Western Blot and Exosomal miRNAs extraction

Protocols

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Western Blot

Introduction

This is the protocol for performing a western blot on exosomes extract to confirm the purity of your sample.

Material

- exosome extract
- Laemmli buffer
- PBS-Tween-glycerin
- SDS PAGE gel
- CD9 marker
- CD63 marker
- Calnexin
- Ponceau red
- PBS-T
- BSA
- milk
- primary antibody
- secondary antibody
- SDS
- ß mercapto

Procedure

1- Take between 10µL and 30 µL from your exosome extract

2- Add H2O to dilute until 150µL (the final volume)

3- Add 37 µL of Laemmli buffer (with Beta-mercaptoethanol) to each sample.

4- Incubate for 10 minutes at 95 °C (to denaturate and break up protein complexes and cause the individual protein to unfold and coat them with negative charge so they will go from top to down (negative to positive) in the gel).

5- Place the gel on the migration chamber and don't forget to balance it.

6- Put the migration PBS-Tween-glycerin buffer between the gel and the balance glass. and then Fill the chamber.

7- Load 48µL of your samples and 7.5µL of marker per well (CD9, CD63 and Calnexin) and run it at 150V for 45 min.

8- Once the migration is done, gently break the glass protecting the gel.

9- Prepare a transfer chamber by wetting nitrocellulose membrane, papier buvard.

10- Prepare a sandwich by:

- putting a papier buvard first on top of it the nitrocellulose membrane
- remove any liquid and bubble with a rolling pin
- put the membrane and on top of that the second papier buvard
- with the rolling pin make everything flat.

11- Launch the trans-blot device with a parameter mixed gel. When it's finished, slowly remove the gel and check if the weight marker has been transferred to the nitro membrane.

12- Add PONCEAU red on it to check if there is protein.

13- Wash it with PBS-T (by adding the PBS-T in plastic cubes + membrane), then put milk for 45 minutes (to block the action) .

14- Add the primary antibody.

15- Wash the nitro membrane with PBS-T 4 times (10minutes each time).

16- Add 7ml of milk+BSA and 1.4 µL of second antibodies to the three membranes and incubate them for 45-60 minutes. (the antibodies we use are very efficient so we can even just put them for 30 minutes but in order to have a better signal it's better to let them incubate for a longer time).

17-Wash the nitro membranes again with PBS-T (3 times).

18- Reveal under x ray

Extraction of miRNA

Introduction

This is the protocol to extract miRNAs from your exosomes.

Material

- exosomes
- ThermoFischer Total Exosome RNA and Protein Isolation Kit
- Acid-Phenol:Chloroform

Procedure

1- Take 165µL in PBS.

2- Add 165µL 2X Denaturing Solution and mix thoroughly.

3- Incubate on ice for 5 minutes.

4- Add 330µL of Acid-Phenol:Chloroform to each sample.

Important: Be sure to withdraw the bottom phase containing Acid-Phenol:Chloroform, not the aqueous buffer that lies on top of the mixture.

5- Mix by vortexing for 30–60 seconds.

6- Centrifuge for 5 minutes at maximum speed (≥10,000 x g) at room temperature to separate the mixture into aqueous and organic phases. Repeat the centrifugation if the interphase is not compact.

7- Carefully remove the aqueous (upper) phase without disturbing the lower phase or the interphase, and transfer it to a fresh tube.

Notes**:**

- Preheat Elution Solution or nuclease-free water to 95°C for use in eluting the RNA from the filter at the end of the procedure.
- Nuclease-free water can be used in place of the elution buffer, especially if concentrating the RNA with a centrifugal vacuum concentrator.
- 100% ethanol must be at room temperature. If the 100% ethanol is stored cold, warm it to room temperature before starting the RNA isolation.

8- Add 1.25 volume of 100% ethanol to the aqueous phase, and mix thoroughly.)

9- For each sample, place a Filter Cartridge into one of the Collection Tubes (supplied in kit).
10- Pipet 310 µL the lysate/ethanol mixture onto the Filter Cartridge.
11- Centrifuge at 10,000 × g for ~15 seconds.
12- Discard the flow-through and repeat until all of the lysate/ethanol mixture has been passed through the filter.
13- Add 310µL miRNA Wash Solution 1 (supplied in the kit) to the Filter Cartridge.
14- Centrifuge at 10,000 × g for ~15 seconds. Then, Discard the flow-through from the Collection Tube and replace the Filter Cartridge into the same Collection Tube.
15- Apply 310 µL Wash Solution 2/3 (working solution mixed with ethanol) and Centrifuge at 10,000 × g for ~15 seconds as in the previous step.
16- Repeat with a second 500 µL of Wash Solution 2/3.
17- After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and centrifuge the assembly at 10,000 × g for 1 minute to remove residual fluid from the filter.
18- Transfer the Filter Cartridge into a fresh Collection Tube (supplied in kit).
19- Apply 50 µL of preheated (95°C) Elution Solution or nuclease-free water to the center of the filter.
20- Centrifuge for ~30 seconds to recover the RNA.
21- Repeat the elution once more with an additional aliquot of 50 µL of Elution Solution or nuclease-free water.
22- Collect the eluate (which contains the RNA) and place it on ice for immediate use, or store it at ≤ –20°C.

Reverse transcription/ qrt-PCR for miRNAs

Introduction

This is the protocol to quantify the targeted miRNAs from your exosomes.

Material

- exosomes
- Takara bio Mir-X™ miRNA FirstStrand Synthesis and TB Green® qRT-PCR Kit
- thermocycle

Procedure

1.In 0.2 ml free nucleus tube, add 5ul of mRQ buffer + 1.25 µL of mRQ Enzyme and 3.75 µl of sample (10 µL the total volume).

2.Put it in the thermocycle for 1 hour at 37°c and then 5 minutes at 80° to activate the enzyme.

3.Add 90 µL of free nuclease H2O (to obtain 100 µl final volume)

4.Store at -20 °C