RapidFire Analyzer provides consistent, rapid and robust peak integration enabling RapidFire-MS based drug discovery efforts at Pfizer La Jolla

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Pfizer La Jolla, Biochemistry
Agilent Innovation Symposium
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Outline

• RapidFire Mass Spectrometry
• References in the Literature
• Utilizing RapidFire Mass Spectrometry at Pfizer La Jolla
• Challenges with data analysis
• RapidFire Integrator Software
• RapidFire Analyzer Software Demo
RapidFire/MS System

• **Ultrafast autosampler & online SPE system**
  – In our applications, it replaces the LC in LC/MS
  – Reusable SPE cartridges
  – Integrates with standard MS instruments (QQQ, TOF, QTOF)
  – Cycle time = 8-15 sec/sample

• **Compatible with biological matrices**
  – Enzyme, substrate, compound
  – Cell culture media
  – Serum, plasma or whole blood
  – Urine
RapidFire Applications

Lead Discovery
Primary screening, secondary screening, SAR, enzymatic and kinetic parameters, IC$_{50}$

*In vitro* ADME
CYP inhibition, metabolic stability, permeability, PgP inhibition, solubility

Forensic Toxicology
Non-endogenous compounds

Multi-omics
Quantitative Proteomics (SISCAPA) targeted & untargeted metabolomics
SPE/MS Sequence

Ultrafast Online SPE

- Sequential, fast & efficient cycling
  8-15 seconds per sample
- Cartridge changer (6 or 12 slots)
  Automatic or programmed switch
- Cartridge packing material
  C4, C8, C18, Phenyl, Cyano, HILIC

Aspirate Sample
Under Constant Vacuum

Load Aqueous

Wash Aqueous or Organic

Reverse Elute Organic

To Waste

To MS

SPE Cartridge

SPE Cartridge

SPE Cartridge

Pfizer
RapidFire 365 – Capacity

12-cartridge capacity → Good match for Benchbot speed

63-plate Capacity → 20,000 samples over 60 hours
# RapidFire 365 – SPE Cartridge

<table>
<thead>
<tr>
<th>Type</th>
<th>Packing</th>
<th>Typical Applications</th>
<th>Part #</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C4</td>
<td>Small molecules, peptides, oligos</td>
<td>G9203A</td>
</tr>
<tr>
<td>A2</td>
<td>C4</td>
<td>Small molecules, peptides, oligos (Lower carryover for some forensic toxicology applications)</td>
<td>G9525A</td>
</tr>
<tr>
<td>B</td>
<td>Cyano</td>
<td>Hydrophobic compounds</td>
<td>G9204A</td>
</tr>
<tr>
<td>C</td>
<td>C18</td>
<td>Proteins, small molecules</td>
<td>G9205A</td>
</tr>
<tr>
<td>D</td>
<td>Graphitic carbon</td>
<td>Hydrophilic compounds, small molecules</td>
<td>G9206A</td>
</tr>
<tr>
<td>E</td>
<td>C8</td>
<td>Proteins, peptides, small molecules</td>
<td>G9207A</td>
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<tr>
<td>F</td>
<td>Phenyl</td>
<td>Aromatic compounds</td>
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<tr>
<td>H</td>
<td>HILIC</td>
<td>Hydrophilic compounds, small molecules</td>
<td>G9209A</td>
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<tr>
<td>O</td>
<td>Blank</td>
<td>Troubleshooting</td>
<td>G9211A</td>
</tr>
<tr>
<td>Z</td>
<td>Custom</td>
<td>Custom applications</td>
<td>G9210A</td>
</tr>
</tbody>
</table>

![Diagram of SPE Cartridge](image)

Inlet
Frit
SPE Packing
Outlet
Frit

1.4 cm

![Diagram](image)

**Table 5. Comparison of a Traditional Method versus RapidFire Mass Spectrometry**

<table>
<thead>
<tr>
<th>Method</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>liquid-liquid salt assisted extraction</td>
<td>4–6 h/batch</td>
</tr>
<tr>
<td>LC/MS instrument run time</td>
<td>5–10 min/injection</td>
</tr>
<tr>
<td>total time</td>
<td>5 1/2–9 1/3 hours/batch</td>
</tr>
<tr>
<td>versus: RFMS</td>
<td>~20 s/sample</td>
</tr>
<tr>
<td>total time</td>
<td>~7 min/batch</td>
</tr>
</tbody>
</table>

*“Herein batch is defined as 20 samples including quality control.”*
Miller VP, **SPE-MS analysis of absorption, distribution, metabolism and excretion assays: a tool to increase throughput and streamline workflow.** *Bioanalysis*. 2012 May;4(9):1111-21
Traditional Biochemical Approaches:

**Advantages:**
- Reagents are readily available (labeled substrates)
- Fluorescent plate readers are robust, reliable, and high throughput

**Disadvantages:**
- Fluorescent and radiolabeled substrates can introduce confounding factors, effect enzyme kinetics, and produce data artifacts
- Radioactivity
  - Requires containment and strict regulations for usage
  - Expensive to use and dispose

Rapid Fire-MS/MS system

**Advantages:**
- Replaces LC in LC/MS
- Simple to use
- 8–15 seconds/sample enables monitoring real-time enzymatic kinetics and HTS
- Compatible with many biological matrices (cells, nucleosomes, tissue extracts)

**Disadvantages:**
- Steep learning curve for implementation in labs with little or no LC-MS/MS experience

LC-MS/MS system

**Advantages:**
- Direct measure of substrate and product
- Only native substrate & enzyme required
- No antibodies, fluorescent or radioactive reagents
- Enables functional biochemical rather than just target binding assays
- Measure multiple species and achieve unparalleled information about biochemical reactions by measuring each methylation state discreetly

**Disadvantages:**
- Slow ~ 4 minutes/sample
- Instrumentation is expensive, not easily scalable to meet demand
- Steep learning curve for implementation in labs with little or no LC-MS/MS experience
RapidFire at Pfizer La Jolla

API 4000 QQQ

Agilent 6495 QQQ

Agilent 6550 QTOF
Biochemical Challenges with Histone Methyltransferases

- HMT’s bind their nucleosome substrates with low nM $K_m$ values
- Low $K_m$ values require low substrate concentration in the assay
  - Results in minimal product generation

Typical HMT Kinetics
Nucleosome substrate

- Target Region

![Graph showing typical HMT kinetics with nucleosome substrate concentration and product generation over time.](image-url)
Biochemical Characterization of a Demethylase

Compound Dose-Response

Time (min)

[Inhibitor]

0 uM

200 uM

Rapid sampling enables real-time kinetic evaluation

Compound IC₅₀

V₀ (counts/minute)

IC₅₀ 0.549 μM

Log [Compound], μM

Kinetic measurement of Demethylase activity on multiple substrates simultaneously

Methyl 0

Methyl 1

Methyl 2
Utilizing RapidFire for HTS

Targeted Full File HTS
<1,000,000 compounds

Targeted Subset Library
<100,000 compounds
Kinase
Fragment
Diversity
Epigenetic
Natural Products
File Mining

Custom Request
<10,000 compounds
Med Chem design
Compound Modeling based on known structure motifs
Data Analysis Challenges

1 96 plate/12 minutes

Sampling rate presents a challenge!
- 120 96well plates/day or
- > 10,000 individual samples/day

Available analysis options:

<table>
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<tr>
<th>Feature</th>
<th>RF Integrator</th>
<th>Mass Hunter/Peak Splitter</th>
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<tr>
<td>Knowledge of Mass Hunter</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>Automatic peak integration</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>Plate based visualizations</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Easy to use with no training</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Review by exception</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Calculations and Thresholds</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>Compatible with API4000</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Accommodates large data sets</td>
<td>Yes</td>
<td>Yes</td>
</tr>
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RapidFire Integrator Summary

- Easy to use

  - Manual peak integration required per plate/sequence basis
  - Unable to view chromatograms on a per plate basis
  - Manual process assigning injections to chromatograms
  - Failed RapidFire batches may prevent peak integration
  - Plate based export is not enabled for multiple sips per plate (typically encountered with kinetic analysis)
RapidFire Analyzer Introduction

✓ Analyzer is a much improved software for RapidFire generated data

✓ Currently in testing; will eventually replace Integrator

✓ Easy to use

✓ Automated peak integration

✓ Plate-based visualization
RapidFire Analyzer Demonstration
RapidFire Analyzer In-depth Features
RapidFire Analyzer Review by Exception

Flag chromatograms that fail based on pre set criteria
### RapidFire Analyzer Summary

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<td>Yes</td>
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<tr>
<td>Flagged sipper errors</td>
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<td>No</td>
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<td>Review by exception</td>
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RapidFire Analyzer correlation with Integrator

![Graph showing the correlation between Analyzer IC50 (uM) and Integrator IC50 (uM). The R square value is 0.9993.]
Summary

✔ Mass Spectrometry enables us to use native substrates in our enzymatic characterization and screening

✔ RapidFire affords us the automation and speed to use Mass Spectrometry like a traditional plate reader

✔ RapidFire Analyzer software is an integral component to our data analysis
Acknowledgements

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