Day2-P16

Comprehensive Evaluation of Multiple Automated High Throughput Extraction Platforms Using Quantitative Real Time PCR Assays

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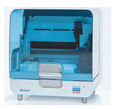
ABSTRACT

- **Background.** Detection of nucleic acid by quantitative polymerase chain reaction (qPCR) and / or quantitative reverse transcriptase PCR (qRT-PCR) continues to be the gold standard in supporting biodistribution, vector shedding, gene expression, and pharmacokinetics bioanalysis studies. These molecular platforms are highly preferred due to their inherent sensitive and robust nature. Furthermore, qPCR and RT-qPCR assays continue to play a central role in clinical diagnostics wherein, clinicians heavily rely on these assays to diagnose (assess levels of viral and bacterial pathogens) and effectively treat patients. Analysis of viral nucleic acid in biological matrices requires efficient extraction of viral nucleic acids. Selection of an appropriate extraction platform is key to the successful method development / validation / testing of any qPCR / RT-qPCR assay. Selections are highly dependent on unique properties of biological matrices. Importantly, introducing automated extraction platforms into a laboratory's workflow increases the efficiency and consistency of the test results. Automated magnetic and silica-based extraction technologies are commonly used in clinical diagnostics and bioanalysis laboratories. In this study, we describe the evaluation of 24 well NucliSENS®easyMag® (BioMerieux; magnetic bead based), 96 well KingFisher™ Flex (Thermo Fisher Scientific; magnetic bead based), 12 well QIAcube Connect (Qiagen; silica based), and 96 well QIAcube HT (Qiagen; silica based) using serum, neat urine, plasma, and peripheral blood mononuclear cells (PBMCs). Viracor evaluated QIAcube Connect HT and KingFisher Flex as alternatives to mid / lower throughput extraction platforms.


QIAcube Connect


QIAcube Connect HT


KingFisher™ Flex


NucliSENS® easyMAG ®

Feature	Kingfisher Flex	QIAcube Connect HT
Extraction Method	Magnetic beads.	Silica-membrane spin columns.
Sample compatibility	50 – 1,000 µL: 96 deep-well plate 200 – 5,000 µL: 24 deep-well plate	200 µL - 5 mL
Throughput (24- 96 samples per run)	Under 60 minutes	75 - 90 minutes
Customization & flexibility	customizable protocols	Locked protocols, less user input
Cross-contamination risk	Lower (closed-tube handling)	Higher (open-tube handling, but dedicated decontamination protocols available)
Kit compatibility & cost	Wide range	Qiagen kits primarily
Footprint	Larger	Smaller

Validation status of assays at Viracor				
Matrices	**EBV**	**VZV**	**HSV-1**	**SARS CoV-2**
Serum	Validated	Validated	Validated	Validated
Plasma	Validated	Not Validated	Validated	Validated
Urine	Validated	Not Validated	Not Validated	Not Validated
PBMC	Not Validated	Not Validated	Not Validated	Not Validated

- **Method Principle**. In this study, Viracor assessed detection of Epstein-Barr virus (EBV), Varicella zoster virus (VZV), Herpes simplex virus 1 (HSV-1), and Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in urine, serum, plasma, and PBMCs. The cell rich matrix (PBMCs) was evaluated on QIAcube Connect using the QIAamp DNA Blood Mini Kit, QIAcube HT using the QIAamp 96 Virus QC HT kit, and KingFisher Flex instruments using the MagMax DNA Multi-Sample Ultra 2.0 kit. The cell free matrices (urine, serum, and plasma) were evaluated on EasyMag, QIAcube HT using the QIAamp 96 Virus QC HT kit, and KingFisher Flex instruments using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit. Each of the matrices was pooled and spiked with commercially procured viral stocks independently at pre-determined concentrations spanning the dynamic range of the assay. The total nucleic concentration in ng/µL in PBMC sample was determined by spectrophotometry at 260 nm. Nucleic acid amplification was performed using the ABI 7500 SDS qPCR instruments in a single well format. A seven-point standard curve (5 copies/reaction to 5 x 10^6 copies/reaction) was created using a dilution of a linearized plasmid used for quantitation.

Matrices	Virus	Base extraction platform	New extraction platform being evaluated	Performance characteristics
Serum Plasma Urine PBMC	EBV, VZV, HSV-1, and SARS-CoV-2	NucliSENS® easyMAG ® NucliSENS® easyMAG ® NucliSENS® easyMAG ® QIAcube Connect	QIAcube Connect HT and KingFisher™ Flex	Stock quantification, Recovery, Precision, DNA and RNA yield,

- Samples were spiked at three levels: High, Medium, and Low positive levels. Unspiked samples were also tested. Five replicates per level per run were set-up. Four total runs were set up. Data from the first run was used for quantifying viral stocks and assigning expected concentrations. Data from Runs 2, 3, and 4 were used for Precision and Accuracy calcs.

RESULTS

Dilution Factors

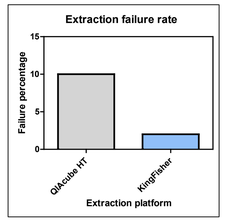
At Viracor, dilution factors are commonly used to convert copies/PCR reaction to copies/mL of original specimen. Overall, QIAcube HT has higher dilution factor compared to other platforms.

DNA Virus (EBV, HSV-1, and VZV)							
EasyMAG		QIAcube Connect (PMBCs)		KingFisher		QIAcube HT	
Stock Quant	mL	Stock Quant	mL	Stock Quant	mL	Stock Quant	mL
Extraction Volume	0.50	Extraction Volume	0.40	Extraction Volume	0.40	Extraction Volume	0.20
Elution Volume	0.1	Elution Volume	0.1	Elution Volume	0.1	Elution Volume	0.1
Eluate volume	0.01	Eluate volume	0.01	Eluate volume	0.01	Eluate volume	0.01
DILUTION FACTOR	20	DILUTION FACTOR	25	DILUTION FACTOR	25	DILUTION FACTOR	50
RNA Virus (SARS-CoV2)							
EasyMAG		QIAcube Connect (PMBCs)		KingFisher		QIAcube HT	
Stock Quant	mL	Stock Quant	mL	Stock Quant	mL	Stock Quant	mL
Extraction Volume	0.50	Extraction Volume	0.35	Extraction Volume	0.35	Extraction Volume	0.20
Elution Volume	0.1	Elution Volume	0.1	Elution Volume	0.05	Elution Volume	0.1
Eluate volume	0.015	Eluate volume	0.015	Eluate volume	0.015	Eluate volume	0.015
DILUTION FACTOR	13.33	DILUTION FACTOR	19.05	DILUTION FACTOR	9.52	DILUTION FACTOR	33.33

False Positivity / Contamination Rate

	QIAcube Connect HT				Total
	SARS	HSV-1	EBV	VZV	
Serum	N/A	1/15	N/A	N/A	4/60
Plasma	N/A	N/A	N/A	1/15	
Urine	N/A	N/A	N/A	N/A	
PBMC	N/A	1/15	1/15	N/A	
	KingFisher				Total
	SARS	HSV-1	EBV	VZV	
Serum	N/A	2/15	N/A	N/A	4/45
Plasma	N/A	1/15	N/A	N/A	
Urine	N/A	N/A	N/A	N/A	
PBMC	N/A	N/A	1/15	N/A	

Extraction Failure Rate

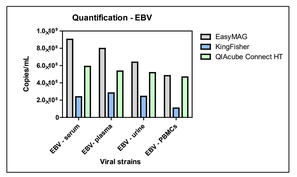


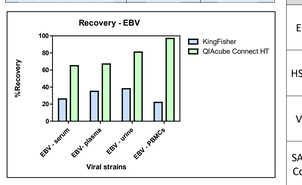
Quantification of Viral Stocks

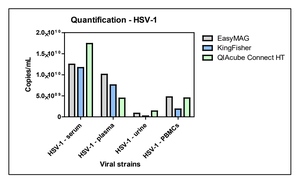
Quantification values of viral stocks (copies/mL)			
Virus - matrix type	EasyMAG	KingFisher	QIAcube Connect HT
EBV - serum	9.04E+08	2.38E+08	5.91E+08
EBV- plasma	7.98E+08	2.83E+08	5.36E+08
EBV - urine	6.39E+08	2.44E+08	5.18E+08
EBV - PBMCs	4.83E+08	1.08E+08	4.68E+08
HSV-1 - serum	1.25E+10	1.17E+10	1.74E+10
HSV-1 - plasma	1.01E+10	7.58E+09	4.42E+09
HSV-1 - urine	8.46E+08	1.86E+08	1.38E+09
HSV-1 - PBMCs	4.73E+09	1.84E+09	4.47E+09
VZV - serum	2.10E+09	2.00E+09	8.13E+08
VZV - plasma	1.98E+09	2.23E+09	5.87E+08
VZV - urine	1.08E+09	6.55E+08	4.65E+08
VZV - PBMCs	3.24E+08	2.07E+08	4.18E+08
SARS CoV-2 - serum	2.40E+07	1.03E+07	5.67E+07
SARS CoV-2 - plasma	5.84E+07	1.46E+07	9.14E+07
SARS CoV-2 - urine	5.25E+07	1.91E+07	7.49E+07
SARS CoV-2 - PBMCs	2.08E+07	1.41E+07	5.12E+07

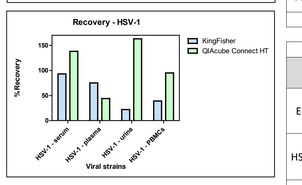
Percent Recovery

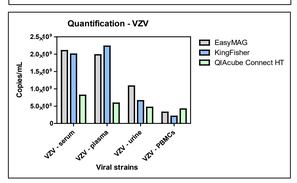
Percentage Recovery		
Virus - matrix type	KingFisher	QIAcube Connect HT
EBV - serum	26.38%	65.36%
EBV- plasma	35.48%	67.19%
EBV - urine	38.19%	81.16%
EBV - PBMCs	22.32%	96.98%
HSV-1 - serum	93.18%	138.46%
HSV-1 - plasma	74.96%	43.72%
HSV-1 - urine	22.05%	162.93%
HSV-1 - PBMCs	38.93%	94.55%
VZV - serum	111.17%	38.73%
VZV - plasma	112.55%	29.60%
VZV - urine	60.53%	45.64%
VZV - PBMCs	63.75%	128.78%
SARS CoV-2 - serum	42.85%	248.89%
SARS CoV-2 - plasma	24.99%	41.05%
SARS CoV-2 - urine	36.31%	142.72%
SARS CoV-2 - PBMCs	67.82%	246.60%

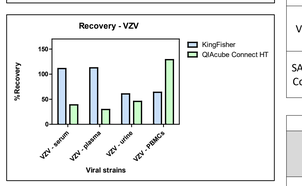


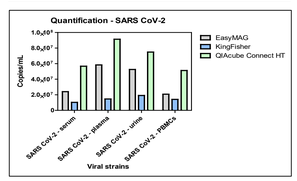


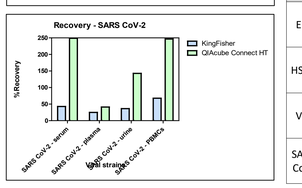












Precision

Serum Precision (<30% Intra-Assay, <40% Inter-Assay)												
		EasyMag	KingFisher					Qiacube HT				
		Run 1	Run 1	Run 2	Run 3	Run 4	InterAssay	Run 1	Run 2	Run 3	Run 4	InterAssay
EBV	High	11%	13%		5%	4%	26%	9%	7%	15%		11%
	Med	8%	5%		7%	6%	26%	13%	7%	4%		16%
	Low	8%	10%		14%	9%	26%	29%	21%	11%		21%
HSV-1	High	9%	16%	7%	2%		15%	21%	6%	26%		18%
	Med	7%	10%	10%	8%		49%	6%	19%	8%		17%
	Low	12%	78%	17%	12%		59%	11%	33%	15%		27%
VZV	High	16%	13%	14%	9%		26%	22%	25%	66%		45%
	Med	31%	34%	29%	30%		32%	31%	36%	15%		36%
	Low	39%	17%	66%	15%		85%	40%	69%	79%		67%
SARS-CoV2	High	7%	31%	4%	6%		31%	24%	16%	53%		44%
	Med	5%	12%	24%	16%		34%	13%	28%	31%		22%
	Low	15%	21%	27%	4%		26%	44%	18%	14%		29%

Plasma Precision (<30% Intra-Assay, <40% Inter-Assay)												
		EasyMag	KingFisher					Qiacube HT				
		Run 1	Run 1	Run 2	Run 3	Run 4	InterAssay	Run 1	Run 2	Run 3	Run 4	InterAssay
EBV	High	10%	8%		24%	4%	33%	17%	14%	12%		14%
	Med	11%	9%		14%	4%	39%	18%	20%	10%		16%
	Low	13%	6%		19%	8%	41%	12%	10%	11%		14%
HSV-1	High	25%	2%	6%	14%		15%	22%	20%	10%		18%
	Med	9%	8%	12%	7%		25%	14%	12%	2%		16%
	Low	11%	7%	6%	6%		29%	19%	6%	11%		18%
VZV	High	6%	7%	13%	10%		20%	26%	13%	40%		42%
	Med	26%	60%	23%	15%		36%	31%	28%	34%		40%
	Low	43%	96%	55%	56%		64%	24%	22%	25%		50%
SARS-CoV2	High	9%	6%	16%	13%		29%	23%	6%	16%		15%
	Med	12%	13%	15%	25%		26%	6%	15%	40%		24%
	Low	11%	28%	16%	26%		24%	25%	24%	24%		25%

Note: Extraction issues in Qiacube HT for SARS-CoV2 in Plasma

Urine Precision (<40% Intra-Assay, <50% Inter-Assay)												
		EasyMag	KingFisher					Qiacube HT				
		Run 1	Run 1	Run 2	Run 3	Run 4	InterAssay	Run 1	Run 2	Run 3	Run 4	InterAssay
EBV	High	16%	12%	N/A	8%	6%	62%	8%	12%	14%		54%
	Med	8%	26%	N/A	14%	6%	59%	32%	22%	18%		57%
	Low	18%	27%	N/A	13%	5%	59%	27%	32%	19%		75%
HSV-1	High	12%	6%	9%	25%	N/A	87%	52%	13%	11%		61%
	Med	12%	18%	22%	17%	N/A	52%	7%	13%	14%		90%
	Low	46%	49%	56%	40%	N/A	48%	28%	12%	14%		82%
VZV	High	9%	17%	8%	8%	N/A	58%	15%	16%	9%		35%
	Med	6%	22%	30%	15%	N/A	74%	40%	10%	23%		42%
	Low	7%	15%	14%	27%	N/A	60%	47%	28%	22%		43%
SARS-CoV2	High	3%	8%	8%	3%	N/A	16%	5%	10%	13%		29%
	Med	12%	10%	11%	7%	N/A	17%	20%	18%	3%		18%
	Low	22%	12%	32%	12%	N/A	28%	18%	11%	3%		21%

PMBC Copies/mL (<40% Intra-Assay, <50% Inter-Assay)												
		Qiacube Connect	KingFisher					Qiacube HT				
		Run 1	Run 1	Run 2	Run 3	Run 4	InterAssay	Run 1	Run 2	Run 3	Run 4	InterAssay
EBV	High	4%	10%		9%	14%	39%	14%	15%	12%	19%	46%
	Med	23%	8%		13%	6%	45%	9%	17%	18%	29%	59%
	Low	8%	12%		16%	8%	41%	22%	16%	14%	14%	40%
HSV-1	High	14%	10%	14%	32%		42%	8%	7%	4%		23%
	Med	9%	14%	46%	19%		34%	2%	5%	8%		16%
	Low	14%	22%	26%	33%		39%	10%	8%	8%		17%
VZV	High	10%	13%	16%	15%		15%	7%	19%	44%		26%
	Med	10%	26%	27%	31%		31%	18%	23%	41%		29%
	Low	36%	48%	34%	61%		73%	16%	29%	22%		24%
SARS-CoV2	High	27%	4%	32%	32%	10%	83%	31%	9%		5%	21%
	Med	16%	16%	49%	8%	24%	61%	23%	8%		12%	23%
	Low	24%	25%	53%	9%	52%	36%	32%	43%	34%	36%	40%

Accuracy = Log_{10}Expected – Log_{10}Observed (≤ 0.70) and > 90% Pos, 100% Neg

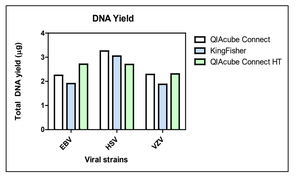
Serum Accuracy							
		EasyMag		KingFisher		Qiacube HT	
EBV	Pos	15/15	100%	45/45	100%	44/44	100%
	Neg	5/5	100%	15/15	100%	15/15	100%
HSV-1	Pos	15/15	100%	45/45	100%	43/43	100%
	Neg	5/5	100%	13/15	87%	12/13	92%
VZV	Pos	15/15	100%	42/42	100%	41/43	95%
	Neg	5/5	100%	15/15	100%	15/15	100%
SARS	Pos	15/15	100%	39/39	100%	37/37	100%
	Neg	5/5	100%	14/14	100%	13/13	100%

Plasma Accuracy							
		EasyMag		KingFisher		Qiacube HT	
EBV	Pos	15/15	100%	45/45	100%	45/45	100%
	Neg	5/5	100%	15/15	100%	15/15	100%
HSV-1	Pos	15/15	100%	44/44	100%	43/43	100%
	Neg	5/5	100%	14/15	93%	14/14	100%
VZV	Pos	15/15	100%	44/44	100%	45/45	100%
	Neg	5/5	100%	15/15	100%	14/15	93%
SARS	Pos	15/15	100%	43/43	100%	31/31	100%
	Neg	5/5	100%	15/15	100%	11/11	100%

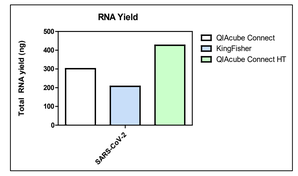
Urine Accuracy							
		EasyMag		KingFisher		Qiacube HT	
EBV	Pos	15/15	100%	37/45	82%	42/45	93%
	Neg	5/5	100%	15/15	100%	15/15	100%
HSV-1	Pos	5/15	33%	12/45	27%	33/45	73%
	Neg	5/5	100%	15/15	100%	15/15	100%
VZV	Pos	15/15	100%	45/45	100%	44/44	100%
	Neg	5/5	100%	15/15	100%	15/15	100%
SARS	Pos	15/15	100%	44/44	100%	36/37	97%
	Neg	5/5	100%	15/15	100%	14/14	100%

PBMC Accuracy							
		Qiacube		KingFisher		Qiacube HT	
EBV	Pos	15/15	100%	45/45	100%	59/59	100%
	Neg	4/4	100%	15/15	100%	19/20	95%
HSV-1	Pos	14/14	100%	45/45	100%	45/45	100%
	Neg	5/5	100%	15/15	100%	14/15	93%
VZV	Pos	14/14	100%	43/43	100%	45/45	100%
	Neg	5/5	100%	15/15	100%	15/15	100%
SARS	Pos	15/15	100%	43/49	88%	48/48	100%
	Neg	5/5	100%	20/20	100%	19/19	100%

DNA Yield



RNA Yield



Conclusions

Parameters	King-Fisher vs QIAcube HT
%-Recovery/Analytical sensitivity	QIA-HT **outperformed** KF in recovery, achieving remarkably **high yields**. This translates to potentially **higher analytical sensitivity**, impacting the assay's LOD and LLOQ.
Accuracy	**Similar** performance
Precision	**QIA-HT exhibited better precision** overall, particularly in Inter-assay tests.
DNA Yield and Purity	**No significant** differences in DNA yields
RNA Yield	HT has significantly **higher RNA** yields
Negatives with Positive Ct Values	**Similar** performance
Sample Extraction Failures	HT has **high** extraction failure (KIT Specific-?)
Cost/reaction	Comparable

Matrices	EBV	HSV-1	VZV	SARS CoV2	Recommended Platform
Serum	1-EM	1-EM	EM	1-EM	EasyMag
	2-HT	2-HT		2-KF	
	3-KF				
Plasma	1-EM	1-EM	EM	1-EM	EasyMag
	2-HT	2-HT		2-KF	
		3-KF			
Urine	EM	?	EM	1-EM	EasyMag
				2-HT	
				3-KF	
PBMC	HT	HT	HT	QC	QIAcube-HT

- EasyMAG outperformed QIAcube Connect HT and KingFisher for serum, plasma, and urine.
- QIAcube HT outperformed QIAcube for PBMCs
- In agreement with historical results, HSV-1 and VZV viral stocks generated anomalous results .
- Alternative extraction methods may be necessary for extracting neat urine samples.

REFERENCES

- Burns MJ, Nixon GJ, Foy CA, Harris N. Standardization of data from real-time quantitative PCR methods – evaluation of outliers and comparison of calibration curves. BMC Biotechnology. 2005 Dec 7; 5:31.
- Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR 3rd, Smith TF. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev. 2006 Jan;19(1):165-256. Review. Erratum in: Clin Microbiol Rev.2006Jul;19(3):595
- MM03, 3rd Edition. Molecular Diagnostics Methods for Infectious Diseases; Approved Guideline. Clinical and Laboratory Standards Institute. Wayne, PA. February 2015.
- MM06-A2, Vol. 30 No.22. Quantitative Molecular Diagnostics Methods for Infectious Diseases; Approved Guideline, Second Edition, Clinical and Laboratory Standards Institute. Wayne, PA. November 2010.
- Molecular Microbiology: Diagnostic Principles and Practice, Second Edition. David H. Persing. ASM Press. 2011. Washington, D.C.
- Motulsky HM and Brown RE. Detecting outliers when fitting data with nonlinear regression-a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics 2006, 7:123.