Technical Manual

STAT5A (Phospho-Ser780) Transcription Factor Activity (ELISA) Assay Kit

- Catalogue Code: TFAB00057
- Indirect ELISA Kit
- Research Use Only

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1. Assay Principles and Advantages

The Assay Genie Transcription factor Activity Assay (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. Assay Genie Transcription Activity Assay 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 Assay Genie Transcription Factor Activity Assay Kits can eliminate these challenges and help expedite the journey from research to publication or product.

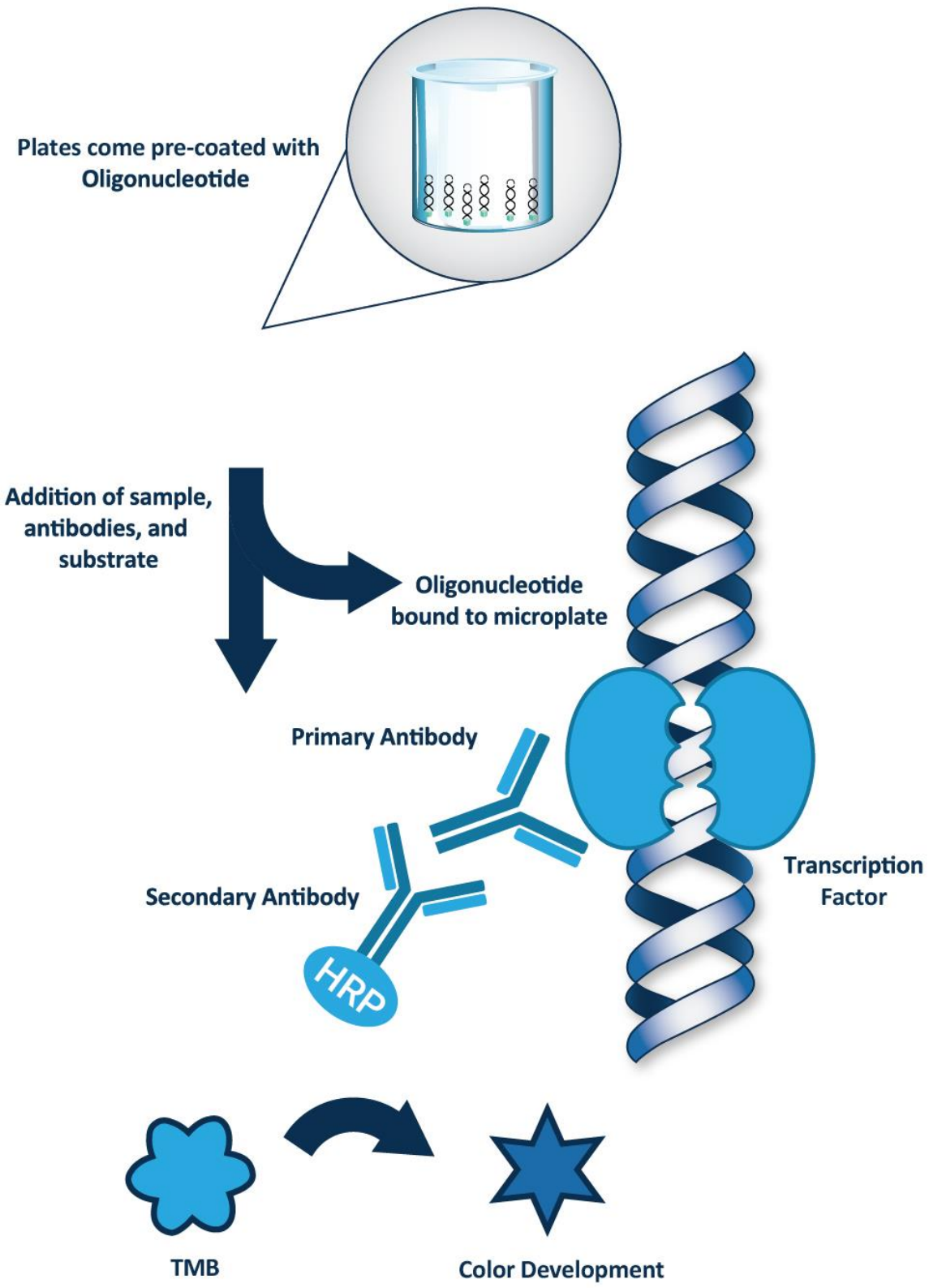


Figure: Transcription Factor Activity Assay Principle

2. Assay restrictions

- This ELISA kit is intended for research purposes only, NOT diagnostic or clinical procedures of any kind.
- 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.

3. 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.

4. Kit Contents

Component	Quantity	Storage	Temperature
96-Well dsDNA Oligonucleotide Coated Microplate	12 x 8	6 Months	4°C
100x Primary Antibody	100 µl	6 Months	4°C
HRP-Conjugated Anti-Rabbit IgG Antibody	12 ml	6 Months	4°C
Nuclear Lysate Positive Control	Lyophilized	Lyophilized: 1 Year	4°C
		Reconstituted: 6 months	-80°C
Wild-Type Consensus dsDNA Oligonucleotide	20 µl	6 Months	-20°C
Mutant Consensus dsDNA Oligonucleotide	20 µl	6 Months	-20°C
10x Wash Buffer	50 ml	6 Months	4°C
2x Binding Buffer	12 ml	6 Months	-20°C
Primary Antibody Diluent	12 ml	6 Months	4°C
Nuclear Wash Buffer	12 ml	6 Months	-20°C
Cytoplasmic Extraction Buffer	6 ml	6 Months	-20°C
Nuclear Extraction Buffer	6 ml	6 Months	-20°C
Ready-to-Use Substrate	12 ml	6 Months	4°C
Stop Solution	12 ml	6 Months	4°C
Adhesive Plate Sealers	2 Sheets	-	-
Technical Manual	1 Manual	-	-

5. Shipping and 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.

6. Additional Materials Required

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)

7. 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 ddH_2O 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 immediately used.

1x Primary Antibody

The Primary Antibody is provided at 100x concentration. It is recommended to make a fresh 1x Primary Antibody solution. 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, Cytoplasmic Extraction Buffer, Nuclear Wash Buffer, Nuclear Extraction Buffer, etc. and store them at the temperatures indicated in the table on the page 7.

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.

8. Sample Preparation and Storage

The Assay Genie Transcription Factor Activity Assay 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, not all 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.

9. Nuclear and Cytoplasmic extraction Protocol

The Assay Genie Transcription Factor Activity Assay 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/phosphatase added in, like PMSF, has 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, 1.5ml 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 (100mM), Protease Inhibitor Cocktail

1. Add 250 ul 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.

Preparation of Complete Cytoplasmic Extraction Buffer

Materials: Cytoplasmic Extraction Buffer, PMSF Stock Solution (100mM), 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 (100mM), 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 5. 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 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. Then, re-suspend the pellet with Complete Nuclear Extraction Buffer equal to 2x the pellet volume.
2. If volume changes are greater than or equal to 50 ul 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 ul, 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.

10. 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 NLPC Negative Control (Blank) 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	+ 1:10 NLPC, + Primary pAb	+ WT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary Ab	...
B	+ 1:20 NLPC, + Primary Ab	+ 1:20 NLPC, + Primary pAb	+ WT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary Ab	...
C	+ 1:40 NLPC, + Primary Ab	+ 1:40 NLPC, + Primary pAb	+ WT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary Ab	...
D	- NLPC, + Primary Ab	- NLPC, + Primary pAb	+ WT Oligo, - NLPC, + Primary Ab	+ Sample, - Primary Ab	...
E	+ 1:10 NLPC, - Primary Ab	+ 1:10 NLPC, - Primary pAb	+ MT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary pAb	...
F	+ 1:20 NLPC, - Primary Ab	+ 1:20 NLPC, - Primary pAb	+ MT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary pAb	...
G	+ 1:40 NLPC, - Primary Ab	+ 1:40 NLPC, - Primary pAb	+ MT Oligo, + NLPC, + Primary Ab	+ Sample, - Primary pAb	...
H	- NLPC, - Primary Ab	- NLPC, - Primary pAb	+ MT Oligo, - NLPC, + Primary Ab	+ Sample, - Primary pAb	...

11. Transcription Factor Activity Assay (ELISA) 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 110 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.

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**
1:20	105 µl	92.4 µl	10.5 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**	**WT Oligo**	**Total Volume**
1:20	105 µl	92.4 µl	10.5 µl	2.1 µl	210 µl

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 3 times with 1x Wash Buffer with gentle shaking in-between.

Primary Antibody/Phospho-Antibody (Primary Ab/pAb)

1. The Primary Antibody and Phospho-Antibody are provided at 100x concentration. Calculate the total volume of antibody needed by:

   Total Working Volume = 100 µl x Number of Wells Using Primary Ab/pAb

   To prepare working Primary Antibody or Primary Phospho-Antibody working solution, divide the total working volume by 100 and add that volume of provided Primary Antibody or Primary Phospho-Antibody to the calculated total volume of Primary Antibody Diluent. Mix thoroughly by inverting several times.

2. Add 100 µl of working Primary Antibody or Primary Phospho-Antibody solution to every well that is being used. Leave the on orbital shaker at room temperature for 2 hours.

3. Wash 3 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 3 times with 1x Wash Buffer with gentle shaking in-between.

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

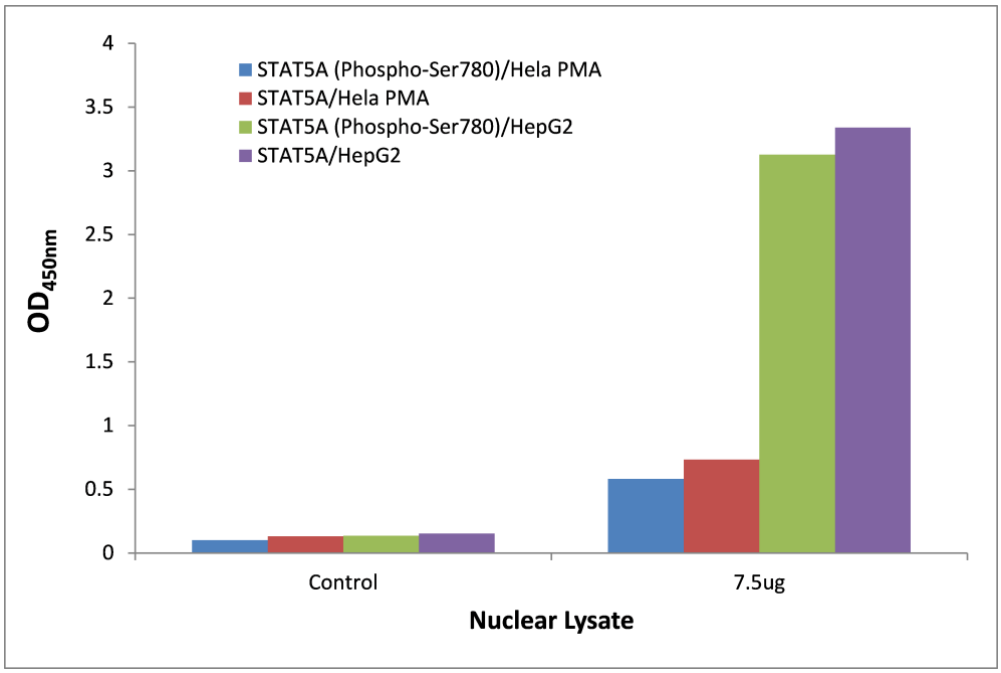
NOTE: *Readings directly at 450 nm without correction may be higher than actual reading, giving less accurate data for concentration determination.*

12. Data Analysis

1. Average the readings for each set of Nuclear Lysate Positive Control, Sample, and Blank control. Subtract the average Blank values from the Nuclear Lysate Positive Control values and the average Sample values to correct for background noise.

2. The OD values from 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.



The Assay Genie STAT5A (Phospho-Ser780) Transcription Factor Activity Assay Kit detects active STAT5A (Phospho-Ser780) in Hela and HepG2 Nuclear Extract. The Hela and HepG2 cells were grown 3 days in DMEM with 10% FBS and harvested for nuclear extract. The Hela cells were stimulated by PMA (200nM) before harvest.

13. Troubleshooting Guide

Problem	Causes	Solutions
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
High background	Inadequate washing between steps	Ensure the proper volume of wash buffer and steps
	Incorrect order or location in addition of reagents steps	Use template provided and ensure protocol is strictly followed
	Cross contamination	Use sterile technique
	Uneven reagent addition or washing of wells	Ensure multi-channel pipette or plate washer is calibrated and not clogged

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

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