



AneuSure[®] Plus v2

Product Instruction Manual

CAT# GT-11108

QF-PCR Kit

Detection of Numerical Aneuploidies of Chromosomes:

21, 18, 13, X and Y + SMA

Produced by

GENETEK BIOPHARMA GmbH

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1. AneuSure® Plus v2 Overview

- Detecting aneuploidy for chromosomes 13, 18, 21, X, and Y and detecting the presence or absence of exon 7 of SMN1 or SMN2 genes
- Can be used on extracted DNA from blood, amniotic fluid, CVS, and cell-free DNA in amniotic fluid
- 29 markers (two for SMN1 and SMN2 genes) amplification in a single reaction
- 5-dye fragment analysis using capillary electrophoresis
- Compatible with Applied Biosystems® 3130/3130xl and 3500/3500xL Genetic Analyzer

1.1. Intended Use

AneuSure® Plus v2 is a QF-PCR kit for rapid detection of chromosomal aneuploidies for chromosomes 13, 18, 21, X and Y as well as detecting the presence or absence of exon 7 of the SMN1 and SMN2 genes for detecting 5q SMA in every fetal sample which is tested for chromosomal aneuploidies by QF-PCR method.

With AneuSure® Plus v2 Kit, amplification of 29 markers (on chromosomes 21, 18, 13, X and Y) in a multiplex PCR reaction is possible. All markers have been selected to have high heterozygosity and are distributed throughout the chromosomes 13, 18, 21, X and Y. The kit also contains 2 sets of primers for quantifying exon 7 of the SMN1 and SMN2 genes. Deletion of exon 7 of the SMN1 gene is seen to be deleted in about 95-98% of 5q SMA cases. DNA template for AneuSure® Plus v2 can be extracted from several sources: amniotic fluid (AF), chorionic villus (CVS) and blood. To prevent sample mix-up and to ensure originality of the fetal sample, we recommend sample from mother be amplified and used as a reference alongside of the fetal sample (please follow national guidelines if available). Another advantage of this kit is the reliable detection of Turner syndrome (Monosomy of X) by using 7/X and 11/X markers, allowing the quantification of chromosome X as well as other sex chromosome markers.

1.2. AneuSure® Plus v2 Markers

STR loci (short tandem repeat) consist of short and repetitive sequence elements, 2 - 7 base pairs in length. These tandem repeats are well distributed throughout the human genome and are a rich source of highly polymorphic markers which can be detected by PCR using primers from their flanking sequences. The STR loci alleles are differentiated by the number of copies of the repeat sequence contained within the amplified region (locus) and can be distinguished from each other using fluorescence detection after capillary electrophoretic separation.

AneuSure® Plus v2 markers cover the whole length of 13, 18, 21, X, and Y chromosomes (Table 1). Markers heterozygosity and SNP in their primer sites have been tested on several thousand DNA samples.

Table 1: Number of markers for each chromosome

Chromosome	Number of markers
Chromosome 13	5
Chromosome 18	5
Chromosome 21	6
X/Y	2
X chromosome	7
Y Chromosome	2

Table 2: Markers used in AneuSure® Plus v2 kit

No.	Marker	Chromosome	Size Range	Chr. Location
1	AMXY	X & Y	102-120	Xp22.2
				Yp11.2
2	D13S325	13	125-190	13q14.11
3	D18S390	18	210-250	18q22.3
4	D21S1809	21	260-280	21q22.2
5	D21S1446	21	285-340	21q22.3
6	D21IFNAR	21	350-408	21q22.1
7	D13S252	13	425-470	13q12.2
8	DXS7132	X	115-150	Xq12
9	D18S391	18	160-200	18p11.31
10	SRY	Y	208-219	Yp11.31
11	D13S634	13	220-251	13q21.33
12	D13S258	13	253-325	13q21.33
13	SMN1, 2	5	330-345	5q13.2
14	D21S1414	21	350-430	21q21.1
15	Y/X b	Y, X	110-135	Yp11.2
				Xq21.31
16	HPRT	X	145-185	Xq26.3
17	D21S1442	21	188-233	21q21.3
18	11/X	11, X	235/247	11q22.3
				Xp11.21
19	D18S1002	18	250-310	18q11.2
20	DXS6803	X	315-344	Xq21.31
21	D21S1411	21	360-375	21q22.3
22	D13S797	13	115-155	13q33.2
23	DYS437	Y	160-200	Yq11.21
24	7/X	7, X	210-243	7q34
				Xq13.3
25	DX-TATC 13.3	X	245-271	Xp21.2
26	D18S535	18	275-325	18q12.3
27	DXS981	X	330-365	Xq13.1
28	D18-GATA178F11	18	370-430	18p11.32

- These sizes are obtained using ABI 3500xL Genetic Analyzer and GT500 internal size standard. Out of range alleles can be observed in different populations. Validate the results for your instrument before testing fetal samples.
- AMXY and Y/X B are the sex determination markers that detect two homologous regions on X and Y chromosomes.

- 7/X and 11/X markers are segmental duplication regions shared between the X and 7 or X and 11 chromosomes and is used for reliable detection of Turner syndrome. The heights of these peaks represent the copy number for each chromosome. Almost equal heights show two copies of the X and two copies of the 7 or 11 chromosomes. We cannot have simultaneous monosomies for chromosomes X and 7 or 11 because a fetus with monosomy of chromosome 7 or 11 will not last to the first month of pregnancy, and therefore, if the heights are equal, it means that there are two X and two 7 or 11 chromosomes and also half height for the X chromosome means there is only one X chromosome. Therefore, if the sex of the sample is female, the conclusion would be the female sample has only one X chromosome or is affected with Turner Syndrome.
- For testing the SMA status of the fetus (being affected or not), two different locations in exon 7 of the SMN1 and SMN2 genes have been selected to quantify the exons 7 on the SMN1 and SMN2 genes. In affected cases, the **SMN1** copy would not be present. If SMN1 copies are absent, then their absence should be specifically checked by other most specific methods like MLPA or GT SMA Detector real time PCR kit from Genetek Biopharma. The kit may also detect copy number variations between SMN1 and SMN2, however, the kit is recommended for the detection of complete absence of both exon 7 of the SMN1 genes.

Table 3: Reliable detection of Turner syndrome

	Normal female	Normal male	Turner
Height ratio of 7/X or 11/X markers	1:1	2:1	2:1
X-STR markers	Homozygote or heterozygote	Hemizygote	Hemizygote
Y-STR markers	Not detected	Detected as a single band	Not detected

- DX-TATC 13.3 marker is located on the short arm of chromosome X and does not exist in other commercial kits. It has been tested on several hundreds of samples and shows reasonable allele frequency.

1.3. Five-dye fragment analysis

ABI 3130, 3130xl, and 3500 and 3500xL Genetic analyzers (Applied Biosystems®) are recommended for the 5-dye capillary electrophoresis of amplified products produced using AneuSure® Plus v2 kit.

Table 4: The fluorescent dyes used in AneuSure® Plus v2 kit

Name	6-FAM	GT2907	GT2712	GT1803	GT500
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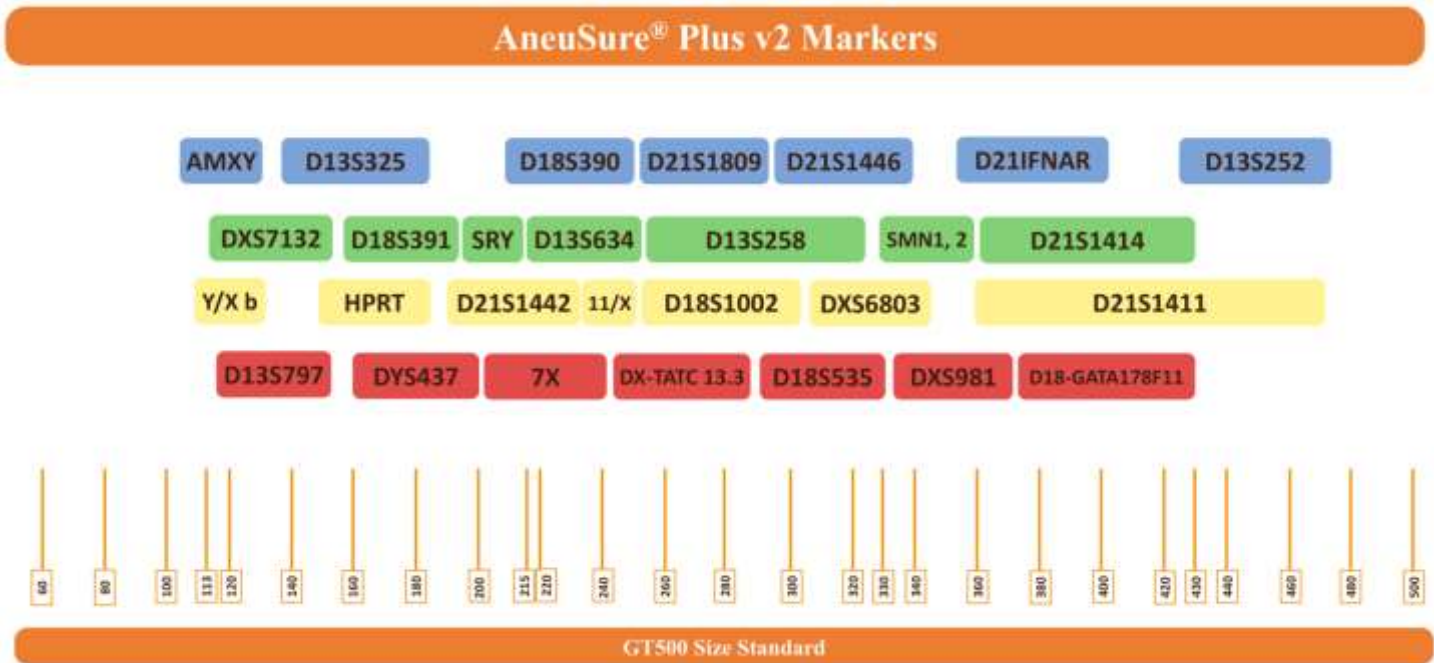


Figure 1. Diagram shows distribution and placement of AneuSure® Plus v2 Kit markers with GT500 Size Standard.

2. PCR

2.1. Storage Condition

- Store at -20 °C
- Keep the primer mix in a dark place (because of fluorescently labelled primers)
- Avoid frequent freeze and thaw (store the materials in small aliquots)
- Low-quality result may be obtained after the expiration date (12 months)






2.2. Materials and equipment


2.2.1. Laboratory condition

Fluorescent based STR kits can amplify a small amount of DNA. So, care should be taken not to contaminate the working area. Primer Mix, PCR Mix and GT HSTaq DNA polymerase should be stored in a separate lab (Pre-PCR area). GT500 Size Standard, GTM5 v2 Matrix Standard are amplicons and should be stored in post-PCR area. In each run, negative control should be added to determine possible and source of contamination. We recommend that DNA from each personnel working in the lab be profiled so in case of contamination, the source can be determined and precautionary measures can be taken.

2.2.2. Material required for Fragment Analysis

Table 5: Provided with the Kit in Box A and Box B. They should be kept separately. Box A in one freezer and Box B is another freezer (PCR product)

BOX-A		
	Tube Label	Tube cap colour
1	PCR Mix	
2	Primer Mix	
3	GT HSTaq	
4	GT QCDF150(Control DNA-50ng/µl)	
5	GT QCW (H2O)	

BOX-B		
	Tube Label	Tube cap colour
1	GT500 Size Standard	
2	GTM5 v2 (Optional)	

Not provided with AneuSure® Plus v2 (but are needed)

- Reagents and equipment for DNA extraction
- Equipment and consumable for amplification (i.e. Thermal Cycler, Micropipette, Filter Tips, etc.)
- Applied Biosystems Genetic Analyzer (ABI 3130/xl or 3500/xL) with Data Collection software for 5-dye system detection
- Applied Biosystems Genetic Analyzer (ABI 3130/xl or 3500/xL) relevant Performance optimized polymers (i.e. POP-4, POP-6 or POP-7) and Capillary Array or equivalent
- Applied Biosystems Hi-Di™ Formamide or equivalent
- GTM5 v2 Matrix Standard for Spectral calibration (GT- 41103) (can be obtained from Genetek Biopharma)

2.2.3. QF-PCR Amplification by AneuSure® Plus v2

- DNA can be extracted from blood, amniotic fluid, CVS and tissue samples. This kit also works for blood samples on filter paper such as DNA Banking Card (DBC™) as well as amniotic fluid cells collected on DNA Banking Card. For instruction on direct PCR method please contact us by email (support@genetek.de).
- 5-10 ng DNA can be used as a template.
- For optimizing and getting the best results, internal validation for each laboratory is recommended.

2.2.4. AneuSure® Plus v2 components

Table 6: PCR reaction set-up

Component	Volume for 1 reaction[μl]
GT QCW (H ₂ O)	10
PCR Mix	7
Primer Mix	1
GT HSTaq	1

2.2.5. AneuSure® Plus v2 protocol

- Bring reagents to room temperature.
- Vortex Primer Mix and PCR Mix, then spin down briefly to remove all residues from the lid. Gently mix the enzyme by inverting or pipetting.
- Prepare a Master Mix calculating number of samples and controls by following the recipe given above. Every preparation can be done at room temperature (no cold condition is required during preparation).
- Mix by pipetting or Vortex Master Mix briefly.
- Transfer 19 μL of Master Mix into each 0.2 ml PCR tube for each sample.
- Add 1 of sample DNA (1-5 ng per reaction) into each PCR tube. Make one positive control PCR tube using the DNA provided in the kit and also for negative control add 1 μl of sterile Direct Q dd H₂O instead of DNA.
- Vortex and spin down each PCR tube. Make sure that no drops are left at the tube wall or lid.
- Place tubes into thermal cycler.
- Use the following PCR program for the amplification of all markers.

Table 7: PCR program

Initial step	Cycling			Final Extension	Storing in Cycler
	Denaturation	Annealing	Extension		
95 °C	95 °C	63 °C	70 °C	70 °C	4 °C
20 min	1 min	90 sec	2 min	17-20 min	∞
27-30 Cycles					

- After completion of PCR, store the PCR products at 2-6°C until analysis with Genetic Analyzer.

Notes:

- PCR product is persistent for about 24h at room temperature. It is better to keep it in a refrigerator and in dark.
- If the time between amplification and capillary electrophoresis is more than one week, the quality of results may be reduced.
- A positive control DNA (sample with known genotype) and a negative control should be run with each multiplex PCR. We recommend using GT QCDF150 as a positive control especially early on during testing our kit or setup. The result for this control DNA can be found from Genetek website and also in our latest user manual.
- According to the quality or quantity of DNA template, you may require changing the number of cycles in PCR program or the amount of DNA used.

Attention:

After PCR is complete, tubes should never be opened in the PCR setup area (pre-PCR area) or near the kit components.

3. Capillary electrophoresis

- ABI 3130/xl and 3500/xL (Applied Biosystems®) Genetic Analyzers are recommended for 5-dye capillary electrophoresis of the amplified PCR products.
- Please make sure your ABI Data Collection software supports 5-dye fragment analysis (according to the instrument user manual).
- AneuSure® Plus v2 Kit is validated using 50 cm capillary array and POP7 as well as on 36 cm array and POP4 sing ABI 3500xL (Applied Biosystems®).
- For more details and optimization, follow the user guide on [DNA Fragment Analysis by Capillary Electrophoresis by Applied Biosystems®](#).

Notes:

- Injection time or voltage can be adjusted according to the amount of PCR product.
- An increase or decrease in the injection time or voltage may result to run product through the capillary.
- PCR products can be injected into the capillary more than one time or the results can be re-analyzed.

3.1. Instrument Preparation Applied Biosystems® 3500/3500xL Genetic Analyzer (before the first use of AneuSure® Plus v2 Kit)

Make sure that maintenance and installation of capillary array, buffers and polymer are done according to Applied Biosystems 3500/3500xL Genetic Analyzer User Guide.

Attention:

Spectral Calibration must be made using GTM5 v2 Matrix Standard, the machine must be calibrated with GTM5 v2 Matrix Standard before using the kit. Please find detailed protocol for spectral calibration with GTM5 v2 Matrix Standard here - CAT# 41103 or contact us at support@genetek.de.

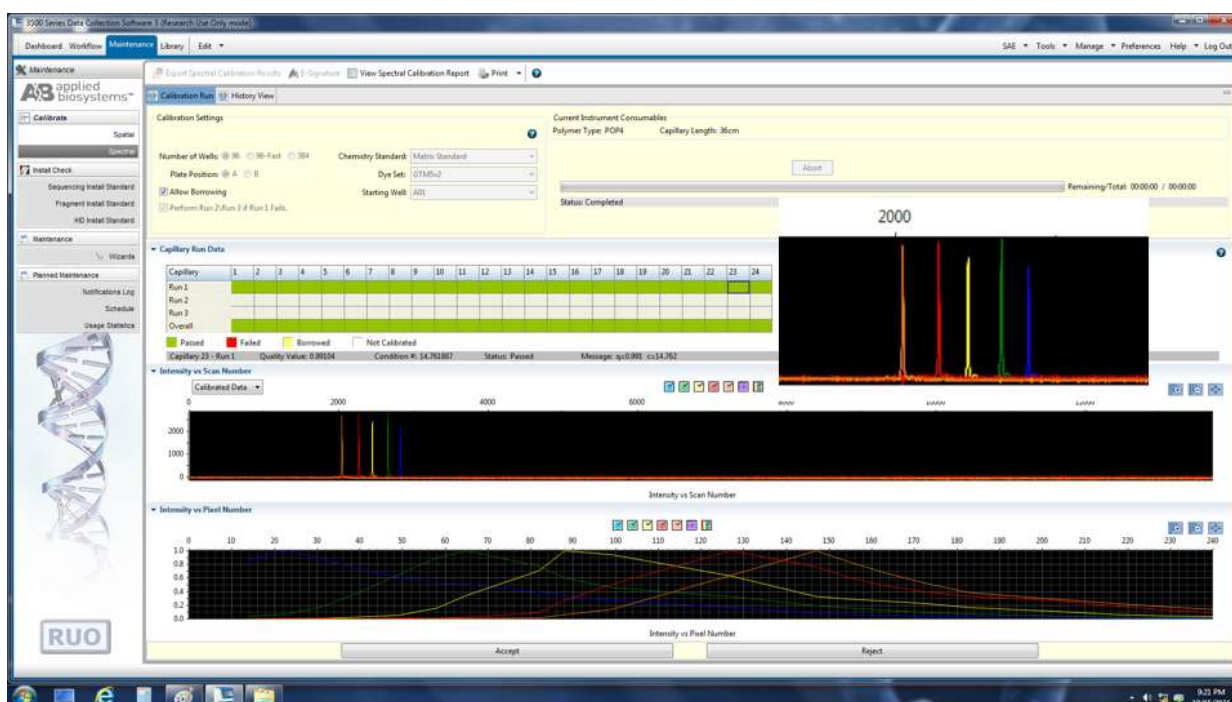


Figure 2. An example of a successful spectral calibration with GT 5-dye system on Applied Biosystems Genetic Analyzer 3500xL

- The Dashboard screen (Figure 3) is launched when 3500 Data Collection Software is opened. Click the Refresh button to make sure that all the information on the Dashboard is up-to-date. Make sure that the Maintenance and Consumables notifications are acceptable.
- Adjust the oven temperature to 60° C, then click “Start Pre-Heat” button. You may proceed for the first injection only after Oven Temperature and Detection Cell Temperature numbers turn green.



Figure 3. Dashboard of Applied Biosystems 3500 Data Collection software.

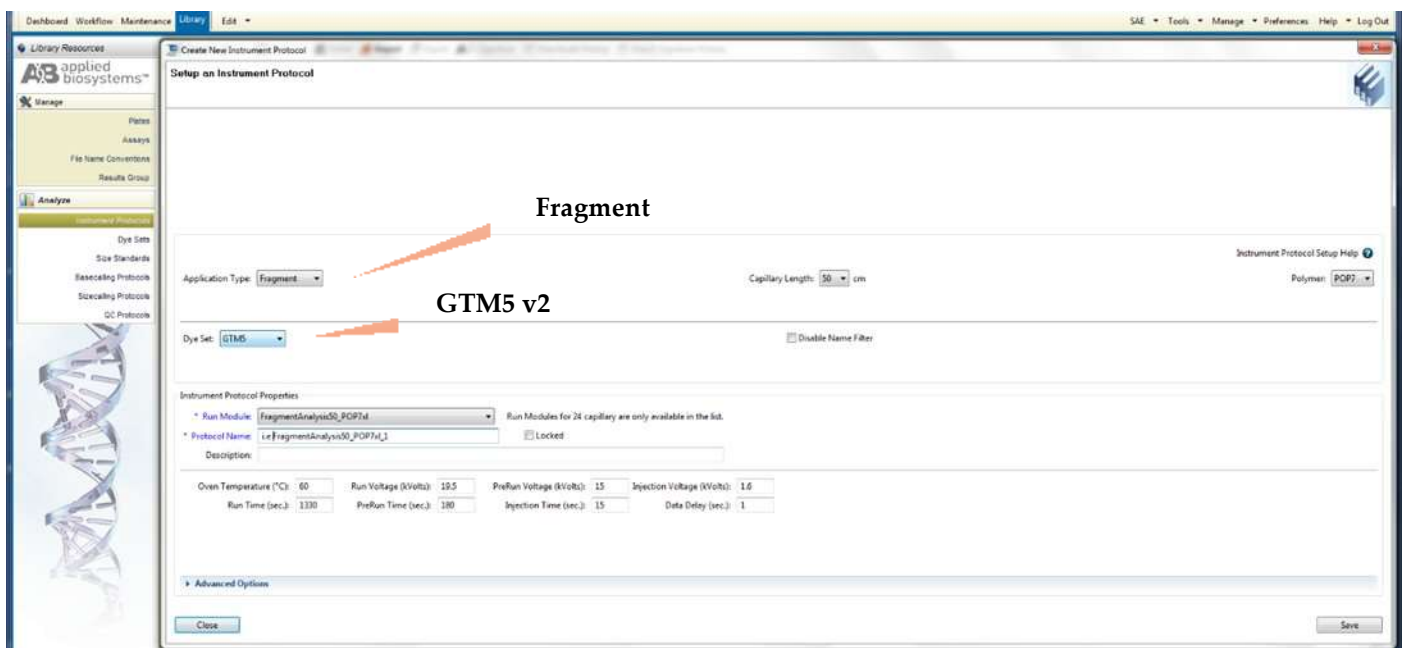


Figure 4. Screenshot for the “Create New Instrument Protocol” window on Applied Biosystems 3500 Data Collection software.

- User can apply settings as shown in the Figure 4. Make sure that you select GTM5 v2 as a *Dye Set* (same name as was used to perform the GTM5 v2 spectral calibration).

Onset of first analysis of AneuSure® Plus v2 system, the user must create an Instrument Protocol, Size Standard, QC Protocol, Assay, File Name Convention and Results Group.

3.1.1. Create a new Instrument Protocol

- a) Navigate to the *Library*
- b) Select “Instrument Protocols”
- c) Select “Create” (Figure 4)

Data Collection Software will store this information (until there is a change in the physical properties of the instrument), and it can be used for consequent runs.

Alternatively, individual lab should validate and define the settings according to their results. For more detailed information, refer to the Applied Biosystems 3500/3500xL Genetic Analyzer User Guide.

3.1.2. Create a New Size Standard for the QC protocol

- a) Navigate to the *Library*
- b) Select “Size Standards”
- c) Select “Create” (Figure 5)

The Data Collection Software will store this information (until there is a change in the physical properties of the instrument), and it can be used for subsequent runs.

- d) Name the Size Standard as “GT500” and as Dye Color select “Orange”

The fragments size in the GT500 Size Standard are 60, 80, 100, 113, 120, 140, 160, 180, 200, 215, 220, 240, 260, 280, 300, 320, 330, 340, 360, 380, 400, 420, 430, 440, 460, 480 and 500.

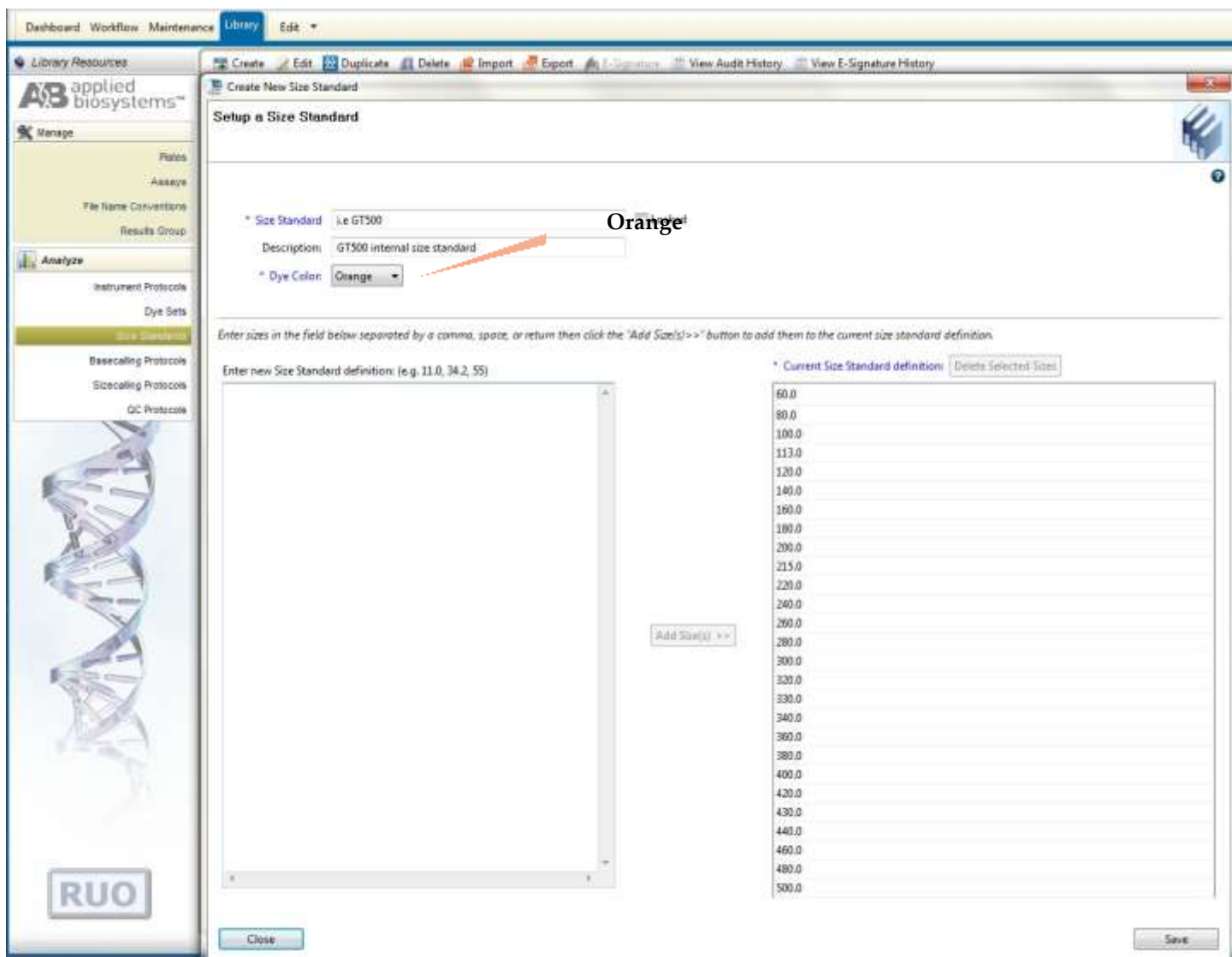


Figure 5. Screenshot for the “Create New Size Standard” window on Applied Biosystems 3500 Data Collection software.

3.1.3. Create a QC protocol

- a) Navigate to the *Library*
- b) Select “QC Protocols”
- c) Select “Create” (Figure 6)

The Data Collection Software will store this information (until there is a change in the physical properties of the instrument), and it can be used for subsequent runs.

- d) Name the protocol as “i.e. GT500” and select the *Size Standard* “GT500”

Users can select settings as shown in the Figure 6 or alternatively may define these settings based on internal validation condition for AneuSure® Plus v2 on the Applied Biosystems® 3500/3500xL Genetic Analyzer.

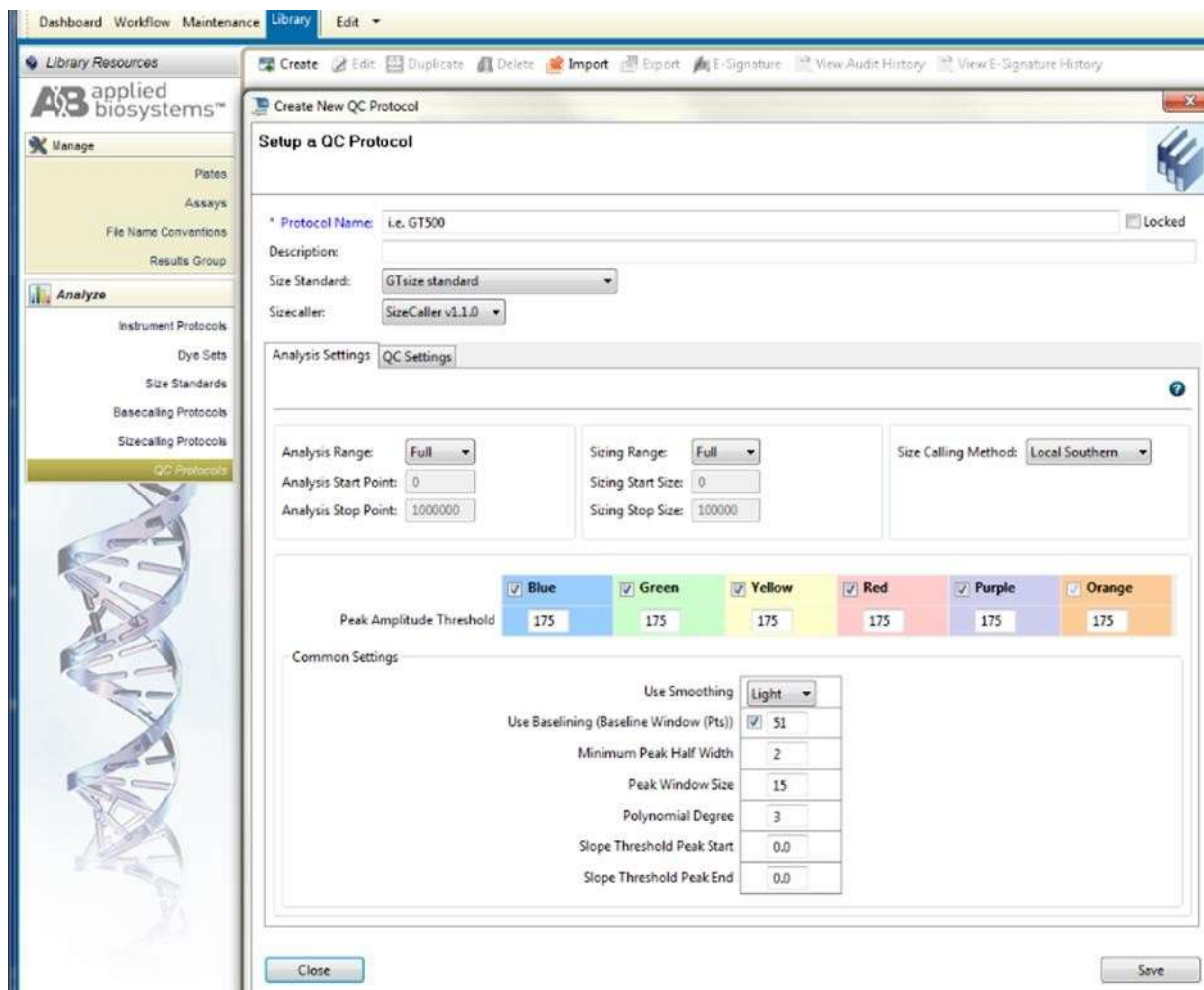


Figure 6. Screenshot for the “Create New QC Protocol” window on Applied Biosystems 3500 Data Collection software.

3.1.4. Create a new Assay

- a) Navigate to the *Library*
- b) Select “Assays”
- c) Select “Create” (Figure 7)

Data Collection Software will store this information (until there is a change in the physical properties of the instrument), and it can be used for subsequent runs.

- d) In the *Create New Assay* window, as shown in Figure 7, choose the *Instrument Protocol* created in Step 3.1.1 and the *QC Protocol* created in Step 3.1.3
- e) Give a name to the assay
- f) Choose the application type “*Fragment Analysis*”

Any named sample on the plate must have an Assay assigned to it.

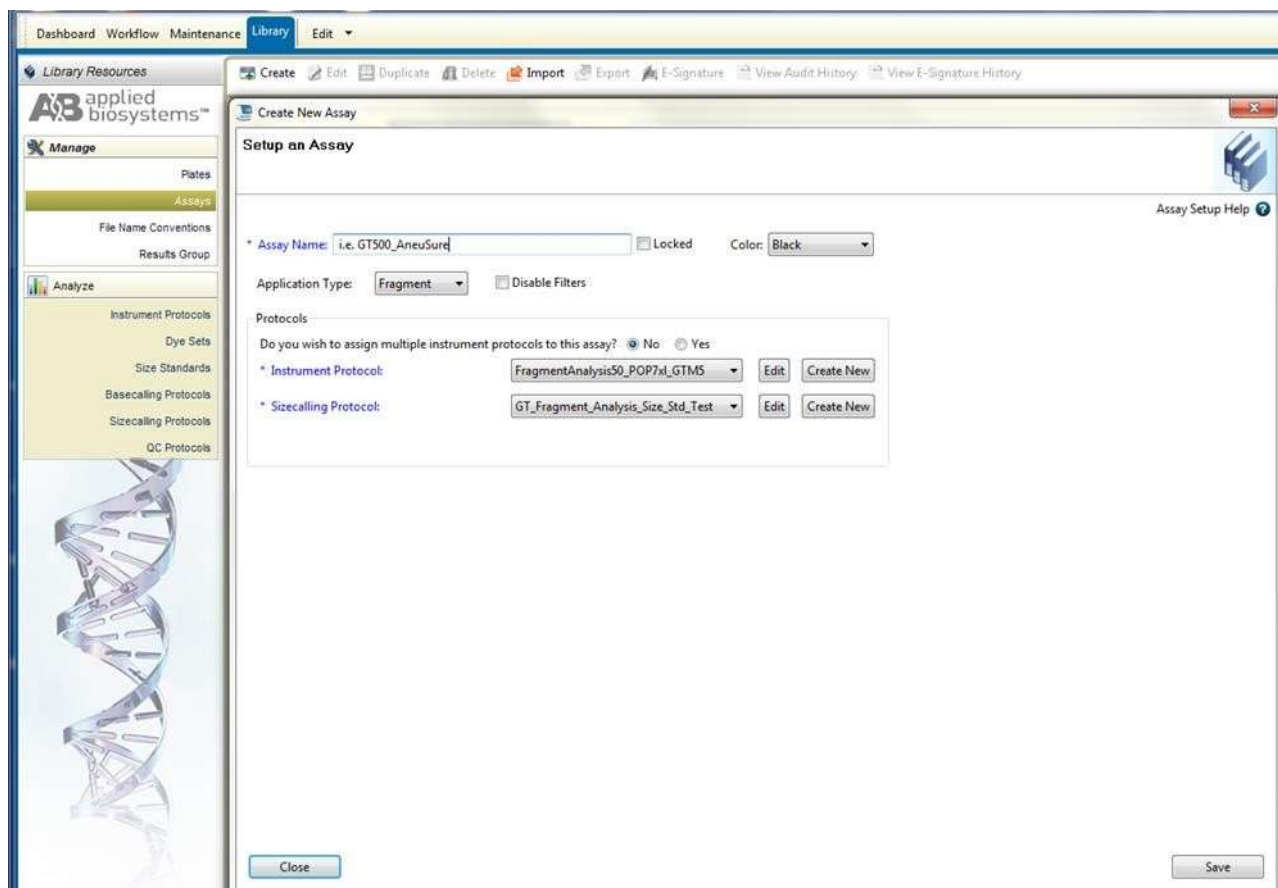


Figure 7. Screenshot for the “Create New Assay” window on Applied Biosystems 3500 Data Collection software

3.1.5. Create a new File Name Conventions

- a) Navigate to the *Library*
- b) Select “*File Name Conventions*”
- c) Select “*Create*” (Figure 8)

Data Collection Software will store this information (until there is a change in the physical properties of the instrument), and it can be used for subsequent runs.

- d) Choose the *File Name Attributes* according to your lab practices

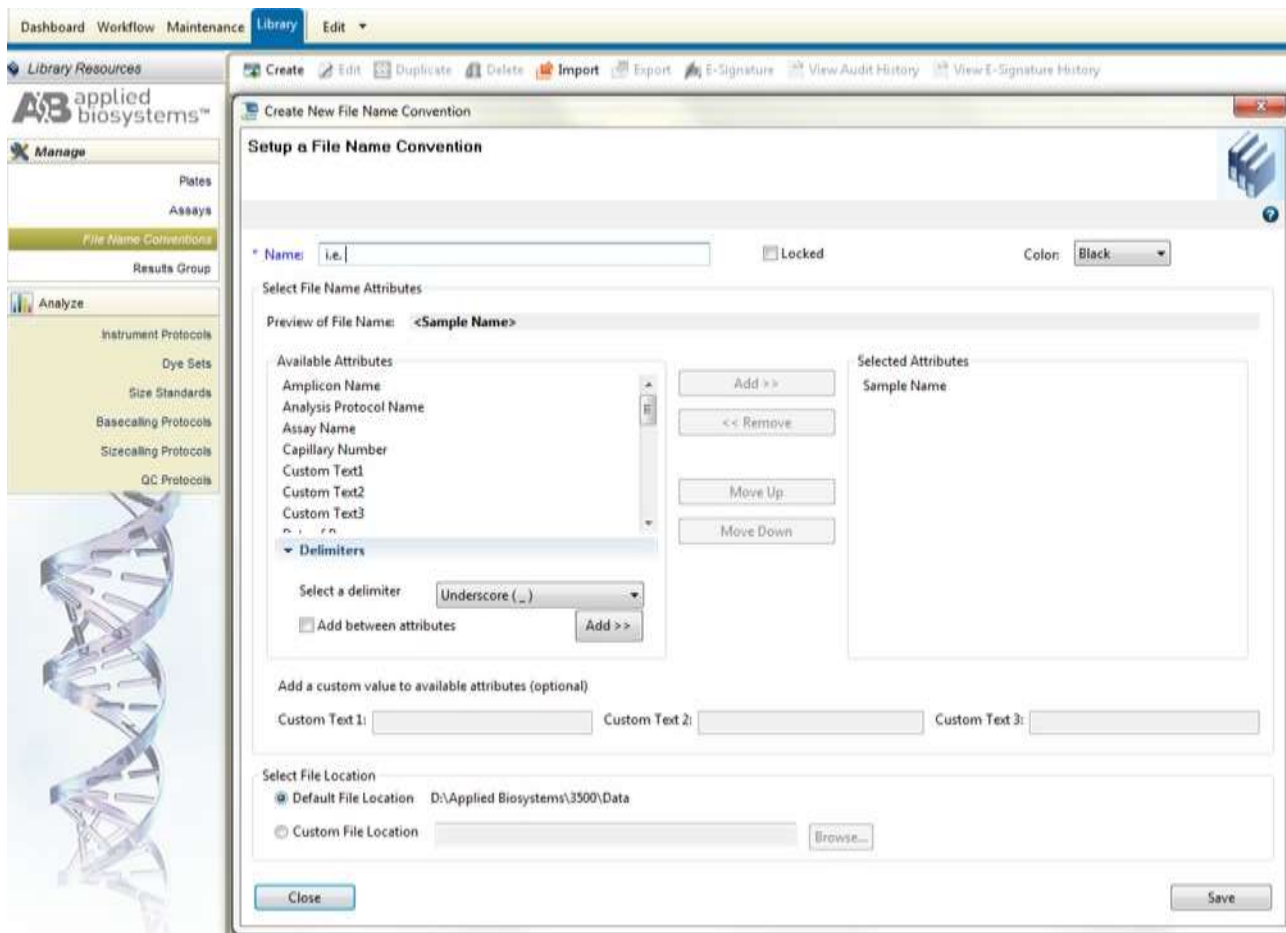


Figure 8. Screenshot for the “Create New File Name Convention” window on Applied Biosystems 3500 Data Collection software

3.1.6. Create a new Result Group

- a) Navigate to the *Library*
- b) Select “*Results Group*”
- c) Select “*Create*” (Figure 9)

Data Collection Software will store this information (until there is a change in the physical properties of the instrument) and it can be used for subsequent runs.

- d) Choose the *Results Group Attributes* according to your lab practices

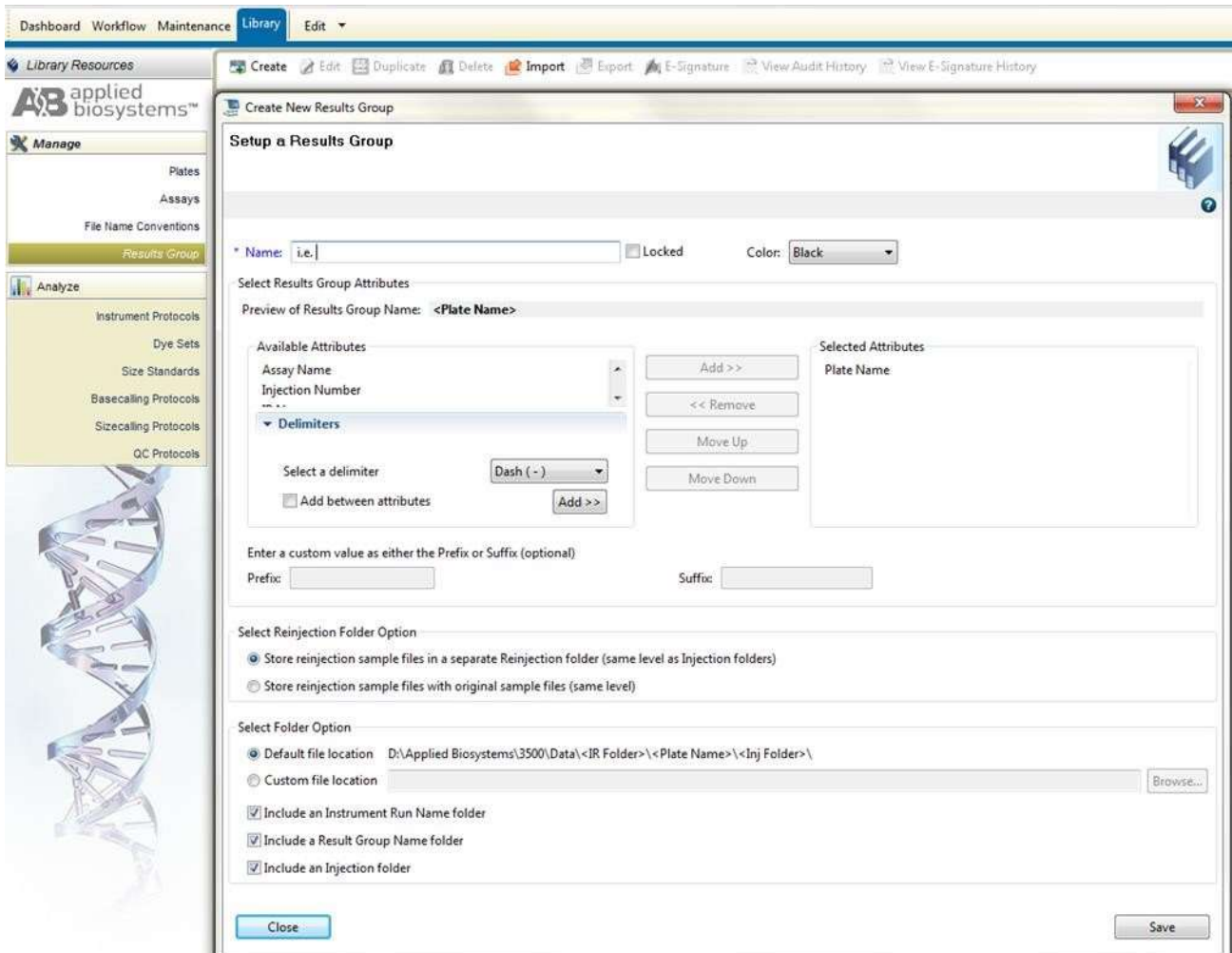


Figure 9. Screenshot for the “Create New Result Group” window on Applied Biosystems 3500 Data Collection software

3.1.7. Create a New Plate

- a) Navigate to the *Library*
- b) From the manage menu select “Plates”
- c) Select “Create” (Figure 10)
- d) Define a name for the plate
- f) Choose plate type “Fragment Analysis” from the drop-down menu



Figure 10. Screenshot for the “Defining plate properties” window on Applied Biosystems 3500 Data Collection software

3.1.8. Select “Assign Plate Contents”

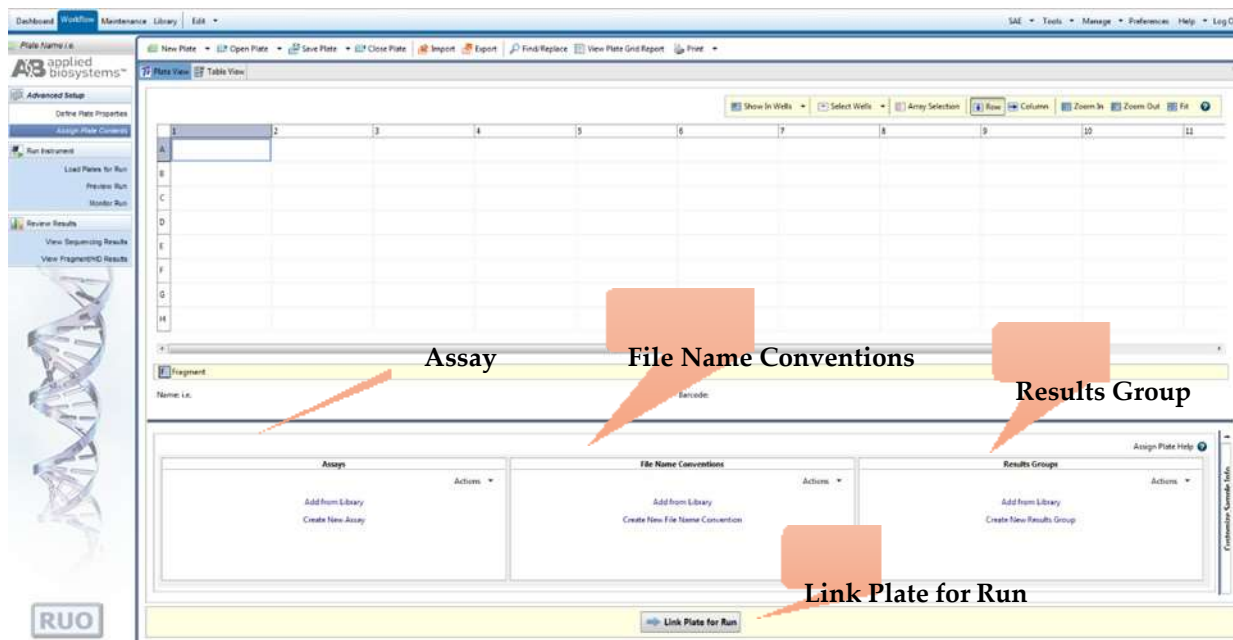


Figure 11. Screenshot for the “Assign Plate Contents” window on Applied Biosystems 3500 Data Collection software

- Define sample names to wells.
- In the *Assign Plate Window* (Figure 11), in the bottom left corner, in a box “Assay”, click *Add from Library* option to select the *Assay* created in Step 3.1.4. Click on the *Add to Plate* button and close the window.
- In the *Assign Plate Window*, in the bottom middle, in the box “File Name Conventions”, click *Add from Library* option to select the *File Name Convention* created in Step 3.1.5. Click on the *Add to Plate* button and close the window.
- In the *Assign Plate Window*, in the bottom right, in the box “Results Groups”, click *Add from Library* option to select the *Results Group* created in Step 3.1.6. Click on the *Add to Plate* button and close the window.
- Select the sample wells, then select the boxes in the *Assay*, *File Name Convention* and *Results Groups* that relevant to those samples.
- Select “*Link Plate for Run*”. It will lead to open *Load Plate* window. Select “Yes”.
- In the *Run Information* window, give a *Run* name (Figure 12). Select “Start Run” after loading the plate.

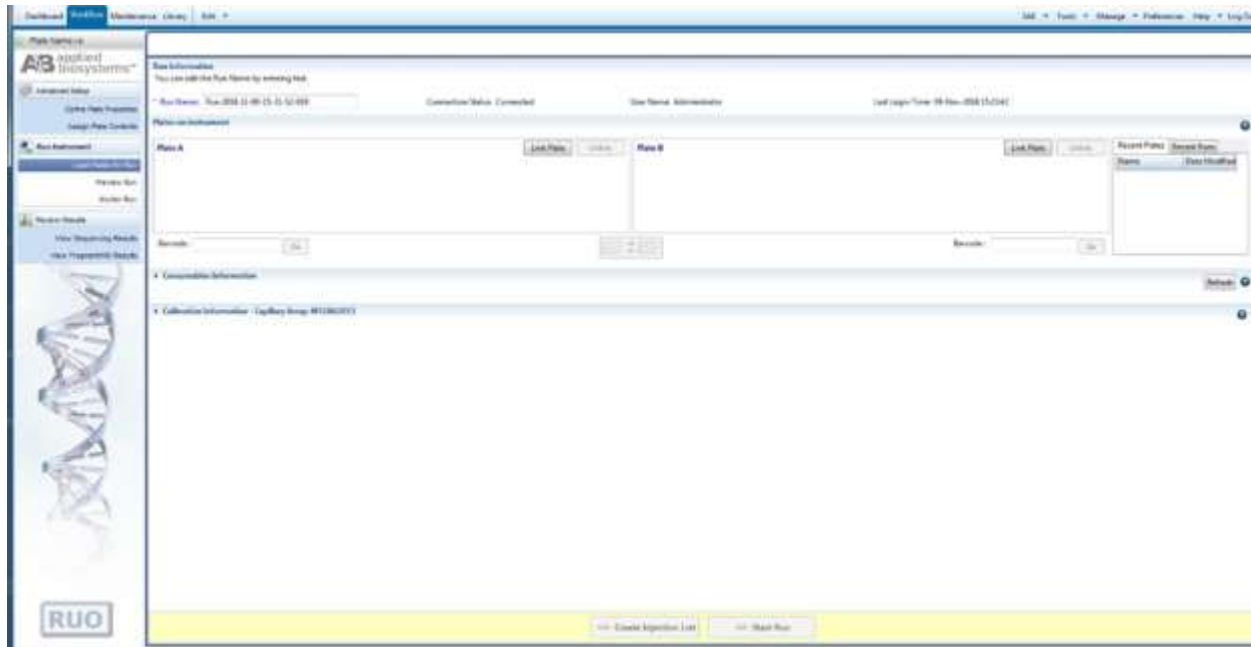


Figure 12. Screenshot for the “Run Information” window on Applied Biosystems 3500 Data Collection software

3.2. Instrument Preparation Applied Biosystems® 3130/3130xl Genetic Analyzer (before the first use of AneuSure® Plus v2 Kit)

Make sure that maintenance and installation of capillary array, buffers and polymer are done according to Applied Biosystems® 3130/3130xl Genetic Analyzer User Guide. Ensure that a spectral calibration is performed with GTM5 v2 Matrix Standard as mentioned above in this instruction in Capillary electrophoresis section. Before starting the electrophoresis for fragment analysis on the ABI Genetic Analyzer the following settings need to be set up in the instrument’s Data Collection Software; **Run Module**, **Instrument Protocol** and **Plate**. The instructions below are from an ABI 3130xl Genetic Analyzer with AneuSure® Plus v2 as an example (Dye set: Any5Dye, GTM5 v2). The procedure is however similar to the other instruments. For further details, refer to the User Guide for the instrument used.

Attention:

Spectral Calibration must be made using GTM5 v2 Matrix Standard, the machine must be calibrated with GTM5 v2 Matrix Standard. Please find detailed protocol for spectral calibration with GTM5 v2 Matrix Standard here - CAT# 41103 or contact us at support@genetek.de.

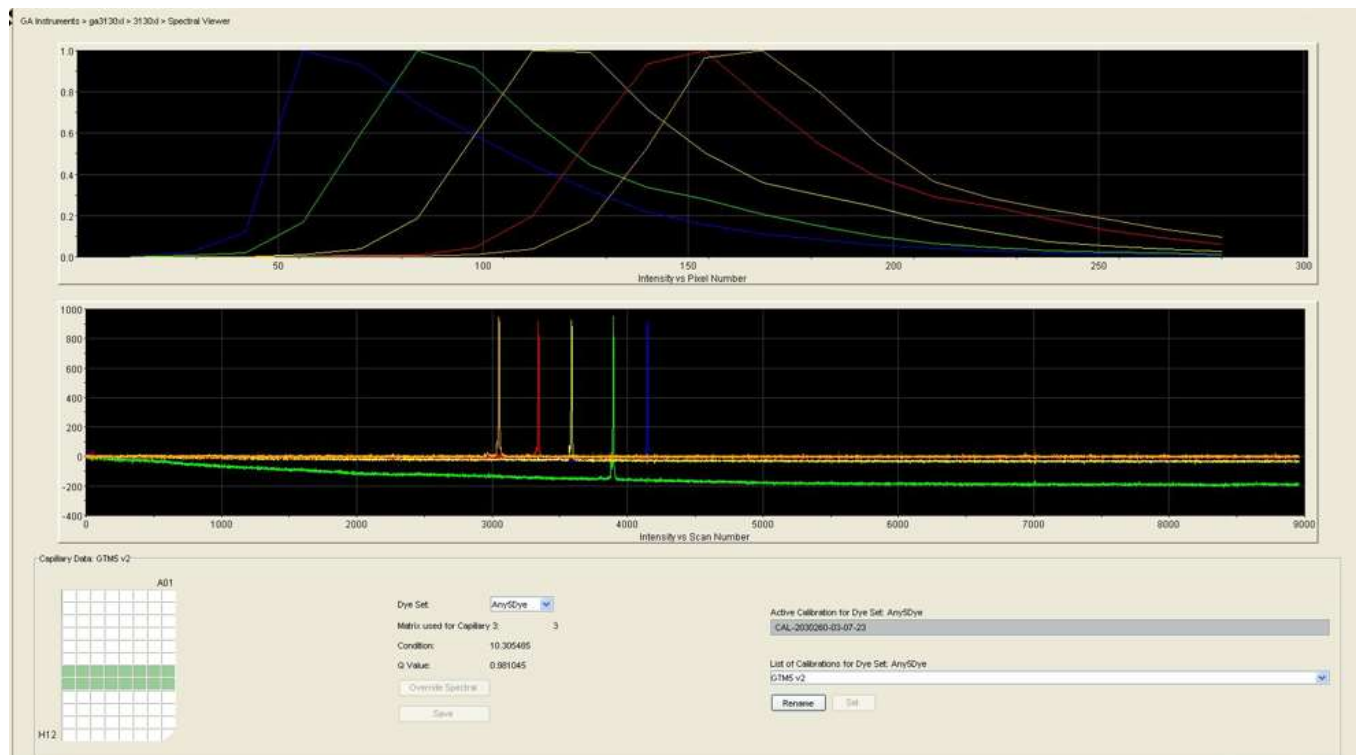


Figure 13. An example of a successful spectral calibration with GT 5-dye system on Applied Biosystems Genetic Analyzer 3130xl

3.2.1. Create a Run Module

In the left navigation window select Module Manager and New. Fill out the Run Module Editor according to the kit instructions for use (IFU).

- a) Name: Enter a name of the Run Module (AneuSure® Plus v2)
- b) Type: Regular
- c) Template: FragmentAnalysis50_POP7 (default template for the capillary array and polymer used)
- d) Click OK

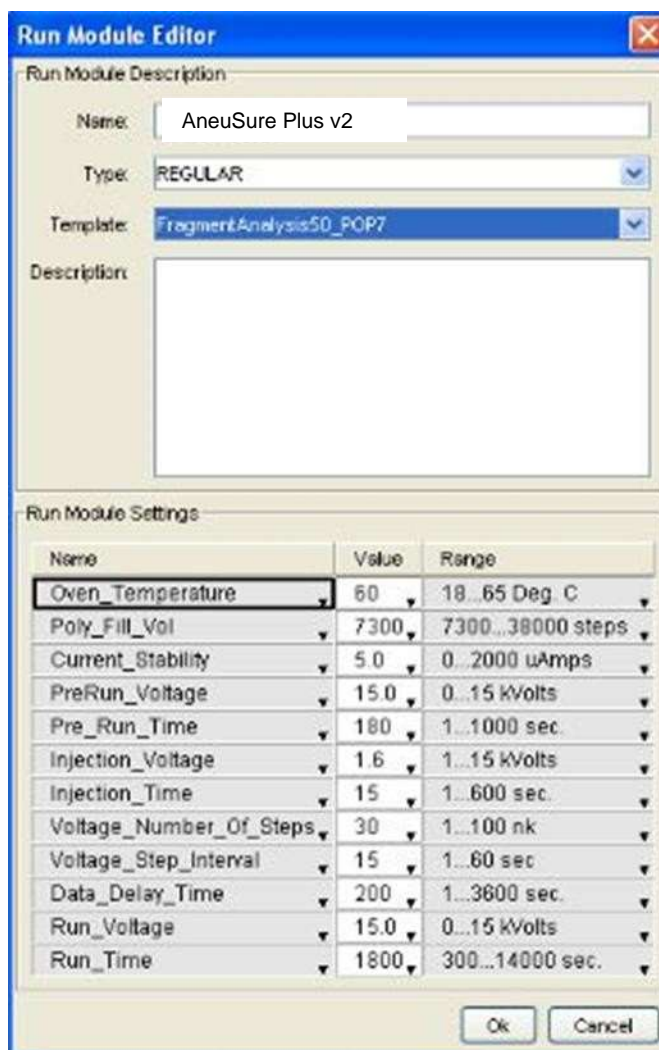


Figure 14. Screenshot for the “Module Manager” window on Applied Biosystems 3130 Data Collection software

3.2.2. Create an Instrument Protocol

From the left navigation window select Protocol Manager and New.

- a) Fill out the Protocol Editor
- b) Name: Enter a name of the Run Module (AneuSure® Plus)
- c) Type: Regular
- d) Run Module: Select the Run Module created (AneuSure® Plus)
- e) Dye Set: Any5Dye
- f) Click OK



The screenshot shows a 'Protocol Editor' dialog box. The 'Name' field contains 'AneuSure Plus v2'. The 'Description' field is empty. The 'Type' dropdown is set to 'REGULAR'. The 'Run Module' dropdown is set to 'AneuSure Plus v2'. The 'Dye Set' dropdown is set to 'Any5Dye'. There is a small icon button to the right of the 'Dye Set' dropdown. At the bottom right are 'OK' and 'Cancel' buttons.

Figure 15. Screenshot for the “Create New Instrument Protocol” window on Applied Biosystems 3130 Data Collection software

3.2.3. Set up a Plate for run

- From the left navigation window select Plate Manager and New.

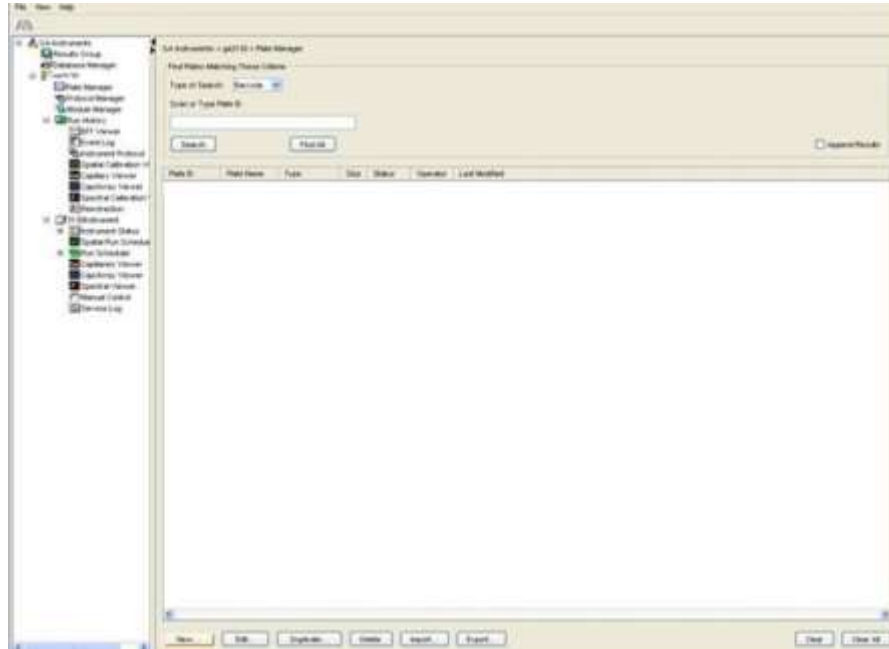


Figure 16. Screenshot for the “Plate Manager” window on Applied Biosystems 3130 Data Collection software

3.2.4. Fill out the New Plate Dialog

- a) Name: Enter a name of the plate
- b) Application: GeneMapper-Generic (used if data is analyzed on a separate computer)
- c) Plate type: 96-Well
- d) Owner Name: enter the name of the owner
- e) Operator Name: enter the name of the operator
- f) Click OK



Figure 17. Screenshot for the “*New Plate Dialog*” window on Applied Biosystems 3130 Data Collection software

3.2.5. Fill out the GeneMapper Plate Editor

- a) Sample name: Enter the sample names
- b) Comment: optional
- c) Instrument Protocol 1: Select the instrument protocol that you created before
- d) Click OK

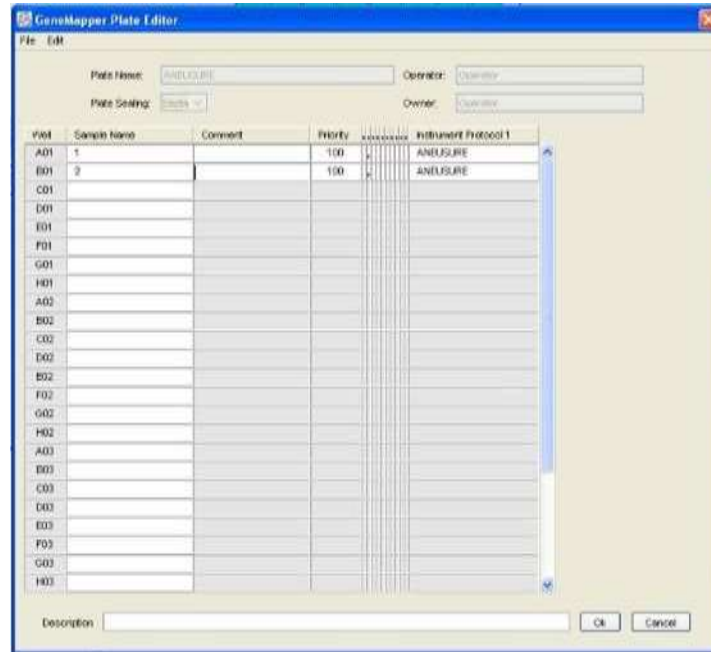
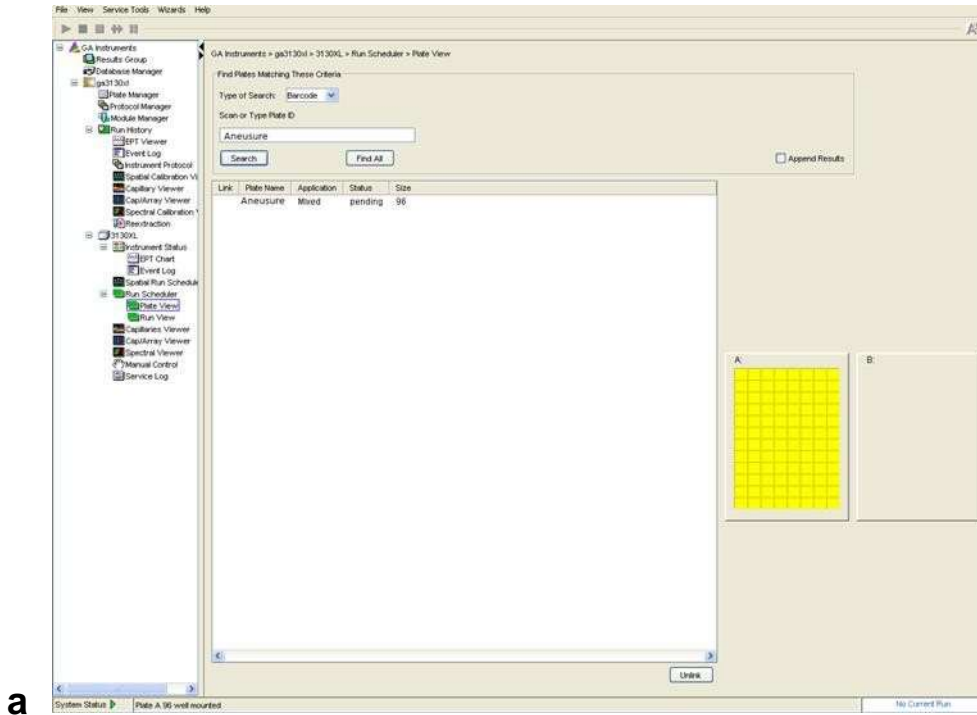


Figure 18. Screenshot for the “GeneMapper Plate Editor” window on Applied Biosystems 3130 Data Collection software

- From the left navigation window, select Run Scheduler, search for AneuSure® Plus v2 (plate name).



- Select the plate created in Step 3 (status pending). Link the plate by clicking on the yellow plate position indicator, which will turn green when linked. Start the run on the green arrow.

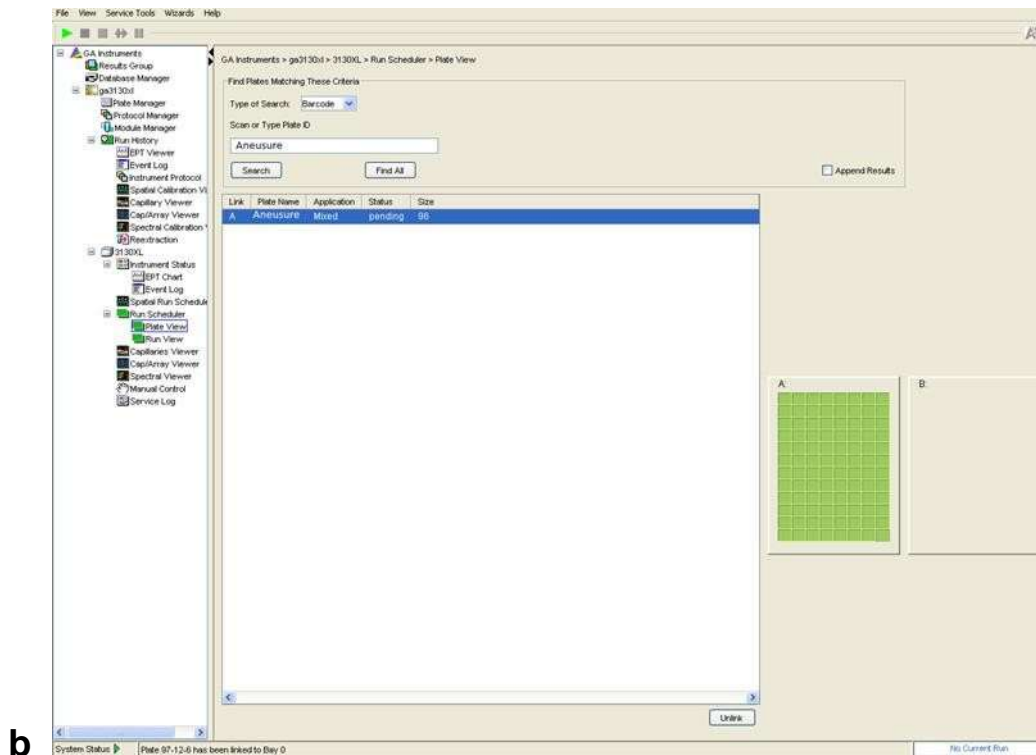


Figure 19 a & b. "Plate view" window on Applied Biosystems 3130 Data Collection software.

- The Process Plates dialog box appears. Click OK to start processing the plate.



Figure 20. “Process Plates dialog” window on Applied Biosystems 3130 Data Collection software.

3.3. Sample preparation for capillary electrophoresis (3500 Series and 3130 Series instruments)

Please note: The Size Standard used in the AneuSure® Plus v2 kit is GT500.

- Vortex and spin 9.5 µL (x number of samples) Hi-Di™ Formamide and 0.5 µL GT500 (x number of samples) in a 1.5 mL tube. For every 8 samples prepare 10 since there may be pipetting error. The amounts below are for 10 injections. Please test using different amount of internal size standard to sample. We have found that using 0.5 µL of sample and 0.5 µL of size standard gives better results but each lab has to test and optimize the Formamide, sample and size standard ratios.
- Pipette 10 µL of the prepared size standard mix to required number of well and add 1 µL PCR product to it and use pipet to mix. Cover the wells with appropriate septa.
- Denature the PCR product by heating the plate in a thermal cycler. Set the cycler as:
 - 95°C for 5 minutes
 - 4°C for 30 seconds
- Place the PCR products on the ice (or cool box at -20) for 3 minutes
- Centrifuge the plate at 1,000xg for 10 seconds to remove any bubbles in the wells.
- Place the plate in the Genetic Analyzer and start run.

Please note: Detection limits for each instrument is different; hence, injection time, injection voltage or the amount of sample mixed with loading mix (Hi-Di™ Formamide and GT500 internal size standard) may need to be adjusted. Use the Module Manager in the data collection software to modify the injection time or voltage in the run module according to your lab validation (as mentioned in the instrument preparation above).

4. Result analysis and Interpretation

4.1. Software for sample analysis

- For AneuSure® Plus, the Applied Biosystems fragment analysis software compatible with your genetic Analyzer is recommended. This kit is compatible with GeneMapper software. Analysis method depends on the software.

Each diagnostic lab should have individual interpretation and reporting procedure and criteria. To develop such procedure use of “**QF-PCR for the diagnosis of aneuploidy best practice guideline**” is recommended here - https://www.cytogenetics.org.uk/prof_standards/professional_standards.html.

4.2. General guideline for the analysis of AneuSure® Plus v2 results

AneuSure® Plus v2 PCR products are observed with 5-dye system on an electropherograms in the GeneMapper® software. For the analysis, import AneuSure® Plus v2 panels. It can be downloaded from our website or contact us at support@genetek.de.

For detailed procedure on fragment analysis on GeneMapper® software please refer to the GeneMapper® *user guide*.

4.2.1. Criteria for Interpretations

- “Size” shows the fragment size. The size may differ between individuals but are usually constant within a person and his/her parents. With QF-PCR, one is not concerned with sizes of each allele unless there is maternal cell contamination, samples mix-up, PCR contamination from previous samples of DNA from those working in the lab or similar issues are involved.
- The area under each peak in electropherogram represents the amount of amplified PCR product.
- The height of each peak represents the activity of each fluorescent component which shows the quantity of the fluorescent compartment of each marker.
- These results are shown as electropherograms in the analysis software. Height and the area related to each peak are observable in this software.
- Negative control should not show any peculiar fragment size of between 100 to 500 bp.
- Quality control DNA (if used) should show expected results as shown here – see Example profile GT QCDF150.
- There should not be excessive bleed-through between dye colors or “*Pull-up*” effect in the electropherograms.
- Successful amplification must result into at least one peak for each marker (except for Y chromosome markers which would be absent in normal female sample)

4.2.2. Quantification of peak-area ratio

QF-PCR amplification of STR markers generates fluorescent product which can be measured by software in terms of -

- *Size* – length of the amplicon in base pair (the picture below shows a 227.48 or 227 bp size for this fragment).
- *Area and Height* – These values represent fluorescent activity, so the amount of the PCR product.

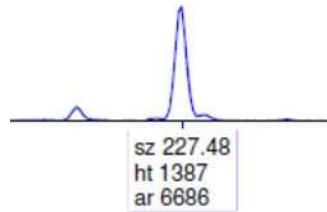


Figure 21. Allele plots generated with GeneMapper® 5.0 for homozygous peak

4.2.3. Peak ratio for Disomy (normal)

- In normal individual heterozygous for the STRs – almost equal amount of fluorescence generated for both alleles should be seen. Hence the ratio between the area and height of the peak count is almost 1:1.

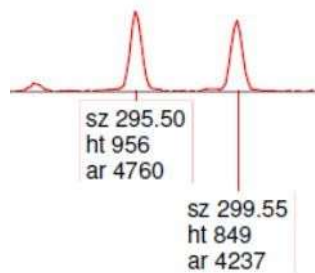
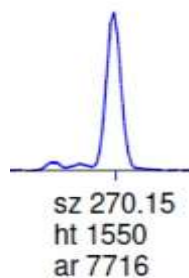


Figure 22. Allele plots generated with GeneMapper® 5.0 for heterozygous peak. It is known that for the same locus, the peak height for the longer repeat size is proportionally smaller than the smaller size (299.55 vs 295.50). So one has to be careful when trying to see if di-allelic trisomy is present or not

- Normal individual being homozygous for a STR would show only one peak. Hence it cannot be quantified, and this is an uninformative marker.



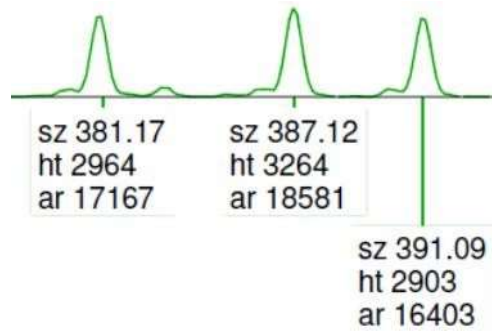
Figures 23. Allele plots generated with GeneMapper® 5.0, peak height and area are shown in the label

- Result for a sample with normal copy number for a chromosome will show homozygous or heterozygous pattern for all STR markers in the AneuSure® Plus v2 Kit. Interpretation and assessment of normal copy number should be based on at least two informative markers for each chromosome.

4.2.4. Peak ratio for Trisomy 21, 18, 13 and Triploidy

- **Trisomy determination in tri-allelic marker**

Results for STR marker on a chromosome show three peaks of almost similar heights and having similar fluorescent intensity then the ratio of the peak would be 1:1:1.



Figures 24. Allele plots generated with GeneMapper® 5.0 for tri-allelic trisomy, peak height and area are shown in the label

- **Trisomy determination in di-allelic marker**

Results for STR marker on a chromosome shows unbalanced two peaks, due to one of the peaks representing two alleles that are shared to one or both parents. Hence the ratio between the two peaks is 2:1 or 1:2.

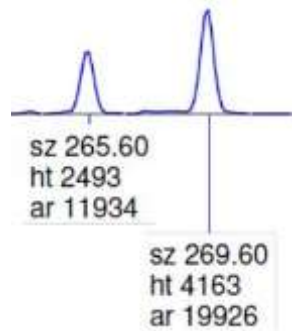


Figure 25. Allele plots generated with GeneMapper® 5.0 for di-allelic trisomy, peak height and area are shown in the label

- Interpretation for Trisomy is only acceptable if at least two markers on the same chromosome have trisomic patterns. Follow up testing with single chromosome marker specific kits is recommended if enough information for interpretation is not provided from the first test. Genetek Biopharma has chromosome specific markers for all 5 chromosomes present in the AneuSure® Plus v2 Kit.

4.2.5. Interpretation for XY markers

- Any conclusion for the copy number of sex chromosome should be made after assessing all the sex chromosome markers together.
- The AMXY marker can be used to determine the presence of a Y chromosome. It amplifies sequence on the X (104 bp) and Y (110 bp) chromosomes and represents the relative amount of X to Y sequence.
- The 7X marker in the AneuSure® Plus v2 Kit is a segmental duplication marker which means it represents sequence from both 7 and X chromosomes. This marker is extremely useful in case of Turner syndrome and in case of Monosomy. See the example profile for Turner Syndrome generated using AneuSure® Plus v2 below.

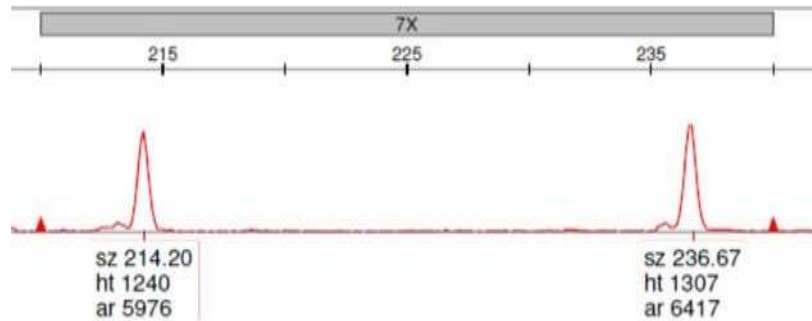


Figure 26. Normal male 7X or monosomy X – height ratio is ≥ 1.8 .

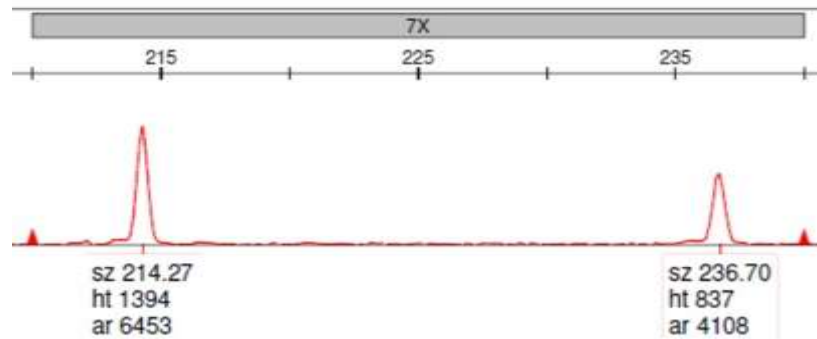


Figure 27. Normal female 7X – height ratio is between 0.8 and 1.4.

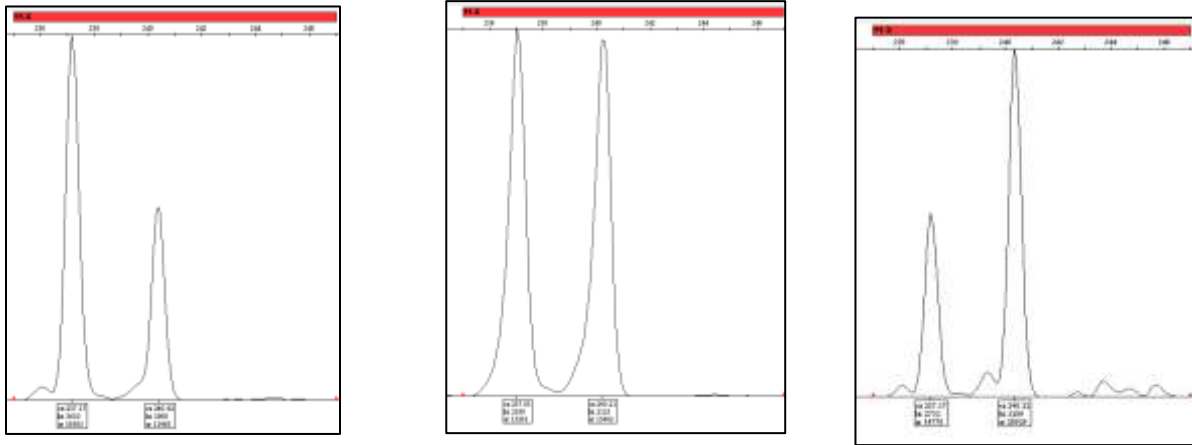


Figure 28. The figure on the left shows a normal male 11/X or monosomy X (Turner Syndrome if the sample is from a female) – height ratio should be $\geq 1.8:1$ since there are two chromosome 7 and one chromosome X. The figure in the middle shows a normal female sample (almost 1:1 ratio). The figure on the right shows a person with 4 X chromosomes since the ratio is 1:2 or 2:4 because there are 2 chromosome 7 and therefore, there is 4 chromosome X.

5. Examples of results

5.1. A sample from an affected individual with 5q SMA (deletion of SMN1).

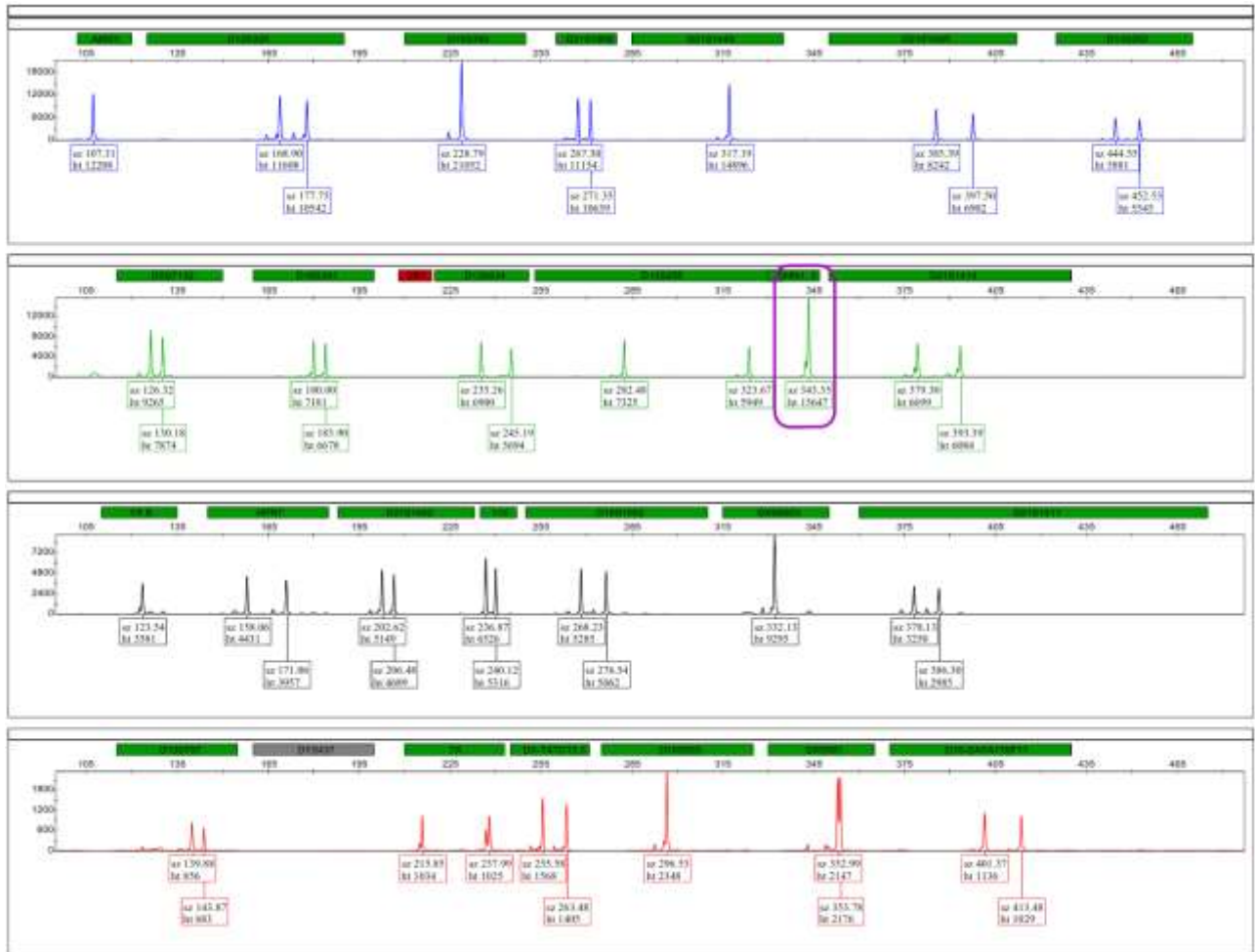


Figure 29. Example of a fetal sample with no aneuploidy. However, we only see a single peak for the SMN1 and SMN2 (fragment size 340 is for SMN1 and 343 is for SMN2). Therefore, the fetus is most probably affected with SMA, and result should have been confirmed by other technique. The MLPA result indicated that the sample was indeed affected with SMA (MLPA data is not shown here).

5.2. A normal female profile (GT QCDF150 included in all AneuSure® Plus v2 Kit)

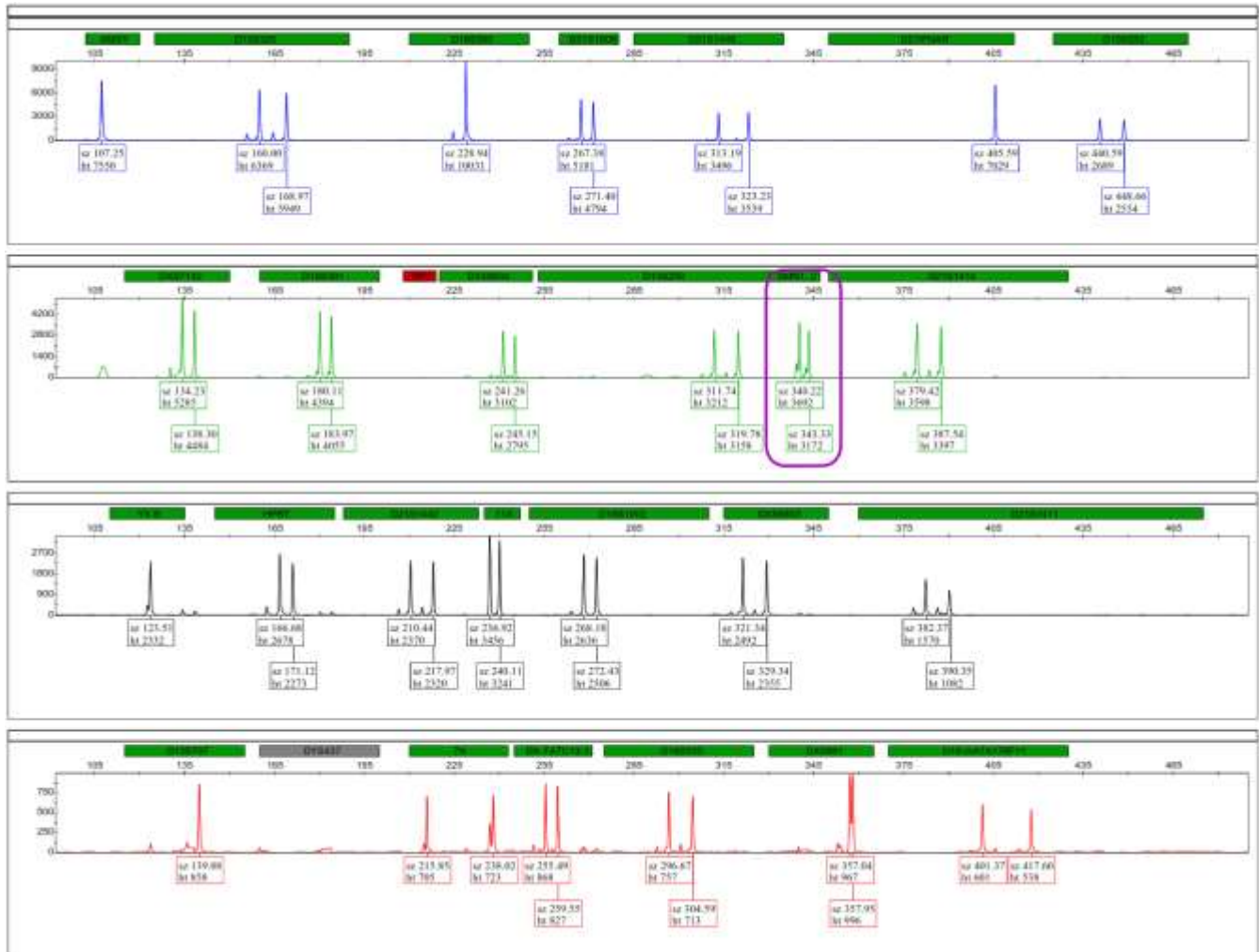


Figure 30. Example of normal female profile (normal for aneuploidy and SMA).

5.3. Trisomy 21 – ‘Down Syndrome’

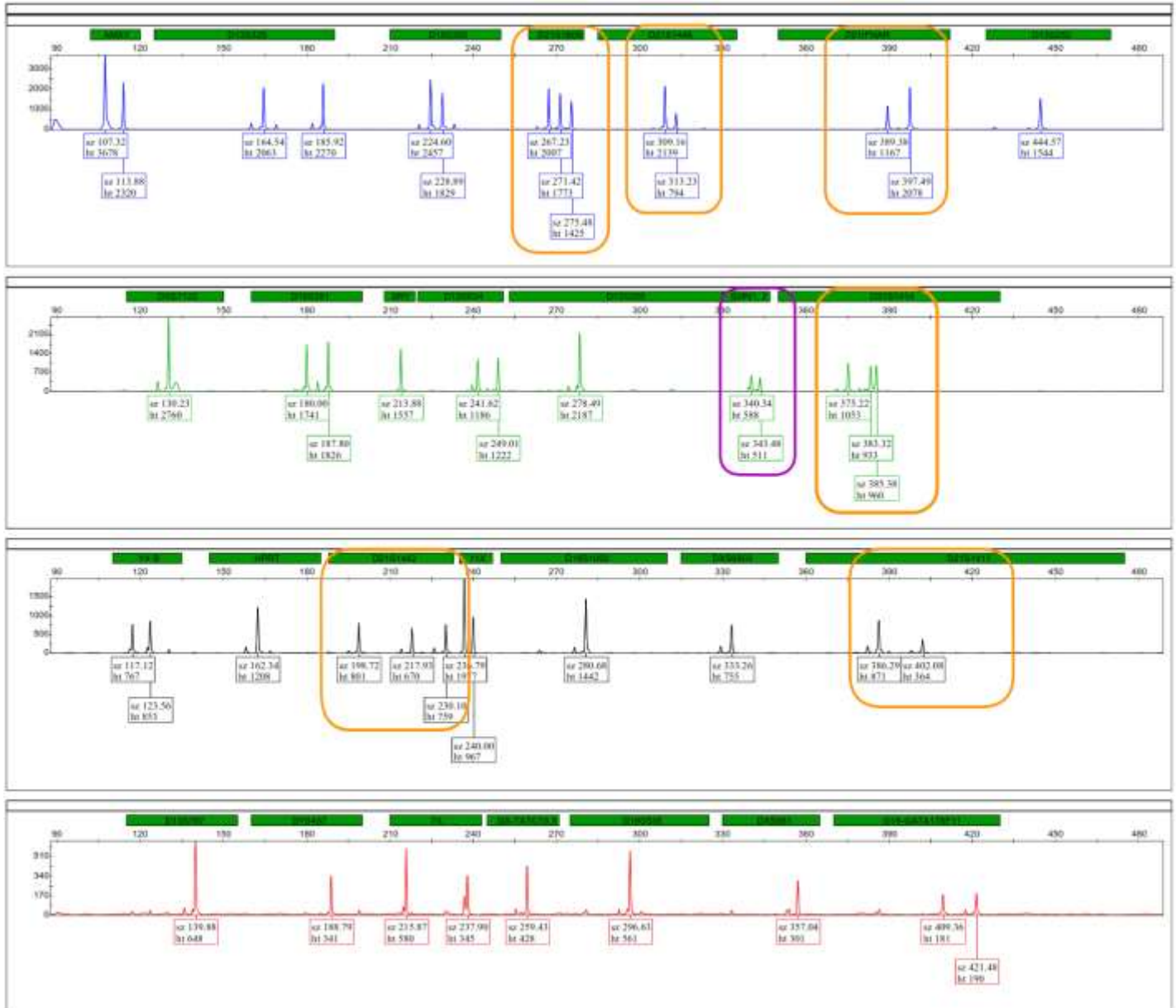


Figure 31. An example of a Down Syndrome profile. Markers showing trisomy are boxed. This is a Tri-allelic trisomy as there are three distinct peaks for most chromosome 21 STR markers.

5.4. Trisomy 18 – ‘Edward Syndrome’

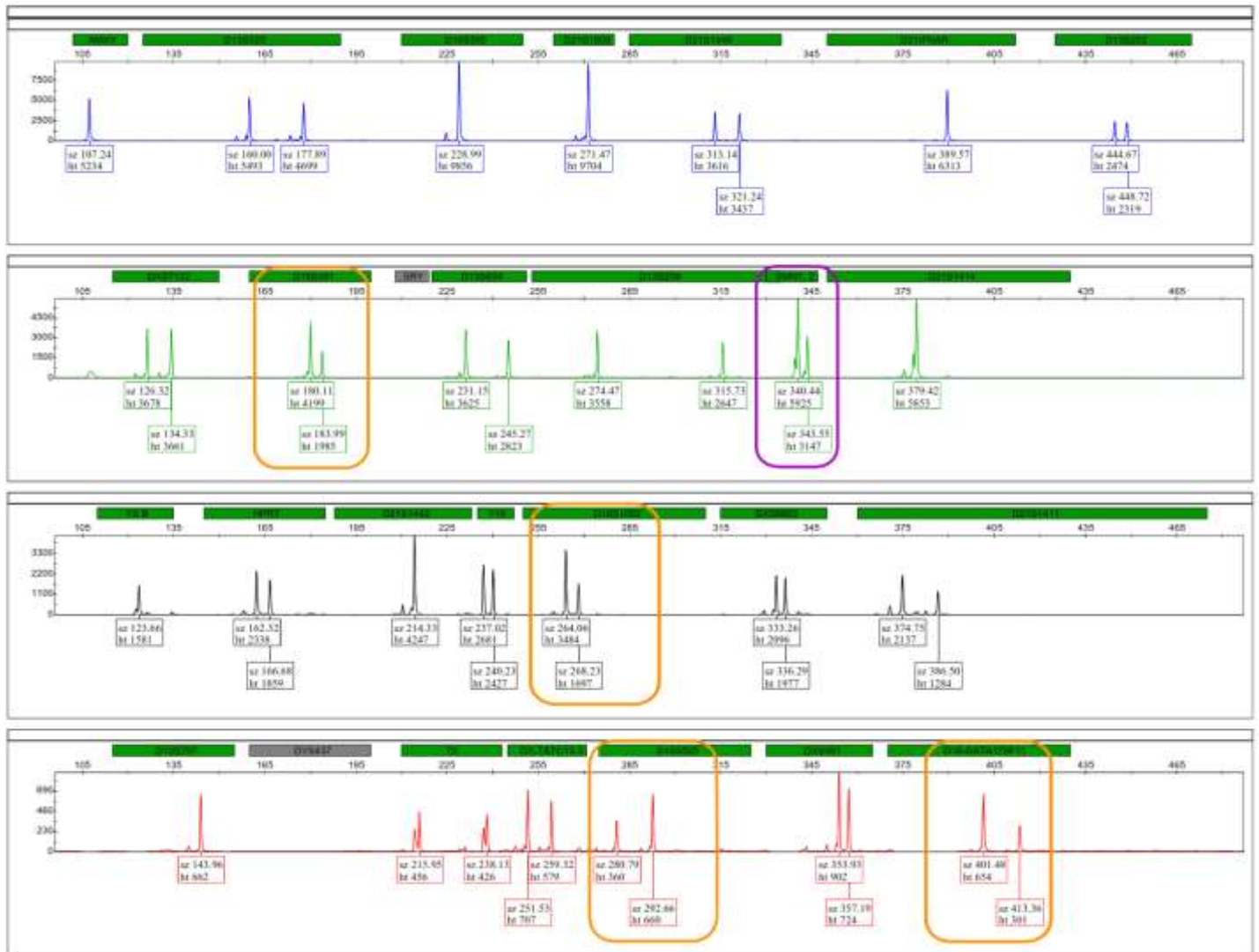


Figure 32. Example of an Edward Syndrome profile. Markers showing trisomy are boxed. This is a Diallelic trisomy as there are two peaks for most chromosome 18 STR markers but one is almost twice the other in height.

5.5. Trisomy 13 – ‘Patau Syndrome’

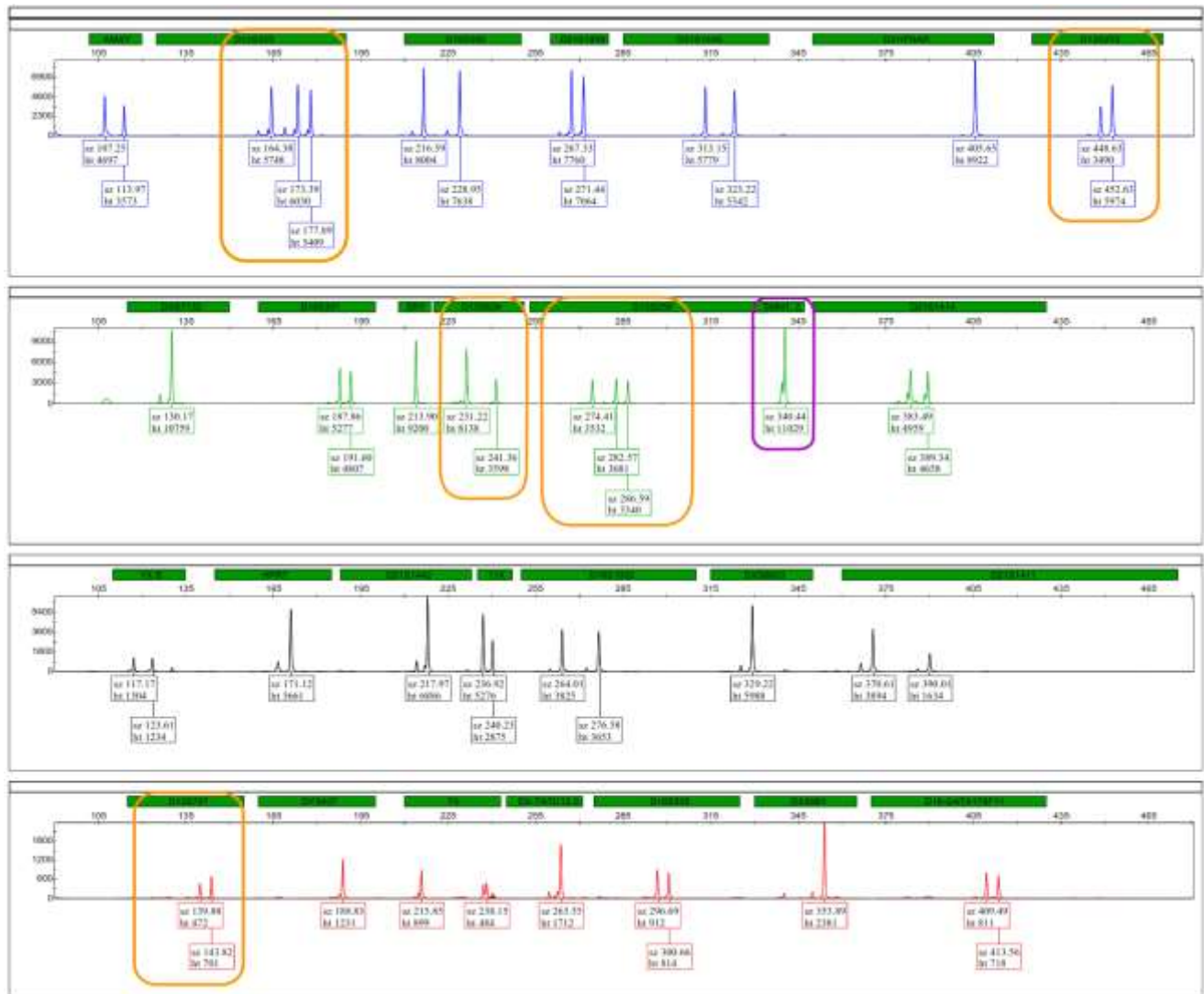


Figure 33. Example of Patau Syndrome profile. Markers showing trisomy are boxed. This individual has only SMN1 copies and no SMN2 copies (otherwise normal for SMA). The sample is a male based on male specific markers like AMXY, XY B, 7/X and 11/X. This individual show trisomy for D21S1411 but since other chromosome 21 markers are normal, it would be regarded as polymorphism and not an issue.

5.6. Turner Syndrome (XO)

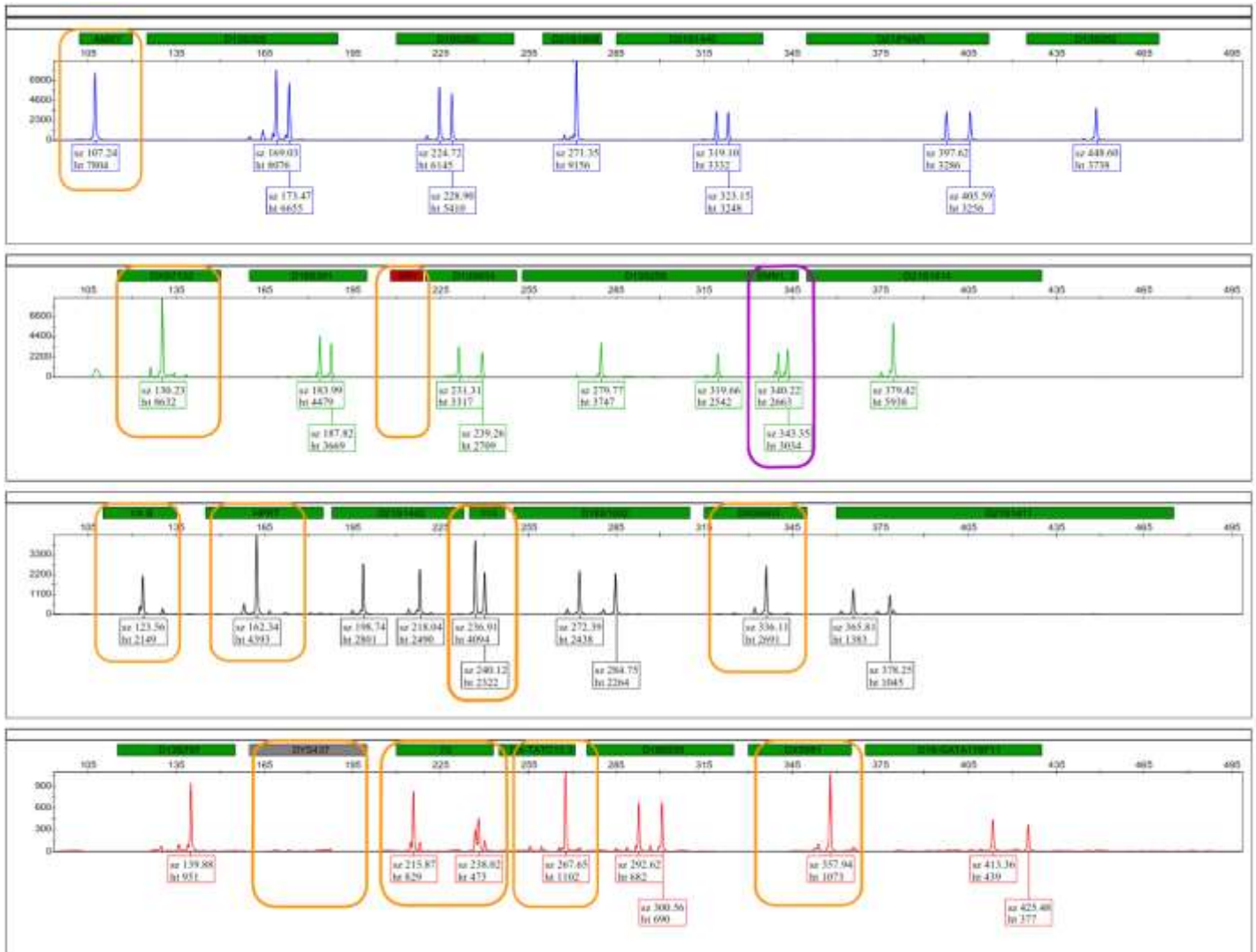


Figure 34. Example of Turner Syndrome profile. Markers showing monosomy of the X chromosome are boxed. This is a female sample as indicated by the AMXY and XY B markers. However, the 11/X and 7/X markers indicate presence of a single chromosome X or monosomy for chromosome X or Turner Syndrome.

5.7. Klinefelter Syndrome (XXY)

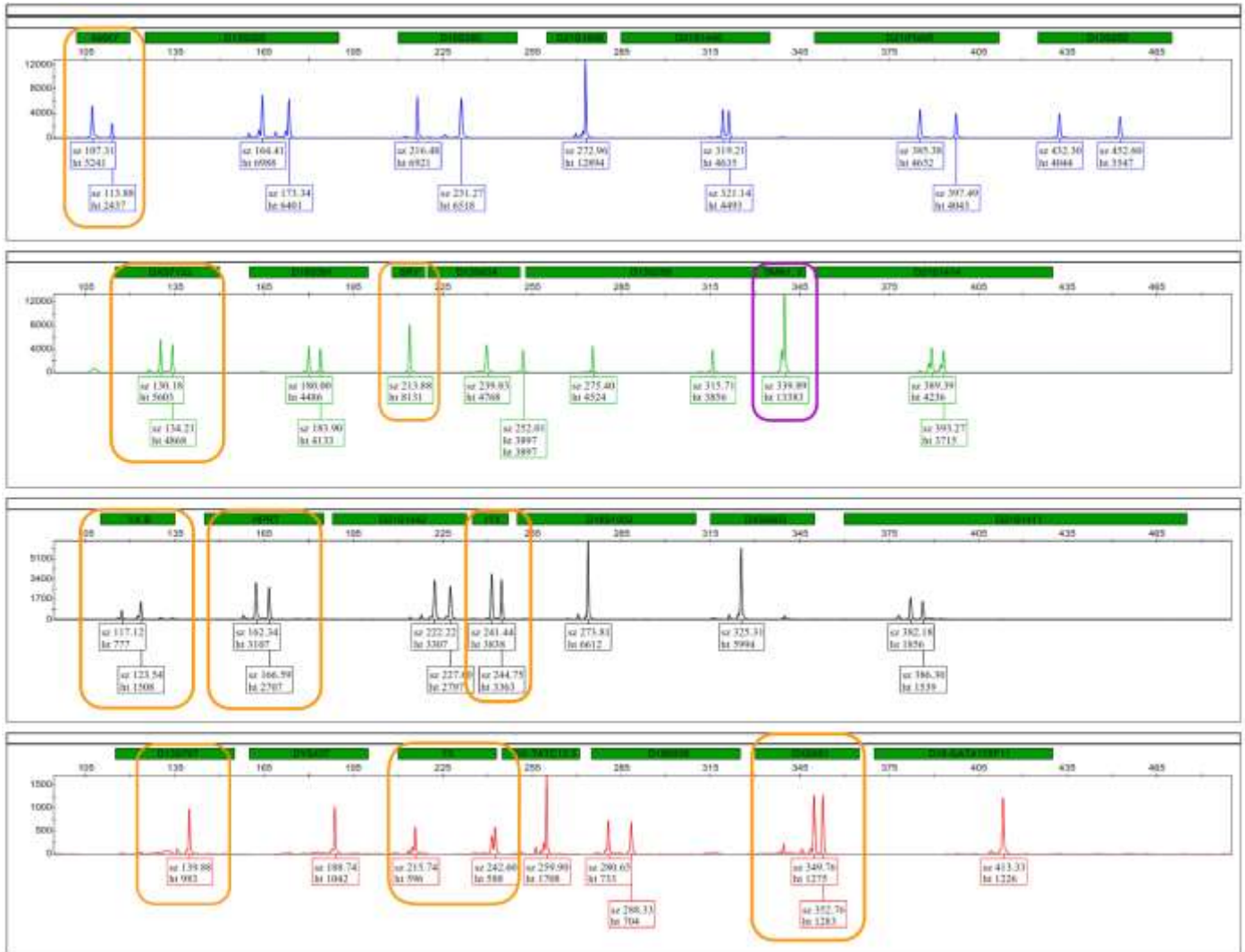


Figure 35. Example of Klinefelter Syndrome profile. Markers showing Klinefelter Syndrome are boxed. The SRY, AMXY and XY B show a male sample but some of the X chromosome markers show two STR peaks indicating a female sample. The two segmental duplication markers, 11/X and 7/X have almost equal heights. This individual has only SMN1 gene.

6. Troubleshooting

For any technical question or issue (not mentioned here) please contact our customer support here – support@genetek.de.

Issue Observed	Possible cause and Solution
No peak detection or faint peaks	<p>PCR reaction mix is not well mixed with enzyme and DNA. Vortex or use pipette to mix the PCR reaction mixture after adding DNA. PCR has to be repeated.</p>
	<p>An air bubble formation in the reaction tube can cause poor mixing of reaction mixture. Use a pipette to remove the air bubble or centrifuge the reaction mixture before thermal cycling.</p>
	<p>Poor amplification due to improper thermal cycling. AneuSure® Plus v2 Kit amplification protocol is validated using Eppendorf Mastercycler® nexus. Individual lab must perform internal validation for different thermal cycler to confirm the cycling protocol.</p>
	<p>Poor capillary electrophoresis injection if faint peaks for GT500 Size Standard is also observed. Re-inject samples or increase injection time.</p>
	<p>Lower quality formamide was used. Use only the recommended formamide.</p>
	<p>Run quality control GT QCDF150 provided with AneuSure® Plus v2 Kit to check efficiency of primer mix and other PCR reagents.</p>
<p>Inhibition of PCR because of too much template or other impurity in DNA extraction. Check the quality and quantity of extracted DNA. Use only the recommended DNA concentration. Make sure DNA is not degraded.</p>	

Extra peaks observed in one or more dye channels

Amplification of STRs can result in artifacts that seem as peaks one base smaller than actual peak due to incomplete addition of the 3' "A" residue.

To avoid this phenomenon, we recommend:

- a) Make sure to perform complete extension step as described in the protocol.
- b) Decrease the amount of DNA template in the reaction, too much DNA can lead to incomplete adenylation.
- c) Make sure reaction is not over amplified, decrease cycle number. Eventually each lab should perform internal validation for cycling condition.

Pull-up or bleed-through because of too high peaks. Make sure that analysis method is performed using GTM5 v2 Dye Set Spectral Calibration.

Check if Spectral Calibration results are acceptable. See instructions in instrument preparation in section 3.

Samples not denatured completely, perform denaturation step as recommended.

Cross contamination with another sample DNA or PCR reagent is contaminated with amplicons. Use aerosol-resistant pipette tips, change gloves for pre- and post- PCR steps.

Store reagents in appropriate (Pre- and Post-) storage space. Do not open pre - PCR reagent tubes in Post-PCR lab.

Long-term stored PCR products are used.

Polymer-caused artifacts, check Polymer expiration date and storage time as mentioned in the manufacture guide.

Off-scale peaks

If off-scale peaks after primer peaks are observed –

- a) Excessive DNA is added as template. Prepare new reaction with diluted DNA to repeat the PCR and capillary electrophoresis.
- b) Excessive size standard in sample. Prepare new reaction using less size standard and repeat electrophoresis run.

No sizing data or size quality fails

- a) Incorrect or no size standard is selected in analysis method or protocol editor. Make sure that size standard option is edited with GT500 Size Standard.
 - b) Incorrect size standard is used. We recommend using GT500 with AneuSure[®] Plus v2 Kit to obtain optimum results.
-

7. Limitations and Disclaimer

Any result obtained from AneuSure® Plus v2 or any other diagnostic Kit should be used and interpreted by qualified persons. GENETEK BIOPHARMA GmbH cannot bear any responsibilities for false use and interpretation being made by any lab. The results obtained by AneuSure® Plus v2 or any other diagnostic Kit should only be used to indicate overall clinical scenario hence GENETEK BIOPHARMA GmbH cannot be responsible for any clinical decisions made by user or client lab.

AneuSure® Plus v2 Kit is designed to detect trisomies and sex-chromosome aneuploidies only for chromosomes 21, 18 and 13 specific. It will not detect other chromosome abnormalities or defect. User must carefully inspect any case of Maternal Cell Contamination and placental mosaicism before interpretation and patient consultation.

Result analysis guideline is generated using set of samples from specific populations. User lab should perform internal validation for any specific population for heterozygosity in each population.

We recommend that individual laboratory perform and develop its own test procedure and interpretation standard operative procedure. Best practice guidelines as mentioned in following section can be used to generate such documents.

AneuSure® Plus Kit is for Research Use Only and user bears all the responsibility for its use in clinical practice. Please consult best practice guidelines when using any QF-PCR kits including AneuSure® Plus kit as mentioned above.

8. General Safety Warnings

- Any procedure should be performed by professional/qualified personal.
- Care should be taken while handling any human origin material, all samples should be considered potentially infectious. Lab technician or person handling the DNA must follow good lab practice and safety guidelines.
- Store all the components as described in the user guide.
- Laboratories should test their own quality check samples for each type of the assay to validate the Kit procedure.







Chemical safety

- Before handling any chemicals, refer to the Safety Data Sheet provided by the manufacturer and follow relevant precautions.
- Minimize the contact with chemicals. Wear appropriate personal protective lab wear i.e. safety glasses, protective clothing, gloves.
- Check for chemical leaks and spills.
- Comply with local regulation regarding chemical storage, handling and disposal.

SDSs

- The SDS for each of the Kit component is available online at GENETEK BIOPHARMA GmbH website <https://genetek-biopharma.com/>
- Any request for specific SDS can also be made from support@genetek.de.

9. Symbols used on labels and packaging

Description	Symbol
Read Instructions before Use	
Do not use after the year, month and date mentioned	
Manufacturer name and address	
Storage temperature limit – Upper and Lower	
Manufacturer's Catalogue number	
Manufacturer's Batch code or Lot number	

10. Further Reading

1. Association for Clinical Cytogenetics and Clinical Molecular Genetics Society. QF-PCR FOR THE DIAGNOSIS OF ANEUPLOIDY BEST PRACTICE GUIDELINES (2012) V3.01.
2. DNA Fragment Analysis by Capillary Electrophoresis User Guide by Applied Biosystems® Publication Number 4474504.
3. Mann, K. and Ogilvie, C. M. (2012), QF-PCR: application, overview and review of the literature. *Prenat Diagn*, 32: 309-314. doi:10.1002/pd.2945
4. Best Practice Guidelines for Internal Quality Control in Genetic Laboratories by Association for Clinical Genetic Science
5. Mattocks CJ, Morris MA, Matthijs G, et al. A standardized framework for the validation and verification of clinical molecular genetic tests. *Eur J Hum Genet*. 2010;18(12):1276-88.
6. Mackie Ogilvie, C., Donaghue, C., Fox, S.P., Docherty, Z., Mann, K. 2005. Rapid prenatal diagnosis of aneuploidy using Quantitative Fluorescence-PCR (QFPCR). *Journal of Histochemistry and Cytochemistry* 53(3):285-28