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Type-it® CNV Probe PCR +qC Kit Handbook

For reliable and precise relative quantification of gene copy number in the human genome using TaqMan® probes

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Kit Contents

Type-it CNV Probe PCR +qC Kit	(100)	(400)
Catalog no.	**206662**	**206664**
Number of 25 µl reactions	**100**	**400**
2x Type-it Probe PCR Master Mix (with ROX™ dye)*	1.3 ml	5.2 ml
Type-it CNV Reference Probe Assay	1 vial (for 100 assays)	4 vials (for 400 assays)
Buffer TE	2 ml	2 ml
RNase-Free Water	1.9 ml	3 x 1.9 ml
Quick-Start Protocol leaflet	1	1

* Contains HotStarTaq® *Plus* DNA Polymerase, Type-it Probe PCR Buffer, and dNTP mix (dATP, dCTP, dGTP, dTTP).

Shipping and Storage

The Type-it CNV Probe PCR +qC Kit is shipped on dry ice and should be stored immediately upon receipt at –20°C in a constant-temperature freezer and must be protected from exposure to light. The kit can be stored under these conditions until the expiration date on the kit box, without showing any reduction in performance.

The 2x Type-it Probe PCR Master Mix should also be protected from exposure to light and can be stored at 2–8°C for up to 1 month (depending on the expiration date), without showing any reduction in performance.

The Type-it CNV Reference Probe Assay should be stored at –20°C, either lyophilized or reconstituted. Avoid repeated (>6 times) freeze–thaw cycles.

For information on the correct reconstitution of the Type-it CNV Probe Reference Assay, see "Reconstitution and use of 25x Type-it CNV Reference Probe Assay", page 13.

Intended Use

The Type-it CNV Probe PCR +qC Kit is intended for molecular biology applications. This kit is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/Support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN® kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the Type-it CNV Probe PCR +qC Kit is tested against predetermined specifications to ensure consistent product quality.

Product Description

Component	Description
2x Type-it Probe PCR Master Mix, which contains:	
HotStarTaq *Plus* DNA Polymerase:	HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*. HotStarTaq *Plus* DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 5-minute, 95°C incubation step.
Type-it Probe PCR Buffer (includes ROX passive reference dye):	Novel PCR buffer for accurate and precise quantification of gene copy number; includes Factor MP for stringent multiplex PCR and Q-Bond® technology for fast cycling.
dNTP mix:	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality
Type-it CNV Reference Probe Assay:	Universal relative quantification control assay for reliable $\Delta\Delta C_T$-based* copy number calculation of the gene of interest by duplex or multiplex PCR. The patent-pending reference assay is based on a multi-copy genetic element that is distributed on different chromosomes in the human genome, and is suitable for detection of all somatic and germline CNVs.
RNase-free water:	Ultrapure quality, PCR grade
Buffer TE:	Molecular biology grade

* The term C_T is synonymous with Cq according to the MIQE guidelines.

Introduction

CNVs are known to affect around 10–20% of the human genome and have been associated with many types of cancer and various complex diseases, such as autism and autoimmune disorders. Relative quantitative PCR ($\Delta\Delta C_T$), with its ease of use, sensitivity, and scalability, is often the method of choice for CNV validation and association studies. For further details on relative quantification, please refer to Appendix G, page 39.

The Type-it CNV Probe PCR +qC Kit provides all the components required for accurate and reliable gene copy number quantification using sequence-specific TaqMan probes for all germline and somatic copy number variations. The kit has been optimized and validated for use both with commercial (e.g., TaqMan Copy Number Assays) and self-designed TaqMan assays for relative copy number quantification of the genes of interest (GOI). The 2x master mix and the Type-it CNV Reference Probe Assay provided with the Type-it CNV Probe +qC Kit are preoptimized for use with TaqMan assays for all GOIs, eliminating the need for time-consuming optimization of duplex and multiplex PCR and validation of single-copy references for each GOI.

The Type-it Reference Probe Assay, which is based on a multi-copy genetic element, is detected in the same tube together with the GOI by duplex or multiplex PCR. This multi-copy genetic element serves as a universal quantification reference to enable reliable $\Delta\Delta C_T$-based relative gene copy number quantification and validation of possible copy number variation (CNV). Compared with commonly used single-copy reference genes, this unique multi-copy reference assay ensures superior reliability in CNV quantification and eliminates the need for optimization and validation of single-copy reference assays for each GOI under investigation.

The 2x Type-it Probe PCR Master Mix provided with the Type-it CNV Probe PCR +qC Kit contains ROX dye, and is suited for use with various real-time cyclers, including the Rotor-Gene® Q cyclers, as well as instruments from Applied Biosystems, Bio-Rad, Cepheid, Eppendorf, Roche, and Agilent (Table 1). Using the optimized protocols described in this handbook and the Quick-Start Protocol provided with the kit, duplex or multiplex PCR is carried out in the presence of ROX passive reference dye.

Table 1. Suitable real-time PCR instruments

Supplier	Thermal cycler
QIAGEN	Rotor-Gene Q
Life Technologies	Applied Biosystems® 7500 (all series), Applied Biosystems 7300 and 7300, ABI PRISM® 7000 and 7700, Applied Biosystems StepOne™ and StepOnePlus™, ViiA7
Agilent/Stratagene	Mx3000P® and Mx3005P®
Bio-Rad	iCycler iQ®, CFX
Roche	LightCycler® 480, LightCycler 2
Eppendorf	RealPlex®

Type-it Probe PCR Master Mix

In contrast with current methods, the Type-it CNV Probe PCR +qC Kit eliminates the need for optimization of the concentrations of primers, Mg^{2+}, and DNA polymerase. The 2x Type-it Probe PCR Master Mix is based on our patented multiplex PCR technology, which is successfully implemented in a variety of QIAGEN PCR solutions. Furthermore, it is specifically optimized for precise quantification of copy number variations by parallel amplification of the provided Type-it CNV reference Probe Assay together with the GOI. The master mix ensures that the target sequence is amplified with the same efficiency and sensitivity in the duplex or multiplex PCR reaction as in a corresponding singleplex reaction.

The 2x Type-it Probe PCR Master Mix contains HotStarTaq *Plus* DNA Polymerase, Type-it Probe PCR Buffer, dNTPs, as well as ROX passive reference dye.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase is a chemically modified form of QIAGEN's *Taq* DNA Polymerase. HotStarTaq *Plus* DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperatures. This prevents the formation of misprimed products and primer dimers during reaction setup and the first denaturation step. Competition for reactants by PCR artifacts is therefore avoided, enabling high PCR specificity and sensitivity and accurate quantification. The enzyme is activated by a 5-minute, 95°C incubation step, which is easily incorporated into existing thermal cycling programs. It also enables reaction setup at room temperature for increased convenience.

Type-it Probe PCR Buffer

The Type-it Probe PCR Buffer has been specifically developed for sensitive, precise, and rapid relative quantification to detect both germline and somatic CNVs in the human genome using sequence-specific probes.

A novel additive in the buffer, Q-Bond, allows short cycling times on standard cyclers and on fast cyclers with rapid ramping rates. Q-Bond also increases the affinity of *Taq* DNA polymerases for short single-stranded DNA, reducing the time required for primer–probe annealing to a few seconds. This allows a combined annealing/extension step of only 30 seconds in multiplex PCR. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times.

In addition to various salts and additives, the buffer also contains a specially optimized combination of K^+ and NH_4^+, which promote a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer-annealing conditions, leading to increased PCR specificity. When using this buffer, primer annealing is only marginally influenced by the $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is usually not required.

The buffer also contains Factor MP, a key component of QIAGEN's proprietary multiplex PCR technology, which facilitates multiplex PCR. This synthetic factor increases the local concentration of primers and probes at the DNA template and stabilizes specifically-bound primers and probes, allowing efficient annealing and extension. The unique composition of the Type-it Probe PCR Buffer prevents different amplification reactions from affecting each other. Furthermore, the need to determine a specific annealing temperature for each primer–probe set is eliminated. This enables fast two-step cycling with a combined annealing/extension step for all targets, with the advantage that several assays can be run in parallel on a single real-time cycler, using the same cycling protocol.

ROX passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR–related variations in fluorescence detection.

The use of ROX dye is necessary for instruments from Applied Biosystems and is optional for Agilent instruments. The master mix supplied with the Type-it CNV Probe PCR +qC Kit contains ROX dye and can be used on all real-time cyclers (see Table 1). Kit performance is not compromised, even when used with real-time cyclers not requiring ROX. When performing multiplex, real-time PCR, we do not recommend using probes that have ROX or Texas Red® fluorophore as the reporter dye, since their performance in the presence of ROX passive reference dye is unpredictable.

Type-it CNV Reference Probe Assay

The Type-it CNV Reference Probe Assay is a TaqMan probe-based assay and targets a multi-copy gene element in the human genome. It allows simultaneous amplification of the Type-it CNV Reference and user-defined GOI by multiplex, real-time PCR using sequence-specific probes for reliable quantification of CNVs.

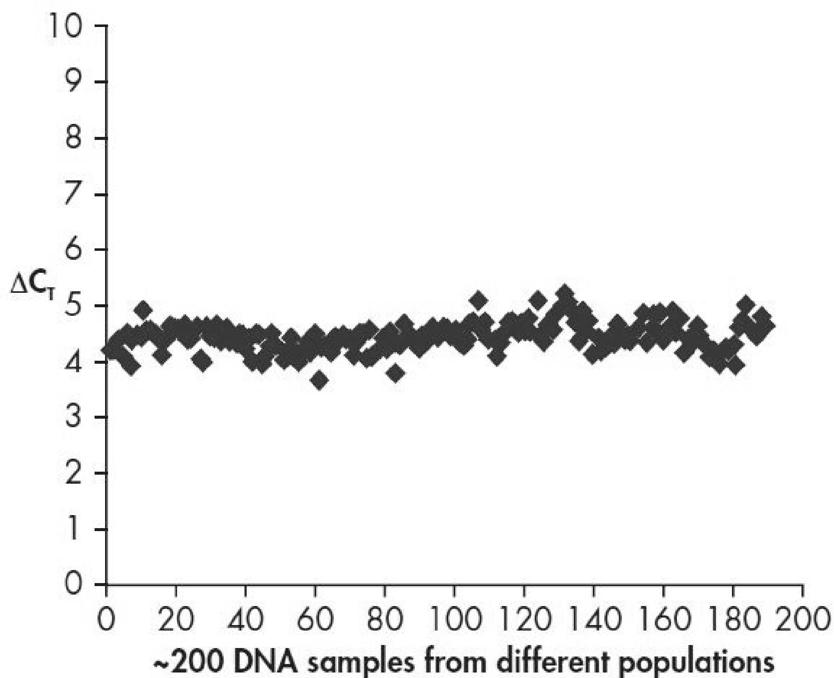


Figure 1. Highly reliable multi-copy quantification control assay. DNA samples from 188 donors were evaluated. ΔC_T values between a nonvariable region of the KRAS gene and the multi-copy genetic element were detected by the Type-it CNV Reference Assay.

The Type-it CNV Reference Probe Assay is consistent among diverse human populations, assuring successful and universal application. The multi-copy genetic element has been quantified and validated with 188 human gDNA

samples from healthy donors and shown to be consistent between genders and across populations. It is therefore highly suitable as a universal quantification control assay for $\Delta\Delta C_T$ analysis.

The Type-it CNV Reference Probe Assay (provided lyophilized) contains a forward and reverse primer and a TaqMan probe, and has been specifically optimized to prevent interference with target primers. The Type-it CNV Reference Probe Assay uses MAX™ as a reporter dye. With excitation/emission maxima of 524/557 nm, the MAX dye has a spectral profile allowing detection in the same channel as HEX™, JOE®, or VIC®, and therefore can be used with all real-time cyclers. To ensure optimal performance of multiplex amplification of the GOI and CNV reference, we recommend that the GOI assay design follows certain design specifications. For more details on GOI target assay design, see Appendix C, page 27.

Sequence-specific probes

The Type-it CNV Probe Kit is optimized for use with hydrolysis (TaqMan) probes, the most commonly used sequence-specific probes for real-time PCR. For more information about sequence-specific probes and their design and handling, see Appendices C and D. TaqMan probes are sequence-specific oligonucleotides with a fluorophore and a quencher moiety attached. The fluorophore is at the 5' end of the probe, and the quencher moiety is usually located at the 3' end or internally. During the extension phase of PCR, the probe is cleaved by the 5'→3' exonuclease activity of *Taq* DNA polymerase, separating the fluorophore and the quencher moiety. This results in detectable fluorescence proportional to the amount of accumulated PCR product.

Comparative method or $\Delta\Delta C_T$ method of relative quantification

The comparative or $\Delta\Delta C_T$ method of relative quantification relies on direct comparison of C_T values of the target sample and a calibrator or control (reference) sample. The Type-it CNV Reference Probe Assay serves as a quantification control and enables fast and efficient relative quantification of gene copy number using TaqMan probe-based multiplex qPCR.

For further information, please see Appendix G, page 39, or refer to our brochure *Critical Factors for Successful Real-Time PCR*. To obtain a copy, contact QIAGEN Technical Services, or visit www.qiagen.com/literature/defaultbrochures.aspx to download a PDF.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- Primers and TaqMan probes for detection of the GOI target must be purchased from an established oligonucleotide manufacturer. Primers should be of standard quality or high-performance liquid chromatography (HPLC) pure, and probes should be highly purified (e.g., HPLC purified). Lyophilized primers and probes should be dissolved in Buffer TE to provide a stock solution of 100 μM; concentration should be checked by spectrophotometry (for details, see Appendix D, page 29).
- Nuclease-free (RNase/DNase-free) consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive detection of viral nucleic acids.
- Cooling device or ice
- Real-time PCR thermal cycler (see Table 1)
- PCR tubes, plates, or Rotor-Discs® (use thin-walled PCR tubes or plates recommended by the manufacturer of your real-time cycler)

Important Notes

Reconstitution of the Type-it CNV Reference Probe Assay

To reconstitute the Type-it CNV Reference Probe Assay (100) to a 25x working solution, briefly centrifuge the tube, add 110 µl Buffer TE (pH 8.0; provided with the kit), and mix by vortexing the tube 4–6 times. If necessary, gently warm the tube to help the primers dissolve. The reconstituted 25x Type-it CNV Reference Probe Assay should be stored at –20°C.

Note: Avoid repeated freeze/thaw cycles; limit them to a maximum of 6 in total. If planning to use small amounts, please aliquot accordingly.

Guidelines for effective relative quantification of the GOI and the Type-it CNV Reference Probe Assay by multiplex PCR

The Type-it CNV Probe +qC Kit works with TaqMan primer–probe assays that have been designed using standard real-time PCR design parameters. Please read the following guidelines for multiplex real-time PCR before starting.

- Choose a reporter dye and quencher compatible with the Type-it CNV Reference Probe Assay (MAX fluorescent dye; detectable using the VIC or Hex channel). Typically, the target-specific probe is labeled with FAM™. The fluorescence spectrum of the GOI assay dye must be well separated from the MAX spectrum or exhibit only minimal overlap. For instrument-specific recommendations such as suitable reporter dyes, please refer to Appendix E, page 30 before starting.
- The Type-it CNV Reference Probe Assay employs MAX as a reporter dye. If using MAX dye for the first time on an instrument, ensure that detection is carried out using the correct channel or filter. For details, see Appendix E, page 30.
- PCR products for real-time PCR using sequence-specific probes should be as short as possible; ideally 60–150 bp. The Type-it CNV Reference Probe Assay has been specifically optimized for multiplexing with typical GOI assays with amplicon lengths of up to 150 bp.
- Always use the same algorithm or software to design the primers and probes. For optimal results, use GOI assays that have been designed using standard software with standard algorithm parameters and reaction conditions. This will typically result in melting temperatures (T_m) suitable for duplexing with the reference assay. For details, see Appendix C, page 27.
- Check the concentration of primers and probes before starting. For details, see Appendix D, page 29.

- Check the real-time cycler user manual for **correct setup of the cycler for multiplex analysis** (e.g., setting up detection of two different dyes from the same well). Be sure to activate the detector for both reporter dyes used.
- Some real-time cyclers require **a calibration procedure for each reporter dye**. Check whether the reporter dye of the GOI assay is part of the standard set of dyes already calibrated on the instrument. If it is not, perform a calibration procedure for the dye before using it for the first time according to the manufacturer's instructions.
- For optimal results, always use the **cycling conditions specified in the protocol**.
- Include appropriate controls in each real-time PCR run to give additional information for interpretation of results. For details, see "Controls".

Controls

Be sure to include positive control samples, as well as a no template control (NTC) as a negative control in each PCR run.

As a calibrator, include at least one genomic DNA control of known copy number for each assay tested in the experiment to ensure accurate relative quantification.

At least one NTC reaction containing all the components of the reaction except the DNA template that contains the GOI target should be included. This enables detection of possible contamination in the reagents.

Protocol 1: Duplex PCR Using Commercial TaqMan Copy Number Assays on all real-time cyclers

This protocol is optimized for relative quantification of DNA copy number in the human genome and is intended for use with the Type-it CNV Probe PCR +qC Kit and TaqMan Copy Number Assays (Life Technologies) on all real-time cyclers (see Table 1).

Important points before starting

- Read the "Important Notes" section on page 13 before starting.
- For each gene of interest (GOI), we recommend using the provided Type-it CNV Reference Probe Assay in the same reaction well as a universal reference assay for reliable $\Delta\Delta C_T$-based quantification of the CNV in the human genome. The TaqMan probe of the reference assay is labeled with MAX dye, which is detected on the HEX/VIC channel.
- To reconstitute the Type-it CNV Reference Probe Assay (100) to a 25x working solution, see page 13.
- It is recommended to use a probe labeled with FAM to detect the gene of interest by duplex PCR on any cycler. For multiplex analyses, we strongly recommend using dual-labeled probes with nonfluorescent quenchers (standard TaqMan or TaqMan MGB™ Probes).
- Always use 30 pg–30 ng template DNA (see Table 2).
- Use the primer and probe concentrations specified in this protocol.
- **Always use** the cycling conditions specified in this protocol. The cycling conditions and the Type-it CNV Reference Probe Assay have been optimized for use with the 20x TaqMan Copy Number Assay (Life Technologies).
- The PCR **must** start with an initial incubation step of 5 min at 95°C to activate HotStarTaq *Plus* DNA Polymerase.
- For data analysis, always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.
- We strongly recommend testing the performance of new TaqMan Copy Number Assays in individual assays before combining them with the Type-it CNV Probe Reference Assay in duplex assays.

Procedure

1. **Thaw the 2x Type-it Probe PCR Master Mix, template DNA (human genomic DNA), Type-it CNV Reference Probe Assay 25x working**

solution, 20x TaqMan Copy Number Assay, and RNase-free water. Mix the individual solutions and place them on ice.

2. **Prepare a reaction mixture according to Table 2.**

   Note: It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. Typically, reaction setup can be performed at room temperature (15–25°C). However, it is recommended to keep samples and reconstituted assays on ice or in a cooling device.

3. **Mix the reaction mixture thoroughly and dispense appropriate volumes into PCR tubes or the wells of a PCR plate or Rotor-Disc.**
4. **Add template DNA (start with 10 ng; use 30 pg–30 ng as the range) to the individual PCR tubes or wells. See Table 2 for details.**
5. **Program the real-time cycler according to Table 3.**

   Note: Data acquisition should be performed during the combined annealing/ extension step.

6. **Place the PCR tubes, Rotor-Disc, or plate in the real-time cycler, and start the cycling program.**
7. **Perform data analysis.**

   Note: Before performing data analysis, specify the analysis settings. Select the analysis settings (i.e., baseline settings and threshold values) separately for the GOI and the Type-it CNV Reference Probe Assay. Optimal analysis settings are a prerequisite for accurate quantification data.

   Note: Only if using the Applied Biosystems 7500, 7500 Fast, or ViiA7 Real-Time PCR Systems, it is recommended to use the 'manual C_T' function instead of the 'auto C_T' function for data analysis. Use a value of 0.01 as a starting point for the threshold setting. For all other cyclers, use the automatic C_T function as a starting point.

Table 2. Reaction setup for duplex PCR using TaqMan Copy Number Assays from Life Technologies

Component	Volume (μl)
Reaction mix	
2x Type-it Probe PCR Master Mix	12.5
25x Type-it CNV Reference Probe Assay solution*	1
20x TaqMan Copy Number Assay for the GOI (FAM labeled)	1.25
RNase-free water	Variable
Template DNA (added at step 4)	Start with 10 ng (use 30 pg–30 ng as the range)
Total reaction volume	25†

* See "Reconstitution and use of Type-it CNV Reference Probe Assay" on page 13.

† If the real-time cycler requires a final reaction volume other than 25 μl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the ABI PRISM 7900, use a reaction volume of 10 μl.

Table 3. Cycling conditions

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			
Denaturation	**30 s**	**95°C**	
Annealing/extension	**30 s‡**	**60°C**	Combined annealing/extension step with fluorescence data collection.
Number of cycles	40		The number of cycles may be increased or decreased, depending on the template used.

‡ For real-time PCR systems that require a minimum annealing/extension time longer than 30 seconds, adjust the time accordingly, as per the requirements of the real-time PCR system in operation.

Protocol 2: Multiplex PCR (up to 4-plex) Using Self-Designed TaqMan Copy Number Assays

This protocol is optimized for relative quantification of DNA copy number in the human genome and is intended for use with the Type-it CNV Probe PCR +qC Kit and self-designed TaqMan copy number assays on all real-time cyclers (Table 1).

Important points before starting

- Read the "Important Notes" on page 13 before starting.
- For each gene of interest (GOI), we recommend using the provided Type-it CNV Reference Probe Assay in the same reaction well as a universal reference assay for reliable $\Delta\Delta C_T$-based quantification of the CNV in the human genome. The TaqMan probe of the reference assay is labeled with MAX dye, which is detected on the HEX/VIC channel.
- To reconstitute the Type-it CNV Reference Probe Assay (100) to a 25x working solution, see page 13.
- It is recommended to use a probe labeled with FAM to detect the gene of interest by duplex PCR on any cycler. For multiplex analyses, we strongly recommend using dual-labeled TaqMan or TaqMan MGB Probes with nonfluorescent quenchers.
- Always use 30 pg–30 ng template DNA (see Table 4).
- Use the primer and probe concentrations specified in this protocol.
- **Always use** the cycling conditions specified in this protocol. The cycling conditions and the Type-it CNV Reference Probe Assay have been optimized for use with GOI assays generating amplicons between 60 bp and 150 bp.
- The PCR **must** start with an initial incubation step of 5 min at 95°C to activate HotStarTaq *Plus* DNA Polymerase.
- For data analysis, always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.
- We recommend preparing a 25x primer–probe mix containing specific primers and probe (recommended concentrations in the 25x primer–probe mix: 10 µM of each primer and 5 µM of the probe) for each GOI (see Appendix A, page 25).
- We strongly recommend testing the performance of new TaqMan primer/probe sets in individual assays before combining them with the Type-it CNV Probe Reference Assay in multiplex assays.

Procedure

1. **Thaw the 2x Type-it Probe PCR Master Mix, template DNA (human genomic DNA), Type-it CNV Reference Probe Assay 25x working solution, 25x primer–probe mix containing primers and probe for the GOI, and RNase-free water. Mix the individual solutions and place them on ice.**
2. **Prepare a reaction mixture according to Table 4.**

   Note: It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. Typically, reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep samples and reconstituted assays on ice or in a cooling device.
3. **Mix the reaction mixture thoroughly and dispense appropriate volumes into PCR tubes or the wells of a PCR plate or Rotor-Disc.**

   Note: For optimal duplex performance of the GOI and Type-it CNV Reference Probe Assays, we recommend using a total reaction volume of 25 µl. If your real-time cycler requires a different reaction volume, adjust the amount of reaction mix including all reaction components accordingly.
4. **Add template DNA (start with 10 ng; use 30 pg–30 ng as the range) to the individual PCR tubes or wells. See Table 4 for details.**
5. **Program the real-time cycler according to Table 5.**

   Note: Data acquisition should be performed during the combined annealing/ extension step.
6. **Place the PCR tubes, Rotor-Disc, or plate in the real-time cycler, and start the cycling program.**
7. **Perform data analysis.**

   Note: Before performing data analysis, select the analysis settings for each probe (i.e., baseline settings and threshold values). Optimal analysis settings are a prerequisite for accurate quantification data. Perform the $\Delta\Delta C_T$ method of relative quantification.

   Note: Only if using the Applied Biosystems 7500, 7500 Fast, or ViiA7 Real-Time PCR Systems, it is recommended to use the 'manual C_T' function instead of the 'auto C_T' function for data analysis. Use a value of 0.01 as a starting point for the threshold setting. For all other cyclers, use the automatic C_T function as a starting point.

Table 4. Reaction setup for duplex, triplex, or 4-plex PCR using self-designed GOI assays

Component	Volume (µl)
Reaction mix	
2x Type-it Probe PCR Master Mix	12.5
25x Type-it CNV Reference Probe Assay solution*	1
25x primer–probe for GOI 1† (FAM labeled)	1
Only for triplex PCR: 25x primer–probe mix for GOI 2† (labeled with dye 3)‡	1
Only for 4-plex PCR: 25x primer–probe mix for GOI 3† (labeled with dye 4)‡	1
RNase-free water	Variable
Template DNA (added at step 4)	Start with 10 ng (use 30 pg–30 ng as the range)
Total reaction volume	25§

* See “Reconstitution and use of Type-it CNV Reference Probe Assay” on page 13.

† **IMPORANT**: A 25x primer–probe mix consists of 10 µM forward primer, 10 µM reverse primer, and 5 µM probe in Buffer TE, resulting in a final concentration of 0.4 µM forward and reverse primer, and 0.2 µM probe.

‡ Suitable dyes for multiplex analysis (dependent on cycler) must be detected in channels/filters different from FAM and MAX/VIC/JOE/HEX channels/filters.

§ If the real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the ABI PRISM 7900, use a reaction volume of 10 µl.

Table 5. Cycling conditions

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			
Denaturation	**30 s**	**95°C**	
Annealing/extension	**30 s***	**60°C**	Combined annealing/ extension step with fluorescence data collection.
Number of cycles	40		The number of cycles depends on the amount of template DNA.

* For real-time PCR systems that require a minimum annealing/extension time longer than 30 seconds, adjust the time accordingly, as per the requirements of the real-time PCR system in operation.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Target signal not detected (or detected late)	
a) Incorrect cycling conditions	Always start with the optimized cycling conditions specified in the protocols.
b) HotStarTaq *Plus* DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq *Plus* DNA Polymerase activation step (5 min at 95°C) as described in the protocols.
c) Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. See Appendix D, page 29, for details on evaluating the concentration of primers and probes. Repeat the assay.
d) Incorrect or no detection step	Ensure that fluorescence detection takes place during the combined annealing/extension step when using TaqMan probes.
e) Insufficient number of cycles	Increase the number of cycles.
f) Wavy curve at high template amounts	Discard all the components of the multiplex assay (e.g., master mix, primers, and probes). Repeat the multiplex assay using fresh components.
g) Probe design not optimal	If the amplification reaction was successful, there may be a problem with the probe. Review the probe design guidelines (see Appendix C, page 27).

	Comments and suggestions
h) Primer or probe concentration not optimal	Use optimal primer concentrations (each primer at a final concentration of 0.4 *μ*M). In most cases, a probe concentration of 0.2 *μ*M gives satisfactory results. Depending on the quality of your probe, results may be improved by adjusting probe concentration within the range of 0.1–0.4 *μ*M. Check the concentrations of primers and probes by spectrophotometry (see Appendix D, page 29). Ensure the correct handling and storage of the Type-it CNV Reference Probe Assay (see "Shipping and Storage", page 4 and "Reconstitution and use of Type-it CNV Reference Probe Assay", page 13).
i) Incorrect detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for each reporter dye. Check whether the chosen combination of reporter dyes is compatible with the selected detection channels or filter sets. Please refer to the instructions for the real-time cycler for more details.

Differences in C_T values or in PCR efficiencies between a duplex assay and the corresponding singleplex assay

a) Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of HotStarTaq *Plus* DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension.
b) Analysis settings (e.g., threshold and baseline settings) not optimal	Check the analysis settings (threshold and baseline settings) for each reporter dye. Repeat analysis using optimal settings for each reporter dye.

	Comments and suggestions
c) Imprecise spectral separation of reporter dyes	Since multiplex assays use multiple probes, each with a fluorescent dye, the increased fluorescent background may affect the shape of the amplification plots obtained with some real-time cyclers. This may lead to differences in C_T values of up to 5% between the multiplex assay and the corresponding singleplex assays; this can usually be avoided by using optimal threshold settings.
No linearity in ratio of C_T value/crossing point to log of the template amount	
a) Template amount too high	When signals are coming up at very early C_T values (e.g., <6), adjust the analysis settings accordingly.
b) Template amount too low	Increase template amount if possible. Note that detection of very low starting copy numbers may not be in the linear range of a standard curve.
Varying fluorescence intensity among replicates	
a) Contamination of real-time cycler with fluorescently labeled probes	Decontaminate the real-time cycler according to the manufacturer's instructions.
b) Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
c) Wavy curve at high template amounts	In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template.

Appendix A: Preparing a 25x Primer–Probe Mix for each GOI

For ease of use, we recommend preparing a 25x primer–probe mix for the GOI containing target-specific primers and probe (Table 6).

Table 6. Preparing 25x primer–probe mix for the GOI assay

Component	Concentration (25x)	Final concentration
Forward primer*	10 µM	0.4 µM†
Reverse primer*	10 µM	0.4 µM†
Probe	5 µM	0.2 µM‡
Buffer TE	–	–

* If using more than one forward or reverse primer per target, use a 4 µM concentration for each primer.

† A final primer concentration of 0.4 µM is optimal in most cases. Depending on assay design and GOI target sequence, performance might be improved by increasing the primer concentration to 0.5 to 1.0 µM. Before adapting primer concentration, verify the concentration of the primer solutions.

‡ A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Appendix B: Starting Template

Both the quality and quantity of nucleic acid starting template affect PCR, in particular the sensitivity and efficiency of amplification.

Quality of starting template

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, ethanol, EDTA, and other chemical solvents than single-step enzyme-catalyzed processes (see Table 7). QIAGEN offers a complete range of nucleic acid preparation systems, ensuring the highest-quality templates for PCR, for example, the QIAamp® and DNeasy® systems for rapid purification of genomic DNA. For more information, contact our Technical Service Departments (see back cover or visit www.qiagen.com).

Table 7. Impurities exhibiting inhibitory effects on PCR

Impurity	Inhibitor concentration
SDS	>0.005% (w/v)
Phenol	>0.2% (v/v)
Ethanol	>1% (v/v)
Isopropanol	>1% (v/v)
Sodium acetate	5 mM
Sodium chloride	25 mM
EDTA	0.5 mM
Hemoglobin	1 mg/ml
Heparin	0.15 i.U./ml
Urea	>20 mM
RT reaction mixture	15% (v/v)

Quantity of starting template

The annealing efficiency of primer to template is an important factor in PCR. Owing to the thermodynamic nature of the reaction, the primer: template ratio strongly influences the specificity and efficiency of PCR and should be optimized empirically. If too little template is used, primers may not be able to find their complementary sequences. Too much template may lead to an increase in mispriming events. As an initial guide, spectrophotometric and molar conversion values for different nucleic acid templates are listed in Tables 8 and 9, respectively.

Table 8. Spectrophotometric conversions for nucleic acid templates

1 A_{260} unit*	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33

* Absorbance at 260 nm = 1

Table 9. Molar conversions for genomic DNA templates

Genomic DNA	Size	pmol/µg	Molecules/µg
Drosophila melanogaster	1.4×10^{8}*	1.1×10^{-5}	6.6×10^{5}†
Mus musculus (mouse)	2.7×10^{9}*	5.7×10^{-7}	3.4×10^{5}†
Homo sapiens (human)	3.3×10^{9}*	4.7×10^{-7}	2.8×10^{5}†

* Base pairs in haploid genome.

† For single-copy genes.

Table 10. Conversion of copy numbers of starting template for different DNA sources

Number of copies of starting template	Human genomic DNA‡
100–1000	0.36–3.6 ng
$>1 \times 10^{3} – 5 \times 10^{4}$	3.6–179 ng
$>5 \times 10^{4}$	>179 ng

‡ Refers to single-copy genes.

Appendix C: Assay and Probe Design

Several important factors need to be considered when designing GOI assays to ensure reliable $\Delta\Delta C_T$-based quantification. These include:

- Optimal primer and probe design
- Use of appropriate primer and probe concentrations
- Correct storage of primers and probes

Assay design

Guidelines for the optimal design of primers and probes are given on the next page.

Table 11. Guidelines for primer/probe design

	Guidelines
Software	■ Use specialized design software (e.g., Primer Express® Software). ■ Pre-existing assays designed with this software under standard settings typically work fine as they are.
Designing new assays	■ Using standard algorithm parameters and reaction conditions of Primer Express or Primer3 Software, the T_m of both primers should be 58–63°C and within 2°C of each other. ■ T_m of probes should be 5–10°C higher than the T_m of primers. ■ Avoid a guanidine at the 5' end of probes, next to the reporter, since this causes quenching. ■ Avoid runs of 4 or more of the same nucleotide, especially guanidine. ■ Choose the binding strand so that the probe has more C than G bases. ■ Design primers or probes in a conserved region of the GOI. ■ Perform appropriate database analysis to identify such a region and verify the functionality of the assay using isolates of a different origin.
Primer sequence	■ Length: 18–30 nucleotides. ■ GC content: 30–70%. ■ Always check the specificity of primers by performing a BLAST® search (www.ncbi.nlm.nih.gov/blast). Ensure that primer sequences are unique for your template sequence. ■ Check that primers and probe are not complementary to each other. ■ Try to avoid highly repetitive sequences. ■ Avoid mismatches between the 3' end of primers and the template sequence.

Table continued on next page.

Table 11. Continued

	Guidelines
	■ Avoid complementarity of 2 or 3 bases at the 3' ends of primer pairs to minimize primer dimer formation. ■ Avoid runs of 3 or more Gs and/or Cs at the 3' end.

Appendix D: Handling and Storage of Primers and Probes

Dissolving GOI primers and probes

- To reconstitute the Type-it CNV Reference Probe Assay (100) to a 25x working solution, see page 13.
- To reconstitute your GOI-specific primers and probe to a 25x working solution, briefly centrifuge the tube, add 110 µl Buffer TE (pH 8.0; provided with the kit), and mix by vortexing the tube 4–6 times. If necessary, gently warm the tube to help the primers dissolve. The reconstituted primers and probes should be stored at –20°C.
- We do not recommend dissolving primers and probes in water. Lyophilized primers should be dissolved in a small volume of low-salt Buffer TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) to make a concentrated stock solution.

Quantification of primers

The given amount and/or concentration after dissolving of commercially supplied primers is often a very rough approximation. Before use, primers should be accurately quantified using a spectrophotometer. After dissolving the primer using the volume of Buffer TE quoted on the oligo vial or datasheet, measure the A_{260} (OD) of a 1 in 100 dilution of the stock solution using a glass cuvette with a 1 cm path-length, and calculate the concentration.* This measured value should be used for subsequent calculations.

Spectrophotometric conversion for primers: 1 A_{260} unit (1 OD) = 20–30 µg/ml

Concentration can be derived from the molar extinction coefficient (Σ_{260}) and A_{260} (OD).

A_{260} (OD) = Σ_{260} x molar concentration of the primer

* To ensure significance, A_{260} readings should be greater than 0.15.

If the $\sum_{260}$ value is not given on the primer data sheet, it can be calculated from the primer sequence using the following formula:

A_{260}(OD)= 0.89 x [(nA x 15,480) + (nC x 7340) + (nG x 11,760) + (nT x 8850)

where n = number of respective bases.

Example:

Primer length: 24 nucleotides with 6 each of A, C, G, and T bases.

Observed A_{260}(OD) of a 1 in 100 dilution = 0.283

$\sum_{260}$ = 0.89 x [(6 x 15,480) + (6 x 7340) + (6 x 11,760) + (6 x 8850)] = 231,916

Concentration = A_{260}(OD)/$\sum_{260}$ = 0.283/231,916 = 1.22 x 10^{-6}M = 1.22 μM

Concentration of primer stock solution = concentration of dilution x dilution factor =1.22 μM x 100 = 122 μM

Storage of primers and probes

Primers should be stored in small aliquots at –20°C. Unmodified primers are stable under these conditions for at least 1 year. Fluorescently labeled probes are usually stable under these conditions for at least 6 months. Repeated freeze–thaw cycles should be avoided, as they may lead to degradation.

Primer and probe quality

The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel; a single band should be seen. Please contact QIAGEN Technical Services or your local distributor for a protocol.

The quality of the fluorescent label and the purity of hydrolysis probes can be determined by comparing fluorescence before and after DNase digestion. Incubate probes with or without 5 units DNase at 37°C for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.

Appendix E: Suitable Reporter Dyes on Different Cyclers

Multiplex, real-time PCR requires the simultaneous detection of two or more different fluorescent reporter dyes. The Type-it CNV Reference Probe Assay provided with the Type-it CNV Probe PCR +qC Kit uses the reporter dye MAX, which has excitation/emission maxima of 524/557 nm, and a nonfluorescent quencher (Iowa Black®). For accurate detection, the fluorescence spectrum of the GOI assay dye must be well separated from the MAX spectrum or exhibit only minimal overlap.

For duplex quantification of gene copy number with the Type-it CNV Reference Probe Assay, we recommend using FAM as a reporter dye for the GOI. This will guarantee optimal duplex performance with the Type-it CNV Reference Probe Assay. Other dyes detected in a different detection channel than MAX may also be suitable.

For optimal results, we recommend using a nonfluorescent quencher for the GOI assay (e.g., Dark Quencher, Black Hole Quencher® [BHQ®] or Iowa Black Quencher). It is **not recommended** to use fluorescent quenchers (e.g., TAMRA™ fluorescent dye). Due to their own native fluorescence, fluorescent quenchers contribute to an overall increase in background and reduce the signal-to-noise ratio.

Before starting, choose the suitable filter or channel for detection of GOI and reference assay reporter dyes using the detection optics of your real-time cycler. If you have not used the reporter dyes FAM or MAX on the real-time cycler before, note that some instruments require a calibration procedure to be performed for a reporter dye when using it for the first time. Additionally, check whether the instrument requires the addition of ROX as a reference dye.

Table 12. Dyes commonly used in quantitative, multiplex, real-time PCR*

Dye	Excitation maximum (nm)	Emission maximum (nm)†
FAM	494	518
TET™	521	538
JOE	520	548
VIC	538	552
MAX	524	557
Yakima Yellow®	526	552
HEX	535	553
Bodipy® TMR	542	574
NED™	546	575
Cy3	552	570
TAMRA	560	582
Cy3.5	588	604
ROX	587	607
Texas Red	596	615
Cy5	643	667

* If there are no specific recommendations in the tables below for your real-time cycler, please refer to the user manual or other technical documentation for your instrument to find out which filters can be used for the recommended reporter dyes in duplex analysis.

† Emission spectra may vary depending on the buffer conditions.

The following tables show potential multiplex-grades for various cyclers that can be used with the Type-it CNV Probe PCR +qC Kit. Limitations in maximum levels of multiplexing are due to cycler.

Table 13. Suitable reporter dyes — Rotor-Gene Q

Type of assay	Green channel	Yellow channel (Type-it CNV Reference Probe Assay)	Red channel	Crimson channel
Duplex	FAM	MAX		
Triplex	FAM	MAX		Quasar® 705
4-plex	FAM	MAX	Cy5	Quasar 705

Table 14. Suitable reporter dyes — Rotor-Gene 3000

Type of assay	Channel 1 (470/510)	Channel 2 (530/555) (Type-it CNV Reference Probe Assay)	Channel 4 (625/665)
Duplex	6-FAM	MAX	
Triplex	6-FAM	MAX	Cy5

Table 15: Suitable reporter dyes — ABI PRISM 7000 and Applied Biosystems 7300

Type of assay	Filter A	Filter B (Type-it CNV Reference Probe Assay)	Filter C	Filter D
Duplex	6-FAM	MAX		ROX (passive reference)
Triplex	6-FAM	MAX	Bodipy TMR NED	ROX (passive reference)

Table 16. Suitable reporter dyes — Applied Biosystems 7500

Type of assay	Filter A	Filter B (Type-it CNV Reference Probe Assay)	Filter C	Filter D	Filter E
Duplex	6-FAM	MAX		ROX (passive reference)	
Triplex	6-FAM	MAX	Bodipy TMR NED	ROX (passive reference)	
4-plex	6-FAM	MAX	Bodipy TMR NED	ROX (passive reference)	Cy5

Table 17. Suitable reporter dyes — Applied Biosystems 7900HT

Type of assay	Target 1	Target 2 (Type-it CNV Reference Probe Assay)	Target 3	Passive reference
Duplex	6-FAM	MAX		ROX
Triplex	6-FAM	MAX	Bodipy TMR NED	ROX

Table 18. Suitable reporter dyes — ABI PRISM 7700

Type of assay	Target 1	Target 2 (Type-it CNV Reference Probe Assay)	Passive reference
Duplex	6-FAM	MAX	ROX

Table 19. Suitable reporter dyes — Applied Biosystems StepOne

Type of assay	Filter 1	Filter 2 (Type-it CNV Reference Probe Assay)	Passive reference
Duplex	6-FAM	MAX	ROX

Table 20. Suitable reporter dyes — Applied Biosystems StepOnePlus

Type of assay	Filter 1	Filter 2 (Type-it CNV Reference Probe Assay)	Filter 3	Filter 4
Duplex	6-FAM	MAX		ROX (passive reference)
Triplex	6-FAM	MAX	Bodipy TMR NED	ROX (passive reference)

Table 21. Suitable reporter dyes — Applied Biosystems ViiA 7,

Type of assay	Filter x1-mx	Filter x2-m2 (Type-it CNV Reference Probe Assay)	Filter x3-m3	Filter x4-m4	Filter x5-m5
Duplex	6-FAM	MAX		ROX (passive reference)	
Triplex	6-FAM	MAX	Bodipy TMR NED	ROX (passive reference)	
4-plex	6-FAM	MAX	Bodipy TMR NED	ROX (passive reference)	Cy5

Table 22. Suitable reporter dyes — Mx3000P, Mx3005P, and Mx4000®

Type of assay	Optical path 1 (FAM filter set)	Optical path 2 (HEX/JOE filter set, (Type-it CNV Reference Probe Assay)	Optical path 3 (ROX filter set, Passive Reference)	Optical path 4 (Cy5 filter set)
Duplex	6-FAM	MAX	ROX	
Triplex	6-FAM	MAX	ROX	Cy5

Table 23. Suitable reporter dyes — iCycler iQ

Type of assay	Channel 1 (filter 490/530)	Channel 2 (filter 530/575) (Type-it CNV Reference Probe Assay)	Channel 4 (filter 635/680)
Duplex	6-FAM	MAX	
Triplex	6-FAM	MAX	Cy5

Table 24. Suitable reporter dyes — Mastercycler ep *realplex*

Type of assay	Channel 1 (520 nm filter)	Channel 2 (550 nm filter)	Channel 4 (605 nm filter), ROX passive reference
Duplex	6-FAM	MAX	ROX

Table 25. Suitable reporter dyes — LightCycler 2.0

Type of assay	Detection channel 1 (530 nm filter)	Detection channel 2 (560 nm filter) (Type-it CNV Reference Probe Assay)	Detection channel 5 (670 nm filter)	Detection channel 6 (705 nm filter)
Duplex	6-FAM	MAX		
Triplex	6-FAM	MAX	Cy5	
Triplex	6-FAM	MAX		Alexa Fluor® 660 Pulsar® 650

Table 26. Suitable reporter dyes — LightCycler 480

Type of assay	Channel 1 (450/500)	Channel 2 (438/533)	Channel 3 (523/568), (Type-it CNV Reference Probe Assay)	Channel 5 (615/670)
Duplex		6-FAM	MAX	
Triplex	Cyan 500	6-FAM	MAX	
Triplex		6-FAM	MAX	Cy5

Appendix F: Rotor-Gene Q Setup for Adjustment of Fluorescence Channel Sensitivity

Adjustment of fluorescence channel sensitivity for the Type-it CNV Reference Probe Assay

We recommend setting the detection range of the yellow channel for the Type-it CNV Reference Probe Assay on a fixed gain to ensure optimal fluorescence gain. Click “Edit Gain” in the “New Run Wizard” dialog box (Figure 2) to open the “Gain for Yellow” dialog box. Set the gain for the yellow channel to a value of 9 (Figure 2).

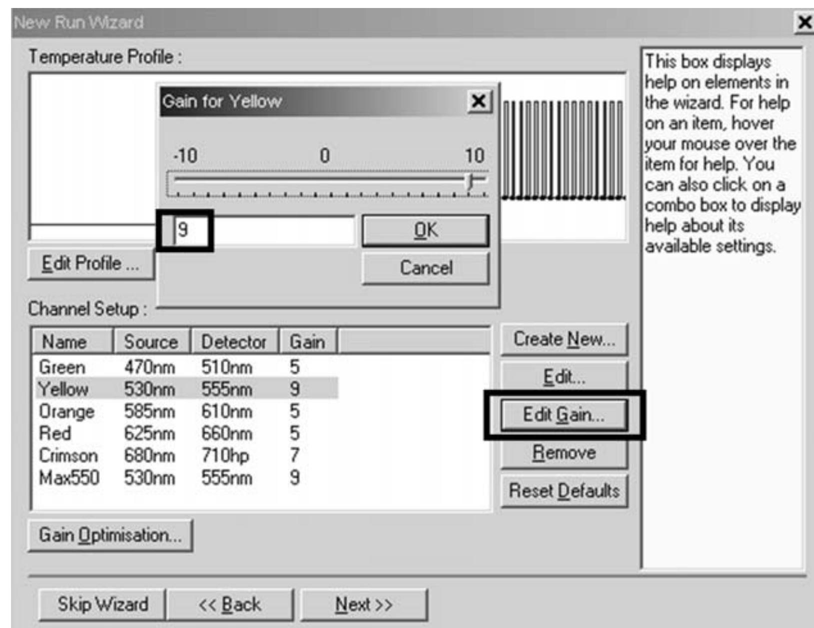
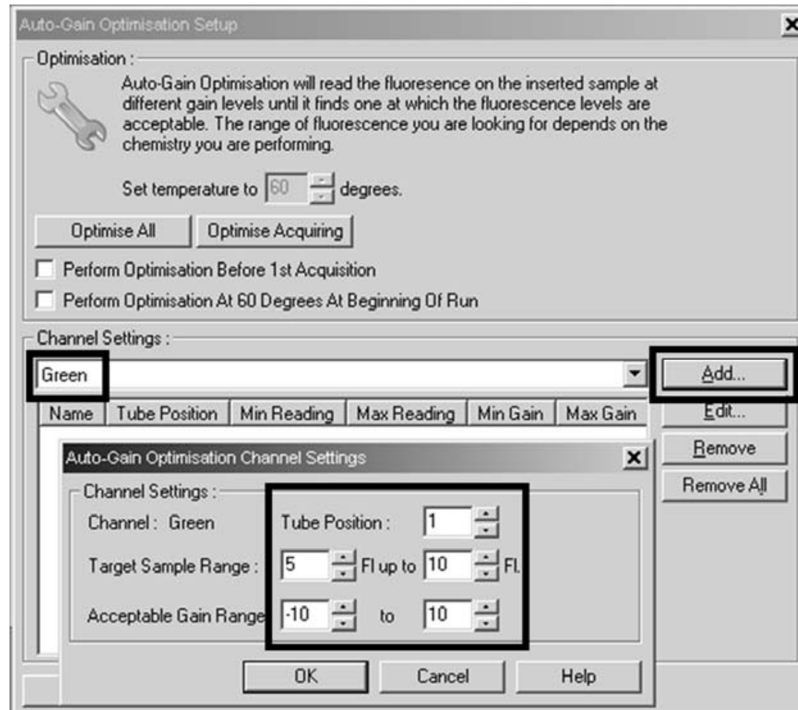


Figure 2. Setting a fixed gain for the Type-it CNV Reference Probe Assay (yellow channel).

Adjustment of fluorescence channel sensitivity for the GOI assay

We recommend determining the detection range of the green channel for the GOI assay according to the fluorescence intensities in the PCR tubes. Click “Gain Optimisation” in the “New Run Wizard” dialog box (Figure 2) to open the “Auto-Gain Optimisation Setup” dialog box. Add channel “Green” from the drop-down menu and adapt the “Auto-Gain Optimisation Settings” as shown in Figure 3A). Adjust the calibration temperature to 60 degrees to match the annealing temperature of the amplification program, and check the box “Perform Optimisation Before 1st Acquisition” (Figure 3B).

A



B

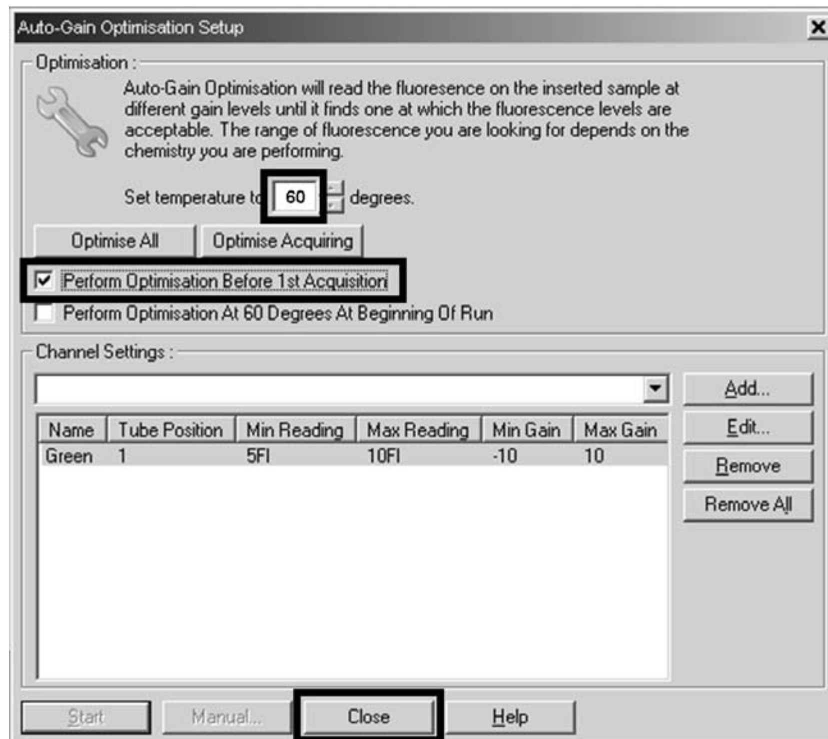


Figure 3. Adjusting the fluorescence channel sensitivity for the GOI assay (green channel) in the “Auto-Gain Optimisation Setup” dialog box.

Appendix G: Relative Quantification

For detailed information on qPCR and qPCR terminology; please refer to our brochure *Critical Factors for Successful Real-Time PCR*. To obtain a copy, contact QIAGEN Technical Services, or visit www.qiagen.com/literature/defaultbrochures.aspx to download a PDF.

Comparative method or $\Delta\Delta C_T$ method of relative quantification

The comparative or $\Delta\Delta C_T$ method of relative quantification relies on direct comparison of C_T values. For a valid $\Delta\Delta C_T$ calculation, the amplification efficiency of the target and the reference a must be approximately equal. Examining how ΔC_T (C_T target – C_T reference) varies with template dilution can help determine if the two amplification reactions have the same PCR efficiency. Evaluating the relative efficiencies of the target amplification and the reference (control) amplification is achieved by plotting standard curves for each amplicon using the same sample. The C_T values generated from equivalent standard curve mass points (target versus reference) are used in the ΔC_T calculation (C_T target – C_T reference). The preparation of standard curves is only required to determine the amplification efficiencies of the target and reference genes in an initial experiment. In all subsequent experiments, no standard curve is required for quantification of the target sequence. If amplification efficiencies are comparable, amounts of target are simply calculated by using C_T values as described below.

First of all, the ΔC_T value for each sample is determined by calculating the difference between the C_T value of the target gene and the C_T value of the reference gene (i.e., the multi-copy genetic element detected by the Type-it Reference Probe Assay). This is determined for each unknown test sample, as well as for a control sample, which, in subsequent analysis, will serve as a calibrator sample.

Guidelines for relative quantification using $\Delta\Delta C_T$ method

- Perform a validation experiment to determine the PCR efficiency for the target and reference gene.
- Perform real-time PCR for the target and reference with DNA derived from different samples.
- Determine the ΔC_T value by subtracting the reference gene C_T value from the target gene C_T value for each sample.
- Define the control sample and determine the $\Delta\Delta C_T$ value by subtracting the calibrator ΔC_T value from the ΔC_T value of each sample.
- Calculate the copy number change of the GOI relative to the control sample by using the formula $R=2^{-\Delta\Delta C_T}$.

The $\Delta\Delta C_T$-based relative quantification method can be used to determine whether the copy number of the GOI is changed in different samples. The relative quantification method is based on the assumption that the copy number of the reference gene is not changed among different samples; therefore any C_T difference of the reference assay reflects the difference of the template DNA amount. Compared to commonly used single-copy genes, multi-copy genetic elements distributed over different chromosomes, such as the one detected by Type-it CNV Reference Probe Assay, are more reliable references for qPCR-based copy number quantification. This is because the relative impact of an occasional copy number or sequence change of the multi-copy genomic element on the C_T difference, and the calculated copy number change for the GOI, is much smaller compared to that of a single-copy reference gene.

The ratio of the copy number change of the GOI (R) in a test sample compared to a control sample can be calculated using the following equation:

$R = 2^{-\Delta\Delta C_T}$

$\Delta\Delta C_T = \Delta C_T$ (Test sample) $- \Delta C_T$ (Control sample)

$=$ (C_T (GOI, Test sample) $- C_T$ (Reference, Test sample)) $-$

(C_T (GOI, Control sample) $- C_T$ (Reference, Control sample))

If $R > 1$, the copy number of the GOI is higher in the test sample than the control sample; if $R < 1$, the copy number of the GOI is lower in the test sample than the control sample.

Alternatively, the PCR efficiency of both the GOI and reference assays can be taken into consideration to calculate the change of the GOI copy number more accurately. In addition, more than one reference assay can be used to ensure even more reliable results. We recommend REST Software for such advanced copy number analysis.

$\Delta\Delta C_T$ value: The $\Delta\Delta C_T$ value describes the difference between the average ΔC_T value of the sample of interest (e.g., microdeletion) and the average ΔC_T value of a reference sample (e.g., unaltered). The reference sample is also known as the calibrator sample and all other samples will be normalized to this when performing relative quantification.

$\Delta\Delta C_T$ = Average ΔC_T (sample of interest) – average ΔC_T (reference sample)

Therefore, it is of high importance to use reproducible and reliable methods, and optimized reagents and assays — as provided in the Type-it CNV Probe PCR +qC Kit — to avoid false-positive and -negative results, implying false gain and false loss, respectively.

For more information on the $2^{-\Delta\Delta C_T}$ method, please refer to (Livak and Schmittgen, 2001).*

Appendix H: Data Analysis

When carrying out data analysis, follow the recommendations provided by the manufacturer of the real-time cycler. Fundamental guidelines for data analysis and some important considerations are given below. Further information can be found in *Critical Factors for Successful Real-Time PCR*. To obtain a copy, contact QIAGEN Technical Services, or visit www.qiagen.com/literature/brochures to download a PDF.

Considerations for duplex/multiplex data analysis

Real-time PCR data are produced as sigmoidal-shaped amplification plots (when using a linear scale), in which fluorescence is plotted against the number of cycles.

- The quantification cycle (Cq value) or threshold cycle (C_T value) serves as a tool for calculation of the starting template amount in each sample. This is the cycle in which there is the first detectable significant increase in fluorescence.
- The optimal threshold setting depends on the reaction chemistries used for PCR. Therefore, an optimal threshold setting established for another kit may not be suitable for the Type-it Probe PCR +qC Kit, and may need to be adjusted.
- The method for determination of C_T values differs depending on the real-time cycler used. Check the handbook or the software help file for your real-time cycler for details on threshold settings.
- Most real-time cyclers contain a function that determines the noise level (background) in early cycles, where there is no detectable increase in fluorescence due to PCR products (usually referred to as the baseline settings). Adjust the settings for this function.
- For duplex or multiplex assays, the analysis settings need to be adjusted for each of the reporter dyes used.
- Depending on your real-time cycler, low levels of signal crosstalk, even between apparently well-separated reporter dyes, may influence duplex results in rare cases. In most cases, low levels of crosstalk can be overcome by optimal analysis settings.

* Livak, K. J. and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. Methods. ***25***, 402.

Ordering Information

Product	Contents	Cat. no.
Type-it CNV Probe PCR +qC Kit (100)	For 100 x 25 µl multiplex PCR reactions: Contains 1.3 ml 2x Type-it Probe PCR Master Mix (with ROX dye), Type-it CNV Reference Probe Assay (for 100 reactions), Buffer TE, and RNase-Free Water	206662
Type-it CNV Probe PCR +qC Kit (400)	For 400 x 25 µl multiplex PCR reactions: Contains 5.2 ml 2x Type-it Probe PCR Master Mix (with ROX dye), Type-it CNV Reference Probe Assay (for 400 reactions), Buffer TE, and RNase-Free Water	206664
Related products		
Type-it CNV SYBR® Green PCR+ qC Kit (100)	For 100 x 25 µl PCR reactions: Contains 1.3 ml 2x Type-it SYBR Green PCR Master Mix, Type-it CNV Reference Primer Assay (for 100 reactions), Buffer TE, and RNase-Free Water	206672
Type-it CNV SYBR Green PCR+ qC Kit (400)	For 400 x 25 µl PCR reactions: Contains 5.2 ml 2x Type-it SYBR Green PCR Master Mix, Type-it CNV Reference Primer Assay (for 400 reactions), Buffer TE, and RNase-Free Water	206674
Type-it CNV SYBR Green PCR Core Kit (400)	For 400 x 25 µl PCR reactions: Contains 5.2 ml 2x Type-it SYBR Green PCR Master Mix, Buffer TE, and RNase-Free Water	206624

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