White paper

Ensuring qPCR data reliability – controlling for contamination

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Those working in the demanding laboratory environment of current SARS-CoV-2 testing can probably relate to Alexander Fleming departing for his holiday, while leaving behind stacks of *Staphylococcus* plates. Ninety two years ago, Fleming returned from his break to find masses of overgrown plates. One plate stood out as different: this plate had an infection of mould and around it, a zone clear of *Staphylococcus*. The age of formal antibiotic discovery began, with countless lives saved as a result.[1]

Scientific progression was similarly influenced during the 1980s, when several research labs purchased yeast tRNA to be used as a carrier for precipitation of the RNA from their species of interest. After the lengthy procedures of construction of cDNA libraries and screening for functional clones, containing genes from their organism of interest that would rescue mutations in *Saccharomyces cerevisiae* strains, many researchers were dismayed to discover they had cloned, rescued and sequenced *S. cerevisiae* yeast genes originating from mRNA contamination in their purchased tRNA. As a result, many molecular biologists abandoned their original research interests and turned their attention to studying fundamental yeast genetics [John Rosamund, personal communication, 1993].

Unfortunately, though entertaining, these may be the only examples of scientific contamination events leading to fortuitous discoveries: science, particularly in regulated fields such as molecular diagnostics and forensics, is advanced through dedication to stringent processes and methods. Problems such as high background, low sensitivity and low specificity can be avoided through stringent adherence to carefully developed optimisation and validation processes.

Designing and implementing controls

To realise the potential of single molecule detection using PCR, two critically important factors must be optimised: the specificity of the individual amplification oligonucleotides to the target sequence, and ensuring that the system is free from any material that could serve as a template for the generation of a false positive signal. Quantitative PCR (qPCR) makes use of the synergistic specificity of the hybridisation of three separate oligos, conventionally two primers and a probe. Only when a template molecule is present should the primers amplify the intended amplicon sequence, to which the probe binds and is subsequently degraded by the polymerase to generate sequence-specific fluorescence signals.

A mainstay of scientific method is the inclusion of carefully considered controls alongside both research and routine procedures. During the initial stages of development of a PCR, qPCR or the combined reverse transcription and qPCR (RT-qPCR) assay, controls are used to:

A) Ensure that no signal is generated in the absence of target sequence by assay components alone. For example, this could be a signal resulting from dimerisation of primer or probe (or both) and subsequent amplification, or contamination of reaction components such as enzymes or buffers;

B) Verify that the design is functional and specific. For example, for pathogen detection, this could be accomplished by ensuring that the target sequence is distinguished from any similar host sequences, or for a topical example, this would ensure that the current SARS-CoV-2 assays do not detect the common cold coronavirus sequences;

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C) Assess assay efficiency and the limits of quantification and detection to ensure adequate sensitivity.

Table 1 provides a summary of the application of controls to assay development.

Once the assay has been developed, optimised and verified, positive and negative controls are run alongside routine clinical samples to ensure reliability of the diagnostic result.

qPCR and RT-qPCR controls: *Interpretation of control data during assay development*

Control	Expected result	Result	Interpretation	Action
No template control (NTC)	Negative	Negative	No contamination or primer dimers evident	• Ensure positive control is correct.
	Negative	Positive	Primer dimers or contamination evident	• Check assay with an intercalating dye and compare product sizes using melt curve analysis.
Target template* (artificial control)	Positive	Positive	Assay functioning	• Ensure negative control is correct. Optimisation may still be required.
	Positive	Negative	Failed reaction	• Repeat using intercalating dye to determine whether primers, probe or both have failed. • Repeat assay using a different template to identify alternative explanations for reaction failure.
No reverse transcription (RNA targets)	Negative	Negative	No DNA amplification	• Ensure positive control is positive and NTC is negative.
	Negative	Positive	DNA amplification (or primer dimers)	• Read in conjunction with NTC. Both samples being positive indicates primer dimers; negative NTC and positive minus RT indicates detection of contaminating DNA. • Redesign assay to span exon junctions, or repeat RNA extraction.
Target template serial dilution to single copies of target/minimum three replicates	Estimate of assay efficiency and reproducibility	Efficiency (95–105%) Replicates highly reproducible	Assay optimised	• Validate in conjunction with negative controls.
	Estimate of assay efficiency and reproducibility	Efficiency <95% or >105% Replicates highly variable	Assay improvements required (where possible)	• Optimise assay conditions (oligo concentrations and/or annealing temperature). • Redesign assay.
Nonspecific template control (SPUD) [Internal positive control]	Positive (of specified Cq)	Negative or higher Cq than expected	Presence of contaminants inhibiting reaction efficiency	• Systematically explore the source of contamination. May occur at any stage from sample preparation to test set-up.

Table 1.

*Validation using control template material provides additional information around assay efficiency and limit of detection. Bustin *et al.*[2], described an exemplary study for the development of a clinical diagnostic assay. They developed a multiplex qRT-PCR for detection of SARS-CoV-2 from clinical samples and included controls for both development and inclusion of quality control assessments when applied to patient material. The negative control was a simple "No Template Control" containing all assay components, with the exception of template. This was run when assays were being validated and when combined in multiplex. The assay description clearly states that no product was amplified in the absence of a template, neither for single nor multiplex assays. This confirmed that the primers of all assays did not interact and that specific template had not found its way into the reaction components.

A comparison of two probe types for detection of FluA, FluB,*SARS-CoV-2 and Human RNaseP*

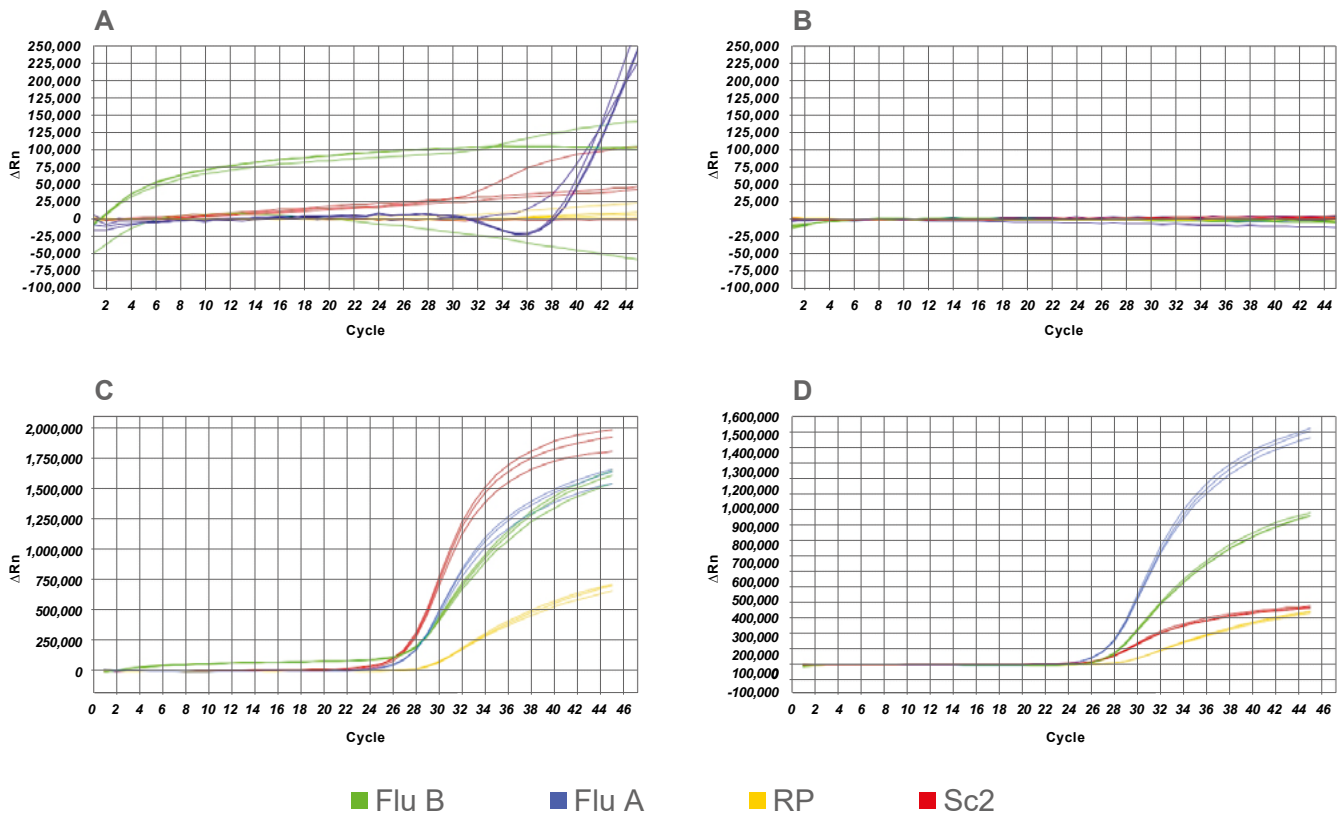

Figure 1. During development of a multiplex assay to detect FluA, FluB, SARS-CoV-2 and Human RNaseP, two probe types (1 and 2) were tested, each in multiplex. The data presented (A and B) show only the NTC data for each assay. Using the probe type 2 shown in B there was no amplification for any target but for probe type 1, shown on A, amplification was evident at late cycles of FluA detection. In view of the amplification used only in this assay, the most likely cause was interaction of assay oligos using probe type 1, with contamination being less likely. This data needs to be taken in context of the positive controls, in which both probe types returned a positive signal (probe type 1 with positive controls are show in Figure C and probe type 2 in Figure D).

Two positive controls were also included in the Bustin *et al.* [2] assay design. The first positive control is an artificial RNA target of sequence that has no similarity to any known, natural sequences. Amplification of this target provides assurance that the reverse transcription (RT) and qPCR steps are functioning correctly. In addition, the inclusion of the RNA control template at a constant concentration provides an indirect assessment of the presence of inhibitory contaminants with the sample. This is following the principle of Nolan *et al.*[3] SPUD Assay in which Cq values are recorded in the presence and absence of sample. Deviations from the Cq measured for the control template are indicative of the sample containing inhibitory material.[4] The final control included in the multiplex is an assay to detect human nucleic acid to provide assurance that sample preparation techniques have yielded template material. These controls are used in conjunction to verify that the assay as a whole functions; however, further assessment is required to determine the quality of the assay.

In the Bustin *et al.*[2] example cited above, a genotyping assay was required to differentiate between emerging pathogen mutations, but the assay requirements were also specific to SARS-CoV-2, thus not detecting other pathogens or human sequences. However, when used to support diagnosis of infectious disease, exquisite detection sensitivity is an additional requirement for many assays.

The limit of quantification of an assay is determined using control template material. Control material may be derived from clinical, artificial or recombinant pathogenic material or a synthetic oligonucleotide template (Figure 2). The advantage of artificial or synthetic templates is that these can be manufactured to copy numbers far exceeding anything required in an assay and quantified accurately using digital PCR.

Assay validation determination using qPCR detection of a standards panel

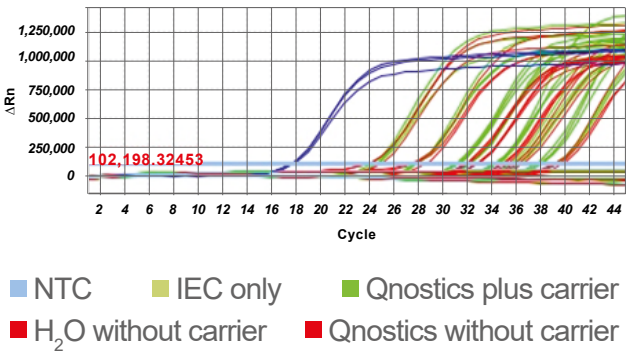

Figure 2. Standards from the Qnostics SARS-CoV-2 Analytical Q Panel 01, (digital (d) target copy/mL ranging from one million to 50 viral copies per mL) were extracted in triplicate using sbeadex™ chemistry in the presence (green) and absence (red) of a PolyC carrier. The blue amplification plots are detection of the internal positive control within the Primerdesign/Novocyt genesig® Real-Time PCR COVID-19 (CE) detection kit . The carrier ensured higher sensitivity of detection, detecting nine copies for all replicates at Cq 38. No amplification was evident in the negative controls.

In this way, controls are designed to identify vulnerabilities in the entire experimental or diagnostic process and ensure accurate interpretation of data. Having optimised and adopted a diagnostic assay, it may be tempting to maximise sample processing by neglecting to run parallel controls, but this amounts to a false economy. Within a diagnostic setting, parallel controls ensure validity of results and ensure that false positives and negatives are correctly identified and designated for further investigation (Table 2). For a diagnostic qRT-PCR assay, reliable identification of affected individuals is reliant on negative controls returning absolutely no signal; therefore, any signal detected in the negative control of an optimised and validated qPCR/RT-qPCR assay indicates that the reaction has become contaminated with the specific template.

qPCR and RT-qPCR controls: *Interpretation of control data during routine testing*

Control	Expected result	Result	Interpretation	Action
No template control (NTC)	Negative	Negative	No contamination	Run or batch is valid.
	Negative	Positive	Contamination evident	Invalidate run.
Target template (artificial control)	Positive	Positive	Assay functioning	Run or batch is valid.
	Positive	Negative	Failed reaction	Invalidate run.
Internal full process control	Positive	Positive	Assay functioning	Sample is valid.
	Positive	Negative	Failed reaction	Sample is invalid.

Table 2.

Sources of contamination

There are many different sources and types of reaction contamination (Table 3). The specificity of qPCR amplification and detection is not generally sensitive to the presence of other oligonucleotides. Indeed, complex multiplex amplification systems, such as syndromic panels, can be constructed by careful oligo design in such a way as to maintain both specificity and sensitivity of analyte detection. The tolerance to the presence of undetected and undetectably low level, non-homologous oligos in any oligo preparation has allowed for the annual production of millions of discrete oligo sequences by commercial manufacturers using shared lab spaces and equipment without the need for scrupulous cleaning between each oligo synthesis and purification event. Greater levels of product segregation, line clearance and cleaning (including lab hygiene concepts) are typically employed for the production of oligos under more stringent constraints for high-quality oligos for molecular diagnostics or therapeutic applications.

This tolerance to the presence of contaminating sequences is not true for the concurrent or collocated production of amplification oligos along with the production of target sequences that have corresponding primer and probe binding sites. Such longmers are often requested by the developers of new assays as part of a non-clinical performance evaluation of the assay. In such cases, the production of these amplifiable sequences should be carried out under strict conditions of segregation from potential amplification sequences since the presence of even single copies of such templates would give rise to false positive amplifications from the use of the amplification oligos. In the high pressure environment of oligo production labs trying to meet the urgent needs of multiple projects, there is an absolute requirement for a streamlined process for the identification of requests for potentially contaminating oligos, followed by the institution of a lab process to synthesise the longmers while mitigating the potential for cross-contamination.

Hugget *et al.*[5] highlight sample or reaction template carryover and contamination of assay components by synthetic oligo as two potential sources of contamination that may result in a false positive result from a clinical diagnostic assay. The article quotes one source of PCR contamination as the potential for transfer of amplification material from previously run PCRs and one of the key factors that led to the development of qPCR was the potential to reduce operator mediated cross contamination of subsequence PCRs by ensuring analysis of reaction products within the tube to eliminate manipulation of concentrated PCR products post reaction. A further advantage of qPCR is the ability to detect single copies of a target. However, while this exquisite sensitivity is a benefit in diagnostic and forensic applications where small amounts of target material must be detected, there is also the potential for it to be a detriment because it also creates vulnerability to the presence of minute concentrations of template contamination. The ramifications of false positives vary between applications but could be devastating in both forensic and diagnostic settings. The recent SARS-CoV-2 pandemic has revealed the dramatic economic impact of an isolating workforce, with accurate testing and subsequent contact notification a requirement to slow viral transmission[6]. False positive results lead to unnecessary self-isolation of healthy individuals and those with whom they have been in contact, as well as instilling a lack of confidence in the testing process.

Sources of contamination and result

Contamination	Source	Result	Action
Positive sample to negative sample	Cross-contamination during sample handling	False positive	May not be detected, indicated if negative control is also contaminated.
Inhibitory materials	Carried over during sample preparation	False negative	Detected by multiplexed Internal or Full Process Control, or separate positive control (for example, SPUD) of known Cq/concentration. Each sample must be checked. Repeat sample processing when inhibitors are evident.
	Contaminated reagents	All reactions delayed (lower Cq than expected)	Verify Cq of positive control is correct for quantity. Replace reagents. Consider inhibition-resistant reagents[7].
Specific template material	PCR product leakage from previous reactions	False positive	If identified as such, implement a deep cleaning regimen and ensure reactions are free from contaminants before proceeding.
	Synthetic oligonucleotides (used as positive controls)	False positive	It is difficult to remove template contamination from such highly concentrated source material and may be necessary to redesign and optimise a new assay. Some labs have had to resort to new physical spaces.
	Artificial control material	False positive	It is difficult to remove template contamination from such highly concentrated source material and may be necessary to redesign and optimise a new assay. Some labs have had to resort to new physical spaces.
Human DNA/RNA	End-use contamination	Potential false positive if the assay targets human sequences	Use new batches of reagent.
	Contamination of buffers occurring during manufacture	Potential false positive if the assay targets human sequences	Supplier must investigate and ensure reagents free from contaminants are provided.
Bacterial DNA/RNA	Contamination of buffers occurring during manufacture	Potential false positive if the assay targets bacterial sequences	A well-recognised challenge since many enzymes are produced in bacterial systems. Specifically manufactured reagents may be required.

Table 3.

It is accepted that the end-user scientist or technician has a responsibility to ensure robust science, implement processes to prevent transfer of material between samples and incorporate solutions that can further protect sample integrity. One example utilises the incorporation of Uracil deoxyuridine triphosphate in the PCR and subsequent incubations with uracil-DNA glycosylases which cleave the N-glycosidic bond between uracil and deoxyribose,[8] leaving an abasic site which causes DNA degradation when the PCR incubation temperature is increased.

However, the end-user is absolutely reliant upon supply of high-quality reaction components and reagents. It is paramount that those supplying products to the scientific discovery and diagnostic processes have failsafe processes in place to ensure that the raw materials, reagents and protocols are reliable, robust and fit for purpose. Yet, it is apparent that contamination continues to be a challenge within molecular biology testing: an internet search using the terms “contaminated molecular biology reagents” returns 5.5 million results. Unlike most pathogenic viruses, bacteria are ubiquitous and, in the absence of strict microbiological control, can be found in aqueous solutions and on lab surfaces or consumables. As such, the nucleic acids from bacteria can be copurified along with target nucleic acids. This can give rise to anomalous results from amplifications of bacterial sequences that target highly conserved regions across multiple bacterial organism families, such as 16S ribosomal RNA. Many enzymes used in amplification technologies are manufactured using recombinant bacteria to express high levels of the enzymes. Although the enzymes are extensively purified as part of the production process, there is still the chance that some bacterial sequences remain in the various enzyme preparations. The introduction of bacterial nucleic acids into either sample preparation or downstream amplifications (or both) could potentially compromise the validity of microbiome or metagenomics analyses. Oligos potentially can be contaminated by the solvents used in their HPLC purification. Some aqueous buffers can support bacterial growth, which can then develop into biofilms in the liquid handling instrumentation used in the preparation of the oligos. If these bacterial sequences are introduced late in the preparation method, they will be carried through into the final oligo preparation. The use of bacteriostatic compositions in the HPLC buffers significantly reduces this form of bacterial DNA contamination.

For the production of oligonucleotides to be used for the determination of human identity, methods and processes should be introduced to minimise the chance of introducing hDNA from the operators in the production of the oligos. The use of appropriate PPE and suitable air handling processes can be used to minimise the opportunity to introduce this form of oligo contamination. As the use of next generation sequencing (NGS) increases, further scrutiny on the risks and mitigations for this type of reagent contamination are expected.

The need for adherence to fundamental contamination control systems for both end-users and kit suppliers was illustrated by the widely reported challenges faced by the team at US CDC when developing and producing the first SARS-CoV-2 detection kits. Shortly after implementation of diagnostic procedures, several centres reported positive results in negative controls. As a pattern developed, it was clear that the provided kits were contaminated with template sequences. It emerged that kits had been assembled in a research lab environment where positive control material was also being handled. Lack of process segregation, including changing lab coats between the laboratory regions exposed to viral control material and the kit assembly regions are most likely to have caused cross-contamination.

Reports of this contamination resulted in widespread confusion and sharing of misinformation, wrongly suggesting that the test itself was contaminated by virus, and therefore, that taking a test was dangerous. This single contamination event resulted in delays to test administration; thus, delaying disease containment, increasing commercial costs due to the wasted product, potentially exacerbating further deaths of those refusing to risk taking the test and increasing an overall lack of trust in those providing information about disease mitigation.

The second major source of potential oligo contamination, revealed by the current SARS-CoV-2 pandemic is the critical area of manufacturing vulnerability. This is mainly due to the high frequency demand for the same

diagnostic assay sequences and reagents by multiple users around the world. A major supplier of oligos issued a stark warning to customers, revealing that they had identified a potential source of contamination: that manufacture of synthetic templates may contaminate other oligos that are produced in the same physical environment. This risk was reported previously[9] but within the context of a single order which contained assay oligos and positive control template oligo. It was highlighted that in these circumstances the template could cross-contaminate the assay primers and/or probes during manufacture. It was believed that rarely would two independent scientists be requiring sequences that would contaminate across orders, between different groups. Despite knowledge of this cross-contamination challenge, at least three major European oligo producers experienced significant contamination of their oligo supply. Wernike *et al.*[10] ordered multiple replicates of the same SARS-CoV-2 detecting assay from three suppliers and demonstrated that the first supplier acquired contamination within a week (end of March 2020 to beginning of April 2020) and by April 2020 the remaining two suppliers also provided contaminated oligos (as noted in Table 1 of the Wernike *et al.*[10], publication). In one case, a negative control reported an extraordinary Cq of 17 cycles, impossible to distinguish from a positive control or sample. Several other reports reveal that this has become a global emergency and oligo manufacturers are being called upon to mitigate the risk of oligo contamination.[11] Within a research setting, these contamination events are irritating, and many researchers split their orders such that primers and probes are ordered before template, or request that the manufacturer process these in different manufacturing facilities. Within a diagnostic or forensic setting, such cross-contamination is potentially catastrophic, with the potential to be life-threatening. The increased risk during a pandemic is that many different groups are requesting the same assays and the same long oligo to use as a synthetic target for assay development. In the light of reports describing "background" amplification evident in negative controls of RT-qPCR assays applied to the detection of SARS-CoV-2, a group of leading molecular biologists, experts in the field of RT-qPCR issued a stark warning.[5]

Unfortunately, the current pandemic environment has provided a perfect storm to illustrate the vulnerabilities of RT-qPCR assays to contamination events. While experienced end-users have been aware of these vulnerabilities from the introduction of PCR, those in routine testing environments may not be. In addition to the utmost care for those in the testing environment[12], recent events have revealed an urgent need for those manufacturing facilities and reagent or kit suppliers to develop and implement operating procedures to mitigate the risks of cross-contamination. SARS-CoV-2 has revealed many weaknesses in systems previously taken for granted (Table 4). Ultimately, it is contingent upon suppliers to respond rapidly to these new challenges and recognise the life and death implications of business decisions. Many have implemented rapid change, at unprecedented rates and are now offering high-quality reagents that are suitable for the rapid and accurate testing protocols that are required to manage and research emerging viruses such as SARS-CoV-2.

Contamination cause and mitigation strategies

Source of contamination	Mitigation
Template carryover between reactions	• Maintain separate areas, supplies and dedicated equipment for assay set-up. • Change aerosol barrier pipette tips between all manual liquid transfers. • Wear clean lab coats and change disposable gloves regularly and when contamination is suspected. • Ensure lab coats are changed when moving between areas where template is and is not present. • Keep reactions capped as much as possible. • Ensure separate aliquots are used for each batch of reactions and dispose of any remaining. • Work surfaces, pipettes and centrifuges should be decontaminated with solutions containing 10% bleach. Residual bleach should be removed with 70% ethanol. • Use a master mix containing uracil N-glycolase (UNG)/uracil DNA glycolase (UDG) to degrade templates created by PCR.
Template contamination of accessory reagents	• Ensure separate aliquots are used for each batch of reactions and dispose of any remaining reagents. • Dispose of reagents and batch test a new supply. Change supplier if the issue persists.
Oligo contamination at source	• Ensure the oligo supplier has stringent operational policies in place to identify templates and a strategy to prevent contamination.

Table 4.

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