Enterovirus
Detection with real time RT-PCR reagents

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Overview:

Ref 2

Enteroviruses cause some of the commonest illnesses in man; some of these infections are severe. Enterovirus (aseptic) meningitis and encephalomyelitis are epidemic in the summer. These summer epidemics include some fatal neonatal diseases and some paralytic diseases (in the past including polio) rarely myocarditis. However, the most common manifestation of summer enteroviral illnesses is asymptomatic carriage, mild febrile illnesses, coryza, pharyngitis, bronchitis, some diarrhea, and some rashes. In any season one or more enterovirus species may circulate in the same community.

Enteroviral meningitis presents as a febrile illness with headache. There may be a preceding pharyngitis and some photophobia and lethargy. The headaches and CSF pleocytosis may persist for a month, although usually only a week.

The febrile, irritable, or lethargic neonate (less than 2 months old) will have cerebral spinal fluid (CSF) obtained for "sepsis" evaluation. In the summer these CSF specimens will be tested for enterovirus by culture or RT-PCR. The CSF will have a variable number of leukocytes present from 0 to thousands/µl. Some of these enteroviral septic neonates will have seizures, a very few CNS damage, and rarely a necrotizing fatal hepatitis or myocarditis. Amongst this relatively large number of febrile neonates there will be infants with bacterial sepsis (streptococcal group B sepsis usually) and a few infants with Herpes simplex infection. These infants are difficult, if not impossible to distinguish clinically until the specific organism is identified.

Primers and probe are available for amplification and detection of enteroviruses.

The Enterovirus primers are specific for a 135bp portion of the 5'UTR segment. This conserved probe sequence detects most, but not all, Enteroviruses. The detection probes for Enterovirus are molecular beacons (1)

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Products

Products	Catalog No.	Quantity
Enterovirus FAM-BHQ1	**PP1900**	**0.055ml**

Primers and probe.

Store at –20C. 25-20 µl reactions

Typical use: Add 2 ul to 20 ul RT-PCR reaction. Use with one or two step RT-PCR reactions. Detect at 510nm.

Enterovirus Plasmid 200 pg/ml	**PLAS1900**	**0.25ml**

Store at –20C.

Typical use: make serial 10 fold dilutions in water for standard curve, diluting 5 ul into 45 ul water.

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Detection Enterovirus RNA

Attostar Enterovirus primers and probes can be used with commercial one or two step RT-PCR master mixes. These two kits work well: Qiagen OneStep RT-PCR and Invitrogen RNA UltraSense One-Step. Each needs to be ordered from their respective manufacturer.

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Use of the Enterovirus plasmids:

Dilute the plasmid in water to prepare a standard curve. Common dilutions would be 10-fold from 200 to 0.02pg/ml. The 0.02 pg/ml plasmid dilution contains 12 copies of plasmid in 2 μl.

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Qiagen OneStep RT-PCR (Catalog number 210210):

www.qiagen.com

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Reagent volumes on RotorGene*

*Similar cycle conditions and reaction volumes may be used on many other thermal cyclers.

For RotorGene, no BSA added.	Reagent volumes for 20ul Qiagen OneStep RT-PCR master mixes (ul) Qiagen #210210 2.5 mM Magesium with no Mg added. With this amt Mg addition final is 5 mM Mg.										
Reaction tube number	1	2	3	4	5	6	7	8	9	10	
25 mM Magnesium	2	4	6	8	10	12	14	16	18	20	µl
RNase free water	2.4	4.8	7.2	9.6	12	14.4	16.8	19.2	21.6	24	µl
Qiagen dNTP Mix	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0	µl
Qiagen OneStep Enzyme Mix	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0	µl
Qiagen OneStep 5X buffer	4.0	8.0	12.0	16.0	20.0	24.0	28.0	32.0	36.0	40.0	µl
Attostar Primer-probe PP1900 (10X)	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	µl
	Dispense 12 µl / tube										
	RNA 8 µl / tube										

Reaction tube number	11	12	13	14	15	16	17	18	19	20	
25 mM Magnesium	22	24	26	28	30	32	34	36	38	40	µl
RNase free water	26.4	28.8	31.2	33.6	36	38.4	40.8	43.2	45.6	48	µl
Qiagen dNTP Mix	8.8	9.6	10.4	11.2	12.0	12.8	13.6	14.4	15.2	16.0	µl
Qiagen OneStep Enzyme Mix	8.8	9.6	10.4	11.2	12.0	12.8	13.6	14.4	15.2	16.0	µl
Qiagen OneStep 5X buffer	44.0	48.0	52.0	56.0	60.0	64.0	68.0	72.0	76.0	80.0	µl
Attostar Primer-probe PP1900 (10X)	22.0	24.0	26.0	28.0	30.0	32.0	34.0	36.0	38.0	40.0	µl
	Dispense 12 µl / tube										
	RNA 8 µl / tube										

Cycle conditions on RotorGene

50C for 30minutes (Reverse transcriptase)
95C for 15 minutes (Activation of Taq and inactivation of Reverse transcriptase)
45 cycles
95C 15 seconds
55C 30 seconds RotorGene Channel Setup FAM/Sybr, Cy5; Gain 7
72C 30 seconds

FAM/Sybr has a source of 470nm and Detector 510nm (LightCycler use F1)
Cy5 has a source of 625nm and Detector 660hp nm
Quasar 670 has the same fluorescent absorption and emission as Cy5.

Reagent volumes for LightCycler

LightCycler with BSA added	Reagent volumes for 20ul Qiagen OneStep RT-PCR master mixes (ul) Qiagen #210210 2.5 mM Magesium with no Mg added. With this amt Mg addition final is 5 mM Mg.										
Reaction tube number	1	2	3	4	5	6	7	8	9	10	
25 mM Magnesium	2	4	6	8	10	12	14	16	18	20	µl
RNase free water	1.4	2.8	4.2	5.6	7	8.4	9.8	11.2	12.6	14	µl
Qiagen dNTP Mix	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0	µl
Qiagen OneStep Enzyme Mix	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0	µl
Qiagen OneStep 5X buffer	4.0	8.0	12.0	16.0	20.0	24.0	28.0	32.0	36.0	40.0	µl
BSA 1 mg/ml	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	µl
Attostar Primer-probe PP1900 (10X)	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	µl
	Dispense 12 ul / reaction tube										
	RNA 8 µl / tube										

Reaction tube number	11	12	13	14	15	16	17	18	19	20	
25 mM Magnesium	22	24	26	28	30	32	34	36	38	40	µl
RNase free water	15.4	16.8	18.2	19.6	21	22.4	23.8	25.2	26.6	28	µl
Qiagen dNTP Mix	8.8	9.6	10.4	11.2	12.0	12.8	13.6	14.4	15.2	16.0	µl
Qiagen OneStep Enzyme Mix	8.8	9.6	10.4	11.2	12.0	12.8	13.6	14.4	15.2	16.0	µl
Qiagen OneStep 5X buffer	44.0	48.0	52.0	56.0	60.0	64.0	68.0	72.0	76.0	80.0	µl
BSA 1 mg/ml	11.0	12.0	13.0	14.0	15.0	16.0	17.0	18.0	19.0	20.0	µl
Attostar Primer-probe PP1900 (10X)	12.0	14.0	16.0	18.0	20.0	22.0	24.0	26.0	28.0	30.0	µl
	Dispense 12 ul / reaction tube										
	RNA 8 µl / tube										

Cycler conditions for LightCycler

50C for 30minutes(Reverse transcripitase)
95C for 15 minutes(Activation of Taq and inactivation of Reveres trascriptase)
45 cycles
    95C 15 seconds
    55C 30 seconds acquire fluorescent signal on F1 gain =5
    72C 30 seconds
40C 30 seconds cool

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Invitrogen RNA UltraSense One-Step RT-PCR (Catalog number 11732-927)

www.invitrogen.com

Reagent volumes on RotorGene

RotorGene	Reagent volumes for 20 ul Invitrogen RNA UltraSense One-Step Quantitatve RT-PCR Cat no 11732-927.										
Reaction tube number	1	2	3	4	5	6	7	8	9	10	
RNA UltraSense Enzyme	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	µl
RNA UltraSense 5X buffer	4.0	8.0	12.0	16.0	20.0	24.0	28.0	32.0	36.0	40.0	µl
Attostar Primer-probe PP1900	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	µl
	Dispense 7 ul/tube										
	Dispense 13 ul RNA/tube										
Reaction tube number	11	12	13	14	15	16	17	18	19	20	
RNA UltraSense Enzyme	11.0	12.0	13.0	14.0	15.0	16.0	17.0	18.0	19.0	20.0	µl
RNA UltraSense 5X buffer	44.0	48.0	52.0	56.0	60.0	64.0	68.0	72.0	76.0	80.0	µl
Attostar Primer-probe PP1900	22.0	24.0	26.0	28.0	30.0	32.0	34.0	36.0	38.0	40.0	µl
	Dispense 7 ul/tube										
	Dispense 13 ul RNA/tube										

Cycle conditions on RotorGene

50C for 30minutes (Reverse transcriptase)
95C for 15 minutes (Activation of Taq and inactivation of Reverse transcriptase)
45 cycles
95C 15 seconds
55C 30 seconds RotorGene Channel Setup FAM/Sybr, Cy5; Gain 7
72C 30 seconds

FAM/Sybr has a source of 470nm and Detector 510nm (LightCycler use F1)

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Reagent volumes on LightCycler

LightCycler with BSA added	Reagent volumes for 20 ul Invitrogen RNA UltraSense One-Step Quantitatve RT-PCR Cat no 11732-927.										
Reaction tube number	1	2	3	4	5	6	7	8	9	10	
RNA UltraSense Enzyme	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	µl
RNA UltraSense 5X buffer	4.0	8.0	12.0	16.0	20.0	24.0	28.0	32.0	36.0	40.0	µl
BSA 1 mg/ml	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	µl
Attostar Primer-probe PP1900	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	µl
	Dispense 8 ul/tube										
	Dispense 12 ul RNA/tube										
Reaction tube number	11	12	13	14	15	16	17	18	19	20	
RNA UltraSense Enzyme	11.0	12.0	13.0	14.0	15.0	16.0	17.0	18.0	19.0	20.0	µl
RNA UltraSense 5X buffer	44.0	48.0	52.0	56.0	60.0	64.0	68.0	72.0	76.0	80.0	µl
BSA 1 mg/ml	11.0	12.0	13.0	14.0	15.0	16.0	17.0	18.0	19.0	20.0	µl
Attostar Primer-probe PP1900	22.0	24.0	26.0	28.0	30.0	32.0	34.0	36.0	38.0	40.0	µl
	Dispense 8 ul/tube										
	Dispense 12 ul RNA/tube										

Cycle conditions on LightCycler:

50C for 30minutes(Reverse transcriptase)
95C for 15 minutes(Activation of Taq and inactivation of Reverse transcriptase)
45 cycles
    95C 15 seconds
    55C 30 seconds acquire fluorescent signal on F1 gain =5
    72C 30 seconds
40C 30 seconds cool

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Extraction / amplification control with Qβ bacteriophage

Adding Qβ bacteriophage (BAC200) to the sample provides RNA for extraction, amplification, and reaction condition RT and PCR controls.

When added to a sample, Qβ bacteriophage adds a known amount of RNA. The Qβ RNA can then be extracted, amplified, and detected as a control. The Qβ bacteriophage controls for the efficiency of RNA extraction, reverse transcription, PCR amplification inhibitors, intact amplification reagents (reverse transcriptase, DNA polymerase, buffers, dNTPs), and instrument function (thermal cycling and fluorescent detection system).

The Qβ RNA may be detected in a separate RT-PCR reaction using FAM labeled probe (PP201). Or the Qβ RNA and test organism RNA may be detected using a multiplex reaction using Quasar 670 labeled probe (PP250) and FAM labeled test organism probe.

Please refer to the Qβ BAC200, PP201, and PP250 product literature.

Brief procedure for use of Qβ as extraction and amplification control:

- Add 5µl Qβ bacteriophage to the sample. Proceed with RNA extraction. Dilutions of the bacteriophage may be made to give a final RT-PCR Ct value that is about 35. At this dilution, the phage is more sensitive, i.e. more likely, to detect a poor extraction or the presence of RT-PCR inhibitors in the reaction.

  Please refer to the Qβ BAC200, PP201, and PP250 product literature.

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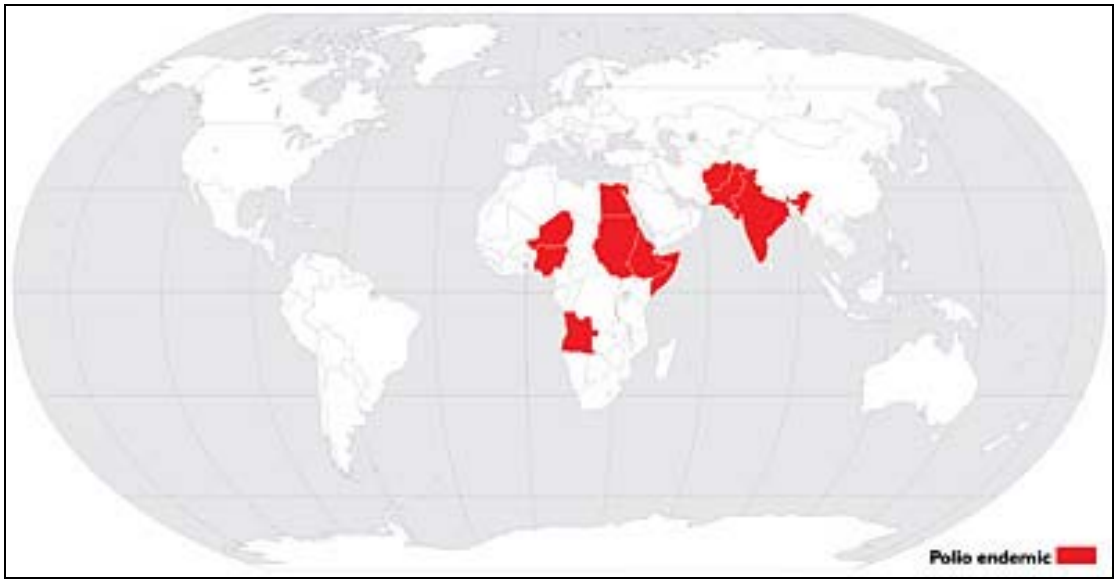
Reference:

1) http://www.molecular-beacons.org/Introduction.html
2) Image from http://www.kennebunkrotary.org/

For technical support, contact Attostar@Attostar.com

Enlarged image:

Countdown to a Polio Free World (2).



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