**Supporting Information**

# The Generality of Kinase-Catalyzed Biotinylation

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## I. Additional Experimental Details

### Materials

The disodium salt of adenosine 5'-triphosphate (ATP-2Na), glycerol, sodium hydroxide (NaOH), potassium chloride (KCl), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), sodium dodecyl sulfate (SDS), glacial acetic, acetonitrile, and the SA-Cy5 conjugate were purchased from Fisher. HPLC grade trifluoroacetic acid (TFA) and acetonitrile were obtained from VWR and EM Millipore, respectively. TEMED were purchased from Acros. $D_2O$ and $CD_3OD$ were obtained from Cambridge Isotope Labs. Ammonium bicarbonate and β-casein protein substrate was bought from Sigma. PKA, CK2, ABL, sodium fluoride, sodium orthovanadate and Abl peptide were purchased from New England Biolabs. All other kinases (AKT1, ASK1, Aurora A, CaMK4, CDK1, CHK1, CK1, DAPK1, ERK1, FLT1, GRK5, GSK3β, HIPK1, JAK3, MARK1, MLK1, MST1, NEK2, PAK1, RSK1, SRC, and TGFβR2), AKT peptide substrate, Autocamtide, CHKtide, IGF1Rtide, GSK3 peptide, Axltide, PAKtide, RSK1 peptide substrate, p34cdc2 peptide, kemptide, CK2 peptide substrate, poly(Glu4,Tyr1), Myelin Basic Protein (MBP), dephosphorylated casein protein, Histone H1 protein and ADP-Glo assay were obtained from Promega. Jak3tide was purchased from Anaspec. Triton X-100 was purchased from Fluka. Coomassie Briliant Blue was obtained from NuSep. Immobilion P and $P^{sq}$ PVDF membrane were purchased from Millipore. Sypro Ruby and Pro-Q diamond stain was obtained from Invitrogen. Ready Gel 16% Tris-tricine gels and acrylamide:bisacrylamide solution (37:1 crosslink) was purchased from Bio-Rad.

### Instrumentation

An HP 8452A Diode array UV-Vis spectrophotometer was used to measure absorbance of ATP-biotin after synthesis.[1] The luminescence values for the ADP-Glo assay were measured with a fluorimeter (GENios Plus Tecan). The SDS-PAGE apparatus was purchased from BioRad (Protean III) and a mini-gel setup was used. Protein transfer was carried out using the Mini-Transblot Electrophoretic Transfer Cell apparatus from Bio-Rad. Peptides were analyzed using Waters 1525 binary HPLC pump and Waters 2998 photodiode array detector. A reverse phase C-18 column (YMC America, INC 250×4.6 mm, 4μm, 8 nm) was used to separate peptide mixtures. Separated proteins after SDS-PAGE were visualized using a Typhoon 9210 scanner (Amersham Biosciences).

**Table S1. Concentrations of Kinase and Substrate used in Kinase reactions**

| Kinase | Biotinylation and Quantification[a] | Kinetic Studies[b] | |
|---|---|---|---|
| | Kinase (nM) | Kinase (nM) | Peptide/Protein Substrate[c] (µg/µL) |
| **ABL** | 125 | 15.0 | 0.2 |
| **AKT1** | 94 | 5.9 | 0.2 |
| **ASK1** | 133 | 4.2 | 0.1 |
| **Aurora A** | 112 | 1.4 | 0.1 |
| **CaMK4** | 101 | 1.3 | 0.2 |
| **CDK1** | 102 | 0.7 | 0.1 |
| **CHK1** | 98 | 3.1 | 0.2 |
| **CK1** | 129 | 16.1 | 0.1 |
| **CK2** | 166 | 166 | 0.2 |
| **DAPK1** | 113 | 2.1 | 0.1 |
| **ERK1** | 182 | 4.6 | 0.1 |
| **FLT1** | 85 | 13.3 | 0.2 |
| **GRK5** | 70 | 10.0 | 0.1 |
| **GSK3β** | 110 | 3.4 | 0.2 |
| **HIPK1** | 113 | 1.4 | 0.1 |
| **JAK3** | 125 | 1.6 | 0.1 |
| **MARK1** | 64 | 0.4 | 0.2 |
| **MLK1** | 104 | 3.9 | 0.1 |
| **MST1** | 96 | 3.0 | 0.2 |
| **NEK2** | 105 | 3.3 | 0.1 |
| **PAK1** | 88 | 10.5 | 0.2 |
| **PKA** | 150 | 53 | 0.2 |
| **RSK1** | 74 | 0.7 | 0.2 |
| **SRC** | 96 | 1.2 | 0.2 |
| **TGFβR2** | 0.118 | 18.4 | 0.1 |

[a] Biotinylation studies are shown in Figure 3 of the manuscript, while HPLC or ProQ quantification studies are shown in Figure 4 and 5 of the manuscript. [b] Kinetic Studies are shown in Table 1 of the manuscript. [c]The peptide or protein substrate used with each kinase for each experiment is shown in Figure 2b of the manuscript.

## II. Complete Gel Images for All Biotin Labeling

**Figure S1 (A).** Reactions with ASK1

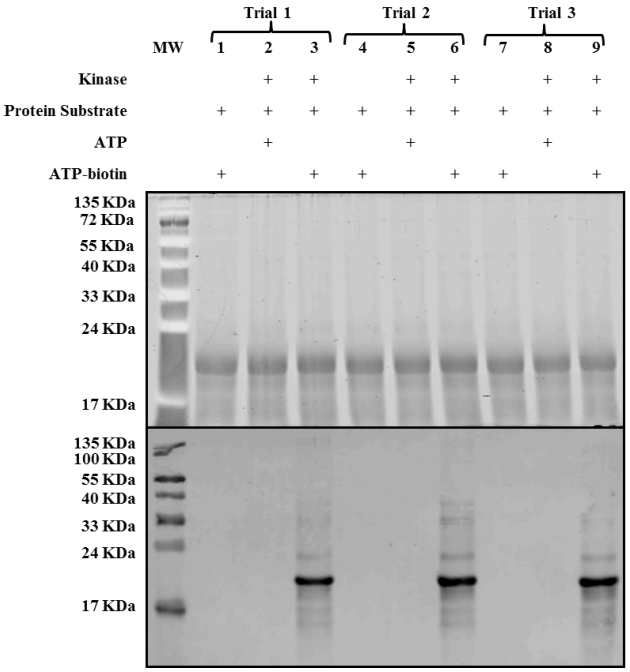



**Figure S1 (B).** Reactions with Aurora A

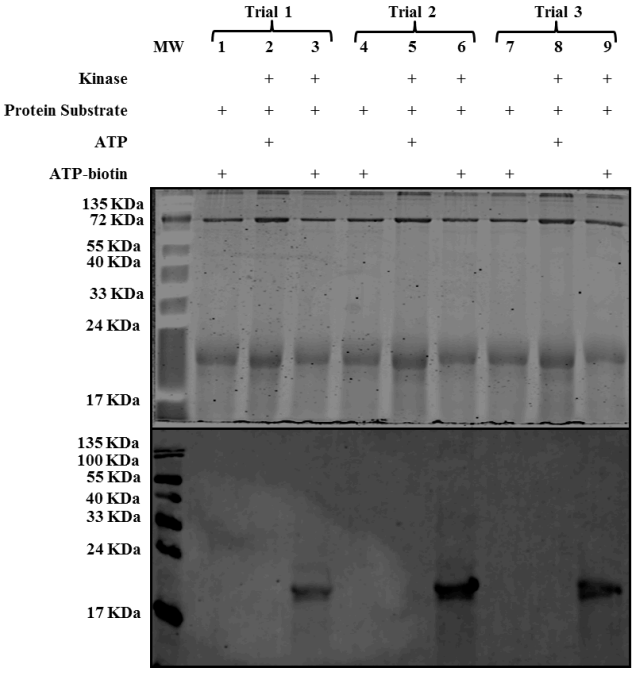


**Figure S1 (C).** Reactions with CDK1

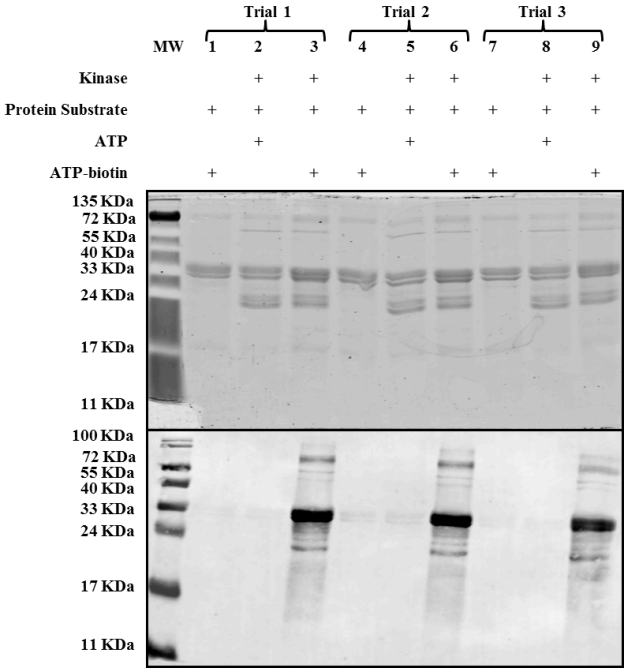



**Figure S1 (D).** Reactions with CK1

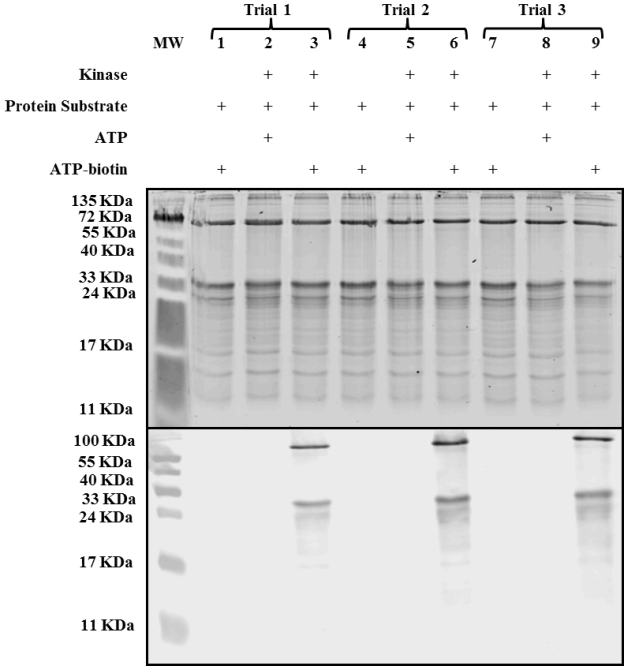


**Figure S1 (E).** Reactions with DAPK1

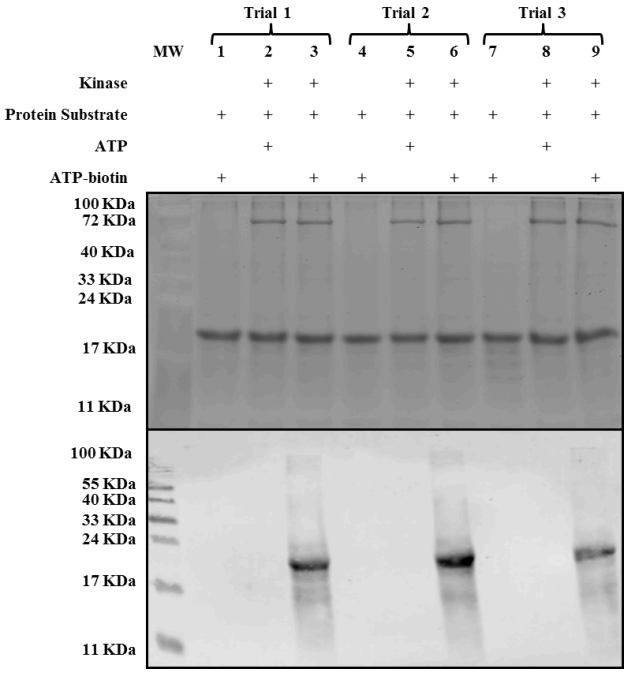



**Figure S1 (F).** Reactions with ERK1

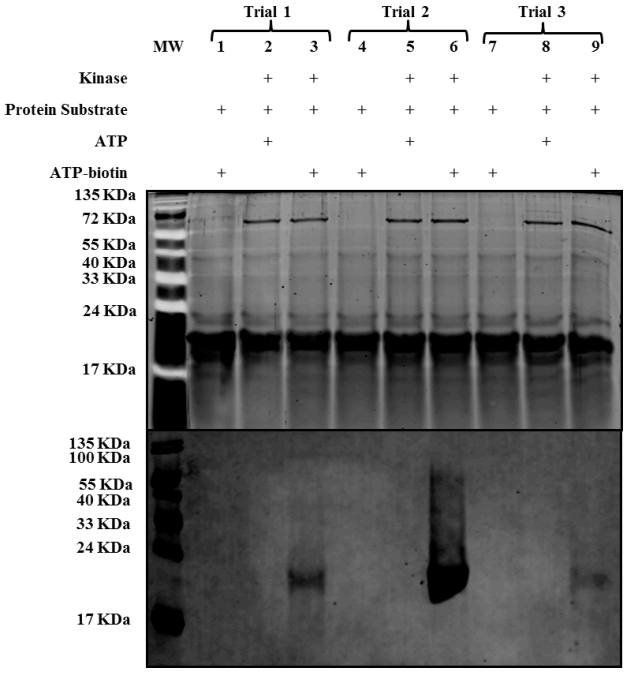


**Figure S1 (G).** Reactions with GRK5

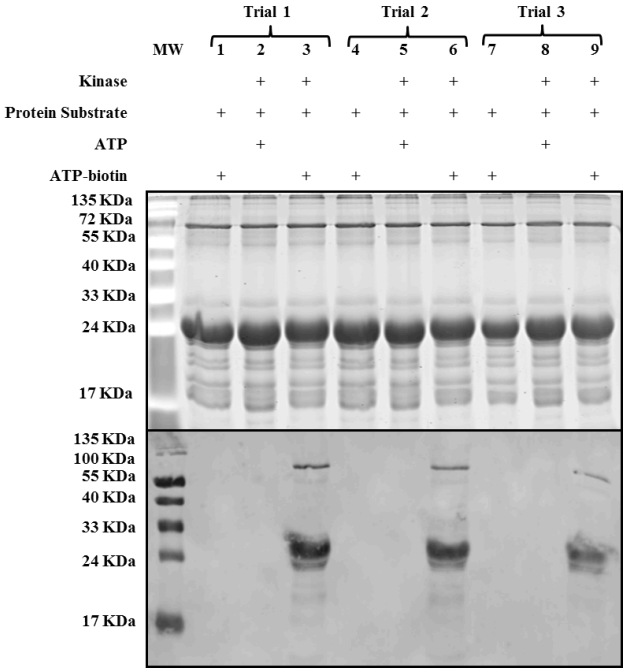



**Figure S1 (H).** Reactions with HIPK1

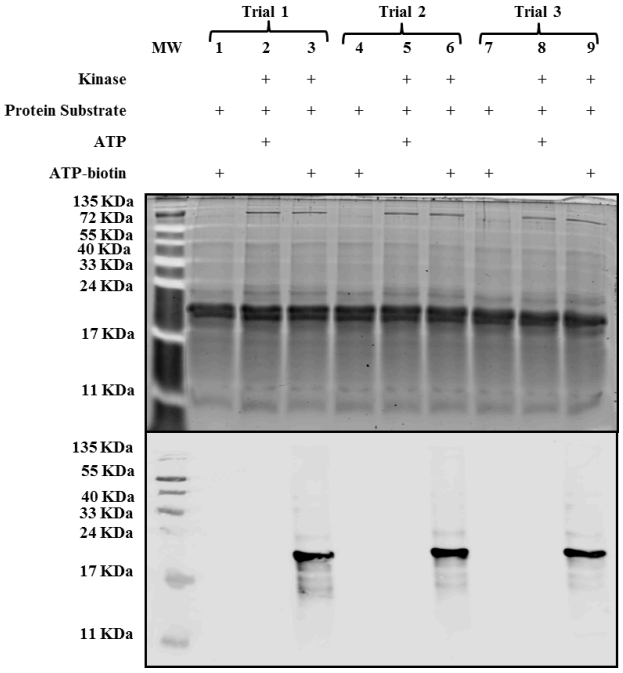


**Figure S1 (I).** Reactions with MLK1

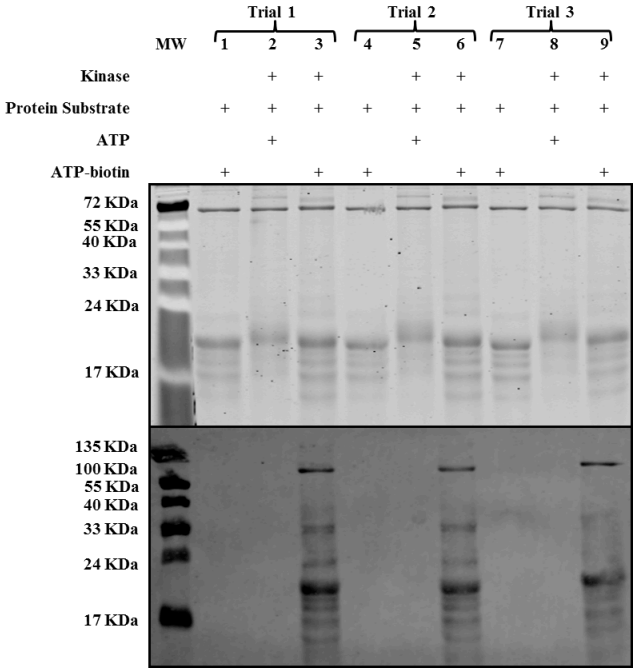



**Figure S1 (J).** Reactions with NEK2

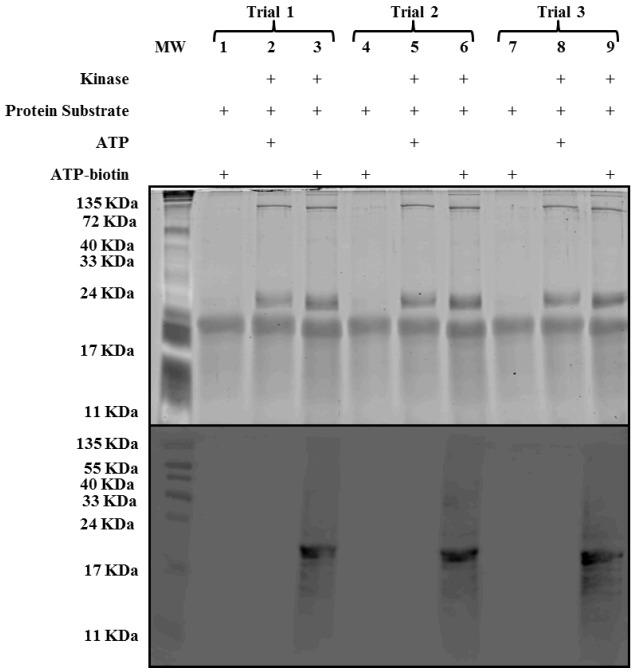


**Figure S1 (K).** Reactions with TGFβR2

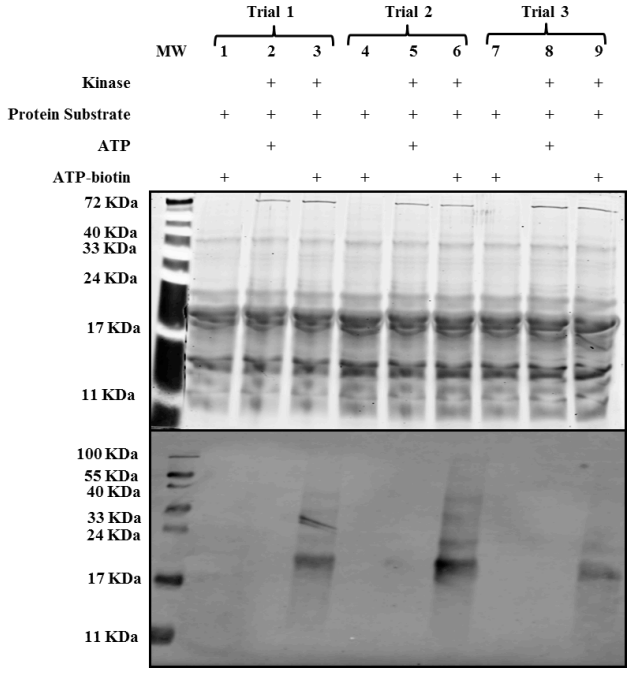



**Figure S1:** Full gel images for protein labeling experiments with eleven different kinases. Phosphorylated proteins or biotinylated phosphoproteins were created by incubating ATP or ATP-biotin with ASK1 and MBP (A), Aurora A and MBP (B), CDK1 and Histone H1 protein (C), CK1 and dephosphorylated casein protein (D), DAPK1 and MBP (E), ERK1 and MBP (F), GRK5 and Casein protein (G), HIPK1 and MBP (H), MLK1 and MBP (I), NEK2 and MBP (J), or TGFβR2 and MBP (K) in the manufacturer provide buffer (1X, Promega). Crude reaction mixtures were separated by 16% SDS-PAGE and transferred in to PVDF membrane. Proteins were visualized with sypro ruby stain (top gel), while biotinylated proteins were observed with SA-Cy5 conjugate (bottom gel). The reaction contents are indicated above each lane. Trial 1, 2, and 3 represented three independent trials. MBP stands for Myelin Basic Protein. Complete gel images for the remaining three kinases (Abl, CK2, and PKA) were reported earlier.[1] Data shown here are reported in the manuscript in Figure 3a.

**Figure S2 (A).** Reactions with AKT

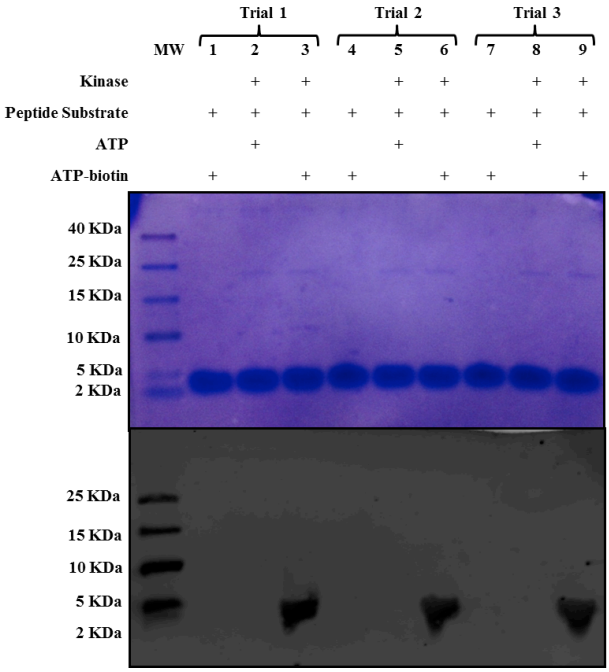



**Figure S2 (B).** Reactions with CaMK4

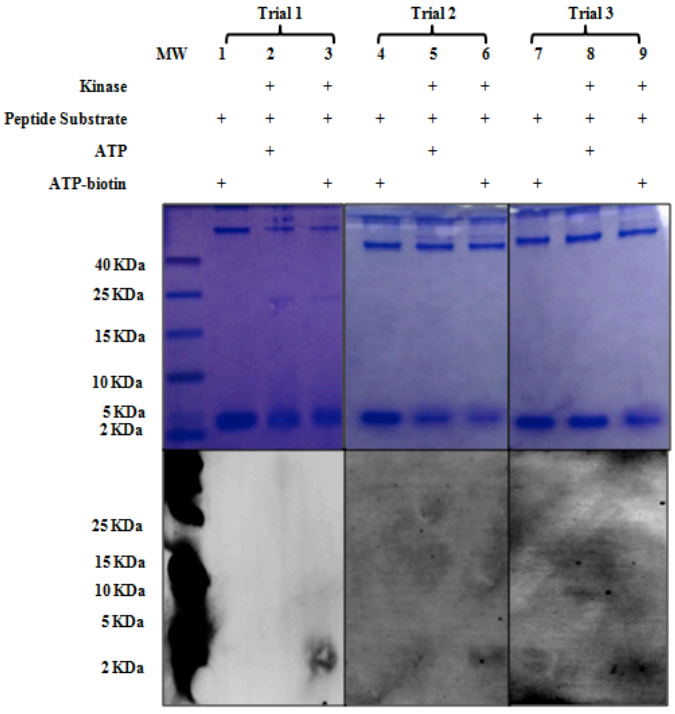


**Figure S2 (C).** Reactions with CHK1

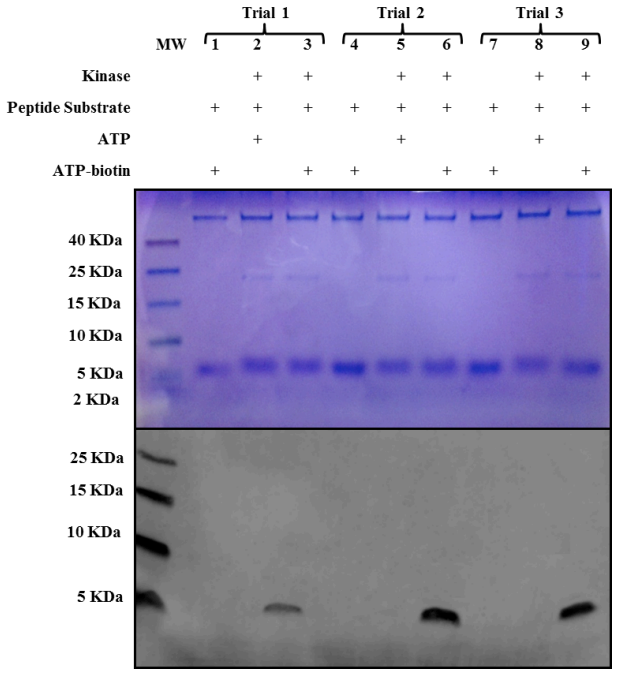



**Figure S2 (D).** Reactions with FLT1

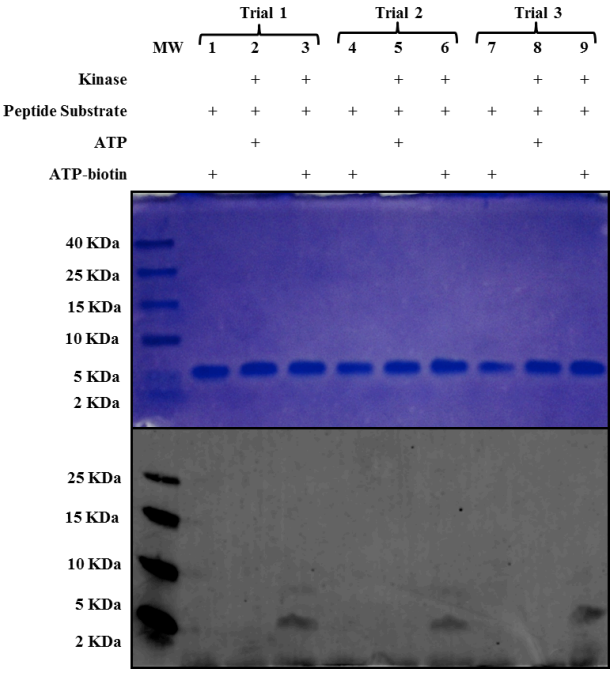


**Figure S2 (E).** Reactions with GSK3β

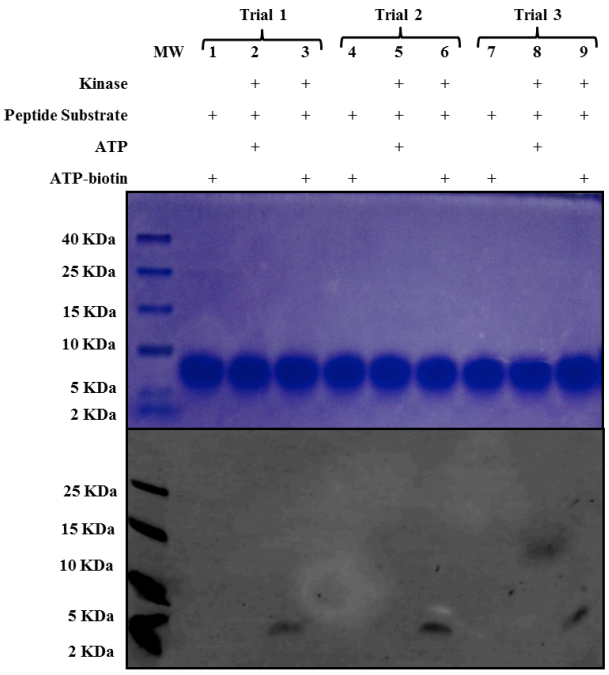



**Figure S2 (F).** Reactions with JAK3

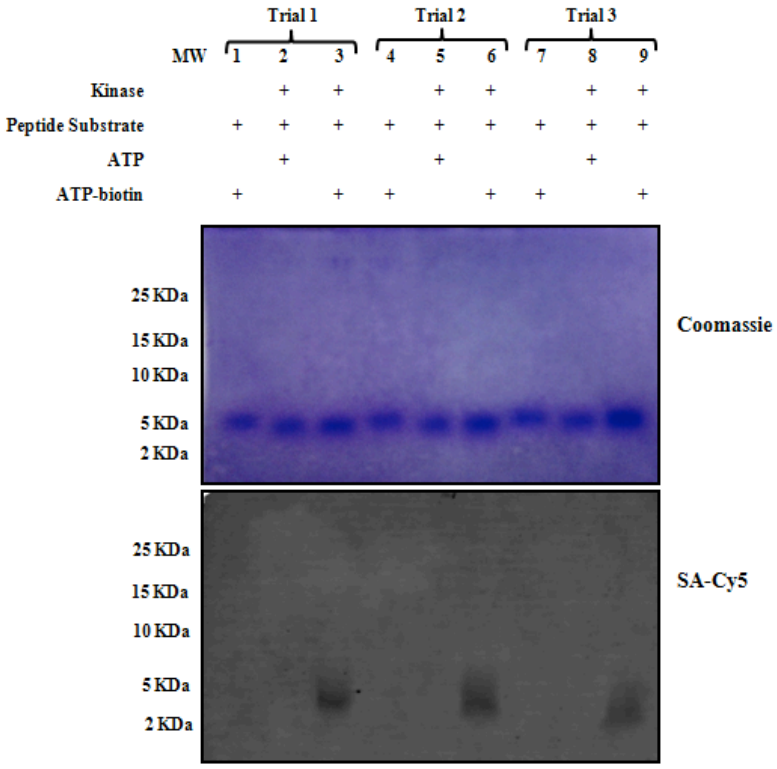


**Figure S2 (G).** Reactions with MARK1

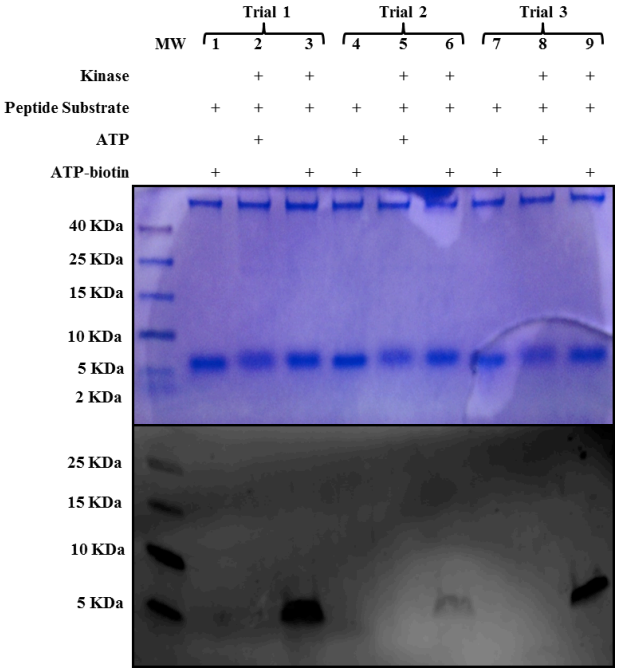



**Figure S2 (H).** Reactions with MST1

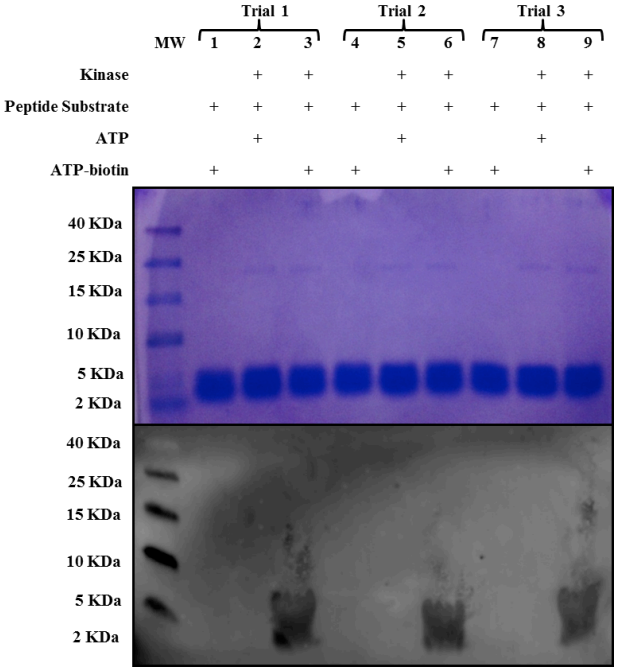


**Figure S2 (I).** Reactions with PAK1

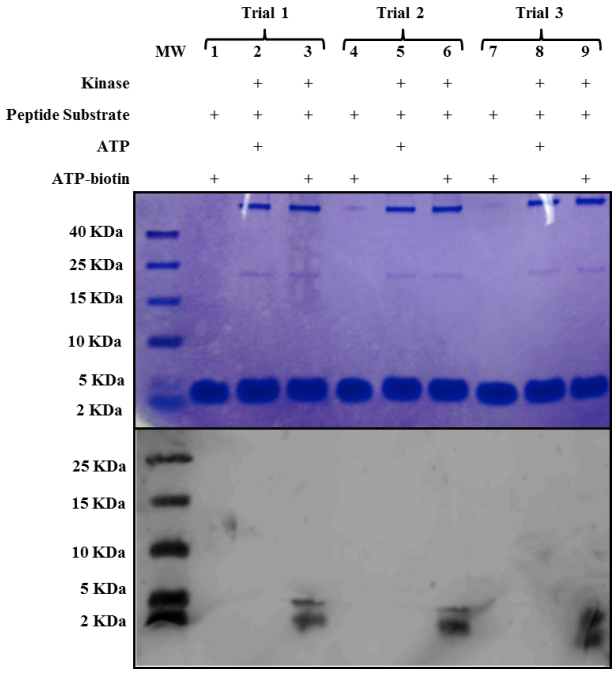



**Figure S2 (J).** Reactions with RSK1

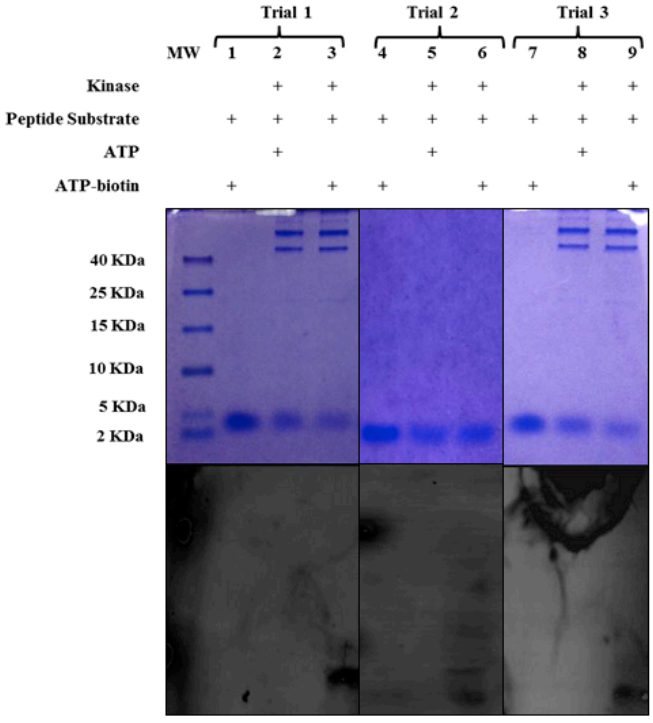


**Figure S2 (K).** Reactions with SRC

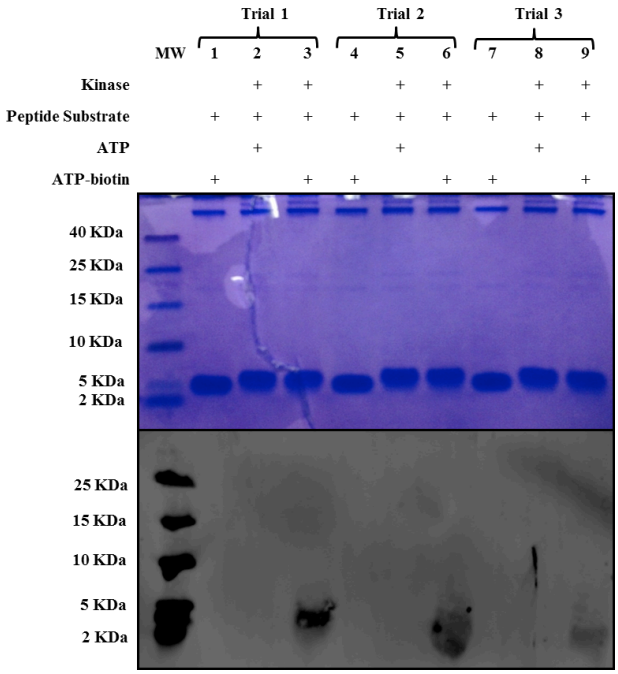



**Figure S2:** Full gel images for peptide labeling experiments with eleven kinases. Phosphorylated peptides or biotinylated phosphopeptides were created by incubating ATP or ATP-biotin with AKT1 and AKT substrate (CKRPRAASFAE) (A), CaMK4 and Autocamtide (KKALRRQETVDAL-amide) (B), CHK1 and CHKtide (KKKVSRSGLYRSPSMPENLNRPR) (C), FLT1 and IGF1Rtide (KKKSPGEYVNIEFG) (D), GSK3β and GSK3 peptide substrate (YRRAAVPPSPSLSRHSSPHQ(pS)EDEEE) (E), JAK3 and JAK3tide peptide (GGEEEEYFELVKKKK) (F), MARK1 and CHKtide (KKKVSRSGLYRSPSMPENLNRPR) (G), MST1 and Axltide (KKSRGDYMTMQIG) (H), PAK1 and PAKtide (RRRLSFAEPG) (I), RSK1 and RSK peptide substrate (KRRRLASLR) (J), or SRC and p34cdc2 peptide substrate (KVEKIGEGTYGVVYK-amide) (K) in the manufacturer provide buffer (1X, Promega). Crude reaction mixtures were separated on a 16% Tris-tricine gel and transferred to a PVDF membrane. Peptides were visualized with coomassie brilliant blue (top gel) and biotinylated peptides were detected with a SA-Cy5 conjugate (bottom gel). Reaction contents are indicated above each lane. Trials 1, 2, and 3 represent three independent trials. Data shown here are presented in Figure 3b of the manuscript.

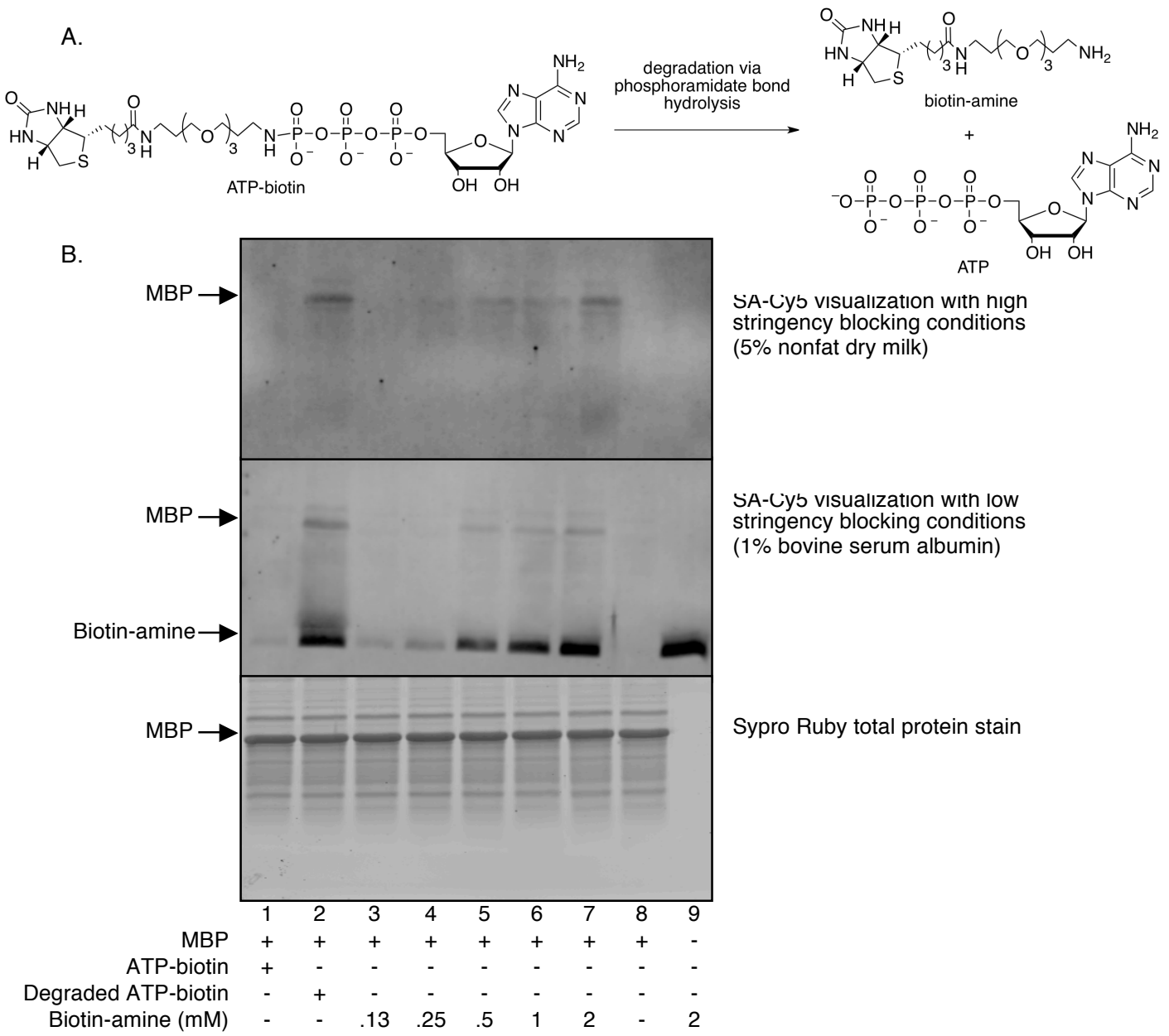



**Figure S3.** Control reactions to assess nonspecific (kinase-independent) biotinylation by ATP-biotin, which was reported in a recent publication.[2] Evidence of the ATP-biotin degradation product, biotin amine (A), was present in Figure 3 of ref. 2, suggesting impure (degraded) ATP-biotin was used. To assess if biotin amine could account for the kinase-independent biotin labeling observed in ref. 2, various concentrations of biotin amine (0.13, 0.25, 0.5, 1.0, or 2.0 mM final concentration) were incubated with MBP under kinase reaction conditions (2 hr, 30°C, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.01% Brij 35), but without kinase enzyme. Gel analysis was performed (16% SDS-PAGE), as described. Because the high stringency blocking conditions typically used during SA-Cy5 (streptavidin-Cy5) staining (5% nonfat dry milk) did not allow visualization of biotin amine (top gel), low stringency blocking (1% BSA) was used (middle gel), similar to the conditions in ref. 2 (1% casein). Degraded ATP-biotin was generated by incubating ATP-biotin at room temperature for 48 hours. Kinase-independent biotin labeling of MBP was present only when high concentrations (≥0.5 mM, lanes 2, 5, 6, and 7) of biotin amine were present. These results are consistent with our prior report stressing use of high purity ATP-biotin (see lane 1).[3] The results also suggest that high stringency blocking conditions should be used with streptavidin stains (SA-Cy5 or SA-HRP, for example).

## III. All Data Related to Quantitative Labeling Studies

**Table S2: Percentage Conversion of Biotinylation with ATP-biotin relative to phosphorylation with ATP with 25 Kinases**

| Kinase | Method[a] | % Conversion[b] |
|---|---|---|
| ABL | HPLC | 85 ± 6% |
| AKT1 | HPLC | 51 ± 4% |
| ASK1 | Gel | 53 ± 8% |
| AurA | Gel | 96 ± 1% |
| CaMK4 | HPLC | 63 ± 3% |
| CDK1 | Gel | 77 ± 2% |
| CHK1 | Gel | 47 ± 4% |
| CK1 | Gel | 82 ± 9% |
| CK2 | HPLC | 51 ± 4% |
| DAPK1 | Gel | 63 ± 4% |
| ERK1 | Gel | 80 ± 11% |
| FLT1 | HPLC | 87 ± 20% |
| GRK5 | Gel | 47 ± 8% |
| GSK3β | HPLC | 44 ± 6% |
| HIPK1 | Gel | 45 ± 2% |
| JAK3 | Gel | 91 ± 2% |
| MARK1 | Gel | 65 ± 9% |
| MLK1 | Gel | 78 ± 6% |
| MST1 | Gel | 55 ± 2% |
| NEK2 | Gel | 94 ± 2% |
| PAK1 | HPLC | 63 ± 2% |
| PKA | HPLC | 61 ± 6% |
| RSK1 | HPLC | 76 ± 7% |
| SRC | HPLC | 96 ± 7% |
| TGFβR2 | Gel | 91 ± 3% |

[a] Protein or polypeptide substrates were used in the gel method, while peptide substrates were used in the HPLC method. The substrates used are indicated in Figure 2B of the manuscript. [b] The raw data associated with these conversion percentages are shown in Figure 4 and 5 of the manuscript and Figures S4 and S5.

**Figure S4 (A).** Reactions with ASK1

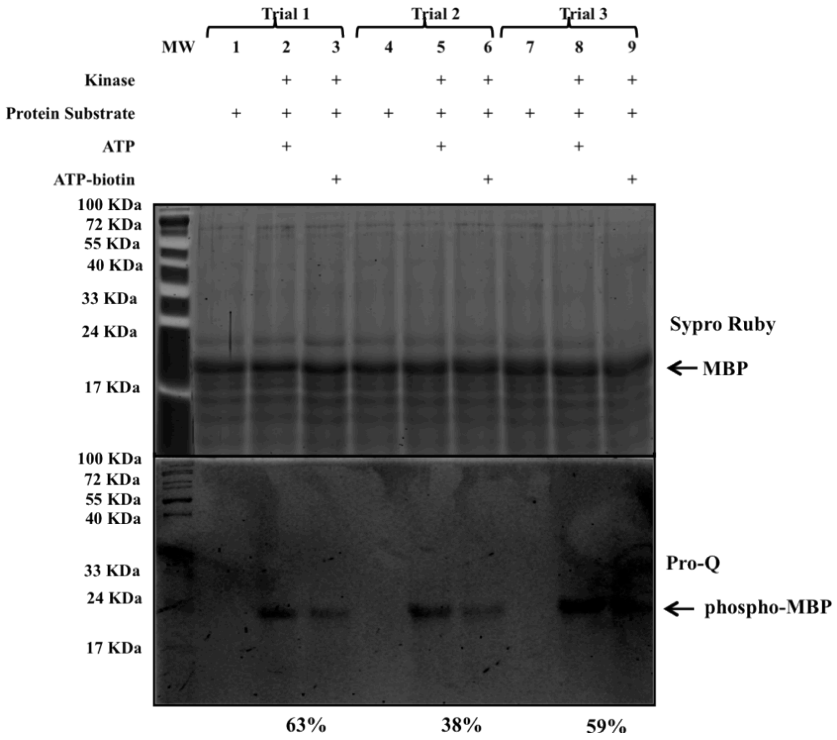



**Figure S4 (B).** Reactions with Aurora A

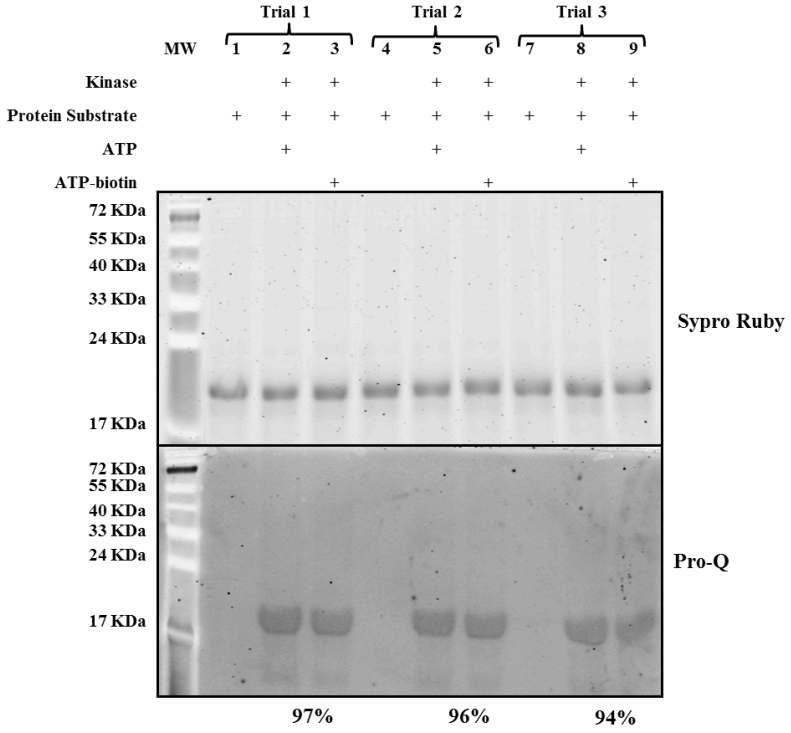


**Figure S4 (C).** Reactions with CDK1

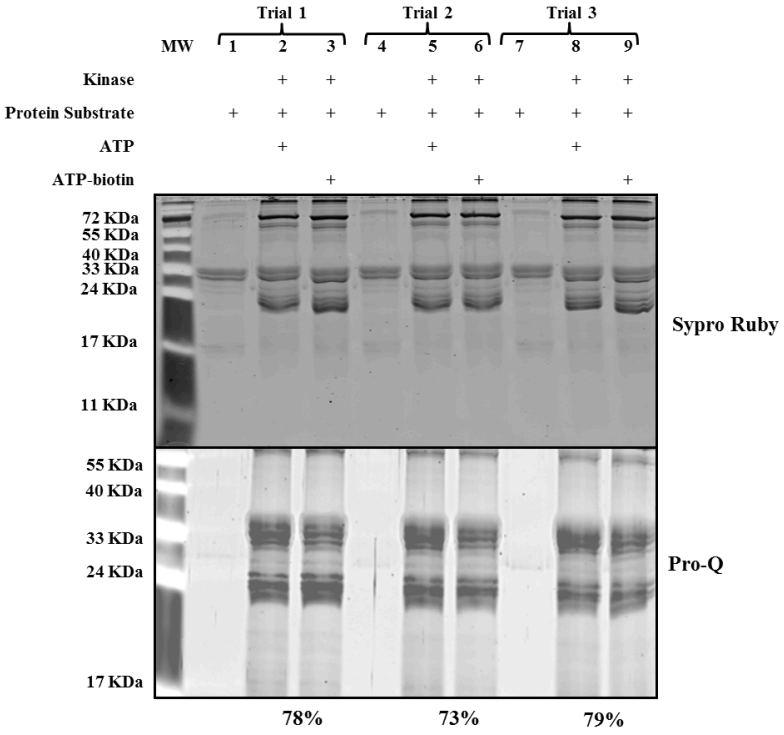



**Figure S4 (D).** Reactions with CHK1

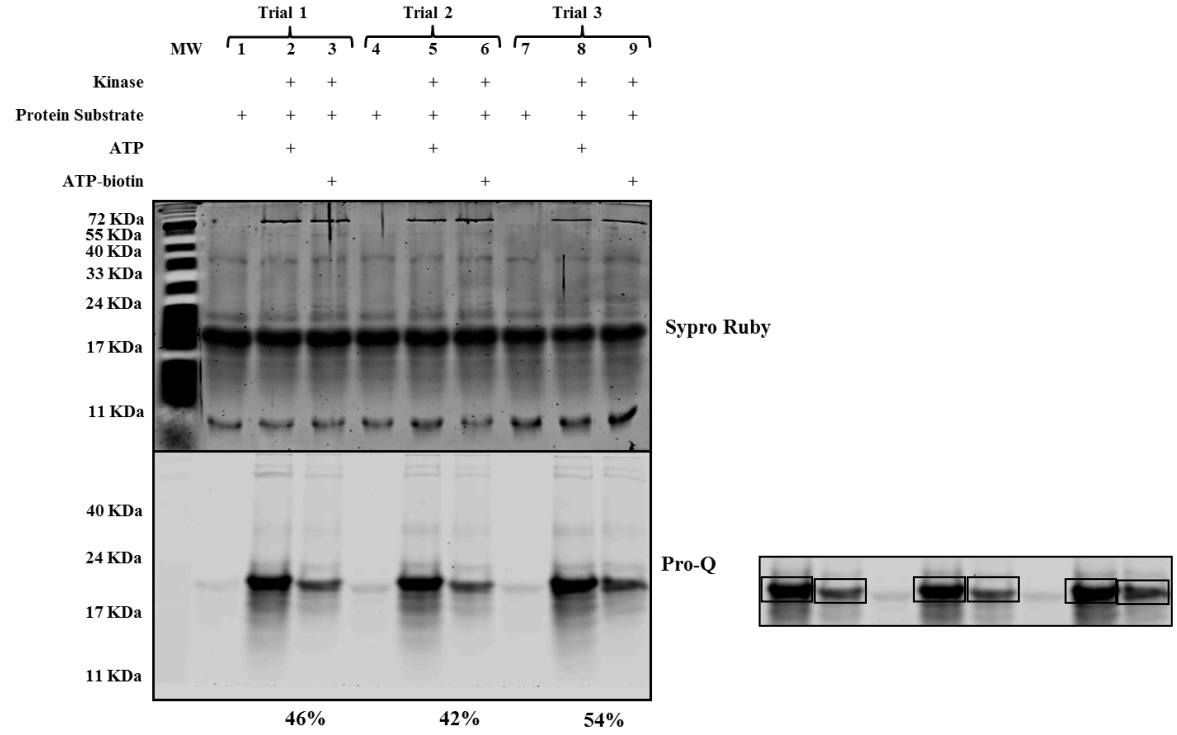


**Figure S4 (E).** Reactions with CK1

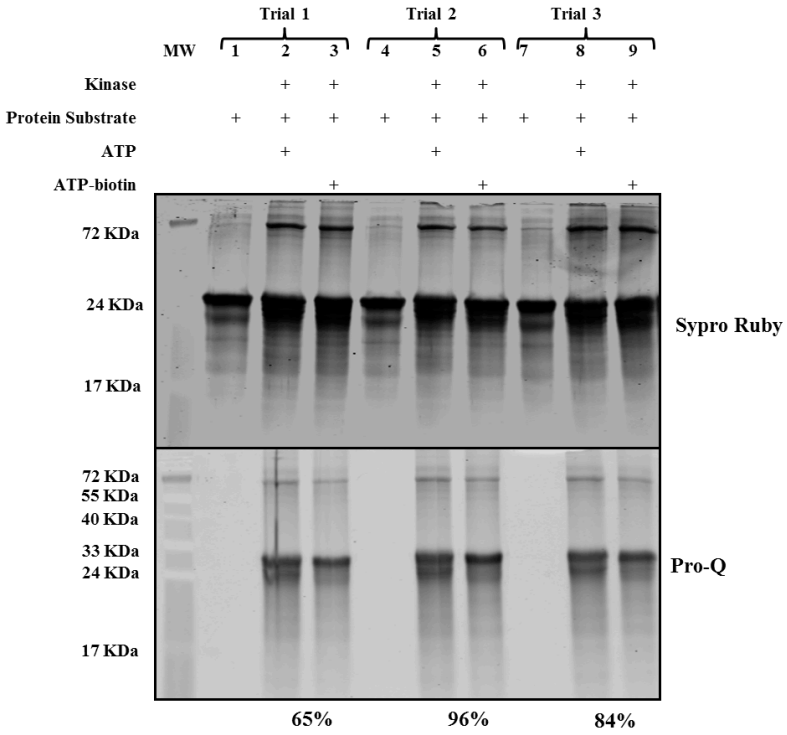



**Figure S4 (F).** Reactions with DAPK1

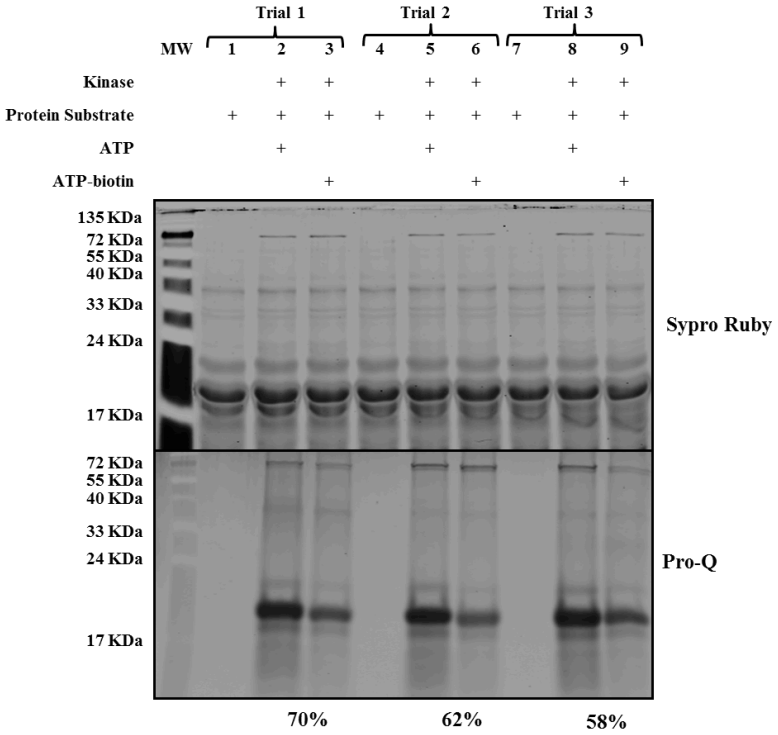


**Figure S4 (G).** Reactions with ERK1

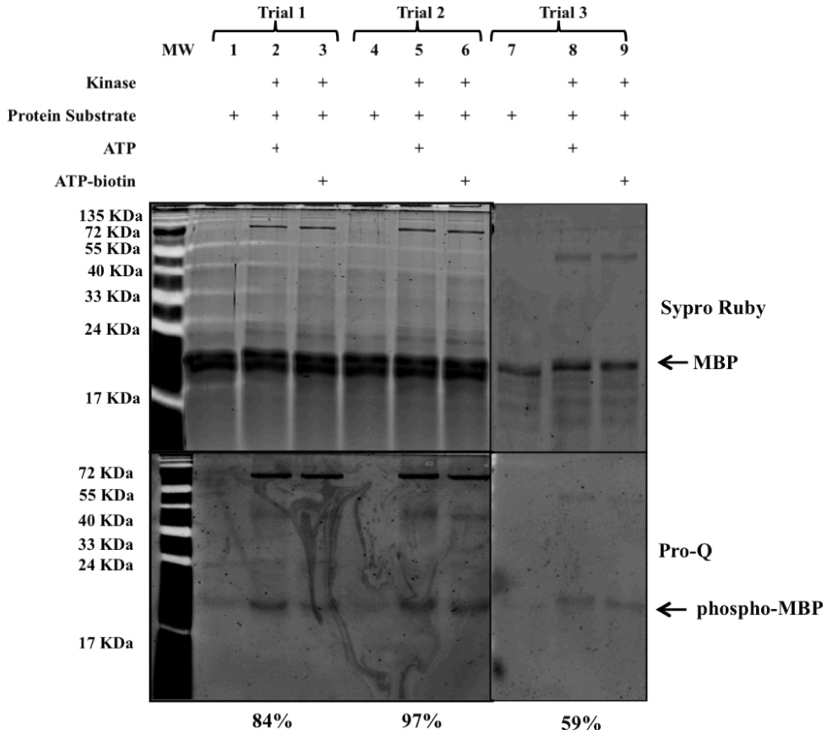



**Figure S4 (H).** Reactions with GRK5

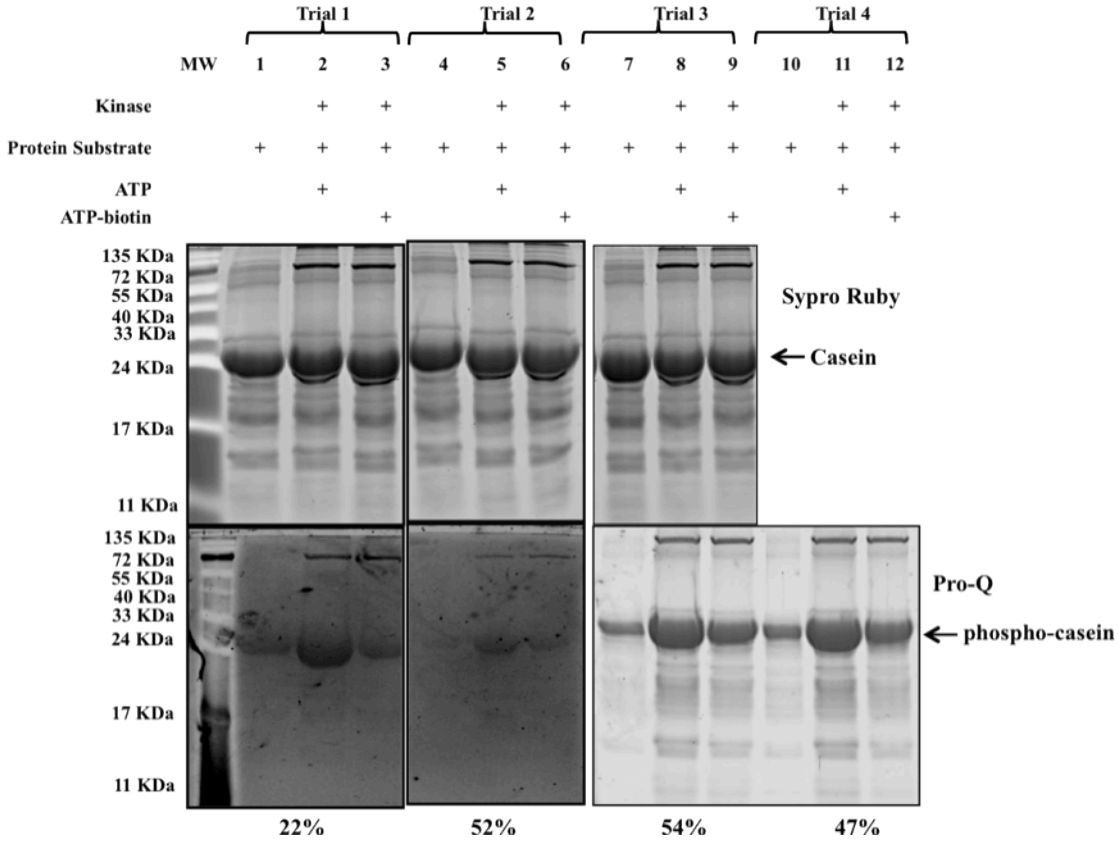


**Figure S4 (I).** Reactions with HIPK1

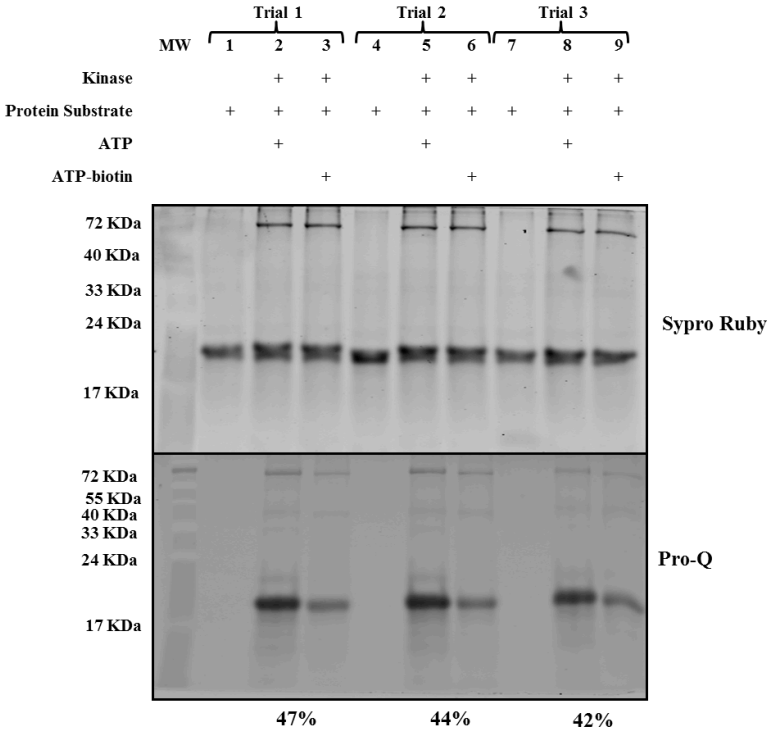



**Figure S4 (J).** Reactions with JAK3

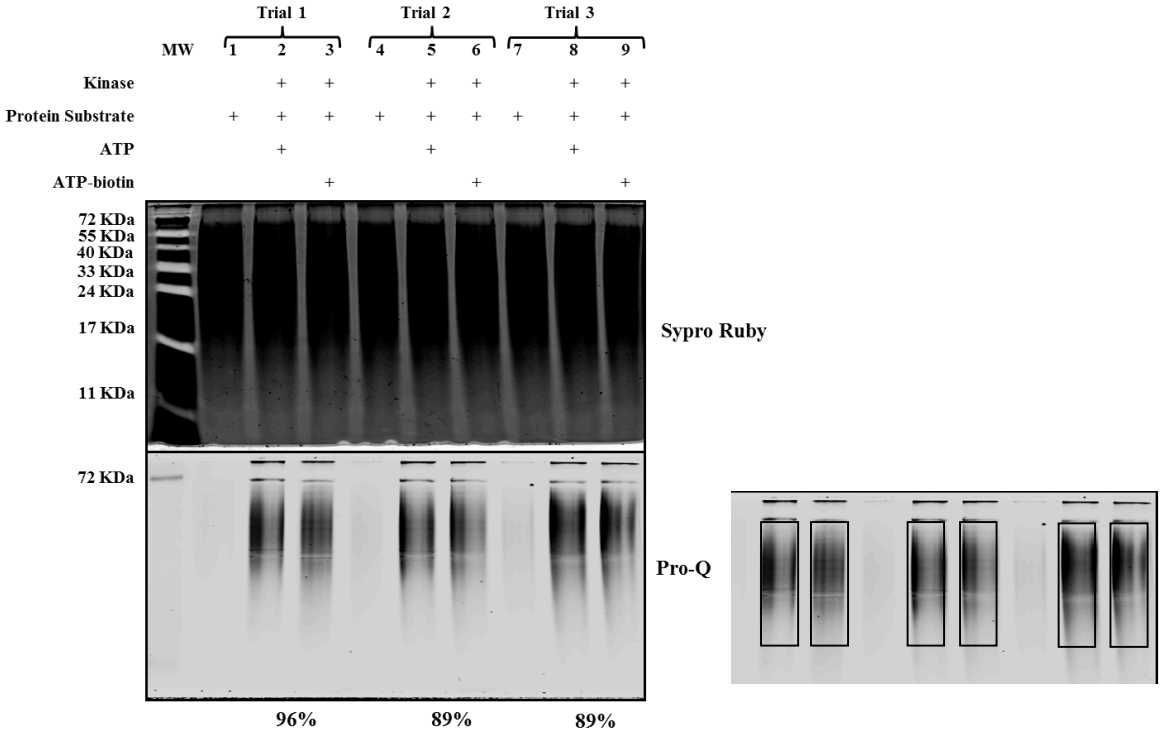


**Figure S4 (K).** Reactions with MARK1

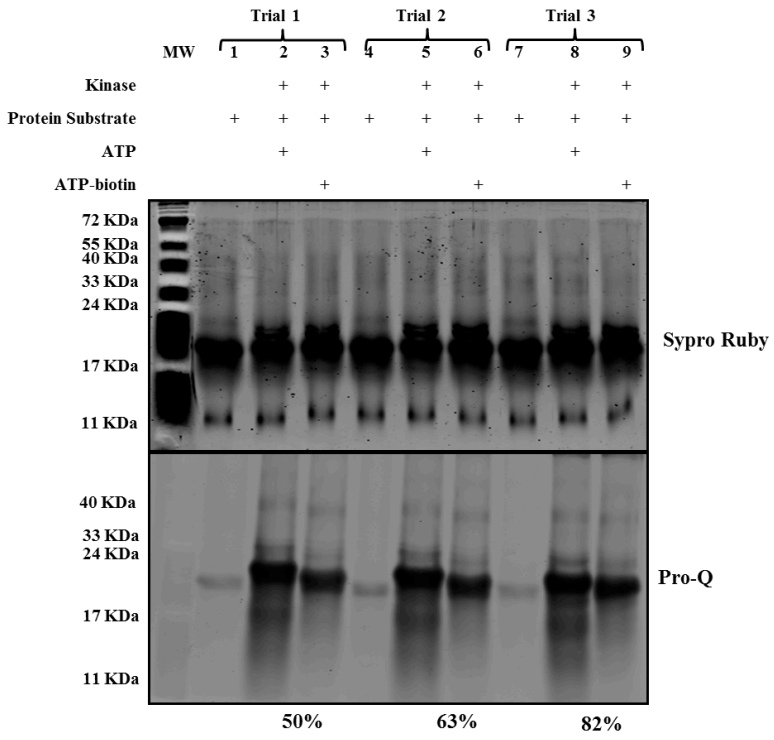



**Figure S4 (L).** Reactions with MLK1

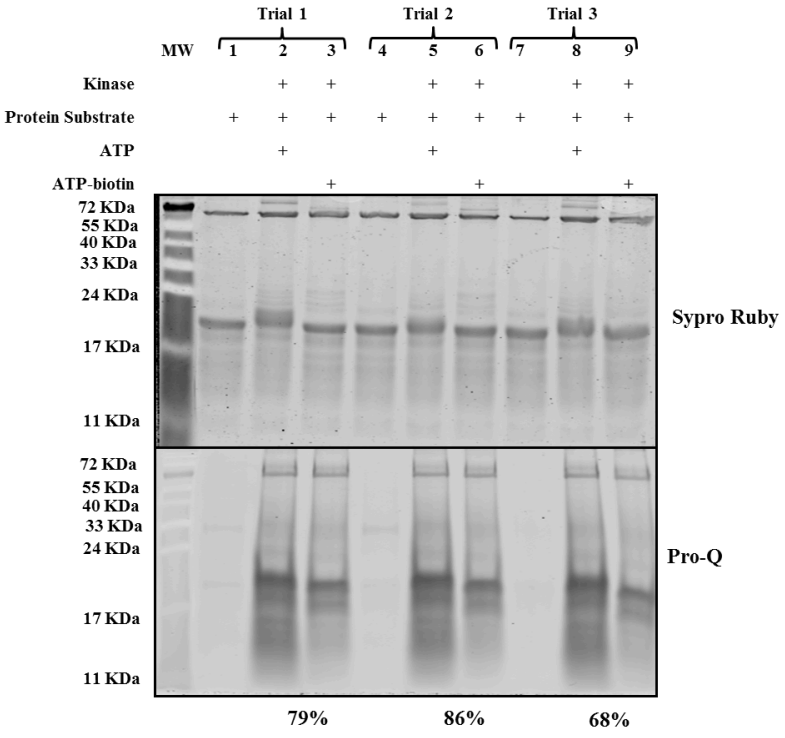


**Figure S4 (M).** Reactions with MST1

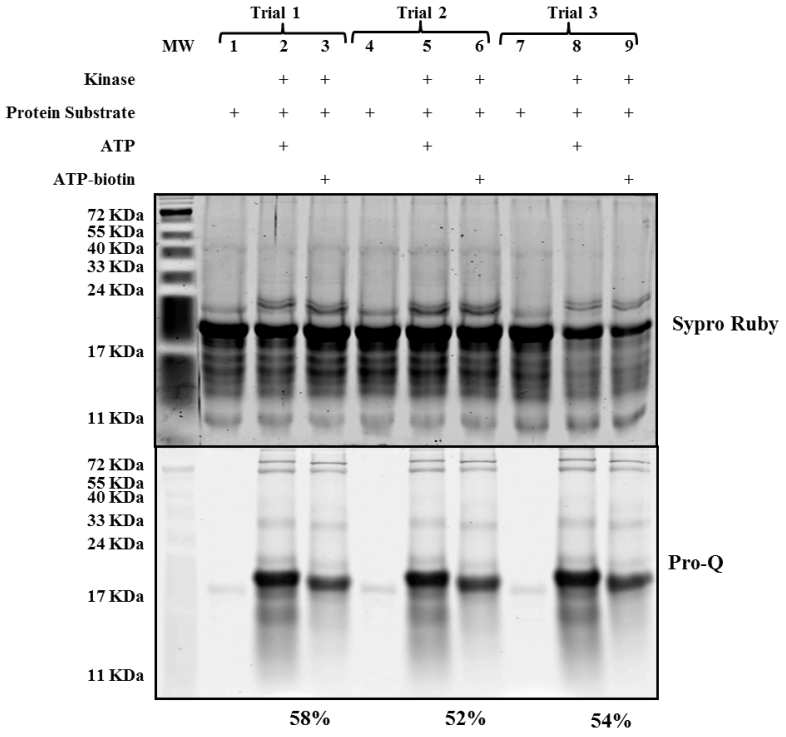



**Figure S4 (N).** Reactions with NEK2

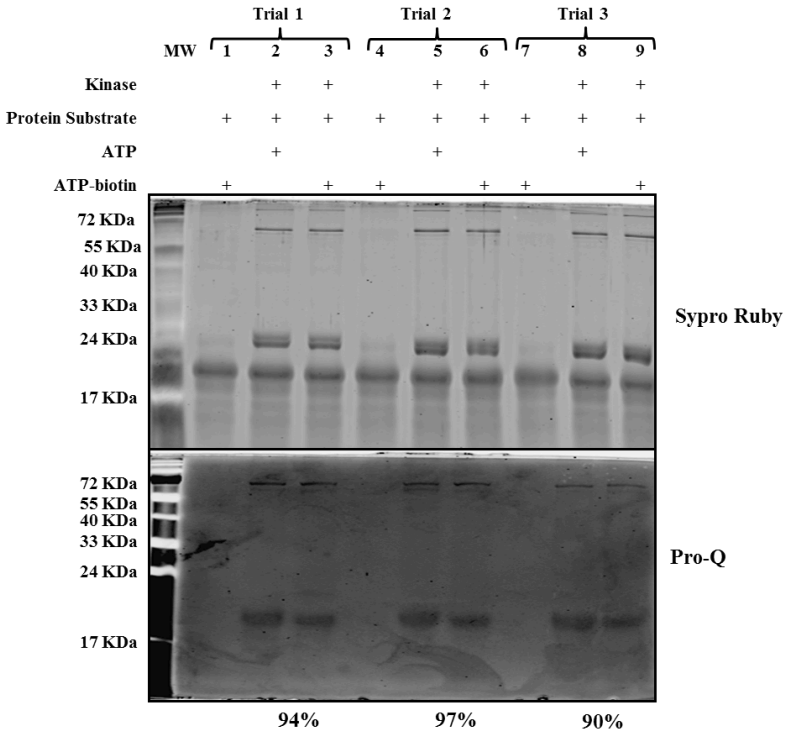


**Figure S4 (O).** Reactions with TGFβR2

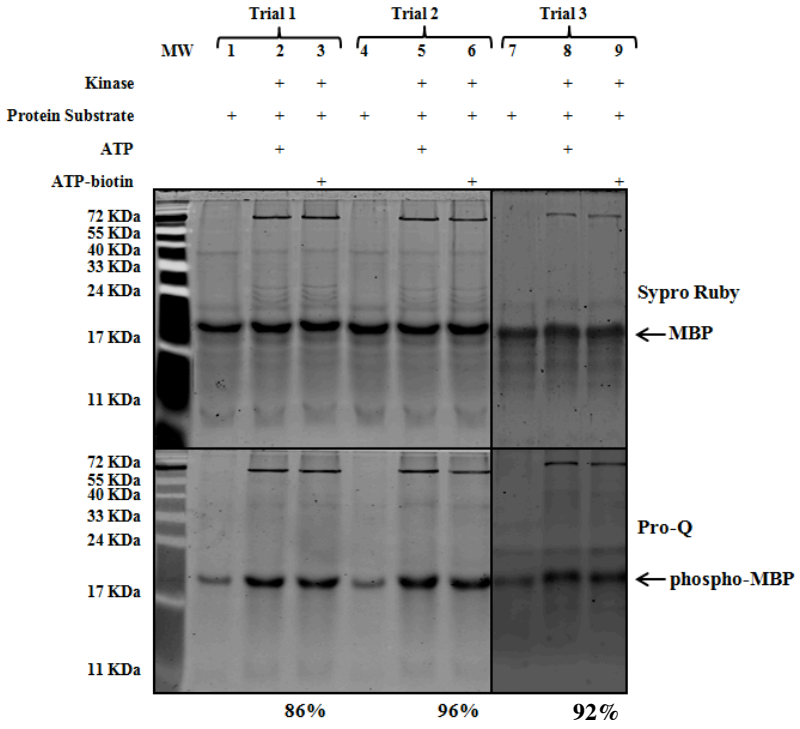
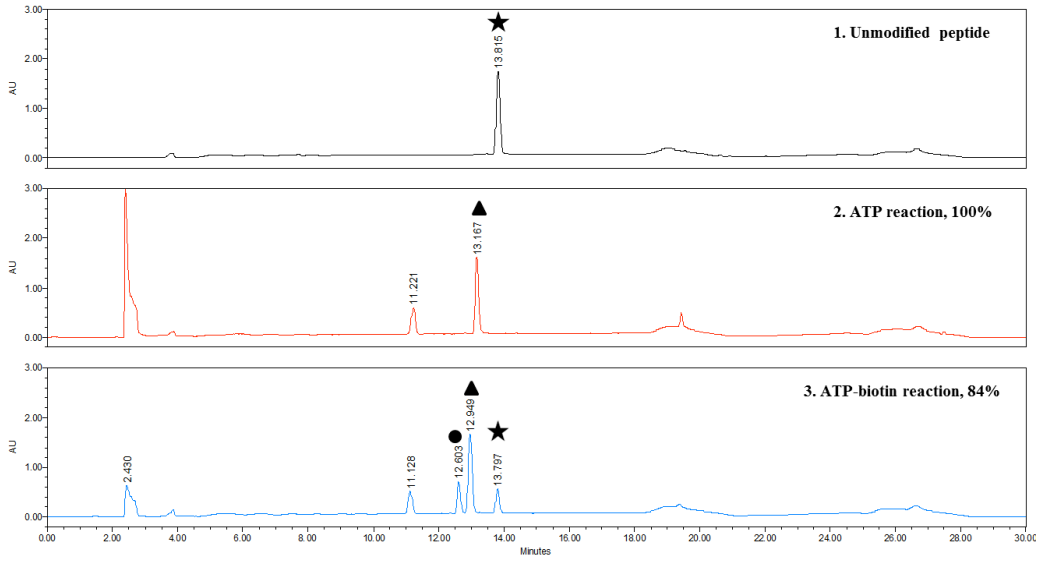


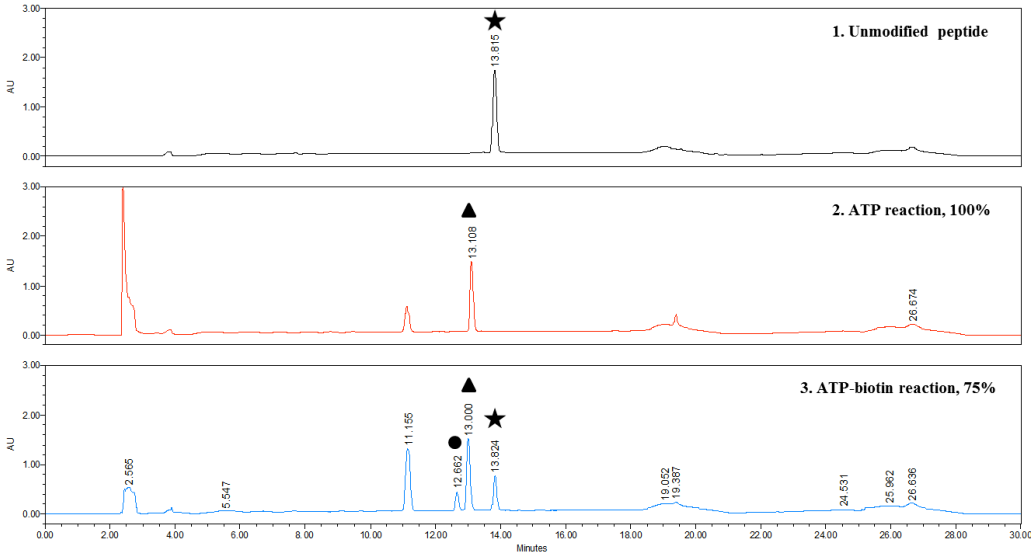

**Figure S4:** Full gel images for protein quantification experiment with fifteen kinases. Phosphorylated proteins or biotinylated phosphoproteins were created by incubating ATP or ATP-biotin with (A) ASK1 and MBP, (B) Aurora A and MBP, (C) CHK1 and MBP, (D) CDK1 and Histone H1 protein, (E) CK1 and dephosphorylated Casein protein, (F) DAPK1 and MBP, (G) ERK1 and MBP, (H) GRK5 and Casein protein, (I) HIPK1 and MBP, (J) JAK3 and poly($Glu_4$, Tyr1)peptide substrate, (K) MARK1 and MBP, (L) MLK1 and MBP, (M) MST1 and MBP, (N) NEK2 and MBP, or (O) TGFβR2 and MBP in the manufacturer provide buffer (1X, Promega). Reaction products were treated with 50% TFA to obtain phosphoproteins for quantification. Crude reaction mixtures were separated on 16% SDS-PAGE. Proteins are visualized with Sypro Ruby Stain (top gel) and phosphoproteins with Pro-Q diamond stain (bottom gel) with reaction contents indicated above each lane. Trial 1, 2, and 3 represent three independent trials. MBP is the abbreviation for Myelin Basic Protein. To represent how protein bands were boxed for quantification, boxes on the protein bands in S4D and S4J are shown. Data shown here are presented in Figure 4 of the manuscript.

**Figure S5 (A).** Reactions with ABL

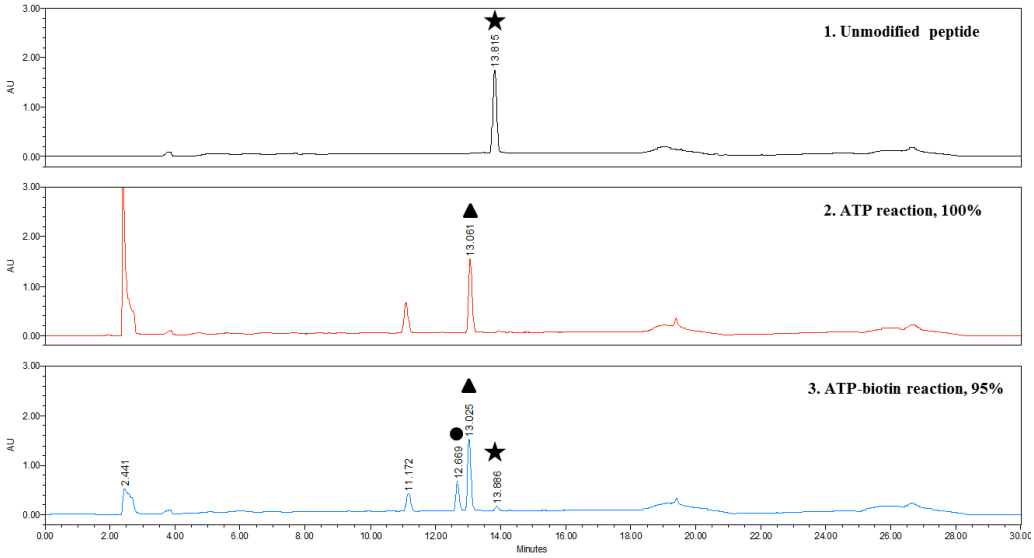
**Trial 1**




**Trial 2**



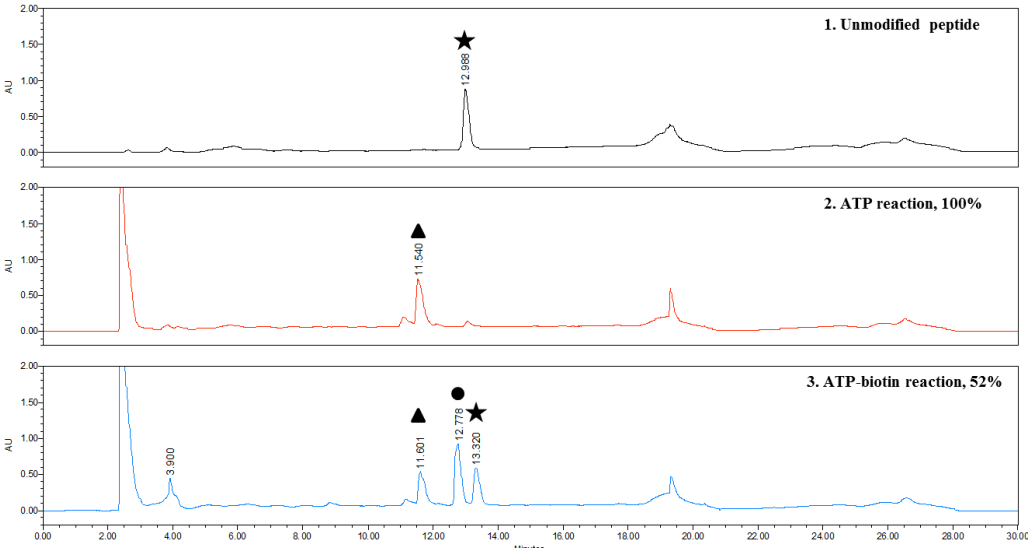

**Trial 3**



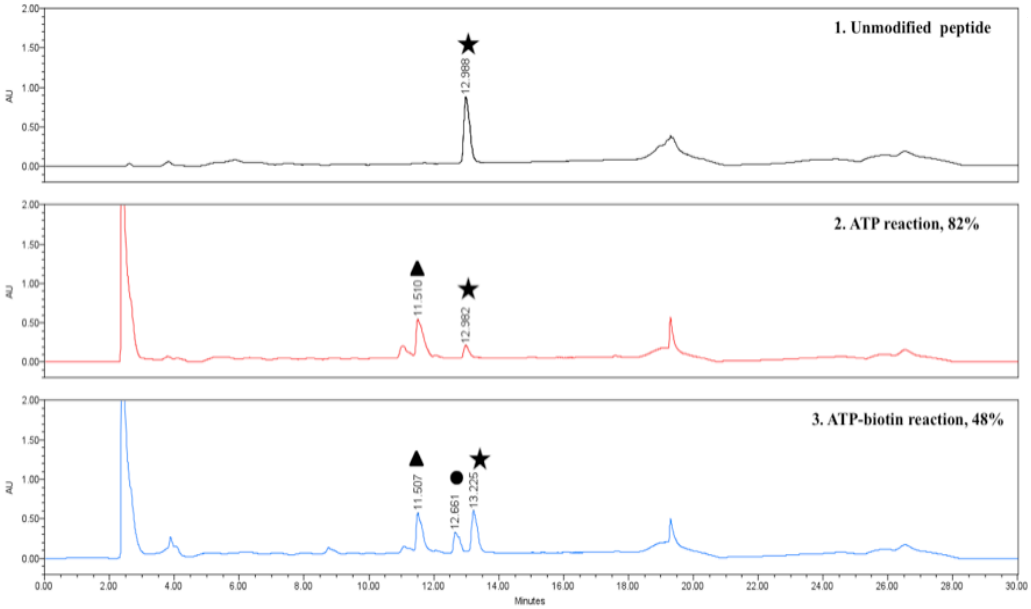

★ Unmodified peptide ▲ Phosphopeptide ● acid-cleaved biotin amine

**Figure S5 (B).** Reactions with AKT1

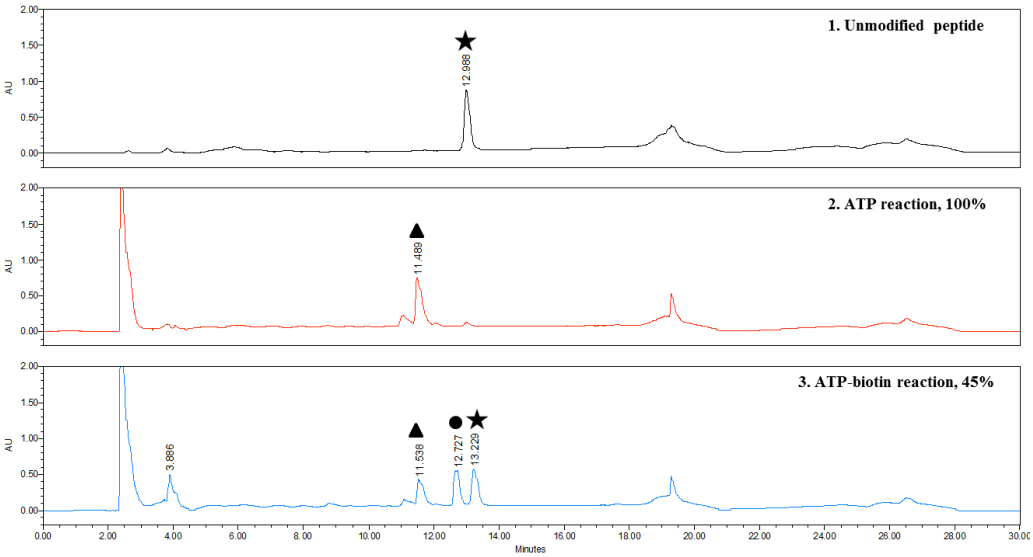
**Trial 1**




**Trial 2**



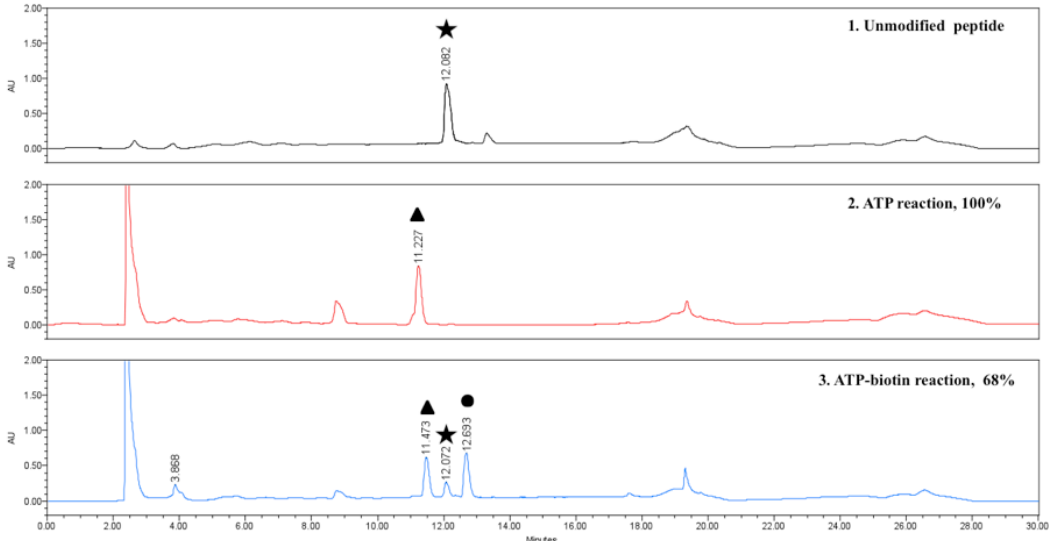

**Trial 3**



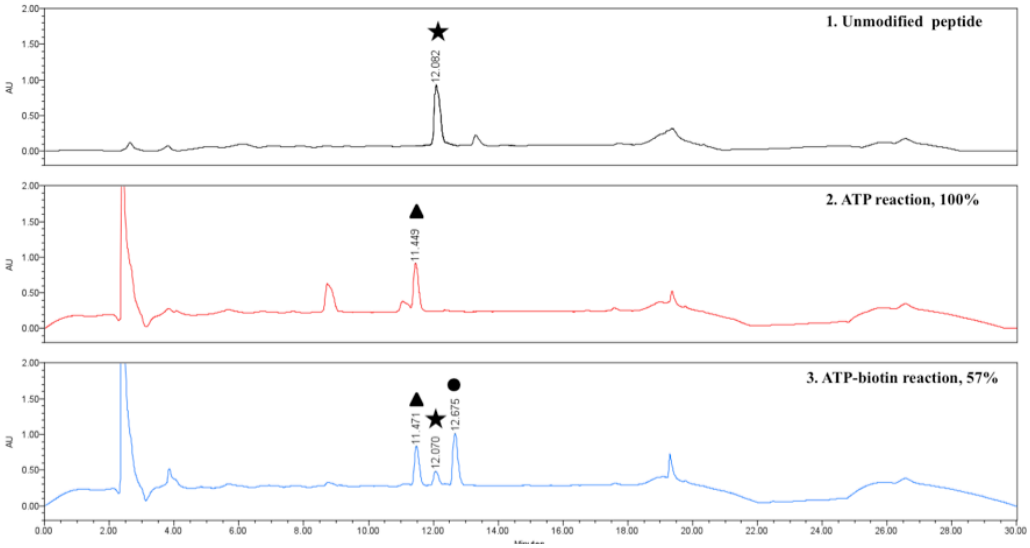

★ Unmodified peptide ▲ Phosphopeptide ● acid-cleaved biotin amine

**Figure S5 (C).** Reactions with CaMK4

**Trial 1**




**Trial 2**




**Trial 3**

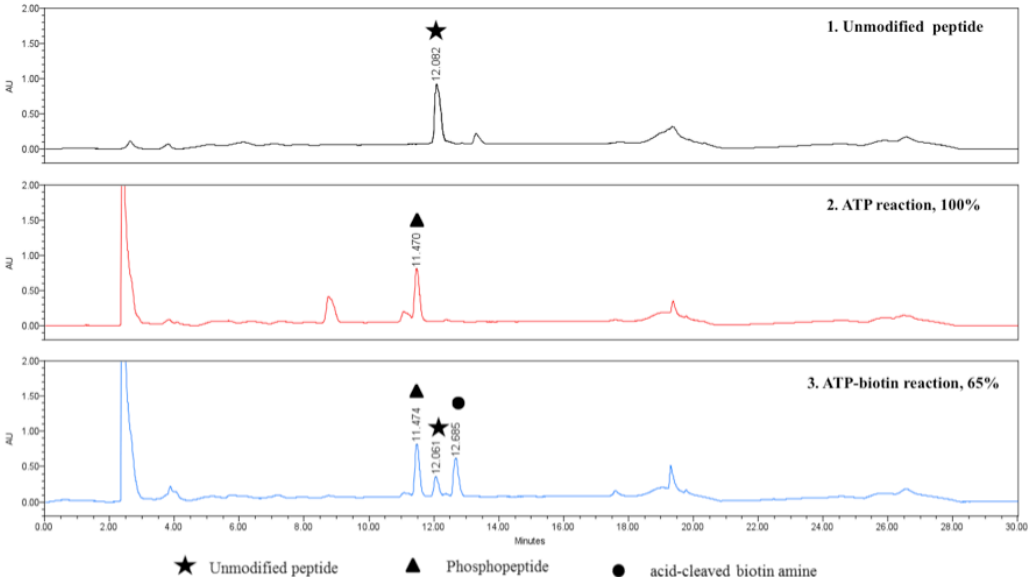
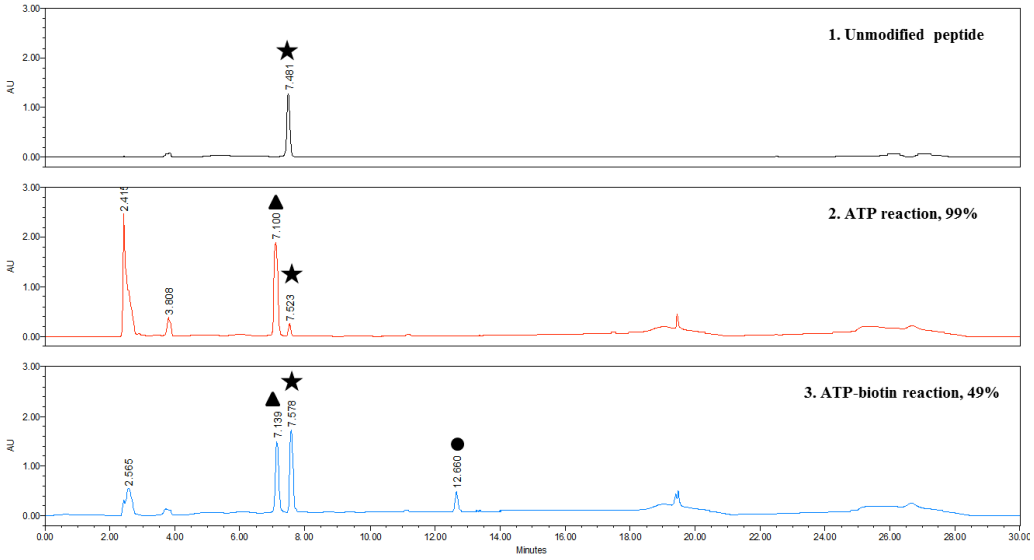
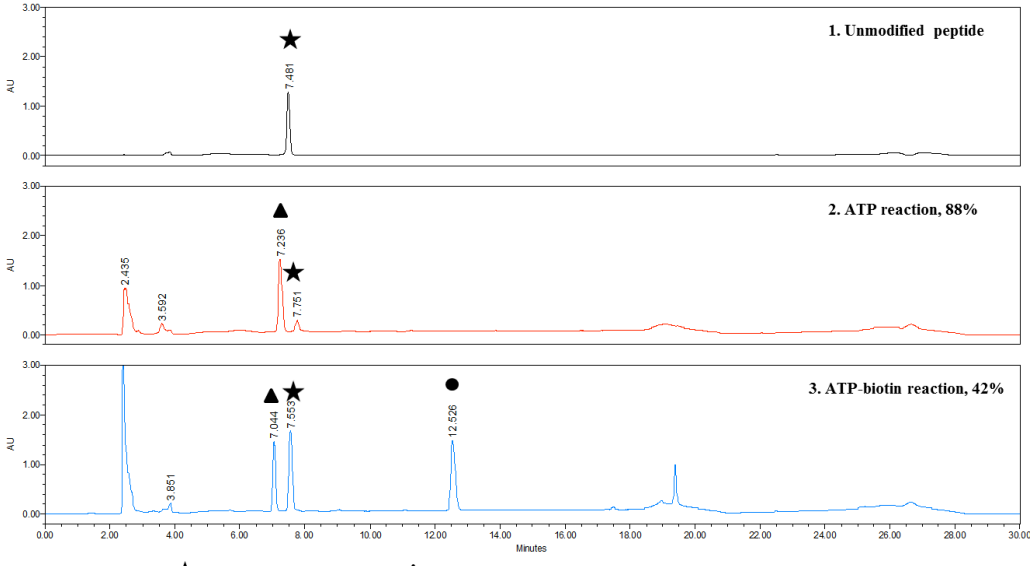


**Figure S5 (D).** Reactions with CK2

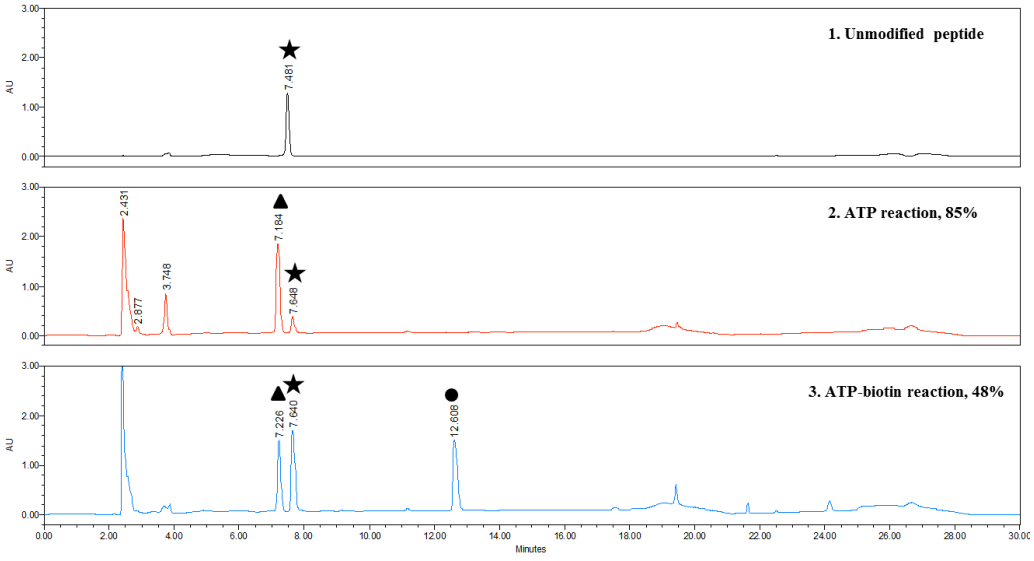
**Trial 1**




**Trial 2**




**Trial 3**




★ Unmodified peptide ▲ Phosphopeptide ● acid-cleaved biotin amine

**Figure S5 (E).** Reactions with FLT1

**Trial 1**

**Trial 2**

**Trial 3**

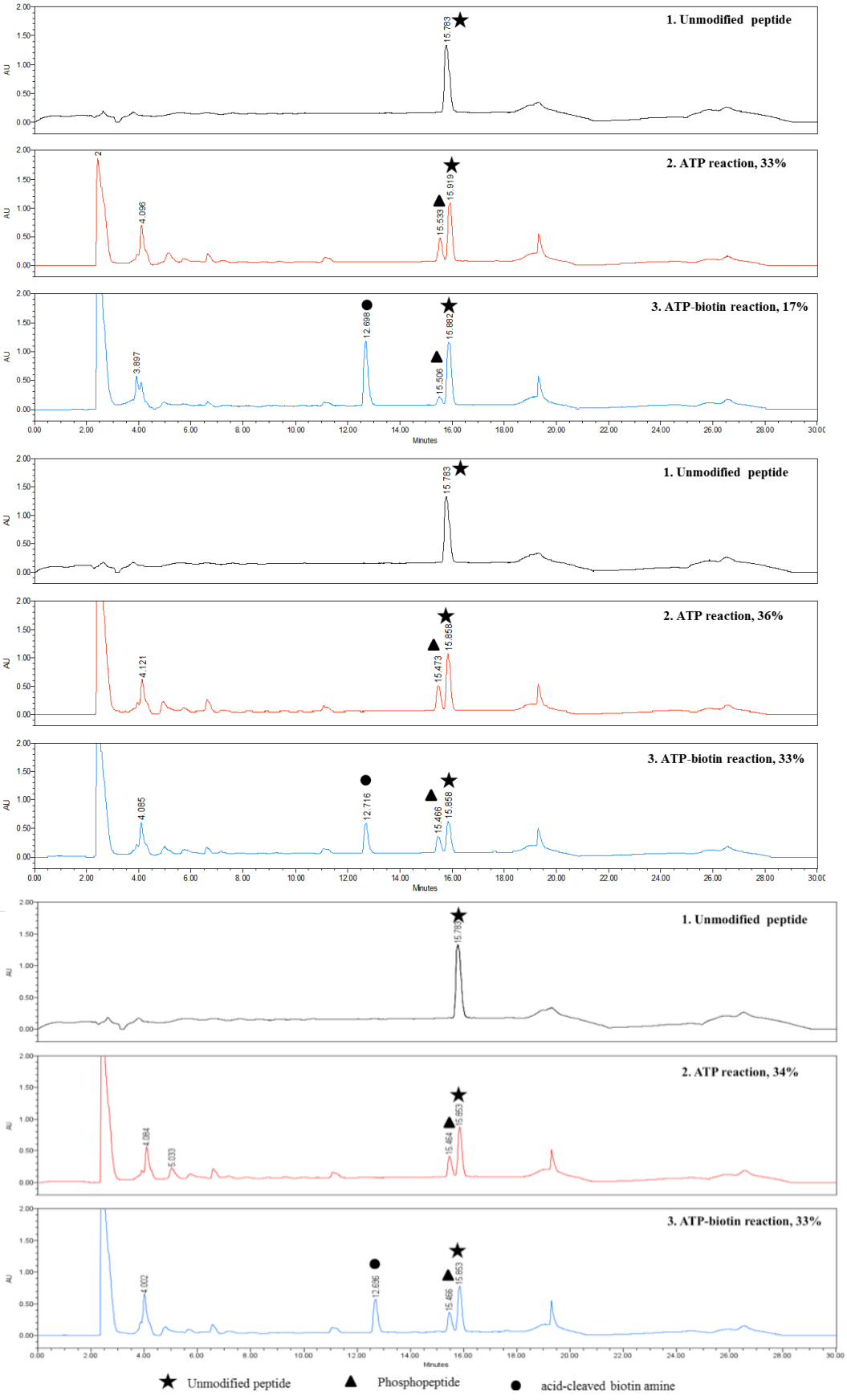
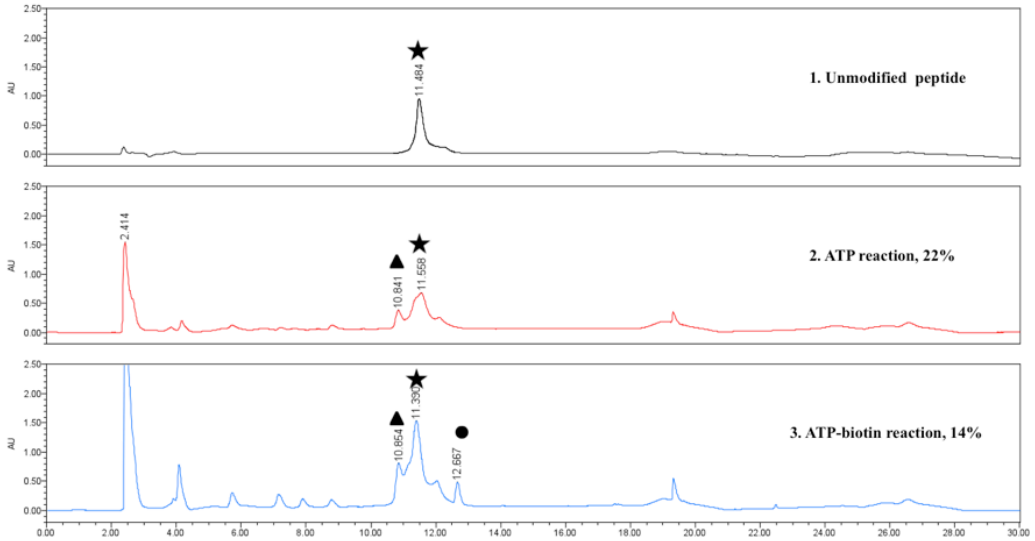
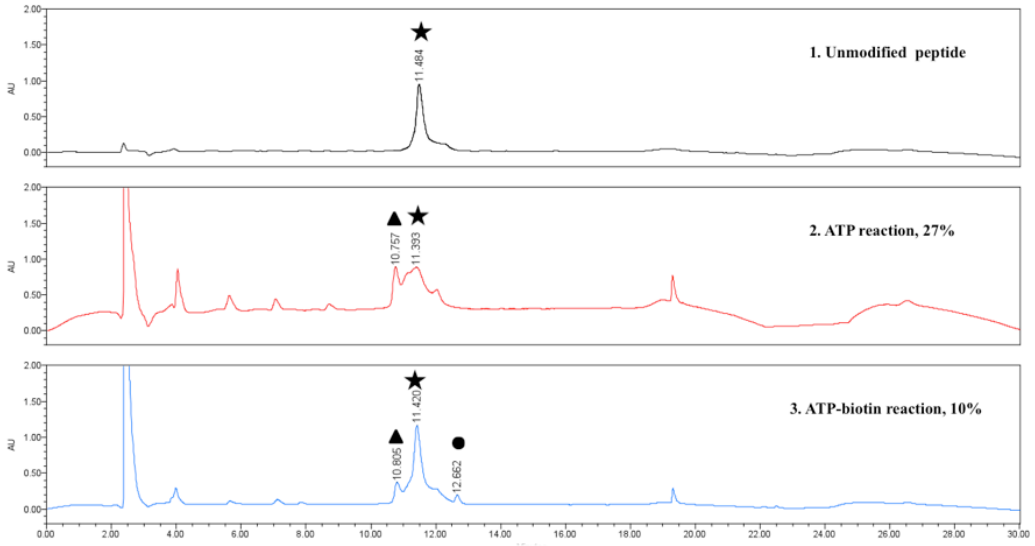


**Figure S5 (F).** Reactions with GSK3β

**Trial 1**




**Trial 2**




**Trial 3**

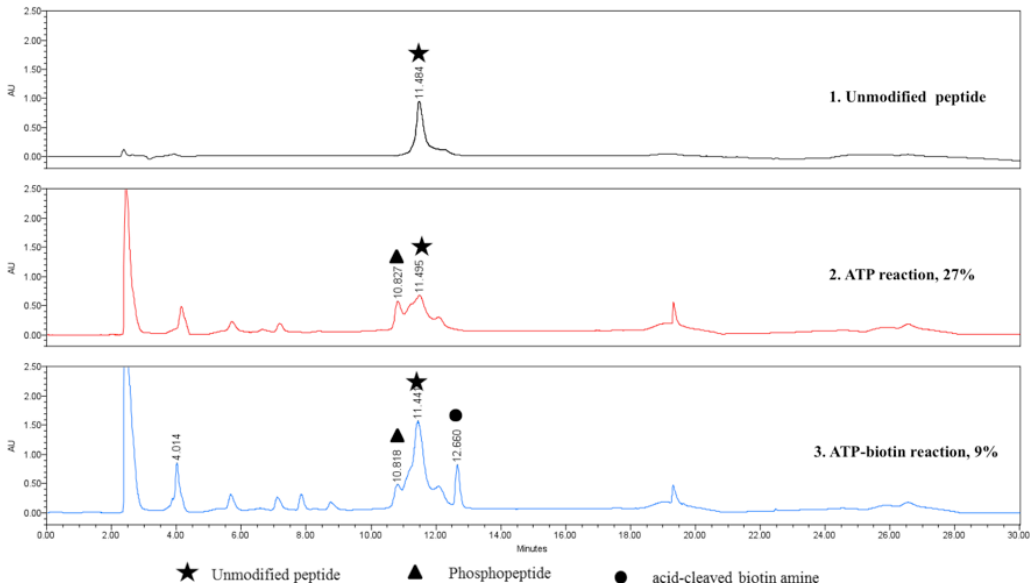


**Figure S5 (G).** Reactions with PAK1

**Trial 1**

**Trial 2**

**Trial 3**

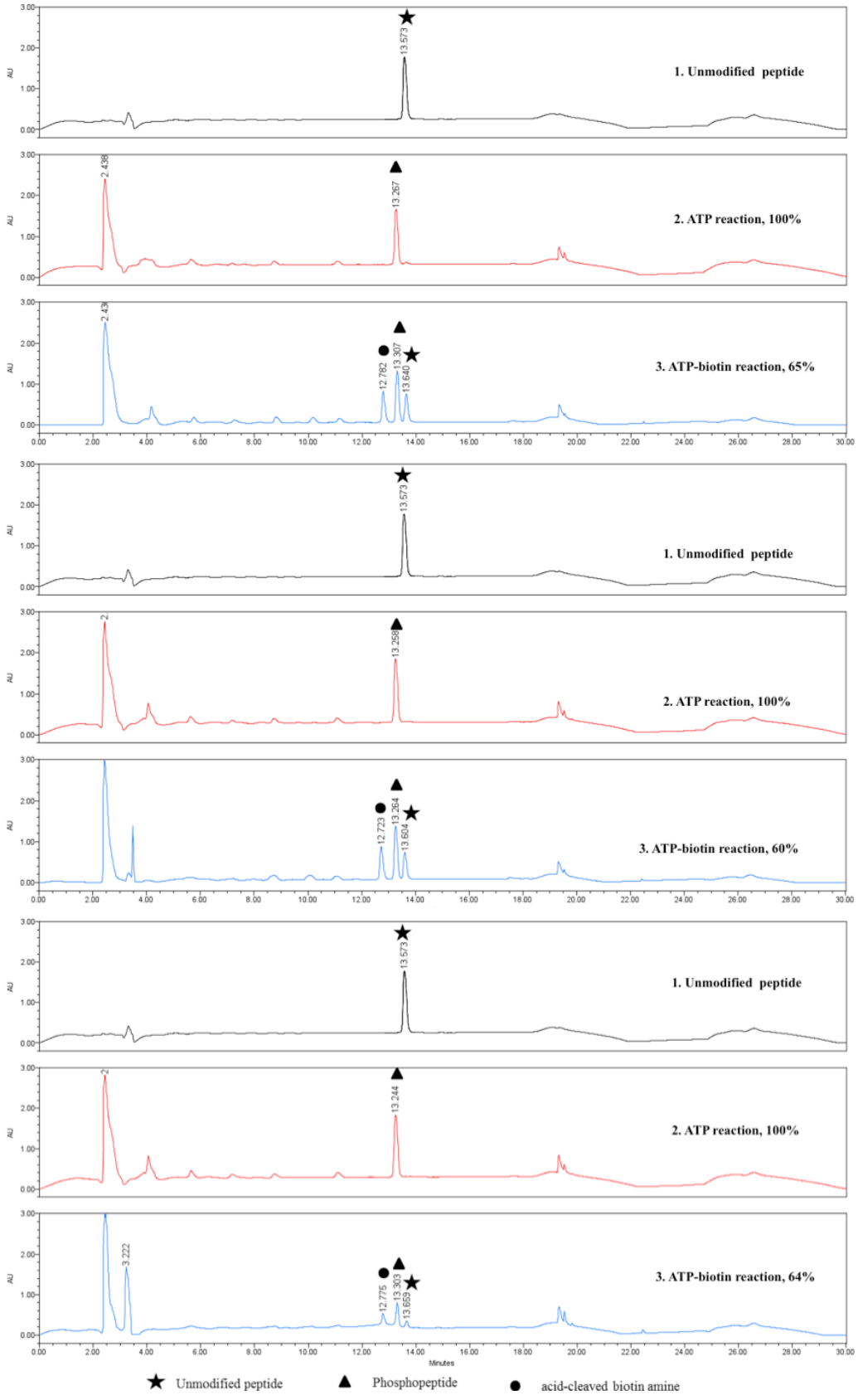
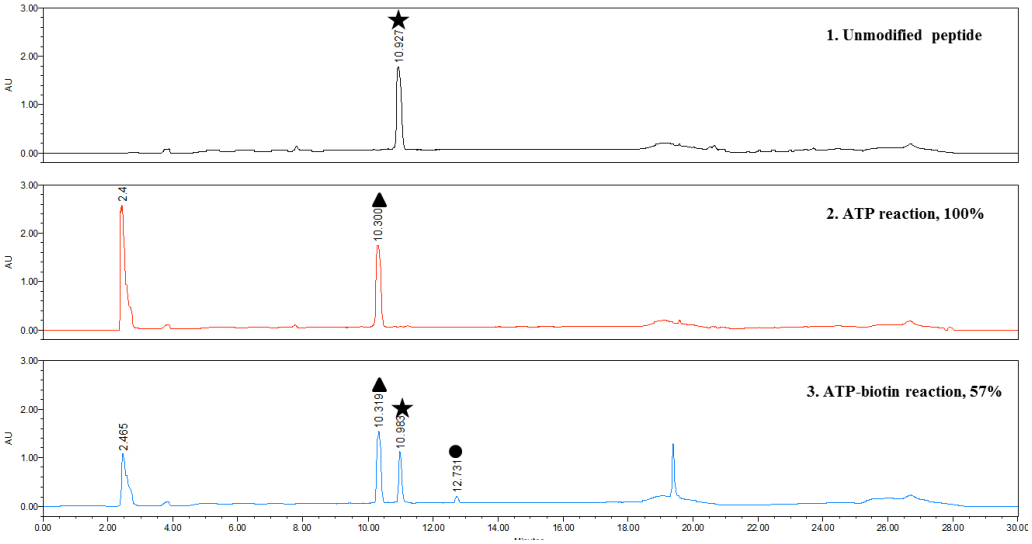
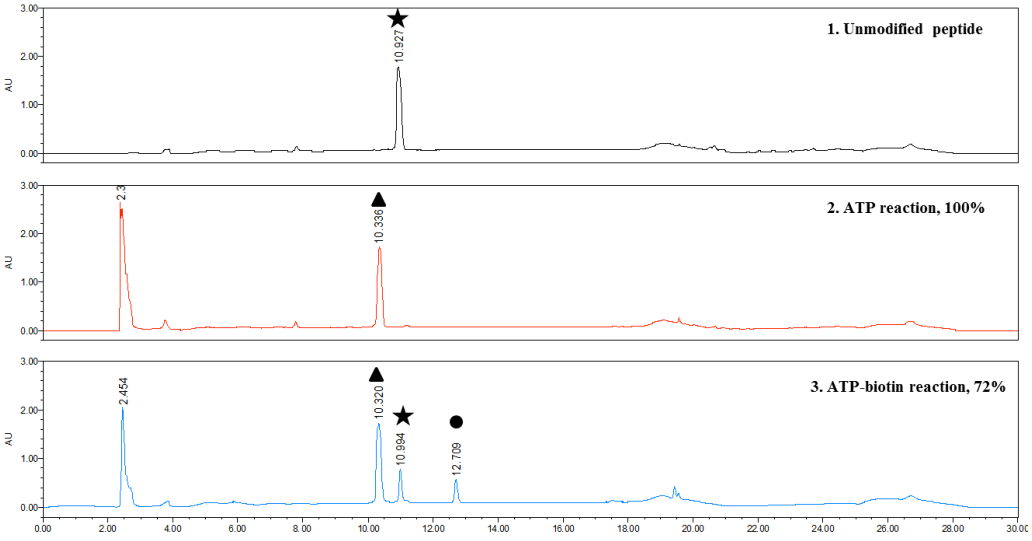


**Figure S5 (H).** Reactions with PKA

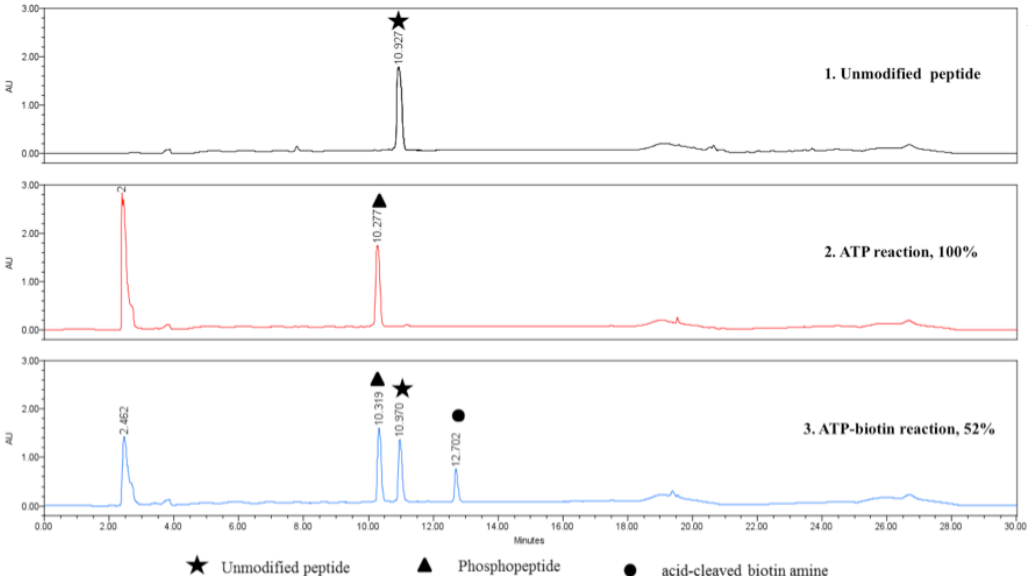
**Trial 1**




**Trial 2**




**Trial 3**




★ Unmodified peptide ▲ Phosphopeptide ● acid-cleaved biotin amine

**Figure S5 (I).** Reactions with RSK1

**Trial 1**

**Trial 2**

**Trial 3**

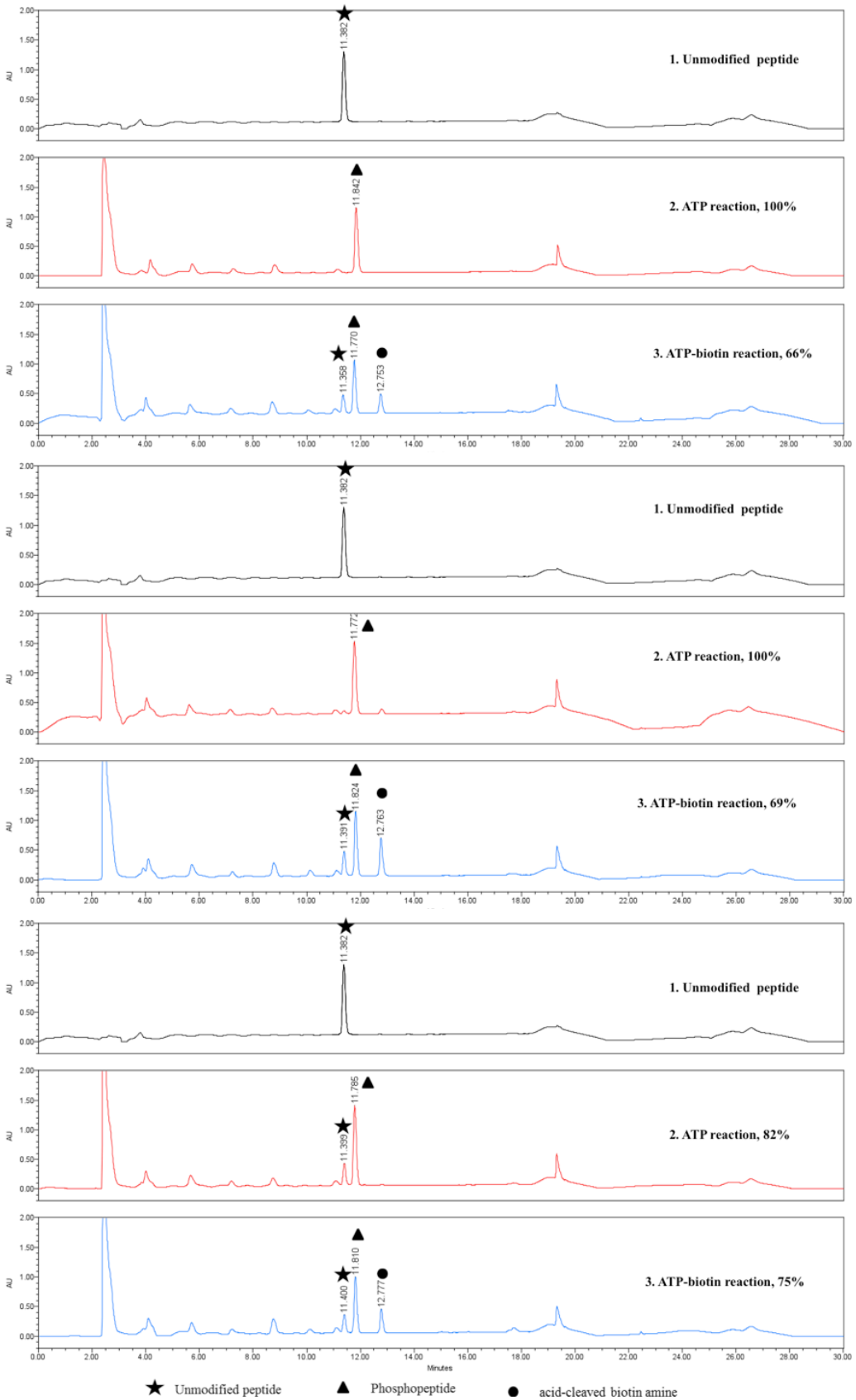


**Figure S5 (J).** Reactions with SRC

**Trial 1**

**Trial 2**

**Trial 3**

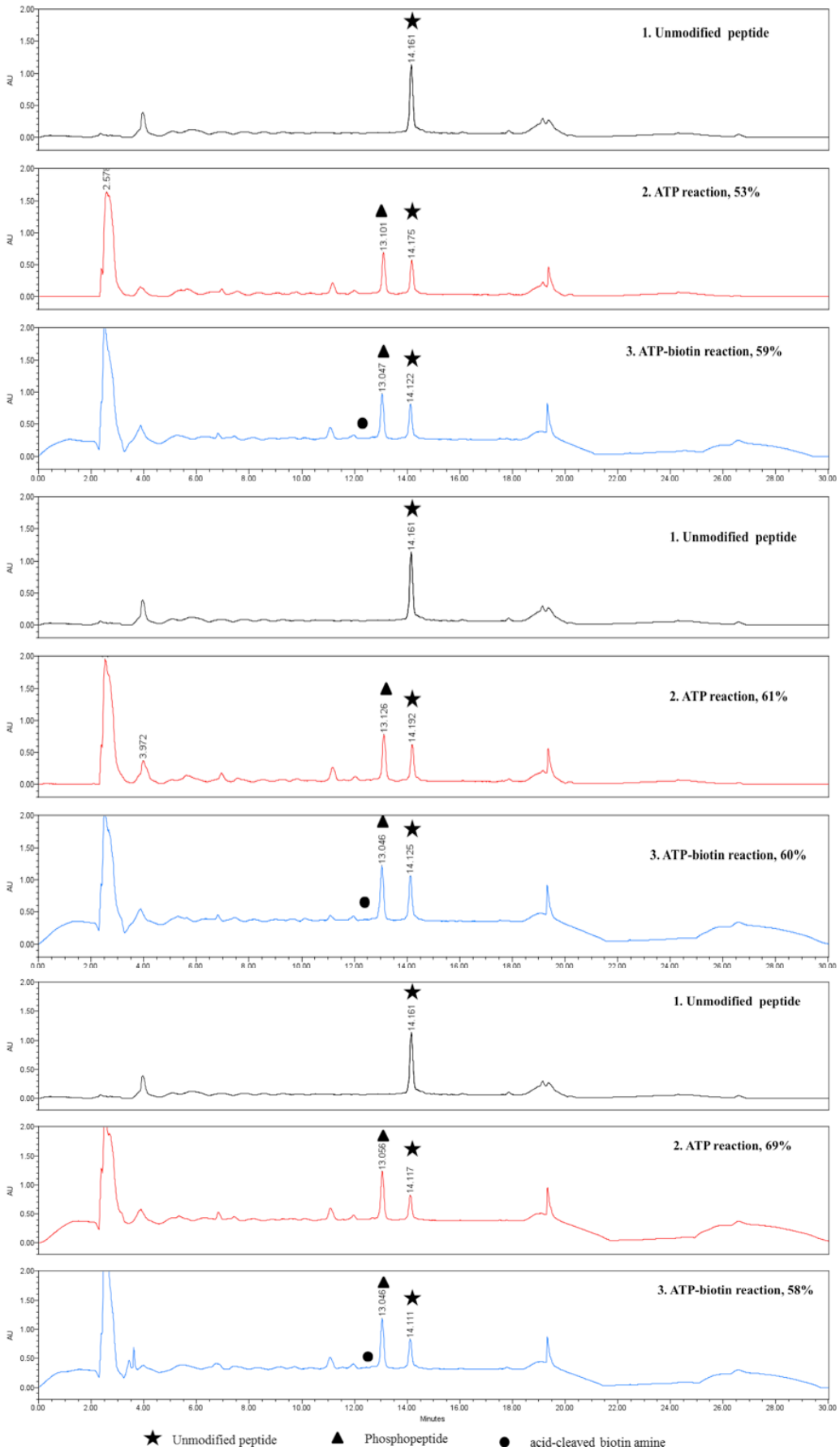
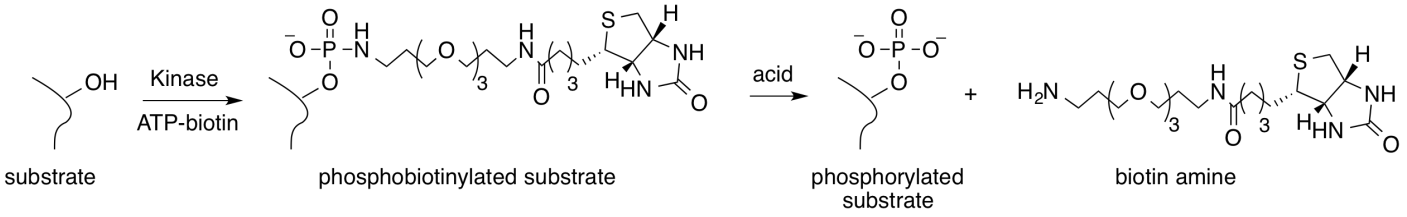


**Figure S5:** HPLC traces for peptide substrates alone (panel 1), or after phosphorylation with ATP (panel 2), or biotinylation with ATP-biotin (panel 3). Reactions were performed using (A) ABL and the ABL peptide substrate (EAIYAAPFAKKK); (B) AKT1 and the AKT (PKB) substrate (CKRPRAASFAE); (C) CaMK4 and the Autocamtide (KKALRRQETVDAL-amide); CK2 and the CK2 peptide substrate (RRREEETEEE); FLT1 and the IGF1Rtide (KKKSPGEYVNIEFG); GSK3β and the GSK3 substrate (YRRAAVPPSPSLSRHSSPHQ(pS)EDEEE); PAK1 and the PAKtide (RRRLSFAEPG); PKA and the kemptide (LRRASLG); RSK1 and the S6K substrate (KRRRLASLR); SRC and the SRC peptide substrate (KVEKIGEGTYGVVYK-amide). Note: For GSK3 reactions, the starting peptide was already phosphorylated, leading to poor separation of mono and di-phosphorylated peptides Three independent trials are shown for each kinase. The peak corresponding to unmodified peptide (star), phosphopeptide product (triangle), or biotin-amine product after acid cleavage (circle) are indicated on each image. The percentage conversion for each ATP or ATP-biotin reaction is indicated in the upper right corner of each image. Data shown here are presented in Figure 5 of the manuscript.

Scheme S1: Acid cleavage of phosphobiotinylated peptides to produce biotin amine, which is observed in the HPLC spectra in Figure S5.



## IV. ADP-Glo Assay-Control Reactions

Because the ATP-Glo assay (Promega) was developed for reactions utilizing ATP, several control reactions were performed with ATP-biotin. The ADP-Glo assay comprises two steps after hydrolysis of ATP to ADP during the kinase reaction [4]. The first step involves incubation of the reaction with the ADP-Glo™ reagent to degrade any remaining ATP and stop the kinase reaction. The second step introduces the reaction to kinase detection reagent (KDR) to convert ADP to ATP and create a luminescence signal with luciferase/luciferin (www.promega.com). To assure that ATP-biotin is compatible with the assay, the susceptibility of ATP-biotin to the ADP-Glo reagent and KDR were tested.

A series of experiments were performed with ATP-biotin without an initial kinase reaction to determine the fate of ATP-biotin with the ADP-Glo and KDR reagents (Figure S6A). No significant luminescence signal was observed when ADP-Glo and KDR were incubated with either ATP (Figure S6A, lane 2) or ATP-biotin (Figure S6A, lane 3), indicating that both were degraded by the ADP-Glo reagent as expected. To test if ATP or ATP-biotin interfered with the detection of ADP in the assay, reactions containing ADP were performed in the presence of ATP and ATP-biotin and in both cases similar luminescence signal observed (Figure S6A, lanes 5 and 6), which was comparable to the reaction with ADP alone (Figure S6A, lane 4). These control reactions showed that ATP and ATP-biotin are performing similarly in the assay.

Next, control reactions were preformed with KDR only to assure minimal interference of ATP-biotin with the luminescence generation step of the reaction. When ATP (Figure S6A, lane 7) or ADP (Figure S5A, lane 8) was treated with only KDR, the signal intensity was the same as when ADP was treated with ADP-Glo reagent and KDR (Figure S6A, compare lane 4 and 8). In contrast, the ATP-biotin/KDR reaction produced only background signal (Figure S6A, lane 9). Also, while a combination of the ATP and ADP almost doubled the signal (Figure S6A, compare lane 12 with lanes 7 and 8), inclusion of ATP-biotin did not significantly affect the luminescence signal of KDR reactions with either ATP or ADP (Figure S6A, compare lanes 10 and 11 to lanes 7 and 8). These control reactions indicate that the ATP-biotin does not produce signal with KDR.

Finally, we performed ADP-Glo assay controls including an initial kinase reaction with PKA and kemptide peptide substrate (Figure S6B). After the kinase reaction, luminescence signal was observed in reactions containing ATP (Figure S6B, lane 8) or ATP-biotin (Figure S6B, lane 9) as cosubstrates. If either enzyme or peptide substrate were omitted from the initial kinase reaction, ATP (Figure S6B, lane 4 and 6) and ATP-biotin (Figure S6B, lane 5 and 7) displayed minimal signal, showing dependence on hydrolysis in the kinase reaction. These results indicated that the ADP-Glo assay shows similar reactivity with ATP-biotin and ATP, and can be used for kinetics experiments.

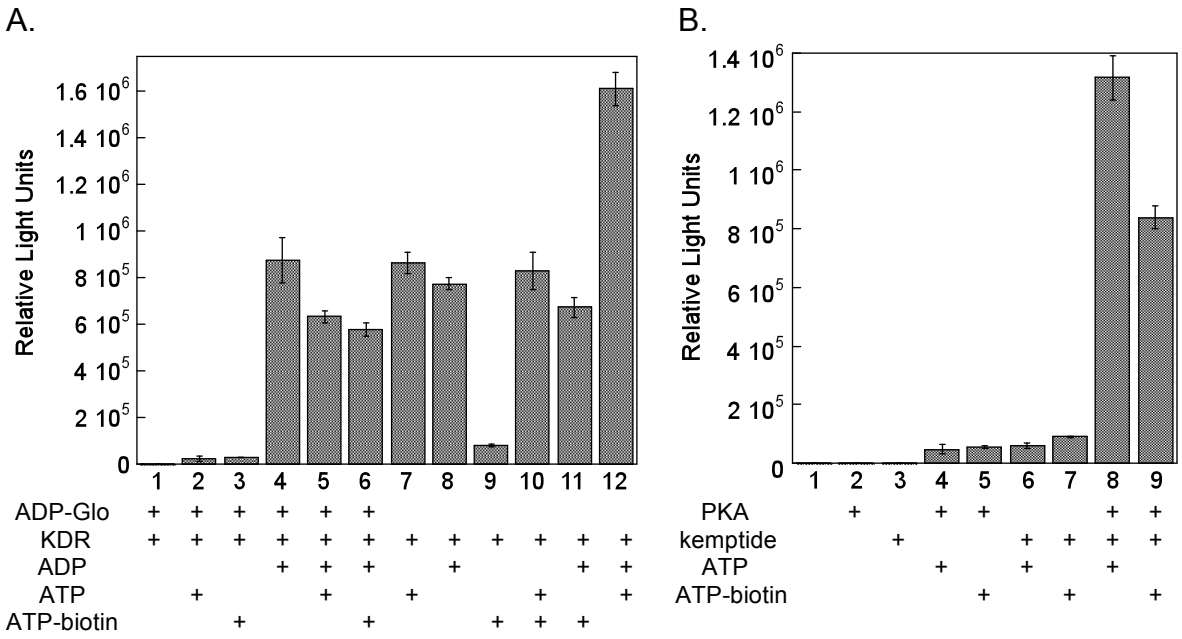
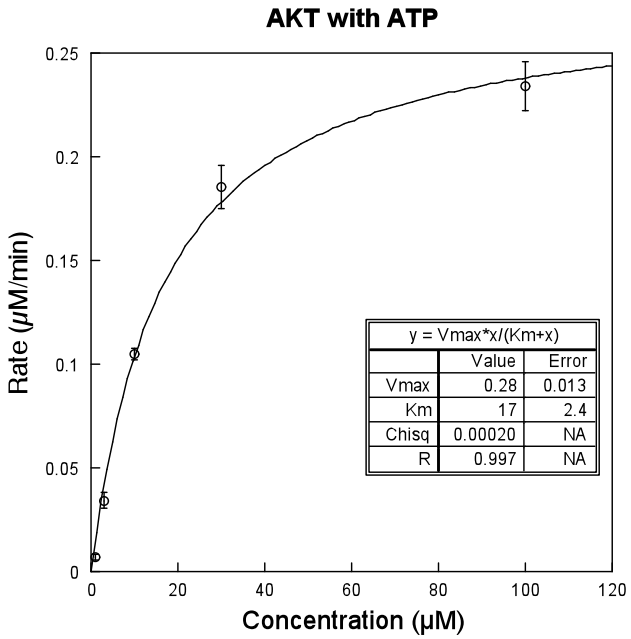
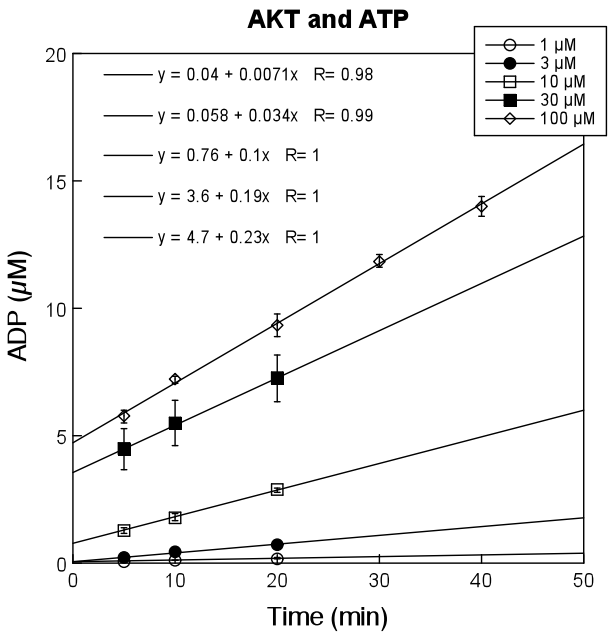


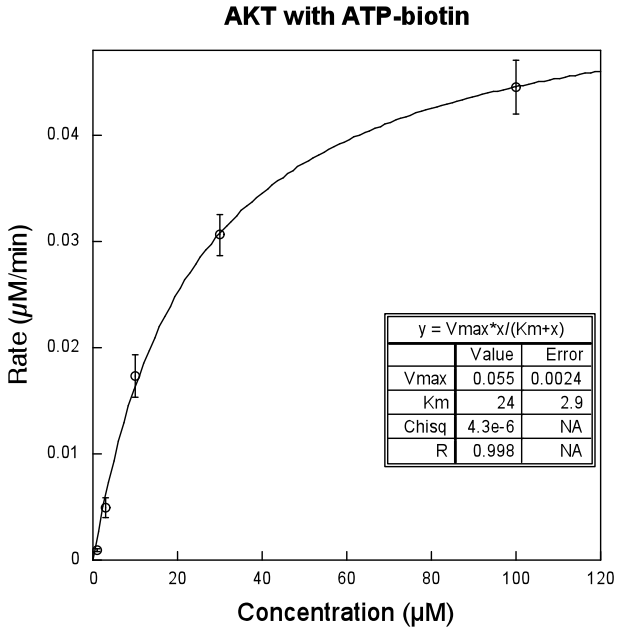

**Figure S6.** Several control experiments were carried out to validate the ADP-Glo assay with ATP-biotin. **(A)** ADP-Glo assays were performed without an initial kinase reaction. Lanes 1-6 included both ADP-Glo reagent and kinase detection reagent, while lanes 7-12 used kinase detection reagent (KDR) only. The presence of ADP, ATP, or ATP-biotin are indicated below each lane. RLU represents relative light units. **(B)** The ADP-Glo assay was performed with an initial PKA kinase reaction either with or without kinase or kemptide substrate. The components of each reaction are listed below the lane, with lanes 8 and 9 containing both kinase and substrate. All lanes included both ADP-Glo reagent and kinase detection reagent (KDR).

## V. Michaelis-Menten Kinetics Plots

**Figure S7 (A).** Reactions with AKT1



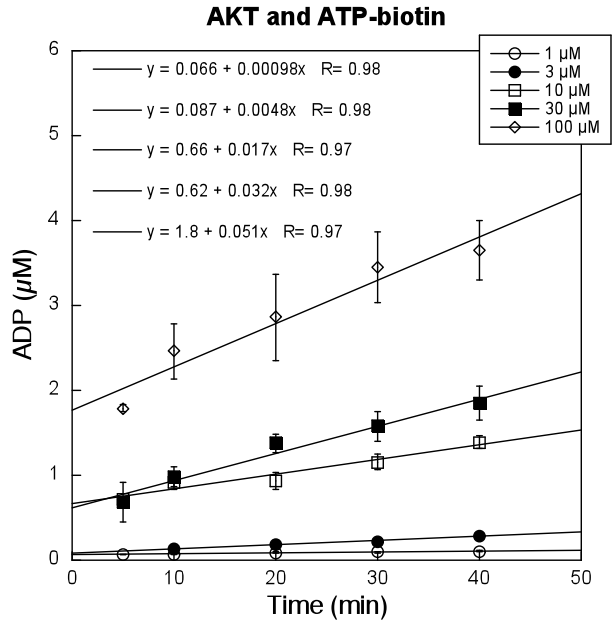



**Figure S7 (B).** Reactions with ASK1

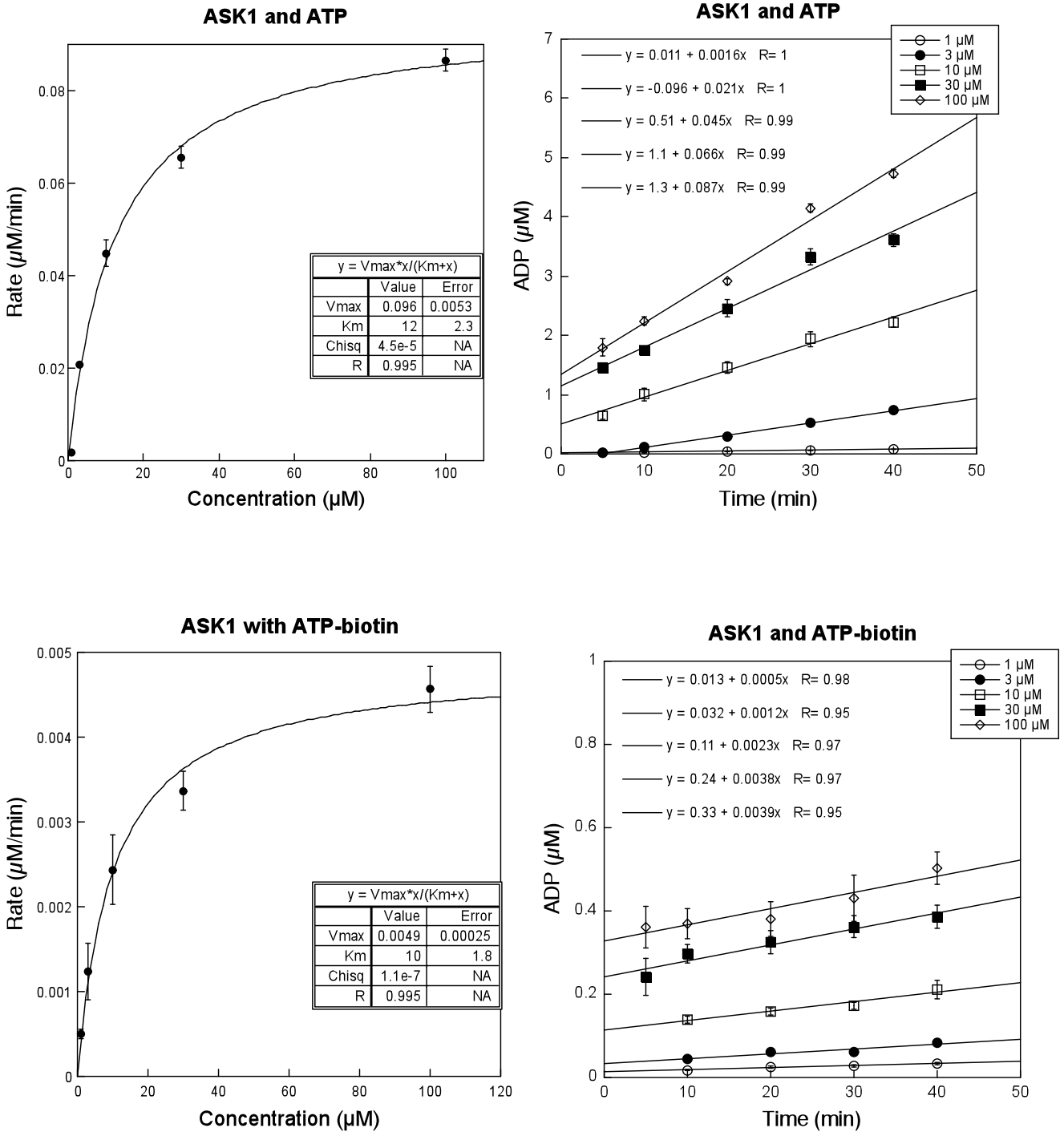
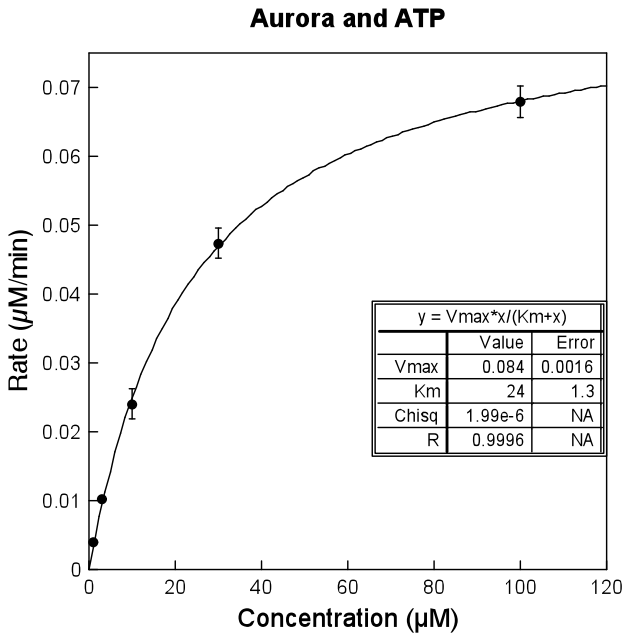
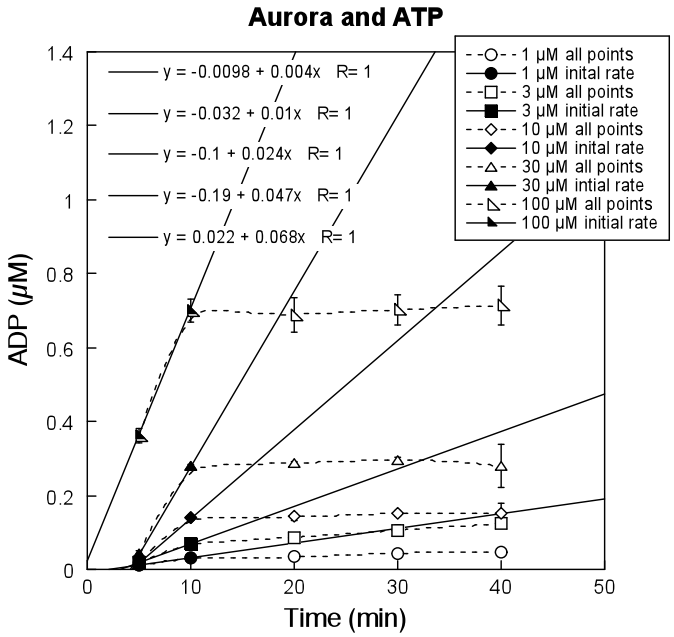
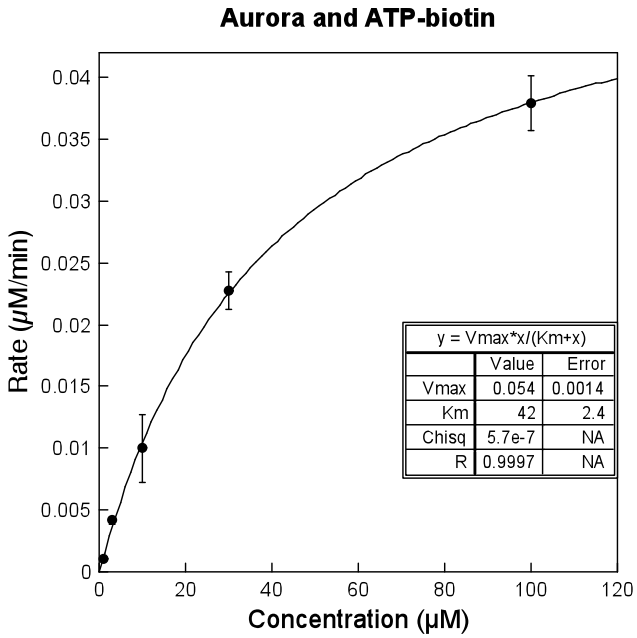


**Figure S7 (C).** Reactions with Aurora A



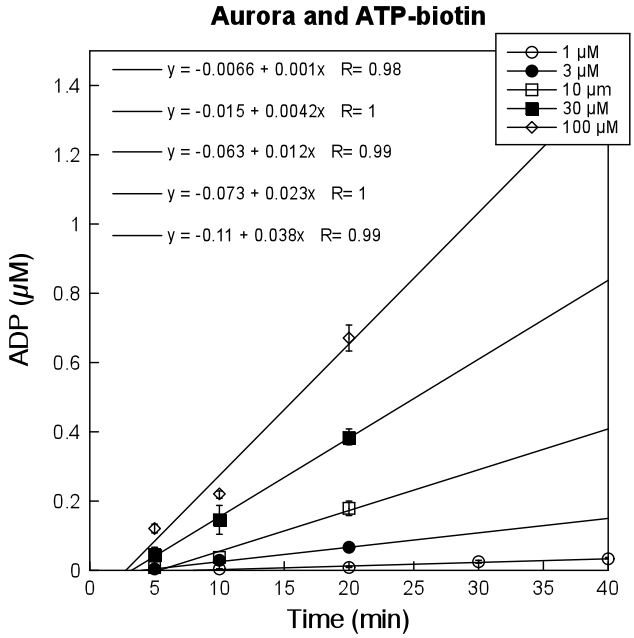
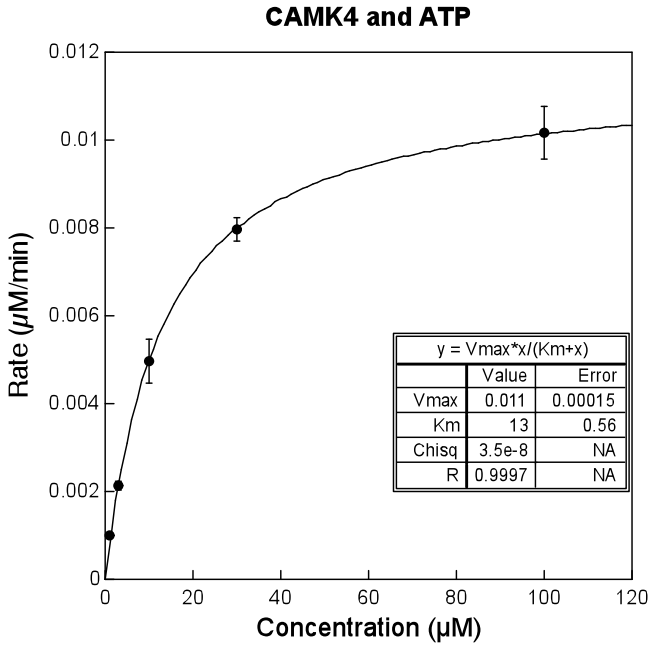
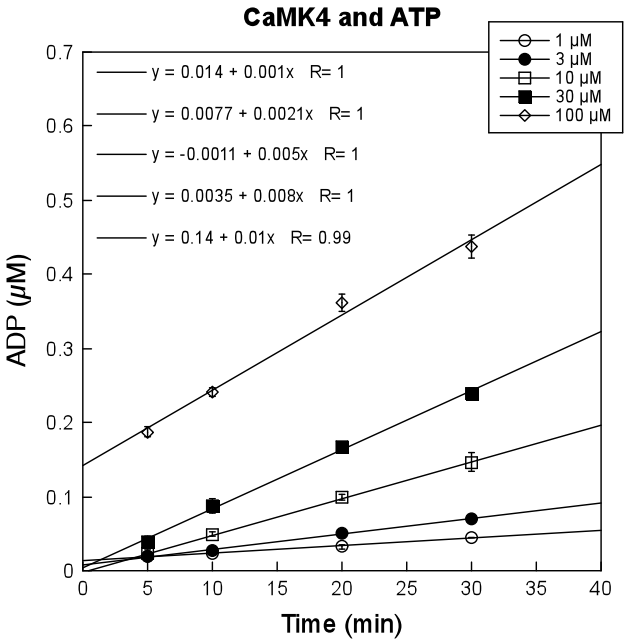
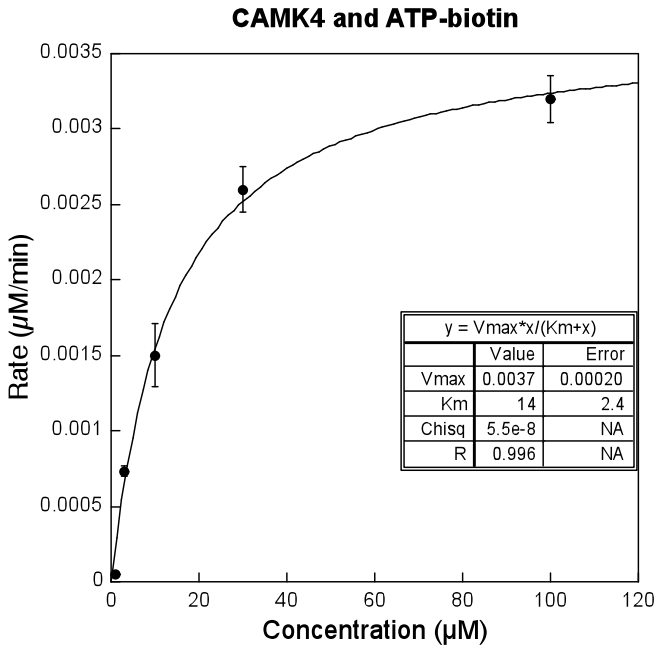



**Figure S7 (D).** Reactions with CAMK4



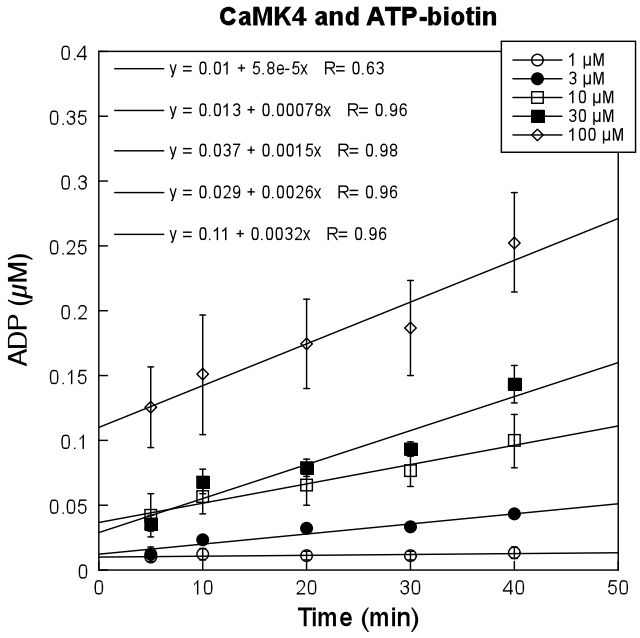
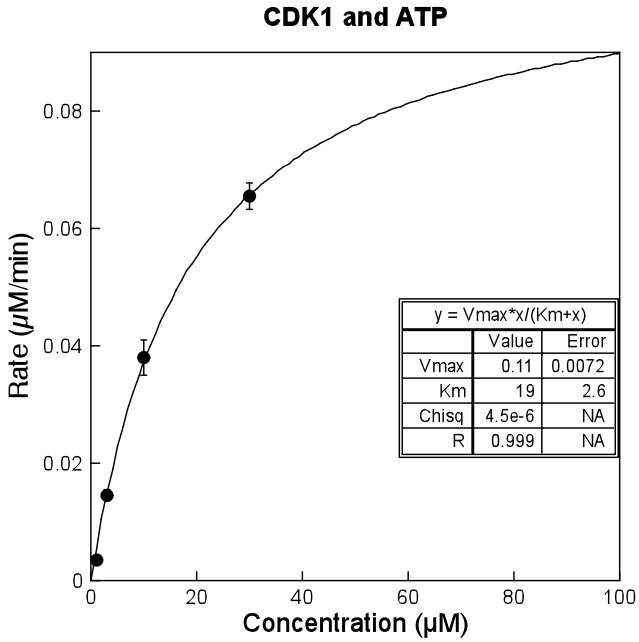
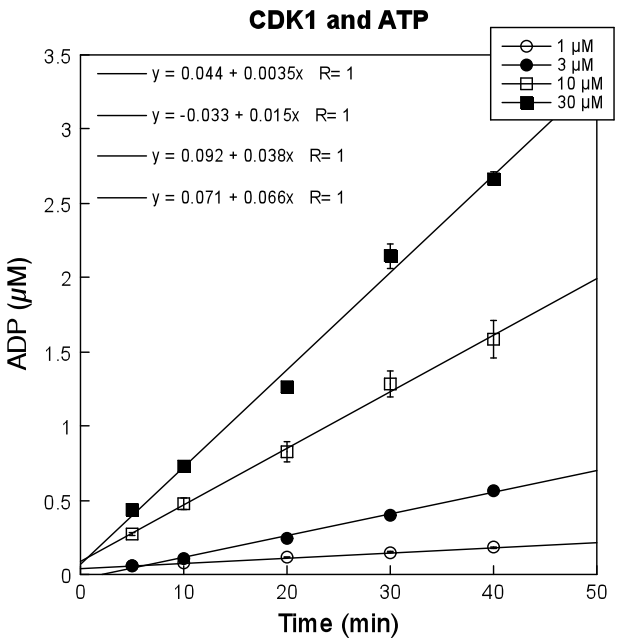
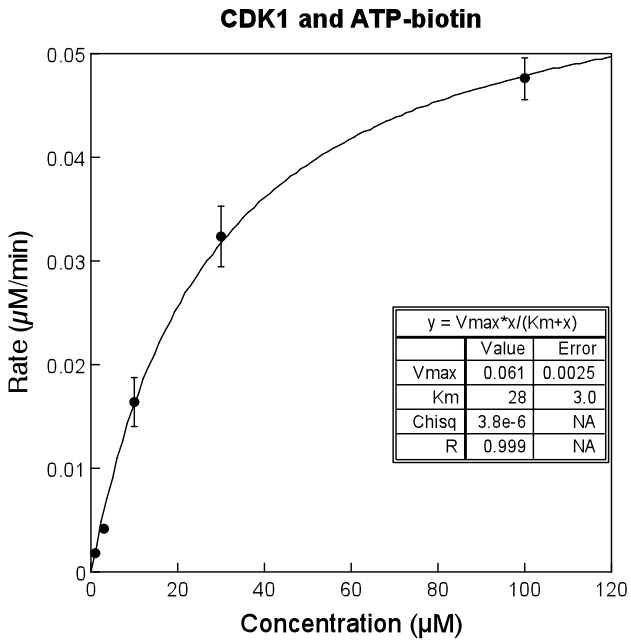



**Figure S7 (E).** Reactions with CDK1



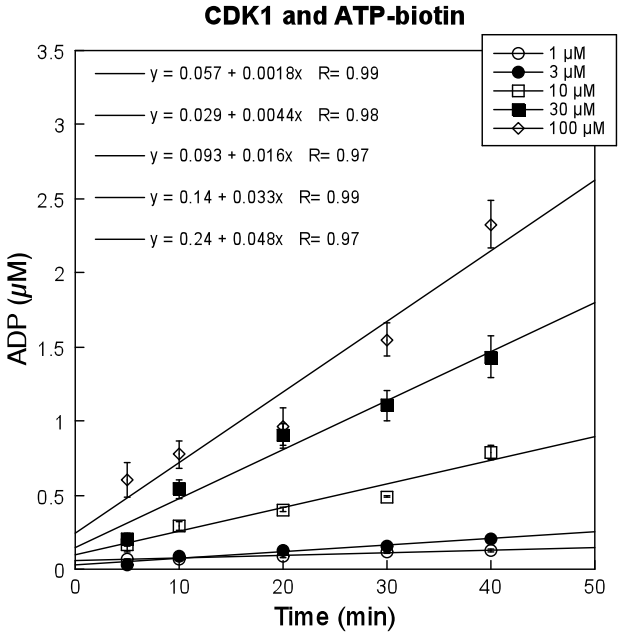
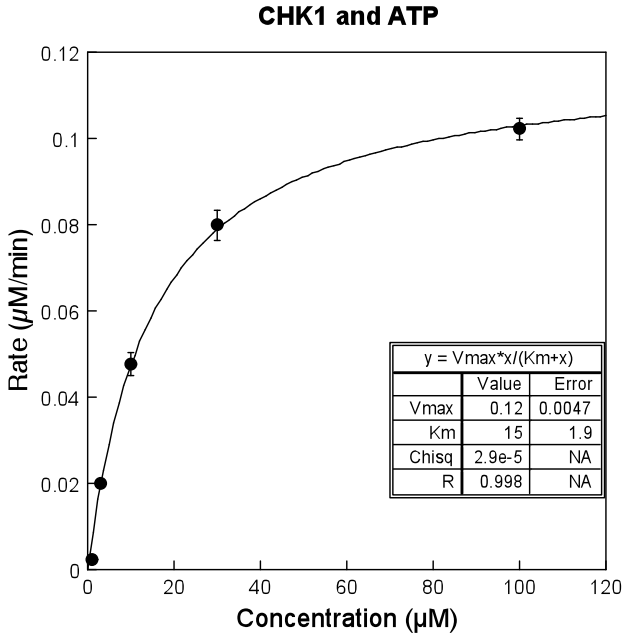
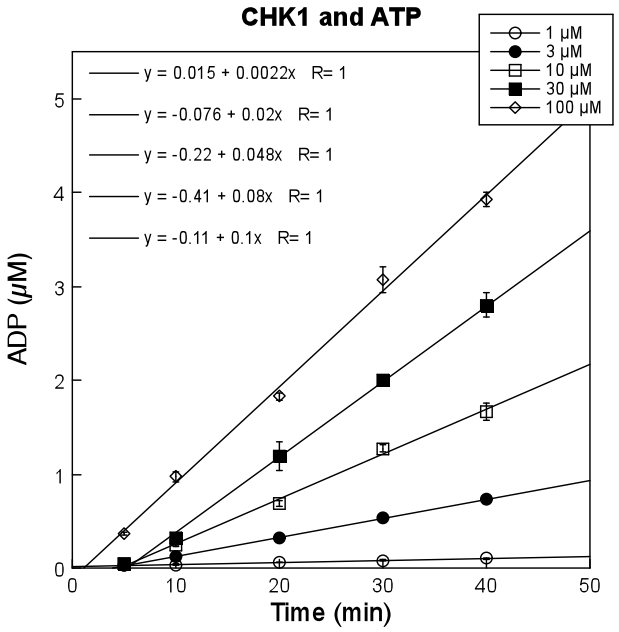
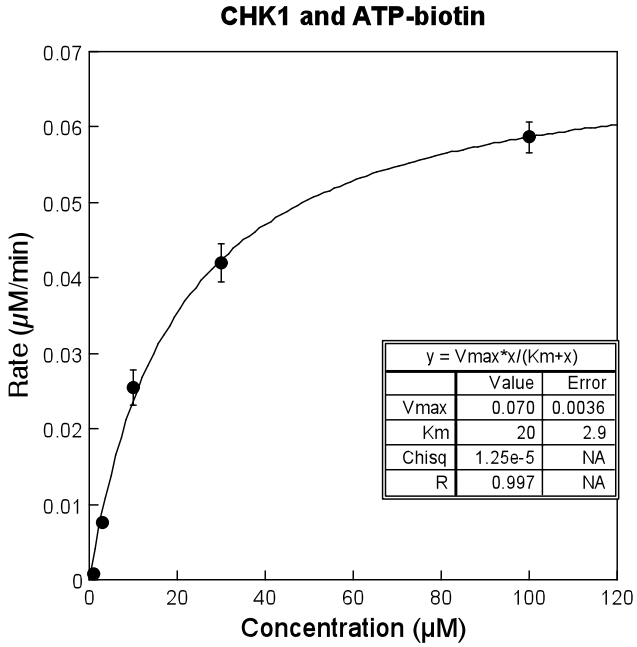



**Figure S7 (F).** Reactions with CHK1



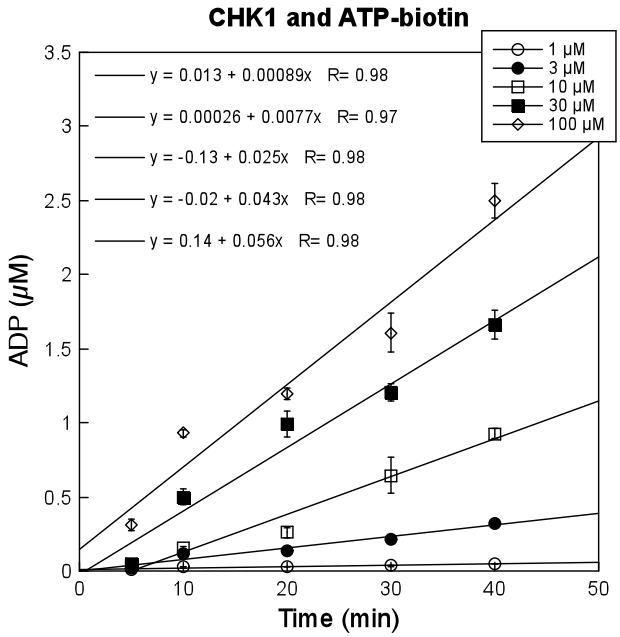
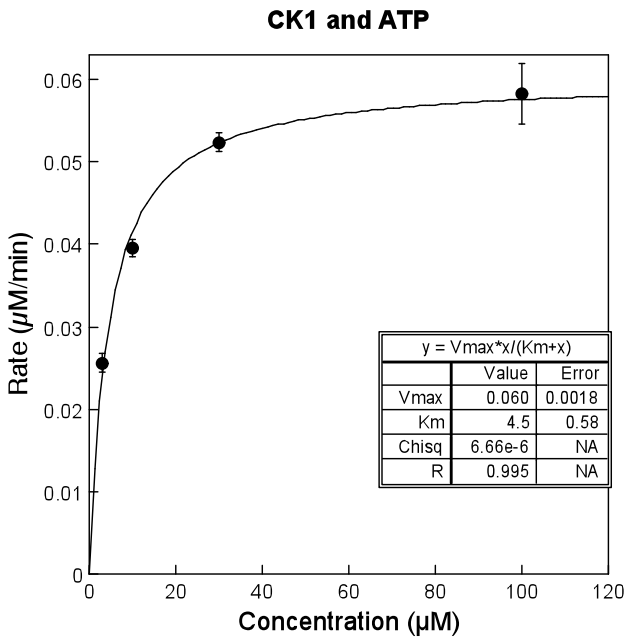
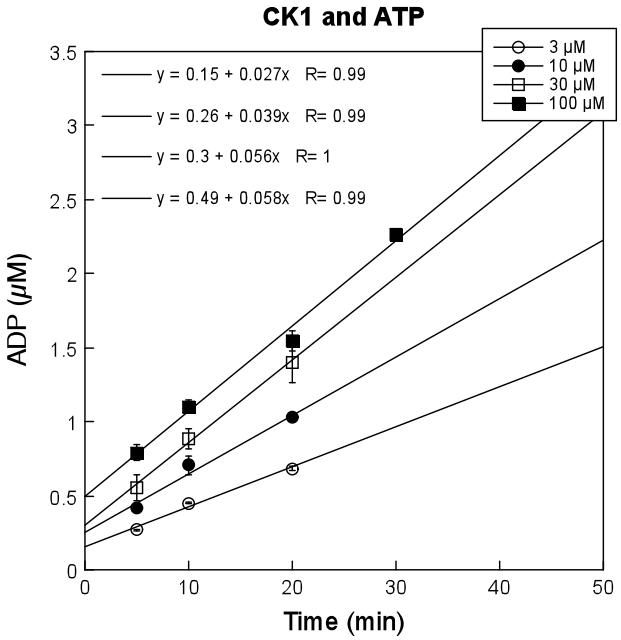
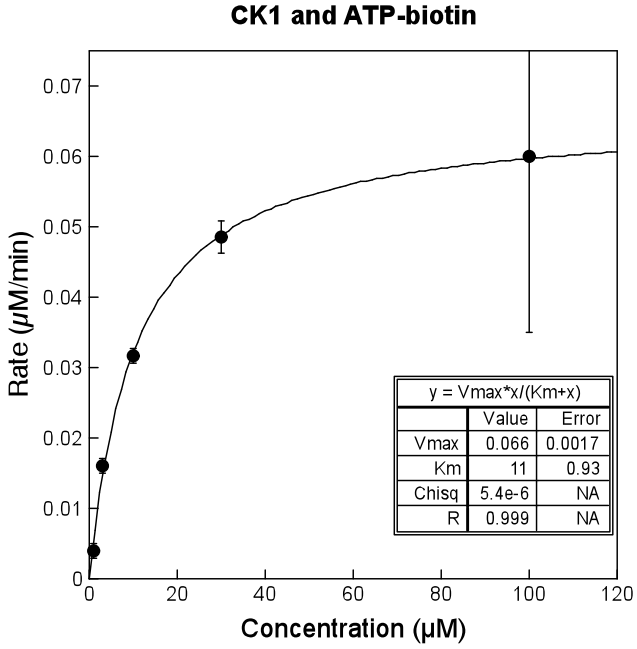



**Figure S7 (G).** Reactions with CK1



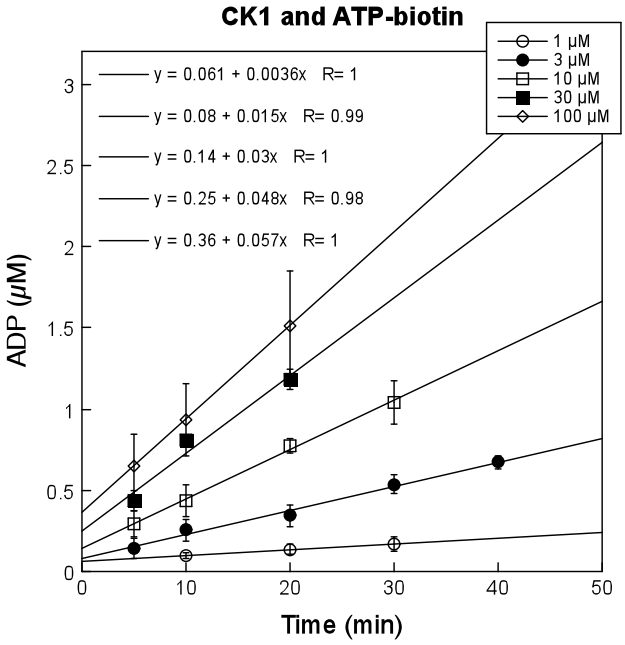
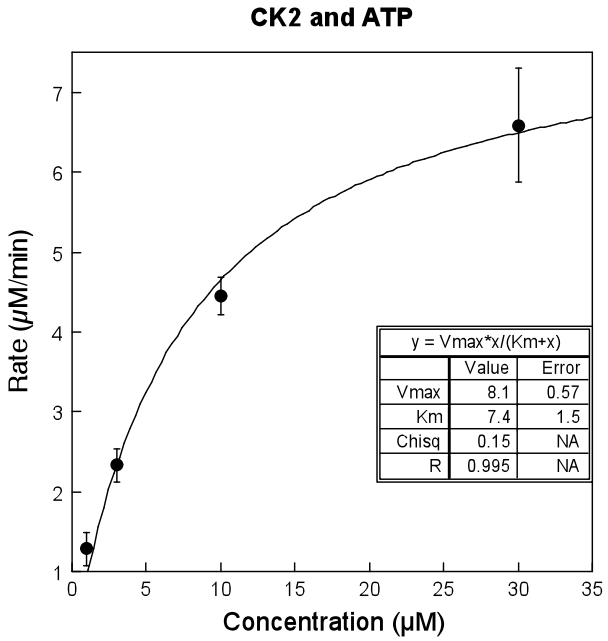
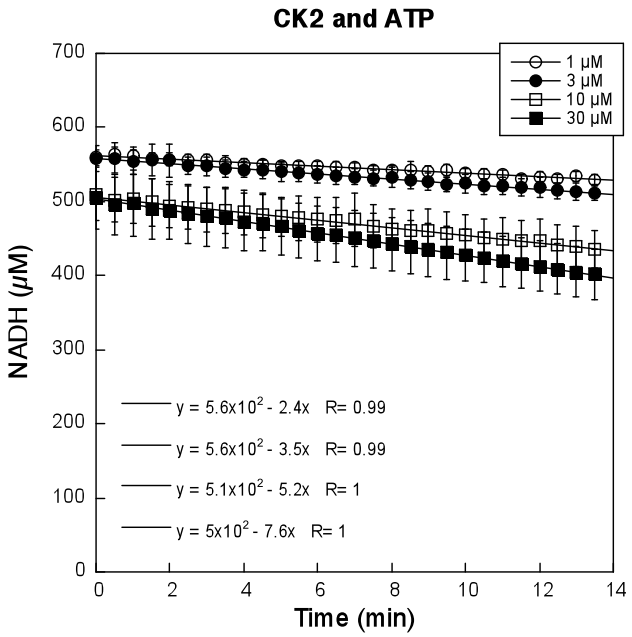
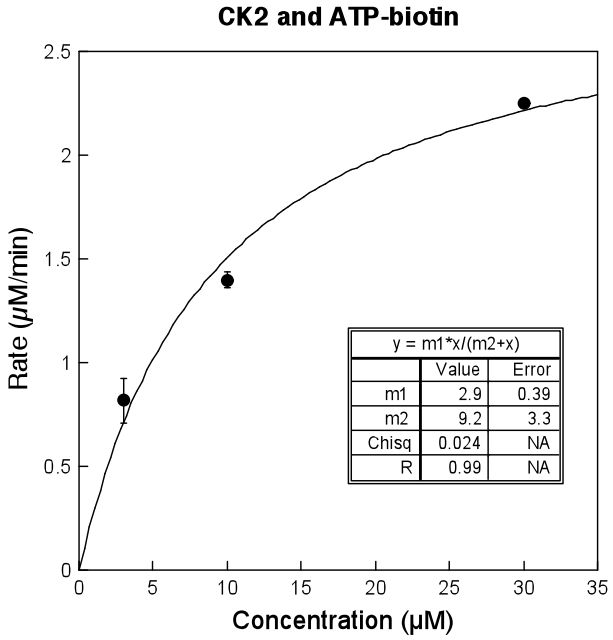



**Figure S7 (H).** Reactions with CK2



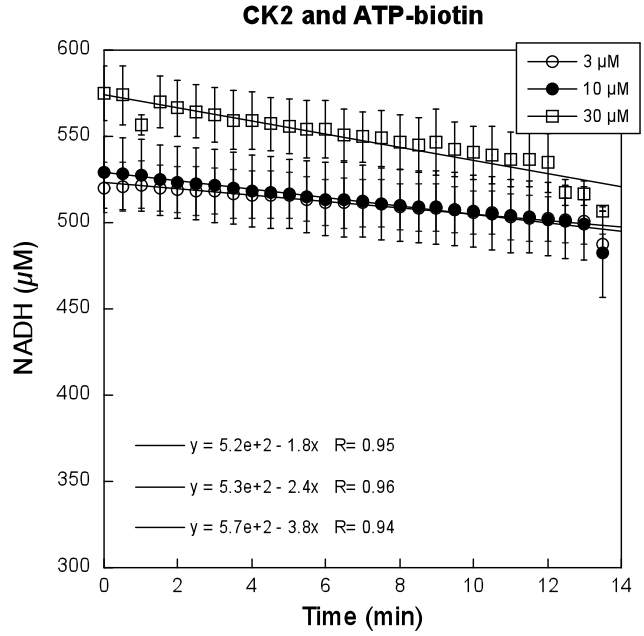
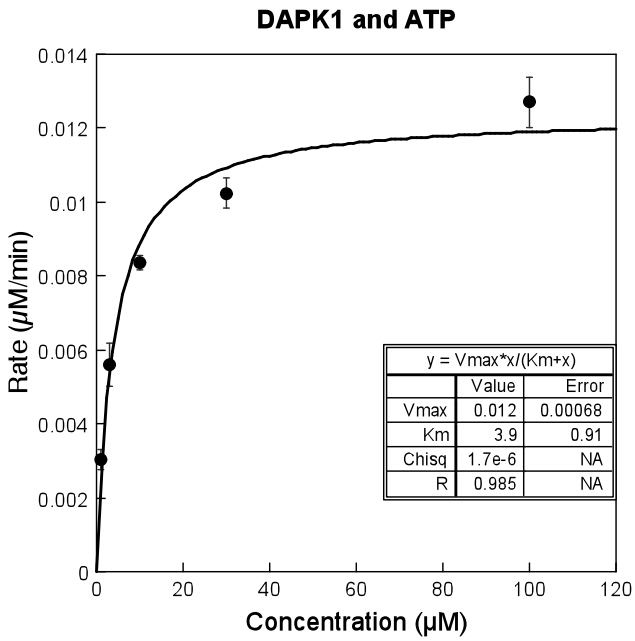
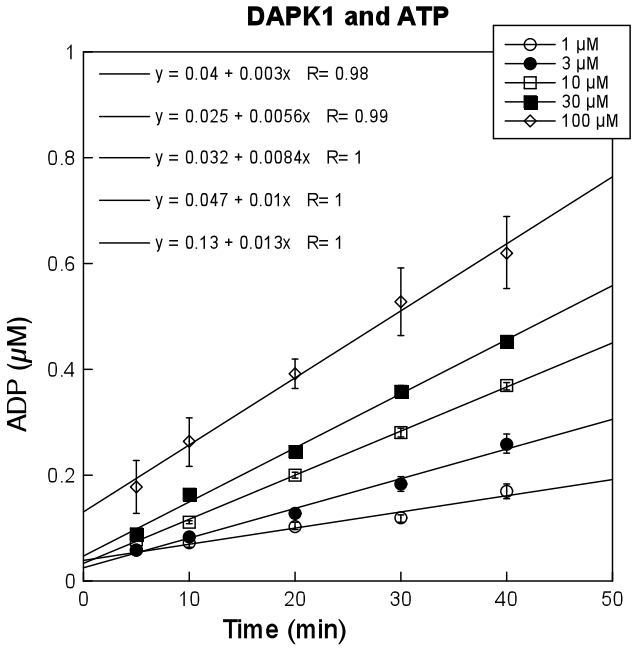
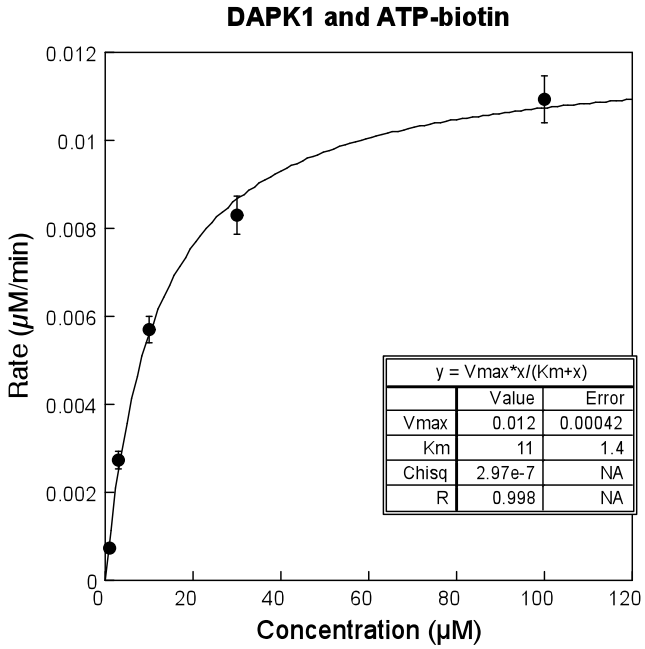



**Figure S7 (I)** Reaction with DAPK1



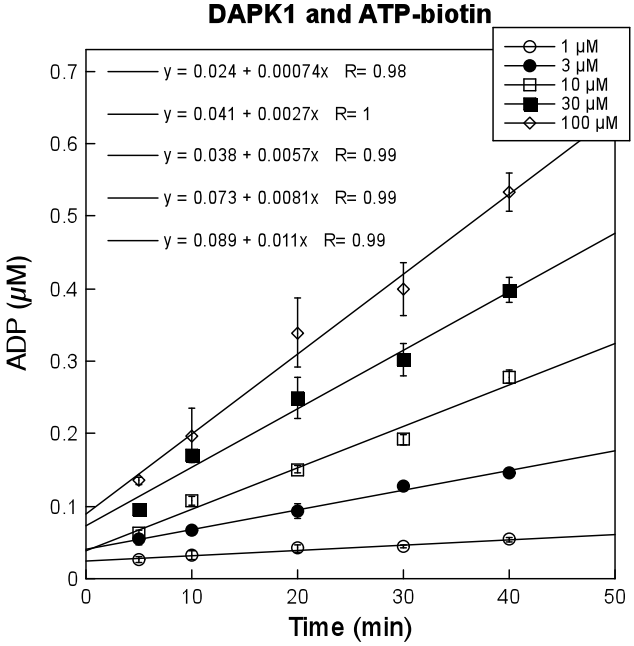
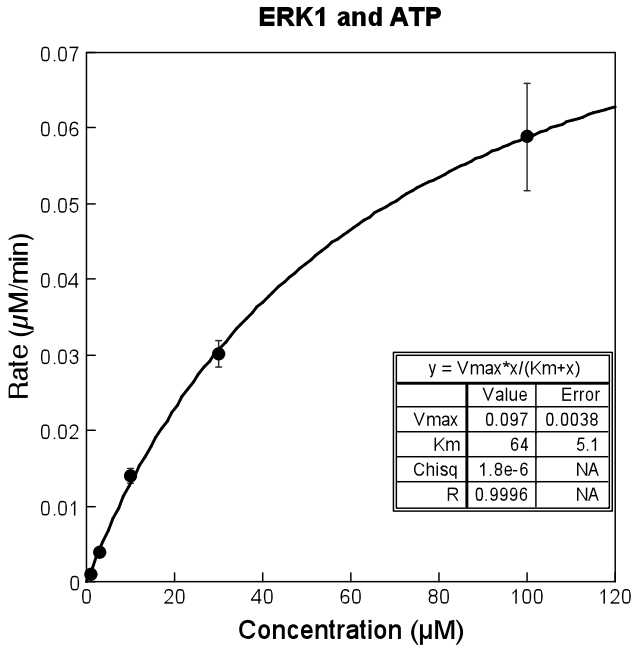
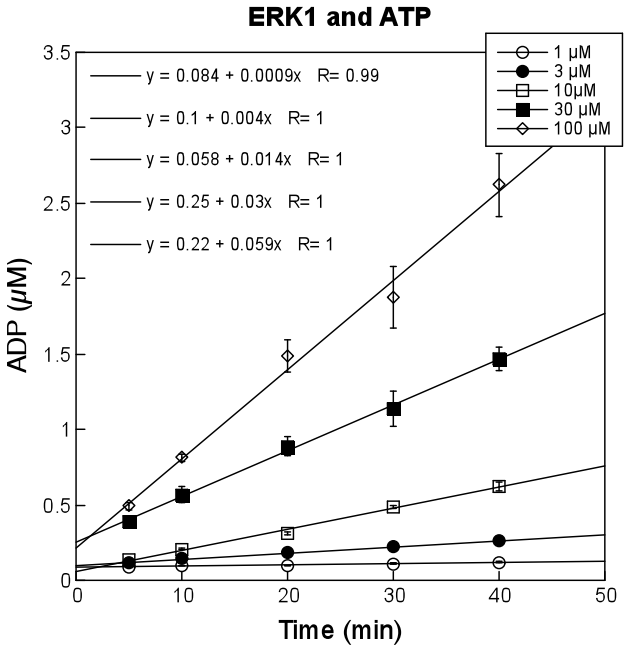
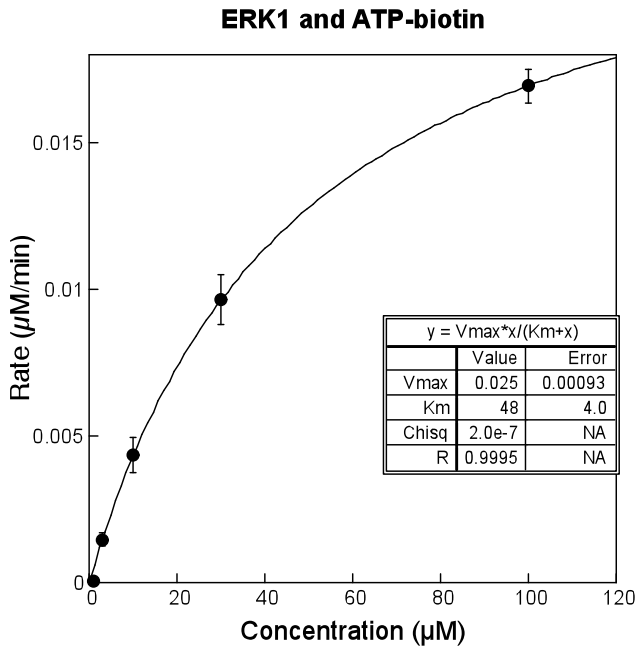
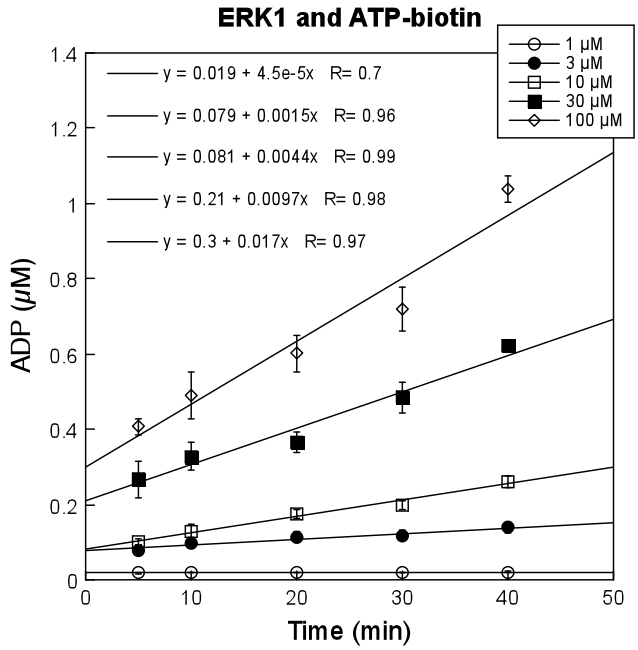



**Figure S7 (J).** Reactions with ERK1

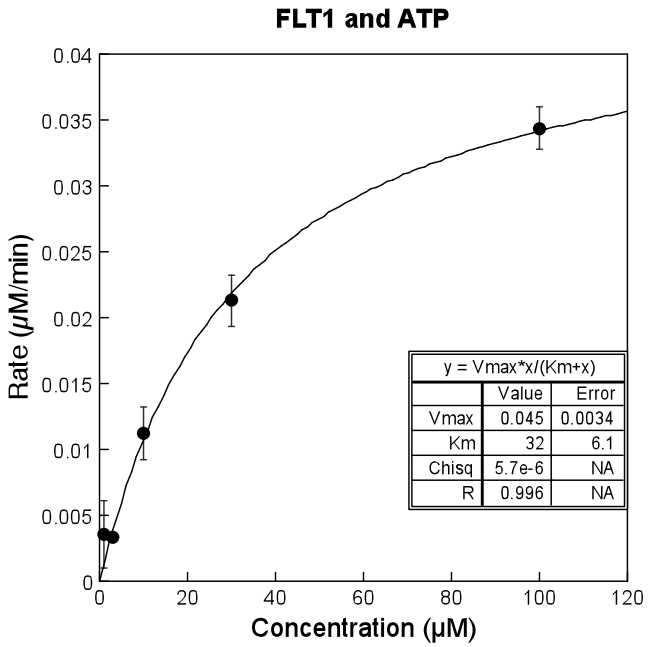


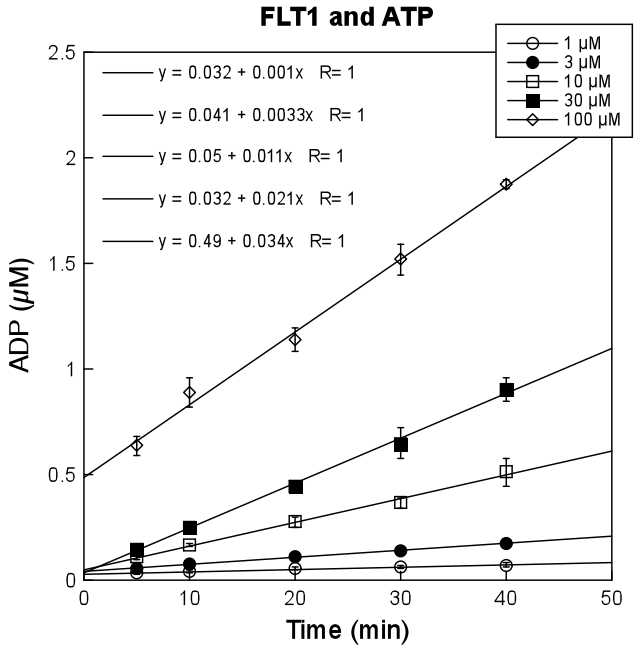



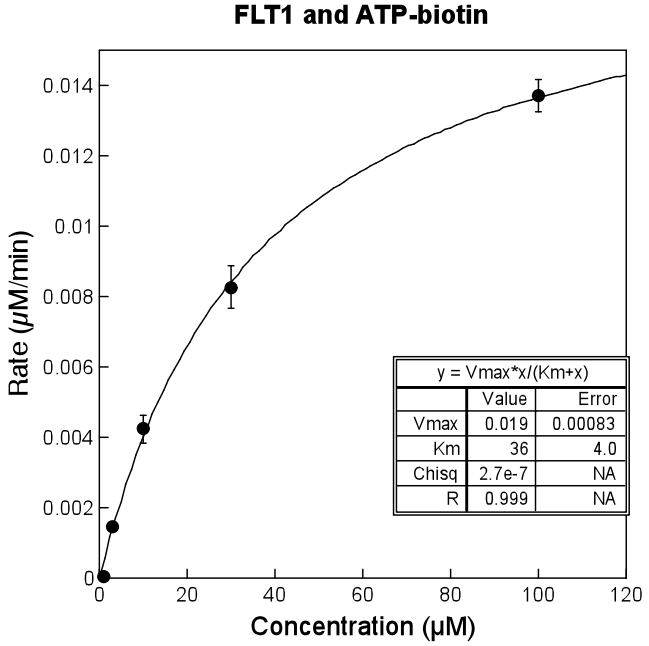



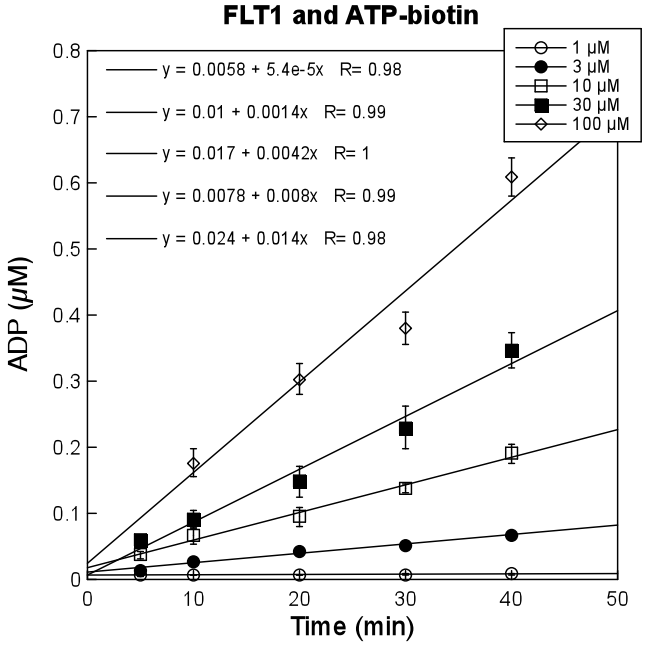



## Figure S7 (K) Reaction with FLT1

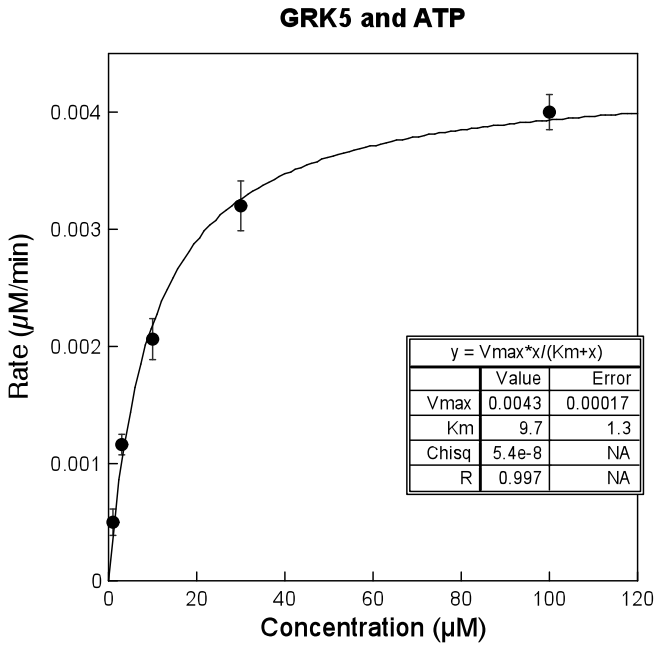


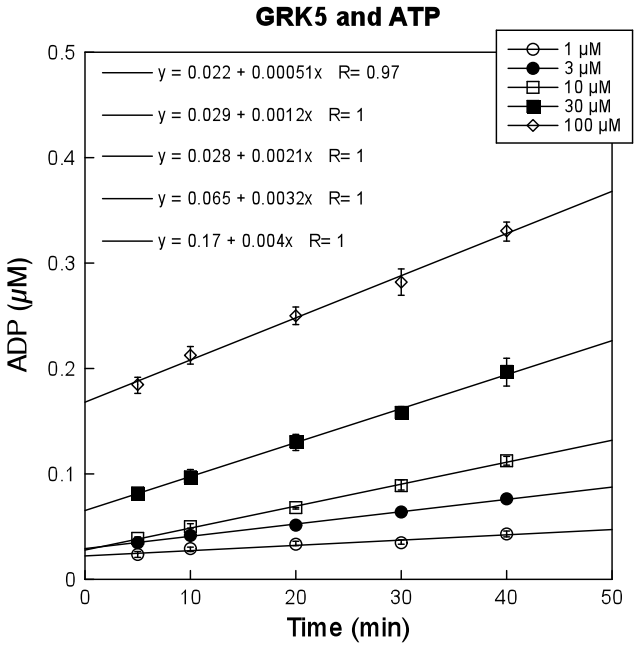



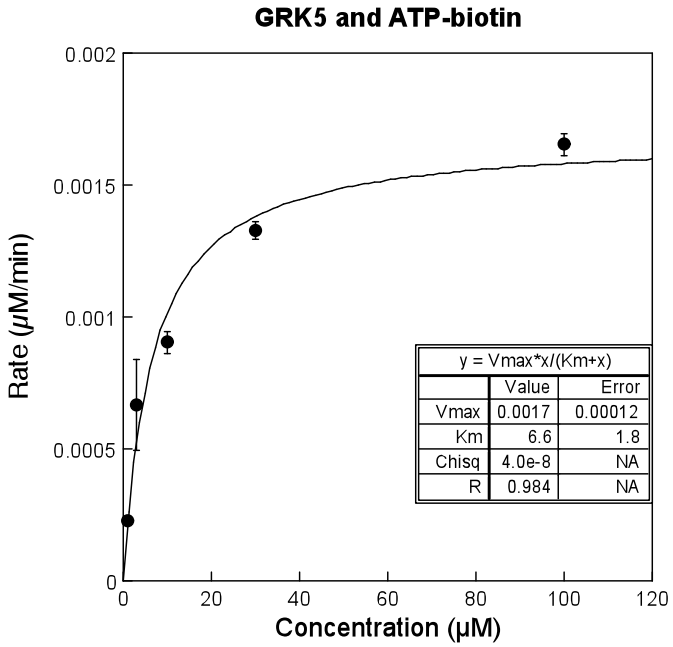



## Figure S7 (L) Reaction with GRK5



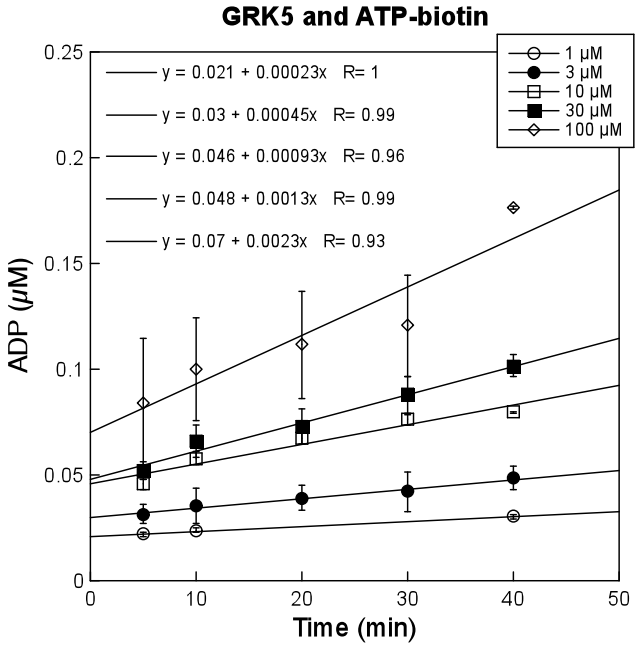
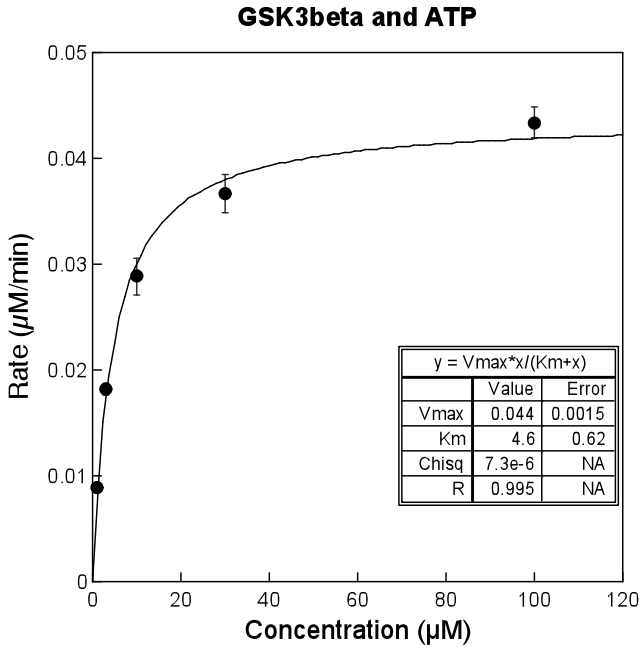
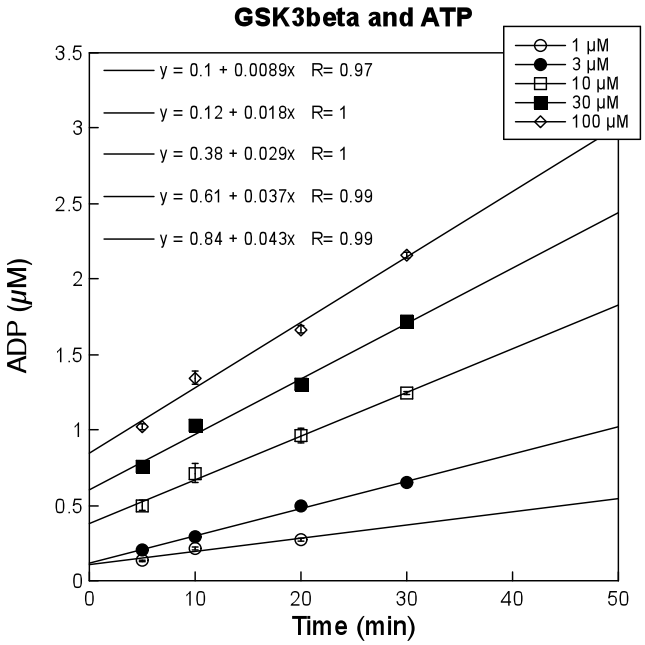
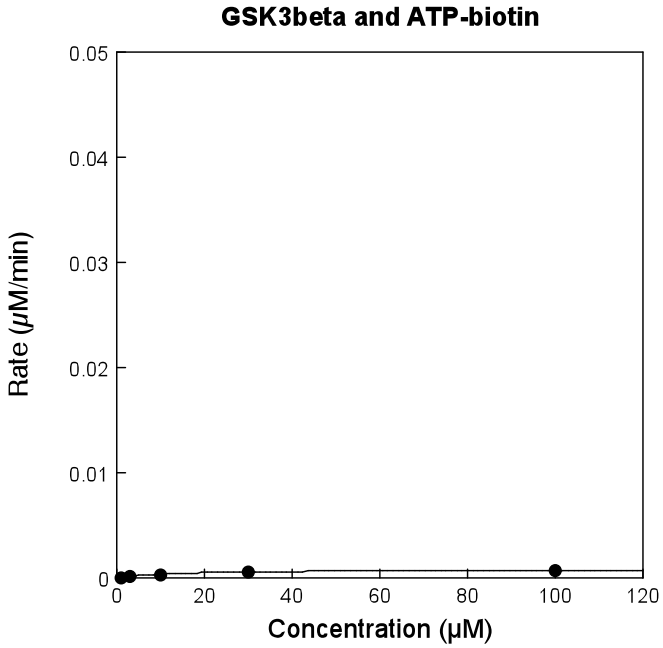



**Figure S7 (M).** Reactions with GSK3β



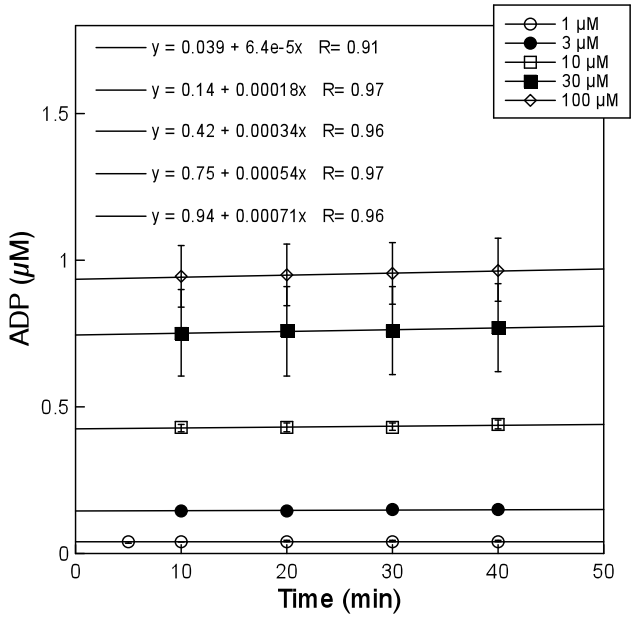
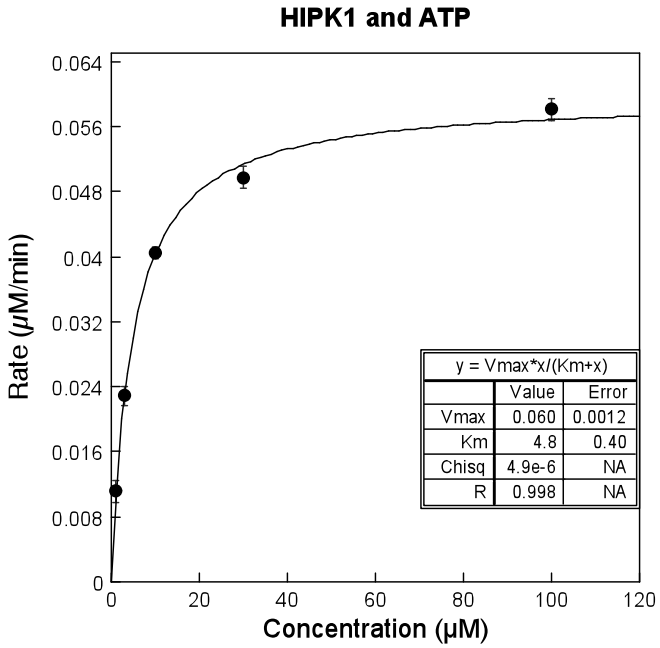
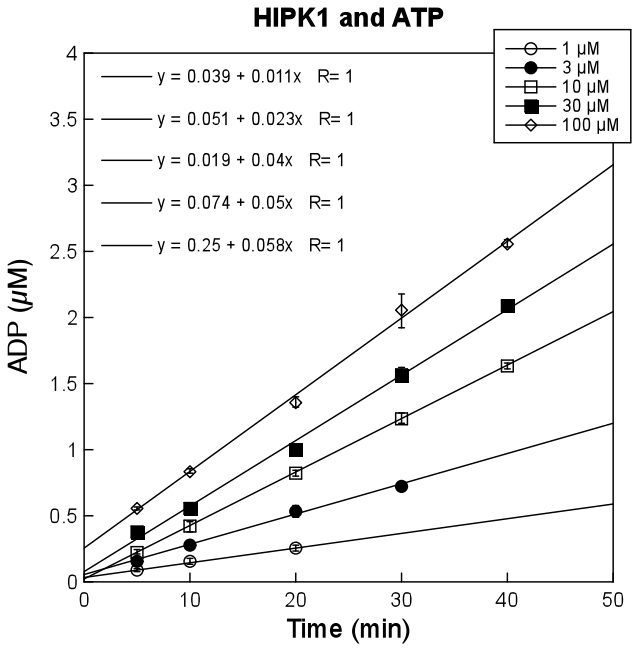
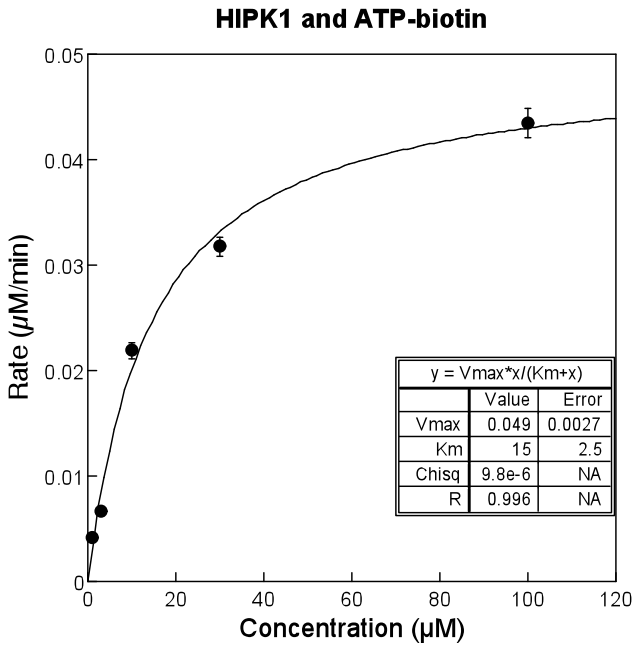



**Figure S7 (N).** Reactions with HIPK1



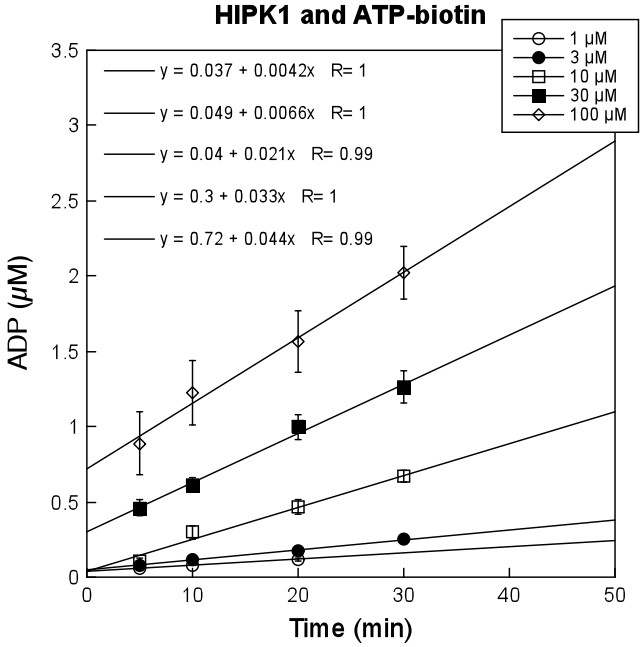
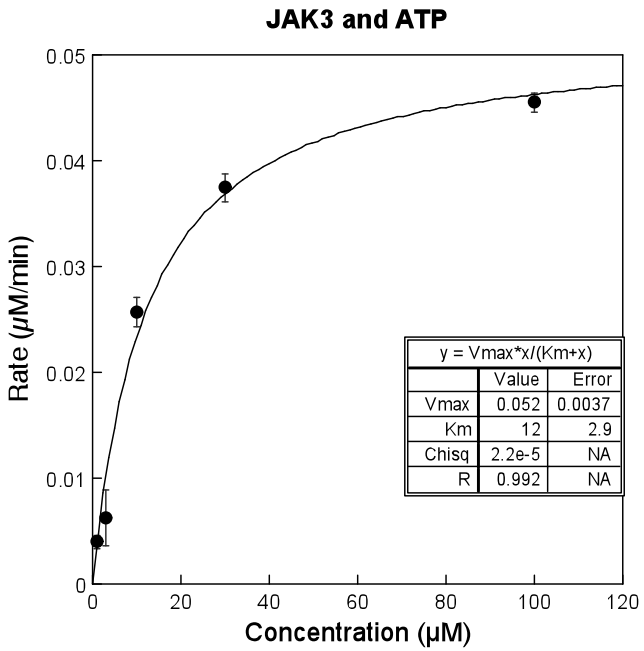
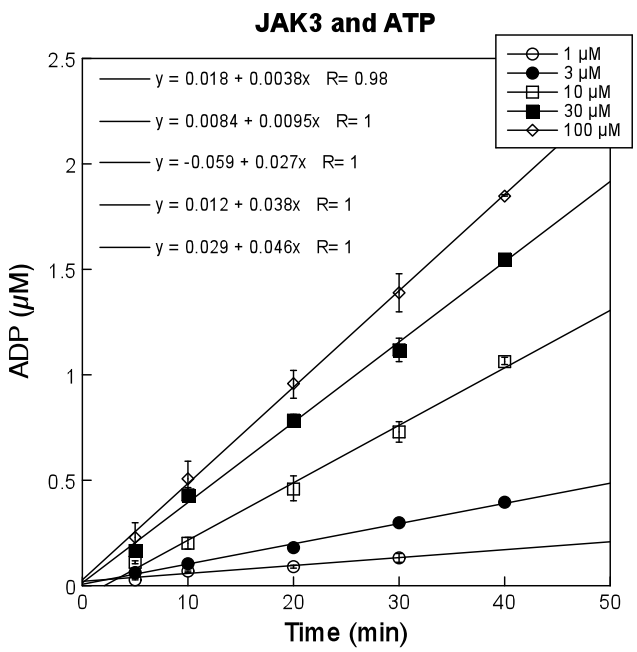
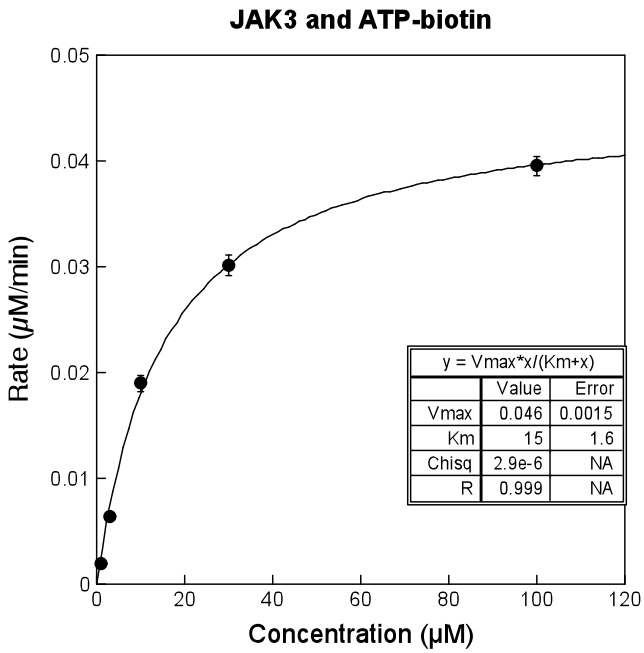



**Figure S7 (O).** Reactions with JAK3



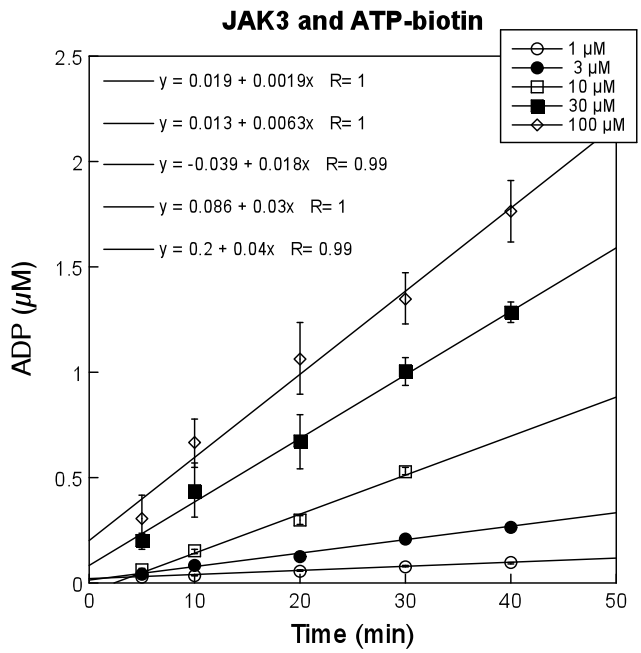
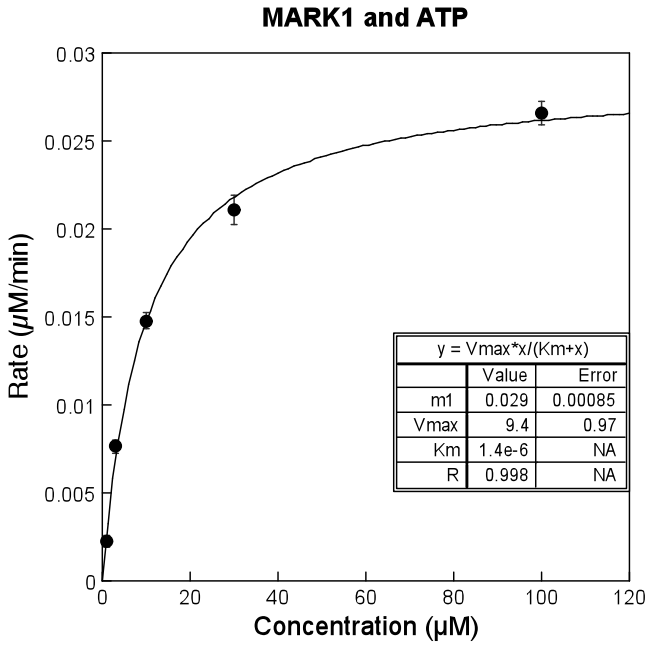
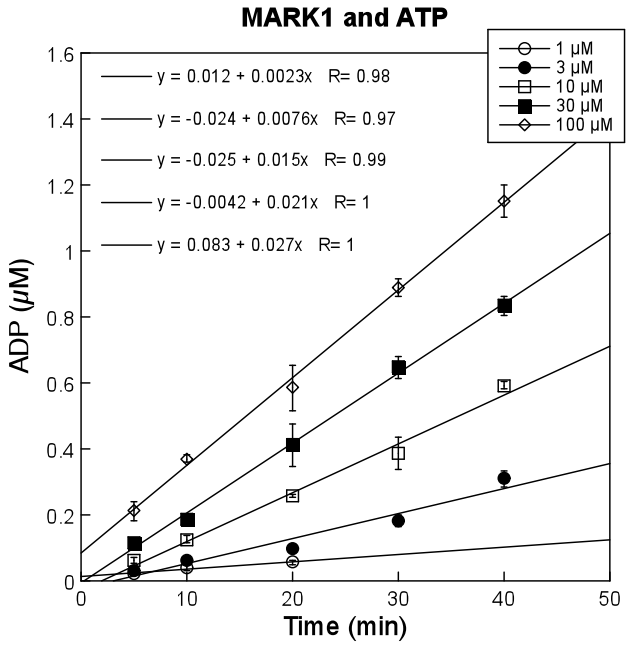
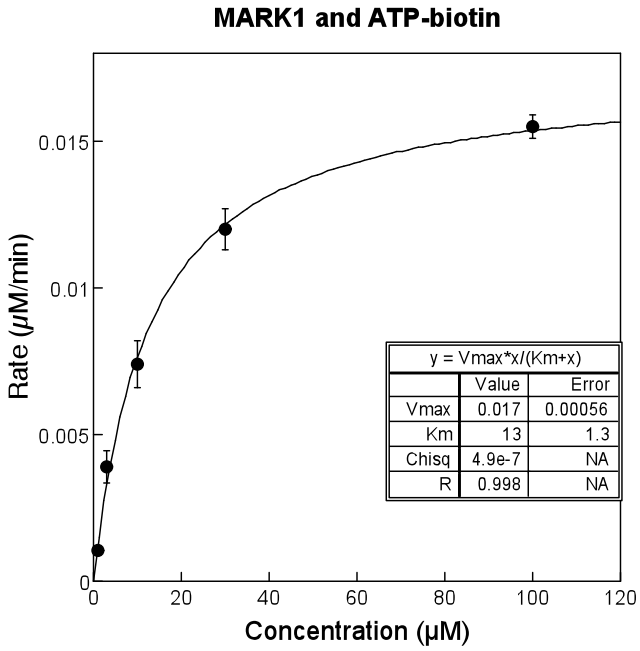



**Figure S7 (P).** Reactions with MARK1



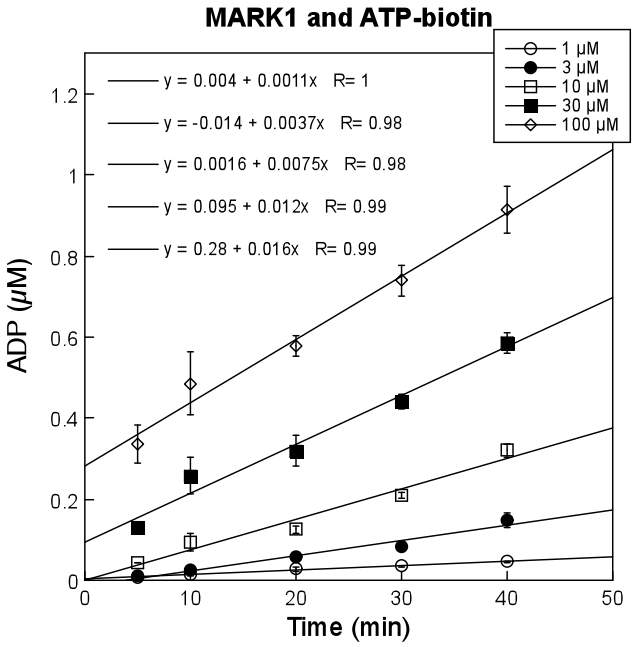
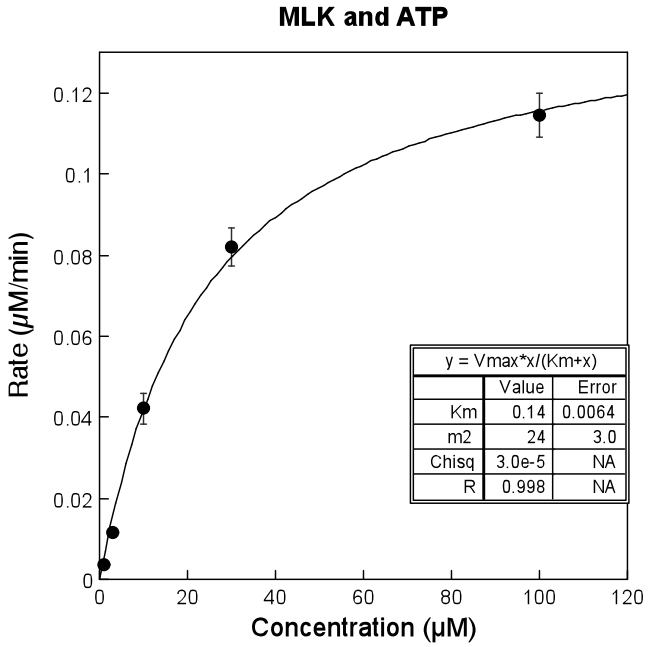
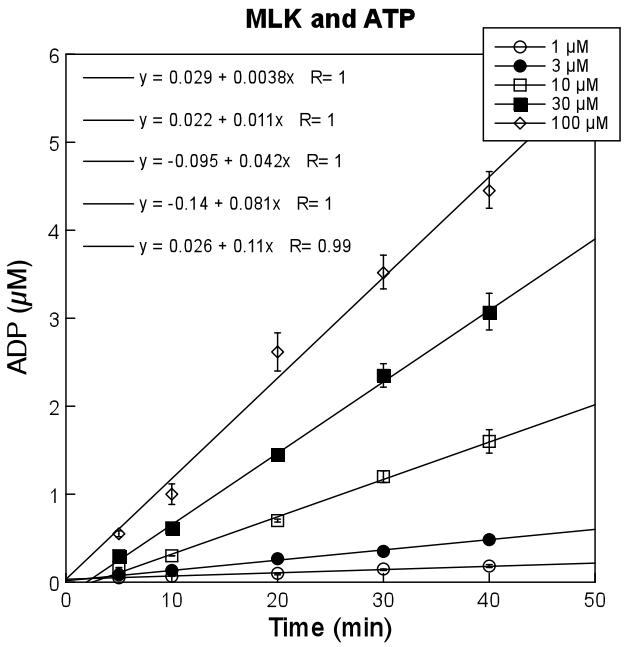
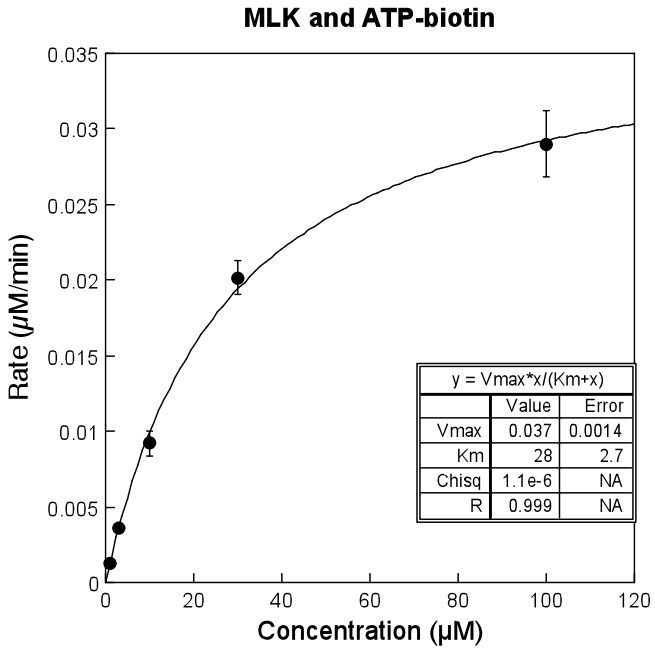



**Figure S7 (Q).** Reaction with MLK1



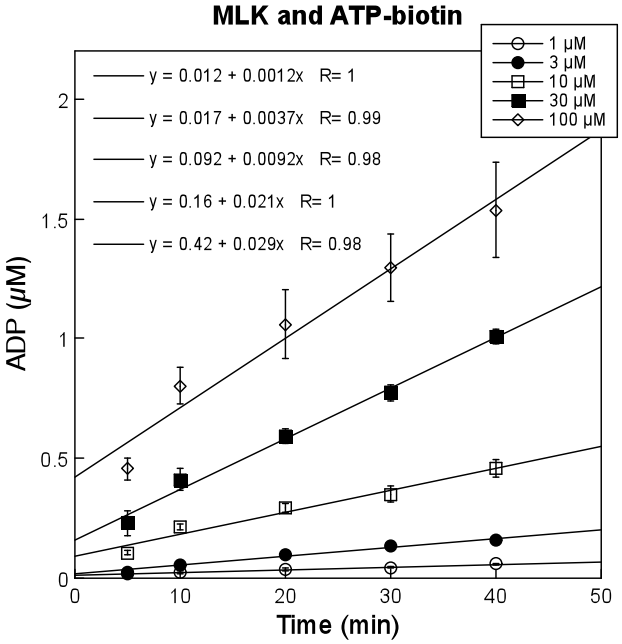
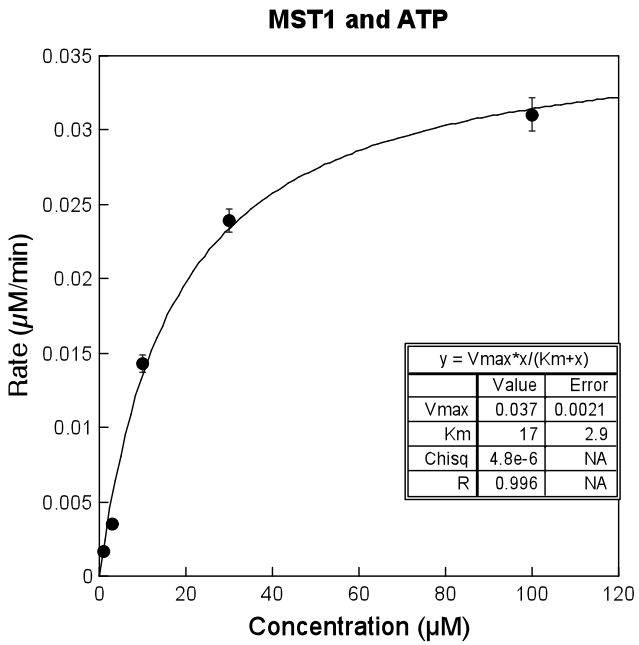
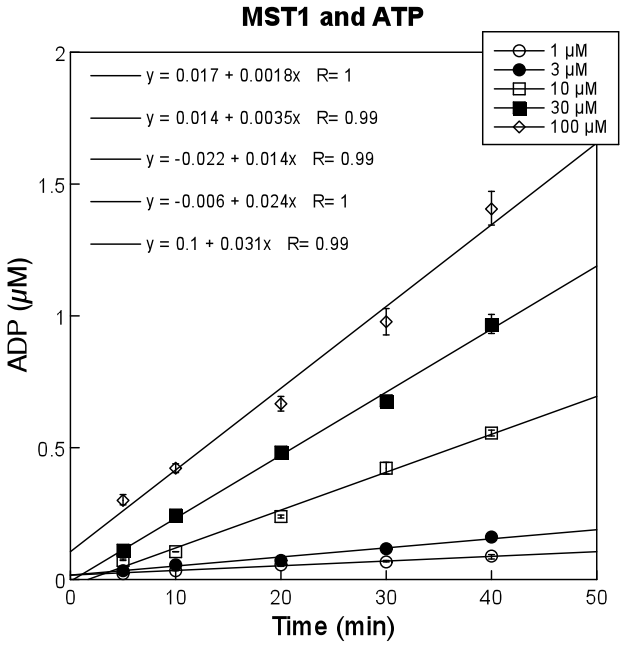
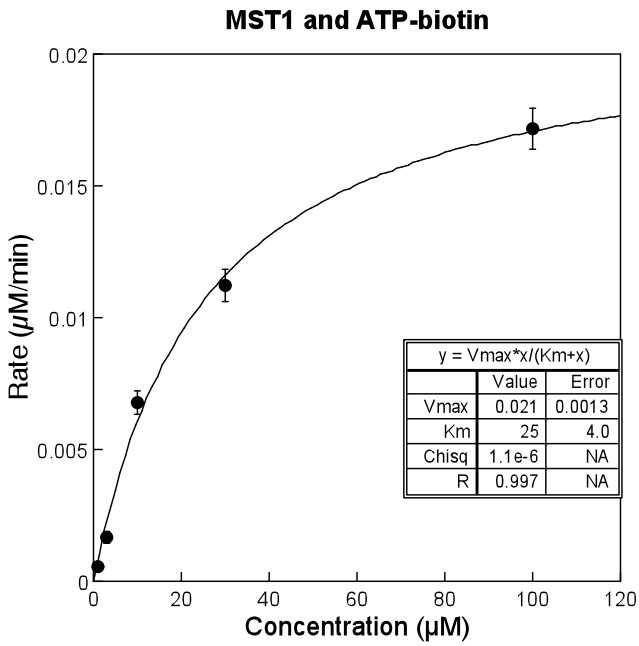



**Figure S7 (R).** Reactions with MST1



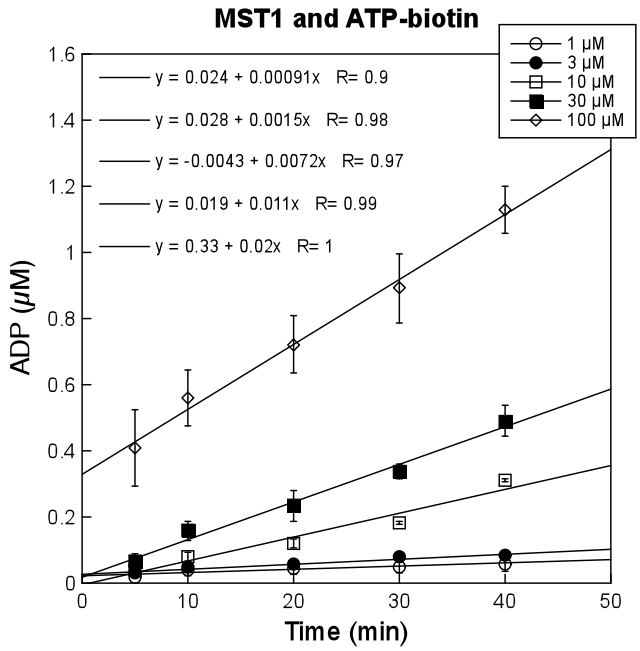
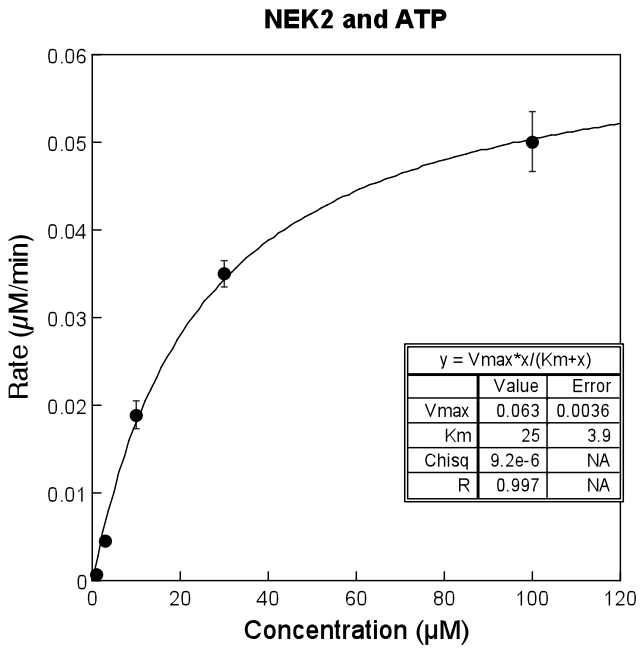
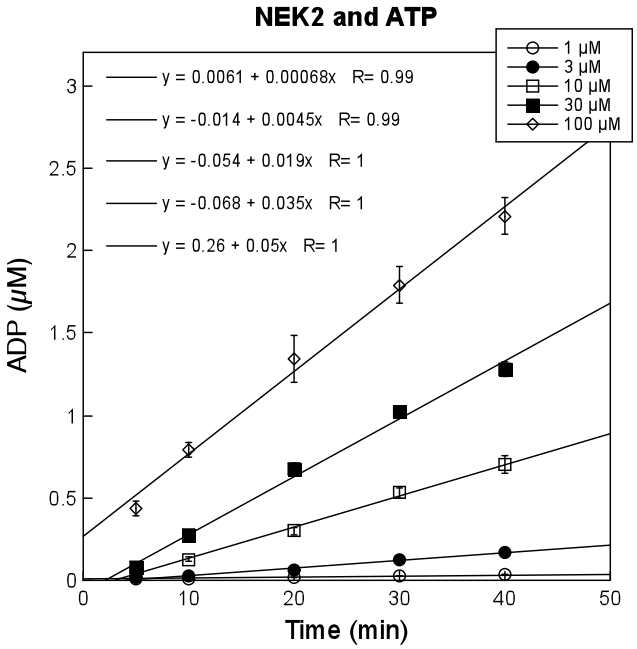
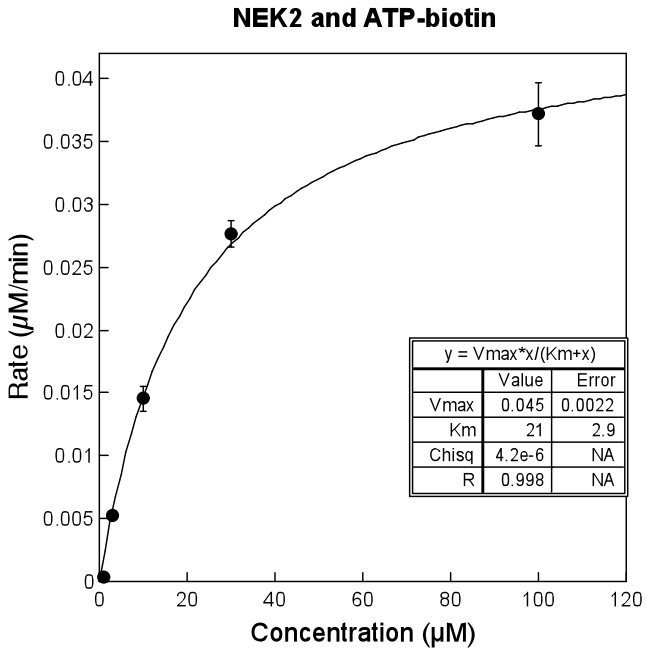



**Figure S7 (S).** Reactions with NEK2



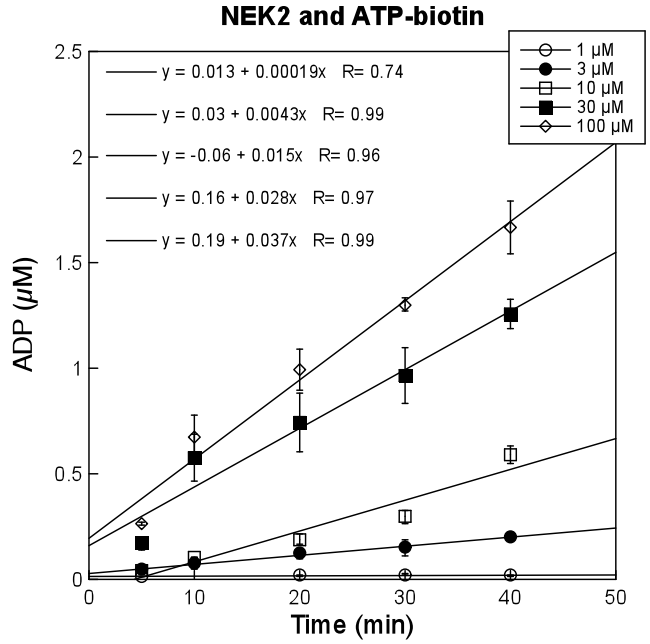
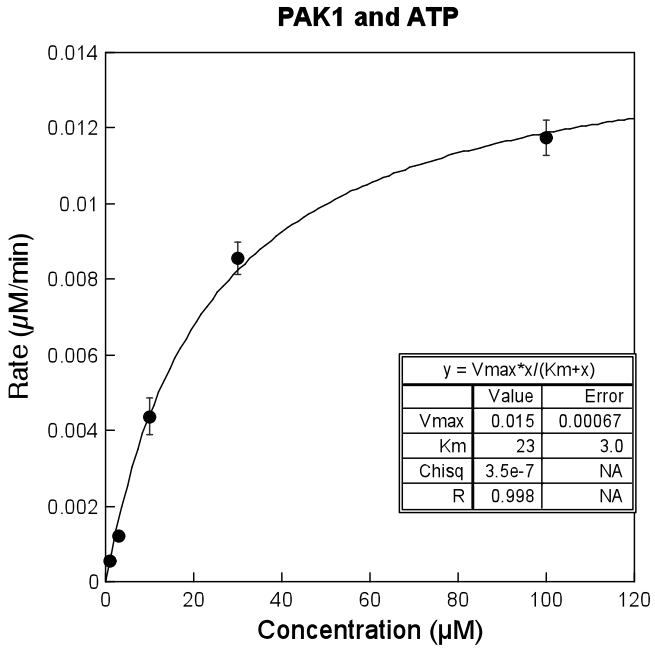
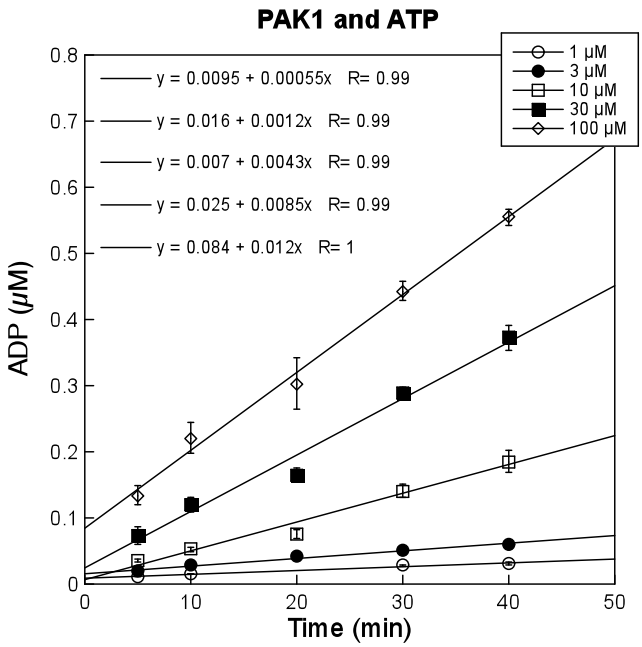
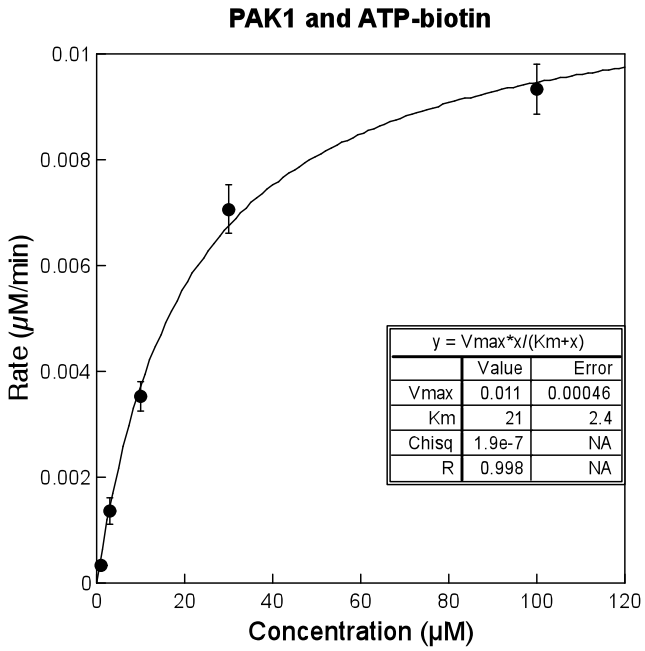



**Figure S7 (T).** Reactions with PAK1



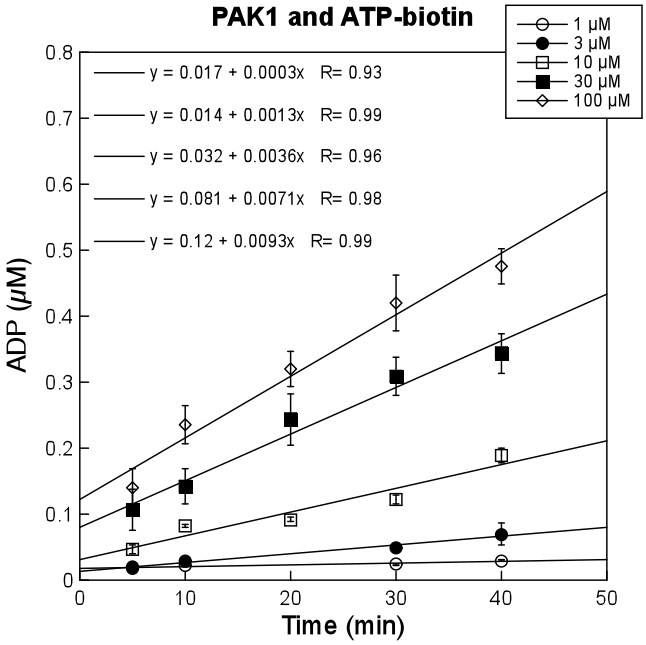
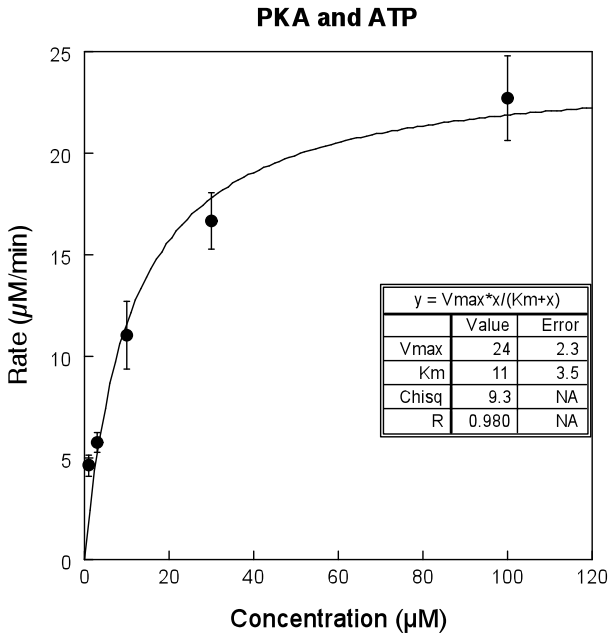
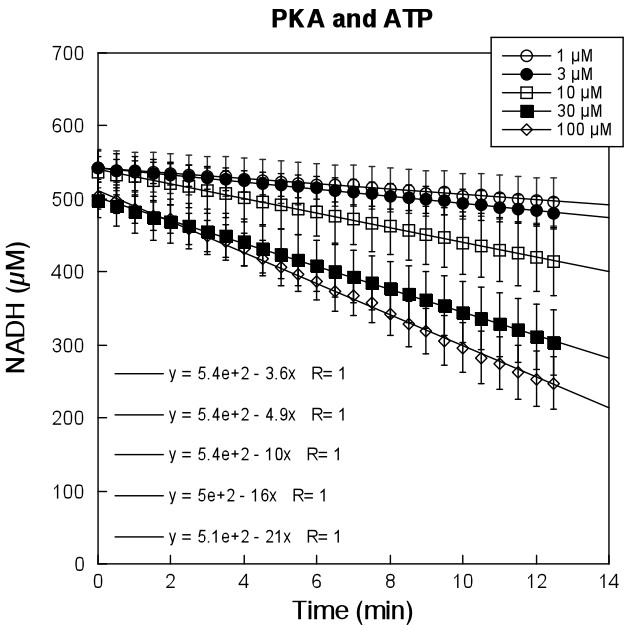
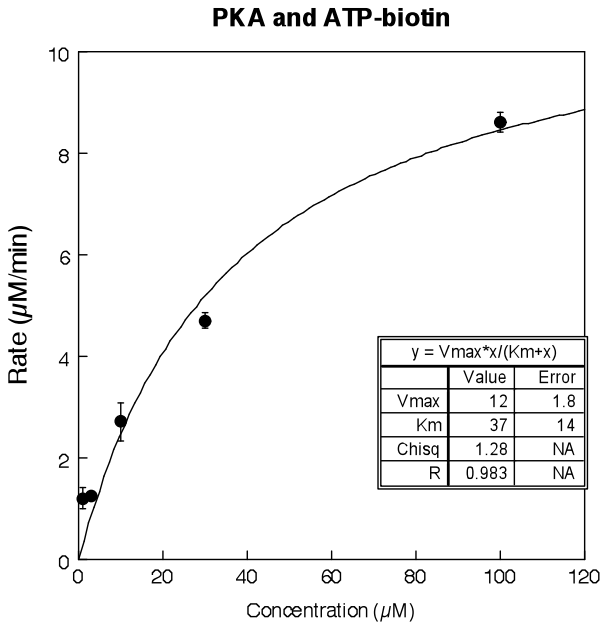



**Figure S7 (U).** Reactions with PKA



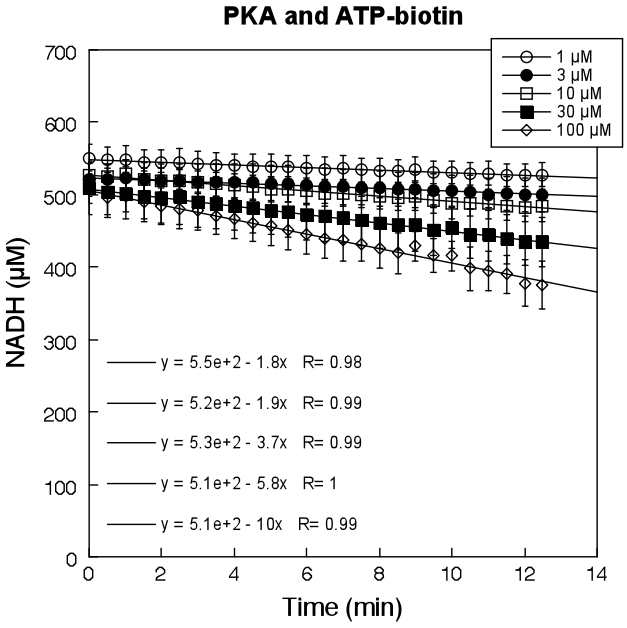
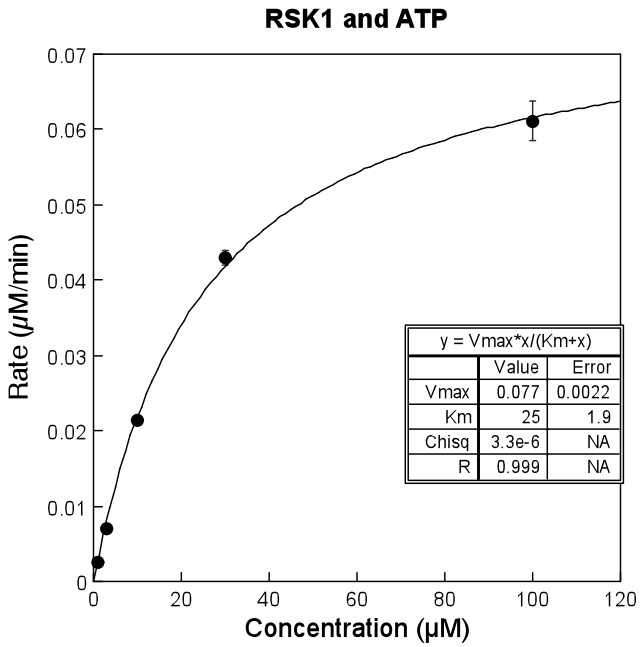
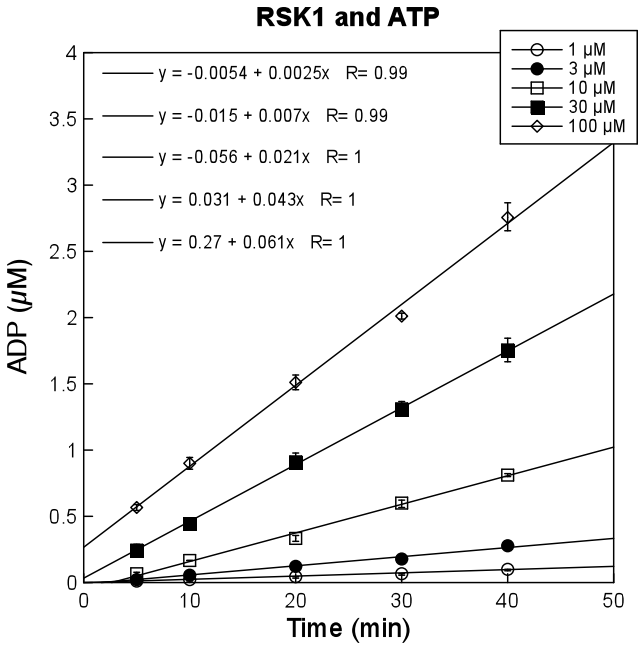
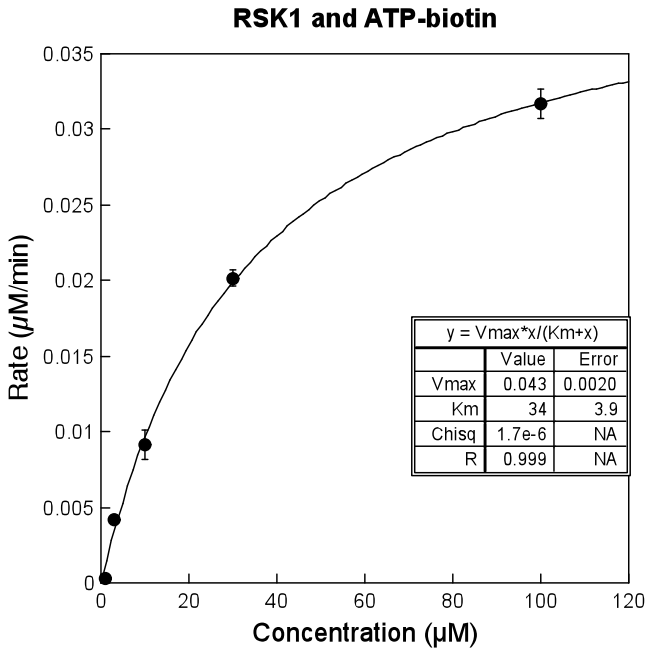



**Figure S7 (V).** Reactions with RSK1



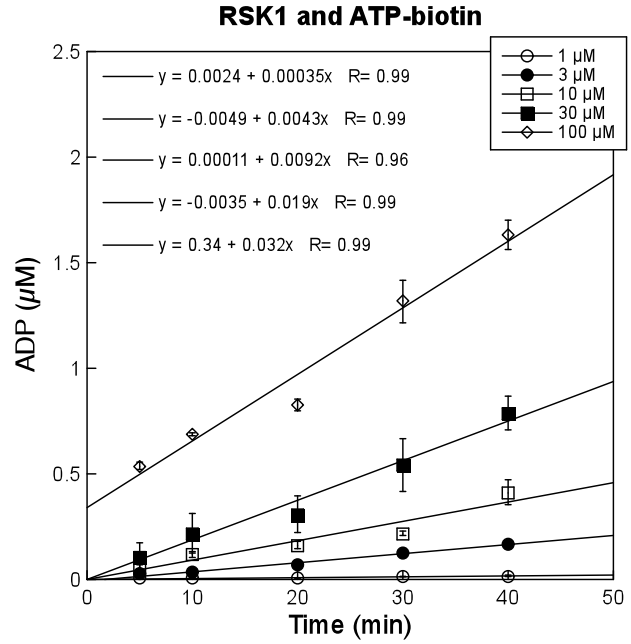
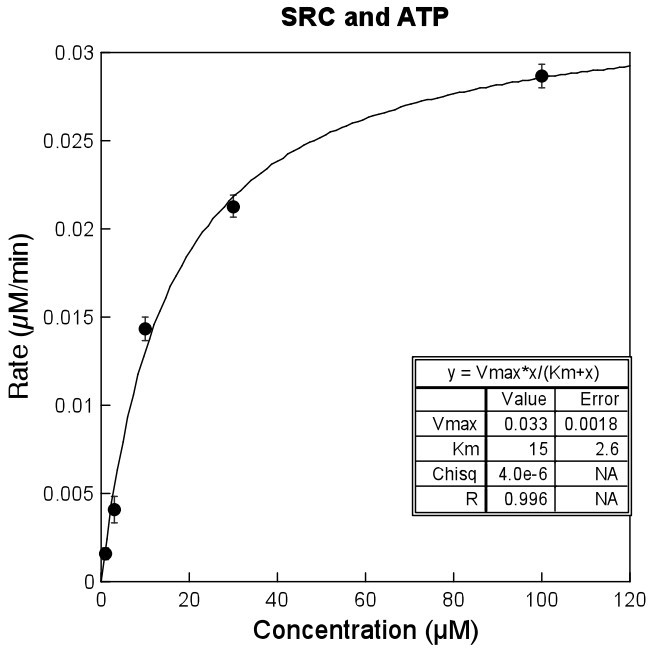
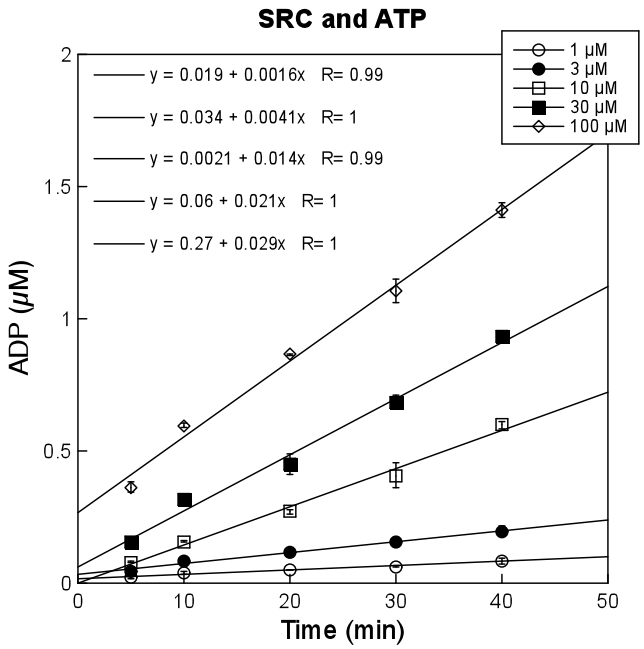
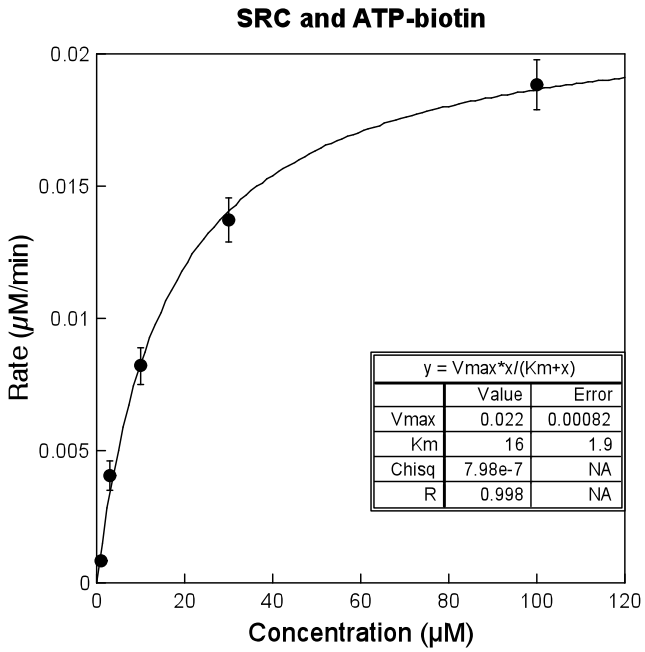



**Figure S7 (W).** Reactions with SRC



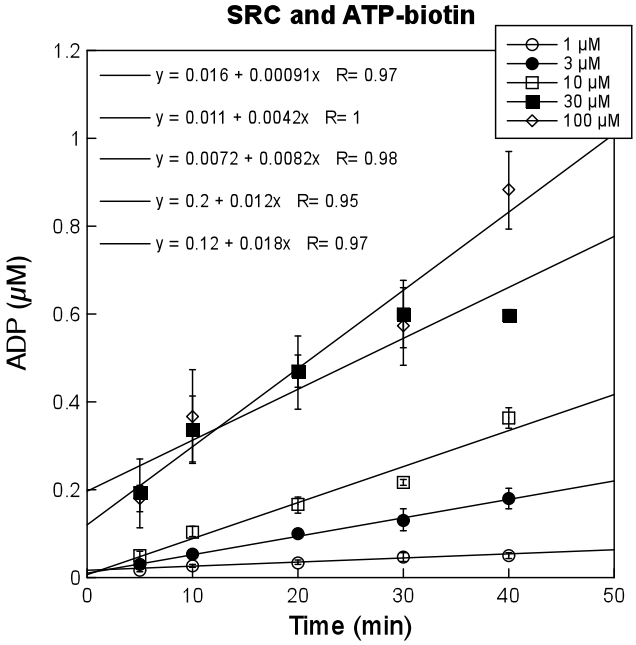
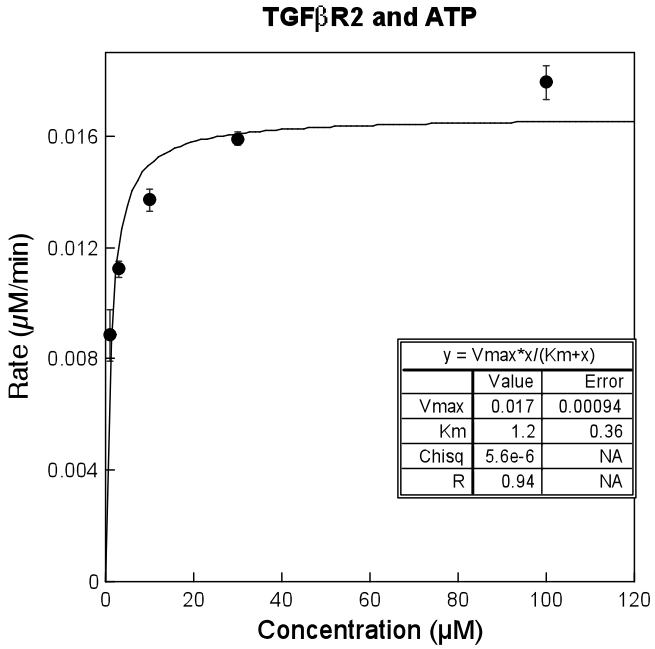
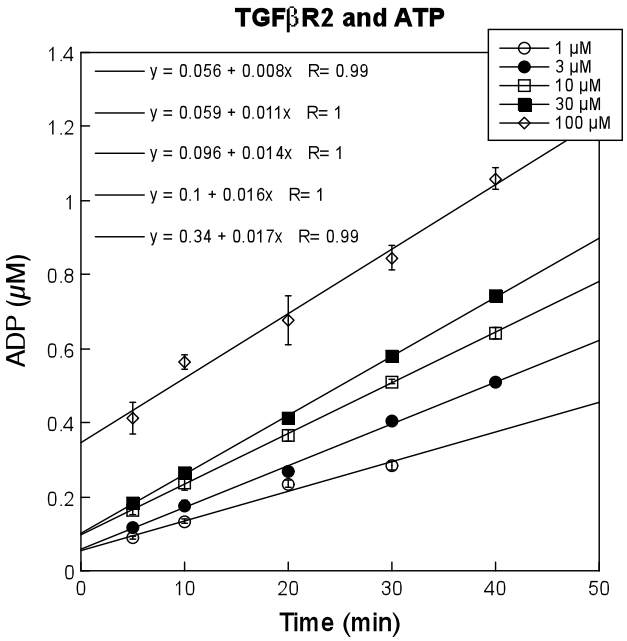
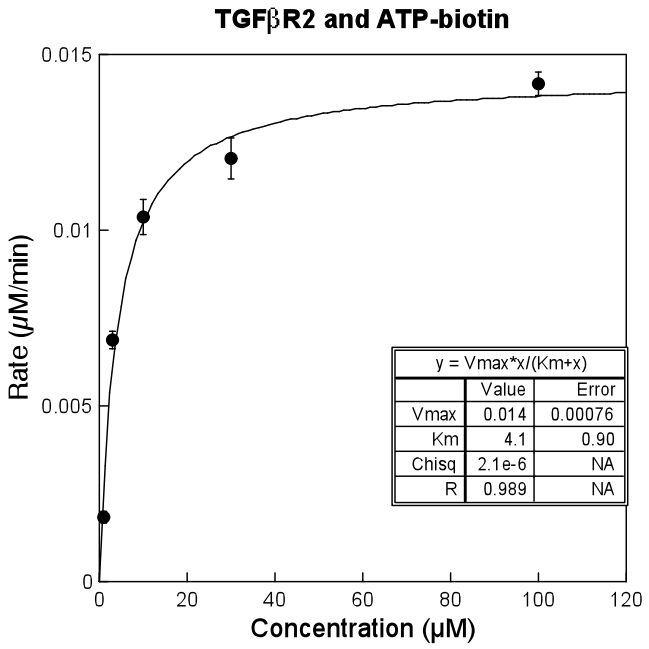



**Figure S7 (X).** Reactions with TGFβR2



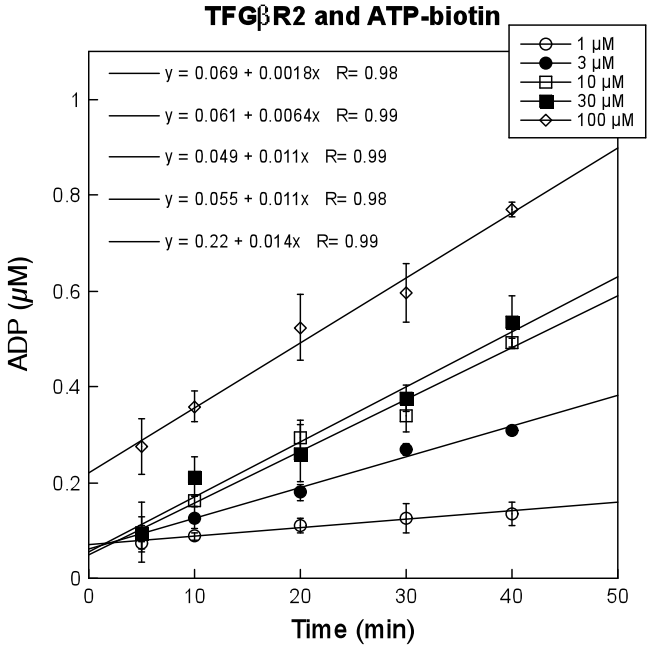



**Figure S7.** Michaelis-Menten (right, rate versus cosubstrate concentration) and rate plots (left, ADP production over time) at each cosubstrate concentration (1, 3, 10, 30, and 100 μM) are shown for each kinase using ATP (top plots) or ATP-biotin (bottom plots) as the cosubstrate. The calculated $V_{max}^{app}$ and $K_M^{app}$, along with the linear equations for the rate at each cosubstrate concentration, are shown in each plot. All plots were generated in KaleidaGraph (Synergy Software). (A) AKT1 and AKT (PKB) peptide substrate (CKRPRAASFAE); (B) ASK1 and MBP; (C) Aurora A and MBP- for this plot only, the rate plot includes all points, showing reaction saturation (dashed lines with open icons), in addition to the initial rate linear equation calculated from the two earliest time points in the initial phase of the reaction (closed icons); (D) CAMK4 and Autocamtide (KKALRRQETVDAL-amide); (E) CDK1 and Histone H1 protein; (F) CHK1 and CHKtide (KKKVSRSGLYRSPSMPENLNRPR); (G) CK1 and dephosphorylated Casein protein; (H) CK2 and CK2 peptide (RRREEETEEE). Only three substrate concentrations were used with ATP-biotin due to technical issues; (I) DAPK1 and MBP; (J) ERK1 and MBP; (K) FLT1 and IGF1Rtide (KKKSPGEYVNIEFG); (L) GRK5 and Casein protein; (M) GSK3β and GSK3 substrate (YRRAAVPPSPSLSRHSSPHQ(pS)EDEEE). The rate plot data are most consistent with product inhibition, which prevented measurement of kinetics with ATP-biotin; (N) HIPK1 and MBP; (O) JAK3 and poly($Glu_4$,$Tyr_1$) peptide substrate; (P) MARK1 and CHKtide (KKKVSRSGLYRSPSMPENLNRPR); (Q) MLK1 and MBP; (R) MST1 and Axltide (KKSRGDYMTMQIG); (S) NEK2 and MBP; (T) PAK1 and PAKtide (RRRLSFAEPG); (U) PKA and kemptide (LRRASLG); (V) RSK1 and S6K substrate (KRRRLASLR); (W) SRC and SRC substrate (KVEKIGEGTYGVVYK-amide); (X) TGFβR2 and MBP. The ADP-Glo™ assay was used for these kinetics measurements for all kinases, except CK2 and PKA where the NADH-dependent enzyme coupled assay was used.

## VI. Docking Analysis Data and Images

**Table S3**: Grid box docking parameters for Autodock.

| | AKT1 | G3Kβ | NEK2 | Src |
|---|---|---|---|---|
| PDB file name | 4EKK | 1PYX | 2W5B | 2SRC |
| Grid Point Spacing (Angstroms) | 0.375 | 0.375 | 0.375 | 0.375 |
| Grid box size | 90 x 90 x 60 | 78 x 56 x 64 | 66 x 82 x 76 | 72 x 74 x 54 |
| Coordinates of Central Grid Point of Maps | 13.470<br>2.170<br>24.916 | 37.075<br>7.019<br>38.174 | 25.023<br>13.966<br>-16.198 | 18.959<br>24.462<br>62.440 |

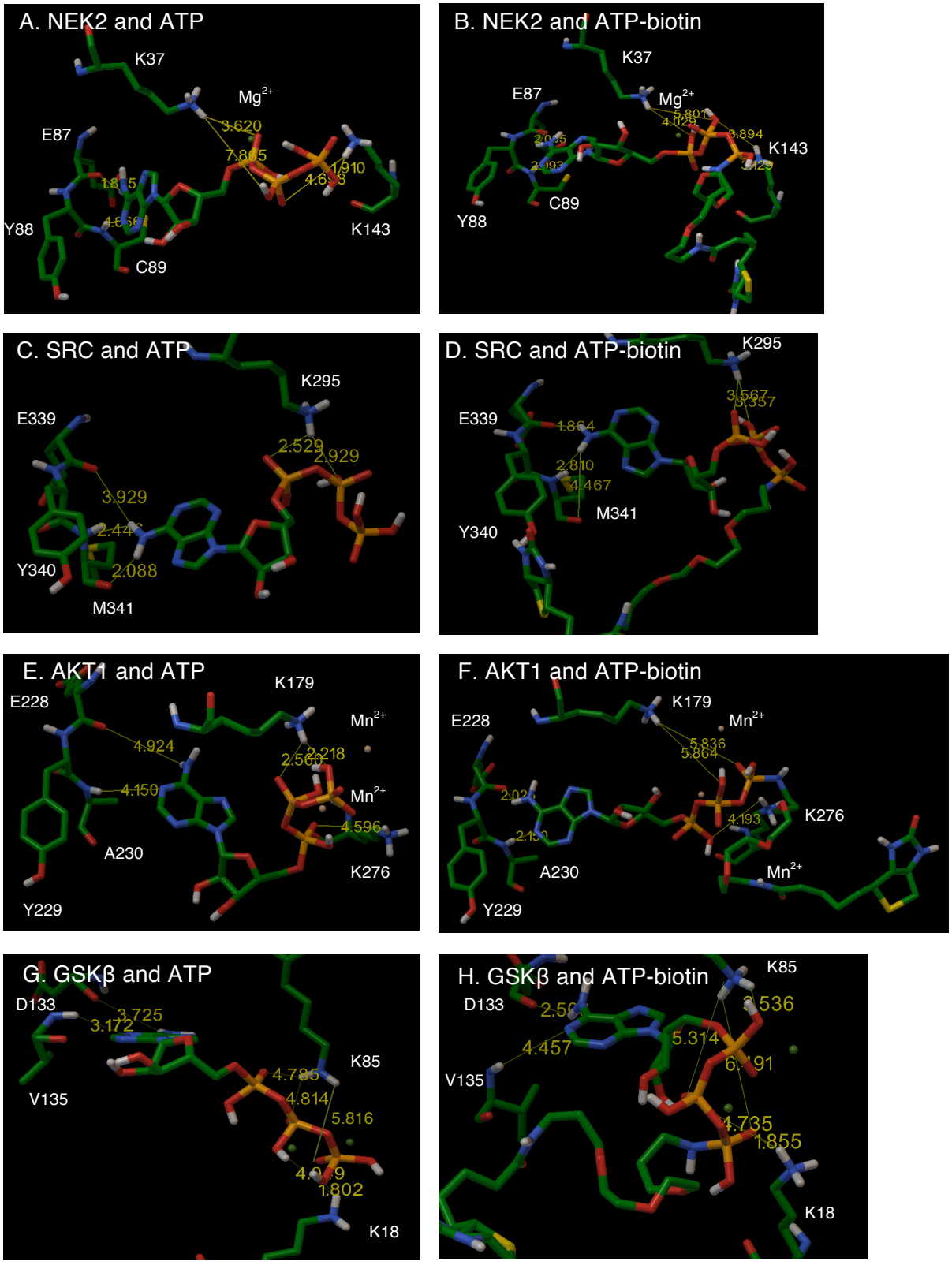



**Figure S8.** Docking images with distance measurements shown between the triphosphates of ATP (A, C, E, and G) and ATP-biotin (B, D, F, and H) and active site residues of NEK1 (A and B), Src (C and D), AKT1 (E and F), and GSKβ (G and H). Docking was performed with Autodock. Atom colors are C = green, O = red, N= blue, P = yellow, H = grey with distances in Angstroms.

## VII. References

[1] C. Senevirathne, M.K. Pflum, Biotinylated phosphoproteins from kinase-catalyzed biotinylation are stable to phosphatases: implications for phosphoproteomics, Chembiochem : a European journal of chemical biology, 14 (2013) 381-387.
[2] D.P. Arora, E.M. Boon, Unexpected biotinylation using ATP-gamma-Biotin-LC-PEO-amine as a kinase substrate, Biochem Biophys Res Commun, 432 (2013) 287-290.
[3] C. Senevirathne, K.D. Green, M.K.H. Pflum, Kinase-Catalyzed Biotinylation of Peptides, Proteins, and Lysates, Current Protocols in Chemical Biology, John Wiley & Sons, Inc.2012.
[4] H. Zegzouti, M. Zdanovskaia, K. Hsiao, S.A. Goueli, ADP-Glo: A Bioluminescent and homogeneous ADP monitoring assay for kinases, Assay and drug development technologies, 7 (2009) 560-572.