



Figure S3. Mass spectrometric analysis reveals quantitative phosphorylation of the Src-YEEI and Hck-YEEI activation loop tyrosines following preincubation with ATP. Src-YEEI and Hck-YEEI were preincubated with ATP for 3 h followed by pepsin digestion and mass spectral analysis as described under Materials and Methods. A) ESI-MS/MS spectra of Src-YEEI peptic peptide IEDNEpY⁴¹⁶TARQGAKF ($[M+H]^+ = 1721.75$ Da), derived from the activation loop, indicates that Tyr416 is phosphorylated (numbering as per crystal structure of c-Src; PDB ID: 2SRC). The mass difference between fragment ions y_9 and y_7 (red) matches that of phosphotyrosine plus threonine for a total mass of 344 Da. B) ESI-MS/MS spectra of the Hck-YEEI peptic peptide ARVIEDNEpY⁴¹⁶TARQGAKF ($[M+H]^+ = 2048.95$ Da), derived from the activation loop, indicates that Tyr416 is phosphorylated. The mass difference between fragment ions y_8 and y_9 (red) corresponds to phosphotyrosine. Fragment b-series ions are also present (blue). We were unable to detect the corresponding unphosphorylated activation loop peptides in either spectrum, suggesting that preincubation with ATP under these conditions leads to stoichiometric phosphorylation of the activation loop.