Rady Children's Hospital San Diego	APPROVAL DATE	MANUAL:
☐ **P O L I C Y** ☒ **PROCEDURE** ☐ **STANDARD OF CARE** ☐ **STANDARDIZED PROCEDURE** ☐ **GUIDELINE** ☐ **OTHER**	**1/16/2019**	**Molecular Diagnostics** TRACKING # **MD077**
	TITLE: **EBV DNA Detection**	
	PERFORMED BY: **Clinical Laboratory Scientists**	

1. PURPOSE

This procedure provides instructions for the qualitative and quantitative detection of Epstein Barr Virus (EBV) in plasma using real-time PCR.

2. PRINCIPLE

The EBV DNA Detection assay is a real-time PCR method using Diasorin EBV Primer Pair Analyte Specific Reagent (ASR) and the Liaison MDX Integrated Cycler instrument. The primer pair sequences amplify and detect a conserved region of the Lmp2A gene in purified extracts of EDTA plasma. The Liaison quantitation algorithm estimates the starting template copies by modeling the concentration of PCR reaction substrates, as determined by a calibration curve.

3. CLINICAL SIGNIFICANCE

The Epstein-Barr virus (EBV), a widespread human herpesvirus with B-lymphocyte tropism, is the causative agent of infectious mononucleosis, and is strongly associated with several human malignancies. Infection with EBV is common and is generally subclinical or presents as a self-limited illness lasting 2 to 3 weeks. As a member of the herpes family, EBV can remain latent for long periods of time, and become reactivated at a later stage. Though the initial infection may result in a relatively benign syndrome, reactivation in an immunocompromised person can lead to more serious results. EBV nucleic acids or proteins have been identified in tissues affected by Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, transplantation-related lymphoproliferative disorders, AIDS-related primary central nervous system lymphoma, and other B- and T-cell lymphomas.

Conventional diagnostic methods are rarely useful in the evaluation of EBV-related disorders in immunosuppressed patients. PCR offers the possibility of expedient detection of EBV from specimens. Monitoring of EBV viral load may help with the identification of post-transplantation lymphoproliferative disorders, and monitoring lymphomas in patients with the acquired immunodeficiency syndrome.

4. SAMPLE REQUIREMENTS

4.1. Whole blood collected in an EDTA tube, 1-2 mL to yield a minimum of 0.3 mL plasma. Transport specimens to the laboratory as soon as possible.

4.2. Centrifuge blood and aseptically transfer plasma to a sterile screw-cap tube within 2 hours of collection.

4.3. Store at or below -70^{o}C upon receipt. Plasma for EBV DNA testing is stable for up to 1 month frozen, 1 week refrigerated, and 24 hours at room temperature.

4.4. Rejection Criteria

4.4.1. Unlabeled or incompletely labeled specimens will be rejected.

4.4.2. Specimens with signs of leakage during transport will be rejected.

4.4.3. Heparinized and ACD plasma.

4.4.4. For rejected specimens, request a new specimen and document the request. Store rejected specimen until a new specimen is received and assign a new specimen number.

5. MATERIALS, EQUIPMENT, AND SUPPLIES

Reagents/Controls	Equipment	Supplies
Simplexa EBV Primer Pair, MOL9002	* 3M Integrated Cycler, Software version 3.0 or higher	Universal Discs, MOL1400
Simplexa 2.5X Universal Master Mix, MOL9010	Single-channel micropipettes with accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL	Universal Disc Cover Tape, MOL1500
Simplexa DNA Extraction and Amplification Control Set	Freezer (manual defrost) at -10 to -30°C	Universal Disc Cooling Plate
Nuclease-free water (Sigma cat. W4502).	Freezer (manual defrost) at -70°C and refrigerator at 2 to 8°C	Universal Disc Applicator Sealer
Negative Control: Pooled EBV Negative plasma	Biosafety cabinet and Dead Air Box	RNase/DNase-free disposable aerosol-barrier pipet tips
Exact Diagnostics EBV Low Run Control, EBVL101	Microcentrifuge(s)	RNase/DNase-free microcentrifuge tubes
Exact Diagnostics EBV High Run Control, EBVH102	Vortex mixer(s)	Reagent Cooling block or ice bucket

***Instrument/Reagent Manufacturer:** Diasorin, Inc. Technical Support: 562-240-6369 or 800.838.4548, option 3

6. REAGENTS AND QC MATERIALS

6.1. Do not use reagents beyond expiration dates.

6.2. Store Simplexa reagents at -10 to -30°C (do not use frost-free freezer).

6.3. Allow Simplexa reagents to thaw at room temperature (20 to 24°C), and then place on ice or in a refrigerated cooler block immediately.

6.4. Once thawed, store Simplexa reagents at 2 to 8°C for no more than 30 days. Do not refreeze.

6.5. If PCR setup will not be performed immediately after the reaction mix is prepared, store reaction mix at 2 to 8°C and use within one hour.

6.6. Do not combine Simplexa reagents from different lots or shipments.

6.7. Store Exact Diagnostics Low and High Run Controls at -70°C. Thaw material at room temperature and vortex briefly prior to use. Once thawed, material is stable for 24 hours when stored at 2-8°C. Do not dilute.

7. QUALITY CONTROL POLICY

7.1. Control samples are tested in the same manner as patient samples.

7.2. At least one Negative control is included with each run.

7.3. Low and High run controls are included with each run.

7.4. New lots of Primer Pairs are evaluated with positive and negative specimens (when available), concurrent with being placed in use. QC or external PT material may be used when positive specimens are not available.

7.5. New lots of 2.5X Universal Master Mix are evaluated with QC material concurrent with being placed in use.

7.6. Reaction inhibition is monitored with the SEAC set.

8. CALIBRATION POLICY

Calibration must be performed at least once every six months, with primer pair lot changes, when quality control repeatedly fails to meet established criteria, and following major service to the instrument. Refer to procedure MD072, Assay Calibration on the Integrated Cycler.

9. PROCEDURE

9.1. Positive Control Preparation

CAUTION: All steps of the control preparation procedure are performed in a biological safety cabinet (BSC). All specimens must be removed from the BSC prior to handling controls.

Step	Action
1.	• Thaw all vials of ExactDx EBV Low and High Run Controls at room temperature. Vortex briefly and quick spin.
2.	• Prepare aliquots by carefully transferring 215 µL of control material into 1.5 mL microcentrifuge tubes. • Label tubes with control name (EBV LPC, EBV HPC), lot number, and expiration date. Store aliquots at -60°C to -80°C.
3.	• Decontaminate all work surfaces and equipment inside the BSC prior to handling specimens.

9.2. Testing Protocol

Step	Action
1.	**Prepare PCR Worksheet** • Attach small specimen label (or write the specimen number) to each location on worksheet. • Add the Negative, Low Positive, and High Positive controls to the worksheet.
2.	**Prepare easyMAG Extractor** • Program a Quant DNA extraction run. The elution volume is 50 uL. • Identify the easyMAG sample position on the PCR worksheet.
3.	**Biosafety Cabinet: Dispense Specimens** • Thaw and briefly vortex specimens and controls. Briefly centrifuge the controls. Place specimens and controls on ice until needed. • Pipet 5 uL of the Internal Control into each location on the easyMAG sample strip. • Pipet 200 uL of specimen and control materials into the appropriate easyMAG Sample Strip location.
4.	**EasyMAG Extraction** • Load Sample Strips on the easyMAG. • Refer to MD065 for instructions to start on-board lysis, add Magnetic Silica, and complete the extraction.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>5.</td><td>Dead Air Box: Prepare Reaction Mix / Add to Universal Disc
• Calculate total reagent volumes for Reaction Mix on worksheet:
<table>
<tr><th>Reagent</th><th>Volume per reaction</th></tr>
<tr><td>Simplexa 2.5X Master Mix</td><td>4.0 uL</td></tr>
<tr><td>Nuclease-free water</td><td>0.4 uL</td></tr>
<tr><td>EBV Primer Pair</td><td>0.4 uL</td></tr>
<tr><td>SEAC Primer Pair</td><td>0.2 uL</td></tr>
</table>
• If frozen, thaw Primers and 2.5X Master Mix at room temperature and store in refrigerated cooling block during use.
• Mix the vials by inverting 6 to 8 times and briefly centrifuge.
• Add Reaction Mix reagents to a 0.6 mL microcentrifuge tube in a cooling block.
• Mix by inversion or by pipetting 8 to 10 times and briefly centrifuge.
• Place a Universal Disc on a Disc Cooling Plate.
• Add 5 µL of the reaction mix to each well of the universal disc.
Note: To reduce the risk of contamination, hold the pipette at a 30-degree angle and insert the tip under the roof of the well to deliver the sample.</td></tr>
<tr><td>6.</td><td>Sample Loading Area: Load Samples on Universal Disc
• Add 5 µL of extracted patient samples and controls to the appropriate well on the Universal Disc.
Apply the Universal Disc Cover Tape to the disc, using the Disc Applicator to seal tape completely.</td></tr>
<tr><td>7.</td><td>PCR Amplification Area: Start a EBV Quant Prediction Run
• Load the sealed Universal Disc in the Integrated Cycler and start the run. Refer to MD063, Operation of Integrated Cycler, for instructions to program and start run.</td></tr>
</table>

9.3. Result Interpretation

Step	Action
1.	**Generate a Report** • Remove the disc and inspect reactions wells for appropriate volume (10 uL). NOTE: Reaction wells containing the incorrect volume must be investigated and the sample must be retested. • From the analysis screen, select the segment to print. • Press the Print Preview button to view and print the ***Quant Prediction Report***.
2.	**Analyze Controls** • The Negative control must have an Estimated Quantity of *Not Detected*. • Both levels of positive control must have an Estimated Quantity that falls within established ranges. **Note:** If the above criteria are not met, refer to **Out-of-Range QC** instructions. Do not proceed to Step 3.

Step	Action
3.	**Analyze Patient Sample Results** • Examine patient sample results after controls are determined to be valid. • For samples with an EBV IU/mL of *Not Detected*, the internal control (SEAC) must be *Detected*. If the SEAC is *Not Detected*, the sample must be retested. • Highlight all samples on the report printout with a numeric value.

9.4. **Out-of-Range QC Protocol**

9.4.1. If the Negative control has a numeric value, contamination is suspected. The run is handled as follows:

9.4.1.1. Results that are Not Detected and SEAC is Detected can be reported.

9.4.1.2. Results with a numeric value are not reported and must be retested.

9.4.1.3. Follow the steps outlined in Procedure MD032, "Reduction of Contamination in the Molecular Laboratory", until evidence of contamination is eliminated.

9.4.2. If both positive QC levels are Not Detected or have numeric values that fall outside established limits, master mix preparation errors or reagent integrity is suspected. The run must be repeated with new reagents if indicated.

9.4.3. If one of 2 positive QC levels is Not Detected, a pipetting error for the affected level is suspected. Retest the control eluate in question with the same reagents to rule out other sources of error before reporting patient results.

9.4.4. If one of 2 positive QC levels falls outside the established limits, observe the QC Results Review function in Beaker. Review the Levy-Jennings (LJ) graph of the affected level for shifts or trends.

9.4.5. A downward trend may indicate deteriorating QC material. Replace the affected level and monitor the subsequent run. Patient results may be reported if the unaffected level is within range.

9.4.6. A shift may indicate a QC lot change or pipetting error for the affected level. Patient results may be reported if the unaffected level is within range.

9.5. **LIS Result Entry**

9.5.1. Qualitative Tests

9.5.1.1. Report printout result is Not Detected: report Negative.

9.5.1.2. Any numeric value: report Positive. If a "Qualitative with Reflex to Quantitative" test was ordered, a quantitative test will be generated. Report the numeric value.

9.5.2. Quantitative Tests

9.5.2.1. A numeric value that is $\geq$ 430 IU/mL or <4,541,006 IU/mL: Report the numeric value in IU/mL and $\log_{10}$. The interpretive comment is *Detected*.

9.5.2.2. A numeric value that is less than 430 IU/mL: report *Detected but Not Quantified*. The interpretive comment is, *EBV is detected but at a level below 2.63 log IU/mL (430 IU/mL). Virus detected at a level below 2.63 log IU/mL cannot be accurately quantified by this assay.*

9.5.2.3. A numeric value that is >4,541,006 IU/mL (>6.66 log IU/mL): Report *Detected but Not Quantified*. The interpretive comment is, *EBV DNA is detected but at a level above 4,541,006 IU/mL (6.66 log IU/mL). Virus detected at a level above 6.66 log IU/mL cannot be accurately quantified by this assay.*

9.5.3. The following comments are attached to all results:

The quantification range of this assay is 430 to 4,541,006 IU/mL (2.63 log to 6.66 logIU/mL).

"This test was developed and its performance characteristics determined by the Molecular Diagnostics Laboratory, Rady Children's Hospital San Diego. It has not been cleared or approved by the U.S. Food and Drug Administration. This test is for clinical purposes and should not be regarded as investigational or for research."

10. ANALYTICAL SENSITIVITY

The lowest actual concentration of analyte that can be detected greater than 95% of the time Limit of Detection (LoD) is 486 IU/mL (2.64 log IU/mL).

11. ANALYTICAL SPECIFICITY

Studies have indicated that the primers did not cross react with other viruses or bacteria including Adenovirus, *Anaplasma phagocytophilum*, *Bartonella henselae*, *Bartonella quintana*, *Borrelia burgdorferi*, *Ehrlichia chaffeensis*, Enterovirus, HBV, HIV-1, HIV-2, HSV-1, HSV-2 HDV, HHV-6, HHV-7, HHV-8, HTLV-1, JCV, Parvovirus, *Toxoplasma gondii*, VZV, CMV, and BKV. Furthermore, nucleic acid sequence databases indicated that the primers did not have significant homology with other pathogens or with human DNA.

12. LIMITATIONS

12.1. Testing personnel must be trained and familiar with testing procedures and interpretation of results prior to performing the assay.

12.2. All results from this and other tests must be used in conjunction with the clinical history, epidemiological data and other data available to the clinician evaluating the patient.

12.3. The prevalence of infection will affect the predictive value.

12.4. Negative results do not rule out EBV infections.

12.5. False-negative results may occur when the infecting organism has genomic mutations, insertions, deletions, or rearrangements or when performed very early in the course of illness.

12.6. False-negative results may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling.

12.7. False-positive results may occur. Repeat testing may be indicated in some settings.

12.8. The performance of this test has not been established with potentially interfering medications for the treatment of infectious mononucleosis.

12.9. This test cannot rule out diseases caused by other bacterial or viral pathogens.

13. INTERFERING SUBSTANCES

The performance of this assay was evaluated for potentially interfering substances that may be present in plasma. Reaction inhibition is monitored with the Simplexa Extraction Amplification Control (SEAC).

14. EXPECTED RESULTS

The expected normal result for EBV DNA Qualitative is NEGATIVE.

15. REFERENCES

Diasorin Molecular EBV Primer Pair, REF MOL9002 Rev. 01

LAISON® MDX Studio Software Revision 1.0, March 31, 2017 PI-MOL1101-UD-D Rev 01PI.MOL1101.