

References

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VIII. Estimating the yield of DIG-labeled nucleic acids

An accurate quantification of DIG-labeled DNA obtained in the labeling reaction is most important for optimal and reproducible results in various membrane or *in situ* hybridization techniques. Too high of a probe concentration in the hybridization mix usually causes background, while too low of a concentration leads to weak signals.

Estimate the yield of DIG-labeling the following way:

Dilution series of the labeling reaction and dilutions of an appropriate standard are both spotted on nylon membranes. The membrane is then processed in a short detection procedure.

Exception: PCR labeled DIG probes are checked via agarose gel (compare to page 39)

Estimating the yield in a spot test with a DIG-labeled control

The estimation of yield can also be performed in a side by side comparison of the DIG-labeled sample nucleic acid with a DIG-labeled control, that is provided in the labeling kits. Dilution series of both are prepared and spotted on a piece of membrane. Subsequently, the membrane is colorimetrically detected. Direct comparison of the intensities of sample and control allows the estimation of labeling yield.

Products required

DIG-labeled controls for estimating the yield of DNA, RNA are available as separate reagents or in the respective labeling kits. The DIG-dUTP/dATP-tailed and 3'-End labeled Oligonucleotide Control is only available in the DIG Oligonucleotide Tailing Kit, 2nd generation, and the DIG Oligonucleotide 3'-End Labeling Kit, 2nd generation.

Reagents available from RAS for this procedure

Reagent	Description	Available as
Labeled Control DNA	Digoxigenin-labeled pBR328 DNA that has been random primed labeled according to the standard labeling procedure; the total DNA concentration in the vial is 25 µg/ml, but only 5 µg/ml of it is DIG-labeled DNA.	▶ DIG-labeled Control DNA* (Cat. No. 11 585 738 910)
Control Oligonucleotide, DIG-ddUTP-labeled	2.5 pmol/µl oligonucleotide, labeled with Digoxigenin-11-ddUTP according to the standard labeling procedure.	▶ Vial 6, DIG Oligonucleotide 3'-End Labeling Kit, 2 nd generation* (Cat. No. 03 353 575 910)
Control Oligonucleotide, DIG-dUTP/dATP tailed	2.5 pmol/µl oligonucleotide, tailed with Digoxigenin-11-dUTP and dATP according to the standard labeling procedure.	▶ Vial 7, DIG Oligonucleotide Tailing Kit, 2 nd generation* (Cat. No. 03 353 583 910)
Labeled Control RNA	Digoxigenin-labeled "antisense"-Neo RNA, transcribed with T7RNA polymerase from 1 µg template DNA, according to the standard labeling procedure. The solution contains approx. 100 µg/ml DIG-labeled RNA and 10 µg/ml unlabeled DNA template.	▶ DIG-labeled Control RNA* (Cat. No. 11 585 746 910)
DNA Dilution buffer	10 mM Tris-HCl, pH 8.0 (20°C), 50 µg/ml DNA from fish sperm.	▶ Vial 9, DIG Oligonucleotide 3'-End Labeling Kit, 2 nd generation* (Cat. No. 03 353 575 910) ▶ Vial 10, DIG Oligonucleotide Tailing Kit, 2 nd generation* (Cat. No. 03 353 583 910)
RNA Dilution buffer	DMPC-treated H ₂ O, 20× SSC and formaldehyde, mixed in a volume ratio of 5 + 3 + 2.	
Blocking Reagent	Blocking reagent for nucleic acid hybridization; white powder.	▶ Blocking Reagent (Cat. No. 11 096 176 001)
Anti-Digoxigenin-AP	Anti-digoxigenin [Fab] conjugated to alkaline phosphatase.	▶ Anti-Digoxigenin-AP, Fab fragments* (Cat. No. 11 093 274 910)
NBT solution	75 mg/ml nitroblue tetrazolium salt in dimethylformamide.	▶ NBT [Cat. No. 11 383 213 001] (dilute from 100 mg/ml)]
BCIP solution	50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), toluidinium salt in dimethylformamide.	▶ BCIP (Cat. No. 11 383 221 001)

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.354.657 and 5.702.888 owned by Roche Diagnostics GmbH.

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Additionally required solutions	Description
Washing Buffer (Bottle 1; dilute 1:10 with double dist. water)	100 mM Maleic acid, 150 mM NaCl; pH 7.5 (+20°C); 0.3% (v/v) Tween 20
Maleic acid Buffer (Bottle 2; dilute 1:10 with double dist. water)	100 mM Maleic acid, 150 mM NaCl; pH 7.5 (+20°C)
Blocking Solution (Bottle 3; dilute 1:10 with 1× Maleic acid Buffer)	1% (w/v) Blocking Reagent for nucleic acid hybridization, dissolved in Maleic acid Buffer. Blocking Solution is cloudy and should not be filtered. It is stable for at least two weeks when stored at 2–8°C, but must then be brought to 15–25°C temperature before use
Detection Buffer (Bottle 4; dilute 1:10 with double dist. water)	100 mM Tris-HCl, 100 mM NaCl; pH 9.5 (20°C)
TE Buffer	10 mM Tris-HCl, 0.1 mM EDTA; pH 8.0 (20°C)

Procedure

- 1 Prepare a 1 ng/μl working solution of DIG-labeled Control DNA (original conc. 5 ng/μl) in DNA Dilution Buffer, by mixing 5 μl DIG-labeled Control DNA with 20 μl DNA Dilution Buffer.

Prepare a 10 ng/μl working solution of DIG-labeled Control RNA (original conc. 100 ng/μl) in RNA Dilution Buffer, by mixing 2 μl DIG-labeled Control RNA with 18 μl RNA Dilution Buffer.

For the DIG 3' -end labeled or tailed control oligonucleotide a predilution is not required.
- 2 Make serial dilutions of the (prediluted) controls, according to the appropriate dilution scheme. Mix thoroughly between dilution steps.

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Dilution Scheme A (for DNA probes)

Tube	DNA (μl)	From Tube #	DNA Dilution Buffer (μl)	Overall Dilution (from Tube D1)	Final Concentration
D1*		-		None	1 ng/μl
D2	2	D1	198	1 : 100	10 pg/μl
D3	15	D2	35	1 : 330	3 pg/μl
D4	5	D2	45	1 : 1000	1 pg/μl
D5	5	D3	45	1 : 3300	0.3 pg/μl
D6	5	D4	45	1 : 10 ⁴	0.1 pg/μl
D7	5	D5	45	1 : 33,000	0.03 pg/μl
D8	5	D6	45	1 : 10 ⁵	0.01 pg/μl
D9	0	-	50	-	0

*working solution of labeled probe or control DNA.

Dilution Scheme B (for Oligonucleotide probes)

Tube	Oligo (μl)	From Tube #	DNA Dilution (from Tube N1)	Overall Dilution (from Tube N1)	Final Concentration
N1*			-	None	100 fmol/μl
N2	3	N1	7	1 : 3.3	30 fmol/μl
N3	2	N1	18	1 : 10	10 fmol/μl
N4	2	N2	18	1 : 33	3 fmol/μl
N5	2	N3	18	1 : 100	1 fmol/μl
N6	0	-	20	-	0

* working solution of labeled probe or control oligonucleotide

Dilution Scheme C (for RNA probes)

Tube	RNA (μl)	From Tube #	RNA Dilution Buffer (μl)	Overall Dilution (from Tube R1)	Final Concentration
R1*		-		None	10 ng/μl
R2	2	R1	18	1:10	1 ng/μl
R3	2	R2	198	1:1000	10 pg/μl
R4	15	R3	35	1:3300	3 pg/μl
R5	5	R3	45	1:10 ⁴	1 pg/μl
R6	5	R4	45	1:3.3 × 10 ⁴	0.3 pg/μl
R7	5	R5	45	1:10 ⁵	0.1 pg/μl
R8	5	R6	45	1:3.3 × 10 ⁵	0.03 pg/μl
R9	5	R7	45	1:10 ⁶	0.01 pg/μl
R10	0	-	50	-	0

* working solution of labeled probe or control RNA



Highly diluted solutions of RNA in H₂O are not very stable. Spots have to be made immediately after preparing the dilutions. Alternatively the RNA can be diluted in RNA dilution buffer (DMPC-treated H₂O, 20× SSC and formaldehyde, mixed in a volume ratio of 5 + 3 + 2) for greater stability.

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- 3** Use table 1 on page 36 to estimate the expected yield of DNA labeling reactions. Predilute an aliquot of the newly labeled experimental **DNA probe** to an expected final concentration of approx. 1 ng/μl.
- or*
- Predilute an aliquot of the newly labeled experimental **oligonucleotide probe** to a final concentration of 100 fmol/μl.
- or*
- Predilute an aliquot of the newly synthesized experimental **RNA probe** to an expected final concentration of approx. 10 ng/μl. In a standard RNA labeling reaction approx. 10 g newly synthesized DIG-RNA probe is transcribed from 1 μg DNA template.
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- 4** Make serial dilutions of the prediluted experimental probe, according to the appropriate dilution scheme:
- ▶ for DNA probes, use dilution scheme A
 - ▶ for oligonucleotide probes, use dilution scheme B
 - ▶ for RNA probes, use dilution scheme C
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- 5** On a narrow strip (approx. 3 × 5 cm) of Positively Charged Nylon Membrane (Cat. No. 11 209 272 001), apply 1 μl spots from probe dilutions:
- ▶ for DNA probes D2–D9
 - ▶ for oligonucleotide probes N2–N6
 - ▶ for RNA probes R3–R10
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- 6** In a row parallel to the probe dilutions, apply 1 μl spots of the corresponding control dilutions (*i.e.*, D2–D9, N2–N6 or R3–R10, made from the appropriate control). Mark location of each probe and control spot with a pencil.
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- 7** Fix the nucleic acid spots to the membrane by doing one of the following:
- ▶ Crosslink with Stratalinker 120 mJ, or
 - ▶ Crosslink with UV light for 3–5 min, or
 - ▶ Bake the membrane at 120°C for 30 min, or
 - ▶ Bake the membrane at 80°C for 2 h
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- 8** Transfer the membrane to a plastic container (*e.g.*, a petri dish) containing 20 ml Washing Buffer. Incubate for 2 min with shaking. Discard the Washing Buffer.
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- 9** Incubate membrane for 30 min in 10 ml Blocking Solution. Discard the Blocking Solution.
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- 10** Prepare 10 ml antibody solution by dilution of Anti-DIG alkaline phosphatase 1:5000, recommended for NBT/BCIP detection, in Blocking Solution. Therefore centrifuge the Anti-DIG-AP for 5 min at 10 000 rpm in the original vial and pipet the necessary amount from the surface.
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- 11** Incubate the membrane for 30 min in 10 ml Antibody Solution.
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- 12** Wash membrane twice (2 × 15 min) with 10 ml portions of Washing Buffer.
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- 13** Equilibrate membrane 2–5 min in 10 ml Detection Buffer.
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- 14 Mix 45 μ l NBT solution and 35 μ l BCIP solution in 10 ml of Detection Buffer. This color substrate solution must be prepared freshly.
 - ! Alternatively, chemiluminescent detection can be performed, using the DIG Luminescent Detection Kit.
- 15 Pour off the Detection Buffer and add the color substrate solution. Allow the color development to occur in the dark. The color precipitate starts to form within a few minutes and continues for approx. 16 h. Do not shake while the color is developing.
- 16 When the spots appear in sufficient intensity, stop the reaction by washing the membrane with TE buffer or double dist. water for 5 min.
- 17 Compare spot intensities of the control and experimental dilutions to estimate the concentration of the experimental probe (See Figure 1).

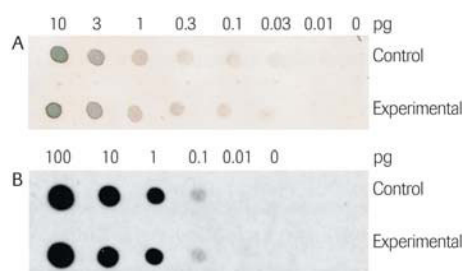


Figure 1: Estimating the Yield of DIG-labeled DNA. Dilutions of the Labeled Control DNA and the newly labeled (experimental) DNA were spotted on, fixed to, and directly detected on a Roche Applied Science Nylon Membrane, with colorimetric (Panel A) or chemiluminescent detection (Panel B).