KAMIYA BIOMEDICAL COMPANY

RPA (Replication Protein A) DNA Repair Kit

For the measurement of RPA70 and RPA32 in cells, tissues, or animals

Cat. No. KT-182

For research use only. Not for use in diagnostic procedures.

PRODUCT INFORMATION

RPA DNA Repair Kit
Cat. No. KT-182

PRODUCT
The ***K-ASSAY®*** RPA DNA Repair Kit is for the measurement of RPA70 and RPA32 in samples. For research use only. Not for use in diagnostic procedures.

INTRODUCTION
Replication Protein A (RPA) is composed of RPA70, RPA32 and RPA14 (70, 32 and 14 kDa subunits, respectively). RPA binds directly to single stranded DNA (ssDNA) where it organizes and protects ssDNA during DNA replication, recombination and repair. The inability to replicate or repair DNA breaks leads to cell cycle arrest and apoptosis. Therefore, accurate monitoring of RPA activation in cells, tissues or animals is crucial for biomedical research and drug development. To date, such research projects are tedious and time consuming, and lack high-throughput screening methods.

The ***K-ASSAY®*** RPA DNA Repair Kit provides a fast, user-friendly format for studying DNA damage and repair protein interactions. RPA DNA Repair Kits are designed specifically for the study of RPA. They contain a 96-well plate to which a single stranded DNA oligonucleotide has been immobilized. RPA contained in nuclear extracts binds specifically to this DNA molecule and is detected through use of an antibody directed against RPA32. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provides a sensitive colorimetric readout that is easily quantified by spectrophotometry. The 96-well plate with individual strips of 8 wells is suitable for manual use or for high-throughput screening applications.

PRINCIPLE
The RPA protein, composed of subunits RPA70, RPA32 and RPA14, binds directly to ssDNA ends and was originally identified as a required factor for SV40 DNA replication in vitro. More recently, it has been indicated that RPA may be involved in various DNA repair pathways. A globular domain at the C-terminus of RPA32 has been shown to interact with uracil DNA glycosylase (UDG), XPA and RAD52, each members of a different DNA repair pathway. RPA32 interacts with UDG, an important enzyme in the base excision repair (BER) pathway, and both UDG and RPA32 are known to co-localize at replication foci, suggesting that RPA also plays a role in coupling BER to DNA replication. RPA32 and RPA70 bind to XPA, a key player in the nucleotide excision repair (NER) pathway, allowing interaction of XPA with sites of DNA damage. Finally, RPA32 has been shown to interact with RAD52.The C-terminus of RAD52 is thought to play an important role in the repair of double-stranded DNA breaks by homologous recombination.

RPA is upregulated and accumulates in the nucleus after treatment with DNA damaging enediyne drugs C-1027 and neocarzinostatin. The importance of RPA in DNA repair has been reported by deletion analysis as the deletion of the C-terminus of the RPA32 subunit can cause inhibition of NER activity *in vitro*.

In addition to its single-stranded DNA end binding activity, RPA has been shown to unwind double-stranded DNA. RPA also binds to and stimulates the activity of helicases. RPA stimulates helicase activity of the Bloom's syndrome protein, which is involved in regulation of genomic instability and cancer predisposition.

RPA also binds to the Werner syndrome gene product (WRN), a DNA helicase belonging to the RecQ family involved in the premature onset of age-related phenotypes.

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

1. RPA activation can be determined by Western blot by using antibodies specific for the RPA 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 RPA can be assayed by gel retardation, also called electrophoretic mobility shift assay (EMSA). In this method, nuclear extracts are incubated with a radioactive oligonucleotide probe. If RPA 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.

RPA 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, the ***K-ASSAY®*** RPA DNA Repair Kit is a high-throughput assay to quantify RPA 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. RPA DNA Repair Kits contain a 96-well plate on which has been immobilized a linear single-stranded oligonucleotide. RPA contained in nuclear extract binds specifically to this oligonucleotide. The primary antibody used in the RPA DNA Repair Kit recognizes an epitope on RPA32 protein that is accessible upon DNA binding. Addition of a secondary HRP-conjugated antibody provides a sensitive colorimetric readout easily quantified by spectrophotometry. 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 RPA 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 RPA activation using as little as 0.15 µg of nuclear extract from untreated Raji cells.

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

COMPONENTS AND STORAGE

Except for the nuclear extract that must be kept at -80ºC, kit components can be stored at -20ºC prior to first use. Then, we recommend storing each component at the temperature indicated in the table below.

Reagent	Quantity	Storage
RPA32 antibody	11 µL	4°C for 1 year
Anti-mouse HRP-conjugated IgG	11 µL	4°C for 1 year
Competitor oligonucleotide	100 µL (2.5 pmol/µL)	-20°C for 1 year
Raji nuclear extract	40 µL (2.5 µg/µL)	-80°C for 6 months
Dithiothreitol (DTT)	100 µL (1 M)	-20°C for 1 year
Protease Inhibitor Cocktail	100 µL	-20°C for 1 year
Lysis Buffer	10 mL	4°C for 6 months
Binding Buffer	10 mL	4°C for 6 months
10X Washing Buffer	22 mL	4°C for 6 months
10X Antibody Binding Buffer	2.2 mL	4°C for 6 months
Developing Solution	11 mL	4°C for 1 year
Stop Solution	11 mL	4°C for 1 year
96-well assay plate	1	4°C for 1 year
Plate sealer	1	

MATERIALS REQUIRED BUT NOT PROVIDED

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Shaking platform
- Microplate spectrophotometer capable of reading at 450 nm (655 nm as optional reference wavelength)
- Nuclear extraction reagents including Hypotonic Buffer, Detergent, Phosphatase Inhibitor Buffer and 10X PBS (see page 7)

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 nuclear 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 Washing Buffer:
Prepare the amount of 1X Washing Buffer required for the assay as follows: For every 100 mL of 1X Washing Buffer required, dilute 10 mL 10X Washing Buffer with 90 mL distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The 1X Washing Buffer may be stored at 4°C for one week. The Tween 20 contained in the 10X Washing Buffer may form clumps, therefore homogenize the buffer by incubating at 50°C for 2 minutes and mixing 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 protein stabilizer contained in the 10X Antibody Binding Buffer may form clumps, therefore homogenize the buffer by warming to room temperature and vortexing for 1 minute prior to use. Dilute both primary and HRP-conjugated secondary antibodies to 1:1,000 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 buffer for diluting both the primary and secondary antibodies.

Developing Solution:
The Developing Solution should be warmed to room temperature before use. The Developing Solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. A blue color present in the Developing Solution indicates that it has been contaminated and must be discarded. Prior to use, place the Developing Solution at room temperature for at least 1 hour. Transfer the amount of Developing Solution required for the assay into a secondary container before aliquoting 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:** The Stop Solution is corrosive. Wear personal protective equipment when handling, i.e. safety glasses, gloves and lab coat.

Raji nuclear extract:
The Raji nuclear extract is provided as a positive control for RPA activation. Sufficient extract is supplied for 20 reactions. This extract is optimized to give a strong signal when used at 5 µg/well. We recommend aliquoting the extract in 5 µL fractions and storing at -80ºC. Avoid multiple freeze/thaw cycles of the extract.

Competitor oligonucleotide:
The RPA competitor oligonucleotide is provided as a competitor for RPA binding in order to monitor the specificity of the assay. Used at 5 pmol/well, the oligonucleotide will prevent RPA 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 Quick Chart for Preparing Buffers in this section). To allow for optimum competition, add the oligonucleotide to the well prior to addition of the nuclear 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 competitor oligonucleotide	Competitor oligonucleotide	2 µL	16 µL	96 µL	N/A
	Binding Buffer	43 µL	344 µL	2.064 mL	N/A
	TOTAL REQUIRED	**45 µL**	**360 µL**	**2.16 mL**	**N/A**
1X Washing Buffer	Distilled water	2.025 mL	16.2 mL	97.2 mL	194.4 mL
	10X Washing 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 buffer for diluting both the primary and secondary antibodies.

ASSAY PROCEDURE

Determine the appropriate number of microwell strips required for testing samples, controls and blanks 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 room temperature 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 Washing Buffer and 1X Antibody Binding Buffer as described above in the section Buffer Preparation and Recommendations. Multi-channel pipettor reservoirs may be used for dispensing the Binding Buffer, Washing Buffer, Antibody Binding Buffer, Developing Solution and Stop Solution into the wells being used.

Step 1: Binding of RPA to the immobilized probe

1. Add 40 µL of Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 40 µL Binding Buffer that contains 5 pmol (2 µL) of the RPA 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 nuclear extract diluted in Complete Lysis Buffer per well.

   Positive control wells: Add 5 µg of the provided Raji nuclear extract diluted in 10 µL of Complete Lysis Buffer per well (2 µL of extract in 8 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 room temperature with mild agitation (100 rpm on a Labline 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, then tap the inverted plate 3 times on absorbent paper towels.

Step 2: Binding of primary antibody

1. Add 100 µL diluted RPA32 antibody (1:1,000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature with mild agitation (100 rpm on a Labline orbital shaker).
3. Wash the wells 3 times with 200 µL 1X Washing Buffer (as described in Step 1, No. 4).

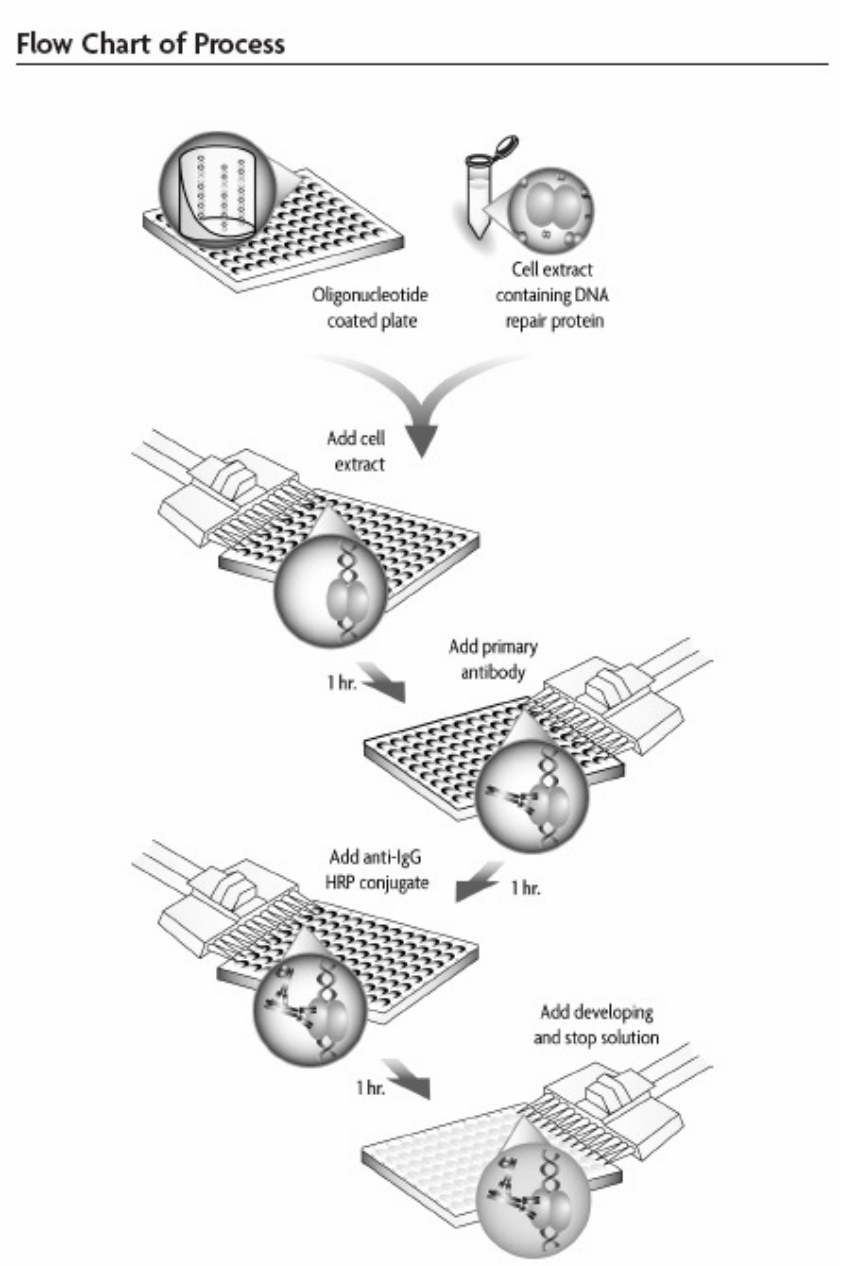
Step 3: Binding of secondary antibody

1. Add 100 µL of diluted anti-mouse HRP-conjugated antibody (1:1,000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature with mild agitation (100 rpm on a Labline orbital shaker).
3. During this incubation, place the Developing Solution at room temperature.

4. Wash the wells 4 times with 200 µL 1X Washing 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 room temperature protected from direct light. Please read 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 presence of the acid, the blue color turns yellow.
4. Read absorbance on a spectrophotometer 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.



Preparation of Nuclear Extract:
If you prefer to make your own buffers, please refer to the following protocol.
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.

PERFORMANCE CHARACTERISTICS

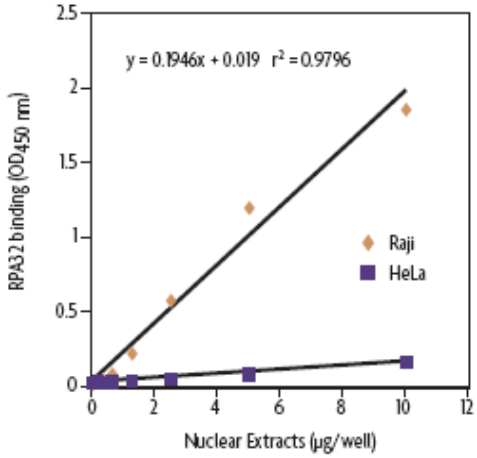
The RPA DNA Repair Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: > 0.15 µg nuclear extract/well.

Range of detection: The RPA DNA Repair Kit provides quantitative results from 0.15 to 10 µg nuclear extract/well (see graph below).

Cross-reactivity: The RPA32 antibody recognizes RPA32 from human, hamster and yeast origins.

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



Monitoring RPA32 binding with the RPA DNA Repair Kit: Different amounts of nuclear extracts from unstimulated Raji and HeLa cells are tested for activity using the RPA DNA Repair Kit. These curves are provided for demonstration only.

TROUBLESHOOTING

Problem	Possible Cause	Recommendation
No signal or weak signal in all wells	Omission of key reagent	Check that all reagents have been added in the correct order.
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity.
	Enzyme Inhibitor present	Sodium azide will inhibit the peroxidase reaction, follow our recommendations to prepare buffers.
	Plate reader settings not optimal	Verify the wavelength and filter settings in the plate reader.
	Incorrect assay temperature	Bring substrate to room temperature.
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette.
High background in all wells	Developing time too long	Stop enzymatic reaction as soon as the positive wells turn medium-dark blue.
	Concentration of antibodies too high	Increase antibody dilutions.
	Inadequate washing	Ensure all wells are filled with Washing Buffer and follow washing recommendations.
Uneven color development	Incomplete washing of wells	Ensure all wells are filled with Washing Buffer and follow washing recommendations.
	Well cross-contamination	Follow washing recommendations.
High background in sample wells	Too much cell extract per well	Decrease amount of nuclear extract down to 2.5 µg/well.
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1:2,000 for primary antibody and 1:5,000 for the secondary antibody. The sensitivity of the assay will be decreased.
No signal or weak signal in sample wells	Not enough nuclear extract per well	Increase amount of cell extract to 10 µg/well.
	RPA is poorly activated or inactivated in nuclear fractions	Perform a time course for RPA activation in the studied cell line.
	Cell extracts are not from human, hamster or yeast origin	Perform study with a human, hamster or yeast model.

FOR RESEARCH USE ONLY

KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
www.k-assay.com