

Monitoring Protein Stability In Vivo Using an Intein-Based Biosensor

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Abstract

Inteins are elements translated within host proteins and removed via a unique protein splicing reaction. In this process, the two peptide bonds flanking the intein are rearranged, releasing the intein and leaving a standard peptide bond in its place. Due to their ability to shuffle peptide bonds in a specific and controlled manner, inteins have proven valuable in protein engineering, leading to the development of numerous impactful technologies. In one application, intein-based biosensors link the activity of a host protein to intein excision. Recently, we developed a biosensor to measure protein stability in vivo, in which the removal of an intein-protein fusion is required for antibiotic resistance. In our protocol, cells expressing our biosensor are logarithmically diluted and spotted on agar plates containing increasing levels of antibiotics. Following incubation, quantitative survival curves can be generated. We also developed a dual protein stability sensor where both antibiotic resistance and fluorescence can be used as readouts and demonstrated that co-expression of the chaperonin GroEL can promote survival and fluorescence. Taken together, our novel intein-based biosensor adds to the available tools to measure protein stability within the cellular environment.

Key features

- Biosensor demonstrates a 100,000-fold difference in survival between stable and unstable test proteins.
- Dual biosensor quantitatively links protein stability to antibiotic resistance and fluorescence simultaneously.

Keywords: Intein, Protein splicing, Biosensor, Chaperone, Protein stability, Protein folding

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Background

Inteins (intervening proteins) are polypeptide segments translated within host proteins that are removed through a protein splicing reaction [1,2]. In the intein-mediated splicing reaction, the intein is released by the formation of a peptide bond between the flanking sequences, termed N- and C-exteins, which form the mature host protein. This reaction can proceed without any external factors, though the rate at which splicing occurs is highly dependent on the conditions present. The unique ability of inteins to rearrange peptide bonds has led to the invention of numerous applications in protein engineering [2,3]. Abundant and widespread within the microbial world [4], prior work has demonstrated that some inteins have adapted to serve as environmental sensors, displaying their capacity to regulate protein function in response to conditions such as metals, heat, salt, oxidative stress, and DNA damage [2,5,6].

To monitor intein activity in response to different conditions, protein splicing reporters are often used. These reporters are flexible in design, as their only requirement is the presence of a cysteine, serine, or threonine as the first residue following the intein [1]. Previously, we developed a kanamycin intein splicing reporter (KISR), with it providing an easy method to detect selective small molecule inhibitors of protein splicing within the cellular environment [7–10]. As inteins are absent in humans but present within human pathogens including *Mycobacterium tuberculosis* and *Cryptococcus neoformans*, splicing inhibitors may serve as novel antimicrobials, making them increasingly important targets to study [11,12]. Kanamycin A is a commonly used aminoglycoside antibiotic that inhibits translation. Bacteria with a mature aminoglycoside phosphotransferase (KanR), however, can survive in the presence of the antibiotic. The KISR reporter makes use of KanR as the exteins flanking an intein, making it so intein splicing activity confers antibiotic resistance to the experimental cells transformed with the reporter [7–10]. In the presence of small molecule inhibitors, the KISR will be unable to splice, leaving the cells vulnerable to the antibiotic. Therefore, this reporter allows for easy observation of what molecules can act as inhibitors and to what degree they inhibit, as stronger inhibitors will lead to reduced growth.

We recently developed an intein-based biosensor that adapts the KISR framework into a tripartite folding biosensor, where a protein of interest (POI) is inserted within the intein between conserved splicing blocks (Figure 1). Tripartite protein folding biosensors serve to measure the stability of a POI by linking it to reporter activity within the cellular environment [13]. Our intein-based tripartite biosensor design, while serving a similar purpose, distinguishes itself from previous biosensors through the insertion of the POI into an intein, which is then flanked with reporter extein sequences [14]. Through splicing activity, the POI is removed as the extein sequences are joined into a functional reporter molecule. This allows the biosensor to avoid the challenges associated with previous designs that kept a POI fused to the reporter, such as limitations on the size of POI insertion or restrictions on reporter function. Initial work with our biosensor was carried out using the *E. coli* maltose binding protein (MBP) as a POI, with it being inserted into the homing endonuclease (HEN) loop of the *Pyrococcus horikoshii* RadA intein (RadA-I) [14]. The HEN loop of RadA-I has been noted to accommodate POI insertion, as previous work has shown that the intein remains splicing active upon insertion of ZsGreen into the loop [15,16]. The RadA-I intein was also selected due to its rapid and accurate splicing across a variety of extein contexts [17–19], which indicated that it could accommodate reporter exteins. The RadA-I-MBP fusion was inserted within KanR (KanR-RadA-I-MBP), with kanamycin resistance serving as the reporter in a manner similar to KISR. This biosensor was then tested in a variety of conditions, where it was observed that a decrease in MBP stability (due to mutations) or inhibition of splicing activity resulted in reduced survival of cells, as they were not provided kanamycin resistance [14]. This supported the viability of our intein-based biosensor approach, as the stability of the POI was directly linked to reporter activity. Further work, which made use of the fluorescent protein TagRFP675 as the POI, showed the potential of our biosensor design as a dual-folding sensor, with both

antibiotic resistance and fluorescence linked to POI stability [14]. Potential applications of our intein-based biosensor are broad due to the relaxed requirements of intein splicing, making this approach viable for linking protein stability to any desired in vivo readout [14].

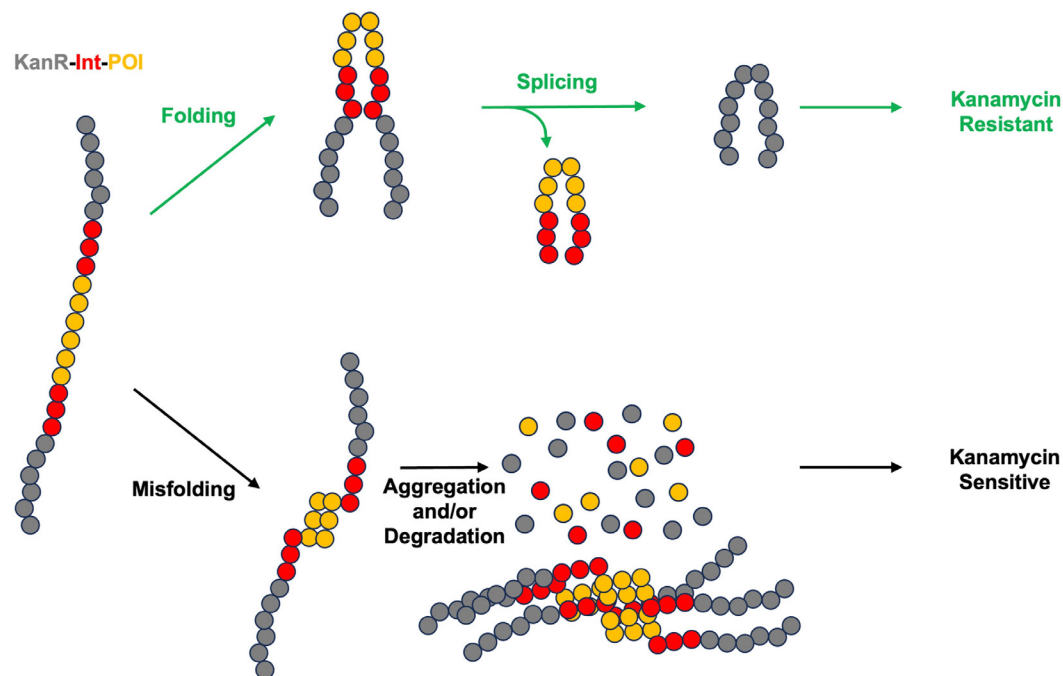


Figure 1. Intein-based protein stability sensor. KanR is colored in gray, intein is colored in red, and the protein of interest (POI) is colored in orange.

Materials and reagents

Biological materials

1. NEB5α *E. coli* (New England Biolabs, C2987H) (Table 1)
2. MC4100(DE3) [20]

Table 1. *E. coli* strains used in this study.

Strain number	Plasmid 1	Plasmid 2			Strain
CWL599	KanR-Int ^{WT} -MBP ^{MUT}	ApR			NEB5a
CWL600	KanR-Int ^{WT} -MBP ^{WT}	ApR			NEB5a
CWL640	KanR-Int ^{MUT} -MBP ^{WT}	ApR			NEB5a
AS917	KanR-Int ^{WT} -MBP ^{MUT}	ApR	pBAD33mut-Empty	CmR	MC4100(DE3)
AS923	KanR-Int ^{WT} -MBP ^{MUT}	ApR	pBAD33-GroEL	CmR	MC4100(DE3)
AS925	KanR-Int ^{WT} -MBP ^{WT}	ApR	pBAD33mut-Empty	CmR	MC4100(DE3)
AS931	KanR-Int ^{WT} -MBP ^{WT}	ApR	pBAD33-GroEL	CmR	MC4100(DE3)
AS933	KanR-Int ^{WT} -TagRFP675	ApR	pBAD33mut-Empty	CmR	MC4100(DE3)
AS939	KanR-Int ^{WT} -TagRFP675	ApR	pBAD33-GroEL	CmR	MC4100(DE3)

Reagents

1. Difco LB broth, Miller (BD, catalog number: 244610)
2. Difco LB agar, Miller (BD, catalog number: 244510)
3. Ampicillin sodium salt (Fisher, catalog number: BP906)
4. Kanamycin sulfate (Fisher, catalog number: BP1760)
5. Chloramphenicol (Fisher, catalog number: BP904100)
6. Ethanol (Sigma-Aldrich, catalog number: E7023)
7. L-Arabinose (Chem-IMPEX, catalog number: 29750)
8. Phosphate-buffered saline (PBS) (10 ×) pH 7.4, RNase-free (Fisher, catalog number: AM9625)

Solutions

1. LB medium (see Recipes)
2. LB agar (see Recipes)
3. 100 mg/mL ampicillin stock solution (1,000 ×) (see Recipes)
4. 50 mg/mL kanamycin stock solution (1,000 ×) (see Recipes)
5. 35 mg/mL chloramphenicol stock solution (1,000 ×) (see Recipes)
6. 20% L-arabinose stock solution (100 ×) (see Recipes)

Recipes

1. LB medium

25 g of LB broth

Bring to 1 L with ddH₂O

Autoclave to sterilize

2. LB agar

40 g of LB agar

Bring to 1 L with ddH₂O

Autoclave to sterilize

3. 100 mg/mL ampicillin stock solution (1,000 ×)

1 g of ampicillin sodium salt

Bring to 10 mL with ultrapure H₂O

Place in 10 mL syringe and pass through 0.22 mm filter to sterilize

Aliquot in 1.5 mL tubes and store at -20 °C

4. 50 mg/mL kanamycin stock solution (1,000 ×)

500 mg of kanamycin sulfate

Bring to 10 mL with ultrapure H₂O

Place in 10 mL syringe and pass through 0.22 mm filter to sterilize

Aliquot in 1.5 mL tubes and store at -20 °C

5. 35 mg/mL chloramphenicol stock solution (1,000 ×)

350 mg of chloramphenicol

Bring to 10 mL with ethanol

Place in 10 mL syringe and pass through 0.22 mm filter to sterilize

Aliquot in 1.5 mL tubes and store at -20 °C

6. 20% L-arabinose stock solution (100 ×)

2 g of L-arabinose

Bring to 10 mL with ultrapure H₂O

Place in 10 mL syringe and pass through 0.22 mm filter to sterilize

Aliquot in 1.5 mL tubes and store at -20 °C

Laboratory supplies

1. 1, 10, 20, 200, and 1,000 µL pipette tips (USA Scientific, Inc. TipOne, catalog numbers: 1111-3800, 1120-1810, 1111-0706, and 1111-2820)
2. 15 mL conical tubes (Greiner Bio-One, catalog number: 188 271)
3. Tissue culture plate 96 wells (Fisher Scientific, catalog number: FB012931)
4. Nonbinding 96-well plates (Greiner Bio-One, catalog number: 655904)
5. Black 96-well plates, black (Corning, catalog number: NBS3991)
6. Petri dishes (Fisher Scientific, catalog number: FB0875712)
7. 1.5 mL cuvette (Fisher Scientific, catalog number: 14955127)
8. 1.5 mL microcentrifuge tubes (USA Scientific, catalog number: 1615-5500)
9. 10 mL syringe (BD, catalog number: 302995)
10. 0.22 mm syringe filters (Fisher Scientific, catalog number: 09-719A)
11. Roll & Grow™ spherical glass plating beads (MP Biomedicals, catalog number: 115000550)
12. Cell scraper (CELLTREAT, catalog number: 229310)

Equipment

1. Benchtop refrigerated shaking incubator (Thermo Scientific, catalog number: SHKE4000)
2. Spectrophotometer (Eppendorf, catalog number: 6131 02779)
3. Amersham Imager 600 (GE, catalog number: 29-0834-61)
4. Microplate reader (Tecan, model: Infinite M200 Pro)
5. P20 Pipetman (Gilson, catalog number: FA10003M)
6. P200 Pipetman (Gilson, catalog number: F144058M)
7. P1000 Pipetman (Gilson, catalog number: FA10006M)
8. Multichannel pipette (Thermo Scientific, catalog number: 46300100)
9. Incubator (Fisher Scientific, model: 11-690-637D)
10. Infrared thermometer (Etekcity, model: 817915020807)
11. Bunsen burner
12. 1 cm grid paper

Procedure

Procedure 1: Single expression of an intein-based biosensor

A. Prepare overnight cultures

1. In sterile conditions, prepare an overnight culture for each of the three NEB5 α strains (CWL599, CWL600, and CWL640) by inoculating a culture tube that contains 5 mL of LB media and 5 μ L of 1,000 \times ampicillin stock solution.
2. Incubate at 37 $^{\circ}$ C with shaking at 250 rpm for approximately 20 h.

B. Prepare agar plates

1. Prepare LB agar.
2. Warm LB agar until liquid, then allow it to cool to approximately 65 $^{\circ}$ C.
3. Control plates: add 1 μ L of 1,000 \times ampicillin stock solution per 1 mL of LB agar.
4. Experimental plates: add 1 μ L of 1,000 \times ampicillin and 1 μ L of 1,000 \times kanamycin stock solution per 1 mL of LB agar.
5. Pour the agar into Petri dishes and allow it to solidify overnight at room temperature.

C. Cell titer standardization

1. Turn on the spectrophotometer to allow it to warm up (Figure 2).

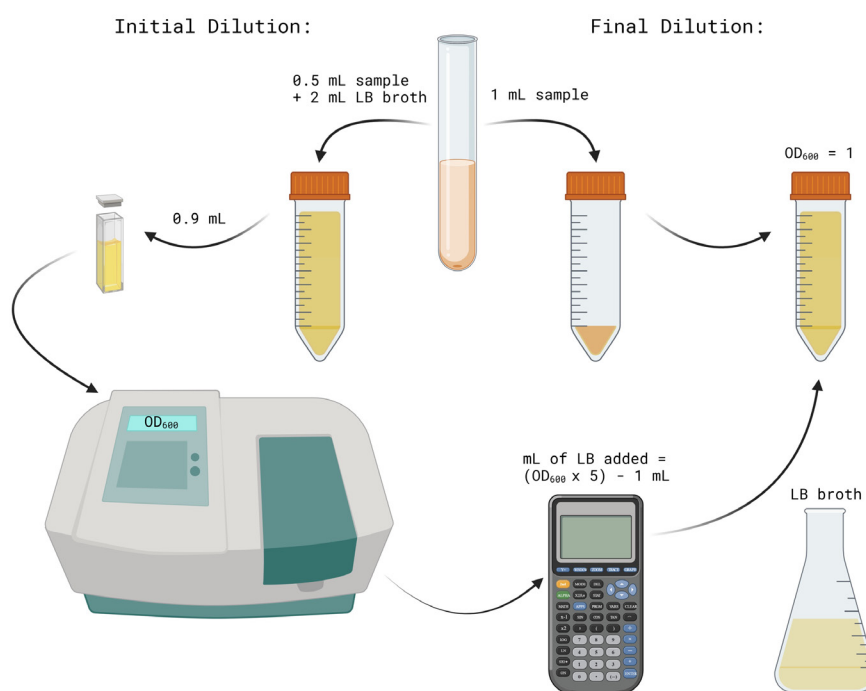


Figure 2. Procedure for normalizing overnight cultures to $OD_{600} = 1$. Image generated using [BioRender](#).

2. Label two 15 mL conical tubes for each 5 mL culture as *initial dilution* and *final dilution* for each sample.
3. Fill the *initial dilution* tubes with 2 mL of LB broth.
4. Transfer 0.5 mL of the CWL 599 culture into its respective *initial dilution* tube. Pipette up and down three times to mix.
5. Repeat step C4 for the remaining samples.
6. Take 1 mL of each *initial dilution* mixture and transfer into its respective *final dilution* tube.
7. Transfer 0.9 mL of each *initial dilution* mixture into 2 mL cuvettes. Measure and record their OD₆₀₀ using the spectrophotometer.
8. If the OD₆₀₀ value is above 1, add LB to the *final dilution* tube to bring the solution to an OD₆₀₀ of 1. The equation to solve for X mL of LB added is: $X = ((OD_{600} \text{ Value}) \times 5) - 1 \text{ mL}$.

D. Serial dilution

1. Turn on the Bunsen burner and work near its base to prevent contamination while the nonbinding 96-well plate lid is open (Figure 3).

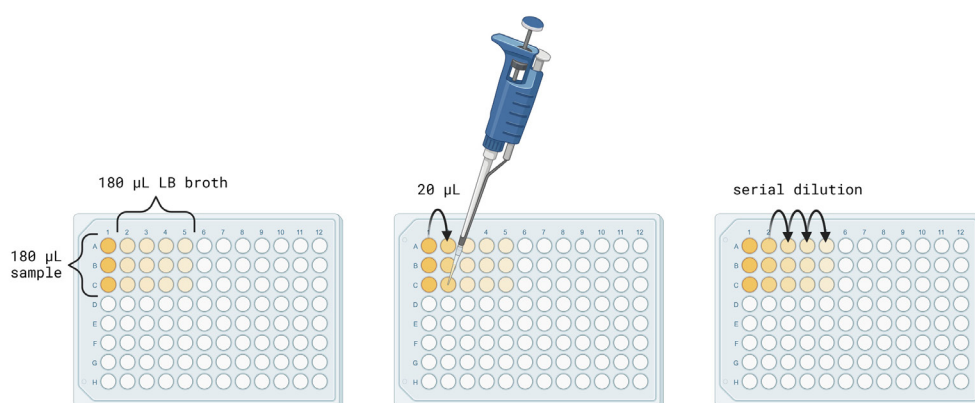


Figure 3. Procedure for making serial dilutions. Image generated using [BioRender](#).

2. Assign row A to CWL 599, row B to CWL 600, and row C to CWL 640 of the nonbinding 96-well plate.
3. Using a P200 Pipetman, pipette 180 µL of LB broth into wells 2–5 in rows A–C.
4. Pipette 180 µL of each cell culture at an OD₆₀₀ value of 1 into its respective row in the empty wells in column 1. Change pipette tips between samples.
5. Using a P20 Pipetman, transfer 20 µL of sample from well A1 into well A2.
6. After drawing up the 20 µL sample, pump the plunger on the Pipetman three times to mix the 20 µL sample into the 180 µL of LB. Discard the pipette tip after mixing.
7. Transfer 20 µL of sample from well A2 into well A3.
8. After drawing up the 20 µL sample, pump the plunger on the Pipetman three times to mix the 20 µL sample into the 180 µL of LB. Discard the pipette tip after mixing.
9. Transfer 20 µL of sample from well A3 into well A4.
10. After drawing up the 20 µL sample, pump the plunger on the Pipetman three times to mix the 20 µL sample into the 180 µL of LB. Discard the pipette tip after mixing.
11. Transfer 20 µL of sample from well A4 into well A5.
12. After drawing up the 20 µL sample, pump the plunger on the Pipetman three times to mix the 20 µL sample

into the 180 μ L of LB. Discard the pipette tip after mixing.

13. Repeat step D11 for the remaining samples.

E. Spot titers

1. Print a 1 \times 1 cm grid on paper using Excel as a template (this can be reused).
2. If plates were stored at 4 $^{\circ}$ C, warm the control and experimental agar plates in a 30 $^{\circ}$ C incubator for 10–15 min before use. If the plates were prepared the previous day, they can remain at room temperature.
3. Label plates with strain, date, and the antibiotic(s) included.
4. Arrange three control plates and three experimental plates on the grid paper.
5. Set a P10 multichannel pipette to six drops of 1.25 μ L.
6. Using a P10 multichannel pipette, draw up CWL 599 samples from row A. Spot them onto each agar plate by lightly touching the tip to the surface. Avoid piercing the agar. Open the plate lid only briefly while spotting to prevent contamination. Discard pipette tips after spotting each row (Figure 4).

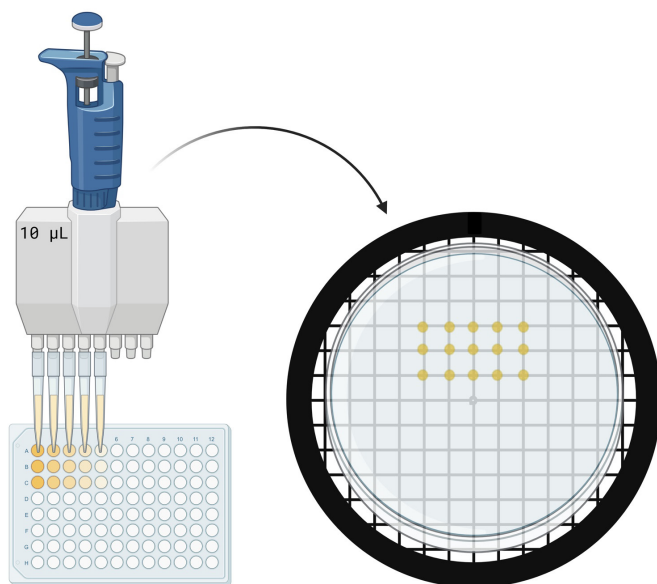


Figure 4. Procedure for spotting cells on agar plates. Image generated using [BioRender](#).

7. Draw up CWL 600 samples from row B and spot them below the CWL 599 spots on each agar plate. Ensure you just touch the pipette tip to the surface. Discard tips after each plate.
8. Draw up CWL 640 samples from row C and spot them below the CWL 600 spots on each agar plate. Ensure you just touch the pipette tip to the surface. Discard tips after each plate.
9. Allow the sample spots to dry at room temperature. Then, invert the plates upside-down and incubate them at 37 $^{\circ}$ C overnight (16–20 h) (Figure 5).

Note: Survival may vary depending on incubation temperature.

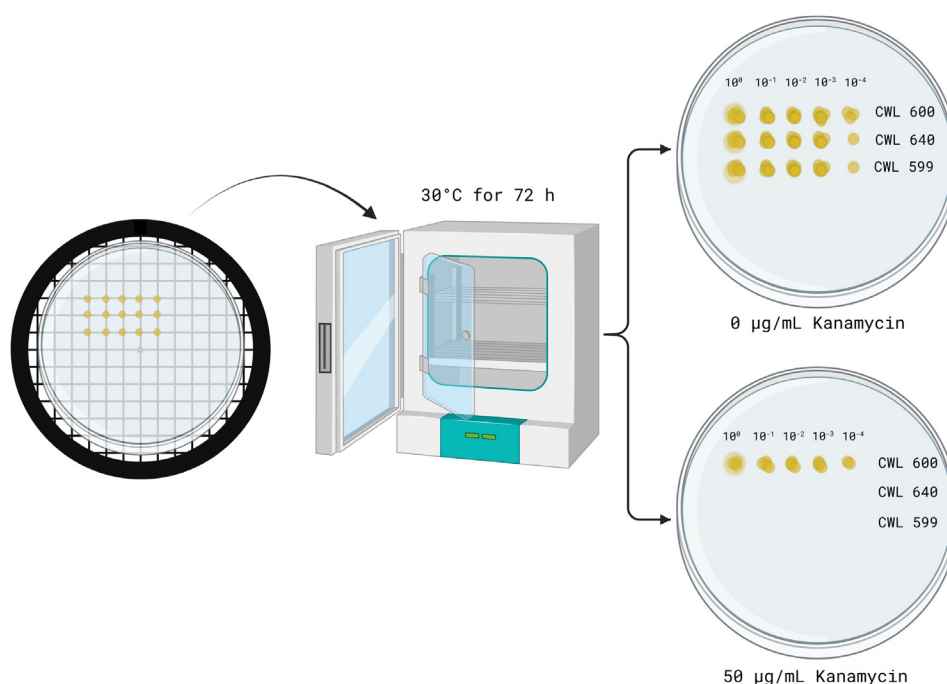


Figure 5. Spot titer plate incubation and expected results. Image generated using [BioRender](#).

Procedure 2: Dual expression of intein- or fluorescence-based biosensor with GroEL chaperone

A. Prepare overnight cultures

1. Under sterile conditions, prepare an overnight culture for each of the six MC4100 strains (AS917, AS923, AS925, AS931, AS933, and AS939) by inoculating 3 mL of LB medium with 6 µL of 1,000 × ampicillin and 3 µL of 1,000 × chloramphenicol stock solutions.
2. Incubate at 37 °C shaking at 250 rpm overnight (16–20 h).

B. Prepare agar plates

1. Prepare LB agar.
2. Autoclave the LB agar for 30 min at 121 °C and allow it to cool to approximately 65 °C.
3. Control plates: Per 1 mL of LB agar, add 2 µL of 1,000 × ampicillin, 1 µL of 1,000 × chloramphenicol, and 10 µL of 100 × L-arabinose stock solution.
4. Experimental plates: Per 1 mL of LB agar, add 2 µL of 1,000 × ampicillin, 1 µL of 1,000 × chloramphenicol, 10 µL of 100 × L-arabinose, and various concentrations of kanamycin from the 1,000 × kanamycin stock solution: 0.1 µL (5 µg/mL), 0.2 µL (10 µg/mL), and 0.4 µL (20 µg/mL).
5. Non-inducing plates for GroEL chaperone: Per 1 mL of LB agar, add 2 µL of 1,000 × ampicillin, 1 µL of 1,000 × chloramphenicol, and various concentrations of kanamycin from the 1,000 × kanamycin stock solution: 0.1 µL (5 µg/mL), 0.2 µL (10 µg/mL), and 0.4 µL (20 µg/mL).
6. Pour agar into Petri dishes and allow it to solidify overnight at room temperature.

C. Cell titer standardization

Follow the same protocol as described in Procedure 1 (nonbinding 96-well plates can be used if necessary).

D. Serial dilution

Follow the same protocol as described in Procedure 1 (nonbinding 96-well plates can be used if necessary).

E. Spot titers

1. Print a 1 × 1 cm grid on paper using Excel as a template (this can be reused).
2. If plates were stored at 4 °C, warm the control and experimental agar plates in a 37 °C incubator for 10–15 min before use. If the plates were prepared the previous day, they can remain at room temperature.
3. Label each plate with the strain, date, and the antibiotic(s) included.
4. Arrange three control plates and three experimental plates on the grid paper.
5. Set a P10 multichannel pipette to 5 µL.
6. Using a P10 multichannel pipette, draw up each sample (AS917, AS923, AS925, AS931, AS933, and AS939) from rows A to F.
7. Spot 5 µL of each sample onto each agar plate by lightly touching the tip to the surface. Avoid piercing the agar. Open the lid only briefly while spotting to prevent contamination. Discard pipette tips after spotting each row.
8. Allow the sample spots to dry at room temperature. Then, invert the plates upside-down and incubate them at 37 °C overnight (16–20 h) (Figure 6). *Note: Survival may vary depending on incubation temperature and kanamycin concentration.*

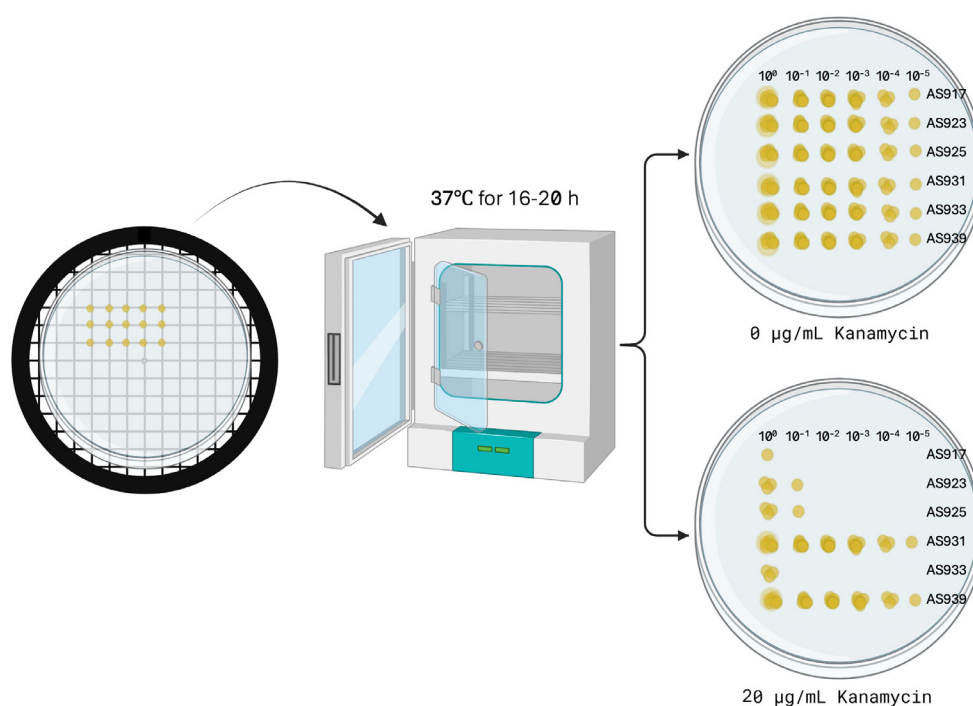


Figure 6. Spot titer plate incubation and expected results. Image generated using [BioRender](#).

F. Fluorescence assay of TagRFP675

1. Spread 50 μL of 1.0 OD_{600} AS933 and AS939 (prepared in Procedure 2, Section C) onto LB agar plates (control, experimental, and non-inducing plates) using glass plating beads (Figure 7).
2. Invert the plates upside-down and incubate them at 37 °C overnight (16–20 h).
3. Turn on the spectrophotometer and microplate reader to allow them to warm up.
4. Using a P1000 Pipetman, add 1 mL of 1 \times PBS (pH 7.4) to each plate and scrape the entire cell layer.
5. Transfer the cell suspension to a 1.5 mL microcentrifuge tube.
6. Centrifuge cells at $10,000 \times g$ for 1 min and discard supernatant.
7. Resuspend the cell pellet in 1 mL of 1 \times PBS (pH 7.4) by pipetting several times to ensure even resuspension.
8. Label two 1.5 mL microcentrifuge tubes as *initial dilution* and *final dilution* for each sample.
9. Add 900 μL of 1 \times PBS (pH 7.4) to each *initial dilution* tube.
10. Using a P200 Pipetman, transfer 100 μL of each resuspended sample into its respective *initial dilution* tube. Pipette up and down three times to mix.
11. Repeat step F10 for the remaining samples.
12. Transfer 1 mL of each *initial dilution* mixture into 2 mL cuvettes. Measure and record their OD_{600} value using a spectrophotometer. If necessary, calculate the final OD_{600} by multiplying by 10 (the dilution factor).
13. Take 100 μL of each *initial dilution* mixture and transfer it into its respective *final dilution* tube.
14. If OD_{600} exceeds 1, adjust the sample to an OD_{600} of 1 by adding 1 \times PBS. Use the formula: $X = 0.1 \times (\text{OD}_{600} \times 10(\text{dilution factor}) - 1)$, where X is the volume of 1 \times PBS (in mL) to add.
15. Using a P200 Pipetman, transfer 100 μL of each 1.0 OD_{600} sample from step F14 into a black 96-well plate. For accuracy, use triplicates (i.e., three wells per sample, filling rows A–C or D–F, if needed). Change pipette tips between samples.
16. Measure and record fluorescence using a microplate reader, setting the excitation wavelength to 598 nm and the emission wavelength to 675 nm.

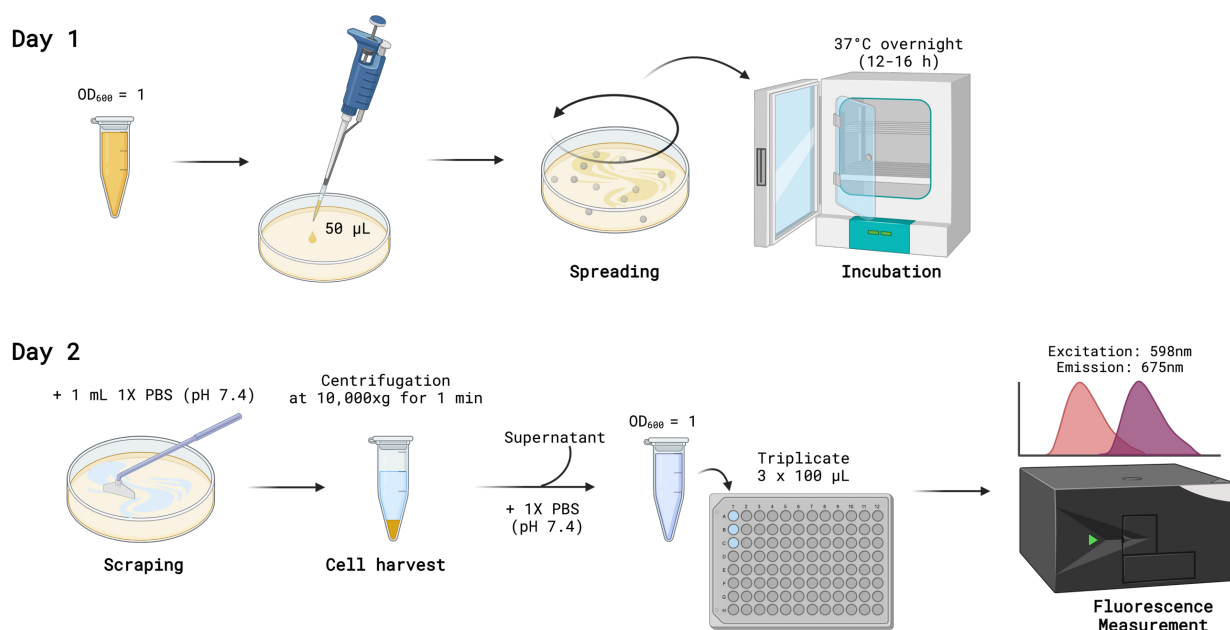


Figure 7. Fluorescence assay of TagRFP675. Image generated using [BioRender](#).

Validation of protocol

This protocol has been used and validated in the following research article:

- Son et al [14]. An intein-based biosensor to measure protein stability in vivo. *Protein Sci.*

General notes and troubleshooting

1. It is critical that a second antibiotic other than kanamycin be used to maintain the intein-based folding plasmid. The backbone, pUC4K, has resistance to ampicillin, while the pBAD33 backbone has resistance to chloramphenicol, both independently of kanamycin.
2. For the intein-based folding sensor, expression of the KanR-Intein-POI fusion protein is constitutive.
3. Survival in the presence of kanamycin for cells containing the intein-based folding sensor is highly dependent on temperature, incubation time, and antibiotic concentrations. Growth also depends on whether the cells, prior to their exposure to kanamycin, were grown in liquid culture or collected from plates, as well as the growth phase of the cells.
4. The initial dilution (Figure 2) may need to be adjusted to ensure that an accurate OD_{600nm} reading is obtained (0.1–1.0). Additionally, based on the measured OD_{600nm} of a strain, the final dilution factor will need to be adjusted to obtain a titer of 10⁰.

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Competing interests

The authors declare no conflicts of interest.

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