

Rodent Pathogen Testing Standard Operating Protocol

Ver 1.01

Modified Date: 12/06/2020

Receipt and Custody of specimens

Blood and ear samples will be collected in the field by Battelle from rodents that meet taxonomic, size, condition, and bleed/ear sample history criteria (including *Peromyscus* spp., other cricetid species, and dipodids). The targeted blood volume of each sample collected will range from 10 μ L to 50 μ L. Ear samples are collected using a 2000 Micron (2 mm) tissue biopsy punch. Each blood or ear sample will be collected by NEON in an individually labeled cryovial. Samples will be shipped to UMass on dry ice with accompanying shipping manifest.

Incoming rodent samples:

- Rodent samples submitted by Battelle will come in large batches. Open the container(s) and ensure all samples listed on the manifest have been received and are in good condition (e.g., not broken or missing; labels are legible). Notify the Contract Officer immediately of any problems with the shipment within 48 hours of receipt.
- An internal label will be assigned to each rodent sample at this point. The data should be input into a database
- After reconciling with internal label and Battelle manifest, samples are then moved forward for Total Nucleic Acid Extraction.

Total Nucleic Acid Extraction

Things to do before starting:

- This protocol was modified from Epicentre MasterPure Complete DNA and RNA Purification Kit Bulk Reagents.
- Total Nucleic Acid Extractions are performed in the “dirty” room, Room B1.
- Total Nucleic Acid extractions are carried out on a bench that is cleaned daily with 10% Bleach.
- All waste from the DNA extraction is placed in a plastic “trash” container on the bench and then emptied in to the biohazard trash.

- There should be 1 extraction control for each set of extractions. This is typically the 30th tube in the set. This no template control allows us to monitor for DNA contamination in the extraction reagents as well as DNA contamination between samples due to human error or a problem with the protocol.
- Turn on and warm Hybridization Oven to 65C.
- Turn on and cool centrifuge to 4C.

For rodent ear samples, part A:

1. Place the tube of rodent ear samples into a TissueLyser reaction-tube holder (24 samples/holder).
2. Place the tube holder and tubes into a -80C freezer overnight (or at least 1 hour). **DO NOT** freeze top and bottom plate of the adaptor, only freeze tube holder.
3. Assemble the TissueLyser Adapter Set 2 x 24 using a bottom plate, the reaction-tube holder (with 2.0 ml safe-lock tubes containing one ear sample and one 5mm bead), and a top plate with sealing strips.
4. Ensure that the top and bottom plate fit securely, and make sure that the reaction tubes are sealed securely.
5. Make sure that two TissueLyser adapter sets are balanced. Distribute the reaction tubes evenly across the TissueLyser. Balance the lid by ensuring 2.0 ml safe-lock tubes are in all 4 corners of both 24 sample holders.
6. Place the assembled TissueLyser Adapter Sets into the TissueLyser holders (arms), and close the handles tightly. **DO NOT** use the TissueLyser with only one TissueLyser Adapter Set, since this will cause machine imbalance and decrease homogenization efficiency.
7. Homogenize the rodent ear samples at 23Hz, 1.5 minutes.

For rodent blood samples, part A:

1. Add 300 μ L of Red Cell Lysis Solution into 50 μ L whole rodent blood tube. Invert three times to mix and then flick the bottom of the tube to suspend any remaining material.
2. Incubate at room temperature for 5 minutes and then vortex briefly. Continue incubating at room temperature for an additional 5 minutes followed again by brief vortexing.
3. Pellet the white blood cells by centrifugation for 25 seconds in a microcentrifuge.
4. Remove most of the supernatant, leaving approximately 25 μ L of liquid. Vortex to suspend the pellet.

For rodent blood and tissue samples, part B:

1. Dilute 1 ul of 50 ug/ul Proteinase K (<http://www.epibio.com>; Proteinase K; MPRK09250 $\mu\text{g}/\mu\text{l}$) into 300 ul of 1X Tissue and Cell Lysis Solution (<http://www.epibio.com>; 2X T&C Lysis Buffer should be diluted to 1X using molecular grade water. 2X T&C Lysis Buffer MTC085H) for each sample. For 30 samples, prepare master mixture: 1X Tissue and Cell Lysis Solution: 300 ul X 31 = 9300 ul; 50 ug/ul Proteinase K : 1 ul X 31 = 31 ul;
2. Take 301ul master mixture for one sample from part A.
3. Mix thoroughly and incubate at 65 C for 15 minutes; vortex every 5 minutes.
4. Place the samples on ice for 10 min.
5. Add 150 ul of MPC Protein Precipitation Reagent (MPC Protein Precipitation; MMP03750 Bulk order) to 301 ul of lysed sample and vortex vigorously for 10 seconds.
6. Pellet the debris by centrifugation at 4 C for 10 minutes at 14,000 RPM in a microcentrifuge.
7. Transfer 350 ul of supernatant to a tube containing 500 ul of ice cold isopropanol. Invert the tube several (30-40) times. Store the protein pellets in the 4C fridge until all testing is complete.
8. Pellet the DNA/RNA by centrifugation at 4 C for 10 minutes at 14,000 RPM in microcentrifuge.
9. Carefully pour off the isopropanol without dislodging the nucleic acid pellet. You can remove all of the residual isopropanol with a pipette.
10. Rinse with 1000 ul 75% ice-cold ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipette.
11. Air-dry your pellets overnight in the bio-safety cabinet, or in the 65C incubator for 20 minutes. Use tube openers to separate tubes to prevent cross contamination. Resuspend the DNA/RNA in 40 ul of molecular grade water.
12. Store samples (29 samples and 1 extraction control in general) at -20C in the freezer box and proceed to the next section for real-time PCR setup.

Design of Assays and Master Mix

- We use multiplexing Taqman real-time PCR to detect rodent pathogen DNAs.
- The multiplexing master mixes are using Agilent Brilliant III Ultra-Fast QPCR (Agilent Cat# 600880). The primers and dual-labeled probes are ordered from IDTDNA. Double-Quenched Probes contain a 5' fluorophore (FAM, HEX or Cy5); 3' IBFQ quencher; and proprietary, internal ZEN or TAO quencher.
- Gene fragments (gBlocks) are ordered from IDTDNA as Taqman PCR positive QC controls. These gene fragments (gBlocks) are double-stranded, sequence-verified genomic blocks.

- Multiplexing master mix are made each in 50 ml conical tube. Six 15ul reaction from the master mix are tested QC at gBlock concentration 5 pg- 0.005pg for positive controls (3 reactions) and water for negative controls (3 reactions).
- If the master mixes pass the QC, they are aliquoted into 1.5 mL tubes and stored at -80C (About 100 reactions per tube, 15 ul per reaction). The master mixes that aren't used as frequently should be stored as smaller aliquots to limit the number of times each aliquot needs to be thawed and frozen (as this may reduce the efficacy of the master mix). Exposure to light can also damage the master mix. Each technician should have their own freezer box in the -20°C freezer and they should remove a few tubes of the master mixes that they will need that week and place them at -20C. When preparing to run a plate, the appropriate mastermix tubes should be placed in a DNA-free tube rack at 4C to thaw.
- Rodent pathogen groups are also listed below:

Base Pathogens:

Base Pathogens Group B-IT1:

Borrelia general species
Borrelia burgdorferi sensu lato
Borrelia miyamotoi
Borrelia mayonii

Base Pathogens Group B-IT2:

Babesia microti
Anaplasma phagocytophilum
 Ehrlichia muris-Like Agent

Option 1 Pathogens:

Option 1 Pathogens Group B-NIT1:

Ehrlichia chaffeensis
Borrelia lonestari
Ehrlichia ewingii

Option 1 Pathogens Group B-NIT2:

Francisella tularensis
Rickettsia rickettsii
Rickettsia parkeri

Rickettsia philipii

Option 2 Pathogens:

Option 2 Pathogens Group Virus:

Powassan virus

Heartland virus

Bourbon virus

Colorado Tick Fever virus

Setup a Sample Loading Map

- All sample are run as a 96-well plate format.
- Use a below sample as a template map. It contains 29 samples, 1 extraction control (EC), 1 super positive control (SPC) and 1 no template control (NTC).
- Print out the map and use this to determine which samples and master mixes need to be thawed at 4C in order to set up this plate.
- This printed map should also be labeled with the file name that contains the results and should be filed in the office according to month as well as a copy by contract/project name (if applicable).

An example of sample loading map:

Sample ID	Well	Triplex	FAM	HEX	Cy5	Well	Triplex	FAM	HEX	Cy5	Well	Triplex	FAM	HEX	Cy5
1	A1	HTT1				A5	IT1				A9	IT2			
2	B1	HTT1				B5	IT1				B9	IT2			
3	C1	HTT1				C5	IT1				C9	IT2			
4	D1	HTT1				D5	IT1				D9	IT2			
5	E1	HTT1				E5	IT1				E9	IT2			
6	F1	HTT1				F5	IT1				F9	IT2			
7	G1	HTT1				G5	IT1				G9	IT2			
8	H1	HTT1				H5	IT1				H9	IT2			
9	A2	HTT1				A6	IT1				A10	IT2			
10	B2	HTT1				B6	IT1				B10	IT2			
11	C2	HTT1				C6	IT1				C10	IT2			
12	D2	HTT1				D6	IT1				D10	IT2			
13	E2	HTT1				E6	IT1				E10	IT2			
14	F2	HTT1				F6	IT1				F10	IT2			
15	G2	HTT1				G6	IT1				G10	IT2			
16	H2	HTT1				H6	IT1				H10	IT2			
17	A3	HTT1				A7	IT1				A11	IT2			
18	B3	HTT1				B7	IT1				B11	IT2			
19	C3	HTT1				C7	IT1				C11	IT2			
20	D3	HTT1				D7	IT1				D11	IT2			
21	E3	HTT1				E7	IT1				E11	IT2			
22	F3	HTT1				F7	IT1				F11	IT2			
23	G3	HTT1				G7	IT1				G11	IT2			
24	H3	HTT1				H7	IT1				H11	IT2			
25	A4	HTT1				A8	IT1				A12	IT2			
26	B4	HTT1				B8	IT1				B12	IT2			
27	C4	HTT1				C8	IT1				C12	IT2			
28	D4	HTT1				D8	IT1				D12	IT2			
29	E4	HTT1				E8	IT1				E12	IT2			
EC	F4	HTT1				F8	IT1				F12	IT2			
SPC	G4	HTT1				G8	IT1				G12	IT2			
NTC	H4	HTT1				H8	IT1				H12	IT2			

Date:
QPCR File Name:

Operator:
Note:

Real Time PCR Plate Setup

- This protocol was modified from Brilliant III Ultra-Fast QRT-PCR Master Mix MasterPure.
1. Thaw the master mix.
 2. Vortex briefly and spin down master mix. Put the master mix on ice.
 3. Prepare a 96 well non-skirt PCR plate.
 4. Add 15 μ l of the master mix solutions into the appropriate wells based on the sample loading map, then bring the tubes back to their storage box at -20°C.
 5. Add 1 ul of DNA/RNA template to each well, following the layout of the map, for example sample 1-29. When adding template, be sure NOT to open tubes of DNA over the plate. Pipette slowly to avoid contamination of other wells. Always hold pipette straight up and down (not tilted) to get the most accurate volume. Make sure that you are getting a NEW TIP for EVERY well and you are ejecting

the tips ABOVE the waste container in the hood (don't let the pipette move below the edge of the container as this may cause contamination of the pipette with DNA). It is also helpful to move the template tube to a new tube rack and place in the spot corresponding to the last well it was added to on the 96-well plate.

6. Add 1ul extraction control (EC), 1 ul super positive control (SPC) and 1 ul water as NTC (No Template Control).
7. Seal the 96-well PCR plate with optically clear film. Make sure to press the film down around all edges and between wells to prevent sample evaporation.
8. Centrifuge the plate using a plate centrifuge, 800 g, 3 min. Proceed to MX3000P instrument instructions.

Instrument Settings and PCR run

Important notes before starting:

- The taqman detection is set up using the Agilent Mx3000P QPCR System. Standard filter sets include FAM/SYBR® Green I (492nm-516nm), HEX/JOE/VIC (535nm-555nm), ROX/Texas Red (585nm-610nm), Cy5 (635nm-665nm).
- ROX is used as a reference dye in all tests.

1. Open the "MxPro" program.
2. Select "Quantitative PCR (Multiple Standards)" as experiment/project type and select "Turn lamp on for warm up?" (warm-up takes about 20 minutes, so do this just before setting up your plate)
3. SETUP plate and thermal profile:
 - a. Hit "All" to select all wells
 - b. Well type- Unknown
 - c. Select HEX, FAM, Cy5 and ROX under "Collect fluorescence data"
 - d. Thermal profile setup tab: Import from the file of the last plate run
 - e. Only RNA virus contains this step: 50 °C for 10 min (1 cycle), 95 °C for 3 min (1 cycle)
 - f. 95 °C for 10 min (1 cycle)
 - g. 95 °C for 15 seconds, 60 °C for 60 seconds, END* (40 cycles). *Need endpoint to tell computer to read dye
4. File → Save As → C → NEON Rodent →
5. Name as: Date_Plate_# for day (20150105_Plate1)
6. You must write this plate name on qPCR map.
7. Carefully place the plate in the Agilent Mx3000P machine qPCR machine with A1 in the top left-hand corner.
8. Be sure to lock the metal cover down on top of the plate.

9. Verify correct settings in the template file.
10. Select RUN tab, be sure to specify if the light should turn off after the run. Select START. If the lamp is not warmed up yet, click warm-up then run.

Interpretation of results

Checklist for inspecting results:

- Extraction control.
 - Negative control.
 - Super positive control.
 - Amplification curve of each sample.
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1. Once the program has finished, save the file and open to the Analysis tab.
 2. Click on an individual well and then click Results.
 - a. Check amplification curve and Ct values. The amplification curves of true positive targets are exponential. The results of the real-time PCR assays are based on cycle threshold (Ct) values obtained from the amplification curves of the targets. The Ct value represents the number of cycles required to reach a particular threshold fluorescence signal level. The fewer cycles it takes to obtain a detectable fluorescence level, the greater the amount of target in samples.
 - b. A positive sample should have an amplification curve and Ct values for FAM, HEX or Cy5. It is generally accepted that a 3-cycle difference in target Ct compared to the negative control Ct will reliably separate a true positive signal from a contamination result (Bustin, 2004). As the PCR protocol involves 40 cycles, Ct 37 is the appropriate 3 cycle different Ct value.
 - c. Ct values above 37, but below 40 may represent cases where target is present at very low levels. In this case, samples should be re-tested using 3 replicates.
 - d. Extraction control: it will result in an amplification curve if contamination occurred during extraction step.
 - e. Negative control: it will result in no amplification curve. If the Ct value of the target in a negative control is less than 3 cycles apart from the Ct of the same target in a sample, that target in that sample fails to qualify as positive and must be disregarded in the analysis.
 - f. Super positive control: it consists of a control DNA template that will result in an amplification reaction if the PCR was correctly performed.
 3. Record Ct value on the sample loading map.
 4. Input the Ct values into the rodent pathogen database along with manifest data.