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Procedure for Immunofluorescence (IF) Staining of Slides with Enriched Populations of Circulating Tumour Cells (CTCs)

1. MATERIALS, EQUIPMENT AND FORM

Reagents & Solutions:	Equipment:	Supplies:
Skim milk powder	Humidified chamber	Coverslips (22x40 and 22x50)
Tween-20	Micropipettors	10, 100 and 1000 µl tips
1x PBS	Coplin jars	Laboratory wipes
Vectashield Mounting Medium with DAPI	Magnetic stirrer	
CD45 Primary antibody: AbCam Rabbit Monoclonal [EP322Y] to CD45 (Cat. # ab40763)	Stir bar	
Texas Red Secondary antibody: Sheep Polyclonal Secondary Antibody to Rabbit IgG – H&L [Texas Red] (Cat. # ab6793)	Aqueous waste container	
Pan-CK antibody: WaveSense Pan Cytokeratin – FITC (4,5,6,8,11,13,18) Cat. # R2105-1	Fluorescence microscope equipped with recommended filters	

2. REAGENTS

<u>1x PBS</u>
Add 100mL of 10x PBS to 900mL of distilled water.

<u>Wash buffer</u>
Add 1mL Tween-20 to 999mL 1x PBS, mix well.
Prepare fresh (can be stored for 2 weeks at room temperature).

<u>5% Skim milk</u>
Add 5g skim milk powder to 100mL wash buffer.
Place on a stirrer until the skim milk dissolves.
Always make fresh.

3. PROCEDURES

SAMPLES

- Samples consist of patient blood samples, or donor blood/buffer samples spiked with cancer cells from culture, and applied to slides using the Wavesense immunomagnetic enrichment assay.
- Following fixation, slides should be kept in a desiccated slide box at -20°C until ready for the IF 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 spreads should be used as positive and negative controls for the IF testing.
- Controls should be run on each day of IF testing.

PREPARATIONS REQUIRED BEFORE STARTING PROCEDURE

- All reagents and stock solutions should be prepared prior to the start of the procedure
- Label the slides correctly: patient ID, date, lab identification, etc., along with name of person doing the procedure
- Prepare fresh solutions prior to each procedure
- To protect the light sensitive fluorophores, work in reduced light conditions as much as possible

PROTOCOL

Treating slide with blocking agent

- Remove the slides from -20˚C freezer and bring them to room temperature.
- Rinse the slide with 1x PBS briefly in a coplin jar.
- Place the slide in a coplin jar containing 5% skim milk and incubate at room temperature for 1 h.
- Wash the slide 5 times with RT 1x PBS/0.1% Tween-20.
- Hold the slide vertically on a kimwipe in order to drain entire wash buffer, do not allow slide to dry.

Primary CD45 antibody assay

- Make a 1:200 dilution of Rabbit and Human CD45 primary antibody in Dako antibody diluent.
- Apply 100μl of primary antibody on to the slide.
- Cover the slide with a 22x50mm coverslip and place in a humidified chamber at room temperature for 45 min.
- Gently remove the coverslip and wash the slide 5 times with RT 1x PBS/0.1% Tween-20in a coplin jar replacing fresh wash buffer after every wash.
- Hold the slide vertically on a kimwipe in order to drain the entire wash buffer, do not allow slide to dry.

Secondary CD45 antibody assay

- Make a working solution of 2.5 μg/mL Sheep anti-rabbit polyclonal antibody (Texas Red) in Dako antibody diluent.
- Apply 100ul of secondary antibody on to the slide.
- Cover the slide with a 22x50mm coverslip and place in a humidified chamber at room temperature for 15 min in dark.
- Gently remove the coverslip and wash the slide 5 times with RT 1x PBS/0.1% Tween-20 in a coplin jar replacing fresh wash buffer after every wash.
- Hold the slide vertically on a kimwipe in order to drain the entire wash buffer, do not allow slide to dry.

PanCK antibody assay

- Apply 100µl Mouse anti-human PanCK FITC conjugate to the slide.
- Cover the slide with a 22x50mm coverslip and place in a humidified chamber at room temperature for 15 min in dark.
- Gently remove the coverslip and wash the slide 5 times with RT 1x PBS/0.1% Tween-20in a coplin jar replacing fresh wash buffer after every wash.
- Hold the slide vertically on a kimwipe in order to drain the entire wash buffer, do not allow slide to dry.

Counterstain assay

- Apply 20µl of Vectashield Mounting Medium with DAPI to the target area and apply coverslip (22x50)
- Keep the slides in a desiccated slide box at -20°C for at least 30 minutes.
- View the slides under appropriate filters under a fluorescence microscope.

Table 2. Summary Protocol

	Action	Temperature	Incubation time
1	Remove the slide from -20 C freezer and bring them to room temperature	RT	
2	Rinse the slide with 1x PBS briefly in a coplin jar	RT	
3	Incubate the slide in a coplin jar containing 5% skim milk	RT	60 minutes
4	Wash the slide 5 times with 1x PBS/0.1% Tween-20	RT	2 minutes each
5	Apply 100 µl of primary antibody on to the slide	RT	
6	Cover the slide with a 22x50mm coverslip	RT	
7	Incubate slide in a humidified chamber	RT	45 minutes
8	Wash the slide 5 times with 1x PBS/0.1% Tween-20	RT	2 minutes each
9	Apply 100 ul of secondary antibody on to the slide	RT	60 minutes
10	Cover the slide with a 22x50mm coverslip	RT	
11	Incubate slide in a humidified chamber in the dark	RT	15 minutes
12	Wash the slide 5 times with 1x PBS/0.1% Tween-20	RT	2 minutes each
13	Apply 100µl Mouse anti-human PanCK FITC conjugate to the slide	RT	
14	Cover the slide with a 22x50mm coverslip	RT	
15	Incubate slide in a humidified chamber in the dark	RT	15 minutes
16	Wash the slide 5 times with 1x PBS/0.1% Tween-20	RT	2 minutes each
17	Apply 20 µl Vectashield Mounting Medium with DAPI to the target area and apply coverslip (22x50mm)	RT	
18	Keep the slides in a desiccated slide box at -20°C Freezer	RT	30 minutes
19	View the slides using appropriate filters under a fluorescence microscope		

References

- Refer to the 3rd edition of the Public Health Agency of Canada's Laboratory Biosafety Guidelines,

- Refer to the Queen's University's Biohazards Safety Manual published by the Department of Environmental Health and Safety, when handling biohazardous materials such as blood and tumor samples