DuoSet® IC

Human/Mouse Active* STAT1 p91

Catalog Number DYC1490-2
DYC1490-5

For the development of transcription factor assays to measure active STAT1 p91.

*This assay can be converted to measure total STAT1 p91 or total STAT1/STAT2 heterodimer. See "Principle of the Assay" on page 2 for details.

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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MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.  
614 McKinley Place NE  
Minneapolis, MN 55413  
United States of America  
TELEPHONE: (800) 343-7475  
TELEPHONE: (612) 379-2956  
FAX: (612) 656-4400  
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.  
19 Barton Lane  
Abingdon Science Park  
Abingdon, OX14 3NB  
United Kingdom  
TELEPHONE: +44 (0)1235 529449  
FAX: +44 (0)1235 533420  
E-MAIL: info@RnDSystems.co.uk

R&D Systems China Co. Ltd.  
24A1 Hua Min Empire Plaza  
726 West Yan An Road  
Shanghai PRC 200050  
TELEPHONE: +86 (21) 52380373  
FAX: +86 (21) 52371001  
E-MAIL: info@RnDSystemsChina.com.cn
PRINCIPLE OF THE ASSAY

This DuoSet® IC activity assay contains the basic components required for the development of a transcription factor assay to measure active STAT1 p91, also called STAT1α. A biotinylated double stranded (ds) oligonucleotide containing a consensus STAT1 binding site is incubated with nuclear extracts. STAT1 p91-ds oligonucleotide complexes are subsequently captured by an immobilized antibody specific for STAT1 p91. After washing away unbound material, detection utilizing Streptavidin-HRP is performed. An unlabeled ds competitor oligonucleotide is provided to demonstrate the specificity of the assay.

Note: This assay may be converted to measure total STAT1 p91 by substituting the Active STAT1 p91 Biotin Labeled ds Oligonucleotide with anti-STAT1 p91 biotinylated antibody (R&D Systems, Catalog # BAF1490). For details, refer to the antibody package insert.

This assay may be converted to measure total STAT1/STAT2 heterodimer by substituting the Active STAT1 p91 Biotin Labeled ds Oligonucleotide with anti-STAT2 biotinylated antibody (R&D Systems, Catalog # BAF1666). For details refer to the antibody package insert.

MATERIALS PROVIDED

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

<table>
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<tr>
<th>Description</th>
<th>Part #</th>
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DYC1490-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*

DYC1490-5 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

- Aprotinin (Sigma # A6279)
- Leupeptin (Sigma # L8511)
- Pepstatin (Sigma # P4265)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Chymostatin (Sigma # C7268)
- Sodium orthovanadate (activated)* (Na₃VO₄) (Sigma # S6508)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- Nuclease-free water
- Deionized or distilled water
- Pipettes and pipette tips
- 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 Na₂HPO₄, 1.5 mM KH₂PO₄, 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.

**Lysis Buffer A** - 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40 Alternative, 2 mM activated sodium orthovanadate, 3 μg/mL Aprotinin, 25 μg/mL Leupeptin, 25 μg/mL Pepstatin, 25 μg/mL Chymostatin, 0.2 mM PMSF. Aliquot and store at ≤ -20°C for up to 12 months.

**Lysis Buffer B** - 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 420 mM NaCl, 0.5 mM DTT, 25% glycerol, 2 mM activated sodium orthovanadate, 25 μg/mL Leupeptin, 25 μg/mL Pepstatin, 25 μg/mL Chymostatin, 0.2 mM PMSF, 3 μg/mL Aprotinin. Aliquot and store at ≤ -20°C for up to 12 months.

**Substrate Solution** - 1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

**Stop Solution** - 2 N H₂SO₄ (R&D Systems, Catalog # DY994).

*Sodium orthovanadate should be activated for maximum inhibition of tyrosine phosphatases. Prepare a 200 mM solution of sodium orthovanadate and adjust the pH to 10.0 using NaOH or HCl. The solution will be yellow. Boil the solution until it turns colorless (approximately 10 minutes), then cool it to room temperature. Adjust the pH to 10.0 again, and repeat boiling and cooling. Repeat this procedure until the solution remains colorless and the pH stabilizes.

**The use of 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

Active STAT1 p91 Capture Antibody (Part 841219) - Each vial contains 180 μg/mL of goat anti-human STAT1 p91 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.*

Active STAT1 p91 Biotin Labeled ds Oligonucleotide (Part 841220) - Reconstitute each vial with 450 μL of nuclease-free water. After reconstitution, aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.* Avoid repeated freeze-thaw cycles.

Active STAT1 p91 Unlabeled ds Oligonucleotide (Part 841221) - Reconstitute each vial with 150 μL of nuclease-free water. After reconstitution, aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.* Avoid repeated freeze-thaw cycles.

Streptavidin-HRP (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2 - 8° C.* DO NOT FREEZE.

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

PREPARATION OF SAMPLES

Keep all solutions and samples on ice while preparing nuclear extracts.

Nuclear Extracts - Wash the cells with PBS, making sure to remove any remaining PBS after centrifugation. Solubilize cells at 2.5 x 10⁶ cells/mL in Lysis Buffer A by pipetting up and down. Centrifuge at 16,000 x g for 5 minutes at 2 - 8° C in a microcentrifuge. Remove cytosolic supernatant and solubilize nuclear pellet in Lysis Buffer B at 1 x 10⁸ cells/mL. Vortex for 10 seconds and incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 5 minutes at 2 - 8° C. Transfer supernatants to a fresh ice cold tube, aliquot, and store at ≤ -70° C.

Note: It is recommended that the protein concentration of nuclear extracts be determined before use. Total protein concentration of nuclear extracts should be 0.5 - 5.0 mg/mL.
**PRECAUTION**

The Stop Solution suggested for use with this kit is an acidic solution. Wear eye, hand, face, and clothing protection when using this material.

**TECHNICAL HINTS AND LIMITATIONS**

- This DuoSet IC activity assay 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.
- The type of double stranded oligonucleotide, substrate, and the concentration of capture antibody can be varied to create an activity assay with a different sensitivity and dynamic range. A basic understanding of activity assay development is required for the successful use of these reagents in activity assays.
- 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 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 sterile conditions and stored at 2 - 8° C or be prepared fresh daily.

**GENERAL ELISA PROTOCOL**

**Plate Preparation**

1. Dilute the Capture Antibody to a working concentration of 1 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 decanting. Invert the plate and blot 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

The sample volume in step 1 is sufficient to perform duplicate analyses. Optimal amounts of nuclear extracts should be determined for each application, however, 10 μg/well will typically give optimal results in this assay.

Note: This assay can be converted to measure total STAT1 p91 or total STAT1/STAT2 heterodimer. See "Principle of the Assay" on page 2 for details.

1. Add 3 μL of Biotin Labeled ds Oligonucleotide to 2 - 40 μg of nuclear extract sample in an Eppendorf tube. Adjust the final volume to 30 μL with Lysis Buffer B and incubate at room temperature for 30 minutes.

A blank containing Biotin Labeled ds Oligonucleotide without nuclear extracts should be performed.

Note: The Unlabeled ds Oligonucleotide is provided for the determination of specificity via a competition assay (see Specificity on page 7). Add 3 μL of Unlabeled ds Oligonucleotide to a reaction containing 3 μL Biotin Labeled ds Oligonucleotide and nuclear extract as prepared above. Adjust the final volume to 30 μL with Lysis Buffer B and incubate at room temperature for 30 minutes.

2. Add 200 μL of Reagent Diluent to each sample. Mix gently.

3. Place 100 μL of sample in each well. Cover with a plate sealer and incubate at room temperature for 2 hours.

4. Repeat the aspiration/wash as in step 2 of Plate Preparation, washing a total of 5 times.

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, washing a total of 5 times.

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 sample and subtract the average oligonucleotide-only blank optical density.

SPECIFICITY

A 7- to 9-fold increase in active STAT1 p91 was observed when nuclear extracts prepared from IFN-α treated Daudi and IFN-γ treated U937 cells were analyzed by this DuoSet IC activity assay. The binding specificity of the biotinylated ds oligonucleotide was demonstrated by competition with the non-biotinylated form of this oligonucleotide. The unlabeled ds competitor oligonucleotide blocked the signal in nuclear extracts of IFN treated cells.

Figure 1: Human U937 and Daudi cells were treated with 100 ng/mL of IFN-γ (R&D Systems, Catalog # 285-IF) for 10 minutes or IFN-α (R&D Systems, Catalog # 11101-1) for 20 minutes, respectively. Nuclear extracts were prepared and assayed at 5 μg/well in the DuoSet IC activity assay. Extracts from treated cells were also incubated with a competitor oligonucleotide (90 pmol), corresponding to the unlabeled ds oligonucleotide.
Electrophoretic Mobility Shift Assay (EMSA) with supershift

Capture antibody specificity was demonstrated by an electrophoretic mobility shift assay (EMSA) with a supershift of the STAT1 p91-DNA complex induced only by anti-STAT1 specific antibodies.

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<tr>
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Figure 2: Human U937 cells were treated with 100 ng/mL of IFN-γ for 20 minutes. Nuclear extracts were prepared and incubated with the Biotin Labeled ds Oligonucleotide for 30 minutes at room temperature. Antibodies (2 μg) were then added to the indicated samples and after an additional 30 minute incubation at room temperature, samples were electrophoresed on a 5% polyacrylamide gel. The shifts in oligonucleotide mobility were detected by chemiluminescence. The goat anti-STAT1(c-term) and goat anti-STAT1 p91 (R&D Systems, Catalog # PAF-ST1) polyclonal antibodies were used as positive controls, whereas goat anti-STAT2 (R&D Systems, Catalog # PAF-ST2) polyclonal antibody served as the negative control.
Detection of Active Mouse STAT1 p91

Active STAT1 p91 in mouse myeloid leukemia (M1) cells treated with mouse IFN-\(\gamma\) was detected with the DuoSet IC activity assay. The results correlate well with Western blot data.

**Figure 3:** Mouse M1 cells were treated with 100 ng/mL of mouse IFN-\(\gamma\) (R&D Systems, Catalog # 485-MI) for 3 hours. Nuclear extracts were prepared and assayed in the DuoSet IC activity assay at 5 \(\mu\)g/well. Nuclear extracts were also analyzed by Western blot using a commercial rabbit anti-phospho-STAT1 antibody.
SENSITIVITY AND LINEARITY

This DuoSet IC activity assay is 5 - 20 times more sensitive than an EMSA. Active STAT1 p91 can be detected in as little as 0.2 μg of nuclear extracts. STAT1 p91 signal increased proportionally over a 40-fold range demonstrating assay linearity.

**Active STAT1 p91 Assay**

![Active STAT1 p91 Assay graph]

**EMSA**

![EMSA graph]

**Figure 4:** Mouse M1 cells were treated with 100 ng/mL of mouse IFN-γ for 3 hours. Nuclear extracts were prepared and assayed in this DuoSet IC activity assay. The same nuclear extracts were analyzed by EMSA.
Figure 5: Human U937 and Daudi cells were treated with 100 ng/mL of IFN-γ or IFN-α, respectively, for the indicated times. The same U937 and Daudi nuclear extract preparations were used for all analyses. Nuclear extracts were prepared and assayed at 5 μg/well in this DuoSet IC activity assay. For immunoprecipitation, 2 μg of STAT1 p91 DuoSet IC Capture Antibody was added to 20 μg of nuclear extract and incubated at room temperature for 1 hour. Protein G was then added to the samples, and after an additional 1 hour incubation, the samples were washed. Samples were solubilized in SDS sample buffer and electrophoresed on a 5 - 15% gradient polyacrylamide gel. Western blots were performed using antibodies recognizing either total or phosphorylated STAT1 p91. EMSA were performed using 10 μg of nuclear extracts incubated with 10 fmol of the Biotinylated ds Oligonucleotide and electrophoresed on a 5% non-denaturing polyacrylamide gel.