Prostatype® RT-qPCR Kit

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

IFU 0019, Revision 8, February 2022, Prostatype Genomics AB
(www.prostatypegenomics.com/technical-documents)

REF PG0007

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Contents

1.   Name and Intended Use

The Prostatype® RT-qPCR Kit is a one-step reverse transcription quantitative PCR (RT-qPCR) test intended for the assessment of the mRNA expression levels of the three genes IGFBP3, F3, and VGLL3, relative to the expression level of gene GAPDH in total RNA extracted from formalin fixed paraffin-embedded (FFPE) human prostate core needle biopsy containing cancer cells.

The output of the Prostatype® RT-qPCR Kit may be used by health care professionals in adjunct to other parameters currently used for prostate cancer prognosis to estimate prostate cancer aggressiveness.

2.   Summary and Explanation

Prostatype RT-qPCR kit is a four-plex one-step RT-qPCR kit, which is custom designed to measure gene expression levels of the three human biomarker genes IGFBP3 (insulin-like growth factor binding protein 3), F3 (coagulation factor III (thromboplastin, tissue factor)), and VGLL3 (vestigial-like family member 3), normalized to the expression level of the gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) in RNA extracted from FFPE human prostate cancer tissue samples. The gene expression signature of IGFBP3, F3 and VGLL3 was reported to be able to estimate prostate cancer patients' overall survival at the time of diagnosis [1]. It was identified and evaluated on a Swedish cohort of 189 PCa patients diagnosed between 1986 and 2001 with nearly complete follow-up overall survival data. The gene expression signature was shown sufficient to categorize the patients into high-risk, intermediate-risk and low-risk subtypes [1]. The gene expression signature was further investigated and then verified to significantly improve prediction accuracy of prostate cancer overall survival when supplementing readily available clinical data altogether in a Swedish population-based new cohort with FFPE prostate core needle biopsy tissue samples. [2]

The Prostatype RT-qPCR kit evaluates the expression levels of three biomarker genes in prostate cancer tissue samples. A reasonably high percentage of cancer epithelial cells shall be recommended, ideally, at least 50% shall be cancer epithelial cells [2]. The cancerous prostatic epithelial cells are reported to contribute to the prognostic value of the three biomarker genes irrespective of their pathological Gleason patterns. This indicates that the gene signature may reflect underlying pathogenetic mechanisms of prostate cancer. [2] This study further provides supporting evidence for the underlying 'stemness' hypothesis in initiation and progression of prostate cancer [1,2]. The amount of tissue input shall be evaluated and recommended by a professional pathologist in order to obtain enough signal, the detailed procedures of sample taking are presented in the Instruction For Use of Prostatype Test System.

In order to generate a prognostic statement, the expression levels of the three biomarker genes measured with the Prostatype RT-qPCR kit shall be utilized by the stand-alone software, the Classification of Prostatic Malignancy Algorithm (CPMA) together with currently used clinical parameters. By presenting the referenced overall survival or treatments, which is evaluated by its comprehensive archived anonymous patient database, it will be possible for the healthcare professionals to predict the outcome of the disease in relation to treatment with a higher precision than before. Additionally, the P-Score estimates the likelihood of having aggressive prostate cancer for individual patients based on their clinical and genetic profile.

3.   Principles of the Procedure

The Prostatype® RT-qPCR is designed as a One-Step RT-qPCR. The Kit provides all reagents to perform the reverse transcription (RT), amplification and detection of mRNA by quantitative real-time polymerase chain reaction (PCR) in one 4-plex reaction mix. Each assessment is conducted in a triplicate reaction. The reagents are ready to use and no additional reagents are required to perform the RT-qPCR reaction.

The mRNA, extracted from FFPE tissue, is reverse transcribed *in vitro* into cDNA using sequence specific primers for the three target genes IGFBP3, F3 and VGLL3 as well as the reference gene GAPDH. The

generated cDNA of the four genes is subsequently amplified and detected using the same sequence specific primers. Individual dual-labeled hydrolysis (Taqman®) probes are used for gene specific detection of the amplification. Individual fluorescence dyes are attached to the four hydrolysis probes allowing independent detection and quantification of the four genes in a single reaction well. Each probe binding to a cDNA target is hydrolyzed during amplification of the target. This leads to different fluorescence signals of specific wavelengths that are detected by the LightCycler® 480 instrument in dedicated optical channels.

With each PCR cycle the fluorescence signals increase and are measured in real-time. The cycle where an individual fluorescence level crosses a defined threshold is called Crossing Point (Cp). The Cp value depends on the initial concentration of the target contained in the sample: The more target RNA that is present in the tested mRNA sample the lower the Cp value will be. The Prostatype® RT-qPCR Kit measures the expression levels of the three target genes IGFBP3, F3, and VGLL3 relative to the expression level of the gene GAPDH. This allows relative quantification independent of the input amount of total mRNA. The relative expression is represented by the so-called Delta Cp value and is calculated as follows: Delta Cp (target gene) = Cp (target gene) – Cp (GAPDH).

The reference gene GAPDH furthermore serves as an internal control to evaluate sample validity and integrity and to ensure that a sufficient amount of total mRNA is utilized. The Prostatype® RT-qPCR Kit is designed to be carried out on a LightCycler® 480-II or a LightCycler® 480-I.

4. Materials Provided

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. The content for each independent package is listed in Table 1.

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.

4.1. Contents

Table 1: Contents of the Prostatype® RT-qPCR Kit.

Reagent	Containers	Volume
Prostatype Master	1 vial	≥ 135 µL
Prostatype Manganese	1 vial	≥ 30 µL
Prostatype Positive Control	1 vial	≥ 19 µL
Prostatype Negative Control	1 vial	≥ 19 µL

4.2. Safety Information

When working with chemicals, always wear a laboratory coat and disposable gloves. The Prostatype® RT-qPCR Kit contains no hazardous ingredients. For more information, please consult the respective material safety data sheets (MSDS) available at our website (www.prostatypegenomics.com/technical-documents).

5. Storage and Stability

Reagents provided with the Prostatype® RT-qPCR Kit are stable until the expiration date when stored and handled as directed. Do not use material past expiration date. Do not mix components from different Prostatype® RT-qPCR Kit lots.
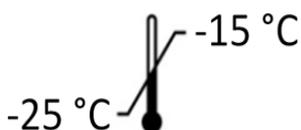
Store all components of the Prostatype® RT-qPCR Kit upon arrival at -25 to -15 °C.

Each component of the Prostatype® RT-qPCR Kit may be thawed and refrozen two times.

6. Materials Required, but not Provided

6.1. General Laboratory Equipment

The following general laboratory equipment is required to perform the Prostatype® RT-qPCR. All laboratory equipment must be installed, calibrated, operated, and maintained according to the manufacturer's recommendations.

- Vortex (e.g. VWR International, catalog no 444-1372, or equivalent)
- Pipettes with adjustable volumes of the following ranges 0.5 – 10 µL, 10 – 100 µL, 100 – 1000 µL (e.g. Eppendorf Reference®, adjustable volume pipette, Eppendorf, catalog no. 4910 000.018, 4910 000.042 and 4910 000.069, or equivalent)
- Repeater pipette or dispenser capable of repetitively dispensing volumes in an adjustable range (e.g. Eppendorf Multipette® plus, Eppendorf, catalog no. 4981 000.019, or equivalent)
- Bench top centrifuge with rotor for 1.5/2.0 mL tubes (e.g. Centrifuge 5417 R, Eppendorf, catalog no. 5407 000.317 with rotor F-45-30-11, Eppendorf, catalog no. 5490 015.002, or equivalent)
- Plate centrifuge capable for PCR plates (e.g. Centrifuge 4-16, Qiagen, catalog no. 81320 and 81031, or equivalent)
- Heating block with mixing function (e.g. Eppendorf ThermoMixer© C, catalog no. 13527550, or equivalent)

6.2. General Laboratory Consumables and Reagents

- 2.0 mL micro tubes with round bottom and with attached PP cap with lid seal mechanism (e.g. Sarstedt SafeSeal, catalog no. 72.695.400 or Eppendorf Safe-Lock™, catalog no. 0030 120.094, or equivalent)
- Pipette tips with aerosol barrier, e.g. ep Dualfilter T.I.P.S.®, Eppendorf:
  - 0.1 – 10 µL, catalog no. 0030 077.504, or equivalent
  - 2 – 100 µL, catalog no. 0030 077.547, or equivalent
  - 50 – 1000 µL, catalog no. 0030 077.571, or equivalent
- Tips for repetitive pipettes, e.g. Combitips plus®, Eppendorf:
  - 0.5 mL, catalog no. 0030 069.226, or equivalent
- Applicator to form a tight seal between a microplate and an adhesive film (e.g. LightCycler® 480 Sealing Foil Applicator, Roche, catalog no. 04706170001)
- DNA decontamination reagent (e.g. DNA AWAY®, VWR, catalog no. 732-2353, or equivalent)
- RNase decontamination reagent (e.g. RNase AWAY®, VWR, catalog no. 732-2351, or equivalent)

6.3. Required Special Equipment and Consumables

The following special equipment and consumables are required to perform the Prostatype® RT-qPCR Kit and cannot be replaced by other equipment.

- LightCycler® 480 Instrument I with 96-well heat block (Roche, catalog no. 05015278001) or LightCycler® 480 Instrument II with 96-well heat block (Roche, catalog no. 05015278001), and with Software version 1.5.x
- LightCycler® 480 Multiwell Plate 96 (Roche, catalog no. 04729692001)
- LightCycler® 480 Sealing Foil (Roche, catalog no. 04729757001)

6.4.   **Installation Requirements**

Installation, calibration, performance verification, and maintenance of the Roche LightCycler® 480 Instrument needs to be performed according to the manufacturer's instructions.

7.   Precautions and Safety

7.1.   **Laboratory Precautions**

This procedure is for professional laboratory use only and assumes familiarity with RNA handling and real-time PCR assays.

Compliance with good laboratory practices is essential to minimize the risk of cross-contamination between samples during and after the RNA extraction, and purification procedure.

Avoid microbial contamination of reagents when removing aliquots from reagent bottles. Prevent the introduction of RNases into samples and controls during the test procedure. Always wear a laboratory coat and disposable gloves. Preferably a working area exclusively used for RNA testing shall be used. Additionally all surfaces, materials and equipment must be treated using a RNase decontamination reagent (e.g. RNase AWAY®) prior to using the Prostatype® RT-qPCR Kit.

The use of sterile pipette tips with aerosol barrier is recommended to prevent cross-contamination of patient samples.

To prevent contamination by amplicons generated from previous PCR we recommend a strict separation of pre-PCR activities (e.g. RNA extraction and purification, PCR setup) and post-PCR activities (e.g. real-time PCR). Further, we recommend that used PCR plates are disposed in a way that no PCR product can be released. For example, used PCR plates should be placed in a re-closable plastic bag or equivalent immediately after removal from the PCR instrument; the bag should 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. Additionally, all surfaces, materials and equipment may be treated using a DNA decontamination reagent (e.g. DNA AWAY®) prior to using the Prostatype® RT-qPCR Kit.

Preparation of the Prostatype® RT-qPCR shall be done at room temperature (15 – 25°C).

7.2.   **Microbiological and Infectious State**

The product does not contain any infectious substances or agents, which cause disease in humans or animals.

8.   Test Procedure

Note: Prior to the test procedure RNA extraction from formalin fixed paraffin-embedded (FFPE) tissue must be performed.

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.

Do not store RNA after extraction and ensure that freshly extracted RNA is used in the Prostatype® RT-qPCR test within the same day.

Note: The Prostatype® RT-qPCR kit has been validated to be available to generate Cp(GAPDH) ≤28 with around 95% possibility by using specimen collected according to following criteria: 1) unstained FFPE tissue sections from core needle biopsies. 2) Sections were of 10 μm thickness. 3) Minimum tumor area used was 30 mm² with tumor content >50 % (1/2).

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.   Setup of the LightCycler® 480 Instrument

A color compensation file needs to be saved on the LightCycler® 480 Instrument before performing the Prostatype® RT-qPCR test. General information about performing color compensation can be found in the LightCycler® 480 Instrument Operator's Manual. The reagents for performing a color compensation experiment required for Prostatype® RT-qPCR Kit (Prostatype® RT-qPCR color compensation plate, Ref: CMC-001) can be purchased at Prostatype Genomics AB.

8.1.1. Saving a New Detection Format on the LightCycler® 480 Instrument I

Note: This section describes the setup using a LightCycler® 480 Instrument I. The setup for a LightCycler® 480 Instrument II is described below.

When running the Prostatype® RT-qPCR the first time on a LightCycler® 480 Instrument I, it is necessary to set up a new detection format according to Figure 1.

- Start the software version 1.5.x.

- Click "Open Tools".

- In the tools window click "Detection Formats".

- Click "New" and give the new detection format a name, e.g. "Prostatype Channels".

- Mark the checkboxes for all of the following filter combinations:

    o Filter Combination 1:   523 – 568

    o Filter Combination 2:   558 – 610

    o Filter Combination 3:   483 – 533

    o Filter Combination 4:   615 – 670

- Change the name of the filter combinations as followed:

    o 523 – 568:   GAPDH

    o 558 – 610:   IGFBP3

    o 483 – 533:   F3

    o 615 – 670:   VGLL3

- Change the filter settings for every combination as followed:

    o Melt factor: 1

    o Quant Factor: 10

    o Max Integration Time (Sec):   2

Figure 1: Filter combination for the Prostatype® RT-qPCR Kit on the LightCycler® 480 Instrument I.

8.1.2. Saving a New Detection Format on the LightCycler® 480 Instrument II

Note: This section describes the setup using a LightCycler® 480 Instrument II. The setup for a LightCycler® 480 Instrument I is described above.

When running the Prostatype® RT-qPCR the first time on a LightCycler® 480 Instrument II, it is necessary to set up a new detection format according to Figure 2.

- Start the software version 1.5.x.

- Click "Open Tools".

- In the tools window click "Detection Formats".

- Click "New" and give the new detection format a name, e.g. "Prostatype Channels".

- Mark the checkboxes for all of the following filter combinations:

    o Filter Combination 1:   533 – 580

    o Filter Combination 2:   533 – 640

    o Filter Combination 3:   465 – 510

    o Filter Combination 4:   618 – 660

- Change the name of the filter combinations as followed:

    o 533 – 580:   GAPDH

    o 533 – 640:   IGFBP3

    o 465 – 510:   F3

    o 618 – 660:   VGLL3

- Change the filter settings for every combination as followed:

    o Melt factor: 1

    o Quant Factor: 10
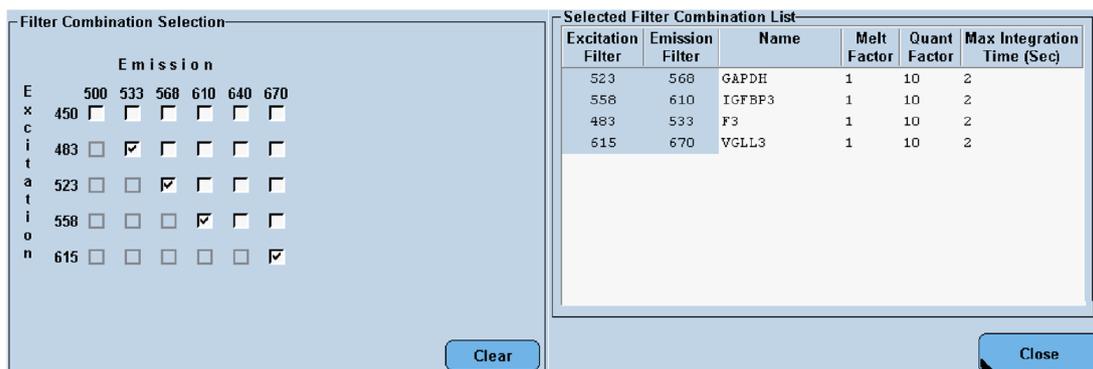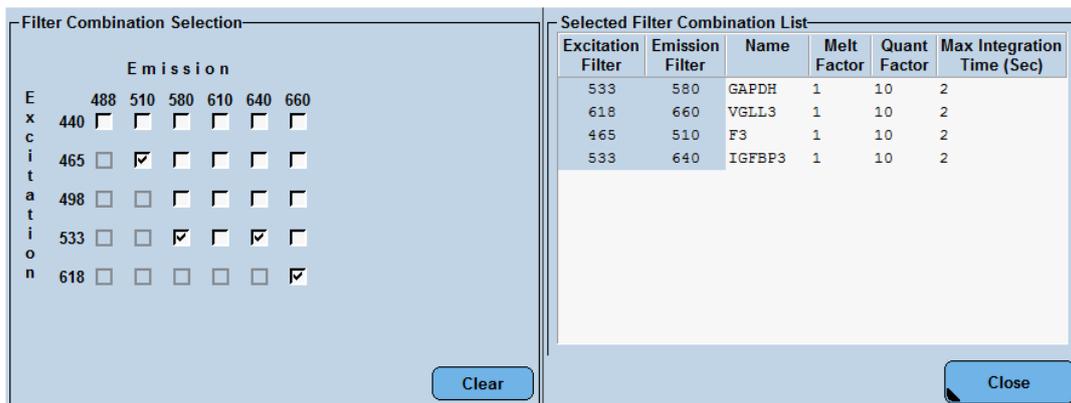
    o Max Integration Time (Sec):   2

Figure 2: Filter combination for the Prostatype® RT-qPCR on the LightCycler® 480 Instrument II.

8.2.   **Prostatype® RT-qPCR Setup**

Each box of Prostatype® RT-qPCR Kit contains sufficient reagents to perform expression analysis of up to four patient samples plus four controls.. Each RNA sample (patient RNA sample, or Prostatype Positive Control, or Prostatype Negative Control) must be tested in triplicate. Prostatype Positive Control and Prostatype Negative Control must be included in triplicate in each independent run.

Note: To obtain the optimal use of the reagents in the Prostatype® RT-qPCR Kit samples should be batched. If samples are tested individually this will use up more reagents and reduce the number of samples that can be tested with the Prostatype® RT-qPCR Kit.

Note: The provided reagents allow running up to three individual PCR runs. Store reagents at -25°C to -15°C between PCR runs.

Note: Brief centrifugation of micro tubes (stated as 'Briefly spin down the tubes') is required in several steps of this instruction to remove drops from the lid or to collect remaining liquid. It is recommended to centrifuge for 10 - 20 sec at 1,000 ± 150 rcf using a Bench-Top centrifuge.

Note: Gently vortexing of tubes and containers is required in several steps of this instruction to ensure homogeneous mixing of liquid. It is recommended to use a vortexer adjusted to medium speed for 15 to 20 sec. This step is critical for proper mixing of the reagents and for obtaining valid results.

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.

Important: A color compensation run needs to be done prior to the first Prostatype® RT-qPCR. An individual color compensation file is required for every LightCycler® 480 Instrument, where the Prostatype® RT-qPCR is performed. General Information about generating a color compensation file can be found in the LightCycler® 480 Instrument Operator's Manual.

8.2.1.   **Thawing of Components**

Thaw one tube Prostatype Master, Prostatype Manganese, Prostatype Positive Control and Prostatype Negative Control at room temperature (15 – 25 °C) for **25 to 35 min**.

Note: While the components are thawing, gently vortex 4 x 15-20 sec to ensure proper mixing.

8.2.2.   **Preparation of Master Mix**

Note: Ensure that the PCR preparation time from beginning of Mastermix preparation to starting the PCR plate does not exceed **90 min**.

Note: One Prostatype Positive Control and one Prostatype Negative Control must be included in each individual run.

Note: Each RNA sample (patient RNA sample, or Prostatype Positive Control, or Prostatype Negative Control) must be tested in triplicate.

- Gently vortex the Prostatype Master tube and the Prostatype Manganese tube. Briefly spin down.

- Transfer the corresponding volumes of Prostatype Master and Prostatype Manganese depending on the number of samples to be tested (number of patient samples + 2 controls) as indicated in Table 2 into a 2.0 mL reaction tube.

- Mix the Master Mix by gently vortexing.

- Briefly spin down the Master Mix to remove drops from the lid.

Note: Do not store Master Mix, use immediately. Refreeze unused Prostatype Master and Prostatype Manganese directly after usage. All components of the Prostatype® RT-qPCR Kit may be refrozen and thawed two times.

Table 2: Preparation of Master Mix.

Number of Samples	Number of Wells in PCR Plate	Volume of Prostatype Master	Volume of Prostatype Manganese
1 sample (+2 controls)	9 wells	132.3 µL	13.5 µL
2 samples (+2 controls)	12 wells	176.4 µL	18.0 µL
3 samples (+2 controls)	15 wells	220.5 µL	22.5 µL
4 sample (+2 controls)	18 wells	264.6 µL	27.0 µL

8.2.3. Plate Preparation

Note: For a higher number of samples to be tested a repeater pipette/dispenser is recommended for repetitive dispensing of the Master Mix.

Set up the PCR plate as follows. A plate layout as shown in Table 3 is recommended.

- Transfer 15 µL Master Mix each into the selected wells of a LightCycler® 480 Multiwell Plate 96 Reaction Plate.
- Gently vortex the patient RNA samples, the Prostatype Positive Control and the Prostatype Negative Control. Briefly spin down.

Note: Positive and Negative Control 4 x 15-20 sec to ensure proper mixing.

- Add 5 µL of the patient RNA samples, the Prostatype Positive Control and the Prostatype Negative Control to respective wells of the PCR plate.
- Ensure that all samples are tested in triplicate.
- Seal the PCR plate with LightCycler® 480 Sealing Foil.
- Briefly spin down the PCR plate with a plate centrifuge for 2 min at 2000 ± 100 rcf.
- Start PCR plate within **30 min** after plate preparation.

Note: Refreeze unused Prostatype Positive Control and Prostatype Negative Control directly after usage. All components of the Prostatype® RT-qPCR Kit may be refrozen and thawed two times.

Table 3: Recommended Plate Layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	PC	PC	NC	NC	NC	S1	S1	S1	S2	S2	S2
B	S3	S3	S3	S4	S4	S4	S5	S5	S5	S6	S6	S6
C	S7	S7	S7	S8	S8	S8	S9	S9	S9	S10	S10	S10
D	S11	S11	S11	S12	S12	S12	S13	S13	S13	S14	S14	S14
E	S15	S15	S15	S16	S16	S16						
F												
G												
H												

PC: Prostatype Positive Control, NC: Prostatype Negative Control, S: Patient Sample

8.2.4. Plate Loading

Note: Start the PCR plate within 30 min after finalization of the plate preparation.

Note: It is recommended to save a run template with the defined cycling settings in the LightCycler® 480 Software.

- Start the software version 1.5.x.
- Create a new experiment, click "New Experiment".
- Load the specified run template file or define the following experiment according to the PCR program parameters detailed in Table 4 and Figure 3 below.
- Select the detection format as set up in 8.1.1 or 8.1.2 (e.g. "Prostatype Channels").
- Click "Subset Editor" and generate a sample subset including all used wells as described in the manual of the LightCycler® 480 Instrument.
- Enter unique sample identifier to each of the three replicates of each sample.
- Save the experiment setup using a unique name.
- Open the tray.
- Place the PCR plate into the frame (position A1 goes to the upper left corner), ensure that the plate fits accurately in the frame. Close the tray.
- Click "Start Run" to start.

Table 4: Standard PCR Program for the LightCycler® 480 Instrument.

Program Parameter	RT-Step	Cycling		Cooling
Analysis Mode	None	Quantification mode		None
Cycles	1	40		1
Segment	1	1	2	1
Target [°C]	63	92	60	40
Hold [hh:mm:ss]	00:20:00	00:00:05	00:00:40	00:00:10
Ramp Rate [°C/s]*	1.6	1.6	1.6	1.6
Acquisition Mode	None	None	Single	None

* Select the detection format as created in section 8.1; activate the filter combinations for GAPDH, IGFBP3, F3 and VGLL3 and set the "Reaction Volume" to "20".

Figure 3: Screenshot from LightCycler® 480 software v1.5 showing the run protocol of the Prostatype® RT-qPCR Kit. A: Details of the RT-Step. B: Details of the Cycling Step. C: Details of the Cooling Step.

8.3. Analysis Settings on the LightCycler® 480 Instrument

8.3.1. General

For reliable quantification the Crossing Point ("Cp") of an amplification curve with the threshold shall be located in the early exponential phase of a PCR (exponential phase = straight segment of a PCR curve when displayed on a logarithmic scale).

It may be required to adjust the threshold level to guarantee reliable Cp values. To do so the horizontal threshold line in the Amplification Plot may be adjusted manually in compliance with the following rules (see Figure 4 below):

1. The threshold shall cross the PCR curves during the early exponential phase.
2. The threshold shall be located above the baseline of the PCR curves.
3. The threshold shall be within a defined upper/lower limit of the "STD Multiplier" as specified for each individual gene in section 8.3.2.

Each amplification signal should be visually inspected for irregular amplification. Signals crossing the threshold due to inconsistent data points (noise peaks) or linear curve shape should not be evaluated. In case of irregular amplification the affected well must be omitted from the analysis by removing the "Include" checkmark in the Sample Table.
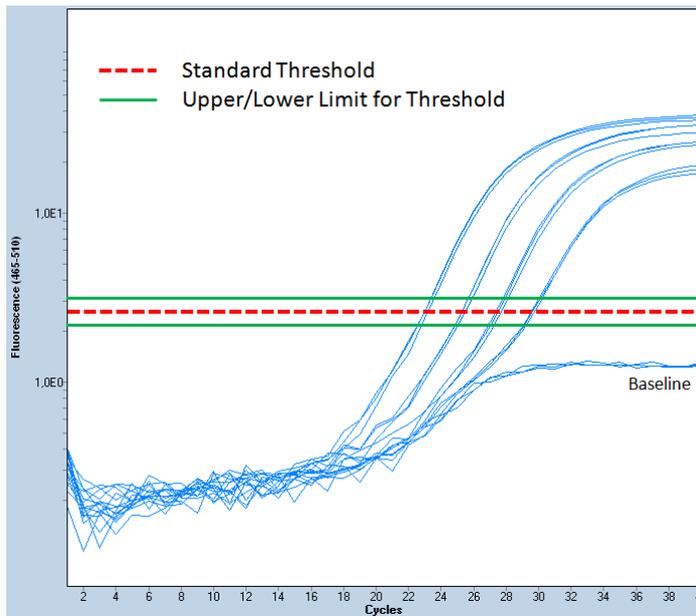
Figure 4: Screenshots of amplification curves using the LightCycler® 480 Instrument (logarithmic scale). The red dotted line shows the threshold using the standard "STD Multiplier" values as indicated in section 8.3.2. The threshold may be adjusted within the indicated upper and lower limit if required.

8.3.2. Analysis Procedure

Note: Perform data analysis only on used wells by generating a respective sample subset as described in the manual of the LightCycler® 480 Instrument.

- After the run has finished click "Analysis" on the LightCycler® 480 Basic Software Module bar opening the "Analysis Overview" window.
- Select "Abs Quant/Fit Points".
- In the "Create new analysis" window choose the respective subset containing all used wells and confirm.
- Activate color compensation by clicking the arrow button next to "Color Comp (Off)" than click "In Use" and select the respective color compensation file.
- Set "First Cycle" to "1" and "Last Cycle" to "40".
- Set the background (click blue "Background" button) to "6 - 11" by setting "Min Offset" to "5" and "Max Offset" to "10" in the "Cycle Range" window.

GAPDH:
- Activate the respective filter combination for GAPDH:
    - LightCycler® 480 I device: "Filter Comb 523 – 568"
    - LightCycler® 480 II device: "Filter Comb 533 – 580"
- Set the noise band to "Noise Band (STD Mult)" and set the "STD Multiplier" manually to "**50.0**" in the "Noise Band" window.
- If required the threshold level may be adjusted manually in compliance with the rules in section 8.3.1. Ensure that the displayed "STD Multiplier" value is between **40.0** and **60.0.**
- Ensure the Threshold value in the "Analysis" window equals the Noise Band value.
- Ensure the number of Fit Points is "2".
- Click "Calculate".
- GAPDH Crossing Point ("Cp") for each sample is displayed in the Sample Table.
- Export the Cp values by clicking with the right mouse button within the Sample Table. Choose "Export". Save the file under the experiment name containing the gene name "GAPDH".

IGFBP3:

- Activate the respective filter combination for IGFBP3:
  - LightCycler® 480 I device: "Filter Comb 558 – 610"
  - LightCycler® 480 II device: "Filter Comb 533 – 640"
- Keep all settings as adjusted before with one exception:
- Set the STD Multiplier manually to "**35.0**" in the "Noise Band" window.
- If required the threshold level may be adjusted manually in compliance with the rules in section 8.3.1. Ensure that the displayed "STD Multiplier" value is between **28.0** and **42.0.**
- Ensure the Threshold value in the "Analysis" window equals the Noise Band value.
- Ensure the number of Fit Points is "2".
- Click "Calculate".
- IGFBP3 Crossing Point ("Cp") for each sample is displayed in the Sample Table.
- Export the Cp values by clicking with the right mouse button within the Sample Table. Choose "Export". Save the file under the experiment name containing the gene name "IGFBP3".

F3:

- Activate the respective filter combination for F3:
  - LightCycler® 480 I device: "Filter Comb 483 – 533"
  - LightCycler® 480 II device: "Filter Comb 465 – 510"
- Keep all settings as adjusted before with one exception:
- Set the STD Multiplier manually to "**80.0**" in the "Noise Band" window.
- If required the threshold level may be adjusted manually in compliance with the rules in section 8.3.1. Ensure that the displayed "STD Multiplier" value is between **64.0** and **96.0.**
- Ensure the Threshold value in the "Analysis" window equals the Noise Band value.
- Ensure the number of Fit Points is "2".
- Click "Calculate".
- F3 Crossing Point ("Cp") for each sample is displayed in the Sample Table.
- Export the Cp values by clicking with the right mouse button within the Sample Table. Choose "Export". Save the file under the experiment name containing the gene name "F3".

VGLL3:

- Activate the respective filter combination for VGLL3:
  - LightCycler® 480 I device: "Filter Comb 615 – 670"
  - LightCycler® 480 II device: "Filter Comb 618 – 660"
- Keep all settings as adjusted before with one exception:
- Set the STD Multiplier manually to "**27.5**" in the "Noise Band" window.
- If required the threshold level may be adjusted manually in compliance with the rules in section 8.3.1. Ensure that the displayed "STD Multiplier" value is between **22.0** and **33.0.**
- Ensure the Threshold value in the "Analysis" window equals the Noise Band value.
- Ensure the number of Fit Points is "2".
- Click "Calculate".
- VGLL3 Crossing Point ("Cp") for each sample is displayed in the Sample Table.
- Export the Cp values by clicking with the right mouse button within the Sample Table. Choose "Export". Save the file under the experiment name containing the gene name "VGLL3".

8.4.   **Validity of Prostatype® RT-qPCR Runs**

The Prostatype® RT-qPCR run is considered valid if criteria set forth for the Prostatype Positive Control and the Prostatype Negative Control in the following tables are met.

If either the Prostatype Positive Control or Prostatype Negative Control (or both) is invalid, the data for patient samples cannot be interpreted. Testing must be repeated for all patient samples included in this run.

Note: Use the median Cp values/Delta Cp values out of the three replicates received for each sample for the following calculations. Median refers to the value in the middle when arranging all values from the lowest to the highest (see example in Table 9 on page 19). When only two values are available the mean/average of those two values is used. For either the controls or patient samples three PCR replicates must be performed and evaluated. In case of an irregular curve, one of the three results might be omitted, such that at least two replicates remain for evaluation.

8.4.1.   **Evaluation of the Prostatype Positive Control**

- Check if results of at least two PCR replicates of the Positive Control are available.
- If no Cp value is reported for a gene use the number "**38**" as a "simulated Cp" in order to calculate a Delta Cp value.
- Identify the median Cp value for GAPDH.
- Check if the validity limit for GAPDH is met:

Table 5: GAPDH Cp Validity Limit of the Prostatype Positive Control.

Gene	Cp Validity Limit
GAPDH	20.00 ≤ Median Cp ≤ 22.00

- Calculate the delta Cp values for IGFBP3, F3 and VGLL3 for every well according to the following formula:

$$Delta\ Cp\ [gene] = Cp\ value\ [gene] - Cp\ value\ [GAPDH]$$

(Wherein gene is IGFBP3, F3 or VGLL3)

- Identify the median Delta Cp values for IGFBP3, F3 and VGLL3.
- Check if the validity limits for IGFBP3, F3 and VGLL3 are met:

Table 6: Delta Cp Validity Limits of the Prostatype Positive Control.

Gene	Delta Cp Validity Limits
IGFBP3	1.90 ≤ Median Delta Cp ≤ 3.90
F3	0.90 ≤ Median Delta Cp ≤ 2.90
VGLL3	2.80 ≤ Median Delta Cp ≤ 4.80

8.4.2. **Evaluation of the Prostatype Negative Control**

- Check if results of at least two PCR replicates of the Negative Control are available
- Identify the median Cp value for GAPDH.
- Check if the validity limits for the Prostatype Negative Control are met:

Table 7: Validity Limits of the Prostatype Negative Control.

Gene	Cp Validity Limits
GAPDH	29.00 ≤ Median Cp ≤ 31.00
IGFBP3, F3, VGLL3	no Cp

8.5. **Validity of Individual Patient Sample Wells**

Only if the external controls are valid according to section 8.4, the results of the internal control for each patient sample well can be evaluated. An individual well is valid, if the criterion for the internal control set forth in Table 8 is met.

- Check if the validity limit for individual patient sample wells is met:

Table 8: Validity Limit of Patient Samples.

Gene	Cp Validity Limit
GAPDH	Median Cp ≤ 28.00

- Ensure that at least two patient sample wells met this criterion.

If a patient sample well exceeds the validity limit, the data for that patient sample well cannot be interpreted. At least two valid patient sample wells are required for further data analysis. Otherwise the patient sample result is invalid and testing of the patient sample must be repeated.

9. Data Analysis for Patient Samples

If all requirements outlined in section 8.4 and 8.5 are met, the results of an individual sample can be calculated and reported. The mRNA expression levels of the three genes IGFBP3, F3, and VGLL3, relative to the expression level of gene GAPDH will be indicated by the median delta Cp determined as outlined below. Table 9 shows an example how to calculate the Median Delta Cp for one gene.

- Calculate the delta Cp values for IGFBP3, F3 and VGLL3 for every well according to the following formula:

$$Delta\ Cp\ [gene] = Cp\ value\ [gene] - Cp\ value\ [GAPDH]$$

(Wherein gene is IGFBP3, F3 or VGLL3)

- If no Cp value is reported the gene was expressed below the detection limit. In order to calculate a Delta Cp, use the number "**38**" as a "simulated Cp".
- Identify the Median Delta Cp values for IGFBP3, F3 and VGLL3.

Table 9: Example for the calculation of the Median Delta Cp for VGLL3 for one sample. A similar calculation must be performed for the genes F3 and IGFBP3.

PCR Replicate	Cp Value GAPDH	Cp Value VGLL3	Calculated Delta Cp	Median Delta Cp VGLL3
Well 1	20.25	25.36	Delta Cp_1 = 25.36 – 20.25 = 5.11	Median {5.11; 4.99; 18.02} = 5.11
Well 2	20.15	25.14	Delta Cp_2 = 25.14 – 20.15 = 4.99	
Well 3	19.98	*no Cp*	Delta Cp_3 = 38 – 19.98 = 18.02	

10. Quality Control

10.1. External Controls

The Prostatype® RT-qPCR Kit contains Prostatype Positive Control and Prostatype Negative Control. These controls must be included in triplicate in each individual PCR run to ensure validity of test results. The provided material is sufficient to perform three individual PCR runs. The Prostatype Positive Control and Negative Control values must be within the validity limits (see Table 5, or Table 6, or Table 7). If a control is out of its specified range, the associated test results are invalid, must not be reported and testing must be repeated.

10.2. Internal Controls

For every patient sample the expression level of the reference gene GAPDH is determined and used to evaluate the sample validity and integrity. The GAPDH level must be above a specific value to guarantee a robust measurement of the target genes (see Table 8). Furthermore the internal control is used to verify that enough amplifiable RNA was extracted during the RNA extraction step.

11. Limitations of the Procedure

The product shall be used by personnel experienced in molecular biological techniques, specifically real-time PCR. General recommendations on the organization and procedures of the laboratory shall be obeyed in order to prevent DNA contamination.

All results from Prostatype® RT-qPCR Kit must be evaluated in conjunction with patient clinical history and other available information.

12. Performance Characteristics

12.1. RNA Input

Compatibility of the Prostatype® RT-qPCR Kit with use of total RNA extracted from paraffin embedded (FFPE) tissue from human prostate core needle biopsy as input was validated by assessment of 16 individual clinical specimen. Total RNA was extracted from unstained prostate core needle biopsies containing at least 50% cancer cells. The specimen collection was done according to following criteria: area ≥ 30 mm², thickness 10 µm. RNA extraction kit as recommended in section 8 was used. Results of Prostatype® RT-qPCR Kit were valid for around 95 % of the specimen.

12.2. Linearity/Precision/Limit of Quantitation

Linearity was evaluated by testing a dilution series of synthetic target RNA covering a range from 2.5×10^6 to 38 molecules/µL. Testing comprised three different Prostatype® RT-qPCR Kit Lots. For GAPDH and F3 assay the linear range covers the concentrations from 2.5×10^6 to 38 copies/µL. For IGFBP3 linearity is given down to 153 copies/µL, and for VGLL3 down to 305 copies/µL. Within the reported range the

assays present linearity with a regression coefficient $R^2 > 0.99$ (see Figure 5). For the linear range slopes have been observed between -3.12 and -3.29. Standard deviation (SD) and coefficient of variation (CV) were calculated within the defined range of linearity. The largest SDs of 0.4 Cp were detected for IGFBP3 at a concentration of 153 copies/μL and for VGLL3 at a concentration of 305 copies/μL. The largest CV was 1.6 % for VGLL3 at a concentration of 3.9×10^4 copies/μL. The Limit of Quantitation of the Prostatype® RT-qPCR is represented by the lower level of the linear range of the respective assay stated above.
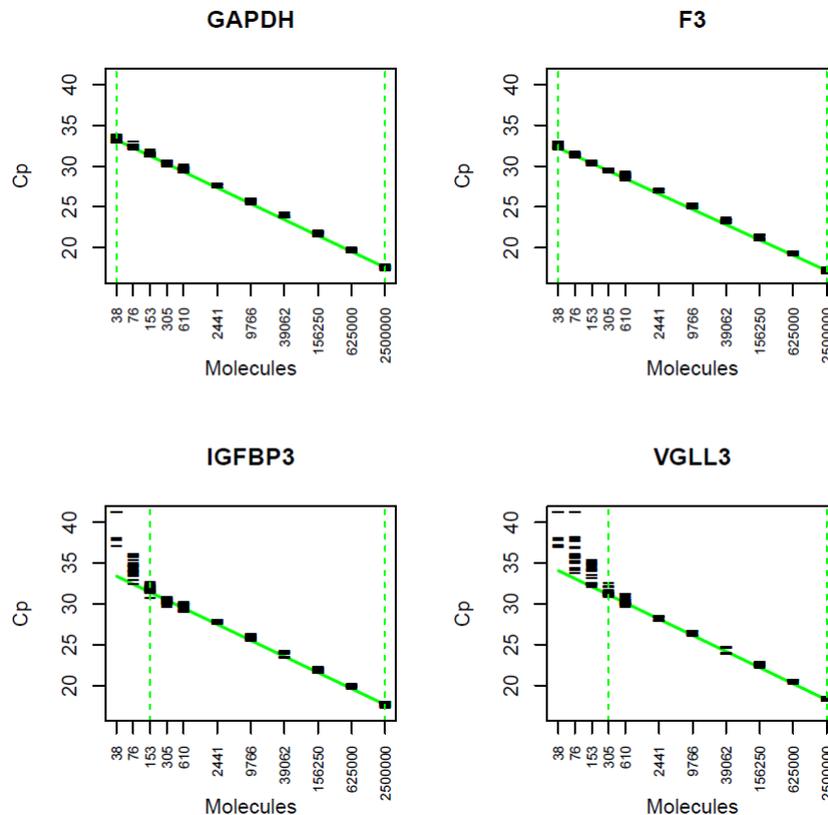

Figure 5: Linearity of the four genes contained in the Prostatype® RT-qPCR. The observed Cp values (black lines) are plotted for the 11 tested target concentrations ranging from 38 to 2.5×10^6 molecules per μL. Each target concentration was assessed by at least 15 PCR replicates. The fitted linear regression is shown as solid green line.

12.3.   Limit of Detection

The limit of detection was evaluated by repeated analysis of synthetic target RNA covering a range from 2.5×10^6 to 38 molecules/μL. The four lowest target concentrations were run in 24 replicates involving three different Prostatype® RT-qPCR Kit lots. The lowest concentrations showing amplification curves for all replicates (100% detection) were as follows: GAPDH: 38 copies/μL, F3: 38 copies/μL, IGFBP3: 76 copies/μL, VGLL3: 153 copies/μL.

12.4.   Relative Sensitivity

Relative sensitivity was evaluated by testing samples containing different proportions of IGFBP3, F3 and VGLL3 target in an equal amount of GAPDH background. Following samples were tested in 6 replicates per sample with different Prostatype® RT-qPCR Kit lots: 800 copies/μL, 400 copies/μL and 200 copies/μL of IGFBP3, F3 and VGLL3 target in an equal amount of 4.0×10^4 copies/μL GAPDH background resulting in proportions of 1:50, 1:100 and 1:200. For each of the four assays, all replicates of the samples (18 PCR wells) showed an amplification curve (100% detection), confirming a relative sensitivity for the three target assays below 1%.

12.5. Limit of Blank

Twenty-one (21) PCR wells containing no RNA targets were tested with different Prostatype® RT-qPCR Kit lots to evaluate the limit of blank. The three target genes (IGFBP3, F3 and VGLL3) showed no amplification curve for all 21 wells. Therefore, the limit of blank corresponds to the maximal observable Cp value of 38 for those genes. For GAPDH 16 of the 21 PCR wells showed an amplification curve with Cp values between 35.2 and 38.0. The limit of blank for GAPDH was estimated by the 95% quantile and equals Cp 35.2.

12.6. Reproducibility

Reproducibility was evaluated by repeated testing of aliquots from eight RNA pools extracted from formalin fixed paraffin-embedded (FFPE) human prostate core needle biopsy containing cancer cells. Testing was performed by four operators at three different testing laboratories across Europe. Each testing site prepared 12 independent PCR plates involving three different Prostatype® RT-qPCR Kit lots. Results of the evaluation are detailed in

Table 10.

Table 10: Results of reproducibility testing. Mean Cp and related standard deviation (SD) and coefficient of variation (CV) are listed for the four genes. All eight RNA pools were measured in 36 independent PCR runs.

RNA Sample	GAPDH			F3			VGLL3			IGFBP3		
	Mean Cp	SD (Cp)	CV (%)	Mean Cp	SD (Cp)	CV (%)	Mean Cp	SD (Cp)	CV (%)	Mean Cp	SD (Cp)	CV (%)
Pool 1	23.9	0.150	0.626	26.2	0.295	1.126	26.4	0.316	1.198	27.2	0.327	1.205
Pool 2	25.4	0.189	0.742	27.6	0.302	1.096	28.4	0.388	1.366	27.6	0.315	1.140
Pool 3	24.4	0.208	0.856	26.2	0.307	1.172	28.9	0.474	1.639	26.5	0.292	1.100
Pool 4	24.6	0.267	1.086	25.6	0.288	1.122	28.1	0.459	1.632	26.5	0.299	1.131
Pool 5	23.4	0.203	0.867	25.3	0.300	1.184	27.1	0.357	1.318	25.4	0.258	1.019
Pool 6	24.2	0.181	0.749	26.5	0.299	1.126	29.5	0.609	1.060	26.4	0.265	1.002
Pool 7	24.0	0.165	0.685	26.2	0.277	1.059	28.8	0.457	1.586	26.1	0.297	1.141
Pool 8	23.6	0.186	0.791	25.1	0.228	0.909	28.2	0.408	1.447	25.7	0.296	1.154

12.7. Interference

Aliquots from three RNA pools unspiked and spiked with potentially interfering substances were processed. No interference was observed with elevated levels of Hematoxylin/Eosin (HE, 0.01% v/v), Xylol/Xylene (0.01% v/v), DNase I (0.06 U/µL), Human genomic DNA (20 ng/µL), Guanidinium Chloride (5 mM), Ethanol (1% v/v) and EDTA (1mM).

13.   Meaning of Symbols

🕮		Consult Instructions for Use
REF		Catalogue Number
IVD		*In Vitro* Diagnostic Medical Device
LOT		Lot Number
⧗		Expiration Date
🏭		Manufacturer
🌡		Temperature Limitation
∑		Sufficient For
▦		Barcode

14. 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.

15. Contact Information

The Prostatype® RT-qPCR kit is manufactured by:

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