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Phospho-Retinoblastoma (pSer^{249}/pThr^{252}) ELISA, Human

ProductInformation

Product Number **CS0060**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

Phospho-Retinoblastoma (Rb) (pSer^{249}/pThr^{252}) ELISA is a phosphorylation site-specific assay, designed to quantitate the amount of retinoblastoma (Rb) protein that is dually phosphorylated at serine 249 and threonine 252. This ELISA does not recognize Rb phosphorylated at sites other than (pSer^{249}/pThr^{252}) or non-phosphorylated Rb. A monoclonal antibody specific for retinoblastoma (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate provided. Rb standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the Rb antigen binds to the immobilized (capture) antibody. After incubation, excess reagents are washed away and an anti-phospho Rb (pSer^{249}/pThr^{252}) is added. This antibody binds to the immobilized Rb (pSer^{249}/pThr^{252}) protein captured during the first incubation. After removal of excess antibody, Anti-Rabbit IgG-HRP) is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess HRP substrate solution is added, which is acted upon by the bound enzyme to produce yellow color. The intensity of this colored product is directly proportional to the concentration of phosphorylated retinoblastoma present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of Phospho-Retinoblastoma (pSer^{249}/pThr^{252}).

For normalization of the Rb content of the samples, an ELISA kit for human Rb (Product No. CS0050) is available in which detection is independent of phosphorylation status.

Retinoblastoma protein (Rb), the tumor suppressor product of the retinoblastoma susceptibility gene, is a 110 kDa protein and plays an important role in regulating cell growth and differentiation. Loss of its function leads to uncontrolled cell growth and tumor development. Mutational inactivation of the Rb gene is found in all retinoblastomas and in a variety of other human malignancies including cancers of breast, lung, colon, prostate, osteosarcomas, soft tissue sarcomas, and leukemia. Central to the role of the Rb protein as a tumor suppressor, is the ability of Rb to halt inappropriate proliferation by arresting cell in the G1 phase of the cell cycle. At the transcriptional level, Rb protein exerts its growth suppressive function by binding to transcription factors including E2F-1, PU.1, ATF-2, UBF, Elf-1, and c-Abl. The binding of Rb protein is governed by its phosphorylation state. Hypo-phosphorylated Rb binds and sequesters transcription factors, most notably, those of the E2F/DP family, inhibiting the transcription of genes required to traverse the G1 to S phase boundary. The cell cycle inhibitory function is abrogated when Rb undergoes phosphorylation, catalyzed by the complex of cyclins/cyclin-dependent protein kinases (cdks). Rb contains at least 16 consensus serine/threonine sequences for cdk phosphorylation, although the significance of all these sites is unclear. It has been demonstrated that phosphorylation of threonine 821 or threonine 826 disrupts interaction with proteins containing the sequence LXCXE. Phosphorylation of these sites as well as serine 807, 811 and 780 also disrupts binding to E2F. The dephosphorylation of the Rb protein returns Rb to its active, growth suppressive state. Removal of phosphates on Rb appears to be carried out by a multimeric complex of protein phosphatase type 1 (PP1) and noncatalytic regulatory subunits at the completion of mitosis.

Reagents

- **Phospho-Retinoblastoma (pSer^{249}/pThr^{252}) Standard, 2 vials, Product No. R 8652**–lyophilized extract from Jurkat cells. *See vial label for quantity and reconstitution volume.*
- **Standard Diluent Buffer, 25 mL, Product No. S 3068**- contains BSA and sodium azide as a preservative.

- **Monoclonal Anti-Retinoblastoma coated 96-well plate, 1 plate, Product No. R 8902** - A plate using break-apart strips coated with monoclonal antibody specific for Rb (regardless of phosphorylation state).
- **Anti-Phospho-Retinoblastoma (pSer^{249}/pThr^{252}) 11 mL Product No. R 8777**
- **Anti-Rabbit IgG-Horseradish Peroxidase (HRP) Concentrate, 100X**, **Product No. I 3033** - contains 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 8912** – contains thymol and BSA. Ready to use.
- **Wash Buffer Concentrate 25X, 100 mL, Product No. W 2639** - *See Reagent Preparation for handling, dilution and storage instructions.*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**.

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL
- Cell extraction buffer (see recommended extraction procedure)
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Graph paper: linear, log-log, or semi-log, as desired
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard
- Absorbent paper towels to blot the plate
- Calibrated beakers and graduated cylinders in various sizes
- Vortex mixer

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
- Cell Extraction Buffer
  10 mM Tris, pH 7.4
  100 mM NaCl
  1 mM EDTA
  1 mM EGTA
  1 mM NaF
  20 mM Na4P2O7
  2 mM Na3VO4
  1% Triton X-100
  10% Glycerol
  0.1% SDS
  0.5% Deoxycholate
  1 mM PMSF (stock is 0.3 M in DMSO).
  PMSF is very unstable and must be added prior to use, even if added previously.
  Protease inhibitor cocktail (Product. No. P 2714).
  Add 250 µL of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 ºC or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 ºC.

Thaw on ice. Add the protease inhibitors just before use.

Procedure for Extraction of Proteins from Cells

The protocol has been applied to several cell lines with the Cell Extraction Buffer above. Researchers may use the procedures that work best in their laboratories. They will have to assay the cell lysates for the satisfactory extraction.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –70 ºC and lysed at a later date).

4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression and phosphorylation of retinoblastoma. For example,
5. 10^8 Jurkat cells grown in RPMI plus 10% FBS can be extracted in 1 mL of Cell Extraction Buffer. Under these conditions, use of 0.1-1 µL of the cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer (see Assay Procedure) is sufficient for the detection of Rb protein.
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 ºC.
7. Aliquot the clear lysate to clean microcentrifuge tubes.

Reagent Preparation

Phospho-Retinoblastoma ($pSer^{249}/pThr^{252}$) Standard
This human Rb standard is lyophilized extract from Jurkat cells. The total Rb protein (phosphorylated and non-phosphorylated) was calibrated against a mass of highly purified recombinant human Rb protein expressed in *E. coli.*
1 unit of phospho-Rb is defined as the amount of Rb ($pSer^{249}/pThr^{252}$) derived from 15,000 Jurkat cells in exponential growth. Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute one vial of Retinoblastoma Standard with Standard Diluent Buffer according to label directions.
2. Mix gently and wait 10 minutes to ensure complete reconstitution.
3. Label as **100 Units/mL human Rb** ($pSer^{249}/pThr^{252}$)
4. Prepare serial standard dilutions as follows:

Tube #	Standard Diluent Buffer	Standard from tube #:	Final Standard Concentration Units/mL
	Reconstitute according to label instructions		100 Units/mL
2	0.25 mL	0.25 mL (1)	10
3	0.25 mL	0.25 mL (2)	5
4	0.25 mL	0.25 mL (3)	2.5
5	0.25 mL	0.25 mL (4)	1.25
6	0.25 mL	0.25 mL (5)	0.625
7	0.25 mL	0.25 mL (6)	0.32
8	0.5 mL	-	0

Mix thoroughly between steps.

5. Use within 1 hour of reconstitution

Anti-Rabbit IgG-HRP concentrate (100X), contains 50% glycerol and is very viscous. To ensure accurate dilution follow instructions below:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Mix: 10 µL IgG-HRP Concentrate +1 mL HRP Diluent (sufficient for one 8-well strip, prepare more as needed)
4. Label as Anti-Rabbit IgG-HRP Working Solution.
5. Return the unused Anti-IgG-HRP concentrate to the refrigerator.

Wash Buffer

1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as Working Wash Buffer.
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability

All components of this kit are stable at 2 to 8 ºC. Any unused reconstituted standard should be discarded or frozen at 70 ºC. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit expiration date. To obtain C of A go to www.sigmaaldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 ºC).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2 - 8°C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay

- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch.

Human Phospho-Retinoblastoma (pSer^{249}/pThr^{252}) Assay Summary

1) **Incubate 100 µL of Standards and Samples (diluted >1:10) for 2 hours at RT. (Optional: Incubate overnight at 4°C)**

   aspirate and wash 4x

2) **Incubate 100 µL of Detection Antibody 1 hour at RT.**

   aspirate and wash 4x

3) **Incubate 100 µL of HRP Anti-Rabbit IgG 30 min. at RT.**

   aspirate and wash 5x

4) **Incubate 100 µL of stabilized Chromogen 30 minutes at RT *in the dark*.**

5) **Add 100 µL of Stop Solution and read at 450nm.**

Total Time 4 hours

1st incubation

a Add 100 µL Standard Diluent to zero wells.

b Add 100 µL standards, samples or controls to the appropriate wells. *Samples in Cell Extraction Buffer must be diluted at least 1:10 (1:25 or 1:50 were found to be optimal) in Standard Diluent Buffer. The dilutions should be optimized for each assay.*

c Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature. *Alternatively, plate may be incubated overnight at 2 to 8 °C.*

d Wash wells 4 times following washing instructions.

e After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

2nd incubation

a Add 100 µL Anti-Phospho-Rb (pSer249/pThr252) detection antibody to all wells (except chromogen blanks).
b Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation

a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: *If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

Stop reaction

a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Blank the plate reader against the Chromogen Blank wells (contain Chromogen and Stop Solution).
c Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution.

Results

The results may be calculated using any immunoassay software package. The four-parameter algorithm provides the best curve fit. If the software program is not readily available, the concentrations of Retinoblastoma may be calculated as follows:

1. Calculate the Average Net OD (nm) for each standard dilution and samples as follows:
   Average Net OD (nm)=
   Average Bound OD (nm) –
   Average Chromogen Blank OD (nm)
2. On graph paper plot the Average Net OD (nm) of standard dilutions against the concentration (Units/mL) of Rb (pSer249/pThr252) for the standards.
3. Draw the best curve through these points to construct the standard curve.
4. The Rb concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by dilution factor of each sample.
6. Samples producing signals higher than the 100 Units/mL standard should be further diluted in *Standard Diluent Buffer and* re-assayed.

Product Profile

Typical Results

The standard curve below is for illustration only and should not be used to calculate results in your assay. Run standard curve in each assay.

Rb (pSer249/pThr252) Standard Units/mL	Optical Density 450 nm
0	0.180
1.6	0.251
3.12	0.327
6.25	0.439
12.5	0.631
25	0.914
50	1.50
100	2.57

Limitations:

- Do not extrapolate the standard curve beyond the 100 Units/mL standard points.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- Although Rb degradation in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance Characteristics

Specificity

Jurkat cells were grown in the presence of the kinase inhibitor, staurosporine, at various concentrations for 24 hours prior to lysis. Lysates were normalized for total Rb content using the Sigma Rb ELISA (Prod. No. CS0050). Levels of Rb phosphorylation at serine 249 and threonine 252 were determined. These data show that staurosporine inhibits the phosphorylation of Rb at serine 249 and threonine 252, possibly through the inhibition of cdks. The results correlated well with immunoblot analysis of the same samples (inset in the graph below). Protein is not detected in cell lysates from SaoS2 cells, an Rb deficient osteosarcoma cell line. The levels of Rb protein detected with this ELISA kit are consistent with results obtained by immunoblot.

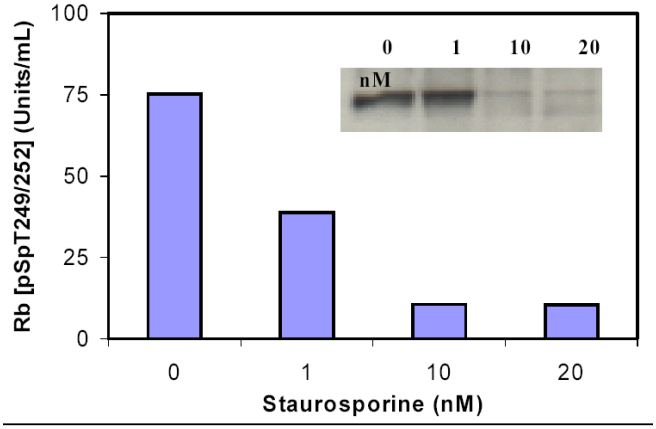

Figure 1 Detection of Rb (pSer^{249}/pThr^{252})

The specificity of this assay for dually phosphorylated pRb (pSer^{249}/pThr^{252}) was confirmed by peptide competition. The data presented in Figure 2 show that only the dual phospho-peptide containing phosphorylated serine 249 and threonine 252, could block the ELISA signal.

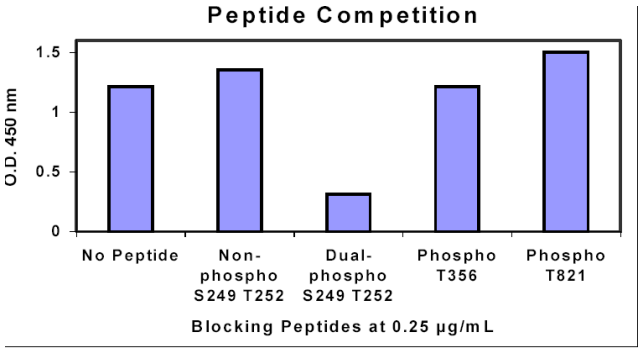

Figure 2

Sensitivity

Sensitivity of this assay is < 0.8 Units/mL. Sensitivity was calculated by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. The sensitivity of this ELISA was compared to immunoblotting using known quantities of pRb (pSer^{249}/pThr^{252}). The data presented in Figure 3 show that the sensitivity of the ELISA is approximately 4x greater than that of immunoblotting. The bands shown in the immunoblotting data were developed using rabbit anti-Rb (pSer^{249}/pThr^{252}) and alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

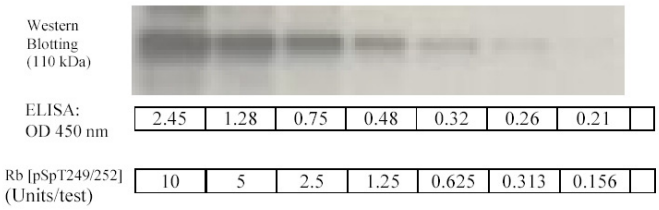

Figure 3 Immunoblotting vs. Elisa

Precision

1. Intra-Assay Precision

Samples of known Insulin Receptor Subunit concentration were assayed in replicates of 14 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	85.17	31.01	10.59
Standard Deviation (SD)	5.29	2.84	6.75
% Coefficient of Variation	6.21	9.16	7.04

2. Inter-Assay Precision

Samples were assayed 28 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (Units/mL)	1.26	15.63	6.53
Standard Deviation (SD)	1.44	0.56	0.07
% Coefficient of Variation	9.21	8.53	5.70

Sample Recovery

The recovery of Rb (pSer^{249}/pThr^{252}) protein added to Rb negative cell lysate (SaoS2 cells, adjusted to 0.1 mg/mL total protein) averaged 105% when diluted in Standard Diluent Buffer.

Parallelism

Natural Rb (pSer^{249}/pThr^{252}) extracted from a Jurkat cell lysate was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against Rb (pSer^{249}/pThr^{252}) standard curve. Parallelism is demonstrated by the figure 4 and indicated that the standard accurately reflects natural retinoblastoma (pSer^{249}/pThr^{252}) content in samples.

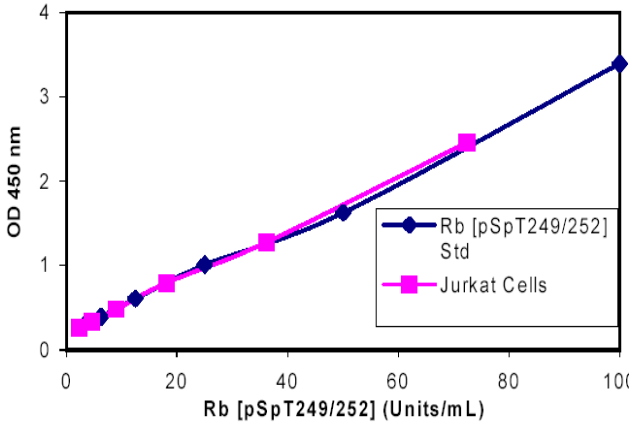

Fig. 4 Parallelism: Rb (pSer^{249}/pThr^{252}) ELISA

Linearity of Dilution

Jurkat cells were lysed with Cell Extraction Buffer. This lysate was adjusted to 100 Units /mL Rb (pSer^{249}/pThr^{252}) protein and serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99 in both cases.

Dilution	Cell Lysate Measured (Units/mL)	Expected (Units/mL)	% Expected
Neat	72.4	72.4	-
1:2	35.6	36.2	98%
1:4	20.2	18.1	112%
1:8	9.9	9.0	109%
1:16	4.7	4.5	105%
1:32	2.5	2.3	109%

References

1. Brown, V.D., et al., Cumulative effect of phosphorylation of pRB on regulation of E2F activity. Mol. Cell. Biol., **19,** 3246-3256 (1999).
2. Harbour, J.W., et al., Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell, **98**, 859-869 (1999).
3. Kaelin, W.G. Functions of the retinoblastoma protein. BioEssays, **21**, 950-958 (1999).
4. Zarkowska, T. and S. Mittnacht, Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem., **272,** 12738-12746 (1997).
5. Knudsen, E.S. and J.Y. Wang, Dual mechanism for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol. Cell. Biol**. 17**, 5771-5783 (1997).
6. Knudsen, E.S. and J.Y. Wang Differential regulation of retinoblastoma protein function by specific cdk phosphorylation sites. J. Biol. Chem. **271**, 8313-8320 (1996).

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