

ab207228 STAT (STAT1alpha, STAT3, STAT5A, STAT5B) Transcription Factor Assay Kit (Colorimetric)

Instructions for use:

For quantitative measurement of STAT1alpha, STAT3, STAT5A, STAT5B activation in human and rat nuclear extracts.

This product is for research use only and is not intended for diagnostic use.

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1. BACKGROUND

STAT (STAT1alpha, STAT3, STAT5A, STAT5B) Transcription Factor Assay Kit (Colorimetric) (ab207228) is a high throughput assay to quantify activation of STAT family members (STAT1alpha, STAT3, STAT5A, STAT5B) at the same time in one assay. This assay combines a quick ELISA format with a sensitive and specific non-radioactive assay for transcription factor activation.

A specific double stranded DNA sequence containing the STAT consensus binding site (5' – TTCCCGGAA – 3') has been immobilized onto a 96-well plate. Active STATs present in the nuclear extract specifically binds to the oligonucleotide. The primary antibodies recognize epitopes of STAT1alpha, STAT3, STAT5A, STAT5B proteins accessible only when the protein is activated and bound to its target DNA. An HRP-conjugated secondary antibody provides sensitive colorimetric readout that at OD 450 nm. This product detects human and rat STAT (STAT1alpha, STAT3, STAT5A, STAT5B). STAT3 can also be detected in mouse.

Key performance and benefits:

- Assay time: 3.5 hours (cell extracts preparation not included).
- Detection limit: < 0.6 µg nuclear extract/well.
- Detection range: 0.3 – 10 µg nuclear extract/well.

STAT (signal transducers and activators of transcription) transcription factors were discovered fourteen years ago as mediators of interferon-induced gene expression. They comprise a family of latent cytoplasmic proteins that are activated to participate in gene control when cells encounter various extracellular polypeptides. Their critical role in development and normal cell signaling has been largely determined through the analysis of transgenic mice lacking individual STAT genes. The STAT family consists of seven members that are activated by virtually every cytokine and growth factor.

INTRODUCTION

The STAT proteins are unique among transcription factors as they contain a SH2 (src-homology 2) phosphotyrosine-binding domain, a common protein-protein interaction domain among signaling proteins. Tyrosine phosphorylation around residue 700 is essential for the dimerization of STATs and the concomitant nuclear translocation of the dimer. Ligand-activated receptors that catalyze this phosphorylation include EGF, PDGF and CSF-1, all receptors with intrinsic tyrosine kinase activity, as well as receptors that lack intrinsic tyrosine kinase activity but to which JAKs are noncovalently associated. Receptors to which JAKs are bound are often referred to as cytokine receptors.

STAT dimers and heterodimers (but not monomers) are competent to bind DNA. The known DNA binding heterodimers are STAT1/STAT2 (strong binding requires the joint presence of p48) and STAT1/STAT3. STATs that form homodimers that bind DNA include STAT-1, -3, -4, -5 (STAT5A and 5B interact in a manner equivalent to a heterodimer) and -6.

In most cases, STAT activation is transient. Inactivation of STAT proteins is carried out by several mechanisms, including dephosphorylation of STAT proteins in the nucleus and degradation through the ubiquitin-proteasome pathway. Inhibitors of the JAK-STAT pathway include suppressor-of-cytokine-signaling (SOCS) proteins/JAK binding (JAB) proteins, STAT-induced STAT inhibitors (SSIs) and protein inhibitors of activated STAT (PIAS).

2. ASSAY SUMMARY

Single Stranded DNA oligonucleotide



Sample



Primary Antibody



HRP-Conjugated Antibody



Substrate

Colored Product



Prepare all reagents, nuclear extracts and controls as instructed. Plate is supplied pre-coated with an oligonucleotide containing STAT consensus binding site.

Add sample (nuclear extracts containing activated transcription factor) to appropriate wells. Incubate plate for 1 hour at RT.

Add primary antibody to wells. Incubate plate for 1 hour at RT.

Aspirate and wash each well. Add HRP-conjugated secondary antibody. Incubate plate for 1 hour at RT.

Aspirate and wash each well. Add developing solution until wells turn medium to dark blue. Add Stop Solution. Measure absorbance at OD 450 nm.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. STORAGE AND STABILITY

Store kit at -20°C (nuclear extract must be kept at -80°C) in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt. After first use, components are stable for 6 months.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

GENERAL INFORMATION

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
	2 Plates		
STAT1alpha antibody	11 μ L	-20°C	-20°C
STAT3 antibody	20 μ L	-20°C	-20°C
STAT5A antibody	11 μ L	-20°C	-20°C
STAT5B antibody	11 μ L	-20°C	-20°C
Anti-rabbit HRP-conjugated IgG (0.25 μ g/ μ L)	2 x 11 μ L	-20°C	4°C
Wild-type oligonucleotide (10 pmol/ μ L)	100 μ L	-20°C	-20°C
Mutated oligonucleotide (10 pmol/ μ L)	100 μ L	-20°C	-20°C
Nb2 nuclear extract (Prolactin stimulated) (2.5 μ g/ μ L)	40 μ L	-80°C	-80°C
Dithiothreitol (DTT)	100 μ L	-20°C	-20°C
Protease Inhibitor Cocktail	100 μ L	-20°C	-20°C
Herring sperm DNA (1 μ g/ μ L)	100 μ L	-20°C	-20°C
Lysis Buffer	10 mL	-20°C	4°C
Binding Buffer	10 mL	-20°C	4°C
10X Wash Buffer	60 mL	-20°C	4°C
10X Antibody Binding Buffer	2 x 2.2 mL	-20°C	4°C
Developing Solution	2 x 11 mL	-20°C	4°C
Stop Solution	60 mL	-20°C	4°C

GENERAL INFORMATION

96-well assay plate	2	-20°C	4°C
Plate sealer	2	-20°C	RT

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 450nm (OD 655 nm can be used as optional reference wavelength)
- MilliQ water or other type of double distilled water (ddH₂O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Rocking Platform

For nuclear extract preparation:

- Hypotonic buffer (20 mM Hepes pH7.5, 5 mM NaF, 10 μ M Na₂MoO₄, 0.1 mM EDTA)
- Phosphatase Inhibitors (NaF, β -glycerophosphatase, PNPP, NaVO₃)
- 10X PBS (0.1 M phosphate buffer pH7.5, 1.5 M NaCl, 27 mM KCl)
- NP-40

Alternatively, you can use our Nuclear Extraction Kit (ab113474) to prepare nuclear extracts.

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample or control will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

Please see Quick Table for Reagent Preparation at the end of this section for a quick reference.

9.1. **Dithiothreitol (DTT, 1 M):**

Ready to use as supplied. Dilute in Lysis Buffer and Binding Buffer as described in section 9.4 and 9.5 respectively. Store at -20°C.

9.2. **Protease Inhibitor Cocktail (PIC):**

Ready to use as supplied. Dilute in Lysis Buffer as described in section 9.4. Store at -20°C.

9.3. **Herring sperm DNA (1 µg/µL):**

Ready to use as supplied. Dilute in Binding Buffer as described in section 9.5. Store at -20°C.

9.4. **Lysis Buffer:**

Prepare **Complete Lysis Buffer (CLB)** by adding 1 µL of 1 M DTT and 10 µL of Protease Inhibitor Cocktail to 1 mL of Lysis Buffer – see Quick Table for Reagent Preparation to see how much is required depending on number of tests. Use the CLB immediately for cell lysis. The remaining amount should be discarded if not used in the same day.

Store undiluted Lysis Buffer at 4°C.

9.5. **Binding Buffer:**

Prepare **Complete Binding Buffer (CBB)** by adding 2 µL of 1 M DTT and 10 µL of 1 µg/µL Herring Sperm DNA to 1 mL of Binding Buffer – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Discard remaining CBB if not used in the same day. Store undiluted Binding Buffer at 4°C.

9.6. **Wash Buffer:**

Prepare **1X Wash Buffer** by making a 1/10 dilution of 10X Wash Buffer in distilled water (ddH₂O) – see Quick Table for Reagent Preparation to see how much is required depending on number of tests. Mix gently to avoid foaming.

1X Wash Buffer can be stored at 4°C for one week. **NOTE:** *Tween 20 contained in the 10X Wash Buffer may form clumps. If this happens, homogenize buffer by vortexing for 2 minutes prior to use.*

Store undiluted 10X Wash Buffer at 4°C.

9.7. **Antibody Binding Buffer:**

Prepare **1X Antibody Binding Buffer (ABB)** by making a 1/10 dilution of 10X Antibody Binding Buffer in distilled water (ddH₂O) – see Quick Table for Reagent Preparation to see how much is required depending on number of tests. Mix gently to avoid foaming.

Discard remaining 1X ABB if not used in the same day. **NOTE:** *BSA contained in the 10X Antibody Binding Buffer may form clumps. If this happens, homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use.*

Store undiluted 10X Antibody Binding Buffer at 4°C.

9.8. **STAT1α Antibody:**

Dilute supplied STAT1α antibody 1/1000 in 1X ABB – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Aliquot and store undiluted STAT1α antibody at -20°C. Avoid multiple freeze/thaw cycles.

9.9. **STAT3 Antibody:**

Dilute supplied STAT3 antibody 1/1000 in 1X ABB – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Aliquot and store undiluted STAT3 antibody at -20°C. Avoid multiple freeze/thaw cycles.

9.10. **STAT5A Antibody:**

Dilute supplied STAT5A antibody 1/1000 in 1X ABB – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Aliquot and store undiluted STAT5A antibody at -20°C. Avoid multiple freeze/thaw cycles.

9.11. **STAT5B Antibody:**

Dilute supplied STAT5B antibody 1/1000 in 1X ABB – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Aliquot and store undiluted STAT5B antibody at -20°C. Avoid multiple freeze/thaw cycles.

9.12. **Anti-rabbit HRP-conjugated Antibody:**

Dilute supplied anti-rabbit HRP-conjugated antibody 1/1000 in 1X ABB – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Aliquot and store undiluted anti-rabbit HRP-conjugated antibody at 4°C. Avoid multiple freeze/thaw cycles.

9.13. **Developing Solution:**

Ready to use as supplied. Warm Developing Solution to room temperature 1 hour prior to use – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Store unused Developing Solution in the dark at 4°C. The Developing Solution may develop a yellow hue over time, but this does not affect product performance. A blue color present in the Developing Solution indicates that it has been contaminated and must be discarded.

9.14. **Stop Solution:**

Ready to use as supplied. Store unused Stop Solution at 4°C.

WARNING: Stop Solution is corrosive. Wear personal protective equipment when handling, i.e. safety glasses, gloves and labcoat.

9.15. Nb2 (Prolactin stimulated) nuclear extract (2.5 µg/µL):

Ready to use as supplied. The Nb2 (prolactin stimulated) extract gives a strong signal for both STAT5A and STAT5B. Extract has been optimized to be used at 5 µg/well. There is enough extract to perform 20 reactions per plate. Aliquot extract in 5 µL fractions and store at -80°C. Avoid multiple freeze/thaw cycles.

9.16. Control oligonucleotides (wild-type & mutated):

Oligonucleotides are provided to monitor the specificity of the assay.

Wild-type oligonucleotide: competes with sample nuclear extracts for STAT (STAT1alpha, STAT3, STAT5A, STAT5B) consensus binding site.

Mutated oligonucleotide: no effect on ability of sample nuclear extracts to bind to STAT (STAT1alpha, STAT3, STAT5A, STAT5B) consensus binding site.

Use wild-type and/or mutated oligonucleotide at 20 pmol/well: dilute 2 µL appropriate oligonucleotide in 31.8 µL of CBB (section 9.5) per well used – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Aliquot undiluted oligonucleotides and store at -20°C. Avoid multiple freeze/thaw cycles.

9.17. 96-well assay plate:

Ready to use as supplied.

Store unused strips in the aluminium pouch at 4°C.

9.18. Plate sealer:

Ready to use as supplied. Store at room temperature.

ASSAY PREPARATION

Quick Table for Reagent Preparation

Reagents to prepare	Components	1 well	1 strip (8 wells)	6 strips (48 wells)	12 strips (96 wells)
Complete Lysis Buffer (CLB)	DTT	0.02 μ L	0.2 μ L	1.2 μ L	2.4 μ L
	PIC	0.23 μ L	1.8 μ L	10.8 μ L	21.6 μ L
	Lysis Buffer	22.25 μ L	178.0 μ L	1.068 mL	2.136 mL
	TOTAL REQUIRED	22.5 μL	180.0 μL	1.08 mL	2.16 mL
Complete Binding Buffer (CBB)	DTT	0.07 μ L	0.54 μ L	3.2 μ L	6.5 μ L
	Herring Sperm DNA	0.34 μ L	2.7 μ L	16.2 μ L	32.4 μ L
	Binding Buffer	33.39 μ L	266.8 μ L	1.6 mL	3.2 mL
	TOTAL REQUIRED	33.8 μL	270 μL	1.62 mL	3.24 mL
Oligo (wt or mutated) in CBB	Wt or mutated oligo	4 μ L	32 μ L	192 μ L	N/A
	CBB	41 μ L	328 μ L	1.97 mL	N/A
	TOTAL REQUIRED	45 μL	360 μL	2.16 mL	N/A
1X Wash Buffer	ddH ₂ O	2.025 mL	16.2 mL	97.2 mL	194.4 mL
	10X Wash Buffer	225 μ L	1.8 mL	10.8 mL	21.6 mL
	TOTAL REQUIRED	2.25 mL	18 mL	108 mL	216 mL

ASSAY PREPARATION

Reagents to prepare	Components	1 well	1 strip (8 wells)	6 strips (48 wells)	12 strips (92 wells)
1X Ab Buffer*	ddH ₂ O	202.5 µL	1.62 mL	9.72 mL	19.44 mL
	10x ABB	22.5 µL	180 µL	1.08 mL	2.16 mL
	TOTAL REQUIRED	225 µL	1.8 mL	10.8 mL	21.6 mL
1° Ab STAT1α 1/1000 Dilution	STAT1α Ab	0.11 µL	0.9 µL	5.4 µL	10.8 µL
	1X ABB	110 µL	900 µL	5.4 mL	10.8 mL
	TOTAL REQUIRED	110.11 µL	900.9 µL	5.4 mL	10.8 mL
1° Ab STAT3 1/1000 Dilution	STAT3 Ab	0.11 µL	0.9 µL	5.4 µL	10.8 µL
	1X ABB	110 µL	900 µL	5.4 mL	10.8 mL
	TOTAL REQUIRED	110.11 µL	900.9 µL	5.4 mL	10.8 mL
1° Ab STAT5A 1/1000 Dilution	STAT5A Ab	0.11 µL	0.9 µL	5.4 µL	10.8 µL
	1X ABB	110 µL	900 µL	5.4 mL	10.8 mL
	TOTAL REQUIRED	110.11 µL	900.9 µL	5.4 mL	10.8 mL
1° Ab STAT5B 1/1000 Dilution	STAT5B Ab	0.11 µL	0.9 µL	5.4 µL	10.8 µL
	1X ABB	110 µL	900 µL	5.4 mL	10.8 mL
	TOTAL REQUIRED	110.11 µL	900.9 µL	5.4 mL	10.8 mL

*Volumes listed refer to preparation of buffer for diluting both primary and secondary antibodies.

ASSAY PREPARATION

Reagents to prepare	Components	1 well	1 strip (8 wells)	6 strips (48 wells)	12 strips (92 wells)
2° Ab 1/1000 Dilution	HRP-conj Ab	0.11 µL	0.9 µL	5.4 µL	10.8 µL
	1X ABB	110 µL	900 µL	5.4 mL	10.8 mL
	TOTAL REQUIRED	110.11 µL	900.9 µL	5.4 mL	10.8 mL
Developing Solution	TOTAL REQUIRED	112.5 µL	900 µL	5.4 mL	10.8 mL
Stop Solution	TOTAL REQUIRED	112.5 µL	900 µL	5.4 mL	10.8 mL

10. SAMPLE PREPARATION

- We recommend using our Nuclear Extraction Kit (ab113474) to prepare nuclear extracts, as it contains all necessary buffers and will help to reduce inconsistencies in the assay that may arise from using homemade or other buffers.
- Alternatively, you can refer to the protocol below.

10.1. Prepare reagents needed:

10X PBS

For 250 mL:

0.1 M Phosphate Buffer, pH 7.5

3.55g Na_2HPO_4 +

0.61g KH_2PO_4

1.5 M NaCl

21.9 g

27 mM KCl

0.5 g

Adjust to 250 mL with ddH₂O. Prepare a 1X PBS solution dilute 10X PBS solution 1/10 in ddH₂O.

Sterilize 1X PBS pH 7.5 solution by filtering through a 0.2 μm filter. Store filter-sterilized solution at 4°C.

PIB (Phosphatase Inhibitor Buffer)

For 10 mL:

125 mM NaF

52 mg

250 M β -glycerophosphate

0.55 g

250 mM PNPP

1.15 g

25 mM NaVO_3

31 mg

Adjust to 10 mL with ddH₂O. Mix the chemical by vortexing. Incubate solution at 50°C for 5 minutes. Mix again. Store at -20°C.

HB (Hypotonic Buffer)

For 50 mL:

20 mM Hepes, pH 7.5

0.24 g

5 mM NaF

12 mg

10 μM Na_2MoO_4

5 μL of 0.1 M solution

0.1 mM EDTA

10 μL of 0.5 M solution

Adjust pH to 7.5 with 1 N NaOH. Adjust volume to 50 mL with ddH₂O. Sterilize by filtering through a 0.2 μm filter. Store filter-sterilized solution at 4°C.

ASSAY PREPARATION

PBS/PIB: prior to use, add 0.5 mL of PIB to 10 mL of 1X PBS.

- 10.2. Use cells from a confluent 100-mm dish / 75 cm² flask. 1 x 10⁷ cells yield approximately 0.5 mg of nuclear extract.
- 10.3. Wash cells with 10 mL of ice-cold PBS/PIB solution.
- 10.4. Add 10 mL of ice-cold PBS/PIB and scrape cells off the dish with a cell scraper. Transfer cells to a pre-chilled 15 mL tube and centrifuge at 300 x *g* for 5 minutes at 4°C in a pre-chilled centrifuge. Discard supernatant.
- 10.5. Resuspend pellet in 1 mL of ice-cold HB buffer by gently pipetting and transfer the cells into a pre-chilled 1.5 mL tube.
- 10.6. Allow cells to swell on ice for 15 minutes.
- 10.7. Add 5 µL 10% NP-40 (0.5% final) and mix by gently pipetting.
- 10.8. Centrifuge homogenate for 30 seconds at 4°C in a microcentrifuge. Discard supernatant.
- 10.9. Resuspend nuclear pellet in 50 µL Complete Lysis Buffer (see section 9.4) and rock the tube gently on ice for 30 minutes on a shaking platform.
- 10.10. Centrifuge for 10 minutes at 14,000 x *g* at 4°C and save supernatant (nuclear extract).
- 10.11. Determine protein concentration of the extract by using a Bradford-based assay. We recommend BCA Protein Quantification Kit (ab102536).
- 10.12. Aliquot and store nuclear extracts at -80°C. Avoid multiple freeze/thaw cycles.

11. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to correct temperature prior to use.
- We recommend to assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- If less than 8 strips are used, cover the unused wells with a portion of the plate sealer while performing the assay. The content of these wells is stable at RT if kept dry and can therefore be used later for a separate assay. Use strip holder for the assay.

11.1. STAT binding to its consensus sequence

11.1.1. Add 30 μ L CBB containing wt or mutated oligo (Step 9.16) to the Competitive Binding Control wells.

11.1.2. Add 30 μ L CBB to each of the other wells.

11.1.3. Prepare sample and control wells:

Competitive Binding control wells = 20 μ L of sample diluted in CLB (use 2 – 20 μ g of nuclear extract/well).

Sample wells = 20 μ L of sample diluted in CLB (use 2 – 20 μ g of nuclear extract/well).

Positive control wells = 2 μ L of provided Nb2 (Prolactin stimulated) nuclear extract + 18 μ L CLB (5 μ g nuclear extract per well).

Blank wells = 20 μ L CLB only.

11.1.4. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at RT with mild agitation (100 rpm on a rocking platform).

11.1.5. Wash each well 3 times with 200 μ L 1X Wash Buffer. For each wash, flick plate over a sink to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.

11.2. Primary antibody binding

- 11.2.1. Add 100 μ L diluted antibodies (1/1000 dilution for STAT1, STAT3, STAT5A and STAT5B in 1X ABB) to all wells being used.
- 11.2.2. Cover plate and incubate for 1 hour at RT without agitation.
- 11.2.3. Wash each well 3 times with 200 μ L 1X Wash Buffer. For each wash, flick plate over a sink to empty the wells, then tap the inverted plate 3 times on absorbent paper towels.

11.3. Secondary antibody binding

- 11.3.1. Add 100 μ L diluted anti-rabbit HRP-antibody (1/1000 dilution in 1X ABB) to all wells being used.
- 11.3.2. Cover plate and incubate for 1 hour at RT without agitation.
- 11.3.3. During this incubation, place Developing Solution at RT.
- 11.3.4. Wash each well 4 times with 200 μ L 1X Wash Buffer. For each wash, flick plate over a sink to empty the wells, then tap the inverted plate 3 times on absorbent paper towels

11.4. Measurement

- 11.4.1. Add 100 μ L RT Developing Solution to all wells being used.
- 11.4.2. Incubate 2 – 10 minutes at RT protected from direct light. Optimal incubation time will vary for each experiment depending on amount of transcription factor present in the sample. Monitor the blue color development in the sample wells until it turns medium to dark blue. Do not overdevelop.

NOTE: *Positive control wells may need to overdevelop to allow adequate color development in sample wells.*

- 11.4.3. Add 100 μ L Stop Solution to all wells being used. The solution within the wells will change from blue to yellow after adding the Stop Solution (due to presence of acid in Stop Solution).
- 11.4.4. Read absorbance on a spectrophotometer at OD 450 nm within 5 minutes, with a reference wavelength of OD 665 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

12. TYPICAL DATA

TYPICAL DATA – Data provided for **demonstration purposes only**.

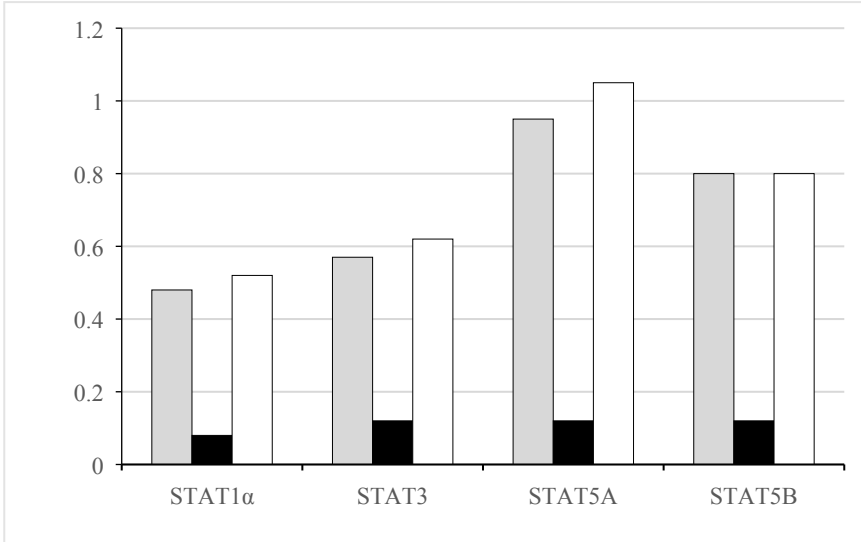


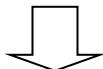
Figure 1 Nuclear extracts from Nb2 cells stimulated with prolactin) were assayed for activity of STAT family members: STAT1alpha, STAT3, STAT5A, STAT5B in the absence (grey) or presence of wild-type (black) or mutated (white) consensus binding oligonucleotides. Note that the wild-type oligonucleotide reduces STAT binding by over 90% while incubation with the mutant STAT competitor oligo has limited effect on STAT binding to DNA. These results are provided for demonstration purposes only.

13. QUICK ASSAY PROCEDURE

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

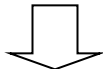
Sample binding to consensus sequence

- Add 30 μ L CBB + wt/mutant oligo to Competitive Binding control wells. Add 30 μ L CBB to each of the other wells used.
- Add 20 μ L sample, positive control and blank to the relevant wells.
- Incubate 1 hour RT with mild agitation (100 rpm on a rocker).
- Wash each well 3 times with 200 μ L 1X Wash Buffer.



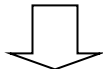
Primary antibody binding

- Add 100 μ L diluted primary antibody (1/1000) to each well.
- Incubate 1 hour RT with no agitation.
- Wash each well 3 times with 200 μ L 1X Wash Buffer.



Secondary antibody binding

- Add 100 μ L diluted HRP antibody (1/1000) to each well.
- Incubate 1 hour RT with no agitation.
- Wash each well 4 times with 200 μ L 1X Wash Buffer.



Measurement

- Add 100 μ L RT Development Solution to each well.
- Incubate 2 – 10 minutes RT protected from light.
- Add 100 μ L Stop Solution into each well.
- Measure OD 450 nm (reference wavelength 655 nm).

14. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in any well	Omission of key reagent	Check that all reagents have been added in the correct order
	Substrate or conjugate in no longer active	Test conjugate and substrate for activity
	Enzyme inhibitor present	Sodium azide will inhibit peroxidase reaction; do not add to buffers
	Plate reader settings not optimal	Verify the wavelength and filter settings in the plate reader
	Incorrect assay temperature	Bring reagents to room temperature
No Signal or weak signal in sample wells	Not enough nuclear extract per well	Increase amount of nuclear extract – do not exceed 50 µg/well
	STAT factors are poorly activated or inactivated	Perform a time course for STAT activation in the studied cell line
	Extracts are not from correct species	This product detects bound STAT factors in human and rat samples
High background in all wells	Developing time too long	Stop enzymatic reaction as soon the positive wells turn medium-dark blue
	Concentration of antibodies too high	Increase antibody dilutions
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
High background in sample wells	Too much nuclear extract per well	Decrease amount of nuclear extract to 1 – 2 µg/well
	Concentration of antibodies too high	Perform antibody titration to determine optimal concentration. Start with 1/2000 for 1° Ab and 1/5000 for 2° Ab. Assay sensitivity will be decreased
Uneven color development	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Well cross-contamination	Follow washing recommendations

15.INTERFERENCES

These chemicals or biological materials will cause interference in this assay causing compromised results or complete failure:

- Sodium azide – it will inhibit the peroxidase reaction. Do not add to any buffer to be used in this assay.

16.NOTES

Technical Support

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