

Comparative Evaluation of the Seegene Seeplex RV15 and Real-Time PCR for Respiratory Virus Detection

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Comparative Evaluation of the Seegene Seeplex RV15 and Real-Time PCR for Respiratory Virus Detection

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Reaction #	FAM channel	JOE channel	Cy5 channel
1	FLA	FLB	
2	RSV		
3	PF2	PF1	PF3
4	ADV	Enterovirus	
5			HRV
6	MPNV		
7	PDV	K-Ras	

Table I

Distribution of primer and probe combinations and probe dye-labels within each of the seven real-time RT-PCR reactions, i.e. reaction 4 had adenovirus and enterovirus primers and probes.

Virus		Sequence	Final Conc. (nM)
Influenze A	Forward	CAAGACCAATCCTGTCACCTCTG	900
$(\mathbf{FL} \mathbf{A})$	Reverse	TGCATTTTGGACAAAGCGTCTAC	900
(I'LA)	Probe	FAM-AGTCCTCGCTCACTGGGCACGGT-BHQ1	200
Influence D	Forward	GGCAACACCTGTTTCCATATTAAG	900
(FL B)	Reverse	GCCTGTTAATGCAGACAAAACTC	900
(I'LD)	Probe	JOE-TCATAGGCAGCTCCGAAGCAAGACATGA-BHQ1	200
Description	Forward	GAAGGGTCAAACATCTGTTTAACAAG	900
Respiratory	Reverse 1	GTTTCAGCTWGTGGGAAGAAAGATA	900
(PSV)	Reverse 2	GTGTCAGCCTGTGGAAAGAAGGATA	900
$(\mathbf{K}\mathbf{S}\mathbf{V})$	Probe	FAM-TGATCCTGCATTRTCACARTACCATCCTCT-BHQ1	200
Densinfly and 1	Forward	ATTCAGACAGGATGGAACCGTTAA	900
$(\mathbf{DE1})$	Reverse	GATACTAAGCTTTGTTGTGACCTCAT	900
(ГГ1)	Probe	JOE-ACCAATGCCTTCAACTGTGTCTCCCGTG-BHQ1	200
Dansinflaanse 2	Forward	AGAGATAACAGGGTTTGAGAATAATTCAT	900
(DE2)	Reverse	CAAATGGAGTTTGGTGATTAAGGGTA	900
(ГГ2)	Probe	FAM-TCCAGATGCTCGATCAACTATGTCCAGAGG-BHQ1	200
D : C 2	Forward	CGATTAGAGGCTTTCAGACAAGATG	900
Parainfluenza 3	Reverse	CTGTTGAGACCGCATGATTGAC	900
(ГГЭ)	Probe	Cy5-CCACTGTGTCACCGCTCAATACCAGCC-BHQ3	200
	Forward	CCCCTGAATGCGGCTAATCC	900
Enterovirus	Reverse	GTCACCATAAGCAGCCAATATAAGAA	900
	Probe	AACACGGACACCCAAAGTAGTCGGTTCC	200
A 1	Forward	GCCCCAGTGGTCTTACATGCACATC	900
Adenovirus	Reverse	GCCACGGTGGGGTTTCTAAACTT	900
(ADV)	Probe	FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1	200
	Forward 1	TGTGCTCRCTTTGAGTCCTC	900
Rhinovirus	Forward 2	TGTGCTCAGTGTGCTTCCTC	900
(HRV)	Reverse	TGAAACACGGACACCCAAAGTA	900
	Probe	Cy5-CCCTGAATGYGGCTAACCTTAAMCCTGC-BHQ3	200
	Forward	CATATAAGCATGCTATATTAAAAGAGTCTC	500
Metapneumovirus	Reverse	CCTATTTCTGCAGCATATTTGTAATCAG	250
(MPNV)	Probe	FAM-TGYAATGATGAGGGTGTCACTGCGGTTG-BHQ1	500
K-Ras	Forward	GCCTGCTGAAAATGACTGAATATAAAC	600
	Reverse	TGATTCTGAATTAGCTGTATCGTCAAG	600
	Probe	JOE-TGCCTACGCCACAAGCTCCAACTACCA-BHQ1	100
	Forward	GCGGGTGCCTTTTACAAGAAC	600
PDV	Reverse	CAGAATAAGCAAAATTGATAGGAACCAT 🦳	600
	Probe	FAM-TCTTTCCTCAACCTCGTCCGTCACAAGT-BHQ1	100

Table II

Primer and probe sequences for each of the ten targets detected by the real-time respiratory RT-PCR system. The ADV primer-probe set is derived from those described in [Heim et al., 2003], and the MPNV set from [Maertzdorf et al., 2004]. The remaining primers and probes were developed in-house.

Single virus samples	n	C _t range [†]	Dual virus samples	n
FLA	9	18.5 - 31.4	ADV & HRV	2
FLB	9	22.7 - 37.6	PF1 & ADV	1
HRV	9	21.2 - 38.3	RSV & ADV	2
ADV	9	23.4 - 38.1	RSV & HRV	1
MPNV	8	22.4 - 35.0	HRV & MPNV	4
RSV	8	25.3 - 38.2	FLA & MPNV	2
PF1	13	23.8 - 37.9	FLA & RSV	4
PF2	6	25.3 - 35.3	PF3 & MPNV	1
PF3	11	25.3 - 37.5	PF2 & ADV	1

Table III

Characteristics of the 100 respiratory extracts tested by Seeplex RV12. Either single targets (left-hand two columns) or two targets (right-hand columns) were detected in each sample by in-house multiplex RT-PCR. The target range of RV12 comprises these 9 viruses, together with two human coronavirus groups (229E/NL63 and OC43/HKU1). Also, RSV-A and RSV-B are detected separately.

† Ct ranges are derived from both single and dual virus samples.



Single virus samples	n	C _t range [†]
FLA	10	23.6 - 34.6
FLB	17^{*}	18.8 - 32.5
HRV	4	23.5 - 36.0
ADV	10	20.4 - 32.4
MPNV	7	20.6 - 31.5
RSV	9	23.4 - 36.4
PF1	9	22.7 - 30.6
PF2	5^*	20.7 - 32.7
PF3	7	23.3 - 32.6

Dual virus samples	n
ADV & PF3	1
FLB & HRV	1
FLB & PF3	1
FLA & PF2	1
RSV & ADV	2^*

Table IV

Characteristics of the 84 respiratory extracts tested by Seeplex RV15. Either single targets (left-hand two columns) or two targets (right-hand columns) were detected in each sample by in-house multiplex RT-PCR. Four FLB, two PF2, and both RSV-ADV dually-infected samples had been tested previously in the RV12 evaluation. All viruses in these repeated samples had been previously detected by the RV12 system. In addition to the 12 targets detected by RV12, RV15 also detects human bocavirus, human parainfluenza 4, and enterovirus.

[†] Ct ranges are derived from both single and dual virus samples.

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Sample #	PCR result (C _t)	RV12
7	FLB (35.77)	None
29	FLB (35.70)	None
83	FLB (32.01)	None
94	FLB (37.56)	None
60	HRV (33.54)	None
43	MPNV (35.32)	None
32	PF1 (35.21)	RSVb
27	HRV (29.34), MPNV (35.62)	HRV, ADV
82	FLA (30.81), MPNV (35.94)	FLA
87	MPNV (26.57), PF3 (33.92)	MPNV
100	PF2 (28.5), ADV (38.11)	PF2
17	ADV (31.65)	ADV, 229E
18	ADV (26.85), RSV (32.64)	ADV, RSVa, HRV
39	RSV (30.24)	RSVa, OC43 , ADV
47	ADV (33.44)	ADV, OC43
50	FLA (28.95)	FLA, HRV
55	HRV (32.69)	HRV, OC43
66	HRV (21.16)	HRV, RSVa
70	FLA (27.98)	FLA, RSVa
71	HRV (31.81)	HRV, RSVa
90	PF3 (31.81)	PF3, RSVb
102	PF2 (29.14)	PF2, HRV
(b)		2

Sample #	PCR result (C _t)	RV15
156	FLB (26.50)	None
158	FLB (30.30)	None
159	FLB (30.41)	None
160	FLB (29.46)	None
170	FLB (31.64)	None
142	ADV (30.02)	None
143	ADV (29.76)	None
150	ADV (23.38)	None
108	PIV-2 (26.98), FLA (28.70)	None
182	ADV (27.20)	HRV, RSV
107	MPNV (26.92)	MPNV, HRV
134	RSV (29.86)	RSVb, HEV
165	FLA (27.03)	FLA, OC43
176	FLB (25.13)	FLB, OC43
183	RSV (31.96)	RSVb, BocV

Table V

Summary of the discrepant results (highlighted in bold type) between the in-house real-time PCR and Seeplex® RV12 (a) and RV15 (b). Samples where Seeplex® missed targets detected by PCR are given first.





Figure 2: Plot showing how peak heights obtained using Seeplex® RV12 and the TapeStation microelectrophoresis platform display negative correlation with the threshold cycles obtained from the RT-PCR method. Data shown is from Influenza A-positive samples. 105x64mm (300 x 300 DPI)

Title Page

Title: Comparative Evaluation of the Seegene Seeplex RV15 and Real-Time PCR for Respiratory Virus Detection

Running Title: Real-Time PCR and Seeplex Systems for Respiratory Virus Detection

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1 Abstract

Respiratory virus infections contribute substantially both to hospitalisations of young
children, and to morbidity in immunocompromised patients such as those with
haemotological malignancies. Their rapid and accurate diagnosis is essential to patient
management.

To evaluate the prospective utility of Seeplex® DPO technology in respiratory virus
diagnosis, a panel of 99 respiratory samples positive by real-time RT-PCR for one or
more viruses was assayed by the Seegene Seeplex® RV12 system. As well as being
able to detect all ten viruses in the real-time RT-PCR system with the exception of
enteroviruses, RV12 can also distinguish between the two subgroups of RSV and
detect two subgroups of coronaviruses.
Seven of the 9 viruses in common with the RT-PCR were detected reliably by RV12.

Eleven samples RT-PCR-positive for Metapneumovirus and 5 samples positive for
Influenza B were not detected by RV12. Seegene developed a second-generation
system, RV15, which not only allowed detection of 3 additional viruses, but also
addressed the potential problems with RV12 specificity. To address these concerns,
84 respiratory samples positive for a range of viruses by real-time PCR were assayed
with RV15. The results of this evaluation improved significantly upon those seen with

19 RV12.

20 The high throughput capabilities and potential lower technical requirements afforded

21 by the Seeplex® system may offer an alternative to real-time RT-PCR systems.

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22 Text23 Introduction

24 Timely and accurate diagnosis of respiratory virus infection is essential to patient care 25 in settings as diverse as haematopoietic stem cell transplant recipients [Jalal et al., 26 2007; Piralla et al., 2009], childhood bronchitis and pneumonia [Shay et al., 2001; 27 Michelow et al., 2004] and the seasonal influenza epidemics implicated in substantial 28 annual mortality [Mook et al., 2008]. As such infections often present with similar 29 symptoms, laboratory analysis is essential. 30 Many laboratories rely still on non-molecular approaches such as direct 31 immunofluorescence and viral culture. However, in the UK, various real-time PCR-32 based systems have been employed for a number of years, their utility having been 33 demonstrated largely by comparison with these more traditional methods [Templeton 34 et al., 2004; Bustin and Mueller, 2005; Freymuth et al., 2006; van de Pol et al., 2007; 35 Mahony et al., 2007; Kuypers et al., 2009] (reviewed in Mahony, 2008). Emerging 36 technologies thus have to demonstrate significant benefit against these existing tools 37 to achieve widespread adoption [Pabbaraju et al., 2008; Arens et al., 2010]. 38 The novel Seeplex® system exploits Dual Priming Oligonucleotides (DPOs) as PCR 39 primers, each comprising a standard primer-length 5' portion which initiates priming, 40 separated by multiple inosine residues from a shorter, 6-12 nucleotide 3' component 41 which determines duplex extension. As well as facilitating melting temperature 42 regulation during primer design, mispriming and PCR artefacts such as primer 43 dimerisation are almost eliminated, allowing simultaneous and efficient amplification 44 of multiple complex targets in single reactions [Chun et al., 2007]. 45 The system summary in Figure 1 shows a standard nucleic acid extraction, a cDNA 46 step, and then a DPO PCR, in which the products of each target virus have different

47 nucleotide lengths. Post-PCR product analysis employs an automated bench-top
48 system with which target detection interpretations are inferred from PCR product
49 sizes.

50 The RV12 system enables simultaneous detection of 12 respiratory viruses in 2

51 reactions per sample. Its performance when compared to traditional diagnostic

52 methods has been demonstrated frequently [Yoo et al., 2007; Drews et al., 2008; Roh

53 et al., 2008; Kim et al., 2009], with potential time and resource savings when

54 compared to more modern methods, particularly in high throughput settings where

55 prompt diagnosis is important. More recently, the RV15 kit has been developed that

56 targets 15 viruses in 3 reactions.

57 No studies evaluating either system against real-time PCR-based respiratory virus

58 detection methods have yet been published. The results of a comparative evaluation of

59 serial iterations of the Seeplex® system with the multiplex PCR-based method that

60 has been used for 5 years as the primary diagnostic assay for respiratory virus

61 detection in a major London hospital are described.

62 Materials and Methods

Samples were nasopharyngeal aspirates, bronchio-alveolar lavages, or respiratory swabs (nose and/or throat) submitted to the laboratory for routine testing. Aspirates and lavages were diluted in saline and centrifuged to separate cellular material from mucoid and liquid components. Swabs were transported in viral transport medium and extracted directly. Two automated nucleic acid extractors were employed at different times during the course of this evaluation - the NucliSENS easyMAG platform (Biomérieux, Marcy l'Etoile, France) and the QIAamp One-For-All Nucleic Acid Kits on the Qiagen BioRobot MDx DSP (Qiagen, Crawley, UK). Immediately prior to extraction, samples were supplemented with a fixed quantity of phocine distemper virus (PDV), obtained from cell culture supernatant and stored in lots at -80°C. Additionally, all samples that underwent easyMAG extraction were pre-incubated at 56°C for 10 minutes with Buffer AL and 30mAU/ml proteinase K (both Qiagen). Nucleic acid extracts were stored at -80°C between extraction and analysis. **Real-Time RT-PCR**. Each sample extract was subjected to seven simultaneous real-time RT-PCRs, with each reaction detecting between one and three targets (Table I). The seventh reaction constituted a duplex control reaction, with primers and probes targeting both a human

80 gene from the *ras* family (K-Ras) [Hilbe et al., 2003] and Protein H (haemagglutinin)

- 81 of the PDV internal control RNA.
- Mastermixes and samples were dispensed robotically into the Rotorgene Gene-Disc
 reaction vessels by a CAS1200 liquid handler (both Qiagen).
- 84 Quantitect Multiplex RT-PCR Mix NoRox (Qiagen), specific primers and probe
- 85 combinations (Table II, all obtained from Sigma-Genosys, Gillingham, UK), and 10µl
 - 86 of nucleic acid extract were mixed in 25µl reactions. Real time cycling, detection and

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post-run analysis were performed on the 72-well Rotorgene platform (Qiagen) with
fixed fluorescence thresholds, allowing direct comparison both between samples and
between runs. Positive controls (a panel of plasmids containing the specific PCR
target as inserts) for each respiratory virus target were included in each run, and
compared to expected threshold cycle (Ct) values. For each sample, Ct values for the
PDV and K-Ras control reactions were compared to expected values before parallel
PCR results were considered valid.

94 Seeplex® RV12.

95 Ninety-nine respiratory extracts stored at -80°C were selected that previously had

96 tested positive by RT-PCR for one or more respiratory virus targets. With the

97 exception of enterovirus and the internal control virus (PDV), which were not

98 detected by RV12, all viral targets in the RT-PCR were represented (Table III).

99 Random hexamer-primed cDNA synthesis products were generated using the

100 Revertaid system (Fermentas, York, UK), according to the manufacturer's

101 instructions, and stored at -20°C until use.

102 Each cDNA preparation was subjected to the RV12 PCR procedure according to the 103 manufacturer's instructions (Seegene, Seoul, South Korea). Briefly, parallel 20µl 104 reactions were set up, each containing RV12 mastermix, 8-MOPS contamination 105 control reagent, and 3µl of cDNA. One of each pair was supplemented with 4µl 106 primer mix A, the other with 4μ l primer mix B. Thermal cycling conditions were as 107 follows: 15 minutes at 94°C, followed by 40 cycles of 94°C for 30s, 60°C for 90s, and 108 72°C for 90s, followed by a single incubation of 10 minutes at 72°C. Completed 109 reactions were analysed using the MultiNA platform (Shimadzu Corporation, Kyoto,

110 Japan), coupled to a dedicated Seegene software template. Analysis output comprised

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both a qualitative interpretation of presence or absence of each virus target, and adetermination of peak intensity.

113 Seeplex® RV15.

114 Eighty-four respiratory extracts stored at -80°C testing positive for one or more

- 115 RT-PCR virus targets (Table IV) were subjected to cDNA synthesis using the Mu-LV
- 116 system (Invitrogen, Paisley, UK), according to the manufacturer's instructions.
- 117 Reaction products were stored at -20°C until use. Eight samples of the 84 had been

118 part of the previous RV12 analysis.

- 119 Each cDNA preparation was subjected to the RV15 PCR procedure according to
- 120 Seegene's instructions. Briefly, parallel 20µl reactions were set up for each sample, as
- 121 per the RV12 kit, but with three reactions per sample (primer mixes A, B & C) rather
 - 122 than the two in the earlier kit. Thermal cycling conditions were as per the RV12 kit.
- 123 Completed reactions were analysed using the Tape Station platform (Lab901,
- 124 Edinburgh, UK) linked to a specific Seeplex® RV15 interpretation software module.
- 125 Again, results were presented both qualitatively and semi-quantitatively, according to
- 126 the migration rate and band intensity of each species present within completed

127 reactions.

Redesigned Influenza B Primers.

129 During the evaluation, a new panel of influenza B primers was developed by Seegene

- 130 to replace those in the RV15 kit. The cDNA preparations of the 19 FLB-positive (by
- 131 RT-PCR) samples analysed by RV15 were subjected to amplification using these
- 132 primers under the same conditions as for the primer mixes of the RV12 and RV15
- 133 kits, but in monoplex reactions. Analysis was performed as per RV15, above.

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134	Results	

135 Seeplex® RV12.

- 136 All targets detected by the RT-PCR were also detected by Seeplex® RV12 in 88 of
- 137 the 99 nucleic acid extracts tested with the Seeplex® RV12 system. In 7 of the
- remaining 11 samples, single infections were missed (4xFLB, one each of HRV, PF1,
- and MPNV), and in four samples where two viruses had been detected by RT-PCR,
- 140 the virus target with the higher C_t was missed by RV12 (2xMPNV, 1xPF3 and
- 141 1xADV). Comparison of MultiNA peak heights and RT-PCR C_t values for each virus
- 142 target revealed a broad negative correlation between the two parameters, with the
- 143 exception of HRV, where no correlation was observed (Figure 2).
- 144 In all instances where PF2 (7 samples), FLA (15), and/or RSV (15) were detected by
- 145 RT-PCR, these virus targets were detected with RV12 also. For each instance where
- 146 PF1, PF3, HRV, and/or ADV were detected by RT-PCR, all except one of each virus
- 147 target were detected by Seeplex® RV12. Each instance of undetected virus
- 148 represented a sample whose C_t was found in the higher part of the range for that virus.
- 149 FLB and MPNV targets were detected less frequently. Four of the 5 FLB-positive
- 150 samples with the highest C_t values, and the 3 MPNV-positive samples with the
- 151 highest C_t values were not detected by RV12.
- 152 Conversely, ADV, RSV, HRV and MPNV were detected by RV12 in extracts where
- 153 the RT-PCR system had failed to do so (in 3, 4, 4, and 1 sample respectively), and
- 154 with the exception of the RSV detections, had high peak heights, consistent with high
- 155 target concentrations in the initial samples.

156 **Seeplex® RV15**.

- 157 Eighty four extracts from respiratory samples RT-PCR-positive for at least one
- respiratory virus were tested by RV15. With the exception of ADV and FLB, all

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viruses detected by RT-PCR were also detected by RV15, including all instances of MPNV (7/7). Inverse correlations between C_t and RV15 band intensity were less pronounced than that seen with RV12. Three of 9 ADV-positive samples were not detected by RV15. All four FLB-positive samples that had also been tested with RV12 remained FLB-positive by RV15. However, a further 5 previously untested FLB-positive samples failed to signal with RV15. Again, viruses were detected by RV15 that were not detected by the RT-PCR: HRV, enterovirus, coronavirus, and bocavirus (1, 1, 2, and 1 sample respectively). The RT-PCR system did not target either coronavirus or bocavirus. Table V details the discrepant results between the RT-PCR and Seeplex® systems. **Redesigned Influenza B Primer Set.** Using the new primer set designed to overcome the FLB-detection limitations seen with RV12 and RV15, all FLB-positive samples in which FLB had been detected by RV15 were FLB-positive by the new primer set, as were four of the five samples FLB-negative by RV15. The single sample where FLB was not detected by Seeplex® had given a C_t of 30 by RT-PCR. The lack of amplification controls in this assay series precluded detection of PCR inhibition.

Discussion

177	An in-house multiplex RT-PCR system and two iterations of a novel PCR-based
178	technology for the detection of respiratory viruses have been compared. In the first
179	instance, 99 samples were evaluated, and in the second, 84. A further panel of 19
180	FLB-positive samples was tested by a revised primer set within the novel Seeplex®
181	system. Whilst direct comparison of the two systems is limited, due to the sample sets
182	having been pre-determined to be positive for one or more target viruses by one
183	system, it can be stated that the overall performance of the Seeplex® RV12 and RV15
184	systems was very good, with several instances of viruses being detected where the
185	RT-PCR system had either failed or had been unable to do so.
186	Furthermore, with each virus, some negative correlation was observed between RT-
187	PCR threshold cycle and Seeplex® peak intensity, suggesting the latter could also be
188	employed semi-quantitatively.
189	The frequency of instances where targets detected by RT-PCR were missed by
190	Seeplex® was low, and may have been influenced by repeated freeze-thaw cycles of
191	affected samples, nearly all of which were at the upper end of the relevant C_t range
192	(i.e. with lower target concentrations). However, both MPNV and FLB targets were
193	detected poorly by RV12, with concomitant low band intensities from samples where
194	these targets were detected. Revision of the primer sets was a key component of the
195	development of RV15. RV15 gave a 100% detection rate of all viruses detected by
196	RT-PCR, including seven MPNV-positive samples, but no improvement in FLB
197	detection. The latter primer set was modified further, and retesting the panel of known
198	FLB-positive samples gave substantially greater success. Although these new primers
199	have since been included in the RV15, further evaluation within a standard Seeplex®
200	multiplex PCR format is required.

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201	A limitation to the Seeplex® system is its internal control facility. The artificial
202	targets included in each PCR mastermix allows validation only of the PCR step,
203	whereas the addition of PDV to each extraction allows the RT-PCR system also to
204	control for both the RNA extraction and the reverse transcription step. Inhibition of
205	the RT-PCR system, detected by poorly-amplifying PDV target, occurs with a not
206	insignificant frequency; it is essential to report that a lack of virus detection is not due
207	to any technical deficiency within the system.
208	In the laboratory setting, the Seeplex® system looks to compare favourably with
209	existing technologies where high throughput, ease of use, low technical requirements,
210	and a fully kit-based format are factors. Equally useful is the ability of the RV15
211	system to detect bocavirus and coronaviruses, whose clinical impact is becoming
212	increasingly clear [Esposito et al., 2008; Brodzinski and Ruddy, 2009; Garcia-Garcia
213	et al., 2010].
214	Since the completion of this work, the Seeplex® RV15 kit now includes the
215	redesigned influenza B primer set, human RNase P whole process control and now
216	comprises a one-step RT-PCR system rather than the two-step systems employed in
217	these evaluations.
218	In summary, the Seeplex® RV15 produces results comparable to the in house system.
219	The high throughput capabilities and low technical requirements afforded by
220	Seeplex® recommend it as a practical alternative to real-time RT-PCR.

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