For general laboratory use.

Print Protocol | Print Changes to Previous Version | Print Safety Precautions | Print License Disclaimer

LightCycler® Multiplex DNA Master

Version 02

Content version: January 2015

Easy-to-use 5× reaction mix optimized for multiplex qPCR, compatible with the LightCyler® 480 or the LightCycler® 96 Real-Time PCR Systems

Cat. No. 07 339 585 001	1 kit 200 reactions of 20 µl final volume each
Cat. No. 07 339 577 001	1 kit 1,000 reactions of 20 µl final volume each

Store the kit at −15 to −25°C!

www.lifescience.roche.com

1. What this Product Does

Number of Tests

The kit is designed for 200 or 1,000 reactions with a final reaction volume of 20 µl each

Kit Contents

Vial/Cap	Label	Function	Catalog Number
1 red cap	qPCR Reaction Mix, 5× conc.	contains qPCR Reaction Buffer, AptaTaq Polymerase, dATP, dCTP, dGTP, and dUTP, $MgCl_2$, and proprietary additives	Cat. No. 07 339 585 001, 1 vial, 880 µl Cat. No. 07 339 577 001, 5 vials, 880 µl each
2 colorless cap	Water, PCR grade		Cat. No. 07 339 585 001, 3 vials, 1 ml each Cat. No. 07 339 577 001, 15 vials, 1 ml each

Storage and Stability

This kit is shipped on dry ice.

Store the kit at –15 to –25°C. This kit is stable until the expiration date printed on the label.

The kit is stable at +2 to +8°C for 4 weeks.

For Vial 1, avoid repeated freeze/thaw cycles (more than 5 ×). Aliquot Vial 1 and freeze or store at +2 to +8°C for a maximum of 4 weeks.

Although we recommend working on ice and preparing the reagents right before use, the working solution is stable at +15 to +25°C up to 4 hours and is therefore ideal for use in automated workflows.

Additional Equipment and Reagents Required

- Standard laboratory equipment
- Nuclease-free pipette tips
- 1.5 ml RNase-free microcentrifuge tubes to prepare master mixes and dilutions.
- To minimize risk of RNase contamination, autoclave all vessels.

For qPCR:

- Real-Time PCR systems such as LightCycler® 480 Instrument* or the LightCycler® 96 Instrument*
- LightCycler® 480 Multiwell Plate 96* or 384*
- Standard swing-bucket centrifuge with rotor for multiwell plates

- For qPCR primer and probe design:
  Universal ProbeLibrary Assay Design Center at
  www.universalprobelibrary.com

- To design and order qPCR assays and panels:
  www.realtimeready.roche.com

Optional

For DNA purification

- MagNA Pure 96 Instrument* including Disposables
- MagNA Pure 96 Internal Control Tube*, optional
- MagNA Pure 96 DNA and Viral NA Kit, Large Volume* or
- MagNA Pure 96 DNA and Viral NA Kit, Small Volume*

Alternatively use other nucleic acid purification instruments and manual sample preparation products such as:

- MagNA Pure LC 2.0 Instrument* with MagNA Pure LC Total Nucleic Acid Isolation Kit*
- MagNA Pure Compact Instrument* with MagNA Pure Compact Nucleic Acid Isolation Kit I
- High Pure Viral Nucleic Acid Kit*

Application

The LightCycler® Multiplex DNA Master is designed for fast, highly sensitive and specific real-time PCR analysis of DNA.

The single vial master mix allows fast and convenient hot start qPCR without the need for upfront polymerase activation incubation. The mix is optimized for use with hydrolysis probes as well as Universal ProbeLibrary (UPL) probes, and does not require optimization of $MgCl_2$ concentration.

Assay Time

The LightCycler® Multiplex DNA Master can be used for multiplex qPCR protocols. For example, a triplex protocol using 45 cycles requires less than 60 minutes when using the LightCycler® 480 System.

2. How to Use this Product

2.1 Before You Begin

Precautions

Always use nuclease-free techniques. Nuclease contaminated reagents and reaction vessels will degrade template nucleic acids. Please follow these guidelines to minimize risk of contamination:

- Wear disposable gloves and change them frequently.
- Avoid touching surfaces or materials that could cause nuclease carry-over.
- Use only reagents provided in this kit. Substitutions may introduce nucleases.
- Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents.
- Use only new nuclease-free aerosol-blocking pipette tips.
- Use a work area specifically designated for nucleic acid work, and if possible use reaction vessels and pipettors dedicated only for work with template nucleic acid.

Sample Material

Use any DNA suitable for qPCR in terms of purity, concentration, and absence of PCR inhibitors. For reproducible isolation of nucleic acids, use either the MagNA Pure LC Instrument*, the MagNA Pure Compact Instrument*, or the MagNA Pure 96 Instrument*, and a dedicated MagNA Pure Nucleic Acid Isolation Kit* (for automated isolation), or a High Pure Nucleic Acid Isolation Kit* (for manual isolation).

For details, see the Roche Catalog or visit the Life Science website: www.lifescience.roche.com

No Template Controls

We highly recommend testing known negative specimens as controls in each run to control for possible contamination. To prepare a no template control, replace the template DNA with PCR grade Water (Vial 2).

Primers

Suitable concentrations of PCR primers range from 0.2 to 0.5 μM final concentration. The recommended starting concentration is 0.5 μM each.

Probes

Suitable concentrations of hydrolysis probes range from 0.05 to 0.5 µM (final concentration in PCR). The recommended starting concentration is 0.25 µM each.

- The optimal probe concentration is the lowest concentration that results in the lowest quantification cycle value (Cq) and adequate fluorescence dynamics for a given target concentration.
- For a hydrolysis probe hybridization complex, the Tm of the hydrolysis probe has to be higher than the Tm of the primers.

$MgCl_2$

The master mix is optimized with a fixed concentration of $MgCl_2$, and should not need concentration optimization for most assays.

2.2 Procedure

LightCycler® 480 or LightCycler® 96 System Protocol

The following procedure is optimized for use with the corresponding LightCycler® System you are using.

⚠ Program the LightCycler® Instrument before preparing the reaction mixes.

For details on how to program the experimental protocol, see the current LightCycler® 480 Instrument Operator's Manual or the LightCycler® 96 System Operator's Guide.

The LightCycler® 480 Instrument II protocol contains the following program:

- **Denaturation** of DNA
- **Amplification** of the DNA
- **Cooling** of the thermal block

A) LightCycler® 480 Instrument II PCR Profile (Multiwell Plate 96 or 384)

Setup				
Detection Format		**Reaction Volume**		**Block Type**
Hydrolysis Probe or UPL Probe		20 µl (10 µl)		96 (384)
Programs				
Program Name		**Cycles**		Analysis Mode
Initial Denaturation		1		None
Amplification		45[1)]		Quantification
Cooling		1		None
Temperature Targets				
Target [°C]	**Acquisition Mode**	**Hold [hh:mm:ss]**	**Ramp Rate [°C/s]**	**Acquisitions [n/°C)]**
Initial Denaturation				
95	None	00:00:30	4.4 (4.8)	–
Amplification				
95	None	00:00:05	4.4 (4.8)	–
60[2)]	Single	00:00:30	2.2 (2.5)	–
Cooling				
40	None	00:00:30	2.2 (2.5)	-

[1)] 45 cycles are suitable for most assays. If the assay shows steep amplification curves and early crossing points, 40 cycles should be sufficient. Reducing the number of cycles will improve uniformity of the product and reduce the time required for the assay.

[2)] The LightCycler® Multiplex DNA Master includes an aptamer for hot start. Anneal / Extend temperatures significantly below 60°C may not be well tolerated because aptamers will inhibit DNA polymerase activity at low temperatures.

Color Compensation Protocol for the LightCycler® 480 Instrument II

For a multicolor, multiplex amplification, the application of a color compensation file is necessary to compensate for optical crosstalk between the different channels.

For the LightCycler® 480 Instrument II, an instrument-specific color compensation file is mandatory for multicolor experiments, and a color compensation object can be generated by performing the following experiment.

The LightCycler® 480 Instrument II protocol contains the following program:

- **Initial Denaturation** of DNA
- **Amplification** of the DNA
- **Temperature Gradient Step** to create the Color Compensation file
- **Cooling** of the thermal block

For details on how to program the experimental protocol, see the LightCycler® 480 Software Operator's Manual, version 1.5.

Programming a Customized Detection Format for the LightCycler® 480 System Filter Combination Selection

The Detection Format in the LightCycler® 480 Software, version 1.5 setup must be customized for the applied multicolor hydrolysis probe format used in the qPCR detection. In the "Tool" module, the "Detection Formats" option allows creating a new detection format specified by the user, including a Detection Format list, a Filter Combination Selection area, and a Selected Filter Combination List. Different filter settings for the LightCycler® 480 II Instrument are defined

Example for 3-color Hydrolysis Probes

Detection Formats	Excitation Filter	Emission Filter
FAM	465	510
Red 610	533	610
Cy5	618	660

For the new customized detection format, set for all selected filters in the "Selected Filter Combination List", the following values:

Melt Factor	1
Quant Factor	10
Max Intregration Time	2

The following table shows the qPCR parameters that must be programmed for a LightCycler® 480 Color Compensation file with a LightCycler® 480 Multiwell Plate 96.

Setup			
Detection Format	**Block Type**		
Customized (see section above)	96		
Programs			
Program Name	**Cycles**	**Analysis Mode**	
Initial Denaturation	1	None	
Amplification	45	Quantification	
Temperature Gradient Step	1	Color Compensation	
Cooling	1	None	
Temperature Targets			
Target [°C]	**Acquisition Mode**	**Hold [hh:mm:ss]**	**Ramp Rate [°C/s]**
Initial Denaturation			
95	None	00:00:30	4.4
Amplification			
95	None	00:00:05	4.4
60	Single	00:00:30	4.4
Temperature Gradient Step			
95	None	00:00:10	4.4
40	None	00:00:10	2.2
95	Continous		5 Acq. /°C
Cooling			
40	None	00:00:30	2.2

Preparation of the Color Compensation Run

Prepare the calibrator qPCR mix for more than one reaction. Multiply the amount in the volume column by the number of reactions you need (minimum of 3 to 5 replicates) plus additional reactions since there will be a slight loss of liquid during the pipetting steps. In order to ensure accuracy, do not pipette volumes less than 1 µl when adding the individual reagents.

For each dye, set up the following reactions:

Component	1× Buffer	1× for each Dye
qPCR Reaction Mix, 5× conc.(Vial 1)	4.0 µl	4.0 µl
Detection mix for each dye	-	X µl (depending on the assay)
Water, PCR grade (Vial 2)	16.0 µl	Y µl (depending on the assay)
Template, such as DNA or positive samples eluates	-	5.0 µl
Total Volume	**20 µl**	**20 µl**

Step	Action
❶	Pipette the replicates of each different calibrator mix into a pre-cooled LightCycler® 480 Multiwell Plate 96.
❷	Seal the LightCycler® 480 Multiwell Plate using a sealing foil.
❸	Place the multiwell plate in a standard swing-bucket centrifuge containing a rotor for multiwell plates with suitable adaptors. Balance it with a suitable counterweight (*e.g.,* another plate), and centrifuge for 2 minutes at 1,500 × g.
❹	Load the multiwell plate into the LightCycler® 480 Instrument II and start the program.

Create Color Compensation Object

When the experiment is finished, open the **Sample Editor**. Select workflow **Color Comp** and designate the used positions of the Multiwell Plate as "Water" for Buffer replicates, and the appropriate dyes respectively (*e.g.*, FAM, Red610, Cy5 for the example mentioned above).

Open the **Analysis** module "Color Compensation", click on **Calculate**, and save the color compensation object in the database.

From this point on, you can apply this 'CC Object' to each dual-, triple- or quadruple-color experiment performed with FAM, Yellow555, Red610, and Cy5 on the same instrument.

B) Protocol for use with the LightCycler® 96 Instrument

Run Editor			
Detection Format			**Reaction Volume**
Select Dyes used in your assays. If using the DNA Process Control, select Cy5 in Channel 4.			20 µl
Programs			
Temp. [°C]	**Ramp Rate [°C/s]**	**Hold [s]**	**Acquisition Mode**
Preincubation (Initial Denaturation)			
95	4.4	30	None
2-Step Amplification			
No. of Cycles: 45[1)]			
95	4.4	5	None
60[2)]	2.2	30	Single

[1)] 45 cycles are suitable for most assays. If the assay shows steep amplification curves and early crossing points, 40 cycles should be sufficient. Reducing the number of cycles will improve uniformity of the product and reduce the time required for the assay.

[2)] The LightCycler® Multiplex DNA Master includes an aptamer for hot start. Anneal / Extend temperatures significantly below 60°C may not be well tolerated because aptamers will inhibit DNA polymerase activity at low temperatures.

Color Compensation Protocol for the LightCycler® 96 Instrument

The LightCycler® 96 Instrument does not require the creation of a color compensation object.

Setup of the PCR Reaction

Follow the procedure below to prepare at least ten 20 µl standard reactions. 10 µl amplifications included in brackets to be used for 384 well plate set-ups:

Do not touch the surface of the LightCycler® 480 Multiwell Plate.

1. Thaw the solutions and, to ensure recovery of all the contents, briefly spin vials in a microcentrifuge before opening.
   Mix carefully by pipetting up and down or vortex briefly. Place samples on ice.
2. Prepare a 20× conc. solution of your primers and a 20× conc. solution of your probes.
3. In a 1.5 ml reaction tube, prepare the PCR Mix and put on ice.
   For best results, prepare not less than 10 reactions in order to reduce pipetting errors. To prepare more reactions, multiply the amount in the "Volume 1 Reaction" column below by the number of reactions to be run, plus at least one additional reaction.

Component	Volume 1 Reaction	Volume 10 Reactions	Final Conc.
Water, PCR grade (Vial 2)	9 µl (4.5 µl)	90 µl (45 µl)	-
qPCR Reaction Mix, 5× (Vial 1)	4 µl (2 µl)	40 µl (20 µl)	1×
Primer Mix, 20×	1 µl (0.5 µl)	10 µl (5 µl)	1×
Probe Mix, 20×	1 µl (0.5 µl)	10 µl (5 µl)	1×
Total volume	**15 µl (7.5 µl)**	**150 µl (75 µl)**	

4. Mix carefully by pipetting up and down or vortex briefly. Place on ice. Although we recommend working on ice and preparing the reagents right before use, the working solution (everything combined except DNA template) is stable at +15 to +25°C up to 4 hours, and is therefore ideal for use in automated workflows.
5. Prepare sample concentration of the DNA.
6. Pipette 15 µl (7.5 µl) qPCR Mix into a precooled multiwell plate.
   Add 5 µl (2.5 µl) of the DNA template.
   Seal multiwell plate with LightCycler® 480 Sealing Foil.
7. Place the Multiwell Plate 96 into a standard swing-bucket centrifuge with a suitable adapter and balance it with a suitable counterweight (*e.g.,* another multiwell plate).
   Centrifuge at 1,500 × *g* for 0.5 - 2 minutes.
8. Load the reaction vessels into the LightCycler® 480 or LightCycler® 96 Instrument.
9. Start the PCR program described above.
   If you use reaction volumes other than 20 µl, be sure to adapt the right volume in the running protocol. To start, we recommend using the same hold times as for the 20 µl volume.

2.3 Quality Control

Each lot of LightCycler® Multiplex DNA Master is tested to meet specifications of the qPCR using a duplex qPCR assay on the LightCycler® 480 Instrument II.

3. Results

The following results were obtained using the LightCycler® Multiplex DNA Master on the LightCycler® 480 Instrument. A duplex reaction using ß-globin specific assay with BHQ-2 internally quenched probe (FAM) and ß2M specific assay with UPL probe (Yellow555).

Human reference cDNA served as the template.

FAM channel (465-510)

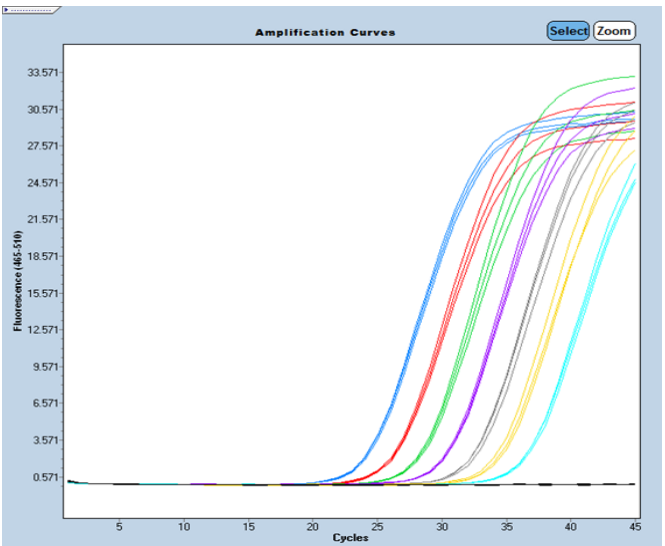

Fig. 1: The FAM channel shows the results for ß-globin. Amplification curves shown were generated from a four-fold dilution series (10 ng, 2.5 ng, 625 pg, 156 pg, 40 pg, 10 pg, and 2.4 pg) of Human Reference cDNA. No template controls are shown in black. Duplex qPCR was performed in a reaction volume of 20 µl per well in a LightCycler® 480 Multiwell Plate 96.

Yellow 555 channel (533/580)

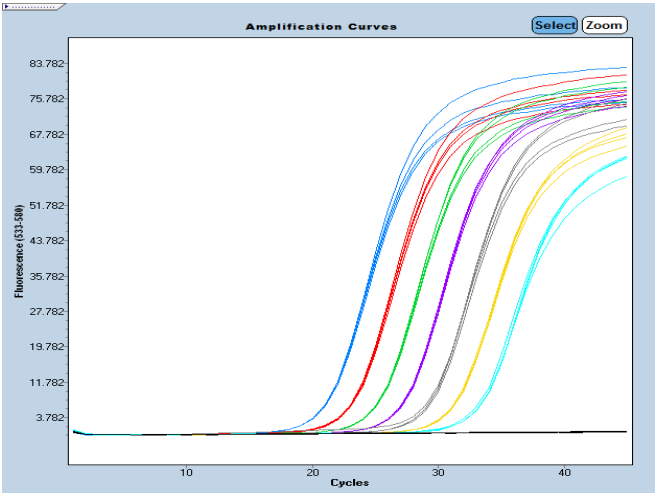

Fig. 2: The Yellow555 channel shows the results for ß2M. Amplification curves shown were generated from a four-fold dilution series (10 ng, 2.5 ng, 625 pg, 156 pg, 39 pg, 10 pg, and 2.4 pg) of Human Reference cDNA. No template controls are shown in black. Duplex qPCR was performed in a reaction volume of 20 µl per well in a LightCycler® 480 Multiwell Plate 96.

4. Troubleshooting

	Possible Cause	Recommendation
Fluorescence intensity varies	Some of the reagent is still in the upper part of the microwell or an air bubble is trapped in the microwell.	Repeat centrifugation, but allow sufficient centrifugation time so all reagent is at the bottom of the microwell and air bubbles are expelled.
	Skin oils or dirt on the surface of the microwell plate.	Always wear gloves when handling the multiwell plate.
Fluorescence intensity is very low	Low concentration or deterioration of dyes in the reaction mixtures because dye was not stored properly.	• Keep dye-labeled reagents away from light. • Store the reagents at –15 to –25°C and avoid repeated freezing and thawing.
	Poor PCR efficiency (reaction conditions not optimized)	• Primer concentration should be in the range of 0.2 to 0.5 µM, probe concentration should be in the range of 0.2 to 0.5 µM and half of the primer concentration. • Check annealing temperature of primers and probes. • Check experimental protocol. • Optimize annealing temperature in the reverse transcription step or in the PCR reaction. • Always run a positive control along with your samples.
	Chosen imaging time is too low	• Choose the appropriate detection format in combination with “dynamic” detection mode or • Increase imaging time when using “manual” detection mode. For details see LightCycler® 480 Software Instrument Operator's Manual.
	qPCR primers and probes are not optimized	• Check sequence and location of the hydrolysis probe on the PCR product. • Check PCR product on an agarose gel
	PCR has not been optimized	• Check primer design (quality). • Check PCR product on an agarose gel

	DNA is degraded during isolation or improper storage	• If possible check DNA quality.
	Pipetting errors and omitted reagents	• Check for missing reagents • Check the pipetting procedure
	Impure sample material inhibits reaction	• Dilute sample 1:10 and repeat the analysis. • Repurify the nucleic acids to ensure removal of inhibitory agents.
Negative control sample gives a positive signal	Contamination	Remake all critical reaction mixes. Be sure to use special Pre-PCR setup working areas.

5. Supplementary Information

5.1 Conventions

5.1.1 Text Conventions

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

Text Convention	Usage
Numbered instructions labeled ❶, ❷ etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Diagnostics.

5.1.2 Symbols

In this document, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

5.2 Changes to Previous Version

Editorial changes

5.3 Ordering Information

Roche offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our homepage www.lifescience.roche.com:

- LightCycler® 480 System: www.lightcycler480.com
- LightCycler® 96 System: www.lightcycler96.com
- Automated Sample Preparation (MagNA Pure LC System and MagNA Pure Compact System): www.magnapure.com
- Manual Sample Preparation of Nucleic Acids: www.lifescience.roche.com/shop/en/us/products/manual-sample-preparation-of-nucleic-acids

	Product	Pack Size	Cat. No.
Instrument and Accessories	LightCycler® 480 Instrument II, 96 well	1 instrument (96 well)	05 015 278 001
	LightCycler® 480 Instrument II, 384 well	1 instrument (384 well)	05 015 243 001
	LightCycler® 480 Block Kit 96 Silver	1 block kit for 96-well PCR Multiwell Plates	05 015 219 001
	LightCycler® 480 Block Kit 384 Silver	1 block kit for 384 -well PCR Multiwell Plates	05 015 197 001
	LightCycler® 480 Multiwell Plate 96, white	5 × 10 plates with sealing foils	04 729 692 001
	LightCycler® 480 Multiwell Plate 384, white	5 × 10 plates with sealing foils	04 729 749 001
	LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
	LightCycler® 480 Software, Version 1.5	1 software package	04 994 884 001
	LightCycler® 96 Instrument	1 instrument	05 815 916 001
	MagNA Pure 96 Instrument	1 instrument plus accessories	06 541 089 001
	MagNA Pure 96 Internal Control Tube	150 tubes (15×10)	05 435 293 001
	MagNA Pure LC 2.0 Instrument	1 instrument plus accessories	05 197 686 001
	MagNA Pure Compact Instrument	1 instrument plus accessories	03 731 146 001
Nuceic Acid Isolation Kits	MagNA Pure 96 DNA and Viral NA Small Volume Kit	3 sets for 192 isolations each	06 543 588 001

Associated Kits

Product	Pack Size	Cat. No.
MagNA Pure 96 DNA and Viral NA Large Volume Kit	3 sets for 96 isolations each	06 374 891 001
MagNA Pure LC Total Nucleic Acid Isolation Kit	1 kit (192 isolations)	03 038 505 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	1 kit for 32 isolations	03 730 964 001
High Pure Viral Nucleic Acid Kit	1 kit for up to 100 purifications	11 858 874 001
Transcriptor Universal cDNA Master	100 reactions	04 379 012 001
Transcriptor First Strand cDNA Synthesis Kit	1 kit for up to 50 reactions, including 10 control reactions	04 379 012 001

5.4 Disclaimer of License

NOTICE: For patent license limitations for individual products please refer to www.technical-support.roche.com.

5.5 Trademarks

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5.6 Regulatory Disclaimer

For general laboratory use.

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If you have questions or experience problems with this or any Roche product for life science, please contact our Technical Support staff. Our scientists are committed to providing rapid and effective help.
Please also contact us if you have suggestions for enhancing Roche product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to the research community worldwide.

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

Visit **www.lifescience.roche.com**, to download or request copies of the following materials:

- Instructions for Use
- Material Safety Data Sheets
- Certificates of Analysis
- Technical Manuals
- Lab FAQS: Protocols and references for life science research

To call, write, fax, or email us, visit www.lifescience.roche.com and select your home country to display country-specific contact information.

0115.07361564001②

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany