

# CHOOSING THE BEST KINASE ASSAY TO MEET YOUR RESEARCH NEEDS

BY MICHAEL CURTIN, B.S., PROMEGA CORPORATION

Finding the best tool for measuring kinase activity can be a challenging task and will depend on your sample type, available instrumentation, and desired throughput. Promega offers a variety of kinase assay systems using several detection methods (fluorescence, luminescence or radioactivity). These assays include add-mix-measure formats suitable for high-throughput and assays that can be used with virtually any kinase substrate combination. Here we provide a guide for choosing the best assay for your research needs.

## Introduction

Protein kinases are enzymes capable of transferring the  $\gamma$ -phosphate group from ATP to a serine, threonine or tyrosine residue in specific substrate proteins. These phosphorylation events modulate the activity of a vast number of proteins, including ion channels, transcription factors, phosphatases and other kinases. Based on sequence analysis, the human genome encodes approximately 518 protein kinases, and approximately one-third of the proteins in a typical mammalian cell are phosphorylated (1). Protein kinases function primarily as components of signaling pathways in which signals perceived at the surface of a cell are transduced through the cell by a series of phosphorylation events that ultimately bring about a cellular response, such as a change in the rate of gene transcription or the modulation of ion channel activity. Protein kinases play critical roles in a variety of cellular functions including cell growth, development, differentiation, membrane transport, and cell death (2,3). Abnormalities in signaling pathways can lead to various pathological conditions including many forms of cancer. For this reason, protein kinases are important targets for both basic research and drug development.

Numerous assay technologies are available; some are best suited for use with purified protein kinases, while others allow the researcher to measure the activity of a particular kinase in a crude tissue or cell extract. Promega offers a variety of assay systems to measure protein kinase activity. Choosing the right assay for your needs depends largely on the source of the kinase activity and the desired throughput. Sources of protein kinase activity include complex mixtures

of protein typically found in cells and cell extracts to highly purified recombinant protein kinases expressed for the purposes of high-throughput screening (HTS) applications.

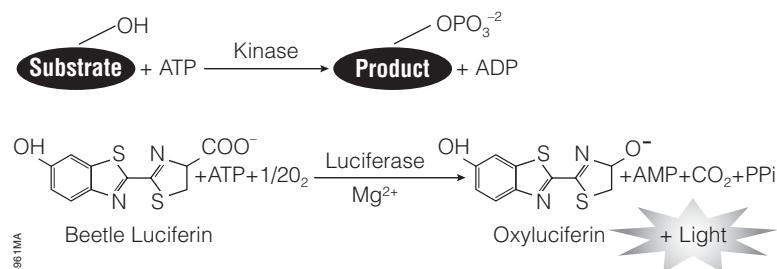
## Measuring Kinase Activity From a Purified Source

Drug development begins with the identification of molecular targets that play a central role in a particular disease state. Protein kinases are attractive targets because of their prominent roles in many pathological states. Screening purified kinases is an integral part of drug discovery. Development of small molecule inhibitors of kinases as new therapeutics has proven successful with the FDA approval of Gleevec® (STI-571) protein tyrosine kinase inhibitor for treating chronic myelogenous leukemia and Iressa® (ZD 1839) and Tarceva® (erlotinib) EGFR tyrosine kinase inhibitors for treating lung cancer. Many pharmaceutical companies continue to search for kinase inhibitors that might prove useful for developing novel therapeutics (4).

When performing HTS of purified kinase targets, researchers look for assays that are fast, relatively easy to use, and produce reliable results. Promega offers several types of assay systems for measuring purified kinase activity that rely on different methods of detection. Systems include radiolabeled assays, such as our SAM<sup>2</sup>® membrane-based assays, fluorescent assays (ProFluor® Kinase Assays) as well as luminescent assays (Kinase-Glo® Assays).

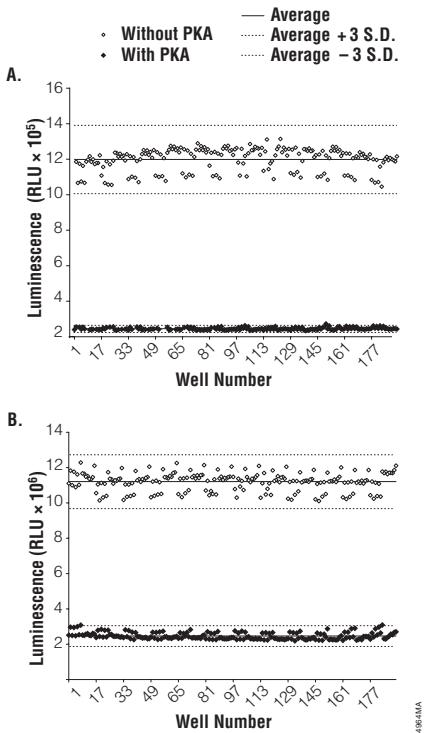
## Luminescent-Based Assays

The luminescent Kinase-Glo® Assays have gained widespread acceptance among HTS users due to their "add and



**Figure 1. The Kinase-Glo® Assay reaction.** The kinase reaction is conducted under the appropriate conditions. ATP remaining at the time that Kinase-Glo® Reagent is added is used as a substrate by the Ultra-Glo™ Luciferase to catalyze the mono-oxygenation of luciferin. The luciferase reaction produces one photon of light per turnover. Luminescence is inversely related to kinase activity.

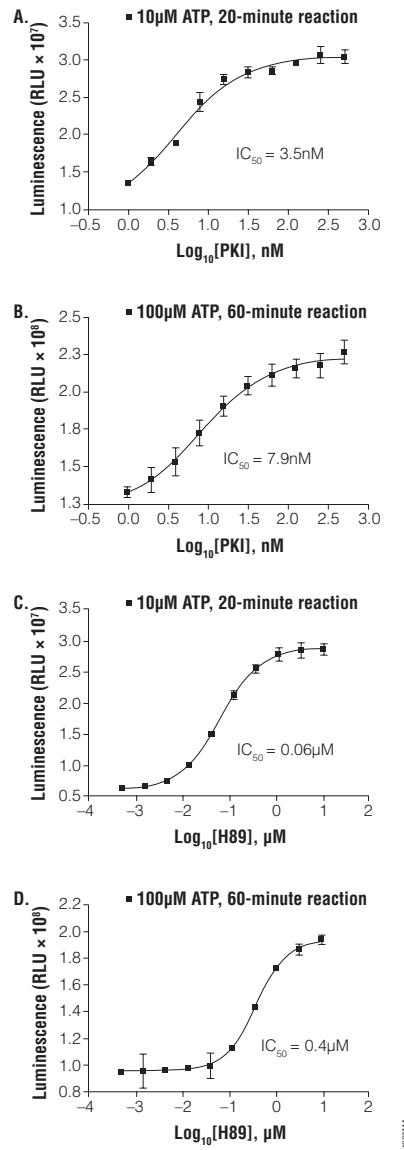
# Choosing a Kinase Assay



**Figure 2. Determining Z'-factor using Kinase-Glo® Plus Assay in a 384-well plate. Panel A.** The assay was performed as described in Technical Bulletin #TB343, with 0.2 units/well PKA and 10 μM ATP for 5 minutes at room temperature (solid symbols) or without PKA (open symbols). **Panel B.** The assay was performed using 0.2 units/well PKA and 100 μM ATP for 30 minutes at room temperature (solid symbols) or without PKA (open symbols). Final volumes of the kinase reactions for the 384-well plate assays were 20 μl. Solid lines indicate the mean, and the dotted lines indicate  $\pm 3$  S.D. Z'-factor values were ~0.8 for 10 μM ATP and 100 μM ATP.

read" format, low false-positive hit rates and the ability to assay a wide variety of kinase-substrate combinations. These luminescent assays are homogeneous HTS methods for measuring kinase activity by quantifying the amount of ATP remaining in solution following a kinase reaction (Figure 1). The assay is performed in a single well of a 96- or 384-well plate by adding a volume of Kinase-Glo® Reagent equal to the volume of solution in the well of a completed kinase reaction and measuring luminescence. Kinase-Glo® and Kinase-Glo® Plus Assays use a recombinant luciferase (Ultra-Glo™ Luciferase) to monitor the change in ATP levels. The luminescent signal is correlated with the amount of ATP present and inversely correlated with the amount of kinase activity. The Kinase-Glo® Assays can also be used to measure activity of kinases with substrates that are prephosphorylated, such as glycogen synthase 3 kinase, or kinases that phosphorylate their substrates on multiple sites, such as IKKs.

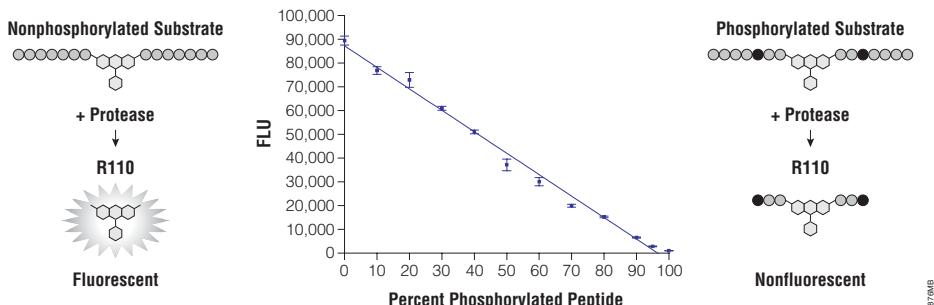
Z'-factor is a statistical value that compares the dynamic range of an assay to data variation in order to assess assay quality for high-throughput applications (5). A Z'-factor value



**Figure 3. Determining the IC<sub>50</sub> for ATP-competitive and noncompetitive inhibitors.** PKA inhibitor (noncompetitive, PKI, and competitive, H89) titrations were performed in solid white, flat-bottom 96-well plates in a total volume of 50 μl as described in Technical Bulletin #TB343 using 0.5 unit/well PKA and the indicated amount of inhibitor. Reactions were carried out at room temperature in 10 μM ATP and 500 μM peptide substrate for 20 minutes or in 100 μM ATP and 500 μM peptide substrate for 60 minutes. Data points are the average of two determinations, and error bars are  $\pm$ S.D. IC<sub>50</sub> results determined using the Kinase-Glo® Plus Assay are 3.5 nM and 7.9 nM for PKI at 10 and 100 μM ATP, respectively. These compare favorably to the IC<sub>50</sub> values reported for these compounds in the literature (7,13). Curve fitting was performed using GraphPad Prism® sigmoidal dose response (variable slope) software.

equal to 1.0 indicates a perfect assay. Assays that produce Z'-factors greater than 0.5 are considered excellent assays. The Kinase-Glo® Assays consistently produce Z'-factor values of greater than 0.7 in both 96- and 384-well formats (Figure 2).

# Choosing a Kinase Assay



**Figure 4. ProFluor® Kinase Assay schematic and graph demonstrating that the presence of a phosphorylated amino acid (black circles) blocks the removal of amino acids by the protease.** The graph shows the average FLU ( $n = 6$ ) obtained after a 30-minute Protease Reagent digestion using mixtures of nonphosphorylated PKA R110 Substrate and phosphorylated PKA R110 Substrate. The total peptide concentration was 5 $\mu$ M in 50 $\mu$ l of Reaction Buffer A to which 25 $\mu$ l of Protease Reagent diluted in Termination Buffer A was added. (FLU = Fluorescence Light Unit, excitation wavelength 485nm, emission wavelength 530nm;  $r^2 = 0.992$ ) As the concentration of the phosphopeptide increases in the reaction, FLU decrease.

**Table 1. PKA Activity As Measured Using the SAM<sup>2®</sup> 96 Biotin Capture Plate.**

Sample	Substrate	PKA	Substrate + PKA
A	12.0	40.2	26,435.0
B	30.1	36.2	24,518.0
C	24.1	22.1	22,017.8
D	28.1	18.1	23,666.7
E	20.4	10.2	25,403.9
F	22.4	32.6	24,695.9
G	18.3	42.8	24,051.2
H	16.3	40.7	26,886.9
Avg.	21.5	30.4	24,709.9
S.D.	6.02	12.09	1,559.11
% CV	28.06	39.81	6.31

The enzyme reaction was performed with substrate only (Substrate), enzyme only (PKA) or substrate plus enzyme. Reactions were terminated as described (10,12), and sample aliquots (5 $\mu$ l) were added to wells of a 96-well biotin capture plate. The wells were washed using a vacuum manifold (4 washes of 2M NaCl, 6 washes of 2M NaCl/1% HPO<sub>4</sub>, 4 washes of water). The plates were dried and counted using a MicroBeta® TriLux liquid scintillation counter (EG&G Wallac, Inc.). Letters A–H represent replicate samples placed in random wells to examine well-to-well variations. Results are expressed in counts per minute (cpm). Avg. = average; S.D. = standard.

Kinase-Glo® Plus differs from the original formulation in that it is linear up to 100 $\mu$ M ATP, while the original is linear to 10 $\mu$ M ATP. Using higher concentrations of ATP in a kinase reaction allows screeners to more easily select for non-ATP binding site inhibitors. To demonstrate the capability of the assay in distinguishing between ATP-competitive and noncompetitive inhibitors, we selected two well known inhibitors of PKA (Figure 3). The compound H89 is reported in the literature as a potent ATP-competitive inhibitor of PKA. Protein kinase

inhibitor peptide (PKI) is an ATP-noncompetitive PKA inhibitor. Titration of PKI in a 96-well plate using 0.5 units of PKA at two different ATP concentrations shows only a twofold change in IC<sub>50</sub>. These values correspond to IC<sub>50</sub> values for PKI (3–5nM) reported in the literature (6). However, titration of H89 using similar assay conditions shows approximately sixfold change in IC<sub>50</sub>. The lower value corresponds to the IC<sub>50</sub> (0.048 $\mu$ M) reported for H89 for PKA at 10 $\mu$ M ATP (7). These data support the notion that this assay can discriminate between ATP-competitive and noncompetitive inhibitors of protein kinases.

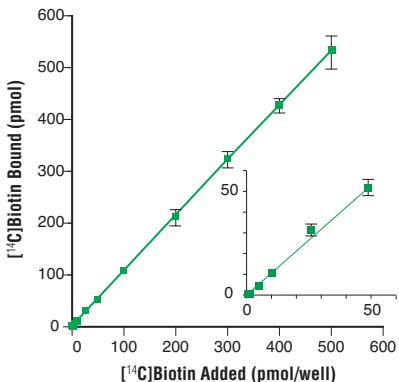
## Fluorescent-Based Assays

The ProFluor® Kinase Assays are fluorescence intensity-based assays that measure kinase activity using purified kinase in a multiwell plate format and involve “add and read” steps only. The user performs a standard kinase reaction with the provided bisamide rhodamine 110 peptide substrate specific for the kinase of interest. The conjugated substrate is nonfluorescent (Figure 4, reference 8). After the kinase reaction is complete, the user adds a termination buffer containing a protease reagent. This simultaneously stops the reaction and removes amino acids specifically from the nonphosphorylated substrate, producing highly fluorescent rhodamine 110. Phosphorylated substrate is resistant to protease digestion and remains non-fluorescent. Thus, fluorescence is inversely correlated with kinase activity. The assays consistently yield excellent Z'-factor values, produce IC<sub>50</sub> data comparable to published data, and allow the flexibility of batch-mode processing.

## Radiolabeled Assays

The SAM<sup>2®</sup> Biotin Capture Membrane can also be adapted to high-throughput applications. Promega offers the SAM<sup>2®</sup> Biotin Capture membrane in formats conducive to high-throughput applications. These include a single-sheet format (7.6 × 10.9cm) and the SAM<sup>2®</sup> 96 Biotin Capture Plate (96 wells).

# Choosing a Kinase Assay

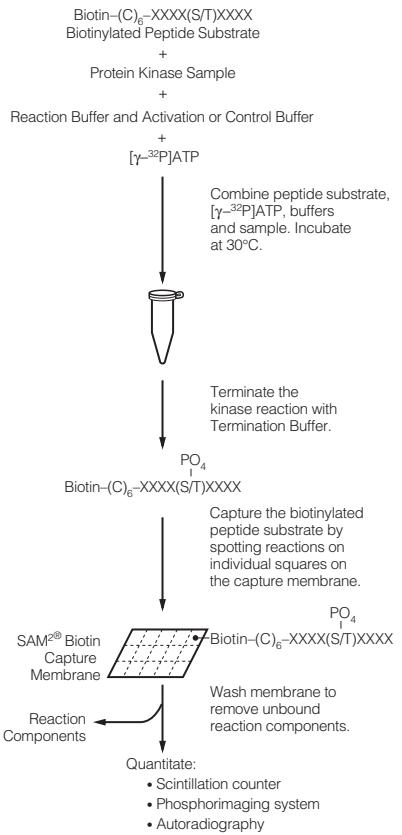


**Figure 5. Linearity of binding of  $[^{14}\text{C}]$ biotin to the SAM2® 96 Biotin Capture Plate.** Samples (5 $\mu\text{l}$ ) of various concentrations of  $[^{14}\text{C}]$ biotin were added to individual wells of a SAM2® 96 Biotin Capture Plate. The plate was washed, dried and counted as described in Table 1. The inset is an enlargement of the 0–50 pmol portion of the graph.

To illustrate the performance of the SAM2® 96 Biotin Capture Plate in a high-throughput situation, we performed a protein kinase A (PKA) assay (Table 1), measuring PKA activity in the presence of enzyme only, in the presence of substrate only and in the presence of both (9). The level of radioactivity determined in the absence of the enzyme ("Substrate") or in the absence of the substrate ("PKA") represents <0.02% of input counts. The range of background counts was extremely low, and the assay was carried out with maximum efficiency; the full washing procedure took only five minutes. Also, the coefficient of variation for enzyme activity did not exceed 8%, indicating highly reproducible results and consistency in the assay performance. When tested with biotin and biotinylated peptides, the binding capacity of SAM2® Plates was linear between 5 and 500 pmol/well, and binding was stoichiometric, as demonstrated in Figure 5 (10). This feature is critical for enzymes such as PTK with substrates that have high  $K_m$  values.

## Measuring Protein Kinase Activity from Complex Mixtures of Protein

The most commonly used assay to quantitate protein kinase activity from crude cell extracts using peptide substrates is the P81 phosphocellulose filter assay (11). This method relies on the capture of peptide substrate by phosphocellulose via electrostatic interactions between the positively charged substrate and the negatively charged P81 filter. P81 phosphocellulose has a number of distinct drawbacks. First, the positively charged, radiolabeled substrate is bound to the P81 filter by weak electrostatic forces, and labeled substrate can be lost during washing. Less stringent washing conditions reduce the amount of peptide lost; however, higher background counts often result, leading to poor signal-to-noise ratios and reduced sensitivity. Secondly, most enzyme preparations contain numerous kinases that will phosphorylate endogenous, positively charged proteins, which are likely to bind to the P81 fil-



**Figure 6. Overview of the SignaTECT® Assay Protocol.**

ter. Additionally, [ $\gamma$ -<sup>32</sup>P]ATP preparations contain radiolabeled contaminants that possess a positive charge at low pH. The binding of these compounds results in higher backgrounds and lower signal-to-noise ratios. Finally, peptide substrates of equal positive charge often exhibit wide variability in binding to phosphocellulose filters. Peptide substrates that do not contain at least two positively charged amino acids, such as arginine or lysine, will not efficiently bind to P81 filters. The addition of these amino acids may alter the specificity of the substrates, making them substrates for other kinases.

The SignaTECT® Protein Kinase Assay Systems overcome the drawbacks of P81 phosphocellulose by using biotinylated peptide substrates in conjunction with the SAM2® Biotin Capture Membrane. This streptavidin-coated membrane is made using a proprietary process that results in a high density of streptavidin (5). The binding of biotin to streptavidin is rapid and very strong ( $K_d = 10^{-15}\text{M}$ ), and the association is unaffected by rigorous washing procedures, denaturing agents, extremes in pH, temperature and salt concentrations. High signal-to-noise ratios are generated even with crude extracts, while the high substrate capacity allows for optimum reaction kinetics. The systems can be used to measure protein kinase activities using low femtomole levels of purified enzyme or crude tissue/cell extracts. As outlined in Figure 6, the assay steps and analysis

# Choosing a Kinase Assay

of results for the SignaTECT® Assay Systems are straightforward and require only common laboratory equipment. Following phosphorylation and binding of the biotinylated substrate to the SAM<sup>2</sup>® Biotin Capture Membrane, unincorporated [ $\gamma$ -<sup>32</sup>P]ATP is removed by a simple stringent wash procedure. This procedure also removes nonbiotinylated proteins that have been phosphorylated by other kinases in the sample. The bound, labeled substrate is quantitated by scintillation counting, phosphorimaging analysis or using autoradiography in combination with a densitometer.

## Summary

The study of kinases and their role in cellular regulation continues to expand as the human genome is sequenced and new kinases are identified as expression products of newly discovered genes. Reagents and assay systems that allow sensitive, accurate and high-throughput analysis of both purified kinases as well as crude extracts will enhance the characterization of these important cellular components and will speed the identification of appropriate therapeutic targets and the development of new and more effective treatments.

Promega offers a wide array of kinase assays to help in this endeavor and we are confident that we have an assay for your particular needs. ■

## References

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## Protocols

*Kinase-Glo® Plus Luminescent Kinase Assay Technical Bulletin #TB343*  
([www.promega.com/tbs/tb343/tb343.html](http://www.promega.com/tbs/tb343/tb343.html))

*Kinase-Glo® Luminescent Kinase Assay Technical Bulletin #TB318* ([www.promega.com/tbs/tb318/tb318.html](http://www.promega.com/tbs/tb318/tb318.html))

*ProFluor® PKA Assay Technical Bulletin #TB315*  
([www.promega.com/tbs/tb315/tb315.html](http://www.promega.com/tbs/tb315/tb315.html))

*ProFluor® Src-Family Kinase Assay Technical Bulletin #TB331* ([www.promega.com/tbs/tb331/tb331.html](http://www.promega.com/tbs/tb331/tb331.html))

*SignaTECT® PKA Assay System Technical Bulletin #TB241*  
([www.promega.com/tbs/tb241/tb241.html](http://www.promega.com/tbs/tb241/tb241.html))

*SignaTECT® PKC Assay System Technical Bulletin #TB242*  
([www.promega.com/tbs/tb242/tb242.html](http://www.promega.com/tbs/tb242/tb242.html))

*SignaTECT® PTK Assay System Technical Bulletin #TB211*  
([www.promega.com/tbs/tb211/tb211.html](http://www.promega.com/tbs/tb211/tb211.html))

*SignaTECT® CaM KII Assay System Technical Bulletin #TB279* ([www.promega.com/tbs/tb279/tb279.html](http://www.promega.com/tbs/tb279/tb279.html))

*SignaTECT® DNA-PK Assay System Technical Bulletin*

#TB250  
([www.promega.com/tbs/tb250/tb250.html](http://www.promega.com/tbs/tb250/tb250.html))

*SignaTECT® cdc2 PK Assay System Technical Bulletin*

#TB227  
([www.promega.com/tbs/tb227/tb227.html](http://www.promega.com/tbs/tb227/tb227.html))

*SAM<sup>2</sup>® Biotin Capture Membrane Technical Bulletin*

#TB547  
([www.promega.com/tbs/tb547/tb547.html](http://www.promega.com/tbs/tb547/tb547.html))

*SAM<sup>2</sup>® 96 Biotin Capture Plate Technical Bulletin #TB249*

([www.promega.com/tbs/tb249/tb249.html](http://www.promega.com/tbs/tb249/tb249.html))

## Ordering Information

Product	Size	Cat.#
Kinase-Glo® Plus Luminescent Kinase Assay	100ml*	V3773
Kinase-Glo® Luminescent Kinase Assay	100ml*	V6713
ProFluor® PKA Assay <sup>1</sup>	8 plate*	V1241
ProFluor® Src-Family Kinase Assay <sup>1</sup>	8 plate*	V1271
SignaTECT® PKA Assay System	96 reactions	V7480
SignaTECT® Protein Kinase C Assay System	96 reactions	V7470
SignaTECT® Protein Tyrosine Kinase Assay System	96 reactions	V6480
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase Assay System	96 reactions	V8161
SignaTECT® DNA-Dependent Protein Kinase Assay System	96 reactions	V7870
SignaTECT® cdc 2 Protein Kinase Assay System	96 reactions	V6430
SAM <sup>2</sup> ® Biotin Capture Membrane <sup>2</sup>	96 samples*	V2861
SAM <sup>2</sup> ® 96 Biotin Capture Plate <sup>2</sup>	5 × 96-well plates*	V7542

\*Available in additional sizes.

<sup>1</sup>Inquire about customization of these assay systems.

<sup>2</sup>For Laboratory Use.

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