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ProductInformation

Phospho-Src [pTyr^{418}] ELISA, Human

Product Number **CS0460**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description
Phospho-Src [pTyr^{418}] ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative determination of Src protein that is phosphorylated at tyrosine residue 418 in cell lysates. A monoclonal antibody specific for Src (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. Standard dilutions containing Src [pTyr^{418}], control specimens, and unknown samples are pipetted into these wells. During the first incubation, the Src antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and an antibody specific for Scr phosphorylated on tyrosine 418 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized Src protein. After removal of excess detection antibody, horseradish peroxidase-labeled (HRP) anti- rabbit IgG is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess anti-rabbit IgG-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of phospho-Src [pTyr^{418}] present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of Src [pTyr^{418}].

Phospho-Src [pTyr^{418}] ELISA is designed to detect and quantify the level of Src protein phosphorylated on tyrosine 418. Although performance characterization of this ELISA kit was done primarily on human cell lines, platelets, and lymphocytes, cross-reactivity of this kit with mouse and rat cells was observed. This assay is intended for the detection of Src [pTyr^{418}] from lysates of cells. For normalizing Src content use Src ELISA, which is independent of phosphorylation state, Sigma Product. No. CS0450).

c-Src (pp60c-src), the cellular homolog of the Rous sarcoma virus protein v-Src, is the protein product of the c-*src* proto-oncogene with molecular weight of 60 kDa. c-Src localizes to the cytoplasm, the plasma membrane, focal adhesions, and cadherin-containing cell junctions. The many functions of this protein include the promotion of cytoskeletal reorganization which influences cell adhesion and migration, the enhancement of cell survival, and the regulation of cell division. c-Src is the prototypical member of a family of homologous non-receptor tyrosine kinases. c-Src, along with Fyn and Yes, are ubiquitously expressed, while the expression of Lck, Lyn, Fgr, Hck, and Blk is limited to hematopoietic cells. Src family proteins are characterized by the presence of Src homology (SH) domains, designated SH1-SH4. c-Src's SH1 domain, located near its C terminus, contains the protein tyrosine kinase domain. The SH2 domain, located near the N terminus, mediates c-Src's association with phosphotyrosine-containing proteins. The SH3 domain, located near the N terminus, mediates c-Src's association with polyproline-containing proteins. The SH4 domain, located at the N terminus, contains a signal for myristoylation that allows c-Src to localize to the plasma membrane. c-Src also contains a C terminal regulatory domain, as well as a unique region located near its N terminus, which allows it to interact with membrane-associated receptors. c-Src mediates signals arising from receptor tyrosine kinase ligand binding, G protein-coupled receptor ligand binding, and integrin engagement. These stimuli regulate c-Src's activity through modulating its phosphorylation state, protein folding, protein: protein interactions, and subcellular localization. Phosphorylation of tyrosine 418 of human c-Src (corresponding to tyrosine 416 in chicken), contained in the activation loop of the kinase domain, is required for full activation. Phosphorylation of this residue is the result of intermolecular autophosphorylation. Phosphorylation of tyrosine 529, located in the C terminal regulatory domain, inhibits c-Src's activity. Phosphorylation of this residue is catalyzed by the kinase CSK (C terminal kinase). Tyrosine 529 phosphorylation induces intramolecular folding in which the phosphorylated tyrosine interacts with Src's SH2 domain, holding the catalytic domain in an inactive state and preventing substrate binding. c-Src's activity can be restored either by tyrosine 529

dephosphorylation by RPTP-α, or through displacement by other SH2 domain-containing proteins. c-Src's inhibition through intramolecular folding involving the C terminal domain is regulated by phosphorylation of several N terminal serine residues by the kinase p34cdc, activating c-Src at the G2 to M phase of the cell cycle. Interestingly, the C terminal regulatory region is deleted in v-Src and in some c-Src mutations associated with human cancers, a feature which is associated with enhanced activation. c-Src is also regulated by the formation of an intramolecular association between its SH3 domain and a polyproline-containing region located between SH2 and kinase domains.

Substrates for c-Src are numerous and include: the focal adhesion, integrin, and cytoskeletal-associated proteins FAK, vinculin, cortactin, paxillin, tensin, ezrin, p130cas, p190RhoGAP, and p120RasGAP; the adherens junction proteins β- and γ-catenin, occludin, and connexin 43; the transcription factor, STAT3; the enzymes, PLCγ and PI3K; and the scaffold protein, Shc. c-Src is currently under investigation in cell cycle control, mitogen-dependent and -independent cell growth, anchorage-dependent and -independent cell growth, cell differentiation, B and T cell development, autocrine regulation of growth factor production, transcriptional regulation, neurobiology, angiogenesis, and cancer studies. c-Src activation, observed in some human cancers, especially those of the breast and colon, is associated with an invasive, proliferative, motile cell phenotype. Drugs that specifically target c-Src are currently under development as potential cancer therapeutic agents.

Reagents

- **Phospho-Src [pTyr^{418}] Standard, Lyophilized, 2 vials, Product No. P 1497 -** Lysates of human platelets isolated from whole blood and, autophosphorylated. Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 7319,** contains sodium azide as preservative.
- **Monoclonal-Anti-Src-Coated 96 well plate, 1ea, Product No. S 6694 -** A plate using break-apart strips coated with monoclonal antibody specific for full-length Src (regardless of phosphorylation state).
- **Anti-Phospho-Src [pTyr^{418}], 11 ml, Product No. P 0997**
  A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.
- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. R 6778-** contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 5788-** contains 3.3 mM thymol. Ready to use.
- **Wash Buffer concentrate, 25X, 100 mL, Product No. W 2639 -** *See Reagent Preparation for handling, dilution and storage instructions*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 μL and 1,000 μL.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.

Cell Extraction Buffer

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM Na_4P_2O7
2 mM Na_3VO_4
1% Triton® X-100
10% glycerol
0.1% SDS
0.5% deoxycholate

1 mM PMSF (stock is 0.3 M in DMSO)
PMSF is very unstable and must be added prior to use, even if added previously.

Protease inhibitor cocktail (Sigma Product No. P 2714)

Add 250 µl of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 °C.

Thaw on ice. Add the protease inhibitors just before use.

Procedure for Extraction of Proteins from Cells

This protocol has been successfully applied to several cell lines of human origin. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –70 °C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals.
5. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of Src [$pTyr^{418}$]. For example, $4x10^7$ Colo 201 cells grown in RPMI plus 10% FBS can be extracted in 1 mL of Cell Extraction Buffer. Under these conditions, use of 0.1-5 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer is sufficient for the detection of Src [$pTyr^{418}$].
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 °C.
7. Aliquot the clear lysate to clean microcentrifuge tubes.

Before assay: extracted cell lysate samples containing protein should be diluted with Standard Diluent Buffer at least 1:10. This dilution is necessary to reduce the matrix effect of the cell extraction buffer.

Reagent Preparation

Standard

Note: This Src [$pTyr^{418}$] standard was prepared from lysates of human platelets isolated from whole blood. Src contained in these lysates was allowed to autophosphorylate in the presence of 4 mM ATP and 1X Autophosphorylation Buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT) for 2 hours at 30 °C.

One Unit of standard is defined as the amount of Src [$pTyr^{418}$] derived from the autophosphorylation of 0.17 µg lysate-derived total protein. Subsequent lots of standard will be normalized to this lot of material to allow consistency of Src [$pTyr^{418}$] quantitation.

1. Reconstitute Standard with *Standard Diluent Buffer*. Refer to standard vial label for instructions.
2. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 100 units/mL Src [$pTyr^{418}$]. Use standard within 1 hour of reconstitution.
3. Prepare serial standard dilutions as follows

Tube #	Standard Buffer	Standard from tube #:	Final units/mL
1	Reconstitute according to label instructions		100 U/mL
2	0.25 mL	0.25 mL (1)	50 U/mL
3	0.25 mL	0.25 mL (2)	25 U/mL
4	0.25 mL	0.25 mL (3)	12.5 U/mL
5	0.25 mL	0.25 mL (4)	6.25 U/mL
6	0.25 mL	0.25 mL (5)	3.12 U/mL
7	0.25 mL	0.25 mL (6)	1.6 U/mL
8	0.25 mL	-	0 U/mL

4. Remaining reconstituted standard should be discarded or frozen at –70 °C. for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Anti-rabbit IgG Horseradish Peroxidase (HRP)
Note: The *Anti-rabbit IgG-HRP* 100X concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution:
1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Within 1 hour of use, dilute 10 µL of this 100X concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-rabbit IgG-HRP Working Solution.
4. Return the unused concentrate to the refrigerator
5. For more strips use the following amounts:

# of 8 well strips	IgG-HRP Concentrate µL	Diluent mL
2	20	2
4	40	4
6	60	6
8	80	8
10	100	10
12	120	12

Wash Buffer
1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as *Working Wash Buffer.*
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability
All components of this kit are stable at 2-8 °C. Any unused reconstituted standard should be discarded or frozen at –70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www.sigma-aldrich.com

Procedure
Precautions
- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8 °C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions
- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

Phospho-Src [pTyr418] ELISA Assay Summary

1) **100 µL of Standards or Samples (samples diluted 1:10 or higher in Standard Diluent Buffer)**
   Incubate 2 hours at RT
   aspirate and wash 4x

2) **Add 100 µL Anti-Phospho-Src [pTyr418]**
   Incubate 1 hour at RT.
   aspirate and wash 4x

3) **Add 100 µL Anti-Rabbit IgG-HRP**
   Incubate 30 min at RT.
   aspirate and wash 4x

4) **Add 100 µL Stabilized Chromogen**
   Incubate 30 minutes at RT
   (in the dark).

5) **Add 100 µL of Stop Solution**
   Read at 450nm.

Total Assay Time - 4 hours

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilution wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch

1st incubation

a Add 100 µL Standard Diluent to zero wells.
b Add 100 µL standards, samples or controls to the appropriate wells.
c Samples prepared in cell extraction buffer or brain homogenate buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample plus 90 µL buffer). *The dilutions should be optimized for each assay.*
d Cell culture supernatants or buffered solutions; dilute 1:2 in *Standard Diluent Buffer* (50 µL buffer + 50 µL sample).
e Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature.
f Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 4 times following washing instructions

2nd incubation

a Add 100 µL Anti-Phospho-Src [pTyr418] detection antibody to all wells (except chromogen blanks).
b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation

a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: *If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

Stop reaction

a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Blank the plate reader against the Chromogen Blank wells (containing Chromogen and Stop Solution).

Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution

Results

1. The results may be calculated using any immunoassay software package.
2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of may be calculated manually.
4. Calculate the Average Net OD (average reading of 2 wells) for each standard dilution and samples as follows:
5. Average Net OD = Average Bound OD – Average Chromogen Blank OD
6. On graph paper plot the Average Net OD of standard dilutions against the standard concentration (units/mL) of Src [$pTyr^{418}$]. Draw the best curve through these points to construct the standard curve.
7. The concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
8. Multiply the values obtained for the samples by dilution factor of each sample.
9. Samples producing signals higher than the 100 units/mL standard should be further diluted and assayed again.

Product Profile

Typical Results
The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Standard Src [$pTyr^{418}$] units/mL	Optical Density 450 nm
0	0.165
1.6	0.200
3.12	0.257
6.25	0.364
12.5	0.594
25	1.044
50	1.840
100	2.889

Limitations
- Do not extrapolate the standard curve beyond the 100 units/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of cell extract and brain homogenate samples have not been thoroughly investigated.
- The rate of degradation of native Src in various matrices has not been investigated.

Performance characteristics

Sensitivity
The analytical sensitivity of this assay is <1 unit/mL of Src [$pTyr^{418}$]. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

The sensitivity of this ELISA was compared to immunoblotting using known quantities of Src [$pTyr^{418}$]. The data presented in Figure 1 show that the ELISA is as sensitive as immunoblotting. The bands shown in the immunoblotting were developed using rabbit anti-Src [$pTyr^{418}$], an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

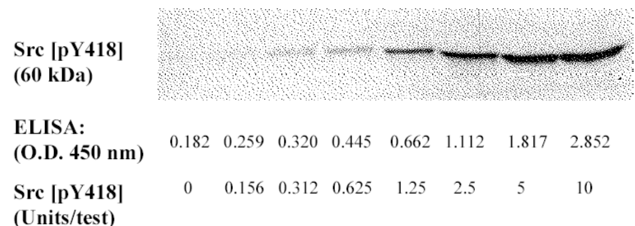

Figure 1 Detection of Src [$pTyr^{418}$]. by ELISA vs immunoblot

Precision
1. Intra-Assay Precision

Samples of known Src [$pTyr^{418}$] concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (units/mL)	68.2	27.8	14.0
Standard Deviation (SD	2.6	0.6	0.4
% Coefficient of Variation	3.8	2.2	2.7

2. Inter-Assay Precision
Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (units/mL)	69.1	28.4	15.6
Standard Deviation (SD)	2.8	1.1	1.3
Coefficient of Variation %	4.0	3.8	8.5

Recovery
To evaluate recovery, extraction buffer was diluted 1:40 with *Standard Diluent Buffer* to bring the SDS concentration to <0.01%. Src [pTyr^{418}] at various levels was spiked into the cell extract and percent recovery calculated over endogenous levels. On average, 98% recovery was observed.

Parallelism
Natural Src [pTyr^{418}] from extracts of platelets and Colo 201 cells cultured in RPMI + 10% FCS were serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the Src [pTyr^{418}] standard curve. Parallelism demonstrated in Figure 2 indicates that the standard accurately reflects natural full length Src [pTyr^{418}] content in samples.

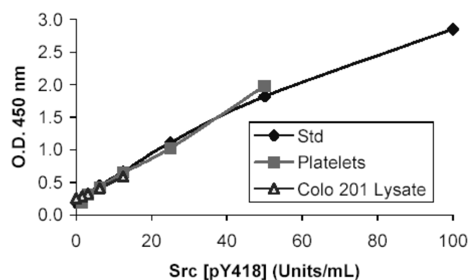

Figure 2 Phospho-Src [pTyr^{418}] ELISA Parallelism

Linearity of Dilution
Colo 201 cells were grown in tissue culture medium containing 10% fetal calf serum and lysed with Cell Extraction Buffer. This lysate was diluted with *Standard Diluent Buffer* over the range of the assay and measured for Src [pTyr^{418}]. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

Dilution	Cell Lysate		
	Measured units/mL	Expected units/mL	% Expected
Neat	100	100	100
1:2	54	50	108
1:4	29	25	116

Specificity

The specificity of this assay for Src phosphorylated at tyrosine 418 was confirmed by peptide competition. The data presented in Figure 3 show that only the phospho-peptide containing the phosphorylated tyrosine could block the ELISA signal. Neither the same sequence containing non-phosphorylated tyrosine at position 418, nor the phospho-peptide containing phosphorylation at tyrosine 529, could block the signal.

The assay was found to have 100% cross-reactivity with Fyn, but not Lyn. Cross-reactivity with other Src family members was not evaluated.

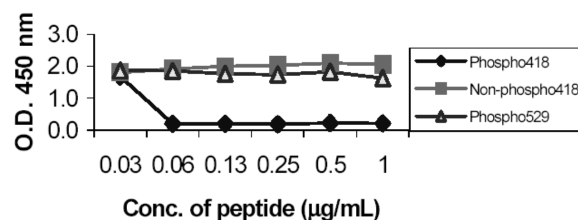

Figure 3 Phospho-Src [pTyr^{418}] ELISA: Peptide Blocking

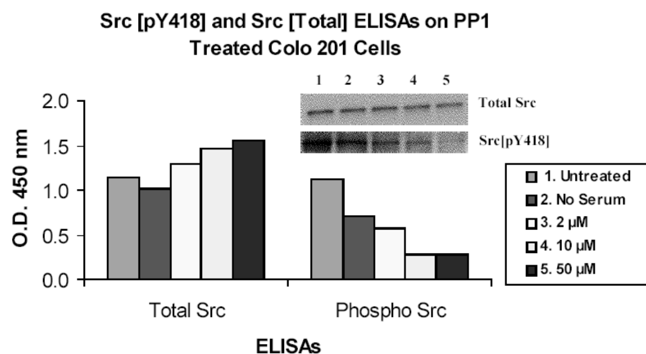

Figure 4

Figure 4 shows that treatment with PP1, a specific inhibitor of Src Kinase, inhibited phosphorylation of Src in Colo 201 cells. This inhibition occurred in a dose-dependent manner as assayed by Src [$pTyr^{418}$] ELISA Colo 201 cells were treated with PP1 at varying concentrations for 3 hours, lysed, and assayed in parallel for both total Src and Src [$pTyr^{418}$] The amount of Src remained comparable, while the levels of phosphorylation at tyrosine 418 decreased with increasing doses of PP1.

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