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The Science of Precision Health

Simoa HD-X Technology Overview

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Field Applications Scientist

August 2019

Simoa - Single Molecule Detection

nature biotechnology

Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations

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The ability to detect single protein molecules[1,2] in blood could accelerate the discovery and use of more sensitive diagnostic biomarkers. To detect low-abundance proteins in blood, we captured them on microscopic beads decorated with specific antibodies (one target protein molecule per bead) and then labeled the immunocomplexes with an enzymatic reporter capable of generating a fluorescent product. After isolating the beads in 50-fl reaction chambers designed to hold only a single bead, we used fluorescence imaging to detect single protein molecules. Our single-molecule enzyme-linked immunosorbent assay (digital ELISA) approach detected as few as ~10–20 enzyme-labeled complexes in 100 μl of sample (~10^{-19} M) and routinely allowed detection of clinically relevant proteins in serum at concentrations (<10^{-15} M) much lower than conventional ELISA[3–5]. Digital ELISA detected prostate-specific antigen (PSA) in sera from patients who have undergone radical prostatectomy at concentrations as low as 14 fg/ml (0.4 fM).

The clinical use of protein biomarkers to differentiate between healthy and disease states, and to monitor disease progression, requires the measurement of low concentrations of proteins in complex samples. Current immunoassays typically measure proteins at concentrations above 10^{-12} M[6]. The serum concentrations of the majority of proteins important in cancer[7], neurological disorders[8,9], and the early stages of infection[10], however, are thought to range from 10^{-16} to 10^{-12} M. For instance, a 1-mm^3 tumor composed of a million cells that each secrete 5,000 proteins into 5 liters of circulating blood translates to a concentration of ~2×10^{-15} M (or 2 fM). Moreover, serum from individuals recently infected with HIV contains 10–3,000 virions per ml, resulting in estimated concentrations of the p24 capsid antigen ranging from 50×10^{-18} M (50 aM) to 15×10^{-15} M (15 fM)[10]. Attempts to develop methods capable of measuring these concentrations of proteins have focused on the replication of nucleic acid labels on proteins[11,12], or on measuring the bulk, ensemble properties of labeled protein molecules[13–16]. The work of Mirkin *et al.*[12,17] and others[18] using labels based on gold nanoparticles and DNA biobarcodes has pushed the detection of proteins into the low femtomolar range; a recent report using this technology demonstrated the detection of 10 fM of PSA in serum[17]. Nonetheless, the sensitivities achieved by methods for detecting proteins still lag behind those for nucleic acids, such as PCR, limiting the number of gene products that have been detected in blood[6,19]. The isolation and detection of single protein molecules provides a promising approach for measuring extremely low concentrations of proteins[1,2]. For example, Todd *et al.*[2] have developed flow-based methods for serially detecting single fluorescently labeled detection antibodies that have been released from immunocomplexes formed on solid substrates. Here, we report an approach for detecting thousands of single protein molecules simultaneously using the same reagents as the gold standard for detecting proteins, namely, the ELISA. This method has been used to detect proteins in serum at subfemtomolar concentrations.

Our approach makes use of arrays of femtoliter-sized reaction chambers (**Fig. 1**)—which we term single-molecule arrays (SiMoAs)—that can isolate and detect single enzyme molecules[20–24]. This approach builds from the work of Walt *et al.*[20–23], who used these arrays to study the kinetics[21] and inhibition[20] of single enzymes. Our objective was to exploit the ability of SiMoAs to trap and detect single enzymes to detect single enzyme-labeled proteins. In the first step of this single-molecule immunoassay (**Fig. 1a**) a sandwich antibody complex is formed on microscopic beads (2.7 μm diameter), and the bound complexes are labeled with an enzyme, as in a conventional bead-based ELISA. When assaying samples containing extremely low concentrations of protein, the ratio of protein molecules (and the resulting enzyme-labeled complex) to beads is small (typically <1:1) and, as such, the percentage of beads that contain a labeled immunocomplex follows a Poisson distribution. At low concentrations of protein, the Poisson distribution indicates that beads carry either a single immunocomplex or none. For example, if 50 aM of a protein in 0.1 ml (3,000 molecules) is captured and labeled on 200,000 beads, then 1.5% of the beads will carry one protein molecule and 98.5% will not carry any protein molecules (**Fig. 1b**)[22]. It is not possible to detect these low numbers of enzyme labels using standard detection technology (for example, a plate reader), because the fluorophores generated by each enzyme diffuse into a large assay volume (typically 0.1–1 ml), and it takes hundreds of thousands of enzyme labels to generate a

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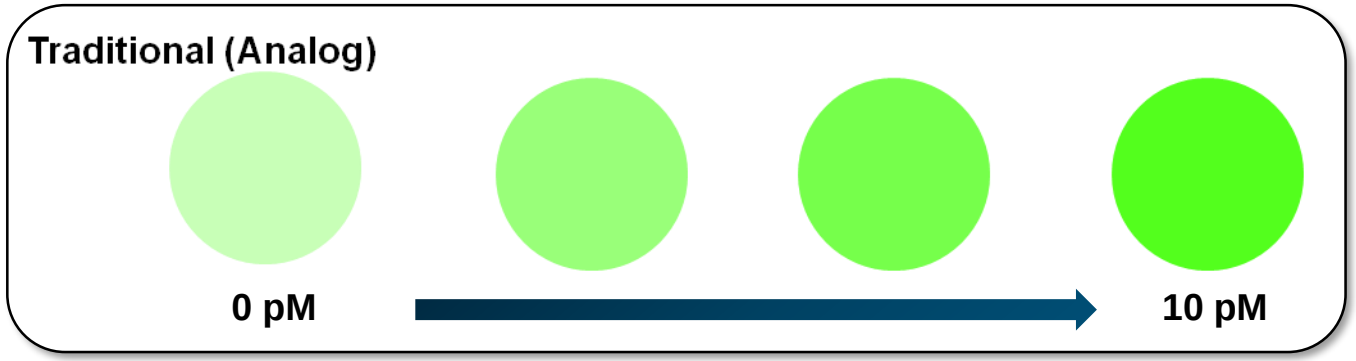
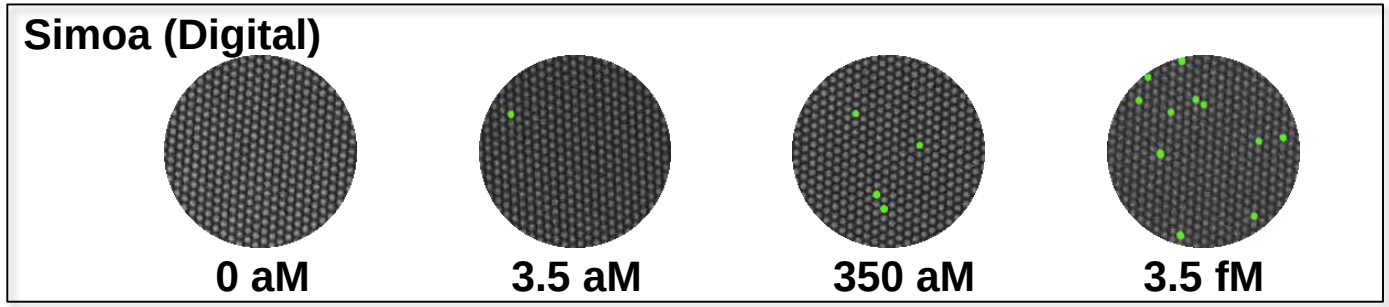
Received 1 February; accepted 29 April; published online XX XXXX 2010; doi:10.1038/nbt.XX

Simoa Bead Assays: Digital vs. Analog Detection

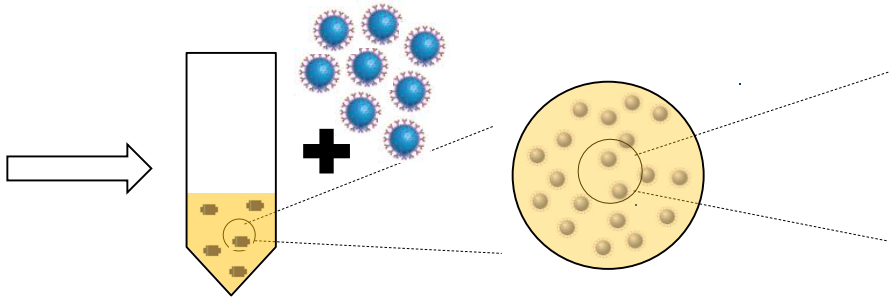


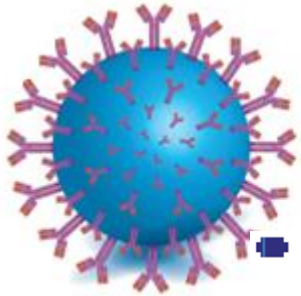
- Reaction volume = 100 $\times 10^{-6}$ L Microliters (mL)
- Diffusion = dilution = low sensitivity
- Millions of molecules needed to reach detection limit



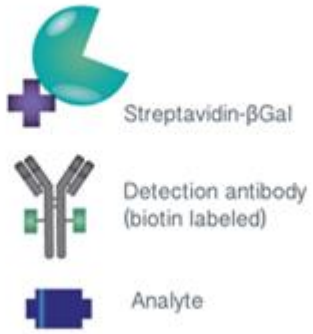
- Reaction volume = 50 $\times 10^{-15}$ L **(2 billion times smaller)** Femtoliters (fL)
- Diffusion defeated = single molecule resolution = ultimate sensitivity
- One molecule needed to reach detection limit

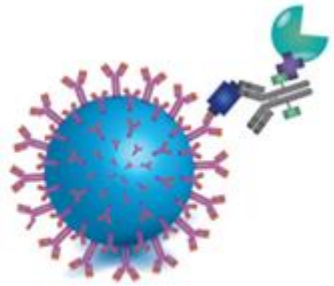
Simoa Bead Assays



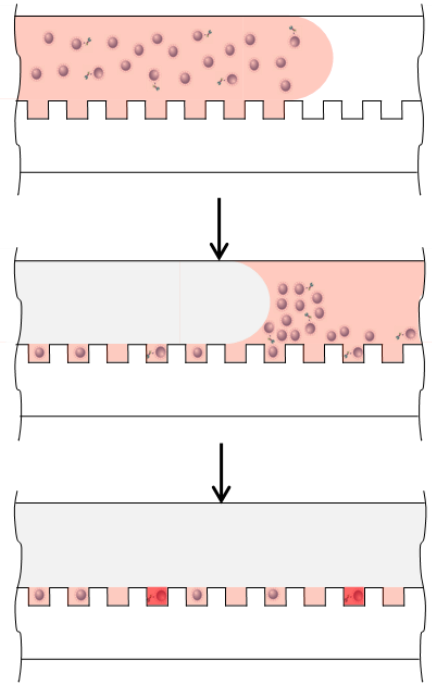


Dye-encoded magnetic beads with capture antibody

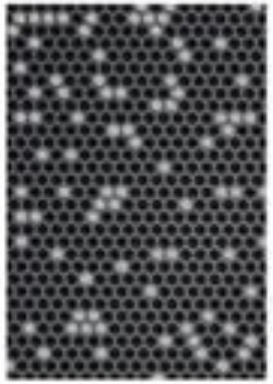




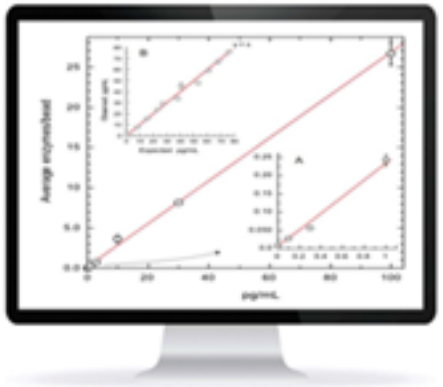
Formation of single immunocomplexes on beads



Load beads onto array and seal with oil



Image



Analyze and Report

[Rissin *et al.*, 2010]
[Kan *et al.*, 2012]

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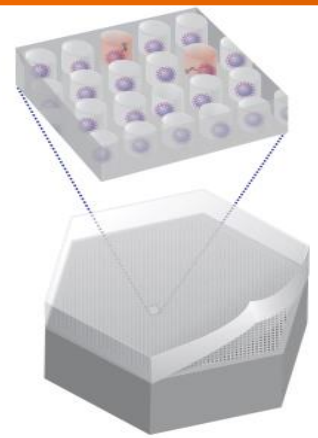
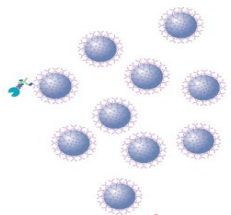
Enzymes are counted - Average Number of Enzymes per Bead (AEB) from fraction of positive beads (Fon)

Analyte Concentration

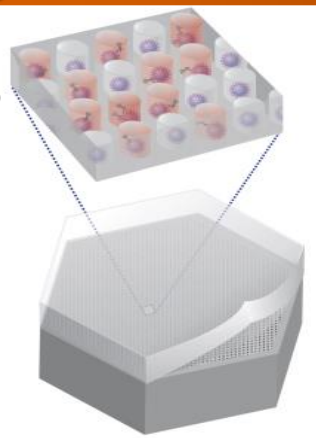
f_{on} = 0.1

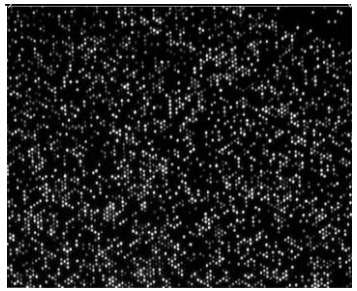


AEB = 0.1

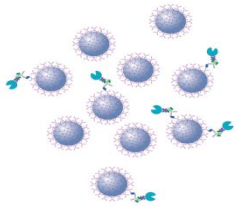


f_{on} = 0.5

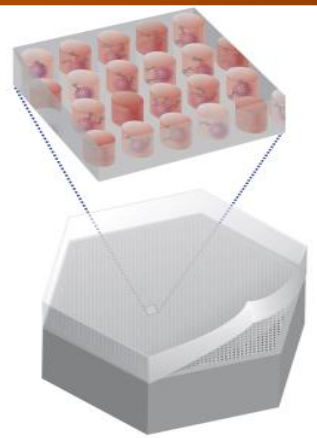


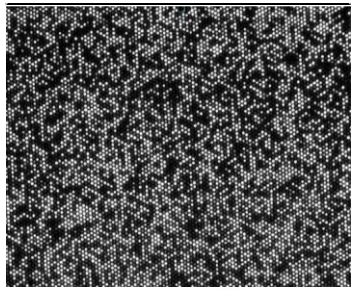


AEB = 0.6

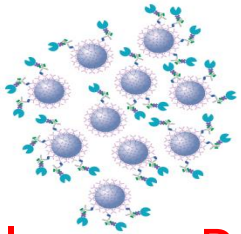


f_{on} >0.99





AEB = ?



The fraction of beads with immunocomplexes follows a Poisson distribution

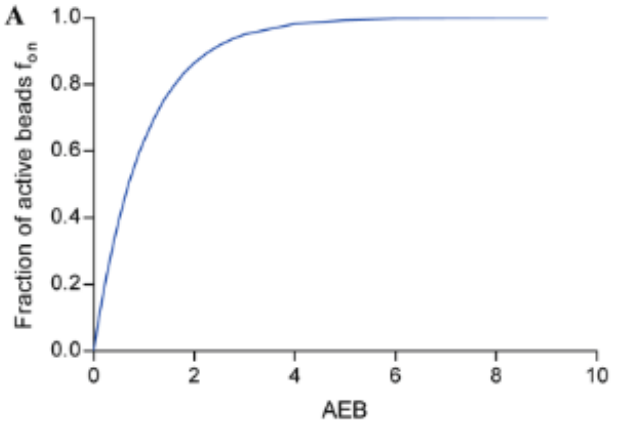
Rissin *et al.*, *Anal.Chem.* 2011, *83*, 2279-2285

Digital and Analog Determination of Protein Concentration

Digital Determination of Protein Concentration

Percentage of active beads < 70% or $f_{on} < 0.7$

$AEB_{digital} = -\ln [1 - f_{on}]$



Analog Determination of Protein Concentration

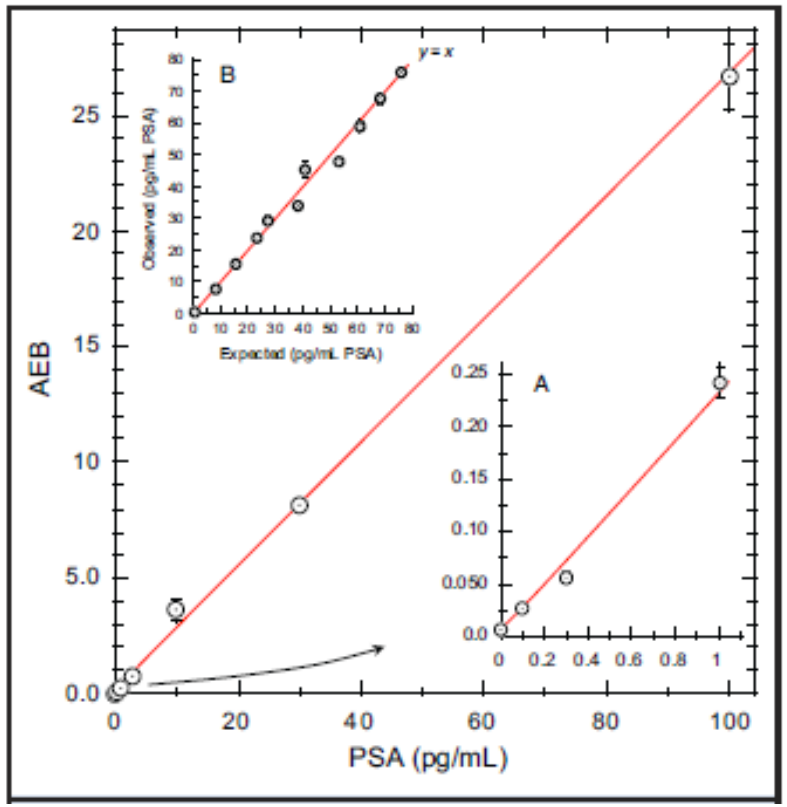
Percentage of active beads > 70% or $f_{on} > 0.7$

- $\bar{I}_{bead}$ is the average fluorescence intensity of the wells containing a bead
- $\bar{I}_{single}$ is the average fluorescence intensity of a single enzyme, calculated from an array in this batch with < 10% active beads or (f_{on} <0.2)

$$AEB_{analog} = \frac{f_{on} \times \bar{I}_{bead}}{\bar{I}_{single}}$$

Rissin *et al.*, *Anal.Chem.* **2011**, *83*, 2279-2285

Digtal and Analog Measurements Extend Dynamic Range



LoD < 0.03 pg/mL

- Combined digital and analog **immunoassay** dynamic range is >4 logs

Wilson *et al.*, *Clin Chem.* **2011**, *57(12)*, 1712-21

Summary of AEB Quantitation

AEB = 0.1

- Digital Readout, AEB calculated using f_{on}, **not** the $\bar{I}_{bead}$

$\bar{I}_{bead}$ from this sample **will** be used in $\bar{I}_{single}$ calculation, f_{on} <0.2

AEB = 0.6

- Digital Readout, AEB calculated using f_{on}, **not** the $\bar{I}_{bead}$

Multiple enzymes on beads are accounted for in data analysis using Poisson distribution equation

$\bar{I}_{bead}$ from this sample **will not** be used in $\bar{I}_{single}$ calculation (F_{on} >0.2)

AEB = 3.0

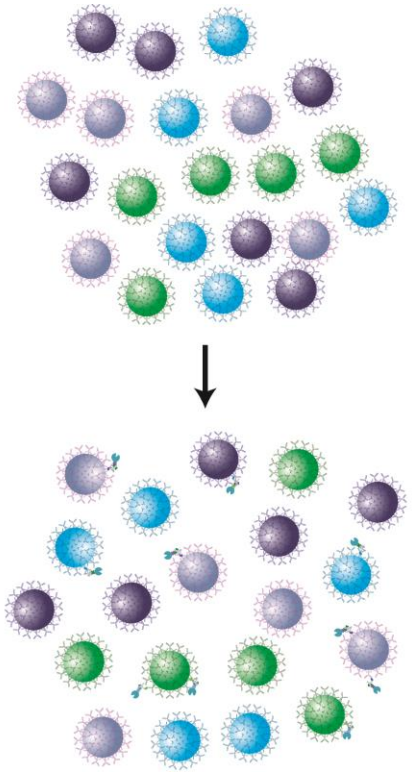
- Analog Readout, uses $\bar{I}_{single}$ and $\bar{I}_{bead}$ in calculation of AEB result for jobs with f_{on} >0.7

All the beads have >1 enzyme

Rissin *et al.*, *Anal.Chem.* **2011**, *83*, 2279-2285

Simoa Bead Multiplexing

I. Formation of multiplex immunocomplex



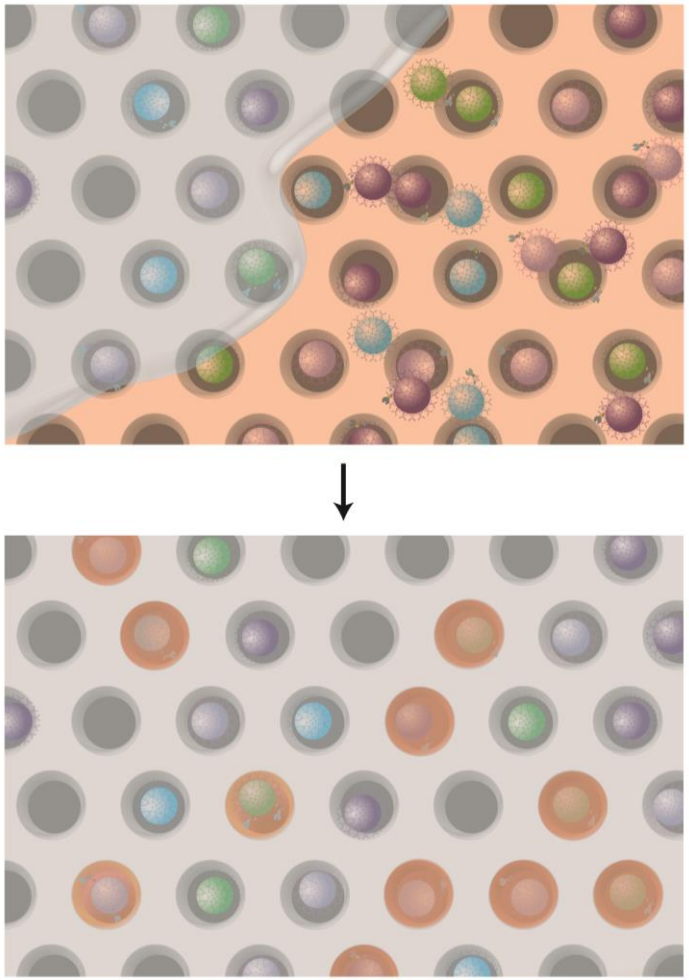
Multiplex beads have different fluorescent coatings

Mixture of subpopulations of encoded capture beads

Capture and enzyme-labeling of multiple, single protein molecules on specific beads

Assay workflow is the same

II. Load, seal and image of multiplex beads



Beads loaded with substrate into arrays of femtoliter wells and sealed with oil

Assay throughput is the same

Arrays imaged fluorescently at multiple wavelengths

Rissin *et al.*, 2013

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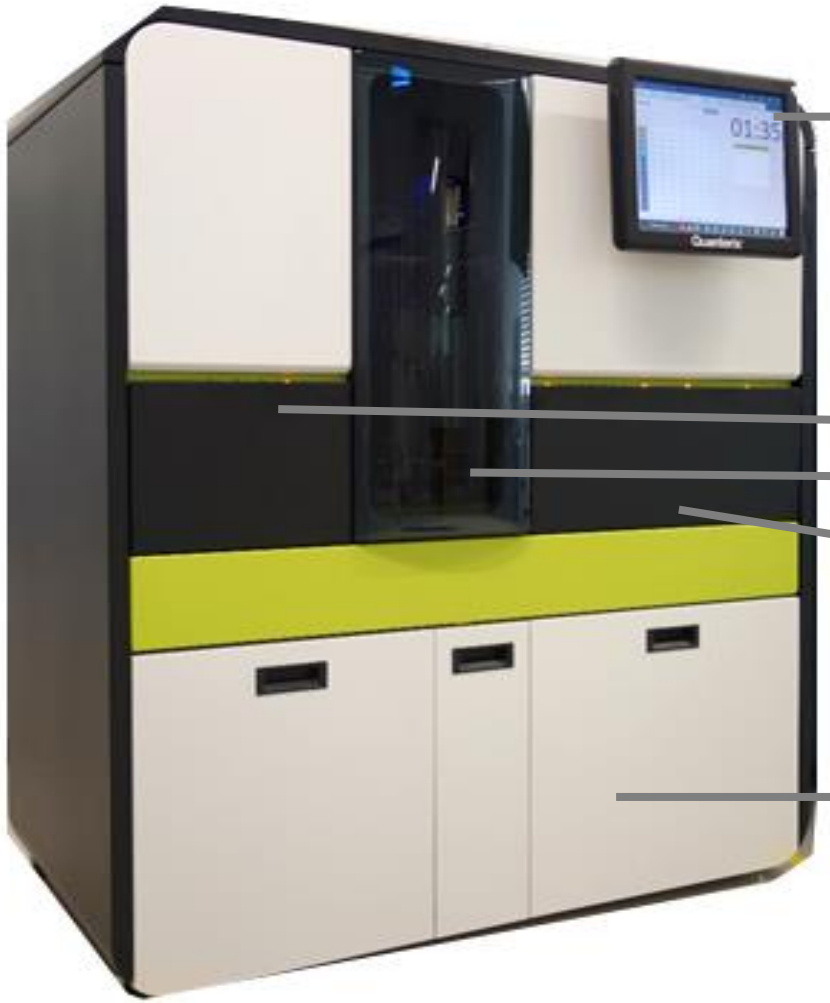
HD-X HARDWARE OVERVIEW

HD-X System Layout: Front View

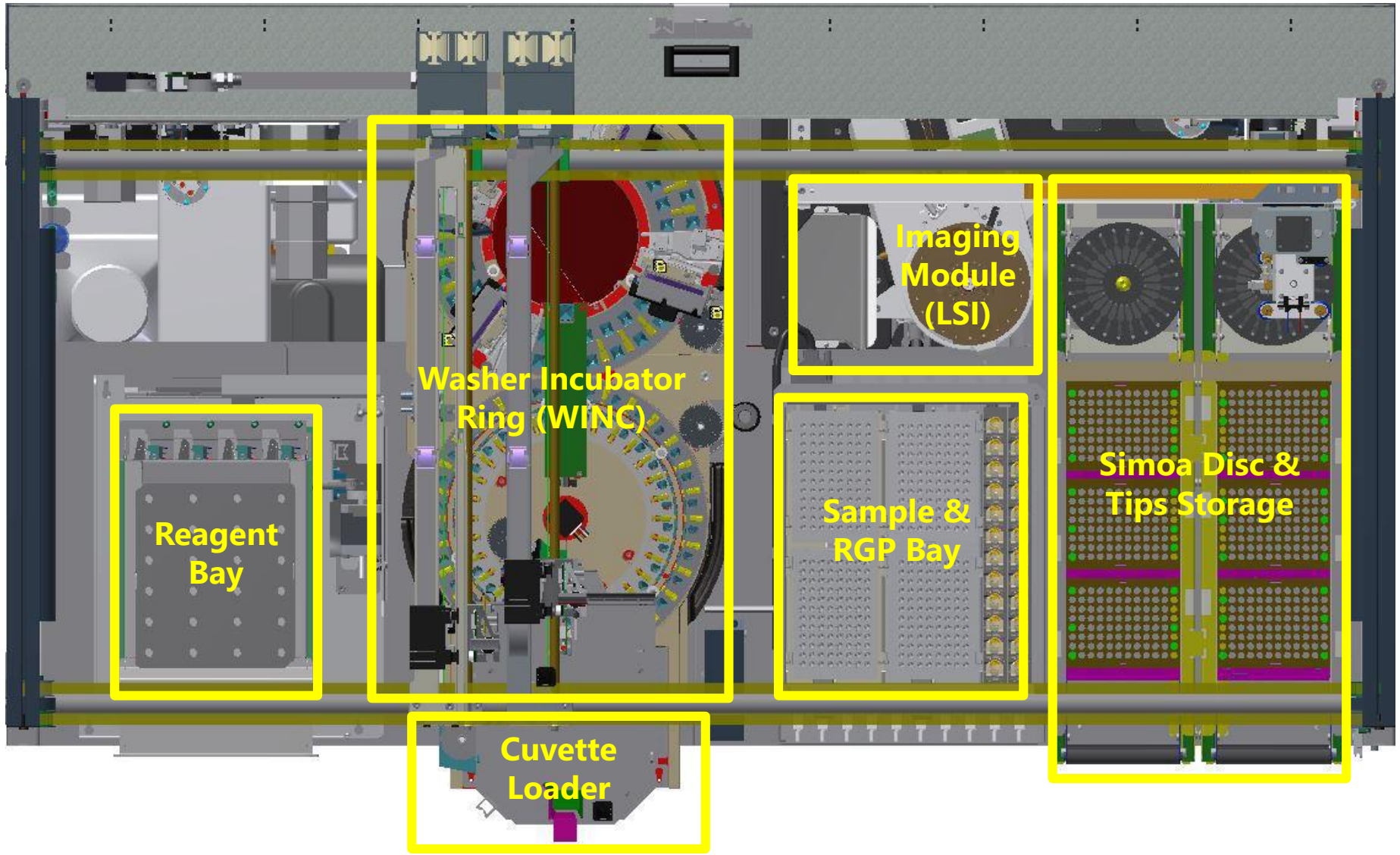


User control interfac
(multi-touch monitor)

Input bays
- Reagent bay
- Cuvette carousel
- Sample bay
- Tips and disc bay

System bay
- Bulk resource supply and waste

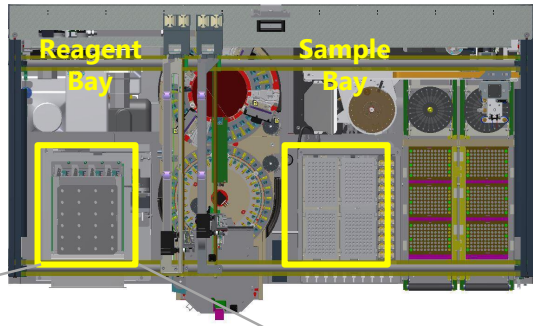
System Module Review



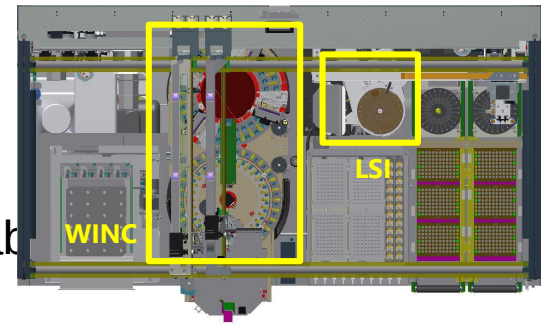
New Features: Reagent & Sample Bays

- Reagent Bay
  - Cooled, 4°C reagent bay
  - Addition of integrated barcode scanner automatically scans and registers reagent bottles when rack is inserted

- Sample & RGP Bay
  - Addition of integrated barcode scanner automatically scans and registers RGP bottles when rack is inserted
  - Cooling has not been added to the sample bay

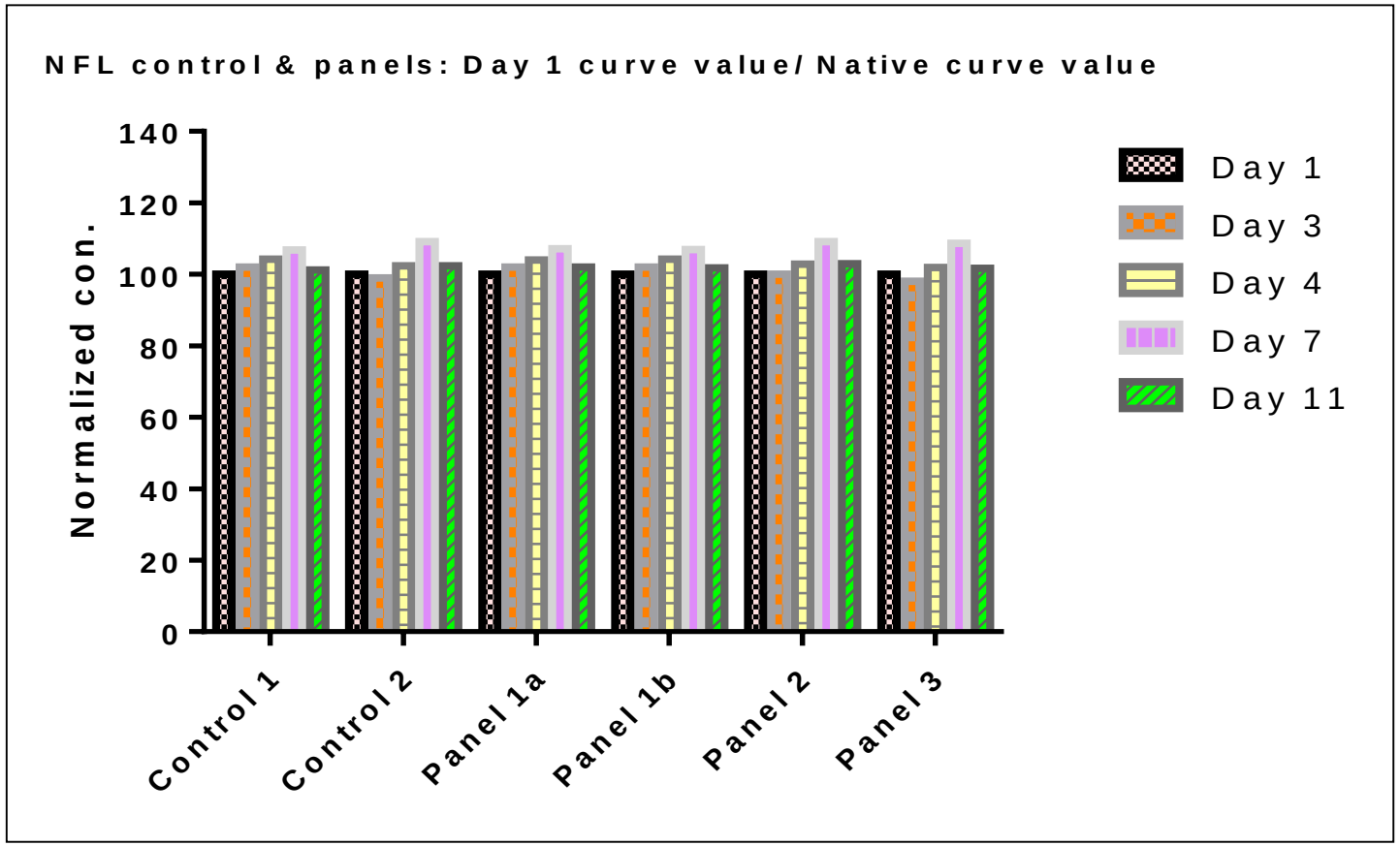


New Features: Thermal Management



- Washer/Incubator Rings (WINC)
  - The location where samples are processed and the immunoassay complex is formed
  - Passive temperature management via heating elements and cooling fans at WINC stab temperature to 30°C despite fluctuations in ambient temperature
  - **Controlling temperature at the WINC improves reproducibility within-run, between-runs and between-labs**
- Load/Seal/Image (LSI) Module
  - The reaction between SBG enzyme and RGP substrate occurs within the LSI resulting in detectable signal
  - Heating has been added to the LSI to maintain this reaction at a constant 30°C to prevent fluctuations in signal intensity due to changes in ambient temperature
  - **Controlling temperature at LSI improves within-run, between-run and between-instrument reproducibility**

Improved thermal management and inter-run reproducibility enables stored calibrator curve capability



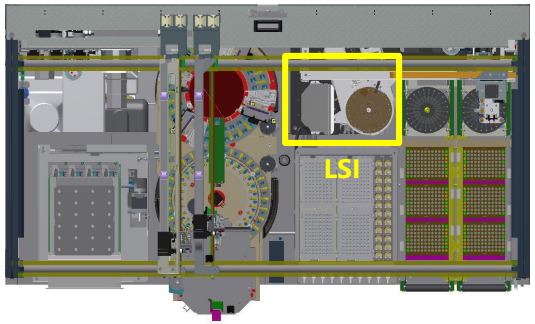
Stored curve precision on HD-X equal to individual plate curves out to 11 days (single reagent lot)

- Stored curve %CV = 5.86%
- Individual plate curve %CV = 5.88%

Stored calibration curves offers users additional throughput and cost savings

- Stored curves will allow users to run samples in place of calibrators within a single kit lot
- Stored curves may be applied to fit the users need
  - Multi-plate Run: Curve included on Plate 1 only, additional plates can include sample only
  - Between-Run: Curve included in Run 1, subsequent runs can included sample only
- Stored Calibrators using current Simoa Assays
  - Current kit claims will not change, customers will need to adopt and evaluate curve storage to validate performance in their own lab

New Features: Load/Seal/Image Module



- LED Light Source
  - A LED light source has replaced plasma lamp used on HD-1
  - Plasma lamps have been the #1 source of variability and instrument failures for HD-1 due to fluctuations in power output as lamps age
  - LED light source provides consistent output and performance across instruments and removes the need for a mechanical shutter

- Magnetic Bead Loading
  - The LSI station is now equipped with a fixed magnet positioned below the array to increase efficiency of bead loading into the wells
  - Increased efficiency reduces variability in bead fill percent and provide an opportunity to develop new assays with increased sensitivity

Engineering Improvements: Drawers and Direct Plumbing

- Cabinet design improvements
  - System buffers and waste now user-friendly drawer design
  - Improved bottle to address issues observed with leaking
  - User experience for maintenance activities significantly improved with easier access and bottle remove/replace
- Optional kits will be available for direct plumb DI water and liquid waste
  - Walk away automation for a 3-plate run (or 3 single plate run)
  - Waste will need to be emptied and consumables replaced between long runs
  - Available post-launch, Q4

HD-X New Features Summary

HD-X includes a series of design changes and updates to the HD-1 hardware and software to improve reliability, ease of use and robustness.

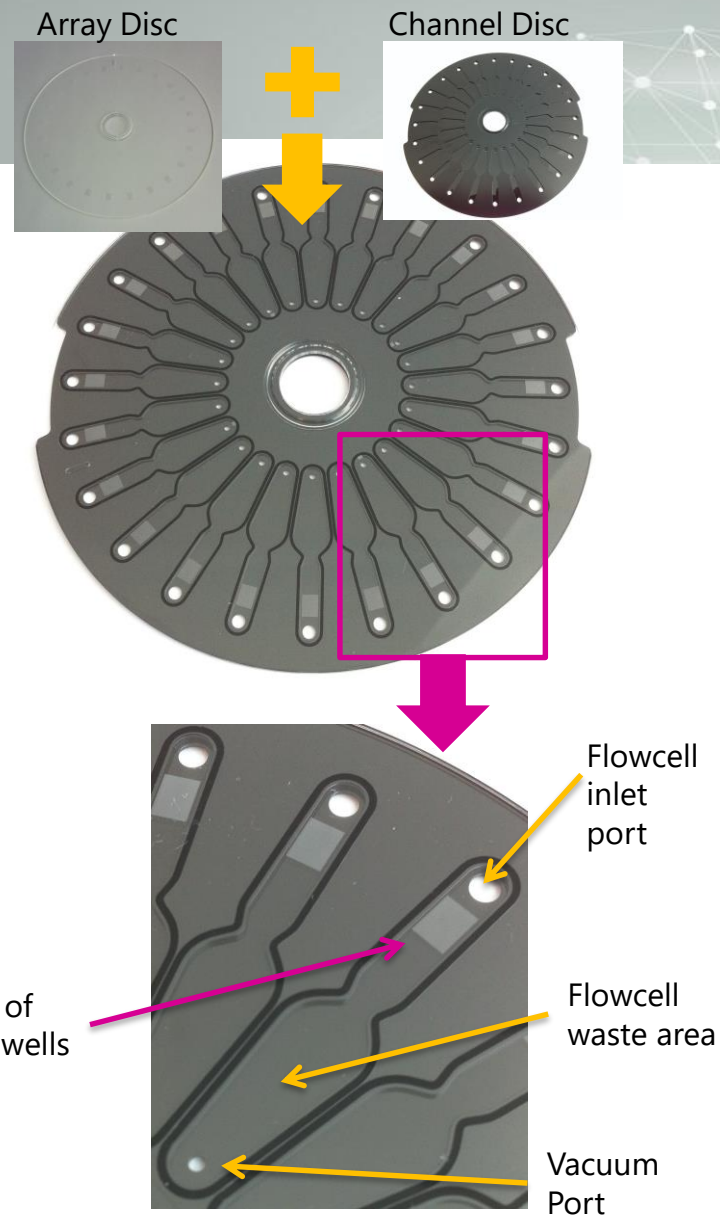
HD- X Feature	Assay, Data, Ease of Use Benefit
LED Light source	More consistent assay signal and instrument uptime. Increased multiplexing (6-plex)
Heated temp controlled assay chamber	More precision in assay, reduced timing concerns
Improved thermal management	Overall improvement in assays C.V.s improves data quality and provides option for reducing frequency of assay calibration (↑ throughput; ↓ cost/sample)
Improved fixed and disposable pipettor design	Increase in overall reliability
Doors replaced draws for buffer and waste reservoirs	Easier to empty and fill
Optional direct plumbing of dI Water and Liquid Waste	Higher throughput runs w/o operator assistance
Magnetic assisted bead loading	Higher bead fill provides path for increased assay sensitivity
Windows 10 compatibility (by end of 2019; FoC upgrade from Windows 7)	Enables network connection to customer domains (after Win7 End of Life Jan2020)
User Account management, enhanced Run Reports	Facilitates customer documentation of 21CFR part11 compliant processes

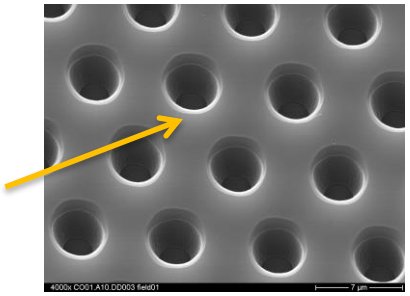
Software improves control and traceability to enable easier integration into a compliant, 21 CFR Part 11 workflow

- Improved User Account Management & Authorization
  - Automated password policy enforcement
  - User account lock out after failed login attempts
  - Admin rights to push password reset on next user login
- Enhanced Reporting
  - User-generated Batch Calibration Reports now includes reagent information and assay definition details
- Enhanced Traceability
  - Assay definition versioning
  - Log messages describing changes in Custom Assay and User Management Screens
- Kiosk mode only
- Windows 7 available at launch, Windows 10 upgrade available Q42019 (FOC)

Simoa Disc

- Each disc has 24 flow cells per disc
- Each flow cell consists of an inlet port, array of microwells, waste chamber and vacuum port
- Each array has 238,000 microwells 4.25 µm in diameter, 3.25 µm in depth and are spaced out 8.0 µm on center hexagonal close pack
- Bead size is 2.7 µm



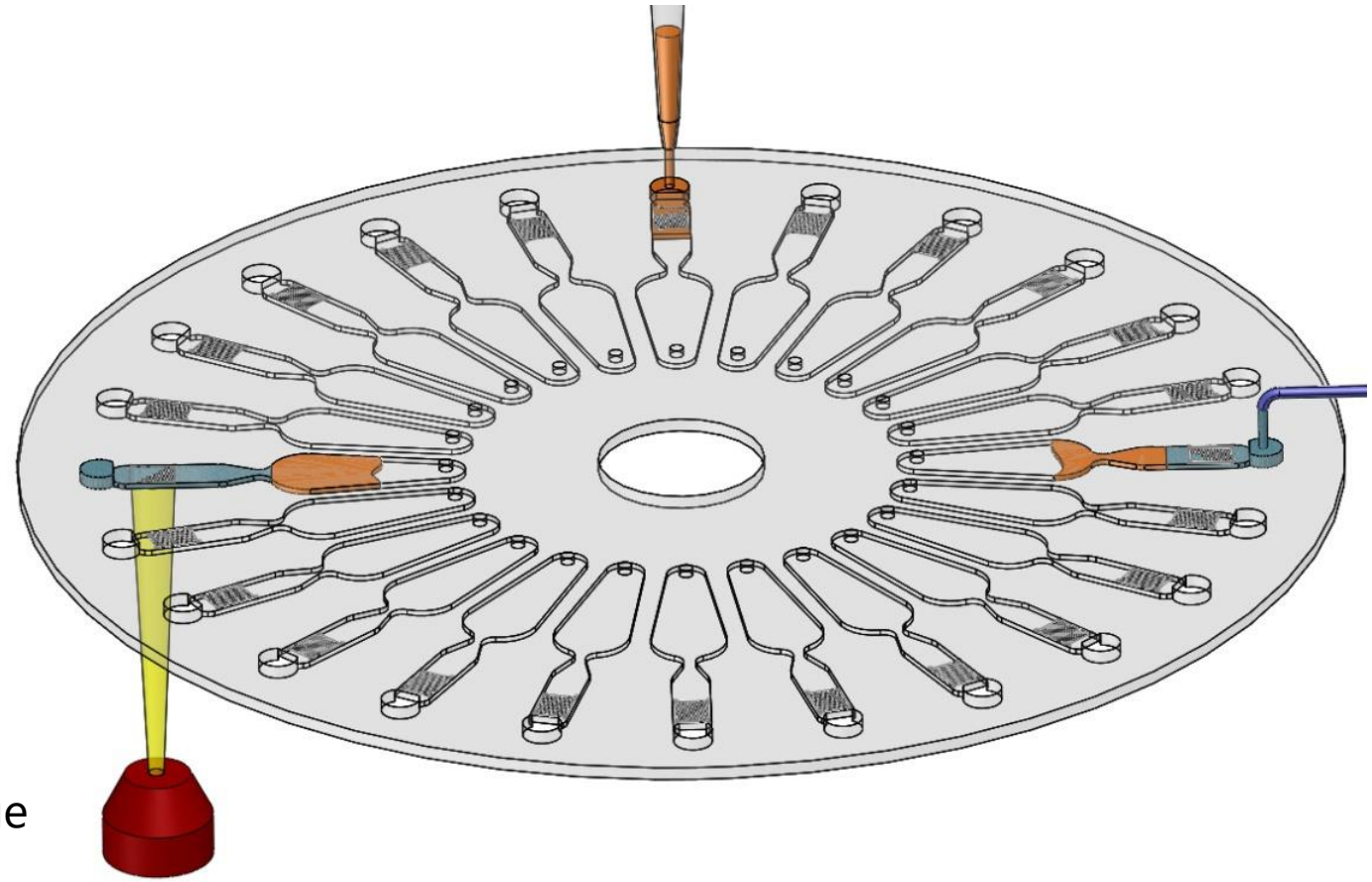


4.25 x 3.25 µm wells
8 µm on center hexagonal close pack

Array of microwells

Kan *et al.*, *Lab Chip* **2012,** *12*, 977-985
Wilson *et al.*, *J Lab Autom.* **2015**, *1-15*

Wells on the Simoa disc are loaded, sealed and imaged



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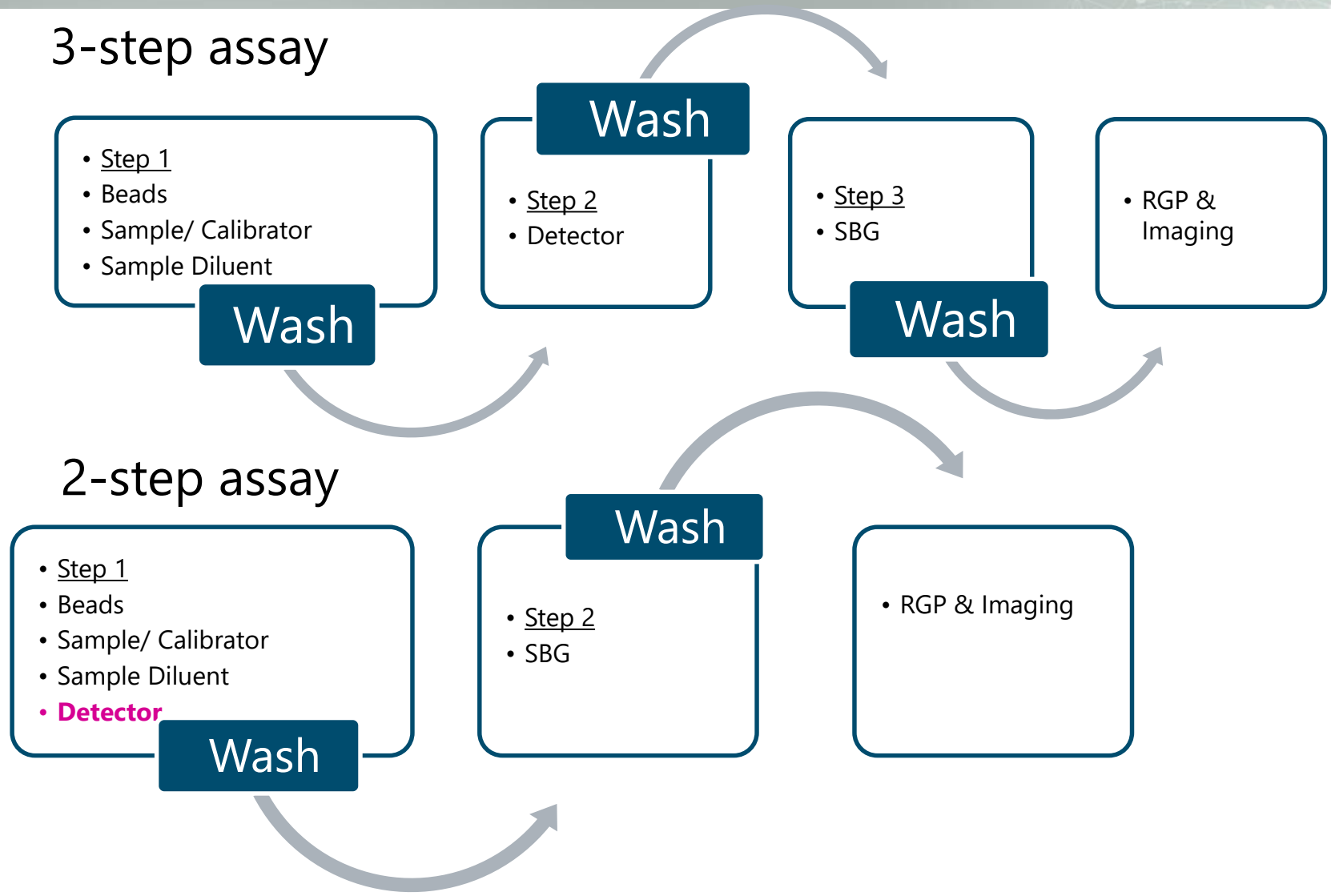
SIMOA ASSAY OVERVIEW

Simoa Assay Overview



Advantage Kits

- Follow setup according to specific assay package insert
  - Reagents (Beads, Detector, SBG) are ready to use
  - Calibrator curve uses calibrator concentrate. Dilute per package insert instructions
  - Contains enough reagents for 1 plate (96 samples)
- In HD-X Software, Import assay definition
  - Reagents scanned in using barcoded labels on ready to use bottles
  - In pop-up menu, select the assay specific reagent

Discovery Kits

- Follow setup according to specific assay package insert
  - Reagents (Beads, Detector, SBG) are concentrated and need to be diluted to working concentration prior to running on instrument. A magnet is required to wash beads
  - Calibrator curve uses calibrator concentrate, similar setup as 2.0 and Advantage Kits. Dilute per package insert instructions
  - Contains enough reagents for 2 plates (188 samples)
- In HD-X Software, Import assay definition from the customer portal
  - Reagents scanned in using generic Discovery barcoded labels
  - In pop-up menu, select the assay specific reagent
  - Assay Setup – Calibrator and Samples setup is the same as premade kits

Quanterix Simoa HD-X Assay Workflow

Load Plate

Pipetting Arm

Sample Transfer to Reaction Vessel

Sample Dilution & Capture Bead Addition

Incubation Step 1

Typical 3 Step Assay as an example

Sample Incubation

Wash Station

3 Wash

Detector Addition

Incubation Step 2

Detector Incubation

3 Wash

SβG Addition

Incubation Step 3

SβG Incubation

6 Wash

Bead Concentration & Substrate Addition

Bead Transfer to Imaging Disc

Image Disc

Disc loading & Imaging details in next slide

Data Analysis

Results

Quanterix™

HD-X WORKFLOW

Simoa Workflow

Step 1. Load consumables, reagents and check system fluids and waste

Step 2. Assay Run Setup

Step 3. Load Samples

Step 4. Press Start HD-1 automates Simoa assay.

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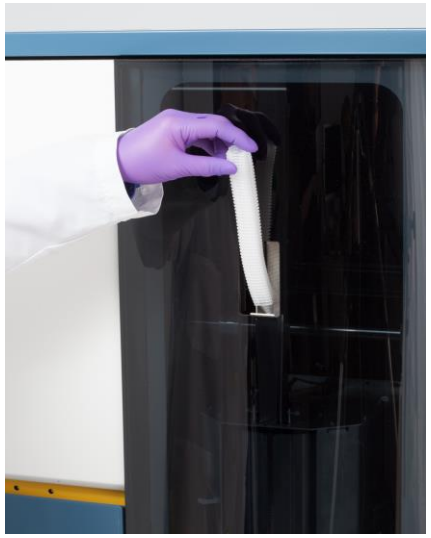
Step 5. Data analysis

Step 1: Load Reagents and Consumables

Load Beads, Enzyme conjugate (SβG), Substrate (RGP)



Load up to 500 cuvettes

6 boxes of 96 tips

2 stacks of 16 Simoa discs

Step 1: Check Liquids and Waste

System fluids

- Wash Buffer 1
- Wash Buffer 2
- System Liquid: DI water

- Solid & Liquid waste

Step 2: Assay Run Setup on Touchscreen GUI

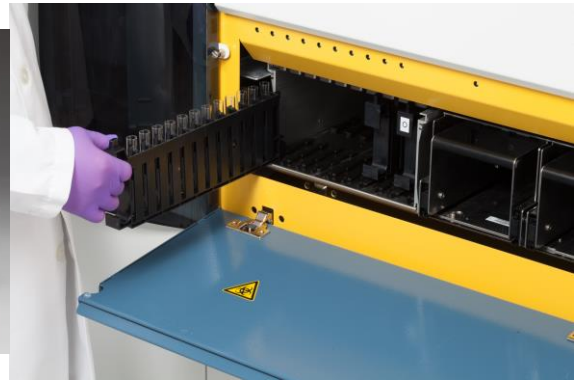
- **Select Assay or Import Assay definition**
- **Set up the software and assign samples and calibrators**

Step 3: Load Samples

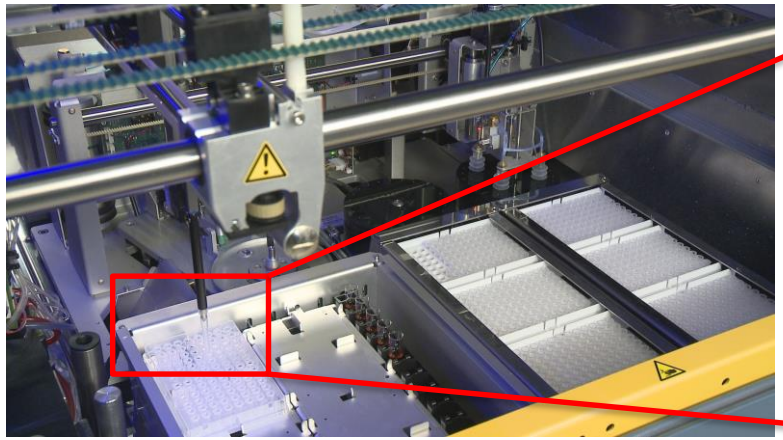
- **Up to four 96-well plates**

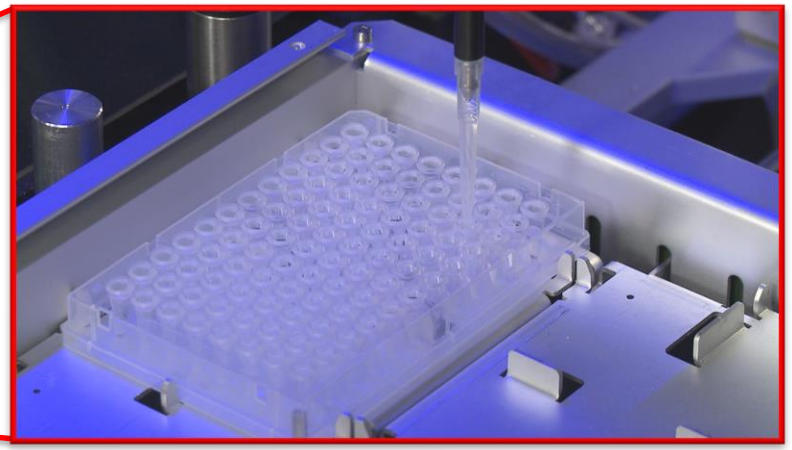
OR

- **Up to 96 primary tubes**

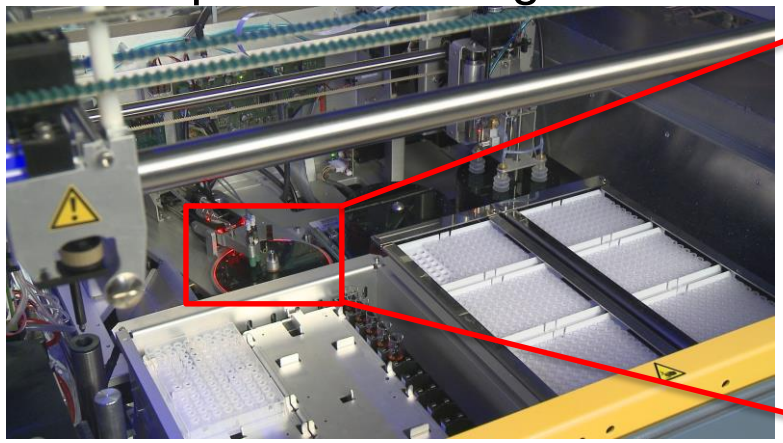


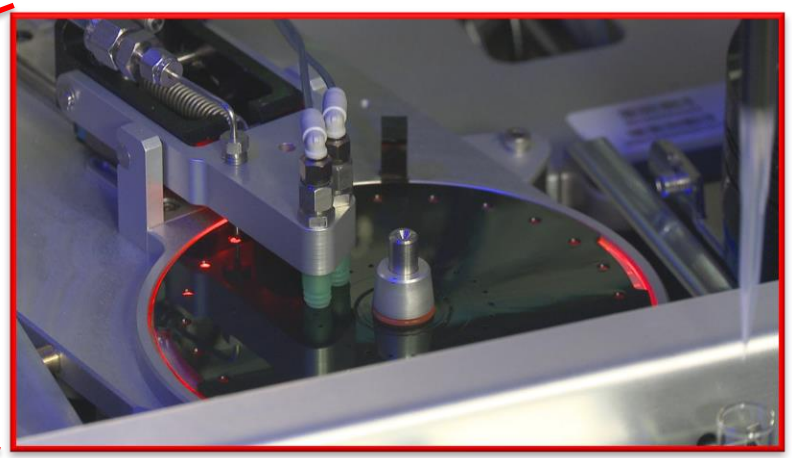
Step 4: Press Start for Automation of Simoa Assay





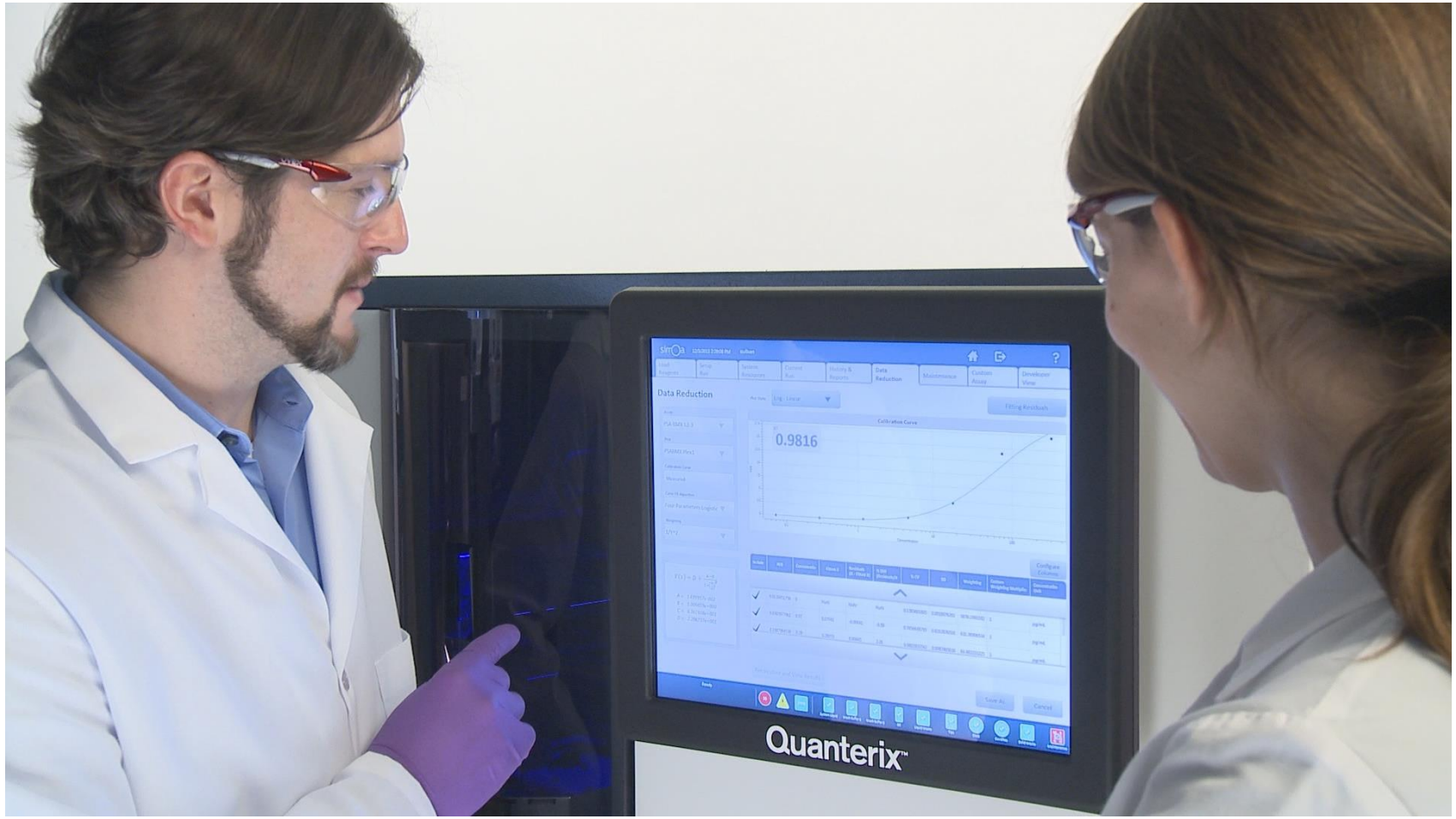
- Sample Processing





- Sample Imaging & Analysis

Step 5: Data Analysis



Samples tested on Simoa

- Serum
- Plasma
- Urine
- CSF
- Tissue extracts
- Interstitial fluid
- Brain extract
- Liver Extract
- Whole Blood
- Breath condensate
- Blood spots
- Tears
- Saliva
- Cell or IVF culture supernatant
- PBMC
- Blood Fractionations (IP)
- Stool

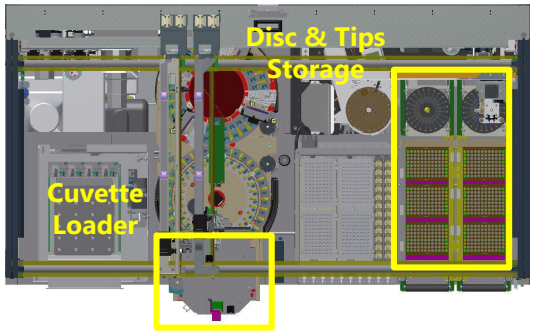
Species tested on Simoa

- Human
- Monkey
- Canine
- Feline
- Rodent

Cuvette Loader & Disc and Tip Storage

- HD-X shares the same consumables as HD-1

- No changes to consumables and consumables loading



Consumables	Item Number
Simoa Disc Kit (rev 2)	103347
Simoa Cuvettes Bulk Pack (rev 2)	103346
Conductive Tips	101726
Conical Well Plates	101457
Reagent Bottle Pack	102411
Sealing Oil	100206
SQT Kit	101537

What's covered in Basic training?

Day 1

- Simoa Technology
- HD-X Workflow
- Customer Portal
- Hands on Kit Assay- Two Runs

Day 2

- Hands on Kit Assay
- Instrument Maintenance/ Best Practices
- Troubleshooting & Customer Support Tools
- Data Analysis Review

What's covered in Homebrew training?

Day 1

- Capture antibody to Bead conjugation
- Detector Antibody labeling
- Bead Aggregation Assay

Day 2

- Review Bead Aggregation Assay results
- HB Assay Dev –Run 1
- Assay Optimization Presentation
- Review run 1 results
- HB Assay Dev- Run 2

Day 3

- Review Homebrew Assay Results
- Homebrew Assay Development- Set up run 3
- Troubleshooting Tips