GE Healthcare

Amersham
TR-FRET Generic Reagents

Product Booklet

Codes:

Cy5 anti-Phosphotyrosine antibody	(1 mg)	PA92000
Cy5 anti-Glutathione S-transferase antibody	(1 mg)	PA92002
Cy5 Streptavidin	(1 mg)	PA92005
Eu (TMT) anti-Phosphotyrosine antibody	(50 µg)	PA92001
	(1 mg)	PA92007
Eu (TMT) anti-Glutathione S-transferase antibody	(50 µg)	PA92003
	(1 mg)	PA92009
Eu (TMT) Streptavidin	(50 µg)	PA92004
	(1 mg)	PA92010

Page finder

1. Legal

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GE Healthcare UK Limited
Amersham Place, Little Chalfont,
Buckinghamshire, HP7 9NA UK

2. Handling

2.1. Safety warnings and precautions

Warning: ***For research use only.*** Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage

Store lyophilized at 2–8°C Following reconstitution the material may be stored at 2–8°C for up to 4 weeks, or at -15°C to -30°C in aliquots to avoid repeat freeze thaw cycles.

2.3. Expiry

Each lyophilized component is stable for at least 4 weeks from the date of despatch when stored under the recommended conditions.

3. Components

PA92000
Cy5 anti-Phosphotyrosine antibody, 1 µg

PA92001
Eu (TMT) anti-Phosphotyrosine antibody, 50 mg

PA92002
Cy5 anti-Glutathione S-transferase antibody, 1 mg

PA92003
Eu (TMT) anti-Glutathione S-transferase antibody, 50 µg

PA92004
Eu (TMT) Streptavidin, 50 µg

PA92005
Cy5 Streptavidin, 1 mg

PA92007
Eu (TMT) anti-Phosphotyrosine antibody, 1 mg

PA92009
Eu (TMT) anti-Glutathione S-transferase antibody, 1 mg

PA92010
Eu (TMT) Streptavidin, 1 mg

4. Description

The products described are generic reagents that will allow the configuration of assays based on homogeneous time resolved fluorescence resonance energy transfer (TR-FRET).

Donor reagents have been labelled with a Terpyridine-bis(Methyl-enamine) Tetraacetic acid (TMT) chelate of europium (1).

Cy™5 has been used as the label for the acceptor reagents.

All reagents are available labelled with either Eu (TMT) or Cy5.

Evaluation of both donor and acceptor orientations will allow the optimisation of assay configurations over a wide range of applications.

5. Protocol

5.1. Introduction

The need to screen large numbers of targets against extensive small molecule libraries has led to the demand for an increase in both the sensitivity and speed of assays. Increasingly homogeneous assays based on fluorescence are becoming the method of choice.

Fluorescence resonance energy transfer (FRET) is the radiationless transfer of excitation energy from a donor to an acceptor molecule. This is a distance-dependent phenomenon occurring over 1–10 nm and as such is comparable with the dimensions of biological macromolecules. FRET is a valuable tool in studying proximity events in biological systems (2).

The majority of organic dyes, used for FRET, have a fluorescence lifetime of less than 10 ns.

Background fluorescence from biological samples, buffer components and plastic ware also have similar lifetimes therefore this can limit assay sensitivity (3).

To overcome this, dyes with considerably longer lifetimes have been used. This technique, known as time resolved fluorescence (TRF) delays the measurement of assay signal until background fluorescence has dissipated. (Figure 1)

Time resolved fluorescence resonance energy transfer (TR-FRET) is a homogeneous technique that combines the benefits of both FRET and TRF.

The fluorescence lifetime of chelates of rare earth elements is typically 100 µs–2 ms, and offers advantages over conventional fluorophores as donors for TR-FRET. The use of Eu (TMT) as a donor and Cy5 as a spectrally matched acceptor will allow improvements in assay sensitivity, in particular the ratio of signal to background (4).

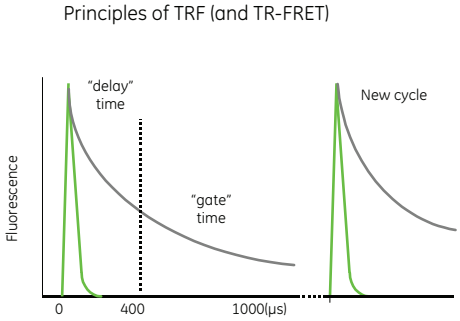

Fig 1. Principles of TRF and TR-FRET

GE Healthcare supply a range of donor and acceptor generic reagents that will allow researchers to perform sensitive, non-radiometric, screening assays using the TR-FRET technique.

5.2. Fluorescent labels

5.2.1. Europium (TMT) chelate

Lanthanide ions have a low extinction coefficient, and the presence of solvent, especially water, quenches their luminescence. Many organic ligands have been synthesized, which can 'chelate' a Lanthanide (III) ion, this diminishes the number of solvent molecules coordinated to the ion, allowing it to be sensitized to generate higher luminescence.

Effective ligands need to have a high coordination number, efficient light harvesting properties, long triplet excited state lifetime, large Stokes shift, low number of coordinated water molecules, solubility in aqueous media.

The aromatic terpyridyl structure (Figure 2), of Eu (TMT) provides the chelated Eu^{3+} ion with a relatively hydrophobic environment

necessary for intense fluorescence output and long fluorescence lifetime. Also the 3-amino group of the ligand is readily converted to the isothiocyanate, allowing labelling of primary amine groups on proteins.

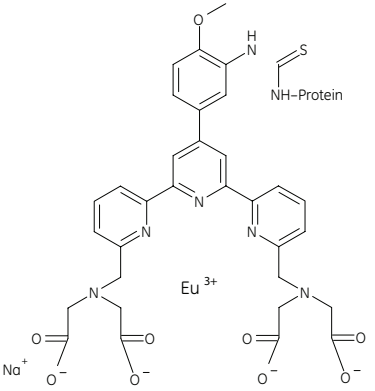

Fig 2. Eu (TMT) utilized for biological labelling

The fluorescence lifetime of an aqueous solution of unconjugated Eu (TMT) is 1.4 ms.

The luminescence intensity of the Eu (TMT) was measured in either water or TRIS buffer in the presence of various additives. The addition of Ca^{2+}, Mg^{2+} and EGTA had no effect on intensity; however, when either Fe or Mn was added, the intensity dropped to zero almost immediately.

In buffers of varying pH little effect was seen on the emission spectra of Eu (TMT) when excited at 340 nm (Figure 3). In addition, the presence of 10% DMSO had little or no effect on the emission spectrum or luminescence intensity observed.

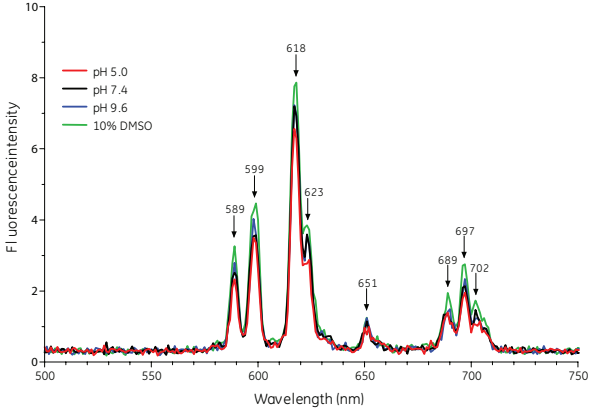

Fig 3. Emission spectra of the Eu (TMT) (unconjugated form).

5.2.2. Cy5

Cy5 is a bright, water soluble dye which is readily conjugated to biomolecules via active ester chemistry. It has a large molar extinction coefficient (250 000) and emission in the far red region of the spectrum which makes it ideal as an acceptor in TR-FRET applications.

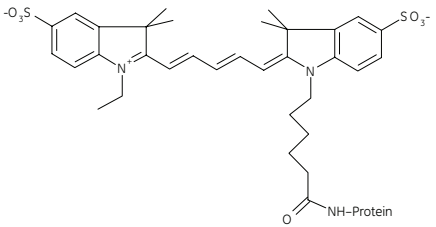

Fig 4. Cy5 utilized for biological labelling

In buffers of varying pH little effect was seen on the emission spectra of Cy5 (Figure 5). In addition, the presence of 10% DMSO had little or no effect on the emission spectrum or luminescence intensity observed.

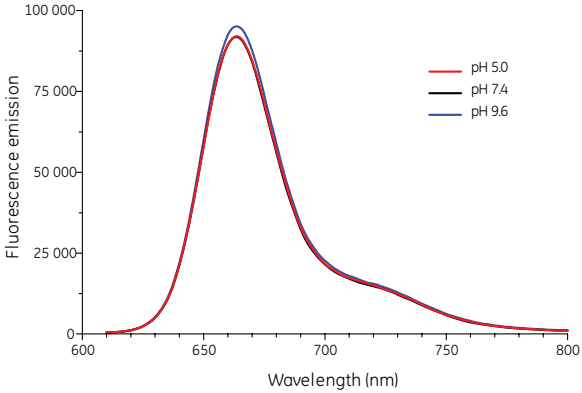

Fig 5. Emission spectra of Cy5 (unconjugated form)

5.3. Eu (TMT) and Cy5 conjugates

5.3.1. Reagent preparation

Lyophilized conjugates of Cy5 are available as 1 mg packs and Eu (TMT) as either 50 µg or 1 mg.

Reconstitute to 1 mg/ml using deionised water; following reconstitution the material may be stored at 2–8°C for up to 4 weeks, or at -15°C to -30°C in aliquots to avoid repeat freeze thaw cycles.

5.3.2. Protein specificity

The anti-phosphotyrosine antibody is a mouse monoclonal antibody (clone PY20) of isotype IgG2b.

The anti-GST antibody has been raised in goat using recombinant glutathione S-transferase from *Schistosoma japonicum* produced in *E.coli*.

Electrophoretically isolated and purified streptavidin is produced from *Streptomyces Avidinii*.

Each reagent has been individually tested to ensure that high binding to antigen or biotin is retained. The functional tests were performed in the following manner:

a) Anti-phosphotyrosine antibodies: Functionality was demonstrated by binding of the antibody conjugate to the phosphotyrosine residue of a biotinylated peptide immobilised on a Streptavidin microplate.

b) Anti-GST antibodies: Functionality was demonstrated by the binding of the antibody conjugate to GST (*Schistosoma japonicum* recombinant, expressed in *E. coli*) which had been adsorbed onto a microplate.

c) Streptavidin: Functionality was demonstrated by binding the conjugate to biotinylated bovine serum albumin which had been adsorbed onto a microplate.

All reagents have been labelled with the optimal number of Eu(MT) or Cy5 molecules to provide the most efficient energy transfer and signal:background ratio.

5.3.3. Quality assurance

All of the labelled conjugates have been tested in TR-FRET model assays. The resulting signal was measured using FARCyte™ fluorescence plate reader with the following instrument settings:

Europium excitation	340 nm
Cy5 emission	670 nm
Delay (lag time)	50 µs
Signal integration	400 µs

Assay details and typical signal:background are shown below:

This information should be used as a guide only and individual assay optimisation is the responsibility of the end user.

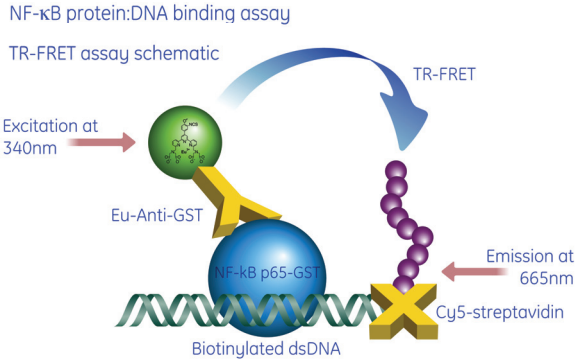

Fig 6. TR-FRET schematic: NF-κB protein: DNA binding assay
(The figure shows one donor:acceptor orientation only)

a) Eu (TMT) anti Glutathione S transferase antibody donor and Cy5 Streptavidin acceptor

0.5 pmoles p65 GST fusion protein and 0.5 pmoles Eu (TMT) anti GST antibody were incubated at room temperature for 60 min, 2 pmoles biotinylated dsDNA NF-κB-specific (HIV-L) consensus sequence added to the reaction mixture and incubated for a further 60 min at room temperature Finally 8 pmoles of Cy5 Streptavidin was added.

A signal:background of 8:1 was observed.

b) Eu (TMT) Streptavidin donor and Cy5 anti Glutathione S transferase antibody acceptor

2 pmoles p65 GST fusion protein and 8 pmoles Cy5 anti GST antibody were incubated at room temperature for 60 min, 1 pmole biotinylated dsDNA NF-κB-specific (HIV-L) consensus sequence added to the reaction mixture and incubated for a further 60 min at room temperature. Finally 0.5 pmoles of Eu (TMT) Streptavidin was added.

A signal:background of 6:1 was observed.

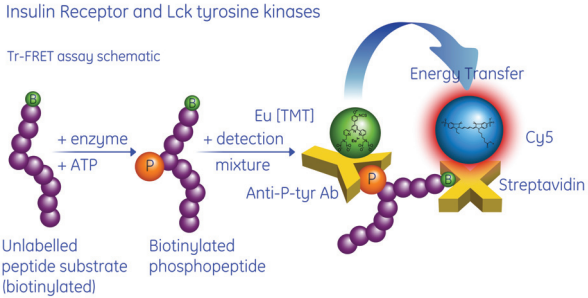

Fig 7. TR-FRET schematic: Detection of phosphorylated peptides (The figure shows one donor : acceptor orientation only)

c) Eu (TMT) anti-Phosphotyrosine antibody donor and Cy5 Streptavidin acceptor

Biotinylated peptide (specific sequence for Lck protein tyrosine kinase) phosphorylated at the tyrosine residue (50 nM), Eu (TMT) anti PY20 antibody (10 nM) and Cy5 Streptavidin (10 nM) were added to a 384 well microplate and incubated for 60 min at room temperature.

A signal:background of 8:1 was observed.

d) Eu (TMT) Streptavidin donor and Cy5 anti Phosphotyrosine antibody acceptor

Biotinylated peptide (specific sequence for Lck protein tyrosine kinase) phosphorylated at the tyrosine residue (50 nM), Cy5 anti PY20 antibody (100 nM) and Eu (TMT) Streptavidin (8 nM) were added to a 384 well microplate and incubated for 60 min at room temperature.

A signal:background of 6:1 was observed.

5.4. Model assays

Example biological applications data has been generated using reagents from the product range.

This data should be used as reference information for customers wishing to establish their own applications. This information should be used as a guide only and individual assay optimisation is the responsibility of the end user.

5.4.1. NF-κB protein:DNA binding assay

Nuclear Factor-κB (NF-κB) is a transcription factor that is considered to be of physiological importance because of its key role as a regulatory molecule in immune response, inflammation, cancer and apoptosis (5, 6). A TR-FRET assay to evaluate the binding interaction between the p65 subunit of NF-κB and a dsDNA NF-κB-specific (HIV-L) consensus sequence has been developed. (Figure 6).

Assays containing NF-κB p65-GST (University of St Andrews, UK) recombinant fusion protein (10 nM) and Eu (TMT) - anti-Glutathione S Transferase antibody (10 nM) were incubated in the dark with agitation for 1 hour at 20–25°C in 10 mM HEPES, 20 mM Sodium Acetate, 0.2 mM EDTA buffer, pH 7.0 containing 5 mM DTT, 1 mg/ml BSA and 0.05% NP40.

Sensitivity of detection was evaluated using a 2^n titration of 40 nM biotinylated dsDNA (containing the NF-κB consensus binding

sequence) and the reaction mix was incubated at 20–25°C for a further 1 hour in the dark with agitation.

For competition and inhibition assays, 20 nM biotinylated NF-κB specific dsDNA was added to the reaction mix and incubated as stated together with competitor dsDNA, IκBα protein (University of St Andrews, UK) or buffer respectively. Finally, Cy5 Streptavidin (10 nM) was added to each reaction well and incubated for a further 15 minutes.

Reactions were performed in a total volume of 100 µl using Corning black 384-well non-binding surface plates. TR-FRET was measured on FARCyte fluorescence plate reader using 340 nm excitation and 670 nm emission filter sets. Lag time was set at 50 µsec and integration time was 400 µsec.

The lowest limit of detection (sensitivity) of NF-κB specific dsDNA in the assay was 5.2 fmol/well (52 pM) as determined by titration (Figure 8). At maximal acceptor concentration (40 nM), signal: background for the assay was 9.6:1.

Competition for binding of specific and non-specific dsDNA to the p65-GST protein (Figure 9) indicated that signal is significantly reduced with the addition of the specific dsDNA competitor containing the
NF-κB p65 consensus binding sequence. The non-specific dsDNA reduced signal only marginally when added at considerable excess, demonstrating binding specificity.

Inhibition of protein:dsDNA binding was evaluated using IκBα protein. IκBα is a NF-kB regulatory protein found in the cell cytoplasm which inhibits DNA binding activity (7). Effect of inhibition by IκBα is shown in Figure 10 and demonstrates that increasing concentrations of the inhibitory protein reduced the signal in the assay. An IC_{50} value for the inhibition of NF-κB specific dsDNA binding to the p65 recombinant protein by IκBα was 15 nM.

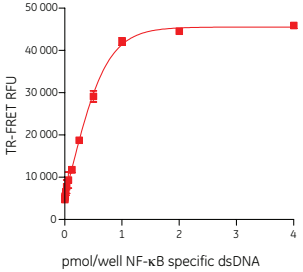

Fig 8. Sensitivity analysis of NF-kB : dsDNA binding assay

Specific biotinylated dsDNA was titrated from 40 nM with a constant 10 nM p65-GST and Eu (TMT) - anti-GST antibody. Cy5-Streptavidin acceptor was added to the biotinylated dsDNA at a 1:2 (w/w) concentration. Data plotted as quadruplicates, mean ± SEM.

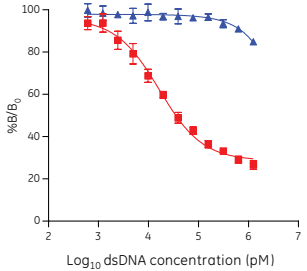

Fig 9. Analysis of the specificity of NF-κB : dsDNA binding

Competition of the biotinylated dsDNA for binding to the p65-GST recombinant protein was demonstrated using unlabelled NF-κB specific dsDNA sequence (■) and an unlabelled non-specific dsDNA

sequence (▲). dsDNA was titrated from a maximum concentration of 1 µM. Data plotted as triplicates, mean ± SEM.

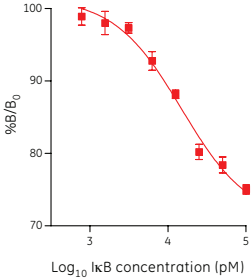

Fig 10. Inhibition of NF-κB : dsDNA binding by IκBα protein

IkBa protein was titrated with a constant 10 nM p65-GST recombinant protein at a maximum concentration of 100 nM. IC_{50} value determined from the assay was 15 nM. Data was plotted as triplicates, mean ± SEM.

5.4.2. Detection of phosphorylated peptides

Phosphorylation of peptide substrates is now a commonly used assay technique for determining activity and for high throughput screening of kinase enzyme targets and the use of time resolved-fluorescence resonance energy transfer (TR-FRET) assays has become a popular method by which this is performed (8). Here we demonstrate in a model system that Eu (TMT) anti Phosphotyrosine antibody and Cy5 Streptavidin can be used to measure the presence of phosphorylated peptide by TR-FRET using FARCyte fluorescence plate reader.

Assays were set up in buffer (50 mM HEPES pH 7.0 containing 10 mM $MgCl_2$, 1 mM DTT and 0.1% (w/v) BSA containing peptide (30 µl) at a final concentration of 2 µM. This 'total peptide' consisted

of two separate biotinylated peptides, both 14 mers where one contained a single Phosphotyrosine residue and was added at 0, 0.31, 0.62, 1.25, 2.5, 5, 10 and 20% of the total. The remainder of the peptide being made up of the same peptide sequence but containing a Tyrosine residue in place of the Phosphotyrosine described above. To the mixture, for detection, was then added 10 µl (20 nM final concentration) Eu (TMT) anti Phosphotyrosine antibody and 10 µl (100 nM final concentration) Cy5 Streptavidin. Tests were therefore performed in a total volume of 50 µl using Corning black 384-well non-binding surface plates. Following the addition of the detection reagents, reactions were incubated for 60 minutes at ambient (22–25°C) with agitation for 60 minutes before TR-FRET signals were measured.

TR-FRET was measured on FARCyte fluorescence plate reader using 340 nm excitation and 670 nm emission filter sets. Lag time was set at 50 µsec and integration time was 400 µsec. The resulting signals observed are shown in Figure 11. As further confirmation of these results, analysis of the data by a statistical method commonly referred to as the 'Z'-factor' (9) indicated that when the phosphorylated peptide was added at 10% of the total peptide, a Z' value of 0.71 was observed.

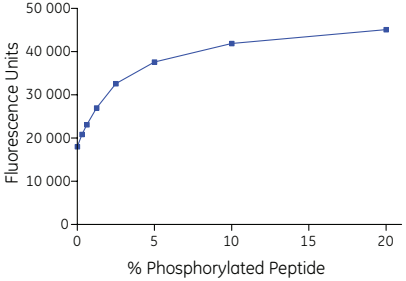

Fig 11. Detection of phosphorylated peptide

Peptide solutions were set up as described and phosphorylated peptide detected by the addition of Eu (TMT) anti Phosphotyrosine antibody and Cy5 Streptavidin. TR-FRET signals were then measured on FARCyte fluorescence plate reader. Data was plotted as means of 24 replicates ± SEM. For Z' analysis the number of replicates tested was 48 in the presence of 10% phosphorylated peptide.

These two application examples demonstrate the ability to use Eu (TMT) and Cy5 as donor and acceptor probes for sensitive determination of TR-FRET signal using FARCyte fluorescence plate reader.

The assays are seen to be effective for competition and inhibition studies.

6. References

1. Saha, A. K. *et al., J. Am. Chem. Soc.* **115**, 11032–11033 (1993).

2. Van Der Meer, B. W. *et al.*, Resonance Energy Transfer, Theory and Data, VCH Publishers, New York , ISBN 1-56081-643-0, (1994).

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6. Baldwin, A. S., *J. Clin. Invest.* **107**(1), 3–6 (2001).

7. Tran, K. *et al., Mol. Cell. Biol.* **17**(9), 5386–5399 (1997).

8. Park, Y-W. *et al, Anal Biochem.* **269**, 94–104 (1999).

9. Zhang, J-H. *et al., J. Biomolecular Screening* **2**, 67–73 (1999).

7. Related products

FARCyte™ Fluorescence Plate Reader	VF129017
Eu (TMT) Isothiocyanate	PA99140
Additional Cy5 reagents: all mono-reactive fluors	
Cy5 NHS Ester, 1 mg	PA15101
Cy5 NHS Ester, 5 mg	PA15105
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Cy5 NHS Ester, 25 mg	PA15106
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Cy5.5 NHS Ester, 5 mg	PA15605
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Cy5Q NHS Ester, 1 mg	PA75101
Cy7Q NHS Ester, 1 mg	PA77101
Cy3 NHS Ester, 5 mg	PA13105
Cy3.5 NHS Ester, 5 mg	PA13605
Cy3B NHS Ester, 5 mg	PA63100
Cy7 NHS Ester, 5 mg	PA17105
Cy5Q NHS Ester, 5 mg	PA75100
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Also available as 10 mg, 25 mg and 50 mg packs on request

Some bis-reactive fluors, available on request

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