





# Custom Assay Service

## Services

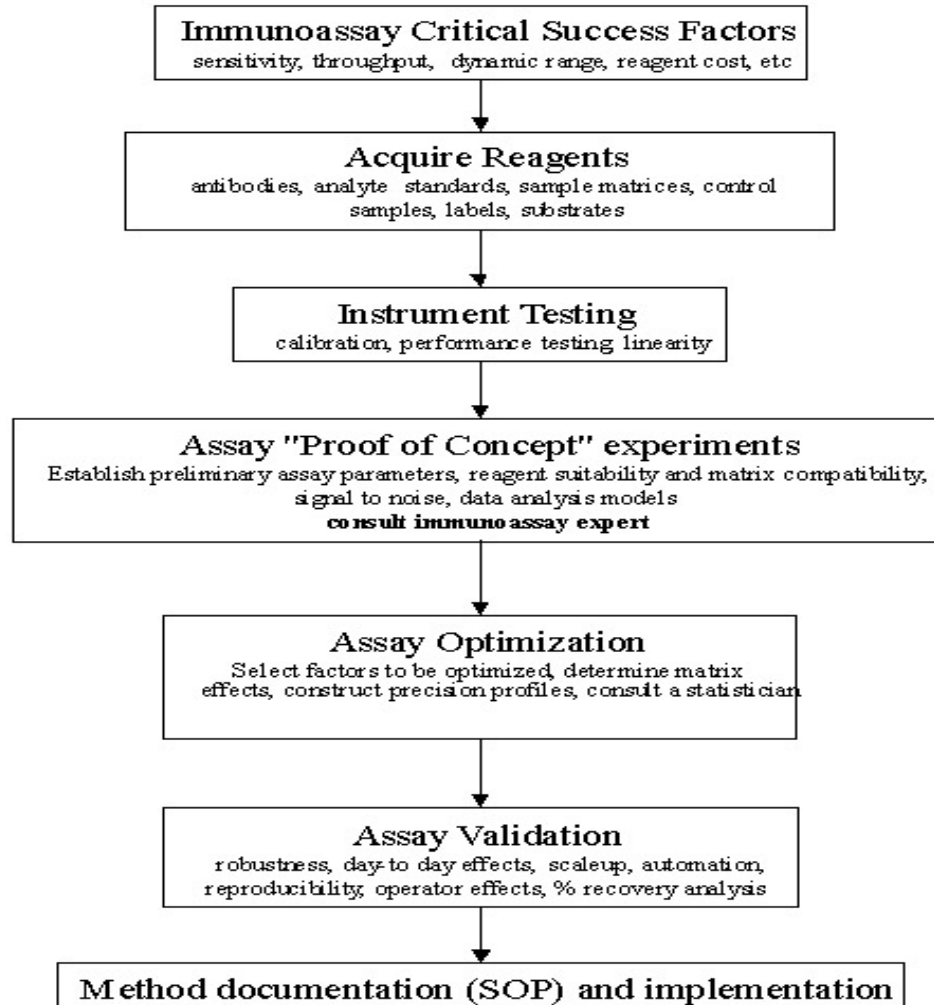
Sample Testing  
Assay Development  
Antibody Derivatization



## Assay Development: Reagents & Prototyping

- Same chemistry as Singulex kitted assays
- Labeling kits are purchased from Singulex
- Yields around 60-80% from 1mg labels
- Identify best working reagents
  - Current ELISA - Transferred ELISA method

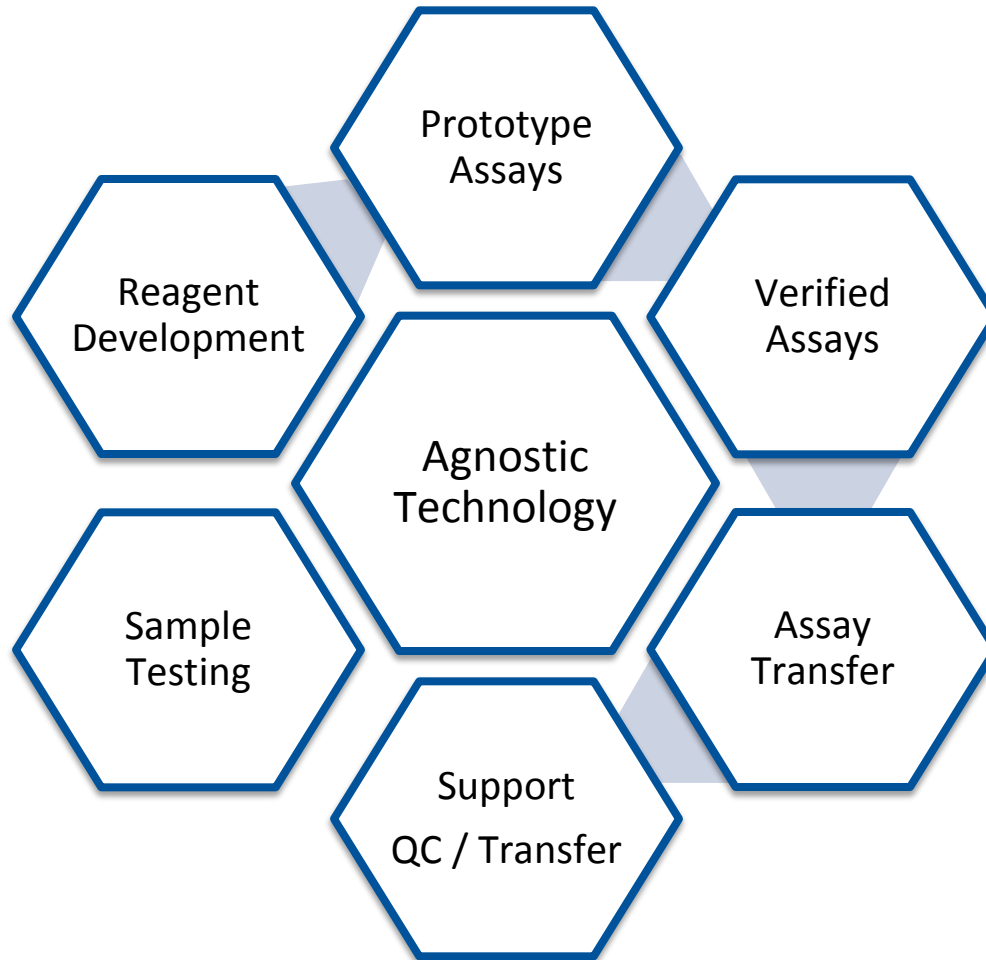
# Assay Development: Reagents & Prototyping



## Assay Development: Evaluation

- What are expected values
- Assessment of available assays
- Limited assessment
  - Standard curve, RoQ, Matrix QC strategy
- Aim to get performance criteria around the following:
  - Standard Curve, Precision, Linearity,  
Recovery/Accuracy, Sensitivity, Short Term Stability

# Assay Development: Process



## Assay Development: Optimization

- Review clinical values
- Reach semi-robust conditions meeting:
  - LLOQ
  - ULOQ – max out EP
- Calibrators and RoQ >4 Log
- Matrix QC strategy & Placement
  - LLOQ, Low, Med, Hi, ULOQ, Sample
- Outside reference protein
  - For lot-lot control
- Minimum Required Dilution
- Matrix effects
- Diluent buffer optimization

## Assay Development: Qualification

- Multiple pool matrix screening
  - Alt. matrix selection, potential interference
- Single-donor screening min. five ♂ & five ♀
- Linearity from >10x ULOQ
- Pilot QC produced
  - Multi-donor or Single donor samples
  - Qualification ranges used and assessed
- QC dilution schema tested
- Scale up of QC for validation
- Aim to get performance criteria around the usual validation criteria
  - Define validation criteria



## Assay Development: Validation

- Std Curve Performance
- Precision
- Linearity
- Recovery / Accuracy
- Sensitivity
- Manual vs. Automated Pipetting Comparison
- Samples Range Verification
- Normal and patient populations
- Short-term stability
- Long-term stability
  - Typically up to 24 months



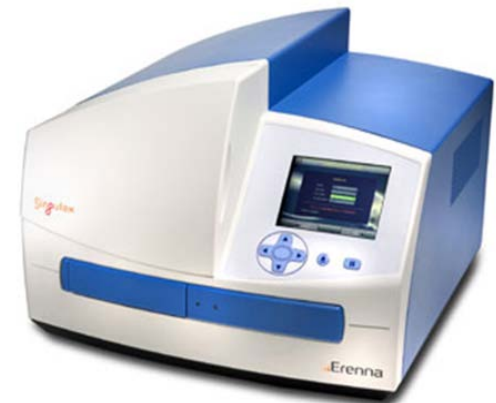
## Assay Development: Overview I

- Optimal Ab conc. determined:
  - ELISA data
    - Capture & Detection optimization.
    - Assay performance
  - Curve Shape
    - 4 or 5-PL
    - ULOQ, LLOQ & LLoRQ, Anchor points
- Start with Standard Diluent only
- DE, EP, and TP signal values
- SgxLink with SoftMaxPro values



## Developing the assay: Overview II

- Run standard curve with varying concentration of Capture and Detection Ab in Standard Diluent
- Passive adsorption of the capture Antibody onto a 96-well plate
  - Optimal assay plate
- Option to use microparticle beads
- Alexa Fluor<sup>®</sup> labeled detection Ab
  - Client or Pacific Biomarkers

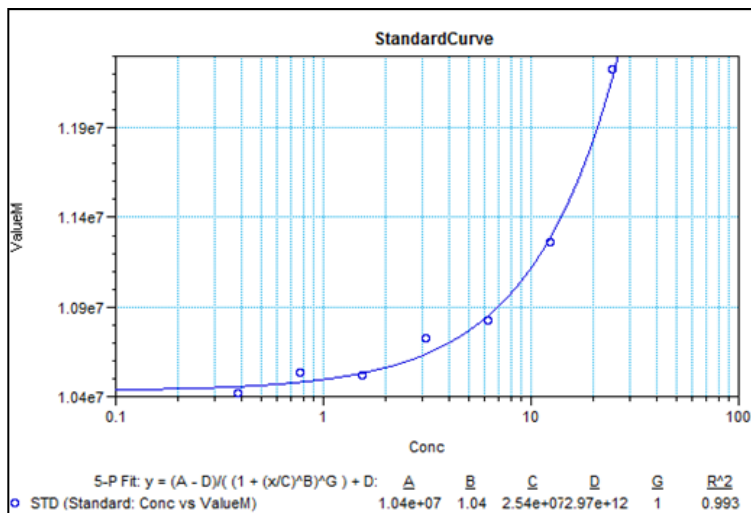


## Developing the assay: Overview III

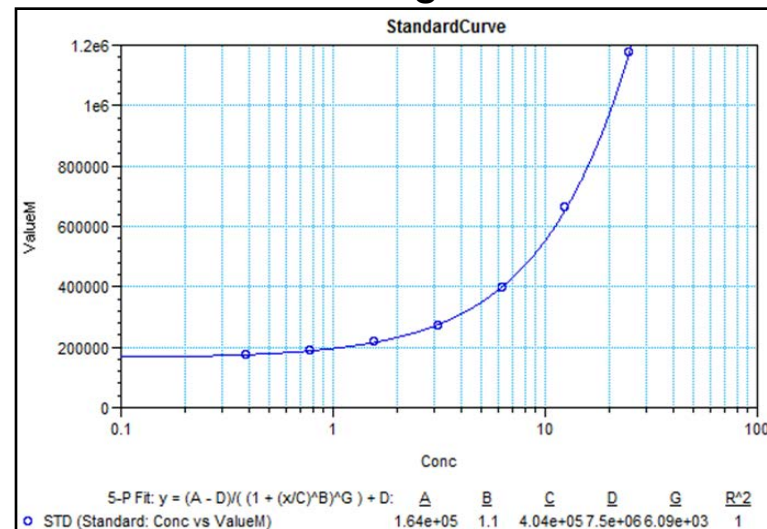
- Design the assay based on client specifications
  - A&P
  - Use 3-6 controls
  - Validation samples
- Goal
  - Smallest volume with highest sensitivity
  - Expand the LLOQ getting greater sensitivity
  - Triplicate or duplicate replicates
  - Pros and cons
    - Maximize plate space
    - Save sample volume
    - Curve robustness
    - Less plate failures

# Curve Comparison

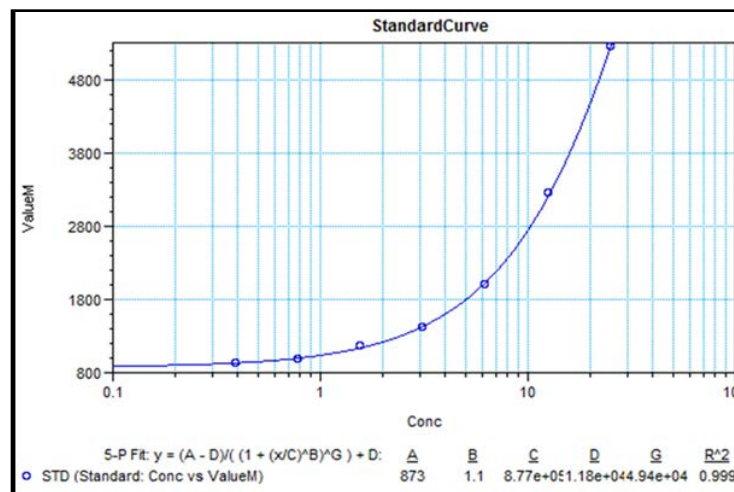
TP Signal



EP Signal



DE Signal



5-PL Curve fitting

Capture Ab = 1.0µg/mL

Detection Ab = 200ng/mL

## Optimizing the assay: DoE

- Detection Ab: 100 – 800ng/mL
- Capture Ab: 100 – 1000ng/mL
- Phase 1: Singulex Standard Diluent only
- Phase 2: Singulex Standard Diluent and Pooled K2 EDTA Plasma run in duplicate
- Phase 3: K2 EDTA Plasma run in triplicate

## Capture Ab Comparison

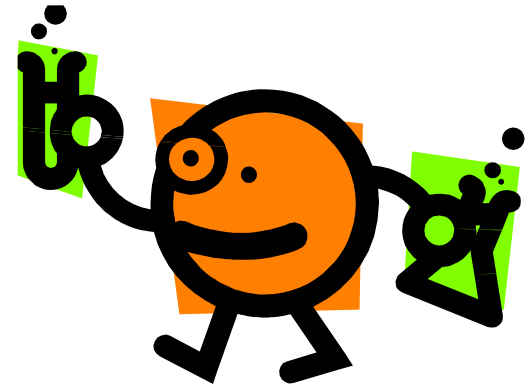
Standard (ng/mL)	0.5ug/mL Capture Ab 9 curves				0.25ug/mL Capture Ab 9 curves				0.125ug/mL Capture Ab 9 curves			
	Mean (ng/mL)	Accuracy (%RE)	SD	Precision (%CV)	Mean (ng/mL)	Accuracy (%RE)	SD	Precision (%CV)	Mean (ng/mL)	Accuracy (%RE)	SD	Precision (%CV)
200	195.5	97.7	5.74	2.9	203.2	101.6	33.81	16.6	216.4	108.2	31.55	14.6
100	104.6	104.6	4.68	4.5	100.2	100.2	4.72	4.7	93.2	93.2	1.93	2.1
50	50.4	100.8	1.55	3.1	51.6	103.2	1.29	2.5	53.7	107.3	0.39	0.7
25	23.6	94.6	0.89	3.8	25.1	100.3	1.21	4.8	25.3	101.1	0.35	1.4
12.5	13.0	104.3	1.26	9.7	12.2	97.2	0.33	2.7	12.5	100.1	0.22	1.8
6.75	6.2	91.5	0.20	3.3	6.1	90.4	0.17	2.8	6.0	88.9	0.14	2.4
3.13	3.2	101.9	0.33	10.2	3.1	100.1	0.12	3.9	3.0	95.3	0.18	6.2
1.56	2.3	149.6	0.81	34.6	1.7	106.2	0.15	9.1	1.6	100.4	0.10	6.6
0.78	1.1	135.3	0.32	30.0	0.9	117.0	0.18	19.8	0.9	113.2	0.16	18.1
0.39	0.5	119.7	0.24	51.9	0.4	102.6	0.10	25.0	0.5	132.5	0.08	14.6
0.2	0.0	0.0	-	-	0.3	125.0	0.10	42.0	0.3	150.0	0.09	29.8
0.0	-	-	0.25	-	-	-	0.00	0.0	-	-	-	-





## Setting the assay parameters

- Capture Ab: 250ng/mL
- Detection Ab: 800ng/mL
- Standard curve
  - 12 point curve in duplicate
  - Top std (ULOQ) = 300 ng/mL
  - Lowest std = 0.29 ng/mL
  - LLOQ = 0.59 ng/mL
  - Neat no MRD
- 60 second well read time
- Prepared three controls using pooled matrix and standard material



## Standard Curve Performances in Plasma

	Target (ng/mL)	Mean (ng/mL)	Accuracy %RE	Precision %CV
ULOQ	360.0	325.68	90.5	19.3
	180.0	174.34	96.9	13.0
	90.0	93.80	104.2	7.5
	45.0	45.17	100.4	8.2
	22.5	22.91	101.8	6.9
	11.25	10.80	96.0	12.4
	5.63	6.65	118.2	24.0
	2.82	2.82	100.3	10.2
	LLOQ	1.40	1.26	90.0
LAP1	0.70	1.04	148.6	39.8
LAP2	0.35	0.88	251.1	66.0
	0.0	0.24	-	53.7

	Target (ng/mL)	Mean (ng/mL)	Accuracy %RE	Precision %CV
ULOQ	300.00	309.52	103.2	13.5
	150.00	147.12	98.1	9.6
	75.00	81.65	108.9	5.8
	37.50	39.18	104.5	6.9
	18.75	19.47	103.8	9.0
	9.38	9.23	98.5	12.0
	4.69	5.01	106.9	6.4
	2.34	2.47	105.4	11.8
	1.17	1.57	134.1	18.7
LLOQ	0.59	0.76	129.8	27.1
LAP	0.29	0.69	236.0	98.3
	0.00	0.81	-	109.7

## Standard Curve Performances in Plasma

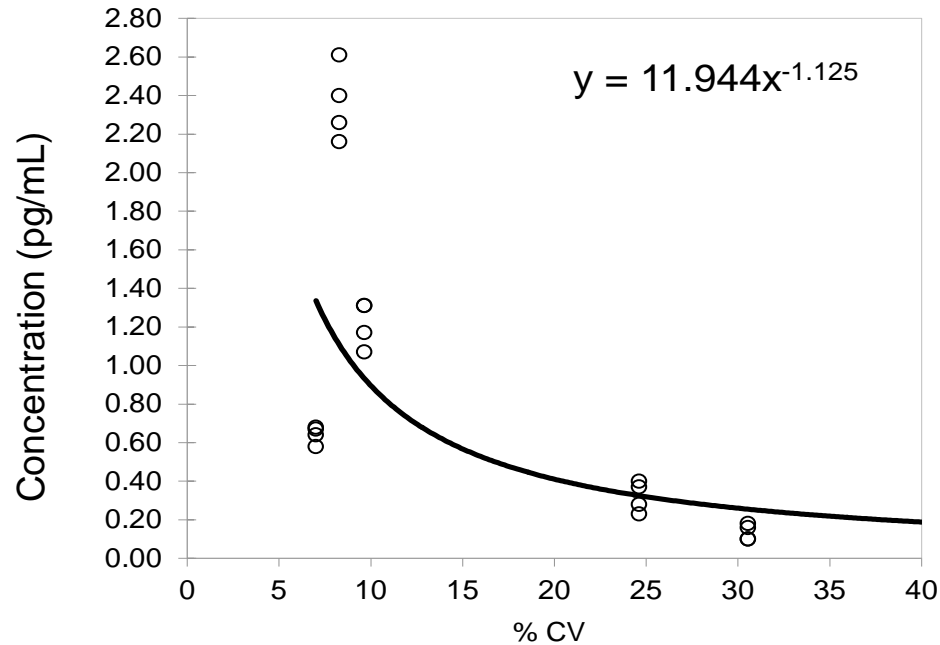
	Standard (ng/mL)	Mean (ng/mL)	Accuracy (%RE)	SD	Precision (%CV)
ULOQ	80.0	74.684	93.4	8.081	10.8
	40.0	35.094	87.7	2.643	7.5
	20.0	26.772	133.9	4.817	18.0
	10.0	10.375	103.8	0.583	5.6
	5.0	5.136	102.7	0.229	4.5
	2.5	2.226	89.1	0.112	5.0
	1.25	1.154	92.3	0.143	12.4
	0.63	0.653	104.4	0.136	20.8
	0.31	0.337	107.7	0.046	13.5
	0.156	0.189	120.7	0.035	18.5
LLOQ	0.078	0.088	112.6	0.014	15.7
LAP	0.039	0.041	104.8	0.018	43.4
	0.0	0.029	-	-	-

## Qualification Examples

- Tech to tech comparison
- QC/sample volumes: 30, 40, and 50 $\mu$ L
- Filtered vs. Unfiltered pooled matrix
  - Filter pool before use
  - Use a 96 well filter plate
- Accuracy & Precision
- Selectivity
- Spike/Recovery
- Linearity
- Diluent selection
- Sample dilution
- LLOQ determination



## Lower Limit of Quantification



LLOQ was determined to be 0.41 pg/mL at 20% CV using the equation derived from a power fit of the data:  $y = 11.944x^{-1.125}$ .

# Testing sample volume

Three levels of controls were prepared using Plasma.

		Mean (ng/mL)	Accuracy (%RE)	SD	Precision (%CV)	n Duplicates
50uL	QCL	3.4	114.9	0.67	19.4	56
	QCM	22.2	110.9	2.95	13.3	56
	QCH	137.4	114.5	31.87	23.2	56
40uL	QCL	3.1	102.5	0.12	3.8	14
	QCM	20.5	102.7	0.72	3.5	14
	QCH	130.0	108.3	3.47	2.7	14
30uL	QCL	2.9	96.5	1.09	37.6	10
	QCM	18.3	91.5	1.32	7.2	10
	QCH	119.7	99.8	16.51	13.8	10

## Overall QC Performance 50 $\mu$ L volume

	Target value	Mean (ng/mL)	Accuracy (%RE)	SD	Precision (%CV)	n Duplicates
QCL	3.0	3.29	109.5	0.63	19.3	80
QCM	20.0	21.33	106.7	2.73	12.8	80
QCH	120.0	133.55	111.3	26.29	19.7	80

## Standard Curve Performance in Serum

	Standard (ng/mL)	Mean (ng/mL)	Accuracy (%RE)	SD	Precision (%CV)
ULOQ	80.000	77.385	96.7	3.228	4.2
	40.000	40.899	102.2	5.089	12.4
	20.000	21.379	106.9	3.992	18.7
	10.000	9.478	94.8	0.777	8.2
	5.000	5.545	110.9	0.471	8.5
	2.500	2.587	103.5	0.284	11.0
	1.250	1.188	95.1	0.089	7.5
	0.625	0.632	101.2	0.073	11.5
	0.313	0.319	102.0	0.062	19.4
	0.156	0.172	109.8	0.017	9.8
LLOQ	0.078	0.079	101.8	0.002	2.2
LAP 1	0.039	0.044	113.8	0.033	73.6
LAP 2	0.020	0.025	128.2	0.037	146.3
	0.0	0.006	-	-	-



## Control comparison of two matrices

		Target Conc. ng/mL	Mean ng/mL	Precision %CV	Accuracy %RE	n
Serum	QCL	0.2	0.209	18.7	104.5	25
	QCM	2.0	2.543	16.1	127.2	25
	QCH	20.0	31.835	28.0	159.2	25
K2 EDTA Plasma	QCL	0.2	0.207	21.8	103.7	15
	QCM	2.0	2.418	14.3	120.9	15
	QCH	21.0	22.513	23.3	107.2	15

## Standard Curve Performance for Validation and In-Validation Runs

Assigned pg/mL	Mean	SD	%CV	%RE
100.00	99.73	1.38	1.4	99.7
50.00	49.66	1.49	3.0	99.3
25.00	26.51	0.70	2.6	106.0
12.50	12.13	0.42	3.5	97.0
6.25	6.27	0.23	3.7	100.2
3.13	3.01	0.14	4.6	96.1
1.56	1.55	0.10	6.3	99.3
0.78	0.82	0.04	5.0	104.6
0.39	0.42	0.02	5.0	107.9
0.20	0.19	0.03	14.4	95.9
0.10	0.10	0.03	24.9	103.8
0.00	0.02	0.02	123.9	-

12 point standard curve – ULOQ is 100 pg/mL and LLOQ is 0.39 pg/mL

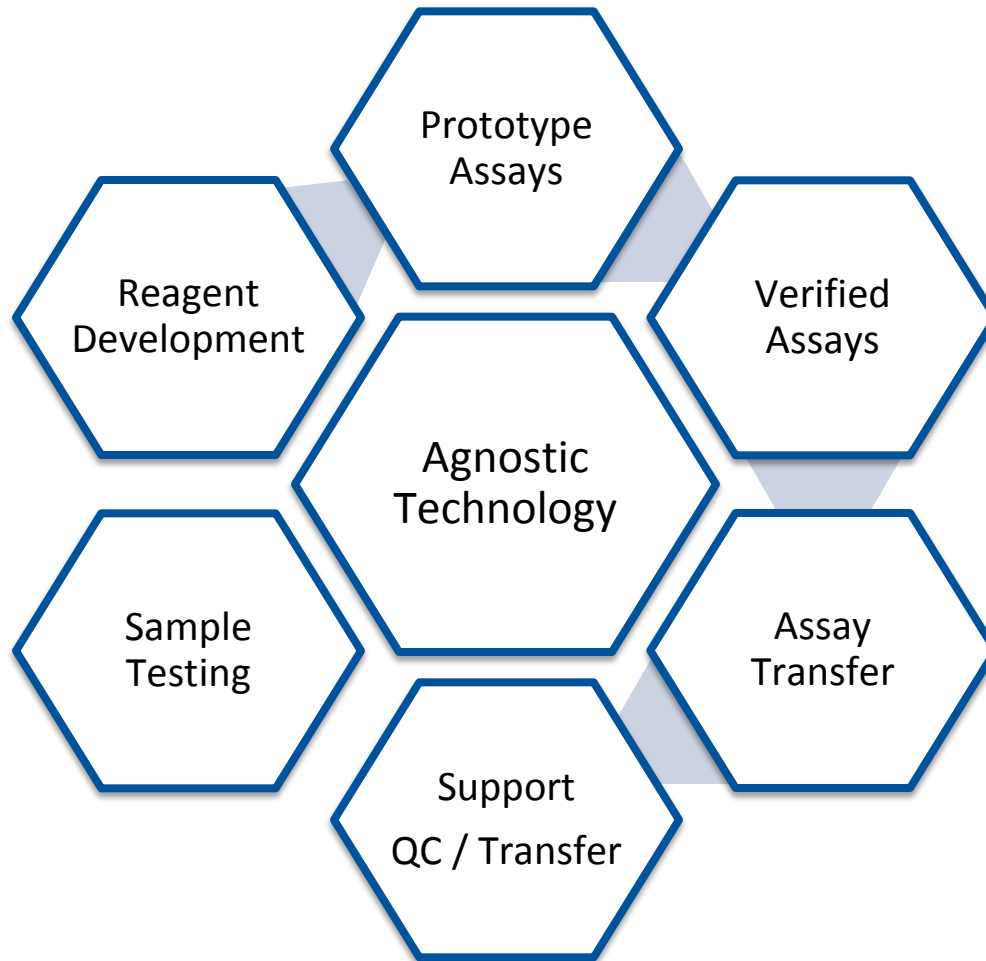
## Clinical Sample Analysis

Validation			
	QC Low	QC Medium	QC High
<b>Mean</b>	2.01	12.68	42.07
<b>SD</b>	0.18	1.38	3.44
<b>%CV</b>	9.0	10.9	8.2
<b>%RE</b>	101.3	98.9	98.6
<b>n</b>	18	18	18

In-sample Validation			
	QC Low	QC Medium	QC High
<b>Mean</b>	2.22	13.72	48.86
<b>SD</b>	0.16	0.98	3.67
<b>%CV</b>	7.4	7.1	7.5
<b>%RE</b>	112.3	107.0	114.5
<b>n</b>	18	18	18

QC Performance			
	QC Low	QC Medium	QC High
<b>Mean</b>	2.12	13.20	45.47
<b>SD</b>	0.20	1.29	4.91
<b>%CV</b>	9.6	9.8	10.8
<b>%RE</b>	106.8	102.9	106.6
<b>n</b>	36	36	36
<b>+2.0SD</b>	2.52	15.78	55.29
<b>-2.0SD</b>	1.71	10.62	35.64

# Assay Development: Summary





# Thank You

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