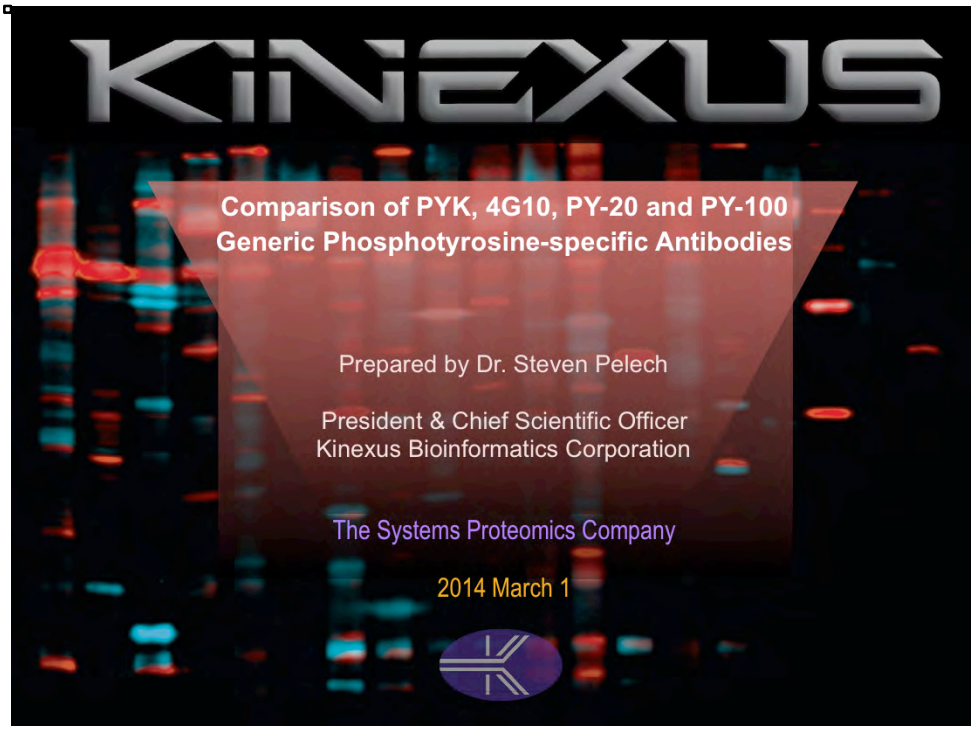
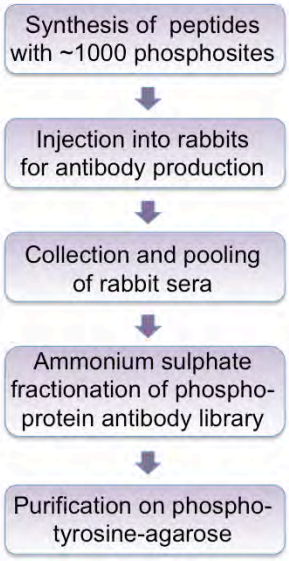


This presentation summarizes the analyses of the PYK rabbit polyclonal antibody produced by Kinexus Bioinformatics Corporation (Product ID: AB-PG001) and how its compares with the three most popular mouse monoclonal generic phosphotyrosine-specific antibodies. PYK is available in a pack size of 25 µg/vial at a concentration of 1 mg/ml for $US 89. As shall be evident, PYK is superior to these other commonly used antibodies in terms of its wider range of phosphotyrosine site detection and greater potency with less non-specific cross-reactivity.

Figure 1. Schema for preparation of generic phosphotyrosine-specific (PYK) antibodies.



The PYK antibody was originally produced from the injection of over 50 rabbits with over 125 long peptides (~25 amino acids) with multiple phosphosites. After 4 months of repeated immunizations, the rabbits were sacrificed and their sera were pooled and subjected to ammonium sulphate precipitation. The immunoglobulin fraction was dissolved in phosphate-buffered saline and applied to columns with phosphotyrosine-agarose for recovery of the PYK antibody.

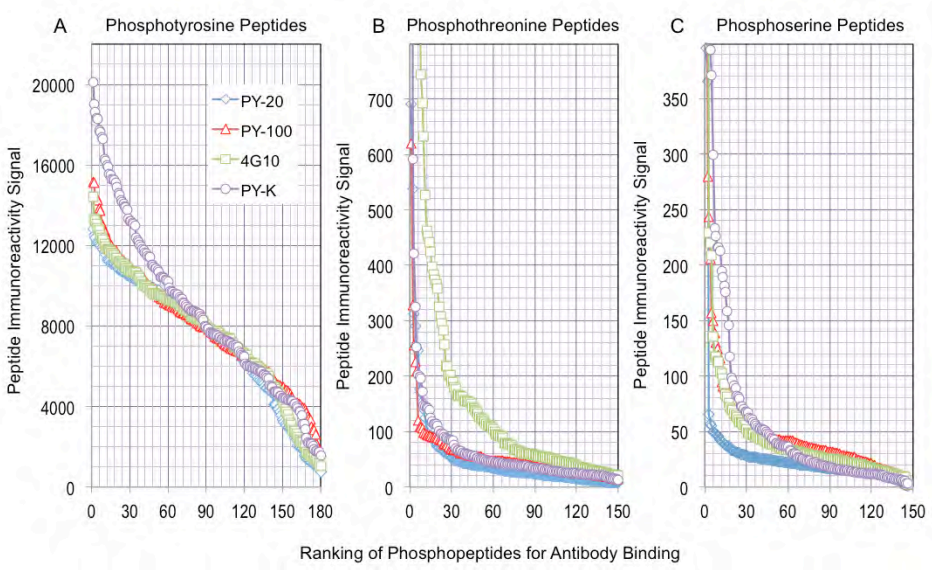
Table 1. Summary of immunoreactivities of generic phosphotyrosine-specific antibodies with phosphopeptide microarrays.

Phosphopeptide Type	Phosphotyrosine				Phosphotyrosine			
Antibody Name	PY-20	PY-100	4G10	PY-K	PY-20	PY-100	4G10	PY-K
Antibody Conc. (µg/ml)	5	1	5	2	5	1	5	2
Microarray	JPT Phosphatase Peptide Microarray				Kinexus Kinex™ KPSM Microarray			
Number of Peptides Tested	6099				184			
Max Signal Recorded	2174	1040	2974	10705	17299	13970	11339	18914
Median	385	208	586	2684	10614	7243	6177	7395
S.D. (from Median)	±440	±207	±517	±1947	±4396	±2760	±2556	±4051
Median / Max Signal	0.18	0.2	0.2	0.25	0.61	0.52	0.54	0.39
Median / Median with P-Tyr	1	1	1	1	1	1	1	1

Phosphopeptide Type	Phosphothreonine				Phosphoserine			
Antibody Name	PY-20	PY-100	4G10	PY-K	PY-20	PY-100	4G10	PY-K
Antibody Conc. (µg/ml)	5	1	5	2	5	1	5	2
Microarray	Kinexus Kinex™ KPSM Microarray							
Number of Peptides Tested	166				146			
Max Signal Recorded	933	572	1393	1532	534	672	822	865
Median	34	38	47	35	26	33	23	23
S.D. (from Median)	±108	±56	±217	±132	±59	±64	±90	±102
Median / Max Signal	0.037	0.066	0.034	0.023	0.049	0.05	0.028	0.026
Median / Median with P-Tyr	0.0032	0.0052	0.0076	0.0048	0.0025	0.0046	0.0038	0.0031

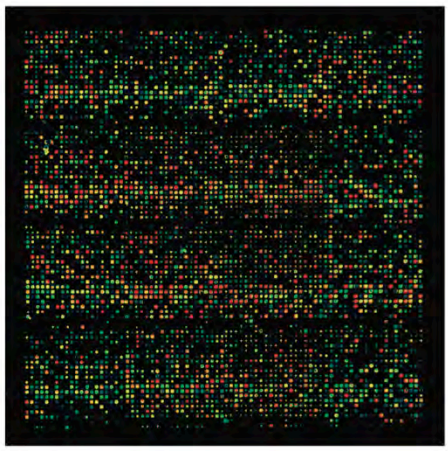
Reactivities of generic phosphotyrosine antibodies for 6099 tyrosine-phosphorylated peptides on the JPT Phosphatase Peptide Microarray (Jerini Peptide Technologies GmbH) and ~500 phosphopeptides on the Kinex™ (KPSM) Phosphopeptide Microarray. This study demonstrates that PYK provides for stronger detection of diverse tyrosine phosphorylated peptides then the monoclonal antibodies when the antibody concentration is considered. The data also reveal that all of these generic phosphotyrosine antibodies display little reactivity with phosphothreonine and phosphoserine-containing peptides.

Figure 2. Phosphoamino acid specificity of generic phosphotyrosine-specific antibodies.



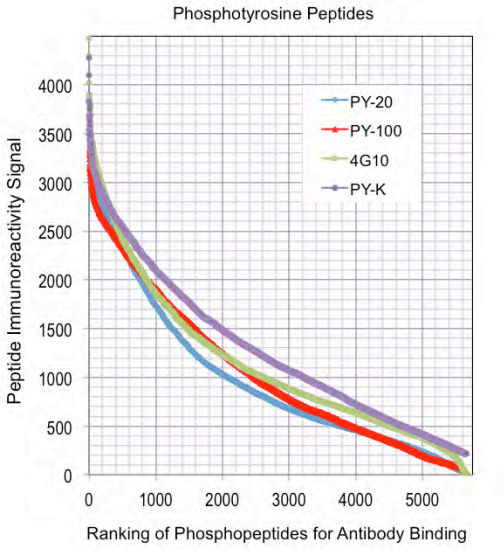
Reactivities of generic phosphotyrosine antibodies for 500 phosphopeptides on the Kinex™ (KPSM) Phosphopeptide Microarray. All of the data for immunoreactive peptide signal intensity were normalized to the median value for each tested for each type of phosphorylated peptide [Phosphotyrosine (Panel A); Phosphothreonine (Panel B); and Phosphoserine (Panel C) on the microarray. In each data set, the peptide signals for each antibody and type of phosphorylated peptide were sorted in order of the strongest signal first. The final concentrations of antibodies used for probing each grid of phosphopeptides on the microarray were ~2 µg/ml PYK, ~5 µg/ml 4G10, ~5 µg/ml PY-20, and ~1 µg/ml PY-100.

Figure 3. Scanned image of the PYK phosphotyrosine-specific antibody probed tyrosine.



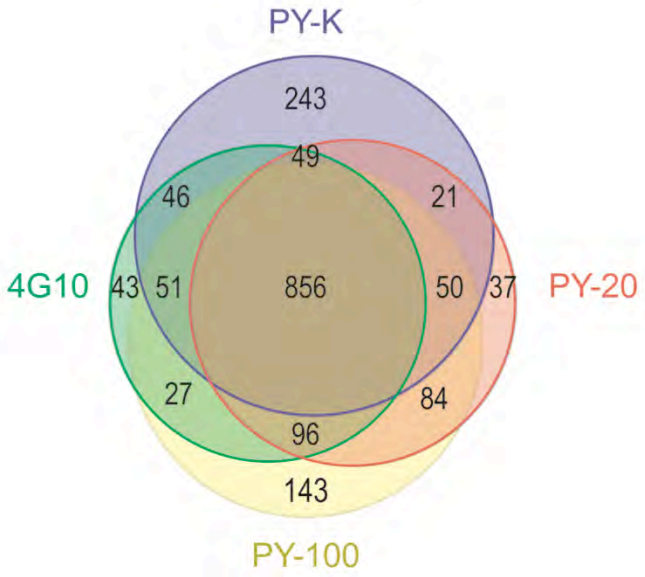
One of three representative fields of the JPT Phosphatase Peptide Microarray (PhosphoSites-Tyrosine) (Jerini Peptide Technologies GmbH) that features approximately 6100 distinct human phosphotyrosine-containing peptides after probing with PYK (~2 µg/ml) for 2 h. The binding of the primary antibodies was detected by Alexa 546-labelled donkey anti-rabbit IgG secondary antibody. Colors of the spots indicate their relative signal intensity, with red as the strongest and blue the weakest.

Figure 4. Phosphotyrosine peptide reactivity of generic phosphotyrosine-specific antibodies.



Reactivities of generic phosphotyrosine antibodies for 6099 phosphotyrosine-containing peptides on the JPT Phosphatase Peptide Microarray. All of the data for immunoreactive peptide signal intensity were normalized to the median value for each tested antibody. In each data set, the peptide signals for each antibody and type of phosphorylated peptide were sorted in order of the strongest signal first. The final concentrations of antibodies used for probing each grid of phosphopeptides on the microarray were ~2 µg/ml PYK, ~5 µg/ml 4G10, ~5 µg/ml PY-20, and ~1 µg/ml PY-100.

Figure 5. VENN diagram tyrosine-phosphorylated peptides detected by generic phosphotyrosine-specific antibodies.



Reactivities of generic phosphotyrosine antibodies for 1746 most immunoreactive phosphotyrosine-containing peptides on the JPT Phosphatase Peptide Microarray. The selected phosphopeptides showed immunoreactivities that were at least one standard deviation higher than the median value determined for each tested antibody are considered. Phosphopeptides that are strongly recognized by two or more antibodies are shown in overlapping circles. Of the 1746 strongly immunoreactive phosphopeptides , PYK detected 1316 (75.4%), 4G10 detected 1,168 (66.9%), PY-20 detected 1,193 (68.3%) and PY-100 detected 1,307 (74.9%).

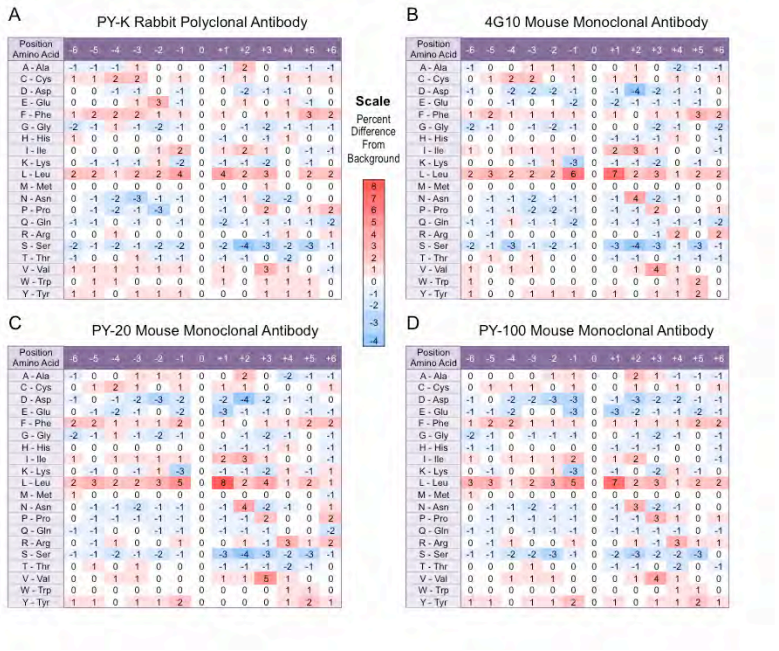
Table 2. Frequency of amino acids surrounding human phosphotyrosine sites.

Position Amino Acid	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6
A - Ala	6.4	6.7	6.5	6.1	7.0	5.6	0	5.7	6.4	5.9	5.9	5.8	6.6
C - Cys	1.5	1.4	1.6	1.5	1.3	1.2	0	1.7	2.1	1.6	1.7	1.9	1.5
D - Asp	5.9	5.6	6.4	6.6	7.0	7.7	0	6.3	6.1	4.7	5.1	5.7	5.2
E - Glu	7.5	8.0	7.8	8.5	7.8	7.2	0	8.5	7.5	5.7	6.9	7.5	7.0
F - Phe	3.1	3.0	3.2	2.8	2.6	3.0	0	3.5	3.1	3.3	3.2	3.2	3.1
G - Gly	6.8	6.5	7.3	7.0	7.7	6.5	0	6.9	6.1	5.9	6.5	7.1	6.9
H - His	3.0	2.2	2.3	2.3	2.2	2.8	0	2.5	2.1	2.3	2.5	2.4	2.3
I - Ile	4.3	4.1	4.0	4.0	3.6	5.7	0	4.7	4.3	5.2	4.3	4.1	4.4
K - Lys	7.3	7.5	6.9	6.9	7.1	6.7	0	5.9	6.9	6.3	7.7	7.6	7.3
L - Leu	8.3	7.9	8.3	7.6	7.9	9.0	0	8.2	7.7	10.0	8.6	7.6	8.0
M - Met	2.1	2.2	2.2	2.4	2.1	2.0	0	2.2	2.2	2.8	2.1	1.9	2.2
N - Asn	3.9	4.2	4.1	4.5	4.8	4.5	0	3.6	4.6	3.4	3.9	3.9	3.9
P - Pro	5.4	5.7	5.5	5.4	6.5	5.7	0	3.9	5.9	7.2	5.9	6.0	6.0
Q - Gln	4.5	4.7	4.1	4.9	4.3	4.2	0	5.3	4.4	4.3	4.4	4.6	4.2
R - Arg	7.0	6.6	6.5	6.3	5.7	5.5	0	5.9	6.3	6.3	6.9	7.0	6.8
S - Ser	7.8	8.0	8.4	8.2	8.4	6.8	0	9.0	8.3	7.8	8.0	8.0	7.8
T - Thr	5.1	5.4	4.9	5.1	4.8	5.1	0	5.2	5.2	5.1	5.0	4.9	5.0
V - Val	5.3	5.1	5.3	5.5	5.4	6.4	0	6.0	5.7	6.8	6.2	5.0	5.6
W - Trp	0.8	0.9	0.8	0.8	0.6	0.8	0	0.8	0.8	0.9	0.9	0.8	0.8
Y - Tyr	3.5	3.7	3.6	3.6	3.3	3.6	100	3.9	3.5	3.7	3.5	3.7	3.6

%
10
9
8
7
6
5
4
3
2
1
0

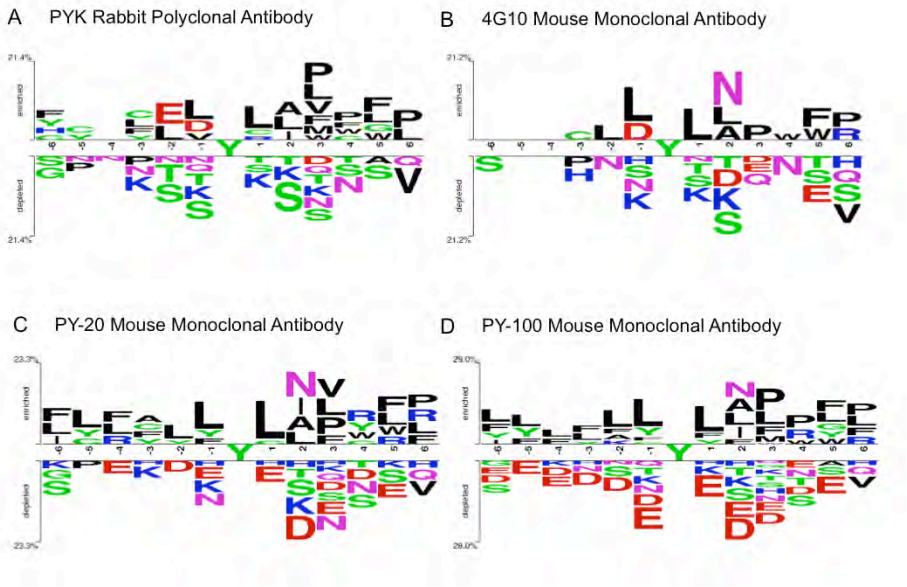
The percent frequency of each of the 20 common amino acids bordering the phosphotyrosine residues in 34,004 experimentally confirmed human phosphosites was determined using data collected from PhosphoNET (www.phosphonet.ca). It is evident that the amino acid residues for Leucine, Serine, Glutamic acid, Lysine, Glycine, Arginine, Alanine and Aspartic acid most commonly flank phosphotyrosine sites in human proteins. It is important that generic phosphotyrosine-specific antibodies will immunoreact favourably with tyrosine phosphosites that are bordered by these amino acid residues.

Table 3. Flanking amino acid specificities of generic phosphotyrosine-specific antibodies.



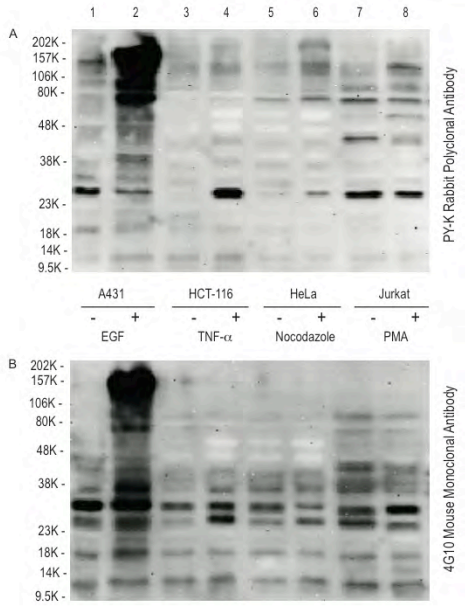
The recognition specificities for amino acid residues flanking phosphotyrosines for binding generic phosphotyrosine antibodies were examined against 6070 phosphotyrosine-containing peptides on the JPT Phosphatase Peptide Microarray. The percent frequency of each amino acid at each residue surrounding the phosphotyrosine residue on all 6070 phosphopeptides was determined as the background, which was subtracted from the observed values for those peptides that exhibited immunoreactivities that were one standard deviation above the median value for each phosphotyrosine antibody preparation that was tested. Red- to pink-coloured cells correspond to amino acids that occur in higher frequency at each amino acid position than expected by random and are favoured, whereas blue-coloured cells identify amino acid residues that are unfavourable. It is evident that 4G10, PY-20 and PY-100, but not PYK, exhibit a bias against detection of tyrosine phosphorylation sites that are flanked by acidic amino acids. As shown in Table 2, many physiological phosphotyrosine sites in proteins feature surrounding Aspartic acid and Glutamic acid residues.

Figure 6. Logo diagram for amino acid specificity preferences of generic phosphotyrosine-specific antibodies.



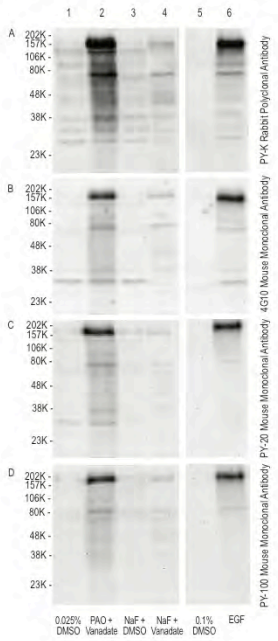
The amino acid residue recognition preferences of the flanking sequences for generic phosphotyrosine-specific antibodies tested on 6070 distinct tyrosine-phosphorylated peptides printed on the JPT Phosphatase Peptide Microarray. The phosphopeptide microarray was probed with PYK (A), 4G10 (B), PY20 (C) and P-Tyr-100 (D) antibodies and the top peptides were selected using the cut-off criteria as described in the text for classifying the positive and negative data sets. The visualization graphs were generated with the Two Sample Logos algorithm [Vacic V., Lakoucheva L.M., and Radivojac P. "Two Sample Logo: A Graphical Representation of the Differences between Two Sets of Sequence Alignments." Bioinformatics, 22(12): 1536-1537. (2006)]. It is evident that the monoclonal antibodies PY-20 and PY-100 displayed very similar specificities, and acidic residues were negative determinants for all three monoclonal antibodies tested.

Figure 7. Immunoreactivities of PYK and 4G10 phosphotyrosine-specific antibodies with human cell lines.



Comparison of the immunoreactivity profiles of PY-K and 4G10 antibodies towards protein-tyrosine phosphorylation induced by various perturbations in a panel of human cancer cell lines. Panel A: PYK (~2 μg/ml); Panel B: 4G10 (~5 μg/ml). Lanes 1 and 2: 16 h serum-starved A431 cervical carcinoma cells treated with vehicle (0.1% DMSO) and 100 ng/ml epidermal growth factor (EGF) for 5 min, respectively; Lanes 3 and 4: HCT-116 colon carcinoma cells treated with vehicle (H_2O) and 2 ng/ml tumour necrosis factor-alpha (TNFα) for 20 min, respectively; Lanes 5 and 6: HeLa cervical carcinoma cells treated with vehicle (0.1% DMSO) and 50 ng/ml nocodazole for 16 h, respectively; Lanes 7 and 8: Jurkat T-cells treated with vehicle (0.1% DMSO) and 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 10 min, respectively. The amount of lysate protein loaded in each lane was 25 μg.

Figure 8. Immunoreactivities of generic phosphotyrosine-specific antibodies with A431 cell lysates.



Selectivity of generic phosphotyrosine-specific antibodies towards protein-tyrosine phosphorylation induced by treatments of various phosphatase inhibitors and EGF in A431 human cervical carcinoma cells. Lane 1: 10% FCS incubated A431 cells treated with 0.025% DMSO for 30 min; Lane 2: 10% FCS incubated A431 cells treated with the combination of the protein-tyrosine phosphatase inhibitors, 25 μM phenylarsine oxide (PAO) and 50 μM Na_3VO_4 for 30 min; Lane 3: 10% FCS incubated A431 cells treated with protein phosphatase inhibitor, 30 mM NaF for 30 min; Lane 4: 10% FCS incubated A431 cells treated with the combination of 30 mM NaF and 50 μM Na_3VO_4 for 30 min; Lane 5: 16 h serum-starved A431 cells treated with 0.1% DMSO for 10 min; Lane 6: 16 h serum-starved A431 cells treated with 100 ng/ml EGF in 0.1% DMSO for 5 min. Panel A, B, C and D were probed with PYK (~2 μg/ml), 4G10 (~5 μg/ml), PY-20 (~5 μg/ml), and PY-100 (1:200; estimated to be ~1 μg/ml), respectively. The amount of lysate protein loaded in each lane was 25 μg.