**Title:**

KRSA: An R Package and R Shiny Web Application for Upstream Kinase Analysis of Kinome Array Data

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**Abstract:**

Phosphorylation by serine-threonine and tyrosine kinases is critical for determining protein function. Array-based approaches for measuring reporter peptides signal levels allow for the testing of differential phosphorylation between conditions for distinct active kinomes. Peptide array technologies like the PamStation12 from PamGene allows for generating high-throughput, multi-dimensional, and complex functional proteomics data. As the adoption rates of such technologies increases, there is an imperative need for software tools that streamline the process of analyzing such data. We present Kinome Random Sampling Analyzer (KRSA), an R package and R Shiny web-application for analyzing kinome array data to help users better understand the complex patterns of functional proteomics in complex biological systems. KRSA is an All-In-One tool that reads, formats, fits models, analyzes, and visualizes PamStation12 kinome data. While the underlying algorithm has been experimentally validated in previous publications, we demonstrate KRSA workflow on dorsolateral prefrontal cortex (DLPFC) in male (n=3) and female (n=3) subjects to identify differential phosphorylation signatures and upstream kinase activity. Kinase activity differences between males and females were compared to a previously published kinome dataset (11 female and 7 male subjects) which showed similar global phosphorylation signals patterns.

**Availability and implementation:**

KRSA R package repository: <https://github.com/kalganem/KRSA>. KRSA R package website: <https://kalganem.github.io/KRSA/>. KRSA R Shiny repository: <https://github.com/kalganem/KRSA_App>. KRSA R Shiny web application: <https://kalganem.shinyapps.io/KRSA/>.

**Supplementary information**

The full analysis script for this manuscript is available online: <https://github.com/kalganem/krsa_manuscript>.

**Introduction:**

Protein phosphorylation marks one of the most important biological mechanisms that underlies various normal cellular functions, acting in complex protein-substrate networks. Phosphorylation cascades are also perturbed in many disease states (Hanahan and Weinberg, 2011; Simpson et al., 2019). As a result, kinases are one of the most studied proteins given their central role in normal and abnormal cell biological mechanisms (Ardito et al., 2017; Lahiry et al., 2010; Pawson and Scott, 2005; Ubersax and Ferrell, 2007). Kinomics, or the study of kinases and kinase signaling, has expanded from individual activity assays, with one peptide to study one kinase, to array or chip-based technology of up to 1000 reporter peptides, called kinase arrays or kinome arrays (Baharani et al., 2017; Diks et al., 2004; Houseman and Mrksich, 2002). The selected reporter peptides are designed to cover a broad range of signaling pathways, with large numbers allowing for a better understanding of kinase interactions and global changes that occur between two states (i.e., disease, cell type). However, analyzing the data from these peptide arrays is a complex process given that many kinases can phosphorylate the same peptide and an individual kinase can phosphorylate many peptides, making accurate interpretation of the data a challenging task. As the use of these kinome arrays becomes more widespread, there is an increasing need for tools that efficiently and accurately analyze these datasets. In particular, analytic tools are needed for nonexpert users of kinome array platforms.

Bioinformatics tools that are specifically designed to analyze kinome array datasets are beginning to emerge. One of these analytic tools is the Kinomics Toolkit, which gives users a platform for exploration of the peptide phosphorylation data, but does not provide upstream kinase predictions (Dussaq et al., 2018). Another tool that was designed specifically to process kinase array data is the PamgeneAnalyzeR package, though this package is primarily focused on the pre-processing steps of kinase array datasets and not the downstream analysis (Bekkar et al., 2020).

However, tools that comprehensively analyze kinome array data, and open sourced, are nonexistent. Current approaches of analyzing kinome array data are relying on manual statistical analyses or proprietary software such as BioNavigator by PamGene.

Prediction of upstream kinase activity and network-based analyses provides a biologically-meaningful springboard for further research that is currently lacking in a user-friendly application. There are existing tools that aim to predict upstream kinases based on an input of enriched genes or phosphopeptides, like KEA (Lachmann and Ma'ayan, 2009) and PTM-SEA (Krug et al., 2019). However, none of these tools are specifically designed to take raw data from PamChip datasets and run a complete analysis pipeline starting from pre-processing to visualizing kinome networks.

A common and validated approach to predicting upstream kinase activity is to analyze the differences between 1) kinases predicted to be upstream of the peptides that are differently phosphorylated between two conditions and 2) kinases predicted to be upstream of the remaining peptides on the chip (Anderson et al., 2014; Isayeva et al., 2015). In a similar statistical approach, we have previously described a method which uses random sampling to identify highly active kinases from kinome array data (Bentea et al., 2019; Dorsett et al., 2017; McGuire et al., 2017). Briefly, we look at overrepresented/underrepresented kinases relative to an expected distribution using random permutation sampling of peptides. This type of analysis is valuable because it separates kinases that are truly differentially active from those who are highly active globally and don’t represent a change between states.

Here we present the Kinome Random Sampling Analyzer, or KRSA, R package which automates many of the steps described above, including parsing kinome array raw files, peptide filtering, random sampling, different visualizations, and kinase network generation. We have also developed a web-based R Shiny application, that is build on top of the KRSA R package, that allow for users with no programming skills to analyze their data. The KRSA Shiny application can be used by biologists and data scientists alike, with no knowledge of statistical software required. KRSA makes analyzing kinome array datasets accessible and eliminates much of the human workload that the previous method required.

This method has been applied to multiple datasets and predictions have been experimentally validated in our laboratory through individual kinase activity assays and inhibitor studies (Bentea et al., 2019; Bentea et al., 2020; Creeden et al., 2020; Dorsett et al., 2017; Flaherty et al., 2019; McGuire et al., 2017; Schrode et al., 2019). An early version of KRSA, containing only the random sampling algorithm, identified altered phosphorylation of peptides and subsequently perturbed kinase activity in the anterior cingulate cortex (ACC) between schizophrenia and control subjects (McGuire et al., 2017). This tool was also used to analyze date from frontal cortex and hippocampus of rats subjected to lateral fluid percussion as a model of traumatic brain injury (TBI) and their sham surgery counterparts to identify differences in kinase activity in these brain regions (Dorsett et al., 2017). We used the platform to explore the kinase activity in cortical neurons differentiated from induced pluripotent stem cells (iPSCs) from a schizophrenia patient with a 4-bp mutation in the DISC1 gene (Bentea et al., 2019). KRSA also was used to analyze kinome signatures of genetic perturbation of NRXN1 and FURIN1 in human induced pluripotent stem cell (hiPSC)-derived neurons (Flaherty et al., 2019; Schrode et al., 2019). KRSA also has been utilized to analyze the kinome signature of mice with a genetic deletion of a specific subunit of cystine/glutamate antiporter system (xCT −/− mice) (Bentea et al., 2020). More recently, KRSA was utilized to investigate the unique kinomic networks of different patient-derived pancreatic ductal adenocarcinoma (PDAC) cell lines (Creeden et al., 2020).

Interest within the neuroscience community in defining sex differences in the brain has increased over the past several decades. Differences in kinase activity and signaling between males and females have been implicated in sex-related variations in neuronal cell survival, outcomes after brain injury, and fear extinction, among other research areas (Armstead et al., 2017; Matsuda et al., 2015; Zhang et al., 2003).

To demonstrate the use of KRSA, we used postmortem brain tissue from the dorsolateral prefrontal cortex (DLPFC) to investigate kinome signature differences between female and male healthy subjects. We also paired this experiment with a previously published postmortem brain kinome array study, hippocampus (HPC), to compare against our findings (Rosenberger et al., 2016).

**Design and Implementation:**

We will briefly describe the design and functionally of the KRSA R package. More details are available in the package vignettes which also hosted online (https://kalganem.github.io/KRSA/). This provides more comprehensive details on all KRSA functions and example datasets. That web page also hosts a complete KRSA workflow starting from reading raw data to visualizing network models. Additionally, we built an R shiny app for KRSA that are built using the R package. The KRSA R Shiny GitHub page has complete details on how to access the app and interact with its user interface (<https://github.com/kalganem/KRSA_App>). The general pipeline of using the KRSA R package can be divided into three main steps; loading raw files, choosing design parameters, exploring upstream kinase analysis results (Figure 1).

**Image processing and formatting**

The PamChip images are pre-processed using BioNavigator to generate numerical values of the median minus background signal intensity. This raw file is then read by KRSA as the main input file. KRSA will read, parse, and reformat the raw file for downstream analyses using the *krsa\_read()* function. The user then has to define the groups within the samples, either using an existing variable in the input file, or creating a new one. *krsa\_extractEndPointMaxExp()* and *krsa\_extractEndPoint()* will extract the end point (last cycle) data points from the processed data, which then will be used to filter out some peptides based on different quality control (QC) parameters. The data will undergrow a couple of QC steps using different functions: *krsa\_qc\_steps()* scales the negative values to the base line and optionally filters out data points with high signal saturation values. *krsa\_filter\_lowPeps()* will filter out peptides with very low signals. All of these QC steps are carried out by dedicated functions with arguments that can be adjusted by the user.

**Model Fitting**

Linear regression slope of the signal intensity as function of exposure time is calculated to represent the peptide phosphorylation intensity. This value is then multiplied by 100 and log2 transformed to represent the final signal of the peptide. All of that is carried out by the *krsa\_scaleModel()* function. This function will return a list of three data frames: modeled data, normalized modeled data by Barcode/Chip, and grouped modeled data. Peptides with a relative low R2 of as results of the linear regression model can be excluded from subsequent analyses using *krsa\_filter\_nonLinear()****.***

**Global Signals Visualization**

The KRSA package can then plot the final signal intensity of selected samples using different figures. Including heatmaps and violin plots. *krsa\_heatmap()* has several data scaling options like scaling the data by row (peptide), column (sample), and no scaling. Also, there is option to specify the different algorithms that will be used for the hierarchical clustering. There is also an optional function, *krsa\_cv\_plot(),* that generates a coefficient of variation (CV) plot that can be used to identify potential outliers in certain groups.

**Differential Phosphorylation Analysis**

Using the final signal values, log2 fold changes (LFCs) are calculated between different samples using two approaches. 1) across chip analysis: using the average signal across all samples and chips. 2) within chip analysis: using the log2 fold change analysis within each chip (it’s recommended to do the within chip analysis if the samples are found within each chip). We use the LFC as the main metric to determine the top differential phosphorylated peptides using either a single cutoff or multiple cutoffs (multiple cutoffs are recommended). By doing multiple cutoff values, we address the bias in the arbitrary chosen cutoff value by doing the upstream kinase analysis on multiple peptide sets and choosing the kinase that are shown to be implicated consistently across the different peptide sets. All of that is done through *krsa\_group\_diff()*.

After calculating the LFCs, there are additional visualization options we can use. Beside the heatmaps and violin plots, we can generate a waterfall plot representing the LFCs values for each peptide using *krsa\_waterfall()*. Another figure is the curve plots which represents the linear model fit for each peptide and colored by the different groups, which can be done by calling *krsa\_curve\_plot()*.

**Upstream Kinase Analysis**

Protein kinases predicted to act on phosphorylation sites within the array peptide sequences were identified using GPS 3.0 and Kinexus Phosphonet (Kinexus Bioinformatics) (Xue et al., 2010). These programs provide predictions for serine-threonine kinases targeting peptide sequences ordered by likelihood of binding. The union of the highest ranked 5 kinases in Kinexus and kinases with scores more than two times the prediction threshold in GPS 3.0 were considered predicted kinases for each peptide and used in KRSA analysis (Bentea et al., 2019). This list was combined with kinases shown in the literature to act on the phosphorylation sites of the peptides via PhosphoELM (<http://phospho.elm.eu.org>) and PhosphoSite Plus (<https://www.phosphosite.org>). The user has the option to use the KRSA built-in curated kinase-substrate mapping files or upload their own mapping files to perform the upstream kinase analysis. The upstream kinase analysis is done through the main function of the KRSA package, *krsa()*,which takes in the list of differentially phosphorylated peptides and kinase-substrate mapping data frame and performs the random sampling analysis. Additionally arguments can be adjusted like the number of iterations and seed number. The *krsa()* function can be run in a parallel fashion across different cores utilizing the *future\_map()* function from the furrr package (https://davisvaughan.github.io/furrr/), which will speed up the process of computation specifically when doing multiple peptide sets.

**Kinase Network Model**

The complexity of cellular signaling ensures that kinases do not act in isolation, but instead as part of an interacting network with other kinases and proteins that regulate biological processes (Yao et al., 2015). The nature of this system means that final KRSA predictions should include potentially interacting kinase families for downstream pathway analysis and hypothesis generation. To accomplish this goal, KRSA connects the initial set of kinase hits with other kinases using protein-protein interaction (PPI) databases. The *krsa\_ball\_model()* is used to generate the network, which utilized the igraph package (https://igraph.org/).

**3. Results**

To elucidate differences in kinase activity between the brains of healthy males and females subjects, we used KRSA to predict differential upstream kinase activity.

**Input and Parameters**

Input files, selected parameters, and the full script is found in the KRSA manuscript GitHub page (<https://github.com/kalganem/krsa_manuscript>).

**Data Description**

To demonstrate the various functionalities of KRSA, we set out to compare kinase activity levels between female and male dorsolateral prefrontal cortex (DLPFC). We analyzed postmortem tissue obtained from 3 male and 3 female control subjects (for demographics, see Supplementary Table S1). We also analyzed a previously published kinome array dataset that studies the changes in protein kinase activity during Alzheimer’s Disease (AD) pathogenesis (Rosenberger et al., 2016). This postmortem study was performed using hippocampal (HPC) brain section samples. From this dataset, we reanalyzed all of data for the control samples (Braak Stages 0-1) for both female and male subjects. Given the Braak Stage 0 samples only contains male subjects and the apparent effect of Braak Staging on the kinome signatures, we limited ourselves to samples with Braak Stage 1, and that resulted into having 18 subjects, 11 female and 7 male (for demographics, see Supplementary Table S2).

**Outputs:**

**Global serine-threonine protein kinase activity in female vs. male (DLPFC)**

In the QC steps, KRSA filtered out 55 peptides were considered undetectable, 2 peptides that were not linearly increasing with exposure time (based on R2 > 0.9), and 11 references/control peptides. The log2 fold change was calculated for the remaining 84 peptides by taking the female group as the “baseline”. Based on the three chosen LFC cutoff values (0.2, 0.3, 0.4), three peptide sets were extracted with the lengths of 56, 44, and 33 respectively. Using the first set of peptides, *krsa\_violin\_plot\_grouped()* was used to visualize the global phosphorylation levels and the results indicate that the signatures are not significantly different between females and. males (Figure 2A, p value = 0.59 using a Mann-Whitney test). A heatmap of phosphorylation intensity at each reporter peptide was generated using *krsa\_heatmap()* and scaled by row (by peptide*)* (Figure 2A).

**Global serine-threonine protein kinase activity of the independent dataset (HPC)**

The same approach was done using the independent cohort. However, since this cohort has a bigger number of subjects, we were able to detect a significant difference between males and females kinome signatures (Figure. 2B, p value = 6.04e-5 using a Mann-Whitney test). The samples signatures showed distinct clustering between males and females in both the heatmap unsupervised clustering (Figure. 2B) and in the principal component analysis (PCA) (Supplementary Figure 2).

**Altered kinase activity in female vs. male (DLPFC)**

The different peptide sets, based on the different LFC cutoff values, were used to perform the upstream kinase analysis step in KRSA*. krsa\_waterfall()* was used to visualize the average LFCs at each peptide (Figure 3A). A small set of peptides were chosen to demonstrate the output of *krsa\_curve\_plot()*, which shows the linear fit models (Figure 3B). *krsa()* was used to run the upstream kinases analysis which generates data frames that contain random sampling distribution, standard deviation, and Z scores for each kinase family for each peptide set. The Z scores were averaged to determine the final score of each kinase family (Supplementary Figure 1). Taking one peptide set as an example, the top kinases include CDK, PDK1, STE7, and others (Table 1). Additionally, krsa\_histogram() were used to visualize the experimental peptide hits relative to the random sampling distribution (Figure 4).

**Altered kinase activity in female vs. male in the independent dataset (HPC)**

We used a similar method to determine the upstream kinase hits for the HPC cohort, and one set of peptides led to the identification of serval different serine-threonine kinase families differentially represented in HPC between female and male control subjects (Table 2).

**Kinase network model of female vs. male DLPFC**

The *krsa\_ball\_model()* function was used to generate a network to connect the kinase hits with other kinases families (Figure 5).

**Discussion**

Unlike many diseases and conditions, where distinct high-magnitude changes in gene expression and subsequent downstream function occur because of the disease processes, differences between healthy male and female brains are theoretically subtler and harder to characterize. As an example of KRSA’s capabilities, we probed for kinase activity differences in the male and female brain using postmortem dorsolateral prefrontal cortex. We also compared our findings to a previously published kinome array study. The samples from that study showed a similar pattern of changes between female and male kinome signatures as an overall higher phosphorylation levels in the female samples. For the HPC cohort, the unsupervised clustering also showed a clear separation between male and female signatures. Using Z-score threshold of 2, we saw an overlap of several kinas families, including STE7, JNK, ERK, DYRK, P38, and PDK1 among the two cohorts. There were kinases that were unique in each cohort like CDK and PEK in the DLPFC dataset, and PKA and AKT in the HPC dataset (Supplementary Figure 3).

In the area of kinomics, there is a need for end-to-end processing of kinome array data in a user-friendly, open source, and interactive environment. The Kinome Random Sampling Analyzer (KRSA) R package and Shiny app fill this gap in the field and serve as a stepping stone for the use and interpretation of kinome array data for laboratory biologists and computational biologists alike.

**Acknowledgments**

We would like to thank Daniel Schnell for helpful discussions on the statistical methods behind KRSA. We also thank the developers of R studio, Shiny, and supporting R packages used in the implementation of KRSA.

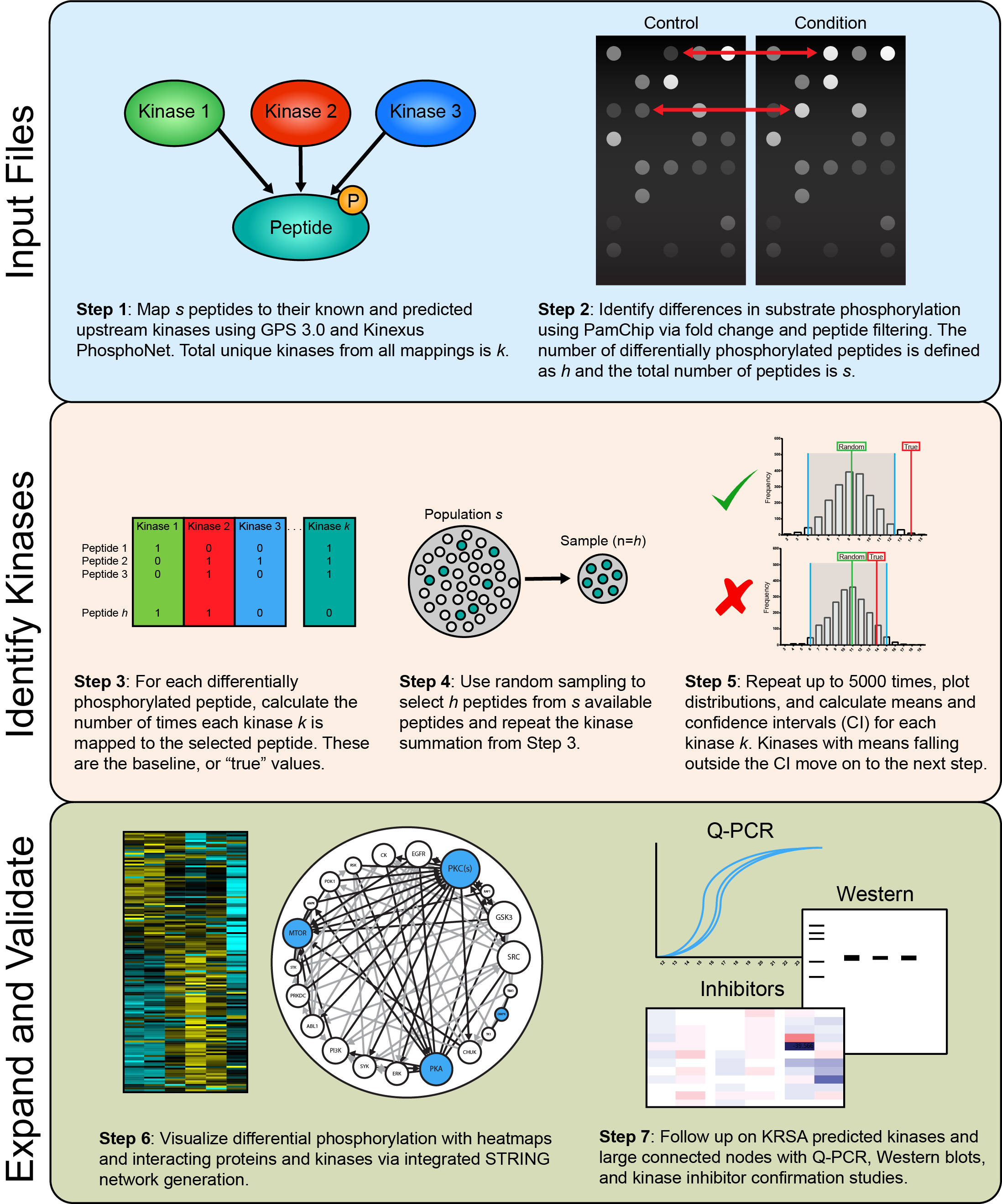
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**Conflict of Interest**

F.N. and R.H. are employed by PamGene International B.V. The remaining authors have declared that no conflicts of interests exist.

**Figures:**

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**Figure 1.** Workflow overview illustrating the primary steps of the KRSA pipeline. The “Input Files” section outlines the initial input files for KRSA, including the raw kinome array data and the peptide-kinase association file as well as the initial filtering step in KRSA. The “Identify Kinases” section describes the random sampling and distribution evaluation methods used to identify differentially active kinases. Finally, the “Expand and Validate” portion of the figure shows the kinase network generation step of KRSA and confirmation experiments that can be used to validate the predictions from KRSA.

**Diagram

Description automatically generated**

**Figure 2.** Global serine-threonine kinase activity in female vs. male DLPFC. (A) Global phosphorylation plots, showing signal intensity (phosphorylation levels) at each reporter peptide, as well as the average phosphorylation values (thick line), and a Mann-Whitney test when comparing females to males. A heatmap generated by KRSA depicting the relative signal intensity at each reporter peptide for the 6 samples on the array (3 females and 3 males) (B) Global phosphorylation plots, showing signal intensity (phosphorylation levels) at each reporter peptide, as well as the average phosphorylation values (thick line), and a Mann-Whitney test when comparing females to males using 18 control samples from the HPC cohort (11 females and 7 males). To highlight differences, the heatmap is normalized per row to present relative changes at each individual peptide between the groups. Red indicates relatively higher levels of phosphorylation and yellow indicates relatively lower levels of phosphorylation.

**Chart

Description automatically generated**

**Figure 3.** Changes in phosphorylation at reporter peptides in female vs. male DLPFC. (A) Waterfall plot showing log2 fold changes (LFC) in phosphorylation at reporter peptides for female vs. male DLPFC. Peptides with positive LFC indicate higher phosphorylation in males, and peptides with negative LFC indicates lower phosphorylation in males. The dashed line indicating the 0.2 LFC cutoff (B) Representative examples of linear model fit of the phosphorylation curves of three reporter peptides in female vs. male DLPFC.

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**Figure 4.** Observed frequency of selected kinases relative to expected random sampling distribution in female vs. male DLPFC. Examples are shown for kinases identified in the reporter peptides more than by random chance alone (BARK2), less than by random chance (ex: ERK, CDK, JNK), as well as for kinases that were not identified to be significantly relevant (ex: COT, MARK, RIPK). KRSA was performed with 2000 iterations and histograms were automatically generated. Gray areas indicate ± 2 standard deviations from the expected distribution mean. The red line indicates the number of mapped peptides for the corresponding kinase based on one of the log2 fold change cutoffs (0.2 shown here). The Z scores for each kinase are derived by calculating how many standard deviations the red line is away from the mean.



**Figure 5.**  Kinase network model of female vs. male DLPFC. The kinase network was obtained in KRSA by growing the kinome array hits with kinase interacting partners as identified using STRING and PhosphoSitePlus. The kinome array hits are color coded by the averaged z score values. Circle size corresponds to the number of interactions, with larger circles having more interactions. Black lines represent interactions with a kinome array direct hit, while gray represent interactions made between associated the other kinase families.

**Tables:**

**Table 1. Predicted kinases and distributions for female vs. male (DLPFC)**

Table

Description automatically generated

**Table 2. Predicted kinases and distributions for female vs. male (HPC)**

Table

Description automatically generated

**Supplementary Data:**

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**Supplementary Figure 1.**  Z scores waterfall plot for the DLPFC cohort. Multiple z scores for each kinase that were calculated using the different peptide sets (that were derived based on the different log2 fold change cutoffs, 0.2, 0.3, and 0.4). The bigger dots represent the averaged Z scores which alos color coded based on the absolute values of the averaged z scores.

**Chart, scatter chart

Description automatically generated**

**Supplementary Figure 2.**  Principal Component Analysis (PCA) of the independent dataset from the AD cohort. Using the subjects in AD cohort dataset (controls only) showing the clustering of samples and the factors that most explain the variance in the kinome signatures. PMI: postmortem interval, Barcode: Chip ID.

Diagram, venn diagram

Description automatically generated

**Supplementary Figure 3.**  Venn diagrams showing overlap of the total of overrepresented/underrepresented kinases for both cohorts. DLPFC from current study, and the HPC cohort study. Filtered kinase with absolute values of Z scores equal or above 2 for both datasets. DLPFC: dorsolateral prefrontal cortex, HPC: hippocampus.

**Supplementary Table S1.** Kinome array subject demographics.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Subject | Sex | Age | pH | PMI (h) |
| M1 | M | 73 | 6.4 | 17 |
| M2 | M | 71 | 6.4 | 13 |
| M3 | M | 71 | 6.4 | 20 |
| F1 | F | 76 | 6.3 | 23 |
| F2 | F | 73 | 5.9 | 25 |
| F3 | F | 77 | 6.6 | 30 |

**Supplementary Table S2.** Kinome array subject demographics for the AD cohort (control subjects only).

|  |  |  |  |
| --- | --- | --- | --- |
| Subject | Sex | Age | PMI (h) |
| TIS163 | M | 62 | 7 |
| TIS164 | M | 82 | 5.5 |
| TIS173 | M | 91 | 4 |
| TIS191 | M | 77 | 7 |
| TIS194 | M | 58 | 5 |
| TIS214 | M | 79 | 6 |
| TIS216 | M | 82 | 5 |
| TIS188 | F | 93 | 4 |
| TIS198 | F | 85 | 7 |
| TIS204 | F | 86 | 6 |
| TIS209 | F | 82 | 4 |
| TIS212 | F | 60 | 6.5 |
| TIS215 | F | 83 | 5 |
| TIS217 | F | 77 | 2.5 |
| TIS221 | F | 92 | 7 |
| TIS223 | F | 60 | 7 |
| TIS227 | F | 50 | 4 |
| TIS228 | F | 83 | 4 |

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