

# Diagnostic and Prognostic Use of DNA Image Cytometry in Cervical Squamous Intraepithelial Lesions and Invasive Carcinoma

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In the fight against cervical malignancy and its precursors, several adjuvant diagnostic methods have been proposed to increase the accuracy of cytologic and histologic diagnoses. Because chromosomal aneuploidy has been accepted as an early key event in tumorigenesis caused by genetic instability, the cytometric equivalent of chromosomal aneuploidy detected by DNA image cytometry (DNA-ICM) may serve as a marker of neoplasia. During the last decade, the appearance of a new generation of hardware with high processing and storage capacities, together with the development of appropriate software, has facilitated the development of high-performance DNA-ICM systems. International consensus on the clinical application of DNA-ICM has been reached. According to the statements of Task Force 8 of the International Consensus Conference on the Fight Against Cervical Cancer, indications for DNA-ICM include the identification of prospectively malignant cells in squamous intraepithelial lesions (SILs) and atypical squamous cells of undetermined significance (ASCUS). The European Society of Analytical Cellular Pathology consensus reports on DNA-ICM have provided standardized technical details on performance, terms, and algorithms for diagnostic data interpretation and quality-assurance procedures. Increasing biologic evidence and clinical data have confirmed the utility of DNA-ICM as an adjuvant method suitable for determining the diagnosis and prognosis of cervical intraepithelial lesions and invasive carcinoma. Patients with ASCUS and low-grade SIL diagnoses that reveal DNA euploidy may return for normal screening intervals, whereas the detection of DNA aneuploidy indicates that these lesions should be removed. Formerly a research tool, today, standardized DNA-ICM has become a useful and low-cost laboratory method to establish objectively and reproducibly an early diagnosis of prospectively progressive cervical intraepithelial lesions at a high-quality level. DNA-ICM may further contribute to the monitoring of treatment in patients with invasive cervical malignancies. *Cancer (Cancer Cytopathol)* 2004;102:41–54. © 2003 American Cancer Society.

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In patients with borderline lesions (mild and moderate dysplasias) of the uterine cervix, cytomorphology alone often is not sufficient for the early and definite cytologic detection of malignancy. Cervical dysplasia describes squamous cells or tissues that potentially may lead to malignancy but do not exhibit sufficient evidence for a definite assumption. Resulting from weakness of morphologic criteria to identify malignant transformation in epithelial cells early and unequivocally, dysplasias are not a disease entity. The widely accepted assumption is that the higher the grade of dysplasia is, the higher the

probability of progression to carcinoma is.<sup>1</sup> However, as a result of insufficient morphologic criteria, neither histologic nor cytologic evaluation can predict whether a lesion will progress to cancer in an individual patient.<sup>2</sup> Rates of regression and progression of cervical dysplasia (positive and negative predictive values) are quite different from one study to another.<sup>1</sup> Insufficient interobserver reproducibility in diagnostic cytology and histology represents another dilemma in the microscopic diagnosis of precancerous cervical intraepithelial lesions.

Because the diagnosis of cervical squamous intraepithelial lesions (SILs) is not only poorly reproducible but also of limited biologic meaning for the individual patient, the number of resulting control procedures usually is high. These range from repeated cytologic smears and biopsies to unnecessary operations (conizations). Missed early diagnoses of cancers may result from cytomorphologic uncertainties. This also results in unnecessary costs and avoidable anxiety for the patients.

In the late 1970s, zur Hausen suggested that there may be an association between human papillomavirus (HPV) and cervical carcinoma.<sup>3</sup> Large numbers of subsequent epidemiologic, clinicopathologic, and molecular studies have linked the presence of specific types of HPV to the development of anogenital carcinoma and its precursors. A recent study estimated the worldwide HPV prevalence in cervical carcinomas at 99.7%.<sup>4</sup> Today, it is widely accepted that HPVs play a critical role in the pathogenesis of most cervical carcinomas and their precursor lesions. The infection of cervical epithelial cells with HPV itself is necessary but still insufficient for neoplastic progression. Other factors, especially genetic alterations, are needed to enter into the process of neoplastic transformation.<sup>5</sup>

Several adjuvant diagnostic methods currently are proposed to increase the diagnostic accuracy and reproducibility of cytology and histology. These range from clinical procedures, like colposcopy, to laboratory methods, like assays for the detection of HPV DNA and HPV typing<sup>6</sup> or DNA image cytometry (DNA-ICM).<sup>1,7,8</sup> In the current review, we focus on the technical performance and application of DNA-ICM in diagnosis and prognosis of cervical SILs and invasive cervical carcinoma.

### **Biologic Background**

Chromosomal aneuploidy is defined as numeric and/or structural aberrations. It is an early key event in tumorigenesis caused by genetic instability.<sup>7,9</sup> The cytometric equivalent of chromosomal aneuploidy, DNA aneuploidy, serves as a marker of neoplasia by assessing large-scale genomic alterations resulting

from genetic instability.<sup>9</sup> DNA-ICM is capable of monitoring the effect of cytogenetic tumor progression on nuclear DNA content. Quantitation of DNA aneuploidy, therefore, may serve as a prognostic marker.<sup>9,10</sup>

Chromosomal aneuploidy has been found in most cervical squamous carcinomas and recently even in high-grade SILs.<sup>11–13</sup> Aberrations of chromosome 1 have been found only in SILs that progressed to invasive carcinoma.<sup>11</sup> The finding of aneuploidy qualifies SIL as high grade, which requires further clinical management.<sup>9,14,15</sup>

The hypothesis that chromosomal aneuploidy itself may be a cause of cancer was proposed first by Boveri at the beginning of the 20th century.<sup>16</sup> During recent decades, this hypothesis has been ignored, because most efforts have been centered on the hypothesis of somatic gene mutations.

Increasing numbers of recently published scientific articles on the relation between aneuploidy and cancer pathogenesis have added to the current understanding about this issue. Chromosomal aneuploidy seems to play a crucial role in cancer development. A recent study using *in situ* hybridization to analyze cervical intraepithelial neoplasias (CINs) has provided sufficient evidence that chromosome 1, 7, and X aneuploidies in SILs are associated with progression toward cervical carcinoma.<sup>17</sup> Using a molecular cytogenetic approach termed comparative genomic hybridization (CGH), Heselmeyer et al. found that the gain of chromosome 3q that occurs in HPV type 16 (HPV-16)-infected aneuploid cells was a pivotal genetic aberration at the transition from severe dysplasia/carcinoma *in situ* to invasive cervical carcinoma.<sup>18</sup> Another study that was published by this working group in 1997 demonstrated that, in patients with advanced-stage (Stage IIB–IV) invasive cervical squamous carcinoma, a recurrent pattern of chromosomal aberration was observed.<sup>19</sup> The most commonly seen aberration, again, was the gain of chromosome arm 3q (in 23 of 30 tumors). During progression to advanced-stage disease, additional chromosomal aberrations are acquired, namely, the gain of chromosome arms 1q and 5p and the loss of chromosomal band 2q36–37. Alterations of many other regions also have been detected in various proportions of cervical malignancies, as shown in Table 1.

From a cytogenetic viewpoint, several categories of chromosomal aberration may occur at different grades of tumor progression<sup>21</sup> that may be divided into primary, secondary, and tertiary aberrations. Primary chromosomal aberrations are the first and are detectable by light microscopy after neoplastic transformation. Usually, one or a few chromosomes are concerned. They mostly result in a peridiploid stem-

**TABLE 1**  
**Genetic Changes in Invasive Cervical Carcinomas<sup>a</sup>**

Gain of genetic material		Loss of genetic material	
Region	Frequency (%)	Region	Frequency (%)
1q	25–45	1q	20–30
1p	22–33	2q	20–33
3p	50	3p13–25.3	39–52
3q	35–77	3q	22
5p	26–34	4p16	20–44
6p	27	4q21–35	20–53
8q	41	5q	20–40
11q	20	6q	22–38
15q	34–41	8p	23–30
17p	30–35	11p15	28
17q	12–45	11q22–24	7–51
20p	23	13q	27–45
20q	16	18p	20–30
Xq	34	18q12.2–21.2	24–37
		19p	20–30
		Xq	44

<sup>a</sup> Adapted from Zhang, 2002.<sup>20</sup>

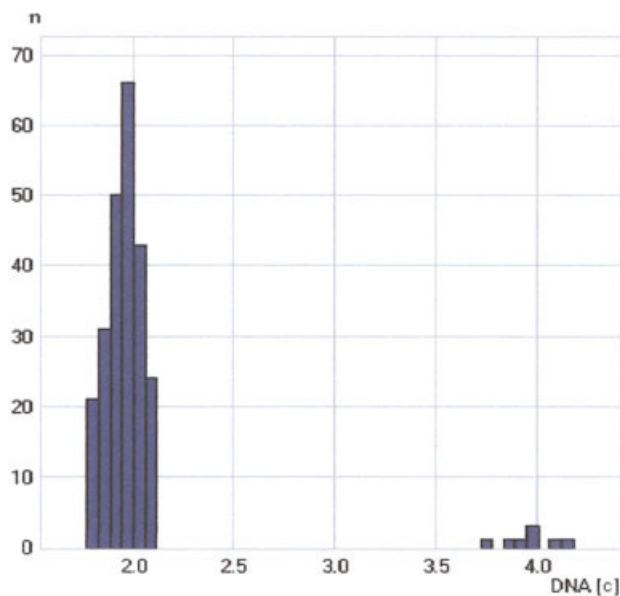
line. Secondary chromosomal aberrations follow the primary aberrations during tumor progression. Rather regularly, tumor type-specific chromosomes are affected, and additional stemlines result. Their net effects on nuclear DNA content mostly are detectable by DNA-ICM. Tertiary chromosomal aberrations occur during further tumor progression; these also are caused by genetic instability. Due to the final loss of stemlines and a large variation in DNA values, their effects on DNA content usually are detectable by DNA cytometry.

A codiscoverer of viral oncogenes in the 1970s, Peter Duesberg, recently observed that the correlation between aneuploidy and malignancy is strong and is evident in almost all solid malignancies. He also observed that the aneuploidy-malignancy correlation explains the both growing list of nonmutagenic carcinogens and why human oncogenes cannot turn human cells into cancer cells.<sup>22</sup> Duensing and coworkers, in a series of experiments on genomic instability, found that high-risk HPV may cause aneusomy through two mechanisms.<sup>23,24</sup> One mechanism involves the ability of viral protein E7 to uncouple the duplication of the centrosome from the cell division cycle, probably by targeting the pRb pathway. The second mechanism is the disturbing effect of E6 on the checkpoint function of the cell cycle by degrading p53. Another study supported this centrosome aberration hypothesis and demonstrated a connection between the severity of the lesions, centrosome aberration, and aneuploidy.<sup>25</sup> The progression from Grade 1 CIN to CIN 2, CIN 3,

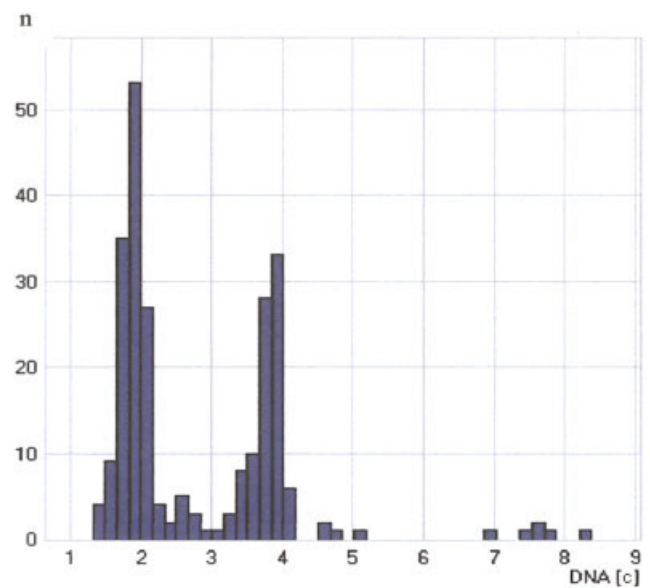
and invasive carcinoma seems to be related to increasing numbers of cells with abnormal centrosome replication and increasing deviation of histograms from the normal DNA-diploid pattern. Most recently, Bollmann and coworkers found that atypical squamous cells of undetermined significance (ASCUS) with DNA rare events > 9c DNA content unit were found exclusively in combination with high-risk HPV infection.<sup>26</sup> These findings support the concept that DNA aneuploidy represents an objective, very early, and highly specific marker of (prospective) neoplasia and that its detection in epithelial dysplasias identifies those lesions that most likely will progress to histologically manifest malignant disease.

### Principle of Method

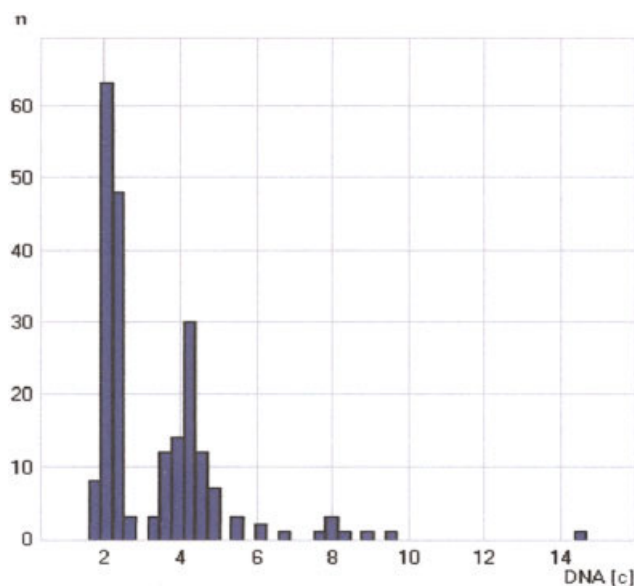
The terms *DNA-ICM* and *DNA flow cytometry (DNA-FCM)* should be used as descriptors to designate the type of nuclear DNA measurement performed. The amount of DNA in the nucleus of a cell (called the '2 c' or 'diploid amount' of DNA) is specific to the type of organism in question. However, within the animal kingdom, with 3 major exceptions, all healthy cells in a given organism contain the same amount of DNA. These include 1) cells that have undergone meiosis in preparation for sexual reproduction and thus contain only 1 c, a haploid amount of DNA, typical of a gamete; 2) cells that are carrying out DNA synthesis in preparation for cell division (mitosis) and thus, for a short period, contain between 2 c and 4 c of DNA; and 3) cells that are undergoing apoptosis and have begun to loose pieces of fragmented DNA. The cell cycle has been divided into different phases: cells in G0 phase are not cycling at all; cells in G1 phase are either recovering from recent division or preparing for the initiation of another cycle; cells are said to be in S phase when they actually are in the process of synthesizing new DNA; cells in G2-phase are those that have completed DNA synthesis and thus possess double the normal amount of DNA; and cells in M phase are in mitosis, undergoing chromosomal condensation and organization that occurs immediately before cytokinesis (resulting in the production of 2 daughter cells, each with 2 c of DNA).<sup>27</sup> In DNA histograms, the following cell cycle phases can be distinguished from each other<sup>9</sup>: 1) G0/G1 phase fraction, all nuclei that belong to a peak that does not represent a duplication of a lower peak (Fig. 4); 2) G2/M phase fraction, all nuclei that belong to a peak in the duplication region of a G0/G1-phase fraction; and 3) S phase fraction, all nuclei with integrated optical density (IOD) values between those of the corresponding G0/G1 phase fraction and its G2/M phase fraction counterpart that do not belong to other stemlines.



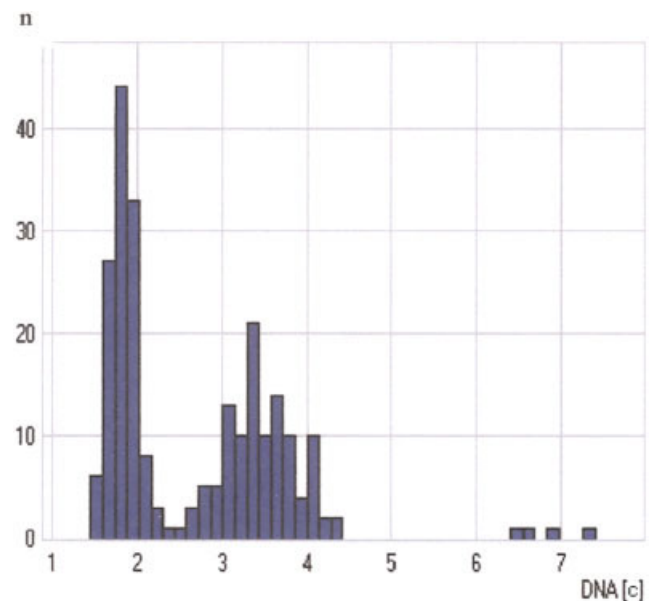
**FIGURE 1.** DNA histogram showing a euploid pattern (stemline at 2.0 DNA content units [c]; 9 c-exceeding events = 0).



**FIGURE 2.** DNA histogram indicating a polyploid pattern (stemlines at 2.0 DNA content units [c] and 4.0 c; 9 c-exceeding events = 0).



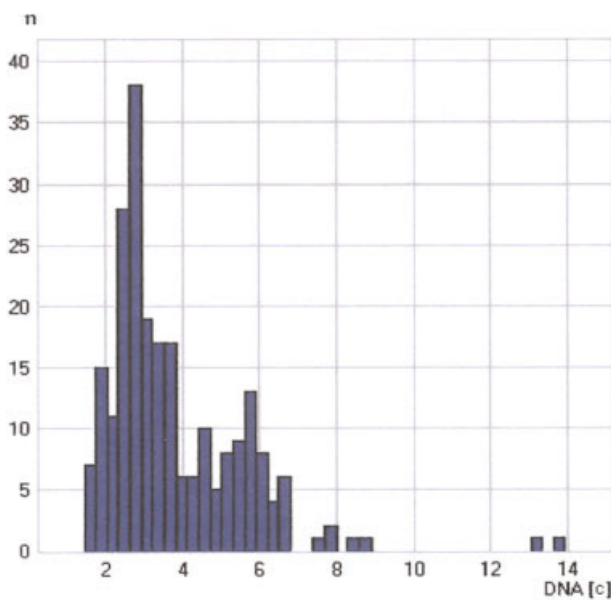
**FIGURE 3.** DNA histogram showing single-cell aneuploidy (9 DNA content unit [c]-exceeding events = 2).



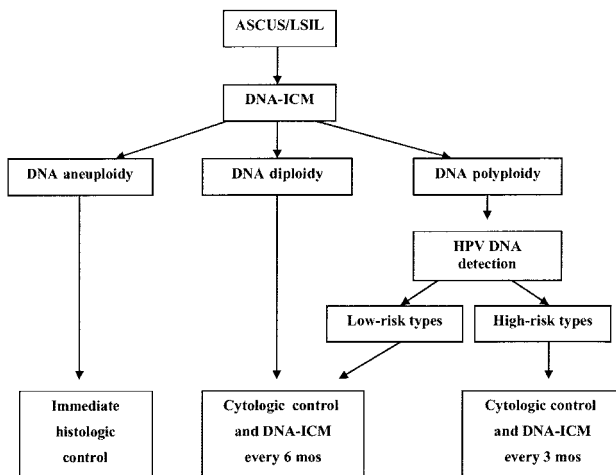
**FIGURE 4.** DNA histogram indicating stemline aneuploidy (stemlines at 1.7 DNA content units [c] and 3.4 c; 9 c-exceeding events = 0).

The nuclear DNA content cannot be measured directly by cytometry. After quantitative DNA staining, the nuclear IOD is the cytometric equivalent of its DNA content in DNA-ICM. Therefore, the DNA content is expressed in a *c scale*, in which 1 c is half of the mean nuclear content of cells from a normal (non-pathologic), diploid population in G0/G1 cell cycle phase (Figs. 1–5).<sup>9</sup> Although ‘chromosomal ploidy’ theoretically is detectable by cytogenetic methods in

each single cell, its DNA content cannot be equated with a certain chromosomal outfit; therefore, the term *DNA ploidy* is the expression of the typical, large-scale genomic status of a cell population. The quantity of nuclear DNA may be influenced by the following mechanisms: replication, polyploidization (Fig. 2), gain, or deletion. Each mechanism affects the size or the number of chromatids. Viral infections may change the nuclear DNA content that is detectable by



**FIGURE 5.** DNA histogram showing stemlines and single-cell aneuploidy (stemlines at 3.0 DNA content units [c] and 6.0 c; 9 c-exceeding events = 2).



**FIGURE 6.** Quality-control flow chart for Papanicolaou smears of atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesions (ASCUS/LSIL).<sup>8</sup> DNA-ICM: DNA image cytometry; HPV: human papillomavirus.

DNA-FCM and DNA-ICM. Among others, the unspecific effects of cytostatic or radiation therapy, vitamin B12 deficiency, apoptosis, autolysis, and necrosis on nuclear DNA content also play a role. Furthermore, the DNA content of a cell is changed regularly throughout the cell cycle.<sup>9</sup> All of these effects have to be taken into consideration when a diagnostic interpretation of DNA histograms is performed. The basic objective of DNA-ICM is to identify DNA stemlines outside the euploid (diploid, tetraploid, or octaploid)

regions as abnormal (or aneuploid) at a defined statistical level of significance (Figs. 4,5). Furthermore, DNA-ICM should provide information about the number of abnormal (aneuploid) DNA stemlines (Fig. 5), the polyploidization of euploid or aneuploid DNA stemlines (Fig. 2), the occurrence of rare cells with an abnormal high DNA content (most likely resulting from genomic alterations) (Figs. 3,5), and cell cycle fractions.

DNA-ICM results in nuclear IOD values in arbitrary units (AU), equivalent but not identical to nuclear DNA content, and the quantitation of nuclear DNA requires a rescaling of IOD values compared with the IOD values from cells with known DNA content, so-called reference cells. By means of reference cells, the AU scale is transformed into a reference unit scale (e.g., 2 c, 4 c, 8 c). Internal reference cells should be used because they have the advantage of sharing all preparatory steps with the analysis cells in the clinical specimens.

Because most interpretations of DNA measurements are population based, the results usually are displayed as DNA histograms (Figs. 1–5). The bin size of such histograms should be adapted to the precision of the actual measurements, i.e., the lower the variability in the reference cells peak, the smaller should be the bin size of histogram classes.

The resolution of DNA-ICM is defined by its precision, which allows the differentiation of 2 separate peaks, and its accuracy in recognizing a peak in an abnormal position using the European Society of Analytical Cellular Pathology (ESACP) consensus thresholds for the minimum precision of measurements, which, given the coefficient of variation (CV) of reference cells, should be < 5%, enabling the recognition of an abnormal DNA stemline that deviates > 10% from the normal diploid position.<sup>9</sup> That means that at least 10% of the genomic DNA has to be lost or gained before this deviation can be detected reliably by means of DNA-ICM. However, in DNA-ICM, a single cell with an abnormally high DNA content (> 10% above the genomic DNA in G2/M-phase) also can be detected reliably.

### Indications for DNA-ICM

In cervical pathology, the application of DNA-ICM is mostly in the differential diagnosis of cytologically doubtful cases, namely, ASCUS or low-grade SIL (LSIL) (the Bethesda system) or Papanicolaou III (Pap III) or Pap IIID (Munich II nomenclature) in German-speaking countries. The effectiveness of diagnostic DNA-ICM for identification of neoplasia/malignancy in cervical preneoplastic diseases has been established sufficiently.<sup>7</sup> For grading the malignancy of invasive

cervical carcinoma, the number of scientific articles still is limited, and their conclusions are not unequivocal.<sup>28-32</sup>

During the last 5 years, international consensus on standardized application of diagnostic DNA-ICM has been reached. Four consecutive ESACP consensus reports provided the scientific methodology for DNA-ICM.<sup>9,33-35</sup> According to the statements of the International Consensus Conference on the Fight Against Cervical Cancer Task Force 18, the indication for DNA-ICM is the identification of potentially malignant cells in SILs and ASCUS.<sup>15</sup>

### **Instrumentation and Software**

DNA quantitations of Feulgen-stained samples first were performed with cytophotometry using ultraviolet light. During the 1960s and 1970s, FCM and scanning microscope photometers were developed, but these instruments still were expensive, required special operational skills, and were available only in special research laboratories. On the other hand, cytometric measurements performed with these systems were too time consuming. Many efforts have been undertaken since the 1970s to develop image-analysis systems for DNA cytometry using the scanning capabilities of video cameras. DNA-ICM is based on the physical principle of light absorption measurement of Feulgen-stained nuclei using a video camera. The camera is provided with a charge-coupled device (CCD) sensor. The CCD is subdivided into thousands of tiny square or rectangular potential wells (6–15  $\mu\text{m}$  per side), each behaving as an independent, true photometric unit that transforms single photons in photoelectrons. Thus, with appropriate enlargement, each nucleus is divided into hundreds of independent, single photometric measurements, which are summarized to give the final integrated optical density.<sup>36</sup> Video-image cytometers are highly efficient, very easy to use, and cost less than one-third of the cost of the previously used instruments. This led to a wide expansion of instrument development for research and clinical applications. The first generations of DNA-ICM systems used for diagnostic purposes were SAMBA (Unilog, France),<sup>37</sup> LEYTAS (Leyden, Netherlands),<sup>38</sup> CAS (Becton Dickinson, San Jose, CA),<sup>39</sup> TAS-plus (Leitz, Wetzlar, Germany),<sup>40</sup> Roche Pathology Workstation (Roche Image Analysis),<sup>7</sup> AccuMed Cyto-Savant (AccuMed, Chicago, IL),<sup>41</sup> CYTOMETER CM-1 (Hund, Wetzlar, Germany),<sup>42</sup> and MIAMED-DNA (Leitz).<sup>43</sup>

The appearance of a new generation of high-resolution video cameras and personal computers with high processing and storage capacities facilitated the development of high-performance DNA-ICM systems. Several systems currently are in use, including CYDOK

(Hilgers; Koönigswinter, Germany),<sup>44</sup> ACAS (Ahrens, Bargteheide, Germany),<sup>45</sup> and AUTOCYTE QUIC DNA (TriPath, Burlington, NC).<sup>46</sup>

The most important optical problems that have great influence on densitometric measurements are glare and shading errors. In most instruments from the first generation, these problems were not solved. During the last decade, glare and shading corrections in most of the commercially available devices are provided by software procedures.<sup>44,47</sup> Because there is a wide variety of DNA-ICM instruments today, we propose using only instruments that meet ESACP performance standards<sup>9,33-35</sup> in clinical routine work to reach a quality-assured and standardized DNA cytometric diagnosis. Appropriate tests and performance standards have been published to assure sufficient densitometric linearity.<sup>36</sup>

Laser scanning cytometry is a new method of image analysis that was developed during the late 1980s and became available commercially in the early 1990s.<sup>48</sup> The method applied to the analysis of populations of more than 100,000 cells per smear is capable of detecting rare DNA events.<sup>26</sup> Its advantage is fast performance and, thus, excellent sample throughput, comparable to that of FCM. Based on the automated scanning principle, all normal cells also are measured; thus, the representative nature of comparably small numbers of dysplastic cells may not be preserved. The visual verification of relevant nuclei or the exclusion of artifacts may be determined only after both the measurement and a restaining step.

### **Sample Preparation and Staining**

Cytologic smears from the ectocervix or endocervix and from the endometrium or imprints (touch preparations) may be investigated. Additional core biopsy samples or paraffin-embedded tissue samples may be used for DNA cytometric investigation after enzymatic cell preparation.

It has been found repeatedly that for diagnostic and prognostic purposes, DNA-ICM is unsuitable when performed directly on tissue sections.<sup>49,50</sup> The measurement of sectioned, individual nuclei of unknown size and shape (such as dysplastic and neoplastic nuclei) does not indicate the amount of DNA (the so-called tomato-salad phenomenon). The proposed procedure for these samples, therefore, is the preparation of monolayer smears by enzymatic cell separation.<sup>7</sup>

Feulgen staining is recommended strictly for absorption cytometry. Other protocols, including Pap staining, are unsuited for DNA-ICM. Usually, prestained Pap smears or monolayer preparations from paraffin blocks undergo rehydration in decreas-

ing ethanol concentrations and refixation in buffered 10% formalin. Hydrolysis in 5 N HCl then is allowed to take place at 27 °C for 60 minutes, followed by staining in Schiff reagent for another 60 minutes at room temperature, rinsing in SO<sub>2</sub>-water, and dehydration at increasing ethanol concentrations. The slides then are covered and kept away from light until measurement.<sup>7</sup>

### Sampling, Measurement, and Scaling

#### *Basic performance standards*

The precision of DNA-ICM measurements must at least allow the identification of DNA stemline(s) as aneuploid if they deviate > 10% from the diploid domain (2 c) or the tetraploid domain (4 c), i.e., if they are outside  $2.0\text{ c} \pm 0.2\text{ c}$  or  $4.0\text{ c} \pm 0.4\text{ c}$ .<sup>9</sup> To achieve this goal with an error probability ( $P$ ) < 0.05, the test statistics require a measurement performance described by the following: 1) a CV of the ratios between modal IOD values of reference cells and nonpathologic G0/G1 cells in a series of measurements of < 5%; 2) a relative standard error of the mean (rSEM;  $\text{rSEM} = \text{CV} / \sqrt{n}$ ) of reference cells in each sample of < 1.5%; 3) linear regression of the mean IOD versus transmission with < 1% deviation from (slope = 1); and 4) linear regression of IOD ratios versus theoretical ratio with < 1% deviation from (slope = 1). Furthermore, a DNA stemline should be identified as polyploid within the duplication position of a G0/G1 phase fraction  $\pm 0.2\text{ c}$  (at 4.0 c) and  $\pm 0.4\text{ c}$  (at 8.0 c), respectively, with an error probability  $P < 0.05$  if the CV of the ratios between modal IOD values of nonpathologic G0/G1 phase and G2/M phase fractions in a series of measurements is < 2.5% (Fig. 2).

The different aspects of the measuring process and of diagnostic interpretation should regularly be subjected to quality-control measures to assure a consistently high level of quality in the diagnostic procedure. Appropriate protocols for such quality assurance have been developed and described elsewhere.<sup>35</sup>

#### *Densitometric measurement*

One important step in setting up the system is to check for glare phenomena. Glare errors should be corrected by software procedures. Measurements of reference cells are needed for rescaling densitometric measurements. Internal reference cells should be used, as discussed above. Lymphocytes, granulocytes, and normal epithelial or stroma cells usually are analyzed as internal standards. At least 30 reference cells should be measured. The CV of the reference cell population should not exceed 5% ( $\text{CV} = \text{standard deviation of the mean} \times 100$ ).<sup>7</sup> Nuclei to be measured are sampled in a systematic, random manner. Only diagnostically relevant cells should be measured, i.e.,

cells of a certain cytologic entity (e.g., all tumor cells or all dysplastic cells) that can be identified by their morphology. A selective sampling for rare nuclei characterized by a high DNA content is allowed only if the occurrence, per se, is of diagnostic relevance.

### Terms and Diagnostic Algorithms

According to ESACP consensus reports, simple and complex algorithms and diagnostic or prognostic classification strategies for DNA histogram interpretation may be used for the following purposes, depending on the material under investigation and the diagnostic or clinical questions: 1) diagnosis of neoplasia, 2) prognostication of neoplasia, and 3) monitoring of therapy.<sup>9,33</sup> Histogram classifications should not be based on subjective interpretations but should be defined by algorithms.

#### *Diagnostic purposes*

The reports of ESACP on standardization of diagnostic DNA-ICM proposed the definitions and algorithms for diagnostic interpretation of DNA histograms that are described in Tables 2,3.<sup>9,33,34</sup>

#### *Prognostic purposes*

According to the ESACP consensus reports,<sup>9,34</sup> classification of the entire DNA histogram based on the position of DNA stemlines may be of prognostic value. The terminology for grading is applicable only to neoplasias, either proven by morphologic investigations or in cases of DNA aneuploidy. The terms defined in Table 4 may be used to classify the histogram for prognostic purposes.

### Clinical Application in Cervical Premalignant Lesions and Invasive Cervical Carcinoma

#### *Diagnosis of malignancy*

DNA aneuploidy is the cytometric equivalent of chromosomal aneuploidy and may serve as a marker of neoplasia by assessing large-scale genomic alterations resulting from genomic instability.<sup>9</sup> Using video image analysis principles on Feulgen-stained smears, DNA-ICM is capable of detecting DNA aneuploidy in the form of aneuploid stemline(s) (Fig. 4,5) and/or rare DNA events (Fig. 5; Table 3)

In a series of 276 Pap smears, 73% of DNA aneuploid, mild or moderate cervical dysplasias developed into carcinoma in situ or higher lesions, and the histologic diagnosis remained dysplastic in 17%. For the detection of invasive lesions, DNA aneuploidy had a sensitivity of 99% and a positive predictive value of 86%.<sup>14</sup>

DNA-ICM has been proposed repeatedly as an adjunctive diagnostic and prognostic method for eval-

**TABLE 2**  
**Terms and Algorithms in Diagnostic Interpretation<sup>a</sup>**

DNA histogram	Frequency distribution of IOD values obtained by quantitative DNA stains and rescaled by IOD values from reference cells in 'c' units; the class width should be twice the standard deviation of the IOD of the G0/G1 phase fraction of reference cells
DNA histogram peak	A statistically significant local maximum in a DNA histogram
Modal value of a histogram peak	The most frequent value in the peak, i.e., the mean value of the histogram class containing the highest number of nuclei; this is close or equal to the mean value of a fitted Gaussian curve according to the principle described above
DNA stemline	The G0/G1 cell phase fraction of a proliferating cell population (with a first peak and a second doubling peak or with nuclei in the doubling region)
DNA stemline ploidy	The modal value of a DNA stemline in the unit c
DNA euploidy	the type of DNA distribution that cannot be differentiated from the distribution of normal cell populations (resting, proliferating, or with polyploidization)
Diploid euploidy	DNA stemlines with a modal value between 1.8 and 2.2 c possess diploid euploidy
Tetraploid euploidy	DNA stemlines with a modal value between 3.6 and 4.4 c possess tetraploid euploidy
DNA aneuploidy	Types of DNA distributions that are different at a statistically significant level from the distributions of normal (resting, proliferating, or with polyploidization) cell populations; DNA aneuploidy either can be seen as DNA stemline aneuploidy or can be indicated by 'rare events'
DNA stemline aneuploidy	Stemline(s) with modal values < 1.80 c or > 2.20 c and < 3.60 c or > 4.40 c possess stemline aneuploidy
'Rare events' in DNA histograms	These are abnormal cells and often are called 5 c- or 9 c-exceeding events (5c-EE or 9c-EE), with nuclear DNA contents higher than the duplicate or quadruplicate of a normal G0/G1 phase population (i.e., not belonging to G2/M phase); they likely represent nonproliferating abnormal cells with different chromosomal aneuploidies and abnormally high number of chromosomes
DNA single-cell aneuploidy	Because most preneoplastic or invasive lesions of uterine cervix are caused by human papillomavirus, which is known for its effects on nuclei causing polyploidization, the 5c-EE cutoff level may not be appropriate for a diagnosis of DNA aneuploidy; thus, DNA single-cell aneuploidy in cervical pathology is defined by the presence of at least 1 cell with a DNA content > 9 c (9c-EE ≥ 1) per slide (Chatelain et al. <sup>52</sup> )
Polyploidization	The (repeated) doubling of a chromosomal set
Euploid DNA polyploidization	The occurrence of peaks in the duplication (×2, ×4, ×8) regions of euploid stemlines; in human tissues, the highest peak usually is at 2 c
Aneuploid polyploidization	The occurrence of peaks in the duplication regions of aneuploid stemlines

IOD: integrated optical density; c: DNA content units.

<sup>a</sup> See Böcking and Haroske et al.<sup>9</sup>**TABLE 3**  
**Algorithms for the Differentiation of Euploidy from Aneuploidy in DNA-Histograms from the Uterine Cervix**

DNA-euploid	diploid: 1.8c ≤ STL ≤ 2.2c, 5cEE = 0 polyploid: 1.8c ≤ STL ≤ 2.2c or 3.6c ≤ STL ≤ 4.4c, 9cEE = 0
DNA-aneuploid	stemline - aneuploidy: STLs neither diploid nor polyploid single cell - aneuploidy: 9cEE > 0

STL: DNA-stemline; 5cEE: 5c Exceeding-Events; 9cEE: 9c Exceeding-Events.

**TABLE 4**  
**DNA Histogram Classification for Prognostic Purposes<sup>a</sup>**

Classification	Definition
Peridiploid	A single DNA stemline with a modal DNA value between 1.8 and 2.2 c
Peritetraploid	A single DNA stemline or a stemline additional to a peridiploid stemline with a modal DNA value between 3.6 and 4.4 c
X-ploid	A single DNA stemline or a DNA stemline additional to a peridiploid or peritetraploid stemline with a modal DNA value outside the thresholds mentioned above ('X' will be substituted by the DNA ploidy value of this stemline, e.g., peritriploid, hyperdiploid, etc.)
Multiploid	Occurrence of more than one abnormal DNA stemline (often called 'Manhattan skyline')

c: DNA content units.

<sup>a</sup> See Böcking<sup>7</sup> and Haroske et al.<sup>9</sup>

uating patients with cervical intraepithelial lesions and invasive cervical carcinoma.<sup>10,14,31,51-55</sup> Many authors have reported high positive predictive values for the development of in situ or invasive carcinoma out of mild-to-moderate cervical dysplasias with proven DNA aneuploidy, varying from 84% to 100%.<sup>14,52,53,56</sup> Intervals between the detection of DNA aneuploidy and histologic follow-up in these studies were up to 3 years.

The frequency of aneuploid DNA patterns in different types of invasive carcinoma of the uterine cervix was investigated by Kashyap and Bhambhani. An increasing trend toward DNA aneuploidy was observed from well differentiated squamous cell carcinoma (SCC) (64%), to moderately differentiated SCC (71%), to poorly differentiated SCC (83%). Eighty-five percent of all endocervical adenocarcinomas in that study also showed aneuploid DNA patterns.<sup>57</sup>

It also has been found that DNA-ICM is useful as an adjuvant diagnostic procedure in patients with endocervical adenocarcinoma. The presence of nuclei with DNA contents > 9 c was observed exclusively in adenocarcinomas with a sensitivity of 96% and a specificity of 100%, indicating that this parameter is suited best for differentiating between malignant and non-malignant endocervical epithelia.<sup>58</sup>



**TABLE 5**  
**Predictive Values of Diagnostic DNA Image Cytometry in Mild or Moderate Cervical Dysplasia for Grade  $\geq 3$  Cervical Intraepithelial Neoplastic Lesions**

Author	No. of patients	Follow-up (mos)	Predictive value (%)	
			Positive	Negative
Böcking et al., 1986 <sup>14</sup>	37	21.3	84	86
Chatelain et al., 1989 <sup>52</sup>	18	$\leq 42.0$	100	83
Kashyap et al., 1990 <sup>53</sup>	42	30.8	93	67
Bollman and Böcking, 1996	497	—	95	98
Hering et al., 2000 <sup>54</sup>	193	$\leq 36.0$	85	77

From the data presented in Table 5, proposals for clinical consequences of DNA-ICM results may be formulated as follows: 1) a high negative predictive value of 95% in cervical smears with ASCUS and LSIL diagnoses revealing DNA euploidy allows patients to return to normal screening intervals; and 2) positive predictive values of 46% for patients who have CIN 3 or higher-grade lesions after 2 months and up to 100% after 3 years for patients who have ASCUS and LSIL with DNA aneuploidy allow the removal of lesions by conization or loop electrical excision procedure (LEEP).

#### **Prognosis of malignancy**

In addition to its diagnostic application, DNA-ICM may be used as a prognostic tool for patients with cervical carcinoma. Although controversy over the prognostic value of DNA-related parameters exists,<sup>28–30</sup> several recent studies have provided more promising data.

In a study of postirradiation cytologic smears from 46 patients with cervical carcinoma, Davey and co-workers found that DNA histograms were correlated significantly with patient outcome.<sup>50</sup> A peridiploid histogram generally denoted a healthy outcome, whereas multiploidy was correlated most often with postirradiation dysplasia. The highest positive predictive value was for DNA aneuploidy; 92% of patients developed recurrences or postirradiation dysplasia. According to the authors, once an abnormal histogram pattern occurs, the patient should be evaluated more aggressively for early diagnosis and management of postirradiation dysplasia and/or local recurrence.

Results from a recent study by Grote et al. in patients with Stage IB/II cervical carcinoma confirmed the high prognostic value of standardized DNA-ICM.<sup>31</sup> A DNA stemline ploidy  $> 2.2c$  was correlated with an unfavorable prognosis. In a Cox proportional hazards regression model, it was found that DNA stemline ploidy and the 5c-exceeding rate (5c-

ER) had prognostic value in both presurgical analysis and postsurgical analysis. In another study of 163 pathologic Stage T1b1–T2b invasive cervical squamous carcinomas, tumors with a DNA index  $> 1.70$  and a 5c-ER  $> 11\%$  represented the poor prognostic group.<sup>32</sup> Thus, in addition to clinical and histopathologic parameters, DNA cytometric indices may contribute to the prognostic evaluation of an individual patient.

It also has been reported that DNA ploidy is a valid indicator of response to radiotherapy in women with cervical malignancies. In 1991, Yu et al. found that tumors with DNA aneuploidy were more radiosensitive than diploid tumors.<sup>59</sup> They concluded that there is a direct correlation between DNA content and radiosensitivity in invasive cervical carcinoma.

Because changes in DNA histograms may indicate therapeutic effects,<sup>60,61</sup> DNA-ICM may have a role in monitoring the response to chemotherapy of patients with invasive cervical carcinoma. To achieve a final conclusion, further studies on this topic will be needed.

The follow-up course and corresponding management strategy for patients with cervical lesions that occur during pregnancy have a great impact not only on the women themselves but also on the outcome of pregnancy. To date, there has been no valid consensus on the follow-up interval and management strategy for such lesions in pregnant women. Except for invasive lesions, which require immediate, appropriate management, DNA-ICM performed in women with ASCUS and SILs may provide relevant information on the prospective biologic behavior of these lesions and may help to establish an appropriate follow-up course for each individual pregnant woman. Women who have cytologic high-grade lesions with a high DNA grade (multiploid aneuploidy) of malignancy in early pregnancy probably should undergo surgical interventions, whereas for women who have cytologic low-grade lesions with a low DNA grade (single cell or single stemline aneuploidy) of malignancy in late pregnancy, colposcopic/cytologic follow-up may be the appropriate management.

#### **Quality assurance of cytologic and histologic diagnoses**

In cases of equivocal cytologic diagnoses, the cytometric detection of DNA aneuploidy speaks in favor of the presence of (prospectively) malignant cells, especially when there are discrepancies between cytologic and histologic diagnoses. In a series of 170 seemingly false-positive routine cervical smears, DNA aneuploidy was found in 47 smears without histologic explanation.<sup>56</sup> Using DNA aneuploidy as a solid marker of malignancy in uterine epithelia, the authors classified those

cases as histologically false-negative, and not false-positive, cytologic diagnoses. In four cytologically suspicious oral lesions, Remmerbach and coworkers demonstrated that the histologic diagnosis had to be revised after the detection of DNA aneuploidy.<sup>62</sup>

DNA-ICM also may be used as a method for quality control in histologic diagnosis. In patients with positive cytologic tumor cell diagnoses and negative histologic follow-up, the detection of DNA aneuploidy should motivate the pathologist to work-up more thoroughly a given conization or biopsy specimen. If no malignancy is found, then the patient should undergo surgery again to remove more cervical tissue and to find the cancer that was missed previously in histologic follow-up.

#### ***Combination with other diagnostic methods***

Adjuvant diagnostic methods currently proposed to increase the diagnostic accuracy of cervical cytology and histology are assays for the detection of HPV and for HPV typing<sup>6,63</sup> and DNA-ICM.<sup>1,7,8</sup> In general, proposed approaches, such as administering HPV tests to women with mild dysplasia to determine whether treatment is necessary, have shown varying levels of effectiveness and are relatively costly.<sup>64–67</sup> Testing for high-risk HPV types actually yielded a wide variation in positive predictive values for the detection of high-grade SIL from 13% to 33.5%.<sup>68–72</sup> Using the Hybrid Capture II test (Digene, Gaithersburg, MD), several studies reported that the specificity of high-risk HPV detection of histologic high-grade SIL ranged from 58% to 85%.<sup>68,69,73</sup> Transient incident infection is common, especially in young women.<sup>70</sup> Thus, specificity remains a concern with HPV testing for primary screening, and more research is needed to determine optimal approaches.<sup>66,74</sup> A recent study in South Africa suggested that specificity may be improved by adjusting the level of HPV DNA used to define a positive result.<sup>75</sup>

Because, to date, DNA-ICM has provided more encouraging data on its diagnostic validity, combining DNA-ICM with HPV testing in patients with high-risk HPV types may improve the specificity of the HPV test and provide more information on the prospective behavior of an individual HPV-positive lesion. In a series of 50 patients with of mild, moderate, and severe cervical dysplasia, Kashyap et al. found that 44.0% of patients showed an aneuploid DNA pattern, whereas positivity for high-risk HPV-16 DNA was found in 46.0% of patients. Follow-up data revealed that 72.7% of patients who had an aneuploid DNA pattern and were positive for HPV-16 experienced progression to carcinoma in situ, compared with only 4.4% of pa-

tients who were positive for HPV-16 and had a euploid DNA pattern.<sup>76</sup>

For an individual patient with a diagnosis of ASCUS or LSIL, the detection of high-risk HPV may lead to uncertainty in management strategy for the health care provider and anxiety for the patient. In such cases, DNA-ICM can subsequently be performed to provide more information on the prospective biologic behavior of the lesion and, thus, an assured management strategy that is reasonable for both the health care provider and the patient.

#### ***Latency period***

Because it may require an interval period ranging from several months up to 10 years for a mild or moderate squamous dysplasia, as diagnosed cytologically on a Pap smear, to develop into histologically proven carcinoma, simultaneously obtained histologic diagnoses may not be adequate to decide correctly on the positive predictive value of cytologic diagnoses or adjuvant methods. In 2001, Sudbø et al. showed that the progression rate from oral dysplasia to invasive oral carcinoma based on the detection of DNA aneuploidy was only 10% after 1 year but increased significantly, to 90%, after 5 years.<sup>77</sup> Thus, this interval should be taken into account in the evaluation of the diagnostic and prognostic value of DNA-ICM.

#### ***Reproducibility of DNA-ICM***

The reproducibility of a method includes two aspects: intraobserver agreement and interobserver agreement. Features that have an impact on reproducibility are the objectivity of diagnostic criteria, the number of diagnostic categories, the study population, and the experience of the individuals who perform the test. Interobserver variability has important implications for diagnostic error and, thus, patient care and medical litigation issues. In cancer screening and diagnosis, cytologic and histologic investigations have contributed greatly to the fight against malignant disease worldwide. Despite its success in screening and early detection of cervical premalignant lesions, the diagnostic accuracy of histology and cytology in pathology of the uterine cervix still has limits. Histomorphologic and cytomorphologic criteria for the diagnosis of different grades of precancerous SILs (dysplasias), even carcinoma in situ of the uterine cervix, are neither defined objectively nor agreed upon internationally. These facts may explain the insufficient intraobserver and interobserver reproducibility of histologic and cytologic diagnoses in pathology of the uterine cervix as reported by many authors.<sup>78–80</sup>

Because the correct performance of DNA-ICM requires cytomorphologic knowledge, it is possible that

both intraobserver reproducibility and (especially) interobserver reproducibility of DNA measurements are not negligible. To date, only a limited number of studies have dealt with the reproducibility of DNA grading. In 1989, Böcking et al. reported intraobserver and interobserver reproducibility rates of 83.9% and 82.2–86.7%, respectively, for DNA grading of malignancy in breast and other cancers.<sup>81</sup> Recently, Nguyen and co-workers reported an interobserver correlation of 94.1% ( $\kappa = 0.84$ ) in DNA measurements performed on 202 routine ASCUS-positive smears (unpublished own data). The comparably high value of interobserver agreement achieved in that study was at least 20% higher than the rates reported in the literature for morphologically based histologic or cytologic diagnoses of cervical dysplasias. Explanations for this high value may include both the high standardization of DNA measurements and diagnostic data interpretation and the objectivity of the method. Compared with the subjective assessment of conventional morphology-based diagnostic methods, DNA histogram interpretation is based on well defined algorithms with proven diagnostic validity in cervical pathology (Figs. 1–5). Therefore, it may be assumed that when it is performed according to standardized procedures, DNA-ICM represents a reliable method with high interobserver reproducibility.

### Economic Aspects

DNA-ICM may contribute to the avoidance of costs that result from repeated and unnecessary cytologic, histologic, and clinical controls, as well as surgical procedures in patients with DNA-euploid ASCUS/LSIL. If DNA aneuploidy is detected, then the indication for a surgical intervention (conization, LEEP-conization, etc.) should be assumed, also to avoid the costs that occur when advanced carcinomas require treatment. DNA-ICM is paid for by German health insurance companies in cases of cytologically doubtful diagnoses (approximately 50 Euros per measurement). It should be pointed out that DNA-ICM is a rather time-consuming method (30–40 minutes per measurement) that requires performance skills and, thus, appropriately trained personnel.

### Quality Control of Diagnostic DNA-ICM

Because a wide variety of hardware and software for diagnostic DNA-ICM exists, and because different algorithms may be used for diagnostic data interpretation, there is a need for 1) standardization of measurement performance, terminology, and diagnostic interpretation of data and 2) quality-control procedures. To obtain results with high diagnostic accuracy and prognostic validity, all steps involved in DNA-ICM

should be performed following a standardized procedure. The 1997 ESACP consensus reports on diagnostic DNA-ICM reserved Part II for dealing with the quality-control issue.<sup>35</sup> The respective steps are specimen preparation and staining, instrumentation, sampling and densitometric measurement, scaling process, and measurement interpretation.

Additional important steps in quality-control procedures in diagnostic ICM are accreditation of measurement devices and the set-up of a remote-control server.<sup>35,82</sup> In the framework of the Prototype Reference Standard Seiden (PRESS) project,<sup>83</sup> several tests have been developed and designed to control the basic technical characteristics of ICM instrumentation; these tests involve inexpensive measures using defined density filters. These different tests have to be performed upon delivery of the machinery by the industrial provider using the appropriate software and at yearly intervals by users. The first system that was officially accredited by the ESACP was the ACAS system (Ahrens). Accreditation of other systems that are in use is encouraged.

The development of the quantitation server EUROQUANT<sup>84</sup> is an important step toward quality control and standardization in diagnostic DNA-ICM.<sup>82</sup> This system is a remote server that was designed to act both as a teleconsultation system and as a cytometric workstation that can be used through Internet technology. Its main functional components are 1) measurement of the performance of DNA ploidy analysis obtained by every end user in an individual case through all steps, leading to methodological recommendations; 2) confirmation or revision of the diagnostic interpretation of the data obtained by the user in individual cases; and 3) rescaling of DNA data for exchange with multicenter data bases.

Concerning quality control, this tool may be used at different levels of the measurement and diagnostic process. All appropriate protocols for quality assurance recommended by ESACP have been implemented into this server. It works completely independently of any commercially available cytometric device. Its functionality is based on the latest scientific knowledge and consensus agreement in the field. Thus, the server is an objective tool for an international methodologic standard.

### Conclusions

Increasing amounts of biologic evidence and clinical data confirm the utility of DNA-ICM as an adjunct method suitable for the diagnosis and prognosis of cervical intraepithelial lesions and invasive cervical carcinoma. Formerly a research tool, standardized DNA-ICM has become a useful and low-

cost laboratory method for objectively and reproducibly establishing an early diagnosis of prospectively progressive cervical intraepithelial lesions at a high-quality level. It may further contribute to the assessment of relevant prognostic parameters and to the monitoring of treatment for patients with invasive cervical carcinoma.

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