

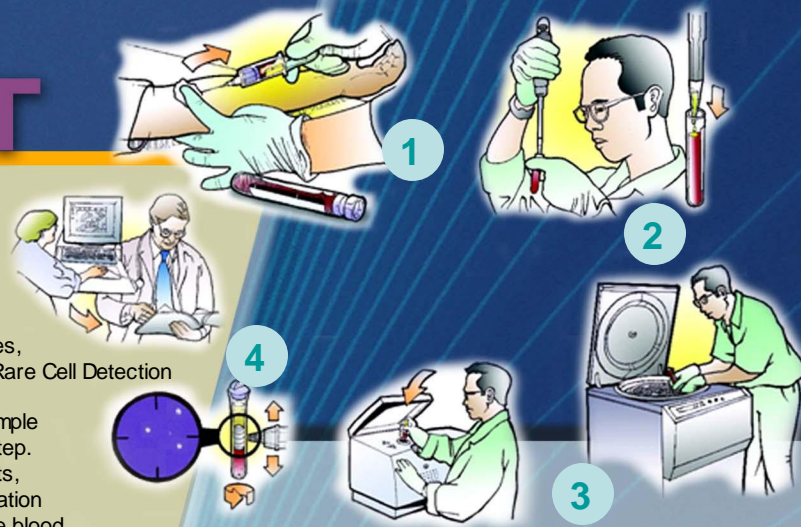
# ABSTRACT

The last decade and a half has seen a dramatic rise in the number of publications focusing on circulating cancer cells as a marker of disease. This pioneering work is establishing the clinical relevance of cancer cells in the blood as a new diagnostic technique for assessing the status of cancer in patients. To date, most have relied on laborious and expensive techniques, such as density-gradient separation and magnetic bead technologies, to capture tumor cells for counting. Battelle has developed a new Rare Cell Detection (RCD) technique that enables counting of tumor cells in the blood using an inexpensive disposable that prepares a relatively large sample of whole blood for automated microscopic examination in a single step. The full technology is composed of a laboratory instrument, reagents, and a custom disposable that allows for the detection and quantification of cells present in concentrations on the order of 1 cell in 8 ml whole blood. While currently in the feasibility stage, Battelle's RCD system will be developed to provide commercial monitoring, staging, and screening for cancer in an efficient, cost-effective manner, not previously achievable. This technology enables an inexpensive test based on a standard Vacutainer™ blood draw and would enable clinicians to evaluate the usefulness of their chosen therapy sooner and with greater frequency than allowed for using current techniques.

The Rare Cell Detection (RCD) system has been developed to its current state through a collaboration between Battelle and the inventors of the base technology. It is the subject of 5 issued and 7 pending patents. It works by staining the cells of interest with specific fluorescent-labeled antibodies, and expanding the buffy coat over a wide focal space for imaging. The buffy coat contains the cancer cells of interest. The buffy coat is expanded by centrifuging the blood sample in a tube that includes a close-fitting float that matches the specific gravity of the buffy coat cells and, thus, expands the buffy layer into a narrow annular space between the float and the tube's inner wall, making it accessible to the image capturing laboratory instrument and software. The tube is then inserted into a custom RCD Reader that scans the tube and captures images of cells of interest. The RCD Reader generates a report that includes cell images and counts.

Experiments were performed to assess sensitivity and specificity of the system. In a blinded study, using normal donor blood and tumor cells from culture, the system identified 73% of cells overall (7 to 121 cells added per sample, n=27). System sensitivity was improved (81.5%) with samples containing very few cells (7 to 20 cells per sample, n=8). Out of ten samples without tumor cells added, two samples were identified as having one cell each, and all others were identified as having zero. A second study using fluorescent calibration beads demonstrated that the system could easily detect 1537 molecules of fluorochrome (on 6-micron beads) in the presence of background fluorescence equivalent to the amount used in the cell spiking study above. Although the fluorescence sensitivity might actually be better, this level should be sufficient to detect most cell surface markers.

Battelle is pursuing multiple partnering options for the development of this technology while also continuing to develop the technology internally. We are in conversations with leading academic and clinical research institutes to obtain grant funding to advance critical research and further establish the technology among thought-leaders, and are actively seeking to license or sell the technology to a strong commercial partner.



# A Platform for Low Cost Quantitation of Rare Cells in Whole Blood

Herbert S. Bresler, Ph.D., Matthew S. Fleming, John S. Laudo, Randy L. Jones, Vincent J. Contini, Albert E. Weller III, Eric R. Navin, David L. Rimm, M.D., Ph.D., Paul Fiedler, M.D., Robert A. Levine, M.D., and Steven C. Wardlaw, M.D.

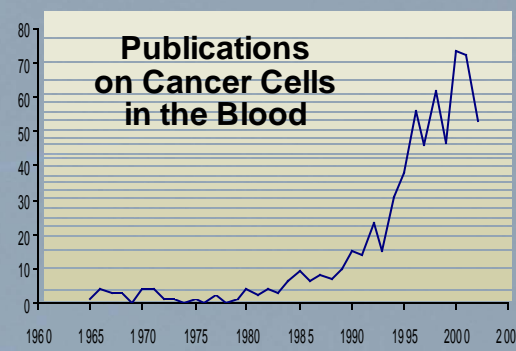
Battelle Healthcare Products, USDx, Inc., Yale University School of Medicine, St. Raphael Hospital



## INTRODUCTION

There has been growing commercial investment and research into the diagnostic utility of detecting and quantifying rare cells in the blood for early cancer detection. Rare cells in whole blood occur in such low concentrations that they are not ordinarily found during peripheral blood screening using commercially available techniques. Some specific cells of interest include epithelial cells originating from carcinomas as well as progenitor cells originating from bone marrow. The detection of these cells may be significant in monitoring, diagnosing, and treating cancers such as colorectal, lung, breast, and prostate. In addition, the load of viral-infected cells could be determined for such viruses as HIV and hepatitis C.

**Rare Cell Detection Technology** Rare Cell Detection (RCD) is a patented technology that enables the detection and quantification of rare cells in whole blood through expansion and imaging of the buffy coat. It is being developed as an easy-to-use, low-cost method for monitoring, staging, and screening for epithelial-based cancers (carcinomas). RCD utilizes a very low-cost disposable that requires only a simple blood draw and minimal handling for analysis.



The last decade and a half has seen a dramatic rise in the number of publications focusing on circulating cancer cells as a marker of disease. In the five years, 1986 - 1990, there were only about 46 publications on detection of circulating cancer cells, compared to 307 published in the last five years, 1998 - 2002.

### A Very Sensitive Assay is Necessary

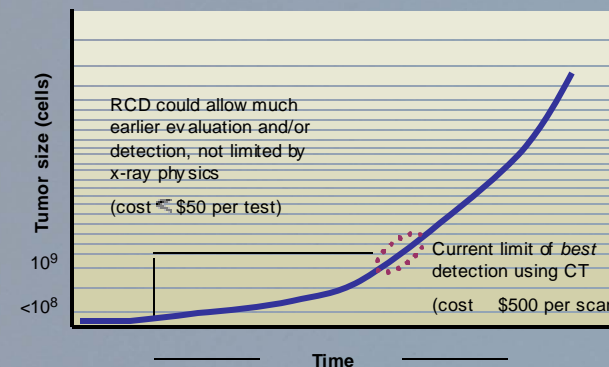
Patient cancer stage	Average cell count Prostate <sup>1</sup>	Average cell count Breast <sup>2</sup>
Control: no cancer	0.8 ± 1.2 cells/7ml	0.085 cell/ml
Localized CAP Stage I or Stage II Breast	5.9 ± 4.7 cells/7ml	0.8 cell/ml
Metastatic CAP Stage IV Breast	46.6 ± 65 cells/7ml	6.1 cell/ml

1. Moreno et al., "Changes in circulating carcinoma cells in patients with metastatic prostate cancer correlate with disease status." *Urology* 2001 Sep; 58(3):386-92  
2. Betsch and Clifton, "Detection of Carcinoma Cells in the Blood of Breast Cancer Patients." *Am J Surg* 2000; 180:446-449

### Potential Clinical Uses of a Rare Event Detection Product

- Therapeutic Monitoring
  - Track progress of chemotherapy, surgery, or other therapy
  - Permit earlier evaluation of chosen regimen
- Recurrence Monitoring
  - Earlier detection of cancer return
- Cancer Staging
  - Determine a patient's stage of cancer advancement
- Screen Population
  - Screen individuals at risk
  - Pre-symptomatic detection

### RCD: Earlier Assessment of Response to Therapy, Progression or Recurrence

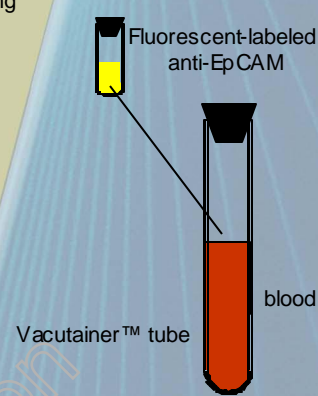


The research presented here focused on the development of an integrated system to find rare events in whole blood. The Rare Cell Detection (RCD) system is based on a novel technology that creates, in essence, a "self-preparing slide" that provides visual access to all the cells of interest from a large volume of whole blood. Rare events can be described as entities found at concentrations as low as approximately 1 event per milliliter of whole blood. The strength in RCD lies not only in the hope of excellent clinical utility, but also in its simplicity that yields a low-cost, easy-to-use system thus allowing for a lower hurdle to market acceptance.

The development team built two functioning laboratory instruments and prepared sufficient reagents and disposables to generate proof-of-principle data. This phase concentrated on the principle of finding the cells with adequate sensitivity (primarily spiked samples). Feasibility has been demonstrated by imaging and enumerating cancer cells from spiked samples and from a limited number of actual cancer patient blood samples. Convincing clinical relevance of tumor cells in the circulation has yet to be demonstrated.

## MATERIALS & METHODS

### Current RCD Procedure: Step 1 - Staining



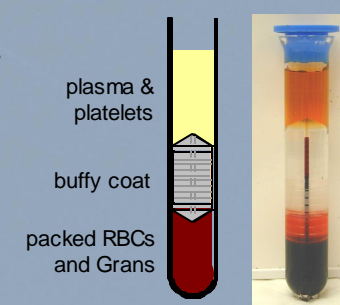
**Process:**

- Fluorescent-labeled anti-EpCAM is added directly to the Vacutainer™ tube containing anticoagulated whole blood
- Tube is re-capped and incubated on a rocker or rotator

**Principles:**

- Antibody binds specifically to cells carrying the surface marker Epithelial Cell Adhesion Molecule, which is found on all epithelial cells (and cancer cells of epithelial origin) but is not found on normal blood cells

### Step 3 - Centrifugation



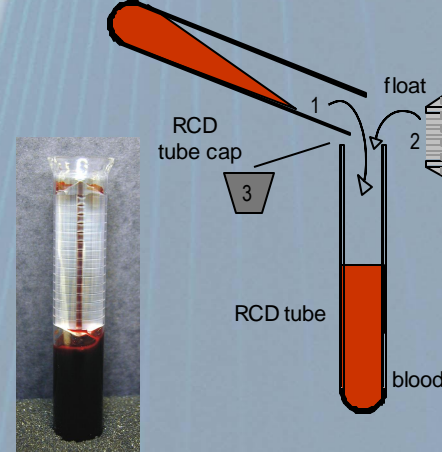
**Process:**

- The tube containing the patient's blood is centrifuged

**Principles:**

- The float moves to buffy coat layer (between plasma and red cells) by virtue of its density
- The buffy coat is approximately 0.5% of total volume and consists mostly of white cells. The targeted epithelial cells are found in the buffy coat with all of the other nucleated cells in the blood
- The buffy coat is spread and captured in a thin layer by the float so it can be inspected by the RCD imaging system

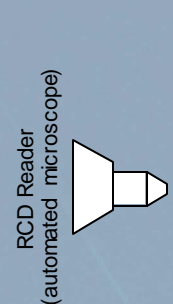
### Step 2 - Tube Preparation



**Process:**

- The blood is transferred from the Vacutainer™ to the RCD tube
- The float is added to the tube
- The RCD tube is capped

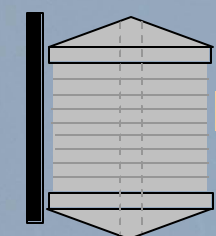
### Step 4 - Enumeration & Reporting



**Process:**

- The tube is placed in the RCD imaging system
- The RCD imaging system examines the buffy coat area around the float identifying objects of interest
- Images of the objects are captured by the system for later manual classification
- The final classification of cells is made based on imaged data

### Design Challenges: Float and Tube



**Float**

- Material selection
  - Non-fluorescent
  - Specific gravity
- Design
  - Ribs
  - Vent

**Tube**

- Extremely thin walls
  - Uniform wall thickness
  - Mold filling
  - Non-fluorescent
- Flexibility and strength
- Clarity maintained

## RESULTS

### Consumable & Chemistry Milestones

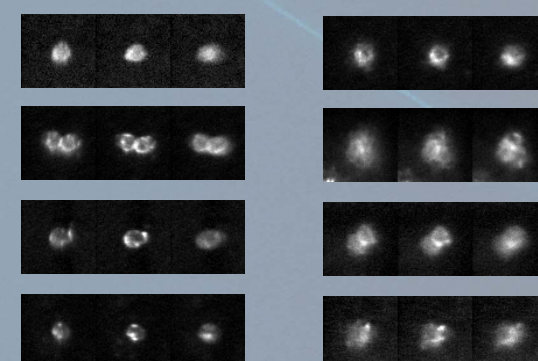
- Novel tube and float design challenges have been addressed in feasibility
- Process for labeling the antibodies with fluorophores is standardized
- Inexpensive antibody used in low quantities
- System sensitivity has been demonstrated to be sufficient for detection of native circulating cancer cells.

### Instrument Milestones

- Semi-automated identification and imaging of cancer cells
- Data logging system
- High quality images of cancer cells in blood

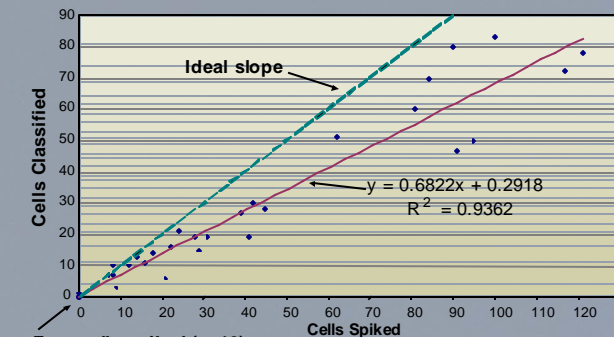
The entire float area was scanned. A digital camera recorded images of objects that fluoresced at the correct wavelength. The RCD Reader performs some filtering on the fly. Of the images captured, only approximately 1/3 were presented to the classifier.

### Images of Spiked Clone A Cells



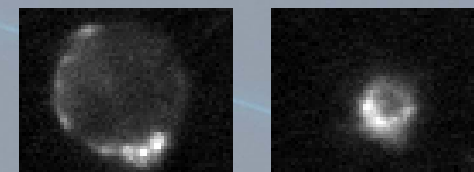
Three focal plane images of each cell are shown.

### Spike Recovery Results (Blinded Study, n = 37)



The RCD system identified 72.8% of the cells spiked into 26 samples. In the 10 additional negative control samples, one cell was identified in each of 2 samples. Identification of cells was more accurate at low cell numbers (81.5% of cells identified with <20 cells per sample, n=8).

### Large "Native" Cell & Clone A Cell



Some unusual cells were observed while running the study. The figure above displays a large native cell (left) and a typical size clone "A" cell (right). This particular sample had 12 such very large cells (approximately 40 microns in diameter). We concluded that these were native epithelial cells, although their origin is uncertain. An additional tube drawn from the same donor on the same day also contained some of these same large cells.

### Calibration Bead Study

R-Phycoerythrin Microbeads (MESF)	Without Alexa-532	With Alexa-532
0	-	-
1535	✓	✓
4654	✓	✓
25142	✓	✓
86665	✓	-

✓ = Beads imaged and identified  
MESF - Molecules of Equivalent Soluble Fluorochrome

Microbeads with levels of R-Phycoerythrin were selected that covered the expected brightness range of native cancer cells (Quantum 27™, Bang's Laboratories, Fishers, IN); beads with 1535, 4654, 25142, and 86665 Molecules of Equivalent Soluble Fluorochrome (MESF). Ten tubes of whole blood from normal donors were used to run two tubes for each bead set and a control beads without fluorochrome. Tubes were run in duplicate, with and without background dye. The excitation wavelength of the system was suitable for both the R-Phycoerythrin and Alexa-532 background dye.

Results demonstrate system sensitivity sufficient to image even the dimmest beads even in the presence of the Alexa-532 background dye and should, therefore, be sufficient for imaging native cancer cells.

## CONCLUSIONS

- Feasibility of the RCD System has been shown:
  - Spike recovery studies as good as or better than previously published results.
  - Native cells identified in blood obtained from study volunteer.
  - Calibration bead study shows sensitivity to be excellent and theoretically good enough to identify even the dimmest cancer cells.
- Results are sufficiently encouraging to warrant additional work:
  - Sensitivity and specificity
  - Cost appropriate to support ~\$50 reimbursement
  - Ease of use, use of standard lab centrifuge, RCD Reader expected to have small footprint

### Drivers for Adoption of RCD Test are Attainable

<b>Clinical Relevance</b>	Clinical relevance of circulating cancer cells in peripheral blood has yet to be established as indicative of the existence or stage of carcinoma. Significant research and investment are being focused on establishing the clinical relevance of circulating cancer cells. Hundreds of papers on circulating cancer cells have been published.
<b>Sensitivity</b>	Required sensitivity must be determined clinically. Current literature indicates that systems will need to detect 1 cancer cell in 10 ml of peripheral blood.
<b>Specificity</b>	Very low false-positive rates will be critical for any technology in this area.
<b>Cost</b>	A test for circulating cancer cells will compete with relatively expensive existing diagnostic tests. Focus groups have indicated that a low-cost test (~\$50) will enable early adoption by patients and physicians, minimizing the effect of reimbursement on the adoption rate.
<b>Ease of Use</b>	Adoption will be significantly influenced by the ease of use of the test by the clinicians and clinical testing laboratory. The most successful tests will use standard physician office practice and will have minimal impact on the clinical laboratory.
<b>Reimbursement</b>	No established reimbursement level exists for tests for circulating cancer cells. Focus groups have indicated that initial tests will likely be paid for by the patient or the hospital out-of-pocket.

### Battelle's Interest in RCD

- Great potential as a platform for cellular diagnostics
- Cancer is first target and represents an attractive market
- Technology is very promising with potential to enable price-sensitive screening market due to low disposable cost
- Options Battelle is pursuing:
  - License/sell technology to a strong commercial partner
  - Actively partner with leading academic research institutes to leverage grants and advance critical research and establish technology among thought-leaders
  - Further develop the technology toward critical milestones
- 5 patents issued, 7 patents pending

