For life science research only.
Not for use in diagnostic procedures.

DIG Northern Starter Kit

Version: 12
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For transcription labeling of RNA with digoxigenin and SP6/T7/T3 RNA Polymerases and chemiluminescent detection with CDP-*Star*, ready-to-use.

Cat. No. 12 039 672 910 1 kit
10 labeling reactions and detection of 10 blots of 10 x 10 cm^2

Store the kit at −15 to −25°C.

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1. General Information

1.1. Contents

Vial / Bottle	Label	Function / Description	Content
1a	DIG Northern Starter Kit, Labeling mix, 5x conc.	▪ Labeling mixture containing optimal concentrations of unlabeled nucleotides and DIG-11-UTP. ▪ Clear viscous solution. ▪ For efficient *in vitro* transcription of linearized template DNA.	1 vial, 40 µl
1b	DIG Northern Starter Kit, Transcription Buffer, 5x conc.	▪ Clear solution. ▪ For efficient *in vitro* transcription of linearized template DNA.	1 vial, 40 µl
2	DIG Northern Starter Kit, SP6 RNA Polymerase	▪ 20 U/µl solution. ▪ Synthesizes RNA from a DNA template.	1 vial, 20 µl
3	DIG Northern Starter Kit, T7 RNA Polymerase	▪ 20 U/µl solution. ▪ Synthesizes RNA from a DNA template.	1 vial, 20 µl
4	DIG Northern Starter Kit, T3 RNA Polymerase	▪ 20 U/µl solution. ▪ Synthesizes RNA from a DNA template.	1 vial, 20 µl
5	DIG Northern Starter Kit, Anti-Digoxigenin-AP, Fab fragments	▪ 750 U/ml clear solution. ▪ Polyclonal sheep anti-digoxigenin, Fab fragments, conjugated to alkaline phosphatase.	1 vial, 60 µl
6	DIG Northern Starter Kit, DNase I, RNase-free	▪ 10 U/µl solution. ▪ Degrades DNA template after transcription reaction.	1 vial, 20 µl
7	DIG Northern Starter Kit, CDP-*Star*, ready-to-use	Chemiluminescent substrate for alkaline phosphatase.	1 bottle, 20 ml
8	DIG Northern Starter Kit, Actin RNA Probe, DIG-labeled	▪ 10 ng/µl solution. ▪ Antisense probe, length 588 bases. ▪ Standard for the quantification of DIG-labeled RNA and as a hybridization control.	1 vial
9	DIG Northern Starter Kit, DIG Easy Hyb Granules	For the hybridization of the DIG-labeled RNA probe.	2 bottles, 100 ml each
10	DIG Northern Starter Kit, Blocking Solution, 10x conc.	▪ Viscous solution. ▪ For chemiluminescent detection.	2 bottles, 100 ml each

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to −25°C, the kit is stable through the expiry date printed on the label.

Vial / Bottle	Label	Storage
1a	Labeling mix, 5x conc.	Store at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.*** ⚠ ***To avoid contamination, aliquot the solutions into 2 to 3 vials.***
1b	Transcription Buffer, 5x conc.	
2	SP6 RNA Polymerase	Store at −15 to −25°C.
3	T7 RNA Polymerase	
4	T3 RNA Polymerase	
5	Anti-Digoxigenin-AP, Fab fragments	Store at +2 to +8°C after opening. ⚠ ***Do not freeze.***
6	DNase I, RNase-free	Store at −15 to −25°C.
7	CDP-*Star*, ready-to-use	Store at +2 to +8°C after opening. ⚠ ***Keep protected from light.***
8	Actin RNA Probe, DIG-labeled	Store at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.*** ⚠ ***Store in aliquots at −15 to −25°C.***
9	DIG Easy Hyb Granules	Store at +15 to +25°C after opening.
10	Blocking Solution, 10x conc.	Store at +2 to +8°C after opening. ⚠ ***For long-term storage, store in aliquots at −15 to −25°C.***

1.3. Additional Equipment and Reagent required

For DNA template preparation using standard method or RT-PCR and PCR

- Thermal cycler
- High Pure Plasmid Isolation Kit*
- Phenol/chloroform
- Expand High Fidelity PCR System*, including buffer
- Primer (sense, antisense)
- Nucleotides (dATP, dCTP, dGTP, dTTP, each 10 mM or Deoxynucleoside Triphosphate Set*)

For DIG RNA labeling

- Water bath (+42°C and +37°C) or heating block
- Ice/water
- Double-distilled water
- EDTA, 0.2 M, pH 8.0

For determination of labeling efficiency

ⓘ *See section,* **Working Solution** *for additional information on preparing solutions.*

- Nylon Membranes, positively charged*
- UV transilluminator, or UV crosslinker, or oven (+120°C or +80°C)
- RNA dilution buffer
- DIG Wash and Block Buffer Set* or
- Washing buffer
- Maleic acid buffer
- Detection buffer
- Lumi-Film*

Suitable gel system for northern blots and formaldehyde gels

See section, **Working Solution** *for additional information on preparing solutions.*

- Gel equipment
- Heatable water bath (+65°C) or heating block (+65°C)
- Ice
- 10x MOPS, pH 7.0 (with NaOH)
- Loading buffer
- Gel solution
- Running buffer

For RNA blotting and fixation

See section, **Working Solution** *for additional information on preparing solutions.*

- Nylon Membranes, positively charged*
- Whatmann 3MM paper
- UV transilluminator, or UV crosslinker, or oven (+120°C or +80°C)
- 20x SSC* or 2x SSC (depending on method)

For hybridization

- Nylon Membranes, positively charged*
- Ice/water
- Water bath
- Shaking water bath or hybridization oven
- Hybridization Bags or temperature-resistant, sealable plastic or glass boxes, petri dishes or roller bottles

  ⚠ ***Do not use open containers with DIG Easy Hyb.***

For stringency washes

- 2x SSC*
- 0.1% SDS*
- 0.1x SSC*

For immunological detection

See section, **Working Solution** *for additional information on preparing solutions.*

- Hybridization Bags or development folders
- DIG Wash and Block Buffer Set* or
- Washing buffer
- Maleic acid buffer
- Detection buffer

For stripping and reprobing of RNA blots

See section, **Working Solution** *for additional information on preparing solutions.*

⚠ ***Always use RNase-free solutions.***

- Water bath
- Hybridization Bags
- Double-distilled water
- Stripping buffer
- 2x SSC*

1.4. Application

The DIG Northern Starter Kit is used for northern blots on nylon membranes.

1.5. Preparation Time

Assay Time

Step	Reaction Time
RNA labeling	1 hour 20 minutes
Hybridization	6 hours or overnight
Immunological detection	1 hour 40 minutes
Chemiluminescent signal detection	5 to 30 minutes

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Templates for labeling reaction

- Linearized plasmid, including the appropriate RNA polymerase promoter sequence (SP6, T3, T7).
  The length of the region to be transcribed should be in the range of 200 to 1,000 bp. Phenolize DNA to avoid RNase contamination.
- Specially prepared PCR product, see section, **Protocols, Preparing DNA template from total RNA using RT-PCR and PCR.**

General Considerations

Precautions

- Work under clean and RNase-free conditions.
- Use DMPC- or DEPC-treated water for preparation of all solutions.
- Autoclave solutions.
- Tween 20* should be added to previously autoclaved or sterile-filtered solutions.
- Rigorously clean and rinse incubation trays with a cleaning agent for removing RNases or bake glass trays 8 hours at +200°C before use.
- Wear powder-free gloves when handling membranes.
- Handle membrane only on the edges with clean forceps.

Standard method for DNA template preparation

① Purify plasmid DNA using the High Pure Plasmid Isolation Kit*.

② Linearize DNA template at a restriction site downstream of the cloned insert. The sequence to be transcribed should be 200 to 1,000 bp in length.

③ To avoid transcription of undesirable sequences, use a restriction enzyme that leaves a 5′ overhang or blunt ends.

④ After restriction digest, purify the DNA by phenol/chloroform extraction, followed by ethanol precipitation.

⑤ Dissolve template DNA in 10 mM Tris, 0.1 mM EDTA, pH 8,0.

⚠ ***Too much EDTA inhibits the transcription reaction; do not use >0.1 mM EDTA in the TE buffer to dissolve the DNA.***

DNA template preparation from total RNA using RT-PCR and PCR

The following steps describe an alternative method for generating templates for *in vitro* transcription labeling of RNA with DIG without cloning.

① Prepare total RNA with one of the standard methods.

② Run an RT-PCR with oligo(dT) primer using the Expand Reverse Transcriptase System.

③ Run the PCR with specially designed primers, including the sequence of the appropriate RNA polymerase promoter.
– Use standard PCR conditions; for best results, use the Expand High Fidelity PCR System*.

RNA polymerase promoter sequences

Promoter	Sequence
SP6 RNA Polymerase	Do not use SP6 promoter consensus sequences for PCR-generated DNA fragments; experiments show that SP6 Polymerase can only initiate efficient transcription if the promoter sequences lie within a plasmid environment.
T7 RNA Polymerase	5′TAATACGACTCACTATAGGG/X-mer or 5′TAATACGACTCACTATAGGA/X-mer
T3 RNA Polymerase	5′AATTAACCCTCACTAAAGGG/X-mer

Factors influencing stringency in hybridization

- The degree of homology of the probe to the target RNA is an important factor for determining the appropriate hybridization conditions.
- Stringency is influenced by temperature; high temperature increases stringency of hybridization, low temperature decreases stringency.
- For RNA:RNA hybridizations with DIG Easy Hyb, use a hybridization temperature of +68°C.
  The temperature may have to be adjusted depending on the GC content and homology of probe to target.

Precautions for immunological detection

- Work under clean, RNase-free conditions for the entire detection procedure.
- If the membrane is to be reprobed, do not allow the membrane to dry at any time.
  Handle membranes only with powder-free gloves.
- Use sufficient volume of all solutions.
- Make sure that membranes do not stick to trays during detection.
  Do not let membranes stick together.
- Shake membranes during the whole procedure.
- When using laboratory trays for the detection procedure, they should be rigorously cleaned before use.
- Work under clean conditions when handling the chemiluminescent substrate solution and avoid phosphatase contamination.
- Perform Anti-DIG-AP binding and chemiluminescent development in separate trays.
- For chemiluminescent detection, seal membranes in plastic bags or development folders.

Safety Information

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Working Solution

The Washing buffer, Maleic acid buffer, Blocking solution, and Detection buffer are also available tested on the absence of DNases and RNases in the DIG Wash and Block Buffer Set*. These solutions are also used in the detection procedure and can be prepared in larger quantities.

DIG RNA labeling

Solution	Composition/Preparation	Storage and Stability	For use in...
Water	Autoclaved, DMPC- or DEPC-treated double-distilled water	Store at +15 to +25°C.	Dilution of solutions.
EDTA	0.2 M ethylenediamine-tetraacetic acid, pH 8.0	Store at +15 to +25°C.	Stops the reaction.

Determination of labeling efficiency

Solution	Composition/Preparation	Storage and Stability	For use in...
RNA dilution buffer	Mix DMPC- or DEPC-treated, double-distilled water: 20x SSC: 37% formaldehyde in a ratio of 5:3:2.	⚠ ***Always prepare fresh.***	Dilution of RNA.
Washing buffer	0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (+20°C), 0.3% (v/v) Tween 20*	Store at +15 to +25°C.	Removal of nonspecific bound antibody.
Maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl, adjust with solid NaOH to pH 7.5 (+20°C)		Dilution of Blocking Solution.
Detection buffer	0.1 M Tris-HCl*, 0.1 M NaCl, pH 9.5 (+20°C)		Adjustment of pH to 9.5.
Preparation of kit working solutions			
Blocking solution,1x	Dilute the 10x Blocking Solution (Bottle 10) 1:10 in Maleic acid buffer.	⚠ ***Always prepare fresh.***	Blocking of nonspecific binding sites on the membrane.
Antibody solution	▪ Centrifuge Anti-Digoxigenin-AP (Vial 5) for 5 minutes at 10,000 rpm in the original vial prior to each use, and pipette the necessary amount carefully from the surface. ▪ Dilute Anti-Digoxigenin-AP 1:10,000 (75 mU/ml) in 1x Blocking Solution.	Store at +2 to +8°C for 12 hours.	Binding to the DIG-labeled probe.

Formaldehyde gel

Solution	Composition/Preparation	Storage and Stability	For use in...
10x MOPS, pH 7.0 (with NaOH)	▪ 200 mM MOPS ▪ 50 mM sodium acetate ▪ 20 mM EDTA	–	Preparation of stock solution.
Loading buffer	▪ 250 µl of 100% Formamide* ▪ 83 µl of 37% formaldehyde ▪ 50 µl 10x MOPS ▪ 50 µl 100% glycerol ▪ 10 µl 2.5% bromophenol blue ▪ 57 µl DEPC/DMPC-treated water	⚠ ***Always prepare fresh.***	Loading samples in the gel.
Gel with 2% formaldehyde	▪ 1.8 g Agarose* ▪ 141.9 ml 1x MOPS ▪ 8.1 ml 37% formaldehyde	–	Separation of samples.
Running buffer	1x MOPS	–	Gel electrophoresis.

Hybridization

Solution	Composition/Preparation	Storage and Stability	For use in...
DIG Easy Hyb Granules working buffer	Reconstitute granules (Bottle 9) by carefully adding 64 ml autoclaved, double-distilled, DEPC- or DMPC-treated water in two portions to the plastic bottle; dissolve by stirring at +37°C. ⚠ ***DIG Easy Hyb Granules must be dissolved under RNase-free conditions.***	Store for 1 month at +15 to +25°C.	Prehybridization and hybridization solution.

Immunological detection

Solution	Composition/Preparation	Storage and Stability	For use in...
Washing buffer	0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (+20°C), 0.3% (v/v) Tween 20*	Store at +15 to +25°C.	Removal of nonspecific bound antibody.
Maleic acid buffer	0.1 M maleic acid, 0.15 M NaCl, adjust with solid NaOH to pH 7.5 (+20°C)		Dilution of Blocking Solution.
Detection buffer	0.1 M Tris-HCl*, 0.1 M NaCl, pH 9.5 (+20°C)		Adjustment of pH to 9.5.
Preparation of kit working solutions			
Blocking solution,1x	Dilute 10x Blocking Solution (Bottle 10) 1:10 with Maleic acid buffer.	⚠ ***Always prepare fresh.***	Blocking of nonspecific binding sites on the membrane.
Antibody solution	▪ Centrifuge Anti-Digoxigenin-AP (Vial 5) for 5 minutes at 10,000 rpm in the original vial prior to each use, and pipette the necessary amount carefully from the surface. ▪ Dilute Anti-Digoxigenin-AP 1:10,000 (75 mU/ml) in 1x Blocking solution.	Store at +2 to +8°C for 12 hours.	Binding to the DIG-labeled probe.

Stripping and reprobing of RNA blots

Solution	Composition/Preparation	Storage and Stability	For use in...
Water	Autoclaved DMPC- or DEPC-treated double-distilled water	Store at +15 to +25°C.	Washing the membrane.
Stripping buffer	▪ 50% Formamide* ▪ 50 mM Tris-HCl*, pH 7.5 ▪ 5% SDS*	⚠ ***Always prepare fresh.***	Removing DIG-labeled probe.
20x SSC stock solution*	3 M NaCl, 300 mM sodium citrate, pH 7.0	Store at +15 to +25°C.	Stock solution for the preparation of 2x SSC.
2x SSC	Dilute 20x SSC stock solution 1:10 with double-distilled, DMPC- or DEPC-treated water.		Washing and storage of membrane.

2.2. Protocols

DNA template preparation

Protocol for using the Expand High Fidelity PCR System*.

See section, **General Considerations***, for additional information on preparing the DNA template.*

1. Add the following reagents in the order listed to an autoclaved, RNase-free reaction tube on ice:

Reagent	Volume [µl]	Final conc.
Autoclaved double-distilled water, DMPC- or DEPC-treated.	variable	–
Expand buffer, 10x conc. containing $MgCl_2$ (supplied with the enzyme)	5	1.5 mM $MgCl_2$, 1x conc.
10 mM dATP, dCTP, dGTP, dTTP	1 each	0.2 mM
Primer 1 (sense)	variable	300 nM
Primer 2 (anti-sense)	variable	300 nM
Expand High Fidelity Enzyme mix	0.75	2.6 U
cDNA	2	–
Final volume	**50**	–

– Mix gently and centrifuge briefly.

2. Place samples in a thermal cycler. The cycling program is given below:

Program	Temp. [°C]	Duration [s]
30 cycles of amplification		
Denaturation	94°C	45
Annealing	60°C	45
Elongation	72°C	90

3. Use your PCR product directly for labeling, see section, **DIG RNA labeling**.

DIG RNA labeling

Label RNA in an *in vitro* transcription reaction with Digoxigenin-11-UTP using the Labeling mixture and optimized Transcription Buffer. This procedure is designed for 1 µg of DNA template. Larger amounts can be labeled by scaling up of all components and volumes.

1. To an autoclaved reaction vial, add 1 µg linearized plasmid DNA or 4 µl PCR product (100 to 200 ng) and RNase-free, DMPC- or DEPC-treated, double-distilled water to a final volume of 10 µl.

2. Add the following on ice:

Reagent	Volume [µl]
Labeling mix, 5x conc. (Vial 1a)	4
Transcription Buffer, 5x conc. (Vial 1b)	4
RNA polymerase (SP6, T7, or T3)	2

– Mix and centrifuge briefly.
– Incubate for 1 hour at +42°C.

3 Add 2 µl DNase I, RNase-free to remove the template DNA.
– Incubate for 15 minutes at +37°C.

4 Stop the reaction by adding 2 µl 0.2 M EDTA, pH 8.0.

Labeling efficiency and size of labeled RNA

In the standard reaction with 1 µg DNA per assay, 67% of the nucleotides are incorporated into approximately 20 µg of newly synthesized DIG-labeled RNA within 1 hour.
The size of the labeled RNA is in the range of 200 to 1,000 bases.

Determination of labeling efficiency

Determination of the yield of DIG-labeled RNA is very important for optimal and reproducible hybridization results. Too high probe concentrations in the hybridization step causes background, while too low concentrations leads to weak signals. The preferential method for determination of labeling efficiency of probes is the direct detection method in comparison to the control RNA (Vial 8).

Direct detection

1 A series of dilutions of DIG-labeled RNA is applied to a small strip of Nylon Membrane, positively charged*.

2 Part of the nylon membrane is preloaded with defined dilutions of control RNA, used as standards.

3 The nylon membrane is subjected to immunological detection with Anti-Digoxigenin-AP and CDP-*Star*, ready-to-use.

4 The intensities of the dilution series of DIG-labeled RNA and control RNA are compared by exposure to an imaging device or X-ray film or Lumi-Film*.

Dilution series

Prepare a dilution series of your labeled probe and your control RNA as described in the following table, where Tube 1 is either a dilution of your labeling reaction to 10 ng/µl (expected yield of a standard labeling reaction is 20 µg of labeled RNA), or the control RNA (Vial 8), which also has the concentration of 10 ng/µl.

Tube	RNA [µl]	From tube No.	RNA dilution buffer [µl]	Dilution	Final conc.
1	–	diluted labeling reaction or Vial 8	–	–	10 ng/µl
2	2	1	18	1:10	1 ng/µl
3	2	2	198	1:100	10 pg/µl
4	15	3	35	1:3.3	3 pg/µl
5	5	3	45	1:10	1 pg/µl
6	5	4	45	1:10	0.3 pg/µl
7	5	5	45	1:10	0.1 pg/µl
8	5	6	45	1:10	0.03 pg/µl
9	5	7	45	1:10	0.01 pg/µl
10	0	–	50	–	0

Determination of labeling efficiency by direct detection

See section, **Working Solution** *for additional information on preparation of solutions.*

The volumes suggested below are for small membrane strips of approximately 3 × 5 cm, processed in a small plastic container, such as a petri dish. Use sufficient buffer volumes to cover the membrane completely during all steps.

1. Apply 1 µl spots of tubes 3 to 10 from your labeled probes and the labeled control to the Nylon Membrane*.

2. Fix the nucleic acid to the membrane by crosslinking with UV light or baking for 30 minutes at +120°C.

3. Transfer the membrane into a plastic container with 20 ml Washing buffer.
   – Incubate while shaking for 2 minutes at +15 to +25°C.

4. Incubate for 30 minutes in 10 ml Blocking solution.

5. Incubate for 30 minutes in 10 ml Antibody solution.

6. Wash 2 × 15 minutes with 20 ml Washing buffer.

7. Equilibrate 2 to 5 minutes in 10 ml Detection buffer.

8. Place membrane with RNA side facing up on a development folder or Hybridization Bag and apply approximately 4 drops CDP-*Star* from the dropper (Bottle 7) to the membrane.
   – Immediately cover the membrane with the second sheet of the folder to spread the substrate evenly and without air bubbles over the membrane.
   – Incubate for 5 minutes at + 15 to +25°C.

9. Seal the edges of the development folder around the damp membrane.
   ⚠ ***Drying of the membrane during exposure will result in dark background.***

10. Expose to an imaging device or to X-ray film or Lumi-Film* for 5 to 25 minutes at +15 to +25°C.

Analyzing the result

Compare the intensity of the spots out of your labeling reaction to the control and calculate the amount of DIG-labeled RNA.

Suitable gel system for DIG northern blots

- Standard protocols for gel electrophoresis can be used as described in the literature.
- Gels lacking ethidium bromide are preferentially used since ethidium bromide can cause uneven background.
- Use formaldehyde denaturation and MOPS/formaldehyde gels; they are easy to handle and reliable.

Target amounts

If you are using...	Then...
total RNA,	load a maximum of 1 µg per lane.
mRNA,	load 100 ng per lane.

Formaldehyde gels

See section, **Working Solution** *for additional information on preparation of solutions.*

Prepare target RNA samples according to the following steps:

1. Add 20 µl (or 2 to 3 volumes) of Loading buffer to the RNA sample.
2. Denature the RNA sample/Loading buffer mix at +65°C for 10 minutes.
3. Chill the RNA sample/Loading buffer mix on ice for 1 minute.
4. Run the gel with 3 to 4 V/cm in RNase-free gel boxes for at least 2 hours (preferably overnight) until the RNAs are well separated.
5. To evaluate the quality of the target RNA after electrophoresis, stain the gel briefly in 0.25 to 0.5 µg/ml ethidium bromide and examine the gel under UV light.

RNA transfer and fixation

- When using formaldehyde gels, rinse gels prior to blotting for 2 × 15 minutes in 20x SSC*.
- All common types of RNA transfer methods are suitable for subsequent DIG hybridization.
- Best results are obtained when gels are blotted by capillary transfer with 20x SSC overnight or at least for 6 hours.
- Use only Nylon Membranes, positively charged*.

⚠ ***Alkali transfer, for example, using 0.4 M NaOH, is not suitable for the transfer of RNA and DIG-labeled Molecular Weight Markers.***

Fixation procedure

Fix the RNA to the membrane by any of the following methods.

⚠ ***Use RNase-free solutions and equipment.***

Method	Steps
UV crosslinking	▪ Place the membrane on Whatman 3MM paper soaked with 2x SSC*. ▪ UV crosslink the wet membrane without prior washing. ▪ After the UV crosslinking, rinse the membrane briefly in double-distilled water and allow to air-dry.
Baking at +120°C	▪ Rinse the membrane 2 × briefly in 2x SSC*. ▪ Bake the nylon membrane at +120°C for 30 minutes or according to the manufacturer's instructions.
Baking at +80°C	▪ Rinse membrane 2 × briefly in 2x SSC*. ▪ Bake at +80°C for 2 hours.

Membrane storage

IF...	THEN...
you want to continue,	use the membrane immediately for prehybridization.
you want to use it later,	store the membrane dry at +2 to +8°C.

Hybridization

Hybridization temperature for northern blots with DIG Easy Hyb is +68°C for 100% homology of the probe to the target sequence. The DIG Easy Hyb working solution used for prehybridization must be prewarmed to +68°C.

1. Prewarm an appropriate volume of DIG Easy Hyb (10 to 15 ml/100 cm² filter) to a hybridization temperature of +68°C.
   – Prehybridize membrane with DIG Easy Hyb for 30 minutes with gentle agitation in an appropriate container.
   ⚠ ***Membranes must move freely, especially if you use several membranes in the same prehybridization solution.***

2. Denature DIG-labeled RNA probe by boiling for 5 minutes and rapidly cooling in ice/water.
   ⚠ ***RNA is degraded by alkali-solutions.***

3. Add 100 ng/ml denatured DIG-labeled RNA probe to prewarmed DIG Easy Hyb (3.5 ml/100 cm² membrane) and mix well.
   ⚠ ***Avoid foaming as bubbles may lead to background.***

4. Pour off prehybridization solution and add probe/hybridization mixture to membrane.
   – Incubate for 6 hours or overnight at +68°C with gentle agitation.

Storage of hybridization solution

In principle, DIG Easy Hyb containing DIG-labeled probe can be stored at −15 to −25°C and be reused when freshly denatured at +65°C for 10 minutes before use.

⚠ ***Do not reuse DIG Easy Hyb containing RNA probes, because successful reuse depends on inherent probe stability and RNase-free working conditions.***

Stringency washes after hybridization

1. Wash 2 × 5 minutes in 2x SSC*, 0.1% SDS* at +15 to +25°C under constant agitation.

2. Wash 2 × 15 minutes in 0.1x SSC*, 0.1% SDS* (prewarmed to wash temperature) at +68°C under constant agitation.

The stringency of the final wash must be determined empirically. Depending on length and homology of the probe, it will be necessary to adjust salt concentration. Fully homologous probes will often require 0.1x wash solution.

Immunological detection

See section, **Working Solution** *for additional information on preparation of solutions.*

Perform the immunological detection on a 100 cm² membrane in a suitable, slightly larger tray.

⚠ ***Perform all incubations at +15 to +25°C with agitation. If the membrane is to be reprobed, do not allow the membrane to dry at any time.***

1. After hybridization and stringency washes, rinse membrane briefly 1 to 5 minutes in Washing buffer.

2. Incubate for 30 minutes in 100 ml Blocking solution.

3. Incubate for 30 minutes in 50 ml Antibody solution.

4. Wash 2 × 15 minutes in 100 ml Washing buffer.

5. Equilibrate 2 to 5 minutes in 100 ml Detection buffer.

6 Place membrane with RNA side facing up on a development folder or Hybridization Bag and quickly apply approximately 1 ml CDP-*Star*, ready-to-use solution out of the dropper bottle (Bottle 7) until the membrane is evenly soaked.
– Immediately cover the membrane with the second sheet of the folder to spread the substrate evenly and without air bubbles over the membrane.
– Incubate for 5 minutes at +15 to +25°C.

7 Squeeze out excess liquid and seal the edges of the development folder around the damp membrane.
⚠ ***Drying of the membrane during exposure will result in dark background.***

8 Expose to an imaging device for 5 to 20 minutes or to X-ray film or Lumi-Film* for 15 to 25 minutes at +15 to +25°C.

i Luminescence continues for at least 24 hours. The signal increases in the first few hours. Multiple exposures can be taken to achieve the desired signal strength.

Stripping and reprobing of RNA blots

i Only membranes which did not dry at any time during the hybridization and detection procedure can be stripped.

1 Rinse membrane thoroughly in autoclaved DMPC- or DEPC-treated double-distilled water.

2 Incubate 2 × 60 minutes at +80°C in Stripping buffer to remove the DIG-labeled probe.

3 Rinse 2 × 5 minutes in 2x SSC*.

4 Prehybridize and hybridize with a second probe or store membrane in a sealed bag.

Storage of stripped membrane

Once the membrane is stripped, store in Maleic acid buffer or 2x SSC* at +2 to +8°C.
⚠ ***Make sure that the membrane does not dry at any time.***

2.3. Parameters

Sensitivity

Rare mRNAs can be detected in 0.1 µg of total RNA.

3. Results

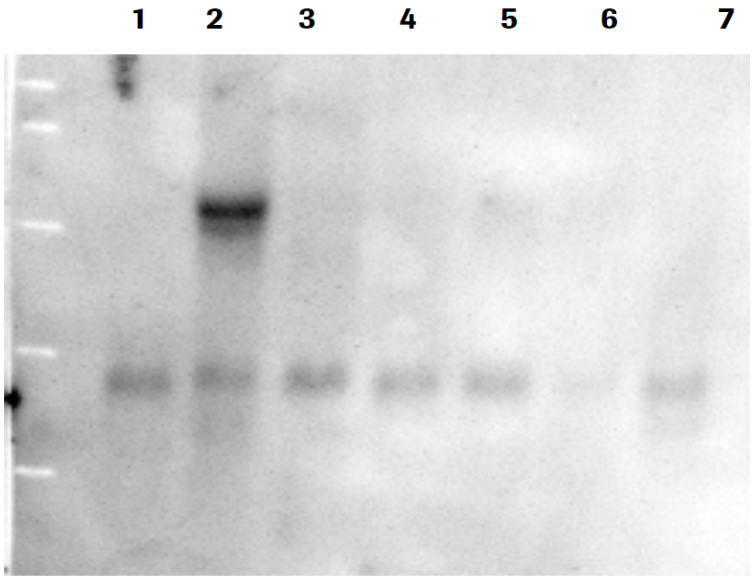
Typical results

The Figure 1 shows the detection of the TSH receptor on thyroid tissue with Graves disease using a Northern blot.

Probe	DIG-labeled TSH receptor (100 ng/ml)
Template	**Lane 1**: 0.1 µg Thyroid RNA **Lane 2**: 0.5 µg Thyroid RNA
Detection	CDP-Star, ready-to-use
Exposure time	10 minutes

The Figure 2 shows different tissues, normalized to Actin, hybridized with TSH-receptor probe (100 ng/ml) in a Northern Blot.

Probe	DIG-labeled TSH receptor probe (100 ng/ml) and DIG-labeled Actin probe (100 ng/ml).	
Template	**Lane**	
	1	Stomach
	2	Thyroid
	3	Spinal cord
	4	Lymph node
	5	Trachea
	6	Adrenal gland
	7	Bone marrow



4. Troubleshooting

Observation	Possible cause	Recommendation
Low sensitivity observed.	Inefficient probe labeling.	Check labeling efficiency, see section, **Determination of labeling efficiency**.
		Purify template DNA by phenolization and ethanol precipitation.
		Make sure that the template was linearized before labeling.
		Do not store template in buffers containing >0.1 mM EDTA.
		Check the amount and quality of target RNA.
	Low probe concentration in the hybridization.	Increase probe concentration to 100 ng/ml.
		Prolong hybridization time to overnight.
High background present.	Concentration of labeled probe too high.	Determine optimal probe concentration as described in section, **Determination of labeling efficiency**; do not use >100 ng/ml.
	Drying of the membrane.	Make sure that the membrane does not dry out at any time.
	Probe sequence.	Check that the probe does not contain crosshybridizing vector sequences.
	Inefficient prehybridization.	Make sure that the membrane was soaked in sufficient prehybridization solution.
	Wrong type of nylon membrane.	Some types of nylon membrane may cause high background; use Nylon Membranes, positively charged*, tested for the DIG System.
	Inefficient stringency washes.	Check temperature of stringency washes; prewarm wash solution to correct temperature.
		Use 0.1x SSC for high stringency wash.
Smear in lanes.	Target concentration too high.	Do not use >1 µg of total or 100 ng of mRNA per lane. *The DIG System is more sensitive than radioactivity and higher RNA concentrations result in detection of degradation products.*

5. Additional Information on this Product

5.1. Test Principle

The DIG Northern Starter Kit generates DIG-labeled, single-stranded RNA probes of defined length by *in vitro* transcription of template DNA in the presence of Digoxigenin-11-UTP using SP6, T7, or T3 RNA Polymerases.

① For RNA labeling, DIG-labeled RNA probes are generated according to the *in vitro* transcription labeling technique.
– The labeling mix contains optimal concentrations of nucleotides and DIG-11-UTP and a specially developed transcription buffer, and is combined with the linearized DNA template and the appropriate RNA polymerase.

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② For hybridization, DIG-labeled RNA probes are used for hybridization to membrane-blotted nucleic acids according to standard methods.

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③ For immunological detection, the hybridized probes are immunodetected with Anti-Digoxigenin-AP, Fab fragments and visualized with the chemiluminescence substrate CDP-*Star*, ready-to-use.
– Enzymatic dephosphorylation of CDP-*Star*, ready-to-use by alkaline phosphatase leads to a light emission at a maximum wavelength of 465 nm (Figure 3) which is recorded with an imaging device or on X-ray film or Lumi-Film*.
– Exposure times are in the range of only 5 to 30 minutes.

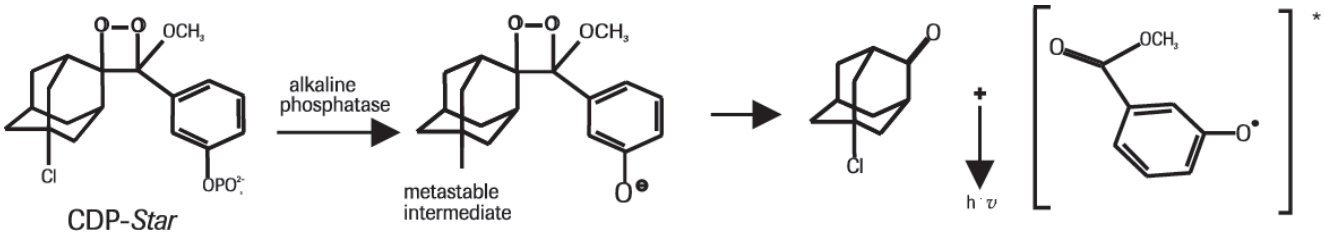

Fig. 3: Reaction of CDP-*Star*.

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Layout changes.
Editorial changes.
Update to include new safety Information to ensure handling according controlled conditions.

6.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Deoxynucleoside Triphosphate Set	4 x 250 µl, 4 x 25 µmol, 100 mM	11 969 064 001
	4 x 1,250 µl, 4 x 125 µmol, 100 mM	03 622 614 001
Expand High Fidelity PCR System	100 U, 1 x 100 U, 40 reactions in a final volume of 50 µl	11 732 641 001
	500 U, 2 x 250 U, 200 reactions in a final volume of 50 µl	11 732 650 001
	2,500 U, 10 x 250 U, 1,000 reactions in a final volume of 50 µl	11 759 078 001
Agarose MP	100 g	11 388 983 001
	500 g	11 388 991 001
Nylon Membranes, positively charged	10 sheets, 20 x 30 cm	11 209 272 001
	20 sheets, 10 x 15 cm	11 209 299 001
	1 roll, 0.3 x 3 m	11 417 240 001
DIG Wash and Block Buffer Set	1 set, 30 blots (100 cm^2)	11 585 762 001
Lumi-Film Chemiluminescent Detection Film	100 films, 7.1 x 9.4 inches, 18 x 24 cm, *Not available in US*	11 666 916 001
Buffers in a Box, Premixed SSC Buffer, 20x	4 l	11 666 681 001
Sodium Dodecyl Sulfate (SDS)	1 kg	11 667 289 001
Tween 20	50 ml, 5 x 10 ml	11 332 465 001
Tris hydrochloride	500 g	10 812 846 001
Formamide	500 ml	11 814 320 001
High Pure Plasmid Isolation Kit	1 kit, 50 purifications	11 754 777 001
	1 kit, 250 purifications	11 754 785 001

6.4. Trademarks

EXPAND and DIG EASY HYB are trademarks of Roche.
All other product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

6.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

6.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany