May 2020

AdnaTest ProstateCancerPanel AR-V7 Handbook

For enrichment of tumor cells from whole blood in cancer research and for detection of AR-V7 expression in enriched tumor cells

For molecular biology applications

REF 396132

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Sample to Insight

QIAGEN

Contents

Kit Contents

Box 1. AdnaTest ProstateCancerSelect

AdnaTest ProstateCancerPanel AR-V7	**(12)**
Catalog no.	**396132**
Number of tests	**12**
Collection Tubes (15 ml)	3 x 5
Collection Tubes (1.5 ml)	24
ProstateSelect Beads	1.2 ml
AdnaTest Lysis/Binding Buffer	2 x 2 ml
Quick-Start Protocol	1

Box 2. AdnaTest RNA Reagent

AdnaTest ProstateCancerPanel AR-V7	**(12)**
Catalog no.	**396132**
Number of tests	**12**
AdnaTest Lysis/Binding Buffer	2 ml
Oligo(dT)$_{25}$ Beads	355 µl
RNA Purification Buffer A	4 ml
RNA Purification Buffer B	4 ml
Tris·Cl Buffer	2 ml

Box 3. AdnaTest ProstateCancerPanel AR-V7

AdnaTest ProstateCancerPanel AR-V7	**(12)**
Catalog no.	**396132**
Number of tests	**12**
AdnaPanel PrimerMix PreAmp AR-V7	100 µl
AdnaPanel PrimerMix CD45	96 µl
AdnaPanel PrimerMix GAPDH	96 µl
AdnaPanel PrimerMix PSA	96 µl
AdnaPanel PrimerMix PSMA	96 µl
AdnaPanel PrimerMix AR	96 µl
AdnaPanel PrimerMix AR-V7	96 µl
AdnaPanel PrimerMix IC	24 µl
AdnaPanel PrimerMix Inhibition Control	96 µl
AdnaPanel Positive Control AR-V7	144 µl
AdnaPanel Internal Control	24 µl
AdnaPanel Inhibition Control	32 µl
Quick-Start Protocol	1

The AdnaTest ProstateCancerPanel AR-V7 reagents are sufficient to analyze 12 blood samples and 4 PCR controls.

Abbreviations

Abbreviation	Definition	Abbreviation	Definition
AdnaMag-L	Magnetic particle concentrator (-large)	GAPDH	Glycerinaldehyde-3-phosphat-dehydrogenase
AdnaMag-S	Magnetic particle concentrator (-small)	kb	Kilobases
AR	Androgen receptor	mRNA	Messenger ribonucleic acid
AR-V7	Androgen receptor splice variant 7	PCR	Polymerase chain reaction
bp	Base pairs	qRT-PCR	Quantitative real-time polymerase chain reaction
C+	Positive control	PSA	Prostate-specific antigen
C–	Negative control	PSMA	Prostate-specific membrane antigen
CD45	Protein tyrosine phosphatase, receptor type, C	RNase	Ribonuclease
cDNA	Complementary deoxyribonucleic acid	rpm	Revolutions per minute
DNA	Deoxyribonucleic acid	RT	Reverse transcription
dNTPs	Deoxynucleotide triphosphates		

Symbols

Use by

Temperature limitation

Catalog number

Manufacturer

Shipping and Storage

The AdnaTest ProstateCancerPanel AR-V7 system is delivered in 3 boxes. The AdnaTest ProstateCancerSelect (Box 1) and the AdnaTest RNA Reagent (Box 2) must be stored at 2–8°C. The components must not be used beyond the expiration date.

AdnaTest ProstateCancerPanel AR-V7 (Box 3), containing the AdnaPanel PrimerMixes and AdnaPanel Controls, must be stored in a constant-temperature freezer at –30 to –15°C. The components must not be used beyond the expiration date.

Intended Use

AdnaTest ProstateCancerPanel AR-V7 is for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of these products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of AdnaTest Prostate ProstateCancerPanel AR-V7 is tested against predetermined specifications to ensure consistent product quality.

Introduction

The AdnaTest ProstateCancerSelect is used for enriching CTCs from whole blood, while the AdnaTest ProstateCancerPanel AR-V7 is used for molecular characterization by analyzing gene expression of AR-V7 and further prostate-cancer–associated genes. The specificity of the detection is at least 90%. In spiking experiments, 5 tumor cells in 5 ml of whole blood are detected at a recovery rate of at least 90%.

Successful CTC detection is based on the combination of combinations principle (COCP). Each AdnaTest has a unique combination of tumor-associated markers and an optimized combination of antibodies for cell selection. By combining a highly specific immunomagnetic cell selection system using an optimized antibody combination, with highly sensitive qRT-PCR technology using a combination of mRNA tumor markers, very high degrees of specificity and sensitivity can be expected. The AdnaTest uses a 2-step process (select and panel) to generate results within 6 hours.

AdnaTest ProstateCancerSelect

AdnaTest ProstateCancerSelect enables the immunomagnetic enrichment of tumor cells via epithelial and tumor-associated antigens. Antibodies against epithelial and tumor-associated antigens are conjugated to magnetic beads for labeling of tumor cells in whole blood. Labeled cells are extracted by a magnetic particle concentrator (AdnaMag-L and AdnaMag-S) and are subsequently lysed (Figure 1, page 8, and Figure 2, page 9). The cell lysate is used for further analysis with AdnaTest ProstateCancerPanel AR-V7.

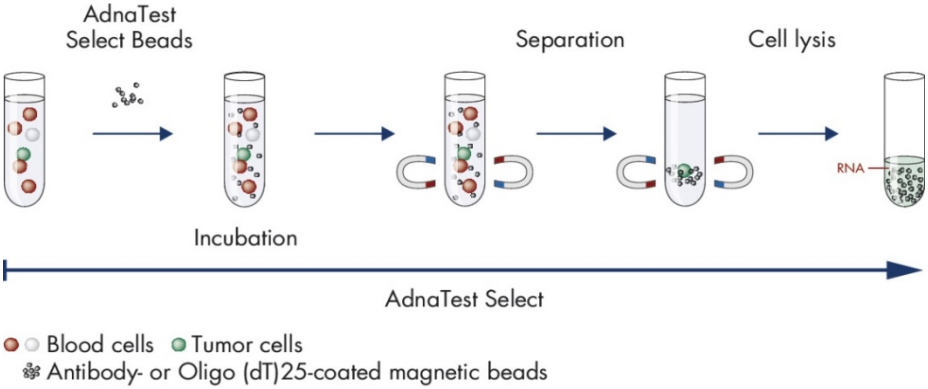

Figure 1. AdnaTest ProstateCancerSelect: Immunomagnetic cell selection with multiple tumor-associated antibodies. In the first step, the CTCs in the blood are enriched (AdnaTest Select). This is achieved using antibody-coated magnetic particles (beads). Several antibodies are used, which bind with high specificity and affinity to the corresponding cancer cells. The enriched cells are lysed and subsequently purified several times to extract mRNA.

AdnaTest ProstateCancerPanel AR-V7

AdnaTest ProstateCancerPanel AR-V7 contains Oligo(dT)$_{25}$ Beads for the isolation of mRNA from the lysate of enriched tumor cells. Reverse transcription results in cDNA, which is subsequently used as template for preamplification and tumor-cell detection and characterization by qRT-PCR. The AdnaPanel PrimerMixes allow amplification of 4 tumor-associated antigens and 2 control genes. Furthermore, the kit contains PrimerMixes for amplification of an internal control and an inhibition control.

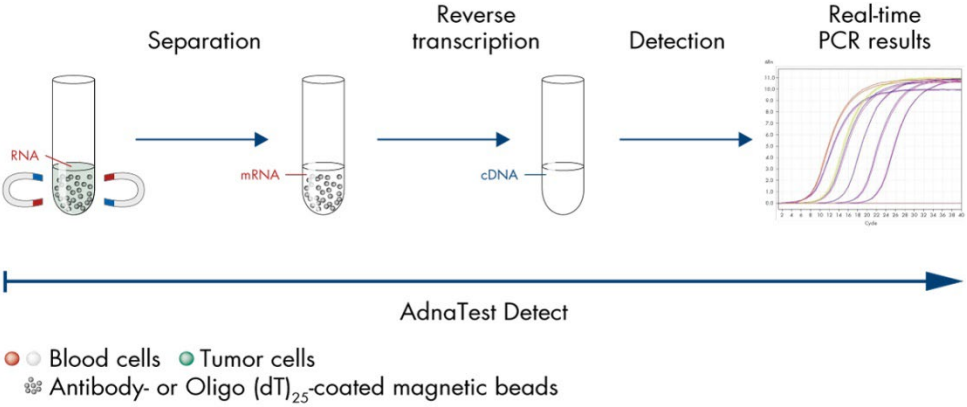

Figure 2. AdnaTest ProstateCancerPanel AR-V7: qRT-PCR of AR-V7 and various prostate cancer associated tumor markers. In a second step, the enriched cells are examined by qRT-PCR for AR-V7 expression and further tumor associated expression patterns. The mRNA strands are reverse transcribed into cDNA. Subsequently, several associated tumor markers can be analyzed using qRT-PCR.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

AdnaTest ProstateCancerSelect

Equipment

- Tube rotator for 15 ml and 1.5 ml tubes (e.g., ELMI Ltd., cat. no. IMIX-03)
- Magnetic particle concentrators
  - AdnaMag-L (cat. no. 399921)
  - AdnaMag-S (cat. no. 399911)

Consumables

- AdnaTube Tubes (cat. no. 399932), when working with BD Vacutainer® ACD-A Tubes (Becton Dickinson GmbH cat. no. 366645 [EU]; 364606 [US])
- Sterile, RNase-free 10 ml glass or plastic pipettes and pipettor
- Sterile, RNase-free 1.5 ml reaction tubes (e.g., Sarstedt, cat. no. 72.690)
- Pipettes and RNase-free pipette tips with aerosol barrier, suitable for pipetting volumes from 100 µl to 1000 µl

Reagents

- Phosphate buffered saline (PBS), pH 7.0–7.3 (e.g., Fisher, cat. no. VX14190169, D-PBS)

AdnaTest ProstateCancerPanel AR-V7

Equipment

- Tube rotator for 1.5 ml tubes
- Magnetic particle concentrator AdnaMag-S
- Thermal block or water bath (65°C)
- Thermal cycler with a heated lid and a heating rate of 2°C/s.
- Quantitative real-time PCR thermocycler

Consumables

- Sterile, RNase-free thin-wall 0.2 ml PCR tubes
- Sterile, RNase-free 1.5 ml reaction tubes
- Pipettes and RNase-free pipette tips with aerosol barrier, suitable for pipetting volumes from 1 µl to 200 µl

Reagents

- Sensiscript® RT Kit (cat. no. 205211; 50 reactions)
- Recombinant RNasin®, RNase-inhibitor, 2500 U (Promega, cat. no. N2511)
- QIAGEN Multiplex PCR Plus Kit (cat. no. 206152)
- miRCURY® LNA® SYBR® Green PCR Kit (cat. no. 339346)
- Crushed ice

Important Notes

Sample preparation

- Blood samples must be taken before the application of therapeutic substances. **Important**: Do not use the AdnaTest ProstateCancerSelect earlier than 7 days after the last therapeutic intervention.
- Blood collection: If sample transportation is less than 4 h, use tubes containing EDTA as anticoagulant (e.g., S-Monovette® K3 EDTA, Sarstedt cat. no. 01.1605.001) to draw at least 7.5 ml whole blood.
- If sample transportation is longer than 4 h, use BD Vacutainer ACD-A Tubes to draw at least 8.5 ml whole blood. Before further processing using the AdnaTest, 5 ml ACD-A blood must be transferred into an AdnaTube.
- Blood must be immediately stored at 2–8°C.
- Samples should be processed as soon as possible, not later than 4 h after blood withdrawal (when using standard EDTA tubes) or within 30 h (when using BD Vacutainer blood collection tubes in combination with AdnaTubes).
- The blood sample must not be hemolyzed.

Handling

- ProstateSelect Beads contain sodium azide as a preservative. Sodium azide is cytotoxic and must, therefore, be removed before using the beads. (See “Protocol: Enrichment of Tumor Cells Using AdnaTest ProstateCancerSelect“, page 14.)
- All components and additional reagents provided by other suppliers must be stored according to their instructions. Observe the safety information of the respective manufacturers.
- Wear protective gloves to avoid contamination with DNA, RNA, and RNases.

- The test must be performed in the denoted sequence and must comply with all specifications stated with respect to incubation times and incubation temperatures.
- Discard samples if the selection beads agglutinate during cell enrichment.
- Perform sample processing (including reverse transcription and subsequent qPCR analysis) in different rooms, if possible, to avoid cross-contamination.
- The use of products from suppliers other than those suggested may adversely affect results.
- Observe the safety and hygiene regulations of the laboratory (e.g., wear lab coats, protective goggles, and gloves).

Protocol: Enrichment of Tumor Cells Using AdnaTest ProstateCancerSelect

Important points before starting

- Before beginning the procedure, read "Important Notes" (page 12).
- Sodium azide must be removed from the ProstateSelect Beads by washing before use, as described in "Preparation of the ProstateSelect Beads", page 14.
- Use the supplied 1.5 ml collection tubes only for the protocol step indicated.

Things to do before starting

- Ensure that the AdnaTest Lysis/Binding Buffer is equilibrated to room temperature (15–25°C). If precipitate is observed, equilibrate the reagent to room temperature and mix until the precipitate is completely dissolved.

Preparation of the ProstateSelect Beads

1. Resuspend the ProstateSelect Beads thoroughly by pipetting.

   Important: Do not vortex.

2. Calculate the volume of ProstateSelect Beads required for all samples to be processed (100 µl per sample), and transfer the calculated volume into a 1.5 ml reaction tube (not provided).

   If more than 10 samples are processed, use additional 1.5 ml reaction tubes (not provided).

3. Place the tube into the AdnaMag-S rack.
4. After 1 min remove the supernatant with a pipette.

   Important: Do not touch the beads when removing the supernatant.

5. Wash the beads as follows:
   5a. Remove the magnet slider from the AdnaMag-S rack.
   5b. Add 1 ml PBS and resuspend the beads by repeated pipetting.
   5c. Place the magnet slider into the AdnaMag-S rack.
   5d. After 1 min, remove the supernatant completely with a pipette.
   5e. Repeat steps 5a–5d twice (3 washes in total).
6. Remove the tube from the AdnaMag-S rack, and resuspend the beads in PBS to the original volume (100 µl per sample). Proceed with "Selection of tumor cells" below.

Selection of tumor cells

1. If using standard EDTA tubes, pipet 5 ml of a blood sample into a 15 ml collection tube. Conversely, if using ACD-A blood in a BD Vacutainer ACD-A Tube, transfer 5 ml blood into an AdnaTube.

   Note: Use of AdnaTubes is mandatory when using BD Vacutainer ACD-A Tubes.
2. Thoroughly resuspend the ProstateSelect Beads (prepared in step 6) by pipetting, and add 100 µl of these beads to each blood sample.
3. Rotate tubes slowly (approximately 5 rpm) for 30 min at room temperature on a device that allows both tilting and rotation.
4. Place tubes into the AdnaMag-L rack without the magnet slider. Swing the AdnaMag-L rack downward to release blood droplets captured in the cap.
5. Insert the magnet slider and incubate the tubes in the AdnaMag-L rack for 3 min at room temperature.
6. Remove blood supernatant completely with a 10 ml pipette without touching the beads.

   Important: Do not touch the beads when removing the supernatant.

7. Wash the bead/cell complexes as follows:
   7a. Remove the magnet slider from the AdnaMag-L rack.
   7b. Add 5 ml PBS. Close the tubes and shake the AdnaMag-L rack gently back and forth 5 times to resuspend the magnetic bead/cell complexes.
   7c. Swing the AdnaMag-L rack with the tubes downward twice to release droplets captured in the cap.
   7d. Place magnet slider into the AdnaMag-L rack. Incubate for 1 min at room temperature.
   7e. Remove supernatant completely with a pipette.
   7f. Repeat steps 7a–7e twice (3 washes in total).
8. Remove the magnet slider from the AdnaMag-L rack.
9. Resuspend the magnetic bead/cell complexes in 1 ml PBS and transfer each sample into a 1.5 ml reaction tube (not provided).
10. Place reaction tubes into the AdnaMag-S rack with an inserted magnet slider.

    Note: The magnet slider of the AdnaMag-S rack can be inserted in 2 positions. Always insert the slider with the white plastic film facing forward, to make sure that the magnets are next to the reaction tubes.
11. After 1 min, remove the supernatant completely with a pipette to optimize the cell lysis.
12. Remove the magnet slider from the AdnaMag-S rack.
13. Add 200 µl AdnaTest Lysis/Binding Buffer (equilibrated to room temperature) to each reaction tube. Resuspend by pipetting at least 5 times.
14. Insert the magnet slider into the AdnaMag-S rack, and incubate for 1 min.
15. Transfer supernatant (cell lysate) into new 1.5 ml reaction tubes (provided).
16. Discard the tubes that contain the beads.
17. Proceed immediately with mRNA isolation (see “Protocol: Isolation of mRNA and Reverse Transcription“, page 17) or store the cell lysates at –30 to –15°C for a maximum of 2 weeks.

Protocol: Isolation of mRNA and Reverse Transcription

This protocol describes the isolation of mRNA and reverse transcription into cDNA.

Important points before starting

- Before beginning the procedure, read “Important Notes” (page 12).
- Use the supplied 1.5 ml collection tubes only for the protocol step indicated.

Things to do before starting

- Ensure that the AdnaTest Lysis/Binding Buffer is equilibrated to room temperature. If precipitate is observed, equilibrate the reagent to room temperature and mix until the precipitate is completely dissolved.
- Equilibrate RNA Purification Buffer A and RNA Purification Buffer B to room temperature. Place Tris·Cl Buffer on ice.
- Thaw 10x Buffer RT and dNTPs (from the Sensiscript RT Kit) at room temperature. Mix by vortexing. Centrifuge briefly and store on ice. Thaw RNase-free water (part of the Sensiscript RT Kit).
- Adjust a thermal block or water bath to 65°C.

Preparation of cell lysates, Inhibition Control sample, and Oligo(dT)$_{25}$ Beads

1. Add 2 µl AdnaPanel Inhibition Control to each sample (200 µl lysate from the AdnaTest ProstateCancerSelect enrichment of tumor cells) and keep on ice.
2. For a nonsample reference, pipet 200 µl AdnaTest Lysis/Binding Buffer into a new reaction tube and add 2 µl AdnaPanel Inhibition Control and keep on ice. Process this Inhibition Control sample as a cell lysate sample.

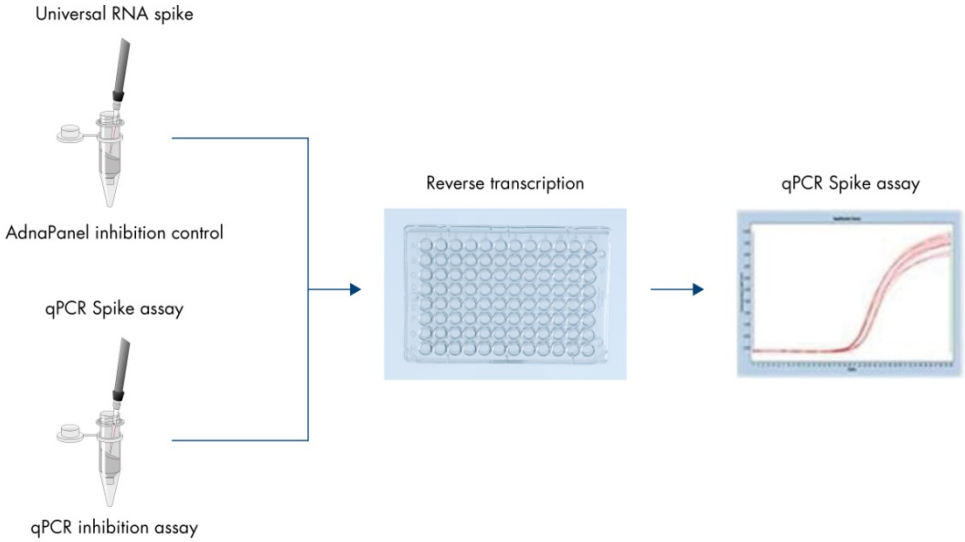

Figure 3. Preparation of AdnaPanel controls.

3. Resuspend the Oligo(dT)$_{25}$ Beads thoroughly by pipetting before use.

   Important: Do not vortex.
4. Calculate the volume of the beads required for all samples to be processed (20 µl per sample, plus 10%) and transfer the calculated volume into an RNase-free 1.5 ml reaction tube (not provided).

5. Place the tube into the AdnaMag-S rack.

   Note: The magnet slider of the AdnaMag-S rack can be inserted in 2 positions. Always insert the slider with the white plastic film facing forward, to make sure that the magnets are next to the reaction tubes.

6. After 1 min, remove the supernatant with a pipette.
7. Wash the beads as follows:
   7a. Remove the magnet slider from the AdnaMag-S rack.
   7b. Add the original volume (step 4, page 18) AdnaTest Lysis/Binding Buffer, and resuspend the beads by repeated pipetting. Resuspend gently to avoid foaming.
   7c. Insert the magnet slider into the AdnaMag-S rack.
   7d. After 1 min, remove the supernatant completely.
   7e. Repeat steps 7a–7d once (2 washes in total).
8. Remove the tube from the AdnaMag-S rack, and resuspend the beads in AdnaTest Lysis/Binding Buffer to the original volume (step 4, page 18). Proceed with "mRNA isolation" below.

mRNA isolation

1. Add 20 µl of Oligo(dT)$_{25}$ Beads (step 8, above) to each tube containing cell lysate (from step 15, page 16).
2. Rotate tubes slowly (approximately 5 rpm) for 10 min at room temperature on a device that allows both tilting and rotation.
3. Place the tubes into the AdnaMag-S rack without the magnet slider. Swing the AdnaMag-S rack downward to release beads and liquid captured in the cap.
4. Insert the magnet slider, wait for 1 min, and then remove the supernatant.

5. Wash the beads with RNA Purification Buffer A as follows:
   5a. Remove the magnet slider from the AdnaMag-S rack.
   5b. Add 100 µl RNA Purification Buffer A to each tube, and resuspend the beads by repeated pipetting. To avoid any loss of beads, rinse lid and tube wall thoroughly.
   5c. Insert the magnet slider into the AdnaMag-S rack.
   5d. After 1 min, remove the supernatant completely.
   5e. Repeat steps 5a–5d once (2 washes in total).
6. Wash the beads with RNA Purification Buffer B as follows:
   6a. Remove the magnet slider from the AdnaMag-S rack.
   6b. Add 100 µl RNA Purification Buffer B to each tube. Resuspend the beads by pipetting, and transfer into new 1.5 ml reaction tubes (provided).
   6c. Insert the magnet slider into the AdnaMag-S rack.
   6d. After 1 min, remove the supernatant completely. This step must be carried out carefully (watch the pellet), because the beads may slide and get removed by mistake.
   6e. Using the same reaction tubes, repeat steps 6a–6d once (2 washes in total).
7. Remove the magnet slider from the AdnaMag-S rack.
8. Add 100 µl ice cold Tris·Cl Buffer to each tube, and resuspend the beads by pipetting.
9. Insert the magnet slider into the AdnaMag-S rack.
10. After 1 min, remove the supernatant completely.
11. Remove the magnet slider from the AdnaMag-S rack.
12. Resuspend the mRNA/bead-complex in 14.75 µl RNase-free water.
13. Transfer the tubes to a thermal block or water bath, and incubate for 5 min at 65°C.
14. Place the tubes on ice immediately for at least 2 min.
15. Proceed immediately (within 5 min) with reverse transcription ("Reverse transcription using the Sensiscript RT Kit", page 21).

    Important: Do not store the mRNA/bead complex.

Reverse transcription using the Sensiscript RT Kit

1. Prepare the RT Master Mix on ice. The RT Master Mix is prepared as shown in Table 1 according to the number of samples.

   The volume of the RT Master Mix should be 10% greater than calculated for the total number of reverse transcription reactions. A reaction without mRNA must always be prepared as a reverse transcription negative control (RT negative control).

2. Vortex the RT Master Mix. Centrifuge briefly and pipet 5.25 µl for each reaction into 0.2 ml PCR tubes.

3. Resuspend the mRNA/bead complexes (from step 12, page 20) carefully with a pipette. Transfer the total volume into the 0.2 ml PCR reaction tube containing the RT Master Mix. Mix thoroughly by repeated pipetting.

Table 1. Reverse transcription reaction setup

Component	Volume
RT Master Mix	
10x Buffer RT	2.0 µl
dNTP Mix (5 mM each dNTP)	2.0 µl
RNase inhibitor, 40 U/µl (Promega)*	0.25 µl
Sensiscript Reverse Transcriptase	1.0 µl
Template RNA†	
mRNA/bead complex or RNase free water	14.75 µl
Total volume	**20.0 µl**

* See “Equipment and Reagents to Be Supplied by User”, page 11.

† For an RT negative control, add 14.75 µl RNase-free water instead of mRNA/bead-complex. The volume of the mRNA/bead-complex may vary slightly. In any case, use the total volume for reverse transcription.

4. Program a thermal cycler for cDNA synthesis according to Table 2.

Table 2. Reverse transcription program

Step	Time	Temperature
Reverse transcription	60 min	37°C
Denaturation	5 min	93°C
Cooling	∞	4°C

5. Place reaction tubes with the cDNA on ice or store at –20 °C for a maximum of 4 weeks.
6. Proceed with "Protocol: Preamplification and Detection of AR-V7 and Prostate-Cancer-Associated Gene Expression", page 23.

Protocol: Preamplification and Detection of AR-V7 and Prostate-Cancer-Associated Gene Expression

Important point before starting

- Before beginning the procedure, read “Important Notes” (page 12).

Things to do before starting

- For preamplification: Thaw 2x Multiplex PCR Master Mix (QIAGEN), AdnaPanel PrimerMix PreAmp AR-V7, and RNase-free water. Vortex, centrifuge quickly, and store on ice.
- For qRT-PCR: Thaw 2x miRCURY SYBR® Green Master Mix (QIAGEN), AdnaPanel PrimerMixes CD45, GAPDH, PSA, PSMA, AR and AR-V7, AdnaPanel PrimerMix Inhibition Control, AdnaPanel Positive Control AR-V7, and RNase-free water. Vortex, centrifuge quickly, and store on ice.

Preamplification PCR

1. The Preamplification PCR Master Mix is prepared as shown in Table 3 (next page) according to the number of samples.

   Note: The volume calculation of the PCR Master Mix should include at least 10% excess volume. The Inhibition Control sample must always be included in this calculation.

2. For each preparation, dispense 43.75 µl Preamplification PCR Master Mix into 0.2 ml PCR reaction tubes. Resuspend the cDNA/bead mix by pipetting, and add 6.25 µl of this to each reaction tube.

   Note: For negative control, add 6.25 µl RT negative control resulting from “Reverse transcription using the Sensiscript RT Kit”, page 21, instead of cDNA.

Table 3. Preparation of the preamplification PCR Master Mix

Component	Volume
2x Multiplex PCR Master Mix	25 µl
RNase-free water	13.75 µl
AdnaPanel PrimerMix PreAmp AR-V7	5 µl
cDNA or RT Negative Control	6.25 µl
Total volume	**50 µl**

3. Program the thermal cycler for PCR, as described in Table 4. Run the thermal cycler with a ramp of 4°C/second. The preamplification PCR is performed for a total of 18 cycles.

Table 4. Preamplification PCR cycling program

	Temperature	Time
Initial activation step	95°C	5 min
3-step cycling (18 cycles)		
Denaturation	95°C	30 s
Annealing	60°C	90 s
Extension	72°C	90 s

4. After completion of the last cycle, transfer samples to –30 to –15°C for a minimum of 15 min, for rapid cooling and inhibition of residual polymerase activity.

   After cooling the samples, proceed directly with sample dilution, or store the samples at –30 to –15°C for future processing.

5. Spin down beads and dilute each sample 1:10 by mixing 20 µl preamplified cDNA (without beads) and 180 µl RNase/DNase-free water in a new reaction tube.

   Important: Do not dilute AdnaPanel Internal Control and AdnaPanel Positive Control AR-V7.

Quantitative real-time PCR

1. Separate qRT-PCR Master Mixes must be prepared for each preamplified and diluted DNA sample, preamplified and diluted RT Negative Control, and AdnaPanel Positive Control AR-V7.

   Important: All qRT-PCR Master Mixes include the cDNA templates, positive control, or negative control. The different primer mixes are added after pipetting the Master Mixes.

   Each qRT-PCR Master Mix is prepared for 7 reactions (6 markers + 1 inhibition control) as shown in Table 5. The volume calculation of the PCR Master Mix should include at least 10% excess volume.

2. For each preparation, dispense 12.0 µl of the PCR Master Mix into qPCR reaction tubes or qPCR plate wells. Add 3.0 µl of corresponding primer mix to each tube/well (Table 7).

Table 5. Preparation of the qRT-PCR Master Mix

Component	Volume for 1 reaction	Volume for 7 reactions
2x miRCURY SYBR® Green Master Mix	7.5 µl*	52.5 µl*
RNase-free water	0–1.5 µl†	0–10.5 µl†
ROX™ reference dye	0–1.5 µl†	0–10.5 µl†
Preamplified sample (dilution 1:10) *or* AdnaPanel Positive Control AR-V7 *or* RT negative control	3 µl	21 µl
Total volume qRT-PCR Master Mix	**12 µl‡**	**84 µl**

* Pay attention to the viscosity of SYBR® Green during pipetting.

† The total amount of ROX and RNAse/DNAse-free water is 10.5 µl per reaction. Depending on the instrument, high or low ROX concentration needs to be used (see Table 6).

‡ The total volume of PCR reaction is 15 µl, including the primer mix.

Table 6. Real-time cyclers requiring high/low concentrations of ROX

High ROX concentration (1:20 dilution of ROX Reference Dye in 1x reaction)	Low ROX concentration (1:200 dilution of ROX Reference Dye in 1x reaction)
ABI PRISM® 7000	Applied Biosystems 7500
Applied Biosystems® 7300	Applied Biosystems ViiA® 7
Applied Biosystems 7900	Applied Biosystems QuantStudio® Systems
Applied Biosystems StepOne®	
Applied Biosystems StepOnePlus	

Table 7. qRT-PCR setup per reaction

Component	Volume
qRT-PCR Master Mix (prepared as per Table 5)	12 µl
AdnaPanel PrimerMix CD45 or AdnaPanel PrimerMix GAPDH or AdnaPanel PrimerMix PSA or AdnaPanel PrimerMix PSMA or AdnaPanel PrimerMix AR or AdnaPanel PrimerMix AR-V7 or AdnaPanel PrimerMix Inhibition Control	3 µl
Total volume	**15 µl**

3. Set up qRT-PCR reactions for the AdnaPanel Inhibition Control and the AdnaPanel Internal Control, as shown in Table 8 and Table 9.

Table 8. Preparation of the qRT-PCR Master Mix (AdnaPanel Inhibition Control)

Component	Volume
2x miRCURY SYBR® Green Master Mix	7.5 µl*
RNase-free water	0–1.5 µl†
ROX reference dye	0–1.5 µl†
AdnaPanel PrimerMix Inhibition Control	3.0 µl
AdnaPanel Inhibition Control	3.0 µl
Total volume	**15 µl**

* Pay attention to the viscosity of SYBR® Green during pipetting.

† Depending on the instrument, the total amount of ROX and RNAse/DNAse-free water is 1.5 µl.

Table 9. Preparation of the qRT-PCR Master Mix (AdnaPanel Internal Control)

Component	Volume
2X miRCURY SYBR® Green Master Mix	7.5 µl*
RNase-free water	0–1.5 µl†
ROX reference dye	0–1.5 µl†
AdnaPanel PrimerMix IC	3 µl
AdnaPanel Internal Control	3 µl
Total volume	**15 µl**

* Pay attention to the viscosity of SYBR® Green during pipetting.

† Depending on the instrument, the total amount of ROX and RNAse/DNAse-free water is 1.5 µl.

4. After pipetting qRT-PCR Master Mix and primers, close the tubes or seal the PCR plate with an adhesive film. Centrifuge for 15 s at ~1000 rpm and run qRT-PCR immediately (If necessary, store in the dark, but not longer than 5 min).
5. Program the thermal cycler for qRT-PCR, as described in Table 10. The qRT-PCR is performed for a total of 35 cycles. Data collection of SYBR® Green signal and melting curve measurements are performed at 78°C. When using the QIAGEN Rotor-Gene® Q in combination with the Rotor-Disc® 100, data collection of SYBR® Green signal is performed at 76°C.

Table 10. qRT-PCR cycling program

Step	Time	Temperature
Initial activation step	10 min	95°C
3-step cycling (35 cycles)		
Denaturation	10 s	95°C
Annealing	10 s	60°C
Extension and data collection	10 s	78°C*
Melt curve		**60–95°C**

* 76°C when using the Rotor-Gene Q in combination with the Rotor-Disc 100.

Run settings

The run settings for Applied Biosystems® StepOne®, and StepOnePlus®, and for QIAGEN Rotor-Gene Q real-time PCR systems are shown in Table 11 and Table 12, respectively. If you are using a thermocycler that is not listed below, please contact our Technical Service support team (for contact information, visit **support.qiagen.com**).

Table 11. Run settings for Applied Biosystems StepOne and StepOnePlus instruments

Parameter	Setting
Threshold	0.48
Baseline Start	3 cycles
Baseline End	10 cycles
Reporter	SYBR®
Quencher	None
Passive reference	ROX

Table 12. Run settings for Rotor-Gene Q

Parameter	Setting
Threshold	0.30
Detection channel	SYBR®

Melting curve analysis

Before calculating the ΔC_T values, a detailed analysis of the melting curves must be performed to exclude signals resulting from primer-dimers or other nonspecific interactions.

Melting temperatures obtained for each amplicon should not differ from the ones in Table 13 (page 30) by more than 2°C. Alternatively, melting points can be compared with the corresponding values in the positive control sample.

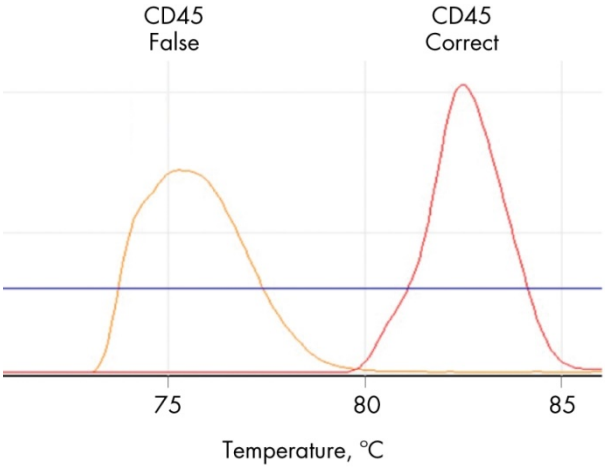

Figure 4. Example melting curves. The left curve shows an incorrect melting temperature for the given PrimerMix. Melting curves should be Gaussian-like, with a clear maximum at the melting temperature.

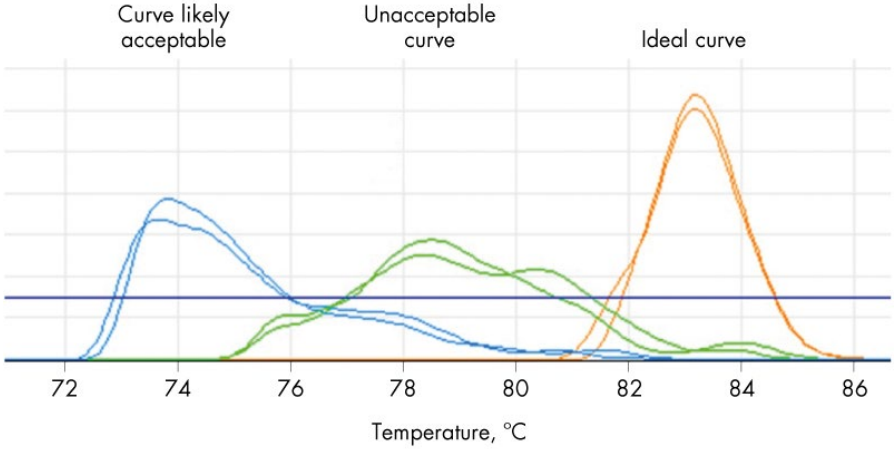

Figure 5. Example melting curve shapes. The green curve is unacceptable and results should be excluded. The blue curve shows a suboptimal shape, but if the melting temperature is correct, then results can be accepted. The yellow curve shape is optimal, and results can be accepted if the melting temperature is also correct.

Table 13. Melting peak temperature ±2.0°C, indicating the correct qPCR product

Target	Melting peak temperature (°C)
CD45	82.48
GAPDH	83.22
PSA	83.07
PSMA	81.13
AR	81.58
AR-V7	79.64
Internal Control	82.32

Data calculation: Method 1

$\Delta\Delta C_T$ = [Cutoff (gene) – Sample C_T (gene)] – [Cutoff (CD45) – Sample C_T (CD45)]

Data calculation: Method 2

ΔC_T = [Cutoff (gene) – Sample C_T (gene)]

The cut-off values (Table 14) were calculated for each gene separately. Table 14 also shows the calculation algorithm for each gene. A test result is regarded as positive if ΔC_T>0.

Table 14. Cutoff values (C_T) and calculation methods

Target name	Cutoff	Calculation method
CD45	24.96	1
GAPDH	24.28	1
PSA	35	2
PSMA	35	2
AR	35	2
AR-V7	35	2
Internal Control	35	2

AdnaPanel Internal Control

The AdnaPanel Internal Control is intended for estimation of plate-to-plate or instrument-to-instrument variations.

AdnaPanel Inhibition Control

The AdnaPanel Inhibition Control and the processed blood samples must not have more than 1 cycle difference. More than 1 cycle difference between AdnaPanel Inhibition Control and sample indicates inhibition of PCR reactions.

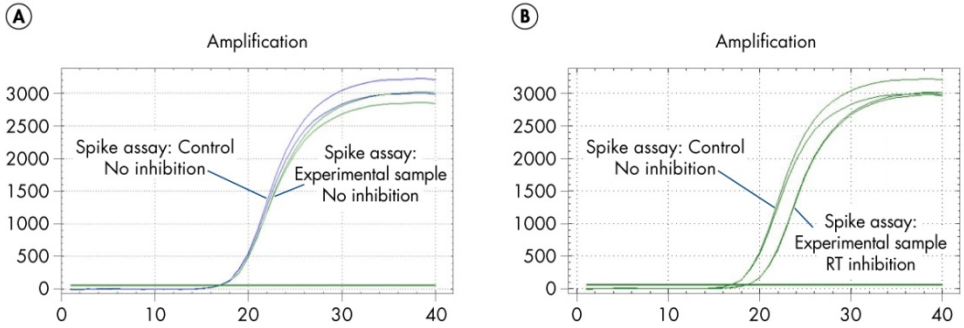

Figure 6. Example amplification curves. A: qPCR amplification plot with no inhibition as the AdnaPanel Inhibition Control in water and in the processed blood sample shows the same Cq value and efficiency. **B**: The RT-reaction is inhibited but not the qPCR reaction, as seen on the shifted Cq value.

Troubleshooting Guide

For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
Blood samples appear different before or during processing	
a) Clotting after addition of Select Beads	Only blood collection tubes indicated in the handbook may be used. If other tubes are used (e.g., tubes containing heparin), blood clotting may be observed during processing, and false-positive or false-negative results cannot be excluded. Even if EDTA is used as anticoagulant, some samples may show clotting or fiber-like aggregates attached to the beads that become visible after the washing steps. This may be observed particularly in late-stage metastatic patient samples and could be due to increased thrombotic conditions in such patients. The results obtained from such samples are invalid and must be discarded.
b) Blood is hemolyzed	Samples transported inappropriately. If blood samples are not shipped in suitable packaging and are in close or direct contact with frozen cool packs, samples partially freeze. Therefore, red blood cells are destroyed. The results obtained from such samples are invalid and must be discarded. For transportation, wrap blood tubes in several layers of plastic films with air cushions, or place them in foam plastic to prevent contact with frozen cool packs.
High C_T values for CD45 and/or GAPDH	
a) Loss of Oligo-dT-Beads during mRNA purification	The Oligo-dT beads tend to slide down the reaction tube during mRNA purification. This phenomenon is generally observed during the washing step using RNA Purification Buffer B and can lead to aspiration and removal of beads during the washing procedure. Using the tubes provided with the kit as indicated in the workflow description should circumvent this issue. Extra care should be taken during pipetting to avoid bead loss.

		Comments and suggestions
b)	Oligo-dT beads not included in the preamplification PCR	The Oligo-dT beads must be included in the preamplification PCR. During reverse transcription, the Oligo-dT fragments on these beads function as primers for the reaction. Therefore, the resulting cDNA remains attached to the beads. Before transferring the cDNA sample to the preamplification PCR Master Mix, it is essential to resuspend the beads carefully.
c)	Cells apoptotic or degraded	Sample storage and transportation is critical. Use only blood collection tubes as indicated, and immediately store samples at 4–8°C after blood withdrawal. Ensure a proper cold chain, and do not process samples that have been stored or transported for more than 4 h (using EDTA tubes) or 30 h (using ACD-A Vacutainers in combination with AdnaTubes).

Ordering Information

Product	Contents	Cat. no.
AdnaTest ProstateCancerPanel AR-V7	For enrichment of tumor cells from whole blood in cancer research, and for detection of AR-V7 expression in enriched tumor cells	396132
Related products		
AdnaTube	12 sample tubes containing EDTA; use only with anticoagulated blood collected in A-CDA blood collection tubes from BD	399932
AdnaMag-L	Magnetic rack for 8 tubes, 15 ml	399921
AdnaMag-S	Magnetic rack for 8 tubes, 1.5 ml	399911
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 150 µl 10x Buffer RT, 100 µl dNTP Mix (contains 5 mM each dNTP), 1.1 ml RNase-free water	205211
QIAGEN Multiplex PCR *Plus* Kit (100)	3 x 0.85 ml 2x Multiplex PCR Master Mix, 2 ml 5x Q-Solution®, 1.2 ml 10x CoralLoad® Dye, and 2 x 1.9 ml RNase-free water	206152
miRCURY LNA SYBR® Green PCR Kit (600)	3 x 1 ml 2x miRCURY SYBR® Green Master Mix, 1 ml ROX Reference Dye, and 2 x 1.5 ml RNase-free water	339346

* Larger kit sizes are available; see **www.qiagen.com**.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
05/2020	Corrected the volumes of collection tubes (15 ml for 3 x 5 tubes and 1.5 ml for 24 tubes) and increased volume of AdnaTest Lysis/Binding Buffer to 2 x 2 ml (from 2 c 1.2 ml). Increased volume of Oligo(dT)$_{25}$ Beads to 355 µl (from 280 µl). Increased volume of AdnaPanel PrimerMix PreAmp AR-V7 to 100 µl (from 60 µl). Added quick-start protocol to kit contents of Box 1 and Box 3. Replaced "Tris-HCl" terminology with "Tris·Cl", per Branding guidelines. In "Reagents" (page 10), bullet 1: Deleted note about Sensiscript RT Kit. Deleted "Patent" section that refers to PCR IP rights from Roche. In "Sample preparation", changed blood storage temperature range to 2–8°C, from 4–8°C. Added "Step" column in Table 2. Reworded step 4 in page 24, for clarity. Added volumes for 1 reaction in Table 5. Added list of real-time cyclers requiring high/low ROX concentrations (new Table 6). In "Ordering Information", replaced cat. no. 206151 (discontinued) with 206152.

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