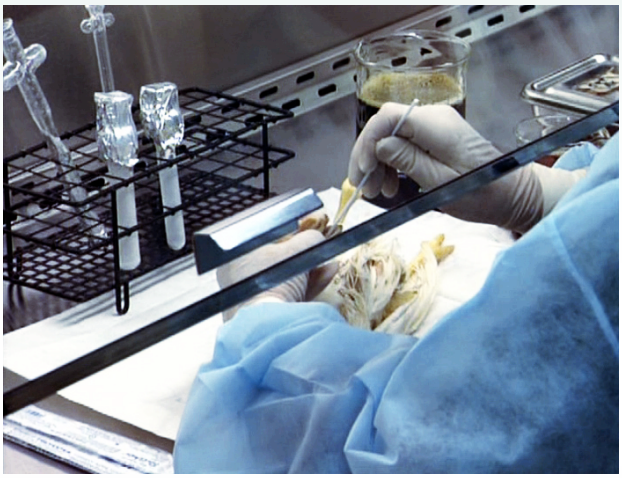
>>USDA High Pathogenic Avian Influenza Diagnostic Training Course

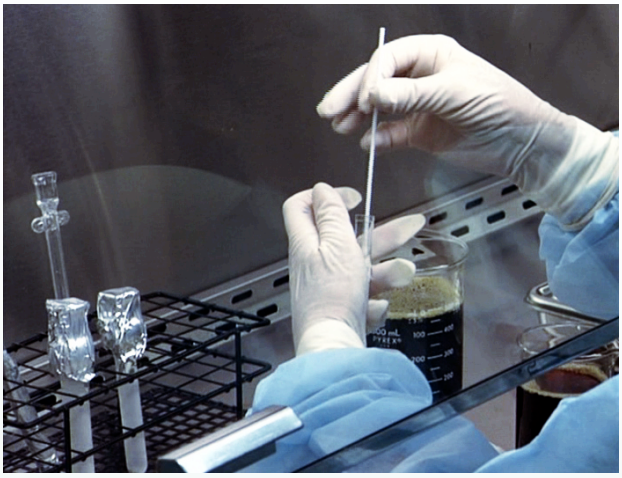
Laboratory Demonstrations Video Text

USDA OIE IICAB IOWA STATE UNIVERSITY

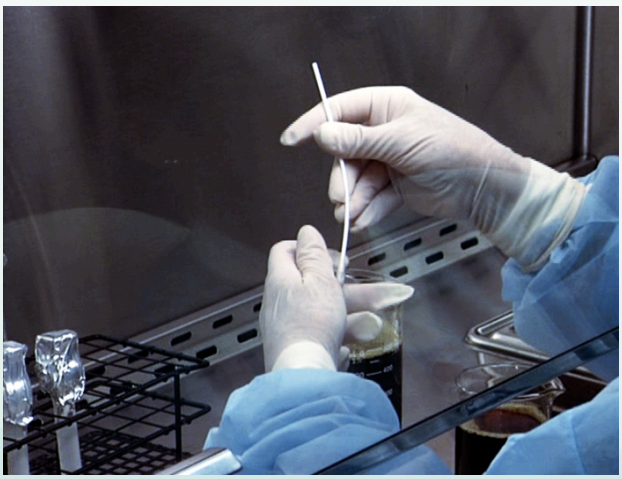
Collection of Tracheal Swabs for Virus Detection



Using a sterile polyester swab with a plastic shaft, insert the swab into the lumen of the trachea.



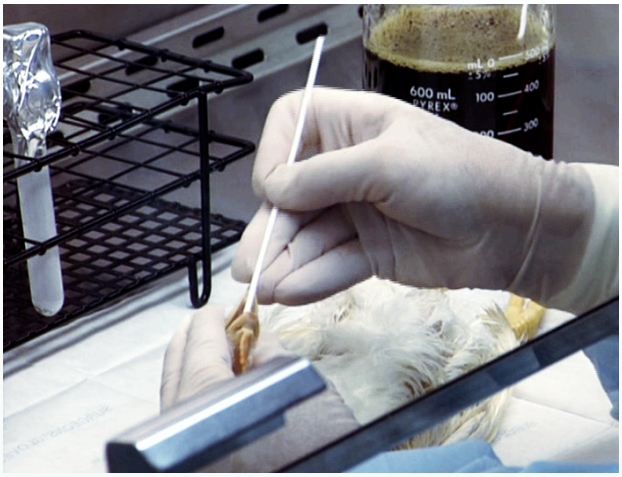
Disperse the contents of the swab into the media by swirling the swab vigorously.



Squeeze the contents of the swab out into the media. Discard the swab into a disinfecting material. You can pool up to 5 swabs from a single flock or species. You should not pool swabs from different species, such as duck with chickens, nor pool swabs from multiple premises or flocks. Also, do not pool tracheal or oropharyngeal swabs with cloacal swabs if intending to test the swabs by PCR assays.

Collection of Oropharyngeal Swabs for Virus Detection

Using a sterile polyester swab, swab around the opening of the trachea.

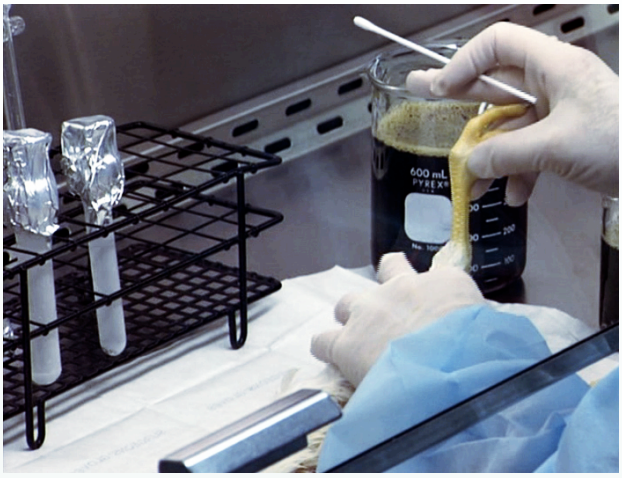


Then swab the Choanal slit found on the dorsal surface of the oral cavity.

Disperse the contents of the swab into the media by swirling the swab vigorously.

Squeeze the contents of the swab out into the media. Discard the swab into a disinfecting material.

Collection of Cloacal Swabs for Virus Detection

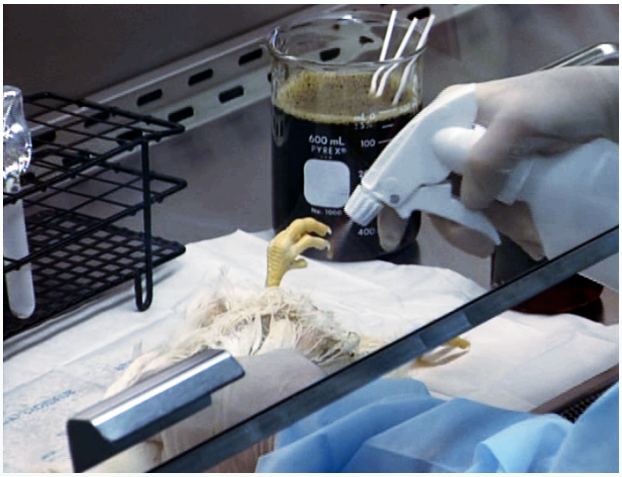


Insert a sterile polyester swab with a plastic shaft into the cloacal opening. Vigorously swab the mucosal lining of the cloaca and lower portion of the large intestine.

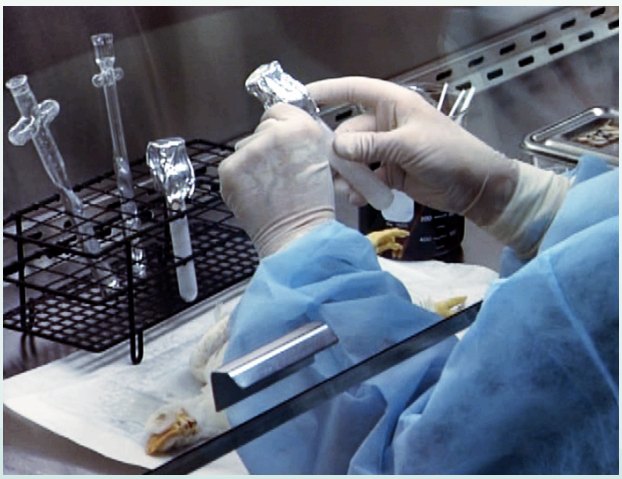
Disperse the contents of the swab into the media by swirling the swab vigorously.

Squeeze the contents of the swab out into the media. Discard swab into disinfecting solution. Do not pool cloacal swab specimens with tracheal or oropharyngeal specimens when the swabs are to be tested by PCR assays.

Collection of Tissue Samples for Virus Detection



Begin by wetting the feathers on the ventral surface with a 70% ethanol solution.



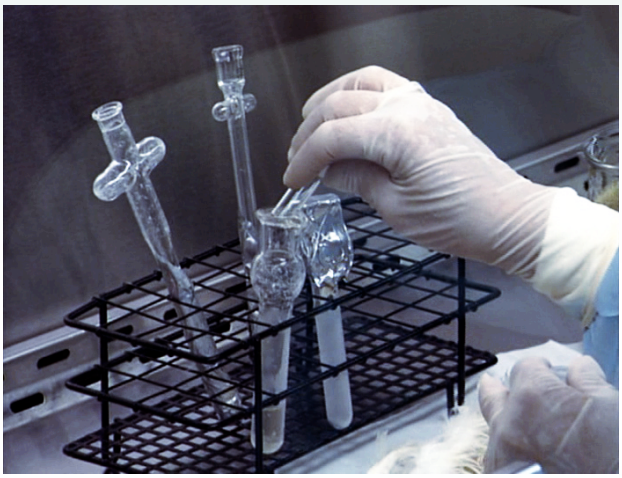
Label sterile plastic bags for each of the organs or organ pools to be collected and unwrap a sterile Tenbroeck tissue grinder for each specimen to be processed. When processing organs, you can pool lung and spleen together, liver and kidney together, and heart, spleen, and bursa together. Do not pool brain with other organs and do not pool the digestive tract with other organs. Also, do not pool tissues from multiple birds as the antibodies from a seropositive bird can neutralize virus from an acutely affected bird.

Pool

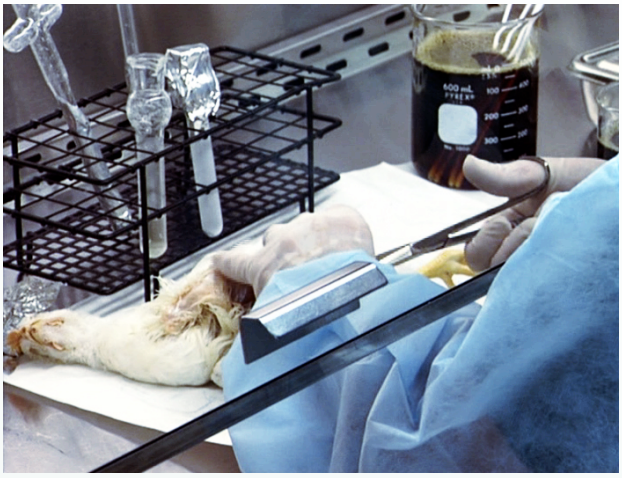
- lung and spleen
- liver and kidney
- heart and spleen and bursa

Do NOT Pool

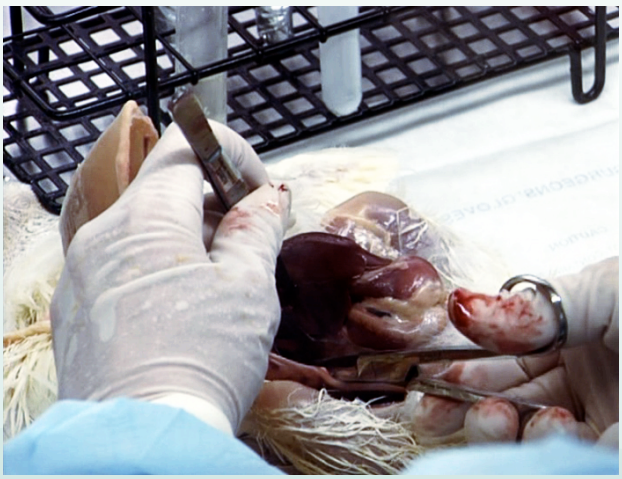
- brain
- digestive tracts
- tissues from multiple birds



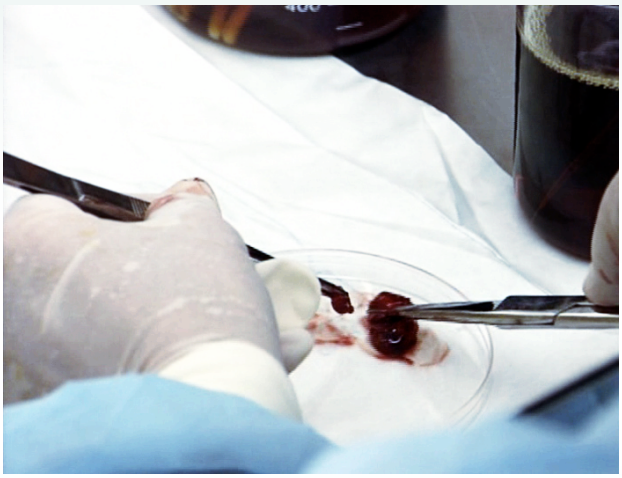
Pour the media-antibiotic solution into the Tenbroeck grinder.



To collect the organs, use a sterile scissors to cut through the skin near the posterior end of the keel bone and reflect the skin and feathers forward toward the head of the bird, exposing the breast muscle. Cut through the abdominal muscle between the posterior end of the keel bone and the vent. Gently lift the keel just enough to observe the posterior abdominal air sacs on either side of the abdomen. Remove the breast to expose the internal organs.

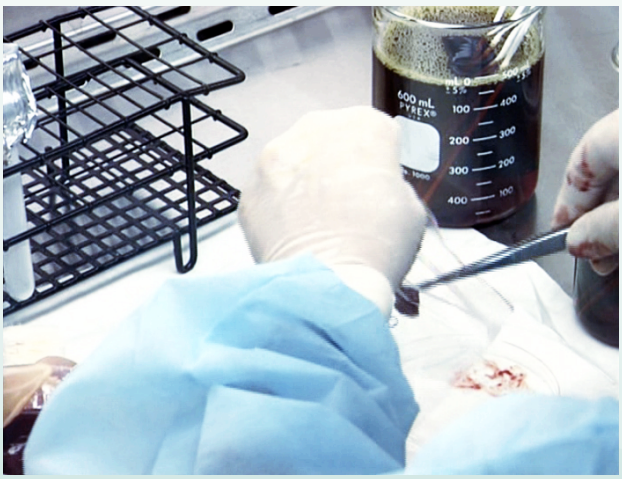


Collect the organs using a sterile scissors and forceps and place on a sterile surface such as a 100 millimeter Petri dish.

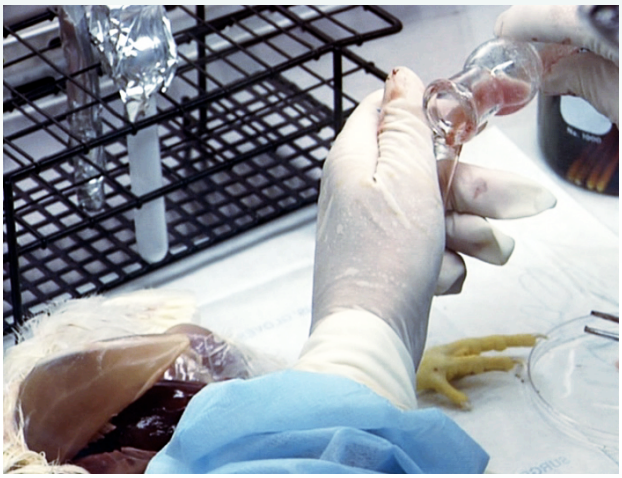


Cut a 0.3-0.5 gram section (sufficient to prepare a 10-15% tissue suspension) of the organ to be processed into the Tenbroeck grinder.

Thoroughly grind the tissue specimens into a homogenous mixture.

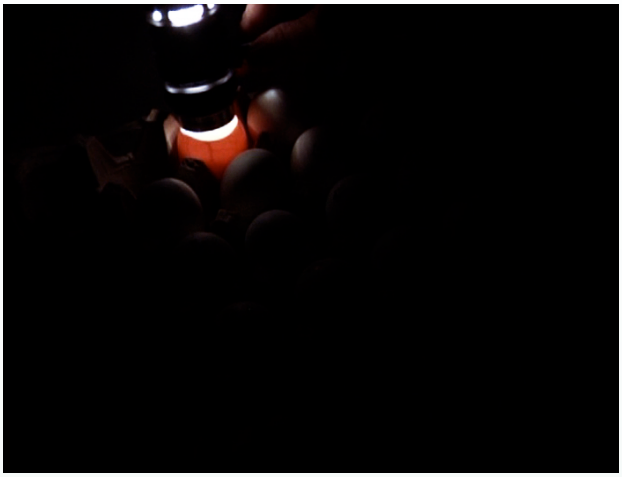


Place the remaining sample into the labeled plastic bag for repository purposes.



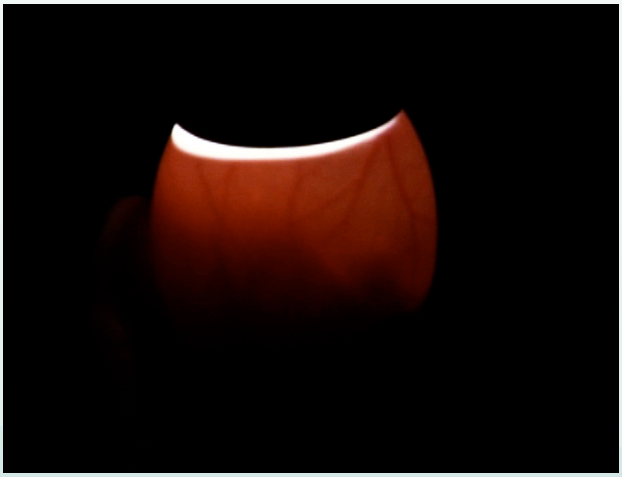
Pour the tissue suspension into a centriguge tube. Prior to egg inoculation, the tissue suspension should be clarified by centrifugation at 1,500 times *g* for 30 minutes and incubated at room temperature for one hour. You can repeat this process for each specimen.

Candling Eggs Used for Virus Isolation

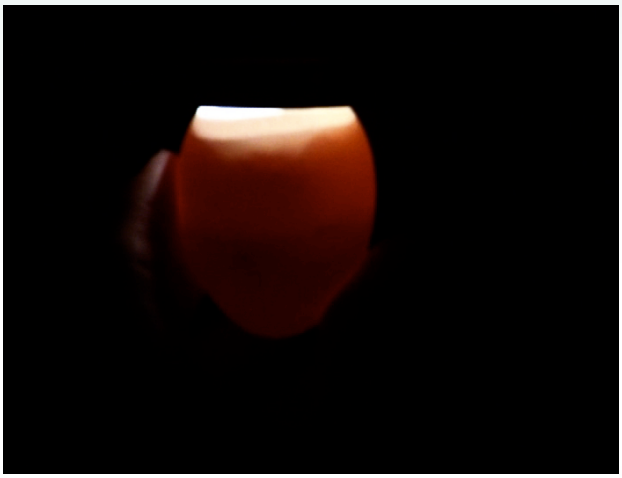


Fertile eggs used to isolate avian influenza or Newcastle disease should be derived from a Specific Pathogen Free (SPF) source flock or a commercial flock negative for antibodies to avian influenza and Newcastle disease.

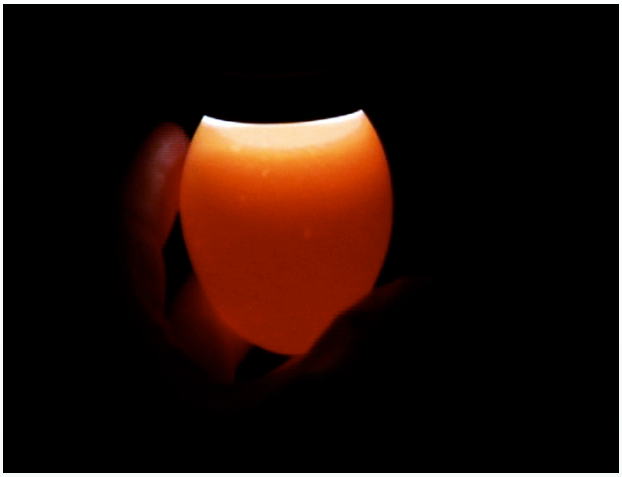
Prior to inoculation, fertile eggs incubated 9 to 11 days should be candled to check for fertility, embryo growth, air sac placement, and chorio-allantoic membrane development and placement.



This is a live embryo. Notice the well developed vein structure throughout the chorio-allanotoic membrane (CAM).



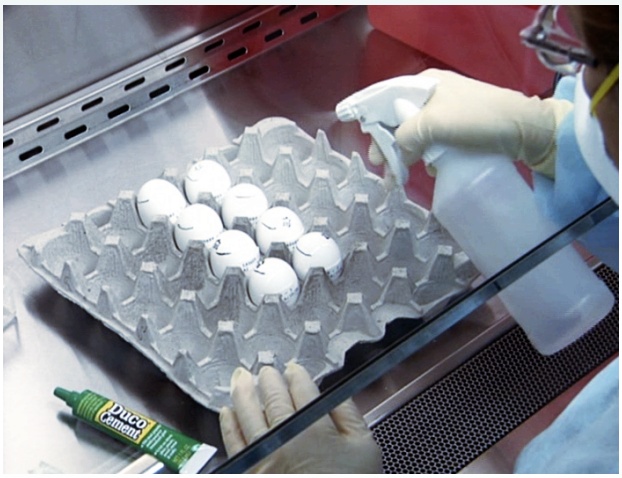
This is a dead embryo. Notice loss of vein structure in the CAM. Depending on the age of the embryo, dead embryos will often be observed to adhere to the egg shell.



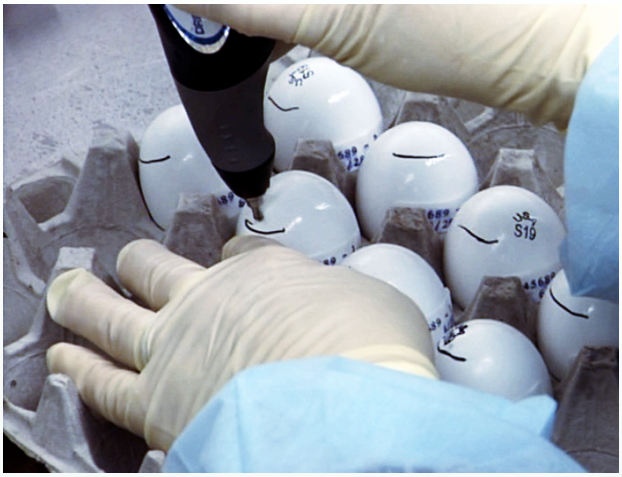
Another example of an egg with a dead embryo. The veins in the developing CAM are not visible.

The CAM in this egg has detached from the shell. This is an example of an egg with poor CAM development that should not be used for virus isolation.

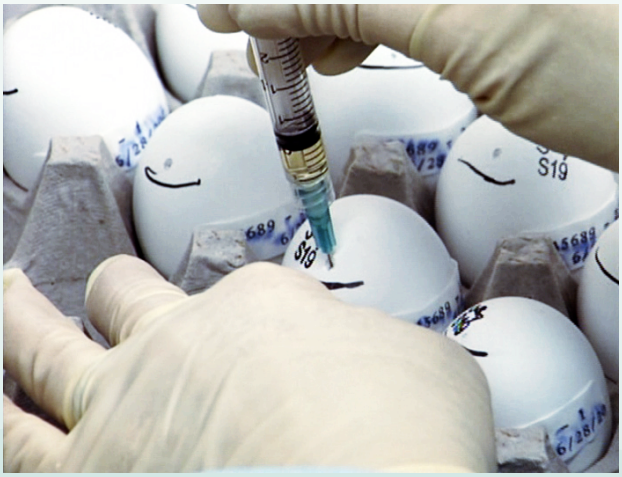
Egg Inoculation: Allantoic Sac Route in a Hood



Disinfect the air cell end of 9 to 11 day old eggs with a tincture of iodine-ethanol mixture or 70% ethanol. Each egg should be candled for viability and the location of the air cell marked on the shell opposite the location of the embryo.



Using a vibrating engraving tool, rotating drill or other suitable tool, drill a small hole in the top of the egg shell where it has been disinfected.

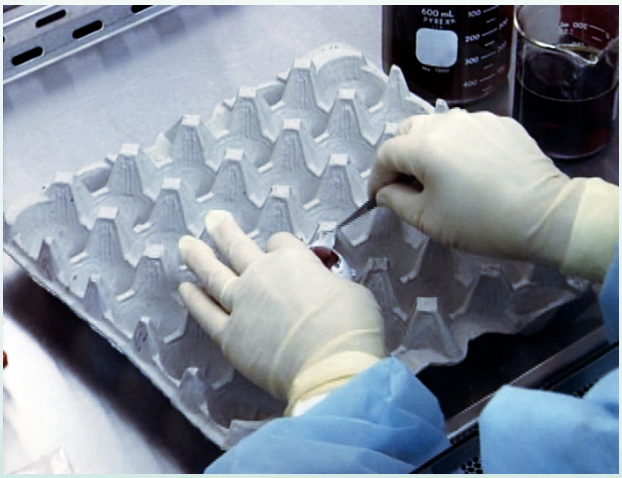


With a syringe fitted with a 25 gauge 5/8” needle, inoculate each egg with 0.3 milliliter of specimen into the allantoic sac. Insert the needle to its full length, vertically through the hole or at a slight angle away from the center of the egg.

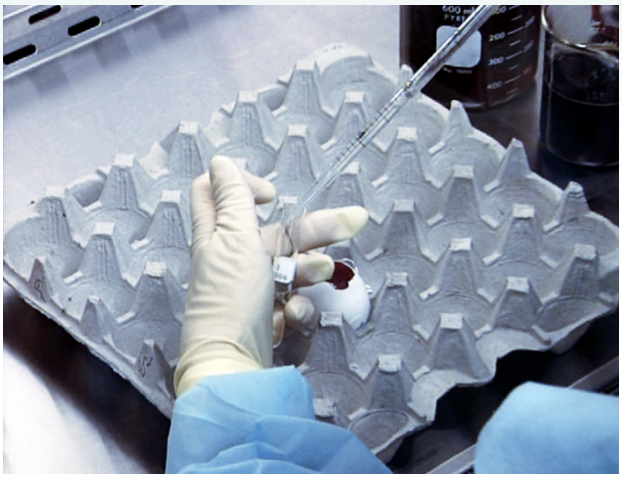
Seal the hole with a small amount of glue or melted paraffin and place back into a humidified incubator set between 36-37° C for 5 days.

Collection of AAF from Eggs with Dead Embryos in a Hood

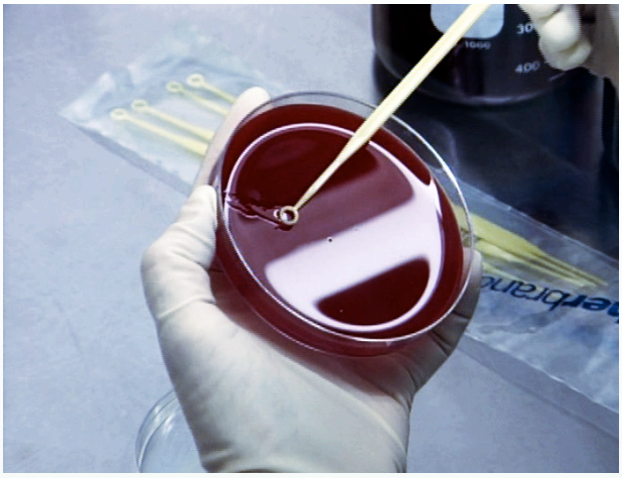
Disinfect the top of the egg with 70% ethanol. Using a sterile forceps, remove the egg shell above the air cell. Place the egg shell fragments into a disinfecting solution such as iodine.



Using a separate pair of sterile forceps, rupture the CAM and amniotic sac surrounding the embryo allowing the AAF to pool over the embryo and membranes. Special care should be taken not to break the yolk sac membrane.

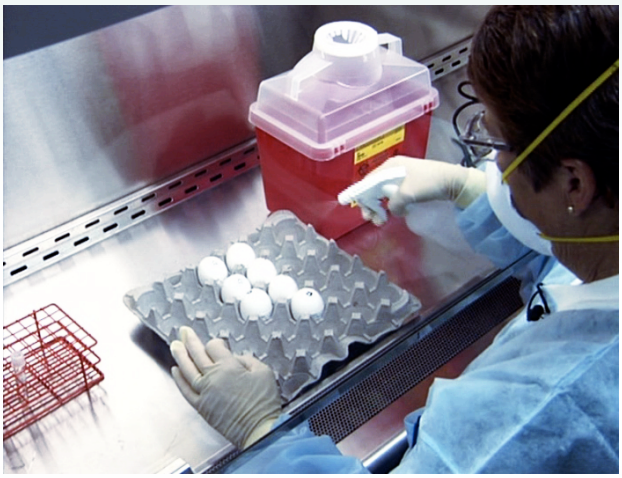


Collect the AAF with a 5 milliliter pipette while holding the embryo away from the pipette tip using the forceps. The fluid should appear clear. Badly contaminated AAF should be discarded. The AAF is placed into an appropriately sized tube and the remaining egg contents discarded. Eggs from only one specimen should be opened in the biological safety cabinet at one time to reduce the chances for cross-contamination.



Check the AAF for bacterial contamination by streaking a loopful of AAF onto a blood or nutrient agar plate. Incubate streaked plates for 24 hours at 37°C and record results.

Collection of AAF from Eggs with Live Embryos in a Hood



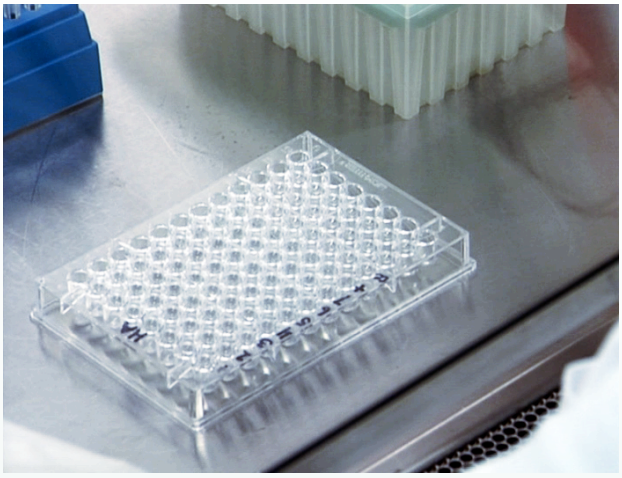
At the end of the incubation period, AAF is collected from eggs with surviving embryos to check for non embryo lethal hemagglutinating viruses. Prior to collecting the AAF, eggs must be refrigerated for 4 to 24 hours to kill the embryo and constrict the blood vessels of the CAM to prevent bleeding. First, the eggs are disinfected with 70% ethanol.

Using a vibrating engraving tool, rotating drill or other suitable tool, drill a small hole just above the air cell.

With a sterile syringe fitted with a 22 gauge one-inch needle, collect the AAF by inserting the needle through the drilled hole above the air cell and directing the needle toward the opposite side of the egg at a slightly downward angle from the horizontal axis. It may take some probing with the needle to locate the fluid. Be careful not to penetrate the yolk sac.

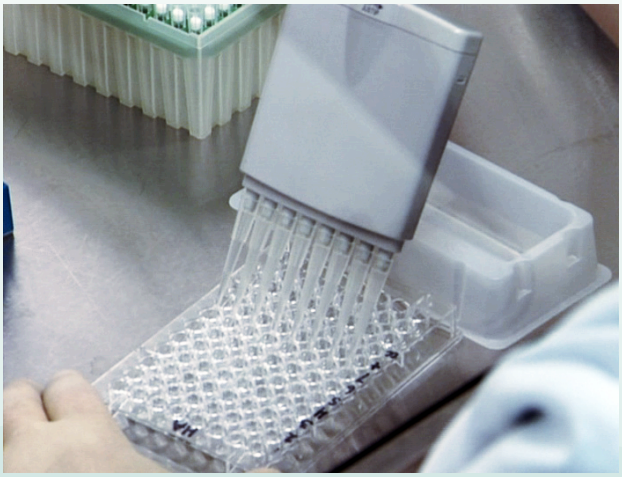
Place the amniotic-allantoic fluid into an appropriate size vial for testing and storage. The AAF from a set of eggs inoculated with the same specimen can be pooled. Discard any fluid that contains blood because the erythrocytes can bind to the virus, reduce the viral titer, and in some cases, lead to a false negative hemagglutination (HA) test result.

Hemagglutination (HA) Test for Detection of Hemagglutinating Viruses in a Hood

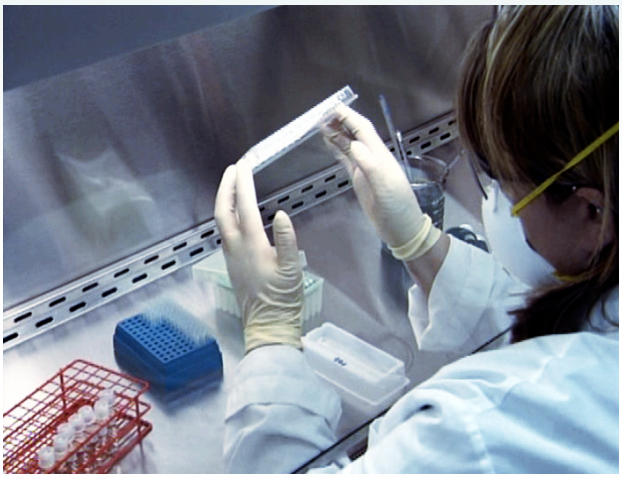


The HA test is commonly used to detect the presence of hemmaglutinating viruses such as orthomyxoviruses and paramyxoviruses in diagnostic specimens following isolation in embryonating eggs and cell cultures. The HA test is performed by making serial two-fold dilutions of the AAF or cell culture fluids in phosphate buffered saline (PBS) followed by the addition of a suspension of washed red blood cells (RBCs) as an indicator to detect HA activity. The HA test as described is performed in "U" bottom microtiter plates.

For the HA test, label one row of 8 wells in the plate for each AAF to be tested, plus an additional row for a positive control and a row for the cell control. The cell control will contain PBS and RBCs only.

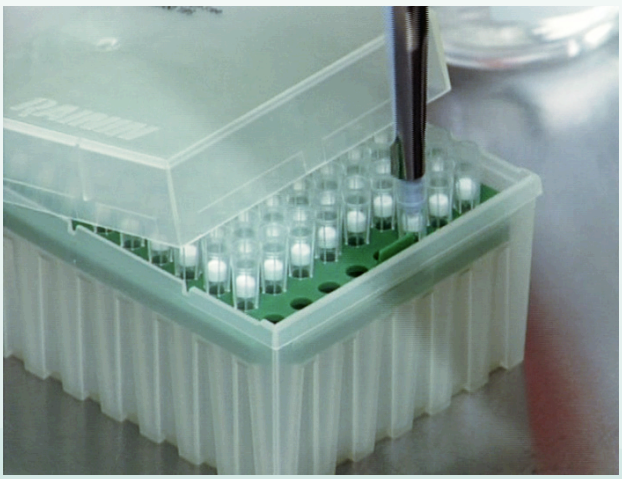


Dispense 50 microliters of PBS to each microtiter plate well being used for the assay, including the positive control row and cell control row.

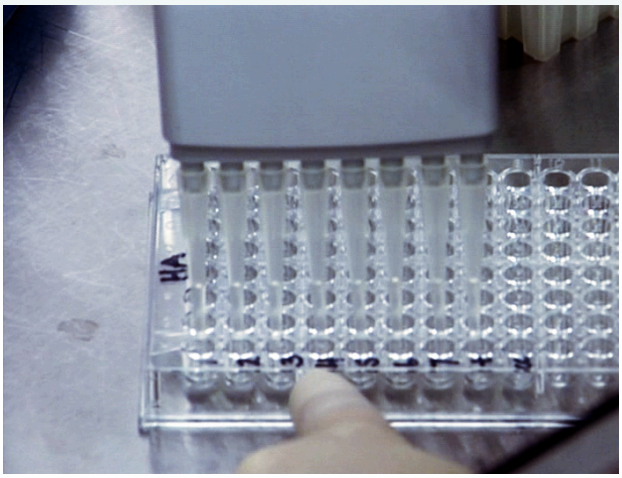


Visually inspect the plate to verify that PBS has been added to the appropriate wells.

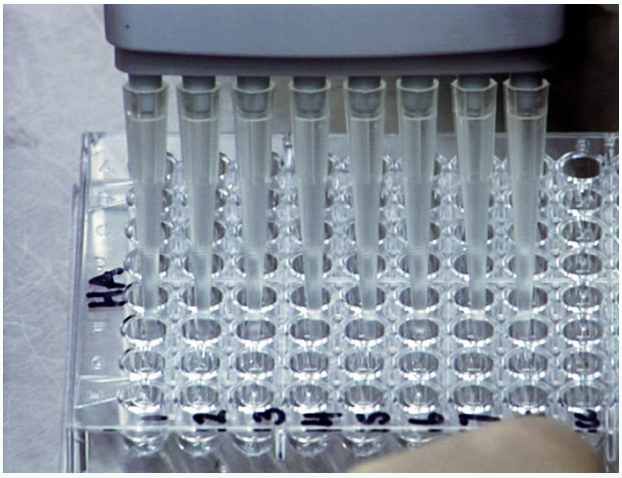
Next, add 50 microliters of AAF to the first well of the respective row of 8 wells. Also add 50 microliters of the positive control antigen to the first well of the positive control row. Note: Because the AAF may contain live virus, it is necessary to use sterile, aerosol resistant, filtered pipette tips to prevent cross contamination of samples.



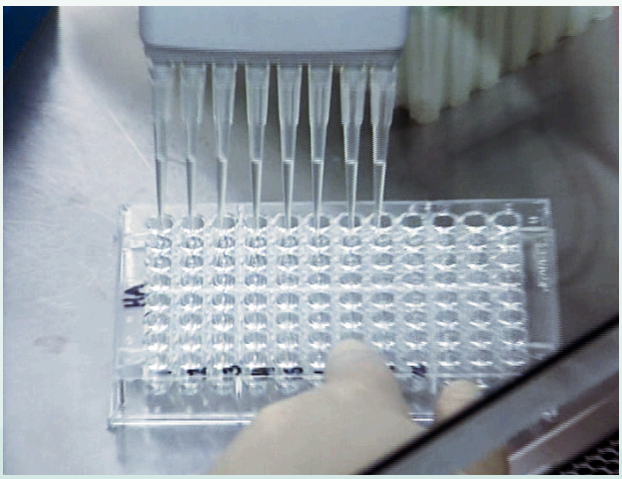
Use a different pipette tip for each sample.



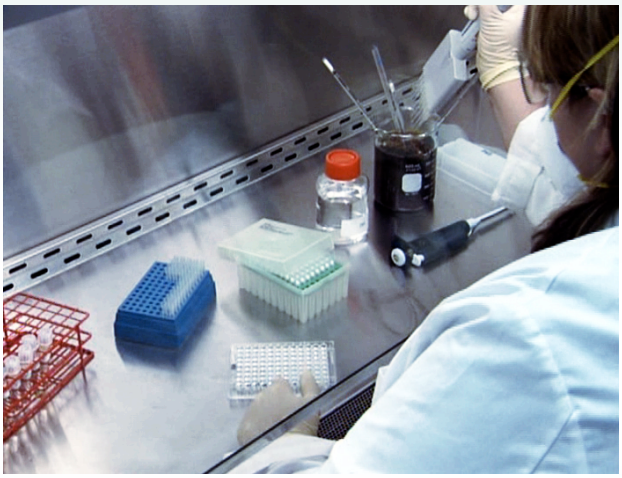
Using a single or multichannel pipettor equipped with non sterile, unfiltered pipette tips, mix the PBS and AAF in the first well of each row. Well contents should be aspirated and expelled (mixed) three to five times to ensure complete mixing. Be sure to keep the pipette tip submerged during mixing to prevent the formation of bubbles.



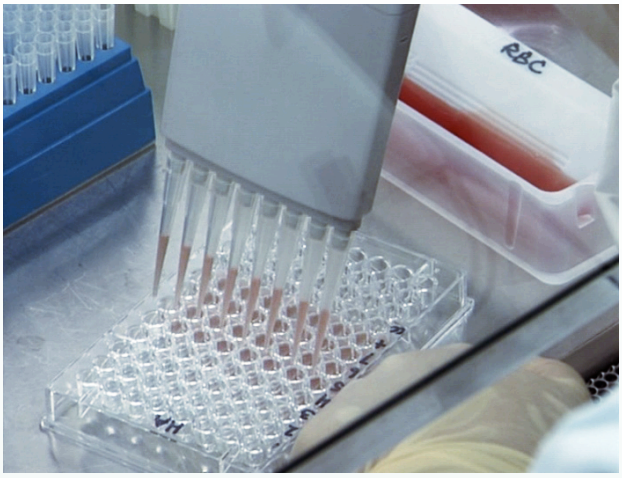
Nonsterile unfiltered tips can be used to make the serial two-fold dilutions of AAF as aerosol contamination will not affect results of the HA test. Approximately 4 to 6 logs of virus are required to achieve hemagglutination of erythrocytes.



Make serial two-fold dilutions of each sample by transferring 50 microliters of the sample dilution from the first well of the row to the second well of the same row, followed by mixing and then transferring 50 microliters from the second well dilution to the third well, etc. Continue this process of mixing and transferring 50 microliters of sample dilution to the subsequent well all the way to the end of the plate row. The first well would contain a 1:2 dilution of AAF, the second well a 1:4 dilution and so on through the last well which would contain a 1:256 dilution of AAF.

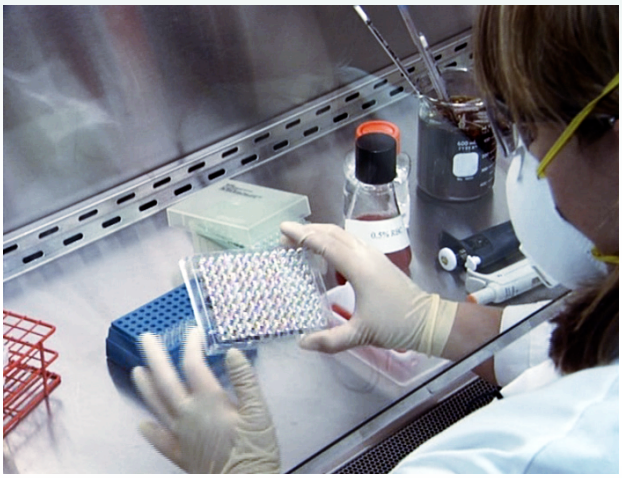


Upon reaching the last well of the row, discard the 50 microliters of the sample dilution in the pipette.



Using nonfiltered, nonsterile pipette tips, add 50 microliters of 0.5% washed erythrocyte suspension to each well. Be sure the erythrocytes are thoroughly suspended and be careful not to allow the pipette tips to come in contact with the sample dilutions to avoid sample carry over.

Place a seal on the top of the plate.



Agitate the plate to thoroughly suspend the erythrocytes. Remove plates from the biological safety cabinet and incubate at room temperature for 20 to 30 minutes or until a distinct button has formed in the cell control well.

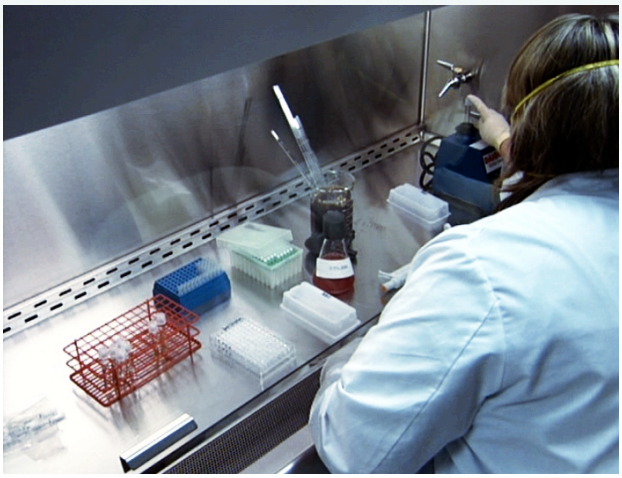
Interpreting HA Test Results

After incubation, determine the endpoint for each sample. The endpoint of the titration is the highest dilution of antigen causing complete hemagglutination. In this example, the endpoint of sample 1 is 1:128. Sample 2 has an endpoint of 1:32. Sample 3 shows an endpoint dilution of 1:64 even though there is partial hemagglutination at the 1:128 dilution. For the hemagglutination-inhibition (HI) test, 4 HA units (HAU) of antigen in 25 microliters is used. For the avian paramyxovirus test, 8 HAU in 25 microliters is used (see HI test for details).

HA Test Back Titration

The back titration is performed to confirm that the correct number of hemagglutinating units (HAUs) are present in the diluted AAF for the hemagglutination-inhibition (HI) test. For avian influenza, 4 hemagglutinating units/25 microliters is used for the hemagglutination-inhibition test. And for avian paramyxoviruses, 8 hemagglutinating units/25 microliters is used. It should be noted that since the HA test is done using 50 microliters, the required number of HAU/50 microliters is 2 times that for 25 microliters.

From the previous HA test we know that Sample 1 had an hemagglutination (HA) endpoint of 1:128 and therefore contained 128 hemagglutinating units per 50 microliters. To calculate a dilution of the sample for the avian paramyxovirus HI test that will contain 8 hemagglutinating units per 25 microliters (or 16 HAUs/50 microliters), divide 128 by 16…which equals 8. Therefore, Sample 1 is diluted 1 to 8 with PBS. Similarly, Sample 2, which had an HA endpoint of 1:32 is diluted 1 to 2 with PBS to achieve 8 hemagglutinating units per 25 microliter, that is, 32 divided by 16 equals 2. Repeat this process for each of the remaining HA positive samples.



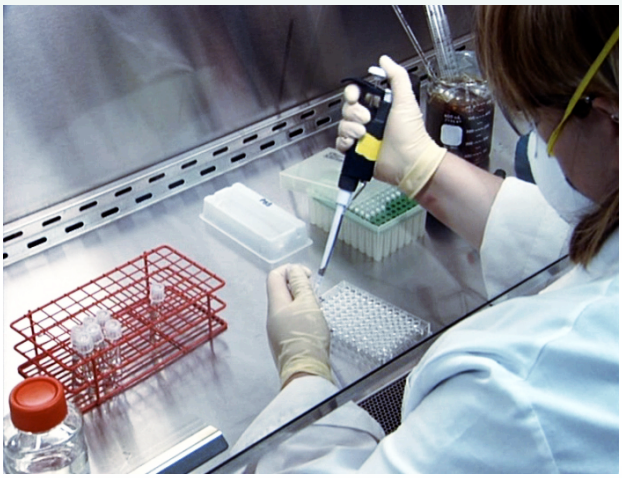
Vortex the sample dilutions before proceeding with the backtitration hemagglutination test.

Perform the HA test with the diluted samples using the same procedure as for the HA test. If the appropriate dilutions were made, the endpoint for each sample should be 16 HAUs per 50 microliters as shown in this example.

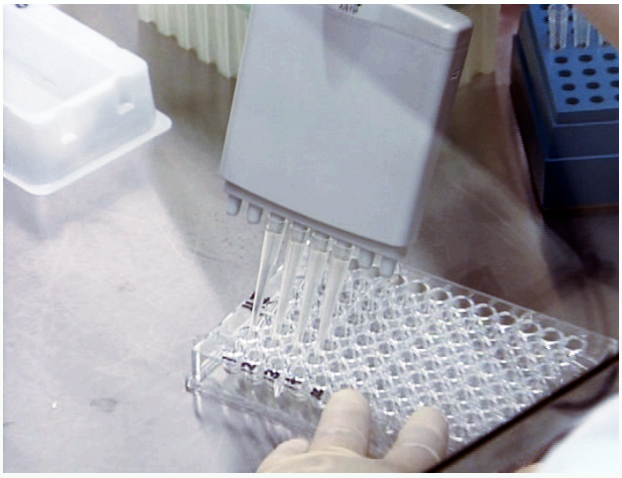
Introduction to the HI Test

The hemagglutination-inhibition (HI) test is a rapid and economical serologic test commonly used to identify hemagglutinating viruses such as orthomyxoviruses and paramyxoviruses. In the HI test, specific antibodies are used to inhibit the agglutination of erythrocytes by hemagglutinating viruses, hence the name hemagglutination-inhibition test. The procedure that will be described is somewhat different from that described in the *OIE Manual of Diagnostic Tests and Vaccines*, but the test as described here has been shown to produce equivalent results. Since most laboratories will not have reagents to identify avian influenza viruses, the procedure described here will be for the identification of avian paramyxovirus type-1 or Newcastle disease virus.

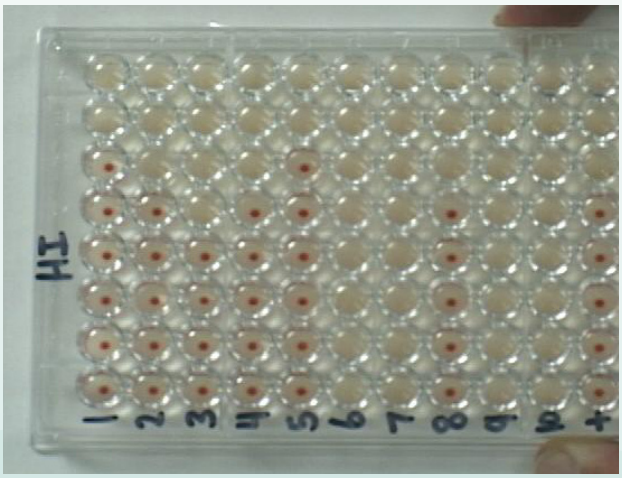
Begin by labeling "U" bottom microtiter plates in rows of 8 wells for each unknown to be tested. Label an additional row to be used for a positive control. Add 25 microliters of the unknown virus sample, diluted to contain 16 hemagglutinating units/50 microliter (which is equivalent to 8 hemagglutinating units per 25 microliters) to each of the 8 wells in the corresponding row. Also, add 25 microliters of diluted Newcastle disease virus to each well in the positive control row.



Add 25 microliters of the standardized antiserum to the first well of each row. The standardized antiserum has been previously absorbed with erythrocytes to remove the natural agglutinins present in some serums. The antiserum is also diluted to achieve an endpoint titer of 1:32 or 1:64 with homologous antigen.



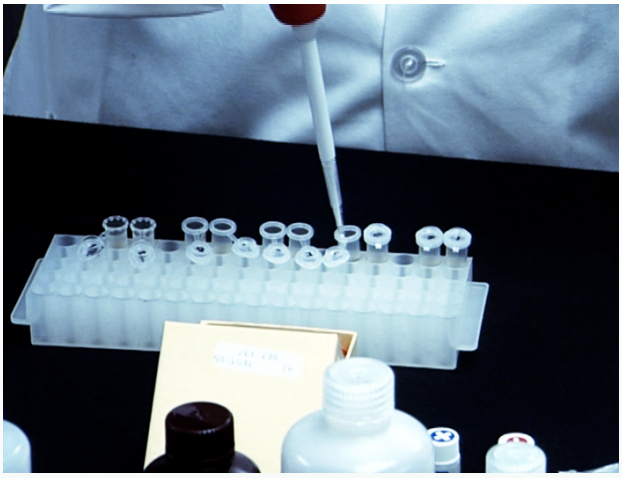
With a single or multichannel pipette set to deliver 25 microliters, make a two-fold serial dilution of the antiserum (in the antigen) across the plate beginning in the first well and continuing through the 8th well. Discard the terminal 25 microliters from the 8th well. At this point each well should contain 25 microliters.



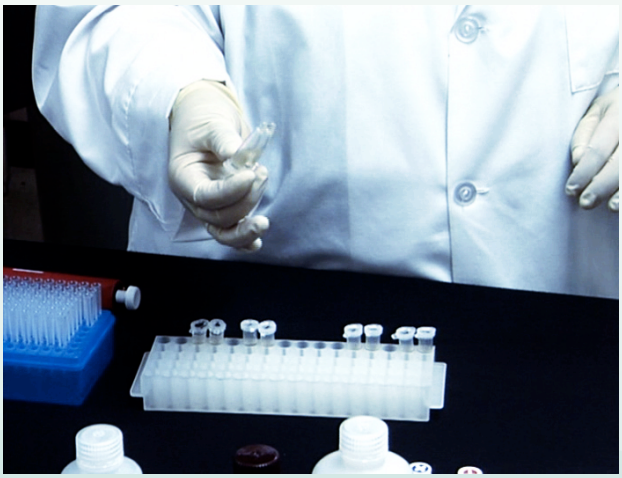
Cover the plate with a plate cover or empty plate and incubate plates in the biosafety cabinet for 30 minutes. Following the 30 minute incubation period, add 50 microliters of a one-half percent washed erythrocytes to all wells. Seal the plate with sealing tape and thoroughly mix to suspend the erythrocytes. Remove plates from the biosafety cabinet and incubate for 20 to 30 minutes at room temperature. The HI test should be read when the erythrocytes in the positive control and cell control wells form a distinct round button in the bottom of the well. To facilitate interpretation of the results, tilt the plate at a 45 degree angle until the erythrocytes in the positive control wells form a tear-drop shape (usually about 20-30 seconds). Record wells where erythrocytes have formed a tear-drop shape as inhibition and wells where the erythrocytes the do not tear-drop as no inhibition. Specimens that do not form a tear-drop but are partially inhibited are recorded as incomplete. Unknowns are identified as Newcastle disease virus if the endpoint of the unknown is within two dilutions of the endpoint of the positive control. Isolates not inhibited by Newcastle disease antiserum should be considered suspect for avian influenza and should be confirmed by other tests.

IDEXX ELISA for Detection of Influenza A Antibodies in Serum

The first step is to dilute the serum samples. The IDEXX kit comes with a dilution buffer. Add 1 milliliter of the dilution buffer to previously labeled dilution tubes. Please note that not every ELISA kit uses the same serum dilution, so be sure to read the manufacturer's instructions carefully for each brand of ELISA kit you use.



With a sterile pipette tip, add 200 microliters or 0.2 milliliters of each serum to a dilution tube to make a 1:500 dilution of the test serum. Again, not every brand of ELISA uses the same serum dilution.



Mix the serum dilutions.

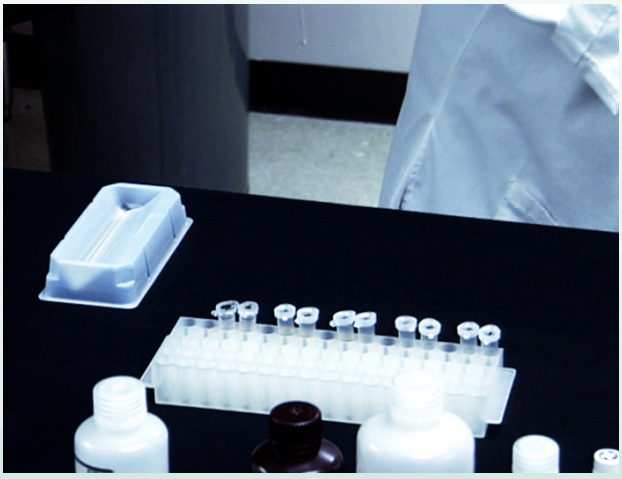
Using the antigen coated plates that come with the kit, start by adding 100 microliters of undiluted negative control serum (provided with kit) into the first two wells (A1 and A2).

Add 100 microliters of undiluted positive control serum (provided with kit) into the next two wells (A3 and A4).

Add 100 microliters of diluted test serum to successive duplicate wells. For example, serum 1 would go in wells A5 and A6, serum 2 would go in wells A7 and A8, and so on. Following the addition of all serums,incubate the plate for 30 minutes at room temperature.

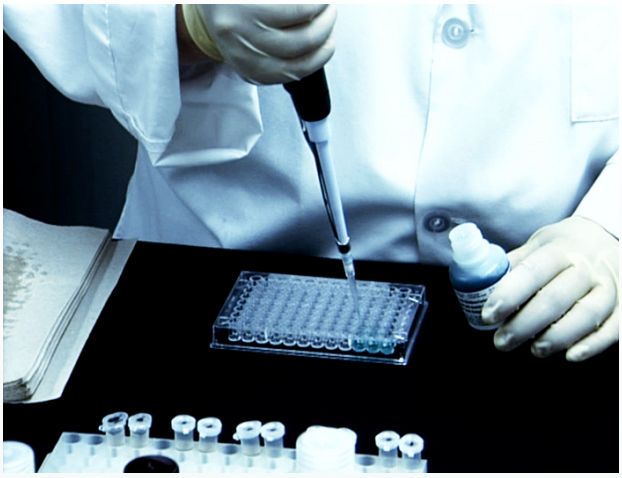
At the end of the incubation period, perform a wash step. First, empty the liquid from the plates by inverting plates and flicking contents into a suitable container for disposal.

With a 300 microliter multichannel pipette, fill each well with distilled water. Empty the contents of the plate as before. Note: An ELISA plate washer can also be used to perform the washes.

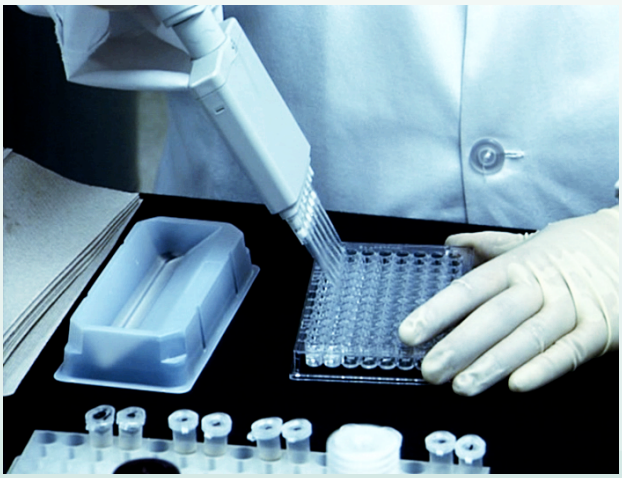


Using the same process, perform a second and third wash.

Tap the plate dry on a paper towel to remove all traces of wash solution.



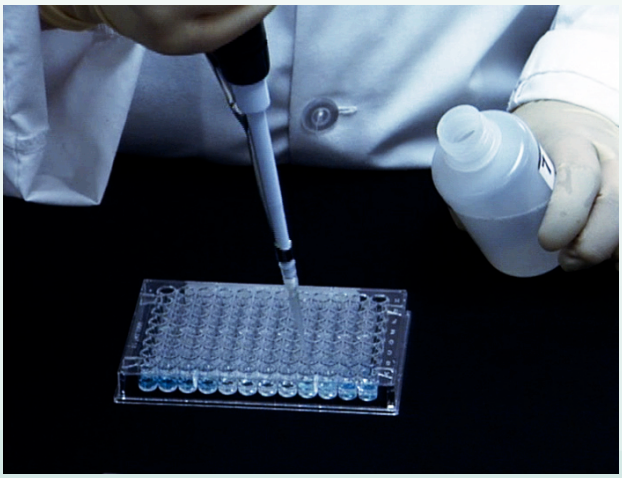
Add 100 microliters of conjugate to each well. Incubate for 30 minutes at room temperature.



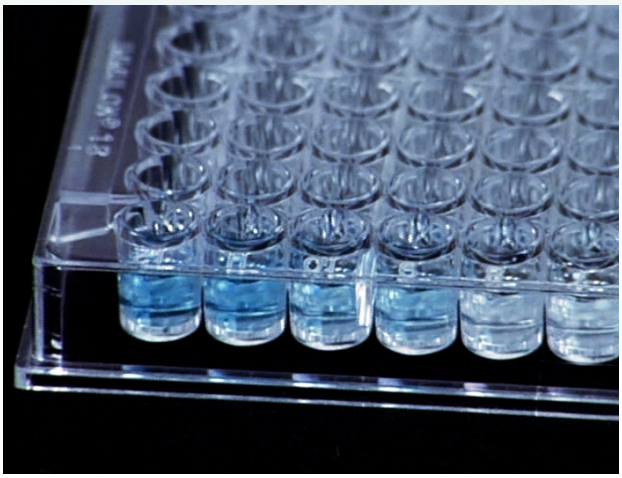
Empty the liquid from the plates and perform another set of three washes with distilled water.

Tap the plate dry on a paper towel to remove all traces of wash solution.

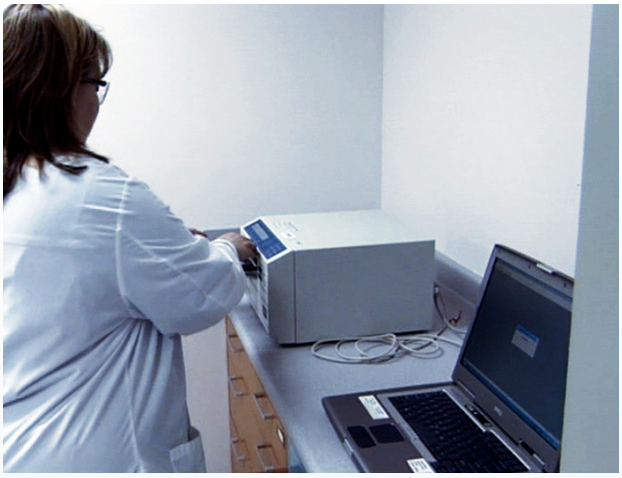
Add 100 microliters of substrate to each well. Incubate for 15 minutes at room temperature.



After the incubation period, add 100 microliters of stop solution to each well.



A blue color change will appear in positive specimens. The darker the color, the stronger the positive.



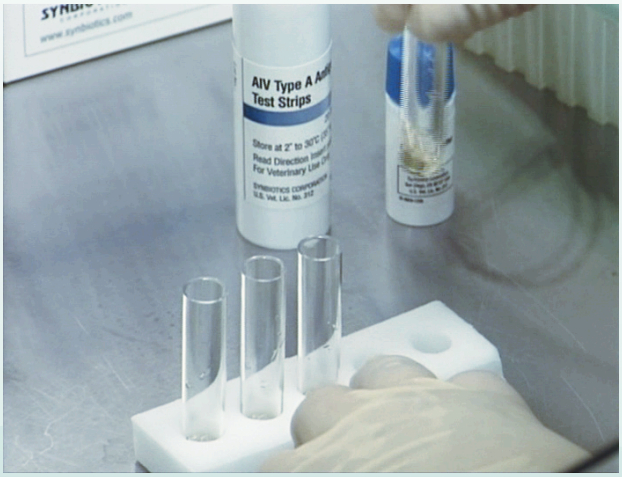
An ELISA reader is used to determine the light absorbency of each sample. Calculate the sample to positive (S/P) ratios by subtracting the average normal control absorbance from each sample absorbance to determine which specimens are positive. Note: Most kits will provide a minimum acceptance value for the positive control that must be achieved for the test to be valid.

Influenza Antigen Detection in a Hood

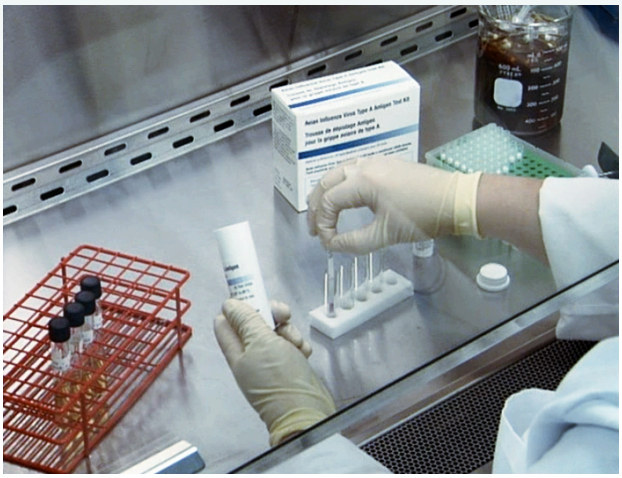
FluDetect by Synbiotics is one example of an antigen detection test kit used as a screening test for type A influenza virus. This test is best suited for testing tracheal, oropharyngeal, and cloacal swabs from recently dead or symptomatic birds or flocks.

Start by adding 8 drops of the extraction buffer into the tubes provided with the kit, one tube per specimen.

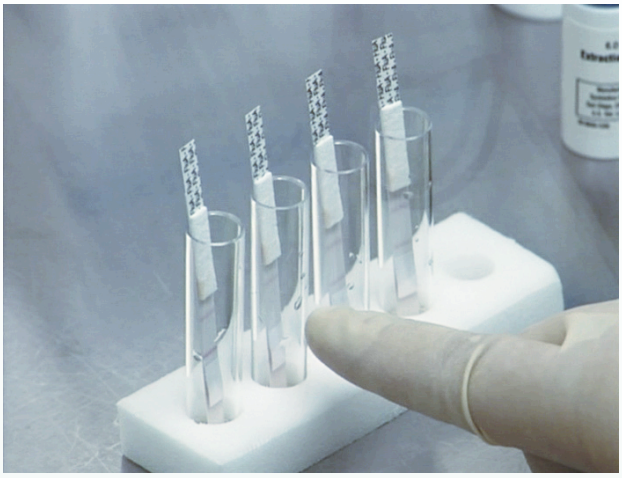
Add 200 microliters of specimen to the corresponding labeled tube. When collecting swabs to use for this test, the manufacturer recommends the use of Brain-Heart Infusion broth (porcine origin).



Mix the samples thoroughly.

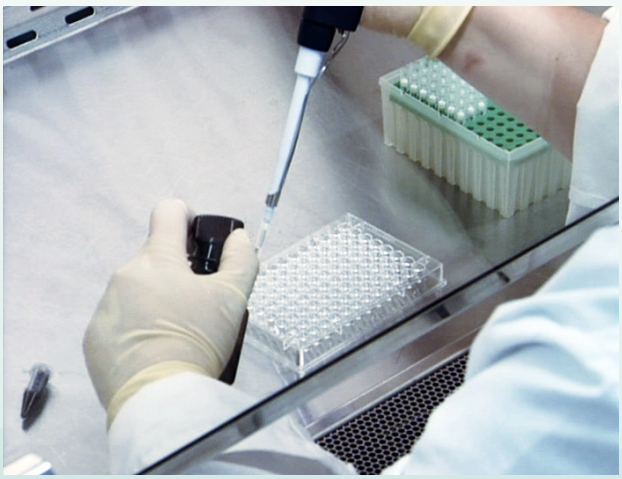


Add one test strip, supplied with the kit, to each specimen tube. Be certain that the pink membrane at the bottom of the strip is completely covered by the solution. Incubate for 15 minutes at room temperature and record test results.



When interpreting results of the test, the top pink line is the control line. If the top pink line develops and the bottom pink line develops at any intensity, the test is positive for type A influenza virus. These specimens show that the first tube from the left is a strong positive, the second tube is a negative, and the third and fourth tubes are both weak positive specimens.

Ambion® Magnetic Bead Extraction of RNA in a Hood

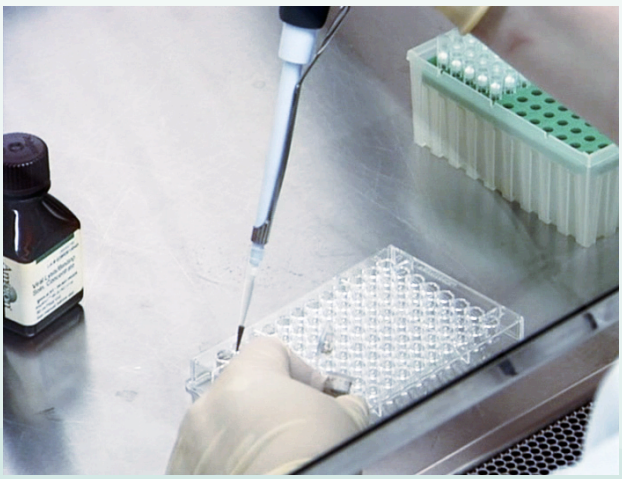


This RNA extraction procedure uses guanidinium thiocyanate, a chemical used to lyse cells and inactivate viruses such as influenza virus. The initial step must be performed in a BSC. Following the addition of the lysis buffer the specimens are no longer infectious and can be safely removed from the BSC.

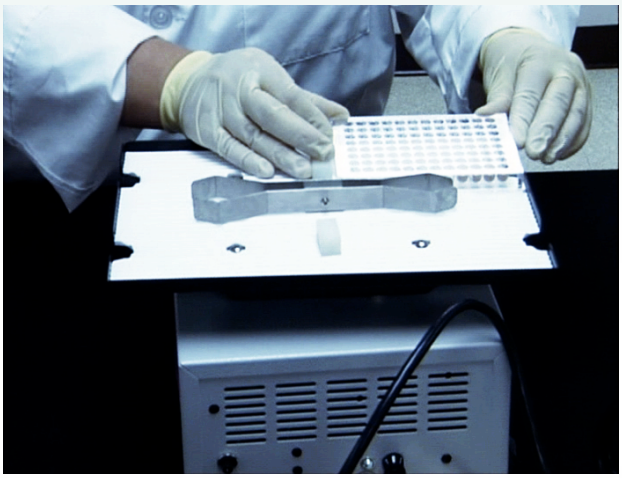
Working in a biological safety cabinet, place 100 microliters of viral lysis/binding solution into each of the sample wells. The lysis/binding solution will need to be prepared according to the manufacturer's instructions or protocol prior to performing this procedure.

Add 50 microliters of the swab specimen into a corresponding well on a 96 well plate, changing pipette tips between each sample.

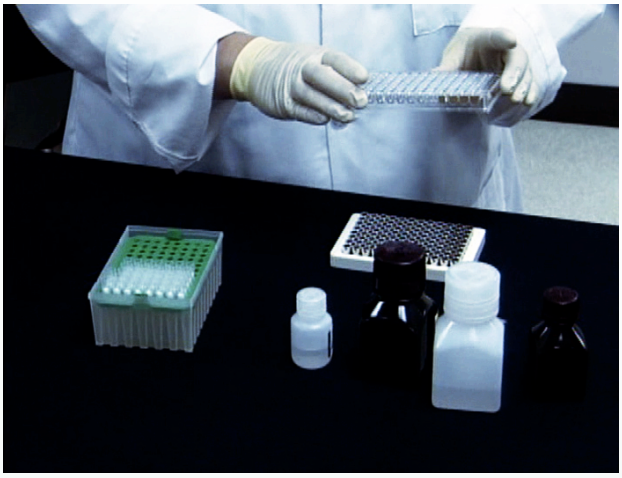
Shake the plate to make sure the lysis/binding solution is thoroughly mixed with the sample.



Next, add 20 microliters of the RNA binding beads using a new pipette tip for each well. The magnetic bead solution will also need to be prepared prior to performing this procedure. Follow the manufacturer's guidelines to properly combine and mix the magnetic bead suspension.

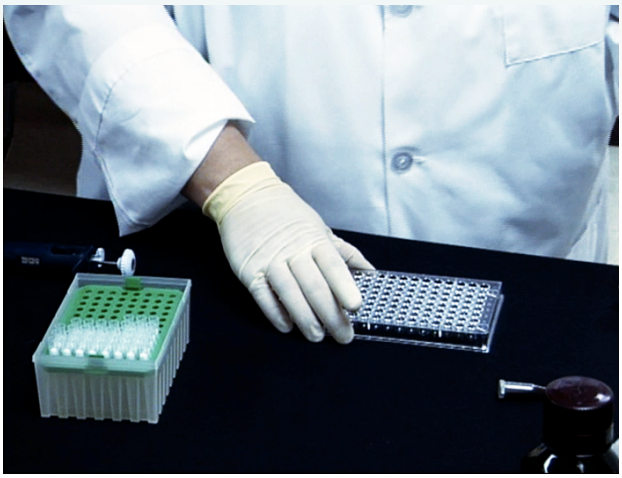


At this point, the virus has been inactivated by the lysis/binding solution so the plate can safely be removed from the biological safety cabinet. Using an orbital shaker, shake the plate on moderate speed for four minutes to mix and suspend the magnetic beads. During this shaking process, the RNA will bind to the magnetic beads.

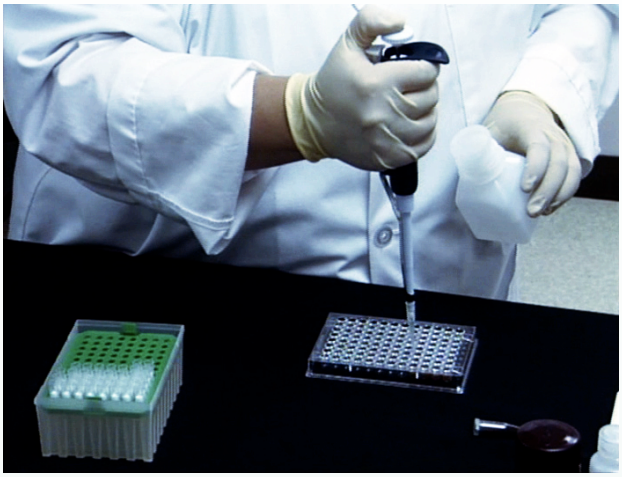


Place the plate onto the magnetic stand for two minutes.

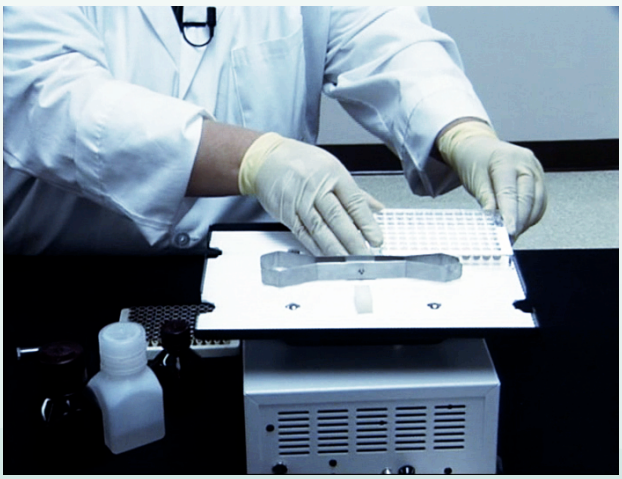
The magnetic beads will migrate towards the magnetic field so that the liquid can be removed with a pipette. Discard the liquid and pipette tips.



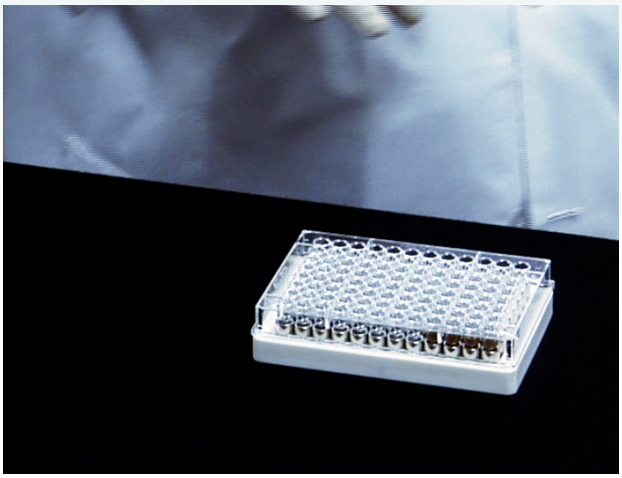
Remove the plate from the magnet so the magnetic beads are released.



Add 100 microliters of Wash Solution 1 to each well. The Wash Solution will need to be prepared prior to performing the procedure.

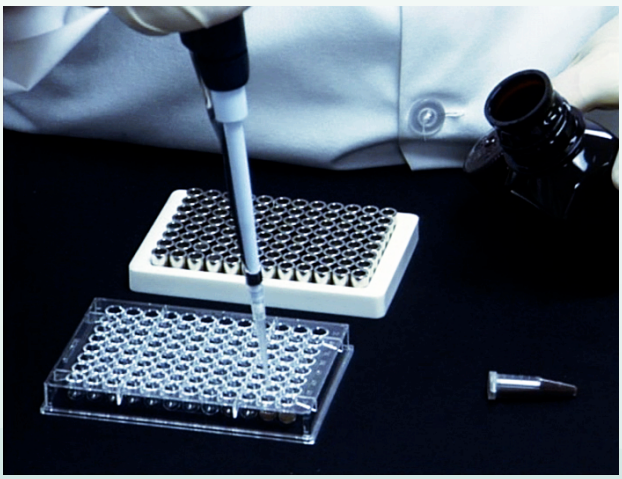


Place the plate back onto the orbital shaker and shake for 30 seconds on moderate speed.

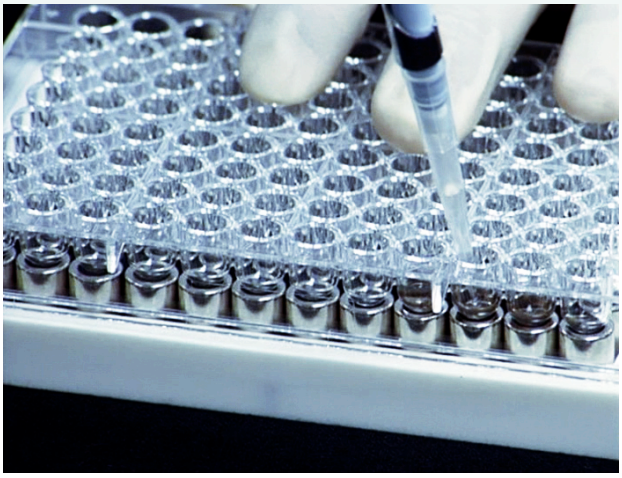


Remove the plate from the shaker and place it on the magnetic stand for one minute.

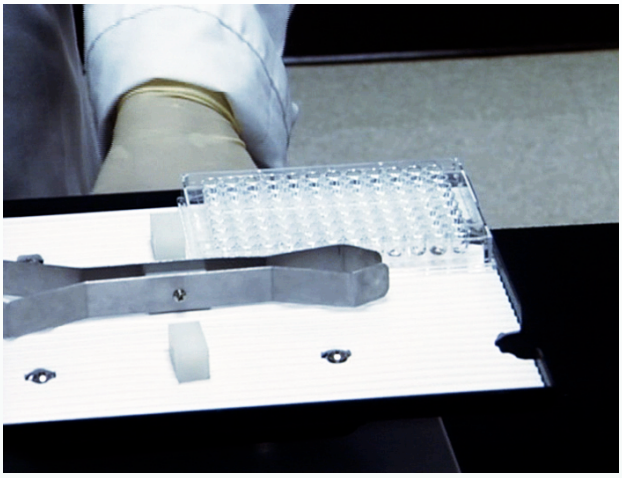
Remove the liquid from the wells. Leave the magnetic beads on the bottom of the well.



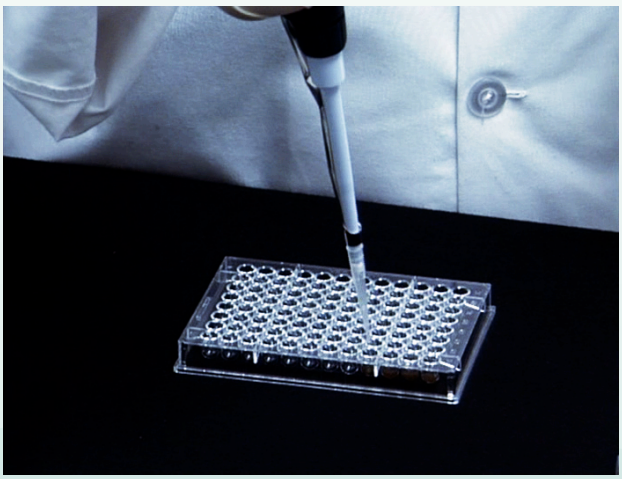
Add 100 microliters of Wash Solution 2. Place the plate on the plate shaker for 30 seconds, then transfer the plate to the magnetic stand before removing the liquid. Repeat the wash step again using Wash Solution 2, the shaker, and magnet.



Following the second wash with Wash Solution 2, remove and discard the wash solution from each well leaving the beads at the bottom of the well.



Place the plate on the shaker for two minutes at a high setting to evaporate any residual ethanol and dry the magnetic beads. It should be noted that residual ethanol is inhibitory to PCR and will affect other down-stream applications.



Once the beads appear dry, add 50 microliters of elution buffer to each well then shake on the orbital shaker for 4 minutes.

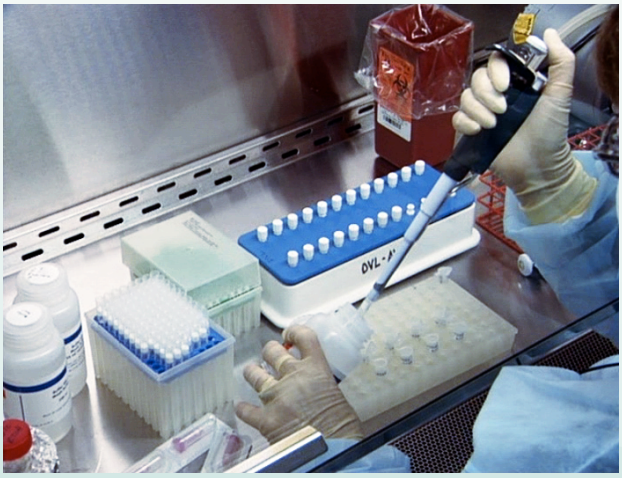
After the 4 minutes, place the plate onto the magnetic stand for 2 minutes. The Elution buffer causes the RNA to release from the magnetic beads. Transfer the RNA solution to an appropriately labeled tube for testing and storage. A separate pipette tip should be used for each specimen.

Qiagen® RNeasy® Mini Columns for RNA Extraction in a Hood

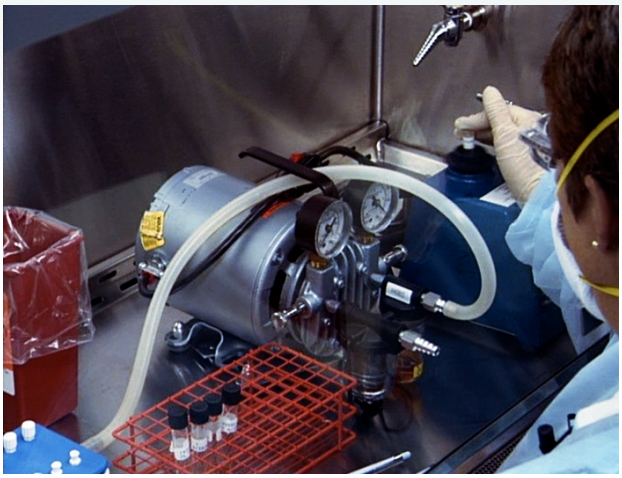
This RNA extraction procedure uses guanidinium thiocyanate, a chemical used to lyse cells and inactivate viruses such as influenza virus. The initial step must be performed in a BSC. Following the addition of the lysis buffer the specimens are no longer infectious and can be safely removed from the BSC.

RNA extraction separates and removes nucleic acid from other cellular components such as proteins and lipids that are inhibitory to PCR.

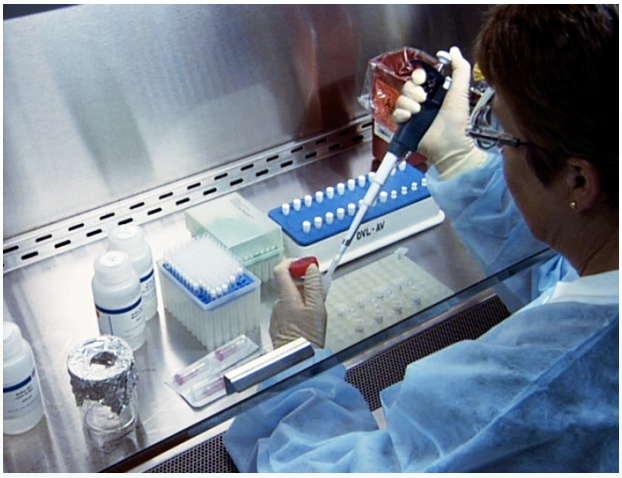
Start by preparing the Qiagen® RNeasy® extraction components according to the manufacturer's instructions. Use a filtered pipette tip to pipette 500 microliters of your sample into a sterile microcentrifuge tube.



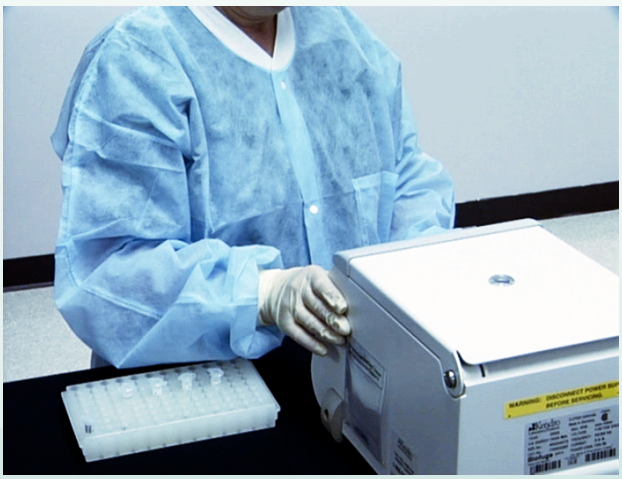
Next, add 500 microliters of RLT lysis buffer to the specimen tube. Do not touch the side of the specimen tube with the pipette tip or insert the tip into the specimen tube. If you do, you will need to change to a new sterile tip.



Thoroughly vortex each sample. Pulse centrifuge the specimen tubes to remove any sample that may be on the lid of the tube after vortexing.

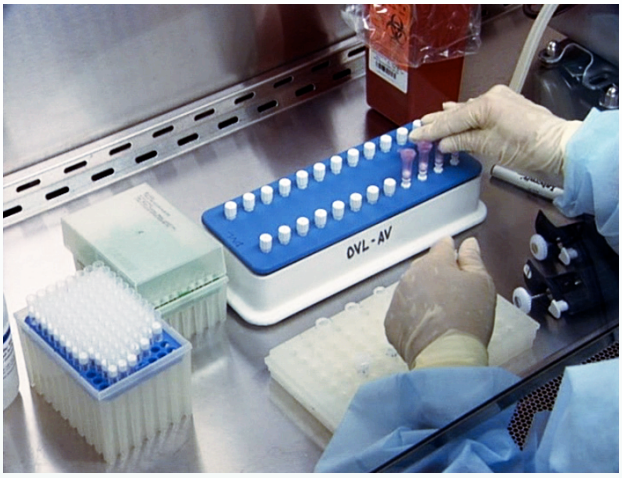


Add 500 microliters of 70% ethanol and vortex each tube.



Centrifuge the lysed sample for 5 minutes at 5000 x ***g*** to clearify the sample.

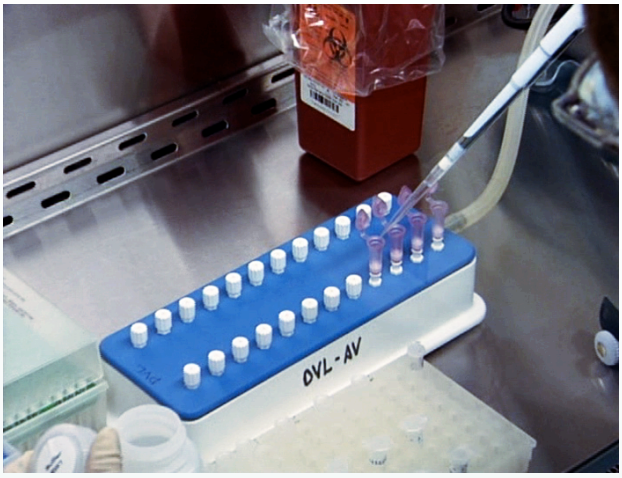
Next, place the appropriate number of RNeasy extraction columns into the column ports on the vacuum manifold and label each column to correspond to the specimen identification.



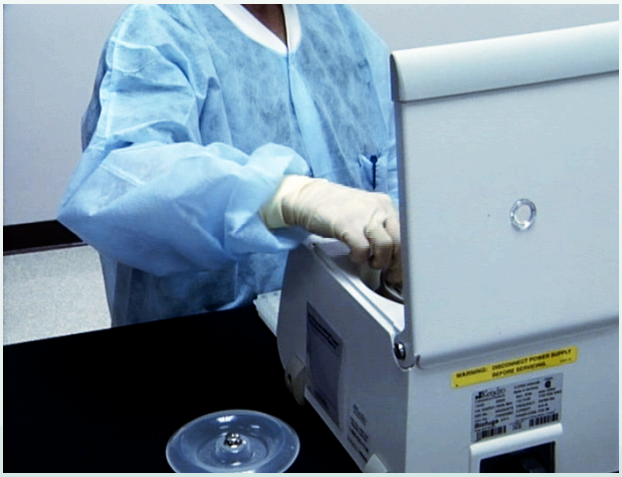
Loosen the lid of each column before turning on the vacuum pump so air can flow through the column when the vacuum is turned on. Turn on the vacuum and push down on the manifold lid so it will form a tight seal with its base.

Using an aerosol resistant pipette tip, individually transfer the liquid in each specimen tube to the corresponding extraction column. Note: Care should be taken not to remove or dislodge the pellet from the bottom of the specimen tube.

After the specimen has been vacuumed from the extraction column, wash the specimen with 700 microliters of previously prepared RW1 buffer.



After the RW1 buffer has been vacuumed from the column, perform two 500 microliter washes with RPE wash solution, making sure that all of the wash solution has been completely vacuumed from the column between each wash step. Note: Any nucleic acid present in the specimen should now be adhering to the white silicon membrane at the bottom of the column.



Place the extraction column into the centrifugation tubes supplied with the columns. Dry the column and remove residual ethanol by centrifugation in a microfuge at full speed for two minutes. Note: Residual ethanol is inhibitory to PCR amplification and other down-stream applications.

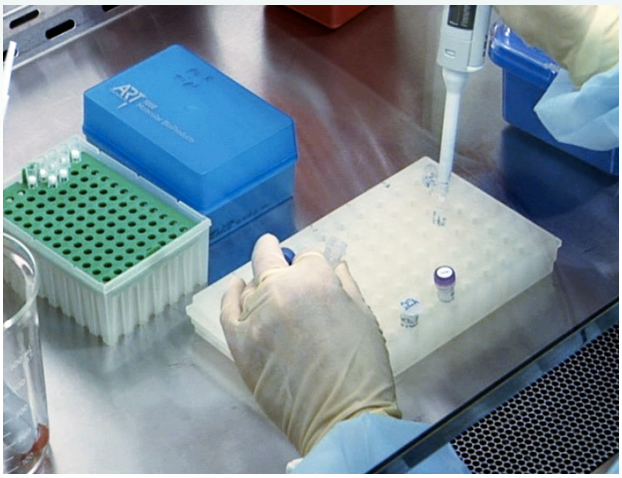
Move the extraction columns to the elution tubes.

Using sterile pipette tips for each tube, add 50 microliters of RNase-free water to each column. Be certain not to touch the silica-gel membrane of the column with the pipette tip. Incubate the tubes at room temperature for 1 to 3 minutes. Centrifuge the tubes for one minute at 10,000 rpm to elute the RNA. Store the sample at 4º C until the sample is tested. RNA that cannot be tested within 24 hours should be stored at -70º C. Multiple freeze and thaw cycles should be avoided.

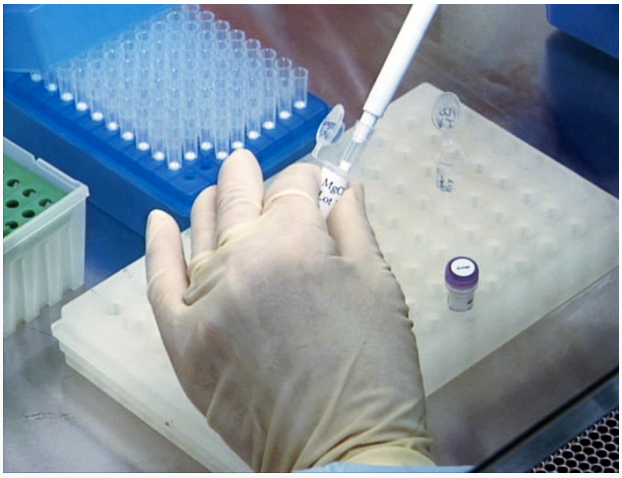
Preparation of Master Mix for Avian Influenza rRT-PCR

This procedure was developed for the Cephied® Smart Cycler. Information on setup and programming the Smart Cycler can be found in the user manual.

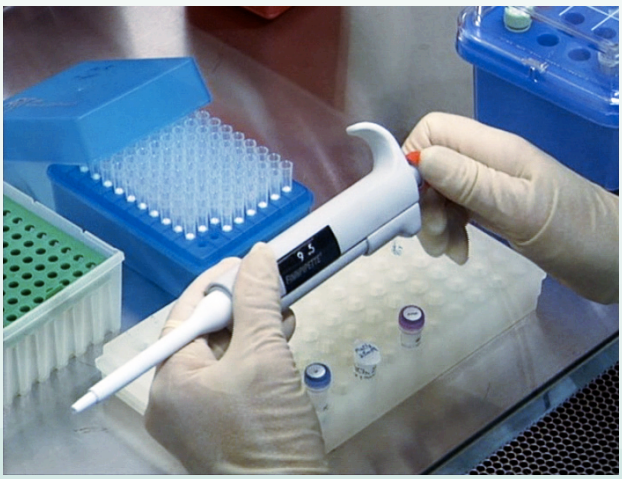
Start by preparing the master mix. You will need to properly calculate the appropriate volume of reagents needed based on the total number of samples to be tested. In this sample experiment, we will be creating master mix for 12 samples. Start by adding 84 microliters of water. Prior to pippetting reagents, pulse vortex each reagent to ensure a homogenous suspension.



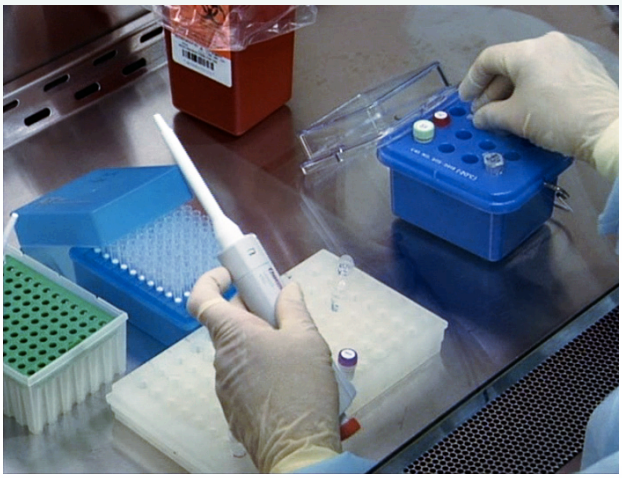
Next add 60 microliters of One-Step RT-PCR 5x buffer.



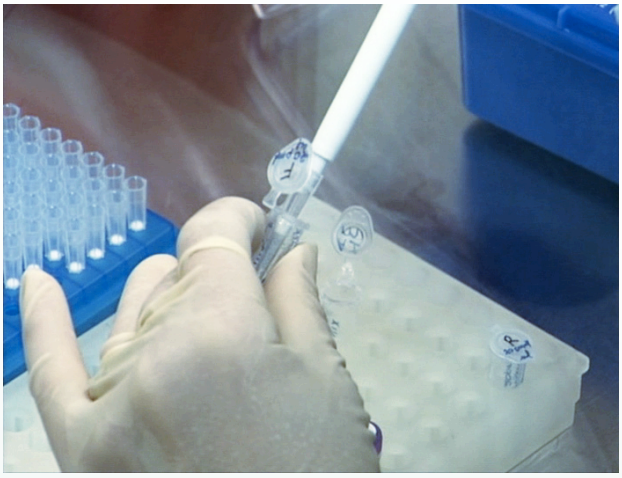
Add 15 microliters of 25 millimolar magnesium chloride.



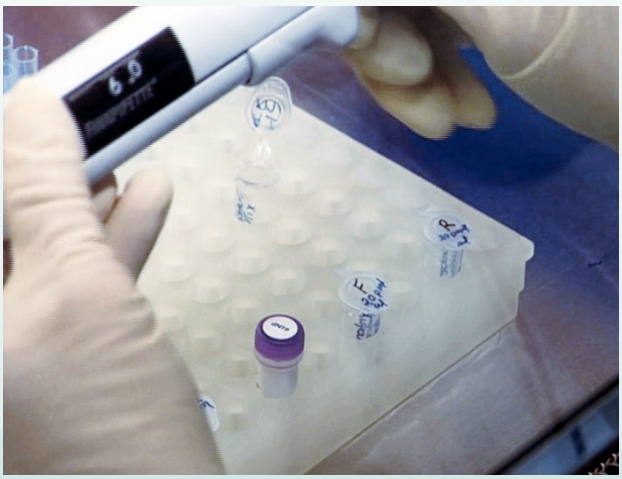
Add 9.5 microliters of dNTPs each at 10 millimolar concentration.



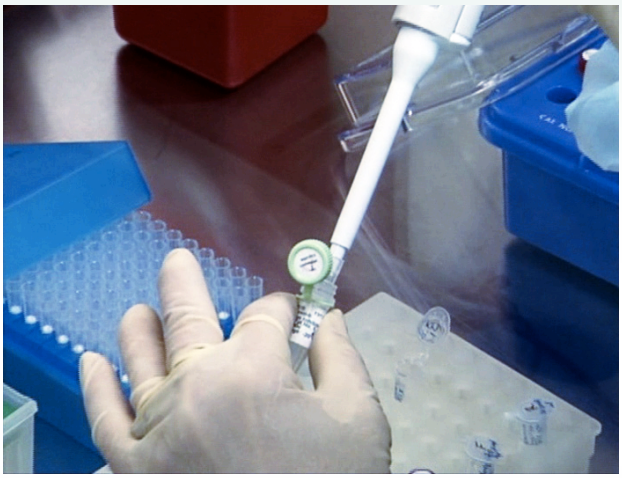
Next add forward and reverse primers, RNase Inhibitor, and enzyme mix. These reagents are perishable so they need to be stored in a refrigerated cooler box until use.



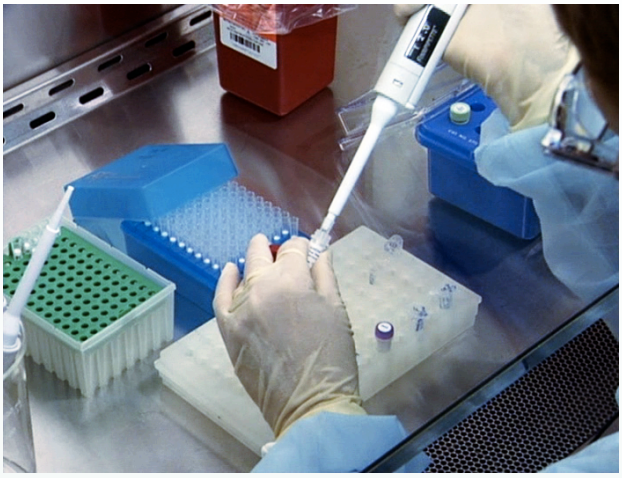
Add 6 microliters of forward primer that has been diluted to 20 picomoles per microliter.



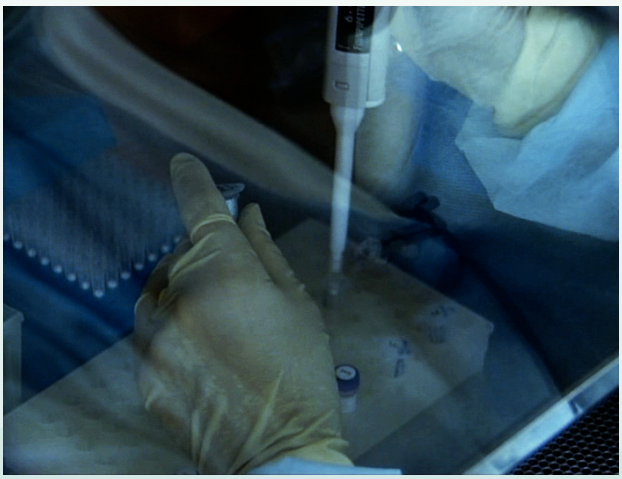
Add 6 microliters of reverse primer that has been diluted to 20 picomoles per microliter.



Add 6 microliters of Promega® RNase Inhibitor at a concentration of 13.3 units per microliter.



Add 12 microliters of Enzyme Mix.



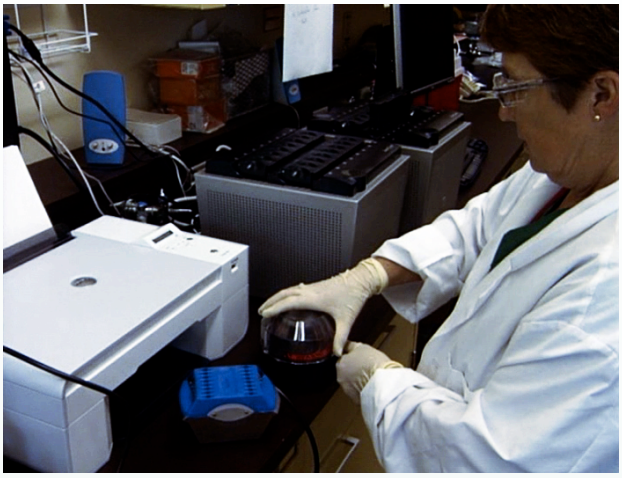
The Probe is light sensitive so you will need to turn off the safety cabinet light before adding the probe to the master mix. Add 6 microliters of the probe that has been diluted to 6 picomoles per microliter. Vortex to ensure uniform mixture, then pulse spin in a microfuge.

To perform the PCR test on the Cephied® Smart Cycler, fill the appropriate number of Smart Cycler tubes with 17 microliters of master mix – remembering to keep the hood light off. In addition to the sample tubes, be certain to include two additional tubes, one for a positive and one for a negative control.

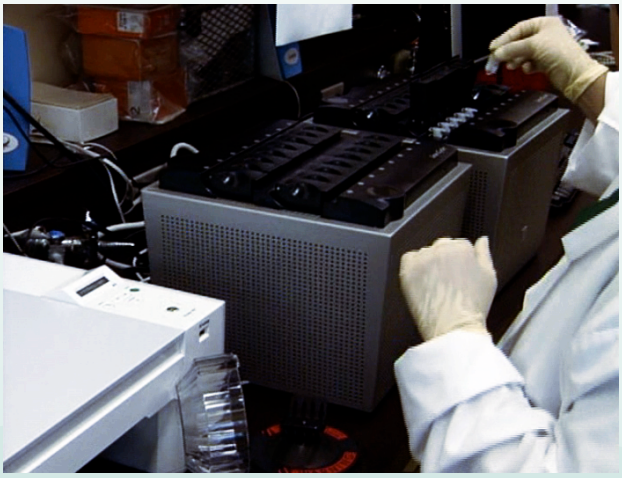
Using sterile pipette tips, add 8 microliters of each RNA sample to a corresponding Smart Cycler tube. Close each tube after adding the RNA. Add 8 microliters of clean water to the negative control tube.

Using a completely different pipette, add 8 microliters of transcribed RNA to the positive AI control tube.

Close and label all tubes.



Before you place the tubes in the Smart Cycler, briefly centrifuge the tubes to remove any air bubbles.



Place the tubes into the Smart Cycler to be tested. Close the Smart Cycler block lids and start the run.