**QUALITY ASSURANCE IN CERVICAL CANCER SCREENING: EVALUATION OF SAMPLE ADEQUACY IN HPV DNA TESTING**

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**Ethics approval statement**

The study received ethical approval from the Local Ethics Committee (Ref. 7438/CEL/2023, addendum 1809/CEL/2024). Women involved in the observational study gave written consent.

**Conflict of interest**

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**Authors' contributions**

Conceptualization, writing, data curation, and editing: Morena d’Avenia. Molecular biology experiments: Morena d’Avenia, Njoku Chinyere R; Citology evaluation: Loredana Santomauro, Michela Iacobellis. Data collection: Morena d’Avenia, Njoku Chinyere R. Statistical Analysis, Tables and Figures: Filippo Dell’Anno, Morena d’Avenia. Writing review: Clementina Cocuzza, Morena d’Avenia, Marianna Martinelli, Arroyo Mühr LS, Filippo Dell’Anno. Supervision and funding acquisition: Michela Iacobellis, Clementina Cocuzza. All authors have read and agreed to the published version of the manuscript.

**Abbreviations**

ASC-H atypical squamous cells-high-grade cannot be excluded

ASC-US Atypical squamous cells of undetermined significance

CCR5 *C-C chemokine receptor type 5*

CC Cervical Cancer

CCS Cervical Cancer Screening

CEL Local Ethic Committee

CI Confidence Interval

CIN Cervical Intraepithelial Neoplasia

Ct Cycles Threshold

EU European Union

FIGO International Federation of Gynecology and Obstetrics

GPSC Good Performance Screening Centers

HPV human papillomavirus

HR High Risk

H-SIL High-grade squamous intraepithelial lesion

IC Internal Control

IFU Instruction for use

LBC Liquid-based cytology

LR Low Risk

L-SIL Low-grade squamous intraepithelial lesion

MPSC Medium Performance Screening Centers

NILM negative for intraepithelial lesion or malignancy

OR odds ratio

PCR Polymerase Chain Reaction

PPSC Poor Performance Screening Centers

PR positive rate

QC Quality Control

RXN reaction

SC Screening out patients’ clinic center

TPSC Top Performance Screening Centers

**Abstract**

**Background:** In HPV-primary screening, sample quality significantly influences test accuracy. Unlike cytology-based screening, no consensus guidelines exist for sample quality assessment in HPV testing. This study aims to evaluate the impact of sample cellularity on HPV testing.

**Methods:** A total of 37,592 liquid-based cytology (LBC) samples from women undergoing screening (aged 30-64, mean 47.8±9 years) were analyzed using Cobas®4800 HPV Test (Roche). Sample adequacy was assessed by the assay’s β-globin internal control and by an independent quantitative cellularity assessment (OncoPredict HPV, Hiantis). HPV positivity rates (PR) were stratified according to β-globin Ct values.

**Results:** Overall HPV-PR was 7.7%. PR reached 9.7% in samples with β-globin ≤ 28Ct, decreasing markedly to 1.4% for β-globin > 34 Ct (p < 0.001). Quantitative analysis showed that Cobas®4800 β-globin Ct = 34 corresponds to approximately 1,5x10^3 nucleated cells/rxn. A subset of 195 HPV-negative samples with β-globin Ct ≥34 was evaluated by LBC: 19% had inadequate cellularity according to LBC guidelines, 8% ≥ ASC-US and 73% NILMs, of which 65% showed cellular atrophy.

**Conclusion:** These findings emphasize the importance of assessing cellularity in HPV-screening in order to avoid potentially false-negative results due to inadequate samples. Future research should focus on establishing standardized cellularity thresholds to improve screening accuracy.

**Keywords:** sample adequacy, HPV-DNA test quality assurance (QA), cervical cancer screening, sample cellularity.

1. **Background**

The primary cause of cervical cancer (CC) is persistent infection with high-risk (HR) human papillomavirus (HPV) genotypes. Molecular HR-HPV assays have improved screening programs through HPV-primary algorithms1,2 based on which HPV-negative women are recalled after 5 years, whilst HPV-positive patients undergo triage with cytology. Despite the improved sensitivity of HPV testing, 5.5 - 16.9% of CCs are reported as HPV-negative, especially in women over the age of 50 years3,4,5,6, often diagnosed at advanced FIGO stages which are linked to a poor prognosis7,8. Recent studies have shown that HR-HPV negative cases represent 5 - 7.8% of all CCs9,10, with only few rare pathological types being truly HPV-negative11,12. The true incidence of HPV-negative invasive CC may therefore be overestimated13,14,15.

False HPV-negative results may arise from analytical and/or pre-analytical issues. Analytical problems can include mutations and deletions in the HR-HPV DNA target that prevent PCR detection16,17, or to rare cases of cervical lesions associated to infection with Low Risk (LR) HPV genotypes18,19. Pre-analytical issues, like the presence of PCR inhibitors in the sample, DNA fragmentation, DNA extraction failure, few abnormal cells in a high background of “normal” cells or samples with inadequate cellular content, can also lead to false negative results9.

Clinical validation studies of new HPV assays20,21 have focused on the clinical sensitivity and specificity of HPV targets’ cut-offs for the detection of CIN2+ lesions22 but less attention has been placed on the assessment of sample adequacy.

Sample quality is well defined when performing liquid-based cytology (LBC) by the Bethesda System which classifies samples as "satisfactory" based on the assessment of samples’ cellularity and the presence of transformation zone components23,24,25. In cervical cytology, a minimum of 5,000-8,000 squamous epithelial cells per slide is recommended23,24,26, as inadequate cellularity can lead to false negative results.

Most commercially available HPV assays are qualitative and arbitrarily estimate the amount of collected cells by applying a Cycle threshold (Ct) cut-off for the amplification of a human internal control (IC) target, as a measure of sample adequacy. Although sample adequacy is essential for the quality assurance of HPV DNA molecular testing in CC prevention, this parameter has not been strictly evaluated nor has a required number of cells been defined in order to increase confidence in “HPV-negative” results, as in the case of cervical cytology. Low-quality samples can in fact result in HPV-DNA false negative results due to low viral loads or other pre-analytical issues9.

The inclusion of an appropriate Sample Adequacy Control is critical in the quality assurance of PCR testing, ensuring the presence of sufficient, good quality human nucleic acids in the reaction27,28. The absence of IC in some HPV assays29,30,31,32 may increase false negatives, as issues in sample collection or preanalytical processing can compromise results9,33,34. Currently, a negative IC result indicates an invalid test outcome33, however, detectable IC amplification at high PCR Ct values, within the indicated assays’ manufacturer’s cut-offs, does not always guarantee the presence of sufficient biological material. Quantitative cellularity assessment or appropriately “selected” Ct values can strengthen confidence in HPV negative results27,28. The aim of this study was to determine whether the cellularity of clinical samples can influence HPV positivity rates in cervical cancer screening (CCS) programs.

1. **Materials and Methods**

*2.1 Sample Collection and study population*

In Apulia (Italy) the CCS program transitioned to primary HPV-based algorithm at the end of 2022. This study analyzed 37,592 consecutive cervical samples collected in PreservCyt® specimen collection medium (Hologic), using the Cervex-Brush® (Rovers) collection device, from women (age range 30-64, mean age 47.8±9 years) living in the Bari metropolitan area (Italy), during the first round of HPV-based primary CCS. Samples, collected from January to December 2023 in 45 different women's screening out-patients’ clinics (SC), were analyzed in a centralized laboratory performing both molecular HPV testing and cytological triage within one week from sample collection.

*2.2 HPV-DNA Assay*

Samples were analyzed using the Cobas®4800 HPV Test (Roche): the test targets the L1 gene35 and reports individually HPV16, HPV18 and a pool of 12 additional HR-HPV types. Human β-globin gene is amplified as IC, confirming the qualitative presence of human nucleated cells in the sample and evaluating any potential PCR inhibition.

Testing was performed strictly following the European assay’s “Instructions For Use” (EU IFU), by loading the primary collection tube onto the Cobas®x480 immediately after vortexing and decapping. From 400 μl starting volume, DNA was extracted and purified in 150 μl of elution buffer from which 25 μl was used for PCR and HPV detection.

The clinical cutoff values for each channel (Ct < 40.5, 40, and 40 for HPV16, HPV18, and HPV “other” HR, respectively) were previously established by Rao et al.35 to achieve the required clinical sensitivity and specificity for the detection of CIN2+ lesions. The study35 did not normalize HPV target cutoff values to the internal control relative quantities. The manufacturer IC cut-off value for valid sample testing was set at Ct 40 for β-globin.

*2.3 Liquid-Based Cytology*

LBC was performed using the ThinPrep® 2000 Processor (Hologic), following the manufacturer's instructions. Cytology results were reported according to the Bethesda System guidelines. LBC results were classified as “negative” if cytology was normal (NILM); as "inadequate" or "non-diagnostic" in cases such as low cellularity (< 5000 cells/slide), excessive blood, mucus, or inflammatory cells, presence of non-cellular amorphous material" or "amorphous debris"; any other result was considered positive. Among NILM samples, those showing atrophy were annotated23.

For primary HPV-based screening, LBC was performed for the triage of HPV-positive samples. For the present study, LBC was also performed on a subset of 195 HPV-negative samples with β-globin Ct ≥ 34. HPV-negative women with ASC-US+ or inadequate cytology were recalled for screening.

*2.4 Testing of Random HPV-Negative Samples with OncoPredict HPV QC Module*

The Quality Control (QC) module of the OncoPredict HPV Quantitative Typing (QT) kit (Hiantis SRL, Milan, Italy) was used on the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using 5 µL of the same DNA eluate on 125 random and negative samples extracted and tested by the Cobas®x480 instrument (24 < b-globin Ct < 40). The QC module accurately quantifies the number of human cells in the sample through quantitative CCR5 gene Real-time PCR reaction and assesses potential PCR inhibition through the separate amplification of a synthetic control target36,37. Standard curves for CCR5 quantification were constructed based on the Ct values of four quantitative standards provided by the kit which were run in triplicate.

*2.5 Evaluation of PreservCyt Volume required for LBC Setup*

A total of 110 ThinPrep® containers with IC amplified at varying Ct values were weighed, before and after cytology preparation.

*2.6 Statistical Analysis*

Data were generated by Cobas®4800 Software v2.2.0. The data archived were imported into Cobas®4800 ArchiveViewer3.0 Software. Selected runs were exported into Microsoft Excel files, and data were consolidated into a single Excel file with barcode, HPV HR Ct, HPV-16 Ct, HPV18 Ct and β-globin Ct. Age and data of women screened at different participating screening out-patients clinics (SC) associated with barcodes were exported from the Laboratory Information System. A merged pseudonymized data file was generated by a Visual Basic for Applications (VBA) code (supplementary table I). Data were evaluated for the distribution of β-globin Ct values, age ranges and the performance of SC in relation to HPV-results. Descriptive statistics were computed and Chi-squared (χ²) tests were used. A logistic regression analysis was performed to identify factors associated with the presence of HPV. Both univariate and multivariate models were applied. The dependent variable was "HPV presence" (positive/negative) and independent variables included β-globin Ct values, the performance of SC and women’s age intervals.

β-globin Ct values were grouped into four categories: ≤ 28 (reference), 28-32, 32-34, and 34-40. Performances of SC were categorized into four groups based on b-globin ranges as indicator of sample collection quality: Top Performance SC (TPSC), Good Performance SC (GPSC), Medium Performance SC (MPSC), Poor Performance SC (PPSC), (Supplementary Table II). Women’s age intervals were arbitrarily divided into four groups: 30–39, 40–49, and 50–59, 60–64 years.

Univariate logistic regression was first conducted to evaluate the association of each independent variable with HPV-result and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Variables with a p-value < 0.05 in the univariate analysis were subsequently included in the multivariate model to adjust for potential confounders. Statistical significance was set at p < 0.05. All analyses were performed using STATA17.

*2.8 Ethics Approval and Consent to Participate*

The study received ethical approval from the Local Ethics Committee (Ref. 7438/CEL/2023, addendum 1809/CEL/2024). Women gave written consent.

1. **Results**

*3.1 HPV Positivity rates related to* β-globin Ct values

The results from the first round of HPV-based primary CCS of women living in the Bari metropolitan area, showed an overall HPV positive rate (PR) of 7.7% (2,891/37,592). Among the HPV positive samples, 2,203 samples (76.2%) were identified as positive for “other” HR-HPV types, 419 samples (14.5%) were positive only for HPV16, 123 (4.2%) only for HPV18, 106 (3.7%) for HPV16 and “other” HR types, 34 (1.2%) for HPV18 and “other” HR types, 2 samples (0.06%) were co-positive for HPV16 and HPV18, and 4 (0.14%) samples showed positivity for a combination of HPV16, HPV18, and “other” HR types.

As part of the laboratory quality assurance assessment, HPV-PR were analysed in association with β-globin Ct values. Data showed a marked decrease in HR-HPV PR as the β-globin Ct values increased. Specifically, PR were highest, at 9.7%, for β-globin ≤ 28 Ct, decreasing to 5.8% for the range 28 < Ct ≤ 32, further dropping to 1.4% in the β-globin Ct range ≥ 34 (Table I). From logistic regression β-globin Ct values showed a strong inverse association with HPV PR (χ²=219.8, p<0.001, Table II). In both univariate and multivariate analyses, samples with a range 28 ˂ β-globin ≤ 32 had significantly lower odds of HPV PR compared to the reference group (β-globin ≤ 28), with ORs of 0.58 (95% CI: 0.53–0.63, p<0.001) and 0.63 (95% CI: 0.58–0.68, p < 0.001), respectively. The association was even stronger for samples with 32 ≤ β-globin < 34, which had ORs of 0.38 (95% CI: 0.27–0.57, p < 0.001) and 0.42 (95% CI: 0.30–0.60, p < 0.001). Samples with 34 ≤ β-globin ≤ 40 range had the lowest odds of HPV-PR, with ORs of 0.13 (95% CI: 0.04–0.41, p < 0.001) in the univariate model and 0.14 (95% CI: 0.05–0.45, p = 0.001) in the multivariate model.

*3.2 Age-related sample cellularity and HPV-PR*

DNA yields and HPV-PR were influenced by patients’ age. Table III shows slight but significant differences in mean age across the b-globin Ct groups (unpaired t-test *p* value < 0.0001). Generally, lower Ct values (indicating higher DNA quantity) were associated with women’s younger mean ages. This trend may suggest that sample quality, as reflected by b-globin Ct, could vary with age. However, the differences are not substantial, suggesting that age may not be the only factor influencing sample cellularity. The association between age ranges and HPV-PR was statistically significant (χ² = 273.0, p < 0.001) (Table IV), confirming previously reported data39,40. In the present study, age was a significant factor in HPV-PR: 11.5% of samples were HPV-positive in the 30–39 yrs age group, compared to 4.8% in the 60–64 yrs age group. The 30–39 years age group had the highest odds of HPV-PR, with ORs of 2.55 (95% CI: 2.20–2.96, p < 0.001) in the univariate model and 2.18 (95% CI: 1.87–2.53, p < 0.001) in the multivariate model. The 40–49 years group had ORs of 1.70 (95% CI: 1.46–1.97, p < 0.001) and 1.50 (95% CI: 1.28–1.74, p < 0.001) in univariate and multivariate analyses, respectively. The 50–59 years age group had lower but still significant odds, with ORs of 1.26 (95% CI: 1.08–1.47, p = 0.003) and 1.19 (95% CI: 1.02–1.38, p = 0.025) in the univariate and multivariate models, respectively, (Table II).

*3.3 Sample Collection Variability Across Screening Out-Patients Clinics*

The study also evaluated the variation in sample cellularity across 45 different women's screening out-patients’ clinics (SC) in the metropolitan area of Bari, where samples were collected for HPV testing. β-globin Ct distributions were assessed in samples collected from different SC, and it was observed that in some clinics the samples collected showed a significantly higher cellularity compared to others as indicated by the distribution of β-globin Ct values. Given that patient age did not significantly differ across centers (patient age mean in clinics 47.5±1.7), these variations are likely to reflect differences in performing sample collection by the health professionals operating in the different SC.

Therefore, we sorted SC in 4 groups (Supplementary Table II). As shown in Table V, some SC had a higher percentage of samples in the β-globin ≤ 28 Ct range as compared to others which had a greater proportion of samples with higher intervals of β-globin Ct values (28 < β-globin ≤ 32, 32< β-globin ≤ 34 and 34 < β-globin ≤ 40). The association between SC performance and β-globin Ct values was statistically significant (χ²= 859.8, p < 0.001), (Supplementary Table III).

The correlation between performance of SC and HPV PR was assessed with a regression model (Table II). GPSC showed reduced odds of HPV-PR compared to TPSC in both univariate (OR=0.77, 95% CI: 0.64–0.93, p = 0.006) and multivariate analyses (OR=0.81, 95% CI: 0.67–0.98, p = 0.028). MPSC had ORs of 0.69 (95% CI: 0.58–0.82, p < 0.001) and 0.76 (95% CI: 0.63–0.91, p= 0.003) in univariate and multivariate models, respectively. PPSC were significantly associated with reduced odds of HPV PR only in the univariate analysis (OR=0.79, 95% CI: 0.66–0.95, p = 0.011), but not in the multivariate model (OR=0.92, 95% CI: 0.77–1.11, p = 0.402) possibly due to the epidemiology of the population living in the suburbs.

This variability across centers underscores the importance of evaluating sample cellularity when performing HPV-based primary screening, as variations in operators’ dependent sample collection from a mucosal surface can influence samples’ cellular yield and directly affect the reliability of HPV testing.

*3.4 Correlation Between Sample Cellularity and β-Globin Ct Values*

The study further evaluated the relationship between β-globin Ct values and a quantitative sample cellularity assessment. The results of testing 125 HPV-negative random samples with OncoPredict HPV QC module36,37 underlined that higher β-globin Ct values are due to a lower initial amount of human DNA or cells in the sample. Moreover, the exogenous PCR inhibition control included in the OncoPredict HPV QC module did not show any evidence of inhibition of the reaction among tested samples, which could account for the higher β-globin Ct values. This is consistent with the hypothesis that β-globin late amplification using Cobas®4800 correlates with samples with a lower cellular content, rather than resulting from PCR inhibition issues.

Graph in Figure 1 plots the number of cells per mL, as a function of β-globin Ct values, calculated by CCR5 standard curve, in 125 samples with b-globin Ct ranging from 25 to 40.

Quantification showed cellularity in the LBC sample and in Cobas®4800 PCR reaction mix respectively ofabout 2x10^5 cells/mL and 1,5x10^4 cells/rxn at a β-globin Ct of 28, 6x10^4 cells/mL and 4x10^3 cells/rxn at a β-globin Ct of 32, 2,5x10^4 cells/mL and 1,5x10^3 cells/rxn at a β-globin Ct of 34, showing a significant drop in nucleated cells with increasing Ct. By a Ct of 39, the cellularity reaches the lowest measurable levels, close to 10 cells/mL and 1 equivalent genome/rxn, indicating that very few nucleated cells are present in the tested sample, but HPV DNA test results for CCS are reported by Cobas®4800 software as “valid” and “HPV-negative”. Moreover, IC Ct value reflects the overall number of nucleated cells in the sample, including inflammatory cells and not just the squamous epithelial cells (the primary target for HPV infection41), which means that Ct values represent total nucleated cell content rather than solely the target cell type for HPV.

*3.5 Cytology and Sample Adequacy of samples with late β-globin amplification*

In a subset of 195 samples with β-globin ≥ 34 Ct, LBC slides were prepared to assess sample adequacy. Cytology revealed that 19% of these samples (37/195) exhibited inadequate cellularity, with less than 5,000 cells per slide, 8% were positive for abnormal cytology (7 ASC-US, 7 L-SIL, 1 ASC-H, 1 H-SIL), and 65% showed normal cytology with marked atrophy, suggesting that atrophic changes in the cervical epithelium may contribute to the reduced cellular yield. The remaining samples (8%) were NILMs but not atrophic.

Within this subset of samples, those found to be HPV-negative with inadequate cytology or positive for ≥ ASC-US were reported as inadequate, and women were invited to repeat the test. Among 16 samples with positive cytology, 4 were HPV positive at the recall visit, 4 missed the recall, 8 were HPV negative (2 with b-globin > 32 Ct). Among inadequate samples (N = 37), 12 women missed the recall, 3 were inadequate again and, 22 were HPV-negative (14 with b-globin >32 Ct), (Supplementary Table I). These further emphasize the need for robust sample collection techniques to avoid missing clinically significant infections and need of recalling systems, (note that 16/53 (30.2%) missed the recall visit).

Moreover, we observed that among samples with β-globin late amplification, very little PreservCyt remained in the ThinPrep® vial after LBC preparation. To investigate this further and to assess potential differences in residual volume, we measured the amount of PreservCyt in 110 ThinPrep® vials, with samples at varying β-globin Ct values, before and after cytology preparation. As shown in Figure 2, there is a strong correlation between the volume aspirated for cytology preparation and the β-globin Ct. This confirms that when the IC Ct is higher, indicating a lower concentration of cells/mL in the sample, the instrument requires to aspirate more liquid to create an adequate LBC slide.

1. **Discussion**

HPV DNA tests validated for CCS, adhering to the Meijer20 and VALGENT21 criteria, focus on achieving clinical sensitivity and specificity for the detection of CIN2+ lesions by HR-HPV positivity. This approach is essential to maintain consistent clinical specificity, as detecting low viral loads typically associated with transient infections with HR-HPV, is usually associated with clinically irrelevant infections which could potentially lead to overtreatment. The findings of this study however demonstrate that the performance of HPV DNA molecular testing is also influenced by the adequacy of the sample starting material. In particular, in a subset of 195 investigated samples with a b-globin Ct ≥ 34, the study findings demonstrated that 33% (4/12) of HPV-negative samples and positive cytology, were found to be HPV-positive at the subsequent recall.

The study findings suggest that, to ensure reliable and accurate COBAS®4800 HPV test results, sample cellularity should meet a minimum threshold of 1,5x10^4 cells/rxn or 2x10^5 cells/mL in the original sample, giving rise to a COBAS®4800 b-globin IC ≤ 28 Ct. In our HPV-primary screening setting, only 50% of all cervical samples achieved amplification within this threshold, while in other settings, such as in Norway38, all LBC samples run on Cobas®4800 were reported as having an IC Ct value ≤ 28.

According to the evaluation conducted in the present study, LBC samples containing not less than 2,5x10^4 cells/mL or 1,5x10^3 cells/PCR rxn (b-globin ≤ 34ct) could be considered acceptable (Table II). Samples with fewer than 2,5x10^4 cells/mL (b-globin > 34ct) should be deemed inadequate for diagnostic purposes and flagged for recollection, since our results demonstrate that low cellularity can significantly affect the performance of COBAS®4800 HPV tests, undermining their ability to accurately detect clinically relevant lesions (82% of probability to miss a positive diagnosis compared to a sample with at least 2x10^5 cells/mL, Table II).

Ensuring sufficient cellular content in samples is therefore critical to maintain the reliability and effectiveness of screening programs, reducing the risk of false-negative results.

Moreover, it is important to note that as different HPV assays require distinct volumes for DNA extraction, elution and amplification, a specific sample quality assessment study is required to determine the acceptable cellular content for each assay.

In particular, low cellularity in cervical samples could be due to several factors, such as:

1. Non-adherence to Standard Operating Procedures42,43: inadequate sample collection, such as improper rotation of the brush, which can result in insufficient cellular material being transferred to the sample resuspension volume44.

2. Pre-analytical Processing Issues:

- If the brush, used for sample collection, is not adequately and quickly rinsed in the solution through proper bristle opening and mixing42,43, many cells may remain on the device, leading to lower sample’s cellularity;

- Inadequate vortexing of the vial before testing may cause cells to remain clumped or settle at the bottom of the tube, rather than being evenly suspended;

- A delay between vortexing and sample processing can exacerbate this problem, as cells may precipitate before the test system aspirates the required volume from the meniscus of the primary vial after its loading on board of the assay’s liquid handler.

3. Test IC target: different assays have different target genes, which may be present in multiple and/or variable number of copies within human cells, influencing sample adequacy assessment and cellular thresholds.

It is possible to improve CCS by adequate sample collection and reliable HPV- primary testing by addressing factors such as improved training of health operators on the procedure of sample collection and on performing pre-analytical and analytical quality controls during sample processing and testing.

Moreover, among the causes of poor sample cellularity, patient’s age and hormonal status must be taken in consideration. In older or postmenopausal and postpartum patients, the cervix may exhibit mucosal atrophic changes. Atrophy often leads to a reduced cellular yield and the formation of cellular clumps, which can negatively impact the quality and quantity of material available for analysis.

Despite these challenges, LBC systems, like ThinPrep®2000, partially mitigate these issues by aspirating a larger volume of medium (ranging from 2–18 mL depending on cellular concentration) and transferring cells onto a slide for optimal microscopic evaluation. This flexibility ensures the presence of a sufficient number of cells for cytological examination. Additionally, LBC protocols include established quality assurance benchmarks based on the number of cells, which provide a reference for sample adequacy and enhance reliability in cytological assessment as previously discussed23,24,25,26.

In contrast, HPV testing lacks similar quality assurance benchmarks for cellularity.

Moreover, standard workflows use a fixed starting sample volume, typically 0.2–0.4 mL of the resuspended cervical sample, used for DNA extraction, irrespective of the starting sample cellularity.

Molecular testing is usually highly sensitive, requiring only a minimal amount of target DNA, but the reduced cellularity in samples, such as for instance from post-menopausal women, can lower DNA yields, as reflected in higher β-globin Ct values. The literature highlights an elevated risk of CC in older women ASCUS and HPV-negative45,46, underscoring the age-related decline in test sensitivity due to sampling challenges. In spite of CC being commonly diagnosed in younger women, due to routine screening, the median age at diagnosis remains 50 years. Women over 65 account for over 20% of new CC cases and 37% of CC deaths in the United States46, underlining the need for improved strategies and quality assurance to prevent CC in older populations.

The results of the present study indicate the importance in establishing clear references and benchmarks for sample adequacy and cellularity assessment within the validation criteria of CCS assays. These standards would help to ensure consistent and accurate test performance across diverse clinical settings and in women of different age groups. Without such references, the risk of false-negative results due to insufficient cellular material remains a challenge, potentially undermining the goals of early detection and prevention of CC.

In screening programs based on self-collected samples, adequate sample cellularity will play an important quality assurance aspect to be considered, which may also increase women’s confidence in self-collection and enhance acceptability for self-sampling in screening47. Studies on self-collection have shown that some women prefer clinician-collected samples due to concerns about the confidence in performing their own sample collection, highlighting the need for confidence-building measures and quality assurance47.

In conclusion, shared guidelines for the definition of minimum sample cellularity for HPV molecular testing through future clinical studies, allowing to establish optimal IC cut-off values across different diagnostic systems, are needed in order to improve confidence in HPV-negative results in CCS. For COBAS®4800, samples with fewer than 2,5x10^4 cells/mL (b-globin > 34ct) should be deemed as inadequate.

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| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **β-globin (Ct values)** | | | | |  |  |
| **HPV** | **β-globin ≤ 28**  N  **(%)** | **28 < β-globin ≤ 32**  N  **(%)** | | **32 < β-globin ≤ 34**  N  **(%)** | **34 < β-globin ≤ 40**  N  **(%)** | **Total**  N  **(%)** | **Chi squared** χ²  **(p-value)** |
| Negative | 16,981 | | 16.7 | 839 | 211 | 34,701 |  |
| **90.3** | | **94.2** | **96.1** | **98.6** | **92.3** | 219.9  ˂0.001 |
| Positive | 1,820 | | 1,034 | 34 | 3 | 2,891 |
| **9.7** | | **5.8** | **3.9** | **1.4** | **7.7** |  |
| **Total** | 18,801 | | 17,704 | 873 | 214 | 37,592 |  |
| **100** | | **100** | **100** | **100** | **100** |  |

**Table I.** *HPV test results sorted by b-globin Ct-value. Numbers and percentages of positive and negative HPV cases for each Ct group of β-globin amplification.*

| **Variables** | **Univariate model**  **(OR [IC 95%];p-value)** | **Multivariate model**  **(OR [IC 95%]; p-value)** |
| --- | --- | --- |
| **β-globin**  **(Ct values)** |  |  |
|  |  |  |
| β-globin ≤ 28 | reference | reference |
| 28˂ β-globin ≤ 32 | 0.58 [0.53-0.63]; <0.001 | 0.63 [0.58-0.68]; <0.001 |
| 32 ˂ β-globin ≤ 34 | 0.38 [0.27-0.53]; <0.001 | 0.42 [0.30-0.60]; <0.001 |
| 34 ˂ β-globin ≤ 40 | 0.13 [0.04-0.41]; <0.001 | 0.14 [0.05-0.45]; 0.001 |
| **Age range** |  |  |
|  |  |  |
| 30-39 years | 2.55 [2.20-2.96]; <0.001 | 2.18 [1.87-2.53]; <0.001 |
| 40-49 years | 1.70 [1.46-1.97]; <0.001 | 1.50 [1.28-1.74]; <0.001 |
| 50-59 years | 1.26 [1.08-1.47]; 0.003 | 1.19 [1.02-1.38]; 0.03 |
| 60-64 years | reference | reference |
| **Sampling Centres (SC)** |  |  |
| TPSC | reference | reference |
| GPSC | 0.77 [0.64-0.93]; 0.006 | 0.81 [0.67-0.98]; 0.028 |
| MPSC | 0.69 [0.58-0.82]; <0.001 | 0.76 [0.63-0.91]; 0.003 |
| PPSC | 0.79 [0.66-0.95]; 0.011 | 0.92 [0.77-1.11]; 0.42 |

**Table II.** *Association between**HPV Positivity Rate (PR) and respectively β-globin Ct values, age range and screening out-patients’ clinics (SC) grouped by estimated performance. Results of logistic regression analyses (univariate and multivariate) are reported as Odds Ratio and IC95%. Top (TPSC), Good (GPSC), Medium (MPSC), Poor Performance SC (PPSC).*

| **Cellularity** | **Age (Mean)** | **StDev** | **N** |
| --- | --- | --- | --- |
| **β-globin ≤ 28ct** |  |  |  |
| Positive | 43.7 | 9.1 | 1820 |
| Negative | 46.1 | 9.1 | 16981 |
| Total | 45.8 | 9.1 | 18801 |
| **28 ˂ β-globin ≤ 32** |  |  |  |
| Positive | 47.0 | 9.7 | 1034 |
| Negative | 49.8 | 9.2 | 16670 |
| Total | 49.7 | 9.2 | 17704 |
| **32 ˂ β-globin ≤ 34** |  |  |  |
| Positive | 48.8 | 10 | 34 |
| Negative | 52.4 | 8.7 | 839 |
| Total | 52.3 | 8.6 | 873 |
| **34 ˂ β-globin ≤ 40** |  |  |  |
| Positive | 44.3 | 14.6 | 3 |
| Negative | 51.9 | 8.9 | 211 |
| Total | 51.9 | 8.9 | 214 |
| **Total** |  |  |  |
| Positive | 44.9 | 9.6 | 2891 |
| Negative | 48.1 | 9.3 | 34701 |
| Grand Total | 47.8 | 9.4 | 37592 |

**Table III.** *Age ± Standard Deviation (StDev) in each b-globin Ct Range. N = sample count in each group. Age unpaired T-Test p value <0.0001 vs β-globin ≤ 28ct group.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Age Range** | | | |  |  |
| **HPV** | **30-39**  N  **(%)** | **40-49**  N  **(%)** | **50-59**  N  **(%)** | **60-64**  N  **(%)** | **Total**  N  **(%)** | **Chi squared** χ²  **(p-value)** |
| negative | 7,459 | 10,996 | 11,790 | 4,456 | 34,701 |  |
| **88.54** | **92.08** | **93.99** | **95.17** | **92.3** | 273.0  ˂0.001 |
| positive | 965 | 946 | 754 | 226 | 2,891 |
| **11.5** | **7.9** | **6.01** | **4.8** | **7.7** |  |
| **Total** | 17,701 | 18,728 | 931 | 232 | 37,592 |  |
| **100** | **100** | **100** | **100** | **100** |  |

**Table IV**. *HPV test results sorted by Age range. Numbers and percentages of positive and negative cases for each age range; the p-value from the chi-square test for each group is compared to the total group.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Performance of Sampling Centres (SC)** | | | |  |  |
| **β-globin (Ct values)** | **TPSC**  N  **(%)** | **GPSC**  N  **(%)** | **MPSC**  N  **(%)** | **PPSC**  N  **(%)** | **Total**  N  **(%)** | **Chi squared** χ²  **(p-value)** |
| β-globin ≤ 28 | 857 | 4,036 | 9,241 | 4,667 | 18,801 |  |
| **57.4** | **55.4** | **51.9** | **42.4** | **50** | 881.6  <0.001 |
| 28 < β-globin ≤ 32 | 609 | 3,221 | 8,192 | 5,682 | 17,704 |
| **40.8** | **44.2** | **46** | **51.6** | **47.1** |
| 32 < β-globin ≤ 34 | 20 | 23 | 293 | 537 | 873 |
| **1.8** | **0.4** | **1.6** | **4.8** | **2.3** |
| 34 < β-globin < 40 | 7 | 0 | 74 | 133 | 214 |
| **0.5** | **0.0** | **0.4** | **1.2** | **0.6** |  |
| **Total** | 1,493 | 7,280 | 17,800 | 11,019 | 37,592 |  |
| 100 | 100 | 100 | 100 | 100 |  |

**Table V.** *DNA yields grouped by b-globin Ct ranges sorted by the performance of* *screening out-patients’ clinics (SC) involved in the study; the p-value from the chi-square test for each group is compared to the total group. SC were sorted in 4 groups on the basis of cellularity of the samples collected: Top (TPSC), Good (GPSC), Medium (MPSC), Poor Performance SC (PPSC).*

**Figure 1.***Sample cellularity/mL vs b-globin Ct by cells/mL assessed by CCR5-qPCR assay.*

**Figure 2.** *PreservCyt (mL) aspirated for LBC setup by ThinPrep® 2000 vs PCR b-globin Ct.*

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