QUANTITATIVE DETECTION OF EPSTEIN-BARR VIRUS (EBV) IN PLASMA AND WHOLE BLOOD SPECIMENS

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BACKGROUND

EBV-associated post-transplant lymphoproliferative disease (PTLD) often occurs within the first year following transplantation. Increasing levels of EBV DNA in both whole blood and plasma have been shown to correlate significantly with subsequent development of PTLD in susceptible patients. As a result, EBV viral load is an important tool in monitoring transplant recipients to provide prognostic information for effective patient management. The NeuMoDx EBV Quant Assay is a "sample to result" type *in-vitro* diagnostic assay incorporating extraction of qPCR-ready DNA from whole blood and plasma specimens, coupled with a sensitive real-time PCR assay to deliver highly accurate results in a completely automated manner. Performance of the NeuMoDx EBV Assay was characterized in both plasma and whole blood on the NeuMoDx 288 and NeuMoDx 96 Molecular Systems.

METHODS

The objective of this study was to demonstrate performance of the NeuMoDx EBV Quant Assay across key analytical performance metrics including analytical sensitivity (LoD), linearity, precision, turnaround time, and equivalency across matrices. Evaluation of the analytical sensitivity was performed using the 1st WHO International Standard for EBV, and the limits of quantitation (LLoQ/ULoQ) were determined using the TAE ≤ 1.0 criterion.

RESULTS

The NeuMoDx EBV Assay demonstrated a limit of detection (LoD) and a lower limit of quantitation (LLoQ) of 75 IU/mL in plasma and of 250 IU/mL in whole blood. The NeuMoDx EBV Assay showed excellent linearity across a 6-log dynamic range ($R^2 > 0.98$) with upper limit of quantitation (ULoQ) of 8 Log_{10} IU/mL. Excellent quantitative precision was demonstrated across 4 levels run on 3 systems over 2 days (360 total specimens), as well as quantitative equivalency across multiple reagent lots. The time to first result for the NeuMoDx EBV Quant Assay was only ~60 min and the automated processing of data provided extremely accurate results. No cross-reactivity was observed against any of the 35 non-target pathogens tested, and the test performed efficaciously in the presence of endogenous and exogenous interfering moieties. Finally, equivalent performance was demonstrated between plasma and whole blood specimens using completely shared assay reagents. Calibrator sets prepared in Basematrix were found to be adequate for accurate quantitation of EBV in both plasma and whole blood matrices, simplifying the entire testing process.

NeuMoDx Molecular System Streamlined Testing

IVD WORKFLOW

EASE OF USE: LOAD SPECIMEN(S), WALK AWAY AND RESULTS IN 60-75 MIN

LOAD SPECIMENS | INITIATE TESTING WITH TWO ON-SCREEN SELECTIONS | QUANTITATIVE RESULT IN ~60 MINUTES

FEATURES

- **Integrated Operation:** Automates all steps of molecular diagnostics starting from raw clinical specimens to providing real-time PCR results in a fully automated process
- **True Random Access:** Ability to mix specimen types and tests
- **High Throughput:** ~300 RNA tests in an 8 hour shift for the N288, ~100 RNA tests in an 8 hour shift for the N96
- **Fast Time to First Results:** ~75 min
- **Continuous Loading:** Specimens and Reagents can be loaded/unloaded at any time
- **Large Walk-Away Window:** Up to 288 samples for the N288, 96 samples for the N96
- **Seamless On Demand Operation:** Automated inventory management of consumables and reagents
- **Long In-Use shelf life:** On-board room temperature stable reagents
- **Real-time PCR:** Five-color fluorescence detection offers real-time PCR multiplexing ability

On board storage of room temperature stable reagents enables easy on demand operation.

Patented microfluidic cartridges: capable of performing independent sample processing and real-time PCR.

EBV Analytical Sensitivity (LoD & LLoQ)

LIMIT OF DETECTION (LoD) and LOWER LIMIT OF QUANTITATION (LLoQ)

The Limit of Detection of the NeuMoDx EBV Assay was confirmed through a hit-rate method using pooled EBV-negative plasma or blood specimens spiked with 1st WHO International Standard for EBV.

The limit of detection of EBV was confirmed to be 75 IU/mL in plasma and 250 IU/mL in whole blood. These values were accepted as the Lower Limits of Quantitation (LLoQ), respectively, meeting the TAE (bias + 2*SD) criterion.

NeuMoDx EBV Assay Limit of Detection - Plasma

Target Conc. (IU/mL)	Target Conc. (Log_{10} IU/mL)	N	# Positive	% Positive	TAE
75	1.87	35	35	100	0.77

Limit of Detection of the NeuMoDx EBV Assay in Plasma. The LoD and LLoQ for the NeuMoDx EBV Quant Assay in plasma was confirmed by processing 35 plasma samples spiked with 75 IU/mL 1st WHO International Standard for EBV with a 100% detection rate.

NeuMoDx EBV Assay Limit of Detection - Whole Blood

Target Conc. (IU/mL)	Target Conc. (Log_{10} IU/mL)	N	# Positive	% Positive	TAE
250	2.40	36	36	100	0.53

Limit of Detection of the NeuMoDx EBV Assay in Whole Blood. The LoD and LLoQ for the NeuMoDx EBV Quant Assay in whole blood was confirmed by processing 36 whole blood samples spiked with 250 IU/mL 1st WHO International Standard for EBV with a 100% detection rate.

EBV Linearity

LINEAR RANGE AND UPPER LIMIT OF QUANTITATION (ULoQ)

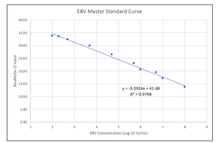
The linearity of the NeuMoDx EBV Assay was determined by diluting either purified EBV stock (Exact Diagnostics LLC, Fort Worth, TX) or NeuMoDx Encapsulated EBV Control in pooled EBV-negative plasma to create a panel spanning 6 Log_{10} units of EBV concentration ranging from 8 Log_{10} IU/mL to 2 Log_{10} IU/mL. The NeuMoDx EBV Assay demonstrated excellent linearity across 6 Log_{10} units, with a ULoQ determined at 8.0 Log_{10} IU/mL.

This data was used to generate a Master Standard Curve for the EBV Assay, which is used by the NeuMoDx Software in combination with a Calibration Coefficient (determined with the EBV Calibrators) to provide a quantitative result. The NeuMoDx EBV Assay uses the same Master Standard Curve and Calibrators prepared in Basematrix for quantitation of both plasma and whole blood specimens.

NeuMoDx EBV Assay Linearity in Plasma

Target Conc. (Log_{10} IU/mL)	n	Mean Conc. (Log_{10} IU/mL)	Abs. Bias	Standard Deviation (SD)	Total Analytical Error (TAE)
8.00	35	8.21	0.21	0.12	0.45
7.00	34	6.68	0.32	0.10	0.52
6.00	35	6.18	0.18	0.07	0.33
6.70	36	6.50	0.20	0.06	0.32
5.70	33	5.46	0.24	0.12	0.47
4.70	36	4.46	0.24	0.15	0.54
3.70	36	3.44	0.26	0.25	0.75
2.70	42	2.72	0.02	0.34	0.69
2.30	36	2.34	0.04	0.30	0.63
2.00	42	2.29	0.29	0.29	0.86

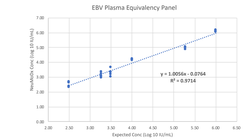
Linear range of the NeuMoDx EBV Assay in K2EDTA Plasma. The NeuMoDx EBV Assay is linear over 6 Log_{10} units.

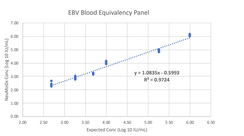


EBV Master Standard Curve. A total of 360 samples were compiled to generate a plot of the threshold cycle at which EBV is detected (Ct) against the known concentration of the target in Log_{10}. The resulting slope of the linear fit, intercept value, and coefficient of determination (R2) is stored in the Assay Definition File (ADF) for the NeuMoDx EBV Assay and used in combination with NeuMoDx EBV Calibrators to assign a quantitative value to test specimens.

EQUIVALENCY BETWEEN PLASMA AND WHOLE BLOOD

In order to validate the Master Standard Curve and demonstrate equivalency between whole blood and plasma, panels were prepared in both plasma and whole blood spanning the quantitative range of the assay. These results show the ability of the NeuMoDx EBV Assay to return an accurate quantitation for both whole blood and plasma specimens using one set of calibrators with the Master Standard Curve.





Confirmation of Linearity in Plasma and Whole Blood. Linearity panels were prepared in both plasma and whole blood using EBV reference materials and secondary standards. The NeuMoDx EBV Assay shows excellent correlation between plasma and blood matrices using the master standard curve and NeuMoDx EBV Calibrators.

Analytical Specificity & Interference

NeuMoDx EBV Assay - Analytical Specificity

BK Polyomavirus	Cytomegalovirus	Human Herpes Virus type-6	Human Herpes Virus type-7	Human Herpes Virus type-8	Hepatitis B Virus
Adenovirus type 5	Hepatitis C Virus	Parvovirus B19	JC Virus	Human Papillomavirus 16	Human Papillomavirus 18
Herpes Simplex Virus type-1	Herpes Simplex Virus type-2	Varicella-Zoster Virus	HIV-1	HIV-2	
Chlamydia trachomatis	Clostridium perfringens	Enterococcus faecalis	Escherichia coli	Klebsiella pneumoniae	Listeria monocytogenes
Mycobacterium avium	Mycoplasma pneumoniae	Neisseria gonorrhoeae	Propionibacterium acnes	Salmonella typhimurium	Staphylococcus aureus
Staphylococcus epidermidis	Streptococcus pneumoniae	Streptococcus pyogenes	Aspergillus niger	Candida albicans	Cryptococcus neoformans

EBV Analytical Specificity (Cross-Reactivity). The analytical specificity of the NeuMoDx EBV Assay was evaluated with EBV-free plasma spiked with 35 phylogenetically similar or commensal organisms found in blood/plasma specimens at high concentrations.* No cross-reactivity was observed in any of the organisms listed in the above table demonstrating 100% analytical specificity.

**Bacteria were spiked at ~ 6E6 CFU/mL; Adenovirus and HIV-2 were spiked at 1E4-1E5 TCID$_{50}$/mL. Remaining viruses were spiked at 1E6-1E7 IU or copies/mL, DNA was spiked at 1E6 copies/mL.*

NeuMoDx EBV Assay Interference - Commensals

Non-Target Organisms	Average Conc. (Log_{10} IU/mL)	Δ Control (Log_{10} IU/mL)
BK Virus, CMV, HHV-6, HHV-7, HHV-8 and HBV	3.31	0.12
Adenovirus 5, HCV, Parvo B19, JC Virus, HPV-16 and HPV-18	3.42	0.23
HSV-1, HSV-2, JC Virus, HIV-1 and HIV-2	3.33	0.14
Chlamydia trachomatis, Clostridium perfringens, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae and Listeria monocytogenes	3.40	0.20
Mycobacterium avium, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Propionibacterium acnes, Salmonella typhimurium and Staphylococcus aureus	3.17	0.02
Staphylococcus epidermidis, Streptococcus pneumoniae, Streptococcus pyogenes, Cryptococcus neoformans, Candida albicans and Aspergillus niger	3.19	0.00

Interfering Substances – Commensal Organisms. The NeuMoDx EBV Assay was performed with EBV positive plasma prepared by spiking negative plasma with EBV targets at 1E3 IU/mL in the presence of the same 35 non-target organisms tested for cross-reactivity. Organisms were tested in pools. Minimal deviation of quantitation from EBV control samples was observed indicating no significant interference from any of the 35 organisms.

NeuMoDx EBV Assay Interference – Endogenous Substances & Disease States

Endogenous/Disease State	Average Conc. (Log_{10} IU/mL)	Δ Control (Log_{10} IU/mL)
Albumin *(120 mg/mL)*	3.20	0.22
Bilirubin *(0.02 mg/mL)*	3.48	-0.05
Hemoglobin *(3 mg/mL)*	3.20	0.22
Triglycerides *(5 mg/mL)*	3.15	0.28
Systematic Lupus	3.23	0.20
Antinuclear Antibody	3.33	0.10
Rheumatoid Arthritis	3.19	0.24

EBV Interfering Substances – Endogenous Substances. The possible interference of endogenous substances and disease state plasma on the NeuMoDx EBV Assay was evaluated with four (4) endogenous substances and three (3) disease state specimens. The NeuMoDx EBV Assay had minimal deviation of quantitation from EBV control samples, with no significant interference.

NeuMoDx EBV Assay Interference – Exogenous Substances

Drug Pools	Average Conc. (Log_{10} IU/mL)	Δ Control (Log_{10} IU/mL)
Azathioprine, Cyclosporine, Foscarnet, Ganciclovir, Valganciclovir hydrochloride	3.30	0.13
Prednisone, Cidofovir, Cefotetan, Cefotaxime, Fluconazole	3.22	0.21
Mycophenolate mofetil, Mycophenolate sodium, Piperacillin, Sirolimus/ rapamycin, Tazzobactam	3.36	0.07
Trimethoprim, Vancomycin, Tacrolimus, Everolimus, Clavulanate potassium	3.32	0.11
Famotidine, Sulfamethoxazole, Valacyloir, Letermovir, Ticarcillin disodium, Leflunomide	3.47	-0.10

Interfering Substances – Exogenous Substances. The NeuMoDx EBV Assay was performed with EBV-positive plasma prepared by spiking negative plasma with EBV targets at 1E3 IU/mL in the presence of 26 drugs commonly utilized by the patient population most at risk of EBV infection. Medications were tested in pools. Minimal deviation of quantitation from EBV control samples was observed indicating no significant interference from any of the 26 medications.

Within Lab Precision

The Within Lab Precision of the NeuMoDx EBV Assay was determined by testing a four-member panel of EBV in plasma over two days, with three testing rounds per day, using two NeuMoDx 288 Molecular Systems and one NeuMoDx 96 Molecular System.

The precision Within-run and Across-runs was characterized and the standard deviation for both was determined to be < 0.35 Log_{10} IU/mL.

NeuMoDx EBV Assay Within Lab Precision

Panel Member	Target Conc. (Log_{10} IU/mL)	Mean Conc. (Log_{10} U/mL)	N	Within System SD	Within Day SD	Within Run SD	Overall (Within Lab) SD
1	5.20	5.30	90	0.27	0.25	0.25	0.27
2	4.20	4.25	90	0.21	0.21	0.12	0.21
3	3.20	3.38	90	0.22	0.20	0.20	0.22
4	2.70	3.03	90	0.30	0.30	0.30	0.33

Within Lab Precision of the NeuMoDx EBV Assay. The NeuMoDx EBV Assay demonstrated excellent within laboratory precision calculated from the quantitative data across target levels, Systems and days with a maximum overall standard deviation < 0.35 Log_{10} IU/mL.

Lot-to-Lot Reproducibility

The NeuMoDx EBV Assay Test Strip and Lysis Buffer 5 are the key components for the NeuMoDx EBV Assay. Excellent lot-to-lot reproducibility was demonstrated spanning three production lots using plasma as the specimen matrix.

NeuMoDx EBV Assay Lot-to-Lot Reproducibility

	Panel Member	Target Conc. (Log_{10} IU/mL)	Mean Conc. (Log_{10} IU/mL)	N	Abs. Bias	Between Lots SD	Within Lot SD	Overall SD
EBV Quant Assay Test Strip	1	5.0	4.97	54	0.02	0.06	0.08	0.10
	2	4.0	3.95	54	0.02	0.08	0.09	0.12
	3	3.0	3.03	54	0.02	0.06	0.10	0.12
	4	2.0	2.02	54	0.03	0.05	0.20	0.20
Lysis Buffer 5	1	5.0	4.97	15	0.18	0.05	0.03	0.06
	2	4.0	3.96	15	0.08	0.22	0.10	0.24
	3	3.0	3.03	15	0.10	0.09	0.11	0.15
	4	2.0	2.13	15	0.04	0.39	0.13	0.41

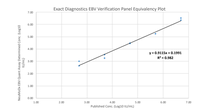
Lot-to-Lot Reproducibility of the NeuMoDx EBV Assay. The NeuMoDx EBV Assay demonstrated excellent reproducibility across target levels and reagent lots for both the EBV Test Strip and Lysis Buffer 5.

Performance Characterization

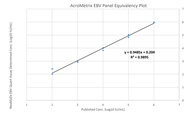
The overall quantitative performance of the NeuMoDx EBV Assay was assessed by testing two commercially available panels of EBV: the AcroMetrix EBV Verification Panel and the Exact Diagnostics EBV Verification Panel. The testing was performed on both the NeuMoDx 288 and the NeuMoDx 96 Molecular Systems.

	Performance Characterization using Commercially Available EBV Panels			
	Equivalency Plot Slope	Equivalency Plot Y-intercept (Bias)	Deming R2 Value	Passing-Bablok p-value
AcroMetrix EBV Panel	0.95	0.20	1.00	0.33
Exact Diagnostics Verification Panel	0.91	0.20	0.99	0.82
Combined Data	0.92	0.25	0.99	0.40

Summary of linearity between NeuMoDx EBV Assay Calculated concentrations and manufacturer-supplied concentrations for EBV Verification Panels



Exact Diagnostics Equivalency Plot
This equivalency plot compares the concentration of EBV calculated by the NeuMoDx EBV Assay to the published concentration of EBV for the Exact Diagnostics EBV Verification Panel.



AcroMetrix Equivalency Plot
This equivalency plot compares the concentration of EBV calculated by the NeuMoDx EBV Assay to the published concentration of EBV for the AcroMetrix EBV Verification Panel.

CONCLUSIONS

The NeuMoDx EBV Quant Assay demonstrated excellent performance using both plasma and whole blood specimens as implemented on the extremely easy to use, rapid, automated NeuMoDx Molecular Systems; sample-to-result solutions for viral load monitoring.

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The NeuMoDx EBV Assay is not available for sale. Performance characteristics for this product have not been established.