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For Professional Use Only

ADIPO-screen kit

Instruction Manual

AmpliSens[®]



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1. INTENDED USE

ADIPO-screen kit is an *in vitro* nucleic acid amplification test based on pyrosequencing technology for genotyping the single-nucleotide polymorphisms (SNPs). **ADIPO-screen** kit is intended for the detection of genetic polymorphisms in genes associated with a liability to obesity.

Certain genetic loci can be united into genetic study **profiles**. Pyrosequencing is a real-time sequencing technique based on the detection of pyrophosphate released during DNA elongation (sequencing by synthesis). Pyrophosphate molecules released upon nucleotide incorporation into the elongating DNA strand undergo a series of enzymatic modifications, as a result of which a chemiluminescent signal is generated. The complex of recorded signals corresponds to the nucleotide sequence of the genetic locus of interest.



For research use only. Not for diagnostic procedures.

2. PRINCIPLE OF PCR DETECTION

Genetic polymorphism detection by pyrosequencing technology includes four stages: DNA extraction from clinical samples, PCR (polymerase chain reaction) of a DNA fragment containing relevant genetic locus, sample preparation, and pyrosequencing reaction.

Upon extraction, PCR of studied genetic locus is performed with the use of specific primers. A biotinylated primer is used for subsequent immobilization of amplification product and sample preparation. The direction of sequencing determines the type of analysis (forward or reverse). Reverse biotinylated primers are used in forward analysis, and forward biotinylated primers are used in reverse analysis.

A PCR product binds to streptavidin-coated Sepharose beads and then is used for subsequent purification of reaction mixture to obtain a single-stranded DNA fragment by serial washes performed with a Vacuum Prep Workstation. Streptavidin Sepharose High Performance reagent (GE Healthcare) is used for amplicon binding. When purification and immobilization of a single-stranded PCR product are completed, relevant genetic locus is sequenced using pyrosequencing technology.

At the PCR stage, the reagent kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT

ADIPO-screen kit is produced in 1 form: **REF** PMQ-006-50-F-CE.

ADIPO-screen kit includes:

<i>Reagent</i>	<i>Description</i>	<i>Locus (polymorphism)</i>	<i>Volume, ml</i>	<i>Quantity</i>
PCR-mix-1 OB-1	colorless clear liquid	FTO (T>A)	0.275	1 tube
PCR-mix-1 OB-2	colorless clear liquid	PPARD (-87T>C)	0.275	1 tube
PCR-mix-1 OB-3	colorless clear liquid	PPARGC1A (S482G G>A)	0.275	1 tube
PCR-mix-1 OB-4	colorless clear liquid	PPARGC1B (A203P G>C)	0.275	1 tube
Polymerase (TaqF)	colorless clear liquid	–	0.03	4 tubes
2.5x PCR-buffer blue	blue clear liquid	–	0.6	4 tubes
Mineral oil for PCR	colorless viscous liquid	–	4.0	2 vials
Seq Primer OB-1-S	colorless clear liquid	FTO (T>A)	0.33	5 tubes
Seq Primer OB-2-S	colorless clear liquid	PPARD (-87T>C)	0.33	5 tubes
Seq Primer OB-3-S	colorless clear liquid	PPARGC1A (S482G G>A)	0.33	5 tubes
Seq Primer OB-4-S	colorless clear liquid	PPARGC1B (A203P G>C)	0.33	5 tubes
Negative Control (C–)	colorless clear liquid	–	1.2	1 tube

ADIPO-screen kit it is intended for performing 55 pyrosequencing reactions for each genetic locus.

4. ADDITIONAL REQUIREMENTS

DNA extraction and amplification

- RNA/DNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol filters (up to 10, 100, 200, and 1000 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for Eppendorf tubes.
- PCR box.

- Personal thermocyclers (for example, Gradient Palm Cycler (Corbett Research, Australia), GeneAmp PCR System 2700 (Applied Biosystems, USA), MaxyGene (Axygen, USA) or equivalent).
- Disposable polypropylene 0,2 or 0,5-ml and 1.5-ml tubes
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.
- Sample preparation and pyrosequencing
- H₂O sterile, vial of 500 ml (manufactured by FBIS CRIE).
- PEERO-prep reagent kit for sample preparation (manufactured by FBIS CRIE) **REF** PMQ-P-CE (contains binding buffer, cleansing solution, 10x denaturation buffer, and 10x washing buffer).
- PyroMark Gold Q24 Reagents or PyroMark Gold Q96 Reagents (QIAGEN, Germany).
- Vacuum Prep Workstation (QIAGEN, Germany).
- Streptavidin Sepharose High Performance 5 ml (GE Healthcare, USA).
- PyroMark Gold Q24 or PyroMark Gold Q96 MD instrument (QIAGEN, Germany) or equivalent.
- Plates for pyrosequencing (QIAGEN, Germany).
- Plate for immobilizing PCR product with Sepharose beads (EIA plate)
- Plate shaker up to 1400 rpm.
- Vortex.
- Heating block.
- Pipettes (adjustable)
- Disposable pipette tips with aerosol filters up to 10 µl and without aerosol filters up to 200 µl.
- 0.2- and 0.5-ml tube racks.
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Parafilm (for example, Parafilm M, Pechiney Plastic. Packaging, USA).
- Disposable powder-free gloves and a laboratory coat.
- Reservoir for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge them briefly.
- Use disposable protective gloves, laboratory coats, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all samples and unused reagents in compliance with the local regulations.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid samples and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- Safety Data Sheets (SDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment, and reagents to the area where the previous step was performed.



Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING



Obtaining samples of biological materials for PCR-analysis, transportation and storage are described in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

ADIPO-screen kit is intended for analysis of DNA extracted by using DNA extraction kits from whole blood.

7. WORKING CONDITIONS

ADIPO-screen kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA extraction

It is recommended to use the following nucleic acid extraction kits:

- RIBO-prep, **REF** K2-9-Et-100-CE.
- DNA-sorb-B, **REF** K1-2-100-CE.



Extract DNA according to the manufacturer's instructions.



Negative control (C-) reagent included in this kit is used for the extraction of negative control sample.

8.2 Preparing PCR

The total reaction volume is 25 µl, the volume of DNA sample is 10 µl. The optimal DNA quantity is 10–30 ng per reaction (use TE-buffer (manufactured by FBIS CRIE) for dilution).

Required reagents:

PCR-mix-1, 2.5x PCR-buffer blue, polymerase (TaqF), mineral oil for PCR.

8.2.1 Preparing tubes for PCR

The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, DNA and control samples into tubes.

1. Prepare a reaction mixture for each studied polymorphism as follows:

Component	Volume/reaction
PCR-mix-1	5*(N+1) µl
2.5x PCR buffer blue	10*(N+1) µl
Polymerase (TaqF)	0.5 (N+1) µl
Total volume per one reaction	about 15 µl

N is the number of reactions to be performed.



Store prepared mixture for no longer than 2 h.

2. Vortex the reaction mixture, and dispense 15 µl into each PCR tube.
3. Add above **1 drop of mineral oil for PCR.**
4. Add **10 µl** of the **DNA samples** with concentration 1–3 ng/µl obtained at the extraction stage.



The Negative control of extraction (Negative Control, (C–)) must be used as the negative control of the analysis of each studied genetic locus. The sample extracted from Negative Control, (C–) should be diluted in the same ratio as DNA extracted from clinical samples.

8.2.2 Amplification

1. Run the following program in the thermocycler (Table 1).
2. When the temperature reaches 95 °C (pause mode), insert tubes into the thermocycler cells and press the button to continue.

It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them in the thermocycler.

Table 1

Amplification program

Gradient Palm Cycler, MaxyGene, GeneAmp PCR System 270			
Step	Temperature, °C	Time	Cycles
0	95	Pause	
1	95	15 min	1
2	95	15 s	45
	60	15 s	
	72	20 s	
4	72	2 min	1
5	10	Storage	

3. When amplification is completed, proceed to preparation of PCR products for pyrosequencing.

8.3 Sample Preparation and Pyrosequencing

PyroMark Q24 System

A. Run Setup for the PyroMark Q24 System

1. Start PyroMark Q24 Software.
2. Test settings:
 - a) select **New Assay** and select **AQ Assay** in the **File** menu.
 - type or paste the **Sequence to Analyze**;
 - click the **Generate Dispensation Order**;
 - b) click **File** and select **Save as**.

Polymorphisms for analysis

<i>Locus</i>	<i>Product</i>	<i>Poly-morphism</i>	<i>rs</i>	<i>Sequence to analyze</i>	<i>Analysis</i>	<i>Genotype variant</i>
FTO	Fat mass and obesity-associated gene	T>A	rs9939609	CT/AAAATTTCACAGC	reverse	TT TA AA
PPARD	Transcription factor PPAR delta	-87T>C	rs6902123	GGT/CGCTGTCT	forward	TT TC CC
PPARGC1A	PPARG coactivator-1a	S482G G>A	rs8192678	CACC/TGGTCTTG	reverse	GG GA AA
PPARGC1B	PPARG coactivator-1b	A203P G>C	rs7732671	GC/GCTTCTTGTCTT	reverse	GG GC CC

3. Create the test template:

- a) select **New Run** in the **File** menu;
 - in the **Instrument Method** menu select **PyroMark Q24 Method 001 Rev. A(1)_1** or method specified in the **PyroMark Gold Q24 Reagents** manual;
 - into each well of the top line of **Plate Setup** window add assays of test samples (press the right mouse button and use the **Load Assay** option)
 - in the second and third line of the **Plate Setup** window add test sample names (notes) by pressing the left mouse button.
- b) select **Save As** in the **File** menu, name the experiment, and save it to USB stick.

Close the test settings window.

4. Select **Pre Run Information** in the **Tools** menu to get a report. Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup.

B. Cartridge preparation**Reagents required:**

PyroMark Gold Q24 Reagents or PyroMark Gold Q96 Reagents (Qiagen), H₂O sterile (FBIS CRIE).



Follow the instructions given in **PyroMark Gold Q24 Reagents** or **PyroMark Gold Q96 Reagents** manuals.

Required volumes of **enzyme** mix (E), **substrate** mix (S), and **nucleotides** (A, T, C, G), are calculated by the software.

1. Dissolve the freeze-dried **enzyme** and **substrate** mixtures in **H₂O sterile** according to the instructions in the kit manual.
2. Load the cartridge with the appropriate volumes of reagents.
3. Seal the cartridge with Parafilm.



Store substrate mixtures and enzyme mixtures at ≤ -16 °C.
Store nucleotides at 4°C. Do not freeze!

C. Sequencing plate preparation

Required reagents: Seq Primers (sequencing primer)

1. Vortex Seq Primers.
2. Add **25 μ l** of **Seq Primer** into the wells of the sequencing plate in accordance with the template of the experiment.
3. Seal the sequencing plate with Parafilm.



Ensure that the well order in the sequencing plate corresponds with the template of the experiment

D. Product preparation to pyrosequencing

Required reagents:

Streptavidin Sepharose High Performance Beads (Streptavidin Sepharose High Performance, GE Healthcare), binding buffer, H₂O sterile, cleansing solution, denaturation solution, washing buffer.



Allow all required reagents and solutions to reach room temperature (22–25°C) before starting.



At first, prepare 1x denaturation solution и 1x washing buffer from 10x stock solutions. Use sterile H₂O as a solvent.

D1. Immobilization of PCR Products

1. Carefully shake the vial containing Sepharose beads until it is homogenous suspension.
2. Prepare the mixture for DNA immobilization calculating per one sample:
 - a) **Sepharose beads – 2 μ l;**
 - b) **Binding buffer – 40 μ l;**
 - c) **H₂O sterile – 28 μ l.**

Calculate the volume for **N** analyzed samples according to Table 3. Include 1 extra reaction when calculating (N+1).

Scheme of preparation of mixture for DNA immobilization

Number of samples (N+1)	Reagent volume for required number of samples, μ l		
	Sepharose beads	Binding buffer	H ₂ O sterile
7	14	280	196
9	18	360	252
11	22	440	308
13	26	520	364
15	30	600	420
17	34	680	476
19	38	760	532
25	50	1,000	700



Thoroughly vortex the mixture before use.

3. Add 10 μ l of PCR product and then 70 μ l of mixture for DNA immobilization per each analyzed well of EIA plate.



Order of analyzed samples in the EIA plate should corresponds to that in the sequencing plate.

4. Seal the whole plate with Parafilm.



Ensure that no leakage is possible between the wells

5. Agitate the EIA plate at room temperature at 1350–1400 rpm for 10 min.

D2. Preparation of samples with Vacuum Workstation

1. Vacuum Workstation setup:

- a) Fill five troughs as follows:

- About **40-50 ml** of **cleansing solution**;
- About **40-50 ml** of **1x denaturation solution**;
- About **40-50 ml** of **1x washing buffer**;
- **40-50 ml** of **H₂O sterile**;
- **70 ml** of **H₂O sterile**.

- b) Enable the vacuum pump and, when vacuum is fed, open the vacuum switch (**On**)

- c) Flash the Vacuum Workstation filter probes in the trough containing 70 ml of H₂O sterile and ensure that water is running to the waste container.

- d) Raise the vacuum tool up and back, beyond 90° vertical, for 2–3 s to drain liquid from the filter probes and ensure that the probes are dry.
- e) Close the vacuum switch (**Off**) and activate the **Parking** position for Vacuum Workstation.

2. Sample preparation:

- a) Remove EIA plate from the shaker, carefully lower the filter probes to the plate;
- b) Feed vacuum to the tool by switching **On** the vacuum and remove the PCR product and mixture for immobilization from the EIA plate;



Sephacose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads

- c) Transfer the vacuum tool to the trough containing cleansing solution and flush the filter probes for 5–10 s.
- d) Transfer the vacuum tool to the trough containing 1x denaturation solution. Flush the filter probes for 5–10 s.
- e) Transfer the vacuum tool to the trough containing 1x washing buffer. Flush the filter probes for 5–10 s.
- f) Raise the vacuum tool up and back, beyond 90° vertical, for 2–3 s to drain liquid from the filter probes.
- g) While the vacuum tool is held over the sequencing plate, close the vacuum switch on the tool (**Off**).
- h) Lower the filter probes to the wells and shake the vacuum workstation gently from side to side to release the beads to the sequencing plate.
- i) Wash the filter probes into the trough containing 40–50 ml H₂O sterile for 5–10 s;
- j) Lower the filter probes into the trough containing 70 ml of H₂O sterile, apply vacuum (**On**) and flash the probes for 10 s.
- k) Raise the vacuum tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes.
- l) Place the tool in the **Parking** position, turn off the vacuum pump, wait for several minutes, and close the vacuum switch on the vacuum tool (**Off**).

3. Primer annealing on the single-stranded DNA fragment:

- a) Heat the Sequencing Plate with the samples at 80°C for 2 min using the prewarmed Plate Holder.



It is recommended to seal the plate with Parafilm and aluminum press to avoid evaporation from the wells

- b) Remove the Plate from the plate holder, and let the samples cool to room temperature for 5 min.

E. Pyrosequencing of PCR product

1. PyroMark Q24 start up:

- a) insert the filled reagent cartridge into the instrument and secure it;
- b) open the plate-holding frame and place the sequencing plate, then close the plate-holding frame and the instrument lid.
- c) Insert the USB stick (containing the run file) into the USB port.
- d) Select **Run** in the main menu of the instrument panel and click **OK**.
- e) When the run file is selected, press **Select** and **Yes** to start the run.
- f) When the run is finished and the instrument confirms that the run file has been saved to the USB stick, press **Close**.

2. Result analysis

- a) Start PyroMark Q24 software;
- b) Select **Open** from the **File** menu and open the result analysis file;
- c) Analyze the results by clicking the **Analyze All Wells** button.
- d) Select the **SNP Analysis Results** option from the **Report** menu and then click **Preview** or **Save** buttons.

PyroMark Q96 MD

A. Run Setup for the PyroMark Q96 MD

1. Switch on the instrument.



Warm up the instrument for 90 min, before starting the work

2. Start up PyroMark MD software.
3. Test settings:
 - a) Select **SNP** in the main menu, then **Simplex Entries**, and then **New Entry** (use the mouse right button);
 - fill the name of the experiment in the **Entry ID** line;
 - type or paste **Sequence to Analyze**
 - click the **Generate Dispensation Order**;
 - b) click **Save** and close the test settings window.
4. Create test template:

Single plate sequencing

- a) select **SNP Runs** in the main menu. In the opened window select **New SNP Run** from the **SNP Runs** folder (use the mouse right button);
- b) set the name and parameters of the experiment in the opened window;
- c) in the **Instrument Parameters** line, select **Instrument Parameters MD 0003CDT** or the method outlined in the kit Manual.
- d) in the **Active Wells** window select the wells corresponding the test samples and click **Activate**.
- e) Select **Setup** and then **Entry**. Click the **Simplex** button, select the required test in the **Entry** line, and mark the analyzed wells using the pencil tool.
- f) Enter names/notes of the test samples in the **Sample ID** and **Notes** tabs.
- g) Select the **General** screen, click **View**, and select **Run**.
- h) A list of the reagents and volumes required for the experiment will appear in the opened window. Print the report and use it for cartridge preparation;
- i) Click **Save** and close the window.

Multiple plate sequencing

- a) Create a test template for each plate as described in the 4a step;
- b) Select **Batch** in the main menu. In the opened window select **New Batch** in the **Batch** option (use the mouse right button);
- c) Select the **Setup** screen, enter the name and parameters of the experiment.
- d) In the main menu click **SNP Runs** and tug each created template to the **Run Name** window;
- e) Enable the **Use Barcode** and **Analyze SNP** options;
- f) Click **Save**;
- g) Select **Setup Information** in the **Batch** menu;
- h) A list of the reagents and volumes required for the experiment will appear in the opened window. Print the report and use it for cartridge preparation;

B. Cartridge preparation

Reagents required: PyroMark Gold Q96 Reagents (QIAGEN), H₂O sterile (FBIS CRIE)



Follow the instructions given in **PyroMark Gold Q96 Reagents Manual**

Required volumes of **enzyme** mix (E), **substrate** mix (S), and **nucleotides** (A, T, C, G), are calculated by the software.

1. Dissolve the freeze-dried **enzyme** and **substrate** mixtures in **H₂O sterile** according to the **PyroMark Gold Q96 Reagents Manual**.

2. Load the Cartridge with the appropriate volumes of nucleotides, enzyme, and substrate mixes.

3. Seal the cartridge with Parafilm.



Ensure that dispensers with capillaries are inserted into cartridge before loading (take the latch aside)



Store substrate mixtures and enzyme mixtures at ≤ -16 °C.
Store nucleotides at +4°C. Do not freeze!

C. Sequencing plate preparation

Required reagents: Seq Primers (sequencing primers)

1. Vortex Seq Primers.

2. Add **12 μ l** of **Seq Primers** into analyzed wells of the sequencing plate in accordance with the template of the experiment.

3. Seal the sequencing plate with Parafilm.



Ensure that the well order in the sequencing plate corresponds with the template of the experiment

D. Product preparation to pyrosequencing

Required reagents:

Streptavidin Sepharose High Performance Beads (Streptavidin Sepharose High Performance, GE Healthcare), binding buffer, H₂O sterile, cleansing solution, denaturation solution, washing buffer.



Allow all required reagents and solutions to reach room temperature (22–25°C) before starting.



At first, prepare 1x denaturation solution and 1x washing buffer from 10x stock solutions. Use H₂O sterile as a solvent.

D1. Immobilization of PCR Products

1. Carefully shake the vial containing Sepharose beads until it becomes a homogeneous suspension.

2. Prepare the mixture for DNA immobilization calculating per one sample:

a) **Sepharose beads – 2 μ l;**

b) **binding buffer – 40 μ l;**

c) **H₂O sterile – 28 μ l.**

Calculate a volume for **N** analyzed samples according to Table 4. Include 1 extra reaction when calculating (**N+1**).

Table 4

Scheme of preparation of mixture for DNA immobilization

Number of samples (N+1)	Reagent volume for required number of samples, μl		
	Streptavidin beads	Binding buffer	H ₂ O sterile
7	14	280	196
9	18	360	252
11	22	440	308
13	26	520	364
15	30	600	420
17	34	680	476
19	38	760	532
25	50	1,000	700
33	66	1,320	924
41	82	1,640	1,148
49	98	1,960	1,372
57	114	2,280	1,596
65	130	2,600	1,820
73	146	2,920	2,044
81	162	3,240	2,268
89	178	3,560	2,492
97	194	3,880	2,716



Thoroughly vortex the mixture before use.

3. Add 10 μl of PCR product and then 70 μl of mixture for DNA immobilization per each analyzed well of EIA plate.



Order of analyzed samples in the EIA plate should corresponds to that in the sequencing plate.

4. Seal the whole plate with Parafilm.



Ensure that no leakage is possible between the wells.

5. Agitate the EIA plate at room temperature at 1350–1400 rpm for 10 min.

D2. Preparation of samples with Vacuum Workstation

1. Vacuum Workstation setup:

- a) Fill five troughs as follows:

- About **40-50 ml** of **cleansing solution**;

- About **40-50 ml** of **1x denaturation solution**;
 - About **40-50 ml** of **1x washing buffer**;
 - **70 ml** of **H₂O sterile**.
- b) Enable the vacuum pump and when vacuum is on, open the vacuum switch (**On**)
 - c) Flash the Vacuum Workstation filter probes in the trough containing 70 ml of H₂O sterile and ensure that water is running to the waste container.
 - d) Raise the vacuum tool up and back, beyond 90° vertical, for 2–3 s to drain liquid from the filter probes and ensure that the probes are dry.
 - e) Close the vacuum switch (**Off**) and activate **Parking** position for Vacuum Workstation.

2. Sample preparation:

- a) Remove EIA plate from the shaker, carefully lower the filter probes to the plate;
- b) Apply vacuum to the tool by switching **On** the vacuum and remove the PCR product and mixture for immobilization from the EIA;



Sepharose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads

- c) Transfer the vacuum tool to the trough containing cleansing solution and flush the filter probes for 5–10 s.
- d) Transfer the vacuum tool to the trough containing 1x denaturation solution. Flush the filter probes for 5–10 s.
- e) Transfer the vacuum tool to the trough containing 1x washing buffer. Flush the filter probes for 5–10 s.
- f) Raise the vacuum tool up and back, beyond 90° vertical, for 2–3 s to drain liquid from the filter probes.
- g) While the vacuum tool is held over the sequencing plate, close the vacuum switch on the tool (**Off**).
- h) Lower the filter probes to the wells and shake the vacuum workstation gently from side to side to release the beads to the sequencing plate.
- i) Lower the filter probes into the trough containing 70 ml of H₂O sterile, apply vacuum (**On**) and flash the probes for 10 s.
- j) Raise the vacuum tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes.
- k) Place the tool in the **Parking** position, turn off the vacuum pump, wait for several minutes and close the vacuum switch on the vacuum tool (**Off**).

3. Primer annealing on a single-stranded DNA fragment:

- a) Heat the Sequencing Plate with the samples at 80°C for 2 min using the prewarmed Plate Holder.



It is recommended to seal the plate with Parafilm and aluminum press to avoid evaporation from the wells.

- b) Remove the Plate from the plate holder, and let the samples cool to room temperature for 5 min.

E. Pyrosequencing of PCR product

1. PyroMark Q96 MD start up:

- a) insert the filled reagent cartridge into the instrument and secure it;
- b) Select **Instrument**, then **Manage** in the program main menu. Click **Open** in the opened **Manage Instruments** window (the lid locking a sequencing plate will raise).
- c) ensure that cartridge dispensers are passable. To do this,
 - insert an empty sequencing plate sealed with Parafilm. Click **Close** in the **Manage Instruments** window and close the lid of the instrument.
 - click **Test** in the **Manage Instruments** window.
 - Click **Open** in the **Manage Instruments** window, open the lid and analyze drops on the Parafilm. Drops order: **D5** – E, **D8** – S, **E5** – A, **E6** – C, **E7** – G, **E8** – T.

Single plate sequencing:

- d) Place the sequencing plate with samples into the instrument. Click **Close** in the **Manage Instruments** window and close the lid.
- e) In the main menu select **SNP** then **SNP Runs**. Open the experiment template in the **SNP Runs** window and press **Run**.
- f) When the reaction is completed click **Close** and proceed to analysis of results.

Multiple plate sequencing

- d) Pull out a plate holder, take the latch aside, and insert plates with analyzed samples.
- e) Select **SNP** then **Batch**. Select the template of the experiment in the **Batch Name** window and click **Play**.
- f) Click **Stop** when sequencing is completed.

2. Result analysis

- a) Start PyroMark MD software;
- b) Select **SNP** then **SNP Runs** from the main menu and open the result analysis file in the **SNP Runs** window;
- c) Analyze the results by clicking the **All** button in the **Analyze** menu.

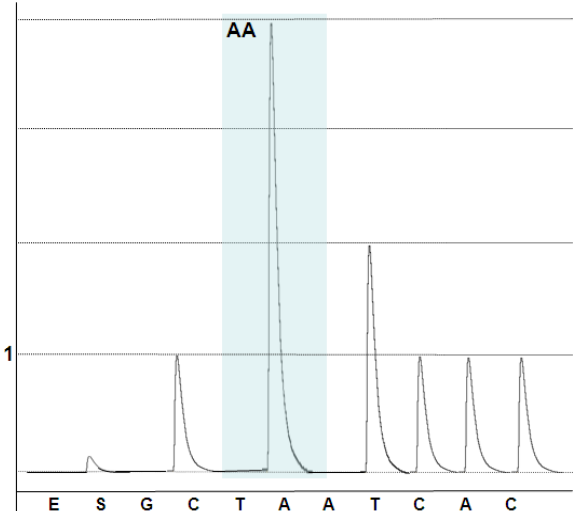
d) To review analysis of results select **Well Info** screen; to export data select the **Report** option.

9. DATA ANALYSIS

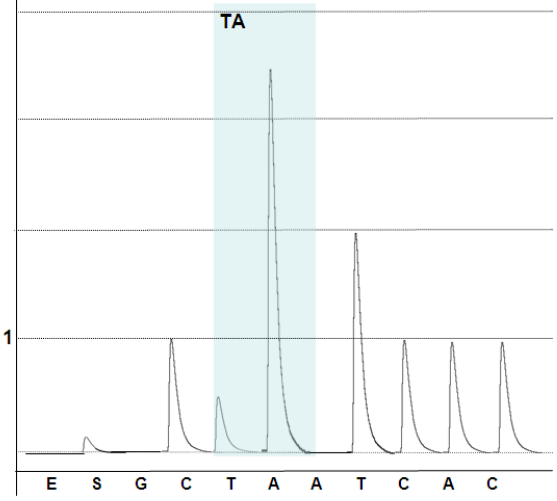
The results are interpreted on the basis of data presented below. For analysis of results, the sequencing direction specified in the Analysis column of Table 2 (forward or reverse strand sequencing) should be taken into account. The SNP allele status is analyzed automatically by the instrument software. As a result of analysis, the genotype is determined based on each locus included in the study profile. Genotype variants are as follows: a homozygote for a frequent allele, a heterozygote, and a homozygote for a rare allele (see the Genotype column in Table 2).

Representative results

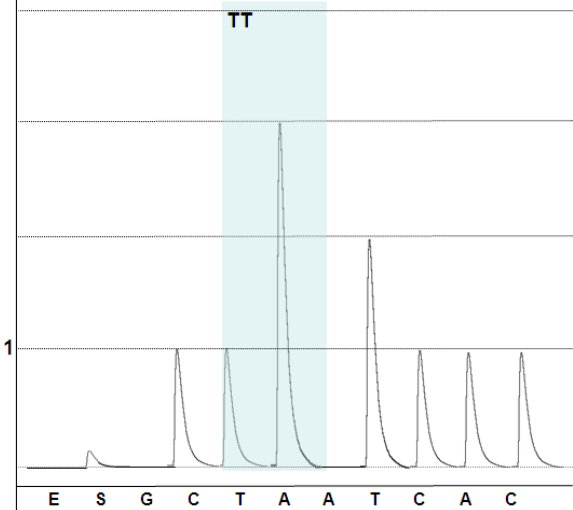
Locus: FTO, polymorphism IVS1 T>A



Genotype: TT

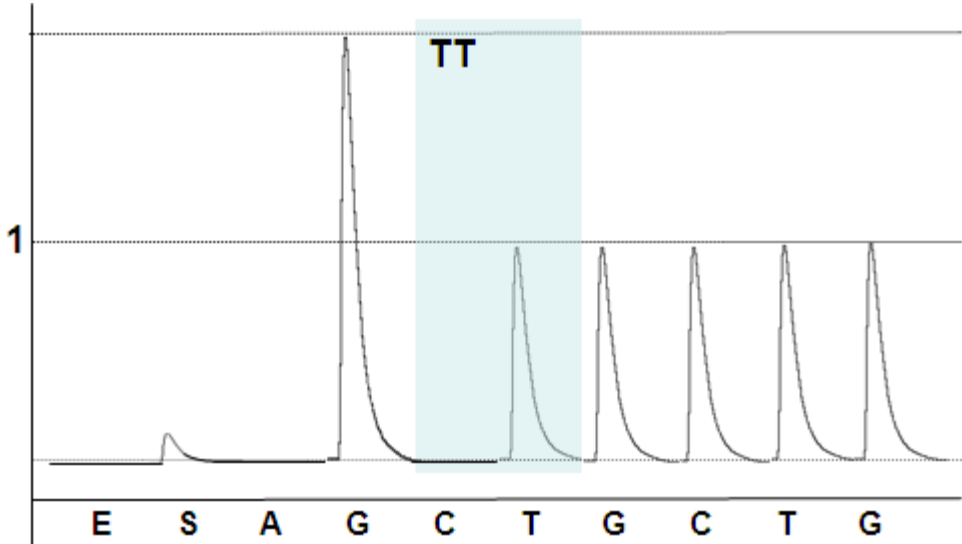


Genotype: TA

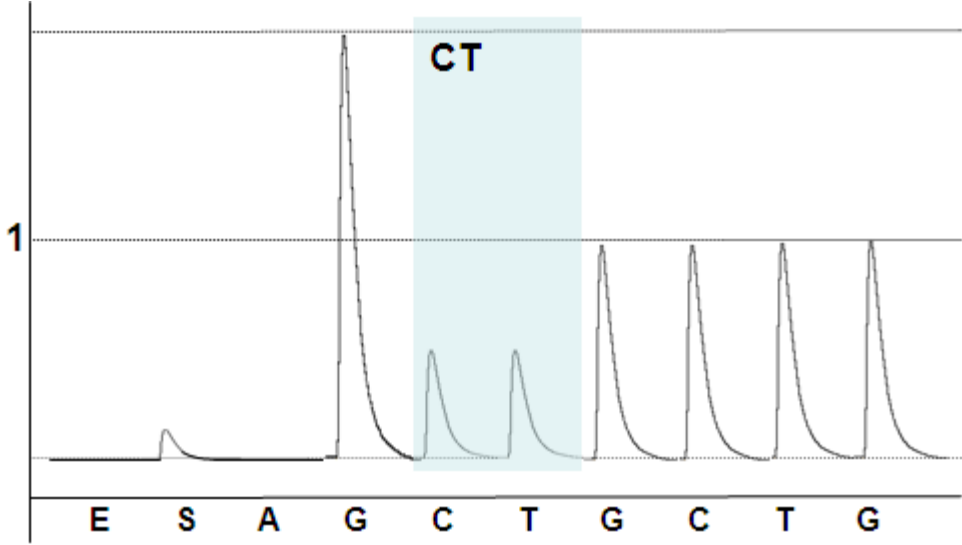


Genotype: AA

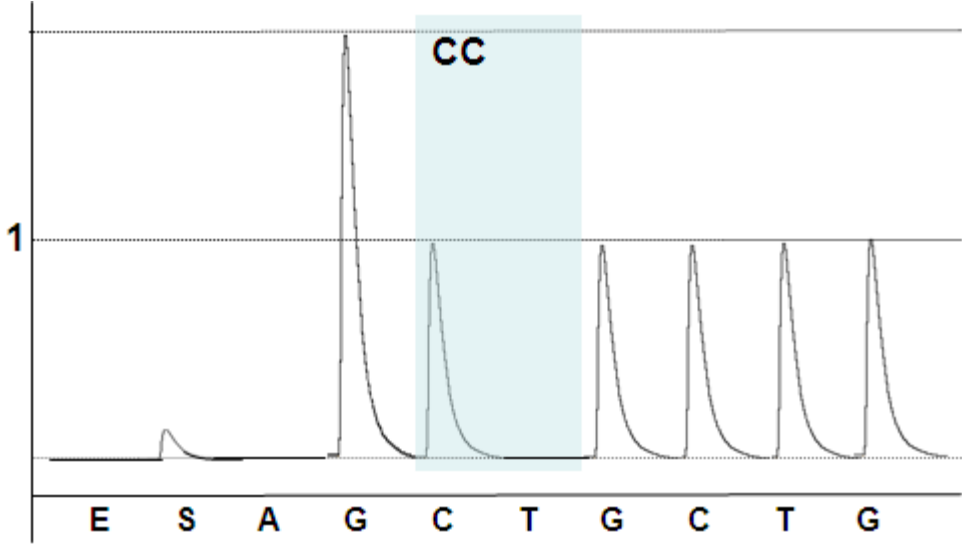
Locus: PPARD, polymorphism -87T>C



Genotype: TT

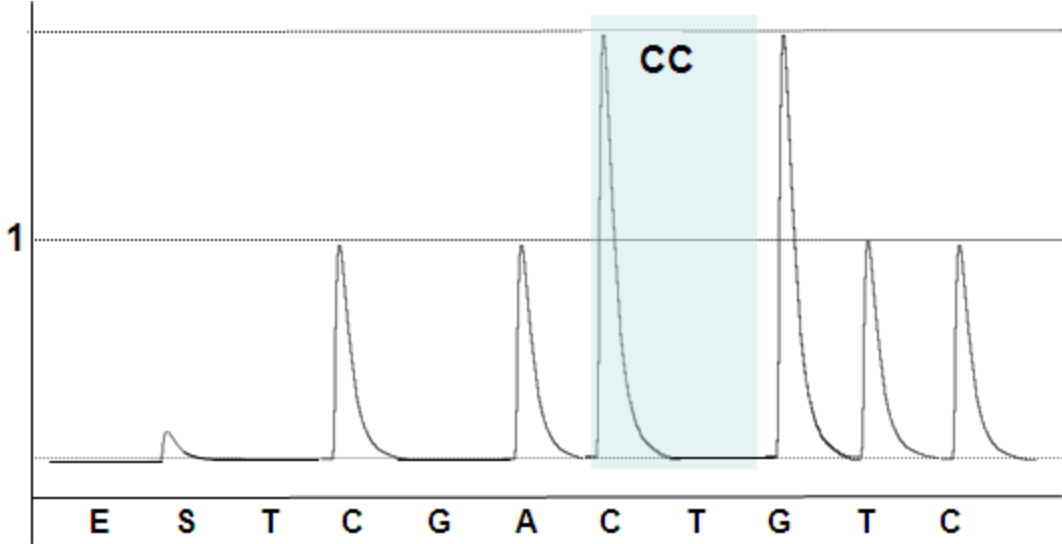


Genotype: TC

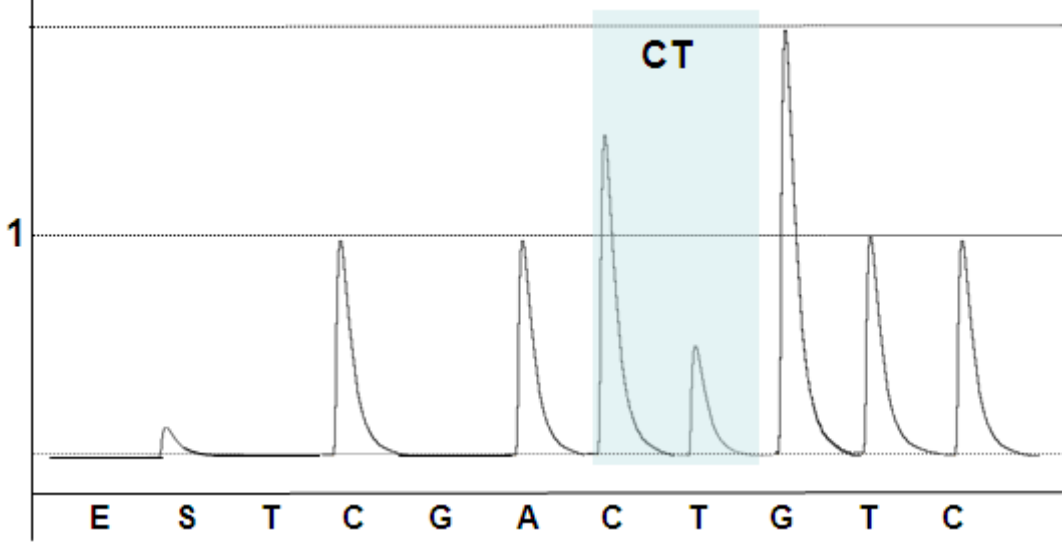


Genotype: CC

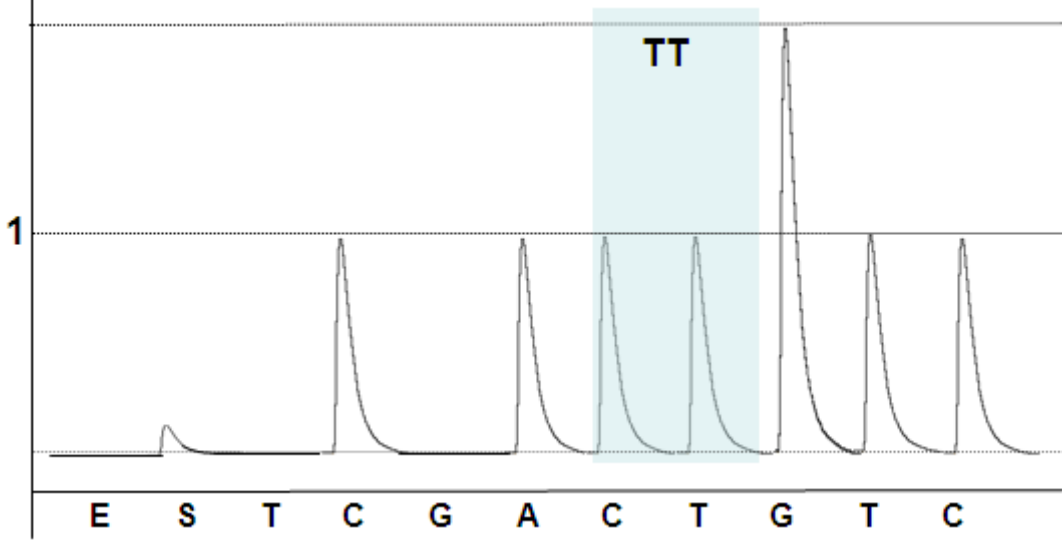
Locus: PPARGC1A, polymorphism S482G G>A



Genotype: GG

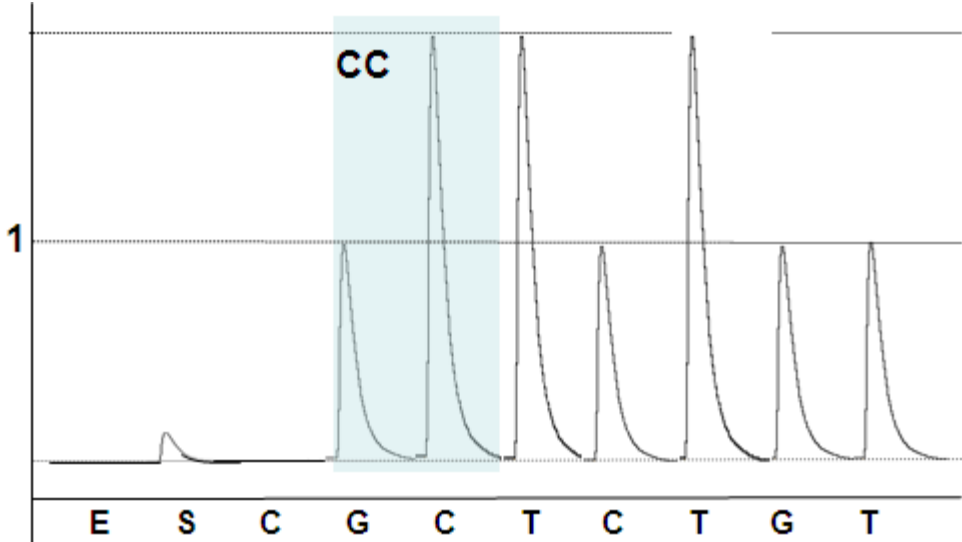


Genotype: GA

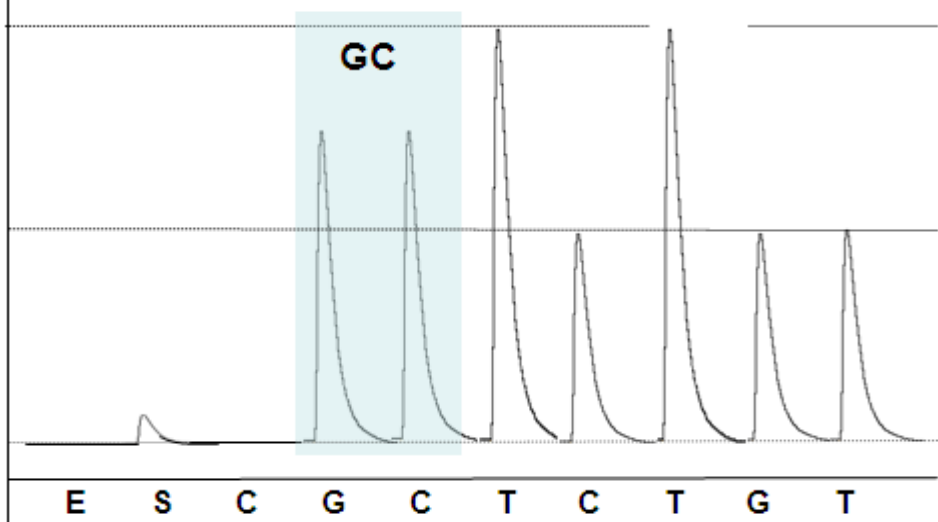


Genotype: AA

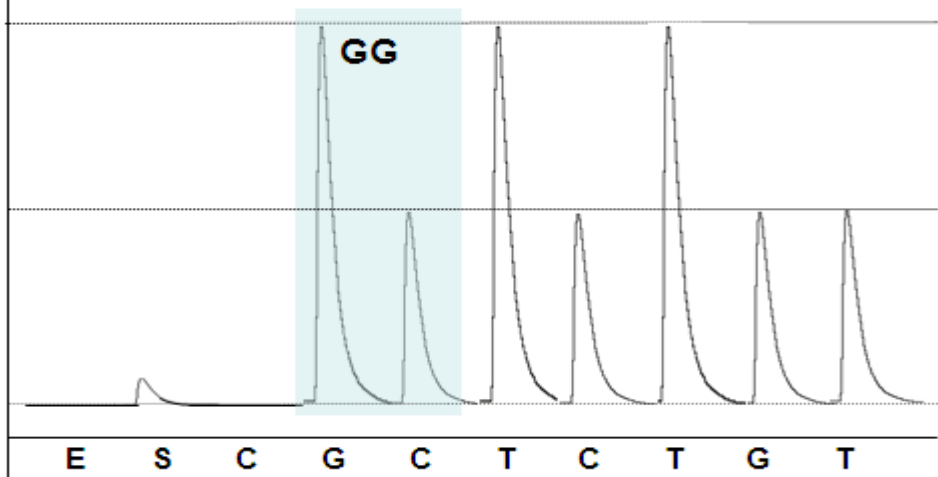
Locus: PPARGC1B, polymorphism A203P G>C



Genotype: GG



Genotype: GC

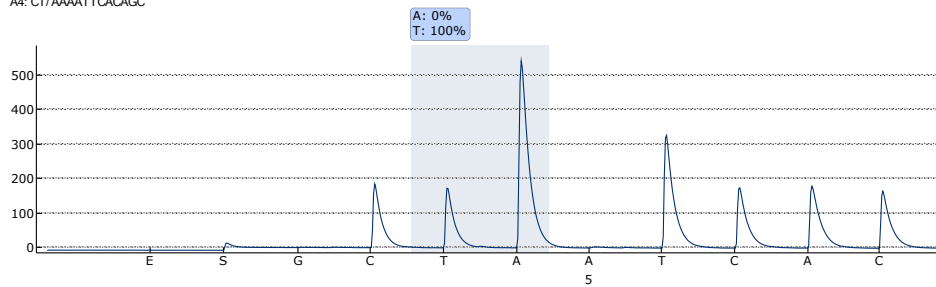


Genotype: CC

Examples of results

Locus: FTO, polymorphism IVS1 A>T

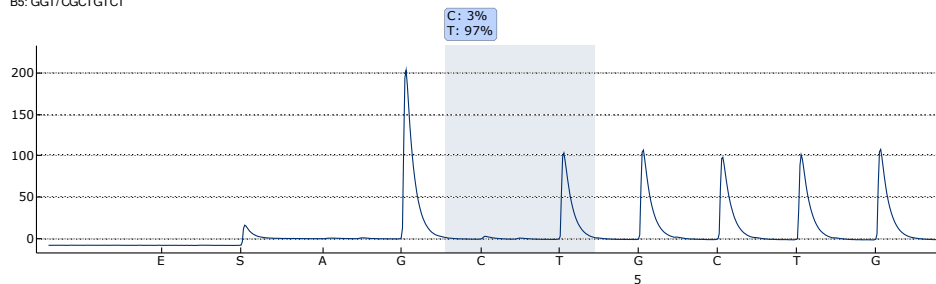
A4: CT/AAAATTCAACGC



Genotype: AA

Locus: PPARD, polymorphism -87T>C

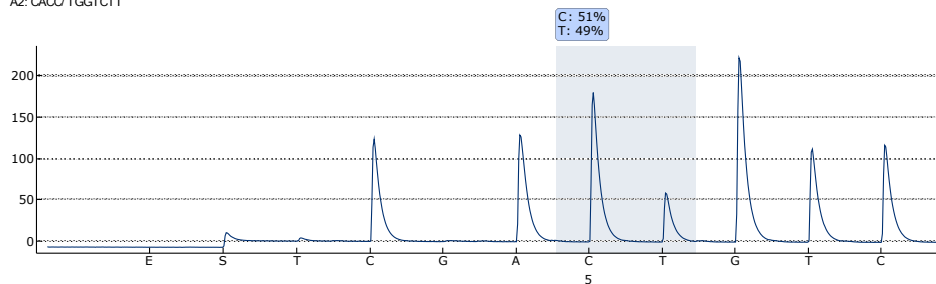
B5: GGT/CGCTGTCT



Genotype: TT

Locus: PPARGC1A, polymorphism S482G G>A

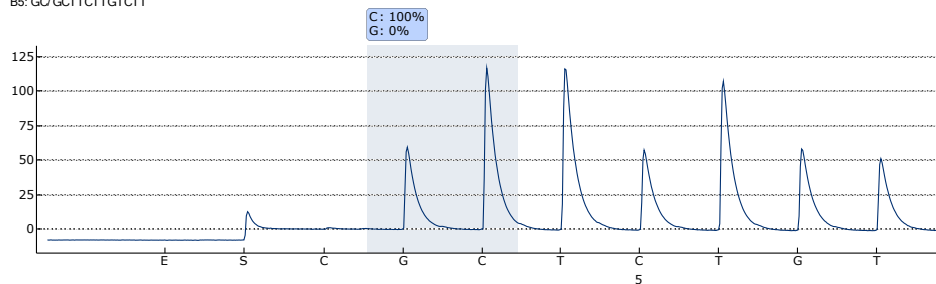
A2: CAC/ TGSTCTT



Genotype: GA

Locus: PPARGC1B, polymorphism A203P G>C


B5: GC/ GCTTCTGTCTT



Genotype: GG

10. TROUBLESHOOTING

Comments and suggestions

Signals in the no template control (negative control)	
a) Cross-talk between wells	Signal from one well is detected in a neighboring well. If re-running samples, avoid placing samples with high signal intensities next to no template control wells
b) PCR contamination	Use sterile pipette tips with filters. Store and extract materials such as specimens, controls, and amplicons separately from PCR reagents
Poor or unexpected sequence	
a) Low quality genomic DNA	Low-quality genomic DNA can cause the PCR to fail. Analyze PCR samples using an electrophoretic technique (using, for example, the QIAxcel® System or agarose-gel electrophoresis)
b) Unexpected rare mutation	A “Check” or “Failed” quality assessment can be caused by an unexpected pattern of peaks. This might indicate an unexpected mutation, which is not analyzed by the standard “Sequence to Analyze”. These samples should be analyzed using the alternative “Sequence to Analyze” considering unexpected mutations
High background	
Incorrect storage of nucleotides	Store nucleotides at 2–8°C. Storage at –15 to –25°C can cause an increase in the background
	If a sequence corresponding to the studied locus is detected in the Negative Control sample, the results for clinical samples are invalid

11. TRANSPORTATION

ADIPO-screen kit should be transported at 2–8 °C for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **ADIPO-screen** kit (except for polymerase (TaqF)) are to be stored at 2–8 °C when not in use. All components of the **ADIPO-screen** kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.



Polymerase (TaqF) is to be stored at temperature from minus 24 to minus 16 °C when not in use.

13. SPECIFICATIONS

The analytical specificity of **ADIPO-screen** kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequence comparison analysis.

14. REFERENCES














1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR diagnostics”, developed by Federal Budget Institute of Science “Central Research

Institute for Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being, Moscow, 2012.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science “Central Research Institute for Epidemiology” ISO 13485-Certified Quality Management System, each lot of the **ADIPO-screen** kit has been tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

	Catalogue number		Caution
	Batch code		Sufficient for
	Research use only		Expiration Date
	Version		Consult instructions for use
	Temperature limitation		Keep away from sunlight
	Manufacturer		Negative control of extraction
	Date of manufacture		
FBIS CRIE	Federal Budget Institute of Science “Central Research Institute for Epidemiology”		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
21.12.16 ME	Text	Corrections according to the template
	1. Intended use	It was added that ADIPO-screen kit is intended for the detection of genetic polymorphisms in genes associated with a liability to obesity