February 2012

Type-it® CNV SYBR® Green PCR Handbook

Type-it CNV SYBR Green PCR+ qC Kit

Type-it CNV SYBR Green PCR Core Kit

For reliable and precise relative quantification of gene copy number in the human genome using SYBR Green based-qPCR

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Contents

Kit Contents

Type-it CNV SYBR Green PCR +qC Kit	(100)	(400)
Catalog no.	**206672**	**206674**
Number of 25 µl reactions	**100**	**400**
2x Type-it SYBR Green PCR Master Mix	1.3 ml	5.2 ml
Type-it CNV Reference Primer 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 SYBR Green PCR Buffer, and dNTP mix (dATP, dCTP, dGTP, dTTP).

Type-it CNV SYBR Green PCR Core Kit	(400)
Catalog no.	**206624**
Number of 25 µl reactions	**400**
2x Type-it SYBR Green PCR Master Mix†	5.2 ml
Buffer TE	2 ml
RNase-Free Water	3 x 1.9 ml
Quick-Start Protocol leaflet	1

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

Shipping and Storage

The Type-it CNV SYBR Green PCR +qC Kit and the Type-it CNV SYBR Green PCR Core Kit are 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 kits can be stored under these conditions until the expiration date on the kit box, without showing any reduction in performance.

The 2x Type-it SYBR Green 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 Primer 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 Reference Primer Assay, see "Reconstitution and use of 25x Type-it CNV Reference Primer Assay", page 12.

Intended Use

Type-it CNV SYBR Green PCR Kits are intended for molecular biology applications. These kits are 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 SYBR Green PCR +qC Kit and the Type-it CNV SYBR Green PCR Core Kit is tested against predetermined specifications to ensure consistent product quality.

Product Description

Component	Description
2x Type-it SYBR Green 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 SYBR Green PCR Buffer:	Novel PCR buffer for accurate and precise quantification of gene copy number; includes Q-Bond® technology for fast cycling.
dNTP mix:	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality
Type-it CNV Reference Primer Assay:*	Universal relative quantification control assay for reliable $\Delta\Delta C_T$-based† copy number calculation of the gene of interest by SYBR Green based-qPCR. 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

* Not supplied with the Type-it CNV SYBR Green Core Kit (400).

† 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 copy number variation (CNV) validation and association studies. For further details on relative quantification, please refer to Appendix E, page 25.

The Type-it CNV SYBR Green PCR +qC Kit provides all the components required for accurate and reliable quantification of all germline and somatic copy number variations using SYBR Green based-qPCR. The 2x master mix and the Type-it CNV Reference Primer Assay provided with the Type-it CNV SYBR Green +qC Kit are preoptimized for use with all genes of interest (GOI), eliminating the need for time-consuming optimization of SYBR Green-based qPCR and validation of reference assays for each GOI under investigation.

The Type-it Reference Primer Assay is based on a multi-copy genetic element and serves as a universal quantification reference to enable reliable $\Delta\Delta C_T$-based relative gene copy number quantification and validation of possible CNVs. 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 SYBR Green PCR Master Mix provided with Type-it CNV SYBR Green PCR Kits is suited for use with various real-time cyclers, including the Rotor-Gene® Q, as well as instruments from Applied Biosystems, Bio-Rad, Cepheid, Eppendorf, Roche, and Agilent (Table 1). The preoptimized protocol described in this handbook and the Quick-Start Protocol provided with the kit enable you to perform reliable SYBR Green-based PCR to detect copy number alterations with maximum precision and ease.

The Type-it CNV SYBR Green PCR Core Kit includes all the components provided with the Type-it CNV SYBR Green PCR +qC Kit, with the exception of the Type-it CNV Reference Primer Assay. Since the Type-it CNV Reference Primer Assay included with the Type-it CNV SYBR Green PCR +qC Kit is provided in excess, the Type-it CNV SYBR Green PCR Core Kit can be purchased separately to utilize any leftover reference assay.

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 SYBR Green PCR Master Mix

In contrast with current methods, Type-it CNV SYBR Green PCR Kits eliminate the need for optimization of the concentrations of primers, Mg^{2+}, and DNA polymerase. The 2x Type-it SYBR Green PCR Master Mix contains HotStarTaq *Plus* DNA Polymerase, Type-it SYBR Green PCR Buffer, and dNTPs.

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 SYBR Green PCR Buffer

The Type-it SYBR Green 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 SYBR Green-based qPCR.

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 annealing to a few seconds. This allows a combined

annealing/extension step of only 30 seconds in 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.

Type-it CNV Reference Primer Assay

The Type-it CNV Reference Primer Assay allows amplification of a multi-copy genetic element that can be used as reference for reliable $\Delta\Delta C_T$-based relative copy number quantification of the GOI.

The Type-it CNV Reference Primer Assay is a universal reference assay for reliable $\Delta\Delta C_T$-based quantification of the CNV in the human genome.

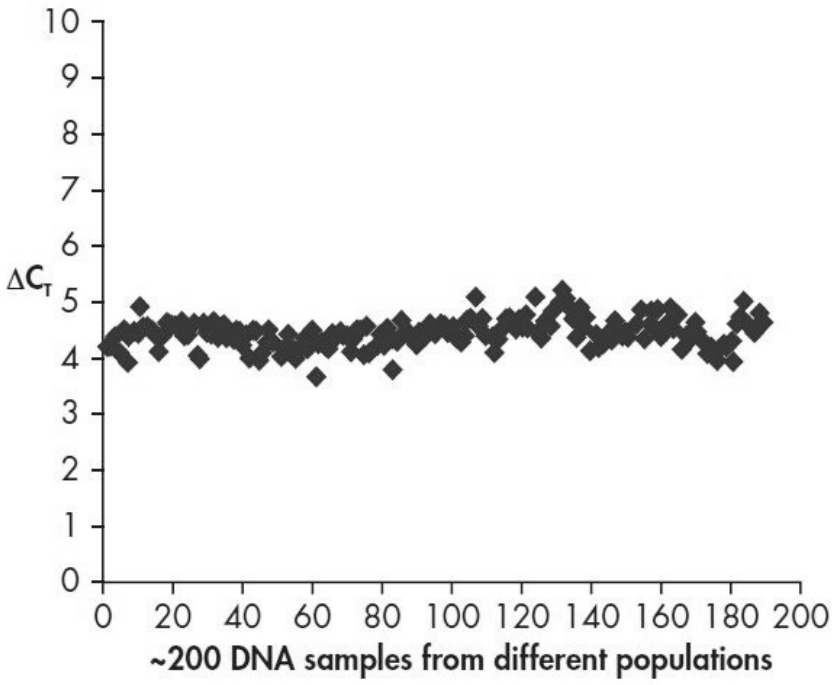

Figure 1. Highly reliable multi-copy quantification control assay. DNA samples from 188 donors were evaluated. ΔC_T values between a nonvariable region of the KRAS gene and the multi-copy genetic element were detected by the Type-it CNV Reference Assay.

The Type-it CNV Reference Primer 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 Primer Assay (provided lyophilized) contains a forward and reverse primer, and has been specifically optimized for highly specific and efficient amplification. To ensure optimal performance 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 22.

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 Primer Assay serves as a quantification control and enables fast and efficient relative quantification of gene copy number using SYBR Green qPCR.

For further information, please see Appendix E, page 25, 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 for the GOI target must be purchased from an established oligonucleotide manufacturer. Primers should be of standard quality or high-performance liquid chromatography (HPLC) pure. Lyophilized primers 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 24).
- 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 Primer Assay

To reconstitute the Type-it CNV Reference Primer 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 Primer 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 CNVs in the GOI by SYBR Green qPCR using the Type-it CNV Reference Primer Assay

The Type-it CNV SYBR Green +qC Kit works with most existing primer assays that have been designed using standard real-time PCR design parameters. However, for optimal performance of an assay in real-time PCR with the Type-it CNV Reference Primer Assay, some considerations need to be made, including considerations on primer design and quality. Please read the following guidelines before starting.

- Check the functionality and PCR efficiency of the GOI-specific primers, using a template containing the amplified target, before starting the relative quantification experiment.
- PCR products for SYBR Green-based qPCR should be as short as possible; ideally 60–150 bp.
- Always use the same algorithm or software to design primers. For optimal results, use GOI assays that have been designed using standard software with standard algorithm parameters and reaction conditions. For details, see Appendix C, page 22.
- Check the concentration of primers before starting. For details, see Appendix D, page 24.
- For optimal results, always use the **cycling conditions specified in the protocol**. The unique composition of Type-it SYBR Green PCR Buffer ensures specific annealing for each primer set. This enables fast two-step cycling with a combined annealing/extension step for the GOI or the Type-it CNV Reference Primer Assay.
- Include appropriate controls in each real-time PCR run to give additional information for interpretation of results. For details, see “Controls” on page 13.

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: Relative Quantification of CNVs Using SYBR Green-Based qPCR on all Real-Time Cyclers

This protocol is optimized for relative quantification of CNVs in the human genome using SYBR Green qPCR, and is intended for use with Type-it CNV SYBR Green PCR Kits and the Type-it CNV Reference Primer Assay on all real-time cyclers (Table 1).

Important points before starting

- Read the "Important Notes" on page 12 before starting.
- For each gene of interest (GOI), we recommend using the provided Type-it CNV Reference Primer Assay as a universal reference assay for reliable $\Delta\Delta C_T$-based quantification of the CNV in the human genome.
- To reconstitute the Type-it CNV Reference Primer Assay (100) to a 25x working solution, see page 12.
- Always use 30 pg–30 ng template DNA (see Table 2).
- Use the primer concentrations specified in this protocol.
- **Always use** the cycling conditions specified in this protocol. The cycling conditions 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.
- We recommend preparing a 25x primer mix containing specific primers (recommended concentrations in the 25x primer mix: 17.5 µM of each primer) for each GOI (see Appendix A, page 20).

Procedure

1. **Thaw the 2x Type-it SYBR Green PCR Master Mix, template DNA (human genomic DNA), Type-it CNV Reference Primer Assay 25x working solution, 25x primer mix for the GOI, 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 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 performance of the 2x Type-it SYBR Green PCR Master Mix and the Type-it CNV Reference Primer Assay, 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 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, select the analysis settings (i.e., baseline settings and threshold values) separately for the GOI and the Type-it CNV Reference Primer Assay. 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 2. Reaction setup

Component	Volume (µl)
Reaction mix	
2x Type-it SYBR Green PCR Master Mix	12.5
25x Type-it CNV Reference Primer Assay solution* or 25x primer mix for GOI†	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 Primer Assay" on page 12.

† The 25x GOI primer mix contains 17.5 µM of each primer, resulting in a final concentration of 0.7 µM of each primer. If using a primer mix of a different concentration, adjust the volume accordingly.

‡ **IMPORANT**: 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	35		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) Mg^{2+} concentration adjusted	Do not adjust the Mg^{2+} concentration in the 2x Type-it SYBR Green PCR Master Mix.
c) 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 protocol.
d) Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers, and template nucleic acid. See Appendix D, page 24, for details on evaluating the concentration of primers. Repeat the assay.
e) Insufficient number of cycles	Increase the number of cycles.
f) Wrong or no detection step	Ensure that fluorescence detection takes place during the combined annealing/extension step.
g) Wavy curve at high template amounts	Discard all the components of the assay (e.g., master mix and primers). Repeat the assay using fresh components.

	Comments and suggestions
h) Primer concentration not optimal	Use optimal primer concentrations (each primer at a final concentration of 0.7 µM). Check the concentrations of primers by spectrophotometry (see Appendix D, page 24). Ensure the correct handling and storage of the Type-it CNV Reference Primer Assay (see "Shipping and Storage", page 4 and "Reconstitution and use of Type-it CNV Reference Primer Assay", page 12).
i) Problems with starting template	Check the concentration, storage conditions, and quality of the starting template (see Appendix D, page 24). If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR using the new dilutions.
j) Insufficient amount of starting template	Increase the amount of template, if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample.
k) PCR product too long	For optimal results, PCR products should be between 60 and 150 bp.
l) No detection activated	Check that fluorescence detection was activated in the cycling program.
m) Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
Differences in C_T values or in PCR efficiencies	
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).

	Comments and suggestions
No linearity in ratio of C_T value/crossing point to log of the template amount	
a) Template amount too high	If signals are appearing 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	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 Mix for each GOI

For ease of use, we recommend preparing a 25x primer mix for the GOI containing target-specific primers (Table 4).

Table 4. Preparing 25x primer mix for the GOI assay

Component	Concentration (25x)	Final concentration
Forward primer	17.5 µM	0.7 µM*
Reverse primer	10 µM	0.7 µM*

* A final primer concentration of 0.7 µM is optimal in most cases. Depending on assay design and GOI target sequence, performance might be improved by varying the primer concentration between 0.5 µM and 1.0 µM. Before adapting primer concentration, verify the concentration of the primer solutions.

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 5). 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 5. 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 6 and 7, respectively.

Table 6. 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 7. 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 8. 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 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 design
- Use of appropriate primer concentrations
- Correct storage of primers

Assay design

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

Table 9. Guidelines for primer 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. ■ Avoid runs of 4 or more of the same nucleotide, especially guanidine. ■ Design primers 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. ■ Avoid complementary sequences within a primer sequence and between the primer pair. ■ Try to avoid highly repetitive sequences. ■ Avoid complementarity of 2 or 3 bases at the 3' ends of primer pairs to minimize primer dimer formation. ■ Avoid mismatches between the 3' end of primers and the template sequence. ■ Avoid runs of 3 or more Gs and/or Cs at the 3' end.

Appendix D: Handling and Storage of Primers

Dissolving GOI primers

- To reconstitute the Type-it CNV Reference Primer Assay (100) to a 25x working solution, see page 12.
- To prepare a 25x working solution of your GOI-specific primers, dissolve the two primers and mix them to achieve a final concentration of 17.5 µM each.
- We do not recommend dissolving primers 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

If the Σ_{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

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

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

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

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

Storage of primers

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

Primer 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.

Appendix E: 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 Primer 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 Primer 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) – ΔC_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.

$\Delta\Delta\mathbf{C_T}$ = 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 SYBR Green 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 F: 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 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 SYBR Green 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

* 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.

fluorescence due to PCR products (usually referred to as the baseline settings). Adjust the settings for this function.

- Whenever possible, select the option for automatic calculation of threshold and baseline for your real-time cycler. **However, note that the default values for data analysis in the cycler software will not always provide the most accurate results.**

Melting curves

All real-time PCR cyclers can perform a melting curve.

To carry out melting curve analysis, the temperature is increased very slowly from a low temperature (e.g., 65°C) to a high temperature (e.g., 95°C). At low temperatures, all PCR products are double stranded, so SYBR Green I binds to them and fluorescence is high, whereas at high temperatures, PCR products are denatured, resulting in rapid decreases in fluorescence.

The fluorescence is measured continuously as the temperature is increased and plotted against temperature. A curve is produced, because fluorescence decreases slightly through the lower end of the temperature range, but decreases much more rapidly at higher temperatures as the melting temperatures of nonspecific and specific PCR products are reached. The detection systems calculate the first derivatives of the curves, resulting in curves with peaks at the respective T_ms. Curves with peaks at a T_m lower than that of the specific PCR product indicate the formation of primer dimers, while diverse peaks with different T_ms or plateaus indicate production of nonspecific products or a smear. See Figure 2 for details.

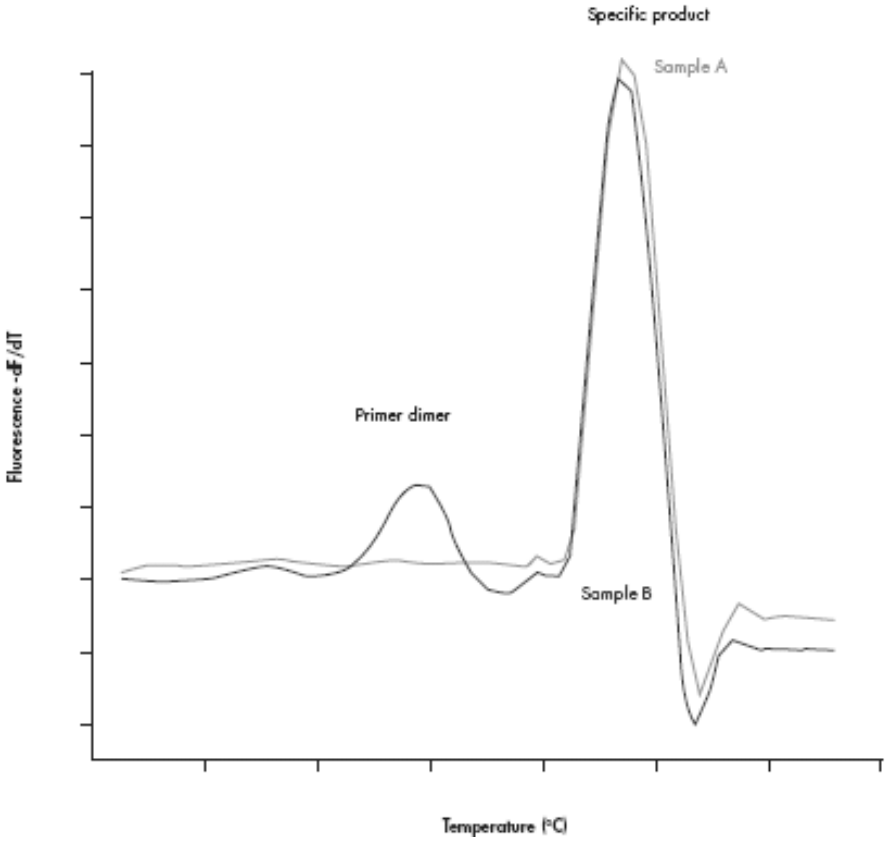

Figure 2. Melting curve analysis. Melting curve analysis of 2 samples (A and B). Sample A yields only 1 peak resulting from the specific amplification product (primer–dimers not coamplified). Sample B shows a peak from the specific product and a peak at a lower temperature from amplification of primer dimers.

Ordering Information

Product	Contents	Cat. no.
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
Related products		
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

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