A novel reference assay for reliable qPCR-based CNV quantification

Nan Fang, Francesca Di Pasquale, Christine Beckmann, Ulla Deutsch, Andreas Missel

QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, Germany

Abstract

Copy number variation (CNV), changes of the DNA copy number in the genome, has been recently shown to be a widespread phenomenon affecting around10–20% of the human genome. The occurrence of CNVs has been associated with various diseases such as autism, autoimmune disorders, and cancer.

The most commonly used molecular biology tools for discovery of CNVs are microarray analysis and next-generation sequencing (NGS). These two high-throughput methods can discover multiple potential CNVs, which subsequently require validation using an independent method. Once validated, the confirmed CNVs can be examined in a large number of samples to identify a statistically significant association between the CNV and the phenotype.

Quantitative PCR (qPCR), with its ease of use, sensitivity, and scalability, is often the method of choice for CNV validation and association studies. The relative quantification principle is used for this application: a reference gene, whose copy number is presumed to be constant among samples to be compared, must first be defined. The copy number of the gene of interest (GOI) is then calculated based on the C_q difference of GOI and reference gene among different samples.

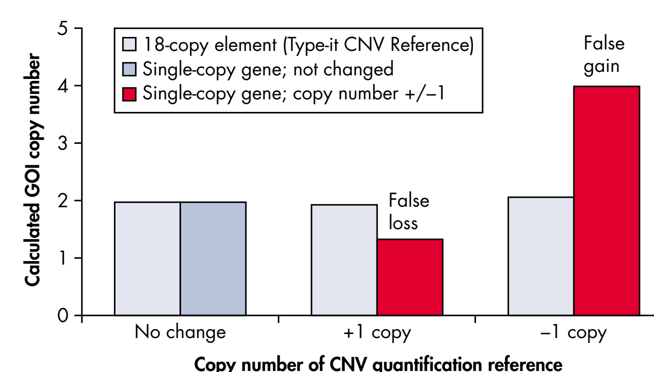
Since the consistent copy number of the reference gene is essential for the qPCR-based CNV quantification, we compared the reliability of commonly used single-copy reference genes, such as TERT (telomerase reverse transcriptase) with a new method using Type-it® CNV Reference Assays in combination with Type-it CNV qPCR Master Mixes, which have been optimized to precisely distinguish small copy number differences. Our results suggest that the combination of Type-it CNV Reference Assays and Master Mixes offer highly sensitive, reliable, and precise CNV quantification results compared with single-copy genes.

Multi-copy element: Reliable quantification reference

More reliable qPCR-based copy number quantification can be achieved by using a multi-copy genetic element that is equally distributed on different chromosomes, instead of a single-copy gene as reference gene for $\Delta\Delta C_q$ analysis.

CNV reference	Copy number, per diploid human genome	C_q change (reference assay)	GOI copy number (real)	GOI copy number (calculated)	CNV calling for GOI
Type-it CNV reference	36	0	2	2.00	NC: Correct
	36+1	–0.04	2	1.94	NC: Correct
	36–1	+0.04	2	2.06	NC: Correct
Single-copy gene	2	0	2	2.00	NC: Correct
	2+1	–0.58	2	1.33	Loss: False
	2–1	+1	2	4.00	Gain: False

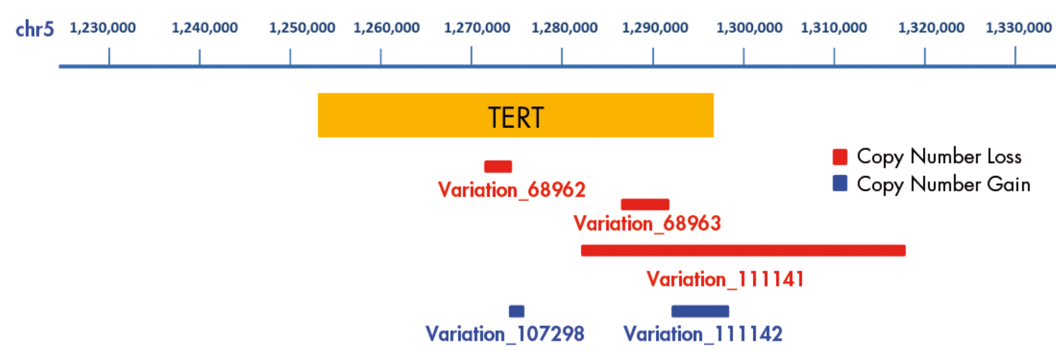
Theoretical calculation. The calculation illustrates the influence of loss or gain of 1 copy from the CNV reference gene on the C_q values of the reference, as well as the copy number calling of the GOI. Assumptions are: 100% qPCR efficiency for reference and GOI; GOI is a single-copy gene whose copy number in test samples is not changed (NC). CNV quantification reference is either a 18-copy genetic element (Type-it CNV Reference) or a single copy gene.



Copy number calling. Loss or gain of one copy from the single-copy reference gene will cause false GOI copy number calling. The GOI copy number calling is not affected by loss or gain of one copy from the multi-copy reference.

Single-copy genes: A less reliable reference for copy number calling

TERT, a single copy gene that is commonly used as reference gene for qPCR-based CNV validation, is subject to copy number variation (3x loss, 2x gain, according to the Database of Genomic Variants; DGV).

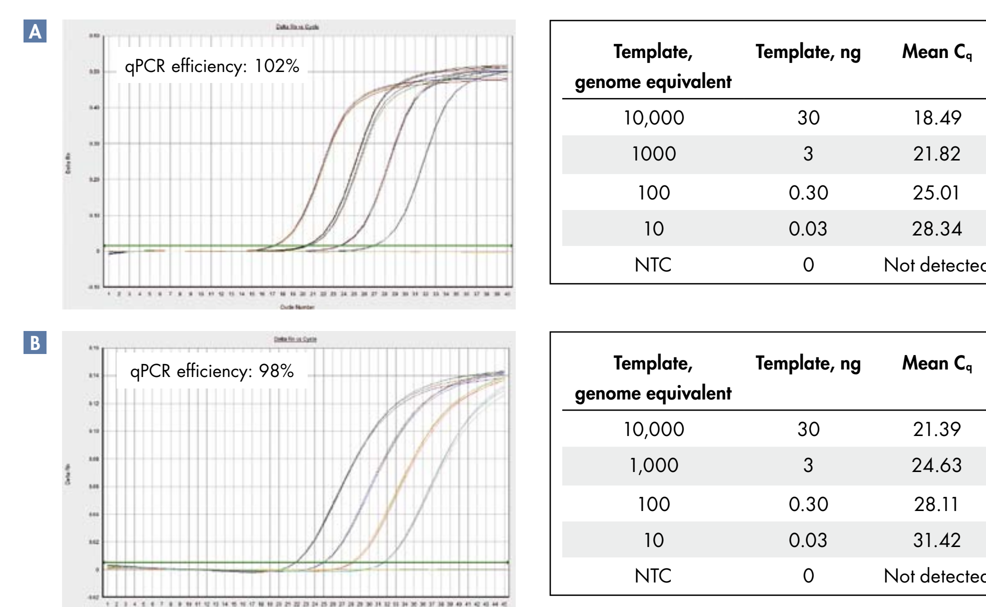


Positions of TERT gene plus 5 known CNVs on chromosome 5. Based on information from Database of Genomic Variants (http://projects.tcag.ca/cgi-bin/variation/gbrowse/hg19/?name=chr5:1211340..1337106).

In addition, there are 517 TERT single nucleotide polymorphisms (SNPs) documented in the dbSNP database (http://www.ncbi.nlm.nih.gov/sites/entrez). A SNP within the CNV reference gene sequences recognized by a qPCR primer or probe can lead to reduced qPCR efficiency and later C_q value and subsequently cause false copy number calling.

High qPCR efficiency and sensitivity with Type-it CNV qPCR Master Mixes and Reference Assays

Using the Type-it CNV Reference Primer Assay and Type-it CNV Reference Probe Assay, results demonstrated a high amplification efficiency over a wide range of input gDNA amounts.



A

Template, genome equivalent	Template, ng	Mean C_q
10,000	30	18.49
1000	3	21.82
100	0.30	25.01
10	0.03	28.34
NTC	0	Not detected

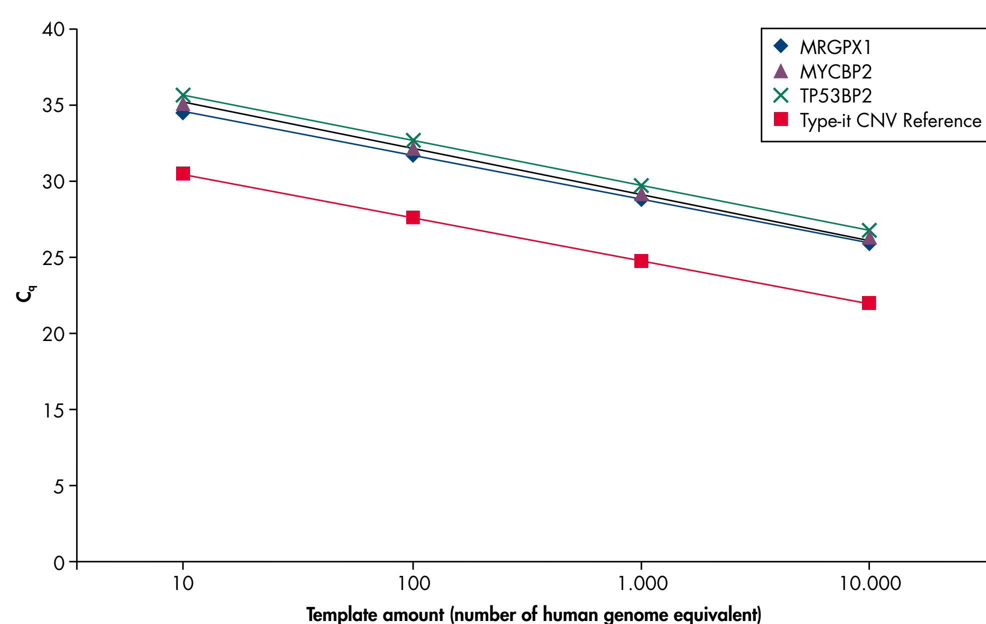
B

Template, genome equivalent	Template, ng	Mean C_q
10,000	30	21.39
1,000	3	24.63
100	0.30	28.11
10	0.03	31.42
NTC	0	Not detected

High qPCR efficiency and sensitivity. Human gDNA equivalent to 10, 100, 1000, and 10,000 genomes (30 pg, 300 pg, 3 ng, and 30 ng, respectively) was amplified on an ABI 7500 cycler using A Type-it CNV Reference Primer Assay with Type-it CNV SYBR® Green Master Mix or B Type-it CNV Reference Probe Assay with Type-it CNV Probe Master Mix. Amplification efficiency was calculated based on the slope of the standard curve. NTC: No-template control.

Probe-based qPCR accurately quantifies GOI and Type-it reference element simultaneously

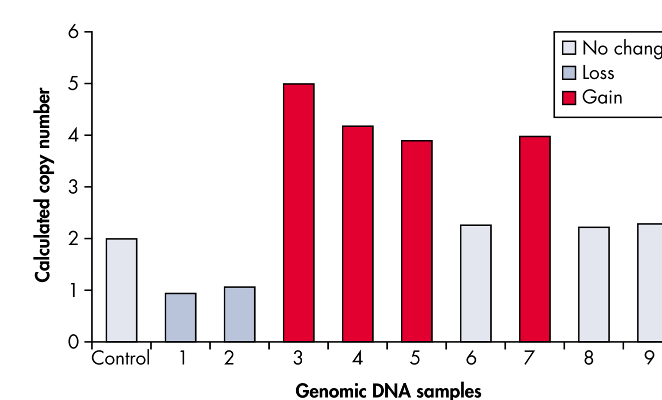
Results demonstrate near 100% qPCR efficiency for both Type-it CNV Reference Assay and GOI Assays within a wide range of template DNA amounts — essential for accurate copy number quantification of GOIs using the $\Delta\Delta C_q$ method. High-efficiency, duplex, probe-based qPCR accurately quantifies both the GOI and the Type-it Reference Element in one qPCR reaction.



Accurate quantification. Duplex qPCR was performed on the Rotor-Gene® Q using Type-it CNV Probe Master Mix and a combination of the Type-it CNV Reference Assay and FAM™-labeled predesigned copy number assays (Supplier L) for GOIs: single-copy genes MRGPRX1, MYCBP2, and TP53BP2. The template was human gDNA equivalent to 10, 100, 1000, and 10,000 genomes (30 pg, 300 pg, 3 ng, and 30 ng, respectively). The C_q difference of Type-it CNV Reference Assay and the three GOI assays reflects the copy number difference (multi-copy vs. single-copy).

Type-it CNV Reference Assays for reliable and accurate qPCR-based CNV validation

- Multi-copy genomic elements are more reliable references for qPCR-based copy number quantification than single-copy genes.
- Type-it CNV qPCR Mastermixes and Assays together enable quick, convenient, and reliable CNV validation and screening.



MRGPRX1 copy number in different Coriell DNA samples. MRGPRX1 copy number was quantified using duplex qPCR in genomic DNA from 9 different Coriell cells (Coriell Institute) and a healthy donor (Control). Type-it CNV Probe PCR Kit (QIAGEN), Type-it CNV Reference Probe Assay (QIAGEN), and a copy number assay for MRGPRX1 from Supplier L were used for duplex PCR reactions. MRGPRX1 copy number was assumed to be 2 in control DNA sample.

The applications presented here are for research purposes. Not for use in diagnostic procedures.

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