**THE EFFECT OF SUBSTRATE CONCENTRATION ON THE IDENTIFICATION OF HEPATITIS C VIRUS PROTEASE INHIBITORS USING A HIGH-THROUGHPUT SCINTILLATION PROXIMITY ASSAY**

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**Introduction**

A Scintillation Proximity Assay (SPA) has been developed for NS3 protease, a virally encoded serine protease which is involved in proteolytic processing of the viral polyprotein necessary for replication of Hepatitis C virus (HCV). NS3 protease is a major target for anti-HCV drug discovery, and a homogenous assay has been developed suitable for high throughput screening for the identification of HCV NS3 protease inhibitors.

The sequence of the peptide substrate used was Biotin-DEECAHLPYK-propionyl-His$_2$-NH$_2$.

When using PVT beads, enough bead had to be added in-vivo complex formation with the viral NS4A protein that acts as a cofactor. This was done by incubating the protease with NS4A cofactor, in the ratio of 100:1 cofactor to protease, in assay buffer at room temperature for 5 minutes, with gentle shaking, prior to addition to assays. Assays were incubated at room temperature for 30 minutes with gentle agitation before being stopped by the addition of 0.5mg streptavidin coated polystyrene beads.

**Method and Results**

**Development of HCV protease assay.** As part of the assay validation, substrate cleavage as a function of HCV NS3 protease concentration and time were investigated (Figures 1 and 2).

**Inhibition of NS3 protease.** The effect of substrate concentration on inhibition of NS3 protease by the N-terminal cleavage product was studied. IC$_{50}$ values were determined using two substrate concentrations; 0.05µM, using labelled peptide only, and 50.05µM, which was approximately twice the $K_m$. The $K_m$ for the peptide substrate was found to be 26µM (data not shown). The high substrate concentration was achieved by the addition of unlabelled peptide that had been biotinylated at the C-terminus. The N-terminal cleavage product acted as a competitive inhibitor of NS3 protease, and consequently, substrate concentration was shown to affect the IC$_{50}$ resulting in a higher IC$_{50}$ value at the higher concentration of substrate. The IC$_{50}$ values at 50.05µM and 0.05µM substrate were 28.0µM and 8.4µM, respectively (Figure 3).

The use of 1.5µM substrate resulted in a high hit rate which was reduced by increasing the substrate concentration to 6.1µM. An increase in substrate concentration had an impact on the assay format since 1mg streptavidin coated PVT SPA bead was not sufficient to capture all the biotinylated species. The binding capacity of the beads is approximately 100pmoles of biotin per mg of bead.

Assays were incubated at 37°C for 30 minutes with gentle agitation and stopped by the addition of 0.5mg streptavidin coated PVT SPA beads. Plates were counted using a TopCount™ microplate scintillation counter. Each data point in this and subsequent figures is the mean (± SEM) of 3 replicates.

<table>
<thead>
<tr>
<th>Type of bead</th>
<th>Streptavidin coated PVT</th>
<th>Streptavidin coated YSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of bead</td>
<td>3.5µg (excess)</td>
<td>1.75µg (limiting)</td>
</tr>
<tr>
<td>Percentage peptide cleavage detected</td>
<td>33.6%</td>
<td>24.3%</td>
</tr>
<tr>
<td>µM</td>
<td>1.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

When using PVT beads, enough bead had to be added to capture all the biotinylated species present. For YSI beads, a limiting amount of bead could be used. The peptide substrate was more hydrophobic than the cleaved product, so in conditions where only a proportion of biotinylated species was captured, binding of the hydrophobic substrate was favoured. This effect was not seen when less hydrophobic YSI beads were used. This experiment highlights the importance of optimising the concentration of assay components prior to screening.

**Conclusions**

- A homogenous assay has been developed for HCV NS3 protease suitable for screening coloured natural product samples.
- Substrate concentration has been shown to effect IC$_{50}$ values and hit rates by altering the proportion of inhibitors identified.
- The importance of optimisation of assay components such as bead concentration is shown.

**References**


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