# Improvements in the Sensitivity of Time Resolved Fluorescence Energy Transfer Assays

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The effect on fluorescence resonance energy transfer (FRET) of multiple labelling of DNA oligonucleotides with donor lanthanide chelate and acceptor  $CyDye^{TM}$  fluors has been investigated. It is shown that using a multiple donor lanthanide chelate with a single acceptor  $Cy^{TM}3$  or Cy5 can increase sensitivity and fluorescence output. The enhanced FRET observed in the multiple donor label system has been utilised in two different DNA based assay formats to demonstrate the advantages over a steady state fluorescence assay and a radiometric assay.

**KEY WORDS:** Fluorescence resonance energy transfer; time resolved fluorescence; multiple labelling; p65 binding; helicase.

#### INTRODUCTION

There is an increasing demand for new leads in drug discovery, which requires the development of sensitive, non-radioactive, homogenous assays which can increase throughput and are amenable to miniaturisation. Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) is a generic technology which is potentially more sensitive than conventional steady state Fluorescence Resonance Energy Transfer (FRET) assays, in some applications.

FRET is a proximity-dependent interaction in which a fluorescent donor molecule transfers excitation energy to an acceptor molecule (this can be a fluorescent or non-fluorescent molecule). Energy transfer occurs primarily because the acceptor dipole resonates or interacts with the donor dipole. Excitation is therefore transferred from the donor to the acceptor molecule without emission of a photon. Energy transfer is dependent on the inverse

sixth power of the intermolecular distance between the donor and acceptor and results in a decrease in donor lifetime and quantum yield and an increase or sensitisation of acceptor fluorescence. The advantages of using TR-FRET include an increased  $R_{\rm o}$  (distance at which 50% of the energy is transferred) and minimal interference from background fluorescence.

This study describes the use of two lanthanide chelates as donor fluors for TR-FRET using cyanine dyes as acceptor fluors. The TR-FRET system is compared with a FRET system for both sensitivity and signal to noise.

We have developed two simple TR-FRET hybridisation assays using a polyaminocarboxylate chelate of terbium [1, 2] and a terpyridine chelate of europium [3] as donor fluors with Cy3 and Cy5 dyes as their respective acceptor fluors. We have demonstrated that sensitivity and signal to noise ratio of the assay are improved by introducing multiple fluors into the system and that the ratio of donor fluor to acceptor fluor influences the signal output.

Data is presented which demonstrates the benefits of the multiple donor TR-FRET assay compared to its

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Fig. 1. Structure of the terbium chelate of carbostyril124-diethylenetriaminepentaacetic acid.

steady state equivalent, by utilising the system to quantify binding of the 65kDa sub-unit of the transcription factor NF- $\kappa$ B (p65) to a fluorescently labelled DNA consensus sequence. Comparison of a TR-FRET Helicase assay with a radiometric equivalent is also reported and this further demonstrates the potential of the assay configuration.

#### **EXPERIMENTAL**

## **Apparatus**

The fluorescence measurements were obtained on a Wallac Victor 1420 Multilabel Counter (EG&G life Sciences) using interference excitation filters of 340/40nm and emission of 572/7.5nm or 670/10nm for time resolved data, and on a Molecular Dynamics Biolumin 960 (Amersham Pharmacia Biotech Ltd.) using 535/10nm and 629/10nm excitation and 569/10nm or 670/10nm emission filters for steady state data depending on the respective fluor.

#### Methods

Evaluation of Sensitivity. Mono and triple amine modified 21mer oligonucleotides were labelled with either a terbium chelate of carbostyril 124-diethylenetriaminepentaacetic acid (cs124DTPA(Tb)) (Fig. 1) or a europium chelate of terpyridine-bis(methylenamine)tetraacetic acid (TMT(Eu)) (Fig. 2). Cy3 was used as the donor fluor for steady state comparison. Absorption and emission spectra for the various fluors used in this study are shown in Fig. 3.

Complementary oligonucleotides (10mers) were labelled with either Cy3 or Cy5 NHS ester via a 5' terminal amine group and used as respective FRET acceptor fluors. All oligonucleotide labelling was performed 'in house' using standard labelling procedures. Constant

donor chelate oligo (5nM) was titrated with acceptor oligonucleotide (10nM to 40pM) in 96 well microtitre plates (200µl final volume). Complementary donor and acceptor oligonucleotides were hybridised at 65°C for 1 hour before measuring TR-FRET or FRET signal. Controls were set up using equivalent acceptor fluor concentrations with non-complementary oligonucleotides.

*p65 Binding Assay.* Evaluation of p65 binding was carried out in 96 well microtitre plates. Triple amine modified NF- $\kappa$ B-specific dsDNA (25nM) labelled with cs124DTPA(Tb) was incubated at room temperature with Cy3 labelled anti-GST polyclonal antibody (25nM) and p65GST recombinant protein (5nM). Specific and non-specific competitor DNA was introduced to the system (up to 12.5μM, final volume 200μl) (Fig. 4) and the TR-FRET signal was measured after 60–90minutes incubation in the dark with agitation. Comparison with a steady

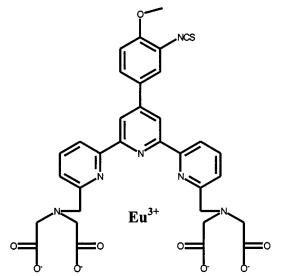
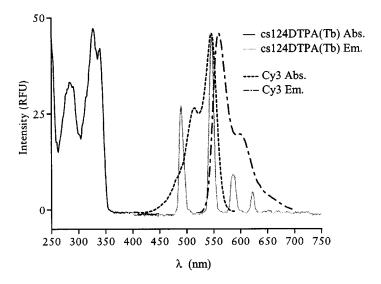
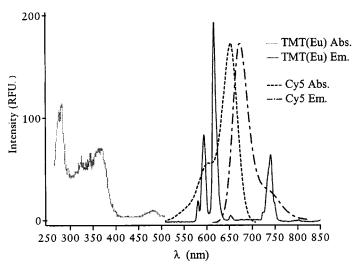


Fig. 2. Structure of the europium chelate of terpyridine-bis(methylenamine)tetraacetic acid.





**Fig. 3.** Absorption (Abs) and emission (Em) spectra for TR-FRET fluorophores used in the study. Overlap of spectra for cs124DTPA(Tb) vs Cy3 (top) and TMT(Eu) vs Cy5 (bottom) are shown.

state system was achieved by substituting the cs124DTPA(Tb) NF- $\kappa$ B-specific dsDNA with Cy5 labelled NF- $\kappa$ B-specific dsDNA.

Helicase Assay. Triple labelled cs124DTPA(Tb) helicase substrate was produced by annealing labelled oligonucleotide to ssDNA from filamentous bacteriophage M13. The assay was configured so that as a result of helicase activity, the cs124DTPA(Tb) labelled oligonucleotide is unwound from the ssM13 (20nM total substrate) and the released oligonucleotide anneals to a complementary Cy3 labelled capture oligonucleotide (20nM) (Fig. 5). Assays were carried out in 96 well microtitre plates (200µl total volume). To demonstrate the TR-FRET assay, the effect of E. coli helicase I concen-

tration upon substrate unwinding was compared to a Scintillation Proximity Assay (SPA) [<sup>3</sup>H] system [4]. This was achieved using a range of helicase concentrations between 5 and 25 fmol/well and incubating at 33°C for 30 minutes before addition of the capture oligonucleotide.

# RESULTS AND DISCUSSION

For all FRET and TR-FRET systems reported, the acceptor fluorescence was monitored. Monitoring of acceptor fluorescence provides a simpler means of FRET detection, particularly for steady state fluors which exhibit small stokes shifts. Discrimination between donor

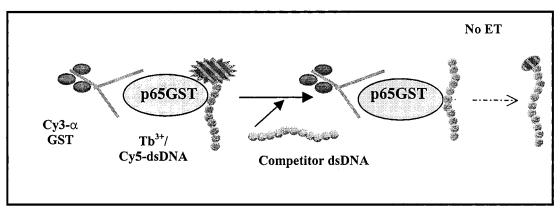


Fig. 4. Illustration of the p65 binding assay showing the Cy3 (Ex. 550nm/Em. 572nm) labelled anti-GST polyclonal antibody and the DNA consensus sequence labelled with either cs124DTPA(Tb) (Ex. 340nm) or Cy5 (Em. 670nm).

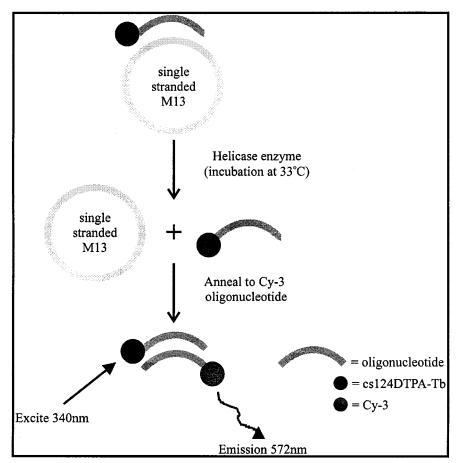
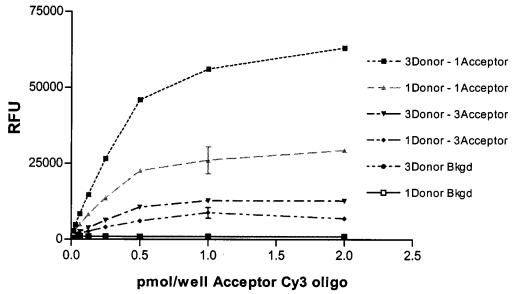


Fig. 5. Illustration of the TR-FRET helicase assay showing cs124DTPA(Tb) labelled oligonucleotide/ M13 substrate and Cy3 labelled complementary capture oligonucleotide.

excitation and emission can be difficult, whereas acceptor sensitised emission is generally at a much longer wavelength than the donor excitation wavelength. The effects of the mono and triple labelled donor and acceptor oligonucleotides on TR-FRET and FRET signal vary depending on the combination of donor to



**Fig. 6.** Comparison of donor vs acceptor fluors for TR-FRET using cs124DTPA(Tb) [Ex. 340nm] as a donor and Cy3 [Em. 572nm] as acceptor. Donor oligo was at a constant 1pmol/well.

acceptor. Multiple donor labelling can increase the specific TR-FRET and FRET signal, provided that only a single acceptor fluor is present (Figs. 6–8). Increasing the number of acceptor fluors reduces signal output and is possibly a result of self quenching. Evidence to substantiate this is found by examining the deactivation of the donor flourescence by the acceptor fluors. This is found to increase with multiple fluors, suggesting a higher energy

transfer than with single fluors, although fluorescence output from the acceptor decreases. Indeed, this quenching is significant enough to reduce the fluorescence output of the triple labelled donor system to below that of the single donor to single acceptor system.

Sensitivity and signal to noise ratio (S/N) is increased in the triple donor to single acceptor system and this effect is similar for both time-resolved and steady

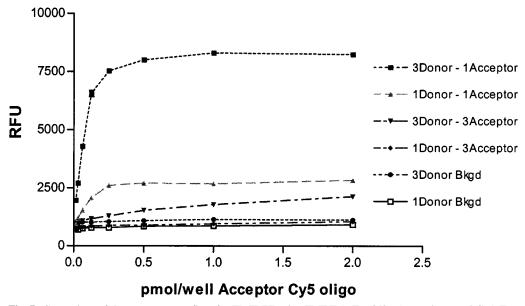


Fig. 7. Comparison of donor vs acceptor fluor for TR-FRET using TMT(Eu) (Ex. 340nm) as a donor and Cy5 (Em. 670nm) as acceptor. Donor oligo was at a constant 1pmol/well.

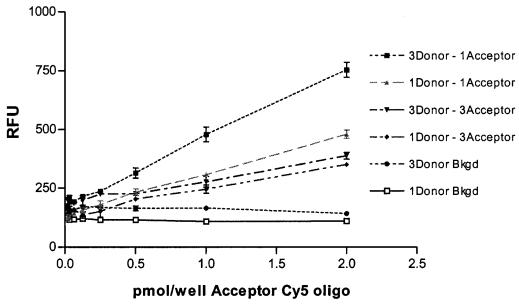


Fig. 8. Comparison of donor vs acceptor fluor for steady state FRET using Cy3 (Ex. 550nm) as a donor and Cy5 (Em. 670nm) as an acceptor. Donor oligo was at a constant 1pmol/well.

state fluor systems compared with the other donor-acceptor combinations. The cs124DTPA(Tb) to Cy3 was the most sensitive FRET fluor pair with a sensitivity (determined as double background signal) being 5.6fmol/well. For TMT(Eu) to Cy5 this was 16.2 fmol/well and 490 fmol/well for the steady state system. In comparison, single donor to triple acceptor fluor sensitivity was 74fmol/well for the cs124DTPA(Tb) to Cy3, a greater than 13 fold decrease in assay sensitivity. TMT(Eu) to Cy5 had a sensitivity of 1.2pmol/well for this combination of donor and acceptor fluors and Cy3 to Cy5 sensitivity was 1.09pmol/well. Table I shows the sensitivities for all possible donor fluor combinations.

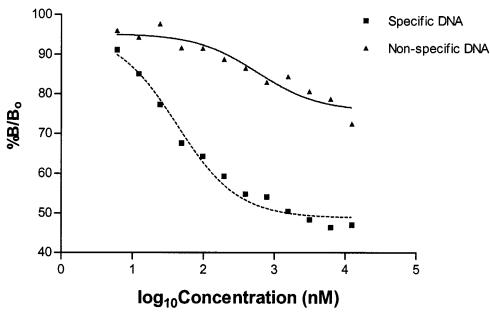
S/N for the triple label cs124DTPA(Tb) to Cy3 was 63:1 compared to 29:1 for a single donor to acceptor system. Using TMT(Eu) as the donor and Cy5 as acceptor the S/N was 8:1 compared to 3.4:1 for the single donor system. The lower S/N ratio for the TMT(Eu) assay is

most likely due to limitations in the sensitivity of the instrument used. This is because Cy5 output at 670nm is close to the detection limits of the photomultiplier tube in the instrument. Detection of smaller wavelengths such as 570nm for Cy3 is therefore more favourable. S/N of the TMT(Eu) assay is however, still greater than the steady state system which produced a S/N of 4:1 for the triple donor system and 2.5:1 for the single donor system.

Specificity of the DNA binding event between Cy3 labelled p65 and cs124DTPA(Tb) or Cy5 labelled dsDNA for the TR-FRET and FRET systems was demonstrated with a competition assay. The assay tested the ability of an unlabelled specific and non-specific DNA sequence to compete with the labelled dsDNA for binding to p65 GST. The results (Fig. 9) demonstrate that up to a 250 fold molar excess of specific DNA in the TR-FRET assay competed with approximately 55% of the p65 GST binding activity. Under the same conditions the non-specific

**Table I.** The Sensitivity of a Simple FRET Hybridisation Assay Depending on the Combination of Donor and Acceptor Fluors in the System (1 or 3)

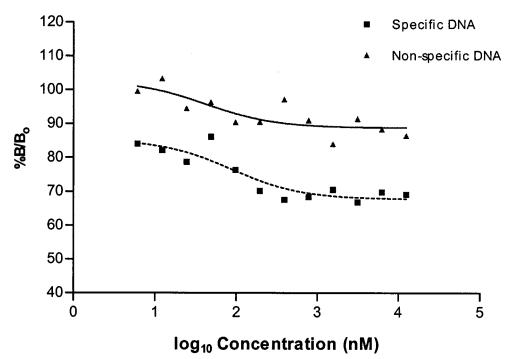
	cs124DTPA(Tb)-Cy3 (340nm/572nm)	TMT(Eu) to Cy5 (340nm/670nm)	Cy3-Cy5 (550nm/670nm)
1 donor-1 acceptor	8.2 fmol/well	103 fmol/well	616 fmol/well
3 donors-3 acceptors	37 fmol/well	1.3 pmol/well	1.36 pmol/well
1 donor-3 acceptors	74 fmol/well	1.2 pmol/well	1.09 pmol/well
3 donors-1 acceptor	5.6 fmol/well	16.2 fmol/well	490 fmol/well



**Fig. 9.** TR-FRET p65 binding assay. Competition binding of specific and non-specific dsDNA, using cs124DTPA(Tb) as donor fluor (Ex. 340nm) and Cy3 (Em. 572nm) as acceptor fluor.

DNA competed with approximately 28% of binding activity. This compares with 32% and 16% competition for the specific and non-specific DNA respectively for the steady state FRET assay (Fig. 10). Using the same con-

centrations of fluor for each assay produces a different shape to the binding curve and is thought to be a result of lower sensitivity of the FRET system. Increasing the fluor concentration in the FRET assay four fold produced

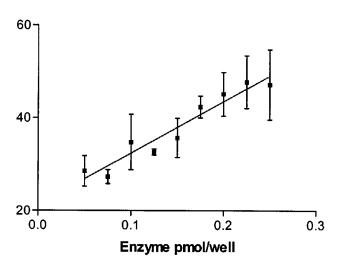


**Fig. 10.** FRET p65 binding assay. Competition binding of specific and non-specific dsDNA, using Cy3 (Ex. 550nm) as donor fluor and Cy5 (Em. 670nm) as acceptor fluor. Concentrations of assay components are identical to those in the TR-FRET system.

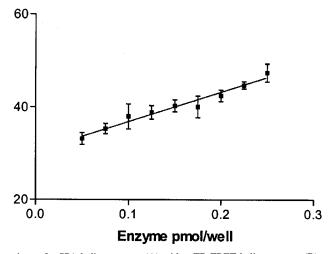
similar data (not shown) to the TR-FRET system and is further evidence that the TR-FRET is the more sensitive assay for this format. Using a multiple label TR-FRET assay involving cs124DTPA(Tb) as a donor and Cy3 as an acceptor fluor therefore has the potential benefit that less reagents would be required (four fold less in the

above example) than a conventional Cy3 to Cy5 assay. It is also likely that the  $R_0$  (distance at which 50% of the energy is transferred from donor to acceptor) is greater for the TR-FRET compared to the FRET format [5]. This assumption is based on previous reports which suggest detection of FRET assays is limited to distances between

A



В



**Fig. 11.** Comparison of a SPA helicase assay (A) with a TR-FRET helicase assay (B). Effect of  $E.\ coli$  helicase I concentration on the unwinding of labelled substrate. The reactions were performed at 33°C for 30 minutes. Values are given as the means and range (n = 3).

donor and acceptor fluors of about 70Å–80Å [6]. Measurable distances for TR-FRET using some of the fluors used in this study have been reported at between 100Å and 200Å [5,6]. Increased energy transfer distance would therefore improve sensitivity in this application, or similar applications where distance between donor and acceptor fluors is large.

A comparison of the TR-FRET helicase assay and the SPA helicase assay are shown in Fig. 11. Both assay formats give similar results in respect of percentage substrate unwound. The SPA assay returned a range of 28.5  $\pm$  3.3% to 47.1  $\pm$  7.6% unwinding of DNA from total substrate compared to  $33.4 \pm 1.3\%$  to  $47.4 \pm 1.9\%$ for the TR-FRET assay. The most significant difference between the assays was the reproducibility of each point. Replicates for data points produced from the TR-FRET assay were closer than the radiometric SPA assay for the concentrations used, so there appears to be less error with the TR-FRET assay. Another significant advantage with the TR-FRET assay is that although both assay formats are homogenous there are less steps involved in the fluorometric assay (2 compared to 3 for the radiometric SPA assay) which would benefit a rapid screen and again reduce reagent usage.

# CONCLUSIONS

Improvements in the sensitivity of a DNA TR-FRET assay can be achieved by introducing multiple donor labels provided that the complementary oligonucleotide has a single acceptor fluor. In some applications TR-FRET assays utilising this multiple donor system, have superior sensitivity over steady state FRET assays at equivalent molar concentrations, particularly where the distance of energy transfer is large and a greater  $R_{\rm o}$  is needed. Up to a four fold increase in sensitivity of a p65 binding assay has been achieved with our TR-FRET system compared to that of a steady state assay. Results of a TR-FRET DNA helicase assay are comparable with that of a radiometric SPA assay with an apparent improvement in error of data points.

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