April 2020

AdnaTest EMT-2/ StemCell Handbook

For enrichment of tumor cells in whole blood for cancer research and detection of cancer-associated gene expression in enriched tumor cells

REF 397082 (AdnaTest EMT-2/StemCell)

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Sample to Insight

QIAGEN

Contents

Kit Contents

AdnaTest EMT-2/StemCell	
Cat. no.	**397082**
Number of tests	**12**
AdnaTest RNA Reagent	**Box 1**
AdnaTest Lysis/Binding Buffer	1 x 2 ml
Oligo(dT)$_{25}$ Beads	1 x 355 µl
RNA Purification Buffer A	1 x 4 ml
RNA Purification Buffer B	1 x 4 ml
Tris-HCL Buffer	1 x 2 ml
AdnaTest CTC-Select	**Box 2**
Collection Tubes (15 ml)	24
Collection Tubes (1.5 ml)	3 x 5
CTC-Select Beads	1 x 1.2 ml
AdnaTest Lysis/Binding Buffer	1 x 2 ml
AdnaTest EMT-2/StemCellDetect	**Box 3**
PrimerMix EMT-2-Detect	1 x 144 µl
Positive Control EMT-2	1 x 40 µl
PrimerMix StemCellDetect	1 x 144 µl
Positive Control StemCell	1 x 40 µl
AdnaWash Leukocyte Reducer	**Box 4**
AdnaTest Leukocyte Reducer	2 x 125 ml

The AdnaTest EMT-2/StemCell reagents are sufficient to analyze 6 PCR controls and 12 blood samples.

Shipping and Storage

The AdnaTest EMT-2/StemCell system is delivered in 4 boxes.

AdnaTest RNA Reagent Box 1 (Box 1) and AdnaTest CTC-Select Box 2 (Box 2) should be stored at 2–8°C. The components must not be used beyond the expiration date.

AdnaTest EMT-2/StemCellDetect Box 3 (Box 3), containing the AdnaTest PrimerMix EMT-2/StemCellDetect and the AdnaTest PositiveControl EMT-2 and AdnaTest PositiveControl StemCell, and AdnaWash Leukocyte Reducer Box 4 (Box 4) must be stored separately at –30 to –15°C. However, for short-term and medium-term storage, AdnaWash Leucocyte Reducer can be stored at 2–8°C. To prevent possible contamination and repeated temperature changes, aliquot the primer mixes, positive controls, and AdnaWash Leukocyte Reducer. The components must not be used beyond the expiration date.

Intended Use

AdnaTest EMT-2/StemCell 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 the 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.

Abbreviations

AdnaMag-L	magnetic particle concentrator (large)
AdnaMag-S	magnetic particle concentrator (small)
Akt-2	protein kinase B
ALDH1	aldehyde dehydrogenase 1
bp	base pairs
CA-125	cancer antigen 125
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EGFR	epidermal growth factor receptor
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
PI3Kα	phosphoinositol-3-kinase
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
TWIST1	transcription factor

Symbols


Use by


Temperature limitation

Catalog number


Consult instructions for use


Manufacturer

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 EMT-2/StemCell is tested against predetermined specifications to ensure consistent product quality.

Introduction

The AdnaTest EMT-2/StemCell system is used for the analysis of epithelial-mesenchymal transition (EMT) and cancer cell stemness characteristics. Tumor cells are immunomagnetically enriched from whole blood using the AdnaTest CTC-Select Beads, and then EMT and StemCell markers are used for molecular characterization. The specificity of the detection is at least 90%. Using EMT-2 markers in spiking experiments, 30 tumor cells in 5 ml of whole blood are detected at a recovery rate of at least 70%. Using a StemCell marker in spiking experiments, 10 tumor cells in 5 ml blood are detected at a recovery rate of at least 70%.

Successful circulating tumor cell (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. Combining a highly specific immunomagnetic cell selection system using an optimized antibody combination with highly sensitive RT-PCR technology using a combination of mRNA tumor markers, the highest degrees of specificity and sensitivity can be expected. The AdnaTest uses a 2-step process (select and detect) to generate results within 5 hours.

AdnaTest EMT-2/StemCell

AdnaTest CTC-Select 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) using AdnaWash Leukocyte Reducer, which reduces leukocyte cross-reactions, and are subsequently lysed (Figure 1). The cell lysate is used for further analysis.

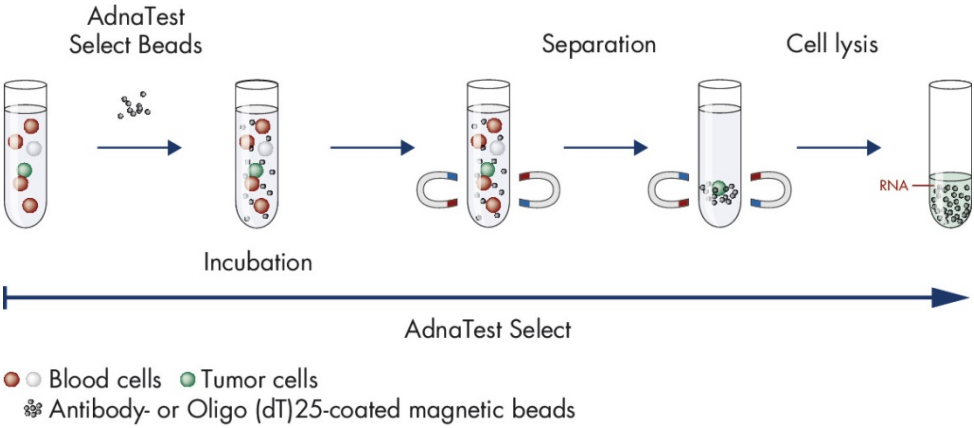

Figure 1. AdnaTest EMT-2/StemCell: Immunomagnetic cell selection with multiple tumor-associated antibodies. In the first step, the CTCs in the blood are enriched (AdnaTest Select). This is achieved using antibody-coated magnetic particles (beads). Several antibodies are used, which bind with high specificity and affinity to the corresponding cancer cells. The enriched cells are lysed and subsequently purified several times to extract mRNA.

In the second step, Oligo $(dT)_{25}$ Beads are used for the isolation of mRNA from the lysate of 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 EMT-2-Detect allows the amplification of 3 tumor-associated antigens and 1 control gene. The PrimerMix StemCellDetect allows the amplification of 1 tumor-associated antigen (Figure 2).

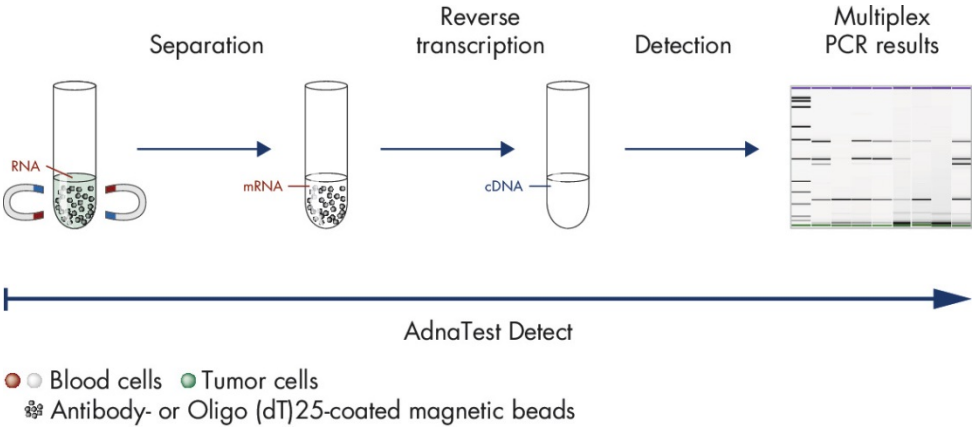

Figure 2. AdnaTest EMT-2/StemCell: Multiplex PCR of various cancer-associated tumor markers. In a second step the enriched cells are examined by RT-PCR for tumor-associated expression patterns. The mRNA strands are reverse transcribed into cDNA. Subsequently, several associated tumor markers can be amplified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

The PrimerMix EMT-2-Detect generates fragments of the following sizes:

- PI3Kα: 551 bp
- Akt-2: 309 bp
- TWIST1: 201 bp
- Actin: 120 bp (internal PCR control)

The PrimerMix StemCellDetect generates a fragment of the following size:

- ALDH1: 161 bp

Note: Fragment sizes may vary slightly. Make sure to use the AdnaTest Positive Control EMT-2 and AdnaTest Positive Control StemCell for assignment of the detected signals.

Optionally, genes associated with breast, colon, prostate and ovarian cancer can also be analyzed using EMT-2 Add-ons (see "Ordering Information", page 48).

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 EMT-2/StemCell

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)
- 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)

Material

- AdnaTube (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)
- Sterile, RNase-free thin-wall 0.2 ml PCR tubes
- Pipettes and RNase-free pipette tips with aerosol barrier, suitable for pipetting volumes from 100 µl to 1000 µl
- Pipettes and RNase-free pipette tips with aerosol barrier, suitable for pipetting volumes from 1 µl to 200 µl

Reagents

- Phosphate buffered saline (PBS), pH 7.0–7.3 (e.g., Fisher cat. no. VX14190169, D-PBS)
- Sensiscript® RT Kit (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, 2.500 U (Promega cat. no. N2511)
- HotStarTaq® Master Mix Kit (cat. no. 203443, 250 U)
- Crushed ice

Important Notes

Sample preparation

- Blood samples must be taken before the application of therapeutic substances. Do not use the AdnaTest EMT-2/StemCell 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 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, cat. no. 399932.
- Blood must be stored at 2–8°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.

Handling

- CTC-Select 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 EMT-2/StemCell”, page 15.)
- All components and additional reagents provided by other suppliers must be stored according to their instructions. Safety advice of the respective manufacturers applies.
- Wear protective gloves to avoid contamination with DNA, RNA and RNases.
- Aliquot the CTC-Select Beads to avoid contamination.

- 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 CTC-Select 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.
- The safety and hygiene regulations of the laboratory must be respected (e.g., wear lab coats, protective goggles, gloves).

Protocol: Enrichment of Tumor Cells Using AdnaTest EMT-2/StemCell

Important points before starting

- Before beginning the procedure, read "Important Notes" (page 13).
- It is necessary to remove sodium azide by washing the CTC-Select Beads prior to use, as described below in "Procedure A: Preparation of the CTC-Select Beads".
- Please use the provided 1.5 ml collection tubes only for the protocol step indicated.

Things to do before starting

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

Procedure A: Preparation of the CTC-Select Beads

1. Resuspend the CTC-Select Beads thoroughly by pipetting.

   Important: Do not vortex.

2. Calculate the volume of CTC-Select 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.

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 steps:
   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 to 5d twice (three 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 "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 CTC-Select 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 min at room temperature on a device allowing both tilting and rotation.
4. Place tubes into the AdnaMag-L rack without the magnet slider. Swing the AdnaMag-L rack downwards to release blood drops 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 the supernatant completely with a 10 ml pipette without touching the beads.

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

7. Wash steps:
   7a. Remove the magnet slider from the AdnaMag-L rack.
   7b. Add 5 ml AdnaWash Leukocyte Reducer. 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 downwards twice to release drops captured in the cap.
   7d. Place the magnet slider into the AdnaMag-L rack and incubate for 3 min 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 rack.
9. Resuspend the magnetic bead/cell complexes in 1 ml AdnaWash Leukocyte Reducer and transfer each sample into a 1.5 ml reaction tube.
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 two 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 3 min remove the supernatant with a pipette.
12. Remove the magnet slider from the AdnaMag-S rack.
13. Resuspend the magnetic bead/cell complexes in 1 ml PBS
14. Place the magnet slider into the AdnaMag-S rack.
15. After 1 min remove the supernatant completely with a pipette to optimize the following cell lysis.
16. Remove the magnet slider from the AdnaMag-S rack.
17. Add 200 µl AdnaTest Lysis/Binding Buffer (equilibrated to room temperature) to each reaction tube. Resuspend by pipetting at least five times.
18. Insert the magnet slider into the AdnaMag-S rack, and incubate for 1 min.

19. Transfer each supernatant (cell lysate) into a new 1.5 ml reaction tube.
20. Discard the tubes with the beads.
21. Continue with mRNA isolation (see "Protocol: Detection of Cancer-Associated Gene Expression in Enriched Tumor Cells Using AdnaTest EMT-2/StemCell", page 19) immediately, or store the cell lysates at –30 to –15°C for a maximum of 2 weeks.

Protocol: Detection of Cancer-Associated Gene Expression in Enriched Tumor Cells Using AdnaTest EMT-2/StemCell

Important points before starting

- Before beginning the procedure, read "Important Notes" (page 13).
- Procedures A–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 precipitate is observed, equilibrate the reagents 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.

   Important: 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 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.

4. After 1 min, remove the supernatant with a pipette.

5. Wash steps:

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

   5b. Add the original volume (step 2, page 19) 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 rack.

   5d. After 1 min, remove the supernatant completely.

   5e. Repeat steps 5a–5d once (2 washes in total).

6. Remove the tube from the AdnaMag-S rack, and resuspend the beads in AdnaTest Lysis/Binding Buffer to the original volume (step 2, page 19). 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 18).

2. Rotate tubes slowly (approximately 5 rpm) for 10 min at room temperature on a device allowing both tilting and rotation.

3. Place the tubes into the AdnaMag-S rack without the magnet slider. Swing the AdnaMag-S rack downwards 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 steps 1:
   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 steps 2:
   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 has to be carried out carefully (watch the pellet), because the beads might slide and could be 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-HCL 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 29.5 µl RNase-free water.
13. Transfer the tubes to a thermal block or water bath, and incubate for 5 min at 50°C.
14. Place the tubes on ice immediately for at least 2 min.
15. Continue immediately (within 5 min) with the reverse transcription (Procedure C: Reverse transcription using the Sensiscript RT Kit).

    Important: 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 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).

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 of RNase-free water instead of mRNA/bead complex. The volume of the mRNA/bead complex may vary slightly. Always use the total volume of this in the reverse transcription reaction.

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 10, page 21) 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.

4. The cDNA is synthesized in a thermal cycler under the following conditions (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 –30 to –15°C for a maximum of 4 weeks.
6. Continue with “Protocol: PCR and Fragment Analysis”, page 24.

Protocol: PCR and Fragment Analysis

Important point before starting

- Before beginning the procedure, read "Important Notes" (page 13).

Things to do before starting

- Thaw HotStarTaq Master Mix, AdnaTest Positive Control EMT-2, AdnaTest Positive Control StemCell, AdnaTest PrimerMix EMT-2-Detect, AdnaTest PrimerMix StemCellDetect, and RNase-free water. Vortex, centrifuge quickly, and store on ice.

Procedure A: Multiplex PCR (EMT-2)

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

   The volume of the PCR Master Mix should be at least 10% greater than the requirement calculated from the number of samples. Note that an AdnaTest Positive Control, RNase-free water as negative control, and the RT control must always be included.

Table 3. Preparation of the multiplex PCR

Component	Volume
Multiplex PCR Master Mix	
HotStarTaq Master Mix	12.5 µl
RNase-free water	4.5 µl
PrimerMix EMT-2-Detect	4 µl
cDNA *or* RT control *or* Negative control (RNase-free water) *or* Positive control, each:	4 µl
Total volume	**25 µl**

2. For each preparation, dispense 21.0 µl of the PCR Master Mix into 0.2 ml PCR reaction tubes. Resuspend the cDNA/bead mix by pipetting, and then add 4.0 µl of it to each reaction tube.

   Note: For negative control, add 4.0 µl of RNase-free water instead of cDNA.

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 (EMT-2)

Step	Time	Temperature
Initial activation step	15 min	95°C
3-step cycling (35 cycles)		
Denaturation	30 s	94°C
Annealing	30 s	60°C
Extension	60 s	72°C
Final extension	10 min	72°C
Cooling	∞	4°C

Procedure B: Singleplex PCR (StemCell)

1. The PCR Master Mix is prepared as shown in Table 5 according to the number of samples.

   The volume of the PCR Master Mix should be at least 10% greater than the requirement calculated from the number of samples. Note that an AdnaTest Positive Control, RNase-free water as negative control, and the RT control must always be included.

2. For each preparation, dispense 21.0 µl of the PCR Master Mix into 0.2 ml PCR reaction tubes. Resuspend the cDNA/bead mix by pipetting, and then add 4.0 µl of it to each reaction tube.

   Note: For negative control, add 4.0 µl of RNase-free water instead of cDNA.

Table 5. Preparation of the singleplex PCR

Component	Volume
Multiplex PCR Master Mix	
HotStarTaq Master Mix	12.5 µl
RNase-free water	4.5 µl
PrimerMix StemCell	4 µl
cDNA *or* RT control *or* Negative control (RNase-free water) *or* Positive control, each:	4 µl
Total volume	**25 µl**

3. A thermal cycler is used for the PCR following the program described in Table 6. Run the thermal cycler with a ramp of 2°C/second. The PCR is performed with a total of 35 cycles.

Table 6. PCR cycling program (StemCell)

Step	Time	Temperature
Initial activation step	15 min	95°C
3-step cycling (35 cycles)		
Denaturation	30 s	94°C
Annealing	30 s	51°C
Extension	30 s	72°C
Final extension	5 min	72°C
Cooling	∞	4°C

Fragment analysis on the Agilent 2100 Bioanalyzer

Perform analysis with the Agilent 2100 Bioanalyzer on a DNA 1000 LabChip®. Follow the instructions in the DNA 1000 LabChip manual and make sure that no beads are transferred into the LabChip. Magnetic beads in the gel can cause false 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. Under **Contexts**, select **Data** 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 StemCell results

The test is considered positive if a PCR fragment of at least 1 tumor-associated transcript is clearly detected.

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

The fragment of the control gene actin must show in all test samples (internal PCR control). An actin signal provides a positive control for a 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, suggesting that a problem occurred during cell separation. 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.

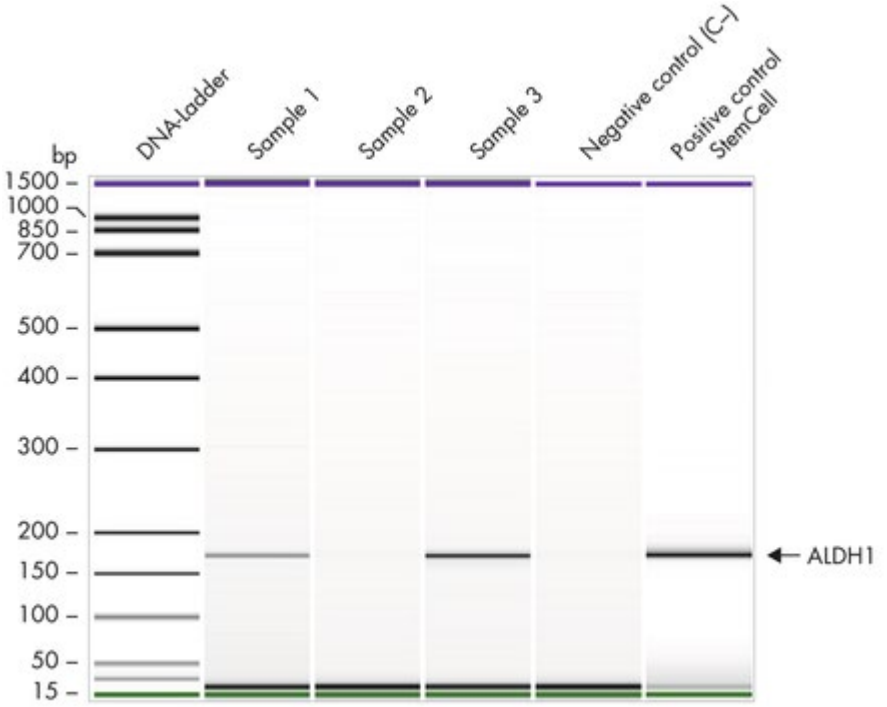

Figure 3. AdnaTest EMT-2/StemCellDetect results of samples using an Agilent 2100 Bioanalyzer: Tumor stem cell-associated gene expression. The first lane shows the DNA size standard (DNA-Ladder). Samples 1 and 3 are positive for ALDH1, and sample 2 is negative. The PCR negative control (C–) and the positive control (StemCell) are shown in the last 2 lanes.

Analysis of the EMT-2 results

The test is considered positive if a PCR fragment of at least 1 transcript associated with EMT-2 (PI3Kα, Akt-2, or TWIST1) is clearly detected. When analysis is performed on the Agilent 2100 Bioanalyzer, peaks with a concentration ≥0.25 ng/µl are positive (Figure 4).

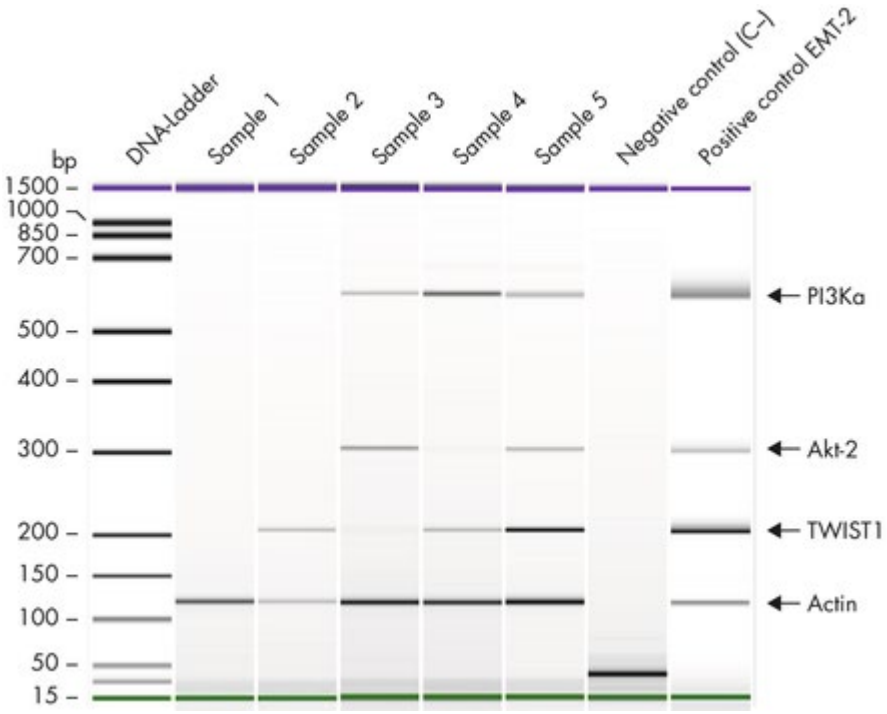

Figure 4. AdnaTest EMT-2/StemCellDetect results of samples using an Agilent 2100 Bioanalyzer: EMT-2–associated gene expression. The first lane shows the DNA size standard (DNA-Ladder). Sample 1 is negative. Sample 2 is positive for TWIST1, sample 3 is positive for Akt-2 and PI3Kα, and sample 4 is positive for PI3Kα and TWIST1. Signals for all 3 EMT-2 markers can be detected in sample 5, and actin is detected in samples 1–5. The PCR negative control (C–) and the positive control (EMT-2) are shown in the last 2 lanes.

The fragment of the control gene actin must be detected in all samples (internal PCR control). An actin signal provides a positive control for a successful cell separation, reverse transcription, and multiplex PCR. No bands larger than 80 bp may be detected in the negative control and RT control samples, because bands larger than 80 bp indicate primer–dimers.

A fragment larger than 1000 bp indicates contamination with genomic DNA, suggesting that a problem occurred during cell separation. 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.

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**).

Appendix A: AdnaTest EMT-2/StemCell Add-on BreastCancerDetect

This protocol requires the use of the EMT-2 Add-on BreastCancerDetect (ordered separately; cat. no. 396212).

Description

The PrimerMix BreastDetect allows amplification of 3 cancer-associated genes and 1 control gene. The primers generate fragments of the following sizes:

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

Note: Fragment sizes may vary slightly. Use the Positive Control Breast (C+) for assignment of the detected signals.

Thing to do before starting

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

Multiplex PCR

1. Prepare the PCR Master Mix as shown in Table 7 according to the number of samples.

   The volume of PCR Master Mix should be at least 10% greater than the requirement calculated from the number of samples. Note that an AdnaTest Positive Control Breast, RNase-free water as negative control (C–), and the RT control must always be included.

Table 7. Preparation of the multiplex PCR

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

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

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

3. Use a thermal cycler for the PCR following the program described in Table 8. Run the thermal cycler with a ramp of 2°C/second. The PCR is performed with a total of 35 cycles.

Table 8. Add-on BreastCancerDetect cycling conditions

Step	Time	Temperature
Initial activation step	15 min	95°C
3-step cycling (35 cycles)		
Denaturation	30 s	94°C
Annealing	30 s	60°C
Extension	60 s	72°C
Final extension	10 min	72°C
Cooling	∞	4°C

4. Continue with the protocol "Fragment analysis on the Agilent 2100 Bioanalyzer", page 27.

Analysis of Add-on BreastCancerDetect results

If you are using the Agilent 2100 Bioanalyzer, peaks with a concentration ≥0.15 ng/µl are positive (Figure 5).

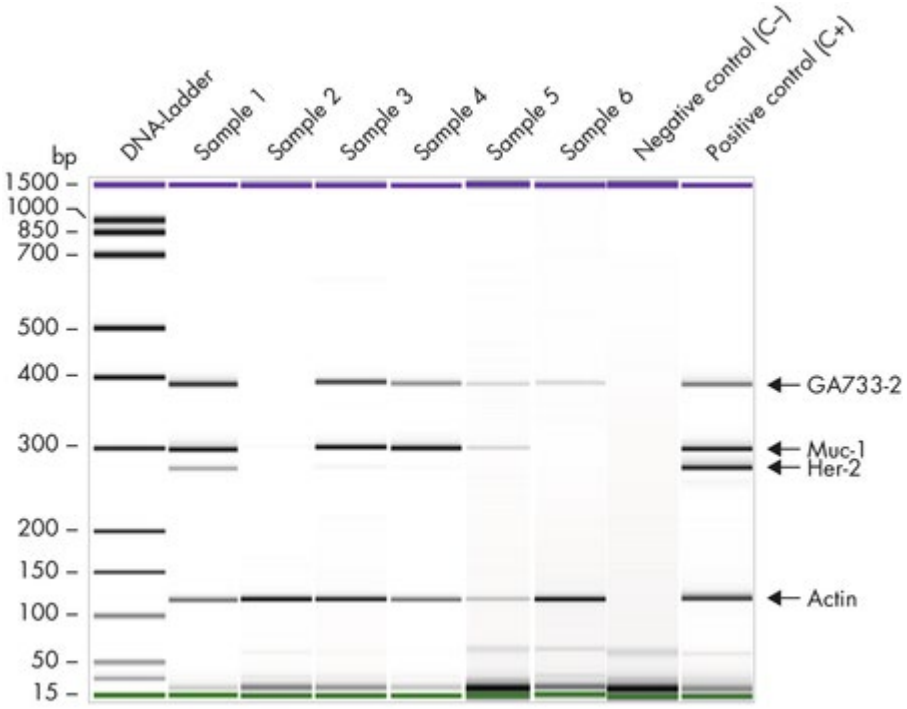

Figure 5. AdnaTest EMT-2/StemCell Add-on BreastCancerDetect: Results of samples analyzed with an Agilent 2100 Bioanalyzer: Breast cancer-associated gene expression. The first lane shows the DNA size standard (DNA-Ladder). Sample 1 is positive for GA733-2, Muc-1, and Her-2; samples 3, 4, and 5 are positive for GA733-2 and Muc-1; and sample 6 is positive for GA733-2. Sample 2 is negative. Actin is detected in samples 1–6.The PCR negative (C–) and positive control (C+) are shown in the last 2 lanes.

The fragment of the control gene actin must be detected in all samples (internal PCR control). An actin signal provides a positive control for multiplex PCR. No bands larger than 80 bp may be detected in the negative control and RT control samples, because bands larger than 80 bp indicate primer–dimers.

A fragment larger than 1000 bp indicates contamination with genomic DNA suggesting that a problem occurred during cell separation. 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.

Appendix B: AdnaTest EMT-2/StemCell Add-on ColonCancerDetect

This protocol requires the use of the EMT-2 Add-on ColonCancerDetect (ordered separately; cat. no. 396222).

Description

The PrimerMix ColonDetect allows amplification of 3 cancer-associated genes and 1 control gene. The primers generate fragments of the following sizes:

- GA733-2 395 bp
- CEA 231 bp
- EGFR 163 bp
- Actin 120 bp (internal control)

Note: Fragment sizes may vary slightly. Use the Positive Control Colon (C+) for assignment of the detected signals.

Thing to do before starting

- Thaw HotStarTaq Master Mix, AdnaTest Positive Control Colon (C+), AdnaTest PrimerMix ColonDetect, and RNase-free water. Vortex, centrifuge quickly, and store on ice.

Multiplex PCR

1. Prepare the PCR Master Mix as shown in Table 9 according to the number of samples. Prepare a volume of Master Mix 10% greater than that required for the total number of PCR reactions to be performed.

   Note: Always include Positive Control Colon (C+), RNase-free water as negative control (C–), and an RT control.

Table 9. Multiplex PCR setup

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

2. Dispense 42 µl Master Mix into 0.2 ml PCR reaction tubes. Resuspend the cDNA/bead mix by pipetting, and add 8 µl to each reaction tube.

   Note: For negative control, add 8 µl RNase-free water instead of cDNA.

3. Program the thermal cycler according to the manufacturer's instructions. PCR is carried out in a thermal cycler for 38 cycles using the cycling conditions described in Table 10. Use a ramp rate of 2°C/second.

Table 10. Add-on ColonCancerDetect cycling conditions

Step	Time	Temperature
Initial activation step	15 min	95°C
3-step cycling (38 cycles)		
Denaturation	45 s	94°C
Annealing	45 s	58°C
Extension	45 s	72°C
Final extension	10 min	72°C
Cooling	∞	4°C

4. Continue with the protocol "Fragment analysis on the Agilent 2100 Bioanalyzer", page 27.

Analysis of Add-on ColonCancerDetect results

If you are using the Agilent 2100 Bioanalyzer, peaks with a concentration ≥0.10 ng/µl are positive (Figure 6).

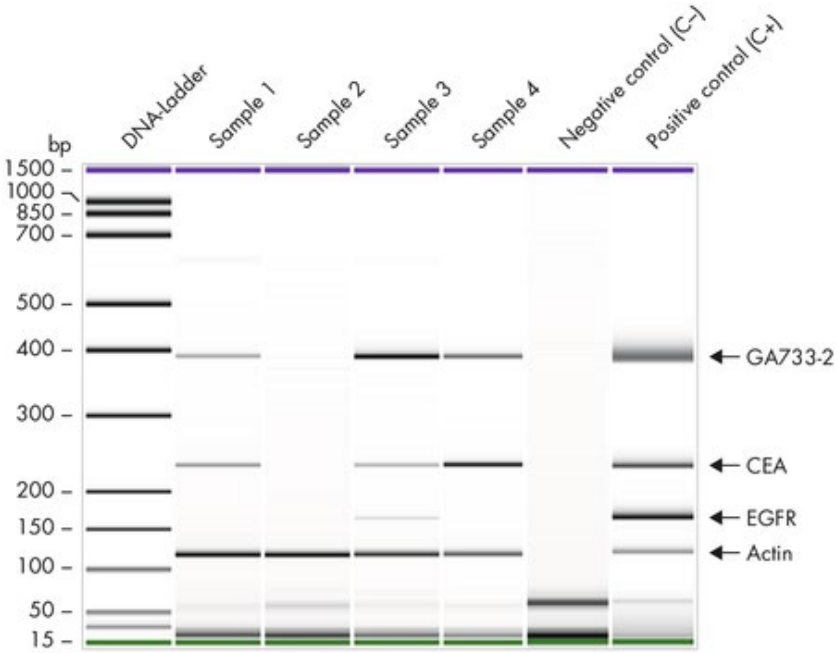

Figure 6. AdnaTest EMT-2/StemCell Add-on ColonCancerDetect: Results of samples analyzed with an Agilent 2100 Bioanalyzer. The first lane shows the DNA size standard (DNA-Ladder). Samples 1 and 4 are positive for GA733-2 and CEA, samples 2 is negative, and sample 3 is positive for GA733-2, CEA, and EGFR. Actin is detected in samples 1–4. The PCR negative (C–) and positive control (C+) are shown in the last 2 lanes.

The fragment of the control gene actin must be detected in all samples (internal PCR control). An actin signal provides a positive control for multiplex PCR. No bands larger than 80 bp may be detected in the negative control and RT control samples, because bands larger than 80 bp indicate primer–dimers.

A fragment larger than 900 bp indicates contamination with genomic DNA, which suggests that a problem occurred during cell separation. 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.

Appendix C: AdnaTest EMT-2/StemCell Add-on ProstateCancerDetect

This protocol requires the use of the EMT-2 Add-on ProstateCancerDetect (ordered separately; cat. no. 396232).

Description

The PrimerMix ProstateDetect allows amplification of 3 cancer-associated genes and 1 control gene. The primers generate fragments of the following sizes:

- PSMA 449 bp
- PSA 357 bp
- EGFR 163 bp
- Actin 120 bp (internal control)

Note: Fragment sizes may vary slightly. Use the Positive Control Prostate (C+) for assignment of the detected signals.

Thing to do before starting

- Thaw HotStarTaq Master Mix, AdnaTest Positive Control Prostate (C+), AdnaTest PrimerMix ProstateDetect, and RNase-free water. Vortex, centrifuge quickly, and store on ice.

Multiplex PCR

1. Prepare the PCR Master Mix as shown in Table 11 according to the number of samples. Prepare a volume of Master Mix 10% greater than that required for the total number of PCR reactions to be performed.

   Note: Always include Positive Control Prostate (C+), RNase-free water as negative control (C–), and an RT control.

Table 11. Multiplex PCR setup

Component	Volume
Multiplex PCR Master Mix	
HotStarTaq Master Mix	12.5 µl
RNase-free water	4.5 µl
PrimerMix ProstateDetect	4.0 µl
cDNA *or* RT control *or* Negative control (RNase-free water) *or* Positive Control Prostate (C+), each:	4.0 µl
Total volume	**25.0 µl**

2. Dispense 21 µl Master Mix into 0.2 ml PCR reaction tubes. Resuspend the cDNA/bead mix by pipetting and add 4 µl to each reaction tube.

   Note: For negative control, add 4 µl RNase-free water instead of cDNA.

3. Program the thermal cycler according to the manufacturer's instructions. PCR is carried out in a thermal cycler for 42 cycles using the cycling conditions described in Table 12. Use a ramp rate of 2°C/second.

Table 12. Add-on ProstateCancerDetect cycling conditions

Step	Time	Temperature
Initial activation step	15 min	95°C
3-step cycling (42 cycles)		
Denaturation	30 s	94°C
Annealing	30 s	61°C
Extension	30 s	72°C
Final extension	10 min	72°C
Cooling	∞	4°C

4. Continue with the protocol "Fragment analysis on the Agilent 2100 Bioanalyzer", page 27.

Analysis of Add-on ProstateCancerDetect results

If you are using the Agilent 2100 Bioanalyzer, peaks with a concentration ≥0.10 ng/µl are positive (Figure 7).

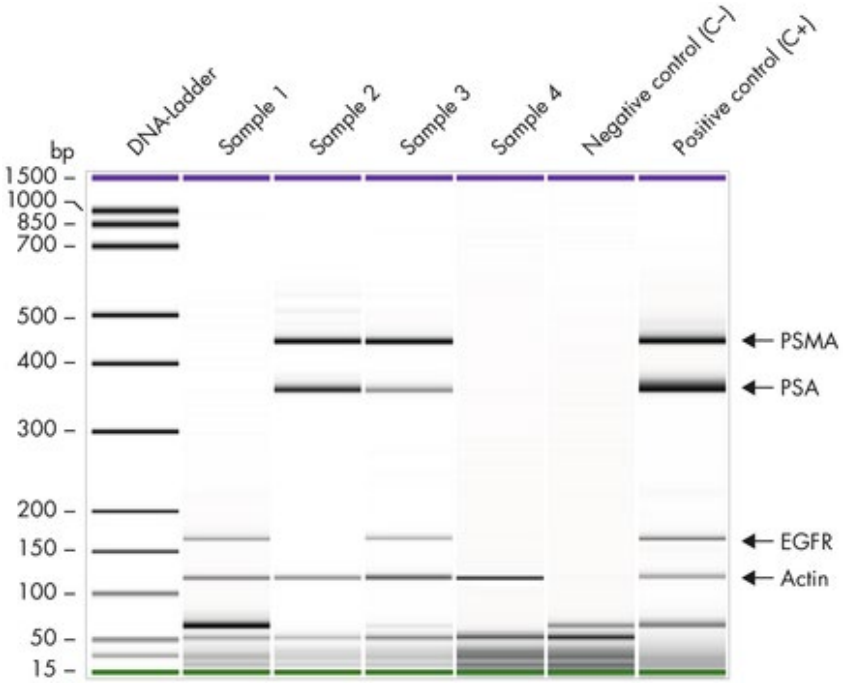

Figure 7. AdnaTest EMT-2/StemCell Add-on ProstateCancerDetect: Results of samples analyzed with an Agilent 2100 Bioanalyzer. The first lane shows the DNA size standard (DNA-Ladder). Sample 1 is positive for EGFR, sample 2 is positive for PSMA and PSA, and sample 3 is positive for PSMA, PSA, and EGFR. Sample 4 is negative. Actin is detected in samples 1–4. The PCR negative (C–) and positive control (C+) are shown in the last 2 lanes.

The fragment of the control gene actin must be detected in all samples (internal PCR control). An actin signal provides a positive control for multiplex PCR. No bands larger than 80 bp may be detected in the negative control and RT control samples, because bands larger than 80 bp indicate primer–dimers.

A fragment larger than 900 bp indicates contamination with genomic DNA, suggesting that a problem occurred during cell separation. 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.

Appendix D: AdnaTest EMT-2/StemCell Add-on OvarianCancerDetect

This protocol requires the use of the EMT-2 Add-on OvarianCancerDetect (ordered separately; cat. no. 396242).

Description

The PrimerMix OvarianDetect allows amplification of 3 cancer-associated genes and 1 control gene. The primers generate fragments of the following sizes:

- CA125 432 bp
- GA733-2 395 bp
- Muc-1 299 bp
- Actin 120 bp (internal control)

Note: Fragment sizes may vary slightly. Use the Positive Control Ovarian (C+) for assignment of the detected signals.

Thing to do before starting

- Thaw HotStarTaq Master Mix, AdnaTest Positive Control Ovarian (C+), AdnaTest PrimerMix OvarianDetect, and RNase-free water. Vortex, centrifuge quickly, and store on ice.

Multiplex PCR

1. Prepare the PCR Master Mix as shown in Table 13 according to the number of samples. Prepare a volume of Master Mix 10% greater than that required for the total number of PCR reactions to be performed.

   Note: Always include Positive Control Ovarian (C+), RNase-free water as negative control (C–) and an RT control.

Table 13. Multiplex PCR setup

Component	Volume
Multiplex PCR Master Mix	
HotStarTaq Master Mix	25 µl
RNase-free water	13 µl
PrimerMix OvarianDetect	4.0 µl
cDNA *or* RT control *or* Negative control (RNase-free water) *or* Positive Control Ovarian (C+), each:	8 µl
Total volume	**50 µl**

2. Dispense 42 µl Master Mix into 0.2 ml PCR reaction tubes. Resuspend the cDNA/bead mix by pipetting and add 8 µl to each reaction tube.

   Note: For negative control, add 8 µl RNase-free water instead of cDNA.

3. Program the thermal cycler according to the manufacturer's instructions. PCR is carried out in a thermal cycler for 37 cycles using the cycling conditions described in Table 14. Use a ramp rate of 2°C/second.

Table 14. Add-on OvarianCancerDetect cycling conditions

Step	Time	Temperature
Initial activation step	15 min	95°C
3-step cycling (37 cycles)		
Denaturation	30 s	94°C
Annealing	30 s	58°C
Extension	30 s	72°C
Final extension	10 min	72°C
Cooling	∞	4°C

4. Continue with the protocol "Fragment analysis on the Agilent 2100 Bioanalyzer", page 27.

Analysis of Add-on OvarianCancerDetect results

Using the Agilent 2100 Bioanalyzer, peaks with a concentration of ≥0.2 ng/µl are positive (Figure 8).

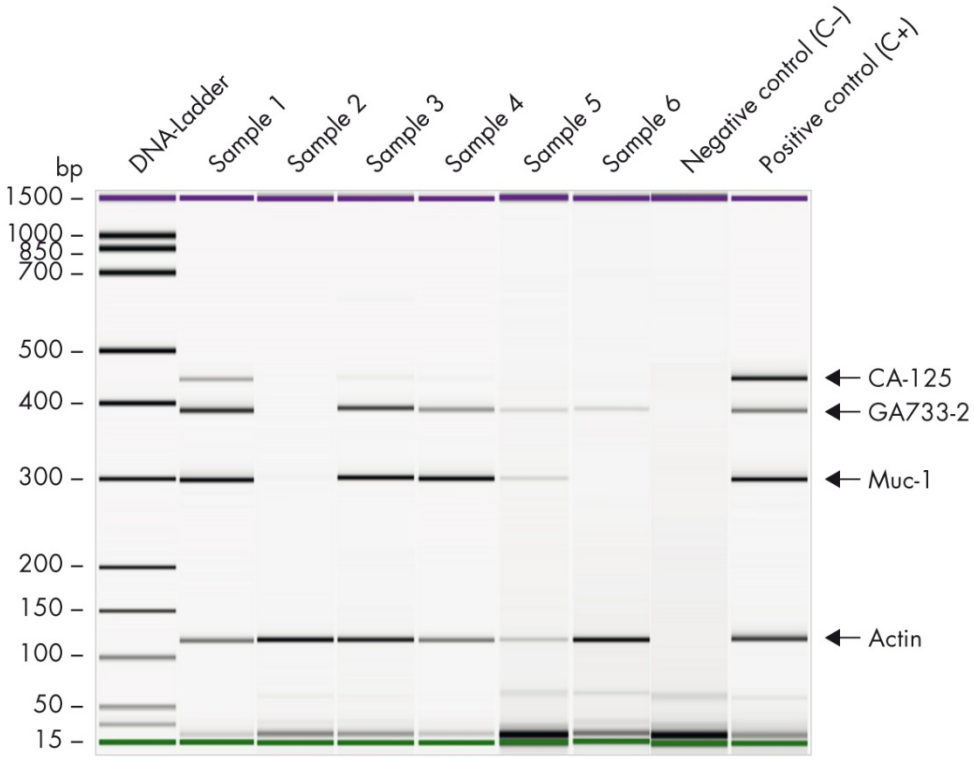

Figure 8. AdnaTest EMT-2/StemCell Add-on OvarianCancerDetect: Results of samples analyzed with an Agilent 2100 Bioanalyzer. The first lane shows the DNA size standard (DNA-Ladder). Sample 1 is positive for CA-125, GA733-2, and Muc-1; samples 3, 4, and 5 are positive for GA733-2 and Muc-1; and sample 6 is positive for GA733-2. Sample 2 is negative. Actin is detected in samples 1–6. The PCR negative (C–) and positive control (C+) are shown in the last 2 lanes.

The fragment of the control gene actin must be detected in all samples (internal PCR control). An actin signal provides a positive control for multiplex PCR. No bands larger than 80 bp may be detected in the negative control and RT control samples, because bands larger than 80 bp indicate primer–dimers.

A fragment larger than 1000 bp indicates contamination with genomic DNA, suggesting that a problem occurred during cell separation. 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.

Ordering Information

Product	Contents	Cat. no.
AdnaTest EMT-2/StemCell	For isolation of CTCs and the subsequent extraction of mRNA from human whole blood for 12 preparations and RT-PCR kit for detection of cancer associated gene expression in enriched tumor cells	397082
Related products		
AdnaTubes	12 sample tubes containing EDTA. Use only with anticoagulated blood collected in ACDA blood collection tubes from BD (Becton Dickinson, 8.5 ml)	399932
AdnaMag-L	Magnetic rack for 8 x 15 ml tubes	399921
AdnaMag-S	Magnetic rack for 8 x 1.5 ml tubes	399911
EMT-2 Add-on BreastCancerDetect	PrimerMix BreastDetect and Positive Control Breast (C+) for analysis of breast cancer associated gene expression in 12 cDNA samples	396212
EMT-2 Add-on ColonCancerDetect	PrimerMix ColonDetect and Positive Control Colon (C+) for analysis of colon cancer associated gene expression in 12 cDNA samples	396222

Product	Contents	Cat. no.
EMT-2 Add-on ProstateCancerDetect	PrimerMix ProstateDetect and Positive Control Prostate (C+) for analysis of prostate cancer associated gene expression in 12 cDNA samples	396232
EMT-2 Add-on OvarianCancerDetect	PrimerMix OvarianDetect and Positive Control Ovarian (C+) for analysis of ovarian cancer associated gene expression in 12 cDNA samples	396242

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Document Revision History

Date	Changes
03/2017	Initial release
04/2020	Increased volume of Oligo(dT)$_{25}$ Beads in the AdnaTest RNA Reagent to 355 µl, from the previous 280 µl. Replaced handbooks with quick-start protocols in kit contents. Replaced EMT-2/StemCellSelect with CTC-Select. In "Analysis of Add-on OvarianCancerDetect results", added statement that a fragment larger than 1000 bp indicates contamination with genomic DNA. In "Sample preparation", changed blood storage temperature to 2–8°C, from 4°C.

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HB-2407-002 04/2020