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# Data-Independent Acquisition Enhancement of a Competitive Activity-Based Protein Profiling Platform for Kinase Inhibitor Screening

Ravi Tharakan | Yanyan Qu | Michele Ceribelli | Patrick J. Morris | Yuhong Fang | Craig J. Thomas | Christopher A. LeClair | Dingyin Tao

National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, Maryland, USA

**Correspondence:** Christopher A. LeClair ([leclairc@mail.nih.gov](mailto:leclairc@mail.nih.gov)) | Dingyin Tao ([dingyin.tao@nih.gov](mailto:dingyin.tao@nih.gov))

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## ABSTRACT

Kinase inhibitors represent a vital class of therapeutic agents widely used in cancer research, immunology, and other disease areas. Mass spectrometry (MS) employing specially designed small-molecule kinase-binding probes has become an essential strategy for identifying novel kinase drug targets. While traditional MS approaches often rely on targeted proteomics (e.g., multiple reaction monitoring [MRM]) or data-dependent acquisition (DDA), data-independent acquisition (DIA) offers broader and more reproducible quantification, especially for low-abundance peptides. In this study, we systematically developed an activity-based protein profiling (ABPP) platform leveraging DIA, through integrated in-house informatics tools for data filtering and motif analysis, to provide an effective kinase profiling workflow. Compared to DDA, the DIA approach yielded more than a 100% increase in identified biotinylated peptides and over 40% improvement in kinase peptide coverage, while reducing the analysis time by half (90 min vs. 180 min per sample). Additionally, there was a modest improvement to the coefficient of variation (CV) in kinase peptide quantification (decrease from 11.41% to 10.70%; mean CV). Shorter liquid chromatography (LC) gradient times (60, 45, and 30 min) were evaluated as a means for increasing sample analysis throughput. Notably, no significant loss in kinase peptide coverage was observed due to shorter gradients, highlighting the capability of DIA to significantly enhance the efficiency and scalability of kinase profiling workflows.

## 1 | Introduction

Protein kinases are important enzymes that are involved in a variety of cell functions and signal transduction pathways by catalyzing the transfer of  $\gamma$ -phosphates from adenosine triphosphate (ATP) to protein recipients [1, 2]. Dysregulation of protein kinases is implicated in numerous diseases, including cancer, immune disorders, and inflammatory disease, making them a heavily investigated class of drug targets [3–5]. Human protein kinases are a large family of proteins (>500) possessing a highly conserved ATP-binding domain, which presents a significant

challenge in the generation of selective kinase inhibitors [6]. Efficacy of the inhibitors may be enhanced by introducing a degree of nonselectivity, but the undesired engagement of off-targets could consequently induce unwanted side effects [7, 8]. Evaluation of kinase selectivity against a comprehensive panel of kinases is essential to better understand kinase inhibitor efficacy, as well as potentially identify new therapeutic targets.

Biochemical assays are classical approaches for kinase selectivity profiling capable of screening large kinase panels in a high-throughput manner [5]. While a valuable tool, these methods

Ravi Tharakan and Yanyan Qu contributed equally.

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typically rely on recombinantly expressed kinases and may not reflect the native kinase activities as observed in biological systems [9, 10]. Alternatively, chemoproteomics approaches have been developed for evaluation of inhibitors binding to kinases under close-to-physiological conditions such as the Kinobeads [11, 12] and KiNativ [13, 14] platforms. Activity-based protein profiling (ABPP) is a chemoproteomic technique that utilizes selective probes to covalently bind proteins within the lysate followed by isolation and identification [6]. Competitive ABPP (cABPP) is a variant where the proteins are exposed to a small-molecule inhibitor prior to the introduction of the covalent probe [15]. Proteins identified from the initial ABPP experiment but decreasing upon cABPP analysis are considered to competitively bind the inhibitor, providing selective targets for drug development.

Kinases generally function through initial binding of ATP and subsequent engagement with a protein target, facilitating hydrolysis of the ATP to adenosine diphosphate (ADP) and concomitant phosphorylation of the target. The capability of both ATP and ADP to bind the active site was exploited to develop selective probes for exploration of the kinome and discovering small molecules with therapeutic interest [16]. The KiNativ platform relies on competition between a biotinylated ATP or ADP probe with small-molecule inhibitors to selectively label kinases in native systems such as cell and tissue lysates. Sample preparation and analysis begin with exposure of the lysed cells to either a small-molecule inhibitor or solvent control, e.g., dimethyl sulfoxide (DMSO). The samples are then treated with either the ATP or ADP probe, which covalently binds to the ATP-binding pocket of the kinase. Nucleophilic attack by the primary amine of the conserved lysine residue within the active site transfers the biotin tag to the protein. Pull-down and isolation of the labelled peptides from the digested peptide mixture are facilitated through streptavidin beads, which strongly bind the biotin tag. The labelled peptides are removed from the beads to provide clean samples, which are injected into a high-performance liquid chromatography-mass spectrometer (HPLC-MS) for identification and quantitation. Through comparison of the relative abundance of kinases for compound-treated and control samples, the kinase selectivity and inhibitor potency can be determined [13]. Various shotgun proteomics or multiple reaction monitoring (MRM) quantitative proteomics methodologies have been incorporated into the KiNativ platform [10, 13, 14, 17].

An inherent limitation of studies using this method is the indiscriminate labelling of lysine residues by the probe, the majority of which are from other ATP-binding proteins. This gives rise to peptides from kinases potentially being missed as they are present at very low abundance in the pull-down fraction [14]. Targeted proteomics approaches have shown advantages in improving sensitivity by monitoring a custom list comprised of kinase peptides that are identified using data-dependent acquisition (DDA)-MS, as reported in previous studies [14, 17]. However, method development of a targeted proteomics assay such as parallel reaction monitoring (PRM) or MRM-MS is usually time-consuming, and the protein coverage is limited to a list of known kinases. Furthermore, DDA analysis is a full scan method where the top intensity ions have higher priority to be fragmented, thus excluding low-abundance peptides contributing to poor reproducibility.

Despite these considerations, the KiNativ platform remained a powerful assay, and we readily utilized it to screen inhibitors for projects selectively targeting kinase-related diseases or to identify off-target effects that introduced inadvertent and undesirable toxicity. Recent advancements in instrumentation and bioinformatics have allowed data-independent acquisition (DIA)-MS to emerge as an alternative label-free quantitative proteomics approach, having the advantage of both PRM-MS and DDA-MS [18–20]. In the DIA mode, all precursors in the defined mass-to-charge ( $m/z$ ) windows are fragmented and measured regardless of intensity, enabling unbiased proteomics quantitation with deeper proteome coverage and better reproducibility [18]. DIA-MS also facilitates a higher analysis throughput when utilizing MS instruments that provide higher ion transmission efficiencies and shorter duty cycles [21]. As such, we incorporated DIA-MS with cABPP to establish an enhanced and robust platform for in-depth chemoproteomic kinase selectivity evaluation.

We initially evaluated and optimized the sample preparation protocol to ensure reproducibility in the isolation of labelled peptides. Assay parameters, including comparison between the ATP and ADP probes, were then assessed to improve performance and further optimize the workflow. Subsequent studies were conducted to determine kinase coverage relative to previously obtained KiNativ data. Herein, we detail how kinome profiling was improved with the use of the proposed DIA-MS platform instead of a traditional DDA-MS method. Insights gained from these efforts supported the potential application of the DIA-based approach in high-throughput kinase selectivity screening.

## 2 | Methods

### 2.1 | Cell Lysate Preparation

Hek293LX cells were originally purchased from Clontech (now Takara Bio USA, San Jose, CA, USA) and maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM), supplemented with GlutaMAX (Gibco, Fisher Scientific, Pittsburgh, PA, USA), 10% fetal bovine serum (FBS) (Cytiva, Marlborough, MA, USA), 1% MWM Non-Essential Amino Acids Solution (Gibco, Fisher Scientific, Pittsburgh, PA, USA), and 1% penicillin/streptomycin (Gibco, Fisher Scientific, Pittsburgh, PA, USA). Two hundred million Hek293LX cells were harvested with trypsin-EDTA, washed once with ice-cold PBS and resuspended in 4 mL of IP Lysis Buffer, supplemented with protease-inhibitors and phosphatase-inhibitors cocktails. After 15 min incubation on ice, cells were lysed using a Dounce homogenizer and the resulting lysate was clarified by centrifugation for 10 min at 13000 rpm. The clarified supernatant was desalted with a Zeba Spin Desalting Column using 1 column for each mL of lysate. Following bicinchoninic acid (BCA) quantification, the lysate was diluted to a concentration of 2 mg/mL with Reaction buffer, and individual 500  $\mu$ L aliquots (corresponding to 1 mg total lysate) were used for further analyses. Cell lysate pellets have been stored at  $-80^{\circ}\text{C}$  for up to 6 months prior to successful use in kinase profiling. However, fresh cell lysate is preferred and was used for experiments herein as better performance and reproducibility are observed. The IP Lysis Buffer, Zeba

Spin desalting column, and Reaction buffer are from Pierce Kinase Enrichment Kits with ActivX Probes (Thermo Scientific, Waltham, MA, USA).

## 2.2 | Compound Binding, Probe Labelling, and Peptide Isolation

The binding, probe labelling, and isolation of labelled peptides from the lysate were performed using Pierce Kinase Enrichment Kits with ActivX Probes according to the manufacturer's recommendation, with minor modifications. Unless otherwise noted, the chemicals and consumables used were from the enrichment kit. A 500  $\mu$ L aliquot of cell lysate (2 mg/mL) was sequentially incubated at room temperature with 20 mM  $MgCl_2$  for 1 min, then DMSO (MilliporeSigma, Burlington, MA, USA) or compound for 10 min, and finally desthiobiotin-ATP or desthiobiotin-ADP probe for 10 min. The lysate mixture was then denatured and reduced by the addition of a prepared solution of urea from the kit and tris(2-carboxyethyl)phosphine (TCEP) (MilliporeSigma, Burlington, MA, USA) in IP Lysis Buffer with final concentrations of 4.9 M and 4.8 mM, respectively, at 55 °C for 45 min. The protein mixture was alkylated with 37 mM iodoacetamide (MilliporeSigma, Burlington, MA, USA) in the dark for 30 min at room temperature and then buffer-exchanged into 2 M urea/20 mM ammonium bicarbonate (pH 8.0) (MilliporeSigma, Burlington, MA, USA) using a Zeba Spin Desalting Column (Pierce, Thermo Scientific, Waltham, MA, USA). After desalting, the lysate proteins were digested overnight at 37 °C with Trypsin/Lys-C (Promega, Madison, WI, USA) at a 1:50 (w/w) enzyme/substrate ratio. The desthiobiotinylated peptides were then captured using Streptavidin Agarose resin. To remove nonspecific binding, the resin was washed four times with each solution (500  $\mu$ L/time) in order with IP Lysis Buffer, 1X PBS (Gibco, Fisher Scientific, Pittsburgh, PA, USA), and MS-grade water (MilliporeSigma, Burlington, MA, USA). Finally, the labelled peptides were eluted from the resin using 50% acetonitrile (MeCN) (Fisher Chemical, Pittsburgh, PA, USA) with 0.1% trifluoroacetic acid (TFA) (MilliporeSigma, Burlington, MA, USA) and stored at –20 °C after lyophilization.

## 2.3 | LC-MS/MS Analysis

LC-MS/MS analysis was performed using an UltiMate 3000-nano LC system coupled to the Orbitrap Fusion Lumos Tribrid mass spectrometer equipped with the Nanospray Flex ion source (Thermo Fisher Scientific, Waltham, MA, USA) [22]. Peptides were resuspended in 25  $\mu$ L of 2% MeCN with 0.1% formic acid (FA) (Thermo Fisher Scientific, Waltham, MA, USA), and 3  $\mu$ L was loaded onto the trap column (AcclaimPepMap 100 C18, 75  $\mu$ m  $\times$  2 cm, particle size: 3  $\mu$ m, 100 Å) by autosampler using loading solvent (2% MeCN in 97.9% MS-grade water with 0.1% FA) at a flow rate of 4  $\mu$ L/min. Elution of peptides from the analytical column (with spray tip, 75  $\mu$ m  $\times$  30 cm, 1.7  $\mu$ m, 100 Å; CoAnn Technologies, Richland, WA, USA) was performed using a 120-min method (~90-min gradient) starting at 98% buffer A (0.1% FA in MS-grade water) at a flow rate of 300 nL/min. The mobile phase was maintained at 2% buffer B (80% MeCN, 19.9% water, and 0.1% FA) for 5 min, 2%–9% B for 2 min, 9%–38% B for 73 min, 38%–50% B for 10 min, 50%–90% B for 5 min, and

maintained at 90% B for 13 min, followed by re-equilibration of the column with 2% B for 10 min. Column oven temperature was set as 40 °C.

For the 60-min gradient, the mobile phase was maintained at 2% buffer B for 5 min, 2%–9% B for 1 min, 9%–38% B for 49 min, 38%–50% B for 6 min, 50%–90% B for 4 min, and then held at 90% B for 6 min. For the 45-min gradient, the mobile phase was maintained at 2% buffer B for 5 min, 2%–9% B for 1 min, 9%–38% B for 36 min, 38%–50% B for 5 min, 50%–90% B for 3 min, and then held at 90% B for 6 min. For the 30-min gradient, the mobile phase was maintained at 2% buffer B for 5 min, 2%–9% B for 0.5 min, 9%–38% B for 24.5 min, 38%–50% B for 3 min, 50%–90% B for 2 min, and then held at 90% B for 5 min.

The mass spectrometer was operated in positive-ionization mode with the Nanospray Flex ion source with spray voltage set at 1800 V, and ion transfer tube temperature set at 250 °C. The MS scan was operated in DIA mode [20], with a precursor range of 390–1010 m/z and an isolation window of 8 m/z with 1 m/z overlap, resulting in 75 windows for each scan cycle. The resolution for MS1 scan was set to 120K. Both standard Automatic Gain Control (AGC) target and auto maximum injection time were selected. The MS2 fragmentation was performed in the Orbitrap with 30K resolution and a normalized collision energy of 30% at HCD activation mode. The AGC target was set at 800%. The MS2 scan range was defined as 145–1450 m/z, and the loop control was set to 3 s.

For samples analyzed using DDA, the method was comparable to a published method we successfully developed using a 180 min gradient [22].

## 2.4 | Bioinformatics Analysis—Data-Dependent Acquisition

The proteomics data from DDA-MS were searched by Proteome Discoverer 3.0 (Thermo Scientific, Waltham, MA, USA) with the Sequest algorithm for peptide identification and quantitation. The MS raw data were searched against a Swiss-Prot human database (version Nov 2022, reviewed database) consisting of 20,328 entries with a precursor ion mass tolerance of 10 ppm and a fragment ion mass tolerance of 0.02 Da. Peptides were searched using fully tryptic cleavage constraints and up to two internal cleavages sites were allowed for tryptic digestion. Fixed modifications consisted of carbamidomethylation of cysteine. Variable modifications considered were Biotin:Thermo-8310, on lysine residues, corresponding to the biotinylated lysine from the ThermoFisher kit, oxidation of methionine residues, and N-terminal protein acetylation. Peptide identification false discovery rates (FDR) were limited to a maximum of 0.01 using identifications from a concatenated database from the non-decoy and decoy databases. Label-free quantification analysis used the “Precursor Ions Quantifier” node from Proteome Discoverer and normalized by total peptide amount. For kinome analysis, further quality control and comparative analysis was performed in R 4.4.0 using the following packages: tidyverse (version 2.0.0) for data formatting and preprocessing; ensembledb (version 2.28.0), EnsDb.Hsapiens.v86 (version 2.99.0), and biomaRt (version 2.60.0) for annotation; cowplot (version 1.1.3),

VennDiagram (version 1.7.3), RColorBrewer (version 1.1-3), pheatmap (1.01.12), and ggExtra (version 0.10.1) for plotting. To process the data, all peptides were exported from Proteome Discoverer and then loaded into an R dataframe using read\_tsv. All non-biotinylated peptides were filtered out by searching for the modification Biotin:Thermo-88310 followed by exclusion of all unlocalized biotinylation. The position of lysine 1 (Lys 1) and lysine 2 (Lys 2) was determined by extracting the sequence surrounding the biotin modification from the peptide using stringr, and regexes was used to match known Lys 1 and Lys 2 motifs [13]. A list of 636 known human kinases was downloaded from the Uniprot/Swiss-Prot database using biomaRt (keyword KW-0418, taxonomy\_id:9606, downloaded 2023.01.24) to remove all non-kinase hits from the kinome data. For Hek293 cell kinase comparison, the total known Hek293 cell proteome was downloaded from a comparative study of common cell lines [4], filtering for proteins detected in all three replicates of the Hek293 cell line. These proteins were then matched to the list of 636 known human kinases to ascertain the expressed Hek293 cell kinases. These results were compared against a kinase list generated from HPLC-MRM data obtained in a previous kinase analysis conducted by KiNativ.

## 2.5 | Bioinformatics Analysis—Data-Independent Acquisition

For DIA proteomics analysis, data were searched by Spectronaut 18.6 in directDIA [23] mode using a FASTA database created from all known human proteins from Swiss-Prot (downloaded 02/21/2024) with the following parameters: precursor q-value cutoff: 0.01; precursor PEP cutoff: 0.2; protein q-value cutoff (experiment): 0.01; protein q-value cutoff (run): 0.05; protein PEP cutoff: 0.75. Decoy sequences were generated with the “mutated” method and preferred fragment source NN predicted fragments, using a library size fraction of 0.1. Post-translational modifications (PTM) were analyzed with a PTM localization probability cutoff of 0.75 and PTM consolidation using sum. Cross-run normalization was performed with filter type “keep if any” for Biotin:Thermo-88310 using local regression normalization. The Biognosys default settings were used for the remainder of the search settings. Further analysis in R was performed with the same software packages as detailed above for DDA with biotinylation and kinase filtering performed in the same manner. For comparisons between DIA and DDA peptides and proteins, the “stripped peptides” were used as the peptide sequence.

## 3 | Results

### 3.1 | An Activity-Based Platform for Kinase Inhibitor Target Screening Using DIA Proteomics

Previous applications of proteomics to determine kinase inhibitor targets have primarily used a targeted MRM method with a preselected list of kinases. While the method allows a specific set of proteins to be consistently targeted in every experiment, it restricts the discovery of novel kinase targets or off-target kinases. As such, these original MRM-based methods are viable when screening a compound library against a set of kinase targets to determine or verify engagement. However, it is often

beneficial or even necessary to identify all kinase targets of an existing drug. For these cases, a more comprehensive, untargeted proteomics method is required to elucidate previously unknown interactions to uncover novel kinase targets that either cause detrimental off-target effects or are advantageous for the repurposing of kinase inhibitors in the treatment of disease states outside the original scope.

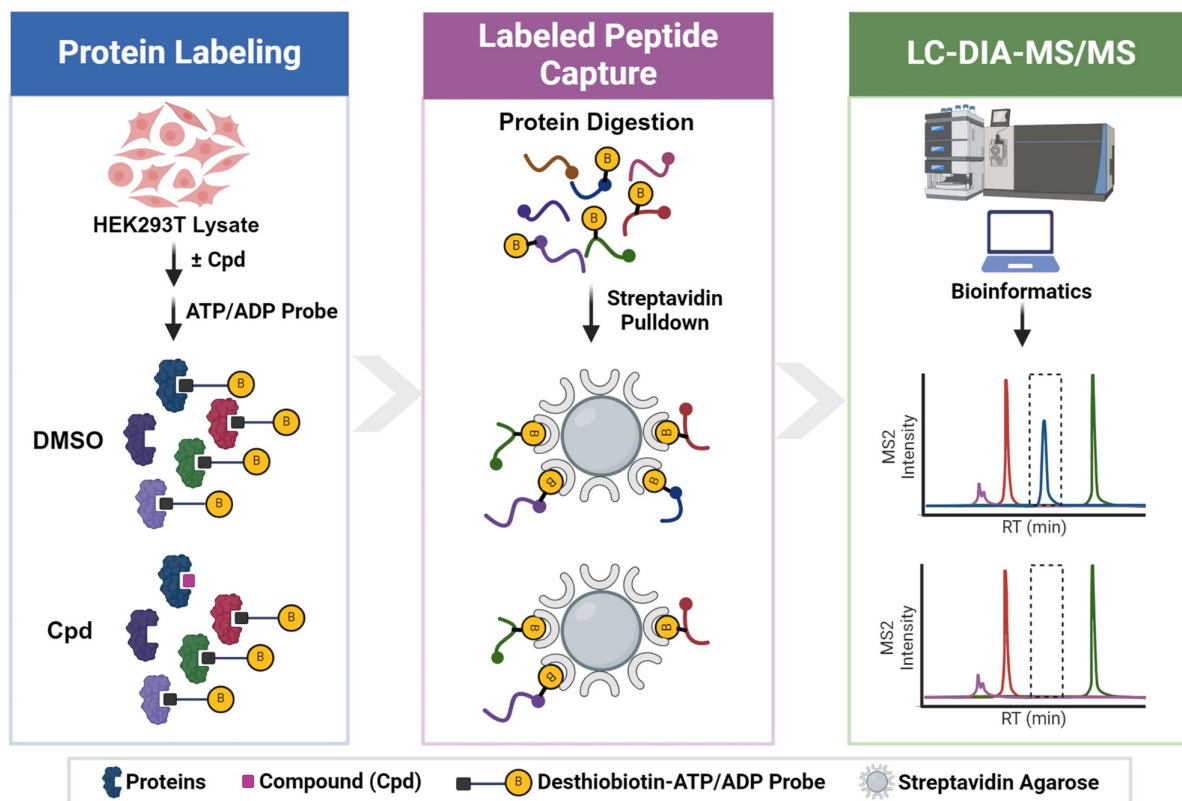
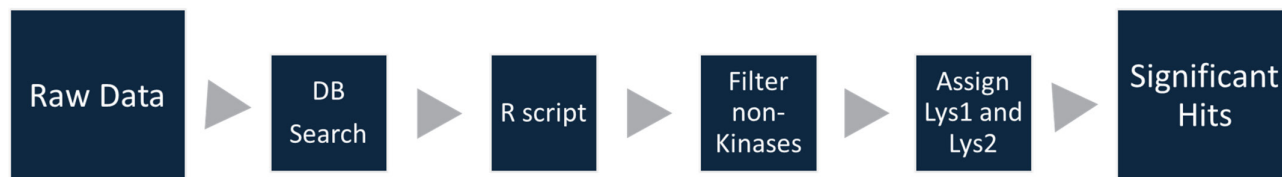
To this end, we developed an untargeted approach for kinase inhibitor screening, which allows general detection of kinase inhibition events in an unbiased manner (Figure 1). The approach utilizes a desthiobiotinylated probe as it retains affinity and specificity for streptavidin, but unlike biotin, it can be easily removed without the need of harsh chemicals or high temperatures, which are not amenable to peptide sample integrity. The probe, comprised of desthiobiotin connected to either ATP or ADP through a linker, initially binds the kinase active site through the ATP or ADP with successive nucleophilic addition by lysine to covalently bind the desthiobiotin linker tag to the kinase. In the same manner as the KiNativ method, a subsequent streptavidin pulldown isolates and enriches the biotinylated peptides of associated kinases after proteomics digestion steps. The identification of kinase inhibitors through activity-dependent proteomics using cABPP preincubates the cell lysates with a compound of interest prior to treatment with the probe. As part of our method development, we established a central data analysis pipeline necessary to confidently detect kinase inhibition events.

Our overall bioinformatics workflow (Figure 1B) first involved searching data generated from either the DDA or DIA method and then postprocessing of the search results is completed in R and Bioconductor, which allows the writing of custom scripts to analyze for specific modifications. After filtering out any peptides not having a biotin modification, we observe >80% of detected and quantifiable tryptic peptides include a biotin modification, demonstrating the specificity of the biotin-streptavidin pulldown. Since our primary interest is kinase inhibition, the next step of the workflow filters out any non-kinases based on comparison to the kinase library. Lys 1 and Lys 2 along with their surrounding motifs are recognized to be conserved in the active site of all known kinases [14]. Furthermore, the uniqueness of each motif enables differentiation between the lysines with Lys 1 having a conserved alanine located two amino acids preceding (AxK) and Lys 2 having a conserved aspartic acid located two amino acids preceding (DxK). Using custom R scripts, we can recover detection rates for each of these lysine motifs using the amino acid sequences of detected peptides. Finally, an “inhibition level” is determined, using the abundance ratio between the treated and the untreated samples. Inhibition level and adjusted *p*-value with experiments performed in triplicate are used to determine which kinases are significantly inhibited.

### 3.2 | Evaluation and Comparison of ADP and ATP Probes

A heatmap analysis was performed on all biotinylated peptides and subsequent mapping to a kinase through an LC-MS DDA-based method to understand the overall characteristics of our dataset (Figure 2A). The ATP and ADP-based probes were



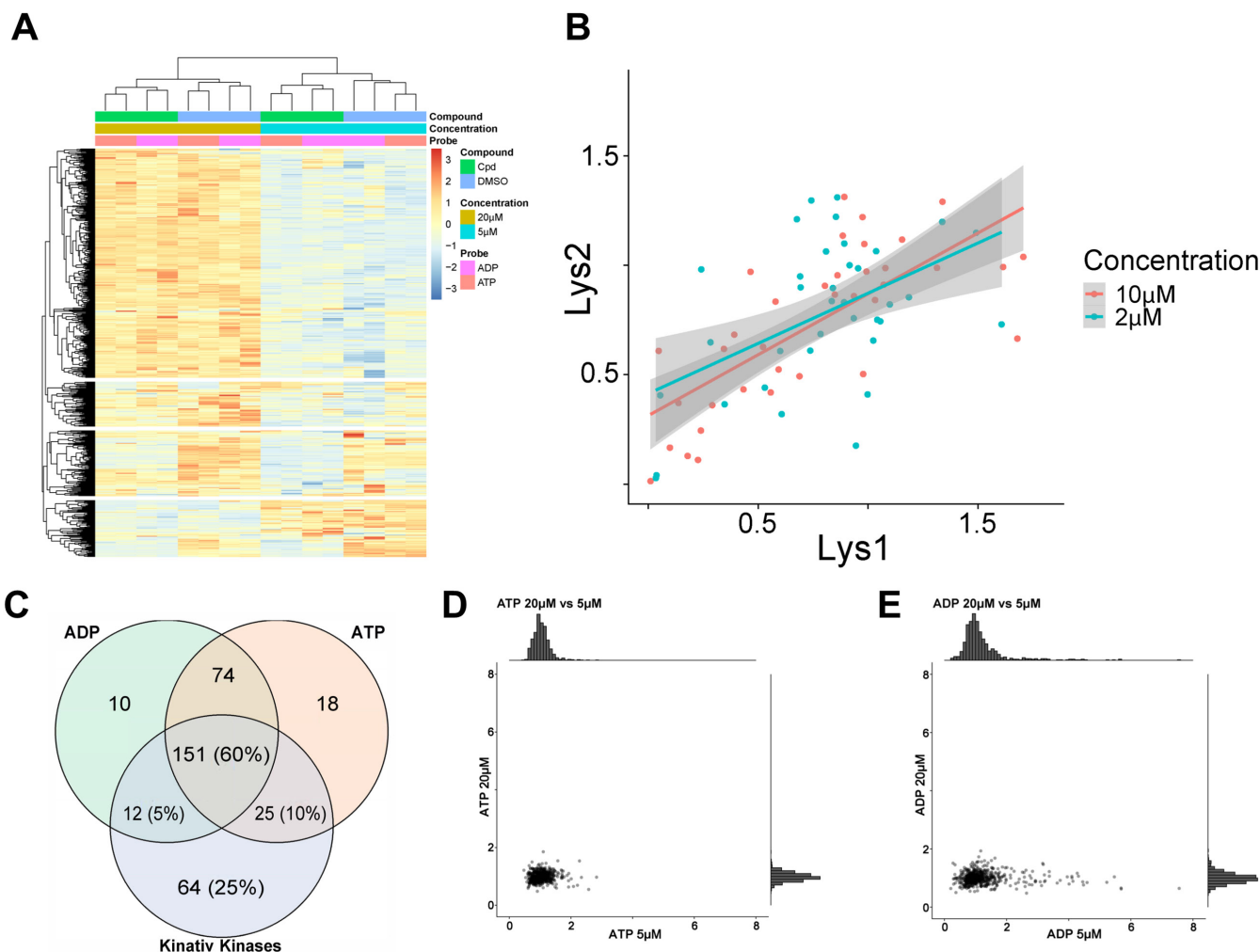
**A****B**

**FIGURE 1** | Workflow for kinase selectivity profiling using KiNativ assay combined with DIA-MS/MS (A) and informatics analysis to determine the hits (B).

compared at two different concentrations (5 and 20  $\mu\text{M}$ ), testing against a novel small-molecule kinase inhibitor (**Cpd**) designed and synthesized in-house for a targeted kinase therapeutic program. Peptides common between all experiments were row-normalized and hierarchically clustered, as were the samples. All replicates of the eight sample groups (**Cpd** or DMSO with either ADP or ATP probe at two concentrations) were clustered together, and the kinase peptides were separated into four clear clusters. Looking at the top large cluster, which comprises approximately half of the identified peptides, the abundance appeared to vary primarily due to probe concentration, suggesting many peptides are highly dependent on the amount of probe present. Whereas the second cluster appeared to depend on both concentration and the probe used. The third cluster, comprising about 25% of all peptides, interestingly had a dependence on the presence or absence of **Cpd** and thus represents the kinases affected by the potential kinase inhibitor, which is our primary focus. The fourth and final cluster was reliant on a combination of probe concentration and presence of the inhibitor. The results of the cluster analysis gave us confidence that a substantial proportion of detected peptides are decreased because the presence of the inhibitor restricts binding of ATP or ADP probe, with the reduction being defined as the peptide “inhibition level.”

Initially, we studied the conserved lysines within the active site of the kinases to determine if our system was biased towards a particular type of kinase inhibition. By analyzing the sequences of the detected peptides through examination of the biotinylation site and the surrounding amino acid motif, we were able to classify all binding as Lys 1, Lys 2, or neither. It was noted that approximately 40% of all peptides contained Lys 1 or Lys 2, leading us to assess for bias towards either conserved lysine by plotting the inhibition levels across all peptides. The analysis compared all peptides for which a biotinylation event corresponding to both Lys 1 and Lys 2 was found (Figure 2B). Overall, we find a general correlation between Lys 1 and Lys 2 inhibition levels, with no evidence of bias towards a specific active site lysine and at two concentrations of kinase inhibitor (2 and 10  $\mu\text{M}$ ).

To assess the breadth of kinome coverage provided by our untargeted DDA method, we compared the kinases identified in our dataset with two reference sets: the comprehensive list of human kinases curated from UniProtKB/Swiss-Prot and the set of kinases targeted by a traditional MRM-based approach (Figure 2C). The analysis revealed that our DDA method detected approximately 50% of all known human kinases, acknowledging that this assessment was only conducted using a



**FIGURE 2** | Evaluation of ATP and ADP probes by a developed HPLC-MS DDA method. (A) Heatmap of all detected biotinylated peptides mapping to kinases, comparing an ADP probe to an ATP probe at several concentrations. Cell lysates were treated with either an ADP-based or an ATP-based probe and then with either a known kinase inhibitor or DMSO control. Abundances of all peptides are plotted. (B) Correlation between inhibition levels of Lysine 1 (Lys1) and Lysine 2 (Lys2) of active site of kinases. All kinases detected with peptides corresponding to both conserved lysines of the kinase active site are plotted. Inhibition level is calculated as the abundance ratio between peptide with and without inhibitor treatment. Kinase inhibitor was tested at two concentrations (2 and 10 μM). (C) Venn diagram of coverage of kinases detected in this experiment, compared to all known kinases from Swiss-Prot, and kinases originally run from KiNativ. Most kinases observed by KiNativ are detected, while approximately half are detected from all kinases. (D and E) Dot plot of distribution of inhibition levels, for ADP (D) and ATP (E). Different concentrations of probe are shown on axes.

single cell line (Hek293), which may limit the full representation of the kinome. Additionally, our method identified over 75% of the 188 kinases targeted by the MRM-based approach and uncovered more than 100 additional kinases not included in the original MRM panel. These findings underscore the capacity of our DDA-based workflow to provide a more comprehensive and unbiased profiling of the human kinome, surpassing the coverage achieved by traditional targeted methods.

We next analyzed the distribution of inhibition levels for each biotinylated peptide common to both ADP and ATP probes at varying concentrations to evaluate the inhibition profiles of the probes (Figure 2D,E). An approximate Gaussian distribution was exhibited for the inhibition levels at both ADP and ATP probe concentrations, facilitating statistical analysis of significantly inhibited peptides. However, we made the unexpected observation that a small proportion of peptides showed an

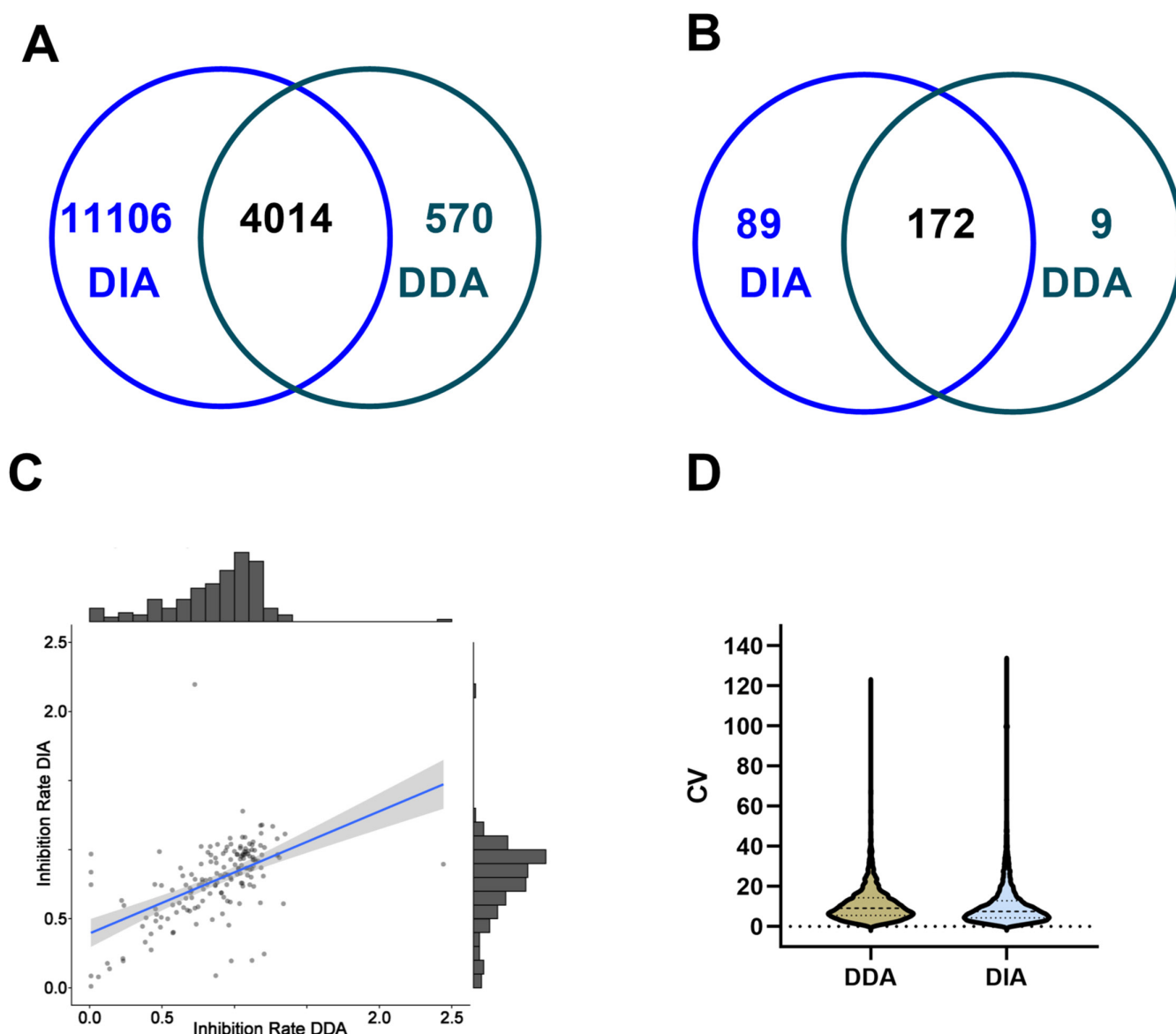
increase in binding of the ADP probe at the lower concentration with inhibitor-treated samples. This suggests that the inhibitor may enhance the binding affinity of the ADP probe in this context. The underlying mechanism for this increase in binding is currently unclear, but given the unexpected result, we decided to proceed with the ATP probe for subsequent experiments, as it did not exhibit significant concentration-dependent changes, thus ensuring consistency across our analyses.

### 3.3 | DIA Method for Kinase Inhibitor Screening

DDA method can provide lower robustness and reproducibility due to the stochastic nature of the mass spectrometer duty cycle. Furthermore, low-abundance peptides may be missed in DDA due to masking by high abundance isoforms. Therefore, we implemented a DIA method for kinome screening. The workflow

was validated by analyzing several samples that had only been treated with the ATP probe, and the comparative experimental results are shown in Figure 3A,B. We found that DIA achieves a >100% increase in the total number of biotinylated peptides detected, with 15,120 peptides in total detected in the DIA sample and 4,584 peptides detected in DDA (Figure 3A). Nearly 90% of the peptides identified by DDA were covered by DIA method. Similarly, a kinase level comparison with our previous list of known kinases showed a 40% increase in kinome coverage using DIA with 261 kinases being detected as opposed to 181 kinases detected by DDA (Figure 3B). Of note is that approximately 95% (172 out of 181) of the kinases originally identified by DDA were shared by DIA method. To see how the inhibitor concentration affected the DIA-based method, we compared results of the DIA and DDA using 1  $\mu$ M of **Cpd**, finding the inhibition level was highly correlated for both methods across

all peptides. Furthermore, a longer “left tail” of distribution is clearly present for both DIA and DDA at 10  $\mu$ M inhibitor, suggesting that several peptides are inhibited at the higher concentration (Figure 3C). Not only does the DIA method provide us with broader coverage of the kinome but it appears to be equally sensitive to inhibited peptides. Finally, we hypothesized that since DIA consistently detects all peptides, the variability in abundance quantification would be lower in DIA. To demonstrate this, we found paired peptides across the DIA and DDA experiments, calculating coefficient of variation (CV) values for each peptide with resulting distribution of CV values plotted in violin plot format (Figure 3D). The analysis satisfyingly shows DIA had a slightly lower mean CV value for quantification (DDA: 11.41%; DIA: 10.70%) and the overall distribution of CV values exhibited a substantial decrease.



**FIGURE 3** | HPLC-MS DIA analysis based kinome screening. (A) Comparison of total biotinylated peptides detected in DIA runs vs. DDA runs. (B) Coverage of all known kinases. Number of total kinases detected in DIA experiment vs. the DDA experiment. (C) Comparison of inhibition rates for DIA vs. DDA. Cells were treated with a kinase inhibitor at the concentration of 1  $\mu$ M, and the inhibition rate was calculated as the abundance ratio between treated and untreated. (D) Comparison of coefficient of variation (CV) values across DIA vs. DDA experiments. Peptides were matched across experiments, and CV values were calculated from abundances measures. Violin plot shows overall distribution of CV values.

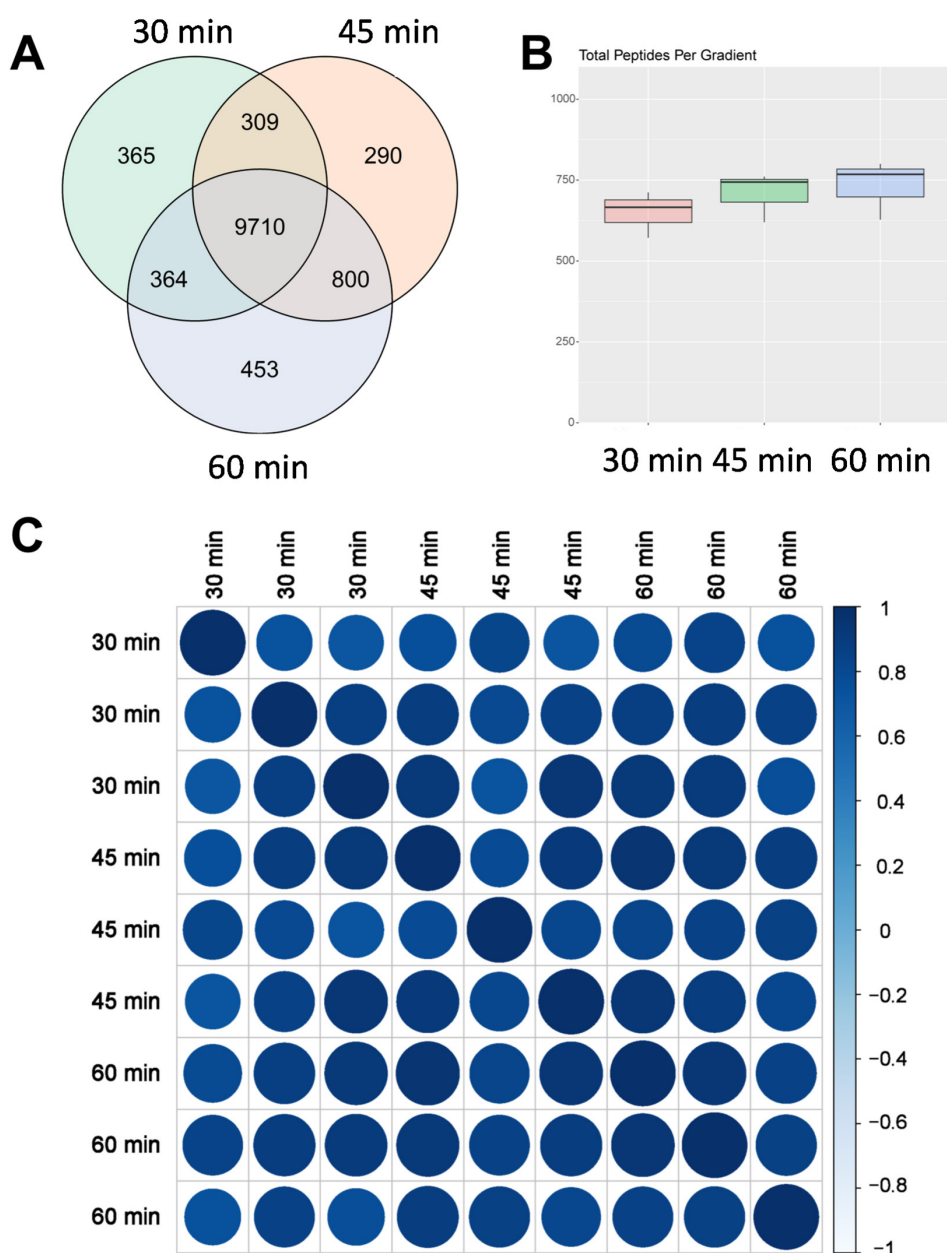
### 3.4 | Feasibility of High-Throughput Kinome Profiling Using Short LC Gradients

To evaluate the scalability of our kinase profiling approach with DIA-MS, we investigated the impact of shorter LC gradients on kinome quantification, testing gradient lengths of 60, 45, and 30 min (Figure 4). A substantial overlap in peptide identifications across all three gradient lengths was observed, with relatively few unique peptides detected at each length (Figure 4A). Importantly, there was no significant decrease in the number of kinase peptides identified as the gradient length decreased (Figure 4B). The robustness of quantification with shorter gradients was also assessed by the performance of a cross-correlation analysis (Figure 4C). The analysis demonstrated broad agreement in kinome quantification

across the different gradient lengths, indicating that shorter gradients do not compromise quantitative accuracy. These findings suggest that employing shorter LC gradients can enhance throughput in kinase profiling without sacrificing data quality, thereby facilitating high-throughput proteomic analyses.

## 4 | Conclusion

In this study, we developed and evaluated a cABPP platform employing a DIA-based method to enhance comprehensive kinome profiling. A DDA method was initially established to achieve unbiased kinome coverage and identify kinase inhibitor targets. We then built upon this initiative by adapting



**FIGURE 4** | Optimization of gradient length for higher-throughput ABPP, comparing a 60-, 45-, and 30-min gradients. (A) Overlap of peptides detected between DIA-based analyses with gradients of varying lengths. (B) Boxplots comparing total number of kinase peptides from three biological replicates of varying gradient lengths. (C) Cross-correlation plot of all kinase peptides detected in any sample of three biological replicates using different gradient methods.



our approach to incorporate DIA, enhancing the robustness and reproducibility of untargeted proteomics. Our findings demonstrate that the DIA-based workflow significantly increases kinome coverage while maintaining consistent quantification across peptides. This approach further enables broad kinome profiling without compromising reproducibility, offering a valuable tool for high-throughput analyses in kinase research.

The development of our current platform used cell lysates, but the workflow can be expanded to screen kinase inhibition targets in intact cells [24]. Future work will be focused on ensuring our ATP probe system is sufficiently cell permeable to reach targets within intact cells such that kinase inhibition activity can be studied in its physiological context, relying on the same principles outlined in this study. The present study also used manual proteomics sample preparation techniques, providing a standard rate of throughput. However, a higher-throughput proteomics pipeline would permit a larger number of kinase inhibitors to be prepared and analyzed in a more rapid fashion, opening up the possibility of comprehensive kinase library screening. We previously developed a high-throughput 384-well plate-based proteomics sample preparation platform, which can be adapted for the methods presented in the current study [25]. The modifications would enable the development of a comprehensive, high-throughput screening workflow for cABPP of small-molecule kinase inhibitors.

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## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [26] partner repository with the dataset identifier PXD072729 (<http://www.ebi.ac.uk/pride/archive/projects/PXD072729>) and <https://doi.org/10.6019/PXD072729>.

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