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

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Protocol status: Working

We use this protocol and it's working

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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 digoxigenin-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 $9 \times 10 \text{ cm}^2$.

Attachments



DIG Northern Starter...

676KB

Guidelines

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



Materials

MATERIALS

⊗ DIG Easy Hyb™ Granules **Roche Catalog #11796895001**

⊗ Blocking Reagent **Roche Catalog #11096176001**

⊗ Anti-Digoxigenin-AP Fab fragments **Roche Catalog #11093274910**

⊗ ROTI®Stock 20x SSC **Carl Roth Catalog #1054.1**

⊗ ROTI®Stock 20 % SDS **Carl Roth Catalog #1057.1**

⊗ Nuclease free water DEPC treated **Carl Roth Catalog #T143.3**

⊗ CDP-Star® ready-to-use **Roche Catalog #12041677001**

⊗ square petri dishes 120 × 120 × 17 mm PS **greiner bio-one Catalog #688161**

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
- 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 stock solution
(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 × 120 × 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 antibody*)

Maleic acid buffer + 0.3 % Tween 20

prepare freshly, e.g. 100 ml 2 x maleic acid buffer + 100 ml H₂O + 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 successive 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



Before start

- 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-labeled 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 × 10 cm² 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 × 120 × 17 mm)
 - wash 2 × 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 × 15 min at 68 °C with constant rotation

Immunological detection of trRNA

- 3 All incubations are performed at RT in a square petridish (120 × 120 × 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 × 15 min in **Washing buffer** in a fresh petridish
- equilibrate the membrane 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 × 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 × 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

- 4
- 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 × 10.0 cm
- signal is detected after 10 - 20 sec exposure time