Northern Blot Protocol (Fluorescence Detection)

Introduction

The Northern blot method is an **established and recognised molecular biology technique** for the analysis of RNA expression in different tissue samples or cell types. It enables **specific detection** and, according to the protocol described here, quantification of **different RNA molecules**, which is crucial for understanding gene regulatory mechanisms and gene expression in different biological contexts. This protocol describes in detail the essential steps of Northern blot analysis, from RNA extraction and gel electrophoretic separation to membrane transfer, hybridisation and detection with specific probes. Careful execution of the method allows **precise and reproducible results** to be obtained, providing valuable insights into gene expression. The Northern blot remains a powerful and adaptable method for simultaneously analysing RNA size and quantity and is therefore an excellent **verification method for existing OMICS technologies**.

This protocol uses **fluorescence detection**, which offers fundamental advantages over other methods such as chemiluminescence detection. Fluorescence detection is characterised by **very high reproducibility and sensitivity** and enables the **acquisition of quantitative data**, while different RNA molecules can be visualized by multiplexing or simultaneous detection. These advantages make it a preferred choice for applications in RNA analysis as it provides **more precise and reliable results**. Following products by ProTec Diagnostics are used with this protocol:

- ✓ ProTec Diagnostics RNA-TRUE Sample Buffer (Cat. No. RTRUESB_1)
- ✓ ProTec Diagnostics RNA-TRUE Marker2000 (Cat. No. RTRUEM2_1) or
- ✓ ProTec Diagnostics RNA-TRUE Marker4000 (Cat. No. RTRUEM2_1)
- ✓ RNA-TRUE Dye500 (Cat. No. RTRUED5_1)
- ✓ RNA-TRUE Dye600 (Cat. No. RTRUED6_1)
- ✓ RNA-TRUE Dye700 (Cat. No. RTRUED7_1)
- ✓ RNA-TRUE Dye800 (Cat. No. RTRUED8_1)
- ✓ RNA-TRUE ChemiDye (Cat. No. RTRUECD_1)

Part I: Preparation of *Biotin* labeled probes for target RNA detection

Required:

- ✓ Specific primer pair
- ✓ PCR block
- ✓ Taq polymerase kit
- ✓ T7 RNA polymerase kit
- ✓ Biotin-labeled UTP for ***fluorescence detection***
- ✓ PCR purification kit
- ✓ EDTA solution (0,2 M)
- ✓ Sodium acetate solution (3 M, pH 5,2)
- ✓ RNase inhibitor [20 U/µl or 40 U/µl]

Step 1. Preparation of a double stranded DNA template using PCR (for subsequent *in vitro* transcription with T7 RNA polymerase)

The PCR product should be ~100 to 400 bases long. The primers should contain a 50:50% GC:AT ratio, have a G or C at its 3` end and be about 20 bases long (the primary annealing part of it!). The reverse primer additionally contains the T7 promoter sequence at its 5' end, the complete T7 Promoter extension for linear template fragments reads:

5´-**GAAATTAATACGACTCACTATA**GGGAGA-3´

Note that if a PCR product is being used, make sure there is an at least 5 bases extension (green) upstream of the T7 RNA polymerase (RNAP) core promoter (**bold**) for optimal polymerase binding. It is useful to have a generic T7 promoter primer which can be used to amplify any template that carries the promoter sequence. As the T7 RNAP has certain base requirements for transcription initiation the first two nucleotides after the core promoter should be GG (red) as these are preferred by T7 RNAP. The 5`cRNA extension is underlined.

Example for a gene specific primer pair:

Forward_primer: 5`-NNNNNNNNNNNNNNNNNNN-3`

Reverse_primer: 5`-**GAAATTAATACGACTCACTATA**GGGAGANNNNNNNNNNNNNNNNNNN-3`

Setting up the PCR reaction mix (100 µl):

10x Taq buffer	10 µl
$MgCl_2$ (50 mM)	4 µl
dNTP mix (10 mM)	2 µl
Forward primer [100 pmol/µl]	2 µl
Reverse primer [100 pmol/µl]	2 µl
Chromosomal DNA template [~0,25 µg/µl]	4 µl
Taq polymerase [5U/µl]	1 µl
Nuclease-free water	75 µl

Touchdown PCR protocol:

Initial denaturation	94°C	5 min	
Melting	94°C	30 sec	
Annealing	63°C	30 sec	10x
Amplification	72°C	1 min	
Melting	94°C	30 sec	
Annealing	60°C	30 sec	10x
Amplification	72°C	1 min	
Melting	94°C	30 sec	
Annealing	57°C	30 sec	10x
Amplification	72°C	1 min	
Final amplification step	72°C	5 min	
	4°C	∞	

Purify the PCR product from an analytical 2% agarose gel or directly from the PCR reaction with a kit of your choice. Make sure that the PCR product to be used is from a single band and of expected size. Always elute your PCR product with **10 mM Tris pH 8.0 buffer** that is to be set up with nuclease free water. The expected template concentration is around 0,5 µg/µl.

Step 2. Preparation of Biotin labeled cRNA detection probes

In vitro transcription with T7 RNA polymerase using the MEGAscript T7 Transcription Kit from Ambion (Cat. No. AM1333) and labelling of the RNA probe with Biotin-16-UTP (10 mM, from Ambion Cat. No. AM8452) for fluorescence detection

Note: The MEGAscript kit contains pyrophosphatase which cleaves the pyrophosphate into two phosphate ions to avoid the inhibitory effect of the pyrophosphate on the T7 RNA polymerase. This drastically increases cRNA yields from typically 5–10 µg per reaction to 100–150 µg.

Setting up the PCR reaction mix (20 µl) (at room temperature, reaction volumes may be scaled up or down if required):

Template DNA	5 µl (approx. 2.5 µg)
NTP (*not* dNTP):	
ATP (75 mM)	1.5 µl
GTP (75 mM)	1.5 µl
CTP (75 mM)	1.5 µl
UTP (75 mM)	1 µl
Biotin-16-UTP (10 mM)	**4 µl**
10x Transcription buffer	2 µl
T7 RNA polymerase	2 µl
RNase inhibitor [20 U/µl]	1.5 µl
	20 µl

Incubate at 37°C in a PCR block for 2h.

Next morning:

Add TURBO DNase Buffer (10x)	2 µl
Add TURBO DNase I [2 U/µl = TURBO DNA-free kit]	1 µl

(One unit is the amount of enzyme required to completely degrade 1 µg DNA in 10 min at 37°C)

- Mix by careful pipetting and incubate for another 30 min at 37°C
- Precipitate the cRNA by addition of 100 µl 3 M Na-Acetate pH 5.2 and 800 µl pure ethanol
- Incubate 2 h at -20 °C or -80 °C
- Centrifuge for 10 min max speed at 4°C
- Discard the supernatant
- Wash with 200 µl of 70% ethanol
- Centrifuge for 10 min at 12.000 g
- Discard the supernatant and air dry the pellet
- Dissolve in 100 µl nuclease-free water and add 1 µl RNase inhibitor
- Store at -80 °C

Labelling checkup:

- Prepare dilutions series up to 10^{-6}
- spot 1 µl from each dilution on a dry membrane
- Perform UV crosslinking (for detailed description see page 8)
- Add 20 ml LI-COR Odyssey Blocking Buffer (PBS) [P/N 927-70001] + 1% SDS & incubate 30 min at RT
- Discard blocking buffer
- Add 15 ml LI-COR Odyssey Blocking Buffer (PBS) [P/N 927-70001] + 0,5 % SDS & add 0.6 µl of CW 800 Streptavidin (1:30.000) [926-32230] and incubate 30 min at RT
- Discard blocking buffer
- Wash 3x 20min with 50 ml PBS+0,1% Tween20 at RT
- place the membrane between two sheets of fresh and clean Whatman paper, gently strike over the stack to remove all residual buffer from the blot. Then let the membrane completely dry at 68°C for ~15 to 20 min

Note : Completely dry membranes will roll up, increasing the signal to noise ration drastically!

- Documentation by LI-COR Odyssey CLx Scanner (or similar detection system)

Part II - Cell harvest and disruption

Required:

- ✓ Cooling Centrifuge
- ✓ Killing buffer (optional: crushed Killing ice)
- ✓ Lysis solution
- ✓ Teflon vessels (5 ml)
- ✓ Steel balls (Ø 7 mm)
- ✓ Bead mill (Mikro-Dismembrator)
- ✓ Liquid nitrogen

Killing buffer	**500 mL**	**500 mL**
Tris-HCl, pH 7.5 (20 mM)	50 mL (0.2 M stock solution, RT)	10 mL (1 M stock)
$MgCl_2$ (5 mM)	5 mL (0.5 M stock solution, RT)	2.5 mL (1 M stock)
NaN_3 (20 mM)	5 mL (2 M stock solution, 4°C)	5 mL (2 M stock)
Aqua dest. (sterile)	440 mL	482.5 mL

Lysis solution	**250 mL**
Guanidinium thiocyanate (4 M)	118.16 g
Na-Acetate, ph 5.2 (0.025 M)	2.03 mL
N-Lauroylsarcosine (0.5 %)	12.5 mL (10% stock solution, RT)
Aqua dest. (sterile)	ad 250 mL

Cell Harvest of 10 to 15 OD (optical density) units from bacterial cell culture. Example: 15 OD units at exponential growth phase OD = 0.5. Volume to be harvested: 15 divided by 0.5 equals 30 ml.

Option 1 – Liquid nitrogen (preferred):
Cool the cells down in liquid nitrogen (dip the Falcon tube 3 times for 5 to 10 seconds with vigorous shaking by hand in between – wear cotton gloves and nitrile gloves at least on your "nitrogen" hand) and immediately centrifuge the cells for 3 minutes at 8.873 g. Discard the supernatant and store the pellets for further preparation in liquid nitrogen or at -80 °C.

Option 2 – Killing ice:
Pre-cool Killing buffer that has been pre-set in 50 ml tubes for approximately 20 mins at -20 °C to obtain Killing ice that you can crush on the rim of the styrofoam box, but never on solid ground! Immediately mix the culture volume with the Killing ice (1:2 v/v). Immediately centrifuge the cells for 3 minutes at 8.873 g. Discard the supernatant and store the pellets for further preparation in liquid nitrogen or at -80 °C.

Both methods are working equally fine resulting in high quality total RNA of high integrity (rRNA precursors are present). Killing ice does contain sodium azide, which can be fatally toxic!

For **mechanic disruption** of the cells resuspend the pellets in 200 µl of pre-warmed Lysis-Buffer (in a water bath at 50 °C) or 200 µl of ice cold Killing buffer. Immediately transfer the suspension to a Teflon vessel that has been pre-cooled in liquid nitrogen. The lower half of the vessel is being filled with liquid nitrogen and also contains a 7 mm diameter steel ball for the cell disruption. Once the cells are completely frozen, close the vessel and put it into a bead mill (here: Mikro-Dismembrator S, Sartorius AG) and disrupt them at 2600 rpm for 2 minutes. Resuspend the frozen lysate powder in 3 (to maximum 4) ml of 56°C pre-warmed Lysis solution (carefully pipet up and down until the solution clears up completely If required: when crystals form use scissors-shortened tips with a wider opening as resuspension is faster then, crystals vanish with time when solution is warming up, but you want to work fast. Split the solution into three (or four) 2 ml safe lock Eppendorf tubes and put immediately to liquid nitrogen. Either store the lysate at -80 °C for further preparation or continue with isolation of RNA immediately.

Part III - Isolation of total RNA

Required:
- ✓ Centrifuge
- ✓ 2 ml reaction tube shaker
- ✓ Acidic phenol mix (PCI)
- ✓ Chloroform mix (CI)
- ✓ Nuclease free water
- ✓ 3 M Na-Acetate solution pH 5.2
- ✓ Isopropanol
- ✓ Ethanol
- ✓ RNase inhibitor (optional) (Promega, N261B)

Acidic-Phenol-Mix-Solution
Mix Roti®-Aqua-Phenol (pH 4.5 - 5) from Carl Roth GmbH & Co KG with chloroform and isoamyl alcohol in a ratio of 50:48:2, or buy the ready-to-use mix Roti®-Phenol/Chloroform/Isoamyl alcohol from Carl Roth GmbH & Co KG.

Chloroform-Mix-Solution
Mix chloroform and isoamyl alcohol in a ratio of 96:4.

3 M Na-Acetate buffer, pH 5.2
3 M Na-Acetate
Adjust the pH with Acidic acid (1.06 mol), **Note:** Do not use HCl!
ad 1 l A. bidest, autoclave

Total RNA is being isolated by the acidic phenol method at room temperature. In the first step phenol mix solution is added to the frozen cell lysate to the maximum (fill up the 2 ml tubes) and samples are shaken for 5 min at max speed. Now centrifuge the tubes for 5 min at 16.000 g at RT. Then transfer 900 µl of the upper aqueous phase to a fresh 2 ml tube (avoid transfer of the DNA and protein contaminated interphase). Add 1 ml of phenol mix solution and repeat the shaking – centrifugation cycle. Now transfer 800 µl of the upper aqueous phase to a fresh 2 ml tube (again keep away from the interphase) and now add 1 ml of chloroform mix solution to get rid of the phenol in your sample. Again repeat the shaking – centrifugation cycle. Now transfer transfer 700 µl of the upper aqueous phase to a fresh 1.5 ml tube and add 100 µl of acetate-solution and 700 µl of pre-chilled isopropanol. Mix by inversion of the tubes and precipitate the RNA for 2 h at -80 °C or over night at -20 °C. Spin down the RNA for 15 min at maximum speed and 4 °C, discard the supernatant and wash the pellet with 1 ml of ice cold 70% ethanol. Centrifuge again for 15 min and completely remove the supernatant.

Caution: Do not dry the pellet too long – this will result in undissolvable RNA!

Only air dry the pellet for 2-3 min at room temperature (no speed vac or heat step!), add 60 to 100 µl of RNase free water (depending on the size of the pellet – normally 3 mm in diameter and opaque). Immediately resuspend the pellet by pipetting it up and down 10 to 15 times and combine the three pellets of one sample at this stage! **Optional: Add 1 µl of RNasin RNase inhibitor** [Promega, N261B]. Let the RNA dissolve on ice for another hour.

Concentration and Quality Check

Either absorption spectroscopy or a spectrometer like the NanoDrop (PeqLab) can be used to measure the RNA concentration.

Required:

- ✓ Silica cuvette (1 cm)
- ✓ UV/Vis Photometer or spectrometer
- ✓ Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit

Absorption spectroscopy (use a silica cuvette (Hellma Suprasil^{R} 10 mm) for all UV applications!):
Dilute your samples 1:10 and 1:20 and measure the absorption at 260 and 280 nm with RNase free water as reference. The ratios of OD260 and OD280 should be between 1.7 and 1.9 (lower values indicate protein contamination and values greater than 2.0 indicate degradation). The concentration can be calculated as follows:

c[µg/ml] = OD260 x dilution factor x constant factor (here: 40 for RNA)

A standard RNA isolation yields concentrations of approximately 1-1.5 µg/µl.

Check the quality of your RNA on an Agilent 2100 Bioanalyzer according to the manufacturer's protocol.

Caution: For all *fluorescence detection methods* you should strictly avoid blue dye contaminations (see RNA sample buffer and do not use a regular ball pen or similar to label your blots, always use a pencil!).
You can also check your RNA quality on your Northern blots by **methylene blue staining** (see page 9)– **BUT** only after fluorescence detection!

Part IV - Denaturing agarose gel electrophoresis of RNA

Required:

- ✓ 10x MOPS buffer
- ✓ 1x MOPS buffer
- ✓ Formaldehyde stock solution (37%)
- ✓ A lot of deionized sterile H_2O
- ✓ **ProTec Diagnostics RNA True Sample Buffer** (**Caution:** Necessary for fluorescence detection!!!)
- ✓ **ProTec Diagnostics RNA TRUE Marker**
- ✓ RNA stock samples
- ✓ RNA Agarose (Sigma Aldrich, Cat. No. 912-36-6)
- ✓ Sterile or better RNase-free low binding microcentrifuge tubes
- ✓ Heating block
- ✓ 500 ml bottle for melting of agarose
- ✓ Gel chambers with gel trays (BIO-RAD Sub-Cell GT DNA Electrophoresis Cell system)
- ✓ Gel caster
- ✓ Water level
- ✓ 20x SSC buffer
- ✓ Denaturation solution
- ✓ Neutralization solution
- ✓ Blotting Apparatus (GE Healthcare)
- ✓ Plastic masks
- ✓ Nylon membrane, positively charged (Use only Nylon Membranes, positively charged, Roche Diagnostics GmbH, REF 11 417 240 001 – 1 roll à 30 cm x 3m)

10x MOPS buffer	**1 L**
200 mM MOPS	41.85 g
50 mM Na-Acetate trihydrate	6.8 g
10 mM EDTA	3.72 g

adjust pH to 7.0 with NaOH and filter sterilize

Preparation of denaturing agarose gels:

- Use 3 g RNA agarose (1.5 % gel)
- Add 145 ml sterile H_2O
- In order to melt the agarose solution: Place the 250 ml bottle for 3 to 5 mins in the microwave oven, adjust „high“, keep the lid slightly opened and remove the bottle every 30 seconds from the oven and mix content thoroughly. Repeat until all agarose has been dissolved.
- Temper gel solution for 30 min at 65 °C in a water bath
- Add 20 ml 10x MOPS buffer
- Add 35 ml formaldehyde (final: 6,5%) under the fume hood, mix well and pour the gel in a fume hood immediately.

Note: You will need 200 ml of gel solution for a gel of approximately 16 x 12 cm and approximately 1 cm thickness. The gel can be much thinner (3-4 mm) because a thinner gel will drastically reduce the blotting times - but handling strongly depends on the experimentator's skills!

During the incubation periods:
Clean all gel apparatus components thoroughly with water and detergent, the gel chamber is only rinsed with sterile A. dest. Assemble and tare the gel cast using the water level and do insert the comb!)

Procedure:

- Leave gels at least for 1 h for solidification (fume hood)
- In the meantime, prepare the RNA loading samples and the **RNA TRUE Marker**
- Mix and spin down thawed RNA stock samples before use

- Transfer the desired RNA amount to be loaded per slot (e. g. 2, 5, or 10 µg depending on the expression level of your target gene) in a fresh 0.5 ml safe lock Eppendorf tube
- Adjust the total volume to 10 µl (5 µg sample) or 20 µl (10 µg sample) by adding sterile deionized water
- Add **5 or 10 µl RNA True Sample Buffer** respectively (the total volume of the loading sample should be 15 or 20 µl.) The RNA True Sample Buffer is stored at - 20 °C in the freezer.
- Adjust heating block to 70 °C
- Transfer the solidified gels in the gel chambers placed under the fume hood.
- Fill gel chambers with 1x MOPS buffer until gels are slightly, but markedly covered with buffer (~4 mm)
- Wait for 5 min to allow soaking of the gels with buffer, and then remove the combs carefully
- Incubate the prepared RNA samples and **RNA True Marker** for 10 min in the heating block at 70 °C to denature secondary structures of the RNAs. After denaturing, transfer loading samples immediately on ice for ~3 min, mix them on a vortexer and spin them down again (short spin).
- Load samples carefully
- Run gels for 120 min (1.5 % gels) at 120 Volts (~12 cm; of course you can adjust the running time to the length of your target RNA and percentage of your gel

Note: Apply 4-5 V per cm between the electrodes, circulate the MOPS buffer and cool the chamber for best results!). Stop the run when the yellow band of the sample buffer has arrived at the lower border of the gel

- Use gels for vacuum blotting

Part V - Vacuum blotting of RNA gels

Denaturation solution	**1L**
50 mM NaOH	2g
10 mM NaCl	0.5844 g
(You can prepare 10x stocks)	

Neutralization solution	**1L**
100 mM Tris/HCl, pH 7.4	12.114 g
(You can prepare 10x stocks)	

20x SSC (saline sodium citrate)
88,23 g NaCitrat-Dihydrat
175,32 g NaCl
ad 850 mL sterile A. dest., adjust to pH 7.0
ad 1 L sterile A. dest., autoclave

Preparations:
Rinse all components of the blotting apparatus with deionized water
Cut membranes to the size about 1 cm bigger than your gel in each axis
The masks should be about 3-4 mm smaller than your gel in each axis

Blotting procedure:
Depending on the size of the gels you may blot one up to 4 gels at the same time

- Soak membranes in **water** for **1 min**
- Assemble the blotting apparatus
- Carefully slide gels from the gel tray and place them accurately on the masks and the lower surface of the membranes (membrane → mask → gel)
- To apply vacuum, switch on the pump and adjust pressure to 80 mbar. Check for constant pressure by compressing the tubes
- Then immediately cover gel surface with **Denaturation Solution** and incubate for **5 min.** *(This causes partial alkaline hydrolysis of the RNA. Large-sized RNA molecules are fragmented to smaller molecules, which results in higher transfer efficiency.)*
- After 5 min, remove Denaturation Solution from the gel surface using a 25 ml pipette
- Now cover gel surfaces with **Neutralization Solution** and incubate for **5 min**
- After 5 min, remove Neutralization Solution from the gel surface using a 25 ml pipette

- Now transfer the RNA by continuous covering of the gel surface with **20xSSC buffer** and incubate for **1 to 3 h depending on the percentage and thickness of the gel**
- **(1 h for a 3 mm and 1.5 % gel - 3 h for a 10 mm and 1.5 % gel)**
- After finishing place membranes on filter paper (crease-free)
- Perform **UV crosslinking** via Stratagene UV Stratalinker 1800, setting **AUTO CROSS LINK, Start – this means 120 mJ/cm²**)
- Store membranes at 4 °C in a sealed plastic bag in a refrigerator or best directly proceed with hybridization

Part VI – Hybridization

Required:

✓ (Pre-)Hybridization solution
✓ RNA probe
✓ **ProTec Diagnostics RNA-TRUE Dye**
✓ Hybridization oven
✓ Hybridization tube

- Place your blot membrane in a hybridization tube; RNA faces inwards
- Add 20 ml of pre-warmed (68 °C) Pre-Hybridization solution
- Incubate under shaking for 1 h at 68 °C in a Hybridization oven
- Prepare the RNA-probe during the Pre-hybridization period
- Fill 15 ml of the Pre-hybridization solution into a 15 ml Falcon tube and add 1 µg of your labelled RNA-probe and mix well
- Add 1 µl of **ProTec Diagnostics RNA-TRUE Dye (**Dye500 /600/700 or 800, respectively)
- Incubate it for 15 min at 65 °C in a water bath and then immediately transfer the probe on ice
- After the Pre-Hybridization period discard the entire solution from the blot and pour the probe into the hybridization tube
- Incubate over night at 68 °C under rolling

Prepare Wash Solution for the next day

Part VII - Fluorescence Detection with the LI-COR Odyssey CLx scanner

Required:

✓ Wash solution and wash buffer
✓ LI-COR Blocking Buffer (PBS) (P/N 927-70001)
✓ PBS + 0,1% Tween20
✓ SDS 10% (sterile filtered)
✓ Hybridized membranes
✓ IR CW800 coupled to streptavidin (P/N 926-32230)
✓ LI-COR Odyssey CLx Scanner (or similar detection system)

Solutions:

(Pre-)Hybridization solution (1 L)

5 x SSC [20x stock → 1:4]	250 ml	
0,1 % Na-N-Lauroylsarcosin [10% stock → 1:100]	10 ml	
0.02 % SDS [10% stock → 1:500]	2 ml	
2 % Blocking Reagent [10% stock → 1:5]	200 ml	
50 % Formamid		500 ml
ad 1 L sterile A. dest		39 ml

Prepare 50 ml aliquots in Falcon tubes and store at -20 °C.

10x Buffer 1 (1 L)

100 mM Maleic acid (***not*** Malic acid!)		116.07 g
150 mM Sodium chloride	87.66 g	
Dissolve in A. dest		
Add Sodium hydroxide to adjust pH to 7.5	~ 72 g	
Add A. dest ad 1L		

10% Blocking Reagent (200 mL)
Dissolve 20 g Blocking Reagent (Roche) in 200 ml Buffer 1
(20ml 10x Buffer 1 ad 200ml sterile A. dest. Mix well, carefully
boil up in microwave ~6x30sec., medium high) and immediately autoclave

20x SSC (saline sodium citrate)
88,23 g NaCitrat-Dihydrat
175,32 g NaCl
ad 850 mL sterile A. dest., adjust to pH 7.0
ad 1 L sterile A. dest., autoclave

Wash solution (1 L) 68°C
Prefill flask with some sterile A. dest.
10 ml 20x SSC (0,2x)
10 ml 10% SDS (0,1%)
ad 1 L sterile A. dest., mix well, and preheat at 68 °C

Wash Procedure
- Recover your probe from the membrane
- Wash with **Wash solution** at **68 °C** for **15 min (50 ml), (Hybridization oven, max. speed)**
- Discard Wash solution and repeat wash step twice

Detection Procedure
- incubate everything in black hybridization boxes
- Add **20 ml LiCor Odyssey Blocking Buffer (PBS) + 1% SDS** & incubate **30 min** at **RT (Rocking platform ~50 rpm)**
- Discard blocking buffer
- Add **15 ml LiCor Odyssey Blocking Buffer (PBS) + 0,5 % SDS** & add **0.6 µl of CW 800 Streptavidin (1:25000)** and incubate **30 min** at **RT (Rocking platform ~30 rpm)**
- Discard blocking buffer
- Wash **3x 20min** with **50 ml PBS+0,1% Tween20** at **RT (Rocking platform ~50 rpm)**
- place the membrane between two sheets of fresh and clean Whatman paper, gently strike over the stack to remove all residual buffer from the blot. Then let the membrane completely dry at 68°C for ~15 to 20 min

Note: Completely dry membranes will roll up, increasing the signal to noise ratio drastically! Fluorescence signals are quantitative and linear on a 6.5 log range

- documentation via Li-Cor Odyssey CLx system

Methylene blue staining

Methylene blue solution (100ml)
100 mg Methylene blue
2 ml Acetic acid (99%)
13.5 ml Na-Acetat (3M, 5.2%)
ad 100 ml A. dest., mix well
store at RT

- incubate the membrane approximately 1 min with methylene blue
- destain the membrane with A. dest. till the bands become visible
- transfer the used methylene blue back into the original bottle for reusing