Western blot protocol

Gel preparation

- Mix together all ingredients for the running gel and the stacking gel (Table is in the protein room)
    - Be careful with PAA - it is very toxic and has to be pipetted with a plastic pipette and has to be deposited into the toxic waste
    - As soon as both TEMED and APS have been added to the gel it starts to become solid, so first do the running gel and after it is solid add TEMED/APS to the stacking gel
- First pour the running gel and put a layer of isopropanol on top of it to avoid bubbles. Let it become solid then remove isopropanol and dry with filter paper
- Then pour the stacking gel on top of the running gel and carefully add a comb. Let is also become solid.
- Let the prepared gels in the glass slides and put them in the fridge with watered towels around them. They are good to use for about a week

Sample preparation

- Inoculate 15ml yeast at 30°C overnight
- If they have reached an OD of 0.6 – 1 the next day you can continue with the next step, otherwise let them grow or if the OD is to high dilute them and let them grow again until they reach the desired OD
- Put 1ml of each sample culture in a micro vial and do not forget the negative control
- Centrifuge for 5 minutes at 13000g
- Remove the supernatant – at this point you can freeze the obtained pallet
- Resuspend the pallet with 50µl 2xSDS + DTT
    - To obtain 2xSDS + DTT (Loading Dye): mix 500µl 6xSDS with 1ml Water and 150µl DTT
- Add glass pearls to each sample
- Shake 5 minutes at 99°C at maximum speed
- Vortex all samples
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- Shake 5 minutes at 99°C at maximum speed
- Centrifuge for 2 minutes 13000g
- Pipette supernatant without the pallet and the glass pearls into a new micro vial

Sample analysis

- Put 15µl of each sample on the gel and 5µl of the ladder
- Run 20 minutes with 80V

- Run at least 1 hour with 120 V
    - Be sure the blue band is at the bottom of the gel
- Remove the glass slides from the gel
- Remove the stacking gel and the blue band at the bottom
- Soak the whatman paper in transfer buffer and the membrane in methanol
- Prepare the blotting by stacking the layers in following way: whatman paper, membrane, gel, whatman paper and remove bubbles
- Blot for 15-20 minutes with the standard protocol
- Put the membrane into a falcon and add 10ml milk, let it roll for 30-60 minutes to block
- Put the milk back into the original falcon and add the first antibody – the anti his – to the membrane, let roll for 1 hour or over night
    - First antibody: 10ml milk, 100µl Tween, 1µl anti his
- Put the antibody back and add 5-10ml TBST to wash the membrane, roll for about 5 minutes
- Wash three times in total
- Add the second antibody – anti mouse – to the membrane, let it roll for 1 hour
    - second antibody: 10ml milk, 100µl Tween, 2µl anti his
- wash again three times with TBST
- mix 270µl of each pico and 25µl of each femto into a micro vial and mix
- apply the mix onto the membrane and place the membrane between two plastic films, remove the bubbles
- take a photo of the membrane
- for proof of loading do a ponceau-coloring – therefore put the membrane into a bowl of ponceau-color and let it move for 20 minutes, rinse with some water