TF-Detect™ AP-1/c-Jun Activity Assay Kit

For rapid and sensitive detection of active c-Jun in human, mouse or rat samples

Cat. No. TF002 (1 plate, 96 reactions)

User Manual

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USER MANUAL

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I. Introduction and Principle

c-Jun, together with Fos and other Jun-related proteins, forms a homodimeric or heterodimeric transcription factor complex AP-1.

AP-1 binds to a TPA DNA Response Element (TRE), the heptamer enhancer motif 5'-TGA[C/G]TCA-3', and regulates gene expression in response to a variety of stimuli including growth factors, tumor promoters, and cytokines. AP-1 controls cellular processes including differentiation, proliferation, apoptosis and stress response.

c-Jun is tightly regulated post-translationally through phosphorylation. It has several phosphorylation sites. The C-terminal one is near the DNA binding domain of c-Jun and its phosphorylation represses c-Jun activity during the resting condition. The other two phosphorylation sites, Ser63 and Ser73, are near the transactivation domain of c-Jun. SAPK/JNKs activate c-Jun by phosphorylating Ser63 and Ser73.

TF-Detect™ AP-1/c-Jun Activity Assay kit enables fast and sensitive detection of Ser73-phosphorylated c-Jun in human, mouse or rat samples in a 96-well format. Double-stranded oligonucleotides containing the AP-1/c-Jun consensus binding site are immobilized in the 96-well plate. The c-Jun proteins present in nuclear extracts are captured by the immobilized oligonucleotides specifically. The Ser73-phosphorylated c-Jun is detected by a phosphor-c-Jun antibody and a HRP-conjugated secondary antibody. The colorimetric signal generated by HRP substrate TMB can be easily quantified by spectrophotometry. The Ser73 phospho-c-Jun antibody that detects Ser73-phosphorylated c-Jun also recognizes Ser100-phosphorylated JunD, as this site is conserved between c-Jun and JunD.

Protocol overview

Rinse 96-well plate (coated with AP-1/c-Jun binding oligonucleotides)
↓
Bind c-Jun (in nuclear extract) to the immobilized oligonucleotides
↓ 1 hour
Wash 3X. Add c-Jun antibody
↓ 1 hour
Wash 3X. Add HRP-conjugated IgG antibody
↓ 1 hour
Wash 3X. Colorimetric reaction

Total ~3.5 hours

Key advantages

- **Multiple species** – Detects human, mouse or rat activated c-Jun.
- **HTS compatible** - Optimized for use with 96-well plate readers for high-throughput screening assays. Single strip (8-well) assay can also be performed.
- **Fast** - 3.5 hours from preparation to detection.
- **Sensitive** – Detect Ser73-phosphorylated c-Jun in as low as 0.2 µg of nuclear lysate (Figure 1).

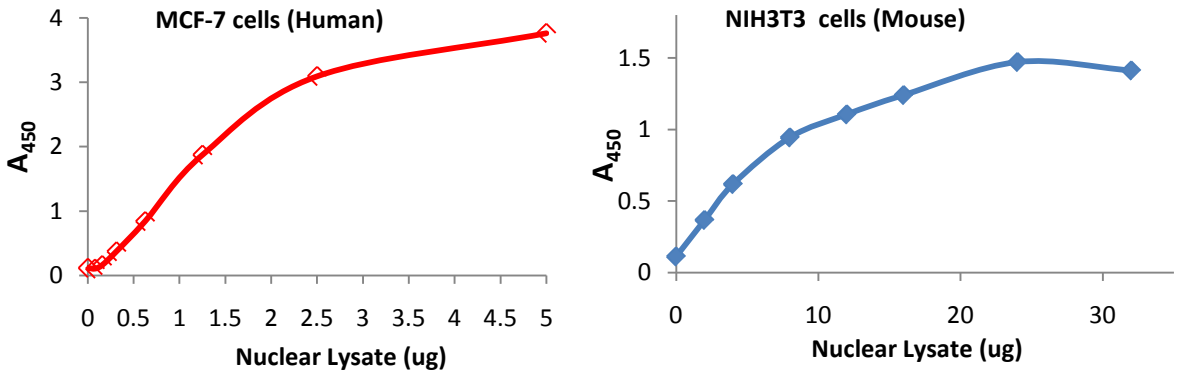

Figure 1. The activities of c-Jun proteins in the nuclear extracts of MCF-7 (left) and NIH3T3 (right) cells were detected using the TF-Detect AP-1/c-Jun Activity Assay Kit. Both cell types were treated with UV light for 20 Sec before harvesting. The cellular nuclear extracts were prepared following the Preparation of Nuclear Extract protocol in the Appendix.

II. Kit Components and Storage

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Components	Quantity	Storage temperature
c-Jun Antibody	15 µl	-20°C Stable for at least 6 months
HRP-Conjugated IgG	15 µl	-20°C Stable for at least 6 months
MCF-7 Nuclear Lysates (Positive Control)	25 µl	-20°C Stable for at least 6 months
Dithiothreitol (DTT) (1M)	100 µl	-20°C Stable for at least 6 months
10x Binding Buffer A	1.5 ml	-20°C Stable for at least 6 months
10x Binding Buffer B	1.5 ml x 2	-20°C Stable for at least 6 months
10x Wash Buffer	25 ml	4°C Stable for at least 6 months
TMB Substrate Solution	12 ml	4°C Stable for at least 6 months
Stop Solution	12 ml	4°C Stable for at least 6 months
96-Well AP-1 Assay Plate (12 Strips)	1 plate	4°C Stable for at least 6 months

Materials required but not provided

5 ml and 10 ml graduated pipettes, beakers, flasks, and cylinders
10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
50 µl to 300 µl adjustable multichannel micropipette, disposable tips, and reservoir
Micro-plate reader capable of reading at 450 nm (620 nm as optional reference wave length)

III. Preparation of Reagents

Prepare a little bit extra than required amount to make sure enough buffers are available for experiments. Required amount of reagents per well:

Reagent	1 well
Nuclear extract sample	10 µl
MCF-7 nuclear lysate (positive control)	2 µl
1x binding buffer A (with DTT at 2 mM)	50 µl
1x wash buffer	2 ml
c-Jun antibody in 1x binding buffer B (1:1000)	100 µl
HRP IgG antibody in 1x binding buffer B (1:1000)	100 µl
TMB substrate	100 µl
Stop solution	100 µl

Nuclear extract samples

We recommend using 10 µl of nuclear extract at 0.2 - 5 µg/µl for each sample well. The total protein amount is 2-50 µg per well. **Note:** The recommended amount is provided as guidance. A broader range of value may be used.

C-Jun positive control

2 µl of the MCF-7 Nuclear Lysate can be used as the positive control.

Binding buffer A and B

Warm up 10x Binding Buffer A and B to room temperature and mix well before use. To prepare 100 ml of 1x Binding Buffer, add 10 ml of the 10x buffer to 90 ml distilled or deionized water. Mix gently to avoid foaming. The 1x Binding Buffers are stable for 30 days at 4°C. Add 200 µl of 1M DTT to 100 ml of 1x Binding Buffer A before use (2mM DTT – final concentration).

Wash buffer

Warm up 10x Wash Buffer to room temperature and mix well before use. If crystals have formed in the 10x buffer, warm and mix gently until they have completely dissolved. To prepare 200 ml of 1x Wash Buffer for one 96-well plate assay, add 20 ml of the 10x Wash Buffer into 180 ml distilled or deionized water. Mix gently to avoid foaming. The 1x Wash Buffer is stable for 30 days at 4°C. It can be stored at -20°C for longer time.

Primary c-Jun antibody

Calculate the amount of primary c-Jun antibody needed to perform the desired experiments and make 1:1000 dilutions with 1x Binding Buffer B (100 µl/well).

Secondary HRP conjugated antibody

Calculate the amount of HRP conjugated antibody needed to perform the desired experiments and make 1:1000 dilution with 1x Binding Buffer B (100 µl/well).

TMB substrate solution

Take appropriate volume of TMB Substrate (100 µl/well) and warm it up to room temperature 1 hour before use. **Note:** Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation. The TMB Substrate Solution may develop a yellow tinge over time. This does not affect the product performance. A blue color in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. After use, discard remaining TMB substrate solution.

Stop solution

Prior to use, take appropriate volume of Stop Solution (100 µl/well) and warm it up to room temperature before use. After use, discard remaining Stop Solution.

IV. Procedure

Determine the number of wells and strips needed. Store the unused strips at 4°C.

1. Mix all reagents thoroughly yet gently to avoid foaming before use.
2. Rinse the 96-well plate with approximately 200 µl Wash Buffer per well. Empty and tap the plate on absorbent pad or paper towel to remove excess buffer. Be careful not to scratch the surface of the 96-well plate.

   Note: Use the plate immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes.
3. Add c-Jun samples to the wells as following:

   Nuclear extract wells: Add 50 µl of Binding Buffer A (with 2mM DTT) to the wells, and then add 10 µl samples (2-50 µg of proteins). Mix well. Duplicated wells for each sample are recommended.

   Positive control wells Add 58 µl of Binding Buffer A (with 2mM DTT) to the wells, and then add 2 µl of MCF-7 Nuclear Lysate per well. Mix well.

   Blank wells: Add 60 µl of Binding Buffer A (with 2mM DTT).
4. Cover the plate with a plate cover. Incubate at room temperature for 1 hour by gently rocking the plate.
5. Remove the plate cover and empty the wells. Wash the plate using 200µl/well of 1X Wash Buffer by gently rocking it for one minute, and then empty and tap the plate on absorbent pad or paper towel to remove excess buffer. Repeat the wash step twice.
6. Add 100 µl of 1:1000 diluted c-Jun primary antibody in 1X Binding Buffer B to each well, including the blank wells. Cover the plate with a plate cover. Incubate at room temperature for 1 hour by gently rocking the plate.
7. Remove the plate cover and empty the wells. Wash the plate with 200 µl/well of 1X Wash Buffer by gently rocking it for one minute. Then empty and tap the plate on absorbent pad or paper towel to remove excess buffer. Repeat the wash step twice.
8. Add 100 µl of 1:1000 diluted HRP conjugated antibody in 1X Binding Buffer B to each well, including the blank wells. Cover the plate with a plate cover. Incubate at room temperature for 1 hour by gently rocking the plate.

9. Remove the plate cover and empty the wells. Wash the plate with 200 µl/well of 1X Wash Buffer by gently rocking it for one minute. Then empty and tap the plate on absorbent pad or paper towel to remove excess buffer. Repeat the wash step twice.
10. Add 100 µl of TMB Substrate Solution (equilibrated to room temperature) to each well (including the blank wells) and mix well. Incubate the plate at room temperature for about 5-15 minutes.
11. Add 100 µl Stop Solution (equilibrated to room temperature) to each well (including the blank wells) and mix well. Read the plate at 450nm within 5 minutes using a microwell plate reader.

V. Appendix

Preparation of Nuclear Extract

1. Aspirate medium from a 10 cm plate and wash the cells with ice-cold PBS.
2. Add 1 ml of ice-cold PBS (per 10 cm plate, 4 - 8 x 10^6 cells). Scrape the cells into PBS and then transfer them into a pre-chilled eppendorf tube.
3. Spin at 4ºC, 2,000 rpm for 5 minutes. Discard the supernatant.
4. Resuspend the cell pellet in 200 µl of **Buffer 1 (**per 10cm plate, scale up or down proportionally for other size culture vessels). Incubate on ice for 15 minutes to allow cells to swell.
5. Add 20 µl of **Buffer 2** to the cells. Vortex for 10 seconds. Then incubate on ice for 5 minutes.
6. Spin at 4ºC, 14,000 rpm for 3 minutes.
7. Transfer the supernatant (cytoplasmic proteins) into a new eppendorf tube.
8. Add another 200 µl of **Buffer 1** to the cell pellet and mix gently.
9. Spin at 4ºC, 14,000 rpm for 3 minutes.
10. Transfer and combine the supernatant in the cytoplasmic protein tube from step 7.
11. Resuspend the pellet with 200 µl of ice-cold **Buffer 3.** Vortex for 30 seconds. (Optional: Sonicate for 2-3 seconds to break down the pellet.) Then rotate vigorously at 4ºC for 30 minutes.
12. Spin at 4ºC, 14,000 rpm for 10 minutes.
13. Transfer the supernatant (nuclear proteins) into a fresh, pre-chilled tube. Measure the protein concentrations. Leave on ice if use immediately, or store aliquots at -80ºC until use.

Buffer 1: 25 mM HEPES, pH 7.4
10 mM KCl
1.5 mM $MgCl_2$
1 mM DTT
10 mM PMSF

Buffer 2: 10% IGEPAL (Igepal CA-630, Sigma)

Buffer 3: 25 mM HEPES, pH 7.4
420 mM NaCl
25% Glycerol
1.5 mM $MgCl_2$
0.2 mM EDTA
1 mM PMSF

VI. Limited Use License and Warranty

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