

Comparative Diagnostic Performance of DNA Ploidy Analysis and p16/Ki67 Dual-Staining with Cytology in hrHPV-Defined Subgroups: A Single-Center Evaluation

Yu Wang, Chao Qi

Pathology Department, Taiyuan Maternity and Child Health Care Hospital, Taiyuan, People's Republic of China

Correspondence: Yu Wang, Pathology Department, Taiyuan Maternity and Child Health Care Hospital, No. 113, Changfeng West Street, Jinyuan District, Taiyuan, 030021, People's Republic of China, Email wangyubk@163.com

Objective: To systematically evaluate the agreement of deoxyribonucleic acid (DNA) ploidy analysis and p16/Ki67 dual-staining (DS) with cytology, and compare their diagnostic performance for detecting cervical abnormalities, particularly in high-risk human papillomavirus (hrHPV)-defined subgroups.

Methods: This cross-sectional study enrolled 877 women undergoing cervical cancer screening. Cervical exfoliated cells were collected for liquid-based cytology, hrHPV testing, DNA ploidy analysis, and p16/Ki67 dual-staining. Agreement between tests was assessed using kappa statistics. Diagnostic performance for detecting cytological abnormalities ($\geq$ ASC-US, $\geq$ LSIL) and histologically confirmed CIN2+ was evaluated using area under the ROC curve (AUC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

Results: DNA ploidy demonstrated stronger agreement with cytology ($\kappa=0.60$, 95% CI: 0.51–0.69) than DS ($\kappa=0.40$, 95% CI: 0.30–0.51). For detecting $\geq$ ASC-US, DNA ploidy achieved significantly higher AUC than DS (0.843 vs 0.705, $P<0.001$), with superior sensitivity (74.7% vs 47.0%, $P<0.001$) and comparable specificity (94.0% vs 94.1%, $P>0.999$). DNA ploidy showed sensitivity comparable to hrHPV testing (74.7% vs 79.5%) but with significantly higher specificity (94.0% vs 84.4%, $P<0.001$). In hrHPV-positive women, DNA ploidy exhibited the strongest agreement with cytology ($\kappa=0.69$) and maintained robust diagnostic performance. For CIN2+ detection, DNA ploidy showed sensitivity of 69.2% and specificity of 62.7%, while DS demonstrated lower sensitivity (53.8%).

Conclusion: DNA ploidy analysis demonstrates stronger agreement with cytology and superior diagnostic performance compared to p16/Ki67 dual-staining, particularly in hrHPV-positive subgroups where effective triage is essential. With sensitivity comparable to hrHPV testing but significantly higher specificity, DNA ploidy offers a balanced, automated approach maintaining high detection rates in cervical cancer screening programs.

Keywords: DNA ploidy, p16/Ki67 dual-staining, cervical cancer

Introduction

Cervical cancer (CC) continues to pose a major public health challenge worldwide, with its primary burden lying in low- and middle-income countries (LMICs), which bear the majority of new cases and deaths.¹ CC inequality, closely linked to socioeconomic development as measured by the Human Development Index (HDI), showing a clear negative correlation: higher HDI is associated with lower incidence and mortality rates.^{2,3} Effective screening is therefore critical for reducing CC burden, particularly in resource-limited settings. Cytology has been the mainstay of screening for decades, but it is limited by subjective interpretation, variable sensitivity (30–87% for Pap smears), and relatively low reproducibility due to inter-observer and sampling variability.⁴

In China, the national cervical cancer screening program has traditionally relied on cytology as the primary modality. However, since the release of the “Healthy China 2030” plan and updated screening guidelines, there has been a progressive transition toward HPV DNA-based testing, particularly in urban demonstration projects and large-scale population screening programs. Nevertheless, cytology remains the dominant method in many resource-limited regions due to infrastructure and

cost constraints.⁵ Although the World Health Organization (WHO) now strongly recommends HPV DNA testing as the preferred high-performance screening method for cervical cancer prevention as part of the 2030 elimination strategy, many LMICs continue to face implementation challenges—including limited laboratory infrastructure, high test costs, and insufficient trained personnel—that have slowed the transition from cytology-based programs. Consequently, cytology remains the primary screening method in many of these settings, despite its known performance limitations.

The introduction of high-risk human papillomavirus (hrHPV) testing has improved screening sensitivity for detecting high-grade cervical intraepithelial neoplasia and more severe lesions (CIN2+), offering over 90% sensitivity for CIN2+ detection. However, its relatively low specificity, often resulting from an inability to distinguish transient from transforming infections, can lead to unnecessary colposcopy referrals and overtreatment, particularly in young women, who have a high prevalence of HPV but also high rates of spontaneous regression.^{6,7} Although cytology has known performance limitations, it remains the primary CC screening method in many LMICs because of its lower cost and infrastructure requirements.

Recent literature highlights that molecular and protein biomarker, particularly when automated, can greatly enhance the sensitivity of CC screening. Their quantitative and stable detection offers a consistent complement to cytology's limitations in subjectivity and inter-observer variability.^{8,9} Among these, DNA ploidy analysis based on cytology slides provides important clinical utility. By offering an objective quantification of nuclear DNA content (a direct marker of malignant potential), it serves as a diagnostically competitive tool. Beyond its inherent merits, the adoption of automated reading systems is what truly optimizes it for clinical and public health use. Automation guarantees the technical consistency and high-volume processing capacity required for reliable, large-scale screening, making it a strategically viable option even in regions with constrained resources.

Among these, DNA ploidy analysis, which uses automated quantitative image cytometry to objectively measure nuclear DNA content and chromosomal abnormalities, has demonstrated competitive accuracy compared to liquid-based cytology (LBC) while offering higher throughput and technical reproducibility, making it particularly suitable for resource-limited settings.¹⁰ Similarly, p16/Ki67 dual-staining cytology (DS),¹¹ which immunocytochemically identifies concurrent expression of proteins indicative of HPV-induced oncogenic transformation (p16 as a cell cycle regulator and Ki67 as a proliferation marker), provides an objective and automated method for triaging HPV-positive women for CIN2+ detection.^{12–15} Among hrHPV infection women with HSIL+, DNA ploidy testing showed higher sensitivity (CIN2+: 79.21% vs 65.35%; CIN3+: 81.48% vs 70.37%) but lower specificity and PPV than LBC, with no significant difference in NPV.¹² In contrast, another study reported that the sensitivity of DS and cytology was 95.7% and 84.7%, respectively, while the specificity was 53.1% and 45.9%.¹³ Both techniques provide objective assessment that reduces dependence on subjective morphological interpretation and observer variability, offering robust alternatives in CC prevention with good clinical performance for detecting CIN2+. The varying performance of DS and DNA ploidy compared to cytology across studies stems from differences in study populations and the subjectivity of cytology. However, direct comparisons of their agreement with cytology and their suitability as clinical substitutes, especially within specific hrHPV-positive subgroups, remain inadequate.

To the best of our knowledge, this study represents the first systematic comparison of DNA ploidy analysis, DS, and cytology. We aimed to evaluate their inter-method agreement and diagnostic performance in detecting cytological abnormalities and histologically confirmed CIN2+, with particular emphasis on hrHPV-defined subgroups. Additionally, we explored the complementary value of combining these automated biomarkers to improve screening accuracy, reduce subjectivity, and advance efficient CC prevention strategies.

Methods

Study Design and Sample Collection

This prospective cross-sectional study was conducted at an outpatient clinic between March 2025 and May 2025, with approval from the Institutional Ethics Committee of the Taiyuan Maternity and Child Health Care Hospital (Approval number: 2024-ER-08), and informed consent obtained from the study participants prior to study commencement. Inclusion criteria were: (1) women aged ≥ 18 years undergoing opportunistic cervical cancer screening at the gynecological outpatient



clinic; (2) sexually active women with an intact uterus; (3) no history of cervical surgery (including conization, loop electrosurgical excision procedure, or hysterectomy); (4) no history of cervical cancer or other gynecological malignancies; and (5) not currently pregnant. Exclusion criteria were: (1) history of cervical treatment within the past 12 months; (2) concurrent participation in other interventional studies; (3) insufficient sample quality for all four tests (cytology, hrHPV, DNA ploidy, and DS); and (4) refusal to provide informed consent. A total of 912 women who underwent opportunistic CC screening in the gynecological outpatient department were consecutively enrolled. During sample collection, cervical exfoliated cells were obtained with a Rovers Cervex-Brush[®] (Rovers Medical Devices, Oss, Netherlands) and immediately rinsed into a vial of PreservCyt solution (Hologic, Bedford, MA, USA), ensuring optimal cellular preservation.

Laboratory technicians blinded to all clinical, cytological and pathological data during the performance and analysis of DNA ploidy, DS, and HPV tests. Cell sample bottles are processed through the ThinPrep automatic standardized procedures for the preparation of cell slides, thin-layer LBC (TCT) smears, in accordance with the manufacturer's instructions. The residual samples were aliquoted for parallel testing by DNA ploidy analysis, DS, and hrHPV testing.

Cytology and Histopathology

Cervical exfoliated cells were obtained using a Rovers Cervex-Brush[®] (Rovers Medical Devices, Oss, Netherlands). The brush was inserted into the cervical os and rotated five times clockwise (360° per rotation) to ensure adequate sampling of the ectocervix and endocervical canal. The brush head was then immediately rinsed into a vial of PreservCyt solution (Hologic, Bedford, MA, USA) by vigorous swirling for 30 seconds. The cytology results were classified according to the Bethesda system (third edition, 2014) into categories including negative for intraepithelial lesion or malignancy (NILM), atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL), atypical squamous cells - cannot exclude HSIL (ASC-H), high-grade squamous intraepithelial lesion (HSIL), and CC. Clinicians determined whether participants required colposcopy and biopsy based on pre-specified referral criteria: any positive result on hrHPV testing, cytology $\geq$ ASC-US, DNA aneuploidy, or positive DS. The colposcopic magnification assessment was conducted, and targeted 2–4 biopsy samples were obtained from the suspicious tissues. Additionally, endocervical curettage (ECC) was performed either for a transformation zone (TZ) II and III when colposcopic uncertainty existed. The histopathological staging was classified into the following categories: Normal, CIN1, CIN2, CIN3, squamous cell carcinoma (SCC), and adenocarcinoma (AD). Cytological and histopathological evaluations were performed blinded to the DNA ploidy and DS results.

HPV Testing

HPV testing was performed using a 24 Human Papillomavirus Genotyping Kit (PCR-Chip Hybridization, BOHUI Innovation Biotech. Group, Beijing, China; China medical device approval No. 20163401108) followed the manufacturer's instructions and generally involved: nucleic acid extraction, PCR amplification, and reverse dot blot hybridization. A total of 24 HPV subtypes were identified and stratified by oncogenic risk based on current WHO/IARC classification: 12 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), with HPV 68 reclassified as probably and HPV 66 possibly high-risk; 3 medium high-risk types (HPV 53, 73, 82); and 7 low-risk types (HPV 6, 11, 42, 43, 44, 81, 83). The determination of positive or negative results was based on the detection of specific hybridization signals at the predefined probe spots on the DNA oligonucleotide array cartridge. While this kit is not currently FDA-approved, it is widely used in Chinese clinical practice and has demonstrated performance comparable to internationally recognized assays in published validation studies.

DNA Ploidy Detection

DNA ploidy analysis was performed using a fully automated imaging system (Motic BA-6000, Xiamen, China) with dedicated reagents for Feulgen staining. TCT smears, fixed in ethanol-acetic acid, and subjected to the Feulgen reaction for stoichiometric DNA quantification based on the Schiff's reagent binding to DNA. The Feulgen staining protocol was performed as follows: slides were hydrolyzed in 5N HCl at room temperature for 60 minutes, then rinsed with distilled water, followed by incubation in Schiff's reagent for 90 minutes in the dark. After three rinses in sulfurous acid solution (2 minutes each), slides were dehydrated through graded alcohols and mounted. Slides were automatically scanned to

capture images and identify intact nuclei. Slides were automatically scanned at 40× magnification to capture high-resolution images and identify intact nuclei. The integrated optical density (IOD) of each nucleus was measured, and the DNA index (DI) was calculated as the ratio of the IOD of a cell to the median IOD of reference diploid cells (2c) within the same sample. A positive result was defined as ≥ 3 cells with $DI \geq 2.5$ or ≥ 5 cells with DI between 1.0–2.5 (aneuploid or hyperdiploid), both thresholds indicating elevated risk of precancerous lesions or CIN, this threshold was pre-defined based on the manufacturer's instructions.

p16/Ki67 Dual-Staining Cytology (DS)

The p16/Ki-67 dual-staining cytology was performed using the p16/Ki-67 Detection Kit (Immunocytochemical Method) (Wondfo, Guangzhou, China; for China medical device regionally filing No. 20240169) according to the manufacturer's instructions. TCT slides were baked at 60°C for 1 hour, dewaxed, hydrated, and washed. Antigen retrieval was performed with EDTA (pH 9.0) at 121°C for 3 minutes. After peroxidase blocking, primary antibodies (p16 and Ki67) were applied overnight at 4°C. HRP- and AP-labeled secondary antibodies were added, followed by DAB (p16, brown) and Fast Red (Ki67, red) development. Hematoxylin counterstaining was followed by dehydration and mounting. ≥ 1 cell with cytoplasmic p16-brown and nuclear Ki67-red staining was considered positive.

Statistical Analysis

All statistical analyses were performed using MedCalc[®] version 22 (MedCalc Software Ltd, Ostend, Belgium). A two-sided P value < 0.05 was considered statistically significant. Continuous variables are presented as median [interquartile range (IQR)], and categorical variables are expressed as frequency (%). The agreement between the two tests was assessed using the Kappa value, and the difference in rates between the two tests was evaluated using the paired chi-square test. Agreement plots were generated using the “reshape2” and “ggplot2” packages in R-4.5.1.

For the all participants and hrHPV subgroups, the area (AUC) under the receiver operator characteristic curve (ROC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with 95% confidence intervals (95% CI) were calculated for each test for detecting cytology $\geq$ ASC-US and $\geq$ LSIL. Differences in AUC between the two tests were tested using the method proposed by DeLong et al.¹⁶ Differences in sensitivity and specificity were assessed using the paired chi-square test, while differences in PPV and NPV were evaluated using the method proposed by Leisenring et al.¹⁷

Additionally, the AUC, sensitivity, specificity, PPV, and NPV with 95% CIs were calculated for each test for detecting CIN2+ and CIN1+.

Results

Basic Information of Participants

A total of 877 cases, following the exclusion of 9 for hrHPV, 15 for DNA ploidy, and 18 for DS tests due to invalid detection results, were included in the final analysis. The cytology result distribution as follows: 794 with NILM, 43 with ASC-US, 23 with LSIL, 6 with ASC-H, and 11 with high-grade squamous intraepithelial lesion or worse (HSIL+). Sixty-four patients underwent colposcopic biopsy, with results showing 40 cases of Normal, 11 of CIN1, and 13 of CIN2+. The median age of participants was 37.0 [IQR: 32.0–45.0]. Among them, 211 (24.1%) were older than 45 years. A total of 190 subjects (21.7%) tested positive for hrHPV. The positive rates of DNA ploidy and DS were 12.5% ($n=110$) and 9.8% ($n=86$), respectively. [Table 1](#) and [Supplementary Table 1](#) detail the distribution of all indicators stratified by cytology and pathology results, respectively.

Agreement Between Tests

The kappa values of inter-rater agreement between DNA ploidy and cytology, and between DS and cytology, were 0.60 (95% CI: 0.51–0.69) and 0.40 (95% CI: 0.30–0.51), respectively. The kappa value between DNA ploidy and DS was the lowest, at 0.38 ([Figure 1A](#)). The discordance rate between DNA ploidy and cytology was 7.9%, and the distribution of cases in hrHPV-negative and hrHPV-positive subgroups is shown in [Figure 1B](#).

Table 1 Basic Characteristics

Cytology	Level	NILM	ASC-US	LSIL	ASC-H	HSIL+	Total
Numbers		794	43	23	6	11	877
Age years, median [IQR]		37.0 [32.0–45.0]	38.0 [31.5–43.8]	37.0 [30.3–45.5]	51.5 [35.0–60.0]	41.0 [39.0–50.8]	37.0 [32.0–45.0]
Age, n (%)	≤45	607 (76.4)	33 (76.7)	17 (73.9)	2 (33.3)	7 (63.6)	666 (75.9)
	>45	187 (23.6)	10 (23.3)	6 (26.1)	4 (66.7)	4 (36.4)	211 (24.1)
hrHPV, n (%)	Negative	670 (84.4)	14 (32.6)	2 (8.7)	1 (16.7)	0 (0.0)	687 (78.3)
	Positive	124 (15.6)	29 (67.4)	21 (91.3)	5 (83.3)	11 (100)	190 (21.7)
HPV multiple infection, n (%)	HPV (-)	631 (79.5)	8 (18.6)	0 (0.0)	1 (16.7)	0 (0.0)	640 (73.0)
	LR-HPV	39 (4.9)	6 (14.0)	2 (8.7)	0 (0.0)	0 (0.0)	47 (5.4)
	Single	111 (14.0)	24 (55.8)	15 (65.2)	5 (83.3)	8 (72.7)	163 (18.6)
	Double	12 (1.5)	4 (9.3)	4 (17.4)	0 (0.0)	2 (18.2)	22 (2.5)
	Triple+	1 (0.1)	1 (2.3)	2 (8.7)	0 (0.0)	1 (9.1)	5 (0.6)
DNA ploidy, n (%)	Normal	746 (94.0)	19 (44.2)	2 (8.7)	0 (0.0)	0 (0.0)	767 (87.5)
	Abnormal	48 (6.0)	24 (55.8)	21 (91.3)	6 (100.0)	11 (100.0)	110 (12.5)
DS, n (%)	DS (-)	747 (94.1)	31 (72.1)	9 (39.1)	3 (50.0)	1 (9.1)	791 (90.2)
	DS (+)	47 (5.9)	12 (27.9)	14 (60.9)	3 (50.0)	10 (90.9)	86 (9.8)

Abbreviations: NILM, Negative for Intraepithelial Lesion or Malignancy; ASC-US, Atypical Squamous Cells of Undetermined Significance; LSIL, Low-Grade Squamous Intraepithelial Lesion; ASC-H, Atypical Squamous Cells - cannot exclude HSIL; HSIL, High-Grade Squamous Intraepithelial Lesion; IQR, Interquartile Range; hrHPV, high-risk Human Papillomavirus; DS, p16/Ki67 dual staining.

In the hrHPV-negative subgroup, statistically significant differences in positive rates were observed between DNA ploidy and cytology ($\geq$ ASC-US threshold), as well as between DS and cytology (all $P < 0.001$), indicating that both biomarker-based assays detect a considerably higher number of abnormalities compared to morphology-based cytology in HPV-negative individuals. Conversely, among hrHPV-positive subgroup, a statistically significant difference was found between DS and cytology ($P < 0.001$), reaffirming the superior sensitivity of DS in identifying true precancerous lesions in hrHPV-infected cohorts. However, the difference in positive rates between DNA ploidy and cytology was not statistically significant ($P = 0.169$), with an adjusted rate difference of -4.2 (95% CI: -9.4 – 1.0), suggesting that in HPV-

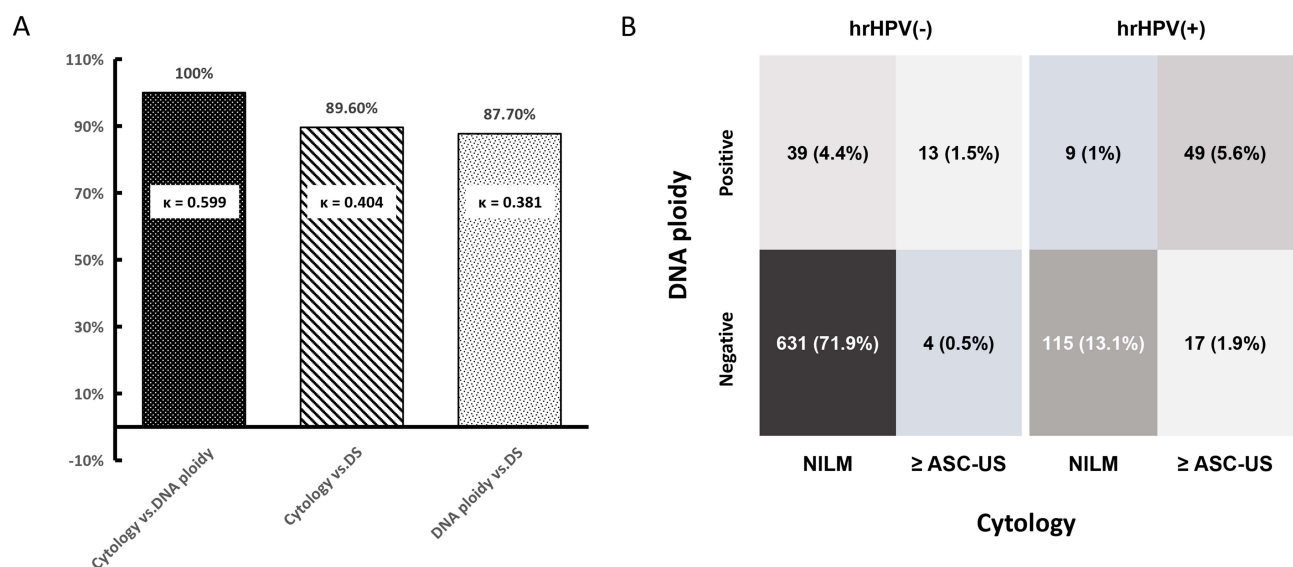


Figure 1 Agreement Between Tests. (A) Agreement among DNA ploidy, DS, and cytology ($\geq$ ASC-US); (B) Distribution of case numbers between DNA ploidy and cytology within hrHPV subgroups.

Abbreviations: DS, p16/Ki67 dual staining cytology; κ , kappa; hrHPV, high-risk Human Papillomavirus; NILM, Negative for Intraepithelial Lesion or Malignancy; ASC-US, Atypical Squamous Cells of Undetermined Significance.

positive settings, DNA ploidy and cytology may perform similarly in detecting additional abnormalities beyond HPV presence itself. The kappa value was the highest in hrHPV-positive subgroup, at 0.69 (95% CI: 0.58–0.80; Table 2).

Performance for Detecting Cytology $\geq$ ASC-US

In detecting cytological abnormalities $\geq$ ASC-US, DNA ploidy analysis demonstrated discriminative ability, with AUC of 0.843, compared to 0.705 for DS ($P < 0.001$; Figure 2 and Table 3). Although the specificity of DNA ploidy was statistically comparable to DS ($P > 0.999$), it exhibited significantly higher sensitivity (74.7% vs 47.0%; $P < 0.001$). This performance advantage of DNA ploidy, higher sensitivity without loss of specificity, was consistently observed across hrHPV-based subgroups (Table 3). Notably, while the sensitivity of DNA ploidy (74.7%) was comparable to that of the hrHPV test (79.5%), its specificity was greater (94.0% vs 84.4%; $P < 0.001$), highlighting its potential as a more balanced test that minimizes false positives without substantially compromising detection rates.

Performance for Detecting Pathological CIN2+ and CIN1+

Cytology ($\geq$ LSIL) consistently provided the best overall performance (highest AUC) for detecting both CIN2+ ($n=13$) and CIN1+ ($n=24$). In the detection of CIN2+, DNA ploidy analysis demonstrated a sensitivity of 69.2% (95% CI: 38.6–90.9%) and a specificity of 62.7% (95% CI: 48.1–75.9%), whereas DS showed the lowest sensitivity at 53.8% (95% CI: 25.1–80.8%) despite having a comparable specificity to DNA ploidy. hrHPV testing showed the highest sensitivity at 84.6%, but this came with the lowest specificity (27.5%) and a low AUC (0.560), indicating a high false-positive rate. It should be noted that these values—the particularly low specificity of hrHPV and the low sensitivity of DS—highlight key limitations of each test in the context of this study population and warrant cautious interpretation. Cytology ($\geq$ ASC-US) yielded a sensitivity of 76.9% (95% CI: 46.2–95.0%) and a specificity of 56.9% (95% CI: 42.2–70.7%). Performance metrics for the detection of CIN1+ are comprehensively detailed in Table 4. For a more sensitive detection strategy, hrHPV testing offered the highest sensitivity (84.6%) with the lower specificity (27.5%), while DNA ploidy analysis emerged as a competitive alternative with well-balanced metrics, particularly for CIN1+ detection (AUC: 0.717, Sensitivity: 70.8%, Specificity: 72.5%).

Table 2 Consistency Analysis Among methods

	A (+), B (+)	A (+), B (-)	A (-), B (+)	A (-), B (-)	Positive rate of A %	Positive rate of B %	Difference % (95% CI)	P-value	Kappa (95% CI)
Cytology* (A) vs DS (B) (all n = 877)									
n	39	44	47	747	9.5	9.8	0.3 (-1.8–2.5)	0.834	0.40 (0.30–0.51)
Cytology* (A) vs DNA ploidy (B) (all n = 877)									
n	62	21	48	746	9.5	12.5	3.0 (1.2–4.3)	0.002	0.60 (0.51–0.69)
Cytology* (A) vs DS (B) (hrHPV (-) n = 687)									
n	7	10	38	632	2.5	6.6	4.1 (2.1–6.0)	< 0.001	0.20 (0.06–0.34)
Cytology* (A) vs DNA ploidy (B) (hrHPV (-) n = 687)									
n	13	4	39	631	2.5	7.6	5.1 (3.3–6.9)	< 0.001	0.35 (0.21–0.50)
Cytology* (A) vs DS (B) (hrHPV (+) n = 190)									
n	32	34	9	115	34.7	21.6	-13.2 (-19.7–6.7)	< 0.001	0.45 (0.32–0.59)
Cytology* (A) vs DNA ploidy (B) (hrHPV (+) n = 190)									
n	49	17	9	115	34.7	30.5	-4.2 (-9.4–1.0)	0.169	0.69 (0.58–0.80)

Notes: * Cytology $\geq$ ASC-US is identified as positive (+), otherwise it is identified as Negative (-).

Abbreviations: CI, confidence interval; DS, p16/Ki67 dual staining; hrHPV, high risk Human Papillomavirus.

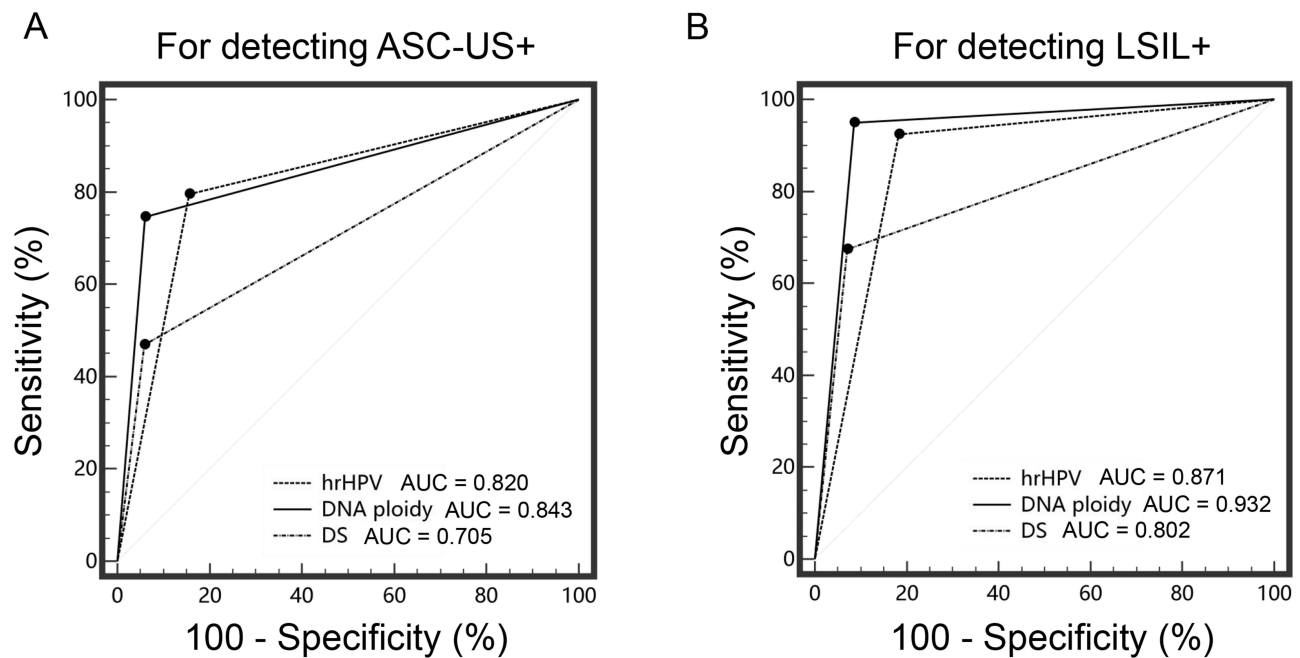


Figure 2 ROC curves of DNA ploidy, DS, and hrHPV for detecting cytological ASC-US+ and LSIL+. (A) For detecting ASC-US; (B) For detecting LSIL.

Abbreviations: ASC-US, Atypical Squamous Cells of Undetermined Significance; LSIL, Low-Grade Squamous Intraepithelial Lesion; ROC, Receiver Operator Characteristic Curve; AUC, the Area under the ROC curve.

Discussion

This prospective cross-sectional study systematically evaluated the performance and clinical utility of two automated, objective biomarkers, DNA ploidy and DS, alongside cytology and hrHPV testing within a CC screening cohort in a single hospital. Our finding is that DNA ploidy analysis emerged as a particularly robust and balanced screening tool, demonstrating superior discriminatory power for cytological abnormalities and a more favorable profile for detecting histological cervical high-grade lesions compared to DS. Moreover, DNA ploidy analysis demonstrated stronger agreement with cytology results

Table 3 Clinical Performance for Detecting Cytology $\geq$ ASC-US

Test	AUC (95% CI)	Sensitivity % (n/N) 95% CI	Specificity % (n/N) 95% CI	PPV % (n/N) 95% CI	NPV % (n/N) 95% CI
For detecting cytology $\geq$ ASC-US (n = 83) in all participants (n = 877)					
hrHPV	0.820 (0.792–0.844)	79.5 (66/83) 69.2–87.6	84.4 (670/794) 81.7–86.8	34.7 (66/190) 30.4–39.3	97.5 (670/687) 96.3–98.4
DS	0.705 (0.674–0.735)	47.0 (39/83) 35.9–58.3	94.1 (747/794) 92.2–95.6	45.3 (39/86) 36.7–54.3	94.4 (747/791) 93.3–95.4
DNA ploidy	0.843 (0.817–0.867)	74.7 (62/83) 64.0–83.6	94.0 (746/794) 92.1–95.5	56.4 (62/110) 48.9–63.6	97.3 (746/767) 96.1–98.1
P^a	< 0.001	< 0.001	> 0.999	0.042	< 0.001
P^b	0.481	0.585	< 0.001	< 0.001	0.720
For detecting cytology $\geq$ ASC-US (n = 17) in hrHPV (-) woman (n = 687)					
DS	0.678 (0.641–0.712)	41.2 (7/17) 18.4–67.1	94.3 (632/670) 92.3–96.0	15.6 (7/45) 8.8–26.0	98.4 (632/642) 97.7–98.9
DNA ploidy	0.853 (0.825–0.879)	76.5 (13/17) 50.1–93.2	94.2 (631/670) 92.1–95.8	25.0 (13/52) 18.2–33.3	99.4 (631/635) 98.5–99.7
P^a	0.003	0.031	> 0.999	0.064	0.014
For detecting cytology $\geq$ ASC-US (n = 66) in hrHPV (+) woman (n = 190)					
DS	0.706 (0.636–0.770)	48.5 (32/66) 36.0–61.1	92.7 (115/124) 86.7–96.6	78.0 (32/41) 64.4–87.5	77.2 (115/149) 72.7–81.1
DNA ploidy	0.835 (0.774–0.885)	74.2 (49/66) 62.0–84.2	92.7 (115/124) 86.7–96.6	84.5 (49/58) 74.1–91.2	87.1 (115/132) 81.7–91.1
P^a	< 0.001	< 0.001	> 0.999	0.342	< 0.001

Notes: P^a , DNA ploidy vs DS. P^b , DNA ploidy vs hrHPV.

Abbreviations: AUC, the area under the receiver operator characteristic curve; CI, confidence interval; ASC-US, Atypical Squamous Cells of Undetermined Significance; hrHPV, high risk Human Papillomavirus; DS, p16/Ki67 dual staining.

Table 4 Clinical Performance for Detecting CIN2+/CIN1+

Test	AUC (95% CI)	Sensitivity % (n/N) 95% CI	Specificity % (n/N) 95% CI	PPV % (n/N) 95% CI	NPV % (n/N) 95% CI
For detecting CIN2+					
hrHPV	0.560 (0.431–0.684)	84.6 (11/13) 54.5–98.1	27.5 (14/51) 15.9–41.8	22.9 (11/48) 18.2–28.4	87.5 (14/16) 64.4–96.4
HPV16/18	0.652 (0.523–0.767)	46.2 (6/13) 19.2–74.9	84.3 (43/51) 71.4–93.0	42.9 (6/14) 24.0–64.1	86.0 (43/50) 78.5–91.2
Cytology (≥ASC-US)	0.669 (0.540–0.782)	76.9 (10/13) 46.2–95.0	56.9 (29/51) 42.2–70.7	31.2 (10/32) 22.7–41.2	90.6 (29/32) 77.7–96.4
Cytology (≥LSIL)	0.690 (0.562–0.800)	61.5 (8/13) 31.6–86.1	76.5 (39/51) 62.5–87.2	40.0 (8/20) 25.7–56.2	88.6 (39/44) 79.4–94.0
DS	0.583 (0.453–0.705)	53.8 (7/13) 25.1–80.8	62.7 (32/51) 48.1–75.9	26.9 (7/26) 16.6–40.6	84.2 (32/38) 74.1–90.9
DNA ploidy	0.660 (0.531–0.774)	69.2 (9/13) 38.6–90.9	62.7 (32/51) 48.1–75.9	32.1 (9/28) 22.2–44.1	88.9 (32/36) 77.5–94.9
For detecting CIN1+					
hrHPV	0.567 (0.437–0.690)	83.3 (20/24) 62.6–95.3	30.0 (12/40) 16.6–46.5	41.7 (20/48) 35.3–48.4	75.0 (12/16) 52.2–89.2
HPV16/18	0.625 (0.495–0.743)	37.5 (9/24) 18.8–59.4	87.5 (35/40) 73.2–95.8	64.3 (9/14) 40.6–82.6	70.0 (35/50) 62.6–76.5
Cytology (≥ASC-US)	0.733 (0.608–0.836)	79.2 (19/24) 57.8–92.9	67.5 (27/40) 50.9–81.4	59.4 (19/32) 47.2–70.5	84.4 (27/32) 70.6–92.4
Cytology (≥LSIL)	0.750 (0.626–0.85-)	62.5 (15/24) 40.6–81.2	87.5 (35/40) 73.2–95.8	75.0 (15/20) 55.5–87.8	79.5 (35/44) 69.6–86.9
DS	0.608 (0.478–0.728)	54.2 (13/24) 32.8–74.4	67.5 (27/40) 50.9–81.4	50.0 (13/26) 35.9–64.1	71.1 (27/38) 60.2–79.9
DNA ploidy	0.717 (0.590–0.822)	70.8 (17/24) 48.9–87.4	72.5 (29/40) 56.1–85.4	60.7 (17/28) 46.8–73.1	80.6 (29/36) 68.3–88.8

Abbreviations: CIN, Cervical Intraepithelial Neoplasia; AUC, the area under the receiver operator characteristic curve; CI, confidence interval; hrHPV, high risk Human Papillomavirus; ASC-US, Atypical Squamous Cells of Undetermined Significance; LSIL, Low-Grade Squamous Intraepithelial Lesion; DS, p16/Ki67 dual staining.

(both ≥ASC-US and ≥LSIL in [Table 2](#) and [Supplementary Table 2](#)) and superior diagnostic accuracy in detecting cytological abnormalities (≥ASC-US or ≥LSIL in [Table 3](#) and [Supplementary Table 3](#)) compared to DS. Furthermore, DNA ploidy demonstrated promising triage potential, particularly among hrHPV-positive women, where it achieved the highest level of agreement with cytological findings (≥ASC-US, kappa = 0.69; ≥LSIL, kappa = 0.66).

The observed agreement between DNA ploidy analysis and cytology ($\kappa=0.60$), compared to that of dual-staining (DS) ($\kappa=0.40$), may be attributed to fundamental differences in what each assay measures. Recent bioinformatics analyses have further elucidated the molecular pathways involved in cervical carcinogenesis, highlighting the complex interplay between viral oncoproteins and host cellular regulatory networks that ultimately lead to genomic instability and aneuploidy.¹⁸ DNA ploidy analysis quantitatively assesses chromosomal instability by measuring nuclear DNA content, with aneuploidy serving as a direct biomarker of genomic disruption often correlated with visually discernible morphological changes in cytology. DS detects whether there are cervical epithelial cells with positive p16 and Ki67 double staining. Since both tests reflect—directly or indirectly—underlying genomic instability, their results show stronger concordance. This genomic instability is a hallmark of HPV-driven carcinogenesis and manifests cytologically as the visible nuclear abnormalities (eg., hyperchromasia, nuclear enlargement, irregular nuclear contours) that pathologist's grade as ASC-US, LSIL, or HSIL.¹⁹ Thus, DNA ploidy and cytology are essentially measuring different facets of the same underlying biological process — one quantitative and molecular,¹⁰ the other qualitative and morphological.²⁰ In contrast, DS detects a specific protein expression phenotype (concurrent p16 and Ki67 positivity) indicative of HPV-driven transformation, which may not always coincide with cytomorphological alterations or DNA ploidy abnormalities, resulting in lower agreement.^{21,22} Although DS has long been used in CC screening,²³ with positivity rate rising alongside severity, this discordance with cytology likely stems from their fundamentally different biological targets. DS detects elevated levels of two specific proteins (p16 and Ki67), which are highly indicative of oncogenic HPV activity.²⁴ However, this protein expression may not always perfectly correlate with the immediate degree of nuclear atypia graded by cytology.²⁵ While highly specific for transformation, this phenotype may not always align perfectly with the morphological spectrum defined by the Bethesda System, leading to lower agreement. This is supported by our subgroup analysis, which showed that in hrHPV-positive women, the difference between DNA ploidy and cytology was not significant ($P=0.169$), whereas DS significantly outperformed cytology. This suggests that in the presence of the causative agent (hrHPV), the genomic instability measured by DNA ploidy aligns more closely with cytological interpretation than the protein expression measured by DS. Conversely, some morphological abnormalities may arise through pathways not fully captured by these two specific protein markers alone.²⁶ The lowest agreement was observed between DNA ploidy and DS (kappa=0.38), underscoring that these two biomarkers capture fundamentally different biological aspects of cervical carcinogenesis.

In our study, DNA ploidy detected abnormalities in 39 hrHPV-negative women, of whom 13 had concurrent cytology $\geq$ ASC-US (true positives by cytology reference) and 26 had negative cytology (NILM). Among these 26 discordant cases (DNA ploidy+/cytology-), the clinical significance is uncertain due to the lack of histological confirmation. It is possible that some represent false positives that would lead to unnecessary colposcopy if used as a standalone triage test. However, it is also possible that some represent early genomic abnormalities that precede morphological changes detectable by cytology or hrHPV testing—a phenomenon known as “genomic instability without visible atypia.” Longitudinal follow-up studies with repeat testing are needed to determine whether these DNA ploidy abnormalities predict future development of cytological or histological abnormalities.

The significantly higher AUC and sensitivity of DNA ploidy for detecting $\geq$ ASC-US (Table 3) and $\geq$ LSIL (Supplementary Table 3) are clinically crucial. An ASC-US+ sensitivity of 74.7% for DNA ploidy versus 47.0% for DS indicates that DNA ploidy would miss substantially fewer women with underlying cytological abnormalities. However, the sensitivity for detecting LSIL+ reached 95.0% (95% CI: 83.1–99.4; Supplementary Table 3). This performance advantage was consistent across hrHPV-defined subgroups, affirming its robustness. Notably, DNA ploidy achieved a sensitivity comparable to that of hrHPV testing (74.7% vs 79.5%) but with significantly higher specificity (94.0% vs 84.4%). In addition, automated DNA ploidy systems can objectively detect abnormal DNA content long before overt morphological changes become apparent to a cytotechnologist, reducing the reliance on subjective interpretation and mitigating inter-observer variability. This addresses a critical weakness of primary hrHPV testing—its relatively low specificity—by offering an objective method that maintains high sensitivity while drastically reducing false positives and potential overtreatment.^{27,28} The performance of all tests in the hrHPV-negative subgroup is important for understanding their role as primary screening tools. Our data shows that both DNA ploidy and DS detected a higher number of abnormalities than cytology in this subgroup ($P < 0.001$). This suggests that these objective biomarkers may identify a subset of women with clinically significant lesions that are missed by both hrHPV testing and cytology.

Additionally, given the high specificity of both Dual-Staining (DS) and DNA ploidy analysis, we adopted a co-testing strategy (“/” combination) where a positive result in either test was interpreted as positive, and a negative result required both tests to be negative. This approach aimed to improve sensitivity for detecting cytological abnormalities ($\geq$ ASC-US). The clinical performance of this strategy is detailed in Supplementary Table 4. Our findings demonstrated that the sensitivity of this combined method for detecting cytological abnormalities was comparable to that of hrHPV testing, while the specificities were 89.2% and 84.4%, respectively. It is important to note that cytology is not the gold standard for diagnosing cervical lesions and is subject to bias. Further evaluation with a larger number of cases with known pathological outcomes is warranted.

Previous studies have reported high CIN2+ sensitivity (82.8%,²⁹ 75.2%,³⁰ 88.1%³¹) for DS. Our study population had a limited number of pathologically confirmed CIN2+ cases ($n=13$). However, our results—which showed a lower sensitivity (53.8%) for DS—underscore how diagnostic performance can vary depending on the population and study protocol. Conversely, our data on DNA ploidy corroborate studies that position aneuploidy as a strong predictor of high-grade disease.

The high agreement and superior performance of DNA ploidy, especially within the hrHPV-positive subgroup, strongly support its potential as a reliable triage test. The high specificity of DNA ploidy (94.0%) is also its most compelling clinical attribute. Here, DNA ploidy demonstrates a strong potential. Its good sensitivity (74.2% for $\geq$ ASC-US in hrHPV+ women) combined with its high specificity (92.7% in the same group) means it can effectively identify those hrHPV-positive women who most need colposcopy while reassuring those who do not. Its fully automated nature offers additional advantages: dramatically increased throughput compared to manual cytology, minimal training requirements for operators, and elimination of inter-observer variability. Our study confirms that hrHPV testing, while sensitive, lacks specificity (27.5% for CIN2+), leading to unnecessary colposcopies, patient anxiety, and potential overtreatment. An ideal triage test must effectively stratify this hrHPV-positive population. Integrating automatic DNA ploidy analysis into a primary hrHPV screening algorithm could be highly efficient: hrHPV testing provides high sensitivity, and subsequent DNA ploidy analysis of positive cases could offer high specificity to identify women who truly need colposcopy.^{12,32} This strategy could help alleviate the burden on healthcare systems in low-resource settings that still rely on cytology, by providing an automated and objective alternative to subjective cytological interpretation.

Regarding the follow-up strategy for abnormal findings, we acknowledge that our referral criteria (any positive test among the four modalities) were designed to maximize sensitivity for this comparative diagnostic study rather than to reflect a specific clinical management algorithm. In clinical practice, a more conservative triage approach—such as reflex testing with DNA ploidy or DS only in hrHPV-positive women—would likely reduce unnecessary colposcopies.

Several limitations of this study should be acknowledged. First, the imbalanced distribution of cytology results, with a predominance of NILM cases and relatively few high-grade lesions (only 11 HSIL+ cases). While this reflects a true screening population, the small number of abnormal cases—particularly for histologically confirmed CIN2+ (n=13)—may limit the reliability and precision of sensitivity estimates for these endpoints. We have reported 95% confidence intervals for all diagnostic performance metrics (Table 3 and Table 4) to reflect this uncertainty. Second, our findings are derived from a single-center population in China, and caution should be exercised when generalizing these results to other LMIC settings. Differences in healthcare access, screening implementation strategies, epidemiological profiles of HPV infection, and population risk characteristics may influence test performance. Third, Verification bias is inherent in any screening study where not all participants undergo the gold standard (colposcopy with biopsy). In our study, only women with at least one positive screening test (hrHPV, cytology $\geq$ ASC-US, DNA aneuploidy, or DS+) were referred for colposcopy. Women negative on all four tests did not undergo biopsy, which may lead to underestimation of false negatives and overestimation of specificity. This limitation is common to diagnostic accuracy studies in cervical cancer screening and reflects real-world clinical practice, where it is unethical to biopsy all screened women. Fourth, we used a regionally approved DS kit (Wondfo); performance characteristics might vary slightly with other commercially available DS kits (eg., CINtec[®] PLUS)³³ Finally, the cost-effectiveness of implementing DNA ploidy versus DS or other triage strategies was not evaluated and warrants future investigation. The future research includes follow-up of these subjects, collecting data from subsequent screening rounds or colposcopy results, to more comprehensively evaluate the screening efficacy and risk stratification of DNA ploidy and DS. Therefore, our conclusions should be considered preliminary and specific to our study population until validated in multi-center, cross-regional studies.

Conclusion

In this study, DNA ploidy analysis demonstrated stronger agreement with cytology and superior diagnostic performance compared to p16/Ki67 dual-staining, with significantly higher sensitivity for detecting cytological abnormalities (74.7% vs 47.0%) and comparable specificity (approximately 94% for both). The stronger concordance between DNA ploidy and cytology ($\kappa=0.60$ vs $\kappa=0.40$) likely reflects their shared biological basis in detecting genomic instability and its morphological correlates, whereas DS captures a specific protein expression phenotype that may not always align with cytological alterations. Notably, DNA ploidy achieved sensitivity comparable to hrHPV testing (74.7% vs 79.5%) but with significantly higher specificity (94.0% vs 84.4%), positioning it as a balanced triage tool—particularly in hrHPV-positive women where it showed the strongest agreement with cytology ($\kappa=0.69$). These findings suggest that automated DNA ploidy analysis could serve as an effective, objective strategy maintaining high detection rates, though validation in larger cohorts with more histologically confirmed endpoints is warranted.

Abbreviations

ASC-H, Atypical Squamous Cells - cannot exclude HSIL; ASC-US, Atypical Squamous Cells of Undetermined Significance; AUC, The area under the receiver operator characteristic curve; CI, Confidence interval; CIN, Cervical Intraepithelial Neoplasia; CC, Cervical cancer; DS, p16/Ki67 dual staining; HSIL, High-Grade Squamous Intraepithelial Lesion; hrHPV, high-risk Human Papillomavirus; IQR, Interquartile Range; LSIL, Low-Grade Squamous Intraepithelial Lesion; NILM, Negative for Intraepithelial Lesion or Malignancy; NPV, Negative Predictive Value; PPV, Positive Predictive Value; ROC, Receiver operator characteristic curve.

Data Sharing Statement

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.



Ethics Approval and Consent to Participate

This study was approved by the Research and Clinical Trial Ethics Committee of the Taiyuan Maternity and Child Health Care Hospital, Taiyuan, China (No.: 2024-ER-08), and it was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the participants prior to the publication of this study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare they have no commercial or financial relationships that could be construed as a potential conflict of interest.

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