150604 EN

AdnaTest EMT-2/StemCellDetect

RT-PCR Kit for detection of gene expression in enriched tumor cells associated with epithelial-mesenchymal transition and cancer cell stemness

For research use only

Manual

REF T-1-537

Contents

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, the QIAGEN Hannover support team will answer you any questions regarding the *AdnaTests* (support@adnagen.com).

Available Add-ons (12 tests):

Article No.	Tumor	Genes
T-1-537-PB	EMT-2 Add-on Breast	GA733-2, Her2, Muc-1
T-1-537-PC	EMT-2 Add-on Colon	GA733-2, CEA, EGFR
T-1-537-PP	EMT-2 Add-on Prostate	PSMA, PSA, EGFR
T-1-537-PO-2	EMT-2 Add-on Ovarian-2	CA125, GA733-2, Muc-1

Purpose

AdnaTest EMT-2/StemCellDetect is used for the analysis of EMT (epithelial-mesenchymal transition) and cancer cell stemness characteristics in immunomagnetically enriched tumor cells by reverse transcription and PCR and is intended for research use only. The specificity of the detection is 90%. Using EMT-2 markers in spiking experiments 20 tumor cells in 5 ml of whole blood are detected at a recovery rate of at least 70%. Using StemCell markers in spiking

experiments 10 tumor cells in 5 ml of whole blood are detected at a recovery rate of at least 70%.
AdnaTest EMT-2/StemCellSelect is used for the enrichment of circulating tumor cells from peripheral blood.

Optionally the analysis of cancer associated genes for the entities Breast, Colon, Prostate and Ovarian is possible (q.v. Order Information, available EMT-2 Add-ons).

Abbreviations and Symbols

AdnaMag-S	Magnetic particle concentrator (-small)
Akt-2	Protein kinase B
ALDH1	Aldehyde dehydrogenase 1
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
C+	Positive control
C-	Negative control
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EMT	Epithelial-mesenchymal transition
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PI3Kα	Phosphoinositol-3-Kinase
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcription
TWIST1	Transcription factor
	Expiry date
	Storage temperature
	Catalogue number
	Consider instructions for use
	Manufactured by

Patents and Registered Trademarks

This test requires licenses of Hoffmann-La Roche AG, Basel. The purchase of *AdnaTests* does not authorize the user to perform the PCR without license. *Dynabeads®* is a registered trademark of Invitrogen and Life Technologies Corporation. The trademarks *Sensiscript* and *HotStarTaq* are registered by QIAGEN, Hilden. *LabChip* is a US registered trademark of Caliper Technology Corp.

Product Description

AdnaTest EMT-2/StemCellDetect 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 subsequently used as template for tumor cell detection and characterization by Multiplex-PCR. The *PrimerMix EMT-2* allows the amplification of three EMT related genes and one control gene. The *PrimerMix StemCell* amplifies ALDH1, which is accepted as tumor stem-cell marker. Taken together the different primer-mixes generate the following fragments:

PrimerMix EMT-2			***PrimerMix StemCell***	
PI3Kα:	551 bp		ALDH1:	161 bp
Akt-2:	309 bp			
TWIST1:	201 bp			
Actin:	120 bp	(internal PCR control)		

Note: Fragment sizes may vary slightly. Please use the *Positive Control (C+)* 1 2 and 1 4 for assignment of the detected signals.

Kit Components

AdnaTest EMT-2/StemCellDetect includes the following components:

Table 1: Kit components

Component	Symbol	T-1-537 (12 tests)
Lysis/Binding Buffer	3	1
Dynabeads Oligo(dT)$_{25}$	4	1
Buffer A	5	1
Buffer B	6	1
10 mM Tris-HCl	7	1
PrimerMix EMT-2	11	1
Positive Control EMT-2	12	1
PrimerMix StemCell	13	1
Positive Control StemCell	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 *AdnaMag-S* (QIAGEN Hannover GmbH, cat. no. T-1-800)
- Thermal block or water bath (50 °C)
- Thermocycler with a heated lid and a heating rate of 2 °C/s.
- Analysis system like the Agilent 2100 Bioanalyzer (Agilent Technologies)or an alternative system

Material:

- 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)
- Pipets and RNase-free pipet tips with aerosol barrier, suitable for pipetting volumes from 1 µl to 200 µl
- Protective gloves

Reagents:

- *Sensiscript* Reverse Transcription Kit (QIAGEN, cat no. 205211, 50 reactions)

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

- Recombinant RNAsin, RNase-inhibitor, 2.500 U (Promega, cat no. N2511)
- *HotStarTaq Master Mix* Kit (QIAGEN, cat no. 203443, 250 U)

Storage

AdnaTest EMT-2/StemCellDetect has to be stored at +4 °C. **However, store the boxes with the *PrimerMixes* and the *Positive Controls* separately at -20 °C**. In order to prevent possible contaminations and repeated temperature changes aliquot the primer mixes. 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 their instructions. Safety advices of the respective manufacturers are valid.
- Wear protective gloves to avoid contamination with DNA, RNA and RNases.

The test 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 incl. reverse transcription and subsequent analysis of amplified PCR products in different rooms, if possible, to avoid cross-contamination.
- **The use of products from other suppliers than suggested 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 isolation of mRNA and reverse transcription, sections D to F deal with Multiplex/Singleplex PCR and fragment analysis.

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 mix 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 *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 close to the reaction tubes.

5. After 1 min remove the supernatant with a pipet.

6. Washing
   a. Remove the magnet slider from the *AdnaMag-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. Insert the magnet slider into the *AdnaMag-S*.
   d. After 1 min remove the supernatant completely.

   Repeat once (two washings in total).

7. Remove the tube from the *AdnaMag-S* and resuspend the beads in *Lysis/Binding Buffer* 3 to the original volume (see step 3).

B. mRNA Isolation

Preparation:

1. Equilibrate Washing *Buffer A* 5 and Washing *Buffer B* 6 to room temperature.
2. Place *10 mM Tris-HCl* 7 on ice.
3. Thaw RNase-free water (part of the *Sensiscript* Reverse Transcriptase-Kit, QIAGEN).
4. Adjust a thermal block or water bath to 50 °C.

Processing:

1. Add 20 µl of *Dynabeads Oligo(dT)$_{25}$* (step A7) to each tube containing cell lysate (*AdnaTest EMT-2/StemCellSelect* manual, step B20).
2. Rotate 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 *AdnaMag-S* without magnet slider. Swing the *AdnaMag-S* downwards to release beads and liquid captured in the cap.
4. Insert magnet slider and remove the supernatants after 1 min.
5. Washing A
   a. Remove the magnet slider from the *AdnaMag-S*.
   b. Add 100 µl Washing *Buffer A* [5] to each tube and resuspend the beads by repeated pipetting. To avoid any loss of beads please rinse lid and tube wall thoroughly.
   c. Insert the magnet slider into the *AdnaMag-S*.
   d. After 1 min remove the supernatant completely.

   Repeat once (two washings in total).
6. Washing B
   a. Remove the magnet slider from the *AdnaMag-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 slider into the *AdnaMag-S*.
   d. After 1 min remove the supernatant completely. This step has to be carried out carefully (watch the pellet) since the beads might be sliding and could be removed by mistake.

   Repeat once in the same reaction tubes (two washings in total).

7. Remove the magnet slider from the *AdnaMag-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 slider into the *AdnaMag-S*.
10. After 1 min remove the supernatants 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 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 (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. The RT Master Mix is prepared as shown in Table 2 according to the number of samples.

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

3. Vortex the RT Master Mix, centrifuge briefly, and pipet 10.5 µl for each reaction into 0.2 ml PCR tubes.

4. Resuspend the mRNA/bead complexes (step B14) carefully with a pipet. Transfer the total volume into the 0.2 ml PCR reaction tube containing the RT Master Mix. Mix thoroughly by repeated pipetting.

Table 2: Reverse Transcription

Component			**Volume**
RT Master Mix	*Sensiscript* Reverse Transcriptase Kit (QIAGEN)	10x Buffer RT	4.0 µl
		dNTPs	4.0 µl
		Sensiscript Reverse Transcriptase (SRT)	2.0 µl
	RNase Inhibitor, 40 U/µl (Promega)		0.5 µl
Samples	mRNA/bead-complex or RT Control (RNase-free water) each[(1)]:		29.5 µl
Total volume			40.0 µl

[1)] **Note:** 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. In any case, use the total volume for reverse transcription!

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. 4 weeks.

D. Multiplex-PCR (EMT-2)

1. Thaw *HotStarTaq Master Mix* (QIAGEN), *PrimerMix EMT-2* 11, RNase-free water and *Positive Control EMT-2* 12, vortex, centrifuge quickly and store on ice.
2. The PCR Master Mix is prepared as shown in Table 4 according to the number of samples.

   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-2* 12, RNase-free water as Negative Control (C-) and the RT Control must always be included.
3. For each preparation dispense 21.0 µl of the Master Mix into 0.2 ml PCR reaction tubes. Resuspend the cDNA/bead mix by pipetting and add 4.0 µl of it to each reaction tube.

Note: As Negative Control add 4.0 µl of RNase-free water instead of cDNA.

Table 4: Preparation of the Multiplex-PCR (EMT-2)

Component		**Volume**
PCR Master Mix	HotStarTaq Master Mix	12.5 µl
	RNase-free water	4.5 µl
	PrimerMix EMT-2 1 1	4.0 µl
Samples	cDNA or RT Control or Negative Control (RNase-free water) or *Positive Control EMT-2* 1 2 each:	4.0 µl
Total volume		25.0 µl

A thermocycler is used for the PCR following the program described in Table 5. Run the thermocycler with a ramp of 2 °C/second. The PCR is performed with a total of 35 cycles.

Table 5: PCR program (EMT-2)

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

E. Singleplex PCR (StemCell)

1. Thaw *HotStarTaq Master Mix* (QIAGEN), *PrimerMix StemCell* 1 3, RNase-free water and *Positive Control StemCell* 1 4, vortex, centrifuge quickly and store on ice.

2. The PCR Master Mix is prepared as shown in Table 6 according to the number of samples.

   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 StemCell* 1 4, RNase-free water as Negative Control (C-) and the RT Control must always be included.

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

Note: As Negative Control add 4.0 µl of RNase-free water instead of cDNA.

Table 6: Preparation of the Singleplex PCR (StemCell)

Component		**Volume**
PCR Master Mix	HotStarTaq Master Mix	12.5 µl
	RNase-free water	4.5 µl
	PrimerMix StemCell 1 3	4.0 µl
Samples	cDNA or RT Control or Negative Control (RNase-free water) or *Positive Control StemCell* 1 4 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. The PCR is performed with a total of 35 cycles.

Table 7: PCR program (StemCell)

95 °C	15 min	
94 °C	30 sec	35 cycles
51 °C	30 sec	
72 °C	30 sec	
72 °C	5 min	
4 °C	∞	

F. Fragment Analysis

Agilent 2100 Bioanalyzer

The analysis with the Agilent 2100 Bioanalyzer (Agilent Technologies) on a DNA 1000 LabChip is recommended. 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 can cause false results.

Start the Bioanalyzer software "2100 expert". Under "Contexts" select "Instrument", click the button "Assay" next to "Assay selection". Choose "electrophoresis > DNA 1000 Series II.xsy". Prepare the chip and start run. For evaluation of the results set a detection threshold as it is described below:

Under "Contexts" select "Data", choose the tab "Assay Properties". On the right select "Global" and "Normal" from the pull down menu. Choose "Sample Setpoints > Integrator > height threshold (FU)" and set this value to "0" (default value is "20") to detect all signals.

Evaluation (EMT-2)

The test is considered positive, if a PCR fragment of at least one EMT-2 associated transcript for (PI3Kα, Akt-2 or TWIST1) is clearly detected.

If you are using the Agilent 2100 Bioanalyzer, peaks with a concentration of ≥ 0.25 ng/µl are positive (Fig. 1).

The fragment of the control gene actin must show in all patient 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 a contamination with genomic DNA. The separation process was not successful and the results have to be discarded if such a fragment is detected.

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

In case assistance is needed to interpret the results, please do not hesitate to contact our support team.

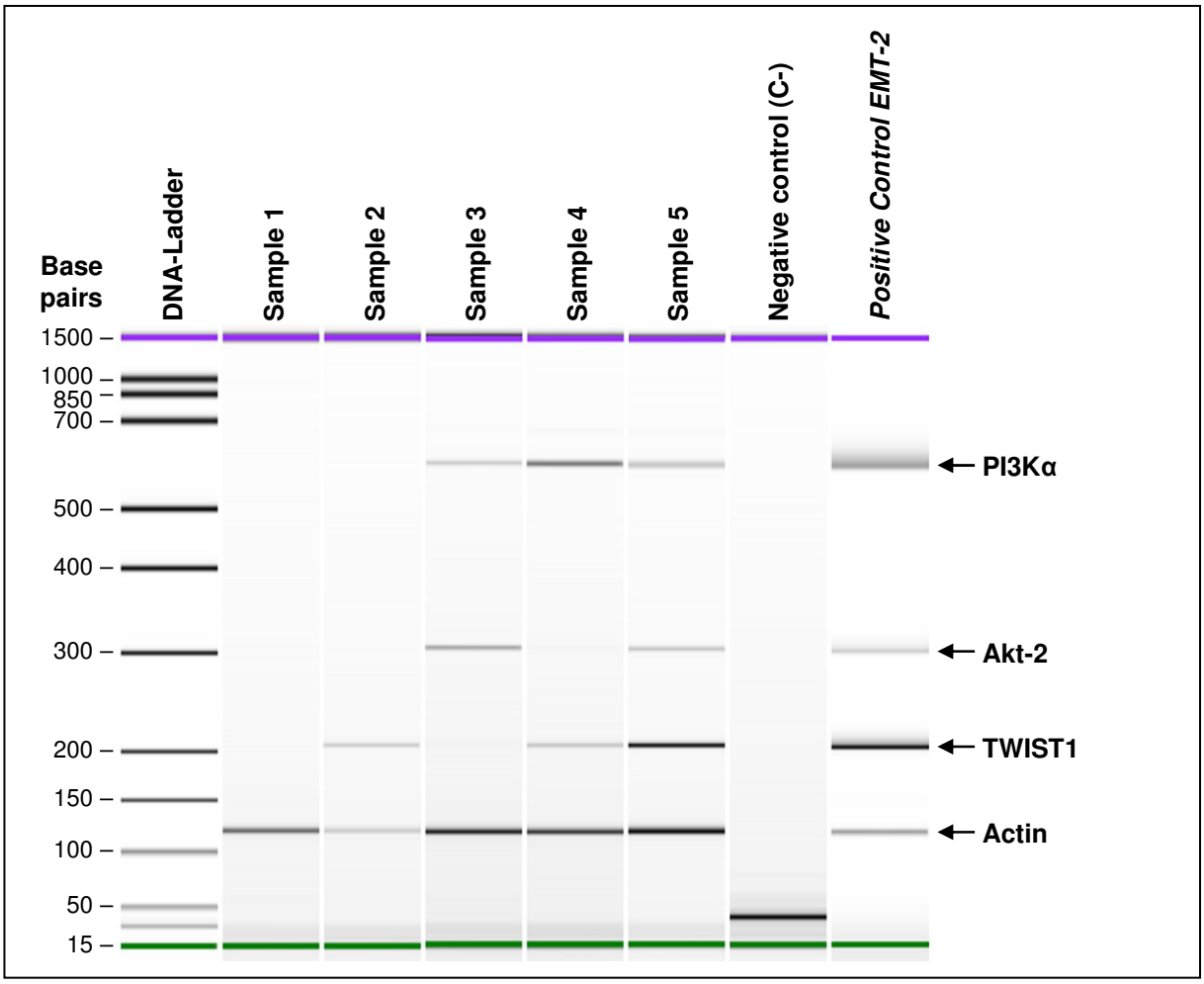

Fig. 1: ***AdnaTest EMT-2/StemCellDetect*** **results of samples using an Agilent 2100 Bioanalyzer: EMT-2 associated gene expression**

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 three EMT-2 markers can be detected in sample 5 and actin is detected in samples 1 to 5. The PCR negative control (C-) and the *Positive Control EMT-2* are shown in the last two lanes.

Evaluation (StemCell)

If you are using the Agilent 2100 Bioanalyzer, peaks with a concentration of ≥ 0.15 ng/µl for ALDH1 are positive (Fig. 2).

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 a contamination with genomic DNA. The separation process was not successful and the results have to be discarded if such a fragment is detected.

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

In case assistance is needed to interpret the results, please do not hesitate to contact our support team.

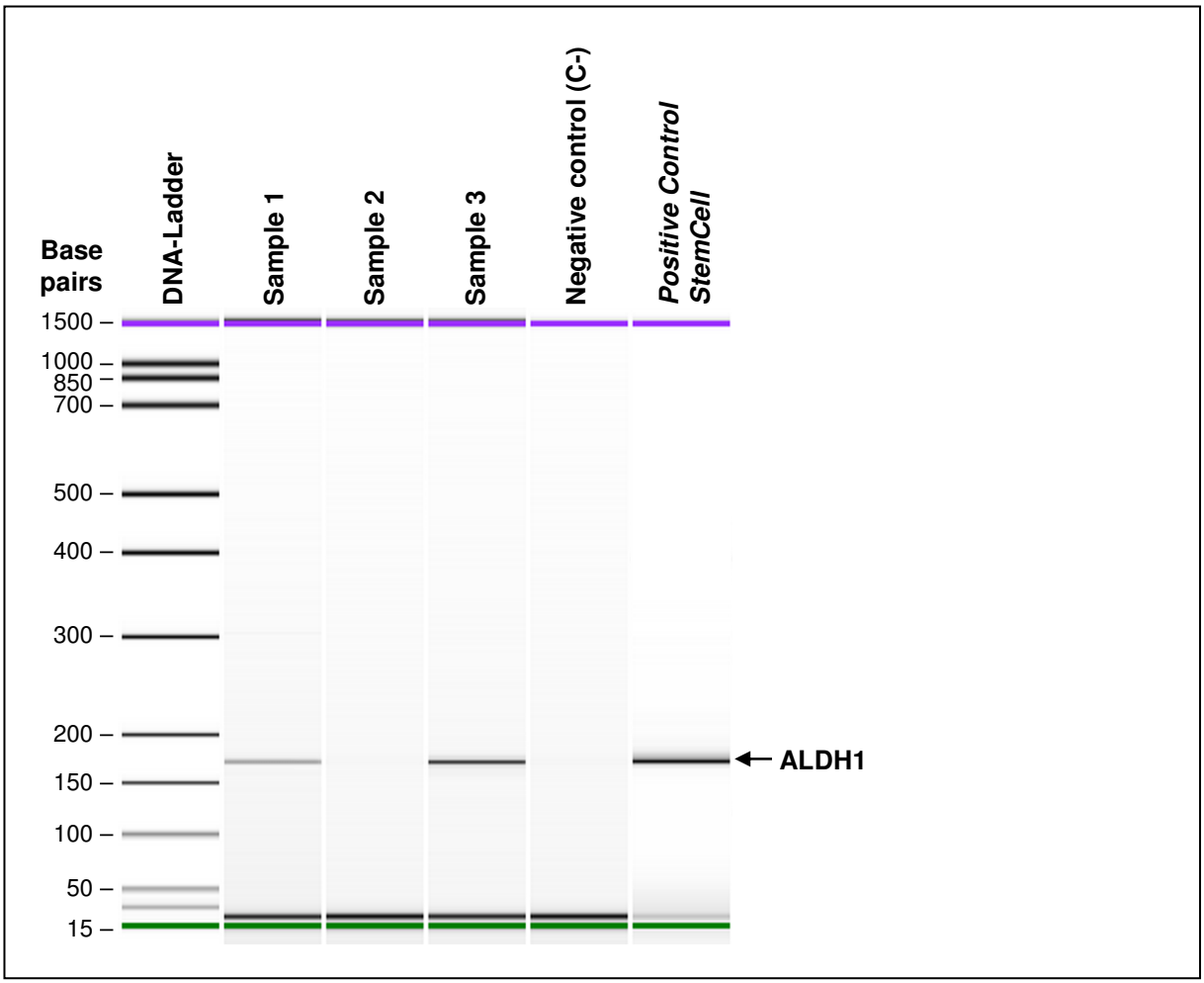

Fig. 2: *AdnaTest EMT-2/StemCellDetect* results of samples using an Agilent 2100 Bioanalyzer: Tumor stem cell associated gene expression

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 two lanes.

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 manual. In case problems still occur, please go to: www.adnagen.com and download our troubleshooting guide in the product section. You will find practical hints for the test procedure and for the correct interpretation of test results.

Do not hesitate to contact our support team when problems continue to exist.

Short Manual

AdnaTest EMT-2/StemCellDetect

Components	*Lysis/Binding Buffer*	3
	Oligo (dT)$_{25}$ Beads	4
	Buffer A	5
	Buffer B	6
	10 mM Tris-HCl	7
	PrimerMix EMT-2	11
	Positive Control EMT-2	12
	PrimerMix StemCell	13
	Positive Control StemCell	14
You need	• 0.2 ml PCR-tubes • 1x 1.5 ml reaction tube per sample • pipets and tips (RNase free) for 1 - 200 µl • *Sensiscript* RT Kit (QIAGEN) • *HotStarTaq Master Mix* Kit (QIAGEN)	

Protocol

- Equilibrate [3], [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 tilting and rotation at approx. 5 rpm.
- Place the reaction tube in *AdnaMag-S* and remove supernatant.
- Wash beads with 2x 100 µl *Buffer A* [5] .

Important: To avoid any loss of beads please rinse lid and tube wall thoroughly.

- Resuspend beads in 100 µl *Buffer B* [6] and transfer into a new 1.5 ml tube.
- Wash beads with 1x 100 µl *Buffer B* [6] .
- Wash beads with 1x 100 µl *Tris-HCl* [7] .
- Resuspend 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 reverse transcription; see Table 8 and Table 9.

Table 8: Reverse Transcription

Component			**Volume**
RT Master Mix	*Sensiscript* Reverse Transcriptase Kit (QIAGEN)	10x Buffer RT	4.0 µl
		dNTPs	4.0 µl
		Sensiscript Reverse Transcriptase (SRT)	2.0 µl
	RNase Inhibitor, 40 U/µl (Promega)		0.5 µl
Samples	mRNA/bead-complex or RT Control (RNase-free water) each[(1)]:		29.5 µl
Total volume			40.0 µl

[1)] **Note:** 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. In any case, use the total volume for reverse transcription!

Table 9: RT program

37 °C	60 min
93 °C	5 min
4 °C	∞

Continue with:

- Multiplex-PCR, Table 10 and Table 11
  → EMT-2 related gene expression
- Singleplex PCR, Table 12 and Table 13
  → Tumor stem cell related gene expression

or store cDNA at -20 °C for max. 4 weeks.

Table 10: Preparation of the Multiplex-PCR (EMT-2)

Component		Volume
PCR Master Mix	HotStarTaq Master Mix	12.5 µl
	RNase-free water	4.5 µl
	PrimerMix EMT-2 1 1	4.0 µl
Samples	cDNA or RT Control or Negative Control (RNase-free water) or *Positive Control EMT-2* 1 2 each:	4.0 µl
Total volume		25.0 µl

- The PCR is performed with a total of 35 cycles.

Table 11: PCR program (EMT-2)

95 °C	15 min	
94 °C	30 sec	⎫
60 °C	30 sec	⎬ 35 cycles
72 °C	60 sec	⎭
72 °C	10 min	
4 °C	∞	

Table 12: Preparation of the Singleplex PCR (StemCell)

Component		Volume
PCR Master Mix	HotStarTaq Master Mix	12.5 µl
	RNase-free water	4.5 µl
	PrimerMix StemCell 13	4.0 µl
Samples	cDNA or RT Control or Negative Control (RNase-free water) or *Positive Control StemCell* 14 each:	4.0 µl
Total volume		25.0 µl

- The PCR is performed with a total of 35 cycles.

Table 13: PCR program (StemCell)

95 °C	15 min	
94 °C	30 sec	35 cycles
51 °C	30 sec	
72 °C	30 sec	
72 °C	5 min	
4 °C	∞	

- For fragment analysis we recommend the use of an Agilent 2100 Bioanalyzer.

QIAGEN GmbH
QIAGEN Strasse 1
D-40724 Hilden
Germany

For support call
Phone: +49 (0) 511 72 59 50 - 50
Fax: +49 (0) 511 72 59 50 - 40
Email: support@adnagen.com
Internet: www.adnagen.com