PathProfiler™

Histone H3 ELISA

Cat. No E300-001

{Includes choice of two (2) Detection Antibody Modules}

Detection Antibody Modules Available	Catalog Number
Total Histone H3 Detection Antibody Module	E300-001A
Phospho Histone H3 (S10) Detection Antibody Module	E300-001B

Components Supplied

- **Histone H3 Pre-Coated 96-well Strip Plate,** 1 each
- **Choice of Two Detection Antibody Modules,** 6 ml each (48-wells)
- **Dilution Buffer A**, 25 ml
- **HRP Solution,** 12 ml
- **TMB Substrate,** 12 ml
- **Stop Solution,** 12 ml
- **20X Wash Buffer,** 50 ml
- **Sealing Tape,** 20 mini-strip films

Introduction

The Bethyl PathProfiler ELISAs are enzyme-linked immunosorbent assays for the detection of total and phosphorylated proteins in cell lysates. These ELISAs allow for rapid screening of multiple samples for expression of specific target protein. Each kit allows for the selection of **two** Detection Antibody Modules, which may be mixed and matched as desired, for detection of the specific targets of the researcher's choice. Each Detection Antibody Module provides sufficient reagent for detection of 48-wells. Additional Detection Antibody Modules may be purchased separately, if desired.

Histone H3 Background

Histone H3 is one of four histone proteins that assemble into an octamer to form the nucleosome core and serve to spool and compact DNA into chromatin. Histones are subject to a complex set of post-translational modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP ribosylation) that are important to the regulation of gene transcription, DNA replication, and DNA repair.

The phosphorylation of Histone H3 at serine 10 has been linked to chromosome condensation as it relates to the activation of transcription, apoptosis, DNA damage repair, and cell division. During interphase, Histone H3 phosphorylation at serine 10 appears to be highly dependent on post-translational modifications of neighboring amino acids. In G2, phosphorylation of serine 10 begins at the pericentromeric chromatin and is completed during prophase of mitosis. Upon exit from mitosis, serine 10 phosphorylation decreases and becomes dephosphorylated. The phosphorylation of Histone H3 (S10) is detected in all eukaryotes during mitosis and is considered as a marker for G2/M phase; however controversy over the function of this modification in relation to chromosome condensation is still not well understood and remains a subject of debate.

Procedure Overview

1. Add 100 µl of diluted sample to appropriate well.

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2. Cover plate and incubate at room temperature (20-25°C) for 1 hour.

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3. Wash plate FOUR times.

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4. Add 100 µl of appropriate Detection Antibody Module to each well.

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5. Cover plate and incubate at room temperature for 1 hour.

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6. Wash plate FOUR times.

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7. Add 100 µl of HRP Solution to each well.

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8. Cover plate and incubate at room temperature for 30 minutes.

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9. Wash plate FOUR times.

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10. Add 100 µl of TMB Substrate Solution to each well.

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11. Develop the plate in the dark at room temperature for 30 minutes.

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12. Stop reaction by adding 100 µl of Stop Solution to each well.

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13. Measure absorbance on a plate reader at 450 nm.

Additional Materials Required

- Reagents and materials for cell lysate preparation, including cell lysis buffer
- Ultrapure water
- Precision pipettors, with disposable plastic tips
- Tubes for diluting samples
- A container to prepare Wash Buffer
- A wash bottle or an automated 96-well plate washer
- Disposable reagent reservoirs
- A standard microtiter plate reader for measuring absorbance at 450 nm

Precautions

- Store all reagents at 2-8ºC. Do not freeze reagents.
- All reagents must be at room temperature (20-25°C) before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Do not let the plate become dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the TMB Solution. Do not expose TMB Substrate solution to glass, foil, or metal. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain preservatives. Wear gloves while performing the assay. Please follow proper disposal procedures.

Cell Lysate Preparation

Note: This protocol has been successfully applied to multiple cell lines, however it may have to be optimized for the investigator's cell type of interest.

Highly Denaturing Cell Lysis Buffer Formulation

- 50 mM Tris-HCl (pH 7.4)
- 150 mM NaCl (Sodium Chloride)
- 1% NP-40
- 0.25% Deoxycholic Acid
- 1 mM EDTA (Ethylene Diamine Tetraacetate)
- 1% SDS (Sodium Dodecyl Sulfate)
- + Protease and Phosphatase Inhibitor Cocktails

Note: Buffer may be stored at room temperature (20-25ºC) for up to three (3) months prior to inhibitor cocktail addition. Add inhibitor cocktails immediately before use.

Protease and Phosphatase Inhibitor Cocktail

- Add to cell lysis buffer immediately before use.
- Suggested inhibitor cocktails include Thermo Scientific, Pierce Protein Research Products, Cat. # 78440, or Sigma-Aldrich, Cat. # P-2714 and P-2850.
- Alternatively, the following components may be used:
  - 1 mM PMSF (Phenylmethylsulfonyl Fluoride)
  - 1 mM AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride)
  - 0.8 µM Aprotinin
  - 50 µM Bestatin
  - 15 µM E-64
  - 20 µM Leupeptin
  - 10 µM Pepstatin A
  - 1 mM NaF (Sodium Fluoride)
  - 2 mM Na_3VO_4 (Sodium Orthovanadate)
  - 1 mM β-glycerophosphate
  - 20 mM $Na_4P_2O_7$ (Sodium Pyrophosphate Decahydrate)
  - 1 mM EGTA (ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid)

Cell Lysis Procedure

1. Prepare and treat cells as desired.
2. For adherent cells, remove media and rinse cells with ice-cold PBS. Harvest cells with trypsin-EDTA or by using a cell scraper. Centrifuge at 500 x g for 5 minutes.

   For suspension cells, harvest by centrifugation at 500 x g for 5 minutes.
3. Wash cells by re-suspending the cell pellet in ice-cold PBS. Pellet cells by centrifugation at 500 x g for 5 minutes.
4. Repeat wash by re-suspending the cell pellet in ice-cold PBS. Pellet cells by centrifugation at 500 x g for 5 minutes.
5. Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible. The cell pellet may be frozen at -70ºC and lysed at a later time, if desired.
6. Add 500 µl of ice-cold Cell Lysis Buffer to ~25 µl of cell pellet.

   Note: 25 µl of HeLa cell pellet is equivalent to ~2.5 X 10^6 HeLa cells. A typical, highly confluent 75cm^2 flask contains ~5 X 10^6 HeLa cells.

   Note: If excess buffer is used for the number of cells lysed, the protein concentration will be low.
7. Shake mixture gently for 15 minutes on ice.

   Note: To increase yields, further sheer DNA by aspirating the cell pellet 5-10 times through a 21 ½ gauge needle or by sonicating the cell pellet for 30 seconds with 50% pulse during the incubation.
8. Transfer mixture to a fresh microcentrifuge tube and centrifuge at ~14,000 x g for 15 minutes at 4ºC to pellet cell debris waste.
9. Transfer the supernatant (cell lysate) to a fresh tube for analysis. Store lysate in single-use aliquots at -70ºC. Avoid multiple freeze-thaw cycles when storing samples.

Sample and Reagent Handling and Preparation

Sample Handling

- Cell lysates may be tested in this ELISA. All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- 100 µl of diluted cell lysate is required per well.
- Samples should be frozen if not analyzed shortly after lysis. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeated freeze-thaw cycles when storing samples.
- If particulate is present in samples, centrifuge prior to analysis.

Sample Dilution

- **Samples must first be diluted prior to testing.** Typically, samples require a 1:10 to 1:100 dilution with Dilution Buffer A. A suggested 1:10 dilution would be to add 10 µl of sample to 90 µl of Dilution Buffer A in a separate tube and mix well. An alternative would be to first add 90 µl of Dilution Buffer A to the sample wells and then to add 10 µl of sample to those wells. Tap the plate gently to mix. Either method provides a final 1:10 dilution of the sample in each well. Each sample may require optimization based on cell concentration and protein expression within the cell lysate.

  Note: For Total Histone H3 and Phospho Histone H3 (S10), optimal total protein concentrations are typically below 20 µg/ml.

1X Wash Buffer Preparation

- Prepare 1X Wash buffer by diluting 20X Wash Buffer in ultra pure water. For example, if preparing 1 L of 1X Wash Buffer, dilute 50 ml of 20X Wash Buffer into 950 ml of ultrapure water. Mix well. Store reconstituted 1X Wash Buffer at 2-8ºC for up to one (1) month. Do not use Wash Buffer if it becomes visibly contaminated during storage.

Assay Procedure

Sample Incubation

- Determine the number of strips required. Leave these strips in the plate frame. Place unused strips in the foil pouch with desiccant and seal tightly. Store unused strips at 2-8°C. After completing assay, keep the plate frame for additional assays.
- Use a Plate Template to record the locations of the Sample Blank (Dilution Buffer A only) and unknown samples within the wells. Use the Plate Template to record the appropriate Detection Antibody Module addition for the desired target protein being analyzed. Each Detection Antibody Module requires a different Sample Blank and unknown sample. For ease in Detection Antibody addition, it is suggested that each 8-well strip used should only measure one Detection Antibody Module protein target.

1. Add 100 µl of appropriately diluted sample to each well. If testing identical samples with multiple Detection Antibody Modules, ensure that samples are added to separate wells for each Detection Antibody Module tested.

   Note: Samples must be diluted prior to testing (see Sample and Reagent Preparation-Sample Dilution section).
2. Add 100 µl of Dilution Buffer A as a Sample Blank to wells that do not contain samples. Ensure that at least one well is used as a Sample Blank for each Detection Antibody Module tested.

   Note: Sample results are typically determined by comparison of absorbance to the Sample Blank.
3. Carefully cover wells with a new adhesive plate cover. Incubate for one (1) hour at room temperature, 20-25°C.
4. Carefully remove adhesive plate cover, discard plate contents and wash FOUR times with Wash Buffer as described in the Plate Washing section.

Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.
2. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure three additional times for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate plate contents from all wells and flood wells with Wash Buffer. Repeat procedure three additional times for a total of FOUR washes. Additional washes may be necessary. Blot plate onto paper towels or other absorbent material.

Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Detection Antibody Module Incubation

- Only remove the required amount of Detection Antibody Module reagent for the number of strips being used.

1. Add 100 µl of appropriate Detection Antibody Module to each well containing sample or blank. Take care to add the appropriate Detection Antibody Module reagent to the desired wells only (as recorded earlier in the Plate Template). Mix well by gently tapping the plate several times.
2. Carefully attach a new adhesive plate cover. Incubate plate for one (1) hour at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with Wash Buffer as described in the Plate Washing section.

HRP Solution Incubation

- Only remove the required amount of HRP Solution for the number of strips being used.

1. Add 100 µl of HRP Solution to each well containing sample or blank.
2. Carefully attach a new adhesive plate cover. Incubate plate for 30 minutes at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times as described in the Plate Washing section.

TMB Substrate Incubation and Reaction Stop

- Only remove the required amount of TMB Substrate Solution and Stop Solution for the number of strips being used.
- Do NOT use a glass pipette to measure the TMB Substrate Solution. Do NOT cover the plate with aluminum foil or metalized mylar. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused

TMB Substrate Solution. If the solution is blue before use, DO NOT USE IT!

1. Pipette 100 µl of TMB Substrate Solution into each well.
2. Allow the enzymatic color reaction to develop at room temperature (20-25ºC) in the dark for 30 minutes. Do NOT cover plate with a plate sealer. The substrate reaction yields a blue solution.
3. After 30 minutes, stop the reaction by adding 100 µl of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.

Absorbance Measurement

Note: Evaluate the plate within 30 minutes of stopping the reaction.

1. Wipe underside of wells with a lint-free tissue.
2. Measure the absorbance on an ELISA plate reader set at 450 nm.

Calculation of Results

- Positive sample values are determined by obtaining higher levels of absorbance than the Sample Blank. Sample values obtained are qualitative to the ELISA performed.
- If desired, phosphorylated detection results can be normalized to total protein detection results by dividing phosphorylated absorbance values by total protein absorbance values. Normalized values from multiple samples can then be evaluated within the same assay.
- Care should be taken when comparing data with absorbance values below 0.2 or above 2.

Performance Characteristics

Typical PathProfiler Data

Bethyl's PathProfiler ELISA kit detects Total Histone H3 and Phospho Histone H3 (S10) in cell lysates (Figure 1). By using Total Histone H3 and Phospho Histone H3 (S10) Antibody Detection Modules, both proteins were detected in the same ELISA. Various cell types were serum starved overnight. Cells were subsequently serum stimulated and treated with 0.5 µg/ml Calyculin A at 37ºC, 5% CO_2 for 15 minutes. Cells were then lysed with Highly Denaturing Lysate Buffer, diluted, and screened in the PathProfiler Histone H3 ELISA using both Total Histone H3 and Phospho Histone H3 (S10) Antibody Detection Modules. While levels of Total Histone H3 remained constant with Calyculin A treatment, an increase in Phospho Histone H3 (S10) was detected upon Calyculin A treatment. Results were obtained within 3 hours.

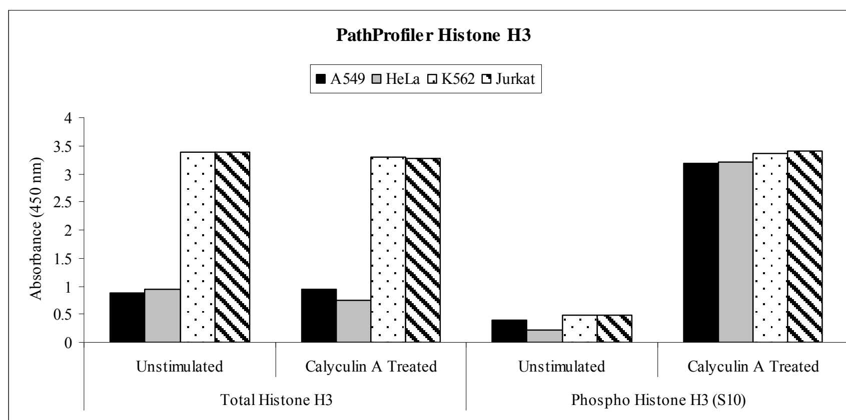

Figure 1: Detection of Total Histone H3 and Phospho Histone H3 (S10) by PathProfiler ELISA. A549, HeLa, K562, and Jurkat cell lines were serum starved overnight. Cells were subsequently serum stimulated and treated with 0.5 µg/ml of Calyculin A at 37ºC, 5% CO_2 for 15 minutes. Cells were lysed and screened using PathProfiler Total Histone H3 and Phospho Histone H3 (S10) Detection Modules (E300-001A and E300-001B, respectively).

PathProfiler Data Correlation to Western Blot

Histone H3 PathProfiler data correlates to protein expression levels as detected by Western blot (Figure 2). Lysates analyzed in the PathProfiler Histone H3 assay (Figure 1) were further analyzed by Western blot using target specific antibodies. Results demonstrate a similar pattern of protein expression detected in Western blot as generated by the PathProfiler Histone H3 ELISA. While levels of Total Histone H3 remained constant with Calyculin A treatment, an increase in Phospho Histone H3 (S10) was detected upon Calyculin A treatment. Unlike the ELISA, which was completed in 3 hours, the Western blot experiment took several hours to complete, not including the required assay optimization.

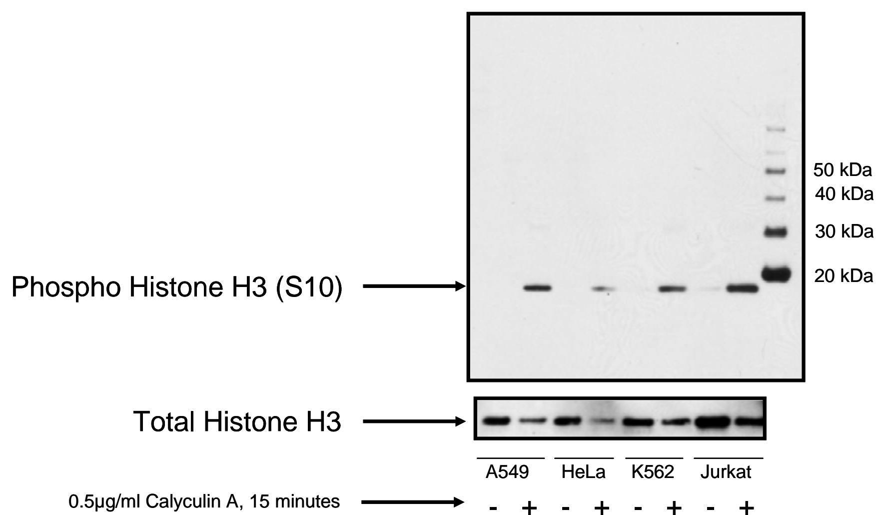

Figure 2: Detection of Total Histone H3 and Phospho Histone H3 (S10) by Western Blot. A549, HeLa, K562, and Jurkat cell lines were serum starved overnight. Cells were subsequently serum stimulated and treated with 0.5 µg/ml of Calyculin A at 37ºC, 5% CO_2 for 15 minutes. Cells were lysed and 1 µg of appropriate cell lysate was loaded into respective wells. Western blots were developed utilizing chemiluminescence with an exposure time of 30 seconds.

PathProfiler Data Correlation to Total Protein Concentration

In order to determine the appropriate total protein concentration of cell lysate required to obtain positive signal results from the PathProfiler Histone H3 ELISA, cell lysates were treated as before and the quantity of total protein in the lysates was determined by BCA assay (Thermo Scientific; Rockford, IL). Lysates were then evaluated in the PathProfiler Histone H3 ELISA using specific total protein concentrations. Results demonstrate the PathProfiler Histone H3 ELISA detects protein expression levels in positive cell lysates at concentrations of 20 µg/ml or less (Figure 3).

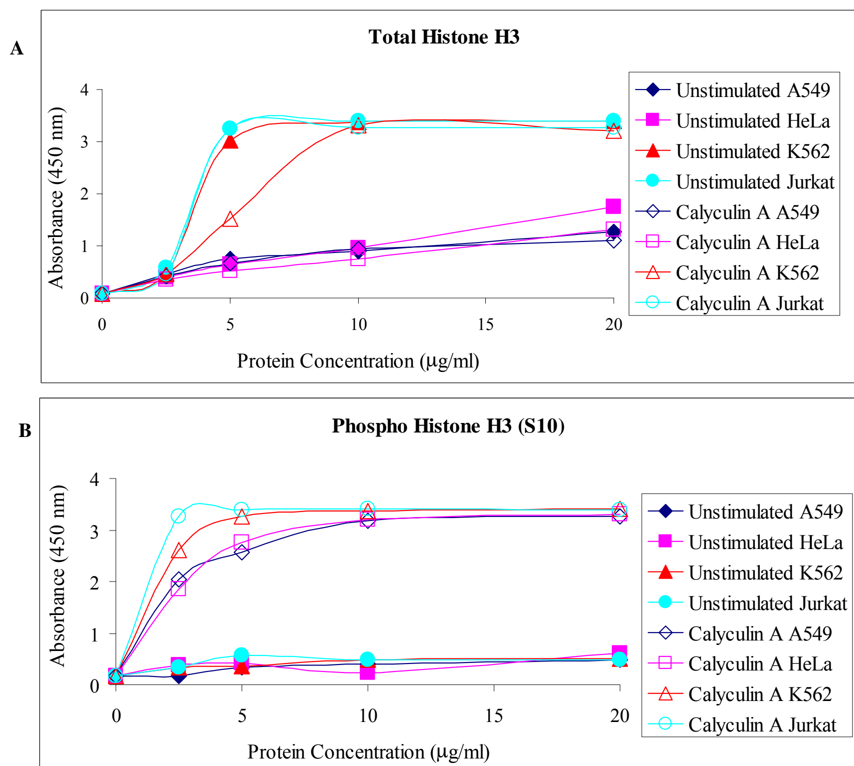

Figure 3: Relationship of Cell Lysate Protein Concentration to Detection of Total Histone H3 and Phospho Histone H3 (S10) by PathProfiler ELISA. A549, HeLa, K562, and Jurkat cell lines were serum starved overnight. Cells were subsequently serum stimulated and treated with 0.5 µg/ml of Calyculin A at 37ºC, 5% CO_2 for 15 minutes. Cells were lysed and protein concentration was determined. Lysates were further screened for **(A)** Total Histone H3 and **(B)** Phospho Histone H3 (S10) expression by PathProfiler Total Histone H3 and Phospho Histone H3 (S10) Detection Modules (E300-001A and E300-001B, respectively).

Assay Range: ≤ 20 µg/ml total protein

Note: Assay range varies based on cell type. Optimal total protein concentrations are typically below 20 µg/ml for Total Histone H3 and Phospho Histone H3 (S10) detection (in positive samples).

Note: Assay range varies based on cell lysis buffer used. Total Histone H3 and Phospho Histone H3 (S10) are nuclear proteins, so highly denatured lysates containing nuclear extracts provide higher levels of protein detection. Minimal sample detection will occur in native lysates.

Background References

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- Nowak S.J. and Corces V.G. (2004) *Trends Genet*, 20(4), 214-21.
- Peterson C.L. and Laniel M.A. (2004) *Curr Biol*, 14(14), R546-51.
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- Zhang K. and Dent S.Y. (2005) *J Cell Biochem*, 96(6), 1137-48.

Warranty

Products are warranted by Bethyl Laboratories, Inc. to meet stated product specifications and to conform to label descriptions when used, handled and stored according to instructions. Unless otherwise stated, this warranty is limited to six months from date of sale. Bethyl Laboratories sole liability for the product is limited to replacement of the product or refund of the purchase price. Bethyl Laboratories products are supplied for research applications. They are not intended for medicinal, diagnostic or therapeutic use. The products may not be resold, modified for resale or used to manufacture commercial products without prior written approval from Bethyl Laboratories, Inc.

Plate Templates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
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