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Expression and Purification of recombinant Bst DNA polymerase (Bst)

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ABSTRACT

Bst is a type I DNA polymerase with strong strand displacement activity isolated from *Geobacillus stearothermophyllus* (previously *Bacillus steathermophylus*). Bst enzyme is a gold standard in isothermal nucleic acid amplification diagnostic techniques, especially in Loop-mediated isothermal amplification (LAMP). LAMP is a low-cost technique, provides a visual detection (when combined with pH indicators) and does not require the use of thermal cyclers. Also, Bst combined with thermostable reverse transcriptase can amplify RNA templates, in a technique known as RT-LAMP. RT-LAMP is useful for RNA virus and transcript detection and can be employed in circumstances that require mass production of diagnostic tests or limited availability of resources. This protocol shows the expression and purification procedure by FPLC of the Bst polymerase for its implementation in diagnostic techniques such as end-point colorimetric and real time fluorometric LAMP and RT-LAMP.

GUIDELINES

During the process of protein purification maintain all samples that contain the protein of interest in a cold environment to avoid protein degradation.

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PROTOCOL integer ID:

63361

Keywords: Bacillus stearothermophylus, polymerase, Bst, protein expression, protein purification **MATERIALS**

Reagents:

🕇 🔯 Chaperone Plasmid Set **Takara Bio USA, Inc. Catalog #3340**

Chemically Competent E. coli One Shot™ BL21(DE3) Invitrogen - Thermo
Fisher Catalog #C600003

- [M] 100 ng/µL pColdI-Bst plasmid Step 3.1

- Stock [м] 30 mg/mL chloramphenicol

- Stock [M] 100 mg/mL carbenicillin

- Stock [м] 1 Molarity (М) IPTG

- Stock [м] 10 % (v/v) Triton X-100

△ 250 µL of SOC medium Step 1.4

- A 8 mL of TFBI solution Step 2.4

2.5 mL of TFBII solution Step 2.5

- Cryotubes with A 500 µL of 30% v/v glycerol (sterilized)

- LB agar plates

- Tubes with 🔼 3 mL Luria-Bertani (LB) medium

- Tubes with 🚨 5 mL Luria-Bertani (LB) medium

- Tubes with 🗸 5 mL Terrific Broth (TB) medium

- Flasks with 🔼 20 mL LB medium

- Flasks with 🔼 50 mL LB medium

- Flasks with 🚨 50 mL TB medium

- Flask with 🚨 100 mL LB medium

- Flasks with 🗸 1 L LB medium

- △ 250 mL lysis buffer A (LB-A) Step 5.5

- 🗸 250 mL Saline Buffer (SB) Step 9.2

- 🗸 500 mL Washing Buffer-AI (WB-AI) Step 10.2

- 🚨 500 mL Elution Buffer-AI (EB-AI) Step 10.5

■ 1 L Desalting Buffer-A (DB-A) Step 11.2

■ A 500 mL Washing Buffer-All (WB-All) Step 12.2

- 🗸 500 mL Storage Buffer-A (SB-A) Step 13.1

⊠ HisTrap HP 5mL Cytiva Catalog #17524801

🔀 HiPrep 26/10 Desalting Column Cytiva Catalog #17508701

🔀 HiTrap Heparin HP affinity column Cytiva Catalog #17040701

⊠ Quick Start™ Bradford 1x Dye Reagent **BioRad Sciences Catalog #5000205**

-

Precision Plus Protein™ Unstained Protein Standards **Bio-rad**Laboratories Catalog #1610363

- Tricine-SDS-PAGE electrophoresis solutions (Step 6.2)
- 8% polyacrylamide gels for Tricine-SDS-PAGE (Step 6.2)

Equipments:

- Thermomixer

Equipment	
Thermomixer® R	NAME
Dry block heating and cooling shaker, 120 V, 60 Hz, 1/cs	TYPE
Eppendorf	BRAND
T3317	SKU

- Orbital shaker

Equipment	
MaxQ™ HP Incubated Tabletop Orbital Shaker	NAME
MaxQ™ HP, 120 V 60 Hz, 6,5 A o 230 V 50/60 Hz, 3,2 A	TYPE
Thermo Scientific	BRAND
SHKE420HP	SKU
https://www.thermofisher.com/order/catalog/product/SHKE420HP	LINK

- Floor model orbital shaker

Equipment	
MaxQ™ HP Incubated and Refrigerated Console Shakers	NAME
MaxQ™ 481 HP, 230 V, 50 Hz	TYPE
Thermo Scientific	BRAND
SHKE481HP	SKU
https://www.thermofisher.com/order/catalog/product/SHKE481HP	LINK

- Centrifuge

Equipment	
Sorvall™ Legend™ XT/XF Centrifuge Series	NAME
Thermo Scientific	BRAND
75004541	SKU
https://www.thermofisher.com/order/catalog/product/75004541	LINK

- Ultrasonic Processor 130W

Equipment	
Ultrasonic Processor	NAME
130-Watt Ultrasonic Processor	TYPE
Cole-Parmer	BRAND
ML-04714-52	SKU
https://www.coleparmer.com/p/cole-parmer-130-watt-ultrasonic-processors/44347	LINK

- Ultrasonic Processor 750W

T50-Watt Ultrasonic Processor CPX750 TYPE Cole-Parmer ML-04711-60 https://www.coleparmer.com/p/cole-parmer-500-and-750-watt-ultrasonic-processors/16401

-Nanodrop

Equipment	
NanoDrop™ One UV-Vis Spectrophotometer	NAME
spectrophotometer	TYPE
Thermo Scientific	BRAND
ND-ONE-W	SKU
https://www.thermofisher.com/order/catalog/product/ND-ONE-W	LINK
Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 SPECIFICATIONS nm (FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10, 64 bit; Voltage: 12 V (DC); Wavelength Range: 190–850 nm	

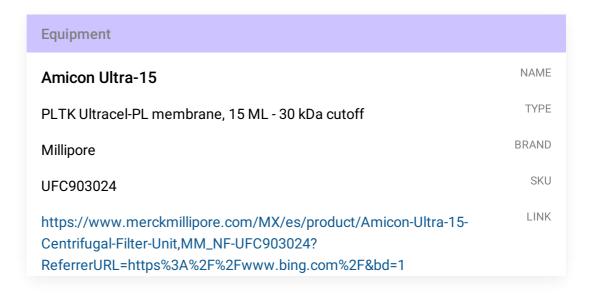
- FPLC system

Equipment	
ÄKTA pure	NAME
Protein purification system	TYPE
Cytiva	BRAND
29046665	SKU
https://www.cytivalifesciences.com/en/us/support/products/akta-pure- 150-l-29046665	LINK

- Spectrophotometer UV/Vis
- Incubator (37°C)
- Water bath (60°C)
- Ultra Low-Temperature Freezer (-80°C)
- Freezer -20°C
- Refrigerator (4°C)
- Analytical balance

Other:

- Ice bath
- Microcentrifuge tubes
- Sterile 0.45 µm membrane filter
- 150 mL Superloop (Cytiva)
- Dialysis membrane
- Ultrafiltration tube (Amicon Ultra-15)



- Image Lab 6.1 Software (Bio-Rad)

BEFORE START INSTRUCTIONS

Ensure to have all the necessary materials and reagents already cleaned, sterilized and filter (in case of the purification solutions).

Preparation of Bst expression cells

20h 45m

- 1 Transformation of chemically competent BL21 (DE3) cells with pKJE7 plasmid.
- 1.2 Transfer the cells to a heat block at 42 °C and incubate for 500:00:53

53s

5m

1.4 at room temperature to the transformed cells and incubate at Add A 250 µL of SOC medium **45** 225 rpm, 37°C, 01:00:00

Note

SOC medium composition

A	В
Tryptone	2%
Yeast extract	0.5%
NaCl	10 mM
KCI	2.5 mM
MgCl2	10 mM
MgSO4	10 mM
Glucose	20 mM

Adjust to pH 7 and sterilize by filtration.

1.5 Plate A 25 µL of transformed cell culture onto LB agar with the corresponding selective agent. Incubate the plates Overnight at 8 37 °C

18h

Note

The pKJE7 plasmid requires [M] 30 μ g/mL chloramphenicol as selective agent.

1.6 Select a single colony of transformed cells and inoculate in

riangle 3 mL Luria-Bertani (LB) medium supplemented with the selective antibiotic. Incubate

18h



1.7 Centrifugate the cell culture at 10000 x g, 4°C, 00:05:00. Remove the supernatant and resuspend the cell pellet in Δ 500 μL LB meduim

5m

1.8 Add 🗸 500 µL of 30% v/v glycerol, mix by pipetting up and down and store at 👃 -80 °C 5m

- 2 Preparation of chemically competent BL21 (DE3) cells harboring pKJE7 plasmid.
- 2.1 Take BL21(DE3) cells harboring pKJE7 plasmid from a frozen glycerol stock using a bacterial inoculating loop and inoculate A 3 mL LB liquid medium with

18h

[M] 30 µg/mL of chloramphenicol . Incubate 🖒 Overnight at 🖒 180 rpm, 37°C

2.2 Inoculate 🚨 1 mL overnight culture in 🚨 100 mL LB medium with IM] 30 μg/mL chloramphenicol and incubate at (5 180 rpm, 37°C, 03:00:00

Note

Monitor the cell growth by measuring the optical density (OD) at 600 nm and remove the cells from incubation when the OD reaches 0.3 to 0.4.

2.3 3 4000 x g, 4°C, 00:10:00

20m

2.4

Gently resuspend the cell pellet in A 8 mL of TFBI solution pre-chilled and incubate 50m

TFBI Solution Composition

A	В
Potassium acetate	30 mM
Rubidium chloride	100 mM
Calcium chloride	10 mM
Manganese chloride	50 mM
Glycerol	15% v/v

Adjust to pH 5.8 with 1M acetic acid and sterilize by filtration.

2.5

Gently resuspend the cell pellet in 🚨 2.5 mL of TFBII solution pre-chilled and incubate





Note

TFBII Solution Composition

A	В
MOPS	10 mM
Rubidium chloride	10 mM
Calcium chloride	75 mM
Glycerol	15% v/v

Adjust to pH $6.5\,\mathrm{with}\,1\mathrm{M}$ sodium hydroxide and sterilize by filtration.

2.6 30m

- 3 Transformation of chemically competent BL21 (DE3)/pKJE7 cells with the pColdI-Bst plasmid, the expression vector for the large fragment of the Bacillus stearothermophilus DNA polymerase (Bst).
- 3.1 Add 🗸 1 µL of plasmidic DNA of [M] 100 ng/µL pColdI-Bst expression vector to Δ 50 μL of competent cells BL21 (DE3)/pKJE7. For the transformation procedure **5** go to step #1

The pColdl-Bst plasmid require [M] 100 µg/mL carbenicillin as selective agent and the pKJE7 plasmid require [м] 30 µg/mL chloramphenicol . Use LB medium supplemented with both antibiotics as selective media.

Small-scale screening cultures

- 4 Preparation of bacterial cultures for Bst expression.
- 4.1 Inoculate 4 5 µL glycerol stock of BL21(DE3)/pKJE7/pColdI-Bst or BL21(DE3)/pKJE7 18h Overnight at
- 4.2 Inoculate Δ 500 µL overnight culture in Δ 50 mL culture medium with antibiotic(s). Use LB or TB according to the medium used for the overnight culture. Incubate \$\mathcal{C}\$ 200 rpm, 37°C for approximately (5) 03:00:00

Monitor the cell growth by measuring the optical density **(OD) at 600 nm** and remove the cells from incubation when the OD is between **0.6**.

Once the culture reaches an OD600 of 0.6, incubate the cell cultures on ice for 00:20:00 before adding the inducer (IPTG).

20m

- 5 Small-scale Bst expression under different induction conditions.
- 5.1 Induce the expression of the Bst under different conditions. Each treatment should be evaluated in triplicate. For example:

[IPTG] Strain Medium Temperature BL21(DE3)/pKJE7* 0.5 mM 16°C LB $0.5\,\mathrm{mM}$ 37°C BL21(DE3)/pKJE7* LB BL21(DE3)/pKJE7* $0.5\,\text{mM}$ 16°C TB BL21(DE3)/pKJE7/pColdI-Bst 0 mM 16°C LB 16°C LB BL21(DE3)/pKJE7/pColdI-Bst 0.1 mM $0.5 \, \text{mM}$ 16°C LB BL21(DE3)/pKJE7/pColdI-Bst 16°C BL21(DE3)/pKJE7/pColdI-Bst 1.0 mM LB 0 mM 37°C BL21(DE3)/pKJE7/pColdI-Bst LB 37°C BL21(DE3)/pKJE7/pColdI-Bst $0.5 \, \text{mM}$ LB 16°C BL21(DE3)/pKJE7/pColdI-Bst $0.5\,\text{mM}$ TB

^{*} BL21(DE3)pKJE7 strain is used as negative expression control.

- 5.2 according to each treatment at (5 180 rpm Incubate at 16 °C or 37 °C 16:00:00
- 5.3 Discard the supernatant, remove 10m Centrifugate the cell cultures at 6000 x g, 4°C, 00:10:00 all the liquid and leave the cell pellet as dry as posible.
- 5.4 Weigh the centrifugation tube with the cell pellet (total weight).

10m

16h

Note

Weigh the empty tube prior centrifugation and subtract it to the total weight to calculate the weight of the cell pellet and hence the biomass produced.

5.5 Resuspend the cell pellet in 🚨 5 mL lysis buffer A (LB-A) (pre-chilled).

Note

Lysis buffer A (LB-A) composition

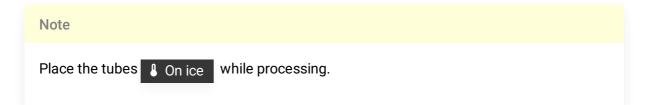
A	В
Tris-HCl pH 7.5	50 mM
EDTA	0.5 mM
2- mercaptoethanol	10 mM
Tergitol NP-40	0.1% v/v
Tween-20	0.1% v/v
PMSF	3 mM

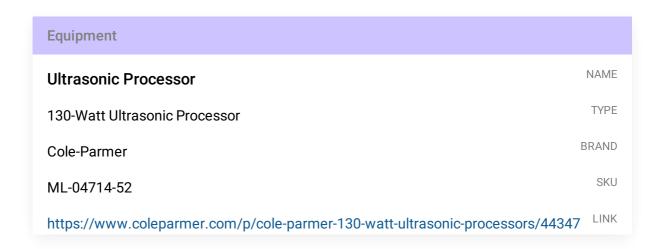
Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

Disrupt cells by ultrasonication at an amplitude of 40%. Apply five cycles of 00:00:15 on 3m 45s 5.6



and 👏 00:00:30 off.





- Centrifugate at \bigcirc 6000 x g, 4°C, 00:15:00 . Recover the supernatant (soluble protein fraction) and discard the pellet.

35m

15m

- For protein clarification, incubate the supernatant at \$60 °C for 00:20:00 in a water bath and centrifugate at 14500 x g, 4°C, 00:15:00 . Recover the clarified supernatant and place it 00 nice .
- 6 Analysis of Bst expression.
- **6.1** Measure total protein concentration by measuring absorbance at 280 nm in a NanoDrop spectrophotometer.

3m

NanoDrop™ One UV-Vis Spectrophotometer Spectrophotometer TYPE Thermo Scientific ND-ONE-W https://www.thermofisher.com/order/catalog/product/ND-ONE-W

intps://www.thermonsher.com/order/catalog/product/ND-ONE-W

SPECIFICATIONS

Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 nm

(FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10, 64 bit;

Voltage: 12 V (DC); Wavelength Range: 190-850 nm

Analyze all clarified supernatant samples by Tricine-SDS-PAGE electrophoresis through a 8% polyacrylamide gel. Load

4 100 µg protein sample per well.

CITATION

Hermann Schägger (2006). Tricine-SDS-PAGE. Nature Protocols.

LINK

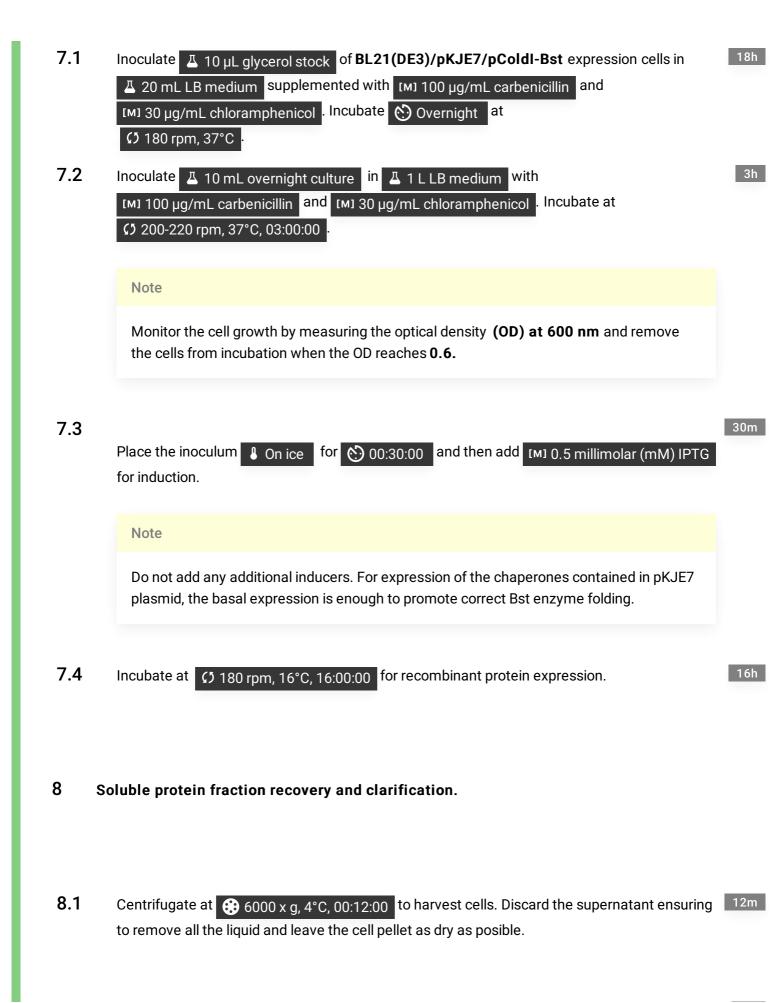
10.1038/nprot.2006.4

6.3 Select the best conditions for protein expression according to the results analysis (biomass, total protein production and electrophoretic profile).

Large-scale production of Bst

2h 6m 20s

7 Expression of recombinant Bst.



Weigh the empty tube prior centrifugation and subtract it to the total weight to calculate the weight of the cell pellet and hence the biomass produced.

- 8.3 4 -80 °C until use (just in case that the purification step is not Store the cell pellet at performed immediately after expression).
- 8.4 Resuspend the cell pellet in 🚨 50 mL lysis buffer A (LB-A) (pre-cooled). If neccesary, defroze the cell pellet in an ice bath before adding the lysis buffer.

5m

Note

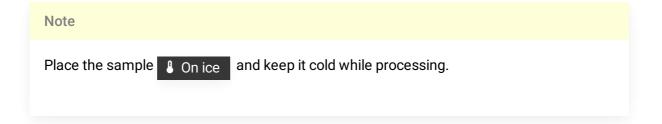
Lysis buffer A composition (LB-A)

A	В
Tris-HCl pH 7.5	50 mM
EDTA	0.5 mM
2- mercaptoethanol	10 mM
Tergitol NP-40	0.1% v/v
Tween-20	0.1% v/v
PMSF	3 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

8.5 Disrupt cells by ultrasonication with an ultrasonic processor at an amplitude of 40% applying 4m 20s pulses of 00:00:10 of ultrasonication and 00:00:00:10 of pause during 00:04:00

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Equipment	
750-Watt Ultrasonic Processor	NAME
CPX750	TYPE
Cole-Parmer	BRAND
ML-04711-60	SKU
https://www.coleparmer.com/p/cole-parmer-500-and-750-watt-ultrasonic-processors/16401	LINK

- 8.6 Centrifugate at \bigcirc 11000 x g, 4°C, 00:30:00 . Recover the supernatant (soluble protein fraction) and discard the pellet.

20m

30m

- 8.7 Incubate the supernatant in a water bath at 60 °C for 00:20:00 for protein clarification.
- 30m
- 8.8 Centrifugate at 11000 x g, 4°C, 00:30:00 . Recover the clarified supernatant and discard the pellet.

Note

Place the supernatant 8 On ice or store at 4°C until use.

Purification of recombinant Bst by FPLC

9 Sample preparation.

Note

Keep all protein samples 8 On ice during the purification process to avoid degradation.

- **9.1** Filter the clarified supernatant through a 0.45 μm membrane.
- **9.2** Dilute the clarified supernatant with saline buffer at a radio of 1:1.

Note

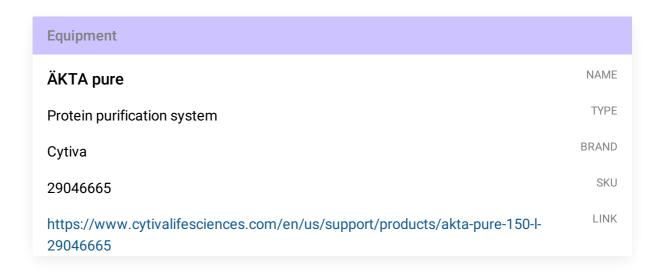
Saline buffer composition (SB).

А	В
Tris-HCl pH 7.5	50 mM
NaCl	100 mM
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

- 9.3 Load the diluted fraction onto a 150 mL Superloop (Cytiva). Store at 4 °C until use.
- 10 Immobilized metal affinity chromatography (Ni²⁺-IMAC).

10.1 Connect a MisTrap HP 5mL Cytiva Catalog #17524801 to a FPLC system.



10.2 Equilibrate the column with 8 column volumes (CV) of washing buffer-AI (WB-AI) at a flow of 2.5 mL/min.

Note

Washing buffer-AI composition (WB-AI).

A	В
Tris-HCl pH 7.5	50 mM
NaCl	100 mM
Imidazole	10 mM
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

10.3 Connect the Superloop charged with the protein fraction and load the sample onto the column at a flow of 2.5 mL/min.

- 10.4 Wash the column with 10 CV of WB-AI at a flow of 2.5 mL/min.
- 10.5 Wash the column with 10 CV of 2% elution buffer-AI (EB-AI) at a flow of 2.5 mL/min.

Elution buffer-AI composition (EB-AI).

A	В
Tris-HCl pH 7.5	50 mM
NaCl	100 mM
Imidazole	500 mM
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

- 10.6 Elute the proteins by passing 5 CV of 100% EB-AI through the column using a flow of 2.5 mL/min.
- 10.7 Analyze all recolected fractions by a 8% Tricine-SDS-PAGE gel electrophoresis. Load Δ 10 μ L protein sample per well.
- 10.8 Pool all elution fractions carrying the recombinant Bst protein. Store at 4 °C until use
- 11 Desalting step.

- 11.1 Connect a System. We HiPrep 26/10 Desalting Column Cytiva Catalog #17508701 to the FPLC system.
- 11.2 Wash the column with 2.5 CV of Mili-Q water. Then, equilibrate the column with 2 CV of desalting buffer-A (DB-A). For both steps use a flow of 10 mL/min.

Desalting buffer-A composition (DB-A).

А	В
Tris-HCl pH 7.5	50 mM
KCI	20 mM
EDTA	1 mM
DTT	1 mM
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

- 11.3 Load the sample onto the column at a flow of 5 mL/min.
- 11.4 Wash the column with 2 CV of DB-A for protein elution at a flow of 10 mL/min.

11.5

Analyze all collected fractions by qualitative Bradford assay using the

- **⊠** Quick Start[™] Bradford 1x Dye Reagent **BioRad Sciences Catalog #5000205** . Pool the fractions with higher protein concentration.
- 11.6 Load the pool of desalted fractions onto a 150 mL Superloop (Cytiva). Store at use.
- 12 Heparin affinity chromatography.
- 12.1 Connect a System. We HiTrap Heparin HP affinity column Cytiva Catalog #17040701 to the FPLC system.
- 12.2 Equilibrate the column with 10 CV of washing buffer-AII (WB-AII) at a flow of 2 mL/min.

Note

Washing buffer-AII composition (WB-AII).

A	В
Tris-HCl pH 7.5	50 mM
EDTA	1 mM
DTT	1 mM
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

12.3 Connect the Superloop charged with the protein fraction and load the sample onto the column at a flow of 2 mL/min.

- 12.4 Wash the column with 5 CV of WB-AII at a flow of 2 mL/min.
- 12.5 Elute proteins by washing the column with a linear gradient of 10 CV of elution buffer-All (EB-All). Use a flow of 2 mL/min.

Elution buffer-AII composition (EB-AII).

A	В
Tris-HCl pH 7.5	50 mM
KCI	1 M
EDTA	1 mM
DTT	1 mM
PMSF	1 mM

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4° C.

- 12.6 Analyze all collected fractions by Tricine-SDS-PAGE electrophoresis through a 8% polyacrylamide gel. Load Δ 10 μL protein sample per well.
- 12.7 Pool all elution fractions carrying the recombinant Bst protein. Store at 4 °C until use.

- 13 Purified Bst enzyme concentration and formulation.
- 13.1 Load the purified Bst enzyme pool onto a dialysis membrane (pre-hydrated). Place the membrane into a beaker with precooled storage buffer-A (SB-A) at a ratio 1:50 (v/v).

Storage buffer-A composition (SB-A).

A	В
Tris-HCl pH 7.5	10 mM
KCI	50 mM
EDTA	0.1 mM
DTT	2 mM
Glycerol	50% v/v

Prepare the buffer with Milli-Q water and adjust to pH 7.5. Store at 4°C.

- Dialyze Overnight at \$ 4 °C with slow agitation.
- Recover the dialized protein, load it onto an Amicon Ultra-15ML 30 kDa cutoff centrifugal filter. Concentrate until a concentration equal or higher than MI 1 mg/mL

Equipment

NAME **Amicon Ultra-15**

PLTK Ultracel-PL membrane, 15 ML - 30 kDa cutoff

BRAND

TYPE

Millipore

SKU

UFC903024

 $https://www.merckmillipore.com/MX/es/product/Amicon-Ultra-15-Centrifugal-Filter-\ ^{LINK}$ Unit,MM_NF-UFC903024?ReferrerURL=https%3A%2F%2Fwww.bing.com%2F&bd=1

Note

Monitor protein concentration measuring absorbance at 280 nm using a NanoDrop spectrophotometer.

Equipment

NanoDrop™ One UV-Vis Spectrophotometer

NAME

spectrophotometer

TYPE

Thermo Scientific

BRAND

ND-ONE-W

SKU LINK

https://www.thermofisher.com/order/catalog/product/ND-ONE-W

SPECIFICATIONS

Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 nm (FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10,

64 bit; Voltage: 12 V (DC); Wavelength Range: 190-850 nm

- Prepare aliquots of \bot 50 μ L of concentrated Bst enzyme
- Add [M] 0.1 % (v/v) triton X-100 to the enzyme aliquots and store at \$ -20 °C
- **13.6** Determine final protein concentration by measuring absorbance at 280 nm in a NanoDrop spectrophotometer.
- Analyze the final Bst enzyme formulation by Tricine-SDS-PAGE electrophoresis through a 8 gel. Load ☐ 3 µL protein sample per well. Load ☐ 3 µL protein ladder

 Precision Plus Protein™ Unstained Protein Standards Bio-rad Laboratories Catalog

#1610363

Analyze the electrophoresis gel by densitometry using the **Image Lab 6.1 Software (Bio-Rad)**. Determine protein concentration for each Bst enzyme aliquot analyzed using the protein ladder as weight standard.

Note

The protein ladder

Precision Plus Protein™ Unstained Protein Standards **Bio-rad Laboratories Catalog** #1610363

includes three reference bands: the 50 KDa with 750 ng, the 20 KDa and 100 KDa bands with 150 ng each per 10 μ L of the protein ladder mix.