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Standard Operating Procedure for Enrichment and Isolation of Circulating Tumour Cells (CTCs) for FISH and IF Analysis Using The WaveSense Immunomagnetic Separation Platform

1. MATERIALS, EQUIPMENT AND FORM

Reagents:	Equipment:	Supplies:
Reagent Alcohol Histological Grade	WaveSense NeoMag Slide Dock	WaveSense EpiSep Hybridization Slides (HS) (NSD II), Catalog #: A3104-10
EpiSep EpCAM (323/A3) magnetic particles	WaveSense Microvial Rotator	Low retention large orifice tips
1.0% BSA/0.1% Sodium Azide/0.01M PBS	WaveSense Magnetic Tube Dock, 2mL (MTD-2)	Sterile Transfer Pipettes
1X PBS pH 7.4	Fluorescence microscope equipped with recommended filters	2.0 mL round bottom Eppendorf tube
Patient Whole Blood	Phase Contrast Microscope	Sterile Serological Pipette
Vectashield with DAPI	Micropipettor	
Methanol	Pipet.aid	6.0 mL sodium heparin BD vacutainer tube
Glacial Acetic Acid	Fluorescence microscope equipped with recommended filters	Coverslips (22x22 and 22x50)
	Centrifuge	
	Water bath (37^{0}C)	
	Coplin jars (plastic)	
	Digital Timer/Alarm	
	Forceps	
	Vortex mixer	
	Calibrated thermometer	
	Graduated cylinder	
	PPE recommended by your Health and Occupational Safety regulations	

2. REAGENTS

<u>1.0% BSA/0.1% Sodium Azide/0.01M PBS (100mL)</u>
100mL of 0.01M PBS
1.0g of BSA (Rockland Cat. # BSA-50)
0.1g of Sodium Azide (Sigma Cat. # S2002)
Store at 4˚C for up to two weeks

<u>Carnoy's fixative (100mL)</u>
66mL methanol
33mL glacial acetic acid
Prepare fresh each day and store at -20˚C

*Please note recent changes regarding wash buffers and tube selection are still under active investigation.

3. PROCEDURES

SAMPLES

- Samples consist of 5mL of patient blood received from clinical trial centers, or volunteer blood spiked with tumour cells from culture.

QUALITY CONTROL

- Ensure that the patient blood has not been frozen.
- Allow all reagents to reach desired temperatures prior to initiating procedure.
- Each EpiSep Hybridization slide should be evaluated against quality parameters determined by the laboratory.

PREPARATIONS REQUIRED BEFORE STARTING PROCEDURE

- Log Patient Sample information into the database.
- All reagents and stock solutions should be prepared prior to the start of the procedure.
- Label the EpiSep HS slides correctly: sample ID, date, lab identification, assay to be performed.
- Prepare fresh solutions prior to each procedure.
- Preheat water baths to 37°C.
- Prepare, label the coplin jars for the prehybridization steps:
    a. 3:1 methanol/glacial acetic acid -20°C
    b. 1.0% BSA/0.1% Sodium azide/0.01M PBS to 37°C

4. PROTOCOL

Part 1

<u>Preparing and washing human whole blood specimens</u>

- Gently invert the sodium heparin BD Vacutainer blood tube 8 times to ensure even mixing.
- Centrifuge in a swinging bucket centrifuge for 10 minutes at 600 x g with the brake speed “OFF” at room temperature.
- Remove plasma and store at -80C.
- Add 1% BSA/0.1% Sodium Azide/0.01M PBS to an equivalent volume of removed plasma.
- Aliquote the specimen into five 2.0mL Eppendorf tubes using a transfer pipette (bore diameter of at least 1.5mm).
- Label the tube appropriately: three aliquots will be used for FISH assays and the remaining two aliquots will be used for Immunofluorescence assays.

Part 2

<u>CTC enrichment using EpCam (323/A3) magnetic particles</u>

- Immediately prior to use, re-suspend the EpCam magnetic particles by vortexing at a medium speed 7 for three 1 second bursts.
- Using a 100µl micropipettor, gently add 25µl of EpCam magnetic particles into each aliquot and incubate the specimen on the microvial rotator for 1 hour at room temperature.

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Sample de-bulking

- Remove the sample from the microvial rotator and place the 2.0mL Eppendorf tube on the Magnetic Tube Dock (MTD-2) for 20 minutes to concentrate the EpCam magnetic particles to the side of the Eppendorf tube.
- Using a transfer pipette (bore diameter of at least 1.5mm), remove the supernatant while the 2.0mL Eppendorf tube remains on the magnetic tube dock.
- Add 1000µl of 1x PBS to the specimen.
- Remove the 2.0mL Eppendorf tube from the Magnetic Tube Dock (MTD-2). Using a transfer pipette (bore diameter of at least 1.5mm), gently re-suspend the EpCam magnetic particles in the remaining 1000µl of 1xPBS.

CTC enrichment and slide preparation

- Place the EpiSep Hybridization Slide on the NeoMag Slide Dock.
- Transfer the specimen entirely to the sample well of the EpiSep Hybridization Slide as quickly as possible without overfilling the well. Wait until the entire sample has absorbed into the slide.
- Record the time elapsed for the sample to clear the well.
- Add 1000µl of 1x PBS to one of the 2,0mL Eppendorf tubes and rinse the tube. Transfer to the second and third tubes, rinsing well each time.
- Apply the PBS rinse to the EpisSep Hybridization Slide on the NeoMag Slide Dock.
- Apply 5-6 drops of 1x PBS to the well (top, bottom, left, and right) to rinse

FISH slide fixation

- Apply 25µl of cold 3:1 methanol/glacial acetic acid fixative to each side of the well (top, bottom, left, and right) for a total volume of 100µl.
- Incubate for 1 minute at room temperature.
- Remove the EpiSep Hybridization Slide absorbent cap and discard in a biohazard container.
- Remove the slide from the NeoMag Slide Dock and submerse in cold 3:1 methanol/glacial acetic acid fixative for 30 minutes at -20°C.
- Heat the slides for 1-2 minutes (but not more than 2 minutes) at 55ºC on a slide warmer.
- Ensure that the CTCs were enriched and captured by checking the slides through the phase-contrast microscope.
- Place the slides in a slide box and store at room temperature for a maximum of three days. Once the slides have been aged store the slides in a desiccated slide box at -20°C.

IF slide fixation

- Apply 25µl of Reagent Alcohol Histological Grade fixative to each side of the well (top, bottom, left, and right) for a total volume of 100µl.
- Incubate for 1 minute at room temperature.
- Remove the EpiSep Hybridization Slide absorbent cap and discard in a biohazard container.
- Remove the slide from the NeoMag Slide Dock and submerse in Reagent Alcohol Fixative for 10 minutes at RT.
- Heat the slides for 1-2 minutes (but not more than 2 minutes) at 55C on a slide warmer.
- Ensure that the CTCs were enriched and captured by checking the slides through the phase-contrast microscope.

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- Store the slides in a desiccated slide box at -20°C.

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Table 2. Summary Protocol

	Action	Temperature	Incubation time
	Part 1		
1	Gently mix the Patient Blood Sample	RT	
2	Centrifuge at 600x g for 10min.		
3	Remove plasma and store at -80ºC		
2	Aliquot the Patient Blood Sample into 2.5mL volume (1 aliquot for FISH assay and 1 aliquot for IF assay)	RT	
3	Add 17.5mL of 0.01M PBS/0.1% Sodium Azide to each of the 50mL conical tubes. Mix the specimens by gentle inversion	RT	
4	Centrifuge at 600x g for 10min.	RT	
5	Remove supernatant	RT	
6	Add up to 20mL 0.01M PBS/0.1% Sodium Azide to the specimen	37ºC	
7	Re-suspend the RBC packed layer	RT	
	Part 2		
7	Re-suspend the EpCam magnetic particles by vortexing at a medium speed 7 three 1 second bursts	RT	
8	Add 50µl of EpCam magnetic particles into the specimen and incubate the specimen in the 50mL Conical Tube on the microvial rotator	RT	60 minutes
9	Debulk Sample	RT	20 minutes
10	Add 1000µl of 0.1% BSA, 0.1% Sodium Azide / 1x PBS to the sample	RT	
11	Re-suspend sample	RT	
12	Place the EpiSep Hybridization Slide on the NeoMag Slide Dock	RT	
13	Transfer the specimen entirely to the sample well of the EpiSep Hybridization Slide	RT	
	Slides for FISH		
14	Apply 100µl of cold 3:1 methanol/glacial acetic acid fixative	RT	1 Minute
15	Remove the EpiSep Hybridization Slide absorbent cap		
16	Remove the slide from the NeoMag Slide Dock and submerse in cold 3:1 methanol/glacial acetic acid fixative	-20°C	30 Minutes
17	Place the slides in a slide box and store at room temperature	RT	Up to 3 days
18	Once the slides have been aged store the slides in a desiccated slide box at -20°C	-20°C	
	Slides for IF		
19	Apply 100µl of Reagent Alcohol Histological Grade fixative	RT	1 Minute
20	Remove the EpiSep Hybridization Slide absorbent cap	RT	
21	Remove the slide from the NeoMag Slide Dock and submerse in Reagent Alcohol	RT	10 Minutes
22	Heat the slides on Slide Warmer	55ºC	1 Minute
23	For IF aliquots store the slides in a desiccated slide box at -20°C	-20°C	
	Part 3		
23	Ensure that the Circulating Tumour Cells were enriched by		

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	checking the slides through the phase-contrast 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

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