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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 that 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 RNA/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 (Spike I and Spike II) that are not present in any known living organism 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 RNA Spike I which has an RNA sequence length of 1000 base pairs. The Spike I assay, which is used for detecting the Universal RNA 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 RNA Spike I Probe

- Universal RNA Spike I template: 10 aliquots of 50 µl, 10^7 copies/µl (0.005 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

Contents TATAA Universal RNA Spike I SYBR®

- Universal RNA Spike I template: 10 aliquots of 50 µl, 10^7 copies/µl (0.005 ng/µl) in 0.1mM EDTA, pH 7

- Spike I Assay primers: 250 rxn* = 250 µl of primer mix
c = 10 µM (per primer)

*rxn = qPCR reaction in 25 µl, concentration = 400nM per primer
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 (RNA)**

• **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 RNA Spike I template at -80°C, and the Spike I 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 of the RNA Spike template, 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 Minimum Information for Publication of Quantitative Real-Time PCR Experiments “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 RNA 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 by 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 with 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.

<table>
<thead>
<tr>
<th>Inhibitors (in H2O)</th>
<th>Absorbance spectrum</th>
<th>Max absorbance</th>
<th>Inhibitory amount (RT)</th>
<th>Inhibitory amount (qPCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% buffered formaldehyde</td>
<td>&lt;250 nm</td>
<td>≈220 nm (buffer)</td>
<td>&gt;0.01% (v/v)</td>
<td>&gt;0.01% (v/v)</td>
</tr>
<tr>
<td>dimethylsulphoxide (DMSO)</td>
<td>200-350 nm</td>
<td>230 nm</td>
<td>&gt;10%</td>
<td>&gt;10%</td>
</tr>
<tr>
<td>EDTA (0.5 M, pH 8)</td>
<td>210-360 nm</td>
<td>254 nm</td>
<td>&gt;0.5 mM</td>
<td>&gt;0.5 mM</td>
</tr>
<tr>
<td>ethanol (100% (A = 0)</td>
<td>&lt;230 nm (A=0)</td>
<td>&lt;230 nm (A=0)</td>
<td>&gt;1% (v/v)</td>
<td>&gt;1% (v/v)</td>
</tr>
<tr>
<td>fulvic acids</td>
<td>200-500 nm</td>
<td>224, 254, 315, 342</td>
<td>&gt;0.01% (w/v)</td>
<td>&gt;0.01% (w/v)</td>
</tr>
<tr>
<td>guanidine HCL</td>
<td>320-450 nm</td>
<td>≈230 nm</td>
<td>&gt;1mM</td>
<td>&gt;1mM</td>
</tr>
<tr>
<td>guanidium thiocyanate (GTC)</td>
<td>220-350 nm</td>
<td>≈260 nm</td>
<td>&gt;1mM</td>
<td>&gt;1mM</td>
</tr>
<tr>
<td>humic acids</td>
<td>200-500 nm</td>
<td>≈224 nm, ≈254 nm</td>
<td>&gt;0.01% (w/v)</td>
<td>&gt;0.01% (w/v)</td>
</tr>
<tr>
<td>chloroform</td>
<td>160-240 nm</td>
<td>&lt;230 nm</td>
<td>&gt;0.1% (v/v)</td>
<td>&gt;0.1% (v/v)</td>
</tr>
<tr>
<td>cholic acid</td>
<td>200-500 nm</td>
<td>several (305, 389...)</td>
<td>&gt;0.1% (w/v)</td>
<td>&gt;0.1% (w/v)</td>
</tr>
<tr>
<td>isopropanol (A=0)</td>
<td>150-200 nm (A=0)</td>
<td>&lt;230 nm (A=0)</td>
<td>&gt;1% (v/v)</td>
<td>&gt;1% (v/v)</td>
</tr>
<tr>
<td>phenol</td>
<td>210-290 nm</td>
<td>230 nm, 270 nm</td>
<td>&gt;0.1% (v/v)</td>
<td>&gt;0.1% (v/v)</td>
</tr>
<tr>
<td>proteins</td>
<td>250-300 nm</td>
<td>≈280 nm</td>
<td>too complex</td>
<td>too complex</td>
</tr>
<tr>
<td>Qiazol™</td>
<td>complex</td>
<td>complex (290 nm)</td>
<td>&gt;0.1% (v/v)</td>
<td>&gt;0.1% (v/v)</td>
</tr>
<tr>
<td>RNA later™</td>
<td>150-250 nm</td>
<td>215 nm</td>
<td>&gt;0.1% (v/v)</td>
<td>&gt;0.1% (v/v)</td>
</tr>
<tr>
<td>sodium dodecyl sulphate 1% (SDS, A=0)</td>
<td>150-350 nm (A=0)</td>
<td>&lt;350 nm (A=0)</td>
<td>0.001% (w/v)</td>
<td>0.001% (w/v)</td>
</tr>
</tbody>
</table>

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 of the RT-qPCR. In such instances, the inhibition and subsequent biasing of the RT-qPCR results may remain unnoticed.
Inhibition test procedure

The test for inhibition is based on an efficient and simple principle: **equal amounts of spike template 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 sample 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 2:** Workflow for inhibition test.

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

**Figure 4:** Higher Cq-values for sample compared to control indicates inhibition in the RT-step.
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 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 5) 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 obtained 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 RNA Spike I template (2*10⁷ copies) to each RT reaction containing RNA extracted from experimental sample.
2. Add 2 µl of TATAA Universal RNA Spike I template (2*10⁷ copies) to a control RT reaction, which instead of experimental RNA contains pure nuclease free H₂O or purified matrix, etc.
Recommendation: Use sufficient amount of Spike template to obtain about $10^4$ molecules in the final qPCR ($C_q < 30$). Too much spike will generate low $C_q$ values, which may cause problems with baseline subtraction on some instruments. With too little Spike template, reproducibility may suffer. We advise adding 2 μl of Spike template per sample as pipetting smaller volumes is less accurate.

Example: If $2 \times 10^7$ copies of RNA Spike template is added to a 20μl RT-reaction, assuming 100% RT efficiency, cDNA is diluted 4 times (from 10 μl to 40 μl) and 2 μl cDNA (5%) is added into the qPCR, a $C_q$-value in the range 15-20 cycle is expected on most qPCR instruments.

3. Reverse transcribe using a standard procedure (any RT kit and any priming method can be used for the TATAA Universal RNA Spike).

4. Perform qPCR on the cDNA from the experimental samples and the control by 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 ($C_q$ and shape of the amplification curve) by comparing the control and experimental sample. Any significant difference indicates RT-qPCR inhibition (Figures 3-6).

$\Delta C_q(\text{experimental sample} - \text{control}) \leq 0.5$ 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.

$\Delta C_q(\text{experimental sample} - \text{control}) > 0.5$ 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.
The TATAA Universal RNA Spike template may be added to any stabilized and homogenized sample prior to RNA purification to test for material loss during isolation, transportation, and storage. Equal quantities of the RNA Spike I template 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 RNA 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:

\[
yield(\%) = \frac{1}{2 \times (Cq_{\text{experimental sample}} - Cq_{\text{control}})} \times 100
\]

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 RNA spike is very low (≈ 0.005 ng / μl) compared to the amount of total RNA present in a typical experimental sample.
Protocol - Estimating extraction yield

1. Add 2 µl of the TATAA Universal RNA Spike I template 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 of the TATAA Universal RNA Spike I template to nuclease free H$_2$O (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. For example; if the elution step is performed with 30 µl TE-buffer, the control sample contains: 2 µl RNA Spike I + 28 µl TE-buffer.

   **Control II (optional):** Add 2 µl TATAA Universal RNA Spike I in the homogenization buffer. The total volume of Control II is equal to the volume used for homogenization of the experimental sample. It is recommended to also add a nucleic acid carrier to this control (such as yeast tRNA), to mimic your experimental sample.

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

4. Reverse transcribe extracted experimental samples and control samples(s).

5. Perform qPCR on the experimental samples 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 an annealing temperature of approximately 60°C.

   **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.

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

   $$\text{yield (\%)} = \frac{1}{2\left(C_q\text{ experimental sample} - C_q\text{ control}\right)} \times 100$$
Example: The total volume of the tissue homogenate per isolation is 400 µl, with added 2 µl of RNA Spike I template (2x10^7 spike molecules), and the elution volume is 40 µl, from which 2 µl of RNA is reverse transcribed into cDNA (10^6 spike molecules). The cDNA is diluted 4x to 40 µl and 2 µl is used per 10 µl qPCR (5% of the RT product transferred). This setup provides 50,000 TATAA Universal Spike molecules per PCR, assuming 100% RT efficiency and 100% extraction yield. Expected Cq-values is 25-30 on most qPCR instruments.

GenEx
A 6 months complimentary license for the qPCR analysis software GenEx Enterprise is included with the TATAA Universal Spike I kit for first time users. 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 RNA Spike I with the Spike I assay provided. If the problem persists, please contact us at info@tataa.com.

Amplification/signal in negative controls
The reagents are probably contaminated.

Samples have same/higher Cq-value than the negative control
It is either possible that too low amounts of RNA Spike template has been used or that complete inhibition is present. Add more RNA Spike and try again. Make sure the quality of the RNA is not compromised due to improper storage before performing qPCR. Make sure the instrument is set optimally.

High spread among replicates
With an RNA Spike of good quality and when using a good pipetting technique, high reproducibility is expected. Low input amounts of RNA Spike or diminished quality can lead to higher variation. Check the accuracy and reproducibility of your pipettes, and make sure the qPCR instrument is performing well.
References


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 the TATAA Universal Spike kits, contact us at info@tataa.com

License information

PCR is covered by several patents owned by Hoffman-La Roche Inc., and Hoffman-LaRoche, Ltd. Purchase of the TATAA Universal DNA/RNA Spike I/II kits 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.
Other products from TATAA

ValidPrime™ - Control and correction of gDNA contamination
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 a market-leading software for qPCR experimental design and data processing, and is supported by the 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 - Validation of correct reference genes
The panel for human, mouse or rat 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 please visit www.tataa.com
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 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.