

Barbara Saxty
MRC Technology 1-3 Burton hole lane, Mill Hill London, NW7 1AD, UK

Application Note 124

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- Fluorescence Polarization screen configured on POLARstar Galaxy
- Rapid detection of false positives
- Identification of interfering compounds and non specific inhibitors

Introduction

Fluorescence polarization (FP) provides a useful tool with which to screen for inhibitors of the interaction of proteins with defined peptides, and many examples have been cited in the literature.¹⁻³ Fluorescence polarization gives a measure of the proportion of peptide found in the bound state in a homogeneous format. However compound interference and non-specific gross structural changes to the protein can give rise to a large number of false positives, which are only identified in later stage biochemical assays. Strategies to eliminate these at an early stage of screening will accelerate the hit to lead process.

Here we present data from an FP screen configured on the POLARstar Galaxy for the interaction of the retinoblastoma tumor suppressor protein (pRB) with E2F peptide. Fluorescein-tagged E2F peptide was used to screen 10,000 small drug like molecules. Hit confirmation strategies based on fluorescence interference and specificity were developed.

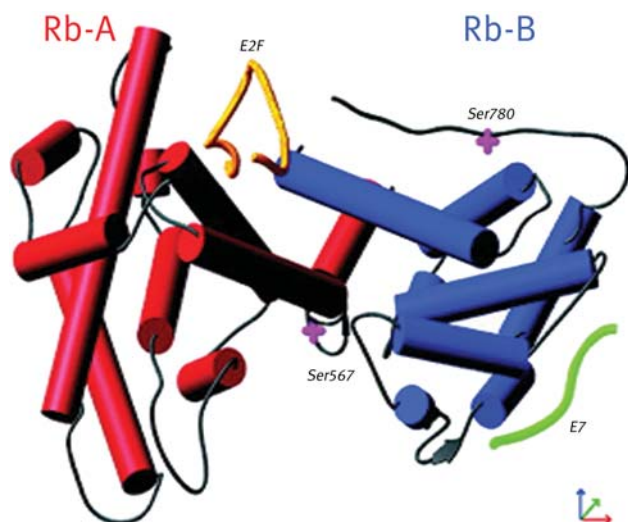


Fig. 1: Model of the interaction of pRB with E2F and E7 peptides

Based on the crystal structure, an FP screen was configured for the interaction of recombinant pRB A/B domains with E2F peptide (depicted in yellow in figure 1).⁴ In addition, a second peptide binding site (E7, depicted in green), distant from the E2F binding pocket, was utilised as an internal control for non-specific inhibitors. Fluorescein-E7 and rhodamine-E2F labelled peptides were synthesised and were used for hit confirmation.

Materials and Methods

Peptides were synthesised and fluoro-tagged using either N-terminal labelling with 5-carboxyfluorescein succinimidyl ester or cysteine labelling with single isomer tetramethylrhodamine-5-maleimide. Following purification on RP-HPLC and confirmation of purity by mass spec, peptides were freeze dried and stored -20°C. Immediately before use peptides were dissolved in assay buffer (50 mM Tris HCl, pH 7.0, 100 mM NaCl, 10 mM DTT, 0.05% NP-40).

Recombinant pRB was expressed in *E. coli* and purified using His-tag. Typical titration binding curves of pRB with the fluoro-labelled peptides are shown (mean±sem, n=3) in figure 2. Fluorescein-E2F showed the greatest degree of polarization, and consequently the best signal to noise. It was chosen as the label of choice for a primary screen. Data were fitted to a one site binding model using Graphpad prism.

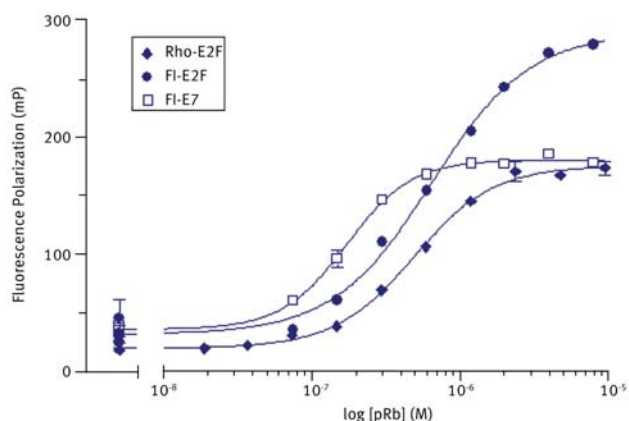


Fig. 2: Binding of fluorescein-E2F, rhodamine-E2F and fluorescein-E7 to pRB

K_d values of (450±70) nM and (380±50) nM were calculated for fluorescein and rhodamine labelled E2F, which were similar to K_d determined for unlabelled peptide using isothermal calorimetry. Fluorescein-E7 showed tightest binding with K_d =(130±20) nM.

The assay was optimised in 384-well black plates (Matrix) and automated using a Beckman Fx liquid handling robot. 1 µM pRB in 50 mM Tris HCl, pH 7.0, 100 mM NaCl, 10 mM DTT, 0.05% NP-40 was mixed with 40 µM compound (4% DMSO) and 0.4 µM fluorescein-E2F (final concentrations). Total reaction volume 50µL. Controls from a test screen of 10,000 compounds are shown in figure 3.

POLARstar Galaxy settings

Fluorescein detection: λ_{ex} 485-12 nm, λ_{em} 520-30 nm
 Rhodamine detection: λ_{ex} 545-7 nm, λ_{em} 580-12 nm
 Default general settings: 50 flashes, 1.0 s positioning delay, 1 cycle
 Software version: fluo32 version 4.31
 Gains were set for ch1 and ch2 using free fluoro-tagged peptide to give mP=35 and k=1

Results and Discussion

Polarized and depolarized signal from fluorescein-E2F with and without pRb present are shown in figure 3 (solid and open blue circles respectively). Specific disruption of binding by E2F protein and peptide are also shown.

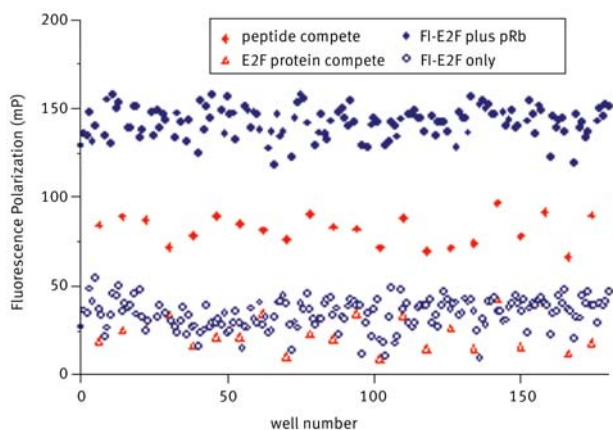


Fig. 3: Controls from a test screen of 10,000 compounds

Addition of E2F protein completely displaces FI-E2F (open red triangle) and the signal is reduced to that of free fluoro-peptide alone. Addition of unlabelled-E2F peptide (solid red diamond) at a concentration which gave 50% inhibition is clearly separated from the control populations. Hits were identified as compounds which reduced the polarization signal to less than mean-3sd of the fluorescein-E2F: pRb control. A summary of the screen data is shown below in table 1.

Table 1: Summary of screen data

Assay Principle		Fluorescence Polarization
Assay Automation		Biomek Fx
Assay Detection		BMG LABTECH
		POLARstar Reader
Assay Parameters	Signal: Noise	6.9
	Signal: Background	4.8
	Z'	0.67
Test Screen 10,000	Z	0.45
	Hit Rate	0.93%

A large proportion (37.5%) of the hits selected from the primary screen were coloured compounds which significantly altered the fluorescence intensity signal, and were potentially interfering with the assay. All hits were included in the hit confirmation assays.

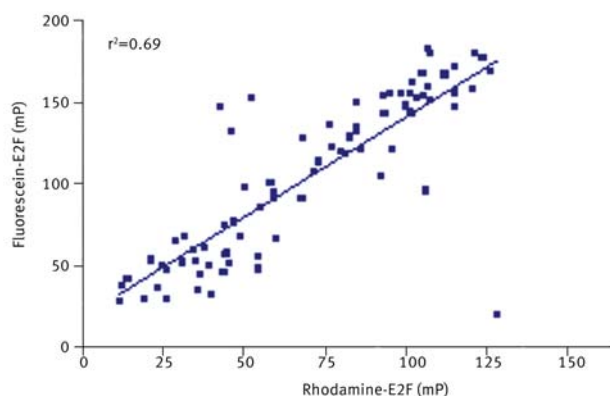


Fig. 4: Identification of fluorescence interfering compounds and correlation between inhibition of rhodamine-E2F and fluorescein-E2F

Hits were replated from master stocks and retested against fluorescein-E2F and rhodamine-E2F. A correlation ($r^2=0.69$) between inhibition of fluorescein-E2F and rhodamine-E2F was observed (figure 4) with a hit confirmation rate of 78%. Notably, 60% of compounds which were potentially interfering with the fluorescein signal were inhibitors with rhodamine-E2F assay, without affecting rhodamine fluorescence intensity signal. Suggesting that deselection of compounds on the basis of fluorescence interference can lead to loss of real inhibitors.

Finally the hits were tested against a second peptide binding site, fluorescein-E7 peptide at 400 nM, as shown in figure 5. The results were compared to inhibition of E2F and a scatter plot is shown.

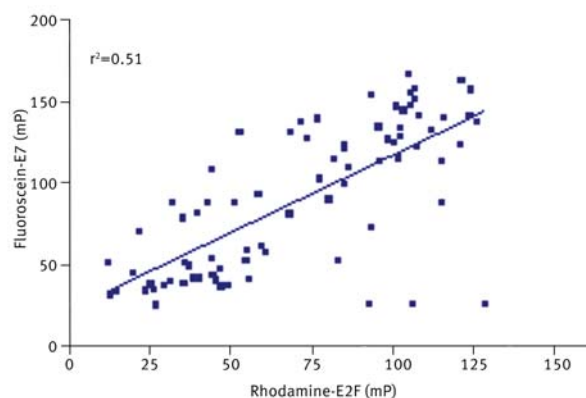


Fig. 5: Correlation between inhibition of fluorescein-E2F and fluorescein-E7 and identification of non-specific hits

A weak correlation was observed ($r^2=0.51$), with 72% of the inhibitors of E2F also inhibiting fluorescein-E7. These compounds were excluded as non-specific inhibitors and were not taken forward in subsequent biochemical assays. 14 hits were taken forward from a total of 80 hits identified in the primary screen.

Conclusion

Binding of fluorescein-E2F to pRb showed a greater degree of polarization compared to binding of rhodamine-E2F, and consequently a better signal to noise ratio and z-factor could be obtained. It was the label of choice for a primary screen. However the use of a red-shift fluoro-label, such as rhodamine for hit confirmation, allowed identification of true inhibitors from potentially interfering compounds. Screening of the hits against the second peptide site, E7, identified non-specific inhibitors, which caused gross structural changes to the protein. These were excluded from further biochemical testing. The combination of rhodamine-E2F and fluorescein-E7 with pRb in the same well allowed rapid selection of specific inhibitors, and minimised reagent usage. Identification of these non-specific inhibitors dramatically reduced the down stream work load.

References

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Germany: BMG LABTECH GmbH
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www.bmglabtech.com

Tel: +49 781 96968-0
 Tel: +61 3 59734744
 Tel: +86 10 6424063
 Tel: +33 1 48862020
 Tel: +44 1296 336650
 Tel: +1 919 806 1735
info@bmglabtech.com