# AN SPA ASSAY FOR RNA HELICASE USING A DUPLEX [$^{33}$P] RNA SUBSTRATE

**[1]Robert Graves*, [1]Betty Howard, [2]Penny Owen and [2]Gerard Brophy**

[1]*Amersham Biosciences Inc., 800 Centennial Avenue, Pistcataway, NJ 08855-1327, USA (Telephone 1 888 SPA HITS, Fax 732-457-8246, e:mail spa.hits@am.amershambiosciences.com)*

[2]*Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England (Telephone +44 1222 526417, Fax +44 1222 526474, e:mail Cardiff.SPA.Group@eu.amershambiosciences.com)*

## Introduction

The Hepatitis C viral (HCV) RNA helicase enzyme (NS3) has become a major focus in the search for drugs to counter infection with this virus[1]. The enzyme is both a proteinase thought to be involved in viral replication, and an RNA helicase involved in RNA splicing, ribosome assembly and the initiation of translation and transcription[2]. An SPA assay for HCV RNA helicase activity has been developed, utilizing a duplex [$^{33}$P]labelled RNA substrate.

## Assay Design and Results

To construct the duplex RNA substrate, the complementary vectors pSP72 and pSP73 (Promega) were linearized using *Nde 1* (pSP72), and *BamH1* (pSP73), and RNA transcripts prepared using an Ambion MAXIscript™ *In Vitro* Transcription Kit. The RNA transcript (p73, 57mer complementary to bps 62-97 of unlabelled strand) from the vector pSP73 was labelled to a specific activity of 2 x $10^9$cpm/µg with [$\alpha^{33}$P]UTP (Amersham Biosciences) and was hybridized to a 10-fold excess of the unlabelled RNA strand (p72, 187bps) transcribed from the vector pSP72. Assay design was similar to the SPA assay for DNA helicase[3]. The duplex RNA substrate and enzyme were incubated in 0.1ml 25mM MOPS pH6.5, 1mM $MnCl_2$, 2mM DTT, 4.5mM ATP and 2units/ml ribonuclease inhibitor. The enzyme-mediated unwinding of the duplex was then measured by adding an excess of biotinylated DNA oligonucleotide (50mer) complementary to the labelled strand in 0.1ml capture buffer (PBS containing 30mM EDTA and 0.1% (w/v) SDS). Following incubation for 30 minutes at 37°C (microplate assay) or 45°C (microtube assay), the biotinylated labelled RNA:DNA hybrids were bound to streptavidin-coated PVT SPA beads and counted. It was necessary to reduce the non-specific binding (NSB) of the duplex RNA substrate to the beads, which was achieved by addition of 1M NaCl and 0.2% (w/v) BSA to the capture buffer (Figure 1).

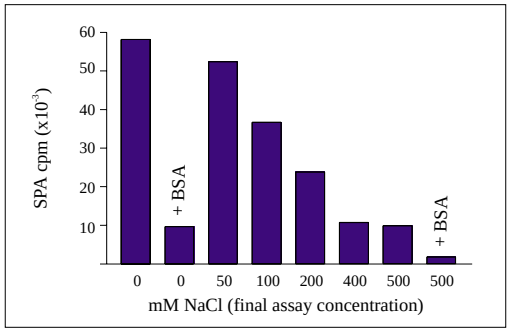


*Figure 1. Reduction in NSB of duplex [$^{33}$P]RNA to SPA beads. 100,000cpm (LSC) duplex RNA was incubated for 30 minutes in microtubes with 0.5mg streptavidin-coated PVT SPA beads in 100µl assay buffer and 100µl capture buffer, with 0-500mM NaCl, +/- 0.1% (w/v) BSA (final concentrations). The microtubes were centrifuged at 9000rpm for 5 minutes and counted for 1 minute in a Packard 1500 TriCarb™ liquid scintillation counter.*

The pH optimum for the helicase activity was 6.5 (Figure 2), which is consistent with published studies[2]. This experiment also included a control where the sense RNA strand (p72) to the capture oligonucleotide was [$^{33}$P]labelled. No specific signal above background was detectable. Also consistent with published work, the enzyme was sensitive to the presence of $MnCl_2$ in the assay buffer (Figure 3), and there was no activity when ATP was omitted (not shown).

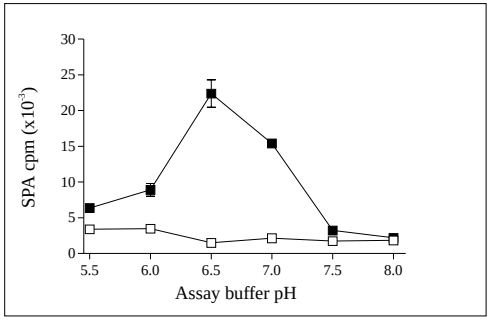


*Figure 2. Optimization of assay buffer pH in RNA helicase assay. (■) 200,000cpm (LSC) duplex [$^{33}$P]p73:p72 RNA substrate was incubated with 0.5µg (1µl) purified NS3 RNA helicase in 100µl assay buffer in microtubes for 30 minutes at 37°C, followed by addition of 100µl capture buffer containing 18 pmol biotin-DNA capture oligonucleotide, and incubation for 30 minutes at 45°C. 0.5mg streptavidin-coated SPA beads were added in 20µl PBS containing BSA (to 0.1% (w/v) final assay concentration). After 10 minutes at room temperature the tubes were centrifuged and counted as in figure 1. (□) Substrate was also prepared with the p72 strand labelled ([$^{33}$P]p72:p73). Results are presented +/- SEM (n=3).*

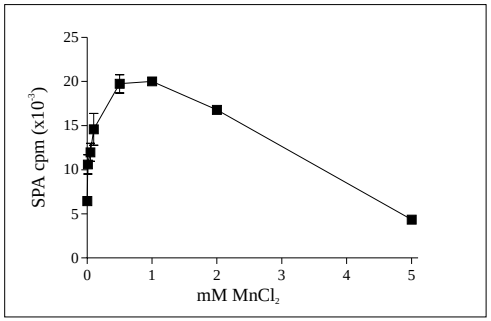


*Figure 3. Effect of assay buffer $MnCl_2$ concentration on HCV RNA helicase activity. Assay conditions were otherwise as for figure 2. (+/- SEM, n=3)*

The maximum helicase activity measured in these experiments represented about 75% total unwinding of the substrate. The activity was sensitive to the presence of ethidium bromide in the assay buffer with an $IC_{50}$ of approximately 6µM (Figure 4).

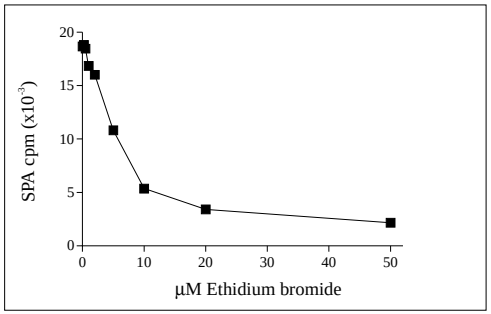


*Figure 4. Effect of increasing concentrations of ethidium bromide in the assay buffer on HCV RNA helicase activity. Assay conditions were otherwise as for figure 2 (+/- SEM, n=3).*

The assay was also adapted to a 96 well microplate, using either overnight bead settling or cesium chloride (CsCl) floatation[4] (Figure 5). The settled assays were performed exactly as in microtubes. For CsCl floatation, NaCl was added from a 5M stock to keep the final volume to 150µl after addition of 'stop'/capture reagents. Beads were then floated by adding 100µl 80% (w/v) CsCl. Optimum results were obtained using bead floating with the plate counted one hour after CsCl addition. When the CsCl-treated wells were recounted after leaving overnight, there was a significant increase in the background counts.

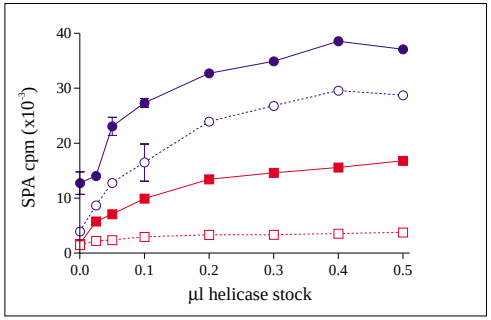


*Figure 5. 96 well microplate based assay for RNA helicase. Following addition of the beads and 'stop'/capture solution, the beads were either settled and the plate counted after 1 hour (□) or overnight (■), or CsCl added and the plate counted after 1 hour (○) or overnight (●). The microplate was counted using a Packard TopCount™. (+/- SEM, n=3)*

In a 384 well microplate the assay volumes were adjusted such that the final volume was 70µl following the addition of the 'stop'/capture reagents. Beads were settled overnight before counting. Signal: noise was highest using the lowest input of substrate counts in the assay (Figure 6). For the microplate assays, the incubation and the capture temperatures were 37°C.

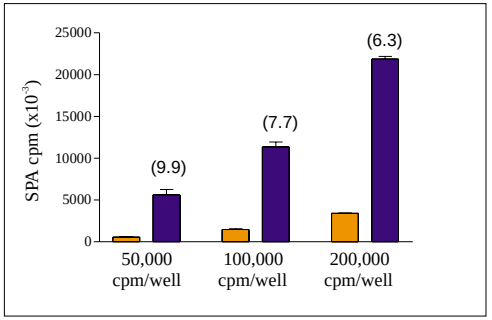


*Figure 6. 384 well microplate assay for RNA helicase. Assay volume was 50µl with (■) or without (■) enzyme (70µl final volume after capture), and the input cpm (LSC) for each well is indicated. The signal:noise ratios are given in brackets. The microplate was counted with a Wallac MicroBeta™. (+/- SEM, n=3).*

## Discussion

A signal increase SPA assay to measure HCV RNA helicase activity has been developed using a duplex RNA substrate in which one of the strands was [$^{33}$P]labelled. The assay was optimized in microtubes, then adapted for use in 96 and 384 well microplates, and in all formats a signal:noise ratio of 10:1 was achievable. The homogenous assay format makes it suitable for high throughput screening applications.

## References

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