# RayBio® Human p53 Transcription Factor Activity Assay Kit

Catalog #: TFEH-p53

**User Manual**
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# RayBio® Human p53 TF Activity Assay Kit Protocol

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Please read the entire manual carefully before starting your experiment.

# I. INTRODUCTION

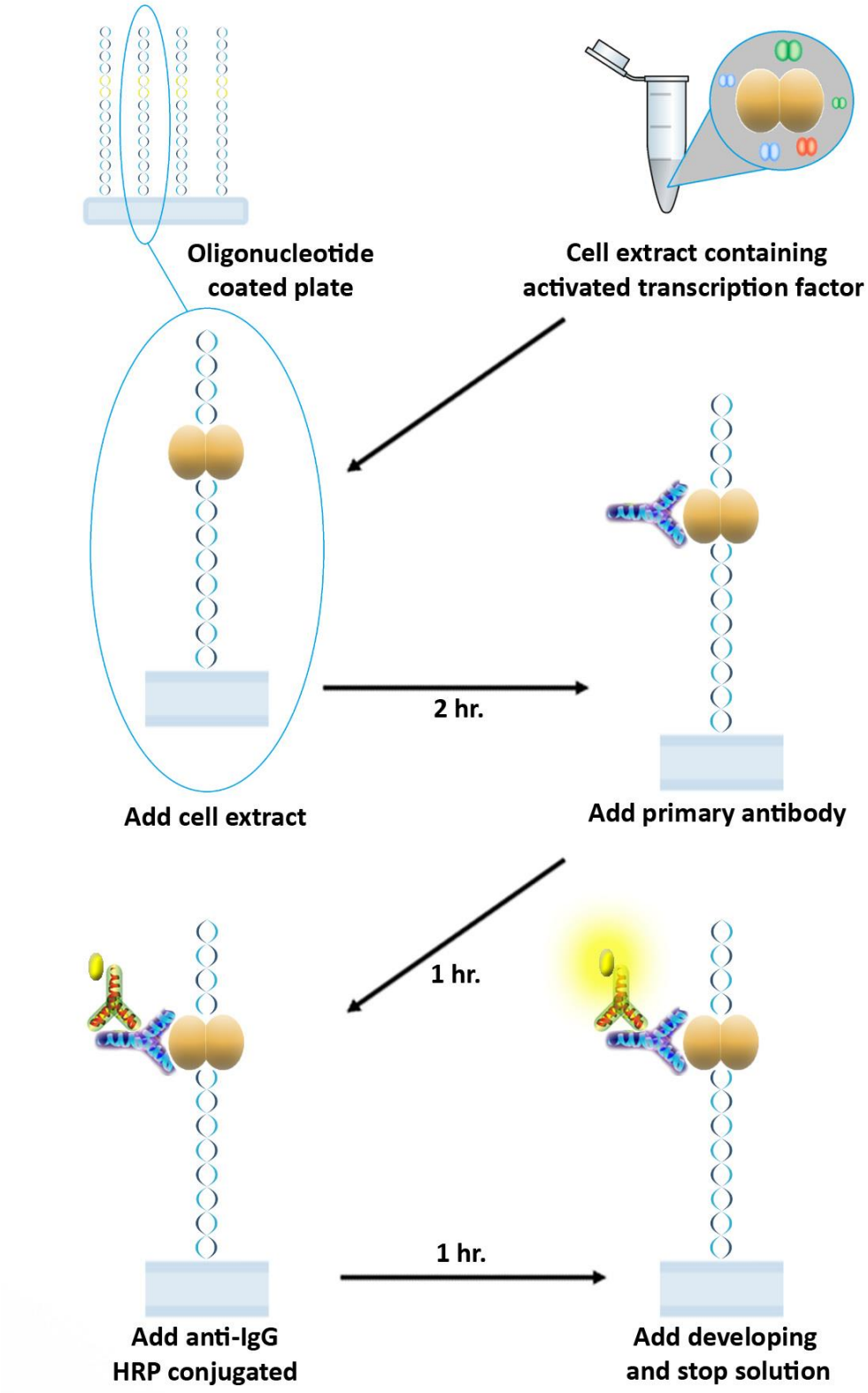
p53 is a master regulating transcription factor of cell fate that activates genes responsible for a cell-cycle checkpoint or apoptosis after exposure to ionizing radiation, UV light, or other DNA-damaging agents. To date over 125 protein-coding genes and noncoding RNAs have been shown to be a direct transcriptional target of p53. The p53 protein has features commonly associated with transcriptional regulators including an amino-terminal transactivation domain consisting of two transactivation subdomain TAD-I and TAD-II, a proline-rich region, a conserved core DNA-binding domain (DBD), a linker region, a tetramerization domain, and an unstructured basic domain in the carboxy-terminus. In normal cells where p53 is found at very low levels, p53 is present in a complex with MDM2, an E3 ubiquitin ligase, which targets p53 for degradation through the ubiquitin pathway. In response to stimuli, this inhibition is relieved and p53 is activated through a number of mechanisms which include phosphorylation, dephosphorylation by protein serine/threonine phoshatase-1, acetylation by the transcriptional coactivator p300/CBP, and induced conformational changes mediated by the prolyl isomerase Pin1. An increase in active p53 protein levels leads to multiple outcomes such as cell cycle arrest, apoptosis, senescence, autophagy, and others. Contrarily, the loss or inactivation of p53 due to damage or mutation, results in the loss of cell-cycle arrest or apoptosis after DNA damage or physiologic stresses. This loss, seen in many human cancers, is expected to lead to increased genetic instability, increased accumulation of mutations, and ultimately oncogenesis.

Accurate monitoring the level of activated p53 in cells, tissues or animal models is required for the investigation of signal transduction pathways both in basic both basic science research investigating signal transduction pathways and applications such as drug development, and simple, quick and high-throughput methods are needed for this purpose.

Traditionally, western blot has been used to detect the expression or modification of p53, electrophoretic mobility shift assay (EMSA) has been used to detect the DNA binding capacity of p53, and transfection of reporter genes such as luciferase and β-galactosidase with p53 binding sites in culture cells have been used in evaluation of p53 reactivity. However, these methods are time consuming, laborious, and sometimes require the use of radioactivity.

The RayBio® p53 TF Activity Assay kit is a non-radioactive transcription factor assay with an ELISA format. It offers an easy, quick, sensitive and high-throughput method to detect the activation of transcription factors. Double-stranded oligonucleotides containing the p53 binding sequence have been coated on 96-well plates. These oligonucleotides specifically capture the active p53 contained in whole cell lysate or nuclear extracts after a short incubation. Subsequently, the primary antibody against p53 recognizes the p53 -DNA complex in each well, and a HRP-conjugated secondary antibody is then used for detection. After washing away any unbound antibody, signal can be obtained easily through a colorimetric assay with a spectrophotometric plate reader at 450 nm. The specificity of the reaction between active p53 and the DNA probe is additionally stringent because of the establishment of specific competitive DNA and non-specific competitive DNA probes in this reaction system.

## II. HOW IT WORKS



## III. STORAGE

Upon receipt, the positive control should be stored at -20°C for short-term storage or -80°C for long-term storage. Opened Microplate Wells or reagents may be stored for up to 1 month at 2-8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge. The remainder of the kit can be stored for up to 6 months at 2-8°C from the date of shipment.

Note: The kit can be used within one year if the entire kit is stored at -20°C upon receipt. Avoid repeated freeze-thaw cycles.

## IV. REAGENTS

| Component | Description | Size |
|---|---|---|
| p53 DNA Probe Microplate | 96 wells (12 strips X 8 wells) coated with p53 probes | 1 plate |
| DNA Binding Buffer | 5X concentrated Buffer | 4 ml |
| Positive Control | Cell nuclear extracts | 1 vial (20 µl) |
| Specific Competitor DNA Probe | Free DNA probes that compete directly with the coated P53 probes. Can bind activated p53. | 1 vial |
| Non-specific Competitor DNA Probe | Free DNA probes with mutations of the coated DNA probe. Cannot bind activated p53. | 1 vial |
| Assay Reagent | 1X solution | 1 vial (200 µl) |
| DTT | 300 mM DTT | 1 vial (200 µl) |
| Wash Buffer Concentrate (20X) | 20X concentrated solution | 25 ml |
| p53 Primary Antibody | Anti-p53 antibody | 1 vial |
| HRP-conjugated Secondary Antibody | Anti-IgG HRP conjugated antibody | 1 vial |
| Antibody Diluent Buffer | Buffer solution for diluting primary and secondary antibodies | 25 ml |
| TMB One-Step Substrate Reagent | 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution | 12 ml |
| Stop Solution | 0.2 M sulfuric acid | 8 ml |

## V. ADDITIONAL MATERIALS REQUIRED

1) Microplate reader capable of measuring absorbance at 450 nm
2) Precision pipettes to deliver 1 μL to 1 mL volumes
3) Adjustable 1-25 mL pipettes for reagent preparation
4) 100 mL and 1 L graduated cylinders
5) Absorbent paper
6) Distilled or deionized water
7) Tubes to prepare positive or sample mixtures

## VI. REAGENT PREPARATION

1. Preparation of samples:

   Prepare nuclear extraction or whole lysate containing targeted protein p53 from cell culture or tissue.

   We recommend using the RayBiotech Nuclear Extraction Kit (Cat#: NE-50) to isolate nuclear proteins for subsequent use in this transcription factor assay.

2. Preparation of transcription factor reaction solutions:

   Thaw the positive control and samples and keep them on ice before adding into wells. Bring all other reagents to room temperature (18 – 25°C) before use. Spin down vials to make sure contents are settled before pipetting.

   Prepare 100 μl transcription factor binding reaction solution for each well with the following: 5x DNA Binding Buffer, Assay Reagent, DTT, Specific Competitor DNA Probe, Non-Specific Competitor DNA Probe, and either Positive Control or samples containing targeted proteins. Fill remaining volume to 100uL with deionized water. Typical examples are shown in the table below.

| | REACTION | | | | |
|---|---|---|---|---|---|
| **COMPONENT** | Positive control | Sample | Specific competitor | Non-Specific competitor | Blank |
| 5x DNA Binding Buffer | 20 µl | 20 µl | 20 µl | 20 µl | 20 µl |
| Assay Reagent | 1.5 µl | 1.5 µl | 1.5 µl | 1.5 µl | 1.5 µl |
| DTT | 1 µl | 1 µl | 1 µl | 1 µl | 1 µl |
| Specific Competitor DNA Probe | - | - | 10 µl | - | - |
| Non-specific Competitor DNA Probe | - | - | - | 10 µl | - |
| Positive Control/Sample containing proteins | 5 µl | * µl | * µl | * µl | - |
| Total volume | bring final volume to **100µl** with deionized water | bring final volume to **100µl** with deionized water | bring final volume to **100µl** with deionized water | bring final volume to **100µl** with deionized water | bring final volume to **100µl** with deionized water |

*Note:*

*Each reaction may be prepared in a labeled microcentrifuge tube or directly in the well of the coated microplate. If the reaction solution is prepared in a tube, add an additional 1-2 µL of deionized water to account for pipetting error when transferring the solution into the well. Once positive control/sample is added, ensure the tube is kept on ice to maintain protein stability. If the reaction solution is prepared directly in the coated plate wells, please add the reagents sequentially as shown in the table to get the best results. Scale volumes accordingly depending on the number of replicates used.*

**Please note that the amount of sample containing the target protein used in this test can be optimized and must be determined by the investigator. To observe the specificity of the DNA binding activity, the amount of protein used in wells containing the sample, specific competitor, and non-specific competitor must be the same.*

*A positive control should be included every time to confirm correct operation of the experiment, however it is not necessary to run the specific competitor and non-specific competitor for each sample every time.*

3. Preparation of primary antibody:

   Once thawed, briefly spin down the p53 Primary Antibody vial before use. Add 100 μl of Antibody Diluent Buffer into the vial to prepare a primary antibody concentrate. Pipette up and down to mix gently (this concentrate can be stored at 4°C for 5 days). The primary antibody concentrate should then be diluted 100-fold with the Antibody Diluent Buffer and used in step 4 of Part VII Assay Procedure.

4. Preparation of secondary antibody:

   Once thawed, briefly spin down the HRP-conjugated Secondary Antibody vial before use. Add 100 μl of Antibody Diluent Buffer into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (this concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should then be diluted 100-fold with the Antibody Diluent Buffer and used in step 6 of Part VII Assay Procedure.

5. Preparation of 1x Wash Buffer:

   Dilute 25 ml of the 20x Wash Buffer Concentrate into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If the Wash Buffer Concentrate (20x) contains visible crystals, warm to room temperature and mix gently until dissolved.

## VII. ASSAY PROCEDURE:

1. Thaw positive control and/or samples containing the target protein on ice. Bring the 96-well plate and other kit components to room temperature (18 - 25°C) before use. If the whole plate will not be used in this assay, place remaining wells back to 2-8°C, or -20°C for long-term storage. It is recommended that all positive control and samples be run in at least duplicate.

2. Add 100 μl of each prepared transcription factor binding reaction solution (see Reagent Preparation step 2) that includes positive control, Specific Competitor, Non-specific Competitor and sample into appropriate wells. Cover wells and incubate for 2 hours at room temperature or overnight at 4°C with gentle shaking.

3. Discard the solution and wash 4 times by filling each well with 300 μl of 1x Wash Buffer (Reagent Preparation step 5) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 μl of diluted p53 Primary Antibody (Reagent Preparation step 3) to each well. Incubate for 1 hour at room temperature with gentle shaking.

5. Discard the solution. Wash as directed in step 3.

6. Add 100 μl of diluted HRP-conjugated Secondary Antibody (see Reagent Preparation step 4) to each well. Incubate for 1 hour at room temperature with gentle shaking.

7. Discard the solution. Wash as directed in step 3.

8. Add 100 μl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

9. Add 50 μl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

## VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

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2. Add 100 μl reaction solution to each well.
   Incubate 2 hours at room temperature or overnight at 4°C.

⇩

3. Add 100 μl diluted primary antibody to each well.
   Incubate 1 hour at room temperature.

⇩

4. Add 100 μl diluted secondary antibody to each well.
   Incubate 1 hour at room temperature.

⇩

5. Add 100 μl TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.

⇩

6. Add 50 μl Stop Solution to each well.
   Read at 450 nm immediately.

## VIII. TYPICAL DATA

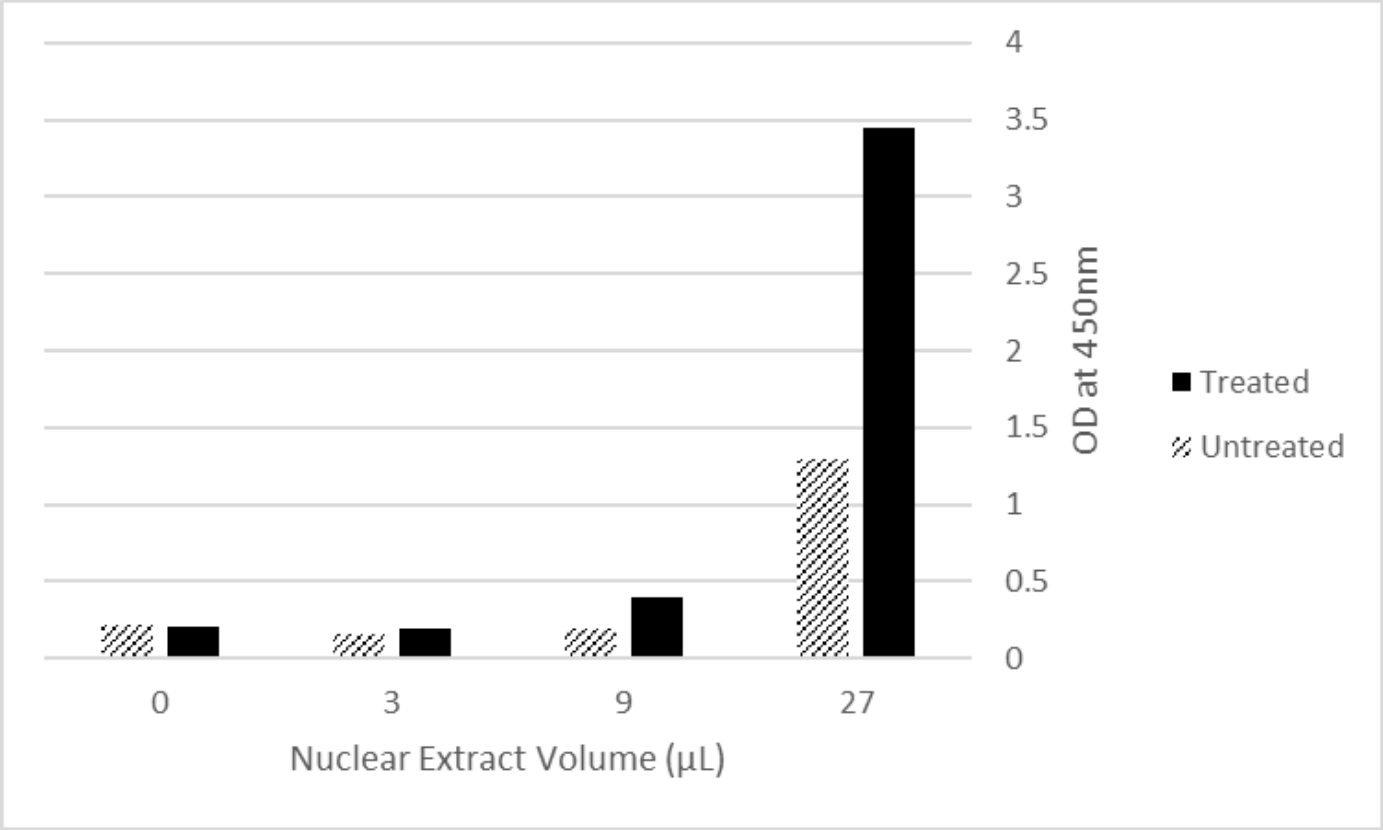


Figure 1: Results of the RayBiotech® p53 Transcription Factor Activity Assay (TFEH-p53) from nuclear extracts of untreated MCF-7 cells and MCF-7 cells treated with $H_2O_2$.

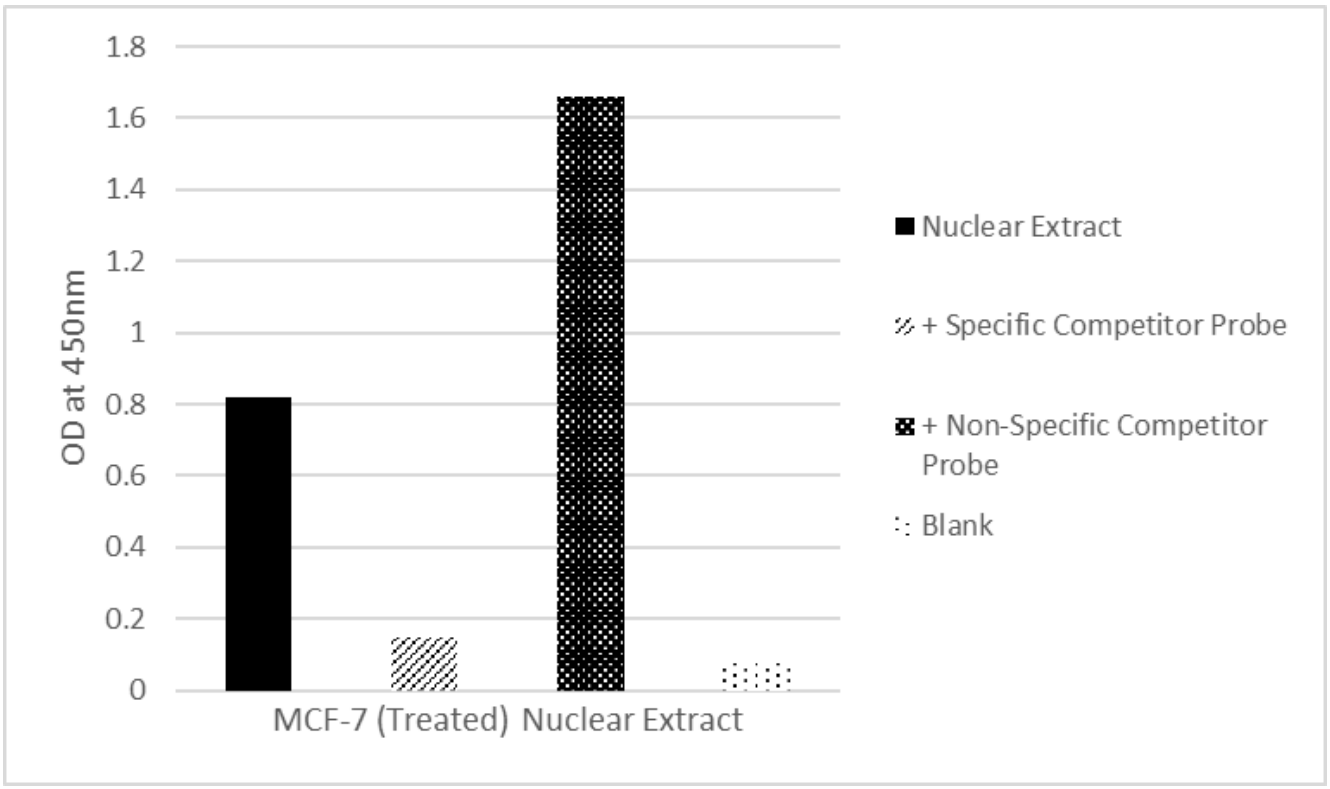


Figure 2: RayBiotech® p53 Transcription Factor Activity Assay (TFEH-p53) from nuclear extracts of MCF-7 cells treated with $H_2O_2$ and the addition of the specific competitor and non-specific competitor. The result shows specific binding of p53 to the p53 DNA binding site.

# TROUBLESHOOTING GUIDE

| Problem | Cause | Solution |
|---|---|---|
| 1. Low signal | 1.Too brief incubation times | 1. Ensure sufficient incubation time; change incubation time in assay procedure step 2 to overnight |
| | 2. Missed key reagent, inadequate reagent volumes or improper dilution | 2. Check to ensure all reagents have been added and check pipettes to ensure correct preparation |
| | 3. Not enough targeted protein per well | 3. Check positive control wells and increase the amount of sample. |
| | 4. Inadequate development in colorimetric assay | 4. Ensure correct developing buffer and enough time used |
| 2. Large CV | 1. Inaccurate pipetting | 1. Check pipettes |
| | 2. Wells cross contamination | 2. Be careful when preparing samples between wells |
| 3. High background | 1. Plate is insufficiently washed | 1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed |
| | 2. Contaminated wash Buffer | 2. Make fresh wash buffer |
| | 3. Incorrect antibody dilution | 3. Check antibody dilutions |

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