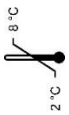
GynTect®

Basic UDI-DI: 426076785GYNTECTYM

Instructions for use

REF	Σ	UDI-DI
GT003-06	6 samples	4260767851017
GT003-10	10 samples	4260767851116



Please read these instructions carefully prior to performing the GynTect® test. Follow the instructions precisely in order to ensure the reliability of the test results.

GynTect® is an *In-Vitro* diagnostic kit for the qualitative detection of six epigenetic markers in bisulfite-converted DNA from cervical samples from women with a positive HPV test result or an unclear Pap test finding. A positive GynTect® test result correlates with the presence of a cervical intraepithelial neoplasia or a cervical carcinoma.

Only for *In-Vitro* Diagnostic (IVD) use by qualified personnel.

Revision 6
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SHIPMENT AND STORAGE

The GynTect® Kit is shipped at room temperature. The temperature limit is monitored by temperature measuring point. Please control the measuring point directly upon arrival of the kit regarding colour changes (see Figure 1), and check if secondary packaging, sealing and primary packaging are undamaged. Upon receipt, the kit must be cooled immediately at 2 °C to 8 °C and stored away from light. Proper shipping and appropriate storage allow the use of the GynTect® Kit and all its components until the indicated use-by date. Under these storage conditions, the use-by date relates to open GynTect® reagents.

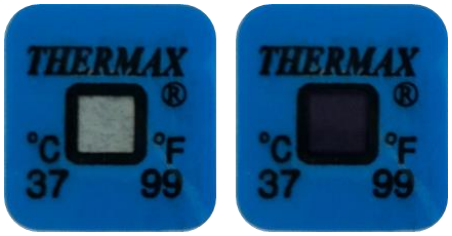

Figure 1 Surveillance of the transport temperature
The temperature measuring point on the GynTect® Kit allows the surveillance of the temperature during transport. The bright silver square in the centre of the point indicates that the temperature during transport did not exceed the allowed transport temperature. In contrast, a black square indicates that the allowed temperature has been exceeded during transport. The performance characteristics of the GynTect® Kit can no longer be guaranteed. In this case please contact oncgnostics GmbH.

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1. INTENDED USE

GynTect® is an *In-Vitro* diagnostic kit for the qualitative detection of six epigenetic markers in bisulfite-converted DNA from cervical samples from women with a positive HPV test result or an unclear Pap test finding. A positive GynTect® test result correlates with the presence of a cervical intraepithelial neoplasia or a cervical carcinoma.

GynTect® is only intended for use by qualified personnel who are familiar with molecular biologic techniques.

2. CLINICAL SIGNIFICANCE

Cervical cancer is the 4th most common cancer in women worldwide, with >600,000 newly diagnosed cases annually [1]. In almost all cervical cancer cases, persistent infection with a high-risk human papillomavirus is observed [2], demonstrating that infection with HPV is a prerequisite for cervical carcinogenesis. HPV-negatively tested women only have an extremely low risk to develop cervical cancer. However, most HPV-positively tested women also do not develop a precancerous lesion or cancer. Only ca. 15% of the women infected with HPV may develop such a cervical precancerous lesion or carcinoma that require treatment [3].

Patients with a positive HPV test result or unclear Pap test findings (Pap II, Pap III and Pap IIID1 and D2) may get the recommendation to get a triage test such as GynTect®, to determine the probability of the presence of a cervical cancerous disease with high accuracy.

The GynTect® result should not be used for final therapy decision, it has to be assessed in combination with other clinical findings.

3. PRINCIPLE OF THE TEST

GynTect® is based on the detection of epigenetic biomarkers, more precisely methylations of specific DNA regions, which correlates with the existence of precancerous lesions or cervical carcinomas [4, 5, 6]. In addition, a bisulphite-specific as well as a methylation-specific reference marker is analysed. The marker regions applied in GynTect® are listed in Table 1.

Table 1 Overview of the GynTect® marker regions

Designation in protocol	Marker region (gene name)
Methylation marker	ASTN1
	DLX1
	ITGA4
	RXFP3
	SOX17
	ZNF671
Control marker	ACHE
	IDS

The marker detection is performed by highly sensitive realtime PCR. The output of the Real-Time instruments is the Cp value (Cross point, cobas z 480 Analyzer) or Cq value (Cycle quantification, CFX96 Real-Time PCR Detection System), both of which correspond to the Cycle threshold Ct value and are also referred to as such below. This value corresponds to the cycle in a Real-Time PCR in which fluorescence rises above a defined threshold value for the first time.

Analysis of a patient sample with GynTect® consists of two steps.
First, the DNA in the cervical smear is treated by a bisulfite reaction, which leads to a "fixation" of the DNA methylation. For the purification following the bisulfite conversion we recommend a shorter and simplified protocol. After elution, the DNA is diluted with water. In the second step the bisulfite-converted DNA is analysed by eight singleplex methylation-specific realtime PCR reactions. The originally methylated DNA regions are selectively amplified using primers which are placed in the tubes of an eight-tube PCR strip.
Realtime detection of the methylation marker and control regions is performed utilizing an intercalating fluorescent dye. In addition, a positive and negative control are carried along to control the PCR. Subsequently, the assay-specific analysis is performed.

Sampling and bisulfite kit are not part of the GynTect® Kit. Special products for sampling and bisulfite treatment are available separately.

The test principle is shown in Figure 2.

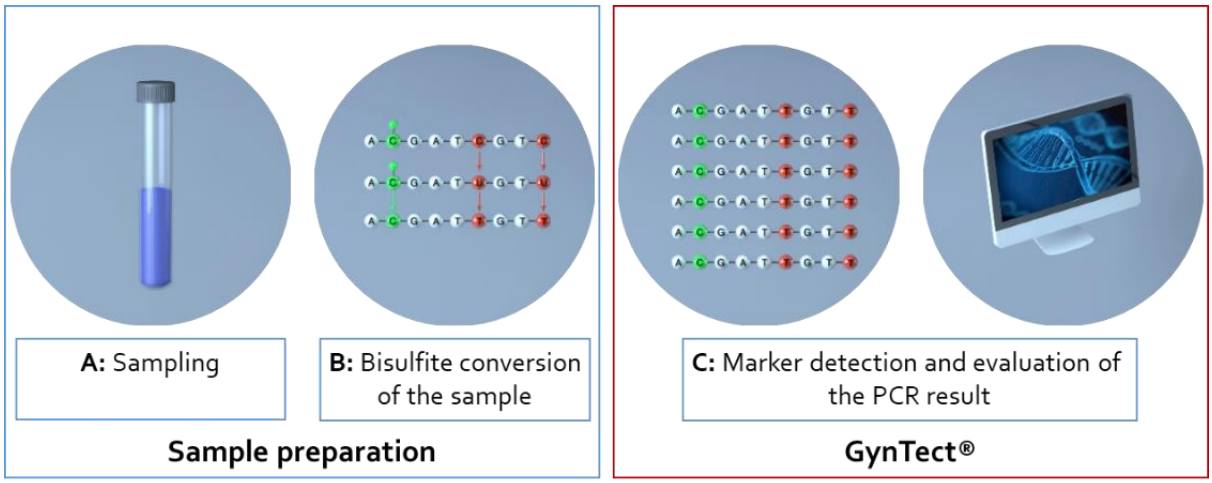


Figure 2 Test principle
***A**: The gynaecologist takes a smear from the cervix uteri of the patient, which is transferred into sample medium.*
***B**: The diagnostic laboratory performs the bisulfite conversion of patient sample.*
***C**: For each sample eight singleplex PCR reactions are performed. Evaluation is achieved by detection of the intercalating fluorescent dye contained in the mastermix and defined melt curve peaks.*

4. DESIGN OF THE GYNTECT® ASSAY

4.1. GynTect® Strips layout

Each GynTect® Strip contains eight primer pairs for the amplification of six methylation-specific markers and two control markers. The layout of the GynTect® Strips is shown in Figure 3.

A complete GynTect® Strip is required for the analysis of a single patient sample.

Figure 3 Layout of the GynTect® Strips
Each position 1 - 6 of the PCR strips contains the primer for one of six methylation-specific markers. Position 7 of the strips contains the primers for bisulfite quality control (ACHE), position 8 the primers for methylation quality control (IDS-M).

4.2. Controls

The GynTect® Kit design comprises several controls. These controls allow the surveillance of critical steps of the test, including sample quality and bisulfite treatment (ACHE), methylation status of the sample (IDS-M) and quality of the PCR reactions.

4.2.1. Quality Control Bisulfite Treatment (ACHE)

This control marker verifies the successful conversion of all non-methylated Cytosine residues to Uracil and thus the quality of the bisulfite treatment. For this purpose, a DNA region located at the human acetylcholine esterase (ACHE) is amplified. Inadequate ACHE amplification indicates that the assay is invalid and needs to get repeated.

4.2.2. Quality Control Methylation (IDS-M)

This control allows the amplification of the imprinted gene IDS, which is methylated on the second female X chromosome (IDS-M). If a Cp value between 20 and 32 is generated in the PCR reaction, the cervical sample is valid. If no amplification is achieved, the assay has to be judged as invalid and needs to be repeated.

4.2.3. Positive Control

A control template is provided for surveillance of the quality of the PCR reaction. During amplification of the control template GynTect® Positive Control (PC) each of the eight markers should provide a Cp value below 38. Otherwise, the PCR reactions are invalid and need to be repeated.

4.2.4. Negative Control

As negative control, reactions with the template GynTect® Water (NTC – No Template Control) are performed. These reactions should be negative for all markers. If specific reaction products occur in any of the reactions, contaminations may have occurred and GynTect® needs to be repeated.

5. REFERENCE MATERIAL

No international reference material is available.

6. KIT CONTENT

Table 2 Content of the GynTect® Kit

Component	Symbol	Content	Volume / Amount	
			GT003-06	GT003-10
GynTect® Mastermix	PCR-MM	2 x PCR mastermix [1]	1 x 1.1 ml	1 x 1.1 ml
GynTect® Strips	STRIPS	PCR strips [2] for patient samples (green caps)	6 strips	10 strips
		PCR strips [2] for positive controls (red caps)	3 strips	1 strip
		PCR strips [2] for negative controls (yellow caps)	3 strips	1 strip
GynTect® Caps	CAPS	PCR cap strips	12 cap strips	12 cap strips
GynTect® Positive Control	CONTROL +	Positive control	1 x 260 µl	1 x 90 µl
GynTect® Water	H_2O	Water	1 x 2 ml	1 x 2 ml
Instructions for use	-	Instructions for use	1	1

[1] Contains all components required for the Polymerase chain reaction (PCR), excl. primer and template.

[2] Contains the primers required for the PCR.

7. CONSUMABLES AND EQUIPMENT (NOT INCLUDED IN THE KIT)

GynTect® may only be performed using the listed material and equipment and only be conducted by qualified personnel. All laboratory equipment has to be installed, maintained, handled and calibrated according to the manufacturer's instructions.

Room temperature is defined as temperature between 15 °C and 30 °C.

Table 3 Required equipment

Equipment	Catalogue no.	Order
EpiTect® Fast Bisulfite Kit (10) *	Z102	order@oncgnostics.com
ThinPrep® PreservCyt® Solution (20 ml)	-	via Hologic Inc.
Cervex-Brush® or Cervex-Brush® Combi	-	via Rovers Medical Devices

* The EpiTect® **Fast** Bisulfite Kit (catalog no. QIAGEN: 59802) must not be confused with the EpiTect® Bisulfite Kit!

The following laboratory equipment and consumables are required to perform the GynTect® assay.

- Centrifuge for 0.5 ml/1.5 ml reaction tubes, ≥ 10,000 xg
- Centrifuge for PCR strips
- Thermal cycler for 0.5 ml reaction tubes
- Vortex mixer / shaker
- Pipettes with different volume ranges and associated filter tips (sterile, DNase-free)
- Reaction tubes for 0.5 ml/1.5 ml (DNase-free)
- Reaction tube stand for 0.5 ml/1.5 ml reaction tubes
- Ethanol 96 – 100%, undenatured
- Real-time PCR device, Detection channel for FAM/SYBR Green

  GynTect® has been validated on

  - cobas z 480 Analyzer (Roche Diagnostics GmbH) with 96-well-block, adapter for PCR strips and LightCycler® 480 SW UDF 2.0.0 (Service Pack 3), evaluation with version 1.5.1.62
  - CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with CFX Maestro software version 2.3
  - CFX Opus 96 (Dx) Real-Time PCR Detection System™ (Bio-Rad Laboratories, Inc.) with CFX Maestro Software Version 2.3

8. STORAGE AND SHELF-LIFE

If transported and stored properly, the GynTect® Kit and its components can be used until the stated date. All reagents contained in the kit are stable until the indicated expiry date after opening, if stored under the indicated conditions and protected against contamination (see Table 4)

Table 4 Storage temperature of the GynTect® Kit and equipment not included in the kit

Equipment	Storage temperature
GynTect® Kit	2 °C to 8 °C
EpiTect® Fast Bisulfite Kit (10) except from Spin Columns, DNA Protect Buffer, Buffer BD	15 °C to 25 °C 2 °C to 8 °C
ThinPrep® PreservCyt® Solution (20 ml)	15 °C to 30 °C
Cervex-Brush® bzw. Cervex-Brush® Combi	15 °C to 30 °C

9. SAFETY INSTRUCTIONS

9.1. General information

When establishing state-of-the-art molecular biology methods, the instructions below must be followed closely to ensure maximum safety for laboratory personnel and to achieve high-quality results:

- As it involves molecular biology processes, such as bisulphite treatment, amplification, and the detection of DNA, this kit is intended only for in vitro diagnostics and should be used only by personnel trained in laboratory practices for in vitro diagnostic.
- Before using the product, read the instructions for use thoroughly. Only the current version is to be taken into account.
- Wear a suitable lab coat, disposable gloves and, if necessary, safety goggles for each step.
- Avoid direct contact with the biological samples, as well as splashing or spraying of the samples.
- The heated lid and incubation block of the thermal cycler can reach temperatures of up to 110 °C. There is a risk of skin burns. Please observe the operating instructions of the device.
- Wash your hands thoroughly after handling samples and reagents.
- Do not use GynTect® if the reagent packaging is damaged. Contact your distributor.
- Do not use the GynTect® Kit after the expiry date and do not use expired reagents.
- Do not mix reagents from different batches and do not mix kit reagents with reagents from other manufacturers.
- Use only materials supplied with the kit or recommended by the manufacturer.
- All required laboratory equipment must be installed, calibrated, handled and maintained according to the manufacturer's instructions.

- Pipetting small volumes of liquid within the microlitre range requires practice. Make sure you pipette the required volumes with the micropipettes as precisely as possible.
- The applicable regulatory requirements for the operator must be complied with.
- Adherence to Good Laboratory Practice (GLP) as outlined, for example, by the U.S. Food and Drug Administration (FDA) or the Organisation for Economic Co-operation and Development (OECD) is assumed. Specifically, recommendations for performing molecular amplification testing should be considered.
- The proper functioning of the PCR devices is only guaranteed at room temperature.

9.2. Spatial division

Due to the high analytical sensitivity of PCR, strict attention should be paid to maintaining the purity of the kit components and samples.

PCR multiplies sections of the DNA in the sample millions to billions of times. Even the smallest amounts of these PCR products (e.g. also spread as aerosol) can lead to a false result if they are carried over into the sample material, into the reagents for bisulphite treatment or into the PCR reagents of this kit.

A clean and well-structured workflow is therefore crucial to prevent incorrect results. To this end, it is necessary to separate the laboratory areas for pre-PCR and post-PCR from each other. Separate equipment, consumables, lab coats and gloves should be available in each area. Never transfer lab coats, gloves or equipment from one area to the other. The Figure 4 shows an example of a laboratory divided into two separate rooms. One area is designated only for bisulphite treatment and preparation of PCR, while in the other area the PCR is carried out.

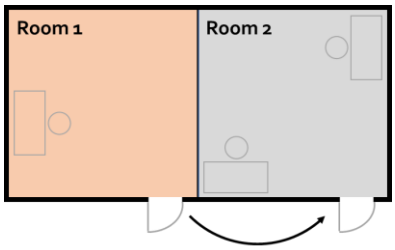


Figure 4 Spatial division
In room 1 bisulfite treatment and PCR preparation (optimal: use a PCR hood) are performed. In room 2 the PCR run is performed, the markers are detected and the results analysed.

9.3. Avoiding contamination

- Lab coats and disposable gloves must be worn during all steps.
- Disposable gloves should be changed frequently and always after (suspected) contamination with reagents or sample material.
- All surfaces, equipment, and supplies must be decontaminated with a suitable cleaning solution (DNA-destroying agents).
- Do not touch the inside of the reaction tubes or their caps.
- When pipetting, filter tips (free of DNase, RNase and human DNA) must always be used to exclude cross-contamination via aerosols generated during pipetting. Tips should always be changed between pipetting steps.
- It is important to perform negative controls to detect possible contamination.

9.4. Handling instructions

- Store the unused components in the original packaging until used.
- All centrifugation steps should be performed at room temperature.
- The workflow can be interrupted after the bisulphite treatment. At this point, the samples can be stored for one week at 2 °C to 8 °C or up to two months at - 15 °C to - 30 °C.
- Never touch the inside of the coloured PCR strip caps while removing them from the strips and dispose them professionally.
- The GynTect® Strips and GynTect® Caps should not be touched without disposable gloves throughout the entire procedure, otherwise non-specific fluorescence signals may occur.
- Labelling of the GynTect® Strips and GynTect® Caps at the wrong positions may yield unspecific fluorescence signals during the PCR runs.
- The GynTect® Strips and GynTect® Caps are intended for single use and cannot be reused.
- Keep the unused GynTect® Strips and GynTect® Caps in their original packaging.

10. DISPOSAL

The unused GynTect® Kit and its components can be disposed of without further special precautions. Patient samples and used reaction tubes must be handled as infectious waste. All reagents must be disposed of in accordance with legal regulations.

11. GynTect® procedure

The following chapter contains a detailed description of the various processing steps, from taking of samples to analysing of the data. The GynTect® Kit GT003-10 contains one positive and one negative control each for one single GynTect® run. All ten samples have to be used in one workflow.

11.1. Timeline for the workflow

Altogether GynTect® may be processed in less than 4 hours with the active handling time requiring circa 2 hours. Prior to performing the first GynTect® assay you should reserve approx. 15 minutes for setting up a PCR template program for the assay.

Figure 5 shows the details of the workflow.

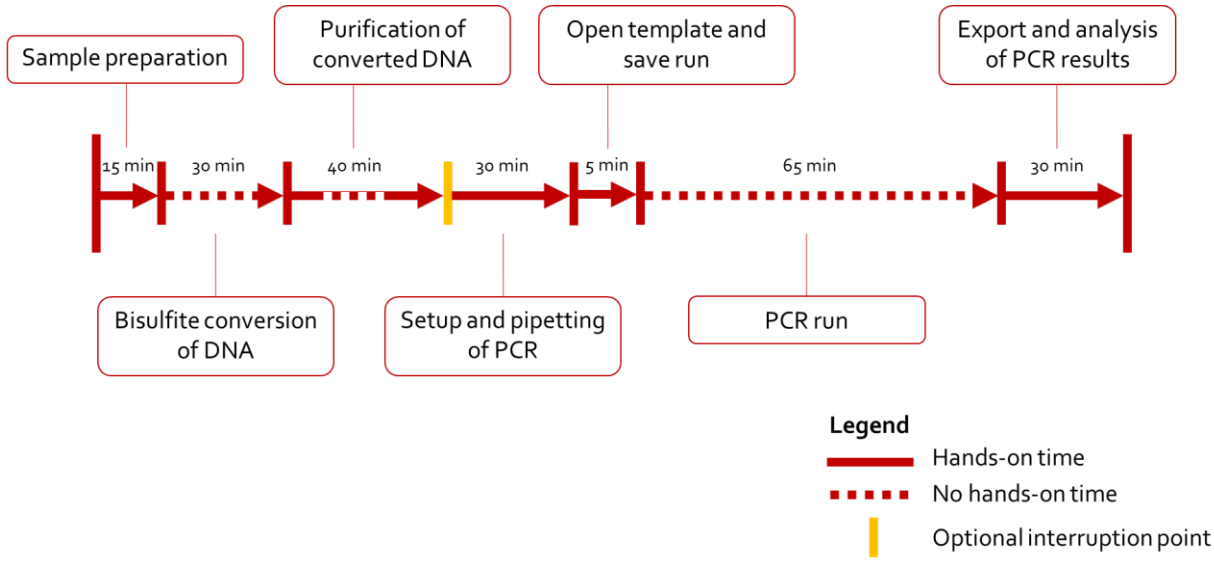


Figure 5 Timeline of the GynTect® workflow

11.2. Sampling

The sampling kit and the bisulfite kit are not part of the GynTect® Kit. The ThinPrep® PreservCyt® Solution vial (Hologic) and the Cervex-Brush® (Rovers Medical Devices) can be purchased from their respective manufacturers. The collection of a cervical sample by the physician is to be performed in accordance with the manufacturer's instructions and in compliance with the generally accepted guidelines for the collection of a cervical smear sample [7].

Important: The brush has to be discarded after sampling and shall not stay in the sample medium otherwise GynTect® performance will be affected.

ThinPrep® PreservCyt® Solution must be used as the smear medium. The use of other sample media was not part of the validation of the GynTect® assay.

Ensuring the good quality of the employed DNA sample is an important prerequisite for the validity of the assay. Improper sampling, bisulphite treatment, and DNA storage may lead to invalid or even false negative results.

Cervical samples can be transported to the laboratory for testing without refrigeration. Samples can be stored for up to 1.5 years at temperature +2 °C to +30 °C.

11.3. Sample preparation

The following steps must be carried out in the sample preparation area (Room 1).

Important: The brush has to be discarded before processing the sample, if the brush is still placed in the sampling vial.

1. Vortex a patient sample for 5 sec at maximum speed and transfer immediately 1 ml of the sample in a 1.5 ml tube.

   Attention: The cells will settle on the bottom of the vial very quickly. The patient sample has to be used within 10 sec after mixing.

2. Centrifuge the sample for 5 min at 10,000 xg.

3. Carefully remove 900 µl supernatant above the pellet. Do not remove or destroy the pellet.

   Attention: The pellet is fixed more or less, depending upon the sample.

4. Vortex the pellet for 3 sec for resuspending. 40 µl of resuspended sample are used for bisulfite treatment.

11.4. Bisulfite treatment of the samples

The bisulfite kit is not part of the GynTect® Kit. GynTect® was validated with the EpiTect® Fast Bisulfite Kit (10) (Qiagen).

Attention: The original protocol of the EpiTect® Fast Bisulfite Kit (10) (Qiagen) was modified. Utilization of this modified protocol is a prerequisite to fulfil the performance parameters indicated.

11.4.1. Bisulfite conversion of the DNA

1. Prepare the buffers of the bisulfite kit to be used according to Table 5.

   Important: Buffer BL is used without *Carrier RNA*. When **Buffer BL** stored or transported at low temperatures a formation of precipitates is possible. In this case, solve precipitation by gentle warming (37 °C) and shaking of **Buffer BL**.

   Table 5 Buffer for EpiTect® Fast Bisulfite Kit (Qiagen)

Buffer	Addition of Ethanol	Storage temperature
Buffer BL *	-	Room temperature
Buffer BW	30 ml Ethanol (96 - 100 %)	Room temperature
Buffer BD	27 ml Ethanol (96 - 100 %)	2 °C to 8 °C

   * Do not add *Carrier RNA*, control of quality (see note)

2. For each sample set up a bisulfite treatment reaction mix using 0.5 ml tubes according to Table 6.

   Important: When **Bisulfite Solution** stored or transported at low temperatures a formation of precipitates is possible. In this case, solve precipitation by gentle warming (37 °C) and shaking of **Bisulfite Solution**.

Table 6 Set-up for bisulfite treatment

Compound	Per reaction
Bisulfite Solution *	85 µl
DNA Protect Buffer	15 µl
Prepared & resuspended sample	40 µl
Total volume per reaction	140 µl

* control of quality (see note)

Attention: Bisulfite Solution cannot be stored. One **Bisulfite Solution** can only be used for one process, leftovers have to be discarded.

3. Vortex the reaction mix for the bisulfite conversion for 3 sec at maximum speed, centrifuge them briefly and leave them at room temperature until further use.

Attention: The **DNA Protect Buffer** changes its colour from green to blue if the pH of the mix is in the correct range. The sample has to be processed within 60 min.

4. Use a thermal cycler with heated lid (100 °C) and program the cycler according to Table 7.

Important: If thermal cycler does not accept a volume as high as 140 µl use next possible programmable volume. The lid does not need to be pre-heated prior to the start of the conversion.

Table 7 Bisulfite conversion using a thermal cycler

Step	Duration	Temperature
Denaturing	5 min	95 °C
Incubation	10 min	60 °C
Denaturing	5 min	95 °C
Incubation	10 min	60 °C
End of reaction	Hold*	20 °C

* Converted DNA may remain in the thermal cycler or stored at room temperature overnight (max. 16 h).

5. Place the 0.5 ml tubes in the heating block of the thermal cycler and start the incubation.

Attention: After bisulfite conversion the samples may be stored overnight (max. 16 hours) at room temperature. Avoid refrigerated storage (at temperatures 2 °C to 8 °C), since the samples may precipitate, preventing further processing.

11.4.2. Purification of the converted DNA

6. Upon completion of the bisulfite conversion, vortex the PCR tube for 3 sec at maximum speed and centrifuge briefly to remove drops from inside the lid.

7. Add **310 µl Buffer BL** to each MinElute® Spin Column.

   Important: Label the MinElute® Spin Column clearly.

8. Add the sample (140 µl) to the spin column and mix briefly by 5 × pipetting up and down.

   Important: Do not touch the membrane in the column with the pipette tip and avoid the formation of bubbles. Make sure that the solution is homogenous and free from striations after 5 x pipetting up and down.

9. Add **250 µl Ethanol** (96 - 100 %) to each spin column, close the lid and mix briefly by 5 x converting the column pivot overhead (180°).

10. Centrifuge each spin column for 30 sec at 18,000 ×g and make sure that the liquid has passed the column into the collection tube.

11. Remove the spin column from the centrifuge, discard the flow through and place the spin column back into the collection tube.

12. For washing, add **200 µl Buffer BW** to the spin column and centrifuge for 30 sec at 18,000 ×g.

13. Add **200 µl Buffer BD** to each spin column, close the lid and incubate for 15 min at room temperature (15 - 30 °C).

14. Upon completion of desulfonation centrifuge the spin column for 30 sec at 18,000 ×g.

15. Add **200 µl Buffer BW** and centrifuge for 30 sec at 18,000 ×g.

16. Remove the spin column from the centrifuge, discard the flow through and place the spin column back into the collection tube.

17. Wash the spin column with **400 µl Buffer BW** and centrifuge for 30 sec at 18,000 ×g.

18. Add **200 µl Ethanol** (96 - 100 %) to each spin column and centrifuge for 30 sec at 18,000 ×g.

19. Place the spin column into a new 2 ml collection tube and centrifuge for 60 sec at 18,000 ×g to remove any residual liquid.

    Attention: Do not skip this step, since residual Ethanol may impair performance of the GynTect® assay.

20. Place the spin column into a clean 1.5 ml tube (not provided), add 20 µl water directly onto the centre of the spin-column membrane and close the lid gently.

    Important: Do not damage the spin-column membrane and do not pipet the water on the side of the spin column.

21. Incubate the spin column at room temperature for 60 sec and then centrifuge the spin column for 60 sec at 8,000 ×g (Elution).

22. Check visually if the eluate has the correct volume.

Optional: At this stage the samples may be stored for up to one week at 2 °C to 8 °C or up to 2 months at - 15 °C to - 30 °C.

11.5. PCR

Before starting the PCR, ensure that the PCR temperature protocol is programmed into the appropriate real-time PCR device to minimise the time between preparation and PCR start. To establish the PCR program on the cobas z 480 Analyzer, proceed as described on page 19. The explanation for PCR on the CFX96 Real-Time PCR Detection System can be found on page 22 onwards.

11.5.1. Preparation and pipetting of the PCR

Important: PCR preparation and pipetting should not take longer than 60 minutes. This step is performed in room 1 (pre-PCR area).

Please note the plate layout described on page 19 or 22 respectively. The positive control (PC) must be positioned in row 11 and the negative control (NTC) in row 12.

1. Add **70 µl GynTect® Water** to each sample.
2. Vortex the samples for 3 sec at maximum speed and centrifuge them briefly.
3. Take the **GynTect® Mastermix** from the kit and use for each sample a **GynTect® Strip** (green lid) and for each control a **GynTect® Strip** (positive control: red lid, negative controls: yellow lid).
4. Place the **GynTect® Strips** on a PCR rack. Please be aware of the orientation of the **GynTect® Strips** (see Figure 6).

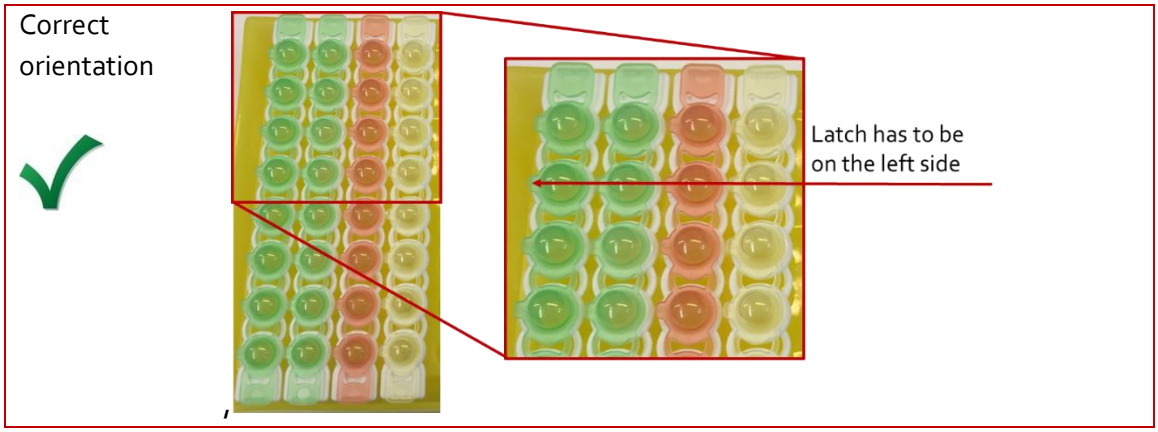


Figure 6 Orientation of the GynTect® Strips

5. Vortex the **GynTect® Mastermix** 3 sec at maximum speed and centrifuge briefly.
6. Remove the coloured and round cap strips from the **GynTect® Strip** and discard them.
7. Add **10 µl** of the **GynTect® Mastermix** to each **GynTect® Strip** tube.

   Note: A blue colouration is observed. This serves as optical inspection and has no influence to GynTect® result.

   Important: Change tips for each strip tube, since the **GynTect® Strips** already contain the primers for PCR.
8. Add **10 µl sample** to each of the eight wells of the **GynTect® Strips**.

Important: Change tips for each strip tube.

Note: Keep the rest of the sample eluates for a repetition of the GynTect® PCR if necessary.

9. Close the **GynTect® Strips** directly after pipetting with flat, clear **GynTect® Caps**.

   Attention: Avoid touching the inside of the **GynTect® Caps** and **GynTect® Strips**. Make sure that the **GynTect® Strips** are tightly closed. The inspection is best performed visually from the side.

10. Vortex the GynTect® Positive Control for 3 sec at maximum speed and centrifuge it off.

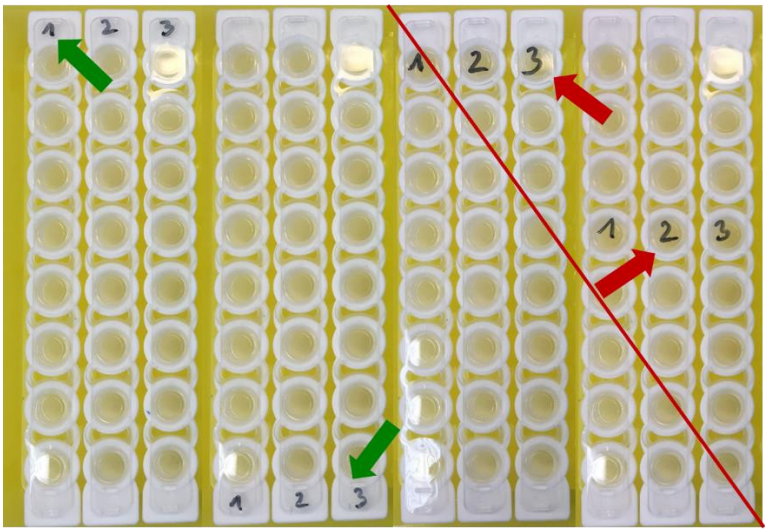
11. Add to each of the eight wells of the **GynTect® Strip** for positive control **10 µl** of **GynTect® Positive Control** and to each of the eight wells of the **GynTect® Strip** for negative control **10 µl** of **GynTect® Water**.

12. Close the **GynTect® Strips** directly after pipetting with flat, clear **GynTect® Caps**.

    Attention: Avoid touching the inside of the **GynTect® Caps** and **GynTect® Strips**. Make sure that the **GynTect® Strips** are tightly closed. The inspection is best performed visually from the side.

13. Label all **GynTect® Strips** after finishing pipetting and closing on the new **GynTect® Caps** latches (see Figure 7).

Correct labelling: Wrong labelling:



Attention:
Please do not write on the **GynTect® Caps** for labelling purposes, because the light excitation and fluorescence emission measurement will be impaired.

Labelling of the **GynTect® Caps** is possible at the latches on both ends.

Figure 7 Labelling of the GynTect® Strips

14. Vortex all closed **GynTect® Strips** for 3 sec at maximum speed and centrifuge them briefly.

11.5.2. Performing the PCR on the cobas z 480 Analyzer

The following section outlines how to perform the GynTect® on the Real-Time PCR systems cobas z 480 Analyzer (marked blue) and CFX96 Real-Time PCR Detection Systems (marked green). However, always follow the manufacturer's instructions for operating PCR devices.

The PCR should be performed in room 2.

11.5.2.1. Creating a PCR template

If you created and saved the PCR template earlier, you can continue with 11.5.2.2 *Starting the PCR run*.

1. Turn on the cobas z 480 Analyzer and its computer. Within 15 sec, select "User defined Workflow" on the computer screen in the BIOS in order to switch to a freely programmable device mode.

2. Select *New Experiment* to create a new Template.
   In the tab *Run Protocol*, set the *Detection Format* at SYBR Green 1 / HRM Dye and set the *Reaction Volume* to 20 µl. Program the temperature protocol according to Table 8 .

   Table 8 PCR temperature protocol on the cobas z 480 Analyzer

Program Name	Number of cycles	Analysis Mode	Target	Acquisition Mode	Hold (hh:mm:ss)	Ramp rate (°C/s)
Initialization	1 x	None	94 °C	None	00:01:00	4.4
Amplification	42 x	Quantification	94 °C	None	00:00:15	4.4
			66 °C	Single	00:00:35	2.2
Melt curve	1 x	Melting Curves	95 °C	None	00:00:15	4.4
			60 °C	None	00:00:20	2.2
			95 °C	Continuous	-	0.11
Cooling	1 x	None	37 °C	None	00:01:00	2.2

3. Save the *Run-Template* under the name *GynTect*.

11.5.2.2. Starting the PCR run

If you saved the PCR template earlier, you can now access it. Check that the correct temperature protocol is set.

1. Under *Subset Editor* generate the recommended plate layout (see Figure 8 and Figure 9).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	-	-	-	-	PC	NTC
B												
C												
D												
E												
F												
G												
H												

Figure 8 Example plate layout for six samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	PC	NTC
B												
C												
D												
E												
F												
G												
H												

Figure 9 Example plate layout for ten samples using GynTect® GT003-10

Important: The plate layout is **not** variable. The positive control (PC) must be positioned in row 11 and the negative control (NTC) in row 12.

2. Define under *Sample Editor* the sample labelling.
3. Place the strips vertically into the PCR device in the defined order.

   Important: Use the adapter for PCR-Strips (orderable by Roche Diagnostics GmbH).
4. Save the PCR run by clicking *disk-button* at desired location using a clear name and start the PCR run by clicking the button *Start Run* in the tab *Run Protocol.*

11.5.2.3. Exporting the data

If you are analysing the PCR run directly on the cobas z 480 Analyzer computer, please continue with 11.5.2.4 *Evaluation setting in the device software*.

After completion of the PCR (Run complete), export the PCR run via  "Export" and save the file to the desired location.

11.5.2.4. Evaluation setting in the device software

If you have exported the PCR run, start the LightCycler® 480 software on another computer and open/import the PCR run. Otherwise, perform the analysis on the computer of the cobas z 480 Analyzer.

1. Choose the algorithm *Abs Quant/Fit Points* in *Analysis* Editor and the possible defined Subset.
2. Set *Background* at 5 to 20 in tab *Cycle Range*, in addition to that choose *Min Offset* 4 and *Max Offset* 19. Assure setting of First Cycle and Last Cycle, the Cycle Range must include 1 to 42.
3. Assure in tab *Noise Band* that *STD Multiplier* is set to 12 and *Noise Band* is calculated automatically.
4. Set in tab *Analysis Threshold* to 0.5 and check that number of *Fit Points* is adjusted to 2.
5. Subsequently confirm all settings by clicking button *Calculate* and perform analysis. Export data table via *Export Table* by right-clicking as .txt-file and save at desired location using a clear name.
6. Go back to *Analysis* Editor and choose the algorithm *Tm Calling* in *Analysis* Editor and the possible defined Subset.
7. Choose checkbox *Height* next to *Tm* as well and confirm all settings by clicking button *Calculate* and perform analysis. Export data table via *Export Table* by right-clicking as .txt-file and save at desired location using a clear name.

Chapter 11.6 describes the analysis and interpretation of the PCR data.

11.5.3. Performing the PCR on the CFX96 Real-Time PCR Detection System

Always follow the manufacturer's instructions for operating PCR devices.

The PCR should be performed in room 2.

11.5.3.1. Creating a PCR template

1. Switch on the PCR device.
2. Program the PCR temperature protocol as described in the Table 9 by selecting and editing the temperature steps and times.

*Table 9 PCR temperature protocol** on the CFX96 Real-Time PCR Detection System*

Programme Name	Step	Number of cycles	Temperature	Time (m:ss)
Initialization	1	1 x	94 °C	1:00
Amplification	2	42 x	94 °C	0:15
	3*		66 °C	0:35
	4		GO TO Step 2	41 x
Melt Curve	5	1 x	95 °C	0:15
	6*	1 x	65 °C 95 °C	0:05 0.5 °C/cycle
Cooling	5	1 x	37 °C	1:00

* *The fluorescence signal is detected via "Plate Read" during Step 3 and Step 6, which is symbolised by the camera symbol.*

** *On the CFX96 Real-Time PCR Detection System, the default ramp rate is 5 °C/sec. This setting was used to validate this IVD test.*

3. Set the reaction volume to 20 µl and the temperature of the *Lid* heater at 105 °C.
4. Save the PCR template using the name *GynTect*.

11.5.3.2. Starting the PCR run

If you saved the PCR template earlier, you can now access it. Check that the correct temperature protocol is set.

1. Place the GynTect® Strips into the PCR device by inserting them vertically into the small wells of the heating block. Select a suitable plate layout (see Figure 10 and Figure 11).

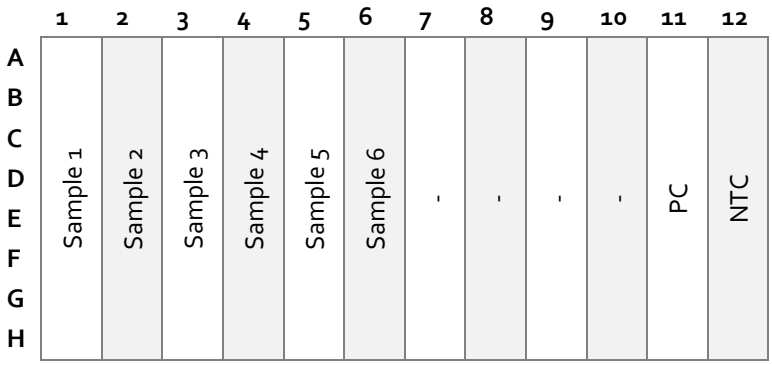


Figure 10 Example of plate layout for 6 patient samples (1 - 6)

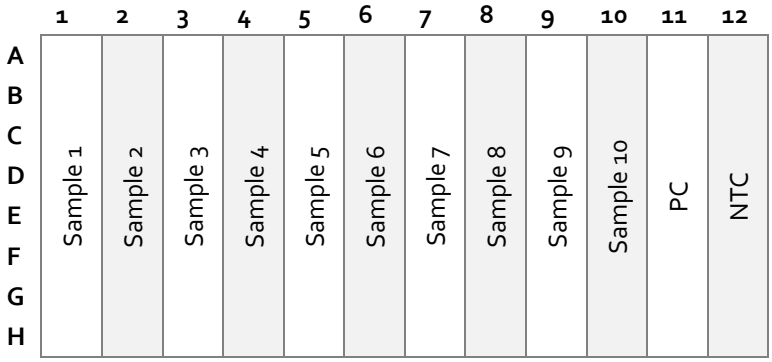


Figure 11 Example of plate layout for 10 patient samples using GynTect® GT003-10

Important: The plate layout is **not** variable. The positive control (PC) must be positioned in row 11 and the negative control (NTC) in row 12.

2. Name the run using a suitable file name. Note that *SYBR/FAM only* are detected before you start the run.

11.5.3.3. Exporting the data

After completing the PCR, export the PCR run (.pcrd file).

11.5.3.4. Evaluation settings in the device software

1. On a computer, open the Bio-Rad CFX Software and first set *Plate Type: BR White* under *User* → *User Preferences* → *Plate* to indicate that white plastic is used.
2. Import the .pcrd file.
3. Define the plate layout under *Plate Setup* → *View/Edit Plate*. Enter the name under *Sample Names*. Unoccupied positions are marked and can be excluded from the analysis by checkmarking *Exclude Wells in Analysis*. Confirm the plate layout under *OK*.
4. Set the analysis settings under *Settings* (see Table 10).

Table 10 Analysis settings on the CFX96 Real-Time PCR Detection System

Parameter	Setting
Cq Determination Mode	Single Threshold
Baseline Setting	Baseline Subtracted Curve Fit
	Apply Fluorescence Drift Correction
Analysis Mode	Target
Cycles to Analyze	1-42
Baseline Threshold	Baseline Cycles → User Defined: Begin: 5; End: 20
	Single Threshold → User Defined: 200

5. Select *Load a Preset View* → *Amplification + Melt* under *Custom Data View* tab. Set manually the threshold in *Melt Peak Chart* to "zero" ("drag-and drop").

6. To export the PCR data select the following settings under *Export* → *Custom Export*

   - Format: Excel 2007 (*.xlsx)
   - Data to Export → check the following boxes
     (Include Run Information Header, Well, Fluorophore, Target Name, Content, Sample Name, Cq, Melt Temperature, Melt Peak Height)

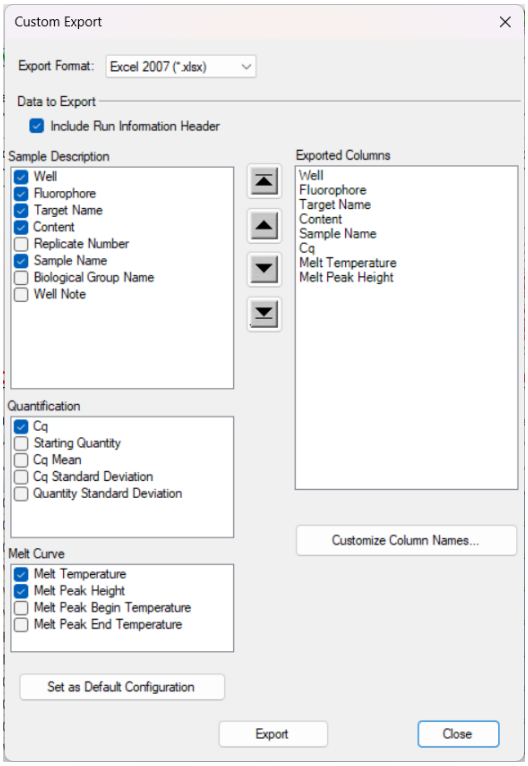


Figure 12 Custom Export

7. Press the *Export* button and save the .xlsx file under a clear name.

11.6. Evaluation and interpretation of PCR data

The following describes the analysis of the exported data.

1. Open a suitable spreadsheet programme and copy the exported PCR data into it.

2. Format the data in a way, e.g. that the results for each sample are written in a column and that all samples are written besides each other.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		PC		NTC	
Marker	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm
ASTN1	37.42	82.99	35.62	84.44	38.76	82.74			31.81	83.58	36.51	83.41	28.42	84.55		
DLX1	33.29	81.43	33.33	81.77	30.54	80.46	34.85	81.92	30.55	81.55	38.30	81.61	28.80	82.75		
ITGA4			39.66	80.75					35.82	81.30			28.19	82.84		
RXFP3					36.77	81.47			33.52	82.68			27.80	82.43		
SOX17			36.87	85.01	39.51	82.86			39.16	82.36			31.19	85.07		
ZNF671	37.50	84.78							29.47	82.98		84.24	27.52	86.08		
ACHE	25.54	79.52	25.58	79.88	23.50	79.70	26.63	79.90	24.77	79.76	29.12	79.80	25.58	80.47		74.47
IDS-M	26.36	80.56	26.49	81.18	24.13	80.78	26.77	80.49	25.55	80.34	29.31	81.42	30.45	86.18		

Validity verification of PCR run

3. The PCR run is **valid** if the positive and negative control fulfil the following criteria (see Table 11).

Table 11 Validity criteria for the GynTect® controls

Marker	Positive Control			Negative Control
	Ct value	Melt temperature range (cobas)	Melt temperature range (CFX)	Ct value
ASTN1	≥ 20, ≤ 38	80 °C – 86 °C	78 °C – 86 °C	No value*
DLX1	≥ 20, ≤ 38	79 °C – 85 °C	77 °C – 85 °C	No value*
ITGA4	≥ 20, ≤ 38	80 °C – 85 °C	78 °C – 85 °C	No value*
RXFP3	≥ 20, ≤ 38	80 °C – 85 °C	78 °C – 85 °C	No value*
SOX17	≥ 20, ≤ 38	81 °C – 87 °C	79 °C – 87 °C	No value*
ZNF671	≥ 20, ≤ 38	80 °C – 87 °C	78 °C – 87 °C	No value*
ACHE	≥ 20, ≤ 38	78 °C – 83 °C	76 °C – 83 °C	No value*
IDS-M	≥ 20, ≤ 38	78 °C – 88 °C	76 °C – 88 °C	No value*

* **Caution:** You should not receive any Ct values for the negative control. If a Ct value is obtained for any of the markers, it should not display a marker-specific melt curve.

Validity and positivity verification of samples

4. The result for the patient sample is **valid** if the control markers ACHE and IDS M fulfil the following criteria (see Table 12).

Table 12 Validity criteria for control markers for patient samples

Marker	Ct value	Melt temperature range (cobas)	Melt temperature range (CFX)
ACHE	≥ 20, ≤ 42	78 °C - 83 °C	76 °C - 83 °C
IDS-M	≥ 20, ≤ 32	78 °C - 88 °C	76 °C - 88 °C

5. The GynTect® result for this sample is considered **invalid**, if a **Ct value > 0, < 20** is generated with a melt temperature in the defined range.

6. For **valid** samples, use the following equation to calculate the ΔCt value for all methylation-specific markers detected with Ct values and melt temperatures in the defined range (see Table 13):

Evaluation ΔCt
$\Delta Ct = Ct_{(Marker\ x)} - Ct_{(IDS\text{-}M)}$

For each sample the methylation-specific markers are rated **positive** if **ΔCt values** generate the values according to Table 13.

Table 13 Criteria for positivity for methylation-specific Marker für patient samples

Marker	Ct value	Melt temperature range (cobas)	Melt temperature range (CFX)	ΔCt value
ASTN1	≥ 20, ≤ 42	80 °C - 86 °C	78 °C - 86 °C	≤ 8,00
DLX1	≥ 20, ≤ 42	79 °C - 85 °C	77 °C - 85 °C	≤ 9,00
ITGA4	≥ 20, ≤ 42	80 °C - 85 °C	78 °C - 85 °C	≤ 9,00
RXFP3	≥ 20, ≤ 42	80 °C - 85 °C	78 °C - 85 °C	≤ 9,00
SOX17	≥ 20, ≤ 42	81 °C - 87 °C	79 °C - 87 °C	≤ 9,00
ZNF671	≥ 20, ≤ 42	80 °C - 87 °C	78 °C - 87 °C	≤ 10,00

Important: Analyse the amplification and melt curves concerning controversial data points and curve characteristics. Samples with controversial curve characteristics have to be scored invalid.

Evaluation of the GynTect® assay

7. For the evaluation of a GynTect® assay attribute the following values for the single positive markers and sum up the values for the six markers:

Table 14 Values for the GynTect® markers

Marker	Value, if marker is positive	Value, if marker is negative
ASTN1	2	0
DLX1	1	0
ITGA4	2	0
RXFP3	2	0
SOX17	2	0
ZNF671	6	0

If the **sum** of all marker values **equals or is higher than 6,** the GynTect® assay of the sample is **positive**.

If the **sum** of all marker values is **equals or is lower than 5,** the GynTect® assay of the sample is **negative**.

A positive GynTect® test result correlates with the presence of a cervical intraepithelial neoplasia or a cervical carcinoma. The GynTect® result should not be used for final therapy decision, it has to be assessed in combination with other clinical findings.

12. PERFORMANCE OF GYNTECT®

The performance data shown here were collected on the cobas z 480 Analyzer.

12.1. Analytical performance

12.1.1. Analytical sensitivity - detection of methylated DNA

The analytical sensitivity of the PCR assay was determined using methylated, bisulfite-converted human genomic DNA. The corresponding detection limits are summarized in Table 15. The dilution series were tested in three independent experiments, each in triplicates. Using normal scrapes, 20 - 50 ng are used for the assay.

Table 15 Analytical sensitivity of the PCR assay – part 1

DNA amount used	Number of cells in assay *	ASTN1 Cp ≤ 42	DLX1 Cp ≤ 42	ITGA4 Cp ≤ 42	RXFP3 Cp ≤ 42	SOX17 Cp ≤ 42	ZNF671 Cp ≤ 42
0.2 ng	30 cells	9 / 9	9 / 9	9 / 9	9 / 9	9 / 9	9 / 9
0.1 ng	15 cells	9 / 9	9 / 9	9 / 9	8 / 8	8 / 8	9 / 9
0.05 ng	7.5 cells	9 / 9	9 / 9	9 / 9	9 / 9	5 / 9	9 / 9
0.02 ng	3 cells	7 / 9	8 / 9	6 / 9	9 / 9	6 / 9	8 / 9
0.01 ng	1.5 cells	5 / 9	5 / 9	6 / 9	8 / 9	5 / 9	6 / 9
0.005 ng	< 1 cell	8 / 9	7 / 9	4 / 9	6 / 9	1 / 9	6 / 9
0.002 ng	< 1 cell	3 / 9	3 / 9	1 / 9	2 / 9	0 / 9	1 / 9

* *one cell contains approx. 6 - 7 pg genomic DNA*

The overall detection limit, at which the methylation markers are detectable in all reactions, lies at 15 cells (0.1 ng).

Furthermore, a DNA mix consisting of methylated bisulfite-converted human genomic DNA and unmethylated bisulfite-converted human genomic DNA was tested in similar experiments. In each assay 20 ng respectively 100 ng DNA were used. The dilution series were tested in three independent experiments, each in triplicates (see Table 16).

Table 16 Analytical sensitivity of the PCR assay – part 2

Percentage methylated DNA	Total amount DNA	ASTN1 ΔCp ≤ 8	DLX1 ΔCp ≤ 9	ITGA4 ΔCp ≤ 9	RXFP3 ΔCp ≤ 9	SOX17 ΔCp ≤ 9	ZNF671 ΔCp ≤ 10
10 %	20 ng	9 / 9	9 / 9	9 / 9	9 / 9	9 / 9	9 / 9
1 %	20 ng	9 / 9	9 / 9	9 / 9	9 / 9	9 / 9	9 / 9
0.1 %	20 ng	5 / 8*	8 / 8*	8 / 8*	6 / 8*	1 / 8*	7 / 8*
0.01 %	20 ng	0 / 9	9 / 9	3 / 9	2 / 9	0 / 9	4 / 9
0 %	20 ng	0 / 9	8 / 9	0 / 9	0 / 9	0 / 9	0 / 9
10 %	100 ng	9 / 9	9 / 9	9 / 9	9 / 9	9 / 9	9 / 9
1 %	100 ng	9 / 9	9 / 9	9 / 9	8 / 9	9 / 9	8 / 9
0.1 %	100 ng	8 / 9	9 / 9	9 / 9	9 / 9	1 / 9	9 / 9
0.01 %	100 ng	0 / 9	8 / 9	1 / 9	1 / 9	0 / 9	4 / 9
0 %	100 ng	0 / 9	9 / 9	0 / 9	0 / 9	0 / 9	0 / 9

* *One out of nine samples was negative for ACHE and has to be excluded.*

The detection limit for the methylation markers were determined at 1 % methylated DNA for a 20 ng sample and at 10 % for a sample with 100 ng DNA per assay.

The results of a spike-in experiment of SiHa cells in patient samples display that in patient samples with a concentration of at least 3×10^5 cells per ml a fraction of 0.1 % methylated cells can be detected reliably.

12.1.2. Analytical specificity – detection of unmethylated DNA

The analytical specificity of the PCR assay was determined using 10 - 12 kb long, unmethylated bisulfite-converted PCR fragments representing the complete human genome. The experiments were performed in a 5-fold determination, the results are summarized in Table 17. Validity of the samples was assured by ACHE. No positive GynTect® test result was obtained up to a concentration of 750 ng unmethylated, bisulfite-converted DNA (biDNA).

Table 17 Analytical specificity of the PCR assay

DNA used	ASTN1 Cp ≤ 42	DLX1 Cp ≤ 42	ITGA4 Cp ≤ 42	RXFP3 Cp ≤ 42	SOX17 Cp ≤ 42	ZNF671 Cp ≤ 42
0 ng unmethylated biDNA	0 / 3	0 / 3	0 / 3	0 / 3	0 / 3	0 / 3
100 ng unmethylated biDNA	0 / 5	1 / 5	1 / 5	0 / 5	0 / 5	0 / 5
250 ng unmethylated biDNA	0 / 5	0 / 5	0 / 5	1 / 5	0 / 5	0 / 5
500 ng unmethylated biDNA	0 / 5	1 / 5	0 / 5	0 / 5	0 / 5	0 / 5
750 ng unmethylated biDNA	1 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5
75 ng methylated gDNA	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5

12.2. Precision

12.2.1. Repeatability

20 different samples were tested three times with GynTect®. In each case a new preparation and bisulfite treatment of the samples was performed. All three experiments were performed by one person, but on different days. 16 samples yielded identical results in 3 / 3 runs. Thus, 80 % of the samples may have a repeatability of 100 % in three experiments.

12.2.2. Reproducibility

20 different samples were tested three times with GynTect® in independent laboratories. In each case a new preparation and bisulfite treatment of the samples was performed as well as different PCR devices (cobas z 480 Analyzer resp. LightCycler II 480) was used. The comparison of two results of each sample generates three valuations per sample (lab a versus lab b, lab b versus lab c, lab a versus lab c). Data thus obtained, 90 % of valuations yielded identical. The evaluation of valid comparison, results in 96.43 % reproducibility.

12.3. Robustness

The following variations in the temperature profile of the PCR run were tested, with no deviations observed in the final GynTect® result (performed with four different samples and a no-template control):

Table 18 Variations of temperature profile

Deviation from original protocol	Effects on positivity GynTect® PC	Average shift of Cp value all Marker
65 °C Annealing Temperature	none	< 1
67 °C Annealing Temperature	none	< 1
92 °C Denaturing Temperature	none	< 1
96 °C Denaturing Temperature	none	< 1
96 °C Denaturing Temperature & 10 sec Denaturing & 30 sec Annealing/Elongation	none	up to 1.12 higher
96 °C Denaturing Temperature & 20 sec Denaturing & 40 sec Annealing/Elongation	none	< 1

The positioning of sample in PCR device has not any impact to GynTect® result.

12.4. Clinical performance

The patient samples used were obtained from European hospitals (Germany, Portugal, Slovakia).

Bisulphite treatment of patient samples was performed as described in chapter 11.4.

For the determination of the clinical performance of GynTect®, 321 patient samples with the following characteristics were investigated: Pap I (n = 199; 62 %), CIN 1 (n = 20; 6.2 %), CIN 2 (n = 28; 8.7 %), CIN 3 (n = 64; 19.9%), cervical carcinoma (n = 10; 3.1 %).

Based on the established cut-off, clinical sensitivity and specificity were calculated.

Table 19 Clinical performance of GynTect®

Finding	GynTect® detection	CI 95 %
Pap I (n = 199)	4.02 %	1.75 % - 7.77 %
CIN 1 (n = 20)	30.00 %	11.89 % - 54.28 %
CIN 2 (n = 28)	39.29 %	21.50 % - 59.42 %
CIN 3 (n = 64)	62.50 %	49.51 % - 74.30 %
CxCa (n = 10)	100 %	69.15 % - 100 %

CI = confidence interval

Table 20 Sensitivity and specificity

Sensitivity for CIN 3+	Specificity for CIN 3+	False-positive rate for Pap I
67.6 %	89.9 %	4.0 %

13. LIMITS OF THE PROCEDURE

- The interpretation of the GynTect® results should always be carried out in conjunction with results of further laboratory diagnostic procedures, as well as taking into account the clinical picture.
- The specifications according to the instructions for use, e.g., pipetting volumes, incubation times, temperatures and preparation steps must be adhered to in order to avoid erroneous results.
- Proper sampling and storage are critical to test results.
- In principle, it cannot be excluded in molecular biological test procedures that further very rare sequence variants could influence the test result, which are not yet covered in the sources consulted for the specificity and sensitivity analysis of the primers and probes.
- Non-specification instrument performance, as well as deviations from the described test procedure, specified storage conditions, materials, equipment, or recommended sample material, may result in differences from results obtained when all specifications are met.
- The provided internal and external controls are aids for the detection of faults. However, they cannot detect every possible fault. It is the user's responsibility to validate any modifications made or, if necessary, the devices used and to ensure compliance with the device specifications.

14. REFERENCES

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[3] Cuzick et al. (2006). Overview of the European and North American studies on HPV testing in primary cervical cancer screening. Int J Cancer. 119(5):1095-1101

[4] Hansel et al. (2014). A Promising DNA Methylation Signature for the Triage of High-Risk Human Papillomavirus DNA-Positive Women. PLOS ONE. Volume 9, Issue 3, e91905

[5] Schmitz et al. (2017). Performance of a methylation specific real-time PCR assay as a triage test for HPV-positive women. Clinical Epigenetics. 9:118

[6] Schmitz et al. (2018). Performance of a DNA methylation marker panel using liquid-based cervical scrapes to detect cervical cancer and its precancerous stages. BMC Cancer. 18:1197

[7] International Agency for Research on Cancer (2008). European guidelines for quality assurance in cervical cancer screening – Second edition

15. LIABILITY

The GynTect® Kit may only be used in accordance with its intended purpose. Oncgnostics GmbH assumes no liability for any other use (e.g., non-compliance with these operating instructions and improper use) and any resulting damage.

16. QUESTIONS AND PROBLEMS

In case of questions and problems with the product, please get in touch with your contact partner at oncgnostics GmbH.

You may reach the technical support of oncgnostics GmbH Monday to Friday between 8.00 a.m. and 4.00 p.m., calling the following number: **+49 (0) 3641 5548500**.

Outside these contact hours, you can reach us by e-mail: **gyntect@oncgnostics.com.**

oncgnostics GmbH
Löbstedter Straße 41
07749 Jena, Germany
Management: Dr. Alfred Hansel, Dr. Martina Schmitz

In case of any of the following errors during processing of the cervical samples, performance of the PCR, analysis of the data and/or failure of the whole GynTect® assay due to one of the controls, proceed as described in the following.

Table 21 Troubleshooting

Problem and cause	Remarks and suggestions
CENTRIFUGE	
No centrifuge available for PCR strips/plates	Shake the 8-well strip vigorously from the wrist until all the liquid is at the bottom of the wells. The inside of the lid must be droplet-free, repeat the process if necessary.
INVALID MELT TEMPERATURES	
The GynTect® Strip was placed in the PCR system rotated by 180°	The melt temperature at position B should correspond to the melt temperature of position G. Well A and B show amplification for all GynTect® Strips except the negative control. The samples may be analysed.
Amplification and melt curves are visible, but the melt temperatures deviate substantially from the requirements given in the manual	Please contact oncgnostics GmbH.

NO / INVALID PCR RESULT	
Ct value of marker IDS-M above the target value	Repeat the test for the sample using a new GynTect® assay. Repeat the test for the sample using a new GynTect® assay in optionally use of a higher sample volume (2 ml to 3 ml). In case of a further negative result for the markers the patient sample cannot be analysed.
Exceeding the target value for GynTect® Positive Control and/or GynTect® Negative Control	Repeat GynTect® for all samples with filled sample eluates (ad 80 µl GynTect® Water).

17. ADDITIONAL NOTES

Regulatory notice to customers in the European Union: Please note your obligation to report to your competent authority and to oncgnostics GmbH any serious incidents that have occurred in connection with the product.

The current version of the safety data sheet for this product is provided in the Download Centre on the website (http://www.oncgnostics.com/en/downloadcenter/) or can be requested by e-mail to **gyntect@oncgnostics.com**.

18. MEANING OF THE SYMBOLS

Symbol	Meaning	Symbol	Meaning
PCR-MM	Mastermix		Temperature limitation
STRIPS	PCR Strips		Use-by
CAPS	Caps		Sufficient for <n> tests
CONTROL +	Positive Control		Manufacturer
H_2O	Water		Consult instructions for use (IFU)
IVD	*In-Vitro* Diagnostic		Store in the dark
LOT	Batch code		Do not re-use
REF	Catalogue Number		CE marking

19. LIST OF CHANGES

Previous version (Release date)	Changes

3.5 (May 2022)	– Restructuring, chapters have been moved within the instructions for use
	– Chapter 11.2 Sampling
	  ○ Storage conditions for cervical samples adapted
	– Chapter 11.5.2 Performing the PCR on the cobas z 480 Analyzer
	  ○ Fixed position of PC (row 11) and NTC (row 12)
	  ○ Screenshots removed
	– Addition of chapter 11.5.3 GynTect® on the CFX96 Real-Time Detection PCR System
	– The following chapters have been added:
	  ○ Chapter 5 Reference material
	  ○ Chapter 13 Limits of the procedure
	  ○ Chapter 14 References
	  ○ Chapter 15 Liability
	  ○ Chapter 17 Additional notes
	  ○ Chapter 19 List of changes

20. SHORT PROTOCOL

In the following please find a copy template of a short protocol in the form of a checklist. Prior to using this short protocol please read the full instructions with all notes and tips in chapter 11.
The bisulfite kit is not part of the GynTect® Kit. Bisulfite treatment of the samples has to be performed using the EpiTect® Fast Bisulfite Kit (10).

SAMPLE PREPARATION

- ☐ Vortex patient sample for 5 sec at maximum speed and transfer 1 ml medium to a 1.5 ml tube
- ☐ Centrifuge sample for 5 min at 10,000 xg
- ☐ Remove 900 µl supernatant above the pellet and discard

BISULFITE TREATMENT OF THE SAMPLES

- ☐ Prepare EpiTect® Fast Bisulfite Kit buffers according to Table a

Table a Buffer composition EpiTect® Fast Bisulfite Kit (Qiagen)

Buffer	Add Ethanol
Buffer BL *	-
Buffer BW	30 ml Ethanol (96 - 100 %)
Buffer BD	27 ml Ethanol (96 - 100 %)

* do not add *Carrier RNA*, control of quality

- ☐ Setup reaction according to Table b, in a 0.5 ml tube, vortex and centrifuge

Table b Bisulfite reaction

Compound	Per reaction
Bisulfite Solution *	85 µl
DNA Protect Buffer	15 µl
Resuspended sample	40 µl
Total volume/reaction	140 µl

* Control of quality

- ☐ Conversion using a thermal cycler, check temperature profile according to Table 7
- ☐ Vortex samples and centrifuge briefly
- ☐ Add **310 µl Buffer BL** to MinElute® Spin Column
- ☐ Add sample and pipet 5 × up and down
- ☐ Add **250 µl Ethanol** (96 - 100 %), mix 5 x by converting column pivot overhead
- ☐ Centrifuge column for 30 sec at 18,000 ×g and discard flow through
- ☐ Add **200 µl Buffer BW** and centrifuge column for 30 sec at 18,000 ×g
- ☐ Add **200 µl Buffer BD** and incubate at room temperature for 15 min
- ☐ Centrifuge column for 30 sec at 18,000 ×g
- ☐ Add **200 µl Buffer BW** and centrifuge column for 30 sec at 18,000 ×g and discard flow through
- ☐ Add **400 µl Buffer BW** and centrifuge column for 30 sec at 18,000 ×g
- ☐ Add **200 µl Ethanol** (96 - 100 %) and centrifuge column for 30 sec at 18,000 ×g
- ☐ Place column in a fresh 2 ml Collection-Tube and centrifuge column for 60 sec at 18,000 ×g
- ☐ Place column in a 1.5 ml Tube, add **20 µl Water** and incubate for 60 sec at room temperature

- [ ] Centrifuge column for 60 sec at 8,000 ×g
- [ ] *Optional breakpoint*: the workflow may be interrupted here, the samples may be stored

PREPARATION AND PIPETTING OF THE PCR

- [ ] Add **70 µl GynTect® Water** to each sample, vortex and centrifuge briefly
- [ ] Vortex and centrifuge briefly **GynTect® Mastermix**
- [ ] Remove coloured caps from **GynTect® Strips** to be used
- [ ] Add **10 µl GynTect® Mastermix** to each reaction well
- [ ] Add **10 µl sample** or **GynTect® Positive Control** or **GynTect® Water** to each of the eight wells of a corresponding **GynTect® Strip**
- [ ] Close **GynTect® Strips** with **GynTect® Caps**
- [ ] Vortex and centrifuge **GynTect® Strips** briefly

PERFORMANCE OF PCR

- [ ] Start PCR device, open software and choose template (GynTect)
- [ ] Name PCR run individually, prepare plate layout and check temperature profile
- [ ] Save PCR run
- [ ] Place **GynTect® Strips** in the device and start PCR run
- [ ] After completing the PCR, make the evaluation settings and export the .txt or .xlsx file

ANALYSIS AND INTERPRETATION OF THE PCR RESULTS

- ☐ Open exported files (.txt or .xlsx) in a suitable spreadsheet program
- ☐ Check the results of **Positive Control** and **Negative Control** for all markers
- ☐ Check all samples for validity and positivity according to Table c

Table c Criteria for validity and positivity

Marker	Ct Value	Melt temperature range (cobas)	Melt temperature range (CFX)	ΔCt (Marker x - IDS-M)	Criteria for
ASTN1	≥ 20, ≤ 42	80 °C - 86 °C	78 °C – 86 °C	≤ 8.00	positivity
DLX1	≥ 20, ≤ 42	79 °C - 85 °C	77 °C – 85 °C	≤ 9.00	
ITGA4	≥ 20, ≤ 42	80 °C - 85 °C	78 °C – 85 °C	≤ 9.00	
RXFP3	≥ 20, ≤ 42	80 °C - 85 °C	78 °C – 85 °C	≤ 9.00	
SOX17	≥ 20, ≤ 42	81 °C - 87 °C	79 °C – 87 °C	≤ 9.00	
ZNF671	≥ 20, ≤ 42	80 °C - 87 °C	78 °C – 87 °C	≤ 10.00	
ACHE	≥ 20, ≤ 42	78 °C - 83 °C	76 °C – 83 °C	-	validity
IDS-M	≥ 20, ≤ 32	78 °C - 88 °C	76 °C – 88 °C		

- ☐ Evaluate the **GynTect® Results**

Table d Values for the GynTect® markers

Marker	Value, if marker is positive	Value, if marker is negative
ASTN1	2	0
DLX1	1	0
ITGA4	2	0
RXFP3	2	0
SOX17	2	0
ZNF671	6	0

Sum of all values ≥ 6

→ **GynTect® positive**

A positive GynTect® test result correlates with the presence of a cervical intraepithelial neoplasia or a cervical carcinoma. The GynTect® result should not be used for final therapy decision, it has to be assessed in combination with other clinical findings.