Northen blotting with DIG Northern Starter Kit (Roche®)

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Abstract

This script is used for northern blotting with the Roche DIG Northern Starter Kit.

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Protocol

Prepartion of needed buffers

Step 1.

It is recommended to prepare all needed buffers before start northern blotting.

List of Buffers:

Solution	Preparation	Storage	Use
DIG Easy Hyb Granules	Reconstitue granules adding 64 mL DMPC treated water, dissolve by stirring at 37° C	at +15° C to 25° C for 1 month	As prehybridization and hybridization solution
Washing buffer	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5 (20° C); 0.3% (v/v) Tween 20	at +15° C to 25° C stable	Removal of unspecific bound antibody
Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl; adjust with NaOH (solid) to pH 7.5 (20° C)	at +15° C to 25° C stable	Dilution of Blocking solution
Detection buffer	0.1 Tris-HCl, 0.1 M NaCl, adjustment of pH to 9.5 (20° C)	at +15° C to 25° C stable	Adjustment of pH to 9.5
Blocking solution	Prepare a 10x stock solution	at 8° C stable	Blocking of unspecific binding sites on the membrane

Preperation of hybridization probe

Step 2.

Producing **5S RNA** hybridization probe:

Primer:

TCTTGGTGTCTTTAGCGTCATGGAAC (#460_5S_6803_fw)

TAATACGACTCACTATAGGGACTTGGCATCGGACATTGTGC (#461_T7_5S_6803_rev)

PCR reaction mix:	
5x Q5 Reaction buffer	10 µL
10 mM dNTPs	1 µL
10 µM forward Primer	2.5 µL
10 µM revers Primer	2.5 µL
template DNA	1 µL
Q5 polymerase	0.5 µL
H_2O	ad 50 µL

PCR setup:			Anneal at 71°C
	1	98°C	30 sec
Loop 30x			
	2	98°C	5 sec
	3	71°C	15 sec
	4	72°C	15 sec
end of Loop			
	5	72°C	2 min
	6	4°C	10 sec

NOTES

Lutz Berwanger 20 Dec 2016

5S RNA hybridization probe is an example. PCR recepies vary of course.

Preparation of denaturating 8% PAA gels

Step 3.

Clean glasplates, spacer and combs with 70% EtOH and RNase Away. Assemble gelchamber in casting frame.

Gel mixture for 10 mL:

8% PAA gel:	
40% PAA (19:1) Rotipage (Roth)	2 mL
Urea	5 g
10x TBE	1 mL
H_2O	ad 10 mL
10x APS	100 µL
TEMED	10 µL

Pour gel and let polymerize at least 2 h at roomtemperature.

DURATION
02:00:00

In-vitro-transcription of hybridization probe

Step 4.

In-vitro-transcription mixture:	
PCR product (0.1-1 µg)	14 µL
T7-Flash 10x reaction buffer	2 µL
DIG RNA labeling mix	2 µL
100 nM DTT	2 µL
RiboGuard RNase Inhibitor	0.5 µL
T7-Flash Enzyme Solution	2 µL

Icubate at 37°C at least 2 h.

DURATION
02:00:00

NOTES
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Start in-vitro-transcription when assembling gel and stop it, when start hybridization step.

RNA preparation and gel loading

Step 5.

Prepare RNA-probes:

- Use 3 µg of previously extracted RNA (see mentioned protocol) and aquibrilate probes on same level with DEPC-treated H_2O and add 2x RNA loadingbuffer (Thermofisher).
- Prepare mixture of 4 µL high range ladder and 2x RNA loadingbuffer (Thermofisher).
- Denature probes and ladder for **5 min at 95°C** and rapidly cool down on ice.
- Load gel and let it run at **25 mA in 1% TBE buffer.**

PROTOCOL

. RNA extraction with PGTX

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NOTES

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It is very important to wash pockets of the gel before loading the gel!

Step 5.1.

Centrifuge for 3 minutes at 4˚C and 4.000 g.

DURATION

00:03:00

Step 5.2.

Resuspend the cell pellet in 1 ml of PGTX. Freeze in liquid nitrogen and store at -20 ˚C.

SAFETY INFORMATION

Wear goggles, a lab coat and gloves when dealing with PGTX and liquid nitrogen.

Step 5.3.

Incubate for 5 min at 95 °C, shaking at 250 rpm in Thermomixer (Eppendorf)

DURATION

00:05:00

Step 5.4.

Rapidly chill 5 min on ice.

DURATION

00:05:00

Step 5.5.

Add 700 µl Chloroform.

REAGENTS

Chloroform by Contributed by users

SAFETY INFORMATION

Wear goggles, a lab coat and gloves when dealing with phenol and chloroform.

Step 5.6.

Let the samples incubate for 10 min at room temperature in a Thermomixer. Vortex from time to time.

DURATION

00:10:00

Step 5.7.

Centrifuge for 15 min at 14.000 g, 4 ˚C. Transfer the upper aqueous phase (**contains RNA**) to a fresh reaction tube and add the same volume (450 µL) of Aqua- P/C/I (Phenol/Chloroform/Isoamylalcohol).

DURATION

00:15:00

SAFETY INFORMATION

Wear goggles, a lab coat and gloves when dealing with phenol and chloroform.

Step 5.8.

Thoroughly mix by vortexing. Centrifuge for 15 min at high speed. Transfer the upper aqueous phase to a 1.5 mL reaction tube.

DURATION

00:15:00

SAFETY INFORMATION

Wear goggles, a lab coat and gloves when dealing with phenol and chloroform.

Step 5.9.

Add 1 volume of isopropanol.

REAGENTS

Isopropanol by Contributed by users

Step 5.10.

Mix and incubate for at least 30 min at -20 ˚C. (Can be left overnight.)

DURATION

00:30:00

Step 5.11.

Centrifuge at 14.000 g for 30 min.

DURATION

00:30:00

Step 5.12.

Wash with 75% chilled ethanol. Avoid resuspending the pellet.

REAGENTS
Ethyl alcohol, Pure 200 proof, for molecular biology E7023 by Sigma Aldrich

Step 5.13.

Discard supernatant.

Step 5.14.

Centrifuge at 14.000 g.

DURATION
00:05:00

Step 5.15.

Repeat washing step with 75% chilled ethanol.

NOTES
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Remove excess of EtOH by using a pipet.

Step 5.16.

Air dry pellet at RT. Do not overdry!

DURATION
00:10:00

Step 5.17.

Resuspend the pellet with 30 µL volume of ddH_2O.

NOTES
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Usually 40 µL DECP-treated H_2O

Step 5.18.

Fill 50 mL Falcon tube with ice. Fill with bacterial liquid culture, up to a volume of 45 mL.

Step 5.19.

Discard supernatant. Resuspend cell pellet in residual water (1 mL). Transfer to 2 mL 'safe lock' tube.

Step 5.20.

DURATION
00:00:15

Semi-Dry blotting with Trans-Blot SD

Step 6.

Preparation of Semidry blotting sandwich:

- Prepare the transfer buffer (1x TBE Buffer)
- Cut the membrane to the dimensions of the gel. Wet the membrane in transfer buffer.
- Cut filter paper (8 peaces of thick filter paper) to the dimensions of the gel. Wet filter paper in transfer buffer.
- Place 4 of the pre-soaked sheets of thick filter paper onto the platinum anode. Roll a pipet or test tube over the surface of the filter paper to exclude all air bubbles.
- Place the pre-wetted blotting media on top of the filter paper.
- Carefully place the gel on top of the transfer membrane, aligning the gel on the center of the membrane.
- Place the other pre-soaked sheets of filter paper onto the gel. Exclude air bubbles as before.
- Carefully place the cathode onto the stack.

Blot with 2 mA/ cm^2 membrane for 45 min.

DURATION
00:45:00

UV-crosslinking

Step 7.

UV-crosslink the wet membrane without prior washing. After crosslinking, membrane can be stored dry at +8°C to +25°C. If you want to go ahead use the membrane immediatley for prehybridization.

Prehybridization

Step 8.

- Prewarm DIG Easy Hyb solution to hybridization temperature (62° C).
- Prehybridize membrane with RNA side facing inwards with DIG Easy Hyb for 30 min with gentle agigation in hybridization flask.

DURATION
00:30:00

NOTES
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Hybridization temperature differs from one probe to each other.

Hybridization

Step 9.

- Denature DIG-labeled RNA probe from **Step 4** by boiling for **5 min** and rapidly cooling in ice/water.
- Add denatured DIG-labeled RNA probe to hybridization flask and incubate for **6 h** or **over night** with gentle aggigation.

Hybridization is follwed by stringency washes:

- Wash **2 x 5 min** in **2x SSC,0.1% SDS** at room temperature under constant aggigation.
- Wash **2 x 15 min** in **0.1x SSC,0.1% SDS** at **62° C** under constant aggigation.

Immunnological detect

Step 10.

Stripping and reprobing of RNA blots

Step 11.