User Manual

TATAA Universal DNA Spike I

SYBR and Probe protocol

Version 1.1 — January 2014

For use in quantitative real-time PCR

tataabiocenter

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Background

Contaminants present in samples are known to inhibit enzymatic reactions and in the context of a reverse-transcription quantitative Polymerase Chain Reaction (RT-qPCR) assay, inhibitors are fully capable of distorting reported measurements. Such enzymatic reactions are a necessary part of the sample preparation prior to the qPCR, such as nuclease/proteinase treatment and subsequent reverse transcription of mRNA to cDNA; inhibition of which often causes erroneous biological readouts, even though the qPCR amplification curves can look perfectly normal. The reason is these upstream reactions are usually exposed to a higher concentration of inhibitors than the qPCR itself. Results may also be compromised by the degradation of DNA during sampling, transport, storage, and other handling processes.

The Universal Spikes from TATAA Biocenter are offered as RNA or DNA templates in two different synthetic sequences that are not present in any known living organism (Spike I and Spike II) and can be used as very effective tools for quality control throughout the entire RT-qPCR experimental workflow.

This manual covers the use of the TATAA Universal DNA Spike I which has a DNA sequence length of 1000 base pairs. The Spike I assay which is used for detecting the Universal DNA Spike I template, is very robust and optimized for high sensitivity for inhibition. The assay amplifies a 300-base region towards the 3′ end of the synthetic template. The measured Cq-values and the shape of the amplification curves reflect the inhibition. The Cq-values of the Spike I assay also reflect losses during extraction, handling, transport, and storage of samples, including freeze-thaw events during RT-qPCR (see following section).

Figure 1: The *Spike I assay* amplifies a 300-base region towards the 3′ end of the synthetic template.

Contents TATAA Universal DNA Spike I Probe

• Universal DNA Spike I:	10 aliquots of 50 µl, 10^5 copies/µl (0.00005 ng/µl) in 0.1mM EDTA, pH 7
• Spike I Assay primers:	250 rxn* = 250 µl of primer mix c = 10 µM (per primer)
• Spike I Assay probe:	250 rxn* = 125 µl of probe, c = 10 µM

*rxn = qPCR reaction in 25 µl, concentration = 400nM per primer, 200nM probe

The Spike I assay has very high PCR efficiency in the absence of inhibitors (E>95%) in tested commercial master mixes and produces a negligible amount of primer-dimer products (No primer-dimer signal in 40 cycles). The limit of detection (LOD) is estimated at around 4 copies of the DNA spike template. The limit of quantification (LOQ, defined as the limiting concentration for which the standard deviation (SD) of 6 qPCR replicates is >0.45), is estimated at 32 copies of the DNA spike template. The probe is available in two versions, either with FAM or with CalFluorGold540 (equivalent to VIC, JOE) reporter, both with BHQ1 quencher.

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Additionally required materials and devices

• Real-time PCR instrumentation
The TATAA Universal Spike I has been validated on the Roche LightCycler 480, Biorad CFX 96/384, Agilent MxPro, Qiagen Rotorgene, ABI 7500 Fast, Eppendorf Realplex, Illumina Eco, Fluidigm BioMark and is expected to perform well on equivalent instruments. If using probe chemistry, the probe signal shall be measured on the instrument's FAM or VIC (JOE) channel, depending on which probe was purchased.

• Master mix
The Spike I assay has been validated in a large number of different commercially available master mixes using conditions recommended by the manufacturers and is expected to perform good in most high quality master mixes. For best results TATAA recommends to use the TATAA Probe GrandMaster® mix for probe based chemistry or the TATAA SYBR® GrandMaster® mix for dye based chemistry.

• Pipettes and tips
It is important to use calibrated pipettes with compatible pipette tips to assure accurate volume handling. Both pipettes and compatible tips are available from www.tataa.com/products.

• Vortex and centrifuge

• Experimental sample (DNA/cDNA)

• Optionally reference cDNA and gDNA
New assays can be validated on cDNA and gDNA libraries available from TATAA (www.tataa.com/products) for mouse, human or rat.

Storage

Store the *TATAA Universal DNA Spike I* template and assay at -20°C for up to one year or at +4°C for up to one month. If using probe; protect the probe from light. Avoid repeated freeze-thaw cycles, use the provided aliquots instead.

A - Test for inhibition

When and what samples to test for inhibition?

Controls for inhibition should be included when performing quantitative analyses using samples known to contain substances interfering with the RT-qPCR. Validation of the purification protocol is required for compliance to the *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments "MIQE" guidelines (Bustin *et al*., 2009); many prominent journals now expect experimental methods to comply with these standards before they will consider publishing a manuscript. Validation using the *Universal DNA Spike I* is effective, easy to perform, and provides an important additional assurance that both the pre-analytical and analytical phases of your experiment are valid. Consider testing for inhibition when:

- a non-validated purification protocol is used
- a validated protocol is used, but the sample matrix is changed (e.g. liver ⇨ brain)
- the amount of input material is increased from that in the validated protocol
- an inexperienced person performs the purification (e.g. precipitation, drying)
- the protocol is customized (e.g. ± washing step)

When experimental samples are heterogeneous it is advisable to monitor every sample for the presence of interfering substances using the *TATAA Universal Spike I*. Examples of such samples include:

- environmental samples (e.g. soil or waste water samples)
- food or animal feed
- stool, faeces, or urine
- blood
- fatty tissue

Spectrophotometry can help to identify samples to be tested for inhibition using the *TATAA Universal Spike I*. The A260/**A280** ratio for pure DNA should be 1.8 (±0.1) and for pure RNA 2.0 (±0.1). The A260/**A230** ratio is expected to be between 2 and 3, however, in practice it is usually ≥ 1.2. It is also important to note that both ratios can to some extent be influenced by the choice of buffer. Many contaminants have absorption spectra that overlap with nucleic acids (Table 2) and will distort the measured ratios.

Inhibitors (in H_20)	Absorbance spektrum	Max absorbance	Inhibitory amount (RT)	Inhibitory amount (qPCR)
10% buffered formaldehyd	<250 nm	≈220 nm (buffer)	>0.01% (v/v)	>0.01% (v/v)
dimethylsulphoxide (DMSO)	200-350 nm	230 nm	>10%	>10%
EDTA (0.5 M, pH 8)	210-360 nm	254 nm	>0.5 mM	>0.5 mM
ethanol (100% (A ≈ 0)	<230 nm (A≈0)	<230 nm (A≈0)	>1% (v/v)	>1% (v/v)
fulvic acids	200-500 nm	224, 254, 315, 342	>0.01% (w/v)	>0.01% (w/v)
guanidine HCL	320-450 nm	≈230 nm	>1mM	>1mM
guandidium thiocyanate (GTC)	220-350 nm	≈260 nm	>1mM	>1mM
humic acids	200-500 nm	≈224 nm, ≈254 nm	>0.01% (w/v)	>0.01% (w/v)
chloroform	160-240 nm	<230 nm	>0.1% (v/v)	>0.1% (v/v)
cholic acid	200-500 nm	several (305, 389...)	>0.1% (w/v)	>0.1% (w/v)
isopropanol (A≈0)	150-200 nm (A≈0)	<230 nm (A≈0)	>1% (v/v)	>1% (v/v)
phenol	210-290 nm	230 nm, 270 nm	>0.1% (v/v)	>0.1% (v/v)
proteins	250-300 nm	≈280 nm	too complex	too complex
Qiazol™	complex	complex (290 nm)	>0.1% (v/v)	>0.1% (v/v)
RNA later™	150-250 nm	215 nm	>0.1% (v/v)	>0.1% (v/v)
sodium dodecyl sulphate 1% (SDS, A≈0)	150-350 nm (A≈0)	<350 nm (A≈0)	>0.001% (w/v)	>0.001% (w/v)

Table 1: Most frequent inhibitors with absorbance spectra and approximate inhibitory levels. RT data tested using Superscript III and TATAA GrandScript cDNA Synthesis Kit. qPCR data obtained using TATAA GrandMaster® mixes, KAPA SYBR® Fast mix and Bio-Rad iQ Supermix.

Instruments such as the DropSense96 (Trinean) are capable of identifying certain contaminants based on the profile of their absorption spectra. However, in order to achieve a measurable effect on the UV/VIS spectra a large quantity of the contaminant is required, frequently much larger than the amounts needed for inhibition. In such instances, the inhibition and subsequent biasing of the RT-qPCR results may remain unnoticed.

Inhibition test procedure

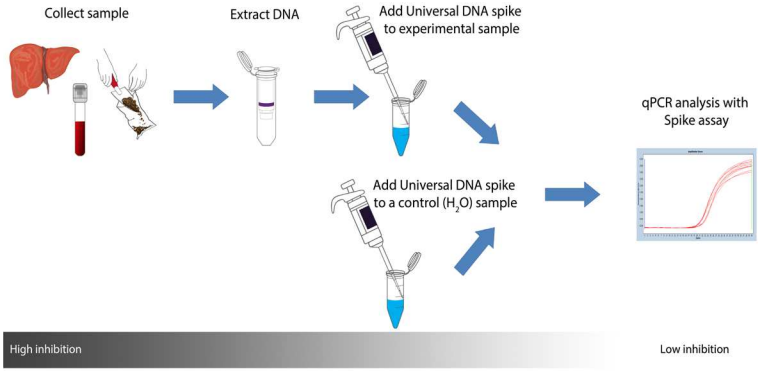

Figure 2: Workflow for inhibition test

The test for inhibition is based on an efficient and simple principle: **equal amounts of spike are added to all experimental samples and to an additional control sample. The control sample is based on nuclease-free water or purified matrix, which is known to be contaminants-free.** All samples have the same volume. The experimental and the control sample are then reverse transcribed and amplified with the Spike I Assay under identical conditions (Figure 2). If the Cq value is greater in an experimental sample than in the control then the analytical process of that experimental sample is inhibited (Figure 3-6). The magnitude of the difference between these Cq values reflects the degree of inhibition.

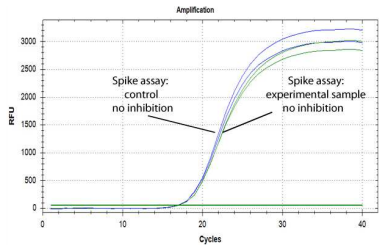

Figure 3: Same Cq-values for sample and control indicates no inhibition.

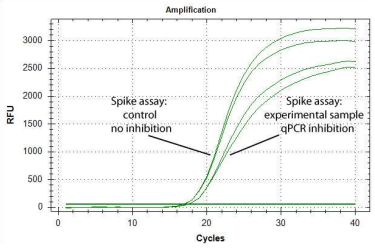

Figure 4: Higher Cq-values in sample compared to control indicates inhibition in the qPCR.

Upstream inhibition: When the experimental sample has higher Cq than the control, but there is no effect on the shape of the amplification curve, only upstream might steps have been inhibited (proteinase K, nuclease treatment), while the qPCR performs as expected. Inhibition shows an assay dependent trend (Ståhlberg *et al.*, 2003), which introduces significant bias.

Inhibited workflow: When the qPCR itself is inhibited the amplification curve typically shows reduced slope compared to the control (Figure 4) in addition to having a higher Cq (Bar *et al.*, 2003). This indicates that interfering agents from the sample are present or that inhibitory reagents from upstream reactions (e.g. phenol extraction, proteinase K or DNase treatment, or reverse transcription) have been carried over to the qPCR.

If the RT or qPCR are inhibited, results are unreliable. The source of inhibition should be identified, the protocol amended and the experimental sample shall be reanalysed (diluted, re-purified or resampled).

Protocol - Test for inhibition

1. Add 2 µl of TATAA Universal DNA Spike I ($2*10^5$ copies) to each qPCR containing DNA extracted from experimental sample.
2. Add 2 µl of TATAA Universal DNA Spike I ($2*10^5$ copies) to a qPCR, which instead of experimental DNA contains pure nuclease free H_20 or purified matrix, etc.

Recommendation: Use sufficient amount of spike to obtain about 10^4 molecules in the final qPCR (Cq <30). Too much spike will generate low Cq values, which may cause problems with baseline subtraction on some instruments. With too little spike, reproducibility may suffer. We advise adding 2 µl of spike per sample as pipetting smaller volumes is less accurate.

Example: If the reaction starts with $2*10^5$ TATAA Universal Spike I molecules, assuming 100% efficiency a Cq in the range 10-15 cycle is expected on most qPCR instruments.

3. Perform qPCR on the DNA from the experimental sample and the control using the provided Spike I assay primer (and probe) mix. Use in-house PCR reagents and recommended primer (and probe) concentrations and use approximately 60°C annealing temperature.

Recommendation: Prepare for a slightly larger amount of reactions to avoid running out of master mix during pipetting. Include a no template control (NTC) to test for contamination of reagents. Using technical qPCR replicates (2-3) is suggested.

4. Analyze amplification data of the Spike I assay (Cq and shape of the amplification curve) by comparing the control and experimental sample. Any significant difference indicates qPCR inhibition (Figures 3-4).

$\Delta Cq^{(\text{experimental sample} - \text{control})} \leq 0.5$ may be caused by technical variation, depending on the performance of the instrument, master mix, and number of replicates. If replicates are available, a t-test can be used for comparison.

$\Delta Cq^{(\text{experimental sample} - \text{control})} > 0.5$ indicates inhibition. The sample should be considered suspect. If a single experimental sample is inhibited, it should be discarded or, preferably, reanalyzed. If experimental samples in general are inhibited the protocol should be further optimized. The easiest option is to reduce the amount of sample material used in the first enzymatic reaction of the workflow.

B - Test extraction yield

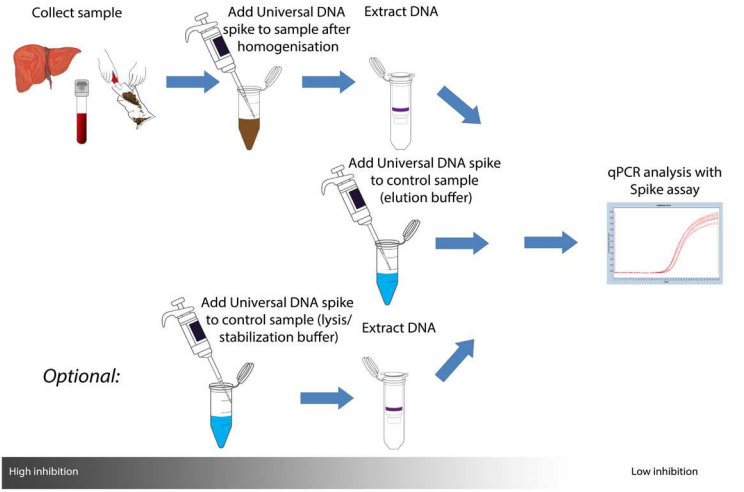

Figure 5: Workflow for extraction yield test

The TATAA Universal DNA spike may be added to any stabilised and homogenised sample prior to DNA purification to test for material loss during isolation, transportation, and storage. Equal quantities of the DNA spike I are added to each experimental sample and to a control sample. The control sample should be based on nuclease free water or elution buffer of the same volume as used for elution/dissolving in the DNA purification protocol. An equal Cq value of the TATAA Universal Spike I in the experimental and control samples reflects 100% yield. If the Cq values differ, the yield of the extraction can be estimated using the following formula:

$$\text{yield (\%)} = \frac{1}{2^{(\text{Cq experimental sample - Cq control})}} * 100$$

Experimental samples that have a very complex matrix may lead to degradation or adsorption of the TATAA Universal Spike I; in such cases a second control sample may be used to address the loss. The spike is added into the homogenisation/lysis buffer without any sample material and processed through all steps of isolation. If the control sample is based on water only, we recommend adding a carrier such as linear polyacrylamid, BSA or yeast tRNA to minimize loss due to adsorption to the purification membrane as the concentration of the DNA spike is very low (≈ 0.00005 ng / µl) compared to the amount of total DNA present in a typical experimental sample.

Protocol - Estimating extraction yield

1. Add 2 µl *TATAA Universal Spike I* to the homogenized and stabilized experimental sample.

Note: TATAA Universal RNA Spike shall be used to evaluate RNA extraction yields and TATAA Universal DNA spike to evaluate DNA extraction yields. Add enough TATAA Universal spike to obtain at least 10^4 molecules in the final qPCRs. More spike may be needed in protocols where yield is poor or when there are large losses.

2. Control I: Add 2 µl *TATAA Universal Spike I* to nuclease free H_2O (elution buffer), where the total volume of this control is equal to volume used for elution of experimental sample at the end of extraction procedure.

Example: If elution step is performed with 30 µl TE buffer, the control sample is: 2 µl TATAA DNA Spike I+ 28 µl TE.

Control II (optional): Add 2 µl *TATAA Universal Spike I* in the homogenization buffer. The total volume of control II is equal to the volume used for homogenization of the experimental sample.

Recommendation: Add a nucleic acid carrier to control II (such as yeast tRNA), to mimic your experimental sample.

3. Extract and purify nucleic acids together with the *TATAA Universal Spike I* from the experimental sample and, optionally, from Control II.

4. Perform qPCR on the experimental sample and on the control sample(s) using the provided Spike I assay primer (and probe) mix. Use in-house qPCR reagents and recommended primer (and probe) concentrations and use approximately 60°C annealing temperature .

Recommendation: Prepare for a slightly larger amount of reactions to avoid running out of master mix during pipetting. Include a no template control (NTC) to test for contamination of reagents. Using technical qPCR replicates (2-3) is suggested.

5. Analyze amplification data of the Spike I Assay by comparing the control(s) and experimental sample. Use the following formula to calculate extraction yield:

$$\text{yield (\%)} = \frac{1}{2^{(\text{Cq experimental sample - Cq control})}} * 100$$

Example: Assume a total volume of tissue homogenate per isolation is 400 µl, with added 2 µl of TATAA Universal Spike I (2x10^5 spike molecules). An elution volume is 40µl, from which 2 µl is used per 10 µl qPCR. This setup provides 10^4 Universal Spike molecules per qPCR, assuming 100% extraction yield. Expected Cq is 20-25 on most qPCR instruments.

GenEx

A 6 months complimentary license for qPCR analysis software GenEx Enterprise is included with the *TATAA Universal Spike I* kit. To get started, send an e-mail to order@tataa.com and state your order number for your *TATAA Universal Spike I* kit together with your customer details. You will receive a key to activate your free license that can be downloaded from www.multid.se. To purchase additional GenEx licenses or for qPCR data analysis services, contact us on order@tataa.com.

Troubleshooting

No 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 step by running the samples on a gel. Run a new test using the DNA Spike I with the Spike I assay provided. If the problem persists, please contact us at info@tataa.com.

Amplification/signal in negative controls

Your reagents are probably contaminated.

Samples have same/higher C_q-value than the negative control

You have used too little spike template or complete inhibition is present. Add more RNA/DNA and try again. Check if the quality of the RNA/DNA is not compromised due to improper storage before performing (RT-)qPCR. Check if the instrument is set optimally.

High spread among replicates

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

References

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T. Bar, A. Ståhlberg, A. Muszta and M. Kubista. Kinetic Outlier Detection (KOD) in real-time PCR Nucl.
Acids Res. 31, e105, 2003.

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Reorder information

The *TATAA Universal RNA Spike (I and II) or DNA Spike (I and II)* kits can be ordered from the TATAA webshop on www.tataa.com, by e-mail to order@tataa.com, or from the TATAA distributor in your country.

Contact

For more information about *TATAA Universal Spike* kits, contact us at support@tataa.com

License information

Other products from TATAA

ValidPrime™ - mouse, human and other vertebrates

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 optimized and specific to a non-transcribed locus of gDNA that is present in exactly one copy per haploid normal genome. The kit also contains a gDNA standard that can be used to test the sensitivity of RT-qPCR assays for gDNA background. ValidPrime™ replaces the need to perform RT(-) controls for all reactions and makes RT-qPCR profiling easier and substantially cheaper.

HL-dsDNase

New generation DNase from Arcticzymes 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.

GenEx

GenEx is market-leading software for qPCR experimental design and data processing, and is supported by all leading qPCR instrument manufacturers. It offers user-friendly optimized workflows for qPCR data pre-processing and analysis, including normalization using spikes and identification of inhibited outliers. Pre-processing includes interplate calibration, efficiency correction, various normalization options, handling of technical replicates and missing data, normalization 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.

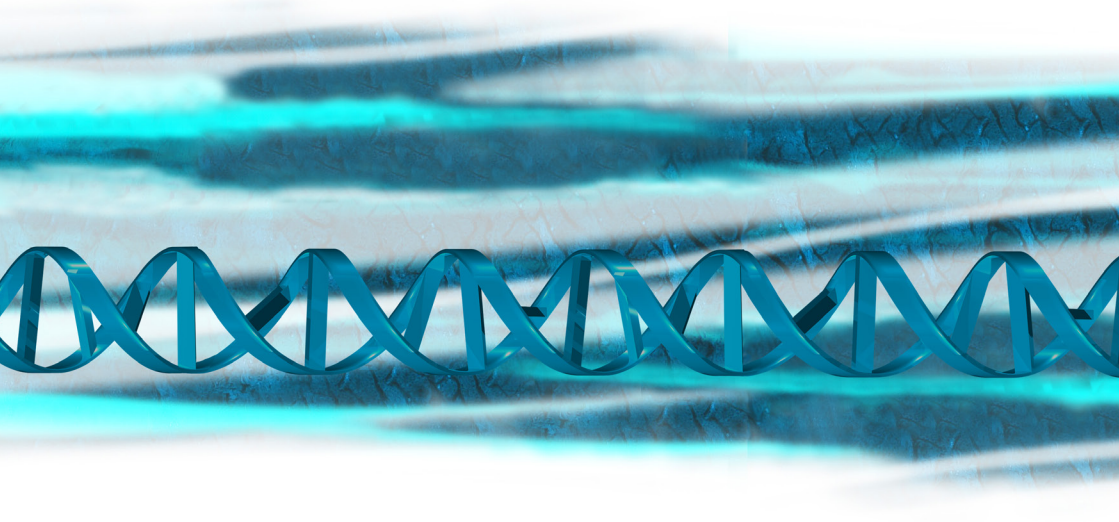
Reference Gene Panel - Human or Mouse

The panel contains primer sets for 12 commonly used human or mouse reference genes. A perfect product for finding the most optimal reference gene for your samples. GenEx Standard software with GeNorm and Normfinder is also included in the kit for first time users.

qPCR training courses at TATAA Biocenter

TATAA Biocenter is leading organizer of hands-on training in qPCR and related technologies. For comprehensive training program see www.tataa.com

Express your genius



TATAA Biocenter, with offices in Gothenburg, San Francisco and Prague is the leading provider of real-time PCR services and the prime organizer of real-time PCR workshops globally. TATAA Biocenter conducts commissioned research and training within 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.

tataabiocenter

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