

## **Non-Radioactive Hybridization and Detection of Atlas cDNA Expression Arrays (CLONTECH) using DIG-Chem-Link Labeled Probes**

### **INTRODUCTION**

The experimental application of arrays has been around for several years. However, with the advent of the human genome project along with many others, the application has become very popular. Arrays are performed on nylon membranes and more recently have been adapted to glass slides. This protocol was developed for the hybridization of PCR products representing different genes, spotted on a membrane (e.g. as provided by Clontech), with a DIG-Chem-Link labeled mRNA probe.

The direct labeling of mRNA pools is extremely sensitive and superior to using cDNA probes. The drawback of all cDNA probes is that there is no guarantee that all the sequences, present in the mRNA pool, will be identically represented in a cDNA probe. Secondary structures in the mRNA are a major obstacle for complete synthesis of the cDNA. The Chem-Link technology provides for the first time a tool to label mRNA pools directly without the necessity of preparing cDNA probes. DIG-Chem-Link is a chemical labeling method, no enzyme is involved. DIG is added via a cis-platin complex to the N<sub>7</sub> position of A and G. The labeled nucleic acid is stable to denaturation by boiling and can be used for all hybridization applications..

**Note:** We recommend to hybridize arrays with a poly A+ RNA probe labeled by DIG Chem-Link.

## EQUIPMENT LIST

This is a reagent set for labeling RNA probes using the DIG-Chem-Link Kit, subsequent hybridization and chemiluminescent detection.

Description	Cat. No.	Size
DIG Chem-Link Labeling and Detection Set	1836463	20 labeling reactions
RNA from yeast	109 233	100 g
Actin RNA Probe	1 498 045	2µg
DIG Easy Hyb Granules	1796 895	6 X 100 ml bottles
DIG Wash and block Buffer Set	1585 762	30 blots
Anti-Digoxigenin-AP, Fab fragments	1093 274	150 U
CDP*, chemiluminescent substrate (ready-to-use)	2041 677	2 X 50ml
20 x SSC	1666 681	4 L
20% SDS Solution	1358 995	100 ml
Lumi-Imager F1 unit	2012 847	Workstation consisting of Lumi-Imager, computer and printer
Lumi-Film Chemiluminescent Detection Film	1 666 916	100 films, 18x24cm
CLONTECH Atlas Human cDNA Expression Array	7740-1	
Your own mRNA or as a test system: INVITROGEN Human Liver polyA <sup>+</sup> RNA	D6080-15	5 µg
mRNA Isolation Kit	1741985	For isolation of at least 70 µg of poly (A <sup>+</sup> ) RNA.

## LABELING REACTION

### Factors influencing the labeling reaction

The efficiency of labeling largely depends on

- The degree of template purification
- The concentration of the two reaction partners: Dig Chem-Link and nucleic acid
- The incubation temperature: 85°C for Chem-Link reaction

**Note:** DIG –Chem-Link can bind to DNA, RNA and nucleotides. The template should be phenol chloroform extracted and ethanol precipitated and dissolved in sterile, RNasefree water for best results.

The following substances (final concentrations given) have been tested and found **not** to inhibit the Chem-Link reaction

- TE buffer: 10 mM Tris-HCl, 1 mM EDTA (pH 7.5 – 8.0)
- 10 mM LiCl
- 10-50 mM NaCl

The following substances (final concentrations given) have been tested and found to interfere with labeling

- Tris > 40 mM
- EDTA > 5 mM
- Mg acetate and NaCl > 100 mM

**Note:** For the screening of arrays, the polyA<sup>+</sup> RNA used as probe, should be absolutely free of DNA, since DIG-Chem-Link can bind to RNA as well as DNA. This could lead to ambiguous hybridization results.

In case residual DNA is present, the mRNA should be treated with DNase (RNase free).

Set up the RNA labeling reaction using the DIG-Chem-Link Labeling reaction mix according to the standard protocol.

Pipette according to the following scheme:

Component	Sample 1
DIG-Chem-Link (Vial 1)	2 $\mu$ l
Your own mRNA or Human Liver polyA <sup>+</sup> RNA (0.5 $\mu$ g/ $\mu$ l; INVITROGEN)	2 $\mu$ g! 4 $\mu$ l
H <sub>2</sub> O DMDC/DEPC treated	14 $\mu$ l
Total reaction volume	20 $\mu$ l

**Note:** Scale up of the amount of template can be performed without loss of labeling efficiency as long as the ratio of DIG-Chem-Link is kept constant, e.g. 1 $\mu$ l of reagent per  $\mu$ g of nucleic acid.

- Mix well and incubate the reaction for 30 min. at 85 °C
- Centrifuge briefly to collect condensate and stop reaction by adding 5  $\mu$ l of stop solution (**vial 2**).

## QUANTIFICATION OF LABELED RNA

It is absolutely necessary to check the labeling efficiency. Therefore the labeled probe should be diluted as follows:

Dilute a small aliquot of the labeling reaction (2 $\mu$ g poly A<sup>+</sup> RNA in 25  $\mu$ l reaction volume) to a final concentration of 1 ng/ $\mu$ l (1  $\mu$ l of probe + 79  $\mu$ l of DMDC or DEPC treated water).

**Note:** The respective tube is referred to as original in the following dillution scheme.

As a control, the DIG-labeled  $\beta$ -actin RNA probe (10 ng/  $\mu$ l) should be used and also diluted to a final concentration of 1ng/ $\mu$ l (5  $\mu$ l of  $\beta$ -actin RNA + 45  $\mu$ l of DMDC or DEPC treated water).

Then proceed with the following dilutions for your labeled probe and the labeled control.

Tube	RNA ( $\mu$ l)	Buffer ( $\mu$ l)	From Tube #	Dilution	Final Concentration
1			Original		1 ng/ $\mu$ l
2	5	495	1	1:100	10 pg/ $\mu$ l
3	15	35	2	1:3.3	3 pg/ $\mu$ l
4	5	45	2	1:10	1 pg/ $\mu$ l
5	5	45	3	1:10	0.3 p/ $\mu$ l
6	5	45	4	1:10	0.1 pg/ $\mu$ l
7	5	45	5	1:10	0.03 pg/ $\mu$ l
8	5	45	6	1:10	0.01 pg/ $\mu$ l
9	0	50	-	-	0

- Apply 1 $\mu$ l spots of dilutions 2-9 to a small piece of membrane (e.g. 5x3cm) for the direct detection method.
- Fix the RNA to the membrane by cross-linking with UV-light or by baking for 30 min. at 120 $^{\circ}$ C. (Roche Molecular Biochemicals Nylon Membrane)

You are now ready to begin the direct detection procedure

Follow the procedure in the Detection section below through development of the film. You can use a petri dish for the direct detection and you can also use reduced volumes. The result should yield at least five spots for each dilution series, showing sensitivity down to 0.1 pg.

Probes can be used for hybridization without further purification.

**Note:** Labeled probes will be stable for at least a year when handled under RNasefree conditions and should be stored at -20  $^{\circ}$ C.

*You are ready now for setting up the hybridization reaction*

## HYBRIDIZATION

### Notes in general:

- Never touch the areas where the cDNAs are immobilized – even when wearing gloves.
- Never allow the array membrane to dry, even slightly, once the prehybridization has been started, until the end of the stringency washes, nor during the detection procedure
- Never let the membrane dry at all, if you intend to reprobe. If you do not intend to reprobe, the membrane may be dried after the last stringent wash.. If the membrane had dried, no complete stripping can be achieved.
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- Continuous agitation of the membrane is necessary during all prehybridization, hybridization and washing steps.

### Prehybridization:

- Prepare DIG-Easy Hyb buffer with 50 µg/ml RNA from yeast by dissolving the RNA directly in DIG-Easy Hyb buffer pre-warmed to 50 °C. (Please prepare this solution fresh, no longer than one hour ahead of use).
- Transfer the membrane to a well sized hybridization bag. Add 12 ml of DIG-Easy Hyb buffer (with 50 µg/ml RNA from yeast) and pre-warmed to 50 °C.
- Seal the bag after removing all trapped air bubbles.
- Incubate the membrane for 30 min at 50 °C with constant agitation.  
**Note:** Alternatively roller bottles can be used.

### Hybridization:

- Prepare DIG-Easy Hyb buffer with 50 µg/ml RNA from yeast by dissolving the RNA directly in DIG-Easy Hyb buffer pre-warmed to 50 °C. (Please prepare this solution fresh, no longer than one hour ahead of use).

- Denature the DIG -labeled probe (probe concentration: 100 ng/ml hybridization buffer, estimated according to the result of the direct detection assay) for 5 min in a boiling waterbath and place on ice immediately for further 5 min.
- Add denatured probe (probe concentration: 100 ng / ml hybridization buffer) to 6 ml DIG-Easy Hyb buffer with 50µg/ml RNA from yeast pre-warmed to 50 °C and mix carefully.
- Decant the prehybridization buffer and transfer the hybridization buffer to the hybridization bag.
- Incubate the membrane at 50 °C over night with constant agitation.  
**Note:** It is important to remove all air bubbles.

### **Post Hybridization/Stringent Washes:**

#### **Preparation of Wash buffers:**

##### Wash buffer 1 (2 x SSC, 0.1% SDS):

Dilute 50 ml 20 x SSC and 2.5 ml SDS (20%) to a total volume of 500 ml with DMDC or DEPC treated water.

##### Wash buffer 2 (0.1 x SSC, 0.1% SDS):

Dilute 2.5 ml 20 x SSC and 2.5 ml SDS (20%) to a total volume of 500 ml with DMDC or DEPC treated water and heat to 68°C.

#### **Washing:**

- Wash each membrane 2 x 5 min. with excess amount of wash buffer 1 at room temperature.
- Wash each membrane 2 x 15 min. with excess amount of wash buffer 2 at 68 °C.

### **DETECTION**

Probe-target hybrids are detected by an enzyme-linked immunoassay. The membrane is blocked to prevent non-specific interaction of the antibody with the filter. An antibody specific for Digoxigenin recognizes the labeled hybrid. Alkaline phosphatase conjugated to the antibody allows detection of the complex. CDP\* is a chemiluminescent substrate for alkaline phosphatase. The light emitted from the blot can be detected with x-ray film or with the Roche Molecular Biochemicals Lumi-Imager™ Workstation.

First, Prepare the buffer solutions for the detection procedure. These buffers are provided in the *DIG Wash and Block Buffer Set*. Prepare the buffers as follows:

1. Find the bottle containing 500ml of *maleic acid buffer (10x)*. Take 100ml of this buffer and add it to 900ml of sterile distilled, RNasefree water. Store at room temperature in a sterile, RNasefree container labeled *Buffer 1*.
2. Find the bottle containing 500ml of *blocking solution (10x)*. Take 50ml of this buffer and add it to 450ml of Buffer 1. Store at 4°C in a sterile, RNasefree container labeled *Buffer 2*.
3. Find the bottle containing 500ml *washing buffer (10x)*. Shake the bottle to suspend the buffer components. Take 50ml of this buffer and add it to 450ml of sterile, RNasefree distilled water. Store at room temperature in a sterile, RNasefree container labeled *washing buffer*.
4. Find the bottle containing 100ml *detection buffer (10x)*. Take 50ml of this buffer and add it to 450ml of sterile distilled, RNasefree water. Store at room temperature in a sterile, RNasefree container labeled *Buffer 3*.

***You are now ready to begin the detection procedure:***

Have solutions ready to use so you can avoid exposing the membrane to air during the detection procedure since any drying of the membrane will result in unacceptable background. The volumes suggested below are for a 100cm<sup>2</sup> blot processed in a plastic container. All of the following incubations are performed at room temperature with shaking. Use sufficient buffer volumes to cover the membrane completely during all steps.

Step	Directions	Time
1	Transfer the membrane into a plastic container with 200 ml <i>Buffer 1</i> . Incubate with shaking at room temperature.	2 min.
2	Pour off <i>Buffer 1</i> and add 200 ml <i>Buffer 2</i> . Incubate with shaking at room temperature. This blocking step can go for 3 hours without detrimental effects.	30 min.
3	When about 5 min. remain in Step 2, prepare the diluted <DIG> alkaline phosphatase reagent. Spin the antibody for 5 min. at full speed in a microfuge. Some lots contain coagulated conjugates that precipitate and can cause a spotty background on the membrane. Take 5µl of the <DIG> antibody conjugate supernatant and add to 50 ml of <i>Buffer 2</i> (1:10,000 dilution). Mix well and replace the buffer from step 2 with this <DIG> antibody solution. Incubate with shaking at room temperature.	30 min
4	Pour off the antibody solution and wash with 200ml <i>washing buffer</i> .	2 x 15 min.
5	Replace the <i>washing buffer</i> with 200ml <i>Buffer 3</i> .	3 min.
6	Prepare the CDP* reagent for use by dilution it 1:100 in <i>Buffer 3</i> (20µl CDP* into 2ml <i>Buffer 3</i> ). Use sterile technique to make the dilution. Incubate the membrane with 1ml CDP* dilution between two sheets of a hybridization bag or plastic sheet protectors. The best way to apply this amount is to drop it over the surface of the blot. Close the sheets as you are applying the substrate to avoid drying of the membrane. Press gently to spread the solution. This step should be done quickly. <b>Do not use saran wrap!!!</b>	5 min.
7	Squeeze out excess liquid, seal the sheets close to the membrane. Avoid air bubbles.	2 min
8	Expose the blot to x-ray film or use the Roche Molecular Biochemicals Lumi-Imager™ Workstation.	5-30 min

## STRIPPING AND REPROBING

**Note:** Never let the membrane dry if several strippings and reprobing are intended.

- Rinse the membrane briefly in DMDC or DEPC treated water
- Wash for 2 x 15 min. with 0.2 N NaOH, 0.1% SDS at 37 °C to remove the DIG labeled probe with constant agitation.
- Rinse thoroughly for 5 min in 2 x SSC
- Prehybridize and hybridize with the next probe.
- The membrane can be sealed wetted with 2 x SSC in a plastic bag and stored at 4 °C when not in use.

## APPENDIX

The Atlas cDNA expression arrays from CLONTECH used in this experiment are nylon membranes (NYTRAN, positively charged) on which 588 cDNAs, nine housekeeping control cDNAs and negative controls have been immobilized in duplicate spots. The genes represent several different functional classes, including transcription factors, cytokines, oncogenes, and tumor suppressor genes. Two identical membranes are included with each purchase for comparative experiments. For the evaluation, refer to the information material from Clontech.

The following controls are applied:

As orientation marks for determination of hybridization signals genomic DNA spots are dotted in the bottom row and in double on the right side.

As negative controls M13, Lambda DNA and pUC18 are spotted in double on the left part of each section on the second last row



Negative controls:	M13 mp18(+) strand DNA	at position 2, 9, 16
	Lambda-DNA	at position 3, 10, 17
	PUC 18	at position 4, 11, 18
Positive controls:	Ubiquitin	at position 5
	Phospholipase A2	at position 6
	HPRT	at position 7

Positive controls continued:	Liver glyceralaldehyde 3-phosphate dehydrogenase	at position 12
	Tubulin alpha	at position 13
	HLA class I histocompatibility Antigen C-4 alpha chain (MHC)	at position 14
	Beta-actin	at position 19
	23-kDa highly basic protein	at position 20
	Ribosomal protein S9	at position 21

### References:

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