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ProductInformation

Phospho-STAT1 [pTyr^{701}] ELISA, Human

Product Number **CS0480**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

Phospho-STAT1 [pTyr^{701}] ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for the quantitative determination of Phospho-STAT1 [pTyr^{701}] in cell lysates. A monoclonal antibody specific for STAT1 (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. Phospho-STAT1 [pTyr^{701}] standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the STAT1 [pTyr^{701}] antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and Anti-phospho-STAT1 [pTyr^{701}] is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized Phospho-STAT1 [pTyr^{701}]. 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-STAT1 [pTyr^{701}] present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of Phospho-STAT1 [pTyr^{701}].

Phospho-STAT1 [pTyr^{701}] ELISA is designed to detect and quantify the level of STAT1 phosphorylated on tyrosine 701 from lysates of human cells or tissues. This kit does not cross react with Phospho-STAT1 [pTyr^{701}] from mouse or rat cells. For normalizing the STAT1 content of the samples, a STAT1 ELISA kit, which is indepentent of phosphorylation status, is available (Product No. CS0470).

STAT1 is a member of the STAT family of proteins, which is comprised of STATs 1, 2, 3, 4, 5A, 5B, and 6. STAT homologs have also been described in *Drosophila sp.* and *Dictyostellium discoidium*. STAT family members are characterized by the presence of a DNA binding domain, an SH3 domain that mediates interaction with polyproline-containing proteins, an SH2 domain that mediates interaction with phosphotyrosine-containing proteins, and a C terminal transactivation (TAD) domain. STAT proteins control the transcription of specific genes in response to cytokine stimulation. Alternative splicing yields two STAT1 isoforms, designated STAT1α and STAT1β, with Mr = 91 kDa and 84 kDa, respectively, which differ at their C termini.

As with all members of the STAT family, STAT1 exists in a latent state in the cytoplasm, and is activated and translocated to the nucleus in response to stimulation. Stimuli which activate STAT1 include interferons-α and -γ, IL-6 family members including oncostatin M and LIF, as well as other cytokines, and the growth factor PDGF. The series of events leading to STAT1 activation arising from interferon-γ stimulation is best characterized. The interferon-γ receptor is composed of two IFNGR1 subunits and two IFNGR2 subunits, which are localized at the plasma membrane as monomers prior to stimulation. Each IFNGR1 subunit has a constitutively associated JAK1, while each IFNGR2 has a constitutively associated JAK2. Upon interferon-γ stimulation, the dimeric ligand binds and dimerizes IFNGR1 subunits, which in turn recruit two IFNGR2 subunits through their extracellular domains. This association allows JAK1 and JAK2 to interact. The interacting JAK proteins transactivate one another by reciprocal tyrosine phosphorylation, and phosphorylate tyrosine 440 of the two IFNGR1 subunits contained in the receptor complex. These phosphorylated tyrosine residues on the IFNGR1 subunits provide paired docking sites for STAT1 via its SH2 domain. STAT1, recruited to the receptor complex, is then phosphorylated at tyrosine residue 701 by the JAKs. This tyrosine phosphorylation promotes STAT1's homo- and heterodimerization mediated by reciprocal phosphotyrosine-SH2 domain interaction and the dissociation of the dimers from the receptor complex. Activated STAT1 is then phosphorylated at serine 727 by an activity with MAPK-like properties, and is

translocated to the cell nucleus. STAT1 nuclear transport is GTP-dependent and involves STAT1's interaction with the protein importin.

Once in the nucleus, STAT1-containing dimers complex with other nuclear proteins including p48, CBP, and p300. The STAT1-containing complexes regulate gene expression, either through interaction with the consensus DNA sequence TTCC(C or G)GAA (the GAS element [interferon-γ activated sequence]) located in the promoter regions of target genes, or through interacting with the interferon-stimulated response element (ISRE). Genes that are upregulated in response to interferon-γ through STAT1 signaling are denoted immediate-early genes, and include those for the transcription factor IRF-1, the type I Fcγ receptor, and guanylate binding protein 1. STAT1 activation of gene transcription is usually transient, being completed in about 15 minutes. A second wave of transcription, observed 6-8 hours following STAT1 activation produces intermediate gene transcripts by a process, which requires additional protein synthesis. Intermediate genes include those for MHC class I and II. STAT1 is currently under investigation in many diverse areas of research including host responses to viral and bacterial infection, immune cell differentiation, and cell growth. STAT1 mediates apoptotic signals arising from interferon-γ stimulation, and may modulate anti-apoptotic signaling arising from NF-κB. STAT1 is also of interest in cancer studies, as this protein is constitutively activated in many tumors, including those of the breast, ovary, lung, head and neck, and in acute myeloid leukemia and erythroleukemia

Reagents

- **Phospho-STAT1 [pTyr⁷⁰¹] Standard, Lyophilized, Product No. P 1622-** recombinant, phosphorylated STAT1 expressed in *E. coli*. Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 7319,** contains sodium azide as preservative.
- **Sample Treatment Buffer, 10 mL, Product No. S 9569**
- **Monoclonal-Anti-STAT1-Coated 96 well plate, 1EA, Product No. S 7194-** A plate using break-apart strips coated with monoclonal antibody specific for full-length STAT1 (regardless of phosphorylation state)
- **Anti-Phospho-STAT1 [pTyr⁷⁰¹], Product No. P 1122**
  A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.

- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. R 7028 -** 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 $Na_4P_2O_7$
  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 phosphorylation of STAT1.

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 STAT1 [pTyr701]. For example, 10^8 HeLa cells grown in DMEM plus 10% FBS and treated with 10 ng/mL IFN-γ for 20 minutes can be extracted in 1 mL of Cell Extraction Buffer. Under these

conditions, use of 1-10 µL of the clarified cell extract diluted to a volume of 100 µL/well in *Standard Diluent Buffer* (See **Assay Method**) is sufficient for the detection of STAT1 [pTyr701].
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

Sample Pre-Treatment
Before assay incubate each sample and control with an equal volume of *Sample Treatment Buffer* on ice for 20 minutes. Dilute this mixture at least 5-fold in *Standard Diluent Buffer*. For example, for duplicate analyses, add 25 µL sample and 25 µL *Sample Treatment Buffer*, then after incubation, add 200 µL *Standard Diluent Buffer*. The dilution chosen should be optimized for each experimental system.

Reagent Preparation

Standard
Phospho-STAT1 [pTyr701]. standard is prepared using purified, full length, recombinant, phosphorylated STAT1 protein
One Unit of standard is equivalent to the amount of STAT1 [pTyr701] derived from 200 pg of STAT1 that was phosphorylated by activated JAK.

1. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 100 Units/mL. Use standard within 1 hour of reconstitution.
2. Prepare serial standard dilutions as follows

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

3. 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-STAT1 [pTyr^{701}] 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-STAT1 [pTyr^{701}]**

   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 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-STAT1 [pTyr^{701}] 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 immunassay 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 phospho-STAT1 [pTyr^{701}]. Draw the best curve through these points to construct the standard curve.
7. The phospho-STAT1 [pTyr^{701}] 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 STAT1 [pTyr^{701}] Units/mL	Optical Density 450 nm
100	3.05
50	1.76
25	0.91
12.5	0.48
6.25	0.33
3.12	0.22
1.6	0.17
0.0	0.11

Limitations
➢ Do not extrapolate the standard curve beyond the 100 Units/mL standard point.
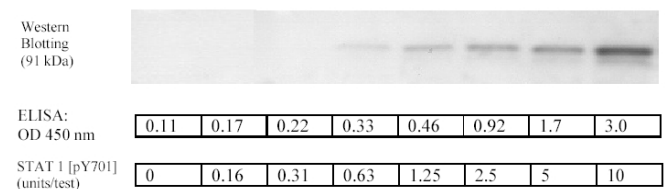➢ The dose response is non-linear in this region and accuracy will be compromised.

➢ 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 influence of various extraction buffers has not been thoroughly investigated. The rate of degradation of native STAT1 or dephosphorylation of STAT1 [pTyr^{701}] in various matrices has not been investigated.
➢ Although STAT1 degradation or dephosphorylation of STAT1 [pTyr^{701}] in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance characteristics

Sensitivity
The analytical sensitivity of this assay is <0.9 Unit/mL of STAT1 [pTyr^{701}]. 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 immunoblot using known quantities of STAT1 [pTyr^{701}]. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately 4x greater than that of immunoblotting. The bands shown in the Western blotting data were developed using rabbit anti-STAT1 [pTyr^{701}]. and an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Western Blotting (91 kDa)

ELISA: OD 450 nm	0.11	0.17	0.22	0.33	0.46	0.92	1.7	3.0
STAT 1 [pY701] (units/test)	0	0.16	0.31	0.63	1.25	2.5	5	10

Figure 1 Detection of Phospho STAT1 [pTyr^{701}] by ELISA and Immunoblot

Precision
1. Intra-Assay Precision
Samples of known concentration of STAT1 [pTyr[701]] were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (units/mL)	49.1	19.2	2.9
Standard Deviation (SD)	2.3	0.9	0.2
% Coefficient of Variation	5.2	4.6	6.0

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)	43.8	19.0	3.0
Standard Deviation (SD)	3.5	1.1	0.2
Coefficient of Variation %	8.0	5.6	7.0

Recovery
To evaluate recovery, Phospho-STAT1 [pTyr[701]] standard was spiked at 3 different concentrations into 10% cell extract buffer. The average recovery was 109%.

Parallelism
Natural STAT1 [pTyr[701]]. from HeLa cell lysate was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the Phospho-STAT1 [pTyr[701]]. standard curve. Parallelism demonstrated in Figure 2 indicates that the standard accurately reflects STAT1 [pTyr[701]]. content in samples.
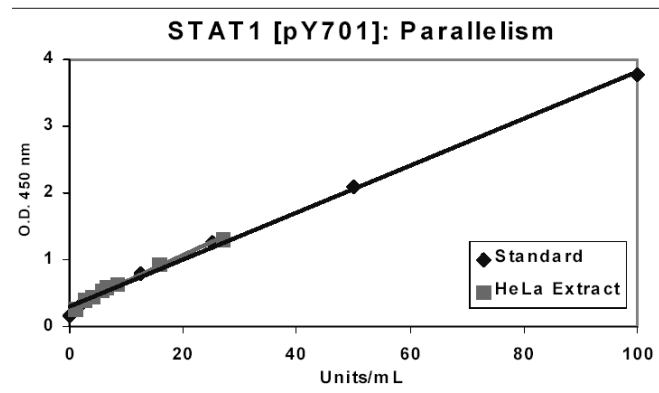

Figure 2

Linearity of Dilution
HeLa cells were grown in cell 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 STAT1 [pTyr[701]]. content. 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	19.9	19.9	100
1:2	11.5	10	115.4
1:4	4.8	5.0	96.6
1:8	2.9	2.5	114.8

Specificity

- The STAT1 [pTyr[701]] ELISA recognizes STAT1 of human origin and does not cross-react with mouse and rat STAT1. This assay recognizes both α and β isoforms of STAT1.
- HeLa cells were treated with IFN-γ at varying concentrations (0.001 to 20 ng/mL) for 20 minutes, then lysed.
- The level of phosphorylation at tyrosine 701 increases with dosage of IFN-γ. The results correlated very well with immunoblot analysis of the same samples (see insert in the graph below). Total STAT1 levels remain constant, while phospho-levels increase with IFN-γ dose.
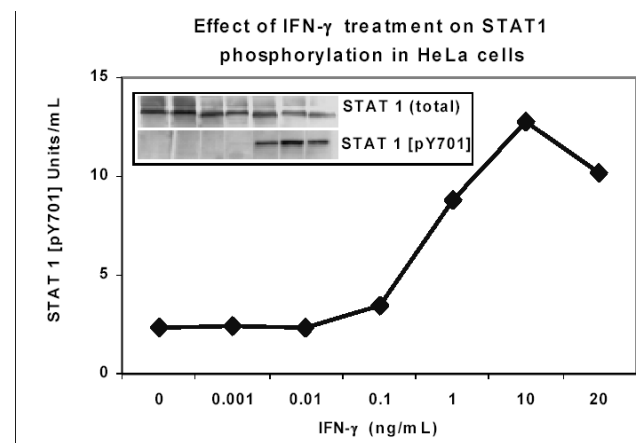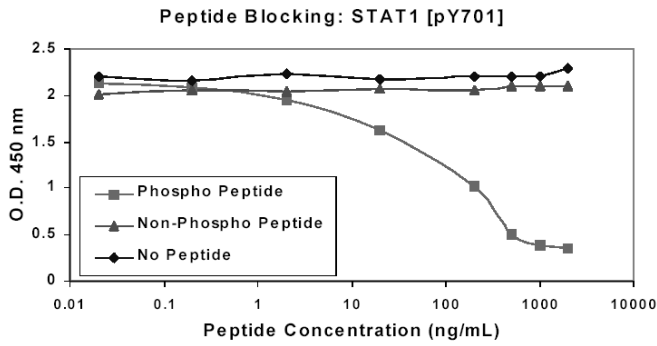

Figure 3

Peptide Competition

The specificity of this assay for phosphorylated STAT1 [pTyr[701]] was confirmed by peptide competition. The data presented in Figure 4 show that the phospho-peptide containing the phosphorylated tyrosine 701 blocks the ELISA signal.

Peptide Blocking: STAT1 [pY701]

Figure 4

References

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