Attachment 12

Real-Time Reverse Transcriptase-Polymerase Chain Reaction for the Detection of Type A Influenza and the Avian H5 and H7 Ha Subtypes In Tracheal and Cloacal Samples

Cepheid Smart Cycler Protocol

Prepared by:
Erica Spackman, Ph.D.
Southeast Poultry Research Lab

U.S. Dept. of Agriculture, Agricultural Research Service
934 College Station Rd.
Athens, GA 30605
706-546-3617
espackman@seprl.usda.gov
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I. MATERIALS

Mention of trade names or commercial products in this procedure is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

General recommendations regarding reagents

This assay was optimized using the using the Qiagen RNeasy kit and Qiagen one-step RT-PCR kit, therefore these reagents are recommended for uniformity, if available. Additionally, it is highly recommended that the PCR primers and probe be highly purified (i.e. HPLC purified).

General

Reagent grade H2O (nuclease free)
Pipetors and tips for volumes between 1µl and 1ml
1.5 ml microcentrifuge tubes Microcentrifuge TE buffer pH 8.3 (Promega #V6231 or V6232) (optional)

RNA Extraction

Qiagen RNeasy Mini Kit (Qiagen #74104 or #74106)
100% Ethanol
70% Ethanol (in nuclease free water)
QiaVac 24 vacuum manifold (Optional)
2-Mercaptoethanol (BME) (Sigma #M-6250)

Trizol LS reagent (Invitrogen #10296028, 200ml)
Chloroform (Sigma #C-2432)
Isopropanol (Sigma #I-9516)
Glycogen 5mg/ml (Ambion #9510)

Real-time RT-PCR

Qiagen one step RT-PCR Kit (Qiagen #210210 or #210212)
Hydrolysis probes (IDT, Idaho Tech/Biochem or Qiagen-Operon) (Table 1)
Primers (IDT, Idaho Tech/Biochem or Qiagen-Operon) (Table 1)
25 mM $MgCl_2$ (Promega #A3511 or #A3513)
RNase Inhibitor (Promega #N2511 or # N2515)
Positive control RNA
Nuclease free H_2O
25µl Smart Cycler tubes (Cepheid #900-0022 or 900-0003)

Table 1. Influenza real-time RT-PCR probe and primer sequences. Protocols for H7 subtype Eurasian, H7 subtype South American strains, the H6 subtype and the H9 subtype strains are available as supplementary protocols.

Specificity		Sequence
Type A influenza-Matrix gene	M+25 5' Primer	5'-AGA TGA GTC TTC TAA CCG AGG TCG-3'
	M+64 Probe	5'-FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1-3'
	M-124 3'Primer	5'-TGC AAA AAC ATC TTC AAG TCT CTG-3'
H7Subtype-North American strains	H7+ 1244 5' Primer	5'-ATT GGA CAC GAG ACG CAA TG-3'
	H7+1281 Probe	5'-FAM-TAA TGC TGA GCT GTT GGT GGC-BHQ1-3'
	H7-1342 3'Primer	5'-TTC TGA GTC CGC AAG ATC TAT TG-3'
H5 subtype-Any strain[a]	H5+1637 Probe	5'-FAM-TCA ACA GTG GCG AGT TCC CTA GCA-BHQ1-3'
	H5-1685 3'Primer	5'-AGA CCA GCT AYC ATG ATT gC-3'
H5 subtype-North American strains	H5+1456 NA 5' Primer	5'-ACG TAT GAC TAT CCA CAA TAC TCA-3'
H5 subtype-Eurasian strains	H5+1456 EA 5' Primer	5'-ACG TAT GAC TAC CCG CAG TAT TCA-3'

a. H5 strain specificity is determined by the 5' primer. Use only one primer in the reaction.

Note on subtype determination: Due to the high level of sequence variation within each HA subtype, a negative RRT-PCR result for a specific subtype does not exclude the possibility that that subtype is present.

Primer and probe handling and dilution

Lyophilized primers and probes must be centrifuged briefly, to ensure that the DNA pellet is at the bottom of the tube, before they are opened and reconstituted. TE buffer should be used for the initial reconstitution of lyophilized primers and probes (Idaho Tech sends TE with probes and primers and it is commercially available). Concentrated stock solutions should be stored at -20°C. Primer stock solutions should be 200µM (200pmol/µl), probes should be 120µM (120pmol/µl). Quantitation information will be supplied for each oligo (primers and probes are DNA oligos) by the manufacturer.

An example of calculation for oligo reconstitution:

You have 17786 pmol of oligo (will be on oligo information sheet from manufacturer).

Need 200pmol/µl for stock concedntration.

Divide pmol of oligo by the pmol/µl needed or: $\frac{17786 \text{ pmol}}{200 \text{ pmol/µl}} = 88.9\text{µl}$

For 200pmol/µl resuspend the pellet in 89µl of TE or nuclease free H2O. The calculation for the probe is the same, except divide the number of probe pmol by 120pmol/µl. Mix gently by tapping the tube and allow the oligo to resuspend for about 10 minutes before use.

Working stocks of primers should be 20pmol/ul (20µM) and working stocks of probes should be 6µM. Dilute the primers 1:10 and dilute the probe 1:20 in nuclease free H_2O (do not use TE buffer) for the working stocks.
Working stocks should be stored at 4°C. The probes are stable at this concentration at 4° C for approximately 1 month. It may be useful to make up several aliquots (5-6) of working stocks of primers and probes which are a volume that can be used in about one month. Store the unused aliquots at -20°C.

Note: the probes are light sensitive; store them in amber tubes if available, and minimize their exposure to light.

Additional information on fluorescent probe handling and storage can found at: www.idahotech.com, www.operon.com and www.idtdna.com.

Suppliers

Biosearch Technologies, Inc.
81 Digital Drive
Novato, CA 94949-5750
1.800.436.6631
WWW.Biosearchtech.com

Promega
2800 Woods Hollow Rd
Madison, WI 53711-5399
1-800-356-9526
www.promega.com

Idaho Technology/Biochem
390 Wakara Way
Salt Lake City, UT 84108
1-800-735-6544
www.idahotech.com

IDT
1710 Commercial Park
Coralville, IA 52241
1-800-328-2661
www.idtdna.com

Qiagen Inc./Operon
28159 Avenue Stanford
Valencia, CA 91355
1-800-426-8157 www.qiagen.com
www.operon.com

II. METHODS

NOTE ON SAMPLE TYPES AND RNA EXTRACTION METHODS
The types of samples collected and the processing of those samples varies by species. The optimal sample types and processing methods for many species are given below.

Table 2. Sample types and optimal processing methods.

Species/ Type	Recommended Specimen	Processing Method	Notes
Gallinaceous Poultry (chickens, turkeys, quail)	Tracheal swab	RNeasy RNA extraction, then RRT-PCR	Virus primarily replicates in the respiratory tract (LPAI)
Waterfowl/ducks	Cloacal Swab	Trizol Reagent RNA extraction, then RRT-PCR	Virus primarily replicates in the intestinal tract. RNA extraction method must be modified for cloacal samples
Any species	Tissue samples	RNA extraction with Trizol Reagent, then RRT-PCR	For HPAI viruses high levels of virus may be in tissues.
Environmental samples	(Swab)	Virus isolation, RRT-PCR not recommended	RRT-PCR can detect inactivated virus

REVISED RNA EXTRACTION PROTOCOL FOR TRACHEAL SWABS (7/03)

RNA Extraction with Qiagen RNeasy Kit- Centrifuge Method
Notes:

- Adaptation of kit for fluid samples from manufacturer.
- All kit supplied buffers and reagents should be prepared in accordance with the kit instructions.
- Use only RNA grade reagents and supplies

1. Vortex the sample (cloacal or tracheal swabs in BHI or other media) for 3-5 seconds and withdraw 500µl and place in a 1.5 ml microcentrifuge tube.

2. Add 500 µl of RLT buffer. Close the tube and vortex the sample for 5 seconds.

3. Add 500 µl of RNA grade 70% ethanol to the tube and mix. Centrifuge the sample for 5 minutes at ~5KXg to pellet any debris.

4. Add 750µl of the supernatant from step 3 to the RNeasy column and centrifuge for 15 seconds at ~12 KXg, empty the flow through from the collection tube and repeat (all of the sample/RLT/70% ethanol mix should be applied to the column).

5. Add 700µl RW1 buffer to the RNeasy column and centrifuge for 15 seconds at ~12 KXg and place the column in a clean collection tube (the collection tube with RW1 flow through may be discarded and replaced with a fresh collection tube).

6. Add 500µl RPE buffer to the RNeasy column and centrifuge for 15 seconds at ~12 KXg, empty the flow through from the collection tube.

7. Repeat step 6 for a total of 2 washes with RPE buffer.

8. Centrifuge the empty RNeasy column an extra 2 minutes at ~14 KXg and discard the collection tube.

9. Place the RNeasy column in an elution tube (or 1.5ml microfuge tube) and add 50 µl nuclease free H_2O to the column. Incubate at room temperature for 1 minute. Elute RNA by centrifuging for 1 minute at ~14KXg. Discard RNeasy column.

RNA Extraction with Qiagen RNeasy Kit- QiaVac 24 Vacuum Manifold Method

Notes:

- Adaptation of the RNeasy kit for fluid samples from manufacturer.
- All kit supplied buffers and reagents should be prepared in accordance with the kit instructions.
- RNeasy column lids should be open whenever vacuum is being applied.
- Use only RNA grade reagents and supplies

1. Vortex the sample (cloacal or tracheal swabs in BHI or other media) for 3-5 seconds and withdraw 500 µl and place in a 1.5 ml microcentrifuge tube.

2. Add 500 µl of RLT buffer. Close the tube and vortex the sample for 5 seconds.

3. Add 500 µl of RNA grade 70% ethanol to the tube and mix. Centrifuge the sample for 5 minutes at ~5KXg to pellet any debris.

4. Place the appropriate number of RNeasy columns in the luer locks of the vacuum manifold, cover any empty positions with the luer caps supplied with the vacuum manifold.

5. Apply vacuum and add the entire sample/RLT/ethanol mixture to an RNeasy column for each sample.

6. Wash by applying 700µl RW1 buffer to each column.

7. Wash again by applying 500µl RPE buffer to the column and repeat for a total of 2 washes with buffer RPE.

8. Shut off the vacuum and place each RNeasy column in a 2ml collection tube. Centrifuge the column for 2 minutes at ~14 KXg and discard the collection tube.

9. Place each column in an elution tube (or 1.5ml microfuge tube) and add 50 µl nuclease free H_2O and incubate at room temperature 1 minute. Elute RNA by centrifuging for 1 minute at ~14KXg.

10. Use 8µl per PCR reaction. Store at -70°C for long term storage.

RNA EXTRACTION FROM CLOACAL SWABS OR TISSUE WITH TRIZOL REAGENT

1. Sample Prparation:
   a. Cloacal Swabs: Vortex vigorously for 7-10 seconds. Centrifuge for 5 min. at

12,000Xg.Extract RNA from the supernatant.

b. Tissues: Make a 10% homogenate of tissue in PBS. Centrifuge for 10 min. at 12,000Xg. Extract RNA from the supernatant.

2. Add 250µl of the supernatant from the sample prepared as described in step 1, to 750µl of Trizol LS reagent. Vortex. Pulse spin to remove liquid from the tube lid.

3. Add 200µl 100% chloroform to the sample/Trizol homogenate. Vortex for 15 sec. Incubate at room temperature for 7 min.

4. Centrifuge at 12,000 x g for 15 min at room temperature.

5. Transfer 400-450µl of the upper aqueous layer to a separate microcentrifuge tube marked with sample number. **Caution: The transfer of organic phase material with the aqueous layer will inhibit the PCR reaction.** Add 500µl of 100% isopropanol. Add carrier to the isopropanol to aid precipitation i.e. glycogen: 1µl of 5mg/ml stock (may be added prior to addition of the aqueous phase from the trizol). Invert tube several times to mix. Incubate at room temperature for 10 min.

6. Centrifuge at 10,000 x g for 10 min at 4 C.

7. Decant liquid. Care should be taken to assure that the RNA pellet is not disturbed. Add 1.0 ml of 70% or 80% ethanol. Mix gently.

8. Centrifuge at 10,000 X g for 5 min at 4 C.

9. Decant ethanol. Invert tube on a clean tissue wipe and allow to air dry for 10 min. or until all visible signs of moisture are gone. It is important not to let the RNA pellet over-dry, as this will decrease its solubility.

10. Hydrate pellet in 100µl of RNase free water and allow to sit at 4 C for 1 hr to overnight.

RNA Handling and Storage

The RNA sample may be stored at 4°C for < 1 week, storage for longer than one week should be at –70°C. Always wear gloves when handling RNA and use only RNase or nuclease free materials and reagents with RNA. Additional RNA handling and storage information can be found in: Sambrook, J. and Russell, D. *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.

Procedure for Real-time RT-PCR for type A Influenza (MA gene), the H5, H6, H7 and H9 HA subtypes

This procedure was designed for the Cephied Smart-Cycler (Cepheid, Sunnyvale, CA). A protocol for the Roche Light Cycler and Idaho Tech R.A.P.I.D. is available. The original reference and validation study for this assay is: Spackman, et. al.; Journal of Clinical Microbiology 40:3256-3260, 2002.

Information on setting-up and programming the Smart Cycler can be found in the Smart Cycler user's manual. The conditions for the influenza primers and probes on the Smart Cycler are shown in tables 2 and 3. The RT step is the same for all primer and probe sets, these conditions are specific for the Qiagen OneStep RT-PCR kit. Cycle times for the PCR phase may vary among different real-time PCR instruments.

Table 3. RT step thermocycling for Qiagen one-step RT-PCR Kit.

RT Step	1 cycle	30 min.	50° C
		15 min.	95° C

Table 4. Thermocycling conditions for gene specific probe and primer sets.

Probe/Primer set		Step	Time	Temp
Type A influenza	45 cycles	Denaturation	1 sec.	94° C
		Annealing[a]	20 sec.	60° C
H7 Subtype North American	40 cycles	Denaturation	1 sec.	94° C
		Annealing[a]	20 sec.	58° C
H5 subtype North American or Eurasian[b]	40 cycles	Denaturation	10 sec.	94° C
		Annealing[a]	20 sec.	57° C
		Extension	10 sec.	72° C

a. Fluorescence is acquired at the annealing step.
b. Use only one H5 subtype 5' primer for the H5 test.

The real-time RT-PCR reactions for type A influenza (M gene) and the H5 and H7 HA subtypes should be setup with the following components and volumes using the appropriate primer and probe set and cycling conditions. Set-up the reactions with the tubes in the cooling block and use aerosol resistant pipet-tips.

1. Prepare the reaction mix (everything but the template) by pipetting: H2O, kit supplied 5X reaction buffer, kit supplied dNTP's and 25mM $MgCl_2$ into a nuclease free microcentrifuge tube using the volumes per reaction for each reagent given in table 4. Next add the RNase inhibitor and enzyme. Add the probe last. Mix by vortexing for 3-5 seconds and centrifuge briefly. Once the probe has been added minimize exposure of the reaction mix to light.

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

Table 5. Real-time RT-PCR reaction mix volumes and conditions for type A influenza (M gene), H5 and H7 HA subtypes.

	Volume Per Reaction	Final Concentration
H_2O	6.95µl	
5X	5	1X
25mM $MgCl_2$	1.25	3.75 mM
Enzyme Mix	1	
Forward Primer	0.5	10 pmol
Reverse Primer	0.5	10 pmol
dNTP's	0.8	320 µM ea. dNTP
Probe	0.5	0.12 µM
Rnase Inhibitor	0.5	13 units
MM per rxn	17	
Template	8	
Total	25µl	

3. Add the template to the smart cycler tubes (8µl per reaction). Note: The template for the positive controls is *in vitro* transcribed RNA from the appropriate gene and the template for the negative controls is H2O.

4. Centrifuge the reaction tubes briefly in the Smart Cycler centrifuge and run the real-time RT-PCR with the conditions described in tables 2 and 3 depending on the probe and primer set used. Note the RT step is the same for all probe and primer sets (the RT step is specific for the Qiagen one-step RT-PCR kit).

III. ANALYSIS OF RESULTS

Positive results on the Smart Cycler may be determined by the Smart Cycler software (shown on the results table in the Smart Cycler software) and are generally reliable; however results should be manually confirmed by examination of the fluorogram.

On the Smart Cycler the default minimum increase in fluorescence for a sample to be classified as positive by the software is 30 units. Because this is an arbitrary threshold, any samples which have an increase in fluorescence between 20 and 40 should be considered suspect and should be re-tested with the type A influenza (M gne) assay and/or subtype specific assays. In general, any questionable samples should be retested. If results of the second test are unsatisfactory additional sampling from the flock or premises should be considered if possible.

Recommendations for evaluating fluorograms

Evaluation of the fluorogram with the following conditions may be helpful in determining results manually:

- ③ All reactions with default settings.

- ③ Remove all reactions with greater than 100 units increase in fluoresce from the graph (this changes the scale, making it easier to identify weak negatives). (Figures 1a and 1b).

- ③ If there are samples which have a "V" shaped fluorescence trace incrementally lower the "background maximum cycles" (analysis settings table) to approximately 2 cycles below the cycle number where the base of the "V" is (Figures 2a and 2b).

Figure 1a. Example of a fluorogram from samples run on the Smart Cycler. All samples shown. Background subtraction is on. All analysis criteria are set to the default values. Note that scale is from 0 to 1000 fluorescence units (Y axis), making it difficult to evaluate weak positive samples.

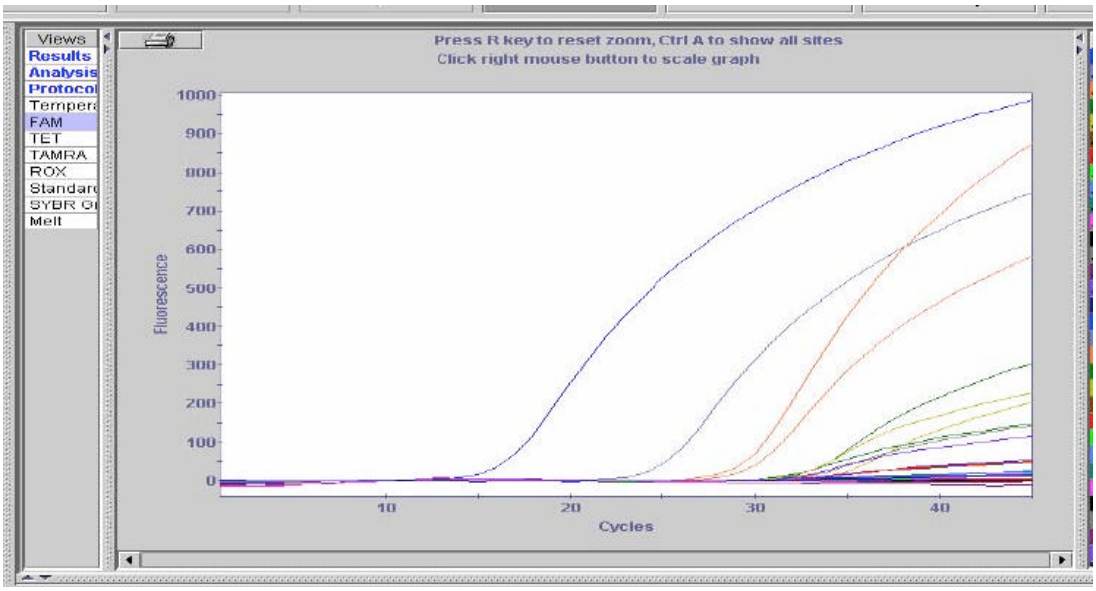


Figure 1b. Same fluorogram as figure 1a, however all samples which increased greater than 100units in fluorescence were removed from the graph. Note that the scale is from 0 to 120 fluorescence units (Y axis) making it easier to recognize weak positives.

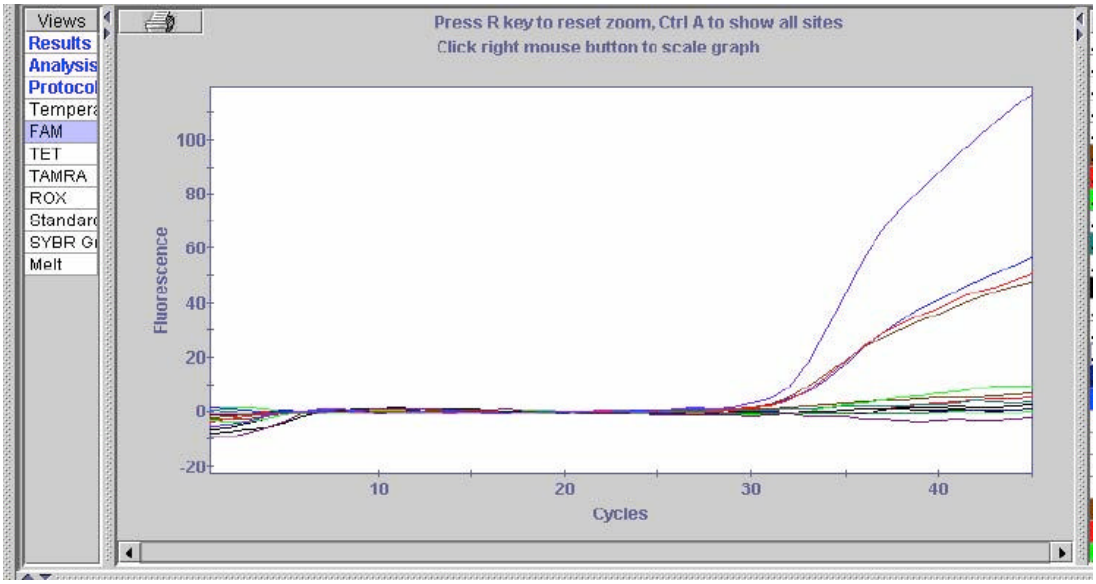


Figure 2a. Example of a "V" shaped fluorescence trace. The background maximum cycle is set to the default of 40 (red circle). All other analysis criteria are set to the default values. The negative control is shown for reference (horizontal line at zero, light blue).

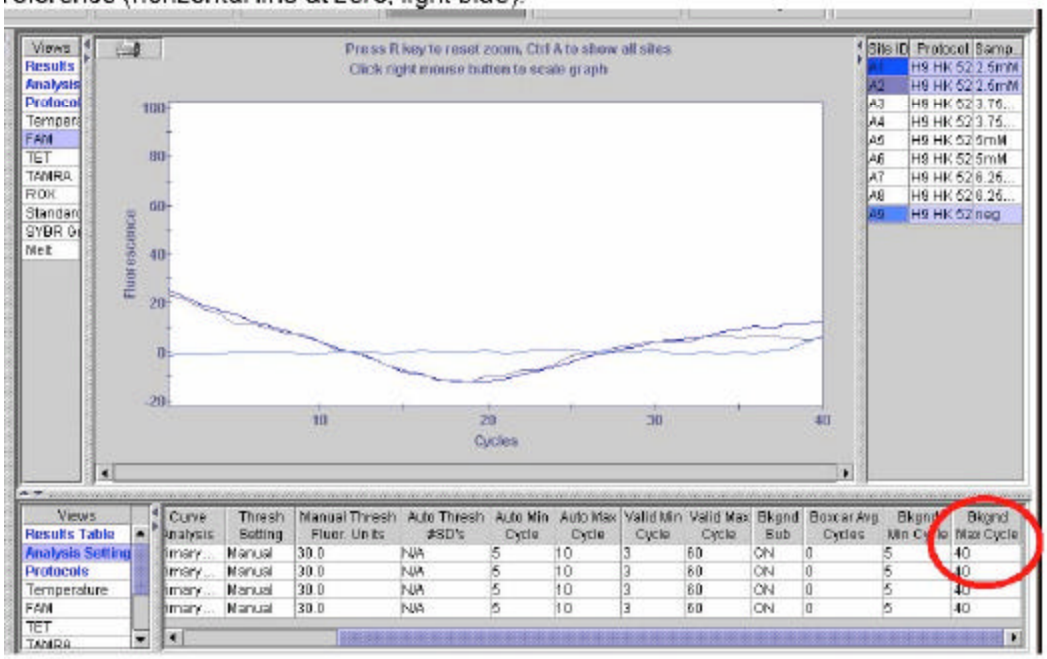
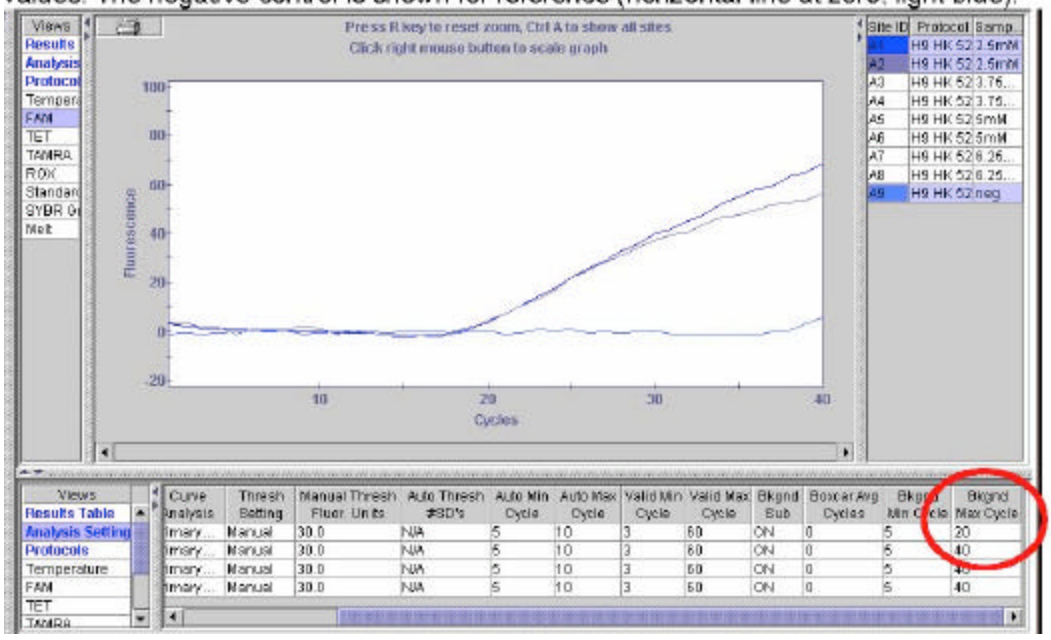


Figure 2b. Same fluorogram as figure 2a, however the background maximum cycles have been reduced to 20 (red circle) to align the background fluorescence at 0 units. All other analysis criteria are set to the default values. The negative control is shown for reference (horizontal line at zero, light blue).



IV. APPENDIX

Troubleshooting

- Positive controls are negative:
  - Control template has degraded.
  - Probe may be old and the fluorescence may be dead.
  - The enzymes may be inactivated.
  - Incorrect thermal-cycling program or fluorescence acquisition (wrong setting or wrong channel being viewed).

- Negative controls are positive:
  - There may be cross contamination among the samples.
  - There may be non-specific probe degradation, use fresh probe and primers.

- Background level too high or too low (should be approximately 100-200 units):
  - The probe concentration may be wrong.
  - The probe may be degraded or too old if the background level is too low.

- Warning message in sample status on results screen.
  - Probe concentration too high (may cause 'railing'; a sharp decrease in fluorescence after a steady increase).

Cross-contamination prevention

Due to the high sensitivity of RT-PCR based assays cross-contamination is an important issue. The following guidelines will help to prevent contamination of PCR samples in the lab:

- ③ Use aerosol resistant pipet tips
- ③ Centrifuge all reagents prior to use, especially freeze-dried materials
- ③ Preparation of samples in a biosafety cabinet
- ③ Use of separate areas (separate biosafety cabinets for RNA extraction and RT-PCR reaction preparation).
- ③ Minimizing sample handling
- ③ Change gloves often

Real-time PCR Basics

The general principle of real-time PCR is the same as standard PCR, however the reaction product can be monitored in real-time with a fluorogenic probe. There are several types of probes for real-time PCR: hydrolysis probes, hybridization probes and molecular beacons. This assay utilizes hydrolysis probes.

In the hydrolysis probe system, a DNA probe which binds the PCR product and which has a fluorogenic reporter dye on one end and a quencher dye on the other end, is added to the PCR reaction (figure 4). As the target PCR product increases the probe binds the amplicons and reporter dye is cleaved from the 5' end of the probe by *taq* polymerase (due to 5' exonuclease activity). As the reporter is cleaved from more and more probe molecules the fluorescence signal from the increases. The fluorescence signal is monitored every cycle, revealing increases in the PCR product as it occurs.

Additional information about Real-time PCR, primers and probes can be found at www.operon.com and www.idtdna.com.

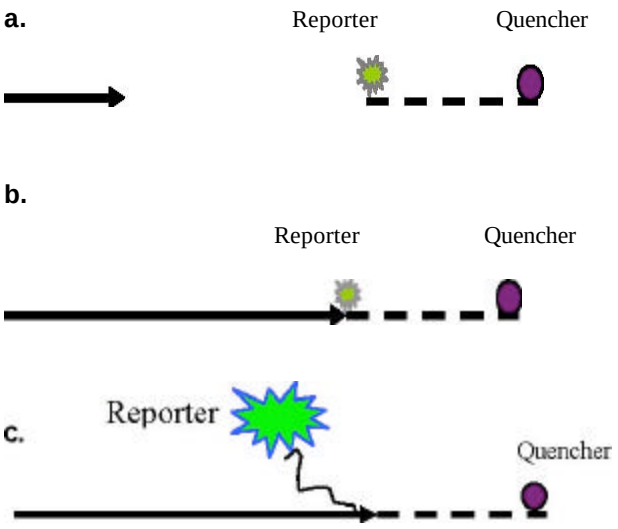


Figure 4. Hydrolysis probe mechanism. **a.** The probe (---) binds the PCR product (-) during amplification. **b.** The polymerase (→) runs into the probe during synthesis of the PCR product. **c.** Taq polymerase cleaves the reporter dye from the probe, increasing the detectable fluorescence of the reporter dye.