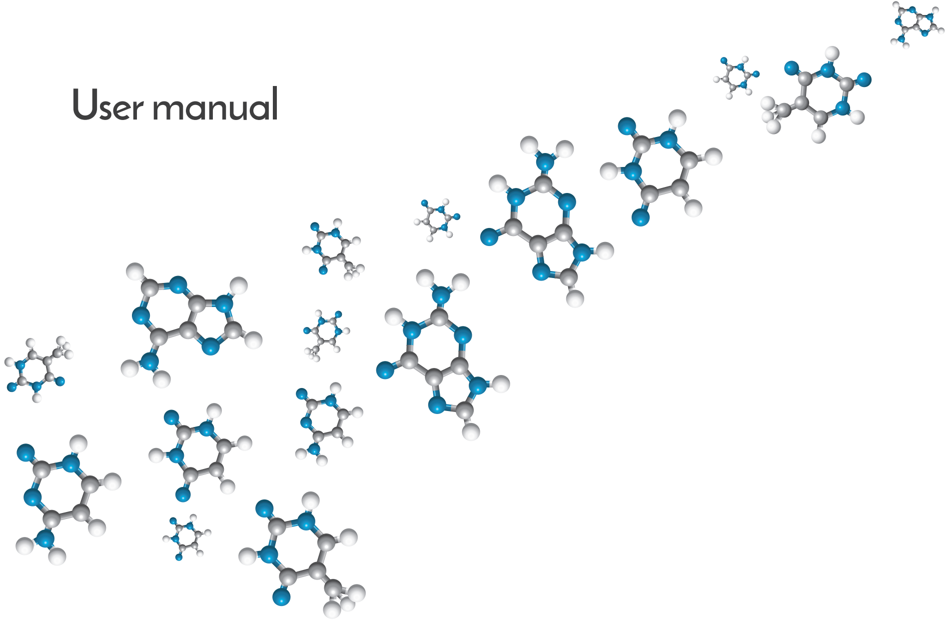
User manual



ValidPrime®

Control for Genomic Background

Version 1.3 - October 2017

SYBR® Protocol

Order #:

Human 250 rxn: A105S25
Human 1000rxn: A105S10
Mouse 250 rxn: A106S25
Mouse 1000rxn: A106S10
Rat 250rxn: A107S25
Rat 1000rxn: A107S10

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Background

For accurate gene expression analysis, the measured Cq-value shall reflect the amount of gene transcripts present in the sample. This requires that the assay is specific and selective for the targeted cDNA and contributions to the signal from primer dimers, pseudogenes and genomic DNA are negligible. To test for primer dimer formation, the qPCR is performed in absence of template (no template control, NTC), and to test for genomic DNA (gDNA) background, the reverse transcription (RT) is performed in the absence of reverse transcriptase (RT(-) control). The NTC is sample independent and is performed only once to validate assay performance, while the amount of gDNA may vary and RT(-) controls are typically measured on all samples. These controls are important but add substantially to the cost of a qPCR study.

ValidPrime® is an assay to test for the presence of gDNA in test samples and when combined with a gDNA control sample, replaces all RT(-) controls. ValidPrime® is highly optimised and specific to a non-transcribed locus of gDNA that is present in exactly one copy per haploid normal genome. The sequence detected by the ValidPrime® assay has no transcriptional activity and is not present in pure cDNA preparations. Therefore, ValidPrime® measures the number of genomic copies present in a sample and can be used for normalisation of samples to cell copy number, as endogenous control for CNV applications, and as control for gDNA background in RT-qPCR. The ValidPrime® kit also contains a gDNA standard that can be used to test the sensitivity of RT-qPCR assays for gDNA background.

The assay has very high PCR efficiency (E > 90% in tested commercial master mixes) and produces negligible amount of primer dimer products. Limit of detection (LOD) is estimated to 4 copies of DNA (0.01 ng of DNA) and limit of quantification (LOQ) is estimated to 32 copies of DNA (0.08 ng of DNA).

In an expression profiling experiment the ValidPrime® assay is added to the list of assays and the gDNA control is added to the list of samples. From the combined measurements of the ValidPrime® assay and the gene of interest (GOI) assays on all samples and on the gDNA control, the genomic background contribution to all RT-qPCR measurements can be assessed. ValidPrime® replaces the need to perform RT(-) controls for all reactions and makes RT-qPCR profiling easier and substantially cheaper. In an expression profiling experiment based on *m* samples and *n* assays, traditional set up requires m RT(-) reactions plus $m \times n$ qPCR controls, while using ValidPrime® only $m + n + 1$ controls are needed (Table 1).

No. of controls		Assays (*n*)									
		1		10		24		48		96	
Samples (*m*)	1	2	3	11	12	25	26	49	50	97	98
	10	20	12	110	21	250	35	490	59	970	107
	24	48	26	264	35	600	49	1176	73	2328	121
	48	96	50	528	59	1200	73	2352	97	4656	145
	96	192	98	1056	107	2400	121	4704	145	9312	193

Traditional RT(-) strategy | ValidPrime®

Table 1: Total number of RT and qPCR controls needed to check for gDNA background using the traditional RT(-) approach compared to the ValidPrime® approach. In an expression profiling experiment based on ***m*** samples and ***n*** assays, traditional set up requires m RT(-) reactions plus m x n qPCR controls, while using ValidPrime® only m + n + 1 controls are needed.

Traditional approach based on RT(-) controls

Presence of genomic background in RT-qPCR expression profiling is conventionally assessed by running an RT(-) control for each sample that is analysed by qPCR for all the GOIs. Any signal observed in these RT(-)-qPCRs is due to presence of contaminating gDNA that is amplified by the qPCR assay designed for the GOI. A common criterion to accept the measured Cq as not being confounded by gDNA contamination is $Cq^{GOI}_{RT(-)} - Cq^{GOI}_{RT(+)} > 5$. The estimated GOI concentration is then accurate to at least 96.9% (Figure 1).

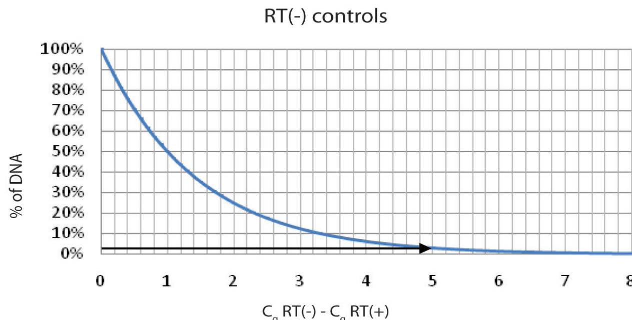


Figure 1: Correlation of ΔCq RT(-)-RT(+) with percentage of gDNA in unknown sample.

If $Cq^{GOI}_{RT(-)} - Cq^{GOI}_{RT(+)} < 5$ the measured $Cq^{GOI}_{RT(-)}$ is confounded. It can be corrected to reflect the RNA concentration using eq. 1 (Laurell *et al.*, 2012):

$$Cq^{GOI}_{RNA} = -\log_2\left(2^{-Cq^{GOI}_{RT(+)}} - 2^{Cq^{GOI}_{RT(-)}}\right)$$

Equation 1.

$Cq^{GOI}_{RT(-)}$ and $Cq^{GOI}_{RT(+)}$ are the Cq-values measured for the RT(+) and RT(-) reactions, and Cq^{GOI}_{RNA} is the Cq-value that would have been obtained for the RT(+) reaction in absence of gDNA contaminations. From Cq^{GOI}_{RNA} the correct transcript amount can be calculated.

ValidPrime®

By using ValidPrime® instead of the traditional RT(-) controls, the same test for gDNA contamination can be performed, and if needed, the same correction for background can be made but with a much smaller number of reactions. The sensitivities of the GOI qPCR assays for gDNA (Cq^{GOI}_{gDNA}) are tested relative to the ValidPrime® assay ($Cq^{ValidPrime®}_{gDNA}$) on the provided gDNA standard. Good performing GOI assays that have been properly designed to exclusively target mRNA by, for example, having intron spanning primers shall not amplify the gDNA standard, while GOI assays that amplify sequences present in multiple copies in the gDNA will have even lower Cq-values than the ValidPrime® assay. All samples are then analysed also with the ValidPrime® assay ($Cq^{ValidPrime®}_{sample}$). The measurement setup is shown in Table 2.

Original data	GOI 1	GOI 2	GOI 3	GOI 4	ValidPrime®
Sample 1	20.1	31.1	22.1	28.2	32.5
Sample 2	20.5	31.2	22.5	28.9	33.2
Sample 3	21.0	31.1	22.9	30.2	32.3
Sample 4	23.1	31.8	22.5	32.3	34.2
Sample 5	23.5	30.8	22.8	32.0	33.1
gDNA standard	25.8	26.9	26.7	26.0	27.0

Table 2: Experimental setup based on five samples assayed for four GOIs and ValidPrime®, including also the gDNA standard. $Cq^{GOI}_{RT(+)}$ is shown in **black**, Cq^{GOI}_{gDNA} in **blue**, $Cq^{ValidPrime®}_{sample}$ in **red**, and $Cq^{ValidPrime®}_{gDNA}$ in **orange**.

$$Cq^{GOI}_{RT(-)} = Cq^{GOI}_{gDNA} + (Cq^{ValidPrime®}_{sample} - Cq^{ValidPrime®}_{gDNA})$$

Equation 2.

From the measured $Cq^{ValidPrime®}_{sample}$, $Cq^{ValidPrime®}_{gDNA}$ and Cq^{GOI}_{gDNA} the expected Cq-values for RT(-) controls ($Cq^{GOI}_{RT(-)}$) are calculated with Equation 2, and, as before, Equation 1 is used to correct for the gDNA background (Table 3).

ValidPrime®	Gene 1			Gene 2			Gene 3			Gene 4		
	$Cq^{GOI}_{RT(+)}$	$Cq^{GOI}_{RT(-)}$	Cq^{GOI}_{RNA}	$Cq^{GOI}_{RT(+)}$	$Cq^{GOI}_{RT(-)}$	Cq^{GOI}_{RNA}	$Cq^{GOI}_{RT(+)}$	$Cq^{GOI}_{RT(-)}$	Cq^{GOI}_{RNA}	$Cq^{GOI}_{RT(+)}$	$Cq^{GOI}_{RT(-)}$	Cq^{GOI}_{RNA}
Sample 1	20.1	31.3	20.1	31.1	32.4	31.9	22.1	32.2	22.1	28.2	31.5	28.4
Sample 2	20.5	32.0	20.5	31.2	33.1	31.7	22.5	32.9	22.5	28.9	32.2	29.1
Sample 3	21.0	31.1	21.0	31.1	32.2	32.0	22.9	32.0	22.9	30.2	31.3	31.1
Sample 4	23.1	33.0	23.1	31.8	34.1	32.1	22.5	33.9	22.5	32.3	33.2	33.4

Table 3: Measured $Cq^{GOI}_{RT(+)}$ and calculated $Cq^{GOI}_{RT(-)}$ using Equation 2, and calculated Cq^{GOI}_{RNA} using Equation 1.

Contents

- Reference standard gDNA. We recommend using not more than 10 ng per qPCR.
  250 rxn* (50 µl, c= 200 ng/µl)
  or 1000 rxns* (100 µl, c= 200 ng/µl)

- ValidPrime® assay primers for:
  250 rxns* (250 µl of primer mix, c=10 µM per primer)
  or 1000 rxns* (1000 µl of primer mix, c=10 µM per primer)

* rxns = qPCR reactions in 25 µl, concentration = 400 nM per primer

Storage

The ValidPrime® kit can be stored for 1 month at +4°C. For long term storage -20°C is recommended. Repeated freeze-thaw cycles should be avoided. Vortex thoroughly and spin down before use.

Additionally required materials and devices

- **qPCR instrumentation**: The ValidPrime® kit has been validated on: Roche LightCycler 480, Biorad CFX, Stratagene MxPro, Rotorgene, ABI 7500 Fast and is expected to perform excellent on related instruments.
- **Master mix or master mix components:** The ValidPrime® kit has been validated in a large number of master mixes using the conditions recommended by the manufacturers, including TATAA SYBR® GrandMaster® Mix with following recommendations: 400 nM final primer concentration, annealing temperature 60°C.
- **Pipettes and tips (available from www.tataa.com)**
- **Vortex and centrifuge**
- **Sample RNA/DNA**
- **Optionally DNase**: With ValidPrime®, the measured $Cq^{GOI}_{RT(+)}$ can be corrected for, if up to 50% of the signal is coming from the gDNA background. If the gDNA background is high it is recommended to reduce it by treating the cDNA with double strand specific DNase that will remove specifically the gDNA without degrading the cDNA. We recommend heat labile dsDNase (HL-dsDNase) that is efficiently heat inactivated and does not digest the PCR product. HL-dsDNase is available from www.tataa.com.
- **Optionally reference cDNA**: Newly designed assays can be validated on cDNA libraries. Several cDNA libraries are available from www.tataa.com.

Amplification protocol

Use the recommended amplification protocol for your master mix. Either a 2- or 3-step amplification protocol can be used. Optimal annealing temperature for the ValidPrime® assay is about 60°C in most master mixes. After amplification it is recommended to perform dissociation curve analysis from 55°C to 95°C according to the instrument manufacturer´s instructions.

Pipetting protocol

Prepare a master mix for each assay using the protocol from the manufacturer, including the recommended concentrations of primers. Prepare for at least one extra reaction so you do not run out of master mix during the pipetting. An NTC is recommended to test for contamination of reagents. We recommend 10 ng of gDNA per 10 µl qPCR, which on most qPCR instruments should produce a Cq for the ValidPrime® assay in the range 20-25 cycles . More than 20 ng of gDNA per 10 µl qPCR can generate high fluorescence background with dye based mixes that causes problems with baseline subtraction.

GenEx

Easiest is to use GenEx for correction of RT-qPCR data for gDNA background and for general qPCR data processing. GenEx is a market leading software for qPCR experimental design and data processing, and is supported by all leading qPCR instrument manufacturers. It offers user friendly optimised workflow for qPCR data pre-processing and analysis. Pre-processing includes interplate calibration, efficiency correction, various normalisation options, handling of technical replicates and missing data, normalisation with paired samples, and correction for gDNA contamination using ValidPrime®. Analyses include absolute quantification, relative quantification and expression profiling. Tutorials are available on: www.multid.se/tutorials.php and free support is offered on www.qpcrforum.com.

A free license for GenEx Enterprise is available for download from www.multid.se and provides the fully functional analysis software for a trial period of 14 days. To purchase GenEx licenses or for qPCR data analysis services, contact us at order@tataa.com.

Trouble shooting

• I do not get any amplification/signal?

The instrument may not have been programmed correctly or there may be a problem with the master mix. Establish if the problem is in the detection or the amplification by running the samples on gel. Run a new test using the gDNA control provided with the ValidPrime® assay.

• My negative controls are amplified?

Your reagents are probably contaminated.

• My samples have same/higher Cq-value than my NTC?

You have used too little cDNA. Add more cDNA and try again. The cDNA may also be of poor quality. Check the quality of the RNA before performing cDNA synthesis.

• My replicates are not tight?

With good quality cDNA and good pipetting technique, very high reproducibility is expected. Low amounts of cDNA can lead to higher variation. Also, low quality cDNA can lead to differences between replicates. Check the accuracy and reproducibility of your pipettes. It is also possible the qPCR instrument is malperforming.

• I get positive ValidPrime® signal even after DNAse treatment?

Often DNAse treatment does not remove all DNA and qPCR will amplify a single molecule. Usually solution based DNase treatment is more efficient than on column based DNase treatment. You may also try the HL-dsDNase from TATAA (www.tataa.com), which has superior performance to competing dsDNases. Usually DNase treatment reduces the gDNA background enough to be accounted for by ValidPrime® and GenEx correction.

References

Henrik Laurell, Jason Iacovoni, Anne Abot, David Svec, Jean-José Maoret, Jean-François Arnal and Mikael Kubista. Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. Nucleic Acids Res. 2012

Mikael Kubista, Vendula Rusnakova, David Svec, Björn Sjögreen and Ales Tichopad. GenEx - Data Analysis Software. In: qPCR in Applied Microbiology. Editor: Martin Filion. Horizon Press, 2012.

License information

PCR is covered by several patents owned by Hoffman-La Roche Inc., and Hoffman-LaRoche, Ltd. Purchase of the ValidPrime® kit does not include or provide a license with respect to any PCR related patents owned by Hoffman-La Roche or others. TATAA Biocenter does not encourage or support the unauthorised or unlicensed use of the PCR process.

Contact and ordering information

To re-order the ValidPrime® kit or for more information about the product and other products available from TATAA Biocenter, please contact us on order@tataa.com or visit our website www.tataa.com or contact your local TATAA distributor in your country.

ValidPrime® Human SYBR®	250 rxn	Order# A105S25
ValidPrime® Human SYBR®	1000 rxn	Order# A105S10
ValidPrime® Mouse SYBR®	250 rxn	Order# A106S25
ValidPrime® Mouse SYBR®	1000 rxn	Order# A106S10
ValidPrime® Rat SYBR®	250 rxn	Order# A107S25
ValidPrime® Rat SYBR®	1000 rxn	Order# A107S10

Other products from TATAA

(HL-)dsDNase

A new generation DNase that is specific to double stranded DNA and can be efficiently inactivated by heating at 55 °C. It can be added to your RT reaction to efficiently remove any gDNA, without degrading single-stranded cDNA. It is completely inactivated by the PCR and does not degrade the double stranded PCR product.

TATAA SYBR® GrandMaster® mix

TATAA SYBR® GrandMaster® Mix is a 2x concentrated ready to use fastmix. With its unique combination of optimised components it delivers maximum PCR efficiency, sensitivity, specificity and robust fluorescent signal using fast, or conventional, cycling protocols with SYBR® Green qPCR.

Reference Gene Panel - Human, Mouse or Rat

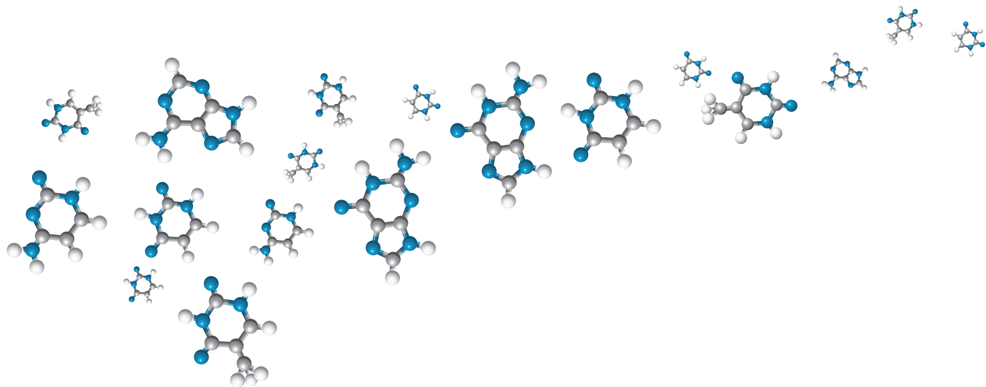
The panel contains primer sets for 12 commonly used reference genes. A perfect product for finding the most optimal reference gene for your samples.

CelluLyser™ - for rapid and easy lysis and cDNA synthesis

The CelluLyser™ Lysis and cDNA Synthesis Kit enables you to generate cDNA from small samples with minimal losses and hands-on time. It is particularly useful for single cell analysis. Using the CelluLyser the entire workflow from cell lysis, to RT and qPCR can be performed without washing steps, thus eliminating material loss.

TATAA Interplate Calibrator - Variation Compensation

For practical reasons many qPCR studies involve the use of samples that are processed in more than a single batch or in which the sample set is extended over time. Even over a short time period, variation between qPCR processing runs is observed due to different baseline subtractions and threshold settings. The TATAA Interplate Calibrator (IPC) is used to compensate for the variation between qPCR runs.



Express your genius

TATAA Biocenter, with offices in Gothenburg, Sweden, and Prague, Czech Republic, is the leading provider of quantitative real-time PCR and NGS services and the prime organiser of quantitative real-time PCR and NGS workshops globally. TATAA Biocenter conducts commissioned research and training within the field of molecular diagnostics and gene expression analysis, along with developing real-time PCR expression panels. TATAA Biocenter has great experience and expertise in high resolution gene expression profiling, pathogen detection, and small sample/single cell analysis.

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