Solutions to common sources of error

in the quantitative PCR workflow

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This reference guide is designed to provide you with solutions to common sources of error in the qPCR workflow. For additional technical resources please refer to www.thermoscientific.com/onebio.

Sample Preparation

Source of Error	Solution	Associated Thermo Scientific Products
Impurities such as salts, proteins, carbohydrates and organics will inhibit the efficiency of RT and PCR steps.	1. Spectrophotometric absorbance scan between 220 nm and 340 nm may reveal contamination by salt, proteins or phenol. 2. Spectrophotometric evaluation may not show low amounts of contamination that will inhibit the downstream enzymatic reactions (RT and qPCR). In this case, a RNA spike control can determine if a step within the RT-qPCR workflow is being inhibited. A DNA spike can be used for qPCR approaches using DNA as starting material. 3.To reduce potential inhibiting contaminants, use DNA/RNA precipitation followed by ethanol washes, or commercially available extraction and purification kits.	1. Thermo Scientific NanoDrop or Thermo Scientific Multiskan GO 2. Thermo Scientific Solaris RNA Spike Control Kit to test for reaction inhibition. 3. Thermo Scientific GeneJET nucleic acid purification kits for high quality RNA, DNA, PCR product and gel purification. Consider also the Thermo Scientific KingFisher system for higher throughput nucleic acid purification.
Genomic DNA contamination increases the likelihood of off-target amplifications and false positives.	1. Avoid nucleic acid extraction techniques that depend upon phenol and chaotropic salts without further solid phase binding & washing steps. Ensure sample size/cell number is within the recommended guidelines so that the lysis capacity of the method is not exceeded – which will lead to poor recovery/ RNA contamination. 2. Treat RNA samples with a DNA degrading enzyme, such as DNase I. 3. Design the detection assay to span an intron if possible. 4. Sample analysis by gel electrophoresis or a microfluidics platform, e.g. Agilent 2100 Bioanalyzer, can reveal gDNA contamination. 5. Incorporate an RT-minus control in RT-qPCR assays to verify that gDNA has been excluded.	1. GeneJET™ nucleic acid purification kits for high quality RNA, DNA, PCR product and gel purification. Consider also the KingFisher system for higher throughput nucleic acid purification. 2A. Thermo Scientific DNase I (RNase free) Cat # EN0521. 2B. RT Enhancer included in Thermo Scientific Verso RT-qPCR Kit degrades any contaminating DNA during the RT step, eliminating the need for a DNase I treatment of the extracted RNA. 3. Solaris™ qPCR Gene Expression Assays are intron-spanning whenever possible.
RNase contamination will result in degraded mRNA, which in turn will bias cDNA production and ultimately detection sensitivity.	1. Use modern nucleic acid extraction kits and techniques that optimize RNA recovery, and minimize RNase contamination. Use automated systems to prevent RNase contamination. 2. Use RNase inhibitors during sample preparation. 3. Change gloves frequently. Use RNase/DNase free plastics or DEPC treated labware. Use RNAse decontaminating solutions and sprays. 4. Sample analysis by gel electrophoresis or a microfluidics platform, e.g. Agilent 2100 Bioanalyzer, can reveal RNA degradation before the reverse transcription step.	1. GeneJET purification kits for high quality RNA, DNA, PCR product and gel purification. Consider also the KingFisher system for higher throughput nucleic acid purification. 2. Thermo Scientific RiboLock RNase inhibitor is included in all Thermo Scientific Maxima and RevertAid cDNA Synthesis Kits. 3. Use of RNAse AWAY eliminates RNase and DNA from laboratory surfaces, and is ideal for decontaminating apparatus, bench-tops, glassware and plasticware.

Reverse Transcription

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Short, non-representative cDNA populations, low yield or otherwise low quality cDNA.	1. The priming strategy directly impacts the efficiency of the reverse transcription step. Decide upon the best priming strategy for your application – gene specific oligo, random hexamers, oligo dT, or a mixture of these. 2. Choose a thermostable, robust, highly processive reverse transcriptase to increase cDNA synthesis through difficult secondary structure, and resist contaminating inhibitors. This will increase overall yield, sensitivity and accuracy of data.	1. The RevertAid cDNA Synthesis Kits include both oligo dT and random hexamers in separate tubes so that you can tailor your master mix priming strategy to your applications. The Thermo Scientific Maxima cDNA synthesis kit provides a mixture of oligo dT and random hexamers optimal for many applications. 2. Maxima™ Reverse Transcriptase was developed through *in vitro* evolution, and engineered to be very thermostable and offer greater resistance to contaminating inhibitors. cDNA synthesis at higher temperatures affords less disruption due to RNA secondary structure, producing longer products (up to 20 kb), more representative coverage and higher yields to increase the detection of lower copy number targets.
RNA template with difficult secondary structure.	1. Increase the temperature of the RT reaction to aid polymerization through RNA secondary structure – this requires the use of a reverse transcriptase that is stable at higher temperatures.	1. Maxima RT is very thermostable and performs well at temperatures that range from 42 to 65°C.
Only a small range of target concentrations is detected.	1. Use of a reverse transcriptase with wide dynamic range for improved efficiency to increase the sensitivity of qPCR.	1. Maxima RT performs well over 10 orders of magnitude of target concentration.
RNA sample contamination by salts or organics will inhibit RT efficiency, lower cDNA yields and bias qPCR outcomes.	1. Choose a reverse transcriptase that is more tolerant of inhibitors typically found in RNA preparations (e.g., salt, phenol, proteins, etc.) 2. Include controls for the reverse transcription step. 3. Avoid phenolic extraction methods, and where possible, use optimized nucleic acid extraction kits and techniques that maximize RNA recovery and purity, and minimize RNase contamination. Use automated systems to prevent RNase contamination. Those studying miRNAs should choose an extraction method that does not result in a size-selective loss of their target molecules.	1. Maxima RT is more tolerant of contaminants such as formamide and guanidine than other commercially available reverse transcriptases. 2. Solaris RNA Spike Control Kit to test for reaction inhibition. 3. GeneJET purification kits for high quality RNA, DNA, PCR product and gel purification. Consider also the KingFisher system for higher throughput nucleic acid purification.
Too little or too much RNA or widely varying amounts of RNA in reverse transcription reactions will result in inefficient or biased results.	1. Carefully quantify the amount of RNA in each sample either spectrophotometrically or by microfluidics platform.	1. NanoDrop or Multiskan GO

Amplification

Source of Error	Solution	Associated Thermo Scientific Products
DNA contamination from previous PCR experiments.	1. Employ dUTP in master mix, in combination with Uracil-DNA-Glycosylase (UNG) to digest any previously amplified DNA that has carried over to the present reaction.	1. Both Maxima and DyNAmo qPCR Master Mixes contain dUTP. UDG (UNG) is also separately available (Cat# EN0361, EN0362).
Impurities such as salts, proteins and organics will inhibit the efficiency of PCR steps.	1. Use non-organic nucleic acid extraction methods to minimize contaminants that inhibit PCR amplification. 2. Spectrophotometric evaluation can reveal contamination by salt, proteins or phenol. 3. In some instances, spectrophotometric evaluation may not detect small amounts of contaminants that are still able to inhibit PCR reactions. Use of an amplification positive control can reveal inhibition due to the presence of impurities. 4. To reduce potential inhibiting contaminants, use DNA/RNA precipitation followed by ethanol washes, or commercially available extraction and purification kits.	1. GeneJET purification kits for high quality RNA, DNA, PCR product and gel purification. Consider also the KingFisher system for higher throughput nucleic acid purification. 2. Nanodrop & Multiskan GO, followed by sample clean-up. 3. Solaris RNA Spike Control Kit to test for reaction inhibition.
Poor primer design can result in low amplification efficiency (less than 90%), primer dimer formation, or off-target amplification.	1. Master mix composition can be optimized to reduce mis-priming. Use optimized commercial master mixes. 2. Validate primer sets (SYBR Green) by melt curves or by checking end-point reactions on a gel. 3. Use pre-designed assays such as primer/probe sets that have guaranteed performance. 4. Use software to aid primer design. 5. SYBR Green analysis - try 3 primer pairs for each new target. 6. Use a hot start DNA polymerase (most commercial master mixes include this).	1. Optimized Maxima and DyNAmo Flash/ ColorFlash qPCR Master Mixes can reduce problems with primer dimers and off-target amplification (hot start polymerase included). 2. Thermo Scientific PikoReal qPCR instrument software includes a melting curve data analysis module. 3. Use high performance Solaris Assays designed using stringent parameters for specificity with optimized Solaris Master Mix. 6. Maxima and DyNAmo Master Mixes include hot start DNA polymerases.
High concentrations of SYBR Green dye can inhibit PCR amplification.	1. Higher concentrations of SYBR Green can be used in combination with DNA polymerases that have been designed to perform well under such conditions.	1. Obtain improved signal intensity and increased sensitivity, without PCR inhibition. DyNAmo ColorFlash/Flash Master Mixes contain an engineered DNA polymerase with a dsDNA binding domain (Tbr) able to tolerate higher SYBR Green concentrations than competitor enzymes.
PCR/qPCR instrument is often in use, or amplification runs are longer than they need to be.	1. Use an optimized Master Mix designed for fast qPCR or validate faster protocols (e.g., Solaris reagents work with faster protocol). 2. Purchase personal qPCR instrument. 3. Purchase dual block PCR instrument to run two runs simultaneously. 4. Purchase fast thermal cycler.	1. DyNAmo Flash & Color Flash Master Mixes can greatly reduce assay times without compromising performance. They are optimized for fast cycling protocols. 2. Low cost PikoReal 24 and 96 Real-Time PCR Systems 3. Thermo Scientific Arktik Thermal Cycler with 48/48 dual block 4. Thermo Scientific Piko 24 or 96 Thermal Cycler, which can perform PCR in 20 minutes.
Evaporation from the well during the PCR run will result in poor amplification.	1. Plate seals must be as effective as possible, use manufacturer's recommended seal. 2. Use plates with a raised rim for improved sealing. 3. Seal plates using plastic sealing tool being careful to seal in all directions.	1.& 2. Thermo Scientific ABsolute qPCR seals and Ultra-Clear qPCR Cap Strips are optimized for use with all Thermo Scientific PCR and qPCR consumables with raised rim design. 3. Thermo Scientific Microplate Heat Sealer (e.g., Cat# AB-1443 ALPS 50V).

Detection Chemistry

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Fluorescent signal is low.	1. Check that amplification was achieved using agarose gel electrophoresis. 2. Use white PCR plastics to optimize light reflection and increase fluorescent detection through minimizing signal loss associated with refraction through clear walled tubes. 3. Use plate seals that have been optimized for optical clarity to improve assay sensitivity. 4. For SYBR Green assays, one option is to use more SYBR Green in master mix in combination with a DNA polymerase designed to be more tolerant of higher SYBR Green concentrations. 5. Use an optimal concentration of ROX for each instrument (if the instrument requires the use of ROX).	1. See sample preparation, reverse transcription and amplification sections. 2. All Thermo Scientific qPCR consumables are available as white and clear options 3. Thermo Scientific Optical Seals (AB-1170) have higher fluorescence transmission than many competitors. 4. Engineered DNA polymerase with dsDNA binding domain in DyNAmo Flash and ColorFlash SYBR Green Kits. 5. Check the instrument compatibility table for the most suitable reagent for each instrument and/or product manuals to add the most optimal ROX concentration. Thermo Scientific qPCR reagents come with ROX, either pre-blended or in a separate tube for many manufacturers' instruments.
Difficulty in determining which pre-designed assay to purchase due to multiple options.	1. Use pre-designed assays that span introns, and detect all known splice variants.	1. Solaris Assays are designed to span introns whenever possible, and detect all known splice variants.

Pipetting

Source of Error	Solution	Associated Thermo Scientific Products
Sample to sample contamination.	1. Use state-of-the-art pipettes or automated pipetters with aerosol-blocking filter tips. 2. Use dUTP in Master Mix, with Uracil-DNA-Glycosylase (UNG) to digest any previously amplified DNA that has carried over to the present reaction.	1. Thermo Scientific Matrix electronic and manual pipetters and filtered pipette tips, Finnpipettes, Versette pipetting workstations. 2. Both Maxima and DyNAmo qPCR Master Mixes contain dUTP. UDG (UNG) is also separately available (Cat# EN0361, EN0362).
Pipetting errors – e.g., skipped or double loading of wells.	1. Use a dye in the Master Mix to aid the addition of reagents to a plate, reduce skipping or double loading of wells, and identify spill-overs. 2. Use additional aids for pipetting. 3. Use channel pipettes to avoid missing single wells.	1. DyNAmo ColorFlash SYBR Green and Probe qPCR kits contain blue master mix and yellow sample buffer. When yellow sample is combined with blue master mix, the reaction turns green to aid tracking progress during plate set-up. For pre-designed probe based assays, Solaris Master Mix contains blue dye. Absolute Blue qPCR Master Mixes for standard cycling protocols. 2. Piko Plate Illuminator is an easy to use pipetting aid. 3. 8, 12, or 16 channel from Matrix and Finnpipettes.

Controls

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Higher than expected variation between technical replicates, or between samples.	1. Use a RNA spike control to determine if a step within the RT-qPCR workflow is being inhibited. A DNA spike can be used for qPCR approaches using DNA as starting material. 2. Check purity and integrity of RNA/DNA samples. 3. Consider trying alternative primer pairs when the r^2 value is less than 0.99 or if the PCR efficiency is <90% or >105%.	1. Solaris RNA spike control kit. 2. NanoDrop or Multiskan GO 3. Review Sample Preparation section for more guidance
Amplification in No Template Control.	1. Use state-of-the-art pipettes or automated pipetters with aerosol-blocking filter tips. 2. Use dUTP in Master Mix, with Uracil-DNA-Glycosylase (UNG) to digest any previously amplified DNA that has carried over to the present reaction. 3. Run endpoint reactions on a gel to determine if product is primer dimer, or other amplicon. Clone and sequence the amplified product to identify contamination.	1. Matrix electronic and manual pipetters and filtered pipette tips, Finnpipettes, Versette pipetting workstations. 2. Both Maxima and DyNAmo qPCR Master Mixes contain dUTP. UDG (UNG) is also separately available (Cat# EN0361, EN0362). 3. Thermo Scientific InsTAclone PCR Product Cloning kit (Cat # K1213) is ideal for rapid, high-efficiency one step cloning directly from a PCR reaction, with standard primer sites flanking the insert for easy sequencing.
Amplification in No RTase control	1. Use state-of-the-art pipettes or automated pipetters with aerosol-blocking filter tips. 2. Use dUTP in Master Mix, with Uracil-DNA-Glycosylase (UNG) to digest any previously amplified DNA that has carried over to the present reaction. 3. Use non-organic nucleic acid extraction methods or those that optimize RNA recovery, and minimize genomic DNA contamination. 4. Treat RNA samples with a DNA degrading enzyme, such as DNase I. 5. Whenever possible, the amplicon should be intron-spanning. 6. Analysis by gel electrophoresis or a microfluidics platform, e.g. Agilent 2100 Bioanalyzer, can reveal gDNA contamination, so remediation may be taken.	1. Matrix electronic and manual pipetters and filtered pipette tips, Finnpipettes, Versette pipetting workstations. 2. Both Maxima and DyNAmo qPCR Master Mixes contain dUTP. UDG (UNG) is also separately available (Cat# EN0361, EN0362). 3. GeneJET purification kits for high quality RNA, DNA, PCR product and gel purification. Consider also the KingFisher system for higher throughput nucleic acid purification. 4. RT Enhancer included in Verso qRT-PCR Kits and Verso cDNA Synthesis Kit degrades any contaminating DNA during the RT step, eliminating the need for a DNase I treatment of the extracted RNA. 5. Solaris qPCR Gene Expression Assays (intron-spanning whenever possible).

For more information, please visit:
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