

# The Valgent4 protocol



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# The Valgent4 protocol: Robust analytical and clinical validation of 11 HPV assays with genotyping on cervical samples collected in SurePath medium

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ABSTRACT

*Background:* The VALidation of HPV GENoyping Tests (VALGENT) is an international initiative designed to validate HPV assays with genotyping capability. The VALGENT4 protocol differs from previous VALGENT installments as the sample collection medium is SurePath, and exclusively includes samples from women ≥30 years of age which is concordant with the majority of HPV primary screening guidelines. Here we present the protocol for the fourth installment of the VALGENT framework.

*Objectives:* In VALGENT4 11 HPV assays will be evaluated using two comparator assays based on PCR with the GP5+/6+ primers.

*Study design:* Overall, the VALGENT4 panel consists of 1,297 routine samples comprised of 998 unselected, consecutive samples, of which 51 samples had abnormal cytology with 13 women diagnosed with ≥CIN2, and 299 consecutive samples enriched for ≥ASCUS cytology (100 ASCUS, 100 LSIL, 99 HSIL) with 106 ≥CIN2 upon follow up. Manipulated and DNA extracted panel samples were characterized with respect to human beta globin (HBB) and overall DNA content and composition to quality assess the panel prior to distribution to the collaborating sites.

*Result:* The relative cellularity (mean CT value of HBB from the Onclarity assay) on the 1,297 LBC samples (CT = 24.8) was compared with 293 un-manipulated routine cytology screening samples (CT = 23.8). Furthermore, the DNA extracted panel samples was characterized using the Exome iPLEX pro assay, which reports amplifiable copies on individual samples as well as copies of five different base pair lengths. Here the data showed a slightly lower number of amplifiable DNA copies (ratio: 0.7, p = < 0.01)) in the VALGENT4 panel samples compared to routine extracted cervical DNA samples

*Conclusion:* The present manuscript details the manipulation, processing and quality assessment of samples used in VALGENT-4. This methodological document may be of value for future international projects of HPV test validation.

## 1. Background

Practically all cervical cancers are derived from an Human papillomavirus (HPV) infection [1–3] and the causal relation is firmly established between the development of cervical cancer and at least 12HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and HPV59, all group 1 carcinogens as classified by IARC) [1,2]. Evidence on the clinical value of HPV testing for triage of borderline cervical

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cytology and test of cure in women who have been treated for pre-invasive cervical lesions is today widely accepted [4–6]. Additionally, randomized controlled trials have provided evidence for the use of HPV-based screening over cytology as a superior and highly reproducible screening modality to prevent both invasive squamous and adeno-carcinomas [5,7]. As a result, primary HPV-based cervical screening is being implemented in several countries globally, substituting conventional and liquid based cytology (LBC) as the standard of care. Together, this has created a rapidly expanding market for molecular HPV tests, and more than 200 different HPV assays are now commercially available [8]. As primary HPV based screening becomes the standard of care, additional focus on evidence of clinical performance of HPV assays for use in cervical cancer screening programs is required.

HPV assays, applicable to cervical screening and disease management contexts, have undergone a rapid technical and scientific evolution over the last decade. The first generation of clinical HPV assays were developed solely to detect oncogenic HPV genotypes using DNA PCR or hybridization techniques and mainly reported the test outcome as either HPV positive or negative, with no individual HPV genotype information available. Newer generations of commercially available HPV assays depend upon a wide variety of DNA and RNA detection techniques and allow for individual reporting of HPV genotypes. Current HPV genotyping assays can basically be divided into three categories (1) Assays with limited genotyping that report separate identification of HPV16 and HPV18 or HPV18/HPV45, combined with pooled detection of the remainder of the oncogenic types, (2) Assays with extended genotyping that report separate identification of ≥5 genotypes combined with one or more bulk detections of the remainder, and (3) Assays with full genotyping, reporting individual identification of all carcinogenic HPV genotypes [9].

Studies have assigned different carcinogenic potential, or risk, to individual HPV genotypes, with HPV16 being the most potent, in particular for squamous carcinomas of the cervix [10–15], followed by HPV18 and to some extent HPV45 associated with adenocarcinomas of the cervix [12,16–18]. Together, up to 70% of all cervical cancers are caused by HPV16 and HPV18. An additional five HPV genotypes (HPV31, 33, 45, 52, & 58) add around 19% to the burden of cervical cancer incidence [16]. Recent study data from long term follow up of a Danish cervical screening cohort, have shown that persistent infections with HPV16, HPV18, HPV31 and HPV33 are associated with an increased risk for development of cervical intraepithelial neoplasia grade 3 or worse (≥CIN3) compared to the remaining oncogenic genotypes [14,19]. The six carcinogenic types HPV35, 39, 51, 56, 59, 68 in contrast contribute 8–9% of the cumulative proportion of HPV-induced cervical cancer [16,20]. Other HPV genotypes incl. HPV66 and 68 are only rarely involved in cervical carcinogenesis, but both are included in all oncogenic HPV assays [16]. Finally, genotype distribution among highly vaccinated cohorts of women have drastically changed affecting the performance of both cytology screening and HPV genotyping [21].

Taken together, knowledge of the genotype(s) in a screening sample may add information on the individual woman´s risk of developing disease [12]. Consequently, HPV technology is progressing from simple plus/minus outcomes towards revealing more detailed information on the HPV infection in question. This fact is reflected in a growing number of national screening guidelines where HPV16/HPV18 genotyping is integrated in screening algorithms [5,22,23].

Fulfillment of the "*Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women of 30 years and older*" [24], is widely accepted as the methodology by which to validate HPV assays for screening use [24,25] and several limited or extended genotyping HPV assays have fulfilled the international guidelines [25–32]. The international validations are based upon the overall clinical performance but not with respect to the clinical performance of detection of individual genotypes *per se*. Assay specific detection of individual genotypes has instead been addressed in studies of defined plasmid based panels with known copy numbers [33,34]. The VALidation of HPV GENoyping Tests (VALGENT) initiative was established to create a framework for comparison and validation of HPV genotyping assays using validated comparator assays, similar to the international guidelines [24] and using a relevant sample population with sufficient disease to confirm clinical performance while including primary screening samples [9]. So far, three installments of VALGENT protocols have occurred, where a broad variety of HPV assays with both extended and full genotyping capability have been evaluated [9].

## 2. Objectives

The fourth installment of the VALGENT framework, the VALGENT4 study, aims to provide high quality comparative data on clinical performance of HPV assays on cervical screening samples. The VALGENT4 protocol differs from previous VALGENT installments as the sample collection medium is BD SurePath™ (Becton, Dickinson and Company), targets DNA assays only, and exclusively includes only samples from women ≥30 years of age which is concordant with the majority of HPV primary screening guidelines. In addition, a high number of assays previously untested on this LBC collection media will be evaluated. Moreover, the VALGENT4 protocol is unique in the VALGENT iterations by proposing a new standard for describing the quality of the sample panel included.

## 3. Study design

### 3.1. Study description

Sample collection, registration, processing and aliquoting was done at the parent site at the Molecular Pathology Laboratory, Dept. Pathology, Copenhagen University Hospital, Hvidovre. The VALGENT4 samples will be provided to participating scientific partners anonymized in concordance with Danish Data Protection Agency regulations. Aliquot panels were shipped refrigerated from parent laboratory to scientific partners.

The VALGENT4 panel was generated using fresh SurePath collected screening samples from the Danish cervical cancer screening program which services a well-screened population with a high background risk of cervical cancer [35]. Moreover, the parent laboratory delivered the panel in two defined versions; pre-extracted and quality described DNA for assays requiring DNA as input material (time between collection and DNA extraction: Mean 28 days, min: 2 days, max: 70 days), and original LBC material (time between collection and aliquoting for panel: Mean 16 days, min: 2 days, and max: 67 days) to those laboratories where assays with full, integrated CE-IVD marked work flows were interrogated. The option to have a panel consisting of extracted DNA limits any variability of various 3rd party DNA extraction platforms on HPV assay performance. Finally, a novel element entailed characterization of all included panel samples using a MALDI-TOF assay which measured the available DNA as well as the relative level of DNA fragmentation in the individual sample.

In total, eleven different HPV genotyping assays from 8 different manufacturers will be evaluated in the VALGENT4 study (Table 1), using GP5+/6 + PCR-EIA with genotyping as comparator in line with previous VALGENT installments.

### 3.2. Sample collection

The VALGENT4 study was embedded into the routine cytology-based operations of the Department of Pathology, Copenhagen University Hospital, Hvidovre, Denmark. This pathology department receives virtually all cervical cytology screening samples from women residing in the Danish Capital Region, covering almost a third of the entire Danish population. Women are invited for screening every three years at ages 23–49 and every fifth year at ages 50–59. An HPV based

**Table 1**
HPV genotyping assays evaluated, concurrent material required and scientific partners under the VALGENT4 study protocol.

| Valgent-4 included Assays | Aliqout | Amplicon length | Scientific partner |
|---|---|---|---|
| BD Onclarity HPV Assay | Original Material | 79–137 bp. | Hvidovre Hospital, Dept. Pathology, Hvidovre, Denmark |
| Genomica CLART HPV4 assay | DNA | 465 bp. | |
| Agena HPV MassArray assay | DNA | 90–122 bp. | |
| Roche cobas 4800 HPV Test | Original Material | ~200 bp | Norwegian HPV Reference Lab,<br>Akershus University Hospital<br>Norway |
| Fujirebio INNO-LiPA Genotyping Extra II test | DNA | 65 bp | Ghent University Hospital, Ghent, Belgium |
| SeeGene Anyplex HPV28 detection test | DNA or Original | ~150 bp | Infection and Cancer Laboratory. Cancer Epidemiology Research Program, Institut |
| Seegene Anyplex II HPV test | Material | ~150 bp | Català d"OncologiaBarcelona, Spain |
| Self-screen HPV-Risk assay | DNA | ~150 bp | Cancer Center Amsterdam, VU University Medical Center, Amsterdam, The Netherlands |
| Genefirst HPV-MPA Genotyping Test | DNA | 150 bp. | Scottish HPV Reference Lab, |
| Liferiver Harmony test | DNA | 100–200 bp. | Royal Infirmary of Edinburgh |
| Liferiver Venus tes | | 100–200 bp. | Scotland |
| Comparator assays | | | |
| GP5+/6 + EIA Luminex | DNA | | DDL Diagnostic Laboratory, Rijswijk, The Netherlands |
| GP5+/6 + PCR EIA kit HPV GP HR | DNA | | International HPV Reference Center, Karolinska University Hospital, Stockholm, Sweden |

"check out test" is offered to women 60–64 years of age prior to exiting the screening program at age 65. The Danish cervical cancer screening program is free of charge at point of delivery. Samples are collected predominately by general practitioners or in some cases by gynecologists. The Combi-brush (Rovers, Oss, Netherlands) is used for collection of cell material and all cytology samples are collected in SurePath medium.

Samples from women aged 30–59 years were included in VALGENT4 study.

The VALGENT4 panel consists of 1297 routine samples collected in two groups as previously described by the VALGENT framework [9]:

- 998 unselected, consecutive samples, of which 51 samples had abnormal cytology (≥atypical squamous cells of undetermined origin, (ASCUS)). By subsequent histological follow-up, 13 women were diagnosed with cervical intraepithelial neoplasia 2 or more (≥CIN2)
- 299 consecutive routine samples enriched for ≥ ASCUS cytology (100 ASCUS, 100 low-grade squamous intraepithelial lesions (LSIL), 99 high-grade squamous intraepithelial lesions (HSIL)). From this population, 106 women were presented with ≥ CIN2 upon follow up.

Three samples were discarded after collection due to lack of general consent after mandatory cross-check in *Danish human biological material in health research projects register* (Vævsanvendelsesregisteret).

### 3.3. Sample processing

SurePath samples nominally contain 10 ml of liquid upon arrival in the laboratory. Currently, the Department of Pathology processes cervical cytology samples using the BD Totalys instrumentation. Here, the cytology processing uses 8 ml of the original sample, which is column purified for debris and mucus, spun down, pelleted, and re-suspended in 1 ml BD Density Reagent. Of this 1 ml medium, 200 µl is used for the cytology slide procedure. In routine, the remaining 800 µl is automatically added the 2 ml of fresh SurePath medium, resulting in a residual volume of 2.8 ml. By calculation, the final volume of the pelleted material in the 2.8 ml holds a 2.3 times higher concentration of cellular material than the original material. In order to approximate the cellularity of the original 10 ml sample, we developed a reconstitution protocol utilizing post-cytology pelleted material mixed with residual original material. The post-cytology pelleted sample was reconstituted by adding 3.6 ml of fresh SurePath medium, resulting in a volume of 6.4 ml (Fig. 1). In addition, the residual, original 2 ml surplus material from the cytology procedure was added to the 6.4 ml of reconstituted sample. Consequently, the nominal volume of the reconstituted sample available for testing in the VALGENT4 protocol was 8.4 ml.

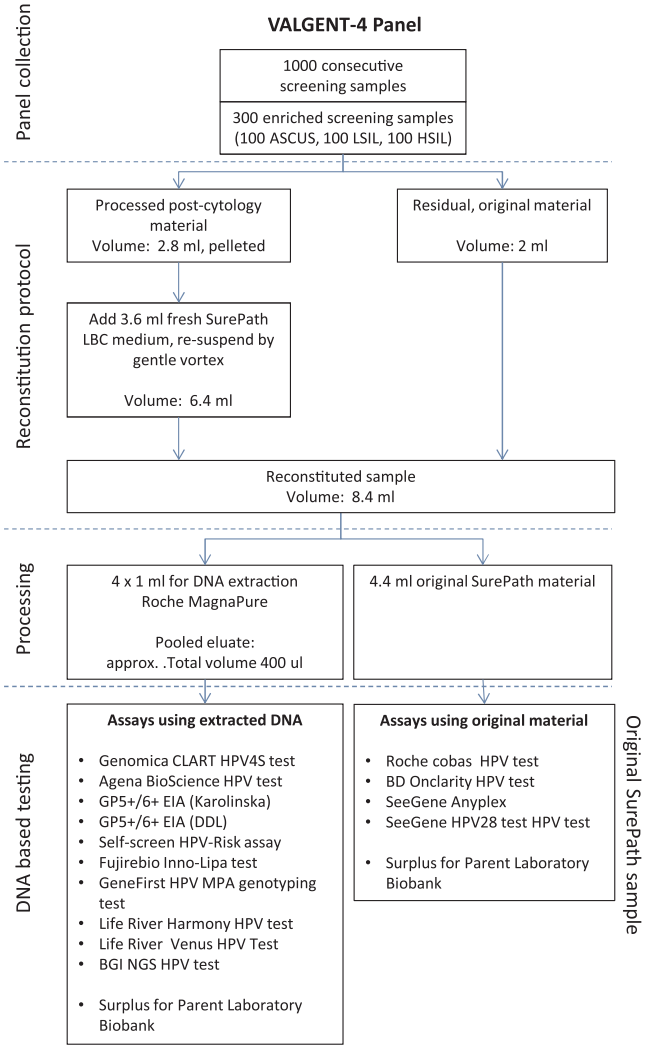


**Fig. 1.** Flowchart.

The reconstituted samples were split into aliquots for DNA extraction and original sample material upon processing of the samples (see processing time above).

### 3.4. DNA extraction

DNA was extracted under strictly controlled conditions using standard operating procedure clinically routine DNA extraction protocol at the parent laboratory based upon the MagNA Pure96 system (Roche Diagnostics, Pleasanton, CA). In short, 4 × 1 ml aliquots of reconstituted material were transferred to four Eppendorf tubes, spun down (14,000 rpm, 5 min), the supernatant removed, and the pellet re-suspended in a mix of 180 µl phosphate buffered saline (10x conc. pH 7.4, Pharmacy product) and 20 µl Proteinase K (Recombinant, PCR grade, Roche Diagnostics). The samples were vortexed and incubated one hour at 56 °C for Proteinase K digestion. Subsequently, the tubes were incubated for one hour at 90 °C to reverse SurePath formaldehyde induced co-valent cross linking. The entire volume was transferred to the MagNA Pure 96 system and extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics). The four DNA eluates were pooled into one tube to a total volume of 400 µl.

### 3.5. Cytology

Cytology was reported according to the Bethesda 2001 criteria, using computer assisted reading by BD Focal Point and Slide Wizard systems. ASCUS cases were triaged (reflex testing) routinely with HPV testing (BD Onclarity). All abnormal cytology findings were adjudicated as per standard operating procedures of the parent laboratory by a senior pathologist. Women with LSIL were recommended for repeated cytology testing after 6 months. Women with inadequate cytology were recommended for repeated sampling and testing within 3 months. Women with normal cytology were returned to routine screening according to age-specific intervals. The complete screening history of the women included in VALGENT4 was retrieved from the Danish Pathology Data Bank (PatoBank). All clinical follow up was managed according to Danish guidelines, and the outcome of the VALGENT4 HPV testing did not affect clinical follow up recommendations. All HPV testing performed in the framework of VALGENT4 was blinded to cytology and clinical follow-up.

### 3.6. Histology

All histology available for disease ascertainment was derived from the clinical follow-up of women whose samples were included in the VALGENT4 cohort. The following screening sample outcomes elicit a recommendation for follow-up with colposcopy and biopsy-taking under the current Danish guidelines:

- Women, ≥30 years with ASCUS and HPV-positivity upon triage testing.
- Women with HSIL, atypical squamous cells – cannot exclude HSIL (ASC-H), atypical glandular cells (AGS) or cytological indications of carcinoma.
- Women with continued ASCUS and LSIL cytological diagnosis, as evaluated by the present sample and screening history.

Danish Gynecology Guidelines recommend biopsy taking on all aceto-white lesions observed, or a random four quadrants biopsy set where no lesions are visible upon colposcopy. The histological follow-up for all women included in the VALGENT4 study was retrieved from the PatoBank.

### 3.7. Data source for clinical outcomes

The PatoBank follow-up period was on average 19 months (min. 18 months; max 20 months) from collection of the VALGENT4 samples. The registration of cervical cytology and histology in the PatoBank has been complete on a national level since 2008.

### 3.8. Participating scientific partner panel testing

All HPV testing within VALGENT4 was blinded to cytology and histology outcomes from the parent laboratory as well as the HPV test results from other scientific partners. All test results were reported to the Scientific Institute of Public Health, Brussels, where the data are compiled, and performance calculations are conducted [9]. All HPV testing was performed according to manufacturer's specifications. Nine different participating scientific partners (including the parent site) are taking part in the VALGENT4 study (Table 1).

### 3.9. Pre-analytical characterization of LBC material and DNA aliquot panel samples

#### 3.9.1. Pre-analytical characterization of cellularity of reconstituted original LBC material

All 1297 panel samples were tested with the BD Onclarity HPV assay (Onclarity) on the automated Viper Lt platform under the VALGENT4 protocol. The Onclarity assay harbors an internal Human Beta globin (HBB) control for assay performance and sample adequacy. To evaluate the resulting cellularity of the resuspension protocol, mean HBB Ct values from the 1297 VALGENT4 samples were retrieved from the Viper Lt and used as a proxy marker for cellularity. As comparator, mean HBB Ct values of unselected, unannotated routine cervical screening SurePath samples were used as a reference group for cellularity (designated "Control-1", N = 293). The Onclarity assay on the Viper Lt Platform workflow has previously been described in detail [27,36]. In summary, 0.5 ml original SurePath material is transferred to a BD CBD medium tube prior to heat treatment for 30 min at 120 °C on the BD pre-warm station. The pre-warmed samples are subsequently transferred to the fully automated Viper Lt platform and tested with the Onclarity assay according to manufacturer's recommendations. Mean HBB values between the two groups were compared using one-way ANOVA testing (IBM SPSS ver. 22).

#### 3.9.2. Amplifiable DNA copies and DNA fragmentation evaluation of DNA extracts

The iPLEX PRO ExomeQC assay (Agena Bioscience, Hamburg) is a quantitative assay which reports average amplifiable human DNA copies in a sample, in addition to reporting the relative number of amplifiable fragments of 100, 200, 300, 400, and 500 base pairs (bp) lengths. Here, the assay was used as a quality control of the DNA extracts derived from the reconstituted VALGENT4 panel. As comparator, DNA from unselected, unannotated, and un-manipulated SurePath cervical screening samples collected from the routine service were used as reference (designated "Control-2", N = 184).

The ExomeQC panel harbors 44 Single Nucleotide Polymorphisms (SNPs), three markers for gender identification and five copy-number markers in a single multiplexed assay. The assay uses the Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) technology. An initial multiplex PCR amplification was set up with 2 µl DNA (duration: 2.5 h), followed by a Shrimp Alkaline Phosphatase (SAP) reaction (dephoshorylize excess Nucleotides, duration: 50 min). This was followed by an iPLEX Pro single base extension PCR reaction in which a mix of oligonucleotide extension primers designed to anneal to the amplified DNA fragments, was added together with an extension enzyme and mass-modified dideoxynucleaotide terminators (duration 3.5 h). The extension products were subsequently desalted with Clean Resin prior to being loaded into the MassArray Dx Nanodispenser RS1000 (Agena Bioscience, Hamburg), which transfers the analyte to a spectroCHIP. Here the sample crystalize with the matrix on the chip, which was then analyzed on the MassARRAY Dx Analyzer 4 (Agena Bioscience, Hamburg). The crystals were irradiated by a laser, inducing desorption and ionization. The MA4 accelerates the samples to a detector that differentiates genetic variants by molecular mass.

**Table 2**
Overall characteristics of the VALGENT4 study population.

| | Screening population | | Enriched population[a] | | Total population | |
|---|---|---|---|---|---|---|
| Characteristics | N | % | N | % | N | % |
| **Age** | | | | | | |
| Mean Age in years | 42.8 (36.0–49.0) | | 40.4 (34.0–45.8) | | 42.2 (36.0–48.0) | |
| 30-39 | 383 | 38.4% | 148 | 49.8% | 531 | 41.0% |
| 40-49 | 408 | 40.9% | 111 | 37.4% | 519 | 40.1% |
| 50-59 | 207 | 20.7% | 38 | 12.8% | 245 | 18.9% |
| 30–59 | 998 | 100% | 297 | 100% | 1,295 | 100% |
| **Cytology** | | | | | | |
| NILM | 947 | 94.9% | 0 | 0% | 947 | 73.0% |
| ASCUS | 6 | 0.6% | 100 | 33.3% | 106 | 8.2% |
| LSIL | 21 | 2.1% | 100 | 33.3% | 121 | 9.3% |
| HSIL. AGC. ASC-H | 24 | 2.4% | 99 | 33.3% | 123 | 9.5% |
| Total | 998 | 100% | 299 | 100% | 1297 | 100% |
| **Histology** | | | | | | |
| No histology | 911 | 91.3% | 55 | 18.4% | 966 | 74.6% |
| Normal | 57 | 5.7% | 82 | 27.4% | 139 | 10.7% |
| CIN1 | 17 | 1.7% | 54 | 18.1% | 71 | 5.5% |
| CIN2 | 5 | 0.5% | 34 | 11.4% | 39 | 3.0% |
| CIN3 | 7 | 0.7% | 64 | 21.4% | 71 | 5.5% |
| Cancer | 1 | 0.1% | 8 | 2.7% | 9 | 0.7% |
| All histologies | 87 | 8.7% | 242 | 80.9% | 329 | 25.4% |
| < CIN2 | 74 | 7.4% | 136 | 45.5% | 210 | 16.2% |
| ≥CIN2 | 13 | 1.3% | 106 | 35.5% | 119 | 9.2% |

[a] Two women enrolled in Valgent4 each had two samples included; therefore therefor 299 samples from 297 women are included in the enriched cohort.

### 3.9.3. Statistical analysis

The Onclarity assay has a three well set-up with nine HPV genotype read-outs with internal HBB control included in each well. The Ct-value of the HBB was calculated as an average of the three HBB Ct values in each of the three wells for every panel and control sample. The Onclarity cut-off for all channels is Ct 34.2. For the ExomeQC analysis, inadequate samples (insufficient number of SNPs detected) and samples with outlier values above 10,000,000 units were excluded in the resulting analysis. Mean Ct, mean available copy numbers, 95% confidence interval, standard deviations as well as the one-way ANOVA test used to calculate the statistical difference in cellularity and DNA integrity parameters between the VALGENT4 panel and Control-1 and 2 samples were calculated using IBM SPSS statistics 22.

## 4. Results

The overall characteristics of the VALGENT4 study population are summarized in Table 2. The mean age was 42.8 years for all included women, and 40.4 and 42.2 years for the unselected, consecutive and enriched subpopulations, respectively. In the 998 consecutive, unselected panel samples, 5.1% (51 samples) had abnormal cytology and as of March 2018, 1.3% (13 samples) had ≥ CIN2 follow-up histology registered in the Danish National Pathology Registry (PatoBank). The enriched population of 100 ASCUS, 100 LSIL and 99 HSIL cytology samples resulted in 106 ≥ CIN2 histology samples as per register update March 2018. In total, 119 ≥ CIN2 cases were registered at data retrieval ≥20 month after baseline.

Comparing the relative cellularity (mean Ct values of HBB control from the Onclarity assay) on the 1,297 LBC panel samples with data from 293 separately collected, un-manipulated routine cytology screening samples (Denominated “Control-1” samples, Table 3A). The mean Ct value of the VALGENT4 samples (Ct = 24.8) was found to be on average 1.0 Ct above the mean Ct value of the Control-1 samples (Ct = 23.8), indicating slightly less cellularity in the panel samples compared to native samples.

For DNA extracted panel samples, characterization was done using the Exome iPLEX pro assay, which reports amplifiable copies on individual samples as well as amplifiable copies at five different base pair lengths (Table 3B). Here, the data showed statistically difference in the number of amplifiable DNA copies comparing the VALGENT4 panel to the Control-2 population (ratio of 0.7, p-value < 0.01). When looking at the relative proportion of different base pair length fragments, the statistical difference was only evident at the longest 400 and 500 bp fragments (Table 3B).

**Table 3A**
Quality assurance of the VALGENT4 LBC samples: Mean HBB Ct value as approx. marker for cellularity.

| Group | N | Mean HBB Ct | Ratio[a] | St.d | 95% Confidence Incidence | | Min | Max | P-value[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Lower | Upper | | | |
| Valgent4 | 1297 | 24.8 | 1.04 | 1.5 | 24.7 | 24.9 | 20.8 | 32.3 | 0.00 |
| Control1 | 293 | 23.8 | 1.00 | 1.5 | 23.6 | 24.0 | 20.5 | 32.0 | |

[a] The Control1 population was used as a reference.
[b] p-value is calculated using the one-way ANOVA test.

## 5. Discussion

The VALGENT framework is an international cooperation aimed at evaluating HPV genotyping tests for clinical use in cervical cancer screening. Here we present the study protocol for the fourth installment of the VALGENT framework, presenting data that provides insight into the underlying quality of the 1,297 VALGENT4 samples. One of the novel elements in VALGENT4 is the resuspension protocol employed which attempts to emulate the original cellularity of un-manipulated screening samples (Fig. 1).

For research use, most cross-sectional studies where HPV tests are applied rely on stored, residual clinical cytology screening material. In contrast, most HPV tests are intended to run on recently collected and un-manipulated original sample material. In addition, for evaluations where multiple assays are applied to samples, the volume of sample may be a rate-limiter. Consequently, to reconcile the need for authenticity with the operational requirements of the study, we developed a

**Table 3B**
Quality assurance of the VALGENT4 DNA samples: Amplifiable copy numbers at average and five different base pair length.

| | Group | N[a] | Mean amplifiable copies | Ratio[b] | Standard deviation | 95% Confidence Incidence | | Min | Max | P-value[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper | | | |
| Avg Copy | Valgent4 | 1,261 | 89,037 | 0.72 | 133,317 | 81,672 | 96,403 | 186 | 1,801,335 | 0.001 |
| | Control2 | 182 | 123,942 | 1.00 | 114,870 | 107,141 | 140,743 | 3,000 | 879,343 | |
| 100 bp | Valgent4 | 1,261 | 34,782 | 1.06 | 84,948 | 30,089 | 39,475 | 369 | 1,633,069 | 0.757 |
| | Control2 | 182 | 32,802 | 1.00 | 37,890 | 27,260 | 38,344 | 780 | 312,634 | |
| 200 bp | Valgent4 | 1,236 | 54,874 | 0.81 | 160,613 | 45,911 | 63,837 | 20 | 4,443,905 | 0.295 |
| | Control2 | 181 | 67,766 | 1.00 | 104,680 | 52,413 | 83,119 | 569 | 795,228 | |
| 300 bp | Valgent4 | 1,261 | 156,201 | 0.77 | 380,175 | 135,198 | 177,205 | 193 | 8,254,550 | 0.124 |
| | Control2 | 182 | 202,641 | 1.00 | 380,425 | 147,000 | 258,282 | 3,669 | 3,960,481 | |
| 400 bp | Valgent4 | 1,260 | 104,438 | 0.67 | 268,635 | 89,590 | 119,285 | 36 | 6,068,401 | 0.014 |
| | Control2 | 182 | 156,585 | 1.00 | 268,209 | 117,356 | 195,813 | 3,726 | 2,904,381 | |
| 500 bp | Valgent4 | 1,249 | 93,544 | 0.59 | 396,191 | 71,550 | 115,537 | 48 | 8,947,189 | 0.031 |
| | Control2 | 181 | 159,193 | 1.00 | 265,673 | 120,227 | 198,159 | 5,843 | 1,804,460 | |

[a] Samples with insufficient number of SNPs were excluded, in addition a sample could have a valid SNPs number, but invalid result in one of the base pair groups, which mean that the numbers in the different base pair groups vary. In addition, samples with outlier values of above 10,000,000 were excluded from both sample sets.
[b] The Control2 population was used as a reference.
[c] The p-value is calculated using the one-way ANOVA test.

reconstitution protocol taking into account the changes introduced to the freshly collected VALGENT4 samples by routine cytology processing. The aim was to reestablish the residual cytology pelleted material to an approximate *per volume cellularity* corresponding to the original cellularity in un-manipulated clinical samples (Fig. 1). This reconstitution protocol was developed with the assistance of Becton, Dickinson and Company, the manufacturer of the SurePath medium, by reverse engineering the concentration and resuspension steps included in standard SurePath processing of original, fresh samples.

As the mean beta globin Ct value of the VALGENT4 samples (Ct = 24.8) was found to be 1.0 Ct above the Control-1 samples (Ct = 23.8), this suggests that the reconstituted panel samples had slightly lower cellularity than freshly collected, un-manipulated screening samples. However, in the context of the clinical positive-negative Onclarity HPV assay cut off (Ct 34.2), the reconstituted panel samples were fully acceptable as clinically, analytical material for HPV analysis. In combination with the ratio data from the ExomeQC analysis, this describes the resulting degree of dilution introduced to the reconstituted panel samples compared to the original sample cellularity. Moreover, the DNA fragmentation analysis showed that fresh, un-manipulated samples had more 400 and 500 bp DNA fragments compared to the reconstituted panel samples. This clear trend downwards between reconstituted samples and original samples in Control-2 was however not unexpected as all sorts of manipulation of cells and DNA leads to some level of deterioration. Whether this has implications for the analytical and clinical performance of HPV assays is unlikely, given that all included assays in the VALGENT4 protocol rely on amplification of less than 500bp HPV fragments (Table 1).

In conclusion, the two QA/QC methods employed indicate that the reconstituted samples used for the VALGENT evaluation contain slightly less analytical material compared to fresh, un-manipulated screening samples. However, the amount of analytical relevant material is reassuringly within the operational boundaries of all included VALGENT4 HPV assays (Table 1).

Besides the reconstitution protocol, VALGENT4 introduced central organization of DNA extraction for all assays running on this input material. This was chosen to eliminate any variation introduced at the level of DNA extraction methodology. Of the 11 assays evaluated in VALGENT4, only BD Onclarity, Seegene Anyplex HPV28/HPV II HR and Roche Cobas HPV assays required original SurePath LBC material as input material for analysis.

Until now, three VALGENT installments have been completed with several genotyping assays tested. The data from these VALGENT studies has showed that the VALGENT framework provides a good base for cross-sectional clinical validation of HPV genotyping assays, by use of well annotated cervical screening samples collected in various screening programs around Europe [9]. A common feature of the VALGENT installments is the use of GP5+/6 + PCR-EIA as the comparator assay to all other included assays. This brings the VALGENT in line with the stipulations within the international validation Guidelines of Meijer et al.

The first VALGENT study, VALGENT-1, included cervical cancer screening samples provided by the AML laboratory (Antwerp, Belgium) and included validation of four full genotyping assays [37–39]. The VALGENT-1 framework included SurePath samples from biobanked screening samples, and thus not freshly collected samples as included here. The second VALGENT study, VALGENT-2, used ThinPrep, involved the collection of samples from the Scottish Cervical Screening Programme via the Scottish HPV Reference laboratory and involved the evaluation of four assays with genotyping capability [40–43]. VALGENT-3 also included ThinPrep collected cervical cancer screening samples, this time from the Laboratory for Molecular Microbiology, Faculty of Medicine, University of Ljubljana, Slovenia [44] and evaluated 10 different HPV assays with different degrees of genotyping capability. VALGENT-1 [37–39] and VALGENT-2 [40–43] have both provided several peer-reviewed papers and publications from VALGENT-3 are underway [32,44]. Formal clinical validation of HPV assays for use in screening has primarily been undertaken on ThinPrep collected samples [24,26,28,30,41,45,46], with only one assay to date, the BD Onclarity, being validated on both ThinPrep [26] *and* SurePath collected samples [27]. Following this, it will be interesting to see how the HPV genotyping assays included in VALGENT4 perform on SurePath collected screening samples in this large-scale performance comparison.

## Ethical and data inspection agency approvals

The study was approved by the Danish Data Inspection Agency J. No. AHH-2017-024, I-Suite: 05356. EU-GDPR compliant data handler agreement was established between the principal site Hvidovre Hospital and the Statistical analysis unit at Sciensano, Brussels. All collected samples were verified for non-compliance in the Danish human biological material in health research projects register (Vævsanvendelsesregisteret).

## Declaration of competing interest

VALGENT is an independent researcher induced research project,

set up by Sciensano, where manufacturers can have their HPV assays evaluated, under condition to provide equipment and kits and to cover costs for laboratory work and statistical analysis. Manufacturers cannot influence publication of manuscripts.

Jesper Bonde attended meetings with various HPV device manufacturers. JB has received honoraria from Hologic/Gen-probe, Roche, Qiagen, Genomica, and BD Diagnostics for lectures. Hvidovre Hospital has ongoing contracts with BD Diagnostics, Genomica, Self-Screen and EU Horizon2020.

Ditte Møller Ejegod attended meetings with various HPV device manufacturers.

Joakim Dillner has previously received research grants to his institution for research on HPV vacciens from an HPV vaccine manufacturer (Merck/SPMSD). The Karolinska University Hospital has ongoing contracts with several manufacturers of HPV diagnostics, including Roche and Genomica.

Daniëlle Heideman: minority stock portfolio in Self-screen B.V., a spin-off company of VU University Medical Center Amsterdam. Self-screen B.V holds patents related to the work, and has developed and manufactured the HPV-Risk Assay. DAMH has been on the speaker's bureau of Qiagen and serves occasionally on the scientific advisory board of Pfizer and Bristol-Meyer Squibb.

Wim Quint: No conflicts of interest to declare.

Miguel Angel Pavon Ribas: received reagents for HPV testing at no cost from Roche, SeeGene, Qiagen and Genomica for research purposes.

Elizaveta Padalko: No conflicts of interest to declare.

Irene Kraus Christiansen: No conflicts of interest to declare.

Kate Cuschieri: No conflicts of interest to declare, KCs institution has received research funding and or associated gratis consumable from the following in the last 3 years: Hologic, Cepheid, Qiagen, Becton-Dickinson, Euroimmun, SelfScreen, LifeRiver, Genomica, Genefirst.

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Copenhagen University Hospital, Hvidovre: Reagents and instrumentation for testing Onclarity, CLART HPV4s and MassArray HPV test were received free of charge from the manufacturers for the duration of the testing. Limited co-funding for the project was obtained as part of the collaboration agreement with the manufacturers Becton, Dickinson and Company, Genomica SAU, and Agena Biotech Gmbh.

Norwegian HPV Reference Lab: No external funding was obtained for this study.

Ghent University: Reagents for testing INNO-LIPA genotyping Extra II test was received free of charge from the manufacturer for the duration of the testing.

Infection and Cancer Laboratory, Barcelona: No external funding was obtained for this study

VU University Medical center: No external funding was obtained for this study.

Scottish HPV reference lab: Reagents and instrumentation for testing LifeRiver Venus, LifeRiver Harmonia, and Genefirst HPV-MPA Genotyping Test were received free of charge from the manufacturers for the duration of the testing. Limited co-funding for the project was obtained as part of the collaboration agreement with the manufacturers Zhanghai Biotech (LifeRiver) and GeneFirst (HPV-MPA Genotyping Test).

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## Author contribution

Design of Protocol: Jesper Bonde, Ditte Møller Ejegod, Lan Xu & Marc Arbyn.

Panel testing and reporting: Joakim Dillner, Daniëlle Heideman, Wim Quint, Miguel Angel Pavon Ribas, Elizaveta Padalko, Irene Kraus Christiansen, Kate Cuschieri, Ditte Møller Ejegod, Jesper Bonde.

Statistical analysis: Marc Arbyn & Lan Xu.

Writing of Manuscript: Jesper Bonde, Ditte Møller Ejegod, Marc Arbyn.

Editing of manuscript: All.

Decision to submit: All.

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