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Western Blots with S. rosetta lysate (version 1)

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol utilizes the preferential lysis of S. rosetta to obtain choanoflagellate-specific proteins from co-culture with feeder bacteria. Protein concentrations are normalized by total protein stain, and the use of LICOR blocking buffer offers increased specificity of antibodies. Additionally, robust detection of specific proteins can be achieved when combined with genome editing of epitope tags into proteins of interest. This protocol was developed with essential input from Alain Garcia de las Bayonas and Flora Rutaganira.

Protocol materials

Pierce™ Coomassie Plus (Bradford) Assay Kit **Thermo Fisher Catalog #**23236	Step 3
Novex wedgewell 10% Tris-Glycine Mini Gel **Thermo Scientific Catalog #**XP00105BOX	Step 12
REVERT Total Protein Stain Kit **LI-COR Catalog #**926-11010	Step 27
Intercept Blocking Buffer **LI-COR Catalog #** 927-70001	Step 35
96 well black assay plate with clear bottom **Corning Catalog #**3603	Step 4

Prepare lysis buffer

1 The following recipe and protocol is based entirely on the **preferential lysis** of *S. rosetta*. Modifications have been made to remove all nucleic acids, and to remove unnecessary components.

	A	B	C	E
	Chemical	[Final]	[Stock]	Chemical Vol
	Water			1.22 ml
	Tris-HCl, pH 8.0	20 mM	1 M	200 µl
	KCl	150 mM	2 M	750 µl
	MgCl2	5 mM	1 M	50 µl
	Sucrose	250 mM	1.75 M (60% w/ v)	1ml 420 ul
	Protease inhibitor t ablet	2 mini tablet / 10 ml		1 ml
	Digitonin	10 mM	20 mM	5 ml
	Pefabloc SC	1 mM	200 mM	50 µl
	DTT	1 mM	1 M	10 µl
	Universal nuclease	3.75 U/µl	250 U/µl	150 µl
	Final Volume			10mL

Notes:
1. Prepare the buffer ahead of time by combining all but the italicized reagents (DTT, Universal Nuclease), splitting into 492 µl aliquots, and storing at -20°C.

2. Just before use, thaw the prepared lysis buffer on ice and then add 0.5 µl of 1 M DTT, 7.5 µl of Universal nuclease to the 492 µl aliquot for a total volume of 500 µl.

Count, Harvest, and Lyse cells

2 Follow the lysis steps of the **preferential lysis** protocol.

Determine protein concentration

3 Using the Bradford assay kit, prepare the BSA standard curves by diluting the standards into fresh lysis buffer following the schemebelow. The detailed protocol can be found **here**. I recommend using the "standard microplate protocol" found in the linked pdf.

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	3,555 µL	45 µL of Stock	25 µg/mL
B	6,435 µL	65 µL of Stock	20 µg/mL
C	3,970 µL	30 µL of Stock	15 µg/mL
D	3,000 µL	3,000 µL of vial B dilution	10 µg/mL
E	2,500 µL	2,500 µL of vial D dilution	5 µg/mL
F	1,700 µL	1,700 µL of vial E dilution	2.5 µg/mL
G	4,000 µL	0	0 µg/mL = Blank

Pierce™ Coomassie Plus (Bradford) Assay Kit **Thermo Fisher Catalog #**23236

4 Pipette 10 µL of each standard or unknown sample into clear bottom 96-well plates 96 well black assay plate with clear bottom **Corning Catalog #**3603 .

5 Add 300 µL of the Assay Reagent to each well, mix by gently taping on all sides for 60 seconds.

6 Incubate plate for 10 minutes at RT.

7 Measure the absorbance at or near 595 nm on a plate reader.

8 Subtract the average blank 595 nm measurement from the standards and sample values.

9 Use the standard curve to determine the protein concentration of each sample.

Run SDS-PAGE

10m

10 Aliquot 20 µg of total protein per sample (variable volume based off concentration) into pcr tubes, and denatured with 4x Laemmli SDS loading buffer (diluted to 1x).

11 Incubate denatured samples for 00:10:00 at Room temperature if your proteins of interest are membrane proteins, or at 80 °C otherwise. Membrane proteins can crash out at higher temperatures.

10m

12 Prepare your gel and running buffer. For smaller membrane proteins (25-100 kD), 10% tris-glycine gels are a good starting point.

Novex wedgewell 10% Tris-Glycine Mini Gel **Thermo Scientific Catalog #**XP00105BOX

Note

The type of gel you use (tris-glycine, bis-tris, percentage) will depend on your protein size and biochemical characteristics. This will require some testing.

13 Load denatured protein samples into the gel. Run according to manufacturers' guidelines.

Prepare the transfer stack

14 In advance, prepare 3.5" x 3.5" squares of filter paper and transfer membrane. (0.45 µM nitrocellulose membranes are a good starting point)

15 While the gel from the previous step runs, prepare the transfer buffer:
3.02 g Tris base (25 mM final); 14.4 g glycine (192mM final); 100 ml methanol (10% final), brought to 1L with ice cold water.

16 Once the gel is done running, prepare the transfer stack. Find a shallow container to place the transfer stack cassette inside. The container should be shallow and wide enough for the cassette to lay open and foryour hands to access it easily.

17 Place foam/fiber pads on both sides of the cassette. Then pour in the transfer buffer until it covers the foam/fiber pads.

18 On one side of the cassette, use clean tweezers and place 2 squares of filter paper. Ensure they're submerged in the transfer buffer. Use the side of a clean P1000 pipette tip to gently rub and remove bubbles from between the filter papers.

Note

From here on, it is essential to remember the order of layers, and the proteins will travel towards anode (positive charge).

Note

For every layer added, bubbles will need to be removed in the same fashion. Any remaining bubbles will result in a poor transfer. Try not to touch the filter papers or membrane directly with gloves, as that may smear the transfer.

19 Take the gel and crack open the plastic housing. Remove the lanes at the top and the thicker section at the bottom (so the gel will lay flat) with the edge of the gel knife, while leaving the gel adhered to one-half of the plastic housing. Place a third filter paper on top of the gel and gently push the filter paper onto the gel until it sticks. Gently pull back the filter paper while ensuring the gel separates with the paper.

Note

You should knick an edge of the gel to ensure you always know the direction its facing.

20 Slowly place the filter paper adhered gel onto the previously submerged filter papers, with the gel facing UP. At this stage there will be plenty of bubbles under the gel. Repeat the bubble removing step slowly until no more bubbles are visible.

Note

bubbles at this step will be small and almost foam-y. Keep an eye out to remove them all from between the gel and paper.

21 Using tweezers, take a square of transfer membrane, and cut off a small corner of the gel. Match the cut corner of the membrane to the cut corner of the gel. While ensuring the gel is submerged or at least recently wetted with buffer, precisely place the membrane over the gel. Lay one edge of the membrane down first and slowly lower the rest of the membrane. Remove bubbles as before.

Note

Be very careful here, as often the membrane will immediately adhere to the gel and you won't be able to reposition the membrane without tearing the gel.

22 Place 3 more filter papers on top, removing bubbles as before.

23 Close the cassette. This is a diagram of how the stack will look once closed.

Transfer

24 Pour any remaining transfer buffer into the transfer tank. Then place the loaded cassette into the tank. Pour the leftover buffer from the container you assembled the cassette in.

25 Put the tank inside a secondary container and surround with ice. Bring the entire set up into a cold room.

26 Run the transfer at 50 V for 30 minutes, then 100 V for 60 minutes

Total protein stain

27 Total protein stains are done with the LICOR 700 total protein stain kit. A detailed protocol can be found **here**.

REVERT Total Protein Stain Kit **LI-COR Catalog #**926-11010

28 Take the membrane from the transfer stack, and place in a clean container. Dry for 00:10:00 at 37 °C 10m

Note

All handling of the membrane should be done with clean tweezers. Do not touch the membrane directly

29 incubate the membrane in TBS or PBS (no detergent) for 00:05:00 at Room temperature with gentle shaking 5m

30 Wash the membrane in water

31 Incubate the membrane in 5 mL Revert 700 Total Protein Stain for 00:05:00 at Room temperature with gentle shaking. Decant afterwards. 5m

32 Wash with 5 mL Revert 700 Wash Solution for 00:00:30 at Room temperature with gentle shaking twice, decanting in between. 30s

33 Wash the membrane in water, and keep the membrane in water while preparing to image.

34 Immediately image the membrane in the 700 nm channel on a membrane imager.

Blocking

35 Take the imaged membrane, and place in a small container for blocking. Block in LICOR Intercept Blocking Buffer, using enough to cover the membrane completely. Incubate for 01:00:00 at 37 °C with gently shaking
Intercept Blocking Buffer **LI-COR Catalog #** 927-70001 1h

36 Decant the blocking buffer, and replace with fresh blocking buffer. Incubate for Overnight at 4 °C with gently shaking 1h

Immunodetection 5m

37 The following day, decant the blocking buffer, and add back a minimal volume of blocking buffer that covers the membrane. This should be done with a serological pipette so you know the final volume.

38 In the blocking buffer, dilute your antibodies. Incubate for 02:00:00 at Room temperature with gentle shaking. 2h

Note

For anti-ALFA tag nanobodies, a 1:10,000 dilution is recommended. The antibody fluorophore should also not be in the 700nm detection wavelength, since bleedover can occur with the total protein stain. The 800nm detection wavelength is best for avoiding cross-talk and has the greatest sensitivity.

39 After incubation, decant the blocking buffer and wash the membrane in TBST or PBST (1x TBS or PBS plus 0.2% Tween-20) for 00:05:00 at Room temperature with gently shaking, repeat two more times for a total of three washes. 5m

40 After the third wash, image the membrane in the 800 nm channel on a membrane imager.