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Procedure for Performing Fluorescence *In* Situ Hybridization (FISH) Using the *TMPRSS2:ERG* Fusion Probe on Slides Enriched for Circulating Tumour Cells

1. MATERIALS, EQUIPMENT AND FORM

Reagents& Solutions:	**Equipment:**	**Supplies:**
100% EtOH	Water baths (75^{0}C, 72 ^{0}C, 37^{0}C)	Thermometer
85% EtOH	Coplin jars (plastic)	Laboratory Wipes
70% EtOH		
2x SSC pH 7.0	Digital Timer/Alarm	Coverslips (22x22 and 22x50)
1x PBS	Forceps	Rubber Cement
1N HCL	Micropipette with tips	1 mL tubes
1x PBS/$MgCl_2$	Vortex mixer	Serological pipettes
Formamide	Microcentrifuge	pH indicator sticks
Distilled H_20	Calibrated thermometer	Stir bar
Pepsin (Porcine Gastric Mucosa) (Sigma-Aldrich, Cat # P6887)	Graduated cylinder	Magnetic stirrer
Pepsin solution	Slide warmer (45^{0}C)	Razor blades
IGEPAL (Sigma Cat # P6887)	PPE recommended by your Health and Occupational Safety regulations	Fluorescence microscope equipped with recommended filters
2x SSC/0.3% Igepal Solution	ThermoBrite Hybridization Instrument	
Nuclease-free H_2O		
Dapi II Counterstain		
LSI/WCPHybridization Buffer		
Squire Lab In-House *TMPRSS2:ERG* Probe		

2. REAGENTS

Pepsin stock solution

- Prepare a 10% stock solution (100mg/ mL) in sterile water.
- Dissolve completely and make 100ul aliquots of stock solution and store them at -20°C for up to 3 months.
- Working solution: **immediately prior to use**, add 6ul of the 10% pepsin solution to 50 mL of pre-warmed 0.01N HCl.

0.01N HCl (50 mL)
0.5 mL of 1N HCl
49.5 mL of dH2O

1M $MgCl_2$ (500 mL)
95.2g MgCl2 (M=95,21g/mol) or
203.3g MgCl2*H2O (Magnesium chloride Hexahydrate, M=203,30g/mol)
add water up to a final volume of 500 mL.

1X PBS/$MgCl_2$ (1000 mL)
Add 50 mL of 1M MgCL2 to 950 mL of 1X PBS.

70% Formamide/2x SSC (50 mL), pH 7.0
35 mL of formamide
15 mL of 2x SSC
Adjust pH with 1N HCL or 1N NaOH

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

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

Add 1.5 mL 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 350 mL of 100% EtOH to a flask and fill with dH_20 to a final volume of 500 mL

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

3. PROCEDURES

SAMPLES

- Samples consist of blood or buffer samples spiked with cancer cells from culture, and applied to slides using the Wavesense immunomagnetic enrichment assay.

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 fixed and stored for 2 days in Carnoy's fixative for at least two days at room temperature.
- 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, 72°C, and 75°C.
- Prepare, label and preheat the coplin jars for the prehybridization steps:
    a. 2x SSC at 37°C
    b. 0.01N HCl at 37°C
    c. 1X PBS at room temperature
    d. 1X PBS/$MgCl_2$ at room temperature
    e. 70% formamide/2x SSC at 72^0C
    f. 70% EtOH at room temperature
    g. 85% EtOH at room temperature
    h. 100% EtOH at room temperature
    i. 70% EtOH at -20^0C
    j. 85% EtOH at $-20\ ^0$C
    k. 100% EtOH at $-20\ ^0$C

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:

<u>Evaluation of Cell Morphology</u>

- Apply 25µl of DAPI II counterstain to the slide and affix a 22x40 coverslip
- Using a fluorescent 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 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

<u>Slide pre-treatment</u>

- Immerse the slide in 2x SSC at 37°C for 30 minutes.

Protease pre-treatment

- Pre-warm the 0.01N HCl solution to 37°C.
- Immediately prior to use, add 6ul of the 10% pepsin solution to 50 mL of 0.01N HCl.
- Incubate slide(s) in pepsin/ 0.01N HCl at 37°C for 5 minutes

Slide Washing/Pre-treatment

- Wash slides in 1X PBS at room temperature for 5 minutes
- Repeat with a second 1X PBS wash for 5 minutes
- Wash slides in 1X PBS/MgCL2 at room temperature, for 5 minutes
- Dehydrate the slide(s) in a series of EtOH solutions (70%, 85% and 100%, 2 minutes each) at room temperature.
- Air-dry the slides.
- Transfer the slide(s) to the 70% formamide/2xSSC at 72°C for 1.5 minutes.
- Immediately dehydrate the slide(s) in a series of **cold** EtOH solutions (70%, 85% and 100%, 2 minutes each) at -20°C
- Air dry the slides

Probe preparation (*3'ERG SpGold; 5'ERG SpGreen; TMPRSS2 SpRed)*

- Vortex an then briefly centrifuge the probe mix
- Prepare the probe mix (per slide)
    - 2µl of each probe
    - 4µl of LSI/WCP hybridization buffer
- 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 8µl of the probe mix to slide and immediately apply coverslip (22mmx22mm).
- Seal the coverslip with rubber cement.

Hybridization

- Once the probe and coverslip are applied to the slide, and the rubber cement has dried, hybridize the slide on a Thermobrite at 37°C for 16 hours (calibrate the temperature of the thermobrite).

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	2xSSC	37°C	30 minutes
3	0.01M HCl with 6µl of pepsin stock solution	37°C	5 minutes
4	1X PBS (Repeat twice)	RT	5 minutes
5	1X PBS/$MgCl_2$	RT	5 minutes
6	70%, 85% and 100% EtOH	RT	2 minutes each
7	Air dry slides		
8	70% formamide/2X SSC	72°C	1.5 minutes
9	70%, 85% and 100% EtOH	-20°C	2 minutes each
10	Air dry slides	RT	
11	Prepare the probe according to the appropriate protocol		
12	Warm up the slides	45°C	5 minutes
13	Apply probe mix to slide and immediately apply coverslip		
14	Seal the coverslip with rubber cement		
15	Target and probe DNA hybridization on Thermobrite	37°C	16 hours
	DAY 2		
16	Carefully remove the rubber cement and coverslip.		
17	2x SSC/0.3% Igepal	72°C	2 minutes
18	2xSSC	RT	5 minutes
19	Air dry in dark		
20	Apply DAPI II counterstain and apply coverslip		