TP53 Transcription Factor Assay Kit

Catalog Number KA1351

96 assays

Version: 02

Intended for research use only

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Introduction

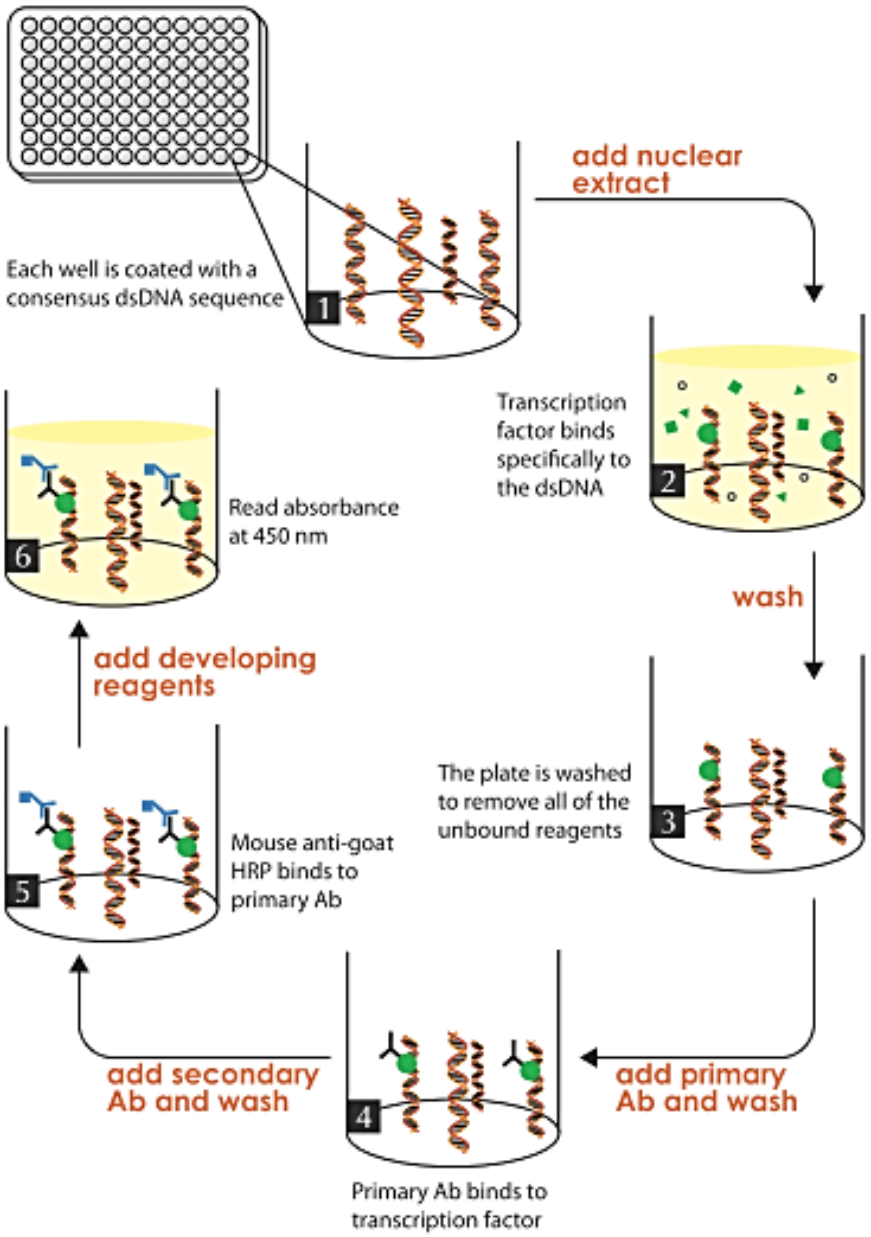
Background

The tumor suppressor protein, p53 is a transcription factor that is commonly referred to as the "guardian of the genome" because of its crucial role in coordinating cellular responses to genotoxic stress.[1] The tumor suppressor activity of p53 is mediated by a variety of mechanisms including cell cycle arrest, apoptosis, and cellular senescence. Approximately 50% of human cancers carry a mutation in the p53 gene; of those tumors that do not have a mutation in the p53 gene, a significant proportion of them have inactivated p53 by alternative mechanisms.[2] Activation of p53 occurs by a variety of internal and external stress signals that result in stabilization of the protein, enhancement of its DNA binding, and transcriptional activity. These changes in p53 are mediated by post-translational modifications of p53 and protein-protein interactions, including ubiquitination, acetylation, phosphorylation, sumoylation, neddylation, methylation, and glycosylation.[3] DNA damage, oncogene activation, ribosomal stress, loss of cell-matrix adhesion, and hypoxia have all been shown to activate p53 resulting in transcription of p53-targeted genes. These p53 target gene products include p21, WAF1, Cip1, MDM2, GADD45, Cyclin G, Bax, and IGF-BP3.[3] There are also genes, which can be repressed by p53, including Bcl-2, Bcl-X, cyclin B1, MAP4, and survivin, some of which are negative regulators of apoptosis. The functions of p53 target genes are diverse, corresponding to p53's activity as a multifunctional protein.[4] Under normal cellular conditions, p53 is maintained at low concentrations and in an inactive form. The regulation of p53 levels and activity involves a complex network of cellular proteins including HPV16, PARP-1, WT1, E1b/E4, MDM2, and others. WT1 or E1B/E4 bind to p53 increasing its stability whereas p53's binding with MDM2 accelerates its degradation through ubiquitination and subsequent degradation.5 When p53 is ubiquitinated it moves out of the nucleus into the cytoplasm where it is rapidly degraded by the proteasome. The MDM2 gene contains a p53 promoter and is therefore transcriptionally regulated by p53 during stress. In this manner p53 itself regulates MDM2 at the level of transcription, where MDM2 protein regulates p53 protein activity.[6]

p53 holds many important clinical implications in the treatment of cancer and is often found to be genetically altered in tumors making it a useful biomarker in carcinogenesis. Restoring endogenous wild-type p53 activity *via* disruption of the MDM2-p53 interaction is of great interest in cancer therapeutics. In addition, the heterogenous autosomal dominant disorder, Li Fraumeni Syndrome, caused by mutations in the p53 gene, is another area of great interest in p53 research.[7]

Principle of the Assay

TP53 Transcription Factor Assay is a non-radioactive, sensitive method for detecting specific transcription factor DNA binding activity in nuclear extracts. A 96-well enzyme-linked immunosorbent assay (ELISA) replaces the cumbersome radioactive electrophoretic mobility shift assay (EMSA). A specific double-stranded DNA (dsDNA) sequence containing the p53 response element is immobilized onto the wells of a 96-well plate. p53 contained in a nuclear extract, binds specifically to the p53 response element. p53 is detected by addition of a specific primary antibody directed against p53. A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm.



General Information

Materials Supplied

List of component

Component	Amount
Transcription Factor Binding Assay Buffer (4x)	3 mL
Transcription Factor Reagent A	120 µL
Transcription Factor p53 Positive Control	150 µL
Transcription Factor Antibody Binding Buffer (10x)	3 mL
Transcription Factor p53 Primary Antibody	120 µL
Wash Buffer Concentrate (400x)	5 mL
Polysorbate 20	3 mL
Transcription Factor p53 Competitor dsDNA	120 µL
Transcription Factor Goat Anti-Mouse HRP Conjugate	120 µL
Transcription Factor p53 96-Well Strip Plate	1 Plate
96-Well Cover Sheet	1 slide
Transcription Factor Developing Solution	12 mL
Transcription Factor Stop Solution	12 mL

Storage Instruction

Component	Storage
Transcription Factor Binding Assay Buffer (4x)	4°C
Transcription Factor Reagent A	-20°C
Transcription Factor p53 Positive Control	-80°C
Transcription Factor Antibody Binding Buffer (10x)	4°C
Transcription Factor p53 Primary Antibody	-20°C
Wash Buffer Concentrate (400x)	RT
Polysorbate 20	RT
Transcription Factor p53 Competitor dsDNA	-20°C
Transcription Factor Goat Anti-Mouse HRP Conjugate	-20°C
Transcription Factor p53 96-Well Strip Plate	4°C
96-Well Cover Sheet	RT
Transcription Factor Developing Solution	4°C
Transcription Factor Stop Solution	RT

Materials Required but Not Supplied

- ✓ A plate reader capable of measuring absorbance at 450 nm
- ✓ Adjustable pipettes and a repeating pipettor
- ✓ A source of UltraPure water; glass Milli-Q or HPLC-grade water are acceptable
- ✓ 300 mM dithiothreitol (DTT)
- ✓ Nuclear Extraction Kit or buffers for preparation of nuclear extracts.

NOTE: The components in each kit lot have been quality assured and warranted in this specific combination only; please do not mix them with components from other lots.

Precautions for Use

✓ Precautions

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

- For research use only. Not for human or diagnostic use.
- Please read the instructions carefully before beginning this assay.
- Kit components may be stored at -20°C prior to use. For long term storage, the Positive Control should be thawed on ice, aliquoted at 25 µL/vial and stored at -80°C. If the assay will be used on multiple days, we recommend each kit component be stored according to the temperatures listed in the booklet.

✓ Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

✓ General Information

- It is not necessary to use all the wells on the plate at one time; however a positive control should be run every time.
- For each plate or set of strips it is recommended that two Blk, two non-specific binding (NSB), and two PC wells be included.

Assay Protocol

Reagent Preparation

- ✓ Transcription Factor Antibody Binding Buffer (10X) - One vial contains 3 mL of a 10X stock of Transcription Factor Antibody Binding Buffer (ABB) to be used for diluting the primary and secondary antibodies. To prepare 1X ABB, dilute 1:10 by adding 27 mL of UltraPure water. Store at 4°C for up to six months.

- ✓ Wash Buffer Concentrate (400X) - One vial contains 5 mL of 400X Wash Buffer. Dilute the contents of the vial to a total volume of 2 liters with UltraPure water and add 1 mL of Polysorbate 20. *NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a pipette. A positive displacement device such as a syringe should be used to deliver small quantities accurately.* A smaller volume of Wash Buffer Concentrate can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 mL/liter of Wash Buffer). Store at 4°C for up to two months.

- ✓ Transcription Factor Binding Assay Buffer (4X) **-** One vial contains 3 mL of a 4X stock of Transcription Factor Binding Assay Buffer (TFB). Prepare Complete TFB Assay Buffer (CTFB) immediately prior to use in 1.5 mL centrifuge tubes or 15 mL conical tubes as outlined in Table 1. This buffer is now referred to as CTFB. *Note: It is recommended that the CTFB be used the same day it is prepared.*

Component	Volume/Well	Volume/Strip	Volume/96-well plate
UltraPure Water	73 µL	584 µL	7,008 µL
4X Transcription Factor Binding Assay Buffer	25 µL	200 µL	2,400 µL
Reagent A	1 µL	8 µL	96 µL
300 mM DTT	1 µL	8 µL	96 µL
Total Required	100 µL	800 µL	9,600 µL

Table 1. Preparation of Complete Transcription Factor Binding Assay Buffer

- ✓ Transcription Factor p53 Positive Control - One vial contains 150 µL of Nutlin-3-stimulated MCF-7 nuclear extract. This nuclear extract is provided as a positive control for p53 activation; it is not intended for plate to plate comparisons. The Positive Control provided is sufficient for 15 reactions and will provide a strong signal (>0.5 AU at 450 nm) when used at 10 µL/well. When using this Positive Control, a decrease in signal may occur with repeated freeze/thaw cycles. It is recommended that the Positive Control be aliquoted at 25 µL per vial and stored at -80°C to avoid loss in signal from repeated freeze/thaw cycles.

Sample Preparation

All buffers and reagents below are required for preparation of Nuclear Extract.

✓ Nuclear Extraction PBS (10x)

1.71 M NaCl, 33.53 mM KCl, 126.8 mM Na_2HPO_4, 22.04 mM KH_2PO_4, pH 7.4

✓ Nuclear Extraction PBS (1x)

Dilute 100 mL of 10x stock with 900 mL distilled H_2O.

✓ Nuclear Extraction Phosphatase Inhibitor Cocktail (50x)

- 0.5 M NaF
- 0.05 M β-glycerophosphate
- 0.05 M Na_3OV_4
- Store at -80°C

✓ Nuclear Extraction PBS/Phosphatase Inhibitor Solution (1x)

Add 200 µL of 50x Phosphatase Inhibitor Solution to 10 mL of 1x Nuclear Extraction PBS, mix well, and keep on ice. Make fresh daily.

✓ Nuclear Extraction Protease Inhibitor Cocktail (100x)

- 10 mM AEBSF
- 0.5 mM Bestatin
- 0.2 mM Leupeptin Hemisulfate Salt
- 0.15 mM E-64
- 0.1 mM Pepstatin A
- 0.008 mM Aprotinin from Bovine Lung
- Made in DMSO, store at -20°C

✓ Nuclear Extraction Hypotonic Buffer (10X)

100 mM HEPES, pH 7.5, containing, 40 mM NaF, 10 µM Na_2MoO_4, and 0.1 mM EDTA.

Store at 4°C

✓ Complete Hypotonic Buffer (1x)

Prepare as outlined in Table 2. The phosphatase and protease inhibitors lose activity shortly after dilution; therefore any unused 1x Complete Hypotonic Buffer should be discarded.

Reagent	150 mm plate~1.5 x 10^7 cells
Hypotonic Buffer (10X)	100 µL
Phosphatase Inhibitors (50X)	20 µL
Protease Inhibitors (100X)	10 µL
Distilled Water	870 µL
Total Volume	1,000 µL

Table 2. Preparation of Complete Hypotonic Buffer.

- ✓ Nonidet P-40 Assay Reagent (10%) - Nonidet P-40 or suitable substitute at a concentration of 10% (v/v) in H_2O. Store at room temperature.

- ✓ Nuclear Extraction Buffer (2X)
  20 mM HEPES, pH 7.9, containing, 0.2 mM EDTA, 3 mM $MgCl_2$, 840 mM NaCl, and 20% glycerol (v/v)
  Store at 4°C

- ✓ Complete Nuclear Extraction Buffer (1X) - Prepare as outlined in Table 3. Some of the phosphatase and protease inhibitors lose activity shortly after dilution; therefore any remaining 1X Extraction Buffer should be discarded.

Reagent	150 mm plate~1.5 x 10^7 cells
Nuclear Extraction Buffer (2X)	75 µL
Protease Inhibitors (100X)	1.5 µL
Phosphatase Inhibitors (50X)	3.0 µL
DTT (10 mM)	15 µL
Distilled Water	55.5 µL
Total Volume	150 µl

Table 3. Preparation of Complete Nuclear Extraction Buffer

- ✓ Purification of Cellular Nuclear Extracts

The procedure described below can be used for a 15 mL cell suspension grown in a T75 flask or adherent cells (100 mm dish 80-90% confluent) where 10^7 cells yields approximately 50 µg of nuclear protein.

1. Collect ~10^7 cells in pre-chilled 15 mL tubes.
2. Centrifuge suspended cells at 300 x g for five minutes at 4°C.
3. Discard the supernatant. Resuspend cell pellet in 5 mL of ice-cold 1x Nuclear Extraction PBS/Phosphatase Inhibitor Solution and centrifuge at 300 x g for five minutes at 4°C. Repeat one time.
4. Discard the supernatant. Add 500 µL ice-cold 1X Complete Hypotonic buffer. Mix gently by pipetting and transfer resuspended pellet to pre-chilled 1.5 mL microcentrifuge tube.
5. Incubate cells on ice for 15 minutes allowing cells to swell.
6. Add 100 µL of 10% Nonidet P-40 (or suitable substitute). Mix gently by pipetting.
7. Centrifuge for 30 seconds (pulse spin) at 4°C in a microcentrifuge. Transfer the supernatant which

contains the cytosolic fraction to a new tube and store at -80°C.

8. Resuspend the pellet in 100 µL ice-cold Complete Nuclear Extraction Buffer (1X) (with protease and phosphatase inhibitors). Vortex 15 seconds at highest setting then gently rock the tube on ice for 15 minutes using a shaking platform. Vortex sample for 30 seconds at highest setting and gently rock for an additional 15 minutes.
9. Centrifuge at 14,000 x g for 10 minutes at 4°C. The supernatant contains the nuclear fraction. Aliquot to clean chilled tubes, flash freeze and store at -80°C. Avoid freeze/ thaw cycles. The extracts are ready to use in the assay.
10. Keep a small aliquot of the nuclear extract to quantitate the protein concentration.

Assay Procedure

✓ Binding of active p53 to the consensus sequence

1. Equilibrate the plate and buffers to room temperature prior to opening. Remove the plate from the foil and select the number of strips needed. The 96-well plate supplied with this kit is ready to use.
   NOTE: If you are not using all of the strips at once, place the unused strips back in the plate packet and store at 2-4°C. Be sure that the packet is sealed with the desiccant inside.
2. Prepare the CTFB as outlined in Table 1.
3. Add appropriate amount of reagent(s) listed below to the designated wells as follows:
   Blk - add 100 µL of CTFB to designated wells.
   NSB - add 100 µL of CTFB to designated wells. Do not add samples or Positive Control to these wells.
   C1 - Add 80 µL of CTFB prior to adding 10 µL of Transcription Factor p53 Competitor dsDNA to designated wells. Add 10 µL of control cell lysate, or sample.
   NOTE: Competitor dsDNA must be added prior to adding the positive control or nuclear extracts.
   S1-S44 - Add 90 µL of CTFB followed by 10 µL of Nuclear Extract to designated wells.
   PC - Add 90 µL of CTFB followed by 10 µl of Positive Control to appropriate wells.
4. Use the cover provided to seal the plate. Incubate overnight at 4°C or one hour at room temperature without agitation (incubation for one hour will result in a less sensitive assay).
5. Empty the wells and wash five times with 200 µL of 1X Wash Buffer. After each wash empty the wells in the sink. After the final wash (wash #5), tap the plate on a paper towel to remove any residual Wash Buffer.

✓ Addition of Transcription Factor p53 Primary Antibody

1. Dilute the Transcription Factor p53 Primary Antibody 1:100 in 1X ABB as outlined in Table 4 below. Add 100 µL of diluted p53 Primary Antibody to each well except the Blk wells.

Component	Volume/Well	Volume/Strip	Volume/96-well plate
1X ABB	99 µL	792 µL	9,504 µL
p53 Primary Antibody	1 µL	8 µL	96 µL
Total required	100 µL	800 µL	9,600 µL

Table 4. Dilution of Primary Antibody

2. Use the adhesive cover provided to seal the plate.
3. Incubate the plate for one hour at room temperature without agitation.
4. Empty the wells and wash each well five times with 200 µL of 1X Wash Buffer. After each wash, empty the contents of the plate into the sink. After the final wash (wash #5), tap the plate three to five times on a paper towel to remove any residual Wash Buffer.

✓ Addition of the Transcription Factor Goat Anti-Mouse HRP Conjugate

1. Dilute the Transcription Factor Goat Anti-Mouse HRP Conjugate 1:100 in 1X ABB as outlined in Table 5 below. Add 100 µL of diluted secondary antibody to each well except the Blk wells.

Component	Volume/Well	Volume/Strip	Volume/96-well plate
1X ABB	99 µL	792 µL	9,504 µL
Goat Anti-Mouse HRP Conjugate	1 µL	8 µL	96 µL
Total required	100 µL	800 µL	9,600 µL

Table 5. Dilution of Conjugate

2. Use the adhesive cover provided to seal the plate.
3. Incubate for one hour at room temperature without agitation.
4. Empty the wells and wash five times with 200 µL of 1X Wash Buffer. After each wash, empty the contents of the plate into the sink. After the final wash (wash #5), tap the plate three to five times on a paper towel to remove any residual Wash Buffer.

✓ Develop and Read the Plate

1. To each well being used add 100 µL of Transcription Factor Developing Solution, which has been equilibrated to room temperature.
2. Incubate the plate for 15 to 45 minutes at room temperature with gentle agitation protected from light. Allow the wells to turn medium to dark blue prior to adding Transcription Factor Stop Solution. (This reaction can be monitored by taking absorbance measurements at 655 nm prior to stopping the reactions; An OD_{655} of 0.4-0.5 yields an OD_{450} of approximately 1). Monitor development of sample wells to ensure adequate color development prior to stopping the reaction. *NOTE: Do not overdevelop; however Positive Control wells may need to overdevelop to allow adequate color development in sample wells.*
3. Add 100 µL of Stop Solution per well being used. The solution within the wells will change from blue to yellow after adding the Stop Solution.
5. Read absorbance at 450 nm within five minutes of adding the Stop Solution. Blank the plate reader according to the manufacturer's requirements using the blank wells

✓ Summary

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when initially performing the assay.

1. Prepare CTFB as described in the Pre-Assay Preparation section, Table 1.
2. Add 90 µL CTFB per sample well (80 µL if adding Competitor dsDNA), 100 µL to Blk and NSB wells).
3. Add 10 µL of Competitor dsDNA (optional) to appropriate wells.
4. Add 10 µL of Positive Control to appropriate wells.
5. Add 10 µL of Sample containing p53 to appropriate wells.
6. Incubate overnight at 4°C or one hour at room temperature without agitation.
7. Wash each well five times with 200 µL of 1X Wash Buffer.
8. Add 100 µL of diluted p53 Primary Antibody per well (except Blk wells).
9. Incubate one hour at room temperature without agitation.
10. Wash each well five times with 200 µL of 1X Wash Buffer.
11. Add 100 µL of diluted Goat Anti-Mouse HRP Conjugate (except Blk wells).
12. Incubate one hour at room temperature without agitation.
13. Wash each well five times with 200 µL of 1X Wash Buffer.
14. Add 100 µL of Developing Solution per well.
15. Incubate 15 to 45 minutes with gentle agitation.
16. Add 100 µL of Stop Solution per well.
17. Measure the absorbance at 450 nm.

Steps	Reagent	Blk	NSB	PC	C1	S1-S44
1. Add reagents	CTFB	100 µL	100 µL	90 µL	80 µL	90 µL
	Competitor dsDNA				10 µL	
	Positive Control			10 µL	10 µL	
	Samples					10 µL
2. Incubate	Cover plate and incubate overnight at 4°C one hour at room temperature without agitation					
3. Wash	Wash all wells five times					
4. Add reagents	Primary Antibody		100 µL	100 µL	100 µL	100 µL
5. Incubate	Cover plate and incubate one hour at room temperature without agitation					
6. Wash	Wash all wells five times					
7. Add reagents	Secondary Antibody		100 µL	100 µL	100 µL	100 µL
8. Incubate	Cover plate and incubate one hour at room temperature without agitation					
9. Wash	Wash all wells five times					
10. Add reagents	Developer Solution	100 µl	100 µl	100 µl	100 µl	100 µl
11. Incubate	Monitor development in wells					
12. Add reagents	Stop Solution	100 µl	100 µl	100 µl	100 µl	100 µl
13. Read	Read plate at wavelength of 450 nm					

Data Analysis

Performance Characteristics

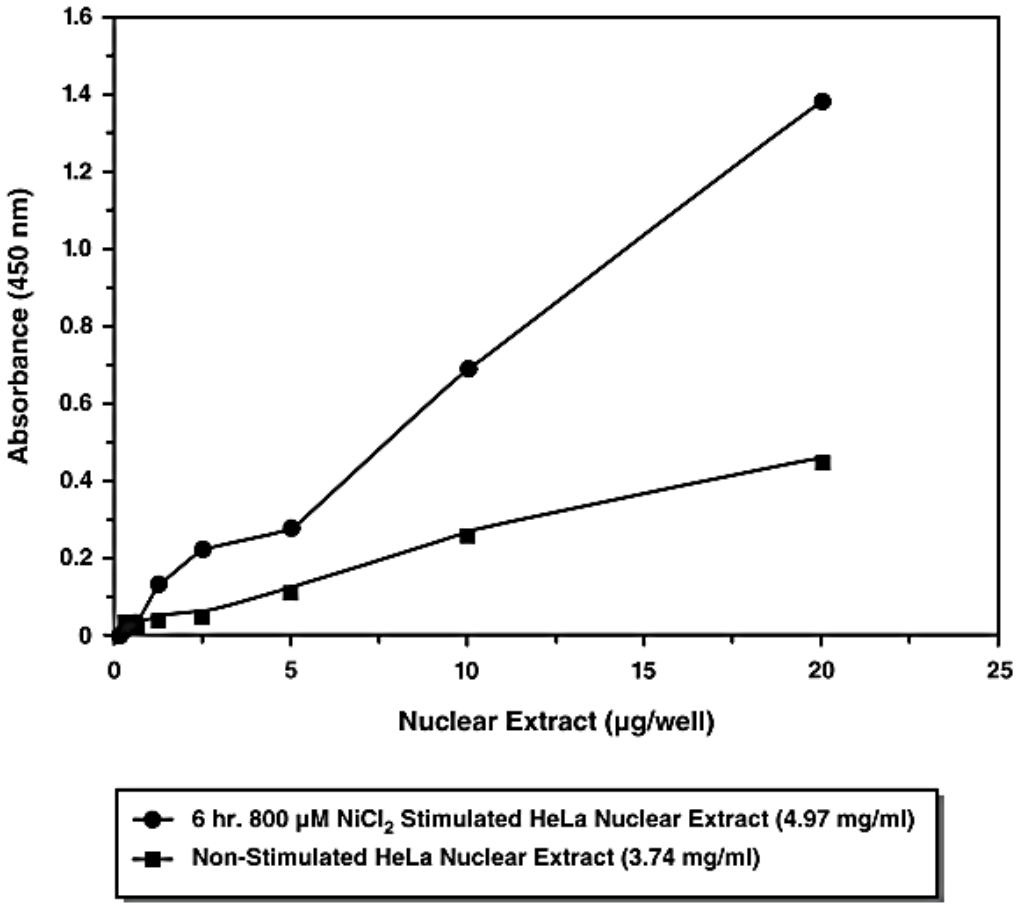


Figure 1. Assay of p53 from nickel chloride stimulate HeLa cell nuclear extracts.

✓ Interferences

The following reagents were tested for interference in the assay.

Reagent	Will Interfere (Yes or No)
EGTA (≤1 mM)	No
EDTA (≤0.5 mM)	No
ZnCl (any concentration)	Yes
DTT (between 1 and 5 mM)	No
Dimethylsulfoxide (≤1.5%)	No

Resources

Trouble shooting

Problem	Possible Causes	Recommended Solutions
No signal or weak signal in all wells	1. Omission of key reagent 2. Plate reader settings not correct 3. Reagent/reagents expired 4. Salt concentrations affected binding between DNA and protein 5. Developing reagent used cold 6. Developing reagent not added to correct volume	1. Check that all reagents have been added and in the correct order. Perform the assay using the positive control 2. Check wavelength setting on plate reader and change to 450 nm 3. Check expiration date on reagents 4. Reduce the amount of nuclear extract used in the assay, or reduce the amount of salt in the nuclear extracts (alternatively can perform buffer exchange) 5. Prewarm the Developing Solution to room temperature prior to use 6. Check pipettes to ensure correct amount of developing solution was added to wells
High signal in all wells	1. Incorrect dilution of antibody (too high) 2. Improper/inadequate washing of wells 3. Over-developing	1. Check antibody dilutions and use amounts outlined in instructions 2. Follow the protocol for washing wells using the correct number of times and volumes 3. Decrease the incubation time when using the developing reagent
High background (NSB)	Incorrect dilution of antibody (too high)	Check antibody dilutions and use amounts outlined in the instructions
Weak signal in sample wells	1. Sample concentration too low 2. Incorrect dilution of antibody 3. Salt concentrations affecting binding between DNA and protein	1. Increase the amount of nuclear extract used; loss of signal can occur with multiple freeze/thaw cycles of the sample; prepare fresh nuclear extracts and aliquot as outlined in product insert 2. Check antibody dilutions and use amounts outlined in the instructions 3. Reduce the amount of nuclear extract used in the assay or reduce the amount of salt in the nuclear extracts (alternatively can perform buffer exchange)

References

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Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44
E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37	NSB	NSB
F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38	PC	PC
G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39	Blk	Blk
H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40	C1	C1

S1-S44-Sample Wells

NSB-Non-specific Binding Wells

PC- Positive Control Wells

Blk- Blank Wells

C1-Competitor dsDNA Wells