**UW Medicine - Pathology**

400-04-01-11

Interphase Fluorescence in Situ Hybridization (IFISH) Protocol for Formalin-Fixed, Paraffin-Embedded Tissue Sections

|  |
| --- |
| Adopted Date: 09/02/04Review Date: 06/12/09Revision Date: 04/16/2013 |

PURPOSE

To analyze paraffin-embedded formalin-fixed tissue sections by IFISH for clinical diagnostic analysis. This protocol works on a variety of tissue types using a variety of IFISH probes.

PROCEDURE

### Specimen Requirements

The specimen is formalin-fixed tissues embedded in paraffin blocks. If required, a control block should be sent for each patient of interest. Controls should be a recent tissue type match, with similar fixation time as the test specimen.

### Materials and Equipment

* + - 1. Water baths
			2. HYBrite warming plate (Vysis)
			3. Slide warmer
			4. 37ºC incubator
			5. Moist chamber

### Reagents and Solutions

* + - 1. Reagents:
				1. Hydrochloric Acid, concentrated (HCl, approx. 37%)
				2. Sterile distilled H2O (Pharmacy services)
				3. 20X SSC (Vysis, Inc. Cat# 30-805850)
				4. NP-40 (Vysis, Inc. Cat # 30-804820)
				5. Sodium Hydroxide
				6. Sodium thiocyanate (NaSCN) (Sigma, cat# 57757)
				7. Sodium Chloride
				8. 200 Proof Ethyl Alcohol
				9. Xylene substitute (Shandon, Cat. #9990505)
				10. DAPI II

2. Preparation of Solutions:

**0.2 N HCl**

Purpose: To extract nuclei and extra-cellular matrix proteins and dissolve histones.

Procedure: Add 17.2 ml concentrated HCl to sterile distilled H2O and adjust to 1 L. Mix well.

Store at room temperature. Shelf life is 1 year.

**20X SSC, pH 5.3 (3.0 M sodium chloride, 0.3 M sodium citrate)**

Purpose: Used in preparation of post-hybridization buffer.

Procedure: Add 66.0 g of 20X SSC to sterile distilled H2O and adjust to 250 ml. Mix well. Adjust pH to 5.3 using concentrated HCl.

Storage at room temperature. Shelf life is 6 mo.

**Post Hybridization Buffer (2X SSC/ 0.3% NP-40 pH 7.0-7.5)**

Purpose: Used to wash excess non-hybridized probe off of sample, leaving behind the hybridized probe attached to the targeted region.

Procedure: Add 3.0 ml of NP-40 to 100 ml of 20X SSC (pH 5.3) and adjust to 1 L with sterile distilled H2O. Mix well. Adjust pH to 7.0-7.5 using NaOH (10 N).

Storage at room temperature. Shelf life is 6 mo.

**Pretreatment Solution (1.0 M Na Thiocyanate)**

Purpose: Increases cell permeability to facilitate subsequent enzyme digestion.

Procedure: Add 81.7 g Na Thiocyanate to sterile distilled H2O and adjust to 1 L. Mix well.

Store at room temperature. Shelf life is 1 year.

**Protease Solution Buffer**

Purpose: Used to dissolve pepsin for protein digestion.

Procedure: Add 9.0 g NaCl to 1.0 L sterile distilled H2O. Mix well. Adjust pH to 2.0 using concentrated HCl.

Store at room temperature. Shelf life is 1 year.

**2X SSC wash buffer**

Purpose: Used during procedure to wash off excess buffers and solutions.

Procedure: Make 10% NaOH by adding 1 ml of NaOH to 9 ml sterile H2O. Mix well. Add 100 ml 20X SSC, pH 5.3 to 900 ml sterile distilled H2O. Mix well. Adjust pH to 7.0 using 10% NaOH.

Store at room temperature. Shelf life is 6 mo.

### Procedure

* + - 1. Upon receiving slides, have Dr. Tom Norwood (or other available pathologist) look at the H&E slide and show you where on the section you will want to focus IFISH scoring. Write down any vital information, or mark on the H&E slide where you should be scoring the FISH probes.
			2. Place 50 ml of pretreatment solution (1.0 Sodium Thiocyanate) in Coplin jar and heat to 85ºC.
			3. Place 50 ml of protease solution with 25 mg of pepsin in a Coplin jar at 37ºC.
			4. Turn slide warmer on and allow it to heat to 45-50ºC.
			5. De-paraffinize your slides by following the below steps:
1. Immerse slides in 3 changes of Xylene substitute for 10 min each.
2. Dehydrate slides in 2 changes of 100% alcohol for 5 min each.
3. Place slides on 45ºC slide warmer for 2-5 min.
	1. Pre-treat slides by following the below steps:
4. Immerse slides in 0.2 N HCl for 20 min.
5. Immerse slides in sterile H2O for 3 min.
6. Immerse slides in 2X SSC wash buffer for 3 min.
7. Immerse slides in 1.0 M Sodium Thiocyanate (pretreatment solution) at 80ºC for 20 min.
8. Immerse slides in sterile H2O for 1-3 min.
9. Immerse slides in 2X SSC wash buffer for 5 min.
10. Wash one more time in fresh Coplin jar of 2X SSC for 5 min.
11. Remove excess buffer by blotting edges of slide on paper towel.
12. Immerse slides in Protease solution at 37ºC for 20 min.
13. Wash slides in 2X SSC twice for 5 min each.
14. Dry slides on a 45ºC slide warmer for 2-5 min.

***Note:* It is not necessary to dehydrate your slides in 85%, 90%, and 100% EtOH in this procedure**!!

* 1. Place slides on pre-warmed Thermobrite at 37ºC.
	2. Add 10 µl of appropriate probe mixture, coverslip, and seal coverslip with paper cement.
	3. Run the program on the Thermobrite that is set for **73**º**C for 5 min**.
	4. Once the Thermobrite has completed with the 73ºC denaturation time, the slides can either remain on the Thermobrite overnight (be sure the strips are moist!), or they can be placed in a moist chamber in the 37ºC oven.

**Day 2:**

* 1. After overnight hybridization at 37ºC, remove slides from either the Thermobrite or moist chamber.
	2. Carefully remove the rubber cement using a watchmaker's forceps and soak the slides in 2X SSC/0.3% NP-40 pH 7.0-7.5 (post-hybridization buffer) at room temperature until the coverslip floats off the slide, if necessary.
	3. Place slides in 2X SSC/0.3% NP-40 at 73ºC for 2.5 min. Agitate slides when placing them in the Coplin jar.
	4. Place slides in post hybridization buffer 2xSSC/0.3% NP40 at room temp for 1 min. (optional)
	5. Remove slides from post-hybridization buffer, air dry
	6. Add 10 µl of DAPI II. Alternatively, DAPI I (for brighter staining of the nucleus), or DAPI III (for dimmer staining of the nucleus) may be used.
	7. Use appropriate scoring rules for the probe set used. **Be sure to analyze the area of the slide that was identified by the pathologist!** Two people should score 100 cells each for each block that was run.

**E. Analyzing**

***Signal Enumeration:***

1. Microscope: An epi-illumiation fluorescence microscope is required. The recommended filter sets are the FITC, Rhodamine, Texas red and DAPI filter. All of the other DNA will fluoresce blue with the DAPI stain.

*Note:*  a double filter which allows enumeration of both the orange and green signal can be used.

1. Assessing slide adequacy: Evaluate slide adequacy using all three filters and the following criteria:
	* The probe signal intensity should be bright, distinct and easily evaluable. Signals should be in either bright, compact, oval shapes or stringy, diffuse, oval shapes.
	* The background should be dark or black and relatively free of fluorescence particles or haziness.
2. Recognition of target signals: use the prescribed filter. Adjust the depth of the focus and become familiar with the size and shape of the target signals and debris. Enumerate the signals only among tumor cells. Tumor cells in general are larger than normal cells, lymphocytes, and epithelial cells. Only enumerate tumor cells within the etched area of infiltrating tumor. Skip those nuclei with signals that require subjective judgment.
	* Skip signals with weak intensity and non-specificity, or with noisy background.
	* Skip nuclei with insufficient counterstain to determine the nuclear border.
	* Enumerate only those nuclei with discrete signals.
	* Skip those nuclei that the nuclear borders are ambiguous.
	* Skip those nuclei with signals that require subjective judgment.
3. Signal Enumeration: Use the 40x objective with the DAPI filter. Scan several areas of tumor cells to account for possible heterogeneity. Select an area of good nuclei distribution. Avoid area of hybridization with weak signals. Use the 100x objective (oil immersion lens) and begin analysis in the upper left quadrant of the selected area. While scanning from left to right, and alternating filters from green to orange, count the number of signals within the nuclear boundary of each evaluable cell according to the guidelines provided below
	* Focus up and down to find all the signals present in the nucleus.
	* Count two signals as one if the distance between the two signals is less than the diameter of one signal.
	* Do not score nuclei that do not have any signals or with signals of only one color. Score only those nuclei with one or more FISH signals of each color.

***Scoring the Slides:***

*Single Enumeration and Scoring of Slides -*

* If control used, count 50 signals for the positive control, negative control and the patient slide (50-100 cells/reader, 2 readers required)
* Count the number of paired signals and unpaired signals.
* ALK FISH must be greater than the diameter of two signals apart to be recorded as separated.
* The other Break Apart FISH inter-signal distance must be 1/2 the width of the nucleus.
* Enter the results into the respective FISH count worksheet. Import the worksheet into the case in PowerPath or use GCS as appropriate.
* Print a copy of the worksheet and enclose the copy with the H&E slide to the pathologist to sign out the case.

Notes for the ALK probe on Tissue:

 1. The Dako ASR probe requires the Texas red fluorescence microscope filter.

 2. If the Dako probe is positive for an ALK rearrangement the case should automatically be reflexed to use the Abbott Molecular IVD probe.

***Import of FISH count Worksheet in PowerPath:***

Do the Fish count on the case using the appropriate FISH worksheet.

Highlight all the test by using the mouse or the Shift key.

Use "CTRL C" to copy. A border will appear around the highlighted worksheet.

Minimize all the open worksheet.

Open the Paint. (Start 🡪 Program 🡪 Accessories 🡪 Paint). Create a shortcut on the desktop.

With a clean screen in Paint, use "CTRL V". This will paste the worksheet image in the paint screen.

In the paint screen go to "File 🡪 save as 🡪 Desktop 🡪 Shortcut to ICC 🡪 (appropriate name of FISH) Fish 🡪 current year "(appropriate name of FISH) Fish" 🡪 current year "(appropriate name of FISH) Fish cases". Type the case number, use the extension "JPG" and click on Save.

Open the "UW Pathology Imaging Suite" on the desktop. In the screen, click on "UW image import" on top left hand screen.

Type the case number and tab over. One or more images in the left part of the screen will appear.

Click on "Add files" on the top right window. Select the file that was just created in the Fish file.

Under "Type of Image" select the "Document Image".

Click on "Import of Image".

***Related Documentation/worksheets:***

ALK FISH Count Worksheet

Break Apart Probe Summaries

Break Apart FISH probe information:

EWS FISH probe: <http://www.abbottmolecular.com/products/oncology/fish/vysis-ewsr1-break-apart-fish-probe-kit.html>

SYT FISH probe (SS18): <http://www.abbottmolecular.com/products/oncology/fish/vysis-ss18-break-apart-fish-probe-kit.html>

FUS FISH probe: <http://www.abbottmolecular.com/products/oncology/fish/vysis-fus-break-apart-fish-probe-kit.html>

ALK FISH probe: <http://www.abbottmolecular.com/products/oncology/fish/vysis-lsi-alk-dual-color-break-apart-rearrangement-probe.html>

REFERENCES

1. From Immunohistochemistry protocol, Department of Anatomic Pathology UW,

[S:\ICC\Procedures\kFISH for HER 2 neu.doc], adopted September 2, 2003

Written By: Director Approval:

(Signature and Date) (Signature and Date)

­­­­­­­­­­­­­­­­­\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

 Cytogenetics Supervisor

**UW Medicine - Pathology**

 **Cytogenetics - UWMC**

**SIGNATURE PAGE FOR POLICIES AND PROCEDURES**

Procedure / Policy Title: Interphase Fluorescence in Situ Hybridization (IFISH) Protocol for Formalin-Fixed, Paraffin-Embedded Tissue Sections

Procedure / Policy Number: 400-04-01-11

|  |  |  |
| --- | --- | --- |
| **STAFF NAME**: (printed) | **STAFF SIGNATURE** | **DATE REVIEWED** |
| Abrenica, Annabelle |  |  |
| Chen, Xiaoqin |  |  |
| Darrin, Delores |  |  |
| DeHoogh-Grigsby, Debi |  |  |
| Donovan, Chris |  |  |
| Kraus, Jean |  |  |
| Liu, Yuhua |  |  |
| Mak, Wai Ling |  |  |
| McInnis, Donna |  |  |
| Mohapatra, Itu |  |  |
| Morgan, Catherine |  |  |
| Pilger, Carrie |  |  |
| Staley, Rong |  |  |
| Stampalia, Ann |  |  |
| Villiers, Catherine |  |  |
| Vogel, Jared |  |  |
| Wang, Sharon |  |  |
| Whalen, Sara |  |  |
| Zhou, Yang |  |  |
|  |  |  |