Look What They've Done To My Shoes!

SCU_China Project 2016

Protocol

CecropinXJ Expression Team

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IPTG Induced CecropinXJ Expression

Procedure

1. Transform the recombinant plasmid pET32a-cecropinXJ into E. coli BL21(DE3) pLYsS competent cells for expression. Incubate the culture overnight at 37°C.
2. Incubate 2ml cultures (LB medium containing 2 µg/ml ampicillin) with 1 colony resistant to ampicillin on a Luria-Bertani (LB) plate containing the recombinant expression plasmid. Incubate the culture overnight at the appropriate temperature 37°C in a shaking incubator.
3. Inoculate 10 ml of LB medium containing 10 µg/ml ampicillin with 100 µl of each overnight culture. Incubate the cultures at 37°C in a shaking incubator until the optical density at 600 nm (OD600) of the culture had reached 0.6-0.8.
4. Induce the culture by adding IPTG to a final concentration of 0.8 mM and continue incubation at 37°C in a shaking incubator for 5 h.

SDS-PAGE Analysis

Procedure

A. Making SDS-PAGE gel

1. Clean and completely dry glass plates, combs, and spacers are required.
2. Assemble gel cassette by following manufacturer instructions.
3. Prepare 15% lower gel (separating gel) by adding the following solutions (wear gloves when prepare gel solution) (total volume= 5 ml)
   1.1 ml ddH_2O
   2.5 ml 30% acrylamide/Bis
   1.3 ml 1.5 M Tris (pH 8.8)
   0.05 ml 20% SDS
   0.05 ml 10% ammonium persulfate (make it fresh and store at 4°C up to a month)
   0.002 ml TEMED (add it right before pour the gel)
4. To avoid polymerization, after adding TEMED, mix well and quickly transfer the gel solution by using 1 ml pipette to the casting chamber between the glass plates and fill up to about 0.7 cm below the bottom of comb when the comb is in place.
5. Add a small layer of isopropanol to the top of the gel prior to polymerization to straighten the level of the gel.
6. Once the gel has polymerized, start to prepare stacking gel (5%) by adding the following solutions (total volume= 3 ml)
   2.1 ml dH_2O
   0.5 ml 30% acrylamide/Bis
   0.38 ml 1 M Tris (pH 6.8)
   0.03 ml 20% (w/v) SDS
   0.03 ml 10% ammonium persulfate
   0.003 ml TEMED (add it right before the gel is poured)

7. Remove the isopropanol layer by using filter paper. Rinse the top layer of the gel with ddH_2O and dry off as much of the water as possible by using filter paper.
8. Add TEMED and mix the stacking gel solution well. Quickly transfer the gel solution by using a 1 ml pipette till the space is full, and then insert the appropriate comb.
9. Allow the top portion to solidify and then carefully remove the comb.
   Note: The gels can be stored with the combs in place tightly wrapped in plastic wrap and put in a second container with wet tissue towel (keep the gels moist) at 4°C for 1 to 2 weeks.

B. Sample preparation

1. 1 ml culture was centrifuged at 4,000 x g for 3 min. The cell pellet was resuspended in 100 µl ddH_2O.
2. Add 25 of µl 5x protein sample buffer to each protein sample, mix and boil the samples at 95 °C heating block module for 10 min.
3. Spin the samples in tabletop centrifuge and leave the samples at room temperature until you are ready to load onto the gel.
   Note: Can store extracted protein samples (containing sample buffer) at -20°C.

C. Electrophoresis

1. Remove the gel cassette from the casting stand and place it in the electrode assembly with the short plate on the inside. Press down on the electrode assembly while clamping the frame to secure the electrode assembly and put the clamping frame into the electrophoresis tank.
2. Pour some 1x electrophoresis running buffer into the opening of the casting frame between the gel cassettes. Add enough buffer to fill the wells of the gel. Fill the region outside of the frame with 1x running buffer.
3. Slowly load the same amount of protein samples into each well as well as load 10 µl of protein marker.
4. Connect the electrophoresis tank to the power supply.

D. Protein detection

Use Coomassie blue staining to determine the expression level of recombinant CecropinXJ through protein bands observation.

Western Blot Analysis

Procedure

A. Making SDS-PAGE gel

1. Clean and completely dry glass plates, combs, and spacers are required.
2. Assemble gel cassette by following manufacturer instructions.
3. Prepare 15% lower gel (separating gel) by adding the following solutions (wear gloves when prepare gel solution) (total volume= 5 ml)
   1.1 ml ddH_2O
   2.5 ml 30% acrylamide/Bis
   1.3 ml 1.5 M Tris (pH 8.8)

0.05 ml 20% SDS
0.05 ml 10% ammonium persulfate (make it fresh and store at 4°C up to a month)
0.002 ml TEMED (add it right before pour the gel)

4. To avoid polymerization, after adding TEMED, mix well and quickly transfer the gel solution by using 1 ml pipette to the casting chamber between the glass plates and fill up to about 0.7 cm below the bottom of comb when the comb is in place.
5. Add a small layer of isopropanol to the top of the gel prior to polymerization to straighten the level of the gel.
6. Once the gel has polymerized, start to prepare stacking gel (5%) by adding the following solutions (total volume= 3 ml)
   2.1 ml dH_2O
   0.5 ml 30% acrylamide/Bis
   0.38 ml 1 M Tris (pH 6.8)
   0.03 ml 20% (w/v) SDS
   0.03 ml 10% ammonium persulfate
   0.003 ml TEMED (add it right before the gel is poured)
7. Remove the isopropanol layer by using filter paper. Rinse the top layer of the gel with ddH_2O and dry off as much of the water as possible by using filter paper.
8. Add TEMED and mix the stacking gel solution well. Quickly transfer the gel solution by using a 1 ml pipette till the space is full, and then insert the appropriate comb.
9. Allow the top portion to solidify and then carefully remove the comb.
   Note: The gels can be stored with the combs in place tightly wrapped in plastic wrap and put in a second container with wet tissue towel (keep the gels moist) at 4°C for 1 to 2 weeks.

B. Sample preparation

4. 1 ml culture was centrifuged at 4,000 x g for 3 min. The cell pellet was resuspended in 100 µl ddH_2O.
5. Add 25 of µl 5x protein sample buffer to each protein sample, mix and boil the samples at 95 °C heating block module for 10 min.
6. Spin the samples in tabletop centrifuge and leave the samples at room temperature until you are ready to load onto the gel.
   Note: Can store extracted protein samples (containing sample buffer) at -20°C.

C. Running the gel (use *1x Running buffer* in the electrophoresis chamber)

1. After flash spinning the samples, load into the wells. Amount depends on the wells you have. Around 20µl are recommended when using the 10wells gel.
2. Be sure to use *ladders* (stored at -20°C), load it into the first right lane directly (no heating).
3. Run with 30V for 30 min, then 80V for 20 min, then 100v.
4. Stop gel if dye front is near the bottom of the gel.

D. Membrane transfer

1. Prepare 1x Transfer buffer. Cut membrane (nitrocellulose). Membrane has to fit gel not your imagination! Mark membrane at its upper right corner.
2. Assemble the “sandwich” for Bio-Rad’ Transblot.

3. Prewet the sponges, filter papers (slightly bigger than gel) in 1x Transfer buffer.
4. black – sponge – filter paper – gel – membrane (with the mark facing the gel on its upper left corner) – filter paper – sponge – white
5. Remove any air bubbles which may have formed between the sandwich components, it is very important for good results. Use a glass tube to gently roll air bubbles out.
6. Place the cassette in module black to black and transfer at constant Voltage (100V) for 90 min.
7. When finished, wash membrane in washing buffer 5 times X 5 min. Membranes could be stored in washing buffer at 4°C.

E. Antibodies and detection

1. Block membranes in Blocking buffer for 1 hour.
2. Incubate with primary antibody diluted in blocking buffer for 1 hour at room temp. or overnight at 4°C.
3. Wash membranes 5 times X 5 min with washing buffer.
4. Detect with ECL kit. And image the bands.

Inhibition Zone Assay

Procedure

A. Prepare the recombinant CecropinXJ solution

1. 10ml culture was centrifuged at 8,000 x g for 5 min after induction.
2. The pellet was resuspended in 10 ml PBS and placed in an ice bath for ultrasonic lysis (200 W, 5 sec, 5 sec).
3. The lysate was centrifuged at 10,000 x g for 5 min and supernatant was collected for further work.

B. the growth situation of *Staphylococcus aureus*

1. Incubate *Staphylococcus aureus* in LB at 37℃.
2. Add a dilution of *Staphylococcus aureus* (20μl; OD600=0.5) to 1.5ml,1.0mland 0.5ml ultrasonic lysate containing recombinant CecropinXJ respectively and add fresh LB to 0.5ml,1.0ml and 1.5ml accordingly.
3. Incubate them at 37℃for 0.5h, 1.0h and 1.5h, test the absorption value of culture at 600nm(OD_{600}) respectively using spectrophotometer.

C. the inhibition zone assay toward *Staphylococcus aureus* (the filter paper method)

1. Soak the filter paper washed in the raw ultrasonic lysate for 10min.
2. Put the filter paper on the medium after plate coating of *Staphylococcus aureus.*
3. Incubate the culture at 37℃overnight and observe the inhibition zone .