AfriDX

COVID-19 diagnostics for Africa

Development of Boon-Enzymes

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Contents

Introduction

What is in this module?

This module will cover the general protocols utilised for the development of novel Boon-Enzymes. Protocols for polymerase chain reaction, gel extraction, klenow assembly, and preliminary expression will be provided, in addition to tips for *E. coli* transformations.

What is a Boon-Enzyme?

The Boon-Enzymes are heterogenous recombinant proteins that has been specifically designed to immobilise onto silica (component: R5 silaffin-tag), provide visual indication of protein expression and allow for protein tracking (component: mCherry), and either amplify targeted DNA/RNA (component: BST 2.0 DNA polymerase, reverse transcriptase X) or specifically bind to antigen targets (component: single-chain variable fragment (scFv)).

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 enzymes/scFv are active under standard LAMP/PCR or lateral flow test/ELISA conditions and can be utilised without further adjustments.

While these enzymes are considered to be modular, it may be simpler to begin by retaining the existing affinity-tag fluorescent protein core and just swapping out the enzyme for further applications.

There are currently two Boon-Enzyme designs, as shown in Figure 1. The reason for the different order is due to the compatibility of the enzyme/scFv with the recombinant additions. Design (1) was utilised by the all the Boon-polymerases (i.e., BST and RTX). This is because both polymerases have their active-site near the C-terminal, and any potential C-terminal addition to these enzymes would severely inhibit the enzyme. N-terminal modifications allow for safe implementation of the Boon-design without serious impact on enzymatic activity. The silaffin tag was positioned at the beginning (N-terminal) to maximise accessibility. In a similar note, the internal histidine-tag was extended from the traditional His6 to His10 (a peptide of 6 or 10 repeating histidine amino acids) to improve its accessibility since it was positioned unfavourably. The histidine-tag was not positioned (based on sequence) next to the R5 silaffin-tag as the two tags were observed to interfere with one another. Design (2) was implemented for the constructs with an scFv. In order to minimise any potential interference with the binding properties of the Boon-scFv, the mCherry was positioned as a linker in

between the light and heavy chains. Lastly, the purification tag was located at the N-terminal.

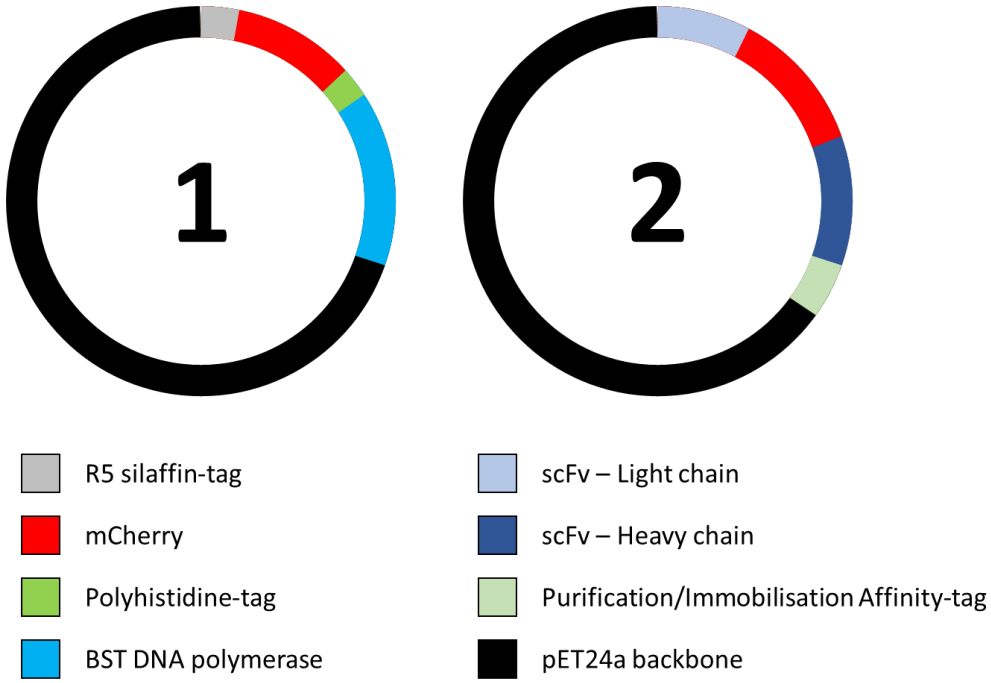

Figure 1: Illustrations of the two current Boon-system architecture (1 – polymerase, 2 – scFv).

What is Klenow Assembly?

Klenow assembly is essentially a variation of the more commonly known Gibson assembly. In principle, these methods can be used to assemble new plasmids by fusing together DNA fragments that have roughly a 15 – 20 bp overlap. The DNA fragments are digested to produce "sticky" overhangs. Due to the overlapping sequence, the digested fragments will anneal to one another. In Gibson assembly, the annealed together plasmid would be ligated before transformed into competent *E. coli*. In klenow assembly, the annealed plasmid is directly transformed into *E. coli*, where the native enzyme of the bacteria performs the ligation step. Overall klenow assembly is a rapid technique that can be harnessed to efficiently produce new Boon-Enzymes, as illustrated in Figure 2.

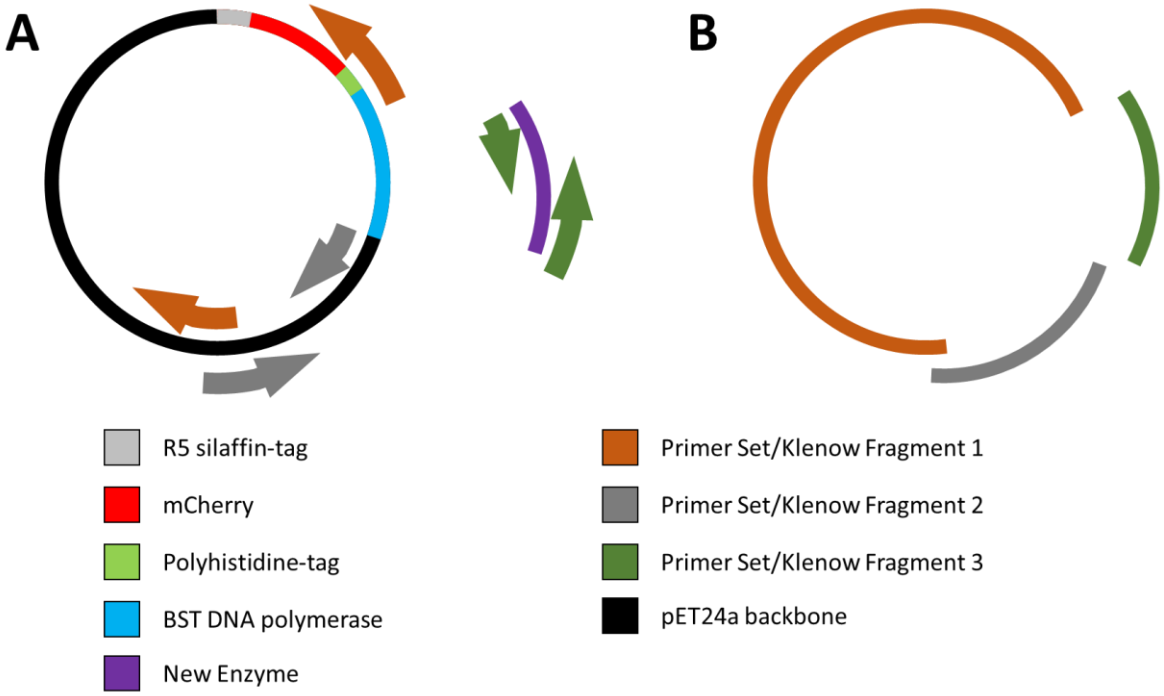

Figure 2: Illustration of an example klenow assembly to swap BST DNA polymerase with a new enzyme. Each fragment would have a 15 – 20 bp overlap with the neighbouring fragment. In this assembly, everything apart from the BST DNA polymerase is kept from the existing plasmid and repurposed to house the new enzyme.

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 3. 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 4. 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.

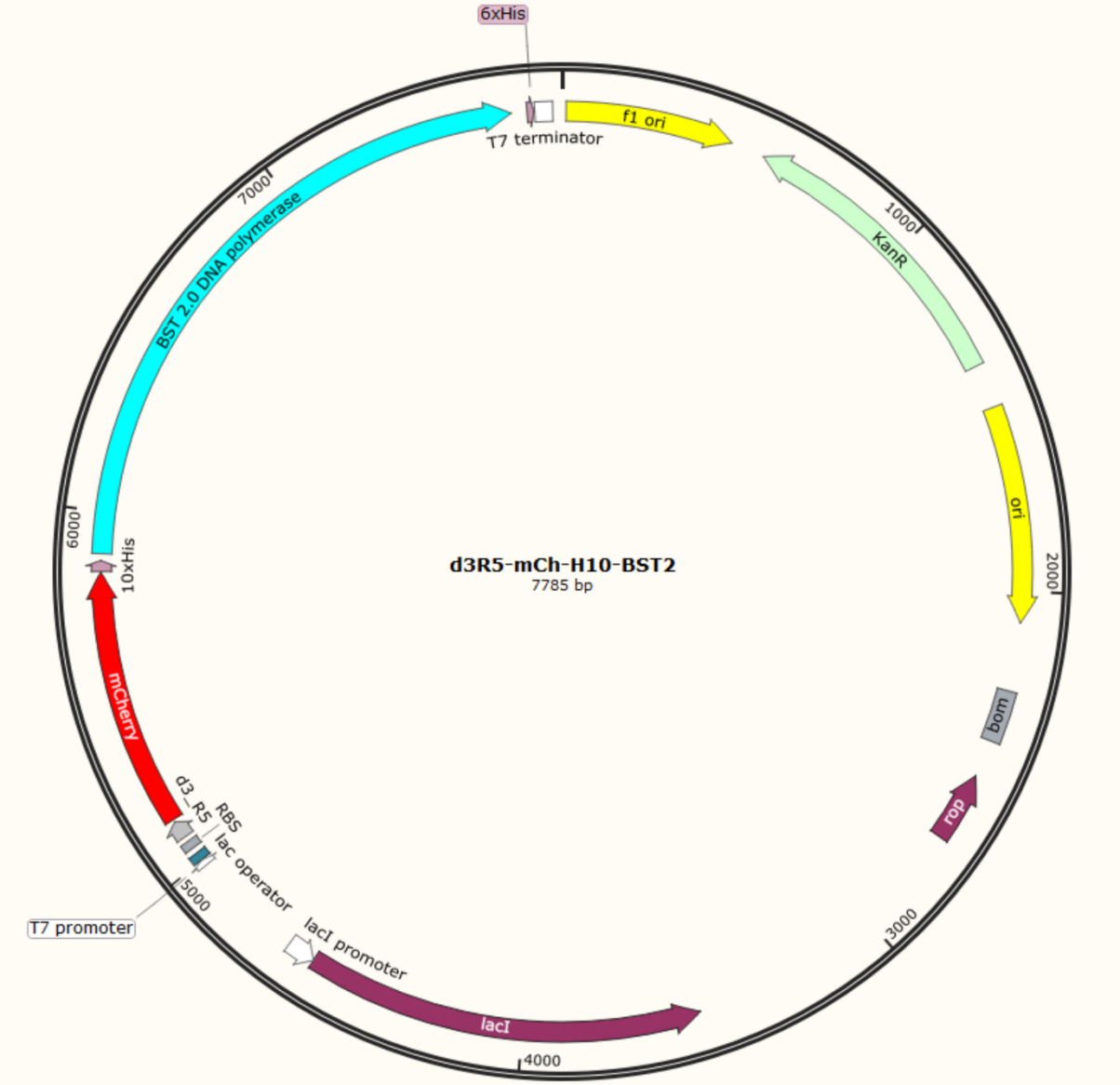

Figure 3: Vector map of the pET24a plasmid used to express d3R5-mCherry-H10-BST2.0

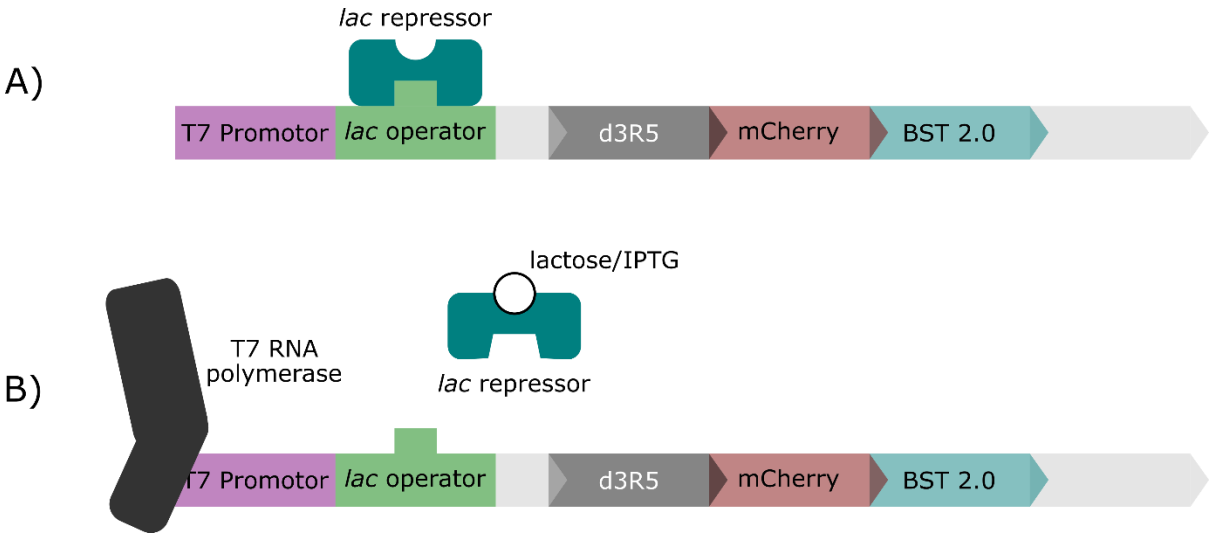

Figure 4: Illustration of the of lac repressor/operator system. A) Inhibited operator that is unable to express protein. B) Relieved expression system that is now capable of expressing protein.

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.

Polymerase Chain Reaction of Klenow Fragments

Preliminary note to user:

- The following protocol was designed using Q5® High-Fidelity DNA Polymerase

Materials:

- Q5® High-Fidelity DNA Polymerase
- Q5® Reaction Buffer
- Forward Primer (One per fragment)
- Reverse Primer (One per fragment)
- dNTP
- Milli-Q® or Nuclease-free water
- 0.2mL PCR tubes
- Ice (Ideal, but not necessary)
- A container for ice (ideal but not necessary)
- Thermocycler
- Pipettes (2µL, 10µL or 20µL, 200µL)
- Pipette tips for the pipettes used (ideally filtered tip, but not necessary)

Polymerase Chain Reaction Protocol:

Step 1: Prepare a 50µL PCR mix, in a PCR tube, using the volumes displayed in Table 1 (Though it is not always necessary, it is often good practice to prepare the reaction on ice):

Table 1: Materials and volumes for PCR mix to generate klenow fragments.

Reagent	Volume	Final Concentration/Mass
Q5 Reaction Buffer (5X)	10µL	1X
dNTP (10mM Each)	1µL	0.2mM Each
Forward Primer (10µM)	2.5µL	0.5µM
Reverse Primer (10µM)	2.5µL	0.5µM
Template DNA	Dependent on Template Concentration	1 - 10ng
Q5 High-Fidelity DNA Polymerase (2U/µL)	0.5µL	1U
Milli-Q®/Nuclease-Free Water	to 50µL	

Step 2: Transfer sample to a thermocycler and run a 30 cycle PCR. The temperature profile is displayed in Table 2:

Table 2: Temperature profile for PCR.

Step	Temperature	Time	Cycles
Initial Denaturation	98°C	10s	1
Denaturation	98°C	10s	30
Annealing[a]	50 - 65°C	30s	
Extension[b]	72°C	30s/kbp	
Final Extension[c]	72°C	120s	1

a) The annealing temperature is dependent on the melting temperature of the primers (Tm). In general, the user can initially attempt to amplify the fragment using an annealing temperature of 50°C. However, if the amplification is dominated by non-specific binding than it is recommend increasing the annealing temperature to a maximum of Tm - 2°C or the annealing temperature recommended online by the NEB Tm Calculator.

b) The length of time is dependent on the size of the amplified fragment. If the size of the fragment (Forward Primer to Reverse Primer) is 1000 base pairs (1000bp or 1kbp) or less, allowing the PCR reaction to extend for 30s. If the amplified fragments in longer than 1kbp, increase the extension time accordingly and round up to the nearest 5 seconds. I.e., For amplification of a 1750bp fragment:

$$30s \times 1.75 = 52.5s \rightarrow 55s$$

c) If the amplified fragment is very long (> 4kbp), increase the final extension time to match the cycle extension time.

Step 3: After the PCR reaction completes either store the samples in a freezer (-20°C) to process the sample at a later time/date or proceed to Section "**Processing Polymerase Chain Reaction Amplified Fragments for Klenow Assembly**".

Processing Polymerase Chain Reaction Amplified Fragments for Klenow Assembly

Preliminary note to user:

- The gel extraction clean-up protocol is dependent on the Gel Extraction kit utilised. Potential kit suppliers include: Qiagen, Thermo Fisher, and New England Biolabs.

Materials:

- Agarose
- 1x TAE Buffer
- SYBR™ Safe DNA Gel Stain
- DNA Ladder
- DNA Gel Electrophoresis Station/Setup (gel mould, well comb, electrophoresis tank, power supply)
- Blue/UV LED gel visualiser
- 1.5mL microcentrifuge tube
- Disposable knife/blade
- Gel Extraction Kit
- Isopropanol
- Ethanol
- Hot Plate/Thermocycler
- NanoDrop
- Pipettes (2µL, 10µL or 20µL, 200µL, 1000µL)
- Pipette tips for the pipettes used

Protocol for Gel Extraction of Klenow Fragments:

Preparing a 1 – 2% (w/v) Agarose Gel

Step 1: Weigh out 250 – 500mg (1 – 2%) of agarose in a 100mL microwavable bottle.

Step 2: Add 25mL of 1X TAE buffer to the agarose bottle. I.e., 25mL of 1X TAE buffer with 250mg of Agarose produces a 1% (w/v) agarose gel. Using the approximate density of water (1g/mL):

$$\frac{Mass\ of\ Agarose}{Volume\ of\ 1X\ TAE\ buffer\ \times\ Density\ of\ Water} \times 100\%$$

$$= \frac{0.25g}{25mL \times 1\frac{g}{mL}} \times 100\%$$
$$= \frac{0.25g}{25g} \times 100\%$$
$$= 0.01 \times 100\% = 1\%$$

Step 3: Microwave the solution at 10 – 15 second intervals with a quirk swirl in between to help dissolve the agarose. It is important to not microwave at too long of an interval to reduce evaporation and to prevent the solution from bubbling out of the bottle. In addition, either leave the bottle open or with a loose lid to prevent a build-up of pressure during heating. Lastly, pick up the bottle with thermal-resistant gloves or even layers of paper towel. DO NOT touch the bottle directly, as it can be very hot.

Step 4: Once the agarose has fully dissolved, allow the bottle to cooldown enough to be touch, but not too cool that that the agarose solution starts to solidify.

Step 5: Transfer the agarose solution to a disposable tube and add 2.5µL of SYBR™ Safe DNA Gel Stain. Most DNA stain are at a stock concentration of 10,000X.

$$\frac{Volume\ of\ DNA\ Gel\ Stain}{Volume\ of\ Agarose\ Solution} \times Stock\ Concentration\ of\ DNA\ Gel\ Stain$$
$$= \frac{0.0025mL}{25mL} \times 10{,}000X$$
$$= 0.0001 \times 10{,}000X$$
$$= 1X$$

It is especially important to transfer the agarose solution to a disposable bottle before adding in the DNA gel stain if working with a carcinogenic stain, such as Ethidium Bromide.

Step 6: Pour the solution into the gel mould and add a well comb that is large enough for approximately 20µL of sample per well. Ensure the well comb is positioned near the top or bottom of the well, as shown in Figure 5.

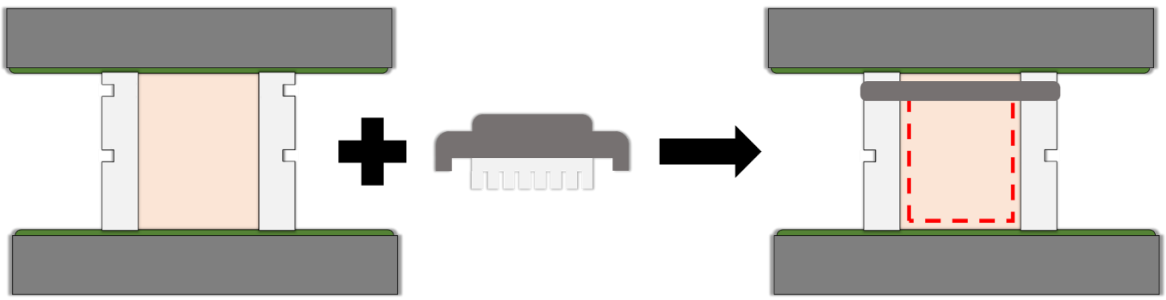

Figure 5: Illustration of setting an agarose gel with the well positioned at the "top."

Step 7: Incubate the setup at room temperature for 15 – 20 minutes, to allow the gel to completely solidify.

Step 8: Remove the well comb and transfer the gel to an electrophoresis tank. Ensure that the "top of the gel" (the edge of the gel closest to the wells) is closer to the negative (black) terminal of the tank. DNA is negatively charged, therefore it will be repelled by the negative terminal and attracted by the positive (red) terminal.

Step 9: Fill the electrophoresis tank with 1X TAE buffer until the agarose gel is fully submerged by a couple of millimetres.

Step 10: Add a 4µL droplet of DNA Loading Dye (6X) to a piece of parafilm.

Step 11: Pipette out 20µL of the PCR fragment and mix the amplicon solution with the Loading Dye droplet until the solution is a homogenous colour.

Step 12: Transfer the mixed 20µL to one of the wells on the agarose gel.

Step 13: Repeat Steps 10 to 12 with another 20µL of the same amplicon sample. It is ideal to use a neighbouring well to the first portion of the fragment sample.

Step 14: Add 6 – 8µL of a DNA Ladder to a neighbouring well.

Step 15: Close the electrophoresis tank and plug in the wiring to the power supply (red wire into the red terminal, black wire into the black terminal).

Step 16: Run the gel electrophoresis at 90V for 45 to 60 minutes.

Step 17: Once the electrophoresis step is done, transfer the gel to a blue/UV led gel visualiser. Ensure the protective cover is on, and then turn on the LEDs.

Step 18: Using the DNA ladder as a reference, observe if there is a strong amplicon band of the expected size. In addition, if a weak band is present observe if there are any bands due to non-specific binding.

Step 19: If the desired band is present, start by zeroing an empty 1.5mL microcentrifuge tube in a microbalance.

Step 20: Using a clean disposable knife/blade, cut out the band and transfer the fragment to the 1.5mL microcentrifuge tube used in Step 19.

Step 21: Weigh the mass of the gel of the fragment and note it down. Important for gel extraction kit steps.

Step 22: The amplicon is now ready to be processed using the user's preferred gel extraction kit. The final elution can also be performed using water.

Step 23: Once the PCR amplicon has been eluted, use a NanoDrop to determine the concentration of the DNA fragment.

Klenow Assembly

Preliminary note to user:

- The following protocol was designed around "fresh" Klenow polymerase. Incubation time can be extended to improve the efficiency of the assembly, especially when using older enzyme.

Materials:

- DNA polymerase I, Large (Klenow) Fragment
- NEBuffer™ 2
- PCR/Klenow Fragments
- Milli-Q®/Nuclease-free water
- 0.2mL PCR tubes
- Ice
- A container for ice
- Thermocycler
- Pipettes (2µL, 10µL or 20µL)
- Pipette tips for the pipettes used (ideally filtered tip, but not necessary)

Protocol for Gel Extraction of Klenow Fragments:

Step 1: Begin by preparing the Klenow Assembly solution (Table 3), on ice, in a 0.2mL PCR tube.

Table 3: Materials and volumes for klenow assembly.

Reagent	Volume	Final Concentration/Mass
DNA polymerase I, Large (Klenow) Fragment (5U/µL)	1µL	5U
NEBuffer™ 2 (10X)	0.5 – 1µL	1X
DNA Fragment X	X	30ng
DNA Fragment Y	Y	30ng
DNA Fragment Z	Z	30ng
DNA Fragment….	…	30ng of each fragment
Milli-Q®/Nuclease-free Water	to 5 – 10µL	

Example:

Reagent	Volume
DNA polymerase I, Large (Klenow) Fragment (5U/µL)	1µL
NEBuffer™ 2 (10X)	1µL
DNA Fragment X – 30ng/µL	1µL
DNA Fragment Y – 45ng/µL	0.67µL
DNA Fragment Z – 15ng/µL	2µL
Milli-Q®/Nuclease-free Water	4.33µL
Total:	10µL

- A total volume of 10µL was used because the volume of Klenow polymerase + DNA fragments is already 4.67µL.
- If this example was a 2-fragment assembly (DNA Fragment X + Y), the start volume (Klenow + DNA Fragment) is 3.67µL. Therefore, a 5µL total solution is still possible.
- If the Klenow assembly appears to be inefficient (low or no bacteria colonies after transformation), 2µL (10U) of Klenow polymerase can be used and/or increase the quantity of DNA fragments to 35 – 40ng per assembly.

Step 2: Transfer the PCR tube to a thermocycler and incubate the reaction at 37°C for 30 minutes. If the Klenow polymerase is old, a 60-minute incubation can be utilised to improve the efficiency of the assembly.

Step 3: Proceed to *E. coli* transformation or store at -20°C and transform the assembly the following day. Transformation should be done with a cloning *E. coli* strain (NEB Turbo, DH5α™). These strains of bacteria are better suited for maintaining and propagating plasmid DNA due to a combination of high transformation efficiency, reduced DNA recombination and inactivated nonspecific endonuclease I.

Transformation I

Preliminary note to user:

- Transformation should be done with a cloning E. coli strain (NEB Turbo, DH5αTM). These strains of bacteria are better suited for maintaining and propagating plasmid DNA due to a combination of high transformation efficiency, reduced DNA recombination and inactivated nonspecific endonuclease I.
- Any direct bacteria work (aliquoting bacteria, plating, etc.) should be conducted under sterile conditions.

Materials:

- LB agar plates with appropriate antibiotic (e.g., kanamycin with pET24a plasmids)
- Competent E. coli designed for cloning
- SOB/SOC media
- Ice
- A container for ice
- Hot plate/water bath
- Media culture incubator with built-in shaker
- Plate culture incubator
- L-shaped spreaders
- 1.5mL microcentrifuge tubes
- Pipettes (10µL or 20µL, 200µL, 1000µL)
- Sterile pipette tips for the pipettes used

Transformation Protocol Tips:

Tip 1: It is recommended to follow the manufactures protocol to maximise the transformation efficiency.

Tip 2: When adding the Klenow assembly to the cell mixture, add the full volume (5 – 10µL).

Tip 3: In addition, after the addition of SOB/SOC media, the 1-hour incubation step at 37°C can be extended to 2 hours to help improve transformation efficiency.

Tip 4: LB agar plates can be stored at 4°C for a maximum of 1 week.

Post-Transformation Screening

Preliminary note to user:

- This is a 2-part protocol comprised on an initial screening followed by DNA sequencing.
- Protocol was designed to screen 12 colonies. Adjust the volumes (for more or less colonies) accordingly.
- The PCR step utilises Q5® High-Fidelity DNA Polymerase, however, a high-fidelity polymerase is not necessary for this protocol.
- Any direct bacteria work (replating, culturing, etc.) should be conducted under sterile conditions.

Materials:

- 1.5mL microcentrifuge tubes
- Centrifuge tubes
- LB agar plates with appropriate antibiotic (e.g., kanamycin with pET24a plasmids)
- Milli-Q®/Nuclease-free water
- Hot plate
- Microcentrifuge
- Centrifuge
- Q5® High-Fidelity DNA Polymerase
- Q5 Reaction Buffer
- Forward Primer
- Reverse Primer
- Sequencing Primers
- dNTP
- 0.2mL PCR tubes
- Thermocycler
- Agarose
- 1x TAE Buffer
- SYBR™ Safe DNA Gel Stain
- DNA Ladder
- DNA Gel Electrophoresis Station/Setup (gel mould, well comb, electrophoresis tank, power supply)
- Gel visualiser/imager
- LB broth
- Antibiotic (e.g., kanamycin with pET24a plasmids)
- Media culture incubator with built-in shaker

- Plate Culture incubator
- Miniprep kit
- Ethanol
- NanoDrop
- Pipettes (2µL, 10µL or 20µL, 200µL, 1000µL)
- Sterile/non-sterile pipette tips for the pipettes used

Post-Transformation Screening Protocol:

Begin by first screening post-transformation colonies with colony PCR.

Step 1: Prepare and label 12 1.5mL microcentrifuge tubes. Each tube should have 40µL of water. If less than 12 colonies are present from the transformation step, screen as many colonies as possible.

Step 2: Prepare fresh LB agar plate. Label the base of the plate (the half of the petri dish that has the agar) in correspondence to the labelled microcentrifuge tube.

Step 3: Begin heating a hot plate to 95 – 100°C.

Step 4: Using a 2, 10, or 20µL pipette with a sterile pipette tip, lightly pick-up a **single** colony from the transformation plate but gently scraping the colony with the pipette tip. Dab the tip into a labelled section of the freshly prepared agar plate, then place the tip into the corresponding microcentrifuge tube and pipette up and down several times.

Step 5: Repeat step 4 for the rest of the colonies being screened. Ensure each colony is transferred to a new section of the plate prepared in step 2.

Step 6: Place the subculture LB agar plate in a 37°C plate culture incubator.

Step 7: Boil the microcentrifuge tube for 10 minutes.

Step 8: Using a microcentrifuge, spin down the samples for 5 minutes at 13krpm, 4°C.

Step 9: Prepare a 132µL PCR master mix, in a microcentrifuge/PCR tube, using the volumes displayed in Table 4 (Though it is not always necessary, it is often good practice to prepare the reaction on ice):

Table 4: Materials and volumes for PCR mix for colony screening.

Reagent	Volume	Final Concentration/Mass
Q5 Reaction Buffer (5X)	30µL	1X
dNTP (10mM Each)	3µL	0.2mM Each
Forward Primer (10µM)[a]	7.5µL	0.5µM
Reverse Primer (10µM)[a]	7.5µL	0.5µM
Q5 High-Fidelity DNA Polymerase (2U/µL)	1.5µL	12U
Milli-Q®/Nuclease-Free Water	82.5µL	

*These volumes are based around having 12 12.5µL PCR samples.

a – It is recommended to use primers that will help to distinguish between the original template(s) and the newly synthesised construct. It can often be useful to screen from the promotor to the terminator region of the plasmid. (e.g., If using a pET-based plasmid design the forward primer to bind to the T7 promotor region and the reverse primer to bind to the T7 terminator region).

Step 9: Aliquot the master mix into 12 PCR tubes with 11µL of solution each.

Step 10: Add 1.5µL of the colony screening sample from step 4 into the corresponding PCR tube from step 9. Ensure to not agitate any pellet that might have formed in the microcentrifuge tube during boiling/spin down. LB agar contamination can inhibit PCR.

Step 11: Transfer sample to a thermocycler and run a 25 cycle PCR. The temperature profile is displayed in Table 5:

Table 5: PCR temperature profile.

Step	Temperature	Time	Cycles
Initial Denaturation	98°C	10s	1
Denaturation	98°C	10s	25
Annealing[a]	50 - 65°C	30s	
Extension[b]	72°C	30s/kbp	
Final Extension[c]	72°C	120s	1

a) The annealing temperature is dependent on the melting temperature of the primers (Tm). In general, the user can initially attempt to amplify the fragment using an annealing temperature of 50°C. However, if the amplification is dominated by non-specific binding than it is recommend increasing the annealing temperature to a maximum of Tm - 2°C or the annealing temperature recommended online by the NEB Tm Calculator.

b) The length of time is dependent on the size of the amplified fragment. If the size of the fragment (Forward Primer to Reverse Primer) is 1000 base pairs (1000bp or 1kbp) or less, allowing the PCR reaction to extend for 30s. If the amplified fragments in longer than

1kbp, increase the extension time accordingly and round up to the nearest 5 seconds. I.e., For amplification of a 1750bp fragment:

$$30s \times 1.75 = 52.5s \rightarrow 55s$$

c) If the amplified fragment is very long (> 4kbp), increase the final extension time to match the cycle extension time.

Step 12: When the PCR nears completion, begin preparing a 1 – 2% (w/v) Agarose Gel

Step 13: Weigh out 250 – 500mg (1 – 2%) of agarose in a 100mL microwavable bottle

Step 14: Add 25mL of 1X TAE buffer to the agarose bottle. I.e., 25mL of 1X TAE buffer with 250mg of Agarose produces a 1% (w/v) agarose gel. Using the approximate density of water (1g/mL):

$$\frac{Mass\ of\ Agarose}{Volume\ of\ 1X\ TAE\ buffer \times Density\ of\ Water} \times 100\%$$

$$= \frac{0.25g}{25mL \times 1\frac{g}{mL}} \times 100\%$$

$$= \frac{0.25g}{25g} \times 100\%$$

$$= 0.01 \times 100\% = 1\%$$

Step 15: Microwave the solution at 10 – 15 second intervals with a quirk swirl in between to help dissolve the agarose. It is important to not microwave at too long of an interval to reduce evaporation and to prevent the solution from bubbling out of the bottle. In addition, either leave the bottle open or with a loose lid to prevent a build-up of pressure during heating. Lastly, pick up the bottle with thermal-resistant gloves or even layers of paper towel. DO NOT touch the bottle directly, as it can be very hot.

Step 16: Once the agarose has fully dissolved, allow the bottle to cooldown enough to be touch, but not too cool that that the agarose solution starts to solidify.

Step 17: Transfer the agarose solution to a disposable tube and add 2.5µL of SYBR™ Safe DNA Gel Stain. Most DNA stain are at a stock concentration of 10,000X.

$$\frac{Volume\ of\ DNA\ Gel\ Stain}{Volume\ of\ Agarose\ Solution} \times Stock\ Concentration\ of\ DNA\ Gel\ Stain$$

$$= \frac{0.0025mL}{25mL} \times 10{,}000X$$

$$= 0.0001 \times 10{,}000X$$

$$= 1X$$

Step 18: It is especially important to transfer the agarose solution to a disposable bottle before adding in the DNA gel stain if working with a carcinogenic stain, such as Ethidium Bromide.

Step 19: Pour the solution into the gel mould and add a single or multiple well combs to produce at least 13 lanes, if possible. Ensure the well comb is positioned near the top or bottom of the well, as shown in Figure 5.

Step 20: Incubate the setup at room temperature for 15 – 20 minutes, to allow the gel to completely solidify.

Step 21: Remove the well comb and transfer the gel to an electrophoresis tank. Ensure that the "top of the gel" (the edge of the gel closest to the wells) is closer to the negative (black) terminal of the tank. DNA is negatively charged, therefore it will be repelled by the negative terminal and attracted by the positive (red) terminal.

Step 22: Fill the electrophoresis tank with 1X TAE buffer until the agarose gel is fully submerged by a couple of millimetres.

Step 23: Add 12 1µL droplets of DNA Loading Dye (6X) to a piece of parafilm.

Step 24: Pipette out 5µL of the PCR fragment and mix the amplicon solution with the Loading Dye droplet until the solution is a homogenous colour.

Step 25: Transfer the mixed 5µL to one of the wells on the agarose gel.

Step 26: Repeat Steps 24 to 25 with the remaining PCR samples.

Step 27: Add 6 – 8µL of a DNA Ladder to a neighbouring well.

Step 28: Close the electrophoresis tank and plug in the wiring to the power supply (red wire into the red terminal, black wire into the black terminal).

Step 29: Run the gel electrophoresis at 90V for 45 to 60 minutes.

Step 30: Once the electrophoresis step is done, transfer the gel to a blue/UV led gel visualiser. Ensure the protective cover is on, and then turn on the LEDs.

Step 31: Using the DNA ladder as a reference, observe if any of the screened colonies has an amplicon band of the expected size.

Step 32: Based on the screening results, choose 1 – 4 colonies to send for sequencing. Without sequencing, you cannot be completely sure that the designed recombinant protein is encoded as expect.

Step 33: Allow the subcultures (step 4-5) to incubate until there are visible colonies growing from the inoculation points.

Step 34: Prepare overnight cultures in centrifuge tubes by adding 5mL of LB broth with 50µg/mL of antibiotic. The number of centrifuge tubes is dependent on then number for colonies being sent for sequencing.

Step 35: Using a 2, 10, or 20µL pipette with a sterile pipette tip, lightly pick-up a **single** colony (one of the colonies chosen for sequencing) from the subculture plate by gently scraping the colony with the pipette tip.

Step 36: Place the tip into the corresponding centrifuge tube and pipette up and down several times.

Step 37: Incubate the overnight cultures at 37°C, 225rpm.

Step 38: The next morning, centrifuge the overnight cultures at 3750rpm, 4°C for 30 minutes.

Step 39: Decant the used LB broth.

Step 40: Using a commercial miniprep kit, process the overnight pellet and harvest the plasmid DNA. Follow the protocol provided by the kit. Water can be used to elute the DNA instead of elution buffer.

Step 41: Measure the concentration and purity of the extracted DNA using a Nanodrop.

Step 42: Prepare and send the chosen samples for sequencing, based on the conditions stated by the service provider.

Step 43: Once sequencing data has been received use software such as Snapgene Viewer and/or ApE to assemble the final Boon-Enzyme sequence and to align it against the expected sequence.

Transformation II for Expression of New Boon-Enzyme

Preliminary note to user:

- Transformation should be done with an expression E. coli strain (BL21, C43). These strains contain the RNA polymerase gene(s) necessary to express recombinant proteins. The choice in E. coli strain can dramatically impact expression. For example, BL21 cells have been optimised to maximise T7-based expression even at the cost of producing more inclusion bodies while C43 has been engineered to assist in the expression of difficult to produce or toxic proteins.
- Any direct bacteria work (aliquoting bacteria, plating, etc.) should be conducted under sterile conditions.

Materials:

- LB agar plates with appropriate antibiotic (e.g., kanamycin with pET24a plasmids)
- Competent E. coli designed for expression
- SOB/SOC media
- Ice
- A container for ice
- Hot plate/water bath
- Media culture incubator with built-in shaker
- Plate culture incubator
- L-shaped spreaders
- 1.5mL microcentrifuge tubes
- Pipettes (10µL or 20µL, 200µL, 1000µL)
- Sterile pipette tips for the pipettes used

Transformation Protocol Tips:

Tip 1: It is recommended to follow the manufactures protocol to maximise the transformation efficiency.

Tip 2: LB agar plates can be stored at 4°C for a maximum of 1 week.

Preliminary Expression of New Boon-Enzyme

Preliminary note to user:

- Any direct bacteria work (aliquoting bacteria, plating, etc.) should be conducted under sterile conditions.

Materials:

- LB broth
- Antibiotic (e.g., kanamycin with pET24a plasmids)
- Sterile 50% glycerol
- 1.5mL microcentrifuge tubes
- Media culture incubator with built-in shaker
- Pipettes (2µL, 10µL or 20µL, 200µL, 1000µL)
- Sterile/non-sterile pipette tips for the pipettes used

Transformation Protocol Tips:

Step 1: Begin by producing an overnight culture (5mL of LB broth with 50µg/mL of antibiotic in a centrifuge tube)

Step 2: Using a 2, 10, or 20µL pipette with a sterile pipette tip, lightly pick-up a **single** colony (from the transformation plate by gently scraping the colony with the pipette tip.

Step 3: Place the tip into the corresponding centrifuge tube and pipette up and down several times.

Step 4: Incubate the overnight cultures at 37°C, 225rpm.

Step 5: The next morning, transfer 0.5mL of the overnight culture into a 1.5mL microcentrifuge tube.

Step 6: Added 0.5mL of 50% glycerol to the 1.5mL microcentrifuge tube mix thoroughly by inverting the tube 5 – 6 times.

Step 7: The glycerol stock can now be stored long-term at -20°C and below. Future overnights can be prepared using 1µL of glycerol stock in 5mL of LB broth and 50µg/mL of antibiotic.

Step 8: The remaining overnight culture can be used to inoculate a large volume of media for expression testing. Initial expression testing can range from 5 hours at 37°C to 24 hours at 18°C.