Evaluation of the use of different cell types as a substrate pool for *in vitro* kinase reaction

Authors

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Abstract

We evaluated the effect of cell lines used to obtain *in vitro* substrates on phosphorylation motifs. Three kinases with representative phosphorylation motifs were evaluated in this study. And, we demonstrated that the difference of cell lines did not affect phosphorylation motifs obtained from *in vitro* kinase reaction.

Introduction

Protein phosphorylation plays an important role in intracellular signal transduction and regulates various biological phenomena such as cell growth and differentiation. Mass spectrometry (MS)-based phosphoproteomics has made it possible to identify thousands of phosphorylation sites in a single measurement[1,2]. However, despite the vast amount of phosphorylation site information stored in public databases[3–5], little is known about the physiological substrates (*in vivo*) of kinases[6].

Perturbation of kinase activity in living cells, either by drug treatment or by knockdown/knockout of specific kinases, makes it possible to assess changes in phosphorylation levels *in vivo*[7,8]. However, these methods also indirectly affect downstream kinases, which hinders direct substrate identification[9]. *In vitro* kinase assays are used to validate direct interactions between a kinase and a candidate substrate. Therefore, direct matching of phosphorylation sites of known *in vitro* substrates is the simplest and most straightforward method to extract reliable site information[10,11]. Recently, we performed *in vitro* kinase reactions using proteins extracted from human cells, followed by enrichment of the phosphopeptides for LC/MS/MS analysis, and identified a total of 198,536 substrates for 385 different kinases[12]. However, a limitation of this screening method is the comprehensiveness

of the *in vitro* substrate profile. That is, if the expressed protein profile of the substrate pool which is used for the *in vitro* kinase assay does not match that of the samples used for the *in vivo* experiments, the number of *in vivo* substrate candidates is necessarily limited. Indeed, the proteome profiles are different in cells. On the other hand, phosphorylation motifs are abstracted information extracted from phosphorylation sites and it has been believed that phosphorylation motifs can be used universally regardless of the concordance of proteome profiles. However, it is unclear whether the extracted phosphorylation motifs depend on the proteome profile of the substrate pool which was used for the *in vitro* kinase assay. Therefore, in this study, we evaluated whether the effect of cell lines used to obtain *in vitro* substrate on phosphorylation motifs.

Method

Cell culture

HeLa cells and MCF7 cells were cultured in DMEM (Fujifilm Wako, Osaka, Japan) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) and 100 µg/mL penicillin/streptomycin (Fujifilm Wako). Jurkat cells were cultured in RPMI1640 (Fujifilm Wako) containing 10% fetal bovine serum and 100 µg/mL penicillin/streptomycin. All cells were maintained in an incubator at 37°C under humidified 5% CO_2 in air.

In vitro kinase assay using cell extracts

Cells were washed and harvested with ice-cold PBS. Proteins were extracted from cells with phase-transfer surfactant[13], and the buffer was replaced with 40 mM Tris-HCl (pH7.5) by ultrafiltration using an Amicon Ultra 10K at 14,000 g. Protein amount was determined with a BCA protein assay kit and the solution was divided into aliquots containing 100 µg. Proteins were reacted with 1 µL of TSAP, and TSAP was inactivated by heating to 75°C for 1 hr. For *in vitro* kinase reaction, proteins were reacted with 1 µl recombinant kinase (Carna Biosciences, Kobe, Japan) or water at 37°C in the reaction buffer (40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 1 mM ATP) for 3 hr. Then, proteins were reduced with 10 mM DTT, alkylated with 50 mM IAA, diluted 2-fold with 50 mM ABC buffer and digested with Lys-C (w/w 1:100) and trypsin (w/w 1:100). Then, phosphopeptides were enriched from the tryptic peptides with HAMMOC[14], desalted using SDB-XC StageTips, and suspended in the loading buffer (0.5% TFA and 4% ACN) for LC/MS/MS analyses.

Global proteome analysis

The aliquots containing proteins were reduced with 10 mM DTT, alkylated with 50 mM IAA, diluted 2-fold with 50 mM ABC buffer and digested with Lys-C (w/w 1:100) and trypsin (w/w 1:100). Then, peptides were desalted using SDB-XC StageTips, fractionated at basic pH[15] and suspended in the loading buffer (0.5% TFA and 4% ACN) for LC/MS/MS analyses.

NanoLC/MS/MS analyses

NanoLC/MS/MS analyses were performed on an Orbitrap Fusion Lumos (Thermo Fisher Scientific), connected to an Ultimate 3000 pump (Thermo Fisher Scientific) and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Peptides were separated on a self-pulled needle column (150 mm length x 100 µm ID, 6 µm opening) packed with Reprosil-C18 AQ 3 µm reversed-phase material (Dr. Maisch, Ammerbuch, Germany). The flow rate was set to 500 nL/min. The mobile phase consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid in 80% acetonitrile. Three-step linear gradients of 5-10% B in 5 min, 10-40% B in 100 min, and 40-100% B in 5 min were employed.

For global proteome analysis, the MS scan range was *m/z* 300-1500. MS scans were performed by the Orbitrap with *r* = 120,000 and subsequent MS/MS scans were performed by the Orbitrap with *r* = 1,5000. Auto gain control for MS was set to 4.00 X 10^5 and that for MS/MS was set to 5.00 X 10^4. The HCD was set to 30.

For phosphopeptides obtained by *in vitro* kinase reaction, the MS scan range was *m/z* 300-1500. MS scans were performed by the Orbitrap with *r* = 120,000 and subsequent MS/MS scans were performed by the Orbitrap with *r* = 1,5000. Auto gain control for MS was set to 4.00 X 10^5 and that for MS/MS was set to 5.00 X 10^4. The HCD was set to 30.

Database searching

For all experiments, the raw MS data files were analyzed by MaxQuant v1.6.17.0 [16]. Peptides and proteins were identified by means of automated database searching using Andromeda against the human SwissProt Database (version 2020-08, 20,368 protein entries) with a precursor mass tolerance of 20 ppm for first search and 4.5 ppm for main search and a fragment ion mass tolerance of 20 ppm. Enzyme was set as Trypsin/P with two missed cleavage allowed. Cysteine carbamidomethylation was set as a fixed modification. Methionine oxidation and acetylation on protein N-term were set as variable modifications. For phosphopeptides, phosphorylations on serine, threonine and tyrosine were also set as variable modifications. The search results were filtered with FDR < 1% at the peptide spectrum match (PSM) and protein levels.

Phosphosites were filtered to accept only those in which the localization score was > 0.75.

Data analysis

The peak area of each peptide in MS1 was quantified using MaxQuant[17]. Missing values were imputed from a normal distribution of log2 intensity using a default setting in Perseus[18]. For the following analysis of phosphoproteome data, the leading proteins were used and only quantitative values derived from monophosphorylated peptides were used. For the following analysis of global proteome data, the lead protein out of Majority protein IDs was used.

PWM score

The probability of observing residue in position from *in vitro* substrates for CK2, ERK1 or PKA is computed as follows:

$$p(x,i) = \frac{f_{x,i} + c(x)}{N + \varepsilon}$$

$$c(x) = p(x) \cdot \varepsilon$$

where $f_{x,i}$ is the frequency of observing residue x at position i and N is the total number of sequences. $c(x)$ is a pseudo count function which is computed as the probability of observing residue x in the proteome, $p(x)$, multiplied by ε, defined as the square root of the total number of sequences used to train the position weight matrix (PWM). This avoids infinite values when computing logarithms. Probabilities are then converted to weights as follows:

$$w_{x,i} = log_2 \frac{p(x,i)}{p(x)}$$

where $p(x)$ = background probability of amino acid x; $p(x,i)$ = corrected probability of amino acid x in position i; $w_{x,i}$ = PWM value of amino acid x in position i. Given a sequence q of length l, a score is then computed by summing log_2 weights:

$$\lambda = \sum_{i=1}^{l} W_{q_i,i}$$

where q_i is the i th residue of q. In this study, the score was computed using the flanking ±7 residues surrounding each phosphosite.

Results and Discussion

In vitro kinase substrates obtained with different cell lines

The Position Weight Matrix (PWM)[19] represents the characteristics of the sequence around the phosphorylation site targeted by a given kinase[20]. Here, the columns of the matrix represent the positions relative to the phosphorylation site, and the rows represent the amino acids. The values of the matrix are log2 transformed probabilities that an amino acid occurs at a position. These probabilities are used to predict the putative substrates, and the PWM scores represent the certainty of those sequences as substrates (see method). This method has been implemented in several kinase substrate prediction methods and applied to *in vivo* kinase substrate identification[20].

The construction of PWMs requires the substrate information as a training set, and the substrate information obtained by *in vitro* kinase reactions against proteins extracted from cells is very useful. However, when using extracted proteins from different cells, the proteome profiles are different. Therefore, the *in vitro* substrate information acquired may depend on the used cell, but the impact of these differences on PWM is not known.

Here, we assessed whether the PWMs based on *in vitro* substrates obtained using cell-extracted proteins are dependent on the cells used to acquire the *in vitro* substrates. We selected three cell lines (HeLa, MCF7, and Jurkat) that are commonly used and known to have different proteome profiles[21].

Then, for the *in vitro* kinase reaction, we selected three kinases which have different substrate specificities (CK2: acidophilic, PKA: basophilic, and Erk1: proline-directed). First, proteins were extracted from each cell. Next, TSAP was added to the proteins to dephosphorylate the endogenous phosphorylation sites, and then TSAP was inactivated by heating. Then, recombinant kinase was added to the protein for kinase reaction, and the kinase-reacted sample was digested into the peptides. The phosphorylated peptides were enriched by hydroxy acid modified metal oxide chromatography and analyzed by LC/MS/MS[14]. The phosphorylated peptides identified in the control sample (kinase (-)), in which distilled water was added instead of recombinant kinase, were excluded from those identified in the sample that reacted with each kinase (kinase (+)), and the final list of peptides phosphorylated by the *in vitro* kinase reaction was determined. To confirm the different profiles of the *in vitro* substrates derived from different cell-extracted proteins, the intensities of the *in vitro* substrates were plotted (Fig. 1). The correlations of intensities between the three replicate experiments were all high ($0.89 < R < 0.98$), and the correlations of intensities

between different cell lines were low (0.66 < R < 0.80) for all kinases. In addition, the intensity ratios of protein or *in vitro* substrate between the two cell lines were plotted, and the intensity ratios between protein and *in vitro* substrate were correlated (Fig. 2). These results indicate that the protein expression levels in each cell bias the *in vitro* substrate information.

PWM from *in vitro* substrates obtained with different cell lines

Next, we evaluated the performance of kinase substrate prediction. For this evaluation, only serine substrates were included because the number of threonine substrates was small. The *in vitro* substrates between each cell line were classified into three classes based on abundance ratio (abundance ratio >2, 2>abundance ratio >1/2, abundance ratio <1/2) (Fig. 3A), and for each class, 400 or 1000 *in vitro* substrates were randomly selected as the training set and PWMs were generated (Fig. 3B). To evaluate the performance of each class-derived PWM in predicting kinase substrates, we performed Receiver Operatorating Characteristic (ROC) curve analysis. As a test set, we randomly extracted 200 known substrates of each kinase from the public database PhosphoSitePlus (positive data set), and combined them with 200 serine peripheral sequences randomly extracted from the human proteome (negative data set). We calculated PWM scores for these test sets using the PWMs from each class. The results showed that the PWMs generated from each class had similar AUCs (Fig. 4). This trend was confirmed for all kinases and cell lines. These results indicate that the prediction of kinase substrates based on PWMs is not affected by differences in *in vitro* substrate abundance between cell lines. This trend was also confirmed using a training set consisting of different numbers of *in vitro* substrates (Fig. 4). Furthermore, increasing the number of *in vitro* substrates in the training set improved the performance of substrate prediction (Fig. 5).

Conclusions

Here, we evaluated the effect of cell lines used to obtain *in vitro* substrates on phosphorylation motifs. Three kinases with representative phosphorylation motifs were evaluated in this study. And, we demonstrated that the difference of cell lines did not affect phosphorylation motifs.

There are more than 10,000 proteins in the cell, and each protein is composed of a diverse amino acid sequence. Therefore, even if the expression levels of some proteins differ, the abundance of a particular motif sequence did not fluctuate greatly and phosphorylation motifs might not be changed by the substrate pool. We anticipate

that the results of our evaluation would be also applicable to kinases with other phosphorylation motifs and the cell extracts would remain one of the substrate pools for the profiling of phosphorylation motifs.

Author contributions

T.N. and Y.I. designed research. T.N. performed research and analyzed data. T.N. and Y.I. wrote the paper.

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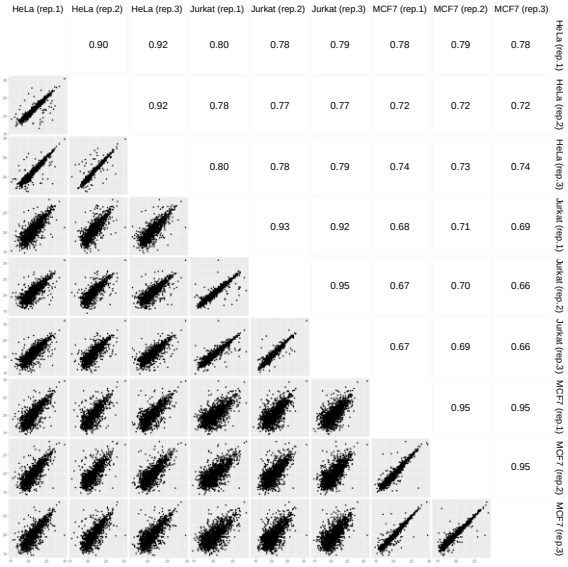
A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0.92 0.78 0.77 0.77 0.72 0.72 0.72
0.80 0.78 0.79 0.74 0.73 0.74
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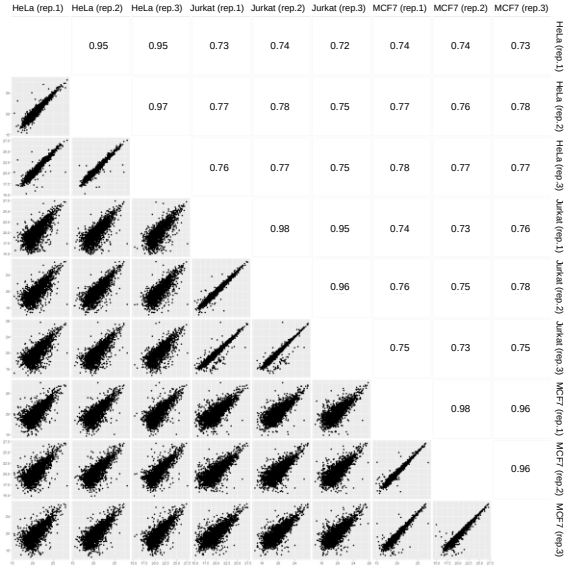
0.95

B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0.97 0.77 0.78 0.75 0.77 0.76 0.78
0.76 0.77 0.75 0.78 0.77 0.77
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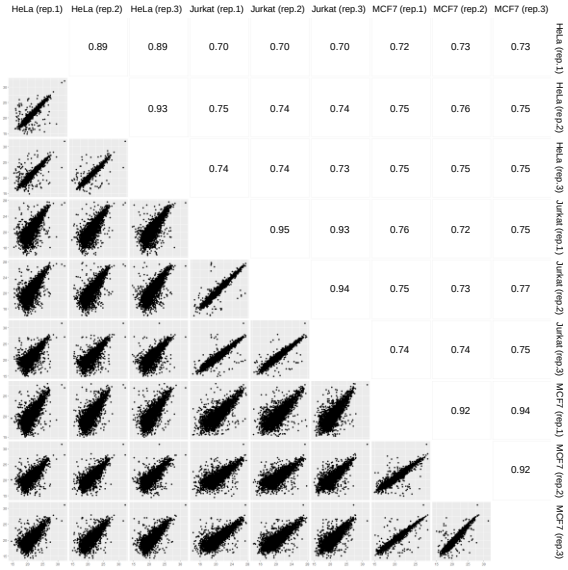
C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0.95 0.93 0.76 0.72 0.75
0.94 0.75 0.73 0.77
0.74 0.74 0.75
0.92 0.94
0.92

Fig. 1 Correlation of intensity of *in vitro* substrate derived from each cell

Pearson correlation coefficients of intensity (log 2) were calculated for phosphorylation sites obtained by *in vitro* kinase reactions for each cell-extracted protein.

(A) CK2 (B) ERK1 (C) PKA

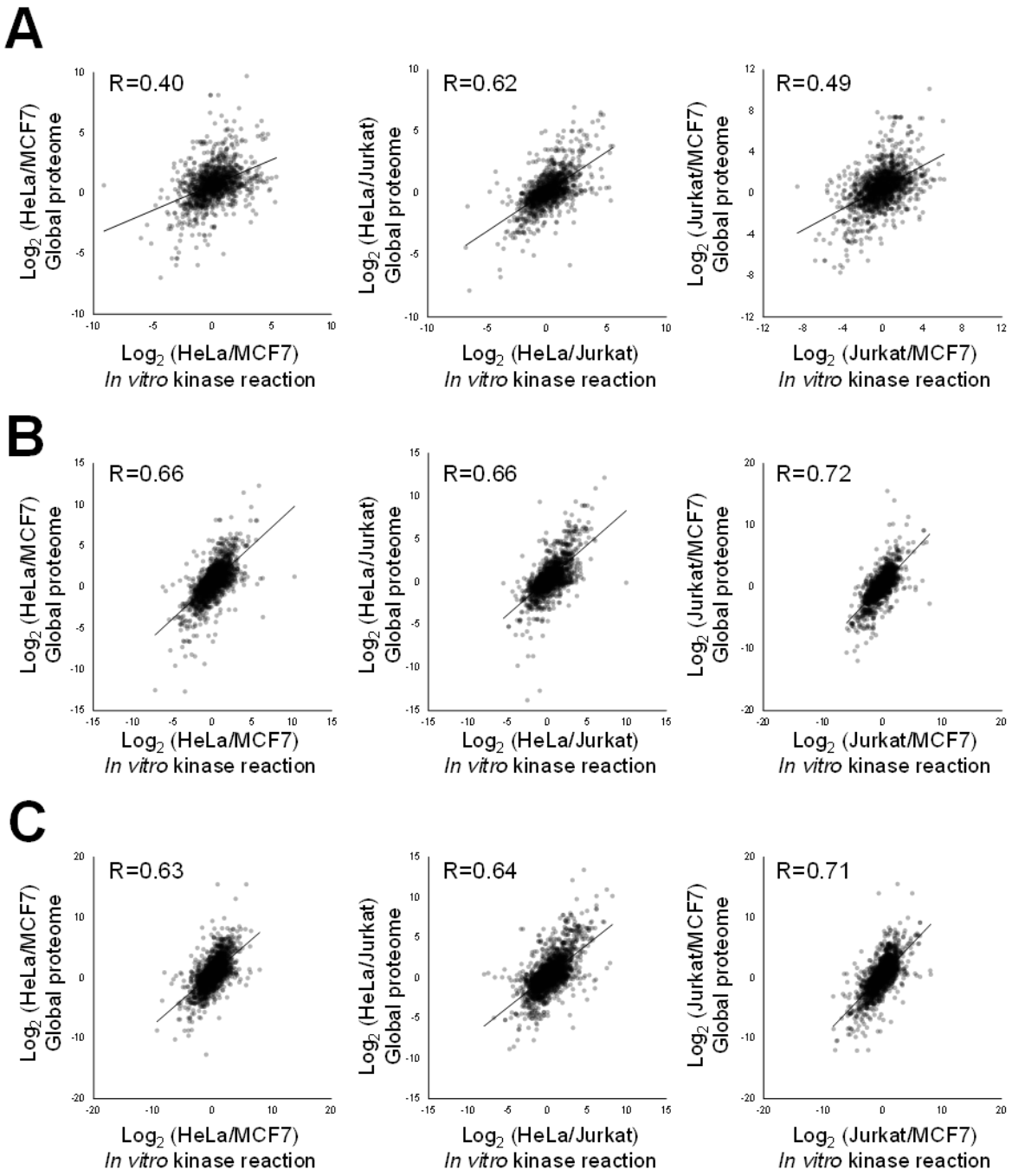


Fig. 2 Correlation between protein expression and *in vitro* substrate abundance
The ratio of given protein expression levels between cell lines was compared with the ratio of the abundance of *in vitro* substrate on the given protein between cell lines. The correlation coefficients in the graphs were calculated by Pearson correlation coefficient. (A) CK2 (B) ERK1 (C) PKA

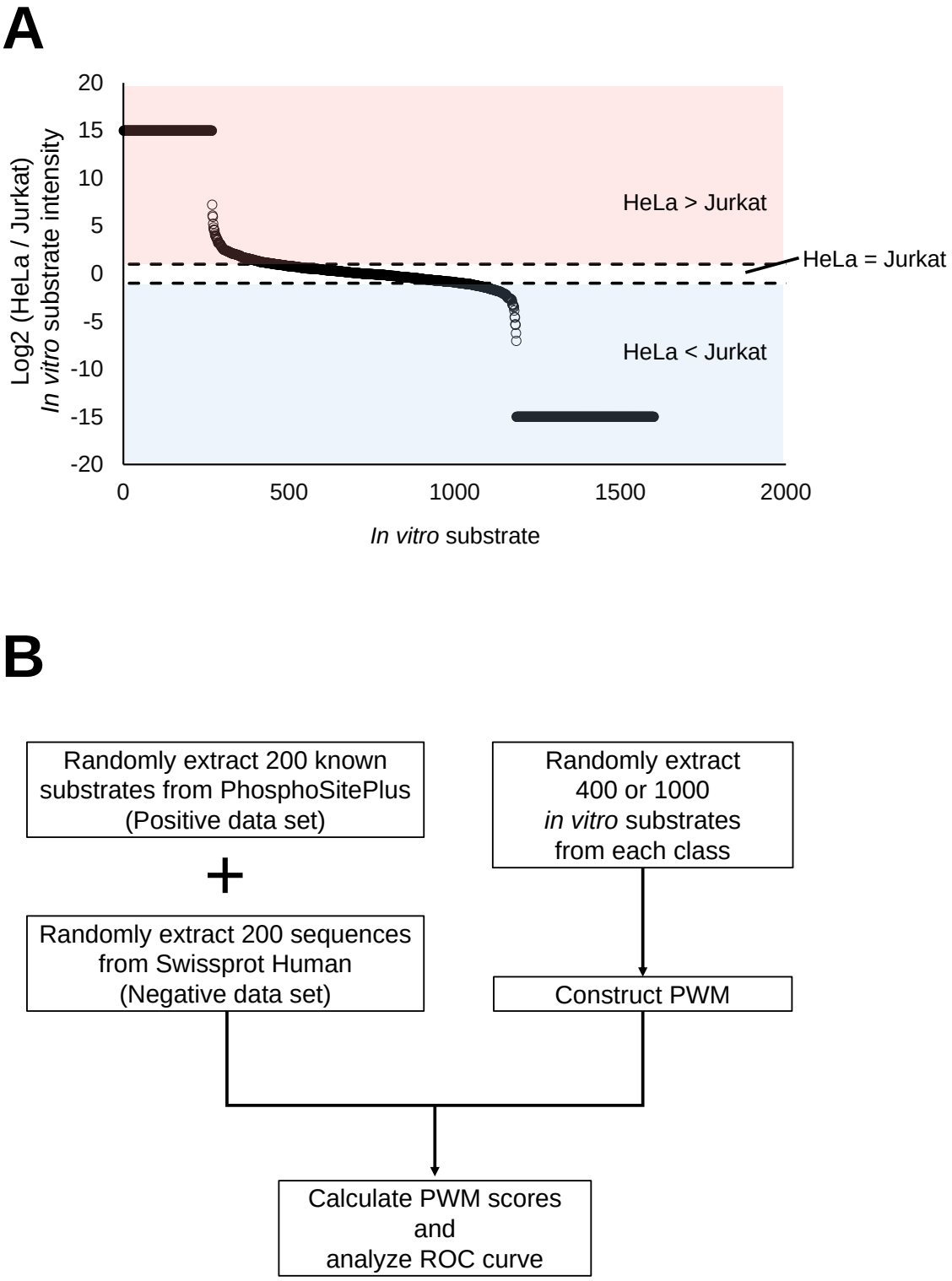


Fig. 3 Evaluation of performance of kinase substrate prediction with each cell-derived PWM

(A) An example of classification based on *in vitro* substrate abundance ratios (B) Workflow

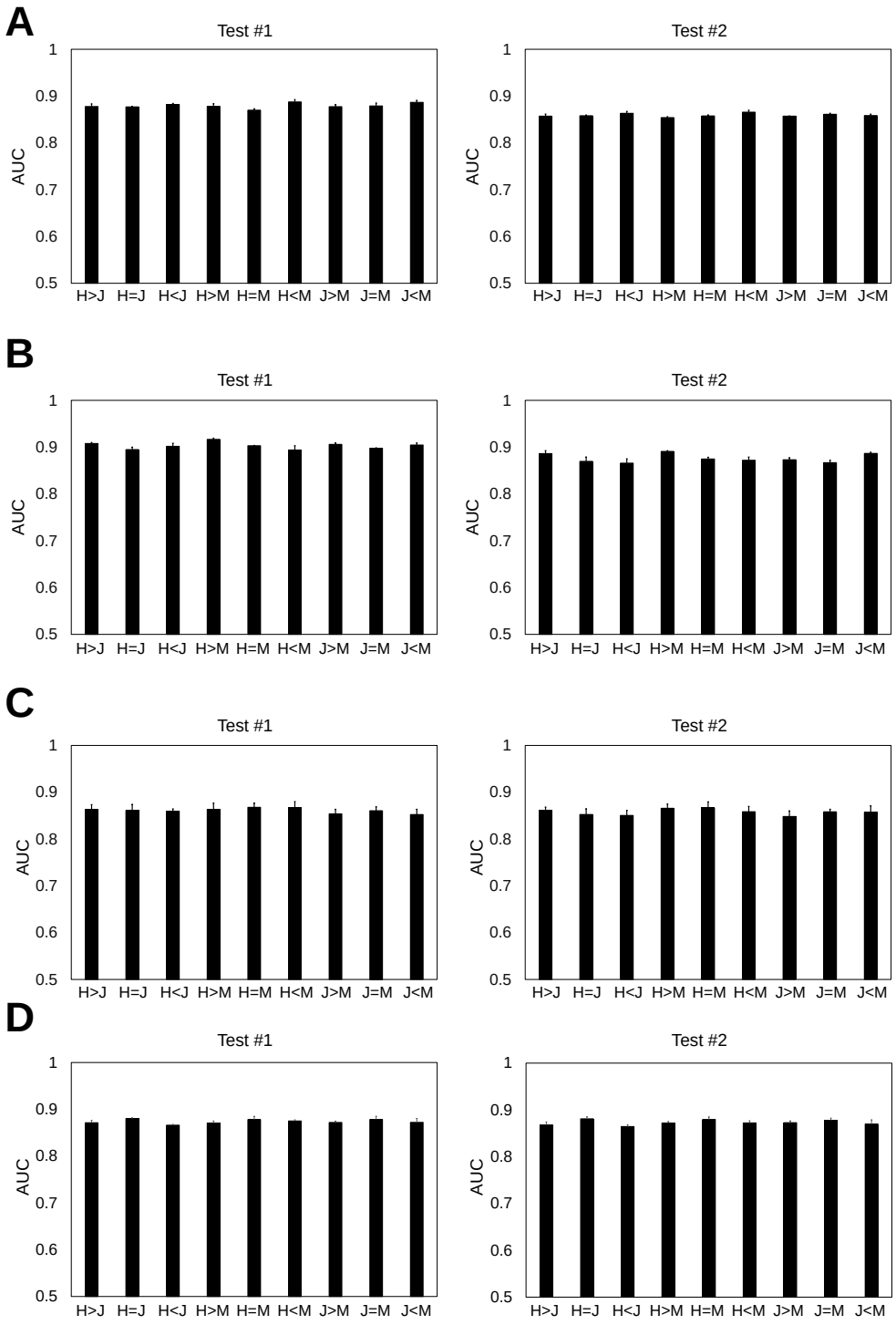


Fig. 4 ROC curve analysis for prediction of kinase substrates with each cell-derived PWM

The performance of kinase substrate prediction for each class was compared. HeLa was represented as H, Jurkat as J, and MCF7 is denoted as M. (A) CK2 (B) ERK 1 (C) PKA (from 400 *in vitro* substrates) (D) PKA (from 1000 *in vitro* substrates)

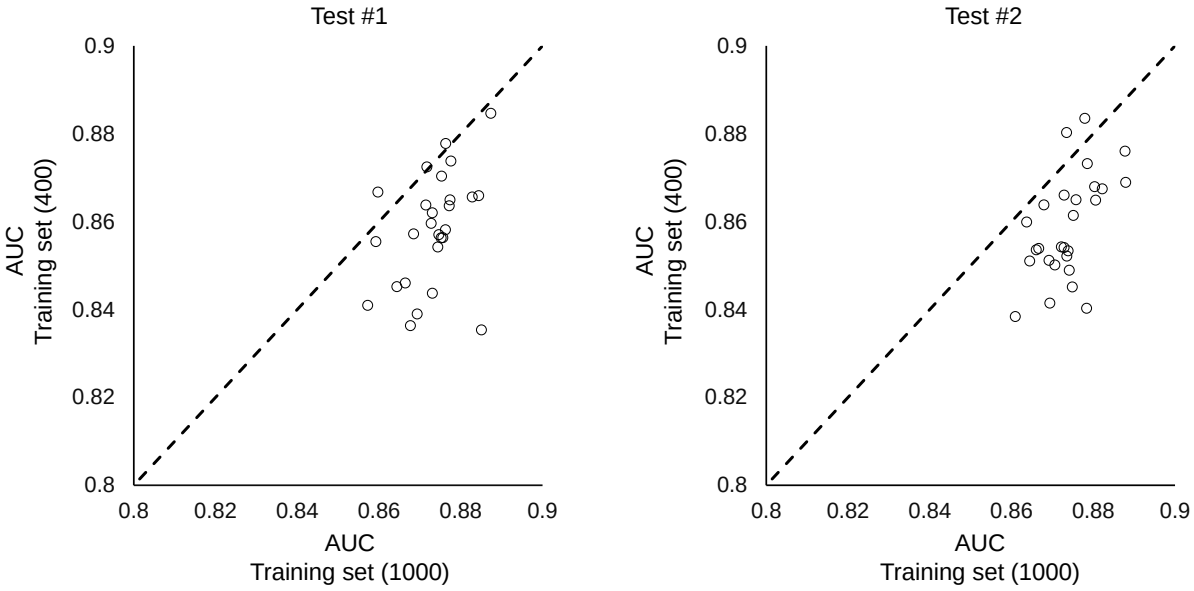
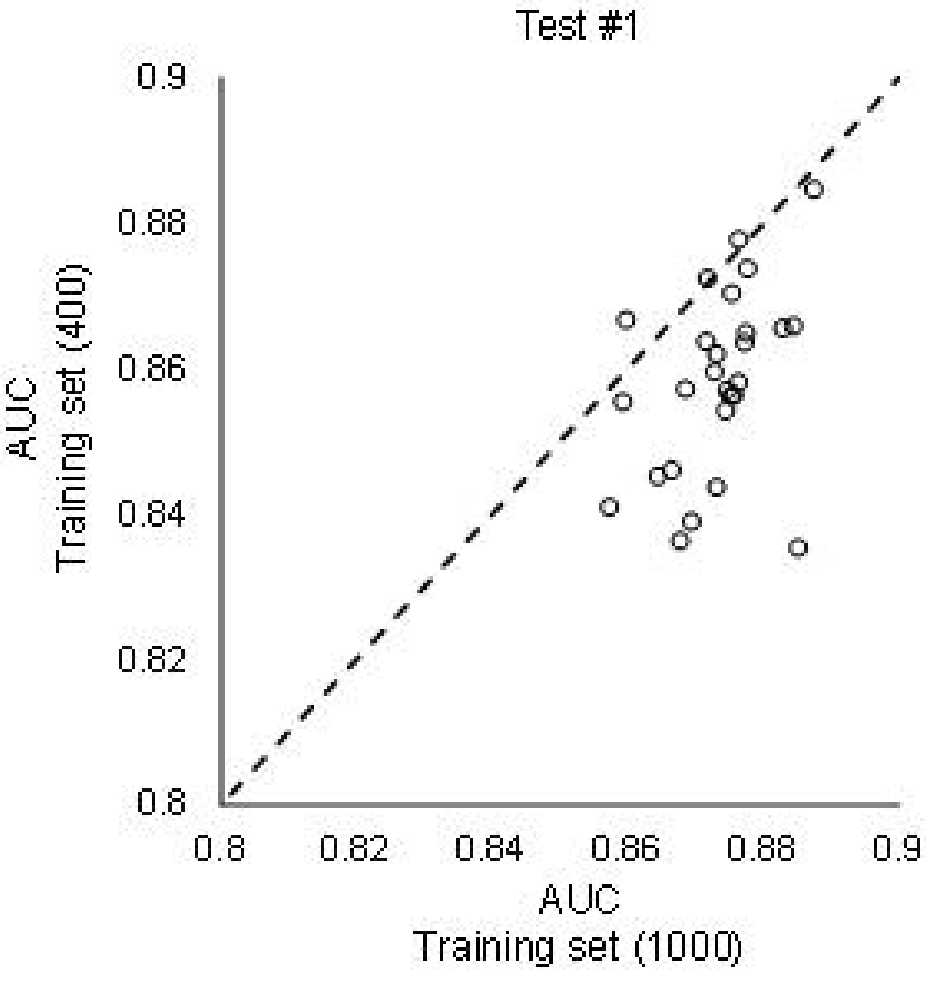


Fig. 5 The number of *in vitro* substrate and kinase substrate predictions for the training set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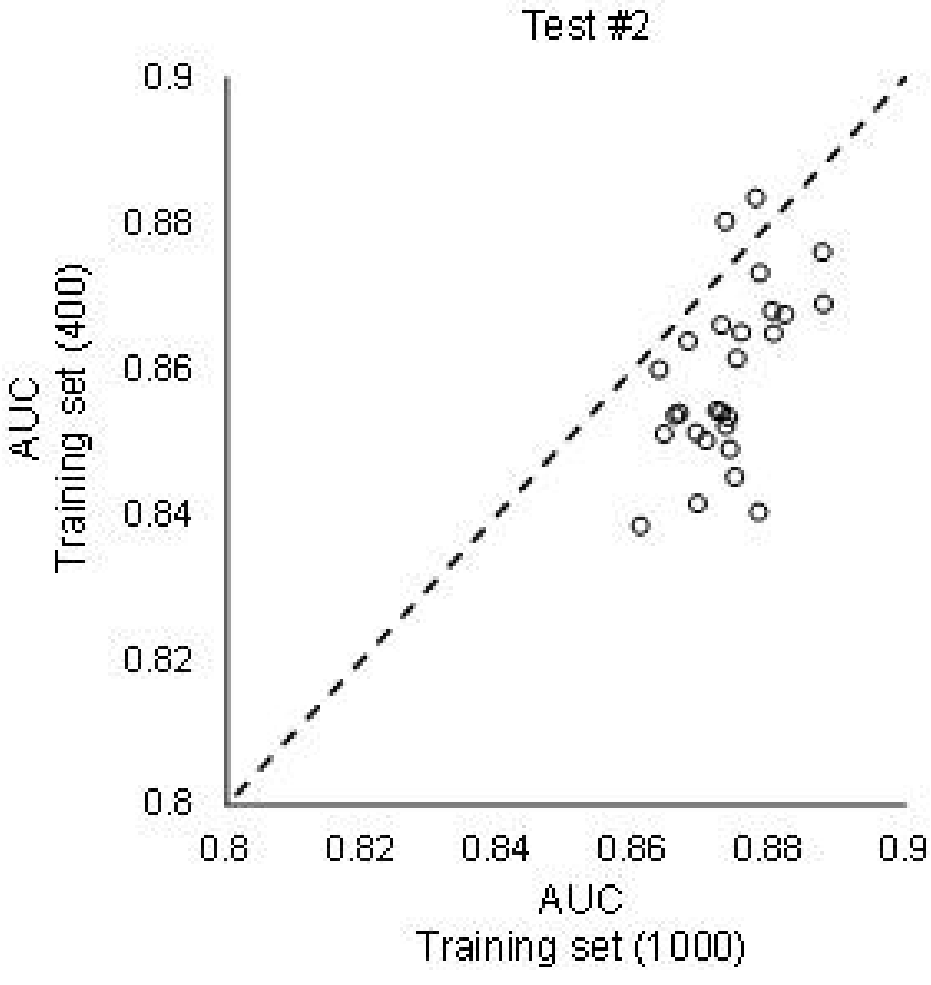
0.88
0.86
0.84
0.82
0.8
AUC
Training set (400)
0.8
0.82
0.84
0.86
0.88
0.9
AUC
Training set (1000)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0.84
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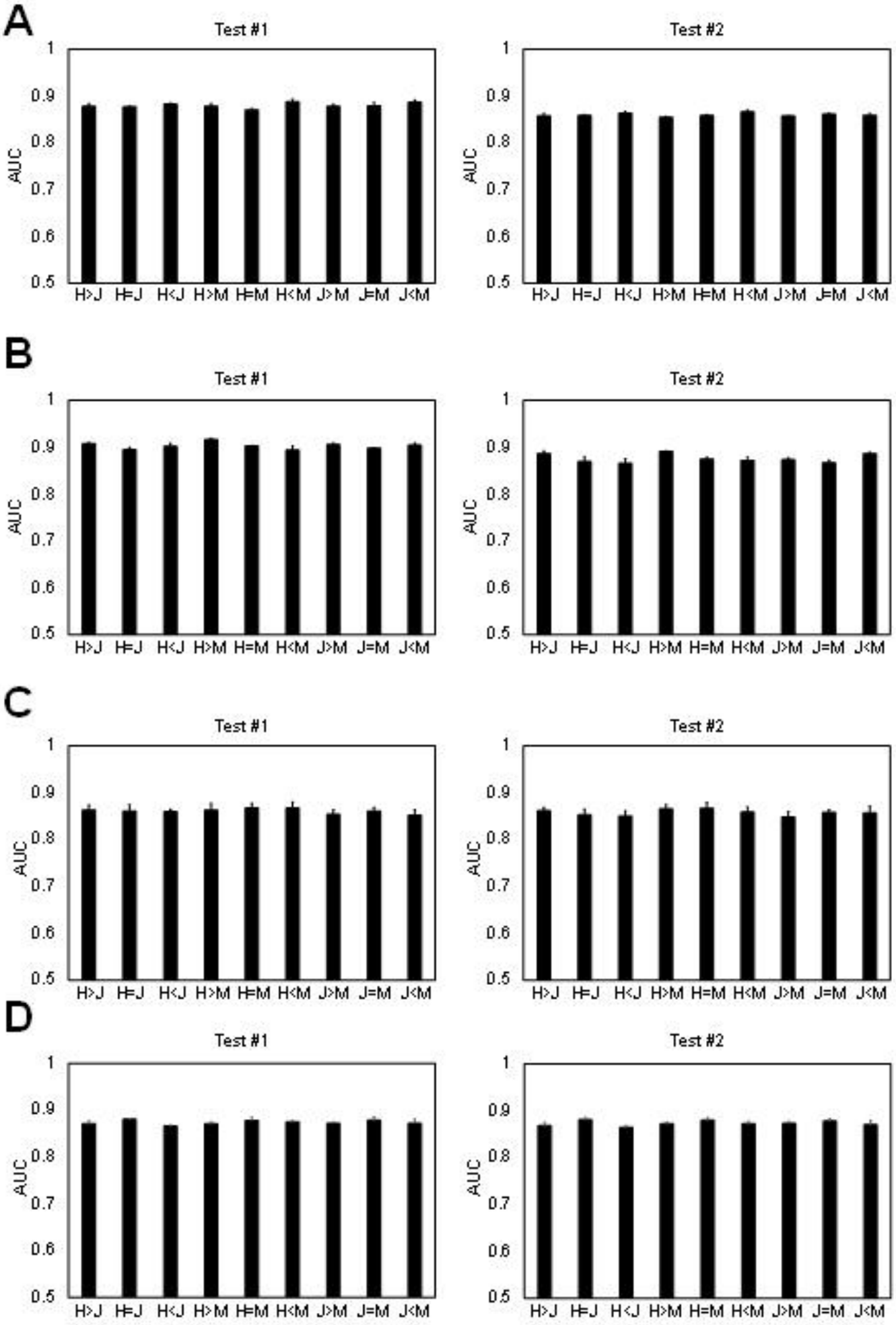
0.86
0.88
0.9
AUC
Training set (1000)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Test #1
Test #2
B
Test #1
Test #2
C
Test #1
Test #2
D
Test #1
Test #2
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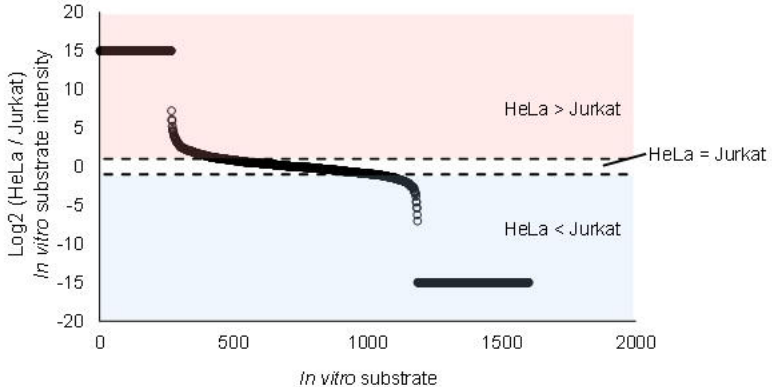
0.9
0.8
0.7
0.6
0.5
H>J
H=J
H<J
H>M
H=M
H<M
J>M
J=M
J<M

A


20
15
10
5
0
-5
-10
-15
-20
Log2 (HeLa / Jurkat)
In vitro substrate intensity
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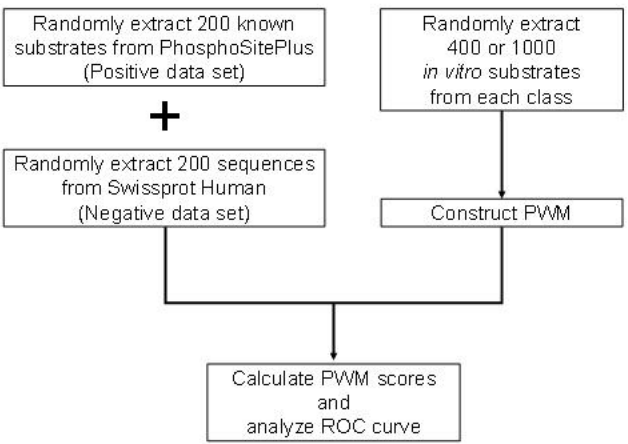
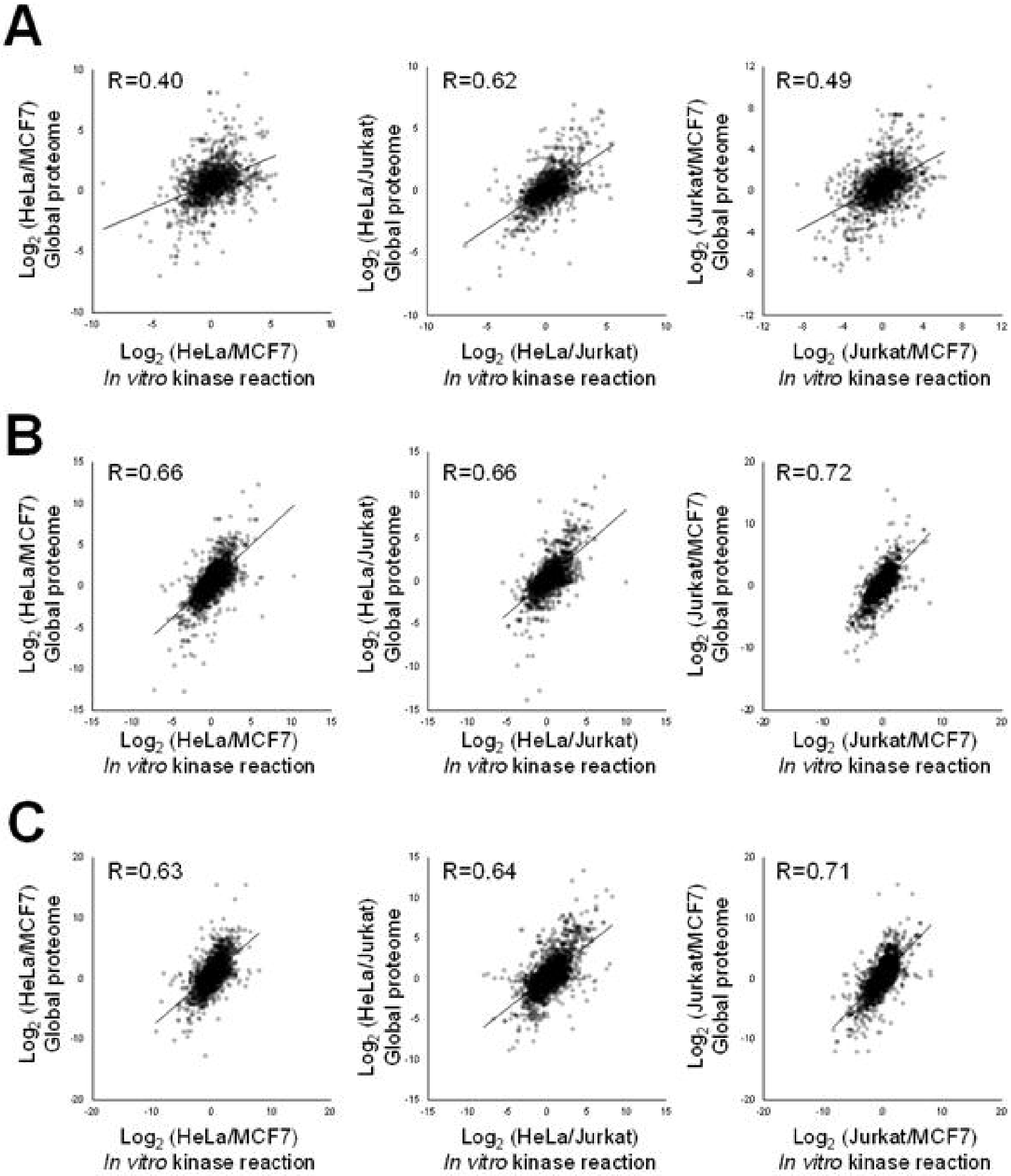
HeLa = Jurkat
HeLa < Jurkat
0
500
1000
1500
2000
In vitro substrate

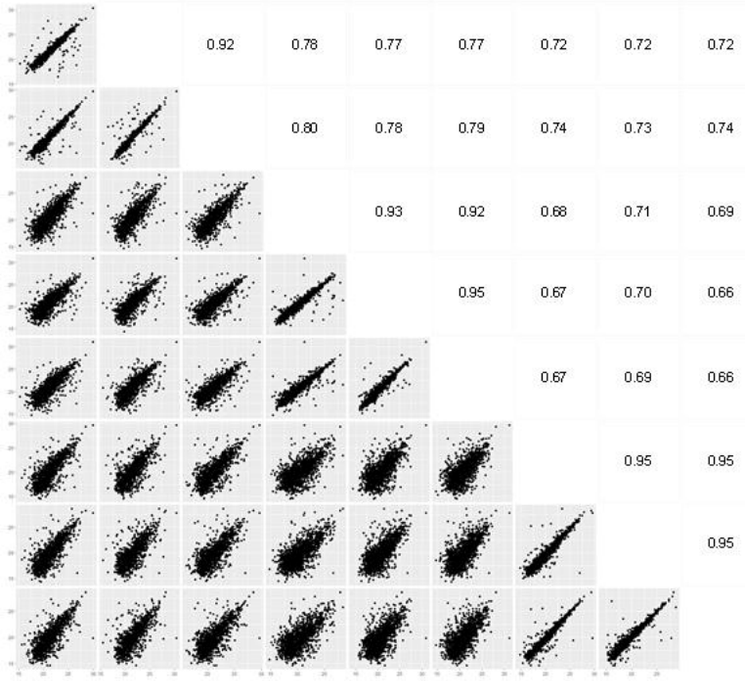
B


Randomly extract 200 known substrates from PhosphoSitePlus (Positive data set)
+
Randomly extract 200 sequences from Swissprot Human (Negative data set)
Randomly extract 400 or 1000 in vitro substrates from each class
Construct PWM
Calculate PWM scores and analyze ROC curve


A
R=0.40
Log2 (HeLa/MCF7) Global proteome
Log2 (HeLa/MCF7) In vitro kinase reaction
R=0.62
Log2 (HeLa/Jurkat) Global proteome
Log2 (HeLa/Jurkat) In vitro kinase reaction
R=0.49
Log2 (Jurkat/MCF7) Global proteome
Log2 (Jurkat/MCF7) In vitro kinase reaction
B
R=0.66
Log2 (HeLa/MCF7) Global proteome
Log2 (HeLa/MCF7) In vitro kinase reaction
R=0.66
Log2 (HeLa/Jurkat) Global proteome
Log2 (HeLa/Jurkat) In vitro kinase reaction
R=0.72
Log2 (Jurkat/MCF7) Global proteome
Log2 (Jurkat/MCF7) In vitro kinase reaction
C
R=0.63
Log2 (HeLa/MCF7) Global proteome
Log2 (HeLa/MCF7) In vitro kinase reaction
R=0.64
Log2 (HeLa/Jurkat) Global proteome
Log2 (HeLa/Jurkat) In vitro kinase reaction
R=0.71
Log2 (Jurkat/MCF7) Global proteome
Log2 (Jurkat/MCF7) In vitro kinase reaction

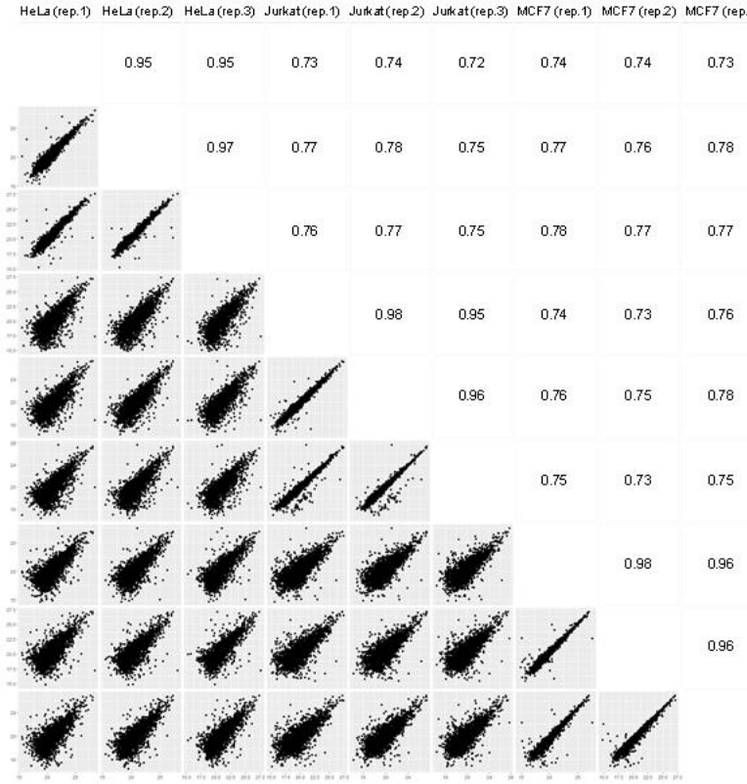
A

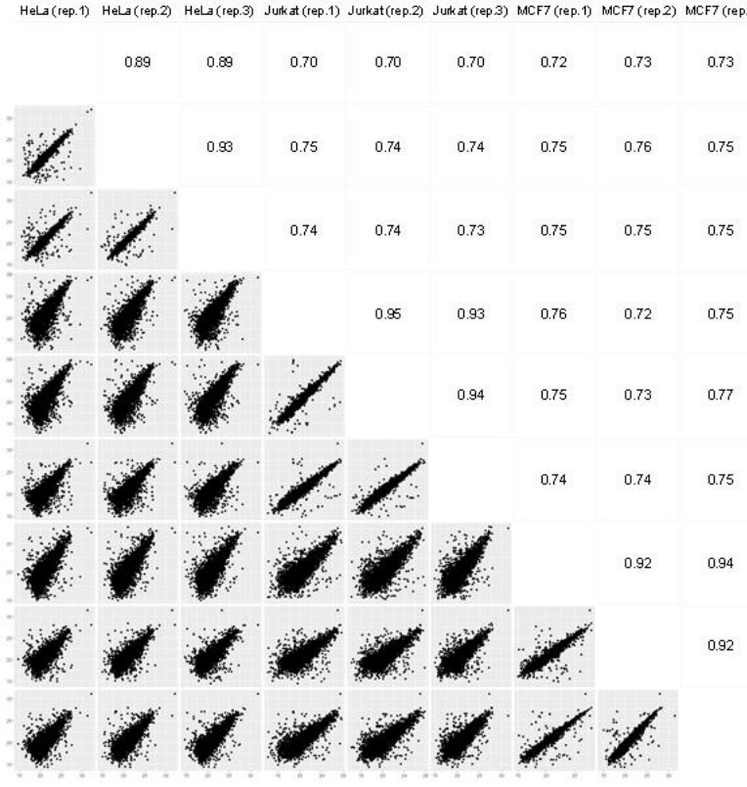
	HeLa (rep.1)	HeLa (rep.2)	HeLa (rep.3)	Jurkat (rep.1)	Jurkat (rep.2)	Jurkat (rep.3)	MCF7 (rep.1)	MCF7 (rep.2)	MCF7 (rep.3)
HeLa (rep.1)		0.90	0.92	0.80	0.78	0.79	0.78	0.79	0.78
HeLa (rep.2)			0.92	0.78	0.77	0.77	0.72	0.72	0.72
HeLa (rep.3)				0.80	0.78	0.79	0.74	0.73	0.74
Jurkat (rep.1)					0.93	0.92	0.68	0.71	0.69
Jurkat (rep.2)						0.95	0.67	0.70	0.66
Jurkat (rep.3)							0.67	0.69	0.66
MCF7 (rep.1)								0.95	0.95
MCF7 (rep.2)									0.95
MCF7 (rep.3)									



B

	HeLa (rep.1)	HeLa (rep.2)	HeLa (rep.3)	Jurkat (rep.1)	Jurkat (rep.2)	Jurkat (rep.3)	MCF7 (rep.1)	MCF7 (rep.2)	MCF7 (rep.3)
HeLa (rep.1)		0.95	0.95	0.73	0.74	0.72	0.74	0.74	0.73
HeLa (rep.2)			0.97	0.77	0.78	0.75	0.77	0.76	0.78
HeLa (rep.3)				0.76	0.77	0.75	0.78	0.77	0.77
Jurkat (rep.1)					0.98	0.95	0.74	0.73	0.76
Jurkat (rep.2)						0.96	0.76	0.75	0.78
Jurkat (rep.3)							0.75	0.73	0.75
MCF7 (rep.1)								0.98	0.96
MCF7 (rep.2)									0.96
MCF7 (rep.3)									



C

	HeLa (rep.1)	HeLa (rep.2)	HeLa (rep.3)	Jurkat (rep.1)	Jurkat (rep.2)	Jurkat (rep.3)	MCF7 (rep.1)	MCF7 (rep.2)	MCF7 (rep.3)
HeLa (rep.1)		0.89	0.89	0.70	0.70	0.70	0.72	0.73	0.73
HeLa (rep.2)			0.93	0.75	0.74	0.74	0.75	0.76	0.75
HeLa (rep.3)				0.74	0.74	0.73	0.75	0.75	0.75
Jurkat (rep.1)					0.95	0.93	0.76	0.72	0.75
Jurkat (rep.2)						0.94	0.75	0.73	0.77
Jurkat (rep.3)							0.74	0.74	0.75
MCF7 (rep.1)								0.92	0.94
MCF7 (rep.2)									0.92
MCF7 (rep.3)