**Traceless production of cyclic peptide libraries in *E. coli*.**

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***ABSTRACT***

**Split intein circular ligation of peptides and proteins (SICLOPPS) is a genetically encoded method for the intracellular production of cyclic peptide libraries of around a hundred million (108) members that utilizes the *Synechocystis sp* PCC6803 (*Ssp*) DnaE split inteins. However, *Ssp* inteins are relatively slow splicing and intolerant of amino acid variation around the splice junction potentially limiting the utility and composition of SICLOPPS libraries. In contrast, *Nostoc punctiforme* (*Npu*) DnaE split inteins not only splice significantly faster, they are also much more tolerant of amino acid variation around their splice junctions. Here we report the use of engineered *Npu* inteins in SICLOPPS for the generation of cyclic peptide libraries and cyclic proteins. Despite their superior splicing characteristics however, we observed a high level of toxicity from the *Npu* SICLOPPS constructs in *E. coli*. The observed toxicity was overcome though incorporation of an SsrA tag to target the spliced *Npu* inteins to the ClpXP complex for degradation. The resulting traceless *Npu* SICLOPPS inteins showed no toxicity to *E. coli*, demonstrating their potential for the production of cyclic peptide libraries for use in a variety of high-throughput screens.**

Cyclic peptides are a privileged class of bio-molecules that are increasingly utilized in early stage drug discovery, with a proven track record in the identification of protein-protein interaction inhibitors.*1-8* As such cyclic peptide libraries are increasingly deployed in high-throughput screening in both academia and industry. Despite this, there are few methods available for the production of cyclic peptide libraries in cells,*9-13* thereby enabling their use in genetic selection or phenotype screens. Split intein circular ligation of peptides and proteins (SICLOPPS),***12*** is a readily accessible method for the intracellular generation of cyclic peptide libraries of over a hundred million members***13, 14*** that uses *Synechocystis sp* PCC6803 (*Ssp*) DnaE split inteins.***15*** Each member of the library is expressed from a unique plasmid, generated using standard molecular biology techniques and a degenerate oligonucleotide. The C- and N-terminal intein fragments (IC and IN respectively) come together to form an active intein that splices to give a head-tail cyclized peptide (Figure 1A). The splice rate of *Ssp* inteins is relatively slow and sensitive to amino acids variation in the n+1 position, which is a significant limitation to SICLOPPS libraries. The intolerance to residue change is potentially a significant problem in library generation, as it suggests that a portion of a given SICLOPPS library do not splice. We therefore aimed to utilize inteins that splice faster and more efficiently, and are more tolerant to extein sequence variations. The *Nostoc punctiforme* (*Npu*) DnaE split inteins not only splice significantly faster, they are also much more tolerant of amino acid variation around their splice junctions, potentially making them potentially more suitable for library generation.*16, 17* The splicing efficiency and sequence tolerance of these inteins has been further improved through the incorporation of site-specific mutations.*18* We therefore sought to assess the potential for using these modified *Npu* inteins for the procustion of SICLOPPS cyclic peptide libraries.

***RESULTS AND DISCUSSION***

We began by building a plasmid encoding *Npu* split inteins in SICLOPPS format (IC-extein-IN), and using it as a template to construct a CX5 SICLOPPS library (where X= any amino acid). We initially assessed the splicing of the CX5 library by SDS-PAGE. The mechanism of SICLOPPS splicing (Figure 1A) involves formation of the active intein through association of IN and IC, followed by splicing to give the cyclic peptide product and the liberation of IN and IC as the by-products. It is not possible to accurately measure the quantity of cyclic peptide produced by SICLOPPS, but as detachment of IC is the last step prior to the formation of the cyclic peptide, the relative quantity of free IC serves as a good indication of the amount of spliced product.*12, 13*

Plasmids encoding a CX5 SICLOPPS library with either *Ssp* or *Npu* inteins were transformed into *E. coli*, and after recovery, expression of the library induced with arabinose. After 6 hours, each set of cells was lysed, and the SICLOPPS inteins isolated using the chitin-binding domain (CBD) present at the C-terminus of both *Ssp* and *Npu* IN. The isolated proteins were visualized by SDS-PAGE; a strong band was observed at the molecular mass associated with unspliced inteins for the *Ssp* library, as well as a band corresponding to IN (Figure 1B). The band associated with IC (and complete splicing) was however very weak, suggesting that a large portion of the *Ssp* CX5 library do not splice (Figure 1B). In contrast, the *Npu* CX5 library showed a weak band corresponding to the full length unspliced intein, with a strong band corresponding to IN and IC (Figure 1B). This suggests that in contrast to the *Ssp* inteins, a large majority of the *Npu* CX5 library splice to give the corresponding cyclic peptides. These observations are in line with previous studies that show *Ssp* inteins are not tolerant of amino acid change at the extein residues around the splice junction (a necessity for building libraries), whereas *Npu* inteins are highly tolerant of extein amino acid variation.*17*

We next assessed the effect of the *Ssp* and *Npu* CX5 SICLOPPS library on the viability of their *E. coli* hosts. We transformed the electrocompetent DH5α cells with plasmids encoding either an *Ssp* or *Npu* CX5 SICLOPPS library and after recovery, plated 100 L of a 1:10000 fold dilution of this solution onto LB-agar plates containing 30 g/mL of chloramphenicol (marker for SICLOPPS plasmid), with or without arabinose (inducer of SICLOPPS). These plates were incubated overnight and the number of surviving colonies quantified. We typically see transformation efficiencies of 107–108 with SICLOPPS plasmids, and in line with this, we observed around 100 colonies on plates containing only chloramphenicol for both *Ssp* and *Npu* libraries. We reasoned that by comparing the number of surviving colonies on chloramphenicol plates with the number of colonies that survive on plates containing both chloramphenicol and arabinose, the portion of each library that affect the viability of the host could be quantified. We therefore carried out a series of experiments where a given SICLOPPS library was transformed into *E. coli* and the resulting recovery mixture plated on the above 2 sets of plates; library members affecting host viability would cause cell death and lead to a reduced number of surviving colonies on plates where SICLOPPS was induced (+ arabinose). We observed 86 ± 7 % of the number of colonies on plates that induced production of the *Ssp* SICLOPPS CX5 library (compared to non-induced plates), indicating that ~14 % of the library members affect host viability (Figure 1C). For the *Npu* inteins however, only 58 ± 4 % of the expected colonies were present on plates containing arabinose (compared to non-induced plates), indicating that ~42 % of the members of the *Npu* SICLOPPS CX5 library affect host viability (Figure 1C). While it is likely that a portion of the cyclic hexamers encoded by a CX5 library are inherently toxic to *E. coli* (e.g. through interference with a critical protein or pathway), both sets of inteins are encoding the same library in the above experiment, the high level of toxicity observed with the *Npu* inteins can only be attributed to the *Npu* inteins themselves. To further assess this observation on the single peptide level, we constructed *Ssp* and *Npu* SICLOPPS plasmids encoding *cyclo*-CLLFVY, a cyclic peptide inhibitor of HIF-1 heterodimerization that was discovered from an *Ssp* SICLOPPS CX5 library.*7* The effect of the SICLOPPS construct that produces this peptide on *E. coli* viability was assessed by drop-spotting ten-fold serial dilutions onto plates containing 30 g/mL chloramphenicol with or without 1.3 M arabinose. Full growth of the bacterial host was observed with both plasmids when SICLOPPS has not been induced (Figure 1D, top plate), but significant toxicity was observed upon the induction of *Npu* SICLOPPS (as indicated by the absence of growth at higher dilutions), with no effect on viability from the *Ssp* SICLOPPS plasmid (Figure 1D, bottom plate).

The reason for the observed toxicity of *Npu* SICLOPPS inteins is unknown, but can only be due to the *Npu* inteins themselves, as this is the only variable between the two conditions in our experiments. The superior splicing and sequence tolerance of *Npu* inteins is well documented, but the above data suggests that they can not be used for SICLOPPS library generation, as a large portion of the library will not be present due to their toxicity to the host. For example, our results suggest that had *Npu* inteins been used in our HIF-1 screen, *cyclo-*CLLFVY would not have been identified, as cells expressing this peptide would not have survived.

We sought to overcome the observed toxicity of SICLOPPS *Npu* inteins by using a protein degradation tag to target the spliced inteins for intracellular degradation. We used the SsrA tag (AANDENYALAA) to for this purpose;*19-21* addition of the SsrA sequence to the C-terminus of a protein has been demonstrated to direct the tagged protein to the ClpXP machinery for degradation, reducing the half-life of the tagged protein to ~5 minutes.*22* The splice time of *Npu* intein has been extensively studies by others, and has been shown to be between 30–60 seconds.*16, 18, 23* We therefore reasoned that the addition of an SsrA tag to the *Npu* inteins would not affect cyclic peptide production, as the *Npu* inteins should splice prior to their degradation, but would cause degradation of spliced IN and IC (Figure 2A). It should be noted that any unspliced SICLOPPS protein would also be degraded after ~5 minutes. We generated a construct encoding SICLOPPS *Npu* inteins with an SsrA tag on the C-terminus of IN (*Npu-*SsrA). *E. coli* were transformed with an *Npu-*SsrA SICLOPPS CX5 plasmid library and expression of the inteins induced with arabinose. After 6 hours, the cells were lysed, and SICLOPPS protein isolated and visualized by SDS-PAGE. In line with our hypothesis, a band was observed for the unspliced SICLOPPS protein, but bands corresponding to IN and IC were not observed, indicating their degradation (Figure 2B). A band corresponding to the unspliced protein was observed (Figure 2B); this is likely to represent the pool of newly expressed protein that is awaiting degradation.

We next assessed the effect of the SsrA tag on the toxicity of the *Npu* SICLOPPS CX5 library to its *E coli* host, using the same approach as detailed above (in Figure 1C) for the *Ssp* and *Npu* inteins. We observed the same number of surviving colonies (± 6 %) on plates containing only chloramphenicol as on plates containing chloramphenicol and arabinose (Figure 2C). This data demonstrates that the addition of the SsrA tag to the *Npu* SICLOPPS inteins eliminates their toxicity to *E. coli*. We next probed this on the single peptide level with *cyclo-*CLLFVY as before. We again monitored host viability by drop-spotting 10-fold serial dilutions of cells containing the above SICLOPPS plasmids onto plates containing 30 g/mL chloramphenicol, with or without 1.3 M arabinose. Both sets of cultures displayed full growth in the absence of arabinose, while only cells encoding *cyclo-*CLLFVY with the *Npu-*SsrA SICLOPPS plasmid survived the induction of SICLOPPS with 1.3 M arabinose (Figure 2D). Together, our data demonstrates that by using an SsrA tag to degrade excess and/or unspliced inteins, the effect of *Npu* SICLOPPS inteins on *E. coli* viability is eliminated.

Although the splicing speed, efficiency and tolerance to extein sequence variation of *Npu* inteins has been extensively documented, it is possible that the reduction in toxicity observed from introduction of the SsrA tag is not due to elimination of spliced IN and IC as hypothesized by us, but instead due to the degradation of unspliced SICLOPPS inteins. In this scenario, SICLOPPS *Npu* inteins are degraded before they splice, resulting in the absence of any cyclic peptides in the host. While it would be challenging to accurately quantify intracellular cyclic peptide levels produced by a SICLOPPS library, we can readily assess the presence of a given cyclic peptide using a phenotype assay for its function. Given the high toxicity observed when replacing *Ssp* inteins with *Npu* inteins for the production of our HIF-1 inhibitor *cyclo-*CLLFVY, and its subsequent reversal upon introduction of an SsrA tag, we utilized this peptide and the bacterial reverse two-hybrid system (RTHS) used for its identification to probe this hypothesis.*7* The reverse two-hybrid system is based on the 434 and chimeric 434/P22 bacteriophage repressors, with the genes encoding target proteins (HIF-1α and HIF-1) integrated onto the chromosome of the host strain. HIF-1α is chromosomally expressed as an N-terminal fusion with a P22 bacteriophage repressor, and HIF-1 as an N-terminal fusions with the bacteriophage 434 repressor. In the absence of IPTG, the operator sites engineered onto the chromosome of the host are unoccupied, allowing expression of two chemically tunable and conditionally selective reporter genes (Figure 3A). The expression of HIS3 (imidazole glycerol phosphate dehydratase) and KanR (aminoglycoside 3’-phosphotransferase for kanamycin resistance) enables survival and growth of the host strain on selective media that lacks histidine and containing kanamycin.*24-26* In the presence of 50 M IPTG the target protein pairs are expressed and their dimerization (HIF-1α with HIF-1 in this case) leads to the formation of a functional 434/P22 repressor that binds chimeric 434/P22 operators on the chromosome of the RTHS strain. The reconstituted repressor inhibits the expression of the downstream reporter genes and prevents survival of the host cell on selective media (Figure 3B). Upon addition of 1.3 M arabinose to the above system, expression of the SICLOPPS construct that produces *cyclo-*CLLFVY is induced; as this cyclic peptide inhibits the HIF-1α/HIF-1 protein-protein interaction, the repressor complex is disrupted, allowing expression of the reporter genes and enabling host survival on selective media (Figure 3C).

Importantly for this experiment, we have previously demonstrated that only spliced *cyclo*-CLLFVY disrupts HIF-1 dimerization in this system;*7* a non-splicing variant of the parent intein formed via mutation of two residues in the C-intein (H24A, F26A)*27* resulted in loss of activity in the HIF-1 RTHS.*7* Unspliced full length, or partially spliced inteins encoding *cyclo-*CLLFVY are therefore unable to disrupt the HIF-1α/HIF-1 protein-protein interaction, with only the spliced cyclic peptide able to restore growth of the HIF-1 RTHS. We used this system to assess whether the effect from the SsrA tag is a result of *Npu* intein degradation prior or post splicing. The HIF-1 RTHS was transformed with one of three plasmids encoding *cyclo-*CLLFVY using *Ssp, Npu,* or *Npu*-SsrA SICLOPPS inteins. The effect of each plasmid on host survival and growth was monitored by drop spotting on selective media lacking histidine and containing kanamycin. In the absence of IPTG (no repressor proteins) and arabinose (no SICLOPPS) the non-induced plasmids did not affected growth of the host RTHS (Figure 3A). Repression of growth was observed for all plasmids on plates containing 50 M IPTG and no arabinose (Figure 3B) demonstrating that the non-induced SICLOPPS plasmids have no effect on the formation of a functional repressor. However, a difference was observed between the 3 plasmids upon induction of SICLOPPS on plates containing 1.3 M arabinose and 50 M IPTG (production of *cyclo-*CLLFVY in the presence of the HIF-1 repressor complex). The toxicity of the *Npu* SICLOPPS inteins observed in our earlier experiments was mirrored here, with the host cell unable to grow on selective media, despite induction of the construct encoding the HIF-1 inhibitor (Figure 3C, top row). In contrast, cyclic peptide expressed from either the *Npu-*SsrA or the *Ssp* plasmids enabled the survival and growth of the HIF-1 RTHS on selective media. This phenotypic assay demonstrates that the *Npu-*SsrA SICLOPPS inteins splice to yield *cyclo*-CLLFVY prior to their degradation by ClpXP.

We next sought to probe whether the effects observed above are limited to the production of cyclic peptides with *Npu* inteins, or also applicable to the production of cyclic proteins with SICLOPPS. We chose green fluorescent protein (GFP) for this purpose, as this protein has previously been cyclized with artificially split inteins.*28* Two SICLOPPS constructs encoding IC-GFP-IN with *Npu* and *Npu*-SsrAinteins were generated and cloned into a pET28a backbone. We assessed the effect of expressing each construct on the viability of the *E. coli* host by monitoring the optical density of the culture at 600 nm (OD600). As previously observed for cyclic peptide production, induction of the *Npu* IC-GFP-IN protein with IPTG had a marked effect on host viability, with a ~50 % reduction in growth rate compared to the non-induced culture (Figure 4A). In contrast, *E. coli* expressing *Npu-*SsrA IC-GFP-IN grew at the same rate as the equivalent non-induced culture (Figure 4B). As discussed previously, it is possible that the observed effects are due to the SsrA tag degrading the IC-GFP-IN protein prior to splicing; in this scenario no plasmid product (GFP) or partially spliced product would be present in the cell. We used western immunoblotting to probe for GFP in cells expressing *Npu-*SsrA IC-GFP-IN. A strong band corrsponding to spliced GFP was observed, with significantly weaker bands for the unspliced and partially-spliced products, indicating that the *Npu* inteins splice prior to SsrA-mediated degradation (Figure 4C). Western immunoblot analysis of non-induced cells showed the absence of any bands corresponding to GFP (Supplementary Figure 1), and similar analysis of the cells expressing cyclic GFP *via Npu* intein demonstrated the presence of a band corresponding to cyclic GFP as expected (Supplementary Figure 1). This data was in line with our observations for cyclic peptide production with *Npu*-SsrA inteins in the above phenotype-based experiments.

Taken together, our data demonstrates that engineered *Npu* inteins may be adapted for the production of SICLOPPS cyclic peptide libraries. But despite their rapid rate of splicing and tolerance to variation in extein sequence, a large portion (42 %) of an *Npu* SICLOPPS library was found to be toxic to their *E. coli* host, significantly reducing their utility. This limitation was overcome through the use of an SsrA tag to target the spliced inteins to the ClpXP machinery for degradation. There was no effect on host viability from the resulting *Npu*-SsrA SICLOPPS inteins when employed for cyclic peptide library, or cyclic protein production. In this study we have utilized three different *E. coli* strains (DH5α, BW27786 and BL21); the observed effects from *Npu* inteins are therefore unlikely to be strain specific. However, it should be noted that prior studies overexpressing *Npu* inteins in *E. coli* ER2566 do not mention observed toxicity.*17* We are currently using these second generation, traceless SICLOPPS inteins for the identification of cyclic peptide inhibitors of a variety of protein-protein interactions.

***METHODS***

All molecular biology reagents were purchased from Fisher Scientific, New England Biolabs or Promega and used in accordance with the manufacturers instructions. All genes were synthesized *de novo* by Integrated DNA Technologies. For reference, the protein sequence of the *Npu-SsrA* SICLOPPS inteins encoding *cyclo-*CAAAAA (underlined) is given below. Insert your extein of choice in this region with a C or S in position 1 (necessary for intein splicing). HHHHHHGENLYFKLQAMGMIKIATRKYLGKQNVYDIGVERYHNFALKNGFIASN**C**AAAAACLSYDTEILTVEYGILPIGKIVEKRIECTVYSVDNNGNIYTQPVAQWHDRGEQEVFEYCLEDGCLIRATKDHKFMTVDGQMMPIDEIFERELDLMRVDNLPNGTAANDENYALAA

In line with Research Councils UK’s data sharing policy, the raw data for these experiments is available upon request from the University of Southampton’s data repository.

**Analysis of SICLOPPS library splicing by SDS-PAGE**

SICLOPPS CX5 libraries were constructed in pARCBD backbone as previously detailed*13* and transformed into electrocompetent DH5α as ligation mixtures. After 1 hour of incubation at 37 oC with shaking, 1 L of the recovery mixture was removed for analysis of transformation efficiency as previously detailed,*13* and the remainder of the recovery mixture was diluted to 10 mL with additional LB, and incubated at 37 oC with shaking until an OD600 of 0.6. Protein expression was then induced, and the cultures incubated with shaking for a further 6h. Inteins were isolated via their affinity tag and visualized by SDS-PAGE as previously detailed.*13*

### Assessing the toxicity of SICLOPPS Library members

A SICLOPPS CX5 library was constructed as a ligation mixture*13* and transformed into electrocompetent DH5α as above. Recovery mixtures were diluted with LB broth and plated at 10-4 and 10-6 dilutions onto LB agar plates containing chloramphenicol (30 g/mL) with and without arabinose (1.3 M). Plates were incubated overnight and the colonies on each set of plates were counted. 10 plates were counted per condition, per set of experiments, and each experiment was repeated 3 times.

**Drop spotting**

Electrocompetent DH5α were transformed with a SICLOPPS plasmid encoding *cyclo-*CLLFVY. After recovery, plating onto LB agar containing 30 g/mL chloramphenicol and overnight incubation, a single colony was used to inoculate a liquid culture containing the 30 g/mL chloramphenicol. After overnight incubation at 37 oC with shaking, the OD600 of each culture was measured, and all cultures were found to be of a similar optical density. Ten-fold serial dilutions of each overnight culture was prepared and drop spotted (2.5 L per spot) onto LB agar plates containing either 30 g/mL chloramphenicol or, 30 g/mL chloramphenicol and 1.3 M arabinose. Plates were incubated overnight at 37 oC.

For experiments using HIF-1 RTHS cells instead of DH5α, the above protocol was repeated, but minimal media selection plates (as previously detailed)*7* were used instead of LB agar.

**Cyclic-GFP Growth assays**

Constructs encoding cyclic GFP with SICLOPPS inteins*28* were synthesized and cloned into pET28a vectors using standard protocols. The resulting plasmids were verified by DNA sequencing. Each plasmid was transformed into chemically competent BL21 cells, and a single colony used to inoculate an overnight culture, which was in turn used to inoculate (1 %) 200 mL of LB. The resulting mixture was incubated at 37 oC with shaking until an OD600 of 0.6 was reached. The sample was divided into two equal portions. One was allowed to grow without induction, while the expression of the SICLOPPS construct was induced in the second sample with IPTG (1 mM). OD600 measurements were taken at 0, 0.5, 1, 2, 3, 4, 5 and 6 hours after induction. Each set of experiments was repeated 3 times.

**Western immunoblotting**

Cyclic-GFP production was induced in *E. coli* as detailed above. Cells were incubated with shaking at 37 oC for 3 hours after induction of expression. Cells were pelleted after this time by centrifugation (3100 rpm, 15 minutes, 4 oC). The pellets were lysed by sonication and cell debris separated by centrifugation (8000 rpm, 40 minutes, 4 oC). Crude cell lysate was separated by 15 % SDS-PAGE and transferred onto nitrocellulose transfer membranes (Protran, Whatman) at 250 mA for two hours. The membranes were blocked in 5 % milk in phosphate buffer saline (PBS) and Tween (0.1 % v/v PBS/Tween) at room temperature for one hour. This was incubated with a mouse anti-GFP primary antibody (Abcam) at 4 oC for 16 hours at a 1:2,000 ratio in 5 % milk in PBS with 0.05 % Tween-20 followed by incubation with anti-mouse secondary antibody at a 1:50,000 dilution for one hour at room temperature. The secondary antibody was detected using an enhanced chemilluminescence (ECL) reagent.

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*Supporting Information Available:* This material is available free of charge *via* the Internet.

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Figure Legends

**Figure 1.** Comparison of SICLOPPS cyclic peptide formation with *Ssp* and *Npu* inteins. A) The mechanism of SICLOPPS. IN and IC interact to form an active intein that splices to cyclize the extein placed in between. In this case a cyclic hexa-peptide library is produced, where X=any proteinogenic amino acid. B) Probing splicing efficiency of a CX5 library by SDS-PAGE. Presence of the IC is indicative of splicing; as can be seen, the majority of the library made with *Ssp* inteins do not splice, where as the *Npu* library splices significantly more efficiently (green arrow). C) Toxicity of *Npu* and *Ssp* SICLOPPS libraries. Around 14 % of *E. coli* transformed with a CX5 SICLOPPS library are not viable upon induction of SICLOPPS, whereas ~42 % of the induced transformants are not viable when using *Npu* inteins with SICLOPPS D) Assessing the effect of *Ssp* and *Npu* inteins on *E. coli* expressing *cyclo*-CLLFVY by drop-spotting serial dilutions (2.5 L of ~10n cells/mL). The effect of *Npu* inteins on *E. coli* viability is demonstrated by the loss of growth at lower dilutions upon the addition of arabinose (red arrow).

**Figure 2.** Reducing the toxicity of *Npu* SICLOPPS inteins using an SsrA tag. A) An SsrA tag is added to the C-terminus of the *Npu* IN, resulting in the intracellular degradation of unspliced and spliced inteins. B) Probing splicing efficiency of a CX5 *Npu*-SsrA library by SDS-PAGE. Please note the absence of IN and IC. C) Toxicity of *Npu* and *Npu*-SsrA SICLOPPS libraries. Around 42 % of *E. coli* transformed with an *Npu* CX5 SICLOPPS library are not viable upon induction of SICLOPPS, whereas the addition of an SsrA tag to the *Npu* inteins eliminates the toxicity from SICLOPPS library members to the *E. coli* host. D) Assessing the effect of *Npu* and *Npu-*SsrA inteins on *E. coli* expressing *cyclo*-CLLFVY by drop-spotting serial dilutions (2.5 L of ~10n cells/mL). The loss of viability from *Npu* inteins is reversed upon addition of an SsrA tag as demonstrated by maintenance of full growth upon the addition of arabinose.

**Figure 3.** Assessing the effect of the *Npu-*SsrA SICLOPPS inteins on the viability of cells expressing *cyclo-*CLLFVY by drop-spotting serial dilutions (2.5 L of ~10n cells/mL). A) In the absence of IPTG and arabinose expression of neither the repressors nor SICLOPPS is induced, therefore full growth is observed for all three plasmids. B) In the presence of 50 M IPTG, the repressors are expressed and come together (due to the interaction of the HIF-1 subunits) to form a functional repressor, which shuts down expression of the reporter construct. Cells fail to fully grow on selective media under these conditions, with no effect from the SICLOPPS plasmids. C) In the presence of 50 M IPTG and 1.3 M arabinose, both the functional repressor and *cyclo-*CLLFVY are produced; this cyclic peptide disrupts the interaction of HIF-1α and HIF-1, hence prevents formation of a functional repressor, which is expected to enable cell survival. However, only cells producing this cyclic peptide using *Ssp* inteins or *Npu-*SsrA inteins survive and grow on selective media.

**Figure 4.** The effect of the SsrA tag on the viability of cells expressing cyclic GFP with SICLOPPS *Npu* inteins. A)Induction of the expression of the *Npu* intein-GFP construct with IPTG reduces the viability of the *E. coli* host by ~50 % over 300 minutes. B) The observed affect on cell viability upon induction of protein expression with IPTG is significantly reduced when using *Npu*-SsrASICLOPPS inteins C) Probing for GFP by western blot in *E. coli* expressing cyclic GFP with *Npu*-SsrA SICLOPPS inteins.