Isolation of CTCs

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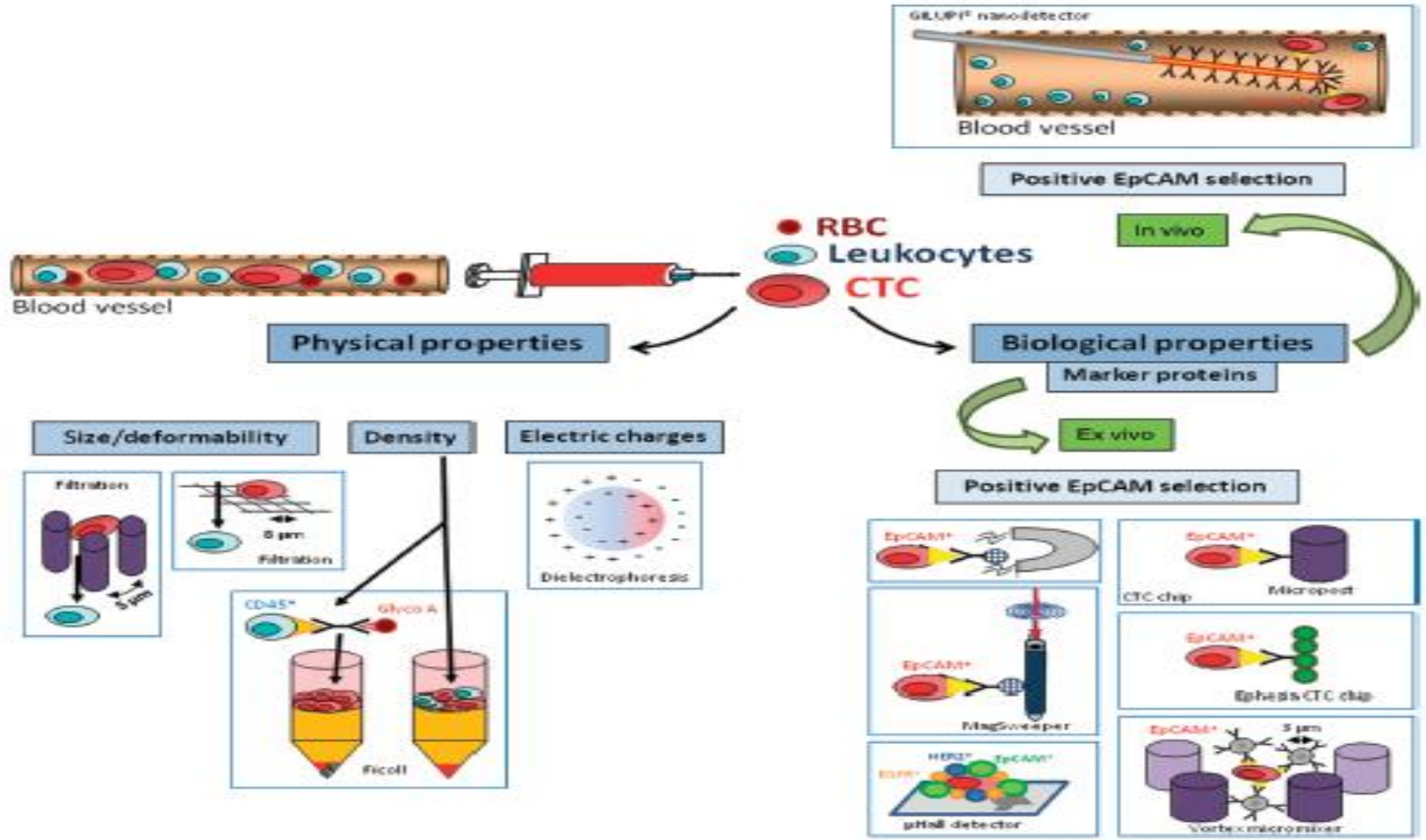
What we know now about CTCs…

- **CTCs are exceedingly rare, with an incidence of approximately 1 CTC per 10^6 -10^9 blood cells**
- During their journey through the vasculature, CTCs are in close association with platelets, macrophages and chemokines, which facilitate their migration
- **Epithelial cells can undergo an epithelial-to mesenchymal-transition (EMT), which induces a substantial change in cell phenotype**
- A sample's CTC count typically depends strongly on the choice of detection methods and may vary from one to several thousand .
- CTC counts can also depend on the timing of blood sampling because a CTC's lifetime in the bloodstream is only 1 to 2 days.
- **Tumor heterogeneity causes considerable variation in the type and number of CTC markers in blood samples**

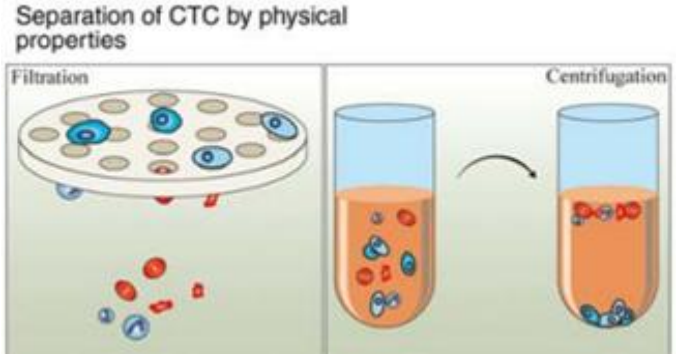
CTC isolation systems



Enrichment of CTCs from the peripheral blood of cancer patients is based on the physical or biological properties of CTCs.

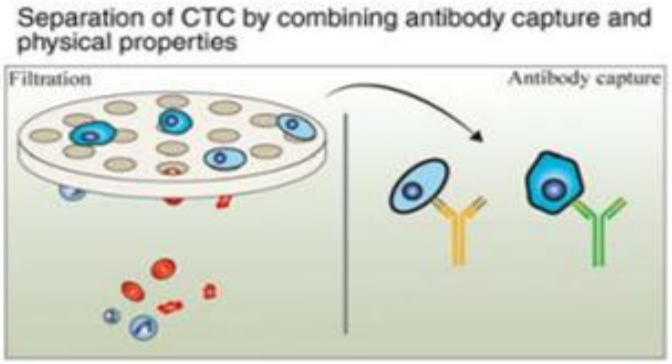
(C. Alix-Panabieres, K. Pantel, Clin Chem, 2013)

Strategies for enrichment of circulating tumor cells



These methods rely on the physical properties of CTCs, such as size, density, and electrophysiological characteristics

These methods rely on specific markers on the surface of CTCs to separate them using antibody capture



These methods combined the use of antibody capture with separation based on physical properties, such as preliminary isolation by size of CTCs, and further screening of CTCs using Ep-CAM antibody capture

Separation of CTCs by physical properties

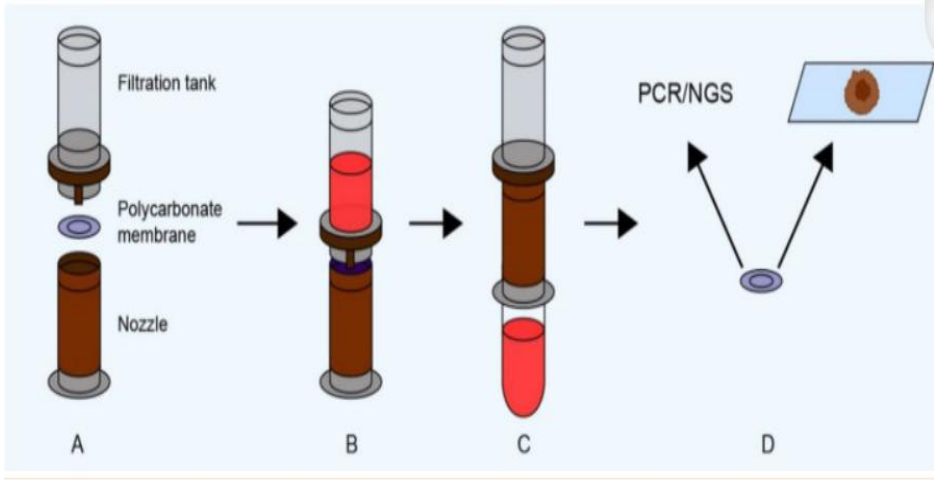
Enrichment methodologies via physical properties are based on unique CTC properties such as their:

- **size of CTCs**

  The diameter of CTCs (~16–20 µm) significantly exceeds that of other blood cells such as red blood cells (RBCs; ~8 µm) and white blood cells (WBCs; ~8–14 µm), and is often used to separate them

Isolation by Size of Epithelial Tumor Cells: ISET

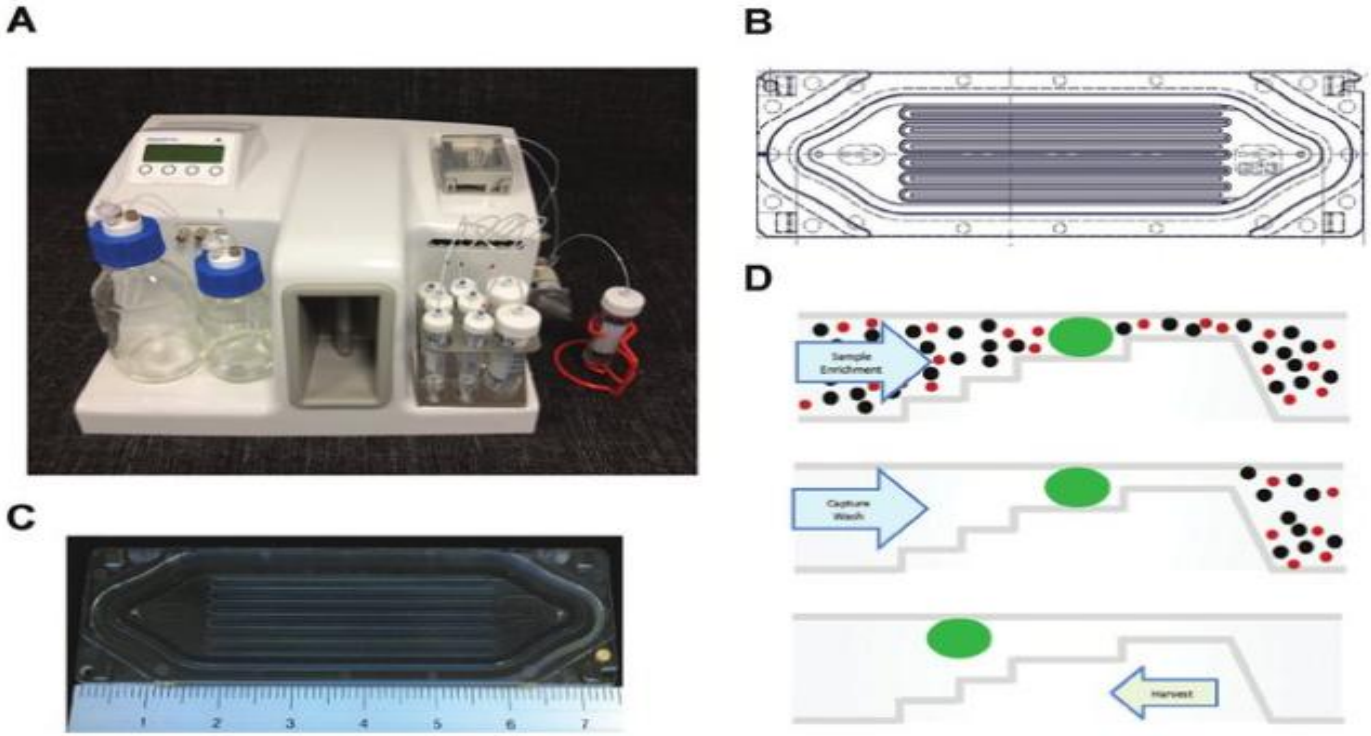
ISET is one of the earliest size-based methods to capture CTCs by directly filtering blood through a calibrated, polycarbonate Track-Etch-type membrane with 8-µm-diameter pores.



This kind of method is characterized by quick, non-selective and label-free separation for CTCs. However, ***one pitfall of this technique is its inability to distinguish monocytes from CTCs in blood samples***

PARSORTIX

The system employs a microfluidic chamber (a Parsortix® cell separation cassette) to capture cells of a **certain size** and deformability from the population of cells present in blood. The cells retained in the cassette are harvested by the Parsortix® PC1 system for use in subsequent downstream assays. Seven-point five mL of whole blood can be processed within 2 h.



The main defect of this method is the difficulty of removing the leukocytes of similar size to CTCs

The Parsortix® PC1 system

FDA cleared for the capture and harvest of intact CTCs from metastatic breast cancer patient blood

ANGLE

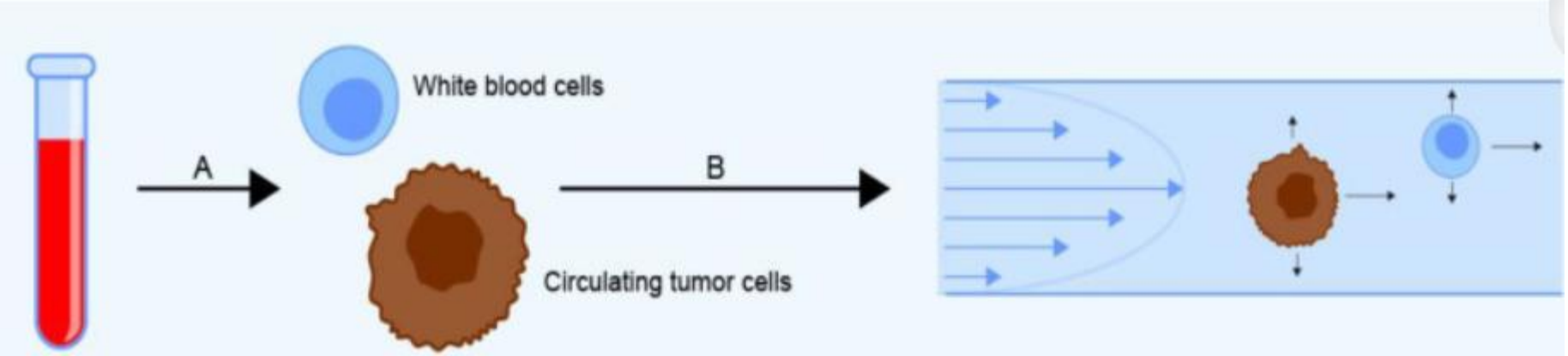
Separation of CTCs by physical properties

Enrichment methodologies via physical properties are based on unique CTC properties such as their:

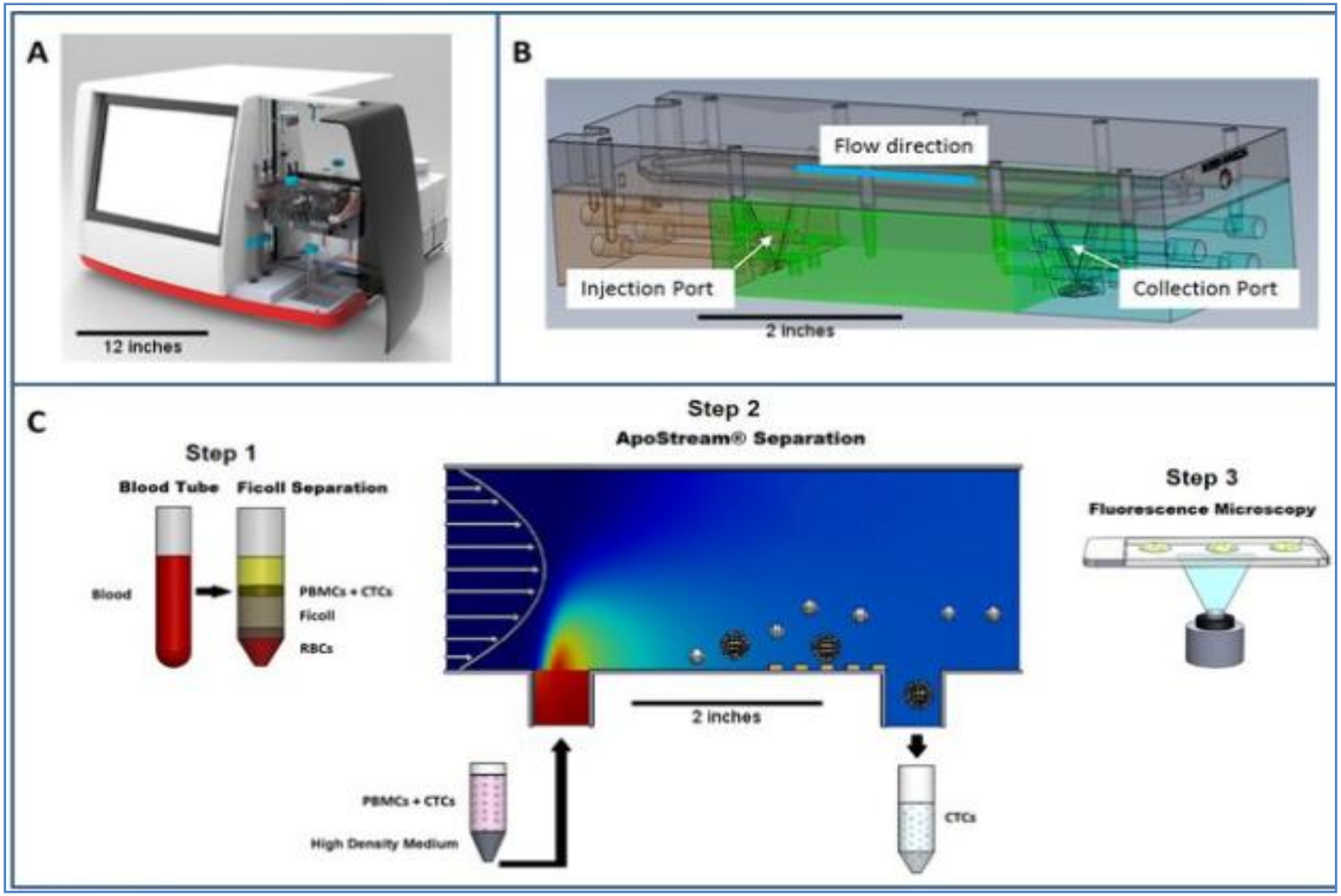
- **membrane capacitance- Electric-charge-based electrophoresis**

CTCs can also be separated by dielectrophoresis (DEP) as CTCs exhibited a distinctive dielectric property. Enrichment based on differences in surface charge and polarizability minimize the injury of captured CTCs, which is favorable to future analysis.

This strategy supports the assumption that tumor cells carry more negative surface charges, or higher zeta-potential, compared to WBCs



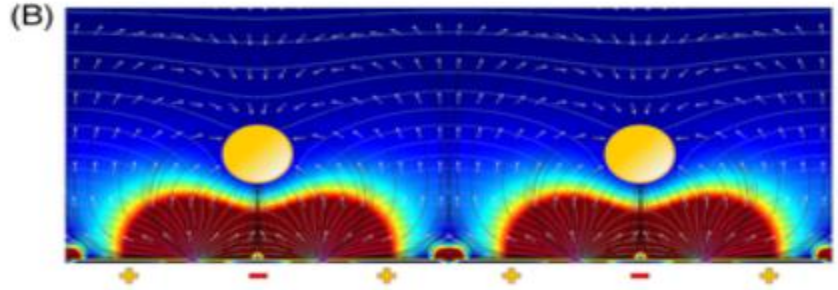
ApoStream®

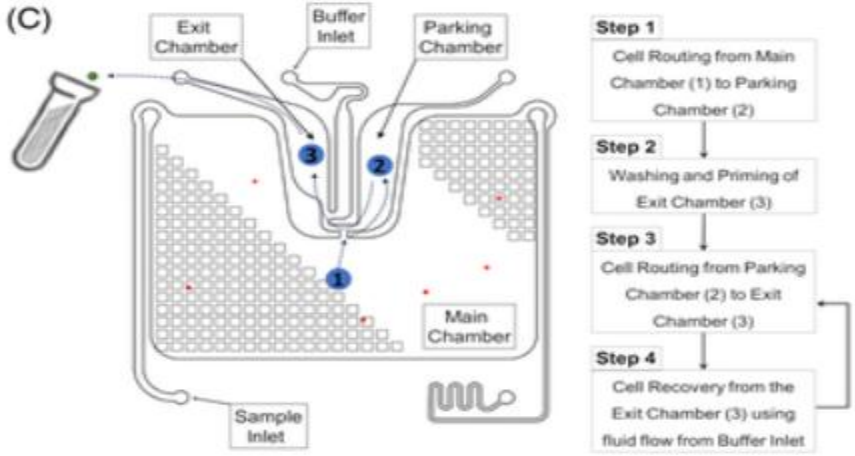


A disadvantage of this method is that there is a partial overlap in the zeta-potential distribution between WBCs and CTCs, resulting in WBCs to remain in the enriched CTCs

https://doi.org/10.1371/journal.pone.0175414

DEPArray™ system: An automatic image-based sorter for isolation of pure circulating tumor cells





DEPArray™ technology, a microchip-based digital sorter, which combines precise microfluidic and microelectronic enabling precise, image-based isolation of single CTCs, which can then be analyzed by Next Generation Sequencing (NGS) methods.

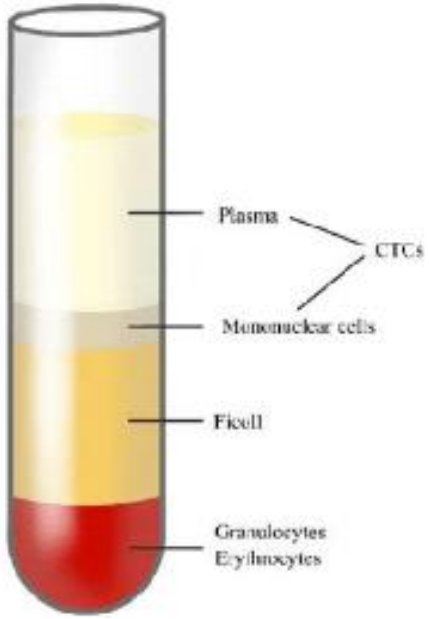
This is a robust image-based cell-sorting technology combines microelectronics and microfluidics in a unique platform

https://doi.org/10.1002/cyto.a.23687

Separation of CTCs by physical properties

Enrichment methodologies via physical properties are based on unique CTC properties such as their:

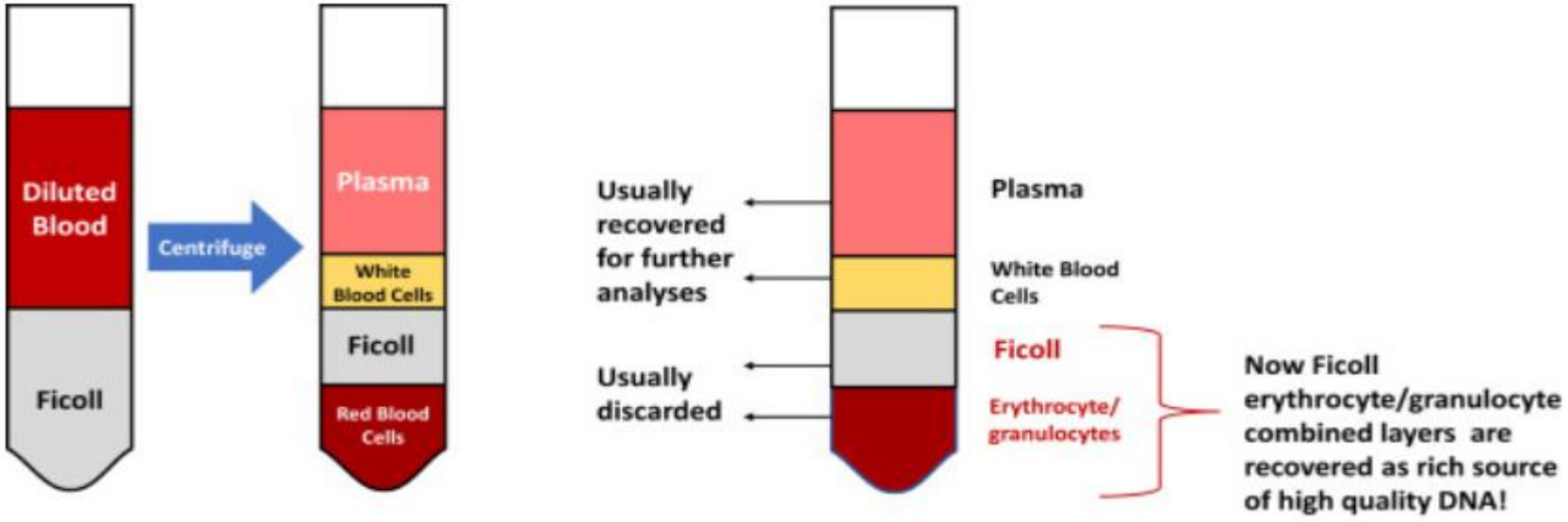
- **Density:** A whole blood sample contains multiple types of cells, e.g., numerous red blood cells (RBCs), nucleated white blood cells (WBCs), and heterogeneous CTCs populations with variable numbers. Dr. Seal (in 1959) found that different cell types in whole blood exhibited the discrepancy in cell density and utilized silicone oil blends to obtain the optimal cell isolation medium.
- The whole blood was separated by centrifugation into the following layers (from bottom to top): erythrocytes, granulocytes, density gradient, buffy coat [the fraction containing mononuclear cells (MNCs), including CTCs], and plasma



Ficoll-Paque

Ficoll-Paque, which are mixtures of high molecular weight sucrose polymers and sodium diatrizoate, can be used for isolating peripheral blood MNCs .

However, due to the cytotoxicity of Ficoll, the formation of cell aggregates may lead to the loss of tumor cells that migrate to the bottom of the medium



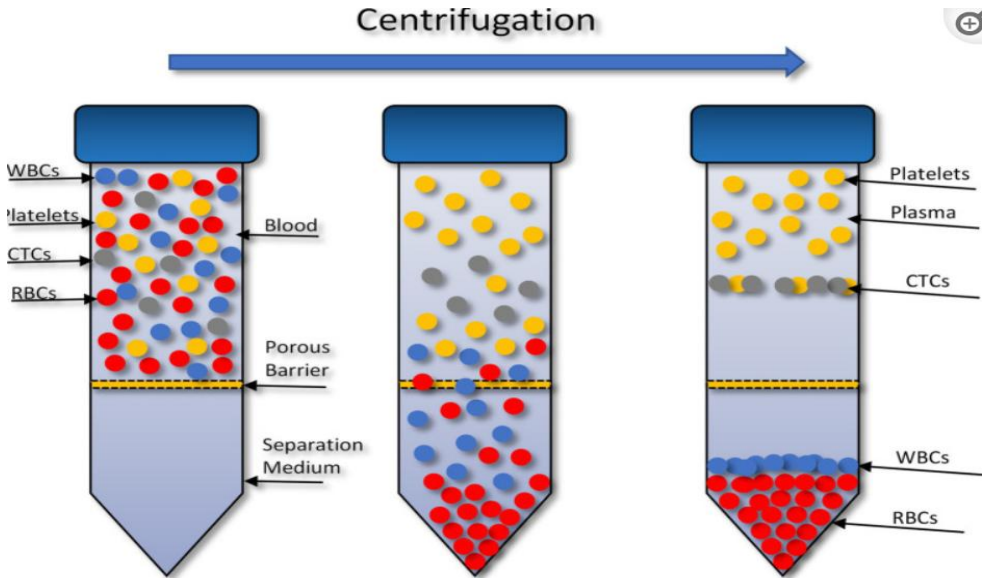
OncoQuick

OncoQuick isolates CTCs through density gradient centrifugation

❑OncoQuick places a permeable apparatus on the separation media in a 50-mL tube to collect CTCs while permitting the erythrocytes and leukocytes to pass through depending on their different buoyancy densities during centrifugation.

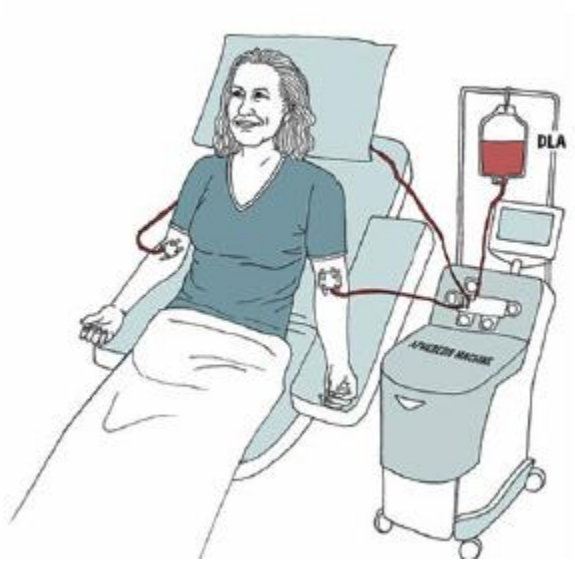
❑The OncoQuick® tube spin at 1,600 *g* for 20 min at 4°C, with a slow acceleration and no brake. Following centrifugation any captured tumour cells resided between the lower separation medium (blue) and the upper plasma (yellow).

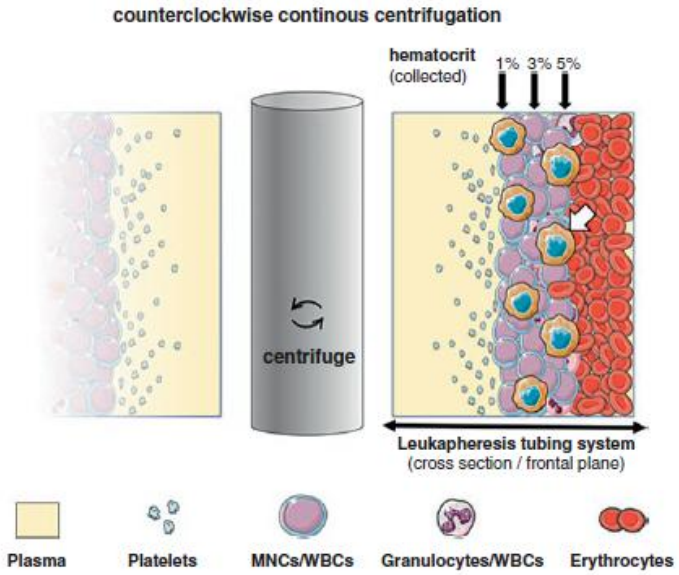
➢*A limitation of this type of enrichment technique is that it cannot exactly get together all the plasma after centrifugation, which results in the loss of potential CTCs. It is likely that CTCs may move into the plasma fraction and the aggregates of CTCs fall to the bottom of the gradient*

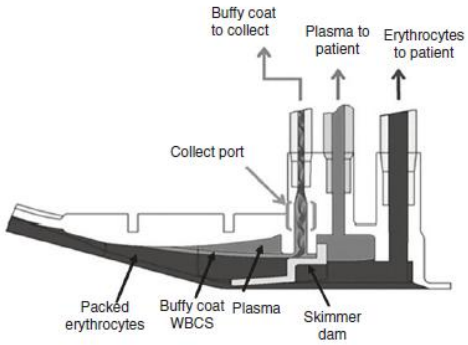


Diagnostic Leukapheresis (DLA)

Leukapheresis separates the WBCs by size and density centrifugation in a continuous-flow procedure.

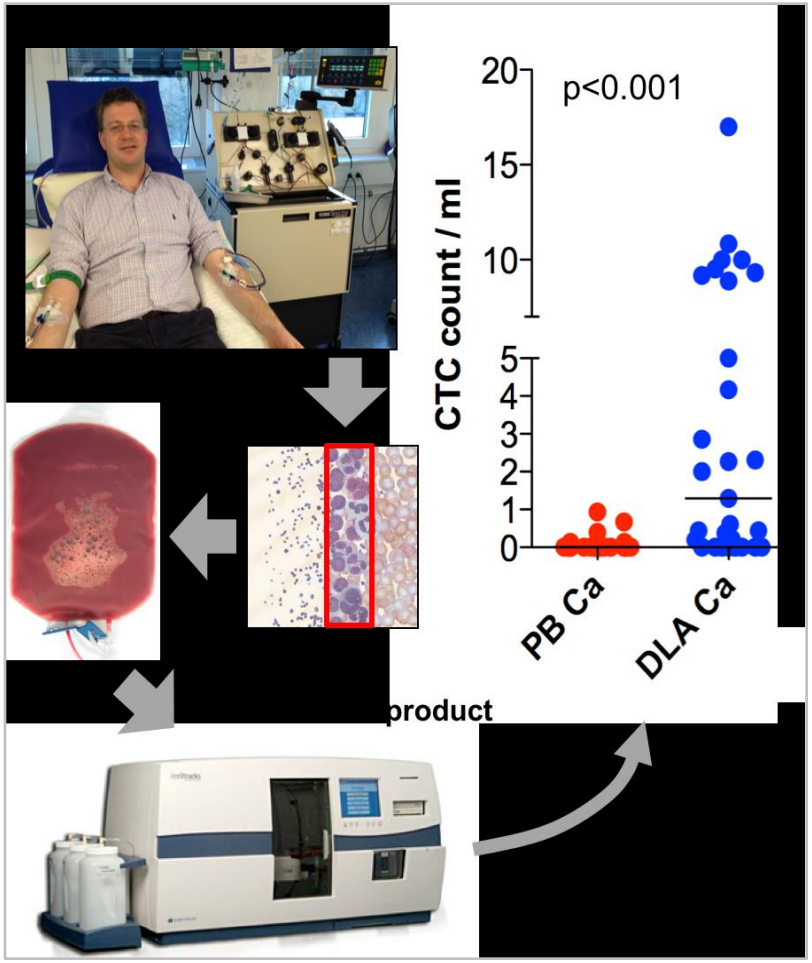






The patients are connected to the apheresis machine via two peripheral venous catheters (PVC), placed in each cubital vein. Through the efferent PVC line, the patient's blood passes through the machine, where the blood becomes anticoagulated and enters the rotating centrifuge. Due to the counterclockwise flow, blood cells with higher density such as erythrocytes migrate to the outside of the channel, plasma drifts to the center, and the buffy coat (WBC) migrates to the middle. The targeted blood fraction can then be drawn into ports located at different distances from the center of the channel via a connector and can be collected into a blood bag.

Diagnostic Leukapheresis (DLA)



Density based cell separation
- Target-cells: 1.05 – 1.088 g/mL
- 2-2.5 Liters of blood
- Duration ~ 1 hour

- 29 DLA products of 23 patients
(PDAC, Breast CA and GI CA)
 Majority cM0 Patients

- 2.3 mL in CS (DLA)
~60 mL blood equivalent

Detection rate 72% (vs. 28% in PB)

 Median of 612 CTCs (29-13102) in Circulation (4.5 L)

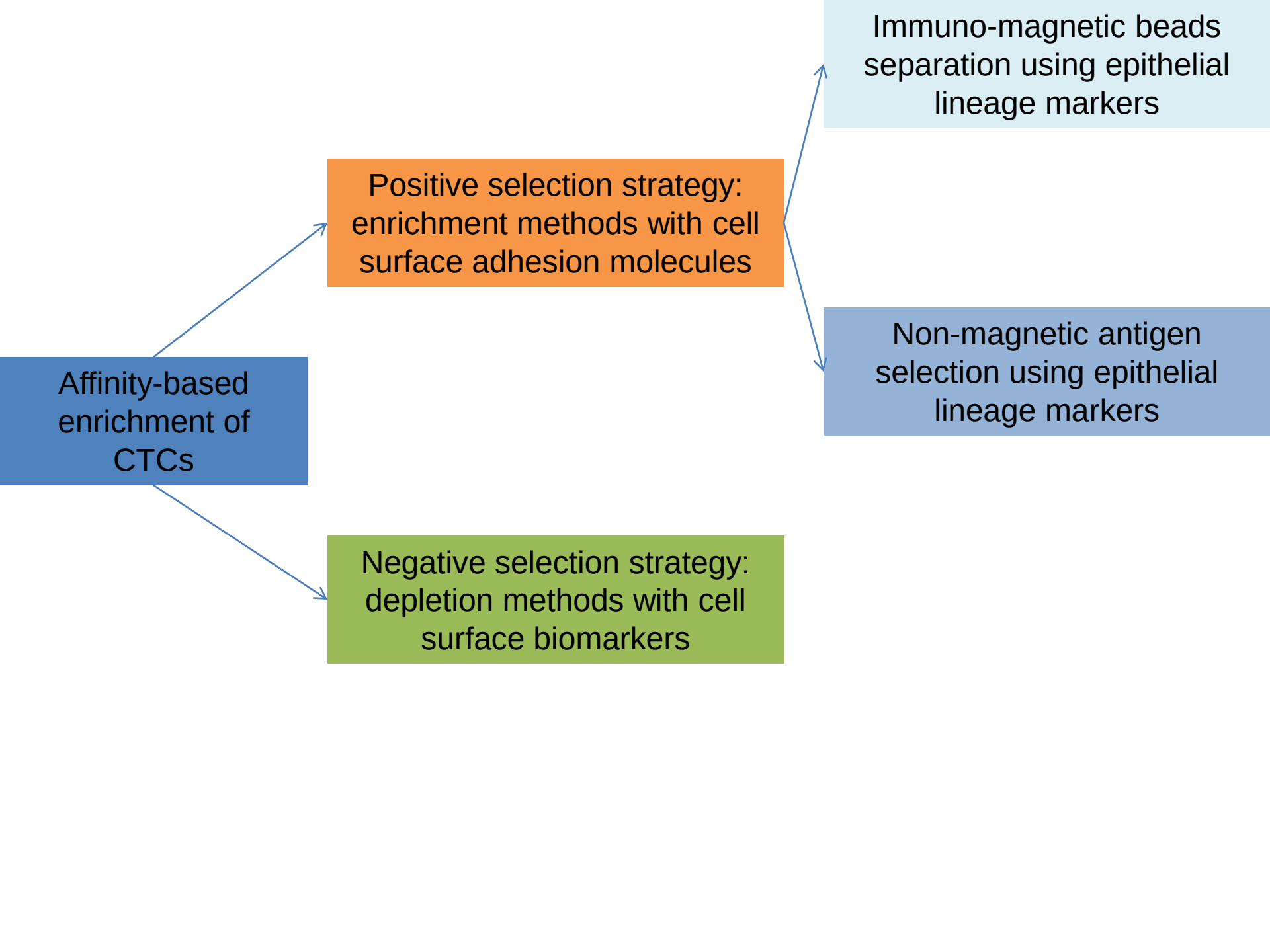
Fischer J C et al. PNAS 2013

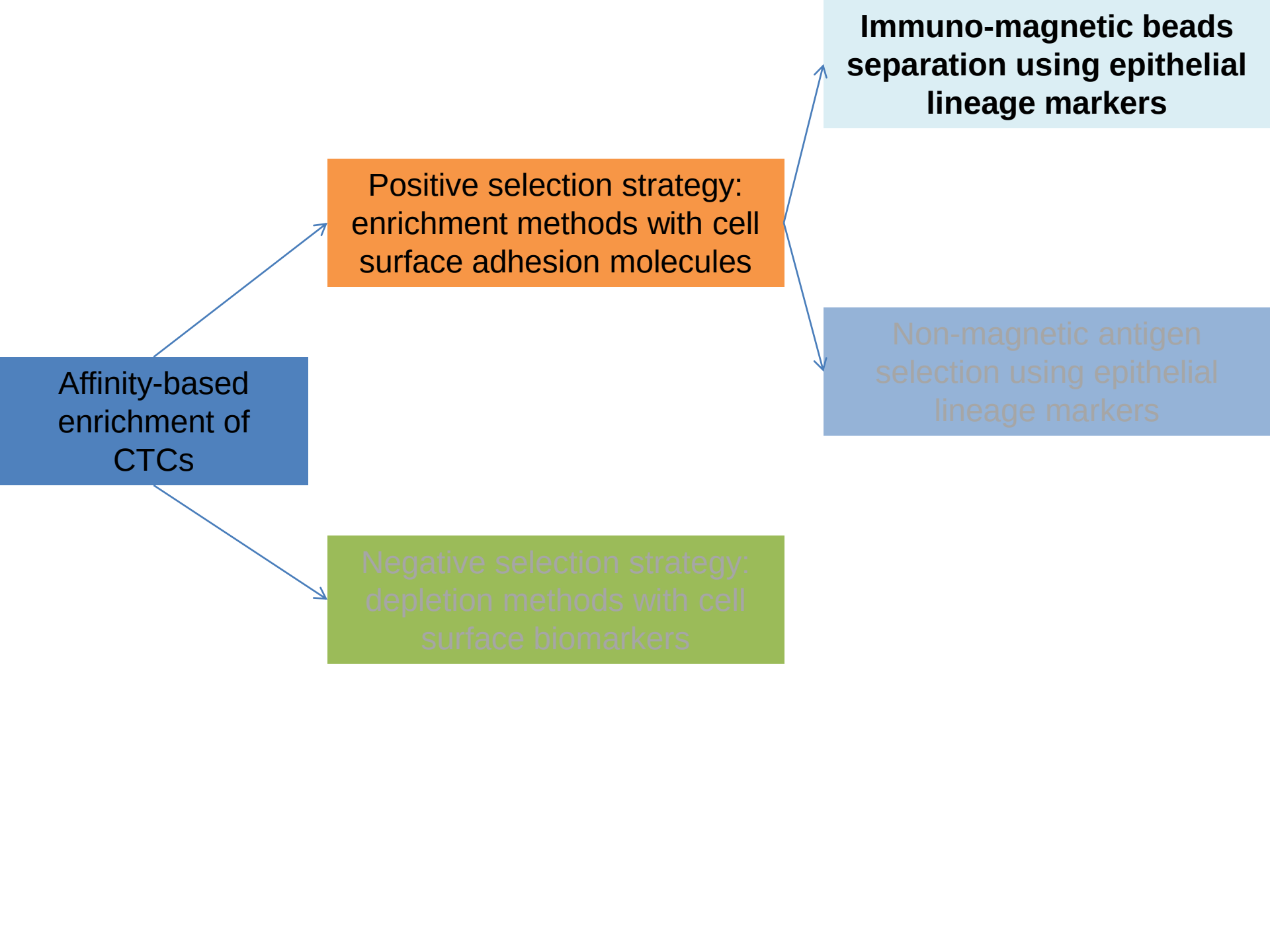
Separation of CTCs by antibody capture (1/2)

- CTCs can also be isolated based on their biological properties, e.g., the presence of cell surface adhesion molecules or deficiency in hematological surface antigen markers could be utilized to distinguish CTCs from blood cells
- The most common technique used for CTC isolation is the immune isolation, which is based on specific CTC cell surface markers.
- EpCAM is an antigen often used in positive selection due to the epithelial origin of most CTCs, while CD45 is used for negative selection as specific antibody for leucocytes .
- However, various other markers such as EGFR, prostate-specific membrane antigen (PSMA) in prostate cancer and HER2 in breast cancer-specific CTCs have also been explored

Separation of CTCs by antibody capture (2/2)

- CTCs do not express the specifically chosen marker in all the cells; therefore, enrichment of CTCs through single positive selection marker generally introduce bias.
- The major drawback of CTC enrichment through negative selection is that the purity of enriched CTCs is compromised. There is also the risk that the CTCs may be entrapped within the bulk of blood cells, resulting in inadvertent loss of CTCs.


Affinity-based enrichment of CTCs
Positive selection strategy: enrichment methods with cell surface adhesion molecules
Negative selection strategy: depletion methods with cell surface biomarkers
Immuno-magnetic beads separation using epithelial lineage markers
Non-magnetic antigen selection using epithelial lineage markers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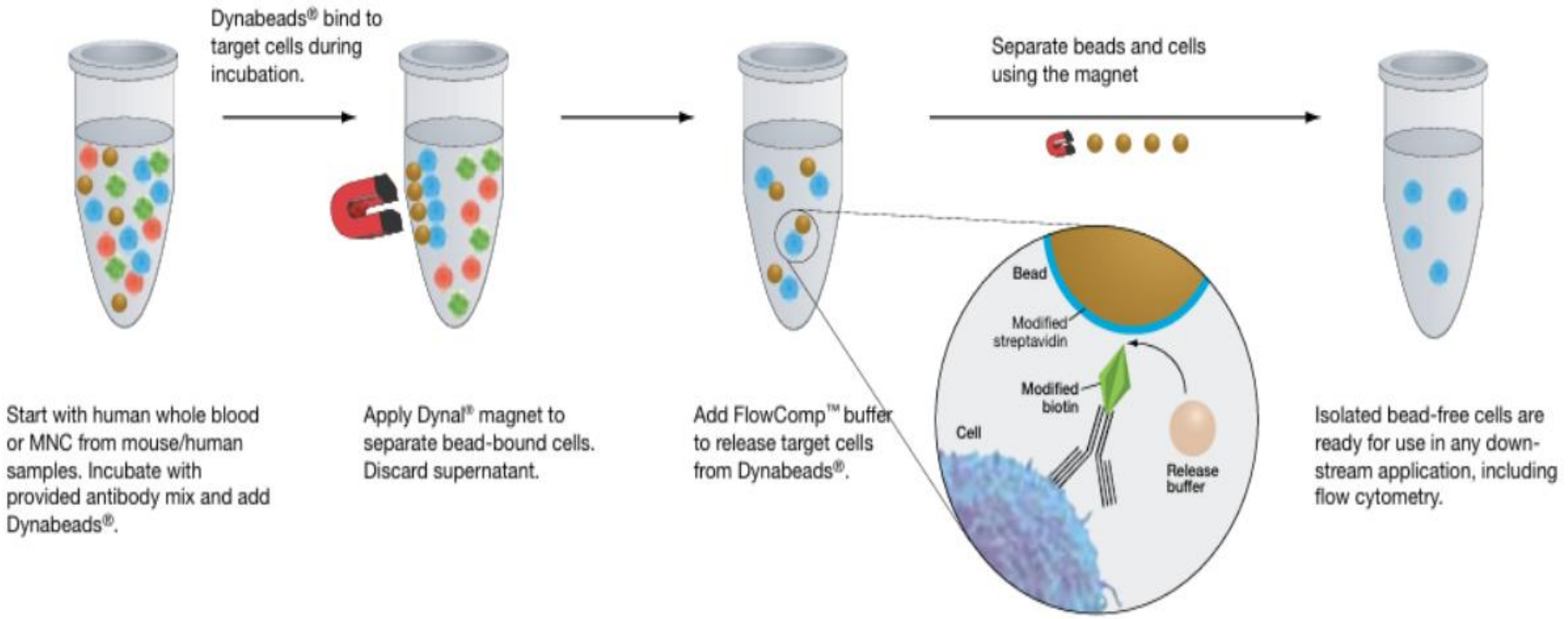
DynaBeads

- DynaBeads® and EasySep are immunomagnetic methods in which antibodies recognizing cell surface antigens are coupled to magnetic beads and used to remove unwanted cells.

- It is not easy to enumerate massive Dynabead-bound cells due to the autofluorescence of the beads, the large number of beads, and the low efficiency of the labeling of cell antibodies when the beads are bound.



EasySep

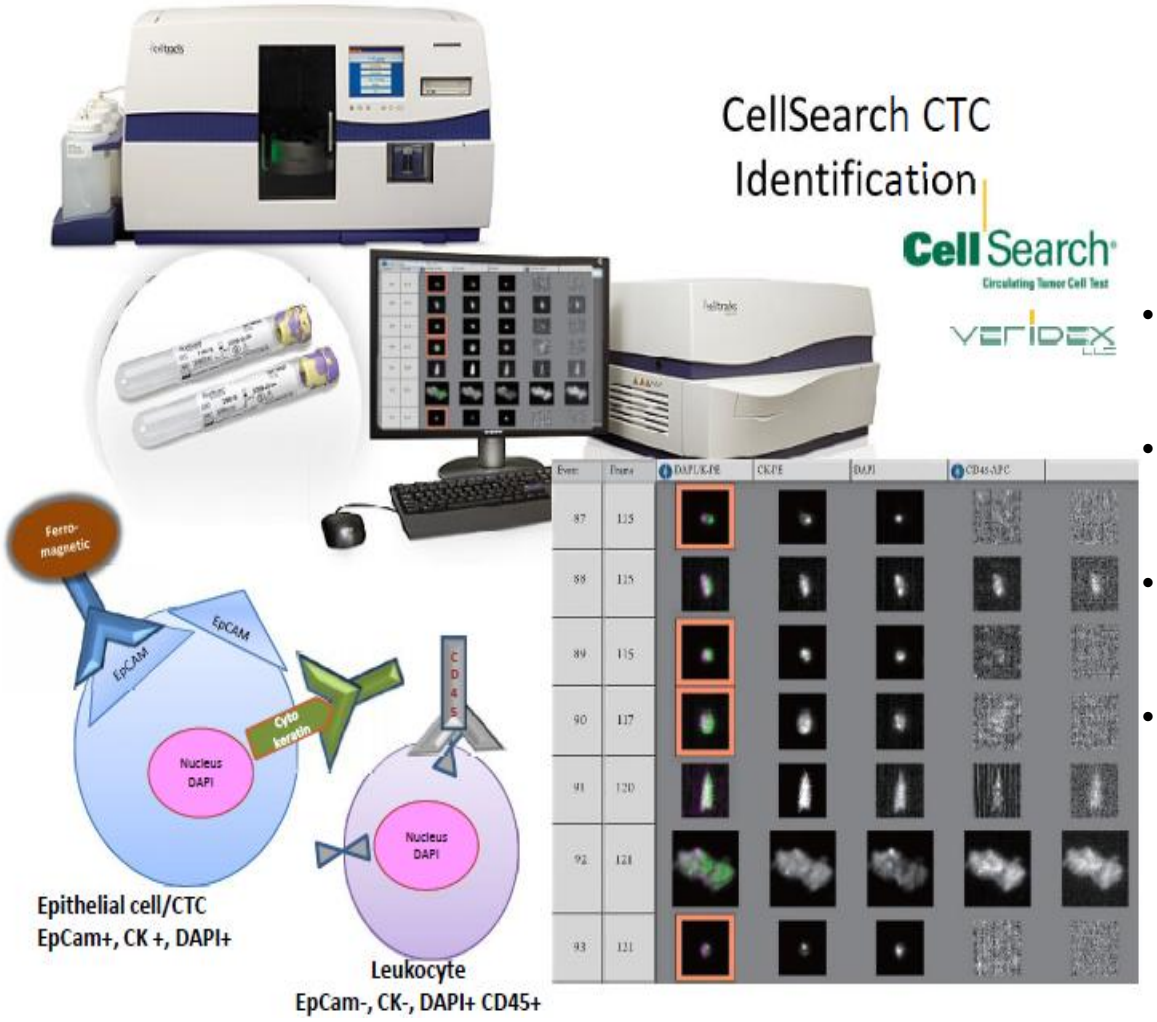
The smaller EasySep nanoparticles do not interfere with downstream immunocytochemical processing and are able to achieve higher purity

1. Add EasySep™ Direct isolation cocktail and EasySep™ Direct RapidSpheres™ to whole blood
2. Place tube in EasySep™ magnet and incubate for 5 minutes*
3. Pour off desired fraction into a new tube and add EasySep™ Direct RapidSpheres™ to enriched cells
4. Place tube in EasySep™ magnet for 5 minutes*
5. Pour off desired fraction into a new tube

*Times are typical for EasySep™ Direct kits. Times for each kit will vary depending on the exact isolation protocol.

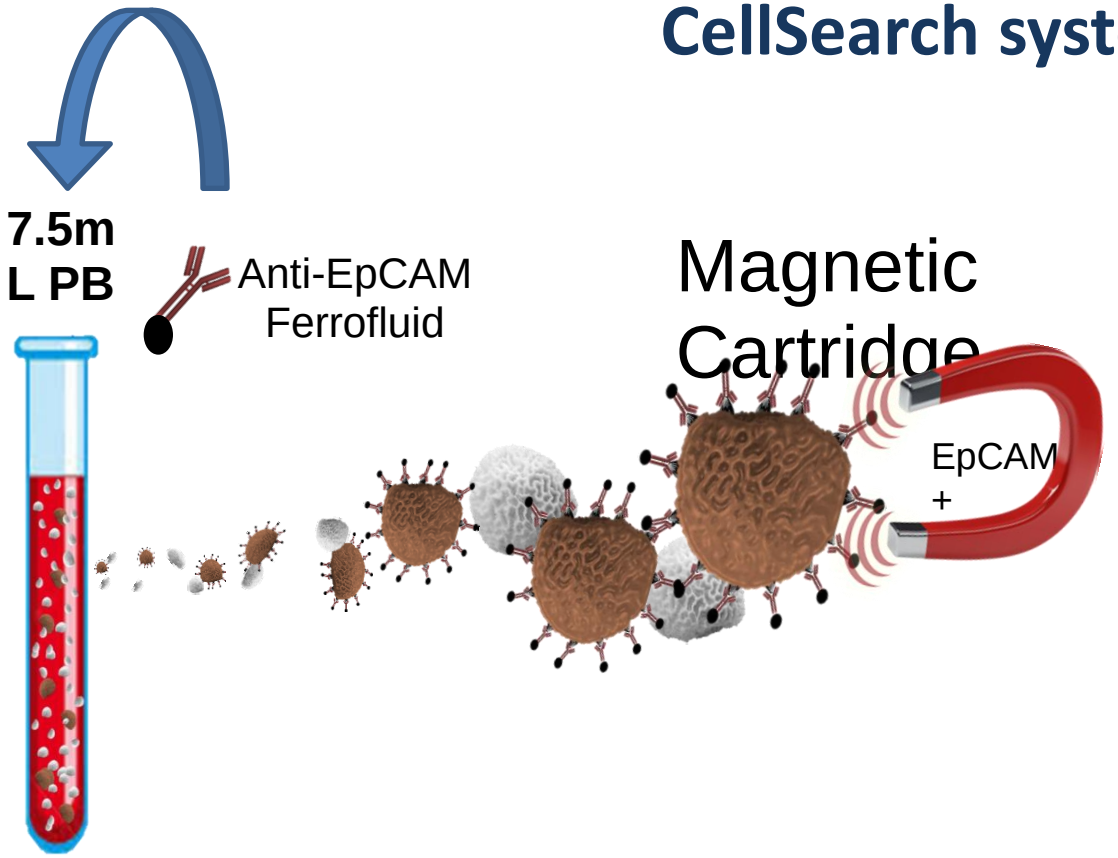
CellSearch – FDA cleared system

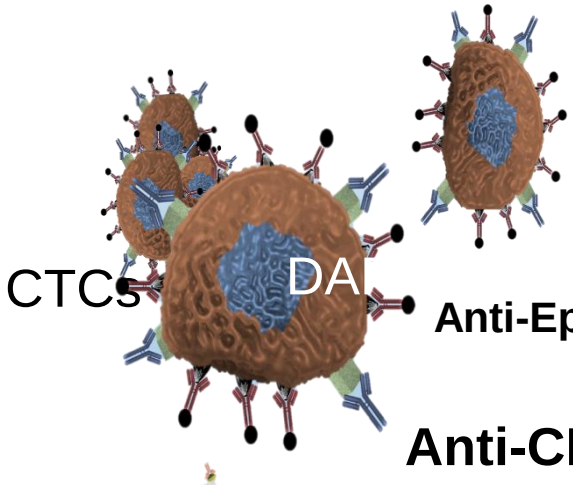
- In 2004, the CellSearch® system was introduced as the first and only Food and Drug Administration (FDA)-approved method for the enumeration of CTCs in 7.5 mL of blood.
- The highest proportion of positive specimens was detected in patients with metastatic prostate cancer, followed by metastatic ovarian cancer and breast cancer

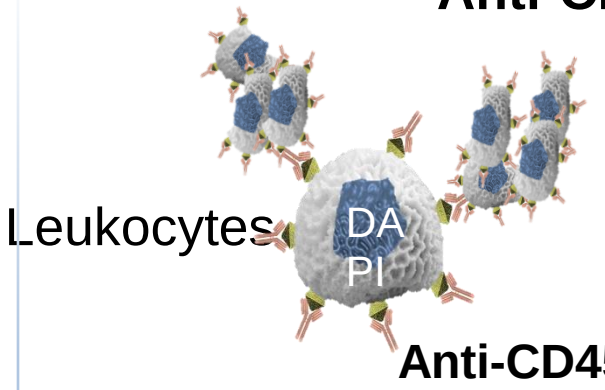


- **Immunomagnetic enrichment**
- **Epithelial cells: Anti cytokeratin staining**
- **Leukocytes: anti CD45**
- **Nuclei: DAPI**

CellSearch system



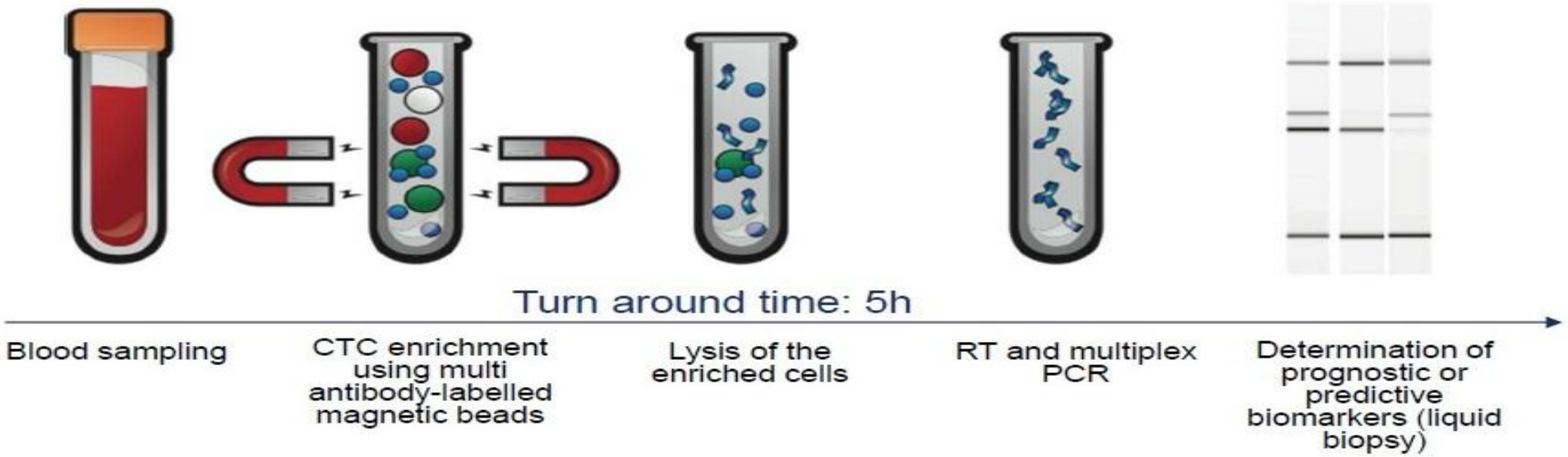




Most nucleated cells in the blood are white blood cells. CTCs are enriched by a magnetic cartridge and are identified by being EpCAM+, CK+ and CD45-. They can afterwards be counted or analyzed.

AdnaTest
(QIAGEN, Hilden, Germany)

The limitation of the fact that CellSearch® detects EpCAM+ cells leading to the loss of EpCAM-cells has been improved in other methods by combining different specific tumor Markers (MagSweeper, AdnaTest® , IsoFlux™ [90], and CTC-µChip).



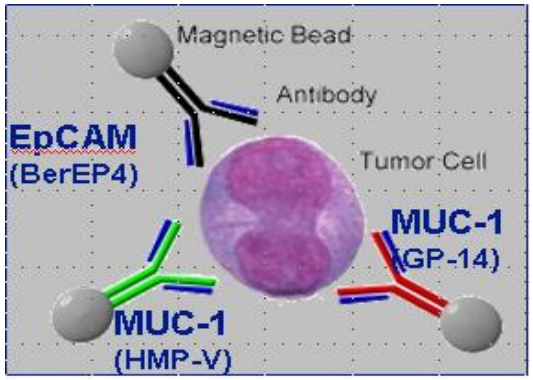
5ml of peripheral blood

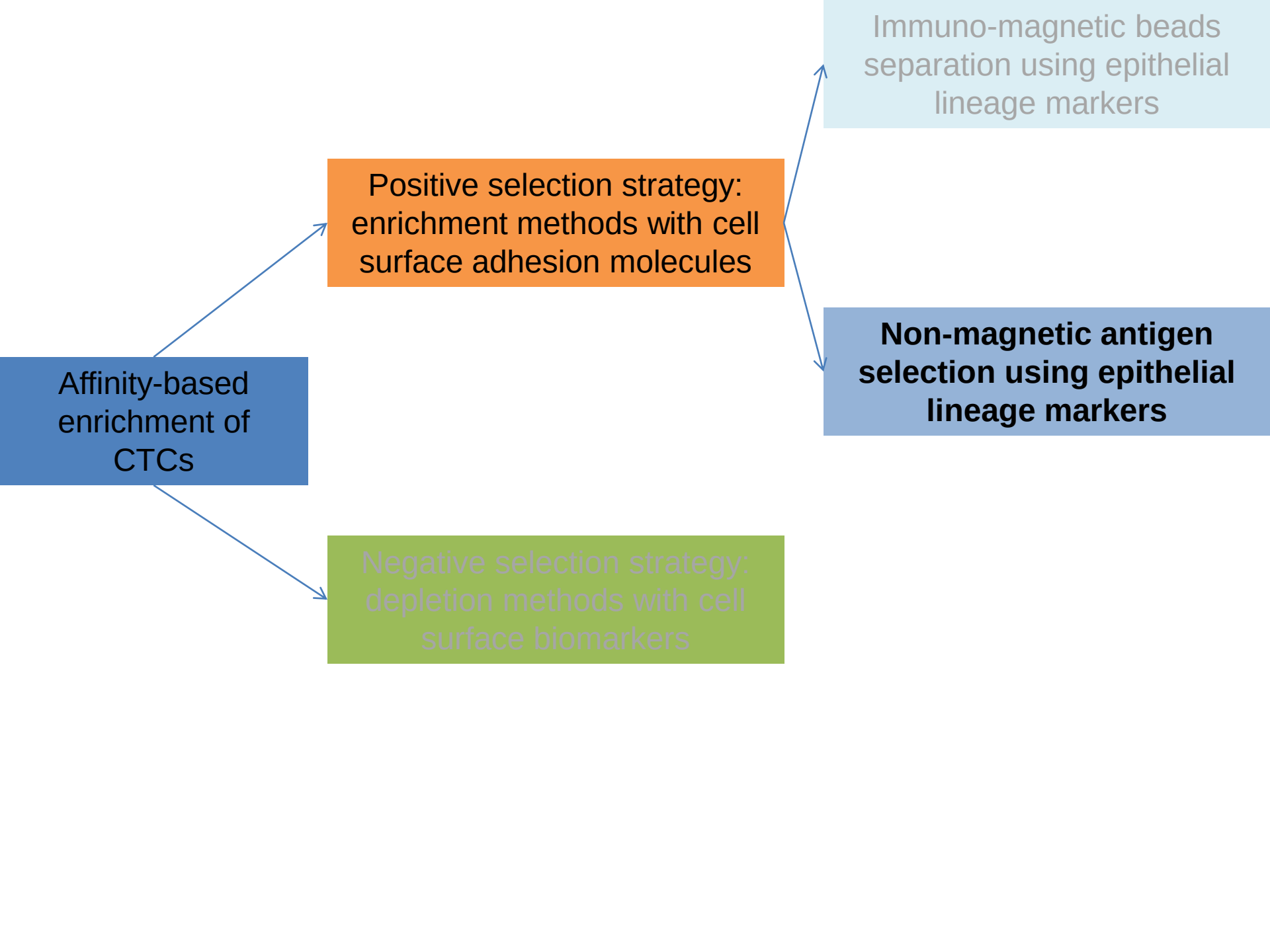
BrCa: EpCAM, MUC-1 and HER2

PCa: PSMA, PSA, EGFR and AR

OvCa: EpCAM, MUC-1, CA125 and ERCC1

CRC: EpCAM, CEA and EGFR

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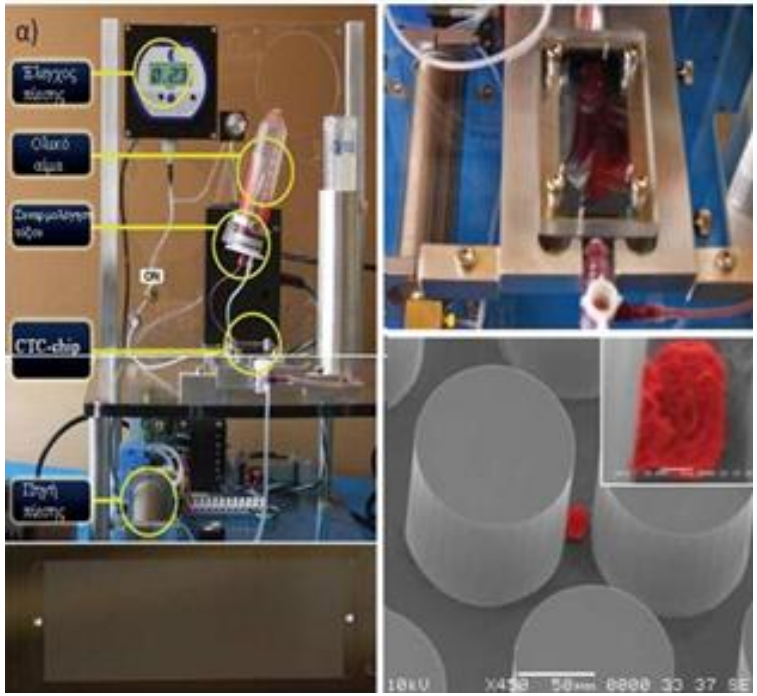
CTC-chip

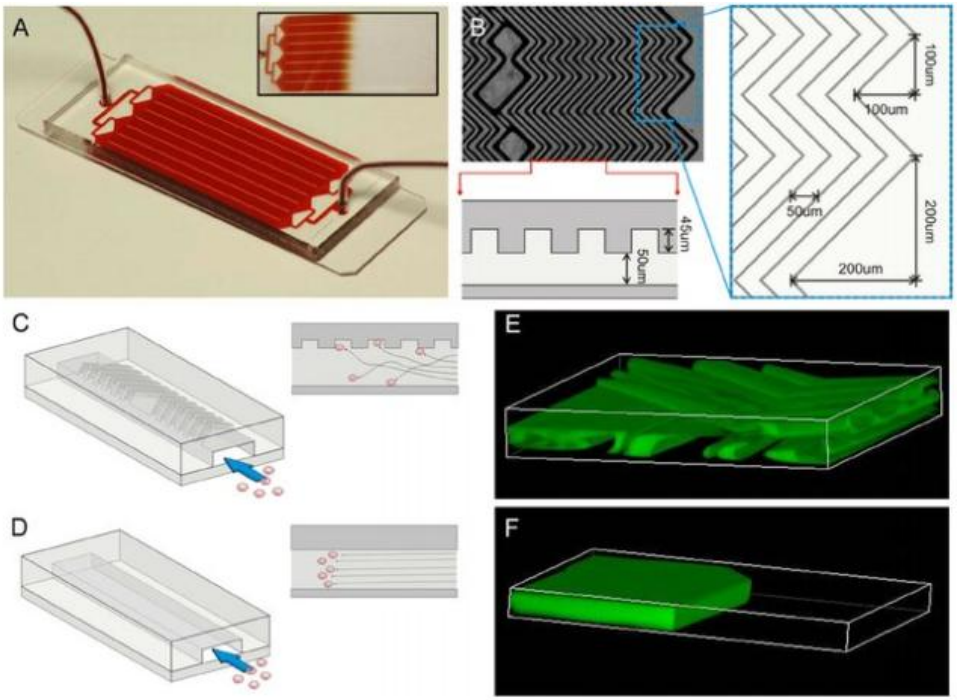


Nagrath S et al. Nature, 2007

- "CTC-chip" is the, which was exploited by Nagrath *et al.* for more efficient and selective isolation of CTCs.
- It is composed of 78,000 microposts conjugated with anti-EpCAM antibody and manipulated under preestablished laminar flow conditions without prelabeling or processing.
- the blood flows through the microfluidic chip, EpCAM-positive CTCs run into the microposts and are retained

A pitfall of the first-generation CTC-chip was that the sophisticated micropost design is difficult to achieve high-throughput production.

Herringbone chip (HB-chip)



❑ HB-chip induces the formation of microvortices generated by passive blending of blood cells to extremely increase the chance of interaction between anti-EpCAM antibodies-coated walls of the channel and CTCs

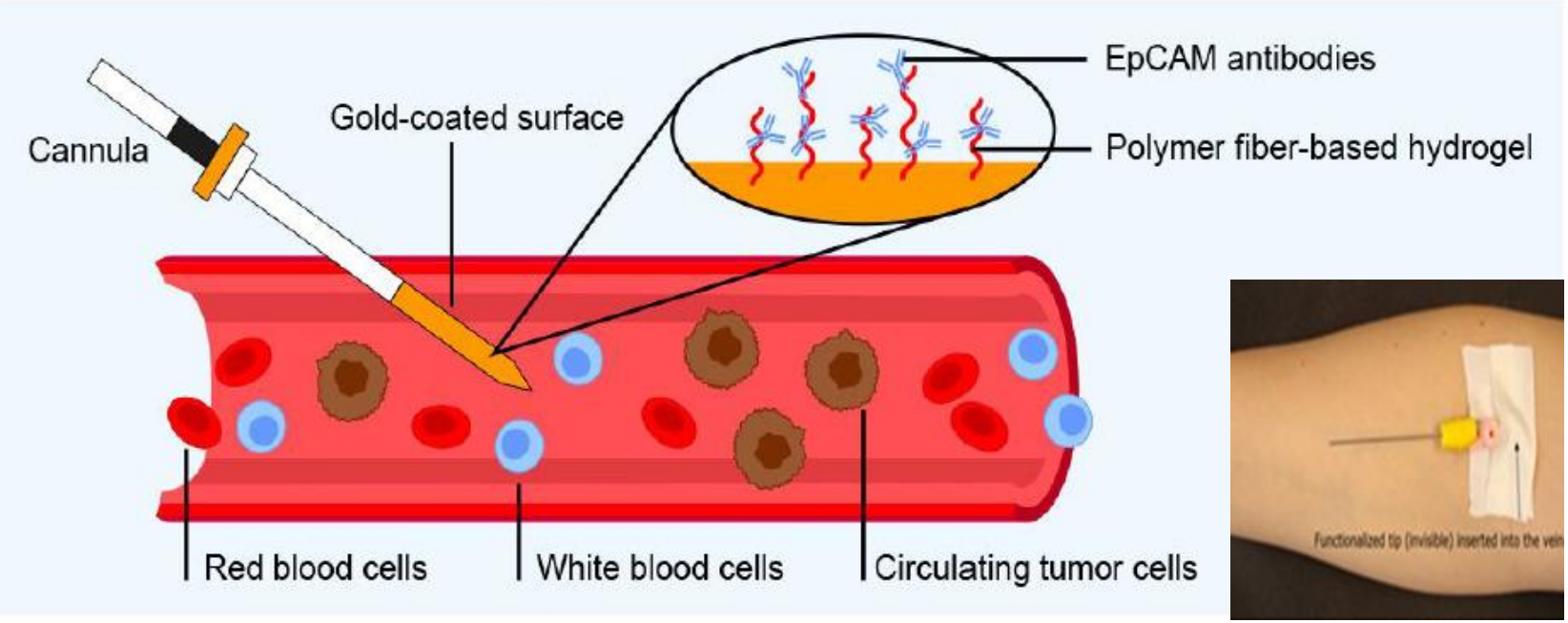
❑ Isolated CTCs are directly used to further analysis on the device.

DOI: 10.1073/pnas.1012539107

The advantage of these chips is that many diverse tumor-specific antigens can be combined for CTC enrichment. Despite the high efficiency of these systems in isolating CTCs, acquiring intact and viable cells from the systems is still a challenge

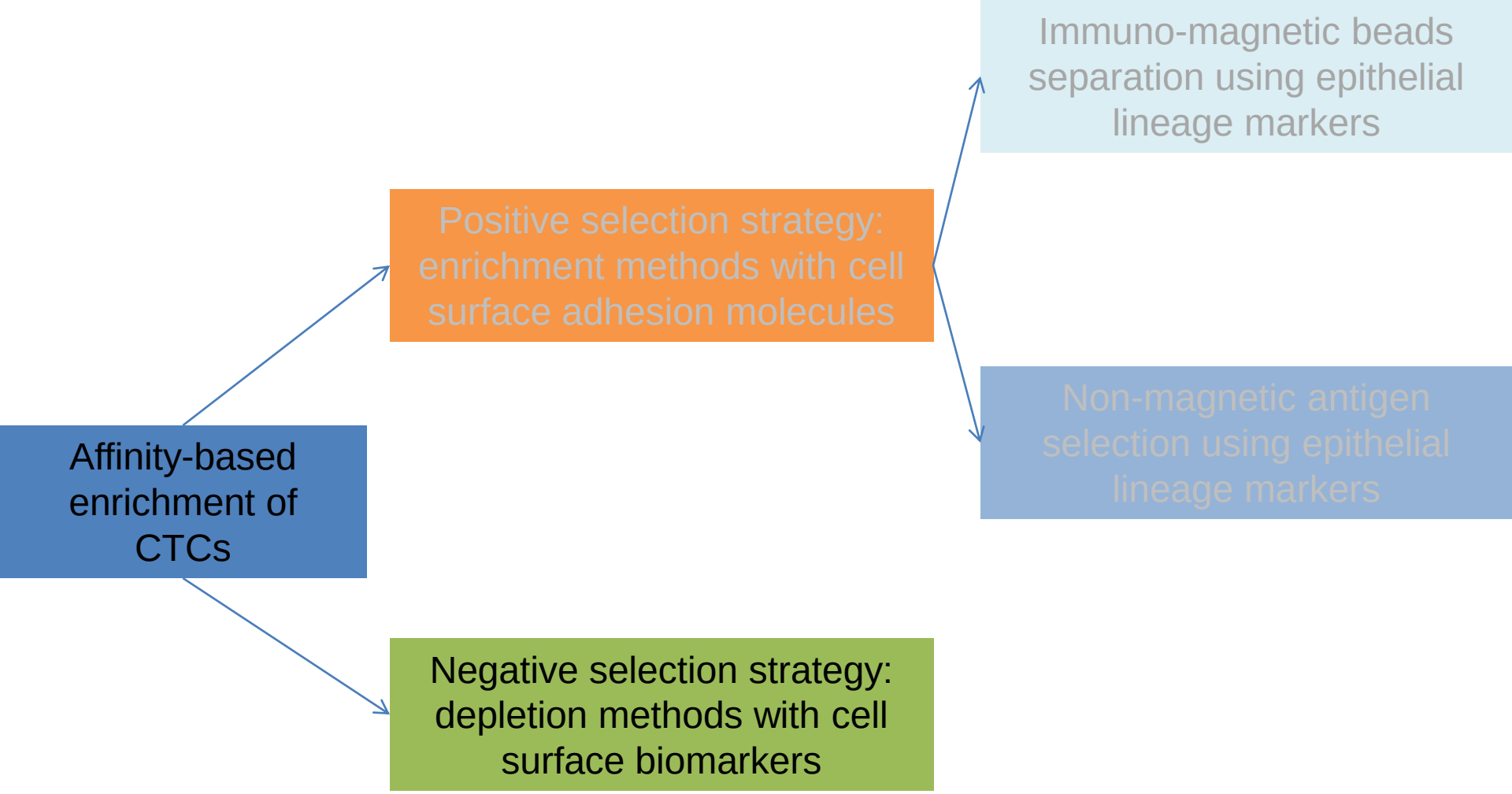
GILUPI Cell Collector / Magwire

EpCAM in-vivo enrichment



❑GILUPI GmbH CellCollector, an in vivo and novel technology, uses an anti-EpCAM wire directly into the peripheral arm vein and captures targeted cells with high efficiency.

❑This technology has the ability to process approximately 1.5 L of blood in 30 minutes, which improves the device's sensitivity, thereby rendering it a promising candidate for future CTC studies



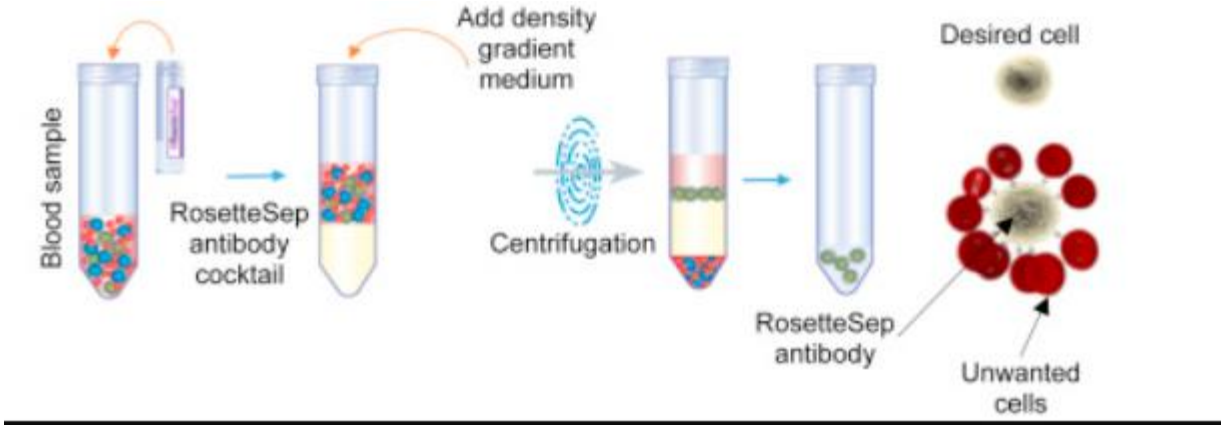
Besides the positive tumor cell markers-based affinity capture of CTC cells, CTCs can also be negatively selected based on blood cell-specific surface markers.

A universe technique for WBC removal is the application of CD45 antibody-labelled magnetic beads. WBCs are depleted by mixing the blood sample with magnetic beads.

To date, several commercially available depletion kits are from StemCell Technologies, Miltenyi Biotec and ThermoFisher Scientific (Waltham, MA, USA)

RosetteSep: immune density cell separation kit

This kit separate and enrich CTCs from normal hematopoietic cells using antibody cocktail for the removal of unwanted cells by changing their density.
The excess cells settle through density gradient centrifugation, and purified tumor cells appear at the interface between the density gradient medium and the plasma



Unneeded cells are cross-linked with RBCs by specific antibodies to form a dense immune rose structure; unlabeled and highly purified target cells are left at the interface between plasma and density gradient centrifuge during density gradient centrifugation

One defect of the negative selection method is that not all nucleated cells in the blood express CD45. And an even more serious pitfall of negative selection is the high loss rate of CTCs due to non-specific bulk effect

Technology	Selection criteria	Assay system	Advantages	Disadvantages
Physical property-based assays				
Density gradient centrifugation	Density	Ficoll-Paque; Percoll; Lymphoprep; OncoQuick	High cell viability; inexpensive	Loss of very small CTCs and cell aggregates; low purity extra enrichment technologies required
Size-based filtration exclusion	Size	ISET; DEF	Quick; non-selective	Inability to distinguish monocytes from CTCs; loss of the CTCs with similar size to WBCs
Deformability-based enrichment of CTCs	Deform-ability	Celsee	Quick; high sensitivity	Loss of relatively small CTCs
Electric-charge-based electrophoresis	DEP	ApoStream	High cell viability; high efficiency	Low purity in some devices
Microfluidic separation based on physical properties of CTCs	Size; deform-ability	Parsortix; ClearCell® FX1	Short processing time of sample	The difficulty of removing the leukocytes of similar size to CTCs; the chip is easy to be blocked
Biological property-based assays				
Immunoaffinity-positive	EpCAM (magnetic beads)	CellSearch; MagSweeper; AdnaTest; Magwire; GILUPI; CellCollector	High recovery; high purity rates	Loss of EpCAM-negative CTCs; problems with the antibody affinity or specificity
	EpCAM (microfluidic chips)	IsoFlux; CTC-chip; HB-chip; CMx; NanaVelcro	High purity rates; high capture efficiency; high cell viability	Long, time-consuming process; sample preprocessing requirement to reduce volume
Immunoaffinity-negative	CD45; CD66b	RosetteSep; SE-iFISH; MINDEC; CTC-iChip	High cell viability; avoid loss of EpCAM -negative CTCs	Inability to deplete CD45-negative endothelial cells; loss of CTC aggregates surrounded by WBCs

Transl Cancer Res. 2020 Mar; 9(3): 2012–2025