

# A Homogeneous TR-FRET NF- $\kappa$ B Protein:DNA Binding Assay

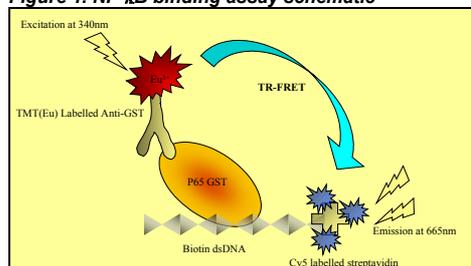
## Introduction

Nuclear Factor- $\kappa$ B (NF- $\kappa$ B) is a transcription factor that is considered to be of physiological importance because of its key role as a regulatory molecule involved in immune response, inflammation, cancer and apoptosis<sup>1,2</sup>.

We have developed a time resolved-fluorescence resonance energy transfer (TR-FRET) assay to evaluate the binding interaction between the p65 subunit of NF- $\kappa$ B and a dsDNA NF- $\kappa$ B-specific (HIV-L) consensus sequence.

The development of a TR-FRET NF- $\kappa$ B binding assay has been reported previously<sup>3</sup> using direct labelled p65 specific dsDNA. We have now further developed the assay to incorporate generic europium [Eu(TMT)] donor and Cy5 acceptor reagents (figure 1).

**Figure 1. NF- $\kappa$ B binding assay schematic**



Assay is configured using a europium chelate labelled anti-GST antibody specific for a p65-GST recombinant protein that interacts with a biotinylated NF- $\kappa$ B-specific dsDNA bound to Cy5 labelled streptavidin

## Method

Oligonucleotide sequences were prepared 'in house' using standard phosphoramidite chemistry. The 19 base-pair stretch of double-stranded DNA's yielded are shown in Table 1.

**Table 1. NF- $\kappa$ B assay dsDNA**

Biotinylated dsDNA (NF- $\kappa$ B-specific)	
5'	Biotin-GATCTAGGACTTTCCGCG 3'
3'	AT CCCTGAAGGCGCCTAG 5'
Unlabelled NF- $\kappa$ B-specific competitor dsDNA	
5'	GATCTAGGACTTTCCGCG 3'
3'	ATCCCTGAAGGCGCCTAG 5'
Unlabelled non-specific competitor dsDNA	
5'	GATCTATTGACTTAAGTG 3'
3'	ATAACTGAATTCACCTAG 5'

Oligo sequences were prepared using standard phosphoramidite chemistry and purified by C18 reverse phase HPLC. Double-stranded DNA was prepared by incubating equimolar amounts of the NF- $\kappa$ B-specific (HIV-L) coding (biotinylated or non-biotinylated) and unmodified non-coding strands in a 75°C water bath for 3-5 minutes before allowing to cool to ambient temperature.

NF- $\kappa$ B p65-GST recombinant protein (10nM) was incubated with Eu anti-GST fusion protein (10nM) in the dark with agitation. The reaction mixture was incubated for 1 hour at room temperature (20-25°C) in 10mM HEPES, 20mM Sodium Acetate, 0.2mM EDTA buffer, pH 7.0 containing 5mM DTT, 1mg/ml BSA and 0.05% NP40.

Sensitivity was evaluated using a 2<sup>n</sup> titration of 40nM of biotinylated NF- $\kappa$ B specific dsDNA. For competition, inhibition, DMSO tolerance and Z' analysis assays, 20nM biotinylated NF- $\kappa$ B specific dsDNA was added to the reaction mix and incubated at room temperature for a further 1 hour in the dark with agitation. Finally, Cy5 streptavidin (10nM) was added to each reaction well and further incubated for 15 minutes. Reactions were performed in a total volume of 100 $\mu$ l using Corning black 384-well NBS plates. TR-FRET was measured on the FARGe™ fluorescence plate reader using

340/35nm excitation and 670/11nm emission filter sets. Lag time was set at 50 $\mu$ s, integration time was 400 $\mu$ s with 30 flashes/well.

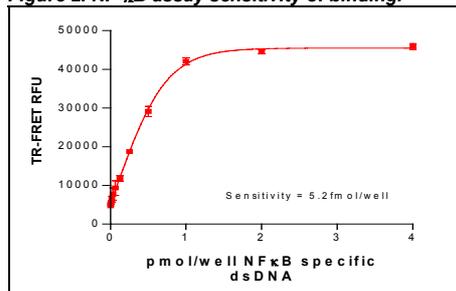
DMSO is a solvent commonly used in assay buffers and preparations. Exposing the sample wells in an assay to varying amounts of the solvent was performed to evaluate of the tolerance of the assay to DMSO. DMSO was added to the sample wells at a maximal concentration of 10% of the well volume.

## Results

Lowest limit of detection (sensitivity) of NF- $\kappa$ B specific dsDNA in the assay was 5.2fmol/well (52pM) as determined by titration with 10nM recombinant protein (figure 2). At maximal acceptor concentration (40nM), signal:noise for the assay was 9.6:1.

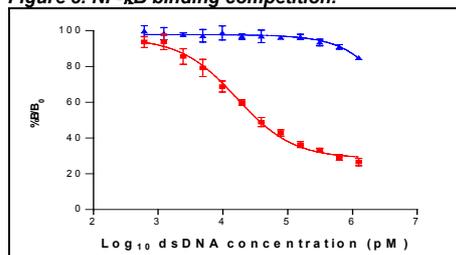
Competition of binding to the p65-GST protein (figure 3) demonstrated that signal is significantly reduced with the addition of the specific dsDNA competitor containing the NF- $\kappa$ B p65 consensus binding sequence, whereas the non-specific dsDNA reduced signal only marginally when added at considerable excess, demonstrating binding specificity.

**Figure 2. NF- $\kappa$ B assay sensitivity of binding.**



Specific biotinylated dsDNA was titrated from 40nM with a constant 10nM p65-GST and europium chelate labelled anti-GST antibody. Cy5 labelled streptavidin acceptor was added to the biotinylated dsDNA at a 1:2 (w/w) concentration. Data plotted as quadruplicates, mean  $\pm$  SEM.

**Figure 3. NF- $\kappa$ B binding competition.**

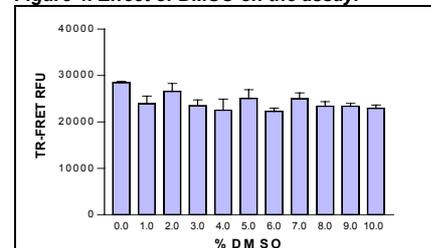


Competition of the biotinylated dsDNA for binding to the p65-GST recombinant protein was demonstrated using unlabelled NF- $\kappa$ B specific dsDNA sequence (■) and an unlabelled non-specific dsDNA sequence (▲). dsDNA was titrated from a maximum concentration of 1 $\mu$ M. Data plotted as triplicates, mean  $\pm$  SEM.

Evaluation of the assay to DMSO tolerance showed that no significant effect was evident even at the highest DMSO content of 10% of the volume in the well (figure 4).

Inhibition of protein:dsDNA binding was evaluated using I $\kappa$ B $\alpha$  protein. I $\kappa$ B $\alpha$  is a NF- $\kappa$ B regulatory protein found in the cell cytoplasm which inhibits its DNA binding activity. Effect of inhibition by I $\kappa$ B $\alpha$  is shown in figure 5 and demonstrates that increasing concentrations of the inhibitory protein reduces the amount of signal in the assay as less binding occurs. IC<sub>50</sub> values for the inhibition of NF- $\kappa$ B specific dsDNA binding to the p65 recombinant protein by I $\kappa$ B $\alpha$  in the assay was 15nM.

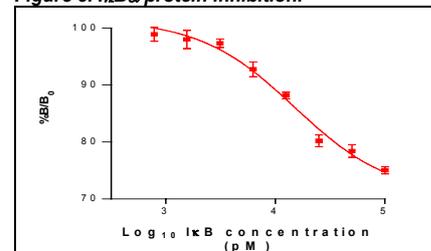
**Figure 4. Effect of DMSO on the assay.**



Evaluation of the effect of DMSO solvent on the assay signal. Data is plotted as quadruplicates  $\pm$  SEM.

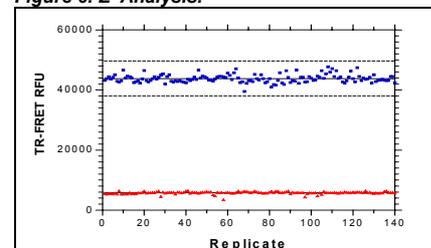
Z' factor analysis was performed as described by Zhang *et al.* Results of the analysis are shown in figure 6. Assays with a Z' factor between 0.5 and 1 are considered to be robust and reliable. The NF- $\kappa$ B protein:DNA binding assay that we have developed has a Z' factor of 0.87.

**Figure 5. I $\kappa$ B $\alpha$  protein inhibition.**



I $\kappa$ B $\alpha$  protein was titrated with a constant 10nM p65-GST recombinant protein at a maximum concentration of 100nM. IC<sub>50</sub> value determined from the assay was 15nM. Data was plotted as triplicates, mean  $\pm$  SEM.

**Figure 6. Z' Analysis.**



10nM of p65-GST recombinant protein was incubated with 10nM of Eu anti-GST antibody and 10nM Cy5 streptavidin in the presence (■) or absence (▲) of NF- $\kappa$ B specific dsDNA. Z' factor analysis was evaluated over 140 sample wells. Z' = 0.87.

## CONCLUSION

- We have demonstrated the ability to measure NF- $\kappa$ B specific dsDNA binding to a p65-GST recombinant protein by TR-FRET using generic reagents and measuring on FARGe.
- The assay is both robust and sensitive using generic TR-FRET reagents.
- The results highlight the potential of the FARGe Fluorescence Plate Reader for measurement of sensitive TR-FRET assays.

## References

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