

TopCount *Topics*

TCA-030

Optimization of ^{33}P Scintillation Proximity Assays Using Cesium Chloride Bead Suspension

Young-Whan Park, Tina Garyantes, Richard T. Cummings,
Merck Research Laboratories, Rahway, New Jersey 07065 USA and
Kelly Carter-Allen, Packard Instrument Company, Meriden, Connecticut 06450 USA

Introduction

Numerous Scintillation Proximity Assays (SPA) have been developed for high throughput screening of kinase inhibitors. These assays typically use a biotinylated peptide substrate containing a specific phosphorylation site. Following reaction of the substrate with the enzyme and $[\gamma\text{-}^{33}\text{P}]\text{ATP}$, the labeled substrate is captured by binding to streptavidin-coated SPA beads. SPA relies on the proximity of the radioactive label in relation to a scintillator impregnated bead to distinguish bound label from free label. Bound label produces a scintillation event in the bead, and free label does not. The path length of the beta emission must be short to achieve good signal-to-noise in a homogeneous format. Due to the high energy and relatively long path length of the ^{33}P beta emission, the best signal-to-noise is achieved by minimizing the bead contact with free label in solution. Typically this is accomplished by settling the beads overnight or centrifuging the plate to compact the beads¹ at the bottom of the plate. In this application note, we discuss an alternative method, using CsCl suspension (flotation), for achieving the optimal signal-to-noise which is rapid and does not require a centrifugation step. An added benefit for detection using the TopCount[®] Microplate Scintillation and Luminescence Counter (Packard) is increased counting efficiency and improved color quench resistance.

Methods

Kinase Inhibition Assay

For the CsCl suspension method, 100 μL kinase reactions were performed in 96-well OptiPlates[™]

(Packard). The microplate contained background wells, control wells with no inhibitor, and a titration series of a known tyrosine kinase inhibitor. Following incubation at room temperature for 40 minutes, 50 μL of streptavidin-coated SPA beads (20 mg/mL) in EDTA quench buffer were added to each well. CsCl solution of 100 μL per well at 7.5 M, 5 M, 2.5 M or 1.25 M was added to determine the optimum concentration required to suspend the beads. Following the addition of CsCl, the microplates were sealed and repeatedly counted in a TopCount for one minute per well at approximately 11 minute intervals.

For the centrifugation method, 100 μL kinase reactions were set up as described above. Following incubation at room temperature for 40 minutes, 100 μL of streptavidin-coated SPA beads (10 mg/mL) in EDTA quench buffer were added to each well. Beads were incubated for two minutes at room temperature, and then centrifuged at 1000 g for five minutes. The microplate was then counted for one minute per well in a TopCount.

Color Quench Correction

Kinase reactions of 100 μL were set up as described above with enzyme at a final concentration of 4.374 nM. In place of inhibitors, twofold dilutions of tartrazine dye (initial concentration of 100 μM) were used to mimic the quench effects of colors found in natural product screening.^{2,3} Following incubation at room temperature for 40 minutes, 50 μL of streptavidin-coated beads (20 mg/mL) were added to each well. Either 7.5 M or 5 M CsCl solution of 100 μL was added to bring the final CsCl concentration to

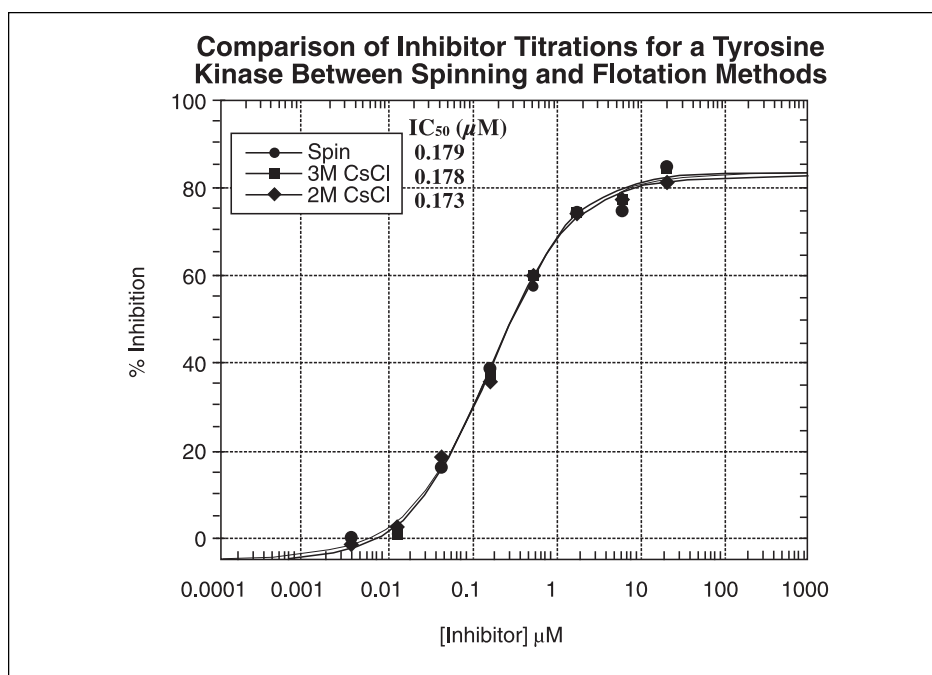


Figure 1.

Comparison of inhibitor titrations for a tyrosine kinase between centrifugation and flotation methods.

3 M or 2 M respectively. A corresponding microplate was prepared for centrifugation using 100 μL of EDTA in place of CsCl. The CsCl microplate was counted at 30 minute intervals after the addition of CsCl, to determine the optimum separation time. CPM, DPM and quench parameter (tSIS) information was collected by counting one minute per well in a TopCount. A previously prepared quench curve was used to automatically determine the counting efficiency and DPM for each well. An analysis of the counting efficiency, quench resistance and DPM recovery was performed comparing the suspension method to the standard centrifugation method.

Results

Comparable IC₅₀ results are shown for both the CsCl suspension and centrifugation methods in Figure 1.

Improved counting efficiency is demonstrated using the CsCl suspension method, especially at

high concentrations of tartrazine dye. These results are shown in Figure 2.

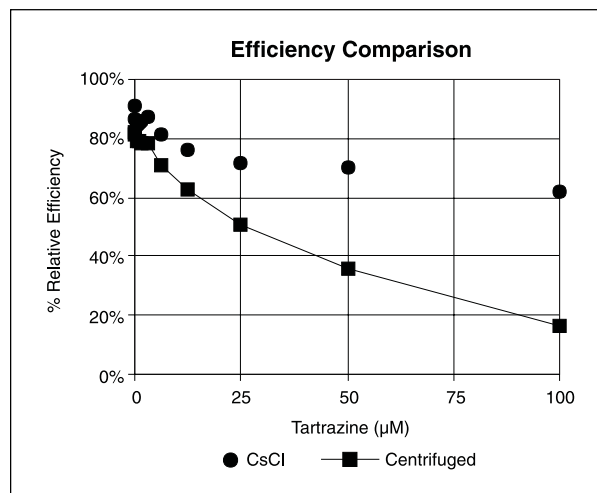


Figure 2.

³³P efficiency comparison between the suspension and centrifugation methods.

Quench Resistance

The tartrazine dye dilution series was counted after centrifugation and by using 3 M CsCl bead suspension. Tartrazine was present at final concentrations of 100 μM , 50 μM , 25 μM , 12.5 μM , 6.25 μM , 3.125 μM , 1.563 μM , 0.781 μM , 0.391 μM , and 0.195 μM . Relative efficiencies were derived from the quench curve. At 100 μM , the centrifuged bead efficiency was reduced to less than 20% relative efficiency compared to unquenched standards. The CsCl suspended bead samples had approximately three times higher counting efficiency at the 100 μM tartrazine concentration (Figures 2 and 3). In practical terms, more strongly colored samples may be assayed with greater accuracy using the suspension method.

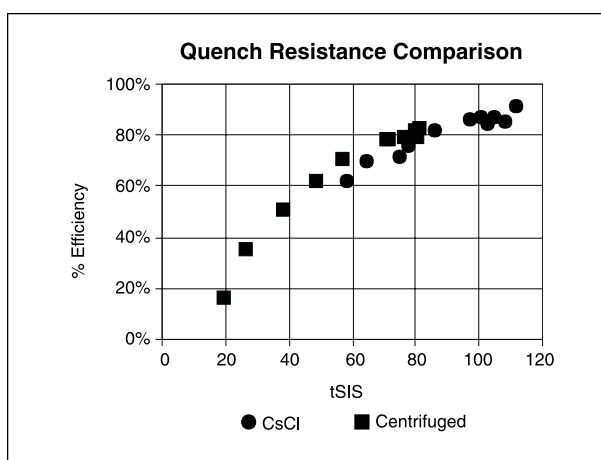


Figure 3.

Quench resistance comparison between the suspension and centrifugation methods.

DPM Recovery

An analysis of the DPM values obtained from automatic quench correction on the TopCount was compared to the expected DPM values for both centrifuged bead samples and CsCl suspended bead samples containing tartrazine dye in concentrations stated above. The average DPM recovery for both sample methods was 97% of the expected value.

Kinetics of Suspension Method

Count rates at various time points for the background and the no-inhibitor control wells were compared. Bead separation is indicated by the decrease in the background as the beads are separated from the free label in solution. Additionally, stabilization of the count rates indicated completion of the separation process. (See Figures 4a and 4b.) 3 M CsCl provided the most rapid separation of the beads, stabilizing the count rates within 60 minutes after addition. Concentrations of 2 M and 1 M of CsCl had slower separation kinetics, but stabilized after two hours. Less than 1 M CsCl did not provide complete separation after three hours, and was not used in further studies.

Signal-to-Noise

Background produced by nonspecific interaction of the free label with SPA beads determines the usable range of the assay. Therefore, minimizing the background will improve the assay sensitivity. Average background wells from the color quench microplates were compared for both CsCl suspended beads (3 M, 90 minutes), and centrifuged beads. (See Table 1.) Average backgrounds for CsCl

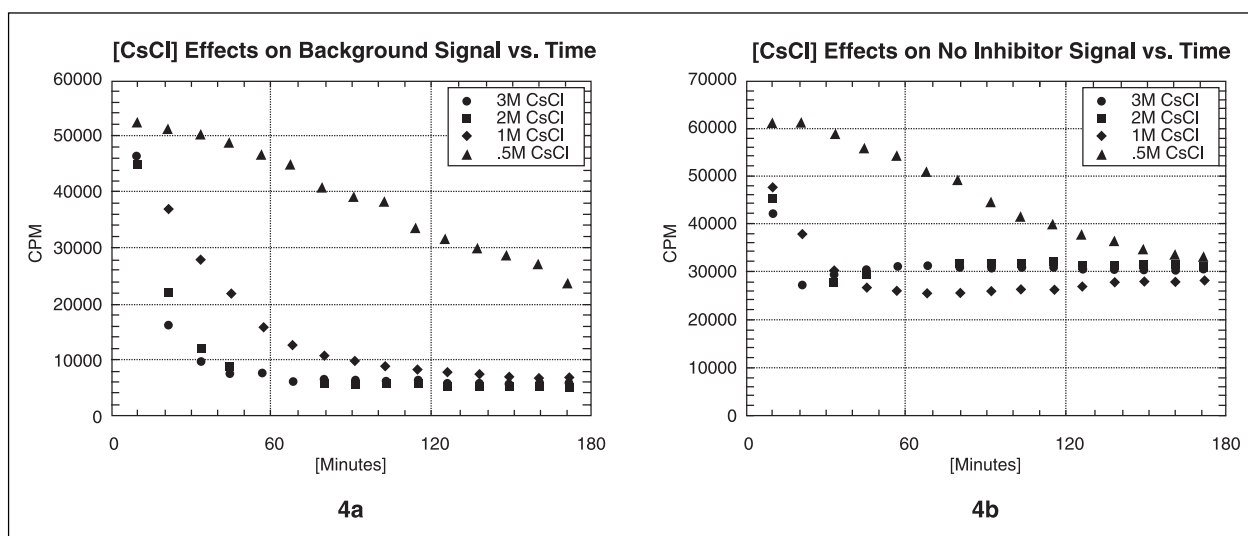


Figure 4 (a and b).

Effect of time on the background (4a) and the no-inhibitor signal (4b) with the CsCl suspension method. Note the high initial CPM which are caused by unbound ^{32}P intermingling with the SPA beads. As the beads begin to float, there is much less of this non-specific scintillation effect.

	Background (DPM)	No Inhibitor (DPM)	Signal-to-Noise Ratio
CsCl Suspended	4309	53814	12.5
Centrifuged	5006	51640	10.3

Table 1.

Signal-to-noise comparison between the CsCl suspension method and the centrifugation method.

suspended beads were slightly lower than centrifuged beads, contributing to an increase in the signal-to-noise ratio.

Conclusions

Suspension of SPA beads using a 3 M CsCl solution is easy, rapid and improves the performance of ³³P SPA kinase assays. It eliminates the overnight bead settling or a centrifugation step, and it enhances the counting efficiency and signal-to-noise of the assay on the TopCount. Liquid handling of the CsCl solution is easily automated, and does not have the viscosity problems of other bead suspension

methods using glycerol. CsCl solutions are well characterized for use density separation methods, and should have relatively little effect on bead binding characteristics.

References

1. Proximity News. Issue number 17. October 1995. A quantitative ³³P SPA assay for p34cdc2 kinase. Amersham International plc.
2. Kit insert, color quench and calibration kit. TRKQ7080Pl/96/01. Amersham International plc.
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