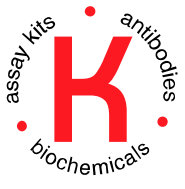


KAMIYA BIOMEDICAL COMPANY

Ku 70/86 DNA Repair Kit

For the quantitative determination of active Ku in nuclear extracts.

Cat. No. KT-132

For research use only, not for use in diagnostic procedures.

PRODUCT INFORMATION

Ku70/86 DNA Repair Kit

Cat. No. KT-132

PRODUCT

The **K-*ASSAY*®** Ku70/86 DNA Repair Kit is an ELISA for the quantitative determination of active Ku in nuclear extracts. It is intended for *in vitro* and research use only.

DESCRIPTION

The Ku protein, composed of the Ku70 and Ku86 heterodimer, binds directly to DNA ends and is part of the non-homologous end joining machinery involved in double-stranded DNA break repair. When a DNA break occurs, the Ku protein binds to keep DNA ends localized and aligned for subsequent repair. These DNA-bound Ku complexes recruit the DNA dependent protein kinase (DNA-PK) to DNA. DNA-PK is responsible for phosphorylating a number of transcription factors including Sp1, p53 and c-Myc, and is thought to signal the arrest of the cell cycle and the recruitment of repair factors. In order to rejoin the incompatible broken DNA ends, DNA nucleases FEN-1 and EXO1 are recruited to remove nucleotides. Processed DNA ends are then rejoined by the DNA Ligase IV-XRCC4 heterodimer.

Ku is upregulated by DNA damaging agents that generate double-stranded DNA breaks such as ionizing radiation. The importance of the Ku heterodimer for double-stranded break repair has been demonstrated by sensitivity to extreme radiation and the presence of VDJ recombination defects in cells that lack Ku.

In addition to its DNA end binding activity, Ku also interacts with specific DNA sequences. Ku has also been shown to bind transcriptional regulatory elements such as c-Myc, transferrin receptor and Grp78, as well as the NRE1 DNA sequence element.

DNA Repair Assays

To date, two methods are widely used to measure Ku activation, either directly or indirectly:

1. Ku activation can be determined by Western blot by using antibodies specific for the Ku protein. This method is time consuming (up to 2 days once the nuclear extracts are prepared), and is not suitable for processing large numbers of samples.

2. The DNA-binding capacity of Ku can be assayed by gel retardation, also called electrophoretic mobility shift assay (EMSA). In this method, nuclear extracts are incubated with a radioactive double-stranded oligonucleotide probe. If Ku is upregulated in the cell extract, it will bind to the probe. Samples are then resolved by electrophoresis on a native polyacrylamide gel, followed by autoradiography. This method is sensitive, but like the previous procedure, it is time consuming (multiple days of gel exposure may be required to achieve sufficient sensitivity) and it cannot be applied to high-throughput screening. Gelshift assays

also require special precautions and equipment for handling radioactivity.

Ku70/86 DNA Repair Kit
Ku is involved in the maintenance of genomic stability, and therefore represents an excellent pharmacological target for developing drugs to treat cancer. However, pharmaceutical research in this field has been hampered by the lack of convenient assays suitable for large numbers of samples.

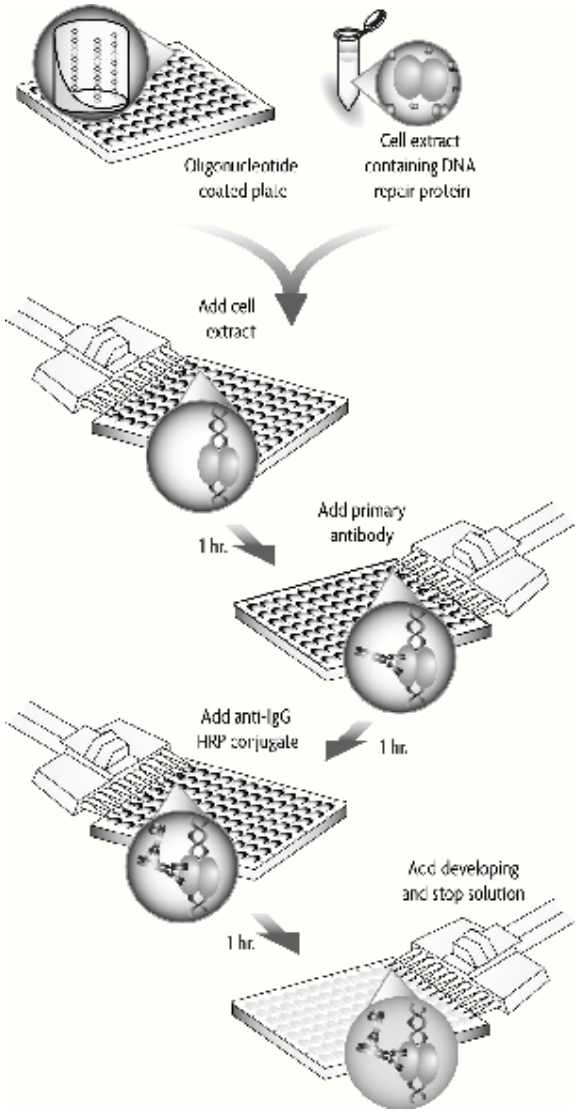
To overcome this problem, KAMIYA BIOMEDICAL COMPANY is introducing a high-throughput assay to quantify Ku activation. The DNA Repair Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for proteins involved in DNA repair. Once the nuclear extracts are prepared, this assay is completed in less than 3.5 hours. As this assay is performed in 96-well plates, a large number of samples can be handled simultaneously, enabling high-throughput automation. This assay is specific for Ku activation and has been shown to be 20-fold faster than the gel-retardation technique. With the 3.5-hour DNA Repair Kit procedure, we could detect Ku activation using as little as 0.15 µg of nuclear extract from untreated Raji cells.

Ku70/86 DNA Repair Kits have many applications including the study of Ku regulation and protein structure/function studies of Ku in areas such as carcinogenesis and aging.

PRINCIPLE
The **K-*ASSAY*** ® Ku70/86 DNA Repair Kit contains a 96-well plate on which has been immobilized a linear oligonucleotide with a blunt end. Ku contained in nuclear extract specifically binds to this oligonucleotide. The primary antibodies used in the Ku70/86 Kit recognize an epitope on either Ku70 or Ku86 protein that is accessible upon DNA binding. Addition of a secondary HRP-conjugated antibody provides a sensitive colorimetric readout easily quantified by spectrophotometry at 450 nm.

WARNINGS AND PRECAUTIONS
- For *in vitro* research use only. Not for internal or external use in humans or animals.
- Stop Solution is corrosive. Wear personal protection equipment when handling assay material such as safety glasses, gloves and labcoat.

Flow Chart of Process



COMPONENTS

Except for the cell extract that must be kept at -80ºC, kit components can be stored at -20ºC

prior to the first use. Then, we recommend storing each component at the temperature indicated in the table below.

Reagents	Quantity	Storage / Stability
Ku70 and Ku86 Antibodies	12 µl (0.1 mg/ml)	4°C for1year
Anti-rabbit IgG-HRP Conjugate	12 µl (0.4 mg/ml)	-20°C for1year
Ku Competitor Oligonucleotide	100 µl (10 pmol/ul)	-20°C for1year
Raji Nuclear Extract	40 µl (5 ug/ul)	-80°C for 6 months
Dithiothreitol (DDT)	100 µl (1M)	-20°C for1year
Protease Inhibitor Cocktail	100 µl	-20°C for1year
Lysis Buffer	10 ml	4°C for 6 months
Binding Buffer	10 ml	4°C for 6 months
10x Wash Buffer	25 ml	4°C for 6 months
10x antibody Binding Buffer	2.5 ml	4°C for 6 months
Developing Solution	12 ml	4°C for1year
Stop Solution	12 ml	4°C for years
96-well assay plate	1	4°C for1year
Plate Sealer	1	

Materials Required but not Provided

- Multi-channel pipette and disposable tips
- Multi-channel pipette reagent reservoirs
- Plate shaker
- Microplate reader capable of reading at 450 nm (655 nm as optional reference wavelength)

PROTOCOLS

BUFFER PREPARATION AND RECOMMENDATIONS

Preparation of Complete Lysis Buffer

We provide an excess of Lysis Buffer in order to perform the assay AND to prepare customized cell extracts. Prepare the amount of Complete Lysis Buffer required for the assay by adding 1 µl of 1 M DTT and 10 µl of Protease Inhibitor Cocktail per ml of Lysis Buffer (see the Quick Chart for Preparing Buffers in this section). Some of the protease inhibitors lose their activity after 24 hours once diluted. Therefore, we recommend using the Complete Lysis Buffer immediately for cell lysis. The remaining amount should be discarded if not used in the same day.

Binding Buffer

This is supplied ready-to-use.

Preparation of 1X Wash Buffer

Prepare the amount of 1X Washing Buffer required for the assay as follows: For every 100 ml of 1X Wash Buffer required, dilute 10 ml 10X Wash Buffer with 90 ml distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. Tween 20 contained in the 10X Wash Buffer may form precipitates. Warm the buffer at 50ºC for 2 minutes and mix to dissolve the precipitates prior to use.

Preparation of 1X Antibody Binding Buffer

Prepare the amount of 1X Antibody Binding Buffer required for the assay as follows: For every 10 ml of 1X Antibody Binding Buffer required, dilute 1 ml 10X Antibody Binding Buffer with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section)*. Mix gently to avoid foaming. Discard remaining 1X Antibody Binding Buffer after use. The BSA contained in the 10X Antibody Binding Buffer may form precipitates. Warm to room temperature (RT) and vortex for 1 minute prior to use. Dilute both primary and HRP-conjugated secondary antibodies to 1:1000 with the 1X Antibody Binding Buffer. Depending on the particular assay, the signal:noise ratio may be optimized by using higher dilutions of both antibodies. This may decrease the sensitivity of the assay.

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

Developing Solution

The Developing Solution should be warmed to RT before use. The Developing Solution is light sensitive. It is, therefore, recommended to avoid direct exposure to intense light during storage. Developing Solution may develop a yellow hue over time. This does not affect product performance. A blue color present in Developing Solution indicates that it has been contaminated and must be discarded. Prior to use, place Developing Solution at RT for at least 1 hour. Transfer the amount of Developing Solution required for the assay into a secondary container before dispensing into the wells (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Developing Solution.

Stop Solution

Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Stop Solution. **WARNING:** Stop Solution is corrosive. Wear personal protective equipment when handling; safety glasses, gloves and labcoat.

Raji nuclear extract

The Raji nuclear extract is provided as a positive control for Ku activation. Sufficient extract is supplied for at least 40 reactions per plate. This extract is optimized to give a strong signal when used at 2.5 µg/well. Aliquot the extract in 5 µl fractions and store at -80ºC to avoid multiple freeze/thaw cycles.

Ku competitor oligonucleotide

The Ku competitor oligonucleotide is provided as a competitor for Ku binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent Ku

binding to the probe immobilized on the plate. Prepare the required amount of competitor oligonucleotide by adding 2 µl of the oligonucleotide to 43 µl of Binding Buffer per well being used (see the Quick Chart for Preparing Buffers in this section). To allow for optimum competition, add the oligonucleotide to the well prior to addition of the cell extract.

Quick Chart for Preparing Buffers

Reagents to prepare	Components	For 1 Well	For 1 strip (8 wells)	For 6 strips (48 wells)	For 12 strips (96 wells)
Complete Lysis Buffer	DTT	0.01 µl	0.1 µl	0.6 µl	1.2 µl
	Protease Inhibitor Cocktail	0.12 µl	0.9 µl	5.4 µl	10.8 µl
	Lysis Buffer	11.12 µl	89 µl	534 µl	1.068 ml
	TOTAL REQUIRED	**11.25 µl**	**90 µl**	**540 µl**	**1.08 ml**
Binding Buffer	**TOTAL REQUIRED**	**45 µl**	**360 µl**	**2.16 ml**	**4.32 ml**
Binding Buffer with Ku competitor oligonucleotide	Competitor oligonucleotide	2.0 µl	16.0 µl	96.0 µl	N/A
	Binding Buffer	43.0 µl	344.0 µl	2.064 ml	N/A
	TOTAL REQUIRED	**45.0 µl**	**360.0 µl**	**2.16 ml**	**N/A**
1X Washing Buffer	Distilled water	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**
1X Antibody Binding Buffer*	Distilled water	202.5 µl	1.62 ml	9.72 ml	19.44 ml
	10X Antibody Binding Buffer	22.5 µl	180 µl	1.08 ml	2.16 ml
	TOTAL REQUIRED	**225 µl**	**1.8 ml**	**10.8 ml**	**21.6 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 the preparation of buffers for diluting both the primary and secondary antibodies.

Ku DNA Repair Assay

Determine the appropriate number of microwell strips required for testing Standards, blanks and samples in duplicate. If less than 8 wells in a strip need to be used, cover the unused wells with a portion of the plate sealer while you perform the assay. The content of these wells is stable at RT if kept dry and, therefore, can be used later for a separate assay. Store the unused strips in the aluminum pouch at 4°C. Use the strip holder for the assay.

Prepare the Complete Lysis Buffer, 1X Wash Buffer and 1X Antibody Binding Buffer as described above in the section Buffer Preparation and Recommendations. Multi-channel pipettor reagent reservoirs may be used for dispensing Binding Buffer, Wash Buffer, Antibody Binding Buffer, Developing Solution and Stop Solution into the wells being used.

Step 1: Binding of Ku to the immobilized probe

1. Add 40 µl Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 40 µl Binding Buffer that contains 20 pmol (2 µl) of the Ku competitor oligonucleotide (see the Buffer Preparation section above for a description of competitive binding).
2. **Sample wells:** Add 10 µl of sample diluted in Complete Lysis Buffer per well. We recommend using 2-10 µg of cell extract diluted in Complete Lysis Buffer per well.

   Positive control wells: Add 2.5 µg of the provided Raji nuclear extract diluted in 10 µl of Complete Lysis Buffer per well (0.5 µl of extract in 9.5 µl of Complete Lysis Buffer per well).

   Blank wells: Add 10 µl Complete Lysis Buffer only per well.
3. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at RT with mild agitation (100 rpm on an orbital shaker).
4. Wash each well 3 times with 200 µl 1X Washing Buffer. For each wash, flick the plate over a sink to empty the wells, and tap the inverted plate 3 times on absorbent paper towels.

Step 2: Binding of primary antibody

1. Add 100 µl diluted Ku70 or Ku86 antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at RT with mild agitation (100 rpm on an orbital shaker).
3. Wash the wells 3 times with 200 µl 1X Washing Buffer (as described in Step 1, No. 4).

Step 3: Binding of secondary antibody

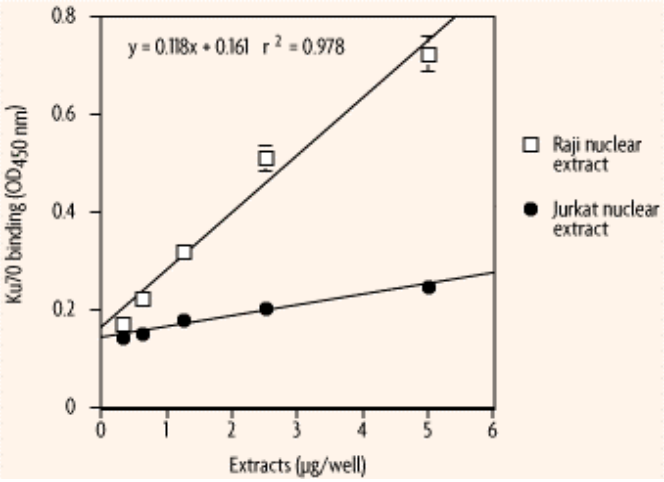
1. Add 100 µl of diluted anti-rabbit HRP-conjugated antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at RT with mild agitation (100 rpm on an orbital shaker).
3. During this incubation, place the Developing Solution at RT.
4. Wash the wells 4 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric reaction

1. Add 100 µl Developing Solution to all wells being used.
2. Incubate 1-5 minutes at RT protected from direct light. Please read the Certificate of Analysis supplied with this kit for the optimal development time for this specific kit lot, which varies from lot to lot. Monitor the blue color development in the sample and positive control wells until it turns medium to dark blue. Do not overdevelop.
3. Add 100 µl Stop Solution. In the presence of acid, the blue color turns yellow.
4. Read absorbance within 5 minutes at 450 nm with a reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

ASSAY PERFORMANCE

Detection limit: < 0.15 µg nuclear extract/well.



Range of detection: The Ku70/86 DNA Repair Kit provides quantitative results from 0.15 to 1.25 µg extract/well for Ku70 and 0.15 to 2.5 µg extract/well for Ku86.

Cross-reactivity: The Ku70/86 DNA Repair Kit contains two antibodies. The Ku70 antibody recognizes Ku70 from human, mouse and rat origins and does not cross-react with Ku86. The Ku86 antibody recognizes Ku86 from human, mouse and rat origins and does not cross-react with Ku70.

Assay time: 3.5 hours. DNA Repair Kits are 20-fold faster than EMSA.

PREPARATION OF NUCLEAR EXTRACT

This procedure can be used for a 15 ml cell suspension in a T75 flask. The yield is approximately 50 µg of nuclear proteins for 10^7 cells.

1. Collect 10 ml of cell suspension in a pre-chilled 15 ml tube.
2. Scrape the cells off the flask in the remaining 5 ml of media with a cell lifter. Transfer cells into the 15 ml tube and spin at 300 x *g* for 5 minutes at 4°C.
3. Discard supernatant. Resuspend cell pellet in 5 ml PBS/PIB and spin at 300 x *g* for 5 minutes at 4°C.
4. Discard supernatant. Resuspend the pellet in 1 ml ice-cold HB buffer by gentle pipetting and transfer the cells into a pre-chilled 1.5 ml tube.
5. Allow the cells to swell on ice for 15 minutes.
6. Add 50 µl 10% Nonidet P-40 (0.5 % final) and mix by gentle pipetting.

7. Centrifuge the homogenate for 30 seconds at 4°C in a microcentrifuge. Remove the supernatant (cytoplasmic fraction) and, if you wish to save this for other uses, transfer it into a pre-chilled microcentrifuge tube. (Store the cytoplasmic fraction at –80°C.)
8. Resuspend the nuclear pellet in 40 µl Complete Lysis Buffer and rock the tube gently on ice for 30 minutes on a shaking platform.
9. Centrifuge for 10 minutes at 14,000 x *g* at 4°C and save the supernatant (nuclear extract). Aliquot and store at –80°C. Avoid freeze/thaw cycles.
10. Determine the protein concentration of the extract by using a Bradford-based assay.

10X PBS	**For 250 ml, mix:**
0.1 M phosphate buffer, pH 7.5	3.55 g Na_2HPO_4 + 0.61 g KH_2PO_4
1.5 M NaCl	21.9 g
27 mM KCl	0.5 g

Adjust to 250 ml with distilled water. Prepare a 1X PBS solution by adding 10 ml 10X PBS to 90 ml distilled water. Sterilize the 1X PBS by filtering through a 0.2 µm filter. The 1X PBS is at pH 7.5. Store the filter-sterilized 1X PBS solution at 4°C.

PIB (Phosphatase Inhibitor Buffer)	**For 10 ml, mix**
125 mM NaF	52 mg
250 mM β-glycerophosphate	0.55 g
250 mM para-nitrophenyl phosphate (PNPP)	1.15 g
25 mM $NaVO_3$	31 mg

Adjust to 10 ml with distilled water. Mix the chemicals by vortexing. Incubate the solution at 50ºC for 5 minutes. Mix again. Store at –20°C.

PBS/PIB

Prior to use, add 0.5 ml of PIB in 10 ml of 1X PBS.

HB (Hypotonic Buffer)	**For 50 ml, mix**
20 mM Hepes, pH 7.5	0.24 g
5 mM NaF	12 mg
10 µM Na_2MoO_4	5 µl of a 0.1 M solution
0.1 mM EDTA	10 µl of a 0.5 M solution

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

STORAGE

Except for Raji nuclear extract that must be kept at -80°C, kit components can be stored at -20°C prior to first use. For storage condition for individual components, see COMPONENTS section.

FOR RESEARCH USE ONLY

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