Western Blot Intein Notebook

Benchling

HORMONIC
UPF_Barcelona

Western blot

MARTES, 18/8/2020

The first step form the Western Blot protocol that Avencia shared is specific for mammalian cells. We need to find another way to lysate the bacterial cells (sample preparation).
There are plenty of ways to do that, as specified at a Sigma-Aldrich article. For bacterial cells we have:

- Freeze/Thaw lysis
- Osmotic shock lysis
- Enzymatic digestion
- Explosive decompression (nitrogen cavitation)

Freeze/thaw lysis (+ enzymatic digestion)

1. Rapidly freeze the cell suspension with liquid nitrogen
2. Thaw the sample
3. Resuspend the cells in a lysis buffer
4. Centrifugate and retain the supernatant

Repeat the cycle several times.

Enzymatic digestion

Enzymatic digestion is often followed by homogenization, sonication or vigurous vortexing in a lysis buffer.
For bacterial cells the prefered enzyme is **lysozyme**, also known as muramidase or N-acetylmuramide glycanhydrolase, that damages bacterial cells walls by catalyzing hydrolysis of 1, 4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins.

MIÉRCOLES, 19/8/2020

Talking with Maria we have concluded that the best option for *E. coli* cell lysis was to work with the sonicator (3rd floor common machines, next to the 30ºC chamber).

In the sonicator we mainly need to specify 3 parameters:

- The sonicating vs. resting time (duty cycle will be set to constant (=100) and checked manually): 30'' sonicating + 1'30'' resting
- The output control (strength of the waves, amplitude)
- The timer (set to maximum to make sure the process is non-stop)

When using the sonicator, we need to bring the sample in a glass of ice with an eppendorf inside, perpendicular to ultrasonic microtip. Guillermo is available to help us using it, and also to help us when doing the western blot.

We need to place the cells in a buffer in order to keep them in a safe space that avoids denaturalization and loss of the proteins. An adequate buffer could be the **S30 buffer** (see protocol).

Once cells are lysated with the sonicator, centrifugate them and keep the supernatant for analysis.

THIS PROCEDURE IS STILL NOT DONE, BUT ONLY EXPLAINED

LUNES, 24/8/2020

S30A buffer preparation

Ingredients:

- 2.72 g Mg Glutamate (L-Glutamic acid hemimagnesium salt tetrahydrate)
- 5.555 g K Glutamate (L-Glutamic acid potassium salt monohydrate)
- 12.5 mL tris base 2 mM

Tris base solution preparation

Tris base [(CH2OH)3CNH2] molar mass/molecular weight

	Atom	# atoms	M. W. (g/mol)	Subtotal mass (g/mol)	E
1	C	4	12.011	48.044	
2	H	11	1.008	11.088	
3	O	3	15.999	47.997	
4	N	1	14.007	14.007	
5				121.136	TOTAL

Tris base powder for 2 M 250 mL solution

	Solution	Concentration [M]	Volume [mL]	M. W. [g/mol]	Mols	Mass [g]
1	Tris base	2	100	121.136	0.2	24.2272

Buffer preparation

Protocol: S30A Buffer

1. Weight the following:

Weights

	Compound	Concentration [mM]	Volume [mL]	M. W.	Mols	Initial concentration [M]	Mass [g]	Initial volume [mL]	Mass [g] / Volume [mL] for 500 mL
1	Mg glutamate	14	1000	388.62	0.014	-	5.44068	-	2.72034
2	K glutamate	60	1000	185.22	0.06	-	11.1132	-	5.5566
3	Tris Base	50	1000	121.136	-	2	-	25	12.5

2. Mix everything in 250 mL of distilled water
3. Put the solution in the pH-meter with a magnetic stirrer and equilibrate the pH to 7.7 (approx. 300 µL of acetic acid)
4. Add distilled water up to 500 mL (done 25/08)

JUEVES, 27/8/2020

Western blot

Cell preparation

1. 8mL of saturated culture from T3+ and T3- were cold centrifugated for 5 minutes, discarted the supernatant and resuspended with 10 mL of COLD S30 buffer.
2. Previous washing is repeated.
3. Centrifugate for 5 minutes and resuspend with 2 mL of cold s30 buffer --> 4mL/mL (concentration of 4 mL of saturated culture for each mL).
4. Aliquot by 1mL in diferent eppendorfs.

Cell sonication lysate

1. Cell lysis: sonication →3 cycles of 0:30 minutes ON + 1:30 minutes OFF with amplitude 0.2 J/s
    a. Place the ependorf in a cubbete of ice and insert the tip to the bottom. once reached, elevate the tip until it doesnt touch the bottom of the eppendorf, nor any wall and still stays inside the liquid.
    b. With the help of the ice center the eppendorf.
    c. after each cycle, reajust the eppendorf position.
    d. Final sample should look more yellowish (in our case it looked more translucid).
    e. Place in ice as much as possible.
2. 14000 xg centrifugation during 15 minutes at 4 ºC
3. Transfer supernatant to a fresh eppendorf tube
4. In a new eppendorf mix 20 µL of supernatant with 5 µL of loading buffer for each sample.
   The loading buffer is at the top of the fridge in fornt of Marc's room, inside a 50 mL Falcon.
   ATTENTION: Loading buffer should be added to the samples under the hood.
5. Boil at 95 ºC for 5 minutes
6. Spin down at maximum speed for 1 minute.

Set up Gel Tank

1. Get the **1X running buffer** from the fridge in front of Marc's room. To get 1x from the one of the fridge that was at 20x, we have diluted 25 mL of the running buffer with 475 mL of distilled water.
2. Remove the withe tape near the bottom of the plate and place it in the cuvette.
3. Fill the tank with running buffer and once filled get rid of the comb to expose the wells.

Load samples

1. Load sample sin an order similar to the below (20 µL/sample):
   Ladder (13 µL) 1 2 3 4 Ladder (13 µL)
   We can find the ladder at the bottom of the freezer, as Nuria is giving it to us. We need to look at the box called "Lama2 3xaav".
2. Connect the tank to the power supply and run the gel at 120 V until the dye front is at the bottom of the gel (approx. 1 hour) In our case 1 hour 15 min.

Dry Transfering

1. Once the gel has run, with a spatula break the plastic case carefully by insering the spatula in the lateral grooves and prying.
2. Cut the gel well fringes and the excess gel. Place the gel in a cubbete and wash it with distilled water for a few seconds.
3. Prepare the iBlot2 dry transfer kit:
    a. Open the kit and place the bottom layer to the macchine.
    b. Lay down the transfer paper, use rolling pin to avioid any bubble.
    c. Lay down the running gel centered, use rolling pin to avioid any bubble.
    d. Wet the paper layer in distilled water and place it on.
    e. Put the last metal layer making sure that the anode and catode of the layers are touching its respective parts of the machine. Again, use rolling pin to avioid any bubble.
    f. Run the iBlot2 with the 7 minute program.
    g. Discard everything BUT the transfer paper. Mark the top side with a pencil.
    h. Cut the sides of the paper (without touching the trasnfered region) so that it fits into the cuvette.

Ponceau staining

1. Place the transfer paper in a cuvette and add the ponceau staining solution (reusable).
2. After coupple of minutes proteins will begin to stain. This indicates protein presence. Cell lysis was done correctly. Take pictures and everything you want now because we will wash it out.
3. Return the ponceau staining solution to the original Falcon.

Fixing and Antibody incubations

1. Prepare 1L of TBST *attach protocol* (in our case 500 mL)
2. In a moving cuvette, wash the transfer paper 3 x 5minutes changing the TBST in between.
3. Prepare the fixing solution 4% milk as follows:
    a. In a 50 mL falcon add 40 mL of TBST.

b. Add 1.6 g of milk powder (4%) and mix well.
c. In a 15 mL falcon add 10 mL of TBST (in our case, x2 were needed)
d. Add 0.4 g of milk powder (4%) and mix well

4. Incuvate the transfer paper with the fixing solution for 15 minutes (?)
5. Discard the liquid and wash the transfer paper 3 x 5 minutes changing the TBST in between.
6. Add the primary antibody to the 15 mL falcon at 1:5000 (10 mL of the TBST 4% milk + primary antibody 1:5000)
7. Incuvate for 4 hours (or overnight at 4ºC) with the primary antibody.

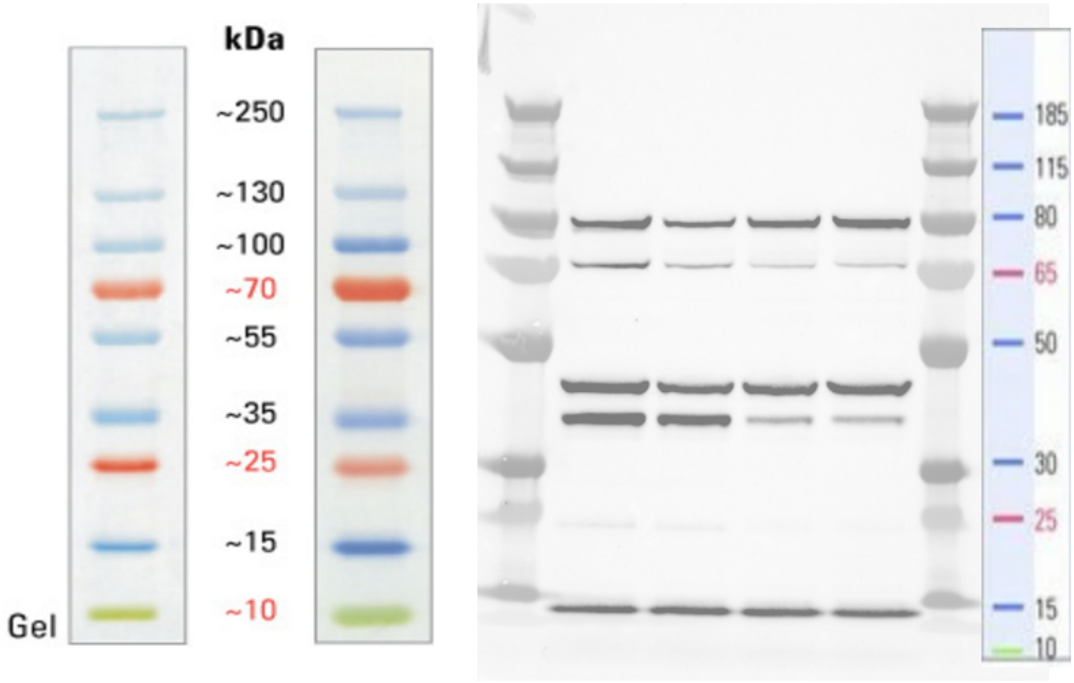
VIERNES, 28/8/2020

1. Return the liquid to its flask (the primary antibody is resuable? we used a reused antibody) and wash the transfer paper 3 x 5 minutes changing the TBST in between.
2. Add the secondary antibody to a 15 mL falcon with 10 mL of TBST with 4% milk (*add protocol or solution properties*).
   a. In a 15 mL falcon add 10 mL of TBST.
   b. Add 0.4 g of milk powder (4%) and mix well.
   c. We added 6-7 µL of the antibody Nuria left us.
3. Incuvate for 1 hour with the secondary antibody.
4. Return the liquid to its flask and wash the transfer paper 3 x 5 minutes changing the TBST in between.

Western Developing

1. Prepare the developing solution mixing the two peroxidase detection reagents, from tehrmofisher kit, at a ratio 1:1 in a 15 mL falcon.
   CAUTION: one reagent is photosensible. Cover the falcon with foil.
2. In the developing machine, place the transfer paper and pour the developin solution. Wait 6 minutes and take a picture.

image.png



Looking at the ladder we can only appreciate 8 bands, when 9 should be depicted (considering this ladder as our reference). We attribute the difference to a bad placement when transfering, so that the 10 kDa band is not appreciable. The 10kDa is present if observe in detail.

Columns 1 and 2 correspond to the T3 + BL21 cells (incubation with T3). Columns 3 and 4 correspond to control T3- BL21 cells incubated with only DMSO. Control cells should express a protein band around 85 kDa, corresponding to the protein complex of the intein plus the split GFP. When incubated with T3, the two parts of GFP should fuse and a band of 37,23 kDa should be present, while the rest of the protein should not be visible as the FlagTag (specific for the antibody) is linked to the GFP.

In the obtained results, we can appreciate 5 bands present at all the columns and an extra one only present at the control. We have, approximately, the following bands:

Table1

	Molecular Weight (kDa)	Column presence	Protein (A)	Explanation	Protein (E)
1	105	1 | 2 | 3 | 4			Incusion Bodies
2	85	1 | 2 | 3 | 4	Intein + split GFP?		whole protein
3	50	1 | 2 | 3 | 4			?
4	41	1+ | 2+ | 3 | 4			eGFP (37kDa)
5	24	1 | 2	GFP?	The fused GFP should appear in columns with T3	eGFP 2 (22kDa)
6	13.5	1 | 2 | 3 | 4			

We should talk with Guillermo about the presence of other proteins. Maybe the BL21 strain he left us has constitutive expression of some proteins.

LUNES, 31/8/2020

BL21 (new and old stock) and DH5α cells were cultivated from glycerol stock in liquid mediums, one with T3 and another one without (6 tubes in total).
Tubes were left under incubation at 37 ºC with vigurous shaking overnight.

MARTES, 1/9/2020

Protocol for Western blot was repeated using the cultures from Monday 31st August. S30A buffer was still in stock from the last time (25/08).

Here are attached only the changes at the protocol:

Cell sonication lysate with urea

1. Cell lysis: sonication → 3 cycles of 0:30 minutes ON + 1:30 minutes OFF with amplitude 0.2 J/s
2. 5alfa t3+ was not as sonicated as the others.
    a. Place the ependorf in a cubbete of ice and insert the tip to the bottom. once reached, elevate the tip until it doesnt touch the bottom of the eppendorf, nor any wall and still stays inside the liquid.
    b. With the help of the ice center the eppendorf.
    c. after each cycle, reajust the eppendorf position.
    d. Final sample should look more yellowish (in our case it looked more translucid).
    e. Place in ice as much as possible.
3. 14000 xg centrifugation during 15 minutes at 4 ºC
4. Transfer supernatant to a fresh eppendorf tube
5. In a new eppendorf mix 20 µL of supernatant with 10 µL of loading buffer and 10 µL of urea.

The loading buffer is at the top of the fridge in fornt of Marc's room, inside a 50 mL Falcon.
ATTENTION: Loading buffer should be added to the samples under the hood.

6. Incubate at 37 ºC for 15 minutes.
7. Spin down at maximum speed for 1 minute.

The wells of the gel were charged following the next figure:

Table2

	Control	BL21	BL21	BL21	BL21	Ladder	BL21 old	BL21 old	DH5α	DH5α
1	T3	+	-	+	-		+	-	+	+
2	Urea	-	-	+	+		-	-	+	-
3										

Load samples

1. Load sample sin an order similar to the below (20 µL/sample):
   We can find the ladder at the bottom of the freezer, as Nuria is giving it to us. We need to look at the box called "Lama2 3xaav".
2. Connect the tank to the power supply and run the gel at 120 V until the dye front is at the bottom of the gel (approx. 1 hour) In our case 1 hour 15 min.

Fixing and Antibody incubations

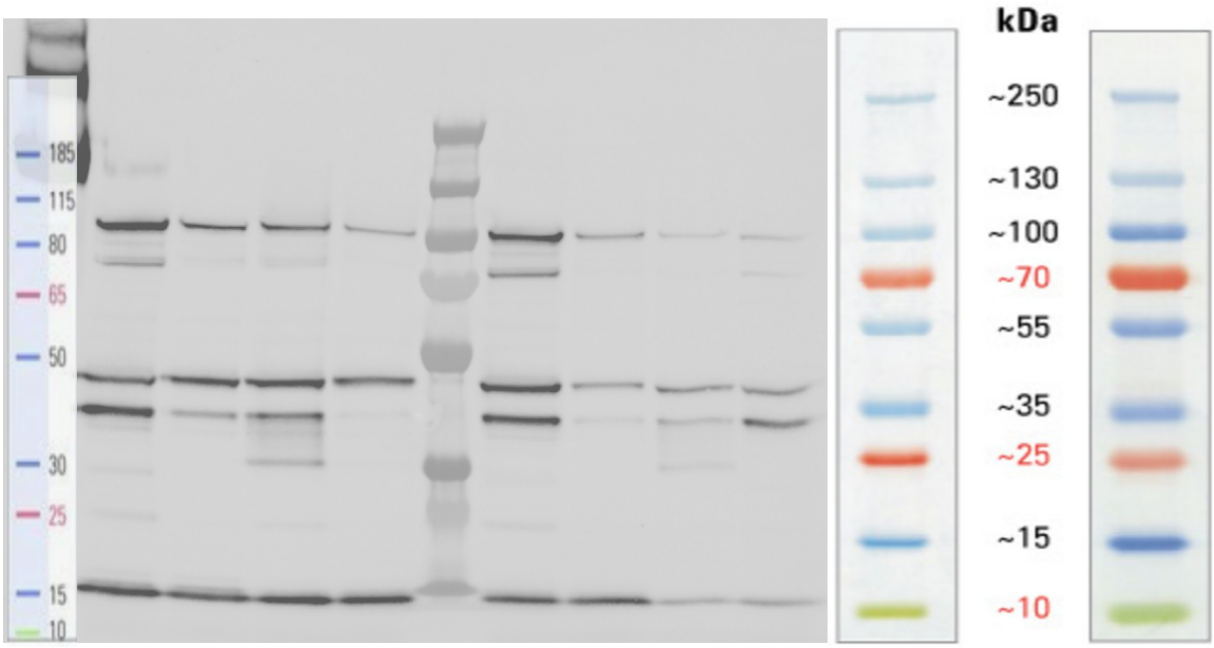
1. ~~Prepare 1L of TBST *attach protocol* (in our case 500 mL)~~
2. In a moving cuvette, wash the transfer paper 3 x 5minutes changing the TBST in between.
3. Prepare the fixing solution 4% milk as follows:
   a. In a 50 mL falcon add 40 mL of TBST.
   b. Add 1.6 g of milk powder (4%) and mix well.
   c. In a 15 mL falcon add 10 mL of TBST (in our case, x2 were needed)
   d. Add 0.4 g of milk powder (4%) and mix well.
4. Incuvate the transfer paper with the fixing solution for 15 minutes (?)
5. Discard the liquid and wash the transfer paper 3 x 5 minutes changing the TBST in between.
6. Add the primary antibody to the 15 mL falcon at 1:5000 (10 mL of the TBST 4% milk + primary antibody 1:5000)
   10 mL · (1/5000) = 0.002 mL antibody = 2 µL antibody (we added 3 µL)
7. Add azida at 0.02% to keep and reuse the antibody for future Western Blots.
   (0.02/100) · 10 mL = 0.002 mL azida = 2 µL azida
8. Incuvate for 4 hours (or overnight at 4ºC) with the primary antibody.

MIÉRCOLES, 2/9/2020

Table3

	Control	BL21	BL21	BL21	BL21	Ladder	BL21 old	BL21 old	DH5α	DH5α
1	T3	+	-	+	-		+	-	+	+
2	Urea	-	-	+	+		-	-	+	-
3										

Captura de pantalla 2020-09-02 a las 13.04.22.png



LUNES, 12/10/2020

Liquid cultures where prepared and left at 37 overnight shaking:

- 4 mL of LB + Amp and IMT3_sfGFP_pUC-Amp with NO T3
- 4 mL of LB + Amp and IMT3_sfGFP_pUC-Amp with 100uM T3
- 4 mL of LB + Amp and IMT3_sfGFP_pUC-Amp with 10uM T3
- 4 mL of LB + Amp and IMT3_sfGFP_pUC-Amp with 10nM T3
- 4 mL of LB + Amp and IMT3_sfGFP_pUC-Amp with NO T3
- 4mL of LB + Chlor and BL21 (DE3) no plasmid (control)

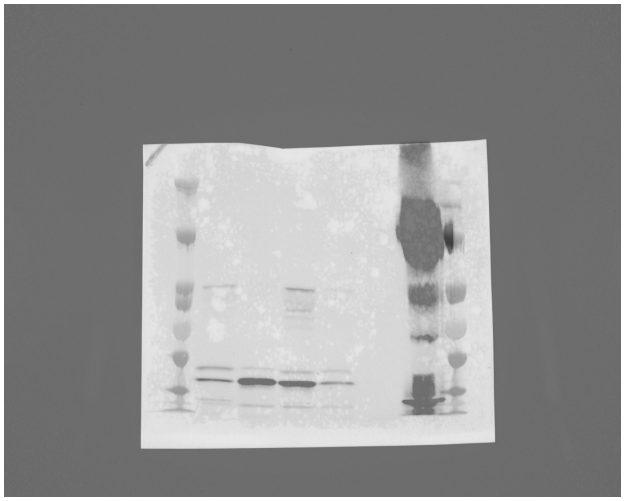
MIÉRCOLES, 21/10/2020

The 5 samples where processed as the previous times, with sonication and boiling at 95ºC with loading buffer.
Treated as the other times with first antibody overnight: Rabbit Anti-DDDK
Secondary antibody: Mouse anti-rabbit (peroxidase)

Samples loaded as:

Ladder 10nMT3 10uMT3 100uMT3NoT3 BL21 (neg control) college's Flagx3 lisiate (Pos Control) Ladder

image.png



ladder