

D³ ULTRA DUET, A NOVEL METHOD FOR DIRECT SPECIMEN TESTING OF RESPIRATORY VIRUSES

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Amended Abstract

Introduction: Diagnostic Hybrids, (DHI), has developed novel reagents and procedures for the qualitative detection of eight respiratory viruses: Influenza A and B, RSV, Adenovirus, MPV, and Parainfluenza 1, 2 and 3 viruses. The D³ Ultra Duet uses a dual fluor labeling strategy in a liquid format that can detect each of these eight viruses in less than 10 minutes after obtaining the cell pellet from a nasal aspirate or swab specimen.

The **standard procedure** for preparing a specimen for DFA (direct fluorescence antibody) staining is to dry a cell suspension onto a glass slide and fix with acetone. These steps are followed by staining with antibodies directly labeled with specific fluors, such as FITC, for 15-30 minutes at 35 – 37°C. After a wash step, a coverslip is placed over the cells and they are examined by fluorescence microscopy. There are several aspects of the DFA technique that are suboptimal. The procedure can take up to 2 hours, primarily because of the time it takes to dry the cells onto the slide. Also, if the slide is not completely dried, the cells can be lost during subsequent processing steps. This can result in an inadequate number of cells to enable detection of the virus.

The **D³ Ultra Duet procedure** uses a proprietary reagent to permeabilize the cells instead of fixing them with acetone. The procedure permeabilizes and stains the cells in one step, compared to the two steps of DFA. Permeabilization dissolves portions of the cell membranes and allows large dye molecules and antibodies access into the cells. The permeabilized cells maintain their three dimensional structure while being stained with counter stain and labeled antibodies. The procedure is done in liquid suspension and because there is no drying step, only a 5 minute 35 – 37°C incubation time is required. After incubation, the cells are rinsed, centrifuged for 2 minutes and loaded onto a proprietary slide for examination. The D³ Ultra Duet assay utilizes a combination of 2 different fluors, Phycoerythrin (PE) and Fluorescein Isothiocyanate (FITC) and contains three, pair-wise sets of fluor-Mab combinations: 1) PE to Flu A Mab and FITC to Flu B Mab; 2) PE to RSV Mab and FITC to MPV Mab; and 3) PE to a pool of PV-1, -2 and -3 Mabs and FITC to Adenovirus Mab. Thus, detection of 8 respiratory viruses can be carried out in just 3 wells compared to the standard DFA that requires 8 different wells.

Study Objectives

Compare the clinical sensitivity and specificity of D³ ULTRA DUET to:

- D³ Ultra Respiratory Virus Screening and ID Kit and MPV ID Kit for direct specimen testing.

- Culture using the DHI D³ respiratory virus screening and ID Kit and the DHI D³ MPV ID Kit for virus detection.

- Point-of-Care (POC) Flu A/B and RSV rapid cartridge tests* for direct specimen testing.

* **POC Cartridge tests** = BinaxNOW® Influenza A & B Test and BinaxNOW® RSV Test.

NOTE: All POC cartridge testing was performed at the Holzer Clinics. Specimens were tested with the POC tests based on requests from the physician. DHI received results upon request.

Methods

Specimens

647 fresh, prospective respiratory specimens were collected using Mattress and Flocked Swabs at the Holzer network of regional Ohio clinics and tested at DHI within 48 hours of collection.

Specimen Processing Procedure

- 2mL of specimen in transport medium were centrifuged for 10 minutes at 1000xg to pellet cells.
- Supernatant was removed and saved for cell culture.
- Cell pellet was washed by re-suspending in 3mL of PBS.
- Cells were centrifuged for 10 minutes at 1000xg to re-pellet cells.
- Supernatant was removed and ~500uL of PBS were added to re-suspend the cell pellet for testing by D³ Ultra Duet method and the Standard Direct Specimen method.

D³ Ultra Duet Procedure

Starting from the cell suspension in step #5 above

- Label one each of the blue (Flu A/Flu B), orange (RSV/MPV) and yellow (PIV Pool/Adeno) 1.7-mL Centrifuge vials with the patient ID.
- Using a transfer pipette, transfer 3 drops (~70-µL) of the cell suspension to each of the three labeled vials from step 1 above.
- Add 2 drops of the corresponding D³ Ultra Duet Antibody Reagent to the labeled vials. Place vials in a 35°C -37°C water bath or incubator for 5 minutes.
- After incubation, using a squeeze bottle or transfer pipette, add approximately 1.5-ml of PBS Buffer to each vial.
- Centrifuge the vials for 2 minutes @ 2000xg.
- Gently decant supernatant into a waste container and blot the vial on adsorbent paper to remove excess liquid.
- To each vial, add 1 drop of re-suspension buffer and break up the cell pellet and mucus by pipetting up and down 5-10 times. Add 20-µL to each well, in the order specified below, changing the tip after each addition. Apply a coverslip to each well and examine at 200x magnification using a fluorescence microscope. Read time to confirm a negative result averages between 1 and 2 minutes per well or up to 3 to 6 minutes/slide.

Testing Order: Well 1 = Flu A/Flu B

Well 2 = RSV/MPV

Well 3 = PIV Pool/Adenovirus

Direct Specimen Procedure

Starting from the cell suspension in step #5 above

- Label one 8-well slide for each specimen.
- Transfer 1 drop (~20-µL) using a transfer pipette of the cell suspension to each of the 8 wells.
- Let the slide air dry for at least 60 minutes.
- Add one drop of each of the D³ Ultra individual MABs and one drop of the MPV MAB to each well.
- Incubate in a 35°C -37°C incubator for 15 minutes
- Gently wash off MAB reagent from slides with PBS.
- Add one drop of Mounting Medium to each well and place a glass cover slip over the wells.
- Examine at 200x magnification using a fluorescence microscope. Read time to confirm a negative result averages between 1 and 2 minutes per well or up to 8 to 16 minutes/slide.

Testing Order: Well 1 = Adenovirus

Well 2 = MPV

Well 3 = Flu A

Well 4 = Flu B

Well 5 = Para 1

Well 6 = Para 2

Well 7 = Para 3

Well 8 = RSV

Culture Methods:

- R-Mix Too cultures were re-fed with 1mL of RM03T.
- Cultures were inoculated in triplicate with 200uL of specimen.
- Cultures were centrifuged for 1 hour at 700xg.
- Cultures were incubated for 48 hours in a 35°C -37°C incubator.
- At 48 hours, 1 monolayer was processed using the D³ Ultra Respiratory Screen reagent and 1 monolayer was processed using the D³ MPV reagent. If the specimen was positive, the third monolayer was scraped and dotted onto an 8-well slide for virus identification using the D³ Ultra Respiratory ID reagents.

Results

Of the 647 samples, 293 were positive by both DFA and D³ Ultra Duet for a respiratory virus. There was 99.3% agreement between the two methods in identifying 46 Flu A, 197 Flu B, 29 RSV, 15 MPV, 1 Adenovirus and 6 Parainfluenza.

2 specimens had dual infections.

Discrepant specimen were either Flu A or B.

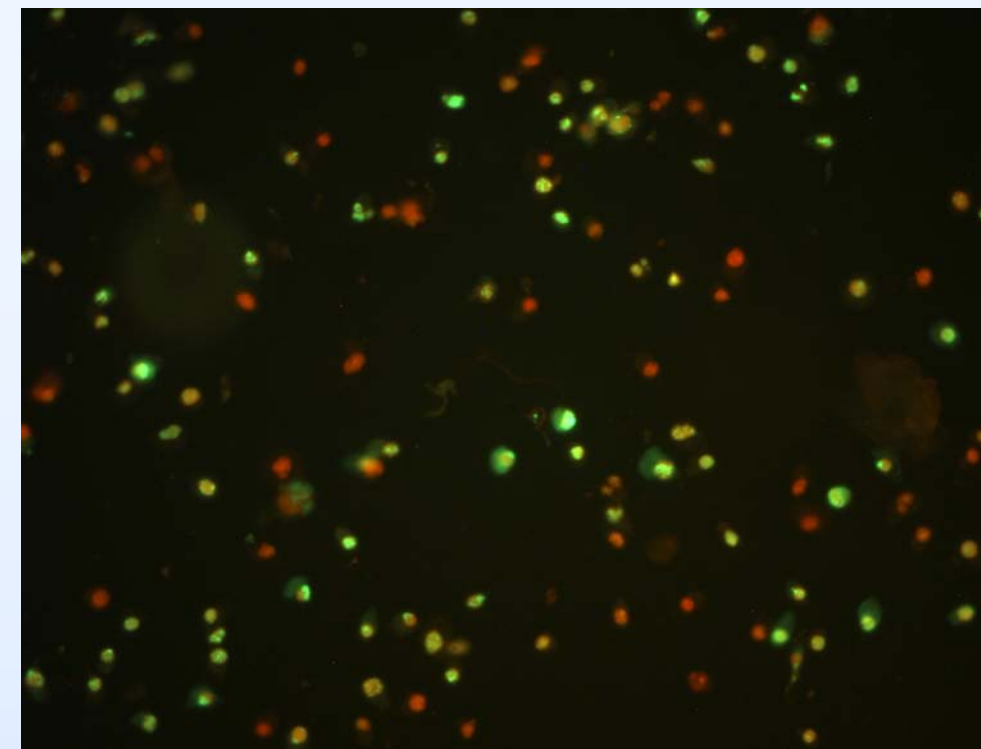
10 samples were QNS.

The Table below summarizes the study results for the D³ Ultra Duet compared to DFA, Culture and POC cartridge tests. The discrepant results of the Ultra Duet compared to the POC tests are discussed in the summary text block to the right.

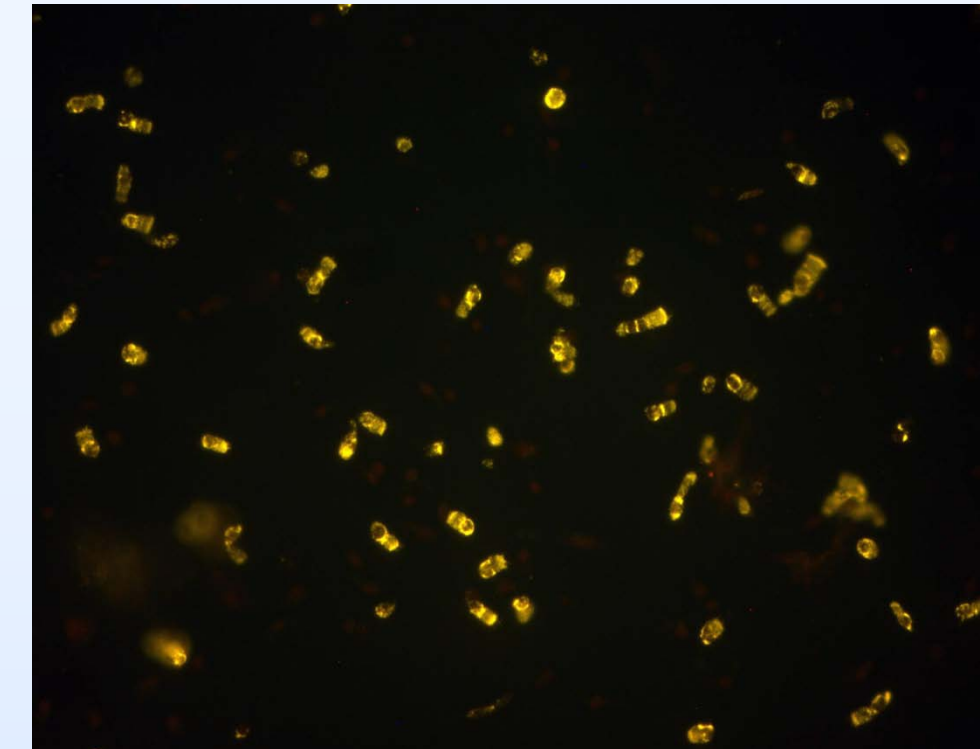
Virus	Ultra Duet compared to DFA		Ultra Duet compared to Culture		Ultra Duet compared to POC	
	PPA	NPA	Sensitivity	Specificity	PPA	NPA
Influenza A	97.6% (46/47)	99.7% (588/590)	82.9% (34/41)	99.0% (494/499)	46.9% (38/81)	96.8% (288/298)
Influenza B	99.5% (197/198)	99.1% (435/439)	86.5% (166/192)	98.3% (342/348)	95.4% (166/174)	89.2% (262/298)
RSV	100.0% (29/29)	100.0% (608/608)	100.0% (11/11)	97.7% (517/529)	80.0% (12/15)	94.9% (282/298)
MPV	100.0% (15/15)	100.0% (622/622)	100.0% (6/6)	98.5% (526/534)	N/A	N/A
Parainfluenza	100.0% (6/6)	100.0% (631/631)	83.3% (5/6)	100% (534/534)	N/A	N/A
Adenovirus	100.0% (1/1)	100.0% (636/636)	100.0% (1/1)	100.0% (539/539)	N/A	N/A

D ³ Ultra Duet	DFA	
	+	-
	+	293
-	2	336

The picture below shows an Influenza B positive clinical specimen. Bright FITC nuclear and cytoplasmic staining occurs in the positive cells, while the negative cell's nuclei fluoresce red.



The picture below shows a Parainfluenza positive clinical specimen. Bright PE cytoplasmic staining occurs in the positive cells, while the negative cell's nuclei fluoresce red.



Summary

- The D³ Ultra Duet method is as sensitive as the standard direct specimen testing method.
- Once the specimen is prepared, time to results for 8 viruses for D³ Ultra Duet is 9 minutes. Time to results for a standard DFA is at least 60 minutes.
- For the 8 major respiratory viruses, D³ Ultra Duet requires only 3 wells to be examined, compared to 8-10 for the standard DFA.

Conclusion: The D³ Ultra Duet provides a rapid alternative to traditional DFA with equivalent sensitivity and specificity. With rapid turn around time, greater sensitivity and specificity, and a menu of 8 viruses, the D³ Ultra Duet is an alternative testing method for virology and microbiology labs that currently use rapid cartridge devices.

Summary of D³ Ultra Duet compared to POC Tests

- Influenza A**
 - 38 specimens were Flu A positive by both methods and confirmed by DFA and/or culture.
 - 43 false positive specimens by POC**
 - 24/43 confirmed negative by culture and DFA.
 - 3 confirmed Flu B, 1 confirmed Adenovirus, 1 confirmed RSV by culture
 - 14/43 were POC Flu A and Flu B positive: 12 confirmed Flu B only by culture. 2 were culture and DFA negative.
 - 10 “false” positive specimens by D³ Ultra Duet**
 - 10/10 confirmed by culture or DFA.
- Influenza B**
 - 166 specimens were Flu B positive by both methods and confirmed by DFA and/or culture.
 - 8 false positive specimens by POC**
 - 4 culture and DFA negative
 - 1 MPV positive by D³ Ultra Duet and culture and 1 Flu A positive by D³ Ultra Duet and culture.
 - 2 confirmed Flu B by culture (DFA & Ultra Duet negative)
 - 36 “false” positive specimens by D³ Ultra Duet**
 - 35/36 confirmed Flu B by DFA and culture
 - 2 were POC RSV positive, 1 was POC Flu A positive
- RSV**
 - 12 specimens were RSV positive by both methods and confirmed by DFA and/or culture.
 - 3 false positive specimens by POC**
 - 1 was Flu B by culture, 2 were negative by DFA.
 - 16 “false” positive specimens by D³ Ultra Duet**
 - 14 were tested only for Influenza A/B
 - 1 was POC RSV negative, 1 was POC Flu A positive