Disclaimer: Use of trade names and commercial sources in this protocol does not imply endorsement by the Centers for Disease Control and Prevention (CDC).

Real-time (TaqMan®) RT-PCR Assay for the Detection of Mumps Virus RNA in Clinical Samples

General Comments

Mumps virus (MuV) causes an acute viral illness with symptoms including fever, headache, muscle aches, tiredness, and loss of appetite, followed by swelling of salivary glands. The parotid salivary glands (which are located within the cheek, near the jaw line, below the ears) are most frequently affected.

TAXONOMY of the FAMILY *PARAMYXOVIRIDAE*

Subfamily *Paramyxovirinae*
- Genus *Respirovirus*
  - Sendai virus
  - Human parainfluenza viruses, types 1 and 3
  - Bovine parainfluenza virus type 3
- Genus *Rubulavirus*
  - **Mumps virus**
  - Human parainfluenza viruses, types 2, 4a and 4b
  - Simian virus 5
- Genus *Morbillivirus*
  - Measles virus
  - Canine distemper virus
  - Rinderpest virus
- Genus *Henipavirus*
  - Hendra virus
  - Nipah virus
- Genus *Avulavirus*
  - Newcastle disease virus
  - Avian paramyxoviruses 1-9

Subfamily Pneumovirinae
- Genus *Pneumovirus*
  - Human respiratory syncytial virus

Primer/Probe Target:

Mumps N Gene (designed at CDC with Primer Express, ABI):

Make a 15 μM stock of each forward and reverse primer; final concentration is 300 nM.
Make a 12.5 μM stock of the probe; final concentration is 250 nM.

Forward Primer (MuN-687F) 5' GTA TGA CAG CGT ACG ACC AAC CT 3'
Reverse Primer (MuN-668R) 5'GCG ACC TTG CTG CTG GTA TT 3'
Probe (MuN-622P) 5'FAM CC GGG TCT GCT GAT CGG CGA T BHQ 3'

gi|9695415|ref|NC_002200.1| The complete genome of mumps virus is 15384 nucleotides in length. Primers amplify a 71bp fragment within the nucleoprotein gene of mumps virus.

The mumps virus genome is a single-strand RNA that contains seven genes encoding the nucleoprotein(N), phosphoprotein (P), membrane (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) proteins. The SH gene is the most variable region of the mumps genome and is used to genotype mumps strains (see standard RT-PCR protocol for mumps for methods used to amplify the SH gene for viral detection and genotyping). Transcripts from the N gene are the most abundant transcript in cells infected with mumps virus. Therefore, the N gene provides a good target for detection of viral RNA from mumps in clinical samples and infected cells.

RNase P (designed at CDC)

Make a 15 µM stock of each forward and reverse primer; final concentration is 300 nM.
Make a 5 µM stock of the probe; final concentration is 100 nM.

Forward (HURNASE-P-F) 5'AGA TTT GGA CCT GCG AGC G 3'
Reverse (HURNASE-P-R) 5'GAG CGG CTG TCT CCA CAA GT 3'
Probe (BHQ1HURNASE-P) 5'FAM-TTC TGA CCT GAA GGC TCT GCG CGBHQ-3'

gi|13937783|gb|BC006991.1| U E G Homo sapiens ribonuclease P/MRP 30kDa subunit, mRNA (cDNA clone. Primers amplify a 64 base pair fragment, base pairs 309 through 373.

The human RNase P gene primer and probe set serves as an internal positive control to monitor sample quality, RNA extraction, and for detection of inhibitors of the PCR reaction. This control cannot by used as a control for reverse transcription (RT) since it will detect both RNA and DNA.

Test Specificity: The mumps virus primer/probe set included in this protocol has been shown to detect mumps strains in genotypes C, H, D, G, and A, including the Jeryl-Lynn vaccine strain. The real time RT-PCR assays showed nearly 100% correlation with virus isolation.

There was no cross-reaction with viral cultures from human specimens positive for parainfluenza virus type 1 (three isolates), parainfluenza virus type 3 (three isolates), respiratory syncytial virus (2 isolates), measles virus, influenza A, influenza B, adenovirus, HSV, or enterovirus.

Protocol Use Limitations:

These protocols were optimized using ABI Taqman® one-step probe RT-PCR chemistry on an ABI 7900HT, 96-well format thermocycler. However, these reactions have been adapted for use on other platforms. CDC can provide mumps RNA controls and proficiency panels for in-house validation of other platforms. If there are questions about other platforms, please contact CDC (prota@cdc.gov).

The sequence of the N gene varies between strains of wild-type viruses. This variability may result in mismatches in the primer of probe sequences with the target sequence. Therefore, this assay may not be able to detect RNA from some wild-type strains.

Positive RT-PCR results should be interpreted in conjunction with signs, symptoms, and recent MMR vaccine history due to the potential detection of the vaccine strain in clinical specimens.

Acceptable specimens: Preferred specimen is an oral/buccal swab; oropharyngeal swabs are acceptable. CSF is an acceptable specimen in the case of suspect mumps meningitis/encephalitis. The nucleic acid extraction procedure has been validated for CSF.

Oral/buccal swab for virus culture: Collect fluid by swabbing the buccal cavity (the space between the cheek and teeth). The parotid duct drains in the space near the upper molars. Massage the parotid gland area just in front of the ear and near the angle of the jaw for 30 seconds prior to collecting secretions with the swab. Swab the area between the cheek and gum by sweeping the swab near the upper molar to the lower molar area. Place the swab in viral transport medium and leave the swab in the medium.

- Swab specimens should be collected only with a Dacron® tip and an aluminum or plastic shaft.
- Swabs must be collected in viral transport medium and stored and shipped cold (i.e. 4°C)
- Swabs with calcium alginate or cotton tips and wooden shafts are unacceptable.

Rejection criteria:
- Specimen not refrigerated
- Use of improper swab or swab not in viral transport medium
- Incomplete specimen labeling/documentation
- Insufficient specimen volume

Assumptions: This procedure assumes a basic familiarity with real-time RT-PCR assays.

Precautions:
- All reactions should be carried out on ice and precautions taken against RNase contamination. Work surface and racks should be cleaned with 10% bleach solution and equipment such as pipettes and centrifuges should be cleaned and decontaminated with products such as “Eliminase” or “RNase away” to minimize risk of contamination.
- Specimens should be processed according to Biological Safety Level 2 guidelines. Specimens that have the potential to contain live virus should be processed in a Biological Safety Cabinet.
- General Practice
    - All PCR steps will occur in designated areas for a) reagent preparation (No DNA), b) specimen processing, c) DNA amplification and d) amplicon detection. Work is directional.
    - Use aerosol barrier tips, disposable gowns that are dedicated to each room, separate micropipettors and equipment for each area.

  - Reagents: will be purchased with previously documented QC when possible. Any solutions made in-house will be validated as functional and free of contamination prior to use.
  - Change gloves often.

Materials

- 20µl and 200µl adjustable pipettes and aerosol barrier tips
- 0.2ml PCR optical reaction tube strips or 96-well reaction plate
- optical strip caps or adhesive plate cover
- sterile, RNase-free, microcentrifuge tubes
- powder-free gloves
- microcentrifuge
- vortex mixer
- PCR set-up hood
- real-time PCR detection system

Reagents:
Materials Needed:

- TaqMan® PCR Core Reagent Kit; 400, 25-µl reactions/kit (Applied Biosystems N-808-0228)
- MultiScribe Reverse Transcriptase; 500 units (Applied Biosystems #4311235)
- RNase Inhibitor x 2; 2000 units@ (Applied Biosystems N-808-0119)
- Nuclease-free water (Ambion #9937)
- Quantitative PCR Human reference total RNA (Statagene #750500)
- MicroAmp optical 96-well reaction plate with barcode (Applied Biosystems #4306737)
- Optical adhesive cover (Applied Biosystems #4311971)
- http://docs.appliedbiosystems.com/pebiodocs/04310299.pdf
- Quantitative PCR Human reference total RNA (Stratagene #750500)
  - NOTE:
    - Store primer and probe stocks at –20°C.
    - Keep probe stocks protected from light.

- Nucleic acid extraction: QIAamp® Viral RNA Mini Kit (Qiagen, catalogue # 52906)

http://www1.qiagen.com/literature/handbooks/PDF/RNAStabilizationAndPurification/FromClinicalSamples/QA_Viral_RNA_Mini/1030454_HB_QA_Viral_RNA_122005.pdf

Follow manufacturer's instructions for sample extraction.

NOTE: Preliminary tests using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science, catalogue #03038505001) demonstrated recovery of detectable mumps virus with a slight increase in the cycle threshold (Ct). This automated system may be acceptable for nucleic acid isolation with a slight loss in sensitivity.

Reagent preparation:

- Keep all reagents on ice during assay set up.
- Vortex all primers and probes.
- Spin all primers and probes and then place on ice or cold block.
- Mix enzymes by pipette or flicking the tube. Do not vortex. Centrifuge briefly and keep on ice or cold block.

Controls for RT-PCR

- No template controls (NTC)
- Mumps *in vitro* transcribed N gene control (10^6 copies, from CDC)
- Every RNA sample should have the endogenous RNaseP reference gene primer/probe set run in parallel with the viral gene target to be tested to ensure the integrity of the RNA and to monitor for inhibition of the PCR reaction.
- PCR Human reference total RNA as control for above.

Procedures

1. Determine the number of reactions (n) based on the number of RNA samples and controls to be tested. Prepare excess reaction volumes (n + 1) for each primer/probe set to allow for pipetting errors and enter this value on the master mix worksheet (see below).
2. Thaw 10X TaqMan buffer, 25 mM Magnesium Chloride, and dNTPs. Once thawed, briefly vortex and centrifuge. Keep components on ice until ready to dispense. Briefly centrifuge enzymes (AmpliTaq Gold, MultiScribe RT, RNase inhibitor) and keep on ice until ready to dispense.
3. Prepare a master mix for each primer/probe set (one mix for mumps N gene, one mix for RNaseP). Add reagents to a 1.5 ml microcentrifuge tube in the order indicated on the master mix worksheet (see below) except for the RNA. Invert, briefly centrifuge, and keep on ice. Master mixes should be prepared fresh immediately before each run.
4. Dispense 22.5 ul of master mix into appropriate wells using a new tip for each master mix. The 96-well plate should be in an Eppendorf PCR-Cooler. See example of sample worksheet below.
5. Add 2.5 ul RNA using a new tip for each well. Total volume in each well should be 25 µl. Prepare duplicate wells for each sample to be tested for mumps N. A single well can be used for RNaseP (see example of sample worksheet below).
6. The following standards and controls should be run on each plate.
   6.1. Make serial ten-fold dilutions of synthetic RNA (MuV N from CDC stock 10^6 copies) in 10 ul volumes in 0.5 ml microcentrifuge tubes on ice using nuclease-free water to obtain working concentrations of 10^5 to 10^2 copies. Dilutions must be made fresh from stock for each run. Add 2.5 µl/well of each standard control RNA.
   6.2. The control for RnaseP is the Quantitative PCR Human reference RNA. Make a 1:1000 dilution of reference RNA stock (1 µg/µl) in a 1.5 ml microcentrifuge tube on ice using nuclease-free water to obtain a working concentration of 1 ng/µl. Dilution should be made fresh from stock for each run. Add 1 µl/well of diluted RNA and qs to sample volume with nuclease-free water as indicated on sample plate worksheet.
   6.3. Include 2.5 ul nuclease-free water as a non-template control (NTC) for all master mix preparations (see example of sample worksheet below).
7. Seal the plate with an optical adhesive cover and centrifuge at 1500 rpm for 3 minutes at room temperature. Wrap the plate in foil and hold at 4^oC until ready to run.

8. Before running on the ABI Prism 7900HT Sequence Detection System, place a compression pad (Applied Biosytems #4312639) on top of the sealed reaction plate.

Thermocycling parameters:

RT Step:	48°C/30 minutes
AmpliTaq Gold Activation:	5°C/5 minutes
PCR (40 cycles)	95°C/15 seconds 60°C/1 minute

Master mix worksheet

Component	Lot # and Exp. Date	Volume/tube (µl)	[Final]	# Rxns.	Total volume (µl)
RNase-free H_2O		9.25	-		
10X TaqMan buffer		2.5	1X		
25 mM magnesium chloride		5.5	5.5 mM		
10 mM dATP		0.75	300 µM		
10 mM dCTP		0.75	300 µM		
10 mM dGTP		0.75	300 µM		
20mM dUTP		0.75	600 µM		
Forward primer		0.5	300 nM		
Reverse primer		0.5	300 nM		
Probe		0.5	(see below)		
AmpliTaq Gold (5 U/µl)		0.125	0.025 U/µl		
MultiScribe RT (50 U/µl)		0.125	0.25 U/µl		
RNase inhibitor		0.5	0.4 U/µl		
Vortex, centrifuge briefly and place on ice. Dispense 22.5 ul/tube					
Add sample RNA or controls		2.5	-		
Total		25			

Probes concentration: Mumps (MuN-622P)= 250 nM; RnaseP (BHQ1HURNASE-P)=100 nM

Interpretation/examination

All clinical samples should exhibit RNaseP reaction curves that cross the threshold line before 40 Ct indicating that nucleic acid extraction and PCR amplification did occur.

For interpretation of Ct values:	Viral gene	RNase P gene
Positive	<40	< 40
Positive	<40	Undet
Negative	Undet	< 40
Indeterminate	Undet	Undet

- Positive or equivocal specimens should be retested if contamination of NTC or the RNA extraction control is evident.
- Samples are tested in duplicate. If a mumps test gives discordant results between the two replicates, the sample should be retested. If one replicate is <38 and the other is Undet, retest. If one is <38 and the other is >38 and <40, no retest is needed.

Failure to detect RNase P in any of the clinical samples may indicate:

- Improper extraction of nucleic acid from clinical materials resulting in loss of RNA or carry-over of RT-PCR inhibitors from clinical specimens
- Absence of sufficient human cells in sample to enable detection
- Improper assay set up and execution
- Reagent or equipment malfunction
- Problems with specimen collection, storage or shipment

Example of Sample Plate Worksheet for ABI 7900HT (24 samples)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17	STD1 10^5	STD1 10^4	STD1 10^3
B	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18	STD1 10^5	STD1 10^4	STD1 10^3
C	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19	STD1 10^2	STD1 10^1	NTC
D	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20	STD1 10^2	STD1 10^1	NTC
E	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21	STD2	STD2	NTC
F	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22			NTC
G	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23			NTC
H	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24			NTC

Legend	
MuV master mix	STD1=mumps control RNA
RNase P master mix	STD2=Control for RNaseP

Comments, suggestions and questions concerning this procedure may be sent to:
CDC c/o Paul Rota prota@cdc.gov