LSBio
an Absolute Biotech Company

Mouse / Human / Rat FOXO4 / AFX1 DNA Binding ELISA Kit

User Manual

Catalog No. LS-F758

It is important that you read this entire manual carefully before starting your experiment.

SAMPLE ONLY

This kit is for Research Use Only. Not for Diagnostic Use.
This kit is not approved for use in humans or for clinical diagnosis.

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Assay Specifications

Target: FOXO4 / AFX1

Synonyms: FOXO4 / AFX1, FOXO4, forkhead box O4, AFX, AFX1, Forkhead box O4, Forkhead box protein O4, MLLT7

Specificity: This kit is for the detection of Mouse / Human / Rat FOXO4 / AFX1. No significant cross-reactivity or interference between FOXO4 / AFX1 and analogs was observed. This claim is limited by existing techniques therefore cross-reactivity may exist with untested analogs.

Sample Types: This kit is intended for use with samples such as Cell Lysates and Nuclear Lysates.

Detection: Colorimetric - 450nm (TMB)

Limitations: This kit is for **Research Use Only** and is not intended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis.

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Assay Principle

The LSBio™ DNA-Binding ELISA Kit contains components necessary for detection of active transcription factors in eukaryotic nuclear or cell lysates. This particular immunoassay utilizes the qualitative technique of an indirect ELISA. Streptavidin is bound to the immunoassay plate and specific biotinylated double-stranded (dsDNA) oligonucleotides are then added to bind to the streptavidin via a high affinity biotin-streptavidin interaction. After subsequent blocking of extraneous binding sites in each well, the sample containing the target of interest can be added. Primary antibody is added to bind activated transcription factors bound to the dsDNA oligonucleotide, which has been immobilized via the plate coated streptavidin. A HRP-conjugated secondary antibody specific for rabbit IgGs is added, which allows for specific binding to the Primary Antibody, and consequently colorimetric detection upon addition of the TMB substrate.

For color development, TMB (3, 3′, 5, 5′-Tetramethylbenzidine) is added to each well. After addition of the substrate, a peroxidase catalyzed reaction will produce a blue TMB Diimine product that is proportional to the target concentration in the sample. Color development is quenched by addition of Stop Solution, or 2 N Sulfuric Acid, which turns the solution yellow. The absorbance can then be read by a spectrophotometer at 450 nm and subsequently allowing for determination of the target concentration in the sample.

Currently the most common methods to detect transcription factor binding to DNA elements and motifs are electrophoretic mobility shift assays (EMSAs), chromatin immunoprecipitation, western blotting, and expression of fused target and reporter genes. These methods are often time consuming, complicated, and make it difficult to achieve satisfactory results. LSBio™ DNA-Binding ELISA Kits can significantly reduce the necessary runtime to within one day and eliminate the need for harmful radioactive labeling while maintaining high sensitivity and signal-to-noise ratio. In the past it was strenuous and inefficient to perform high-throughput screening for hundreds of different samples or transcription factors. Today our revolutionary LSBio™ DNA-Binding ELISA Kits can eliminate these challenges and help expedite the journey from research to publication or product.

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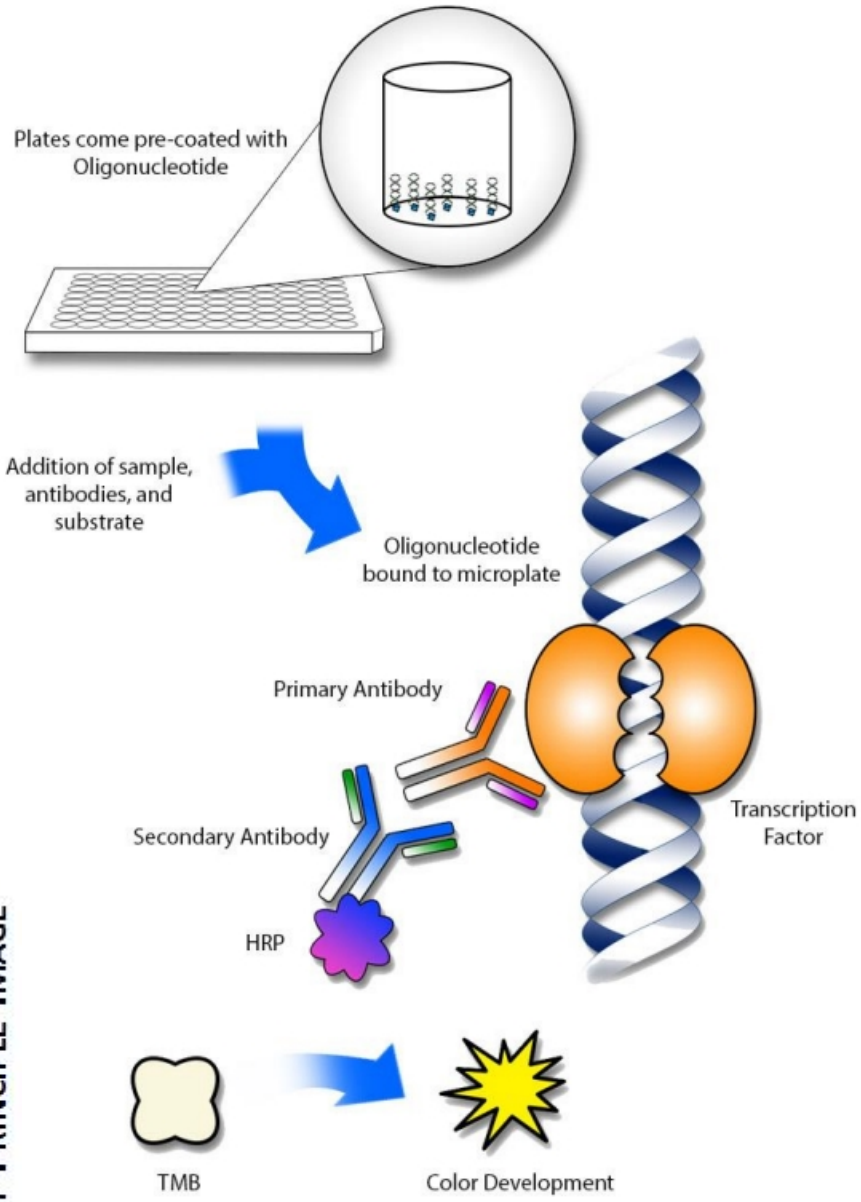
Assay Principle Image



Assay Restrictions

This ELISA kit is intended for **Research Use Only** and is not intended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis.

Materials included in this kit should NOT be used past the expiration date on the kit label.

Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.

Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time, or temperature may cause differences in binding affinity of the materials provided.

The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any biological samples. However the possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.

Health and Safety Precautions

This kit and its components should be handled by those trained in laboratory techniques and used according to the principles of good laboratory practice.

Reagents provided in this kit may be harmful if ingested, inhaled, or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.

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Kit Components

Component	Quantity	Container
12 x 8-Well dsDNA Coated Microplate	1	-
100x Primary Antibody	100 µL	Yellow
HRP-Conjugated Anti-Rabbit IgG Antibody	2 vials of 6 mL	Amber
Nuclear Lysate Positive Control	Lyophilized	Red
Wild-Type Consensus dsDNA Oligonucleotide	20 µL	Green
Mutant Consensus dsDNA Oligonucleotide	20 µL	Purple
10x Wash Buffer	50 mL	Clear
2x Binding Buffer	12 mL	Clear
Primary Antibody Diluent	12 mL	Clear
Nuclear Wash Buffer	12 mL	Clear
Cytoplasmic Extraction Buffer	6 mL	Amber
Nuclear Extraction Buffer	6 mL	Amber
Ready-to-Use Substrate	12 mL	Brown
Stop Solution	12 mL	Clear
Adhesive Plate Sealers	4	-
Technical Manual	1	-

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Kit Storage

Note: After receiving the kit please open and store the kit components at the temperature indicated in the table below. If used frequently reagents may be stored at 4°C. Reconstituted Nuclear Lysate Positive Control must be aliquoted and stored at -80°C. All kit components are stable for 6 months when stored as recommended.

Component	Temperature
12 x 8-Well dsDNA Coated Microplate	4°C
100x Primary Antibody	4°C
HRP-Conjugated Anti-Rabbit IgG Antibody	4°C
Nuclear Lysate Positive Control	Lyophilized: 4°C Reconstituted: -80°C
Wild-Type Consensus dsDNA Oligonucleotide	-20°C
Mutant Consensus dsDNA Oligonucleotide	-20°C
10x Wash Buffer	4°C
2x Binding Buffer	-20°C
Primary Antibody Diluent	4°C
Nuclear Wash Buffer	-20°C
Cytoplasmic Extraction Buffer	-20°C
Nuclear Extraction Buffer	-20°C
Ready-to-Use Substrate	4°C
Stop Solution	4°C

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Other Required Supplies

The following materials and/or equipment are NOT provided in this kit but are necessary to successfully conduct the experiment:

- Microplate reader able to measure absorbance at 450 nm (with correction wavelength set to 540 nm or 570 nm)
- Micropipettes capable of measuring volumes from 1 µL to 1 mL
- Deionized or sterile water (ddH_2O)
- PMSF (Sigma Cat. #78830)
- Protease Inhibitor Cocktail (Sigma Cat. #P-2714)
- Glycerol (Acros Cat. #158920100)
- Sterile 1x PBS and 5 M NaCl for nuclear lysate preparation
- Squirt bottle, manifold dispenser, multichannel pipette reservoir, or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent paper or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 mL
- Bench-top centrifuge (optional)
- Bench-top vortex (optional)
- Orbital shaker (optional)

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Assay Planning

Before using this kit researchers should consider the following:

1. Read this manual in its entirety in order to minimize the chance of error.
2. Confirm that you have the appropriate non-supplied equipment available.
3. Confirm that the species, target antigen, and sensitivity of this kit are appropriate for your intended application.
4. Confirm that your samples have been prepared appropriately based upon recommendations (see **Sample Preparation**) and that you have sufficient sample volume for use in the assay.
5. When first using the kit appropriate validation steps should be taken before using valuable samples. Confirm that the kit adequately detects the target antigen in your intended sample type(s) by running control samples.
6. If the concentration of target antigen within your samples is unknown a preliminary experiment should be run using a control sample to determine the optimal sample dilution.
7. Ensure that the kit is properly stored and do not use it beyond its expiration date.
8. When using multiple lots of the same kit do not substitute reagents from one kit to another. Review each manual carefully as changes can occur between lots. To control for inter-assay variability include a carry-over control sample.

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Reagent Preparation

The following reagents will need to be prepared prior to start of the assay:

1x Wash Buffer
The Wash Buffer is provided at 10x concentration. To prepare 1x Wash Buffer, add 50 mL of 10x Wash Buffer into 450 mL of ddH_2O for a final volume of 500 mL of 1x Wash Buffer.

Nuclear Lysate Positive Control
The Binding Buffer is provided at 2x concentration. It is recommended to make fresh 1x Binding Buffer for the reconstitution of Nuclear Lysate Positive Control. Add 60 µL of 2x Binding Buffer to 60 µL ddH2O to make 120 µL of 1x Binding Buffer. Add 100 µL of 1x Binding Buffer into the Nuclear Lysate Positive Control tube. The Nuclear Lysate Positive Control should be kept on ice at all times. Aliquot and store at -80°C (long term storage) and avoid freeze/thaw cycles if not used immediately.

1x Primary Antibody
The Primary Antibody is provided at 100x concentration. It is recommended to make a fresh 1x Antibody solutions. Add 100 µL of 100x Primary Antibody into 9.9 mL of Primary Antibody Diluent to make enough 1x Primary Antibody solution for one 96-well microplate.

Aliquoting of Buffers and Reagents
If you do not plan on using the whole kit in one sitting it is recommended to aliquot the buffers and reagents, reconstituted Nuclear Lysate Positive Control, 2x Binding Buffer, 100x Protease and Phosphatase Inhibitors, Cytoplasmic Extraction Buffer, Nuclear Wash Buffer, Nuclear Extraction Buffer, etc. and store them at the temperatures indicated [see **Kit Storage**].

HRP-Conjugated Anti-Rabbit IgG Secondary Antibody, Ready-to-Use Substrate, Stop Solution, Primary Antibody Diluent, Wild-Type (WT) Consensus dsDNA Oligonucleotide, Mutant (MT) Consensus dsDNA Oligonucleotide, Nuclear Wash Buffer, Cytoplasmic Extraction Buffer, Nuclear Extraction Buffer are ready-to-use.

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Sample Preparation and Storage

The LSBio™ DNA-Binding ELISA Kit allows for the detection and qualitative analysis of endogenous levels of activated transcription factors in a variety of nuclear and cell lysates. All preparations of experimental samples should maintain the natural and active form of the target transcription factor. In this kit all necessary buffers and reagents are provided for nuclear extraction from cell culture.

Tissue homogenates and heterogeneous mixtures may contain contaminants which interfere with the assay, hence it is best to test for interference by using at least two different dilutions of the sample. If testing demonstrates good correlation between concentration/dilution factor and OD reading, purification may not be required. However if good correlation is not achieved or seen further purification is advised. Moreover if samples contain any visible precipitate they must be centrifuged for 10 minutes at ≥10,000 x g prior to use in the assay.

It is always recommended to make several dilutions to obtain the best OD reading. Ideal OD readings will fall within the detectable range of the assay, which is dependent on the spectrophotometer used. It is up to the investigator to determine an appropriate dilution factor and recommended to run each dilution in duplicates. A minimum of 100 µL of sample or diluted sample is required for each well; please adjust dilution volumes accordingly.

If samples are ready to be used within 24 hours aliquot and store at 4°C. If samples are to be saved for future or long term use aliquot into multiple tubes and store at -80°C. Avoid repeated freeze/thaw cycles to prevent loss of biological activity of transcription factors in experimental samples.

If a sample contains any visible precipitate or pellet it must be clarified prior to use in the assay.

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Nuclear and Cytoplasmic Extraction Protocol

The LSBio DNA-Binding ELISA Kit includes some of the necessary buffers for nuclear and cytoplasmic extraction from cultured cells. These buffers must be supplemented with PMSF (not included) and Protease Inhibitor Cocktail (not included) immediately prior to use. For the Protease Inhibitory Cocktail, we recommend one from Sigma-Aldrich (Cat. # P-2714).

Many transcription factors may not be readily expressed in normal cell culture therefore cell stimulation is often needed to increase the expression of target protein. Use this protocol for cytoplasmic and nuclear extraction following your own cell stimulation/cell culture protocol.

Preparation of Stock Solutions and Buffers

The PMSF Stock Solution, Inhibitor PBS Buffer, Complete Cytoplasmic Extraction Buffer, and the Complete Nuclear Extraction Buffer should all be prepared prior to extraction. PMSF is unstable and must be added fresh just prior to use. Buffers with protease/phophatase added in, like PMSF, have a 24-hour shelf-life at 4°C.

Note: This is just a recommended protocol for your convenience. You may need to optimize the cell extraction procedure for your own experiments and applications.

Preparation of PMSF Stock Solution (100 mM)

Materials: PMSF, DMSO, 1.5 mL microfuge tube

1. Add 0.175 g of PMSF to 10 mL of DMSO.
2. Mix, aliquot into 1 mL tubes, and store at -20°C.

Preparation of PPI (Protease/Phophatase Inhibitor) Buffer

Materials: 1x PBS, PMSF Stock Solution (100 mM), Protease Inhibitor Cocktail

1. Add 250 µL of the Protease Inhibitor Cocktail for every 5 mL of 1x PBS.
2. Add the appropriate amount of PMSF stock solution (100 mM) to the Protease Inhibitor Cocktail/PBS mix for a final concentration of 1 mM PMSF to make the PPI Buffer.

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Preparation of Complete Cytoplasmic Extraction Buffer

Materials: Cytoplasmic Extraction Buffer, PMSF Stock Solution (100 mM), Protease Inhibitor Cocktail

1. Add 250 µL of the Protease Inhibitory Cocktail per 5 mL of Cytoplasmic Extraction Buffer.
2. Add sufficient volume of PMSF stock solution to this mix of Protease Inhibitory Cocktail and Cytoplasmic Extraction Buffer for a final concentration of 1 mM PMSF. This will be the Complete Cytoplasmic Extraction Buffer.

Preparation of Complete Nuclear Extraction Buffer

Materials: Nuclear Extraction Buffer, PMSF Stock Solution (100 mM), Protease Inhibitor Cocktail

1. Add 250 µL of the Protease Inhibitory Cocktail per 5 mL of Nuclear Extraction buffer.
2. Add sufficient volume of PMSF stock solution to this mix of Protease Inhibitory Cocktail and Nuclear Extraction Buffer for a final concentration of 1 mM PMSF. This will be the Complete Nuclear Extraction Buffer.

Cytoplasmic Extraction Procedure

1. For suspension cells, collect cells by centrifuging at 500 x g for 5 minutes. Wash once with cold 1x PBS, and proceed to step five. For adherent cells, wash plates twice with cold 1x PBS.
2. Add 0.5 mL of cold PPI Buffer to each plate. Dislodge cells with a cell scraper, and collect in a pre-chilled 50 mL tube.
3. Wash plates once more with cold PPI Buffer to collect remaining cells and put into the same 50 mL tube.
4. Centrifuge the cell suspension at 500 x g for 5 minutes at 4°C.
5. Re-suspend the pellet in 5x the pellet volume of Complete Cytoplasmic Extraction Buffer. Transfer to a pre-chilled 2 mL tube and keep on ice for 5 minutes.
6. Centrifuge the tube at 3000 x g for 4 minutes at 4°C. Transfer the supernatant to new pre-chilled 2 mL tube and save the pellet. The supernatant is the cytoplasmic lysate. We

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recommend adding enough glycerol for a glycerol concentration of 10%. The cytoplasmic lysate can then be stored at -80°C.

Nuclear Extraction Procedure

1. After transferring out the cytoplasmic lysate, wash the remaining pellet twice by re-suspending the pellet in 1 mL to 2 mL of Nuclear Wash Buffer. Centrifuge at 3000 x g for 4 minutes and discard the supernatant. Resuspend the pellet with Complete Nuclear Extraction Buffer equal to 2x the pellet volume.
2. If volume changes are greater than or equal to 50 µL after re-suspension, add 1/10th pellet volume of 5 M NaCl and incubate the tube for 30 minutes on a shaking platform at 4°C. If volume changes are less than 50 µL, proceed to incubation step indicated above.
3. Centrifuge the tub at maximum speed for 10 minutes at 4°C. This supernatant is the nuclear extract.
4. Determine the concentration of the nuclear extract via Bradford Assay or other preferred methods.
5. Aliquot the nuclear extract and store at -80°C. Avoid freeze/thaw cycles if not used within 24 hours.

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Plate Setup

The 96-well microplate provided with this kit is ready to use and coated with streptavidin bound to biotinylated oligonucleotides which will allow activated transcription factor binding. It is not necessary to rinse plates prior to assay. It is recommended to assay all unknown samples and controls in duplicates. If not all the strips are used at once, keep unused strips sealed and store at 4°C.

A number of controls are included to ensure kit and data quality. It is recommended to run the Nuclear Lysate Positive Control (NLPC) as well as to perform a Primary Antibody negative control to determine background noise. The Wild-Type Consensus dsDNA Oligonucleotide (WT Oligo) and Mutant Consensus dsDNA Oligonucleotide (MT Oligo) controls are optional and used to determine binding specificity of activated transcription factors in samples. The following is an example of a setup that can be used.

	1	2	3	4	...
A	+ 1:10 NLPC, + Primary Ab	+ WT Oligo, + NLPC, + Primary Ab	+ Sample, + Primary Ab	+ Sample, + Primary Ab	...
B	+ 1:20 NLPC, + Primary Ab	+ WT Oligo, + NLPC, + Primary Ab	+ Sample, + Primary Ab	+ Sample, + Primary Ab	...
C	+ 1:40 NLPC, + Primary Ab	+ WT Oligo, + NLPC, + Primary Ab	+ Sample, + Primary Ab	+ Sample, + Primary Ab	...
D	- NLPC, + Primary Ab	+ WT Oligo, - NLPC, + Primary Ab	+ Sample, + Primary Ab	+ Sample, + Primary Ab	...
E	+ 1:10 NLPC, - Primary Ab	+ MT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary Ab	+ Sample, + Primary Ab	...
F	+ 1:20 NLPC, - Primary Ab	+ MT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary Ab	+ Sample, + Primary Ab	...
G	+ 1:40 NLPC, - Primary Ab	+ MT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary Ab	+ Sample, + Primary Ab	...
H	- NLPC, - Primary Ab	+ MT Oligo, - NLPC, + Primary Ab	+ Sample, - Primary Ab	+ Sample, + Primary Ab	...

ImmunoAssay Protocol

If possible all incubation steps should be performed on an orbital shaker to allow added solutions to equilibrate and mix properly. Aside from the Nuclear Lysate Positive Control all provided solutions should be brought to ambient temperature prior to use.

Ensure all 1x Wash Buffer is removed at end of each wash step by blotting a dry towel. DO NOT leave any 1x Wash Buffer in the wells prior to proceeding to the next steps as it may affect assay results.

Nuclear Lysate Positive Control (NLPC)

1. The Nuclear Lysate Positive Control is lyophilized; reconstitute by adding 100 µL of 1x Binding Buffer. It is advised to run the positive control in duplicate or triplicate. The suggested dilutions for Nuclear Lysate Positive Control in 1x Binding Buffer are 1:10, 1:20, 1:40, and Blank.

Dilution	2x Binding Buffer	ddH_2O	Nuclear Lysate Positive Control	Total Volume
1:10	105 µl	84 µl	21 µl	210 µl
1:20	105 µl	94.5 µl	10.5 µl	210 µl
1:40	105 µl	99.75 µl	5.25 µl	210 µl
Blank	105 µl	105 µl	0 µl	210 µl

2. Add 100 µL of Nuclear Lysate Positive Control dilutions to the appropriate wells. For the negative Nuclear Lysate Positive Control well, add 100 µL of 1x Binding Buffer.

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Primary Antibody Negative Controls (-Primary Ab)

1. In the Primary Antibody negative controls, the Primary Antibody is left out to correct for any background noise. The Primary Antibody negative controls should be performed for both the Nuclear Lysate Positive Control and samples. Follow the volumes below for Primary Antibody negative controls for the Nuclear Lysate Positive Controls.

Dilution	2x Binding Buffer	ddH_2O	Nuclear Lysate Positive Control	Total Volume
1:10	105 µl	84 µl	21 µl	210 µl
1:20	105 µl	94.5 µl	10.5 µl	210 µl
1:40	105 µl	99.75 µl	5.25 µl	210 µl
Blank	105 µl	105 µl	0 µl	210 µl

2. Add 100 µL of Nuclear Lysate Positive Control dilutions or Sample dilutions to the appropriate Primary Antibody negative control wells. For the negative Nuclear Lysate Positive Control and negative Sample wells, add 100 µL of 1x Binding Buffer.

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Wild-Type and Mutant Consensus Oligonucleotides (WT/MT Oligo) (Optional)

The Wild-Type Oligonucleotide and Mutant Oligonucleotide controls are optional and used to determine binding specificity of active transcription factors in samples. If active transcription factors in samples are binding specifically to the Wild-Type sequence, there will be a reduction in signal in the Wild-Type control but not in the Mutant control. If they are binding non-specifically, there will be reduced signal from both Wild-Type and Mutant Oligonucleotide controls.

1. We recommend a final concentration of 0.5 nmol of Wild-Type (WT Oligo) or Mutant (MT Oligo) Oligonucleotide in each well. The suggested dilutions for the Wild-Type Oligonucleotide Control follow the recommended positive control with addition of 2 µL of WT Oligo in each Nuclear Lysate Positive Control working solution.

Dilution	2x Binding Buffer	ddH_2O	Nuclear Lysate	WT Oligo	Total Volume
1:10	105 µl	81.9 µl	21 µl	2.1 µl	210 µl
1:20	105 µl	92.4 µl	10.5 µl	2.1 µl	210 µl
1:40	105 µl	97.65 µl	5.25 µl	2.1 µl	210 µl
Blank	105 µl	102.9 µl	0 µl	2.1 µl	210 µl

2. Add 100 µL of WT Oligo Control Dilution into the appropriate WT Oligo Control wells.
3. The suggested dilutions for the MT Oligo Control follow the recommended positive control with addition of 2 µL of MT Oligo in each positive control.

Dilution	2x Binding Buffer	ddH_2O	Nuclear Lysate	MT Oligo	Total Volume
1:10	105 µl	81.9 µl	21 µl	2.1 µl	210 µl
1:20	105 µl	92.4 µl	10.5 µl	2.1 µl	210 µl
1:40	105 µl	97.65 µl	5.25 µl	2.1 µl	210 µl
Blank	105 µl	102.9 µl	0 µl	2.1 µl	210

4. Add 100 µL of MT Oligo Control Dilution into the appropriate MT Oligo Control wells.

Unknown Sample

Transcription Factors are expressed differently across various tissues, cell types, growth stages, and culture conditions. Carefully determine the amount of sample used; we recommend 5 µg or more of cell lysate per well. If the sample concentrations are unknown create several dilutions. It is recommended to perform a Primary Antibody negative control for sample wells to determine background noise. It is also recommended to run your samples in duplicates or triplicates.

1. Determine the volume and dilution necessary for your application. Using 2x Binding Buffer, add appropriate volume so that the final working Sample Dilution contains 1x Binding Buffer.

   Total Working Volume = 100 µL x Number of Sample Wells x 2

2. Add 100 µL of diluted samples to corresponding wells. For negative sample wells, add 100 µL of 1x Binding Buffer. Incubate plate on orbital shaker at room temperature for 2 hours.
3. Wash three times with 1x Wash Buffer with gentle shaking in-between.

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Primary Antibody (Primary Ab)

1. The Primary Antibody is provided at 100x concentration. Calculate the total volume of antibody needed by:

   Total Working Volume = 100 µL x Number of Wells Using Primary Ab

   To prepare working Primary Antibody working solution, divide the total working volume by 100 and add that volume of provided Primary Antibody to the calculated total volume of Primary Antibody Diluent. Mix thoroughly by inverting several times.
2. Add 100 µL of working Primary Antibody solution to every well that is being used except the Primary Antibody negative controls for Nuclear Lysate Positive Controls and samples. For the Primary Antibody negative controls, add 100 µL of Primary Antibody Diluent. Leave the on orbital shaker at room temperature for 2 hours.
3. Wash three times with 1x Wash Buffer with gentle shaking in-between.

HRP-Conjugated Anti-Rabbit IgG Antibody

1. The HRP-Conjugated Anti-Rabbit IgG Antibody is ready to use. Calculate the total volume of antibody needed by:

   Total Working Volume = 100 µL x Number of Total Wells Used

2. Add 100 µL of HRP-Conjugated Anti-Rabbit IgG Antibody to each well that is being used. Incubate on orbital shaker at room temperature for 1 hour.
3. Wash three times with 1x Wash Buffer with gentle shaking in-between.

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Developing Plate

TMB (3, 3′, 5, 5′-Tetramethylbenzidine), the reagent in Ready-to-Use Substrate is provided as a ready-to-use solution. Warm to room temperature before use. Stop Solution is also provided as a ready-to-use solution.

1. Add 50 µL of Ready-to-Use Substrate to every well that is being used. Keep those wells away from light and leave on orbital shaker for 10 to 30 minutes until there is distinctive blue color development from the wells. Closely monitor color development as some wells may develop faster than others. The reaction should be terminated when the well with greatest blue color ceases to continue developing.
2. When color development is sufficient, add 100 µL of Stop Solution to each well that is being used. Leave on orbital shaker for 1 minute or shake by hand to ensure color development is completely stopped. There will be a noticeable color change from blue to yellow.
3. The plate is now ready to read. Within 30 minutes of adding Stop Solution, determine the optical density or absorbance of each well by reading on a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from readings at 450 nm

NOTE: Readings directly at 450 nm without correction may be higher than actual reading, giving less accurate data for concentration determination.

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Data Analysis

1. Average the readings for each set of Nuclear Lysate Positive Control, Primary Antibody negative control, Sample, and Primary Antibody Negative Control for Sample. Subtract the average Primary Antibody Negative Control values from the Nuclear Lysate Positive Controls of the same dilutions and subtract Primary Antibody Negative Controls for Sample from the Sample Dilutions of the same dilutions to correct for background noise.
2. The OD values from Primary Antibody Negative Control wells and wells without Nuclear Lysate Positive Control should be lower than 0.2. The OD values for the Nuclear Lysate Positive Control dilutions should generate a gradient for qualitative analysis for your Sample dilutions.
3. Relative Sample concentration can then be determined by comparing to positive control data or between samples. Make sure to account for any dilutions. Note: This assay is not meant to allow for quantitative analysis.

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Troubleshooting Guide

Problem	Possible Cause	Solution
No Signal or Weak Signal	Incorrect nuclear lysate	Choose different cell line
	Incorrect lysate preparation or storage	Add protease and phosphatase inhibitors, keep everything on ice, and store at -80°C and avoid freeze/thaw cycles
	Key reagents missing	Consult manual and ensure all steps are followed
	Incorrect volume of reagents added	Consult manual and ensure all steps are followed
	Incorrect storage of plate and/or reagents	Keep everything at specific temperature
High Background	Inadequate washing between steps	Ensure the proper volume of wash buffer and steps
	Too much primary or secondary antibody	Reduce concentration
	Buffer/Reagent contamination	Ensure sterile techniques are used to maintain quality of reagents
	Too much nuclear lysate	Use higher dilutions
	Too much substrate	Reduce substrate used
	Substrate Reagent incubation time is too long	Reduce incubation time until adequate color development
Uneven Color Development	Inadequate washing between steps	Ensure the proper volume of wash buffer and steps
	Cross contamination	Use sterile technique
	Uneven reagent addition or washing of wells	Ensure multi-channel pipette or plate washer is calibrated and not clogged

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Common Cell Stimulating Methods

UV Irradiation

1. Grow cells to 90% confluence.
2. Wash cells once by using basic media (without FBS).
3. Add 3 mL basic media each petri dish (diameter: 100mm).
4. Open the petri dish in the UV cross-linker and set 10 mJ/cm2 and push start button.
5. Put dish back into incubator and incubate 0.5 to 3 hours at 37°C.
6. Harvest for cell lysate.

Phorbol 12-Myristate 13-Acetate (PMA) Stimulation

1. Grow cells to 90% confluence.
2. Wash cells once by using basic media (without FBS) and starve cells 18 to 24 hours in 5 mL basic media at 37°C.
3. Wash cells once by using basic medium and add 3 mL basic media for each petri dish.
4. Add PMA to final concentration of 200 nM.
5. Put dish back into incubator and incubate 0.5 to 3 hours at 37°C.
6. Harvest for cell lysate.

Serum Stimulation

1. Grow cells to 90% confluence.
2. Wash cells once by using basic media (without FBS) and starve cells 18 to 24 hours in 5 mL basic media at 37°C.
3. Wash cells once by using basic medium and add 3 mL basic media with 20% FBS for each petri dish.
4. Put dish back into incubator and incubate 1 to 6 hours at 37°C.
5. Harvest for cell lysate.

H2O2 Stimulation

1. Grow cells to 90% confluence.
2. Wash cells once by using basic media (without FBS).

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3. Add 3 mL basic media to each petri dish.
4. Add H_2O_2 to final concentration of 400 nM.
5. Put dish back into incubator and incubate 10 to 30 minutes at 37°C.
6. Harvest for cell lysate.

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Assay Usage and Support

This kit is for **Research Use Only** and is not intended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis. This kit should not be used beyond the expiration date printed on the lot specific kit label.

Warning: This reagent may contain sodium azide and sulfuric acid. The chemical, physical, and toxicological properties of these materials have not been thoroughly investigated. Standard Laboratory Practices should be followed. Avoid skin and eye contact, inhalation, and ingestion. Sodium azide forms hydrazoic acid under acidic conditions and may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent accumulation.

The LifeSpan Guarantee: LifeSpan guarantees the integrity of all components contained with an immunoassay kit, and that the standards provided will produce a standard curve sufficient for the quantification of target antigen concentrations that fall within the specified range of the kit. Due to the variable nature of sample types and preparations, LifeSpan cannot guarantee that the target antigen will be detectable in customer-supplied samples. For this reason, LifeSpan strongly recommends that customers conduct validation experiments, using positive control samples generated in a similar manner to the experimental samples, before using valuable research specimens. Due to the perishable nature of ELISA kits, orders of greater than 5 units of a single catalog number cannot be returned upon shipment, and are not eligible for refund.

Technical Support: LifeSpan's knowledgeable staff scientists are available to answer any questions about this kit. Email your detailed questions to Technical.Support@LSBio.com.

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