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Financial Disclosure

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We have no financial disclosures.

Basic Introduction to Circulating Tumor cells (CTCs) & The CellSearch™ System

Objectives

- Evaluate the use of CTCs and CellSearch (Veridex, Raritan, NJ) in the management of patients with cancer
- Discuss the cytopathologist's role in measuring CTCs
- Describe the integration of CTC assessment into the practice of cytopathology
- Identify and evaluate CTCs in peripheral blood

Definition

- Circulating tumor cells (CTCs): cancer cells shed from either the primary tumor or its metastases
  - Epithelial cells derived from solid tumors
  - Metastasic disease is responsible for most cancer deaths (>90%).

History of CTCs

- Tumor cells were first identified in the blood stream of patients in (1869) by Thomas Ashworth
- Engel, 1955: cancer cells in the peripheral blood of pts with various types of cancer.
- Hematologists,Cytologists & Surgeons: background in Papanicolou & Romanowsky stains: morphologic criteria for cancer cells

ACTA CYTOLOGICA

THE JOURNAL OF EXFOLIATIVE CYTOLOGY

Volume 9 · 1965

The Williams & Wilkins Company
Baltimore, Maryland 21202

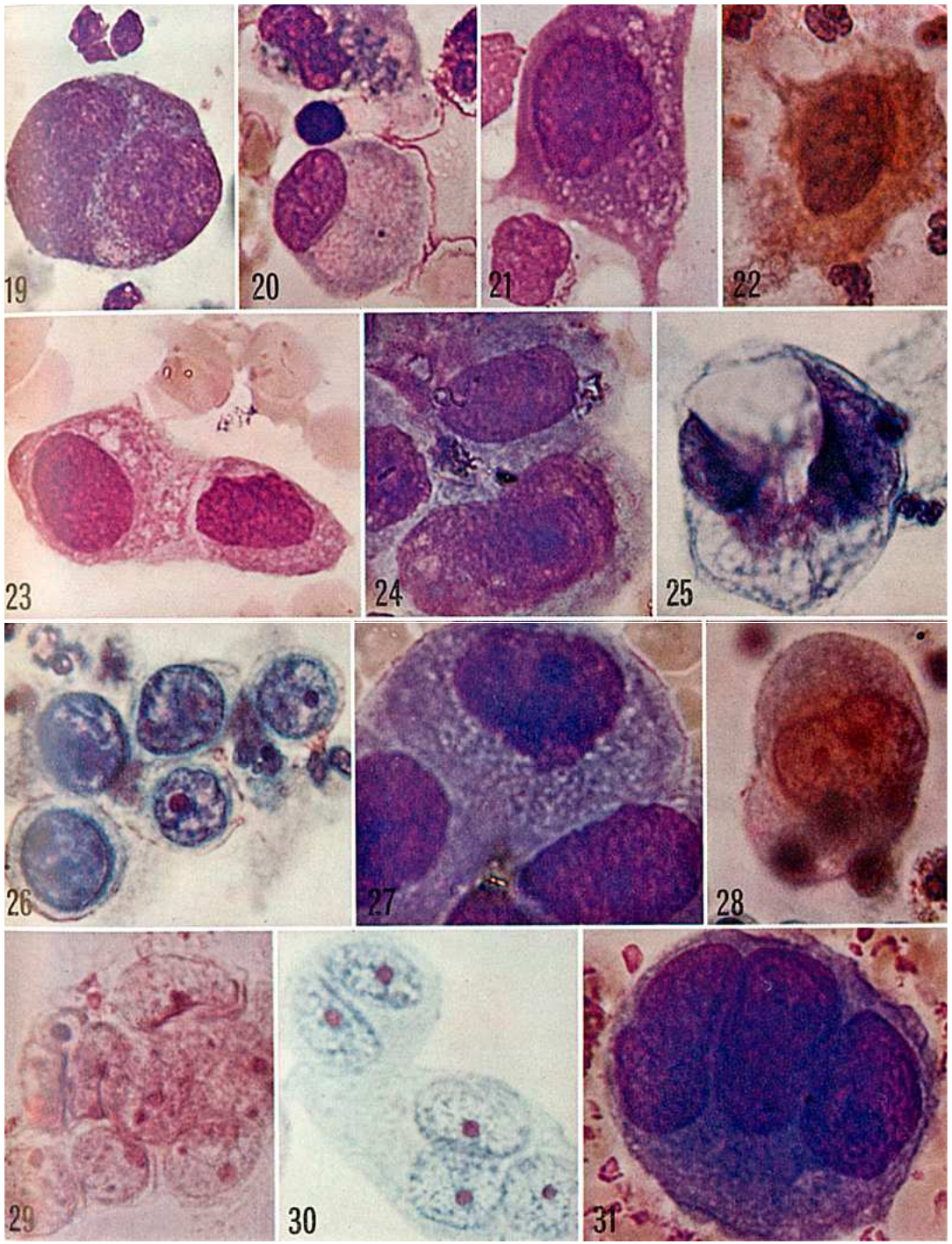
Symposium on Tumor Cells in the Circulating Blood

PART I

ELI M. NADEL, M.D.

GUEST EDITOR

Slide Seminar The Circulating Cancer Cell Cooperative (CCCC): NCI Identification of CTC: Morphologic criteria



National Cancer Institute
Diagnostic Research Branch

- The Circulating Cancer Cell Cooperative 1962:
- Morphology, techniques and patient selection

  - Conclusion : “More extensive well-controlled studies, improved techniques, sharper criteria for recognition of tumor cells are required.”
- Immunofluorescence technique by Coons etal: labeling of antibodies with fluorochromes improved the specificity of detection of CTC.
- Value to cytologic diagnosis of CTC

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer

Massimo Cristofanilli, M.D., G. Thomas Budd, M.D., Matthew J. Ellis, M.B., Ph.D., Alison Stopeck, M.D., Jeri Matera, B.S., R.Ph., M. Craig Miller, B.S., James M. Reuben, Ph.D., Gerald V. Doyle, D.D.S., W. Jeffrey Allard, Ph.D., Leon W.M.M. Terstappen, M.D., Ph.D., and Daniel F. Hayes, M.D.

Utility CTC Measurement

- CTC: Rare in healthy women and in patients with benign breast disease (<1 per 7.5ml blood).
- Monitoring CTC (counts) can predict prognosis in many solid tumors, breast, prostate and colorectal cancers.
- Measuring changes in CTC counts help monitor patient outcome.
- Molecular characterization of CTCs (HER2, EGFR) help select patient's targeted therapy and limit metastases.

Utility CTC Measurement

- The role of CTCs in blood is still under active investigation, their biological significance/ therapeutic relevence is debated.
- Identification, enumeration and molecular characterization of CTCs could expand our understanding of the biology of metastases.
- Several strategies have been used for CTC enumeration.

Techniques for CTC Enumeration

Antibody-based cytometric assays (intact tumor cells)

- IF based technology with monoclonal antibodies to epithelial specific antigens EpCAM, CK
- Epithelial tumors are detected

Molecular (nucleic acid-based assays): PCR/RT-PCR) techniques

- DNA or mRNA: transcripts for EpCam, CK; highly sensitive but lacks specifiity

Enrichment: isolate CTCs

Techniques for CTC Enumeration

- CellSearch system (Immunocon &Veridex,LLC): immunomagnetic/ immunofluorescent based technology to capture of epithelial cells (EpCAM) by ferrofluid
- CTC-chip: Microposts/columns coated with EpCAM ab on silicon chip
- FAST(Fiber-optic Array Scanning Technology): digital microscopy to scan labeled cells, 300,000 cells/min
- Oncoquick: density gradient
- MACS (Magnetic Activated Sorting system): immunobeads capture of epithelial cells
- Microfilter Device: ISET (isolation by size), polycarbonate filter with pore size 8um. Live CTC capture measuring telomerase activity
- The Adna-Test Breast Cancer, RARE, Epispot

CellSearch™ System

- Automated, standardized technology for CTC detection
- Based Immunomagnetic and immunofluorescence
- CellSearch system validated in multiple clinical trails
- FDA approved for CTC detection

System Overview

- Instruments (CellTrack Auto autoprep system)
- Specimen collection, processing and Quality control
- Enumeration of CTCs for predicting progression-free and overall survival in patients with metastatic breast, colorectal and prostate cancer
- Clinical trial background and conclusions
- Interpretation of Results
- Limitations

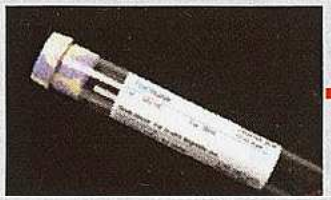
CellSearch™ System

- **Sample preparation system**
  - Cell Search Epithelial kit (Veridex Corporation, Warren NJ)
    Anti Epcam antibodies:
    Anticytokeratin antibodies conjugated to phycoerythrin (PE) 8,18 &19)
    Antibody to CD45 conjugated to allophycocyanin (APC): WBC,
    Nuclear dye (DAPI, 4'6-diamidino-2-phenylindole)
    Controls: Breast cancer cell line (SKBr3)
  - CellTracks AutoPrep system: Automated
- **Sample evaluation**
  - CellSpotter Analyser (Veridex, immunocon): CTC Identification and enumeration.
- **Interpretation of images:** operators (cytotec &pathologist)

CellSearch™ System

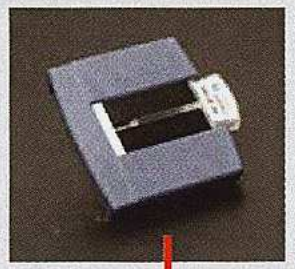
VERIDEX LLC

cellsave® preservative tube



cellsave® autoprep® system

MagNest®



CellSearch™ Circulating Tumor Cell Kit

Off- Line Molecular Analysis

celltracks® analyzer II

Immunomagnetic CTC Selection

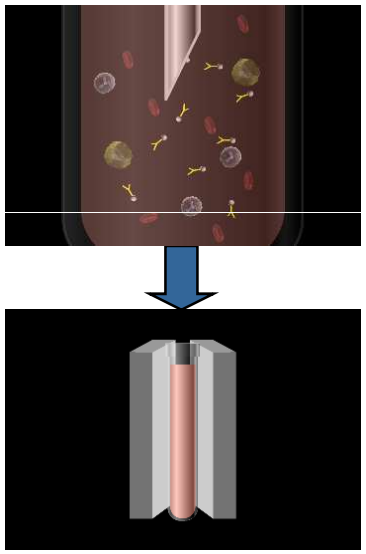
Off-Line

7.5 ml blood from CellSave™ Tube + Buffer

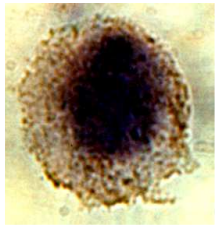
Centrifuge

Processing by the CellTracks™ AutoPrep System

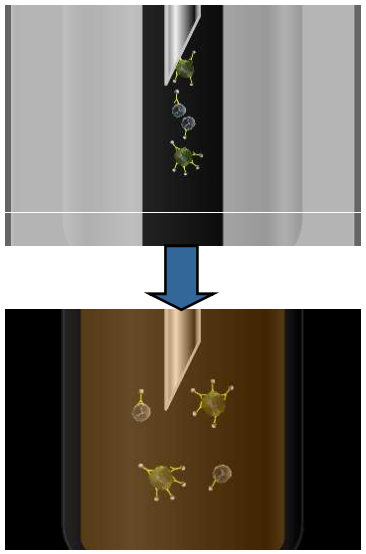
Aspirate plasma
Add buffer
Add ferrofluid.



Magnetic incubation

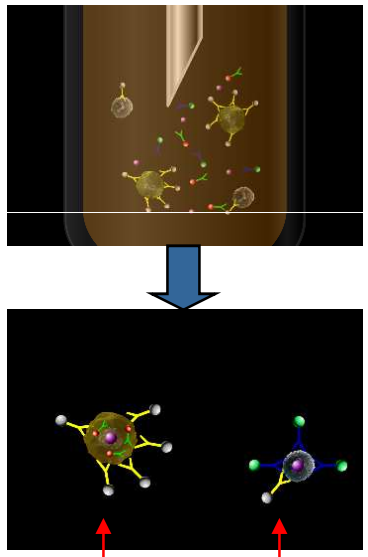


Aspirate fluid and un-labeled cells



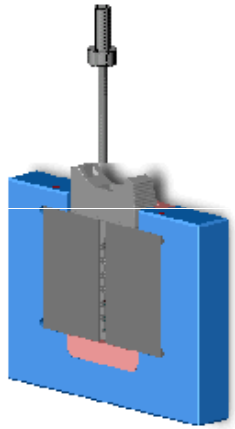
Remove magnets.
Re-suspend target cells in buffer

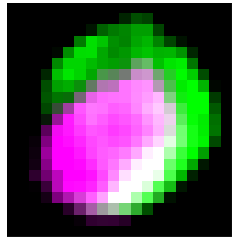
Permeabilize and add Staining Reagents



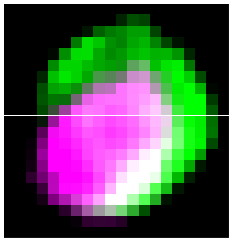
CTC leukocyte

Transfer to MagNest™

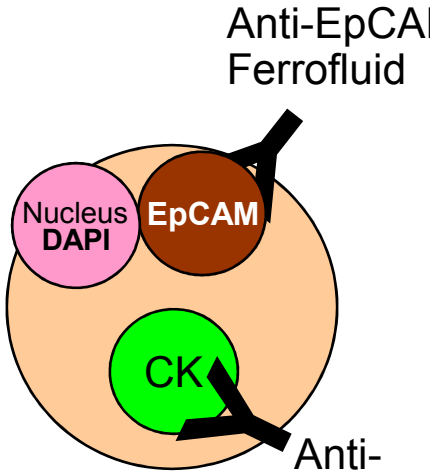


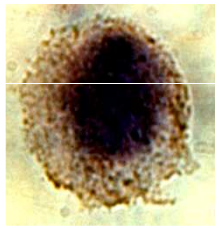


Immunomagnetic Labeling and Immunofluorescent Identification of Cells



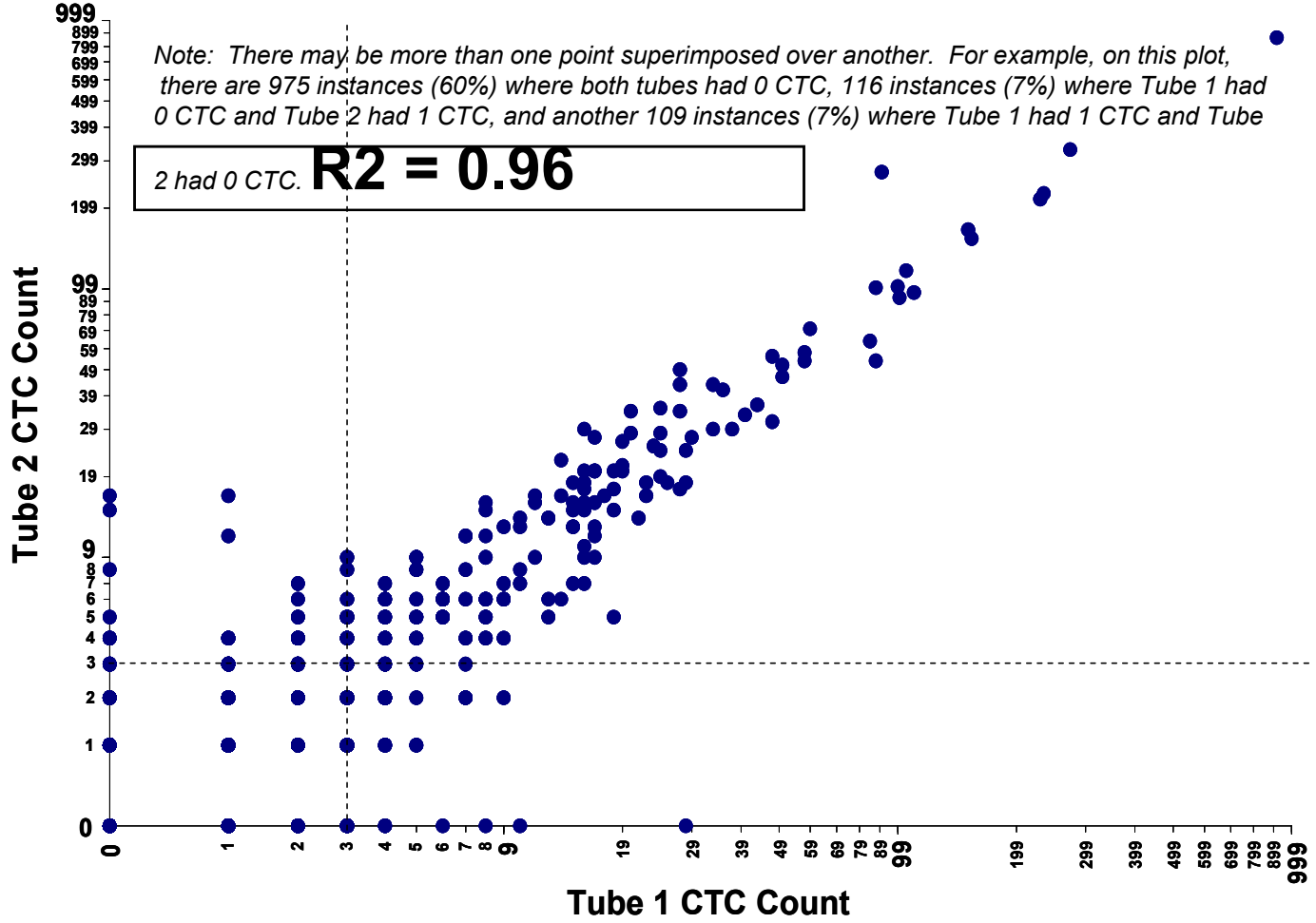
composite





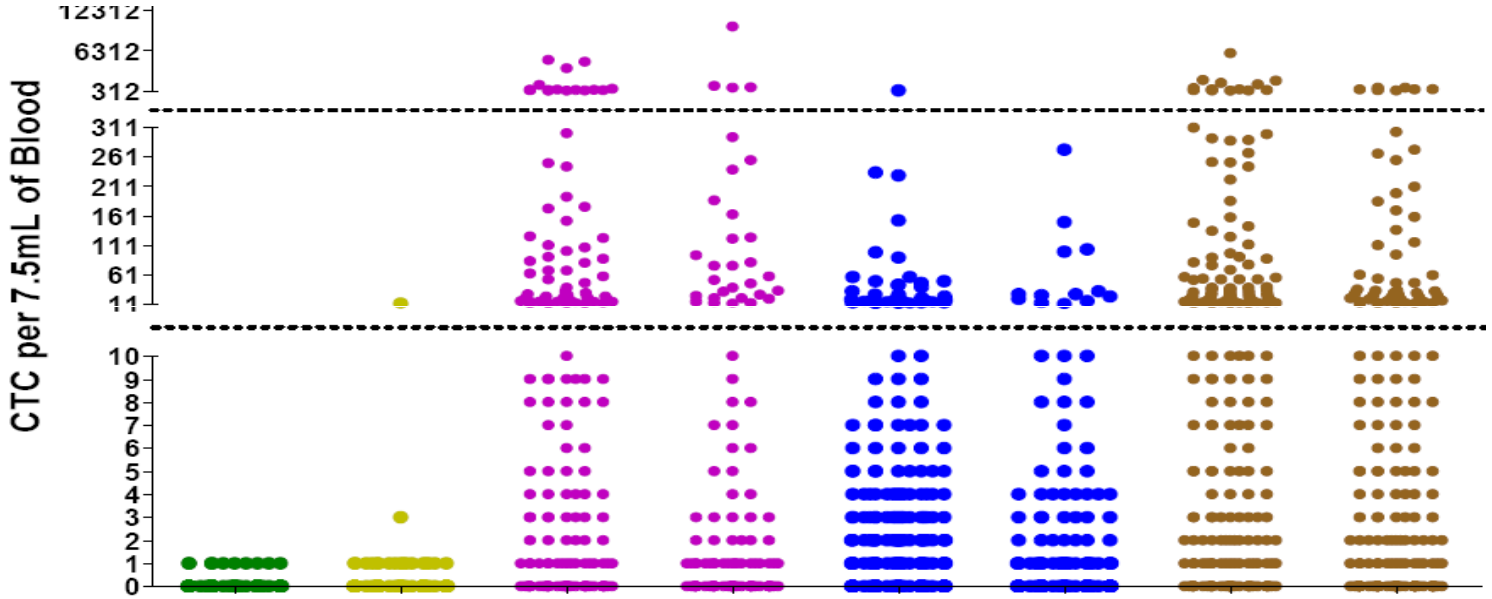
Circulating Tumor Cell

CellSearch™ MCRC Reproducibility



Frequency of CTCs: CellSearch™ System

Figure 1. Frequency of CTC in Controls (Subjects without Cancer) and Patients with Metastatic Breast[1] (MBC), Metastatic Colorectal[2] (MCRC) or Metastatic Prostate Cancer[3] (MPC) before Initiation of a new line of Therapy (Baseline) and ~2-5 weeks After the Initiation of Therapy.



	Healthy Controls	Benign Diseases	MBC Patients[1] Baseline	MBC Patients[1] 3-5 Weeks	MCRC Patients[2] Baseline	MCRC Patients[2] 3-5 Weeks	MPC Patients[3] Baseline	MPC Patients[3] 2-5 Weeks
# of Cases	295	255	177	132	413	334	219	203
N (%) ≥1 CTC	10 (3%)	19 (7%)	125 (71%)	73 (55%)	196 (47%)	89 (27%)	170 (78%)	123 (61%)
N (%) ≥2 CTC	0 (0%)	2 (1%)	108 (61%)	53 (40%)	138 (33%)	48 (14%)	152 (69%)	106 (52%)
N (%) ≥3 CTC	0 (0%)	2 (1%)	102 (58%)	47 (36%)	108 (26%)	41 (12%)	139 (63%)	93 (46%)
N (%) ≥4 CTC	0 (0%)	1 (0%)	94 (53%)	42 (32%)	90 (22%)	34 (10%)	129 (59%)	86 (42%)
N (%) ≥5 CTC	0 (0%)	1 (0%)	88 (50%)	40 (30%)	75 (18%)	26 (8%)	125 (57%)	80 (39%)

[1]MBC reference population information on page 7 of the clinical IFU.
[2]MCRC reference population information on page 27 of the clinical IFU.
[3]MPC reference population information on page 46 of the clinical IFU.

Clinical Trial

3 Prospective multi-institutional clinical trials assessed the performance of the CellSearch™ Assay

- Metastatic Breast Cancer (MBC) > 5 cutoff
- Metastatic Colorectal Cancer (MCRC) >3 cutoff
- Metastatic Prostate Cancer (MPC) >5 cutoff
- Selection of CTC cutoff : Prospectively identified in patients in a training set and confirmed in a validation set

Metastatic Breast Cancer (MBC) cutoff ≥ 5 CTC

Circulating tumor cells, Disease Progression,and Survival in Metastatic Breast Cancer, Cristofanilli et al, Sem Oncol. 2006

Cristofanilli M, Budd GT, Ellis MJ, et al: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 351:781-791, 2004

MBC Clinical Trial Design

177 MBC (metastatic breast cancer)(20 centers)

- Measurable disease, any type or line of therapy (first line, chemo Rx)
- (67%ER/PR+, HER2 52%)
-145 healthy and 200 pts with benign disease
- Imaging and CTC analysis (prior to initiation of therapy)

CTC performed, 1 follow-up(~ 4 weeks) and 12 weeks

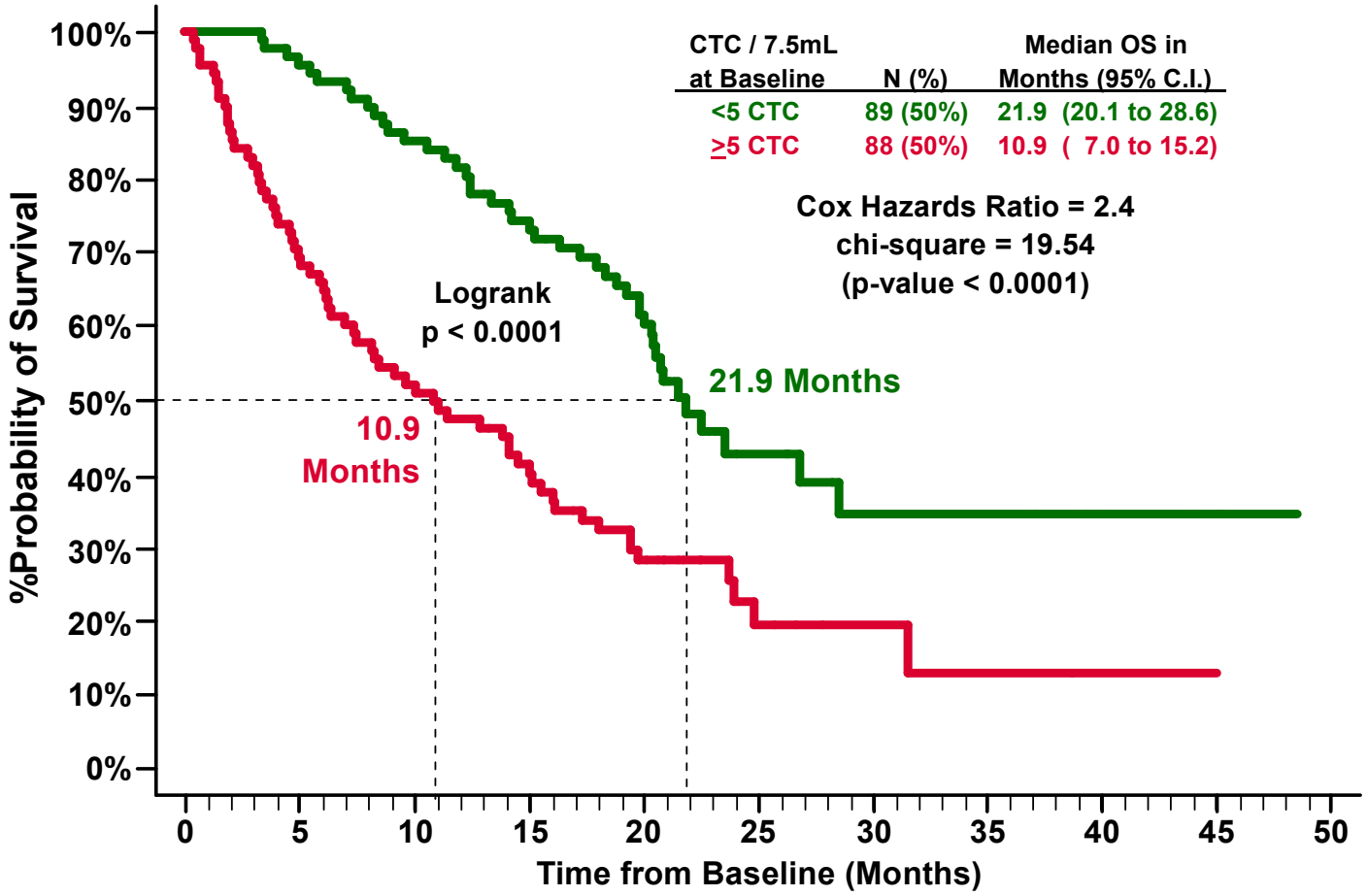
Duration of CTC: 6 months or until progression

Clinical follow up: 50 months

Imaging and clinical progression of disease at 12 weeks*

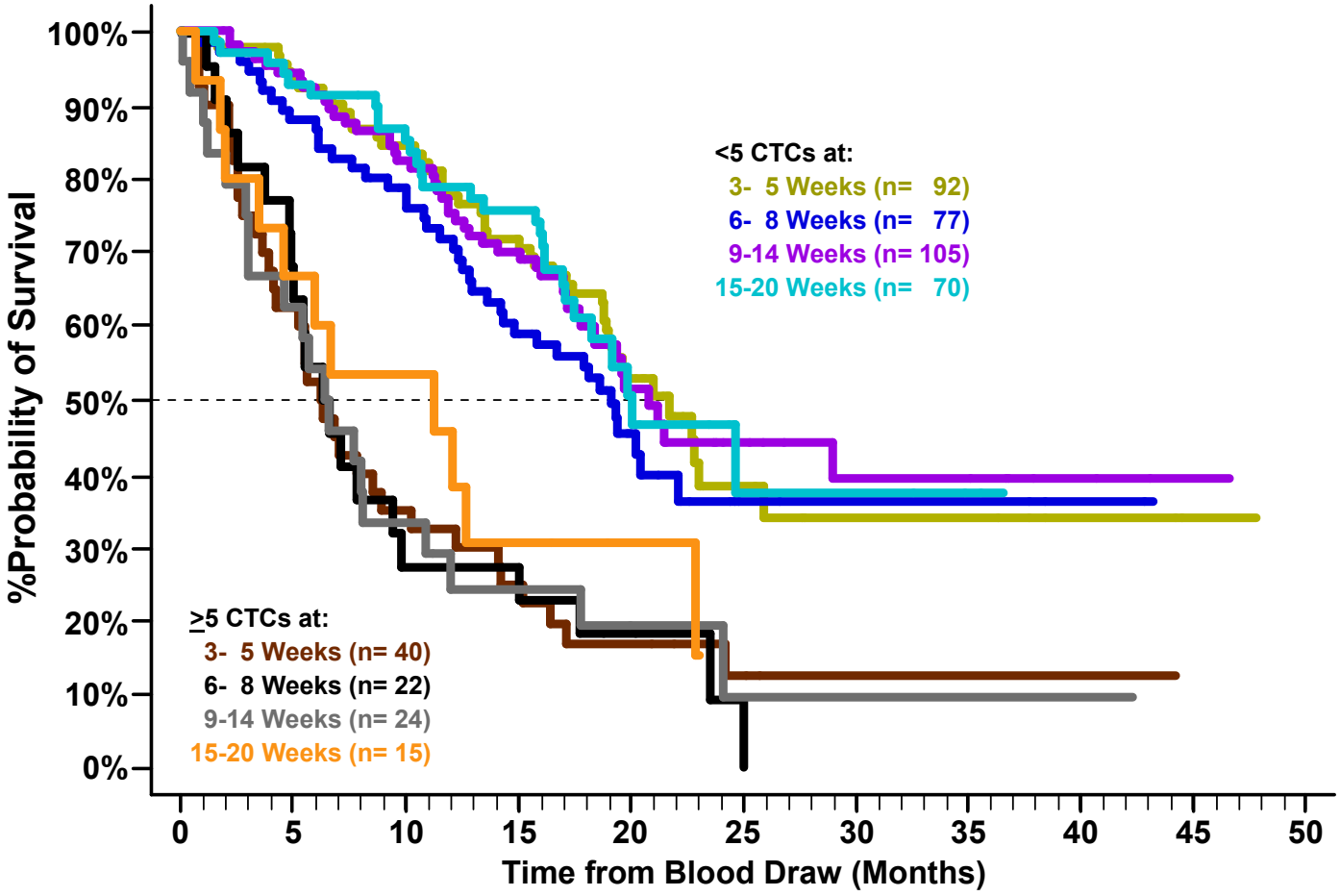
**Circulating tumor cells, disease Progression,and Survival in Metastatic Breast Cancer, Cristofanilli et al, NEJM 2004*

Predictive Value: OS of MBC Patients with <5 or ≥5 CTC at Baseline (N=177)



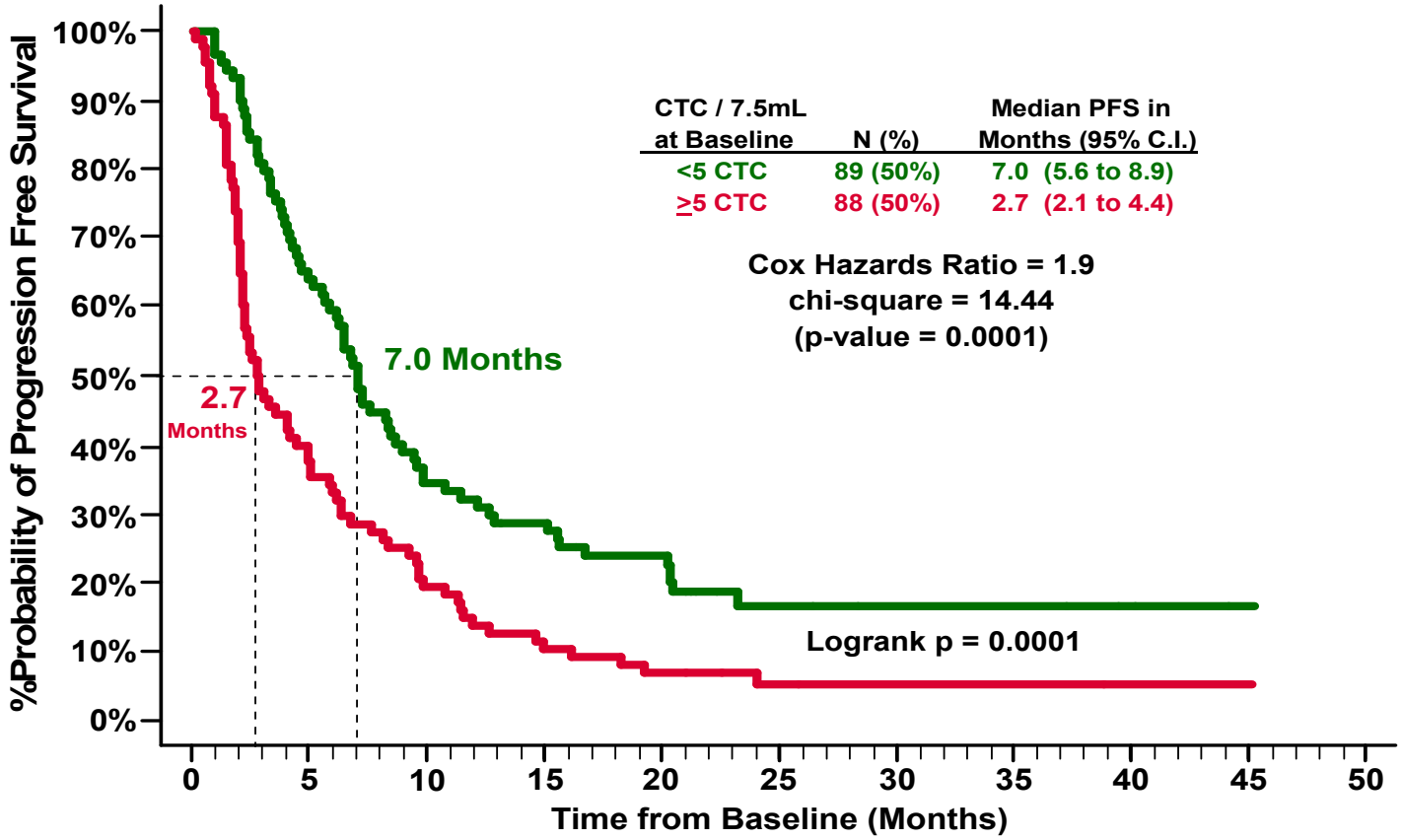
CellSearch™ *Cristofanilli M, Budd T,Ellis M, et al, N Eng J Med. 2004*

Predictive Value: OS of MBC Patients with <5 or ≥5 CTC at different times of Follow-Up



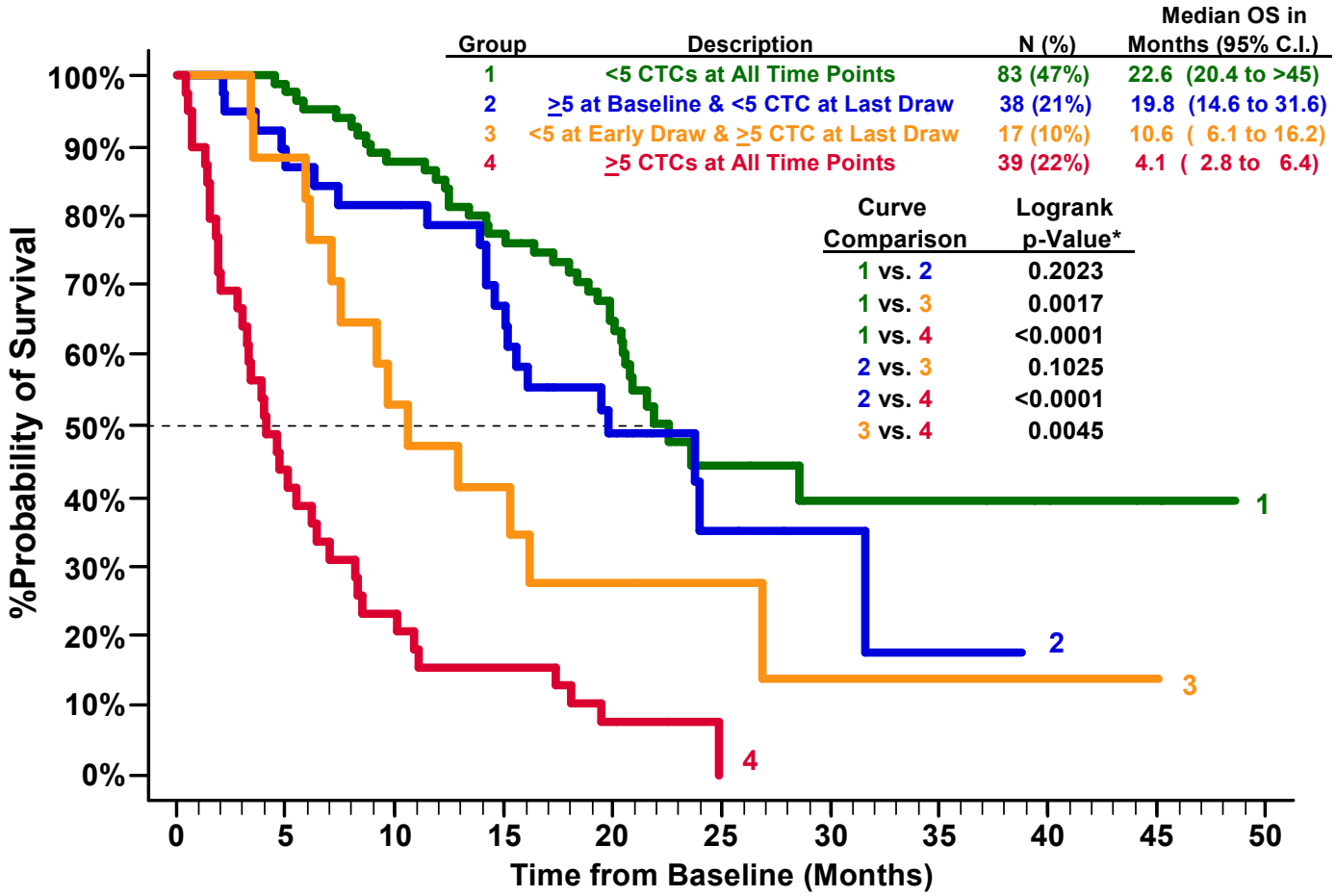
Cristofanilli M, Budd T,Ellis M, et al, N Eng J Med. 2004

Predictive Value: PFS of MBC Patients with <5 or ≥5 CTC at Baseline (N=177)



Cristofanilli M, Budd T,Ellis M, et al, N Eng J Med. 2004

A Reduction in CTC Below 5 After the Initiation of Therapy Predicts Longer OS whereas an Increase in CTC Count to 5 or above Predicts a Shorter OS



* *Cristofanilli M, Budd T,Ellis M, et al, N Eng J Med. 2004*

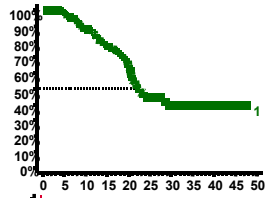
Metastatic Colorectal Cancers (MCRC) Cut off ≥ 3 CTCs

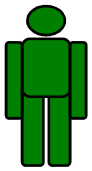
Cohen SJ, Punt CJ, Iannotti N et al: Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol 2008 Jul 1;26(19):

Metastatic Prostate Cancers (MPC)-K073338 Cut off ≥ 5 CTCs

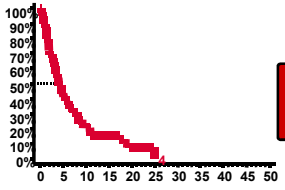
de Bono JS, Sher HI, Montogomery RB et al: Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer, Clin Can Res 2008 oct.

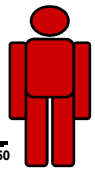
MBC, MCRC, & MPC
Median Overall Survival Comparison
(in months)



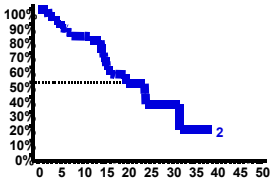


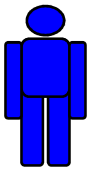
<cut-off at all time points



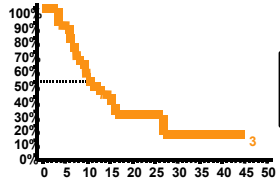


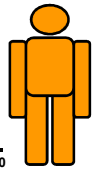
≥cut-off at all time points





≥cut-off at baseline and
<cut-off at final draw





<cut-off at early draw
and ≥cut-off at final draw

MBC	MCRC	MPC
22.6	18.6	>26
4.1	3.9	6.8
19.8	11.7	21.3
10.6	7.1	9.3

MBC, MCRC, and MPC Summary & Conclusion

MBC

- 177 patients
- Cut-off = ≥5 CTC
- Patients with ≥5 CTC at baseline = 50% (88/177 evaluable patients)
- Should be used for serial monitoring
- Predicts PFS and OS
- Combination of CTC and imaging may provide the most accurate assessment of patient prognosis

MCRC

- 430 patients
- Cut-off = ≥3 CTC
- Patients with ≥3 CTC at baseline = 26% (108/413 evaluable patients)
- Should be used for serial monitoring
- Predicts PFS and OS
- Combination of CTC and imaging may provide the most accurate assessment of patient prognosis

MPC

- 231 patients
- Cut-off = ≥5 CTC
- Patients with ≥5 CTC at baseline = 57% (125/219 evaluable patients)
- Should be used for serial monitoring
- Predicts PFS and OS
- Combination of CTC and PSA may provide the most accurate assessment of patient prognosis

CellSearch™ System Limitations

- Results should be used in conjunction with diagnostic tests (lab, imaging), physical exam and medical history.
- Not proven to affect overall health outcomes in patients with metastatic carcinoma
- Potential for monitoring patients
- Insufficient evidence as a marker of disease progression.

Yale CTC Experience

CellSearch (Veridex device) 2006

- >1000 CTC tests
- Clinicians (Oncologists): breast, colorectal and lung cancers
- Guide treatment, research use
- CTCs investigated for HER2/neu protein expression in breast cancer patient's

Interpretation

- Pathologist and cytotechnologist (certified by Veridex)
- CTC are defined as:

  -Nucleated cells lacking CD45 and express CK (8,18 & 19).

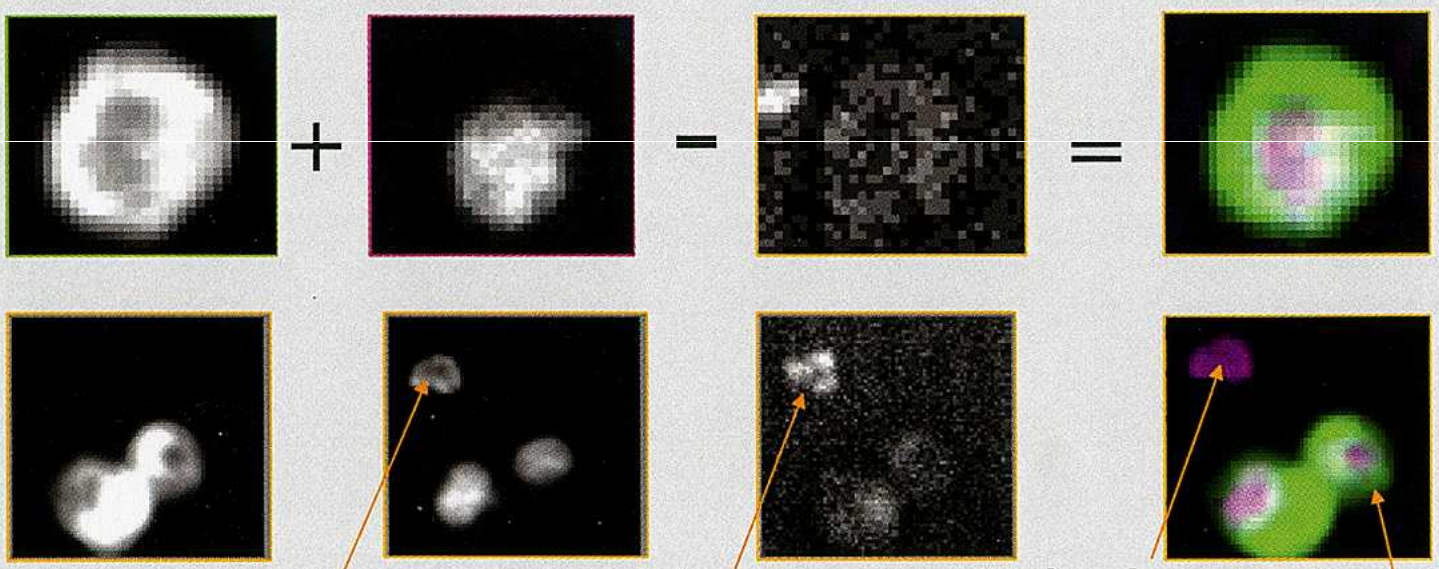
  -Morphology (round or oval with a nucleus within the cytoplasm).

  -Size (4um)

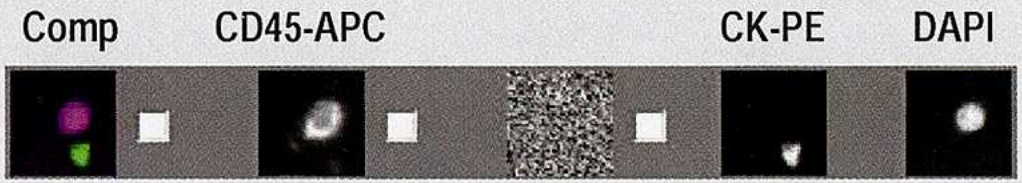
  -Heterogeneity (morphology and size).

Tumor Cell

Cytoplasm	Nucleus	Cell Membrane	Composite
CK-PE pos	DAPI pos	CD45-APC neg	Tumor Cell



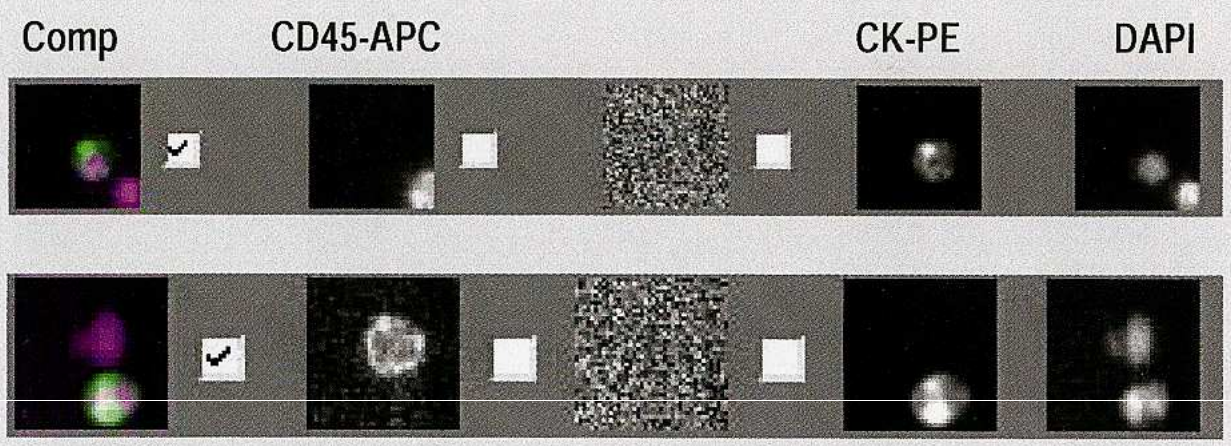
Leukocytes



Leukocytes (CK-PE-/DAPI+/CD45-APC+)

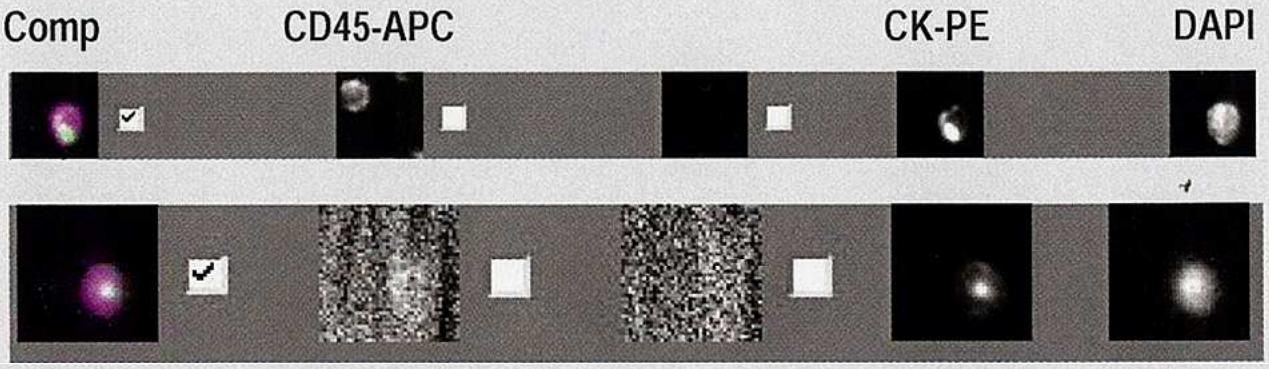
- CK-PE staining in this example is not associated with a nucleus, so it is not a tumor cell
- The nucleic acid staining is associated with staining in the CD45-APC channel, which means that this cell is a leukocyte
- No boxes should be checked

Tumor Cell and Leukocyte in Same Frame



- A single frame may contain more than one cell
- In the examples above, note that the DAPI channel presents two clearly identifiable nuclei
  - one nucleus corresponds to a CK-PE + cell
  - one nucleus corresponds to a CD45-APC + cell
  - the composite box should be checked in both instances to count the tumor cell

Tumor Cells with Dim PE and Bright DAPI

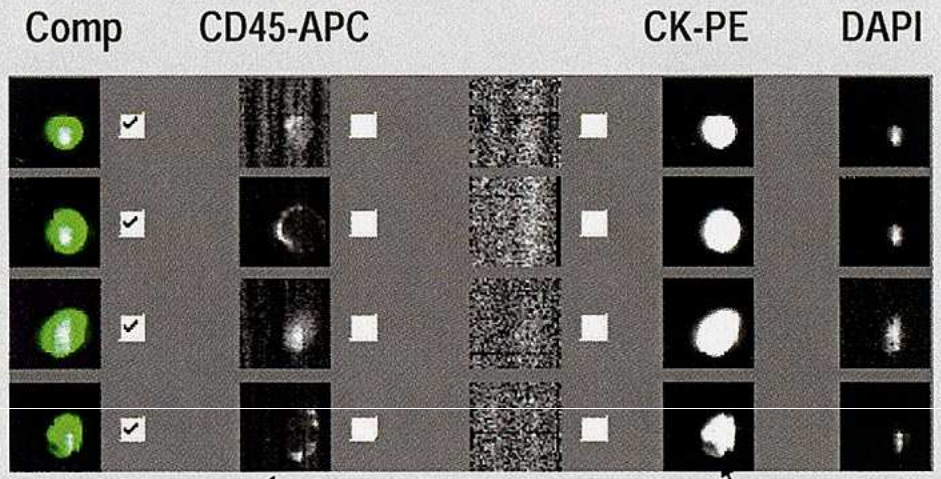


- If the CK-PE image is dim, the DAPI image may appear larger than the CK-PE image
  - Carefully examine CK-PE
  - Dim region in CK-PE is part of the entire cell

Note CD45-APC channel in first example:

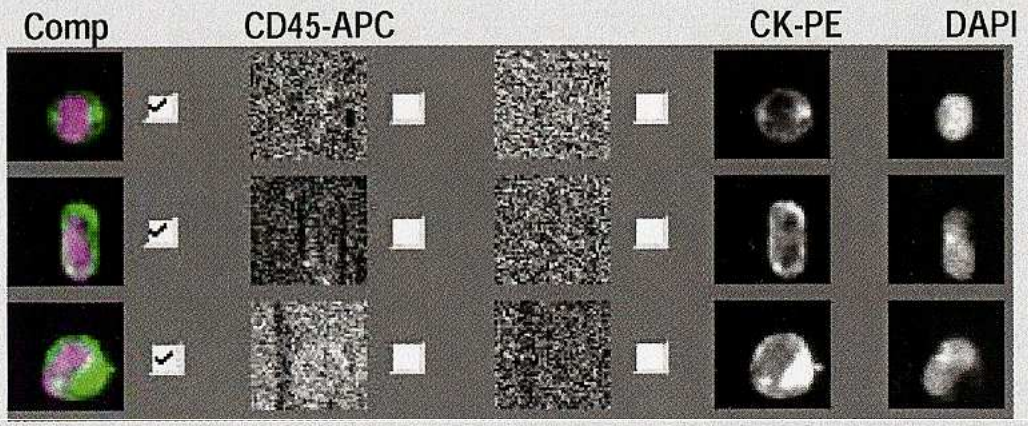
A leukocyte is also visible, but no nucleus is visible in the DAPI channel for the leukocyte.

Tumor Cells: Cytoplasmic Image in APC



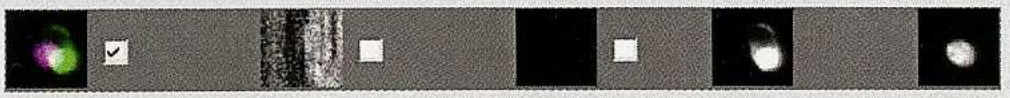
- If image shows a very bright CK-PE image and a dim, irregular or jagged, membrane pattern staining in the CD45-APC channel and all other tumor cell criteria are present, classify the cell as a tumor cell

Typical Tumor Cells



CK+, bright or dim

Tumor cells- nucleus more than 50% in

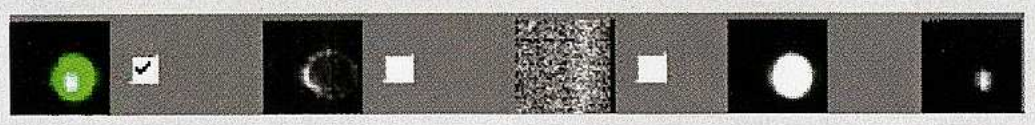


CK+, bright or dim

Tumor cells, nuclear shape in APC

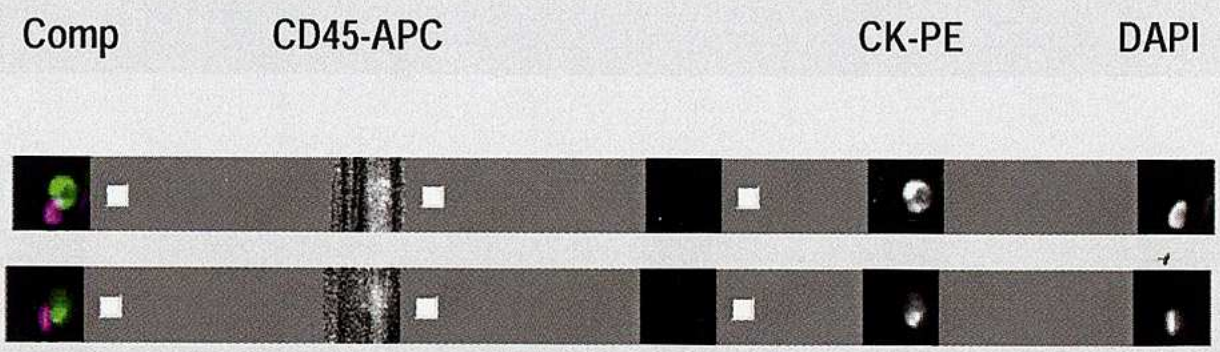
Very bright CK+

Tumor cells, cytoplasmic shape in APC



Very bright CK+

Suspicious Objects



"Detached Nuclei" Suspicious Cells

- Cytoplasm area does not surround the nucleus
- Nucleus appears to overlap the cytoplasm

Note: If many images in the sample display this appearance, it is also possible that the microscope stage has malfunctioned

Suspicious objects should not be counted as tumor cells because their significance has not been established.

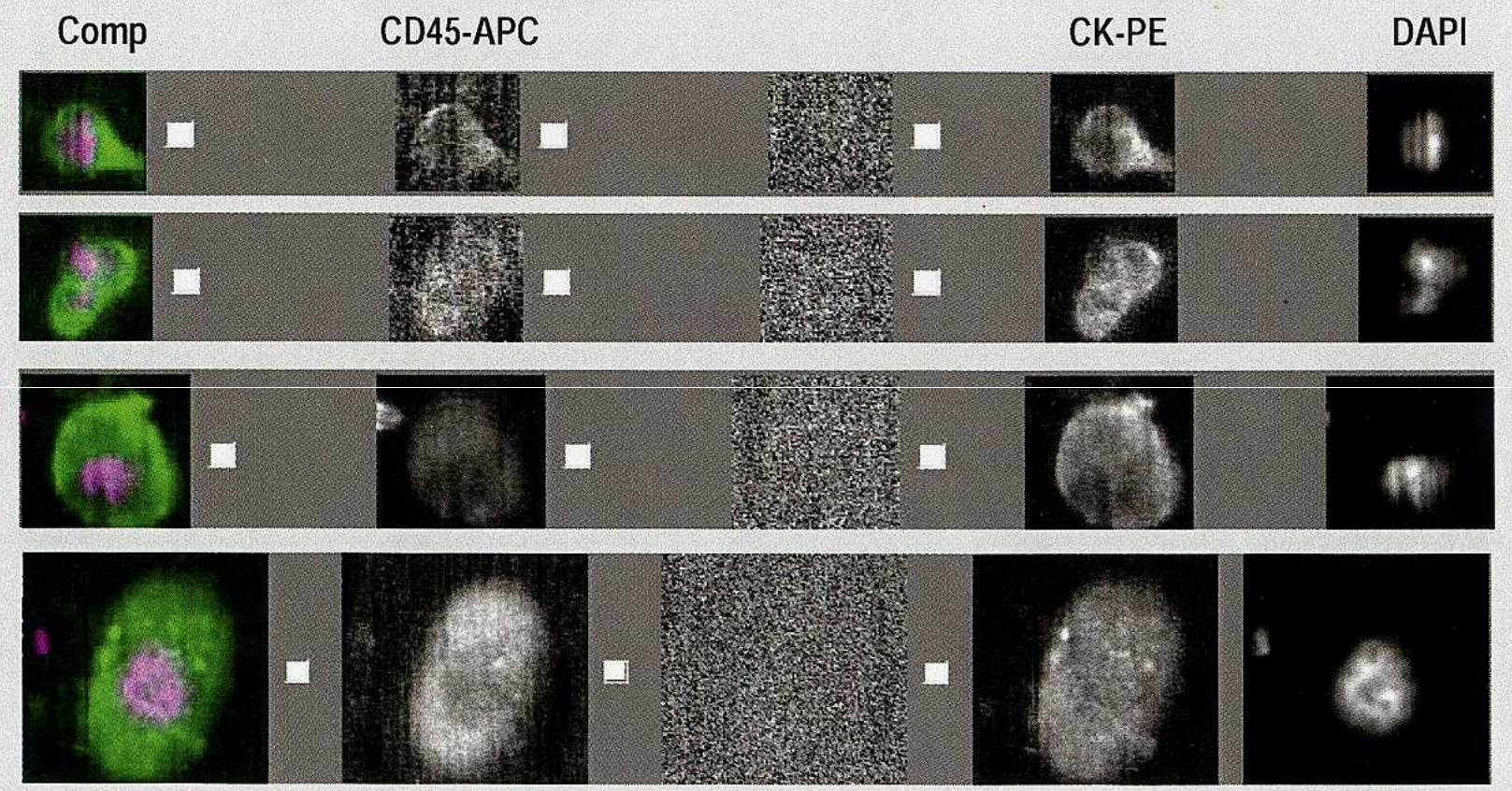
Suspicious Objects

"Speckled" or "Punctate" Suspicious Cells

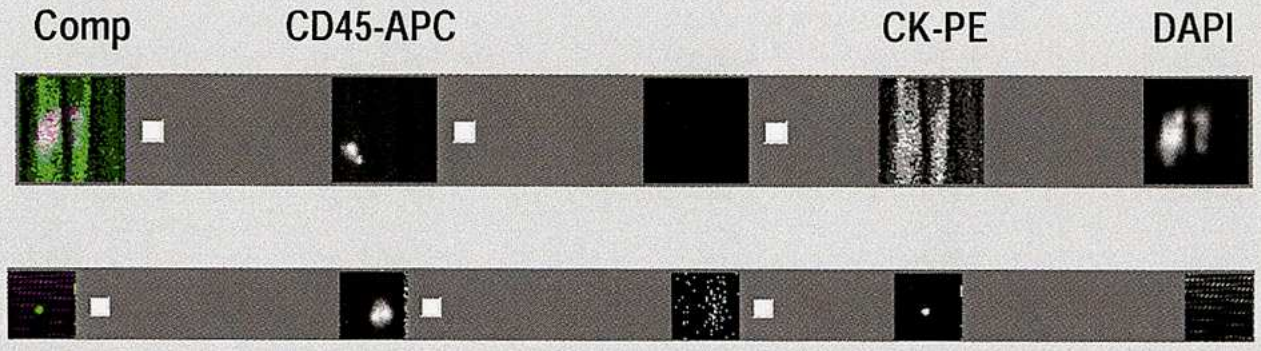
- delineated nuclear image
- irregular, speckled cytoplasmic staining

Note: Suspicious objects should not be counted as tumor cells because their significance has not been established.

Not Classified Cells

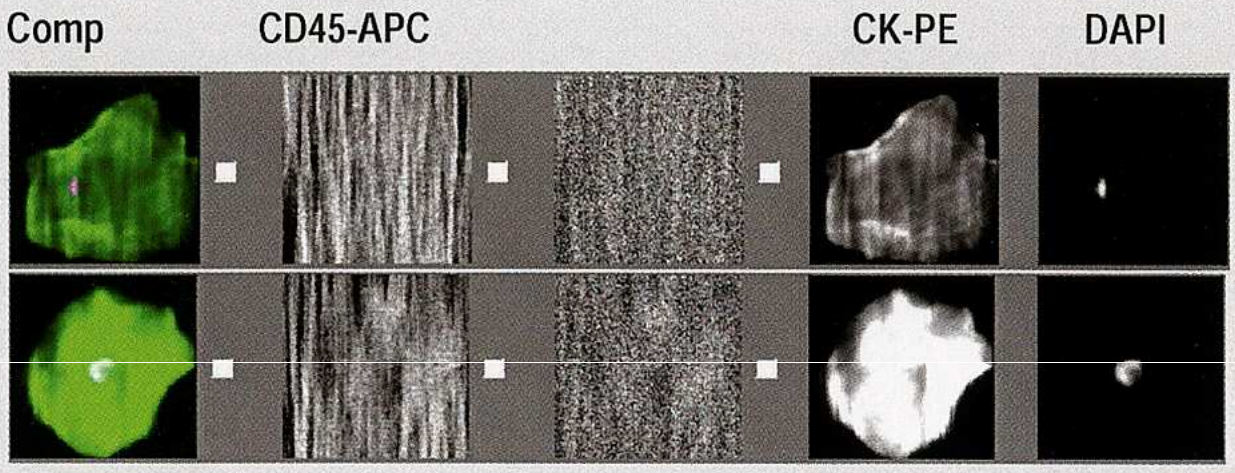


Computer Noise



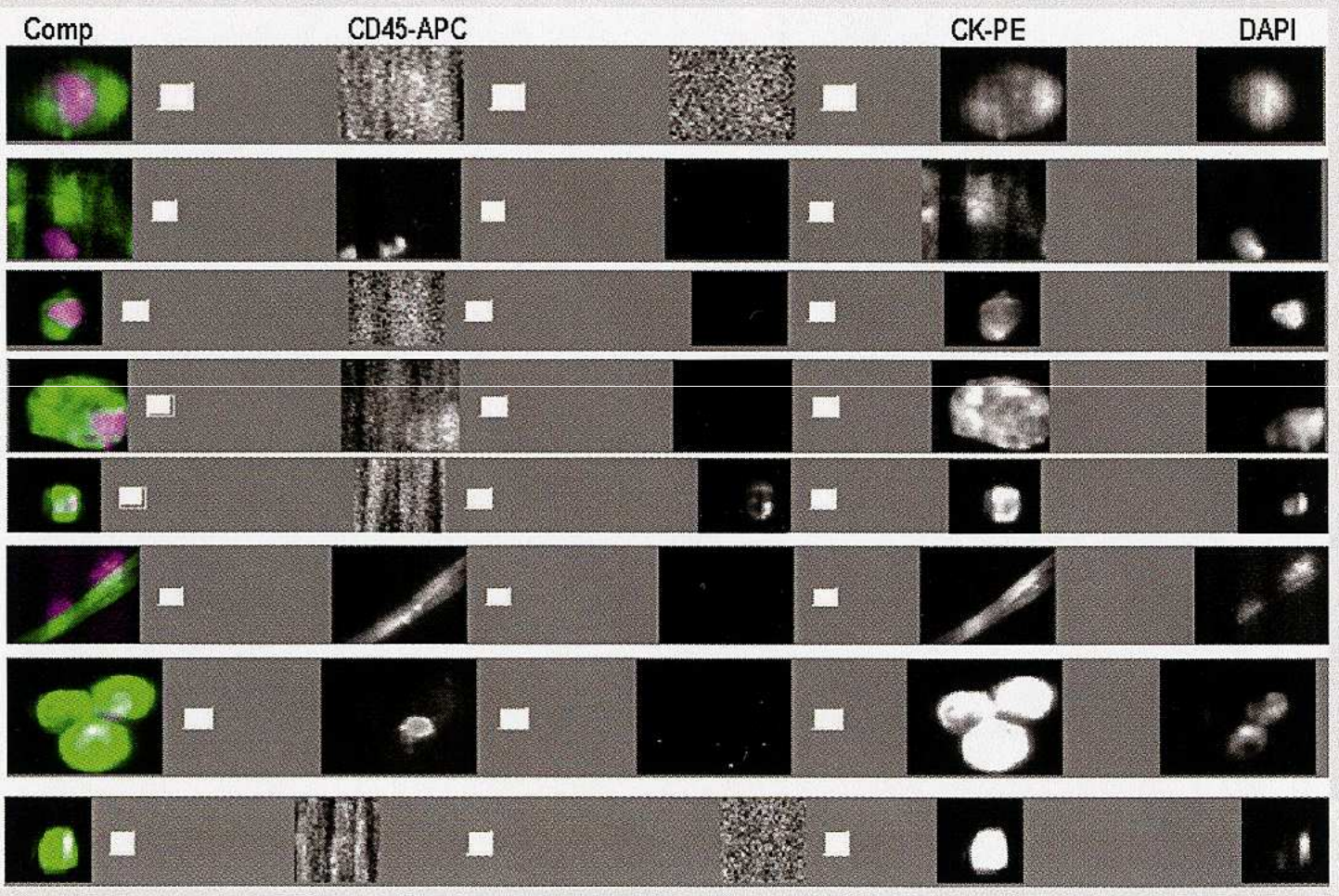
- Caused by over-amplification of the CK-PE or DAPI signals
- Easily recognized as non-cellular events.

Squamous Cells



- Easily identified by their low nuclear to cytoplasmic ratio
- “Corn flake” cytoplasmic appearance
- Very large, polygonal cells with round nuclei

Cell Interpretation Practice



Research Mode

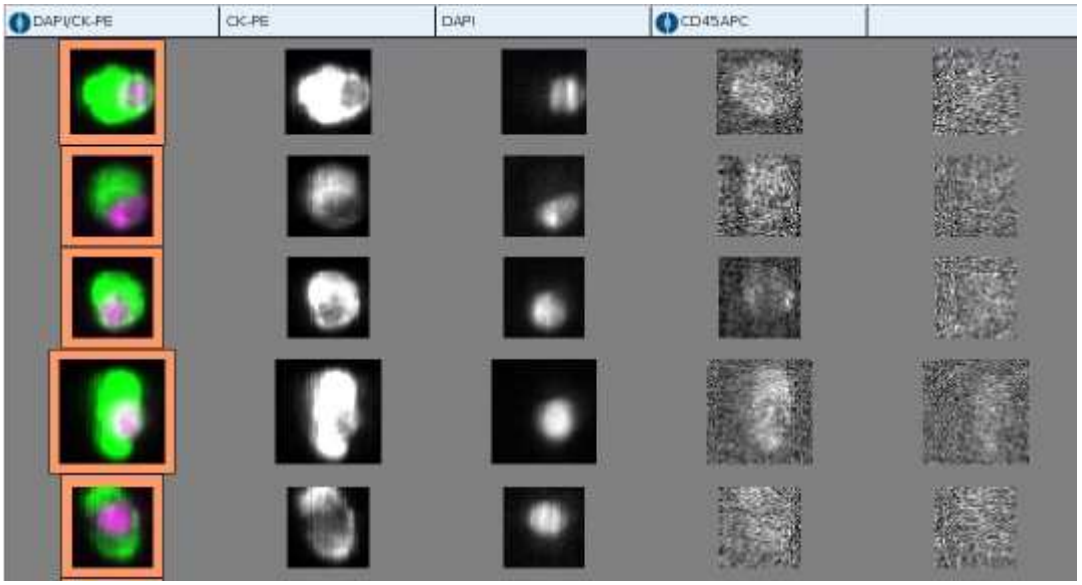
PE+/DAPI+/APC-/FITC+

Click the composite box and the FITC box to count the target cell as positive for the additional marker.

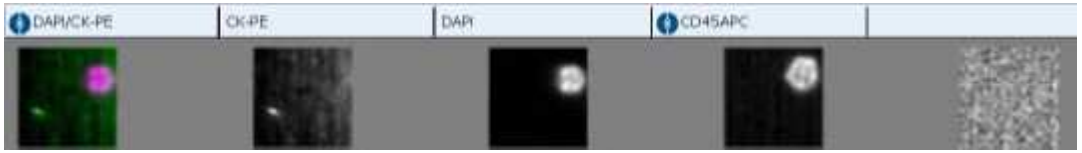
Cell Analysis

CTC's

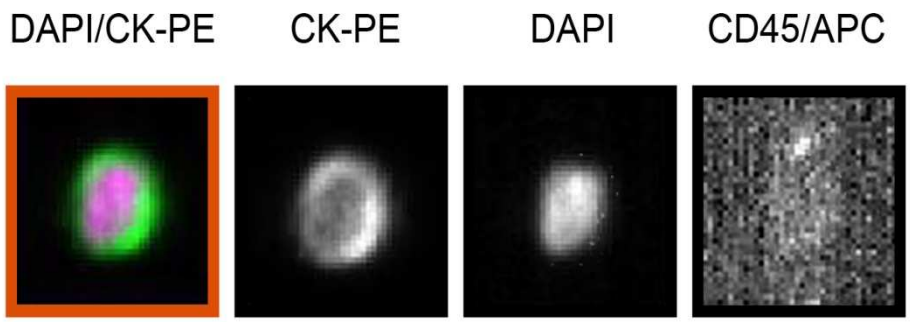
CK-PE+/DAPI+/CD45-APC-

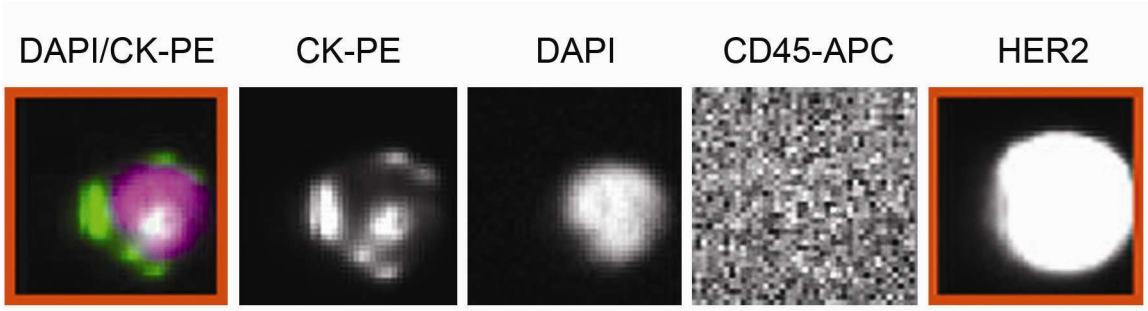


CD45-APC

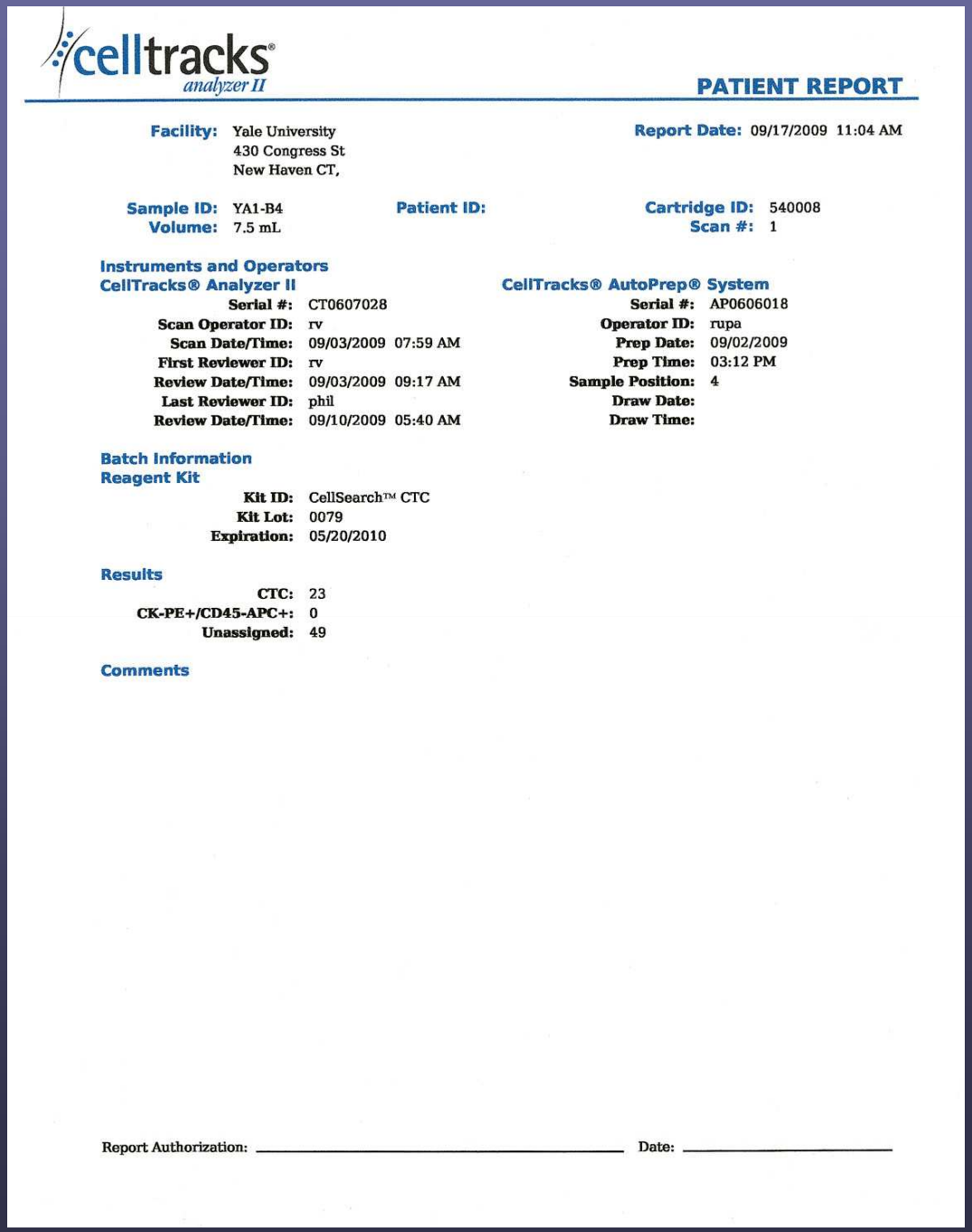


CTCs


DAPI/CK-PE
CK-PE
DAPI
CD45/APC


DAPI/CK-PE
CK-PE
DAPI
CD45-APC
HER2

CTC REPORT



celltracks® analyzer II

PATIENT REPORT

Facility: Yale University
430 Congress St
New Haven CT,

Report Date: 09/17/2009 11:04 AM

Sample ID: YA1-B4 **Patient ID:** **Cartridge ID:** 540008
Volume: 7.5 mL **Scan #:** 1

Instruments and Operators

CellTracks® Analyzer II

Serial #:	CT0607028
Scan Operator ID:	rv
Scan Date/Time:	09/03/2009 07:59 AM
First Reviewer ID:	rv
Review Date/Time:	09/03/2009 09:17 AM
Last Reviewer ID:	phil
Review Date/Time:	09/10/2009 05:40 AM

CellTracks® AutoPrep® System

Serial #:	AP0606018
Operator ID:	rupa
Prep Date:	09/02/2009
Prep Time:	03:12 PM
Sample Position:	4
Draw Date:	
Draw Time:	

Batch Information

Reagent Kit

Kit ID:	CellSearch™ CTC
Kit Lot:	0079
Expiration:	05/20/2010

Results

CTC:	23
CK-PE+/CD45-APC+:	0
Unassigned:	49

Comments

Report Authorization: _____ Date: _____

Summary

CTC detection in peripheral blood in clinical practice

- Low frequency (rare):1CTC among million red blood cells
- Standardized methods with high degree of reproducibility
- Currently, most data on the prognostic value, available for breast, prostate and colon cancers.
- Multicenter analysis and validation is needed to confirm clinical significance.

Summary
CellSearch™ System

- Valuable tool for monitoring cancer patient status and outcome. FDA approved.
- Employs immunomagnetic-enrichment based protocols focused on CTC number as the indicator of patient status or outcome.
- Multi center trial: The number of CTCs was a significant independent predictor of OS and PFS in patients with MBC, MCRC and MPC
- American Society of Clinical Oncology (ASCO): recommendation 2007: CTC test should not be used to make diagnostic or treatment decisions in patients with MBC

Future Potential and Applications: CTCs

- Guide prognosis: Metastatic and early stage cancer patients
- Measure response to anticancer Rx: predictive biomarker
- Select patients for adjuvant chemotheray
- Detect recurrent disease
- ‘Real time biopsy’: Surrogate for Tumor biology
- Molecular characterization: Discover and identify new targets for therapeutic manipulation

Conclusion

- CTC level (< 5): Favorable, this may imply a good response to treatment.
- Caution is warranted because of the lower sensitivity of the CTC test.
- Radiologic disease progression should not be ignored on the basis of a favorable CTC level.
- Favorable CTC level with overt radiologic progression may still suggest a better outcome

Conclusions

The CellSearch System (Veridex)

- Morphology skills highly similar to those of the Cytopathologist

  - Interpretation and Enumeration of CTCs.

  - Protein expression patterns of CTC (ER,PR,HER2, EGFR), additional prognostic information.

- Cytopathology lab with trained cytotechnologists and cytopathologists

  - Natural location for this technology in the healthcare delivery system.

Acknowledgements:

Yale University School of Medicine, Dept of Pathology

- David L. Rimm
- David Chhieng
- Diane Kowalski
- Lab Manager: Kevin Schofield
- Cytotechnologists: Brett Minger, Philip Galullo, Kristina Gordy, Rupa Vyas

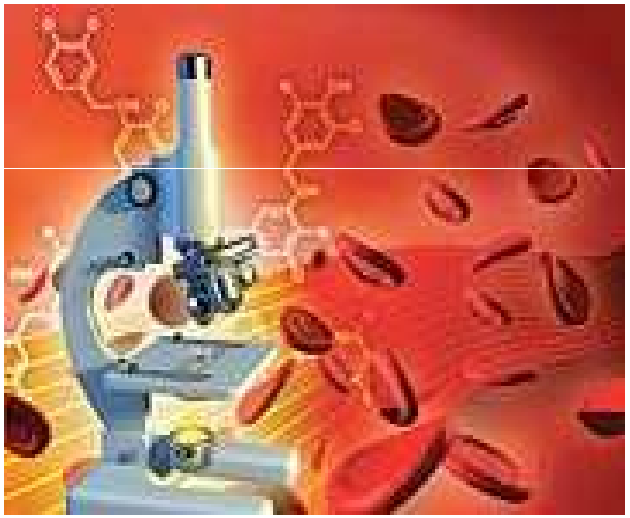
Veridex

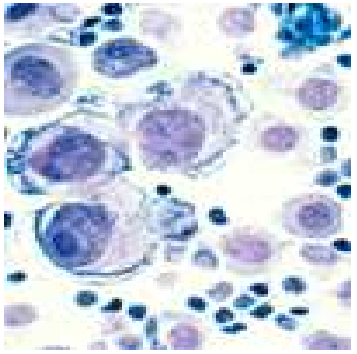
Brian Zuchelkowski

Vera Gibson

History

- Breast Group CEC/CTC Enumeration Study





What does it cost?

Product Code	Item	Price
7900000	CellSearch™ Epithelial Cell Kit (RUO) – 16 tests per kit	$2,800/kit
7900001	CellSearch™ Epithelial Cell Kit (IVD) – 16 tests per kit	$2,800/kit
7900002	CellSearch™ Epithelial Cell Control Kit RUO – 24 tests per kit	$240/kit
7900003	CellSearch™ Epithelial Cell Control Kit IVD – 24 tests per kit	$ 240/kit
7900004	CellSearch™ Profile Kit (RUO) – 16 tests per kit	$1,040/kit
7900006	CellSearch™ Tumor Phenotyping Reagent HER-2/neu (RUO)	$600.00/kit
7900007	CellSearch™ Tumor Phenotyping Reagent MUC-1 (RUO)	$600.00/kit
7900011	CellSearch™ Tumor Phenotyping Reagent EGFr (RUO)	$600.00/kit
7900009	CellSearch™ Circulating Endothelial Cell Kit (RUO)	$2800.00/kit
9528****	CellSave® Preservative Tubes – 100 tubes per pack	$495/Pack
7043****	CellTracks® AutoPrep Instrument Buffer – 20L per pack	$30/package

Lab cost around $175 per test
CPT Codes:
88346 x 3 (immunofluorescent study) = $380 x 3 = $1140
88361 x 2 (morphometric analysis, IHC) = $505 x 2 = $1010
88313 x 1 (special stain) = $210
Charge $2360 per test

Total Costs

Medicare Reimbursement Avg: $777.53
Labor/Overhead: $386.00
Labor/Overhead + Cost per test = $386.00 + $175.00 = $561.00

Tests Requirements

- High Complexity Tests
- Pathologist and cytotechnologist (certified by Veridex)
- Cell Interpretation Proficiency Assessment
- PT Test Requirement


APRIL 23, 2000
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