

Animaltype Sheep

**Reagent kit for multiplex analysis of
12 microsatellite markers and Amelogenin
locus of domestic Sheep (*Ovis aries*)**

Instructions for Use

For Research Use only



08. November 2021



11-14101-0096



Biotype GmbH
Moritzburger Weg 67
01109 Dresden
GERMANY

Content

| | |
|---|-----------|
| 1. Product Information..... | 3 |
| 1.1. Product Description | 3 |
| 1.2. Ordering information for Animaltype Sheep kit and its components ... | 4 |
| 1.3. Kit Components | 4 |
| 1.4. Storage conditions..... | 5 |
| 1.5. Main features of the kit..... | 6 |
| 1.6. Related materials..... | 6 |
| 2. Dry Matter Dilution..... | 7 |
| 2.1. Control DNA of OM-1 and OF-2 | 7 |
| 2.2. Size standard S550 | 7 |
| 3. PCR Amplification..... | 7 |
| 3.1. Setting a reaction..... | 7 |
| 3.2. Amplification conditions | 8 |
| 4. Electrophoresis on ABI PRISM 3130/3130XL Analyzer | 9 |
| 4.1. Matrix creation/spectral calibration..... | 9 |
| 4.2. Capillary electrophoresis conditions..... | 12 |
| 4.3. Creating an Instrument Protocol..... | 13 |
| 4.4. Preparation and loading of amplification products | 13 |
| 4.5. Starting the device..... | 14 |
| 4.6. Optimizing the intensity of signals | 15 |
| 5. Electrophoresis on ABI PRISM® 3500/3500XL Analyzer..... | 16 |
| 5.1. Matrix creation/spectral calibration..... | 16 |
| 5.2. Create Instrument Protocol..... | 18 |
| 5.3. Create Assay | 19 |
| 5.4. Preparation and loading of amplification products | 19 |
| 5.5. Starting the device..... | 20 |
| 5.6. Optimizing the intensity of signals | 21 |
| 6. Data Analysis | 22 |
| 6.1. Import of files for analysis in GeneMapper program | 22 |
| 6.2. Size Standard S550 | 25 |
| 6.3. Size ranges of microsatellite markers | 26 |
| 6.4. Amplification of Control DNA | 26 |
| 6.5. Interpretation of results | 27 |
| 7. Trademarks and Disclaimers..... | 29 |

1. Product Information

1.1. Product Description

Animaltype Sheep is a set of reagents for molecular genetic characterization of domestic sheep for the analysis of kinship and DNA individualization of animals based on multiplex PCR analysis of 12 loci containing short tandem repeats (STR), also known as microsatellite loci, and the Amelogenin gene as a sex marker. The 13 analyzed loci comprise the standard marker panel recommended by the International Society for Animal Genetics (ISAG): McM042, INRA006, McM527, ETH152, CSRD247, OarFCB20, INRA172, INRA063, MAF065, MAF214, INRA005, INRA023 and AMEL. With restriction to the loci CSRD247, INRA005, INRA006, INRA023, INRA063, MAF065, McM527 and OarFCB20 this kit can be used for genotyping of Goat samples. PCR primers have been designed for the multiplex amplification of all 13 loci in one tube. The size of all amplifiable PCR products is <320 bp (taking into account all known alleles). Analysis of PCR results is carried out by capillary electrophoresis method using automatic genetic analyzers with laser-induced fluorescence detection. Animaltype Sheep uses 5 fluorescent dyes characterized by different emission wavelengths to enable simultaneous detection in different fluorescence channels. The primers are labeled with four fluorescent dyes that are detected in the Blue, Green, Yellow and Red channels. The S550 size standard is labeled with a fifth fluorescent dye and is detected in a separate Orange channel simultaneously with PCR products. To obtain a complete STR profile of the sample, 10 nanograms of non-degradation DNA are sufficient. The optimal amount is from 10 nanograms and higher. The reaction mixture in the kit is aliquoted in reaction stripped tubes of 0.2 ml and delivered in lyophilized form, due to which reaction mixtures can be stored at room temperature for at least 18 months without sensitivity loss. The reaction components are activated by adding a certain amount of activator solution to each test tube. Total reaction volume is 25 µl. The maximum volume of DNA solution introduced into the reaction can be 20 µl.

The kit is validated for GeneAmp® 9700, GeneAmp® 2720. PCR products can be analyzed using ABI PRISM® 310/3100/3100Avant/3130/3130XL/3500/3500XL (Applied Biosystems, Foster City, CA-US) and NanoFor® 05M (Syntol, Moscow, RU) genetic analyzers.

| Locus | Repeating Unit Structure |
|-----------|--------------------------|
| McM042 | (AC) _n |
| INRA006* | (CA) _n |
| McM527* | (TG) _n |
| ETH152 | (AC) _n |
| CSRD247* | (CA) _n |
| OarFCB20* | (TG) _n |
| INRA172 | (TG) _n |
| INRA063* | (AC) _n |
| MAF065* | (CA) _n |
| MAF214 | (GT) _n |
| INRA005* | (GT) _n |
| INRA023* | (AC) _n |

Table 1: Description of microsatellite markers Animaltype Sheep. The loci marked with the * are suitable for genotyping goat samples.

1.2. Ordering information for Animaltype Sheep kit and its components

The Animaltype Sheep kit is available in 8-piece vials, which are stripped together with separately attached lids.

Strips 8 x 0.2 ml, vials with separately attached caps

| Name | Reactions in the kit | Catalog number |
|------------------|-----------------------------|----------------|
| Animaltype Sheep | 12 x 8 tubes (96 reactions) | 11-14101-0096 |

1.3. Kit Components

| | |
|--|---------------------------|
| Reaction Mix 96 x 0.2 ml | 12 strips with 8 wells |
| Activator 5X (blue lid) | 500 µl |
| Deionized water (white lid) | 1.7 ml |
| Control DNA OM-1, (lyoph., green lid) | 1 tube (20 reactions) |
| Control DNA OF-2, (lyoph., green lid) | 1 tube (20 reactions) |
| Size standard S550, (lyoph., yellow cover) | 1 tube (120 applications) |

Strips with reaction mixtures are reaction tubes of 0.2 ml, combined into strips of 8 pcs. and are designed for polymerase chain reaction. The bottom of the tubes contains all lyophilized components of polymerase chain reaction including Taq polymerase, dNTP mixture, reaction buffer, primer mixture. Due to the fluorescent-marked primers, the lyophilized dry vitreous reaction mixture at the bottom of the tubes is transparent.

The activator solution is used to dilute the lyophilized reaction mixture. It contains a buffer solution and magnesium ions Mg^{2+} as an activator for the polymerase chain reaction.

Deionized water is used to dilute the components of the set and bring the reactions to the working volume.

Control DNA OM-1 is a high-molecular lyophilized genomic DNA of a male sheep with a known genotype for all the studied loci (Table 2, Fig. 2).

Control DNA OF-2 is the high-molecular lyophilized genomic DNA of a female sheep with a known genotype across all the investigated loci (Table 2, Fig. 3). The control DNA is designed to control the stages of amplification, electrophoresis and data analysis. It is supplied in lyophilized form. It requires dilution with water before use (item 2.1).

Size standard S550 is a lyophilized mixture of fluorescent-labeled DNA fragments of different sizes, labeled with spectral analogue LIZ, detected in the Orange channel. The S550 size standard contains 26 DNA fragments of different sizes (n.a.): 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 220, 230, 240, 260, 280, 300, 320, 340, 360, 380, 400, 420, 440, 450, 500 and 550 bp. Standard S550 is used at the stage of capillary electrophoresis, is introduced into each capillary simultaneously with the investigated sample and serves as a reference internal standard for building a mobility curve of the amplified fragments of the investigated sample. Due to high density of fragments of S550 standard high accuracy and reproducibility of determination of size of amplified fragments of the investigated sample is provided. Before use, it is necessary to add water (item 2.2).

1.4. Storage conditions

All components except activator solution and deionized water are supplied in dry condition. For this reason, no special temperature conditions are required during transport. Fluorescently labeled primers and size standard

S550 are sensitive to light and must be stored in a dark place. Users can store the kits at room temperature for several months without losing sensitivity. After dilution of lyophilized components with water they should be stored at 2 - 8°C for a month. For longer storage, freezing at -20°C is recommended.

1.5. Main features of the kit

Number of simultaneously analyzed markers - 13; List of simultaneously analyzed loci: McM042, INRA006, McM527, ETH152, CSRD247, OarFCB20, INRA172, INRA063, MAF065, MAF214, INRA005, INRA023 and AMEL; Number of fluorescent markers used in the kit - 5; Optimum number of DNA input: 10 ng, Sensitivity limit: 1 ng.

Quality Assurances

The high quality of each component of the kit is checked and controlled during production. Each released lot of lyophilized reagents is regularly checked for compliance with the declared characteristics within 18 months.

1.6. Related materials

Necessary materials that are not included in the set are supplied free of charge:

Matrix standard CS5 for ABI PRISM® 3130 and ABI PRISM® 3130xl and Nanofor® 05. Bins and panels for GeneMapper™.

Materials supplied by other companies

| Reagent | Supplier | Catalogue Number |
|------------------------------|--------------------|------------------|
| Hi-Di™ Formamide | Applied Biosystems | (P/N 4311320) |
| 10 x Genetic Analyzer Buffer | Applied Biosystems | (P/N 402824) |
| Polymer (POP-4) | Applied Biosystems | (P/N 402838) |

2. Dry Matter Dilution

2.1. Control DNA of OM-1 and OF-2

Add 20 µl of deionized water supplied with the kit to each test tube with dry control DNA (2 test tubes with green caps). Mix thoroughly on vortex and collect the solution at the tubes by short centrifugation at the bottom of the tube. For PCR, add 1 µl of control DNA to the reaction tube. After dilution, control DNA should be stored at 2 - 8 °C for a month. For longer storage it is recommended to store in a frozen form. Repeated defrosting should be avoided.

2.2. Size standard S550

Before use, add 120 µl of deionized water to the S550 dry dimensional test tube (yellow cap tube). Mix thoroughly on the vortex and collect the solution at the tube bottom by short centrifugation. After dilution, the S550 size standard should be stored at 2 - 8 °C for a month. For longer storage it is recommended to store in a frozen form. Repeated defrosting should be avoided. For capillary electrophoresis, add 1 µl of S550 standard to each well of the plate containing the formamide and PCR product.

3. PCR Amplification

3.1. Setting a reaction

Include 5 µl Activator in each tube. Then apply up to 20 µl solution of the studied genomic DNA. The optimal amount of DNA to be amplified is 10 ng. The amount of DNA to be amplified depends on its concentration. The maximum possible volume of DNA solution is 20 µl. If necessary, bring the total reaction volume to 25 µl with deionized water supplied as part of the kit.

| Components of the reaction | Volume per 1 reaction |
|----------------------------|------------------------------|
| Activator solution | 5 µl |
| Genomic DNA (10 ng) | up to 20 µl |
| Deionized water | up to the final volume 25 µl |

For long-term storage, dilution of DNA in buffers (pH > 7) containing small amounts of EDTA (e.g. TE with ≤ 0.1 mM EDTA) is recommended. A high concentration of EDTA in DNA solution may cause a decrease in reaction efficiency due to magnesium ion chelation. After application of all components, the reaction mixture should be thoroughly stirred to homogeneous state 5-8 times by pipetting or using vortex. If necessary, collect the solution at the bottom of the tube by short centrifugation. Thorough stirring is necessary for maximum reaction efficiency. Two **positive controls** (1 µl of control DNA OM-1, 1 µl of control DNA OM-2 supplied with the kit) and one **negative control** (deionized water instead of DNA) should be amplified with each series of tested samples.

3.2. Amplification conditions

The amplification conditions listed below are recommended as standard parameters.

| PCR Parameters | |
|----------------|-------------------|
| 94°C | 3 min |
| 95°C | 10 sec |
| 57°C | 30 sec, 35 cycles |
| 68°C* | 60 sec |
| 68°C | 6 min 30 sec |
| 20°C | ∞ |

* The recommended heating rate from 57°C to 68°C is max. 1°C/1 sec.

After completing the PCR program, the amplified products can be stored for a week at 4°C to 8°C in a light-safe place. In case the amplified PCR product should be stored for more than a week, freezing at -20°C is recommended.

4. Electrophoresis on ABI PRISM 3130/3130XL Analyzer

When working with the ABI PRISM® genetic analyzer, and subsequent analysis of fluorescent profiles in the GeneMapper™ program, it is necessary to follow the user instructions from the equipment manufacturer. For correct visualization of the five fluorescent Animaltype Sheep labels, it is necessary to perform spectral calibration for the "any5dyes" dye set using the CS5 matrix standard.

4.1. Matrix creation/spectral calibration

The analysis of Animaltype Sheep amplification products on the genetic analyzer is only possible after calibration with the 5-colour matrix standard CS5 (not supplied with the kit, ordered separately by catalog number: 11-14102-0050). The matrix standard contains a mixture of 5 fragments of different size marked with different fluorescent dyes. These dyes are used in the PCR tagging kit and the S550 size standard. To prepare a working solution of matrix standard CS5, add 50 µl of deionized water to a tube containing lyophilized CS5 (violet lid tube) and incubate at room temperature for 2 min. Then thoroughly mix the solution on the Vortex and collect the solution by short centrifugation at the bottom of the tube. The ready solution can be stored in a dark place at 2 °C - 8 °C for up to 2 weeks. For longer storage time, the solution should be frozen. Repeated defrosting should be avoided.

Preparation of the calibration standard matrix (ABI 3130 /4 capillary)

| | |
|---------------------|-------|
| Hi-Di™ Formamide | 40 µl |
| Matrix Standard CS5 | 4 µl |

Add 10 µl of mixture to wells A01-D01 of 96-well plate. If necessary, remove bubbles from the bottom of the wells by centrifuging.

Preparation of the calibration standard matrix (ABI 3130 XL/16 capillary)

| | |
|---------------------|--------|
| Hi-Di™ Formamide | 160 µl |
| Matrix Standard CS5 | 16 µl |

Add 10 µl of mixture to wells A01 to H02 of 96-well plate. If necessary, remove bubbles from the bottom of the wells by centrifuging.

Spectral calibration

Step A - Create Instrument Protocol for Spectral Calibration

Open **Protocol Manager** in **Data Collection Software**, go to the **Instrument Protocol** tab and select **New** to open the **Protocol Editor**. Enter the following settings in the **Protocol Editor (Instrument Protocol)** window:

| Parameter | Value |
|--------------|----------------------------------|
| Name | for example, Spectral36_POP4_CS5 |
| Type | SPECTRAL |
| Dye Set | any5dye |
| Polymer | POP4 |
| Array Length | 36 |
| Chemistry | Matrix Standard |
| Run Module | Spect36_POP4_1 |

Select **OK** and close **Protocol Editor**.

Step B - Plate creation

Go to **Plate Manager** of **Data Collection Software** to select the button **New**. The **Plate Dialog** window will open.

Enter the following parameters in the New Plate dialog box:

| | |
|-------------|--------------------------------|
| Name | for example, Spectral_any5_CS5 |
| Application | Spectral Calibration |
| Plate Type | 96-Well |

Select **OK**. A new **Plate Editor** table will appear.

| Enter in position A01 | |
|-----------------------|---------------------------------|
| Sample Name | for example, CS5 |
| Priority | for example, 100 |
| Instrument Protocol | Spectral36_POP4_CS5 (see above) |

Identify cell A01 in its entirety. Select the **Fill Down Special** command in the **Edit** menu. The program will fill with the entered values to the corresponding number of cells for one capillary load. For example, from A01 to A04 (**ABI Prism® 3130 / 4 capillaries**) or from A01 to H02 (**ABI Prism® 3130XL / 16 capillaries**). Select **OK** to complete the plate creation and exit the **Plate Editor**.

Step C - Carrying out spectral calibration

Go to the **Run Scheduler - Plate View** tab and select **Find All**. Select the specified name of the created plate (for example, Spectral_any5_CS5). Link the selected name with the plate installed in the device. Start the device.

Step D - Evaluation of spectral calibration results.

Check the status of each capillary (pass or fail) after the device is finished. Open **Instrument Status**, go to the **Event Log** tab. The **Event Messages** window displays the status of all capillaries. Each capillary must have a Q-value of at least **0.95**. The peak heights should be at least 1,000 rfu, but below 5,000 rfu (optimal range between 2000 and 4000 rfu). Additionally, you can view the fluorescent calibration profile for each capillary in the **Spectral Viewer** window. Calibration should be successful for a minimum of 3 of 4 capillaries (or 12 of 16 capillaries, respectively). When using CS5 as a matrix standard, the calibration profile window shall reflect the following peak sequence (left to right) **blue-green-yellow-red-orange**. If calibration is successful, it is recommended that it be renamed with a more convenient name. To do this, press the **Rename** button, enter a new calibration name (e.g. **CS5_[date]**) and press **OK**. Note that for each virtual filter set (**dye set**) the last calibration automatically becomes active. If you plan to use the results of previous calibrations, you should activate them before starting the instrument.

4.2. Capillary electrophoresis conditions

Before performing the first Animaltype Sheep amplification product analysis on a genetic analyzer, you must create the corresponding **Run Module**. To do this, go to the **Module Manager** of the Data Collection Software and press the **New** button. The **Run Module Editor** window will be opened. Create the module by editing Injection Time and Run Time with the following parameters:

| Parameter | Value |
|-------------------------|-------------|
| Oven Temperature | 60 |
| Poly Fill Volume | 4840 |
| Current Stability | 5 |
| PreRun Voltage | 15 |
| PreRun Time | 180 |
| Injection Voltage | 3 |
| Injection Time | 5 |
| Voltage Number of Steps | 40 |
| Voltage Step Interval | 15 |
| Data Delay Time | 1 |
| Run Voltage | 15 |
| Run Time | 1300 |

Press **Save As** and enter a convenient name for the created module (for example, Animaltype Sheep). Press **OK** and exit the module editor by pressing **Close**.

4.3. Creating an Instrument Protocol

Go to the **Protocol Manager** section of **Data Collection Software**. In the **Instrument Protocol** window, click the New button to open the **Protocol Editor**. It is necessary to enter the following parameters:

| Parameter | Value |
|-------------|------------------|
| Name | e.g. Run36_Sheep |
| Type | REGULAR |
| Run Module* | Animaltype_Sheep |
| Dye Set | Any5Dye |

* for the value of the parameter, see item 4.2.

Press the **OK** button to save the changes and exit the protocol editor.

4.4. Preparation and loading of amplification products

To load samples into the device it is necessary to prepare a mixture of Hi-Di™ formamide and S550 size standard (diluted in accordance with paragraph 2.2) in the following ratio:

| Component | Volume per sample |
|-------------------|-------------------|
| Hi-Di™ formamide | 10.0 µl |
| SizeStandard S550 | 1.0 µl |

When calculating the volume of the mixture components, note that all wells where injection will take place, including those without samples, should be filled with Hi-Di™ formamide mixture. After stirring, add 10 µl of mixture to each well of the plate. Then add 1 µl of PCR product or allele ladder to the wells. If necessary, remove the bubbles from the bottom of the plate wells by short centrifugation. Cover the plate with a rubber mat and load it in accordance with the ABI PRISM® Genetic Analyzer user manual. The Animaltype Sheep kit does not require temperature denaturation of the samples!

4.5. Starting the device

Capillary electrophoresis on the ABI PRISM® genetic analyzer is performed according to the user's manual provided by the manufacturer. To obtain correct results, an appropriate **Spectral calibration** (see section 4.1), **Run Module** (see section 4.2), and **Instrument Protocol** (see section 4.3) must be performed.

Step A Creating the plate

Before starting the analysis, you should create a **Plate** that describes the location of the samples on the 96-well plate and provides instructions for the instrument. Go to the **Plate Manager** of the **Data Collection Software** and click the **New** button. The **Plate Dialog** box will appear. Enter the following parameters:

| Parameter | Value |
|-------------|---------------------------------------|
| Name | For example: Animaltype_Sheep _[date] |
| Application | select GeneMapper |
| Plate Type | 96-Well |

Click **OK**. A new **Plate Editor** table will appear.

Step B Fill the table

| Parameter | Value |
|---------------------|---|
| Sample Name | The sample name |
| Priority | is usually 100 (the order of analysis in ascending order) |
| Sample Type | Sample / Allelic Ladder / Positive Control / Neg. Control |
| Size Standard | S550 |
| Panel | Animaltype_Sheep_Panels_Vx |
| Analysis Method | Animaltype_Sheep |
| Bins | Animaltype_Sheep_Bins_Vx |
| Results Group | Select the appropriate Results Group |
| Instrument Protocol | e.g. Run36_Sheep |

For convenience, it is better to enter all sample names first. Then, for the first sample, set all the necessary parameters. Select all columns with the mouse cursor. In the **Edit** menu, select **Fill Down**. The program will fill in all selected cells with values. After these actions, edit the Sample Type column by selecting between Allelic Ladder / Positive Control / Negative Control values.

Step C Start the device and information about the device status

Go to the **Run Schedule** section and click the **Find All** button. Find the name of the created plate in the list, select it and link it with the image of the plate installed in the device. Start the plate analysis.

Fluorescent sample profiles can be viewed in real time in the **Capillaries Viewer** or **Cap/Array Viewer** section. If system errors occur, information about them will appear in the **Event Log (Error Status)** section.

4.6. Optimizing the intensity of signals

To increase the intensity of peaks it is possible to increase the injection time up to 15 sec and/or increase the voltage up to 15 kV. This needs to be done by editing the Run Module (4.2.).

5. Electrophoresis on ABI PRISM® 3500/3500XL Analyzer

When working with the ABI PRISM® genetic analyzer, and subsequent analysis of fluorescent profiles in the GeneMapper™ program, it is necessary to follow the user instructions from the equipment manufacturer. For correct visualization of the five fluorescent Animaltype Sheep labels, it is necessary to perform spectral calibration for the "Any5Dye" dye set using CS5 matrix standard.

5.1. Matrix creation/spectral calibration

Analysis of Animaltype Sheep amplification products on the genetic analyzer is possible only after calibration with 5-color matrix-standard CS5 (not supplied with the kit, ordered separately by catalog number: 11-14102-0050). The matrix standard contains a mixture of 5 fragments of different sizes marked with different fluorescent dyes. These dyes are used in the PCR tagging kit and the S550 size standard. To prepare a working solution of matrix standard CS5, add 50 µl of deionized water to a tube containing lyophilized CS5 (violet lid tube) and incubate at room temperature for 2 min. Then thoroughly mix the solution on the Vortex and collect the solution by short centrifugation at the bottom of the tube. The ready solution can be stored in a dark place at 2 °C – 8 °C for up to 2 weeks. For longer storage time, the solution should be frozen. Repeated defrosting should be avoided.

Preparation of a matrix standard for calibration (ABI 3500 /8 capillaries)

| | |
|---------------------|-------|
| Hi-Di™ Formamide | 80 µl |
| Matrix Standard CS5 | 8 µl |

Add 10 µl of mixture to the A01-H01 wells of the 96-well plate. If necessary, remove bubbles from the bottom of the wells by centrifuging.

Preparation of the calibration standard matrix (ABI 3500XL/ 24 capillary)

| | |
|---------------------|--------|
| Hi-Di™ Formamide | 240 µl |
| Matrix Standard CS5 | 24 µl |

Add 10 µl of mixture to wells A01 to H03 of 96-well plate. If necessary, remove bubbles from the bottom of the wells by centrifuging.

Spectral calibration

Step A - Create a Dye Set for spectral calibration

Go to the **Library** section in **Data Collection Software**. In the **Analyze** section, select the **Dye Sets** tab. In the menu that opens, click **Create** (a new Dye Set creation dialog box will open). In the window that appears, specify the parameters of the new Dye Set:

| | |
|-------------------------|------------------|
| Dye Set Name | CS5 |
| Chemistry | Matrix standard |
| Dye Set Template | Any5Dye Template |
| Arrange Dyes Parameters | leave unchanged |
| Matrix Condition Number | 20.0 |
| Minimal Quality Score | 0.8 |

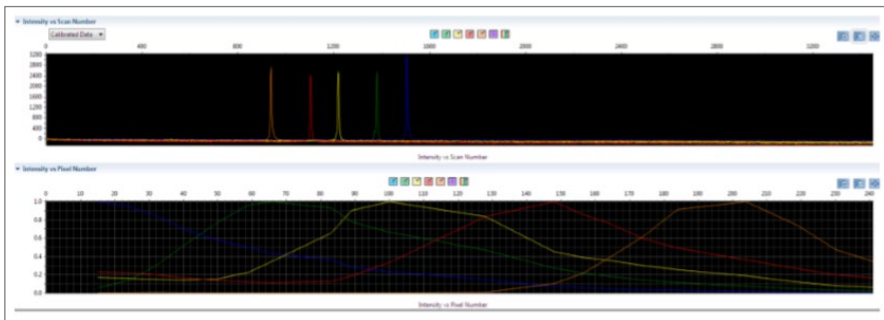
Press the **Save** button. The new Dye Set (CS5) will appear in the list.

Step B - Perform spectral calibration

Go to **Maintenance** section in **Data Collection Software**. In the **Calibrate** section, select the **Spectral Calibration** tab. In the window that opens, select the **number of wells** in the plate used and the plate's **Plate Position**. In Chemistry Standard select Matrix standard. In the **Dye Set** item select CS5. Start the calibration process by pressing the **Start Run** button.

Step C - Evaluation of Spectrum Calibration Results

Check the status of each capillary (pass or fail) after the device is finished. The peak heights should be at least 500 rfu, but below 10,000 rfu (optimal range between 1,000 and 5,000 rfu). Calibration shall be successful for a minimum of 6 of 8 capillaries (or 18 of 24 capillaries, respectively). When using CS5 as a matrix standard, the calibration profile window (Intensity vs Pixel Number) should show the following peak sequence (left to right) **blue-green-yellow-red-orange**.



If calibration is successful, save the results by pressing the **Accept** button.

5.2. Create Instrument Protocol

Go to the **Library** section in **Data Collection Software**. In the **Analyze** section, select the **Instrument Protocols** tab. In the menu that opens, click **Create** (a new Instrument Protocol creation dialog box will open). In the window that appears specify the parameters of the new Instrument Protocol:

| | |
|------------------|--|
| Application Type | HID |
| Dye Set | CS5 |
| Run Module | e.g. "HID36_POP4" (select the protocol corresponding to system parameters) |
| Protocol Name | Animaltype_Sheep |

Edit the Run conditions for Injection voltage to 3 s and adjust run time to analyze up to 550 bp.

Press the **Save** button.

5.3. Create Assay

Go to the **Library** section in **Data Collection Software**. In the **Manage** section, select the **Assays** tab. Click Create button in the menu that opens (a new Assay creation dialog box will open). In the window that appears specify the parameters of the new Assay:

| | |
|---------------------|------------------|
| Assay Name | Animaltype_Sheep |
| Application Type | HID |
| Instrument Protocol | Animaltype_Sheep |

Press the **Save** button.

5.4. Preparation and loading of amplification products

To load samples into the device it is necessary to prepare a mixture of Hi-Di™ formamide and S550 size standard (diluted in accordance with paragraph 2.2) in the following ratio:

| Component | Volume per sample |
|--------------------|-------------------|
| Hi-Di™ formamide | 10.0 µl |
| Size standard S550 | 1.0 µl |

When calculating the volume of the mixture components, note that all wells where injection will take place, including those without samples, should be filled with the mixture. After stirring, add 10 µl of mixture to each well of the plate. Then add 1 µl of PCR product to the wells. If necessary, remove the bubbles from the bottom of the plate wells by short centrifugation. Cover the plate with a rubber mat (septum) and load it in accordance with the ABI PRISM® Genetic Analyzer user manual. The Animaltype Sheep kit does not require temperature denaturation of the samples!

5.5. Starting the device

Capillary electrophoresis on the ABI PRISM® Genetic Analyzer is performed according to the user's manual provided by the manufacturer. To obtain correct results, an appropriate **Spectral calibration** (see section 5.1), **Instrument Protocol** (see section 5.2), and **Assay** (see section 5.3) must be performed.

Step A Creating the plate

Before starting the analysis, you should create a **Plate** that describes the location of the samples on the 96-well plate and provides instructions for the instrument. To do this, go to the **Create New Plate** menu of **Data Collection Software**. A dialog box will appear in which you should specify the parameters of the new plate:

| | |
|------------|--------------------------------|
| Name | For example, Animaltype_[date] |
| Plate Type | Fragment |

Press the **OK** button. A new table with the scheme of the examined plate will appear.

Step B Filling the table

For all the analyzed wells of the plate it is necessary to specify

| | |
|---------------------|---|
| Sample name | name of the object |
| Sample type | type of sample |
| Assay | Animaltype_Sheep (add from library - select Animaltype_Sheep) |
| Filename convention | file name structure (add from library - select My_FNC) |
| Results group | file saving options (folder where the results will be saved). -> add from library - select My_Fragment_Analysis_Result_Group) |

Left click to select all filled samples, then check the Assay, Filename convention and Results group options previously selected.

Step C Start the instrument and status information

After creating the layout of the samples on the plate, click on the **Link Plate for Run** button. In the window that opens, specify the position of the plate in the device and start electrophoresis by clicking on the **Start Run** button.

5.6. Optimizing the intensity of signals

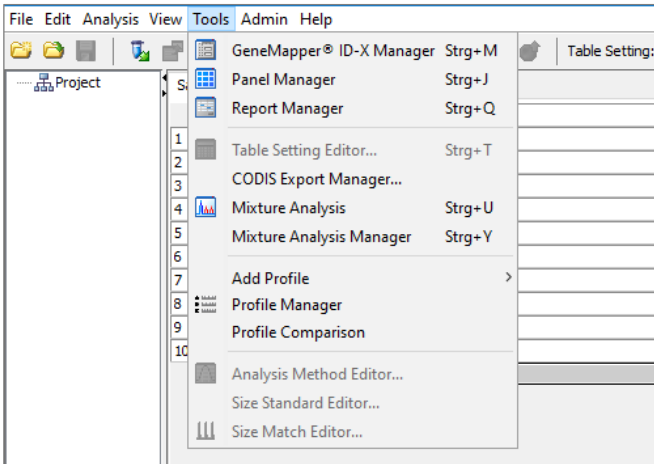
To increase the intensity of peaks, it is possible to increase the injection time up to 15 sec and/or increase the voltage up to 15 kV. It is possible to intensify the signal by cleaning the PCR product from primers and salts. The amount of size standard in this case should also be reduced. When working with highly sensitive ABI PRISM® 3500 Genetic Analyzers, undesirable effects may occur due to excessive signal fluorescence of amplified products at the stage of electrophoresis analysis. In this case, it is recommended to reduce the amount of genomic DNA material introduced into the reaction. In addition, reduction of excessive fluorescence signal level of amplified products can be achieved by reducing the time of injection of samples to 2-4 seconds in the **Run Module**.

6. Data Analysis

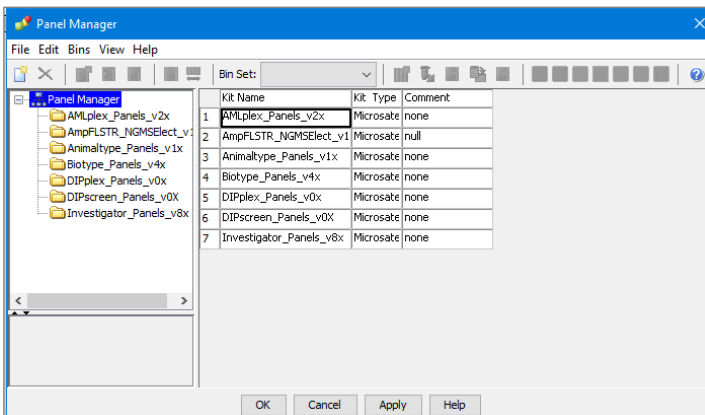
6.1. Import of files for analysis in GeneMapper program

To analyze data in the GeneMapper™ program, you should import the necessary files with bins, panels, size standard and method of analysis according to the following instructions:

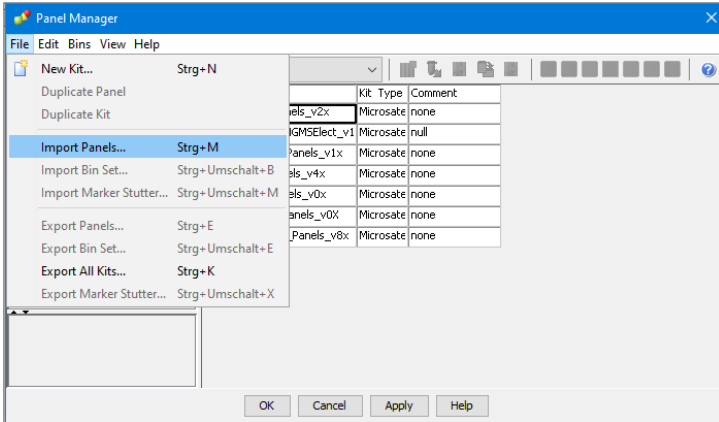
1. Select menu item Tools->Panel Manager.



2. In the upper left segment of an open window, place the cursor on the Panel Manager position.



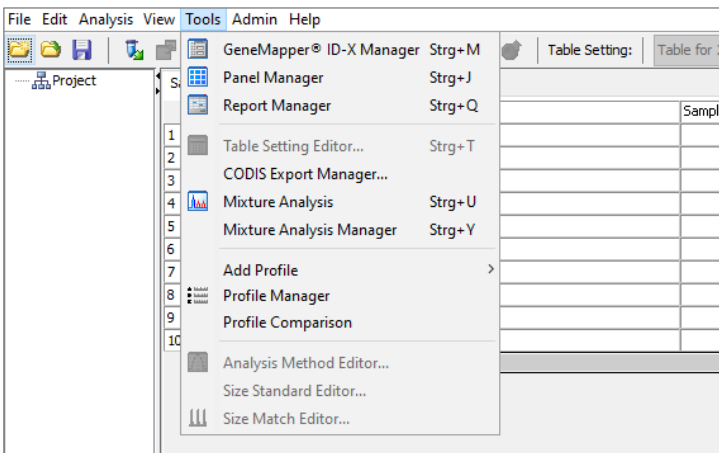
3. In the menu select File -> Import panels.



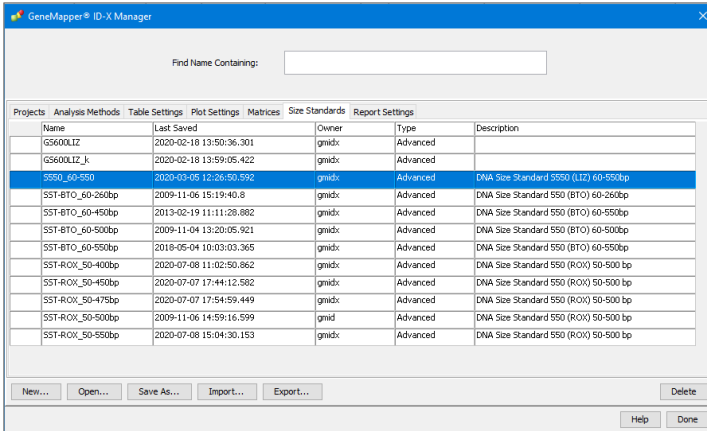
4. In the opened window, find and select a file with panels (e.g. Animaltype_Sheep_Panels_Vx). Load by pressing the Import button.

5. After import of the Panel, select the Panel and import the related Bin Set by choosing **Import Bin Set**. Import Animaltype_Sheep_Bins_Vx.

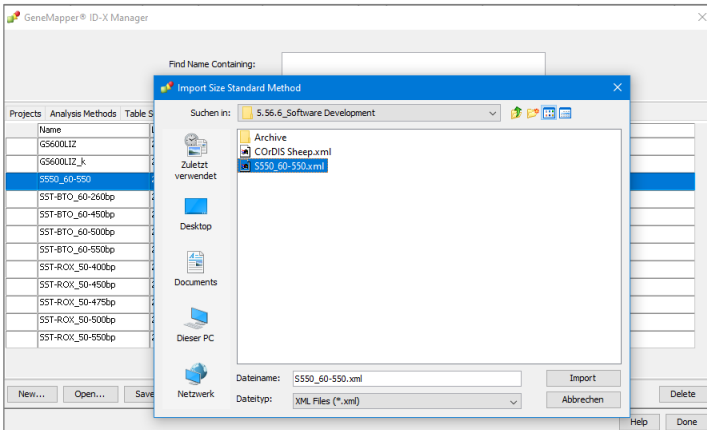
6. Select menu item Tools -> GeneMapper™ Manager.



7. In the window that opens, select the Size Standards tab.



8. Press the Import button and select the S550 size standard file. Load the file by pressing the Import button.



9. Go to the Analysis Method tab, press the Import button, select the file Animaltype_Sheep, upload the file by pressing the Import button.

10. When analyzing data in GeneMapper™, use the following parameters:

| Parameter | Value |
|-----------------|----------------------------|
| Analysis Method | Animaltype_Sheep |
| Panel | Animaltype_Sheep_Panels_Vx |
| Bins | Animaltype_Sheep_Bins_Vx |
| Size Standard | S550 |

6.2. Size Standard S550

Below is an example of an electropherogram with S550 size standard fragment signals in an Orange detection channel. Designations of 26 DNA fragments are given according to their size: 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 220, 230, 240, 260, 280, 300, 320, 340, 360, 380, 400, 420, 440, 450, 500 and 550 bp.

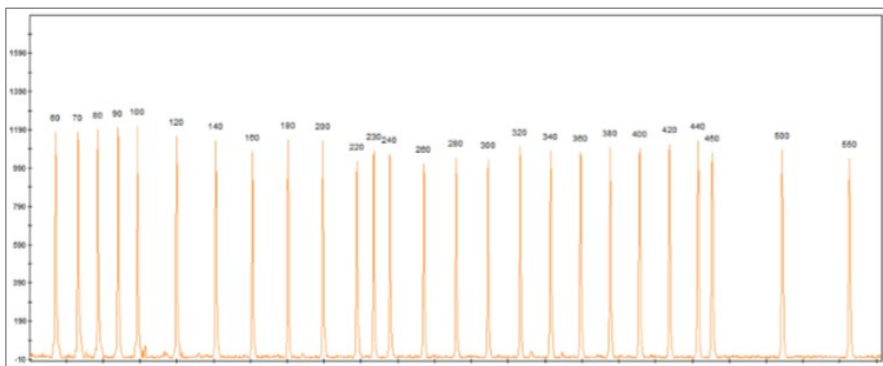


Figure 1: S550 size standard electropherogram. Size of fragments in bp.

6.3. Size ranges of microsatellite markers

| Locus | Range of allele size | Allele of Control DNA OM-1 | Allele of Control DNA OF-2 | Marking Color |
|----------|----------------------|----------------------------|----------------------------|---------------|
| McM042 | 81-107 | 87/93 | 87/89 | Blue |
| INRA006 | 106-134 | 110/116 | 110/116 | Blue |
| McM527 | 158-182 | 166/178 | 174/178 | Blue |
| ETH152 | 184-200 | 186/192 | 186/186 | Blue |
| CSR247 | 205-261 | 211/227 | 213/213 | Blue |
| OarFCB20 | 75-115 | 99/105 | 91/105 | Green |
| INRA172 | 126-172 | 154/160 | 160/160 | Green |
| INRA063 | 165-215 | 189/195 | 169/175 | Green |
| MAF065 | 115-139 | 127/127 | 121/131 | Black |
| MAF214 | 175-267 | 189/223 | 189/189 | Black |
| INRA005 | 123-155 | 127/129 | 147/151 | Red |
| INRA023 | 190-224 | 200/216 | 200/200 | Red |
| AMEL | X-Y | XY | XX | Green |

Table 2: Range of allele size

6.4. Amplification of Control DNA

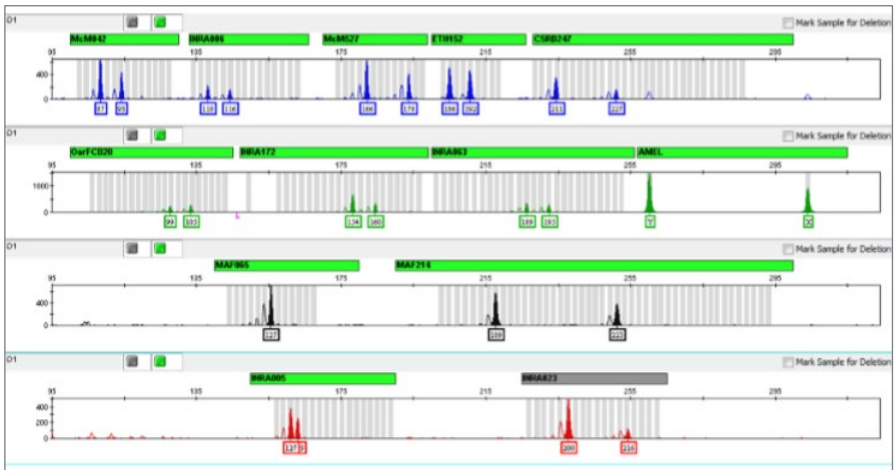


Figure 2: Control DNA OM-1. 1 µl of control DNA was injected into the reaction.

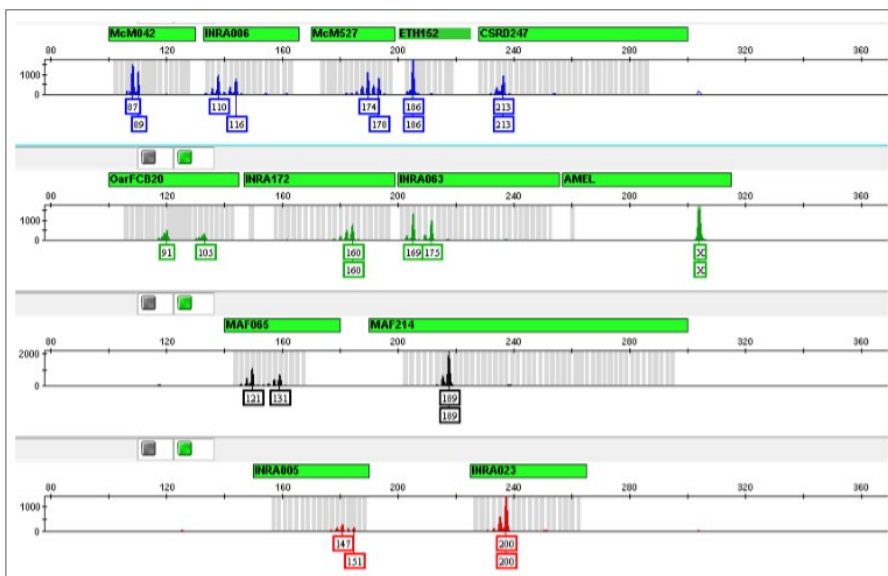


Figure 3: Control DNA OF-2. 1 µl of control DNA was injected into the reaction.

6.5. Interpretation of results

As a result of capillary electrophoresis on the ABI PRISM® Genetic Analyzer, the GeneMapper™ software generates a fluorescent profile reflecting the electrophoretic mobility of PCR products. Using the S550 size standard, the sizes of the amplification products are determined with an accuracy of < 1 bp. The GeneMapper™ software allows the determination of allelic variants for each marker. The obtained genotypes of different animals can be compared to confirm or exclude kinship. One or two allelic PCR-products can occur for each STR marker corresponding to homogeneous and heterozygous condition of the locus, respectively. The difference in the size of alleles is usually a multiple of 2 bp that reflects differences in number of dinucleotide repetitions. For correct determination of the genotype, the nature of stutters must be taken into account. Stutters are artificial by-products of PCR amplification of microsatellite markers. For dinucleotide markers, which include all loci of the Animaltype Sheep set except AMEL, stutters of -2 bp size are typical in relation to the main product. The intensity of the stutter

signal can reach 50% of the allele product intensity. If the allele size differs by 2 bp, the stutter of the longer allele is superimposed on the shorter allele, significantly increasing its signal level (Figure 4 B).

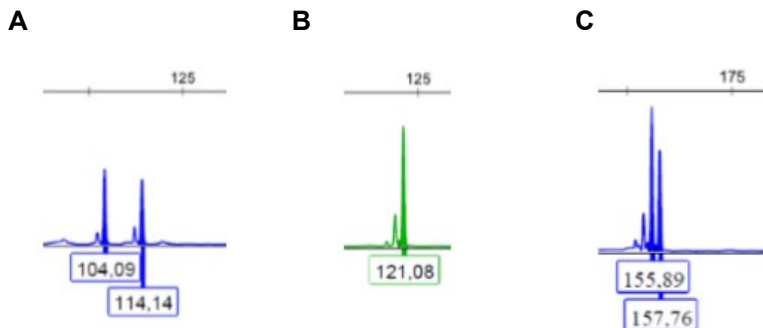


Figure 4:

A - Example of heterozygous genotype 104/114,

B - Example of homozygote 121,

C - Example of heterozygote 155/157 with stutter overlaying on allele 155.

7. Trademarks and Disclaimers

GeneAmp®, ABI PRISM®, GeneMapper™, Hi-Di™ and POP-4™ are trademarks of Applied Biosystems division (Foster City, US-CA) of Thermo Fisher Scientific Ltd. Nanofo® is a trademark of Syntol (Moscow, RU). Other registered names, trademarks, etc. used in this document, even if not specifically marked as such, are not to be considered unprotected by law.

The intended use of Animaltype Sheep is to differentiate individual animals of domestic sheeps. It can be used for kinship analysis, for quality assurance and biodiversity assessment in herd breeding or for traceability of animals and mutton. The test kit is for research use only (RUO). The manufacturer gives no warranty for any application which is regulated by law in the countries of end-users. Thus, the end-users assume sole responsibility for regulatory compliance of the genetical analysis and its application(s).



Biotype GmbH
Moritzburger Weg 67
01109 Dresden
Tel.: +49 351 8838 400
Fax: +49 351 8838 403
info@biotype.de
www.biotype.de

Customer Service & Support
support@biotype.de