Use of Thermomixer comfort and ThermoStat plus in HPV diagnostics using chromogenic in situ hybridization (CISH)

Zofia Borda¹, Pierre Rogalla² and Sven Hauke²

¹ 42 life science, Fischkai 1, 27572 Bremerhaven, Germany, www.42ls.com
² ZytoVision, Fischkai 1, 27572 Bremerhaven, Germany, www.zytovision.com

Summary

On a worldwide basis, cervical carcinoma is one of the most frequent tumor-related causes of death in women. Substantially responsible for the development of cervical carcinoma is a persistent infection with human papilloma viruses (HPV) of the “high-risk type”. A method for detection of such HPV infections in fixed tissue sections is provided by the ZytoFast® HPV-ISH system which is based on the method of chromogenic in situ hybridization (CISH). This study examined the suitability of the Eppendorf Thermomixer comfort and the Eppendorf ThermoStat plus with slide adapter for in situ hybridization in HPV diagnostics. It was shown that Thermomixer comfort and ThermoStat plus yield comparable results with regard to signal intensity and unspecific background as does the standard method with a heating plate and incubator. Moreover, because of the digitally programmable temperature steps, Thermomixer comfort and ThermoStat plus facilitate maximum reproducibility of the experimental conditions and require substantially less effort.

Introduction

Despite routine medical checkups, cervical carcinoma remains one of the most frequent tumor-related causes of death in women throughout the world. Today, it is scientifically undisputed that the origin of cervical carcinoma is substantially the result of an infection with human papilloma virus (HPV) (1). Since the papilloma virus infection is a sexually transmitted disease, HPV infections can be detected in large segments of the sexually active population. However, in 80-90 % of all cases they can be successfully combated by the immune system (2). Only in approx. 2 % of all infected women an aggressive cervical carcinoma develops over the long-term (3). The papilloma viruses can be subdivided into 2 groups:
The less aggressive “low-risk types of HPV” (e.g., HPV type 6, 11, 42, 43 and 44) cause a cellular growth disorder which is associated with the formation of anogenital warts. However, the risk of degeneration into malignant cells is small.

The group of “high-risk types of HPV” (e.g., HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) leads to pronounced cellular growth disorders, which, without medical treatment, are associated with a significantly elevated risk of developing cancer. The high-risk HPV types 16 and 18 are responsible for the majority (approx. 70 %) of all malignant cervical tumors (4). A progression from initial dysplasia to malignant carcinoma occurs only in the presence of a persistent HPV infection in which the dsDNA HPV genome, or parts thereof, is integrated into the host genome and leads to cellular deregulation (1).

A proper differential diagnosis of such HPV infections can, for example, be performed with the ZytoFast HPV-ISH system (ZytoVision, Germany). By using the method of chromogenic in situ hybridization (CISH), HPV-infected cells in tissue sections are made visible to light microscopy by means of colored precipitates.
HPV detection with CISH, methodically very similar to the widespread use of immunohistochemistry, has the advantage of being able to evaluate the results simultaneously with the morphological evaluation of the tissue.

During this study, sample material was examined for HPV infections by using the ZytoFast HPV-ISH system. Concerning the quality of the CISH signals, different incubation strategies for incubating the microscopic slides were compared: 1) The classical method in which denaturation occurs on a heating plate and hybridization occurs in a humidity chamber in the incubator and 2) alternatively the continuous use of the Eppendorf Thermomixer comfort and the ThermoStat plus with the exchangeable thermoblock for “slides”.

**Introduction**

HPV detection with CISH, methodically very similar to the widespread use of immunohistochemistry, has the advantage of being able to evaluate the results simultaneously with the morphological evaluation of the tissue.

During this study, sample material was examined for HPV infections by using the ZytoFast HPV-ISH system. Concerning the quality of the CISH signals, different incubation strategies for incubating the microscopic slides were compared: 1) The classical method in which denaturation occurs on a heating plate and hybridization occurs in a humidity chamber in the incubator and 2) alternatively the continuous use of the Eppendorf Thermomixer comfort and the ThermoStat plus with the exchangeable thermoblock for “slides”.

**Materials and Methods**

The ZytoFast HPV Screening ISH Kit (ZytoVision, Germany) was used to detect human papilloma viruses of types 6, 11, 16, 18, 31, 33 and 35 in tissue sections. Three different methods were used for the incubation of the slides during denaturation and hybridization: The classical method with denaturation on a precision heating plate (Harry Gestigkeit, Germany) and hybridization in a humidity chamber in an incubator (Fisher Scientific, USA). This method was compared to incubation results obtained by using Thermomixer comfort or ThermoStat plus with exchangeable thermoblock for “slides” (Eppendorf, Germany). In situ hybridization was performed according to the instructions of the manufacturer:

**Sample material**

For the experiments 5 μm sections of 2 different formalin-fixed and paraffin-embedded cervical biopsies were used, which had previously been fixed overnight at 60 °C on Histobond® microscopic slides (Paul Marienfeld, Germany).

**Deparaffinization**

Deparaffinization occurred by incubating the sections for 10 min at 70 °C, then at room temperature (RT) 2 x 10 min in 100 % xylol and then washing them for 1 x 5 min in 100 % ethanol. Afterwards, the sections were dried.

**Proteolytic pretreatment**

For proteolytic pretreatment all sections were incubated with pepsin solution for 15 min at 37 °C in a humidity chamber in an incubator. The reaction was stopped for 10 sec in 100 % ethanol and then washed for 30 sec in dH₂O. Afterwards, the sections were dried.

**Denaturation and hybridization**

The HPV probe was pipetted on the sections; the samples then were covered with a coverslip (CS) and sealed with Fixogum (Marabu, Germany). For denaturation and hybridization the slides were incubated for 10 min at 75 °C or 1 h at 37 °C. After hybridization, the CS were removed and the slides were washed in 1 x washing buffer for 5 min at RT, 5 min at 55 °C and 5 min at RT. After an additional washing step for 2 min at RT in dH₂O, the slides were air dried.

**Detection and color development**

The reaction with the AP anti-biotin antibody occurred for 30 min at 37 °C. Then the slides were washed at RT for 2 x 2 min in 1 x washing buffer and 1 x 2 min in dH₂O and then air dried. For color development the sample was incubated with AP substrate (BCIP/NBT) for 30 min at 37 °C, washed at RT 3 x 2 min in dH₂O, air dried and embedded with glycerol. Both the antibody and the color substrate were placed on the sections by using in situ Frames (Eppendorf, Germany). For the incubation steps the Thermomixer comfort and the ThermoStat plus were programmed with the particular temperature values. The Thermomixer comfort also was used to examine the effect of different mixing speeds (0, 300, 900 and 1400 rpm) on the intensity of the CISH signals and the required hybridization time. To guarantee a humid environment in the exchangeable thermoblock for “slides” during incubation, the “reservoir pads” of the block were saturated with dH₂O.

Analysis of the hybridization results occurred on an Axiostar plus light microscope (Carl Zeiss, Germany). From each of the two samples, this entailed evaluating 2 different areas on each of 2 sections with regard to the signal/background ratio and the absolute signal intensity.
Results

The analysis of a total of 24 sections confirmed that all three incubation methods (heating plate/incubator, ThermoStat plus and Thermomixer comfort) yield comparable results with regard to signal intensity and unspecific background (Fig. 1). In some samples incubation with the Thermomixer comfort yielded somewhat stronger signal intensities at comparable background staining (data not shown). In addition to the experiments which were performed in accordance with the ZytoFast HPV Screening ISH Kit, the Thermomixer comfort was also used to examine the effect that dynamic mixing during incubation has on the quality of the hybridization results. For the buffers and reaction conditions used in this kit, continuous mixing during hybridization neither permits a shortening of the incubation time given by the manufacturer nor leads to an intensification of the signals (results not shown). This result can be explained by the fact that the ZytoFast system uses a probe cocktail for HPV detection consisting of short labeled oligonucleotides as well as optimized antibodies. Under standard conditions this guarantees an optimal penetration of the tissue and, thereby, the best possible signal intensity.

Figure 1: Light microscopic images of different tissue sections that were examined for HPV infections by CISH analysis. In each case an overview image of the tissue section and a detailed enlargement thereof can be seen. Cells that exhibit a dark color have tested positive for human papilloma viruses (HPV) with the ZytoFast HPV Screening ISH Kit. The incubation steps either were performed with the Eppendorf Thermomixer comfort (A), Eppendorf ThermoStat plus (B), or on a heating plate followed by incubation in humidity chamber in an incubator (C).
Discussion

The results demonstrate that both Eppendorf Thermomixer comfort and Eppendorf ThermoStat plus in combination with the exchangeable thermoblock for “slides” are very well suited for performing CISH analyses and deliver results that are comparable to those of the standard method which uses a heating plate/incubator. In contrast to the standard method, using the Eppendorf Thermomixer comfort or the ThermoStat plus requires significantly less manipulation during chromogenic in situ hybridization (CISH). This is mainly due to the programmable temperature change of the devices. After denaturation has been completed, it is no longer necessary to transfer the samples into a humidity chamber and in an incubator for hybridization. In addition to reducing the number of processing steps required, the programmable transition between the denaturation and hybridization step (especially in protocols with co-denaturation of probe and sample) also facilitates a defined temperature change and, thereby, offers maximum reproducibility of the experimental conditions. Overall, use of the Thermomixer comfort or the ThermoStat plus, especially in laboratories with only a small sample throughput, is a sensible alternative to the “classical” approach with heating plate/incubator, especially from the perspective of available space and investment costs.

References


Ordering information

<table>
<thead>
<tr>
<th></th>
<th>Order no. International</th>
<th>Order no. North America</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thermomixer comfort</strong>, (Thermomixer R), without thermoblock</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100-240 V, 50-60 Hz</td>
<td>5355 000.01</td>
<td>022670107</td>
</tr>
<tr>
<td>120 V, 50-60 Hz</td>
<td>—</td>
<td>022670158</td>
</tr>
<tr>
<td>220 V, 50-60 Hz</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td><strong>ThermoStat plus</strong>, without thermoblock</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100-240 V, 50-60</td>
<td>5352 000.010</td>
<td>022670204</td>
</tr>
<tr>
<td>120 V, 50-60 Hz</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td><strong>Exchangeable thermoblock for 4 slides</strong></td>
<td>5368 000.010</td>
<td>022670590</td>
</tr>
</tbody>
</table>

ZytoFast® is a registered trademark of ZytoVision GmbH, Bremerhaven, Germany