AfriDX

COVID-19 diagnostics for Africa

Manufacturing Engineered Boon2-BST

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Introduction

What is in this module?

This module will cover both the expression and purification protocol for the Engineered Boon2-BST. Two methods will be presented for protein expression (autoinduction and standard IPTG) to allow the user to utilise the protocol that best fits into their environment. The purification protocol aims to allow the user to purify the protein using silica immobilisation.

What is Boon2-BST?

Boon2-BST is a heterogenous recombinant protein that has been specifically designed to immobilise onto silica (component: Δ3R5 silaffin-tag), provide visual indication of protein expression and allow for protein tracking (component: mCherry), and amplify targeted DNA using loop-mediated isothermal amplification (component: BST 2.0 DNA polymerase). Genetically linking these components together produced a final construct of Δ3R5-mCherry-H10-BST2.0.

Each component of the heterogenous protein is presumed to be "active" at all times. The silaffin-tag will immobilise onto silica by simply incubating the two together in phosphate-buffered saline (PBS) under basic conditions and room temperature. The mCherry gives the protein a pink colour that can be seen without any specialty equipment but can be tracked in spectrophotometers with an excitation of 587 nm and an emission of 607 nm. Finally, the polymerase is active under standard LAMP conditions, and can be utilised for amplification without further adjustments.

It should be noted that though a polyhistidine-loop is present, it is not utilised for protein purification. It was retained within the construct to allow the user with a secondary form of purification for troubleshooting purposes.

How is Protein Expression Achieved?

The genetic information of the Boon2-BST has been encoded in a commonly used *in vitro* expression vector called pET24a, as shown in Figure 1. This vector uses a *lac* repressor (lacI) to inhibit the activation of the T7 expression system (used to express Boon2-BST) by binding to the *lac* operator. The expression system can be freed by introducing lactose (autoinduction) or Isopropyl β-d-1-thiogalactopyranoside (IPTG) (standard expression) to bind to the *lac* repressor, as shown in Figure 2. Once the repressor has been inhibited, T7 RNA

polymerase can bind to the T7 promotor and begin RNA synthesis for protein expression. It is important to be able to control when expression occurs since protein expression is a high energy consuming process. If started too early, the growth rate of the *E. coli* would be severely hampered, minimising the final output of protein. By delaying the expression, the user is providing the bacteria with the necessary time and energy to reach exponential growth phase allowing for high output of both bacteria count and expressed protein.

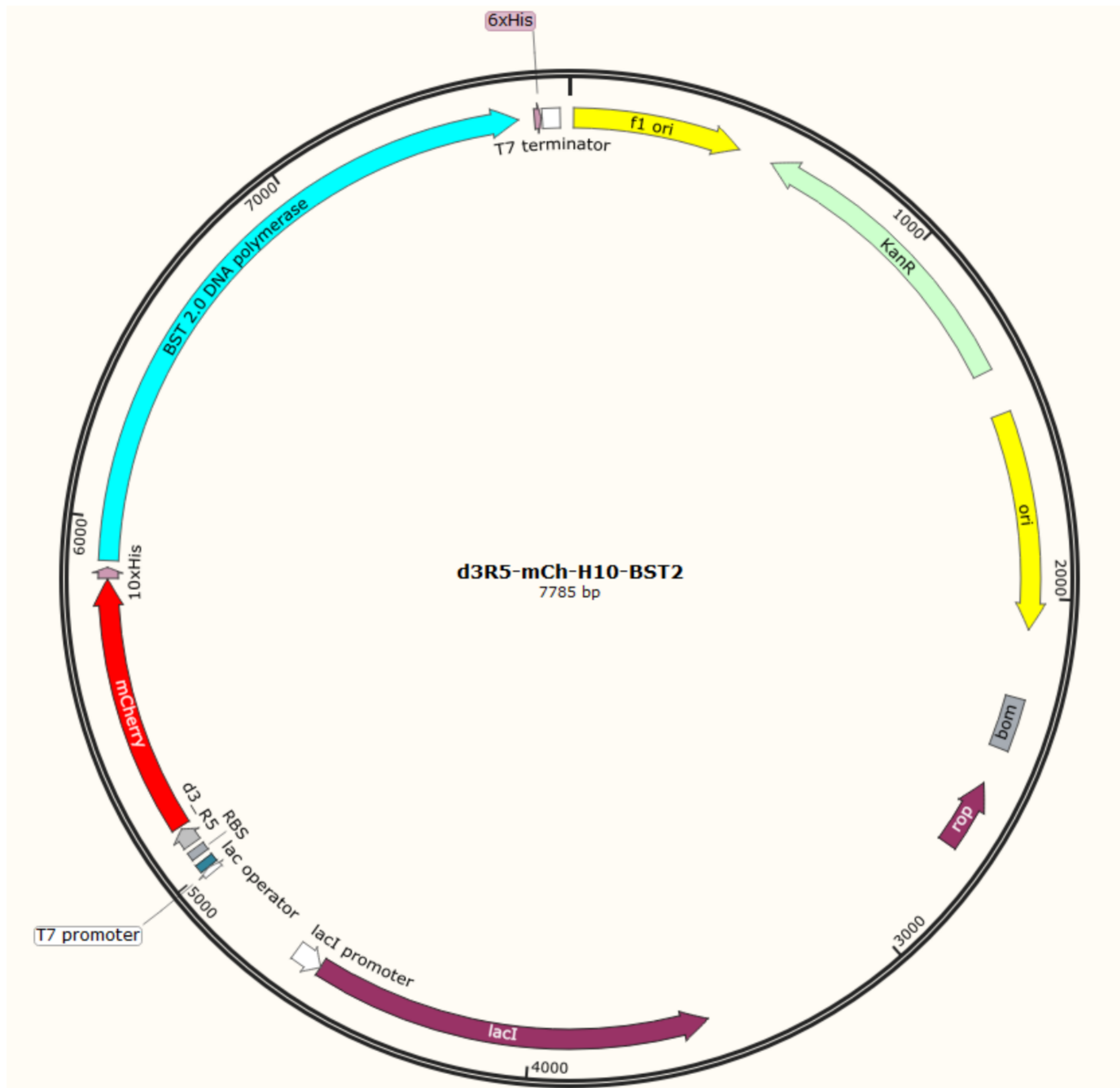

Figure 1: Vector map of the pET24a plasmid used to express d3R5-mCherry-H10-BST2.0

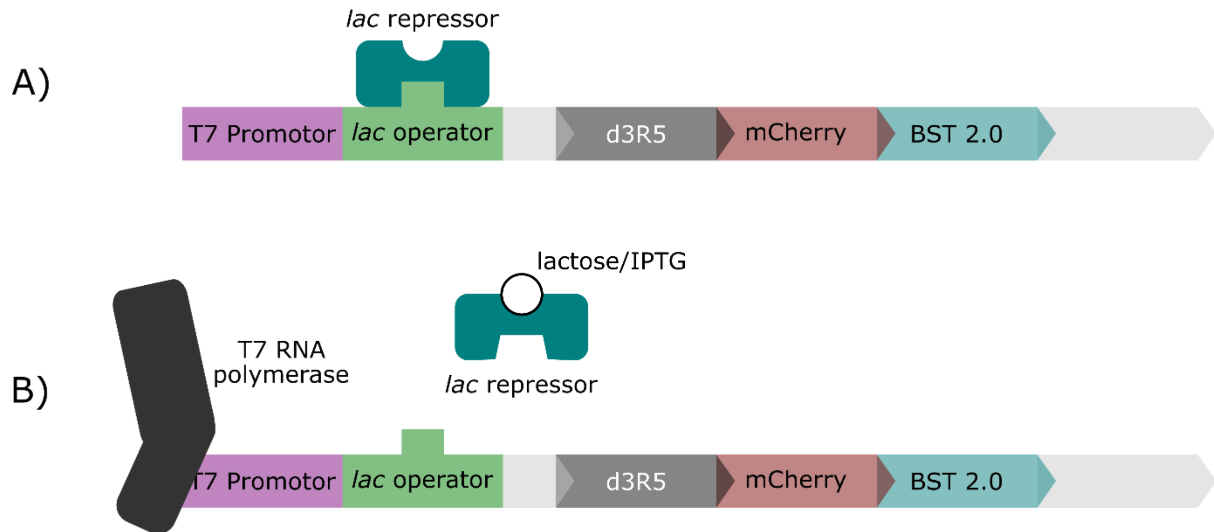

Figure 2: Illustration of the of lac repressor/operator system. A) Inhibited operator that is unable to express protein. B) Relieved expression system that is now capable of expressing protein.

How does the silica immobilise the protein?

The affinity between the Δ3R5 silaffin-tag and the silica is purely electrostatic. Under the slightly basic conditions that the user will be operating in (pH 7.2) the silica is negatively charged. Meanwhile, under the same conditions, the silaffin-tag is positively charged due to the presence of both lysine and arginine amino acids (sequence: **K**SGSYSGS**K**GS**KRR**I**L**). Simply incubating at room temperature is enough for the electrostatic attraction to immobilise the two together.

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Autoinduction Protein Expression Protocol for the Engineered Boon2-BST DNA Polymerase

Preliminary note to user:

- The following protocol was designed using BL21 (DE3) as the host *E. coli* strain
- All the steps of the "protein expression protocol" section should be performed under sterile conditions until step 11 (with the exception of the incubation steps)

Materials:

- Sodium phosphate dibasic (Na_2HPO_4)
- Potassium phosphate monobasic (KH_2PO_4)
- Sodium chloride (NaCl)
- Triptone
- Yeast extract
- Glycerol
- Glucose
- Lactose
- Water
- LB Broth
- Bunsen Burner or Biosafety cabinet (for environment sterility)
- 2 autoclavable 1 litre bottle
- 3 autoclavable 250 mL bottles
- Conical Flask
- Centrifuge Tubes
- Pipettes
- Kanamycin
- Glycerol stock of BL21 (DE3) transformed with the pET24a – Boon2-BST plasmid

Preparing autoinduction media:

Prepare the following for autoclaving.

Solution 1 (2x Media): Weigh out and add the following to a 1 litre autoclavable bottle:

- 6 g of Na_2HPO_4
- 3 g of KH_2PO_4
- 5 g NaCl
- 20 g of Triptone
- 5 g Yeast Extract
- 500 mL of water

Solution 2 (50% vol/vol glycerol): Pour the following into a 250 mL autoclavable bottle:

- 50 mL of glycerol
- 50 mL of water

Solution 3 (10% weight/vol glucose): Add/Pour the following into a 250 mL autoclavable bottle:

- 5 g of glucose
- 100 mL of water

Solution 4 (5% weight/vol lactose): Add/Pour the following into a 250 mL autoclavable bottle:

- 10 g of lactose
- 200 mL of water

Solution 5: Pour at least 500 mL of water into a 1 L autoclavable bottle.

Autoclave all 5 solutions.

Reconstitute autoinduction media: Under sterile conditions add the following to the sterilised 2x media (solution 1):

- 12 mL of sterile 50% glycerol
- 5 mL of sterile 10% glucose
- 40 mL of sterile 5% lactose
- Top up to 1 L using sterile water

Protein Expression Protocol of Engineered Boon2-BST:

The protein expression process starts with an overnight culture.

Step 1: Using 70% ethanol, sterilise the benchtop/biosafety cabinet to be used.

Step 2: Using 70% ethanol, sterilise and clean any pipettes that are to be used.

Step 3: Add 5 mL of LB Broth to a sterile centrifuge tube.

Step 4: Add kanamycin to the LB Broth for a final concentration of 50 µg/mL. (I.e. if the stock concentration of the kanamycin is 100 mg/mL, add 2.5 µL of the stock to 5 mL of LB Broth.)

$$\frac{50\ \frac{\mu g}{mL} \times 5\ mL}{100\ \frac{mg}{mL}} = 2.5\ \mu L$$

Step 5: Inoculate the prepared LB Broth with 1 µL of the Boon2-BST bacterial glycerol stock.

Step 6: Incubate the culture overnight (12 to 16 hours) at 37 °C and 225 rpm.

The following steps are performed the next morning.

Step 7: Add the desired culture volume worth of autoinduction media to a sterile conical flask (I.e. 50 mL, 100 mL, 200 mL, etc). The maximum volume is dictated by the volume of the flask. Standard protocols utilise an air-to-volume ratio of 10:1. This means that if a 2 L flask is being used, the max desired volume of LB Broth should be 200 mL.

Step 8: Add kanamycin to the autoinduction media for a final concentration of 50 µg/mL. (I.e. if the stock concentration of the kanamycin is 100 mg/mL and the desired culture volume is 200 mL, add 100 µL of the stock to the LB Broth.)

$$\frac{50\ \frac{\mu g}{mL} \times 200\ mL}{100\ \frac{mg}{mL}} = 100\ \mu L$$

Step 9: Inoculate the prepared culture flask with the overnight culture at a ratio of 100:1. (I.e. if the desired culture volume is 200 mL, add 2 mL of the overnight culture.)

Step 10: Incubate the large culture at 37 °C and 225 rpm for 10 hours.

Step 11: Aliquot the culture equally into centrifuge tubes.

Step 12: Centrifuge the culture at 3750 rpm for 30 minutes.

Step 13: Decant the supernatant. The pellets can be stored at -20 °C long term or 4 °C short term.

Standard IPTG Protein Expression Protocol for the Engineered Boon2-BST DNA Polymerase

Preliminary note to user:

- The following protocol was designed using BL21 (DE3) as the host *E. coli* strain
- All the steps should be performed under sterile conditions until step 13 (with the exception of the incubation steps)

Materials:

- Conical Flask
- Bunsen Burner or Biosafety cabinet (for environment sterility)
- LB Broth
- Centrifuge Tubes
- Pipettes
- Kanamycin
- Isopropyl β-d-1-thiogalactopyranoside (IPTG)
- Glycerol stock of BL21 (DE3) transformed with the pET24a – Boon2-BST plasmid

Protein Expression Protocol of Engineered Boon2-BST:

The protein expression process starts with an overnight culture.

Step 1: Using 70% ethanol, sterilise the benchtop/biosafety cabinet to be used.

Step 2: Using 70% ethanol, sterilise and clean any pipettes that are to be used.

Step 3: Add 5 mL of LB Broth to a sterile centrifuge tube.

Step 4: Add kanamycin to the LB Broth for a final concentration of 50 µg/mL. (I.e. if the stock concentration of the kanamycin is 100 mg/mL, add 2.5 µL of the stock to 5 mL of LB Broth.)

$$\frac{50\ \frac{\mu g}{mL} \times 5\ mL}{100\ \frac{mg}{mL}} = 2.5\ \mu L$$

Step 5: Inoculate the prepared LB Broth with 1 µL of the Boon2-BST bacterial glycerol stock.

Step 6: Incubate the culture overnight (12 to 16 hours) at 37 °C and 225 rpm.

The following steps are performed the next morning.

Step 7: Add the desired culture volume worth of fresh LB Broth to a sterile conical flask (I.e. 50 mL, 100 mL, 200 mL, etc). The maximum volume is dictated by the volume of the flask. Standard protocols utilise an air-to-volume ratio of 10:1. This means that if a 2 L flask is being used, the max desired volume of LB Broth should be 200 mL.

Step 8: Add kanamycin to the LB Broth for a final concentration of 50 µg/mL. (I.e. if the stock concentration of the kanamycin is 100 mg/mL and the desired culture volume is 200 mL, add 100 µL of the stock to the LB Broth.)

$$\frac{50\ \frac{\mu g}{mL} \times 200\ mL}{100\ \frac{mg}{mL}} = 100\ \mu L$$

Step 9: Inoculate the prepared culture flask with the overnight culture.

Step 10: Incubate the large culture at 37 °C and 225 rpm until an OD_{600} of 0.6 to 0.8 is reached.

Step 11: Once an appropriate OD_{600} has been reached, induce protein production by adding IPTG for a final concentration of 1 mM. (I.e. if the stock concentration of the IPTG is 0.8 M and the culture volume is 200 mL, add 250 µL of the stock to the culture.)

$$\frac{1\ mM \times 200\ mL}{0.8\ M} = 250\ \mu L$$

Step 12: Incubate the induced culture at 37 °C and 225 rpm for 4.5 hours.

Step 13: Aliquot the culture equally into centrifuge tubes.

Step 14: Centrifuge the culture at 3750 rpm for 30 minutes.

Step 15: Decant the supernatant. The pellets can be stored at -20 °C long term or 4 °C short term.

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Protein Purification Protocol for the Engineered Boon2-BST DNA Polymerase

Preliminary note to user:

- The following protocol was designed for purifying Boon2-BST from BL21 (DE3) cells
- For greater lysis efficiency, probe sonication can be used.

Materials:

- An induced *E. coli* cell pellet
- Lysozyme
- 1X PBS (pH 7.2)
- Microcentrifuge Tubes
- Pipettes
- 60 µm silica gel

Protein Purification Protocol of Engineered Boon2-BST:

Step 1: Suspend the cell pellet with 1 mg/mL of lysozyme in 1X PBS (pH 7.2). The final volume of the suspension is dependent on the volume of the bacteria culture used to form the pellet. For this protocol, a ratio of 1 mL of lysozyme per 10 mL *E. coli* culture is used.

Step 2: Incubate the lysis solution at room temperature for 30 minutes.

Step 3: Prepare a solution of 60 µm silica gel with a concentration of 25 mg/mL using 1X PBS (pH 7.2). It should be noted that vortexing and inverting the tube/container can assist in suspending the silica gel. In addition, this solution can be prepared and set aside during the lysis incubation step.

Step 4: After the lysis incubation, aliquot the cell lysis samples into microcentrifuge tubes.

Step 5: Centrifuge the lysis samples for 30 minutes at 13 krpm.

Step 6: Collect the cell lysate supernatant from the centrifuged samples. The solution should be pink in colour.

Step 7: Prepare the silica for immobilisation by first preparing microcentrifuge tubes with 20 mg of 60 µm silica gel (0.8 mL from the stock prepared in **Step 3**).

Step 8: Centrifuge the prepared silica at 7.5 krpm for 5 minutes.

Step 9: After centrifugation, remove the supernatant, leaving only silica gel in the tube.

Step 10: Add the cell lysate directly to the silica, at a ratio of 1 mL of cell lysate to 20 mg of silica gel.

Step 11: Suspend the silica in the cell lysate using a vortexer, and afterwards lay the tube flat on its side to spread out the settling microparticles.

Step 12: Incubate the silica with the cell lysate for 30 minutes at room temperature. To ensure even absorption across the silica microparticles, vortex the sample every 10 minutes.

Step 13: After silica immobilisation, first vortex the sample and then centrifuge at 7.5 krpm for 5 minutes.

Step 14: Remove the supernatant, leaving behind silica with immobilised Boon2-BST. The silica itself should be visibly pink, while the supernatant is colourless.

Step 15: Perform 2 rounds of washing on the silica immobilised Boon2-BST.

Washing Steps:

- Add 1 mL of 1X PBS (pH 7.2)
- Suspend the immobilised protein with a vortex
- Centrifuge the protein at 7.5 krpm for 5 minutes
- Removes the “wash” buffer

Step 16: Suspend the immobilised Boon2-BST in 1X PBS (pH 7.2) to its working concentration. A ratio of 5µL of buffer to 1 mg of silica gel is used.

Step 17: Collect and combine the immobilised Boon2-BST. The working stock can be stored at 4°C both short and long term.