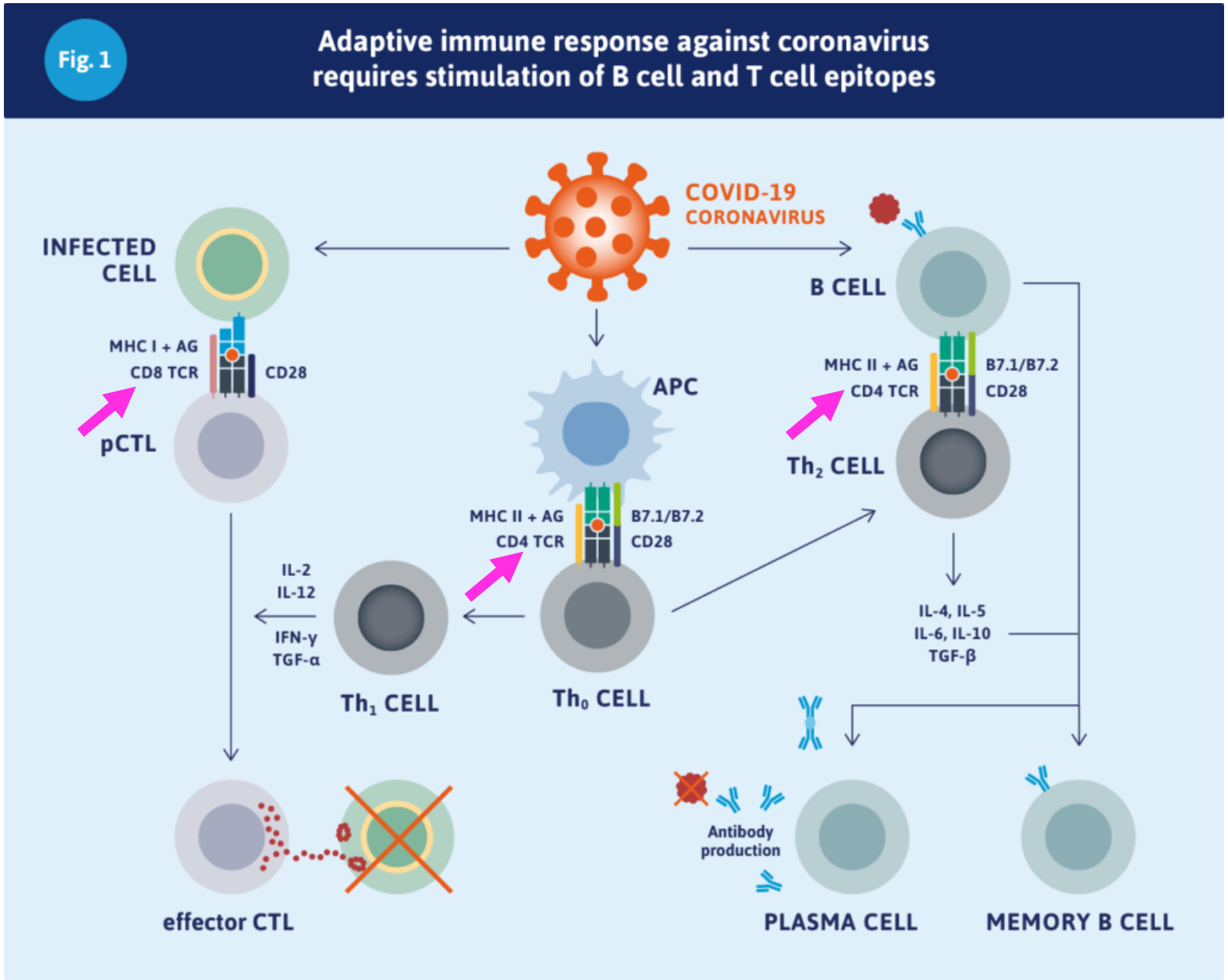



B

| | Primary response | Secondary response |
|---|---|---|
| Lag after immunization | Usually 5–10 days | Usually 1–3 days |
| Peak response | Smaller | Larger |
| Antibody isotype | Usually IgM>IgG | Relative increase in IgG and, under certain situations, in IgA or IgE (heavy-chain isotype switching) |
| Antibody affinity | Lower average affinity, more variable | Higher average affinity (affinity maturation) |

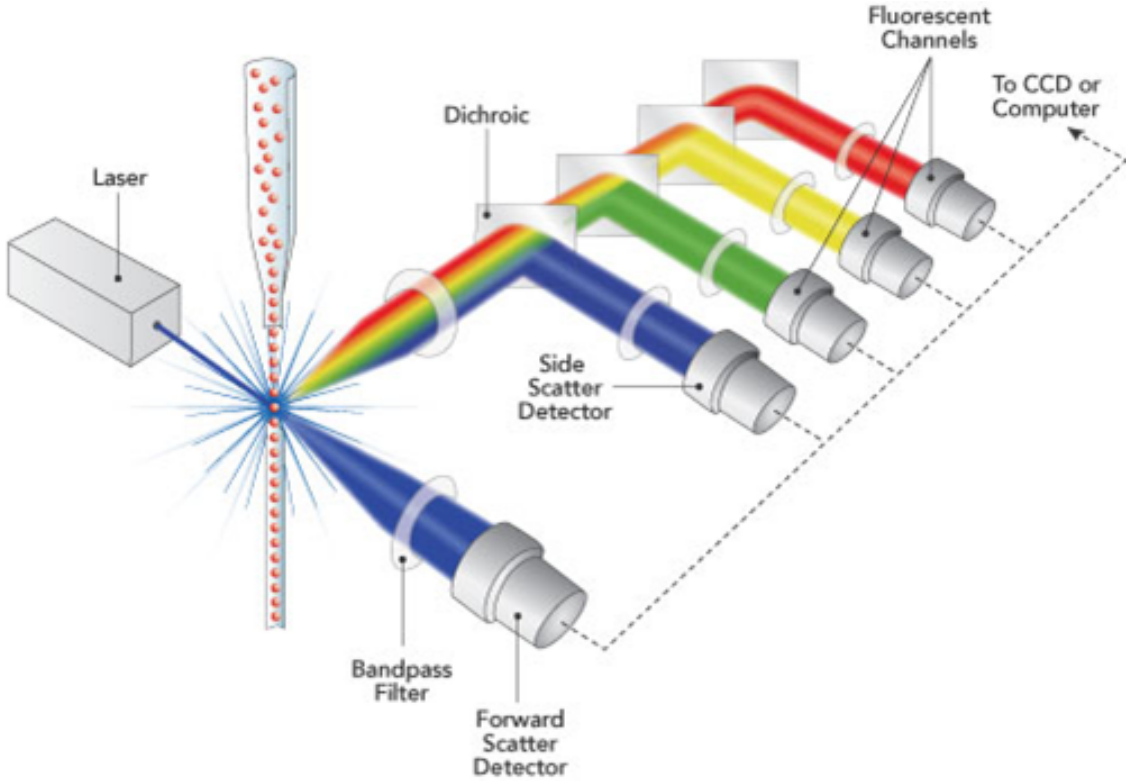
# Measurement of Immune Responses



# Flow cytometry is one of the most powerful measurement tools

- Flow cytometry is a widely used technique for single-cell and particle analysis.
- The sample is focused to ideally flow one particle or cell at a time through a laser beam, where the light scattering and fluorescent intensity are measured.
- Cell/particle type can be labelled by different fluorophores, to enable quantification and/or sorting within a complex mixture

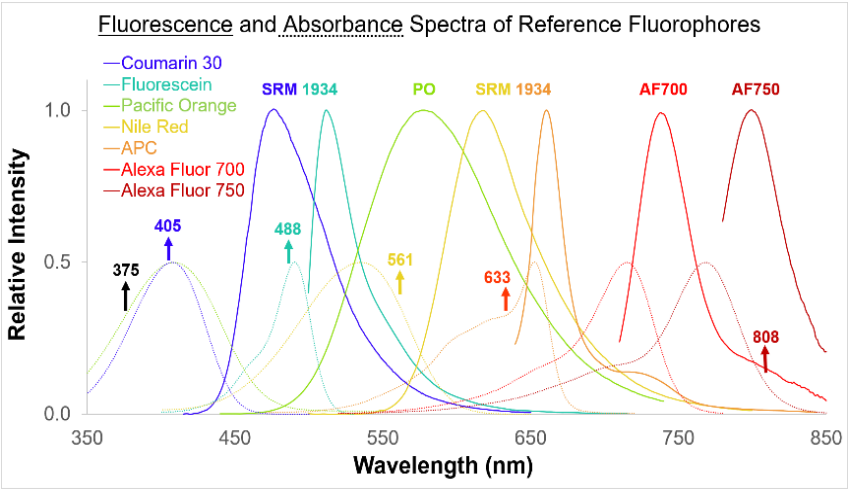


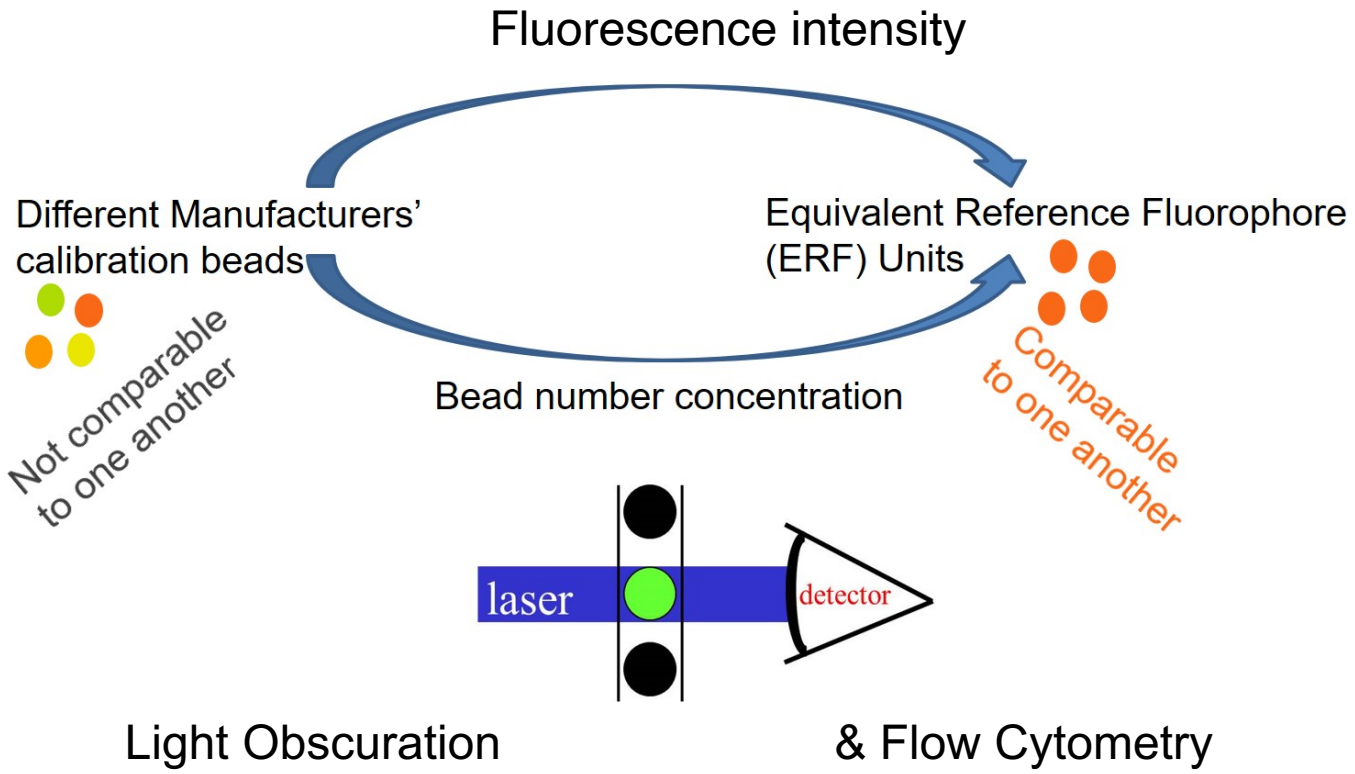
Flow cytometry is an essential tool for basic immunological research, the clinical discovery of potential vaccine and therapeutics, the development and approval of drugs and devices, disease diagnosis, and therapeutic treatment and monitoring.

- Enables single cell analysis in mixed cell samples
- Operates up to thousands of particles/cells per second
- Allows simultaneous multiparametric analysis of the physical, chemical, and biological characteristics
- Enables quantitation and sorting of cells in suspension

# NIST Flow Cytometry Quantitation Consortium

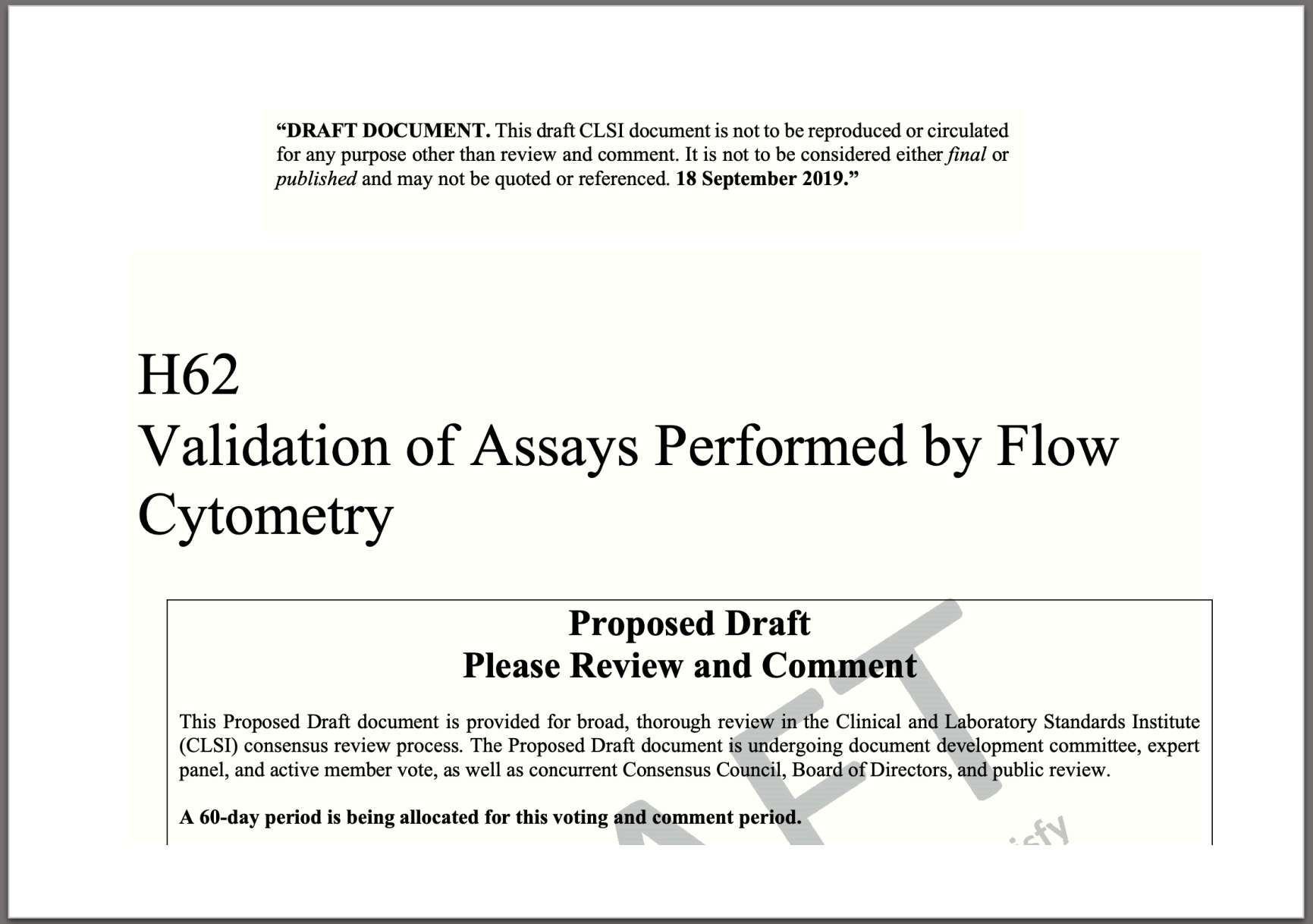
## Assignment of ERF Units – SI Traceable Calibration Beads



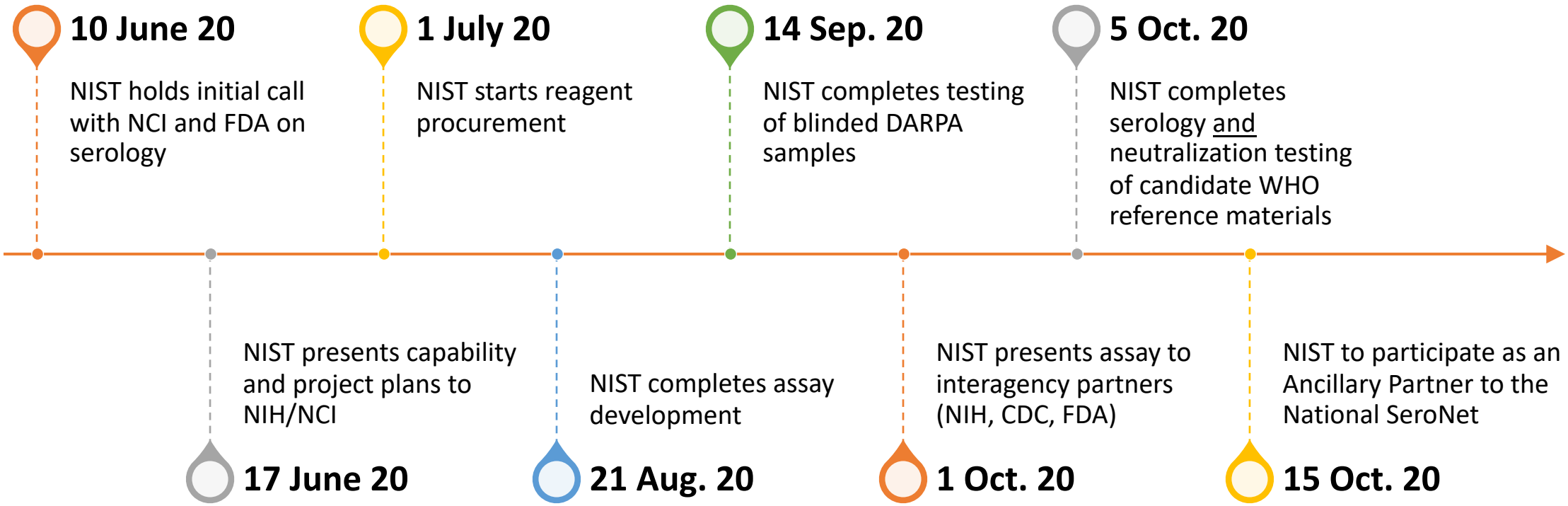

**NIST enables quantitative flow cytometry:**

- We provide measurement service to calibrate the fluorescence signal from microsphere/calibration beads in terms of a unit of equivalent number of reference fluorophores (ERF)
- The ERF unit gives the number of reference fluorophores in solution which produce the same fluorescence signal as a fluorescently labeled microsphere




NIST supports the development of documentary standards

# Timeline


10 June 20
NIST holds initial call with NCI and FDA on serology
17 June 20
NIST presents capability and project plans to NIH/NCI
1 July 20
NIST starts reagent procurement
21 Aug. 20
NIST completes assay development
14 Sep. 20
NIST completes testing of blinded DARPA samples
1 Oct. 20
NIST presents assay to interagency partners (NIH, CDC, FDA)
5 Oct. 20
NIST completes serology and neutralization testing of candidate WHO reference materials
15 Oct. 20
NIST to participate as an Ancillary Partner to the National SeroNet

# Measurement Challenges and NIST Approach

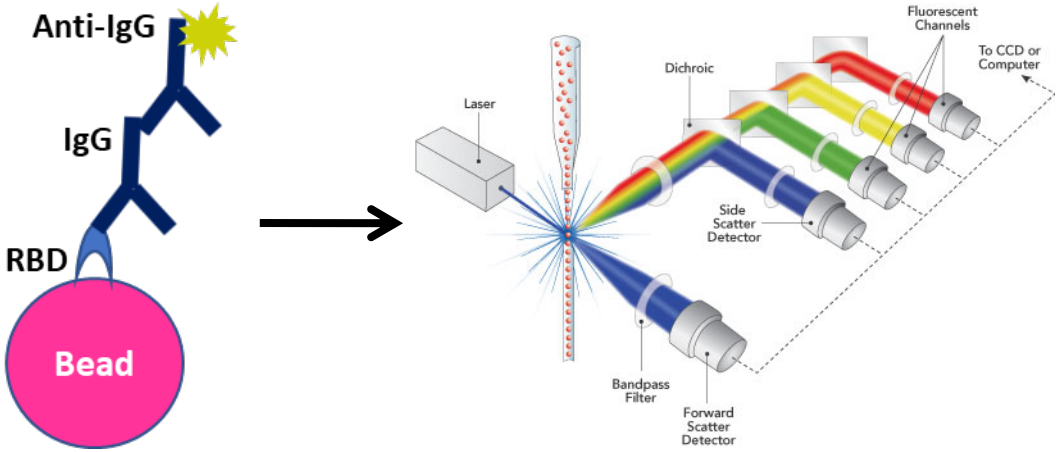
**FDA De-Lists 27 Serology Assays for SARS-CoV-2 Testing**
May 21, 2020 - 360Dx
NEW YORK – The US Food and Drug Administration has removed 27 tests from the list of SARS-CoV-2 serology assays that notified under its Policy D that it intended to seek Emergency Use Authorization. The makers of the tests either voluntarily withdrew them or the firms failed to submit the necessary validation data by a recently issued deadline.

**Coronavirus (COVID-19) Update: FDA Issues Warning Letters to Companies Inappropriately Marketing Antibody Tests, Potentially Placing Public Health at Risk**
*Agency Continues Effort to Help Protect Public Health from Fraudulent Products*
June 17, 2020

https://www.fda.gov/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas

**Needs: Robust assays and reference materials are needed to advance serology**

**Response: Developing and validating Multiplexed bead-based fluorescent immunoassay**



# No appropriate control panels available for assessing the quality of serology

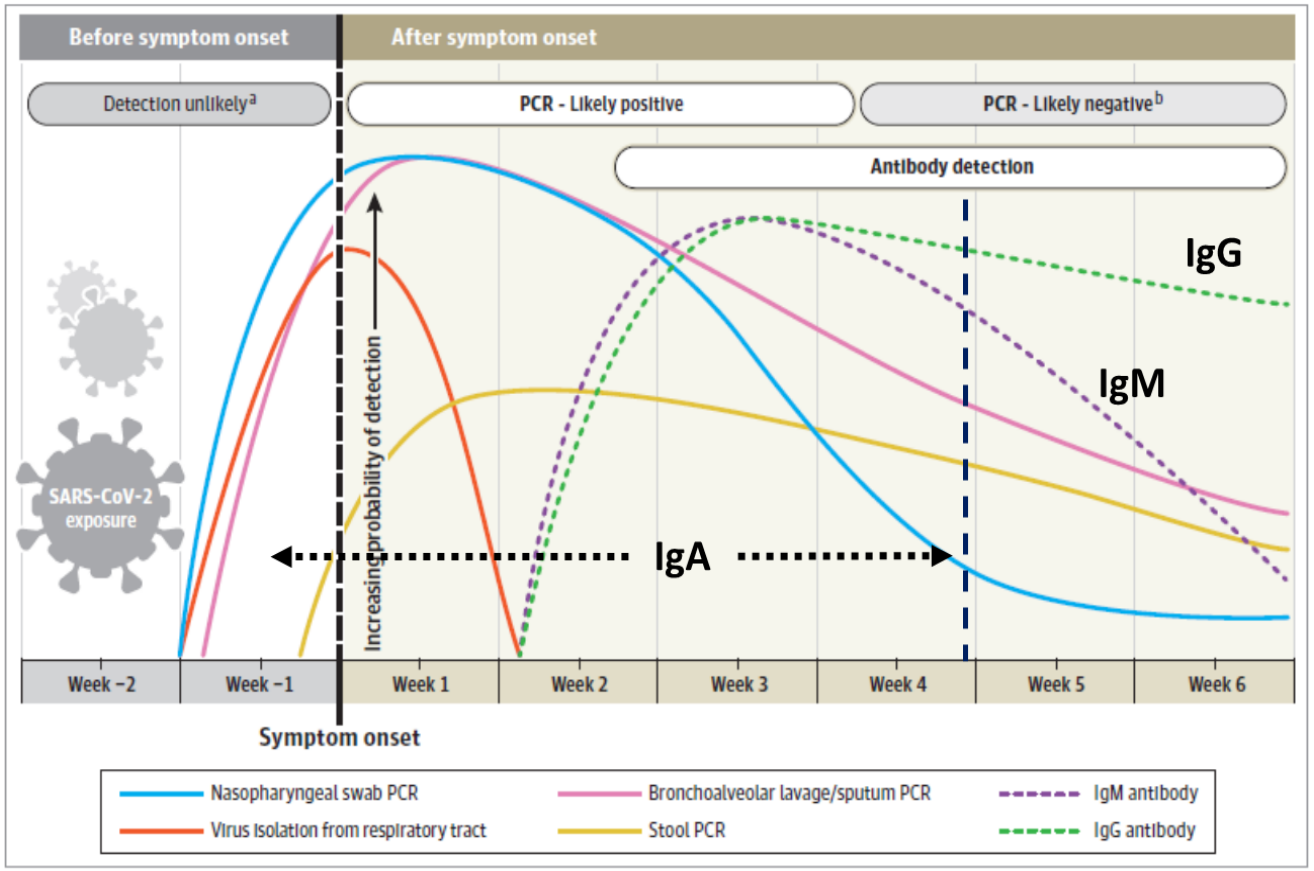
**Functional Activity:**

| Panel ID | Catalog Number | CDC[1] IgM ELISA | CDC[1] IgG ELISA | UCI[2] Array |
|---|---|---|---|---|
| P2-1 | NR-53248 | 400 | > 6400 | |
| P2-2 | NR-52915 | 100 | 1600 | |
| P2-3 | NR-52737 | 100 | > 6400 | |
| P2-4 | NR-52746 | 100 | > 6400 | |
| P2-5 | NR-52905 | 400 | > 6400 | |
| P2-6 | NR-52906 | 100 | 400 | |
| P2-7 | NR-52911 | 100 | 1600 | |
| P2-8 | NR-52760 | < 100 | 1600 | |
| P2-9 | NR-52557 | | < 100 | hCoVpos |
| P2-10 | NR-52562 | | < 100 | hCoVpos |

[1]Centers for Disease Control and Prevention
[2]University of California, Irvine

# The body produces many types of antibodies (IgG, IgM, IgA, etc) in response to the infection

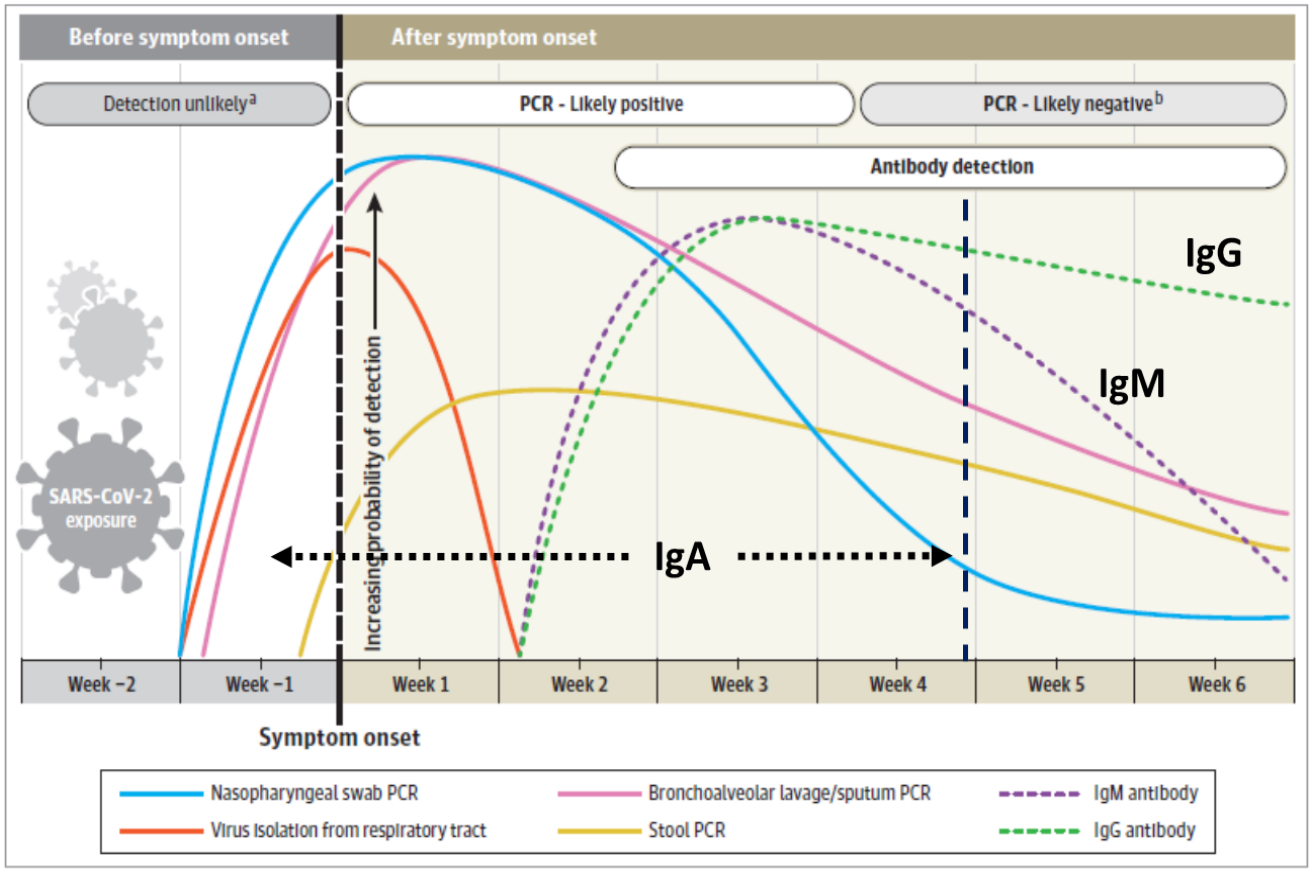
Figure. Estimated Variation Over Time in Diagnostic Tests for Detection of SARS-CoV-2 Infection Relative to Symptom Onset



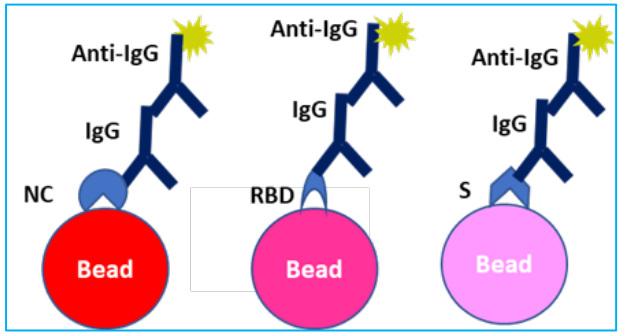

- Human antibodies are classified into 5 isotypes (IgM, IgD, IgG, IgA, and IgE) according to their H chains
- Roles of antibody:
  - Secreted antibodies can bind to and inactivate pathogens (neutralization).
  - Facilitate phagocytosis of foreign substances by phagocytic cells (opsonization)
  - Activation of the complement system to destroy pathogens through lysis and enhanced chemotaxis.
- IgG is most abundant antibody isotype in the blood. IgA is abundant in serum, nasal mucus, saliva

# Design of multiplex (first) flow cytometry serology assays

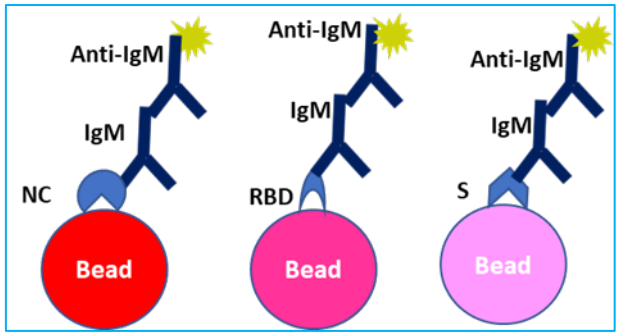
Figure. Estimated Variation Over Time in Diagnostic Tests for Detection of SARS-CoV-2 Infection Relative to Symptom Onset



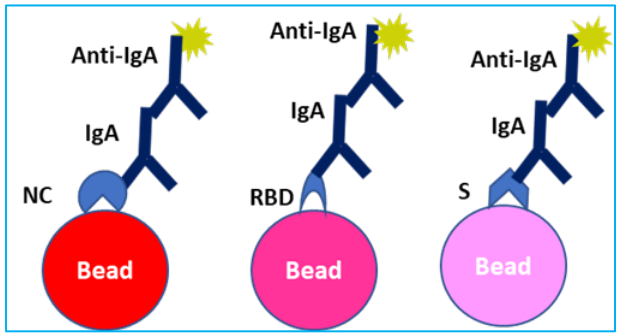

IgG assay



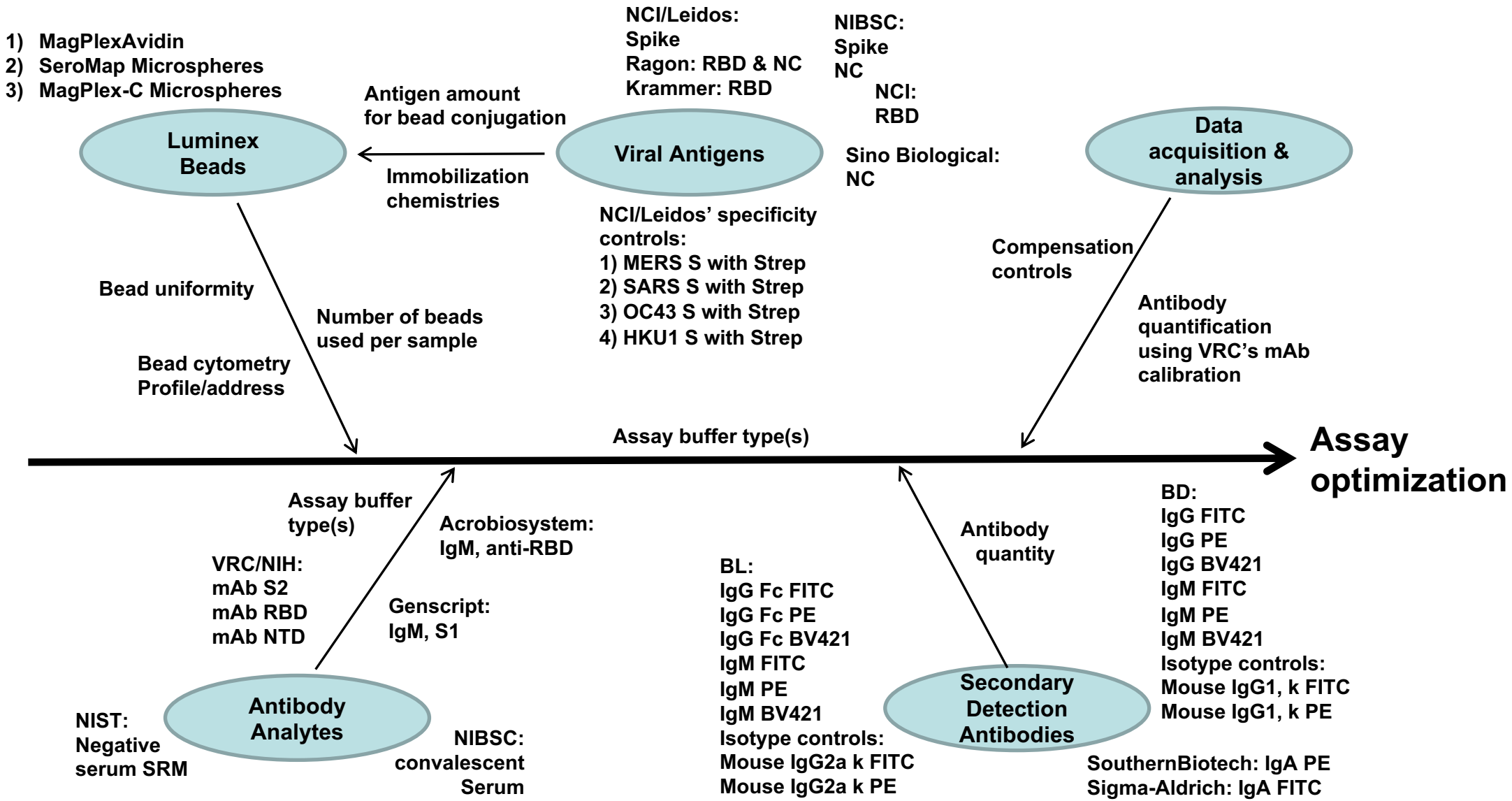

IgM assay



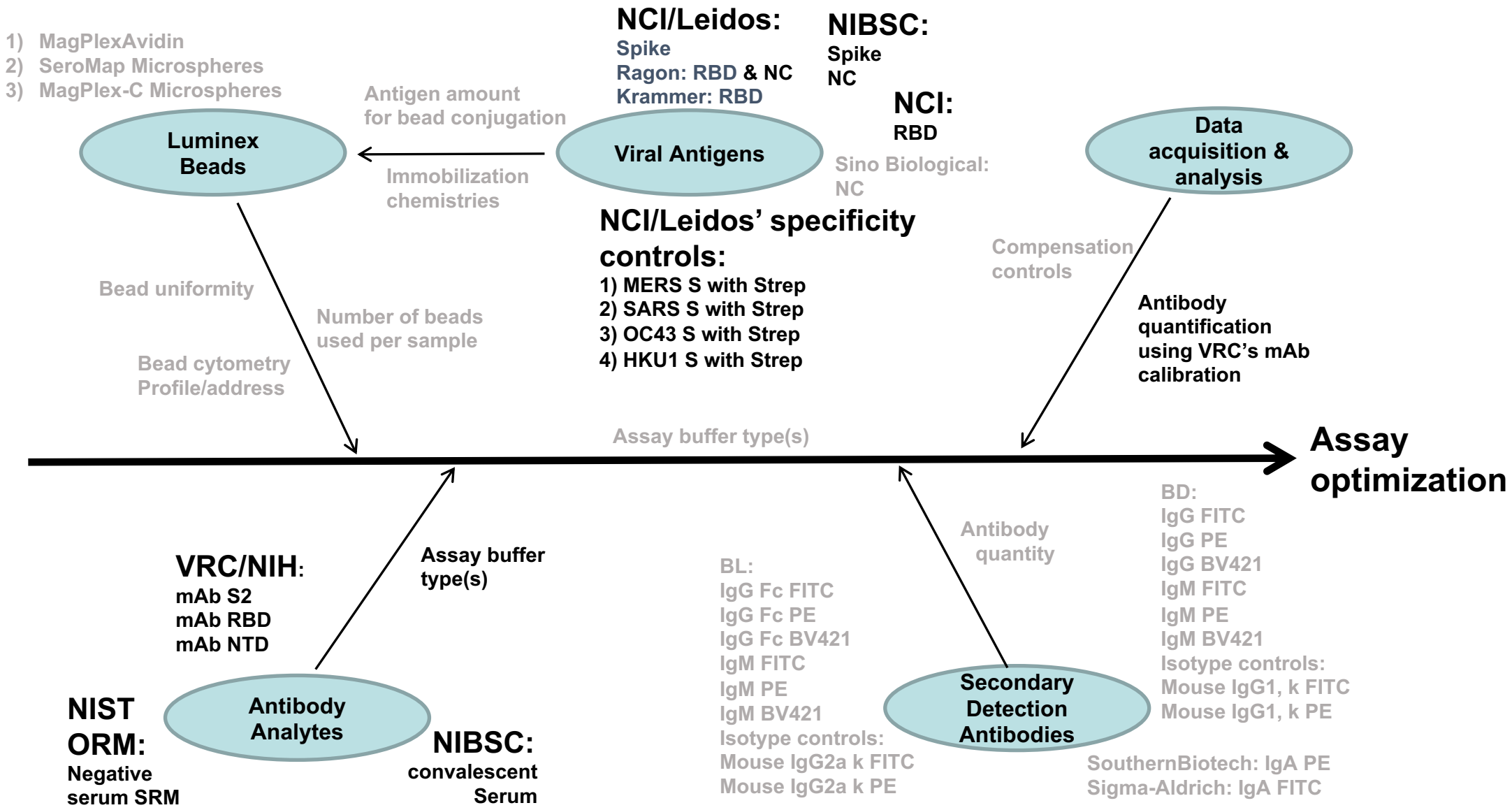

IgA assay



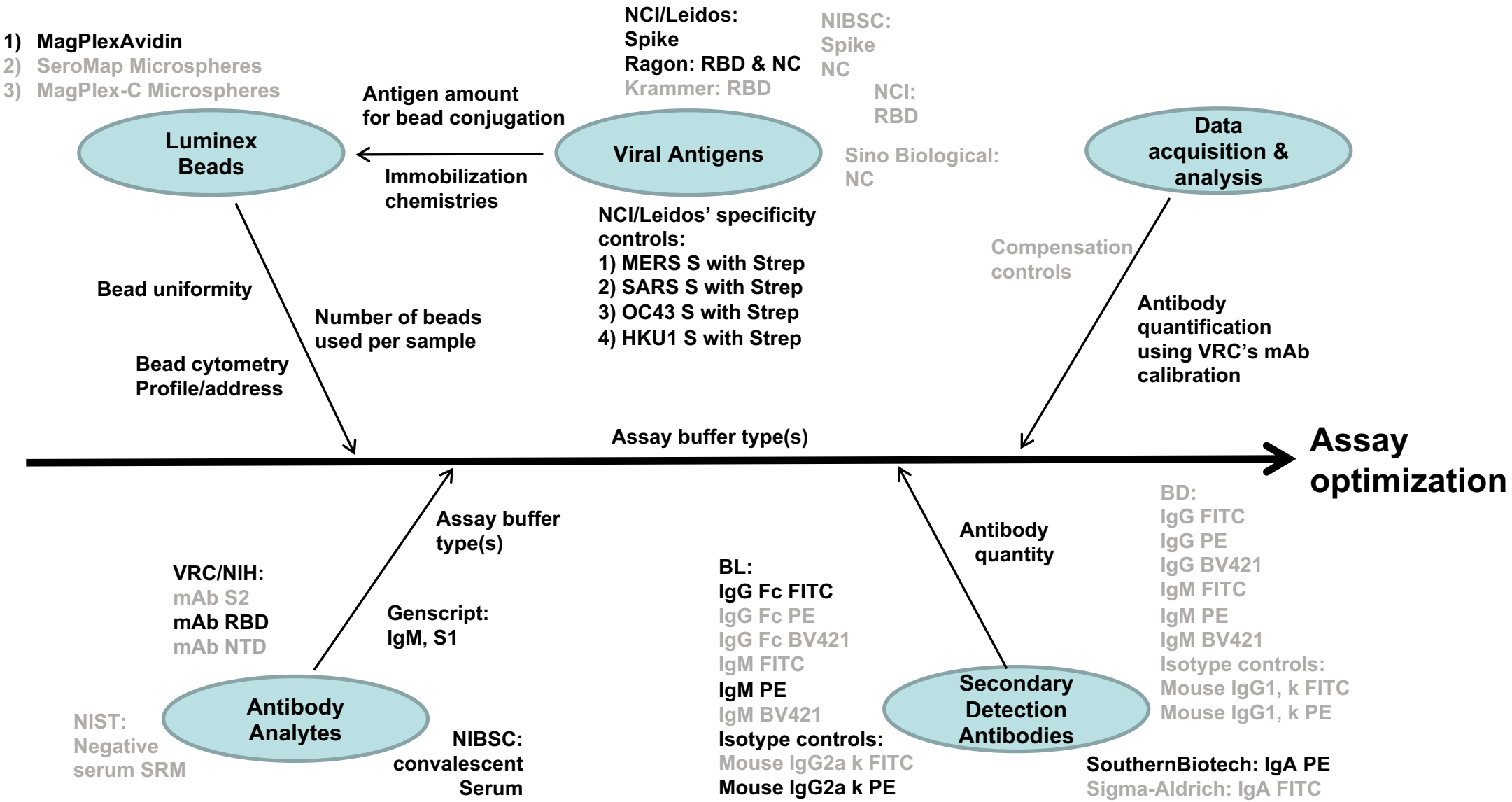
# Serology Assay Development and Optimization



# Key partners provided critical reagents and control materials

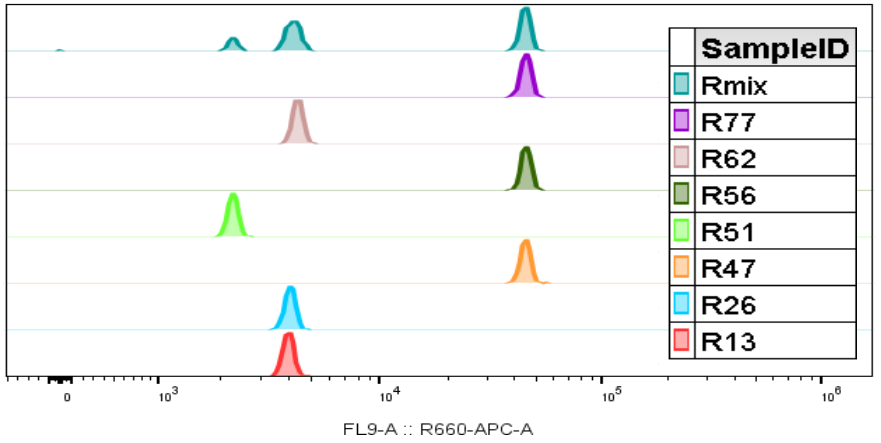


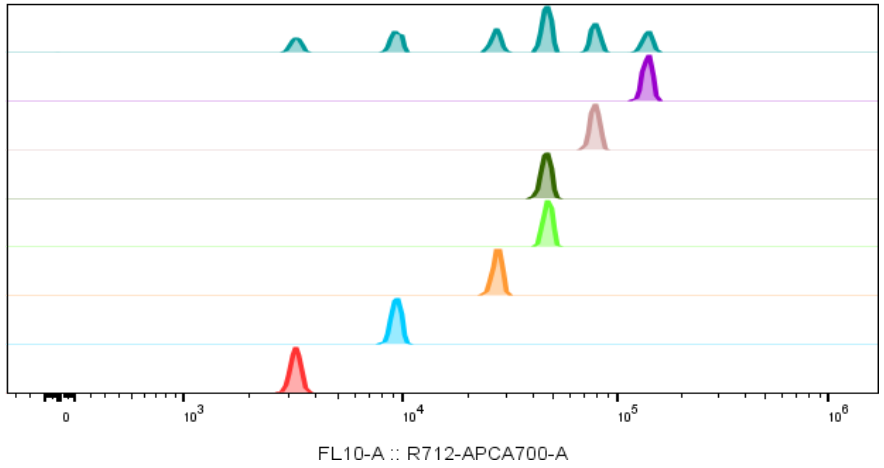
# Many parameters and reagent tested to optimize the assay

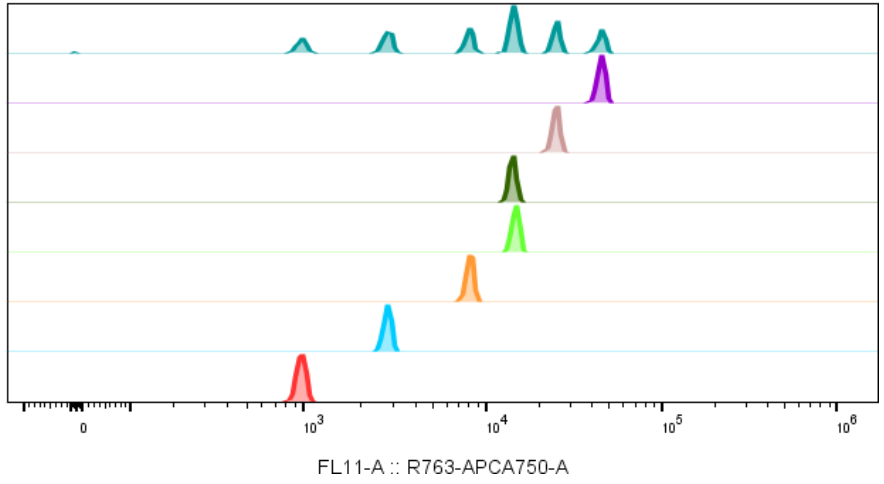


# Optimizing Magplex bead addresses and suitable cytometer detection channels

| Bead Address (Antigen) | R660 APC | R712 APC 700 | R763 APC 750 |
|---|---|---|---|
| R13 (SARS2 NC) | × | ✓ | ✓ |
| R26 (SARS2 RBD) | × | ✓ | ✓ |
| R47 (MERS Spike) | × | ✓ | ✓ |
| R51 (SARS2 Spike) | ✓ | × | × |
| R56 (SARS1 Spike) | × | × | × |
| R62 (OC43 Spike) | × | ✓ | ✓ |
| R77 (HKU1 Spike) | × | ✓ | ✓ |



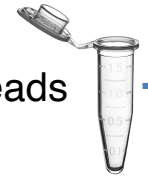



30mins

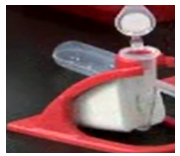
1hr 20mins

3hrs

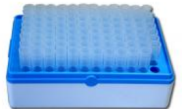
NC/RBD/Spike beads



Magnetic tube separator



0.2x10$^6$ per ml each bead



*NOTE*: Steps 1 and 2 can be automated to significantly improve throughput and consistency

50µl per well

Add 50µl diluted sample per well

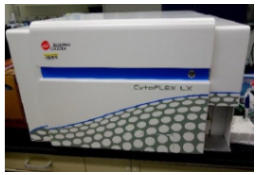
96-well plate

Incubate 30 mins at RT, 750rpm in the dark


Wash 2x with magnetic plate separator

50µl 2$^{nd}$ Ab.

Resuspend beads with 120µl PBT buffer per well



Acquire 2 mins per sample

# Automating assay using **P-CAMP** (a unique NIST biofoundry)

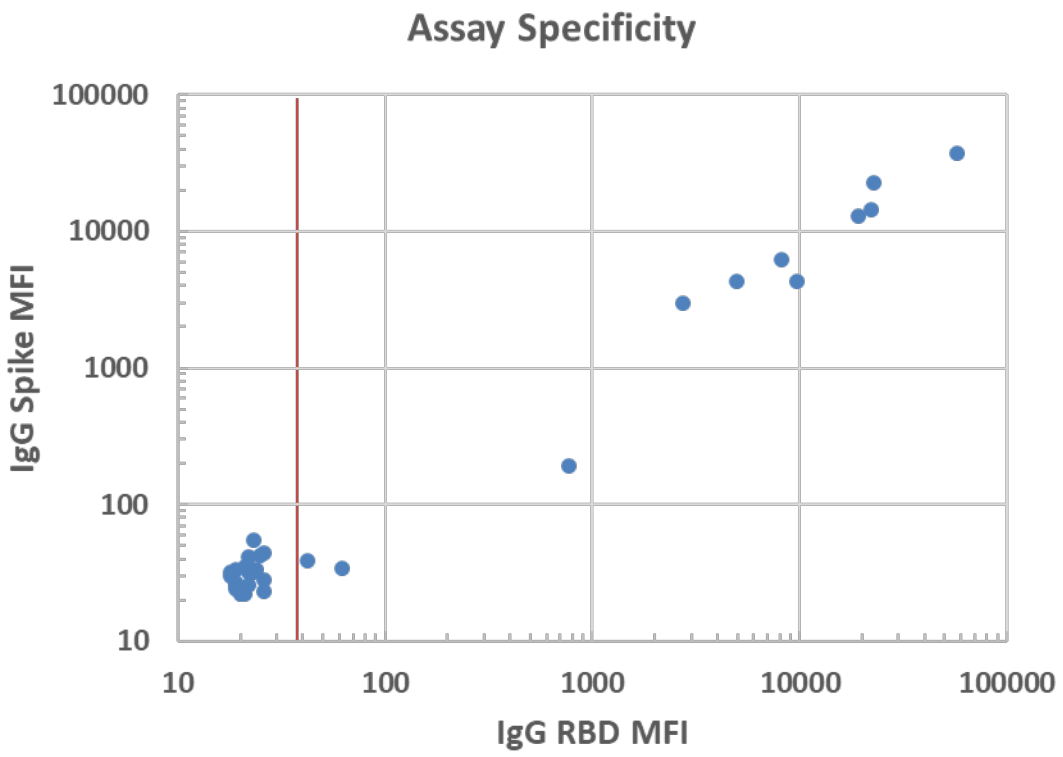
# Plate Design of Quantitative Serological Assays

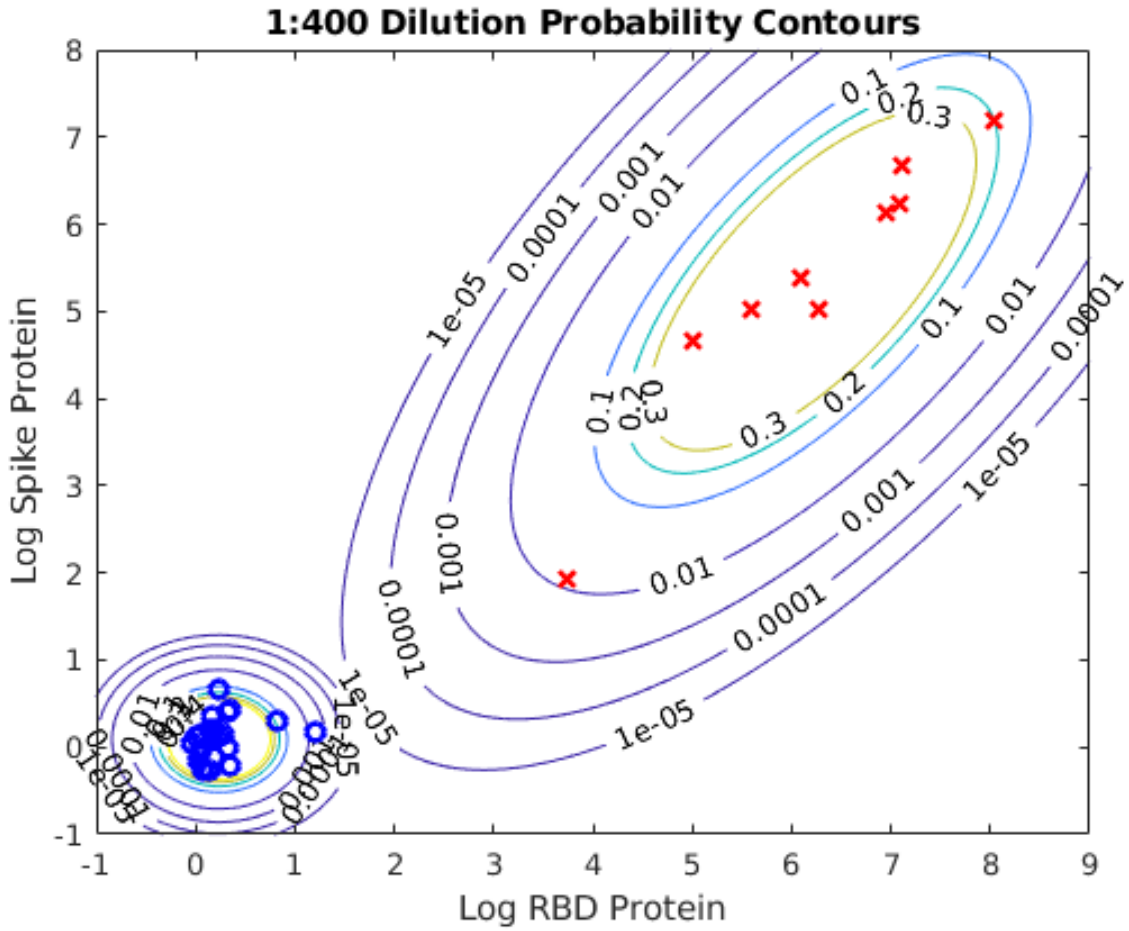
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | (+) Ctrl | (+) Ctrl | (-) Ctr | (-) Ctr | 1:800 Spl 1 | 1:800 Spl 1 | 1:1600 Spl 1 | 1:1600 Spl 1 | | | | |
| B | 1:50 Spl 1 | 1:50 Spl 1 | 1:100 Spl 1 | 1:100 Spl 1 | 1:200 Spl 1 | 1:200 Spl 1 | 1:400 Spl 1 | 1:400 Spl 1 | 1:800 Spl 1 | 1:800 Spl 1 | 1:1600 Spl 1 | 1:1600 Spl 1 |
| C | 1:50 Spl 2 | 1:50 Spl 2 | 1:100 Spl 2 | 1:100 Spl 2 | 1:200 Spl 2 | 1:200 Spl 2 | 1:400 Spl 2 | 1:400 Spl 2 | 1:800 Spl 2 | 1:800 Spl 2 | 1:1600 Spl 2 | 1:1600 Spl 2 |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | 1:50 Spl 7 | 1:50 Spl 7 | 1:100 Spl 7 | 1:100 Spl 7 | 1:200 Spl 7 | 1:200 Spl 7 | 1:400 Spl 7 | 1:400 Spl 7 | (+) Ctrl | (+) Ctrl | (-) Ctrl | (-) Ctrl |

Assay readout of a sample dilution residing in a linearity range of a calibration curve is used for quantifying antibody titer

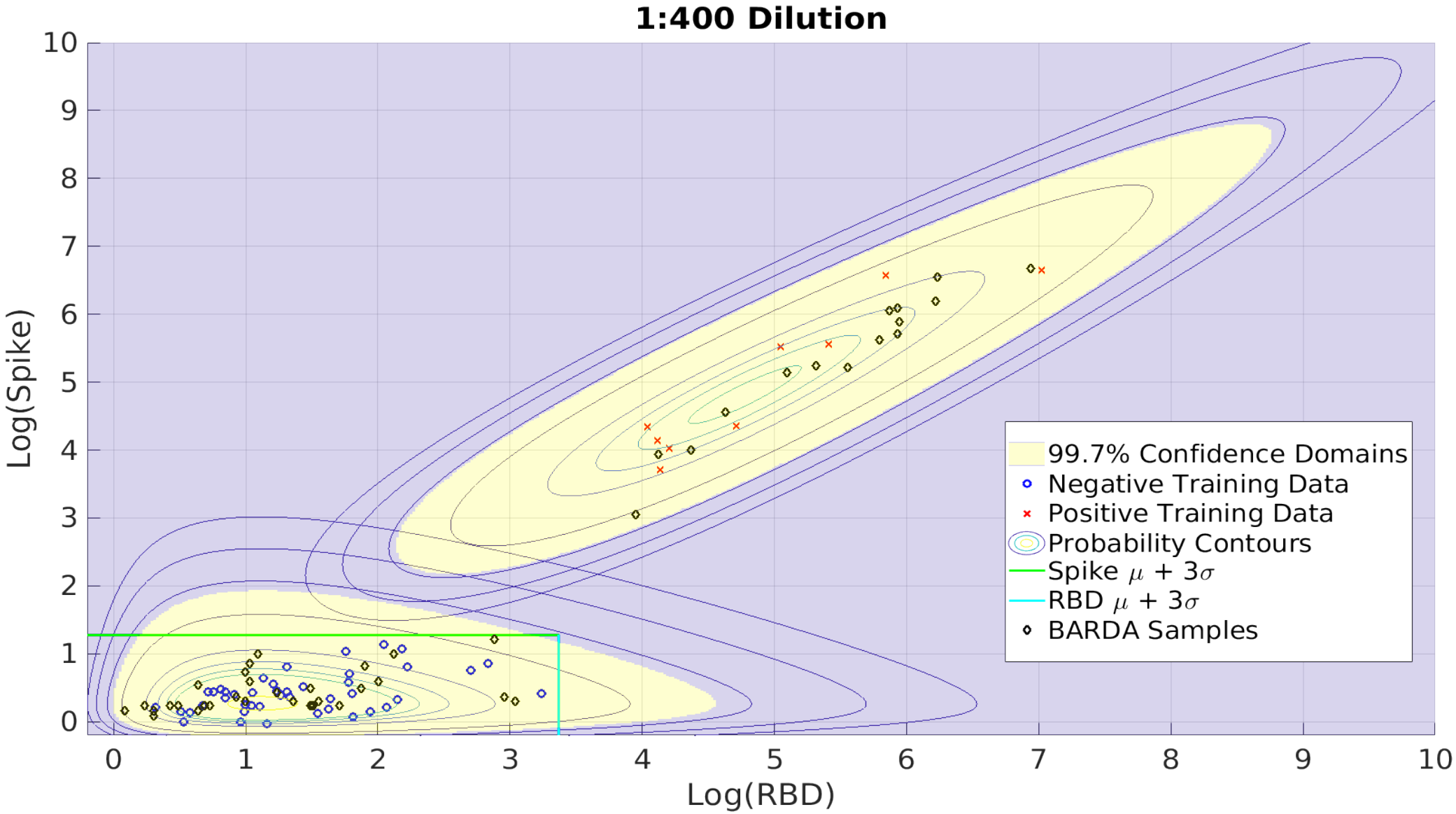
# Demonstration of NIST Serology Assay Performance

**1:400 dilution, 23 negative and 9 positive samples**

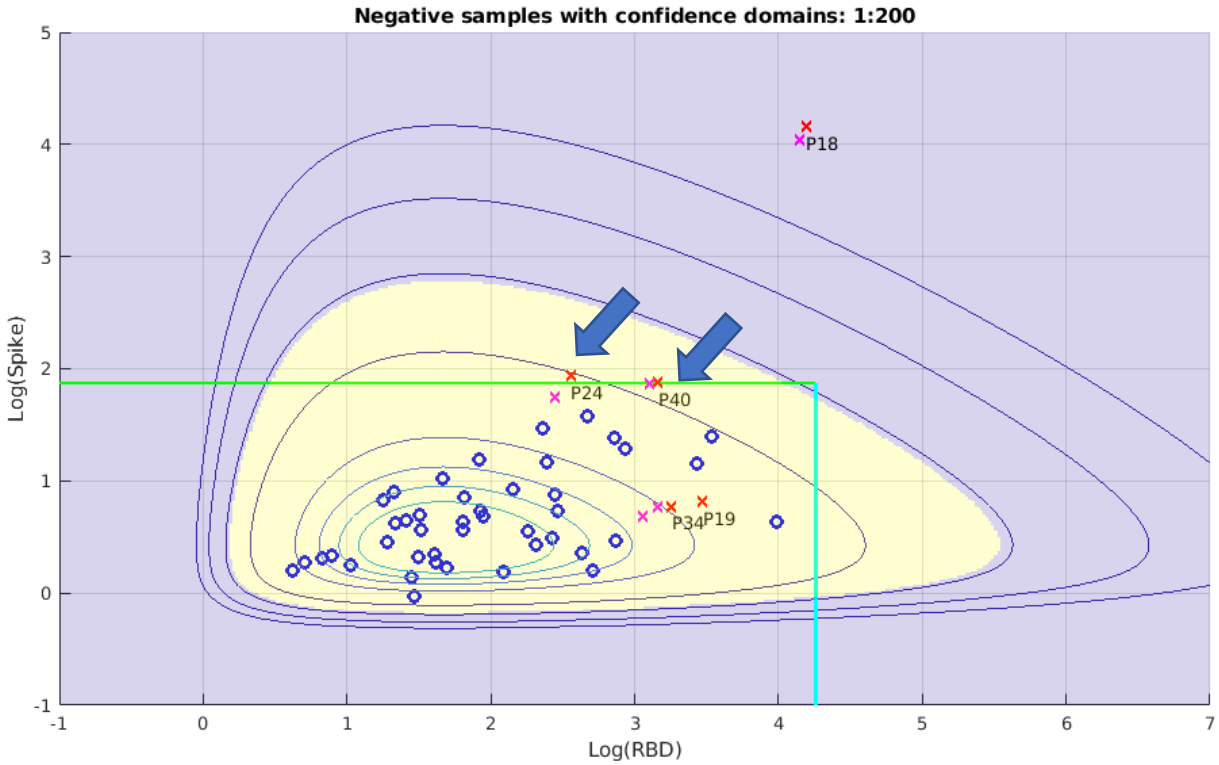


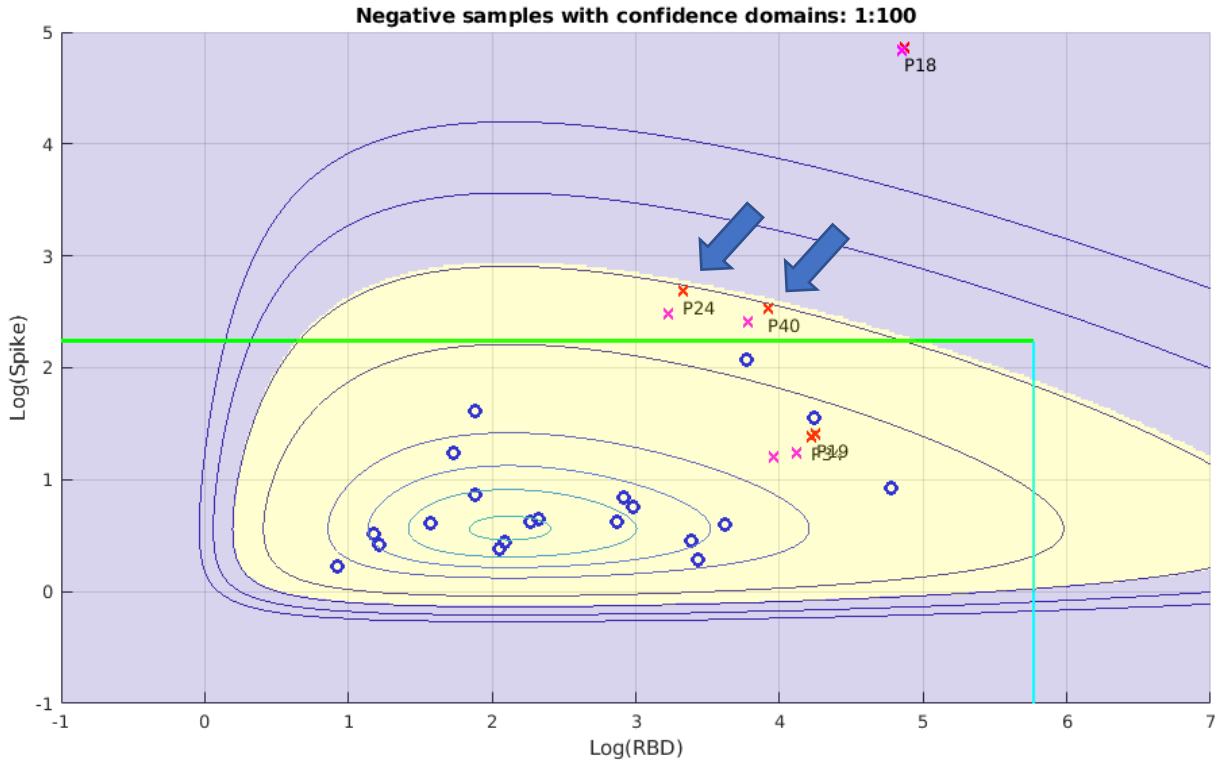



# IgM Assay Validation: Identifying Questionable Samples



# IgM Assay Validation: Evaluating Questionable Samples at low dilutions



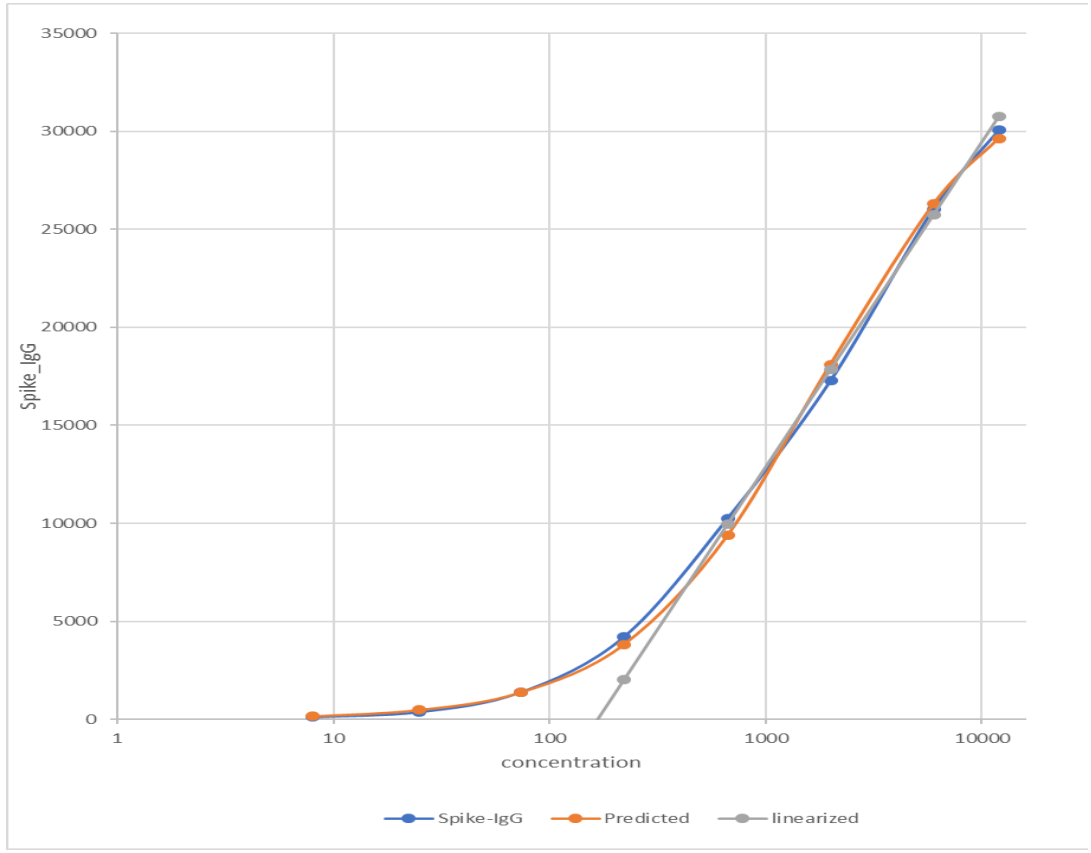

- P24 and P40 were in the negative range with higher uncertainty.
- Results from BARDA (Sept 14): they represented plasma samples from blood donors obtained last October (pre-pandemic), tested positive for other hCoV.

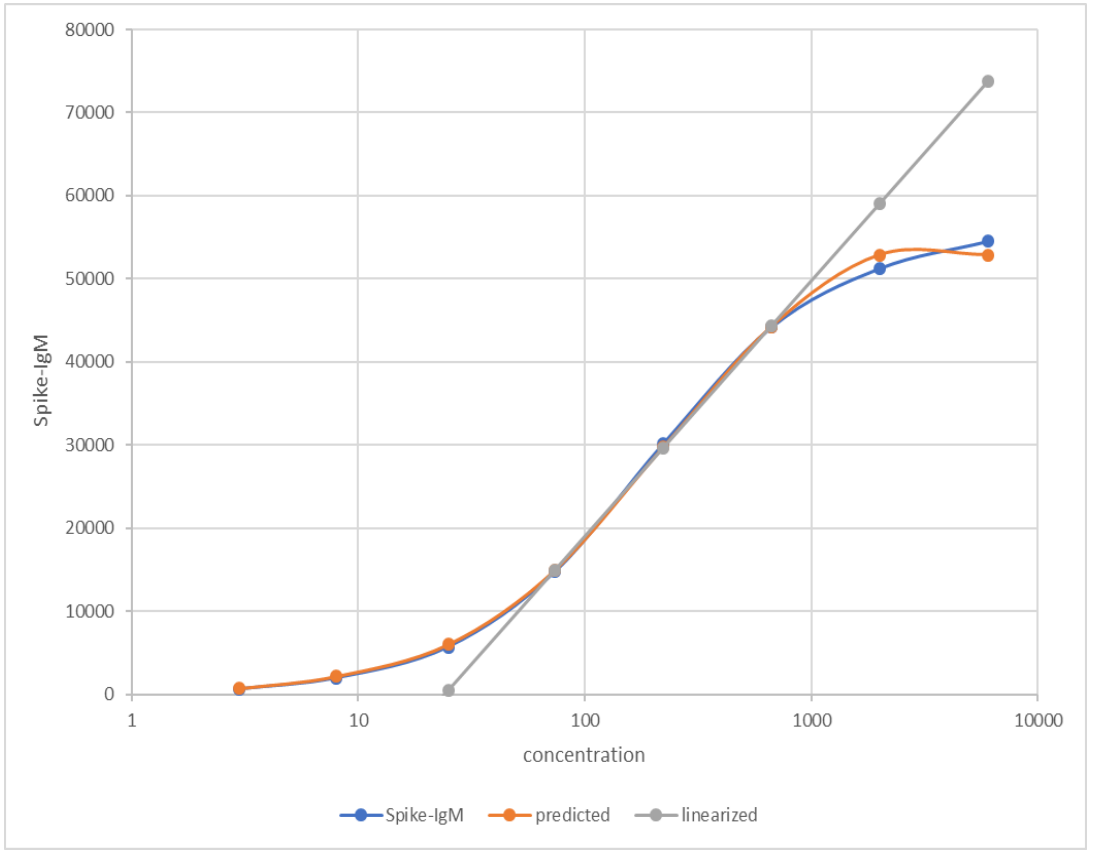
# Assay Performance

- Sensitivity: smallest amount of substance in a sample that can accurately be measured by an assay (100% for IgG assay; 100% for IgM assay)
- Specificity: the ability to measure the intended substance, rather than others, in a sample; proportion of true-negatives which actually test negative (100% for IgG assay; 94% for IgM assay)
- Positive predictive value: portion of true positives in test outcome positives (100% for IgG assay; 88% for IgM assay)
- Negative predictive value: portion of true negatives in test outcome negatives (100% for IgG assay; 100% for IgM assay)
- Accuracy: Blinded assay (BARDA provided answer keys)

Denise Lo, Bruce Kaplan and Allan Kirk, Nature Review Nephrology 2014
doi:10.1038/nrneph.2013.281

# Path to IgG and IgM Quantification



# Ongoing and future work

1

Contribute to the development of global and national standards

2

Develop neutralization assays critical for vaccine and therapeutics development

3

Bridge various standards via NIST scheme for quantitative analysis

## 1st WHO International Standard for anti-SARS-CoV-2 antibody

**Material**: Antibody, human convalescent plasma

**Intended use**: Primary calibrant for serological assays

**Anticipated delivery date**: December 2020

# NIST Participation in WHO-NIBSC Serology Study

Develop serological antibody standard

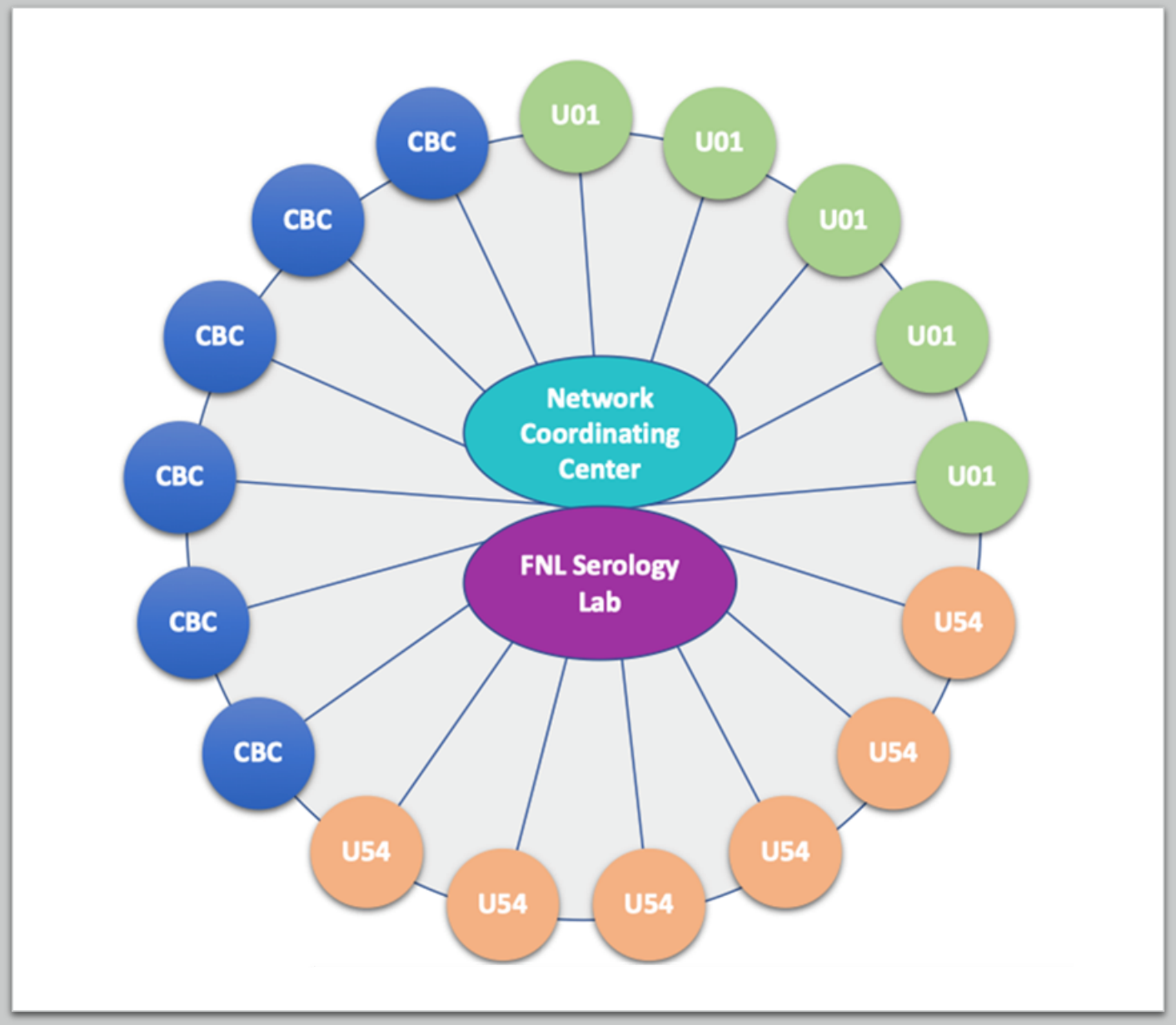
- assess the suitability of different antibody preparations
- characterize the antibody preparations in terms of reactivity/specificity
- assess each preparation's potency and commutability
- recommend to the WHO ECBS, the suitable antibody preparation(s) as the standard

# NIST Contribution to NCI's Serology Sciences Network

- NIST and FDA to participate as ancillary partners

- Develop qualified assay standards for the serology community
  - Reference antigens
  - Reference antibodies for assay quantification
  - Positive controls
  - Antibody panels

- Help evaluate/validate panel used for FDA approval process

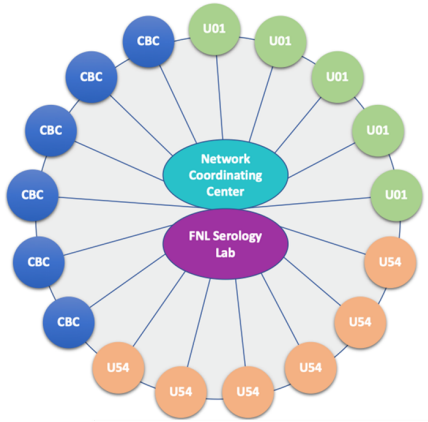


# NIST Participation in WHO-NIBSC Serology Study

World Health Organization

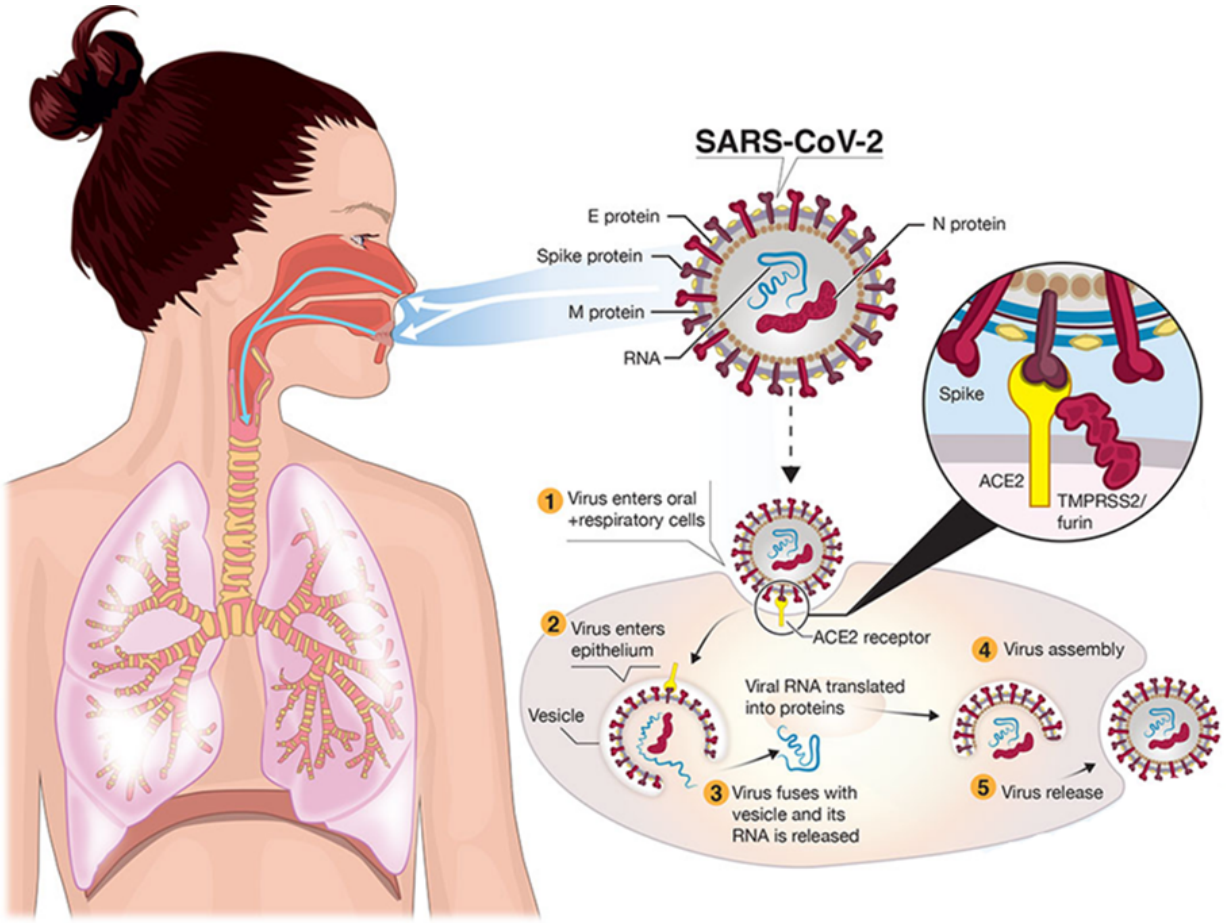
1st WHO International Standard for anti-SARS-CoV-2 antibody

Bridging

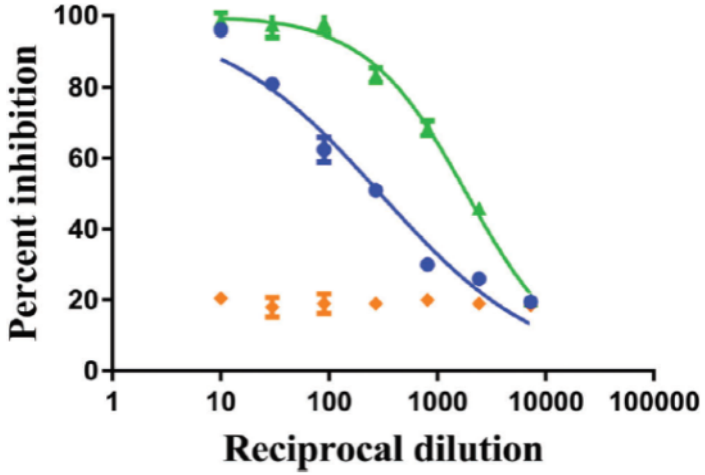



**US Serology Standards and Control Panels**

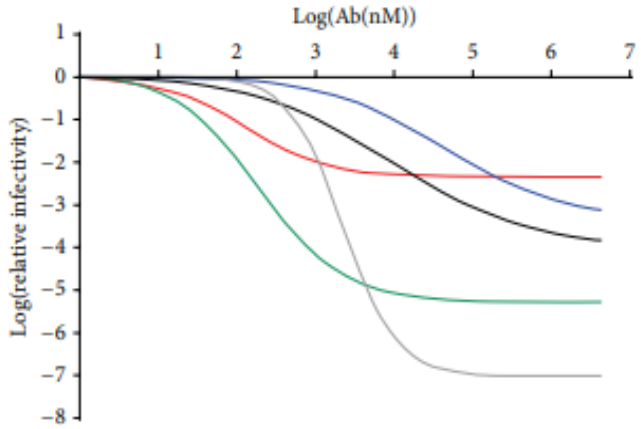
# Development of SARS-CoV-2 Neutralization Antibody and Infectivity Assays




Potency




Efficacy



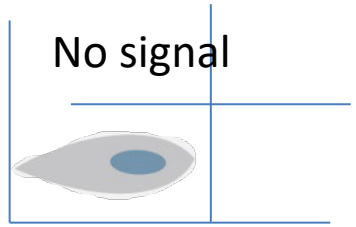
# SARS-CoV-2 Neutralization Antibody Assays

## Cell-based assays

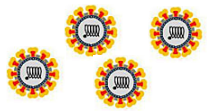
Fluorescent labeled pseudovirus + NAb

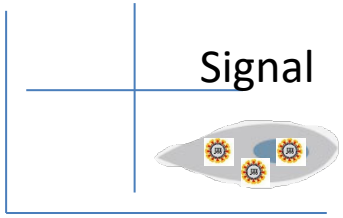
VeroE6 cells or primary epithelial cells

Flow cytometry results




Fluorescent labeled pseudovirus only

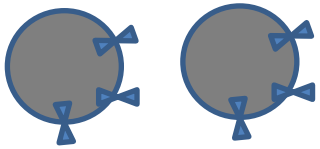


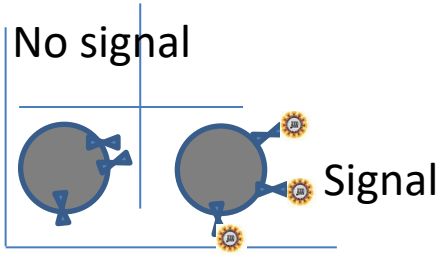



## Surrogate NAb Assay

Fluorescently labeled ACE2

Beads coated with RBD + NAb





# Acknowledgements


- MML: financial and operational support
- TPO: quickly establishing a large number of urgent MTAs
- RPO: for rapid review to ensure appropriate use of biological and patient samples
- Division/MML/NIST biosafety offices: ensuring operation safety