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We use this protocol and it's working

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Expression and purification of recombinant gp32 protein

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ABSTRACT

The gp32 is a single strand binding protein (SSB) that plays a role in genetic recombination, replication and repair from the bacteriophage T4. The gp32 is used as part of an isothermal DNA amplification based on the recombination process, the RPA reaction. RPA uses 4 enzymes: UvsX, UvsY, Bsu and Gp32. It's an isothermal amplification technique that can run at 37°C. In this protocol we are producing a recombinant gp32 that has a 6xHIS-tag using a E. coli expression system. The protocols for the production of the other proteins are also available in protocols.io.

MATERIALS

Binding buffer, pH 7.5

[M] 50 millimolar (mM) Tris-HCl, pH 7.5
[M] 20 millimolar (mM) Imidazole, pH 7.5
[M] 1 Molarity (M) KCl
[M] 5 % (v/v) Glycerol
[M] 0.01 % (v/v) 2-Mercaptoethanol (BME)

Buffer with lower [KCl], pH 7.5

[M] 50 millimolar (mM) Tris-HCl, pH 7.5
[M] 20 millimolar (mM) Imidazole, pH 7.5
[M] 100 millimolar (mM) KCl
[M] 5 % (v/v) Glycerol
[M] 0.01 % (v/v) 2-Mercaptoethanol (BME)

Elution buffer (for resin purification), pH 7.5

[M] 50 millimolar (mM) Tris-HCl, pH 7.5
[M] 200 millimolar (mM) Imidazole, pH 7.5
[M] 100 millimolar (mM) KCl

[M] 5 % (v/v) Glycerol
[M] 0.01 % (v/v) BME

Elution buffer (for FPLC purification), pH 7.5
[M] 50 millimolar (mM) Tris-HCl, pH 7.5
[M] 500 millimolar (mM) Imidazole, pH 7.5
[M] 100 millimolar (mM) KCl
[M] 5 % (v/v) Glycerol
[M] 0.01 % (v/v) BME

Storage buffer, pH 7.5
[M] 10 millimolar (mM) Tris-HCl, pH 7.5
[M] 300 millimolar (mM) NaCl
[M] 1 millimolar (mM) DTT
[M] 0.1 millimolar (mM) EDTA

Ladder:
Pageruler Prestained Protein Ladder **Thermo Fisher Scientific Catalog #26616**

Equipment:
Sonicator OMNI Sonic Ruptor 400
Protein purification system FPLC AKTA START

DAY1: Transformation of competent cells

1d

1 Quantify the plasmid containing the LbCas12a gene and determine the volume that contains 100 ng of the plasmid

2 Defrost the aliquot of BL21(DE3) chemically competent cells On ice. Softly pipette 100 ng of the plasmid in the aliquot and let the tube rest On ice for 00:30:00. 30m

3 Incubate the tube at 42 °C for 00:00:30. 30s

4 Quickly return the tube On ice and incubate for 00:05:00. 5m

5 Add the mixture to a microcentrifuge tube with 800 µL SOC medium or LB broth and incubate at 37 °C for 00:45:00 45m

6 Centrifuge the tube 4500 rpm, Room temperature, 00:08:00. 8m

7 Discard 800 µL of the supernatant and gently resuspend the pellet with the remaining supernatant.

8 Add the resuspension to an LB agar plate previously supplemented with [M] 0.05 mg/mL Kanamycin and spread the recently transformed cells. Incubate plate Overnight at 37 °C.

DAY2: Preparation of pre-inoculum 1d

9 For verification that the colonies in the plate contain the desired plasmid with the protein sequence, perform a PCR colony using universal T7 primers and the PCR protocol for Phusion®High-Fidelity DNA Polymerase (NEB, M0530). Use the following thermocycling procedures for the gp32 plasmid:

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	98	3 min	1
Denaturation	98	30 sec	25
Annealing	60	30 sec	
Extension	72	32 sec	
Final Extension	72	5 min	1
Hold	4	∞	

Run the PCR product in a 1% agarose gel and verify if there is a band in the desired weight (gp32 insert = 1088 bp)

10 Select an isolated bacterial colony from the plate and inoculate a test tube with 10 mL LB medium and [M] 0.05 mg/mL Kanamycin. Incubate the tube Overnight at 220 rpm, 37°C

DAY 3-A:Protein expression in small scale

1d

11 Inoculate 50 µL from the pre-inoculum to an Erlenmeyer flasks with 50 mL LB medium and [M] 0.05 mg/mL Kanamycin (ratio 100:1). Incubate at 220 rpm, 37°C until OD_{600} = 0.5 - 0.6 (3-4 hours).

12 Add IPTG to a final concentration of [M] 0.5 millimolar (mM) to each flask and incubate at 220 rpm, 18°C, 16:00:00.

13 Centrifuge the cell culture 8000 rpm, 4°C, 00:05:00. Discard the supernatant. At this point, you may store the cells pellet at -20°C until you are ready to run the purification.

5m

DAY 4-A:Protein purification in resin

1d

14 Resuspend the cell pellet in 5 mL Binding buffer. Then add lysozyme to a final concentration of [M] 0.1 µg/µL.

15 Incubate the cells at 220 rpm, Room temperature , 00:20:00 and add 10% SDS to a final concentration of 0.02%.

16 Add ~ 100 µL of glass beads and shake vigorously in a vortex for 00:20:00 at room temperature. You can do this by fixing a 15 mL tube to the vortex rubber platform with tape.

20m

17 Centrifugate at 13500 rpm, 4°C, 00:07:00. Collect the supernatant and label it as a Soluble fraction. The pellet is the Insoluble fraction. Collect small fractions of each one to run an acrylamide gel afterwards.

18 **Prepare the resin**. Homogenize resin with its storage buffer by shaking the bottle and transfer it to a new tube. You will use 330 µL of resin for each 1 mL of soluble fraction. Let the slurry sediment or spin it down. Remove the storage buffer and wash the resin in **Binding buffer.** Wash the resin with the same volume as the obtained soluble fraction. Repeat this wash step 3 times.

19 Add the soluble fraction to the resin. Homogenize the mixture gently in an orbital shaker for 20 min (~60 RPM) at room temperature.

20 Let the resin sediment for 10 minutes. Collect a small fraction of the supernatant to run an acrylamide gel afterwards, discard the remainder. Resuspend resin with 1 mL of **Binding buffer.** Homogenize the tube gently with finger taps. Don't flip the tube (1st washing step).

21 Spin down for a few seconds and discard supernatant. Resuspend resin with 1 mL of **Binding buffer.** Homogenize the tube gently with finger taps. Don't flip the tube (2nd washing step).

22 Spin down for a few seconds and discard supernatant. Resuspend resin with 1 mL of **Elution buffer (mM Imidazole).** Homogenize the tube gently with finger taps. Don't flip the tube. Incubate for 10m.

23 Spin down for a few seconds and collect supernatant. Resuspend resin with 1 mL of **Elution buffer (500mM Imidazole).** Homogenize the tube gently with finger taps. Incubate for 00:10:00. Collect small fractions of elutions to run an acrylamide gel afterwards. 10m

Run a 12% acrylamide gel at 200 V to evaluate all the samples you just generated:Lysis sample, Soluble fraction, Insoluble fraction, Flowthrough, 1st washing step, 2nd washing step and Eluted fraction.

DAY 3-B: Protein expression in medium scale 1d

24 Inoculate 2.5 mL from the pre-inoculum to an Erlenmeyer flask with 250 mL LB medium and [M] 0.05 mg/mL Kanamycin, use 4 flasks to obtain 1L of cell culture. Incubate at 220 rpm, 37°C until OD_{600} = 0.5 - 0.6 (3-4 hours).

25 Add IPTG to a final concentration of [M] 0.5 millimolar (mM) to each flask and incubate at 220 rpm, 18°C , 16-19 hours.

26 Centrifuge the cell culture 4000 rpm, 4°C, 00:20:00. Discard the supernatant. At this point, you may store the 1-2 grams of cell pellet at -20°C until you are ready to run the purification. 20m

DAY 4-B: Cells Lysis

4h

27 Resuspend all the cell pellets (from a total of 1 L of culture) in 100 mL Binding buffer. Add PMSF to a final concentration of [M] 0.1 millimolar (mM). Add lysozyme to a final concentration of [M] 0.1 µg/µL.

28 Incubate the cells on an orbital shaker at 220 rpm, Room temperature , 00:20:00.

29 Sonicate on ice until the lysate turns translucid. Use 5 cycles of 00:15:00 power 60% ON, pulse 10. Then 00:15:00 power OFF, with the lysate on ice. 30m

30 Centrifuge 6000 rpm, 4°C, 00:20:00 to separate the insoluble fraction (pellet) from the soluble fraction. Transfer the soluble fraction to a new and clean tube on ice. Collect small fractions of each one to run an acrylamide gel afterwards. 20m

DAY 4-B: Protein Purification with FPLC

31 Prepare the 5 mL HisTrap column in the FPLC system. Wash the tubes, pumps system and the column with 7 column volumes (c.v.) of distilled and filtrated water. Then equilibrate the column

with 7 c.v. of **Binding buffer**.

32 Load the soluble fraction to the FPLC system at a flow of 1 mL/min. Collect a small fraction of each step and signal change to run an acrylamide gel afterwards. Wash the column with 5 c.v. of **Binding buffer**, until the UV and conductivity signal stabilizes.
Then load the **Buffer 100mM KCl** at 2 mL/min to reduce the salt concentration until the UV and conductivity signal stabilizes (5-7 c.v).

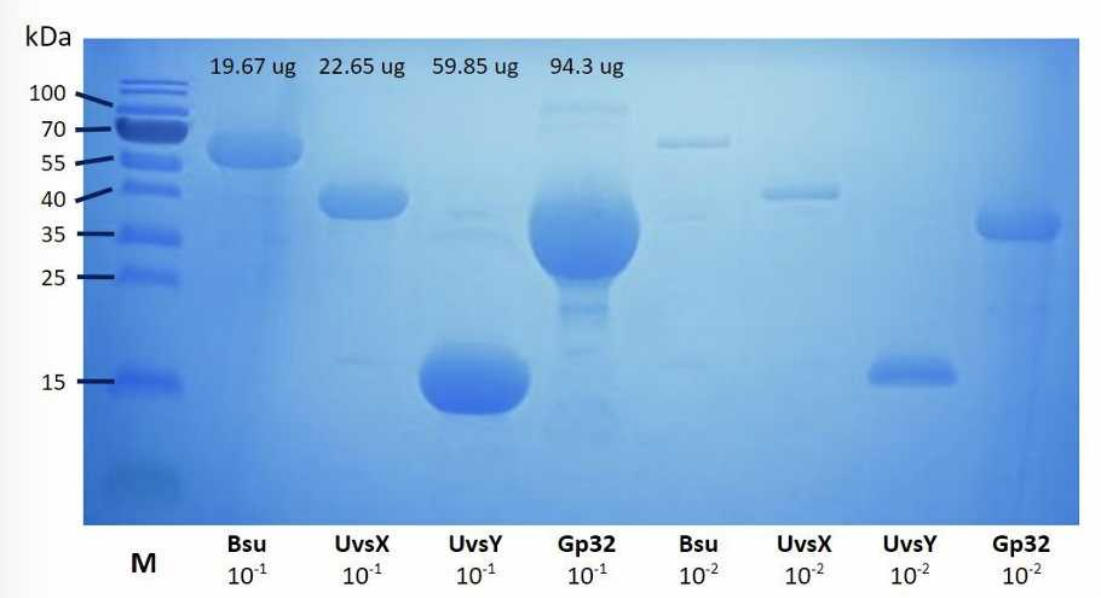
33 **Washing:** Load the column with 7% of pump B (**Elution Buffer**), which is equivalent to ~50 mM Imidazole, until the signal stabilizes.
Elution: Load the column with 38% of pump B (**Elution Buffer**), which is equivalent to ~200 mM Imidazole, until the signal stabilizes.

Start collecting the elution in 8 mL tube fractions immediately after the UV signal increases. After approximately 40 mL, the UV signal will stabilize at a low value. Then load the column with 3 c.v. of 100% of pump B (**Elution Buffer**), which is equivalent to 500 mM Imidazole, until the signal stabilizes again.

34 **Wash the column for storage.** Load the column with **Buffer 100 mM KCl** at 2 mL/min 5 c.v. Then, load the column with 7 c.v of distilled and filtrated water. To storage the column, load it with 5 c.v. of **ethanol 20%** and storage it at 4°C. Clean the FPLC system with distilled and filtrated water. Finally, remove the rest of the water from the system with **ethanol 20%** and keep the system with it until next use.

35 Determine the fractions with the Bsu polymserase by running a SDS-PAGE in a 8% acrylamide gel. The gp32 protein weights 34.3 kDa.

36 Pool the gp32 fractions and concentrate the eluted fractions with the protein with Amicon Ultra-15 Centrifugal Filter Unit **Emd Millipore Catalog #UFC910024** **10kDa**.
Reconstitute the concentrate so it is stored with the components detailed in **Storage Buffer** to decrease the Imidazol to 20 mM. Add glycerol to a 20%, homogenize, make aliquots of 400 µL of the protein and storage them at -80°C.


kDa
19.67 ug
22.65 ug
59.85 ug
94.3 ug
100
70
55
40
35
25
15
M
Bsu 10-1
UvsX 10-1
UvsY 10-1
Gp32 10-1
Bsu 10-2
UvsX 10-2
UvsY 10-2
Gp32 10-2