

**ab207221**  
**NFκB p65**  
**Transcription Factor**  
**Assay Kit**  
**(Chemiluminescent)**

Instructions for use:

For quantitative measurement of NFκB p65 activation in human, mouse and rat nuclear extracts.

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

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## 1. BACKGROUND

NFκB p65 Transcription Factor Assay Kit (Chemi-luminescent) (ab207221) is a high throughput assay to quantify NFκB p65 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 NFκB p65 consensus binding site (5' – GGGACTTTCC – 3') has been immobilized onto a 96-well plate. Active NFκB p65 present in the nuclear extract specifically binds to the oligonucleotide. NFκB p65 is detected by a primary antibody that recognizes an epitope of NFκB p65 accessible only when the protein is activated and bound to its target DNA. An HRP-conjugated secondary antibody provides a sensitive chemiluminescent readout that can be quantified using by luminescence. This product detects only human, mouse and rat NFκB p65.

Key performance and benefits:

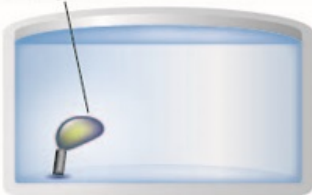
- Assay time: 3.5 hours (cell extracts preparation not included).
- Detection limit: < 40 ng nuclear extract/well.
- Detection range: 0.039 – 2.5 µg nuclear cell extract/well.

The transcription factor NFκB is implicated in the regulation of many genes that code for mediators of the immune, acute phase and inflammatory responses. NFκB is composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family. There are five subunits of the NFκB family in mammals: p50, p65 (RelA), c-Rel, p52 and RelB. These proteins share a conserved 300 amino acid sequence in the N-terminal region, known as the Rel homology domain, that mediates DNA binding, protein dimerization and nuclear localization. Various dimer combinations of the NFκB subunits have distinct DNA binding specificities and may serve to activate specific sets of genes such as adhesion molecules, immunoreceptors and cytokines. The p50/p65 heterodimers are some of the most common dimers found in the NFκB signaling pathway. RelA (p65) heterodimers with both p50 and p52 participate in target gene transactivation.

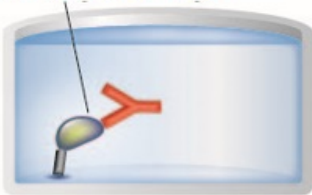
**Single Stranded DNA oligonucleotide**



**Sample**



**Primary Antibody**



**HRP-Conjugated Antibody**



**Substrate**      **Colored Product**



## 2. ASSAY SUMMARY

Prepare all reagents, nuclear extracts and controls as instructed. Plate is supplied pre-coated with an oligonucleotide containing NFkB p65 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 Chemiluminescent Reagent.

Measure signal immediately using a luminometer or CCD camera system.

### 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 that 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)
	1 Plate	5 Plates		
NFκB p65 antibody	11 μL	55 μL	-20°C	-20°C
Anti-rabbit HRP-conjugated IgG (0.25 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 (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
Herring sperm DNA (1 μg/μL)	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
Chemiluminescent Reagent	2 mL	10 mL	-20°C	4°C
Reaction Buffer	4 mL	20 mL	-20°C	4°C
96-well NFκB chemi 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 luminometer or CCD camera-couple imaging system
- MilliQ water or other type of double distilled water (ddH<sub>2</sub>O)
- 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  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>, 0.1 mM EDTA)
- Phosphatase Inhibitors (NaF,  $\beta$ -glycerophosphatase, PNPP, NaVO<sub>3</sub>)
- 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 5 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 (ddH<sub>2</sub>O) – 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 (ddH<sub>2</sub>O) – 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. **NFκB p65 antibody:**

Dilute supplied NFκB p65 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 NFκB p65 antibody at -20°C. Avoid multiple freeze/thaw cycles.

### 9.9. **Anti-rabbit HRP-conjugated Antibody (0.25 µg/µL):**

Dilute supplied anti-rabbit HRP-conjugated antibody 1/500 in 1X ABB. Prepare a further 1/20 dilution in 1X ABB to achieve a 1/10,000 final antibody dilution – 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.

### 9.10. **Chemiluminescent Reagent:**

Equilibrate Chemiluminescent Reagent to room temperature 1 hour prior to use.

Prepare **Chemiluminescent Working Solution** by making a 1/2 dilution of Chemiluminescent Reagent in Reaction Buffer – see Quick Table for Reagent Preparation to see how much is required depending on number of tests.

The Chemiluminescent Working Solution is stable for several hours. After the Chemiluminescent Working Solution is aliquoted into the wells, discard the remaining solution.

### 9.11. **Jurkat (TPA + CI) nuclear extract (2.5 µg/µL):**

Ready to use as supplied. Extract has been optimized to be used at 1.25 µg/well. There is enough extract to perform 80 reactions per plate. Aliquot extract in 5 µL fractions and store at -80°C. 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 NFκB consensus binding site.

Mutated oligonucleotide: no effect on ability of sample nuclear extracts to bind to NFκB 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.

### 9.13. **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.

## ASSAY PREPARATION

### Quick Table for Reagent Preparation

Reagents to prepare	Components	1 well	1 strip (8 wells)	6 strips (48 wells)	12 strips (92 wells)
Complete Lysis Buffer (CLB)	DTT	0.11 $\mu$ L	0.9 $\mu$ L	5.4 $\mu$ L	10.8 $\mu$ L
	PIC	0.23 $\mu$ L	1.8 $\mu$ L	10.8 $\mu$ L	21.6 $\mu$ L
	Lysis Buffer	22.2 $\mu$ L	177.3 $\mu$ L	1.064 mL	2.128 mL
	<b>TOTAL REQUIRED</b>	<b>22.5 <math>\mu</math>L</b>	<b>180.0 <math>\mu</math>L</b>	<b>1.08 mL</b>	<b>2.16 mL</b>
Complete Binding Buffer (CBB)	DTT	0.07 $\mu$ L	0.54 $\mu$ L	3.2 $\mu$ L	6.5 $\mu$ L
	Herring Sperm DNA	0.34 $\mu$ L	2.7 $\mu$ L	16.2 $\mu$ L	32.4 $\mu$ L
	Binding Buffer	33.4 $\mu$ L	267 $\mu$ L	1.6 mL	3.2 mL
	<b>TOTAL REQUIRED</b>	<b>33.8 <math>\mu</math>L</b>	<b>270 <math>\mu</math>L</b>	<b>1.62 mL</b>	<b>3.24 mL</b>
Oligo (wt or mutated) in CBB	Wt or mutated oligo	2 $\mu$ L	16 $\mu$ L	96 $\mu$ L	N/A
	CBB	31.8 $\mu$ L	254 $\mu$ L	1.524 mL	N/A
	<b>TOTAL REQUIRED</b>	<b>33.8 <math>\mu</math>L</b>	<b>270 <math>\mu</math>L</b>	<b>1.62 mL</b>	<b>N/A</b>
1X Wash Buffer	ddH <sub>2</sub> O	2.025 mL	16.2 mL	97.2 mL	194.4 mL
	10X Wash Buffer	225 $\mu$ L	1.8 mL	10.8 mL	21.6 mL
	<b>TOTAL REQUIRED</b>	<b>2.25 mL</b>	<b>18 mL</b>	<b>108 mL</b>	<b>216 mL</b>

## ASSAY PREPARATION

Reagents to prepare	Components	1 well	1 strip (8 wells)	6 strips (48 wells)	12 strips (92 wells)
1X Ab Buffer*	ddH <sub>2</sub> O	157.5 µL	1.26 mL	7.56 mL	15.12 mL
	10x ABB	17.5 µL	140 µL	840 µL	1.68 mL
	<b>TOTAL REQUIRED</b>	<b>175 µL</b>	<b>1.4 mL</b>	<b>8.4 mL</b>	<b>16.8 mL</b>
1° Ab 1/1000 Dilution	NFκB p65 Ab	0.055 µL	0.44 µL	2.64 µL	5.06 µL
	1X ABB	55 µL	440 µL	2.64 mL	4.95 mL
	<b>TOTAL REQUIRED</b>	<b>55.06 µL</b>	<b>440.4 µL</b>	<b>2.64 mL</b>	<b>4.96 mL</b>
2° Ab pre-dilution (1/500)	HRP-conj Ab	0.1 µL	0.8 µL	4.8 µL	9.6 µL
	1X ABB	50 µL	400 µL	3.195 mL	4.79 mL
	<b>TOTAL REQUIRED</b>	<b>50.1 µL</b>	<b>400.8 µL</b>	<b>3.2 mL</b>	<b>4.8 mL</b>
2° Ab 1/20 Dilution	Pre-diluted HRP-conj Ab	2.75 µL	22 µL	132 µL	253 µL
	1X ABB	52.25 µL	418 µL	2.51 mL	4.81 mL
	<b>TOTAL REQUIRED</b>	<b>55 µL</b>	<b>440 µL</b>	<b>2.64 mL</b>	<b>5.06 mL</b>
Chemi-lumi Working Reaction	Chemi Reagent	18.7 µL	150 µL	0.9 mL	1.8 mL
	Reaction Buffer	37.5 µL	300 µL	1.84 mL	3.6 mL
	<b>TOTAL REQUIRED</b>	<b>56.2 µL</b>	<b>450 µL</b>	<b>2.7 mL</b>	<b>5.4 mL</b>

\*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

0.1 M Phosphate Buffer, pH 7.5

1.5 M NaCl

27 mM KCl

#### For 250 mL:

3.55g Na<sub>2</sub>HPO<sub>4</sub> +

0.61g KH<sub>2</sub>PO<sub>4</sub>

21.9 g

0.5 g

Adjust to 250 mL with ddH<sub>2</sub>O. Prepare a 1X PBS solution dilute 10X PBS solution 1/10 in ddH<sub>2</sub>O.

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)

125 mM NaF

250 M β-glycerophosphate

250 mM PNPP

25 mM NaVO<sub>3</sub>

#### For 10 mL:

52 mg

0.55 g

1.15 g

31 mg

Adjust to 10 mL with ddH<sub>2</sub>O. Mix the chemical by vortexing. Incubate solution at 50°C for 5 minutes. Mix again. Store at -20°C.

#### HB (Hypotonic Buffer)

20 mM Hepes, pH 7.5

5 mM NaF

10 µM Na<sub>2</sub>MoO<sub>4</sub>

0.1 mM EDTA

#### For 50 mL:

0.24 g

12 mg

5 µL of 0.1 M solution

10 µL of 0.5 M solution

Adjust pH to 7.5 with 1 N NaOH. Adjust volume to 50 mL with ddH<sub>2</sub>O. 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 cm<sup>2</sup> flask. 1 x 10<sup>7</sup> 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 control 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. NFκB p65 binding to its consensus sequence

11.1.1. Add 30 μL CBB containing wt or mutated oligo (Step 9.12) to the Competitive Binding Control wells.

11.1.2. Add 30 μL CBB to each of the other wells.

11.1.3. Prepare sample and control wells:

**Competitive Binding control wells** = 20 μL of sample diluted in CLB (use 0.2 – 2 μg of nuclear extract/well).

**Sample wells** = 20 μL of sample diluted in CLB (use 0.2 – 2 μg of nuclear extract/well).

**Positive control wells** = 0.5 μL of provided nuclear extract + 19.5 μL CLB (1.25 μg nuclear extract per well).

**Blank wells** = 20 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, then tap the inverted plate 3 times on absorbent paper towels.

## ASSAY PROCEDURE

### 11.2. Primary antibody binding

- 11.2.1. Add 50  $\mu\text{L}$  of diluted NF $\kappa$ B p65 antibody (1/1000) 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  $\mu\text{L}$  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 50  $\mu\text{L}$  diluted anti-rabbit HRP-antibody (1/10,000 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 Chemiluminescent Reagent at RT.
- 11.3.4. Wash each well 4 times with 200  $\mu\text{L}$  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 50  $\mu\text{L}$  RT Chemiluminescent Working Solution to all wells being used. Minimize exposure of the plate to light. Pop any bubbles that remain in the wells to ensure accurate measurements and then read the plate immediately.
- 11.4.2. Read chemiluminescence using a luminometer or CCD camera system. You may need to customize the plate geometry to work with your plate reader.

Plate length = 127,890  $\mu\text{m}$       Plate height = 15,000  $\mu\text{m}$

Plate width = 85,500  $\mu\text{m}$       Height tolerance = 500  $\mu\text{m}$

## 12. 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  $\mu\text{L}$  CBB + wt/mutant oligo to Competitive Binding control wells. Add 30  $\mu\text{L}$  CBB to each of the other wells used.
- Add 20  $\mu\text{L}$  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  $\mu\text{L}$  1X Wash Buffer.



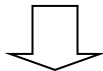
### Primary antibody binding

- Add 50  $\mu\text{L}$  diluted primary antibody (1/1000) to each well.
- Incubate 1 hour RT with no agitation.
- Wash each well 3 times with 200  $\mu\text{L}$  1X Wash Buffer.



### Secondary antibody binding

- Add 50  $\mu\text{L}$  diluted HRP antibody (1/10,000) to each well.
- Incubate 1 hour RT with no agitation.
- Wash each well 4 times with 200  $\mu\text{L}$  1X Wash Buffer.



### Measurement

- Add 50  $\mu\text{L}$  RT Chemiluminescent Working Solution to each well.
- Read chemiluminescence immediately.

# RESOURCES

## 13. 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 or CCD camera settings not optimal	Verify plate reader is set for luminescence measurement mode or check filter settings on the CCD camera
	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 40 µg/well
	NFκB p65 is poorly activated or inactivated in nuclear fractions	Perform a time course for NFκB p65 activation in the studied cell line
	Nuclear extracts are not from correct species	This product specifically detects bound NFκB p65 in human and mouse samples
	Salt concentration too high in binding reaction	Reduce amount of extract per well or dialyze before use
High background in all wells	Measurement time too long	Reduce integration or exposure time on luminometer or CCD camera
	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 0.01-0.05 µg/well
	Concentration of antibodies is too high	Perform antibody titration to determine optimal concentration. Start using 1/2000 for 1ry Ab and 1/50000 for the 2ry Ab. Assay sensitivity will be decreased

### 14. 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.

### 15. NOTES



## **Technical Support**

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For all technical or commercial enquiries please go to:

[www.abcam.com/contactus](http://www.abcam.com/contactus)

[www.abcam.cn/contactus](http://www.abcam.cn/contactus) (China)

[www.abcam.co.jp/contactus](http://www.abcam.co.jp/contactus) (Japan)