APPLICATION NOTE

IMAP FP kinase assays on the SpectraMax M5 Multi-Mode Microplate Reader

Introduction

Protein kinases are central to the regulation of many cellular processes. In recent years they have emerged as one of the most important classes of drug targets for cancer and many other diseases. IMAP® Technology from Molecular Devices enables rapid, non-radioactive assay of a wide array of kinases and is suited to both assay development and high-throughput screening. IMAP technology is based on binding of phosphate through immobilized metal coordination complexes on nanoparticles. When IMAP binding entities bind to phosphopeptides generated by a kinase reaction, molecular motion of the peptide is altered and fluorescence polarization for the fluorescent label attached to the peptide increases (Figure 1). This homogeneous assay is easy to perform and applicable to many kinases regardless of peptide substrate sequence.

The SpectraMax® M5 Multi-Mode Microplate Reader is ideally suited for assay development and high-throughput screening using IMAP assays. This monochromator-based instrument allows users to select optimal wavelengths for any chosen fluorophore without the need to purchase additional filter sets. This application note describes how to run IMAP fluorescence polarization (FP) kinase assays and calibration curves with green- and red-labeled fluorescent substrates using the SpectraMax M5 microplate reader and SoftMax® Pro Software. Enzyme dilution curves were performed for Lck, a tyrosine kinase with a key role in T-cell signaling, and Akt1/PKBα, a serine-threonine kinase involved in phosphatidylinositol 3-kinase signaling and cell survival. Inhibition of Akt1/PKBα by staurosporine was also assayed. High Z' factor values are obtained with both FAM- and TAMRA-labeled peptide substrates, with data comparable to those obtained on the filter-based Analyst® HT Multi-Mode Microplate Reader.

Materials

- IMAP Screening Express Kit with Progressive Binding System (Molecular Devices Cat. #R8125)
  - IMAP Progressive Binding Reagent
  - IMAP Progressive Binding Buffer A (5X)
  - IMAP Progressive Binding Buffer B (5X)
  - IMAP Reaction Buffer (5X)
- Lck kinase (Upstate Cat. #14-442) Cat. #R7159)
  - FAM-p34cdc2-derived peptide (Molecular Devices Cat. #R7157)
  - TAMRA-p34cdc2-derived peptide (Molecular Devices Cat. #R7309)
  - FAM-p34cdc2-derived phosphopeptide calibrator (Molecular Devices Cat. #R7271)

Benefits

- **Homogeneous FP assays provide an HTS-friendly format**
- **Precise, reproducible data with robust Z' factor values**
- **Preconfigured protocol in SoftMax Pro Software**

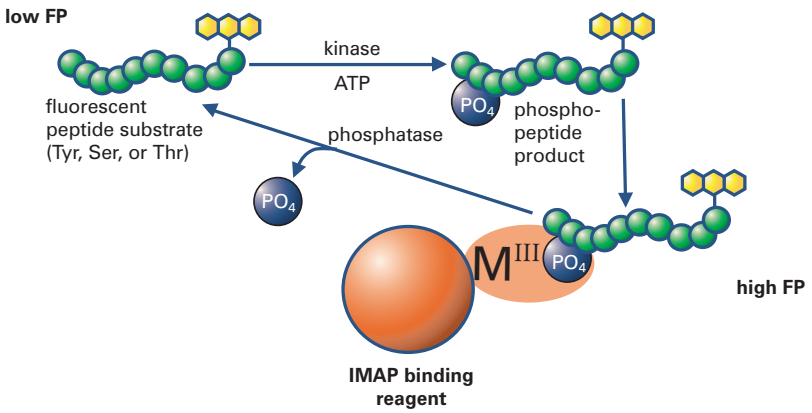

Figure 1. IMAP FP assay principle. Diagram of the IMAP FP kinase assay.

- Akt1-PKBα kinase (Upstate Cat. #14-276)
  - FAM-Crosstide (Molecular Devices Cat. #R7110)
  - FAM-phospho-Crosstide (Molecular Devices Cat. #R7159)
- Adenosine 5' triphosphate (ATP), 50 mM stock in purified water (Sigma Cat. #A6559)
- DL-Dithiothreitol (DTT), 100 mM stock in purified water (Sigma Cat. #D9779)
- Staurosporine (Biomol Cat. #EI-156)
- Black 384-well polystyrene microplate (Corning Cat. #3710)
- SpectraMax M5 Multi-Mode Microplate Reader with SoftMax Pro Software (Molecular Devices)

Methods

Kinase reactions

Step 1: Prepare Complete Reaction Buffer (CRB) by supplementing 1X IMAP Reaction Buffer with DTT at a final concentration of 1 mM (1:100 dilution of 100 mM DTT stock).

Step 2: Make a 400 nM (4X) stock of FAM- or TAMRA-labeled peptide substrate in CRB (1:50 dilution of 20 µM peptide stock).

Step 3: Make a 20 µM (4X) stock of ATP in CRB (1:2500 dilution of 50 mM ATP stock).

Step 4: Prepare an enzyme dilution series at 4X final desired concentrations for the assay. For the kinase inhibition assay, use a constant concentration of enzyme and make a dilution series of staurosporine or other kinase inhibitor. Use CRB for dilutions.

Step 5: Set up kinase reactions by pipetting the following into quadruplicate enzyme assay wells:

- 5 µL CRB or staurosporine
- 5 µL enzyme (for no-enzyme background samples, substitute 5 µL CRB)
- 5 µL of 20-µM ATP stock
- 5 mL of 400-nM peptide stock

Step 6: Incubate at room temperature for 1 to 1.5 hours.

Note: For more detailed instructions, please refer to the IMAP product insert.[1] Assay data for many kinases are available in Molecular Devices' IMAP Assay Archive at http://www.moleculardevices.com/assayarchive/.

Calibration standard (% Phosphorylated)	µL Peptide stock	µL Phospho-peptide stock
0	100	0
12.5	87.5	12.5
25	75	25
50	50	50
100	0	100

Table 1. Preparation of calibration standards.

Read type	Endpoint	
Read mode	Fluorescence polarization	
Wavelengths	FAM Ex 485 nm Em 525 nm Cutoff 515 nm	TAMRA Ex 530 nm Em 590 nm Cutoff 570 nm
Sensitivity	Readings: 100 PMT: high or medium	
Automix	Off	
AutoCalibrate	On	
Assay plate type	384-well Costar black	
Wells to read	[Determined by user]	
Settling time	Delay: 100 ms	
AutoRead	Off	

Table 2. IMAP FP settings in SoftMax Pro for SpectraMax M5 microplate reader.

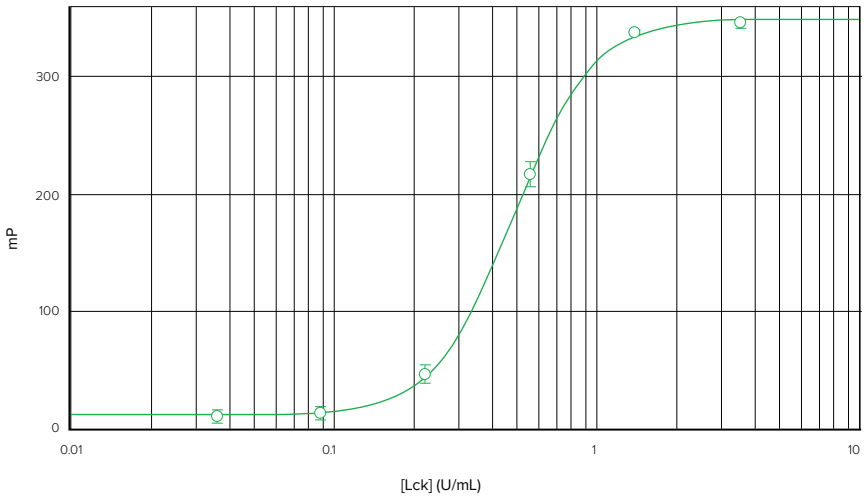

Figure 2. IMAP FP kinase assay on SpectraMax M5 microplate reader. Lck kinase dilution curve with FAM-p34cdc2-derived peptide from 0.04 to 3.5 units/mL enzyme, read on SpectraMax M5 (4-parameter curve fit). Error bars here and on all subsequent graphs are standard deviations.

Calibration standards

Step 1: Make a 100-nM peptide stock in CRB (use the same peptide substrate as in the kinase assay).

Step 2: Make a 100-nM phosphopeptide stock in CRB (use the phosphorylated version of the substrate used in the kinase assay).

Step 3: Combine peptide and phosphopeptide stocks to make calibration standards as indicated in Table 1. Amounts given are sufficient to set up quadruplicate samples.

Step 4: Pipet 20 µL each calibration standard into quadruplicate wells, including a set of buffer-only background samples containing 20 µL CRB.

Binding reaction

Step 1: Prepare Progressive Binding Buffer by combining 75% Progressive Binding Buffer A with 25% Progressive Binding Buffer B.

Step 2: Prepare Binding Solution by diluting Progressive Binding Reagent 1:600 in Progressive Binding Buffer.

Step 3: Pipet 60 µL of Binding Solution into each assay and calibration standard well (including buffer background samples).

Step 4: Incubate at room temperature for 1 hour, protected from light.

Set up template in SoftMax Pro and read plate on the SpectraMax M5 microplate reader:

Note: IMAP FP protocols for use with FAM- and TAMRA-labeled substrates are available in SoftMax Pro 5 in the Binding Assays protocol folder.

Step 1: Open the SoftMax Pro IMAP FP protocol specific to the fluorophore used. FAM and TAMRA protocols are available; adjust wavelength settings if a fluorophore other than FAM or TAMRA is used. Settings for the SpectraMax M5 microplate reader are shown in Table 2.

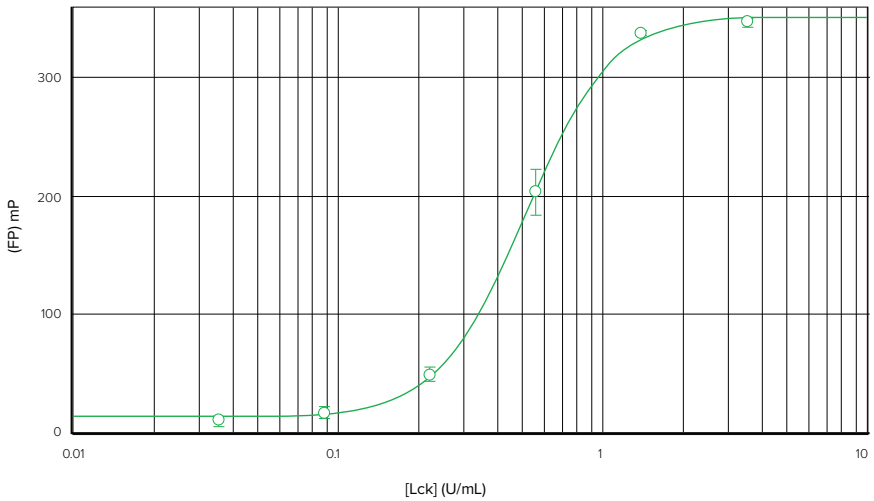

Figure 3. IMAP FP kinase assay on Analyst HT. Lck kinase dilution curve with FAM-p34cdc2-derived peptide from 0.04 to 3.5 units/mL enzyme, read on Analyst HT (4-parameter curve fit).

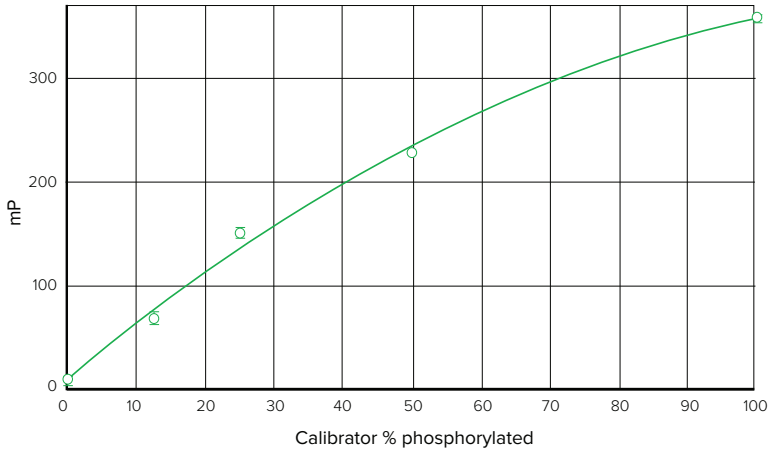

Figure 4. IMAP with FAM-labeled peptide. Calibration standard curve with FAM-p34cdc2-derived peptide and phosphopeptide, read on SpectraMax M5 (quadratic curve fit).

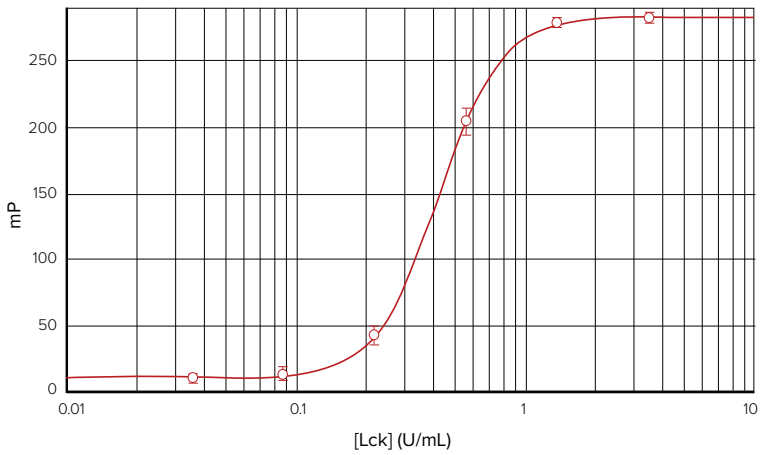

Figure 5. Lck kinase assay with TAMRA-labeled peptide on SpectraMax M5 microplate reader. Lck kinase dilution curve with TAMRA-p34cdc2-derived peptide, read on SpectraMax M5 (4-parameter curve fit).

Step 2: Set up an experiment template designating background samples, kinase assay samples, and calibration standards. Kinase assay samples and calibration standards may be assigned to eight preconfigured 'Sample' groups in the SoftMax Pro template. Assigning buffer-only controls to the corresponding 'Background' groups enables automatic subtraction of backgroundfluorescence prior to calculation of milli-polarization (mP) values.

Step 3: Place the microplate in the plate reader carriage—be sure to include a purple plate adapter since the plate is read from the top—and click Read.

Results

The SoftMax Pro IMAP FP protocol automatically calculates average parallel and perpendicular values, mP, total intensity, standard deviations, and CV. In the group table for each set of assay samples, background subtracted mP is calculated using background samples designated in the template as references. Percent phosphorylation can also be calculated by extrapolating from an appropriate calibration curve.

Figure 2 shows a Lck kinase dilution curve obtained using the FAM-p34cdc2-derived peptide. When the plate was read on the SpectraMax M5 microplate reader, an enzyme concentration range of 0.04 to 3.5 units/mL yielded a delta mP of 303, with a Z' factor of 0.87.[2] Figure 3 shows the same assay plate read on the Analyst HT multi-mode reader. Here the delta mP was 336, and Z' factor was 0.95. On both instruments the EC_{50} was 0.5 units/mL.

Values for mP may be converted to percent phosphorylation if you set up a separate calibration curve using non-phosphorylated and phosphorylated peptide controls (Figure 4). First create a new graph ('Graph#1' is the default name assigned by the software) and plot mP vs. percent phosphorylated (concentration) for your calibrator sample group. The default name for this plot is 'Plot#1.' In the calibration standards group table, insert a new column with the following formula: InterpX('Plot#1@Graph#1@IMAP FP_FAM',AvgbkgsubmP). 'IMAP FP_FAM' is the experiment title.

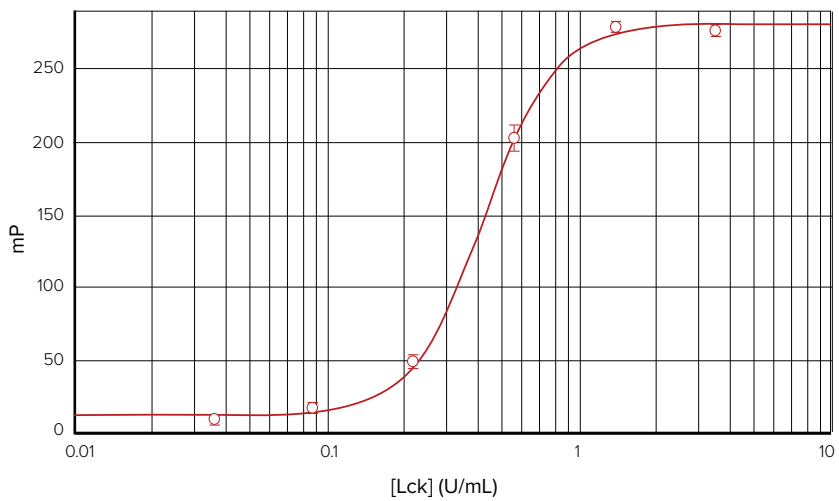

Figure 6. Lck kinase assay with TAMRA-labeled peptide on Analyst HT. Lck kinase dilution curve with TAMRA-p34cdc2-derived peptide, read on Analyst HT (4-parameter curve fit).

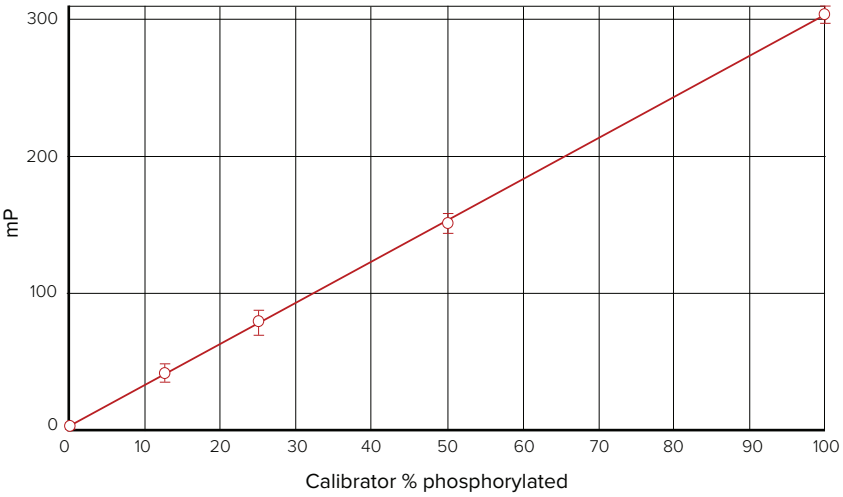

Figure 7. IMAP with TAMRA-labeled peptide. Calibration standard curve with TAMRA-p34cdc2-derived peptide and phosphopeptide, read on SpectraMax M5 (linear curve fit).

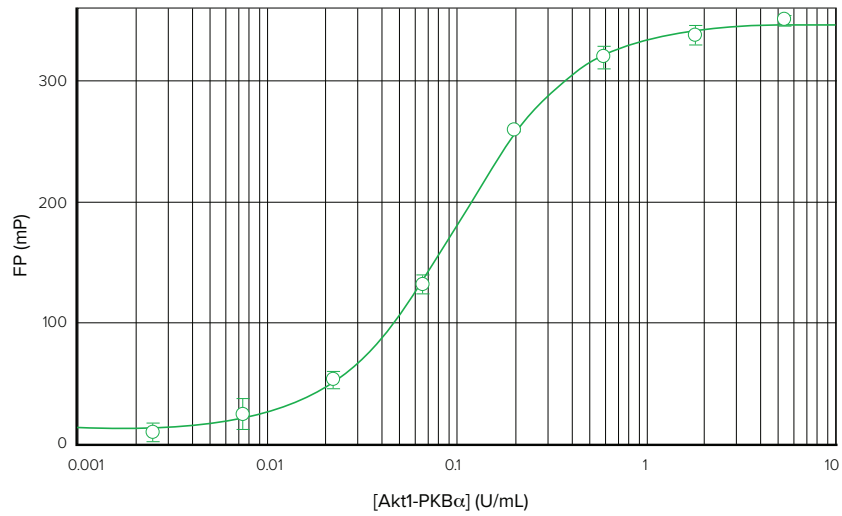

Figure 8. Akt/PBKα kinase assay with FAM-labeled peptide on SpectraMax M5 microplate reader. Akt1/PKBα kinase dilution curve with FAM-Crosstide substrate, read on SpectraMax M5 (4-parameter curve fit).

To determine performance of the IMAP kinase assay with red fluorophore, another Lck kinase dilution curve was performed using the TAMRA-p34cdc2-derived peptide (Figure 5). Here, an enzyme concentration range of 0.04 to 3.5 units/mL yielded a delta mP of 258, with a Z' factor of 0.95 when read on the SpectraMax M5 microplate reader. In Figure 6, the same assay plate was read on the Analyst HT reader, with a delta mP of 270 and Z' factor of 0.93. On both instruments the EC_{50} was 0.4 units/mL, a value close to the EC_{50} with FAM-labeled substrate. A corresponding calibration curve was also set up (Figure 7). Similarity of results with TAMRA-labeled peptide substrate on both instruments to those obtained with FAM-labeled substrate allow users to take advantage of the benefits of red-labeled fluorophores, including minimizing background fluorescence emitted by test compounds.

To demonstrate performance of the IMAP FP assay on the SpectraMax M5 microplate reader with another kinase, an enzyme dilution curve for Akt1/PKBα was obtained using FAM-Crosstide as a substrate. In Figure 8, an enzyme concentration range of 0.002 to 5.4 units/mL yielded a delta mP of 339 and a Z' factor of 0.92. Inhibition of Akt1/PKBα by staurosporine is shown in Figure 9; here the delta mP was 199 with a Z' factor of 0.93. The calculated IC_{50} for staurosporine for this assay was 19.7 nM. The delta mP was lower here than in the enzyme dilution curves because a concentration of kinase was chosen for the inhibition assay that gave about 75% maximal phosphorylation.

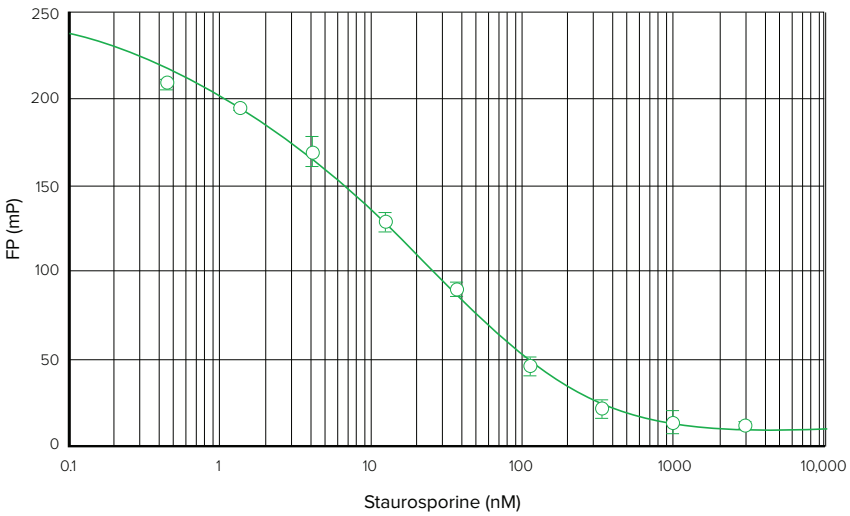

Figure 9. Kinase inhibition on SpectraMax M5 microplate reader. Akt1/PKBα kinase inhibition curve with staurosporine and FAM-Crosstide substrate, read on SpectraMax M5. A five-parameter curve fit, available in SoftMax Pro 5, was used here.

Conclusion

As researchers seek to understand the roles that kinases play in cell signaling and disease, as well as identify kinase modulators that may serve as therapeutics, they are increasingly looking to non-radioactive, homogenous assays that are well suited to both assay development and high-throughput screening. Homogeneous FP assays provide an HTS-friendly format for identifying kinase activators, inhibitors, and substrates. IMAP FP kinase assays run on the SpectraMax M5 microplate reader yield precise, reproducible data with robust Z' factor values. The dual monochromator system allows users to optimize the assays using a wide range of fluorescently labeled peptide substrates without the need to purchase additional filter sets. Results for IMAP FP kinase assays with either green- or red-labeled substrates are very similar to results obtained with the filter-based Analyst microplate readers. IMAP FP protocols in SoftMax Pro 5 provide a convenient way to obtain and perform calculations on FP data and are easily adaptable to any fluorophore.

References

1. IMAP Akt Assay Kit Product Insert (Molecular Devices product #R8058).
2. Zhang, J.H., Chung, T.D.Y., and Oldenburg, K.R. (1999). A simple statistical parameter for use in evaluation and validation of high-throughput screening assays. *J Biomol Screen* 4(2): 67-73.

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