Validation of Legiolert*

for the enumeration of *Legionella pneumophila* from water

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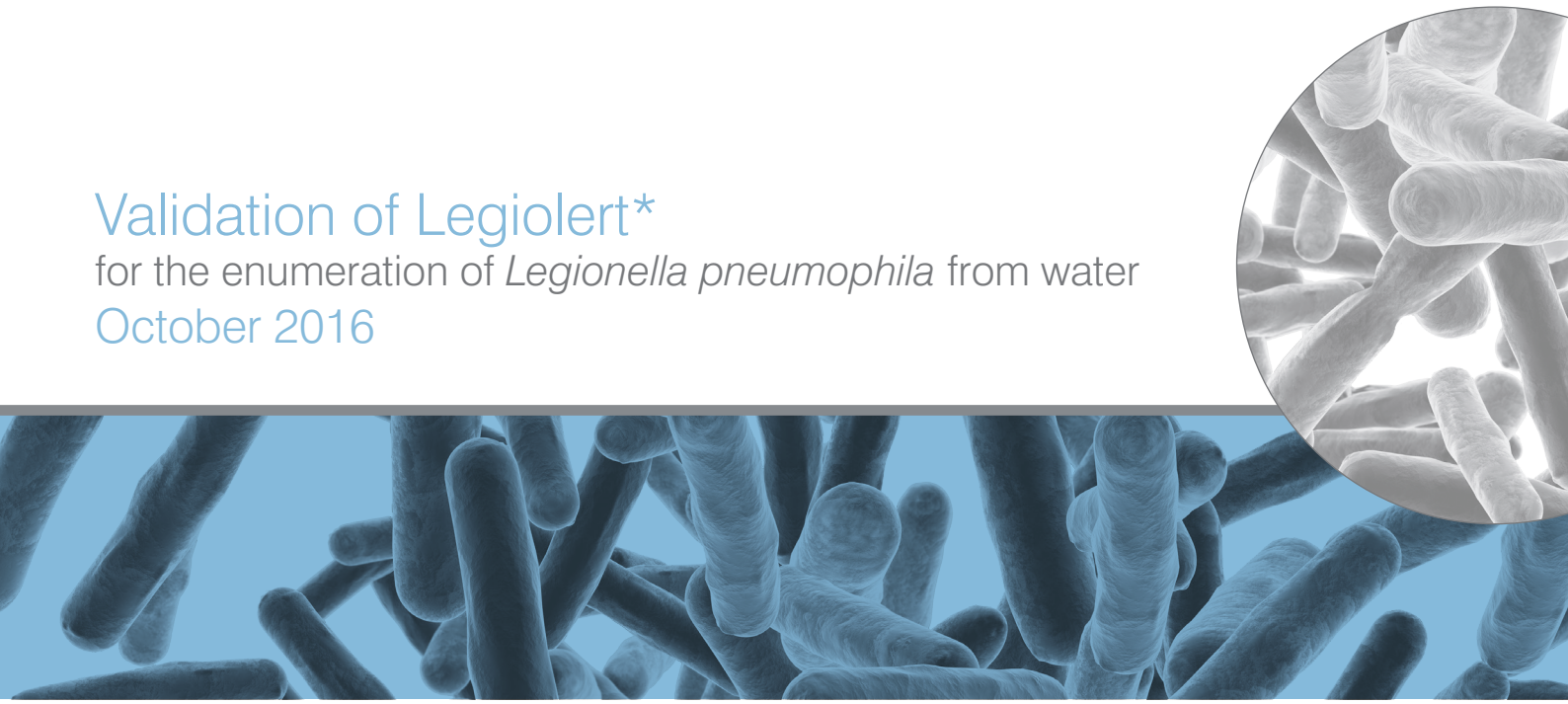

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Validation of Legiolert* for the Enumeration of *Legionella pneumophila* from Water

Executive Summary

The Legiolert method is a test specifically designed for the MPN enumeration of *Legionella pneumophila* from potable water samples and non-potable water, such as cooling tower water samples. Legiolert is presented as a powdered reagent in blister pack format for testing 100 ml water samples or smaller sample volumes made up to 100 ml. It utilizes IDEXX's selective formulation to detect *L. pneumophila*, which coupled with a specifically designed MPN reaction pouch (Quanti-Tray*/Legiolert) allows quantification of *L. pneumophila*. The Legiolert method was validated according to ISO/TR 13843:2000 using natural and spiked drinking water samples.

The method was challenged with isolates from naturally contaminated samples. Assessment of the sensitivity, specificity and selectivity of Legiolert was achieved through the identification of positive isolates from positive brown and/or turbid wells and subcultures from negative wells for each sample were selected for isolation for any potential bacteria present. The results of evaluations for sensitivity, specificity, selectivity and robustness of counting are summarized in the table below.

Sensitivity	98%
Specificity	> 99%
Selectivity	- 0.18
False positive rate	< 0.01%
False negative rate	4.2%
Efficiency	> 99%
Repeatability	< 0.01
Reproducibility	< 0.01

The Legiolert method was compared with the ISO 11731-2 method using membrane filtration and culture on selective media, following the requirements of ISO 17994:2014. Data from 271 routine German potable water samples (covering a range of water hardness tested by four laboratories accredited for the analysis of *Legionella*) were analysed. The outcome of the ISO 17994 two-tailed analysis is summarised below.

MRD%	S.D.	W	X_L	X_U	Conclusion
1.23	106.52	12.94	- 11.71	14.17	Methods not different

MRD% = Mean relative difference expressed as a percentage
S.D. = Standard deviation

ISO/TR 13843:2000(E) Water Quality – Guidance on validation of microbiological methods. Geneva: International Organization for Standardization.

ISO 11731-2 (2004) Water Quality – Detection and Enumeration of *Legionella* – Part 2: Direct membrane filtration method for waters with low bacterial counts. Geneva: International Organization for Standardization.

SO 17994 (2014) Water Quality – Requirements for the comparison of the Relative Recovery of Microorganisms by Two Quantitative Methods. Geneva: International Organization for Standardization.

Validation of Legiolert* for the Enumeration of *Legionella pneumophila* from Water

1 Introduction

The Legiolert method is a test specifically designed for the MPN enumeration of *Legionella pneumophila* (*L. pneumophila)* from potable water samples and non-potable water, such as cooling tower water samples.

Legiolert is presented as a powdered reagent in blister pack format for testing 100 ml water samples or smaller sample volumes made up to 100 ml. It utilizes IDEXX's selective formulation to detect *L. pneumophila*. Legiolert, coupled with a specifically designed Quanti-Tray*/Legiolert*, is incubated at 39°C ± 0.5°C with humidity for 7 days. When *L. pneumophila* are present in a water sample and tested using Legiolert, they produce any combination of brown pigment and turbidity, which represents a confirmed detection result. Enumeration is achieved by the most probable number (MPN) technique.

The Quanti-Tray/Legiolert used in this test is specifically designed for Legiolert to produce quantitative bacterial counts from 100 ml samples using target organism-specific media. The medium/sample mixture is added to a Quanti-Tray/Legiolert pouch which is then sealed in a Quanti-Tray Sealer PLUS prior to incubation. The pouch is designed so that after sealing there are 96 wells containing reagent/sample mixture. The Sealer PLUS is a motor-driven, heated roller instrument designed to seal a Quanti-Tray. The number of positive wells is counted and from an appropriate table the MPN of *L. pneumophila* is determined. The format of the pouch allows MPN values up to 2273 being recorded.

2 Scope of application of Legiolert

Legiolert is designed for the analysis of drinking and similar potable waters (e.g. hospital waters) and the analysis of non-potable waters, such as cooling tower water samples.

3 Target organism identification (ISO/TR 13843 sections 10.2.1 and 9.2)

In the Legiolert method *L. pneumophila* are those bacteria that produce any degree of brown colour and/or turbidity.

3.1 Pure culture challenge (ISO/TR 13843 section 10.2.1)

Definitions of reactions by target and non-target bacteria were confirmed by challenging Legiolert with pure cultures of reference strains of *L. pneumophila* and non-*L. pneumophila* species (sourced from ATCC) and selected Gram-negative and Gram-positive bacteria (sourced from ATCC and NCTC with the inclusion of some environmental isolates) inoculated into mains or bottled tap water. Typical reactions of these reference and environmental strains are presented in Table 1.

Table 1 **Strains of *Legionella pneumophila*, other species of *Legionella*, and selected Gram-negative and Gram-positive bacteria used to test typical positive and negative reactions in Legiolert**

Bacterium	Source	Calculated inoculum dose	Reaction in Legiolert
Legionella pneumophila SG-1	ATCC[1] 33152 WDCM[2] 00107	3 cfu[4]	Typical brown pigment
Legionella pneumophila SG-1	ATCC 33153	2 cfu	Typical brown pigment
Legionella pneumophila SG-1	ATCC 33153	12 cfu	Typical brown pigment
Legionella pneumophila SG-1	ATCC 43106	39 cfu	Typical brown pigment
Legionella pneumophila SG-1	ATCC 43110	36 cfu	Typical brown pigment
Legionella pneumophila SG-2	ATCC 33154	3 cfu	Typical brown pigment
Legionella pneumophila SG-3	ATCC 33155	32 cfu	Typical brown pigment
Legionella pneumophila SG-4	ATCC 33156 WDCM 00180	31 cfu	Typical brown pigment
Legionella pneumophila SG-5	ATCC 33216	42 cfu	Typical brown pigment
Legionella pneumophila SG-6	ATCC 33215	25 cfu	Typical brown pigment
Legionella pneumophila SG-7	ATCC 33823	28 cfu	Typical brown pigment
Legionella pneumophila SG-8	ATCC 35096	32 cfu	Typical brown pigment
Legionella pneumophila SG-9	ATCC 35289	33 cfu	Typical brown pigment
Legionella pneumophila SG-10	ATCC 43283	22 cfu	Typical brown pigment
Legionella pneumophila SG-11	ATCC 43130	26 cfu	Typical brown pigment
Legionella pneumophila SG-12	ATCC 43290	3 cfu	Typical brown pigment
Legionella pneumophila SG-13	ATCC 43736	2 cfu	Typical brown pigment
Legionella pneumophila SG-14	ATCC 43703	28 cfu	Typical brown pigment
Legionella pneumophila SG-15	ATCC 35251	36 cfu	Typical brown pigment
Legionella birminghamensis	ATCC 43702	2600 cfu	No reaction in Legiolert
Legionella cincinnatiensi	ATCC 43753	2300 cfu	No reaction in Legiolert
Legionella erythra	ATCC 35303	4300 cfu	No reaction in Legiolert
Legionella feeleii	ATCC 35072	4100 cfu	No reaction in Legiolert
Legionella hackeliae	ATCC 35250	2200 cfu	No reaction in Legiolert
Legionella jordansis	ATCC 33623	3600 cfu	No reaction in Legiolert
Legionella bozemanii	ATCC 33217	6200 cfu	No reaction in Legiolert
Legionella gormanii	ATCC 33297	5000 cfu	No reaction in Legiolert
Legionella oakridgensis	ATCC 33761	4100 cfu	No reaction in Legiolert
Legionella wadsworthii	ATCC 33877	4200 cfu	No reaction in Legiolert
Legionella sainthelensi	ATCC 35248	1600 cfu	No reaction in Legiolert
Legionella maceachernii	ATCC 35300	6200 cfu	No reaction in Legiolert
Legionella tucsonensis	ATCC 49180	2500 cfu	No reaction in Legiolert
Legionella lansingensis	ATCC 49751	5700 cfu	No reaction in Legiolert
Legionella dumoffii	ATCC 33279	9600 cfu	No reaction in Legiolert
Legionella micdadei	ATCC 33218	2400 cfu	No reaction in Legiolert
Legionella gratiana	ATCC 49413	1200 cfu	No reaction in Legiolert
Legionella steigerwaltii	ATCC 35302	2300 cfu	No reaction in Legiolert
Legionella santicrucis	ATCC 35301	1100 cfu	No reaction in Legiolert
Legionella cherrii	ATCC 35252	1000 cfu	No reaction in Legiolert
Legionella spiritensis	ATCC 35249	3700 cfu	No reaction in Legiolert

Aeromonas hydrophila	Environmental	1100 cfu	No reaction in Legiolert
Bacillus spp.	Environmental	3000 cfu	No reaction in Legiolert
Bacillus subtilis	ATCC 6633 WDCM 00003	4500 cfu	No reaction in Legiolert
Enterobacter aerogenes	ATCC 13048 WDCM 00175	1300 cfu	No reaction in Legiolert
Enterococcus faecalis	ATCC 19433 WDCM 00010	9300 cfu	No reaction in Legiolert
Enterococcus faecalis	ATCC 29212 WDCM 00087	9500 cfu	No reaction in Legiolert
Escherichia coli	ATCC 11775 WDCM 00090	1700 cfu	No reaction in Legiolert
Escherichia coli	ATCC 25922 WDCM 00013	1600 cfu	No reaction in Legiolert
Escherichia coli	NCTC[3] 9001 WDCM 00155	1800 cfu	No reaction in Legiolert
Klebsiella pneumoniae	ATCC 31488	1300 cfu	No reaction in Legiolert
Klebsiella pneumoniae	Environmental	1600 cfu	No reaction in Legiolert
Pseudomonas aeruginosa	ATCC 10145 WDCM 00024	1900 cfu	No reaction in Legiolert
Pseudomonas aeruginosa	ATCC 15442	1500 cfu	No reaction in Legiolert
Pseudomonas aeruginosa	ATCC 25668 WDCM 00144	1400 cfu	No reaction in Legiolert
Pseudomonas aeruginosa	ATCC 27853 WDCM 00025	1700 cfu	No reaction in Legiolert
Pseudomonas aeruginosa	NCTC 12951 WDCM 00207	1200 cfu	No reaction in Legiolert
Pseudomonas putida	ATCC 12633 WDCM 00117	1900 cfu	No reaction in Legiolert
Serratia fonticola	Environmental	2300 cfu	No reaction in Legiolert
Serratia marcescens	ATCC 13880	2300 cfu	No reaction in Legiolert
Staphylococcus aureus	NCTC 8532	7500 cfu	No reaction in Legiolert

[1] American Type Culture Collection.
[2] World Data Centre for Microorganisms.
[3] National Collection of Type Cultures (UK).
[4] Colony forming units.

3.2 Sensitivity, specificity and selectivity studies (ISO/TR 13843 section 9.2)

In terms of microbiological methods these characteristics are defined by ISO/TR 13843 as:

Sensitivity - the fraction of the total positives correctly assigned in the presumptive count;

Specificity - the fraction of the total negatives correctly assigned in the presumptive count;

Selectivity - the ratio of the number of target colonies to the total number of colonies in the sample volume.

For Legiolert, these characteristics relate to the number of positive wells for *L. pneumophila* that actually contained the target bacterium and the number of negative

wells that are truly negative for the target bacterium. Assessment of the sensitivity, specificity and selectivity of Legiolert was achieved through the identification of positive isolates from positive brown and/or turbid wells generated using naturally contaminated samples. These samples would reflect the range of potentially inhibiting background flora to be expected for *Legionella* analysis. After incubation, positive wells were selected from each sample (2 – 3 wells from each sample). These were selected to ensure that the whole range of positive reaction encountered were included. Similarly, negative wells for each sample were selected for isolation for any potential bacteria present. As a result, 639 positive wells and 331 negative wells were subcultured for isolation of any bacteria present. Isolates were identified as *Legionella* or non-target bacteria by sub-culturing to both BCYE and blood agar. Isolates exhibiting growth on BCYE only were considered *Legionella* and isolates growing on both media were considered non-targets. Volumes of 10 µl were removed from targeted wells using aseptic technique and inoculated onto BCYE and Blood Agar. A 2-zone streak pattern was performed in order to assist visualisation of individual colonies. Plates were incubated for min 2 days at 36 ± 2°C and observed for growth. Candidate isolates were sero-grouped using the Oxoid latex sero-grouping kit. Negative results were also confirmed using the Pro-lab Direct Fluorescent antibody kit (*L. pneumophila* SG 1-14).

3.2.1 Calculation of sensitivity, specificity, selectivity, false positive rate and false negative rates

For each parameter the identification data were divided into four categories:

a = number of positive wells found to contain *L. pneumophila* (true positives);

b = number of negative wells found to contain *L. pneumophila* (false negatives);

c = number of positive wells found not to contain *L. pneumophila* (false positives);

d = number of negative wells found not to contain *L. pneumophila* (true negatives).

The sensitivity, specificity, selectivity, false positive rate and false negative rates for *L. pneumophila* from the data were calculated as follows:

Sensitivity = a / (a+b)
Specificity = d / (c+d)
Selectivity = $\log_{10}$ [(a+c) / (a+b+c+d)]
False positive rate = c / (a+c)
False negative rate = b / (b+d)

A further parameter, efficiency (E), which gives the fraction of wells correctly assigned, was calculated as E = (a+d) / (a+b+c+d).

3.2.2 *Legionella pneumophila*

Isolates were obtained from all of the 639 positive wells sub-cultured. All were identified as *L. pneumophila*.

3.2.3 Negative wells

Three hundred and thirty-one (331) negative wells were sub-cultured, of which 14 yielded colonies that were identified as *Legionella* by sub-culturing to both BCYE and

blood agar. *Legionella* isolates were confirmed as *L. pneumophila* by antibody-mediated serotype latex agglutination. All 14 were isolates were identified as *L. pneumophila*.

3.3 Determination of characteristics related to sensitivity and specificity

In the context of the Legiolert test, *L. pneumophila* are organisms that produce a brown coloration and/or turbidity and the outcomes for the calculated parameters are:

Sensitivity = a / (a+b) = 639 / (639+14) = 0.98
Specificity = d / (c+d) = 317 / (0+317) = > 0.99
Selectivity = $\log_{10}$ [(a+c) / (a+b+c+d)] = $\log_{10}$ [(639+0) / (639+14+0+317)] = - 0.18
False positive rate = c / (a+c) = 0 / (639+0) = < 0.01
False negative rate = b / (b+d) = 14 / (14+317) = 0.042
Efficiency (E) = (a+d) / (a+b+c+d) = (639+317) / (639+14+0+317) = > 0.99

The outcomes of the analyses of the data show that for *L. pneumophila* the Legiolert method is very sensitive and specific for the target bacterium, with a false-positive rate of less than 0.01 (i.e. < 1 %). A false-negative rate of 0.042 was also recorded. This may have been due to stressing of the bacteria resulting in a slower response in the medium. The method is very selective with a value of - 0.18, which is better than the guidance value of - 1 suggested by ISO/TR 13843 for colony count methods. The method can also be said to be highly efficient for *L. pneumophila* with an efficiency value of > 0.99.

4 Counting uncertainty (ISO 13843 section 10.2.1 and Annex B)

Repeatability and reproducibility are two estimates of the reliability that can be achieved with an analytical method. These can be assessments of the whole method using appropriate natural samples, or of the counting uncertainty associated with reading the results of a method. The result produced by any method will be dependent upon the ease with which a count of colonies or positive MPN reactions can be made by analysts. This will be affected by the distinctiveness of colonial morphology of target and non-target organisms on an enumeration agar, or the clarity of positive and negative reactions in broth-based MPN tests. Assessments of counting uncertainty can, therefore, provide an indication of any potential problems that could occur with wide adoption of a method.

Repeatability (r) and reproducibility (R) are defined as follows:

repeatability	closeness of the agreement between the results of successive measurements of the same measurand carried out under the same conditions of measurement
Reproducibility	closeness of the agreement between the results of measurements on the same measurand carried out under changed conditions of measurement

When applied to counting of microbiological colonies on an agar plate or positive reactions in an MPN test, the same or changed ‘condition’ is the counting analyst. Thus, in this context, repeatability is the agreement in counts obtained by repeated counting by one analyst, and reproducibility is the agreement in counts obtained by repeated counting by two or more analysts. The assessment of reproducibility is generally more informative than the assessment of repeatability.

Annex B of ISO/TR 13843 provides guidance on the assessment of counting repeatability and reproducibility using relative standard deviations (RSD) of repeated counts. It also recommends that, when using pure cultures, RSDs should ideally be less than 0.02 (i.e. not more that 2 % deviation). Counting uncertainty studies were conducted by IDEXX analysts and the data are presented in Appendix A. The counting was done so that each analyst was not aware of other analysts' counts and the number of positive wells was recorded separately for each analyst. The counting was undertaken after 7 days incubation at 39°C. The calculated RSDs for positive wells from Legiolert trays using naturally contaminated samples (38 samples) or samples inoculated with a selection of 12 reference strain of *Legionella pneumophila* covering a range of serotypes (36 samples representing three for each strain at three differing count levels each) were:

Natural samples	Repeatability	< 0.01
	Reproducibility	< 0.01
Spiked samples	Repeatability	< 0.01
	Reproducibility	< 0.01

The values for the counting of positive wells of *L. pneumophila* from both natural and spiked samples are well below the guidance value of < 0.02 (ISO/TR 13843 section B1), indicating that very reliable counting of positive wells can be achieved with Legiolert.

5 Upper working limit (ISO/TR 13843 sections 10.2.4 and 6.3.3)

The Legiolert test is an MPN method with a counting range of 0 to 2273, determined by the number of wells in the Quanti-Tray/Legiolert pouch. According to ISO/TR 13843 section 6.3.3 no upper limit can be set for MPN methods for practical reasons and for statistical reasons "*because precision does not depend in a simple way on the number of particles introduced in the detection set*".

6 Precision (ISO/TR 13843 sections 10.2.5 and 9.5.5)

The precision of an MPN method is described by the 95% confidence intervals calculated for each MPN value (ISO/TR 13843 section 9.5.5). The confidence intervals for counts from Legiolert in the Quanti-Tray/Legiolert are available using MPN Generator program available from IDEXX.com (https://www.idexx.com/water/mpn-generator.html).

7 Relative recovery (ISO/TR 13843 section 10.2.6 and ISO 17994)

The Legiolert method was compared with the ISO 11731-2 method (currently being revised as ISO/DIS 11731:2015) using direct membrane filtration, following the requirements of ISO 17994. Three hundred and fifty-seven routine German potable water samples covering a range of water hardness were submitted by laboratories accredited for the analysis of *Legionella* and tested according to ISO 11731-2 by IDEXX. Of these samples, 271 yielded data appropriate for analysis of relative counts of *L. pneumophila*. MPN values were rounded to the nearest whole integer. ISO 11731-2 plates were counted after 7 – 10 days of incubation and confirmed according to ISO 11731-2. Paired confirmed count data for the two methods were natural logarithm (ln) transformed and the relative difference in counts for each pair (x_i) calculated as $[\ln(a_i) - \ln(b_i)] \times 100$ % (a_i and b_i being the paired confirmed counts for the trial and reference methods respectively). From the data a mean relative

difference and the standard deviation were calculated. Evaluation of equivalence is based on the mean relative difference and the expanded uncertainty (W) based on the standard deviation of the mean ($W = 2s/\sqrt{n}$), from which the lower (X_L) and higher (X_U) limits of the "confidence interval" are calculated. Based on this statistical approach and a one-sided evaluation, the outcomes of the comparative study are presented in Table 2 (raw data available upon request).

The comparison of Legiolert MPNs counted with confirmed ISO 11731-2 resulted in an outcome of "methods not different" as the "confidence limits" (X_L and X_U) are both within + or - 20% (proposed by ISO 17994 for environmental samples). The mean relative difference is also close to zero.

Table 2 Outcomes of the comparisons of performance for Legiolert and ISO 11731-2 for the enumeration of *Legionella pneumophila* from various waters

MRD%	S.D.	W	X_L	X_U	Conclusion
1.23	106.52	12.94	- 11.71	14.17	Methods not different

MRD% = Mean relative difference expressed as a percentage
S.D. = Standard deviation

8 References

ISO/TR 13843:2000(E) Water Quality – Guidance on validation of microbiological methods. Geneva: International Organization for Standardization.

ISO 11731-2 (2004) Water Quality – Detection and Enumeration of *Legionella* – Part 2: Direct membrane filtration method for waters with low bacterial counts. Geneva: International Organization for Standardization.

ISO 17994 (2014) Water Quality – Requirements for the comparison of the Relative Recovery of Microorganisms by Two Quantitative Methods. Geneva: International Organization for Standardization.

ISO/DIS 11731 (2015) Water Quality – Enumeration of *Legionella*. Draft International Standard, Geneva: International Organization for Standardization.

Appendix A Counting uncertainty for fluorescing wells from Legiolert inoculated with either naturally contaminated samples or samples spiked with *Legionella pneumophila* (see Section 4)

Appendix A1 Repeatability data for reading of positive wells from naturally contaminated samples

Analyst A: Legiolert reads @ 7 days +ve wells					
Sample	**Read 1**	**Read 2**	**Mean**	**St Dev**	**RSD sq**
2016-358	28	28	28	0.00	0.00
2016-359	2	2	2	0.00	0.00
2016-360	9	9	9	0.00	0.00
2016-361	2	2	2	0.00	0.00
2016-362	4	4	4	0.00	0.00
2016-363	12	12	12	0.00	0.00
2016-364	25	25	25	0.00	0.00
2016-365	75	75	75	0.00	0.00
2016-366	16	16	16	0.00	0.00
2016-367	94	94	94	0.00	0.00
2016-368	88	88	88	0.00	0.00
2016-369	53	53	53	0.00	0.00
2016-370	96	96	96	0.00	0.00
2016-371	86	86	86	0.00	0.00
2016-372	4	4	4	0.00	0.00
2016-373	16	16	16	0.00	0.00
2016-374	17	17	17	0.00	0.00
2016-375	21	21	21	0.00	0.00
2016-377	13	13	13	0.00	0.00
2016-378	10	10	10	0.00	0.00
2016-379	8	8	8	0.00	0.00
2016-380	24	24	24	0.00	0.00
2016-381	8	8	8	0.00	0.00
2016-382	4	4	4	0.00	0.00
2016-383	15	15	15	0.00	0.00
2016-384	20	20	20	0.00	0.00
2016-385	5	5	5	0.00	0.00
2016-386	10	10	10	0.00	0.00
2016-387	51	51	51	0.00	0.00
2016-391	1	1	1	0.00	0.00
2016-392	2	2	2	0.00	0.00
2016-393	2	2	2	0.00	0.00
2016-396	1	1	1	0.00	0.00
2016-399	6	6	6	0.00	0.00
2016-400	6	6	6	0.00	0.00
2016-402	24	24	24	0.00	0.00
2016-405	2	2	2	0.00	0.00
2016-407	1	1	1	0.00	0.00
				Sum RSD	**0.00**
				RSDc	**0.00**

Appendix A2 Repeatability data for reading of positive wells from artificially contaminated samples

Analyst A: Legiolert reads @ 7 days +ve wells					
Sample	**Read 1**	**Read 2**	**Mean**	**St Dev**	**RSD sq**
J20-e1	16	16	16	0.00	0.00
J20-e2	61	61	61	0.00	0.00
J20-e3	95	95	95	0.00	0.00
J21-e1	20	20	20	0.00	0.00
J21-e2	50	50	50	0.00	0.00
J21-e3	96	96	96	0.00	0.00
J22-e1	10	10	10	0.00	0.00
J22-e2	59	59	59	0.00	0.00
J22-e3	96	96	96	0.00	0.00
J23-e1	10	10	10	0.00	0.00
J23-e2	45	45	45	0.00	0.00
J23-e3	96	96	96	0.00	0.00
J24-e1	16	16	16	0.00	0.00
J24-e2	47	47	47	0.00	0.00
J24-e3	96	96	96	0.00	0.00
J25-e1	18	18	18	0.00	0.00
J25-e2	56	56	56	0.00	0.00
J25-e3	96	96	96	0.00	0.00
J26-e1	6	6	6	0.00	0.00
J26-e2	17	17	17	0.00	0.00
J26-e3	68	68	68	0.00	0.00
J27-e1	10	10	10	0.00	0.00
J27-e2	60	60	60	0.00	0.00
J27-e3	96	96	96	0.00	0.00
J28-e1	11	11	11	0.00	0.00
J28-e2	32	32	32	0.00	0.00
J28-e3	92	92	92	0.00	0.00
J29-e1	16	16	16	0.00	0.00
J29-e2	66	66	66	0.00	0.00
J29-e3	96	96	96	0.00	0.00
J30-e1	9	9	9	0.00	0.00
J30-e2	19	19	19	0.00	0.00
J30-e3	85	85	85	0.00	0.00
J31-e1	10	10	10	0.00	0.00
J31-e2	42	42	42	0.00	0.00
J31-e3	96	96	96	0.00	0.00
				Sum RSD	**0.00**
				RSDc	**0.00**

Appendix A3 Reproducibility data for reading of positive wells from naturally contaminated samples

Three analysts – Legiolert reads @ 7 days +ve wells						
Sample	**A**	**B**	**C**	**Mean**	**St Dev**	**RSD sq**
2016-358	28	28	28	28	0.00	0.00
2016-359	2	2	2	2	0.00	0.00
2016-360	9	9	9	9	0.00	0.00
2016-361	2	2	2	2	0.00	0.00
2016-362	4	4	4	4	0.00	0.00
2016-363	12	12	12	12	0.00	0.00
2016-364	25	25	25	25	0.00	0.00
2016-365	75	75	75	75	0.00	0.00
2016-366	16	16	16	16	0.00	0.00
2016-367	94	94	94	94	0.00	0.00
2016-368	88	88	88	88	0.00	0.00
2016-369	53	53	53	53	0.00	0.00
2016-370	96	96	96	96	0.00	0.00
2016-371	86	86	86	86	0.00	0.00
2016-372	4	4	4	4	0.00	0.00
2016-373	16	16	16	16	0.00	0.00
2016-374	17	17	17	17	0.00	0.00
2016-375	21	21	21	21	0.00	0.00
2016-377	13	13	13	13	0.00	0.00
2016-378	10	10	10	10	0.00	0.00
2016-379	8	8	8	8	0.00	0.00
2016-380	24	24	24	24	0.00	0.00
2016-381	8	8	8	8	0.00	0.00
2016-382	4	4	4	4	0.00	0.00
2016-383	15	15	15	15	0.00	0.00
2016-384	20	20	20	20	0.00	0.00
2016-385	5	5	5	5	0.00	0.00
2016-386	10	10	10	10	0.00	0.00
2016-387	51	51	51	51	0.00	0.00
2016-391	1	1	1	1	0.00	0.00
2016-392	2	2	2	2	0.00	0.00
2016-393	2	2	2	2	0.00	0.00
2016-396	1	1	1	1	0.00	0.00
2016-399	6	6	6	6	0.00	0.00
2016-400	6	6	6	6	0.00	0.00
2016-402	24	24	24	24	0.00	0.00
2016-405	2	2	2	2	0.00	0.00
2016-407	1	1	1	1	0.00	0.00
					Sum RSD	**0.00**
					RSDc	**0.00**

Appendix A4 Reproducibility data for reading of positive wells from artificially contaminated samples

Three analysts – Legiolert reads @ 7 days +ve wells						
Sample	**A**	**B**	**C**	**Mean**	**St Dev**	**RSD sq**
J20-e1	16	16	16	16	0.00	0.00
J20-e2	61	61	61	61	0.00	0.00
J20-e3	95	95	95	95	0.00	0.00
J21-e1	20	20	20	20	0.00	0.00
J21-e2	50	50	50	50	0.00	0.00
J21-e3	96	96	96	96	0.00	0.00
J22-e1	10	10	10	10	0.00	0.00
J22-e2	59	59	59	59	0.00	0.00
J22-e3	96	96	96	96	0.00	0.00
J23-e1	10	10	10	10	0.00	0.00
J23-e2	45	45	45	45	0.00	0.00
J23-e3	96	96	96	96	0.00	0.00
J24-e1	16	16	16	16	0.00	0.00
J24-e2	47	47	47	47	0.00	0.00
J24-e3	96	96	96	96	0.00	0.00
J25-e1	18	18	18	18	0.00	0.00
J25-e2	56	56	56	56	0.00	0.00
J25-e3	96	96	96	96	0.00	0.00
J26-e1	6	6	6	6	0.00	0.00
J26-e2	17	17	17	17	0.00	0.00
J26-e3	68	68	68	68	0.00	0.00
J27-e1	10	10	10	10	0.00	0.00
J27-e2	60	60	60	60	0.00	0.00
J27-e3	96	96	96	96	0.00	0.00
J28-e1	11	11	11	11	0.00	0.00
J28-e2	32	32	32	32	0.00	0.00
J28-e3	92	92	92	92	0.00	0.00
J29-e1	16	16	16	16	0.00	0.00
J29-e2	66	66	66	66	0.00	0.00
J29-e3	96	96	96	96	0.00	0.00
J30-e1	9	9	9	9	0.00	0.00
J30-e2	19	19	19	19	0.00	0.00
J30-e3	85	85	85	85	0.00	0.00
J31-e1	10	10	10	10	0.00	0.00
J31-e2	42	42	42	42	0.00	0.00
J31-e3	96	96	96	96	0.00	0.00
					Sum RSD	**0.00**
					RSDc	**0.00**