Version 090112

AdnaTest ProstateCancerDetect

RT-PCR Kit for detection of prostate cancer associated gene expression in enriched tumor cells

For in vitro diagnostic use

Manual

Article no. T-1-521

Contents

Order Information 3

Purpose 3

Abbreviations and Symbols 4

Patents and Registered Trademarks 4

Product Description 5

Kit Components 6

Additional Materials Needed 6

Storage 8

Application Information 8

Protocol 9

- A Preparation of Dynabeads Oligo(dT)$_{25}$ 9
- B mRNA Isolation 10
- C Reverse Transcription 12
- D Multiplex PCR 14
- E Fragment Analysis 15

Evaluation 17

References 20

Troubleshooting 21

Short Manual 23

Order Information

On the website www.adnagen.com the addresses of distributors and information about our products can be found. Our distributors will provide you also with technical support.
Furthermore, AdnaGen's support team will answer you any questions regarding the *AdnaTests* (support@adnagen.com).

AdnaTest ProstateCancerSelect and

AdnaTest ProstateCancerDetect can be ordered as listed below.

	Specifications	Order no.
AdnaTest ProstateCancerSelect	12 Selections	T-1-520
AdnaTest ProstateCancerDetect	12 Detections	T-1-521

Purpose

AdnaTest ProstateCancerDetect has been developed for the detection of prostate cancer-associated gene expression in immunomagnetically enriched tumor cells by reverse transcription and PCR for in vitro diagnostic use only.
AdnaTest ProstateCancerSelect is used for the enrichment of circulating tumor cells from peripheral blood.

Abbreviations and Symbols

bp	base pairs
cDNA	complementary deoxyribonucleic acid
C+	positive control
C-	negative control
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
⌛	expiry date
🌡	storage temperature

Patents and Registered Trademarks

This test requires licenses of Hoffmann-La Roche AG, Basel. The purchase of *AdnaTests* does not relieve the user to perform the PCR without license.
Dynabeads is a registered trademark of Dynal Biotech ASA, Oslo, Norway.
The trademarks *Sensiscript* and *HotStarTaq* were registered by Qiagen, Hilden.
LabChip is a US registered trademark of Caliper Technology Corp.

Product Description

AdnaTest ProstateCancerDetect contains oligo $(dT)_{25}$-coated beads for the isolation of mRNA from the lysate of pre-enriched tumor cells. Reverse transcription results in cDNA, which is the template for tumor cell detection and characterization by multiplex-PCR. With the *PrimerMix ProstateDetect* three tumor associated antigens and one control gene are amplified. The primers generate fragments of the following sizes:

PSMA : 449 bp
PSA : 357 bp
EGFR : 163 bp
actin : 111 bp (internal PCR control).

Kit Components

AdnaTest ProstateCancerDetect includes the following components (number of tubes):

Table 1: Kit Components

Component	Symbol	T-1-521 (12 tests)
Lysis/Binding Buffer	3	1
Dynabeads Oligo(dT)$_{25}$	4	1
Buffer A	5	1
Buffer B	6	1
10 mM Tris-HCl	7	1
PrimerMix ProstateDetect	8	1
Positive Control (C+)	9	1

The reagents are sufficient to analyze 6 PCR controls and 12 blood samples.

Additional Materials Needed

Equipment:

- Tube rotator for 1.5 ml tubes
- Magnetic particle concentrator MPC-S (Dynal MPC-S, Invitrogen, cat. no. 120-20D)
- Thermomixer or water bath (65 °C)

- Thermocycler with a heated cover and a heating rate of 2 °C/s.
- Agarose gel electrophoresis and image documentation system or an alternative analysis system like the Bioanalyzer Agilent 2100 (Agilent Technologies).

Material:

- Sterile, RNase-free thin-wall 0.2 ml PCR-tubes
- Sterile, RNase-free 1.5 ml reaction tubes
- Pipets (1-200 µl), RNase-free pipet tips with aerosol barrier
- Protective gloves
- Agarose gels, for instance a precast 4 % agarose gel with ethidium bromide (SIGMA, cat no. P 6097)

Reagents:

- *Sensiscript* Reverse Transcription Kit (Qiagen, e.g. cat no. 205211, 50 reactions)
- Recombinant RNAsin, RNase-inhibitor, 2.500 U (Promega, cat no. N2511)
- *HotStarTaq Master Mix* Kit (Qiagen, e. g. cat no. 203443, 250 U)

Storage

AdnaTest ProstateCancerDetect has to be stored at 4 °C. **However, remove *PrimerMix ProstateDetect* 8 and *Positive Control* 9 (C+) from the kit and store them separately at -20 °C**. In order to prevent possible contaminations and repeated temperature changes aliquot the primer mix. All components must not be used beyond the expiry date.

Application Information

- The test must be performed by personnel skilled in molecular biological techniques.
- All components and additional reagents provided by other suppliers have to be stored according to the instructions. Safety advices of the respective manufacturers are valid.
- Wear gloves to avoid contamination with DNA, RNA and RNases.
- Processing has to be performed in the denoted sequence and has to comply with all specifications stated in respect of incubation times and incubation temperatures.
- Perform sample processing and subsequent analysis of amplified PCR products in different rooms, if possible, to avoid cross-contamination.
- The use of products from other suppliers may cause inferior results.
- The safety and hygiene regulations of the laboratory must be respected (e. g. wear lab coats, protective goggles, gloves).

Protocol

Sections A to C describe the mRNA-isolation and the reverse transcription.

A Preparation of Dynabeads Oligo(dT)$_{25}$

1. Equilibrate *Lysis/Binding Buffer* [3] to room temperature.

   Note: Check that the *Lysis/Binding Buffer* contains no precipitate. If any precipitate is observed, equilibrate the buffer to room temperature and shake until it is completely dissolved.

2. Resuspend the *Dynabeads Oligo(dT)$_{25}$* [4] thoroughly by pipetting before use; do not vortex!
3. 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 a RNase-free 1.5 ml reaction tube.
4. Place the tube into the MPC-S.

   Note: In the MPC-S the magnet can be inserted in two positions. Always use the front position to make sure that the magnet is close to the reaction tube.

5. After 1 min remove the supernatant with a pipet.
6. Washing
   a. Remove the magnet from the MPC-S.
   b. Add the original volume (step 3) *Lysis/Binding Buffer* [3] and resuspend the beads by repeated pipetting. Resuspend gently to avoid foaming.
   c. Place the magnet into the MPC-S.
   d. After 1 min remove the supernatant with a pipet.

   Repeat once (two washings in total).

7. Remove the tube from the MPC-S and resuspend the beads in *Lysis/Binding Buffer* to the original volume (20 µl beads per sample plus 10 %, as calculated in step 3).

B mRNA Isolation

Preparation

I. Equilibrate Washing *Buffer A* [5] and Washing *Buffer B* [6] to room temperature.
II. Place the *10 mM Tris-HCl* [7] on ice.
III. Thaw RNase-free water (part of the *Sensiscript* Reverse Transcriptase-Kit, Qiagen).
IV. Adjust a thermomixer or water bath to 65 °C.

Processing

1. Aliquot 20 µl of the beads (coming from step A7) to each tube containing cell lysate (from step B16 of the *AdnaTest ProstateCancerSelect* Manual).
2. Place the tubes for 10 min at room temperature on a tube rotator and rotate the tubes slowly (approx. 5 rpm) for 10 min at room temperature on a device allowing both tilting and rotation.
3. Place the tubes into the MPC-S.
4. After 1 min remove the supernatants.
5. Washing A
   a. Remove the magnet from the MPC-S.
   b. Add 100 µl Washing *Buffer A* [5] to each tube and resuspend the beads by pipetting.
   c. Insert the magnet into the MPC-S
   d. After 1 min remove the supernatants completely.

   Repeat once (two washings in total).

6. Washing B
   a. Remove the magnet from the MPC-S.
   b. Add 100 µl Washing *Buffer B* [6] to each tube, resuspend the beads by pipetting and transfer into new 1.5 ml reaction tubes.
   c. Insert the magnet in the MPC-S and incubate for 1 min.
   d. Remove the supernatants completely.

   Repeat once in the same reaction tubes (two washings in total).
7. Remove the magnet from the MPC-S.
8. Add 100 µl ice cold *10 mM Tris-HCl* [7] to each tube and resuspend the beads by pipetting.
9. Insert the magnet into the MPC-S.
10. After 1 min remove the supernatants completely.
11. Remove the magnet from the MPC-S.
12. Resuspend the mRNA/bead-complex in 14.75 µl RNase-free water.
13. Place the tubes into a thermomixer or water bath and incubate for 5 min at 65 °C. Mix with approx. 650 rpm.
14. Place the tubes on ice immediately for at least 2 min.
15. Continue immediately (within 5 min) with the reverse transcription (Section C).

Do not store the mRNA/bead complex!

C Reverse Transcription

(Sensiscript Reverse Transcriptase Kit, Qiagen*)*

1. Thaw 10 x buffer RT and dNTPs at room temperature, mix by vortexing, centrifuge briefly, and store on ice. Prepare the RT Master Mix on ice.
2. Prepare a RT Master Mix according to the number of the reactions needed (Table 2).

   The volume of the Master Mix should be 10 % larger than calculated for the total number of reverse transcription reactions. A negative control reaction without mRNA must always be prepared (RT Control).
3. Vortex the RT Master Mix, centrifuge briefly, and distribute 5.25 µl into individual 0.2 ml PCR tubes.
4. Resuspend the mRNA/bead complexes (from step B14) carefully with a pipet. Transfer the total volume of 14.75 µl into the 0.2 ml PCR reaction tube containing the RT Master Mix. Mix thoroughly and centrifuge briefly. RT Control: Add 14.75 µl RNase-free water instead of mRNA.

Table 2: Reverse Transcription

Components			Volume
RT Mastermix	*Sensiscript* Reverse Transcriptase Kit (Qiagen)	10x Buffer RT	2.0 µl
		dNTPs	2.0 µl
		Sensiscript Reverse Transcriptase (SRT)	1.0 µl
	RNase Inhibitor, 40 U/µl (Promega)		0.25 µl
Sample	mRNA/bead-complex or RNase-free H_2O (as RT Control)		14.75 µl
Total volume			**20.0 µl**

5. cDNA is synthesized in a thermocycler under the following conditions (Table 3).

Table 3: RT program

37 °C	→	60 min
93 °C	→	5 min
4 °C	→	∞

6. Place reaction tubes with the cDNA on ice or store at -20 °C for max. 14 days.

D Multiplex PCR

1. Thaw *HotStarTaq Master Mix* (Qiagen), distilled water and Positive Control (C+) [9], vortex carefully, centrifuge quickly and store on ice. Thaw *PrimerMix ProstateDetect* [8], vortex, spin down and place on ice.
2. Depending on the number of samples the PCR Master Mix is prepared according to Table 4.
   The volume of the Master Mix should at least be 10 % larger than the requirement calculated from the number of samples. Note that a Positive Control (C+) [9], a Negative Control (water/C-) and the RT Control must always be prepared.
3. For each preparation dispense 21.0 µl of the Master Mix into each 0.2 ml PCR reaction tube. Resuspend the cDNA/bead mix by pipetting and add 4.0 µl of this suspension (step C6).
   Note: For C-, 4.0 µl of distilled water is added instead of cDNA.

Table 4: Preparation of the multiplex PCR

Components			**Volumes**
PCR Master Mix	*HotStarTaq Master Mix* Kit (Qiagen)	*HotStarTaq Master Mix*	12.5 µl
		Distilled water	4.5 µl
	PrimerMix ProstateDetect [8]		4.0 µl
Samples	cDNA or RT Control or Negative Control (water/C-) or *Positive Control* (C+) [9] each:		4.0 µl
Total volume			25.0 µl

A thermocycler is used for the PCR which follows the program described in Table 5. Run the thermocycler with a ramp of 2 °C/second.

Table 5: PCR program

95 °C	**15 min**	
94 °C	**30 sec**	**42 cycles**
61 °C	**30 sec**	
72 °C	**30 sec**	
72 °C	**10 min**	
4 °C	∞	

E Fragment Analysis

Bioanalyzer Agilent2100

The analysis with the Bioanalyzer Agilent 2100 (Agilent Technologies) on a DNA 1000 LabChip is recommended. Carry out the instructions of the DNA 1000 LabChip manual. Make sure that no beads are transferred into the LabChip. Magnetic beads in the gel can cause erroneous results. When using the Agilent Bioanalyzer 2100 set a detection threshold as it is described below:

Start the Bioanalyzer Software *Bio Sizing* and create a *Default Assay*. Under Instrument select *Assay* > Electrophoresis > *ds1000* > *DNA 1000 Series II*. Under *Data* choose *Assay properties* > *global normal* > *height threshold (FU)* set the *Min Peak Height* to 0 to detect all signals. Under *Global Advanced* set the *Ladder* Peak Height to 0.

Agarose Gel

Alternatively the PCR products are analyzed by electrophoresis on a 4 % agarose gel. Apply 10.0 µl of each product. Additionally apply a 100 bp DNA ladder as size marker according to the manufacturers instructions.

To make sure that the fragments can be discriminated accurately run the gel over a distance of at least 5 cm. Electrophoresis conditions: 100 V, ≥ 1 h.

Evaluation

The test is considered positive, if a PCR fragment of at least one tumor associated transcript is clearly detected.

If you are using the Bioanalyzer Agilent 2100 peaks with a concentration of ≥ 0.10 ng/µl are positive (Fig. 1). Peaks that are not detected at the above setting are negative (concentraton: < 0.10 ng/µl).

To evaluate the results obtained by agarose gel electrophoreses refer to Fig. 2.

<u>In addition, the following criteria must be fulfilled:</u>

- The fragment of the control gene actin must occur in all patient samples (internal PCR control). An actin signal provides a positive control for three procedures:
  a. cell separation was successful.
  b. the reverse transcription and
  c. the multiplex PCR were successful.
- The Negative Control (water/C-) and the RT Control samples must not show any bands of more than 80 base pairs (primer dimers).
- A 500 bp fragment indicates a contamination with genomic DNA. The separation process was not

successful and the results have to be discarded if such a fragment occurs.

Note: The *AdnaTest ProstateCancerSelect* and *AdnaTest ProstateCancerDetect* are optimized to exclude low level expression of tumor associated transcripts. Any change in the protocol or the use of the highly sensitive Bioanalyzer Agilent 2100 might sometimes lead to the detection of weak expression by cells of healthy donors below the cut-off level.

Any deviation from the protocol might lead to false negative or false positive results.

In the case that assistance is needed to interpret the result, please, contact our support team.

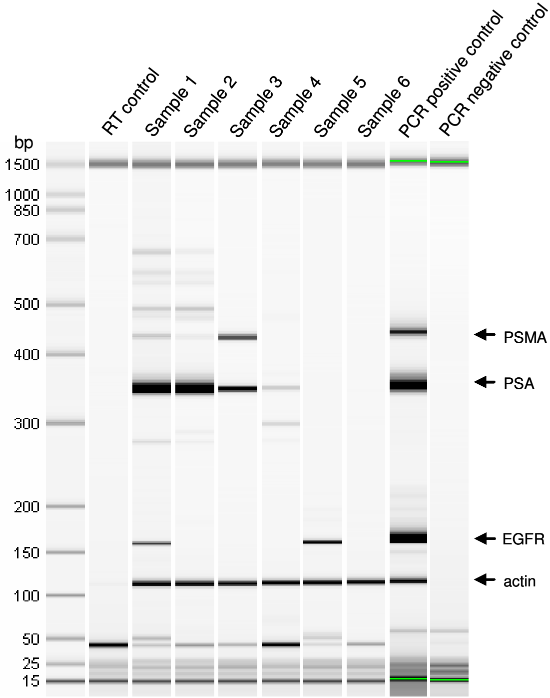

Fig. 1: *AdnaTest ProstateCancerDetect* on patient samples analyzed with the Bioanalyzer 2100 (Agilent):
DNA size standards (ladder) and the RT Control are shown in the first two lanes. Sample 1 is positive for PSA and EGFR, sample 2 only for PSA and sample 3 for PSA and PSMA. The sample 4 is weakly positive for PSA and sample 5 only for EGFR. The sample 6 is negative and shows only an actin fragment as internal control like the other samples. The PCR positive control and negative control are shown in the last two lanes.

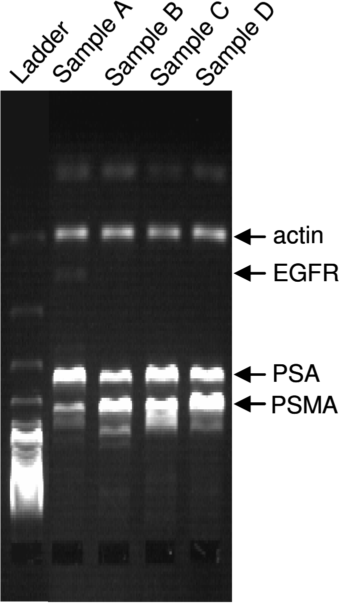

Fig. 2: Analysis by gel electrophoresison on 4 % agarose gel.
Sample A is positive for all three marker (PSA, PSMA and actin). Samples B till D are positive for PSA and PSMA. All samples show an actin fragment as internal PCR control.

References

For references please refer to our website.

http://www.adnagen.com

Troubleshooting

A failure of the gene expression analysis may have various reasons. It is essential that all assay steps are always executed precisely according to the manuals.

In case that there are still problems, the following table gives you comments on the possible causes and suggests corrections. Since the problems may as well have their origin in the cell enrichment process, the Table 6 also refers to *AdnaTest ProstateCancerSelect.*

Do not hesitate to contact us when problems continue to exist.

Table 6: Troubleshooting

Problem	Possible causes	Suggestions for correction
No bands incl. actin, for all samples	Pipetting error	Repetition
	Reagents problems	Control of reagents (storage etc.)
	RNase contamination	Verify that RNase-free material (pipets, tips, reaction tubes etc.) and reagents were used. Wear gloves and change them regularly.

	Poor quality of the blood sample	Verify that blood samples were taken in approved collection tubes only. Verify that blood samples were not haemolysed and that blood withdrawal took place before medication. Discard samples if the selection beads agglutinate during cell enrichment. Blood samples must be processed within 4 h (EDTA) or 48 h (*AdnaCollect*) after withdrawal. Blood samples must be kept on ice or at 4 °C.
	Band could not be identified because of insufficient separation	Check gel concentration, buffers, separation time and the applied voltage.
RT and C- controls show fragments larger than 80 bp	Contamination	Exchange all reagents. Aliquot all reagents before use. Use filter tips. If possible, keep the preparation of samples and the reaction setup locally separated from the analysis of PCR products.
Diffuse bands in the agarose gel	Gel electrophoresis conditions are not optimal	Check concentration of agarose gel. Check electrophoresis buffer.
Bands larger than 1000 bp	Contamination with genomic DNA	Repeat tumor cell enrichment and RT-PCR

Short Manual

AdnaTest *ProstateCancerDetect*

Component	*Lysis/Binding Buffer*	3
	Oligo(dT)$_{25}$ Beads	4
	Buffer A	5
	Buffer B	6
	Tris HCl	7
	PrimerMix ProstateDetect	8
	Positive Control (C+)	9
You need	0.2 ml PCR-tubes 1x 1.5 ml Reaction tube per sample 1-200 µl pipets and tips 4 % Agarose gel Sensiscript RT Kit (Qiagen). HotStarTaq Master Mix Kit (Qiagen).	

Protocol

- Equilibrate 3, 4, 5 and 6 to room temperature and place 7 on ice.
- Wash 20 µl *Oligo(dT)$_{25}$ Beads* 4 per sample 2x with 20 µl *Lysis/Binding Buffer* 3 per sample.
- Add 20 µl washed *Oligo(dT)$_{25}$ Beads* 4 to each sample.
- Incubate for 10 min at room temperature under rotation at 5 rpm.
- Place the reaction tube in MPC-S and remove the supernatant.
- Wash the beads with 2x 100 µl *Buffer A* 5.
- Resuspend the beads in 100 µl *Buffer B* 6.
- Wash the beads with 1x 100 µl *Buffer B* 6.
- Wash the beads with 1x 100 µl *Tris-HCl* 7.

- Resuspend the beads in 14.75 µl RNase free water.
- Incubate for 5 min at 65 °C and place on ice for at least 2 min.
- Continue with the reverse transcription (RT), see Table 7 and Table 8.

Table 7: Reverse Transcription

Components			**Volume**
RT Mastermix	*Sensiscript* Reverse Transcriptase Kit (Qiagen)	10x Buffer RT	2.0 µl
		dNTPs	2.0 µl
		Sensiscript Reverse Transcriptase (SRT)	1.0 µl
	RNase Inhibitor, 40 U/µl (Promega)		0.25 µl
Sample	mRNA/bead-complex or RNase-free H_2O (as RT Control)		14.75 µl
Total volume			**20.0 µl**

Table 8: RT program

37 °C	→	60 min
93 °C	→	5 min
4 °C	→	∞

- Continue with the Multiplex PCR (Table 9) or store the RT products at -20 °C for max 14 days.

Table 9: Multiplex PCR

Components			Volumes
PCR Master Mix	*HotStarTaq Master Mix* Kit (Qiagen)	*HotStarTaq Master Mix*	12.5 µl
		Distilled water	4.5 µl
	PrimerMix ProstateDetect [8]		4.0 µl
Samples	cDNA or RT Control or Negative Control (water/C-) or *Positive Control* (C+) [9] each:		4.0 µl
Total volume			25.0 µl

Table 10: PCR program

95 °C	**15 min**	
94 °C	**30 sec**	**42 cycles**
61 °C	**30 sec**	
72 °C	**30 sec**	
72 °C	**10 min**	
4 °C	∞	

- For fragment analysis use the Bioanalyzer 2100 (Agilent). Alternatively, analyze the fragments and the samples in a 4 % agarose gel (80-100 V for ca. 60 min).

AdnaGen AG
Ostpassage 7
D-30853 Langenhagen
Germany

Phone	+49 (0) 511 725950-50
Fax	+49 (0) 511 725950-40
E-mail	support@adnagen.com