ELISA Kit
Catalog #KHO5621

JAK2
[pYpY1007/1008]

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INTRODUCTION

Janus Activating Kinase 2 (JAK2) is a 130 kDa tyrosine kinase involved in cytoplasmic signal transduction. Ligand binding to a variety of cell surface receptors (e.g., cytokine, growth factor, GPCRs) leads to an association of those receptors with JAK proteins, which are then activated via phosphorylation on tyrosines 1007 and 1008 in the kinase activation loop. Activated JAK proteins phosphorylate and activate STAT (signal transducers and activators of transcription) proteins, which then dimerize and translocate to the nucleus. Once in the nucleus, STAT proteins bind to DNA and modify the transcription of various genes, which can lead to various responses such as cell proliferation, cell survival, immune responses, and differentiation.

JAK2 specifically responds to receptors from the IFN-γ receptor family and single-chain and IL-3 cytokine families. When ligands bind to these receptors, two JAK2 proteins associated with the receptor undergo conformation changes which put them in close proximity leading to activation by transphosphorylation. JAK2 is regulated by protein tyrosine phosphatases, degradation by suppressors of cytokine signaling, and downstream by STAT protein inhibitors. Various chemical inhibitors are also under investigation for specific and non-specific inhibition of JAK2.

The JAK tyrosine kinase family includes JAK1, JAK2, JAK3, and TYK2 all of which contain two homologous kinase domains. One, the JH1 domain is catalytically active while the other domain, JH2 is inactive. The inactive domain may play a role in regulating JAK2 activity as deletion of this domain has been shown to cause constitutive activation of JAK2.

The Invitrogen JAK2 [pYpY1007/1008] ELISA is designed to detect and quantify the level of JAK2 protein phosphorylated at dual tyrosine residues 1007 and 1008. This assay is intended for the detection of JAK2[pYpY1007/1008] from lysates of human, and mouse cells or tissues. Invitrogen also offers a JAK2 (Total) ELISA kit (Cat. # KHO5521), which quantifies JAK2 independently of phosphorylation status and allows normalization of phosphorylated JAK2 to total JAK2.

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READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

The Invitrogen JAK2 [pYpY1007/1008] kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for JAK2 (regardless of phosphorylation state) has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing JAK2 [pY1007/1008], control specimens, and unknowns, are pipetted into these wells and then a rabbit antibody specific for JAK2 phosphorylated at tyrosine 1007 and tyrosine 1008 is added to the wells. During the first incubation, the JAK2 antigen binds to the immobilized (capture) antibody and the JAK2 [pYpY1007/1008] rabbit antibody serves as a detection antibody by binding to the immobilized JAK2 [pY1007/1008] protein. After the first incubation step and washing to remove excess protein and 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 JAK2 [pYpY1007/1008] present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 to 8°C.*

Reagent	***96 Test Kit***
JAK2 [pYpY1007/1008] Standard: Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; red dye*; 25 mL per bottle.	1 bottle
*Antibody Coated Well*s, 12x8 Well Strips.	1 plate
JAK2 [pYpY1007/1008] Detection Antibody. Contains 0.1% sodium azide; blue dye*; 6 mL per bottle.	1 bottle
Anti-Rabbit IgG HRP (100x). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
HRP Diluent. Contains 3.3 mM thymol; yellow dye*; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25X); 100 mL per bottle.	1 bottle
*Stabilized Chromoge*n, *Tetramethylbenzidine (TMB)*; 25 mL per bottle.	1 bottle
*Stop Solutio*n; 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	3
* In order to help our customers avoid any mistakes in pipetting the ELISAs, we provide colored *Standard Diluent Buffer*, *Detection Antibody*, and *HRP Diluent* 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. 11).
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 Concentrate (25X)* 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 $Na_4P_2O_7$
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)
Protease inhibitor cocktail (e.g., Sigma Cat. # P-2714) (reconstituted according to manufacturer's guideline). Add 100 µL per 1 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^{o}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 JAK2 [pYpY1007/1008]. For example, 5 x 10^{7} HEL cells grown in RPMI-1640 plus 10% FBS and treated with 100 µM Sodium Vanadate for 30 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 50 µL/well in *Standard Diluent Buffer* (See **Assay Method**) is sufficient for the detection of JAK2 [pYpY1007/1008].
5. Transfer extract to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4^{o}C.
6. Aliquot the clear lysate to clean microfuge tubes. These samples are ready for assay. Lysates can be stored at -80^{o}C. Avoid multiple freeze/thaw cycles.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of JAK2 [pYpY1007/1008] Standard

Note: This *JAK2 [pYpY1007/1008] Standard* is prepared from phosphorylated recombinant JAK2 [pYpY1007/1008]. One Unit of standard is equivalent to 3.3 μg of HEL cell lysate treated with 100 μM sodium vanadate for 30 mintutes.

1. Reconstitute *JAK2 [pYpY1007/1008] 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 JAK2 [pYpY1007/1008] 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 JAK2 [pYpY1007/1008].
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution off JAK2 [pYpY1007/1008] 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^{o}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 HRP (100X)

Please Note: The *Anti-Rabbit IgG HRP (100X)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Anti-Rabbit IgG HRP (100X)* to reach room temperature. Gently mix. Pipette *Anti-Rabbit IgG HRP (100X)* 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)	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)* to the refrigerator.

D. Dilution of Wash Buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* 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 50 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 50 µL of standards, controls, and diluted samples (>1:5 dilution for cell extract) to the appropriate microtiter wells.
4. Pipette 50 µL of *JAK2 [pYpY1007/1008] Detection Antibody* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.

5. Cover wells with *plate cover* and incubate for **3 hours at room temperature.**
6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
7. Add 100 µL *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.)
8. Cover wells with the *plate cover* and incubate for **30 minutes at room temperature**.
9. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
10. Add 100 µL 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 JAK2[pYpY1007/1008] 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 JAK2 [pYpY1007/1008].

Standard JAK2 [1007/1008] (Units/mL)	Optical Density (450 nm)
100	3.347
50	2.379
25	1.517
12.5	0.837
6.25	0.533
3.12	0.370
1.6	0.264
0	0.179

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 JAK2 or dephosphorylation of JAK2 [pYpY1007/1008] in various matrices has not been investigated. Although JAK2 degradation or dephosphorylation of JAK2 [1007/1008] in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

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PERFORMANCE CHARACTERISTICS

SENSITIVITY

The analytical sensitivity of this assay is <1.5 Unit/mL of JAK2 [pYpY1007/1008]. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

Human HEL cells were treated with 100 µM sodium vanadate for 30 minutes and lysed with cell extraction buffer. The sensitivity of this ELISA was compared to Western blotting using known quantities of JAK2 [pYpY1007/1008]. 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-JAK2 [pYpY1007/1008], and an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Figure 1: Detection of JAK2 [pYpY1007/1008] by ELISA vs Western Blot

ELISA: (O.D. 450 nm)	2.260	1.946	1.544	1.015	0.662	0.406	0.324	0.211
HEL Lysate (µg/test)	20	10	5	2.5	1.25	0.625	0.312	0

PRECISION

1. Intra-Assay Precision

Samples of known JAK2 [pYpY1007/1008] concentrations were assayed in replicates of 12 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	80.28	26.11	11.96
SD	2.33	1.77	0.44
%CV	2.91	6.78	3.66

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	85.67	27.32	12.48
SD	5.91	2.40	0.83
%CV	6.90	8.80	6.65

SD = Standard Deviation
CV = Coefficient of Variation

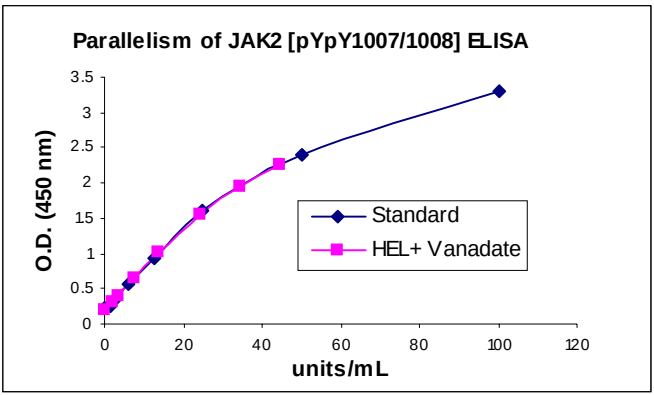
RECOVERY

To evaluate recovery, JAK2 [pYpY1007/1008] Standard was spiked at 3 different concentrations into 5% Cell Extraction Buffer. The average recovery was 124%.

PARALLELISM

Natural JAK2 [pYpY1007/1008] from sodium vanadate treated HEL cell lysate was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the JAK2 [pYpY1007/1008] standard curve. Parallelism demonstrated by the figure below indicates that the standard accurately reflects JAK2 [pYpY1007/1008] content in samples.

Figure 2



LINEARITY OF DILUTION

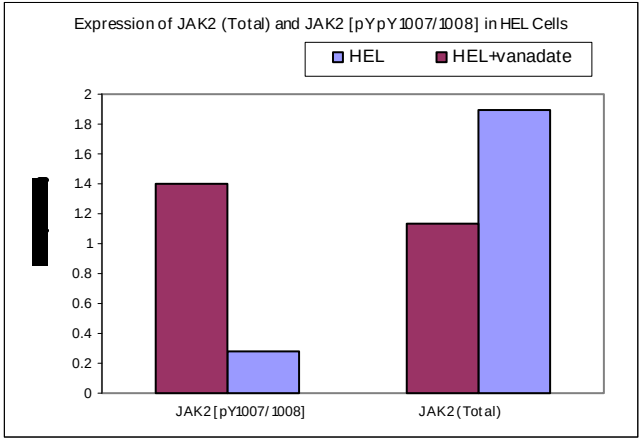
HEL cells were grown in tissue culture medium containing 10% fetal bovine serum, treated with 100 μM sodium vanadate for 30 minutes and lysed with Cell Extraction Buffer. This lysate was diluted in *Standard Diluent Buffer* over the range of the assay and measured for JAK2 [pYpY1007/1008]. Linear regression analysis of sample values versus the expected concentrations yielded a correlation coefficient of 0.99.

	Cell Lysate		
Dilution	**Measured (Units/mL)**	**Expected (Units/mL)**	**% Expected**
1/4	24.51	24.51	100
1/8	13.82	12.26	113
1/16	7.75	6.13	126
1/32	3.59	3.06	117

SPECIFICITY

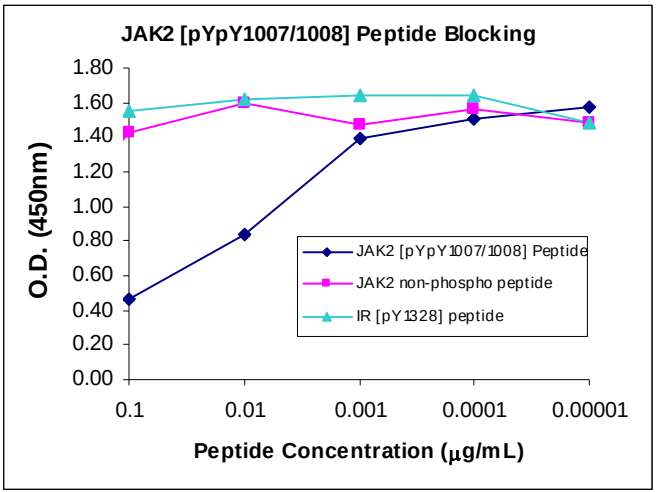
In Figure 3, HEL cells were treated with 100 µM sodium vanadate for 30 minutes. Untreated cells were used as a negative control. Cell extracts were prepared and analyzed with the JAK2 [pY1007/1008] ELISA and the Invitrogen JAK2 (Total) ELISA. The results show that the phosphorylation of JAK2 is increased in sodium vanadate treated HEL cells, whereas the total level of JAK2 remains relatively constant in treated vs. untreated control.

Figure 3



The specificity of this assay for phosphorylated JAK2 was confirmed by peptide competition. The data presented in Figure 4 show that only the phospho-peptide containing the phosphorylated tyrosines 1007 and 1008 block the ELISA signal. The non-phosphorylated peptide sequence did not block the signal. Additionally, an IR phosphorylated tyrosine peptide did not block the ELISA signal, therefore confirming the specificity of the antibody for pYpY1007/1008 only.

Figure 4



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Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

NOTES

JAK2 [pYpY1007/1008] Assay Summary