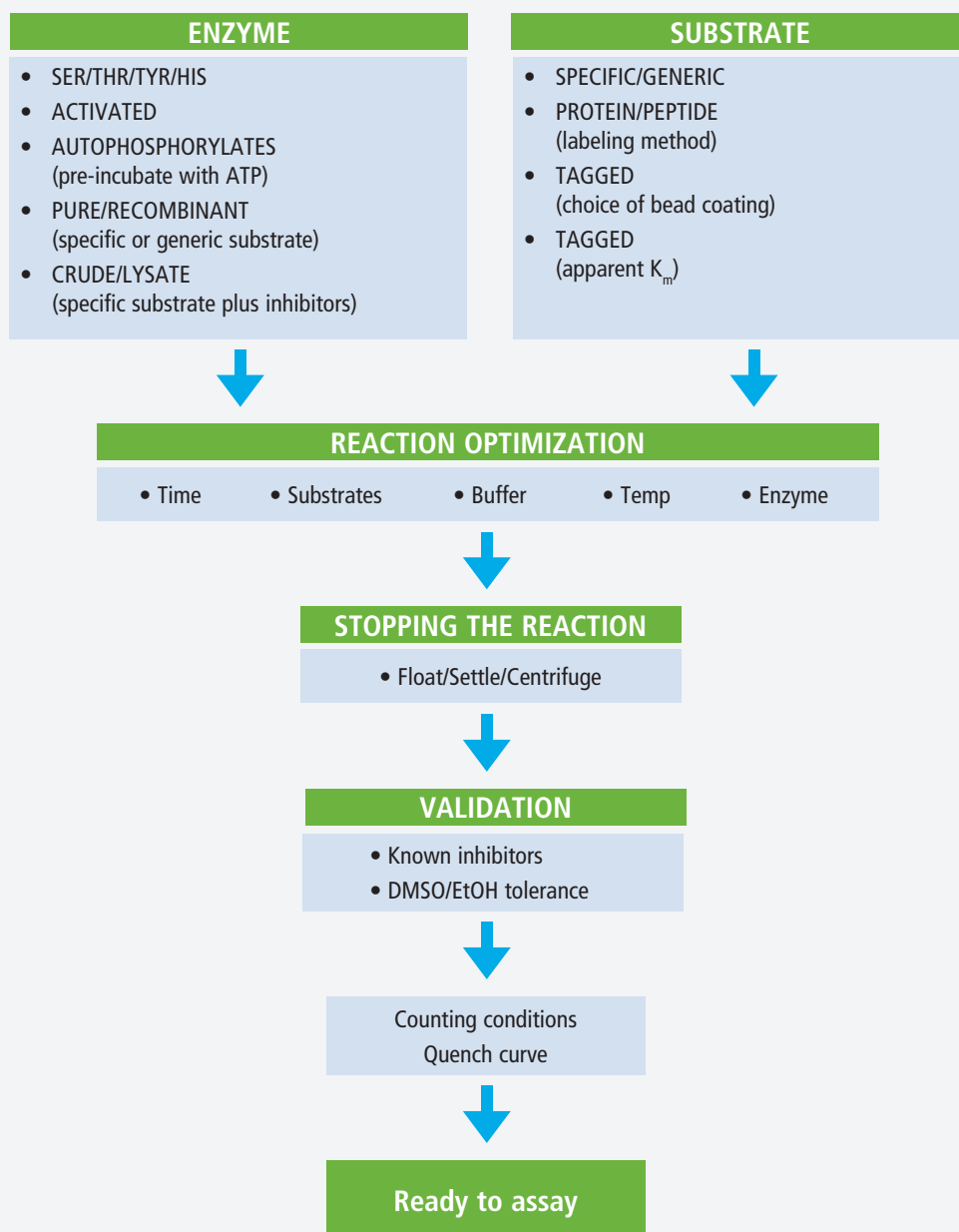


SPA KINASE ASSAY DESIGN



KINASE

Kinases can be obtained as either pure (recombinant) enzymes, or as partially purified material out of crude cellular lysates. The source of the kinase enzyme may affect the choice of substrate. For example, when using a highly purified recombinant kinase, the substrate protein or peptide can be specific or generic, because only one kinase activity is present in the assay mix. When using a crude cellular lysate, there are more than likely multiple kinase activities present in the reaction mix. In this instance, the generic substrate may be phosphorylated by a number of different kinases, whereas a specific substrate will give information only about the kinase of interest.

The kinase enzymes must be available in an activated form, whether they are recombinant or in cellular lysates. Does the reaction buffer include known co-factors?

SPA kinase assays have been developed with a wide range of kinases, including serine/threonine, tyrosine, and histidine. Some kinases are known to autophosphorylate. To avoid this affecting assay performance, pre-incubation in the absence of labeled ATP can eliminate increased background due to radiolabeling of the kinase.

When using relatively crude kinase preparations it is highly likely that a competing phosphatase will also be present. An inhibitor should be included to block the phosphatase activity. Proteases may also be present and thus it is important to add protease inhibitors to the reaction to prevent kinase or substrate degradation.

SUBSTRATE

As with any SPA, the substrate needs to be captured by the bead. The choice of bead coating will be determined by the tag available on the chosen substrate used in the reaction. The majority of assays have used biotin as a tag, but there are also examples of His-tag and GST fusion substrates.

When adding a tag to a substrate, there may be an effect on the K_m . This should always be checked when moving from one tag to another, or from an untagged to a tagged substrate.

REACTION

The concentration of the various components in the assay needs to be optimized. Some components will have a direct effect on each other, for example the protein or peptide substrate concentration will affect the mass of bead required due to the finite capacity per unit mass of bead. The total amount of ATP in the reaction may affect the reaction rate. It is also important to look at the ratio of labeled to unlabeled ATP. If the amount of unlabeled ATP is high, the signal will be compromised. With low amount of unlabeled ATP, inhibitor screens may be biased towards competitive inhibitors of ATP binding. The amount of labeled ATP will also affect the level of background. For 96-well format 0.2 to 0.5 μCi is a commonly used range of labeled ATP. The mass of enzyme used should be determined by titration. Time and temperature optima can be easily established with the SPA format.

STOPPING THE REACTION

The exact composition of the stop reagent will vary depending on the core bead type (PVT or YSi) and on the surface coating of the bead. An EDTA stop that is appropriate for streptavidin-coated beads will not work with the copper chelate beads. YSi beads have a relatively high affinity for ATP and as such require larger amounts (50 mM) of unlabeled ATP are needed in the stop reagent.

COUNTING CONDITIONS

Due to the relatively high energy of the ^{33}P , the non-proximity effect seen in kinase reactions can be significant. To minimize this, the beads need to be packed. This can be achieved by settling under gravity, floatation with 2 M CsCl (only for PVT beads) or by centrifugation.

For optimal performance, check the window settings on the counting instrument.

VALIDATION

The assay should be assessed for tolerance against solvents used for compound addition and against other potentially interfering substances. Known inhibitors can be used to compare IC_{50} values with the literature. Reaction specificity can be checked by using alternative methodology, such as HPLC. Kinetic analysis can also be used for assay validation.

Please visit www.perkinelmer.com/SPA for additional information.

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