# A Comparative Study of Cytomorphology and Flow Cytometry to Detect Malignant Cells in the Body Cavity Fluid

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***Background:*** *Many types of cancer metastasize to the epithelial linings of the body cavity causing malignant fluid to accumulate in such spaces. Cytomorphological evaluation is considered essential in the diagnosis of malignant body fluid. Nevertheless, the accuracy of cytomorphological results is subjective and can vary depending on the cytopathologists' experience.*

***Objective:*** *To determine if DNA content analysis using propidium iodide (PI), anti-CD45 (leukocyte common antigen) and anti-AE1/AE3 (pan-cytokeratin) as analyzed by flow cytometry could be used to detect and differentiate malignant cells in the body fluid when compared to cytomorphological evaluation.*

***Material and Method:*** *A cross-sectional, laboratory-based, observational study on 90 specimens was conducted. Flow cytometric analysis was done. Sensitivity, specificity, positive predictive value, negative predictive value and accuracy were reported.*

***Results:*** *The DNA index (DI) cut-off value, as determined by the receiver operating characteristic curve of 1.215 or more, had 51.7% sensitivity and 89.1% specificity to detect malignant cells. When DI was combined with AE1/AE3 positivity, the sensitivity increased to 62.1% with 80.3% specificity. When such techniques were used in adjunct to cytospin preparation, the sensitivity and specificity increased to 89.7% and 65.6%, respectively. Twelve specimens (13.3%) had positive results by flow cytometry but negative cytomorphological results by pathologists, 4 of which were later confirmed as cancer from pleural biopsy. Eleven specimens (12.2%) had false negativity, 6 of which were unspecified metastatic carcinoma. Four specimens with negative flow cytometric results were cerebrospinal fluid (CSF) specimens with a low cell count. Subgroup analysis in the cases of non-CSF fluid showed 72% sensitivity and 72.1% specificity.*

***Conclusion:*** *Immunophenotypic analysis using DI and AE1/AE3 in conjunction with cytospin preparation had a moderately high sensitivity to detect malignant cells in the body fluid (~90%). Non-CSF specimens yielded better results than CSF. Further modifications are ongoing in order to increase the detection capabilities of our screening panel.*

***Keywords:*** *Body cavity fluid, Flow cytometry, Effusion, DNA content analysis, Cytomorphology*

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In everyday practice, cytomorphological evaluation has frequently been requested in pleural effusion, ascites or cerebrospinal fluid (CSF) specimens obtained from patients with a suspicion of malignancy. Nevertheless, the accuracy of cytomorphological results is subjective and can vary depending upon the cytologists' skills and experiences. DNA flow cytometric analysis has been previously reported to be a versatile, fast and accurate technique for analysis of DNA content in solid tumors[1-4], thus speeding the diagnostic process for patients. The role of flow cytometry to detect hematologic malignancy in the body cavity fluid remains problematic as it requires the utilization of multiple monoclonal antibody reagents[5-7], thus making flow cytometric analysis expensive. This has led to the need for a new analytic protocol, which uses fewer reagents for the detection of malignant body cavity fluid, while maintaining a high accuracy.

Neoplasms are the important causes identified in 30% to 60% of all pleural effusions[3]. Nearly all types of tumors have been reported to involve the pleura and the most common causes were lung cancer, breast cancer and lymphoma. In at least 5-10% of the cases,

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the primary cause cannot be identified[8]. Malignant ascites accounts for around 10% of all cases of ascites and occurs in association with a variety of neoplasms, especially breast, bronchus, ovary, stomach, pancreas and colon cancer[9,10]. Up to 20% of patients with malignant ascites have tumors of unknown primary origin[11]. Carcinomatous meningitis occurs in 4%-8% of patients with solid tumor and 5%-15% of patients with hematologic malignancy. The most common causes are lung cancer, breast cancer and melanoma while 1% to 7% of the cases have tumors of unknown primary[12].

The specimens of interest in the present study were pleural fluid, ascitic fluid and cerebrospinal fluid (CSF). The primary aim of the present study was to determine sensitivity, specificity and accuracy of flow cytometric analysis, using DNA index, in the detection of malignant body cavity fluid. The secondary aim was to determine whether flow cytometry of the body cavity fluid can be used in conjunction with cytospin preparation using Wright's stain to increase the detection sensitivity of malignant cells.

## Material and Method

### *Study design and patient samples*

The present study design was a laboratory-based, cross-sectional, observational study. All patients, aged 18 years or more, who were to have either a therapeutic or diagnostic paracentesis of CSF, pleural effusion, or ascites by indication, were eligible to enter the present study. The present study took place between November 2010 and January 2012. Specimens were excluded from the study if they did not have concomitant cytomorphological analysis by pathologists. All patients provided a written informed consent. The present study was approved by the institutional review board/ethics committee of Faculty of Medicine Siriraj Hospital.

### *Specimen processing*

Specimens were collected in sterile specimen bottles; one bottle was sent to pathology laboratory for cytomorphological evaluation by pathologists and the other bottle was added with heparin 1:10,000 mL and sent to flow cytology laboratory. At least 30 mL of ascites and pleural fluid and 2 mL of CSF were required. For ascites and pleural fluid, which may have many red blood cells present, mononuclear cells (MNC) were separated from red blood cells using density-gradient centrifugation technique (Lymphoprep®, Axis-Shield PoC, Oslo, Norway). MNC were then washed with PBS twice. For CSF, specimens were centrifuged at 2,500 rpm for 5 minutes and the whole cellular component was used for analysis due to a low cell yield. Peripheral blood MNC from healthy volunteers were added to the specimen in a 1:1 ratio for use as euploidy control. Cells were then split into 2 tubes for further staining. One tube was stained with fluorescein isothiocyanate (FITC)-labeled anti-AE1/AE3, an antibody cocktail to detect surface cytokeratin (CK) expression in epithelial-derived cells. Another tube was stained with FITC-labeled anti-CD45, an antibody to leukocyte common antigen on white blood cells. Both antibodies were stained as directed by the manufacturer (Becton-Dickinson, California, USA). Propidium iodide (PI) staining using cycle analysis kit (Becton-Dickinson, California, USA) was performed as directed by the manufacturer.

### *Cytomorphological analysis by pathologists*

Specimens were sent to Pathology Laboratory and processed by centrifugation. Cells were stained with Papanicolaou's stain and Diff-Quick stain. The final reports by cytopathologists were obtained from the hospital charts.

### *Cytomorphological analysis by Wright's staining*

One portion of specimen was separated and then centrifuged. Cells were smeared on glass slide and air-dried. Slides were stained by Wright's staining technique. Results were read by the principal investigator (WL).

### *Flow cytometry analysis*

Processed specimens were analyzed with 4-color flow cytometer (FACSCalibur, Becton-Dickinson, California, USA). Cells were analyzed at least 30 minutes after adding PI to the samples. For pleural effusion and ascites, 20,000 events were required. For CSF, all cells are used for analysis. Compensation setting for flow cytometry DNA content analysis was used as described a priori[13]. DNA aneuploidy was considered when there was an additional G0-G1 peak other than normal diploidy.

The DNA index (DI) was calculated from the following equation:

$$DI = \frac{Mean\ channel\ number\ of\ aneuploid\ G_0/G_1}{Mean\ channel\ number\ of\ diploid\ G_0/G_1}$$

### *Statistical analysis*

Data were described as median and range when data was continuous and as absolute and relative

frequency when data was categorical. Inter-method results were compared using pathologists' cytomorphological results as gold standard. All result readers were blinded from each other's results. Specimens with inconclusive cytomorphology results were excluded from inter-method comparison. Receiver operating characteristic (ROC) curve was analyzed for optimal DNA index cut-off. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of methods were calculated.

**Results**

The characteristics of the 90 specimens enrolled are shown in Table 1. The specimens recruited were slightly male predominance. Approximately half of the specimens were pleural fluid, while the other half of the specimens consisted of CSF and ascites equally. Pre-test clinical diagnosis was in favor of malignant effusion (71.1%).

For flow cytometric analysis, DNA index (DI) was used to analyze DNA content of the cells in the specimen. Euploidy specimens have only a diploid peak with an insignificant small tetraploid peak, resulting in DNA index equal to 1.0. Aneuploidy specimens have another hyperdiploid peak, thus DNA index can be obtained as described in the study method. ROC analysis (as shown in Fig. 1) to determine the optimal DI cut-off showed that DI greater or equal to 1.215 yielded 51.7% sensitivity and 89.1% specificity to detect malignant cells in the body cavity fluid.

Flow cytometric analysis combining DNA content analysis with surface expression of pan-cytokeratin and leukocyte common antigen in this study is shown as in Fig. 2. CD45 was expressed mostly on lymphocyte cells and lymphoma cells. Flow cytometric analysis showed CD45 positivity in euploidy control, thus CD45 could not be used as a positive marker for malignant cells, but could be used for internal control. Pan-cytokeratin antigens were expressed in epithelial-derived cells, which were negative in normal specimen but positive in carcinoma. In the present study, the authors used anti-AE1/AE3 as marker for pan-cytokeratin expression, thus carcinoma cells showed AE1/AE3 positivity.

In order to increase sensitivity of flow cytometric detection capability of flow cytometry method, we defined a positive specimen as having DNA index greater or equal to 1.215 or AE1/AE3 positivity. Sensitivity, specificity and accuracy results are shown in Table 2. Subgroup analysis of specimens with a pretest diagnosis of solid malignancy yielded a higher sensitivity, but a lower specificity.

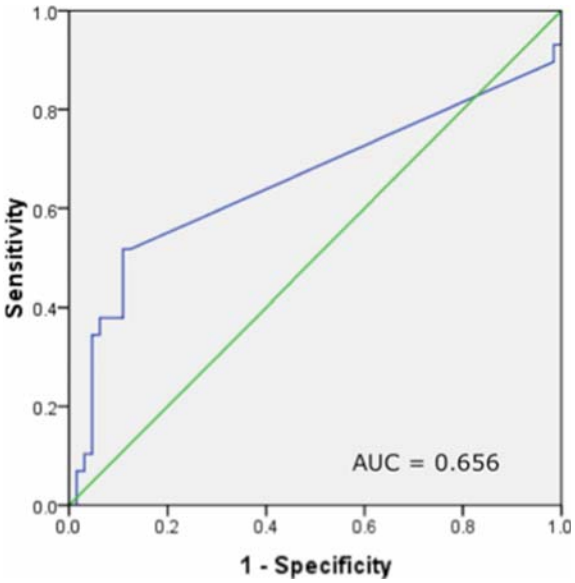


**Fig. 1** ROC curve analysis of DNA index

**Table 1.** Characteristics of the study specimens

| Characteristics | n = 90 |
|---|---|
| | Number of cases (%) |
| Male sex | 56 (62.2) |
| Specimen type | |
| CSF | 22 (24.4) |
| Pleural | 46 (51.1) |
| Ascites | 22 (24.4) |
| Age | Years |
| Median | 60.0 |
| Range | 18-87 |
| Clinical diagnosis | Number of cases (%) |
| Non-malignancy | 26 (28.9) |
| Hematologic malignancy | 29 (32.2) |
| Other malignancy | 35 (38.9) |

Flow cytometry analysis showed no positive CSF specimen. Further analysis showed that the mean cell count yielded from CSF specimens was $3.76 \times 10^3$ cells/uL, thus making flow cytometric analysis inaccurate. Subgroup analyses of the non-CSF specimen, as shown in Table 2, increased flow cytometric analysis sensitivity while maintaining a moderate specificity. Discordant results between flow cytometric analysis and standard cytomorphology by pathologists are shown in Table 3.

Cytospin preparations by Wright's stain were also analyzed as compared to standard cytomor-

phology. To determine whether flow cytometric analysis could be used in addition to cytospin analysis to increase sensitivity of malignant cell detection, positive specimens were defined as flow cytometry positivity or cytospin positivity. Sensitivity, specificity and accuracy results are shown in Table 4.

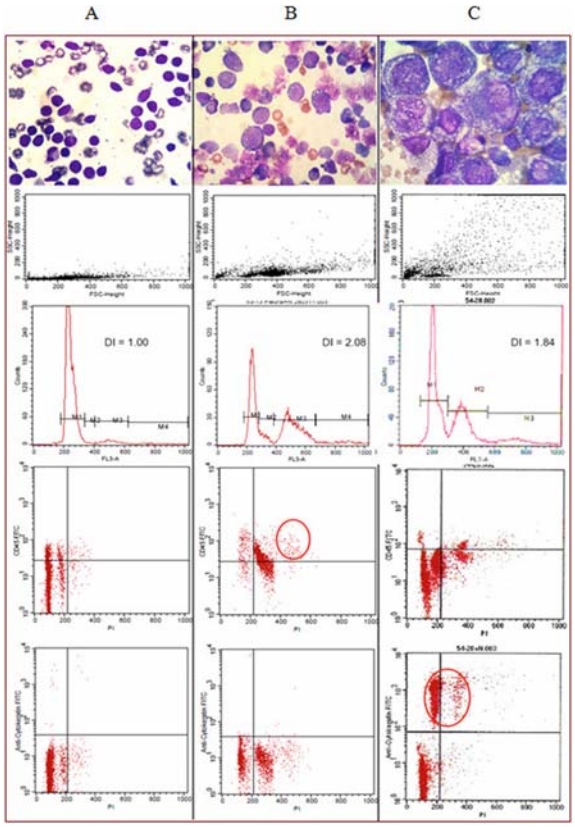


**Fig. 2** Comparative figures between cytomorphology and flow cytometric analysis in representative negative (A) and positive cases; diffuse large B-cell lymphoma (B) and adenocarcinoma (C)

## Discussion

In the present study, DNA content analysis was tested for its ability to detect malignant cell in the body cavity fluid as compared to cytomorphological evaluation. The study of 90 specimens revealed that the DI cut-off value, as determined by the ROC curve of 1.215 or more, had 51.7% sensitivity and 89.1% specificity to detect malignant cells. The authors results were comparable to previous studies on DNA content analysis by flow cytometry[1-3]. Flow cytometry positivity in benign fluid occurred in 5 specimens including 1 parapneumonic effusion, 1 disseminated tuberculosis and 3 cirrhosis. Previous study showed that 3% of benign effusion could be detected by DNA flow cytometry, which was comparable to the 5.5% in the present study.

With respect to false positivity and negativity of flow cytometry, it was of interest to find 4 cases with positive flow cytometry but negative standard cytomorphology who had pleural biopsies proven to be lung cancer (1) and breast cancer (3). In three additional cases including 2 hepatocellular carcinoma and 1 pancreatic carcinoma with positive flow cytometric analysis and negative cytomorphology results, no pathological abnormalities of body cavity lining specimens were evident to confirm the presence of malignant cells. Such specimens were positive by DI criteria, but not AE1/AE3. The negative results by AE1/AE3 may be explained by a low reactivity to AE1/AE3 by hepatocytes and hepatocellular carcinoma[14,15] due to its expression of CK8/18, which is not reactive to anti-AE1/AE3 clone.

False negativity occurred in 4 CSF specimens

**Table 2.** Sensitivity, specificity and accuracy of DNA index, combined with AE1/AE3 positivity as compared with standard cytomorphology by pathologists, including subgroup analysis in specimens with a pretest clinical diagnosis of solid malignancy and non-CSF specimens

| | FCM DI ≥1.215 or AE1/AE3 + (n = 90) | Clinical solid malignancy (n = 35) | Non-CSF specimen (n = 68) |
|---|---|---|---|
| True disease (Positive by gold standard) | 29 | 20 | 25 |
| Parameters | Value% (95% CI) | Value% (95% CI) | Value% (95% CI) |
| Sensitivity | 62.1 (42.3-79.3) | 75.0 (50.9-91.3) | 72.0 (50.6-87.9) |
| Specificity | 80.3 (68.2-89.4) | 53.3 (26.6-78.7) | 72.1 (56.3-84.7) |
| Positive Predictive Value | 60.0 (40.6-77.3) | 68.2 (45.1-86.1) | 60.0 (40.6-77.3) |
| Negative Predictive Value | 81.7 (69.6-90.5) | 61.5 (31.6-86.1) | 81.6 (65.7-92.3) |
| Accuracy | 74.0 (64.9-83.1) | 65.7 (50.0-81.4) | 72.1 (61.4-82.8) |

**Table 3.** Discordant result between DNA content analysis by flow cytometry as compared to standard cytomorphological result by pathologists. Percentages in this table were percentages of discordant results in each type of specimen

| | Flow cytometry negative while cytomorphology by pathologist positive (n = 11) | Number of cases (%) | Flow cytometry positive while cytomorphology by pathologist negative (n = 12) | Number of cases (%) |
|---|---|---|---|---|
| Specimen type | CSF | 4 (18.2) | CSF | 0 |
| | Pleural effusion | 3 (6.5) | Pleural effusion | 5 (10.9) |
| | Ascitic fluid | 4 (18.2) | Ascitic fluid | 7 (31.8) |
| Final diagnosis | CSF | | Pleural effusion | |
| | - Lymphoma | 4 | - Parapneumonic effusion | 1 |
| | Pleural effusion | | | |
| | - Lung cancer | 1 | - Lung cancer | 1 |
| | - Lymphoma | 2 | - Breast cancer | 3 |
| | Ascites | | Ascites | |
| | - CA ovary | 2 | - Cirrhosis | 3 |
| | - Hepatocellular carcinoma (HCC) | 2 | - Disseminated TB | 1 |
| | | | HCC | 2 |
| | | | - CA pancreas | 1 |

**Table 4.** Sensitivity, specificity, and accuracy of flow cytometry, cytospin by Wright's stain, and combination of flow cytometry and Wright's stain

| n = 90<br>Parameters | FCM DI ≥ 1.215 or AE1/AE3 +<br>Value % (95% CI) | Cytospin Wright's stain<br>Value % (95% CI) | FCM + or cytospin +<br>Value % (95% CI) |
|---|---|---|---|
| Sensitivity | 62.1 (42.3-79.3) | 75.9 (56.5-89.7) | 89.7 (72.6-97.8) |
| Specificity | 80.3 (68.2-89.4) | 82.0 (70.0-90.6) | 65.6 (52.3-77.3) |
| Positive Predictive Value | 60.0 (40.6-77.3) | 66.7 (48.2-82.0) | 55.3 (40.1-69.8) |
| Negative Predictive Value | 81.7 (69.6-90.5) | 87.7 (76.3-94.9) | 93.0 (80.9-98.5) |
| Accuracy | 74.0 (64.9-83.1) | 80.0 (71.7-88.3) | 73.3 (64.2-82.4) |

with a positive cytomorphological results for lymphoma due to a low cell count. Low CSF cell yield thus makes an interpretation of CSF difficult and precludes an accurate flow cytometric analysis. Subgroup analysis in non-CSF specimens showed an increased sensitivity from 62.1% to 72.0% with some decrease in specificity as compared to CSF specimens. False negativity in pleural effusion and ascitic fluid occurred in 7 specimens (lung cancer 1, lymphoma 2, ovary cancer 2, and hepatocellular carcinoma 2). These discordances may result from an aberrant loss of surface AE1/AE3 expression and no DNA content abnormalities.

Cytospin preparation by Wright's stain yielded moderate sensitivity and high specificity, which were comparable to flow cytometric analysis. When flow cytometry positivity or cytospin positivity was defined as a positive specimen, sensitivity and specificity were 89.7% and 65.6% respectively. The result was impressive in terms of negative predictive value, which was quite high at 93.0%. Thus, flow cytometry can be used in conjunction with cytospin technique to increase the sensitivity in detection of malignant cells in the body cavity fluid.

The present study utilizes flow cytometer, which is a rapid instrument that can be used to simultaneously determine DNA contents and surface antigenic expression of a single cell. When DI was combined with AE1/AE3 positivity, the sensitivity increased to 62.1% with 80.3% specificity. In this study, a number of malignant cytolomorphologically negative specimens were found to be positive by flow cytometry and subsequently confirmed by additional tissue

biopsy. Nevertheless, there is still room for improvements in the flow cytometry protocol, such as improvement in the CSF detection protocol and the addition of another broader antibody panel.

**Conclusion**

Immunophenotypic analysis using DI and AE1/AE3 is useful for the detection of malignant cells in the body cavity fluid with moderate sensitivity and high specificity. A combination of cytospin preparation by Wright's stain and flow cytometry gives the highest sensitivity (90%). The detection capability of flow cytometry may be improved with further modifications of the screening antibody panel.

**Contributors**

WL contributes to study design, data collection and analysis, and drafting of the manuscript. OP performs flow cytometric analysis. CUA supervises the project and makes a critical revision of the manuscript.

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**Potential conflicts of interest**

None.

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# การเปรียบเทียบการตรวจหาเซลล์มะเร็งในน้ำจากช่องเยื่อบุต่างๆ ของร่างกายด้วยวิธีโฟลซัยโตเมทรีและการตรวจพิเคราะห์ทางสัณฐานวิทยา

**วศิเทพ ลิ้มวรพิทักษ์, อรทัย พรหมสุวิชา, จิรายุ เอื้อวรากุล**

***ภูมิหลัง:*** *การวินิจฉัยภาวะเซลล์มะเร็งแพร่กระจายสู่น้ำในช่องเยื่อบุต่างๆ ของร่างกายใช้การตรวจพิเคราะห์ทางสัณฐานวิทยาเป็นหลัก ซึ่งหลายครั้งวิธีการตรวจดังกล่าวอาจไม่สามารถตรวจพบเซลล์มะเร็งในปริมาณน้อยๆ หรือไม่อาจสรุปได้ว่าสารคัดหลั่งนั้นมีเซลล์มะเร็งอยู่หรือไม่ ขึ้นกับประสบการณ์และทักษะของผู้ตรวจวินิจฉัย*

***วัตถุประสงค์:*** *เพื่อศึกษาว่าการตรวจปริมาณสารพันธุกรรม (ดัชนีดีเอ็นเอ) ร่วมกับการตรวจการแสดงออกของแอนติเจนบนผิวเซลล์ด้วยวิธีโฟลซัยโตเมทรี สามารถนำมาใช้ตรวจวินิจฉัย และแยกชนิดเซลล์มะเร็งได้หรือไม่*

***วัสดุและวิธีการ:*** *ศึกษาไปข้างหน้าโดยเก็บตัวอย่างจากผู้ป่วยที่มีน้ำจากช่องเยื่อบุต่างๆ ของร่างกาย และวัดดัชนีดีเอ็นเอร่วมกับการแสดงออกของแอนติเจน 2 ชนิด คือ leukocyte common antigen และ AE1/AE3 ด้วยเทคนิคโฟลซัยโตเมทรี*

***ผลการศึกษา:*** *จากตัวอย่างตรวจ 90 ตัวอย่าง พบว่าค่าดัชนีดีเอ็นเอที่เหมาะสมที่สุดคือ มากกว่าหรือเท่ากับ 1.215 โดยมีความไวร้อยละ 51.7 ความจำเพาะร้อยละ 89.1 เมื่อนำไปใช้ร่วมกับการแสดงออกของ AE1/AE3 บนผิวเซลล์ พบว่าความไวเพิ่มขึ้นเป็นร้อยละ 62.1 และมีความจำเพาะร้อยละ 80.3 เมื่อใช้ผลค่าดัชนีดีเอ็นเอหรือการแสดงออกของ AE1/AE3 หรือผลการย้อมสีไรท์ร่วมกัน จะมีความไวร้อยละ 89.7 และความจำเพาะร้อยละ 65.6 โดยมี 12 ตัวอย่างที่ได้ผลบวกโดยโฟลซัยโตเมทรีแต่ได้ผลลบจากสัณฐานวิทยา ซึ่งใน 4 ตัวอย่างนั้นมีมะเร็งแพร่กระจายจริงจากการตรวจตัดชิ้นเนื้อเยื่อหุ้มปอด การตรวจโฟลซัยโตเมทรีของน้ำไขสันหลังได้ผลไม่ดีนักเนื่องจากมีปริมาณเซลล์ออกมาน้อย การวิเคราะห์เฉพาะกลุ่มตัวอย่างที่ไม่ใช่น้ำไขสันหลังได้ผลดีกว่า โดยพบความไวของโฟลซัยโตเมทรีร้อยละ 72 และความจำเพาะร้อยละ 72.1*

***สรุป:*** *การตรวจหาเซลล์มะเร็งในน้ำจากช่องเยื่อบุของร่างกายด้วยดัชนีดีเอ็นเอ ร่วมกับการแสดงออกของ AE1/AE3 บนผิวเซลล์ด้วยโฟลซัยโตเมทรี และการตรวจทางสัณฐานวิทยามีความไวสูง (ประมาณร้อยละ 90) ตัวอย่างตรวจที่ไม่ใช่ น้ำไขสันหลังให้ผลดีกว่าน้ำไขสันหลัง การพัฒนาเทคนิคการตรวจด้วยโฟลซัยโตเมทรีจะช่วยให้สามารถตรวจพบ เซลล์มะเร็งได้ดียิ่งขึ้นต่อไปในอนาคต*