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Phospho-p38 MAPK ($pThr^{180}/pTyr^{182}$) ELISA

ProductInformation

Product Number **CS0020**
Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

Phospho-p38 MAPK ($pThr^{180}/pTyr^{182}$) ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) for quantitative determination of p38 MAPK protein phosphorylated on threonine 180/tyrosine 182 ($pThr^{180}/pTyr^{182}$) in cell lysates. A monoclonal antibody specific for p38 MAPK (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate provided. p38 MAPK ($pThr^{180}/pTyr^{182}$) standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the p38 MAPK antigen binds to the immobilized (capture) antibody. After incubation, excess reagents are washed away and an antibody specific for p38 MAPK ($pThr^{180}/pTyr^{182}$) is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized p38 MAPK protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG (Anti-Rabbit IgG-HRP) is added. It binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess Anti-Rabbit IgG-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 p38 MAPK ($pThr^{180}/pTyr^{182}$) present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of p38 MAPK ($pThr^{180}/pTyr^{182}$). The ELISA detects p38 MAPK ($pThr^{180}/pTyr^{182}$) in human, monkey and mouse. The researcher may normalize his results using a p38 MAPK ELISA (Product No. PM0100) which measures p38 MAPK independent of phosphorylation status.

p38 is a mitogen-activated protein kinase (MAPK), also known as a CDC-2-related protein kinase or CSBP (cytokine suppressive anti-inflammatory drug binding protein).[1] p38 MAPK is activated in response to a variety of extracellular stimuli including osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS), anisomycin, UV light and growth factors. The activation of p38 MAPK is mediated by several upstream kinases including MAP kinase kinase 3 (MKK3), MAP kinase kinase 6 (MKK6) and MAP kinase kinase 4 (MKK4, also known as SEK1 and JNKK1). These kinases phosphorylate p38 at threonine 180 and tyrosine 182 in the TGY motif, resulting in p38 activation. The dually phosphorylated form of p38 (threonine 180 and tyrosine 182) is a High Osmolarity Glycerol response kinase (HOG), a 43 kDa endogenous, active form of p38 α, β, and γ required for growth under high osmolarity conditions. This phosphorylated form is present in the variety of cells following treatment with a broad range of extracellular stimuli, including UV B irradiation of human skin, and ischemia.[2-5]

The p38 signaling transduction pathway plays an essential role in regulating many cellular processes including inflammation, cell differentiation, cell growth and death. Regulation of CDC25B phosphorylation by p38 is a critical event for initiating the G2/M checkpoint after ultraviolet radiation. Targets of p38 include transcription factors ATF-2, Max, MEF2C, CHOP, MAPKAPK2 (MAPK-activated protein kinase-2), and PRAK (p38-related/ activated protein kinase, mitogen-activated protein kinase activated protein kinase 5 (MAPKAPK5).[6-8]

Reagents

- **Phospho-p38 MAPK ($pThr^{180}/pTyr^{182}$) Standard, Human, 2 vials, Product No. P 7995** - lyophilized, full length recombinant p38 MAPK protein, expressed in *E. coli* and phosphorylated by MKK6. Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer**, **25 mL**, **Product No. S3943** - containing BSA and sodium azide as preservative.

- **Monoclonal Anti-p38 MAPK-coated 96 well plate, one plate, Product No. P 8995** - A plate using break-apart strips coated with monoclonal antibody specific for full-length p38 MAPK.
- **Anti-Phospho-p38 MAPK (pThr^{180}/pTyr^{182}), 11 mL, Product No. P 7870** – A detection antibody, produced in rabbit, which recognizes p38 phosphorylated on threonine 180 and tyrosine 182. Ready to use.
- **Anti-Rabbit IgG-HRP Concentrate (100X), 1 vial, 0.125 mL, Product No. R 0528** – 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 (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. S2818** - Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450nm
- 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 4ºC 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 recommended Cell Extraction Buffer and procedure are optimized to achieve effective protein phosphorylation. Researchers may use the procedures that work best in their hands. In such cases, they should assay their lysates for the satisfactory extraction and/or phosphorylation.

- Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent). Wash twice with cold PBS.
2. 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).

3. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals.
4. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of p38 MAPK. For example, 10^8 Jurkat cells grown in RPMI plus 10% FBS and treated with 100 µM anisomycin can be extracted in 1 mL of Extraction Buffer.
5. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 ºC.
6. Aliquot the clear lysate to clean microcentrifuge tubes.

Before assay: extracted cell lysate samples containing p38 MAPK ($pThr^{180}/pTyr^{182}$) protein should be diluted with Standard Diluent Buffer at least 1:10 (dilutions 1:20 to 1:50 may be required). This dilution is necessary to reduce the matrix effect of the cell lysate buffer. Example: 0.1-1 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer is sufficient for the detection of p38 MAPK ($pThr^{180}/pTyr^{182}$).

Reagent Preparation

Phospho-p38 MAPK ($pThr^{180}/pTyr^{182}$) Standard

1. Reconstitute one vial of Standard with Standard Diluent Buffer according to label directions.
2. Mix gently and wait 10 minutes to ensure complete reconstitution.
3. Label as **100 U/mL p38 MAPK ($pThr^{180}/pTyr^{182}$)**.
4. Prepare serial standard dilutions as follows:

Tube#	Standard Buffer	Standard from tube #:	Final concentration p38 MAPK ($pThr^{180}/pTyr^{182}$) U/mL
1	Reconstitute according to label instructions		100 U/mL
2	0.25 mL	0.25 mL (1)	50
3	0.25 mL	0.25 mL (2)	25
4	0.25 mL	0.25 mL (3)	12.5
5	0.25 mL	0.25 mL (4)	6.25
6	0.25 mL	0.25 mL (5)	3.12
7	0.25 mL	0.25 mL (6)	1.6
8	0.5 mL	-	0

Mix thoroughly between steps.

5. Use within 1 hour of reconstitution.

One Unit of standard = the amount of p38 MAPK ($pThr^{180}/pTyr^{182}$) derived from 40 pg of p38 MAPK that was phosphorylated by MKK6. Subsequent lots of standard will be normalized to this lot of material to allow consistency of p38 MAPK ($pThr^{180}/pTyr^{182}$) quantitation.

Anti-Rabbit IgG Horseradish Peroxidase (HRP), 100X Concentrate

Solution 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 with 1 mL HRP Diluent (sufficient for one 8-well strip, prepare more if needed)
4. Label as **Anti-Rabbit IgG-HRP Working Solution.**
5. Return the unused Anti-Rabbit IgG-HR concentrate to the refrigerator.

Wash Buffer

1. Equilibrate Wash Buffer Concentrate 25X to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X with 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.sigma-aldrich.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.
- Stabilized Chromogen (TMB) is light sensitive. Avoid prolonged exposure to light. Avoid contact with metal, it may cause color development.

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.

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.

I. 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 (1:50 or 1:100 may be necessary) 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. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

II. 2nd incubation
   a. Add 100 µL Anti- Phospho-p38 MAPK ($pThr^{180}/pTyr^{182}$) (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 4 times following washing instructions.
   d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

III. 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 4 times following washing instructions.
   d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

IV. 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 reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

V. 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.*

VI. 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 p38 MAPK ($pThr^{180}/pTyr^{182}$) may be calculated as follows:

1. Calculate the Average Net OD (average reading of 2 wells) for each standard dilution and samples as follows:
   Average Bound OD – Average Chromogen Blank OD = Average Net OD
2. On graph paper plot the Average Net OD (nm) of standard dilution against the concentration (U/mL) of p38 MAPK ($pThr^{180}/pTyr^{182}$) for the standards.
3. Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
4. The p38 ($pThr^{180}/pTyr^{182}$) concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply value(s) obtained for sample(s) by dilution factor to correct for the dilution in step I.b.
6. Samples producing signals higher than the highest standard (100 Units/mL) should be further diluted in *Standard Diluent Buffer* and re-analyzed, multiplying the concentration by the appropriate dilution factor.
7. Values of p38 MAPK ($pThr^{180}/pTyr^{182}$) should be normalized for total p38 MAPK content. Use p38 MAPK ELISA (Product No. PM0100) to quantitate total p38 MAPK concentration in samples and controls.

Product Profile

Typical Results

The standard curve below is for illustration purposes only and **should not be used** to calculate results in your assay. Run standard curve in each assay. The following data were obtained for the various standards over the range of 0 to 100 Units/mL p38 MAPK ($pThr^{180}/pTyr^{182}$):

OD 450 nm	p38 MAPK ($pThr^{180}/pTyr^{182}$) Standard U/mL
0.160	0
0.212	1.6
0.258	3.12
0.361	6.25
0.554	12.5
0.887	25
1.508	50
2.978	100

Limitations:

- Do not extrapolate the standard curve beyond the 100 U/mL standard point.
- The dose response is non-linear in this region and accuracy is compromised.
- Dilute samples >100 Units/mL with *Standard Diluent Buffer;* re-analyze these and multiply results by the appropriate dilution factor.
- Other buffers and matrices have not been investigated.
- Although p38 MAPK degradation or dephosphorylation of p38 MAPK ($pThr^{180}/pTyr^{182}$) 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

This p38 MAPK (pThr^{180}/pTyr^{182}) ELISA kit is specific for measurement of human, monkey and mouse p38 MAPK phosphorylated at threonine 180 and tyrosine 182. The kit does not detect non-phosphorylated p38 MAPK protein, as shown in Figure 1.

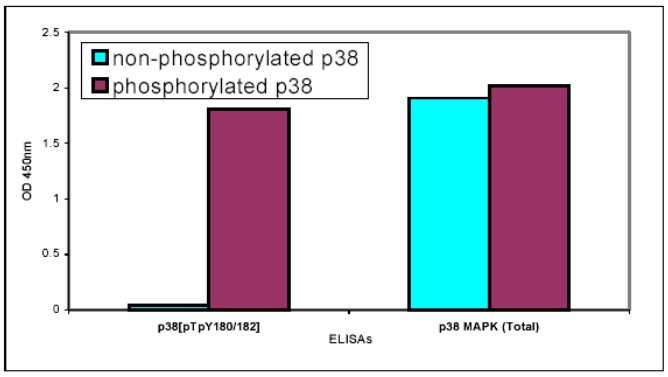


Fig. 1

Recombinant p38 MAPK was phosphorylated using MKK6 enzyme *in vitro*. Non-phosphorylated p38 MAPK was used as control. The phosphorylated and non-phosphorylated p38 MAPK were analyzed with p38 MAPK (pThr^{180}/pTyr^{182}) and p38 MAPK ELISA's. The p38 MAPK ELISA detected both phosphorylated and non-phosphorylated p38 MAPK whereas the Phospho-p38 MAPK (pThr^{180}/pTyr$^{/182}$) ELISA detects only phosphorylated p38.

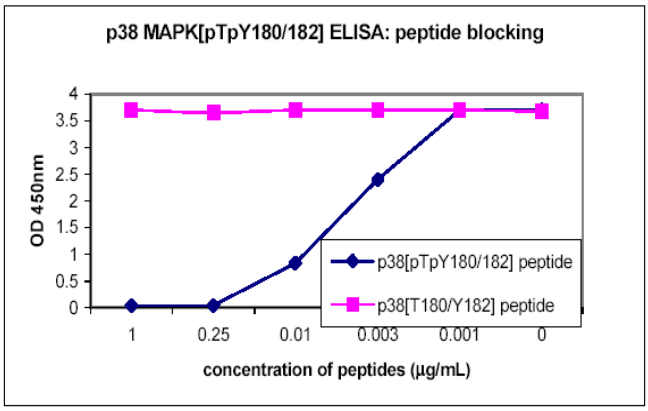


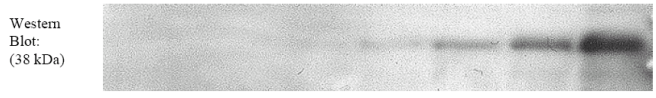
Fig. 2

The specificity of this assay for p38 MAPK phosphorylated at threonine 180 and tyrosine 182 was confirmed by peptide competition. The data presented in (Figure 2) show that only the phospho-peptide containing the phosphorylated threonine and tyrosine could block the ELISA signal. The same sequence containing non-phosphorylated threonine and tyrosine at position 180/182 did not block the signal.

Sensitivity

Sensitivity of this assay is <0.8 U/mL of human p38 MAPK (pThr^{180}/pTyr$^{/182}$). 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 p38 MAPK (pThr^{180}/pTyr$^{/182}$).



ELISA: OD 450 nm	0.160	0.212	0.258	0.361	0.554	0.887	1.508	2.978
p38 MAPK pYpY180/182 (Units/test)	0	0.15	0.31	0.63	1.25	2.5	5	10

Detection of p38 MAPK (pThr^{180}/pTyr^{182})by ELISA vs. immunoblot

The data presented above show that the ELISA is approximately 10 times more sensitive than the immunoblot. The bands shown in the immunoblot were developed using rabbit anti-p38 MAPK (pThr^{180}/pTyr$^{/182}$), an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Precision

1. Intra-Assay Precision

Samples of known p38 MAPK (pThr^{180}/pTyr$^{/182}$) concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (U/mL)	55.4	17.4	4.2
Standard Deviation (SD)	2.4	0.7	0.2
% Coefficient of Variation	4.3	3.9	4.7

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

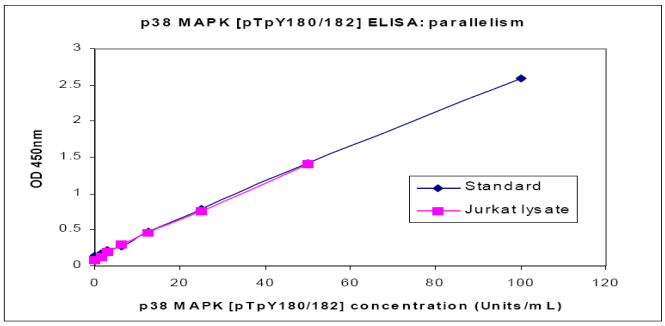
	Sample 1	Sample 2	Sample 3
Mean (U/mL)	56.5	16.5	3.8
Standard Deviation (SD)	5.0	1.1	0.4
% Coefficient of Variation	8.8	6.8	9.7

Sample Recovery

To evaluate recovery, unstimulated Jurkat cell lysates were prepared and adjusted to 200 µg/mL total protein. Recombinant p38 MAPK (pThr^{180}/pTyr^{182}) at 3 levels was spiked into the cell extract and percent recovery calculated over endogenous levels. On average, 97% recovery was observed.

Parallelism

Natural p38 MAPK (pThr^{180}/pTyr^{182}) anisomycin treated Jurkat cell lysate was serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the p38 MAPK (pThr^{180}/pTyr^{182}) standard curve. Parallelism demonstrated by the figure below indicated that the standard accurately reflects p38 MAPK (pThr^{180}/pTyr^{182}) content in samples.



Linearity of Dilution

Extract Buffer was spiked with p38 MAPK (pThr^{180}/pTyr^{182}) 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.

Cell Lysate			
Dilution	**Measured (U/mL)**	**Expected (U/mL)**	**% Expected**
Neat	48.8	48.8	100
1:2	27.4	24.4	110
1:4	15.5	13.7	112
1:8	7.13	7.74	92
1:16	3.61	3.56	101

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AH/JK 4/04