



Immunoassay Kit
Catalog #KHO0621

STAT4
[pY693]*

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*Patent Pending

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INTRODUCTION

STAT4 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 discoideum*. STAT family members are characterized by the presence of a DNA binding domain, an SH3 domain which mediates interaction with polyproline-containing proteins, an SH2 domain which 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 STAT4 isoforms, designated STAT4 α and STAT4 β , the latter which lacks the C-terminal 44 amino acids encompassing the putative transcription activation domain.

STAT4 plays a critical role in the IL-12 signaling pathway. Upon binding of IL-12, both chains of the IL-12 receptor dimerize and activate the associated JAKs and TYK2. Phosphorylated IL-12 receptor subsequently recruits STAT4 to a specific docking site where it gets phosphorylated. The phosphorylated STAT4 dimerizes and translocates into nucleus, where it binds to a cognate binding sequence within IL-12 responsive genes and activates transcription. Genes that require STAT4 for transcriptional activation include IFN- γ , IL-18R α , ERM, IRF-1, and CD25.

STAT4 has been extensively investigated as the mediator of IL-12 responses in both human and murine T lymphocytes. STAT4 knockout mice have shown decreased IFN- γ levels, and as a result mice have impaired resistance to several intracellular pathogens. STAT4 also appears to play a role in chronic obstructive pulmonary disease that is associated with Th1 cell mediated inflammatory process.

The Invitrogen STAT4 [pY693] ELISA is designed to detect and quantify the level of STAT4 protein phosphorylated at tyrosine residue 693. This assay is intended for the detection of STAT4 [pY693] from lysates of human, mouse, and rat cells or tissues.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

The Invitrogen STAT4 [pY693] kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for STAT4 (regardless of phosphorylation state) has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing STAT4 [pY693], control specimens, and unknowns, are pipetted into these wells. During the first incubation, the STAT4 antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody specific for STAT4 phosphorylated at tyrosine 693 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized STAT4 [pY693] protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG (anti-rabbit IgG-HRP) is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the 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 STAT4 [pY693] present in the original specimen.

REAGENTS PROVIDED

Note: Store all reagents at 2 to 8°C.

<i>Reagent</i>	<i>96 Test Kit</i>
<i>STAT4 [pY693] Standard:</i> Refer to vial label for quantity and reconstitution volume.	2 vials
<i>Standard Diluent Buffer.</i> Contains 15 mM sodium azide; red dye*; 25 mL per bottle.	1 bottle
<i>STAT4 Antibody-Coated Wells,</i> 96 wells per plate.	1 plate
<i>Rabbit Anti-STAT4 [pY693] (Detection Antibody).</i> Contains 15 mM sodium azide; blue dye*; 11 mL per bottle.	1 bottle
<i>Anti-rabbit IgG-Horseradish Peroxidase (HRP) Concentrate,</i> (100x). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
<i>HRP Diluent.</i> Contains 3.3 mM thymol; yellow dye*; 25 mL per bottle.	1 bottle
<i>Wash Buffer Concentrate (25x);</i> 100 mL per bottle.	1 bottle
<i>Stabilized Chromogen,</i> Tetramethylbenzidine (TMB); 25 mL per bottle.	1 bottle
<i>Stop Solution;</i> 25 mL per bottle.	1 bottle
<i>Plate Covers,</i> adhesive strips.	3
* In order to help our customers avoid any mistakes in pipetting the ELISAs, we provide colored <i>Standard Diluent Buffer</i> , <i>Detection Antibody</i> , and <i>HRP Diluent</i> to help monitor the addition of solutions to the reaction wells. This does not in any way interfere with the test results.	

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Cell extraction buffer (see Recommended Formulation, p. 10).
4. Distilled or deionized water.
5. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
6. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
7. Glass or plastic tubes for diluting and aliquoting standard.
8. Absorbent paper towels.
9. Calibrated beakers and graduated cylinders in various sizes.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.

3. 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.
4. If particulate matter is present, centrifuge or filter prior to analysis.
5. All standards, controls and samples should be run in duplicate.
6. Samples that are greater than the highest standard point should be diluted with *Standard Diluent Buffer* and retested.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. Cover or cap all reagents when not in use.
9. **Do not mix or interchange different reagent lots from various kit lots.**
10. Do not use reagents after the kit expiration date.
11. Read absorbances within 2 hours of assay completion.
12. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
13. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. *Never* insert absorbent paper directly into the wells.
14. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with *Wash Buffer* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed. If your automated washer allows, 30 second soak cycles should be programmed into the wash cycle.

PROCEDURE FOR EXTRACTION OF PROTEINS FROM CELLS

A. Recommended Formulation of Cell Extraction Buffer:

10 mM Tris, pH 7.4

100 mM NaCl

1 mM EDTA

1 mM EGTA

1 mM NaF

20 mM $\text{Na}_4\text{P}_2\text{O}_7$

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)

Protease inhibitor cocktail (e.g., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 500 μL per 5 mL Cell Extraction Buffer.

This buffer is stable for 2 to 3 weeks at 4°C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at -20°C. When stored frozen, the Cell Extraction Buffer should be thawed on ice. This buffer (minus protease inhibitor cocktail and PMSF) can be obtained from Invitrogen Cat. # FNN0011. **Important:** add the protease inhibitors just before using. The stability of protease inhibitor supplemented Cell Extraction Buffer is 24 hours at 4°C. PMSF is very unstable and must be added prior to use, even if added previously.

B. Protocol for Cell Extraction

This protocol has been successfully applied to several cell lines. Researchers should optimize the cell extraction procedures for their own applications.

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash cells 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 -80°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. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of STAT4 [pY693]. For example, 5×10^7 Jurkat cells grown in RPMI-1640 plus 10% FBS and treated with 100 ng/mL IFN- α for 15 minutes can be extracted in 1 mL of 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 STAT4 [pY693].
5. Transfer extract to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C .
6. Aliquot the clear lysate to clean microfuge tubes. These samples are ready for assay. Lysates can be stored at -80°C . Avoid multiple freeze/thaw cycles.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of STAT4 [pY693] Standard

Note: This STAT4 [pY693] standard is prepared from phosphorylated recombinant STAT4 C-terminal region. One Unit of standard is equivalent to 0.5 μg of Jurkat cell lysate treated with 100 ng/mL IFN- α for 15 minutes.

1. Reconstitute *STAT4 [pY693] Standard* with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 100 Units/mL STAT4 [pY693]. Use the standard within 1 hour of reconstitution.
2. Add 0.25 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 50, 25, 12.5, 6.25, 3.12 and 1.6 Units/mL STAT4 [pY693].
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of STAT4 [pY693] Standard

Standard:	Add:	Into:
100 Units/mL	Prepare as described in step 1	
50 Units/mL	0.25 mL of the 100 Units/mL std.	0.25 mL of the Diluent Buffer
25 Units/mL	0.25 mL of the 50 Units/mL std.	0.25 mL of the Diluent Buffer
12.5 Units/mL	0.25 mL of the 25 Units/mL std.	0.25 mL of the Diluent Buffer
6.25 Units/mL	0.25 mL of the 12.5 Units/mL std.	0.25 mL of the Diluent Buffer
3.12 Units/mL	0.25 mL of the 6.25 Units/mL std.	0.25 mL of the Diluent Buffer
1.6 Units/mL	0.25 mL of the 3.12 Units/mL std.	0.25 mL of the Diluent Buffer
0 Units/mL	0.25 mL of the Diluent Buffer	An empty tube

Remaining reconstituted standard should be discarded or frozen in aliquots at -80°C for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

C. Storage and Final Dilution of Anti-Rabbit IgG Horseradish Peroxidase (HRP)

Please Note: The *Anti-rabbit IgG-HRP (100x concentrate)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Anti-rabbit IgG-HRP (100x concentrate)* to reach room temperature. Gently mix. Pipette *Anti-rabbit IgG-HRP (100x concentrate)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. 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.

For Example:

# of 8-Well Strips	Volume of Anti-Rabbit IgG-HRP (100x concentrate)	Volume of Diluent
2	20 μL solution	2 mL
4	40 μL solution	4 mL
6	60 μL solution	6 mL
8	80 μL solution	8 mL
10	100 μL solution	10 mL
12	120 μL solution	12 mL

2. Return the unused *Anti-rabbit IgG-HRP (100x concentrate)* to the refrigerator.

D. Dilution of Wash Buffer

Allow the 25x concentrate to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the 25x *Wash Buffer Concentrate* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 100 μL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 μL of standards, controls, and diluted samples (>1:10 dilution for cell extract) to the appropriate microtiter wells. Tap gently on side of plate to thoroughly mix. (See **REAGENT PREPARATION AND STORAGE**, Section B.)
4. Cover wells with *plate cover* and incubate for **2 hours at room temperature**.

5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
6. Pipette 100 μ L of *anti-STAT4 [pY693] (Detection Antibody)* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
7. Cover wells with *plate cover* and incubate for **1 hour at room temperature**.
8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
9. Add 100 μ L *anti-rabbit IgG-HRP Working Solution* to each well except the chromogen blank(s). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
10. Cover wells with the *plate cover* and incubate for **30 minutes at room temperature**.
11. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
12. Add 100 μ L of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
13. Incubate for **30 minutes at room temperature and in the dark**. **Please Note: Do not cover the plate with aluminum foil or metalized mylar.** The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells

exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.

14. Add 100 μL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
15. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 μL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*.
16. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
17. Read the STAT4 [pY693] concentrations for unknown samples and controls from the standard curve plotted in step 16. **Multiply value(s) obtained for sample(s) by the appropriate dilution factor to correct for the dilution with *Standard Diluent Buffer*.** (Samples producing signals higher than the highest standard (100 Units/mL) should be further diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration by the appropriate dilution factor.)

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 100 Units/mL STAT4 [pY693].

<u>Standard STAT4 [pY693] (Units/mL)</u>	<u>Optical Density (450 nm)</u>
100	3.184
50	1.962
25	1.074
12.5	0.608
6.25	0.355
3.12	0.229
1.6	0.166
0	0.094

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 100 Units/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >100 Units/mL with *Standard Diluent Buffer*; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various extraction buffers has not been thoroughly investigated. The rate of degradation of native STAT4 or dephosphorylation of STAT4 [pY693] in various matrices has not been investigated. Although STAT4 degradation or dephosphorylation of STAT4 [pY693] in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

This kit is for research use only.

Not for human therapeutic or diagnostic use.

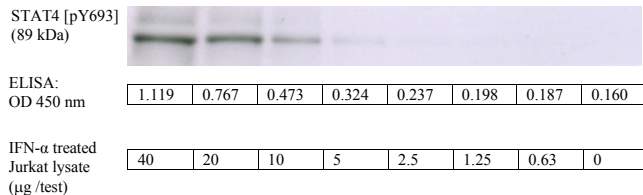
PERFORMANCE CHARACTERISTICS

SENSITIVITY

The analytical sensitivity of this assay is <1 Unit/mL of STAT4 [pY693]. 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 Western blotting using known quantities of STAT4 [pY693]. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately 2x greater than that of Western blotting. The bands shown in the Western blotting data were developed using rabbit anti-STAT4 [pY693], and an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Figure 1: Detection of STAT4 [pY693] by ELISA vs Western Blot: Jurkat Cells Stimulated by IFN- α



PRECISION

1. Intra-Assay Precision

Samples of known STAT4 [pY693] concentrations were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	53.76	22.08	10.80
SD	2.97	1.11	0.93
%CV	5.53	5.04	8.61

SD = Standard Deviation
CV = Coefficient of Variation

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)	50.08	21.43	11.1
SD	4.4	1.23	0.78
%CV	8.79	5.75	7.06

SD = Standard Deviation
CV = Coefficient of Variation

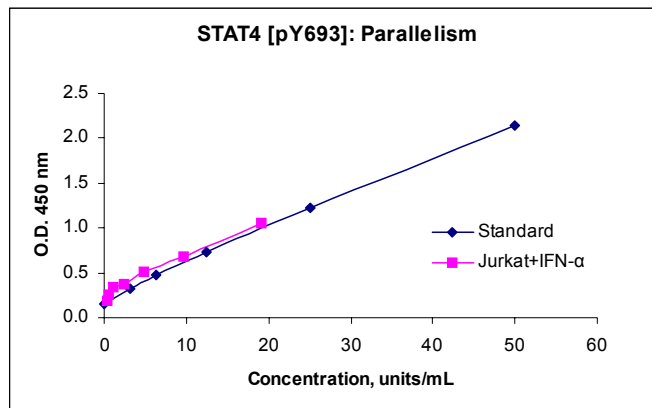
RECOVERY

To evaluate recovery, STAT4 [pY693] Standard was spiked at 3 different concentrations into 10% Cell Extraction Buffer. The average recovery was 95%.

PARALLELISM

Natural STAT4 [pY693] from IFN- α treated Jurkat cell lysate was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the STAT4 [pY693] standard curve. Parallelism demonstrated by the figure below indicates that the standard accurately reflects STAT4 [pY693] content in samples.

Figure 2



LINEARITY OF DILUTION

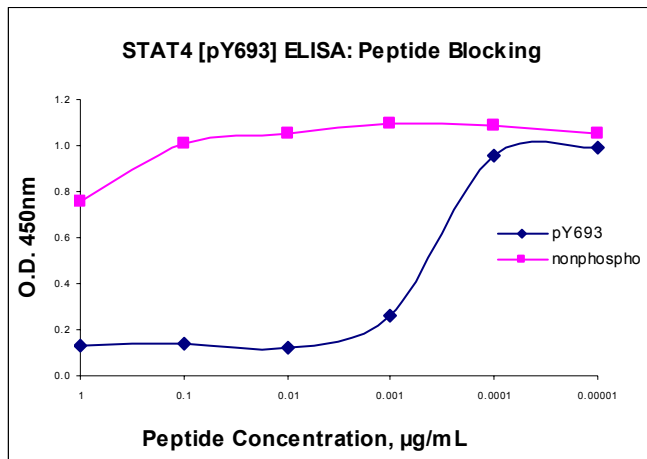
Jurkat cells were grown in tissue culture medium containing 10% fetal bovine serum, treated with 100 ng/mL IFN- α for 15 minutes and lysed with Cell Extraction Buffer. This lysate was diluted in *Standard Diluent Buffer* over the range of the assay and measured for STAT4 [pY693]. Linear regression analysis of sample values versus the expected concentrations yielded a correlation coefficient of 0.99.

Dilution	Cell Lysate		
	Measured (Units/mL)	Expected (Units/mL)	% Expected
Neat	14.37	14.37	100
1/2	8.41	7.18	117
1/4	4.25	3.59	118
1/8	1.89	1.79	105
1/16	0.97	0.89	108

SPECIFICITY

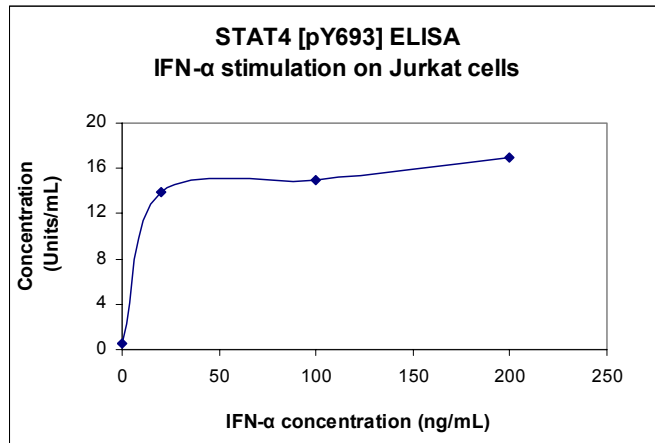
The specificity of this assay for phosphorylated STAT4 [pY693] was confirmed by peptide competition. The data presented in Figure 3 show that only the phospho-peptide containing the phosphorylated tyrosine 693 blocks the ELISA signal. The non-phosphorylated peptide sequence did not block the signal.

Figure 3



Jurkat cells were treated with IFN- α (Cat. # PHC4014) at varying concentrations (20 to 200 ng/mL) for 15 minutes and then lysed. Lysates were assayed by the STAT4 [pY693] ELISA (Cat. # KHO0621) at 400 μ g/mL. Figure 4 shows that the STAT4 [pY693] ELISA detected phosphorylated STAT4 in IFN- α treated Jurkat cells, but not the non-phosphorylated STAT4 in untreated Jurkat cells.

Figure 4



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STAT4 [pY693] Assay Summary

Incubate 100 μ L Standard or Cell Extract (>1:10)
for 2 hours at RT



aspirate and wash 4x

Incubate 100 μ L of Detection Antibody
for 1 hour at RT



aspirate and wash 4x

Incubate 100 μ L of HRP Anti-Rabbit Antibody
for 30 minutes at RT



aspirate and wash 4x

Incubate 100 μ L of Stabilized Chromogen
for 30 minutes at RT



Add 100 μ L of Stop Solution and read at 450 nm



Total time: 4 hours

