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Bachelor's thesis

Verification of the CINtec PLUS Cytology Test (Roche): A Dual-Stain Technology for Epithelial Cells from Cervical Smears in the Context of Cervical Cancer Diagnosis

Institut de Pathologie et de Génétique
Av. Georges lemaître 25, 6041 Charleroi
Malaisse Jérémy & Chavee Sigrid
De Munck Jolien (Crabbe Annelies)

Solenn Bosmans

Departement GDT

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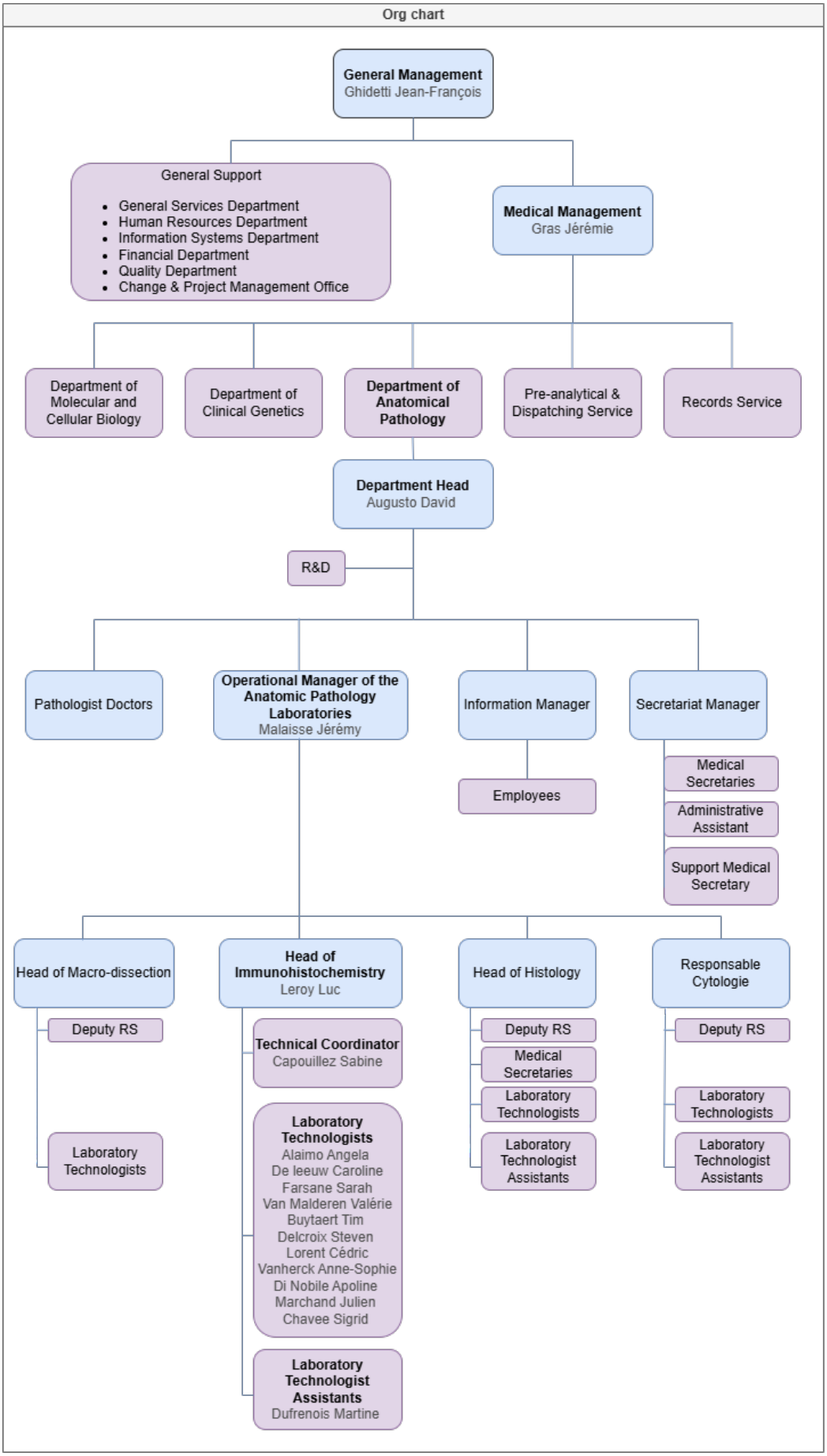
Organizational chart



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List of abbreviations

ASC-US	atypical squamous cells of undetermined significance
CDK	cyclin-dependent kinase
CINx	cervical intraepithelial neoplasia grade x
DAB	3,3’-Diaminobenzidine
DNA	deoxyribonucleic acid
HPV	human papillomavirus
hr-HPV	high-risk human papillomavirus
HRP	horseradish peroxidase
HSIL	high-grade squamous intraepithelial lesions
ICC	immunocytochemistry
IHC	immunohistochemistry
IPG	Institute de Pathologie et de Genetique
IVDR	In Vitro Diagnostic Regulation
LBC	liquid-based cytology
LSIL	low-grade squamous intraepithelial lesions
NILM	negative for intraepithelial lesions or malignancy
Pap	Papanicolaou
pRb	retinoblastoma proteins

Verification of the CINtec PLUS Cytology Test (Roche)

Bosmans Solenn[1]
[1]Institut de Pathologie et de Génétique, Gosselies, Belgium

De Munck Jolien

Abstract (English)

Cervical cancer remains a significant public health problem in Belgium, with high numbers of morbidity and mortality. Current screening methods, such as cytology (Papanicolaou) and molecular human papillomavirus testing, often produce false positives. This results in unnecessary follow-up procedures and increased healthcare costs.

To meet these challenges, the CINtec PLUS Cytology test (Roche) will be introduced at the Institut de Pathologie et de Génétique (IPG). This test is a dual-stain immunocytochemical assay that detects the co-expression of p16 and Ki-67 in cervical epithelial cells, allowing for a more specific identification of cells undergoing abnormal proliferation.

Before its implementation, several verification steps were required. As a diagnostic assay, CINtec PLUS underwent a type 1 verification. Trueness, repeatability, and reproducibility were assessed, as recommended by the practical guidelines.

Trueness was evaluated by comparing CINtec PLUS and Pap cytology results on the same samples. The results showed complete concordance between the two methods. For repeatability and reproducibility testing, all slides from the same case consistently showed the same staining result. These findings demonstrate that CINtec PLUS results are not influenced by procedural or analytical variability.

Additionally, a robustness testing was done to evaluate interpretation reliability outside the recommended staining timeframe of seven days. Robustness testing confirmed the accurate interpretation of slides up to thirty days after staining.

A hematoxylin counterstaining optimization was also conducted to streamline lab workflow. Slides stained with the manufacturer's counterstaining (Hematoxylin I) and a proposed alternative (Hematoxylin II) were compared. The alternative hematoxylin showed reduced background staining and improved visibility of superficial cells. All tested cases showed consistent results, validating the use of this alternative.

In conclusion, the CINtec PLUS Cytology test demonstrated strong technical performance across all evaluated parameters. Its integration into routine at IPG is feasible and supports more accurate detection of cervical lesions. This can reduce unnecessary follow-ups and streamline patient management by helping pathologists focus on patients who truly need follow-up or treatment.

Keywords: Anatomopathology, cervical cancer, cervical smear, CINtec PLUS Cytology, human papillomavirus, immunocytochemistry, Ki-67, liquid-based cytology, p16, verification.

Verification of the CINtec PLUS Cytology Test (Roche)

Bosmans Solenn[1]
[1]Institut de Pathologie et de Génétique, Gosselies, Belgium

De Munck Jolien

Abstract (Dutch)

Baarmoederhalskanker blijft een belangrijk volksgezondheidsprobleem in België, met hoge cijfers wat betreft morbiditeit en mortaliteit. De huidige screeningsmethoden, zoals cytologie (Papanicolaou) en moleculaire humaan papillomavirus testen, geven vaak vals-positieve resultaten. Dit leidt tot onnodige vervolgonderzoeken en verhoogde zorgkosten.

Om deze uitdagingen aan te pakken, wordt de CINtec PLUS Cytologietest (Roche) geïntroduceerd in het Institut de Pathologie et de Génétique (IPG). Deze test is een dubbele immunocytochemische kleuring die de co-expressie van p16 en Ki-67 detecteert in cervicale epitheelcellen, waardoor specifiekere identificatie mogelijk is van cellen met abnormale proliferatie.

Voor de implementatie waren verschillende verificatiestappen vereist. Als diagnostische test onderging CINtec PLUS een verificatie van type 1. Hierbij werden juistheid, herhaalbaarheid en reproduceerbaarheid beoordeeld, zoals aanbevolen in de praktische richtlijnen.

De juistheid werd geëvalueerd door de resultaten van CINtec PLUS en Papanicolaou-cytologie op dezelfde stalen te vergelijken. De resultaten toonden volledige overeenstemming tussen beide methoden. Voor de testen van herhaalbaarheid en reproduceerbaarheid vertoonden alle glaasjes van eenzelfde staal consequent hetzelfde kleuringsresultaat. Deze bevindingen tonen aan dat de resultaten van CINtec PLUS niet worden beïnvloed door procedurele of analytische variatie.

Daarnaast werd een robuustheidstest uitgevoerd om de betrouwbaarheid van de interpretatie buiten het aanbevolen kleuringsvenster van zeven dagen te evalueren. De robuustheidstest bevestigde dat correcte interpretatie van glaasjes mogelijk bleef tot dertig dagen na kleuring.

Ook werd een optimalisatie van de hematoxyline tegenkleuring uitgevoerd om de workflow in het labo te verbeteren. Glaasjes tegengekleurd met het reagens aanbevolen door de fabrikant (Hematoxyline I) werden vergeleken met een voorgesteld alternatief (Hematoxyline II). De alternatieve hematoxyline toonde minder achtergrondkleuring en een betere zichtbaarheid van oppervlakkige cellen. Alle geteste gevallen gaven consistente resultaten, wat het gebruik van dit alternatief valideert.

Samengevat toonde de CINtec PLUS Cytologietest een sterke technische prestatie op alle geëvalueerde parameters. De integratie ervan in de routine bij IPG is haalbaar en ondersteunt een nauwkeurigere detectie van cervicale letsels. Dit kan onnodige vervolgonderzoeken verminderen en het patiëntbeheer stroomlijnen door pathologen te helpen zich te richten op patiënten die daadwerkelijk verdere opvolging of behandeling nodig hebben.

Trefwoorden: Anatomopathologie, cervixkanker, baarmoederhalskanker, uitstrijkje, CINtec PLUS Cytologie, humaan papillomavirus, immunocytochemie, Ki-67, l, p16, liquid-based cytology, verrificatie.

I. Introduction

The Institut de Pathologie et de Génétique (IPG) is the largest laboratory in Wallonia. Their role is to contribute to the diagnosis, monitoring, and treatment of diseases by analyzing biological samples. To ensure the highest standards of quality and reliability in medical biology analyses, IPG is accredited by the Belgian Accreditation Body (BELAC), in accordance with the ISO 15189 standards that specifies the quality and competence requirements applicable to medical biology laboratories. This accreditation guarantees that the institute follows the practical guidelines to implement a quality system in anatomical pathology laboratories, meeting both ISO 15189 requirements and BELAC expectations. Furthermore, IPG ensures full compliance with the European *In Vitro* Diagnostic Regulation (IVDR), to reinforce its commitment to excellence in patient care (*Belac*, n.d.,; *ISO 15189:2012*, 2014).

Among the various diseases, cervical cancer is diagnosed at IPG. The institute plays a crucial role in the early detection and monitoring of this disease through advanced cytological and molecular testing. It is organized into two departments: anatomopathology and molecular biology. The anatomopathology department includes several services, such as immunology, histology, and cytology. The cytology service is primarily focused on cervical cancer screening, which represents 90% of the tests performed. Cervical cancer is most often linked to a persistent human papillomavirus (HPV) infection. Although the high prevalence of these infections, especially among younger women, cytological and molecular tests often lead to unnecessary follow-up procedures and overtreatment. While these screening methods and follow-ups are essential for early detection, their low specificity can result in undue anxiety, invasive diagnostic procedures, and unnecessary medical interventions for patients, of whom would have naturally cleared the infection without any other issue (*Cervical Cancer Screening*, 2024; *HPV And Cancer*, 2023).

This context shows the importance of a test that identifies cells with a higher risk of developing cancer more specifically. The CINtec PLUS test offers a more specific way to identify high-risk cases and may reduce unnecessary follow-ups and overtreatment. In recent years, patients and clinicians have occasionally requested this test at IPG, but they were redirected to other laboratories that offer the service. In 2016, a training session on this test took place at IPG, but it wasn't implemented at that time due to the lack of reimbursement and the relatively high cost per test. Given the socio-economic context of Charleroi, this cost could have made it unaffordable for many patients and possibly discouraged them from opting for this test. Additionally, the disadvantages outweighed their potential benefits.

Recently, however, the increasing demand for more advanced screening tools and the constant need to improve cervical cancer detection have led IPG to reconsider the integration of the CINtec PLUS Cytology test into their services. Before fully adopting this test, several verification steps are essential to ensure quality standards and deliver reliable results. This bachelor thesis aims to verify the technical performance of the CINtec PLUS Cytology test before introducing it at IPG.

II. Literature

Human papillomavirus (HPV) and cervical cancer

Cervical cancer remains a major public health problem in Belgium, with 641 new cases diagnosed in 2022 and 231 associated deaths. In 95% of cases, this type of cancer is linked to a persistent infection with oncogenic types of human papillomavirus (HPV), a virus that is primarily sexually transmitted (*Dépisatge Du Cancer Du Col De L'utérus - CCR*, n.d.; Simoens, n.d.).

HPV infections are very common, and most people will be infected at least once in their lifetime. HPV viruses can be classified into mucosal and cutaneous types and further categorized as high-risk or low-risk. About 90% of the infections with HPVs are low-risk, transient and are easily cleared by the immune system. On the other hand, high-risk HPVs (hr-HPVs) are more likely to evolve into a malignant form. When not cleared by the immune system, hr-HPVs DNA will integrate into the host genome, leading to expression of oncogenes and promoting malignant transformation. The produced oncoproteins cause continuous proliferation, deregulation of cell cycle control, suppression of apoptosis and other oncogenic processes (*Basic Information About HPV and Cancer*, 2024; Longworth & Laimins, 2004; MüNger et al., 2004; Porter & Marra, 2022; Roche, 2016).

Cervical cancer screening in Belgium

In Belgium, HPV screening depends on age and follows national guidelines to meet both efficacy and cost-effectiveness. For women under 30, a Papanicolaou (Pap) or cytological test is recommended as the primary screening method. Cells gently collected from the surface of the cervix and surrounding area are placed on a glass slide, alcohol-fixed, and stained to highlight nuclear details, like chromatin patterns, membrane irregularities and intranuclear invaginations. The morphology of these stained cells are then analyzed under a microscope to identify signs of cervical cancer or cellular abnormalities that could potentially develop into malignancy, such as atypical squamous cells (ASCs), squamous intraepithelial lesions (SILs) and squamous cell carcinoma (Figure 1) (*Definition of Papanicolaou Test - NCI Dictionary of Cancer Terms*, n.d.; *Détection Du Cancer Du Col De L'utérus En Belgique : Introduction Du Test HPV Comme Test De Dépistage Primaire | INAMI*, n.d.; Munger, 2015; Simoens, n.d.).

ASCs include atypical squamous cells of uncertain significance (ASC-US), which are cells that appear abnormal, but it is unclear whether the changes are caused by an HPV infection. Further testing, such as an HPV or molecular test, may be required. Another category is atypical squamous cells where a high-grade squamous intraepithelial lesion cannot be excluded (ASC-H). These cells also appear abnormal but have a higher likelihood of progressing to pre-cancerous cells and may require additional testing and possibly treatment.

SILs are divided into two categories: low-grade squamous intraepithelial lesions (LSIL) that consist of mildly abnormal cells defined as mild dysplasia or cervical intraepithelial neoplasia grade 1 (CIN1) and high-grade squamous intraepithelial lesions (HSIL), in which the cells appear severely abnormal. HSILs are less likely than LSILs to be cleared by the immune system without treatment and can also be referred to as CIN2 or CIN3 (*ASC-US Vs. ASC-H? What Is the Difference?» Incyte Diagnostics*, n.d.; *PAP Smear: Test, Purpose, and Results*, n.d.). The different types of abnormal cells mentioned are illustrated in Figure 1. Additionally, Figure 2 provides an overview of cervical cancer progression and demonstrates how persistent hr-HPV infections can lead to the progression of CIN1 or LSIL to invasive cancer.

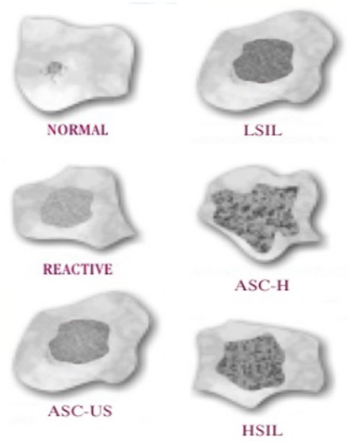

Figure 1: Comparison of cervical epithelial cells: normal, Low-grade Squamous Intraepithelial Lesion (LSIL), reactive, Atypical Squamous Cells of Undetermined Significance (ASC-US), atypical squamous cells where a high-grade squamous intraepithelial lesion cannot be excluded (ASC-H), and High-grade Squamous Intraepithelial Lesion (HSIL). Figure from: ASC-US Vs. ASC-H? What Is the Difference?» Incyte Diagnostics, n.d.).

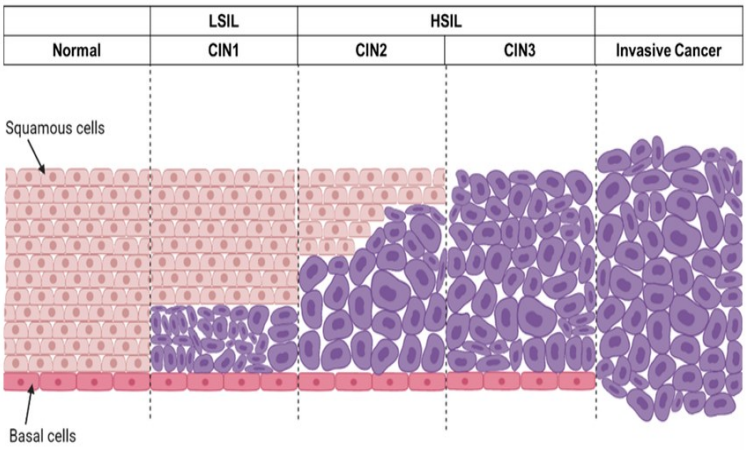

Figure 2: Stepwise progression of squamous lesions: from normal to invasive cancer. This figure illustrate the development of squamous lesions, beginning with normal squamous intraepithelial cells, followed by Low-grade Squamous Intraepithelial Lesion (LSIL, CIN1), and progressing to High-grade Squamous Intraepithelial Lesion (HSIL, CIN2 or CIN3) leading to invasive squamous cell carcinoma. Figure from: An Overview of the Progression of Cervical Cancer. Squamous Cells Are. . ., n.d.

In addition to the aforementioned categories of abnormal cells, Negative for Intraepithelial Lesion or Malignancy (NILM) is also a possible outcome. This indicates that no abnormalities were found in the sample and the cells appear normal, with no signs of intraepithelial lesions or malignancy. This result is considered a negative screening outcome, implying that no further investigation or treatment is necessary at this stage (MyPathologyReport.ca, 2023).

For women between 29 and 65 years old, primary HPV testing is recommended as the preferred method for cervical cancer screening. This molecular test detects HPV DNA in a cervical smear specifically targeting types 16 and 18, which are most strongly associated with cervical cancer. In women above 65, routine screening is typically not recommended. In some cases, co-testing, which combines cytology and HPV testing, may still be considered for women with specific risk factors (*Détection Du Cancer Du Col De L'utérus En Belgique : Introduction Du Test HPV Comme Test De Dépistage Primaire | INAMI*, n.d.; Malloy et al., 2000; Simoens, n.d.).

Challenges in cervical screening

Given the high prevalence of transient HPV infections, especially among younger women, cytology and HPV testing often shows false-positive results, leading to unnecessary follow-up procedures and overtreatment, such as colposcopy or repeated HPV and/or Pap tests. This is because transient infections with minor cellular changes, which are unlikely to progress into cancer, may still lead to positive test results. In younger women, a positive cytology result typically prompts an additional HPV test to identify hr-HPV types. However, it's important to note that only persistent hr-HPV infections, lasting several years, carry the potential to alter cervical cells and result in precancerous lesions and even then, progression to cancer is not inevitable (*Cervical Cancer Screening*, 2024; *HPV And Cancer*, 2023).

Reimbursement for cervical cancer screening in Belgium

In terms of reimbursement, women under 25 are not eligible for cervical screening reimbursement, as routine testing is not recommended in this age group due to the high prevalence of transient HPV infections. For women aged 25 to 29, a cytological test is recommended which are reimbursed every three years. Starting from January 1, 2025, women aged 30 to 64 will continue to benefit from free screening, but the interval between tests will increase from three to five years. For women over 65, screening is only recommended and reimbursed upon a doctor's request and if no tests have been reimbursed in the past 10 years (*Détection Du Cancer Du Col De L'utérus En Belgique : Introduction Du Test HPV Comme Test De Dépistage Primaire | INAMI*, n.d.; Simoens, n.d.).

Principle of the CINtec PLUS Cytology Test (Roche)

Given the challenges in screening for cervical cancer due to the high prevalence of transient HPV infections, the CINtec PLUS Cytology test offers a more specific method for diagnosis. This liquid-based cytology (LBC) test is a qualitative immunocytochemistry (ICC) assay developed for use on the Benchmark Ultra (Roche). This test uses dual-staining technology to simultaneously detect the biomarkers p16 and Ki-67 in cervical cells. The co-expression of these two markers is a reliable indicator of a HPV infection, which can lead to the development of precancerous or cancerous lesions. This dual staining specifically identifies deregulated cells that have lost normal growth control, allowing them to growth uncontrollably, a sign of malignant transformation. When such cells are detected, colposcopy becomes essential for further investigation and clinical management, such as biopsy, follow-up or treatment if necessary. This targeted approach minimizes unnecessary procedures, improving both patient care and comfort (*CINtec® PLUS Cytology Kit,* n.d.; Hammer et al., 2020; Roche, 2016; Roche 2024; Sano et al., 1998).

To better understand why this dual-marker test is so effective, it's important to explore the role of the specific biomarkers used, p16 and Ki-67. In normal cells, retinoblastoma (pRb), a tumor suppressor protein, binds to the transcription factor E2F, which blocks the transcription of genes responsible for cell cycle proliferation, leading to cell cycle arrest. Cyclin-dependent kinases (CDK) are responsible for phosphorylating pRb. In a normal context, this phosphorylation leads to the dissociation of pRb from E2F, allowing E2F to activate genes required for cell cycle progression and proliferation (Roche, 2016; Roche, 2024).

After mitosis, p16, which is found both in the cytoplasm and the nucleus during certain phases of the cell cycle, is expressed. This protein inhibits the CDKs, preventing them from phosphorylating pRb. As a result, pRb remains bound to E2F, which blocks the activation of genes needed for cell cycle progression, leading to cell cycle arrest (Figure 3). Therefore, p16 expression has an antiproliferative effect, acting as a marker of cell cycle arrest, especially during the G0 phase (Park & Soslow, 2020; Roche, 2016; Sano et al., 1998).

Ki-67, on the other hand, is a nuclear protein expressed in proliferating cells (during all phases except G0) and is thus a marker of active cell division (Albagli & Pelczar, 2019; *KI-67 REGULATES GLOBAL GENE EXPRESSION AND PROMOTES SEQUENTIAL STAGES OF CARCINOGENESIS – IGMM*, n.d.; Roche, 2016).

Because of their opposing roles in the cell cycle, p16 being associated with cell cycle arrest and Ki-67 with proliferation, these markers are not co-expressed in the same cell under non-pathological conditions.

However, when an oncogenic HPV infects a cell, the normal regulation of the cell cycle is disrupted. Hr-HPV leads to the expression of E7, which binds to pRb, preventing the formation of the E2F:pRb complex. This results in uncontrolled cell proliferation and the overexpression of p16 (Figure 4). Consequently, this uncontrolled cell proliferation induced by the infection also leads to an overexpression of Ki-67 (Bergeron & Laboratoire Pasteur-Cerba, 2021; *KI-67 REGULATES GLOBAL GENE EXPRESSION AND PROMOTES SEQUENTIAL STAGES OF CARCINOGENESIS – IGMM*, n.d.; Roche, 2016; Sano et al., 1998).

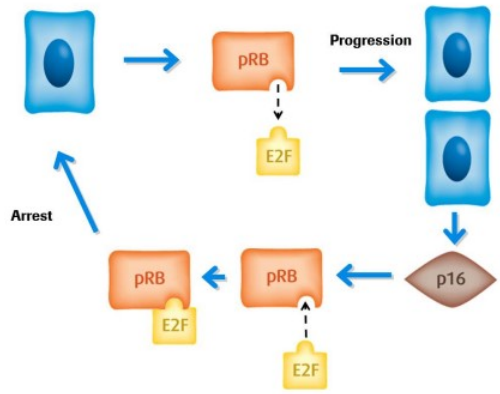

Figure 3: The retinoblastoma protein (pRb), E2F transcription factor and p16 in normal cell cycle function. This figure illustrates the activation of the p16 gene after mitosis in a cell. The expression of p16 leads to the binding of the retinoblastoma protein and the E2F transcription factor. This results in the inhibition of genes related to cell proliferation and progression, leading to cell cycle arrest. The separation of the retinoblastoma protein and the E2F transcription factor allows the cell to resume proliferation. Figure from: Roche, 2016.

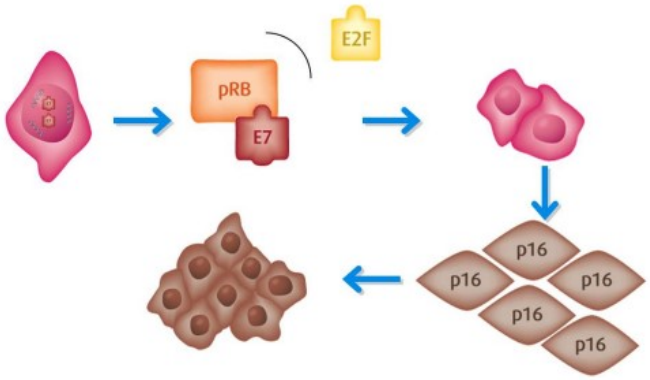

Figure 4: Retinoblastoma protein (pRb), E2F transcription factor, p16 and oncoprotein E7 in oncogenic HPV transformation. This figure illustrates the binding of the oncoprotein E7 produced by human papillomavirus (HPV), which leads to uncontrolled cellular proliferation. This uncontrolled proliferation results in the activation of the p16 gene and subsequent overexpression of p16. Figure from: Roche, 2016.

Clinical applications of the CINtec PLUS Cytology Test

The CINtec PLUS Cytology test is particularly recommended for identifying women at risk of CIN2 or worse. It is especially useful when Pap cytology results are inconclusive, such as in cases of ASC-US, LSIL, or for patients testing positive for hr-HPVs. Unlike conventional cytology and HPV testing, which can lead to unnecessary follow-ups due to transient infections or minor cellular changes, the CINtec PLUS test provides specific information on the presence of deregulated cells, allowing for better clinical decision-making (Boon et al., 2022; Hammer et al., 2020; *HPV And Pap Test Results: Next Steps After an Abnormal Test*, 2024; National Cancer Institute, 2022; Roche, 2016).

Validation and verification

When a method or test is developed by a company, it must undergo validation to ensure that it meets quality standards and is appropriate to answer the intended clinical question. The CINtec PLUS Cytology Test Kit has already been validated by Roche. When a laboratory wants to add a test to its catalog, it must verify the test to ensure that it produces the same results in the lab as the company. Verification is a type of validation that confirms that the laboratory can correctly perform the method or test and demonstrates its reliability (Patel & Shortliffe, 2014; Tracy, 2021).

In the practical guidelines, the required number of slides to be tested for each parameter is specified for each situation (Table 2, Attachments A). Since the CINtec PLUS Cytology test is a diagnostic test, it is categorized as type 1 verification. For trueness assessment, five positive and five negative cases should be selected. For repeatability and reproducibility, three slides per case should be analyzed, for a total of nine slides from three different cases. No cases are required for robustness evaluation (Caignau, n.d.).

III. Experimental Section

A. Material & method

Sample collection - ThinPrep Pap Test PreservCyt solution (Hologic)

A cervical smear is most commonly requested for a Pap or HPV test but may also be used for ICC testing, such as with the CINtec PLUS Cytology Kit. When taking cervical smear, a Cervix-Brush is inserted into the cervical canal to collect an adequate cellular sample. The brush is then placed into a vial containing Thinprep Pap Test PreservCyt solution to release and preserve the collected cells (Hologic, 2021a; IPG, 2023).

Cytological slide preparation - ThinPrep Genesis Processor (Hologic)

For the verification of the CINtec PLUS Cytology Kit, 31 samples of cervical smear (collected cells in ThinPrep solutions) were selected, which correspond to cases where a Pap test was requested.

Slides were prepared using the ThinPrep Genesis Processor (Hologic), which following the company's instructions for the ThinPrep 5000 and ThinPrep 2000 Processors, has been validated to provide equivalent results. The "lame" and "Gyn" programs were selected, and the sample was processed according to the manufacturer’s instructions (Hologic, 2021b) (Figure 114, Attachments F). After preparation, slides were immersed in 96% ethanol for approximately 30 minutes, carefully wiped, and left horizontally to dry for 60 minutes. The dried slides were stored at room temperature, protected from light and should be stained within seven days (Roche, 2024).

This procedure was repeated for all cases. An attempt was made to prepare at least two slides per case, however, in some instances, only one slide could be prepared due to an insufficient number of cells in the ThinPrep solution. Because of this issue, repeatability testing was performed using two slides per case for three cases, resulting in a total of six slides. For reproducibility, as recommended in the practical guidelines, three slides per case were prepared for three cases, totaling nine slides.

For the trueness assessment, 24 cases were used. When two slides could be prepared from a sample, the second slide was used for counterstaining optimization. Additionally, three slides of the same sample were done to evaluate robustness after seven, fifteen, and thirty days to assess the impact of delayed staining.

Immunocytochemical staining - CINtec PLUS Cytology Test on Benchmark Ultra (Roche)

The CINtec PLUS Cytology test is performed on LBC slides obtained from a cervical smear. The detection of p16 relies on mouse monoclonal primary antibodies that bind to secondary anti-mouse antibodies coupled to goat HQ haptens (HQ linker). The presence of multiple HQ haptens fixed to the secondary antibody enables the attachment of several tertiary anti-HQ hapten antibodies, to ensure the amplification of the signal. These tertiary anti-HQ hapten antibodies, conjugated to horseradish peroxidase (HRP), from which the latter converts 3,3'-Diaminobenzidine (DAB) into a brown precipitate in the cytoplasm (Figure 5).

Similarly, the detection of Ki-67 relies on rabbit monoclonal primary antibodies that bind to secondary anti-rabbit antibodies fixed to goat HP haptens (HP linker). The presence of multiple HP haptens fixed to the secondary antibody enables the attachment of several tertiary anti-HP hapten antibodies, which amplifies the signal. These tertiary anti-HP hapten antibodies, conjugated to alkaline phosphatase, ensure the conversion of the Fast Red substrate into a red precipitate in the nucleus (Figure 6) (*CINtec® PLUS Cytology Kit*, n.d.; Roche, 2016; Roche, 2024).

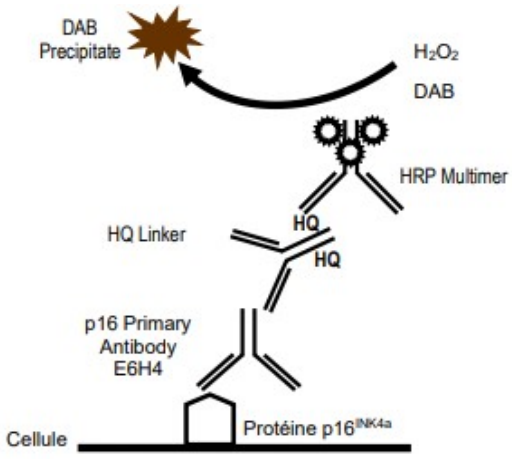

Figure 5: Detection of human p16 with the CINtec PLUS Cytology kit using mouse monoclonal primary antibodies and anti-mouse secondary antibodies conjugated to HQ haptens, resulting in brown precipitate formation from DAB conversion in the cytoplasm. Figure from: Roche, 2024.

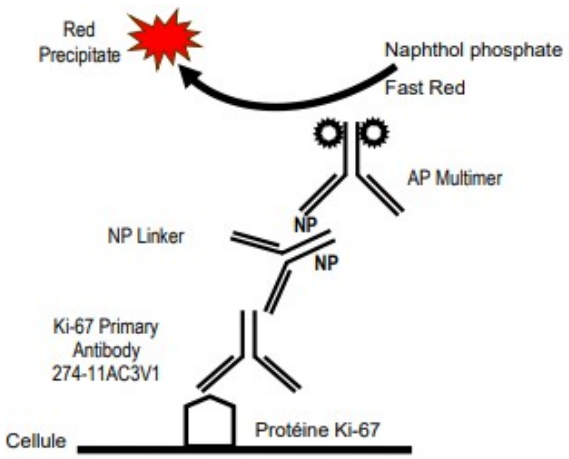

Figure 6: Detection of human Ki-67 with the CINtec PLUS Cytology kit using rabbit monoclonal primary antibodies and anti-rabbit secondary antibodies conjugated to HP haptens, resulting in red precipitate formation from Fast Red conversion in the nucleus. Figure from: Roche, 2024.

Table 1: CINtec PLUS Cytology protocol (Roche), protocol x.

Reagents	Time (min)
Antibody cocktail	16
HQ Linker	12
HRP Multimer	8
NP linker	8
AP Multimer	8
Hematoxylin I	8
Bluing	4

Additionally, hematoxylin and Bluing reagent are used for counterstaining. Table 1 shows the order in which the different reagents are applied to the slide by the device, as well as the duration for which they remain on the slide. The manufacturer provides reagents with an optimal dilution for efficient staining results.

Each CINtec PLUS Cytology slide should include internal controls for each antibody. This means that the slide must contain at least one cervical epithelial cell stained only with p16 and at least one cervical epithelial cell stained only with Ki-67, to confirm that the staining was successful. Since these markers are separately expressed in normal cells, such cells should be visible on the slide. Additionally, cervical epithelial cells stained only with the blue counterstain should be present. These are superficial cells that no longer undergo division and serve as reference cells for comparison with the other stained cells. In figure 7 the different internal are shown.

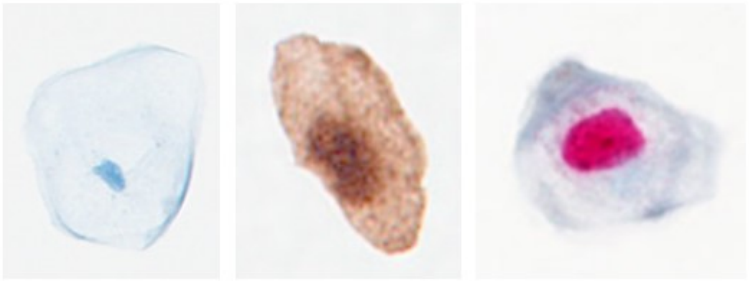

Figure 7: Internal Controls in CINtec PLUS Cytology: Blue counterstained cell (left), p16-positive cell (brown cytoplasm, middle), and Ki-67-positive cell (red nucleus, right).

Trueness and optimization

At the immunohistochemistry (IHC) laboratory at IPG, where the test will be performed after verification, Hematoxylin II is already in use. However, the manufacturer specifically recommends the use of Hematoxylin I. To streamline the workflow, save space in the fridge and in the reagent tray, an optimization from Hematoxylin I (protocol x) to Hematoxylin II (protocol y) is therefore necessary. For trueness and comparison of both hematoxylins, when two slides could be prepared, the first was labeled using protocol x, while the other followed protocol y.

The slides for trueness and optimization were loaded onto the slide trays of the Benchmark Ultra (Figure 115, Attachments F), and the run was started according to the manufacturer's instructions. After the run, the slides were mounted following a two-step protocol. First, a CC/Mount (Diagnostics BioSystems) aqueous mounting medium was applied, followed by an incubation at 58°C for one hour. Afterward, the slides were incubated in xylene for approximately 20 minutes to remove the excess of CC/Mount, followed by the placement of a coverslip using a xylene-based medium with the Tissue-Tek Film Coverslipper (Sakura) (Figure 116, Attachments F). Once the slides were dried, they were examined under the microscope and scanned with the NanoZoomer S210 (Hamamatsu) at 20X.

Repeatability, reproducibility and robustness

To conduct a complete verification, additional tests were performed for repeatability, reproducibility, and robustness. Six slides done for repeatability were stained in the Benchmark Ultra (Roche) on the same day, by the same operator, and using the same device. For reproducibility, the slides from the different cases were stained on different dates, using different Benchmark Ultra devices, loaded by a different laboratory technologist. For the robustness assessment, the slides were stained after 7, 15 and 30 days. All slides for robustness testing were stained on the same device.

The slides were stained, washed, and mounted analogous as those done for trueness and optimization. However, instead of being placed in the Tissue-Tek Film Coverslipper (Sakura), they were mounted manually using a xylene-based medium. These slides were also scanned with the NanoZoomer S210 (Hamamatsu) at 20X.

Table 3 in Attachments A summarizes the number of slides prepared per case. The slides used for repeatability, reproducibility and robustness assessments are also indicated in it. It also indicated which case was stained with protocol x and/or y.

Technical risk analysis

A new technical risk analysis had to be conducted because the CINtec PLUS Cytology test involves techniques that differ from those typically used in the lab and includes different services, leading to new potential risks. Each potential risk in every step, from the input criteria (namely a cervical smear in ThinPrep solution) to the output criteria (namely a mounted, stained cytological slide), was documented. Additionally, solutions and preventive measures were given to minimize each potential risk.

B. Results

After staining, double mounting, and scanning, the stained cells were examined. All the scans can be found in Attachments B, C, D and E. Each slide contains at least one internal control for p16, one for Ki-67 and reference cells.

Trueness

The slides stained for trueness showed that all cases with a NILM pathologist Pap conclusion were negative for the CINtec PLUS test. Only one case with an LSIL pathologist Pap conclusion, namely case 2b, tested positive for CINtec PLUS. Additionally, seven out of eleven cases (cases 3c, 3d, 3e, 3f, 3g, 3h, and 3n) with an HSIL pathologist Pap conclusion were positive for CINtec PLUS, as illustrated in Figures 8 and 9, which show the scan of positive HSIL cases.

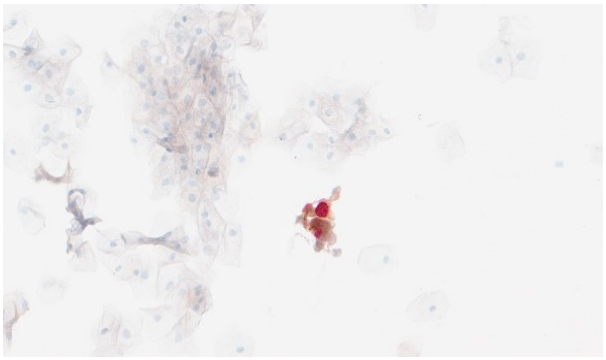

Figure 8: Scan of cervical smear cells from case 3f stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification. The figure illustrates CINtec PLUS-positive cells.

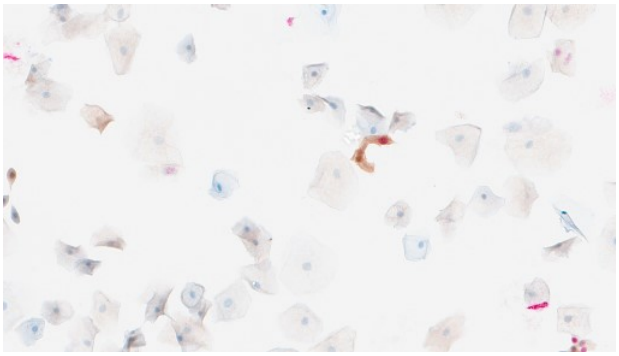

Figure 9: Scan of cervical smear cells from case 3h stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification. The figure illustrates CINtec PLUS-positive cells.

Optimization

An optimization of the counterstaining reagent was carried out to evaluate whether Hematoxylin II, already in use at the IHC laboratory, could reliably replace the manufacturer recommended Hematoxylin I.

For the cases where two slides were prepared, one stained with protocol x (Figure 10) and the other with protocol y (Figure 11). Slides stained with protocol y (Hematoxylin II) generally showed reduced background staining and clearer, better-defined cells.

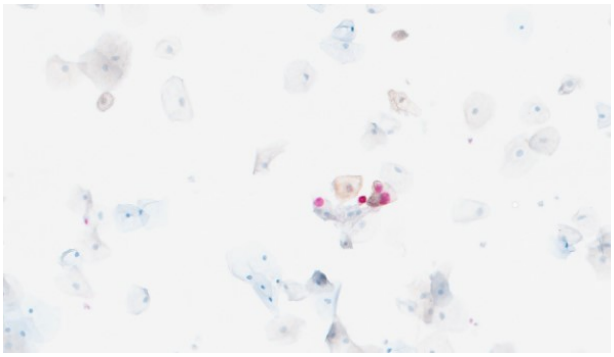

Figure 10: Scan of cervical smear cells from case 2b stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

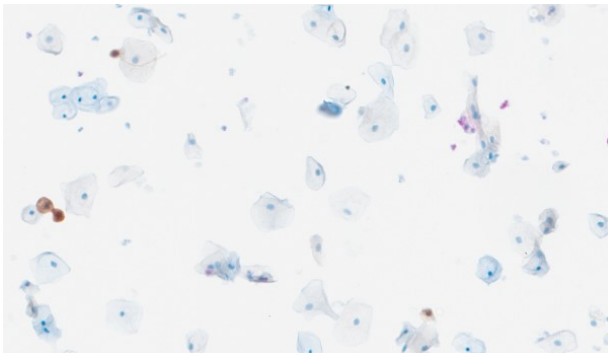

Figure 11: Scan of cervical smear cells from case 2b stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Repeatability

For the samples where two slides were prepared to assess repeatability, the results were always identical for the same case. This means that if one slide from a sample was negative for CINtec PLUS, the other slide from that same sample was also negative and vice versa (Figure 13). Additionally, the staining intensity for p16 and Ki-67 was consistent on both slides (Figure 12).

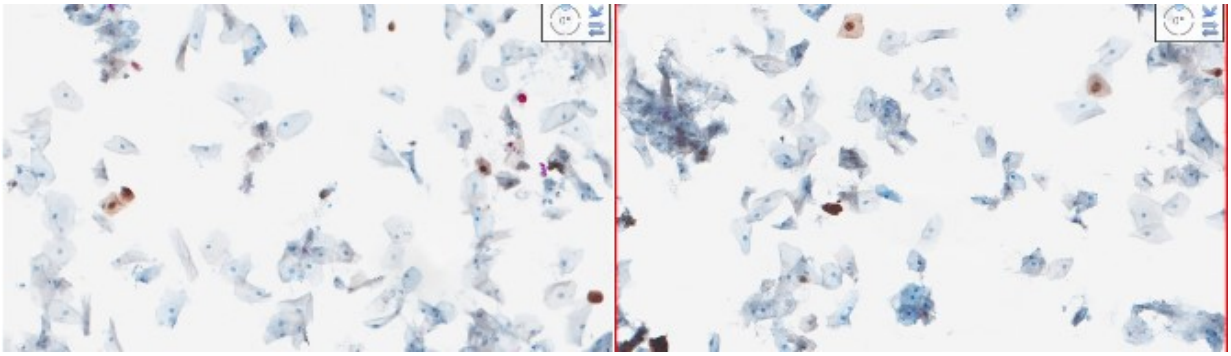

Figure 12: Scan of slide one (left) and slide two (right) of cervical smear cells from case 3p stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification. The figure illustrates p16 and Ki-67-positive cells on the two slides.

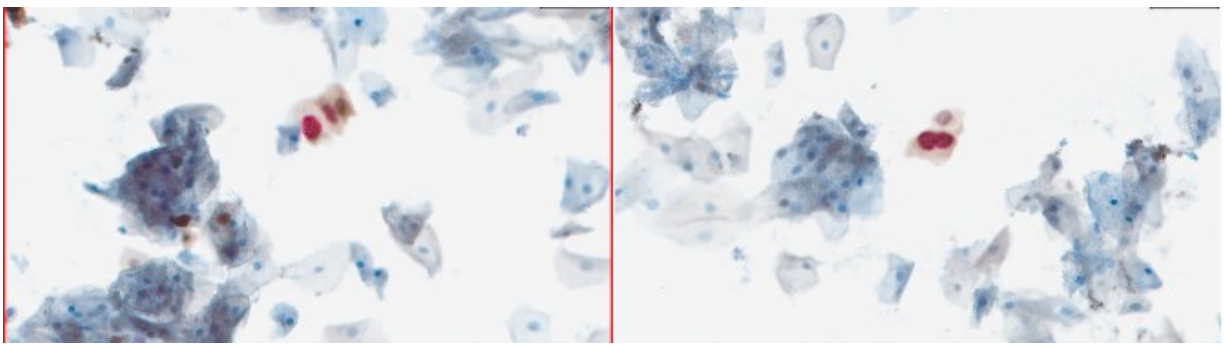

Figure 13: Scan of slide one (left) and slide two (right) of cervical smear cells from case 3p stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification. The figure illustrates CINtec PLUS-positive cells on the two slides.

Reproducibility

For the samples where three slides were prepared to assess reproducibility, the results were always identical. This means that if one slide from a sample was negative for CINtec PLUS, the other slides from that same sample were also negative and vice versa (Figure 16). Furthermore, the staining intensity for p16 and Ki-67 was consistent across all slides (Figure 14 & 15).

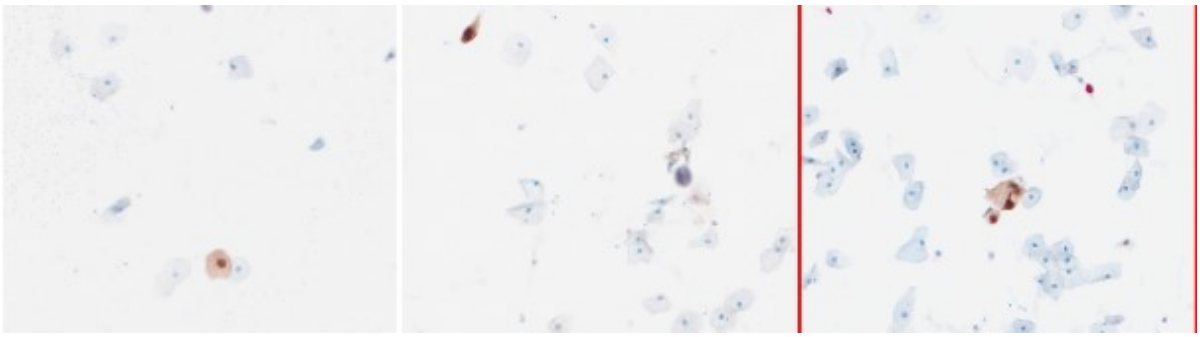

Figure 14: Scan of slide one (left), slide two (middle) and slide three (right) of cervical smear cells from case 3l stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification. The figure illustrates p16-positive cells on all three slides.

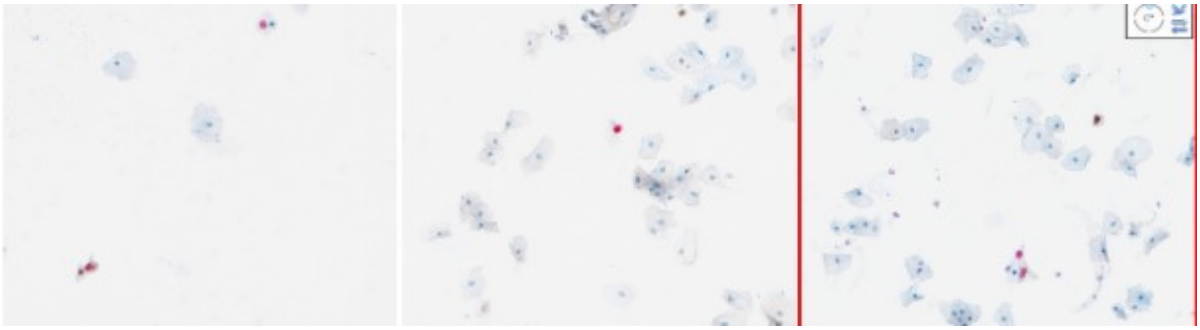

Figure 15: Scan of slide one (left), slide two (middle) and slide three (right) of cervical smear cells from case 3l stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification. The figure illustrates Ki-67-positive cells on all three slides.

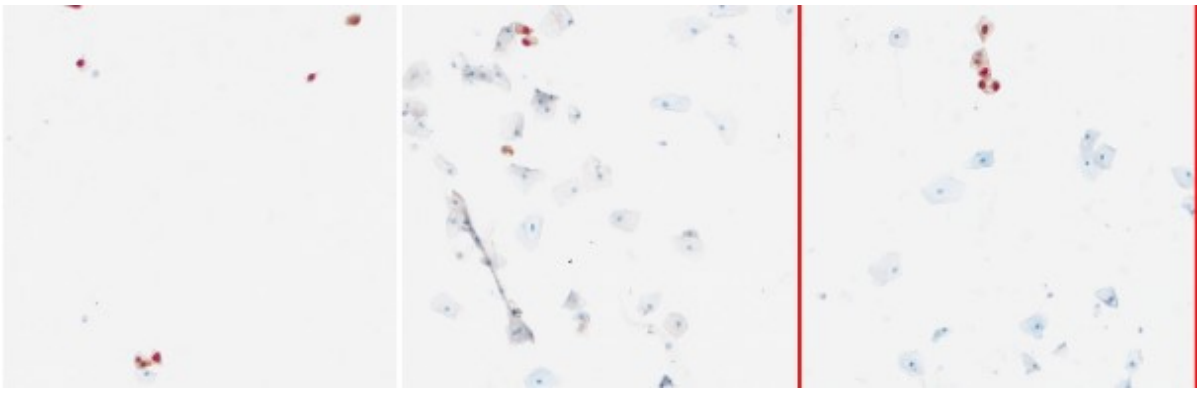

Figure 16: Scan of slide one (left), slide two (middle) and slide three (right) of cervical smear cells from case 3l stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification. The figure illustrates CINtec PLUS-positive cells on all three slides.

Robustness

For the sample where three slides were prepared and stained after 7, 15 and 30 days for robustness, the results were identical. This means that if one slide from a sample was negative for CINtec PLUS, the other slides from that same sample were also negative and vice versa (Figure 19). Furthermore, the staining intensity for p16 and Ki-67 was consistent across all slides (Figure 17 & 18).

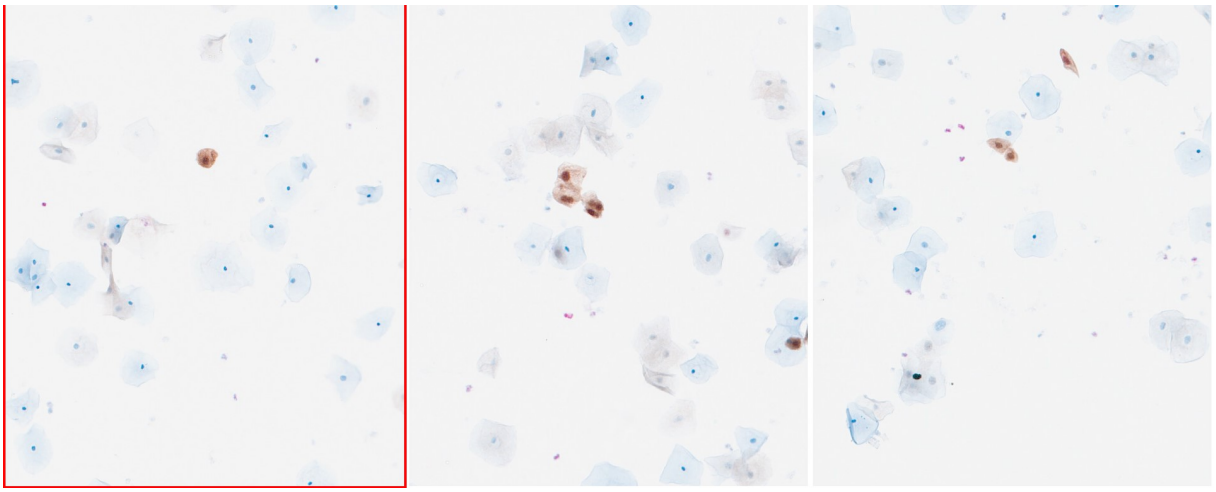

Figure 17: Scan of slide one (left), slide two (middle) and slide three (right) of cervical smear cells from case 2d stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification. The figure illustrates p16-positive cells.

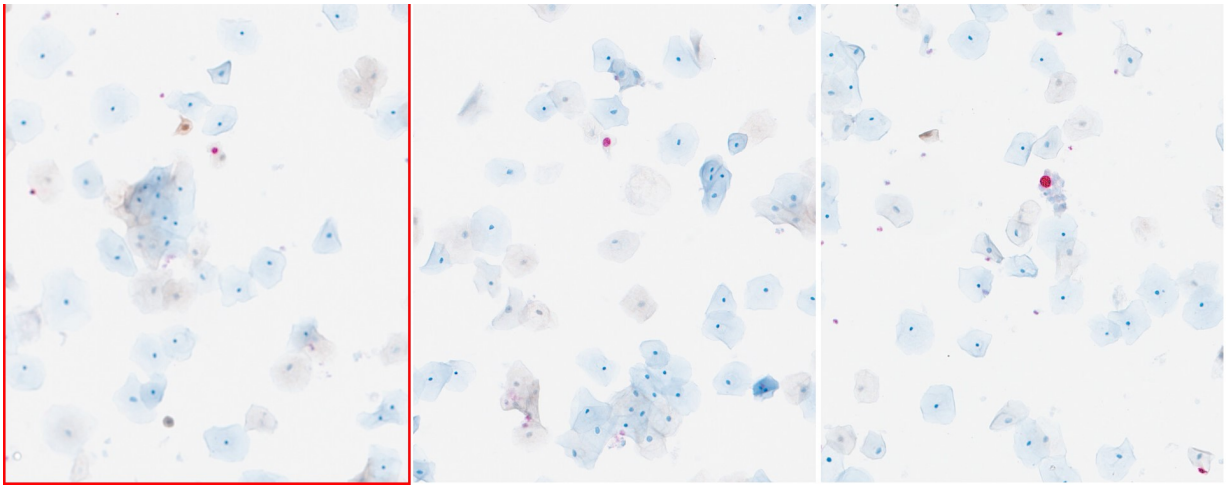

Figure 18: Scan of slide one (left), slide two (middle) and slide three (right) of cervical smear cells from case 2d stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification. The figure illustrates Ki-67-positive cells.

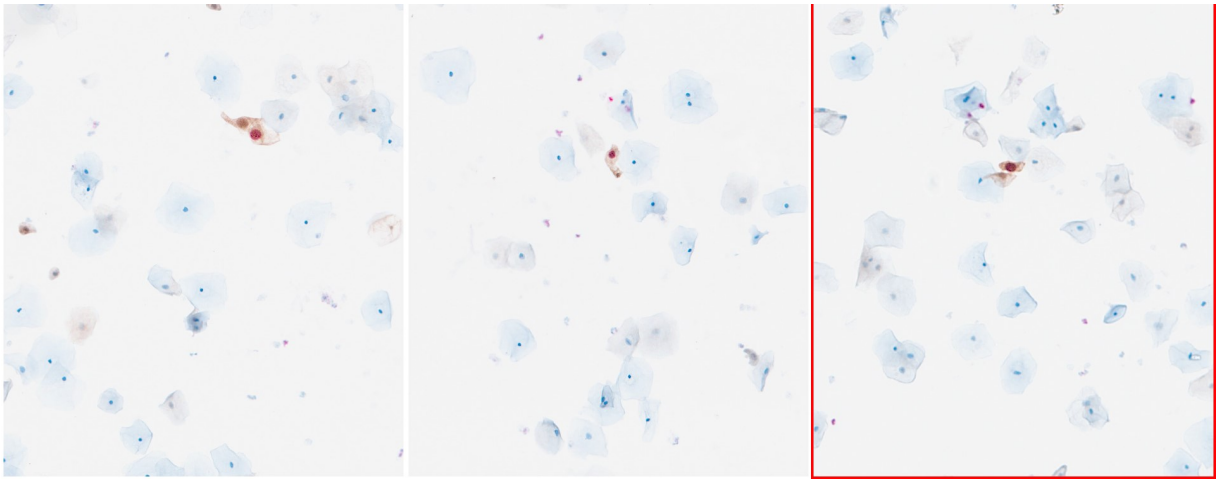

Figure 19: Scan of slide one (left), slide two (middle) and slide three (right) of cervical smear cells from case 2d stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification. The figure illustrates CINtec PLUS-positive cells.

C. Discussion

Trueness

The trueness of the method is demonstrated by its ability to reliably and correctly highlight the specified elements, in accordance with the manufacturer's expectations. This is achieved by comparing the results obtained with CINtec PLUS to the Pap test conclusions.

None of the slides with a NILM Pap pathologist's conclusion tested positive for CINtec PLUS, which suggests that there is no significant disruption in the cell cycle or oncogenic transformation. Since Pap NILM results indicate the absence of intraepithelial lesions or malignancy, this aligns with the expected lack of p16 and Ki-67 co-expression, reinforcing the normal regulation of the cell cycle in these samples.

In contrast, only case 2b, with an LSIL Pap conclusion, tested positive for CINtec PLUS. This shows that patient 2b has cell cycle disruption and suggests signs of possible oncogenic transformation. The fact that only one of the five LSIL cases tested positive for CINtec fits with the idea that, while LSIL lesions show mild changes, they are generally less likely to turn into invasive cancer compared to HSIL lesions and that most HPV infections are transient. LSIL is usually linked to a lower risk of cancer, as many cases go back to normal without treatment.

Additionally, seven out of eleven cases with an HSIL Pap conclusion tested positive for CINtec PLUS. It also fits with the fact that HSIL cases have cells with more pronounced abnormalities and are at a higher risk of progression to cervical cancer.

The trueness was considered acceptable, as the staining results aligned with the Pap test conclusions.

Optimization of the hematoxylin counterstaining

The objective of this optimization was to demonstrate that Hematoxylin II (protocol y) can replace Hematoxylin I (protocol x) to harmonize protocols on the Benchmark Ultra and facilitate the routine work of laboratory technologists, without negatively impacting the staining technique. Protocol y generally results in less background staining, leading to better interpretation of p16-positive cells. Additionally, p16- and Ki-67-negative cells appear more clearly defined and visible.

Pathologists and CINtec PLUS Cytology trainers from Roche confirmed that slides stained using protocol y showed the same results than those obtained with the Roche recommended protocol. They also noted that superficial cells, identified by their blue counterstaining, were more easily distinguishable.

The optimization confirmed that IPG can reliably use hematoxylin II for the counterstaining of the CINtec PLUS to facilitate the routine work. Given these findings, protocol y was selected for repeatability, reproducibility and robustness assessments.

Repeatability

Repeatability is assessed to determine whether the technique produces the same result when staining is repeated under identical conditions, during the same manipulation, on the same cervical smear sample.

The fact that the samples showed consistent results and staining intensity demonstrates the repeatability of the technique, meaning that the method reliably provides consistent outcomes under the same conditions. This minimizes variability and ensures that any observed differences in staining in the laboratory are due to biological or technical factors rather than procedural inconsistencies.

Reproducibility

Reproducibility is assessed to determine whether the technique produces consistent results when applied to the same sample using the same method, but under different analytical conditions. The results from the same samples showed identical outcomes and the same staining intensity for p16 and Ki-67, regardless of the conditions under which the slides were analyzed.

Such reproducibility is essential for ensuring that the technique can be reliably used in different analytical settings or by different operators without affecting the quality of the results. The uniformity of the staining across slides ensures that any differences observed in the staining patterns are biologically or technically relevant, rather than appearing from inconsistencies in the method or analytical conditions.

Robustness

The robustness of the test was evaluated by staining slides beyond the manufacturer's recommended timeframe. The two slides stained after 15 and 30 days showed the same results as the one stained after 7 days, which is the maximum recommended timeframe before staining.

The fact that the slides showed the same results and staining intensity suggests that, if by mistake the slides were not stained within the recommended seven-day period, they could still be interpreted by pathologists if they remain within a 30-day timeframe.

The presence of internal controls on each slide as recommended by the manufacturer ensures a reliable interpretation of the results. The consistency across all results supports the accuracy of the analysis.

Recap of main objectives

This study assessed the introduction of the CINtec PLUS Cytology test at IPG by verifying its technical performance through various validation parameters. All tests confirmed that CINtec PLUS meets the required standards and functions correctly in the laboratory, making its implementation in routine diagnostics a viable option.

The test offers a more specific screening method by targeting cells that proliferate uncontrollably. Follow-up efforts can then be focused on patients who show true signs of cellular transformation. This ensures that women requiring closer monitoring and potential treatment are accurately identified, while unnecessary procedures for those with insignificant abnormalities are minimized.

Cervical cancer remains a significant health challenge, often made more challenging by overtreatment and unnecessary follow-ups. The CINtec PLUS test addresses these issues by reducing false positives results in cervical cancer diagnosis, thereby streamlining patient management by helping pathologists focus on patients who truly need follow-up or treatment.

IPG plays a central role in advancing cancer detection in Wallonia. Integrating CINtec PLUS into its routine screening protocols would reinforce the institute’s commitment to delivering the highest standards of diagnostic care.

IV. Conclusion

This study verified the technical performance of the CINtec PLUS Cytology test at IPG through a type 1 verification, including trueness, repeatability, reproducibility, robustness and hematoxylin counterstaining optimization. The results confirmed that the test meets all required standards and can be reliably implemented in the laboratory's routine workflow.

By more specifically detecting cells at risk of malignant progression, CINtec PLUS improves the accuracy of cervical cancer screening and helps reduce unnecessary follow-up procedures. Its use at IPG will support more targeted patient management and contribute to a more efficient and reliable screening strategy.

Language editing assistance was provided by ChatGPT.

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VI. Attachments

Attachments A: Tables

Table 2: Minimum number of samples to be tested and repetition factor to verify/validate accuracy and precision, considering the intended objective, the origin of the reagents/test, the testing method, and the execution mode (Caignau, n.d.).

	CE-IVD selon la notice ou CE-IVD *CE-IVD* modifié AVEC référence											
	INITIALE AVEC EXPÉRIENCE[1] OU HISTORIQUE						INITIALE SANS EXPÉRIENCE[1]					
		Justesse[2]	Fidélité[2,3]		Robustesse[2]	Autres[2,4]		Justesse[2]	Fidélité[2,3]		Robustesse[2]	Autres[2,4]
			Intra	Inter					Intra	Inter		
1 Coloration de base automatique		5e x 1l.[5]	1e x 3l.[7]	1e x 3l.[7]		U/S/F : > X[12]		5e x 1l.	1e x 3l.[7]	1e x 3l.[7]		U/S/F : > X[12]
	CA[6]	*≥ 80 %*	*≥ 90 %*	*≥ 90 %*			*CA*[6]	*≥ 80 %*	*≥ 90 %*	*≥ 90 %*		
2 Coloration de base manuel		5e x 1l.	N/A	1e x 5l.		U/S/F : > X		5e x 1l.	N/A	1e x 5l.		U/S/F : > X
	CA[6]	*≥ 80 %*		*≥ 90 %*			*CA*[6]	*≥ 80 %*		*≥ 90 %*		
1 Histochimique automatique		5e x 1l.	1e x 3l.[7]	1e x 3l.[7]		I/U/S/F/CC : > X		5e x 1l.	1e x 3l.[7]	1e x 3l.[7]		I/U/S/F/CC : > X
	CA[6]	*≥ 80 %*	*≥ 90 %*	*≥ 90 %*			*CA*[6]	*≥ 80 %*	*≥ 90 %*	*≥ 90 %*		
2 Histochimique manuel		5e x 1l.	N/A	1e x 5l.		I/U/S/F/CC : > X		5e x 1l.	N/A	1e x 5l.		I/U/S/F/CC : > X
	CA[6]	*≥ 80 %*		*≥ 90 %*			*CA*[6]	*≥ 80 %*		*≥ 90 %*		
3 IHC/HIS type 1 panel 15 AC	par AC	5e (+) 5e (-)	3e x 3l.[8] (1 AC)	3e x 3l.[9] (1 AC)		I/U/S/F/CC : > X	par AC	10e (+) 10e (-)	3e x 3l.[8] (1 AC)	3e x 3l.[9] (1 AC)		I/U/S/F/CC : > X
(validation de la méthode)	*CA*[6]	*≥ 90 %*	*≥ 90 %*	*≥ 90 %*			*CA*[6]	*≥ 90 %*	*≥ 90 %*	*≥ 90 %*		
IHC/HIS type 1 (à partir du 16ème AC)	par AC	2e (+) 2e (-)				I/U/S/F/CC : > X	par AC	2e (+) 2e (-)				I/U/S/F/CC : > X
	CA[6]	*≥ 75 %*					*CA*[6]	*≥ 75 %*				
4 IHC/HIS type 2a	Matrice initiale	7e (+) 7e (-)	3e x 3l.	3e x 3l.	5e(+) 5e(-)	I/U/S/F/CC : > X 5e(+) et 5e(-) entre observateurs	Matrice initiale	15e(+) 15e (-)	3e x 3l.	3e x 3l.	5e(+) 5e(-)	I/U/S/F/CC : > X 5e(+) et 5e(-) entre observateurs
(vér/val spécifique)	*CA*[6]	*≥ 90 %*[10]	*≥ 90 %*	*≥ 90 %*	*≥ 90 %*	*≥ 90 %*	*CA*[6]	*≥ 93%*[10]	*≥ 90 %*	*≥ 90 %*	*≥ 90 %*	*≥ 90 %*
IHC/HIS type 2b	Matrice initiale	10e (+) 10e (-)	3e x 3l.	3e x 3l.	5e(+) 5e(-)	I/U/S/F/CC : > X 5e(+) et 5e(-) entre observateurs	Matrice initiale	20e(+) 20e (-)	3e x 3l.	3e x 3l.	5e(+) 5e(-)	I/U/S/F/CC : > X 5e(+) et 5e(-) entre observateurs

Table 3: Cases classified by malignancy (NILM, LSIL, HSIL) and collected for CINtec PLUS Cytology verification at IPG, summarizing the number of slides prepared per case, the corresponding staining protocol (Hematoxylin or Hematoxylin II), and the slides used for repeatability, reproducibility, and robustness assessments.

NILM	**Slides**	**Protocol**	**LSIL**	**Slides**	**Protocol**	**HSIL**	**Slides**	**Protocol**
Case 1a	2	x + y	Case 2a	2	x + y	Case 3a	2	x + y
Case 1b	2	x + y	Case 2b	2	x + y	Case 3b	1	y
Case 1c	1	y	Case 2c*	2	y	Case 3c	1	y
Case 1d	1	y	Case 2d***	2	y	Case 3d	1	y
Case 1e	1	y	Case 2e**	3	y	Case 3e	1	x
Case 1f	1	y	Case 2f	1	x	Case 3f	2	x + y
Case 1g	2	x + y	Case 2g	2	x + y	Case 3g	2	x + y
Case 1h	2	x + y	Case 2h	2	x + y	Case 3h	2	x + y
						Case 3i**	3	y
						Case 3j	1	y
						~~Case 3k~~****	~~2~~	~~y~~
						Case 3l**	3	y
						Case 3m*	2	y
						Case 3n	2	x + y
						Case 3o	2	x + y
						Case 3p*	2	y

*Repeatability
**Rebroductibiity
***Robustness (7 days, 15 days and 30 days)
****~~Robustness (arc ↔ arcless)~~

Attachments B: CINtec PLUS Cytology slide scans for trueness and optimization

Case 1a – protocol x

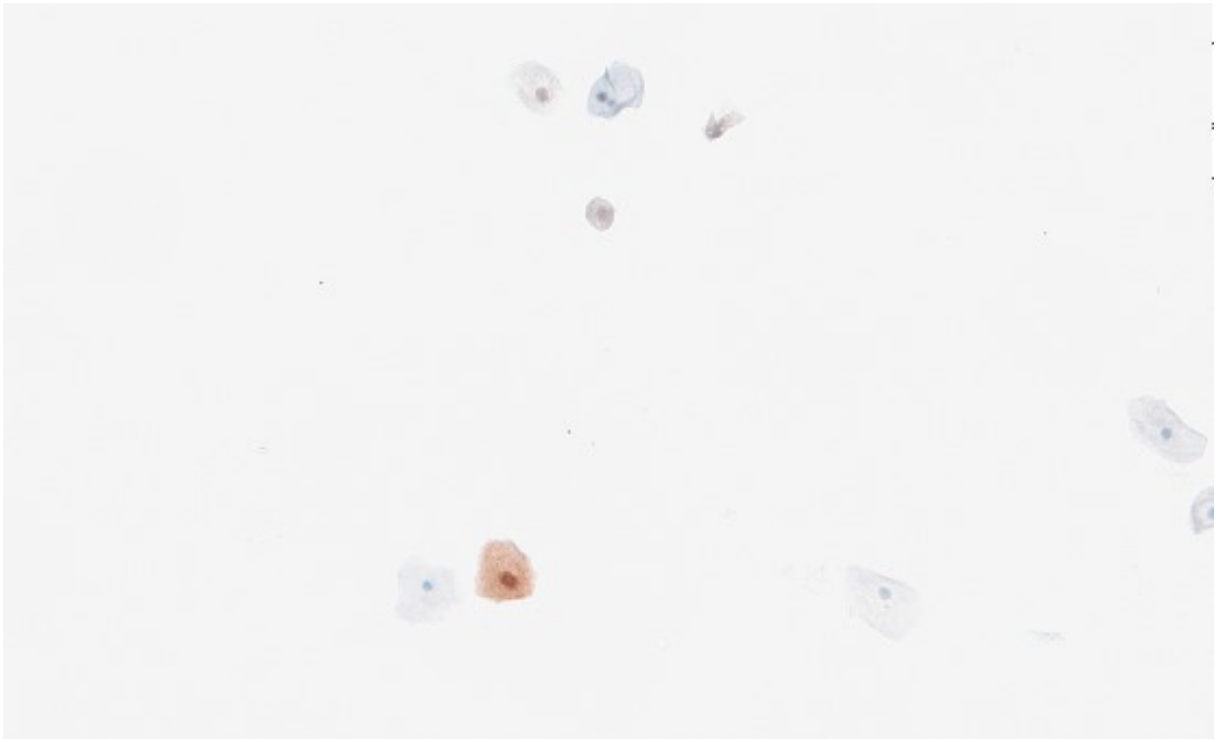

Figure 20: Scan one of slide one of cervical smear cells from case 1a stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

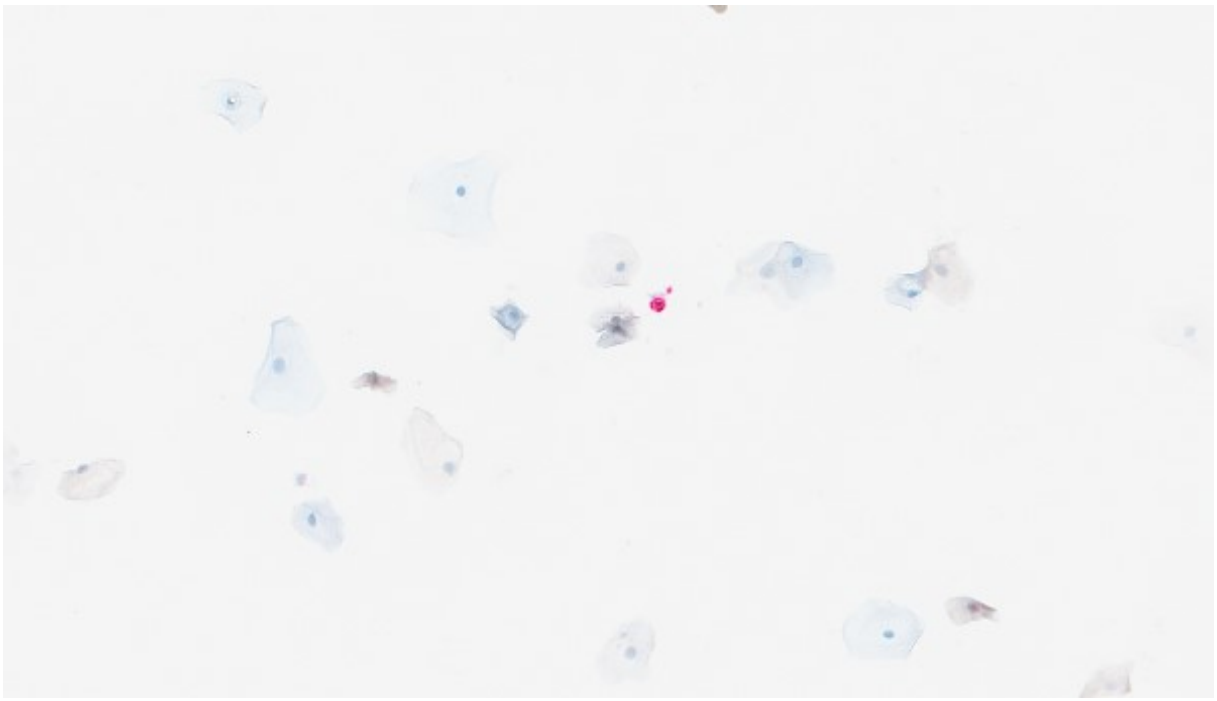

Figure 21: Scan two of slide one of cervical smear cells from case 1a stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1a – protocol y

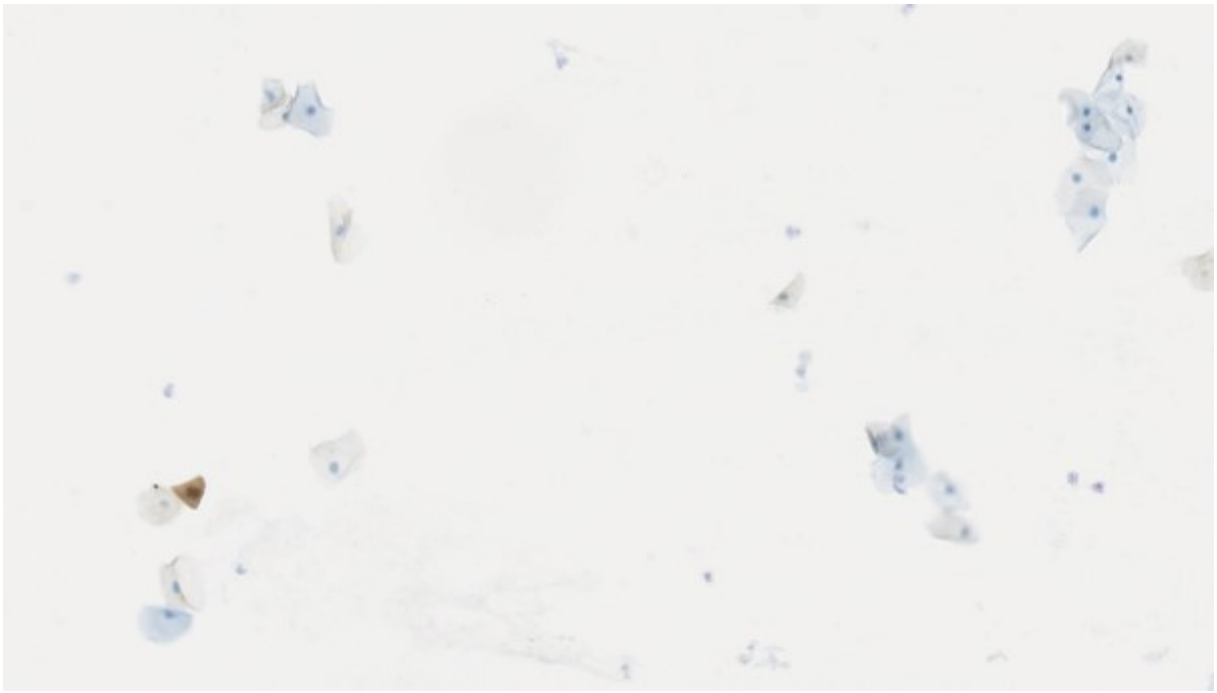

Figure 22: Scan one of slide two of cervical smear cells from case 1a stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

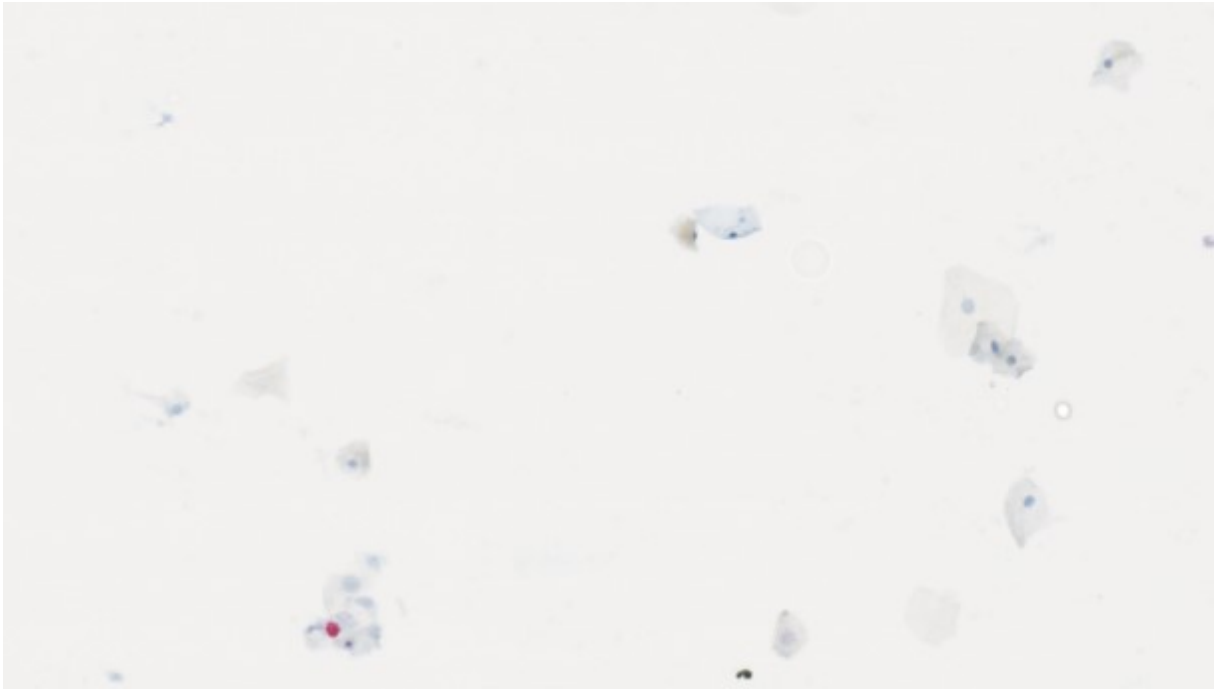

Figure 23: Scan two of slide two of cervical smear cells from case 1a stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1b – protocol x

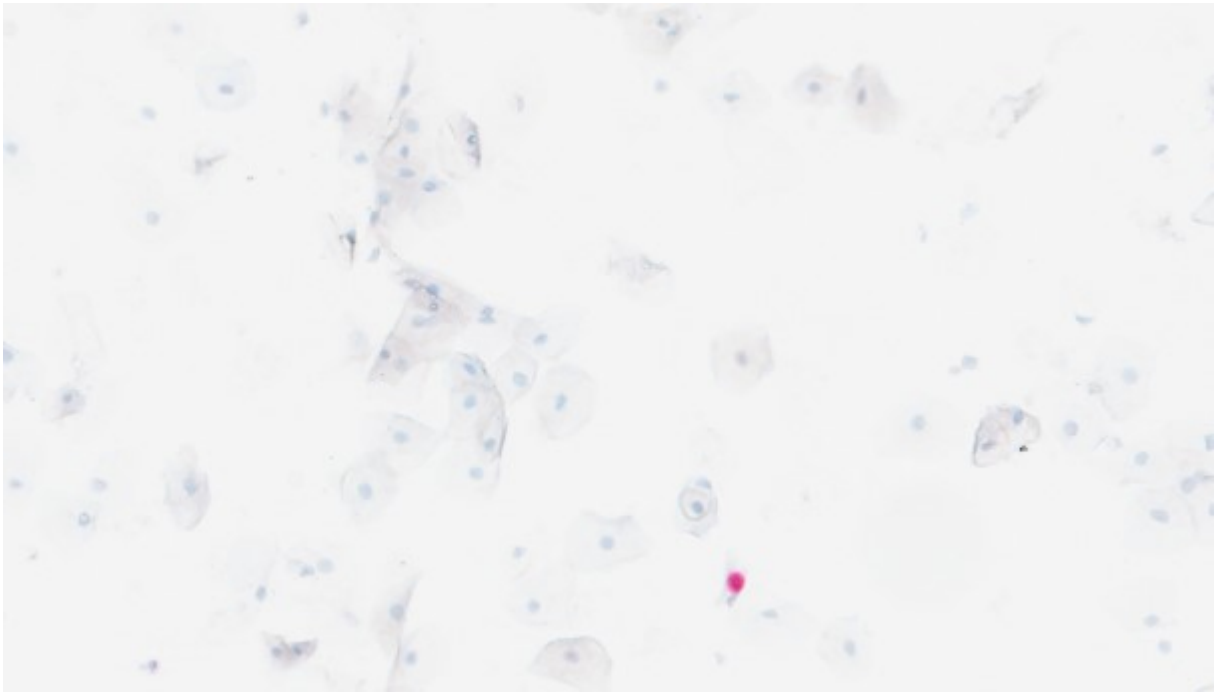

Figure 24: Scan one of slide one of cervical smear cells from case 1b stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

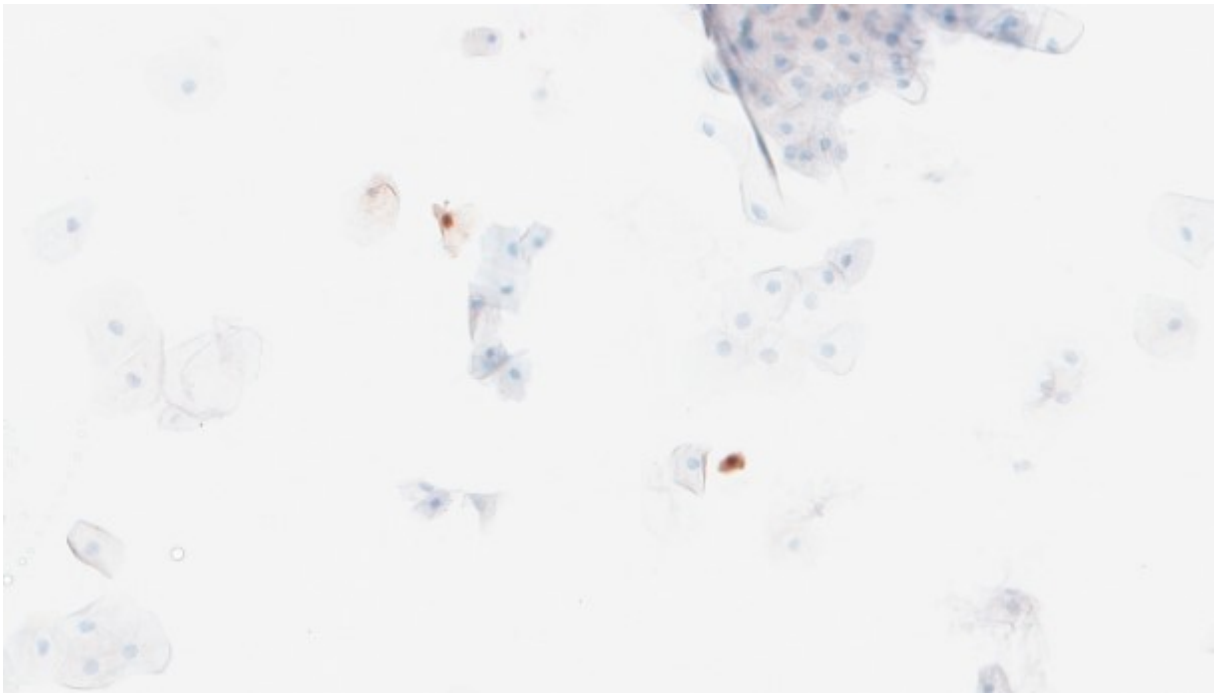

Figure 25: Scan two of slide one of cervical smear cells from case 1b stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1b – protocol y

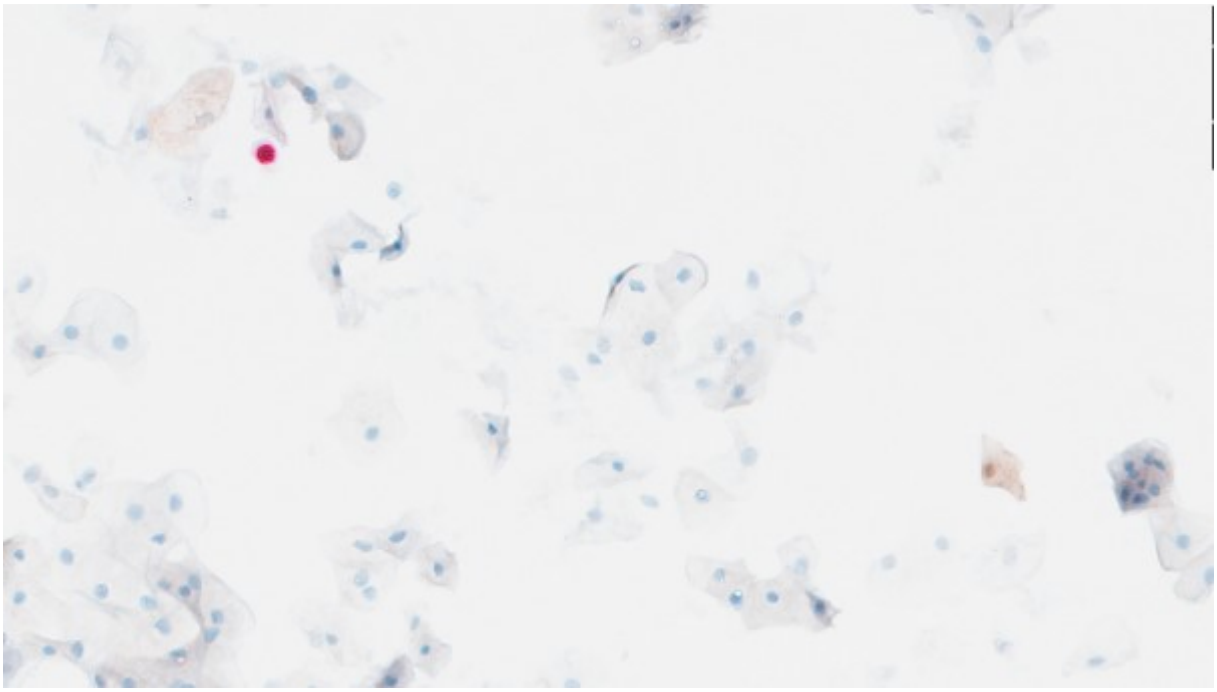

Figure 26: Scan one of slide two of cervical smear cells from case 1b stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

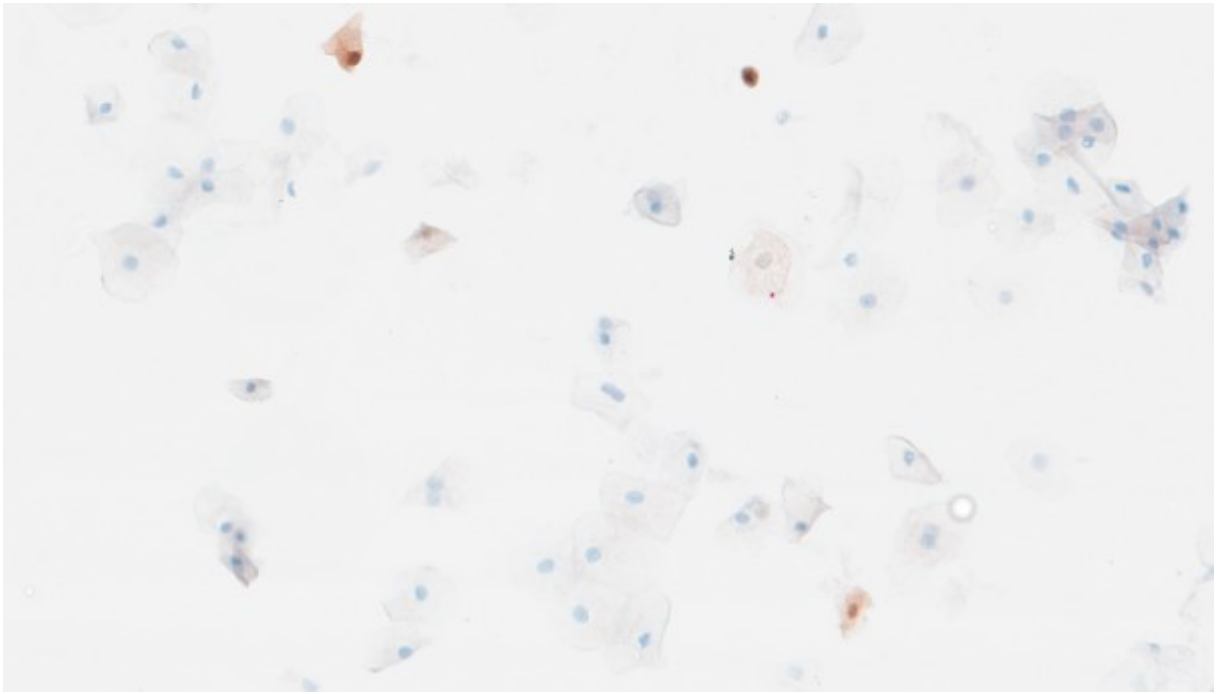

Figure 27: Scan two of slide two of cervical smear cells from case 1b stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1c – protocol y

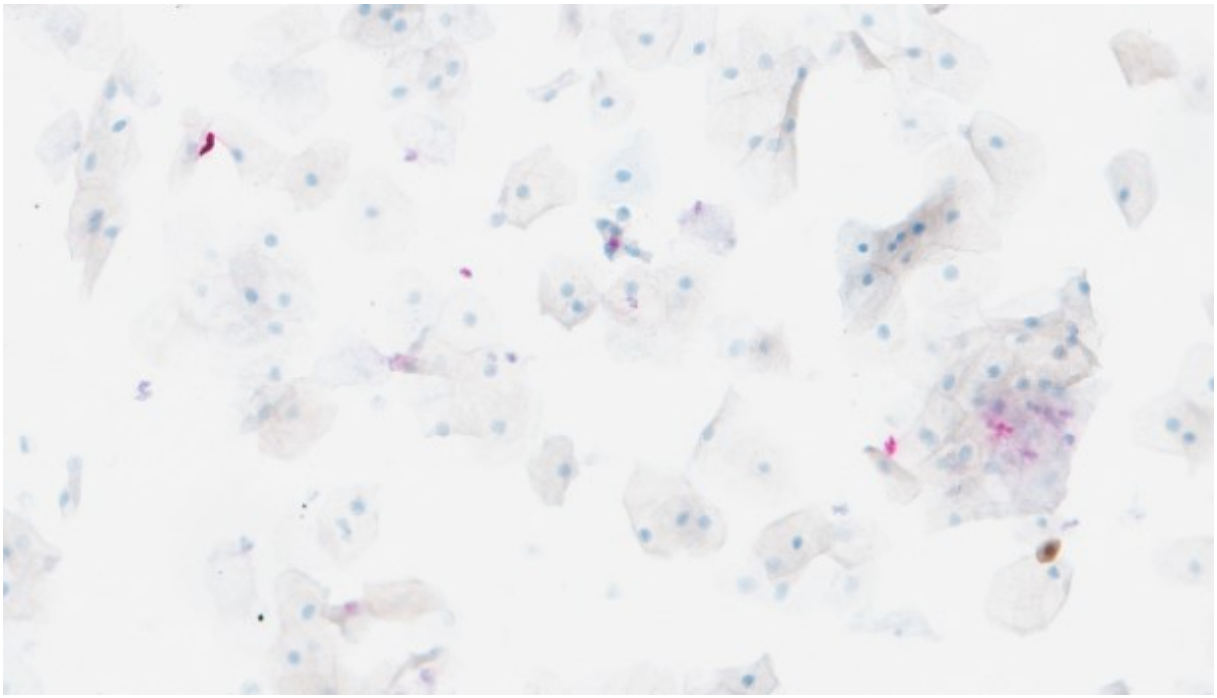

Figure 28: Scan one of cervical smear cells from case 1c stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

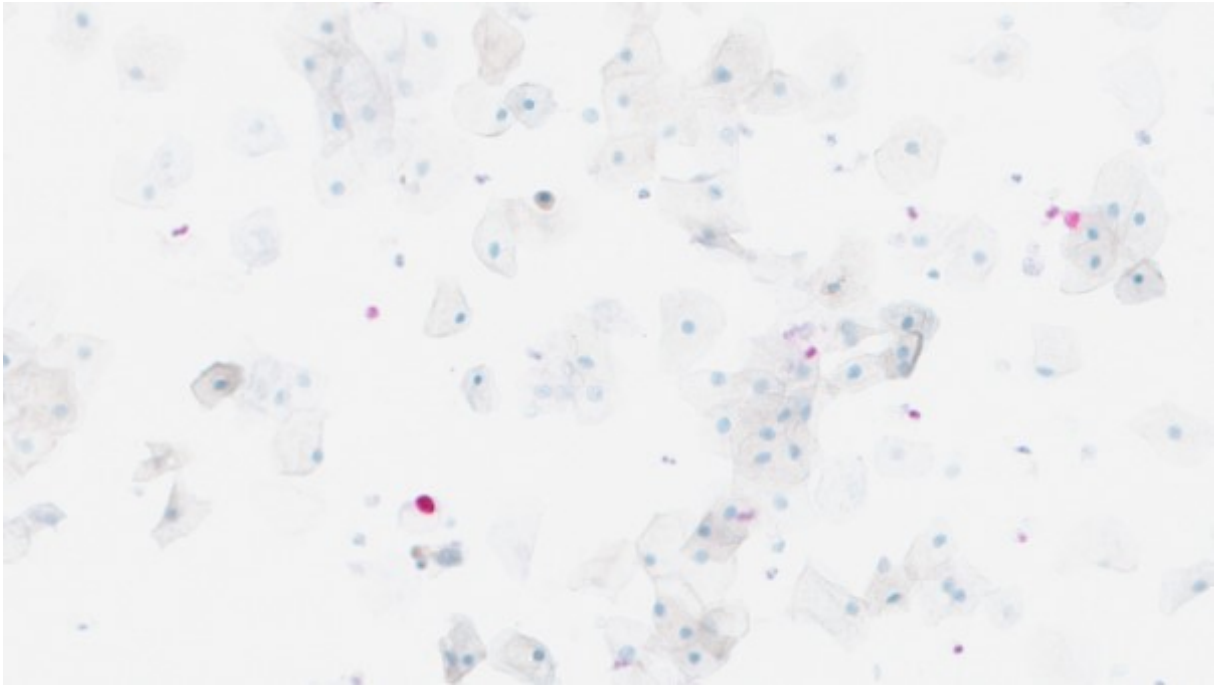

Figure 29: Scan two of cervical smear cells from case 1c stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1d – protocol y

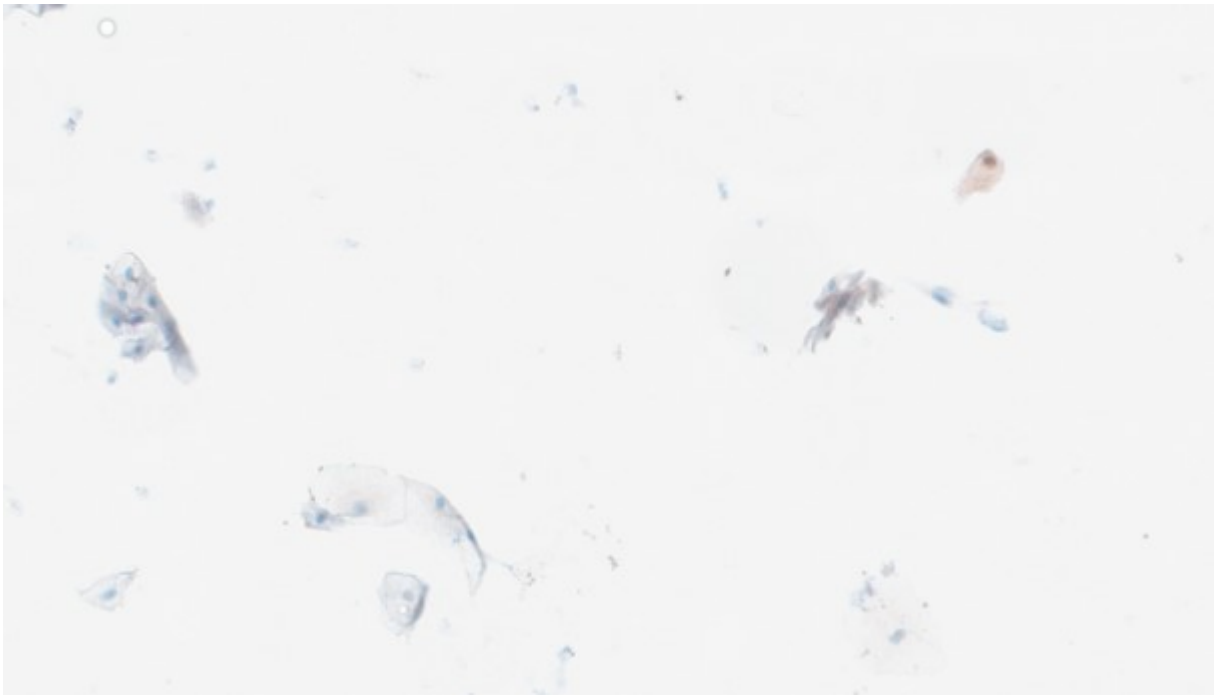

Figure 30: Scan one of cervical smear cells from case 1d stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

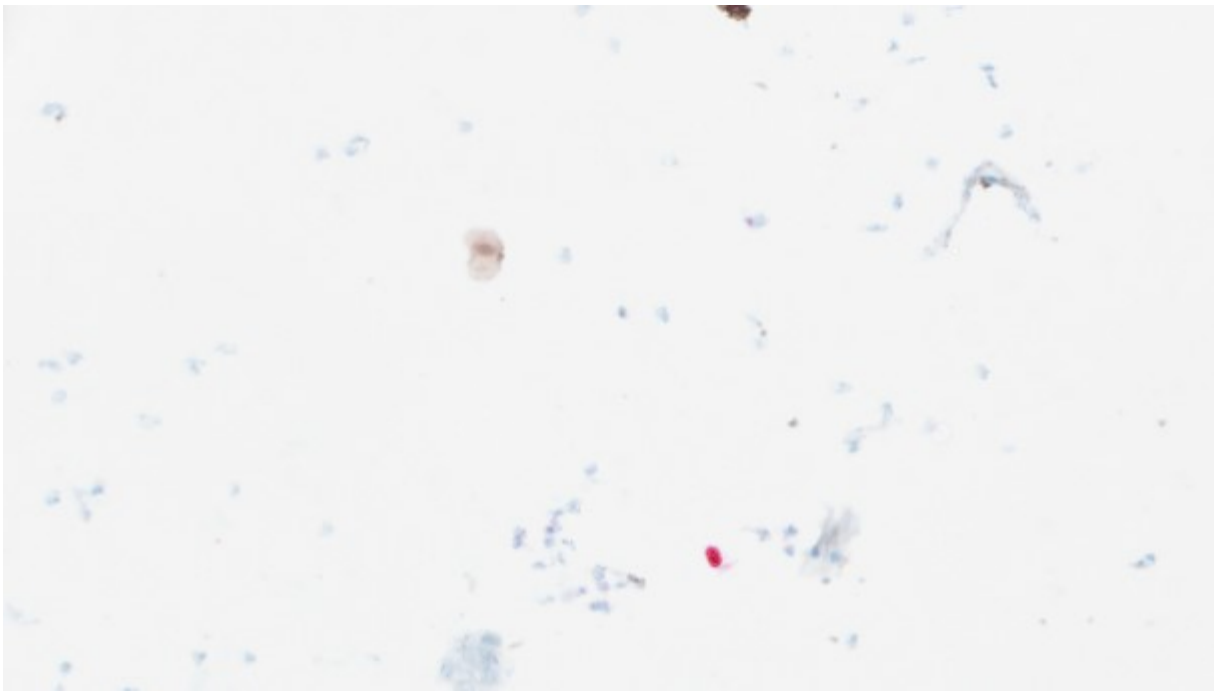

Figure 31: Scan two of cervical smear cells from case 1c stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1e – protocol y

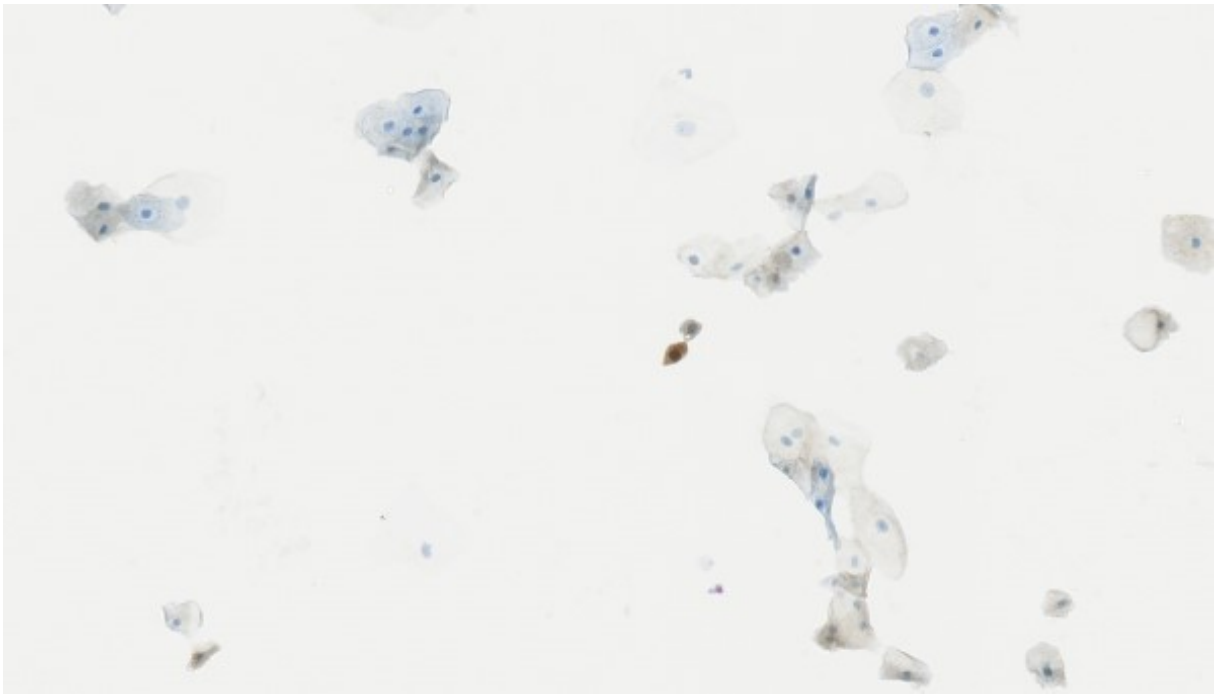

Figure 32: Scan one of cervical smear cells from case 1e stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

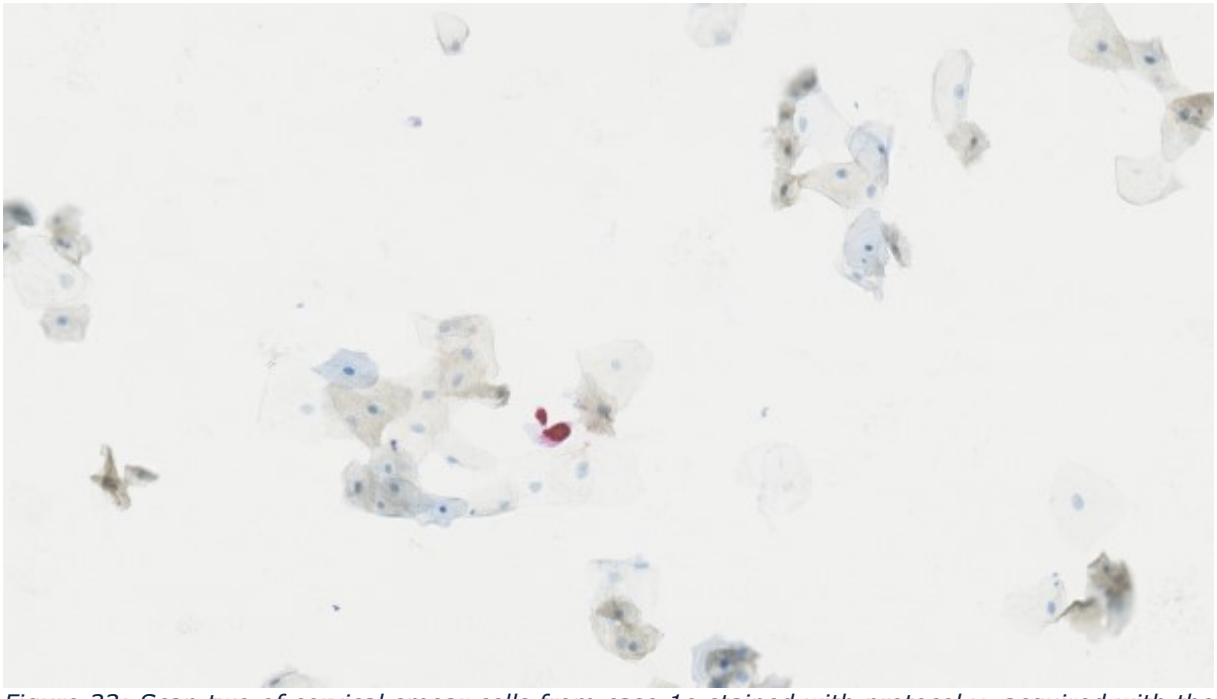

Figure 33: Scan two of cervical smear cells from case 1e stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1f – protocol y

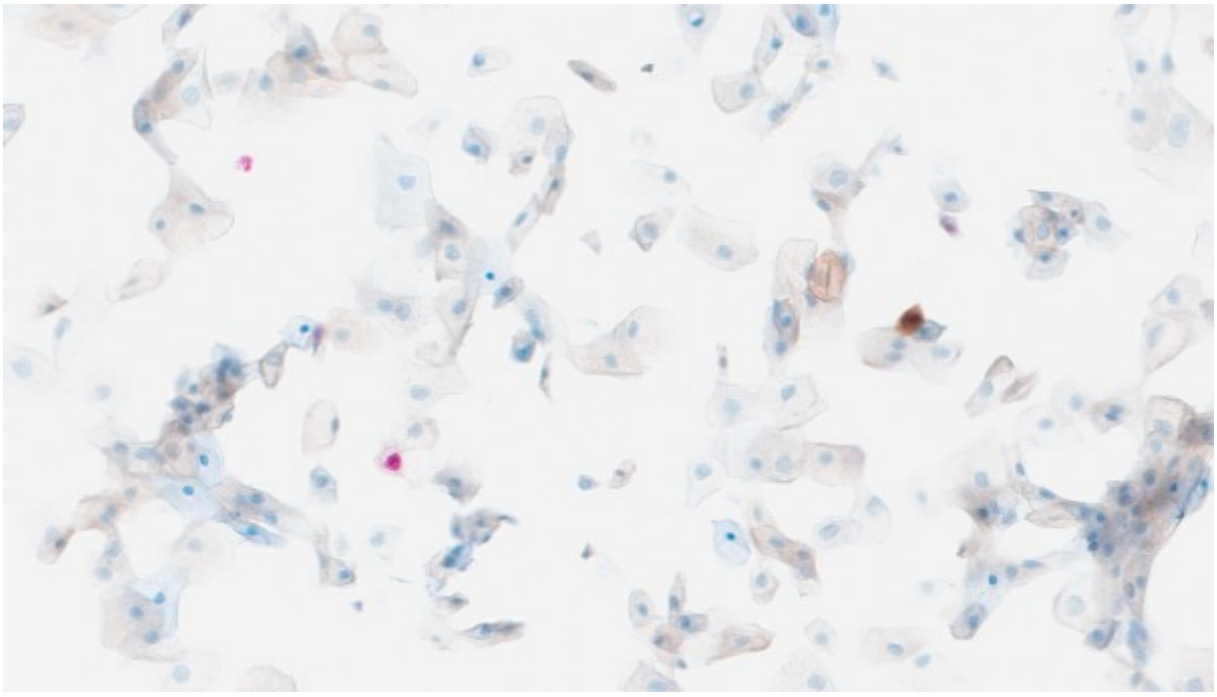

Figure 34: Scan one of cervical smear cells from case 1f stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

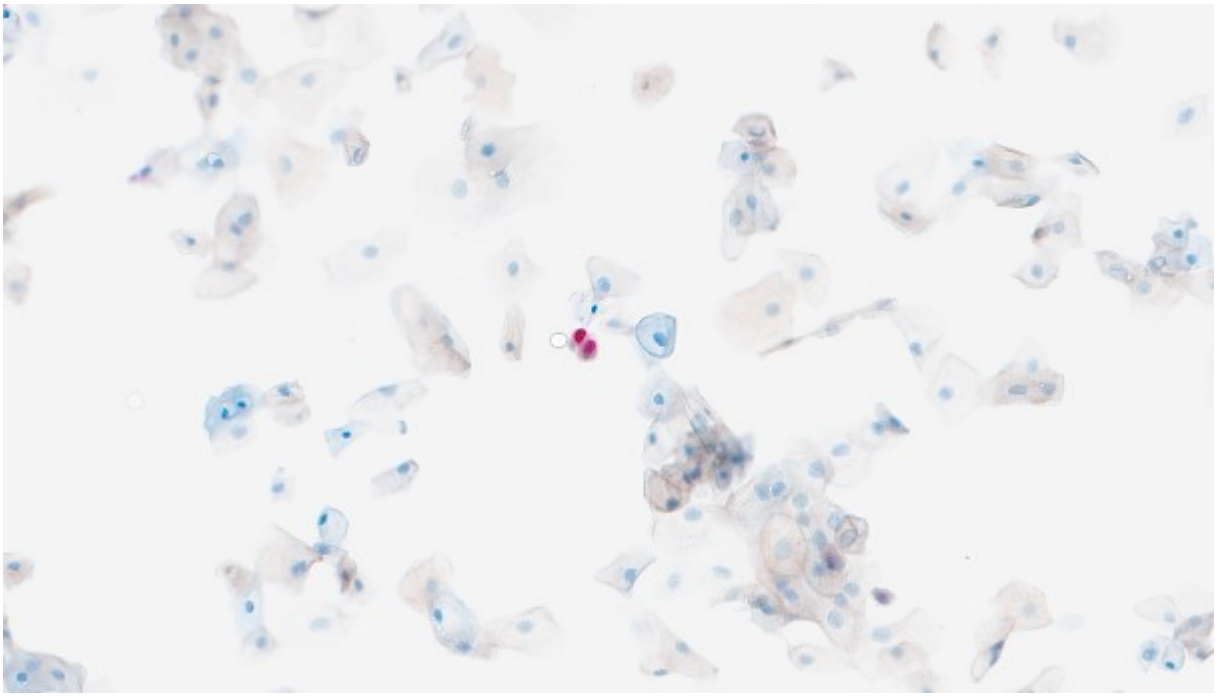

Figure 35: Scan two of cervical smear cells from case 1f stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1g – protocol x

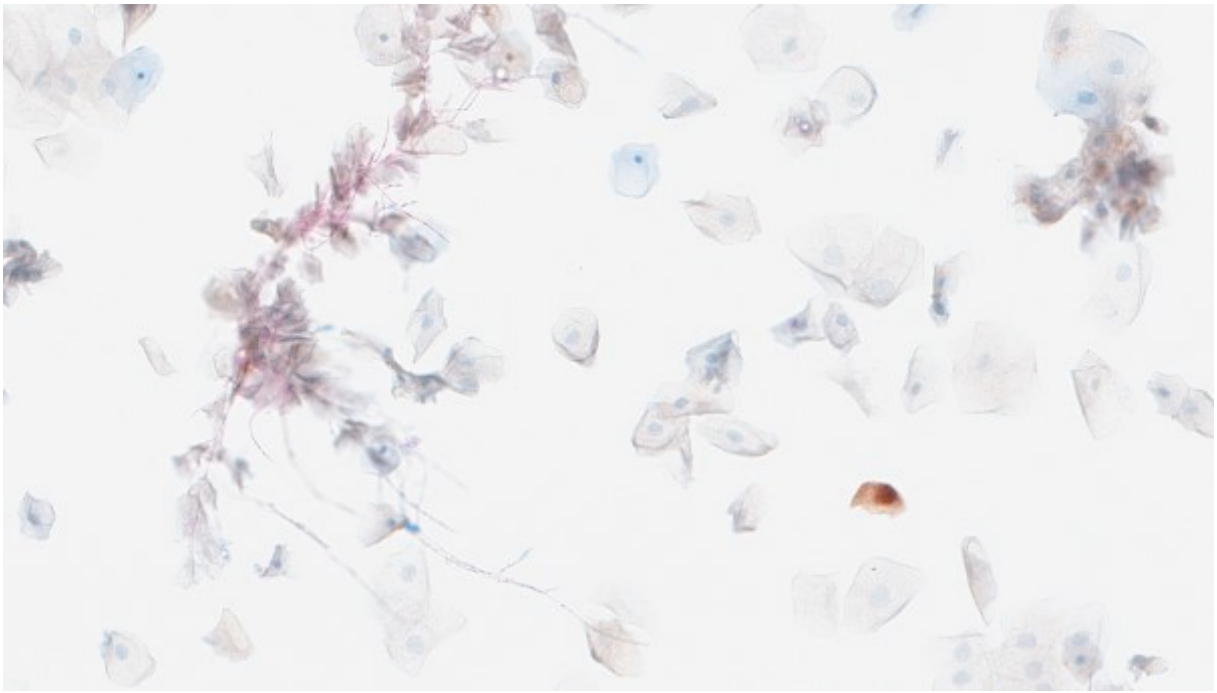

Figure 36: Scan one of cervical smear cells from case 1g stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

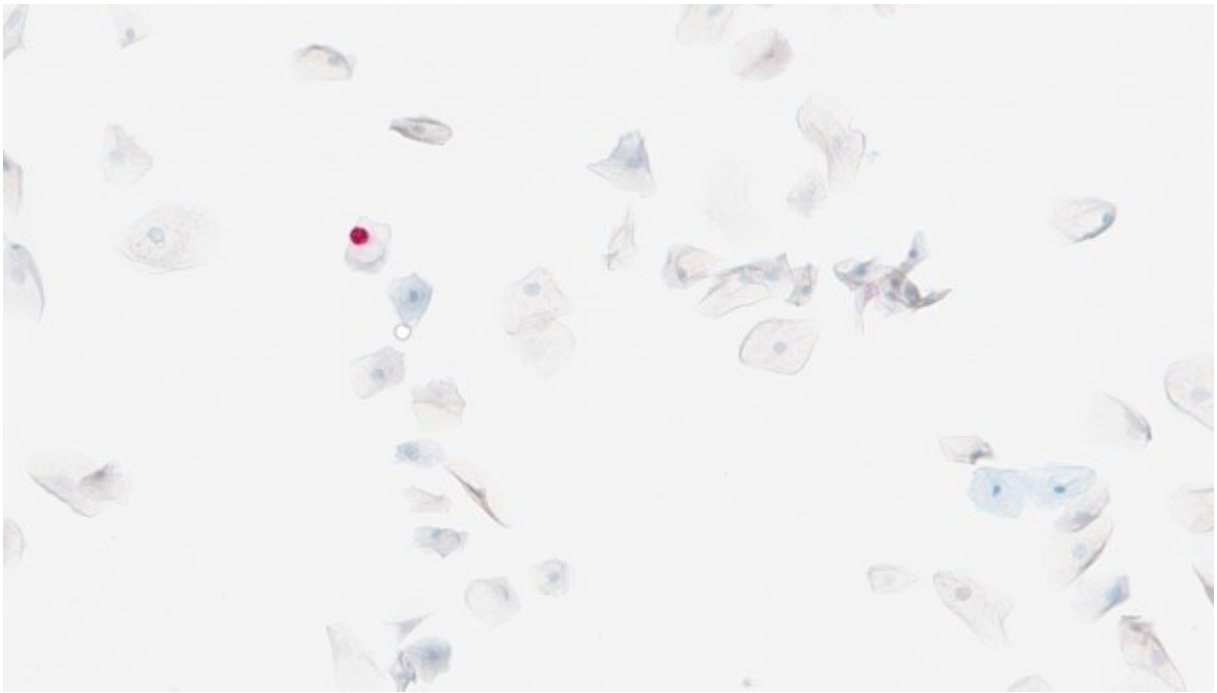

Figure 37: Scan two of cervical smear cells from case 1g stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1g – protocol y

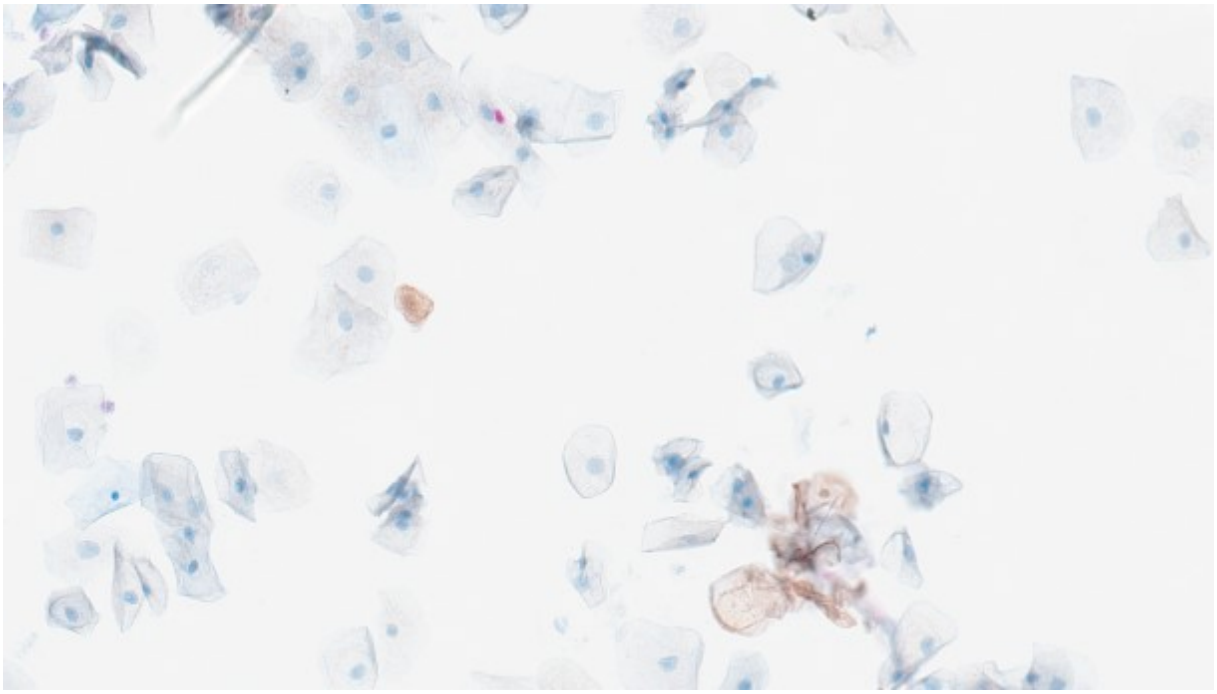

Figure 38: Scan one of cervical smear cells from case 1g stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

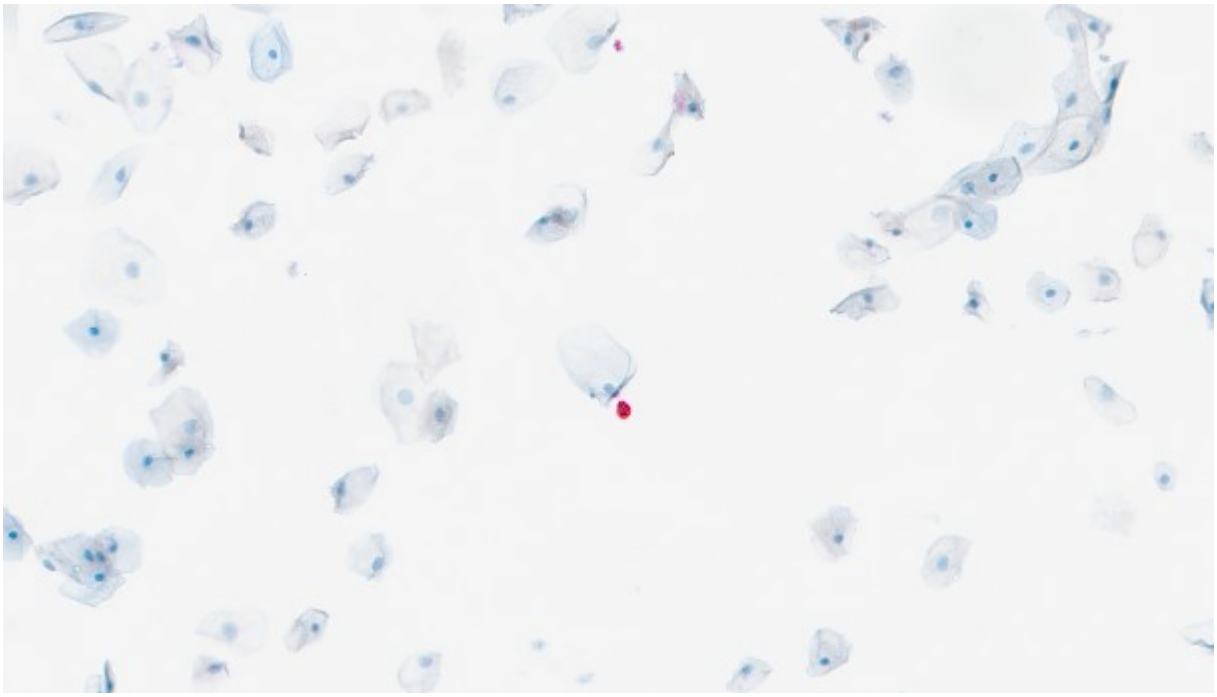

Figure 39: Scan two of cervical smear cells from case 1g stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1h – protocol x

Figure 40: Scan one of cervical smear cells from case 1h stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

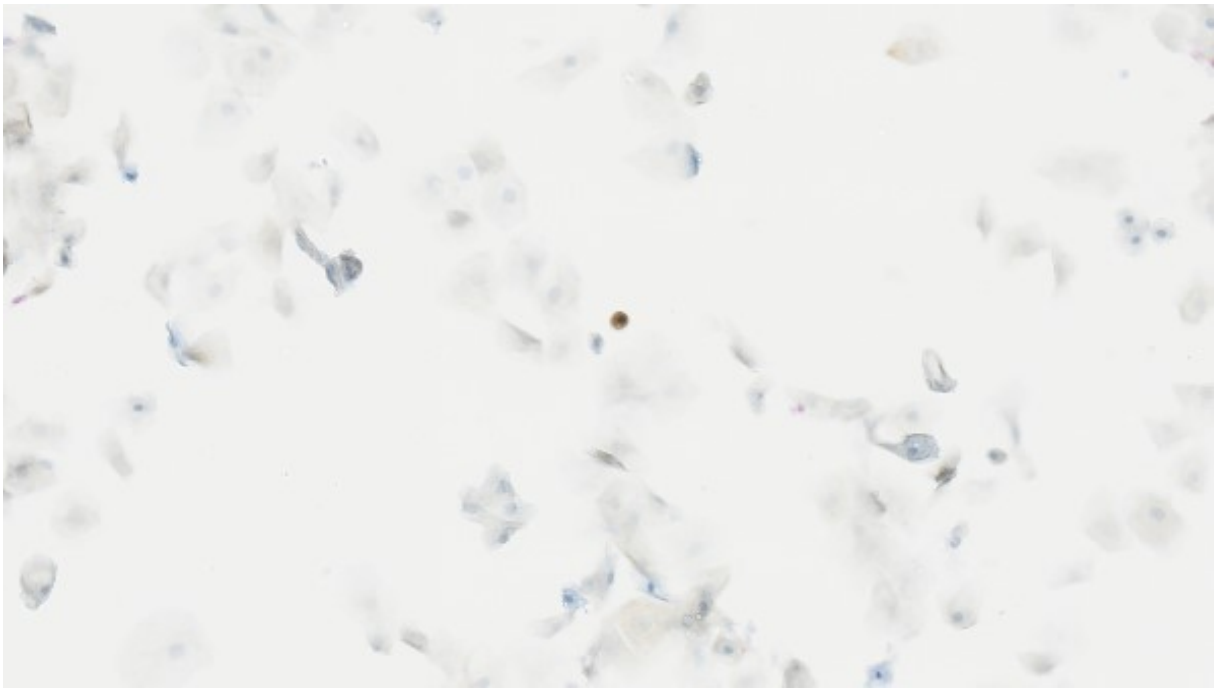

Figure 41: Scan two of cervical smear cells from case 1h stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 1h – protocol y

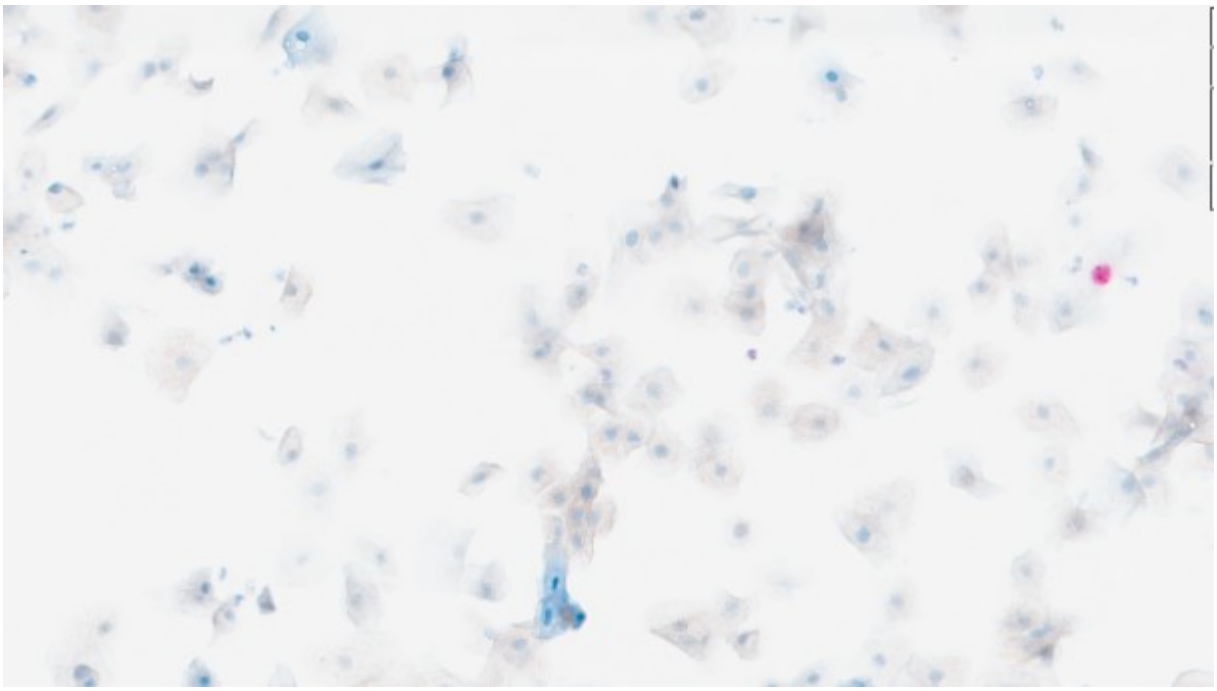

Figure 42: Scan one of cervical smear cells from case 1h stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

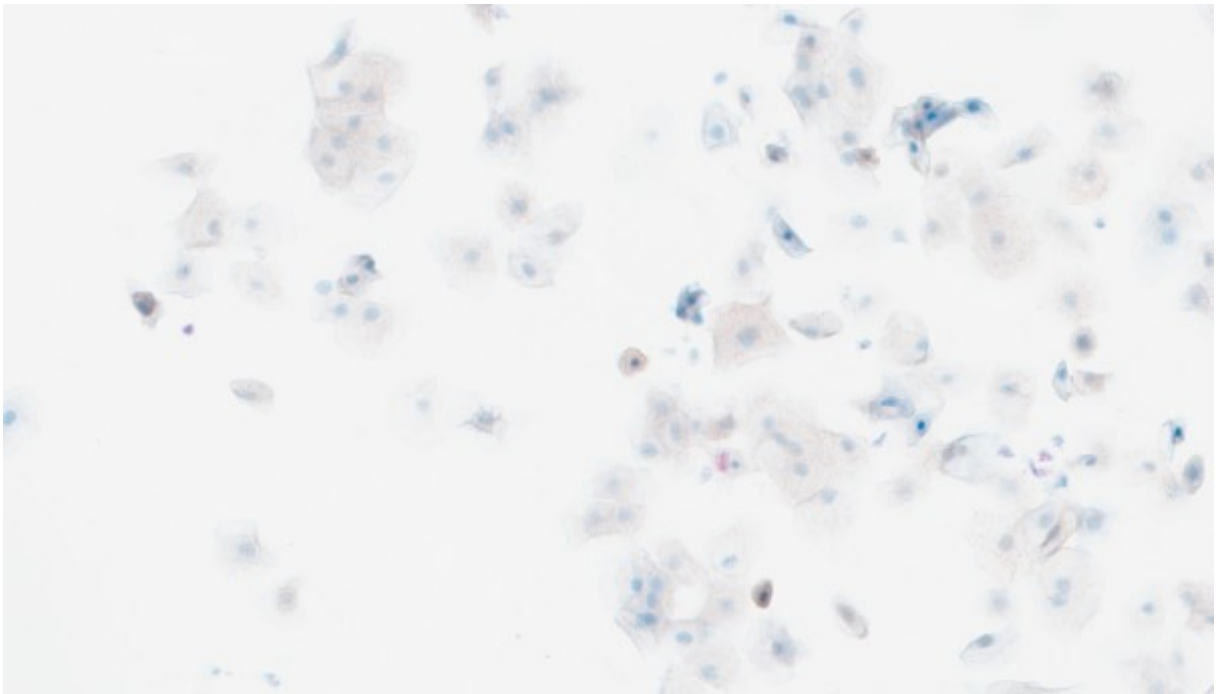

Figure 43: Scan two of cervical smear cells from case 1h stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2a – protocol x

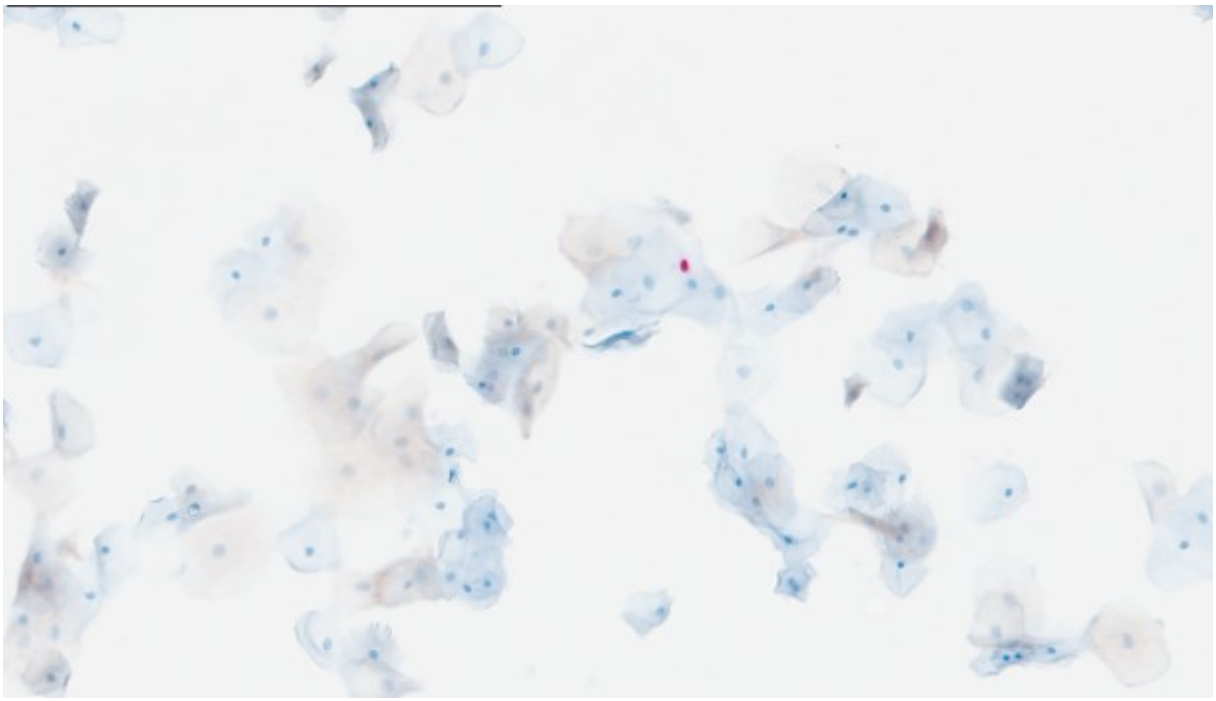

Figure 44: Scan one of cervical smear cells from case 2a stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

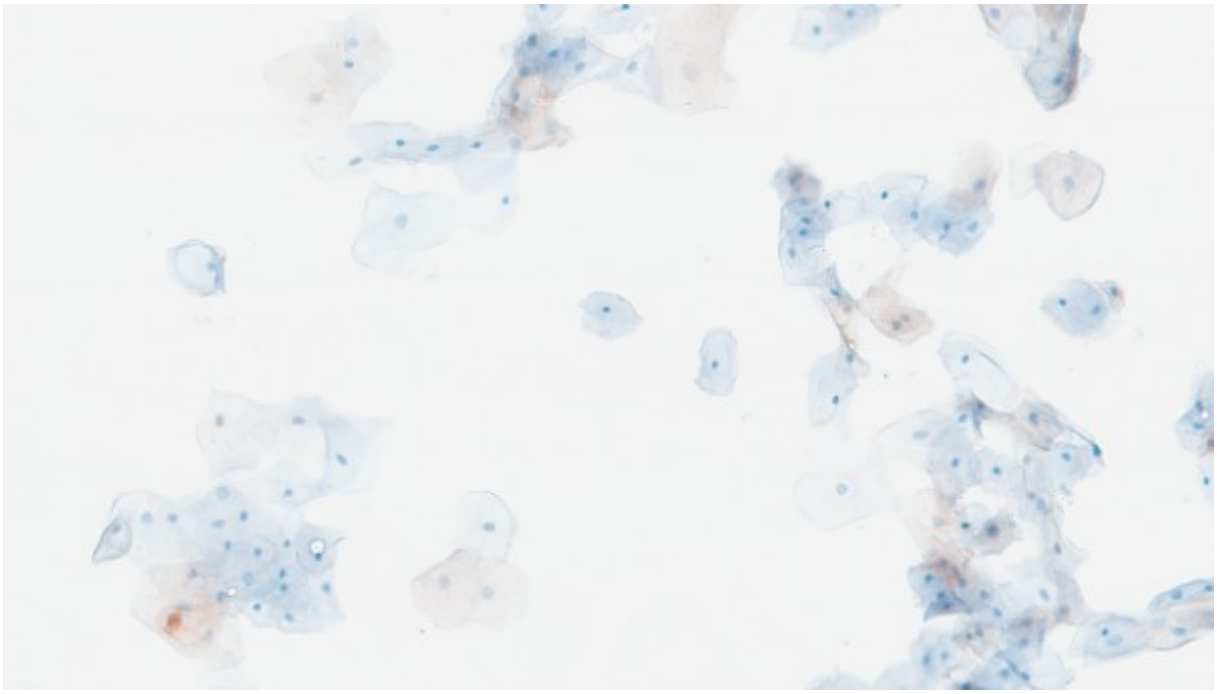

Figure 45: Scan two of cervical smear cells from case 2a stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2a – protocol y

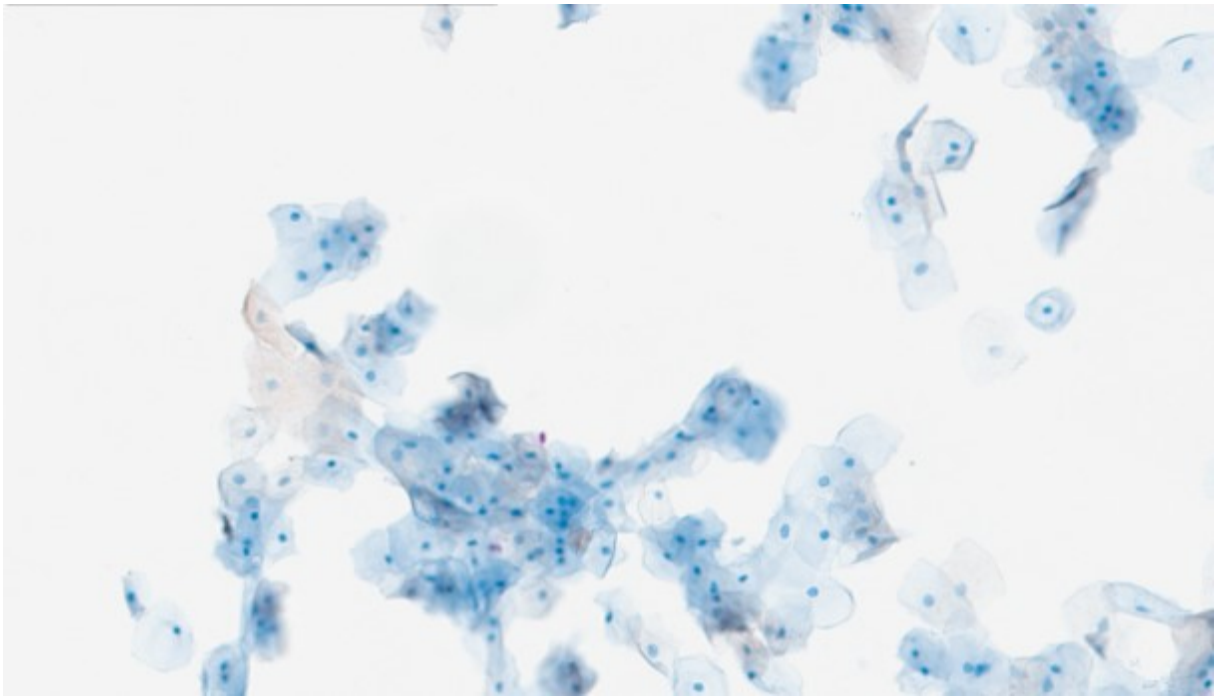

Figure 46: Scan one of cervical smear cells from case 2a stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

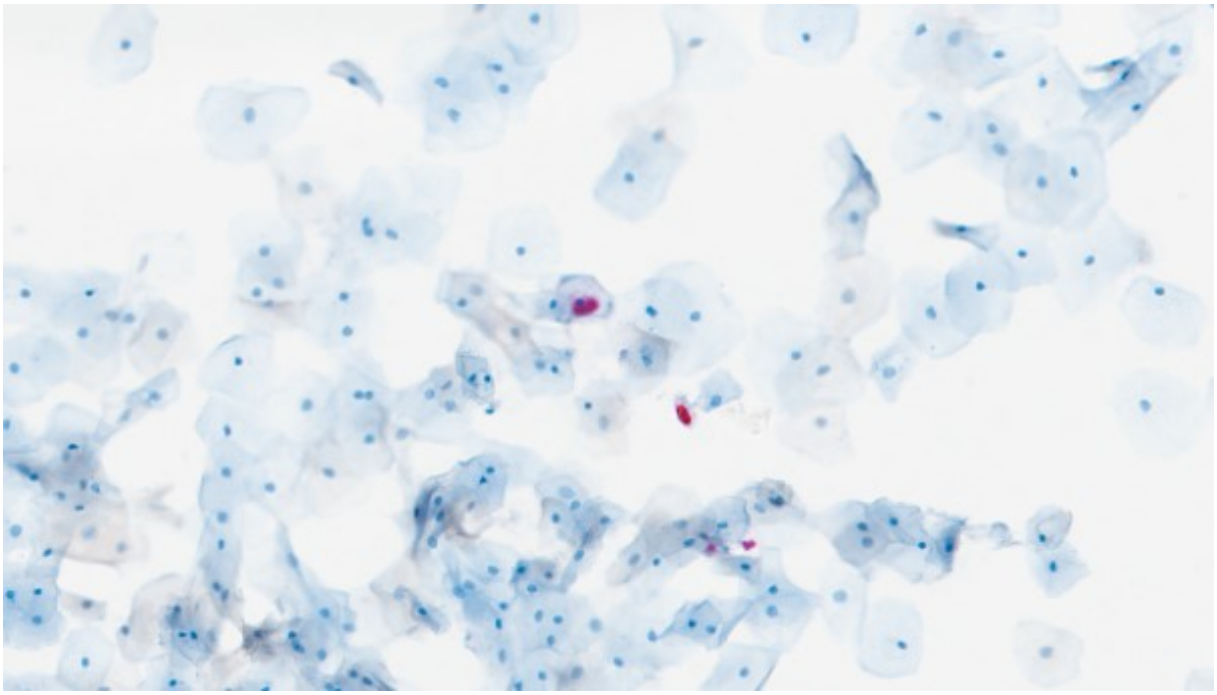

Figure 47: Scan two of cervical smear cells from case 2a stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2b – protocol x

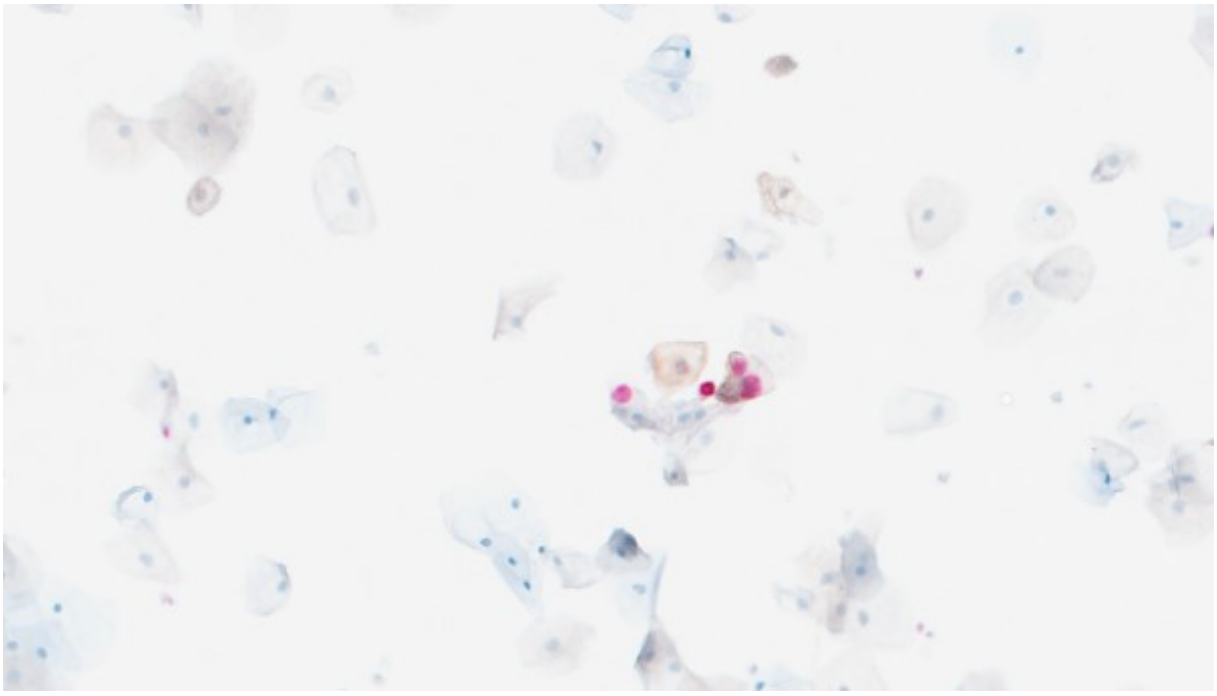

Figure 48: Scan one of cervical smear cells from case 2b stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

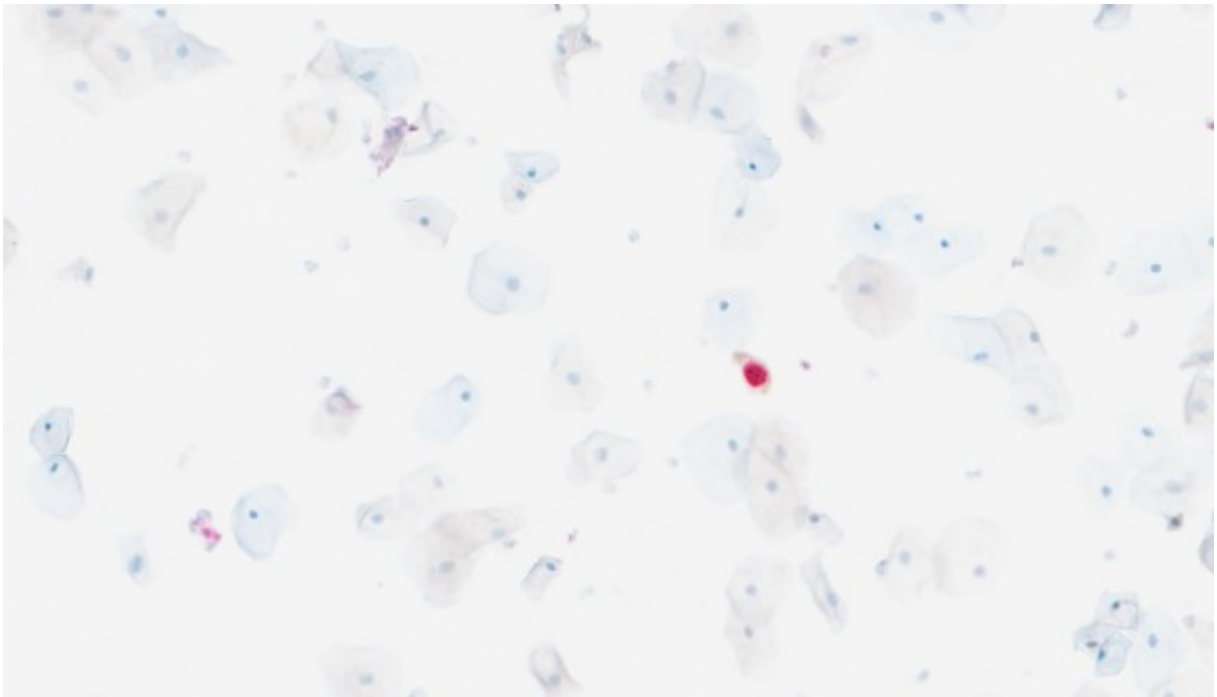

Figure 49: Scan two of cervical smear cells from case 2b stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2b – protocol y

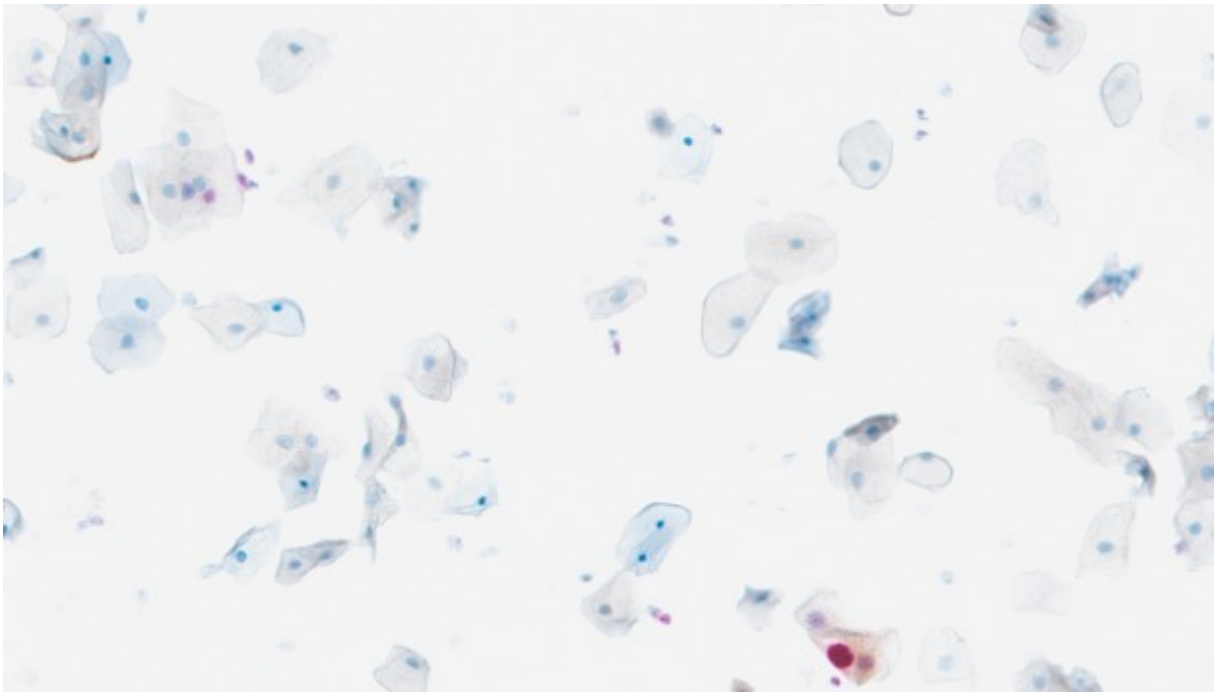

Figure 50: Scan one of cervical smear cells from case 2b stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

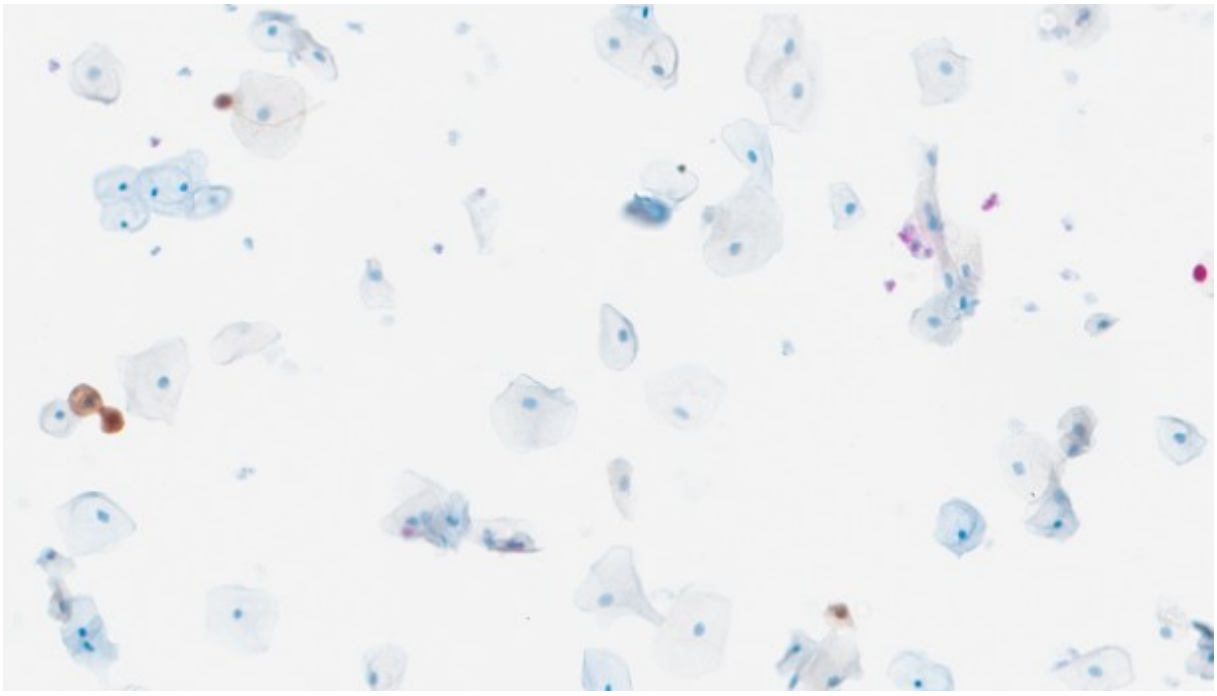

Figure 51: Scan two of cervical smear cells from case 2b stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2f – protocol x

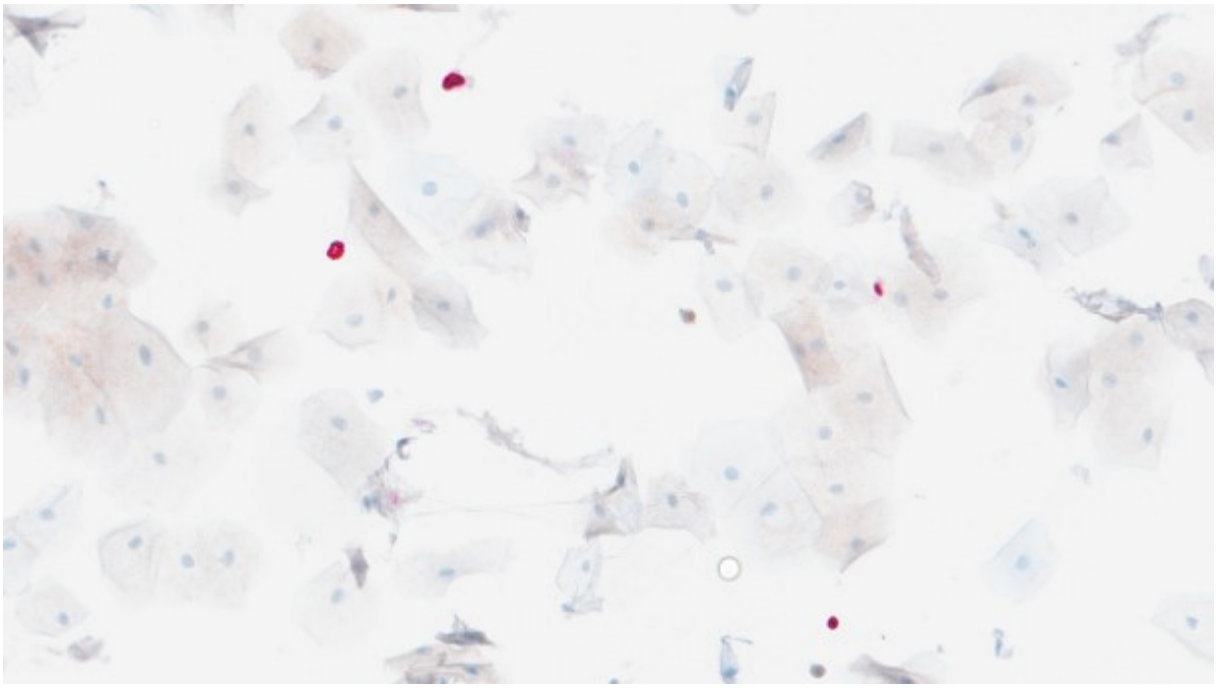

Figure 52: Scan one of cervical smear cells from case 2f stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

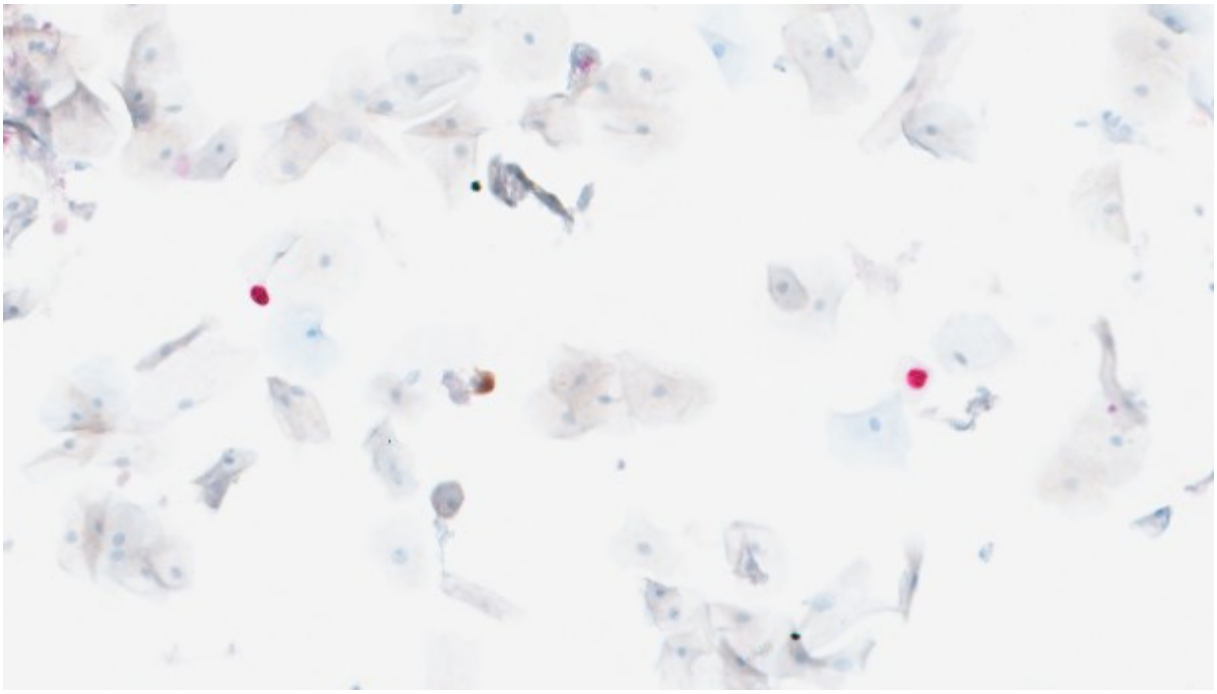

Figure 53: Scan two of cervical smear cells from case 2f stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2g – protocol x

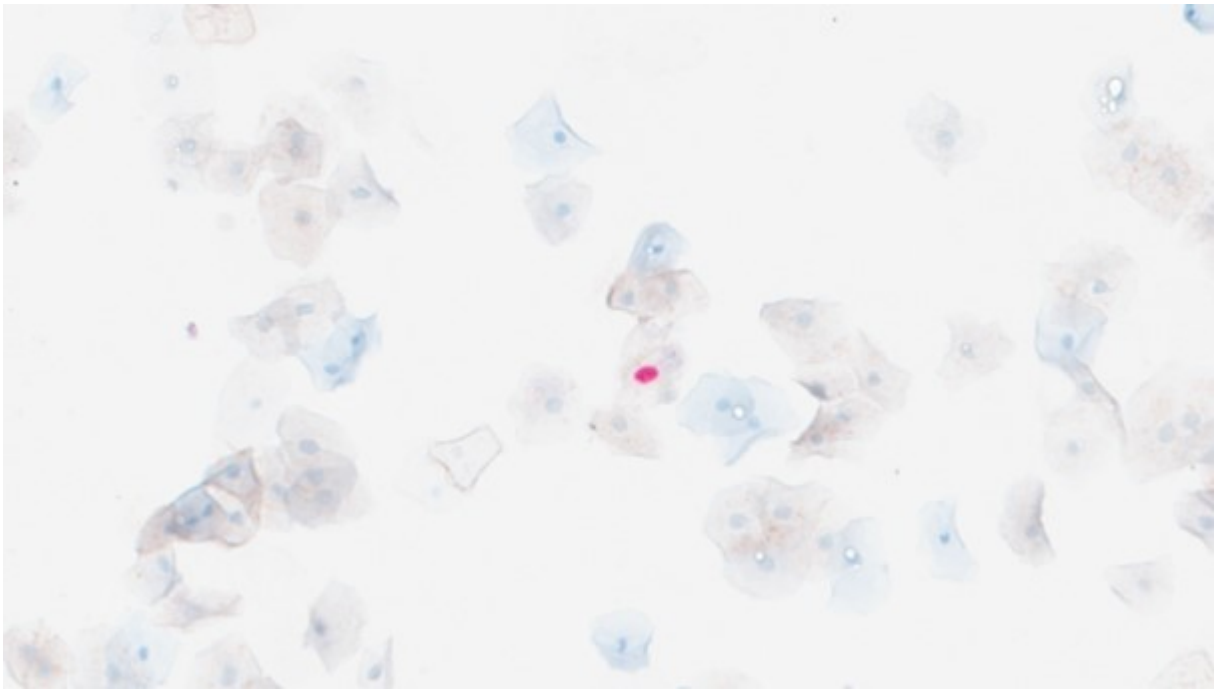

Figure 54: Scan one of cervical smear cells from case 2g stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

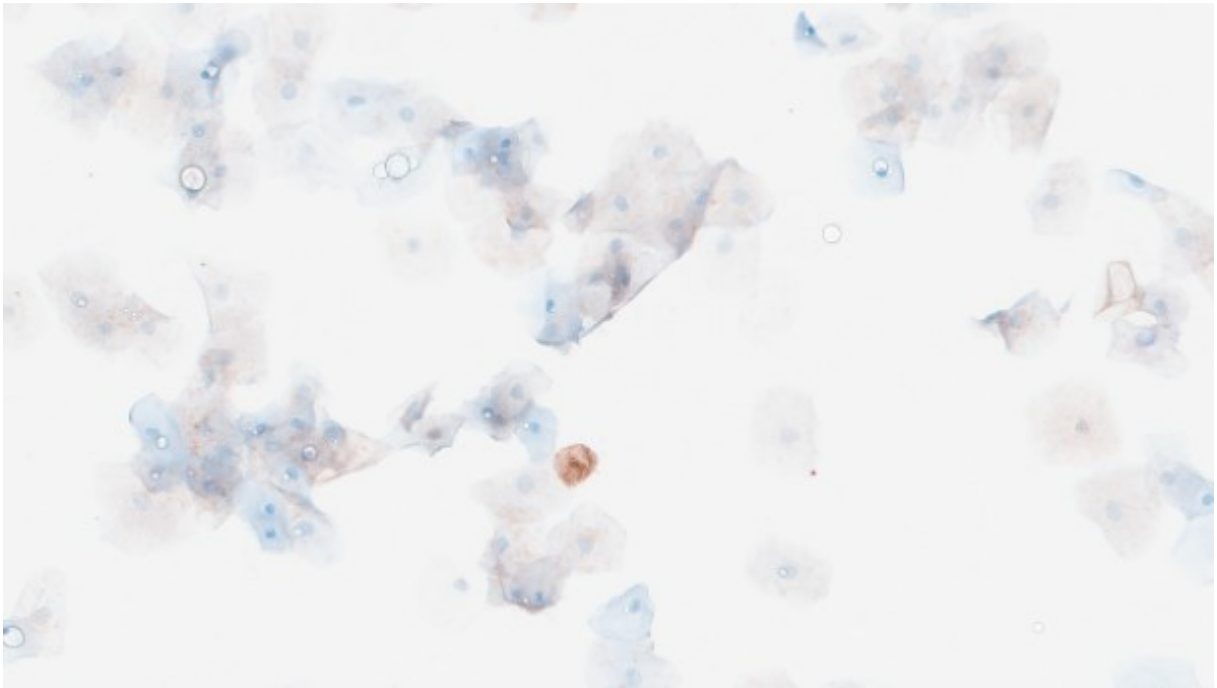

Figure 55: Scan two of cervical smear cells from case 2g stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2g – protocol y

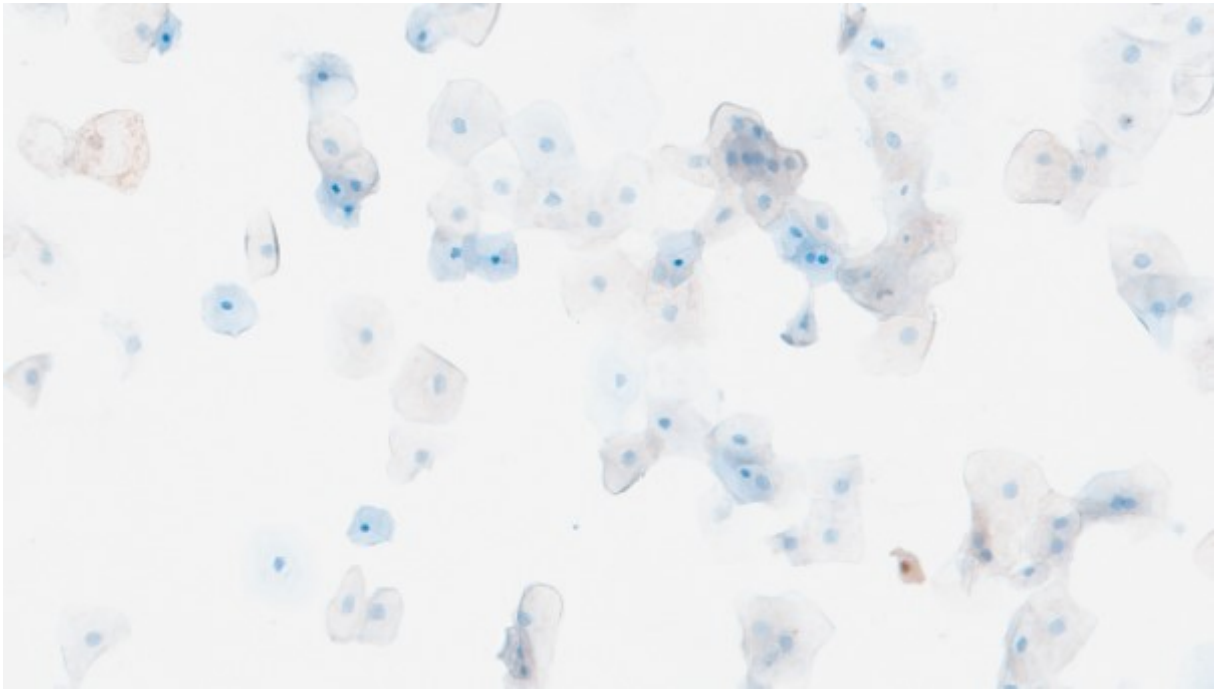

Figure 56: Scan one of cervical smear cells from case 2g stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

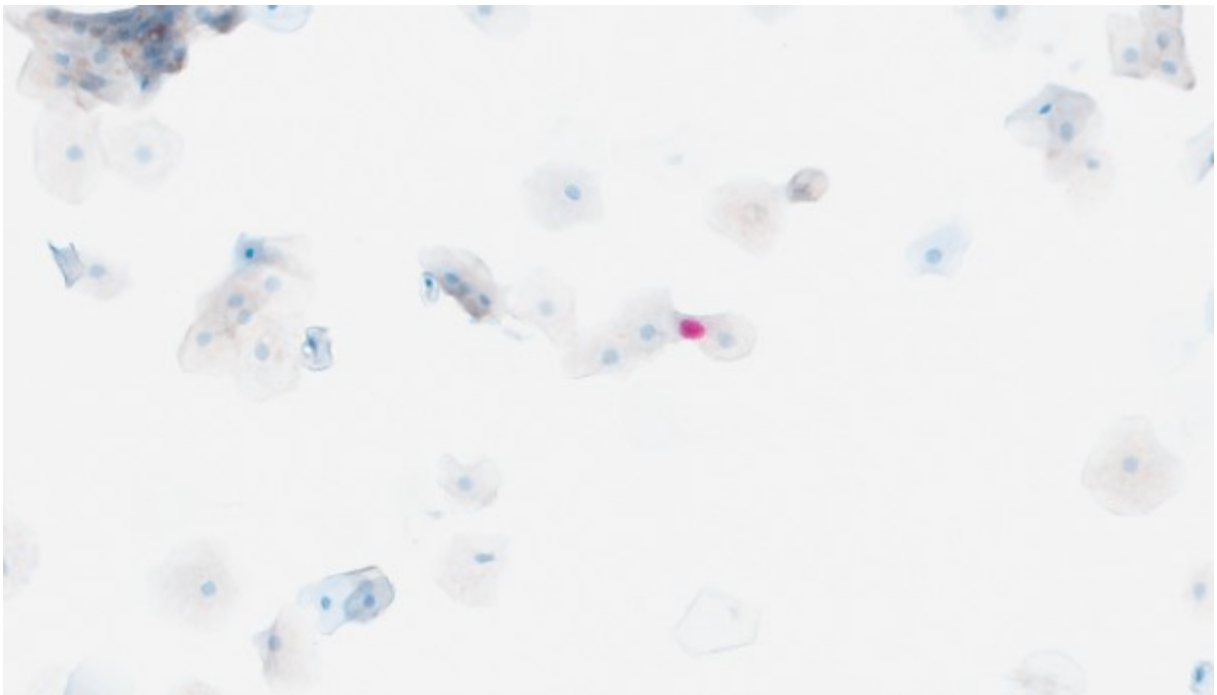

Figure 57: Scan one of cervical smear cells from case 2g stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2h – protocol x

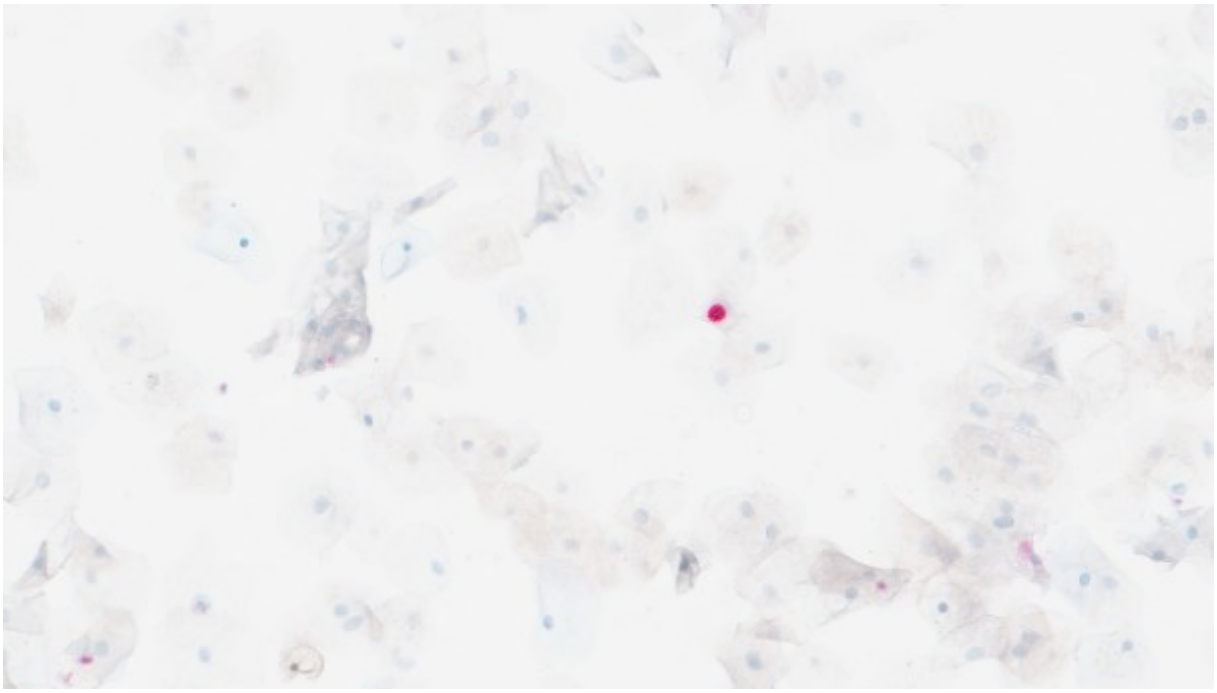

Figure 58: Scan one of cervical smear cells from case 2h stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

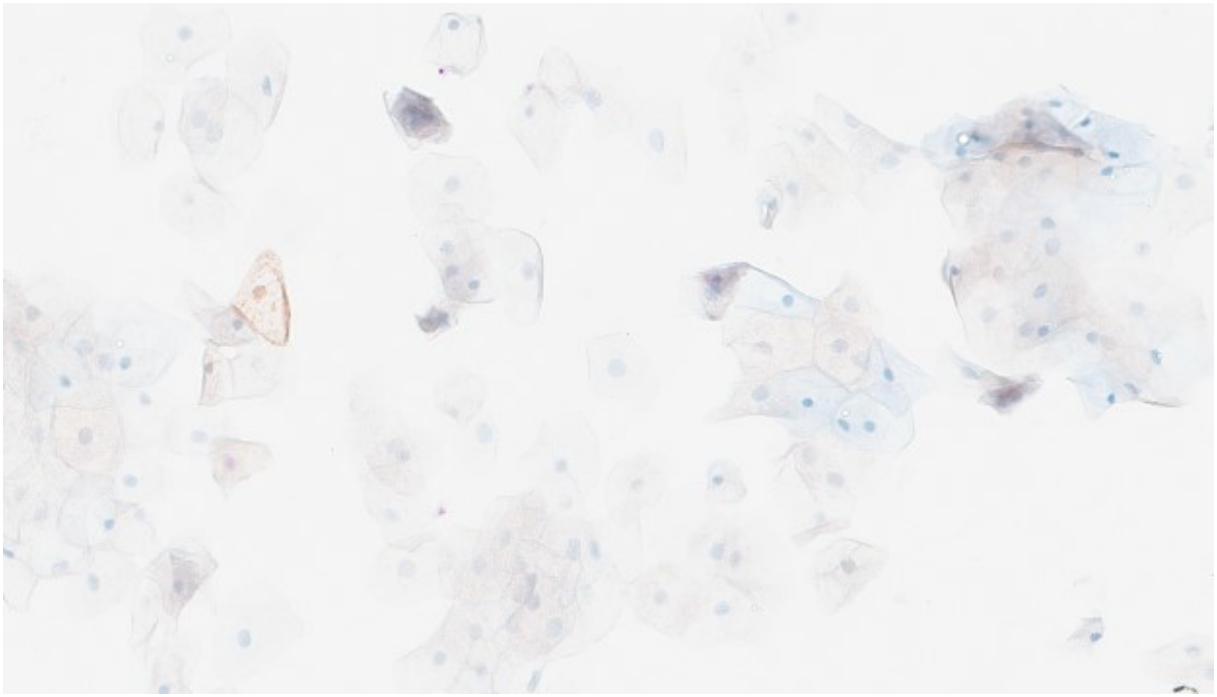

Figure 59: Scan two of cervical smear cells from case 2h stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 2h – protocol y

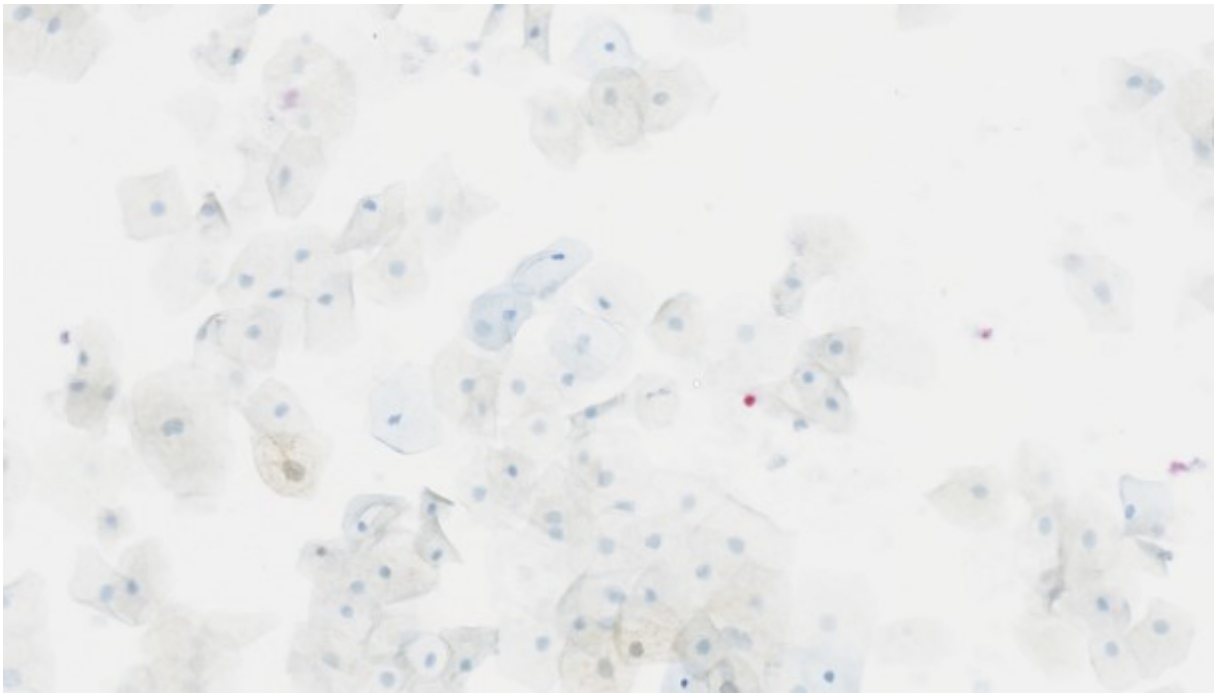

Figure 60: Scan one of cervical smear cells from case 2h stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

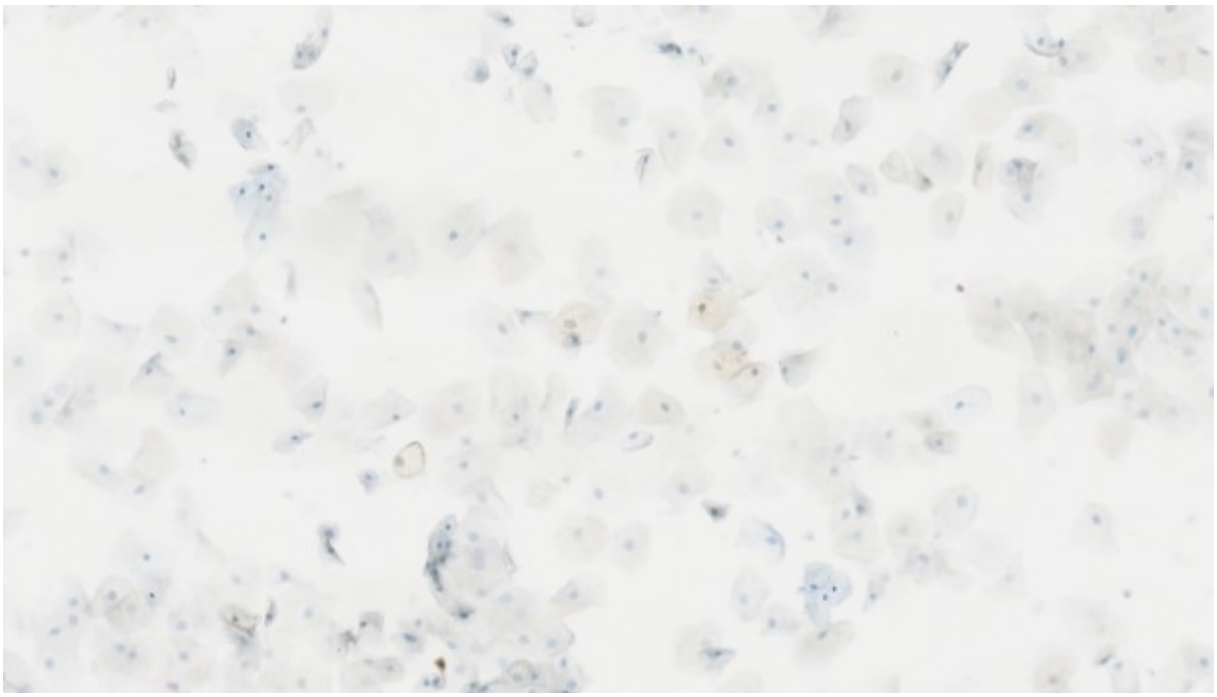

Figure 61: Scan two of cervical smear cells from case 2h stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3a – protocol x

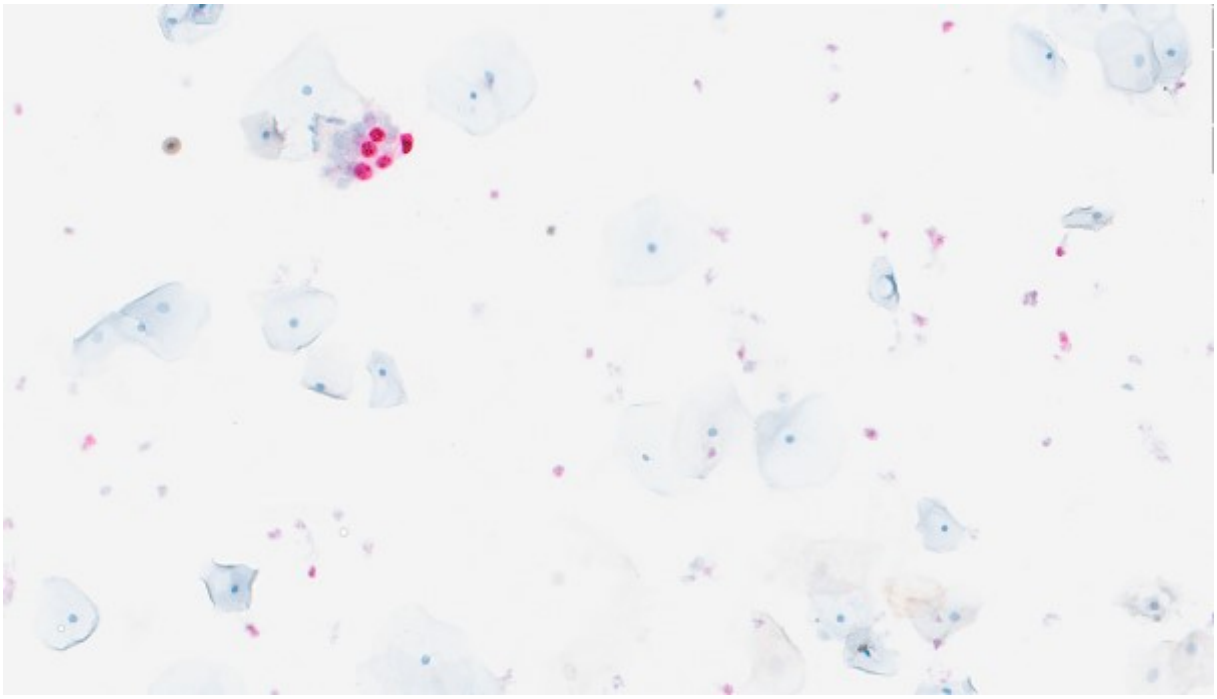

Figure 62: Scan one of cervical smear cells from case 3a stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Figure 63: Scan two of cervical smear cells from case 3a stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3a – protocol y

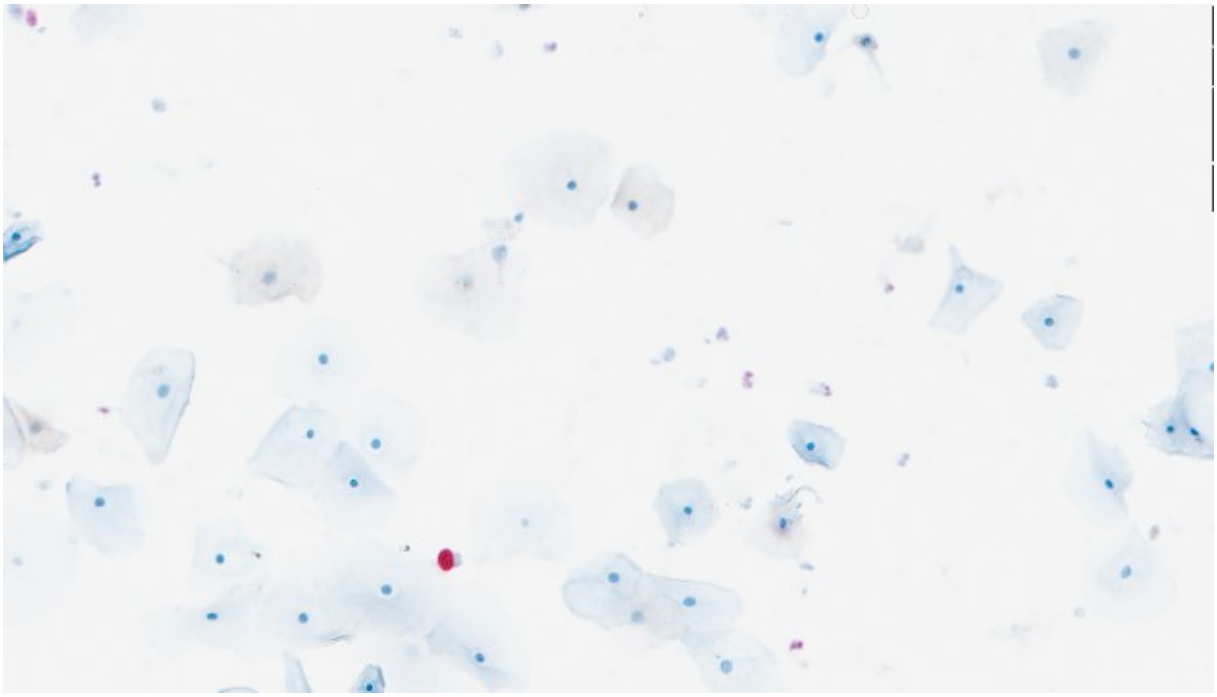

Figure 64: Scan one of cervical smear cells from case 3a stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

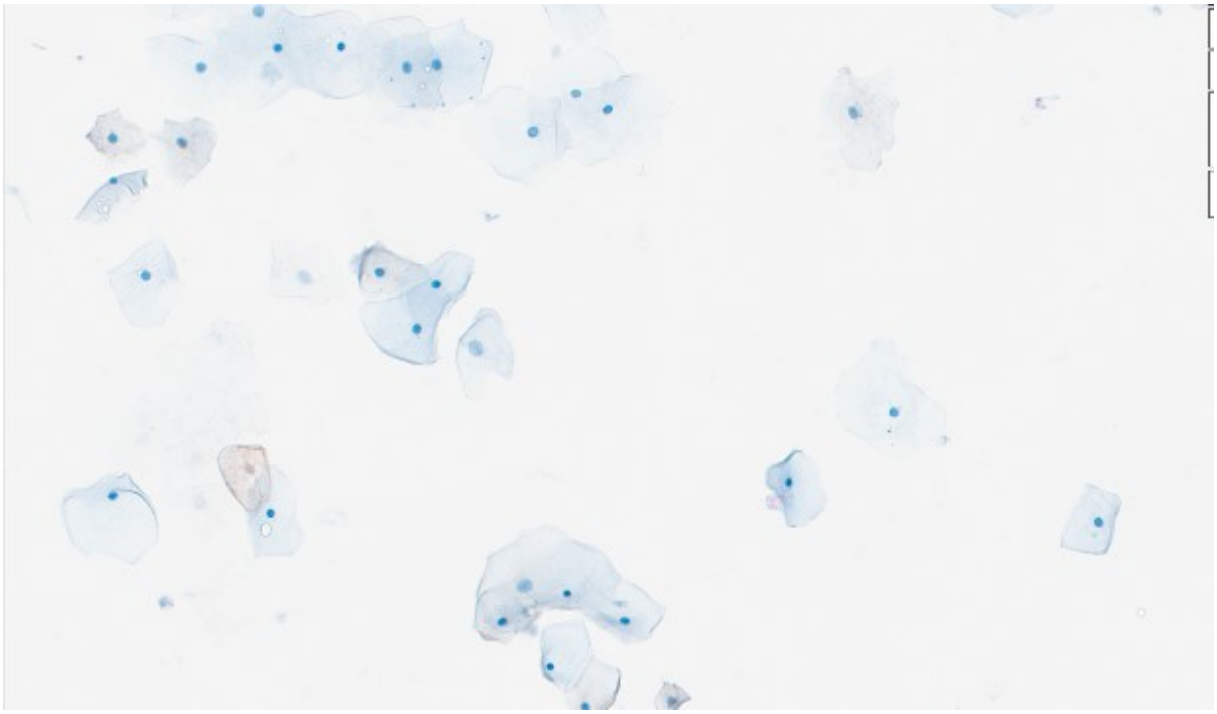

Figure 65: Scan two of cervical smear cells from case 3a stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3b – protocol y

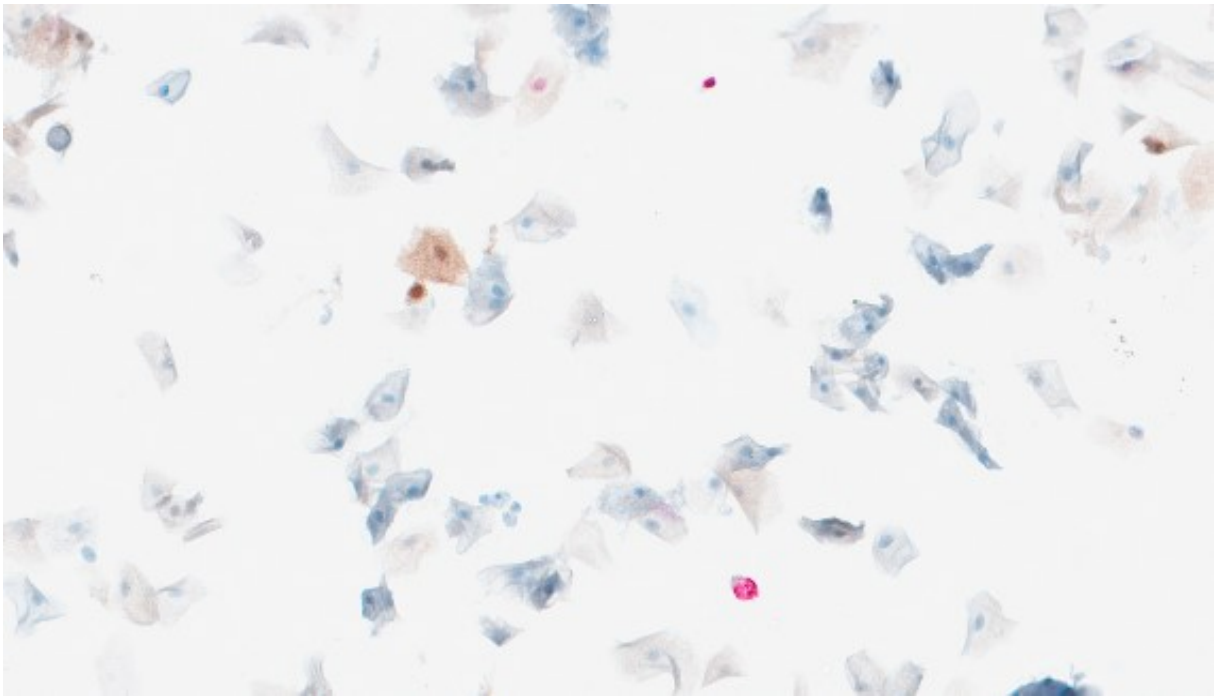

Figure 66: Scan one of cervical smear cells from case 3b stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

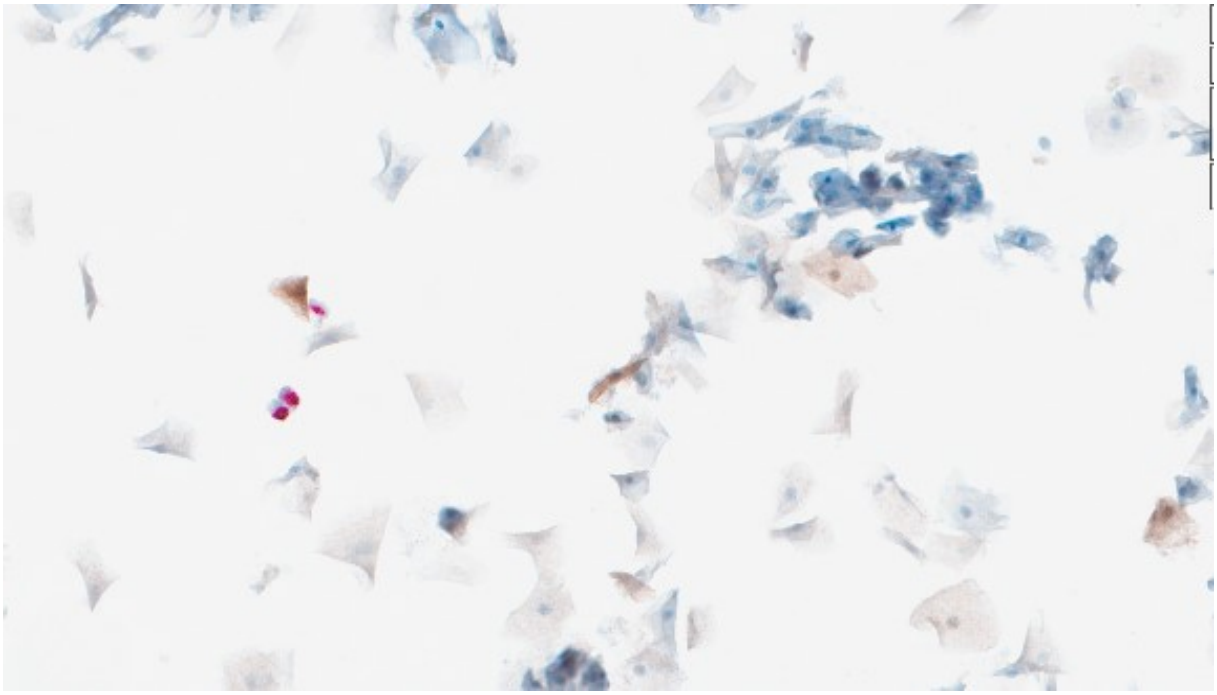

Figure 67: Scan two of cervical smear cells from case 3b stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3c – protocol y

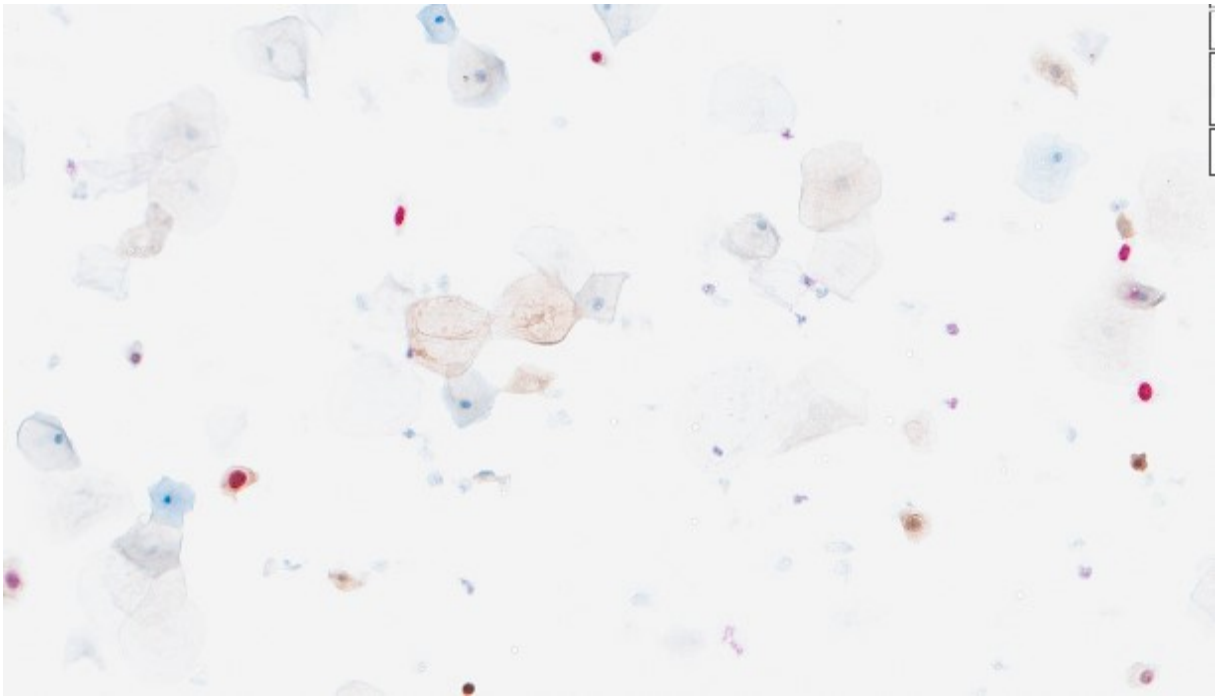

Figure 68: Scan one of cervical smear cells from case 3c stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

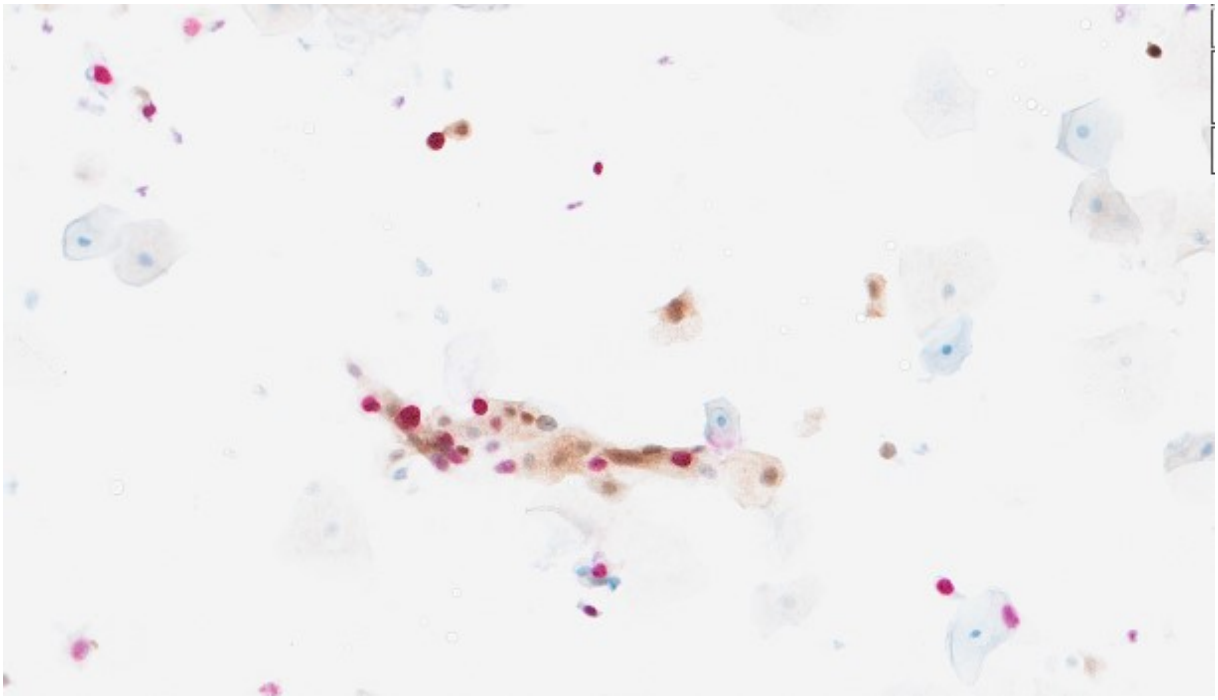

Figure 69: Scan two of cervical smear cells from case 3c stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3d – protocol y

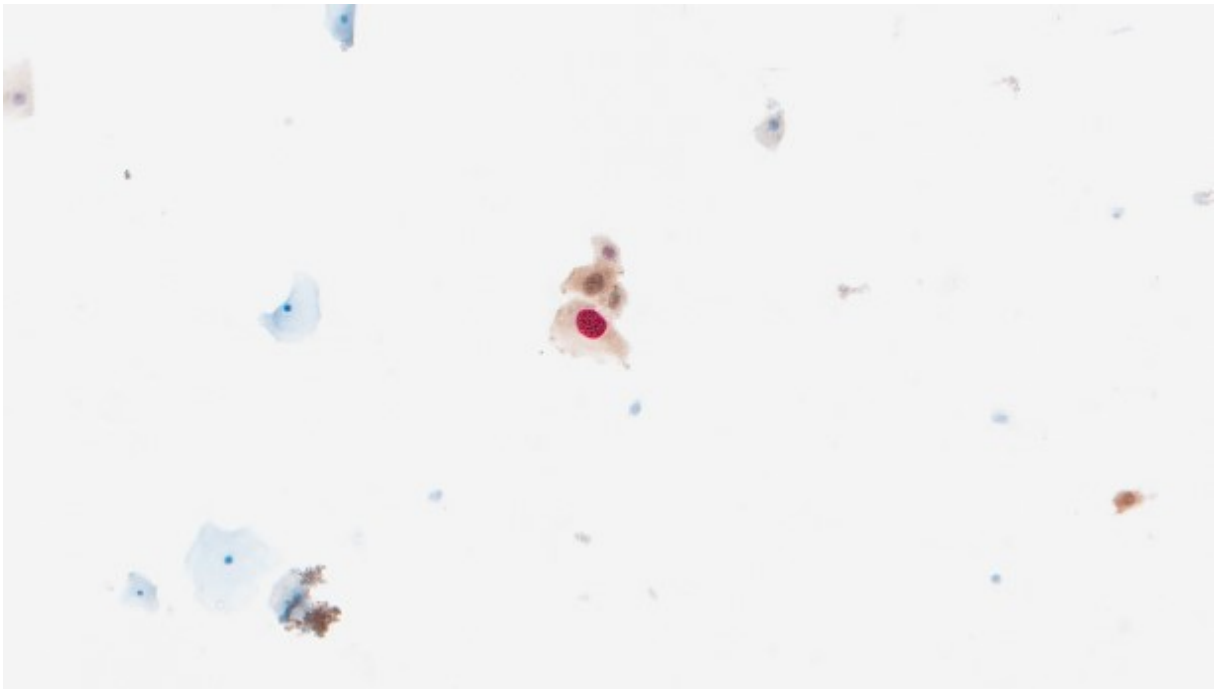

Figure 70: Scan one of cervical smear cells from case 3d stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

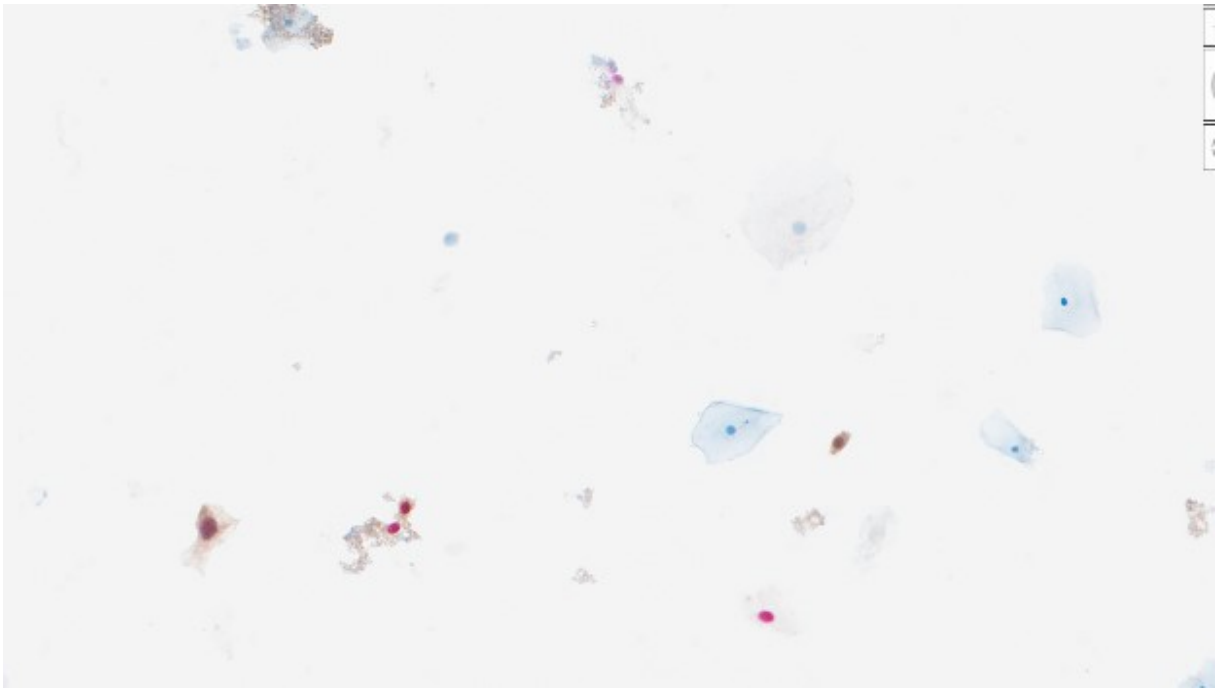

Figure 71: Scan two of cervical smear cells from case 3d stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3e – protocol x

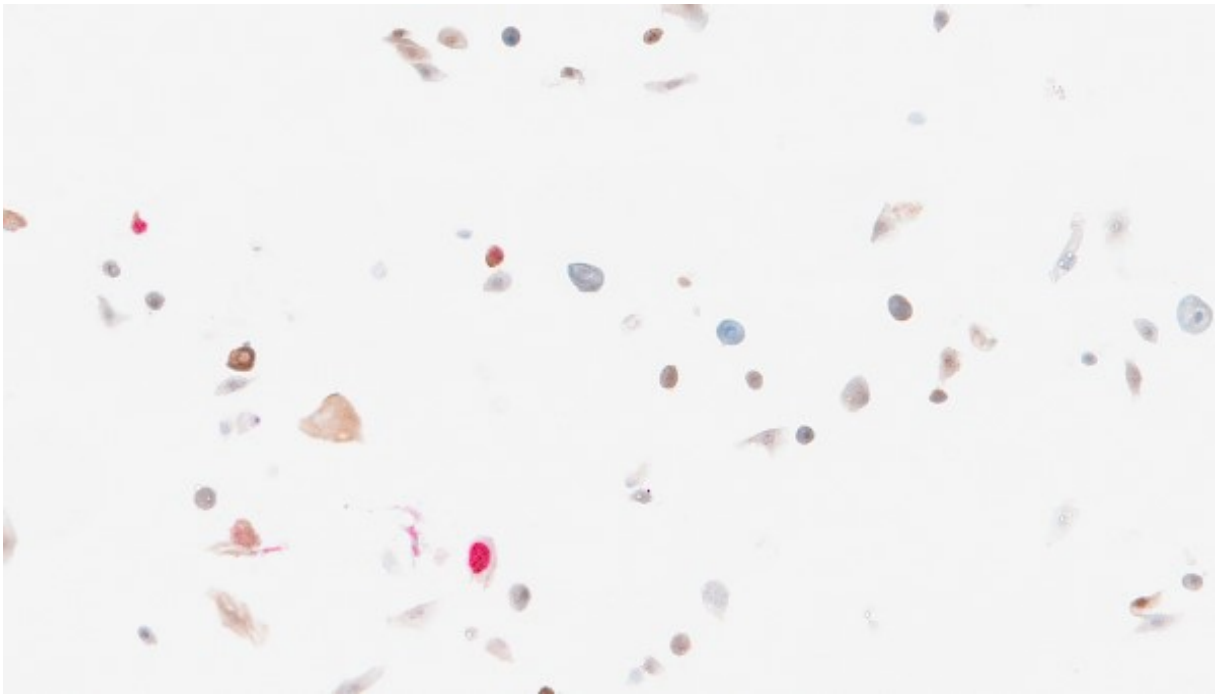

Figure 72: Scan one of cervical smear cells from case 3e stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

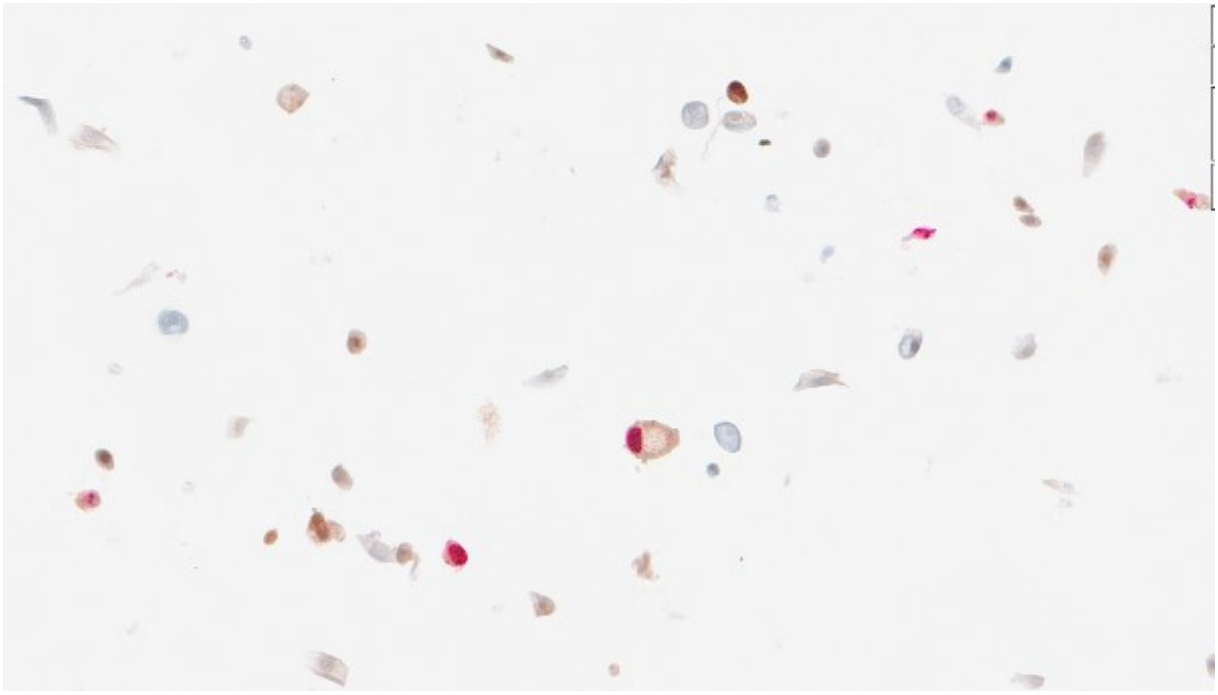

Figure 73: Scan two of cervical smear cells from case 3e stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3f – protocol x

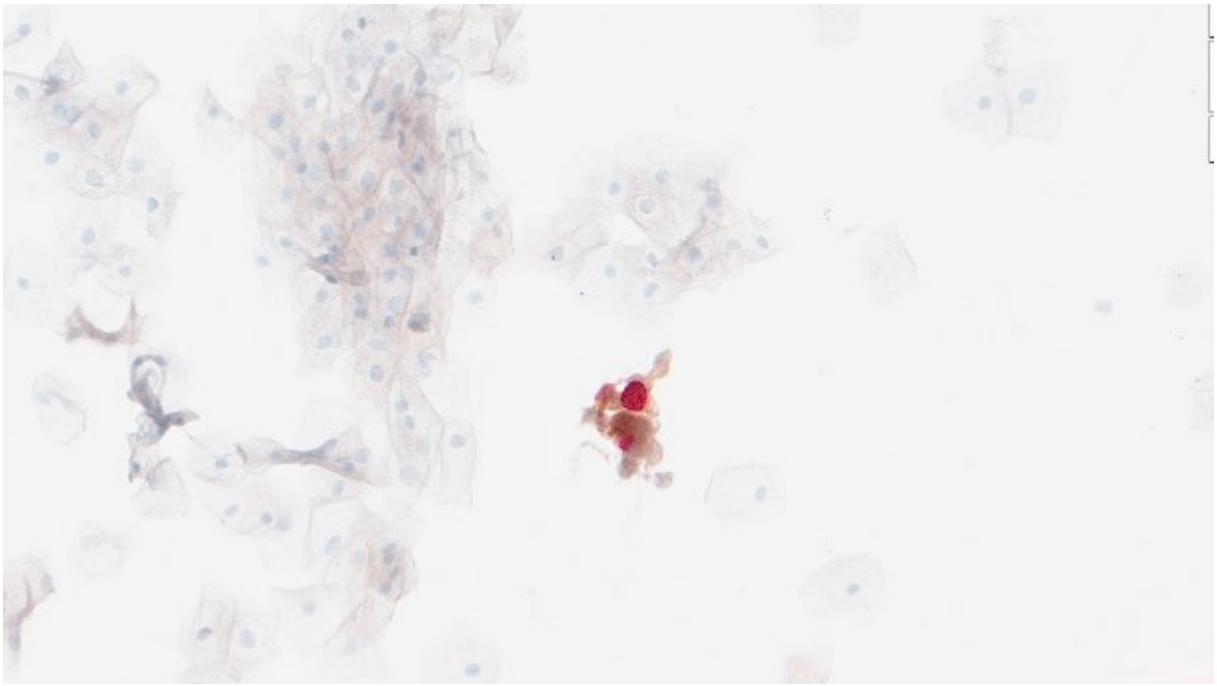

Figure 74: Scan one of cervical smear cells from case 3f stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

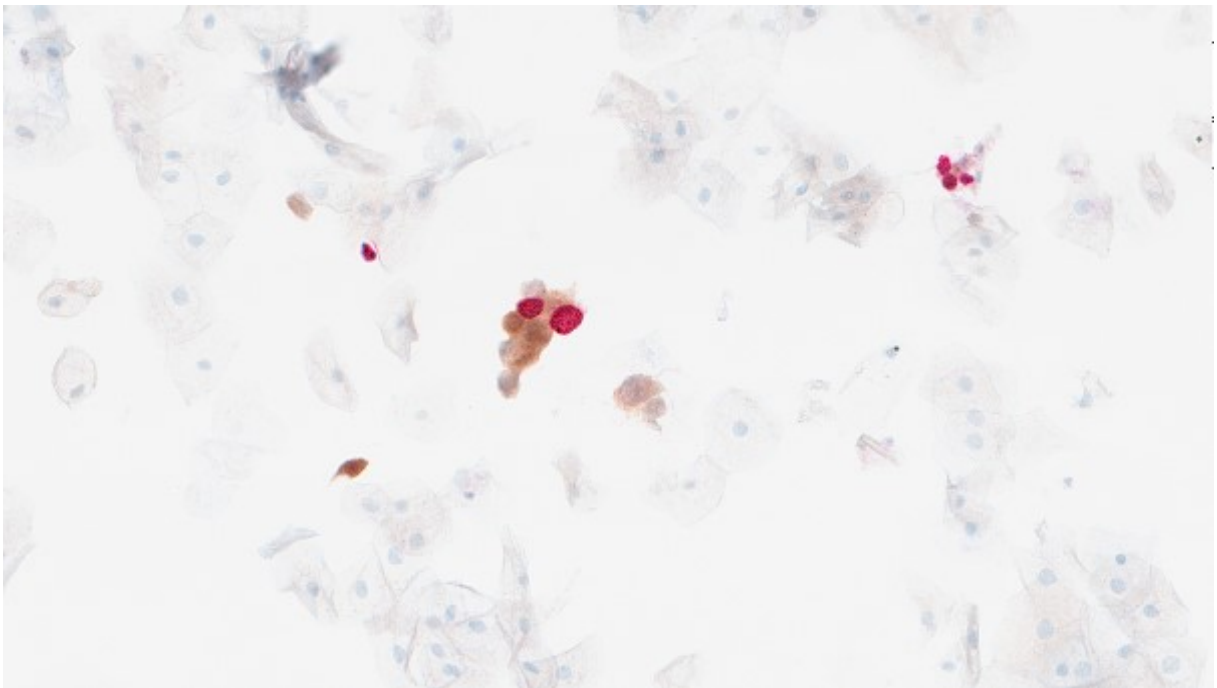

Figure 75: Scan two of cervical smear cells from case 3f stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3f – protocol y

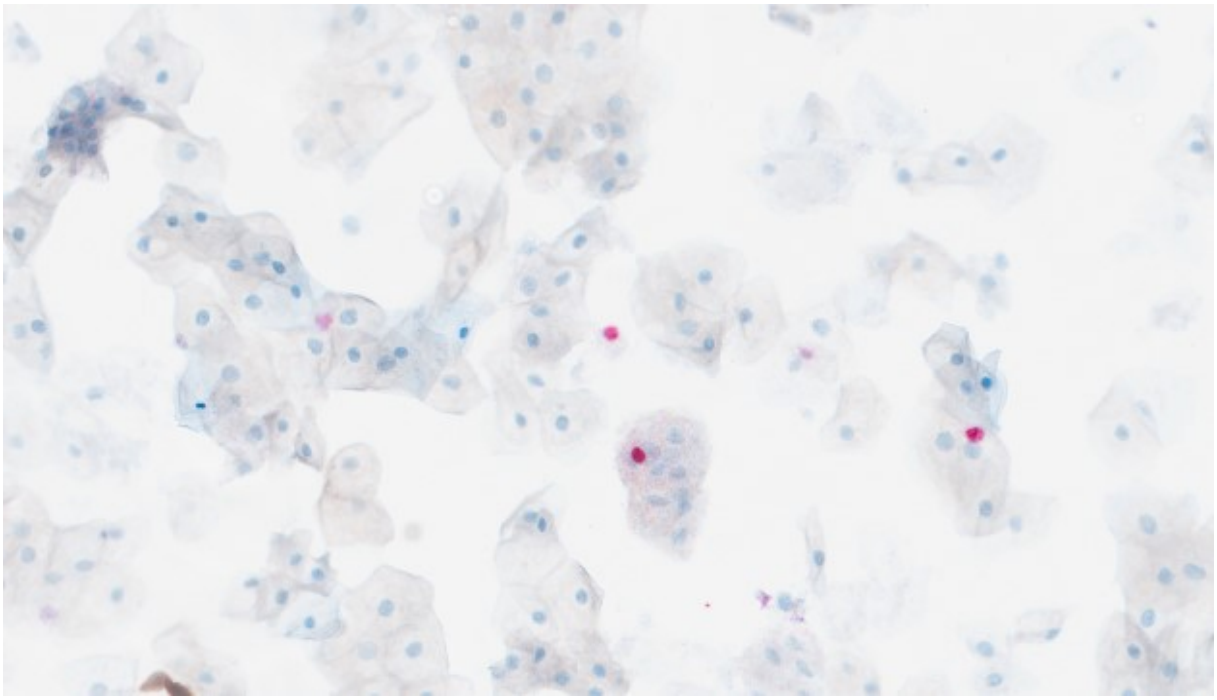

Figure 76: Scan one of cervical smear cells from case 3f stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

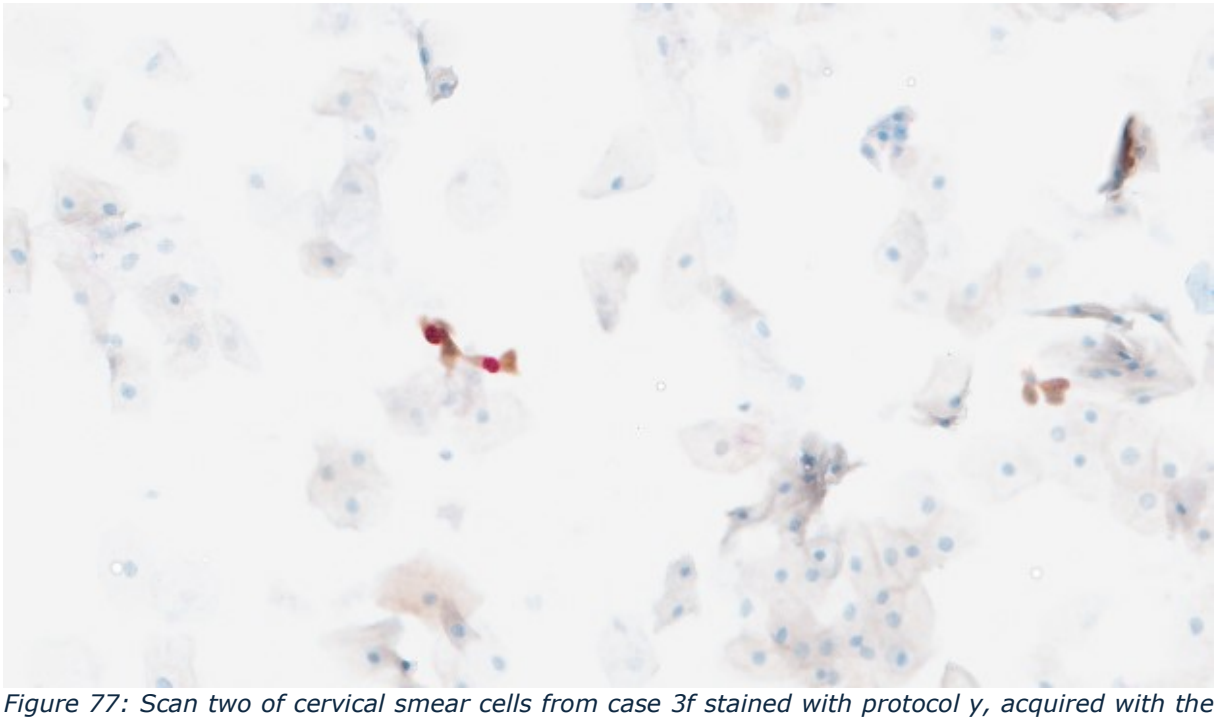

Figure 77: Scan two of cervical smear cells from case 3f stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3g – protocol x

Figure 78: Scan one of cervical smear cells from case 3g stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

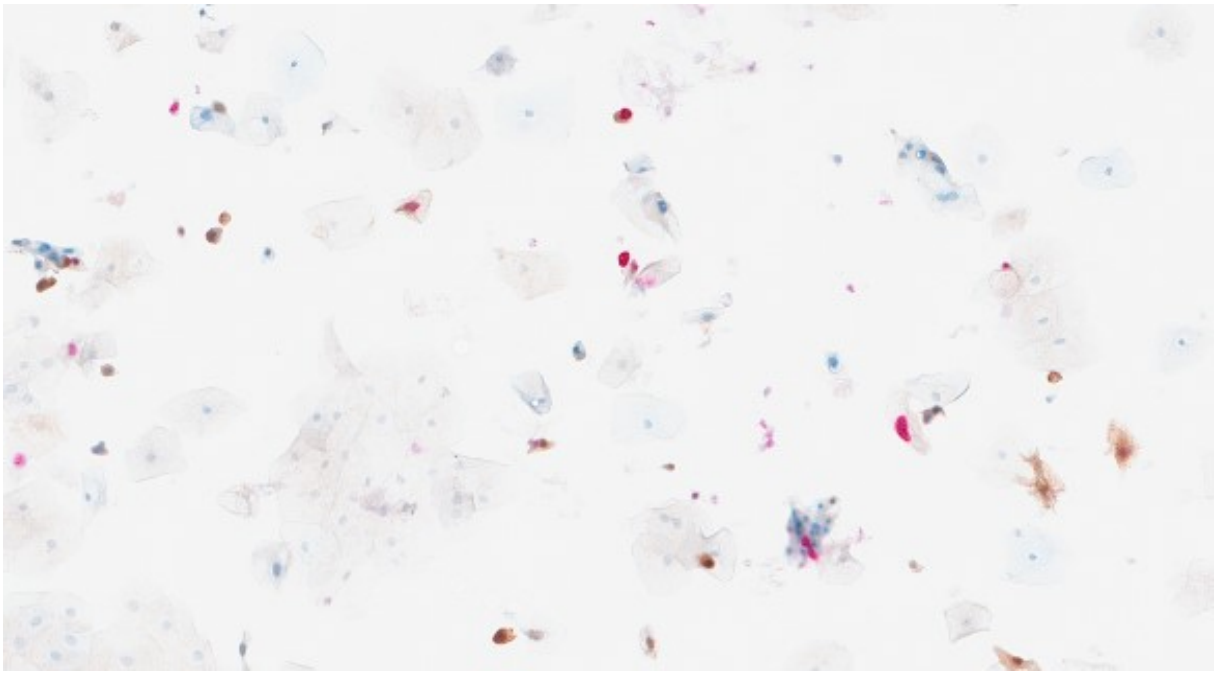

Figure 79: Scan two of cervical smear cells from case 3f stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3g – protocol y

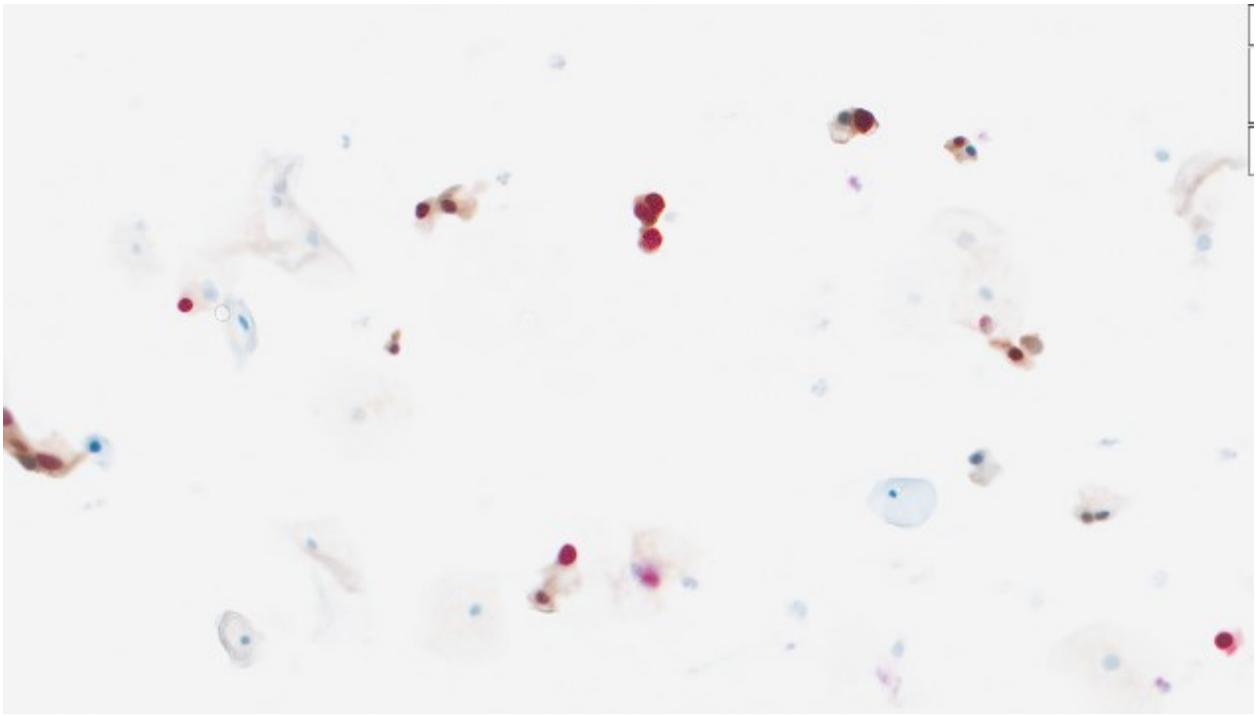

Figure 80: Scan one of cervical smear cells from case 3g stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

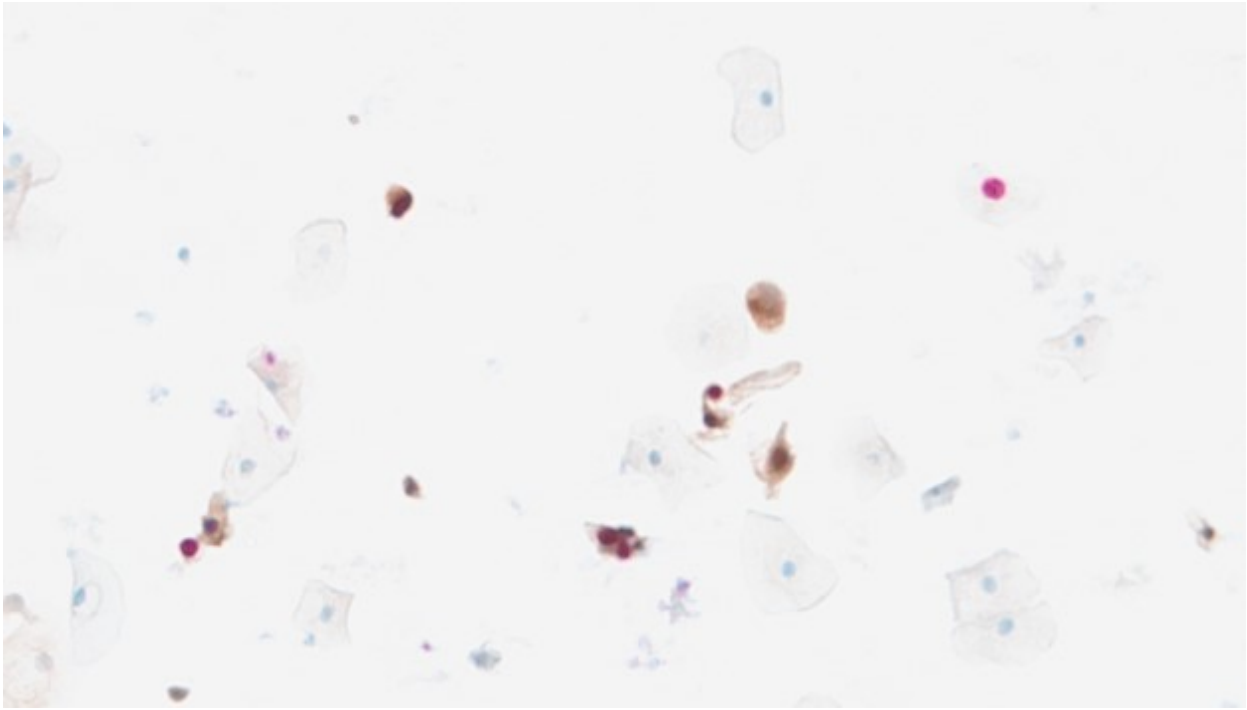

Figure 81: Scan two of cervical smear cells from case 3g stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3h – protocol x

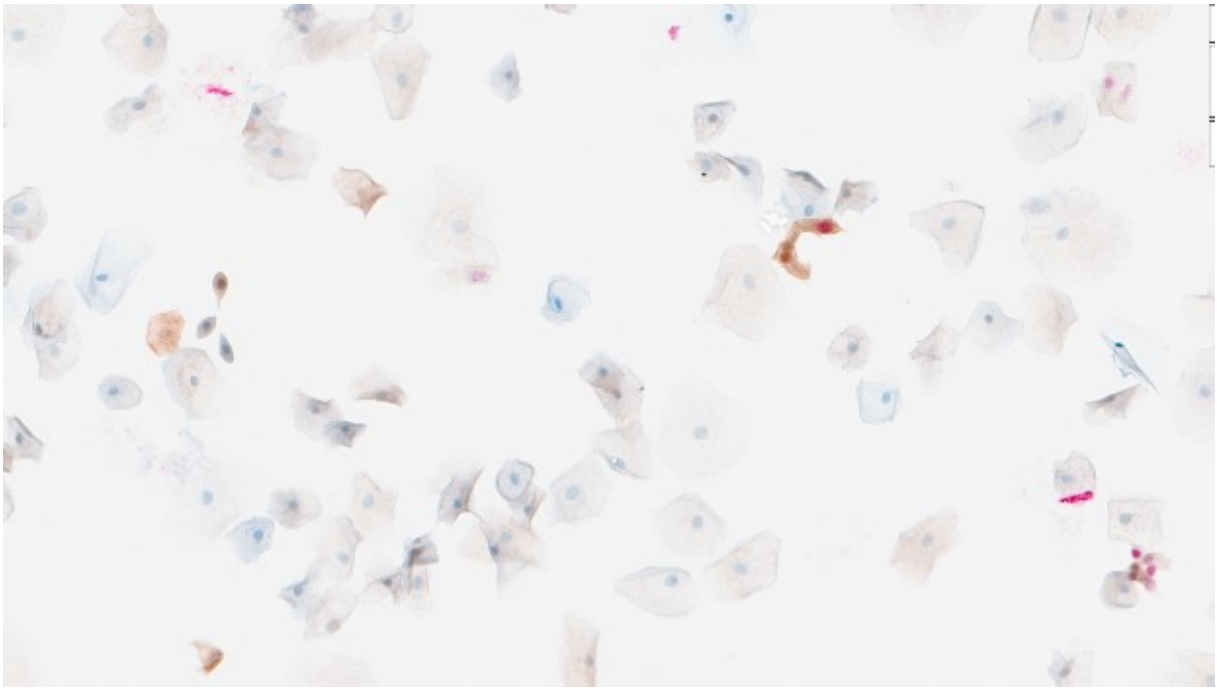

Figure 82: Scan one of cervical smear cells from case 3h stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

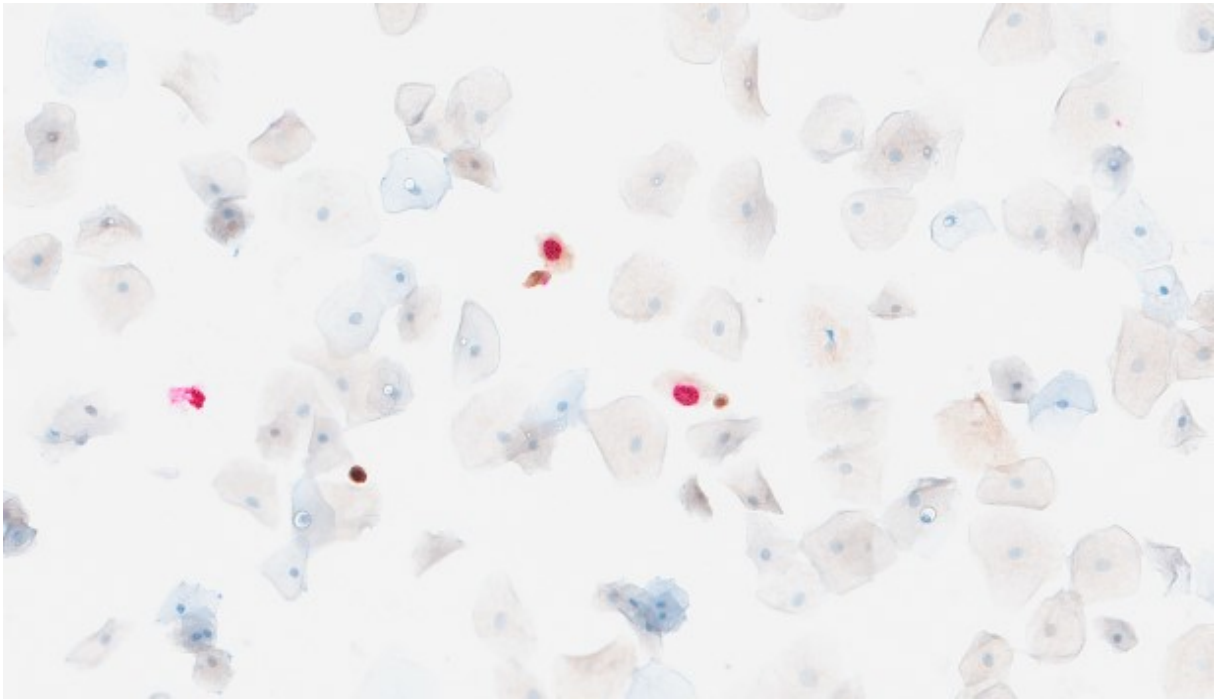

Figure 83: Scan two of cervical smear cells from case 3h stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3h – protocol y

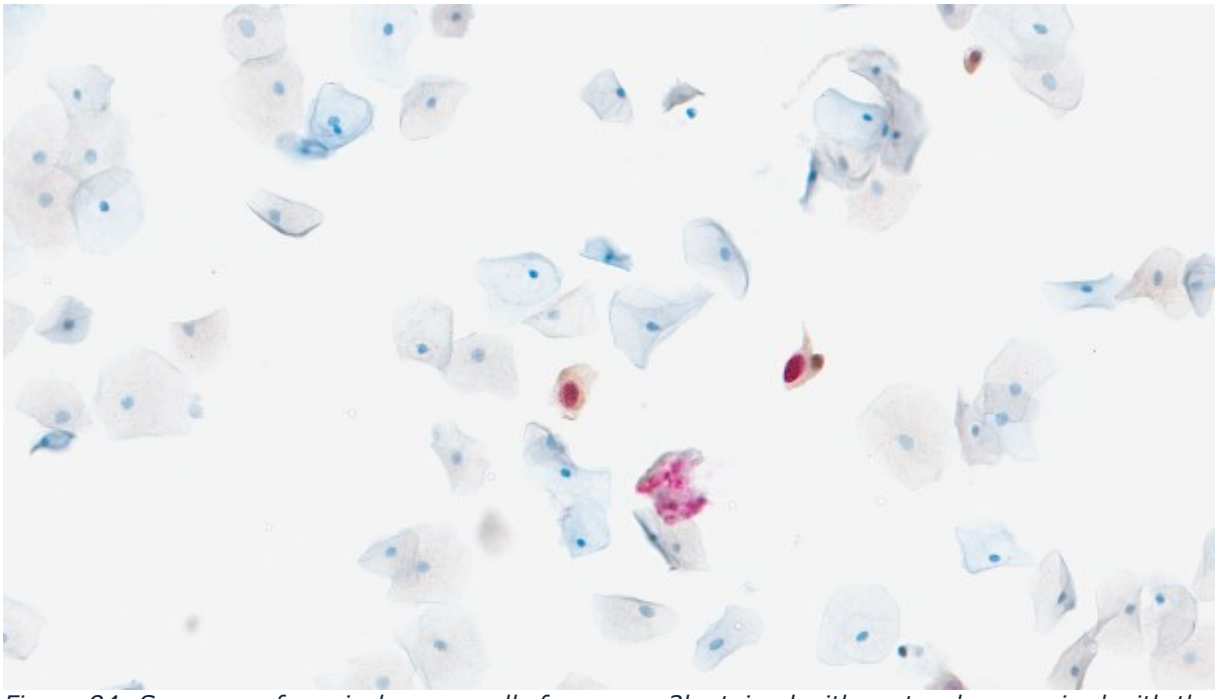

Figure 84: Scan one of cervical smear cells from case 3h stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

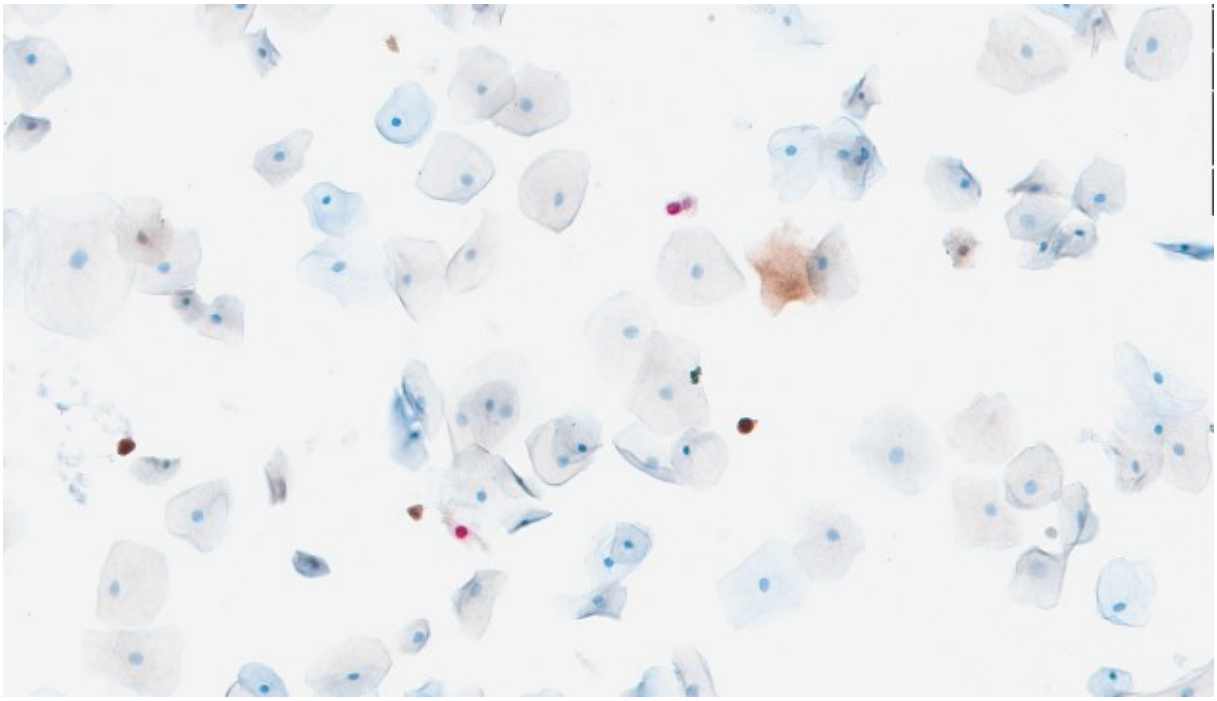

Figure 85: Scan two of cervical smear cells from case 3h stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3j – protocol y

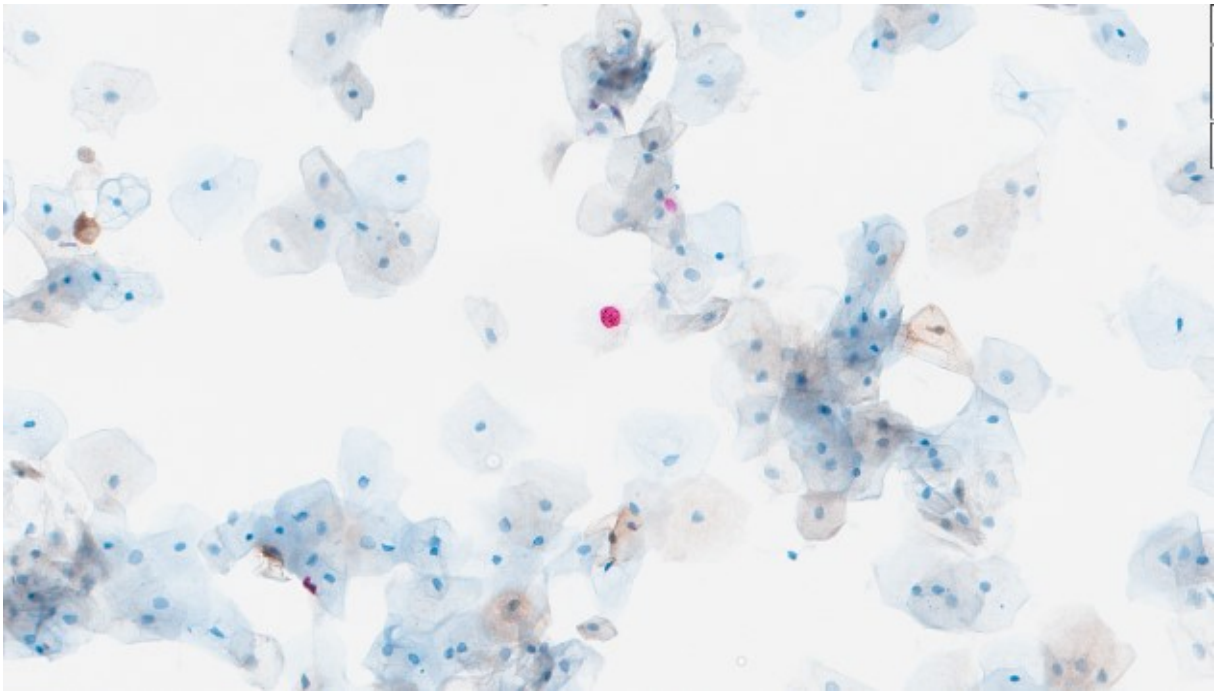

Figure 86: Scan one of cervical smear cells from case 3j stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

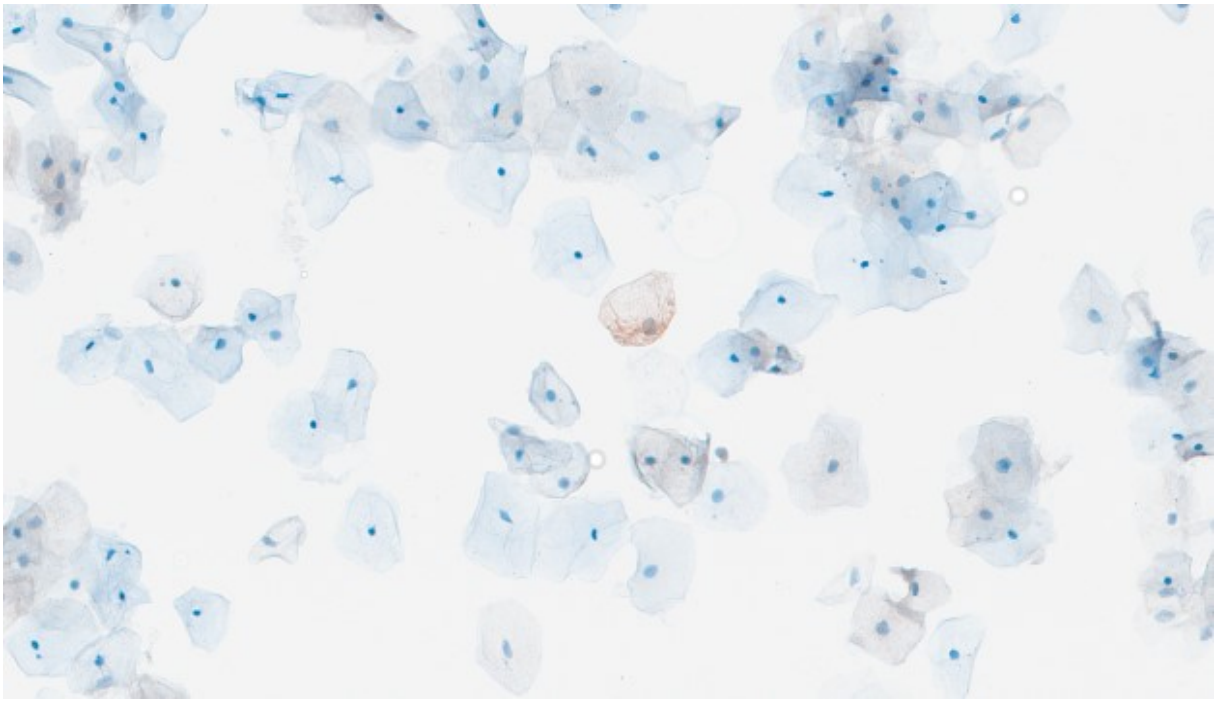

Figure 87: Scan two of cervical smear cells from case 3j stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3n – protocol x

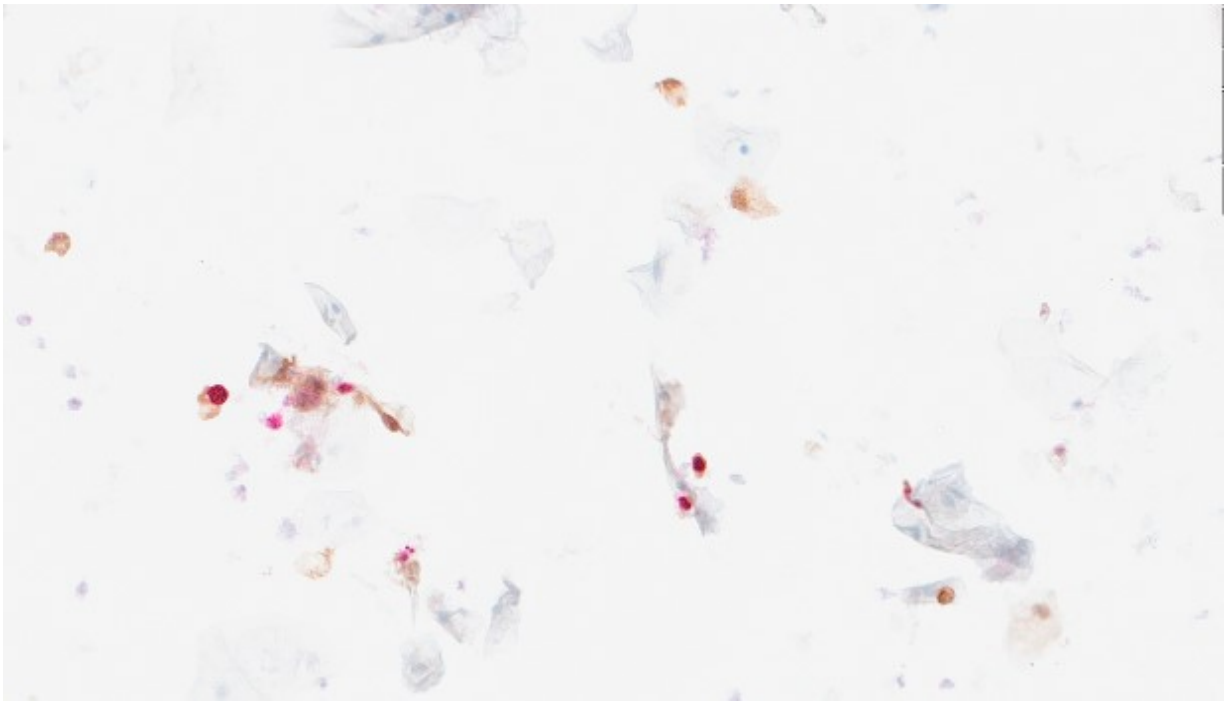

Figure 88: Scan one of cervical smear cells from case 3n stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

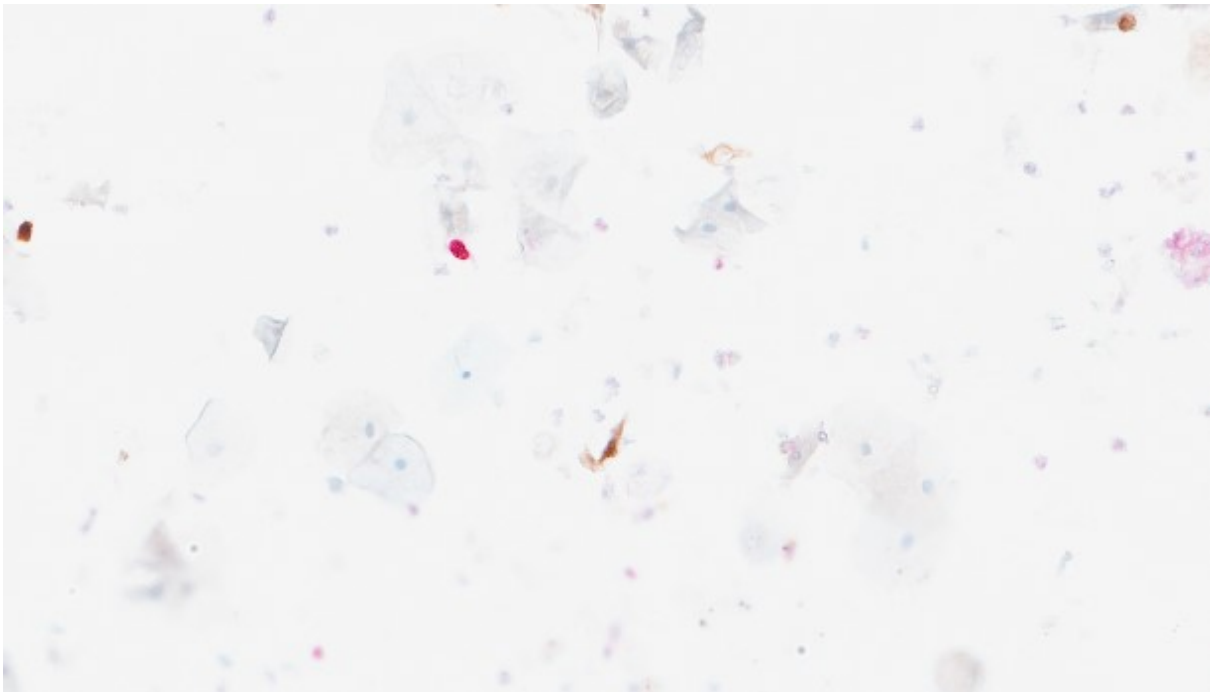

Figure 89: Scan two of cervical smear cells from case 3n stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3n – protocol y

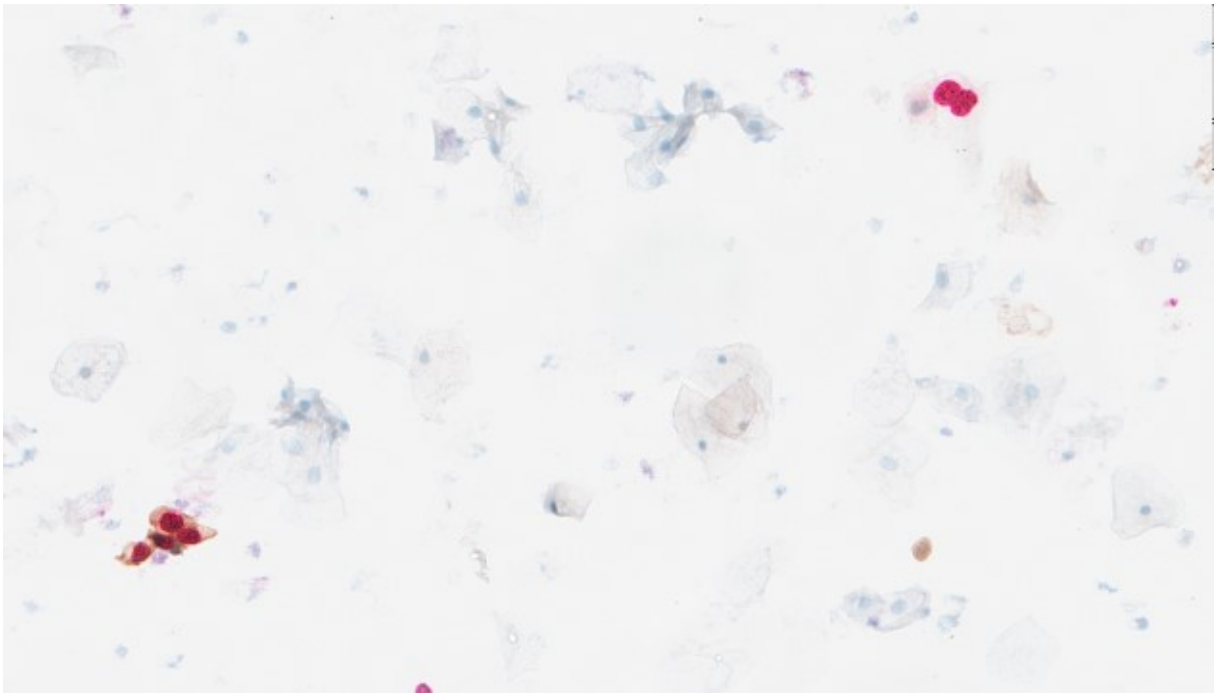

Figure 90: Scan one of cervical smear cells from case 3n stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

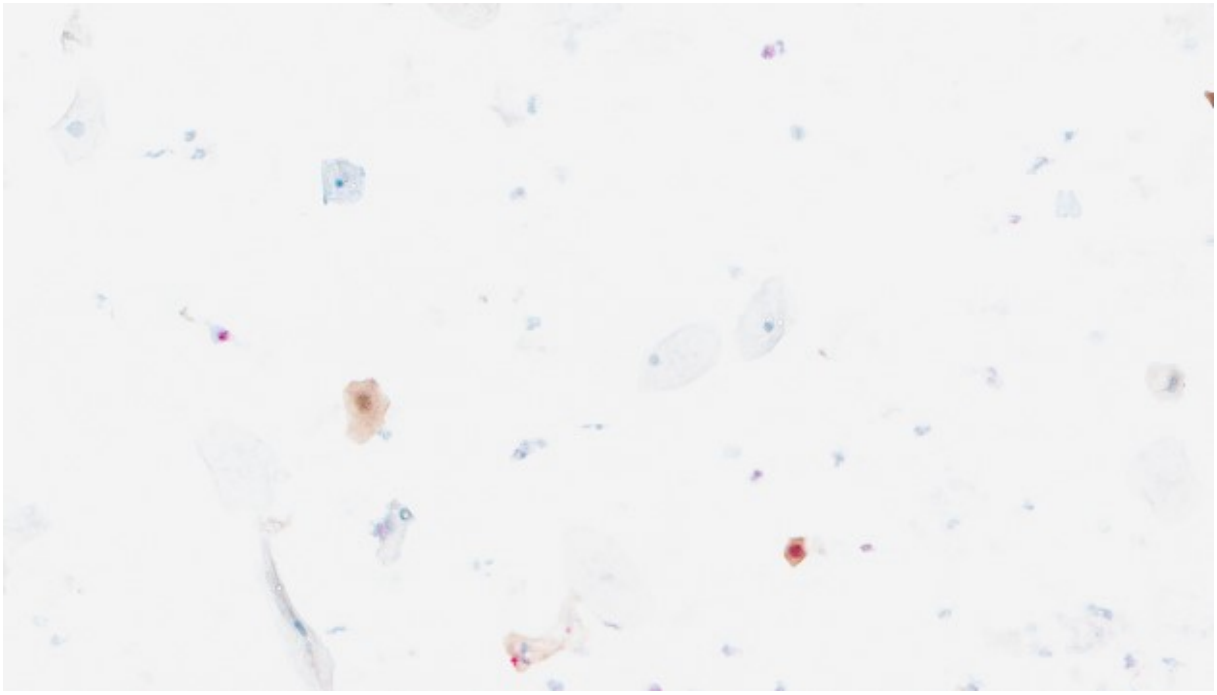

Figure 91: Scan two of cervical smear cells from case 3n stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3o – protocol x

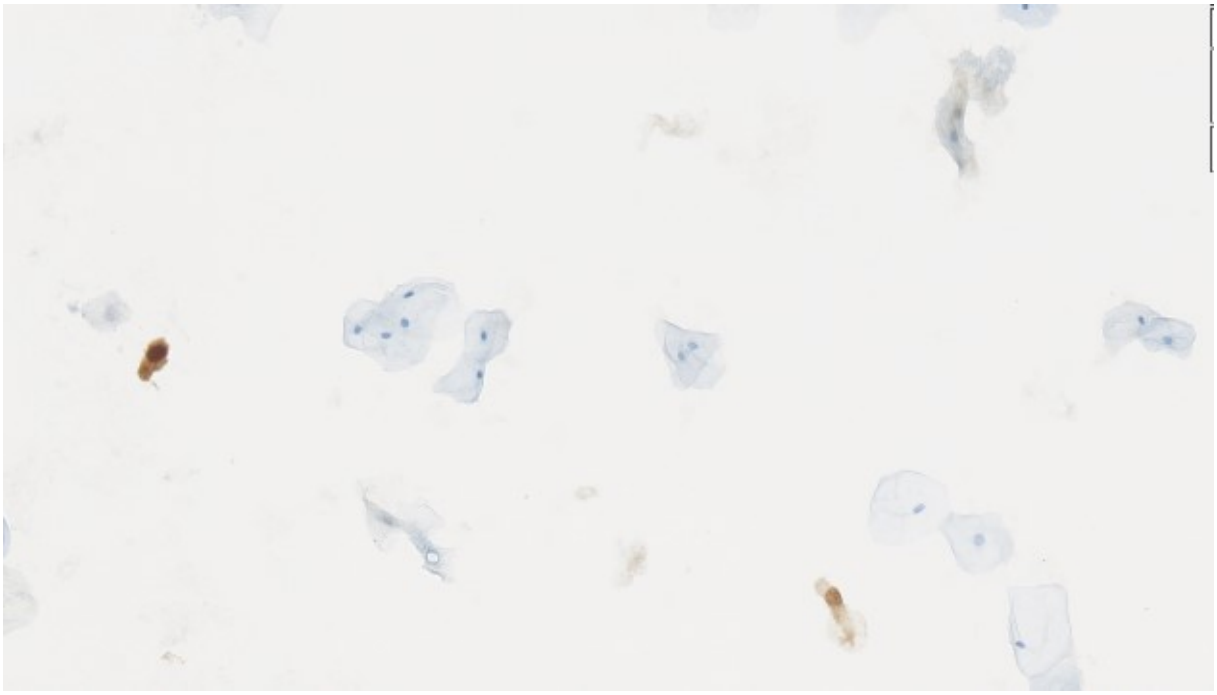

Figure 92: Scan one of cervical smear cells from case 3o stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

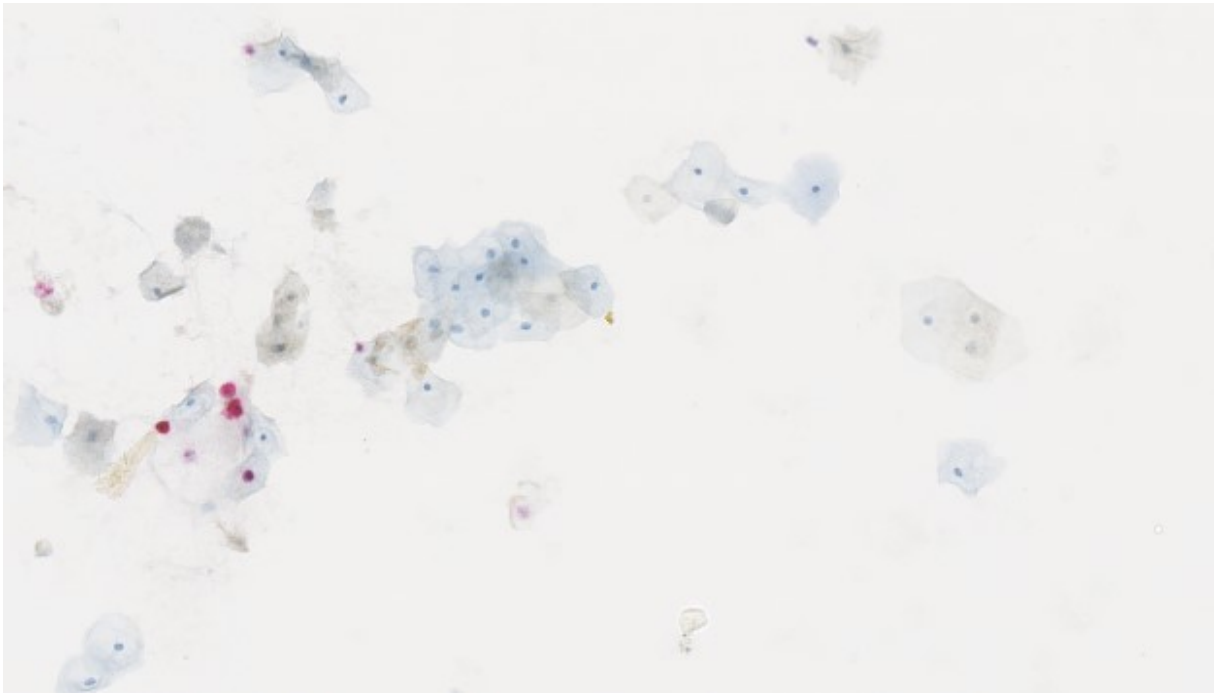

Figure 93: Scan two of cervical smear cells from case 3o stained with protocol x, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3o – protocol y

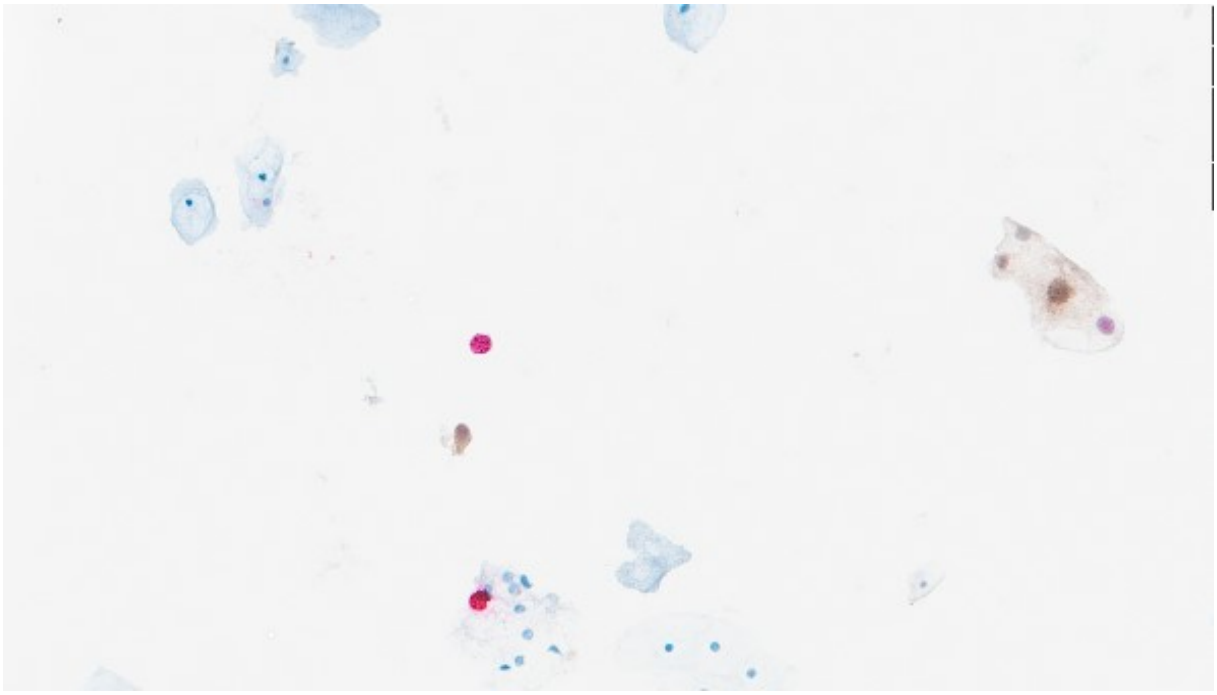

Figure 94: Scan one of cervical smear cells from case 3o stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Figure 95: Scan two of cervical smear cells from case 3o stained with protocol y, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Attachments C: CINtec PLUS Cytology slide scans for repeatability

Case 2c

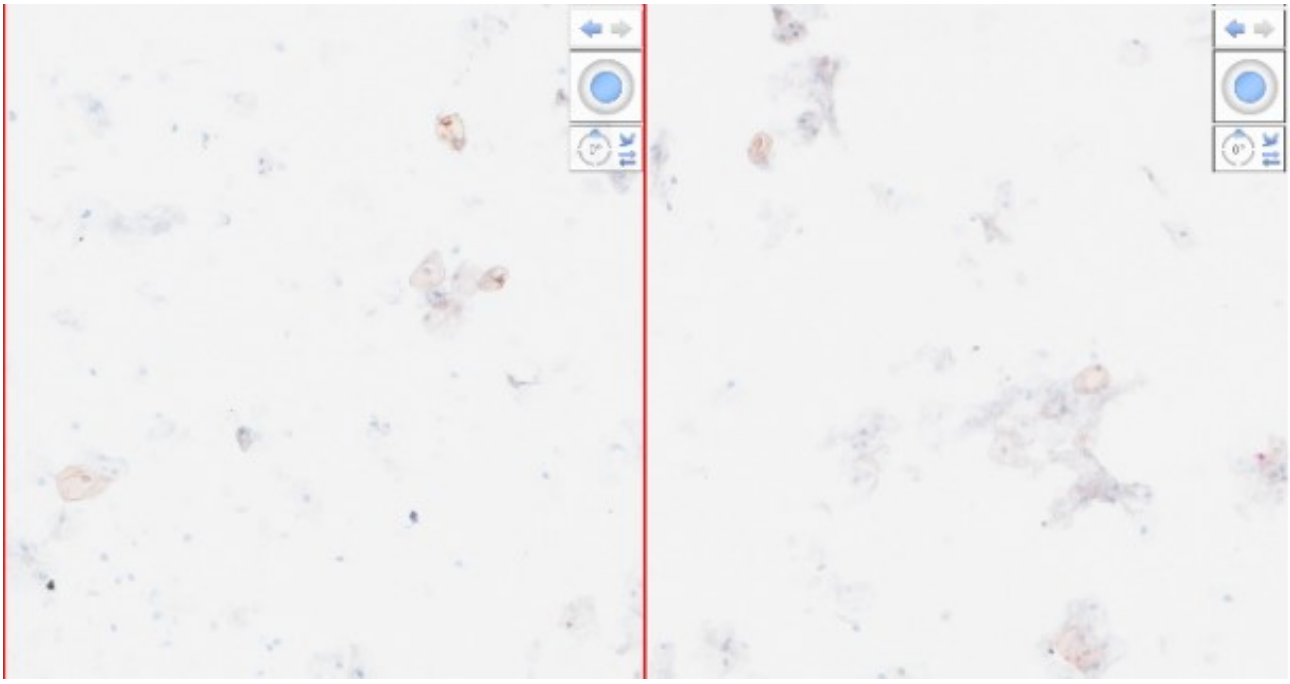

Figure 96: Scan of slide 1 (left) and slide 2 (right) of cervical smear cells from case 2c stained with CINtec PLUS Cytology protocol y for repeatability, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

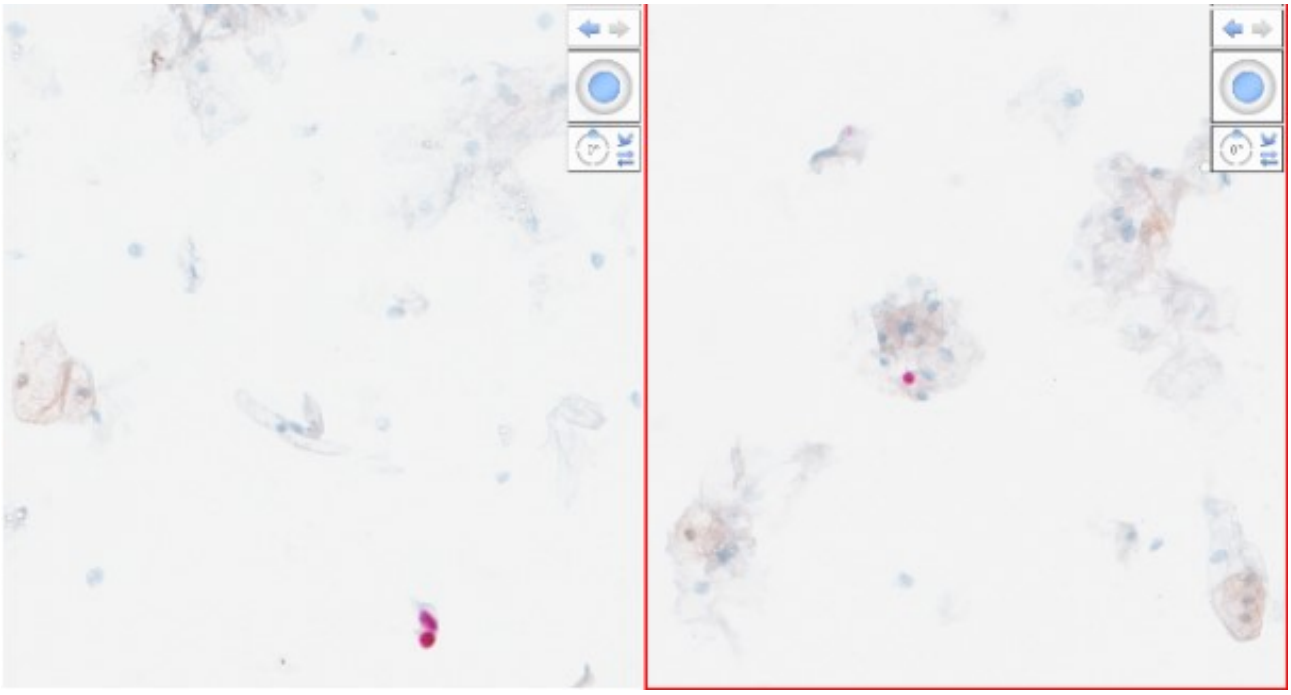

Figure 97: Scan of slide 1 (left) and slide 2 (right) of cervical smear cells from case 2c stained with CINtec PLUS Cytology protocol y for repeatability, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3m

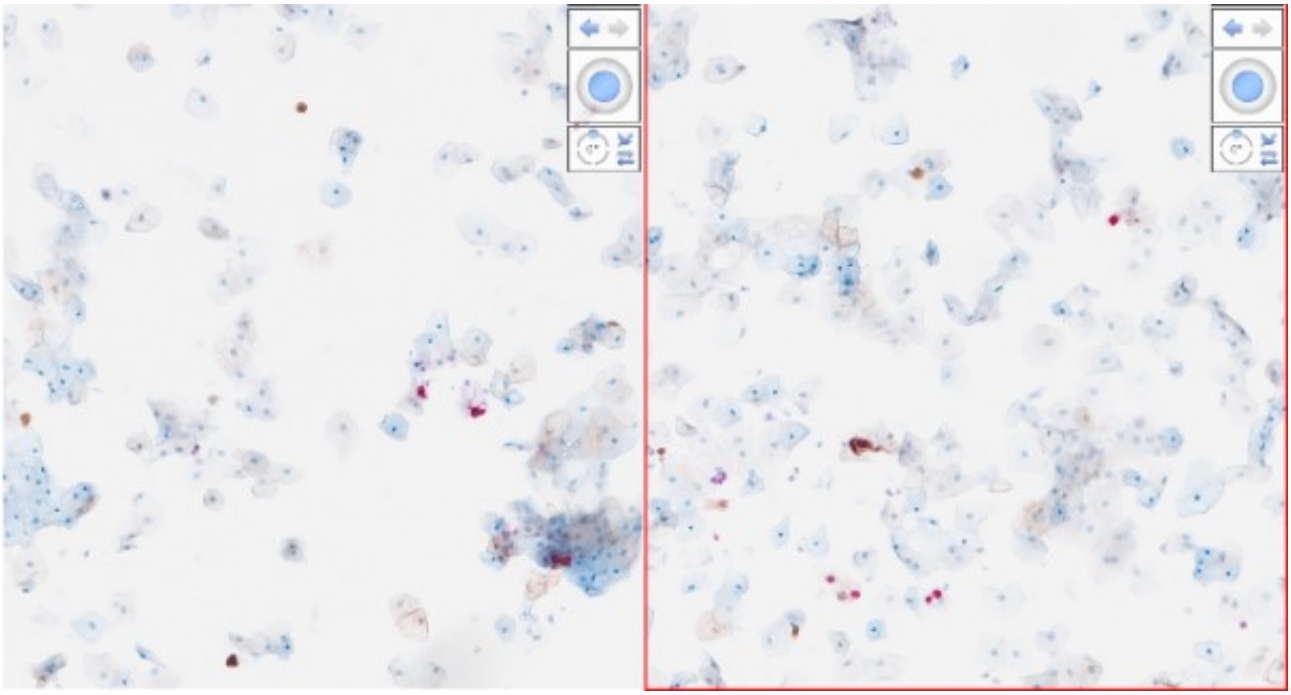

Figure 98: Scan of slide 1 (left) and slide 2 (right) of cervical smear cells from case 3m stained with CINtec PLUS Cytology protocol y for repeatability, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

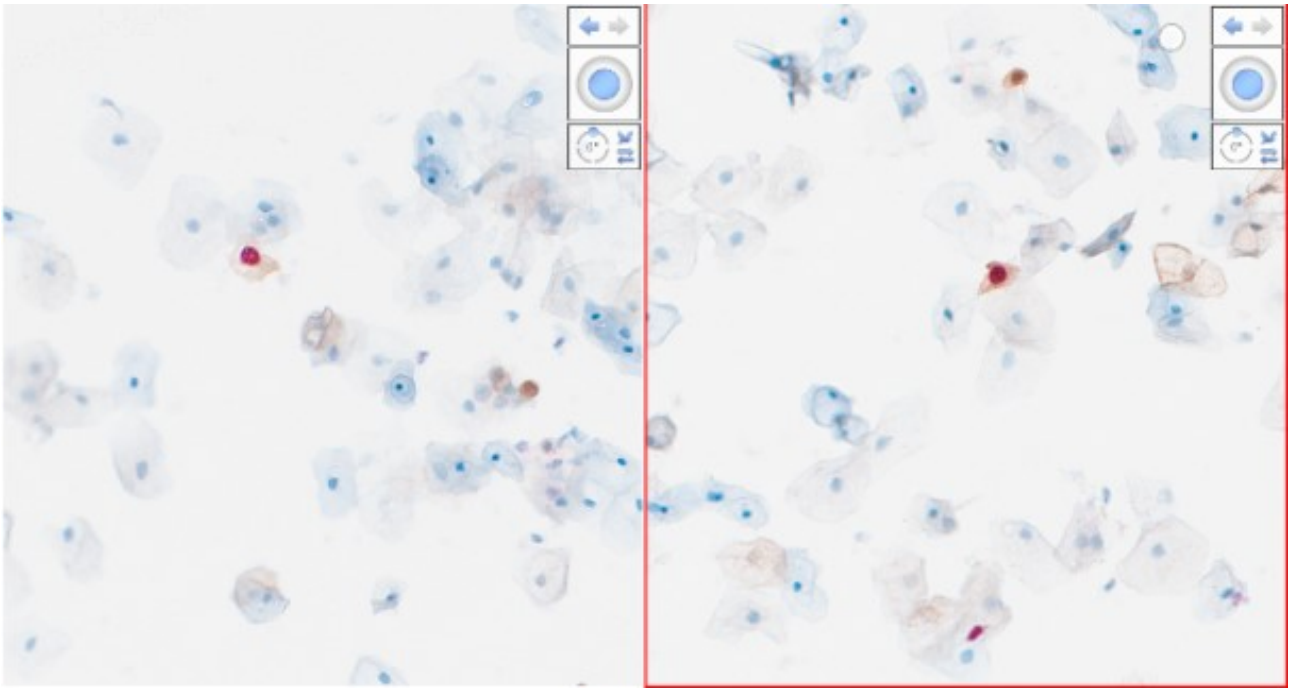

Figure 99: Scan of slide 1 (left) and slide 2 (right) of cervical smear cells from case 3m stained with CINtec PLUS Cytology protocol y for repeatability, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Case 3p

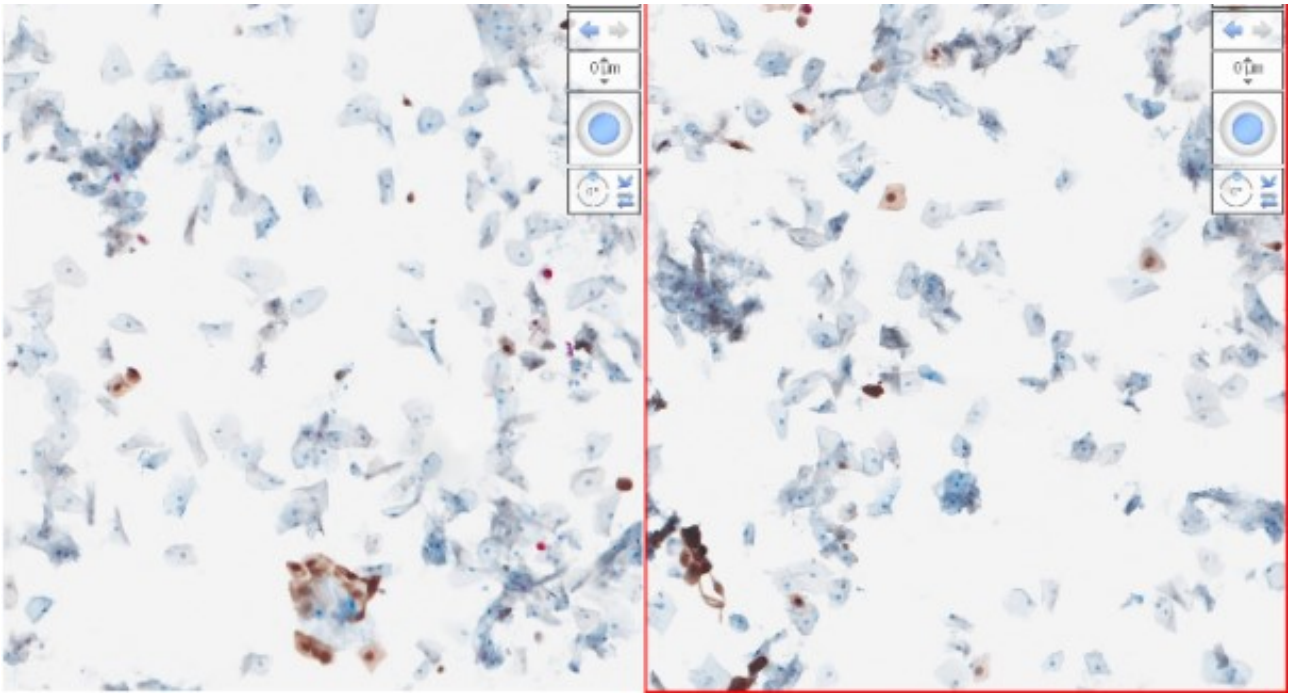

Figure 100: Scan of slide 1 (left) and slide 2 (right) of cervical smear cells from case 3p stained with CINtec PLUS Cytology protocol y for repeatability, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

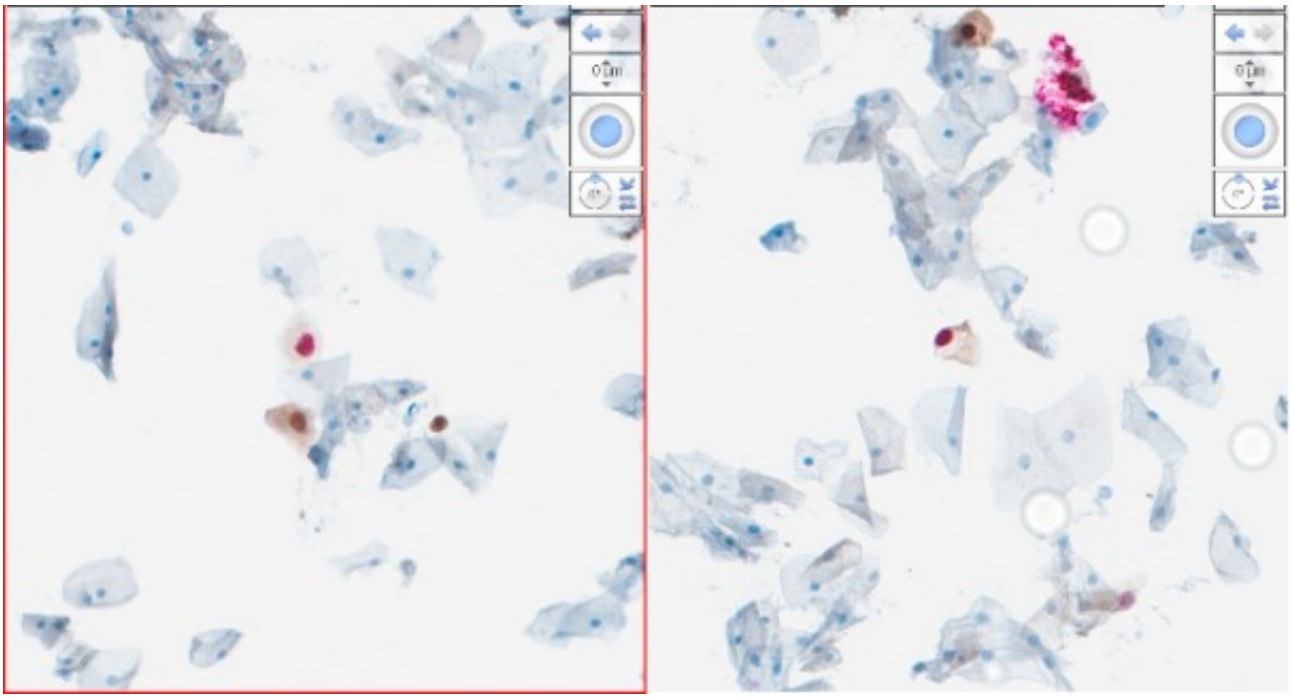

Figure 101: Scan of slide 1 (left) and slide 2 (right) of cervical smear cells from case 3p stained with CINtec PLUS Cytology protocol y for repeatability, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

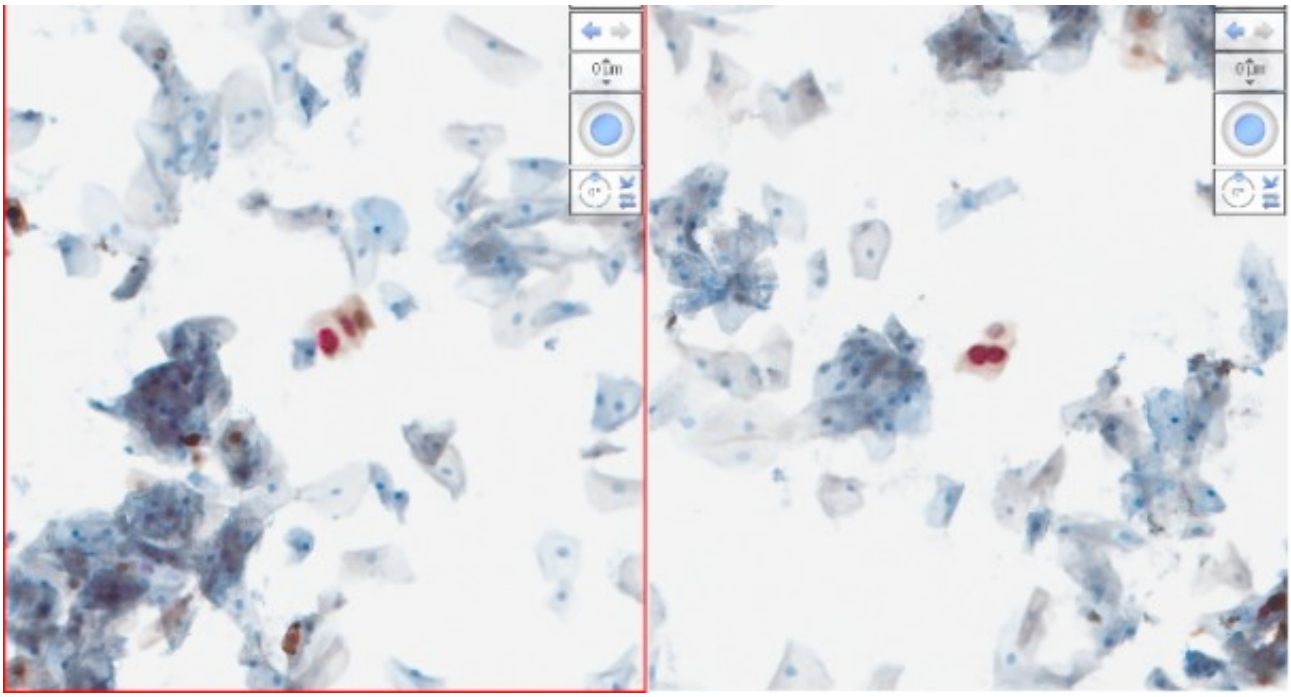

Figure 102: Scan of slide 1 (left) and slide 2 (right) of cervical smear cells from case 3p stained with CINtec PLUS Cytology protocol y for repeatability, acquired with the NanoZoomer S210 (Hamamatsu) at 20x magnification.

Attachments D: CINtec PLUS Cytology slide scans for reproducibility

Case 2e

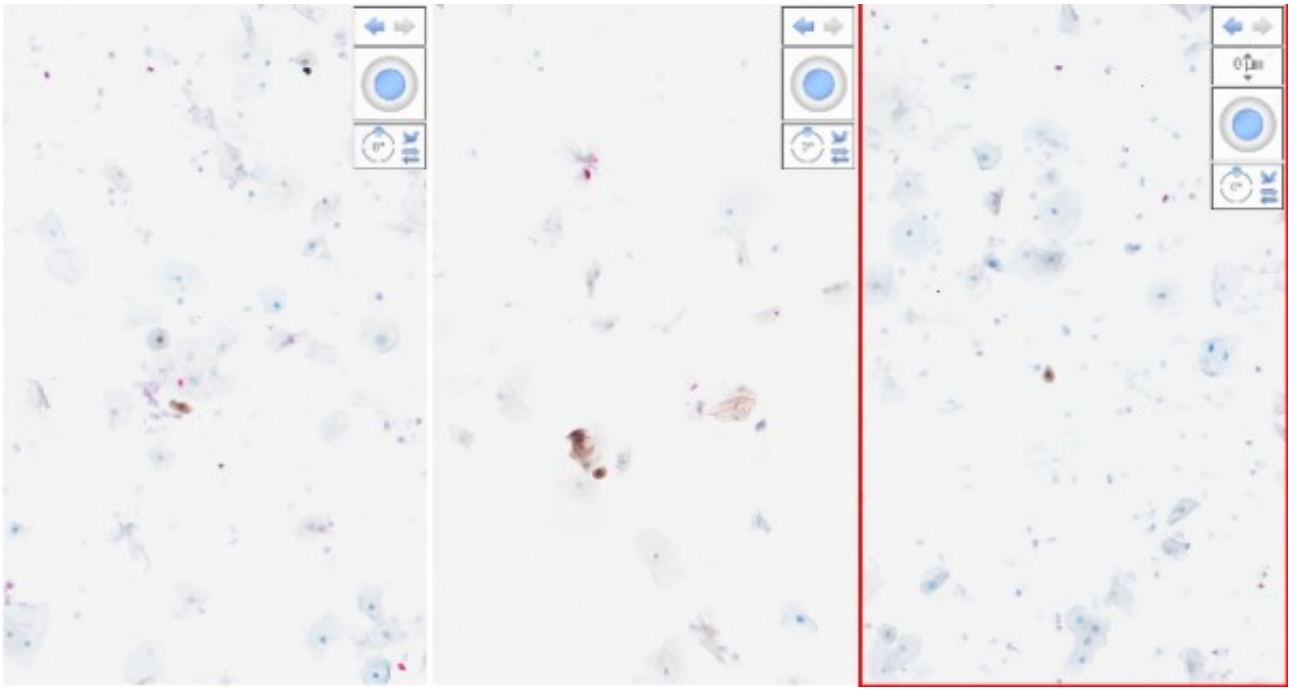

Figure 103: Scan of slide 1 (left), slide 2 (middle) and slide 3 (right) of cervical smear cells from case 2e stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

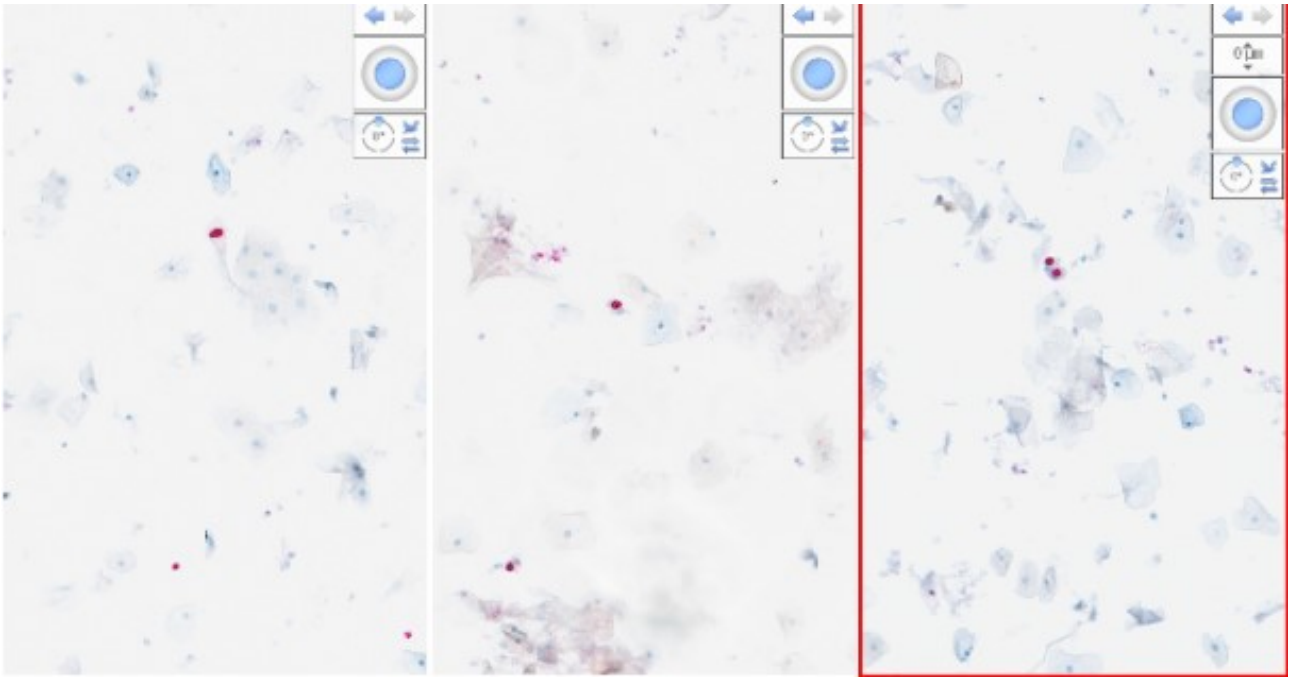

Figure 104: Scan of slide 1 (left), slide 2 (middle) and slide 3 (right) of cervical smear cells from case 2e stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

Case 3i

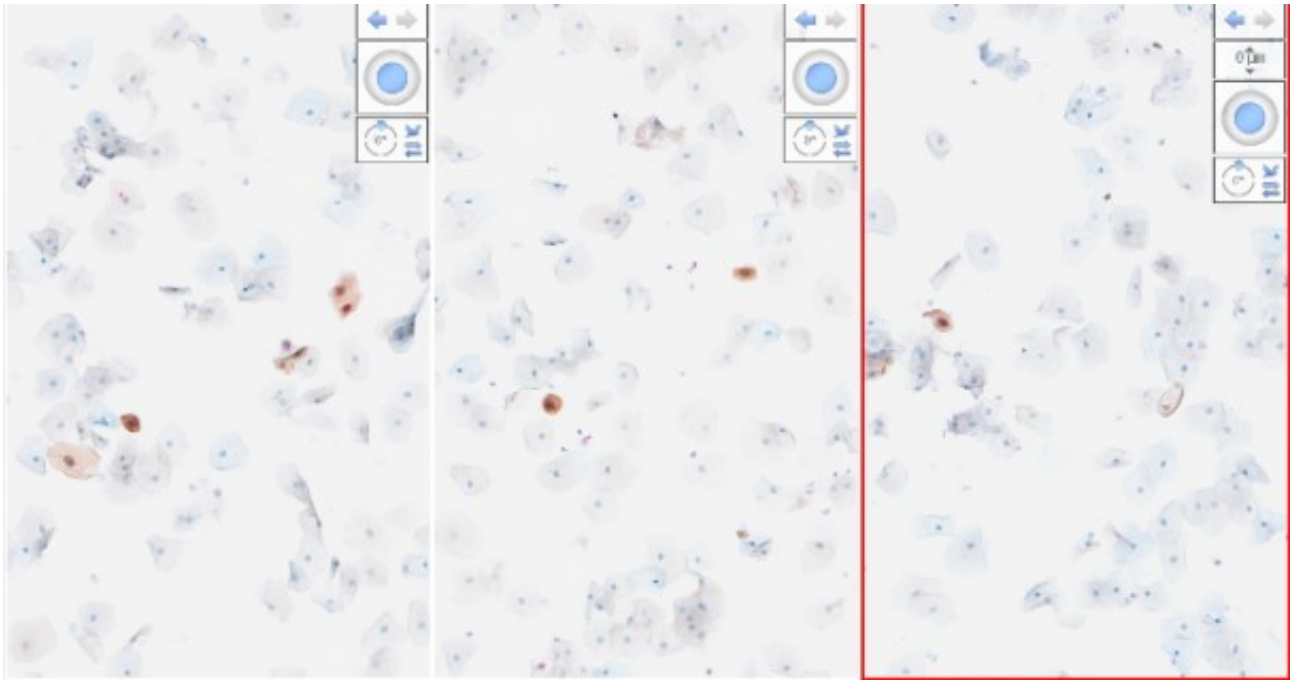

Figure 105: Scan of slide 1 (left), slide 2 (middle) and slide 3 (right) of cervical smear cells from case 3i stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

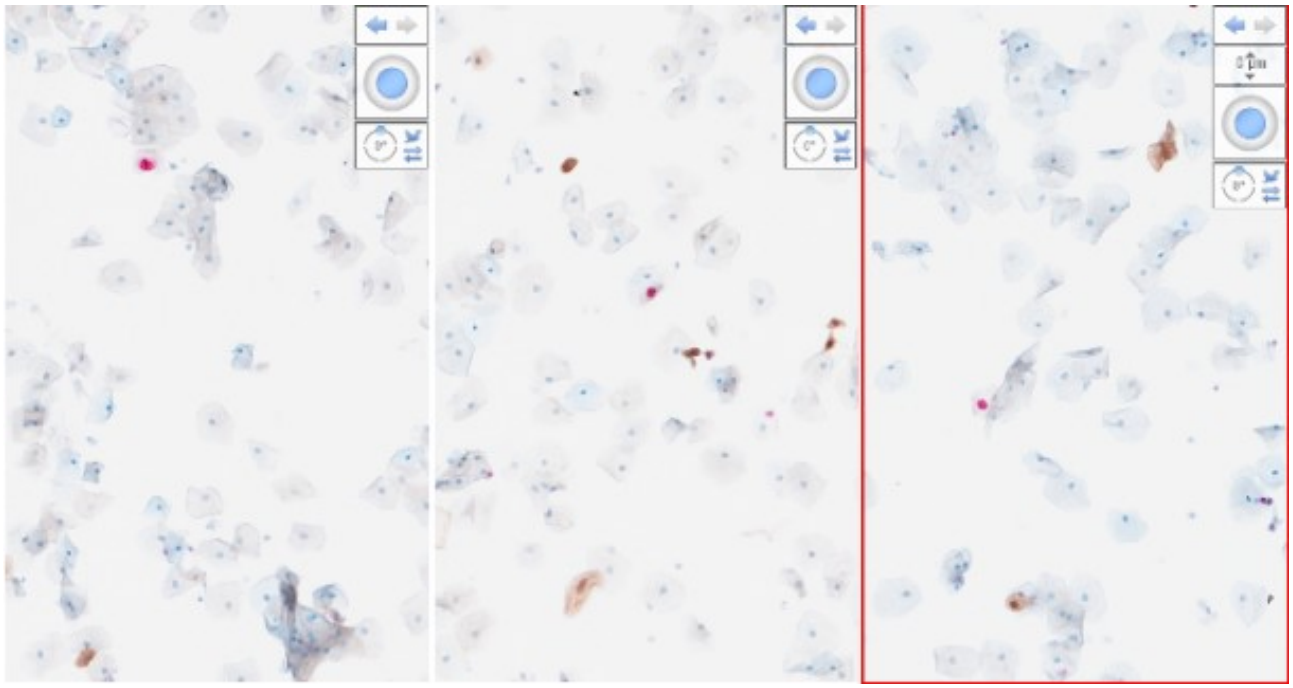

Figure 106: Scan of slide 1 (left), slide 2 (middle) and slide 3 (right) of cervical smear cells from case 3i stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

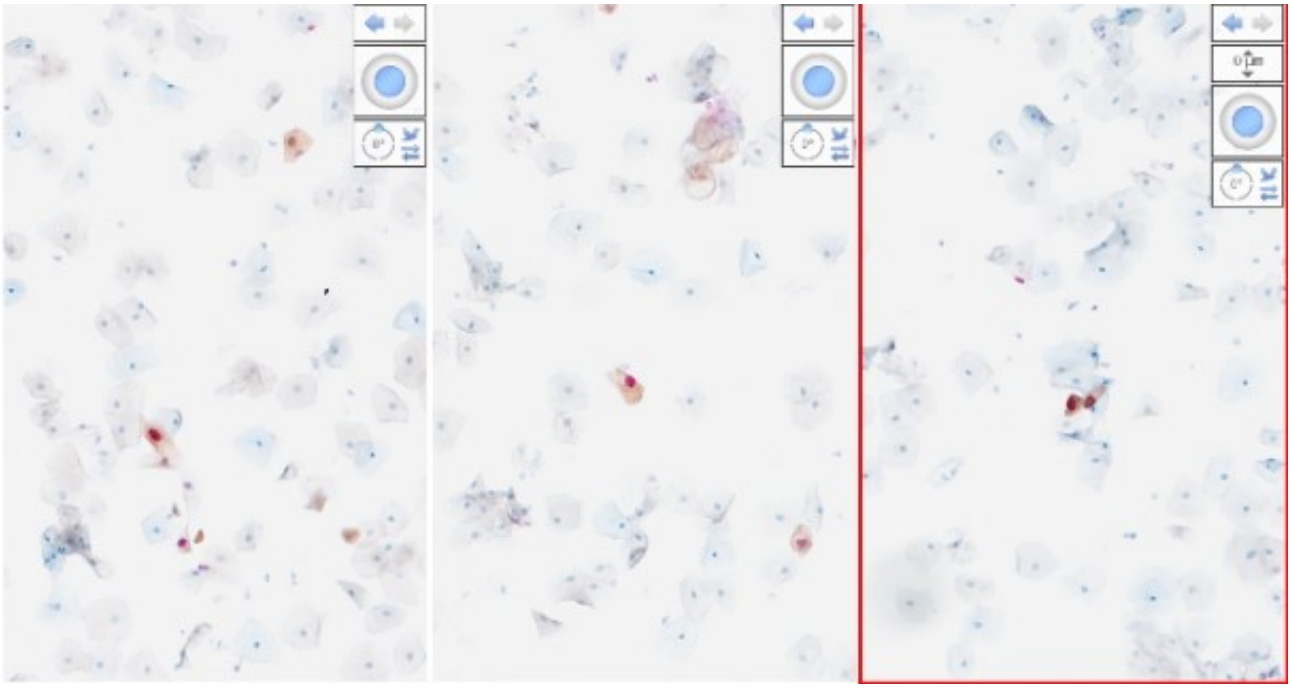

Figure 107: Scan of slide 1 (left), slide 2 (middle) and slide 3 (right) of cervical smear cells from case 3i stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

Case 3l

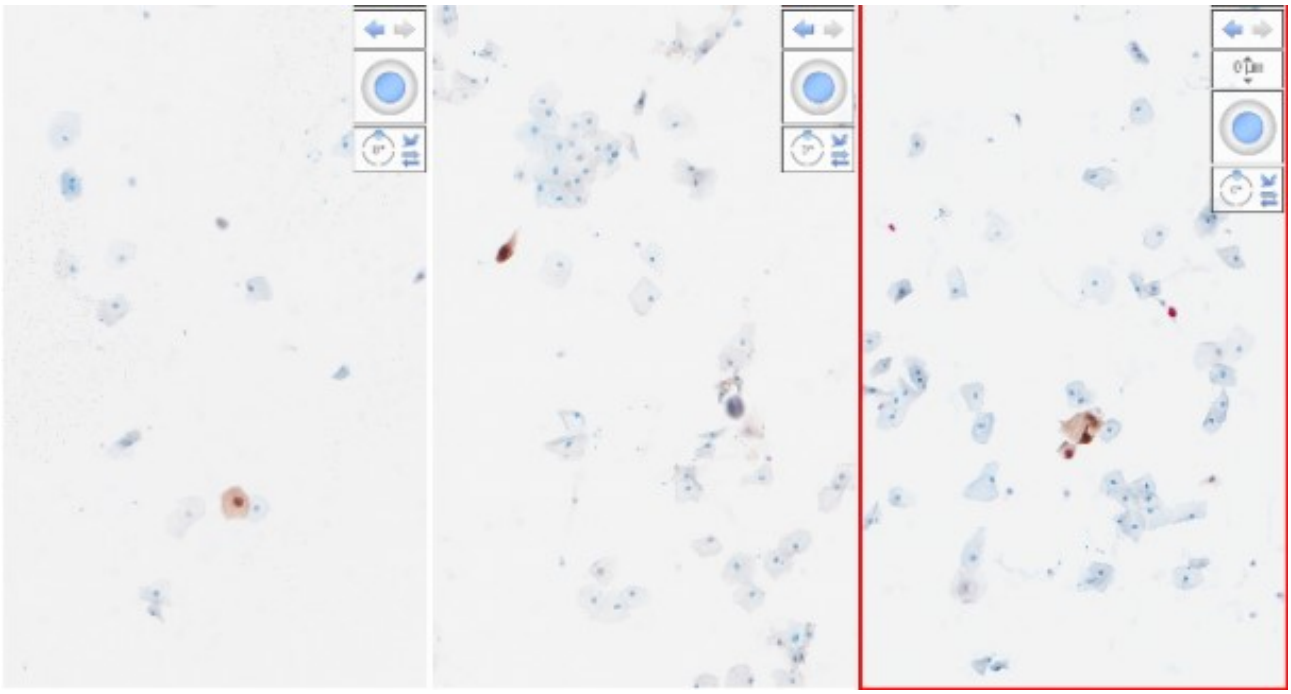

Figure 108: Scan of slide 1 (left), slide 2 (middle) and slide 3 (right) of cervical smear cells from case 3l stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

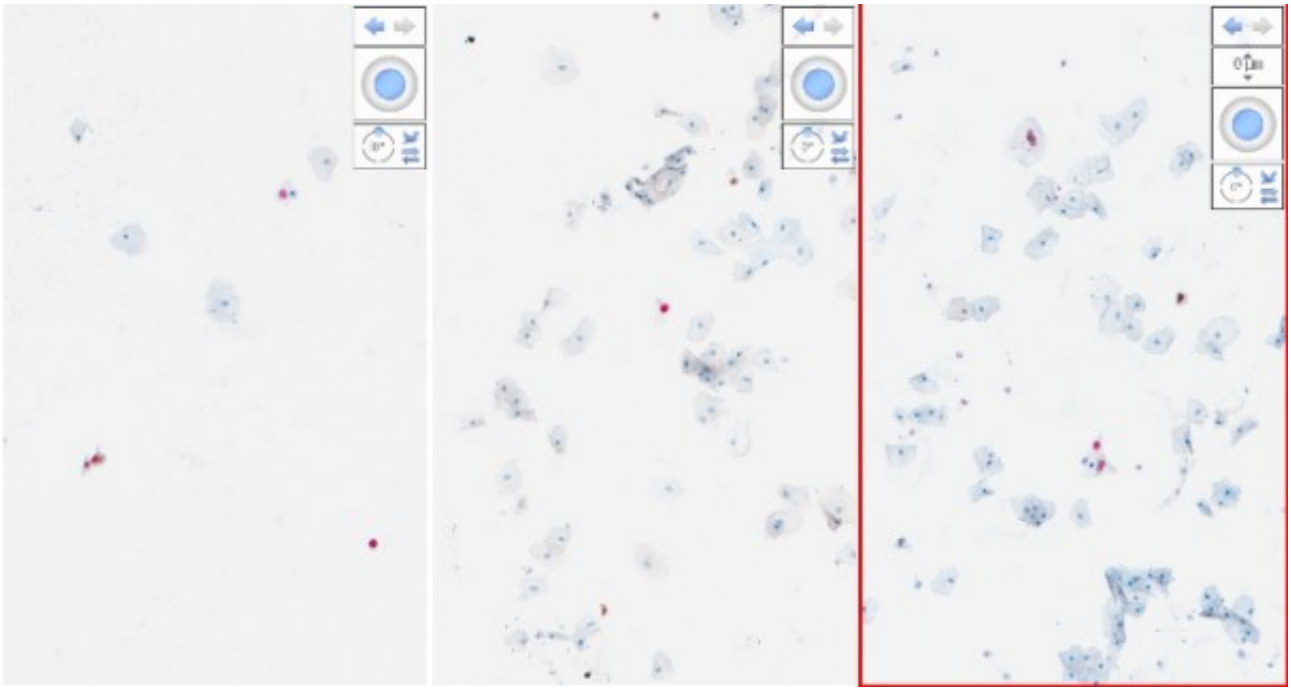

Figure 109: Scan of slide 1 (left), slide 2 (middle) and slide 3 (right) of cervical smear cells from case 3l stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

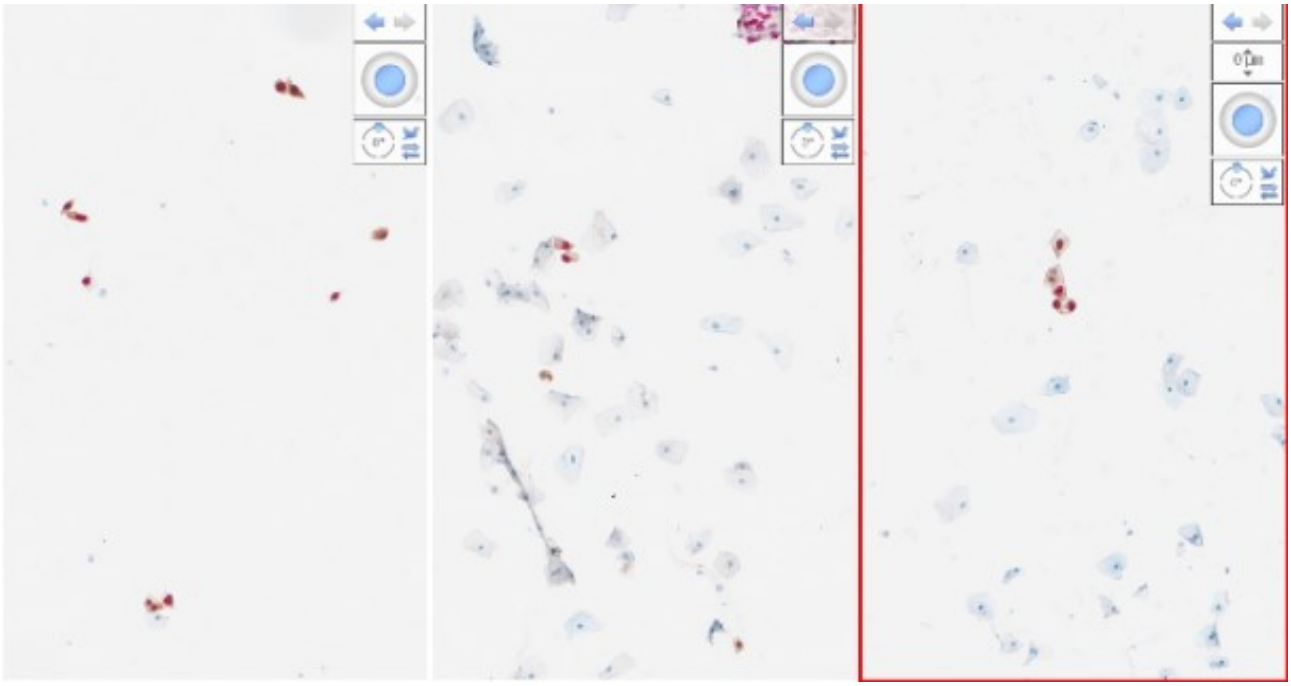

Figure 110: Scan of slide 1 (left), slide 2 (middle) and slide 3 (right) of cervical smear cells from case 3l stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

Attachments E: CINtec PLUS Cytology slide scans for robustness

Case 2d

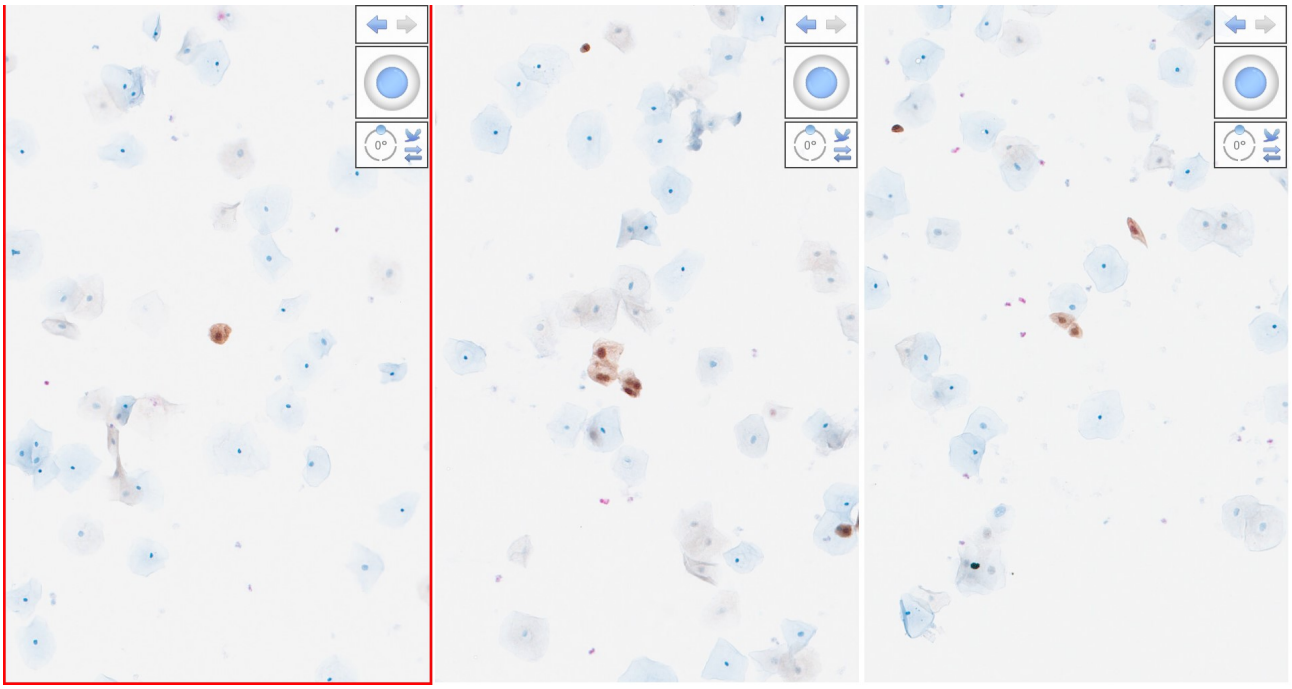

Figure 111: Scan of slide 1 (stained after 7 days), slide 2 (stained after 15 days) and slide 3 (stained after 30 days) of cervical smear cells from case 2e stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

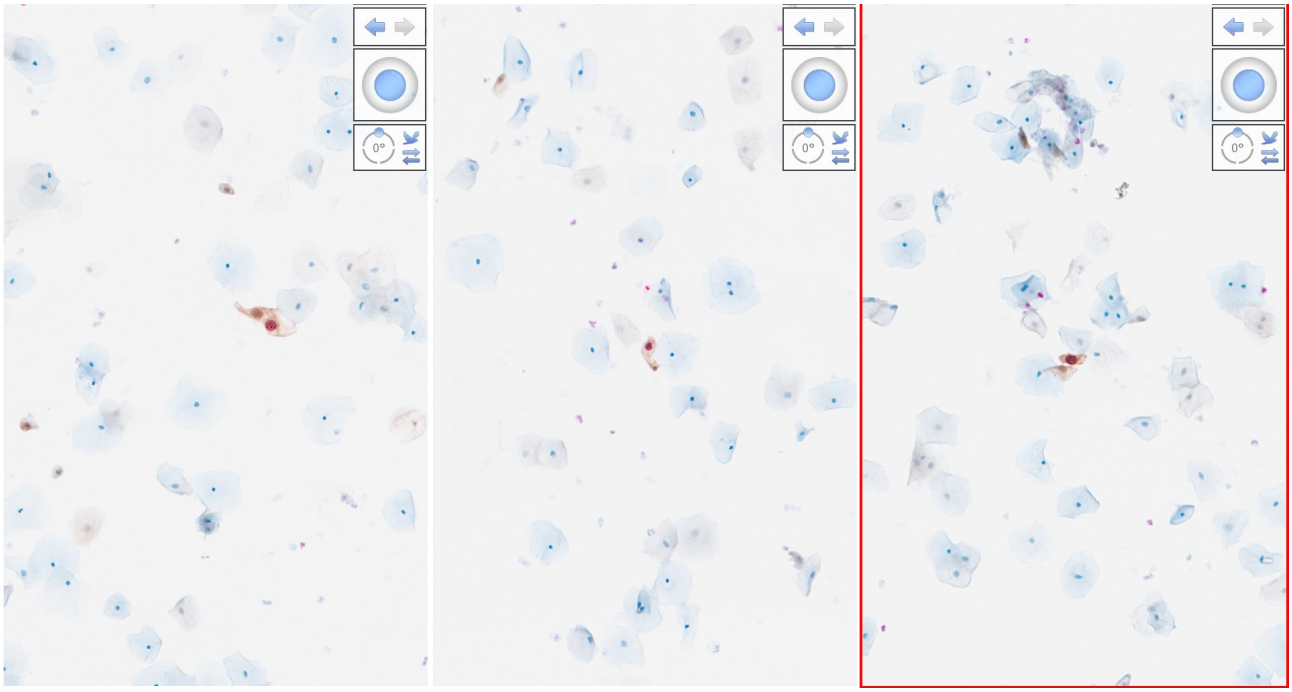

Figure 112: Scan of slide 1 (stained after 7 days), slide 2 (stained after 15 days) and slide 3 (stained after 30 days) of cervical smear cells from case 2e stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

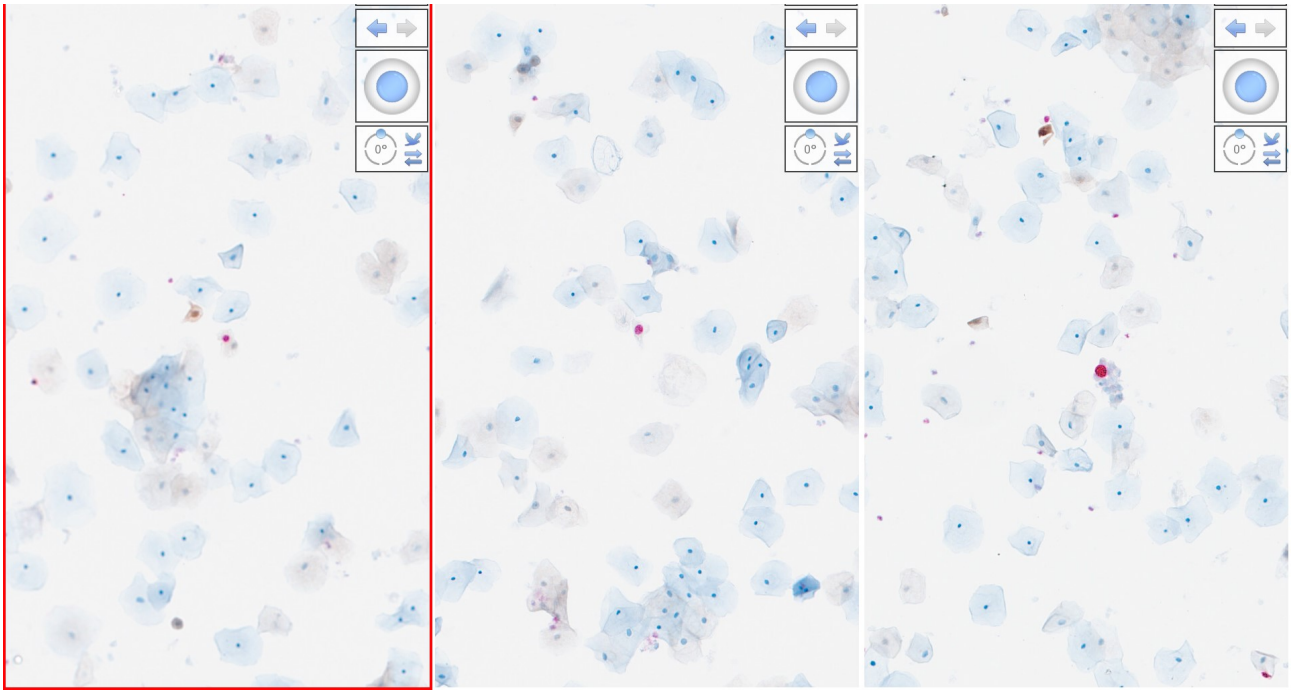

Figure 113: Scan of slide 1 (stained after 7 days), slide 2 (stained after 15 days) and slide 3 (stained after 30 days) of cervical smear cells from case 2e stained with CINtec PLUS Cytology protocol y for reproducibility, acquired with the NanoZoomer S210 (Hamamatsu) at 10x magnification.

Attachments F : Devices

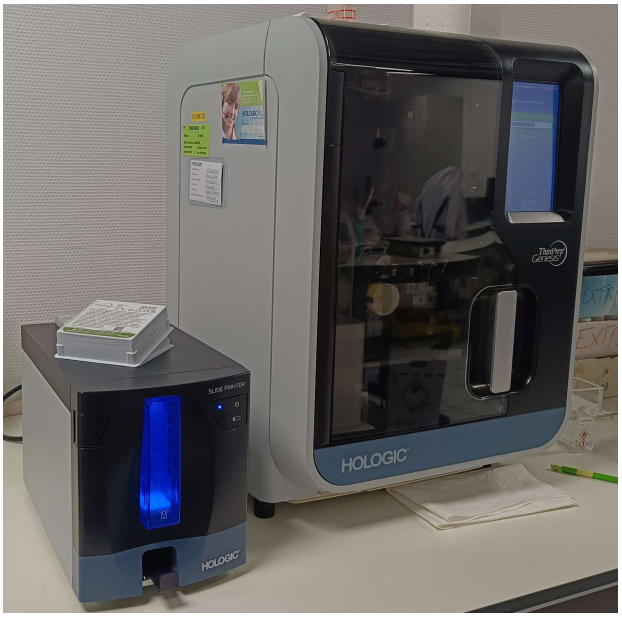

Figure 114: The ThinPrep Genesis Processor and Slide Printer (Hologic) in the cytology laboratory at IPG.

Figure 115: The Benchmark Ultra (Roche) in the IHC laboratory at IPG.

Figure 116: The Tissue-Tek Film Coverslipper (Sakura) in the IHC laboratory at IPG.