Monitoring the Expression, Purification, and Processing of GST-Tagged Proteins Using the Experion™ Automated Electrophoresis System

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Introduction

Expression and purification of recombinant proteins are prerequisites for their structural and functional characterization. Over the past 20 years, affinity tag systems have become the predominant approach for obtaining highly purified target proteins. Among the widely used affinity tags, the glutathione S-transferase (GST) sequence has been shown to stabilize and, in some cases, improve the solubility of recombinant proteins (Terpe 2003); however, this 26 kD tag is immunogenic and may interfere with the structure or biological activity of target proteins, thereby necessitating its removal. Thrombin is a protease commonly employed for this purpose and, if biotinylated, it can be removed from a digestion mixture using *Strep*-tag/StrepTactin chromatography or streptavidin agarose (Terpe 2003). Nevertheless, complications may arise during the purification of GST fusion proteins and subsequent affinity tag removal, such as copurification of host proteins that interact with the GST domain (Lichty et al. 2005) or poor efficiency and specificity of protease activity (Chang et al. 1985, Jenny et al. 2003). The purification of GST fusion proteins, therefore, must somehow be monitored at each step.

We have used the Experion automated electrophoresis system, in combination with the Experion Pro260 analysis kit, to monitor the expression of GST-tagged proteins in *E. coli*, and to analyze samples from the purification and processing of these proteins. Our results demonstrate that the Experion system offers data with quality comparable to that of data generated by traditional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) while eliminating the laborious procedures associated with gel electrophoresis. The accurate protein quantitation information provided directly by Experion software has been applied to the development of an optimized scheme for manufacturing a recombinant, tag-free protein of interest.

Methods

Materials

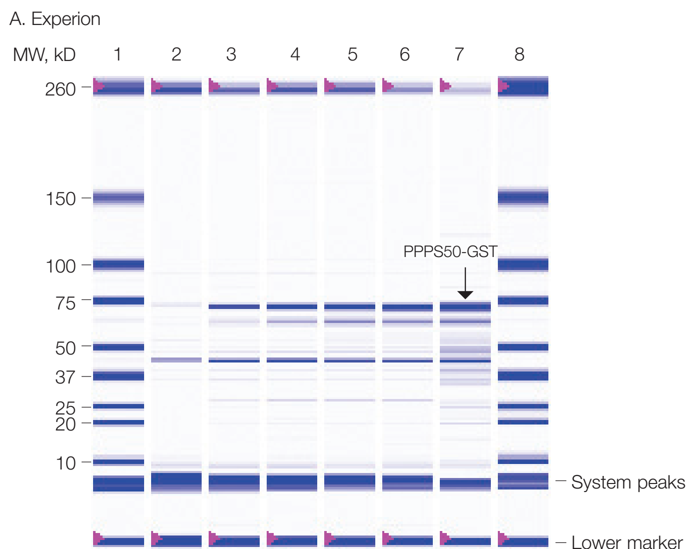
A GST fusion of a proprietary recombinant protein, PPPS50 (50 kD), was used in these studies. The thrombin cleavage capture kit, GST•Bind buffer kit, and Lysonase bioprocessing reagent were purchased from EMD Biosciences, Inc., and the GST MicroSpin purification module was purchased from GE Healthcare. Chemicals used in buffer preparation were obtained from Sigma-Aldrich, Inc. or from VWR International. Media and containers for bacterial cultures were purchased from VWR International.

Expression of Fusion Protein PPPS50-GST

E. coli BL21(DE3) cells harboring the PPPS50-GST expression plasmid were grown at 28°C in Terrific Broth containing 100 µg/ml ampicillin. Expression was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM when culture density (OD 600) reached 0.5–0.6. For expression studies, 200 µl samples were collected upon IPTG induction and at 1, 2, 3, 4, and 22 hr after induction, and stored at –20°C. For purification of PPPS50, cells were harvested 3 hr after induction by centrifugation at 5,000 x g for 20 min at 4°C; the cell paste was frozen and stored at –20°C.

Purification of PPPS50-GST

The frozen *E. coli* BL21(DE3)-PPPS50-GST cell paste (0.4 g) was resuspended in 4 ml 1x GST bind/wash buffer (from the GST•Bind buffer kit) containing 2 µl/ml Lysonase. This cell suspension was incubated at room temperature for 20 min with gentle rotation. Insoluble cell debris was removed by centrifugation at 16,000 x g for 20 min at 4°C in a microcentrifuge. PPPS50-GST fusion protein was purified from 200 µl crude extract using a GST MicroSpin column following the manufacturer's instructions. Samples were taken during this purification process, frozen, and stored at –20°C.



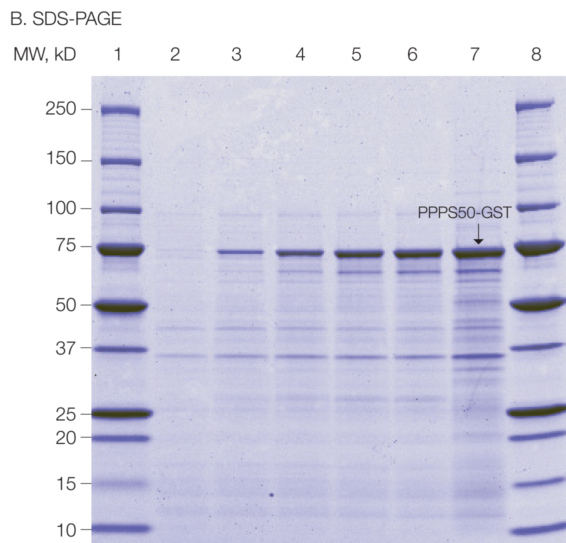


Fig. 1. PPPS50-GST expression in *E. coli* BL21(DE3) cell cultures following induction by IPTG. A, simulated gel image generated by the Experion system showing separation of the Pro260 protein ladder in lanes 1 and 8; B, SDS-PAGE analysis, showing separation of the Precision Plus Protein™ standard in lanes 1 and 8. In both images, lane 2 contains culture sample taken immediately upon IPTG induction; lanes 3–7, culture samples taken 1, 2, 3, 4, and 22 hr after induction by IPTG. The position of the PPPS50-GST fusion protein is indicated.

Thrombin Digestion of PPPS50-GST

To determine the efficiency of fusion protein cleavage by biotinylated thrombin, digestion reactions containing increasing amounts of the enzyme were prepared. The original 1 U/µl biotinylated thrombin stock was diluted to give a series of solutions containing 0.01–0.2 U/µl enzyme, and for each concentration, a 10 µl digestion reaction was prepared to contain 1 µl biotinylated thrombin solution, 1 µl 10x thrombin cleavage buffer, and 64 µg purified PPPS50-GST fusion protein. The reactions were incubated at room temperature for 2 hr. In a separate reaction, 5 U biotinylated thrombin was mixed with 8 mg purified PPPS50-GST and 10x thrombin cleavage buffer into a final volume of 250 µl. Aliquots of this reaction (50 µl) were removed after 0.5, 1, 2, 3, and 20 hr of incubation at room temperature. The extent of fusion protein cleavage in each reaction was determined using the Experion Pro260 analysis kit and by SDS-PAGE.

Stepwise Purification of PPPS50

Tag-free PPPS50 was purified in a stepwise fashion that involved cleavage of purified fusion protein PPPS50-GST with biotinylated thrombin followed by thrombin removal with streptavidin agarose, desalting with Bio-Gel® P-6 support, and removal of the GST tag by affinity chromatography using GST MicroSpin columns.

First, purified PPPS50-GST (8 mg) was mixed with 25 µl 10x thrombin cleavage buffer and 5 U biotinylated thrombin in a final volume of 250 µl. Incubation was carried out at room temperature for 18 hr. A 30 µl sample was taken at the time of completion. To the rest of the reaction, 160 µl streptavidin agarose (50% slurry, from the thrombin cleavage capture kit) was added. This mixture was incubated at room temperature for 30 min with gentle shaking. The streptavidin agarose was then removed with a spin filter according to the manufacturer's instructions. A 27 µl sample was taken from the filtrate for analysis with the Experion system and by SDS-PAGE.

The thrombin-free sample was desalted on Micro Bio-Spin™ 6 columns, which are packed with Bio-Gel P-6 support, and then applied onto a freshly equilibrated GST MicroSpin column. The released GST tag was captured by the column, and the tag-free PPPS50 was collected in the flow-through fraction. Samples of both the bound and flow-through fractions were analyzed with the Experion system and by SDS-PAGE.

Experion Pro260 Analysis

Samples were prepared according to the instructions provided in the Pro260 analysis kit instruction manual for protein separation under reducing conditions. Samples (4 µl), which

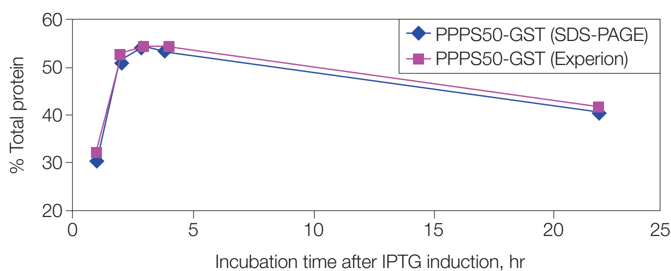


Fig. 2. Abundance of PPPS50-GST fusion protein in *E. coli* BL21(DE3) cells following induction by IPTG. The expression levels of PPPS50-GST were monitored at various time points using the Experion system or SDS-PAGE.

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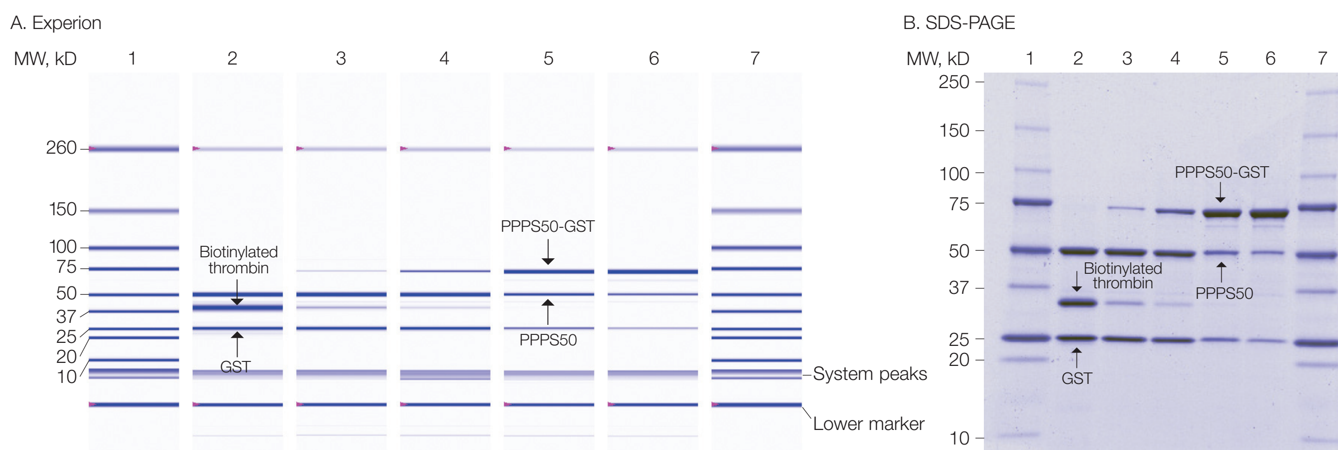

Fig. 3. PPPS50-GST cleavage by biotinylated thrombin. A, simulated gel image generated by the Experion system, showing separation of the Pro260 protein ladder in lanes 1 and 7; B, SDS-PAGE analysis, showing separation of the Precision Plus Protein standard in lanes 1 and 7. In both images, lanes 2–6 show the products from cleavage of 64 µg PPPS50-GST by 1, 0.2, 0.1, 0.02, or 0.01 U biotinylated thrombin after incubation at room temperature for 2 hr. The positions of biotinylated thrombin, PPPS50-GST, tag-free PPPS50, and the GST tag are indicated.

were taken at various time points from the IPTG-induced culture, the thrombin digestion reactions, and the stepwise purification, were used directly, without dilution. Protein purity and relative quantitation values were automatically calculated by Experion software.

SDS-PAGE Analysis

Samples were prepared by mixing 4 µl of each protein solution with an equal volume of 2x Laemmli sample buffer containing 5% β-mercaptoethanol, and were heated at 95°C for 5 min prior to being loaded onto gels. SDS-PAGE was performed in a Criterion™ electrophoresis cell with 4–20% Criterion Tris-HCl precast gels under denaturing conditions. Gels were stained for 1 hr at room temperature with Bio-Safe™ Coomassie G-250 stain, and destained in water overnight. Gels were imaged with a GS-800™ densitometer and analyzed with Quantity One® 1-D analysis software.

Results and Discussion

Monitoring Expression Levels

The production of the 76 kD fusion protein, PPPS50-GST, by *E. coli* BL21(DE3) cells was visualized with the Experion system and by conventional SDS-PAGE (Figure 1), and its expression level was quantitated at various time points upon or after IPTG induction (Figure 2) using Experion or Quantity One software. The in vivo production of PPPS50-GST was found to plateau 3 hr after IPTG addition, when it accounted for ~50% of the total protein present. As shown in Figure 2, results obtained from the Pro260 analysis are consistent with those determined by a GS-800 densitometer and Quantity One software. In addition, the speed of the Experion Pro260 analysis (~30 min for 10 samples) facilitated faster access to results and real-time monitoring of protein expression.

Monitoring Digestion of PPPS50-GST With Biotinylated Thrombin

Digestion of PPPS50-GST by biotinylated thrombin released the GST tag and the target protein, PPPS50. PPPS50-GST was cleaved quantitatively by undiluted biotinylated thrombin within 2 hr of incubation, while digestions using lower concentrations of biotinylated thrombin for the same period of time resulted in mixtures of full-length fusion protein and cleavage products (Figure 3). The postreaction removal of highly concentrated protease was problematic (data not shown). However, the enzyme could be effectively removed following a 20 hr incubation in which PPPS50-GST was fully processed by thrombin at one-fifth of its original concentration, and where no secondary digestion products were observed (Figure 4). The latter condition was adopted to minimize loss of target protein and to facilitate sample handling during removal of biotinylated thrombin with streptavidin agarose. Both the Experion system and SDS-PAGE offered high-quality data suitable for the kinetic study of this digestion. In fact, the two data sets were nearly superimposable (Figure 5).

Monitoring Purification of Tag-Free PPPS50

A protocol for the large-scale purification of PPPS50 was generated using information gained from the above preliminary tests. Figure 6 shows separations of samples taken at various stages during this stepwise protein purification process and the effective separation of the GST tag from the target fusion protein.

Note that, when using the Experion system to analyze protein samples, buffer exchange or desalting of samples prior to analysis may be necessary for optimal detection sensitivity or if absolute quantitation is desired. With the Experion system, charged protein molecules are electrokinetically injected into

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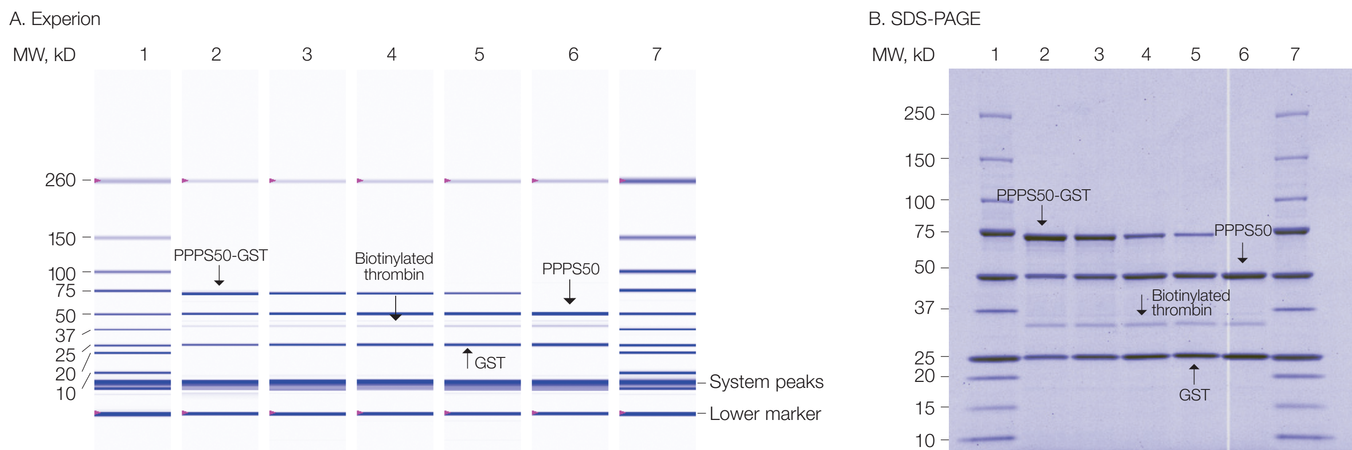

Fig. 4. Time dependence of PPPS50-GST cleavage by biotinylated thrombin. A, simulated gel image generated by the Experion system, showing separation of the Pro260 protein ladder in lanes 1 and 7; B, SDS-PAGE analysis, showing separation of the Precision Plus Protein standard in lanes 1 and 7. In both images, lanes 2–6 contain samples taken at 0.5, 1, 2, 3, or 20 hr of incubation at room temperature. The positions of PPPS50-GST, tag-free PPPS50, biotinylated thrombin, and the GST tag are indicated.

the separation channel of the Pro260 microfluidic chip; therefore, the loading of various proteins may be influenced by other cellular or buffer components present in the sample. In Figure 6, lanes 8 and 9 in both images show the separation of samples before and after desalting, respectively.

The expression level of PPPS50-GST in *E. coli* BL21(DE3) cells, and the purity of this fusion protein following purification on a GST MicroSpin column, as well as the purity of tag-free PPPS50, were determined with the Experion system and by densitometric scanning of SDS-PAGE gels (Table 1). The purity values obtained by the Experion system were generally in good agreement with those obtained by SDS-PAGE and Quantity One software analysis. The largest discrepancy was observed for the purity of PPPS50-GST in crude *E. coli* extracts (Table 1) and was likely due to inherent differences in the resolution offered by the two analytical methods. Whereas the Experion system demonstrated better resolution for 50 kD and larger proteins, SDS-PAGE provided excellent separation of smaller proteins. Accordingly, PPPS50-GST may have overlapped with other protein species of similar molecular weight on a 4–20% Tris-HCl gel, thereby contributing to its apparently higher abundance when detected by SDS-PAGE.

Using an internal 260 kD marker that is included in the Pro260 sample buffer at a known concentration, the Experion system provides automatic relative quantitation of protein samples, a feature that is not available with traditional SDS-PAGE. On the basis of the data reported by Experion software, the yield of PPPS50 from the postpurification processing of PPPS50-GST was ~87%; from 1 g fusion protein, ~870 mg PPPS50 could be obtained at a final concentration of 606 ng/µl.

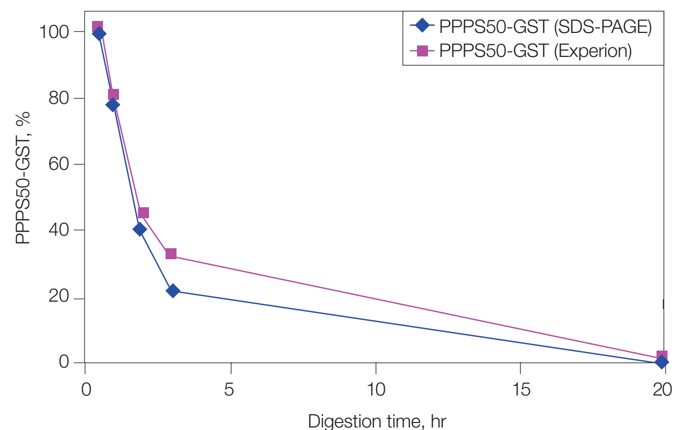

Fig. 5. Kinetics of PPPS50-GST cleavage by biotinylated thrombin at room temperature. Samples from the proteolytic reaction were taken at the indicated time points and analyzed with the Experion system or by SDS-PAGE. The relative amount of PPPS50-GST, calculated from the corrected peak area (Experion software) and the peak density (Quantity One software), was expressed as a percentage of the amount at 0.5 hr, and plotted against reaction time.

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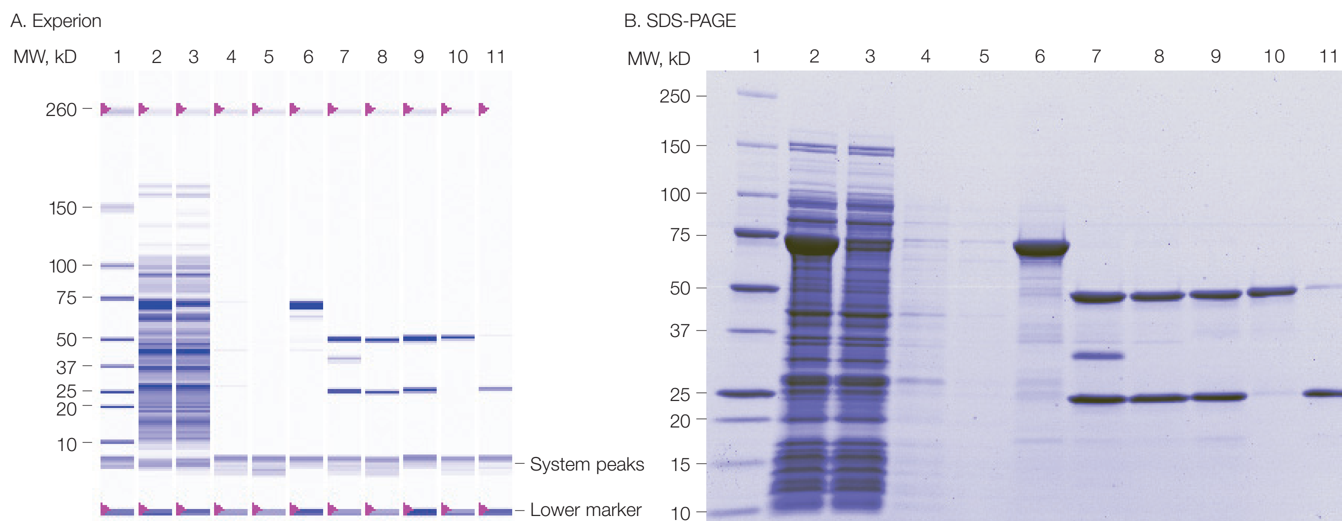

Fig. 6. Purification and processing of PPPS50-GST. A, simulated gel image generated by the Experion system showing separation of the Pro260 protein ladder in lane 1; B, SDS-PAGE analysis, showing separation of the Precision Plus Protein standard in lane 1. In both images, lane 2 contains a separation of crude *E. coli* extract; lane 3, flow-through from a GST MicroSpin column; lanes 4 and 5, fractions of unbound proteins washed from the column; lane 6, PPPS50-GST eluate; lane 7, products from biotinylated thrombin digestion of PPPS50-GST; lane 8, sample after biotinylated thrombin removal with streptavidin agarose; lane 9, sample after desalting with Micro Bio-Spin 6 columns; lane 10, tag-free PPPS50 collected in the flow-through fraction from a GST MicroSpin column; and lane 11, GST tag captured by the GST MicroSpin column.

Table 1. Stepwise purification and processing of PPPS50-GST.

	Experion System		SDS-PAGE	
Sample	Purity	Fold Purification	Purity	Fold Purification
Crude extract	14.0%	1.0	18.4%	1.0
PPPS50-GST	84.9%	6.1	86.6%	4.7
PPPS50	95.7%	6.8	97.3%	5.3

Conclusions

Qualitative and quantitative information about protein samples is valuable to the development of protein purification methods as well as to many downstream applications. The Experion system integrates protein separation, detection, and analysis within a single platform to reduce the amount of sample, reagents, and hands-on time required to perform routine qualitative and quantitative protein analyses. This microfluidics-based system offers great convenience in sample analysis and a throughput unmatched by traditional SDS-PAGE. We have presented its use in protein expression and purification protocol development, as well as in the quality control of process-scale protein production. We are currently investigating the potential applications of the Experion system to other fields of protein chemistry, such as the monitoring of enzymatic assays and protein-protein interaction studies.

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