The Development of a Novel Mobility Shift Analysis System for Kinase Small Molecule Drug Discovery using the SCIEX BioPhase 8800

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1. Abstract

The Mobility Shift Assay (MSA) for separation of substrate and product in various forms of enzymatic reaction has been popular with drug discovery for over 20 years. Caliper Lifesciences/Perkin Elmer (Revvity) successfully commercialized this methodology enabling many drug discovery companies to execute screens and medicinal chemistry support across many therapeutic areas and target classes. Carna Biosciences was one of the early adopters of the technology to support its own kinase drug discovery programs but also to provide support to its commercial kinase production service and to offer compound profiling across the kinome.

Perkin Elmer (PE) announced its EZ Reader II instrument, including consumables and support, will be completely discontinued as of the end of 2024, leaving many MSA-dedicated companies with no MSA options.

Here we show how Carna Biosciences identified and operationalized the SCIEX BioPhase 8800 instrument to perform MSA for kinase drug discovery. This process started from scratch and touched on every aspect of the separation and analysis of kinase substrate and peptide products, including buffer conditions, separation variables, and even software peak recognition and integration.

The result is that the new system provides a way forward to maintain the benefits of MSA analysis of enzyme reactions in a medium-throughput method, as well as maintaining consistency with historical data.

Further optimization of the detection conditions to improve the throughput and automate the data analysis are in progress aiming to initiate the actual service launch from 2024 Spring.

2. MSA – the basics

The Mobility Shift Assay requires a difference in the charge/mass ratio of the analytes, and uses a potential difference across a fluid or semi-fluid medium to provide separation. As analytes flow through capillary channels, the potential difference causes separation so that individual analyte populations can be detected as individual 'pools' or 'peaks' of fluorescence. Fluorescence is usually measured by a laser or LED, and either an intrinsic fluorophore (such as Trp) or an extrinsic fluorophore (such as Ethidium Bromide or Fluorescein).

There are many separation conditions within this compact system, the variation of which can affect the resolution of these peaks, including:
• analyte type (peptide, protein, nucleic acid, overall charge, 3D shape, molecular weight)
• capillary nature (diameter, length, material)
• separation buffer (ionic strength, chaotrophic agents, buffer capacity, viscosity)
• applied potential difference (high voltage, low voltage, voltage difference)
• flow rate

PE had optimized conditions for standard analytes on its EZ Reader II, however for any new system or analyte, where these conditions have changed, the optimization of the remaining variables is required, and is key to a successful MSA.

Fig 1A. The BioPhase 8800   Fig 1B. An 8-channel cartridge

3. Carna's New MSA System: SCIEX BioPhase 8800

SCIEX started as a triple quad mass spectrometry instrumentation and software company. It later expanded into LCMS and capillary electrophoresis equipment and software. The BioPhase 8800 (Figure 1A), which is routinely used for protein and nucleic acid analysis was chosen by Carna as the EZ Reader II replacement. The BioPhase 8800 has:
• A reusable 8 capillary channel cartridge compatible with 384-well plates, to allow for simultaneous multichannel analysis (Figure 1B)
• A capillary system allowing small volumes (~ 8 nL) to be analyzed and a potential difference to be applied
• A detection system compatible with fluorescein labelled peptide substrates
• Software for analysis of substrate/product pairs to calculate a substrate conversion rate and export of the data. The SCIEX software has an advantage over the old PE analysis software: it integrates the analyte peaks to calculate area, rather than measuring peak height, so that measurements are actually more reflective of the substrate/product ratios, than the PE system.
Carna also integrated the BioPhase 8800 with a separate robotic arm and stacking system, so that higher throughput of multi-plate runs were possible.

4. Optimization of conditions

Each of the separation conditions mentioned can be optimized in the BioPhase 8800, and the Assay and Profiling team at Carna Japan have examined each individual variable:

• Capillary System - The physical characteristics of the capillary system are different from the PE microfluidic system, being longer (30 cm) and having sample volumes of 8 nL. This directly impacts the resolution of the substrate/product peaks. It was found that altering the base flow rate changed the time period between peaks, as well as effecting peak broadening. Images of lower pressure vs higher pressure with integrated values are shown in Figure 2 A and 2 B.
• Separation Buffer – PE provided a 'separation buffer' for peptide analysis, which did not allow for correct separation within the BioPhase 8800. Using standard BioPhase buffers as a starting point, the final buffer was optimized with the concentration of cationic ions (Figure 2C).
• Combination of 2 parameters – Both pressurization and adding cationic ion made significant differences in the resolution of the analyte peaks (Figure 2D).
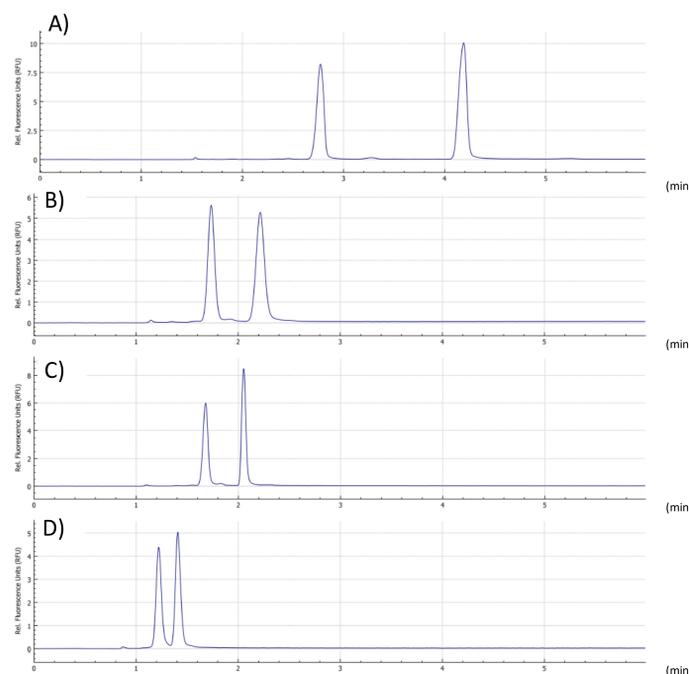

Fig 2. Peak change by separation conditions. A) Lower pressure, B) Higher pressure, C) Addition of cationic ion, and D) combination of Higher pressure and cationic ion.

5. New Data are consistent with Historical Data

• The key determinant of success for the PE to BioPhase 8800 migration is that data produced by the BioPhase 8800, should be equivalent to 20 years worth of historical data produced on the PE system: both for internal QC activities and for profiling activities for external clients.
• Once conditions were optimized for each substrate and product peptide pair, as described above, data produced from the PE system (height of peaks) and the BioPhase 8800 (peak integration), were compared. Both systems produce data that are very similar, and within the range of any normal day-to-day experimental variation. Examples of different kinase/peptide/reference compound combinations and respective IC50 curves can be seen in Figures 3, 4, and 5. Table 1 represents comparison data of IC50s of reference compound against each kinase.
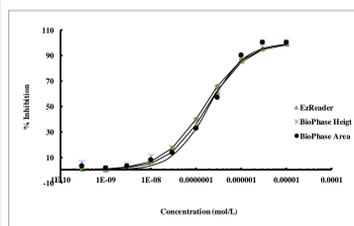
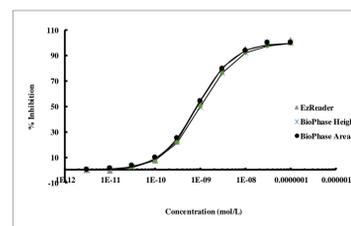Fig 3. IC50 comparison of SRM Substrate; BLK/Lyntide

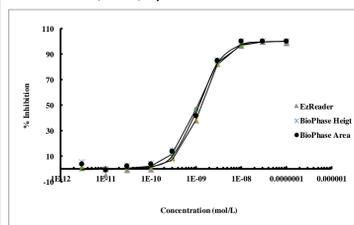Fig 4. IC50 comparison of FGFR3[K650M] Substrate; CSKtide

Fig 5. IC50 comparison of ROCK1 Substrate; LIMKtide

Table 1. IC50 summary of 3 kinases

Substrate	Kinase	EZ Reader	BioPhase Height	BioPhase Area
BLK/Lyntide	SRM	182	153	196
CSKtide	FGFR3 [K650M]	0.872	1.00	0.844
LIMKtide	ROCK1	1.30	1.08	1.15

Staurosporine was used for the reference.
IC50 unit; nM.

6. IC50 Correlation data of 47 substrates

Importantly, the kinase reaction conditions are unchanged – only the conditions of the detection step have been optimized for the new system. Excellent correlation between the new and historical data was therefore observed (Table 2 and Figure 6A and 6B).

Table 2. IC50 comparison of all substrate used in the profiling study

Kinase Name	Substrate Name	Reference Compound	IC50 (nmol/L)	
			EzReaderII	BioPhase8800
ABL_1mM	ABLtide	Staurosporine	1713	1728
ACK	WASP peptide	Staurosporine	3.0	3.0
AKT1	Crosstide	Staurosporine	2.0	2.0
ALK(F1174L)	Srctide	Staurosporine	2.2	2.3
AMPKα1/β1/γ1	SAMS peptide	Staurosporine	0.32	0.32
AurB/INCENP_1mM	Kemptide	Staurosporine	74	85
BUB1/BUB3	H2A peptide	Staurosporine	256	286
CaMK1δ	Synapsin peptide	Staurosporine	2.8	2.7
CDC7/ASK	MCM2 peptide	Staurosporine	16	17
CDK2/CycA2_1mM	Modified Histone H1	Staurosporine	14	12
CDK7/CycH/MAT1	CTD3 peptide	Staurosporine	12	16
CDK9/CycT1	CDK9 substrate	Staurosporine	8.0	8.6
CK2α1/β	CK2tide	TBB	50	54
CLK1	DYRKtide-F	Staurosporine	15	14
DAPK1	DAPK1tide	Staurosporine	2.9	2.4
EEF2K	EEF2Ktide	A-484954	378	325
EPHA1	Blk/Lyntide	Staurosporine	21	20
Erk5	EGFR-derived peptide	Staurosporine	211	228
FLT1_1mM	CSKtide	Staurosporine	6.4	5.4
GSK3β	CREBtide-p	Staurosporine	5.7	5.9
Haspin	Histone H3 peptide	Staurosporine	5.4	5.2
IKKβ	Modified IkBα-derived peptide	Staurosporine	325	432
IKKε	IkBα peptide	Staurosporine	0.9	1.0
IRAK4	IRAK1 peptide	Staurosporine	8.5	8.3
JAK1_1mM	JAK1 substrate peptide	Staurosporine	5.8	6.0
MAP2K7_Cascade	Modified Erktide	Staurosporine	975	1080
MARK4	CHKtide	Staurosporine	0.5	0.5
MNK1	RS peptide	Staurosporine	28	28
MST2_1mM	IRS1	Staurosporine	3.4	3.4
NEK9	CDK7 peptide	Staurosporine	253	254
PDHK2	PDHKtide	VER-246608	31	28
PDK1	T308tide	Staurosporine	4.8	3.9
PIM1	S6K2 peptide	Staurosporine	12	12
PKCα	PKC peptide	Staurosporine	0.30	0.27
PKD1	GS peptide	Staurosporine	1.3	1.5
PLK3	CDC25ctide	GW843682X	29	29
QIK_1mM	AMARA peptide	Staurosporine	8.8	9.0
ROCK1_1mM	LIMKtide	Staurosporine	45	39
RSK4_1mM	S6K peptide(N-FL)	Staurosporine	0.77	0.73
SGK2	SGKtide	Staurosporine	25	25
skMLCK	MLCtide	Staurosporine	36	34
SPHK2	Sphingosine	PF-543	389	453
TAK1-TAB1_1mM	LRRKtide	Staurosporine	32	35
TAOK2	TAOKtide	Staurosporine	35	36
TBK1_1mM	CKtide	Staurosporine	6.3	6.9
TNIK	Moesin-derived peptide	Staurosporine	1.3	1.4
WNK3	SPAKtide	K252a	1446	1390

Forty-seven peptide substrates were tested both in EZ Reader II and BioPhase8800. The IC50s of reference compounds in both systems are shown.
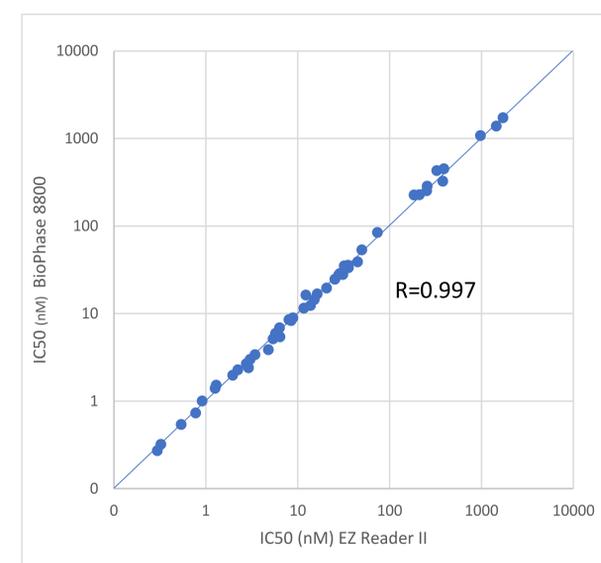

R=0.997

Fig 6A. IC50 correlation

7. The Future

The BioPhase 8800 has enabled a new generation of kinase assay analysis. Using its MSA expertise, Carna has developed new protocols and conditions for the BioPhase 8800, which are scalable for throughput, and produce data which are equivalent to historical data. Carna can quickly and accurately QC their own recombinant protein products and are also able to continue their exceptionally high-quality kinase inhibitor profiling service.

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