A Brief Comparison Of The Various Techniques And Methods For Isolation Of Circulating Tumor Cells (CTC’s)  11.15.13

This document is the result of many questions and concerns expressed over the past few years regarding the techniques and methods available, to date, for the isolation of CTC’s (circulating tumor cells) and to explore their potentials and weaknesses.

**Background:** It is well known that all primary tumors consist of two major categories of cells: The stroma cells and the malignant cells. The stroma consist of normal cells (pericytes, lymphocytes, endothelial cell, fibroblast, etc.) which simply support and feed the tumor itself.

The malignant (cancerous) cells are composed of a non-homogenous population which includes several subgroups with different hallmarks. Very few of the cancerous cells have developed the ability to metastasize (CTC’s) and even fewer, from the CTC’s, have developed the ability to form a new, distant metastatic tumor (Tumor Initiating Cells (TIC’s) or Cancer Stem Cells like-CSC’s). The prevalence of CSC’s inside the primary tumor is very small and the markers to identify them are very close to the surrounding cells. But, the CTC’s include the larger proportion of the CSC’s and they have major differences from the surrounding cells (the blood origin cells). This is the reason why CTC’s have become so popular and important.

All techniques of identification and enumeration of CTC’s can be categorized into one of the following groups:

- Groups where the separation ability is based on the difference in size of CTC’s and other blood origin cells.
- Groups where the separation method is based on specific cell surface markers.
- Groups where the separation method is based on specific intracellular markers.

At this point we need to keep in mind the few hallmarks of the CTC’s, which are:

1. CTC’s are not a homogenous population and there are many variants between them
2. The viability of the CTC’s is very fragile.
3. The number of CTC’s is respectfully small compared with the number of blood origin cells.
4. CTC’s include the entity of CSC’s or dynamic CSC’s (aka plasticity of CSC’s), which are the only subgroups that can be expanded (cultivated).

Based on the above hallmarks of CTC’s, we can better compare the various groups of isolation techniques in order to define the pros and cons of each.

We start with the first group of separation technique [based on size]. It includes multiple assays where the basic principle is to pass the cells through a narrow passage, which the CTC’s cannot fit through. The CTC’s will be collected before the narrow passage area. Such a method will either force the CTC’s to pass from the gradient solutions of the polymerized phase (ficol) or pass through small holes in a solid phase surface (Millipore chip). In order for the CTC’s to pass through the separation phase they will need to be forced to flow through the narrow passage.
The advantages of this technique are:

1. It is easy
2. It allows you to have viable cells after separation

The disadvantages of these methods are:

1. Due to the force (either due to centrifugation or pumping) many of the cells are destroyed (viability decreased up to 70% of CTC's).
2. The CTC’s that are isolated do not represent all of them. There will be many that may pass through the narrow passage due to the (Endothelial Mesenchymal Transition (EMT) and they become more flexible and bendable, therefore different.
3. There are no selection of CSC’s available.
4. This is an enrichment method since many blood origin cells will still remain in and around the area of CTC’s and that produce contamination and noise.

Hence, it is not considered the best method and approach if someone wishes to make a more comprehensive analysis of the CTC’s and especially the CSC’s.

The second group of separation technique is done by selecting a cellular membrane antigen (marker) with high prevalence found on CTC’s. This method uses either immunobead or they use flow cytometry and sorting. By immunobead antibodies conjugated with iron biomolecules, they are attracted from the antigens and inside a strong magnetic field the cells having the antigen on the surface will stay in the bottom and all other cells are removed. The flow cytometric sorting actually interrogates each cell by passing in a laser beam after the cells have been stained with similar antibodies like previous, but instead of using iron biomolecules they are conjugated with fluorochrome and then bleached. Then the sorter deviates only the cells that have been bleached into a separate chamber. This isolates the remainder of unwanted cells from the CTC’s.

The advantage of methods one and two are:

1. Relative easy to perform.
2. High selectivity of each marker (better in sorting than using beads)

The disadvantages are for each method:

- Immunobead
  1. The selectivity of this method is depended on the size of the bead with the iron. The small size bead (50um) can pass easily between cells and identify the antigen and strongly bond to that, but in the magnetic field it cannot produce enough momentum to force the selected cell to stay at the bottom of the vial. The large size beads (250um) can hardly pass between the blood origin cells, but when they find the CTC’s they can easily force them towards the magnetic force field.
  2. Due to the strong magnetic force field many times the CTC’s are disrupted under the pressure of the surrounding cells.
3. Based on one or a few membrane markers it is impossible to include all CTC’s since many of those may not express the antigen of choice.

4. Again, many blood origin cells will remain to the sample causing contamination and noise.

5. The selection rate is extremely high with equally high viability of the cells, but again we have only a subset of CTC’s since only one or a few markers have been selected.

The **third group** of separation technique includes mainly flow cytometric sorting where a scientist immobilizes the cells, fixates them, makes them permeable in order to use immunoglobulins that are conjugated with fluorochromes. Then this method will allow us to use a larger panel of makers, but still the disadvantages of the previous group remains. This is that the isolated CTC’s are not viable any more due to the immobilization step. Hence, the isolated CTC’s do not represent the total number and they cannot be expanded any more.

An exception to this group uses PCR based method in which an antigen of choice has been selected and primers for the relevant gene have been designed and an end-point PCR performed or real time PCR which simply identifies the existence of the mRNA of an encoded antigen and thus a standard curve has been performed. It is now possible to calculate the number of CTC’s that expresses this antigen.

**The advantage of that method is:**

1. Good accuracy and sensitivity

**The disadvantages are:**

1. There are no more cells to work with.
2. Since only one of several antigens have been selected then again only one subset of CTC’s can be identified.
3. There is still no possibility to recognize the CSC’s.

So, all these methods have advantages and major disadvantages they are struggling with in order to generate a good solid outcome which can result in good clinical application.

Keep in mind the last purpose discussed, also take into consideration the hallmarks of CTC’s and CSC’s, in combination of the pros and cons of each method. At this point it would be rational to conclude the following strategy to isolate, enumerate and have viable CTC’s so they can be expanded (at least the subset that have such features), therefore:
**The following procedures are used on all samples tested by R.G.C.C., Ltd. Labs in Greece**

It is better to perform negative selection of cells derived from the blood sample, so that all the blood origin cells will be removed. This approach can be done through flow cytometric sorting. This method provides:

1. Flow cytometric sorting to perform negative selection of cells.
   a. Accuracy
   b. Sensitivity
   c. Viable cells
   d. Close to 100% of all CTC’s can be harvested
   e. Purity of the isolated cells

2. Then the cells that remain can be expanded.

3. The mRNA of the isolated cells can be used for detecting all genome expression profiles through micro-arrays analysis. Then it is possible to have all the aberrant gene expression rates of all genes. From these data it is possible to detect the expression of genes that are related with:
   a. Sensitivity to specific drugs
   b. Development of resistant phenotype
   c. Metastases (invasion, migration, neo-angiogenesis etc)
   d. Self-repair mechanism (resistance to radiation, hyperthermia etc)
   e. Progression rate
   f. Immortalization of cancer cells
   g. Usage of target therapies (TKI, Moab etc)
   h. Metabolism of a pro-drug to its active substance.

4. All this information needs to be further validated since the relation between gene expression and protein expression is not always linear. Hence the cells are exposed to agents in order to confirm the information that the gene expression (epigenetic) profile reveals.

All of these beneficial steps have been selected by R.G.C.C. labs in order to avoid the negative points of the previous methods and develop a procedure that is accurate, fast and where the end result offers good results to clinicians who work with cancer patients.

Simultaneously the same methodology is used by R.G.C.C. in order to reveal new drug targets for cancer and develop new potent anticancer agents.

Any further developments that will result in even better options, methods and techniques, RGCC is ready to adopt and include into the existing algorithm of work-flow in order to improve the quality of analysis and provide a higher level of services.

Sincerely,

Ioannis Papasotiriou, M.D., PhD
EXCITING NEWS FOR RGCC, Ltd OF GREECE 04.23.12

We are in the pleasant position to announce that we have passed the assessment and validation from the National Organism of Accreditation and they have accredited our lab with the ISO 17025:2008 for the following methods:

1. CTC/CSC isolation and immunophenotyping
2. Cancer cell culture viability/cytotoxicity assays after exposure to substances
3. Gene expression assays (mainly related with cancer stemness)

I hope that this will help our physicians their patients and other clients understand our level of commitment to quality and remove any doubts that may have existed concerning the validity of the assays since this accreditation includes inter-laboratory validation (performance of the assays from different labs which we have no interest or relation).

We will utilize such tools as often as needed to promote the quality and sincerity of our work.

ISO 17025 identifies the high technical competence and management system requirements that guarantee your test results and our calibrations are consistently accurate.

This accreditation is recognized and accepted throughout the international scientific and laboratory communities as the standard of excellence.