# Validation report for Horserace Betting Levy Board (HBLB) of *Taylorella equigenitalis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae kits*

Validation Report
Issue 1

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## 1. INTRODUCTION

*Taylorella equigenitalis* (*T. equigenitalis*) is the causative agent of the communicable venereal disease contagious equine metritis (CEM). In stallions, there are no clinical symptoms of the infection, yet the bacteria can be present in penile/preputial smegma and as such may be transmitted through natural mating or artificial insemination; a principal source of infection as a stallion will mate with numerous mares and infection can last for months and even years in some cases. In contrast, clinical symptoms in mares varies from nothing to vaginal discharge associated with endometritis, cervicitis, vaginitis and even abortion of unborn offspring. A vaccination against CEM/*T. equigenitalis* is not currently available.

The CEM organism (CEMO) can spread rapidly leading to infection of epidemic proportions, therefore it is of vital importance to employ a quick, accurate testing method to help prevent occurrence and spread of CEM. Traditional microbiological methods can be employed to determine the presence of the CEMO however *T. equigenitalis* is a slow growing, microaerophilic bacteria which generally takes 3-6 day to grow; negative reports should only be certified after 7 days of incubation with no growth. In addition, traditional microbiology can only detect viable bacteria, yet *T. equigenitalis* may have died during transit to the laboratory or exposure to sunlight or inappropriate temperatures thus giving way to the possibility of generating false negative results.

There is a need within the equine industry from both a legislative and duty of care stand point to detect certain pathogenic species quickly and accurately. Traditional culture methods are slow, time consuming and often non-specific. Consequently, these shortfalls have been overcome by utilising real-time quantitative Polymerase Chain Reaction (qPCR). Until recently only two qPCR assays have been developed and approved for use by the Horserace Betting Levy Board (HBLB): one which identifies *T. equigenitalis* DNA only and the other identifying DNA from *T. equigenitalis* along with the other microorganisms *Klebsiella pneumonia* (*K. pneumonia*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) designed as a multiplex. Both of these kits have been developed by Qiagen and are being marketed as 'cador *T. equigenitalis* PCR kit' and 'cador TKP PCR' respectively.

Although the cador reagents have been utilised within the industry its use is limited by the closed nature of the assay, this being that it can principally be used only on the Rotor-Gene Q also from Qiagen. In addition to this the cador kits had been removed from the market in 2014, the cause for this is unknown, however they have more recently been re-released.

During the period where no kit was available, the HBLB looked to other sources for this assay and as such Primerdesign Ltd already having kits for K. pneumoniae, and P.

aeruginosa began developing a kit for *T. equigenitalis*; all detection kits from Primerdesign having a guarantee of detecting down to 100 copies.

In the UK, isolation of *K. pneumoniae* and/or *P. aeruginosa* is not notifiable by law. However, if infection occurs in stallions, it is advisable for the owner, or a person authorised to act on their behalf, to inform the relevant breeders' association. As such, owners and veterinary staff often chose to test the horse for these microorganisms alongside *T. equigenitalis* detection.

1. *T. equigenitalis* occurs widely in the non-thoroughbred population, and to a limited extent in thoroughbreds, in mainland Europe.
2. *Klebsiella pneumoniae* (*K. pneumoniae*). There are many capsule types of *K. pneumoniae*, most of which do not cause venereal disease. However, types 1, 2 and 5 may be sexually transmitted. Therefore, when *K. pneumoniae* is identified from breeding stock, tests to determine the capsule type(s) present must be undertaken.
3. *Pseudomonas aeruginosa* (*P. aeruginosa*). Not all strains of *P. aeruginosa* cause venereal disease but there is no reliable method to differentiate between the strains. Therefore, all isolates should be considered as potential venereal pathogens.

## 2. OBJECTIVES

To validate the *T. equigenitalis*, *K. pneumoniae* and *P. aeruginosa* genesig® detection kits and show:

- Reproducibility – evidence that the testing methods used will produce the same results no matter who is using the equipment as long as they follow the same protocol.
- Repeatability – test-retest reliability is the variation in measurements taken by a single person or instrument on the same sample under the same conditions.
- Sensitivity – the probability of correctly identifying the presence of an organism with corresponding culture data.
- Specificity – the probability of correctly identifying the absence of an organism with corresponding culture data.
- Consistency – evidence that the testing methods will produce the same results as the current Qiagen solution.

## 3. MATERIALS, REAGENTS AND EQUIPMENT

Primerdesign Ltd is regularly audited by the British Standards Institute to ensure that high quality standards are being met in all areas of the business. This includes Primerdesign's design and manufacturing process, the quality control systems, customer care, administration and management systems. Primerdesign has achieved ISO 13485:2003 and EN ISO 13485:2012 accreditation in addition to our ISO 9001:2008 accreditation.

### 3.1 Materials

Materials were purchased from approved suppliers in accordance with the internal quality procedures (QP09), where in brief, suppliers must be approved to supply the item/material or component. Upon receipt of the following items, all were checked for obvious defects and only accepted when it was clear no defects were apparent. Items were passed on to the responsible department and stored as detailed by the supplier, until required for use. Details of materials used, lot numbers, supplier information and expiry dates can be found in the Validation Plan – HBLB Issue 03, Section3.1

### 3.2 Reagents

Details of reagents used, lot numbers, supplier information and expiry dates can be found in the Validation Plan – HBLB Issue 03, section3.2

### 3.3 Equipment

In accordance with internal quality procedure QP16 Control of Monitoring & Measuring Equipment, all equipment is monitored and measured to ensure it is fit for purpose and maintained to ensure conformity of products. As such qPCR equipment is calibrated as per the manufacturer's instruction; for BioRad CFX machines calibration is internally controlled and requires no manual input; Qiagen recommend all RotorGene equipment is inspected bi-annually; all PD pipettes are calibrated annually, but rotated on a bi-annual basis. Calibration details can be found in the Validation Plan – HBLB Issue 03, Section3.3.

## 4 RESULTS

## 4.1 Assay Development of Primerdesign T. equigenitalis, P. aeruginosa, K. pneumoniae Detection kits

### 4.1.1 Assay Design

The purpose of this experiment was to ensure all the components of the *T. equigenitalis*, *P. aeruginosa* and *K. pneumoniae* kits work with ~100% efficiency and are specific for their target with no mis-priming.

Assay design (encompassing the design of specific primers: forward and reverse, and a specific double-dye hydrolysis probe; specificity conferred at all 3 oligonucleotide annealing sites) was performed by the Primerdesign expert design team, using Beacon Designer software, which encodes a Primerdesign proprietary algorithm. Basic design criteria for forward and reverse primers as well as the double-dye hydrolysis probe can be found in Table 1.

| Primer Design Parameters | Details |
|---|---|
| Primer length | 19 - 27bp |
| Tm | 56.75 ± 2°C |
| Amplicon length | 80 – 130bp |
| Hairpin maximum ΔG | -1kcal/mol |
| Self-dimer maximum | -3kcal/mol |
| Run/Repeat maximum length | 5bp |
| Multiplexing maximum | -6kcal/mol |
| Maximum bases between primer and probe | 40 |
| 3' End maximum ΔG | -5kcal/mol |
| Maximum primer pair Tm mismatch | 2°C |
| Cross dimer maximum ΔG | -3kcal/mol |
| **Probe Design Parameters** | **Details** |
| Probe Length | 20 – 30bp |
| Tm | 10 ± 3°C |

**Table 1. Design criteria for new primers (forward and reverse) and double-dye hydrolysis probes.**

The Beacon Designer software ranks "best assays" based upon the number of design criteria met by each assay; assays meeting the most criteria being ranked highest. The top ranking

assay was assessed to ensure that it contained a random distribution of bases (A, T, G, C), with all bases present. Assays with any repeat patterns and/or non-complex sequences were discarded. Resulting amplicons were no more than 3.5kb from the 3' end of the target of interest.

Secondary structure analyses were then performed and only assays with 75% of the forward and reverse primer structure free from secondary structure were passed. Primers (forward and reverse) were then synthesised by Eurogentec using RP-cartridge-GOLD purification and delivered to Primerdesign Ltd in wet form at a concentration of 100pmol/µl.

Probes were synthesised and HPLC-purified by IDT with a FAM fluorophore (for target of interest) or HEX fluorophore (for internal extraction control) and ZEN quencher. These were delivered to Primerdesign Ltd in lyophilised form and resuspended in 1 ml RNase/DNase free water. The concentration was calculated using the data supplied on the accompanying datasheet.

Primers (forward and reverse) and probe for each pathogen were pre-mixed and dried according to Primerdesign's standard SOPs. When resuspended appropriately with RNase/DNase free water a final concentration of 3 pMoles per reaction for each of the primers and the probe was achieved.

### 4.1.2 QC Testing for the Pathogen Kit: Wet-testing of Primers

Each genesig® kit was quality control tested by way of running real-time PCR experiments as detailed in the Validation Plan. The primers (both forward and reverse, without probe) were tested to ensure that they amplified the corresponding synthetic template with a Cq value of 17.5 ± 2. At least two of the three technical replicates were required to show amplification and each replicate was required to be within 0.5Cq values of each other. Thereafter melt curve analyses were carried out to ensure that the primer pairs were specific for the amplicon of interest and did not amplify non-specific products. A further criterion was that the Tm was required to be >5°C compared to the theoretical Tm. No template controls (NTC, where the synthetic template was replaced with RNase/DNase free water) were required to show no amplification.

The results of the QC testing of the primer pairs (with no probe included) show that all criteria were met, as detailed in Table 2 below. All primer pairs amplified a product in all technical replicates, with the average Cq value of 17.2, 16.5 and 16.5 for *T. equigenitalis*, *P. aeruginosa* and *K. pneumoniae* respectively (Figure 1), thus meeting the Cq criteria of 17.5 ± 2. All NTC reactions for all pathogen detection kits gave either no amplification, or of those that did show amplification, all were after 40 cycles and showing only non-specific product formation.

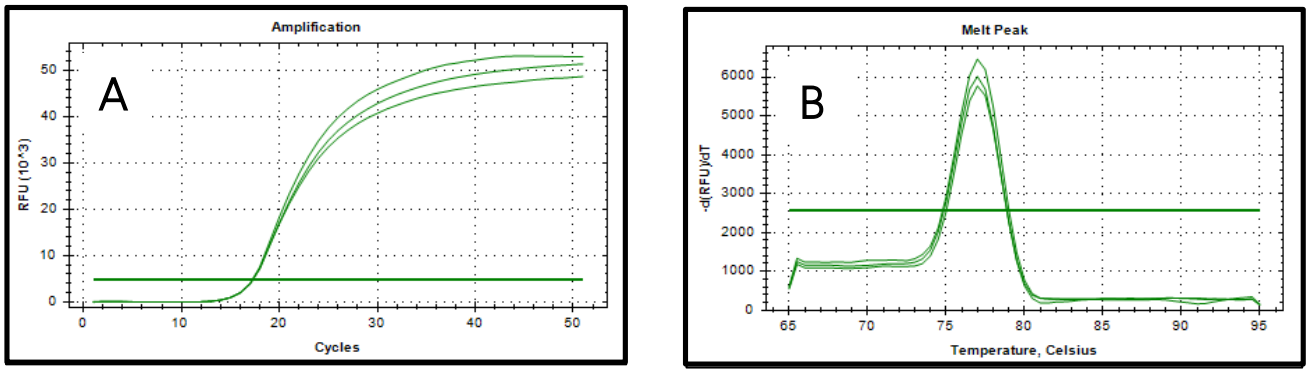
These were therefore considered insignificant (Bustin et al, 2009). All melt curves showed a real melting temperature (Tm) for the corresponding amplicon of >5°C than the theoretical Tm, see Figure 1.

Data generated from this section of results shows that the primer sets for *T. equigenitalis*, *P. aeruginosa* and *K. pneumoniae* are fit for purpose, specific for the appropriate synthetic template and pass this section of quality control.

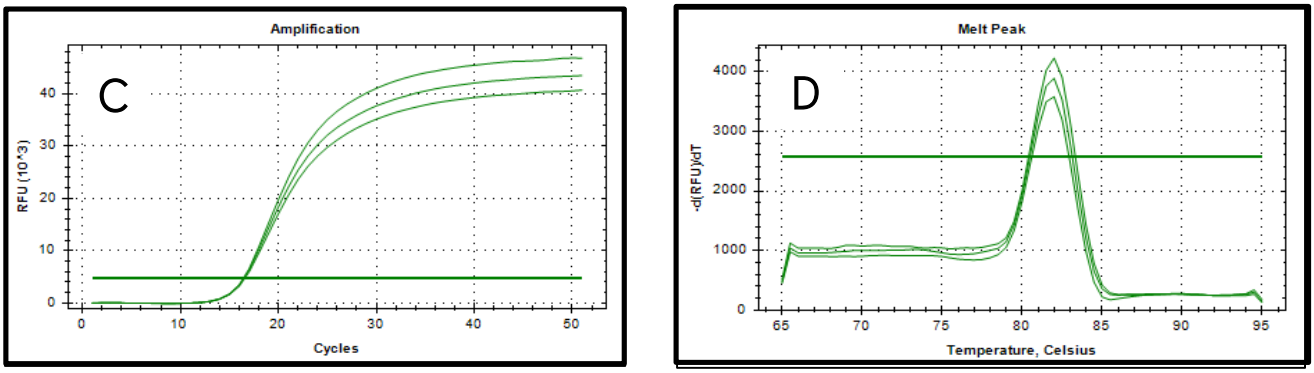
| Detection Kit | Technical Replicate Cq values | Average Cq values | NTC Technical Replicate Cq values | Theoretical Tm | Actual Tm | PASS? |
|---|---|---|---|---|---|---|
| *T. equigenitalis* | 17.3 | 17.2 | 41.7 | 68.2°C | 77°C | YES |
| | 17.2 | | 41.0 | | | |
| | 17.2 | | 44.5 | | | |
| *P. aeruginosa* | 16.4 | 16.5 | 47.0 | 75.1°C | 82°C | YES |
| | 16.4 | | N/A | | | |
| | 16.5 | | 49.4 | | | |
| *K. pneumoniae* | 16.5 | 16.5 | N/A | 74.3°C | 81°C | YES |
| | 16.4 | | 50.4 | | | |
| | 16.4 | | 47.7 | | | |

Table 2. Results of Sybr green/primer only analysis of *T. equigenitalis*, *P. aeruginosa* and *K. pneumoniae* pathogen detection kits.

*T. equigenitalis*




*P. aeruginosa*




*K. pneumoniae*

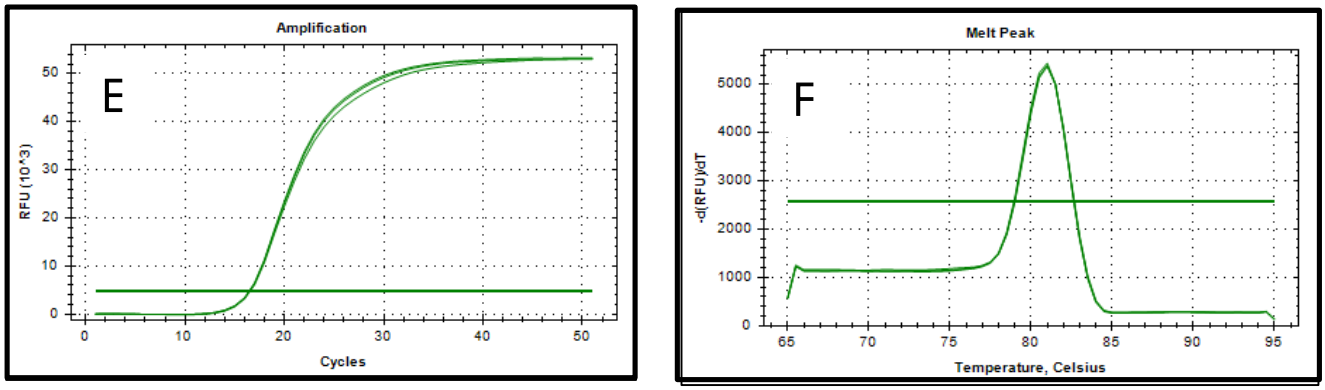



**Figure 1. Data generated by the assays using Sybr green/primer only analysis** of: A) *T. equigenitalis* amplification plots showing all three technical replicates; B) Melt curve analysis of PCR product amplified by *T. equigenitalis* specific primers; C) *P. aeruginosa* amplification plots showing all three technical replicates; D) Melt curve analysis of PCR product amplified by *P. aeruginosa* specific primers; E) *K. pneumoniae* amplification plots showing all three technical replicates; F) Melt curve analysis of PCR product amplified by *K. pneumoniae* specific primers.

### 4.1.3 QC Testing for the Pathogen Kit: Wet testing of Primers with Probe

Having established the specificity of the primers alone, the probe for each assay was then premixed with the forward and reverse primers for the appropriate microorganism and PCR was performed. This was to ensure that the addition of the probe did not adversely affect the functionality of the assay.

It is typical to see a shift in Cq value of 1-2 Cqs on the addition of a probe to a primer only assay. However, the results seen should still be within the predicted value of 17.5 ± 2 Cq when using the appropriate synthetic template and conditions as described in the Validation Plan.

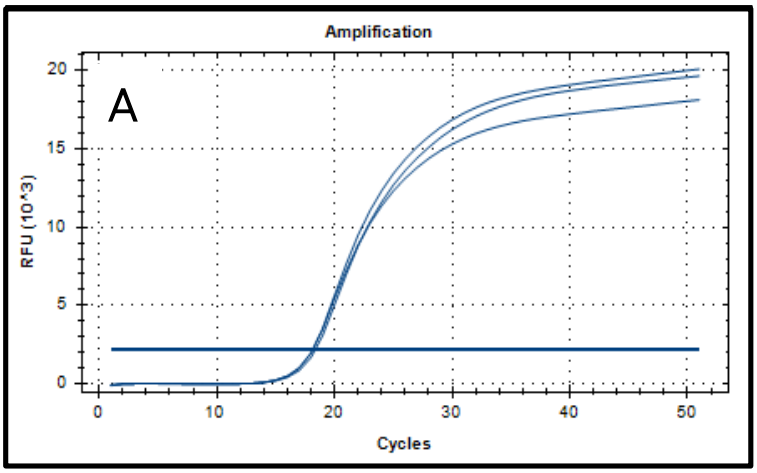
Three technical replicates were performed and data was only accepted and analysed if the Cq values of the technical replicates, for each pathogen, were within 0.5 of each other; with amplification in at least 2 out of the 3 replicates. Table 3 shows the results gained for probe analysis of each pathogen kit and the plots can be seen in Figure 2 below.

The results of this set of analysis show that all technical replicates for each microorganism detection assay amplified and, as predicted, the average Cq value was within the required range of 17.5 ± 2, being 19.2, 18.2 and 18.8 for *T. equigenitalis*, *P. aeruginosa* and *K. pneumoniae* respectively. No NTC control reaction produced any amplification. Consequently, the pathogen detection kits for *T. equigenitalis*, *P. aeruginosa* and *K. pneumoniae* have been shown to contain components that all work on synthetic template as per the internal Primerdesign SOPs and the Validation Plan.

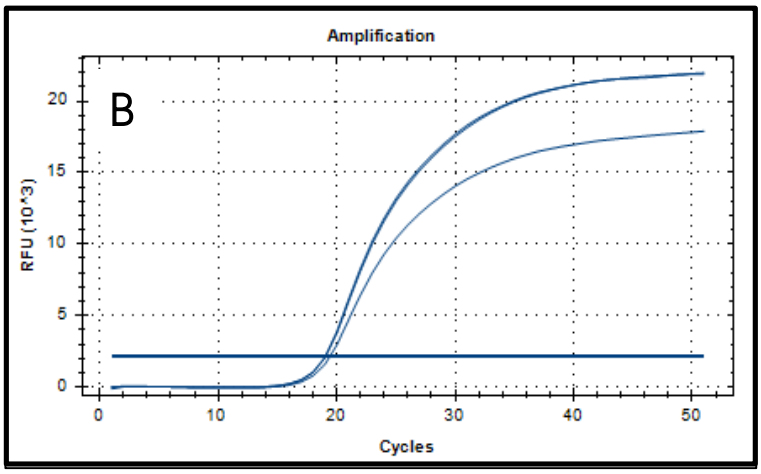
| Detection Kit | Technical Replicate Cq values | Average Cq values | NTC Technical Replicate Cq values | PASS? |
|---|---|---|---|---|
| *T. equigenitalis* | 19.1 | 19.2 | N/A | YES |
| | 19.4 | | N/A | |
| | 19.1 | | N/A | |
| *P. aeruginosa* | 18.1 | 18.2 | N/A | YES |
| | 18.1 | | N/A | |
| | 18.4 | | N/A | |
| *K. pneumoniae* | 18.8 | 18.8 | N/A | YES |
| | 18.7 | | N/A | |
| | 19.0 | | N/A | |

Table 3. Results of primers and probe analysis of *T. equigenitalis*, *P. aeruginosa* and *K. pneumoniae* pathogen detection kits.

*T. equigenitalis*




*P. aeruginosa*




*K. pneumoniae*

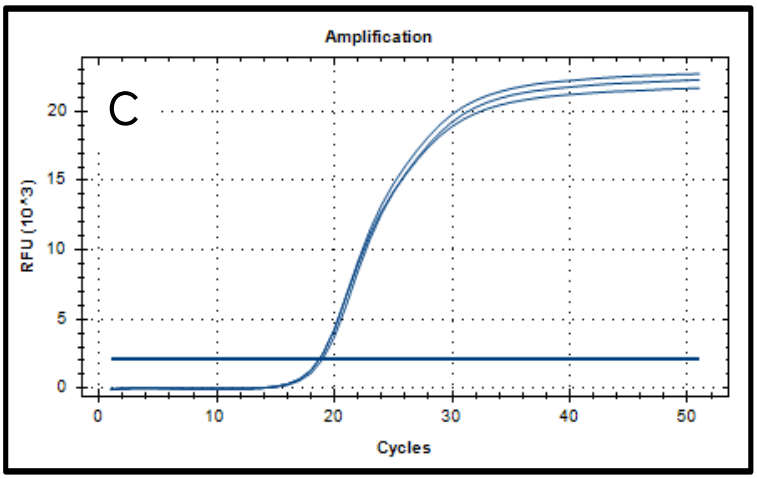



**Figure 2. Initial testing of the primer/probe assays of newly developed kits.** Amplification plots from primer and probe assay showing all three technical replicates produced good amplification for A) *T. equigenitalis*. B) *P. aeruginosa* and C) *K. pneumoniae*

## 4.2 Primerdesign Assay Sensitivity Testing

The purpose of this set of experiments was to ascertain the PCR efficiency of each of the microorganism detection kits on synthetic template. PCR efficiency is a measure of the performance of the assays, where an efficiency percentage of 100% demonstrates that the primers are priming optimally at each PCR cycle, thus achieving the expected logarithmic increase in amplification. When conducted across a broad dynamic range using a standard curve, an optimal PCR efficiency demonstrates that the PCR assay is functioning equally well in the presence of both high and low copy numbers of target DNA. The standard curve method also allows for verification that assay sensitivity remains within acceptable limits. In addition to the PCR efficiency calculations, the limits of detection (LOD) were assessed for each assay using synthetic DNA template.

### 4.2.1. Copy Number Standard Curves

In order to ascertain the efficiency of each of the assays, a six-point standard curve of 10-fold serial dilutions of synthetic template was prepared as described in the Validation Plan (Table 4) and subsequent PCR was performed.

| Standard Curve | Copy Number per µl | Copy Number per Reaction |
|---|---|---|
| Dilution Point 1 | $2 \times 10^5$ | 1,000,000 |
| Dilution Point 2 | $2 \times 10^4$ | 100,000 |
| Dilution Point 3 | $2 \times 10^3$ | 10,000 |
| Dilution Point 4 | $2 \times 10^2$ | 1,000 |
| Dilution Point 5 | 20 | 100 |
| Dilution Point 6 | 2 | 10 |

**Table 4: Description of the standard curve performed for each of the three assays with regard to the template copy number pertaining to each dilution point.**

Amplification of specific product (i.e. giving a Cq value <40; Bustin et al, 2009) was seen in all dilutions of *T. equigenitalis* synthetic template, for all technical replicates. However, according to the inclusion criteria whereby all technical replicates should be within 0.5 Cq values of each other to be included in the analysis, dilution 6 (2 copies per µl/ 10 copies per reaction) technical replicates fall outside the inclusion criteria with Cq values of 36.4, 35.9 and 35.2 with a spread of 1.2Cq's. As a consequence, the Grubb's test was employed to determine if the outlier (Cq of 35.2) could be excluded from analysis. Accordingly, the data shown in Table 5 was assessed and the calculated G score for the data generated from the technical replicates for dilution 6 was 1.05. According to the Grubbs' Critical Value Table, the G score is below the corresponding critical value of N=3 and therefore this data point cannot be excluded from the analysis. Consequently, the biological data set (all three technical replicates for dilution 6) should be excluded from analysis in the standard curve.

| | |
|---|---|
| n=1 Cq value | 36.4 |
| n=2 Cq value | 35.9 |
| n=3 Cq value | 35.2 |
| Average Cq value | 35.8 |
| Standard Deviation | 0.6 |
| G Score ((Mean Cq – Low outlier)/Stdev) | 1.05 |
| Exclude Outlier? | No |
| Exclude biological replicate? | Yes |

**Table 5. *T. equigenitalis* dilution 6 technical replicate data.** When applying the Grubbs' test to assess whether potential outliers can be removed in a controlled fashion, data shows this is not possible and all technical replicates from this biological group must be excluded from analysis.

The remaining 5 dilutions can be used to construct a standard curve (Fig 3A). Figure 3A shows amplification plots for the *T. equigenitalis* assay using dilution 1 – 5; 1,000,000 copies per reaction to 100 copies per reaction. This is in agreement with the guarantee that all Primerdesign genesig® detection kits can detect down to 100 copies of target template under optimal PCR conditions. The efficiency of the reaction, as calculated from the standard curve data, was calculated to be near perfect at 100.6% with a $R^2$ value 0.999. The $R^2$ value shows that there is excellent linearity of the standard curve, thus excellent performance at high and low copy number, as well as reproducibility between technical replicates. In addition, the standard curve allowed determination of the quantitative range for the assay which is considered as 1000000 to 100 copies per reaction. The final dilution within this range equates to a Cq of 32 and thus data beyond Cq 32.3 is not considered quantitative. This data can however give rise to a qualitative positive or negative result as detailed in the Validation Plan.

Whilst the technical replicates for the final dilution (10 copies/reaction) of the *T. equigenitalis* standard curve fell outside the inclusion criteria for quantitative analysis, and therefore the quantitative range for *T. equigenitalis*, positive amplification from all three replicates was observed (Fig 4A). In addition, a further dilution was performed equating to 1 copy per reaction (Fig 4B). For the one copy analysis, of the three technical replicates, only one replicate amplified the specific amplicon with a Cq value of 36.6. This is considered as a negative result as 2 replicates failed to amplify. Together this data confirms sensitivity of the assay of 10 copies and. that the sensitivity of the assay extends beyond the quantitative range. This routine observation gave rise to the two tiered approach to positive calling, the quantitative range and the qualitative range.

The increased spread of Cqs between technical replicates in the presence of low template copy numbers is a very common phenomenon and entirely expected. Under these

circumstances the reactions are individually influenced by stochastic effects. These are random factors that can influence the instigation and initial progression of the PCR when template copy numbers are limited, leading to disparities in the resulting replicate Cqs. Such factors include, for example, the proximity of the primers to the template and consequently the number of cycles undergone prior to the PCR becoming established; minor variations in reagent/component concentrations that are inconsequential at higher copy number or genuine copy number variability between replicates due to the paucity of template in the stock sample. The latter example may be one explanation as to why 2 of 3 replicates failed to amplify with the 1 copy dilution. The ability to reliably add 1 copy per reaction is somewhat limited, however, this data set alone cannot distinguish whether the result is due to template variability or the limits of detection for the assay being between 10 and 1 copies (LOD data in section 4.2.3).

Amplification with Cq values of <40 was seen in all dilutions of *P. aeruginosa* synthetic template. In this instance all technical replicates amplified at each dilution point, including point 6, and all technical replicates amplified the specific product within 0.5Cq values (Figure 3B). As such a standard curve could be constructed from all data points which gave a calculated PCR efficiency of 96.5% with an $R^2$ value of 0.999 (Figure 3B). The quantitative range was thus defined as 1000000 to 100 copies, where 100 copies equates to a Cq of 32.2. In spite of the quality of the 10 copy data for this assay, the quantitative range was not further extended to the 10 copy level. The reason for this decision is that the Cq for 10 copy detection is beyond 35 which is considered within the industry as the limits of reproducible detection. That is, when template copy numbers are low enough as to give a Cq beyond 35 the reaction is far more vulnerable to stochastic effects, as described above for *T. equigenitalis*. As a result, it was expected that, particularly with the added complexity of biological samples, the technical replicates would be liable not to fall within the 0.5 Cq range specified within the Validation Plan when detecting at low abundance level. This would result in the loss of potentially adequate data that otherwise might fall within the qualitative range of the kit as defined by the LOD study. However, to further test the sensitivity of the assay, prior to the LOD study, a further dilution was tested with the addition of 1 copy per reaction (Figure 4C). All replicates amplified in this range showing exquisite sensitivity of the assay. As expected, the plots did not fall within 0.5 Cqs but this is acceptable when the Cq is later than 32.2.

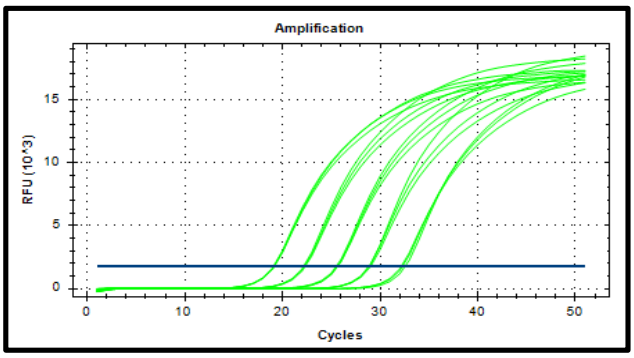
In the case of *K. pneumoniae*, again amplification was observed for dilution 1 (1000000 copies per reaction) up to and including dilution 5 (100 copies per reaction) with Cq values of <40 and with all technical replicates amplifying the specific product within 0.5Cq values (Figure 3C). The resulting standard curve gave a calculated PCR efficiency of 92.8% with a $R^2$ value of 0.988. (Figure 3C). The quantitative range was defined as between 1000000 and 100

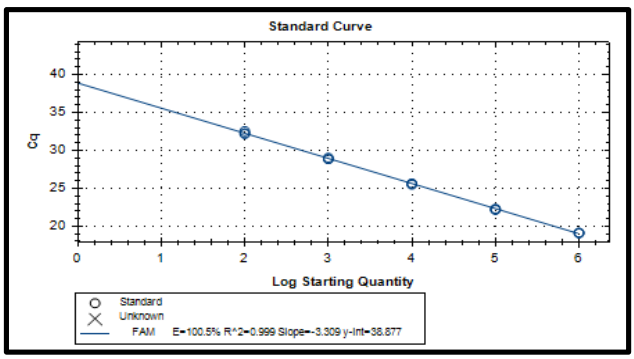
copies, where the end of the quantitative range equates to a Cq of 32.5. As no technical replicates of dilution 6 gave Cqs within 0.5 Cqs of another replicate the Grubb's test was not applied. As with the other assays, 10 and 1 copy detection were analysed (Figure 4D and E) which showed amplification of all technical replicates in both instances, thus demonstrating that this assay has excellent sensitivity.

These data sets add further confirmation that all three of the assays are performing as per the requirements of the Validation plan, if not exceeding expectation, and are therefore fit for purpose. The experiments have proved that the assays are performing extremely well as the PCR efficiencies are all well within the acceptance criteria, the standard curves demonstrate excellent performance at both high and low copy numbers for all assays as well as the low copy number dilutions highlighting excellent sensitivity in all three assays. The tests have allowed definition of the quantitative range for each kit which has been limited only by the fundamental limits of the technology.

*T. equigenitalis*

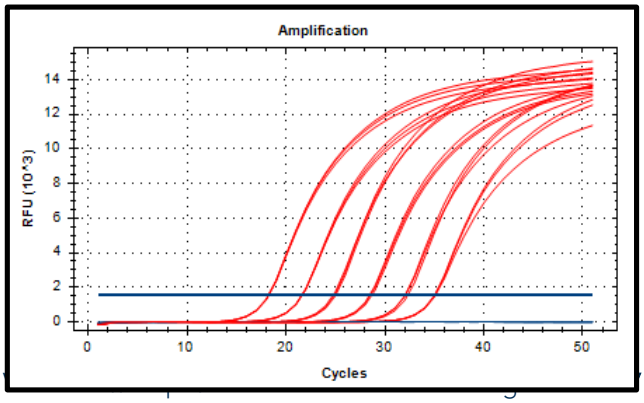
A

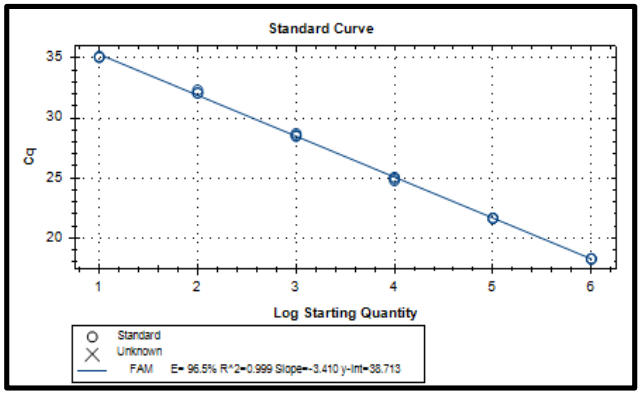



*P. aeruginosa*

B



*K. pneumoniae*

C

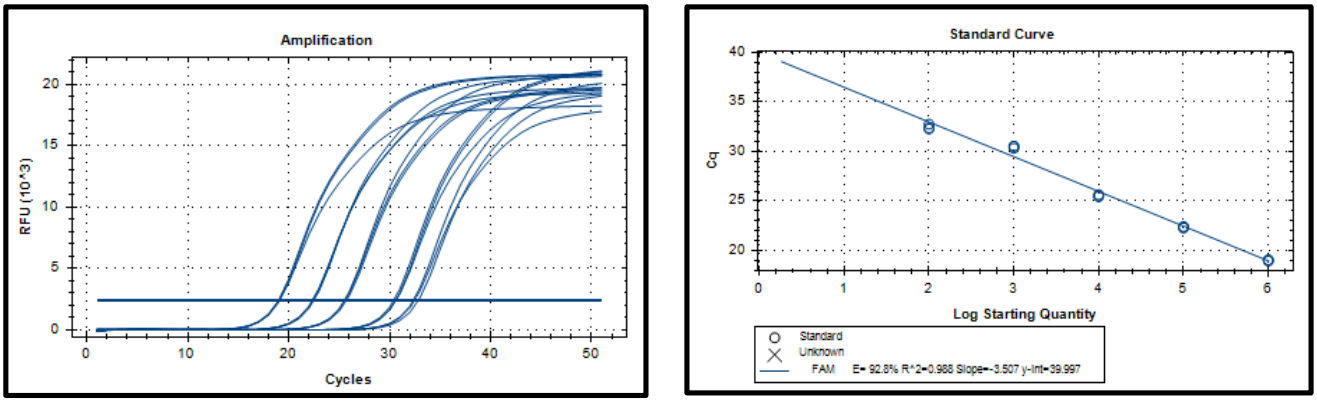
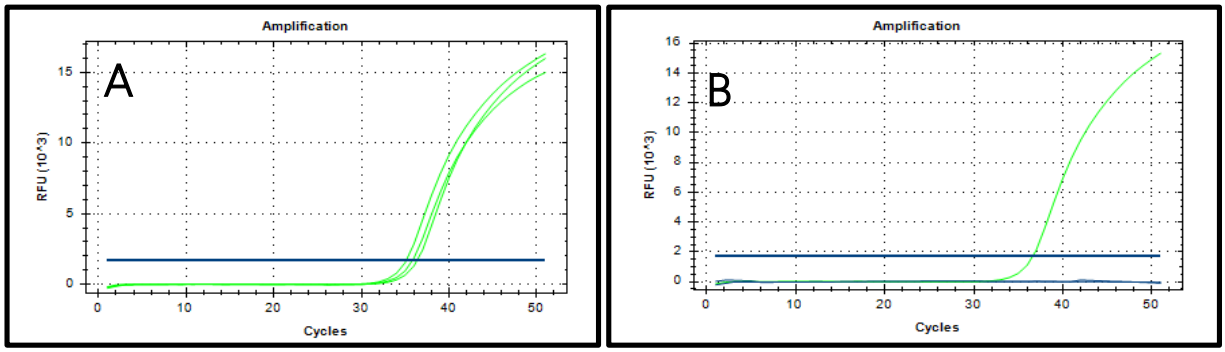



**Figure 3: Standard curves for A) *T. equigenitalis* (green); B) *P. aeruginosa* (red) and C) *K. pneumoniae* (blue).** Right hand panels show amplification plots achieved from dilution 1 (1000000 copies per reaction) to dilution 5 (100 copies per reaction) for *T. equigenitalis* and *K. pneumoniae*. Dilution 1 to dilution 6 (10 copies per reaction) is shown for *P. aeruginosa*. The right hand panel shows the amplification plots for each assay. The left hand panel shows the Cqs plotted against copy number where the gradient is used to calculate PCR efficiency. The PCR efficiency and $R^2$ value for each assay is shown on the associated plot.

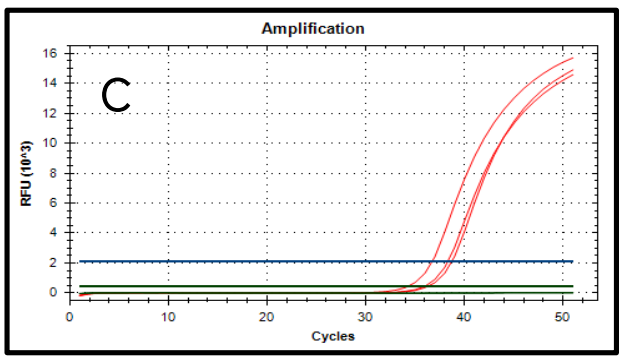
10 copies of template

1 copy of template

*T. equigenitalis*




*P. aeruginosa*




Included in standard curve in Figure 3B

*K. pneumoniae*

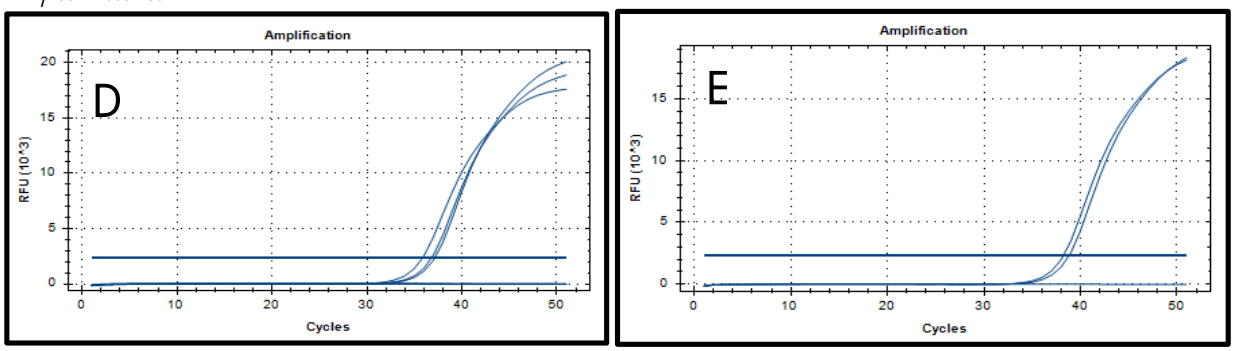



**Figure 4: Amplification plots of serially diluted synthetic template at low copy number.** *T. equigenitalis* (green) at A) 10 copies and B) 1 copy; *P. aeruginosa* (red) at C) 1 copy; *K. pneumoniae* (blue) at D) 10 copies and E) 1 copy Whilst amplification of the technical replicates in each case falls outside the inclusion criteria for quantitative analysis due to the spread observed in the Cq values of the replicates, all 3 assays show a positive signal (ie minimum 2 of 3 replicates with Cq value <40) with 10 copies with *P. aeruginosa* and *K. pneumoniae* showing positive signals in all replicates with 1 copy of template and *T. equigenitalis* demonstrates potential to amplify one copy. As such, all kits are suitable for qualitative analysis.

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### 4.2.2 Colony Forming Unit Standard Curve

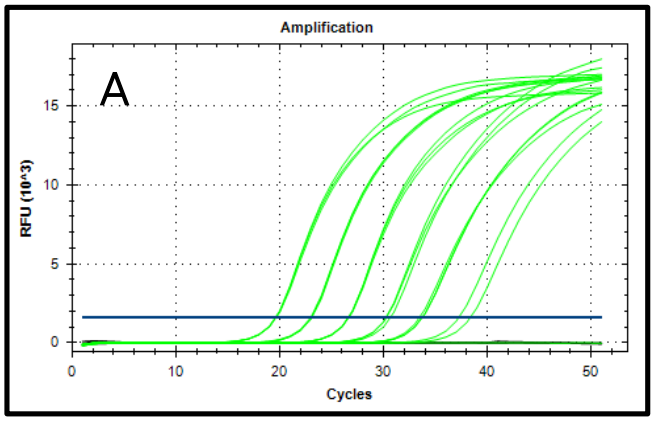
After validating the assays with synthetic templates, they were then required to be validated with known positive bacterial samples. This was to verify that the assays functioned equally well on biological samples and that the sensitivity was unaffected. The bacterial samples were prepared as a standard curve that was intended to be representative of true colony numbers to mirror the known copy number standard curve. As such, the test was aiming to ensure that the LOD is the same for the biological samples as for the positive control template. Each dilution was plated and cultured according to the Validation Plan with the aim of confirming the real time PCR result against the counted colonies.

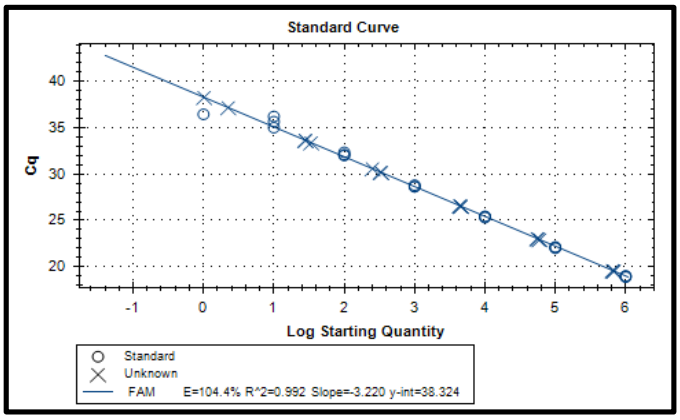
Figure 5 below shows the results of the real time PCR analysis. For each of the 3 assays it was demonstrated that the PCR efficiency and assay sensitivity was equal whether using synthetic template or extracted biological material. As previously described, the replicates show excellent conformity down to the lower limits of the quantitative range, which is then disrupted at later cycles. This agrees with the template standard curves and confirms that the quantitative range established in those experiments is suitable for use with biological samples. In addition, further amplification at lower copy numbers could be observed with all three assays, albeit that only *T. equigenitalis* demonstrated clean Cq separation (delta Cq) between dilution 5 and dilution 6. Amplification from dilution 6 was earlier than expected for *P. aeruginosa* and *K. pneumoniae*, and 1 dilution 6 replicate did fail to amplify for both the *T.*

*equigenitalis* assay and *P. aeruginosa*. At such a low copy numbers it is not concerning that one replicate failed and, according to the Validation Plan, 2 of 3 replicates showing amplification is a positive result. As such, the qualitative calling established with the template standard curves in line with the Validation Plan can also be said to be confirmed as appropriate.

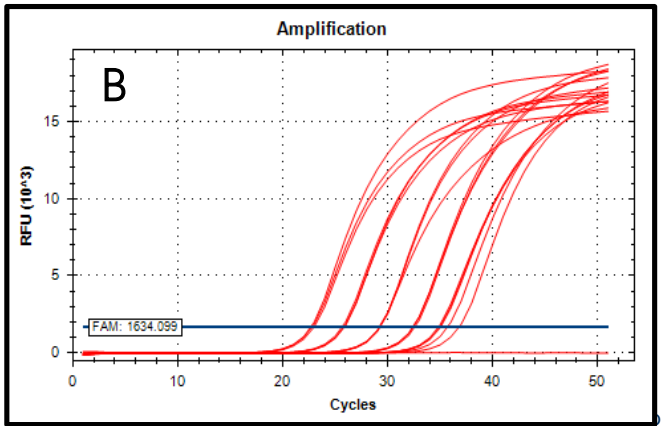
With respect to assay performance on biological samples, it is clear from the right hand panels in Figure 5 that none of the 3 assays deviate in PCR efficiency whether acting on synthetic template or biological samples. In each case the replicates of dilution point 1-5 of the biological standard curve are located at uniform distances from the synthetic standards. This shows that the PCR efficiency is not significantly altered by the use of biological sample compared to template. It also allows accurate Cq calculation for these samples from the template standard curve. As such, all aspects of assay performance have been confirmed with biological samples and the kits have all performed excellently.

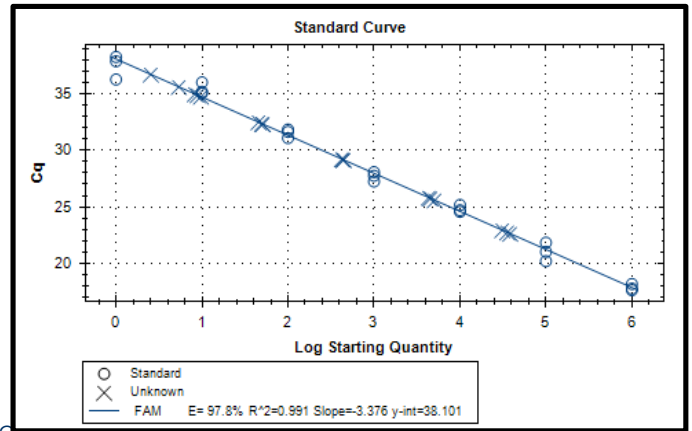
*T. equigenitalis*




*P. aeruginosa*



*K. pneumoniae*

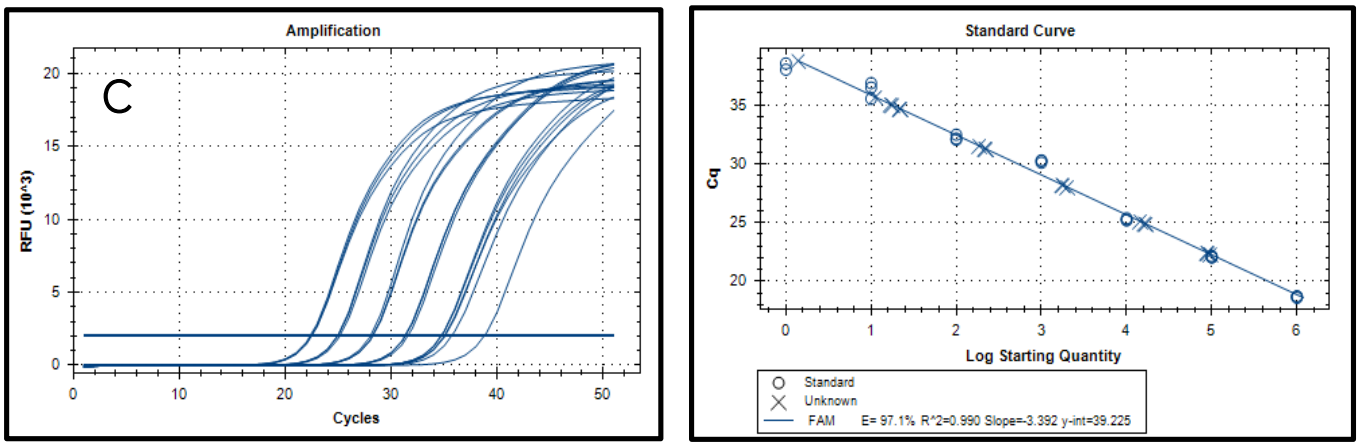



**Figure 5: Standard curves with biological samples.** Left hand panel shows the amplification plots achieved for the biological sample 6-point standard curve conducted on A) *T. equigenitalis*, B) *P. aeruginosa* and C) *K. pneumoniae*. The right hand panels show the line of best fit generated for each assay using a template standard curve, this line being the basis of the PCR efficiency calculations. In each case replicates from the template standard curve points are shown as o; the biological sample standard curves are overlaid and each replicate marked as x

Another aspect that was investigated was whether plated cultures from the same standard curve as that used in the biological standard PCR experiment showed the same copy numbers as those calculated for the PCR samples. The copy numbers added to the PCR replicates are straight forward to calculate using the known copy number standard curve and calculated Cq values for each dilution point (Table 6). Theoretically the copy numbers observed in Table 6 (PCR result) should show correlation with those in Table 7 (CFU counts) i.e. PCR calculated copy numbers and colony numbers cultured from the same starting dilution series of the relevant microorganism. However, it can clearly be seen that this was not the case. There were in addition, technical challenges with regard to counting the colonies with accuracy as in the cases of *P. aeruginosa* and *K. pneumoniae*. In these cases, the colonies were too abundant to count, leaving a paucity of data for these organisms (Table 7). In contrast, *T. equigenitalis* showed very poor growth by culturing but the qPCR results showed that it was clearly abundant. Although some cells did grow, there was also no correlation between the dilution series copy numbers plated and the colony counts. *T. equigenitalis* is known to be difficult to culture which may account for this discrepancy, however Table 6 and Table 7 together clearly show the sensitivity and advantages of a qPCR approach over culturing. A further consideration for the discrepancy between the qPCR and culturing data is the fundamental difference in the manner in which the two techniques report and the factors contributing to the reported values. For example, qPCR will report and amplify on dead cells, those of all stages of the cell cycle and free DNA as well as cells capable for forming a colony. In contrast, when culturing, only those cells that are in the correct phase and condition to be able to form a colony will report. The processes required and potential

losses involved in the culturing of live organisms, as opposed to processing nucleic acid, may have a profound impact on the final counts realised using each technique. As a result, the qPCR technique appears to be exceptionally more sensitive and reliable. It is open to fewer external and biological influences and does not require such an experienced workforce to undertake with accuracy.

| | *T. equigenitalis* | | *K. pneumoniae* | | *P. aeruginosa* | |
|---|---|---|---|---|---|---|
| Dilution point | *Cq* | *Calculated copy number* | *Cq* | *Calculated copy number* | *Cq* | *Calculated copy number* |
| 1 (1:10) | **19.6** | **655196.7** | 22.4 | **76769.0** | 22.8 | **32955.6** |
| 2 (1:100) | 23.0 | **52850.9** | 25.0 | **13584.2** | 25.7 | **3811.9** |
| 3 (1:1000) | 26.6 | **4444.2** | 28.1 | **1361.6** | 29.2 | **480.3** |
| 4 (1:10000) | 30.3 | **324.6** | 31.4 | **451.7** | 32.4 | **38.3** |
| 5 (1:100000) | 33.6 | **37.7** | 34.8 | **16.6** | 34.9 | **9.8** |

**Table 6: Calculation of copy number per reaction for biological sample standard curve**. Template standard curves for each assay completed with known copy numbers of template were used to calculate the copy number present of each organism at each dilution point within the quantitative range, using the Cqs obtained in the biological standard curves.

| | Counts (100ul) | | | Counts per ml | | |
|---|---|---|---|---|---|---|
| Dilution | *T. equigenitalis* | *K. pneumoniae* | *P. aeruginosa* | *T. equigenitalis* | *K. pneumoniae* | *P. aeruginosa* |
| 1:1000000 | 6 | 367 | 516 | 60 | 3670 | 5160 |
| 1:100000 | 1 | * | * | 10 | - | - |
| 1:10000 | 2 | * | * | 20 | - | - |
| 1:1000 | 5 | * | * | 50 | - | - |
| 1:100 | 112 | * | * | 1120 | - | - |
| 1:10 | 1276 | * | * | 12760 | - | - |

* Uncountable as CFU count too high to count manually

**Table 7: Results from plated cultures used in the dilution series parallel with real time PCR.** The test was intended to demonstrate whether colony counting showed good copy number correlation with that calculated by real time PCR. This was found not to be the case.

### 4.2.3 Limits of Detection

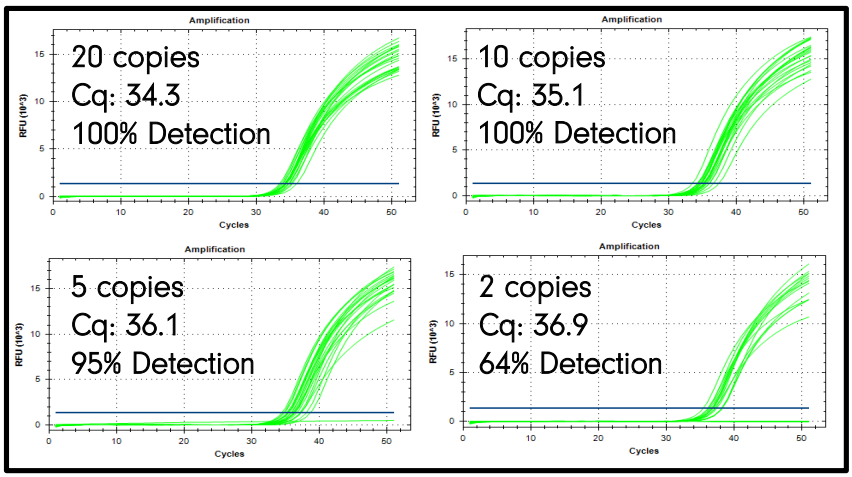
The limits of detection (LOD) are a measure of the sensitivity of each of the *T. equigenitalis*, *K. pneumoniae* and *P. aeruginosa* kits. The test is completed with known copy number template with 22 replicates at 20, 10, 5 and 2 copies per reaction. All of these copy numbers will give Cqs outside of the quantitative range and data is expected to be spread with respect to Cqs. However, The LOD is determined as the copy number at which 95% of replicates amplify prior

to Cq 40 and the spread of data is not of concern. This test is a measure of accurate sensitivity.

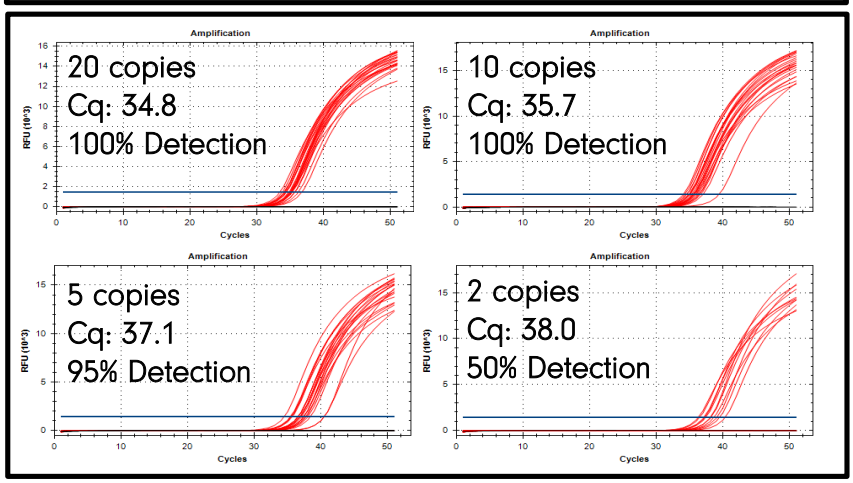
The calculated LOD for *T. equigenitalis*, *K. pneumoniae* and *P. aeruginosa* was 5 copies in each instance. Data for all copy numbers tested can be seen in Figure 6 below, including plots and mean Cqs. For each of the three assays, both 20 copies and 10 copies gave 100% detection with Cqs that were, with limited exceptions, not close to approaching 40. The 20 copy replicates were, as expected, more tightly reproducible, with a lower spread of Cqs compared with the 10 copy detection in each instance. K. pneumoniae also gave a 100% detection rate on 5 copies whereas *T. equigenitalis* and *P. aeruginosa* both passed the LOD of 5 copies with a 95% detection rate. That is, there was one failed amplification from 22 replicates. With regard to *T. equigenitalis* at the 2 copy level, some good amplification can be seen however 8 of 22 replicates failed to amplify which equates to a 36% failure rate. With *P. aeruginosa* amplifying at 2 copies 11 replicates failed to give a positive amplification plot. This equates to a 50% failure rate at 2 copies. Marginally exceeding both of the previous assays was *K. pneumoniae* which showed the lowest 2 copy failure rate at 18%

Each of the three assays showed excellent sensitivity in this analysis ensuring maximum pathogen detection and sensitivity during real world testing.

*T. equigenitalis*




*P. aeruginosa*



*K. pneumoniae*

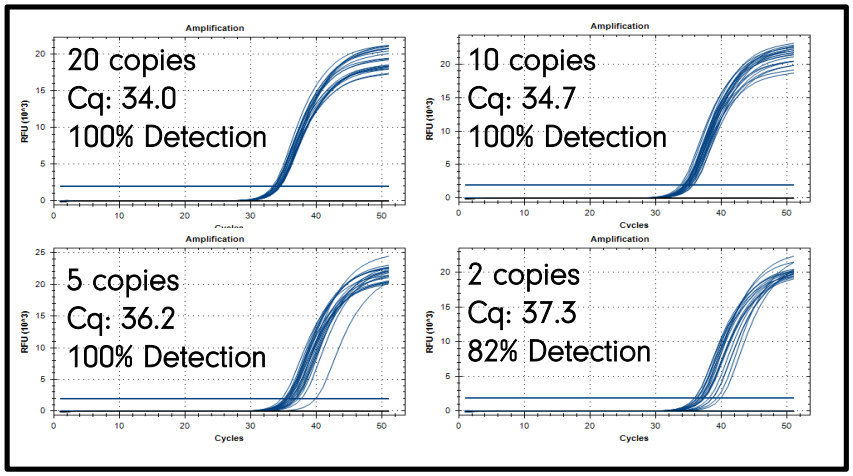



**Figure 6: Limits of detection.** The limits of detection for *T. equigenitalis* (green), *P. aeruginosa* (red) and *K. pneumoniae* (blue) were calculated by testing the percentage of replicates that amplified at 20, 10, 5 and 2 copies. Mean Cq for all amplified copies is situated in the left, top corner of each plot.

## 4.3 SPECIFICTY OF PRIMERDESIGN KITS

### 4.3.1 Cross Reactivity

It was necessary to determine the specificity of each of the assays and verify that they did not cross-react with biological material from each of the other microorganisms under scrutiny. As such, each assay was tested for its ability to amplify on biological template extracted from *T. equigenitalis*, *P. aeruginosa* and *K. pneumoniae*.

The cross reactivity result is summarised in Table 8 below which clearly demonstrates that none of the assays cross react with biological materials pertaining to either of the 2 alternative assays, i.e. the *T. equigenitalis* kit detects excellently on *T. equigenitalis* DNA but is called negative for amplification on *P. aeruginosa* and *K. pneumoniae* DNA (Figure 7); the *P. aeruginosa* kit detects excellently on *P. aeruginosa* DNA but is negative for amplification on *T. equigenitalis* and *K. pneumoniae* DNA (Figure 8); and the *K. pneumoniae* kit detects excellently on *K. pneumoniae* DNA but is called negative for amplification on *P. aeruginosa* and *T. equigenitalis* DNA (Figure 9). It can be noted that a single positive plot was identified with the *T. equigenitalis* kit and *K. pneumoniae* DNA and the *K. pneumoniae* kit with both *T. equigenitalis* and *P. aeruginosa* DNA. As only 1 of 3 replicates showed amplification in each instance, this is reported as negative for pathogen detection as detailed by the Validation Plan. The late Cqs may represent a vanishingly small level of contamination in a single well that could lead to this result. The Cq is far from that expected from genuine amplification, as

demonstrated by the Cq achieved on genuine template and from the internal extraction control, both of which are considerably earlier than that of any of the single plots.

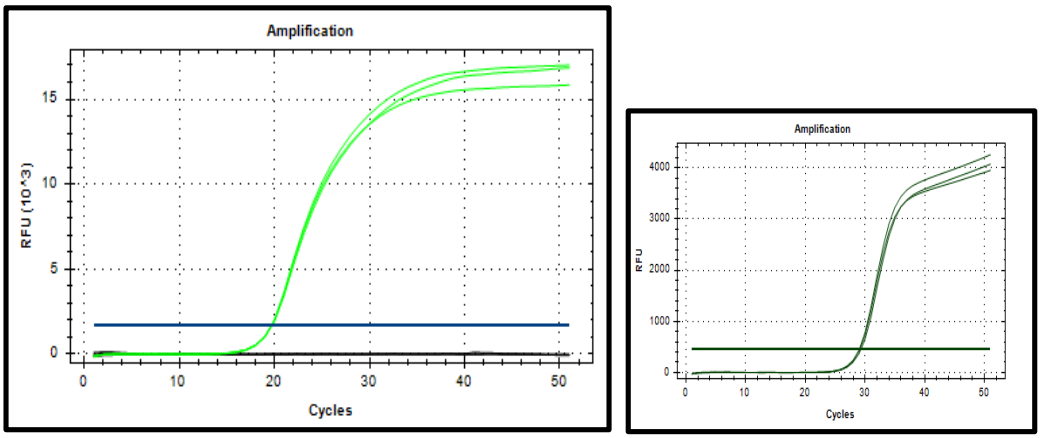
The right hand panels within Figures 7, 8 and 9 show the same 3 replicates as the associated left hand panels, where the left hand panels show the GREEN channel data generated by the assays of interest (i.e. Pathogen kits) and the right hand panels show the YELLOW channel signal from the same wells. The YELLOW channel signal is generated from the internal extraction control that was multiplexed with the pathogen kits. The internal extraction control was added to the biological samples prior to DNA extraction to show that the extraction and PCR had been successful and hence support negative results. The internal extraction control template is co-extracted with the pathogen DNA and so, as it reliably amplified in all replicates recorded as negative, the negative results obtained can be confidently relied upon to be truly negative.

| TEST KIT | *T. equigenitalis* | *P. aeruginosa* | *K. pneumoniae* |
|---|---|---|---|
| MICROORGANISM | | | |
| *T. equigenitalis* | ✓ | X | X |
| *P. aeruginosa* | X | ✓ | X |
| *K. pneumoniae* | X | X | ✓ |

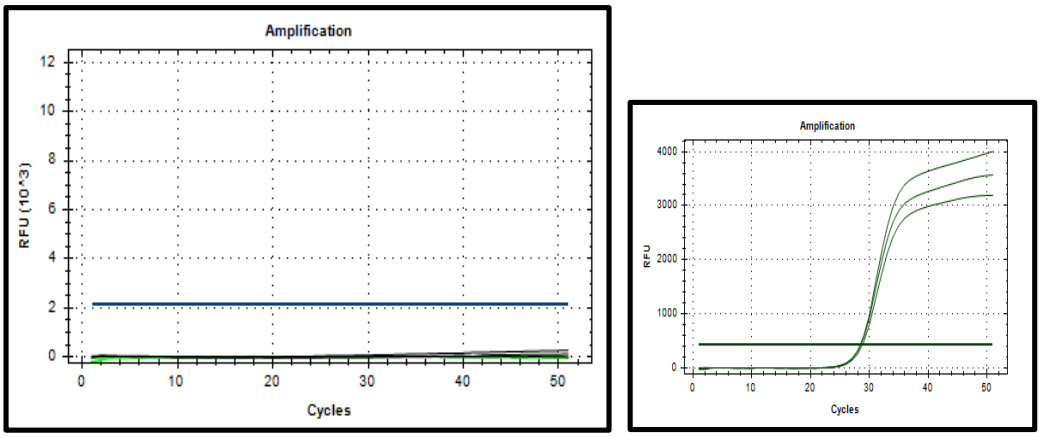
**Table 8: Summary of results for the cross reactivity testing**. Each assay (vertical columns) was tested against DNA from each of the associated microorganisms (horizontal rows) to ensure amplification only occurred on samples specific to the assay

✓= Amplification; X = No amplification

A


Amplification
RFU (10^3)
Cycles
Amplification
RFU
Cycles

B


Amplification
RFU (10^3)
Cycles
Amplification
RFU
Cycles

C

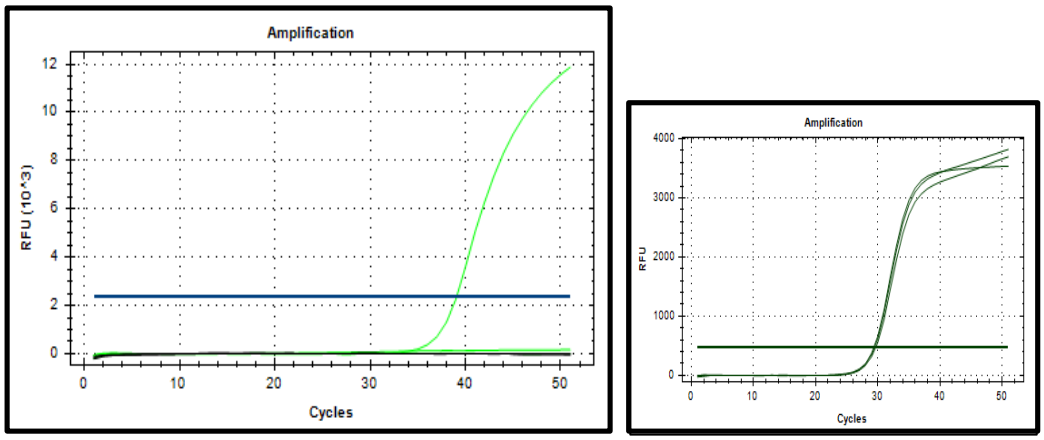

Amplification
RFU (10^3)
Cycles
Amplification
RFU
Cycles

**Figure 7. Specific detection of *T. equigenitalis* with *T. equigenitalis* detection kit.** Amplification plots showing specific detection of *T. equigenitalis* (A) when using the *T. equigenitalis* targeted detection kit (green curves) with *T. equigenitalis* biological sample. The *T. equigenitalis* detection kit did not amplify any signal from *P. aeruginosa* (B) or *K. pneumoniae* (C) biological sample. Internal extraction control reactions were run as a duplex with the target of interest to show successful extraction of biological material from the respective microorganisms. NTC (black) did not show any amplification of signal.

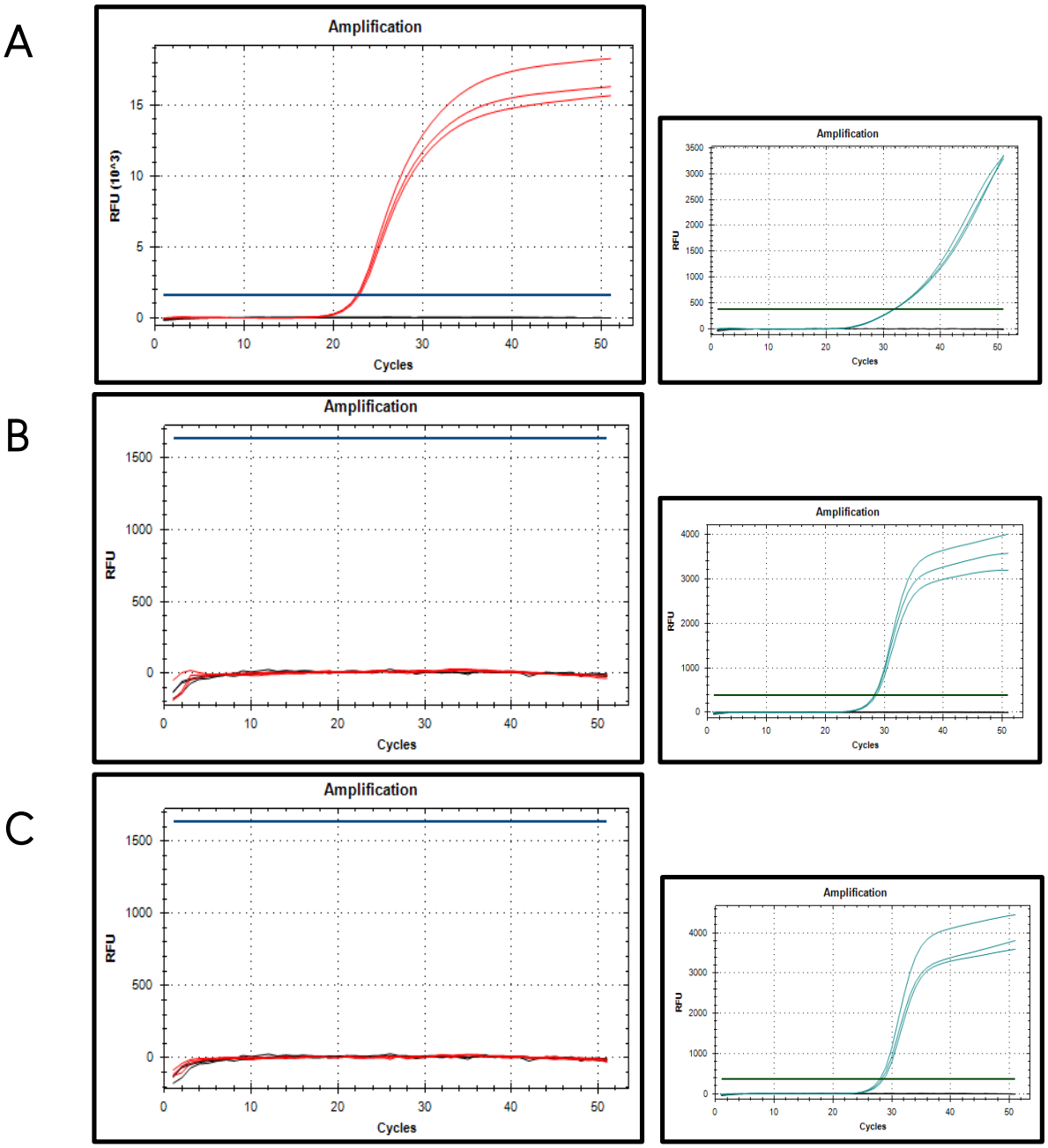


**Figure 8. Specific detection of *P. aeruginosa* with *P. aeruginosa* detection kit.** Amplification plots showing specific detection of *P. aeruginosa* (A) when using the *P. aeruginosa* targeted detection kit (red curves) with *P. aeruginosa* biological sample. The *P. aeruginosa* detection kit did not amplify any signal from *T. equigenitalis* (B) or *K. pneumoniae* (C) biological sample. Internal extraction control reactions were run as a duplex with the target of interest to show successful extraction of biological material from the respective microorganisms. NTC (black) did not show any amplification of signal.

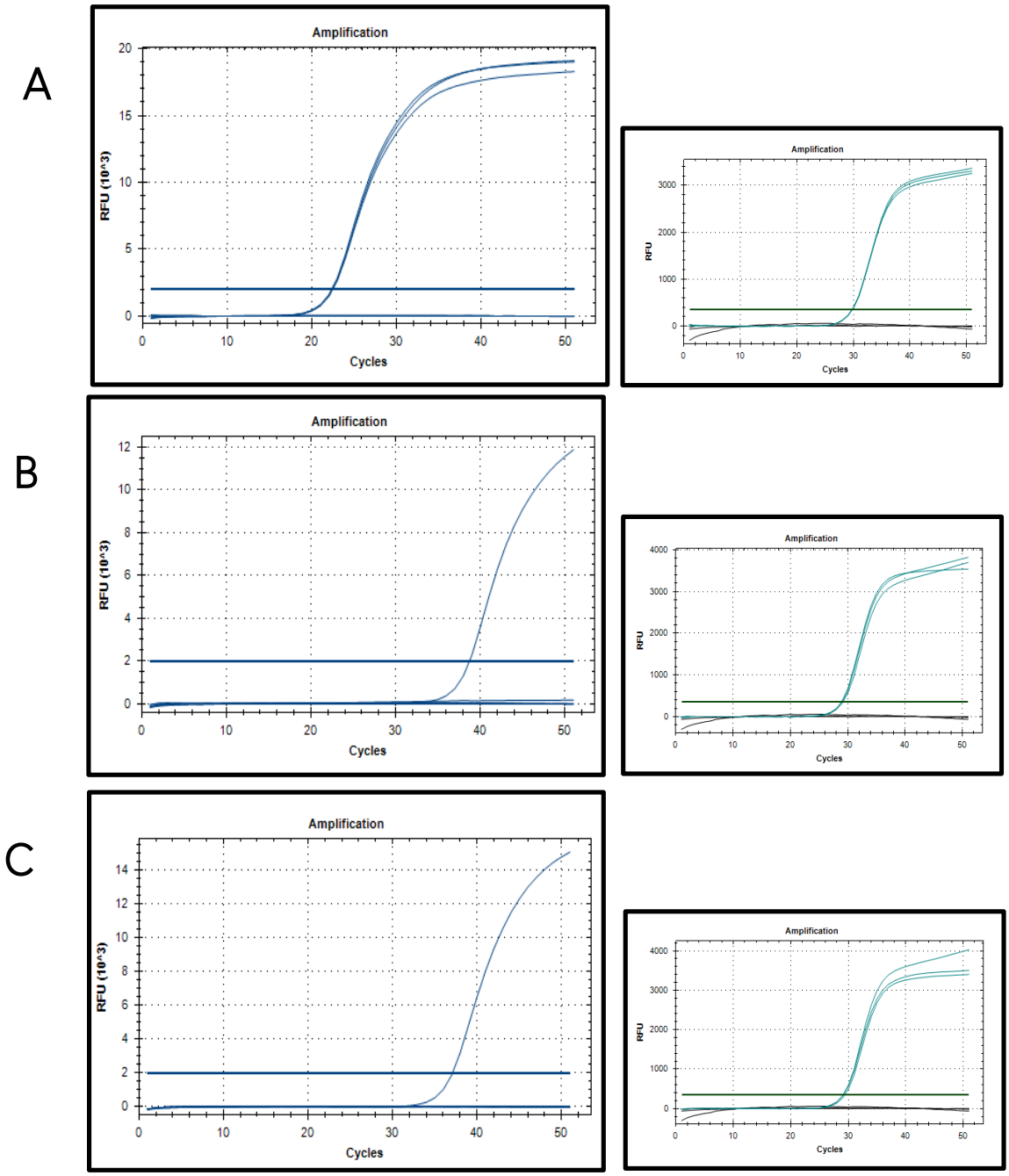


**Figure 9. Specific detection of K. pneumoniae with K. pneumoniae detection kit.** Amplification plots showing specific detection of *K. pneumoniae* (A) when using the *K. pneumoniae* targeted detection kit (blue curves) with *K. pneumoniae*. Biological sample. The *K. pneumoniae* detection kit did not amplify any signal from *T. equigenitalis* (B) or *P. aeruginosa* (C) biological material. Internal extraction control reactions were run as a duplex with the target of interest to show successful extraction of biological material from the respective microorganisms. NTC (black) did not show any amplification of signal.

### 4.3.2 Swab Specificity

Horse genitalia is an environment for a plethora of different microorganisms. It is therefore important that when validating new test kits real swabs are used containing the background flora as well as the target organisms of interest. Due to the paucity of naturally positive samples, swabs were taken and known samples spiked with *T. equigenitalis, P. aeruginosa, K. pneumoniae* or combinations of these organisms. The sample IDS and their positive/negative status for each organism was then blinded and the 50 samples were tested by qPCR using the Qiagen cador kits and the Primerdesign kits (work completed at equine specialist lab partner). The data was analysed and, once all experiments on these samples were completed and all results called, the calls were un-blinded to evaluate the performance of the Qiagen kits compared to that of the Primerdesign kits.

Prior to beginning the analysis, observation of the initial results clearly showed that the original calling criteria set out in the Validation Plan were too stringent for complex biological samples. As such the criteria that all replicates must be within 0.5Cq values when amplification occurs within the defined quantitative range was relaxed to 1Cq. The majority of the data from both manufacturers kits showed amplification at late cycles, flattening of the amplification plots, low end point fluorescence and there was some reduced pairing in the replicates at Cqs close to but not within the qualitative range (Figure 10). Thus the PCR appeared to be potentially sub optimal due to the samples. There are multiple factors that can make biological samples more difficult to amplify than synthetic template. For example, templates are not extracted so do not have the risk of chemical carry over from the process that can cause PCR inhibition, biological samples often have low abundance of target mixed in a high background of co-extracted DNA, the transportation and subsequent storage requirements can add chemical contaminants to the extracted samples and the environment from which the samples are acquired can also introduce contaminants that lead to sub-optimal PCR. Both the Qiagen and the Primerdesign kits would both have lost a significant amount of good quality data had the criteria been adhered to rigidly which was not considered in the interest of the study. An example of the issue can be seen in Figure 10 where this sample would have been eliminated from the analysis, however it can clearly be seen that the three replicates are all correct. Further justification for this decision can be found in the discussion section.

A further deviation from the Validation Plan was that our equine specialist lab partner ran 40 cycles of PCR as opposed to the 50 cycles specified. As the calling criteria for a positive is a Cq ≤40 this deviation will not have had an impact on the final data sets or the positive/negative calling. The choice of 50 cycles over 40 was due to standard practice at Primerdesign and to aid late calling. All non-conformances were recorded in the Primerdesign quality system.

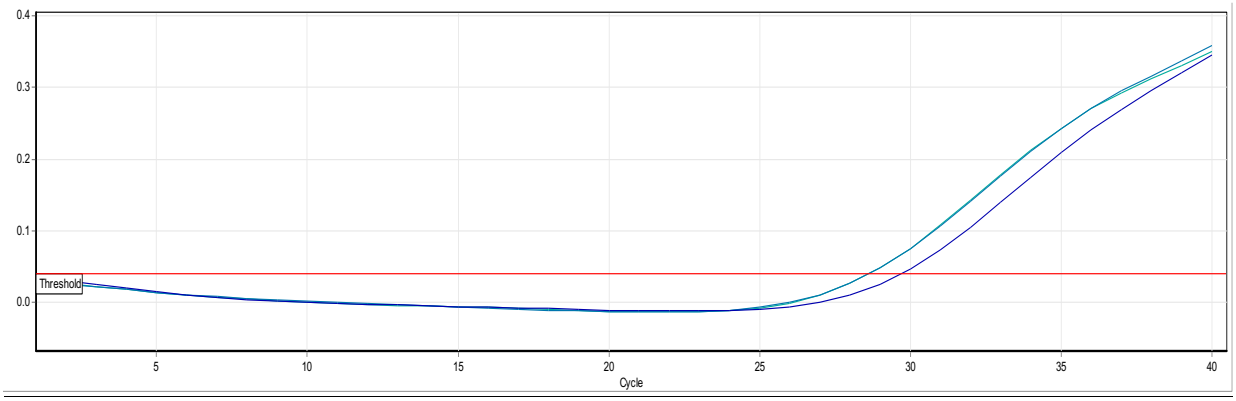



**Figure 10: Amplification plot showing the reason that the calling criteria in the validation plan were altered upon observation of the initial data.** Two amplification plots overlie perfectly but the third is delayed by more than 0.5Cq. The Grubb's test would have eliminated this point from the analysis when the lift off point, end point fluorescence and curve shape show that it is the identical product as that in the other two replicate wells.

The calls for each organism with each kit can be seen in Table 9, in addition, the comparison data is detailed in Table 10. The calling data is simply reported as positive or negative, whereas the comparison data is reported as 'matched' if the Qiagen and Primerdesign calls correlate or 'unmatched' if they do not. All negative results were confirmed using the internal extraction control (IEC) amplification plots on the YELLOW channel where all samples showed a positive IEC signal for each kit tested, thus validating negative calls.

In the most part, the Qiagen and Primerdesign kits showed good agreement for all three assays with an overall discrepancy of 13.3% over all 3 assays (Table 10). The *K. pneumoniae* assay showed the greatest correlation, followed by *T. equigenitalis* with *P. aeruginosa* showing least correlation. This was encouraging in the first instance, however a major reason for a large proportion of the discrepancies was revealed upon un-blinding the samples. When verifying the correct and incorrect calls made by both the Qiagen and Primerdesign kits, it became clear that the accuracy and sensitivity of the Primerdesign kits was greater than that of the Qiagen kits. That is, more correct calls were made by the Primerdesign kits than the Qiagen alternative, as Primerdesign had an overall call error rate (3 assays on 50 samples so 150 calls) of 15.3%, compared to 21.3% for the Qiagen kit (Figure 11). For comparison

purposes, the Primerdesign kit reported no false negatives and 8 false positives with *T. equigenitalis*, compared to no false negatives and thirteen false positives for the Qiagen kit. Interestingly, both kits made similar false positive readings with the *T. equigenitalis* kit and both demonstrated a number of false negatives for *P. aeruginosa*, where again a large percentage of the same samples were miscalled. This may suggest that there was an element of low level contamination with the *T. equigenitalis* DNA in some samples, possibly from the positive controls run with the samples, rather than a failure in the kits. Indeed, the amplification plots for many of these false positives was both late and comparatively weak but appeared as positive compared to the NTC wells.

With regard to *P. aeruginosa*, while both kits showed some sensitivity issue, a greater number of correct, positive calls were made by the Primerdesign kits suggesting increased sensitivity compared to the Qiagen alternative. To demonstrate, there were 9 false negatives and 1 false positive called by the Primerdesign *P. aeruginosa* assay compared to 21 false negatives and 2 false positives for the Qiagen kit.

Lastly, to consider *K. pneumoniae*. This was the most comparable of the 3 assays tested and assay performance with regard to false positive and negative calling was extremely similar. Both kits showed fewer incorrect calls with *K. pneumoniae* compared to the other 2 assays, with 5 incorrect calls made for both kits. The Primerdesign kit showed 3 false negatives and 2 false positive and conversely the Qiagen kit showed 2 false negatives and 3 false positives. As with the other assays, frequently both kits failed to make the correct call on the same samples. This might suggest a sample bias as opposed to kit failure or, for example, limitations of the technology as a whole as opposed to variation in kit performance.

| | Primerdesign calls | | | Qiagen calls | | |
|---|---|---|---|---|---|---|
| Sample ID | *T. equigenitalis* | *K. pneumoniae* | *P. aeruginosa* | *T. equigenitalis* | *K. pneumoniae* | *P. aeruginosa* |
| A1 | POSITIVE | POSITIVE | POSITIVE | POSITIVE | POSITIVE | NEGATIVE |
| B1 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| C1 | POSITIVE | POSITIVE | POSITIVE | POSITIVE | POSITIVE | NEGATIVE |
| D1 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| E1 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| F1 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| G1 | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| H1 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| A2 | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE | NEGATIVE |
| B2 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| C2 | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE | NEGATIVE |
| D2 | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | POSITIVE | NEGATIVE |
| E2 | NEGATIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| F2 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| G2 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| H2 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| A3 | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| B3 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| C3 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| D3 | NEGATIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| E3 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | POSITIVE |
| F3 | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE | NEGATIVE |
| G3 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| H3 | NEGATIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| A4 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| B4 | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE |
| C4 | NEGATIVE | NEGATIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| D4 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| E4 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| F4 | NEGATIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE | NEGATIVE |
| G4 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |

| | | | | | | |
|---|---|---|---|---|---|---|
| H4 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| A5 | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| B5 | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE |
| C5 | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE |
| D5 | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE |
| E5 | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE |
| F5 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| G5 | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE | NEGATIVE |
| H5 | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE |
| A6 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| B6 | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE |
| C6 | POSITIVE | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE |
| D6 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| E6 | POSITIVE | NEGATIVE | POSITIVE | POSITIVE | NEGATIVE | NEGATIVE |
| F6 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE |
| G6 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| H6 | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE |
| A7 | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| B7 | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE |

Table 9: Calls from blinded swab samples using the Qiagen and Primerdesign kits.

| Sample ID | *T. equigenitalis* | *K. pneumoniae* | *P. aeruginosa* |
|---|---|---|---|
| A1 | MATCH | MATCH | UNMATCHED |
| B1 | MATCH | MATCH | MATCH |
| C1 | MATCH | MATCH | UNMATCHED |
| D1 | MATCH | MATCH | MATCH |
| E1 | MATCH | MATCH | MATCH |
| F1 | MATCH | MATCH | MATCH |
| G1 | MATCH | MATCH | UNMATCHED |
| H1 | MATCH | MATCH | MATCH |
| A2 | MATCH | MATCH | UNMATCHED |
| B2 | MATCH | MATCH | MATCH |
| C2 | MATCH | MATCH | UNMATCHED |
| D2 | MATCH | UNMATCHED | UNMATCHED |
| E2 | UNMATCHED | MATCH | MATCH |
| F2 | MATCH | MATCH | MATCH |
| G2 | MATCH | MATCH | MATCH |
| H2 | MATCH | MATCH | MATCH |
| A3 | MATCH | MATCH | UNMATCHED |
| B3 | MATCH | MATCH | MATCH |
| C3 | MATCH | MATCH | MATCH |
| D3 | UNMATCHED | MATCH | MATCH |
| E3 | MATCH | MATCH | UNMATCHED |
| F3 | MATCH | MATCH | UNMATCHED |
| G3 | MATCH | MATCH | MATCH |
| H3 | UNMATCHED | MATCH | MATCH |
| A4 | MATCH | MATCH | MATCH |
| B4 | MATCH | MATCH | MATCH |
| C4 | UNMATCHED | UNMATCHED | MATCH |
| D4 | MATCH | MATCH | MATCH |
| E4 | MATCH | MATCH | MATCH |
| F4 | UNMATCHED | MATCH | UNMATCHED |
| G4 | MATCH | MATCH | MATCH |
| H4 | MATCH | MATCH | MATCH |
| A5 | MATCH | MATCH | UNMATCHED |

| | | | |
|---|---|---|---|
| B5 | MATCH | MATCH | MATCH |
| C5 | MATCH | MATCH | MATCH |
| D5 | MATCH | MATCH | MATCH |
| E5 | MATCH | MATCH | MATCH |
| F5 | MATCH | MATCH | MATCH |
| G5 | MATCH | MATCH | UNMATCHED |
| H5 | MATCH | MATCH | MATCH |
| A6 | MATCH | MATCH | MATCH |
| B6 | MATCH | MATCH | MATCH |
| C6 | MATCH | MATCH | MATCH |
| D6 | MATCH | MATCH | MATCH |
| E6 | MATCH | MATCH | UNMATCHED |
| F6 | MATCH | MATCH | MATCH |
| G6 | MATCH | MATCH | MATCH |
| H6 | MATCH | MATCH | MATCH |
| A7 | MATCH | MATCH | MATCH |
| B7 | MATCH | MATCH | MATCH |

Table 10: Table summarising the concordance of positive/negative sample calling for *T. equigenitalis*, *K. pneumoniae* and *P. aeruginosa* between Primerdesign and Qiagen assays on blinded swab samples

| | Primerdesign Run 1 | | | Primerdesign Run 2 | | | Qiagen Run 1 | | | Qiagen Run 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample ID | T | K | P | T | K | P | T | K | P | T | K | P |
| A1 | | | | | | | | | | | | |
| B1 | | | | | | | | | | | | |
| C1 | | | | | | | | | | | | |
| D1 | | | | | | | | | | | | |
| E1 | | | | | | | | | | | | |
| F1 | | | | | | | | | | | | |
| G1 | | | | | | | | | | | | |
| H1 | | | | | | | | | | | | |
| A2 | | | | | | | | | | | | |
| B2 | | | | | | | | | | | | |
| C2 | | | | | | | | | | | | |
| D2 | | | | | | | | | | | | |
| E2 | | | | | | | | | | | | |
| F2 | | | | | | | | | | | | |
| G2 | | | | | | | | | | | | |
| H2 | | | | | | | | | | | | |
| A3 | | | | | | | | | | | | |
| B3 | | | | | | | | | | | | |
| C3 | | | | | | | | | | | | |
| D3 | | | | | | | | | | | | |
| E3 | | | | | | | | | | | | |
| F3 | | | | | | | | | | | | |
| G3 | | | | | | | | | | | | |
| H3 | | | | | | | | | | | | |
| A4 | | | | | | | | | | | | |
| B4 | | | | | | | | | | | | |
| C4 | | | | | | | | | | | | |
| D4 | | | | | | | | | | | | |
| E4 | | | | | | | | | | | | |
| F4 | | | | | | | | | | | | |
| G4 | | | | | | | | | | | | |

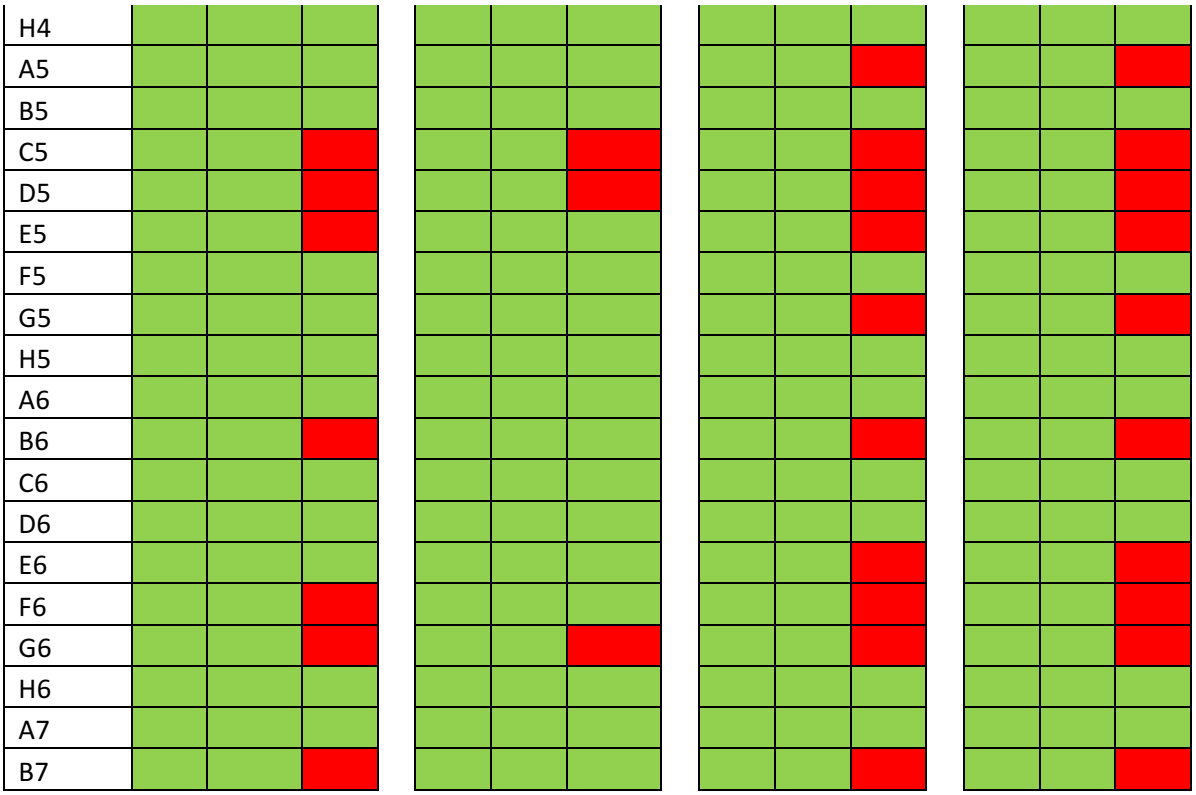



**Figure 11: Accuracy of calling when samples were un-blinded.** Correct calls are shown as green and incorrect calls are shown as red. Data is shown from the specificity and repeatability experimental data sets. T: *T. equigenitalis*, K: *K. pneumoniae* and P: *P. aeruginosa*.

## 4.4 Repeatability

Repeatability testing was undertaken to confirm that the Primerdesign kits gave the same result on the same samples when used by the same individual in the same laboratory. Whilst the Validation Plan outlined the need only to test 22 samples, all 50 samples were repeated for completeness. Again, while not stated in the Validation Plan, the Qiagen kits were also tested for repeatability for comparison purposes and, as described above, 40 cycles of PCR were completed as opposed to the 50 specified.

The repeatability experiment showed excellent correlation between two individual runs. Of the samples and assays that showed discrepancies, 4 were within the *T. equigenitalis* assay, 3 were within the *K. Pneumoniae* assay and 4 were within the *P. aeruginosa* assay (Table 11). There was an overall 'match' rate for the Primerdesign kits of 92.7% over the 150 tests compared to an overall 'match' rate of 93.4% for the 3 Qiagen assays when similarly

repeated (Data not shown). This percentage difference equates to 1 more mismatch in the Primerdesign comparison over the same comparison of the Qiagen experimental results.

Of the tests that did not show correlation, 4 of these were due to improved calling of *P. aeruginosa* in the second experimental replicate such that false negative calls reduced from 9 to 5 for the Primerdesign kit; and 2 related to a small increase in the calling of false positives for *T. equigenitalis*. These areas for these two kits (false negatives and positives respectively) have already been highlighted as areas of relatively high variability and have been consistently difficult for the Qiagen kit also. This data therefore again points to the difficult samples as a significant potential source of variation, and therefore some variation in these calls between runs is not unexpected. Lastly, as the high degree of conformity covers both the correct and incorrect calls it can be seen that there is genuine consistency in the calls which allows a high degree of confidence in the repeatability of all of the kits. It also means that the limits of the kit can be easily established for the end user.

| Sample ID | *T. equigenitalis* | *K. pneumoniae* | *P. aeruginosa* |
|---|---|---|---|
| A1 | MATCH | MATCH | MATCH |
| B1 | MATCH | MATCH | MATCH |
| C1 | MATCH | MATCH | MATCH |
| D1 | MATCH | MATCH | MATCH |
| E1 | MATCH | MATCH | MATCH |
| F1 | MATCH | MATCH | MATCH |
| G1 | MATCH | MATCH | MATCH |
| H1 | MATCH | MATCH | MATCH |
| A2 | MATCH | MATCH | MATCH |
| B2 | MATCH | MATCH | MATCH |
| C2 | MATCH | MATCH | MATCH |
| D2 | MATCH | MATCH | MATCH |
| E2 | UNMATCHED | MATCH | MATCH |
| F2 | MATCH | MATCH | MATCH |
| G2 | MATCH | MATCH | MATCH |
| H2 | MATCH | MATCH | MATCH |
| A3 | MATCH | MATCH | MATCH |
| B3 | MATCH | MATCH | MATCH |
| C3 | MATCH | MATCH | MATCH |
| D3 | MATCH | MATCH | MATCH |
| E3 | MATCH | MATCH | MATCH |
| F3 | MATCH | MATCH | MATCH |

| | | | |
|---|---|---|---|
| G3 | MATCH | MATCH | MATCH |
| H3 | UNMATCHED | MATCH | MATCH |
| A4 | MATCH | MATCH | MATCH |
| B4 | UNMATCHED | MATCH | MATCH |
| C4 | MATCH | UNMATCHED | MATCH |
| D4 | UNMATCHED | UNMATCHED | MATCH |
| E4 | MATCH | MATCH | MATCH |
| F4 | MATCH | MATCH | MATCH |
| G4 | MATCH | UNMATCHED | MATCH |
| H4 | MATCH | MATCH | MATCH |
| A5 | MATCH | MATCH | MATCH |
| B5 | MATCH | MATCH | MATCH |
| C5 | MATCH | MATCH | MATCH |
| D5 | MATCH | MATCH | MATCH |
| E5 | MATCH | MATCH | UNMATCHED |
| F5 | MATCH | MATCH | MATCH |
| G5 | MATCH | MATCH | MATCH |
| H5 | MATCH | MATCH | MATCH |
| A6 | MATCH | MATCH | MATCH |
| B6 | MATCH | MATCH | UNMATCHED |
| C6 | MATCH | MATCH | MATCH |
| D6 | MATCH | MATCH | MATCH |
| E6 | MATCH | MATCH | MATCH |
| F6 | MATCH | MATCH | UNMATCHED |
| G6 | MATCH | MATCH | MATCH |
| H6 | MATCH | MATCH | MATCH |
| A7 | MATCH | MATCH | MATCH |
| B7 | MATCH | MATCH | UNMATCHED |

**Table 11: Table to show the correlation of sample calls when Primerdesign kits are used in a repeat experiment.** Correlation between calls from replicate experiments shown as 'Match'. Uncorrelated results shown as 'Unmatched'

### 4.5 Reproducibility of Primerdesign Kit Results

A selection of 22 samples of extracted DNA used in the repeatability study were sent to Primerdesign to test each kit for reproducibility. This test set out to prove that the kit performed extremely similarly for different individuals at different laboratories with a different qPCR machine (still a Rotorgene) using the same samples. Unfortunately, there was not sufficient sample volume remaining to repeat the experiment exactly as our equine specialist lab partner had run it. As such the samples were diluted 1:3 prior to running them with the Primerdesign kits in house. In addition to the dilution, it was also unfortunate that the machine failed to read the YELLOW channel in this run. Having tested these samples on a number of occasions previously it is known that the IEC was present and functioning. With good correlation, confidence remained high in the negative results acquired and this omission is not considered to have significantly influenced calling. The results were analysed and compared to the calls made in the first specificity analysis (comparing to Qiagen) and the repeatability study (Table 12).

When calculating the 'match' rate between the diluted 22 sample data and the specificity run data, a 'match' rate of 82% was observed. When comparing the 22 samples with the repeatability data, the 'match' rate was 88%. This was not as high as might have been expected from the repeatability data, however, the test conditions for the generation of the reproducibility data were not identical, as described above. The low abundance of target DNA in the original samples made calling the data after the dilutions especially challenging for both assays. It was expected that some positives would no longer be detected; for example, an increase in *P. aeruginosa* false negatives was expected due to the assay's propensity for false negative reports. Upon testing however, samples, such as A1 and B1, which were routinely demonstrating false positive results with both the Qiagen and Primerdesign *T. equigenitalis* kits, were also found to no longer produce positive signals. In this instance, samples were suspected of trace contamination from positive template which it appears was diluted out, thus giving an 'unmatched' result in comparison to the previous occasions.

It is believed that further samples suffered from being diluted (C2 and B7 with the *P. aeruginosa* and *K. pneumoniae* kits respectively) such that replicates did not correlate with one another. This is not uncommon for qPCR under such circumstances but ideally these samples would have been repeated as opposed to the test being eliminated from the analysis as void. In such cases, whilst there were amplification plots that suggested a matching result, these tests had to be discarded for lack of reproducibility between the

replicates and are marked in the table as 'UNMATCHED' in a white box (Table 12). The sample IDs marked as red show samples that showed variability in previous analyses.

While the reproducibility data was good and mismatches in calling could be largely associated with previously reported instances of variability, it is strongly suspected that the circumstances surrounding the testing has led to an underestimation of the true level of reproducibility in these assays. Taking those factors into account as well as the eliminated replicates, the data actually shows the kits to have excellent capacity for reproducibility. This aspect of assay performance is in fact key to the Primerdesign product range from which the 3 assays stem. With a large number of similar tests being used globally, Primerdesign is extremely confident in the performance of these kits and believe that the reported data is not as accurate as it would have been if undiluted sample material had still been available.

| | Reproducibility run vs specificity run | | | Reproducibility run vs repeatability run | | |
|---|---|---|---|---|---|---|
| Sample ID | *T. equigenitalis* | *K. pneumoniae* | *P. aeruginosa* | *T. equigenitalis* | *K. pneumoniae* | *P. aeruginosa* |
| A1 | UNMATCHED | MATCH | MATCH | UNMATCHED | MATCH | MATCH |
| B1 | UNMATCHED | MATCH | MATCH | UNMATCHED | MATCH | MATCH |
| D1 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| F1 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| A2 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| C2 | MATCH | MATCH | UNMATCHED | MATCH | MATCH | UNMATCHED |
| H2 | UNMATCHED | UNMATCHED | MATCH | UNMATCHED | UNMATCHED | MATCH |
| D3 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| E3 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| G3 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| A4 | MATCH | MATCH | UNMATCHED | MATCH | MATCH | UNMATCHED |
| D4 | UNMATCHED | UNMATCHED | MATCH | MATCH | MATCH | MATCH |
| B5 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| D5 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| G5 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| A6 | MATCH | MATCH | UNMATCHED | MATCH | MATCH | UNMATCHED |
| C6 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| D6 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| F6 | MATCH | MATCH | UNMATCHED | MATCH | MATCH | MATCH |
| G6 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| H6 | MATCH | MATCH | MATCH | MATCH | MATCH | MATCH |
| B7 | MATCH | UNMATCHED | UNMATCHED | MATCH | UNMATCHED | MATCH |

**Table 12: Reproducibility comparison of Primerdesign kit calls from 22 samples previously run at our equine specialist lab partner**. The samples were thus run at Primerdesign to demonstrate reproducibility lab to lab. The

calls were compared to the specificity data generated (left) and the repeatability data (right). Red sample IDs show known variable samples.

## 4. DISCUSSION

The data reported in this document strongly supports the claim of accuracy, specificity, repeatability and reproducibility of the Primerdesign *T. equigenitalis*, *K. pneumoniae* and *P. aeruginosa* qPCR based pathogen detection kits. The initial design process was undertaken by experts in qPCR assay design with stringent design criteria and a wealth of experience. This was successfully confirmed by the first primer only and primer/probe analyses which demonstrated that the kits all contain primers specific to their target sequences that function with outstanding efficiency. The addition of the probe adds extra security to the user as the no template controls are consistently flat with these assays and this approach, making diagnostic calling easier and more precise. The kits have been shown to have a quantitative range of between 1000000 and 100 copies in each instance, where 100 copies equates to a Cq of approximately 32. Within this range it can be expected that copy number can be accurately calculated from a standard curve and that replicates will show excellent concordance. Primerdesign guarantees 100 copy detection for all pathogen detection kits which is in line with the determined quantitative range and the minimum requirements outlined by the design criteria. However, it is often found that kits can exceed this level of sensitivity and this was observed for all three of the kits where the limit of detection was calculated as 5 copies in each case, where 95% of replicates showed amplification prior to Cq 40. Further to this, all assays showed the capacity to amplify with significant reproducibility down to 2 copies and even 1 copy was amplified in 3 replicates for the *K. pneumoniae* and *P. aeruginosa* assays. This demonstrates the exquisite sensitivity of the assays and the technology. In spite of the assays demonstrated sensitivity, it is well established within the qPCR industry that cleavage probe assays have a limit of detection of approximately Cq35 with regard to clean, reproducible replicates. This limit was defined as 32 and 32.5 for the tested kits. The reason for this relates to the stochastic effects that influence PCR amplification in the presence of very low template copy numbers. These are uncontrolled influences, as discussed in section 4.2.1, that lead to reduced reproducibility between replicates under these conditions. As such, replicates that amplify later than the quantitative range can be used with less stringency to make qualitative Yes/No calls. This is the qualitative range. The use of cleavage probes allows data interpretation at late Cqs in the qualitative range, thus extending the usability of the kits. This is due to the specificity conferred by the probe whereby a fluorescent signal is only produced when the probe binds to a genuine product.

After the initial assay validation, the assays were further validated on biological samples to ensure that functionality was retained. It was also critical to ensure that that there was no cross reactivity between each assay the DNA of the other remaining kits. All three assays

performed perfectly whereby no cross reactivity was observed, leading forward into blind sample analysis.
The quantitative and qualitative ranges described above were set as boundaries, where different criteria for accepting data as valid were applied, taking into account the limitation of qPCR as a technology. While it was relatively straight forward to adhere to the data pass criteria within the defined quantitative and qualitative boundaries (as laid out in the Validation Plan) when using clean, simple synthetic templates, it was found on first sight of the blinded sample data that the added complexity of the biological sample material was influencing the PCR process and as such the pass criteria became too stringent for the data at hand. This was true for Primerdesign and Qiagen data such that no bias would be introduced when taking the decision to relax the criteria. The criteria described in the plan will enable the acquisition of results that are the best possible results from a qPCR experiment. However, biological samples often contain low copy numbers of target, a high background of co-extracted DNA, derived from the sampled horse or commensal microorganisms; as well as contaminants from storage and DNA extraction procedures that can act as PCR inhibitors. The complexity of biological samples is discussed in section 4.3 where it is reported that these factors led to a decline the quality of the amplification plots and their reproducibility between replicates. As such, the calling was altered to allow greater variability between replicate Cqs than outlined in the Validation Plan i.e. replicates to be within 0.5Cqs when Cqs are within the quantitative range. Many samples, covering all 3 tests and both kit types (Qiagen and Primerdesign) in these key studies would have otherwise been excluded based on one replicate that was outside of this 0.5 Cq criteria which was not a true reflection of the data. This most often occurred at Cqs approaching the limits of the quantitative range, e.g. Cq 30 when the end of the range is 32. The Grubb's test would have excluded the samples in many instances which, it was felt, would not be in the interest of the study. To ensure that these 'outliers' were true amplicons, additional scrutiny of the shape of the amplification plot, lift off point and end point fluorescence was applied.

For further justification, the plan required only 2 of the 3 replicates to amplify for the sample to be called positive and only 1 plot was outside of the criteria in all instances, thus not making it unreasonable to accept the 2 overlaying replicates. Another factor was that Cq calling per se was not straight forward given the shape of the plots from both suppliers (flattened rather than sigmoidal), low end point fluorescence seen as a result of late amplification and absence of a plateau phase in the vast majority of samples. All data sets presented in this manner independent of kit type or assay. The described data makes setting a threshold for Cq calculation relatively arbitrary, such that the actual Cq obtained is of less value compared to the amplification plots themselves. Further, the threshold set at 10% of the end point fluorescence, as outlined in the Validation Plan, is only applicable where a clear plateau is observed. As such, due to the lack of plateau in the samples, the threshold could

not be set according to the Validation Plan for each individual sample but was levelled using the positive control plots. The position of the threshold dictates the Cq called and therefore it's position can have a profound impact on the data obtained. It was not felt that the thresholds chosen were inappropriate for the analysis, simply that the 10% rule could not be applied to each sample. The 10% rule was however applied to the positive control sample and the position relative to the samples checked such that it was placed at the beginning of the logarithmic phase of the amplification plots. This suggests that relaxing the replicate requirements would not have a negative impact on the calling of the data. The horse breeding industry requires a call of low, medium or high infection rate which can still be easily achieved with the applied minor change in calling. With these arguments in place a change in the plan to fit the acquired data appeared to be the most appropriate response.

With the calling amended, the positive /negative calls for all experiments was completed prior to un-blinding the samples. The blind testing clearly demonstrated that Primerdesign kits were equal to or better than the Qiagen kit for accuracy on real world samples for all three assays. On the first set of data on the 50 samples the Primerdesign kit gave a total miscall percentage of 15.3% compared to 21.3% for the Qiagen kit. The same analysis was applied to the repeat whereby the Primerdesign kits gave a miscall percentage of 14.6% compared to 27.3% for the Qiagen kit. The Primerdesign kits have thus been shown to be considerably more accurate than the Qiagen multiplex and that the data was extremely repeatable since the error rate is extremely similar. Both kits had a tendency to miscall the same samples suggesting a sample related problem as opposed to kit failure or, in the case of false negatives, that both kits equally reached the limits of what could be reported upon. With persistent false positives it was hypothesised that there may have been a contamination issue.

The differences in call accuracy reported above will clearly have influenced the comparison between Primerdesign calls and Qiagen calls, showing this as disparity between the kits. It is thus important to remember that the disparity in this instance is highlighting the improved performance of the Primerdesign kit as opposed to failure next to the Qiagen assays. Obviously where both kits failed there will be agreement which confirms that the use of Primerdesign kits will not mean any loss of accuracy but that the kits equally failed on those samples. The striking difference was the improvement in the positive calling of *P. aeruginosa* with the Primerdesign assay over Qiagen which was a definite strength for that kit. In spite of this, both *P. aeruginosa* assays had a propensity to call false negatives. As both kits shared some degree of the same issue there may have been genuine design hurdles to overcome. Interestingly, the Primerdesign *P. aeruginosa a*ssay generally showed slightly later Cqs for the same copy number compared to the other kits which may explain the false negatives in this case. If design was genuinely less optimal then it is likely that Qiagen faced the same

problem with the same result, however the scale of their problem was considerably worse for the Qiagen kit. The reason for the reduced sensitivity may be design or could be that the multiplex format of the Qiagen kit further decreased sensitivity compared to a singleplex approach. Indeed, there are pros and cons to a multiplex vs singleplex approach. Multiplexing allows a reduction in the number of reactions to be run for each sample, this is particularly advantageous when sample is excessively limited or there are very large numbers of samples to screen i.e. high throughput screening programmes. However, a multiplexed approach is more complex whereby three sets of primers and probes are present in the reaction simultaneously. This gives rise to an increased possibility that the primers may interact with each other, thus making them unavailable for PCR and reducing the sensitivity in the presence of low copy numbers of target. To aid in preventing this, the primer concentration for each assay is often reduced, which can also potentially lead to a reduction in overall sensitivity. Another factor that can affect results is in circumstances when multiple assays are amplifying at the same time. Under these conditions it is possible for one assay to consume reagents at a faster rate and therefore deplete resources for amplification of the assay with lower abundance target DNA. This also results in a loss of sensitivity for the latter assay. These reasons alone may mean that some end users may prefer a more robust singleplex solution that Primerdesign can supply.

Further positive data supplied included repeatability tests. Whilst it was not in the Validation Plan to compare run to run variability on the Qiagen test it was used to put the error rate from the Primerdesign assays into context. During this analysis the Qiagen kit showed 1 fewer mismatches over the 150 tests (3 assays with 50 samples) than did the Primerdesign assays This shows that both manufacturer's kits showed excellent repeatability and that they performed equally well in this regard. The kits also performed well in the reproducibility test, although the need to dilute the samples may have had a negative impact on the results. Had the samples had high loads of target DNA the dilution would have been easily compensated for, with a Cq shift of only 1.5 Cqs. However, with some of these samples that was sufficient to gain a mismatched reading. The mismatching was predominantly on samples that showed vanishingly small levels of the target DNA in the comparison experiments. In spite of these challenges, an excellent reproducibility percentage concordance was achieved, though it was expected to be closer to that of the repeatability test.

To summarise. The assay designs have been validated with synthetic template and biological samples. The assays show excellent performance according to PCR efficiency calculations and LOD based sensitivity testing. None of the assays showed cross reactivity with biological template from the other two assays, thus showing total specificity for the target microorganism. Concordance between PCR results and cultured samples was not achieved but this expected to be for technical reasons and does not undermine the performance of the

qPCR kits. Swab testing proved that the Primerdesign kits had increased accuracy compared to the Qiagen multiplex as well as increased sensitivity. The Primerdesign kits showed excellent run to run consistency which was equal to that demonstrated by the Qiagen multiplex. Lab to lab trials were not ideal but the kits still showed good concordance between calls and those that did not agree could be easily explained.

This report has set out to advocate the use the of Primerdesign *T. equigenitalis*, *K. pneumoniae* and *P. aeruginosa* qPCR based pathogen detection kits in the routine screening for these sexually transmitted infections in breeding horses. Primerdesign wish to be recommended by the HBLB as an approved supplier of these kits within the horse breeding industry as an alternative choice to the current Qiagen 'cador *T. equigenitalis* PCR kit' and 'cador TKP' qPCR options. There are a number of reasons why it is felt that recommendation of an alternative to the Qiagen kits would be advantageous to the horse breeding community. Firstly, the report itself details that the Primerdesign kits exceed the performance of the Qiagen option in sensitivity and accuracy of calling. These are vital criteria in the diagnostic space and are a genuine and valuable improvement on the current testing option. In addition, the Primerdesign kits give the user more flexibility in the choice of qPCR platform that they can use with the kits and in whether a sample need be tested with one, all or a combination of the tests without using a more complex multiplex solution. It is understood that a multiplex solution can be advantageous for some users, however the increased complexity may have led to reduced sensitivity and accuracy and a higher risk of failure in the PCR compared to a singleplex approach. As a result, the Primerdesign assays may be a more robust option. In addition, the Primerdesign kits give genuine cost savings to the end user and are otherwise equally matched to the Qiagen kits with respect to ease of use and skill required in data interpretation.

Lastly, at time of writing the Qiagen kits are the only recommended kits within the horse breeding industry, giving no choice to the end user and no alternative in case of a problem with supply or product withdrawal from the market. Indeed, Qiagen have already removed the kits from the market once as recently as 2014, leaving no alternative option prior to their reintroduction. This creates doubt over the long term supply of the kits and questions as to why the kits were withdrawn as a product. As such, a viable off the shelf alternative with equal if not better performance, will protect the industry from withdrawn supply and prevent potentially considerable disruption and expense for breeders should supply of Qiagen kits be halted close to or during the breeding season.

Primerdesign prides itself on the quality of our design and manufacturing. These three kits are part of a greater collection of over 400 pathogen, biohazard, food and water testing kits that fall under the genesig® umbrella and which are sold worldwide. The company was founded

on these kits and they remain a corner stone of the business. It is an area in which we are extremely experienced and competent and the kits excel as a result. We strongly believe that addition of our product to the recommendations list would be truly beneficial to end user and the industry as a whole.