**UW Medicine - Pathology**

400-04-01-10

Interphase Fluorescence in Situ Hybridization (IFISH) for Bladder Cancer Recurrence Using the Vysis Urovysion Kit

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| Adopted Date: 03/16/04  Review Date: 06/12/09  Revision Date: 04/16/13 |

PURPOSE

The Vysis UroVysion Bladder Cancer Recurrence Kit is designed to detect aneuploidy aneusomy for chromosomes 3, 7, 17, and loss of the 9p21 locus via fluorescence in situ hybridization (FISH) in urine specimens from subjects with transitional cell carcinoma of the bladder. Results from the UroVysion Kit are intended for use as a noninvasive method for monitoring for tumor recurrence in conjunction with cystoscopy in patients previously diagnosed with bladder cancer. This is an FDA approved assay.

PROCEDURE

### Specimen Requirements

* + - 1. The UroVysion Kit is designed for use on voided urine specimens. Perform urine collection (40-100 ml) at the physician’s office. Mix voided urine 2:1 (v:v) with Carbowax (2% polyethylene glycol in 50% ethanol) or other 50% ethanol-based preservative (Cytolyte is OK) and transfer to a 50-ml centrifuge tube(s) or other tightly capped plastic container. If urine is not shipped immediately after collection, refrigerate immediately and ship via overnight courier within 24 hr.
      2. The recommended storage and shipping conditions are on ice packs. Urine stored in Carbowax has been shown to be stable for 1 week; however, it is recommended that specimens be processed to the point of fixed cell pellets (see below, PROCEDURE: Sample Processing) within 72 hr of collection. Under no circumstances should urine specimens be stored or shipped at temperatures at or above 37°C.
      3. A normal control specimen is obtained and processed identically to the patient samples.

### Materials and Equipment

* + - 1. UroVysion kit (Cat# 30-161070), which includes
         1. Vysis UroVysion DNA Probe Mixture

Vysis P.N.: 30-171070 (20 Test); 36-171070 (100 Test)

Quantity: 60 µl (20 Test); 300 µl (100 Test)

Storage: -20°C in the dark

Composition: Fluorophore-labeled DNA probes for chromosomes 3, 7, and 17, and locus 9p21 in hybridization buffer. The hybridization buffer is made up of dextran sulfate, formamide and SSC.

* + - * 1. DAPI II Counterstain

Quantity: 300 µl (20 Test); 1000 µl (100 Test)

Storage: -20°C in the dark.

Composition: 125 ng/ml DAPI (4,6-diamidino-2-phenylindole) in 1,4-phenylenediamine, glycerol, and buffer

* + - * 1. NP-40 (Detergent)

Quantity: 4 ml (2 x 2 ml)

Storage: -20°C to 25°C.

Composition: NP-40 (non-ionic detergent)

* + - * 1. 20X SSC salts

Quantity: 66 g for making 250 ml of 20X SSC solution

Storage of salts: -25°C to 30°C

Composition: sodium chloride and sodium citrate

* + - 1. Materials NOT included in UroVysion kit
         1. Pepsin (2500-3500 units/mg) (Sigma P6887)
         2. 6 N HCl (Sigma-Aldrich)
         3. Phosphate Buffered Saline 1X (Gibco 14190-136)
         4. Magnesium Chloride (MgCl2) (Sigma-Aldrich M2670), 100 g
         5. 10% neutral buffered formalin (Fisher Scientific SF100-4)
         6. Slides (Allegiance M6177), w/ 10 mm circle
         7. Coverslips (VWR) 12 mm 1 oz round

### Reagents and Solutions

* + - 1. Preparation of Working Reagents

a. **20X SSC** (3 M sodium chloride, 0.3 M sodium citrate, pH 5.3)

To prepare 20X SSC pH 5.3, add together:

66 g 20X SSC

200 ml purified H2O

250 ml final volume

Mix thoroughly. Measure pH at room temperature with a pH meter. Adjust pH to 5.3 with concentrated HCl. Bring the total volume to 250 ml with purified H2O. Filter through a 0.45 µm pore filtration unit. Store at room temperature for up to 6 mo.

1. **Pepsin Buffer**
   1. Add 2ml of 6N HCl to 998ml Sterile water (Baxter Travenol). Store in plastic water bottle at room temp (Expires after 1 year.)
      1. **Pepsin Wash** (0.5 mg/ml Pepsin pH 1.0-2.0)
   2. weigh 25 mg Pepsin and add to coplin jar.
   3. Add 50 ml Pepsin buffer (see b. above).
   4. Place coplin jar in 37°C waterbath.
   5. Discard solution after using 1 day.
      1. **2 M Magnesium Chloride (100X)**
2. Mix together 4.07 g MgCl2 in 10 ml of DD-H2O in a 15 ml Falcon centrifuge tube.
3. Store refrigerated. Good for 1 year.
   1. **1% Formaldehyde Wash**
      1. Mix in a Coplin jar 12.5 ml of 10% neutral buffered formalin, 37 ml of 1X PBS, and 0.5 ml of 100X MgCl2.
      2. Use at room temperature. Discard after using 1 year. Store at 2-8°C when not in use.
         1. **Ethanol Washing Solutions**
4. Prepare v/v dilutions of 70%, 80%, and 95% using 100% ethanol and purified H2O.
5. Dilutions may be used for one week unless evaporation occurs or the solution becomes diluted due to excessive use.
6. Store at room temperature in tightly capped containers when not in use.
   1. **0.4X SSC/0.3% NP-40**
      1. To prepare, add together:
         * 20 ml 20X SSC, pH 5.3
         * 877 ml Purified H2O
         * 3 ml NP-40
         * 1000 ml Final Volume
           1. Mix thoroughly. Measure pH at room temperature using a pH meter. Adjust pH to 7.5 ± 0.2 with 1 N NaOH.
           2. Adjust volume to 1 liter with purified H2O.
           3. Store solution at room temperature for up to 6 mo.

**2X SSC/0.1% NP-40**

* 1. To prepare, add together:
     + 100 ml 20X SSC, pH 5.3
     + 849 ml Purified H2O
     + 1 ml NP-40
     + 1000 ml Final Volume
  2. Mix thoroughly. Measure pH at room temperature using a pH meter. Adjust pH to 7.0 ± 0.2 with 1N NaOH.
  3. Adjust volume to 1 liter with purified H2O. Mix thoroughly.
  4. Store solution at room temperature for up to 6 months.

### Procedure

* + - 1. Sample Processing

1. Accession as a Neoplasia FISH (NF). Centrifuge urine in a 50 ml centrifuge tube at 1800 rpm (600 g) for 10 min at room temperature.
2. Remove the supernatant to within approximately 1–2 ml of the cell pellet, being careful not to disturb the pellet.
3. Re-suspend the pellet in the remaining supernatant and transfer the contents to a 15 ml conical centrifuge tube. If cell pellet is large and/or chalky, rinse the 50 ml tube with 10 ml of 1X PBS and transfer the contents to the 15 ml tube. If not, skip to step f.

***Note****:* Pellets from the same patient specimen may be combined.

1. Centrifuge sample(s) at 1800 rpm (600g) for 10 min at room temperature.
2. Remove the supernatant to within approximately 0.5 ml of the cell pellet.
3. Re-suspend pellet in the remaining 0.5 ml of supernatant. Slowly add 1-5 ml of fresh fixative (3:1, methanol:acetic acid), dropwise at first, with frequent agitation. Add fix to make total volume 10ml.
4. Let fixed specimens stand at –20°C for a minimum of 30 min.

***Note****:* Specimens may be stored overnight or longer (up to 10 days) at this step.

1. Centrifuge sample(s) at 1800 rpm (600 g) for 5 min at room temperature. Carefully remove the supernatant.

***Note****:* If pellet is not visible or barely visible, further washing of the pellet is not recommended in order to avoid cell loss. Instead, proceed to stepk***,*** below. If sample has been stored overnight or longer, resuspend in fresh fixative prior to slide preparation.

1. Wash pellet by re-suspending in 1-5 ml fixative.
2. Centrifuge sample(s) at 1800 rpm (600 g) for 5 min at room temperature. Repeat above steps **h and i** twice.
3. After centrifugation of cell suspension in fixative: If cell pellet is very small and hardly visible, CAREFULLY remove as much fixative as possible, leaving approximately 100 µl solution. If cell pellet is easily visible, remove as much fixative as possible and add 0.5-1 ml fresh fixative to the cell pellet.
4. Proceed immediately with the slide preparation procedure.
   1. Slide Preparation (For both patient and control)

***Note:*** To save time, place full coplin jars in water baths: Pepsin wash at 37°C and 2X SSC at 73°C.

1. Use 10 mm circle slides. Re-suspend the cell pellet and make one slide and determine if cell density is sufficient.
2. Alternatively, apply 3 µl, 10 µl and 30 µl of cell suspension on three slide circles (circle #1, 2, and 3). And allow samples to air dry. Examine slide under a light microscope using a 10X phase objective. Select the hybridization area (circle #1, 2 or 3) in which ~50-100 cells are visible in the field. If cell density is too high, dilute the cell suspension sample with fixative and redrop slide. ***Note****:* If an excessive amount of debris is present, follow pretreatment procedure and then select hybridization area.
   1. Slide Pretreatment
      1. Allow slide(s) to completely dry at room temperature.
      2. Immerse slide(s) in 2X SSC for 2 min (2-2.5 min.) at 73±1°C.
      3. Immerse slide(s) in Pepsin Wash for 12 min (±2 min) at 37±1°C.
      4. Wash slide(s) in 1X PBS for 5 min (±1 min) at room temperature.
      5. Fix slides in 1% Formaldehyde wash for 5 min (±1 min) at room temperature.
      6. Wash slides in 1X PBS for 5 min (±1 min) at room temperature.
      7. Dehydrate slide(s) by immersing in 70% ethanol solution at room temperature. Allow the slide(s) to stand in the ethanol wash for at least 1 min. Repeat ethanol series, followed by 100% ethanol.
      8. Dry slides at 25°C (air drying station) for 3 min or until completely dry.
   2. Hybridization
3. Remove UroVysion probe from freezer and thaw
4. Turn on Thermobrite and soak pads underneath the lid if slides will be on for more than 20 minutes, set to program #1 (73°C, 39°C) and fill two troughs with distilled H2O from a squeeze bottle.
5. Place slide(s) on Thermobrite surface, apply 3 µl of probe to each target, followed by a clean 12 mm round coverslip. Avoid air bubbles.
6. Using a syringe without needle, apply paper cement to seal all edges of the coverslip. Close Thermobrite lid and run program #1. Move slides to 37C incubator
7. Following hybridization, wash slides by removing the coverslips and placing up to 4 slides at one time into a 73ºC Coplin jar of 0.4X SSC + 0.3% NP-40 for 2 min.
8. Move slides from hot wash solution into a room temp Coplin jar of 2X SSC + 0.1% NP-40 for 0.5-2 min.
9. Dry (protected from light). Apply 10 µl of DAPI II counter stain to each target area with a pipetter, and cover with a 12-mm coverslip.

### Scoring and Interpretation

1. If morphologically abnormal cells\* (cells suspicious for malignancy) are present use the scanning method below:
   1. The slide is scanned for 25 informative, morphologically abnormal\* cells and the FISH signal pattern in those cells recorded.

If any of the following conditions are seen:

* + 1. Four or more cells are seen with 2 or more hypersomic signals per cell (CEP 3, 7, 17) [polysomy]..2
    2. Twelve or more cells with no 9 signals.\*\* (LSI 9p21) [homozygous loss].1,2

The case is considered positive and the analysis is complete. The normal (negative) control is scanned for 25 consecutive, informative cells regardless of morphology. Note: A positive control (Vysis kit 30-80570 Probe Check) is run once with each new probe lot to validate lot. If, after scanning, the 25 cells have fewer than the above numbers of abnormal cells then at least 75 more cells are counted or sample is exhausted. Results at or near the cut off should be interpreted with caution and should be repeated if indicated by faculty. Uninformative IFISH should be repeated.

\* Morphologically abnormal cells are defined in comparison with the predominant cell type in the control sample by one or more of the following parameters: Larger size, irregular shape, cell clusters and a mottled or “patchy” DAPI staining pattern. See Vysis kit instructions. \*\* Assuming adequate hybridization of LSI 9p21 signals are seen in other cells in both the patient and the control.

1. Notes:
2. Use the counting guides in the Vysis kit instructions for signal scoring guidelines.
3. Accounting for Tetraploid cells: If a cell has four of each signal, the cell is considered uninformative.

REFERENCES

* 1. Sokolova IA, Halling KC, Jenkins RB, Burkhardt HM, Meyer RG, Seelig SA, King W. The development of a multitarget, multicolor fluorescence in situ hybridization assay for the detection of urothelial carcinoma in urine. J Mol Diagn 2(3):116-123, 2000.
  2. Vysis UroVysion Bladder Cancer Recurrence Kit Protocol 30-608385 Revision F 3/2002.
  3. Bubendorf L, Grilli B, Sauter G, Mihatsch MJ, Gasser TC, Dalquen P. Multiprobe FISH for enhanced detection of bladder cancer in voided urine specimens and bladder washings. Am J Clin Pathol. 116(1):79-86, 2001.

Written By: Director Approval:

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**SIGNATURE PAGE FOR POLICIES AND PROCEDURES**

Procedure / Policy Title: Interphase Fluorescence in Situ Hybridization (IFISH) for Bladder Cancer Recurrence Using the Vysis Urovysion Kit

Procedure / Policy Number: 400-04-01-10

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