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Not for use in diagnostic procedures.



Universal ProbeLibrary Reference Gene Assays

 **Version 07**

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Reference gene-specific primers and probe for quantification of gene expression levels using dual color real-time PCR

Universal ProbeLibrary Set, Human Reference Gene Assays **Cat. No. 05 046 114 001**

1 set containing 10 different human reference gene assays,
for 100 reactions of 50 μ l or 250 reactions of 20 μ l for each reference gene

Universal ProbeLibrary Human PBGD Gene Assay	Cat. No. 05 046 149 001
Universal ProbeLibrary Human HPRT Gene Assay	Cat. No. 05 046 157 001
Universal ProbeLibrary Human ACTB Gene Assay	Cat. No. 05 046 165 001
Universal ProbeLibrary Human PGK1 Gene Assay	Cat. No. 05 046 173 001
Universal ProbeLibrary Human G6PD Gene Assay	Cat. No. 05 046 246 001
Universal ProbeLibrary Human PPIA Gene Assay	Cat. No. 05 189 268 001
Universal ProbeLibrary Human TBP Gene Assay	Cat. No. 05 189 284 001
Universal ProbeLibrary Human β2M Gene Assay	Cat. No. 05 189 390 001
Universal ProbeLibrary Human GUSB Gene Assay	Cat. No. 05 190 525 001
Universal ProbeLibrary Human GAPD Gene Assay	Cat. No. 05 190 541 001
Universal ProbeLibrary Mouse ACTB Gene Assay	Cat. No. 05 046 190 001
Universal ProbeLibrary Mouse GAPD Gene Assay	Cat. No. 05 046 211 001
Universal ProbeLibrary Rat GAPD Gene Assay	Cat. No. 05 046 220 001
Universal ProbeLibrary Rat ACTB Gene Assay	Cat. No. 05 046 203 001

1 set for each assay, for 200 reactions of 50 μ l or 500 reactions of 20 μ l

-  Store the vials at -15 to -25°C
-  Keep the probe vials away from light!

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1. What this Product Does

About this Product

Universal ProbeLibrary Reference Gene Assays are used to quantify the expression level of a gene of interest in relation to an endogenous reference gene or housekeeping gene in a dual color assay. Universal ProbeLibrary (UPL) assays for human, mouse, and rat targets can be combined with a Universal ProbeLibrary reference gene assay for a dual color assay.

ProbeFinder Software supports the design of multiplex PCR assays for your gene of interest (GOI) in combination with one of the UPL reference gene assays that are available for the selected organism. ProbeFinder Software is available free at the web-based Assay Design Center at <http://www.universal-probelibrary.com>.

Universal ProbeLibrary probes included in the reference gene assays are 12-mer probes that are partially modified with Locked Nucleic Acids (LNA). They are labeled with LightCycler® Yellow 555 to enable dual color assays together with the standard UPL probes, which are labeled with FAM. Probes labeled with LightCycler® Yellow 555 can be detected using PCR instruments with excitation filters of 470 nm to 530 nm and emission filters of 550 nm to 640 nm.

Number of Tests **The Universal ProbeLibrary Set, Human Reference Gene Assays** contains enough primers and probe for 100 reactions in a 50 µl real-time PCR setup or 250 reactions in a 20 µl setup for each of the ten reference genes.

Each Universal ProbeLibrary Reference Gene Assay package contains enough primers and probe for 200 reactions in a 50 µl real-time PCR setup or 500 reactions in a 20 µl setup.

Contents **The Universal ProbeLibrary Set, Human Reference Gene Assays** contains primers and probes for ten different human reference gene assays.

For each of the reference genes in the Human PBGD Gene Assay, Human HPRT Gene Assay, Human ACTB Gene Assay, Human PGK1 Gene Assay, Human G6PD Gene Assay, Human PPIA Gene Assay, Human TBP Gene Assay, Human β2M Gene Assay, Human GUSB Gene Assay, and Human GAPD Gene Assay, the set contains two vials, one vial with a primer mix and one vial with a probe. Each primer mix vial contains 100 µl of a primer pair solution with a concentration of 20 µM for each primer, and each probe vial contains 100 µl of a 10 µM solution of the reference gene specific probe.




Each Universal ProbeLibrary Reference Gene Assay package contains four vials, two vials with a primer mix and two vials with a probe. Each primer mix vial contains 100 µl of a primer pair solution with a concentration of 20 µM for each primer, and each probe vial contains 100 µl of a 10 µM solution of the reference gene-specific probe.

③ Universal ProbeLibrary probes included in the reference gene assays are labeled with LightCycler® Yellow 555 at the 5'-end and with a dark quencher dye near the 3'-end, to enable dual color assays together with the standard UPL probes, which are labeled with FAM. Probes labeled with LightCycler® Yellow 555 can be detected using PCR instruments with excitation filters of 470 nm to 530 nm and emission filters of 550 nm to 640 nm. In a dual color assay application with standard Universal ProbeLibrary assays, the following combination of filters is recommended: standard FAM or SYBR Green I filters and the next possible emission filter of a longer wavelength (usually 560, 568, or 640 nm) as the second channel.

1. What this Product Does, continued

Storage and Stability

Store the vials at -15 to -25°C until the expiration date printed on the label. For short-term storage (up to 1 month) primers and probe may be stored at $+2$ to $+8^{\circ}\text{C}$.

-  Primer and probe vials are shipped on dry ice.
-  Keep the probe vials away from light!
-  Avoid repeated freezing and thawing. After the first thawing, store the primers and probe aliquoted.

Additional Equipment and Reagents Required

Additional reagents and equipment required to perform quantitative real-time PCR using Universal ProbeLibrary assays include:

- Standard laboratory equipment
- For first-strand cDNA synthesis:
 - Transcriptor First Strand cDNA Synthesis Kit*
- For use in combination with the LightCycler[®] Carousel-based System:
 - LightCycler[®] 2.0 Instrument*, LightCycler[®] 1.5 Instrument, or lower versions
 - LightCycler[®] TaqMan[®] Master*
 - LightCycler[®] Capillaries*
 - LC Carousel Centrifuge 2.0* (optional)
- For use in combination with the LightCycler[®] 480 Instrument:
 - LightCycler[®] 480 Instrument 96* or 384*
 - LightCycler[®] 480 Probes Master*
 - LightCycler[®] 480 Multiwell Plate 96* or 384*
 - Standard swing-bucket centrifuge containing a rotor and adapters for multiwell plates
- For use in combination with other real-time PCR instruments:
 - FastStart Universal Probe Master (Rox)*, (for use with instruments requiring a reference dye)
 - FastStart TaqMan[®] Probe Master* (for use with instruments not requiring a reference dye)
- For PCR product carryover prevention (optional):
 - LightCycler[®] Uracil-DNA Glycosylase* (for use with all LightCycler[®] kits and reagents using the FastStart enzyme)
 - Uracil-DNA Glycosylase, heat-labile* (for use with other real-time PCR reagents)

Application

The 165 probes of the Universal ProbeLibrary enable rapid and flexible quantification of virtually any transcript in the transcriptomes of any sequenced organism by real-time PCR assays on the LightCycler® 1.5, 2.0, and 480 Instruments or other commercially available real-time PCR instruments. Performance of the assay with the selected Universal ProbeLibrary probe follows established real-time PCR protocols.

Gene-specific expression quantification assays in combination with the UPL reference gene assays for dual color analysis are easily designed using the web-based ProbeFinder software accessible at www.universalprobelibrary.com or via the Roche Applied Science home page, www.roche-applied-science.com. For details of the assay design procedure, please refer to Chapter 2.5.

Dual Color Assay

The Universal ProbeLibrary Reference Gene Assays enable relative quantification in multiplex PCR experiments. The ProbeFinder software allows design of multiplex PCR with your gene of interest (GOI) and one of the UPL reference gene assays for the organisms human, mouse, and rat. In order to design a multiplex PCR, the multiplex option must be selected in the target specification window on the Assay Design Center. The ProbeFinder software will subsequently design UPL assays for your GOI, while at the same time subjecting each of these designed assays to an *in silico* test to evaluate the ability of each assay to be multiplexed with one of the available UPL reference gene assays for the relevant organism. The output page will show the UPL assays and which UPL reference gene assays these may be multiplexed with. To perform the *in silico* multiplex PCR test, the software will take the following parameters into consideration:

- primer-primer interactions – to avoid formation of primer-dimers
- primer-probe interactions – to avoid probe-primer competition for binding sites or binding to each other
- probe-probe interactions – to avoid probe-probe binding
- probe-amplicon interactions – to avoid the "wrong" probe generating signal on the "wrong" amplicon
- *in silico* PCR with all 4 primers – to avoid amplification of undesired cDNA fragments

2. How to Use this Product

2.1 Before You Begin

Precautions

To reduce the risk of contaminating your PCR reaction with PCR amplicons generated in previous reactions (thus generating false results), as well as the risk of degrading template nucleic acid, follow the recommendations below:

- Always wear a clean lab coat. Use separate lab coats when setting up PCR reactions and handling PCR products.
- Change gloves whenever you suspect they have been contaminated.
- Maintain dedicated areas for PCR setup, PCR amplification, and gel electrophoresis of PCR products.
- Always use nuclease-free reagents, buffers, and consumables.
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Pipet carefully and do not splash or spray PCR samples.
- Keep reactions and components capped whenever possible. Always spin tubes before opening.
- Use pipette tips with aerosol filter inserts to avoid aerosol-mediated contamination of your pipetting device.
- Clean laboratory benches and equipment regularly.
- Prevent carryover contamination by using dUTP and Uracil-DNA N-Glycosylase.

Sample Material

Universal ProbeLibrary assays are designed for the amplification of cDNA.

RNA Preparation

Before setting up a Universal ProbeLibrary assay, convert RNA to cDNA using a reverse transcription reaction. To obtain accurate and reproducible results, use a high quality RNA preparation method for reverse transcription. Total RNA should be non-degraded and free of contaminating DNA, RNases, and inhibitors. For reproducible isolation of nucleic acids, use one of the following:

- MagNA Pure 96 Instrument with a dedicated MagNA Pure 96 reagent kit for high-throughput automated isolation
- MagNA Pure LC Instrument with a dedicated MagNA Pure LC reagent kit for medium-throughput automated isolation
- MagNA Pure Compact Instrument with a dedicated MagNA Pure Compact RNA isolation kit for low-throughput automated isolation
- High Pure RNA Isolation Kit for manual isolation

For further information, visit the Roche Applied Science home page at www.roche-applied-science.com.

Total RNA quality can be verified using gel electrophoresis to show that it is non-degraded. An $OD_{260/280}$ measurement should also be performed; high quality RNA has an $OD_{260/280}$ ratio of 1.8 to 2.0.

As a general guideline, a cDNA concentration of 5 ng equivalent of total RNA/PCR reaction is sufficient for detecting medium abundant mRNA. The optimal RNA amount is dependent on the abundance of your transcript. For less abundant transcripts, a higher amount of total RNA is required.

To avoid DNA contamination, Roche recommends using a DNase-treated RNA preparation. Whenever non-intron-spanning assays are performed, the use of DNase-treated RNA preparations is mandatory.

cDNA Preparation

Preparing high quality first strand cDNA is essential for real-time PCR. Reverse transcription of RNA can be primed using random hexamer, oligo(dT), or gene-specific primers. Because the positions of the genes in UPL assays can be throughout the entire length of transcripts, Roche recommends using a combination of random hexamer and oligo(dT) priming to avoid 3'-bias in the cDNAs.

Since reverse transcription is an essential step for obtaining good results, Roche recommends using the Transcriptor First Strand cDNA Synthesis Kit to prepare your cDNA.

⚠ To minimize the risk of PCR inhibition, the input volume of sample cDNA for the PCR should not exceed 5% of the total reaction volume. Roche recommends diluting the cDNA at least 1:5 for subsequent input into a single reaction. For initial experiments, run undiluted, 1:10 diluted, and 1:100 diluted cDNA template in parallel to determine the optimal template amount.

Negative Control

Always run negative controls with the samples. To prepare negative controls:

- Replace the template cDNA with PCR grade water to reveal contamination problems (no template control).
- Omit addition of reverse transcriptase to the cDNA synthesis reaction to indicate false positive results due to the presence of DNA in the RNA sample (RT minus control).

Alternatively, untranscribed sample RNA can be used in PCR as a RT minus control. In the PCR reaction, use a RNA sample amount that is equivalent to the cDNA sample to be tested.

Primers

Use PCR primers for your GOI assay at a final concentration of 400 nM each. The primer concentration may be optimized by varying the final concentration from 200 - 900 nM in increments of 100 nM.

⚠ To assure optimum performance of your Universal ProbeLibrary assay, always use highly purified (e.g., HPLC purified) PCR primers.

⚠ Optimize the primer concentration first, then determine the probe optimization using the optimized primer concentrations.

🔍 The optimal primer concentration is the lowest concentration that results in the lowest C_p (or C_t) and an adequate fluorescence for a given target concentration.

Universal ProbeLibrary Probes

Use the probes at a final concentration of 200 nM.

- ③ The Universal ProbeLibrary probe concentration may be optimized by varying the final concentration from 100 – 500 nM in increments of 100 nM.
- ③ The optimal probe concentration is the lowest concentration that results in the lowest C_p (or C_t) and an adequate fluorescence for a given target concentration.

Before Running a Multiplex Experiment with UPL Reference Gene Assays

The probes used for UPL reference gene assays contain the dye LightCycler[®] Yellow 555, whereas standard UPL probes contain a FAM dye. Most real-time PCR instruments that are able to read multiple dyes can distinguish these two dyes from each other. However, in most cases, the performance of a multiplex PCR with these two dyes requires that a calibration or color compensation is carried out with the real-time PCR instrument. Typically, this will require that the instruments are presented for these two dyes individually in order to optimize the algorithm used to separate the overlapping emission spectra for the dyes. For best results, consult the operator manual for your instrument to obtain specific instructions on how to perform the color compensation or calibration.

Testing the Ability of Two Assays for Multiplexing

Designing a multiplex PCR experiment is a complex task. It is recommended to perform an *in vitro* validation of the designed multiplex PCR.

The initial validation should preferably include the following setup with 7 PCR reactions for each designed multiplex PCR:

- PCR1. GOI¹⁾ – RG²⁾ multiplex
- PCR2. RG singleplex
- PCR3. GOI singleplex
- PCR4. RG primers with GOI probe
- PCR5. GOI primers with RG probe
- PCR6. NTC RG singleplex
- PCR7. NTC GOI singleplex
 - ¹⁾ Gene Of Interest (GOI)
 - ²⁾ Reference Gene (RG)

Ideally, the assays should have the same C_p values when run in single- and multiplex. Furthermore, signal should not be generated when running a PCR using the UPL reference gene primers with the GOI probe or a PCR using the GOI primers with the UPL reference gene probe.

Finally, the designed multiplex assay may be validated by performing a template (cDNA) dilution series - *i.e.*, at least three individual PCRs with tenfold or twofold dilutions of the cDNA.

Avoiding GOI Assay Inhibition by a Strong Reference Gene Assay

Occasionally, the GOI assay may show a slightly higher Cp value in multiplex than in singleplex. This may occur when an assay for a relatively high expressed reference gene (low Cp values) is multiplexed with an assay for a relatively low expressed GOI (high Cp values).

In these instances, it is recommended to try to inhibit the reference gene assay by using lower concentrations of the reference gene primer mix. Perform a titration of the reference gene primers using a dilution series and run it in multiplex with the GOI assay.

Analyze the resulting amplification curves for both the reference gene and the GOI assay. Identify the multiplex PCR experiment with the reference gene primer concentration where the GOI assay in multiplex performs similarly to the GOI assay in singleplex and the reference gene assay still maintains sufficient signal and good Cp values.

Choosing another of the GOI and/or reference gene assays suggested by ProbeFinder software could be a time-saving alternative to the titration experiment with the reference gene primers.

2.2 Procedure for Use with LightCycler® 480 Systems

Preparation of the PCR Mix Use the **LightCycler® 480 Probes Master** for performing UPL assays on LightCycler® 480 Instruments. Follow the procedure below to prepare one 20 µl reaction using the LightCycler® 480 Probes Master.

Ⓞ Do not touch the surface on the LightCycler® 480 Multiwell Plate when handling it.

- 1 ▪ Thaw the solutions and briefly spin vials in a microcentrifuge before opening.
▪ Mix carefully by pipetting up and down, and store on ice.
- 2 ▪ Prepare the PCR mix in a suitably sized tube on ice. The total volume will depend on the number of samples.
Ⓞ When setting up the PCR mix, compensate for pipetting losses. Roche recommends preparing the PCR mix with 10% overdosage (one extra sample for every 10).
▪ Prepare the PCR Mix for one 20 µl reaction by adding the following components in the order listed below:

Component	Conc.	Vol.	Final Conc.
Primer Mix (UPL Ref. Gene Assay)	20 µM	0.4 µl	400 nM
Probe (UPL Ref. Gene Assay)	10 µM	0.4 µl	200 nM
Primer (GOI) forward	20 µM	0.4 µl	400 nM
Primer (GOI) reverse	20 µM	0.4 µl	400 nM
UPL probe (GOI)	10 µM	0.4 µl	200 nM
LightCycler® 480 Probes Master	2×	10.0 µl	1×
Water	-	3.0 µl	-
Total Volume		15.0 µl	

- 3 ▪ Mix carefully by pipetting up and down. Do not vortex.
▪ Pipet 15 µl of the PCR mix into each well of the LightCycler® 480 Multiwell Plate.
▪ Pipet 5 µl template (cDNA) to the PCR mix of each well.
▪ Seal the Multiwell Plate with LightCycler® 480 Sealing Foil.
- 4 Centrifuge for 2 minutes at 1,500 × *g* in a standard swing-bucket centrifuge, containing a rotor for multiwell plates with suitable adaptors.
- 5 Load the Multiwell Plate into the LightCycler® 480 Instrument.
- 6 Start the LightCycler® 480 Instrument run by using the PCR program as described below.

2.2 Procedure for Use with LightCycler® 480 Systems, continued

LightCycler® 480 Instrument Protocol for the 96 Multiwell Plate Format

For more details on how to program the experimental protocol, see the Operator's Guide for the LightCycler® 480 Instrument.

The following table shows a typical protocol for using the LightCycler® 480 System with Multiwell Plates 96 and a 20 µl reaction volume with the **LightCycler® 480 Probes Master**. It should contain the following programs:

- Pre-incubation for activation of FastStart Taq DNA Polymerase and denaturation of the template cDNA
- Amplification of the target DNA
- Cooling the plate and thermal block cycler unit

Setup			
Detection Format	Block Type	Reaction Volume	
Dual Color Hydrolysis Probe / UPL Probe	96	20 µl	
Filter combination: dynamic Mode FAM (483 – 533) and VIC/Hex/Yellow555 (523 - 568) or FAM (465 – 510) and VIC/Hex/Yellow555 (533-580) respectively, for LightCycler® 480 Instrument Version I or II			
Programs			
Program Names	Cycles	Analysis Mode	
Pre-Incubation	1	None	
Amplification	45	Quantification	
Cooling	1	None	
Temperature Targets			
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)
Pre-Incubation			
95	None	00:10:00	4.4
Amplification			
95	None	00:00:10	4.4
60	None	00:00:30	2.2
72	Single	00:00:01	4.4
Cooling			
40	None	00:00:30	2.2

⚠ For data analysis, color compensation (CC) is required.

- LightCycler® 480 Instrument I: Requires an instrument-specific CC object generated by the user. For details, refer to the Operator's Guide for the LightCycler® 480 Instrument.
- LightCycler® 480 Instrument II: Use the universal CC object that is provided in the software.

LightCycler® 480 Instrument Protocol for the 384 Multiwell Plate Format

For more details on how to program the experimental protocol, see the Operator's Guide for the LightCycler® 480 Instrument.

The following table shows a typical PCR protocol for using the LightCycler® 480 System with Multiwell Plates 384 and a 20 µl reaction volume with the **LightCycler® 480 Probes Master**. It should contain the following programs:

- Pre-incubation for activation of FastStart Taq DNA Polymerase and denaturation of the template cDNA
- Amplification of the target DNA
- Cooling the plate and thermal block cycler unit

Setup			
Detection Format	Block Type	Reaction Volume	
Dual Color Hydrolysis Probe / UPL Probe	348	20 µl	
Filter combination: dynamic Mode FAM (483 – 533) and VIC/Hex/Yellow555 (523 - 568) or FAM (465 – 510) and VIC/Hex/Yellow555 (533-580) respectively, for LightCycler® 480 Instrument Version I or II			
Programs			
Program Names	Cycles	Analysis Mode	
Pre-Incubation	1	None	
Amplification	45	Quantification	
Cooling	1	None	
Temperature Targets			
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)
Pre-Incubation			
95	None	00:10:00	4.8
Amplification			
95	None	00:00:10	4.8
60	None	00:00:30	2.5
72	Single	00:00:01	4.8
Cooling			
40	None	00:00:30	2.5

⚠ For data analysis, color compensation (CC) is required.

- LightCycler® 480 Instrument I: Requires an instrument-specific CC object generated by the user. For details, refer to the Operator's Guide for the LightCycler® 480 Instrument.
- LightCycler® 480 Instrument II: Use the universal CC object that is provided in the software.

2.3 Procedure for Use with LightCycler® Carousel-Based Systems

Preparation of the Master Mix Use the LightCycler® TaqMan® Master for performing UPL assays on LightCycler® Carousel-Based Instruments. Prepare the 5× Master Mix of the **LightCycler® TaqMan® Master** as described below:

- ① Thaw one Vial of “Reaction Mix” (Vial 1b, red cap).
- ② Briefly centrifuge one Vial 1a (“Enzyme”, white cap) and one thawed Vial 1b (“Reaction Mix”, red cap, from Step1).
- ③ Pipet 10 µl from Vial 1a (white cap) into Vial 1b (red cap).
 - Ⓢ Each Vial 1a contains enough enzyme for three vials of Reaction Mix.
- ④ Mix gently by pipetting up and down. Do not vortex.
- ⑤ Re-label Vial 1b (red cap) with the new label (Vial 1: Master Mix) provided with the kit.

The volume of the resulting Master Mix (5× conc.) is sufficient for 32 reactions with a final reaction volume of 20 µl.

Preparation of the PCR Mix Follow the procedure below to prepare one 20 µl reaction using the **LightCycler® TaqMan® Master**.

⚠ Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries.

- ①
 - Thaw the solutions and briefly spin vials in a microcentrifuge before opening.
 - Mix carefully by pipetting up and down and store on ice.
- ②
 - Prepare the PCR mix in a suitably sized tube on ice. Total volume will depend on the number of samples.
 - ⚠ When setting up the PCR mix, compensate for pipetting losses. Roche recommends preparing PCR mixes with 10% overdosage (one extra sample for every 10).
 - Prepare the PCR Mix for one 20 µl reaction by adding the following components in the order mentioned below:

Component	Conc.	Volume	Final conc.
Primer Mix (UPL Ref. Gene Assay)	20 µM	0.4 µl	400 nM
Probe (UPL Ref. Gene Assay)	10 µM	0.4 µl	200 nM
Primer (GOI) forward	20 µM	0.4 µl	400 nM
Primer (GOI) reverse	20 µM	0.4 µl	400 nM
UPL probe (GOI)	10 µM	0.4 µl	200 nM
LightCycler® TaqMan® Master, (vial 1, red cap)	5×	4.0 µl	1×
Water, PCR-grade (vial 2, colorless cap)		9.0 µl	
Total volume		15.0 µl	

- 3 Mix carefully by pipetting up and down. Do not vortex.
- 4
 - Pipet 15 µl of the PCR mix into each LightCycler® Capillary.
 - Add 5 µl of the cDNA template.
- 5 Seal each capillary with a stopper.
- 6
 - Centrifuge the LightCycler® Sample Carousel containing the capillaries in the LC Carousel Centrifuge.
 - Alternatively, place the adapters containing the capillaries in a standard benchtop microcentrifuge. Centrifuge at $700 \times g$ for 5 s (3,000 rpm in a standard benchtop microcentrifuge). Transfer the capillaries to the sample carousel of the LightCycler® Instrument.
- 7 Start the LightCycler® Instrument run by using the PCR program as described below.

Instrument Protocol for LightCycler® Carousel-Based Systems

For more details on how to program the experimental protocol, see the Operator's Guide for your LightCycler® Instrument.

The following table shows a typical PCR protocol that uses the LightCycler® TaqMan® Master. It should contain the following programs:

- Pre-incubation for activation of FastStart Taq DNA Polymerase and denaturation of the cDNA
- Amplification of the target DNA
- Cooling the rotor and the thermal chamber

 For more details on how to program the experimental protocol, see the Operator's Guide for your LightCycler® Instrument.

Programs			
Program Name		Cycles	Analysis Mode
Pre-Incubation		1	None
Amplification		45	Quantification
Cooling		1	None
Temperature Targets			
Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition Mode
Pre-Incubation			
95	00:10:00	20	None
Amplification			
95	00:00:10	20	None
60	00:00:30	20	None
72	00:00:01	20	Single
Cooling			
40	00:00:30	20	None

⚠ For data analysis, color compensation (CC) is required. For a detailed description of how to create a color compensation object, refer to Technical Note No. LC 21/2007 "Color Compensation for Hydrolysis Probe Assays" that is available on our Online Technical Support Site at www.technical-support.roche.com.

- Ⓞ Channel settings for data analysis:
- Channel 530 and 560 when using the LightCycler® 2.0 Instrument
 - Channel 530 and 640 when using the LightCycler® 1.5 Instrument or lower version

2.4 PCR Protocol for use with other Real-time PCR Instruments

General Remarks Universal ProbeLibrary assays are compatible with all real-time PCR instruments capable of detecting fluorescein, FITC, FAM, and/or SYBR Green I. Use a standard real-time PCR protocol for hydrolysis probes, taking into account recommendations in the supplier's pack insert for each respective master mix.

Universal ProbeLibrary reference gene assays are compatible with real-time PCR instruments with excitation filters of 470 nm to 530 nm and emission filters of 550 to 640 nm.

Preparation of the PCR Mix Depending on the real-time PCR instrument you use, PCR reagents containing a reference dye (Rox) may be required. Roche recommends using the **FastStart Universal Probe Master (Rox)** with instruments requiring a reference dye, and the **FastStart TaqMan® Probe Master** with instruments that do not require a reference dye. For detailed information on how to use the FastStart Universal Probe Master (Rox) or the FastStart TaqMan® Probe Master, refer to the corresponding Instructions for Use.

Follow the procedure below to prepare one 20 µl reaction.

- 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 and store on ice.

- 2 Prepare the PCR Mix for one 20 µl reaction by adding the following components in the order listed below:

Component	Conc.	Volume	Final Conc.
Primer Mix (UPL Ref. Gene Assay)	20 µM	0.5 µl	500 nM
Probe (UPL Ref. Gene Assay)	10 µM	0.5 µl	250 nM
Primer (GOI) forward	20 µM	0.5 µl	500 nM
Primer (GOI) reverse	20 µM	0.5 µl	500 nM
UPL probe (GOI)	10 µM	0.5 µl	250 nM
Mastermix [FastStart Universal Probe Master (Rox) or FastStart TaqMan® Probe Master]	2 ×	10.0 µl	1 ×
Water	-	2.5 µl	-
Total volume		15.0 µl	

- 3 ▪ Mix carefully by pipetting up and down. Do not vortex.
▪ Pipet 15 µl of the PCR mix into each reaction device of your real-time PCR system.
▪ Pipet 5 µl of template (cDNA) to the PCR mix in each reaction device.
- 4 Continue according to instructions of your real-time PCR system.

PCR Protocol

Use a standard real-time PCR protocol for hydrolysis probes. For details on how to program the experimental protocol, refer to the Operator's Manual for your real-time PCR instrument.

- ⚠ For best results, be sure the instrument is correctly calibrated. Set the detection channel of your real-time PCR instrument to either SYBR Green I or FAM (*i.e.*, 530 nm) for detection of UPL probes labeled with FAM. For the Universal ProbeLibrary reference gene assays, use the VIC/HEX channel (or the next possible emission filter moving to longer wavelengths, for example, 560 nm or 568 nm).
- Ⓢ When performing UPL assays on Applied Biosystems' Fast Real-time PCR Systems, the use of the Fast Mode protocol may generate sub-optimal results.

The selection of Universal ProbeLibrary probes and the design of primers for real-time PCR of gene transcripts is made using the web-based ProbeFinder software available from the Universal ProbeLibrary Assay Design Center at www.universalprobelibrary.com.

③ The ProbeFinder software has an extensive help function that can be visited whenever in doubt.

-
- 1
 - Open your web browser and go to www.universalprobelibrary.com.
 - Select *Assay Design Center* from the navigation bar on the left side of the screen.
 - On the *Assay Design Center* start screen, select your organism of interest.
-

- 2
 - On the subsequent page, submit a sequence string or a sequence ID for your gene of interest (GOI).
 - For dual color assays, click the box "Design multiplex PCR with reference gene." Select "Any" if you want ProbeFinder to find the most suitable reference gene assay for your gene of interest, or select one of the provided assays in the drop-down menu.
 - Choose whether you want to design an intron-spanning assay. The intron-spanning assay option is active by default. Deselect the "Automatically select an intron-spanning assay" option if necessary.
 - Click the "Design" button.
-

- 3
 - ProbeFinder Software will design appropriate primers and select the best matching UPL probe for your target assay. The optimal assay is subsequently presented on the Result Screen together with the most suitable reference gene assay.
-

- 4
 - If for some reason the designed assay is different from what you desire, alternative and often just as efficient assays are available by clicking "More Assays".
-

③ For a more detailed description of the ProbeFinder software read the Universal ProbeLibrary Assay Design Guide that is available at www.universalprobelibrary.com.

3. Troubleshooting

🕒 For further troubleshooting hints, visit our Online Technical Support Site at www.technical-support.roche.com.

	Possible Cause	Recommendation
No positive fluorescent signal during real-time PCR	Insufficient template amount	<ul style="list-style-type: none"> Add more cDNA when working with very low abundance transcripts, or Include more RNA in your reverse transcription reaction.
	Transcript not present in sample material	
	Incomplete reverse transcription	Use a combination of oligo(dT) and random hexamer for priming of the reverse transcription reaction.
	PCR inhibition	The amount of sample cDNA pipetted into the PCR reaction should not exceed 5% of the total reaction volume. For this reason, Roche recommends diluting the cDNA at least 1:5 fold for a single PCR reaction.
	Wrong detection channels or parameters	Check your PCR protocol.
Log-linear phase of amplification just begins at the end of the cycling program	Number of cycles in the PCR protocol is too low	Run a PCR protocol with 45 or even 50 cycles.
	Insufficient template amount	<ul style="list-style-type: none"> Add more cDNA when working with very low abundance transcripts, or Include more RNA in your reverse transcription reaction.
RT minus control with positive signal	Amplification of remaining genomic DNA in the RNA sample preparation	<ul style="list-style-type: none"> Improve your RNA preparation, <i>e.g.</i>, with a DNase treatment. Check your UPL assay design. For cDNA amplification, the design of an intron-spanning assay helps to avoid false positive signals caused by contaminating genomic DNA. When using the option "Automatically select an intron-spanning assay", UPL assays designed by ProbeFinder target an intron-spanning site.
Negative control sample with positive signal	Contamination	<ul style="list-style-type: none"> Use Uracil-DNA glycosylase for PCR product carryover prevention. Check your PCR setup workflow.

4. Additional Information on this Product

How this Product Works The Universal ProbeLibrary probes enable quantification of gene expression levels of a large number of organisms (human, primates, mouse, rat, *C. elegans*, *Drosophila*, Zebrafish, Rice, Maize, Yeast, *Arabidopsis*) and other organisms included in the NCBI Reference Sequence Database.

The Universal ProbeLibrary uses the hydrolysis probe format for real-time PCR detection. Hydrolysis probe assays are homogenous 5′ nuclease assays, with a single 3′ non-extendable hydrolysis probe which is cleaved during PCR amplification. They are used to detect the accumulation of a specific target DNA sequence. Hydrolysis probes contain two labels in close proximity to each other: a fluorescent reporter dye at the 5′-end and a quencher label at or near the 3′-end. During PCR, when the probe is intact, the fluorescent signal is suppressed by the quenching label. When the probe is hybridized to its target sequence, the 5′→3′ exonuclease activity of the Taq DNA Polymerase cleaves the hydrolysis probe. Separating the reporter and the quencher "unquenches" the fluorescent reporter dye. During each PCR cycle, more of the released fluorescent dye accumulates, boosting the fluorescent signal.

Universal ProbeLibrary Principle

The Universal ProbeLibrary is a powerful system for quantifying the expression level of virtually any transcript of a given organism using real-time qPCR assays. It comprises 165 pretested, real-time PCR detection probes and ProbeFinder Software for the design of gene-specific assays.

Universal ProbeLibrary (UPL) probes are labeled at the 5′ end with fluorescein (FAM) and at or near the 3′ end with a quencher dye. The extensive transcript coverage of UPL probes is due to their short length of just 8 to 9 nucleotides of carefully selected sequences. In order to maintain the hybridization stability and specificity required for qPCR probes, Locked Nucleic Acids (LNAs) are incorporated into the sequence of each UPL probe. LNAs are DNA nucleotide analogs with increased binding strengths compared to standard DNA nucleotides.

Design of a gene-specific UPL assay is performed using ProbeFinder Software. User-defined target information is used to select the suitable Universal ProbeLibrary probe matched to a set of target specific PCR primers. The UPL probe and the PCR primers together constitute the PCR assay for a given target gene.

ProbeFinder Software is available free at the web-based Assay Design Center at www.universalprobelibrary.com.

Universal ProbeLibrary assays for human, mouse, and rat targets can be combined with a Universal ProbeLibrary reference gene assay for a dual color assay. The multiplex option on the sequence input window allows you to design assays for your target gene(s) of interest, in combination with the available reference genes for the selected organism.

Universal ProbeLibrary probes included in the reference gene assays are 12-mer probes that are partially modified with LNA. They are labeled with LightCycler® Yellow 555 to enable dual color assays together with the standard UPL probes, which are labeled with FAM.

Quality Control

Primers and probes of Universal ProbeLibrary Reference Gene Assays are analyzed by anion-exchange HPLC and MALDI-MS to ensure purity and quality. Each Universal ProbeLibrary Reference Gene Assay has passed a real-time PCR performance test.

5. Supplementary Information



5.1 Conventions

Text Conventions To make information consistent and memorable, the following text conventions are used in this document:

Text Convention	Usage
Numbered stages labeled ①, ②, etc.	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ①, ②, etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

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.

Abbreviations

In this document, the following abbreviations are used:

Abbreviation	Meaning
C _p	crossing point
C _t	threshold cycle
GOI	Gene of Interest
LNA	Locked Nucleic Acid
NA	Nucleic acid (RNA or DNA)
PCR	Polymerase Chain Reaction
qPCR	quantitative real-time PCR
RG	Reference Gene
RT-PCR	Reverse transcription polymerase chain reaction
UNG	Uracil-DNA Glycosylase
UPL	Universal ProbelLibrary

5.2 Changes to Previous Version

- Update of real-time PCR protocols
- Editorial changes

5.3 Ordering Information

	Product	Pack Size	Cat. No.
Universal Probe Library Reference Gene Assays	Universal ProbeLibrary Set, Human Reference Gene Assays	1 set 100 reactions of 50 µl or 250 reactions of 20 µl for each reference gene	04 683 650 001
	Human PBGD Gene Assay	200 × 50 µl assays	05 046 149 001
	Human HPRT Gene Assay	200 × 50 µl assays	05 046 157 001
	Human ACTB Gene Assay	200 × 50 µl assays	05 046 165 001
	Human PGK1 Gene Assay	200 × 50 µl assays	05 046 173 001
	Human G6PD Gene Assay	200 × 50 µl assays	05 046 246 001
	Human PPIA Gene Assay	200 × 50 µl assays	05 189 268 001
	Human TBP Gene Assay	200 × 50 µl assays	05 189 284 001
	Human β2M Gene Assay	200 × 50 µl assays	05 189 390 001
	Human GUSB Gene Assay	200 × 50 µl assays	05 190 525 001
	Human GAPD Gene Assay	200 × 50 µl assays	05 190 541 001
	Mouse ACTB Gene Assay	200 × 50 µl assays	05 046 190 001
	Mouse GAPD Gene Assay	200 × 50 µl assays	05 046 211 001
	Rat GAPD Gene Assay	200 × 50 µl assays	05 046 220 001
	Rat ACTB Gene Assay	200 × 50 µl assays	05 046 203 001
Universal ProbeLibrary Sets and Single Probes	Universal ProbeLibrary Probes	2 vials of Universal ProbeLibrary probe per package, 125 µl, 10 µM each	For details, visit www.universal-probelibrary.com
	Universal ProbeLibrary Set, Human	1 set of 90 Universal ProbeLibrary probes, 125 µl, 10 µM, each	04 683 633 001
	Universal ProbeLibrary Set, Mouse	1 set of 90 Universal ProbeLibrary probes, 125 µl, 10 µM, each	04 683 641 001
	Universal ProbeLibrary Set, Rat	1 set of 90 Universal ProbeLibrary probes, 125 µl, 10 µM, each	04 683 650 001
	Universal ProbeLibrary Extension Set, Probes #91 - #165	1 set of 75 probes, 125 µl, 10 µM, each	04 869 877 001
Instruments and Accessories	LightCycler® 480 Instrument II	1 instrument (96 well) 1 instrument (384 well)	05 015 278 001 05 015 243 001
	LightCycler® 480 Multiwell Plate 96, white	5 × 10 plates (including sealing foils)	04 729 692 001
	LightCycler® 480 Multiwell Plate 384, white	5 × 10 plates (including sealing foils)	04 729 749 001
	LightCycler® 2.0 Instrument	1 instrument plus related products and data station	03 351 414 001
	LightCycler® Capillaries (20 µl)	1 pack (5 boxes, each with 96 capillaries and stoppers)	04 929 292 001
	LC Carousel Centrifuge 2.0	1 centrifuge plus rotor and bucket	03 709 507 001 (115 V) 03 709 582 001 (230 V)

5.3. Ordering Information, continued

	Product	Pack Size	Cat. No.
Associated Kits and Reagents	LightCycler® 480 Probes Master	5 × 1 ml (5 × 100 reactions, 20 µl each)	04 707 494 001
		10 × 4 ml (10 × 500 reactions, 20 µl each)	04 887 301 001
		1 × 50 ml (5,000 reactions, 20 µl each)	04 902 343 001
	LightCycler® TaqMan® Master	1 kit for 96 reactions of 20 µl final reaction volume	04 535 286 001
		1 kit for 480 reactions of 20 µl final reaction volume	04 735 536 001
	FastStart Universal Probe Master (Rox)	2 × 1.25 ml for 250 reactions of 20 µl final reaction volume	04 913 949 001
		10 × 1.25 ml for 1,250 reactions of 20 µl final reaction volume	04 913 957 001
		10 × 5 ml for 5,000 reactions of 20 µl final reaction volume	04 914 058 001
	FastStart TaqMan® Probe Master	2 × 1.25 ml for 100 reactions of 50 µl final reaction volume	04 673 409 001
		10 × 1.25 ml for 500 reactions of 50 µl final reaction volume	04 673 417 001
10 × 5 ml for 2,000 reactions of 50 µl final reaction volume		04 673 433 001	
LightCycler® Uracil-DNA Glycosylase	50 µl 100 U (2 U/µl)	03 539 806 001	
Uracil-DNA Glycosylase, heat-labile	100 U	11 775 367 001	

5.4 Disclaimer of License

NOTICE: This product may be subject to certain use restrictions. Before using this product please refer to the Online Technical Support page (<http://technical-support.roche.com>) and search under the product number or the product name, whether this product is subject to a license disclaimer containing use restrictions.

5.5 Trademarks

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5.6 Regulatory Disclaimer

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www.roche-applied-science.com/support

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