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

PCR DIG Probe Synthesis Kit

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For generating highly sensitive probes labeled with DIG-dUTP (alkali-labile) in PCR.

Cat. No. 11 636 090 910 1 kit
25 reactions of 50 µl final volume each. One reaction can produce enough labled probe to analyze 650 cm^2 of blot membrane.

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Label	Function / Description	Content
1	PCR DIG Probe Synthesis Kit, Enzyme mix	▪ 105 U; 3.5 U/µl ▪ Enzyme storage buffer: 20 mM Tris-HCl, pH 7.5 (+25°C), 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Tween 20 (v/v), 0.5% Nonidet P-40 (v/v), 50% glycerol (v/v). ▪ For PCR labeling.	1 vial, 30 µl
2	PCR DIG Probe Synthesis Kit, PCR DIG probe synthesis mix, 10x conc.	▪ Nucleotide mix containing 2 mM each dATP, dCTP, dGTP, 1.3 mM dTTP, 0.7 mM DIG-11-dUTP, alkali-labile; pH 7.0. ⚠ ***Contains a mixture of nucleotides, including DIG-dUTP. The concentration of DIG-dUTP differs from a similar reagent, the PCR DIG Labeling Mix*. Do not interchange these two different nucleotide mixes. The PCR DIG probe synthesis mix (Vial 2) contains a higher concentration of DIG-dUTP to achieve maximal DIG incorporation and probe sensitivity.*** ▪ For PCR labeling.	1 vial, 125 µl
3	PCR DIG Probe Synthesis Kit, PCR buffer with $MgCl_2$, 10x conc.	▪ Reaction buffer containing 15 mM $MgCl_2$. ▪ For PCR labeling.	1 vial, 1 ml
4	PCR DIG Probe Synthesis Kit, dNTP-stock solution, 10x conc.	▪ Solution containing 2 mM each dATP, dCTP, dGTP, dTTP, pH 7.0. ▪ To adjust the DIG-dUTP concentration in the amplification/labeling reaction.	1 vial, 125 µl
5	PCR DIG Probe Synthesis Kit, Control template	▪ 1 ng plasmid DNA (100 pg/µl) in Tris/EDTA buffer, pH 8.0. ▪ The 5 kb plasmid carries the cDNA for human tissue type plasminogen activator (tPA). ▪ Template for the control reaction.	1 vial, 50 µl
6	PCR DIG Probe Synthesis Kit, Control PCR primer mix	▪ 50 pmol of control PCR primer 1 and 2 (2 µM each). ▪ PCR primers for the control reaction.	1 vial, 25 µl

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
1	Enzyme mix	Store at −15 to −25°C.
2	PCR DIG probe synthesis mix, 10x conc.	
3	PCR buffer with $MgCl_2$, 10x conc.	
4	dNTP-stock solution, 10x conc.	
5	Control template	
6	Control PCR primer mix	

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- Nuclease-free, aerosol-resistant pipette tips
- Pipettes with disposable, positive-displacement tips
- Sterile reaction tubes for preparing reaction mixes and dilutions
- Standard benchtop microcentrifuge
- Thermal cycler
- PCR vessels, such as thin-walled PCR tubes

For PCR

- Mineral oil (optional)
- PCR primers
- Template DNA
- Water, PCR grade

For checking the labeling efficiency

- Agarose gel electrophoresis apparatus
- Agarose
- TAE* or TBE* buffer
- Ethidium bromide

1.4. Application

The PCR DIG Probe Synthesis Kit is specifically designed for a variety of applications:

- Generation of highly sensitive hybridization probes suitable for the detection of low- (single) copy target sequences.
- Alternative to random-primed labeling when either the amount of template DNA is limited or only a specific part of the target sequence is required for hybridization.
- The nucleotide concentration in the kit is optimized for the identification of single-copy genes in genomic blots following hybridization to DIG-labeled PCR products.
  Human single-copy genes are typically detectable in 10 µg of genomic DNA.
- PCR products can be directly generated and labeled from small amounts of genomic DNA (100 ng to 1 µg), and subsequently used as hybridization probes.
- The alkali-labile DIG-11-dUTP formulation in the kit enables simple removal of the DIG label following chemiluminescent detection and allows the subsequent re-hybridization of blots with multiple DIG-labeled probes.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Use purified DNA containing the sequence to be labeled:
- Plasmid DNA: 100 to 500 pg, or
- Genomic DNA: 1 to 50 ng

⚠ ***Template concentration during PCR is the most critical factor in producing specific probes. For most templates, use no more than the amounts shown. Too much template will lead to coamplification of primary extension products (those copied past the priming sites). These primary extension products may contain repetitive sequences or unrelated products from secondary priming sites (if prepared from genomic DNA) or vector sequences (if prepared from plasmid DNA). In subsequent hybridization assays, the probe-target hybrid will be a smear because the probe will cross-hybridize with vector or genomic DNA sequences.***

⚠ ***For best results, use cloned inserts as template. Genomic DNA can be more difficult to use.***

ⓘ *Purity of template is not as critical for PCR labeling as for other types of labeling. For best results, use one of the following kits to prepare your template:*

If you are isolating...	Then prepare template with the...
genomic DNA from blood, buffy coat, cultured cells, tissue, mouse tail, yeast, bacteria, or paraffin-embedded tissue sections,	High Pure PCR Template Preparation Kit*
plasmid DNA from bacterial cultures,	▪ High Pure Plasmid Isolation Kit*, or ▪ Genopure Plasmid Midi/Maxi Kits* ▪ Any quick preparation methods, even simple boiling of cells.
viral DNA,	High Pure Viral Nucleic Acid Kit*

Control Reactions

Unlabeled positive control

Identical to experimental sample, except the reaction mix contains no DIG-dUTP.

⚠ ***Include this control reaction in every experiment. It is required for evaluating probe labeling efficiency.***

Labeled positive control

Substitutes the tissue plasminogen activator (tPA) template and primers included in the PCR DIG Probe Synthesis Kit for the experimental template and primers. It produces a labeled probe that recognizes human tPA sequences. The tPA probe generated in the control reaction recognizes a restriction length polymorphism (RFLP) for an EcoRI site in human DNA. This results in the detection of variable fragment patterns depending on the human DNA used. One of the following fragment patterns of human single-copy tPA gene should be detected:
- 2.9 kb + 1.7 kb
- 2.5 kb + 1.7 kb
- 2.9 kb + 2.5 kb + 1.7 kb

ⓘ *This control reaction is optional for the experienced user.*

General Considerations

Labeling efficiency

- Optimal reaction conditions depend on the template DNA and primer, incubation times and temperatures, and the concentration of Mg^{2+} and enzyme.
- To achieve best results, optimize the concentration of the template DNA and primer for each new primer/template pair.

PCR parameters

- Optimize PCR parameters, such as cycling conditions, template concentration, primer sequence, and primer concentration, for each template and primer set in the absence of DIG-dUTP before attempting incorporation of DIG.
- Use the isolated target DNA and the designed PCR primers to perform a series of test PCRs, for example, using different concentrations of Mg^{2+} and different thermal cycler programs.

DIG-dUTP concentration

DIG-labeled nucleotides in the template slow the DNA polymerase and eventually reduce the ability of the polymerase to synthesize full length products that contain the primer sequences needed for the start of the next round of amplification. As the template gets longer, this effect increases. Also, DNA templates with high GC content are not efficiently amplified in the presence of the “standard” final concentration of DIG-dUTP (70 µM). For such templates, reduce the concentration of DIG-dUTP in the reaction by diluting the PCR DIG probe synthesis mix (Vial 2) with the dNTP-stock solution, 10x conc. (Vial 4). For details, see section, **Protocols, Preparation of reaction mix**.

i *Lower concentrations of DIG will not influence the final sensitivity of the hybridization probe.*

Safety Information

For customers in the European Economic Area

Contains SVHC: octyl/nonylphenol ethoxylates. For use in research and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation.

2.2. Protocols

Preparation of reaction mix

For each experimental or control sample, add the following components to a sterile microcentrifuge tube. Place the tube on ice during pipetting.

1. Thaw the reagents and store on ice.
   – Briefly vortex and centrifuge all reagents before setting up the reactions.

2. Prepare a 10x-concentrated solution (1 to 10 µM) of the forward and reverse PCR primer.

3. For each 50 ml reaction, add the components in the order listed below to a sterile reaction tube on ice:

Component	Volume required for			Final conc.
	DIG-labeled experimental probe [µl]	Unlabeled control probe [µl]	DIG-labeled tPA control probe [µl]	
Water, PCR Grade	Add up to 50	Add up to 50	29.25	–
PCR buffer with $MgCl_2$, 10x conc. (Vial 3)	5	5	5	1x[2]
PCR DIG probe synthesis mix (Vial 2)[1]	5	–	5	200 µM dATP, dCTP, dGTP, 130 µM dTTP, 70 µM DIG-dUTP
dNTP-stock solution (Vial 4)[1]	–	5	–	200 µM each dNTP
Forward PCR primer, 10x conc.	5	5	5[3]	0.1 – 1 µM
Reverse PCR primer, 10x conc.	5	5	–	0.1 – 1 µM
Enzyme mix (Vial 1)	0.75	0.75	0.75	–
Template DNA	Variable	Variable	5[4]	1 to 50 ng genomic DNA
				100 to 500 pg plasmid DNA
Total Volume	**50 µl**	**50 µl**	**50 µl**	

4. Mix the reagents and centrifuge briefly to collect the sample at the bottom of the tube.

[1] Adjusting the DIG-dUTP concentration:
– The final concentration of DIG-dUTP (70 µM) when using the undiluted PCR DIG probe synthesis mix (Vial 2) works well for labeling probes up to 1 kb long.
– For labeling probes 1 to 3 kb long, reduce the final concentration of DIG-dUTP to 35 µM. Mix equal parts of the PCR DIG probe synthesis mix (Vial 2) and dNTP-stock solution (Vial 4).
– For labeling short probes (<1 kb) of high GC content, it might also be necessary to reduce the DIG-dUTP concentration to 35 µM.
– For labeling probes >3 kb, reduce the final concentration of DIG-dUTP to 35 µM and use the Expand Long Range enzyme mix* instead of the Expand High Fidelity mix included in the PCR DIG Probe Synthesis Kit. Depending on the specific target sequence it might be necessary to gradually lower the concentration of DIG-dUTP to a minimum of 7 µM. In this case, mix the PCR DIG probe synthesis mix (Vial 2) with the appropriate volume of dNTP-stock solution (Vial 4), for example, mix one part PCR DIG probe synthesis mix and nine parts dNTP-stock solution to achieve a DIG-dUTP final concentration of 7 µM.

[2] Depending on the results of your optimization experiments, adjust the concentration of $MgCl_2$ to achieve optimal amplification.

[3] Use the Control PCR primer mix (Vial 6); final primer concentration is 0.2 µM.

[4] Use the Control template (Vial 5).

PCR

Optimal PCR conditions depend on the specific combination of template, primers, and thermal cycler. For best results, optimize the PCR for the template and primer pair. Use the following conditions as a starting point for initial experiments. They are designed for amplification of a 3 kb fragment.

1. Optional step: Depending on the thermal cycler, overlay the reaction reagents with mineral oil to prevent evaporation of the reagents during the thermal cycling process.

2. Place your samples in the thermal cycler and start PCR.

Program	Cycles	Temp. [°C]	Duration [s]
Initial denaturation	1	95	120
Pre-Incubation	1 – 10	95	30
Annealing		60	30
Elongation		72	40
Pre-Incubation	11 – 30	95	30
Annealing		60	30
Elongation		72	40 + 20 seconds for each additional cycle[1]
Final elongation	1	72	420

3. Use 5 µl of the reaction mixture to verify the results of the reaction.

4. Store the remaining reaction mixture short-term at +2 to +8°C or long-term at −15 to −25°C.

If stored at −15 to −25°C, labeled probes are stable for at least 1 year.

PCR-generated probes are very pure and can be used directly in the hybridization reaction. No further cleanup is required.

[1] The increased elongation time is only required for long (>3 kb) probes. For amplification of shorter probes, use 40 seconds elongation time for all 30 cycles.

Checking the labeling efficiency

1. Run 5 µl of each reaction on an agarose mini gel in TAE or TBE buffer, along with a DNA molecular weight marker.

2. Stain the gel with 0.5 µg/ml ethidium bromide.

3. Examine the bands on the gel. A successful labeling reaction is described in the table.

Probe	Characteristic
tPA control probe	Size: 500 – 550 bp *Actual size of the amplicon is 442 bp. The presence of DIG in DNA makes it run slower in the gel than unlabeled DNA.*
Labeled experimental probe and unlabeled control probe	Visible on gel.
Unlabeled control probe	Predicted size
Labeled experimental probe	Migrates slower (appears larger) than the unlabeled control probe due to the presence of DIG.
Ethidium bromide staining of the labeled DNA	Equal to or slightly less than that of the unlabeled control DNA due to the presence of DIG.

4 If the characteristics in Step 3 are met, the probe has been labeled successfully.
Use the probe at the recommended concentration (2 µl/ml hybridization solution) in blot hybridization protocols.

If the amount of labeled PCR product band is very strong on the gel, use as little as 0.5 µl probe per ml hybridization buffer. If the signal is very faint, use up to 4 µl probe per ml hybridization buffer.

Downstream procedures

1. Standard protocols for gel electrophoresis, denaturation and neutralization of the gel, and transfer and fixation of DNA to a membrane are described in the literature.
   Gels lacking ethidium bromide are preferred as it can cause uneven background. All common types of DNA transfer methods are suitable for subsequent DIG hybridization. Best results are obtained when gels are blotted by capillary transfer with 20x SSC on Nylon Membranes, positively charged*.*

2. Detailed procedures for using the DIG-labeled probe for the detection of human genomic DNA on a Southern blot or dot blot are also described in the literature. Use DIG Easy Hyb buffer* and Hybridization Bags for best results. For detailed information about the hybridization protocol see the DIG Easy Hyb buffer Instructions for Use.
   Do not allow the membrane to dry at any time. If the membrane dries or sticks to a second membrane, for example, during simultaneous processing of blots, the assay will have a high background.

3. For the chemiluminescent detection of the DIG-labeled probe use:
   – DIG Luminescent Detection Kit* or
   – Anti-digoxigenin-AP, Fab fragments* and CDP-Star* or CSPD*
   – DIG Wash and Block Buffer Set*

4. Analyze the hybridized membrane immediately using the immunological detection procedure or store the membrane sealed in a plastic bag for later analysis.
   – The immunological detection procedure involves blocking the membrane and using the anti-DIG-AP conjugate* to detect DIG labeled RNA on the membrane.
   – Use a chemiluminescent substrate such as CSPD* or CDP-Star* to visualize the antibody-probe complexes on the membrane.

5. Expose the membrane to X-ray film or Lumi-Film*, or an imaging device for 10 to 30 minutes.
   – The membranes can be stripped and re-used for hybridization, provided the membrane did not dry at any time throughout the procedure.

Detailed detection procedures are included in the DIG Luminescent Detection Kit Instructions for Use for easy and sensitive detection of DIG-labeled probes.*

3. Troubleshooting

Observation	Possible cause	Recommendation
Low yield of DIG-labeled PCR product.	PCR is not optimized.	Always optimize the PCR parameters: cycling conditions, template concentration, primer sequence, and primer concentration for each template and primer set in the absence of DIG-dUTP, before attempting incorporation of DIG.
	Too much DIG-dUTP in reaction.	Reduce the concentration of DIG-dUTP in the reaction. ℹ *This is especially important for long templates, see section,* **General Considerations**.
Cloudy hybridization background.	Probe concentration too high in the hybridization solution.	Reduce probe concentration to 1 µl probe per ml DIG Easy Hyb buffer*. ⚠ ***Evaluate the amount of labeled probe in the PCR product. If the amount of labeled PCR product band is very strong on the gel, use as little as 0.5 µl probe per ml hybridization buffer.***
Hybridization smear	Template concentration too high during PCR.	For best results, use only small amounts of template. Optimal amounts are 100 to 500 pg plasmid DNA or 1 to 50 ng genomic DNA. ⚠ ***Higher concentrations of template may lead to large amounts of primary extension products in the labeled probe.***
	Target concentration on the blot too high.	Use 1 to 5 µg genomic DNA per lane or <1 ng plasmid DNA per lane.

4. Additional Information on this Product

4.1. Quality Control

For lot-specific certificates of analysis, see section **Contact and Support.**

5. Supplementary Information

5.1. 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	
ℹ *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.

5.2. Changes to previous version

Concentration of the control template has been corrected.
The range of the amount of template plasmid DNA has been adapted accordingly.
Layout changes.
Editorial changes.
New information added related to the REACH Annex XIV.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Buffers in a Box, Premixed TAE Buffer, 10x	4 l	11 666 690 001
Buffers in a Box, Premixed TBE Buffer, 10x	4 l	11 666 703 001
High Pure PCR Template Preparation Kit	1 kit, up to 100 purifications	11 796 828 001
High Pure Plasmid Isolation Kit	1 kit, 50 purifications	11 754 777 001
	1 kit, 250 purifications	11 754 785 001
Genopure Plasmid Midi Kit	1 kit, 20 preparations	03 143 414 001
High Pure Viral Nucleic Acid Kit	1 kit, up to 100 isolations	11 858 874 001
Expand Long Range dNTPack	175 U, 1 x 175 U, 50 reactions in a final volume of 50 µl	04 829 034 001
	700 U, 1 x 700 U, 200 reactions in a final volume of 50 µl	04 829 042 001
	3,500 U, 5 x 700 U, 1,000 reactions in a final volume of 50 µl	04 829 069 001
Buffers in a Box, Premixed SSC Buffer, 20x	4 l	11 666 681 001
DIG Easy Hyb	500 ml	11 603 558 001
DIG Luminescent Detection Kit	1 kit, 50 blots with a size of 10 x 10 cm^2	11 363 514 910
Anti-Digoxigenin-AP, Fab fragments	150 U, 200 µl	11 093 274 910
CDP-*Star*, ready-to-use	2 x 50 ml	12 041 677 001
DIG Wash and Block Buffer Set	1 set, 30 blots (100 cm^2)	11 585 762 001
PCR DIG Labeling Mix	2 x 250 µl, 2 x 25 PCR assays of 100 µl final volume each	11 585 550 910
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
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

5.4. Trademarks

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

5.5. License Disclaimer

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

5.6. Regulatory Disclaimer

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

5.7. Safety Data Sheet

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

5.8. 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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