

MULTIPLEX MICROSATELLITE ANALYSIS WITH 5' DYE-LABELLED MARKER SETS USING FAM, YAKIMA YELLOW, ATTO 550, ATTO 565, AND ATTO 633 ON AN ABI 3130XL GENETIC ANALYZER

INTRUDUCTION

Multiplex PCR with dye-labelled primers is a widely used method for fragment length analysis (VNTR-, SSR-, and Microsatellite Analysis) and is typically followed by capillary electrophoresis on ABI PRISM 310, 3100, 3130 or 3730 Genetic Analyzers. These automated capillary electrophoresis systems can separate, detect, and analyze up to 96 samples of fluorescent labelled DNA fragments in one run.

It separates a mixture of DNA fragments according to their lengths, provides a profile of the separation, determines the length of each fragment (in base pairs), and estimates the relative concentration of each fragment in the sample. For highest efficiency, fragments labelled with five different dyes can be separated in parallel.

As different multiplex PCR kits are available for several applications, researchers are interested in the development of specific multiplex marker sets for their own projects. A wide variety of dyes for these purposes are available on the market. We have re-labelled an existing microsatellite marker set with 14 loci, specific for sheep DNA, with the dyes FAM, Yakima Yellow, ATTO 550, ATTO 565, and ATTO 633. All required steps for the setup of a 5' dye-labelled marker set analysis on the ABI 3130 XL Genetic Analyzer are described.

AIM OF THE PROJECT

The aim of the project was the setup of multiplex PCR analysis with a 5' dye-labelled marker set on the ABI 3130 XL Genetic Analyzer using the dyes FAM, Yakima Yellow, ATTO 550, ATTO 565, and ATTO 633 ordered from Eurofins Genomics.

The project included the production of a new matrix standard, setup of a new matrix on the ABI 3130 XL Genetic Analyzer and re-labelling of an existing marker set for sheep genotyping. For comparison of both dye sets, PCR conditions, primer sequences and concentrations were not changed and PCR was performed on identical samples.

APPLICATION/METHOD

1. Creation of a new dye-specific matrix on the ABI 3130XL

1.1 General remarks

To ensure best analytical results when using a new marker set with dyes which are not covered by the commercially available matrix dye sets, a new matrix must be created on the ABI 3130XL. A matrix dye set contains dye-labelled fragments representing the 5' dyes used in subsequent analyses. For each dye only one fragment should be present. The size of the fragments differed by 25 - 30 base pairs.

1.2. PCR and control of PCR products

We have designed primers specific for pBluescript sequences. The length of the amplified fragments was 200 bp (FAM), 175 bp (Yakima Yellow), 145 bp (ATTO 550), 116 bp (ATTO 565), and 91 bp (ATTO 633). After PCR, all fragments were initially analysed separately using Filter Set G5 with a matrix originally created with an ABI "Multi-Capillary DS-33 Matrix Standard Kit". Based on the signal intensities observed during this analysis, all five PCR products were then mixed together in different ratios and further analysed. As no size standard can be run in parallel for matrix generation, only raw data could be interpreted (see Fig.1 and 2).

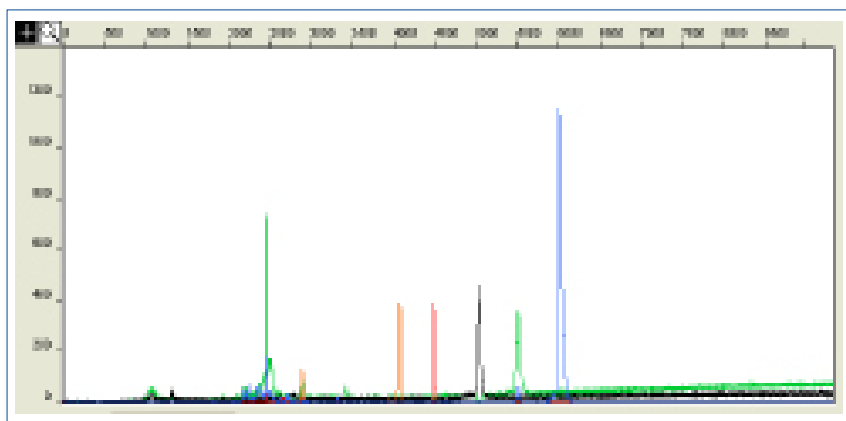


Fig.1 Raw data generated by GeneScan Software: Separation of five PCR products (Scan region 3800 to 5900) amplified with primers labelled with ATTO 633 (orange), ATTO 565 (red), ATTO 550 (yellow/black), Yakima Yellow (green) and FAM (blue): Peak heights of the first four dyes are balanced, blue peak is significantly higher.

Conclusion: In final mixture only 50 % FAM-labelled PCR product should be

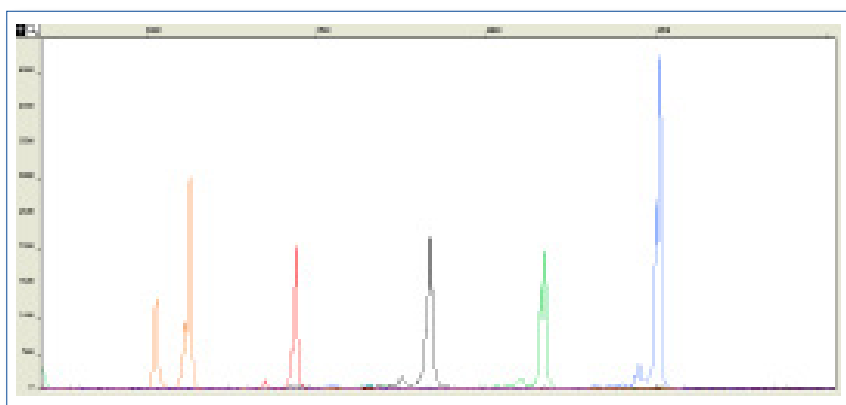


Fig.2 Raw data generated by GeneScan Software: Separation of five PCR products (Scan region 3000 to 4500) amplified with primers labelled with ATTO 633 (orange), ATTO 565 (red), ATTO 550 (yellow/black), Yakima Yellow (green) and FAM (blue). This mixture was finally used for the matrix run.

1.3. PCR and control of PCR products

To set up a matrix run on the ABI 3130XL (Fig. 7), a new protocol in the "Data Collection Software 3.0" must be created:

1. In the "Run Module Editor", the values which given by Data Collection Software 3.0 were chosen.
2. In the Protocol Editor type "spectral", Dye Set "G5", Polymer "POP4", Array Length "36", Chemistry "Matrix Standard" and Run Module "APEC_36_POP4" were chosen.
3. With the "Edit Param..." button the "Edit Parameter" window can be opened.
4. Matrix condition number bounds can be changed in the Edit Parameter window: If the lower value is too high (e.g. 8.5) a matrix can not be created. When lowered to 3, all electropherograms are accepted by the data collection software.

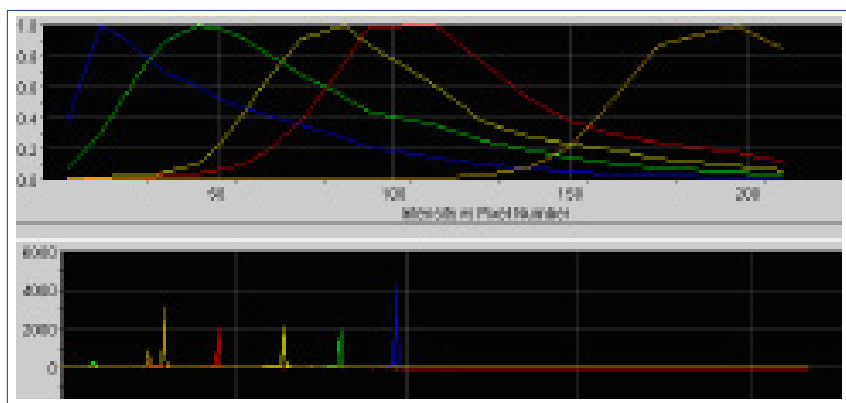


Fig.7 Graphs of a successful matrix run: If two fragments are present as seen for the orange channel, the scan range should be modified to fade out the first fragment.

For capillary electrophoresis, 1 µl matrix standard was added to 10 µl HIDI Formamide, incubated for 2 minutes at 95°C, cooled in a freezer for 5 minutes, and separated in all 16 capillaries. After all 16 electropherograms were accepted by the data collection software, this new matrix was re-named in order to avoid further analyses with non-matching dye sets and matrices. This is recommended, especially if different G5 matrices have been created on the same instrument.

1.4. Quality check of the new matrix

After the matrix was created, the five PCR products were separated by capillary electrophoresis to check the quality of the new matrix. Every PCR product was only detected in the corresponding channel. No "pull-ups" or other artefacts were detected (see Figure 8).

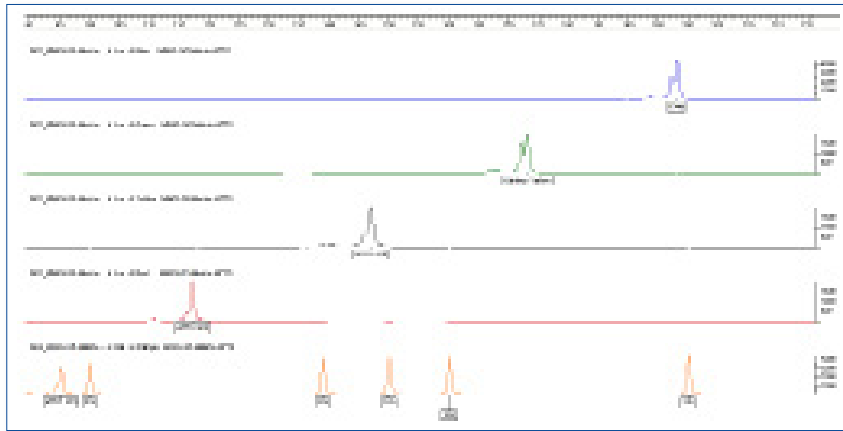


Fig.8 Separation of five PCR products labelled with FAM, Yakima Yellow, ATTO 550, ATTO 565 and ATTO 633 in the presence of ABI Size Standard LIZ 500 (additional peaks in orange channel): Every peak is only detected in the corresponding channel, no artefacts are

2. Multiplex PCR for sheep genotyping

Primers from an existing marker set were re-labelled, as shown in table 1. PCR was performed with two multiplex reactions - one reaction with primer pairs for 11 loci and one reaction with primer pairs for 3 loci under identical conditions (primer concentrations, PCR cycles). After capillary electrophoresis with the new matrix, data was analysed with GeneScan® 3.7 and Genotyper® 3.7 software.

Channel	Former Dye Set	New Dye Set
Blue	FAM	FAM
Green	VIC	Yakima Yellow
Yellow	NED	ATTO 550

Table 1 Re-labelling of primers.

RESULTS

Complete profiles could be generated for all analysed samples. The peak heights were comparable to those from PCR products with FAM/VIC/NED/PET labelled primers, especially at the ATTO 550 and 565 labelled PCR products, the peaks are higher than the corresponding NED and PET labelled PCR product peaks. An example of these profiles is shown in figure 9.

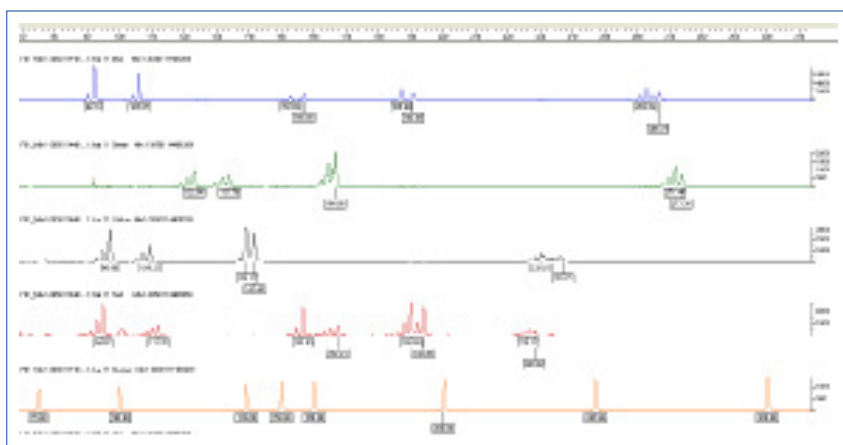


Fig.9 Separation of PCR products from 14 dinucleotide-repeat microsatellites, labelled with FAM, Yakima Yellow, ATTO 550, and ATTO 565 in the presence of ABI "Size Standard LIZ

Dye-labelled PCR products with high concentrations where signal intensities are too high, artefacts like "pull-ups" from one channel to the neighbouring channel can usually be detected. However, using our new matrix - even at peaks with 6000 to 7400 rfu - no artefacts were detected, as shown in figure 10.

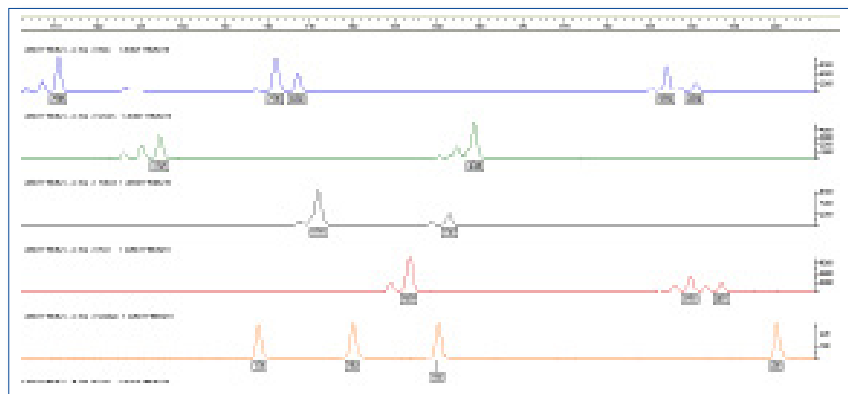


Fig.10 Separation of PCR products, labelled with FAM, Yakima Yellow, ATTO 550, ATTO 565, and ATTO 633 in the presence of ABI "Size Standard LIZ 500": No "pull-ups" could be detected at peak heights of up to 7500 rfu.

When performing capillary electrophoresis on ABI 3130XL instruments, the fragment lengths determined by GeneScan® or Genemapper® software depends on three major factors:

1. The length of the PCR product
2. The PCR product sequence
3. The dye used for labelling

Also the fragments present in the internal size standard as well as the used polymer and capillary can influence the fragment lengths.

During our experiments all conditions were identical, except the dyes used. We found that the measured fragment lengths differ between the PCR products labelled with the different dyes, as listed in table 2, examples are shown in figure 11.

Channel	Former Dye Set	New Dye Set	Fragment Length	Differences in measured fragment length
Blue	FAM	FAM	90 to 300 bp	No differences
Green	VIC	Yakima Yellow	120 to 270 bp	Yakima Yellow labelled fragments appeared 1 bp shorter
Yellow	NED	ATTO 550	100 to 110 bp	ATTO 550 labelled fragments appeared 6 bp longer
Yellow	NED	ATTO 550	140 to 170 bp	ATTO 550 labelled fragments appeared 5 bp longer
Yellow	NED	ATTO 550	220 to 240 bp	ATTO 550 labelled fragments appeared 4 bp longer
Red	PET	ATTO 565	85 to 100 bp	ATTO 565 labelled fragments appeared 1 bp longer
Red	PET	ATTO 565	150 to 240 bp	ATTO 565 labelled fragments appeared 0,5 bp longer

Table 2 Differences in measured fragment lengths of identical PCR products labelled with different dyes.

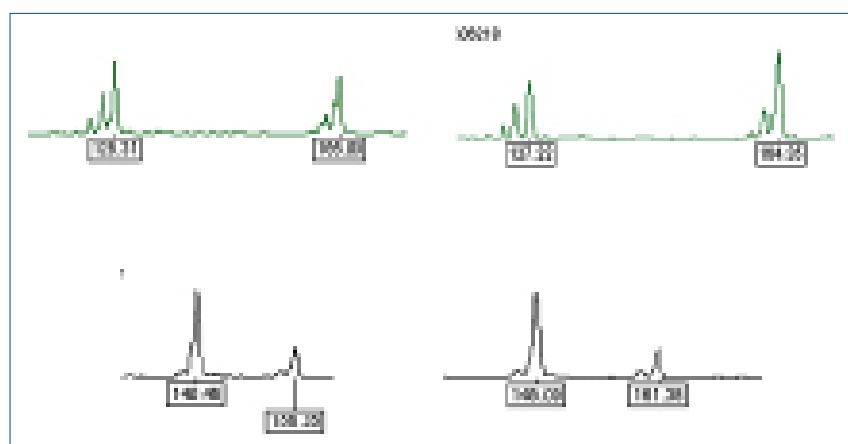


Fig.11 Differences in determined fragment lengths of identical PCR products: Fragments labelled with Yakima Yellow (b) seem about 1 bp shorter than products labelled with NED (a) and fragments labelled with ATTO 550 (d) seem about 6 bp longer than products labelled with VIC (c).

CONCLUSION

For high-throughput multiplex microsatellite analysis with 5' dye technology on ABI 310/3100/3130/3730 instruments, 5' labelled PCR primer are used. With the combination of FAM, Yakima Yellow, ATTO 550, ATTO 565, and ATTO 633, [an exceptional set of dyes for this kind of analyses is now available.](#)

Using traditionally available dye combinations, signals from the dyes which were detected in the blue and green channels are stronger than those from the dyes detected in the yellow channel and especially in the red channel.

We found in our study that these problems are solved with the combination FAM, Yakima Yellow, ATTO 550, ATTO 565, and ATTO 633. The strong fluorescent signals from the ATTO dyes allows very sensitive analyses over all channels with well balanced DNA profiles between the dye channels.

[Therefore the tested dye combination is an excellent alternative to the dye combination FAM/VIC/NED/PET/LIZ.](#) PCR products labelled with FAM, Yakima Yellow, ATTO 550 and ATTO 565 can also be separated with LIZ labelled size standards.

For best results, a matrix with PCR products labelled with the five dyes should be created on the instrument.

We obtained [best results by creating the matrix under dye set G5.](#) As the fragment lengths can be different depending on the dye used, existing macros on bin-sets must be re-defined.

LITERATUR/ARTICLES

Genetic diversity in swiss goat breeds based on microsatellite analysis, N. Saitbekova, C. Gaillard, G. Obexer-Ruff, G. Dolf G Obexer-Ruff, G Dolf *Animal Genetics* Volume 30 Issue 1, Pages 36 - 41

Genetic relationships in Swiss sheep breeds based on microsatellite analysis, N. Stahlberger-Saitbekova, J. Schläpfer, G. Dolf & C. Gaillard *Journal of Animal Breeding and Genetics* Volume 118 Issue 6, Pages 379 - 387

ABI 3130XL user manual