Label-free, Native Analyte Screening via the RapidFire™ High-throughput Mass Spectrometry Platform

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Senior R&D Manager, RapidFire™
Agilent Technologies, Inc.
November 9, 2011

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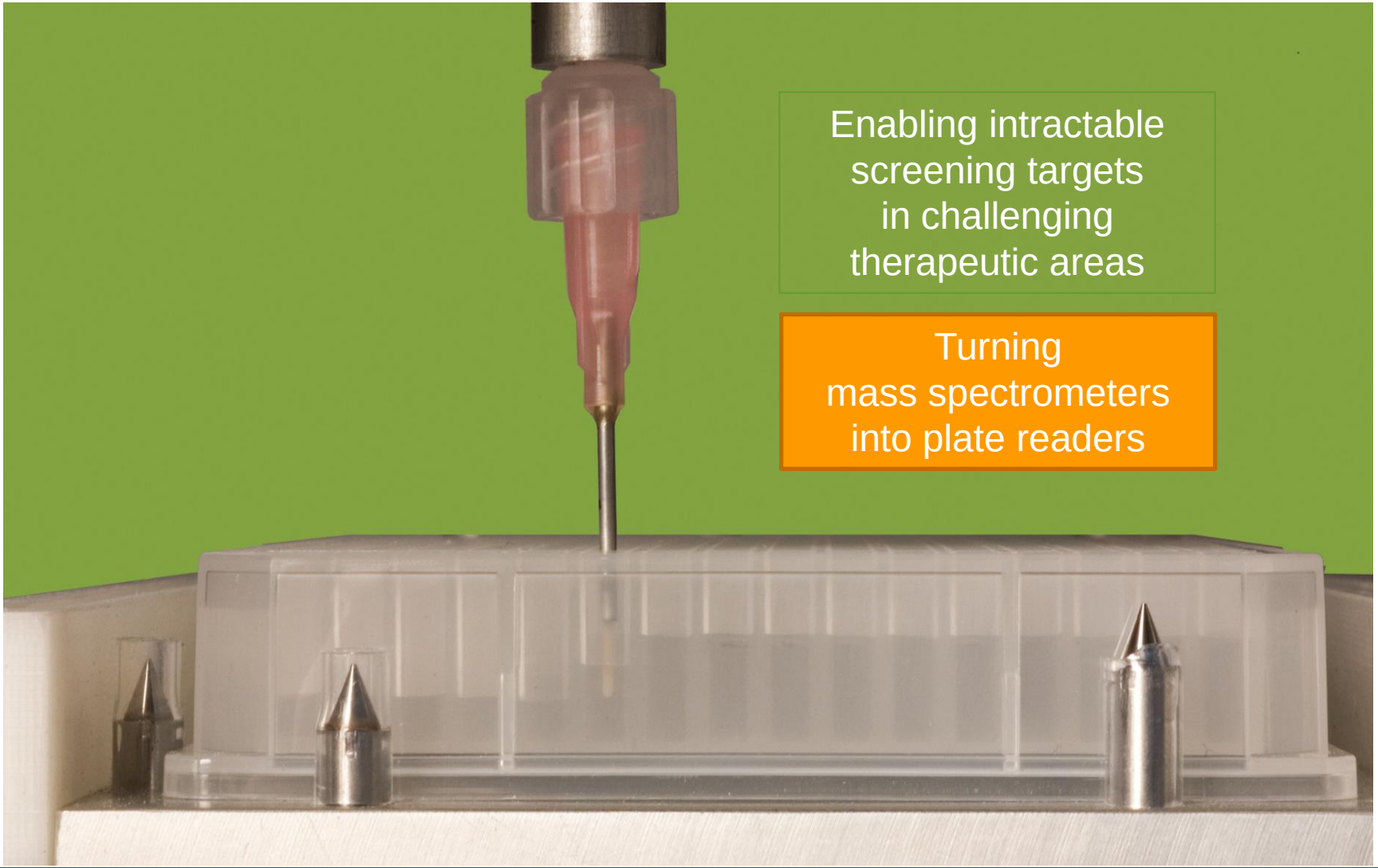
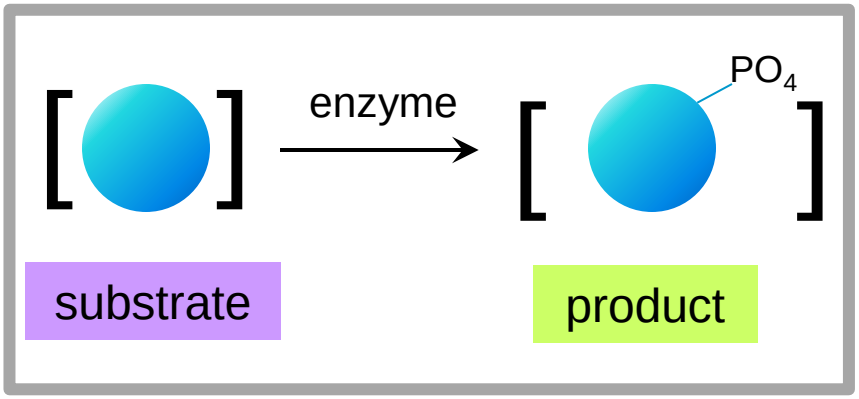
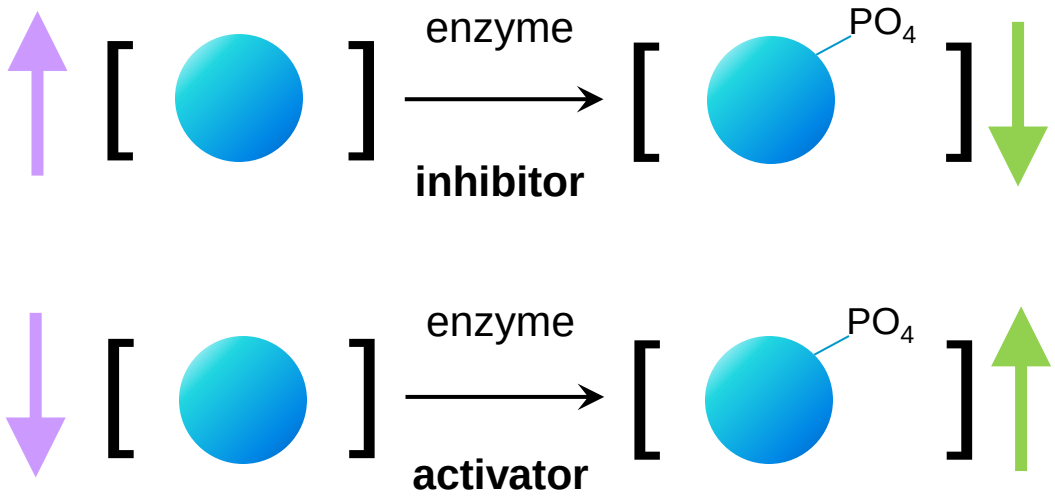


Functional Biochemical Assays



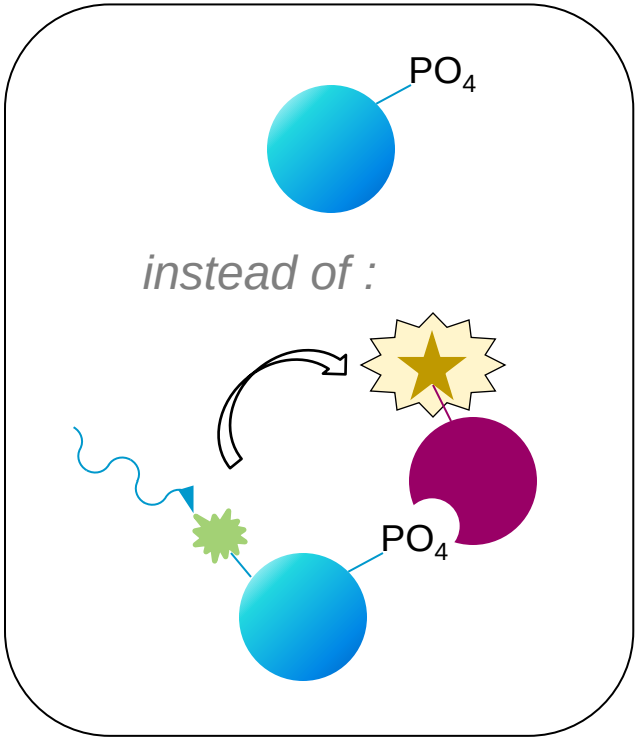
$$\% \text{ conversion} = \left[\frac{(\text{AUC MW2})}{(\text{AUC MW1} + \text{AUC MW2})} \right]$$

AUC: area under the curve



Advantages of mass spectrometry

- True label-free detection
- Direct, quantitative measurements
- **Native** reaction substrates & products
  - (no radioactivity, no surrogate analytes, no indirect or secondary components)
- Functional biochemical assays
  - (rather than target binding assays)



- How to bring innovation while maintaining automation?

Limitations of MS

Molecules must be charged

Desalting step required

Sample purification is

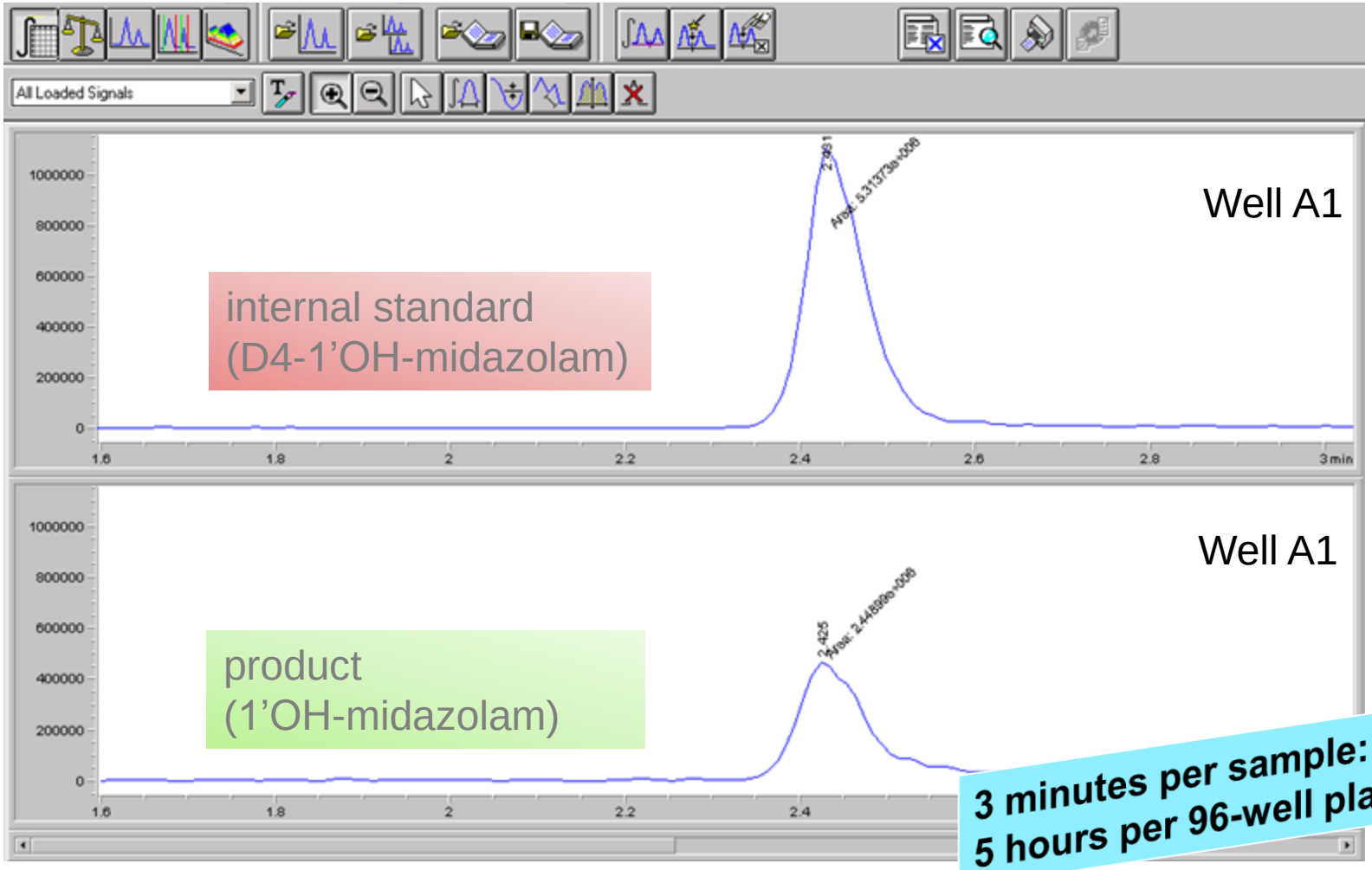
- Serial
- slow

Instrumentation is expensive,

- not easily scalable
- to meet demand

- How to bring innovation while maintaining automation?

From HPLC … (high performance liquid chromatography)



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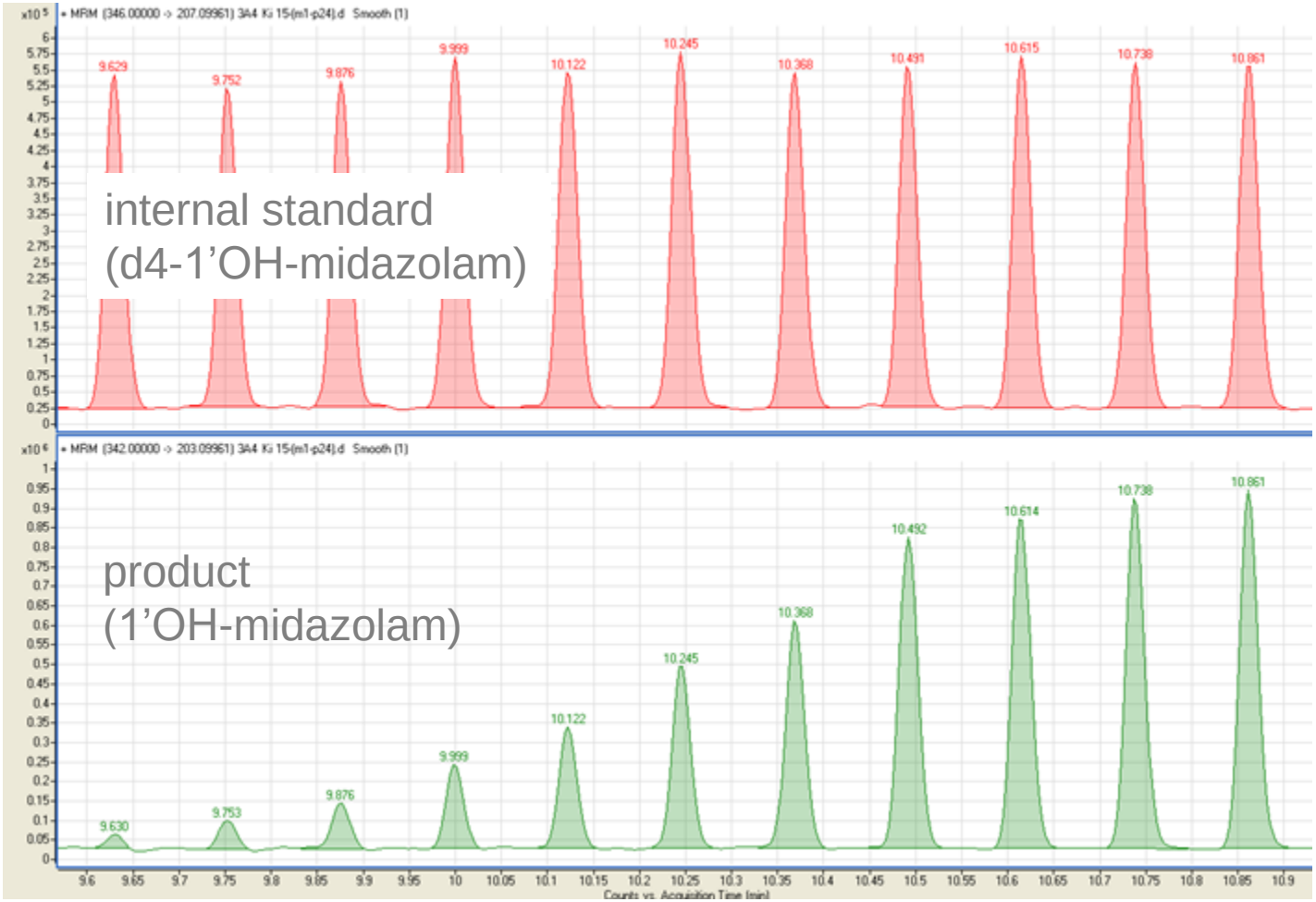
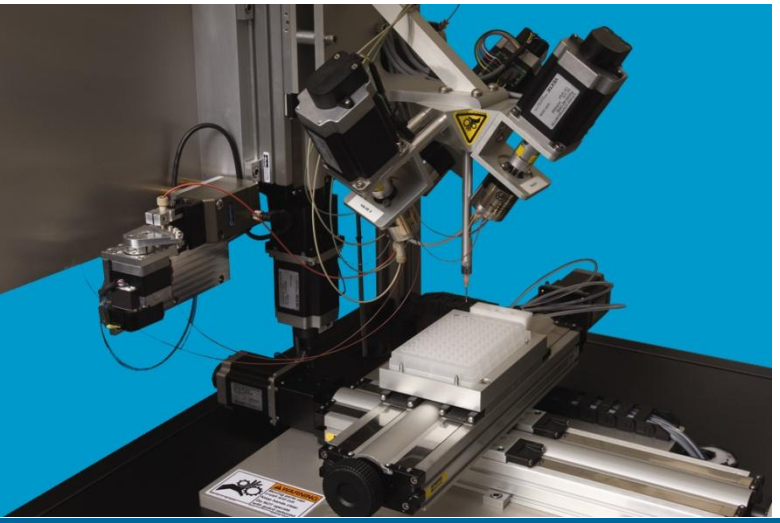
… to RapidFire



RapidFire Mass Spectrometry



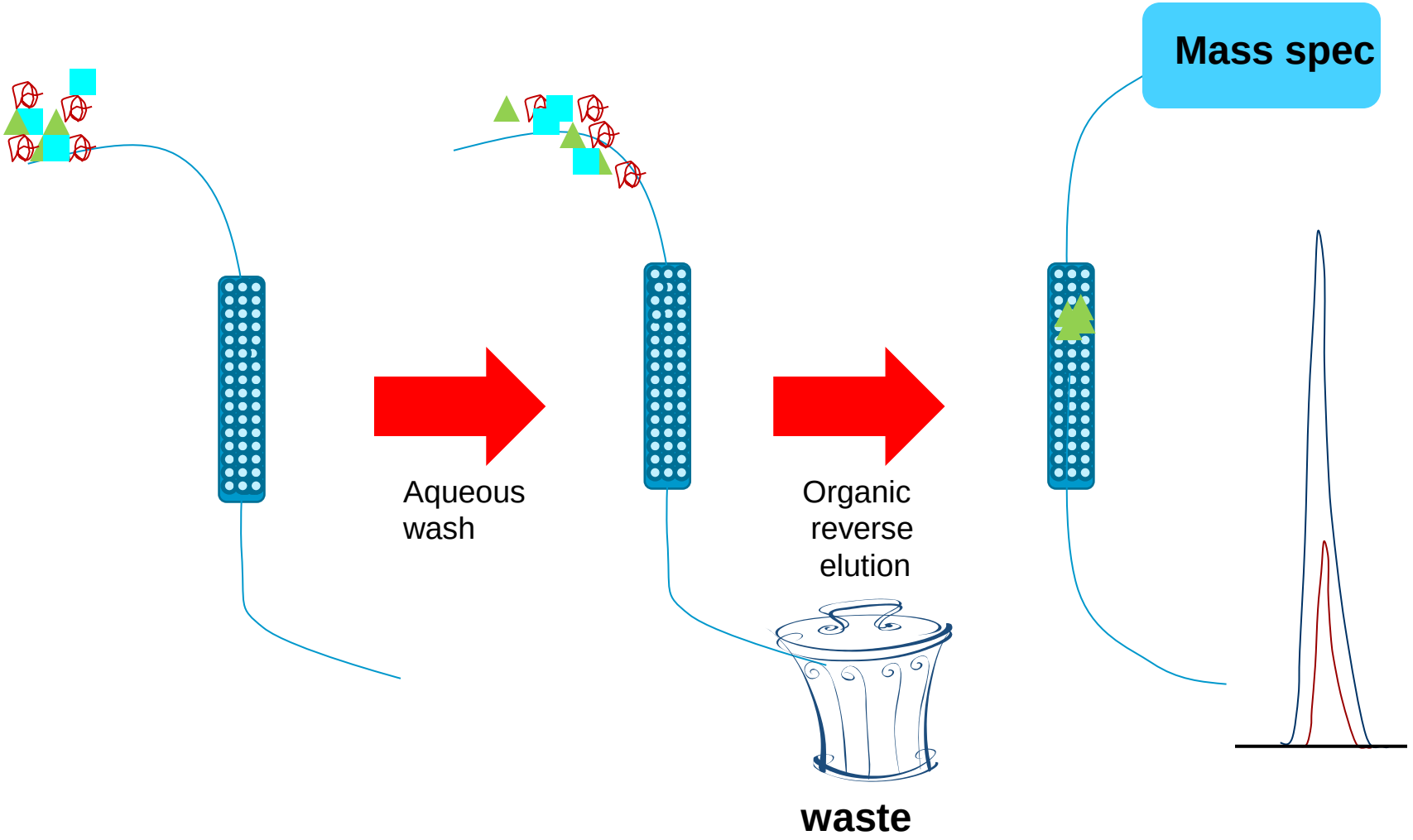
Fast sample purification system

- Integrated, automated, micro-scale solid-phase extraction (μSPE)
- Replaces LC in LC/MS
- cycle time: 6–10s/sample

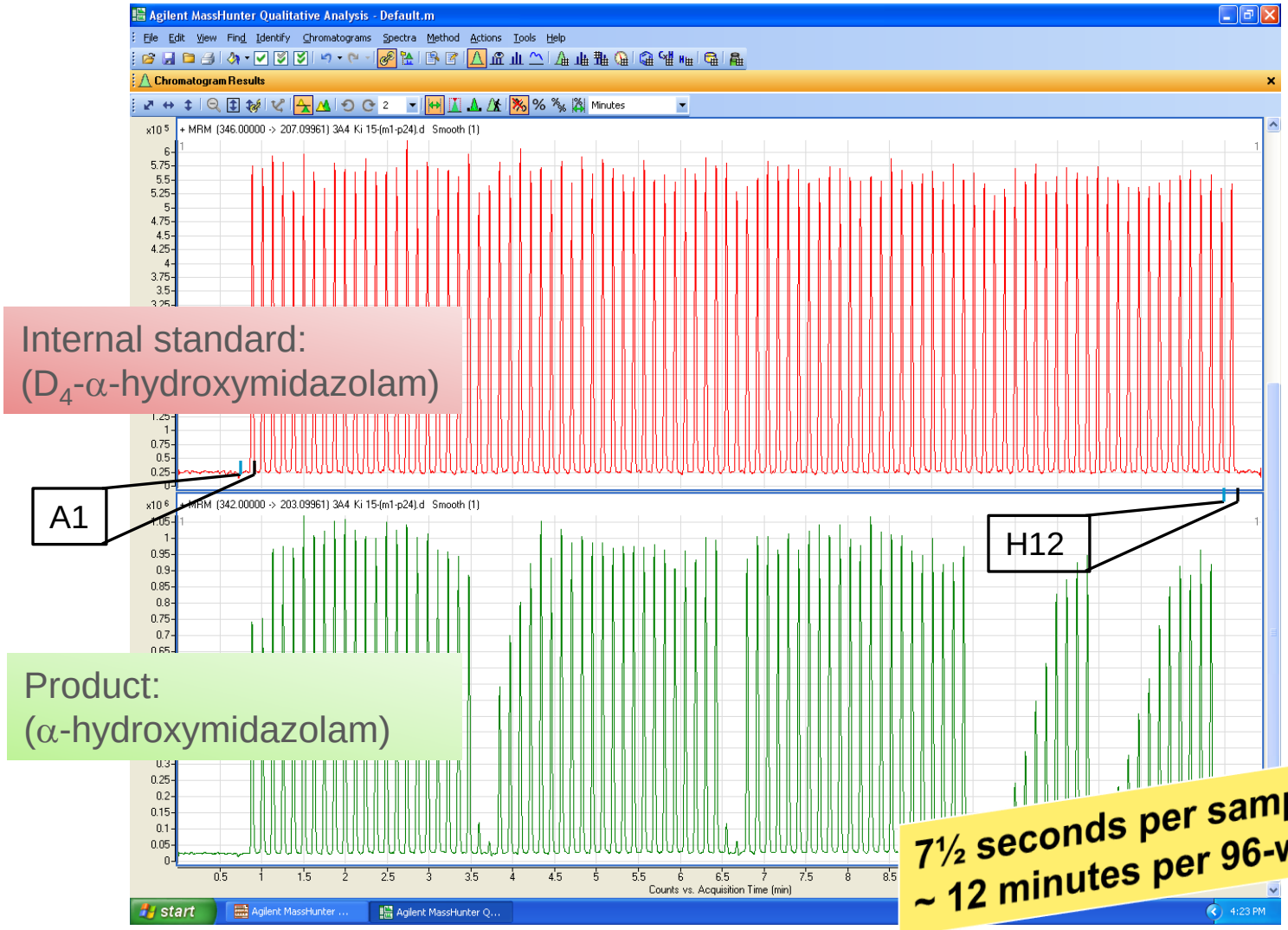
Compatible with many biological matrices

- Microsomal preparation
- Cell culture supernatant
- Tissue extract
- Plasma, whole blood, urine

SPE-MS/MS Analysis



…..To RapidFire



Internal standard:
(D_4-α-hydroxymidazolam)

A1

H12

Product:
(α-hydroxymidazolam)

7½ seconds per sample:
~ 12 minutes per 96-well plate

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Applications of the RapidFire Platform

1) Native Analyte Detection

- surrogate substrates can introduce confounding factors, effect enzyme kinetics, and produce data artifacts

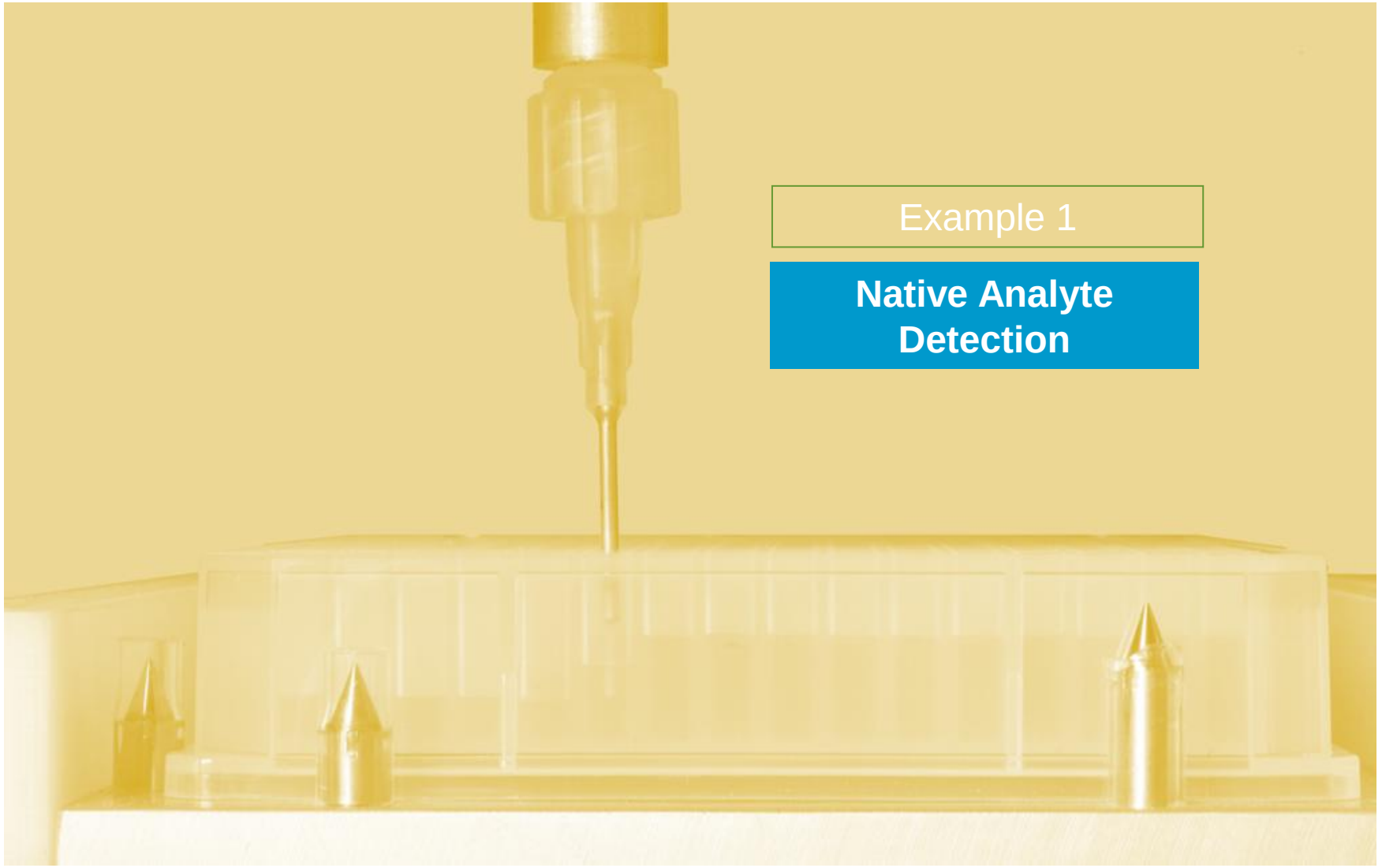
2) Replace Intractable Assays

- assays may present challenges in workflow, may be resource intensive, may be cost prohibitive, may present regulatory issues (radioactivity)

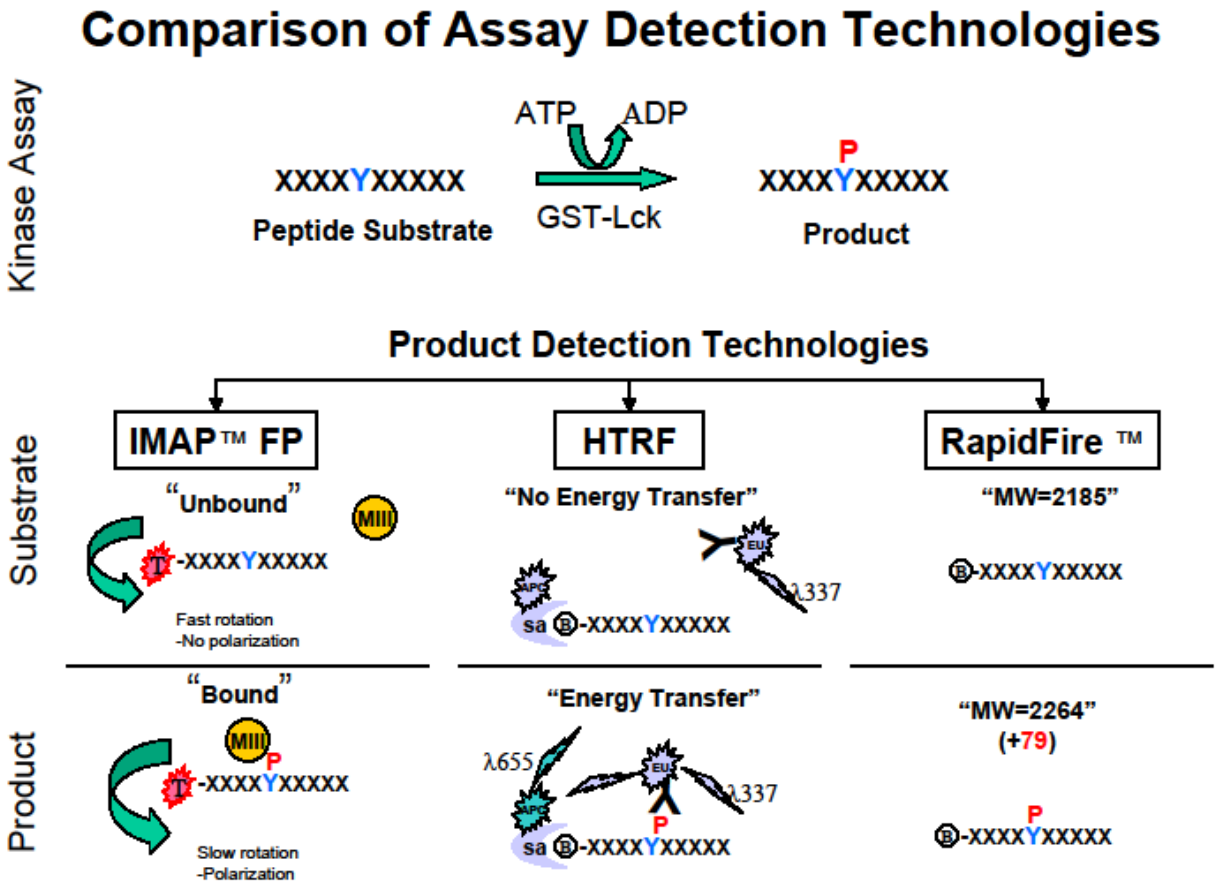
3) Enable Target Classes

- multiple modification events on the same substrate are impossible to track by many common optical and radioactive methodlogies

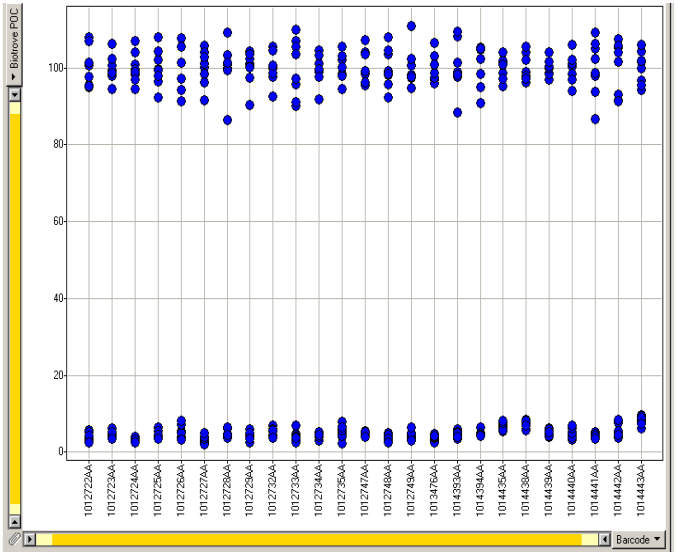
1) Native Analyte Detection



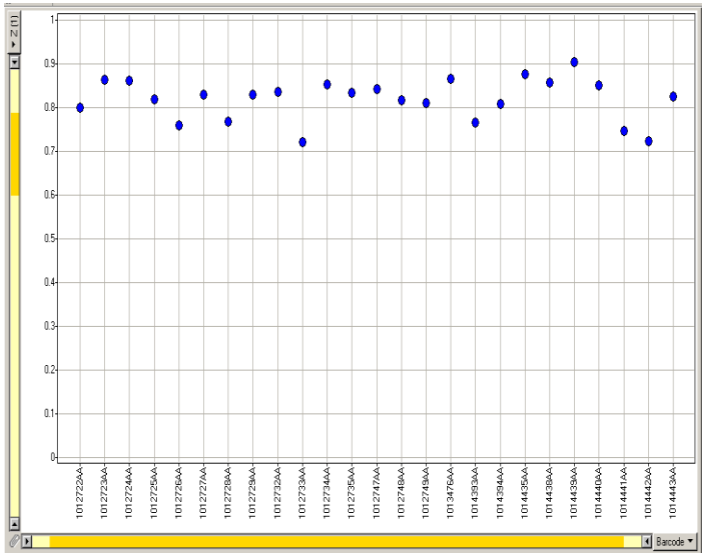
Example 1a: Amgen – Lck Kinase
Introducing Confounding Factors



Control Wells



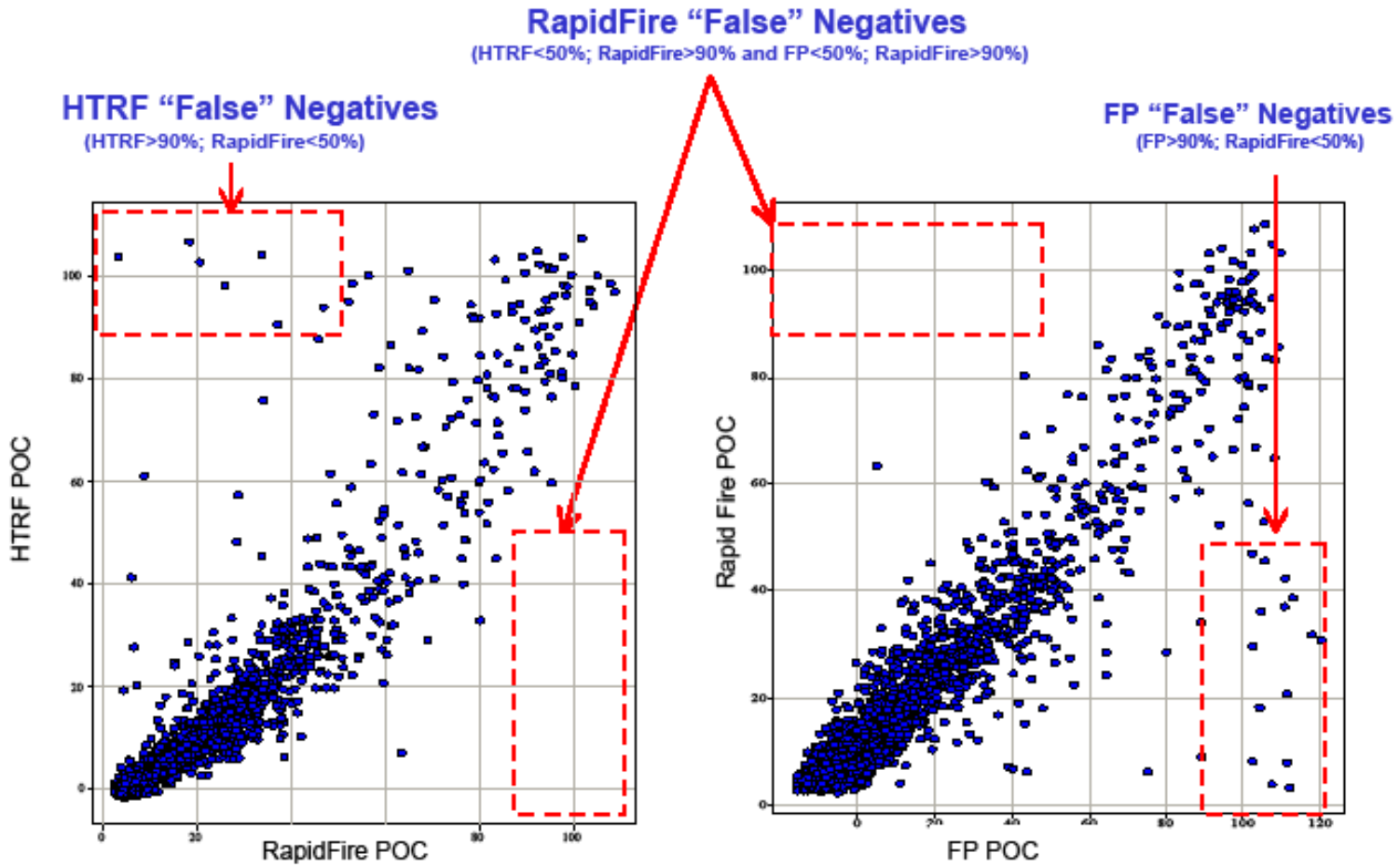
Z Factor



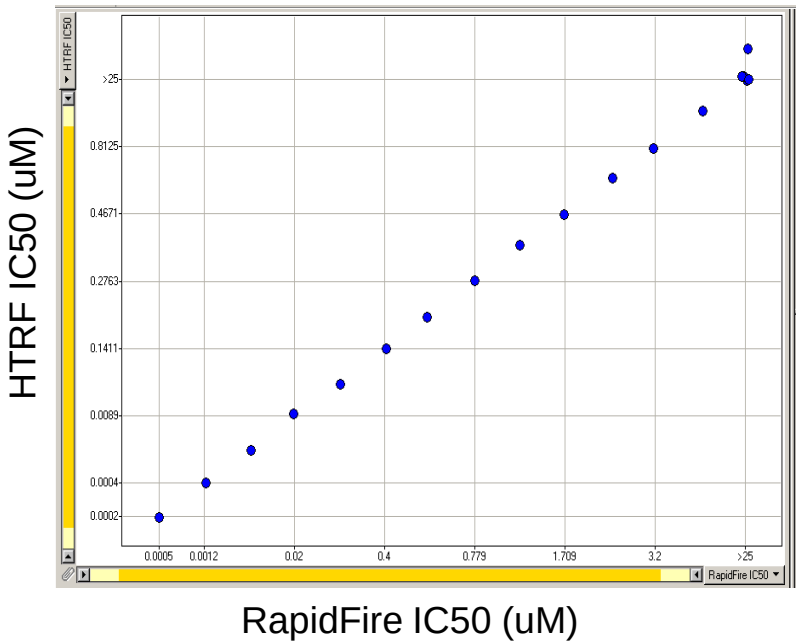
Summary of Statistics

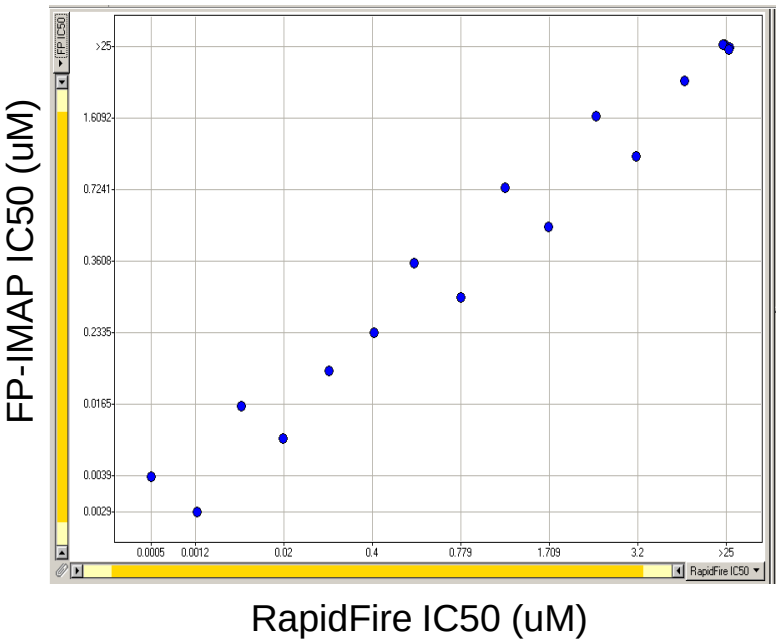
	#compounds	Z factor	Repeat Confirmation Rate	RapidFire™ Confirmation Rate	Signal to bkg
FP	2000	0.75	97.5%	96%	*102 mP*
HTRF	2000	0.85	99.5%	99.00%	10
RapidFire™	2000	0.82	-	-	15

Correlation of Single Point HTS Data:
HTRF, FP-IMAP™ and RapidFire™



IC_{50} SAR Rank Order Correlation Analysis





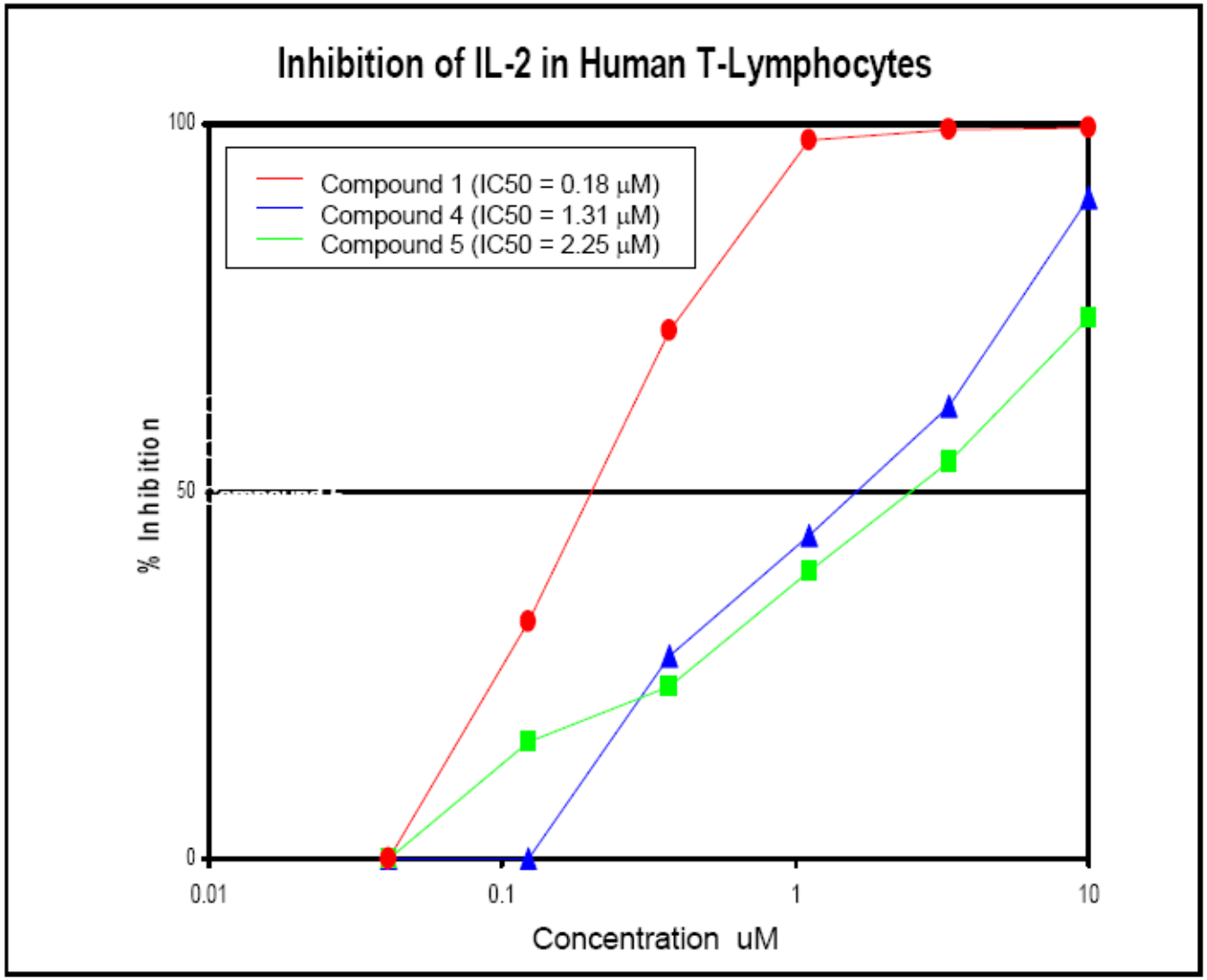
- Rank SAR was preserved when IC50s from RapidFire were compared to either HTRF and FP-IMAP

Summary of HTS POC Data:
Discrepancies Between HTRF, FP-IMAP™ and RapidFire™

Compound	RapidFire	HTRF	FP
1	3	104	-10
2	18	107	104
3	21	103	111
4	26	98	24
5	34	104	42
6	37	91	110
7	47	94	102
8	3	0	112
9	4	1	108
10	8	1	111
11	8	4	103
12	30	15	103
13	31	11	120
14	32	10	118
15	36	21	105
16	39	38	113
17	42	24	111
18	46	88	106

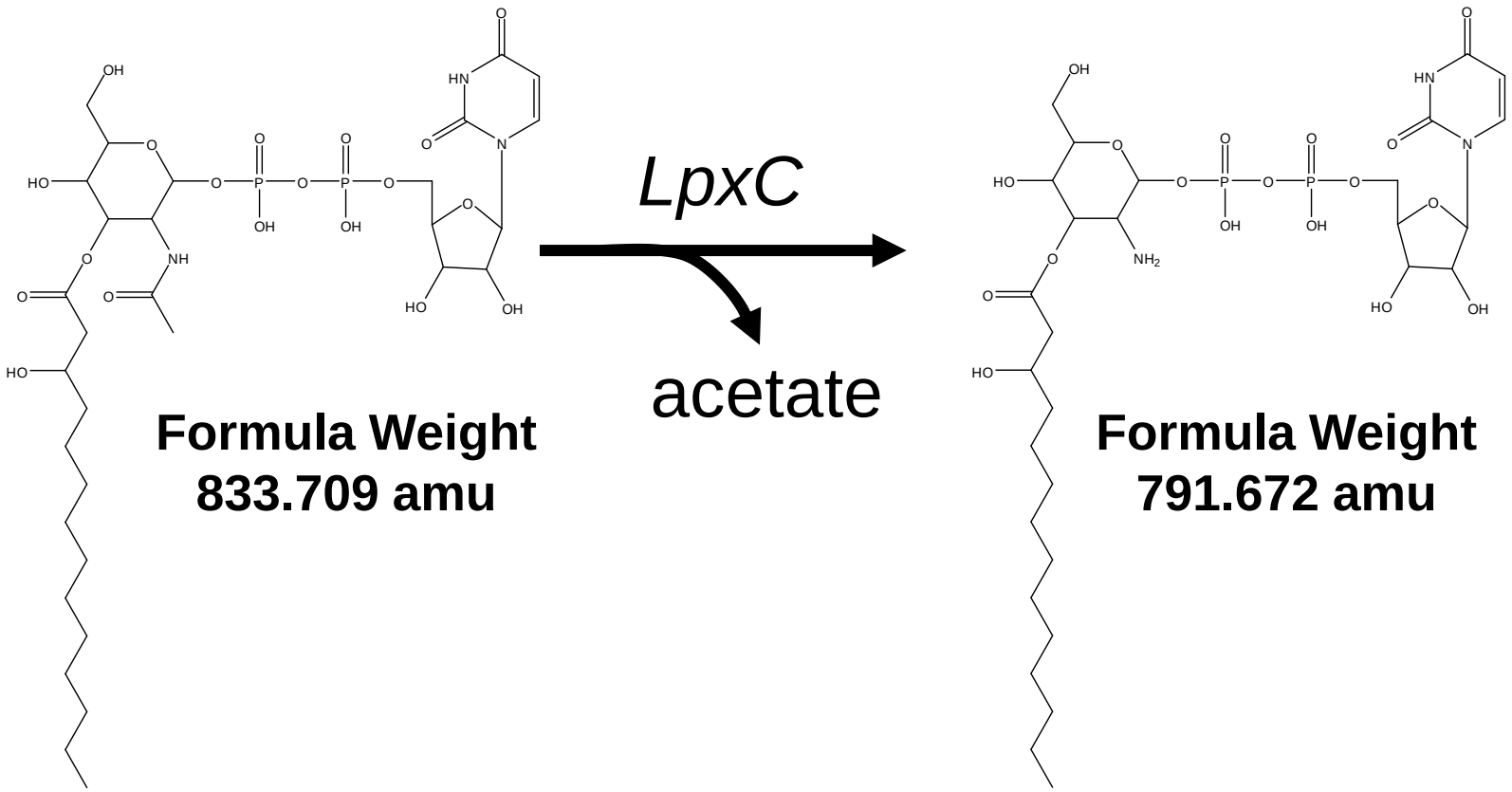
18 Compounds were identified as hits (<50 POC) using RapidFire but were non-hits (>50 POC) for HTRF and/or FP

Cellular Activity of Hits “Uniquely” Identified by RapidFire



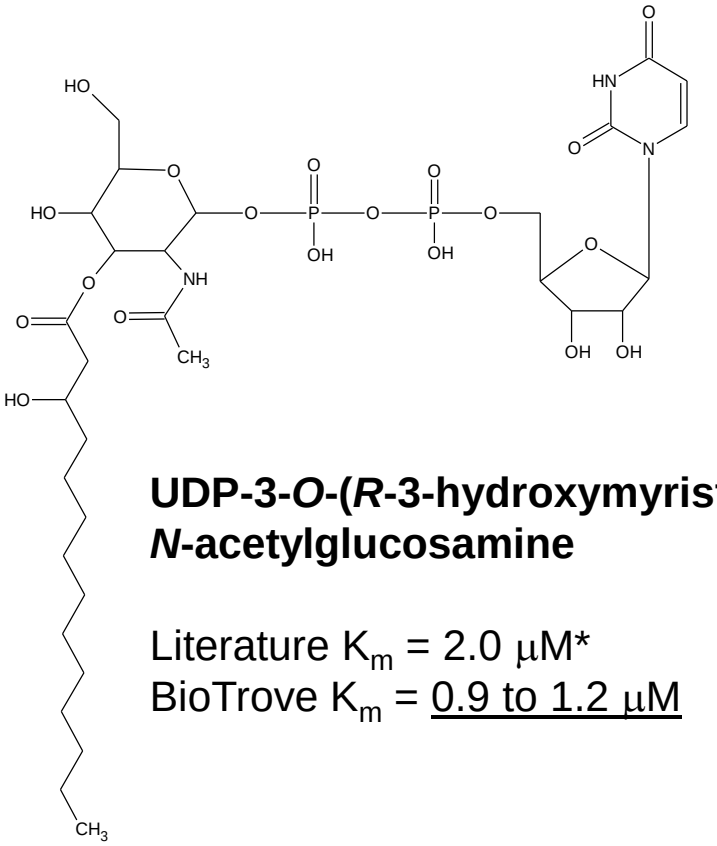
Example 1b: Schering Plough - LpxC Assay

Affecting Enzyme Kinetics



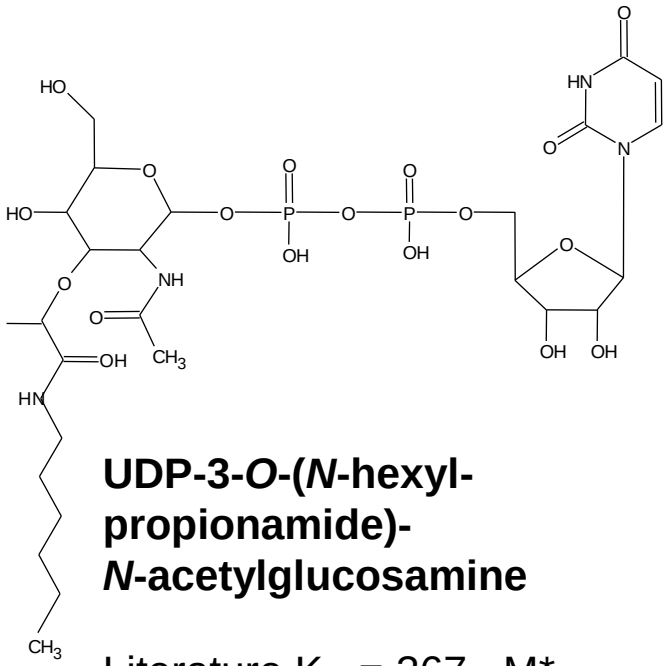
Formula Weight
833.709 amu

Formula Weight
791.672 amu



UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine

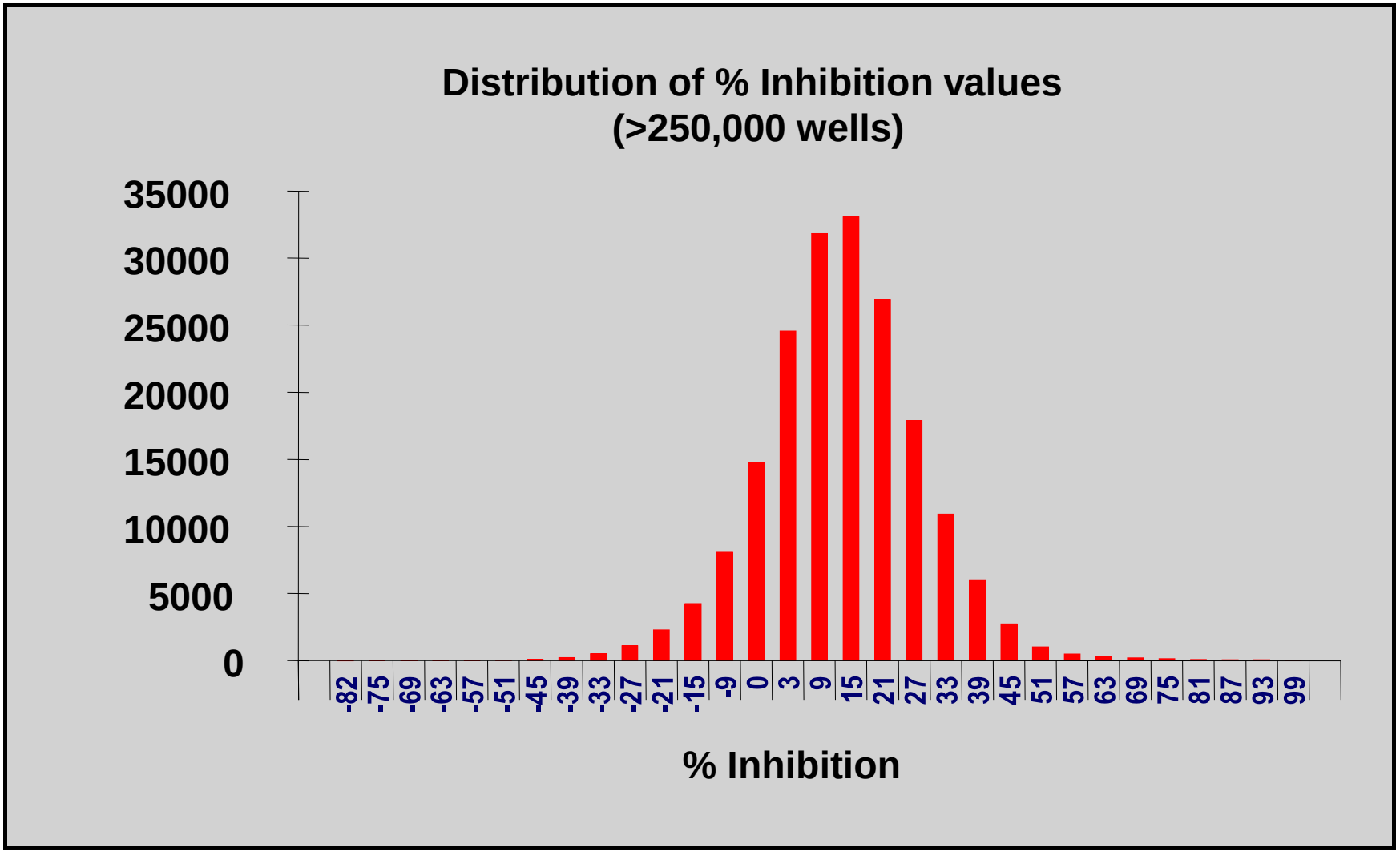
Literature K_m = 2.0 μM*
BioTrove K_m = 0.9 to 1.2 μM

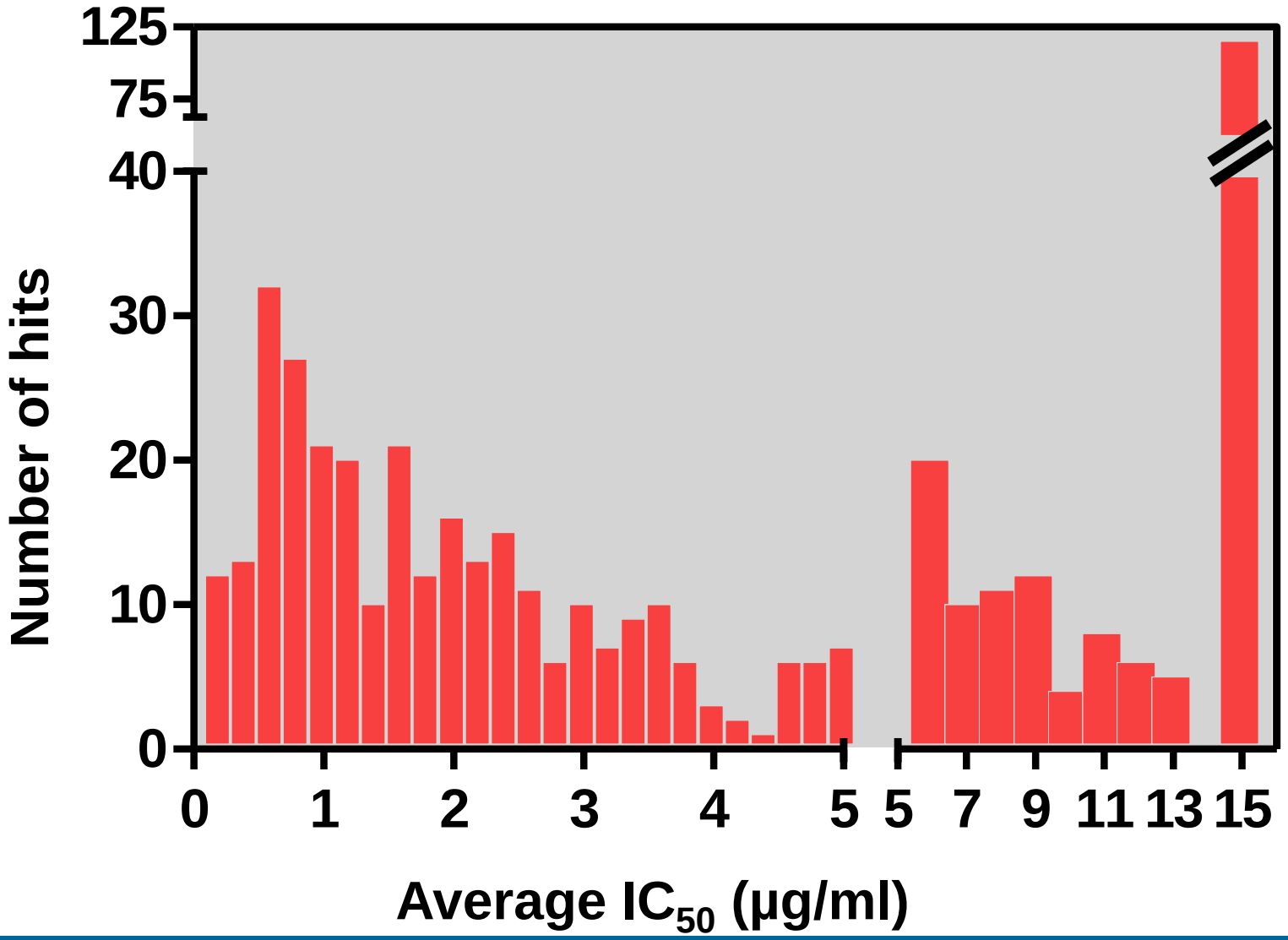


UDP-3-*O*-(*N*-hexyl-propionamide)-*N*-acetylglucosamine

Literature K_m = 367 μM*

* A Fluorescence-Based Homogeneous Assay for Measuring Activity of UDP-3-*O*-(*R*-3-Hydroxymyristoyl)-*N*-acetylglucosamine Deacetylase
Wen Wang, Mita Maniar, Rakesh Jain, Jeff Jacobs, Joaquim Trias, Zhengyu Yuan
Analytical Biochemistry **290**, 338-346 (2001)


Distribution of % Inhibition values
(>250,000 wells)
35000
30000
25000
20000
15000
10000
5000
0
-82
-75
-69
-63
-57
-51
-45
-39
-33
-27
-21
-15
-9
0
3
9
15
21
27
33
39
45
51
57
63
69
75
81
87
93
99
% Inhibition


125
75
40
30
20
10
0
Number of hits
0
1
2
3
4
5
5
7
9
11
13
15
Average IC50 (µg/ml)

Three patent applications have been filed by SPRI

1. **20070167426**: Compounds for the treatment of inflammatory disorders and microbial diseases
2. **20070129378**: Compounds for the treatment of inflammatory disorders and microbial diseases
3. **20060178366**: Compounds for the treatment of inflammatory disorders

The concentrations of substrate and product in the reaction mixtures are determined with proprietary RapidFire®high-throughput mass spectrometry (HTMS). Assay mixtures are partially purified with reverse phase chromatography, where they are washed with water containing 5 mM ammonium formate and eluted onto the mass spectrometer in 80% acetonitrile, 20% water, and 5 mM ammonium formate. The mass spectrometry peak areas of the substrate and product are measured to determine the concentration of these analytes. The assay signal is the percentage of substrate that is converted to product. Percent inhibition, %I, in test samples is determined from the following equation: %I = 100 X (TSB - SampleSignal) (TSB).

Example 1c: Sirtris – SIRT1 Assay

Introducing Data Artifacts

FORTUNE

Can red wine help you live forever?

Turns out there's something to it. Fortune's David Stipp recounts the amazing, real story of the scientist and startup that have a shot at making it happen.

FORTUNE Magazine
By David Stipp, Fortune
January 19 2007

NEW YORK (Fortune) -- If you haven't heard of resveratrol, you're probably too young to have had the experience of gazing in the bathroom mirror in the morning and thinking, "damn."

Resveratrol is the ingredient in red wine that made headlines in November when scientists demonstrated that it kept overfed mice from gaining weight, turned them into the equivalent of Olympic marathoners, and seemed to slow down their aging process. Few medical discoveries have generated so much instant buzz - even Jay Leno riffed about it in his opening monologue.

sirtris™
PHARMACEUTICALS

News Release

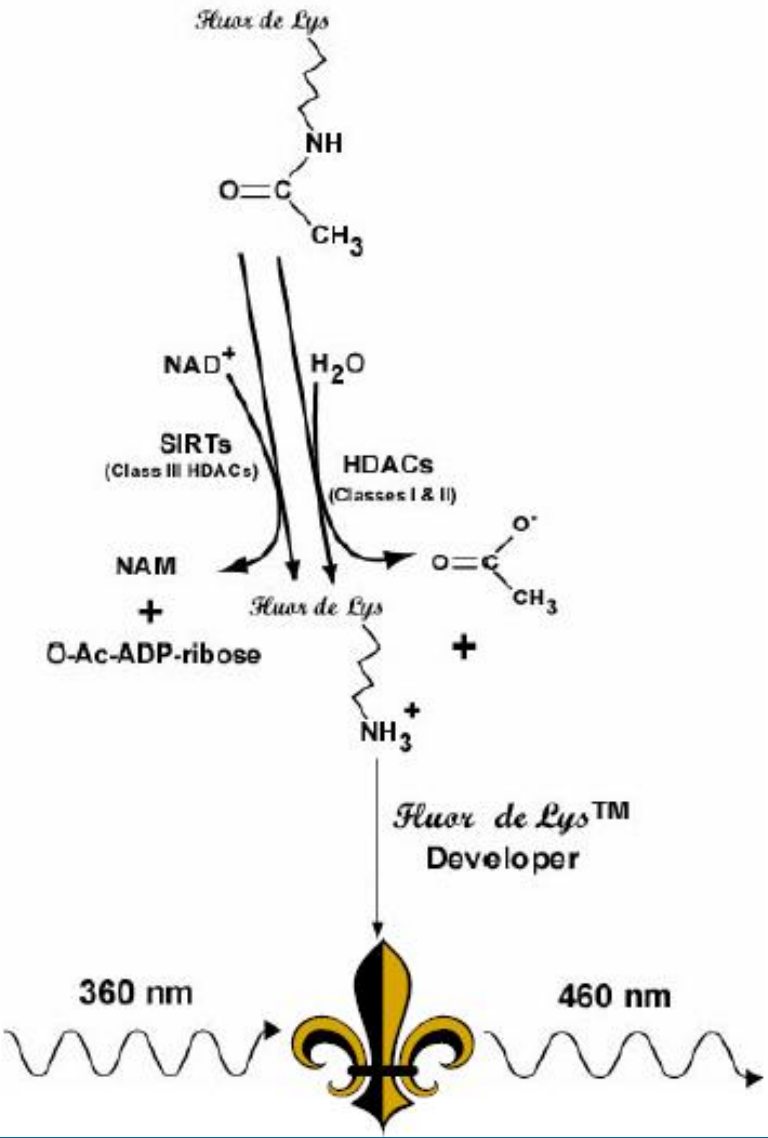
GlaxoSmithKline to acquire Sirtris Pharmaceuticals, a world leader in 'Sirtuin' research and development

Issued – Tuesday 22 April 2008, London, UK, Philadelphia PA, Cambridge, MA – GlaxoSmithKline (NYSE: GSK) and Sirtris Pharmaceuticals Incorporated (Nasdaq: SIRT) announced today that they have entered into a definitive agreement pursuant to which GlaxoSmithKline will acquire Sirtris Pharmaceuticals for approximately USD720 million (or approx. GBP362 million) through a cash tender offer of USD22.50 (or approx. GBP11.33) per share.

The Resveratrol Saga

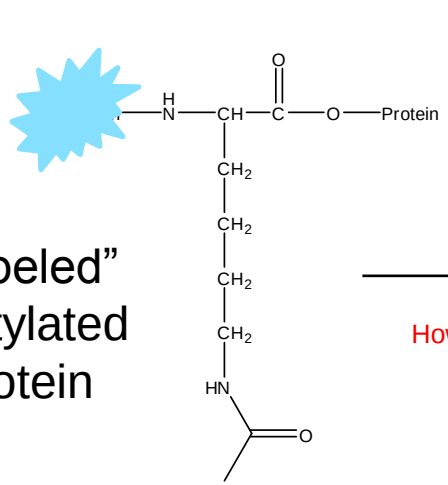
"Pfizer scientists have thrown down the gauntlet…claim that the reported Sirtris compounds do not do what they are claimed to do… suggest that Sirtris' earlier findings are due to an experimental artifact…almost certainly the case that there are problems with the Sirtris compounds"

Fluor de Lys Assay From Enzo



Labeled vs. Un-labeled Sirtuin Assay

Free fluorophore

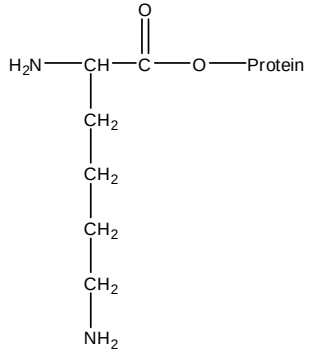


"Labeled" Acetylated Protein

SIRT1/Chymotrypsin

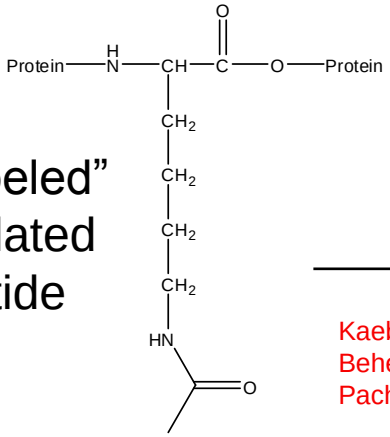
Resveratrol

Howitz *et. al*., *Nature* (**2003**) 425:191-196



Reaction Activation

De-Acetylated Peptide

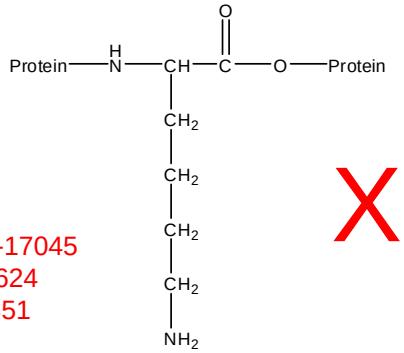


"Unlabeled" Acetylated Peptide

SIRT1/Chymotrypsin

Resveratrol

Kaeberlein *et. al*., *J. Biol. Chem.* (**2005**) 280:17038-17045
Beher *et. al*., *Chem. Biol. Rug Des.* (**2009**) 74:619-624
Pacholec *et. al*., *J. Biol. Chem.* (**2010**) 285:8340-8351



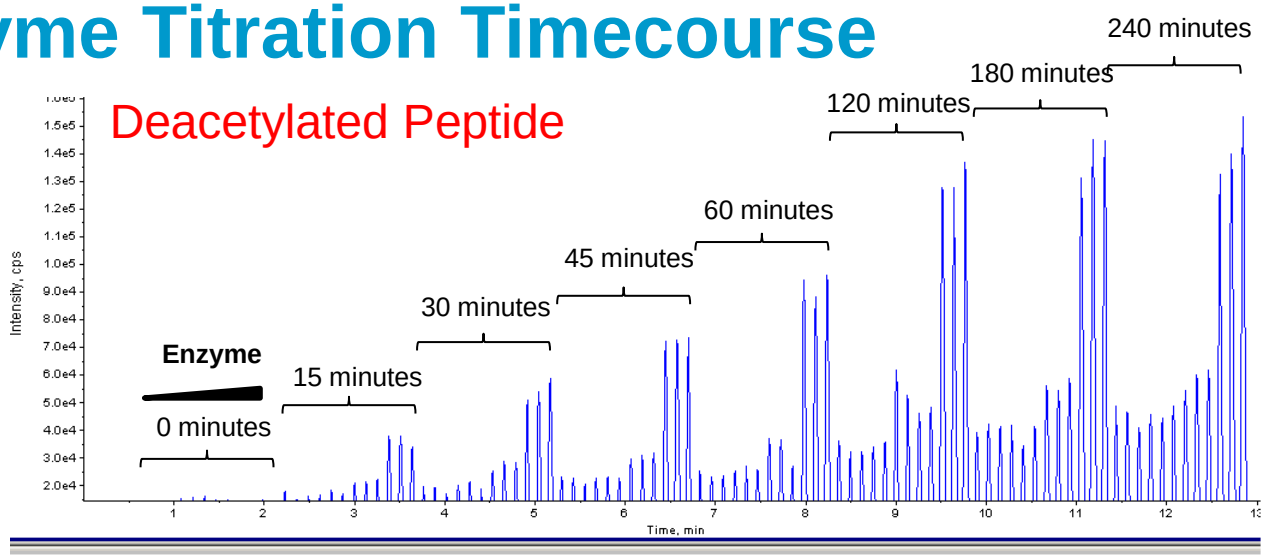
X No Reaction Activation

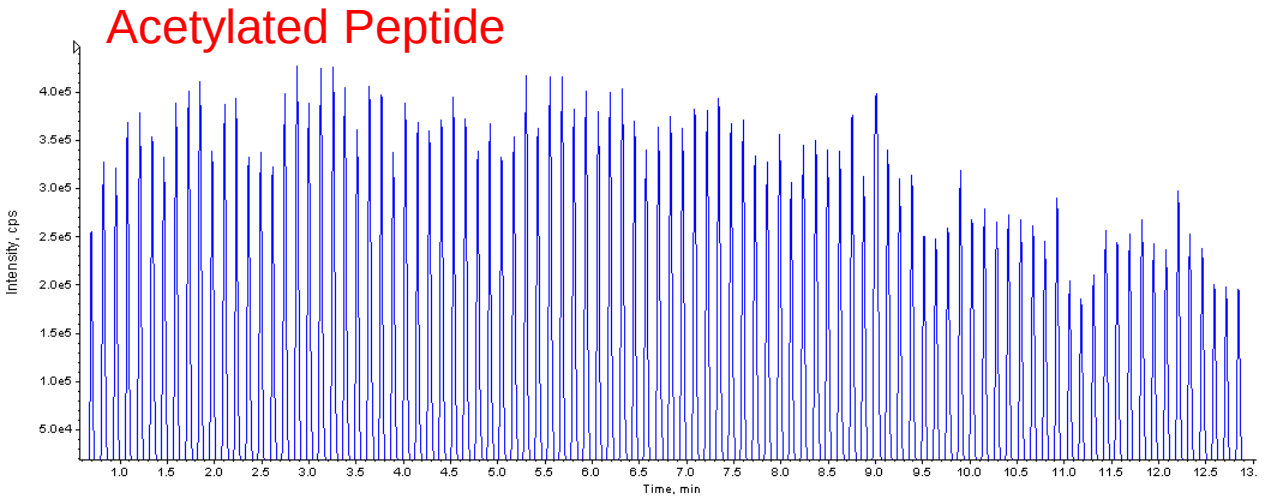
De-Acetylated Peptide

SIRT1 - Enzyme Titration Timecourse

Reaction Conditions:

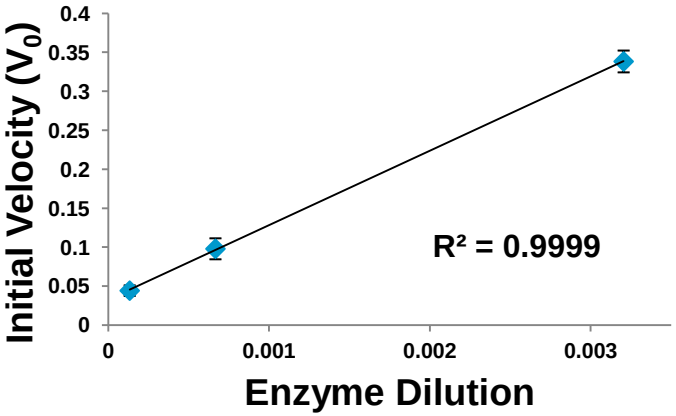
50 mM Tris pH 7.5
137 mM NaCl
2.7 mM KCl
1 mM $MgCl_2$
0.05% BSA
5 mM DTT
100 μM NAD^+
10 μM p53 peptide
(Anaspec cat # 62121)

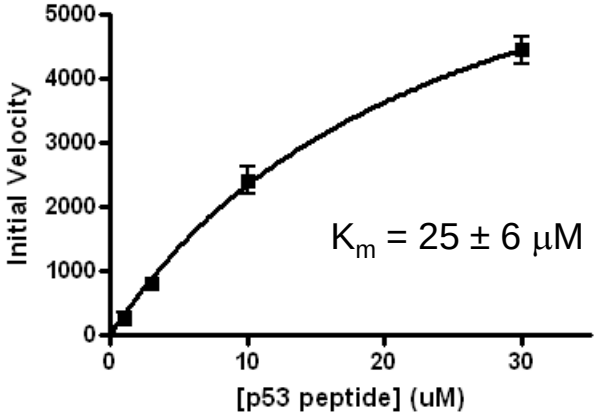


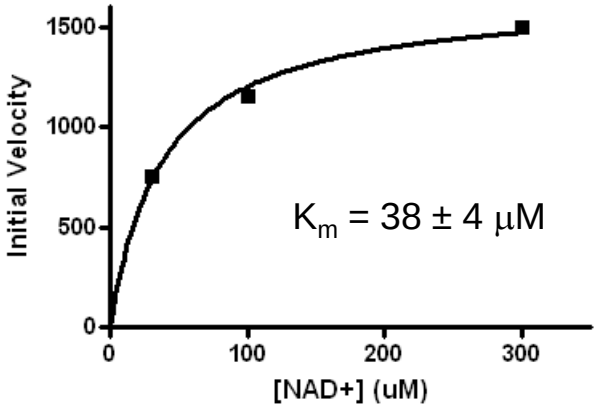


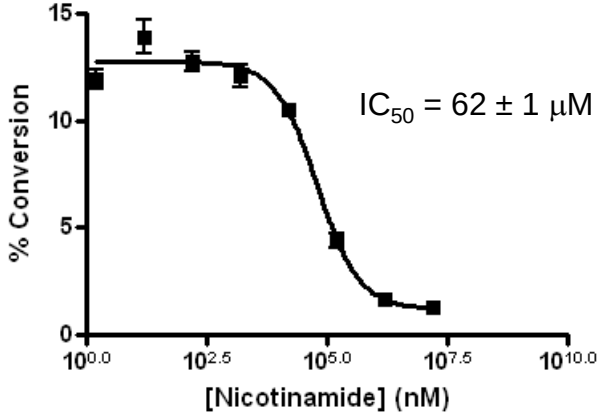
~12.5 minute analysis time

SIRT1 - Enzymatic Parameters (K_m, IC_{50}, etc…)









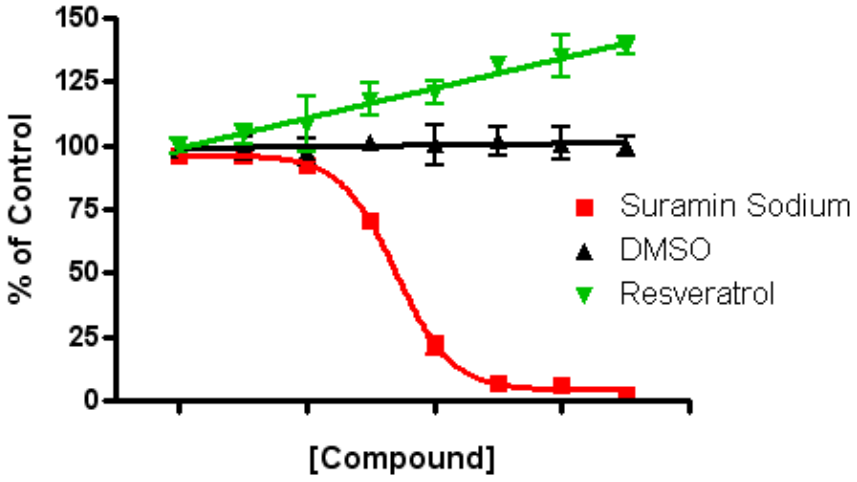
Literature IC_{50} value ~ 50 µM

Bitterman *et. al*., *J. Biol. Chem*. (**2002**) 277: 45099-45107

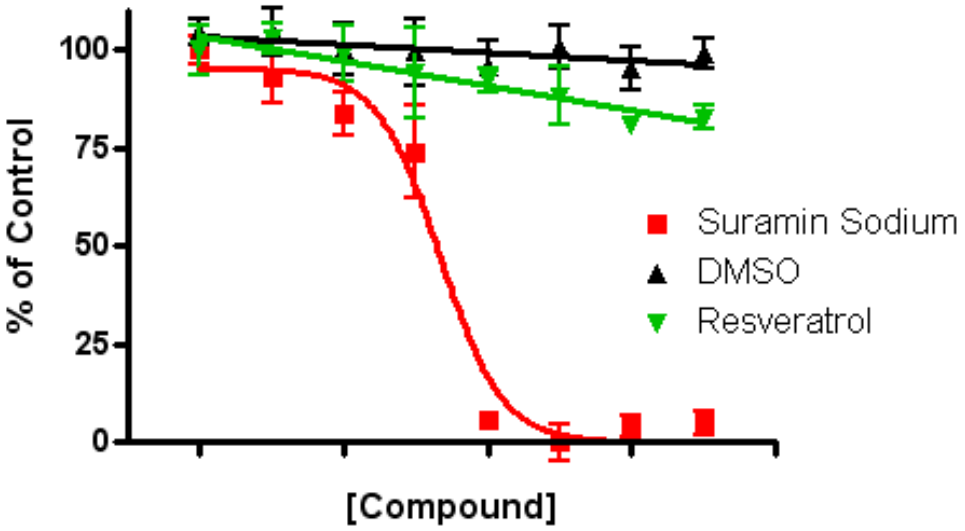
Marcotte *et. al*., *Anal. Biochem.* (**2005**) 332:90-99

SIRT1 - Substrate Dependant Activation by Resveratrol

"Labeled" Peptide

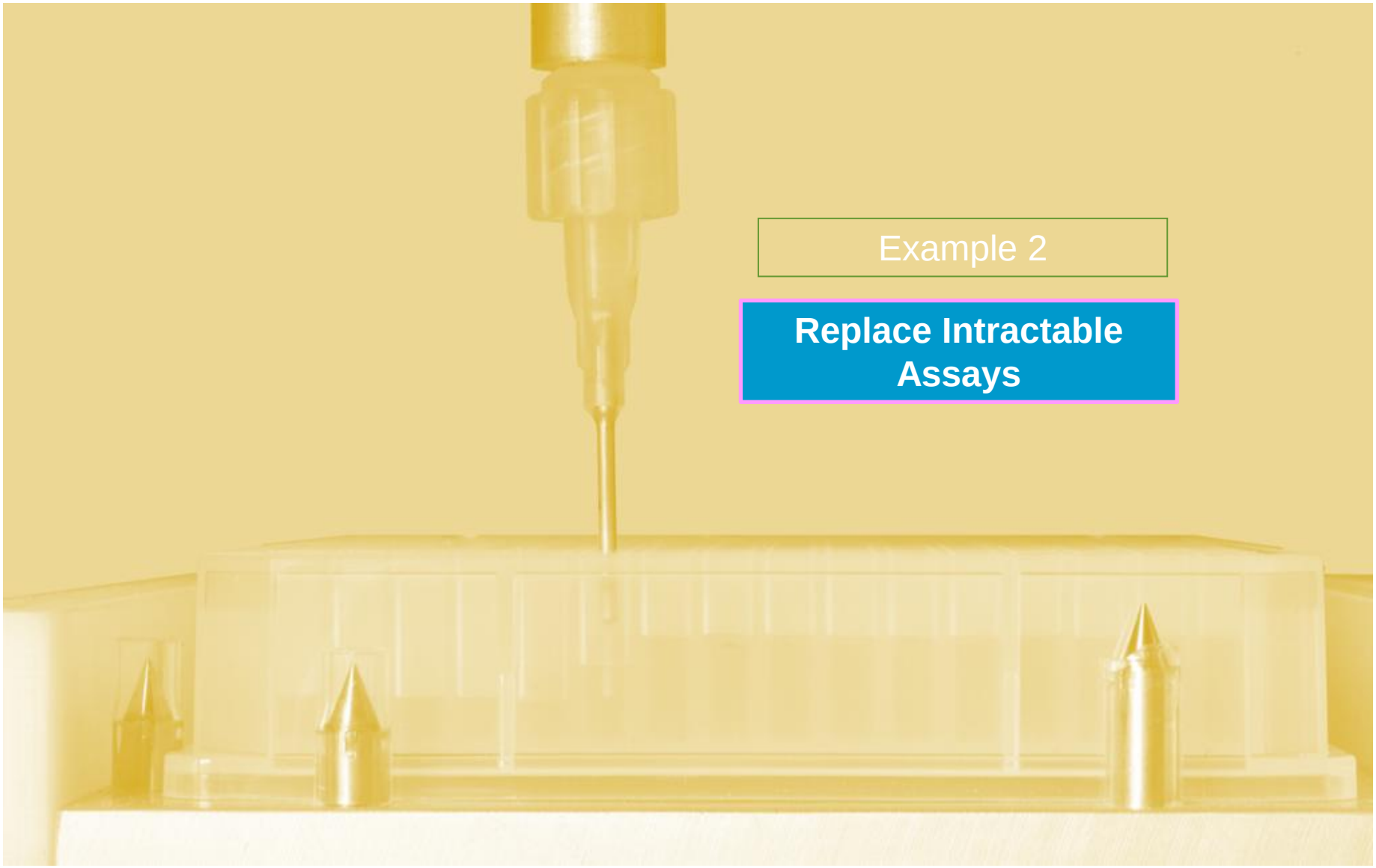


"Unlabeled" Peptide

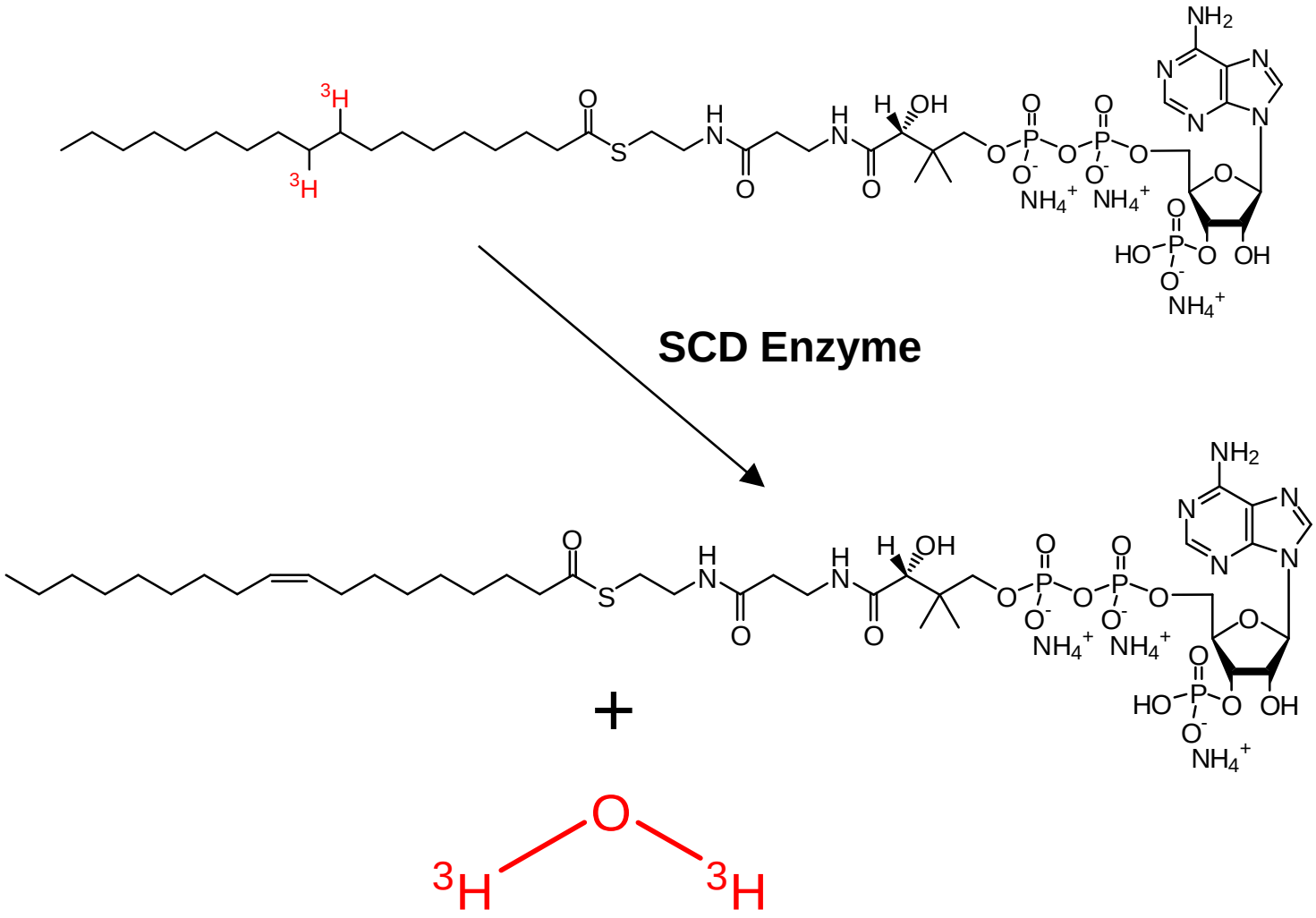


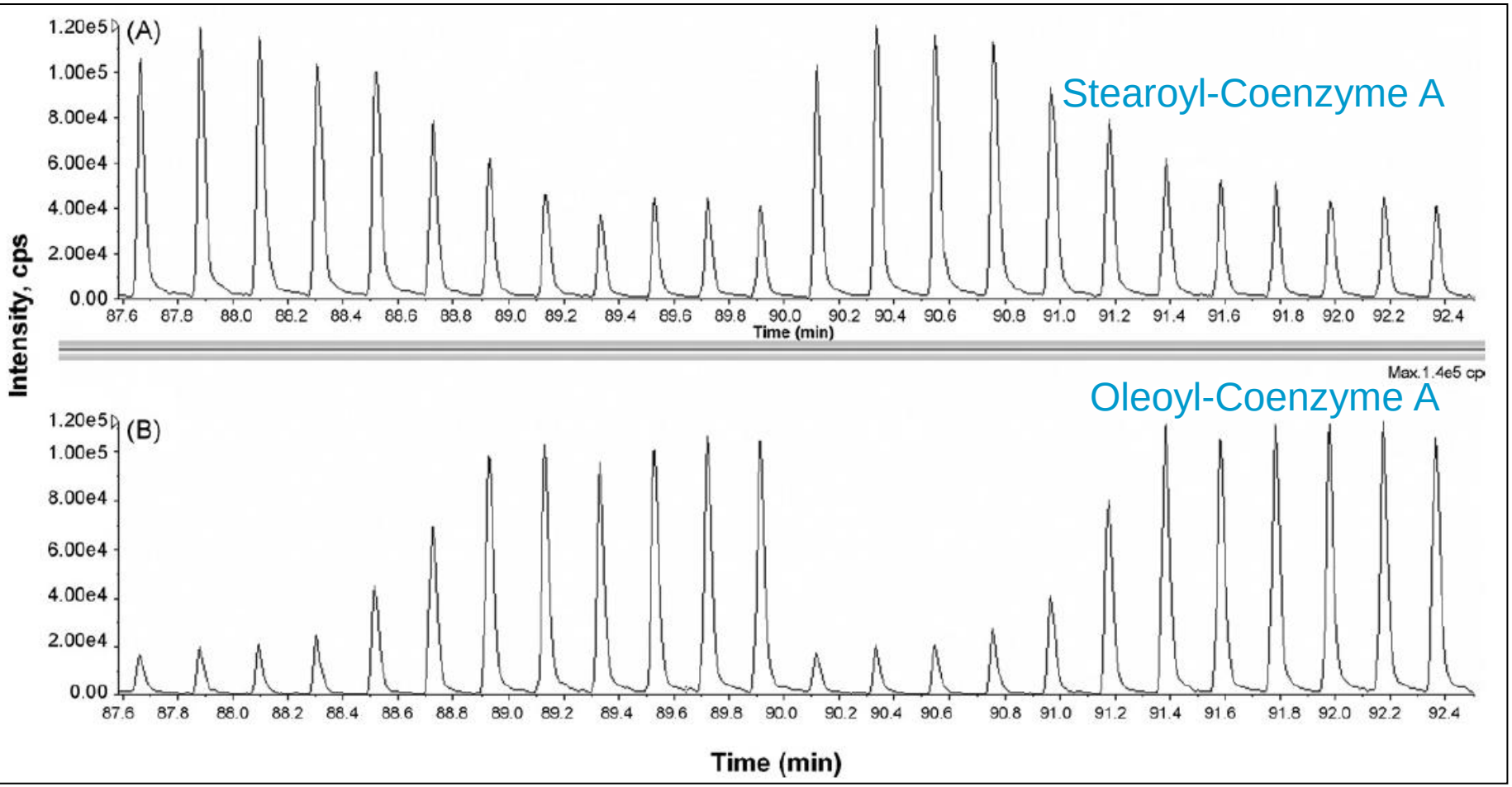
Milne *et. al*., *Nature* (**2007**) 450:712-716

2) Replace Intractable Assays



Example 2a: Pfizer – Stearoyl-Coenzyme A Desaturase




(A)
Stearoyl-Coenzyme A
(B)
Oleoyl-Coenzyme A
Intensity, cps
Time (min)
Max. 1.4e5 cps
1.20e5
1.00e5
8.00e4
6.00e4
4.00e4
2.00e4
0.00
87.6 87.8 88.0 88.2 88.4 88.6 88.8 89.0 89.2 89.4 89.6 89.8 90.0 90.2 90.4 90.6 90.8 91.0 91.2 91.4 91.6 91.8 92.0 92.2 92.4

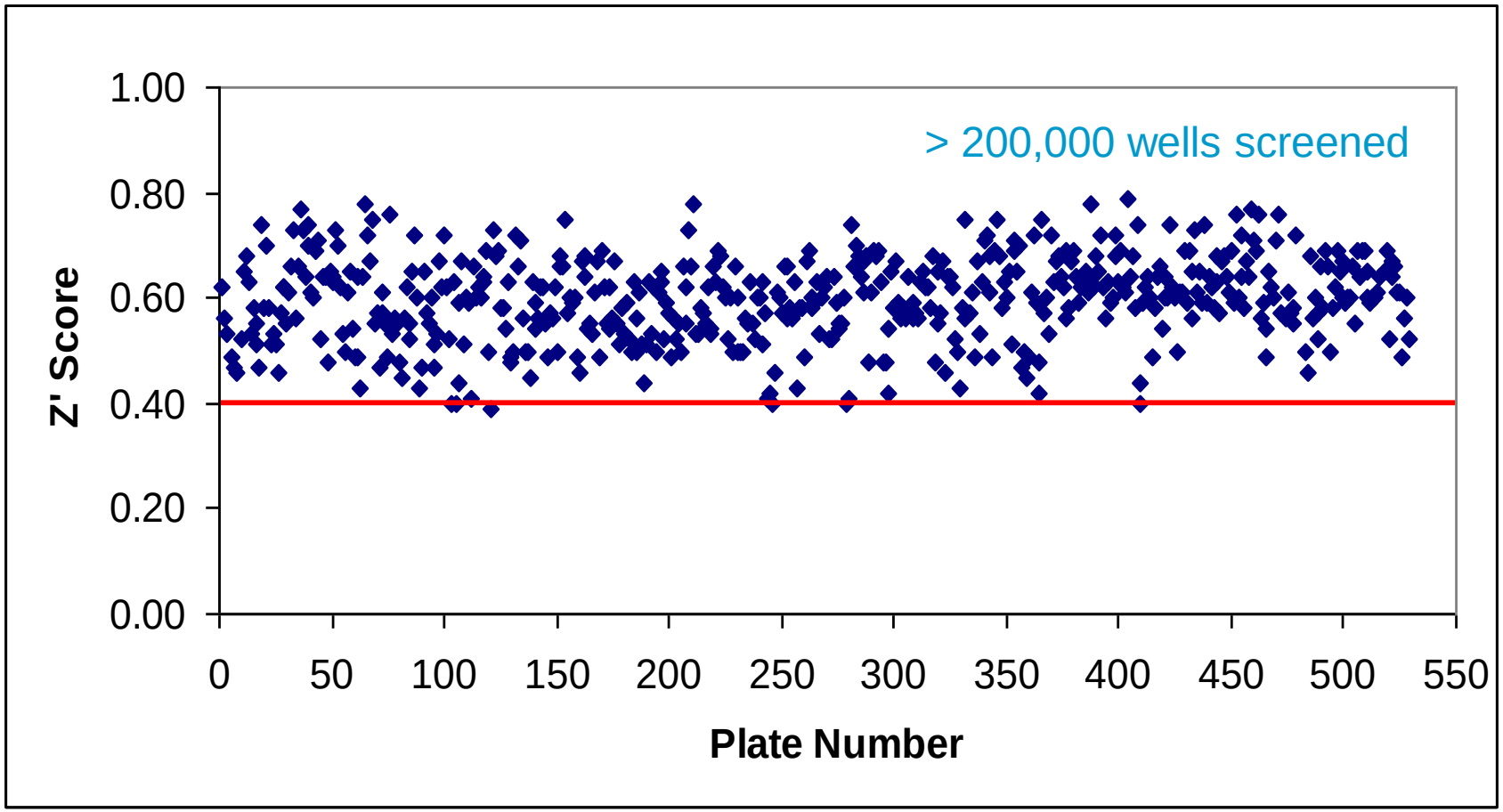
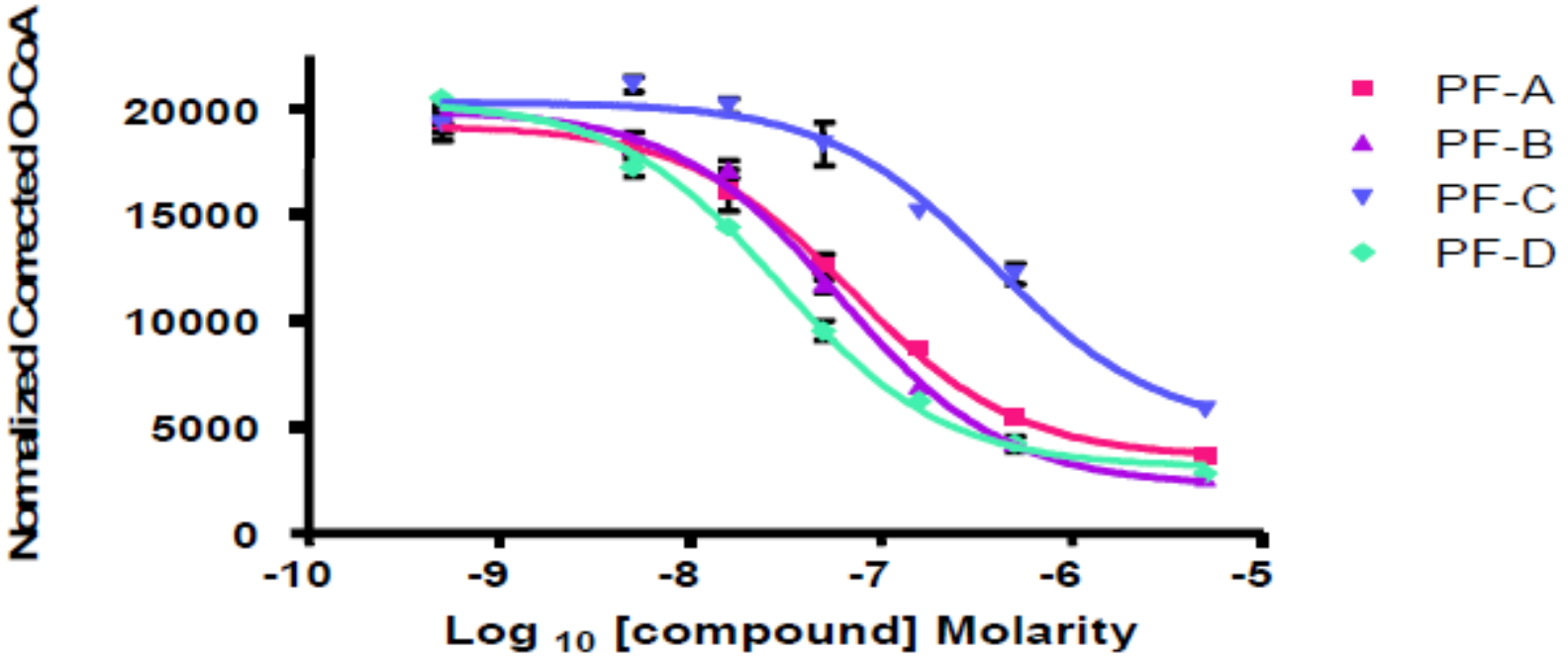


Fig. 5 – SCD1 assay quality as determined by Z′ values. Total number of 384-well plates analyzed was 518. Average Z′ score was 0.597 with median Z′ score of 0.60.

SCD HTMS HIT TO LEAD

IC50 Analysis on Historical Compounds



	EC50
PF-A	7.030e-008
PF-B	6.080e-008
PF-C	3.884e-007
PF-D	2.964e-008

Example 2b: Amgen – 2-Oxoglutarate Oxygenase Enzymes

Protein Hydroxylases

- i.e. Factor inhibiting HIF-1α (FIH)

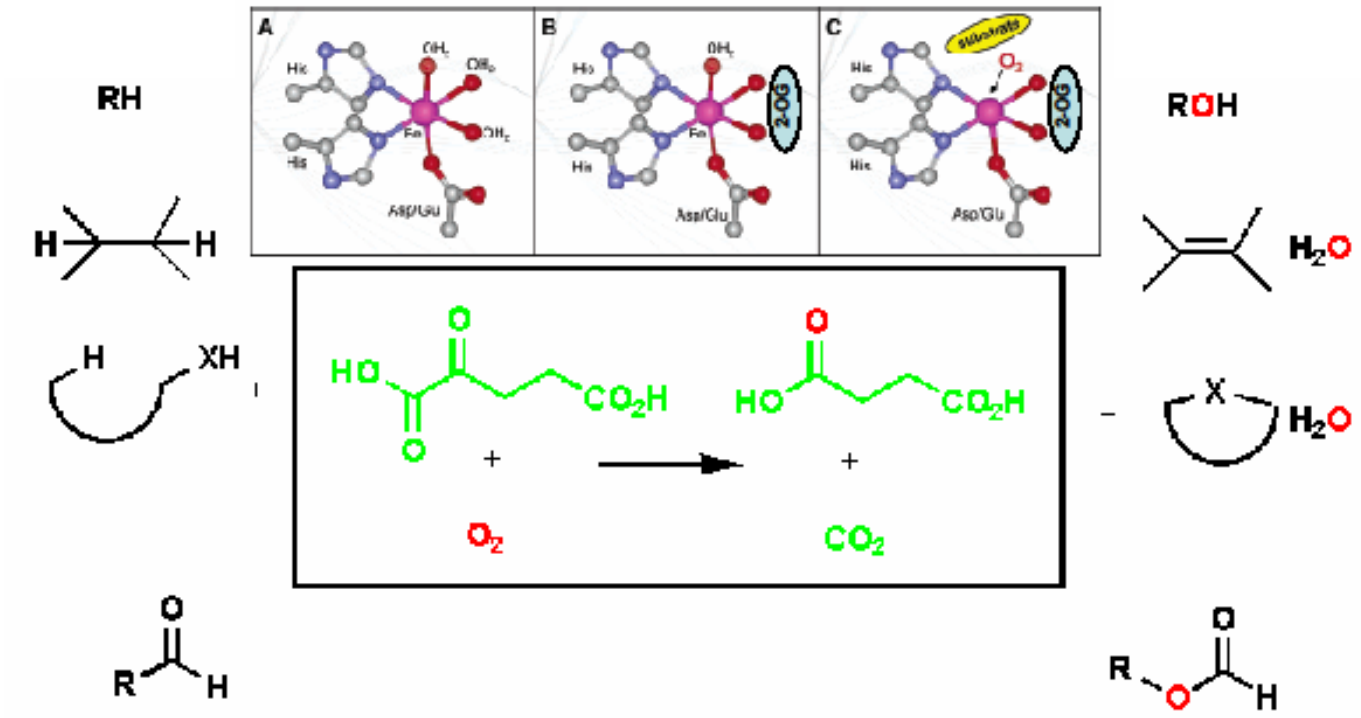
Small Molecule Hydroxylases

- i.e. Phytanoyl-CoA hydroxylase

DNA Demethylases

- i.e. AlkB

2OG Utilizing Enzymes Catalyse many Reactions

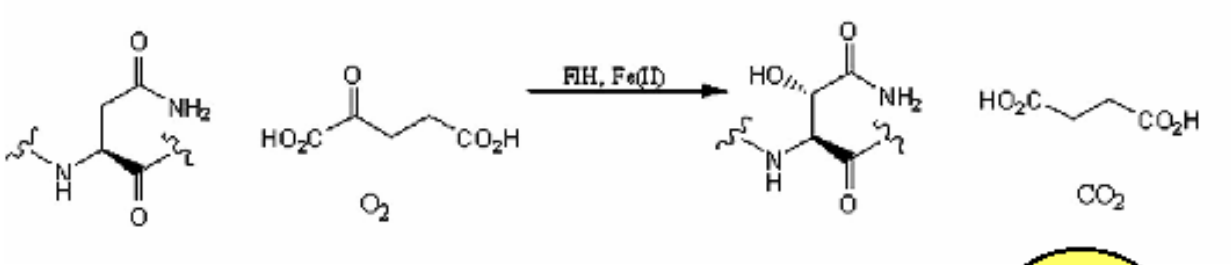


Classically, these enzymes have been investigated using O_2 consumption or CO_2 liberation assays, however the mass change of the product makes them amenable to MS analysis

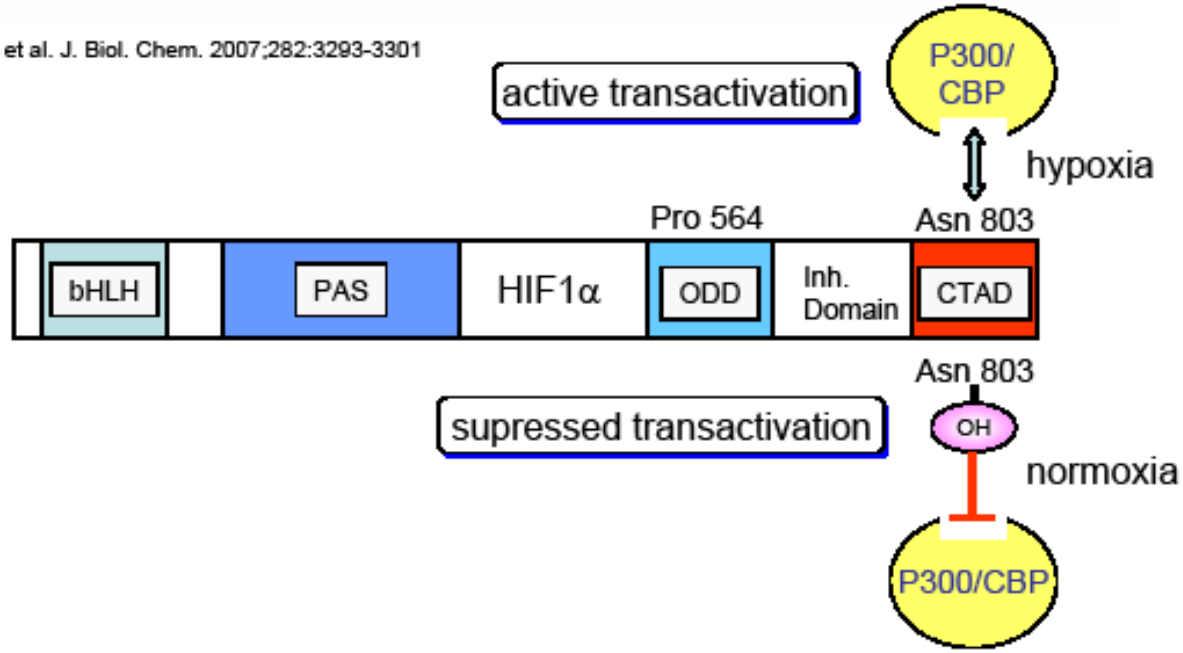
Miquel Costas,† Mark P. Mehn,‡ Michael P. Jensen,‡ and Lawrence Que, Jr.*,‡

Chem. Rev. 2004, 104, 939–986

Factor Inhibiting HIF (FIH) is a 2-OG Oxygenase

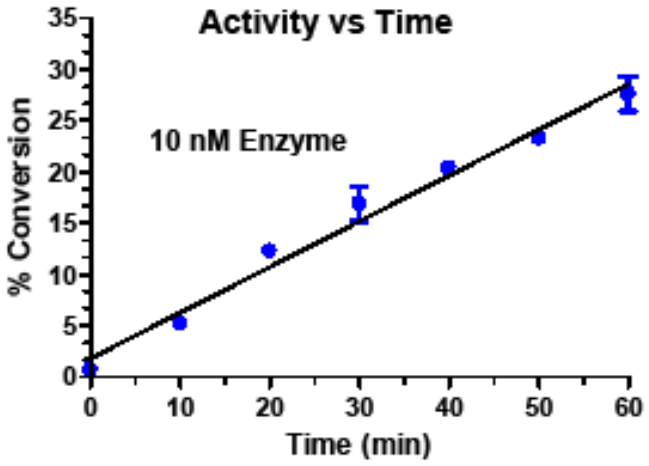


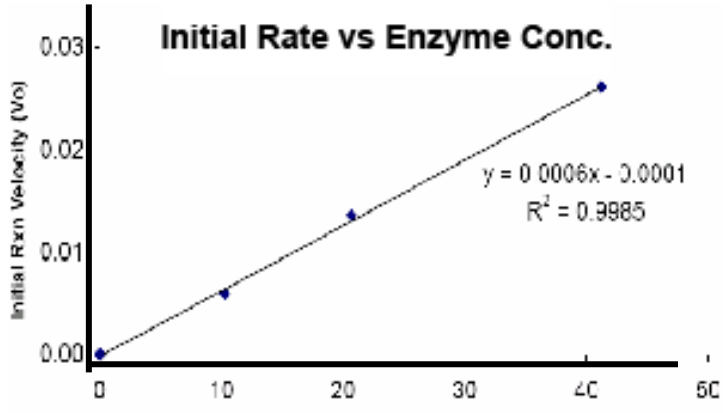
Hewitson, K. S. et al. J. Biol. Chem. 2007;282:3293-3301

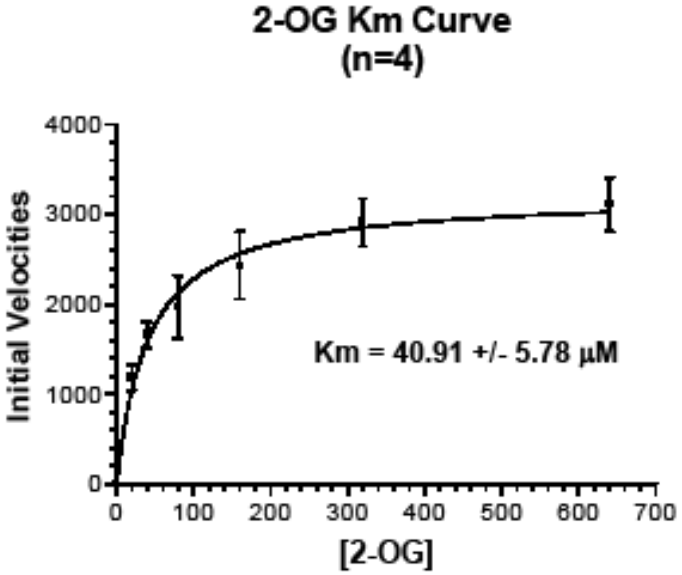


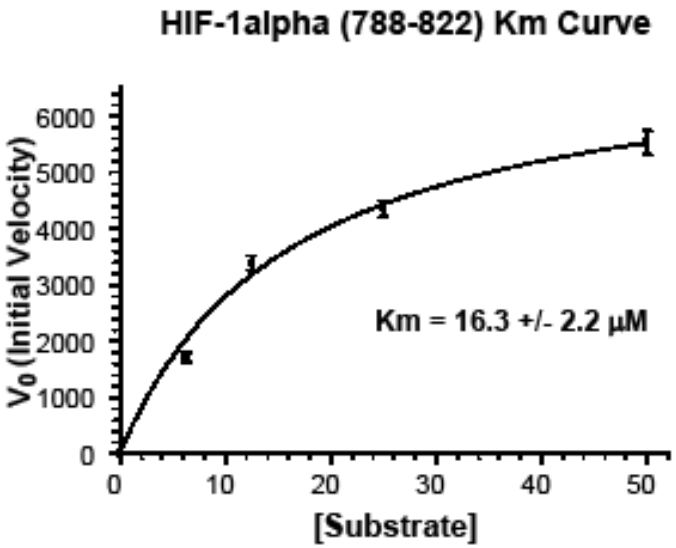
Inhibition of hydroxylation of the CTAD domain of HIF 1α at low O2 tension results in active transactivation by recruitment of P300

HT-MS FIH Assay Development

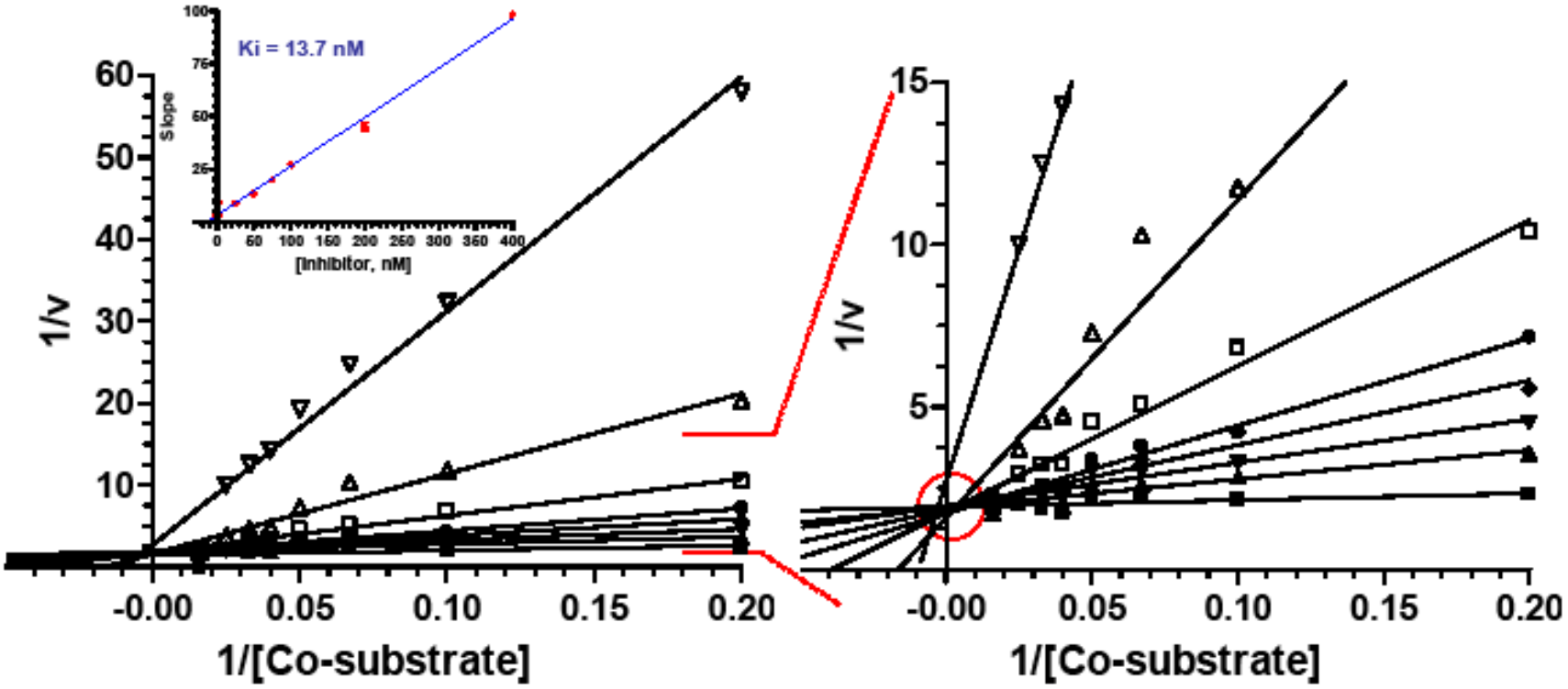






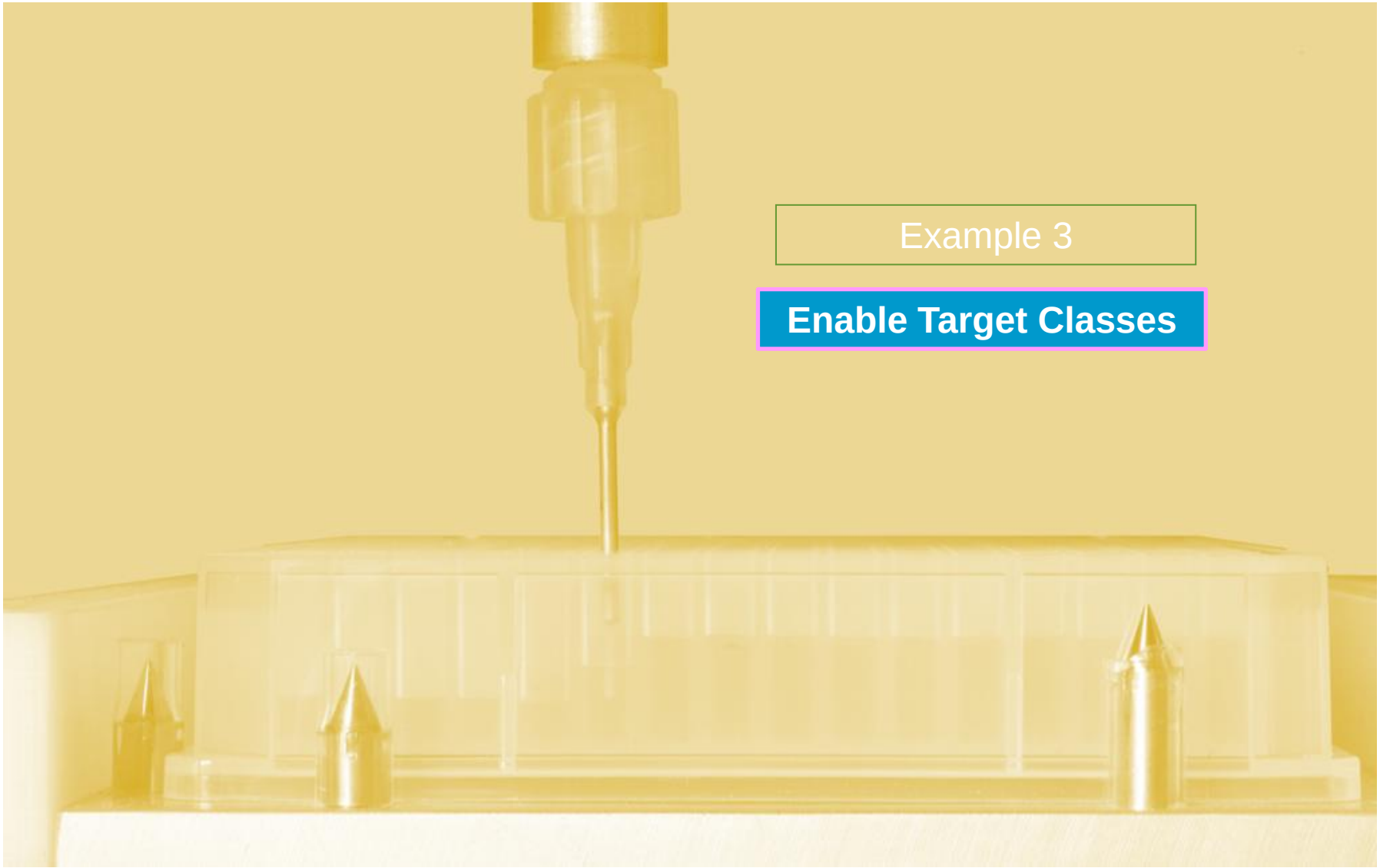


Mechanism of FIH Inhibition Determined by HT-MS

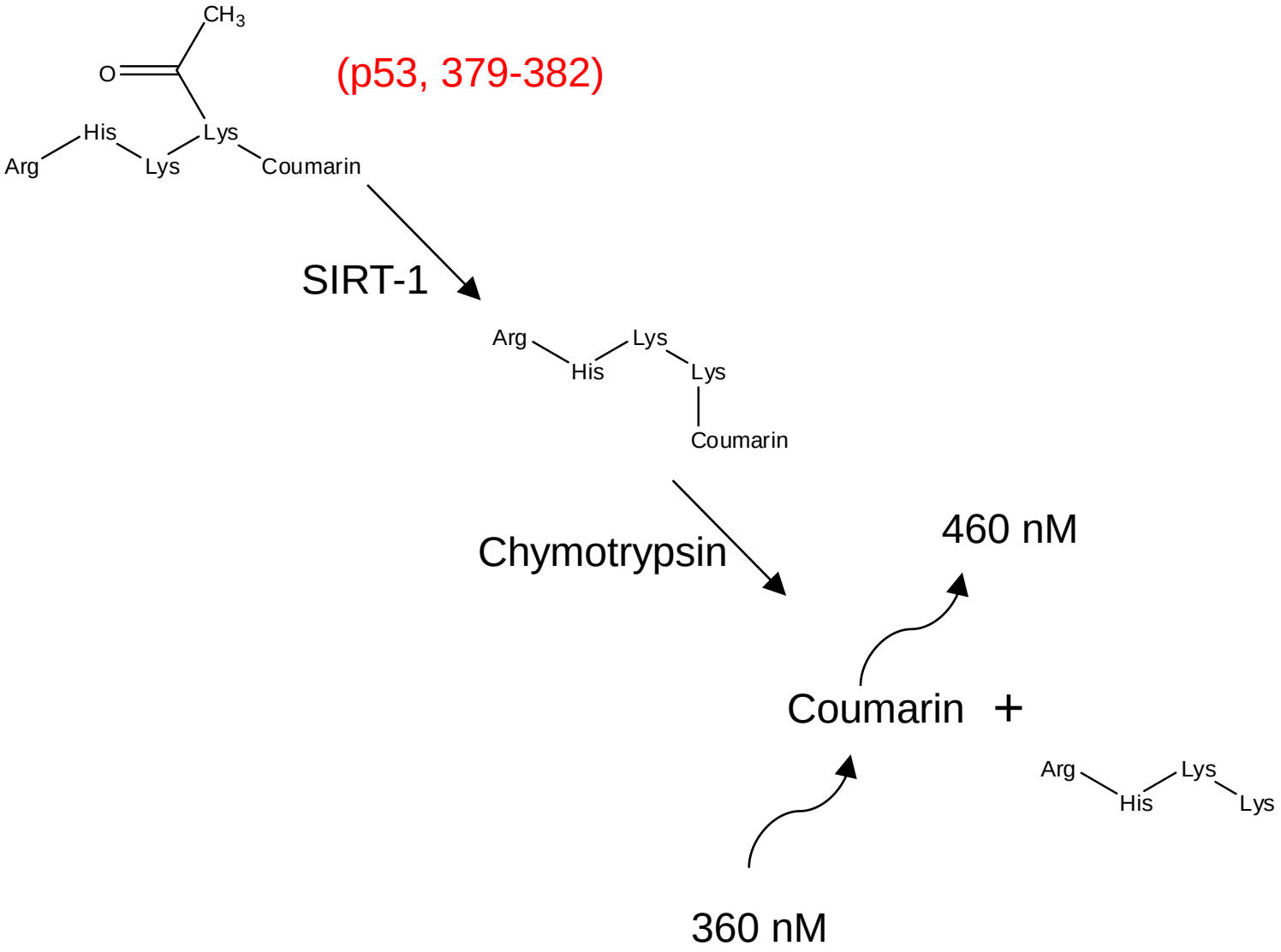


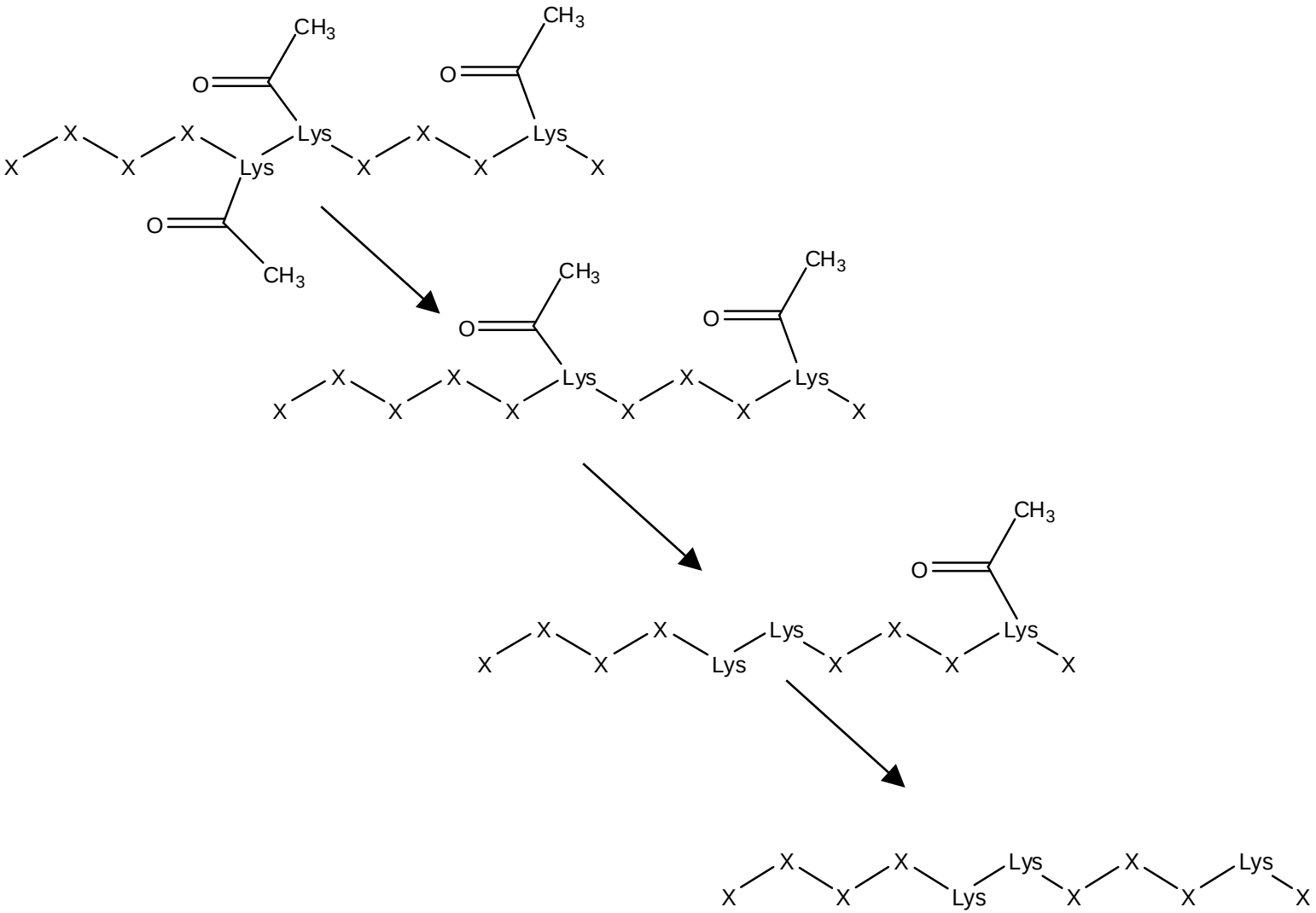
The sensitivity and precision of HT-MS readily allowed mechanistic analysis of inhibitors and determination of true Ki

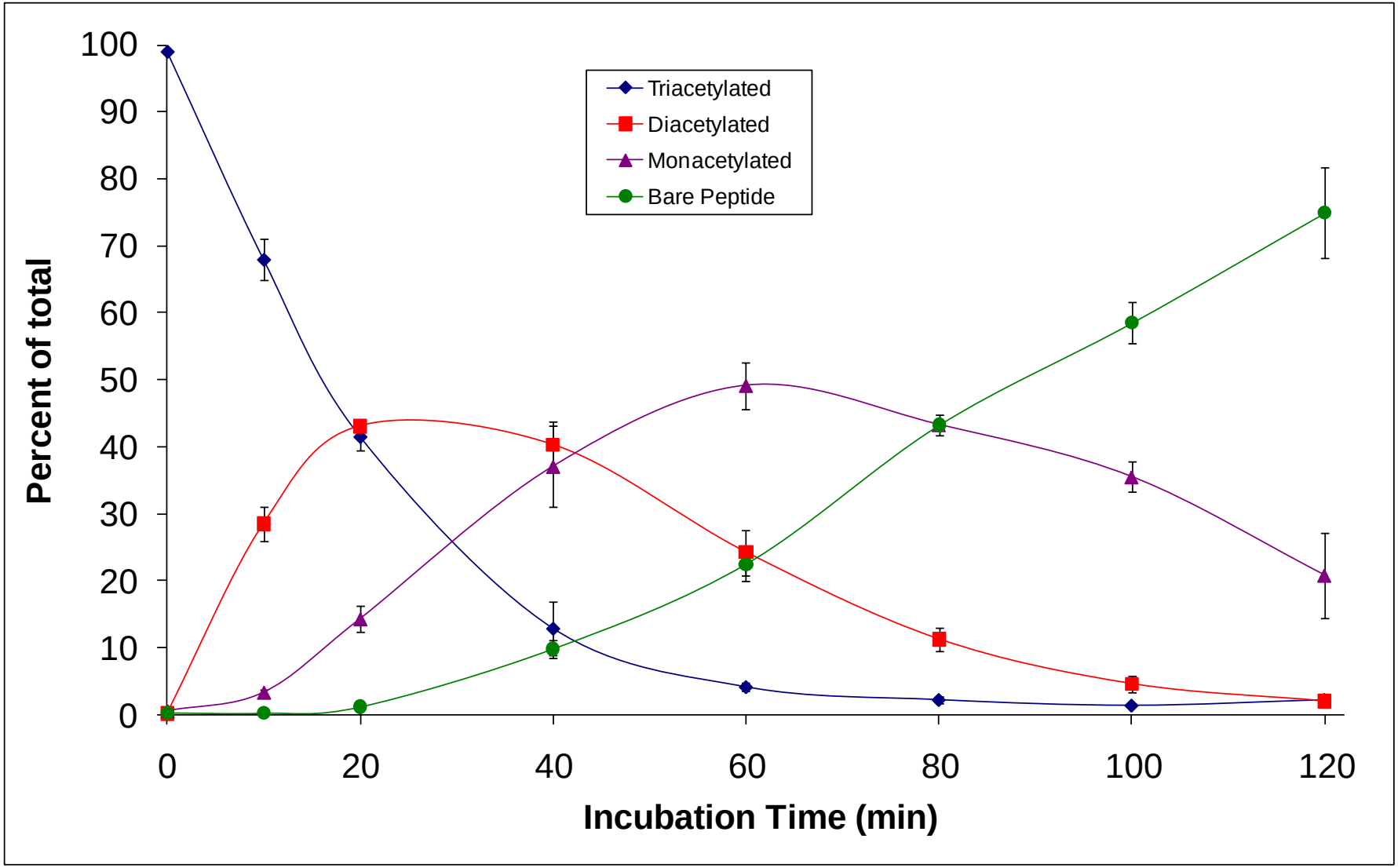
3) Enable Target Classes

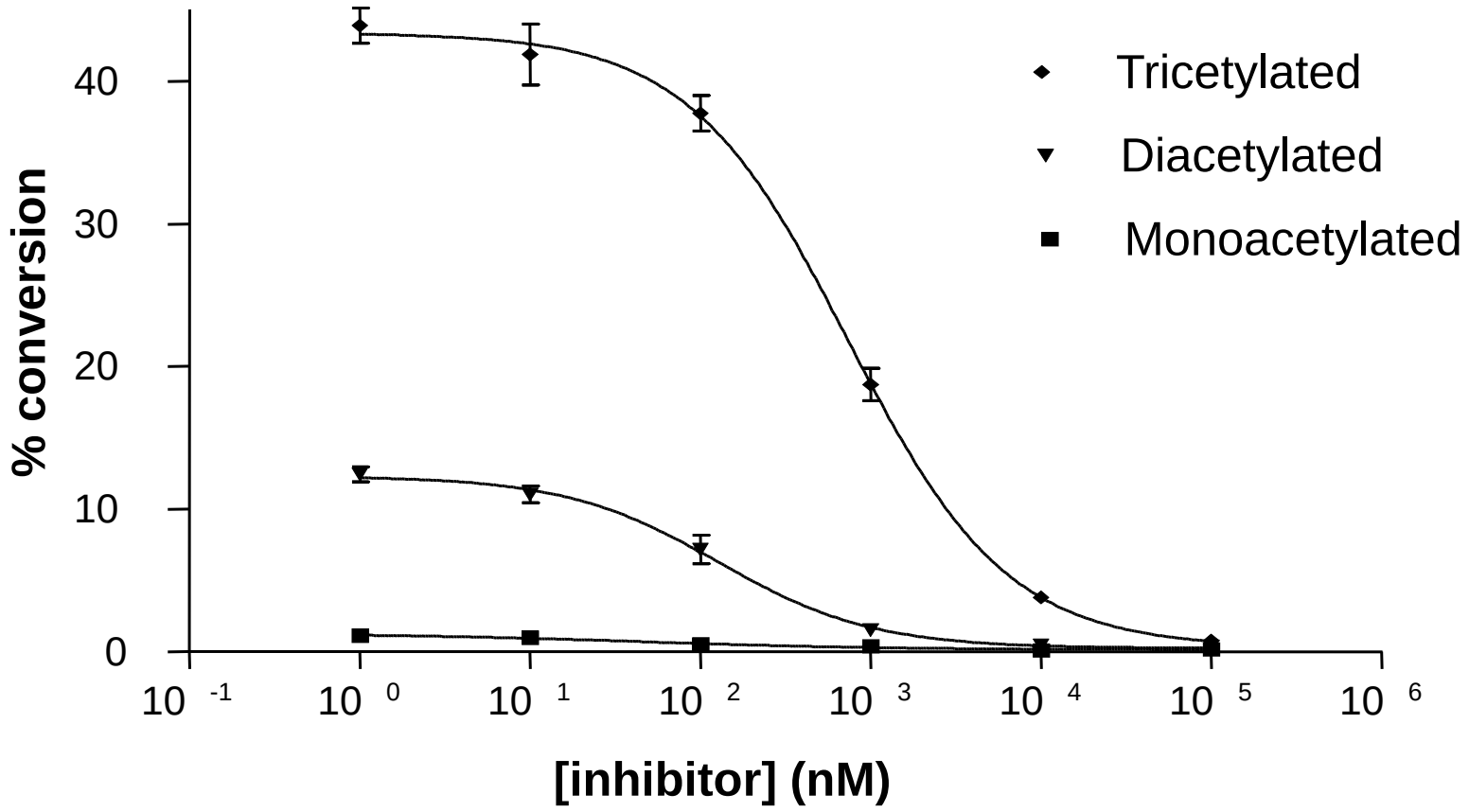


Example 3a: Sirtris – SIRT1 Assay




CH3
O
X
Lys


Triacetylated
Diacetylated
Monacetylated
Bare Peptide
Percent of total
Incubation Time (min)
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120

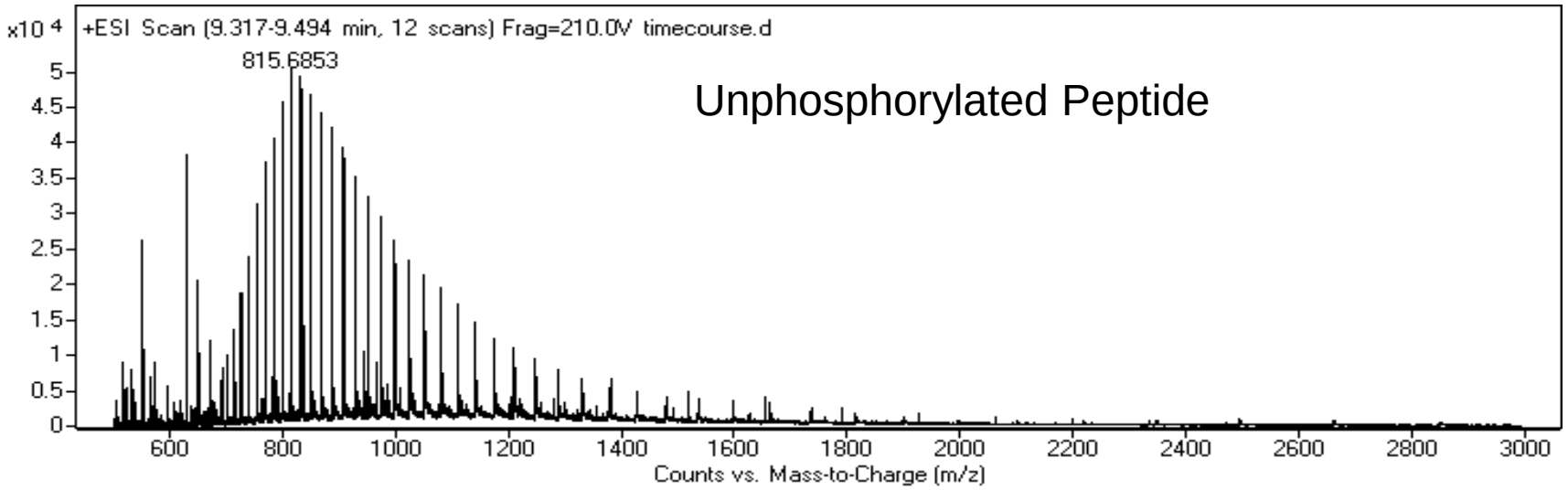


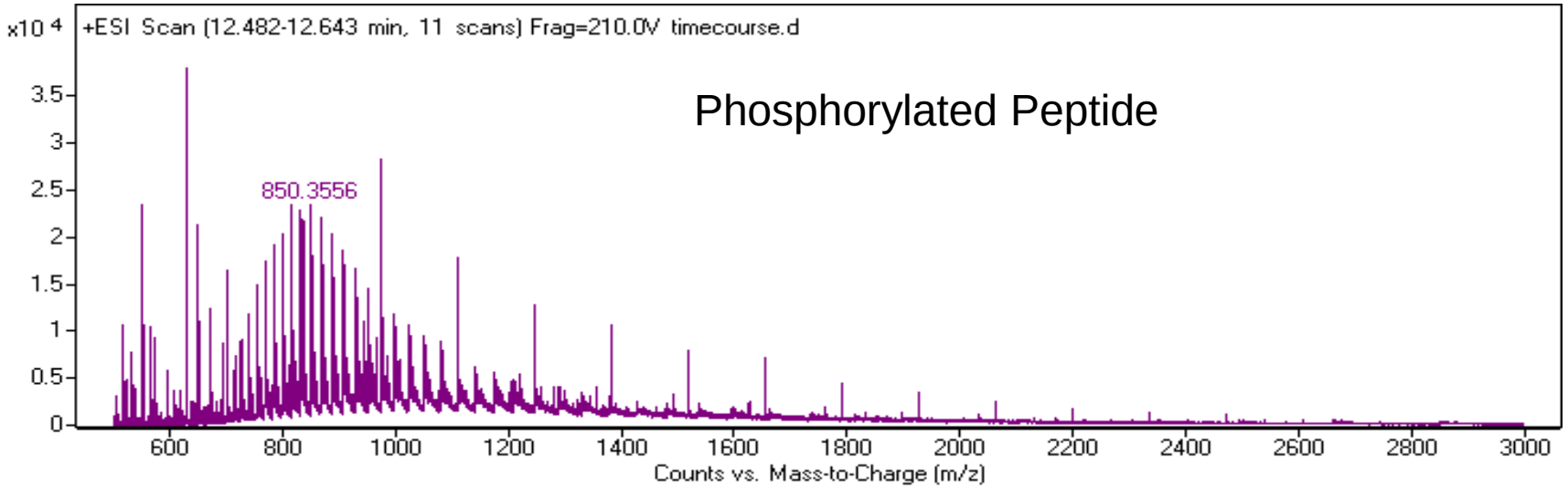
- Monoacetylated IC_{50}= 46.3 nM
- Diacetylated IC_{50}= 127.4 nM
- Triacetylated IC_{50}= 735.6 nM

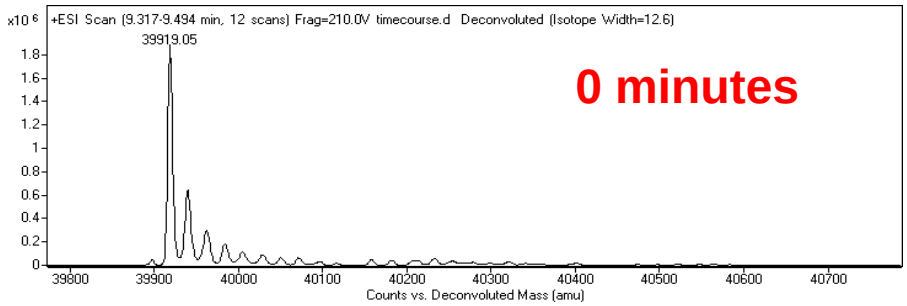
Example 3b: Glaxo SmithKline – Whole Protein Kinase

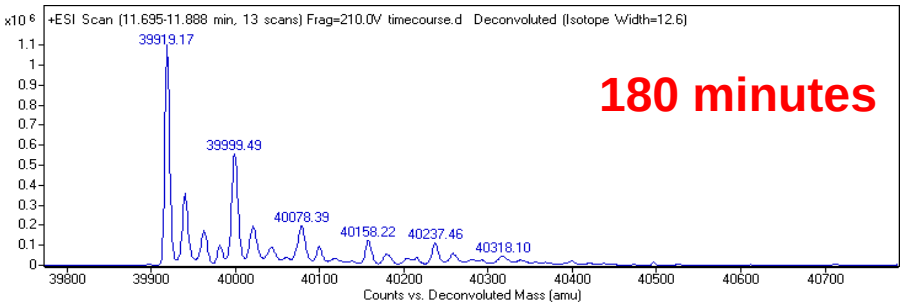
Triple Quadrupole (QqQ)
Mass Spectrometry

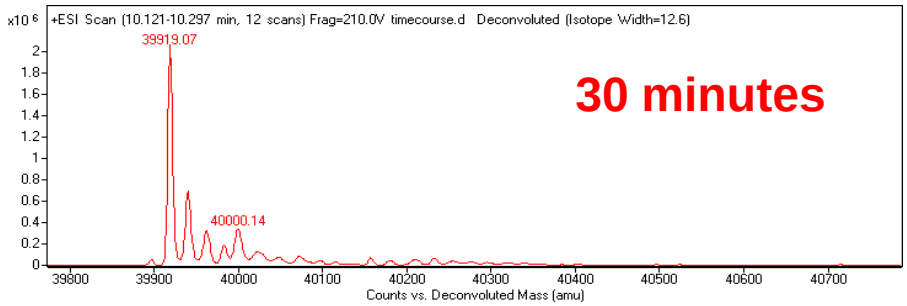
Time of Flight (TOF)
Mass Spectrometry

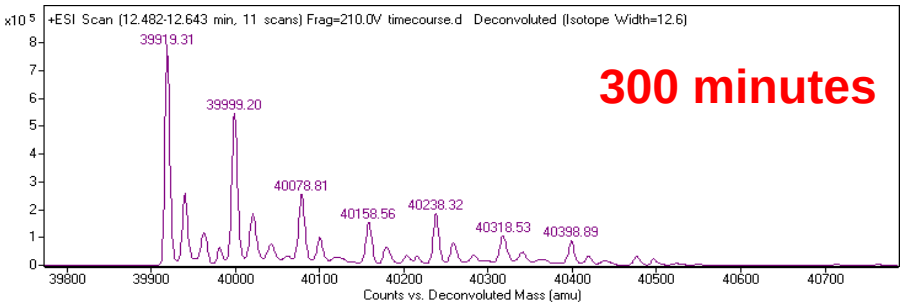

x10 4
+ESI Scan (9.317-9.494 min, 12 scans) Frag=210.0V timecourse.d
815.6853
Unphosphorylated Peptide
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
3000
Counts vs. Mass-to-Charge (m/z)

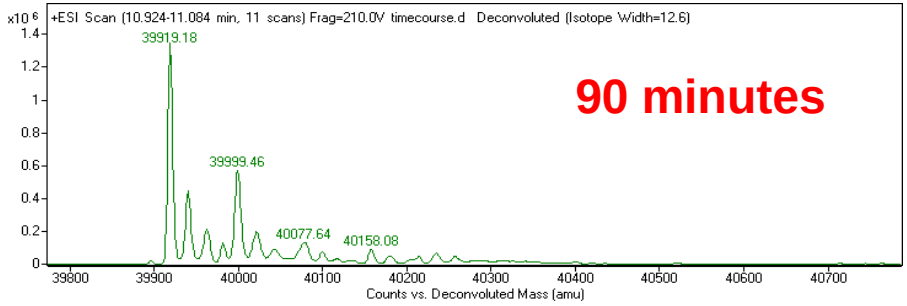

x10 4
+ESI Scan (12.482-12.643 min, 11 scans) Frag=210.0V timecourse.d
Phosphorylated Peptide
850.3556
3.5
3
2.5
2
1.5
1
0.5
0
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
3000
Counts vs. Mass-to-Charge (m/z)

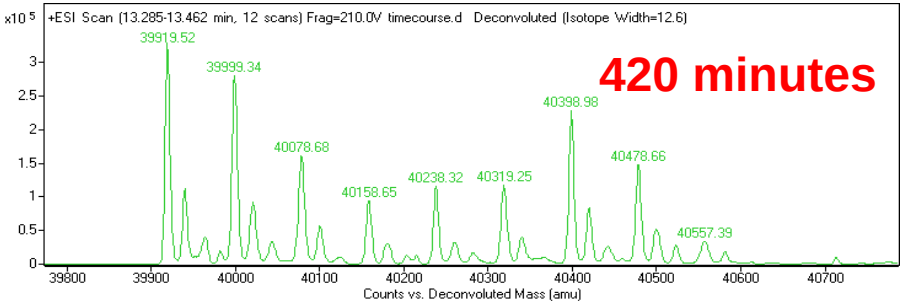

+ESI Scan (9.317-9.494 min, 12 scans) Frag=210.0V timecourse.d Deconvoluted (Isotope Width=12.6)
x10 6
39919.05
0 minutes
Counts vs. Deconvoluted Mass (amu)

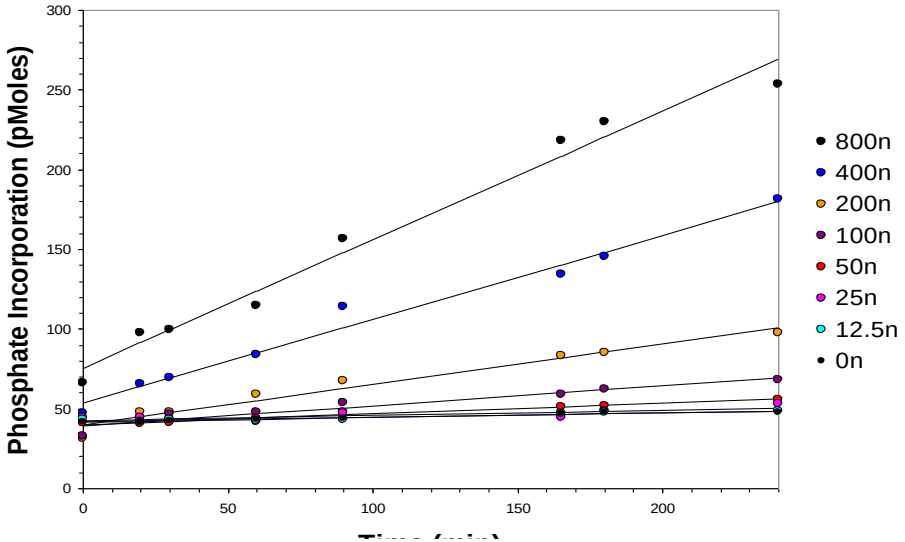

+ESI Scan (11.695-11.888 min, 13 scans) Frag=210.0V timecourse.d Deconvoluted (Isotope Width=12.6)
x10 6
39919.17
39999.49
40078.39
40158.22
40237.46
40318.10
180 minutes
Counts vs. Deconvoluted Mass (amu)

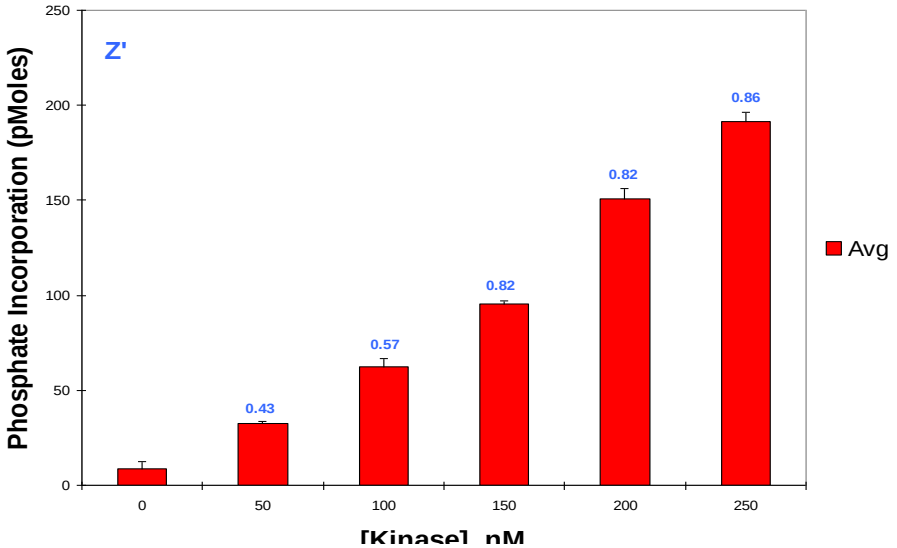

+ESI Scan (10.121-10.297 min, 12 scans) Frag=210.0V timecourse.d Deconvoluted (Isotope Width=12.6)
x10 6
39919.07
40000.14
30 minutes
Counts vs. Deconvoluted Mass (amu)

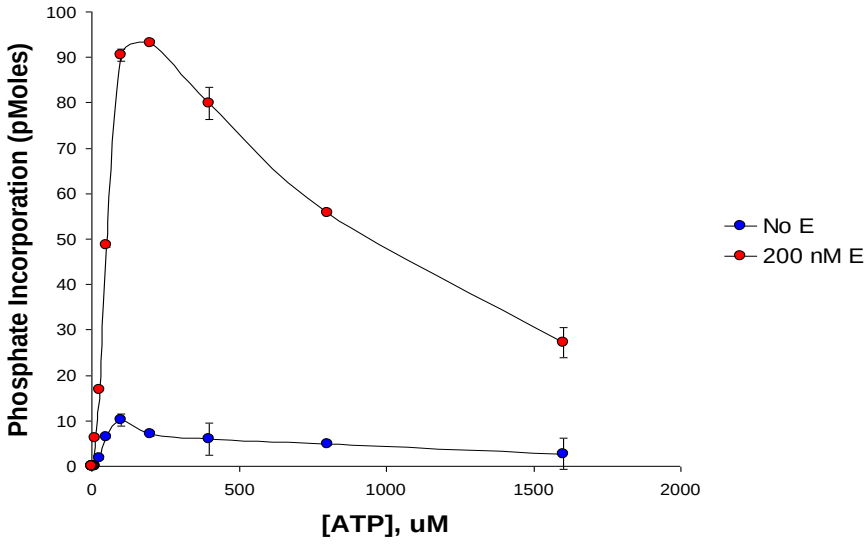

+ESI Scan (12.482-12.643 min, 11 scans) Frag=210.0V timecourse.d Deconvoluted (Isotope Width=12.6)
x10 5
39919.31
39999.20
40078.81
40158.56
40238.32
40318.53
40398.89
300 minutes
Counts vs. Deconvoluted Mass (amu)

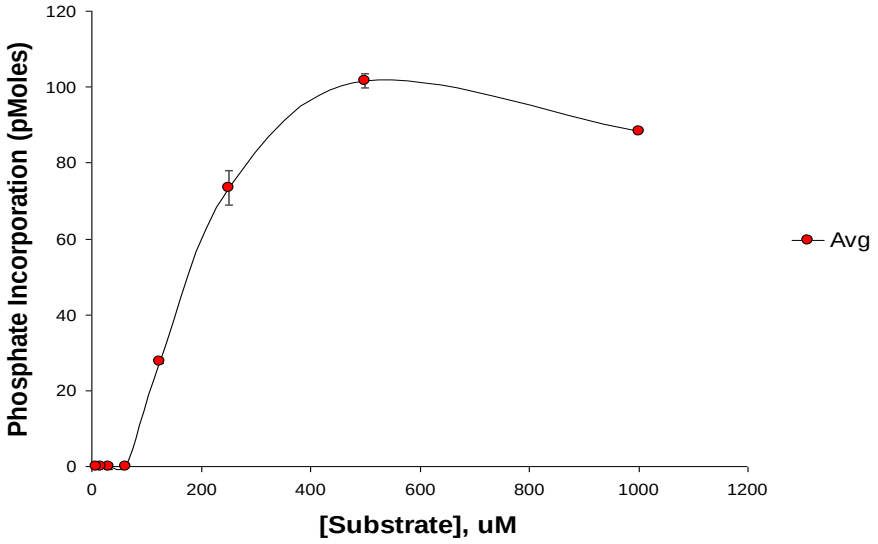

+ESI Scan (10.924-11.084 min, 11 scans) Frag=210.0V timecourse.d Deconvoluted (Isotope Width=12.6)
x10 6
39919.18
39999.46
40077.64
40158.08
90 minutes
Counts vs. Deconvoluted Mass (amu)

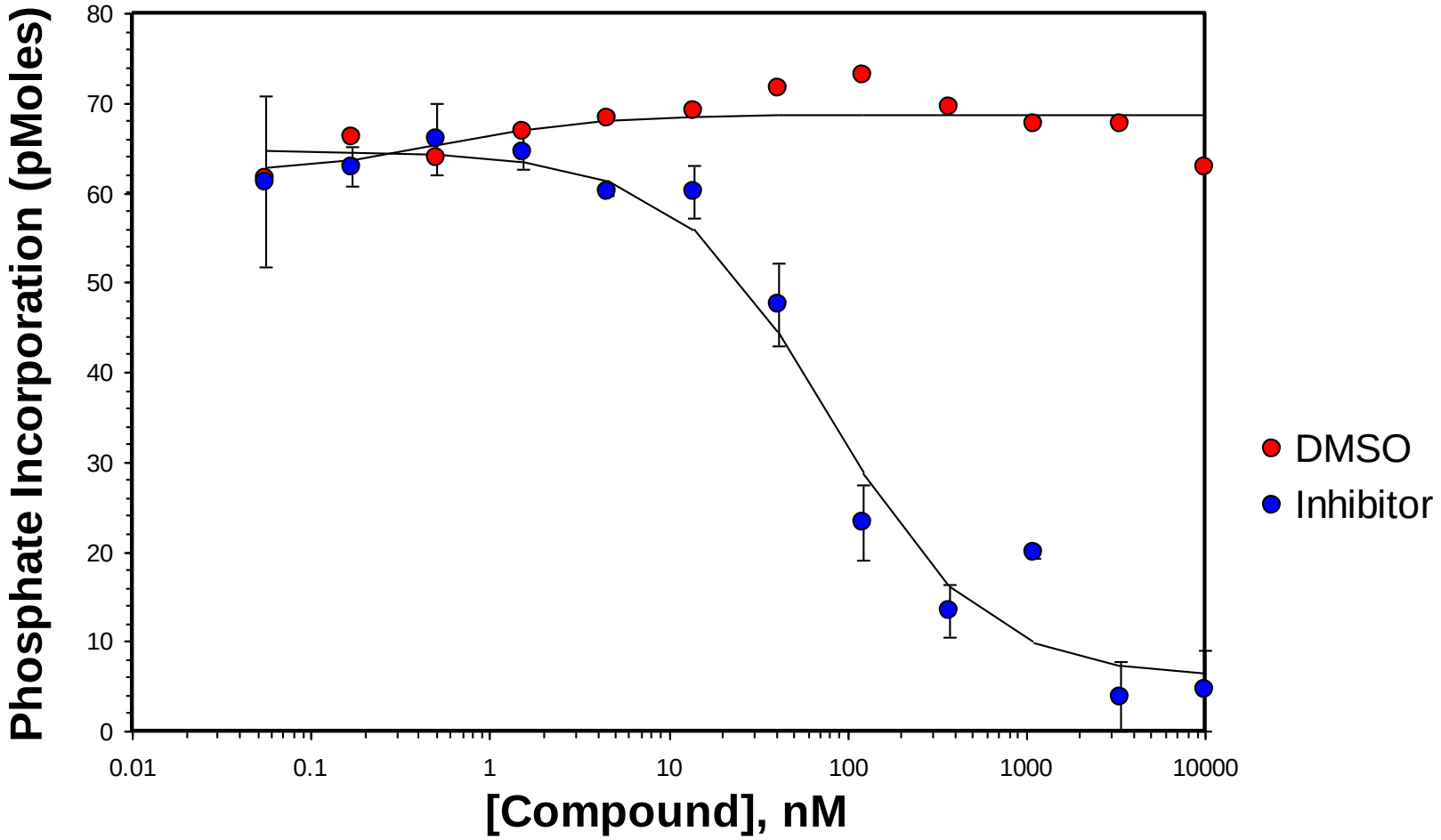

+ESI Scan (13.285-13.462 min, 12 scans) Frag=210.0V timecourse.d Deconvoluted (Isotope Width=12.6)
x10 5
39919.52
39999.34
40078.68
40158.65
40238.32
40319.25
40398.98
40478.66
40557.39
420 minutes
Counts vs. Deconvoluted Mass (amu)


Phosphate Incorporation (pMoles)
800n
400n
200n
100n
50n
25n
12.5n
0n


Z'
Phosphate Incorporation (pMoles)
0.43
0.57
0.82
0.82
0.86
Avg


Phosphate Incorporation (pMoles)
No E
200 nM E
[ATP], uM


Phosphate Incorporation (pMoles)
Avg
[Substrate], uM


Phosphate Incorporation (pMoles)
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
100
1000
10000
[Compound], nM
DMSO
Inhibitor

RapidFire Publications & Presentations

PROTEIN MODIFICATION

- Protein kinases (ATK1/PKBa, Lck Kinase)
- Protein hydroxylases (FIH)
- Diubiquitinase

ONCOLOGY

- Farnesyltransferase
- Phosphatidylserine decarboxylase
- Sphingosine Kinase

INFLAMMATION/PAIN

- Prostaglandin-E_2 synthase
- Fatty acid amide hydrolase
- Lipoxygenases (5-LOX, 15-LOX)

ANTI-INFECTIVES

- UDP-3-O(R-3-hydroxymyristoyl)-deacetylase [LpxC]

NEUROLOGY

- Phytanoyl-CoA hydroxylase
- Acetylcholinesterase

EPIGENETICS

Histone acetylases/deacetylases (sirtuins, HDACs, HATs)
Protein methylases/demethylases (LSD-1, JMJD2)
DNA demethylases

METABOLIC DISORDER/DIABETES

11b-hydroxysteroid dehydrogenase
Diacylglycerol acyltransferase
Stearoyl-CoA desaturase
GM3 synthase
Acetyl -CoA carboxylase
Serine palmitoyltransferase
ATP citrate lyase

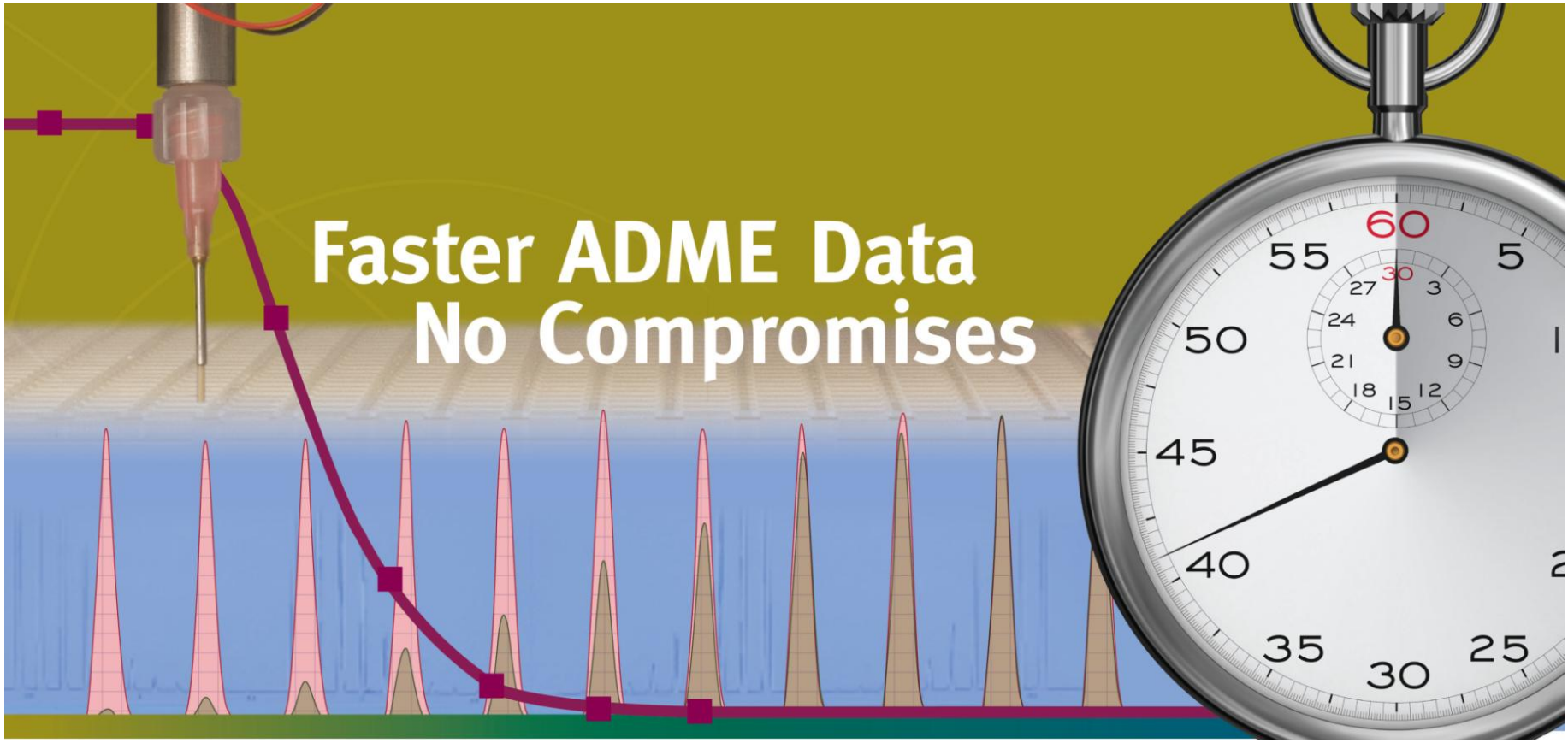
CARDIOVASCULAR DISEASE

Phosphatidylethanolamine N-methyltransferase
Phospholipase A2
Cystathionine synthase

***Assays have been demonstrated in whole cells, biological fluids or animal tissues**

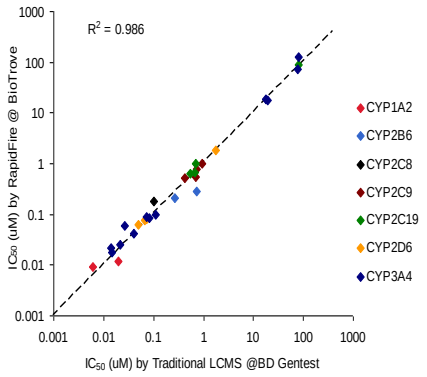
RapidFire 300 applications for *in vitro* ADME

(absorption-distribution-metabolism-excretion)

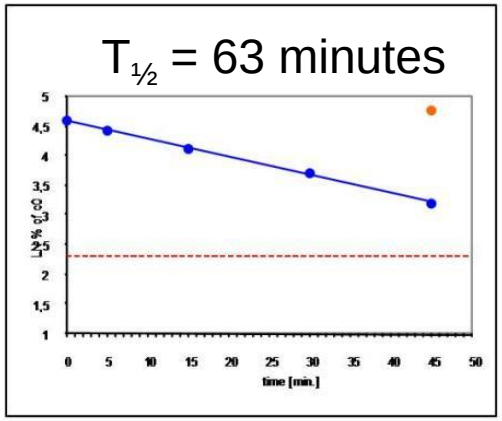


AbsorptionDistributionMetabolismExcretion

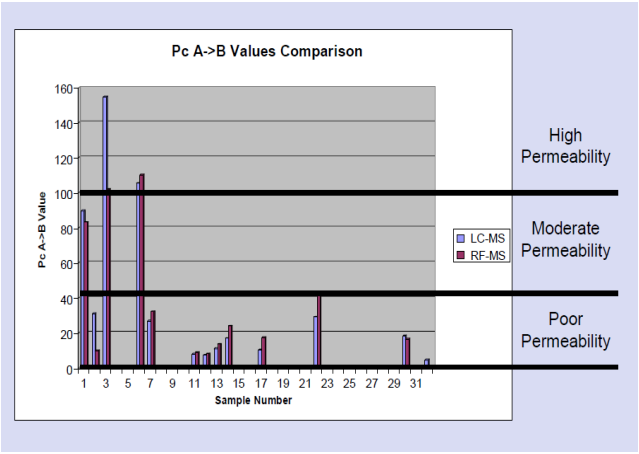
CYP450 Inhibiton



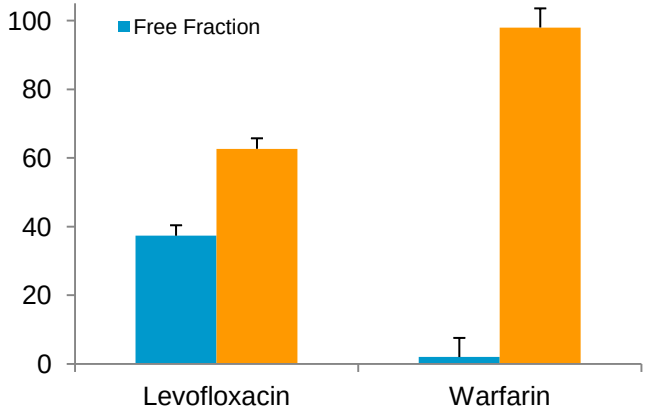
Metabolic Stability



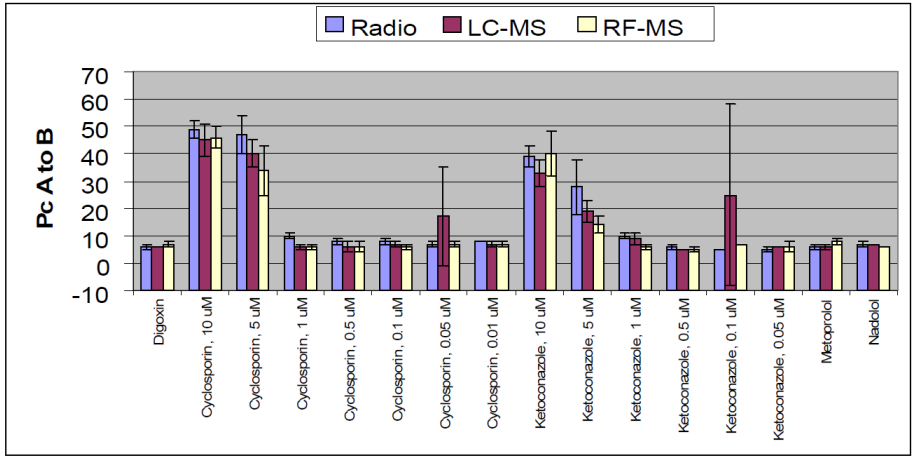
Permeability (PAMPA/Caco-2)



Plasma Protein Binding



P-Glycoprotein Inhibition

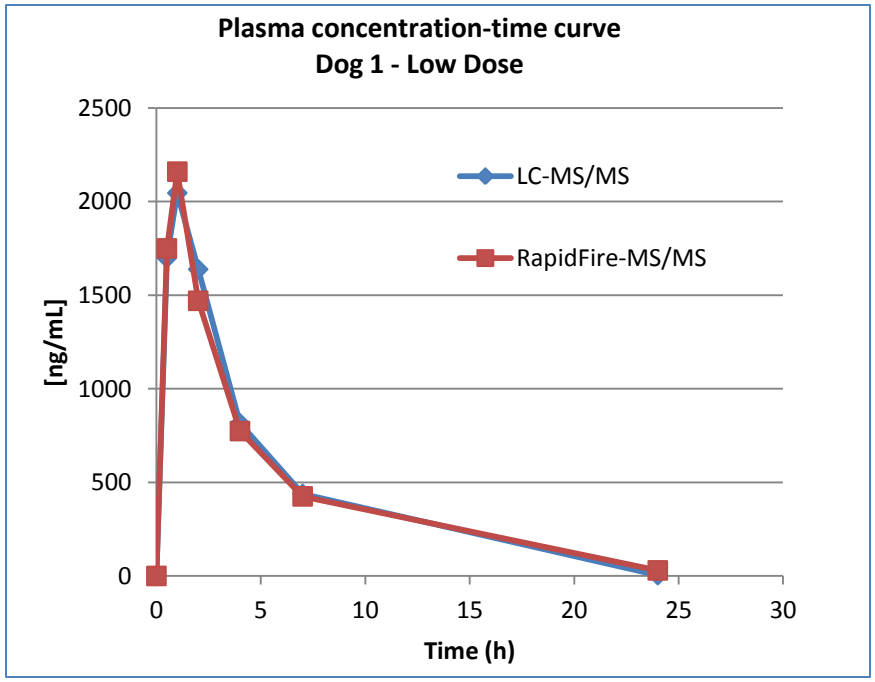


Pharmacokinetics

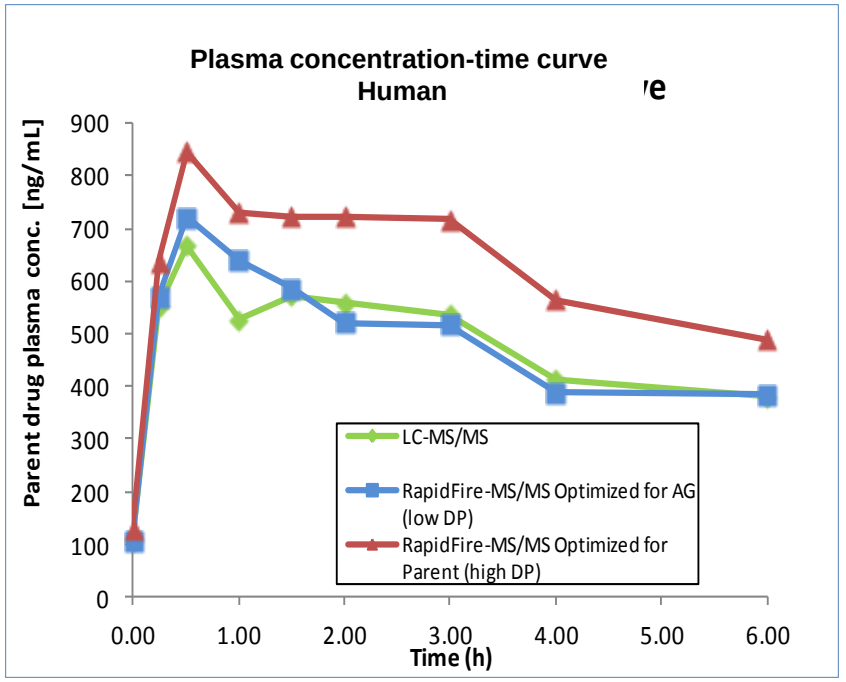
Bioanalytical and Biotransformation Challenges in Meeting Global Regulatory Expectations & New Technologies for Drug Discovery Challenges

Applied Pharmaceutical Analysis 2010
September 19 – 22, 2010, Baltimore, MD

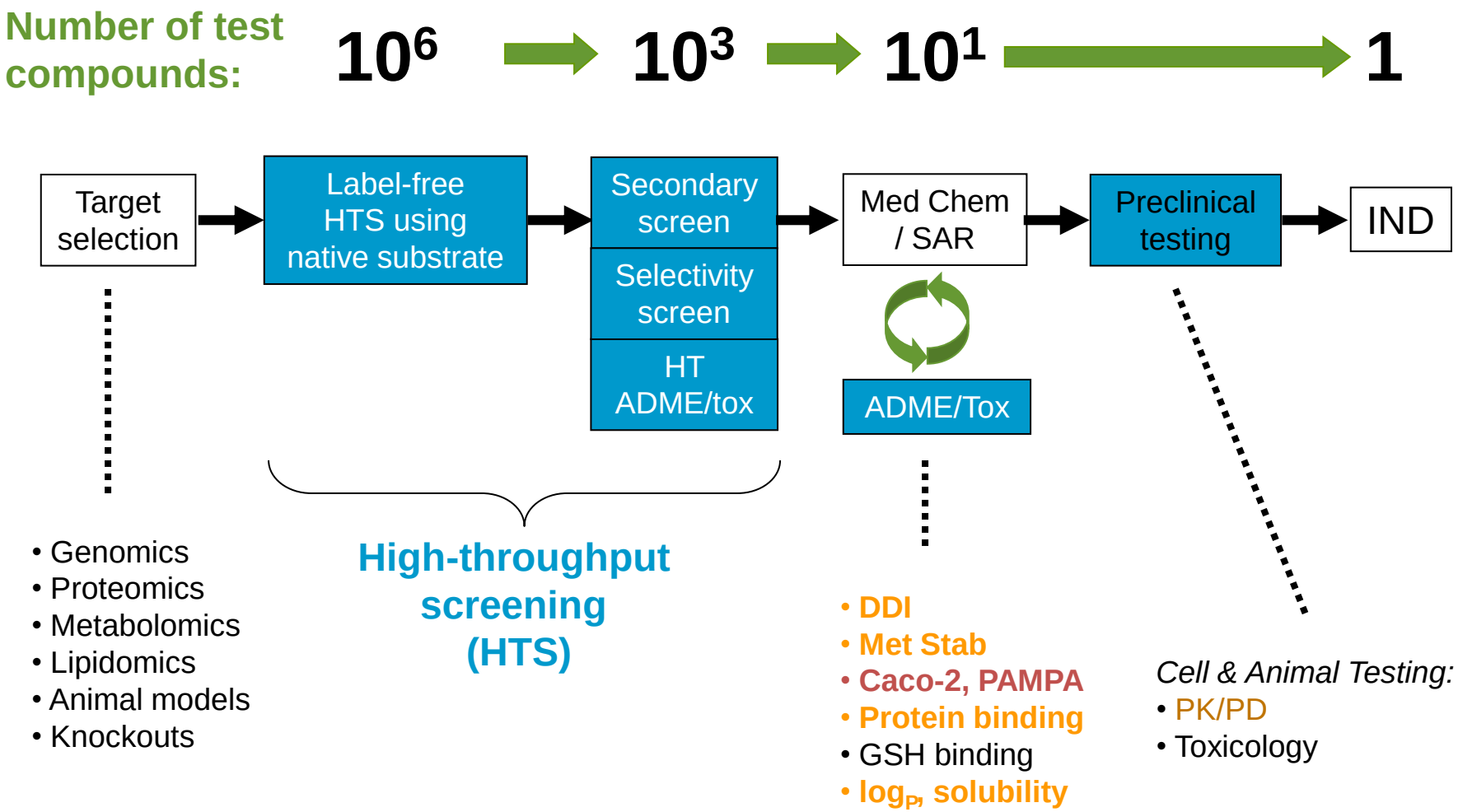
J&J Compound #1



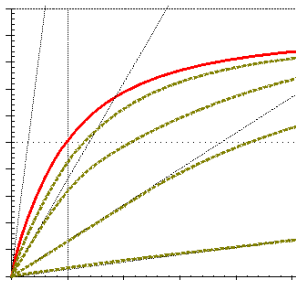
J&J Compound #2

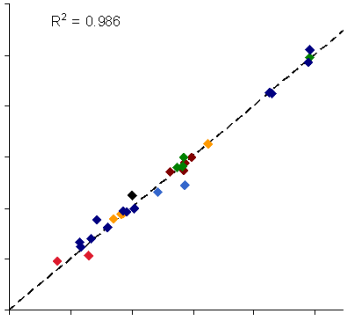


Drug Discovery with RapidFire



RapidFire resolves bottlenecks…





Improved data quality:

- Throughput allows for
  - multiple concentrations: run full IC_{50}s
  - multiple time points
- Avoids surrogate substrates, indirect and coupled assays

Maximal productivity:

- Fastest data turnaround
  - 10x data generated per FTE compared to LC-MS
- Rapid assay development in HTS ⇒ faster time to answer

Strong correlation with traditional technologies:

- LC-MS/MS: CYP450 inhibition, metabolic stability, etc
- Optical-probe or radioactivity-based detectors

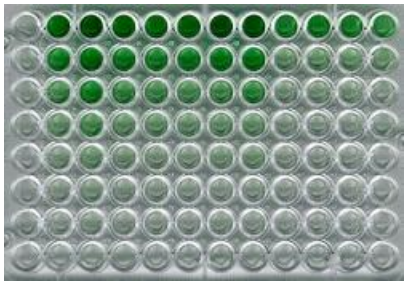
Advantages of RapidFire

Lower operational costs:

- Solvents: 6 s @3.0 mL/min = 0.25 mL (~2¢/sample)
- Cartridge: $200/cartridge or
  ~ 3000 samples/cartridge (~7¢/sample)

Minimal reagent & disposal cost in HTS:

- Only native substrate & enzyme are required
- No antibodies, luminescent or radioactive reagents, kits



Fits existing workflows

- Designed to operate similar to a plate reader

RapidFire Customer Solutions

Assay Development And Screening Services

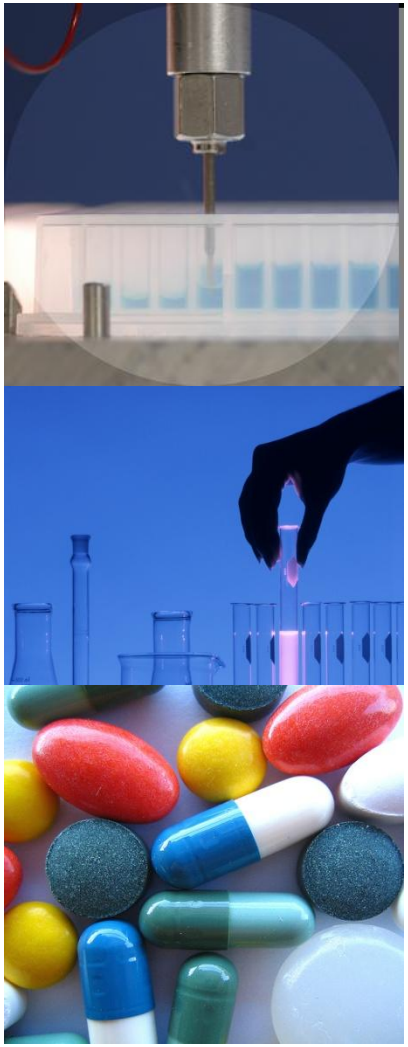
Contract Research Group

- Assay Development
- HTS Screening
- *In vitro* ADME assay screening

Instrument Sales

RapidFire Instrument Group

- RF 360 (TOF, QQQ compatible)
- RF 300 (QQQ compatible)
- RF 200 (QQQ compatible)



Customers & Collaborators who have published or presented RapidFire data

- Glaxo SmithKline
- Amgen
- Astra Zeneca
- Genzyme
- Becton Dickinson
- Merck
- Roche
- Pfizer
- Takeda
- Sirtris
- Bayer
- Exelixis
- Boehringer Ingelheim
- Schering-Plough
- OSI Pharmaceuticals
- Johnson & Johnson
- Bristol-Myers Squibb
- Novartis
- Biogen Idec
- MIT

Please Contact Us with RapidFire Application Questions

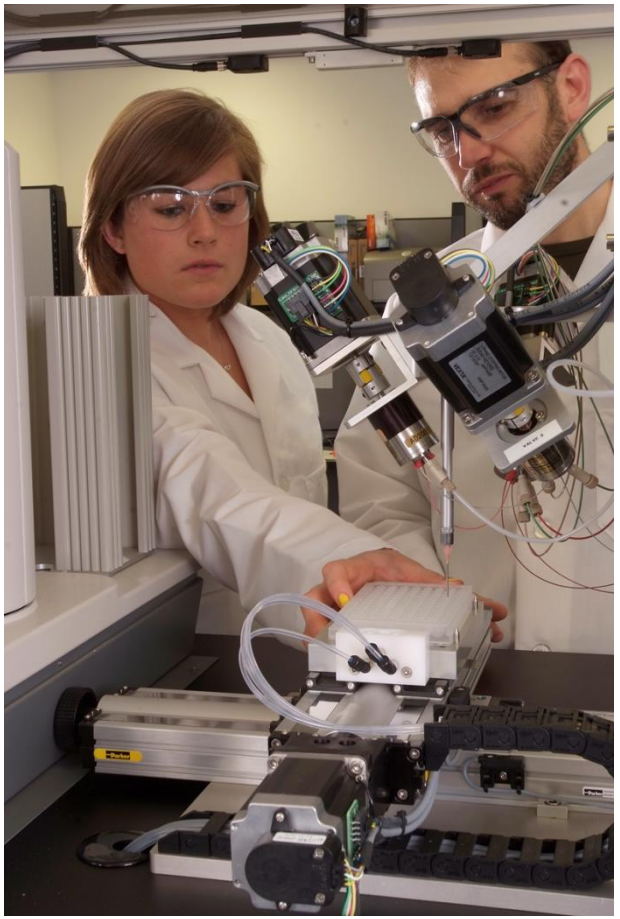
William A. LaMarr, Ph.D.

Senior Manager, R&D

RapidFire

william.lamarr@agilent.com

(781)928-2718



http://www.chem.agilent.com/en-US/Products/Instruments/ms/rapidfire/pages/default.aspx