For general laboratory use.
FOR *IN VITRO* USE ONLY.

PCR DIG Probe Synthesis Kit

For generation of highly-sensitive probes labeled with DIG-dUTP (alkali-labile) in the polymerase chain reaction (PCR)

Cat. No. 1 636 090

Kit for 25 polymerase chain reactions (50 µl)

Version 4, August 2000

Store at −15 to −25° C

1. Kit contents

Caution

Vial 2 (PCR DIG probe synthesis mix)

This mix from this kit contains a mixture of nucleotides, including the DIG-dUTP. The concentration of DIG-dUTP provided here differs from a separate yet similar reagent, the PCR DIG labeling mix (Cat. No. 1 585 550). These nucleotide mixes **should not be interchanged**. Vial 2 (PCR DIG probe synthesis mix) contains a higher concentration of DIG-dUTP to achieve maximal DIG incorporation, and maximal probe sensitivity. Only vial 2 should be used with this PCR DIG Probe Synthesis Kit.

Bottle/ Cap	Label	Content including function	Cat. No. (if available separately)
1	Enzyme mix, Expand[1] High Fidelity	• 30 µl enzyme mix (105 units) • 3.5 units/µl • Storage buffer; 20 mM Tris-HCl, pH 7.5 (25°C), 100 mM KCl, 1 mM dithiothreitol (DDT), 0.1 mM EDTA, 0.5% Tween[2] 20 (v/v)*, 0.5% Nonidet[3] P40 (v/v)* , 50% glycerol (v/v). • Enzyme mix for the labeling of PCR product	1 732 641
2	PCR DIG probe synthesis mix, 10 × conc.	• 125 µl • Mixture containing dATP, dCTP, dGTP (2 mM each); 1.3 mM dTTP; 0.7 mM DIG-11-dUTP, alkali-labile; pH 7.0. • Nucleotide mix for the labeling of PCR product	
3	PCR buffer with $MgCl_2$, 10 × conc	• 1 ml • Expand High Fidelity buffer 10 × conc. with 15 mM $MgCl_2$ • Buffer for the PCR labeling reaction	
4	dNTP stock solution, 10 × conc.	• 125 µl • Mixture contains dATP, dCTP, dGTP, dTTP (2 mM each), pH 7.0. • Solution for the possible dilution of the PCR labeling reaction	1 581 295
5	Control template	• 50 µl • 1 ng plasmid DNA [20 pg/ml] in Tris/EDTA buffer; pH 8.0. • The 5 kb plasmid contains the cDNA for human tissue type plasminogen activator (tPA). • Template for the control reaction	
6	Control PCR primer mix	• 25 µl • [50 pmol] of control PCR primer 1 and 2 (2 mM each). • Primer for the control reaction	

2. Product overview

Kit description

The PCR DIG Probe Synthesis Kit contains all reagents required for the direct digoxigenin (DIG)-labeling of DNA fragments generated by the polymerase chain reaction (PCR) process (1, 2). The polymerase chain reaction is ideally suited to prepare specific and efficiently labeled hybridization probes. The kit enables the synthesis of highly sensitive probes by incorporation of DIG-dUTP into the PCR product. The nucleotide concentration in the PCR DIG Mix ensures the detection of single-copy genes in genomic blots after hybridization to DIG-labeled PCR products.

The kit includes the new enzyme mix Expand[1)] High Fidelity (e.g. Cat. No. 1 732 641) to ensure maximal yield and highest accuracy. Furthermore a dNTP stock solution is supplied to allow adjustment of the concentration of labeled nucleotide. A plasmid containing a cDNA for a human single-copy gene and the respective primers serve as a control for the efficiency of the PCR labeling reaction.

Synthesis principle

PCR products can directly be amplified and labeled from low amounts of plasmid or genomic DNA and subsequently be used as hybridization probes without further purification.

Basic steps

Please refer to the following table.

Stage	Description
PCR labeling	DIG-labeled DNA probes are generated according to the PCR labeling technique.
Hybridization	DIG-labeled probes are used for hybridization to membrane blotted nucleic acids according to standard methods. The use of the alkali-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for rehybridization with a second DIG-labeled probe.
Immunological detection	The hybridized probes are immunodetected with anti-digoxigenin-AP, Fab fragments and are then visualized with a chemiluminescent substrate (CDP-*Star*® or CSPD®) or by colorimetric detection with e.g. NBT/ BCIP.

Application

The kit is especially designed for generation of highly sensitive hybridization probes suitable for detection of low (single) copy target sequences. Besides this the alkali-labile form of DIG-dUTP generated a probe which is easily used for rehybridizations of (genomic) blots by merely stripping of.

Labeling efficiency

Optimal reaction conditions are dependent on template DNA and primer. In particular incubation times and temperatures, concentration of Mg^{2+} and enzyme but also concentration of template DNA and primer should be optimized for optimal results for each new primer/ template pair (3).

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Sample material

Partially purified DNA containing the sequence to be labeled. Use either:

- Plasmid DNA, 10–100 pg (optimal amount, 10 pg)
- Genomic DNA, 1–50 ng (optimal amount, 10 ng)

Note: Purity of template is not as critical for PCR labeling as for other types of labeling.

Number of tests

1 kit is sufficient for 25 polymerase chain reactions.

Quality Control

The PCR DIG Probe Synthesis Kit is function-tested in PCR. Amplification products are assayed in genomic Southern blots. Under PCR conditions described the control reaction generates an amplification product of 442 bp. Due to multiple incorporation of DIG-dUTP during the PCR process the molecular weight of the PCR product is increased significantly compared to the unlabeled PCR product. After hybridization of the PCR product to 5 µg human genomic DNA and chemiluminescent detection a specific fragment pattern is detected.

Kit storage/ stability

The unopened kit is stable at -15 to -25°C until the expiration date printed on the label. Shipping conditions on dry ice.

Sensitivity

The nucleotide concentration in the PCR DIG probe synthesis mix ensures the identification of single-copy genes in genomic blots after hybridization to DIG-labeled PCR products. Human single-copy genes typically are detectable in 5 µg of genomic DNA.

Advantages

Benefits	Feature
Small amounts of template DNA	Because incorporation of DIG-dUTP into the PCR product produce highly sensitive probes.
Purity of template DNA is not as critical for PCR labeling	As for other types of labeling the purity of template is not critical, especially random primed labeling. For example, suitable templates include partially purified plasmid prepared by any of a variety of "quick preparation" methods, even simple boiling of cells.
The labeling reaction requires less optimization than other methods	• Can efficiently use GC-rich regions as template • For most templates, no optimization of $MgCl_2$ concentration is required; most labeling reactions will work with the standard concentrations of 1.5 mM $MgCl_2$.
Large quantities of DIG-labeled probes.	Yield of labeled probe is very high.
dNTP stock solution	Because of their unique sequences, some large fragments (larger than 1 kb) amplify poorly when high concentrations of DIG-dUTP are used. If a template produces yields which are insufficient, we supply dNTP stock solution to dilute the DIG-dUTP. Please see „Procedure" and „Trouble Shooting" for additional instructions.
Evaluation of labeling efficiency	Very quickly by gel electrophoresis
No purification of labeled PCR product necessary	PCR-generated probes are very pure and can be used directly in the hybridization reaction.
The PCR DIG Probe Synthesis Mix contains the alkali-labile form of DIG-dUTP.	Subsequent rehybridizations of (genomic) blots by merely stripping off the DIG molecule under alkaline conditions.

3. Procedures and required materials

Before you begin

Critical Hints about PCR Labeling

Please refer to the following table:

PCR amplification parameters	Optimize PCR amplification 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.
DIG-dUTP concentration	According to the length of the probe being labeled the concentration of DIG-dUTP has to be adapted: • < 1 kb, use a 1:3 ratio of DIG-dUTP : dTTP (standard labeling mix vial 2). ***Note:*** DNA containing a high GC content may require a 1:6 ratio of DIG-dUTP : dTTP. • > 1 kb, use a 1:6 ratio of DIG-dUTP : dTTP. Mix equal parts of PCR DIG probe synthesis mix (vial 2) and dNTP stock solution (vial 4) • > 3 kb, use a 1:6 ratio of DIG-dUTP : dTTP and substitute the Expand Long Template enzyme mix for the Expand High Fidelity enzyme mix included in the PCR DIG Probe Synthesis Kit. ***Note:*** You may eventually need to reduce the DIG-dUTP : dTTP ratio as low as 1:10.

Unlabeled positive control

Identical to experimental sample except the reaction mix contains no DIG-dUTP.
Note: Always 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 Probe Synthesis Kit) for your experimental template and primers. Produces a labeled probe that recognizes human tPA sequences.

The tPA probe generated in the control PCR recognizes a restriction length polymorphism (RFLP) for an *Eco* RI site in human DNA (5). 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

Note: As you gain experience with the labeling kit, you may choose not to run this control reaction.

3.1 PCR reaction

Additional equipment and reagents required

- all equipment needed for PCR reactions
- sterile double dist. water
- mineral oil (optional, depending on your PCR equiment)
- Primer:
  - 1–10 µM solution of upstream primer
  - 1–10 µM solution of downstream primer
- Template DNA

Procedure

Cycling conditions depend on the combination of template, primers, and thermal cycler. The conditions given below may not be optimal for your template/primer combination, but are a good starting point for initial experiments.

Step 1 — Add the following components to a sterile microcentrifuge tube on ice:

Reagent	DIG labeled probe	Unlabeled DNA control	Labeled kit control	Final conc.
Sterile double dist. water	variable	variable	29.25 µl	-
PCR buffer with $MgCl_2$, 10 x conc., (vial 3)	5 µl	5 mµl	5 µl	1x
PCR DIG Labeling Mix (vial 2)	5 µl	-	5 µl	200 µM dNTP
dNTP stock solution (vial 4)	-	5 mµl	-	200 µM dNTP
Upstream and downstream primer	variable	variable	5 µl (vial 6)	0.1 -1 µl each
Enzyme mix (vial 1)	0.75 µl	0.75 mµl	0.75 µl	2.6 units
Template DNA	variable	variable	5 µl (vial 5)	
Total	**50 µl**	**50 µl**	**50 µl**	

Step 2 — Mix the reagents and centrifuge briefly to collect the sample at the bottom of the tube.

Step 3 — Overlay with 100 µl mineral oil to reduce evaporation of the mix during amplification.
Note: If your thermal cycler has a top heater, the oil overlay is not necessary.

Step 4 — Place samples in the thermal cycler and start PCR.

Cycle number	Reaction	Temperature	Time period
	Initial denaturation	95°C	2 min
Cycles 1 - 10	Denaturation Annealing Elongation	95°C 60°C 72°C	30 s 30 s 40 s
Cycles 11 - 30	Denaturation Annealing Elongation	95°C 60°C 72°C	30 s 30 s 40 s + additional 20 s for each sucessive cycle
	Note: The increased elongation time is only required for long (3 kb) fragments. For amplificatin of shorter fragments, continue to use the 40 s elongation time for all 30 cycles.		
	Final elongation step	72°C	7 min
	Hold	4°C	

Step 5 — The PCR labeled probe should be stored:

short term	at 2-8°C until the PCR product is used for hybridization
long term	at -18 to -25°C, stable for at least one year.

Analysis of labeled probe

The best way to check the success of the reaction is to run a portion (5 µl) of each reaction on an agarose mini gel and then stain the gel with ethidium bromide (EtBr).

- The tPA control probe will have an apparent size of 500–550 bp.
  Note: The actual size of the amplicon is 442 bp. The presence of DIG in DNA makes it run slower in the gel than unlabeled DNA. The agarose concentration of the gel determines the extent to which the migration rate deviates from its "true" value.
- Your unlabeled control probe should be the predicted size.
- Both the labeled experimental probe and the unlabeled control probe should be clearly visible on the gel.
- Your labeled experimental probe should migrate slower than your unlabeled control probe (due to the presence of DIG).
- The EtBr staining of the labeled DNA will be somewhat less than the unlabeled control DNA.
  Note: When you are using a high ratio of DIG-dUTP : dTTP (1:3), the reaction will make less labeled probe than unlabeled probe. The polymerase is slowed by the presence of the DIG hapten.

3.2 DNA electrophoresis, transfer, and fixation

General

Standard protocols for gel electrophoresis, denaturation and neutralization of the gel are described in Sambrook et al. (6). Gels lacking ethidium bromide are preferred, because ethidium can cause uneven background problems. All common types of DNA transfer methods are suitable for subsequent DIG hybridization (7,8).

In our experience, best results are obtained when gels are blotted by capillary transfer with 20x SSC on nylon membranes, positively charged* (Cat. No. 1 209 272).

Note: Alkali transfer (e.g., in 0.4 M NaOH) is not suitable for the transfer of DIG-labeled molecular weight markers*.

Overview

Stage	Description
1	Gel electrophoresis.
2	Denaturation and neutralization of DNA in the gel.
3	Fixation of DNA on the membrane.

Electrophoresis

We recommend the following:

- Prepare a suitable electrophoresis gel as thin as possible.
- Load small amounts of the target DNA samples:

genomic DNA	1–5 µg per lane
plasmid DNA	< 1 ng per lane
DIG-labeled Molecular Weight Marker* ***Note:*** The needed will depend on the expected size of your hybridization product. Make sure to load enough marker to produce prominent bands that are about the same size as your hybrid.	2-5 µl per lane

- Run the gel until the DNA bands are well separated.
- To assess the quality of the target DNA, stain the gel briefly in 0.25–0.50 µg/ml ethidium bromide, then destain with water.
- Examine the gel under UV light.

Fixation procedure

After the transfer, while the blot is still damp, fix the DNA to the blot by either of the following methods:

IF you want to...	THEN...
UV-crosslinking (nylon membrane)	• 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.
bake at 120°C (nylon membrane)	• wash the membrane briefly in 2x SSC. • bake the nylon membrane at 120°C for 30 min or according to the manufacturer`s instructions.

Storage of membrane

Please refer to the following table.

IF...	THEN...
you want to go ahead.	use the membrane immediately for prehybridization.
you want to work later on	store the membrane dry at 2 to 8°C.

3.3 Hybridization with the DIG-labeled Probe

Hybridization

The procedures for using the DIG-labeled probes for the detection of human genomic DNA on a Southern blot can also be used for the detection on Dot blots.

We recommend to use

- DIG Easy Hyb buffer* (Cat. No. 1 603 558) and
- Hybridization bags* (Cat. No. 1 666 649)

for best results. For detailed information about the Hybridization protocol please see the DIG Easy Hyb buffer package insert.

Probe concentration

The standard probe concentration is 2 µl probe per ml hybridization buffer.

- If the labeled PCR product band on the evaluation gel was very faint, use up to 4 µl probe per ml hybridization buffer.
- If the labeled band was very strong, use only 1 µl (or even as little as 0.5 µl) probe per ml hybridization buffer.

Overview

Do not allow the membrane to dry at any time from the beginning of prehybridization through probe-hybrid visualization. If the membrane dries or sticks to a second membrane (e.g., during simultaneous processing of blots), the assay will have a high background.

Stage	Description
1	Prehybridization of blot.
2	Hybridization with DIG-labeled probe.
3	Stringency washes.

Storage of membrane

After finishing the last high stringency wash, you may air dry the membranes and store it in a sealed bag at 2 to 8°C for later detection.

3.4 Chemiluminescent detection

Chemiluminescent detection

For the chemiluminescent detection of the labeled DIG-probe we recommend to use

- CDP-*Star* *(Cat. No. 2 041 677) or CSPD* (Cat. No. 1 755 633)
- DIG Block and Wash Buffer Set* (Cat. No. 1 585 762) and
- Anti-digoxigenin-AP, Fab fragments * (Cat. No. 1 093 274)

Overview

This table lists the single steps of the chemiluminescent detection.

Stage	Description
1	Washing and blocking of membrane.
2	Antibody binding.
3	Washing and equilibration of membrane.
4	Chemiluminescent reaction.
5	Film exposure.

For detailed information about the detection protocol please see the package insert of CDP-*Star* or CSPD.

3.5 Stripping and reprobing of DNA blots

General

The alkali-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for rehybridization experiment.

Additional reagents required

- 0,2 N NaOH, 0,1% SDS (w/v)
- 2x SSC

Protocol

Please refer to the following table.

Note: When stripping and rehybridization of blots is planned, the membrane should not dry off at any time.

Step	Action
1	Rinse membrane briefly in **double distilled water**.
2	Wash for 2x 15 min in **0.2 N NaOH, SDS, 0.1%** (w/v) at 37°C under constant agitation.
3	Equilibrate briefly in **2x SSC**.
4	Prehybridize and hybridize with the next probe according to the protocol.

4. Appendix

Trouble shooting table

This table describes various trouble shooting parameters for DIG-labeling and detection

Problem	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 [1]	Reduce the concentration of DIG-dUTP in the reaction. This is especially important for long templates, for further information see chapter 3.
Cloudy hybridization background	Probe concentration too high in the hybridization solution.	Reduce probe concentration to 1 µl probe per ml DIG Easy Hyb buffer. ***Note:*** Evaluate the amount of labeled probe in the PCR product. If the amount of labeled PCR pro-duct 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. Ideal amounts: 10 pg plasmid DNA or 10 ng genomic DNA. ***Note:*** Our experience indicates that higher concentrations of template lead to large amounts of primary extension products in the labeled probe.
	Target concentration too high on the blot	We recommend to use 1–5 µg per lane genomic DNA or < 1 ng plasmid DNA per lane

References

1 Saiki, R. et al. (1985) *Science* **230,** 1350–1354.
2 Lion, T. & Haas, O.A. (1990) *Anal. Biochem.* **188,** 335–337.
3 Rolfs, A. et al. (1992) PCR: Clinical Diagnostic and Research, Springer Verlag, Berlin.

Related products

Kits

Product	Pack Size	Cat. No
DNA Isolation Kit for Cells and Tissue	10 isolations for 400 mg tissue or 5 x 10^7 cells	1 814 770
High Pure Plasmid Isolation Kit	50 purifications 250 purifications	1 754 777 1 754 785
High Pure PCR Product Purification Kit	1 kit (50 purifications) 1 kit (250 purifications)	1 732 668 1 732 676
High Pure PCR Template Preparation Kit	1 kit (100 reactions)	1 796 828
Plant DNA Isolation Kit for convenient isolation of plant genomic DNA	up to 150 purifications	1 667 319

Single reagents

Product	Pack Size	Cat. No.
Agarose MP	20 g 100 g 500 g	1 444 964 1 388 983 1 388 991
Blocking reagent	50 g	1 096 176
CDP-Star	1 ml 2 ml	1 685 627 1 759 051
CDP-Star, ready-to-use	2x 50 ml	2 041 677
CSPD	1 ml 2 ml 4 ml	1 655 884 1 759 035 1 759 043
CSPD, ready-to-use	2x 50 ml	1 755 633
NBT/BCIP ready to use tablets	20 tablets	1 697 471
NBT/BCIP Stock solution	8 ml	1 681 451
DIG Easy Hyb (ready-to-use hybridization solution without formamide)	500 ml	1 603 558
DIG Easy Hyb Granules	1 set (6 x 100 ml)	1 796 895
DIG Quantification Test Strips	50 strips	1 669 958
DIG Wash and Block Buffer Set	30 blots (10 x 10 cm^2)	1 585 762
DNA Molecular Weight Marker, Digoxigenin-labeled: DNA MWMII DNA MWMIII DNA MWM V DNA MWM VI DNA MWM VII DNA MWM VIII	 5 µg (500 µl) 5 µg (500 µl) 5 µg (500 µl) 5 µg (500 µl) 5 µg (500 µl) 5 µg (500 µl)	 1 218 590 1 218 603 1 669 931 1 218 611 1 669 940 1 449 451
Hybridization bags	50 bags	1 666 649
Lumi-Image F1	240 Volt 120 Volt	2 015 170 2 012 847
Nylon Membrane, positively charged (20 x 30 cm) (10 x 15 cm) (0.3 x 3 m roll)	 10 sheets 20 sheets 1 roll	 1 209 272 1 209 299 1 417 240

* available from Roche Molecular Biochemicals
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E-mail Adress	Country
argentina.biochem@roche.com	Argentina
biochem.au@roche.com	Australia
Gerhard.Muehlbauer@roche.com	Austria
biochem.be@roche.com	Belgium
Valent@mbox.cit.bg	Bulgaria
africhem@camnet.cm	Cameroon
biochem.ca@roche.com	Canada
biochem.cn@roche.com	China
Info@medisell.com.cy	Cyprus
Bm-comp@bm-comp.cz	Czech Republic
dk.biochem@roche.com	Denmark
ou.melestrum@neti.ee	Estonia
pharsc.et@telecom.net.et	Ethiopia
helsinki.biochem_diagnostics@roche.com	Finland
biochem.fr@roche.com	France
mannheim.biochemInfo@roche.com	Germany
Bm_roche@hotmail.com	India
h.hajian@tebtech.com	Iran
tubanegin@istn.irost.com	Iran
Dyn@netvision.net.il	Israel
it.biochem@roche.com	Italy
biochemicals@rdj.co.jp	Japan
pharmakp@net2000ke.com	Kenya
Bmskorea@chollian.net	Korea
react@ncc.moc.kw	Kuwait
Raitis@invitros.lv	Latvia
Sakkijha@rdleb.com	Lebanon
Gintaras@eksma.lt	Lithuania
diagnostics@prophac.lu	Luxembourg
Vccl@vol.net.mt	Malta
Aiouche.echo@dounia.net.ma	Morocco
biocheminfo.nl@roche.com	Netherlands
biochem.nz@roche.com	New Zealand
bofungwu@linkserve.com.ng	Nigeria
biochem.se@roche.com	Norway
biochem.pt@roche.com	Portugal
Topdiag@fx.ro	Romania
biochem.sg@roche.com	Singapore
roche.diagnostics@siol.net	Slovenia
south_africa.bioboffin@roche.com	South Africa
biochem.es@roche.com	Spain
biochem.se@roche.com	Sweden
BiochemInfo.CH@roche.com	Switzerland
Jean-Marie.kindbeiter@roche.com	Tunisia
bmuae@emirates.net.ae	United Arab Emirates
uk.biochem@roche.com	United Kingdom
biochemts.us@roche.com	USA
Mvalentiner@telcel.net.ve	Venezuela
dusica@eunet.yu	Yugoslavia
biochemts.row@roche.com	All other countries

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Argentina 541 954 5555; **Australia** (02) 9899 7999; **Austria** (01) 277 87; **Belgium** (02) 247 4930; **Brazil** +55 (11) 3666 3565; **Bulgaria** +35929625408; **Cameroon** 237-370269; **Canada** (450) 086 7050; (800) 361 2070; **Chile** 00 56 (2) 22 33 737 (central) 00 56 (2) 22 32 099 (Exec); **China** 86 21 6427 5586; **Colombia** 0057-1-3412797; **Cyprus** +357-2-311362; **Czech Republic** (0324) 45 54, 58 71-2; **Denmark** +45 363 999 58; **Egypt** 20-2-3619047; **Estonia** 372-7-447600; **Ethiopia** 251-1-552799; **Finland** +358 9 525 333 66; **France** 04 76 76 30 87; **Germany** (0621) 759 8568; **Greece** 3 (01) 67 40 238; **Hong Kong** (852) 2485 7596; **India** +91-22-8379906; **Indonesia** 62 (021) 252 3820 ext. 755; **Iran** +98-21-8072374 / +98-21-8797027; **Israel** 972-6- 6380569; **Italy** 039 247 4109-4181; **Japan** 03 3432 3155; **Kenya** +254-2-750112; **Korea** 82-2-3471-6500; **Kuwait** +965-4837859; **Latvia** 371-787828309; **Lebanon** Fax: 00961-1-399667; **Lithuania** 370-2-729715; **Luxembourg** +352-496098; **Malta** Fax: +356-341087; **Morocco** Fax: +212-2-944040; **Malaysia** 60 (03) 755 5039; **Mexico** (5) 227 8967; **Netherlands** (036) 539 4911; **New Zealand** (09) 276 4157; **Nigeria** +234-1-521767; **Norway** (47) 23 373300; **Philippines** (632) 810 7246; **Poland** +48 (22) 22 66 84 305; **Portugal** (01) 4171717; **Republic of Ireland** 1 800 40 90 41; **Romania** +40-1-2123763; **Russia** (49) 621 759 8636 Fax: (49) 621 759 8611; **Saudia Arabia** +966-1-4010364; **Singapore** 0065 272 9200; **Slovenia** +386 61 1363528; **South Africa** (011) 886 2400; **South Korea** 02 569 6902; **Spain** (93) 201 4411; **Sweden** (08) 404 8800; **Switzerland** +41 (41) 799 6161; **Taiwan** (02) 736 7125; **Thailand** 66 (2) 274 07 08 (12 line); **Turkey** 0090 212 216 32 80; **United Arab Emirates** +971-4-694351; **United Kingdom** (0800) 521578; **USA** (800) 428 5433. **Venezuela** Fax: +0058-4810697; **Yugoslavia** +381 11 137163.

Enzo

Roche

Roche Diagnostics GmbH
Roche Molecular Biochemicals
Sandhofer Strasse 116
D-68305 Mannheim
Germany