# ab207215
# NFATc1 Transcription Factor Assay Kit (Colorimetric)

Instructions for use:

For quantitative measurement of NFATc1 activation in human and mouse nuclear extracts.

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

Version 2a, Last Updated 31 January 2024

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## 1. BACKGROUND

NFATc1 Transcription Factor Assay Kit (Colorimetric) (ab207215) is a high throughput assay to quantify Nuclear Factor of Activated T cells 2 (NFAT2 or NFATc1) activation. 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 NFATc1 consensus binding site (5' – AGGAAA – 3') has been immobilized onto a 96-well plate. Active NFATc1 present in the nuclear extract specifically binds to the oligonucleotide. NFATc1 is detected by a primary antibody that recognizes an epitope of NFATc1 accessible only when the protein is activated and bound to its target DNA. An HRP-conjugated secondary antibody provides sensitive colorimetric readout at OD 450 nm. This product detects human and mouse NFATc1.

Key performance and benefits:

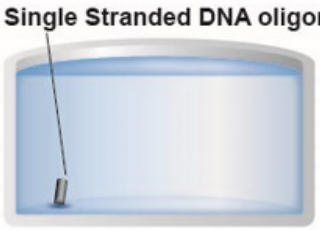
- Assay time: 3.5 hours (cell extracts preparation not included).
- Detection limit: < 0.6 µg nuclear extract/well.
- Detection range: 0.6 – 10 µg nuclear extract/well.

Nuclear Factor Activated T cells (NFAT) proteins are transcription factors that were first identified as inducers of the immune response. These proteins also play varied roles in cell differentiation and adaptation for vascular endothelial cells or skeletal muscle cells. There are four NFAT family members, NFAT1-4 (NFAT2 is also called NFATc or NFATc1), from which numerous isoforms are generated by alternative splicing. NFAT mRNAs are found in peripheral blood lymphocytes, spleen (NFAT1 and 2) and thymus (NFAT4). NFATs modulate the expression of numerous cytokines such as interleukines, GM-CSF, IFNα, IFNγ and CD40L. In resting cells, NFATc1 is confined to the cytoplasm, where it is maintained in a phosphorylated state by the action of constitutive kinases. Upon stimulation, NFATc1 is dephosphorylated by calcineurin and migrates to the nucleus. NFATc1 dephosphorylation is stimulated by Ca2+-coupled membrane receptors, such as T cell and B cell receptors, and the CD40 and G protein-associated receptors (thrombin or H1 histamine receptors).

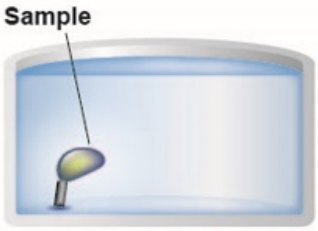
NFATc1 can also be activated by calcium ionophores. Cyclosporin A and FK506 immunosuppressor drugs inhibit calcineurin activity on NFAT. When calcium levels drop, calcineurin becomes inactive and NFATc1 is rephosphorylated by kinases and exported back into the cytoplasm.

NFAT phosphoproteins share two conserved domains: a DNA-binding domain (DBD) displaying limited similarity to the Rel protein family DBD, and modulating interactions with AP-1 dimers; and a NFAT homology region (NHR), upstream of the DBD that regulates translocation and DNA-binding activity. Transactivation domains can be found at the N- and C-terminal ends of the NFAT proteins. NFATs bind to the DNA consensus motif 5´-T/AGGAAA-3´ as monomers. NFATs can cooperatively interact with AP-1 and GATA proteins for DNA binding. Vitamin D3 receptor heterodimers (RXR:VDR) can abolish NFAT modulation of IL-2 by binding to a site which overlaps the NFAT distal site. Sites in IL-2 and GM-CSF promoters can accommodate NFAT and the Ets-family member, Elf-1.

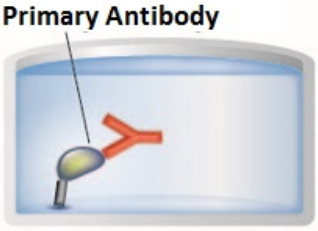
## 2. ASSAY SUMMARY



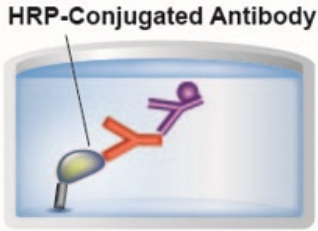

Prepare all reagents, nuclear extracts and controls as instructed. Plate is supplied pre-coated with an oligonucleotide containing NFATc1 consensus binding site.



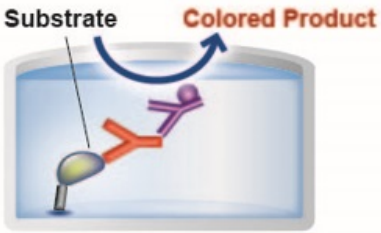

Add sample (nuclear extracts containing activated transcription factor) to appropriate wells. Incubate plate for 1 hour at RT.




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 -20ºC (nuclear extract must be kept at -80ºC) 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.

## 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) |
|---|---|---|---|---|
| | 1 Plate | 5 Plates | | |
| NFATc1 antibody (0.2 µg/µL) | 22 µL | 110 µL | -20°C | 4°C |
| Anti-mouse HRP-conjugated IgG (0.4 µg/µL) | 11 µL | 55 µL | -20°C | 4°C |
| Wild-type oligonucleotide (10 pmol/µL) | 100 µL | 500 µL | -20°C | -20°C |
| Mutated oligonucleotide (10 pmol/µL) | 100 µL | 500 µL | -20°C | -20°C |
| Jurkat nuclear extract (PHA treated) (2.5 µg/µL) | 40 µL | 200 µL | -80°C | -80°C |
| Dithiothreitol (DTT) | 100 µL | 500 µL | -20°C | -20°C |
| Protease Inhibitor Cocktail | 100 µL | 500 µL | -20°C | -20°C |
| Lysis Buffer | 10 mL | 50 mL | -20°C | 4°C |
| Binding Buffer | 10 mL | 50 mL | -20°C | 4°C |
| 10X Wash Buffer | 22 mL | 110 mL | -20°C | 4°C |
| 10X Antibody Binding Buffer | 2.2 mL | 11 mL | -20°C | 4°C |
| Developing Solution | 11 mL | 55 mL | -20°C | 4°C |
| Stop Solution | 11 mL | 55 mL | -20°C | 4°C |
| 96-well assay plate | 1 | 5 | -20°C | 4°C |
| Plate sealer | 1 | 5 | -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 (dd$H_2O$)
- 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 µM $Na_2MoO_4$, 0.1 mM EDTA)
- Phosphatase Inhibitors (NaF, β-glycerophosphatase, PNPP, $NaVO_3$)
- 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 9.3 and 9.4 respectively. Store at -20°C.

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

Ready to use as supplied. Dilute in Lysis Buffer as described in section 9.3. Store at -20°C.

9.3. **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.

9.4. **Binding Buffer:**

Ready to use as supplied – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Store undiluted Binding Buffer at 4°C.

9.5. **Wash Buffer:**

Prepare **1X Wash Buffer** by making a 1/10 dilution of 10X Wash Buffer in distilled water ($ddH_2O$) – 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.

9.6. **Antibody Binding Buffer:**

Prepare **1X Antibody Binding Buffer (ABB)** by making a 1/10 dilution of 10X Antibody Binding Buffer in distilled water ($ddH_2O$) – 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.

9.7. **NFATc1 Antibody (0.2 µg/µL):**

Dilute supplied NFATc1 antibody in 1/500 in 1X ABB – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

Aliquot and store undiluted NFATc1 antibody at 4°C. Avoid multiple freeze/thaw cycles.

9.8. **Anti-mouse HRP-conjugated Antibody (0.4 µg/µL):**

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.

9.9. **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.

9.10. **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 lab coat.*

9.11. **Jurkat (PHA treated) nuclear extract (2.5 µg/µL):**

Ready to use as supplied. 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 -80ºC. Avoid multiple freeze/thaw cycles.

9.12. **Control oligonucleotides (wild-type & mutated):**

Oligonucleotides are provided to monitor the specificity of the assay.

Wild-type oligonucleotide: competes with sample nuclear extracts for NFATc1 consensus binding site.

Mutated oligonucleotide: no effect on ability of sample nuclear extracts to bind to NFATc1 consensus binding site.

Use wild-type and/or mutated oligonucleotide at 20 pmol/well: dilute 2 µL appropriate oligonucleotide in 43 µL of BB (section 9.4) 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.

9.13. **96-well assay plate:**

Ready to use as supplied.

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

9.14. **Plate sealer:**

Ready to use as supplied. Store at room temperature.

### 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 | 0.01 µL | 0.1 µL | 0.6 µL | 1.2 µL |
| | PIC | 0.12 µL | 0.9 µL | 5.4 µL | 10.8 µL |
| | Lysis Buffer | 11.12 µL | 89.0 µL | 534 µL | 1.068 mL |
| | **TOTAL REQUIRED** | **11.25 µL** | **90.0 µL** | **540 µL** | **1.08 mL** |
| Binding Buffer (BB) | **TOTAL REQUIRED** | **45.0 µL** | **360 µL** | **2.16 mL** | **4.32 mL** |
| Oligo (wt or mutated) in BB | Wt or mutated oligo | 2 µL | 16 µL | 96 µL | N/A |
| | BB | 43.0 µL | 344 µL | 2.064 µL | N/A |
| | **TOTAL REQUIRED** | **45.0 µL** | **360 µL** | **2.16 µL** | **N/A** |
| 1X Wash Buffer | $ddH_2O$ | 2.025 mL | 16.2 mL | 97.2 mL | 194.4 mL |
| | 10X Wash Buffer | 225 µL | 1.8 mL | 10.8 mL | 21.6 mL |
| | **TOTAL REQUIRED** | **2.25 mL** | **18 mL** | **108 mL** | **216 mL** |

| Reagents to prepare | Components | 1 well | 1 strip (8 wells) | 6 strips (48 wells) | 12 strips (92 wells) |
|---|---|---|---|---|---|
| 1X Ab Buffer* | $ddH_2O$ | 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 1/500 Dilution | NFATc1 Ab | 0.22 µL | 1.6 µL | 10.8 µL | 21.6 µL |
| | 1X ABB | 110 µL | 900 µL | 5.189 mL | 10.78 mL |
| | **TOTAL REQUIRED** | **110.2 µL** | **901.6 µL** | **5.2 mL** | **10.8 mL** |
| 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** |

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

## 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 $ddH_2O$. Prepare a 1X PBS solution dilute 10X PBS solution 1/10 in $ddH_2O$.

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 $ddH_2O$. 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 $ddH_2O$. Sterilize by filtering through a 0.2 µm filter. Store filter-sterilized solution at 4°C.

**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 $cm^2$ flask. 1 x $10^7$ 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 9.3) 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. NFATc1 binding to its consensus sequence

11.1.1. Add 40 µL CBB containing wt or mutated oligo (Step 9.12) to the Competitive Binding Control wells.

11.1.2. Add 40 µL CBB to each of the other wells.

11.1.3. Prepare sample and control wells:

**Competitive Binding control wells** = 10 µL of sample diluted in CLB (use 2 – 10 µg of nuclear extract/well).

**Sample wells** = 10 µL of sample diluted in CLB (use 2 – 10 µg of nuclear extract/well).

**Positive control wells =** 2 µL of provided Jurkat nuclear extract + 8 µL CLB (5 µg nuclear extract per well).

**Blank wells** = 10 µL 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 µL 1X Wash Buffer. For each wash, flick plate over a sink to empty the wells, and then tap the inverted plate 3 times on absorbent paper towels.

11.2. **Primary antibody binding**

11.2.1. Add 100 µL diluted antibody (1/500 dilution 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 µL 1X Wash Buffer. For each wash, flick plate over a sink to empty the wells, and then tap the inverted plate 3 times on absorbent paper towels.

11.3. **Secondary antibody binding**

11.3.1. Add 100 µL diluted anti-mouse 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 µL 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 µL 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 µL 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**.

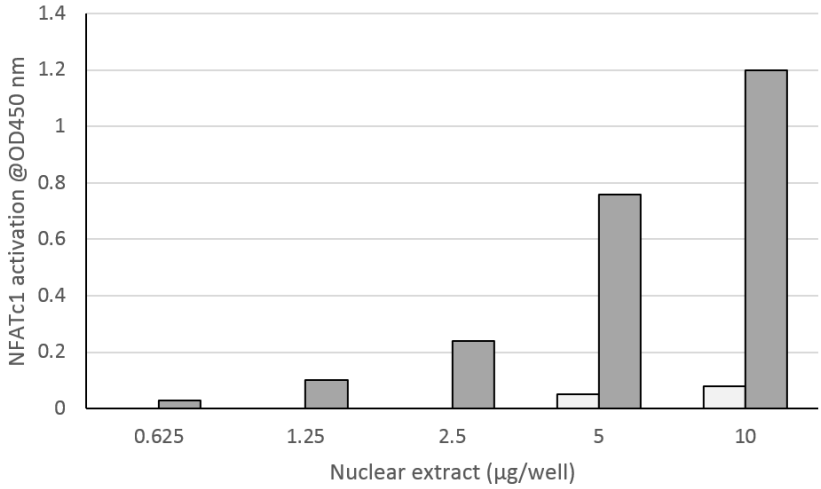


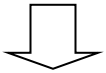

**Figure 1** Different amounts of nuclear extract from untreated (light grey) and PHA treated (dark grey) Jurkat cells were tested for NFATc1 activation. This data is provided for demonstration only.

## 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.*

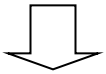
**Sample binding to consensus sequence**

- Add 40 µL CBB + wt/mutant oligo to Competitive Binding control wells. Add 40 µL CBB to each of the other wells used.
- Add 10 µL 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 µL 1X Wash Buffer.



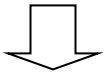
**Primary antibody binding**

- Add 100 µL diluted primary antibody (1/500) to each well.
- Incubate 1 hour RT with no agitation.
- Wash each well 3 times with 200 µL 1X Wash Buffer.



**Secondary antibody binding**

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



**Measurement**

- Add 100 µL RT Development Solution to each well.
- Incubate 2 – 10 minutes RT protected from light.
- Add 100 µL 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 |
| | NFATc1 is poor activated or inactivated | Perform a time course for NFATc1 activation in the studied cell line |
| | Extracts are not from correct species | This product detects bound NFATc1 in human and mouse 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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