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Hybridization and detection of small RNA samples with DIG-labeled probes

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1 Works for me dx.doi.org/10.17504/protocols.io.us6ewhe

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ABSTRACT

This protocol can be used to hybridize small RNAs (50 - 200 nt) - either from total RNA isolates or from in vitro transcripts - with digoxigenein-labeled probes, followed by detection with chemiluminescence using CDP-star. The protocol follows mainly the instructions given in the DIG Northern Starter Kit, Version 8.0 (Roche).

It is adjusted to Northern Blots with membrane size of around 9 x 10 cm^2 .

ATTACHMENTS

DIG Northern Starter Kit.pdf

GUIDELINES

Pay attention to the standard precautions needed for working with RNA (e.g. always ware cloves; clean pipettes, surfaces, trays with decontaminant (e.g. RNase away), use RNase free solutions)

MATERIALS

NAME	CATALOG #	VENDOR
DIG Easy Hyb™ Granules	11796895001	Roche
Blocking Reagent	11096176001	Roche
Anti-Digoxigenin-AP Fab fragments	11093274910	Roche
ROTI®Stock 20x SSC	1054.1	Carl Roth
ROTI®Stock 20 % SDS	1057.1	Carl Roth
Nuclease free water DEPC treated	T143.3	Carl Roth
CDP-Star® ready-to-use	12041677001	Roche
square petri dishes 120 x 120 x 17 mm PS	688161	greiner bio-one

MATERIALS TEXT

Hybridization:

DIG Easy Hyb

- reconstitute DIG Easy Hyb Granules with 64 ml sterile, nuclease free water (add 32 ml of water to the bottle and incubate at 37°C with shaking until powder is solved, then add another 32 ml of water), takes time!

10 x TBE buffer

- 108 g/l Tris
- 55 g/l Boric acid

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- 100 ml/l 0.5 M EDTA, pH 8.0

- Hybridization oven
- Hybridization tubes 150 mm length
- Thermoblock

Stringency washes:

2 x SSC + 0.1 % SDS
prepare freshly from 20xSSC and 20% SDS stocksolution
(5 ml 20xSSC and 250 µl 20% SDS in 50 ml Water)

0.1 x SSC, 0.1 % SDS:
5 ml 20 x SSC and 5 ml 20 % SDS in 1 L water
(stored at RT; solution might get slightly turbid due to SDS precipitation, but gets clear when heated)

- hybridization oven
- table shaker
- square petridishes (120 x120 x 17 mm)

Immunological detection

Maleic acid buffer *(Dilution of blocking solution)*
- 0.1 M maleic acid
- 0.15 M NaCl

adjust with solid NaOH to pH 7.5 (20°C)

prepare 1 L of 2 x maleic acid buffer (23.2 g maleic acid, 17.54 g NaCl), add NaOH plates to pH6.0 - 6.5 , afterwards proceed carefully with 5 M and 1 M NaOH, because pH starts to rise rapidly when getting near 7.5!
Solid NaOH takes a while to get into solution, thus buffer preparation takes time!
Autoclave

Washing buffer *(removal of unspecific bound atibody)*
Maleic acid buffer + 0.3 % Tween 20

prepare freshly, e.g. 100 ml 2 x maleic acid buffer + 100 ml H_2O + 0.6 ml 20% Tween

Detection buffer *(pH adjustment)*
- 0.1 M Tris-HCl
- 0.1 M NaCl

adjust to pH 9.5 (20 °C)

prepare a 10 x stock solution (58.44g NaCl, 121.14 g Trizma-base, pH9.5) and autoclave

10 x Blocking solution *(blocking of unspecific binding sites on the membrane)*
- prepare a 10% (w/v) solution of blocking reagent powder in maleic acid buffer, e.g add 10 g to 100 ml maleic acid buffer in a 250 ml Schott flask
- dissolve the powder by succesive cycles of heating in a microwave
- 10 x blocking solution is stored at 4 - 8°C

CDP-Star, ready-to use solution
- store bottles at 4-8 °C

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BEFORE STARTING

- make sure that you have all buffers and solutions prepared; reconstitution of DIG Easy Hyb granules and preparation of maleic acid buffer and 10 x blocking solution is time consuming!
- make sure that you have your DIG-labled probes ready: to receive sufficient amount of probe (~ 1 µg), in vitro transcription of small RNA fragments takes 4-5 h!

Hybridization

1
- prewarm an appropriate volume of DIG Easy Hyb to 62 °C (5 ml per hybridization tube)
- wet the Northern Blot membrane (9 x 10 cm^2 size) in 0.5 x TBE buffer
- pre-hybridize the membrane with prewarmed DIG Easy Hyb solution in a hybridization tube (role the membrane with the RNA facing inside) for 30 min at 62 °C in an hybridization oven with constant rotation; don't forget to use a balance tube when you have just one tube
- membrane should move freely (use just 1 membrane per tube to avoid stacking of membranes)
- denature the DIG-labeled probe (in vitro transcript of trRNA sequence) for 5 min at 95°C in a thermoblock, cool down on ice and mix with 5 ml prewarmed DIG-Easy Hyb
- remove pre-hybridization solution and add probe/hybridization mixture to the membrane
- hybridize o/n at 62 °C

Overnight

stringency washes

2 All washing steps are done with 25 ml volume
- place membrane (RNA facing upwards) in a cleaned square petridish (120 x 120 x 17 mm)
- wash 2 x 5 min in 2 x SSC + 0.1% SDS at RT under constant agitation on a table shaker (90 rpm)
- meanwhile prewarm an appropriate volume of 0.1 x SSC + 0.1 SDS at 68 °C in the hybridization oven
- clean the hybridization tube and re-use it to wash the membrane for 2 x 15 min at 68 °C with constant rotation

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Immunological detection of trRNA

3 All incubations are performed at RT in a square petridish (120 x 120 x 17 ml), with 25 ml solution each, on a table shaker (90 rpm)

- after stringency washes, rinse the membrane shortly (1-5 min) in ***Washing buffer***
- prepare a ***5 x blocking solution*** by diluting 10 x Blocking solution 1: 2 in maleic acid buffer (50 ml falcon tube, always prepare fresh)
- incubate for at least 30 min in 5 x blocking solution in a fresh, cleaned petridish
- centrifuge the anti-digoxigenin-AP antibody for 5 min at 10 000 rpm and 4 °C prior to use
- pipet the necessary amount carefully from the surface
- dilute antibody 1 : 10000 (75 mU/ml) in fresh 5 x blocking solution (5 µl in 50 ml 5 x blocking solution)
- incubate the membrane for 30 min in ***Antibody solution*** to bind the antibody to the DIG-labeled probe
- wash 2 x 15 min in ***Washing buffer*** in a fresh petridish
- eqilibrate the menbrane 2 - 5 min in ***Detection buffer***
- place the membrane (with RNA side facing up) on a development folder or hybridization bag; we put the membrane between the sheets of a transparent, clear disposable waste bag (Sekuroka, 300 x 200 mm, 40 µm, PP)
- lift one sheet up and quickly apply approx. 1 ml ***CDP-Star, ready-to-use*** solution out of the dropper bottle until the membrane is evenly soaked
- Immediately cover the membrane with the second sheet to spread the substrate evenly and without air bubbles over the membrane
- incubate for 5 min at RT; for trRNA detection, a prolonged (1 h) incubation time led to stronger signals
- squeeze out excess liquid, but keep the membrane damp; drying of the membrane will result in dark background!
- signals were detected by using the Bio-Rad Gel Doc imaging system, program chemiluminescence- high sensitivity, image area same as for the corresponding PAA-gel (13.4 x 10.0 cm) and exposure times 20 - 200 sec (trRNA)
- rinse the membrane briefly in 0.5 x TBE, dry completely and store between the sheets of Whatman paper for hybridization with 5S-RNA probe (no stripping of membrane required)

Immunological detection of 5S-RNA

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- membranes hybridized with trRNA probes could be reprobed with the 5S-RNA specific probe without stripping of the membrane; the 5S-RNA signal is extremely strong and therefore detectable at exposure times too short for the detection of the trRNA signal
- take the dried membrane and start with the hybridization protocol (Step 1)
- hybridize the membrane with the in vitro transcript of the 5S-RNA sequence at 62 °C

Overnight

- proceed with the protocol steps 2-3
- no incubation necessary after application of CDP-Star ready-to-use solution, immediately expose the membrane to the Gel Doc imager, program chemiluminescence- high sensitivity, image area 13.4 x 10.0 cm
- signal is detected after 10 - 20 sec exposure time

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