Amplification and Detection of Nucleic Acid by the Real-Time RT-PCR Procedure

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Components of rRT-PCR

- Isolation of nucleic acid – RNA extraction
- Reverse transcription – RNA is transcribed to cDNA by an enzymatic reaction
- Amplification of the gene/target specific nucleic acid by PCR
- Detection of the amplified PCR product via a fluorogenic probe – interpretation of results
PCR is conducted with cDNA following reverse transcription
One-Step Real-time RT-PCR

- The RT and PCR procedures are conducted in a single tube
- The RT reaction is conducted first with target specific primers to produce cDNA
- The RT enzyme is inactivated and the double stranded cDNA is denatured with a 15 min. 95°C incubation following the RT procedure
- Multiple cycles (40-45) of PCR
Gene specific cDNA is synthesized from extracted RNA by the reverse transcriptase (RT) procedure.

Extracted RNA

RT enzyme produces cDNA with target specific primers

Double stranded cDNA
Polymerase Chain Reaction (PCR)

- Reagent products (master mix) synthesize multiple copies of new DNA ($10^6$)
- Amplification of the synthesized DNA is detected by the thermal cycler instrument and software
- Fluorescence, a result of amplification, is emitted by the target specific probe and recorded at each cycle in real-time
- The USDA AI procedure uses hydrolysis/Taqman probes with FAM reporter and Black Hole quencher dyes
Hydrolysis/Taqman probes
The two strands of DNA are separated during the denaturation step by heating reagent mixture to 95°C
Taq

5' 3'

Forward Primer

Reporter

Probe

Quencher

5'

Strand 1 of cDNA nucleic acid

3'

Strand 2 of cDNA nucleic acid

Reverse Primer
Forward Primer → Taq → Reporter → Probe → Quencher → Reverse Primer

Strand 1 of cDNA nucleic acid

Strand 2 of cDNA nucleic acid
Taq

Forward Primer

Reporter

Probe

Quencher

Strand 1 of cDNA nucleic acid

5'

Strand 2 of cDNA nucleic acid

Reverse Primer

3'
Results interpretation

Positive

Negative
Wet Reagents

- “Wet reagents” is the term to describe combining liquid master mix reagents separately for primers, probe, enzyme, etc.
- The current use of “wet” reagents for RRT-PCR for AI and NDV requires individual labs to purchase and do their own quality control (potential variability between lots of reagents)
- “Wet” reagents also have a finite lifespan, particularly the fluorescent probe
Development of Dried Down Reagents

- Lyophilize the primers, probes, buffers, and Mg+ into a bead format
  - Provide increased stability of reagents
  - Simplify testing procedure (reduced # of steps)
  - Increase quality control with standardized testing
- Potential to include reagents for multiplex testing, including internal controls
- Enzymes are not included at this time because of performance issues
Preparing Reagent Master Mix

• Reagent preparation must be conducted in a dedicated containment device
• Dedicated calibrated pipettors with matching filtered tips
• Equipment (gloves, etc.) from extraction/amplification area - NO
• Enzymes should remain cold
• Fluorescent probes should be protected from direct light
• Ready? - Have all needed reagents?
Preparing Reagent Master Mix

Arrange reagents in order of reagent list to eliminate errors

Vortex and pulse spin most reagents (esp. MgCl₂) prior to distribution – NOT ENZYMES

When working with small volumes rinse pipette tip one time to assure entire reagent has been added
Add water 1st

Add 5X Buffer 2\textsuperscript{nd}

Add enzymes and probes last

Insert pipette tip just below surface when pipetting enzyme

One quick vortex to thoroughly mix reagents upon completion of master mix
Dedicated Space and Equipment

RNA extraction, preparation of master mix and RNA transfer should be conducted dedicated primary containment devices and with pipettors dedicated to each procedure.

- **RNA extraction**
  - BSC and extraction pipettors

- **Preparation of master mix**
  - BSC or PCR workstation
  - Clean master mix pipettors

- **RNA transfer**
  - BSC or PCR workstation
  - RNA transfer pipettors
Preparation of clean reagents, extraction and RNA transfer should not be conducted in the same laboratory space as electrophoresis of amplified RNA.

Pipettors
- Ideally 3 sets
  - 1. RNA extraction, RNA transfer, and preparation of clean reagents
- 2 sets – increases possibility of false +
  - 1. RNA extraction and transfer
  - 2. clean reagents
- ONLY USE AEROSOL RESISTANT TIPS
- Use powder-free gloves
- Change gloves often
- Don’t store reagents or RNA in -20C frost-free freezers
Sample Storage

- Swab materials
  - 4°C for 3-4 days, more than 4 days (-70°C)

- Tissue samples
  - -20°C short term storage, Long term -70°C

- Sample RNA
  - 4°C less than 24 hrs., more than 24 hrs. -70°C

- Control RNA
  - 4°C up to 2 weeks, Long term -70°C (aliquot)

- Probe
  - 4°C up to 2 weeks, Long term -20 or -70°C (aliquot)

- Avoid multiple freeze thaw cycles for everything
Real-time RT-PCR Materials

- A set of RT-PCR reagents
  - Enzyme and RNase inhibitor in a bench top cooler
  - 5X buffer
  - dNTPS
  - Positive control (AIV M or H5 RNA)
  - Nuclease free water
  - Forward primer (AIV M+25 or H5+1456)
  - Reverse primer (AIV M-124 or H5-1685)
  - Probe (AIV M+64 or H5+1637)
RNA Transfer

Mix reagents by vortexing for 3-5 seconds and centrifuge briefly.

Add 17µl of the master mix to each of your Smart Cycler tubes (add the mix to the bottom of the cup at the top of the reaction tube).

Add 8µl of template to the Smart Cycler tubes, close and label each tube as follows:

1. Positive control: *in vitro* transcribed RNA from the target gene
2. Negative control: nuclease free water.
3. test sample 1
4. test sample 2
Setting up rRT-PCR

- Centrifuge the reaction tubes briefly in the Smart Cycler centrifuge.

- Place the reaction tubes into the Smart Cycler and run with the "AIV Matrix" or "H5" program.
  - The program has already been programmed into the smart cycler.
Real-time RT-PCR Materials

- For each lab group
  - Smart Cycler tube cooling block
  - Smart Cycler tubes
  - RNA samples
  - Dedicated pipets and aerosol resistant pipet tips
  - 1.5ml tubes
Prepare the reaction master mix in a 1.5ml tube

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<tr>
<th>Component</th>
<th>Volume in µl</th>
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<td>H₂O</td>
<td>83.4</td>
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<tr>
<td>5X</td>
<td>60</td>
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<tr>
<td>25mM MgCl₂</td>
<td>15.0</td>
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<tr>
<td>Enzyme Mix</td>
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<tr>
<td>Forward Primer</td>
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<tr>
<td>dNTP’s</td>
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<tr>
<td>Probe</td>
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<tr>
<td>Rnase Inhibitor</td>
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4 people/group
Prepare the reaction master mix in a 1.5ml tube

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Demonstration of RNeasy RNA Extraction and Master Mix Prep

- Group 1 – RNeasy Extraction
  - 4 people in each subgroup
  - 2/vaccum pump

- Group 2 – magnetic bead extraction and wet mix set-up
  - 4 people in each subgroup
  - Matrix or H5 assay
  - Frig wet mix until RNA is extracted
Thank You For Your Attention