Label Free High-Throughput Whole Protein Kinase Screening Assay

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Introduction

Protein kinases are involved in the regulation of multiple cellular processes by catalyzing phosphorylation of target substrates. Dysregulation of kinase activity has been found in a variety of diseases; therefore, protein kinase inhibitors offer an opportunity to target these diseases. To facilitate the screening and characterization of kinase inhibitors, we developed a mass spec-based high throughput screening method to measure their effect on kinase activity, permitting the use of whole protein substrates which advantageously allows detection of both competitive and non-competitive inhibitors. In addition, this assay does not rely on site-specific antibodies, allowing detection of phosphorylated serine, threonine, or tyrosine residues. Lastly, mass spectrometry facilitates quantitative interrogation of complex kinetics in proteins with multiple phosphorylation sites.

Multiple phosphorylation whole protein kinase assay facilitated by high-throughput mass spectrometry interface.

Assay Conditions

- All assays were monitored using the RapidFire high-throughput mass spectrometry system software interfaced to an Agilent 6220 Accurate-Mass TOF.

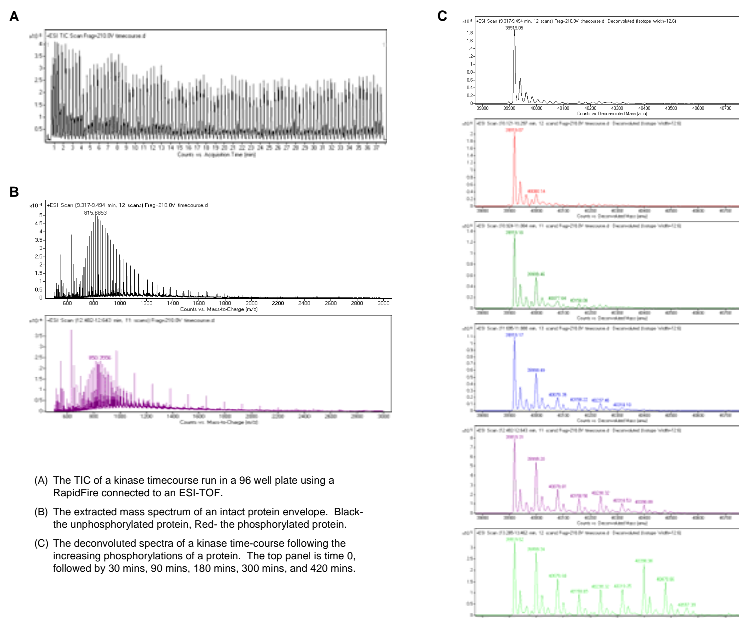
RapidFire Conditions

- Buffer A = 100% water with 0.09% formic acid
- Buffer B = 100% acetonitrile with 0.09% formic acid
- SPE Column A (reversed-phase C_8 chemistry)

- MassHunter was used to integrate and deconvolute the data. Excel with macros was then used to convert the data to amount of phosphate incorporation. Analysis of a 96-well plate of data was achieved in under an hour.

# of phosphorylations	Deconvoluted masses
0	39918.7
1	39998.698
2	40078.696
3	40158.694
4	40238.692
5	40318.69
6	40398.688
7	40478.686
8	40558.684

RapidFire System



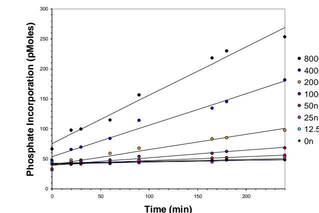
(A) The TIC of a kinase timecourse run in a 96 well plate using a RapidFire connected to an ESI-TOF.

(B) The extracted mass spectrum of an intact protein envelope. Black- the unphosphorylated protein, Red- the phosphorylated protein.

(C) The deconvoluted spectra of a kinase time-course following the increasing phosphorylations of a protein. The top panel is time 0, followed by 30 mins, 90 mins, 180 mins, 300 mins, and 420 mins.

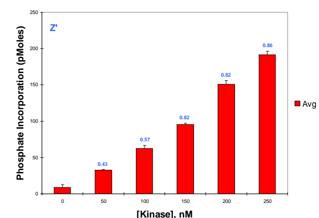
Results

Enzyme linearity curves



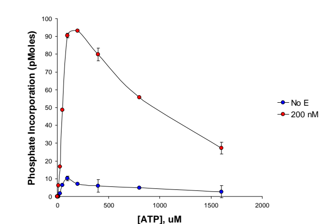
Enzyme linearity was performed at various incubation times using different concentrations of kinase and fixed concentrations of substrate and ATP. The optimal incubation period to achieve maximum signal and a linear time course was chosen at 4 hrs.

Enzyme titration



The kinase concentration was varied from 0nM to 250nM for 4 hrs. 1μM substrate and a non limiting ATP concentration (200μM) was used.

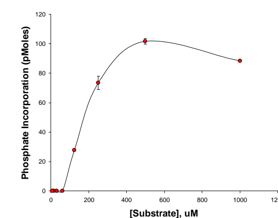
ATP titration



The assay was run for 4hrs with ATP concentrations ranging from 0μM to 400μM (2-fold serial dilution) and using 100nM kinase and 1μM substrate. The inhibition observed was confirmed offline using traditional methodologies.

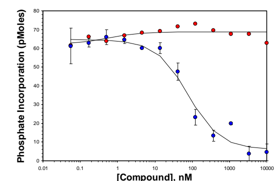
Results

Substrate titration



Substrate concentration was varied from 0nM to 1000nM (2-fold serial dilution) for 4 hrs. Substrate inhibition was observed at higher concentrations. 100nM kinase and 200μM ATP was used. The km was calculated using Michealis-Menton equation to be 351nM.

IC50 curve



To further validate the kinase assay, an inhibition study was conducted. The assay used 1μM substrate, 150nM kinase, and 100μM ATP. Inhibitor concentration ranged from 0.05 to 10000nM (3-fold serial dilution). The IC50 was calculated to be 78nM.

Conclusions

BioTrove's RapidFire in conjunction with Agilent 6220 ESI-TOF MS provides a platform for:

- **Label-free screening of whole protein kinase assays**
- **Quantitative measurement of multiple modifications to full length native proteins**
- **Sustained throughputs of 10-12 seconds/sample**
- **Due to selectivity of MS, this platform should be applicable to assays beyond protein phosphorylation**
  - **Deacetylation**
  - **Methylation**
  - **Ubiuitination**
  - **Hydroxylation**
  - **Modification to DNA or antibodies**