

## Appendix 1: CanPatrol multiplex mRNA *in situ* analysis for CTC enumeration

### *Peripheral-blood collection and primary enrichment*

Collect two 5 mL tubes of venous blood using a No. 21 gauge needle into ethylenediaminetetraacetic acid (EDTA)-anticoagulant vacutainers. Invert the tubes 5–8 times, then transfer the blood into SurExam RNA-save storage tubes via the provided connecting device; invert 10 times to ensure complete anticoagulation and lysis. Keep the tubes at room temperature (RT) for 30 min or at 4 °C, and perform CTC isolation within 4 h.

### *Nanofiltration enrichment*

The pre-processed cell suspension was passed through an 8 µm polycarbonate nanopore filtration membrane under gentle negative pressure ( $\approx 0.4$  bar), retaining larger CTCs on the filter surface. The membrane was then rinsed with 10 mL PBS to remove residual plasma and small blood cells.

### *On-membrane fixation, dehydration, and permeabilization*

- (I) Fixation: flood membrane with 400 µL 4% paraformaldehyde for 15 min at RT;
- (II) Dehydration: sequentially treat the membrane with 400 µL of 100%, 70%, and 50% ethanol (2 min each);
- (III) Washing: rinse three times with 1 mL PBS (2 min each);
- (IV) Permeabilization: add 100 µL translantant (SurExam) and incubate at RT for 5 min; wash three times with 1 mL PBS.

### *Protease digestion*

Apply 200 µL protease working solution (1:60 dilution in PBS) and incubate at RT for 1 h. Wash three times with 1 mL PBS (2 min each).

### *Multiplex probe hybridization*

Overlay 150 µL mRNA probe cocktail:

- Epithelial: CK8, CK18, CK19, EpCAM;
- Mesenchymal: Twist, vimentin;
- Leukocyte: CD45;
- Extra: EGFRvIII.

Hybridize at 40 °C for 3 h in a humidified chamber.

Wash twice with 1 mL pre-warmed washing buffer (42 °C, 2 min each).

### *Signal amplification and color development*

- (I) Pre-amplification: 150 µL preamp solution, 40 °C, 30 min;
  - (II) Amplification: 150 µL amp solution, 40 °C, 30 min;
  - (III) Wash twice with washing buffer;
  - (IV) Signal amplification: 150 µL horseradish peroxidase (HRP)-conjugated amplifier, 40 °C, 30 min;
  - (V) Color development: 150 µL tyramide signal amplification (TSA) substrate, incubate in the dark at 40 °C for 30 min.
- Wash twice with PBS.

### *Nuclear counterstaining and scanning*

Add 10  $\mu$ L 4',6-diamidino-2-phenylindole (DAPI) (1  $\mu$ g/mL) and incubate at RT for 5 min. Wash once with PBS. Scan the entire membrane using a Metafer-ISH automated fluorescence microscope (MetaSystems, 20 $\times$  objective).

### *CTC definition*

- DAPI-positive nucleus;
- CD45-negative;
- Positive signal for  $\geq 7$  tumor probe (CK8/18/19, EpCAM, Twist, or vimentin);
- Cell diameter  $\geq 8$   $\mu$ m;
- Manual review by two independent operators; discrepancies resolved by re-scanning.