Worldwide Pharmaceutical Sciences

Molecules to Market

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Improving the Accuracy, Speed, and Reproducibility of Titer Determination for the Clone Selection Process

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OUTLINE

- Selection of a high throughput, automatable assay for titer evaluation to support clone selection and culture process development
- A High level initial evaluation (precision, accuracy) of potential titer assays using 1L Bioreactor samples
  - Historical methods: Protein A HPLC and ELISA
  - Novel techniques
    - Guava RapidQuant™ system: “ELISA on a bead” read by a Flow Cytometer
    - FortéBio Octet: A novel system that determines concentration by measuring the binding rate to a sensor surface, Protein A in this case, and comparing to external standards
- FortéBio Qualification Experiments, with comparison to UV (gold standard for concentration determination)
- Bridging Studies, using Real Clone Selection Samples to compare FortéBio Octet to ELISA and Protein A HPLC

Clone Selection and the Timeline Crunch

Timelines are forever squeezed and the number of projects to be evaluated per year continues to increase.

- The time required from Transfection to Clone Selection is a Critical Parameter.
- Choosing the optimum clone also involves evaluating cell cultivation conditions for the lead clones.
- Automation and DOE have improved the clone selection process but the process is primarily driven by titer results. Thus improving the accuracy and precision of the titer results should make the clone selection process more efficient.

Selection of an Automatable High-Throughput Titer Assay

- Our charter was to select and automate a titer assay for integration with a Cell Line Development (CLD) robot that would enable Culture Process Development

- Method requirements:
  - Plate based assay
  - Capability for full automation
  - Throughput capability of > 1 plate per hour
  - Data turnaround of < 12 hours
  - Large linear range, 1 dilution had to cover all samples
  - Rugged
  - Low sample consumption

Accurate and Rapid Titer Determination Remains a Challenge for Clone Selection

- Protein A HPLC and ELISA are commonly used for titer evaluation, however each have their own challenges
- Protein A HPLC is often impractical early in the process due to low titers, low sample volumes, and high sample load.
- ELISA is often the method of choice because it is easily automatable. Unfortunately, experiments show that it has less than desirable precision, accuracy, and turnaround time.
  - Sample load prevents doing a dilution series of every sample, this greatly compromises the value of ELISA especially in early development when expression levels are unknown.
  - Linear range can be the real downfall in this case as high expressers are labeled mediocre due to the Hook Effect or saturation.

Alternative Titer Method - Guava RapidQuant™ System for human IgG

"ELISA on a bead" read by a Flow Cytometer

capture bead + antibody in hybridoma supernatent

bead-bound analyte + fluorescently labeled detector antibody

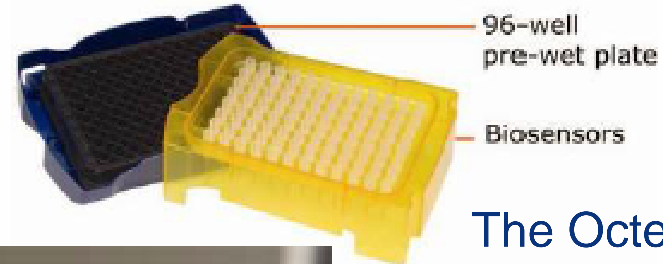
bead-bound fluorescence

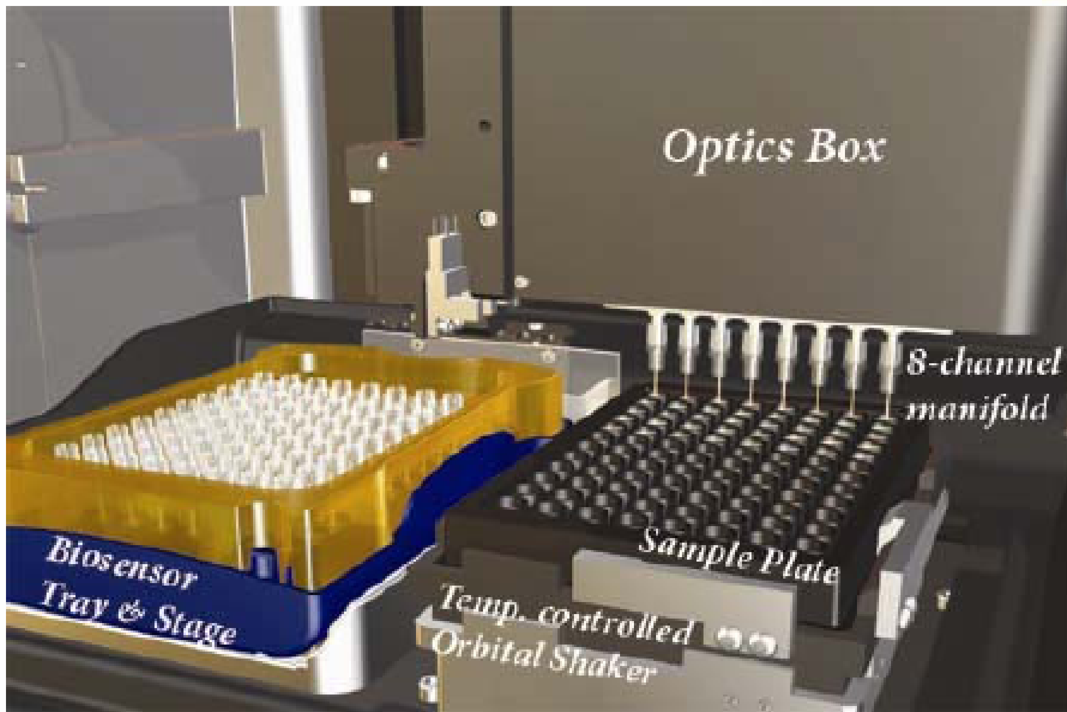
bead detection on Guava platform

Incubate beads & supernatant sample in microplate
→ Add detector antibody & incubate
→ Add buffer
→ **Acquire data on Guava EasyCyte System**

Alternative Titer Method - FortéBio Octet System with Protein A BioSensors

Octet Instrument





The Octet System measures the binding rate to a sensor surface (Protein A in this case) under controlled conditions and determines concentration by comparison to external standards.

fortéBIO

. Confidential Information

FortéBio Standard Curve

The binding plots for a Std. Curve consisting of 8 levels:

120, 90, 60, 30, 15, 7.5, 3.75, 0.9375 µg/mL

Sensor A Sensor B Sensor C Sensor D Sensor E Sensor F Sensor G Sensor H

Binding (nm)

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

0 20 40 60 80 100 120

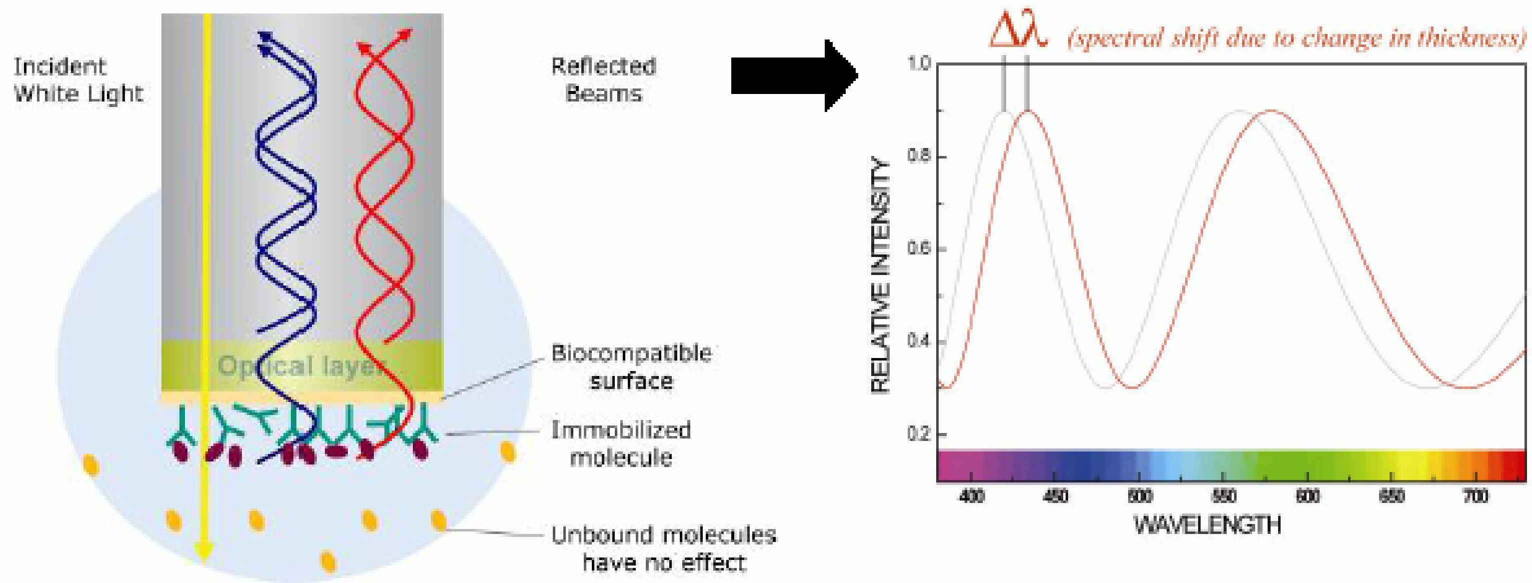
Time (sec)

FortéBio Octet Q system

BioLayer Interferometry (BLI)

Proprietary new technology for label-free detection

- A layer of molecules attached to the tip of an optic fiber creates an interference pattern at the detector.
- Any change in the number of molecules bound causes a measured shift in the pattern



Titer Method Comparison (96 samples)

Method requirements:

- Capability for full automation
- Throughput capability of 1 plate per hour
- Data turnaround of <12 hours

	Guava Rapid-Quant	Protein A ELISA	FortéBio Octet Q	Normal Protein A HPLC
Run Time/ harvest (inc. prep)	25 min (90 min) (clarified)	~3 hours* (6 hours*) *can run in parallel	25 min (no prep)	**6 hours** (clarified)
Sample consumed	1-2 µL	1-2 µL	None*	20 µL
Automation obstacles	Medium, Cleaning Cycles	Low	Low, Tray handling	Low
Linear Range	0.5 – 20 µg/mL	0.8 -50 ng/mL	2 -200 µg/mL	0.25 – 8 mg/mL
Cost per Plate	**~$200**	$30	**~$225**	Very low
Automating Data Output	???	good	good	varied

Evaluation of Alternate Techniques for Titer Determination

- 4 Titer assays were chosen for initial evaluation
  - Guava RapidQuant™ system for human IgG
  - FortéBio Octet system using Protein A biosensors
  - Sandwich ELISA (4 different ELISAs were screened including the one used by our Cell Line Development Group, the best one was chosen for use in the comparison)
  - Protein A HPLC
- The following parameters were evaluated: Accuracy, Precision, Linear Range, Sample Consumption, and "Automatability"
- Protein A HPLC was chosen as the "gold standard" against which the other assays were compared

Experiment #1 – Evaluation of Potential Methods with 1L Bioreactor Samples

- Experiment #1
  - Evaluation of settled Bioreactor broths using Protein A HPLC, ELISA, Guava, and FortéBio
  - The following samples were analyzed against a mAb#1 standard curve by the 4 assays
    - mAb#2 (an IgG_2), 2 bioreactors were sampled
    - mAb#3 (an IgG_1)
    - mAb#4 (an IgG_2)

Experiment #1 – Evaluation of Potential Methods with 1L Bioreactor Samples

- The same sample was used as a standard for all assays
- 8-12 Replicate dilutions were analyzed by ELISA
- 6 replicate dilutions (between 2 plates) were analyzed by FortéBio
- 6 replicate dilutions (between 2 plates) were analyzed by Guava
- Accuracy was evaluated against Protein A HPLC
- The precision of each assay (relative standard deviation for replicate analyses) was also evaluated

Experiment #1 – Evaluation of Potential Methods with 1L Bioreactor Samples

Sample Name	Protein A HPLC Titer (% of HPLC value)	ELISA Titer (% of HPLC value)	ELISA %RSD	Guava Titer (% of HPLC value)	Guava %RSD	FortéBio Titer (% of HPLC value)	FortéBio %RSD
mAb#2	100	126.0	15.0	94.7	8.4	94.3	2.6
mAb#2	100	126.0	7.9	89.5	6.0	95.0	2.1
mAb#3	100	85.7	5.2	107	4.1	95.7	2.3
mAb#4	100	103.0	16.0	n/a	n/a	95.1	4.4

Experiment #1 – Summary

- Guava
  - Assay can meet throughput needs,
  - Accuracy and Precision are better than ELISA
  - Instrument Prep prior to run will complicate automation
- FortéBio Octet with Protein A BioSensors
  - Fastest assay
  - Most accurate and precise assay that can meet throughput needs
  - Largest linear range
  - Easily automatable
  - Zero sample prep (except dilution if necessary)

Experiment #2A – Qualification of FortéBio for Clone Selection and Culture Process Development

- Experiment #2A
  - Protein A eluates were analyzed using UV(280nm) and then gravimetrically spiked into media and analyzed by FortéBio on multiple days to evaluate: linearity, precision, mAb to mAb response, day to day variability, lot to lot variability
  - The following samples were all analyzed against the mAb#2 curve from day 1
    - mAb#2 - an IgG2
    - mAb#3 - an IgG1
    - mAb#5 - an IgG2
    - mAb#6 - an IgG4

Experiment #2A - Qualification

mAb#2 Curve from Day 1 used for all Samples

FortéBio - 3 replicates on day 1, 2, 4, and 5

	UV conc* µg/mL	Forté avg 4 days, 3 reps	Pooled %RSD	(Forté/ UV) *100
mAb#2	117.7	119.7	2	102
mAb#2	88.3	86.8	1	98
mAb#2	58.9	62.9	4	107
mAb#2	29.4	31.4	6	107
mAb#2	14.7	15.1	6	102
mAb#2	7.4	7.7	4	105
mAb#2	3.7	4.0	4	110
mAb#2	0.9	1.1	7	121
mAb#3	1.1	1.3	7	115
mAb#3	4.5	4.9	4	109
mAb#3	9.0	9.7	8	108
mAb#3	18.0	19.5	7	108
mAb#3	36.1	41.9	4	116
mAb#3	72.2	83.4	6	116
mAb#3	108.3	122.9	4	114

*Concentration after gravimetric dilution

Experiment #2A - Qualification

mAb#2 Curve from Day 1 used for all Samples

FortéBio - 3 replicates on day 1, 2, 4, and 5

	UV conc µg/mL	Forte avg 4 days 3 reps	%RSD	(Forté/ UV) *100
mAb#5	119.4	101.1	4	85
mAb#5	89.6	72.2	3	81
mAb#5	59.7	52.1	4	87
mAb#5	29.9	26.0	5	87
mAb#5	14.9	14.0	6	94
mAb#5	7.5	7.0	6	94
mAb#5	3.7	3.5	8	95
mAb#5	0.9	0.9	13	98
mAb#6	1.7	1.8	9	107
mAb#6	6.7	7.0	4	105
mAb#6	13.3	14.9	5	112
mAb#6	26.6	29.9	5	112
mAb#6	53.2	59.0	5	111
mAb#6	106.5	116.3	7	109

Expanding the Scope of the Qualification

- At this point it was becoming clear that FortéBio Octet w/ Protein A BioSensors was clearly superior to ELISA
  - 10x savings in hand-on time and turnaround time
  - FortéBio provides accuracy and precision approaching that of Protein A HPLC for samples that would have had to be analyzed by ELISA due to titer and throughput issues.
- Utilizing FortéBio for IgG quant could greatly improve the Clone Selection process.
  - Worked with Cell Line Development to expand the qualification to encompass all Cell Line Development sample types from transfection to culture optimization
    - Additional Matrices
    - Additional Sample Types

Experiment #2B - Matrix Effects

For Accuracy the Samples Should be in the Same Matrix as the Standard Curve

- mAb#2 was diluted, to 10 & 100 µg/mL, in media (x) and media (x) spiked with various additives to double the normal level

Diluent	Sample Conc.	%RSD – 8 reps	% Recovery vs. control
media (x) (control)	105.1	2.6	
Additive (P)	114.1	3.9	108.5
Additive (Q)	112.3	2.5	106.9
Additives (Q+R)	118.4	2.2	112.7
Additive (S)	111.1	2.7	105.7
media (x) (control)	10.0	2.6	
Additive (P)	11.0	1.3	109.7
Additive (Q)	10.5	2.1	104.8
Additives (Q+R)	11.3	1.9	112.4
Additive (S)	10.5	1.4	104.8

Linearity and precision appear unaffected

Experiments #3A and 3B, Bridging Studies by our CLD group using Real Clone Selection Samples

- Our Cell Line Development group wanted to see how the assay would behave in their hands
  - Worked with them to set up 2 large bridging studies using real samples that they executed independently
- Experiment 3A: Comparison to ELISA
  - Chose 88 samples from early in the clone selection process for analysis by FortéBio and ELISA in duplicate
- Experiment 3B: Comparison to Protein A HPLC
  - Chose 24 samples from later in the clone selection process for analysis by FortéBio and Protein A HPLC

Exp. 3A - CLD Evaluation - FortéBio vs. ELISA

%RSD of 88 Samples in Duplicate (note the poor reproducibility for ELISA)**

(%RSD = 100 * St. Dev. / Average)

**** the missing x's are from when 1 or both ELISA values reported "max" or "min"**

% RSD for Replicate Analysis

% RSD: 0.0, 10.0, 20.0, 30.0, 40.0, 50.0

sample #: 0, 20, 40, 60, 80, 100

ELISA % RSD

ForteBio %RSD

Exp. 3A - CLD Evaluation
ELISA Results** vs. FortéBio Results

** only samples with 2 valid ELISA values

Concentrations normalized to 100% ForteBio

$y = 0.4007x + 8.9076$

$R^2 = 0.751$

ELISA (y-axis): 0, 10, 20, 30, 40, 50, 60

Forte (x-axis): 0, 20, 40, 60, 80, 100

Classic example of the “Hook Effect” that plagues ELISA

Exp. 3A - CLD Evaluation
ELISA Results*** vs. FortéBio Results

*** only samples with 2 valid ELISA values and FortéBio results below 50% (which is the top of the ELISA Linear Range, to eliminate the Hook Effect)

normalized ForteBio concentration < 50%

$y = 0.7645x + 0.9487$

$R^2 = 0.9036$

ELISA: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45

ForteBio: 0, 10, 20, 30, 40, 50, 60

Exp. 3B - CLD Evaluation
HPLC Results vs. ForteBio Results

24 samples ran by ForteBio and Protein A HPLC

ForteBio vs HPLC - all Values normalized to 100

$y = 0.9012x - 1.3384$

$R^2 = 0.9711$

Method Comparison – 96 Samples

	ELISA	HPLC	ForteBio
Total time	6-18 hours	12 hours	30 min
Hands on time	90 min	90 min	7-10 min
Precision (%RSD)	5-20%	1-5%	Typically < 5%
Linear range	Linear Range for given dilution (64 fold) 0.8 – 50 µg/mL	Narrow Linear Range for given inj. Vol. (24 fold) 0.25 – 8 mg/mL	Wide Linear Range, (200 fold) 2 – 200 µg/mL
Main Concerns	Relatively poor reproducibility/ accuracy, "Hook Effect"	Clone Selection samples are below LOQ, column fouling	**Cost per Analysis ~ $2.00 * (sensor reuse software upgrade reduces cost 5 - 10x)**
Other concerns	repetitive motion risk	Waste generation ~1L/ 96 samples, response drift as column ages	single supplier

FortéBio Octet Q system
Summary

Advantages:

- Fastest Detection method at <30 min/ plate, ~20 sec/ sample. Sample clarification is not necessary, no sample prep involved except possibly dilution (depending on concentration)
- Linear Range of Detection of 2 µg/mL – 200 µg/mL typically < 5%RSD; (0.8 µg/mL – 350 µg/mL typically < 10% RSD)
- The data analysis is automated and software outputs in Word or Excel with 2 clicks.

Disadvantages:

- Operating costs are $2.25/ sample** including the standards and controls.

** A BioSensor Re-use Upgrade is now available that allows up to 10 reads per tip at the cost of 16 less wells per plate

BioSensor Regeneration aka *Protein A Plus*™

- Upgrade allows 8 FortéBio protein A BioSensors to be regenerated and reused over an entire plate**
  - **2 columns of the well plate are lost for regeneration and re-equilibration buffers
  - Purified mAb was diluted to 100 µg/mL and used to fill an entire plate that was read 3 times, with and without sensor regeneration

channel	% response no regen	%RSD	% response - 10 reads	% RSD	% resp. - 5 reads
A	100.8	1.3	97.8	1.1	98.1
B	96.8	1.1	96.8	0.4	99.0
C	98.8	1.2	99.9	0.5	99.1
D	100.9	1.1	98.1	0.5	98.8
E	103.8	1.1	103.0	0.6	104.1
F	102.9	2.0	104.7	0.8	104.5
G	99.0	2.0	100.7	0.7	100.0
H	98.9	1.4	99.0	0.6	96.5

BioSensor Regeneration

- BioSensor Regeneration

Channel Response: regen vs non-regen

Legend: no regen; 10 reads per; 5 reads per

% recovery: 105.0, 102.5, 100.0, 97.5, 95.0

Channel: A, B, C, D, E, F, G, H

Instrument to Instrument Variability and Channel Bias

- Channel Bias and Instrument to Instrument Variability
  - Each channel is read and "light plumbed" independently and the light intensities can not be completely controlled
  - This leads to a channel bias that is fixed and consistent
- Purified mAb diluted to 100 µg/mL was used to fill 4 entire plates and read on 4 of our FortéBio instruments

	Channel Response 100 * channel avg/ total avg			
	Unit 1	**Unit 2**	**Unit 3**	**Unit 4**
Ch. A	101.0	102.3	100.2	99.8
Ch. B	99.2	101.3	97.5	95.4
Ch. C	99.1	99.2	97.8	100.3
Ch. D	98.3	98.2	102.2	99.9
Ch. E	98.0	101.9	103.1	100.4
Ch. F	100.1	95.8	101.7	100.7
Ch. G	99.3	102.6	98.5	103.7
Ch. H	105.0	98.6	99.0	99.8

Conclusion Slide

- Utilizing FortéBio for IgG quantitation in place of ELISA will greatly improve the Clone Selection process.
  - 10x savings in hand-on time and turnaround time
  - FortéBio provides accuracy and precision approaching that of Protein A HPLC for samples that would have had to be analyzed by ELISA due to titer and throughput issues.
  - The BioSensor Regeneration upgrade successfully reduces cost per analysis up to 10x with comparable performance (down from $2.25 per sample)
- The cell line development group has completely switched from ELISA to FortéBio except for the robots
- We are currently working with Thermo® to add FortéBio to our Cell Line Development robots

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  - Gregory Schneider

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Backup Slides

CLD Evaluation
ELISA Procedure (96 Samples)

- Prepare coating buffer, blocking buffer and wash buffer (30 min)
- Coat plate with capture antibody (overnight)
- Add blocking buffer to plate (1 hr or overnight)
- Dilute samples 3 times @ 1:10 (30 min)
- Add standard curve and samples to each ELISA plate
  - Varies from 15 min to overnight incubation
- Wash 3 times each plate (10 min)
- Dilute and add secondary antibody
  - Varies from 15 to 30 min incubation
- Wash 3 times each plate (10 min) } 10 min
- Prepare and add ABTS }
- Read plate using TECAN reader
- Hands on time = 1.5 hr
- Total Assay time = 1.5 days

CLD Evaluation
HPLC Procedure (96 Samples)

- Prepare buffers
- Clarify samples (40 min – spin and filter)
- Write Sequence
- Purge Sample lines
- Washing steps after every 10 samples (42 min)
- 6 minutes run time per sample (9.6 hrs; 16.6 hrs w/ cleaning cycles for 96 samples)
- Manually calculate concentrations (10 min)
- Creates ~ 1000mL HPLC waste per plate
- Hands on time = 1.5 hr; total assay time = 17 hours

CLD Evaluation
FortéBio Procedure

- Prepare standard curve (once per week) in media
  - Ex. 120, 90, 60, 30, 15, 7.5, 3.75, and .9375 µg/mL (used for application A w/100x dilution, corresponds to 0.1 – 12 mg/mL)
- Soak sensor tips (2 min)
- Dilute samples if necessary (direct analysis for 2-160µg/mL)
- Analyze 8 samples in 2 minutes
  - 96 samples in ~25 min
- Automatically calculates sample concentrations and exports to Excel with 2 clicks