

# SELECTION OF DONOR AND ACCEPTOR REAGENTS IN A TR-FRET ASSAY

Tyrosine kinase assays using biotinylated peptides (b-peptide) as substrates have usually been assayed with europium-labeled (Eu)  $\alpha$ -phosphotyrosine antibodies as donor reagents and streptavidin-allophycocyanin conjugates (SA-APC) as the acceptor reagent largely because SA-APC was the only readily available acceptor reagent suitable for this assay.

ProZyme now offers a line of APC conjugates, including  $\alpha$ -phosphotyrosine antibody (PY20 MAb), which allows the assay to be formulated with SA-Eu as the donor. This study compares the results of PY20-Eu/SA-APC *vs.* SA-Eu/PY20-APC reagent pairs in a model assay with a biotinylated phosphotyrosine-containing peptide (b-phos-peptide). Assay results are compared both with respect to Signal/Noise (S/N) ratios obtained and reagent costs.

## PROCEDURE

### REAGENTS

#### Assay 1 PY20-Eu/SA-APC

##### Negative Control Wells

12.3 ng/ml (5 nM) b-peptide, Pierce #29914  
 0.90  $\mu$ g/ml (6 nM PY20) PY20-Eu, PKI #AD0066  
 0.34  $\mu$ g/ml (2.3 nM SA) SA-APC, PZI #PJ25S

##### Positive Control Wells

12.7 ng/ml (5 nM) b-phos-peptide, Pierce #29934

0.90  $\mu$ g/ml (6 nM PY20) PY20-Eu, PKI #AD0066

0.34  $\mu$ g/ml (2.3 nM SA) SA-APC, PZI #PJ25S

#### Assay 2 SA-Eu/PY20-APC

##### Negative Control Wells

12.3 ng/ml (5 nM) b-peptide  
 0.13  $\mu$ g/ml SA-Eu (2.3 nM SA) PKI #AD0062  
 2.4  $\mu$ g/ml (6 nM PY20) PY20-APC, PZI #PJ254

##### Positive Control Wells

12.7 ng/ml (5 nM) b-phos-peptide  
 0.13  $\mu$ g/ml (2.3 nM SA) SA-Eu, PKI #AD0062  
 2.4  $\mu$ g/ml (6 nM PY20) PY20-APC, PZI #PJ254

For each assay, 12 negative and 12 positive control wells, each containing 100  $\mu$ l of assay mixture in 96-well Costar plates were incubated for 2 hours and read in a Wallac Victor<sup>2</sup> 1420 Multilabel Counter (PerkinElmer) with the Lance™ 665/615 protocol. For each assay, separate determinations were made in black and in white plates.

For each well, Channel A (665 nm) to Channel B (615 nm) ratio (A/B) was calculated, and the mean and standard deviation (STD) of this ratio were calculated for each set of 12 values. Each plate was counted five times. Data reported in are the average of these five determinations of each value. Please see TechNote TNPJ100.25 *Signal, Background and Spectral Overlap Compensation in FRET Assays* for a discussion of the calculations.

Table 1 - Comparison of Assay Direction and Plate Color

	Assay 1	Assay 2	Assay 1	Assay 2
Donor	PY20-Eu	SA-Eu	PY20-Eu	SA-Eu
Acceptor	SA-APC	PY20-APC	SA-APC	PY20-APC
Plate Color	Black	Black	White	White
Negative Control	.003421	.004375	.003846	.006098
Std Dev	.000312	.000489	.000114	.000238
Positive Control	.336411	.368960	.325801	.36564
Std Dev	.005477	.006011	.003936	.003576
% Relative Std Dev (positive control)	1.6	1.6	1.20	0.98
S/B	98	84	85	60
S/N	63	63	78	100

## RESULTS

Table 1 shows that both assays in black plates give comparable results. Although Assay 1 gives a slightly better S/B, S/N for the assay is actually identical to that for Assay 2.

In addition, while it has been reported that black plates give superior results in TR-FRET assays, we find this is true only when comparing S/B values. S/N values, which are the more appropriate indicators of assay sensitivity, were improved by the use of white plates. See TechNote TNPJ100.11 *Detection of Tyrosine Kinase Phosphorylated Peptide with FRET* for additional data.

### Assay Costs

Assay costs were calculated for each Assay Format, based on published 1-mg prices for each reagent assuming 96-well plates containing 0.1 ml of assay mix (Table 2).

Table 2 - Cost Per Well

	Assay 1	Assay 2
<b>Peptide (nM)</b>	5	5
<b>Donor</b>	PY20-Eu	SA-Eu
Conc (nM)	6.0	2.3
Conc (µg/ml)	0.90	0.13
Cost (\$)/mg	6,930	7,350
Cost (\$)/well	0.62	0.09
<b>Acceptor</b>	SA-APC	PY20-APC
Conc (nM)	2.3 (SA)	6.0 (PY20)
Conc (µg/ml)	.34	2.40
Cost (\$)/mg	300	650
Cost/well	.01	.16
<b>Total Cost \$/well</b>	<b>0.63</b>	<b>0.25</b>

## DISCUSSION

The binding events in these assays are mediated in one instance by the association of biotin and SA, and in the other by phosphotyrosine and  $\alpha$ -phosphotyrosine antibody. The association of biotin and SA occurs with a much higher affinity than that of phosphotyrosine and PY20. To achieve optimal FRET signal, the substrate should be saturated with both donor and acceptor molecules. The higher the affinity of the donor/acceptor conjugate for the substrate, the smaller the excess of conjugate required to effectively saturate the substrate.

In these assays using 5 nM b-phos-peptide, 2.3 nM Streptavidin was required to provide optimal FRET, while 6 nM PY20 was required to give optimal antibody binding (optimal reagent concentrations will vary somewhat depending upon the specific assay conditions, conjugates, substrates, etc.)

In TR-FRET assays, donor quenching due to FRET is relatively small, and in these assays did not exceed 10 - 20% of the donor signal. Consequently, S/N ratios are not very sensitive to the specific direction of the assay—that is, whether the reagent pair used is SA-Eu/PY20-APC or PY20-Eu/SA-APC. However, because the optimum signal for the SA component is obtained at a much lower concentration than the optimum signal for the PY20 component, the assay is less expensive when the Eu is bound to the SA because the Eu is the most expensive FRET component of the assay.

In general, TR-FRET assays in which Eu is conjugated to the binding component with the highest affinity for the substrate will provide the best combination of performance and cost-effectiveness. While the data shown

here suggest that performance in this configuration is equivalent in black plates and enhanced in white plates, more extensive data (TechNote TNPJ100.50 *Detection of Tyrosine Kinase Phosphorylated Peptide with FRET*) show that this configuration provides improved signal:noise. At the same time, by reducing the usage of the more expensive Eu-based reagents, this configuration significantly reduces per-well costs. In any specific assay, other factors including steric effects and the number of moles of Eu-chelate bound per mole of binding component must also be taken into account.

## CONCLUSIONS

Assay 2 gives equivalent or slightly better S/N ratios than Assay 1, and does so at less than half the reagent cost per well. Some increase in S/N can be obtained in either experiment by using white plates instead of black plates. These results suggest that Assay Direction can be an important economic consideration during assay development.

## TECHNICAL SERVICE

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[<http://www.prozyme.com/technical/index.html#technotes>](http://www.prozyme.com/technical/index.html#technotes)

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