TransAM™
p53 Transcription Factor Assay Kits

(version D1)

Catalog Nos. 41196 & 41696

Revision	Date	Description of Change
D1	January, 2024	The p53 antibody has been replaced by a new rabbit monoclonal antibody which will react with p53 of human and African green monkey origin. The new antibody will perform similarly to the previous one in the assay. There is no change to the protocol. Please refer to the TDS with the lot# of antibody received in the kit for performance data.

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Overview

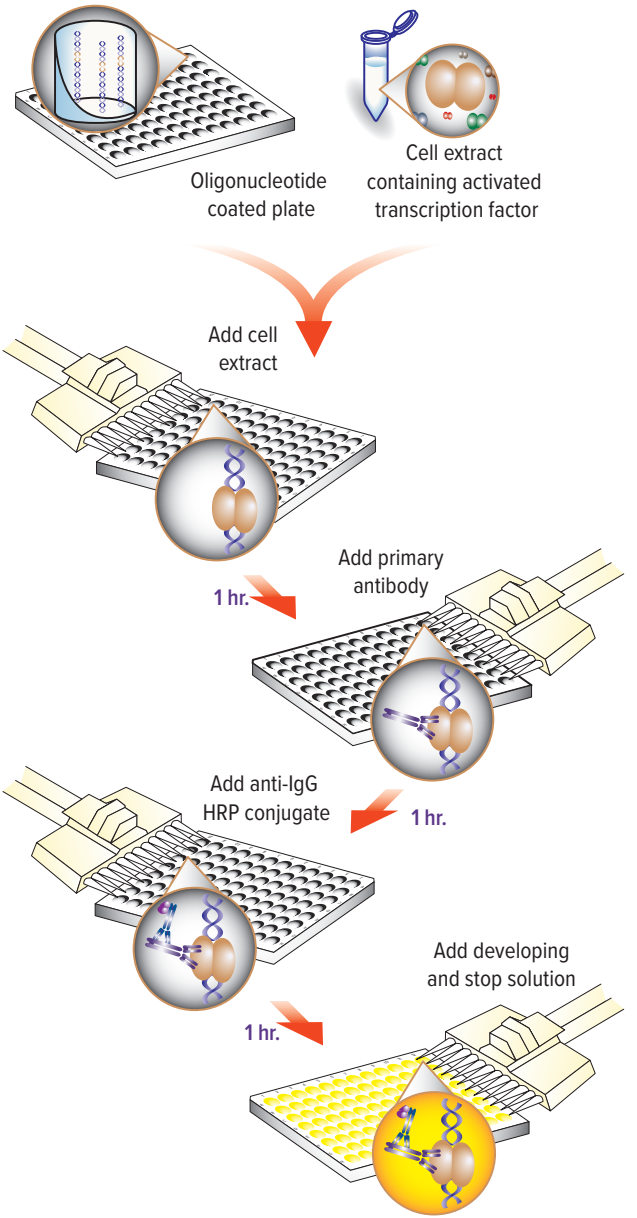
The tumor suppressor p53 acts primarily as a transcriptional activator that controls the expression of many genes implicated in cell growth control, DNA repair, apoptosis, redox regulation, nitric oxide production and protein degradation. Mutational inactivation of the p53 gene product is one of the most common genetic events that occur in human cancers. Therefore, accurate monitoring of p53 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.

With its patented TransAM™ method*, Active Motif introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM Kits combine a fast, user-friendly format with a sensitive, specific assay. TransAM p53 Kits are designed specifically for the study of the p53 activation pathway. They contain a 96-well plate to which an oligonucleotide containing the p53 consensus binding site has been immobilized. p53 contained in nuclear extracts binds specifically to this oligonucleotide and is detected through use of an antibody directed against p53. 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. TransAM p53 Kits are available in two sizes:

product	format	catalog no.
TransAM p53	1 x 96-well plate	41196
	5 x 96 well plates	41696

* Technology covered by AAT-filed patents and licensed to Active Motif.

Flow Chart of Process



p53 Transcription Factor

The tumor suppressor protein p53 is a transcription factor that switches on a series of protective genes when the cell is exposed to stressful events. Many solid tumors contain defective forms of p53 that are unable to stop cells from proliferating when, for example, their DNA has been damaged. Therefore, p53 functions to selectively destroy stressed or abnormal cells, thereby protecting the organism from cancer development[1]. Stress events include radiation, low pH, heat shock, hypoxia, genotoxins, DNA damage, RNA polymerase II block and oxidant injury[2]. Two human p53 homologues, p73 and p63 were recently identified with roles in stem cell identity, neurogenesis, natural immunity and homeostatic control[3-5]. These homologues can drive gene expression from promoters similar to that bound by p53, but neither of these have been found to be highly mutated in cancers, nor is p73 bound to viral oncoproteins that neutralize p53 protein activity, so their function in regulating p53-dependent cancer progression is unclear.

p53 possesses a modular architecture with an N-terminal transactivation domain, a strongly conserved core DNA-binding domain, a tetramerization domain, and a regulatory C terminus. The p53 DNA-binding domain comprises several hot spot regions for mutation that inactivate p53 in more than half of all human tumors[6]. Tetrameric p53 binds specifically to a DNA consensus sequence consisting of two consecutive palindromic 10-bp half-sites 5´-RRRCWWGYYY-3´ (R = A or G, Y = C or T, W = A or T), which can be separated from 0 to 13 bp[7]. The tetramer assembly stabilizes the p53 monomer folding and increases the DNA-binding activity of p53. p53 stays inactive in the nucleus when bound to MDM2 protein, an E3 ubiquitin ligase that targets both p53 and itself for ubiquitination. MDM2 represses p53 activity by inducing its nuclear export and degradation in proteasomes[8, 9]. Stress signals, such as DNA damage, activate protein kinases that lead to p53 phosphorylation of numerous sites and subsequent activation of p53 by inhibiting p53-MDM2 interaction[10]. MDM2 gene expression is regulated by p53, creating a feedback loop in which p53 activates expression of MDM2, which keeps p53 levels low during normal growth and development.

Transcription Factor Assays

To date, three methods are widely used to measure p53 activation, either directly or indirectly:

1. p53 activation can be determined by Western Blot by using antibodies specific for p53 protein. This method is time consuming (up to 2 days once the nuclear cell extracts are prepared), and is not suitable for processing large numbers of samples.

2. The DNA-binding capacity of p53 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 containing the consensus sequence for p53 binding. If p53 is active in the nuclear 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.

3. Another method used to assay p53 activation is based on reporter genes, typically luciferase or β-galactosidase, placed under the control of a promoter containing a p53 consensus binding site. The promoter can be artificial, made of several 10-bp half-sites and a TATA box, or natural, like promoter sequences from cell cycle check point or pro-apoptotic proteins. However, the procedure is limited by the following issues: (i) reporter gene assays have to be repeated several times to obtain statistically reliable data; and (ii) reporter gene assays are sensitive to confounding factors that may influence the expression level of the reporter gene, and therefore assays have to be carefully standardized. Reporter gene assays are sensitive and easy to perform with a large number of samples, but require efficient cell transfection with the reporter plasmid.

TransAM p53

p53 is involved in cell proliferation and therefore represents an excellent pharmacological target to develop drugs for inhibiting angiogenesis and metastasis in cancers. However, pharmaceutical research in this field has been hampered by the lack of convenient assays suitable for high sample number experiments.

To overcome this, Active Motif is introducing a high-throughput assay to quantify p53 activation[11]. The TransAM Kit combines a fast and user-friendly ELISA format with a sensitive and specific assay for transcription factors. TransAM p53 Kits contain a 96-well plate on which has been immobilized an oligonucleotide that contains a p53 consensus binding site (5´-GGACATGCCCGGGCATGTCC-3´). p53 contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used in TransAM p53 Kit recognizes an accessible epitope on p53 protein 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 p53 activation and has been shown to be 5-fold more sensitive and 20-fold faster than the gel-retardation technique. With the 3.5-hour TransAM procedure, we could detect p53 activation using as little as 0.6 µg of nuclear extract from H_2O_2-treated cells. A comparable assay using EMSA required 5 µg of nuclear extract and a 3-day autoradiography.

TransAM has many applications including the study of drug potency toward p53 binding, p53 transcriptional activity regulation and protein structure/function studies of p53 and its mutated variants in areas such as angiogenesis, metastasis, tumorigenesis, and many more.

Kit Performance and Benefits

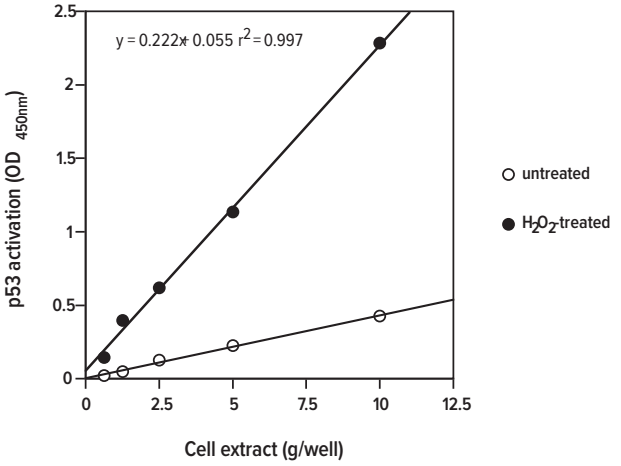
The TransAM p53 Kit is for research use only. Not for use in diagnostic procedures.

Detection limit: < 0.6 µg nuclear extract/well. TransAM p53 is 5-fold more sensitive than EMSA.

Range of detection: TransAM provides quantitative results from 0.6 to 10 µg of nuclear extract/well (see graph below).

Cross-reactivity: TransAM p53 detects p53 from human and African green monkey origin.

Assay time: 3.5 hours. TransAM is 20-fold faster than EMSA.



Monitoring p53 activation with the TransAM p53 Kit: Different amounts of nuclear extracts from untreated (opened squares) and H_2O_2-treated (filled circles) MCF-7 cells are tested for p53 activation by using the TransAM p53 Kit. These curves are provided for demonstration only.

Kit 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.

Reagents	Quantity 1 plate / 5 plates	Storage / Stability
p53 antibody	11 µl / 55 µl (0.2 mg/ml)	-20°C for 6 months
Anti-rabbit HRP-conjugated IgG	11 µl / 55 µl (0.4 mg/ml)	4°C for 6 months
Wild-type oligonucleotide AM21	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
Mutated oligonucleotide AM21	100 µl / 500 µl (10 pmol/µl)	-20°C for 6 months
MCF-7 (H_2O_2) nuclear extract	40 µl / 200 µl (2.5 mg/ml)	-80°C for 6 months
Dithiothreitol (DTT)	100 µl / 500 µl (1 M)	-20°C for 6 months
Protease Inhibitor Cocktail	100 µl / 500 µl	-20°C for 6 months
Poly [d(I-C)]	100 µl / 500 µl (17 µg/ml)	-20°C for 6 months
Lysis Buffer AM2	10 ml / 50 ml	4°C for 6 months
Binding Buffer AM5	10 ml / 50 ml	4°C for 6 months
10X Wash Buffer AM2	22 ml / 110 ml	4°C for 6 months
10X Antibody Binding Buffer AM2	2.2 ml / 11 ml	4°C for 6 months
Developing Solution	11 ml / 55 ml	4°C for 6 months
Stop Solution	11 ml / 55 ml	4°C for 6 months
96-well p53 assay plate	1 / 5	4°C for 6 months
Plate sealer	1 / 5	

Additional materials required

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform
- Microplate spectrophotometer capable of reading at 450 nm (655 nm as optional reference wavelength)

For Nuclear Extract preparation

- Hypotonic Buffer
- Phosphatase Inhibitor Buffer
- 10X PBS
- Detergent (NP-40)

Buffer Preparation and Recommendations

Preparation of Complete Lysis Buffer

We provide an excess of Lysis Buffer AM2 in order to perform the assay AND to prepare customized cell extracts. Our Nuclear Extract Kit can also be purchased separately (Cat. Nos. 40010 & 40410). 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 AM2 (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.

Preparation of Complete Binding Buffer

Prepare the amount of Complete Binding Buffer AM5 required for the assay by adding 1 µl of DTT and 10 µl of 17 µg/ml poly[d(I-C)] per ml of Binding Buffer (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Complete Binding Buffer.

Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 100 ml of 1X Wash Buffer required, dilute 10 ml 10X Washing Buffer AM2 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. The Tween 20 contained in the 10X Wash Buffer AM2 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 AM2 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 AM2 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 1:1000 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 buffer for diluting both the primary & 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 labcoat.

Nuclear extract

The MCF-7 (H_2O_2) nuclear extract is provided as a positive control for p53 activation. Sufficient extract is supplied for 25 reactions per plate. This extract is optimized to give a strong signal when used at 5 µg/well. We recommend aliquoting the extract in 10 µl fractions and storing at -80°C. Avoid multiple freeze/thaw cycles of the extract. Various cell extracts are available from Active Motif (see Appendix, Section B. Related Products).

Wild-type and mutated consensus oligonucleotides

The wild-type consensus oligonucleotide is provided as a competitor for p53 binding in order to monitor the specificity of the assay. Used at 20 pmol/well, the oligonucleotide will prevent p53 binding to the probe immobilized on the plate. Conversely, the mutated consensus oligonucleotide should have no effect on p53 binding. Prepare the required amount of wild-type and/or mutated consensus oligonucleotide by adding 2 µl of appropriate oligonucleotide to 43 µl of Complete 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.

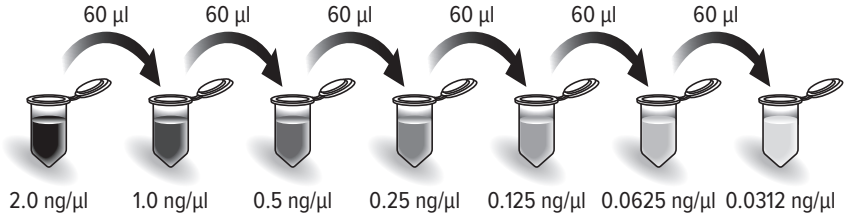
Optional- Preparation of standard curve

For those who wish to quantify the amount of p53 in their samples, Active Motif offers recombinant p53 for use as a protein standard (see Appendix, Section B. Related Products).

1. Begin with a 100 ng/µl working stock of recombinant protein (use the TransAM Complete Lysis Buffer to dilute the protein). Set up a standard curve in duplicate using the following concentrations: 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.0312 and 0 ng/µl. Note: The preceding range is provided as guidance, a broader range of values may be used.
2. Make up a 2.0 ng/µl solution by adding 2.0 µl of the 100 ng/µl working stock to 98 µl of Complete Lysis Buffer. Next, pipette 60 µl of Complete Lysis Buffer into the 7 remaining

tubes. Use the 2.0 ng/µl solution to prepare a dilution series as indicated below. Be sure to mix each tube thoroughly before each transfer. The 2.0 ng/µl standard serves as the high standard, while Complete Lysis Buffer alone serves as the 0.0 ng/µl.

3. 10 µl from each tube will be aliquoted to the wells in Step 1, No. 2 of the protocol and will correspond to the following quantities of p53: 20, 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.0 ng/well.



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 AM2	11.12 µl	89 µl	534 µl	1.068 ml
	TOTAL REQUIRED	**11.25 µl**	**90 µl**	**540 µl**	**1.08 ml**
Complete Binding Buffer	DTT	0.05 µl	0.4 µl	2.4 µl	4.8 µl
	Poly [d(I-C)]	0.45 µl	3.6 µl	21.6 µl	43.2 µl
	Binding Buffer AM5	44.5 µl	356 µl	2.136 ml	4.272 ml
	TOTAL REQUIRED	**45 µl**	**360 µl**	**2.16 ml**	**4.32 ml**
Complete Binding Buffer with wild-type or mutated oligonucleotide	Wild-type or mutated oligo	2 µl	16 µl	96 µl	N/A
	Complete Binding Buffer	43 µl	344 µl	2.064 ml	N/A
	TOTAL REQUIRED	**45 µl**	**360 µl**	**2.16 ml**	**N/A**
1X Wash Buffer	Distilled water	2.025 ml	16.2 ml	97.2 ml	194.4 ml
	10X Wash Buffer AM2	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 Ab Binding Buffer AM2	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 & secondary antibodies.

p53 Transcription Factor Assay

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, Complete Binding Buffer, 1X Wash 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 Complete Binding Buffer, Wash Buffer, Antibody Binding Buffer, Developing Solution and Stop Solution into the wells being used.

Step 1: Binding of p53 to its consensus sequence

1. Add 40 µl Complete Binding Buffer to each well to be used. If you wish to perform competitive binding experiments, add 40 µl Complete Binding Buffer containing 20 pmol (2 µl) of the wild-type or mutated consensus oligonucleotide (see the Buffer Preparation section above for a description of comptitive 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. A protocol for preparing nuclear extracts can be found on pgae 12.

   Positive control wells: Add 5 µg of the provided MCF-7 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.

   OPTIONAL – Protein standard wells: Add 10 µl of the appropriate protein standard diluted in Complete Lysis Buffer to each well being used (see page 8, Preparation of standard curve).
3. Use the provided adhesive cover to seal the plate. Incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform).
4. Wash each well 3 times with 200 µl 1X Wash 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 p53 antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. Wash the wells 3 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 3: Binding of secondary antibody

1. Add 100 µl of diluted HRP antibody (1:1000 dilution in 1X Antibody Binding Buffer) to all wells being used.
2. Cover the plate and incubate for 1 hour at room temperature without agitation.
3. During this incubation, place the Developing Solution at room temperature.

4. Wash the wells 4 times with 200 µl 1X Wash Buffer (as described in Step 1, No. 4).

Step 4: Colorimetric reaction

1. Transfer the amount of Developing Solution required for the assay into a secondary container. Add 100 µl Developing Solution to all wells being used.
2. Incubate 5-10 minutes at room temperature 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 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.

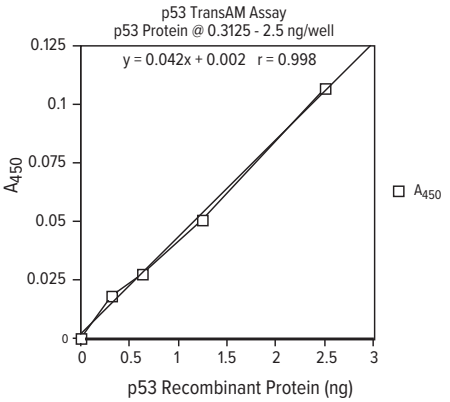
OPTIONAL – Calculation of results using the standard curve

If you have generated a standard curve using Active Motif's recombinant p53 protein, average the duplicate readings for each standard, control, and sample and subtract the optical density (OD) obtained from the zero standard.

Plot the OD for the standards against the quantity (ng/well) of the standards and draw the best fit curve. The data can be linearized using log/log paper and regression analysis may also be applied.

To quantify the amount of p53 in the samples, find the absorbance value for the samples on the y-axis and extend a horizontal line to the standard curve. At the intersection point extend a vertical line to the x-axis and read the corresponding standard value. Note: If the samples have been diluted, the value read from the standard curve must be multiplied by the dilution factor.

Example curve:
The following standard curve is provided for demonstration only. A standard curve should be made every time an experiment is performed.



Preparation of Nuclear Extract

For your convenience, Active Motif offers a Nuclear Extract Kit (Cat. Nos. 40010 & 40410). This kit contains buffers optimized for use in TransAM Kits, which serves to reduce inconsistencies in the assay that may arise from using homemade or other buffers. If you prefer to make your own buffers, please refer to the following protocol.

This procedure can be used for a confluent cell layer of 75 cm^2 (100-mm dish). The yield is approximately 0.5 mg of nuclear proteins for 10^7 cells.

1. Wash cells with 10 ml of ice-cold PBS/PIB.
2. Add 10 ml of ice-cold PBS/PIB and scrape the cells off the dish with a cell lifter. Transfer the cells into a pre-chilled 15 ml tube and spin at 300 x *g* for 5 minutes at 4°C.
3. Resuspend the pellet in 1 ml of ice-cold HB buffer by gentle pipetting and transfer the cells into a pre-chilled 1.5 ml tube.
4. Allow the cells to swell on ice for 15 minutes.
5. Add 50 µl 10% Nonidet P-40 (0.5 % final) and mix by gentle pipetting.
6. Centrifuge the homogenate for 30 seconds at 4°C in a microcentrifuge.
7. Resuspend the nuclear pellet in 50 µl Complete Lysis Buffer and rock the tube gently on ice for 30 minutes on a shaking platform.
8. Centrifuge for 10 minutes at 14,000 x *g* at 4°C and save the supernatant (nuclear cell extract). Aliquot and store at –80°C. Avoid freeze/thaw cycles.
9. 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 50°C for 5 minutes. Mix again. Store at –20°C.

PBS/PIB

Prior to use, add 0.5 ml of PIB to 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.

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Appendix

Section A. Troubleshooting Guide

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 Wash Buffer and follow washing recommendations
Uneven color development	Incomplete washing of wells and follow washing recommendations	Ensure all wells are filled with Wash Buffer
	Well cross-contamination	Follow washing recommendations
High background in sample wells	Too much nuclear extract per well	Decrease amount of nuclear extract down to 1-2 µg/well
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1:1000 for primary antibody and 1:5000 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 nuclear extract not to exceed 40 µg/well
	p53 is poorly activated or inactivated in nuclear fractions	Perform a time course for p53 activation in the studied cell line
	Extracts are not from human origin	Perform study with a human model

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

Active Motif North America

Toll free: 877.222.9543
Direct: 760.431.1263
Fax: 760.431.1351
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