Performance Characteristics of Host-Cell DNA Quantification Methods

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Confidence in the reliability and comparability of results is a primary requirement for measurements made in both industrial and clinical environments. The increased uptake and wider application of molecular biological methods requires assurance of the accuracy, reliability, and comparability of the techniques. However, there are a number of difficulties. The variety of available technologies and platforms coupled with a lack of certified reference materials and standard methods are significant challenges to ensuring comparability of measurements. The pace of technological innovation is a further issue in development of standardized approaches.

Although all the current methods available for host-cell DNA (HCD) determination fulfill legal and safety requirements, there is a growing need in industry for detailed information on their performance characteristics. We designed this study to investigate performance characteristics and comparability of HCD quantification methods, thus enabling end users to choose the most appropriate method for their analytical requirements. The work was funded by the Measurement for Biotechnology program (www.mfbprog.org.uk), which is one of several initiatives supporting the United Kingdom's National Measurement System (NMS). Ensuring measurement comparability is one of the fundamental aims of the NMS, which seeks to enable sustainable development and effective application of biotechnology in a number of commercial sectors including the biopharmaceutical industry (1).

PRODUCT FOCUS: BIOPHARMACEUTICALS

PROCESS FOCUS: PRODUCT PURIFICATION

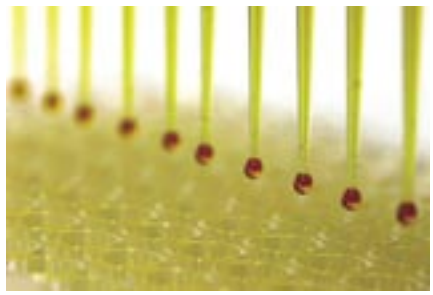
WHO SHOULD READ: ANALYTICAL AND PROCESS SCIENTISTS, QA/QC

KEYWORDS: DNA DETECTION METHODS, HOST-CELL IMPURITIES, QUALITY CONTROL, ANALYTICAL TESTING, COMPARABILITY

LEVEL: INTERMEDIATE

Regulating Host-Cell Impurities

Biopharmaceutical products are produced by fermentation using either microbial or eukaryotic cells grown in complex media. Crude preparations of drug substance from fermentation contain a number of biological molecules derived from the host expression cells, which are present as impurities. These impurities can be present as HCD, among other materials, and they may be copurified with the drug substance during product purification. It is known that certain host-cell–derived biological



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molecules may have toxic activity. Therefore, removal of the host cell materials is desirable to prevent potential adverse effects (2). Complete removal of all host-cell–related impurities is technically demanding and unnecessary for product safety. Regulatory authorities define acceptable levels of by-products including HCD. Several guidance documents detail acceptable levels of host-cell–derived DNA in final biopharmaceutical products. FDA requirements state an upper limit of 100 pg per therapeutic dose (3) or up to 10 ng/dose in the case of some biopharmaceuticals requiring large doses (such as monoclonal antibodies). The World Health Organization (WHO) also has published guidelines (4). A statement on the amount of HCD in a drug product is required from manufacturers to determine whether the residual HCD level is within acceptable limits for a particular product.

Methods for Quantification of Host-Cell DNA

Several methods exist for quantifying levels of host-cell DNA in biopharmaceutical products. Among these are PicoGreen analysis (Molecular Probes, www.invitrogen.com), hybridization techniques, qPCR, and the Threshold assay (Molecular devices, MDS Analytical Technologies, www.moleculardevices.com).

PicoGreen Analysis: The PicoGreen double-stranded (ds) DNA quantitation assay allows measurement of the concentration of dsDNA in a sample using fluorometers or fluorescence microplate readers (**5**). The PicoGreen reagent is an asymmetrical cyanine dye. Free dye is essentially nonfluorescent, but upon binding to dsDNA, the dye exhibits > 1,000-fold fluorescence enhancement (Figure 1). There is a linear relationship between the fluorescence detected on the fluorometer and the concentration of dsDNA in a sample. This assay is not sequence specific and therefore measures all dsDNA in a sample.

Hybridization assays (Figure 2) involve binding of DNA probes to denatured and immobilized host-cell DNA. Probes are labeled with radioactive tags or fluorescent dyes and bind to complementary targets during hybridization. Signal detection is achieved with autoradiography or by phosphor- or fluorescence-imaging systems, and the signal detected is proportional to the amount of DNA immobilized on a filter (**6**). Depending on the probe used, this assay can be either specific or nonsequence specific.

Figure 1: Stages in the PicoGreen process

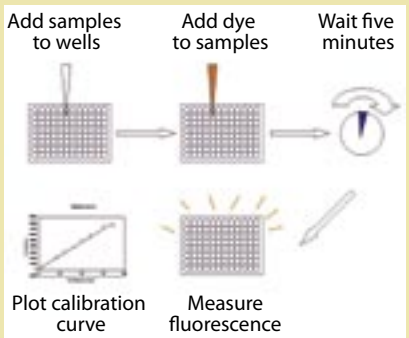
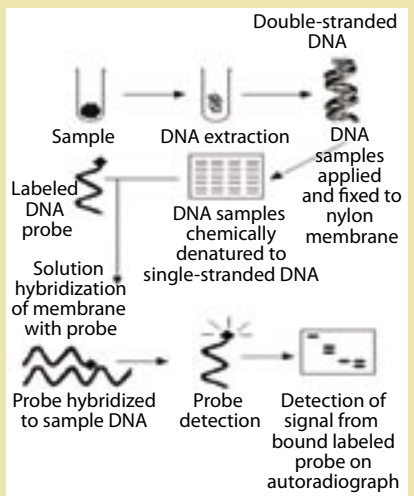


Figure 2: Tthe hybridization process



qPCR or *real-time* PCR (rtPCR, Figure 3) is an extension of the polymerase chain reaction (PCR) and exploits the ability to monitor the progress of PCR as it occurs (in real time) to determine the quantity of target in the reaction. Data are collected throughout the process to monitor the increase in PCR product formation, enabling quantitative determination of the starting amounts of DNA in a sample. A range of different chemistries can be used to detect host-cell DNA when using qPCR, including the commonly used SYBR Green I dye (Molecular Probes) and sequence-specific reporters such as hybridization and 5´-nuclease (TaqMan assay, www2.appliedbiosystems.com) probes (**7**). This assay is sequence specific.

The Threshold Assay: The Threshold total DNA assay (Figure 4) quantitatively measures picograms of single-stranded DNA (ssDNA). This quantification is based on a capture technique whereby a biotinylated single-stranded binding (SSB) protein and an anti-ssDNA antibody conjugated to urease bind simultaneously to the single-stranded DNA present in a sample. The complexes that are formed are then captured on a biotinylated membrane in a filtration step using the strong affinity of streptavidin for biotin. The urease conjugated to the anti-ssDNA antibody is used to detect and quantify the DNA. After filtration, the membrane is placed in a reader containing the substrate urea. The urease hydrolyzes the urea, which results in a pH shift that correlates with the amount of host-cell DNA in the sample (**8**).

Figure 3: Diagram detailing stages of the qPCR process

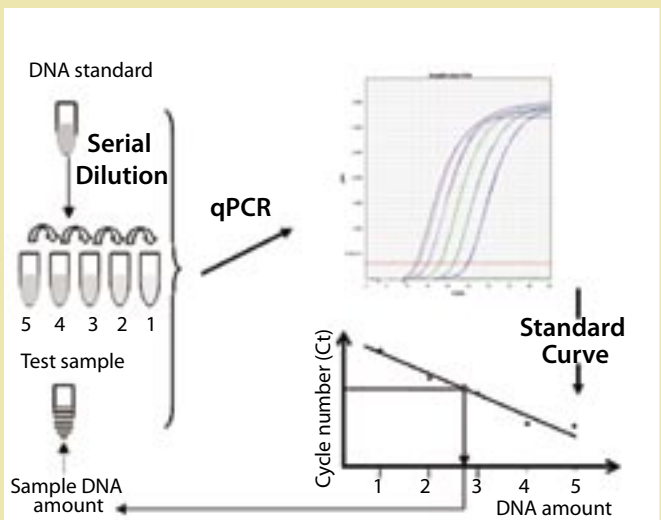
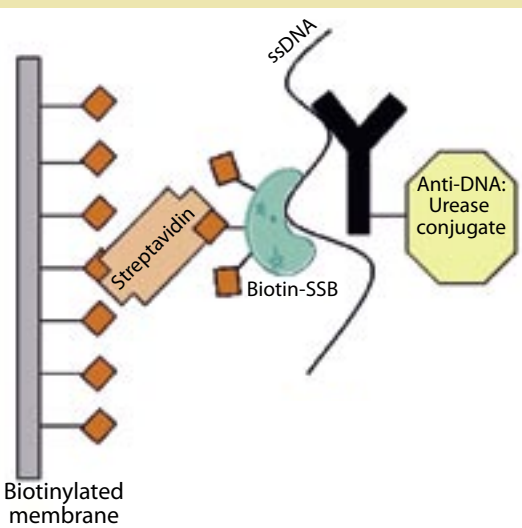


Figure 4: Diagram of the Threshold total DNA assay



Performance Characteristics of HCD Quantification Methods

Performance characteristics are extremely important in influencing a laboratory's decision to set up and implement a particular method. A wide range of factors influences the choice of analytical methodology including cost, throughput, regulatory compliance, and sensitivity. As part of this study, a cross-section of laboratories ranked nine factors in order of importance (Figure 5). Reliability was consistently the most important factor influencing choice of analytical methodology.

An in-depth study of performance characteristics for current HCD quantification methods was carried out as part of this study, and the results are detailed in Table 1. Both advantages and disadvantages exist for each method considered. Hybridization and PicoGreen techniques are inexpensive in terms of set up costs and the cost of running one experiment, which is beneficial to laboratories operating on lower budgets. However, with hybridization methods one experiment is very time consuming, which has an impact on efficiency, labor costs, and labor intensiveness. PicoGreen is inexpensive and user-friendly, but the technique is nonspecific. It detects all dsDNA and so is widely used in industry for general quantification of DNA — and less widely for HCD quantification.

In the current advanced scientific and technological environment, qPCR and the Threshold system are emerging as the accepted industry methods for quantification of HCD. Although some qPCR instruments are expensive to buy, a range of different instruments are available to suit different budgets. On the other hand, the Threshold system is high in cost of capital equipment as well as cost per test, making the Threshold a worthwhile investment only for laboratories that use the instrument routinely. Nevertheless, the Threshold can also be used for host-cell-protein quantification, and this flexibility may be useful in the routine testing environment. qPCR has the highest throughput and (with correct analyst training) is also the quickest in terms of time needed to set up an experiment. In combination with a robot, speed can be increased and labor intensiveness reduced even further.

In terms of dynamic range and limit of detection/sensitivity, qPCR outperforms all methods (Figure 6). qPCR has a wide dynamic range and a very high sensitivity compared with the other methods. Threshold, on the other hand, has a very small dynamic range but is designed to detect very low amounts of target and thus is suited to trace-level HCD analysis. Although the wide dynamic range of qPCR makes it a versatile method for a number of applications that require DNA quantification a significant benefit of the Threshold system is that the sampling volume is much larger than that of qPCR. Threshold sampling requires a 500-µL aliquot whereas qPCR requires only a few µL of sample for analysis. Therefore, Threshold analysis may provide a more precise estimation of the total DNA present in a sample through minimizing the effect of sampling variability on the quantification result.

Figure 5: Graphic representation of the significance of factors influencing choice of analytical methodology for HCD determination

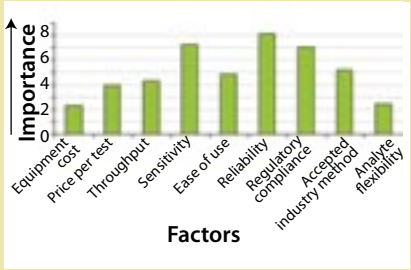


Figure 6: Schematic representation of the dynamic range of DNA quantification methods

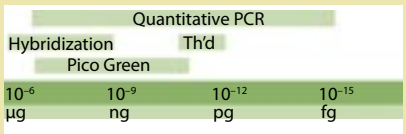
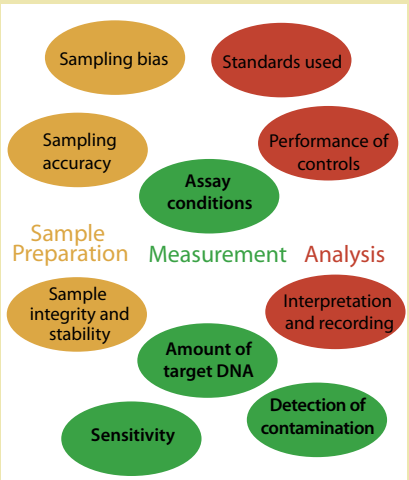


Figure 7: Illustration of the stages of the analytical process where variability may occur



Sources of Variability in HCD Quantification Methods

Measurement uncertainty can be defined as an estimated range of values within which the true value of a measurement lies. The range of values gives an indication of the reliability of a measurement result. The experimental result may then be reported as $x \pm y$, where x is the reported measured value and $\pm y$ is the degree of uncertainty associated with the measurement result (**9**). Sources of variability in analytical processes and methodology give rise to measurement uncertainty. A number of factors may contribute to the overall accuracy of HCD determination. Figure 7 represents schematically some of the stages where variability may occur.

Measurement Uncertainty in Sample Preparation: To place confidence in the results of an analysis, it is advisable to give some consideration to the sources of uncertainty and variability in the method as a whole. In terms of HCD analysis, the early stages of the process are similar for both the qPCR and Threshold methods, and thus we consider the sources of uncertainty during sample preparation for these techniques together (Figure 8).

Influence of DNA Standards: The DNA standard used to anchor these quantitative measurements exerts one of the most significant influences on the analytical result. We questioned several laboratories about the DNA standards routinely used as calibrators for HCD analyses. Several different sources of materials were reported, including DNA stocks prepared and quantified in-house, a standard provided by the Threshold kit

Round-robin studies of this type can provide valuable information on PERFORMANCE characteristics and robustness of methods with the eventual aim of standardizing methods and developing standard operating procedures.

Figure 8: Diagram detailing some potential sources of variability in sample preparation

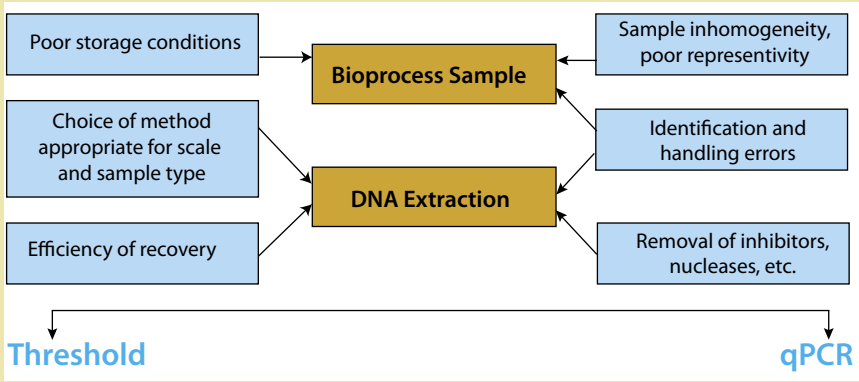
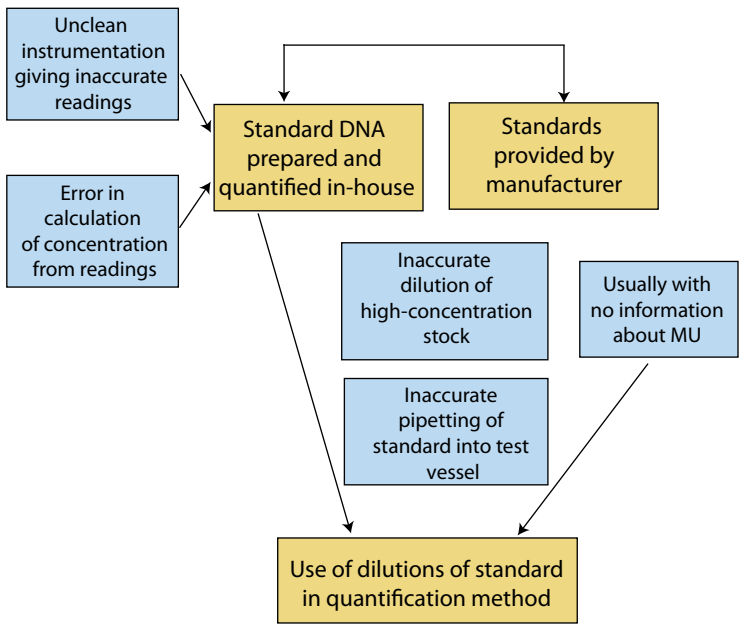


Figure 9: Schematic representation of the possible sources of measurement uncertainty associated with the use of DNA standards



manufacturer, a single-stranded DNA (m13 mp18) from Takara (www.takara-bio.com), and genomic DNA purchased from Sigma-Aldrich (www.sigmaaldrich.com). It is unusual for such DNA preparations to be provided with a stated uncertainty, and thus the accuracy of the value assigned is unclear. The calf thymus standard supplied at 5 ng/µL by the Threshold manufacturer is characterized and quantified using standard ultraviolet (UV) spectrophotometry before being diluted and aliquotted, which is the procedure used to make the majority of in-house standards. The variety of standards used and potential inaccuracies in the measurement process using the samples (Figure 9) may lead to poor interlaboratory consistency of quantitative results.

Measurement Uncertainty in qPCR Analysis: The various stages involved in measuring the amount of DNA in unknown samples using qPCR may all introduce inaccuracies into the final results. The processes are broken down and shown together with some of the potential problems inherent in each process (Figure 10).

Measurement Uncertainty in the Threshold Assay: Similarly, the processes involved in Threshold measurement may all contribute variability to the analytical process, and the various stages are considered in Figure 11.

Evidently, a number of factors can contribute to measurement uncertainty in HCD analysis using both qPCR and the Threshold assay. Generally, test variability for both methods is heavily influenced by the operator, the equipment, equipment calibration, and the test environment in which the process is being carried out. Repeatability comes from comparing results produced on the same sample by the same analyst using the same method and equipment over a short period of time and therefore excludes the influence of these factors. By contrast, reproducibility includes those factors along with estimates made using the same sample measured by different analysts in different laboratories, possibly using different instruments and methods, and possibly over longer periods. Therefore, both repeatability and reproducibility are valuable measurements to take into account when considering method performance (**10**).

Details of the Round-Robin Study

An integral part of this study was to carry out a round-robin assessment of the common HCD quantification approaches in industry. In this particular instance, the type of round-robin study carried out can be best described as a collaborative trial used to assess the performance of a

Table 1: Performance characteristics of the various host cell DNA quantification methods

	qPCR	Threshold	Hybridization	PicoGreen
Cost per test	~ £1–2	~ £10	~ £0.50	~ £0.10
Price of capital equipment	~ £20,000–~ £120,000	~ £5,000	~ £9,000	~ £10,000
Limit of detection	10 fg (bacterial 5 pg (mammalian)	2 pg (total DND assay)	10 ng (14)	0.2 ng
21 CFR Part 11 compliant	Yes	No, but many INDs filed with the FDA	No	No
Throughput (including control of samples)	32 capillaries, 384-well plate	8 wells/stick, up to 4 sticks in one run	Slot blot, typically 72 wells	96–384 well plates
Ease of use	No commercial kits available; complex design and interpretation	All reagents supplied in kit format	No complete reagent kits available	All reagents supplied in kit format
Time/speed	1 hour plate set up; 35 min–2 h for run; 30 min data analysis	3 h total (including data analysis, excluding sample pretreatment	At least 48 h	Up to 1 h
Accepted industry method	Used industry-wide	Over 300 currently in industry use	Less widely used	Used routinely for DNA quanitification and less widely for HCD
Dynamic range	10 fg–1 µg, depending on genome	2–200 pg	10 ng–2,500 ng (14)	0.2–1,000 ng
Analyte specificity	ss and dsDNA	ss/dsDNA or protein	ss and dsDNA	dsDNA
Sequence specificity	Sequence specific	Mainly total DNA, but some sequence-specific assays available	Sequence-specific or total DNA depending on the probe used	Non sequence specific

Table 2: Summary of the details of the analytes provided to round-robin participants

Sample Number	Analyte Type, with Estimated In-House Values
1	0
2	0
3	~165 pg
4	~165 pg
5	~210 pg
6	~210 pg

test method to identify the common sources of uncertainty and the extent of comparability in quantitative DNA measurement (11). In this study, laboratories were sent same sample asked to follow exactly the same method protocol to carry out sample analysis. For the scientific community, round-robin studies of this type can provide valuable information on performance characteristics and robustness of methods with the eventual aim of standardizing methods and developing standard operating procedures.

Analyte Requirements: After initial consultation of end users regarding methods for quantitative HCD, it was clear that the majority of potential participants in the study were using qPCR and Threshold analysis. The samples prepared for the round-robin therefore needed to meet several criteria:

- Concentration relevant to the HCD application
- Concentration appropriate for both analytical methods
- Samples stable for the duration of the study
- Analytes amenable to quantification by both methods.

Because the dynamic ranges of the qPCR and Threshold methods are somewhat different (Figure 6), this limited the concentrations of test samples that could be provided for analysis. Table 1 gives details of the sample concentrations.

Sample Preparation: Samples were prepared at LGC (Teddington). The analytical target was prepared from strain *Escherichia coli* (Migula) Castellani and Chalmers MG1655 (ATCC #3755726, www.atcc.org), which was provided freeze-dried and stored in an airtight vial. The freeze-dried cells were recovered using a DIFCO maximal recovery media and then streaked onto nutrient agar plates. Colonies produced on the plates after incubation were inoculated in LB broth, and cultures of the *E. coli* were propagated. DNA was extracted from the cultures using a commercial QIAmp DNA mini-kit from Qiagen (www.qiagen.com). To ensure that pure high molecular weight DNA had been extracted, purity checks were carried out by running agarose gels. The DNA stocks were quantified using an ND-1000 spectrophotometer from NanoDrop (www.nanodrop.com) and diluted down to the required final analyte concentrations. Each analyte stock was aliquotted into low-retention plastic microcentrifuge tubes and then freeze-dried. The homogeneity and stability of test samples prepared at each concentration level were confirmed by qPCR. Resuspension buffer for the freeze-dried samples and primers and probes for the qPCR assay were also supplied to participants. The qPCR assay used was an *E. coli* 5´-nuclease assay (12).

Participants received a total of six unknown samples as freeze-dried pellets (Table 2). A single aliquot was provided to qPCR participants, and three replicate aliquots were provided to them using the Threshold method. Those participants were requested to resuspend the freeze-dried pellets in the buffer provided and to measure and report the total amount of DNA in the sample. In addition, qPCR participants were provided with a high concentration DNA stock to quantify

in-house and then use as the calibrator for qPCR analysis of the six freeze-dried unknowns.

The samples were randomly assigned to participants using the RAND function within Microsoft Excel. Samples were packaged on dry ice and shipped to participants by courier.

Results

A total of 18 sample sets were distributed to participants, and 12 result sets were returned. Of those, three sets were from Threshold analysis, and the remaining nine were from qPCR analysis.

Quantification of Unknowns: Figure 12 represents the consensus data taken from all nine sets of qPCR results. Both the median and mean values are very close to those expected from initial in-house estimation of test materials. These results are promising, considering the range of instruments and reagents used.

The raw results of individual participants were assessed to derive measures of repeatability and reproducibility for each analyte, including the relative standard deviation under both repeatability and reproducibility conditions (RSDr and RSDR, respectively). The relative standard deviations are presented graphically in Figure 13.

As expected, the repeatability within a laboratory was much tighter than the comparability of results between laboratories. The agreement between the qPCR results overall was closer than expected, especially given the expected sampling variability (most laboratories analyzed between 5 and 10 µL of the very low concentration sample).

Unfortunately the number of participants returning data was small for the Threshold method. In addition, the results of the Threshold analysis did not permit an effective comparison of the performance of the two methods. From the results it would seem that clearly something has affected analysis of the samples by the Threshold method, possibly acid hydrolysis caused by heating the samples in the presence of Tris in the resuspension buffer.

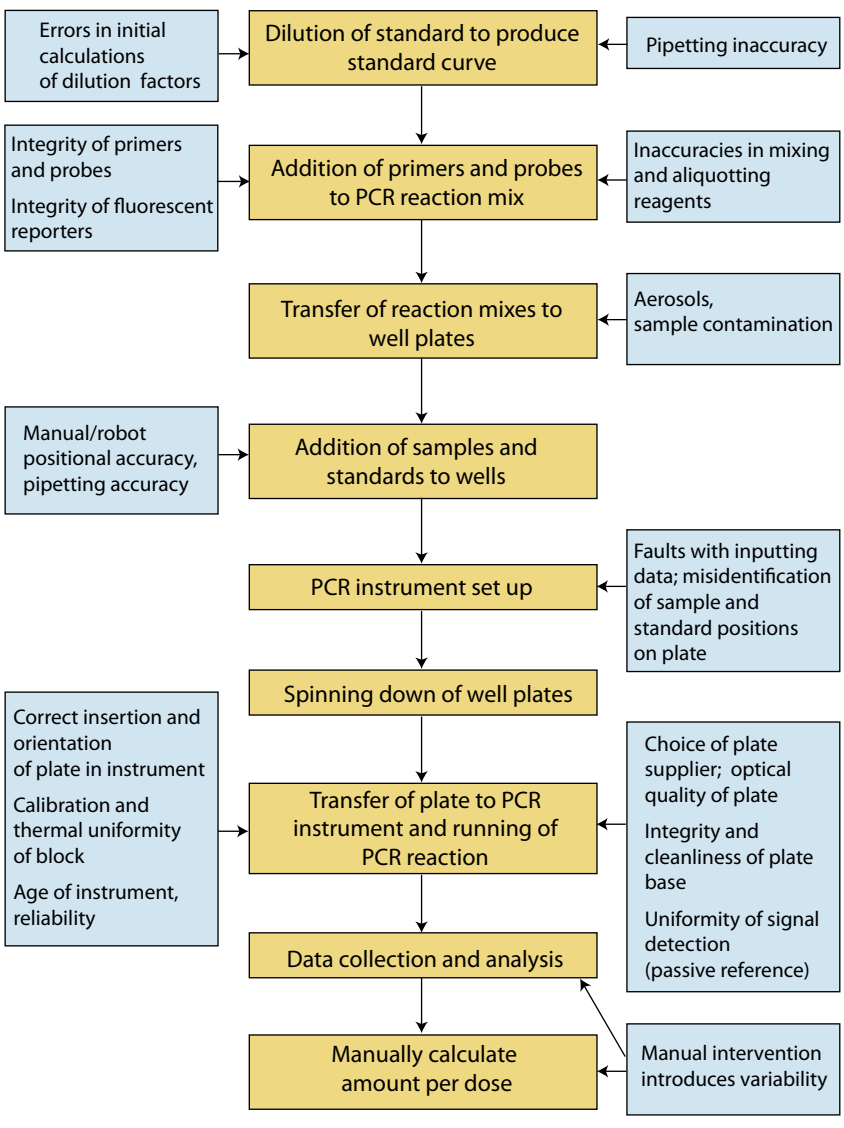
In summary, there was only one sample type, and there were only a small number of laboratories carrying out analysis of the Threshold samples. Therefore, the results are not representative of the in-house method performance on routine samples.

Standard DNA Comparability: To assess the potential comparability between methods, the relative amount of DNA was measured in a range of commonly used standards in-house at LGC. In the absence of reference materials for quantitative analysis, the results are intended to demonstrate comparative amounts of DNA in each sample rather than an accurate determination of each standard tested. Those standards are shown in Table 3 together with the measured values, and the results of the analysis are shown graphically in Figure 14. The concentration of standards was determined using PicoGreen analysis as detailed in English et al. (**13**), with an independent batch of Cambio standard DNA used as the calibrator. Values of the high-concentration standards were normalized against the Threshold high-calibrator concentration by using the ratio of the expected standard concentration to the concentration of the Threshold standard, enabling the results to be compared more easily.

As can be seen from the graph representing the relative amounts of DNA in each sample, the values are all similar. Although some samples are from the same batch, each is an independent vial of material.

Samples 4–8 were past the batch expiry date, and it should also be

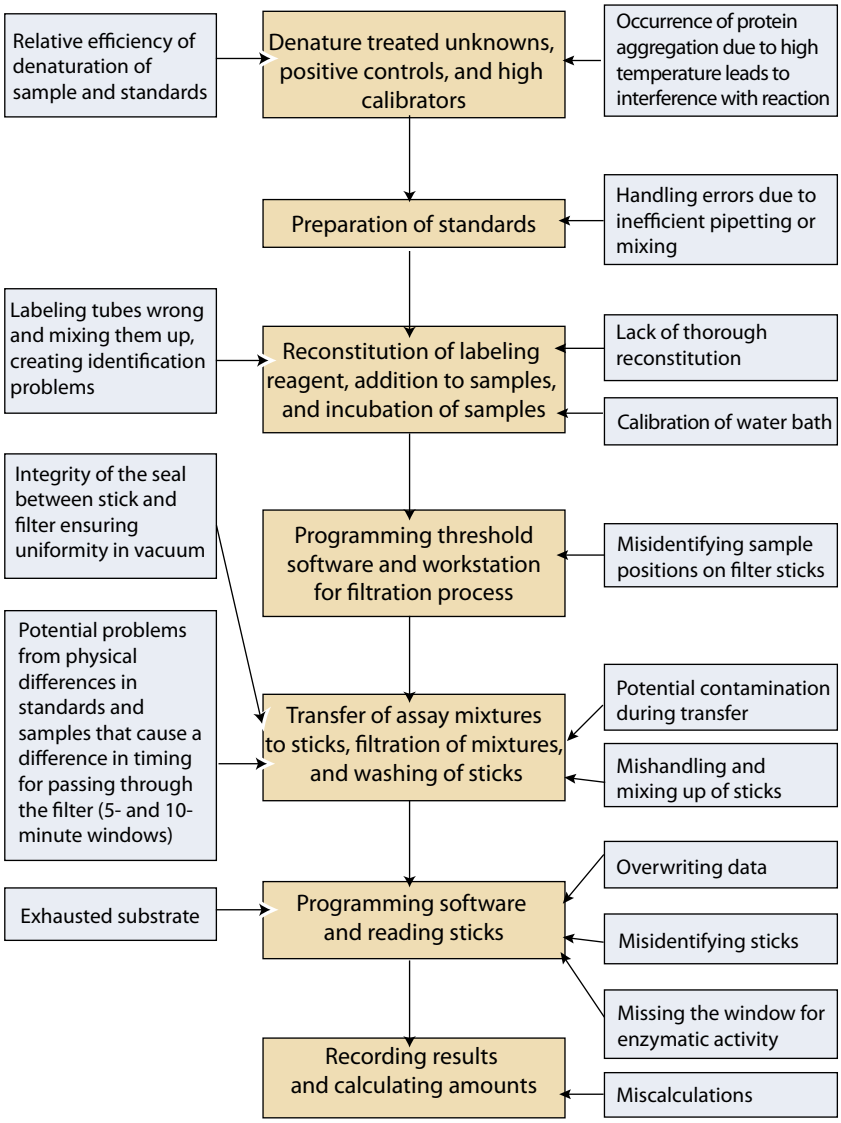
Figure 10: Breakdown of stages involved in qPCR analysis, together with potential sources of measurement variability



noted that the concentration of samples 4–11 was measured at the limit of detection of the PicoGreen assay, reflected in the larger standard deviation for the Threshold standard results. It is important to note that an independent batch of the Cambio standard was used to anchor the quantification. Thus, the relative amounts of DNA in the standards are the feature of interest, because no one standard can be deemed to be the "true value." Hence, a difference from the "expected concentration" of 0.005-ng/µL does not indicate bias in the other standards analyzed. Despite these caveats, the results demonstrate the broad comparability of standards from a range of sources.

In summary, results from the round-robin indicated that the overall mean and median values for each sample were surprisingly close to the expected results, where qPCR was concerned. However, the results of the Threshold analysis did not permit an effective comparison of the performance of the two methods. Nevertheless, the repeatability and reproducibility of the qPCR results were assessed, and as expected, the within-laboratory repeatability was much tighter than the comparability of results between laboratories. The overall agreement between the qPCR results was closer than anticipated, particularly given the expected sampling variability of the method.

Figure 11: Diagram showing breakdown of the Threshold method to the component stages, together with potential sources of analytical uncertainty



Ensuring Confidence

This investigation highlights the importance of interlaboratory comparability studies for ensuring confidence in results. Information provided by studies such as these allow end users to make informed decisions about the choice of analytical methodology to best suit their needs. It is apparent that the current absence of certified reference materials for DNA quantification precludes full metrological traceability of the HCD techniques. Development of relevant certified reference materials (CRMs) would

Figure 12: Graphical representation of the summary qPCR results, showing the mean and median values for each analyte, together with the associated variability; as ± SD (means) and MAD (medians). The expected values from in-house estimation are also shown.

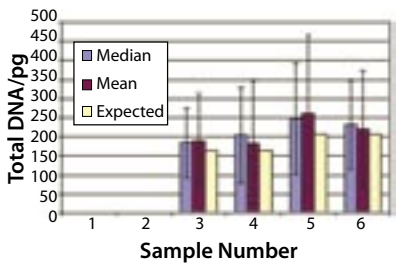


Figure 13: qPCR results displayed as overall RSDr and RSDR for each analyte

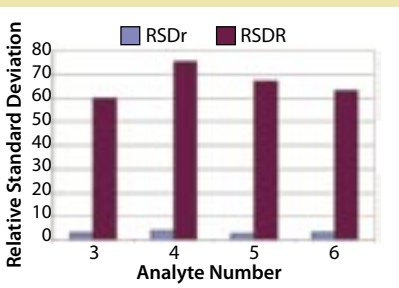


Figure 14: Relative amount of DNA in different aliquots of commonly used DNA standards, shown with error bars of ±1 SD (see Table 3)

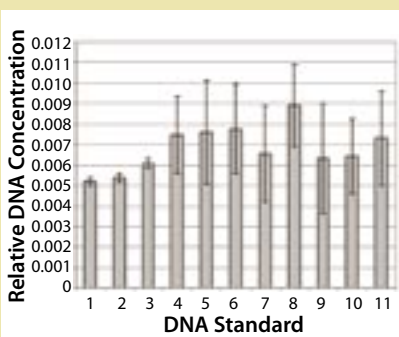


Table 3: Measured concentrations and details of a number of batches of two commonly used DNA standards (see Figure 14)

Standard ID	Details	Expected concentration in µg/mL	Actual concentration	Concentration normalized to 5 ng/µL	Standard Deviation
1	Cambio DNA	1	1.0402	0.0052	0.0002
2	Cambio DNA	1	1.0732	0.0054	0.0002
3	Cambio DNA	1	1.2170	0.0061	0.0002
4	Threshold HC	0.005	0.0075	0.0075	0.0019
5	Threshold HC	0.005	0.0076	0.0076	0.0025
6	Threshold HC	0.005	0.0078	0.0078	0.0022
7	Threshold HC	0.005	0.0065	0.0065	0.0023
8	Threshold HC	0.005	0.0089	0.0089	0.0020
9	Threshold HC	0.005	0.0063	0.0063	0.0027
10	Threshold HC	0.005	0.0064	0.0064	0.0018
11	Threshold HC	0.005	0.0073	0.0073	0.0023

enable evaluation of the range of HCD methods and provide valuable resources to facilitate in-house method validation and quality management processes. Provision of universally applicable standards would also potentially increase the comparability of measurements between laboratories.

Acknowledgments

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