Developing an Antibody-free Kinase Assay for Protein Substrates using AlphaScreen Technology

Introduction

Protein kinases are directly implicated in many human diseases including inflammatory diseases and cancer; thus, kinase inhibitors show great promise as new therapeutic drugs. Herceptin and Iressa are representative examples of kinase inhibitors currently used to fight breast or non-small cell lung cancers[1,2].

Kinases can be divided into two main families based on the amino acid they phosphorylate: tyrosine or serine/threonine. As a tool to measure kinase activity, various homogeneous and non-radioactive technologies take advantage of generic antibodies directed against phosphotyrosine residues (ex. PY20, PT66, p-Tyr100). However, comparable generic antibodies are not available for the detection of phosphoserine and phosphothreonine residues. Additionally, recognition of phosphoserine/threonine residues is known to be highly sequence specific and assays to measure the activity of serine/threonine kinases often work best when complete phosphorylation domains are used as substrates instead of short peptides. This is a challenge for HTS technologies such as TR-FRET, where assay partners must lie in the same plane within 7 nm of one another, or fluorescence polarization, where kinase peptide substrates are limited to approximately 20 amino acids.

To address these two limitations, PerkinElmer, Inc. has recently developed the AlphaScreen™

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PhosphoSensor Kit. This kit is composed of Streptavidin-coated Donor beads and the newly developed PhosphoSensor-coated Acceptor beads. The latter are covered with a Lewis metal (LM^{3+}) that gives them the ability to detect phosphate groups irrespective of the sequence of the phosphorylated residues (see Figure 1b). Additionally, as with all AlphaScreen assays, because it allows assay partners to be separated by as much as 200 nm, this new assay is well suited for either large peptide or complete protein substrates.

Using the phosphorylation by JNK3 of a GST fusion of the c-Jun domain, residue 1-79, two different assays were developed to highlight the potential of AlphaScreen technology to detect serine/threonine phosphorylation of large proteins:

1) A conventional AlphaScreen assay using an anti-phospho sequence-specific antibody bound to AlphaScreen Protein A-coated Acceptor beads (Figure 1a).
2) An antibody-free assay using the AlphaScreen PhosphoSensor Kit (Figure 1b).

This application note compares the results obtained by each format and provides a representative example of the development of a serine/threonine kinase assay using the new PhosphoSensor Kit.

Materials and methods

Table 1 lists the reagents and consumables used in this work, together with their suppliers.

GST-cJun biotinylation

500 µl of protein (1 mg/ml) were mixed with 500 µl of 0.1 M MES buffer pH 6.5, 2.25 µl of a 10 mg/ml

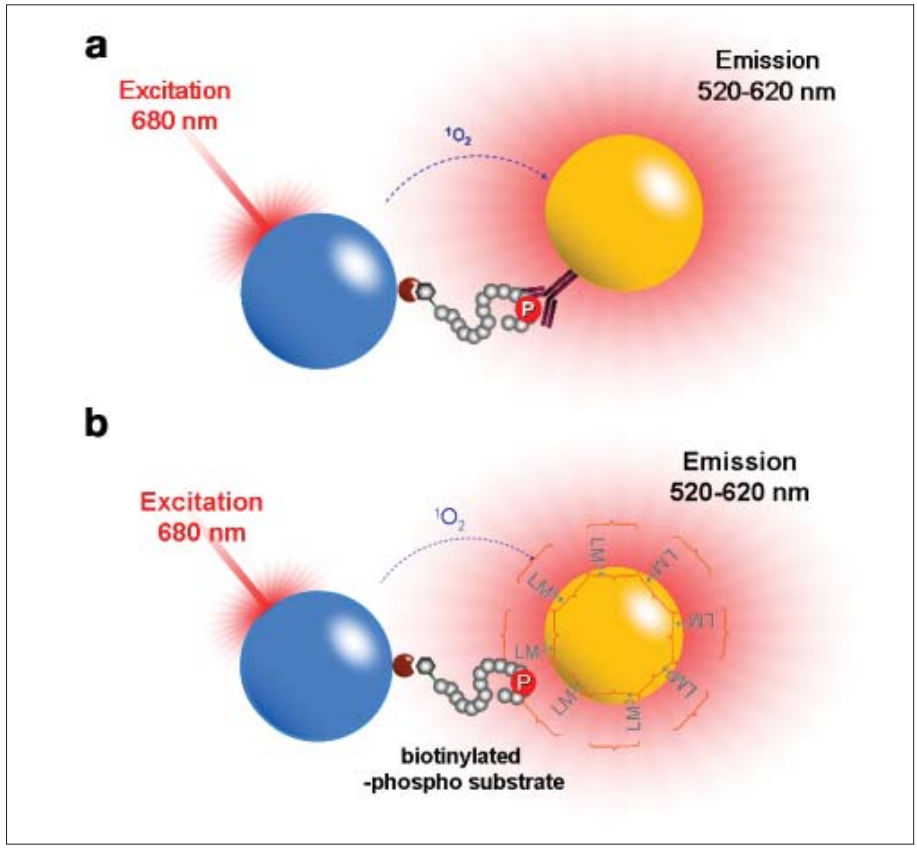

Figure 1. ***Principles of AlphaScreen detection of kinase activity.*** Following the phosphorylation of a peptide or protein substrate, biotinylated phospho-product can be captured simultaneously by Streptavidin-coated Donor beads and anti-phospho antibody-coated Acceptor beads (antibody-based detection) (a) or by PhosphoSensor-coated Acceptor beads (antibody-free detection) (b). When irradiated with a laser at 680 nm, beads brought into proximity will generate a signal between 520-620 nm which can be read on an EnVision AlphaScreen reader.

Table 1. Reagents and Consumables.

Item	Supplier	Cat. Number
AlphaScreen IgG (Protein A) Detection Kit	PerkinElmer	6760617
AlphaScreen PhosphoSensor Kit	PerkinElmer	6760307
AlphaScreen TruHits™ Kit	PerkinElmer	6760627
OptiPlates™ 384	PerkinElmer	6007390
TopSeal-A	PerkinElmer	6005250
GST-cJun (1-79) Fusion Protein	Stratagene	205145
Recombinant JNK3/SAPK1b	UpState	14-501
Recombinant Anti-phospho cJun (Ser73) Antibody	UpState	06-659
Staurosporine	Sigma-Aldrich	S4400
SP600125	Calbiochem	420119
JNK III Inhibitor	Calbiochem	420130
EX-Link™ Biotin-BMCC	Pierce Biotechnology	21900
D-Salt Polyacrylamide Desalting Columns	Pierce Biotechnology	43240

solution of Biotin-BMCC added, and the reaction incubated for two hours at 37 °C. The resultant biotinylated GST-cJun was desalted on a PD-10 column equilibrated in PBS with 0.01% Tween®-20. Biotinylated GST-cJun was titrated using the AlphaScreen TruHits assay to determine the concentration of biotinylated protein detectable by the Streptavidin-coated Donor beads.

Kinase assays

As discussed later in the results and discussion section, prior to performing the comparison between kinase assays with and without the anti-phospho cJun antibody, optimum amounts of the kinase reagents (Biotinylated-GST-cJun, JNK3 and ATP) as well as $MgCl_2$ and EDTA were determined by titration. For the final antibody-based kinase assay, 5 nM biotin-GST-cJun, 30 nM anti(phospho) cJun antibody, 3 nM active JNK3 and 10 µM of ATP were added to kinase assay buffer (20 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 100 µM Na_3VO_4, 0.01% Tween-20) for a final volume of 20 µl. Reactions were then incubated for 2 hours at room temperature (RT). Detection was performed by the addition of 5 µl of mixed beads (Protein A-coated Acceptor 20 µg/ml and Streptavidin-coated Donor 20 µg/ml) diluted in detection buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 80 mM EDTA, 0.3% BSA), followed by a one-hour incubation at RT in the dark.

For the final antibody-free kinase assay, 100 nM biotin-GST-cJun, 10 nM of active JNK3 and 10 mM of ATP were first added to kinase assay buffer (25 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 1 mM DTT, 0.01% Tween-20) for a final volume of 15 ml. Reactions were then incubated for 2 hours at RT. The reaction was stopped by the addition of 2.5 mM of EDTA in 5 ml of detection buffer (10 mM Tris-HCl pH 7.0, 100 mM NaCl, 0.1% Tween-20), for a period of time of 30 minutes at RT. Detection was then performed by the addition of 5 ml of mixed beads (PhosphoSensor Acceptor 20 mg/ml and Streptavidin-coated Donor 20 mg/ml) diluted in detection buffer, followed by a one-hour incubation at RT in the dark. Detection was performed by the addition of 5 µl of mixed beads (Protein A-coated Acceptor 20 µg/ml and Streptavidin-coated Donor 20 µg/ml, final concentrations in 25 µl detection reaction volume) diluted in detection buffer (20 mM Tris-HCl pH 7.4, 200 mM

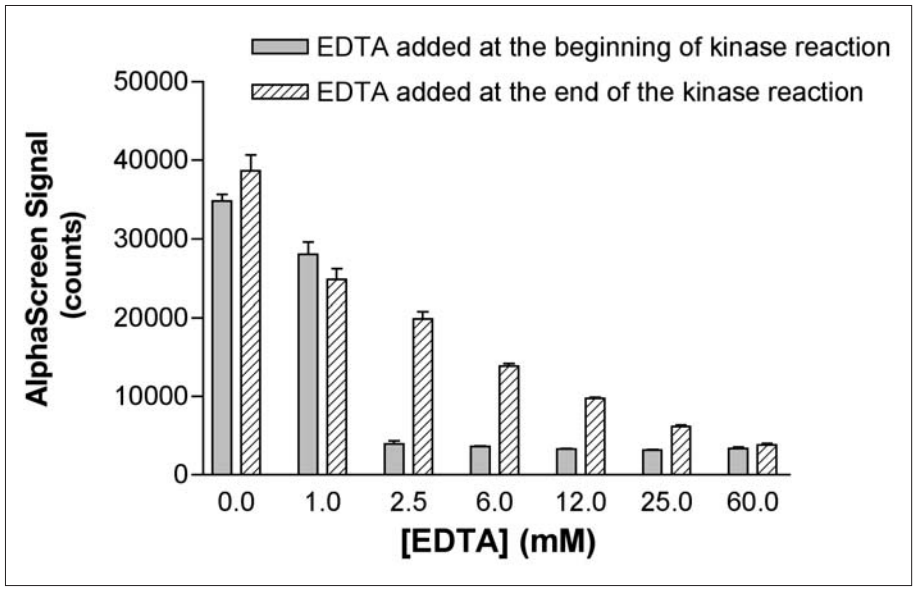

Figure 2. ***EDTA titration using the Antibody-free assay configuration.*** This experiment was performed using 10 nM of JNK3, 100 nM of biotin-GST-cJun and 10 µM of ATP in the presence of 1 mM of $MgCl_2$.

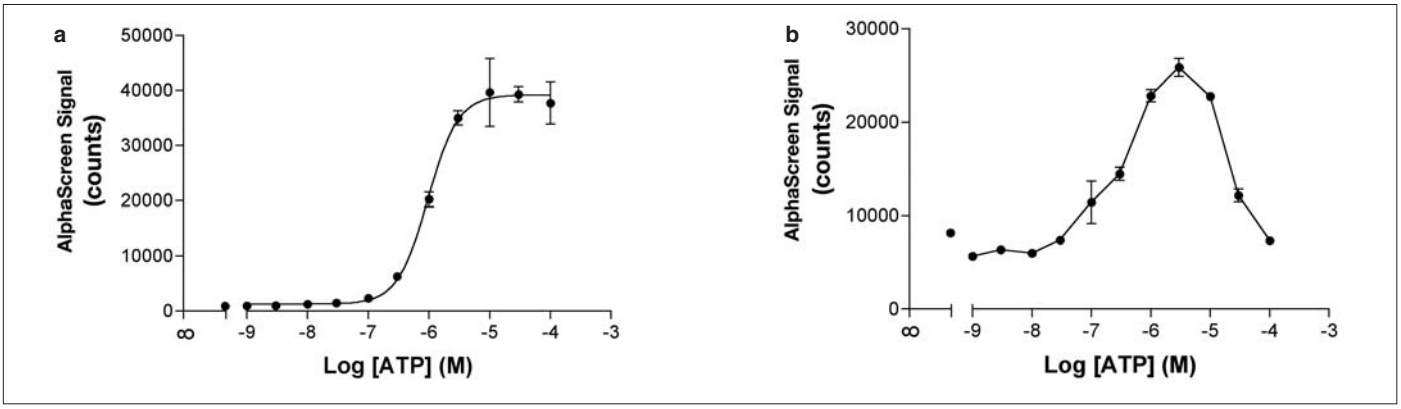

Figure 3. ***ATP titration using the Antibody-based Assay (a) and the Antibody-free Assay (b).*** 5 nM of biotin-GST-cJun and 3 nM of active JNK3 were used for the antibody-based assay as well as 30 nM of anti- (phospho)cJun. For the antibody-free assay, 100 nM of biotin-GST-cJun and 10 nM of active JNK3 enzyme were used.

NaCl, 80 mM EDTA, 0.3% BSA), followed by a one-hour incubation at RT in the dark.

For both assays, plates were read on an EnVision AlphaScreen reader (PerkinElmer cat. no. 2102-0010) and the data was analyzed using GraphPad Prism™ software.

Results and discussion

Determination of $MgCl_2$ and EDTA concentrations

Phosphorylation by kinases requires ATP to be complexed with Mg^{2+} ions to catalyze the release of energy contained in ATP. In order to stop phosphorylation prior to detection, AlphaScreen assays employ EDTA to chelate Mg^{2+} ions. For the antibody-based detection, EDTA has no effect on the detection by Protein A-coated Acceptor beads. Thus, for this assay, we used an excess of EDTA (80 mM) in the detection buffer. However, EDTA at high concentration can interfere with the PhosphoSensor Acceptor beads because of the very same chelating properties. To limit the amount of EDTA required, we limited $MgCl_2$ to 1 mM as this amount was still able to generate 80% of maximum signal (data not shown). As shown in Figure 2, the optimal concentration of EDTA was found to be about 2.5 mM – a concentration high enough to fully terminate the reaction if added at the beginning of the assay but low enough to still yield a signal 4.5 times that of background when added in the termination step.

Determination of ATP, enzyme and substrate concentrations

Based on previous AlphaScreen assay experience[3], fixed concentrations of biotin-GST-cJun, enzyme, and anti-(phospho) cJun (antibody-based detection only) were used to determine the optimal concentration of ATP in both assay formats. As shown in Figure 3, the AlphaScreen signal increased in an ATP dose-dependent manner, reaching a maximum at 10 µM and 3 µM of ATP for the antibody-based and antibody-free detection formats, respectively. Unlike the antibody-based format, the antibody-free assay showed higher sensitivity to ATP due to competition between ATP and phospho-peptide for binding to the PhosphoSensor beads

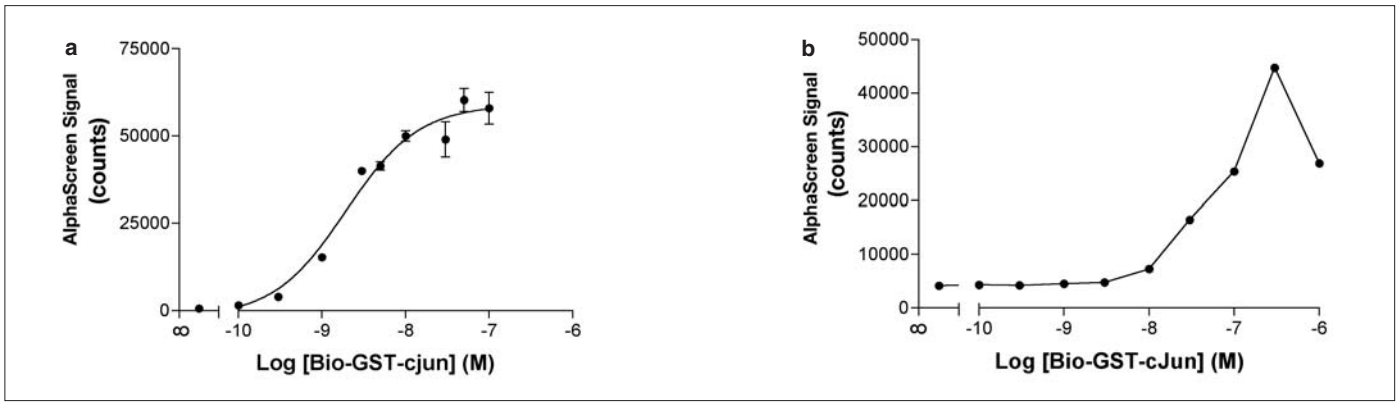

Figure 4. ***Substrate titration using the Antibody-based configuration (a) and the Antibody-free assay (b).*** Both assays were performed using 10 µM of ATP and 3 nM or 10 nM of active enzyme for the antibody-based and antibody-free assay format, respectively.

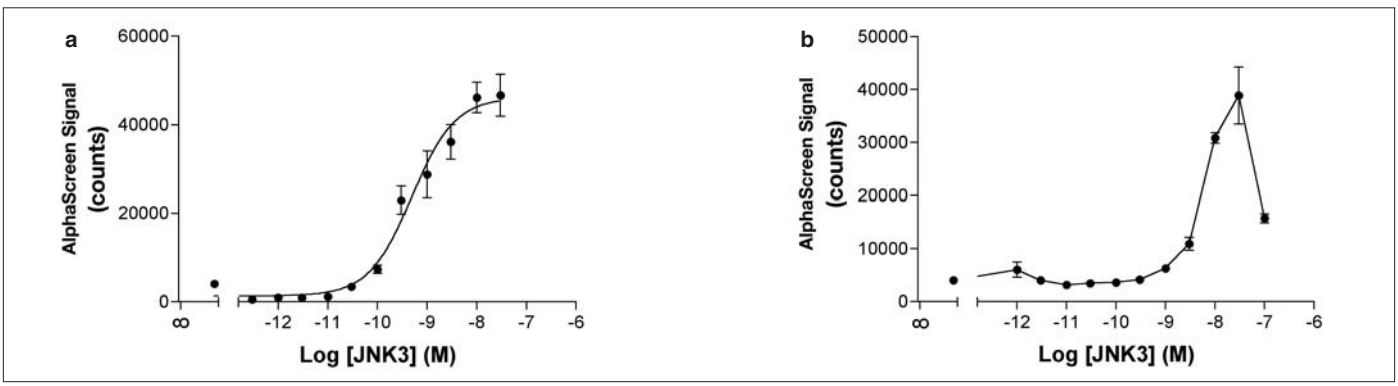

Figure 5. ***Enzyme titration using the Antibody-based assay (a) and the Antibody-free approach (b).*** The experiments were performed using 10 µM of ATP and 5 nM of biotinylated substrate for the antibody-based assay and 100 nM of biotinylated substrate for the antibody-free assay.

(see Figure 3b). In some cases, this phenomenon can be alleviated by increasing the concentration of PhosphoSensor Acceptor beads or by performing a dilution step before the addition of the beads.

The optimal concentration of substrate was evaluated using 10 µM of ATP for both assay configurations. As shown in Figure 4, generation of maximum signal using the PhosphoSensor beads required 10 times more substrate than with the antibody-based beads (30 nM with antibody versus 300 nM without antibody) due to the higher affinity of the antibody-based capture beads.

We then performed an enzyme titration curve for JNK3. We chose 100 nM instead of 300 nM of substrate concentration to perform the antibody-free detection to lower the cost of the assay though 300 nM did generate a higher signal window. As shown in Figure 5, the AlphaScreen signal increased with enzyme concentration, reaching a maximum signal at 10 nM of enzyme for the antibody-based assay and 30 nM in the antibody-free assay. With the PhosphoSensor Acceptor beads, a signal decrease is observed at high enzyme concentrations that may reflect a single-ended saturation by an excess of phosphorylated product preventing pairing of Acceptor and Donor beads. Or, since JNK3 is phosphorylated in its active form, it is also possible that a high concentration of enzyme can compete with the binding of the phosphorylated substrate. Based on these results, 3 nM and 10 nM of JNK3 were used in subsequent experiments for the antibody-based and antibody-free platforms, respectively.

Comparison of the potency of JNK3 inhibitors using AlphaScreen antibody-based versus antibody-free detection formats

Using the optimized assay parameters described above, we evaluated the potency of the inhibitors staurosporine, SP600125, and JNK III Inhibitor. Staurosporine is a generic kinase inhibitor while SP600125 is a reversible ATP-competitive inhibitor[4,5]. The JNK III inhibitor is known to disrupt specifically the c-Jun/JNK complex[6]. As expected and shown in Figure 6, increasing enzymatic inhibition produced decreasing AlphaScreen signal. The rank order of potency for Staurosporine, SP600125, and the JNK III Inhibitor are in agreement in both assay configurations. However, the difference between the assays in the absolute IC_{50} for each inhibitor is due to the difference in substrate and enzyme concentrations used in each assay. The inhibitors did not interfere with either the antibody-based or the antibody-free AlphaScreen detection (data not shown).

Using the optimized assay conditions, the AlphaScreen antibody-based detection generated a signal window superior to the antibody-free detection. The signal to background was 46 for the antibody-based versus 5.9 for the antibody-free with an average maximum signal of 140,000 and 26,000 counts for antibody-based and antibody-free assays, respectively.

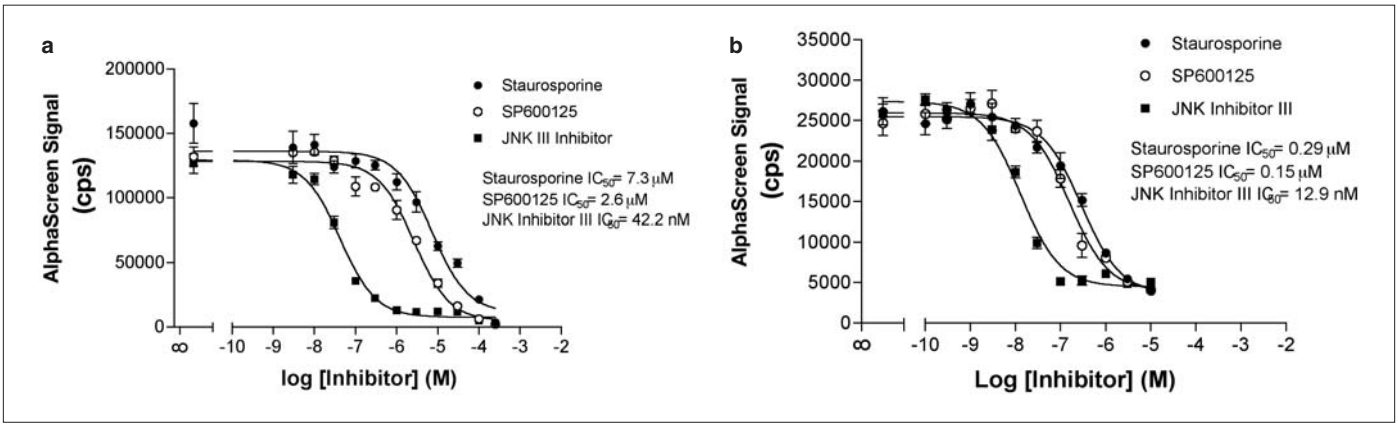

Figure 6. ***Enzyme inhibition using conventional kinase inhibitors using the Antibody-based assay (a) and the Antibody-free PhosphoSensor assay (b).*** 10 µM of ATP, 3 nM of JNK3, 5 nM of biotin-GST-cJun, and 30 nM of anti- (phospho)cJun IgG were used to perform the antibody-based assay. 10 µM of ATP, 10 nM of JNK3, and 100 nM of biotin-GST-cJun were used for the antibody-free experiment.

Conclusion

The majority of the 500 kinases encoded in the human genome represent serine kinases[7]. Despite the lack of generic anti-phospho serine antibodies, the results presented here demonstrate the ability of the AlphaScreen technology to provide sensitive and robust assays that enable the identification and characterization of new serine/threonine kinases. AlphaScreen represents the first non-radioactive homogeneous technology capable of measuring phosphorylation of large protein substrates. Nevertheless, when specific anti-phospho antibodies are available, the antibody-based AlphaScreen assay provides a better signal window. When specific antiphospho antibodies are not available or are deemed too costly, the newer AlphaScreen technology with PhosphoSensor Acceptor beads provides an excellent signal window with comparable inhibitor pharmacological profiles. We thus expect this new AlphaScreen antibody-free kinase assay to provide a major enabling tool for kinase deorphanization projects.

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