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Procedure for Performing Fluorescence *In* Situ Hybridization (FISH) on Slides Enriched for Circulating Tumour Cells (Previously Stained Using Immunofluorescence)

1. MATERIALS, EQUIPMENT AND FORM

Reagents& Solutions:	**Equipment:**	**Supplies:**
100% EtOH	Water bath (75^{0}C and 37^{0}C)	Thermometer
85% EtOH	Coplin jars (plastic)	Laboratory Wipes
70% EtOH	Digital Timer/Alarm	Coverslips (22x22 and 22x50)
2x SSC pH 7.0	Forceps	Rubber Cement
Distilled H_20	Micropipette with tips	1mL tubes
IGEPAL (Sigma Cat # P6887)	Vortex mixer	Serological pipettes
2x SSC/0.3% Igepal Solution	Microcentrifuge	pH indicator sticks
Nuclease-free H_2O	Calibrated thermometer	Stir bar
Dapi II Counterstain	Graduated cylinder	Magnetic stirrer
LSI/WCPHybridization Buffer	Slide warmer (45^{0}C)	Razor blades
	PPE recommended by your Health and Occupational Safety regulations	Fluorescence microscope equipped with recommended filters
	ThermoBrite Hybridization Instrument	

2. REAGENTS

2xSSC (100mL) pH7.0
10mL of 20xSSC
90mL of dH2O
Adjust pH with 1N HCL or 1N NaOH

2xSSC/0.3% IGEPAL (500mL)
498.5mL of 2xSSC (pH 7.0)
1.5mL of IGEPAL (Sigma-Aldrich, Cat # P6887)

Add 1.5mL of IGEPAL with a serological pipette. Pipette up and down to rinse the pipette. Mix thoroughly with a magnetic stir bar and heat the solution to 40-50°C until the IGEPAL has been completely dissolved. Store in the dark at room temperature for up to 30 days. If sediment appears during storage, discard and prepare a fresh wash solution.

70% EtOH
• Add 350mL of 100% EtOH to a flask and fill with dH_20 to a final volume of 500mL

85% EtOH
• Add 425mL of 100% EtOH to a flask and fill with dH_20 to a final volume of 500mL

3. PROCEDURES

SAMPLES

- Samples consist of blood containing a population of circulating tumour cells (CTCs) applied to slides using the Wavesense immunomagnetic enrichment assay, and subjected to previous analysis by immunofluorescence (IF).
- This protocol applies to slides that have been fixed by immersion in Reagent Alcohol for 30 minutes at room temperature prior to the IF protocol.

QUALITY CONTROL

- Control slides must be run concurrently with patient slides to monitor assay performance and to assess the accuracy of signal enumeration. Cell line or normal blood metaphase spreads should be used as positive and negative controls for the FISH testing.
- Controls should be run on each day of FISH testing.
- The criteria for slide adequacy must be satisfied and the signal enumeration results should be within the specific established guidelines.
- Ensure that all reagents have reached desired temperatures prior to initiating procedure.
- Each hybridized slide should be evaluated against quality parameters determined by the laboratory.

PREPARATIONS REQUIRED BEFORE STARTING PROCEDURE

- Slides must be stored at -20ºC following IF analysis.
- All reagents and stock solutions should be prepared prior to the start of the procedure.
- Label the slides correctly: probe ID, date, lab identification gene or chromosome location, etc., along with name of person doing the procedure.
- Prepare fresh solutions prior to each procedure.

Day 1:

- Preheat water baths to 37°C and 75°C.
- Prepare, label and preheat the coplin jars for the prehybridization steps:
    a. 2x SSC at room temperature
    b. 2x SSC at 37°C
    c. 70% EtOH at room temperature
    d. 85% EtOH at room temperature
    e. 100% EtOH at room temperature.

Day 2:

- Preheat water bath to 72°C.
- Prepare, label and preheat the coplin jars for the post-hybridization steps:
    a. 2x SSC/0.3% Igepal at 72°C
    b. 2x SSC at room temperature

PROTOCOL

Day 1:

Evaluation of Cell Morphology

- Using a fluorescence light microscope equipped with a DAPI filter, evaluate the suitability of the cells for FISH based on the observed staining results
- Good quality cells should exhibit bright, uniform DAPI staining, and have smooth, round edges
- Carefully remove the coverslip and wash in 2x SSC at room temperature for 5 minutes before proceeding to the next step

Removing Coverslip (From IF Assay)

- Immerse the slide in 2x SSC at RT for 10-30 minutes until the coverslip easily falls off.

Slide pre-treatment

- Immerse the slide in 2x SSC at RT for 15 minutes
- Immerse the slide in 2x SSC at 37°C for 30 minutes.
- Dehydrate the slide(s) in a series of EtOH solutions (70%, 85% and 100%, 2 minutes each) at room temperature.
- Air-dry the slide(s).

Probe preparation

- Vortex an then briefly centrifuge the probe mix
- Prepare the probe mix as per manufacturer's directions
- Vortex and centrifuge
- Denature the probe at 75°C for 5 minutes
- Centrifuge
- Before applying the probe warm up the slide(s) at 45°C for 5 minutes.
- Apply 10µl of the probe mix to slide and immediately apply coverslip (22mm x 22mm).
- Seal the coverslip with rubber cement.

Co-denaturation

- Once the probe and coverslip are applied to the slide, and the rubber cement has dried, warm the slide on a Thermobrite at 80°C for 2 minutes (calibrate the temperature of the thermobrite) for denaturation of target and probe DNA.
- Hybridize for 16 hours at 37°C.

Day 2:

Washing the slide

- Carefully remove the rubber cement and coverslip by securing the coverslip between your index finger and thumb, slowly peeling off the rubber cement with forceps. Use a razor blade to carefully lift the coverslip off the slide without dragging it across the slide's surface.
- Immediately immerse the slide(s) in 2xSSC/0.3% Igepal at 72°C for 2 minutes (+ 0.2°C/ per slide, up to a maximum of 73°C).
- Immerse the slide(s) in 2xSSC at room temperature for 5 minutes.
- Air dry in the dark keeping the slide in an upright position.

- Apply 20µl of Vysis DAPI II counterstain to the target area and apply coverslip (22x50).
- Keep the slides at -20°C for at least 30 minutes.

Table 2. Summary Protocol

	Action	Temperature	Incubation time
	Day 1		
1	Evaluate cell morphology using DAPI II stain		
2	Remove coverslip (2x SSC)	RT	10-30 minutes
3	2x SSC	RT	15 minutes
4	2x SSC	37°C	30 minutes
5	70%, 85% and 100% EtOH	RT	2 minutes each
6	Air dry slides	RT	
7	Prepare the probe according to the appropriate protocol		
8	Warm up the slides	45°C	5 minutes
9	Apply probe mix to slide and immediately apply coverslip		
10	Seal the coverslip with rubber cement		
11	Place the slide on a thermobrite	80°C	1.5 minutes
12	Target and probe DNA hybridization	37°C	16 hours
	DAY 2		
13	Carefully remove the rubber cement and coverslip.		
14	2x SSC/0.3% Igepal	72°C	2 minutes
15	2xSSC	RT	5 minutes
16	Air dry in dark		
17	Apply DAPI II counterstain and apply coverslip		