The pursuit of robust quantification of oligonucleotides by hybridization assays

Jill Uhlenkamp, PhD
08 June 2023

labcorp

Assay reagent quality

Assay development case study

- Developing a dual probe hybridization assay for an aptamer therapeutic
- Assay framework was provided by the client
- Biotin- and Digoxigenin-labeled probes were obtained from a commercial vendor

labcorp

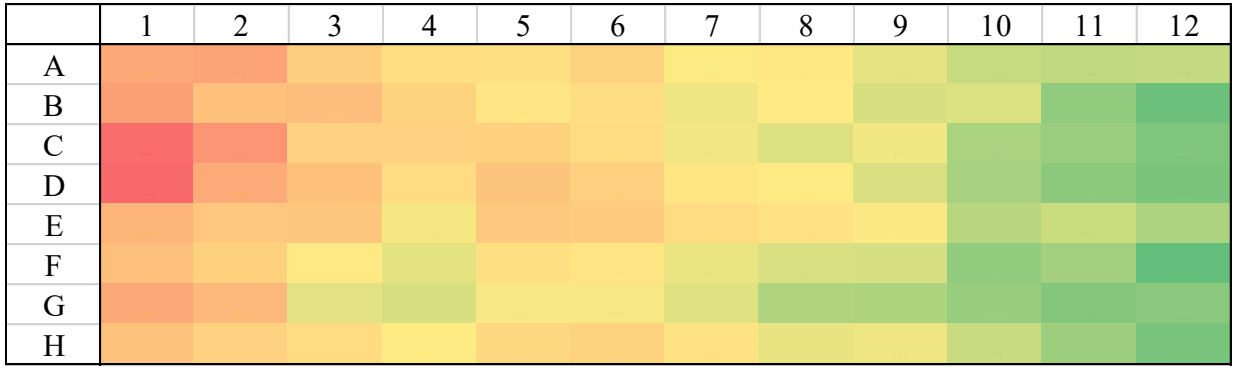
Assay reagent quality – impact on assay performance

Assay development summary

Phase 1 – Fine tune from provided method

- Commercial probes
- 7 runs over 6 days for optimization
- 2 runs on Qualification Day 1
  - Fail for QC bias

Intra-Run A&P – 6 QCs at each level				
QC bias (# out of 6)				
	0-10%	10-20%	>20%	Fail
ULOQ	0	1	5	5
HQC	1	4	1	1
MQC	6	0	0	0
LQC	6	0	0	0
LLOQ	6	0	0	0



Whole plate precision (% CV) approximately 5%

Assay reagent quality – impact on assay performance

Assay development summary

Phase 1 – Fine tune from provided method

- Commercial probes
- 7 runs over 6 days for optimization
- 2 runs on Qualification Day 1
  - Fail for QC bias

Phase 2 – Re-optimization

- Commercial probes
- 14 runs over 6 days to adjust assay parameters
  - Probe concentrations increased
- 4 runs over 2 Qualification days
  - Fail for QC bias, selectivity, stability

A&P – 3 QCs at each level				
QC bias (# out of 3)				
	0-10%	10-20%	>20%	Fail
ULOQ	0	2	1	1
HQC	0	2	1	1
MQC	2	1	0	0
LQC	0	2	1	1
LLOQ 1	0	2	1	0
LLOQ 2	2	0	1	0

Dilution linearity				
Bias (# out of 5)				
	0-10%	10-20%	>20%	Fail
In Range	0	4	1	1

Assay reagent quality – impact on assay performance

Assay development summary

Phase 1 –
Fine tune from provided method

- Commercial probes
- 7 runs over 6 days for optimization
- 2 runs on qualification day 1
    - Fail for QC bias

Phase 2 –
Re-optimization

- Commercial probes
- 14 runs over 6 days to adjust assay parameters
- Probe concentrations increased
- 4 runs over 2 qualification days
    - Fail for QC bias, selectivity, stability

Phase 3 –
Investigation

- **Fresh vs. frozen samples, location effects, incubation times**
- **38 total runs over 3 phases without transitioning to validation**

labcorp

Assay reagent quality – impact on assay performance

Assay development summary

Phase 4 –
New probes produced by client

- High probe concentration → vendor supply issue and quality concerns
- 2 runs over 2 days for optimization
  - Range remains 50-fold, shifted up by factor of 2
- 8 runs to successfully qualify assay for validation
- 10 total runs to re-optimize and complete the development

labcorp

Assay reagent quality – impact on assay performance

Assay development summary

Phase 4 – New probes produced by client

- High probe concentration → vendor supply issue and quality concerns
- 2 runs over 2 days for optimization
  - Range remains 50-fold, shifted up by factor of 2
- 8 runs to successfully qualify assay for validation
- 10 total runs to re-optimize and complete the development

Dilution linearity				
Bias (# out of 6)				
	0-10%	10-20%	>20%	Fail
In Range	5	1	0	0

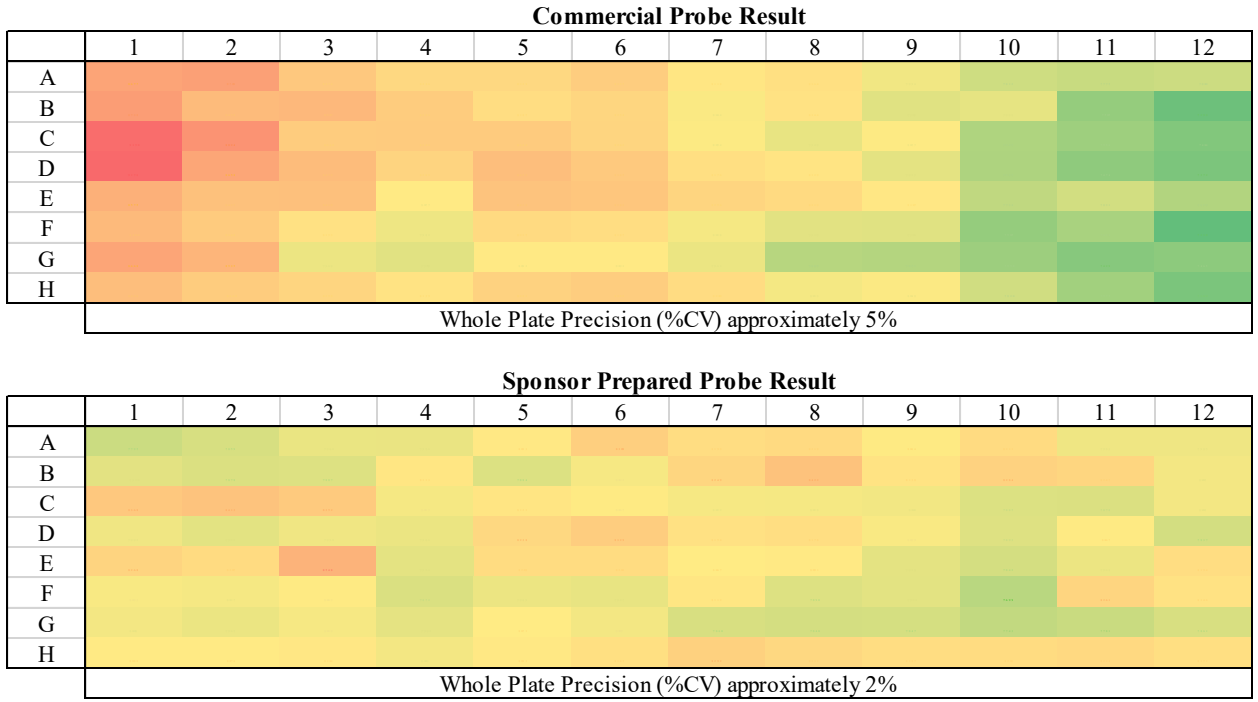
QC bias results					
		0-10%	10-20%	>20%	Fail
Run A: A&P 6 QCs at each level	ULOQ	5	1	0	0
	HQC	3	3	0	0
	MQC	5	1	0	0
	LQC	1	5	0	0
	LLOQ	4	2	0	0
Run B: A&P 3 QCs at each level	ULOQ	2	1	0	0
	HQC	3	0	0	0
	MQC	1	2	0	0
	LQC	3	0	0	0
	LLOQ	2	1	0	0
Run C: 2 QCs at H/M/L	HQC	2	0	0	0
	MQC	1	1	0	0
	LQC	1	1	0	0

labcorp

Assay reagent quality – impact on assay performance

Phase 4 – New probes produced by client

- High probe concentration → vendor supply issue and quality concerns
- 2 runs over 2 days for optimization
  - Range remains 50-fold, shifted up by factor of 2
- 8 runs to successfully qualify assay for validation
- 10 total runs to re-optimize and complete the development



Assay procedure control

Assay development case study

- Dual probe hybridization assay for an ASO therapeutic
- Biotin- and Digoxigenin-labeled probes were obtained from a commercial vendor
- Attempt to transfer a fully validated assay
  - Streptavidin plate and probe lots previously noted as consequential to assay performance
  - Hybridization temperature was changed from 37°C to ambient temperature for better sensitivity

labcorp

Assay procedure control – impact on assay performance

Assay development summary

- Many assay parameters verified/unchanged
  - MRD, probe concentrations
- Could not achieve the same LLOQ
  - Quantitative range shifted and shrunk
- Hybridization temperature changed from 37°C to ambient room temperature to achieve a lower LLOQ

Parameter	Transferred assay	In our lab	Adjusted assay
Quantitative Range	1.5 – 200 nM	4 – 150 nM	1 – 100 nM
Sample Incubation Temp.	37°C	37°C	ART

Assay Development Summary

Assay procedure control – impact on assay performance

Assay development summary

- Many assay parameters verified/unchanged
  - MRD, probe concentrations
- Could not achieve the same LLOQ
  - Quantitative range shifted and shrunk
- Hybridization temperature changed from 37°C to ambient room temperature to achieve a lower LLOQ

Assay Development Summary

Parameter	Transferred assay	In our lab	Adjusted assay
Quantitative Range	1.5 – 200 nM	4 – 150 nM	1 – 100 nM
Sample Incubation Temp.	37°C	37°C	ART

24 hr Room Temperature Stability				
	LQC (3 nM)		HQC (75 nM)	
Sample	Result	%Bias	Result	%Bias
1	4.93	64.3	111	48.0
2	4.78	59.3	101	34.7
3	4.98	66.0	99.9	33.2

labcorp

Assay procedure control – impact on assay performance

Assay development summary

- Many assay parameters verified/unchanged
  - MRD, probe concentrations
- Could not achieve the same LLOQ
  - Quantitative range shifted and shrunk
- Hybridization temperature changed from 37°C to ambient room temperature to achieve a lower LLOQ

Assay Development Summary

Parameter	Transferred assay	In our lab	Adjusted assay
Quantitative Range	1.5 – 200 nM	4 – 150 nM	1 – 100 nM
Sample Incubation Temp.	37°C	37°C	ART

ART Sample Incubation

24 hr Room Temperature Stability				
	LQC (3 nM)		HQC (75 nM)	
Sample	Result	%Bias	Result	%Bias
1	4.93	64.3	111	48.0
2	4.78	59.3	101	34.7
3	4.98	66.0	99.9	33.2

37°C Sample Incubation

24 hr Room Temperature Stability				
	LQC (30 nM)		HQC (225 nM)	
Sample	Result	%Bias	Result	%Bias
1	33.7	12.3	243	8.0
2	31.5	5.0	231	2.7
3	28	-6.7	209	-7.1

labcorp

Assay procedure control – impact on assay performance

Assay development summary

- Many assay parameters verified/unchanged
  - MRD, probe concentrations
- Could not achieve the same LLOQ
  - Quantitative range shifted and shrunk
- Hybridization temperature changed from 37°C to ambient room temperature to achieve a lower LLOQ

Parameter	Transferred assay	In our lab	Adjusted assay	Final assay
Quantitative Range	1.5 – 200 nM	4 – 150 nM	1 – 100 nM	10 – 300 nM
Sample Incubation Temp.	37°C	37°C	ART	37°C

ART Sample Incubation

24 hr Room Temperature Stability				
	LQC (3 nM)		HQC (75 nM)	
Sample	Result	%Bias	Result	%Bias
1	4.93	64.3	111	48.0
2	4.78	59.3	101	34.7
3	4.98	66.0	99.9	33.2

37°C Sample Incubation

24 hr Room Temperature Stability				
	LQC (30 nM)		HQC (225 nM)	
Sample	Result	%Bias	Result	%Bias
1	33.7	12.3	243	8.0
2	31.5	5.0	231	2.7
3	28	-6.7	209	-7.1

Assay Development Summary

Enhanced assay sensitivity

Assay development case study

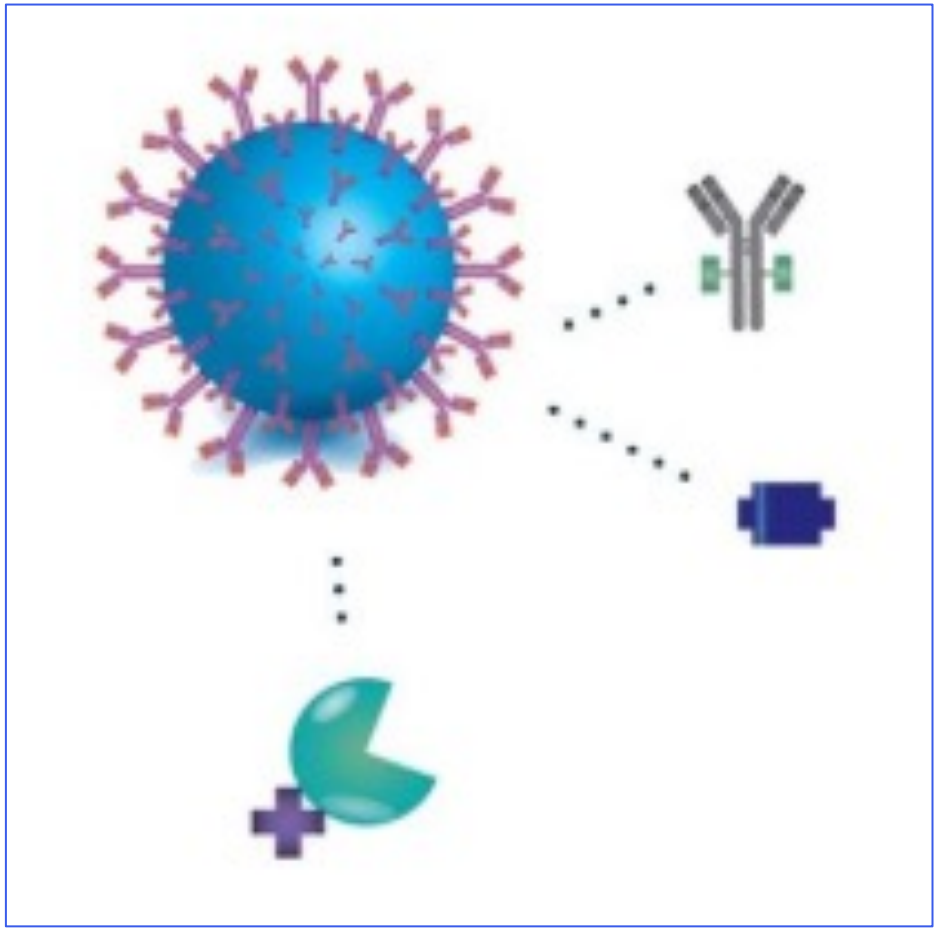
- Compare MSD ECL assay to Quanterix Simoa®
- Assays developed for 21-mer non-proprietary analyte
- Probe design
  - MSD ECL
    - 3’ biotin for capture
    - 5’ dig for detection
  - Quanterix Simoa®
    - 5’ amine modification with spacer to couple to the beads
    - 3’ biotin for detection with streptavidin-labeled enzyme

labcorp

Enhanced assay sensitivity with non-traditional LBA platform

Quanterix Simoa® summary

- Traditional sandwich assay format in solution on magnetic beads
  - Streptavidin-labeled enzyme produces fluorescence
- Sample is loaded to a disk and travels across an array of small wells
- Fluorescence image is captured, fluorescent beads are counted
  - At higher sample concentrations, number of binding events per bead can be determined

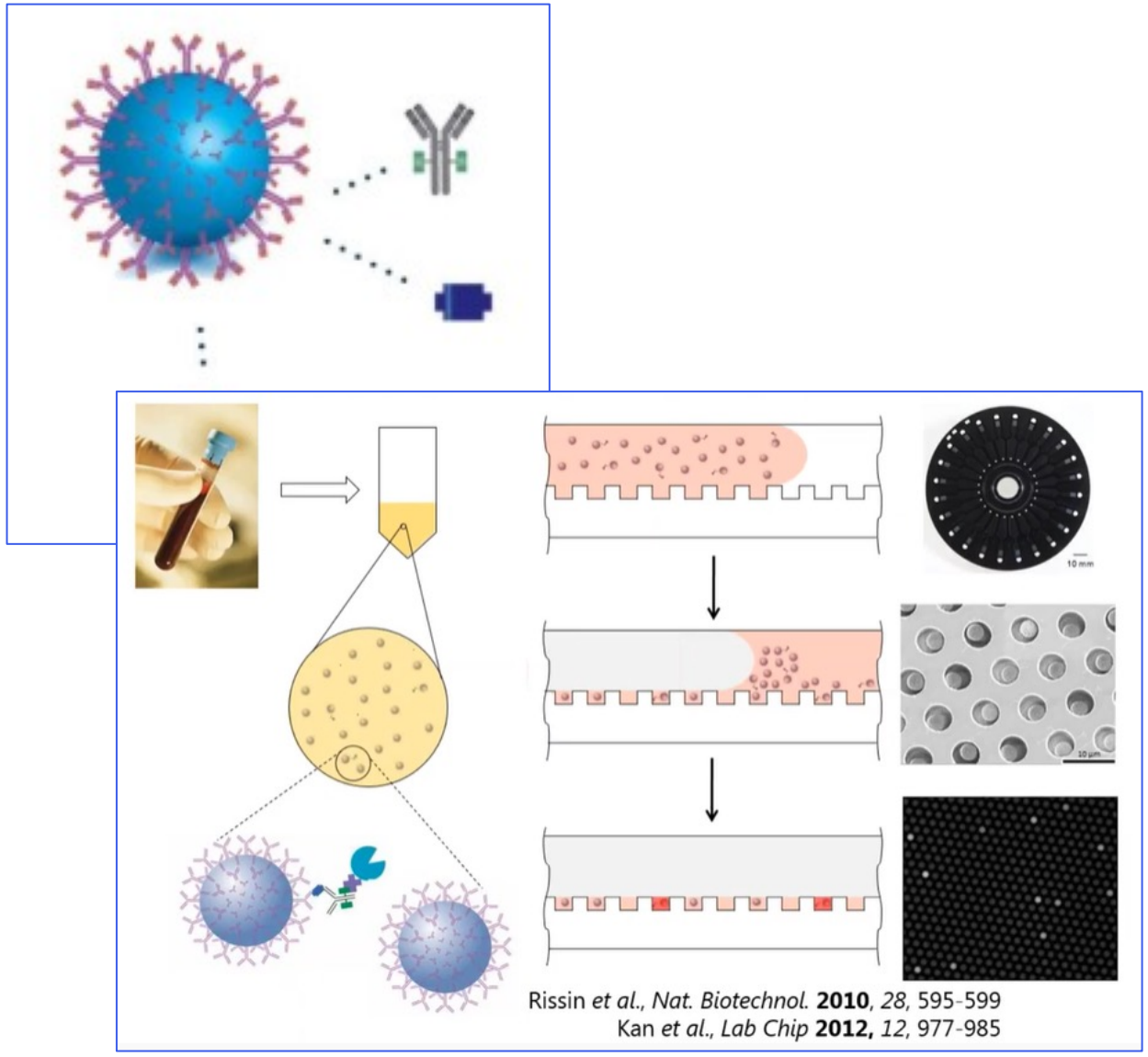


labcorp

Enhanced assay sensitivity with non-traditional LBA platform

Quanterix Simoa® summary

- Traditional sandwich assay format in solution on magnetic beads
  - Streptavidin-labeled enzyme produces fluorescence
- Sample is loaded to a disk and travels across an array of small wells
- Fluorescence image is captured, fluorescent beads are counted
  - At higher sample concentrations, number of binding events per bead can be determined

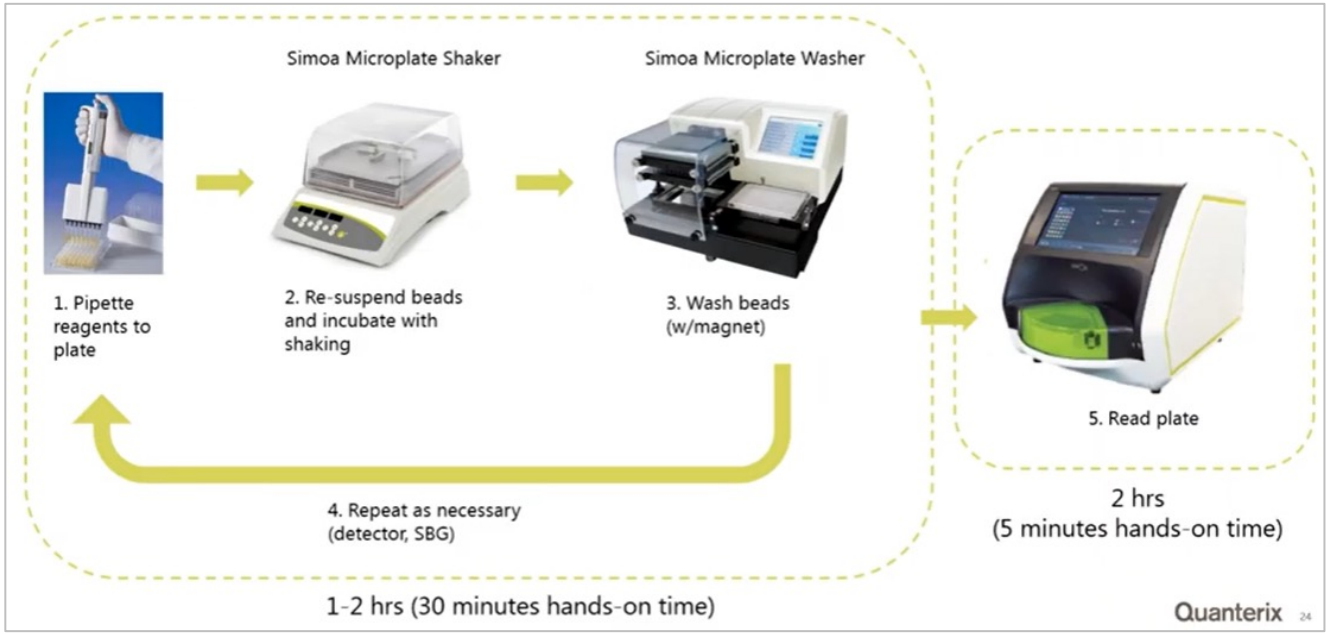


Rissin *et al.*, *Nat. Biotechnol.* **2010**, *28*, 595-599
Kan *et al.*, *Lab Chip* **2012**, *12*, 977-985

Enhanced assay sensitivity with non-traditional LBA platform

Quanterix Simoa® summary

- Traditional sandwich assay format in solution on magnetic beads
  - Streptavidin-labeled enzyme produces fluorescence
- Sample is loaded to a disk and travels across an array of small wells
- Fluorescence image is captured, fluorescent beads are counted
  - At higher sample concentrations, number of binding events per bead can be determined



Enhanced assay sensitivity with non-traditional LBA platform

Simoa® Technology and ECL Comparison

Simoa® Method outline (in human serum)	ECL Method outline (in human serum)
1. Dilute all samples in buffer	1. Hybridization: Incubate capture probe + samples (1.5 hour incubation)
2. Hybridization: Combine capture beads + diluted samples + detector probe and incubate (2 hour incubation)	2. Add hybridized probe/samples to streptavidin coated MSD plate and incubate (1-2 hour incubation)
3. Wash beads and incubate with SBG enzyme (1 hour incubation)	3. Wash plate and add digoxigenin-labeled detector probe (1 hour incubation)
4. Wash beads and load onto instrument	4. Wash plate and add Sulfo Tag-labeled anti-digoxigenin detection antibody (1 hour incubation)
5. Plate reading (2-3 hours) Total Assay Time = 7-8 hours Readout signal: Fluorescence Reported raw data: AEB (Average number of Enzyme per Bead)	5. Wash plate and read Total Assay Time = 5-6 hours Readout signal: electrochemiluminescence Reported raw data: RLU

labcorp

Enhanced assay sensitivity with non-traditional LBA platform

Simoa® Technology and ECL Comparison
Accuracy & Precision

Simoa® Range I	Concentration (fM)	%Bias	%TE
ULOQ	10,000	-3.9	15.3
HQC	7500	-10.4	17.4
MQC	550	-9.8	16.9
LQC	60	-17.5	35.6
LLOQ	30	**-30.6**	**54.2**

Samples were tested in duplicates

Simoa® Range II	Concentration (fM)	%Bias	%TE
ULOQ	8000	7.5	19.6
HQC	6000	3.8	13.6
MQC	900	2.1	12.8
LQC	200	15.1	37.0
LLOQ	100	4.4	14.1

Samples were tested in triplicates

ECL	Concentration (fM)	%Bias	%TE
ULOQ	2,000,000	5.5	13.0
HQC	1,600,000	4.4	9.3
MQC	100,000	-2.3	7.0
LQC	10,000	-6.4	11.8
LLOQ	4000	-10.5	18.7

Samples were tested in duplicates

labcorp

Enhanced assay sensitivity with non-traditional LBA platform

Simoa® Technology and ECL Comparison
Final Results

Parameter	Simoa®	ECL
Lower Limit of Quantitation	100 fM	4000 fM
Upper Limit of Quantitation	8000 fM	2,000,000 fM
Sample Requirement per Test	25 µL	150 µL
MRD	1/14	1/3.3
Curve Fit; Weighting Factor	4-PL; $1/y^2$	4-PL; $1/y^2$
Method Selectivity	Pass	Pass
Dilution Linearity	1/500,000	1/40,000

labcorp

Enhanced assay sensitivity with non-traditional LBA platform

Simoa® Technology and ECL Comparison Final Results

Parameter	Simoa®	ECL
Lower Limit of Quantitation	100 fM	4000 fM
Upper Limit of Quantitation	8000 fM	2,000,000 fM
Sample Requirement per Test	25 µL	150 µL
MRD	1/14	1/3.3
Curve Fit; Weighting Factor	4-PL; $1/y^2$	4-PL; $1/y^2$
Method Selectivity	Pass	Pass
Dilution Linearity	1/500,000	1/40,000

40x improvement in sensitivity

labcorp

Conclusions

- **Assay procedure details**
- **Reagent characteristics**
  - Probe quality
  - Probe design (LNA, other modifications)
- **Seeking new formats to meet assay demands**
  - Simoa® or other technologies

labcorp

Acknowledgments

- **Labcorp Indianapolis Immunochemistry Services Scientists**
  - Peter Wallace, Russ Garton, James Cyran
  - Bingbing Wang, Erica Simmons, Barry Peterson
- **Labcorp Indianapolis Immunochemistry Services Leadership**
  - Eric Thomas
  - Mark O'Dell
  - Dan Sikkema
- **Labcorp Scientific Affairs**
  - Peter Murphy
- **Quanterix Corporation**
  - David Rissin and David Duffy
  - Carly Trulock
  - Tech Support (Gaurav Chavan, Perla Gallo, Jia Lu, Jillian Mason, Jennifer DeMent)
- **Other Scientific Support**
  - Rob Nelson

labcorp

Thank you!
Questions?