DuoSet® IC

Human/Mouse Phospho-STAT3 (Y705)

Catalog Number DYC4607-2
DYC4607-5

For the development of sandwich ELISAs to measure Signal Transducer and Activator of Transcription 3 (STAT3) phosphorylated at Y705 in cell lysates.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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PRINCIPLE OF THE ASSAY

This DuoSet® IC ELISA contains the basic components required for the development of sandwich ELISAs to measure Signal Transducer and Activator of Transcription 3 (STAT3) phosphorylated at Y705 in cell lysates. An immobilized capture antibody specific for STAT3 binds both phosphorylated and unphosphorylated STAT3. After washing away unbound material, a biotinylated detection antibody specific for STAT3 phosphorylated at Y705 is used to detect the protein, utilizing a standard Streptavidin-HRP format.

MATERIALS PROVIDED

Store the unopened kit at 2 - 8° C. Do not use past kit expiration date.

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC4607-2**	**Cat. # DYC4607-5**
Phospho-STAT3 (Y705) Capture Antibody	842631	2 - 8° C	1	2
Phospho-STAT3 (Y705) Detection Antibody	842632	2 - 8° C	1	2
Phospho-STAT3 (Y705) Standard	842633	2 - 8° C	3	5
Streptavidin-HRP	890803	2 - 8° C	1	1

DYC4607-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC4607-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

*Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol on page 6.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- NP-40 Alternative (EMD/Calbiochem # 492016)
- Protease Inhibitor Cocktail (Sigma # P8340)
- Sodium Deoxycholate (Amresco # 0613)
- Sodium Dodecyl sulfate (Sigma # L4390)
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates [Costar EIA Plates (Catalog # 2592 or R&D Systems, Catalog # DY990) are suggested]
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2 - 7.4, 0.2 μm filtered.

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2 - 7.4 (R&D Systems, Catalog # WA126).

Reagent Diluent - 5% BSA* in Wash Buffer.

IC Diluent #15 - 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40 Alternative, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate.

Substrate Solution - 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

*The use of R&D Systems Reagent Diluent Concentrate 2 (Catalog DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2 - 8° C.

Tween is a registered trademark of ICI Americas.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-STAT3 (Y705) Capture Antibody (Part 842631) - Each vial contains 720 μg/mL of mouse anti-human STAT3 antibody when reconstituted with 200 μL of PBS. After reconstitution, store at 2 - 8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Phospho-STAT3 (Y705) Detection Antibody (Part 842632) - Each vial contains 3.6 μg/mL of biotinylated rabbit anti-human phospho-STAT3 (Y705) antibody when reconstituted with 1.0 mL of Reagent Diluent. After reconstitution, store at 2 - 8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Phospho-STAT3 (Y705) Standard (Part 842633) - Each vial contains 250 ng/mL of recombinant human phospho-STAT3 (Y705) when reconstituted with 500 μL of Reagent Diluent. **Use within one hour of reconstitution. Use a fresh standard for each assay.** A seven point standard curve using 2-fold serial dilutions and a high standard of 50,000 pg/mL is recommended.

Streptavidin-HRP (Part 890803) - 1 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2 - 8° C. **DO NOT FREEZE.**

PREPARATION OF SAMPLES

Cell Lysates - Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer #15, and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay. If needed, further dilutions should be made in Reagent Diluent.

Note: *In this ELISA, it is recommended that 10 - 30 μg of total cell protein is diluted into 100 μL of Reagent Diluent.*

*Provided this is within the expiration date of the kit.

PRECAUTION

The Stop Solution suggested for use with this kit is an acidic solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

TECHNICAL HINTS AND LIMITATIONS

- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the standard reflect the environment of the samples being measured. The diluents suggested in this protocol should be suitable for most cell lysates.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2 - 8° C or be prepared fresh daily.

GENERAL ELISA PROTOCOL

A plate layout is provided to record standards and samples assayed.

Plate Preparation

1. Dilute the Capture Antibody to the working concentration of 4.0 μg/mL in PBS without carrier protein. Immediately coat a 96 well microplate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 μL of Reagent Diluent to each well. Incubate at room temperature for 1 - 2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 μL of sample or standards in Reagent Diluent per well. Use Reagent Diluent as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.

   Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 50,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Immediately before use, dilute the Detection Antibody to a working concentration of 100 ng/mL in Reagent Diluent. Prepare only as much Detection Antibody as required to run each assay. Add 100 μL of the diluted Detection Antibody to each well. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using Reagent Diluent. Add 100 μL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of Plate Preparation.
7. Add 100 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

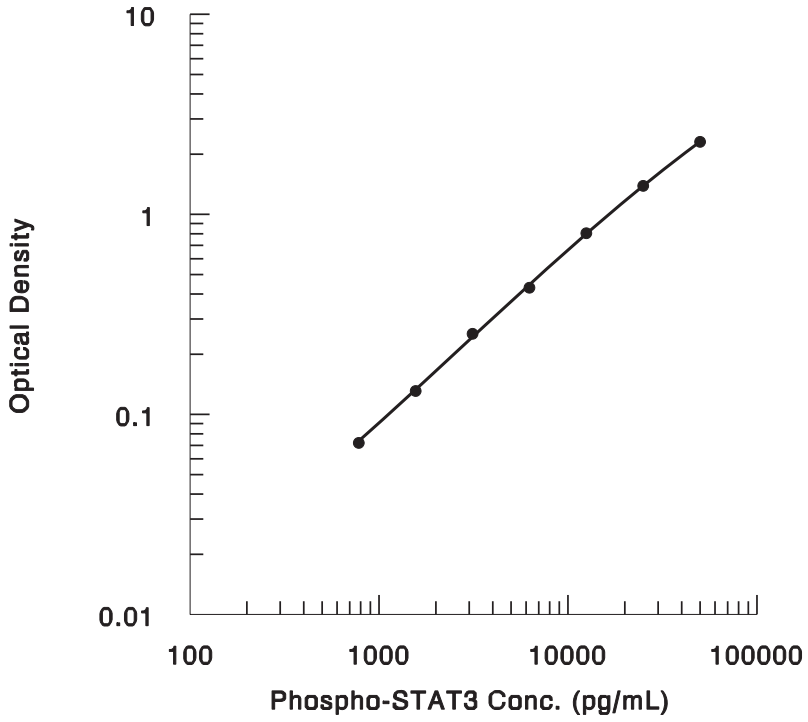
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample then subtract the average zero standard optical density. Results may be normalized to total protein or cell number.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the phospho-STAT3 (Y705) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using this Human/Mouse Phospho-STAT3 (Y705) DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



CALIBRATION

This DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-STAT3 (Y705) produced at R&D Systems.

SPECIFICITY

The Human/Mouse Phospho-STAT3 (Y705) DuoSet IC ELISA specifically recognizes STAT3 phosphorylated at Y705. The specificity of the capture antibody was demonstrated by competition from recombinant human (rh) STAT1, rhSTAT2, and rhSTAT3. Competition for the capture antibody was only observed with rhSTAT3 being able to block endogenous STAT3 from Daudi human Burkitt's lymphoma cells.

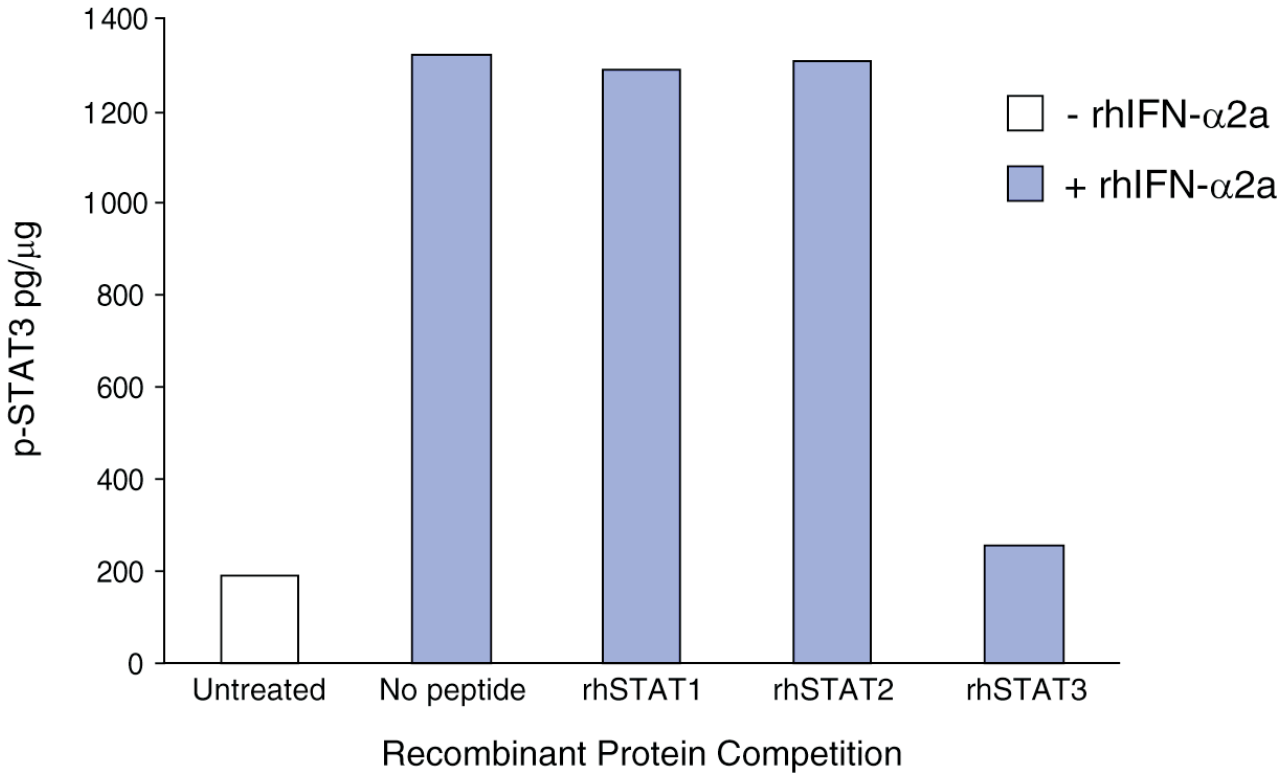


Figure 1: A lysate was prepared from Daudi human Burkitt's lymphoma cells untreated or treated with 500 U/mL of rhIFN-α2a (R&D Systems, Catalog # 11100-1) for 15 minutes. 500 ng/mL of rhSTAT1, rhSTAT2, or rhSTAT3 protein was added to 30 µg of lysate and analyzed using this DuoSet IC ELISA.

The Human/Mouse Phospho-STAT3 (Y705) DuoSet IC ELISA specifically recognizes STAT3 phosphorylated at Y705. The specificity of the detection antibody was demonstrated by competition from the phosphorylated peptide corresponding to STAT1 (Y701), STAT2 (Y689), and STAT3 (Y705). Competition for the detection antibody was only observed with the phosphorylated peptide to STAT3 (Y705), which was able to block detection of endogenous STAT3 from Daudi human Burkitt's lymphoma cells.

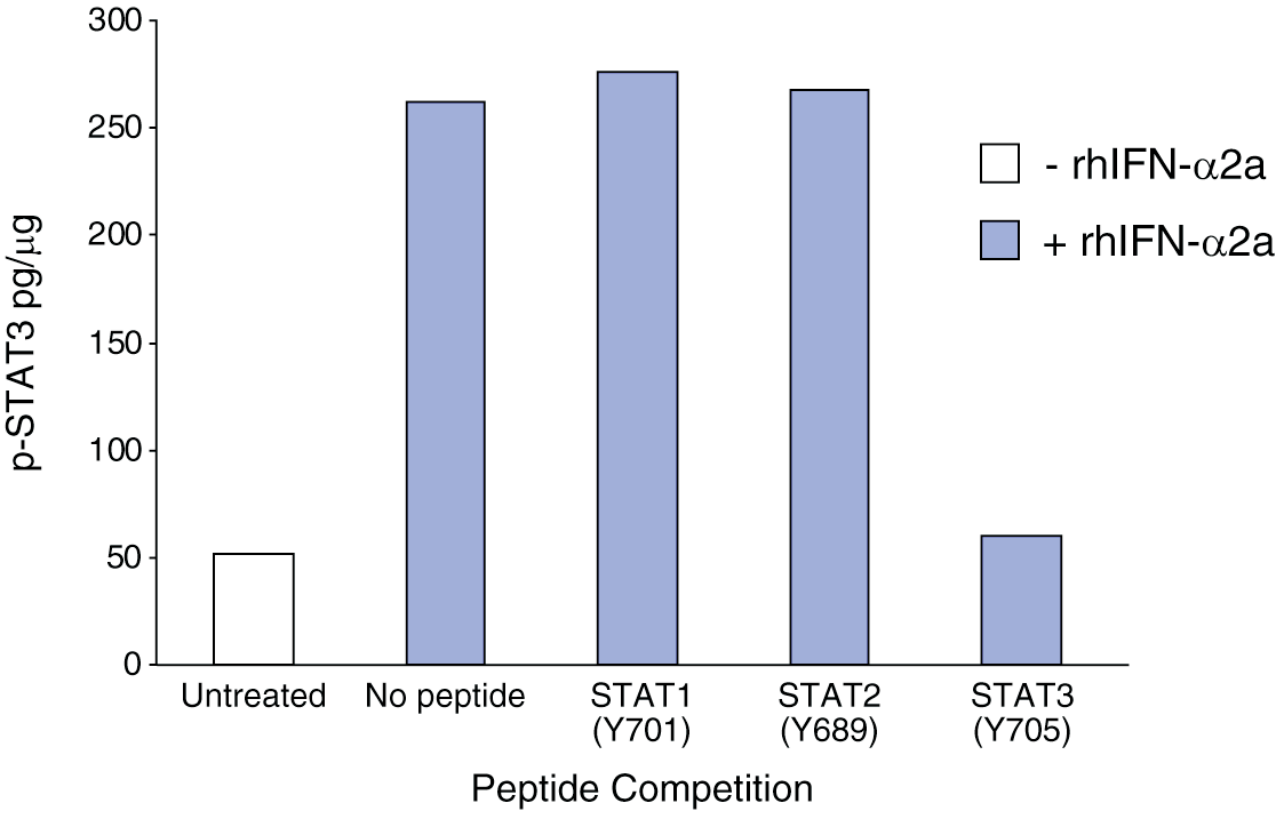


Figure 2: A lysate was prepared from Daudi human Burkitt's lymphoma cells untreated or treated with 500 U/mL of rhIFN-α2a for 15 minutes. 40 ng/mL of phosphorylated peptide corresponding to STAT1 (Y701), STAT2 (Y689), or STAT3 (Y705) was added with the detection antibody and analyzed using this DuoSet IC ELISA.

QUANTIFICATION

Amounts of phosphorylated STAT3, as quantified by the Human/Mouse Phospho-STAT3 (Y705) DuoSet IC ELISA, are consistent with the relative levels of phosphorylated STAT3 determined by qualitative Western blot analysis.

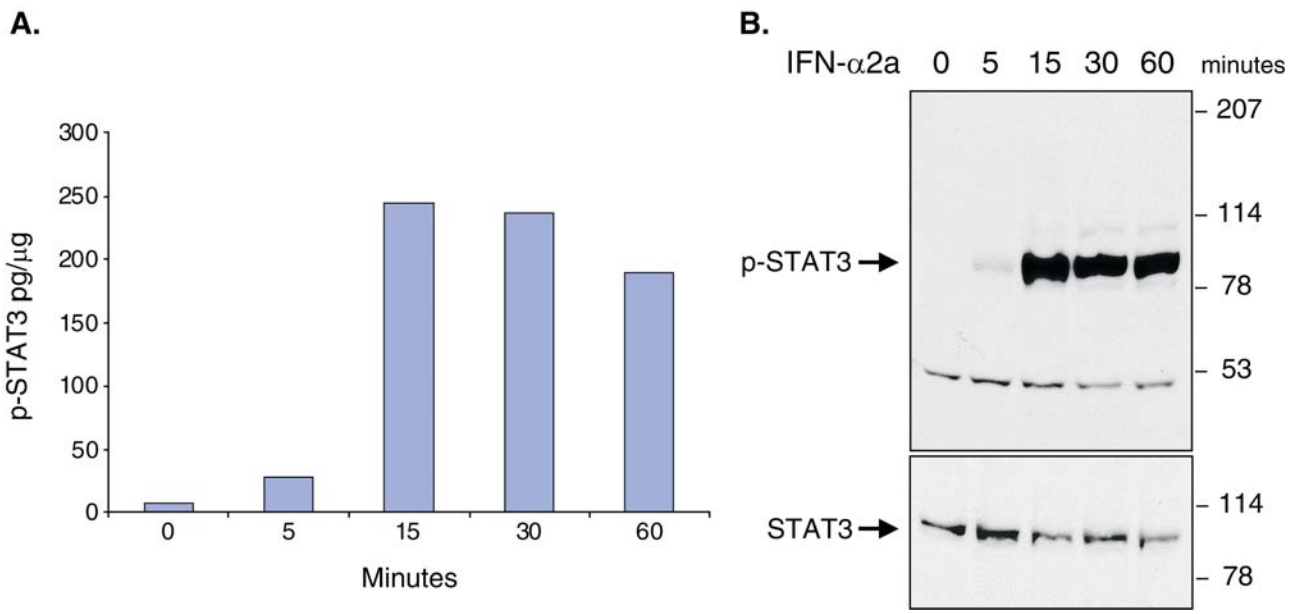


Figure 3: Time course of phospho-STAT3 activation.

A. A lysate was prepared from Daudi human Burkitt's lymphoma cells treated with 500 U/mL of rhIFN-α2a for the indicated periods of time. 30 µg of lysates from each time point were quantified with this DuoSet IC ELISA.

B. The same extracts were immunoblotted with either the Phospho-STAT3 (Y705) Detection Antibody (upper panel) or anti-STAT3 antibody (R&D Systems, Catalog # MAB1799; lower panel).

The DuoSet IC ELISA results correlate well with the relative levels and kinetics of phosphorylated STAT3 (Y705) detected by Western blot. The immunoblot with anti-STAT3 antibody indicates that the total levels of STAT3 remained constant during induction with rhIFN-α2a.

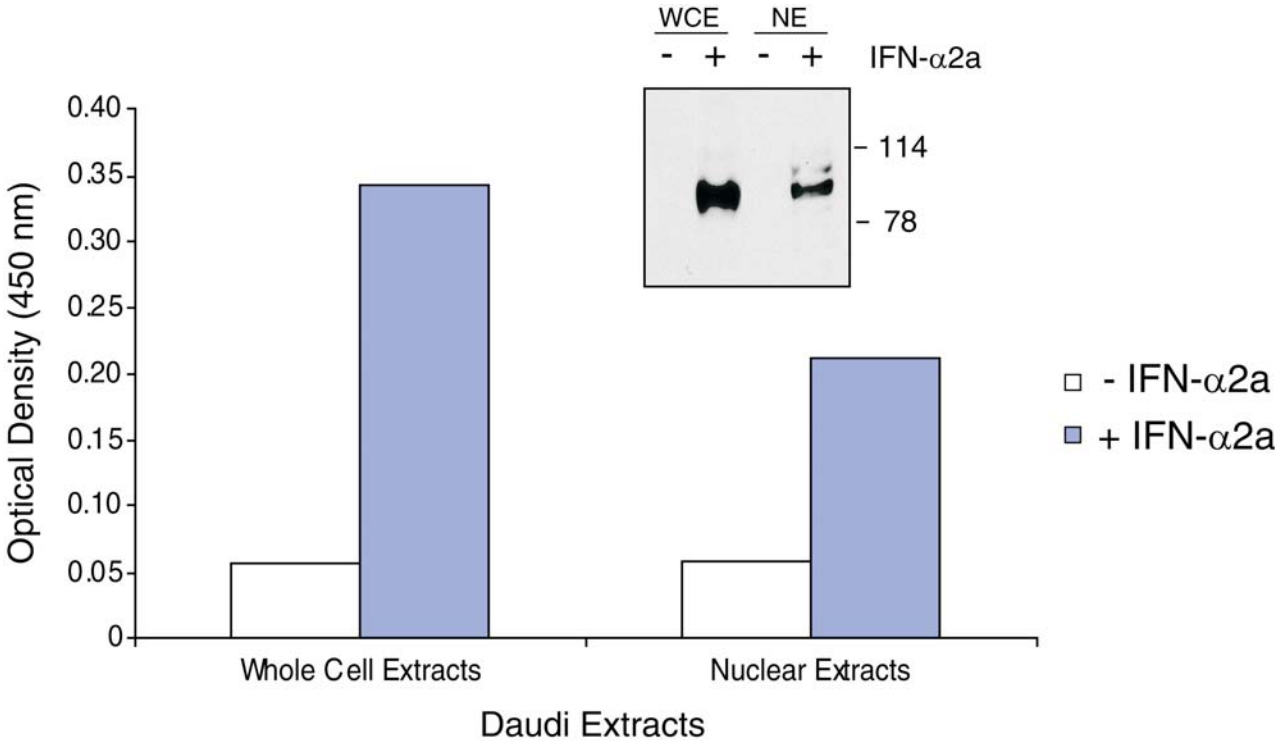


Figure 4: Comparison of μg amounts of whole cell extracts and nuclear extracts in phospho-STAT3 (Y705). Whole cell extracts (WCE) or nuclear extracts (NE) were prepared from Daudi human Burkitt's lymphoma cells treated with 500 U/mL of recombinant human IFN-α2a for 15 minutes. 30 μg of WCE and 10 μg of NE were quantified with this DuoSet IC ELISA. The same extracts were immunoblotted with the Phospho-STAT3 (Y705) Detection Antibody (inset).

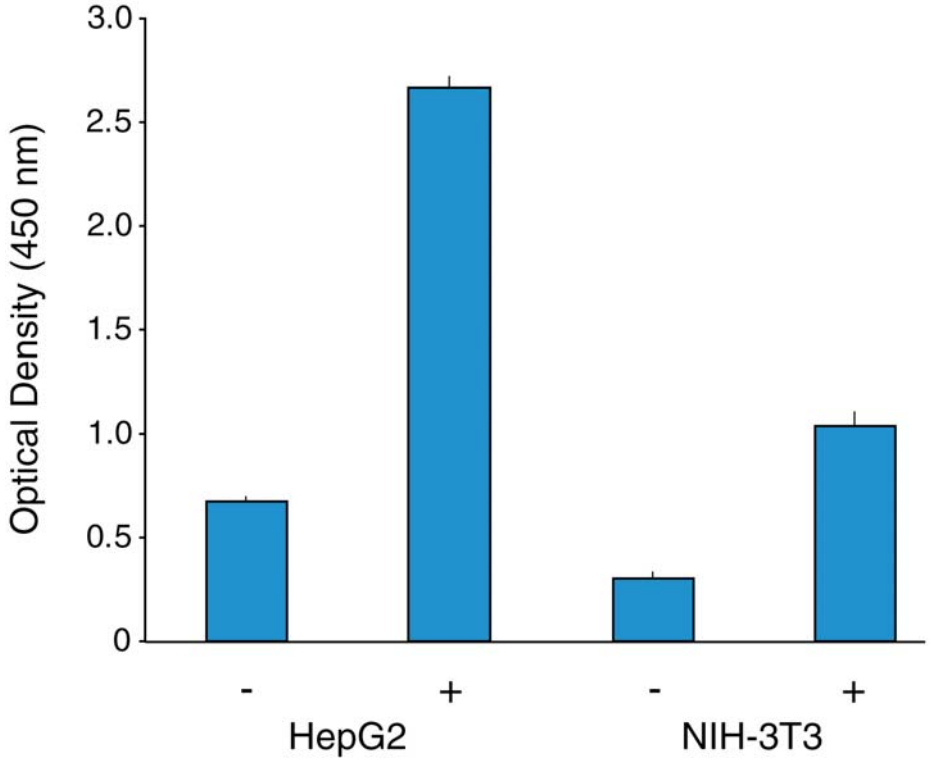
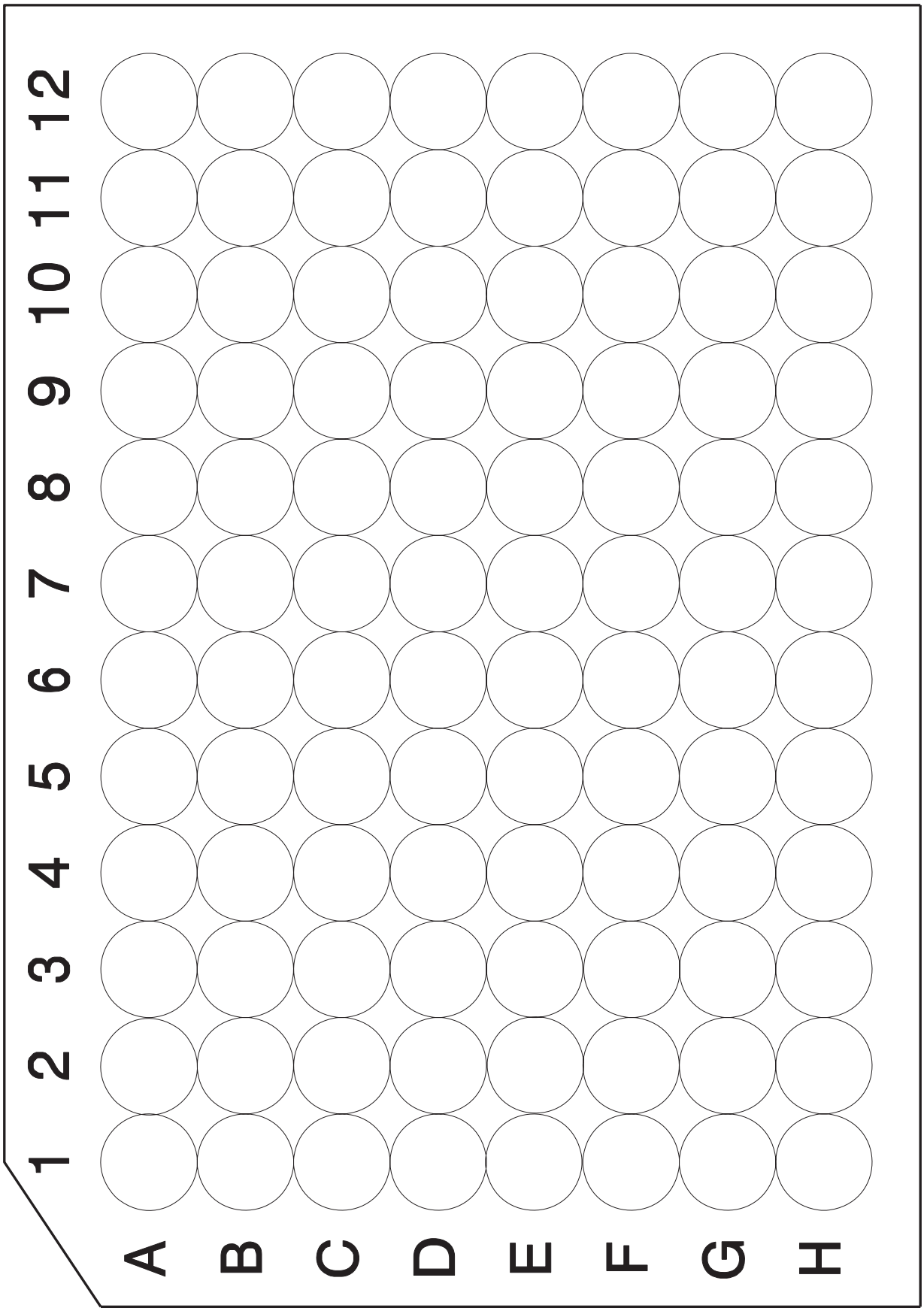


Figure 5: Detection of phosphorylated STAT3 (Y705) in human and mouse cell lines. Lysates were prepared from HepG2 human hepatocellular carcinoma cells stimulated for 15 minutes with 100 ng/mL of rhIL-22 (R&D Systems, Catalog # 782-IL) or NIH-3T3 mouse embryonic fibroblasts stimulated for 15 minutes with 100 ng/mL of rhPDGF-AB (R&D Systems, Catalog # 222-AB). 30 μg of unstimulated (-) or stimulated (+) extracts were quantified with this DuoSet IC ELISA.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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