



AdnaTest *EMT-1/Stem CellDetect*

RT-PCR kit for detection of the EMT-1/Stem Cell and breast cancer associated gene expression in circulating tumor cells

For research use only

Manual

Article no. T-1-533

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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 EMT-1/Stem Cell can be ordered as listed below.

	Specifications	Order no.
<i>AdnaTest EMT-1/Stem Cell</i>	12 Tests	T-1-533

Purpose

AdnaTest EMT-1/Stem CellDetect is used for the analysis of EMT-1/Stem Cell characteristics in immunomagnetically enriched circulating tumor cells (*AdnaTest EMT-1/Stem CellSelect*) by reverse transcription and PCR in addition to breast cancer associated gene expression (Muc-1, Her-2, GA733-2). The kit is for research use only.

Abbreviations and Symbols

bp	base pairs
cDNA	complementary deoxyribonucleic acid
C+	positive control
C-	negative control
DNA	deoxyribonucleic acid
MPC-S	magnetic particle concentrator (-small)
EMT	epithelial/mesenchymal transition
ALDH1	aldehyde dehydrogenase1
PI3K α	Phosphoinositol-3-Kinase
Akt-2	Proteinkinase B
Twist 1	Transcriptions factor
GA733-2	gastrointestinal tumor associated antigen 733-2
Her-2	human epidermal growth factor receptor 2
Muc-1	Muc-1 gene
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
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 EMT-1/Stem CellDetect 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 EMT-1* three EMT related genes and one control gene are amplified. The *PrimerMix Stem Cell* amplifies ALDH1, which is accepted as tumor stem-cell marker. The *PrimerMix Breast Detect* can be used to determine breast cancer associated gene expression. The different primer-mixes generate the following fragments:

EMT-1

Akt-2 : 306 bp
Twist 1 : 203 bp
PI3K α : 595 bp
Actin : 119 bp (internal PCR-control)

Stem Cell

ALDH1: 165 bp

Breast cancer related gene expression profile

GA733-2 : 395 bp
Muc-1 : 293 bp
Her-2 : 270 bp
Actin : 114 bp (internal PCR control).

Kit Components

AdnaTest EMT-1/Stem CellDetect includes the following components (number of tubes):

Table 1: Kit Components

Component	Symbol	T-1-509 (12 tests)
<i>Lysis/Binding Buffer</i>	3	1
<i>Dynabeads Oligo(dT)₂₅</i>	4	1
<i>Buffer A</i>	5	1
<i>Buffer B</i>	6	1
<i>10 mM Tris-HCl</i>	7	1
<i>PrimerMix Breast Detect</i>	8	1
<i>Positive Control (C+)</i>	9	1
<i>Gel Calibrator</i>	10	1
<i>PrimerMix EMT-1</i>	11	1
<i>Positive Control EMT-1</i>	12	1
<i>PrimerMix Stem Cell</i>	13	1
<i>Positive Control Stem Cell</i>	14	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 (50 °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)

Note: The *Sensiscript* Reverse Transcription Kit (cat no. 205211) will suffice for 25 samples only because double volume is required for each reaction.

- 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 EMT-1/Stem CellDetect has to be stored at 4 °C. **However, store the two boxes with the *PrimerMixes* and *Controls* separately at -20 °C.** In order to prevent possible contaminations and repeated temperature changes aliquot the primer mixes and the gel calibrator. 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 50 °C.

Processing

1. Aliquot 20 µl of the beads (coming from step A 7) to each tube containing cell lysate (from step B 16 of the *AdnaTest BreastCancerSelect* 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.

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.

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 29.5 µl RNase-free water.
13. Place the tubes into a thermomixer or water bath and incubate for 5 min at 50 °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 10.5 µl into individual 0.2 ml PCR tubes.
4. Resuspend the mRNA/bead complexes (from step B 14) carefully with a pipet. Transfer the total volume of 29.5 µl into the 0.2 ml PCR reaction tube containing the RT Master Mix. Mix thoroughly and centrifuge briefly. RT Control: Add 29.5 µl RNase-free water instead of mRNA.

Table 2: Reverse Transcription

Components			Volume
RT Mastermix	<i>Sensiscript</i> Reverse Transcriptase Kit (Qiagen)	10x Buffer RT	4.0 µl
		dNTPs	4.0 µl
		<i>Sensiscript</i> Reverse Transcriptase (SRT)	2.0 µl
	RNase Inhibitor, 40 U/µl (Promega)	0.5 µl	
Sample	mRNA/bead-complex or RNase-free H ₂ O (as RT Control)		29.5 µl
	Total volume		

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 EMT-1 Multiplex PCR

1. Thaw *HotStarTaq Master Mix* (Qiagen), distilled water and *Positive Control EMT-1* [12], vortex carefully, centrifuge quickly and store on ice. Thaw *PrimerMix EMT-1* [11], 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 be at least 10 % larger than the requirement calculated from the number of samples. Note that a *Positive Control EMT-1* [12], 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 C 6).

Note: For water/C-, 4.0 µl of distilled water is added instead of cDNA

Table 4: Preparation of the EMT-1 multiplex PCR

Components	Volumes	
PCR Master Mix	HotStarTaq Master Mix	12.5 µl
	Distilled water	4.5 µl
	<i>PrimerMix EMT-1</i> [11]	4.0 µl
Samples	cDNA or RT Control or Negative Control (water/C-) or <i>Positive Control EMT-1</i> [12] each:	4.0 µl
Total volume		25.0 µl

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

Table 5: EMT-1 PCR program

95 °C	15 min	} 35 cycles
94 °C	30 sec	
60 °C	30 sec	
72 °C	45 sec	
72 °C	10 min	
4 °C	∞	

E Stem Cell Multiplex PCR

1. Thaw *HotStarTaq Master Mix* (Qiagen), distilled water and *Positive Control Stem Cell* [14], vortex carefully, centrifuge quickly and store on ice. Thaw *PrimerMix Stem Cell* [13], vortex, spin down and place on ice.
2. Depending on the number of samples the PCR Master Mix is prepared according to Table 6.
The volume of the Master Mix should be at least 10 % larger than the requirement calculated from the number of samples. Note that a *Positive Control Stem Cell* [14], 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 (step C 6) mix by pipetting and add 4.0 µl of this suspension.

Note: For water/C-, 4.0 µl of distilled water is added instead of cDNA

Table 6: Preparation of the Stem Cell multiplex PCR

Components		Volumes
PCR Master Mix	HotStarTaq Master Mix	12.5 µl
	Distilled water	4.5 µl
	<i>PrimerMix ALDH1-Detect</i> [8]	4.0 µl
Samples	cDNA or RT Control or Negative Control (water/C-) or <i>Positive Control (C+)</i> [9] each:	4.0 µl
Total volume		25.0 µl

A thermocycler is used for the PCR following the program described in Table 7. Run the thermocycler with a ramp of 2 °C/second.

Table 7: Stem Cell PCR program

95 °C	15 min	} 35 cycles
94 °C	30 sec	
51 °C	30 sec	
72 °C	30 sec	
72 °C	5 min	
10 °C	∞	

F Multiplex PCR of breast cancer related gene expression

1. Thaw HotStarTaq Master Mix (Qiagen), distilled water and *Positive Control (C+)* [9], vortex carefully, centrifuge quickly and store on ice. Thaw *PrimerMix BreastDetect* [8], vortex, spin down and place on ice.
2. Depending on the number of samples the PCR Master Mix is prepared according to Table 8.
The volume of the Master Mix should be at least 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 42.0 µl of the Master Mix into each 0.2 ml PCR reaction tube. Resuspend the cDNA/bead mix by pipetting and add 8.0 µl of this suspension (step C 6).

Note: For water/C-, 8.0 µl of distilled water is added instead of cDNA.

Table 8: Preparation of the multiplex PCR of breast cancer related gene expression

Components			Volumes
PCR Master Mix	<i>HotStarTaq Master Mix Kit (Qiagen)</i>	<i>HotStarTaq Master Mix</i>	25.0 µl
		Distilled water	13.0 µl
	<i>PrimerMix BreastDetect</i> [8]		4.0 µl
Samples	cDNA or RT Control or Negative Control (water/C-) or <i>Positive Control (C+)</i> [9]		each: 8.0 µl
	Total volume		

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

Table 9: PCR program of breast cancer related gene expression

95 °C	15 min	} 35 cycles
94 °C	1 min	
60 °C	1 min	
72 °C	1 min	
72 °C	10 min	
4 °C	∞	

G 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 and for the analyzes of the EMT-1 product apply 10.0 µl of the *Gel Calibrator* [10]. Vortex the *Gel Calibrator* before use. 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

EMT-1

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

Using the Agilent 2100 Bioanalyzer, peaks with a concentration of ≥ 0.25 ng/µl for PI3Kα, Akt-2 and Twist are positive.

In addition, the following criteria must be fulfilled:

- The Negative Control and the RT Control samples must not show any bands larger than 80 base pairs (primer dimers).
- The actin fragment must be present in all samples analysed (internal PCR control).

Note: The *AdnaTest EMT-1/Stem CellDetect* is optimized to exclude illegitimate expression of the EMT transcripts. Any changes in the protocol might lead to loss of specificity.

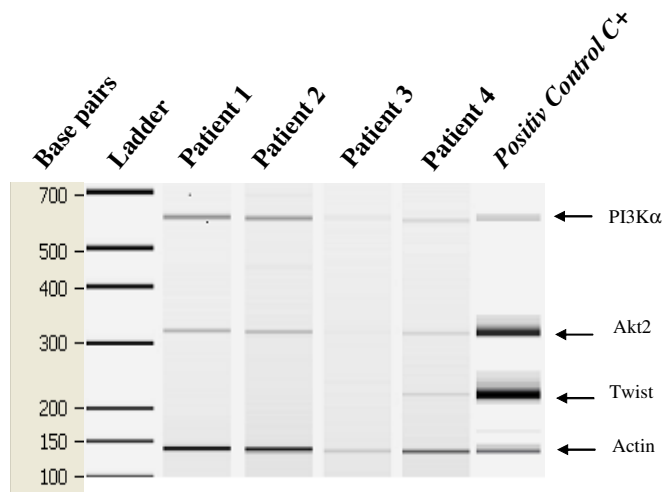


Fig. 1. EMT-1 positive samples

The figure shows EMT samples analyzed with the Agilent 2100 Bioanalyzer. Patients 1 and 2 are positive for PI3K α and Akt2. Patient 3 is negative because the concentrations of the genes PI3K α , Akt2 and Twist are below cut off (0.25 ng/ μ g). Patient 4 is positive for all markers because their concentrations are all above cut off.

Stem Cell

Using the Agilent 2100 Bioanalyzer, peaks with a concentration of ≥ 0.15 ng/ μ l for ALDH1 are positive.

In addition, the following criteria must be fulfilled:

- The Negative Control and the RT Control samples must not show any bands larger than 80 base pairs (primer dimers).

Note: The AdnaTest EMT-1/Stem CellDetect is optimized to exclude illegitimate expression of the ALDH1 transcripts. Any change in the protocol might lead to loss of specificity.

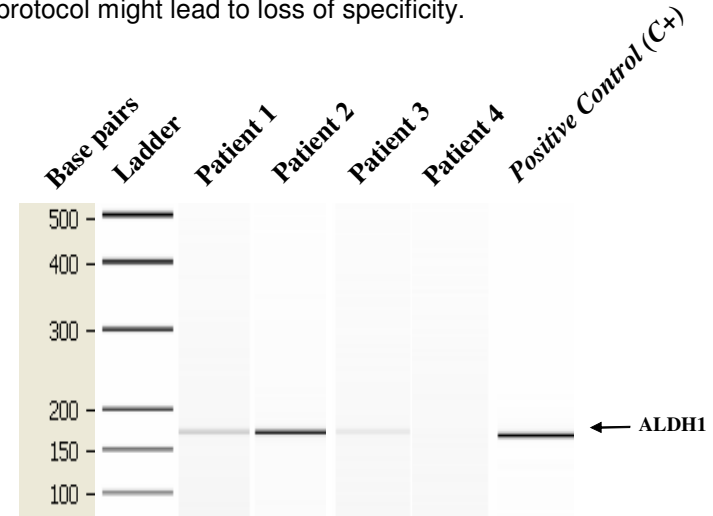


Fig. 2. Stem Cell positive samples

The figure shows fragment analyzes with the Agilent 2100 Bioanalyzer. Patient 1 and 2 are positive for ALDH1 (165 bp). Patient 3 and patient 4 are negative since the concentration of ALDH1 is < 0.15 ng/ μ l.

Breast cancer related gene expression profile

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

Using the Bioanalyzer Agilent 2100 peaks with a concentration of ≥ 0.15 ng/ μ l for Muc-1, Her-2 and GA733-2 are positive.

In addition, the following criteria must be fulfilled:

- The actin fragment must be present in all samples analysed (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 fragment larger than 1 kb 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 Emt-1/Stem CellSelect* and *AdnaTest EMT-1/Stem CellDetect* 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 illegitimate expression levels in healthy donors.

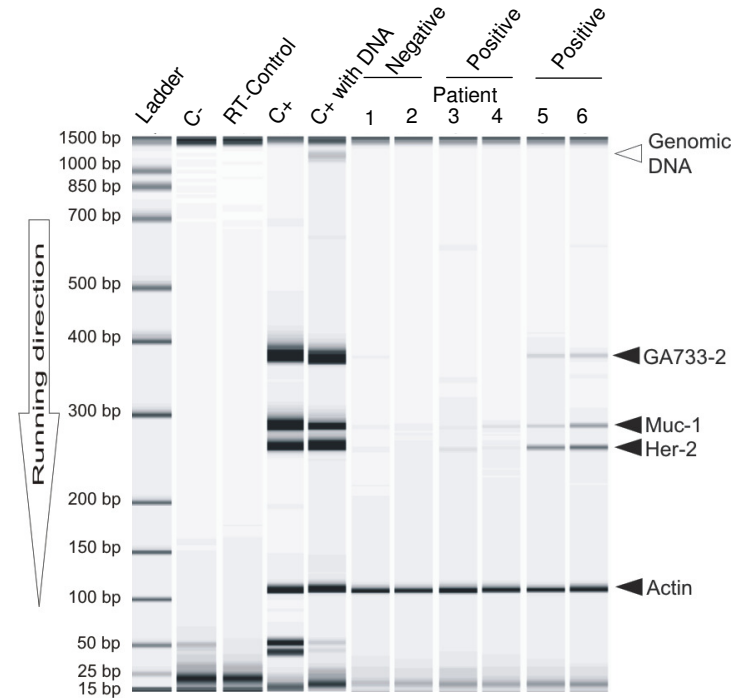


Fig. 3: Breast cancer related gene expression profile

DNA size standards (ladder), the PCR control (C-), the RT Control, the Positive Control (C+) and cDNA of a cell line that was contaminated with genomic DNA are shown. Sample 1 and 2 are negative. Sample 5 and 6 are positive of the three tumor associated markers GA733-2, Muc-1 and Her-2. Sample 3 is positive for Her-2 and sample 4 is positive for Muc-1.

Evaluation Agarose Gel

EMT-1

Fragments stained equal or more intensive than the *Gel Calibrator* 10 are positive.

The *Gel Calibrator* helps to evaluate the agarose gel. The staining intensity of the *Gel Calibrator* indicates fragments that can neither be judged positive nor negative.

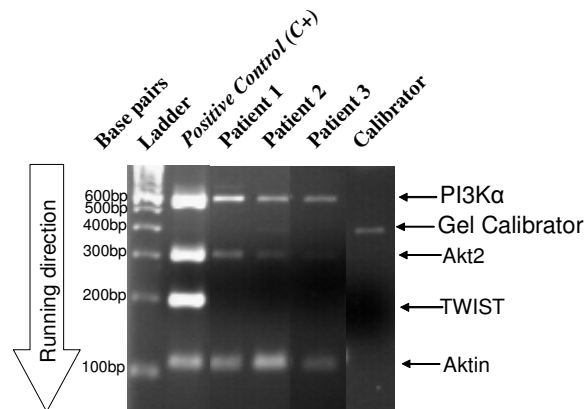


Fig. 4. Analysis of *AdnaTest EMT-Detect* on 4% agarose gel

Patient 1 und 2 are positive for PI3K α und Akt2. Patient 3 is positive for PI3K α .

Stem Cell

ALDH1 is considered positive in an agarose gel if a signal shows up.

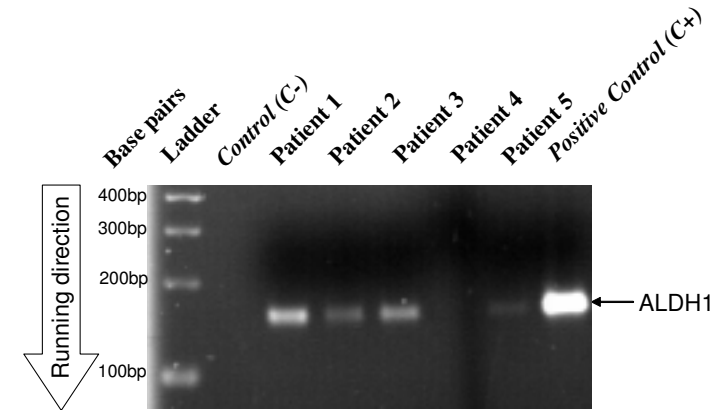


Fig. 5. Analysis of *AdnaTest ALDH1-Detect* on 4% agarose gel

Patient 1, 2 and 3 are positive for ALDH1 (165 bp). Patient 4 and patient 5 are negative.

Breast cancer related gene expression profile

Breast cancer related genes are considered positive in an agarose gel if a signal shows up.

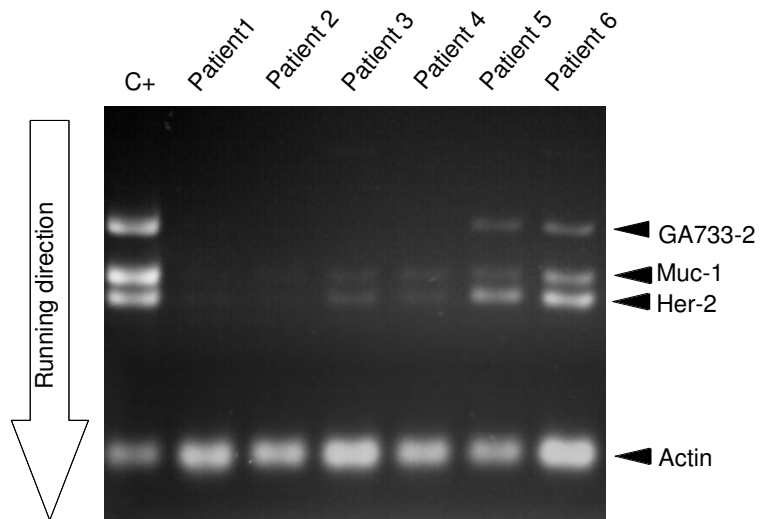


Fig. 6: Analysis of breast cancer related gene expression profile by gel electrophoresis on 4% agarose gel

Patient 1 und 2 are negative for breast cancer related genes. Patient 4, 3, 5 und 6 are positive for breast cancer related genes.

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.

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 also refers to *AdnaTest EMT-1/Stem CellSelect*.

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

Table 10: Troubleshooting

Problem	Possible causes	Suggestions for correction
No bands incl. actin, for all samples	Pipetting error	Repeat test
	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. Check the fill level. The blood should reach the mark "7,0" 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 24 h (<i>AdnaCollect</i>) after withdrawal. Blood samples must be placed on ice and stored closed 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 Emt-1/Stem CellDetect

Component	<i>Lysis/Binding Buffer</i>	3
	<i>Oligo(dT)₂₅ Beads</i>	4
	<i>Buffer A</i>	5
	<i>Buffer B</i>	6
	<i>Tris HCl</i>	7
	<i>PrimerMix BreastDetect</i>	8
	<i>Positive Control (C+)</i>	9
	<i>Gel Calibrator</i>	10
	<i>PrimerMix EMT-1</i>	11
	<i>Positive Control EMT-1</i>	12
	<i>PrimerMix Stem Cell</i>	13
	<i>Positive Control Stem Cell</i>	14
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** and transfer into new 1.5 ml reaction tubes.
- Wash the beads with 1x 100 µl *Buffer B* **6**.
- Wash the beads with 1x 100 µl *Tris-HCl* **7**.
- Resuspend the beads in 29.5 µl RNase free water.
- Incubate for 5 min at 50 °C and place on ice for at least 2 min.

Continue with the reverse transcription (RT), see

- Table 11 and Table 12.

Table 11: Reverse Transcription

Components			Volume
RT Mastermix	<i>Sensiscript</i> Reverse Transcriptase Kit (Qiagen)	10x Buffer RT	4.0 µl
		dNTPs	4.0 µl
		<i>Sensiscript</i> Reverse Transcriptase (SRT)	2.0 µl
		RNase Inhibitor, 40 U/µl (Promega)	0.5 µl
Sample	mRNA/bead-complex or RNase-free H ₂ O (as RT Control)		29.5 µl
	Total volume		

Table 12: RT program

37 °C	→	60 min
93 °C	→	5 min
4 °C	→	∞

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

Table 13: EMT-1 Multiplex PCR

Components		Volumes
PCR Master Mix	HotStarTaq Master Mix	12.5 µl
	Distilled water	4.5 µl
	<i>PrimerMix EMT-1</i> [11]	4.0 µl
Samples	cDNA or RT Control or Negative Control (water) or <i>Positive Control EMT-1</i> [12] each:	4.0 µl
Total volume		25.0 µl

Table 14: EMT-1 PCR program

95 °C	15 min	} 35 cycles
94 °C	30 sec	
60 °C	30 sec	
72 °C	45 sec	
72 °C	10 min	
4 °C	∞	

Table 15: Stem Cell Multiplex PCR

Components		Volumes
PCR Master Mix	HotStarTaq Master Mix	12.5 µl
	Distilled water	4.5 µl
	PrimerMix ALDH1-Detect [8]	4.0 µl
Samples	cDNA or RT Control or Negative Control (water) or Positive Control (C+) [9] each:	4.0 µl
	Total volume	25.0 µl

Table 16: Stem Cell PCR program

95 °C	15 min	} 35 cycles
94 °C	30 sec	
51 °C	30 sec	
72 °C	30 sec	
72 °C	5 min	
10 °C	∞	

Table 17: Multiplex PCR of breast cancer related gene expression profile

Components			Volumes
PCR Master Mix	HotStarTaq Master Mix Kit (Qiagen)	HotStarTaq Master Mix	25.0 µl
		Distilled water	13.0 µl
	PrimerMix BreastDetect [8]	4.0 µl	
Samples	cDNA or RT Control or Negative Control (water/C-) or Positive Control (C+) [9] each:	8.0 µl	
	Total volume	50.0 µl	

Table 18: PCR program of breast cancer related gene expression profile

95 °C	15 min	} 35 cycles
94 °C	1 min	
60 °C	1 min	
72 °C	1 min	
72 °C	10 min	
4 °C	∞	

- For fragment analysis use the Bioanalyzer 2100 (Agilent). Alternatively analyze the fragments, the samples and the Gel Calibrator [10], in a 4 % agarose gel (80-100 V for ca. 60 min).

AdnaGen AG
Ostpassage 7
D-30853 Langenhagen
Germany

Phone ++49 (0) 511 72 59 50 - 50

Fax ++49 (0) 511 72 59 50 - 40

E-mail support@adnagen.com