March 2018

AdnaTest BreastCancerSelect and BreastCancerDetect Handbook

Σ 12 (catalog number 395412)

Σ 12 (catalog number 396412)

For enrichment of tumor cells from the whole blood of breast cancer patients and detection of breast cancer associated gene expression in enriched tumor cells
For in vitro diagnostic use
Version 1

IVD

CE

REF 395412 (AdnaTest BreastCancerSelect)
396412 (AdnaTest BreastCancerDetect)

QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY

R2 MAT 1112927

Sample to Insight

QIAGEN

Contents

Intended Use

The AdnaTest BreastCancerSelect is an in vitro diagnostic method intended for the immunochemical enrichment of circulating tumor cells from anti-coagulated whole blood samples obtained from breast cancer patients, through a combination of epithelial and tumor associated antigens.

The AdnaTest BreastCancerDetect is an in vitro diagnostic assay intended for the analysis of expression profiles of tumor cells by reverse transcription and multiplex PCR and subsequent densitometric analysis of the PCR products by automated capillary electrophoresis utilizing the Agilent® 2100 Bioanalyzer.

The AdnaTest BreastCancerSelect/Detect is not intended for screening purposes and is not to be used as a diagnostic test to confirm the presence of breast cancer.

The product is intended to be used by professional users, such as technicians and physicians who are trained in molecular biological techniques.

Summary and Explanation

AdnaTest BreastCancerSelect enables the immunomagnetic enrichment of tumor cells via epithelial and tumor associated antigens. AdnaTest BreastCancerDetect is used for analysis of breast cancer associated gene expression in immunomagnetically enriched tumor cells by reverse transcription and PCR.

Principle of the Procedure

AdnaTest BreastCancerSelect

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).

The cell lysate is used for further analysis with AdnaTest BreastCancerDetect.

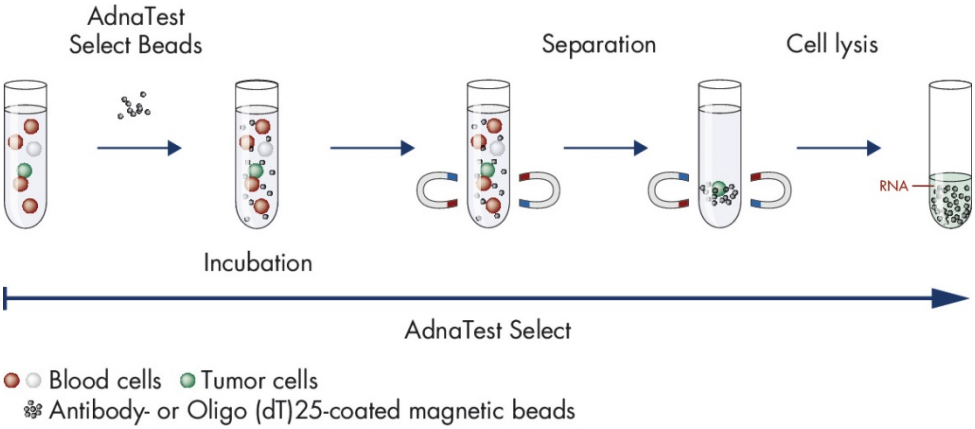

Figure 1. AdnaTest BreastCancerSelect: Immunomagnetic cell selection with multiple tumor associated antibodies. In the first step, the CTCs in the blood are enriched (AdnaTest Select). This is achieved using antibody-coated magnetic particles (beads). Several antibodies are used, which bind with high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

AdnaTest BreastCancerDetect

AdnaTest BreastCancerDetect contains Oligo $(dT)_{25}$ Beads for the isolation of mRNA from the lysate of pre-enriched tumor cells. Reverse transcription results in cDNA, which is subsequently used as template for tumor cell detection and characterization by multiplex PCR. The AdnaTest PrimerMix BreastDetect allows amplification of three tumor associated antigens and one control gene.

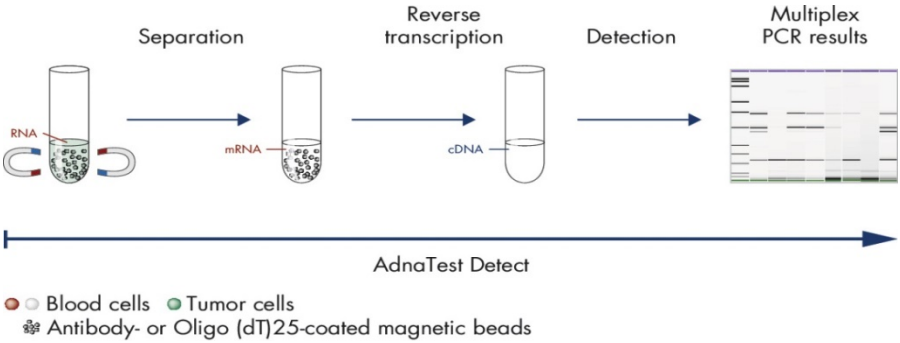

Figure 2. AdnaTest BreastCancerDetect: Multiplex PCR of various cancer associated tumor markers. In a second step the enriched cells are examined by RT-PCR for tumor associated expression patterns. The mRNA strands are reverse transcribed into cDNA. Subsequently, several associated tumor markers can be amplified using multiplex PCR and visualized.

The AdnaTest PrimerMix generates the following fragments:

AdnaTest PrimerMix BreastDetect

- GA733-2: 395 bp
- Muc-1: 299 bp
- Her-2: 265 bp
- Actin: 120 bp (internal PCR control)

Note: Fragment sizes may vary slightly. Make sure to use the AdnaTest Positive Controls for assignment of the detected signals.

Materials Provided

Kit contents

AdnaTest BreastCancerSelect			
Catalog number			**395412**
Number of tests			**12**
Collection Tubes	Collection Tubes (15 ml)	COL TUBE	3 x 5
Collection Tubes	Collection Tubes (1.5 ml)	COL TUBE	24
Red	BreastSelect Beads	BSB	1 x 1.2 ml
Red	AdnaTest Lysis/Binding Buffer	LBB	1 x 2 ml
	Handbook		1

AdnaTest BreastCancerDetect			
Catalog no.			**396412**
Number of tests			**12**
AdnaTest RNA Reagent Box			**Box 1**
Red	AdnaTest Lysis/Binding Buffer	LBB	1 x 2 ml
Orange	Oligo(dT)$_{25}$ Beads	OdT	1 x 280 µl
White	RNA Purification Buffer A	BA	1 x 4 ml
White	RNA Purification Buffer B	BB	1 x 4 ml
Purple	Tris-HCL Buffer	TB	1 x 2 ml
AdnaTest BreastCancerDetect			**Box 2**
Blue	AdnaTest PrimerMix BreastDetect	PMB	1 x 144 µl
Orange	AdnaTest Positive Control Breast (C+)	CONTROL +	1 x 56 µl
	Handbook		1

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

Materials Required but Not Provided

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 BreastCancerSelect

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
- 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 BreastCancerDetect

Equipment

- Tube rotator for 1.5 ml tubes (e.g., ELMI Ltd., cat. no. IMIX-03)
- Magnetic particle concentrator AdnaMag-S (cat. no. 399911)
- Thermal block or water bath (50°C)
- Thermal cycler with a heated lid and a heating rate of 2°C/s.
- Agilent 2100 Bioanalyzer (Agilent Technologies)

Consumables

- Sterile, RNase-free thin-wall 0.2 ml PCR tubes
- 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 1 µl to 200 µl

Reagents

- Sensiscript® RT Kit (QIAGEN, cat. no. 205211, 50 reactions)
  - **Note**: The Sensiscript RT Kit (cat. no. 205211) is sufficient for only 25 samples because twice the volume is required for each reaction.
- Recombinant RNasin®, RNase-inhibitor, 2500 U (Promega, cat. no. N2511)
- HotStarTaq® Master Mix Kit (QIAGEN, cat. no. 203443, 250 U)
- Crushed ice

Warnings and Precautions

For in vitro diagnostic use

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.

Discard sample and assay waste according to your local safety regulations.

These tests must be performed by personnel skilled in molecular biological techniques.

Patents

AdnaTest BreastCancerDetect requires licenses from Hoffmann-La Roche AG, Basel. The purchase of AdnaTest BreastCancerDetect does not authorize the user to perform the PCR without license.

Reagent Storage and Handling

Storage

The AdnaTest BreastCancer system is delivered in three boxes. AdnaTest BreastCancerSelect (cat. no. 395412) and the AdnaTest RNA Reagent Box 1 (cat. no. 396412, Box 1) must be stored at 2–8°C. The components must not be used beyond the expiration date.

AdnaTest BreastCancerDetect Box 2 (cat. no. 396412, Box 2), containing the AdnaTest PrimerMixes and AdnaTest Positive Controls, must be stored separately at –30 to –15°C. To prevent possible contamination and repeated temperature changes, aliquot the primer mix. The components must not be used beyond the expiration date.

Handling

- BreastSelect Beads contain sodium azide as preservative. Sodium azide is cytotoxic and must, therefore, be removed before using the beads. (See "Protocol: Enrichment of Tumor Cells Using AdnaTest BreastCancerSelect", page 13.)
- 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 in respect of incubation times and incubation temperatures.
- Discard samples if the selection beads agglutinate during cell enrichment.
- Perform sample processing, including reverse transcription and subsequent analysis of amplified PCR products, in different rooms, if possible, to avoid cross-contamination.
- The use of products from suppliers other than those suggested may adversely affect the results.
- Observe the safety and hygiene regulations of the laboratory (e.g., wear lab coats, protective goggles and gloves).

Specimen Handling and Storage

Sample preparation

- Blood samples must be taken before the application of therapeutic substances. Do not use the AdnaTest BreastCancerSelect earlier than 7 days after the last therapeutic intervention!
- Blood collection: If sample transportation is less than 4 hours, 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 of whole blood.
- If sample transportation is longer than 4 hours, use BD Vacutainer ACD-A Tubes (Becton Dickinson GmbH, cat. no. 366645 [EU]; 364606 [US]) to draw at least 8.5 ml of whole blood. Before further processing using the AdnaTest, 5 ml ACD-A blood must be transferred into an AdnaTube Sample Tube (cat. no. 399932).
- Blood must be stored at 4–8°C immediately.
- Samples should be processed as soon as possible, but not later than 4 hours after blood withdrawal when using standard EDTA tubes or within 30 hours when using BD Vacutainer blood collection tubes in combination with AdnaTubes.
- The blood sample must not be hemolyzed.

Protocol: Enrichment of Tumor Cells Using AdnaTest BreastCancerSelect

Important points before starting

Before beginning the procedure, read "Warnings and Precautions" (page 10), "Reagent Storage and Handling" (page 11) and "Specimen Handling and Storage" (page 12).

- It is necessary to remove sodium azide by washing the BreastSelect Beads prior to use, as described below in "Procedure A: Preparation of the BreastSelect Beads".
- Please use the provided 1.5 ml collection tubes only for the protocol step indicated.

Things to do before starting

- Ensure that AdnaTest Lysis/Binding Buffer is equilibrated to room temperature. If a precipitate is observed, equilibrate the reagent to room temperature and mix until the precipitate is completely dissolved.

Procedure A: Preparation of the BreastSelect Beads

1. Resuspend the BreastSelect Beads thoroughly by pipetting; do not vortex!
2. Calculate the volume of BreastSelect 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.
4. After 1 minute remove the supernatant with a pipette.

   Note: Do not touch the beads when removing the supernatant!
5. Wash steps:

   5a. Remove the magnet slider from the AdnaMag-S.

5b. Add 1 ml PBS and resuspend the beads by repeated pipetting.
5c. Place the magnet slider into the AdnaMag-S.
5d. After 1 minute remove the supernatant completely with a pipette.
5e. Repeat steps 5a to 5d twice (three washes in total).

6. Remove the tube from the AdnaMag-S, and resuspend the beads in PBS to the original volume (100 µl per sample). Proceed with "Procedure B: Selection of tumor cells", below.

Procedure B: Selection of tumor cells

1. When using standard EDTA tubes, pipet 5 ml of a blood sample into a 15 ml Collection Tube (provided).
   When using ACD-A blood in a BD Vacutainer ACD-A Tube, transfer 5 ml of blood into an AdnaTube.

   Note: AdnaTubes are mandatory when using BD Vacutainer ACD-A Tubes.
2. Resuspend the BreastSelect Beads thoroughly (prepared in step 6 of Procedure A) by pipetting, and add 100 µl of these beads to each blood sample.
3. Rotate tubes slowly (approximately 5 rpm) for 30 minutes at room temperature on a device allowing both tilting and rotation.
4. Place tubes into the AdnaMag-L without the magnet slider. Swing the AdnaMag-L downwards to release blood drops captured in the cap.
5. Insert the magnet slider and incubate the tubes in the AdnaMag-L for 3 minutes at room temperature.
6. Remove blood supernatant completely with a 10 ml pipette without touching the beads.

   Note: Do not touch the beads when removing the supernatant!
7. Wash steps:
   7a. Remove the magnet slider from the AdnaMag-L.
   7b. Add 5 ml PBS. Close the tubes and shake the AdnaMag-L gently back and forth 5 times to resuspend the magnetic bead/cell complexes.
   7c. Swing the AdnaMag-L with the tubes downwards twice to release drops captured in the cap.

7d. Place the magnet slider into the AdnaMag-L and incubate for 1 minute at room temperature.

7e. Remove supernatant completely with a pipette.

7f. Repeat steps 7a to 7e twice (three washes in total).

8. Remove the magnet slider from the AdnaMag-L.

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 with an inserted magnet slider.

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

11. After 1 minute remove the supernatant completely with a pipette to optimize the following cell lysis.

12. Remove the magnet slider from the AdnaMag-S.

13. Add 200 µl AdnaTest Lysis/Binding Buffer (equilibrated to room temperature) to each reaction tube. Resuspend by pipetting at least five times.

14. Insert the magnet slider into the AdnaMag-S, and incubate for 1 minute.

15. Transfer supernatant (cell lysate) into new 1.5 ml reaction tubes (provided).

16. Discard the tubes with the beads.

17. Continue with mRNA isolation (see “Protocol: Detection of Breast Cancer Associated Gene Expression in Enriched Tumor Cells Using AdnaTest BreastCancerDetect”, page 16) immediately, or store the cell lysates at –20°C for a maximum of 2 weeks.

Protocol: Detection of Breast Cancer Associated Gene Expression in Enriched Tumor Cells Using AdnaTest BreastCancerDetect

Important points before starting

- Before beginning the procedure, read "Warnings and Precautions", (page 10) and "Reagent Storage and Handling" (page 11).
- Procedures A to C describe the isolation of mRNA and reverse transcription.
- Please use the provided 1.5 ml collection tubes only for the protocol step indicated.

Things to do before starting

- Ensure that AdnaTest Lysis/Binding Buffer is equilibrated to room temperature. If a 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-HCL 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 50°C.

Procedure A: Preparation of Oligo(dT)$_{25}$ Beads

1. Resuspend the Oligo(dT)$_{25}$ Beads thoroughly by pipetting before use; do not vortex!
2. 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).

3. Place the tube into the AdnaMag-S.

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

4. After 1 minute remove the supernatant with a pipette.
5. Wash steps:
   5a. Remove the magnet slider from the AdnaMag-S.
   5b. Add the original volume (step 2, page 16) AdnaTest Lysis/Binding Buffer and resuspend the beads by repeated pipetting. Resuspend gently to avoid foaming.
   5c. Insert the magnet slider into the AdnaMag-S.
   5d. After 1 minute remove the supernatant completely.
   5e. Repeat steps 5a to 5d once (two washes in total).
6. Remove the tube from the AdnaMag-S, and resuspend the beads in AdnaTest Lysis/Binding Buffer to the original volume (step 2, page 16). Proceed with "Procedure B: mRNA isolation".

Procedure B: mRNA isolation

1. Add 20 µl of Oligo(dT)$_{25}$ Beads (step 6, above) to each tube containing cell lysate (step 15, page 15).
2. Rotate tubes slowly (approximately 5 rpm) for 10 minutes at room temperature on a device allowing both tilting and rotation.
3. Place the tubes into the AdnaMag-S without the magnet slider. Swing the AdnaMag-S downwards to release beads and liquid captured in the cap.
4. Insert the magnet slider and remove the supernatant after 1 minute.
5. Wash steps 1:
   5a. Remove the magnet slider from the AdnaMag-S.
   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.

5d. After 1 minute remove the supernatant completely.

5e. Repeat steps 5a to 5d once (two washes in total).

6. Wash steps 2:

   6a. Remove the magnet slider from the AdnaMag-S.

   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.

   6d. After 1 minute remove the supernatant completely. This step must be carried out carefully (watch the pellet) since the beads may slide and could be removed by mistake.

   6e. Repeat steps 6a to 6d once in the same reaction tubes (two washes in total).

7. Remove the magnet slider from the AdnaMag-S.
8. Add 100 µl ice cold Tris-HCL Buffer to each tube, and resuspend the beads by pipetting.
9. Insert the magnet slider into the AdnaMag-S.
10. After 1 minute remove the supernatant completely.
11. Remove the magnet slider from the AdnaMag-S.
12. Resuspend the mRNA/bead-complex in 29.5 µl RNase-free water.
13. Transfer the tubes to a thermal block or water bath, and incubate for 5 minutes at 50°C.
14. Place the tubes on ice immediately for at least 2 minutes.
15. Continue immediately (within 5 minutes) with reverse transcription (Procedure C: Reverse transcription using the Sensiscript RT Kit).

    Note: Do not store the mRNA/bead complex!

Procedure C: 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 negative control reaction without addition of mRNA must always be prepared (RT control).

2. Vortex the RT master mix. Centrifuge briefly and pipet 10.5 µl for each reaction into 0.2 ml PCR tubes.
3. Resuspend the mRNA/bead complexes (step 14, above) 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	4.0 µl
dNTP Mix (5 mM each dNTP)	4.0 µl
RNase inhibitor, 40 U/µl (Promega)	0.5 µl
Sensiscript Reverse Transcriptase	2.0 µl
Template RNA*	
mRNA/bead complex or RNase free water	29.5 µl
Total volume	**40.0 µl**

* As RT control, add 29.5 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. cDNA is synthesized in a thermal cycler under the following conditions (Table 2).

Table 2. Reverse transcription program

Temperature	Time
37°C	60 minutes
93°C	5 minutes
4°C	∞

5. Place reaction tubes with the cDNA on ice or store at –20 °C for a maximum of 4 weeks.

Continue with "Protocol: Multiplex PCR (AdnaTest BreastCancerDetect)", page 20.

Protocol: Multiplex PCR (AdnaTest BreastCancerDetect)

Important point before starting

- Before beginning the procedure, read "Warnings and Precautions" (page 10) and "Reagent Storage and Handling" (page 11).

Things to do before starting

- Thaw HotStarTaq Master Mix (QIAGEN), AdnaTest PrimerMix BreastDetect, AdnaTest Positive Control Breast and RNase-free water. Vortex, centrifuge quickly and store on ice.

Multiplex PCR

1. The PCR master mix is prepared as shown in Table 3 according to the number of samples.

   The volume calculation of the PCR master mix should include at least 10% excess volume. Note that an AdnaTest Positive Control Breast, RNase-free water as negative control and the RT control must always be included.

2. For each preparation dispense 42.0 µl of the PCR master mix into 0.2 ml PCR reaction tubes. Resuspend the cDNA/bead mix by pipetting and add 8.0 µl of this to each reaction tube.

   Note: As negative control add 8.0 µl of RNase-free water instead of cDNA.

Table 3. Preparation of the multiplex PCR

Component	Volume
Multiplex PCR master mix	
HotStarTaq Master Mix	25 µl
RNase-free water	13 µl
AdnaTest PrimerMix BreastDetect	4 µl
cDNA or RT control or Negative control (RNase-free water) or AdnaTest Positive Control (C+) each:	8 µl
Total volume	**50 µl**

3. A thermal cycler is used for the PCR following the program described in Table 4. Run the thermal cycler with a ramp of 2°C/second. The PCR is performed with a total of 35 cycles.

Table 4. PCR cycling program

	Temperature	Time
Initial activation step	95°C	15 minutes
3-step cycling (35 cycles)		
Denaturation	94°C	30 seconds
Annealing	60°C	30 seconds
Extension	72°C	60 seconds
Number of cycles:	35	
Final extension:	72°C	10 minutes
Cool-down:	4°C	∞

Interpretation of Results

Fragment analysis on the Agilent 2100 Bioanalyzer

Analysis is performed with the Agilent 2100 Bioanalyzer (Agilent Technologies) on a DNA 1000 LabChip®. Follow the instructions of the DNA 1000 LabChip manual and make sure that no beads are transferred into the LabChip. Magnetic beads in the gel may cause invalid results.

1. Start the Bioanalyzer software **2100 expert**. Select **Instrument** under **Contexts** and then click the **Assay** button next to **Assay Selection**.
2. Select **Electrophoresis > DNA 1000 Series II.xsy**. Prepare the chip and start the run.
3. For evaluation of the results, set a detection threshold:
   3a. Select **Data** under **Contexts** and then click the **Assay Properties** tab. Select **Global** and **Normal** from the drop-down menu on the right.
   3b. Select **Sample Setpoints > Integrator > height threshold (FU)** and set this value to **0** (default value is **20**) to detect all signals.

Analysis of the results for AdnaTest BreastCancerDetect

The test is considered positive if a PCR fragment of at least one tumor associated transcript (GA733-2, Muc-1 or Her-2) is clearly detected.

Using the Agilent 2100 Bioanalyzer, peaks with a concentration of ≥0.15 ng/µl are positive (Figure 3).

The fragment of the control gene actin must be detected in all patient samples (internal PCR control). An actin signal provides a positive control for successful cell separation, reverse transcription and multiplex PCR. Negative control and RT control samples must not show any bands larger than 80 base pairs (primer-dimers).

A fragment larger than 1000 bp indicates contamination with genomic DNA. The separation process was not successful and the results are invalid in this case.

IMPORTANT: If the protocol is not followed exactly, this may result in false-negative or false-positive results.

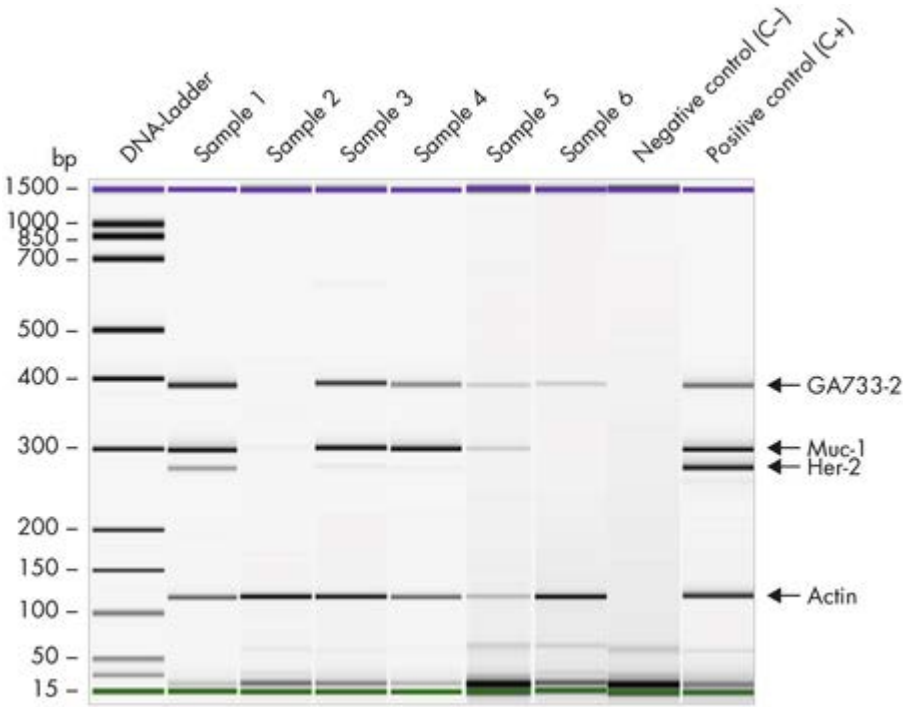

Figure 3. AdnaTest BreastCancerDetect results of multiplex PCR samples analyzed with an Agilent 2100 Bioanalyzer. The first lane shows the DNA size standard (DNA-Ladder). Sample 1 is positive for GA733-2, Muc-1 and Her-2. Samples 3, 4 and 5 are positive for GA733-2. Muc-1 in sample 2 is negative. The only band detected in sample 6 is for GA733-2. Actin is detected in samples 1 to 6. The PCR negative (C-) and positive control (C+) are shown in the last two lanes (Agilent 2100 Bioanalyzer).

Troubleshooting guide

See 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**).

Quality Control

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

Limitations

All reagents may exclusively be used in in vitro diagnostics.

The product is only to be used by personnel specially instructed and trained in in vitro diagnostics procedures.

It is important that the operator reads the instructions for use thoroughly before using the system.

Strict compliance with the instructions for use is required for optimal PCR results.

Check the expiration dates printed on the box and labels of all components. Do not use components beyond their expiration date.

Any diagnostic results that are generated must be interpreted in conjunction with other clinical or laboratory findings.

Performance Characteristics

Recovery

Two and 5 cultured MCF7 breast cancer cells were spiked into blood samples from healthy donors to determine the recovery rates achieved with AdnaTest BreastCancerSelect/Detect (Table 7).

Table 7. AdnaTest BreastCancerSelect/Detect recovery rate of tumor cells spiked into blood samples from healthy donors

	Number of positives	Total number of samples
Two tumor cells spiked into 5 ml blood	159 (91%)	175
Five tumor cells spiked into 5 ml blood	168 (98%)	171

The recovery rate is 91% for detection of 2 tumor cells spiked into 5 ml of blood from healthy donors. Five cells in 5 ml of blood from healthy donors can be successfully detected in 98% of all cases.

Specificity

AdnaTest BreastCancerSelect/Detect was used to analyze 233 healthy donors to determine the rate of false-positives at the given cut-off (0.15 ng/µl fragment concentration for each gene profile included, except for actin). This demonstrated a specificity of 97% for AdnaTest BreastCancerSelect/Detect (Table 8).

Table 8. Determination of specificity

Controls	Total number of samples	Number of false positives	Specificity (%)
Healthy donors	233	7 (3%)	97

Reproducibility

Twenty blood samples from healthy donors were spiked with 10 MCF-7 breast cancer cells per sample. Blood samples were analyzed by two operators using AdnaTest BreastCancerSelect/Detect to determine the reproducibility. The intra-assay and the inter-assay reproducibility were 100% (Table 9).

Table 9. Reproducibility of AdnaTest BreastCancerSelect/Detect

Operator	Positive AdnaTest result/samples	Intra-assay reproducibility (%)	Inter-assay reproducibility (%)
A	10/10	100	100
B	10/10	100	100

Precision

To determine the precision, aliquots of cDNA were pooled and analyzed using AdnaTest BreastCancerDetect. Two operators analyzed 30 cDNA samples, consisting of 3 independent measurements of 10 samples. The intra-assay and inter-assay precision were 100% (Table 10).

Table 10. Precision of AdnaTest BreastCancerDetect

Operator	Positive AdnaTest result/samples	Intra-assay precision (%)	Inter-assay precision (%)
A	30/30	100	100
B	30/30	100	100

Interfering substances

Anticoagulants

When drawing and transporting blood, use of anticoagulants is mandatory. However, heparin and citrate lead to aggregate formation after addition of AdnaTest immunomagnetic beads, which can result in a lack of test results or false test results. However, EDTA and ACDA (citrate/dextrose/adenine solution A) are compatible with AdnaTest immunomagnetic beads.

Hemolysis

Hemolysis in blood samples (plasma fraction appears red) is, in most cases, due to incorrect transportation or storage conditions. Such samples may give false-negative results and should be discarded.

Chemotherapeutics, targeted therapy drugs and anti-hormonal regimens

Chemotherapeutics (e.g., taxanes, cisplatin, oxaliplatin, 5-FU, anthracycline, irinotecan etc.) are potent cytotoxins and cause damage or rapid cell death in a blood sample. This results in a high likelihood of false-negative results when using AdnaTest immunomagnetic beads. After administration of these substances, the human body needs around 5–7 days to detoxify them Table 9. Blood samples drawn during this time must not be used with AdnaTest immunomagnetic beads.

Table 9. Half-lives of chemotherapeutics

Drug	Half life	Reference
5-Fluouracil	Up to 20 minutes	**www.drugs.com/pro/fluorouracil-injection.html**
Docetaxel	Up to 11.1 hours	**www.drugs.com/pro/docetaxel.html**
Cis-platinum	Up to 30 minutes	**www.drugs.com/pro/cisplatin.html**
Carbo-platinum	Up to 5.9 hours	**www.drugs.com/pro/carboplatin.html**
Paclitaxel	Around 25.4 hours	**www.drugs.com/pro/paclitaxel.html**

The same precaution is also recommended for targeted therapy regimens such as antibodies (Herceptin®, bevacizumab, cetuximab etc.), tyrosine kinase blockers (e.g., olaparib, Iressa®, Erbitux®, lapatinib, etc.) and anti-hormonal drugs (e.g., tamoxifen, abiraterone, enzalutamide, etc.) administered as a single drug or in combination with chemotherapeutics.

In clinical trials demonstrating the prognostic value of circulating tumor cells (CTC) identified and characterized using AdnaTest immunomagnetic beads, no negative interference of chemotherapeutics, targeted therapies or anti-hormonal therapies was observed, provided the waiting period of at least 7 days after administration of the drug was complied with. Furthermore, a negative impact of common co-medications (e.g., Aspirin, ibuprofen, aprepitant, steroids, etc.) is unlikely but is being monitored.

Interfering conditions

Blood clotting

In the context of clinical trials, we observed blood clotting after incubation with AdnaTest immunomagnetic beads – most frequently in blood samples from patients in a late disease state. Blood samples that exhibit clotting are difficult to process during the AdnaTest workflow due to increased viscosity and are difficult to pipet. They also contain an unacceptably high number of contaminating leukocytes, which leads to false-positive results. Such samples must be discarded.

Benign organic disease and chronic inflammatory conditions

Benign organic disease and chronic inflammation, such as arthritis, benign prostatic hyperplasia (BPH), Crohn's disease etc., do not lead to false-positive AdnaTest results.

Acute allergy

With acute allergic conditions, there is an increased number of contaminating leucocytes after CTC enrichment using AdnaTest immunomagnetic beads. Therefore, false-positive results cannot be fully excluded.

Clinical studies

In a performance evaluation study conducted at the Clinics for Gynecology and Obstetrics, University of Essen, Germany (Tewes et al. 2009), patients with metastatic breast cancer were tested using AdnaTest immunomagnetic beads and followed up during therapy. Of 42 patients enrolled to date, circulating tumor cells persisted during therapy in 52% (22/42) of patients. In an analysis of overall survival, a marked difference in survival for the AdnaTest positive and AdnaTest negative cohorts was observed – log-rank p=0.005 (Figure 1).

Reference

Tewes, M. et al. (2009) Molecular profiling and predictive value of circulating tumor cells in patients with metastatic breast cancer: an option for monitoring response to breast cancer related therapies. Breast Cancer Res Treat. **115**, 581.

Abbreviations

AdnaMag-L	Magnetic particle concentrator (-large)
AdnaMag-S	Magnetic particle concentrator (-small)
bp	Base pairs
C+	Positive control
C–	Negative control
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
GA733-2	Gastrointestinal tumor associated antigen 733-2
Her-2	Human epidermal growth factor receptor 2
kb	kilobases
mRNA	Messenger ribonucleic acid
Muc-1	Muc-1 gene
PCR	Polymerase chain reaction
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcription

Symbols

	Contains reagents sufficient for <N> tests
	Use by
	Temperature limitation
	Catalog number
	Consult instructions for use
	Manufacturer
	In vitro diagnostic medical device
	Material number
	Global Trade Item Number

Ordering Information

Product	Contents	Cat. no.
AdnaTest BreastCancerSelect	For isolation of CTCs and the subsequent extraction of mRNA from human whole blood for 12 preparations	395412
AdnaTest BreastCancerDetect	RT-PCR kit for detection of breast cancer associated gene expression in enriched tumor cells	396412
Related products		
AdnaTubes	12 sample tubes containing EDTA. Use only with anticoagulated blood collected in A-CDA blood collection tubes from BD	399932
AdnaMag-L	For 8 tubes, 15 ml	399921
AdnaMag-S	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
HotStarTaq Master Mix Kit (250 U)	3 x 0.85 ml HotStarTaq Master Mix (contains 250 units HotStarTaq DNA Polymerase, PCR Buffer with 3 mM $MgCl_2$, and 400 µM of each dNTP) and 2 x 1.7 ml RNase-Free Water	203443

* The Sensiscript RT Kit (50) is sufficient for only 25 samples using AdnaTest BreastCancerDetect because twice the volume is required for each reaction.

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.

Handbook Revision History

Document	Changes	Date
HB-2397-001	Initial release	March 2017
HB-2397-002	Correction in "Protocol: Detection of Breast Cancer Associated Gene Expression in Enriched Tumor Cells Using AdnaTest BreastCancerDetect". The volume of RNase-Free Water in step 12 of "Procedure C: Reverse transcription using the Sensiscript RT Kit" was corrected from14.75 µl to 29.5 µl.	March 2018

Limited License Agreement for AdnaTest BreastCancerSelect and AdnaTest BreastCancerDetect

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the protocols provided with the product, this handbook, and additional protocols available at **www.qiagen.com**. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see **www.qiagen.com.**