Prostatype® Test System

To achieve reliable results, the instructions for use must be read carefully prior to use and followed precisely.

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Contents

1.   Name and Intended Use

The Prostatype® Test System (PrTS) is a product intended for prognostic evaluation of newly diagnosed prostate cancer patients. Through an RT-qPCR test, clinical data and an algorithm, the product offers a decision support tool regarding the choice of treatment for each patient. The PrTS consists of two parts:

1. The Prostatype® RT-qPCR Kit: a test designed for use on formalin-fixed, paraffin-embedded (FFPE) tumor samples. After RNA extraction, a set of reagents is added to amplify four genes; one reference gene and three cancer-related genes.
2. The Classification of Prostatic Malignancy Algorithm (CPMA): This software product contains an algorithm capable of identifying similar (reference) patients and providing their survival in relation to the treatment as an individualized decision support for each prostate cancer patient in question. It also calculates the P-score: a mathematical model estimating the aggressiveness of a patient's prostate cancer based on his genetic and clinical parameters.

The intended use of the PrTS is the following: The PrTS is a test system which contains the Prostatype® RT-qPCR Kit and the evaluation software CPMA. The Prostatype® RT-qPCR Kit is used to perform a 4-plex one-step reverse transcription quantitative PCR test intended for the assessment of mRNA expression levels of the three genes IGFBP3, F3, VGLL3 relative to the expression level of gene GAPDH in total RNA extracted from FFPE human prostate core needle biopsy containing cancer cells. The output of Prostatype® RT-qPCR Kit is entered into the CPMA together with other clinical information. The output of the CPMA is a Prostatype® Test System Report presenting the most similar reference patients, selected from the CPMA reference patient database. Additionally, a numerical score (P-score) is provided to estimate the aggressiveness of prostate cancer. Taken together, the Prostatype® Test System Report can be used by healthcare professionals in adjunct to other parameters currently used for prostate cancer prognosis.

2.   Summary and Explanation

The Prostatype® Test System contains (1) the Prostatype® RT-qPCR Kit and (2) the software CPMA. Based on a comparison of genetic and clinical data of a newly diagnosed prostate cancer patient, the CPMA conducts a similarity search in a database of reference patients and identifies the three most similar historical patients. Information regarding the reference patients' survival range and treatment is provided. The P-score provides an individual estimation of prostate cancer aggressiveness. This information can be used – together with other parameters – to identify treatment options and the test system thereby intends to provide a decision support for doctors and prostate cancer patients.

The Prostatype® RT-qPCR Kit is a 4-plex one-step reverse transcription (RT) quantitative Polymerase Chain Reaction (qPCR) test for assessment of expression levels of the three genes IGFBP3, F3 and VGLL3 relative to the expression of GAPDH in RNA extracted from FFPE needle biopsies of human prostate cancer. This three-gene expression signature has been identified and evaluated in a Swedish cohort study with 189 prostate cancer patients that received their diagnosis between 1986 and 2001. The gene signature has been shown to categorize prostate cancer patients into high-risk, intermediate-risk and low-risk subtypes (1). A follow-up study in a new cohort of patients revealed that the precision of prostate cancer survival prediction could be further improved by combining genetic data with clinical parameters including the patient's age at diagnosis, PSA value, Gleason score, primary Gleason pattern, and Tumor stage (2).

When using the Prostatype® RT-qPCR Kit, it is recommended to ensure that a high percentage of cancer cells are present in the tissue used for RNA extraction. To obtain optimal, reliable results, the tissue shall contain a minimum of 50% cancer cells (2). It has been reported that expression levels of the three biomarker genes in prostate cancer epithelial cells are independent of their Gleason pattern. Thus, the expression signature may mirror mechanisms underlying prostate cancer pathology strengthening the hypothesis that cancer stem cells are involved in prostate cancer initiation and progression (1, 2). The

amount of tissue to be used for the Prostatype® RT-qPCR assay shall be evaluated by a pathologist or a professional with equivalent training.

After the relative expression of the three biomarker genes has been determined and analyzed using the Prostatype® RT-qPCR Kit, the genetic data is combined with clinical parameters of the respective patient. Based on this data a similarity search in a database containing genetic and clinical information of historical prostate cancer patients is performed using the CPMA software. The software is based on a k-Nearest Neighbors (kNN) algorithm and protected by a username/password combination preventing unauthorized access to the database. The CPMA algorithm identifies the three most similar reference patients and displays their overall survival time along with the treatment they underwent.

3. Principles of the Procedure

3.1. Prostatype® RT-qPCR Kit

The Prostatype® RT-qPCR Kit is used to perform a 4-plex, one-step RT-qPCR test. The kit contains all reagents required for reverse transcription, amplification and detection with quantitative PCR in a 4-plex reaction mix. After extraction of RNA from the FFPE tissue, the mRNA is reverse transcribed using sequence specific primer pairs for the genes IGFBP3, F3, VGLL3 and GAPDH. Subsequently, the generated cDNA is amplified and detected using individual dual-labeled hydrolysis probes (Taqman®). The hydrolysis probes are labeled with an individual fluorophore and fluorescence quencher, which prevents fluorescence detection. During the amplification step, each probe that binds to a cDNA target is hydrolyzed due to the exonuclease function of the *Taq* polymerase and the quencher is spatially separated from the fluorophore leading to the emission of fluorescence signals at specific wavelengths. The signals are detected by the LightCycler® 480-II or -I in specific channels. Thereby, each one of the four genes can be detected and quantified independently in a single reaction well.

As the amplicons increase exponentially with each PCR cycle, fluorescence signals increase as well and are detected in real-time. When the intensity of a particular fluorescence signal crosses a pre-defined threshold, the corresponding cycle is referred to as Crossing Point (Cp). The Cp value depends on the initial concentration of the target gene. A higher initial amount of target RNA results in a lower Cp value. Based on the Cp values of the three target genes (IGFBP3, F3 and VGLL3) and the reference gene (GAPDH), the relative target gene expression (Delta (Δ) Cp) can be calculated with the following formula:

$$\Delta Cp \text{ (target gene)} = Cp \text{ (target gene)} - Cp \text{ (GAPDH)}$$

Apart from normalization of target gene expression levels, the reference gene GAPDH functions as an internal control and its corresponding Cp value in each sample is used to assess sample validity and integrity as well as to ensure that the utilized amount of RNA in the sample was sufficient.

3.2. CPMA (Classification of Prostatic Malignancy Algorithm)

The CPMA software has been designed in accordance with ISO13485 and IEC 62304:2006/AMD1:2015. The software converts the genetic information obtained with the Prostatype® RT-qPCR Kit – in combination with clinical parameters – into prognostic information intending to assist clinicians in making patient-specific and relevant treatment decisions.

The software compares the newly entered patient data with each individual historical patient within a reference database and identifies the three most similar patients in the database. Thereby, the result is based on authentic patients. The CPMA employs a kNN algorithm for the similarity assessment between the patients in the reference database and the newly entered patient. This algorithm transforms, scales and weighs individual parameters to obtain optimal results.

4. Materials

For a comprehensive list of materials provided and required for the Prostatype® Test System, please refer to the **Instructions for Use (IFU) of the Prostatype®-RT-qPCR-Kit** (3). It is imperative that a Roche LightCycler® 480-II or a Roche LightCycler® 480-I is used for carrying out the Prostatype® RT-qPCR test. For installation, calibration, performance verification and maintenance of the Roche LightCycler® 480 instrument, manufacturer's instructions need to be followed.

5. Instructions for the Preparation of Tissue Samples for Analysis with the Prostatype® Test System

The Prostatype® Test System uses RNA isolated from FFPE-prostate cancer core needle biopsies as input material. To obtain reliable results with the Prostatype® RT-qPCR test, high quality and integrity of the RNA are crucial. Adequate preparation of the biopsy material prior to RNA extraction is a critical step and includes three steps:

5.1. Sectioning of FFPE tissue blocks

From the FFPE tissue blocks, separate sections are prepared according to conventional histopathological protocols with the following modifications:

- Use DNase/RNase-free water.
- After drying the tissue section, the paraffin-melting step is omitted.

NOTE: To be suitable for use for the Prostatype® RT-qPCR Kit, FFPE tissue blocks should not exceed an age of 11 years.

NOTE: Tissue embedded in unbuffered formalin should not be used, as it does not produce reliable results.

Prepare the following sections:

- One tissue section of 5 μm thickness: This section will be used for Hematoxylin and Eosin (H&E) staining. The staining procedure shall be carried out in accordance with standard protocols.
- Nine sequential tissue sections of 10 μm thickness: These sections will remain unstained and the tissue will be used for RNA extraction.
  NOTE: Collect the sections on glass slides without DNase/RNase contamination!

5.2. Marking and quantification of the cancer area

Using a traditional microscope or a digital scanner (e.g. iScan Coreo, Ventana Medical Systems, Inc.), mark the cancer tissue in the H&E-stained tissue section.

NOTE: Marking should be performed by a pathologist or a professional with equivalent training to avoid erroneous marking of the tumor region.

NOTE: The cancer area should be between $10 – 30 \text{ mm}^2$. When marking the cancer area manually, the marked area per section should be 1.5 – 4.5 mm in length on up to 9 FFPE sections to avoid that the amount of harvest tissue is below specifications.

NOTE: To obtain good results, the marked area should contain at least 50% cancer cells.

Conventional microscope:

- Mark the cancer area on the H&E-stained slide.

- Calculate the cancer area.

Digital scanner:

- Scan images at 20x magnification.
- Mark and calculate the cancer area using an image processing software (e.g. Image Viewer, Ventana Medical Systems, Inc.).
- Transfer the cancer area marking from the scanned image to the original H&E-stained slide using a microscope.

The obtained sample serves as a map and is used as a template in the following step.

5.3. Scraping of cancer cells

In this step, the H&E-stained slide and the unstained sections are used as input material. Furthermore, a disposable scalpel and a DNase/RNase-free microcentrifuge tube are required.

- Overlap the H&E-stained section with an unstained section.
- Identify and carefully mark the cancer area on the unstained section with the scalpel blade using the H&E-stained slide as a map.
- Scrape the cancer area using the scalpel and collect the scraped tissue in a microcentrifuge tube.
  NOTE: This step should be executed with high precision and a certain amount of practice is required to find the right angle when holding the scalpel in order to scrape the tissue coherently.
  NOTE: A new disposable scalpel and a separate microcentrifuge tube shall be used for each sample.
  NOTE: For optimal test results, we recommend scraping of 30 mm^2 tissue containing a minimum of 50% cancer cells. To achieve a sufficient amount of tissue, the pathologist can combine tumor samples from one or several biopsies of the same patient.

6. Extraction of RNA from Tissue Samples

To extract RNA from the scraped prostate cancer tissue, we recommend the use of the semi-automated Maxwell® 16 LEV RNA FFPE Purification Kit with the instrument or Roche High Pure FFPE RNA Micro Kit according to the manufacturer's instructions with the following modifications.

Suggested modifications for the Maxwell® 16 LEV RNA FFPE Purification Kit:

1. After adding mineral oil, the incubation at 80°C for 2 min is carried out under shaking at 1200 rpm.
2. Digestion of the tissue: Incubation at 56°C for 15 min (For New tissues that are used within one year, the shaking speed is 1200 rpm for 5 min, and then 650 rpm for 10 min followed by incubation at 80° for 1 hour are carried out under shaking at 650 rpm.

Suggested modifications for Roche High Pure FFPE RNA Micro Kit:

1. For specimens collected less than one year prior to RNA extraction, add 350 μL Lysis Buffer and 350 μL Binding Buffer, in the homogenization step to reduce viscosity.
2. Centrifuge the High Pure Micro filter for 4 minutes instead of 2 minutes at maximum speed before the RNA elution step.

7. Instructions for Use of the Prostatype® RT-qPCR Kit

Detailed instructions regarding the use of the Prostatype® RT-qPCR Kit as well as instructions how to analyze the obtained raw data are provided in the **IFU of the Prostatype® RT-qPCR Kit** (3). In short, RNA extracted from scraped FFPE-samples is used as starting material for the Prostatype® RT-qPCR

Test. Each box of Prostatype® RT-qPCR Kit contains sufficient reagents to perform expression analysis of up to four patient samples plus four controls. Inside the kit box, there are four small independent packages. Each package can be used to perform expression analysis for one patient sample along with one positive control and one negative control, thus avoiding repeating freeze-thawing of the reagents. Each patient sample and control shall be tested in triplicates. ΔCp values and median ΔCp values are calculated as described in detail in the **IFU of the Prostatype® RT-qPCR Kit**. Since analysis of the test is based on determining median Cp and median ΔCp values instead of calculating mean values, outlier handling is not relevant when using the Prostatype® RT-qPCR Kit.

Note: If more than 1 patient needs to be analyzed in one run the kit reagents of similar kind can be pooled after thawing. DO NOT pool reagents from different batches.

8. Performance of the Prostatype® RT-qPCR Kit

The Prostatype® RT-qPCR Kit has been validated to be able to generate Cp (GAPDH) ≤28 with around 95 % possibility by using specimen collected to the following criteria:

- Unstained FFPE tissue sections from core needle biopsies were used.
- Sections were of 10 μm thickness.
- Minimum tumor area used was 30 mm^2 with tumor content >50%.

For samples with a tumor area between 15mm^2 and 30mm^2 and 50% cancer cells, it is still encouraging to continue performing RNA extraction and Prostatype® RT-qPCR test, since there is >90% possibility to obtain Cp(GAPDH) ≤28 from these specimens. For specimens with a tumor area <15mm^2 and 50% cancer cells, there is around 80% possibility to generate Cp(GAPDH) ≤28. Samples with <5mm^2 tumor area are not suitable for testing with Prostatype® RT-qPCR Kit.

8.1. Detailed performance characteristics

For detailed information regarding Limit of Quantification, Limit of detection, Precision, Reproducibility, Interference, and Relative Sensitivity please refer to *Section 12* in the **IFU of the Prostatype® RT-qPCR Kit.**

9. Instructions for Use of the CPMA database

After obtaining the Cp values for the four genes by using the Prostatype® RT-qPCR Kit, the data is analyzed as described in detail in the **IFU of the Prostatype® RT-qPCR Kit** and median ΔCp values are calculated for the three genes IGFBP3, F3 and VGLL3 (3). In the next step, a similarity search is performed based on the gene expression data in combination with clinical parameters in order to provide prognostic information. The similarity search is based on the software CPMA, which searches the reference patient database for the three most similar historical patients. The result is presented as the survival range of the three reference patients.

9.1. Performing a CPMA database search

In order to perform the database search, the following steps must be executed:

- Double click on the CPMA-Software icon to start the software.
- Log in to the software using your username and password provided by Prostatype Genomics AB.
- To enter a new patient into the database, click on **New patient** at the upper left corner and enter patient ID into the corresponding text box.
- Enter the following numeric values into the respective text boxes: Gleason score, primary Gleason pattern, Tumor stage, PSA value, Age at diagnosis, median ΔCp IGFBP3, median ΔCp F3 and median ΔCp VGLL3

- If applicable, enter information regarding the patient's treatment or comments in the "*Comment*" text box.
- Enter a valid kit code in the "*Kit id*" text box.
  NOTE: Each Prostatype® RT-qPCR Kit provides a unique kit code that can be used for 16 individual samples.
- Click **OK**, verify your input and sign with your CPMA username and password.

To view a patient in the list, conduct the following steps:

- Double click on the corresponding patient ID or select **View patient** in the lower left corner.

  NOTE: The result depends on quality and correctness of input data. Incorrect input data can result in inaccurate prognostic information!

  NOTE: In case of incorrectly entered patient data, or if the status of a previously entered patient needs to be set as obsolete, the *correction enabling code* functionality can be applied to resolve this mistake (see section *9.2. Correction enabling codes* for details).

9.2. Correction enabling codes

If errors in the entered patient data are detected after the input has been signed, the *correction enabling code* functionality can be used to correct this issue. In order to use this feature, a correction enabling code needs to be obtained from Prostatype Genomics AB.

To perform corrections of entered data:

- Log in to the software using your username and password.
- Click **Tools** and select **Correction enabling code**.
- Contact the Prostatype Genomics AB (info@prostatypegenomics.com) and provide the three-letter code identifying your system/CPMA and the date for when you would like to perform the correction.
- You will receive an unlock-code (valid on the date you specified) from the CPMA administrator.
- Type the received code at the effective date to access the correction functionality.
  NOTE: Only one patient can be edited per code and per day!
- Select the patient to edit and click **Set patient obsolete** in the upper left corner
- Enter the correct data for the patient into the CMPA.
- Sign the input with your user name and password.

The previous, incorrect patient input is now marked as obsolete. The new patient data can be compared with the three most similar reference patients as described in section *9.1. Performing a CPMA database search*.

NOTE: Log out of the CPMA and close the software directly after use to prevent unauthorized utilization of the software.

9.3. Backing up patient data

Regular backup of patient data to a safe location is recommended according to the following instructions:

- Locate the folder containing the encrypted database files (*.cpma). The default root folder is "[root:]/ProgramData/ProstatypeGenomicsAB/CPMA").
- Copy this folder into the desired location, i.e. a securely stored or password-protected external drive.

9.4. CPMA maintenance

No maintenance is required for CPMA to remain functional.

10. Limitations, Precautions and Safety

To protect yourself as well as to avoid contamination of reagents and samples, always wear a laboratory coat and disposable gloves when using the Prostatype® Test System. This measure is also critical when scraping cancer tissue for RNA extraction.

The Prostatype® RT-qPCR Kit does not contain any hazardous ingredients. The respective material safety data sheets (MSDS) are available on our website and provide more detailed information (www.prostatypegenomics.com/technical-documents).

To obtain reliable results, it is *imperative*, to adhere to the instructions given in the IFU. Deviations from these guidelines may cause the generation of incorrect or invalid data or may lead to the failure to obtain test results.

- The Prostatype® Test System is for use in a professional laboratory only and assumes familiarity with RNA handling and real-time PCR assays.
- Good laboratory practice is essential to minimize the risk of cross-contamination between samples during and after RNA extraction and purification.
- General recommendation on the organization and procedures of the laboratory shall be adhered to in order to prevent DNA contamination.
- Avoid microbial contamination of reagents when removing aliquots from reagent bottles.
- The use of sterile pipette tips with aerosol barrier is recommended to prevent cross-contamination of patient samples.
- Always wipe all working areas, surfaces, equipment and materials with a DNA decontamination agent and an RNase decontamination agent before starting the procedure.
- Preferably, a working area exclusively used for RNA testing shall be used.
- Samples intended for analysis with the Prostatype® Test System are to be selected by a professional pathologist or a professional with equivalent training.
- The Prostatype® Test System uses specimen obtained from unstained FFPE tissue sections from prostate core needle biopsies. The tissue sections shall be of 10 µm thickness.
- An optimal amount of 30 mm^2 tissue containing at least 50% cancer cells is recommended for each patient sample.
- Do not store RNA after extraction and ensure that freshly extracted RNA is used in the Prostatype® RT-qPCR test within one day.
- For each individual qPCR, a Prostatype® Positive and Negative Control has to be tested on the same 96-well PCR-plate.
- All patient samples as well as the Prostatype® Positive and Negative Control shall be analyzed in triplicates.
- The Prostatype® RT-qPCR Kit should only be used by professional laboratory personnel that has been trained and certified by Prostatype Genomics according to the corresponding training document (4).
- Preparation of the Prostatype® RT-qPCR shall be performed at room temperature (15-25 °C).
- The raw data (Cp values) need to be analyzed according to the instructions provided in the **IFU of the Prostatype® RT-qPCR Kit** to assess validity of the test run and to generate ∆Cp values and median ∆Cp values.
- The Prostatype® RT-qPCR test needs to be carried out on a Roche LightCycler® 480-II or a Roche LightCycler® 480-I.
- To prevent contamination by amplicons generated from previous PCR reactions, a strict separation of pre-PCR activities (e.g. RNA extraction, PCR setup) and post-PCR activities (e.g. real-time PCR) is strongly recommended. We further recommend that used PCR plates should be placed in a re-sealable plastic bag or equivalent immediately after removal from the PCR instrument. The bag shall be closed and disposed in a dedicated waste container. Never store a used PCR plate outside of the PCR instrument. Never open a used PCR plate.

11. Meaning of Symbols

[i]	Consult Instructions for Use
REF	Catalogue Number
IVD	*In Vitro* Diagnostic Medical Device
LOT	Batch Code
⧗	Expiration Date
▲	Manufacturer
⌡	Temperature Limitation
∇Σ	Contains sufficient for <n> tests
▦	Barcode

12. References

1. Peng Z, Skoog L, Hellborg H, Jonstam G, Wingmo IL, et al. (2014) An expression signature at diagnosis to estimate prostate cancer patients' overall survival. Prostate Cancer Prostatic Dis 17:81-90.

2. Peng Z, Andersson K, Lindholm J, Bodin I, Pramana S, et al. (2014) Operator dependent choice of prostate cancer biopsy has limited impact on a gene signature analysis for the highly expressed genes IGFBP3 and F3 in prostate cancer epithelial cells. PLoS One 9: e109610.

3. Peng Z, Andersson K, Lindholm J, Dethlefsen O, Pramana S, et al (2016) Improving the prediction of prostate cancer overall survival by supplementing readily available clinical data with gene expression levels of IGFBP3 and F3 in formalin-fixed paraffin embedded core needle biopsy material. PLoS One 11: e0145545.

4. Prostatype® RT-qPCR Kit Instructions for Use. Prostatype Genomics. 2022

13.   Contact Information

The Prostatype® Test System is manufactured by:

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For further information and support please contact us by sending an email to: info@prostatypegenomics.com by calling the phone number: +46 (0) 8-20 87 00.