



## Kinome 2.0<sup>plus</sup> Compound Profiling Kits

### > *staurosporine selectivity, potency and reproducibility case study*

#### 1.0 background

Kinome 2.0<sup>plus</sup> Compound Profiling kits include protein arrays consisting of glass slides spotted with 336 human kinases and related proteins. The kinases are tethered using a proprietary tag which allows orientation of the kinases in a reproducible manner and which ensures that the proteins are folded and functional. Also included in the Kinome 2.0<sup>plus</sup> kit are fluorescently labeled Broad Specificity Ligands (BSLs). In the absence of a competitor, the BSLs bind to a wide range of arrayed kinases and this binding can be measured using a conventional microarray scanner. During the assay, the user's label-free ATP-competitive kinase inhibitor competes directly for binding and can displace the BSL from arrayed kinases. The magnitude of BSL binding therefore depends on the concentration and affinity of the competing compound and this competitive binding assay enables the determination of compound selectivity across a wide range of kinases in a single experiment. In addition, if the compound is tested at multiple concentrations, these data can be plotted against the binding of labeled ligand, and the IC<sub>50</sub> of the drug for each assayable kinase can be determined. The K<sub>d</sub> for the binding of labeled ligand for each kinase is supplied and can be used to convert the IC<sub>50</sub> to the inhibition constant, K<sub>i</sub>.

#### 1.1 screening strategy

Kinome 2.0<sup>plus</sup> Compound Profiling kits can be used to determine selectivity and potency for a wide range of kinases under identical experimental conditions. Selectivity assays give a measure of the specificity of a compound whilst potency determines the affinity of the compound for the kinases. The Kinome 2.0<sup>plus</sup> kit contains 10 identical Kinome arrays and sufficient Broad Specificity Ligand (BSL) to use all 10 arrays, either for selectivity or potency experiments. One kit will allow up to nine compounds to be assayed on individual arrays versus one control array using one BSL reporter. Alternatively, for greater confidence, four compounds can be screened in parallel on pairs of arrays and the binding compared with that on two control arrays.

Presented below is a case study showing results obtained using Kinome 2.0<sup>plus</sup> kits to determine the selectivity and potency of staurosporine. Staurosporine exhibits a broad range of specificity and potency for different kinases. With any compound that inhibits a kinase, it is important to know which other kinases are targeted by the molecule. In the first selectivity experiment, staurosporine was assayed in parallel with Gleevec™, Erlotinib and Gefitinib (Iressa™) at a concentration of 10 μM against the panel of kinases which bind BSL3-Cy3. In a separate experiment, the





effect of staurosporine on BSL2 binding is shown. Finally, competitive binding curves have been constructed to determine the potency of staurosporine inhibition for different kinases on Kinome 2.0 arrays using BSL3.

## 2.0 compound selectivity with BSL3

### 2.1 methods

Full instructions for performing drug selectivity experiments are included in the Protein Function Arrays Product Manual. In brief, compounds were dissolved in DMSO to give 10 mM stock solutions. A working solution of labeled ligand (BSL3-Cy3) was prepared by diluting the ligand 1 in 1000 in Ligand Assay Buffer and the working solution divided into 5 aliquots. The compounds were then diluted into the ligand working solutions to give 10  $\mu$ M solutions. A separate application note (“Recommendations for Compound Concentration in Selectivity Assays”) gives advice on the selection of doses for selectivity and potency experiments using Sense Proteomic Kinome arrays. An equivalent volume of DMSO was added to the control tube. After mixing, 4 mL of each solution was transferred into each of two wells of a QuadriPERM™ (Greiner BioOne) dish. Arrays were then removed from their storage solution and placed in clean Pap jars (Evergreen Scientific) containing 30 mL of Ligand Assay Buffer and were shaken for 5 min on ice. Arrays were then removed from the wash solutions and one array placed in each of the diluted inhibitor solutions in chambers of the QuadriPERM dishes. The assay dishes were then covered in foil to prevent photobleaching and were shaken gently at 20°C for 75 min. After incubation, the arrays were washed for 5 min in fresh Ligand Wash Buffer, centrifuged dry and scanned using a GenePix® 4200AL (Molecular Devices Inc.) microarray scanner. Each array image was saved as a 16-bit TIF file and was analyzed using ArrayPro® Analyzer (Media Cybernetics Inc.) using the GAL file present on the Analysis Tools CD provided with the kit. Data was exported from ArrayPro Analyzer as an Excel™ worksheet and was then pasted into the Kinome 2.0 Selectivity Analysis Workbook provided on the Analysis Tools CD.

### 2.2 selectivity data for staurosporine, Gleevec, Erlotinib and Gefitinib (Iressa)

The Kinome 2.0 Selectivity Analysis Workbook provides a quick and easy method to analyse, sort, and review data obtained from selectivity experiments. Once the data is pasted into the workbook, mean values and standard deviations for the replicates of each feature and percentage inhibition are automatically calculated. In total, 185 kinases were assayable in this experiment (i.e. the BSL3-Cy3 signal was above the **default threshold value** which is equal to the average binding to the four control proteins plus 3 x the average Standard Deviation of binding to the control proteins - a measure of non-specific binding). Within the Selectivity Analysis Workbook it is possible to filter the data by setting thresholds for three criteria: labeled ligand (BSL3-Cy3) binding to the controls (dRFU), the level of inhibition desired (% inhibition) and a confidence level (*p*-value) filter. The filters select the most significant differences between the control array and the drug





treated arrays for presentation. In this study, filters were set to identify events which showed both greater than 30% inhibition for those kinases above the default threshold value and those which showed differences between the controls and drug treated with *p*-values from t-tests less than 0.05 (confidence level >95%).

The number of kinases inhibited by the compounds is shown in Table 1 below.

**Table 1. Inhibition of labeled ligand, BSL3-Cy3, binding to kinases on Kinome 2.0 arrays by small molecule inhibitors.**

	Gefitinib (Iressa)	Gleevec	Erlotinib	Staurosporine
Kinases inhibited	8	3	10	110

The data in Table 1 clearly shows that the compounds assayed each have very different selectivities. As expected, the broad specificity inhibitor staurosporine inhibited labeled ligand binding to a wide range of kinases whereas Gleevec only inhibited three kinases above the set thresholds (see above).

A list of the top 40 kinases inhibited by staurosporine at 10  $\mu$ M and the percentage inhibition for each are shown in Table 2 below. The data are compared where possible with data from a SelectScreen™ assay (Invitrogen) for kinase inhibition with 10  $\mu$ M staurosporine. The SelectScreen assay measures kinase activity by phosphorylation of peptide substrates. In all cases there is very close correlation between the percentage inhibition obtained in the two sets of data showing that the binding assay gives comparable results to activity assays for kinase inhibition.





**Table 2. Top 40 kinases exhibiting greater than 30% inhibition of BSL3-Cy3 binding in the presence of staurosporine at 10  $\mu$ M. Highlighted in yellow are kinases for which data is compared between the Sense Proteomic and SelectScreen Kinase assays.**

	Sense Proteomic data			SelectScreen data*	
	Kinase Group	Protein symbol	Average %inhibition	Protein symbol	Average %inhibition
1	Other	AURKB	99		
2	CAMK	PRKD2	99	PRKD2 (PKD2)	99
3	Other	CAMKK2	99		
4	AGC	RPS6KB1	99	RPS6KB1 (p70S6K)	100
5	Other	AAK1	98	AURKB (Aurora B)	100
6	CAMK	PIM2	98	PIM2	97
7	AGC	STK32A	98		
8	AGC	SGK	98	SGK (SGK1)	98
9	n.a.	STK6	97	STK6 (Aurora A)	99
10	Other	STK18	97		
11	CMGC	SRPK2	97	SRPK2	73
12	Other	PACE-1	96		
13	AGC	SGK2	96	SGK2	101
14	AGC	RPS6KA2	96	RPS6KA2 (RSK3)	101
15	CAMK	CHEK1	95	CHEK1 (CHK1)	101
16	CAMK	STK17B	94		
17	AGC	RPS6KA1	94	RPS6KA1 (RSK1)	98
18	TK	RET_aa 658-1114	93		
19	STE	PAK4	92	PAK4	97
20	CAMK	STK29	92		
21	AGC	SGKL	91	SGKL (SGK3)	99
22	AGC	RPS6KA4	90	RPS6KA4 (MSK2)	104
23	CAMK	PIM1	89	PIM1	98
24	Atypical	CABC1	88		
25	TKL	ACVR1	88		
26	CMGC	GSK3B	86	GSK3B (GSK3 beta)	101
27	CAMK	SNARK	86		
28	Atypical	ADCK4	85		
29	AGC	PRKX	85		
30	CMGC	CDK9	85		
31	Other	TOPK	84		
32	CAMK	C20orf97	84		
33	TK	ACK1	84		
34	Atypical	BCKDK	83		
35	TK	RET	83	RET	101

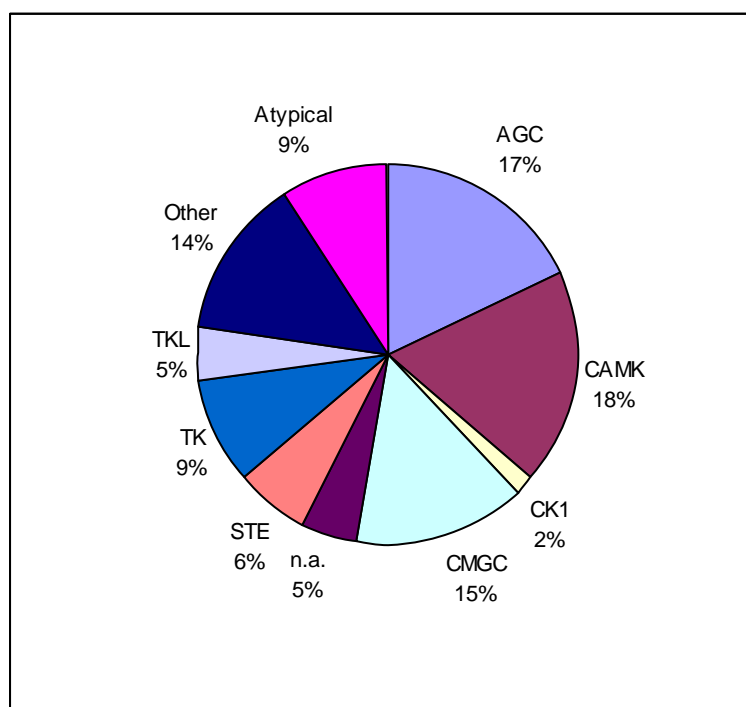


36	Other	NEK11	83
37	STE	MAP2K5	82
38	CK1	VRK3	82
39	Other	STK16	81
40	Other	MGC16169	80

\*Data from [www.invitrogen.com/Downloads/SelectScreen\\_Data\\_193.pdf](http://www.invitrogen.com/Downloads/SelectScreen_Data_193.pdf).

Using the Analysis Workbook it is possible to sort the data by Kinase Type or Kinase Group (see Kinome 2.0 arrays product description on the Analysis Tools CD) to determine which kinase sub-groups as defined by the classifications of Manning *et al.* (Science, 2002, 298:1912) and Park *et al.* (PNAS, 2005, 102:8114)<sup>a</sup> are most inhibited by the compounds. Figure 1 shows the distribution of all 110 kinases inhibited by staurosporine by more than 30% arranged according to kinase group.

Figure 1. Inhibition of kinases by staurosporine arranged by kinase group.



### 2.3 drug selectivity conclusions

The compound selectivity results show clear differences between the selectivity of Gefitinib, Gleevec, Erlotinib and staurosporine. Staurosporine as expected showed the least specificity and inhibited a broad range of kinases from different kinase groups. Of the 40 inhibited kinases listed above 22 showed more than 90% inhibition



with 10  $\mu\text{M}$  staurosporine. The data obtained using the competitive binding assay gave very comparable results to those obtained using a kinase activity assay (SelectScreen) and it can therefore be concluded that binding and activity assays for kinase inhibition give comparable results. The Kinome 2.0<sup>plus</sup> competitive binding assay however allows parallel analysis of more than 150 kinases in a single assay.

### 3.0 compound selectivity with BSL2 and staurosporine

#### 3.1 methods

Compound selectivity using BSL2 as the reporter ligand were performed as described in section 2.1. In this experiment the effect of staurosporine at a final concentration of 10  $\mu\text{M}$  was determined. Five kinome 2.0 arrays were assayed in parallel to determine the reproducibility of inhibition.

#### 3.2 selectivity data for staurosporine

Staurosporine is a broad specificity kinase inhibitor. To demonstrate reproducibility five staurosporine treated arrays were compared with a single control array. A data filter of >30% inhibition and >95% confidence was applied. The five compound treated arrays gave extremely consistent results with 21 or 22 kinases being inhibited on all the arrays. Table 3 shows a comparison of the percentage inhibitions for the kinases inhibited more than 30% on any of the five staurosporine treated arrays. In most cases the coefficient of variation of percentage inhibition between the five arrays is less than 8% and the average for all 22 inhibited kinases was only 4.2% indicating that essentially the same results can be obtained on different arrays within an assay.





**Table 3. Shows percentage inhibition for kinases by 10  $\mu$ M staurosporine. The mean, standard deviations and coefficients of variation (CV%) demonstrate the consistency of selectivity on the five arrays.**

symbol	Stauro1 %inhibition	Stauro2 %inhibition	Stauro3 %inhibition	Stauro4 %inhibition	Stauro5 %inhibition	Mean	StDev	CV%
ABL2	42	37	39	43	45	41	3.2	7.7
ACK1	39	36	36	36	37	37	1.3	3.4
ACVR1	80	80	79	80	81	80	0.6	0.8
AURKB	66	66	62	67	66	66	1.9	2.9
BLK	84	84	83	85	85	84	1.1	1.3
BMPR1A	45	44	44	48	49	46	2.2	4.7
BMPR1B	53	52	52	56	56	54	1.9	3.5
DDR1	51	48	44	44	46	47	2.9	6.1
DDR1_aa 444-913	61	60	61	59	62	61	1.2	2.0
FES	90	90	90	91	92	91	0.7	0.7
FGFR2	85	85	85	85	85	85	0.4	0.4
FGFR2_aa 399-821	89	89	89	89	91	90	0.9	1.0
FRK	47	52	46	50	50	49	2.6	5.3
JKK	77	81	81	76	78	78	2.3	2.9
MAP2K5	79	79	79	79	80	80	0.5	0.6
NEK11	74	73	69	73	75	73	2.2	3.0
NEK3	52	53	49	46	50	50	2.9	5.7
NLK	63	61	59	63	64	62	2.0	3.3
RET	28	36	30	37	38	34	4.4	13.0
RET_aa 658-1114	82	80	81	78	81	80	1.3	1.6
RIOK2	31	31	31	27	33	31	2.3	7.6
STK6	60	63	41	59	58	56	8.8	15.7
	Average=						8.8	4.2

### 3.3 selectivity analysis with BSL2 conclusions

The data show that determination of selectivity is extremely consistent with all the compound treated arrays showing inhibition of the same kinases in all but two cases. In these two exceptions, inhibition was detected but was just below the threshold set. These results suggest that accurate data would be achievable with only single arrays in a selectivity assay, however, using single arrays per compound or concentration is less likely to detect inconsistencies in assay performance and it is therefore recommended that duplicate arrays are used.

### 4.0 compound potency

Following drug selectivity, the potency of drug interactions can be determined by constructing competitive binding curves for the inhibition of kinases by small molecule inhibitors. These experiments on Sense Proteomic's arrays allow comparison of compound potencies against a wide range of kinases under identical conditions.

### 4.1 methods

Full instructions for performing drug potency experiments are included in the Protein Function Arrays Product Manual. In brief, staurosporine was dissolved in DMSO to give a 10 mM stock solution. A working solution of labeled ligand (BSL3-





Cy3) was prepared by diluting the ligand 1 in 1000 in Ligand Assay Buffer and the working solution divided into 10 x 5 mL aliquots. The staurosporine was then serially diluted in DMSO to give a 4-fold dilution series of concentrated solutions and these were then added to the BSL3-Cy3 working solutions to give a range of concentrations from 100  $\mu$ M downwards. After mixing, 4 mL of each solution was transferred into a well of a QuadriPERM (Greiner BioOne) dish. Arrays were then removed from their storage solutions, placed in clean Pap jars (Evergreen Scientific) in 30 mL of Ligand Assay Buffer and were shaken for 5 min on ice. Arrays were then removed from the wash solutions and one array placed in each staurosporine solution in the filled chambers of the QuadriPERM dishes. The assay dishes were then covered in foil to prevent photobleaching and shaken gently at 20  $^{\circ}$ C for 75 min. After incubation, the arrays were washed for 5 min in fresh Ligand Wash Buffer, centrifuged dry and scanned using a GenePix 4200AL microarray scanner. Each array image was saved as a 16-bit TIF file and was analyzed using ArrayPro Analyzer (Media Cybernetics Inc.) using the GAL file present on the Analysis Tools CD provided with the kit.

#### 4.2 drug potency results

Data was exported from ArrayPro Analyzer as an Excel<sup>TM</sup> worksheet and then pasted into the Kinome 2.0 Potency Analysis Workbook provided on the Analysis Tools CD. Algorithms in the workbook calculate the  $IC_{50}$  for the compound for each kinase and use the  $K_d$  values for binding of BSL3-Cy3 to each kinase, if available, to determine the  $K_i$  for the compound interactions. The software plots a potency curve for each compound interaction and these were sorted according to score<sup>b</sup>. A total of 168 curves gave  $K_i$  values, but using the curve score this list was reduced to 78 high quality curves. Curves were obtained for all of the kinases listed in Table 4 which were identified as the top hits in the selectivity assay. The curve fitting parameters  $R^2$ ,  $IC_{50}$  and  $K_i$  are shown for each of the top 40 kinase hits. Most of the curves in this list have  $R^2$  values greater than 0.9 indicating high confidence curve fits. The kinases exhibited a broad range of affinities for staurosporine from 200 pM for CAMKK2 to 576 nM for C20orf97.

**Table 4. Top 40 kinases exhibiting > 30% inhibition of BSL3-Cy3 binding in comparison to results obtained for these kinases in the potency assay.**

			%inhibition			%inhibition	
	Symbol	Kinase group	in selectivity assay	$IC_{50}$ [nM]	$R^2$	$K_i$ [nM]	in potency assay
1	AURKB	Other	99.0	8.7	0.99	4.6	99.5
2	PRKD2	CAMK	98.9	15.7	0.99	10.3	99.5
3	CAMKK2	Other	98.7	0.5	0.91	0.2	99.8
4	RPS6KB1	AGC	98.7	14.1	0.98	6.4	99.7
5	AAK1	Other	98.3	569.0	0.98	6.1	99.6
6	PIM2	CAMK	98.1	33.0	0.96	11.5	99.6
7	STK32A	AGC	98.0	0.5	0.93	0.3	98.6

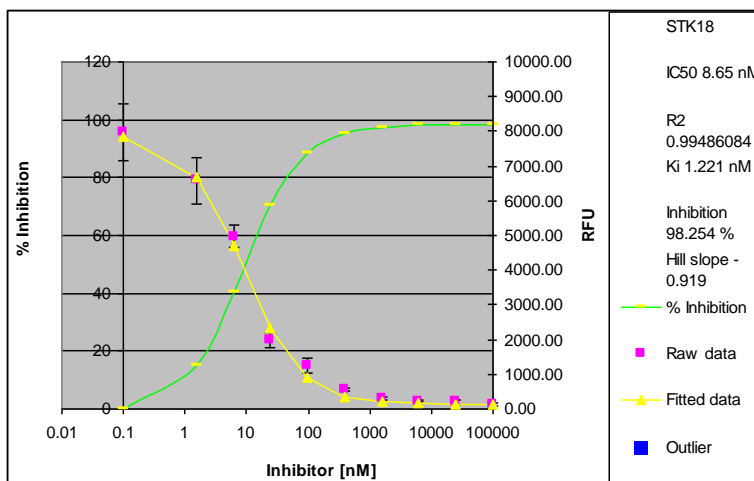
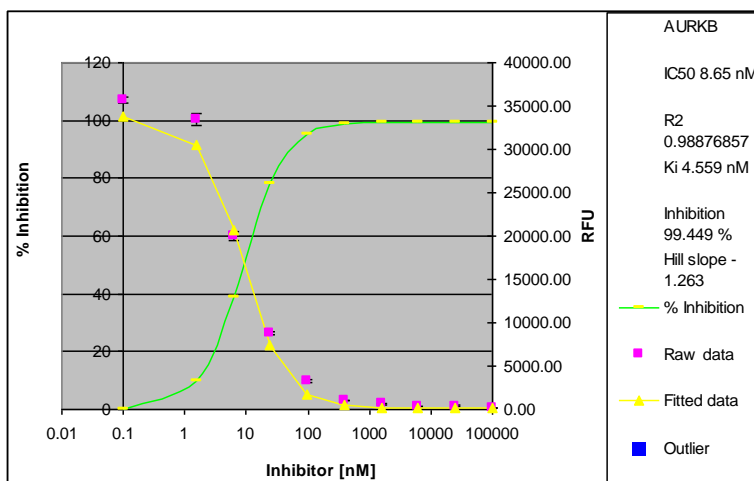


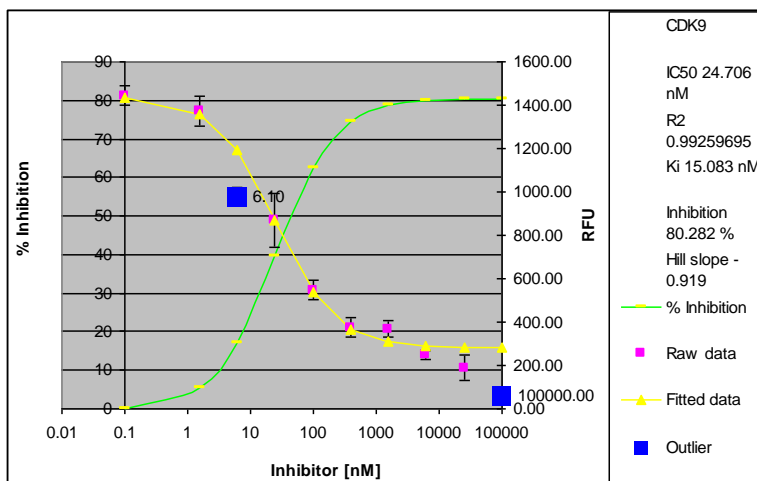
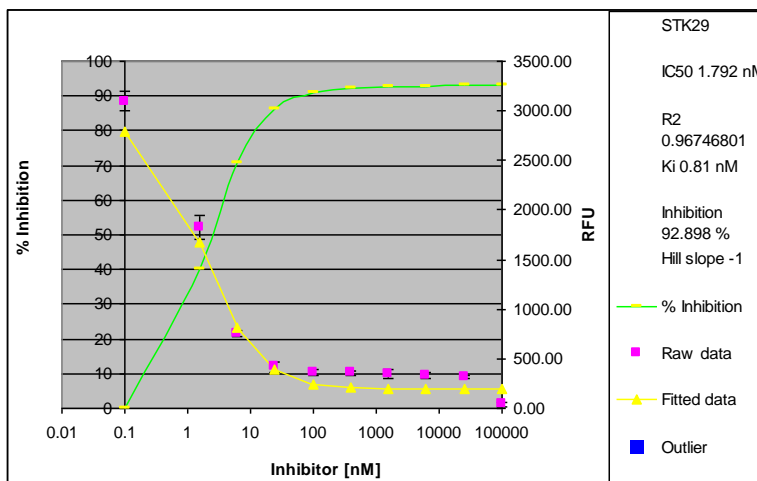
8	SGK	AGC	97.8	59.7	1.00	9.3	99.3
9	STK6	n.a.	97.3	1.9	0.97	1.0	98.8
10	STK18	Other	97.3	8.9	0.99	1.3	98.3
11	SRPK2	CMGC	96.9	0.5	0.92	0.3	99.6
12	PACE-1	Other	96.4	0.5	0.92	0.3	98.5
13	SGK2	AGC	96.4	106.5	0.98	39.8	99.3
14	RPS6KA2	AGC	96.0	9.0	1.00	3.1	96.6
15	CHEK1	CAMK	95.3	2.5	0.99	1.8	97.6
16	STK17B	CAMK	94.4	370.3	1.00	7.4	99.1
17	RPS6KA1	AGC	94.3	18.1	0.99	12.3	94.7
18	RET_aa 658-1114	TK	92.5	5.2	0.95	2.7	95.1
19	PAK4	STE	92.1	0.8	0.95	0.7	89.3
20	STK29	CAMK	92.1	1.8	0.97	0.8	98.9
21	SGKL	AGC	91.2	23.7	0.98	7.1	96.2
22	RPS6KA4	AGC	90.3	80.1	0.99	13.5	92.4
23	PIM1	CAMK	88.8	101.5	0.94	56.6	94.8
24	CABC1	Atypical	88.2	57.4	0.96	1.6	88.4
25	ACVR1	TKL	87.5	1101.1	0.99	177.7	97.5
26	GSK3B	CMGC	86.3	204.1	0.89	38.6	89.0
27	SNARK	CAMK	86.2	0.5	0.91	0.3	91.1
28	ADCK4	Atypical	85.0	372.3	0.93	437.9	91.0
29	PRKX	AGC	84.5	6.9	0.91	4.2	91.1
30	CDK9	CMGC	84.5	24.7	0.99	15.1	80.3
31	TOPK	Other	84.5	0.6	0.92	0.4	93.1
32	C20orf97	CAMK	84.1	794.3	0.91	576.0	87.0
33	ACK1	TK	83.9	689.6	0.95	124.3	92.0
34	BCKDK	Atypical	83.2	79.8	0.93	1.2	87.6
35	RET	TK	83.2	6.8	0.97	2.5	89.1
36	NEK11	Other	82.8	1188.7	0.99	276.5	96.1
37	MAP2K5	STE	82.1	38.2	0.85	27.6	88.7
38	VRK3	CK1	81.8	149.9	0.94	1.6	92.7
39	STK16	Other	81.0	404.8	0.87	239.7	89.1
40	MGC16169	Other	79.9	0.7	0.88	0.5	91.2

The data in Table 4 also shows a comparison of the % inhibition in the selectivity and potency assays using BSL3. It is readily seen that the percent inhibition in each assay is very similar showing that these kinases were nearly maximally inhibited at 10  $\mu$ M staurosporine as used in the selectivity assay. Some representative curves and their accompanying parameters for curve fitting are shown in Figure 2 below.



Figure 2. Shows fitted curves for four of the kinases listed in Table 4.





### 4.3 drug potency conclusions

Kinome 2.0<sup>plus</sup> kits can be used to produce data for potency of compounds for many kinases in parallel in a single experiment with same-day results. In this example  $K_i$  values for the interaction of staurosporine with 168 kinase targets were determined. The reproducibility of the arrays enables high quality curve fitting. The data show that staurosporine exhibits a wide range of potencies for different kinases and allows unprecedented analysis of the potency of this broad spectrum inhibitor for a large panel of kinases. Insufficient  $K_i$  data was available from other sources to compare results with the broad range of  $K_i$  values generated in this experiment.





## 5.0 overall conclusions and product summary

Kinome 2.0<sup>plus</sup> protein array kits have been used to determine the selectivity and potency of staurosporine against a broad range of kinases in an array format. Both experiments yielded high quality data which in the case of selectivity was comparable to that obtained in activity assays using the SelectScreen technology (Invitrogen). Protein arrays provide a facile and quick method to rapidly screen for inhibitor kinase interactions and to determine the potency of inhibitors against a large kinase panel in parallel.

## 6.0 ordering details

PFA1003 - Kinome 2.0<sup>plus</sup> protein function (10) array kit - compound profiling v1  
PFA1002 - Kinome 2.0 protein function (10) array kit

## 7.0 trademarks and legal

Gleevec is a trademark of Novartis AG.  
Iressa is a trademark of Astra Zeneca plc.  
Cy and CyDye are trademarks of GE Healthcare Biosciences.  
Excel and MS are trademarks of the Microsoft Corporation.  
SelectScreen is a trademark of Invitrogen Corporation.  
ArrayPro is a trademark of Media Cybernetics Inc.  
GenePix is a registered trademark of Molecular Devices Inc.  
QuadriPERM is a trademark of Greiner-BioOne.





To find out more go to [www.senseproteomic.com](http://www.senseproteomic.com)

mail us at [info@senseproteomic.com](mailto:info@senseproteomic.com)

or call us +44(0) 1628 513500

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<sup>a</sup> The Kinase annotation is based on Manning *et al.* Science (2002) 298:1912 and Park *et al.* PNAS (2005) 102:8114. Kinases are grouped in 10 different categories AGC, CAMK, CK1, CMGC, STE, TK, TKL, others, non assigned and atypical kinases.

AGC: containing PKA, PKG and PKC families

CAMK: Calcium/Calmodulin dependent kinases

CK1: Casein Kinase

CMGC: containing CDK, MAKpK, GSK3 and CLK families

n.a.: not assigned

STE: homologs of yeast Sterile7, Sterile 11 and Sterile 20 kinases

TK: Tyrosine kinase

TKL: Tyrosine Kinase like

Other

Atypical

<sup>b</sup> The scoring algorithm in the Binding Curve Analysis Workbook is calculated on the  $R^2$  values for the curve fit, the percentage inhibition and the noise (a measure of data variability). Only those curves which exceed the set scores are displayed. The score cut-off can be set by the user to filter the data to show only the best fitting curves and data.

