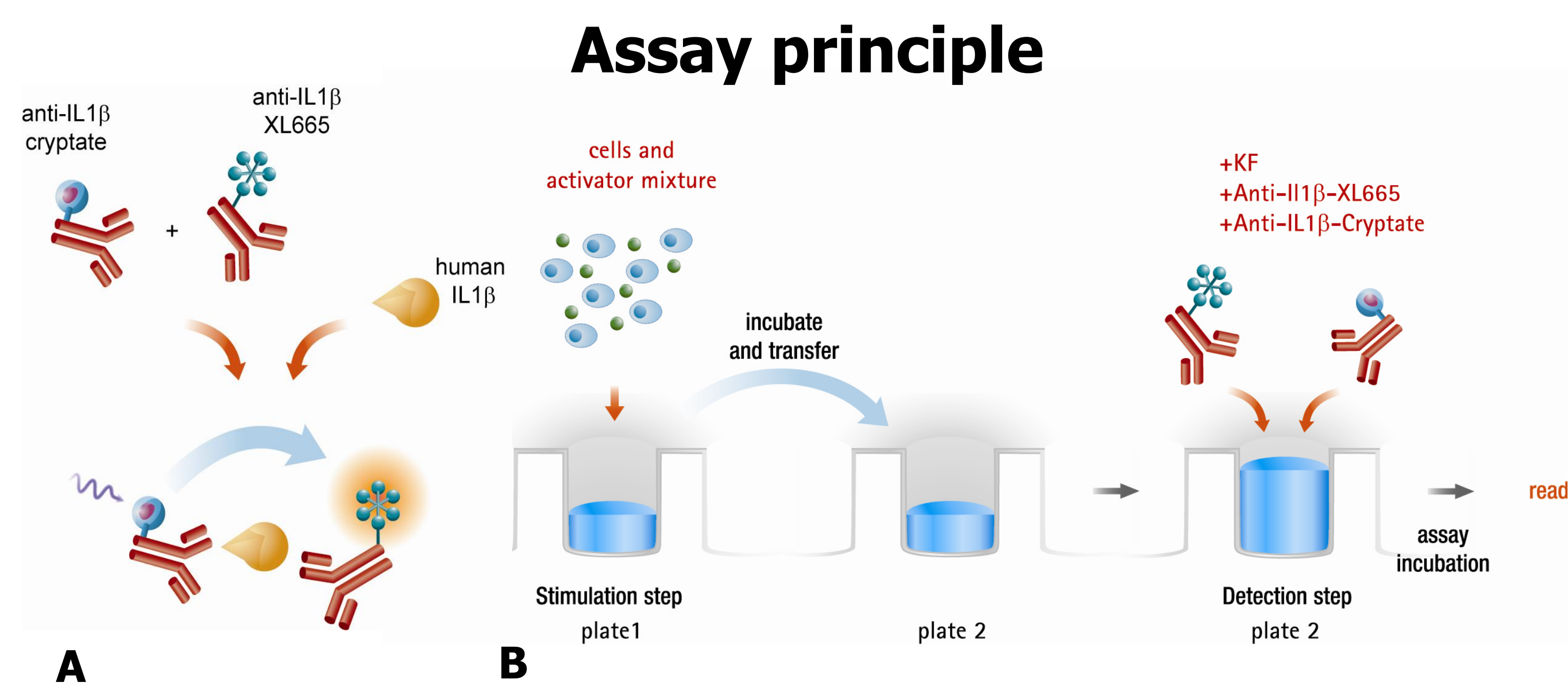


## Introduction

The detection of cytokines in cell culture medium is a widely used method for screening of inflammatory modulators and a number of assay kits are available for this purpose. We describe the results from a recent screen employing the use of the HTRF® detection platform from Cisbio to identify compounds that modulate interleukin 1-beta (IL-1β) secretion without affecting release of tissue necrosis factor alpha (TNFα). Subsequent experiments demonstrated false positives. These were due to compound fluorescence seen in the HTRF assay when detecting IL-1β due to the presence of serum in the IL-1β HTRF assay.

- 50,000 compounds screened using the Cisbio HTRF human IL-1β kit
- 1,000 compounds taken through to hit confirmation
- 250 compounds screened as concentration response curves in both the IL-1β and the TNFα assay



**Figure 1. Assay principle for detection of human IL-1β (Panel A)**

The supernatant assay protocol was used (B) and run in two separate microplates; a culture plate for cell stimulation and an assay plate in which cytokine detection was carried out. The TNFα assay works on the same principle with the same detection reagents but different recognition antibodies.

### Compound interference in time resolved fluorescence assays

The use of a fluorophore with a long emission life time such as europium (donor emission wavelength = 620 nm) is designed to minimize compound interference artefacts which arise from compound fluorescence interference that is relatively short lived. Higher 620 nm values would result in smaller ratio values as generated from the equation below. In this assay scenario, low ratio values were indicative of an active compound.

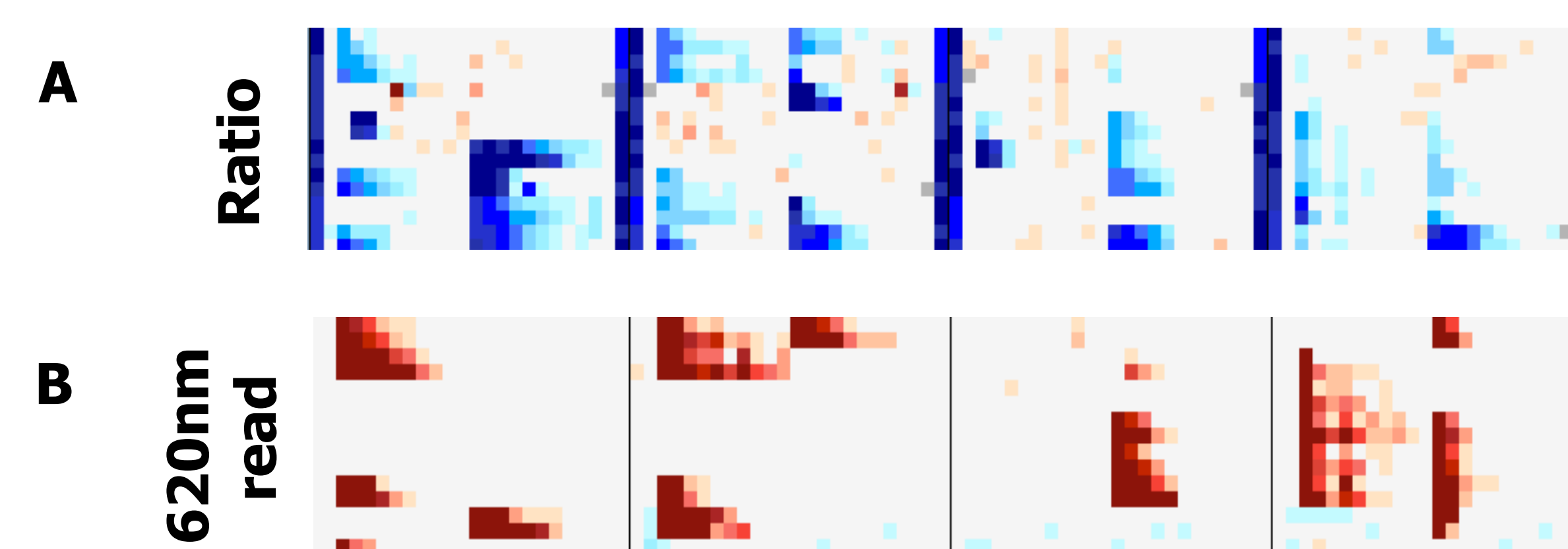
$$\text{Ratio} = \left( \frac{665\text{nm (acceptor}_{Em})}{620\text{nm (donor}_{Em})} \right) \times 10^4$$

Assay hits were expected to be the result of a consistent, and low, 620 nm value and a reduced 665 nm reading. During the screening process, compounds were highlighted in the database where the 620 nm value was 1.5 fold greater than the plate median. Although any spurious compounds were flagged at primary and hit confirmation screens, no compound was discarded at this stage so as not to eliminate any potential compounds that may have a degree of activity being masked by any changes in 620 nm values.

## Results

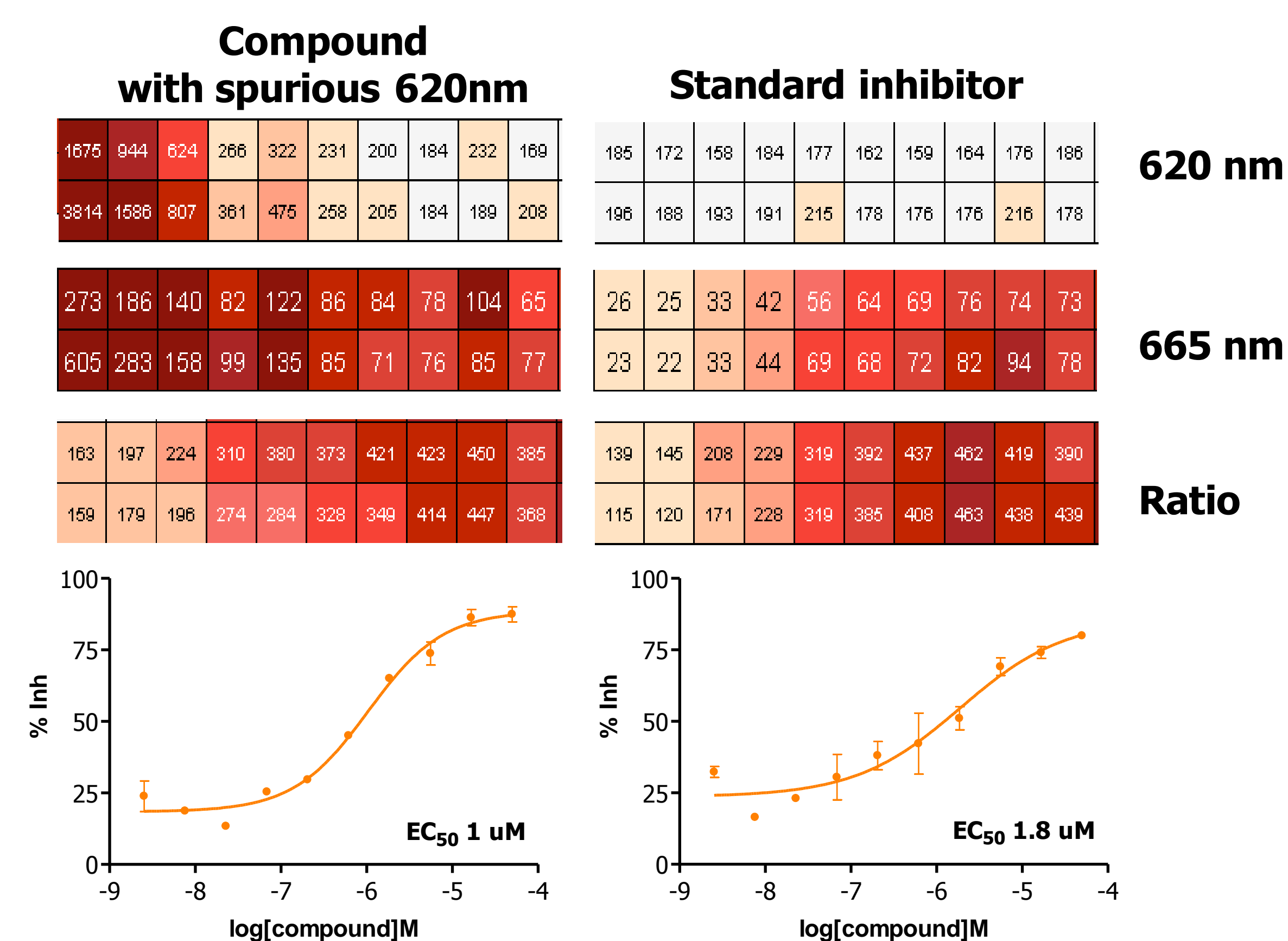
### Compounds were identified giving a concentration dependent response in the 620 nm read

At the potency determination phase it was noted that a number of the hit compounds showed elevated 620 nm responses. This finding was not seen in the TNFα assay, indicating an IL-1β assay specific anomaly.



**Figure 2. Genedata ratio depiction of the hits in CRC format (Panel A).**

A standard inhibitor was present in the bottom right position on each plate. Data shows a number of potentially active compounds (A). However, when the 620 nm read is investigated further (B) it reveals that some of these compounds displayed a high, often concentration dependent, 620 nm value, leading to a low ratio rather than reduced IL-1β secretion.



**Figure 3. Comparison of a high 620 nm false positive with the standard inhibitor**

The data shows how the test compound increased 620 nm values and inverted the expected 665 nm values to generate a ratio profile consistent with an active compound.

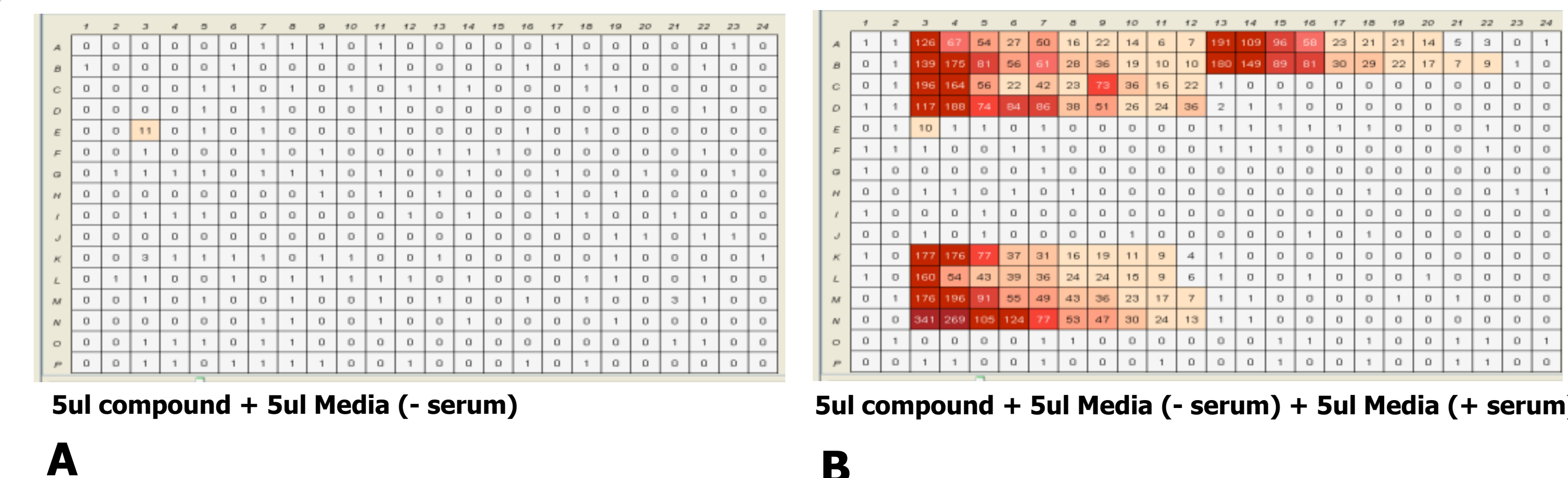
### Investigation of possible causes of the false positive readings

There are a number of possible causes, such as compound auto-fluorescence, kit reagent interference or media component interference.

To investigate the issue further and conclude whether the problem is kit or compound related, fluorescence readings were taken at the appropriate wavelengths for:

- compound alone
- compound + media, no serum
- compound + media, with serum
- compound + media, with serum + kit reagents

Media used: Gibco RPMI + Glutamax, Serum: 10% BioSerum, 0.2 μM PES

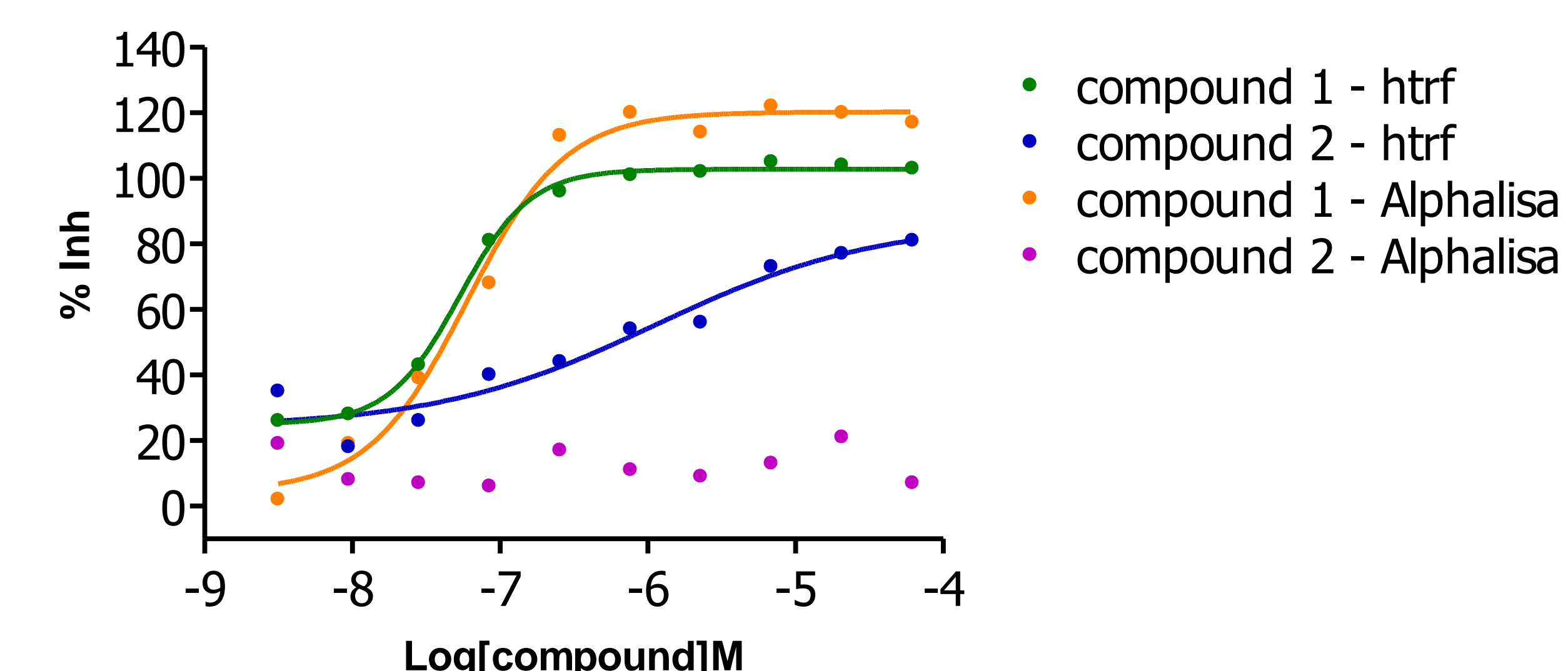


**Figure 4. Genedata visualization of a compound plate showing false positive compounds detected from the screen**

Compound fluorescence was measured in the absence (A) and presence (B) of serum, both in the absence of HTRF kit components. Plates were read on an EnVision™ using the same protocol as used for the screen. The compounds are clearly fluorescent when incubated in the presence of serum. As these compounds did not show a similar fluorescence pattern in the TNFα assay it would be unlikely that the issue is with the kit reagents as the basic principle is the same with both assays, only the antibodies differ and in our hands serum was excluded from the TNFα protocol.

### Perkin Elmer AlphaLISA® IL-1β detection kit

All compounds with putative activity were tested using an orthogonal IL-1β assay platform (AlphaLISA, Perkin Elmer) to confirm the inhibition of cytokine secretion. A number of compounds had confirmed activity in both assay formats and for these compounds the 620 nm emission reading was consistent across the concentration range tested. However, a significant number of compounds, largely from the same chemical series, were active in the HTRF format only and all showed elevated 620 nm emission values.



**Figure 4. AlphaLISA screening results for selected compounds**

Compound 1 was identified as a potential active in the HTRF assay and where the 620 nm was within the normal range was confirmed using AlphaLISA with comparable IC<sub>50</sub> values 53 nM and 59 nM, respectively. For compound 2 the activity using HTRF was associated with increased 620 nm emission and did not confirm in the AlphaLISA platform, indicating the activity was due to a fluorescence artefact.

## Conclusions

- a number of apparent hit compounds were subsequently identified as being false positives through the use of an orthogonal IL-1β platform (AlphaScreen®, Perkin Elmer)
- compounds can interact with serum to generate a long-lived fluorescence in the same region of the spectrum as europium emission