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

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Respiratory virus infections contribute substantially both to hospitalizations of young children, and to morbidity in immunocompromised patients such as those with hematological 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 10 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 nine viruses in common with the RT-PCR were detected reliably by RV12. Eleven samples RT-PCR-positive for Metapneumovirus and five samples positive for influenza B were not detected by RV12. Seegene developed a second-generation system, RV15, which not only allowed detection of three 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 RV12. The high throughput capabilities and potential lower technical requirements afforded by the Seeplex® system may offer an alternative to real-time RT-PCR systems. *J. Med. Virol.* ***83:1469–1475, 2011.*** © 2011 Wiley-Liss, Inc.

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INTRODUCTION

Timely and accurate diagnosis of respiratory virus infection is essential to patient care in settings as diverse as hematopoietic stem cell transplant recipients [Jalal et al., 2007; Piralla et al., 2009], childhood bronchitis and pneumonia [Shay et al., 2001; Michelow et al., 2004] and the seasonal influenza epidemics implicated in substantial annual mortality [Mook et al., 2008]. As such infections often present with similar symptoms, laboratory analysis is essential.

Many laboratories rely still on non-molecular approaches such as direct immunofluorescence and viral culture. However, in the UK, various real-time PCR-based systems have been employed for a number of years, their utility having been demonstrated largely by comparison with these more traditional methods [Templeton et al., 2004; Bustin and Mueller, 2005; Freymuth et al., 2006; Mahony et al., 2007; van de Pol et al., 2007; Kuypers et al., 2009; reviewed in Mahony, 2008]. Emerging technologies thus have to demonstrate significant benefit against these existing tools to achieve widespread adoption [Pabbaraju et al., 2008; Arens et al., 2010].

The novel Seeplex® system exploits Dual Priming Oligonucleotides (DPOs) as PCR primers, each comprising a standard primer-length 5′ portion which initiates priming, separated by multiple inosine residues from a shorter, 6–12 nucleotide 3′ component which determines duplex extension. As well as facilitating melting temperature regulation during primer design, mispriming and PCR artifacts such as primer dimerization are almost eliminated, allowing simultaneous and efficient amplification of multiple complex targets in single reactions [Chun et al., 2007].

The system summary in Figure 1 shows a standard nucleic acid extraction, a cDNA step, and then a DPO PCR, in which the products of each target virus have different nucleotide lengths. Post-PCR product analysis employs an automated bench-top system with which target detection interpretations are inferred from PCR product sizes.

The RV12 system enables simultaneous detection of 12 respiratory viruses in two reactions per sample. Its performance when compared to traditional diagnostic methods has been demonstrated frequently [Yoo et al., 2007; Drews et al., 2008; Roh et al., 2008; Kim et al., 2009], with potential time and resource savings when compared to more modern methods, particularly in high throughput settings where prompt diagnosis is important. More recently, the RV15 kit has been developed that targets 15 viruses in three reactions.

No studies evaluating either system against real-time PCR-based respiratory virus detection methods have yet been published. The results of a comparative evaluation of serial iterations of the Seeplex® system with the multiplex PCR-based method that has been used for 5 years as the primary diagnostic assay for respiratory virus detection in a major London hospital are described.

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 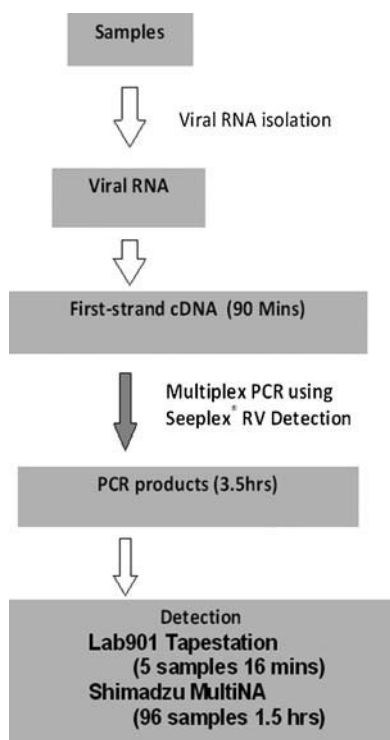and lavages were diluted in saline and centrifuged to separate cellular material from mucoid and liquid components. Swabs were transported in viral transport medium and extracted directly. Two automated nucleic acid extractors were employed at different times during the course of this evaluation—the 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 min with Buffer AL and 30 mAU/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 gene from the *ras* family (K-Ras) [Hilbe et al., 2003] and Protein H (hemagglutinin) 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).

Quantitect Multiplex RT-PCR Mix NoRox (Qiagen), specific primers and probe combinations (Table II, all obtained from Sigma-Genosys, Gillingham, UK), and 10 µl of nucleic acid extract were mixed in 25 µl reactions. Real-time cycling, detection, and 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 (C_t) values. For each sample, C_t values for the PDV and K-Ras control reactions were compared to expected values before parallel PCR results were considered valid.

Fig. 1. Workflow diagram of the Seeplex® diagnostic system.

TABLE I. Distribution of Primer and Probe Combinations and Probe Dye-Labels Within Each of the Seven Real-Time RT-PCR Reactions, That Is, Reaction 4 Had Adenovirus and Enterovirus Primers and Probes

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 II. Primer and Probe Sequences for Each of the 10 Targets Detected by the Real-Time Respiratory RT-PCR System

Virus		Sequence	Final conc. (nM)
Influenza A (FLA)	Forward	CAAGACCAATCCTGTCACCTCTG	900
	Reverse	TGCATTTTGGACAAAGCGTCTAC	900
	Probe	FAM-AGTCCTCGCTCACTGGGCACGGT-BHQ1	200
Influenza B (FLB)	Forward	GGCAACACCTGTTTCCATATTAAG	900
	Reverse	GCCTGTTAATGCAGACAAAACTC	900
	Probe	JOE-TCATAGGCAGCTCCGAAGCAAGACATGA-BHQ1	200
Respiratory syncytial virus (RSV)	Forward	GAAGGGTCAAACATCTGTTTAACAAG	900
	Reverse 1	GTTTCAGCTWGTGGGAAGAAAGATA	900
	Reverse 2	GTGTCAGCCTGTGGAAAGAAGGATA	900
	Probe	FAM-TGATCCTGCATTRTCACARTACCATCCTCT-BHQ1	200
Parainfluenza 1 (PF1)	Forward	ATTCAGACAGGATGGAACCGTTAA	900
	Reverse	GATACTAAGCTTTGTTGTGACCTCAT	900
	Probe	JOE-ACCAATGCCTTCAACTGTGTCTCCCGTG-BHQ1	200
Parainfluenza 2 (PF2)	Forward	AGAGATAACAGGGTTTGAGAATAATTCAT	900
	Reverse	CAAATGGAGTTTGGTGATTAAGGGTA	900
	Probe	FAM-TCCAGATGCTCGATCAACTATGTCCAGAGG-BHQ1	200
Parainfluenza 3 (PF3)	Forward	CGATTAGAGGCTTTCAGACAAGATG	900
	Reverse	CTGTTGAGACCGCATGATTGAC	900
	Probe	Cy5-CCACTGTGTCACCGCTCAATACCAGCC-BHQ3	200
Enterovirus	Forward	CCCCTGAATGCGGCTAATCC	900
	Reverse	GTCACCATAAGCAGCCAATATAAGAA	900
	Probe	AACACGGACACCCAAAGTAGTCGGTTCC	200
Adenovirus (ADV)	Forward	GCCCCAGTGGTCTTACATGCACATC	900
	Reverse	GCCACGGTGGGGGTTTCTAAACTT	900
	Probe	FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1	200
Rhinovirus (HRV)	Forward 1	TGTGCTCRCTTTGAGTCCTC	900
	Forward 2	TGTGCTCAGTGTGCTTCCTC	900
	Reverse	TGAAACACGGACACCCAAAGTA	900
	Probe	Cy5-CCCTGAATGYGGCTAACCTTAAMCCTGC-BHQ3	200
Metapneumovirus (MPNV)	Forward	CATATAAGCATGCTATATTAAAAGAGTCTC	500
	Reverse	CCTATTTCTGCAGCATATTTGTAATCAG	250
	Probe	FAM-TGYAATGATGAGGGTGTCACTGCGGTTG-BHQ1	500
K-Ras	Forward	GCCTGCTGAAAATGACTGAATATAAAC	600
	Reverse	TGATTCTGAATTAGCTGTATCGTCAAG	600
	Probe	JOE-TGCCTACGCCACAAGCTCCAACTACCA-BHQ1	100
PDV	Forward	GCGGGTGCCTTTTACAAGAAC	600
	Reverse	CAGAATAAGCAAAATTGATAGGAACCAT	600
	Probe	FAM-TCTTTCCTCAACCTCGTCCGTCACAAGT-BHQ1	100

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.

Seeplex® RV12

Ninety-nine respiratory extracts stored at −80°C were selected that previously had tested positive by RT-PCR for one or more respiratory virus targets. With the exception of enterovirus and the internal control virus (PDV), which were not detected by RV12, all viral targets in the RT-PCR were represented (Table III).

Random hexamer-primed cDNA synthesis products were generated using the Revertaid system (Fermentas, York, UK), according to the manufacturer's instructions, and stored at −20°C until use.

Each cDNA preparation was subjected to the RV12 PCR procedure according to the manufacturer's instructions (Seegene, Seoul, South Korea). Briefly, parallel 20 µl reactions were set up, each containing RV12 mastermix, 8-MOPS contamination control reagent, and 3 µl of cDNA. One of each pair was supplemented with 4 µl primer mix A, the other with 4 µl primer mix B. Thermal cycling conditions were as follows: 15 min at 94°C, followed by 40 cycles of 94°C for 30 sec, 60°C for 90 sec, and 72°C for 90 sec, followed by a single incubation of 10 min at 72°C. Completed reactions were analyzed using the MultiNA platform (Shimadzu Corporation, Kyoto, Japan), coupled to a dedicated Seegene software template. Analysis output comprised both a qualitative interpretation of presence or absence of each virus target, and a determination of peak intensity.

Seeplex® RV15

Eighty-four respiratory extracts stored at −80°C testing positive for one or more RT-PCR virus targets (Table IV) were subjected to cDNA synthesis using the Mu-LV system (Invitrogen, Paisley, UK), according to the manufacturer's instructions. Reaction products were stored at −20°C until use. Eight samples of the 84 had been part of the previous RV12 analysis.

Each cDNA preparation was subjected to the RV15 PCR procedure according to Seegene's instructions.

TABLE III. Characteristics of the 100 Respiratory Extracts Tested by Seeplex RV12

Single virus samples	n	C_t range[a]
FLA	9	18.5–31.4
FLB	9	22.7–37.6
HRV	9	21.2–38.3
ADV	9	23.4–38.1
MPNV	8	22.4–35.0
RSV	8	25.3–38.2
PF1	13	23.8–37.9
PF2	6	25.3–35.3
PF3	11	25.3–37.5

Dual virus samples	n
ADV and HRV	2
PF1 and ADV	1
RSV and ADV	2
RSV and HRV	1
HRV and MPNV	4
FLA and MPNV	2
FLA and RSV	4
PF3 and MPNV	1
PF2 and ADV	1

Either single targets (left-hand two columns of the top part) or two targets (two columns of the bottom part) were detected in each sample by in-house multiplex RT-PCR. The target range of RV12 comprises these nine viruses, together with two human coronavirus groups (229E/NL63 and OC43/HKU1). Also, RSV-A and RSV-B are detected separately.
[a]C_t ranges are derived from both single and dual virus samples.

Briefly, parallel 20 μl reactions were set up for each sample, as per the RV12 kit, but with three reactions per sample (primer mixes A, B, and C) rather than the two in the earlier kit. Thermal cycling conditions were as per the RV12 kit. Completed reactions were

TABLE IV. Characteristics of the 84 Respiratory Extracts Tested by Seeplex RV15

Single virus samples	n	C_t range[a]
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 and PF3	1
FLB and HRV	1
FLB and PF3	1
FLA and PF2	1
RSV and ADV	2[*]

Either single targets (left-hand two columns of the top part) or two targets (two columns of the bottom part) 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.
[a]C_t ranges are derived from both single and dual virus samples.

analyzed using the Tape Station platform (Lab901, Edinburgh, UK) linked to a specific Seeplex® RV15 interpretation software module. Again, results were presented both qualitatively and semi-quantitatively, according to the migration rate and band intensity of each species present within completed reactions.

Redesigned Influenza B Primers

During the evaluation, a new panel of influenza B primers was developed by Seegene to replace those in the RV15 kit. The cDNA preparations of the 19 FLB-positive (by RT-PCR) samples analyzed by RV15 were subjected to amplification using these primers under the same conditions as for the primer mixes of the RV12 and RV15 kits, but in monoplex reactions. Analysis was performed as per RV15, above.

RESULTS

Seeplex® RV12

All targets detected by the RT-PCR were also detected by Seeplex® RV12 in 88 of the 99 nucleic acid extracts tested with the Seeplex® RV12 system. In 7 of the remaining 11 samples, single infections were missed (4 × FLB, one each of HRV, PF1, and MPNV), and in four samples where two viruses had been detected by RT-PCR, the virus target with the higher C_t was missed by RV12 (2 × MPNV, 1 × PF3, and 1 × ADV). Comparison of MultiNA peak heights and RT-PCR C_t values for each virus target revealed a broad negative correlation between the two parameters, with the exception of HRV, where no correlation was observed (Fig. 2).

In all instances where PF2 (7 samples), FLA (15), and/or RSV (15) were detected by RT-PCR, these virus targets were detected with RV12 also. For each instance where PF1, PF3, HRV, and/or ADV were detected by RT-PCR, all except one of each virus target were detected by Seeplex® RV12. Each instance of undetected virus represented a sample
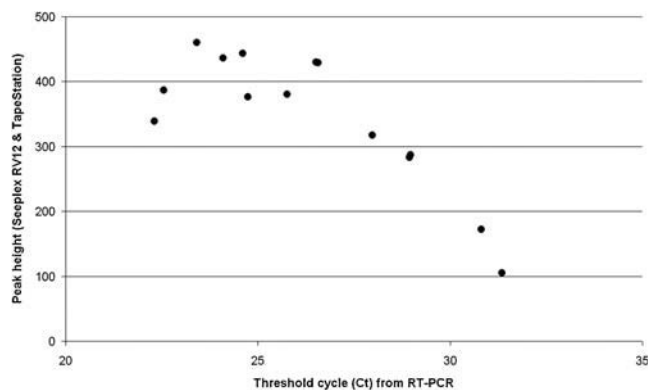

Fig. 2. Plot showing how peak heights obtained using Seeplex® RV12 and the TapeStation microelectrophoresis platform display negative correlation with the threshold cycles obtained from the RT-PCR method. Data shown are from influenza A-positive samples.

whose C_t was found in the higher part of the range for that virus. FLB and MPNV targets were detected less frequently. Four of the five FLB-positive samples with the highest C_t values, and the three MPNV-positive samples with the highest C_t values were not detected by RV12.

Conversely, ADV, RSV, HRV, and MPNV were detected by RV12 in extracts where the RT-PCR system had failed to do so (in 3, 4, 4, and 1 sample, respectively), and with the exception of the RSV detections, had high peak heights, consistent with high target concentrations in the initial samples.

Seeplex® RV15

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 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 nine 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 five 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

An in-house multiplex RT-PCR system and two iterations of a novel PCR-based technology for the detection of respiratory viruses have been compared. In the first instance, 99 samples were evaluated, and in the second, 84. A further panel of 19 FLB-positive samples was tested by a revised primer set within the novel Seeplex® system. Whilst direct comparison of the two systems is limited, due to the sample sets having been pre-determined to be positive for one or more target viruses by one system, it can be stated

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)

Sample #	PCR result (C_t)	RV12
(a)		
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**

Sample #	PCR result (C_t)	RV15
(b)		
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**

Samples where Seeplex® missed targets detected by PCR are given first.

that the overall performance of the Seeplex® RV12 and RV15 systems was very good, with several instances of viruses being detected where the RT-PCR system had either failed or had been unable to do so.

Furthermore, with each virus, some negative correlation was observed between RT-PCR threshold cycle and Seeplex® peak intensity, suggesting the latter could also be employed semi-quantitatively.

The frequency of instances where targets detected by RT-PCR were missed by Seeplex® was low, and may have been influenced by repeated freeze–thaw cycles of affected samples, nearly all of which were at

the upper end of the relevant C_t range (i.e., with lower target concentrations). However, both MPNV and FLB targets were detected poorly by RV12, with concomitant low band intensities from samples where these targets were detected. Revision of the primer sets was a key component of the development of RV15. RV15 gave a 100% detection rate of all viruses detected by RT-PCR, including seven MPNV-positive samples, but no improvement in FLB detection. The latter primer set was modified further, and retesting the panel of known FLB-positive samples gave substantially greater success. Although these new primers have since been included in the RV15, further evaluation within a standard Seeplex® multiplex PCR format is required.

A limitation to the Seeplex® system is its internal control facility. The artificial targets included in each PCR mastermix allows validation only of the PCR step, whereas the addition of PDV to each extraction allows the RT-PCR system also to control for both the RNA extraction and the reverse transcription step. Inhibition of the RT-PCR system, detected by poorly amplifying PDV target, occurs with a not insignificant frequency; it is essential to report that a lack of virus detection is not due to any technical deficiency within the system.

In the laboratory setting, the Seeplex® system looks to compare favorably with existing technologies where high throughput, ease of use, low technical requirements, and a fully kit-based format are factors. Equally useful is the ability of the RV15 system to detect bocavirus and coronaviruses, whose clinical impact is becoming increasingly clear [Esposito et al., 2008; Brodzinski and Ruddy, 2009; Garcia-Garcia et al., 2010].

Since the completion of this work, the Seeplex® RV15 kit now includes the redesigned influenza B primer set, human RNase P whole process control and now comprises a one-step RT-PCR system rather than the two-step systems employed in these evaluations.

In summary, the Seeplex® RV15 produces results comparable to the in-house system. The high throughput capabilities and low technical requirements afforded by Seeplex® recommend it as a practical alternative to real-time RT-PCR.

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