4.8 Indirect Fluorescent Antibody (IFAT) Procedure

(Modified from LaPatra et al., 1989)

The fluorescent antibody test (FAT) is a serological method for confirming the identity of viral isolates. The indirect FAT (IFAT) technique uses pathogen specific monoclonal anti-virus antibody (MAb) as the primary antibody (usually mouse IgM), and fluorescein isothiocyanate (FITC) conjugated antisera (usually goat or rabbit anti-mouse IgG) to the immunoglobulin used for the primary antibody. There are four basic steps for IFAT: preparing and fixing cell monolayers; infecting the cell monolayers with the virus; staining the slides with antibody reagents; reading and interpreting the slides.

A. Preparation of Materials

1. Sterilization

a. Place 12 to 18 mm diameter cover glasses in a heat resistant container and sterilize at 120 ^{o}C for 30 minutes.

b. Include dissecting forceps.

2. Dry completely in drying oven.

3. Prepare the antibody reagents according to the instructions provided by the supplier.

4. Appropriate QA/QC testing should be performed periodically on the antibodies, antisera, and cell lines to ensure accurate results are obtained during this assay.

B. Preparation of Cell Monolayers on Cover Glasses

1. Working in a clean hood, place one cover glass per well in a 24-well plate. At least four cover glasses are made for each of the unknown samples, positive control (reference) viruses, and negative control (uninoculated) cell lines. One cover glass from each of these groups is then stained and examined at approximately 8, 12, 24, and 48 hours as necessary for confirmation. Observing the cultures at multiple time points allows for visualization of the virus at the most appropriate stage of infection of the cell monolayer.

2. Using the cell line specified for the suspect virus, seed each well of the plate with the appropriate volume of that cell suspension as in Section 2, 4.3.B “Seeding Procedures for Plates.”

3. Incubate at 20 to 25^{o}C for 18 to 24 hours or until the cells are 80 to 100% confluent as this

minimizes excessive cell loss during fixation and staining.

C. Virus Sample Inoculation and Incubation

1. Preparation of the Suspect and Known Virus Sample

a. Using a pipette, stir and scrape the bottom of the suspect virus well or reference virus-infected flask (Section 2, 4.7.C.2 "Procedure for Producing Reference Viruses") to be subcultured to dislodge the cell layer.

b. Aspirate the fluid and cell debris from the well or flask and place in a sterile tube for centrifugation.

c. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 X g.

d. Use supernatant from this tube for inoculation of the cover glass cell cultures.

2. Inoculation of the Cover Glass Cell Cultures with the Suspect and Known Virus Samples

a. Aspirate media from each well of the 24-well plate containing a cover glass, leaving a small amount to inhibit cell dehydration.

b. Inoculate 50 to 100 µL of supernatant prepared in "Preparation of the Suspect and Known Virus Sample" (above) from each sample onto four monolayered cover glasses in each of four labeled wells.

c. Inoculate 50 to 100 µL of reference virus onto each of four monolayered cover glasses, one of which will be used at each time period.

d. One negative (uninoculated) control monolayered cover glass is made for each cell line used, one of which will be stained and examined at each time period.

e. Absorb 1 hour without rocking at 15 to 18^{o}C, sample removal is not necessary.

f. Add 1 mL MEM-5 (Section 2, 4.9.F "MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)") or Leibovitz's L-15 (Section 2, 4.9.H "Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines") per well as appropriate for the cell lines being used.

3. Incubate at 15 to 18^{o}C.

D. Cover Glass Fixation and Mounting

1. Working under the hood at room temperature, aspirate media from each well to be examined at that time period (sample plus positive and negative control cover glasses). Disinfect the aspirated fluid prior to disposal.

2. Add 1 mL PBS (Section 2, 3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") to each

well and let stand for five minutes.

3. Aspirate PBS and disinfect prior to disposal.

4. Repeat steps 2 and 3.

5. Add 1 mL cold (4^{o}C) methanol and let stand for five minutes.

6. Leave some or all of the methanol in the well. Tip the plate, without spilling fluid, and use sterile forceps to remove the cover glass from the well. Leaving some fluid in the well helps decrease the adherence of the cover glass to the bottom of the plate.

7. Allow cover glasses to air dry on a labeled absorbent surface such as lab paper.

8. If not stained immediately, store at 4^{o}C for short term (days to weeks) or at -20^{o}C for long term (weeks to months).

E. Staining and Viewing the Cover Glasses

1. Cover fixed cell cultures with 0.1 mL of mouse monoclonal anti-virus antiserum (MAb) specific for the suspect virus and incubate at room temperature for 30 minutes in a dark humidified chamber.

2. Flood the cells twice for five minutes each time with PBS.

3. Filter the goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugated antibodies through a 0.45 µm membrane before use.

4. Cover fixed cell cultures with 0.1 mL of the second antibody (FITC conjugate) and incubate at room temperature for 30 minutes in a dark humidified chamber.

5. Flood the cells twice for five minutes each time with PBS.

6. Counter stain with a 0.01% solution of Evans blue (Section 2, 3.7.D.5.b “Evans Blue”) for one minute, rinse with PBS and air dry in the dark.

7. Place a drop of pH 9 fluorescent antibody (FA) mounting fluid (Section 2, 3.7.D.2 “FAT Mounting Fluid (pH 9.0)”) on the cells and place the cover glass cells down on a clean microscope slide.

8. Examine at 400 to 1000x magnification on a microscope equipped with epifluorescent illumination.

F. Results

1. Positive controls should contain focal clusters of cells exhibiting apple green cytoplasmic fluorescence (see Figure 4.17); no fluorescence should be observed in the negative controls.

2. With no problems identified in the assay and with fluorescence of the suspect sample which appears similar to the positive control at one of the time points, the suspect sample is considered **POSITIVE** for the virus specific to those antibodies.

3. With no problems identified in the assay but without appropriate fluorescence at any of the four time periods, the suspect sample is considered **NEGATIVE** for the virus specific to those antibodies.

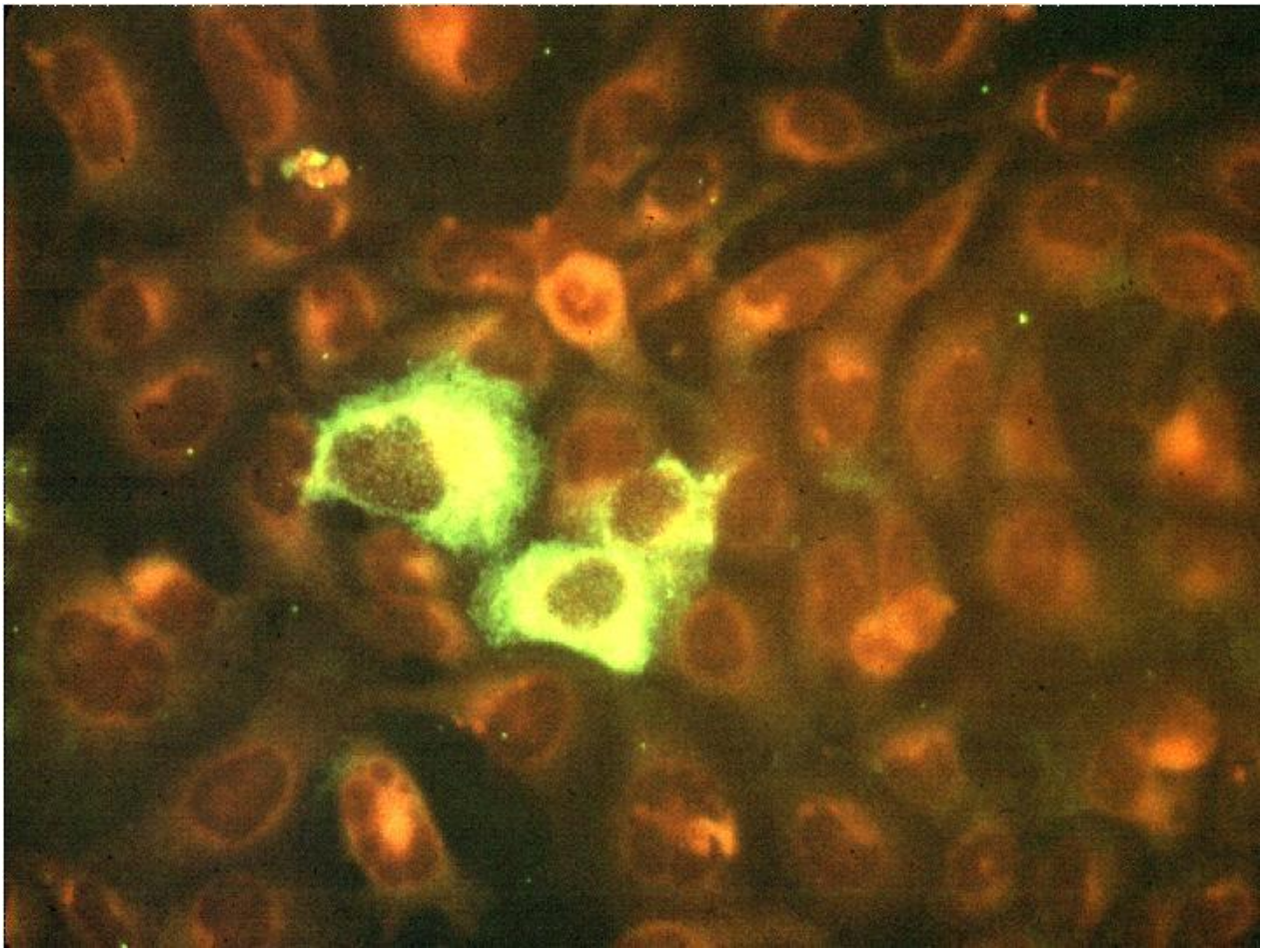

Figure 4.17. Typical fluorescence of positive IFAT for IHNV on EPC cell monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.