

Instruction of Use:

I. Intended Use

The Vita-Cap™ N9 RCE kit is for the functional enrichment of viable tumor progenitor and other rare stem cells from whole blood, lymph, bone marrow and other bodily fluids. Enriched cells can be analyzed by flow cytometry and microscopy for immuno-phenotyping and counting of CTCs and CTPCs, as well as other molecular analyses, including proteomics, gene expression profiling, genomic and mutational analyses.



Vita-Cap™ N9:
Processing 1 -
3 mL blood

- **For research use only.**
- **Not for diagnostic or therapeutic use.**

Product Numbers:

104.01N (Vita-Cap™ N9)

104.02R (Vita-Cap™ R9)

104.03G (Vita-Cap™ G9)

Product Specifications	
Each kit Contains:	6 tubes of Vita-Cap™ N9 coated with the plain CAM film (N9), red fluorescent CAM (R9) or green fluorescent CAM (G9). 1 tube of 10x anticoagulant blood diluents. 1 tube of Cell Releasing CAM Enzyme. *
No. Cells Processed:	6 samples of 1-mL to 3-mL whole blood or bone marrow plus 4-mL of anticoagulant working medium.
Starting Sample:	Whole blood, bone marrow and peritoneal effusions.
Downstream Applications:	Flow cytometry, Fluorescence microscopy, FISH, etc; Quantitative RT-PCR, DNA microarray, SAGE, proteomics and genomics.

The Vita-Cap™ N9 RCE kit is for the functional enrichment of viable tumor progenitor and other rare stem cells from whole blood, lymph, bone marrow and other bodily fluids. Enriched cells can be analyzed by flow cytometry and microscopy for immuno-phenotyping and counting of CTCs and CTPCs, as well as other molecular analyses, including proteomics, gene expression profiling, genomic and mutational analyses. Usually,
 * CAM-avid cells released from CAM scaffolds by Cell Releasing CAM Enzyme plus Trypsin/EDTA shall be concentrated to a small volume, 100 µL PBS containing the enriched cell fraction. The 100 µL cell suspension can be processed for cellular analyses such as fluorescence microscopy, FISH and flow cytometry.
 ** CAM-avid cells released from CAM scaffolds by Cell Releasing CAM Enzyme plus Trypsin/EDTA shall be concentrated to a small volume, 10 µL PBS containing the enriched cell fraction. The 10 µL cell suspension can be lysed for protein, mRNA or DNA isolation, followed

by proteomics, quantitative RT-PCR, DNA microarray, SAGE and genomic analyses. The sensitivity of qRT-PCR detection can be 1 tumor cell in 1 mL of blood.

II. Summary and Explanation

Vita-Cap™: The Vita-Cap™ rare cell enrichment (RCE) device employs the Vitatex proprietary functional cell adhesion matrix (CAM*: a porous layer of extracellular matrix polymer coated with blood-borne adhesion molecules) that mimics the tumor interstitial microenvironment. The inner surface of the Vita-Cap™ tube is coated with a scaffold layer that is comprised of plain or fluorescently labeled CAM¹. Circulating tumor cells (CTCs: defined using expression of the epithelial cell adhesion molecule [EpCAM] and tumor markers, i.e., CA125 for ovarian tumor; CA19-9 for pancreatic tumor; HER2 for breast tumor; or PSMA for prostate tumor), and their subpopulation named circulating tumor progenitor cells (CTPCs: defined using expression of the cancer stem cell marker CD44v6 and the invasiveness marker seprase) in blood, adhere to the CAM surface due to their high avidity for the extracellular matrix (ECM) and subsequent ingestion of CAM (CAM⁺)². Since the proclivity to degrade and ingest ECM is one of the hallmarks of invasive and metastatic cells, CAM⁺ cells captured in Vita-Cap™ tubes represent a unique way to identify CTCs and CTPCs.

For rare cell enrichment, venous blood is collected in a standard blood collection heparin tube. The specimen is either transported immediately or stored/shipped at 4°C within 48 hours to the laboratory for processing. Optimally, 2-mL of blood is diluted with 4 mL anticoagulant cell dilute working buffer and introduced into the Vita-Cap™ tube and incubated on a tube roller at 37°C for two hours. Unlike blood cells, circulating rare progenitor cells, particularly those cells which are tumorous, epithelial, endothelial and stem-like in origin, differentially attach themselves to the CAM-coated beads (scaffolds) on the inner surface of the Vita-Cap™ tube. Invasive, viable cells such as metastatic tumor cells ingest proteins in the CAM scaffolds and remain attached while non-invasive blood cells are subsequently washed off of the tube inner surface. Once washed, attached (rare) cells can be released from CAM scaffolds by enzymatic digestion, and the identity of the enriched rare cells characterized by multi-parameter flow cytometry and microscopy. CTCs and CTPCs resolved by flow cytometry can be validated by fluorescence microscopy with double positivity in epithelial / tumor cell surface markers and their ability to ingest fluorescent CAM (CAM⁺).

Alternatively, the attached rare cells that are released from CAM scaffolds can be concentrated and their DNA, RNA and protein can be extracted with appropriate buffers for molecular analysis of cancer or other diseases.

III. Materials Supplied

* US Patents [7,785,814](#), [7,687,241](#), [7,374,898](#), [7,250,492](#); U.S. Patent Application numbers: 10/220,347, 11/010,122, 10/978,029; and international patents and applications covering European, Australia, Canada, Japan and China.

¹ Vita-Cap™ coated with plain CAM (Vita-Cap™ N9), red fluorescently labeled CAM (Vita-Cap™ R9), and green fluorescent CAM (Vita-Cap™ G9) are available.

² Invasive tumor cells can be identified as CAM⁺ only if CAM is fluorescently pre-labeled with red fluorescent dyes (series R) or green fluorescent dyes (series G).

- **104.01N (Vita-Cap™ N9); 104.02R (Vita-Cap™ R9); 104.03G (Vita-Cap™ G9) for processing 1- to 3-mL of whole blood each tube** are supplied in a set of 6 tubes.
- **One tube of 10x Cell Dilute Stock.** It is supplied in a single tube, shipped with the kit at ambient shipping temperatures.

NOTE: The 10x Cell Dilute Stock must be stored at 4°C immediately upon receipt. For processing each 1 - 3 mL of blood in a Vita-Cap™ N9 tube, dilute 0.4 mL of the 10x Cell Dilute Stock with 3.6 mL of complete cell culture (CCC) medium (See Section IV) to make 4-mL cell dilute working solution that will be added into one Vita-Cap™ N9 tube prior to adding anticoagulated blood to the tube.

- **One tube of Cell Releasing CAM Enzyme,** for enzymatic digestion of CAM and release of progenitor cells, is supplied in each box.

NOTE: Vitatex CAM Enzyme must be stored in a freezer (-20°C) immediately upon receipt. Immediately prior to use, dilute the frozen enzyme by adding 6 mL of 1x phosphate buffered saline (PBS), pH 7.4.

NOTE: 1:1 mixture of CAM enzyme solution and Trypsin-EDTA (see below) will be used to elute rare cells adhering on the CAM scaffold. The un-used **Enzyme** working solution can be stored at 4°C for a few weeks.

IV. Additional Materials Required but Not Supplied

Cell Isolation Procedures:

- For collection of anti-coagulated venous blood, BD Vacutainer® 10.0 mL sodium heparin tube (BD catalog # 367874) is recommended; others such as BD Vacutainer® 6.0 mL sodium heparin tube (BD catalog # 367878) or 6.0 mL lithium heparin tube (BD catalog # 367886) are also workable.
- Rotator capable of horizontal rotation of Vita-Cap™ tubes. (See Figure on the right). Example: the Stovall Low Profile Roller catalog #: ROLAAUV1S <http://www.slscience.com/low-profile-roller> (See Figure on the right), the Stuart Digital tube rollers SRT9D <http://www.stuart-equipment.com/product.asp?dsl=85> rotator, or the Barnstead Thermolyne Labquake® shaker/Rotisserie, part number 3,625,485.
- A temperature-controlled incubator (to house the rotator) that maintains a temperature of 37°C. Example: Stuart Incubator S160D <http://www.stuart-equipment.com/product.asp?dsl=75>. (See Figure on the right).
- Low-speed, swing-out bucket centrifuge for pelleting cells.
- Sterile 15 mL polystyrene conical centrifuge tubes, i.e., BD catalog # 352095.
- Complete cell culture (CCC) medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Cellgro catalog # 10-013-CV) and RPMI 1640 (Cellgro catalog # 10-040-CV) supplemented with 10% calf serum (Hyclone,



Thermo Scientific catalog # SH30072.03), 5% Nu-serum (BD catalog # 355504), 2 mM L-glutamine (Cellgro catalog # 25-005-CI), 1 unit/ml penicillin and 10 µg/ml streptomycin (Invitrogen catalog # 15140-122).

- Sterile 1x phosphate buffered saline (PBS), pH 7.4.
- Sterile 0.2% BSA (bovine serum albumin, Sigma catalog # A9085) in 1x PBS, pH 7.4.
- Trypsin-EDTA solution, i.e., Invitrogen catalog # 25200056, a 1:1 mixture of CAM enzyme solution and Trypsin-EDTA will be used to elute rare cells adhering on the CAM scaffold.
- A vacuum aspirator for gentle removal of supernatant waste.
- For preparation of nuclear cells, red cell lysis buffer (154mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA) pH 8.0 to be mixed with whole blood at a ratio of 1:25 and at 20-30°C. Commercial products currently available include RBC Lysis Buffer 10X (BioLegend catalog # 420301), RBC Lysis Buffer 10X (Imgenex catalog # 10089), and RBC Lysis Buffer (StemCell Technologies catalog # 07850).

Cellular analyses using fluorescence microscopy and flow cytometry:

- Antibody reagents are used for staining of epithelial tumor (Epi) cells, tumor progenitor (TP) cells and hematopoietic lineage (HL) cells, i.e., see publications (Lu et al., 2010; Fan et al., 2009; Paris et al., 2009).

NOTE: Vitatex has generated antibody reagent sets for staining CTCs and subsequent counting by flow cytometry. These include:

- Mouse anti-human epithelial tumor cell surface antigens (Epi), including EpCAM and ESA, catalog # **MABS2002**. Alexa488-conjugated mouse anti-human Epi, catalog # **MABS2002Alexa488**.
- Mouse or rat anti-human tumor progenitor cell surface antigens (TP), including CD44v6 and seprase, catalog # **MABS2003**. PE-conjugated rat anti-human TP, catalog # **MABS2003PE**.
- Mouse anti-human hematopoietic lineage (HL) or WBCs (CD45), conjugated with APC.
- 7-aminoactinomycin (7AAD: Molecular Probe, catalog # A-1310) for staining cell nuclei.

NOTE: (a) Preparation of 7AAD stock solution – add 500 µL DMSO into 1 tube containing 1 mg 7AAD powder, vortex, divide into 25 µL aliquots in new sterile 1.5 mL tubes, and store stock solution tubes in a -20°C freezer. The stock solution in each 1.5 mL tube contains 50 µg 7AAD in 25 µL DMSO. (b) Preparation of 7AAD working solution – the 7AAD working solution is made by adding 975 µL PBS into each stock solution tube. 10 µL of 7AAD working solution is sufficient for staining cellular nuclei in one sample. The 7AAD working solution can be stored in a refrigerator for one week.

NOTE: Other nucleic acid dyes such as DAPI can be used to replace 7AAD.

- BD FACS Lysing solution, 10x (BD catalog # 349202).
- Polystyrene Tube with Cell-Strainer Cap, 5 mL (BD catalog # 352235).
- A flow cytometer.

RNA, DNA or Protein Extraction Procedures:

- RNA extraction.
 - Commercial RNA extraction kit such as RNeasy Mini Kit (Qiagen Inc., catalog # 74104, www.qiagen.com) or QIAamp RNA Blood Mini (Qiagen Inc., catalog #52304).
- DNA extraction.
 - Commercial Wizard DNA Purification Kit (Promega, Madison, WI) for gene copy number changes of the isolated CTCs, as described (Paris et al., 2009). Another DNA isolation kit tested was QIAamp DNA Mini Kit (Qiagen Inc., catalog # 51304, www.qiagen.com).

V. Specimen Collection, Rare Cell Enrichment and Identification / Enumeration by Flow Cytometry

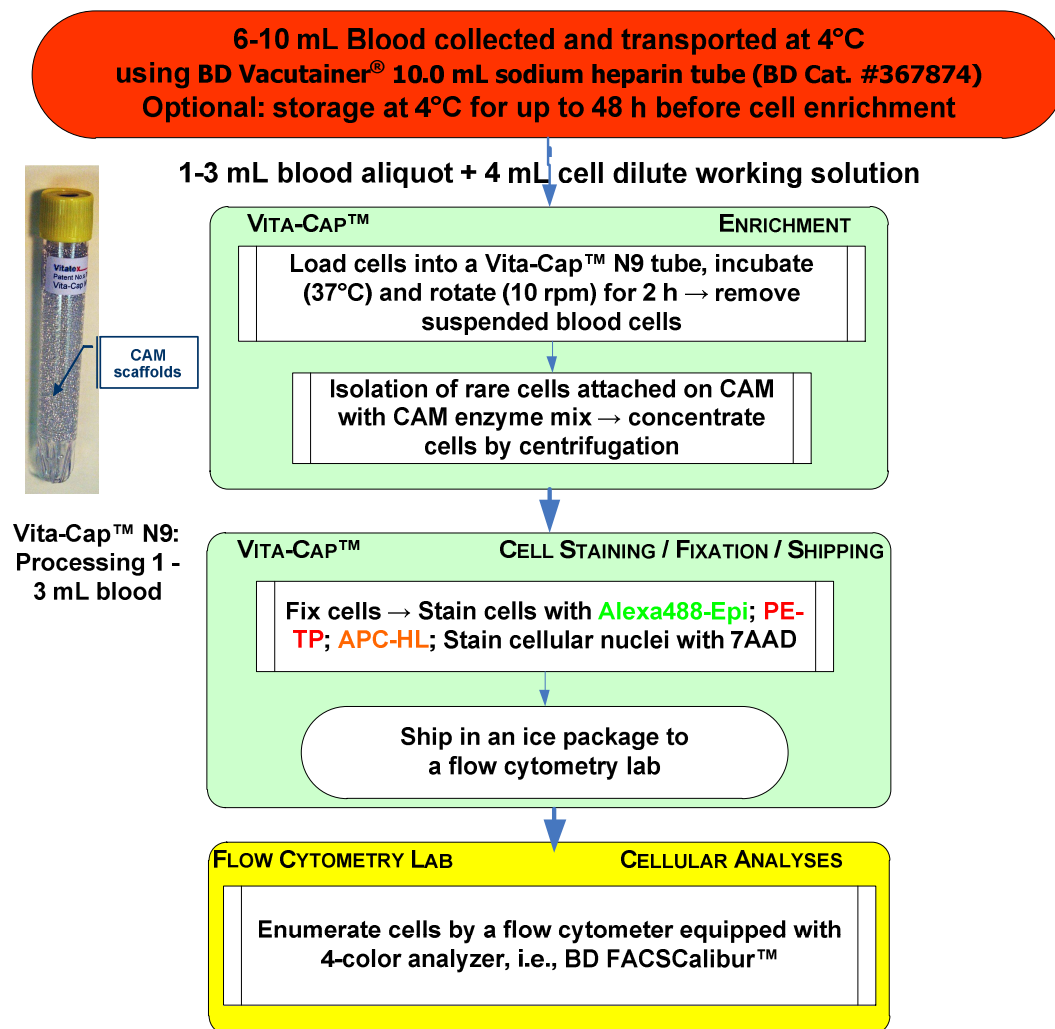


Figure 1: Vita-Cap™ enrichment of CTCs in blood, their identification and enumeration using flow cytometry.

NOTE: The methods and procedures described here can be applied to analysis and enumeration of circulating endothelial cells and circulating endothelial progenitor cells in whole blood, as well as different rare cells in bone marrow.

NOTE: Based on our recent publications (Lu et al., 2010; Fan et al., 2009; Kennedy et al., 2009; Paris et al., 2009) and Vitatex internal R&D, rare cells in the blood of cancer patients are enriched using Vita-Cap™ RCE™ tubes and characterized by flow cytometry. CTCs are nucleic acid (NA) and epithelial / tumor markers (Epi) positive but hematopoietic lineage (HL) markers negative – $NA^+ / EpCAM^+CA125^{+3} / CD45^-$. Circulating tumor progenitor cells

³ Tumor marker CA125 is used for ovarian cancer samples; PSMA (FOLH1) for prostate cancer; HER2 for breast cancer; CA19-9 for pancreatic cancer and other GI cancers. CA125 is used throughout the text when a tumor marker is used to combine with other markers.

(CTPCs) are CTCs that exhibit tumor progenitor (TP) markers (CD44v6⁺SEP⁺) – NA⁺ / EpCAM⁺CA125⁺ / CD45⁻ / CD44v6⁺SEP⁺. Technically, **CTCs and CTPCs are identified using the following criteria.**

- (1). Positive selection procedure for cellular nuclei using the nucleic acid dye 7AAD to exclude contaminating non-cellular particulates and platelets;
- (2). Positive staining for Alexa488 (or fluorescein)-conjugated antibodies against the epithelial lineage marker EpCAM / ESA and the tumor marker CA125, PSMA, HER2 or CA19-9 (Epi);
- (3). Positive staining for PE-conjugated antibodies against epithelial stem marker CD44v6 and invasiveness marker seprase (SEP);
- (4). Negative selection procedure using APC-antibodies against the hematopoietic lineage (HL) markers, including leukocyte common antigen CD45.

Vita-Cap™ - Cell Isolation Protocol:

1. **Specimen Collection:** Using your institution's recommended procedure for standard blood venipuncture, collect blood into one or more BD Vacutainer® 10.0 mL sodium heparin tube (BD catalog # 367874). Transport tubes to laboratory.

NOTE: Blood collection tubes may be stored for up to 48 h at 4°C or shipped at 4°C to the laboratory within 48 h prior to rare cell enrichment steps, although internal evaluation showed that, when blood was stored at 4°C for five days, ~40% of CTCs were recovered.

NOTE: All pipetting steps from this point should be conducted in a laminar flow hood using sterile technique.

2. **Incubation of blood in Vita-Cap™ N9 tube:** Add 4 mL of Cell Dilute Working Solution to each Vita-Cap™ N9 tube. Add 1 - 3 mL of anticoagulated blood into each Vita-Cap™ N9 tube, seal the tube, and invert the sealed tube 5 times to mix. Place the sealed tube on a rotator and rotate at 10 cycles per minute at 37°C. Incubate for 2 hours for tumor cell attachment to occur.
3. **Removal of unattached cells:** After incubation, decant unattached cells with liquid into a waste container. Wash tubes three (3) times by pipetting 3 mL of 1x PBS into tube and gently inverting tube three (3) times, discarding wash solution each time.
4. **Enzymatic release of tumor cells captured by the CAM:**
 - a. Dissolve Vitatex CAM Enzyme by adding 6-mL of 1x PBS, pH 7.4. Prepare 1:1 mixture consisting of 6-mL CAM enzyme and 6-mL Trypsin-EDTA solution (a total of 12-mL).
 - b. Add 2 mL of the working enzyme solution into each washed Vita-Cap™ tube, seal the tube, place on rotator in incubator, and rotate at 37°C for 10 minutes to dissolve CAM and release tumor cells into suspension.
 - c. Add 5 mL of the CCC medium into each Vita-Cap™ tube to stop enzymatic action and elute cells into suspension by gently inverting tube five (5) times.

- d. Concentrate cells by centrifuging each Vita-Cap™ tube at 1,000 rpm for 3 min and remove supernatant by gentle aspiration, retaining the last 300 µL containing the enriched cell fraction.

NOTE: Glass beads may be found to fall off from the wall. Since beads are much larger and heavier than cells, cells in suspension can be transferred by using fine pipette tips to avoid glass beads.

VI. Flow Cytometry Protocol

5. Staining with antibody conjugates and nucleic acid dye: From Step 4-d.) above, loosen the cell pellet thoroughly by flicking the tube.

- a.) Fixation and RBC lysis: Fixation and RBC lysis can be done by treating cells with 1 mL of 1x FACSLyse buffer, at 20-25°C for 15 min, to lyse the RBCs and mildly fix the stained cells. Add 3 mL of PBS containing 0.2% BSA, mix and concentrate cells by centrifuging the tube at 350xg or 1,000 rpm for 5 min, and remove supernatant by gentle aspiration, retaining the last 100 µL containing the enriched cell fraction. [Fixed cell suspension could be stored at 4°C at this [point](#).]
- b.) Antibody and nucleic acid dye staining: Add antibody reagents⁴ and 7AAD stock solution⁵, and stain cells at 20-25°C (only un-fixed live cells should be stained on ice) in the dark, for 30 min.
- c.) Washing of stained cells: Add 3 mL of sterile PBS containing 0.2% BSA. Collect cells by centrifugation at 350xg for 5 min. Remove supernatants and save the last 500-µL containing fixed, stained cell suspension in sterile PBS containing 0.2% BSA.

6. Preparation for flow cytometric cell counting: Particulates in fixed, stained cells must be filtered away using a Polystyrene Tube with Cell-Strainer Cap (BD catalog # 352235). Collect cells by centrifugation at 350xg for 5 min. Count cells with a flow cytometer.

V. Specimen Collection, Rare Cell Enrichment and RNA/DNA/Protein Extraction of Isolated Rare Cells

NOTE: The methods and procedures described here can be applied to analysis and enumeration of circulating endothelial cells and circulating endothelial progenitor cells in whole blood, as well as different rare cells in bone marrow.

⁴ Add 8 µL Alexa488-antibodies against epithelial tumor (Epi) markers; 8 µL PE-antibodies against tumor progenitor (TP) markers; 30 µL APC-antibodies against hematopoietic lineage (HL) markers, to the 100 µL cell suspension.

⁵ For flow cytometric measurement of cellular nuclei: 7AAD is used to stain nucleic acid of all fixed cells.

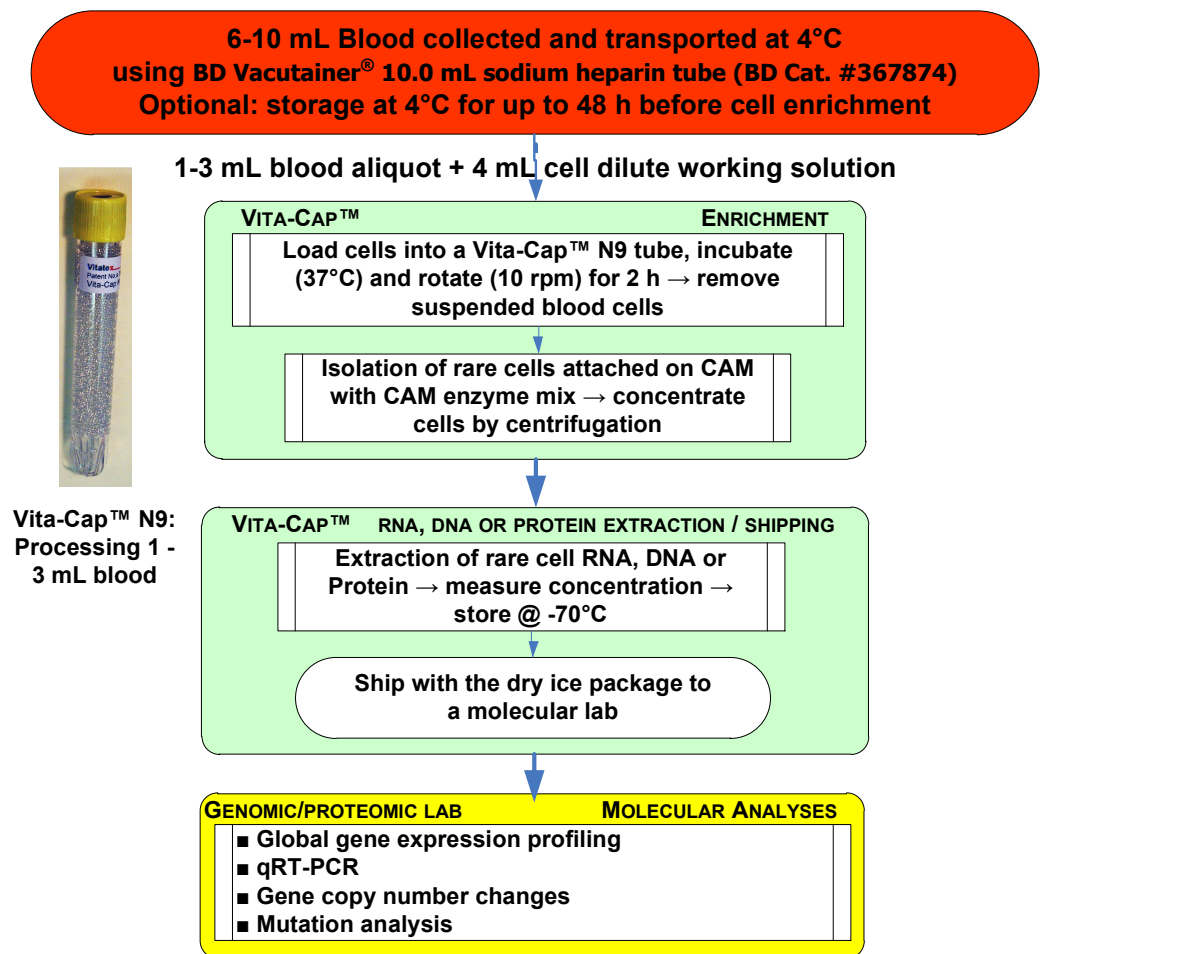


Figure 2: Vita-Cap™ enrichment of CTCs in blood, and extraction of RNA/DNA/Protein for molecular analyses.

VI. RNA Extraction Protocol

- 7. Disrupt the cells:** From Step 4-d.) above, loosen the cell pellet thoroughly by flicking the tube. Add 350 µL of Buffer RLT in RNA Mini kit, and vortex or pipet to mix. **RNeasy spin columns and RNA extraction procedures according to manufacturer’s instruction** [RNeasy Mini Kit (Qiagen Inc., catalog # 74104, www.qiagen.com)].

VII. DNA Extraction Protocol

- 8. Disrupt the cells by adding Buffer RLT:** From Step 4-d.) above, loosen the cell pellet thoroughly by flicking the tube. Extract DNA using Wizard DNA Purification Kit (Promega, Madison, WI), following the manufacturer’s instruction.

NOTE: To study cell population with a defined phenotype, CAM-adherent cells released from the tube by CAM enzyme solution can be further sorted with FACS. Alternatively, negative

selection to remove hematologic lineage cells from the enriched cells can be performed using magnetic particles conjugated with anti-HL antibodies. A specific cell population can then be extracted for their RNA or DNA or protein for molecular analyses of pure cell population.

References

Fan, T., Zhao, Q., Chen, J.J., Chen, W.T., and Pearl, M.L. (2009). Clinical significance of circulating tumor cells detected by an invasion assay in peripheral blood of patients with ovarian cancer. *Gynecol. Oncol.* *112*, 185-191.

Kennedy, A., Dong, H., Chen, D., and Chen, W.T. (2009). Elevation of seprase expression and promotion of an invasive phenotype by collagenous matrices in ovarian tumor cells. *Int. J Cancer* *124*, 27-35.

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