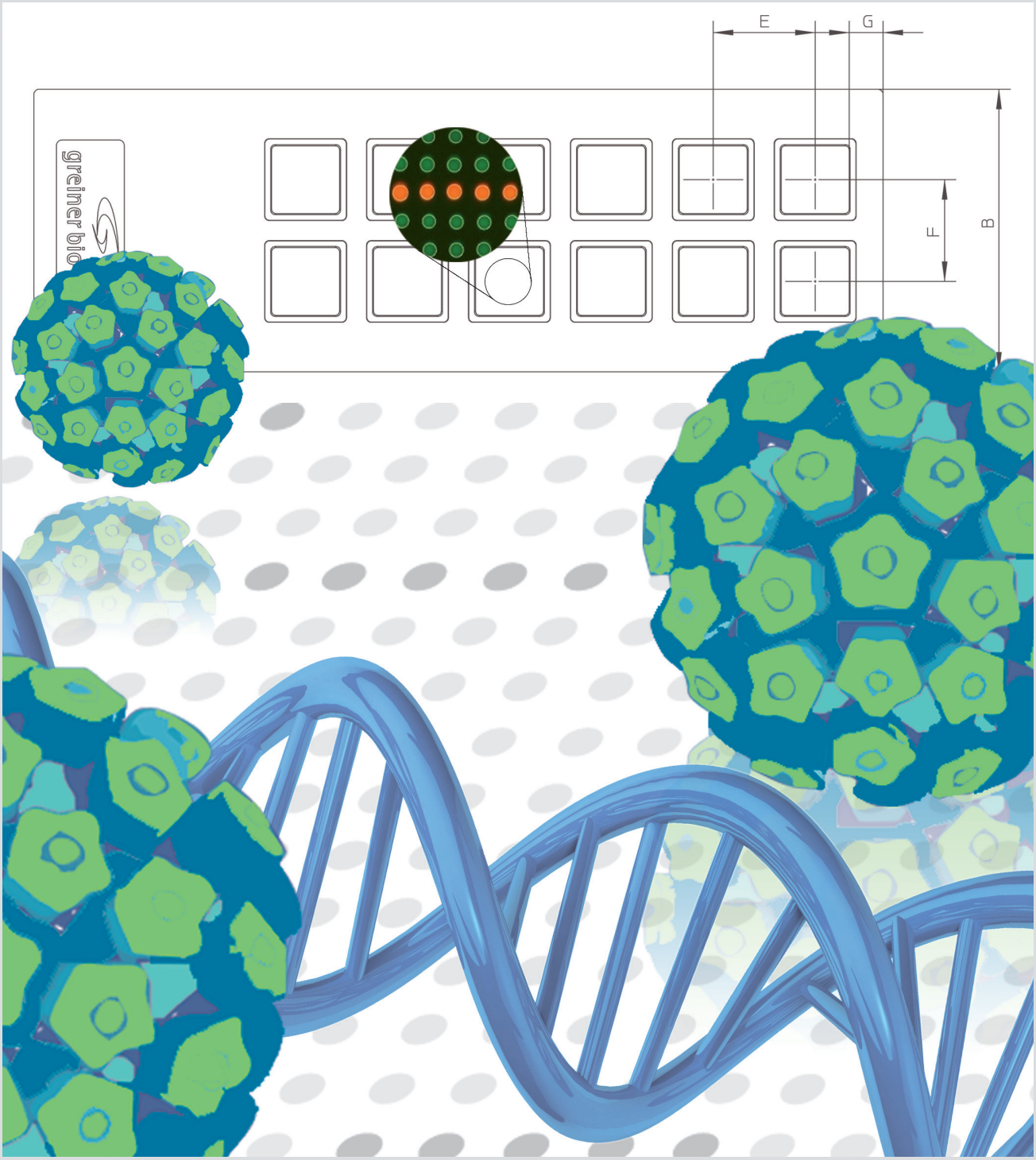
# Application Note

## Isolation of DNA from cervical specimens with the chemagic Prepito for the detection of human papillomaviruses (HPV) using the PapilloCheck® HPV-genotyping assay



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## Introduction

The underlying cause of cervical cancer is persistent infection with certain types of human papillomaviruses (HPV)[1]. Cervical HPV types are classified into a high risk (high-risk HPV, hrHPV) and a low risk group (low-risk HPV, lrHPV). Whereas the high-risk HPV types are associated with an increased risk of developing cervical cancer, low-risk HPV types mainly cause benign genital warts[2]. Because of this central role played by HPV in the development of cervical cancer and cervical disease, diagnostic assays are being developed to detect HPV-DNA in cervical specimens.

Papill**oCheck**® from Greiner Bio-One is a CE-IVD certified diagnostic kit for the detection and genotyping of 24 human HPV types in DNA preparations from human cervical smears. It allows the simultaneous identification of HPV types by amplifying a fragment of the HPV genome via polymerase chain reaction (PCR) and hybridising the PCR products to specific probes of a DNA-microarray. For PCR-based diagnostic tests and even more for such a multiplex HPV genotyping system, the DNA isolation process is a critical step[3]. Alternative extraction methods have an impact on the performance of the subsequent HPV detection and the HPV type-specific profiles obtained[4]. Therefore, robust methods for DNA extraction from either liquid-based cytology (LBC) media and collection media used for HPV testing are needed to reduce the amount of invalid HPV tests and to guarantee intra-assay reproducibility[5,6].

Papill**oCheck**® has been validated using DNA prepared with the oCheck® DNA Extraction Kit (Greiner Bio-One, Frickenhausen, Germany). This application note aimed at comparing this manual silica column-based method and an automated magnetic bead-based protocol of the **chemagic Prepito** (chemagen AG, Baesweiler, Germany) for the isolation of human and viral DNA from cervical smears stored in four different commercial specimen collection media, namely Papill**oCheck**® Collection Kit (Greiner Bio-One), PreservCyt® (Hologic, Bedford, MA, USA), SurePath™ (BD, Franklin Lakes, NJ, USA) and STM™ (Qiagen, Hilden, Germany) medium. The **chemagic Prepito** turned out to be a suitable option for automated DNA extraction from all tested collection media for the subsequent Papill**oCheck**® HPV detection.


[1] Walboomers, J. et al (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999 Sep;189(1):12-9.
[2] Bosch F.X. et al. (2008). Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine. 2008 Aug 19;26 Suppl 10:K1-16.
[3] Dunn, S.T. et al (2007). DNA extraction: an understudied and important aspect of HPV genotyping using PCR-based methods. J Virol Methods. 2007 Jul;143(1):45-54.
[4] Roberts C.C. et al. (2011). PCR assays with the linear array HPV genotyping PCR assay and influence of DNA extraction method on HPV detection. J Clin Microbiol. 2011 May;49(5):1899-906.
[5] Peevor R. et al. (2011). Development of optimal liquid based cytology sample processing methods for HPV testing: minimising the ‚inadequate' test result. J Virol Methods. 2011 May;173(2):374-7.
[6] Donà M.G. et al. (2011). Comparative evaluation of different DNA extraction methods for HPV genotyping by linear array and INNO-LiPA. J Med Virol. 2011 Jun;83(6):1042-7.


## Material and Methods

**Table 1:** List of materials and suppliers

| | | |
|---|---|---|
| **Automated DNA Extraction** | | |
| chemagic Prepito | art. No. 540 | PerkinElmer chemagen Technologie GmbH |
| Prepito Pathogen NA Kit* | art. No. 2022 | PerkinElmer chemagen Technologie GmbH |
| Prepito Pathogen NA Extension Kit** | art. No. 2023 | PerkinElmer chemagen Technologie GmbH |
| **Manual DNA extraction** | | |
| oCheck® DNA Extraction Kit | Cat. No. 515040 | Greiner Bio-One |
| **HPV detection** | | |
| Papill**oCheck**® | Cat. No. 465060 | Greiner Bio-One |
| CheckScanner™ | Cat. No. 862070 | Greiner Bio-One |
| CheckReport™Software | Cat. No. 862080 | Greiner Bio-One |

*The Prepito NA Body Fluid Kit (art. No. 2021) or the Prepito Viral NA/gDNA Kit (art. No. 2020) can also be used for the extraction. Dedicated machine protocols must be used.
**Includes two lysis buffers and Proteinase K to perform the extraction from cervical specimens stored in SurePath™ medium.

**Samples:** All together 210 cervical specimens were extracted in parallel with both the oCheck® DNA Extraction Kit and the **chemagic Prepito**. Finally each DNA extract was analysed with the Papill**oCheck**® HPV genotyping assay. The sample set consisted of 48 samples fixed in PreservCyt®, 48 in Papill**oCheck**® Collection Kit, 48 in STM™ and 66 in SurePath™ medium.

**Manual DNA extraction - oCheck® DNA Extraction Kit:** Extractions were performed according to the manufacturers protocol, in brief: samples stored in Papill**oCheck**® Collection Kit or PreservCyt® medium were directly processed. Cervical samples fixed in STM™ collection medium were prepared by diluting 100 µl of sample with 150 µl water. The resulting 250 µl were used for the subsequent DNA isolation process. For samples fixed in SurePath™ medium, 250 µl sample were centrifuged for 5 min at 11000 g. The pellet was resuspended in 250 µl water and subsquently used for DNA extraction. For pre-lysis, 80 µl buffer L1, 2.4 µl carrier RNA and 20 µl Proteinase K solution was added to 250 µl of the sample and incubated for 30 minutes at 56°C. Lysis was carried out by adding 250 µl buffer L4 and incubation for 15 minutes at 70°C. After adjusting the conditions for DNA binding to the silica membrane by adding 300 µl ethanol, the DNA in the lysate was bound to the spin column in two steps and washed with 500 µl buffer W1 and 600 µl buffer W2. After drying the spin column, DNA was eluted with 100 µl pre-heated (70°C) elution buffer and analysed with the Papill**oCheck**® HPV genotyping assay.

**Automated DNA extraction - chemagic Prepito:** Extractions were performed according to the manufacturers protocol, in brief: samples stored in Papill**oCheck**® Collection Kit or PreservCyt® medium were directly processed. For cervical

samples fixed in STM™ collection medium, an aliquot of 100 µl sample was diluted with 150 µl water before using only 200 µl for the subsequent automated DNA isolation process. According to the **Prepito Pathogen NA Kit** protocol, 10 µl Proteinase K and 4 µl Poly (A) RNA was added to each 200 µl sample aliquot. Following this, the reaction vessels were filled with 150 µl Magnetic Beads and the Elution Tubes with 100 µl Elution Buffer. Any other buffer needed during the run was dispensed automatically. Since sample fixed in SurePath™ medium turned out to be critical, a protocol was established to isolate human and viral DNA from cervical swabs stabilized in this medium using the **Prepito Pathogen NA Extension Kit**: 200 µl of sample fixed in SurePath™ medium was centrifuged for 5 min at 11000 g. The supernatant was discarded and the pellet was resuspended in 200 µl water. 110 µl Lysis Buffer EL 1, 20 µl Proteinase K and 2.4 µl carrier RNA was added. After an incubation step of 30 min at 56° C, 340 µl Lysis Buffer EL 2 was added followed by another incubation step of 15 min at 70°C. This premix was processed with a dedicated machine protocol without automated dispensing of lysis buffer.


**Figure 1:** chemagic Prepito (PerkinElmer chemagen Technologie)

Papill**oCheck**® **HPV genotyping:** Papill**oCheck**® was performed according to the manufacturers protocol. Assay principle: after the extraction of viral and human genomic DNA from a cervical specimen, a 350 bp fragment of the E1 gene of the HPV genome is amplified by PCR in the presence of a set of HPV-specific primers. In the same reaction, a fragment of the human "house keeping gene" ADAT1 (human tRNA-specific adenosine deaminase1) is amplified to monitor the presence of human sample material in the cervical specimen (sample control) and an internal control-template present in the Papill**oCheck**® MasterMix is amplified to monitor the performance of the PCR (PCR control). The PCR products are then hybridised to specific DNA probes and on-chip controls attached to the Papill**oCheck**® chip surface and unbound DNA is removed in the subsequent washing steps. Finally, the Papill**oCheck**® chip is automatically scanned, analysed and evaluated using the CheckScanner™ and CheckReport™ Software, respectively. The CheckReport™Software allows the visualization, analysis and evaluation of the results and automatically shows both the corresponding values (signal to-noise ratio, SNR) of the detected HPV types and the controls in a detailed report. Papill**oCheck**® identifies 18 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82) and 6 low-risk HPV types (6, 11, 40, 42, 43, 44) in parallel.

**Data Evaluation:** The Papill**oCheck**® assay features a comprehensive range of on-chip controls. Several control systems monitor all critical steps of both the assay and chip processing, including presence of human material in the cervical specimen and successful DNA extraction (sample control), quality of the PCR reaction (PCR control) and the efficiency of the hybridisation (hybridisation control), as well as printing quality (orientation control and printing control). For the comparison of the extraction methods the sample control and PCR control were used as evaluation criteria. Mean SNR values of the control signals and standard deviations were calculated for cervical samples analyzed. Calculations were performed separately for each collection medium used. Furthermore, the positive concordance and negative concordance of the HPV results were calculated. Positivity was defined as a positive HPV results for a certain sample, independent of the HPV type detected. For comparison of the genotyping result the following definitions apply: type-specific profiles were rated identical when exactly the same genotyping result was obtained with both extraction systems. For samples positive for multiple HPV types, genotyping results were rated as concordant, when at least the most dominant types were detected with both extraction methods used.


**Figure 2:** Papill**oCheck**® (Greiner Bio-One)


**Figure 3:** oCheck® DNA Extraction Kit (Greiner Bio-One)

## Results

**Performance of sample and PCR controls:** DNA was extracted from cervical samples stored in four different fixatives using manual (oCheck® DNA Extraction Kit) and automated extraction (**chemagic Prepito/Prepito Pathogen NA Kit**) in parallel. For automated extraction of samples fixed in SurePath™ medium, samples had to be pre-treated for efficient DNA extraction. As pre-treatment an additional off-board lysis protocol was established and reagents for this treatment are now supplied by the **Prepito Pathogen NA Extension Kit**. The efficiency and quality of the extraction was assessed by comparing the signal-to-noise ratios (SNR values) of two on-chip controls – sample control and PCR control. Mean SNR values and standard deviations are displayed in **Figure 4** stratified for the different media and methods used.

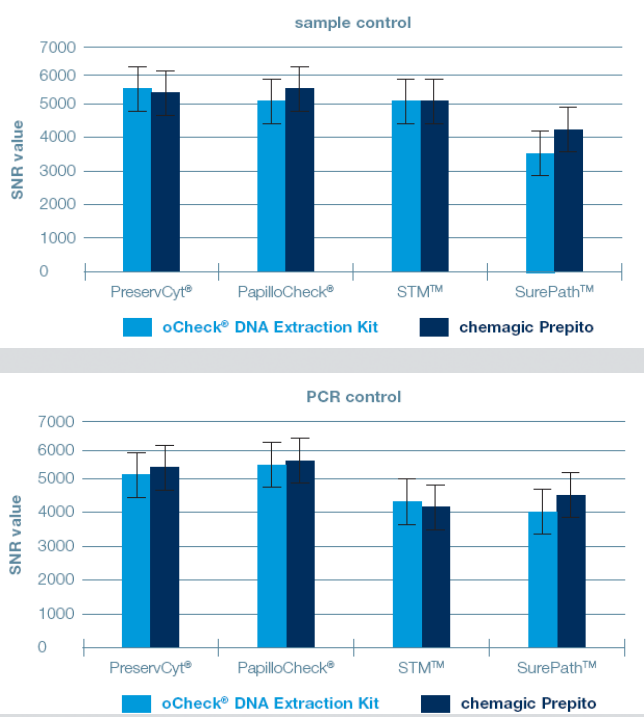


**Figure 4:** Performance of sample and PCR controls

Comparable results were obtained from both DNA isolation methods, independent of the collection media used. For both controls, no significant performance differences were observed comparing the two DNA extraction methods.

**Concordance of HPV detection and genotyping:** To further investigate the influence of the DNA isolation method on the HPV result, the positive and negative concordances were calculated. HPV positivity and negativity (see Data Evaluation in the Materials and Methods section) for all samples tested are summarized in **Table 2**, stratified for the DNA extraction method used.

**Table 2:** Papill**oCheck**® HPV results obtained for samples stored in the different media

| | | Manual DNA Extraction (oCheck® DNA Extraction Kit) | | |
|---|---|---|---|---|
| | | HPV positive | HPV negative | total |
| Automated DNA Extraction (chemagic Prepito) | HPV positive | 81 | 4 | 85 |
| | HPV negative | 5 | 120 | 125 |
| | total | 86 | 124 | 210 |

Again, both extraction methods performed equally well. The positive and negative concordance of HPV results for all samples tested was as high as 94.2 % and 96.8 %, respectively. For five samples, DNA extraction with the oCheck® DNA Extraction Kit led to HPV positive results, whereas the application of the **chemagic Prepito** led to HPV negative results. For almost the same amount of samples (four samples) it was vice versa. Discordant results were equally distributed across the different collection media with one exception – for the Papill**oCheck**® Collection Kit medium, no discordant results were obtained. Further analysis of the results revealed that in each discordant case an HPV-specific signal near the detection limit of the Papill**oCheck**® assay was obtained using one of both extraction methods, but missed using the other. In general, for samples with virus loads very near the detection limit of an HPV assay, any minor variation within the procedure can be responsible for discrepant HPV results. Comparing the type-specific profiles of the HPV positive samples, no discordant genotyping result was obtained using the two extraction methods.

## Conclusion

In comparison to the validated manual DNA extraction method, automated DNA isolation using the **chemagic Prepito** has proved to be a reliable and efficient option for cervical specimens that will subsequently be analyzed using Papill**oCheck**®. Applying the **Prepito Pathogen NA Kit**, Papill**oCheck**® results were highly concordant for samples stored in all tested commercial sample collection media. Samples stored in Papill**oCheck**® Collection Kit, PreservCyt® and STM™ medium were directly processed, SurePath™ samples were pre-treated with the dedicated **Prepito Pathogen NA Extension Kit**.

## Acknowledgement

All experiments were designed by Dr. M. Schleichert and performed by D. Wallner and M. Formanek at Lambda GmbH, Rainbach (Austria), a subsidiary of Greiner Bio-One GmbH. This application note and the adaptation of the protocol for cervical samples stored in SurePath™ was completed in close cooperation with Dr. C. Henze and Dr. U. Schmitz from PerkinElmer chemagen Technologie GmbH, Baesweiler (Germany).

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