



AneuSure[®] Max Plus v2

Product User Manual

CAT# GT-11112

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® Max Plus v2 Overview

- Detecting aneuploidy for chromosomes 13, 18, 21, X, and Y and detecting 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
- 35 markers amplification in a single reaction
- 6-dye fragment analysis using capillary electrophoresis
- Compatible with Applied Biosystems® 3500/3500xL Genetic Analyzer

1.1. Intended Use

The AneuSure® Max Plus v2 kit is an in vitro diagnostic product for the detection of aneuploidies for chromosome 13, 18, 21, X and Y as well as detecting presence or absence of exon 7 of SMN1 and SMN2 genes for detecting 5q SMA in every fetal sample which is tested for chromosomal aneuploidies by QF-PCR. With AneuSure® Max Plus v2 Kit, amplification of 35 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 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® Max Plus v2 can be extracted from several sources: amniotic fluid (AF), chorionic villus (CVS), and blood. To prevent sample mix-up and to ensure the fetal sample's originality, we recommend that the sample from the mother be amplified and used as a reference (please follow national guidelines if available). Another advantage of this kit is the reliable detection of Turner syndrome (Monosomy of X) by using 7X and 18X markers, allowing the quantification of chromosome X as well as other sex chromosome markers.

1.2. AneuSure® Max 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 that 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® Max Plus v2 markers cover the whole length of 13, 18, 21, X, and Y chromosomes (Table 1). Marker heterozygosity and SNPs 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	7
Chromosome 18	7
Chromosome 21	8
X/Y	2
X chromosome	8
Y Chromosome	2

Table 2: Markers used in AneuSure® Max 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	234/247	11q22.3
				Xp11.21
19	D18S1002	18	250-310	18q11.2
20	DXS6803	X	315-344	Xq21.31
21	D21S1411	21	360-475	21q22.3
22	D13S797	13	115-155	13q33.2
23	DYS437	Y	160-200	Yq11.21
24	7X	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
29	D21S1437	21	100-160	21q21.1
30	D21S1435	21	166-225	21q21.3
31	D13S628	13	230-295	13q31.3
32	18/ X	18, X	315-340	18q12.1
				Xq21.1
33	D18S978	18	348-390	18q12.3
34	D13S742	13	400-493	13q12.12

- These sizes are obtained using ABI 3500xL Genetic Analyzer and GT600 internal size standard. Out of range can be observed in different populations. Validate the result for your instrument.
- AMXY and Y/X B are the sex determination markers that detect two homologous regions on X and Y chromosomes.
- 7X, 11X, and 18/X markers are segmental duplication regions shared between the X and 7, 11 or 18 and X chromosomes, respectively, and are 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 chromosomes. We cannot have simultaneous

monosomies for chromosomes X and 7 or X and 18 because a fetus with monosomy of chromosome 7 will not last to the first month of pregnancy, and a monosomy for chromosome 18. 18 shows aneuploidy for other chr. 18 markers. For Turner syndrome, one expects to see homozygosity for all X-related markers as well as reduced heights for chromosomes 7 and 18. A fetus with trisomy 18 and Turner syndrome will not viable, so it is not an issue during aneuploidy screening.

Table 3: Reliable detection of turner syndrome

	Normal female	Normal male	Turner	Trisomy 18
Height ratio of 7/X and 11/X markers	2:2	2:1	2:1	2:2 or 2:1
Height ratio of 18/X markers	2:2	2:1	2:1	3:2 (female) 3:1 (male)
X-STR markers	Homozygote or heterozygote	Hemizygote	Hemizygote	Homozygote or heterozygote
Y-STR markers	Not detected	Detected as a single band	Not detected	Not detected (female) Detected (male)

- DX-TATC 13.3 marker is located on the short arm of the chromosome X and does not exist in other commercial kits. It has been tested on several hundred samples and shows reasonable heterozygosity.
- Segmental duplications (SD) are used to support STR findings for aneuploidies. When aneuploidy is suspected, relevant SD marker should also show aneuploidy for the given chromosome.

1.3. Six-dye fragment analysis

ABI 3500, 3500xL and SeqStudio Genetek Analyzers (Applied Biosystems®) are recommended for 6-dye capillary electrophoresis of amplified products.

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

Name	6-FAM	GT2907	GT2712	GT1803	GT2107	GT600
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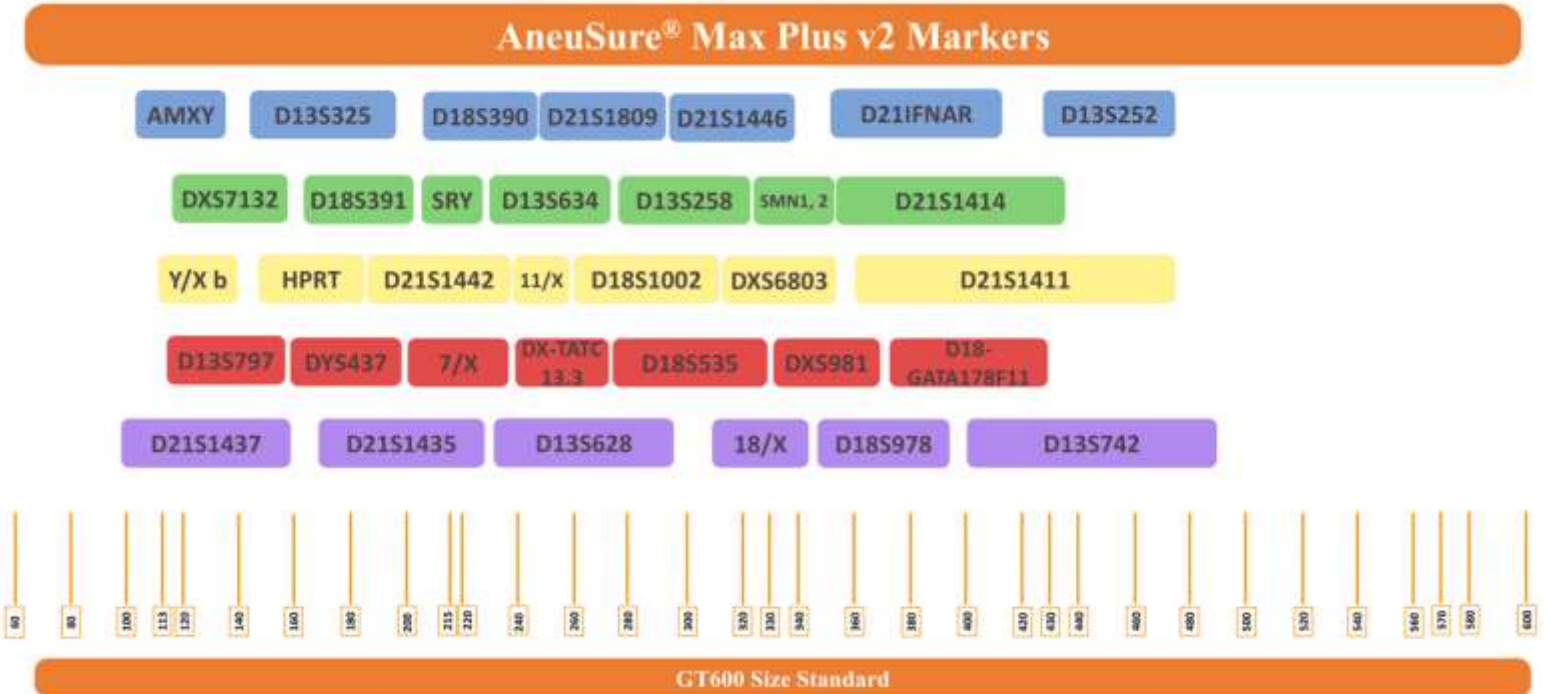


Figure 1. Diagram shows distribution and placement of AneuSure® Max Plus v2 Kit markers with GT600 Size Standard.

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 results may be obtained after the expiration date (12 months)








2.2. Materials and equipment

2.2.1. Laboratory conditions

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). GT600 Size Standard, GTM6 v2 Matrix Standard are amplicons and should be stored in a post-PCR area. In each run, a negative control should be added to determine the possible source of contamination. We recommend that DNA from each personnel working in the lab be profiled so that 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			BOX-B		
	Tube Label	Tube cap colour		Tube Label	Tube cap colour
1	PCR Mix		1	GT600 Size Standard	
2	Primer Mix		2	GTM6 v2 (Optional)	
3	GT HSTaq				
4	GT QCDF150(Control DNA-50ng/µl)				
5	GT QCW (H2O)				

Not provided with AneuSure® Max 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 (3500/xL) with Data Collection software for 5-dye system detection
- Applied Biosystems Genetic Analyzer (ABI 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
- GTM6 v2 Matrix Standard for Spectral calibration (GT-41104) (can be obtained from Genetek Biopharma)

2.2.3. QF-PCR Amplification by AneuSure® Max 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® Max Plus v2 components

Table 6: PCR reaction set-up

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

2.2.5. AneuSure® Max 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 μ L 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 μ l 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 3500/xL (Applied Biosystems®) Genetic Analyzers are recommended for 6-dye capillary electrophoresis of the amplified PCR products.
- Please make sure your ABI Data Collection software supports 6-dye fragment analysis (according to the instrument user manual).
- AneuSure® Max 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.
- PCR products can be injected into the capillary more than one time, or the results can be re-analyzed.

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

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

Attention:

Spectral Calibration must be made using GTM6 v2 Matrix Standard, the machine must be calibrated with GTM6 v2 Matrix Standard before using the kit. Please find detailed protocol for spectral calibration with GTM6 v2 Matrix Standard here - www.genetek-biopharma.com or contact us at support@genetek.de.

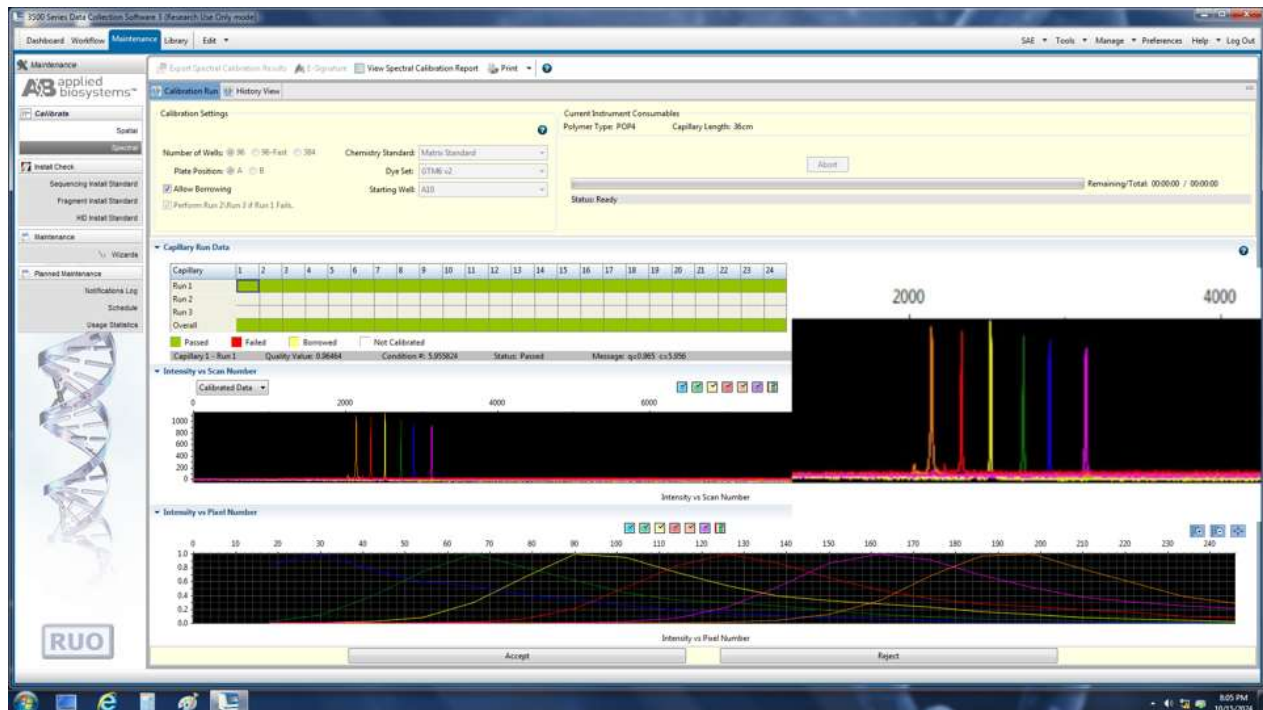


Figure 3. An example of a successful spectral calibration with GT 6-dye system on Applied Biosystems Genetic Analyzer 3500xL

- The Dashboard screen (Figure 4) 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 4. Dashboard of Applied Biosystems 3500 Data Collection software

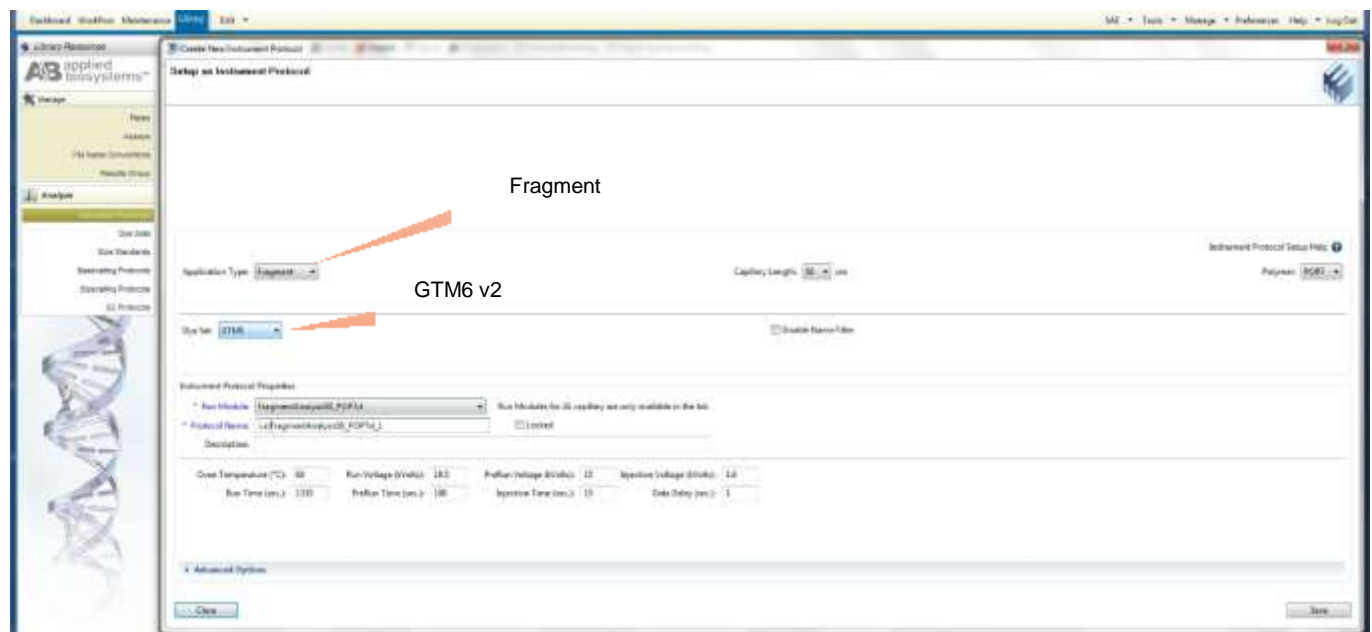


Figure 5. Screenshot for the “Create New Instrument Protocol” window on Applied Biosystems 3500 Data Collection software

- User can apply settings as shown in the Figure 5. Make sure that you select GTM6 v2 as a Dye Set (same name as was used to perform the GTM6 v2 spectral calibration).

Onset of first analysis of AneuSure® Max Plus v2 system, 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 5)

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 labs 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 6)

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 “GT600” and as Dye Color select “Orange”.

The fragments size in the GT600 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, 500, 520, 540, 560, 570, 580, and 600 bp.

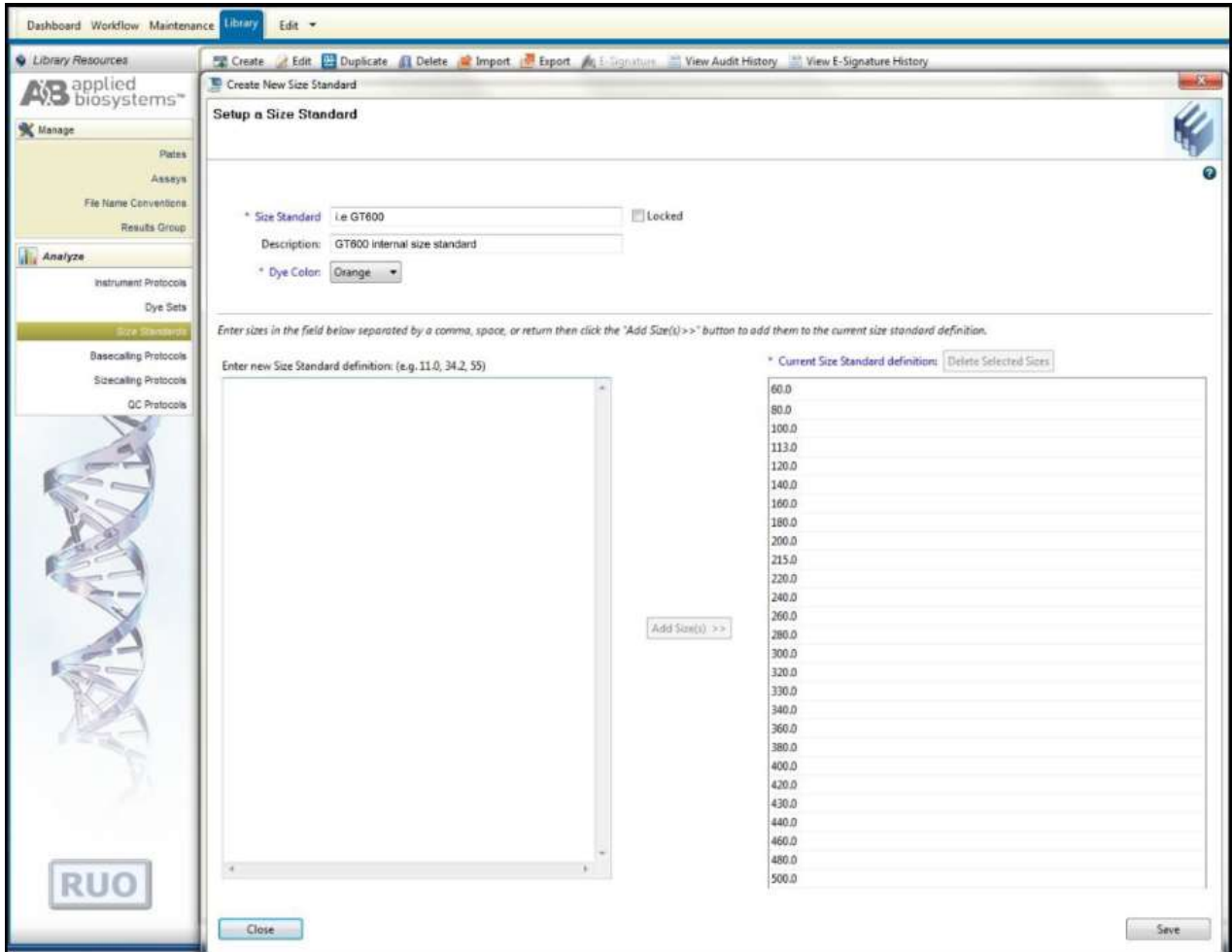


Figure 6. Screenshot for the “Create New Size Standard” window on Applied Biosystems 3500 Data Collection software

3.1.3. Create a QC protocol

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

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. *GT600*” and select the *Size Standard* “GT600”

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

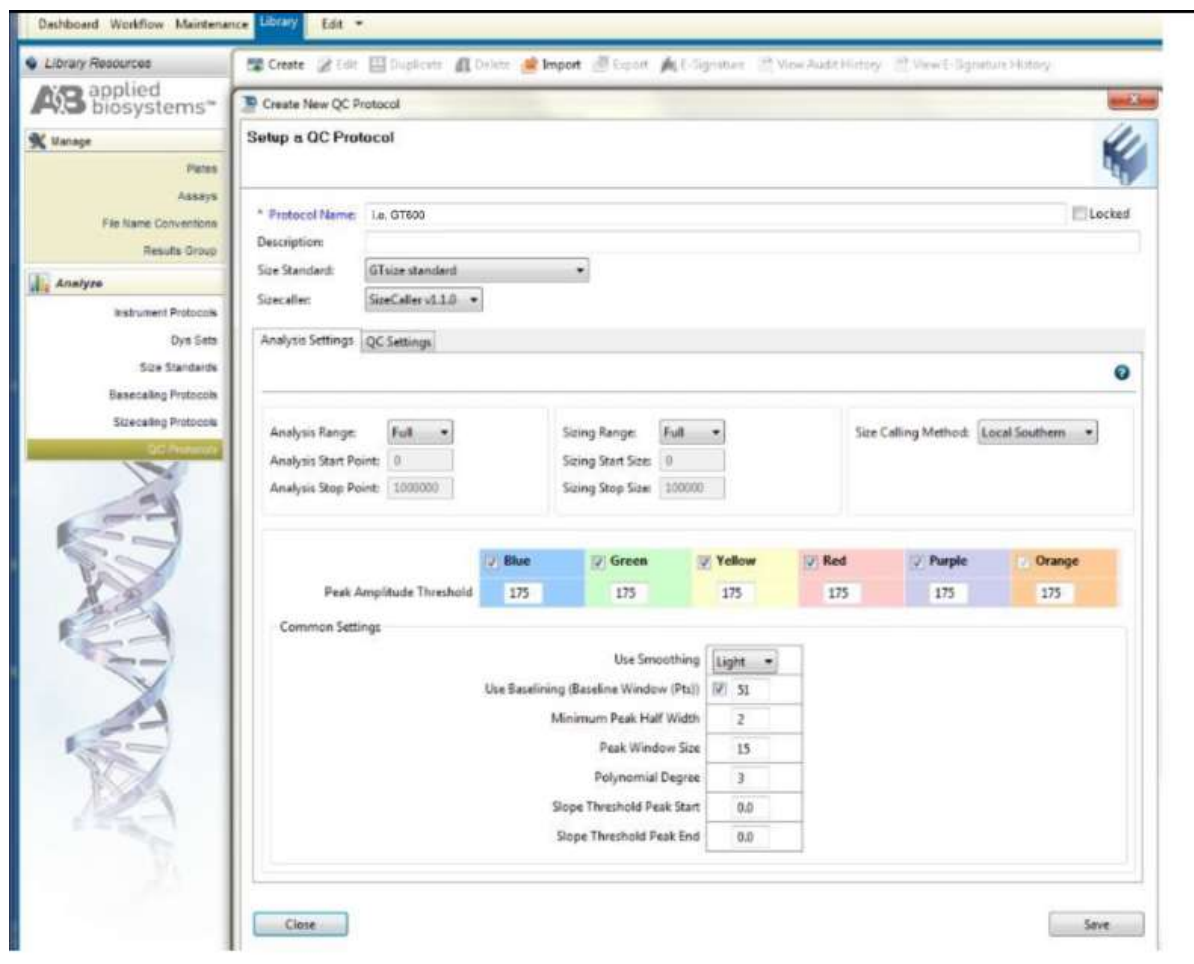


Figure 7. Screenshot for the “Create New QC Protocol” window on Applied Biosystems 3500 Data Collection software

3.1.4. Create a new Assay

- a) Navigate to the Library
- b) Select “Assays”
- c) Select “Create” (Figure 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) In the *Create New Assay window*, as shown in Figure 8, 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.

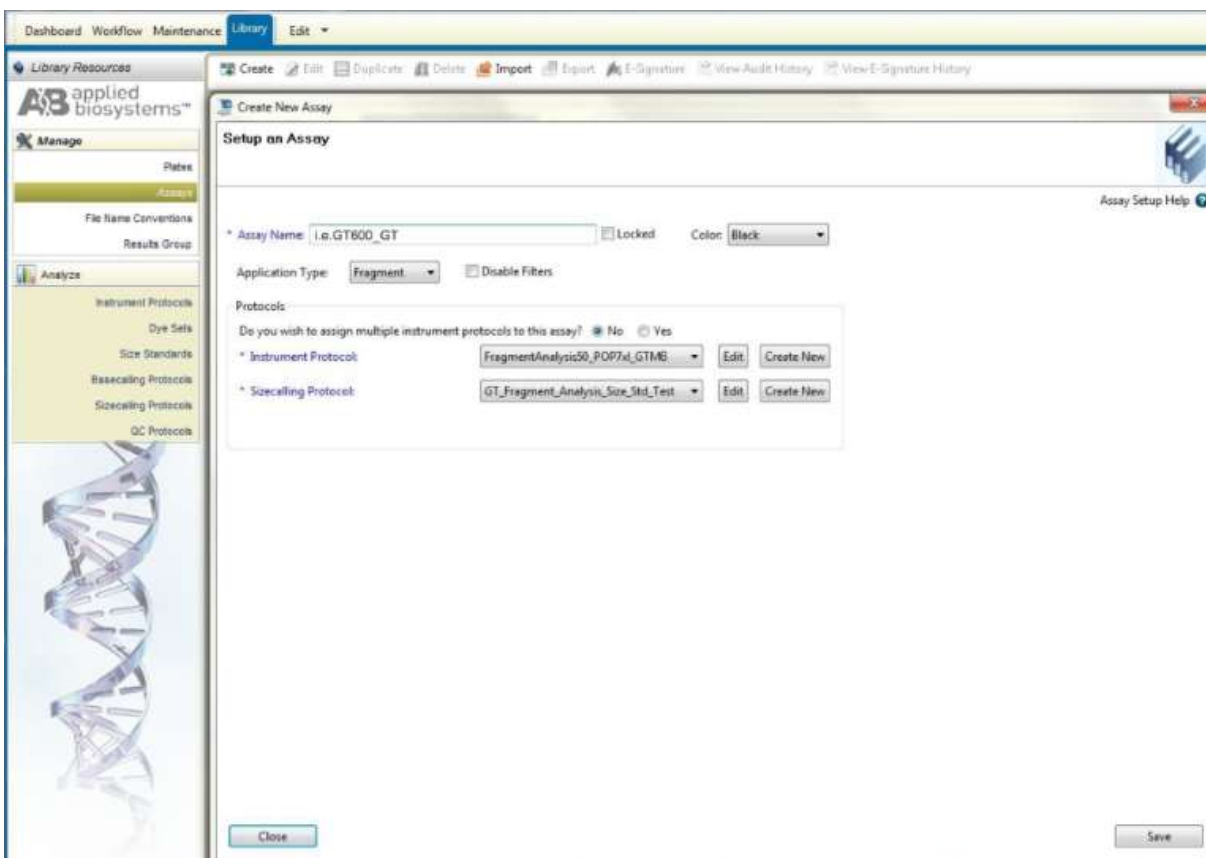


Figure 8. Screenshot for the “Create New Assay” window on Applied Biosystems 3500 Data Collection software

3.1.5. Create a new File Name Conventions

- a) Navigate to the Library
- b) Select “File Name Conventions”
- c) Select “Create” (Figure 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 File Name Attributes according to your lab practices

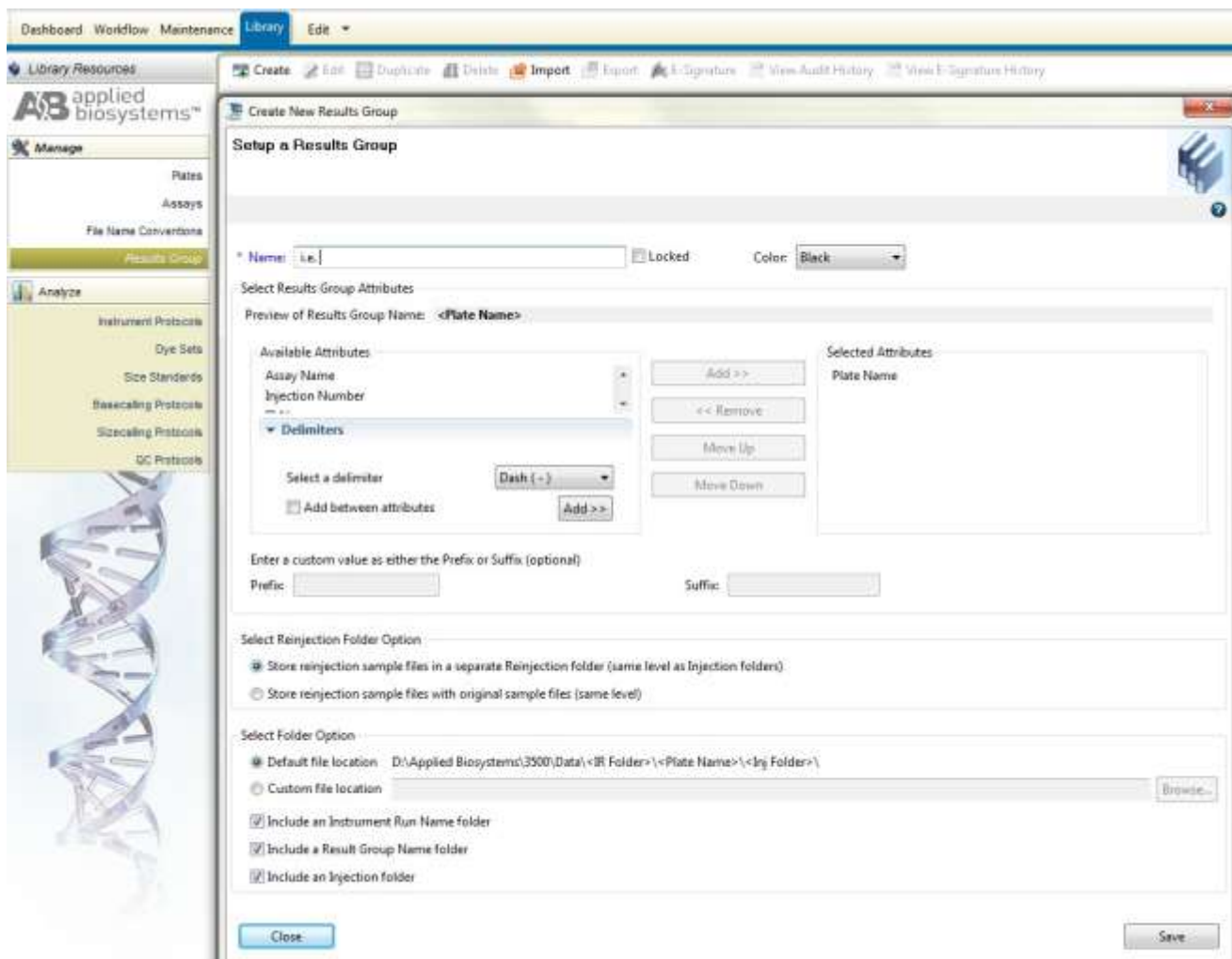


Figure 9. Screenshot for the “Create New File Name Convention” window on Applied Biosystems 3500 Data Collection software.

3.1.6. Create a new Result Group

- a) Navigate to the Library
- b) Select “Results Group”
- c) Select “Create” (Figure 10)

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

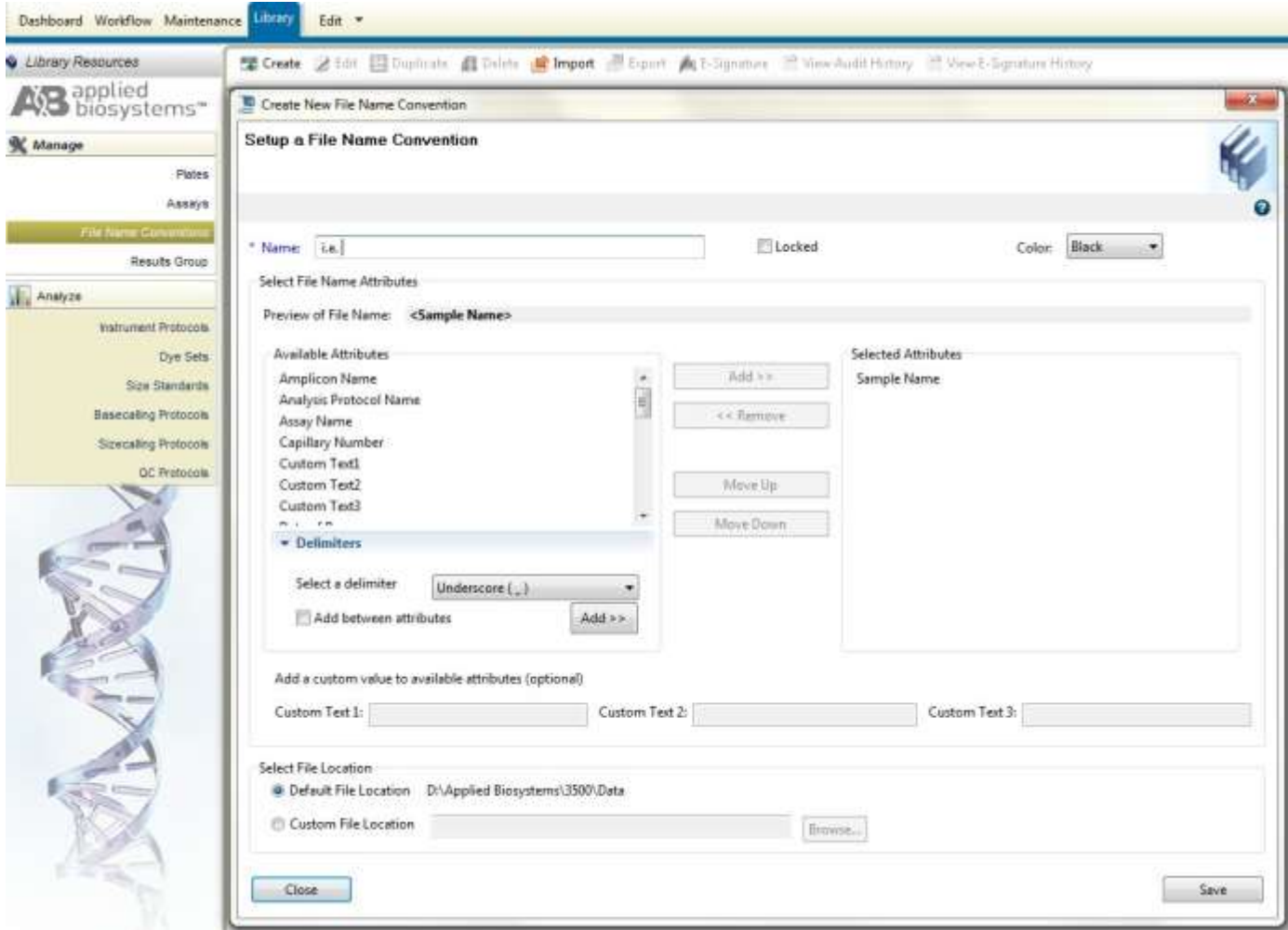


Figure 10. Screenshot for the “Create New Result Group” window on Applied Biosystems 3500 Data Collection software

3.1.7. Create a New Plate

- a) Navigate to the *Library*
- b) From the manage menu select “Plates”
- c) Select “Create” (Figure 11)
- d) Define a name for the plate
- f) Choose plate type “*Fragment Analysis*” from the drop-down menu



Figure 11. Screenshot for the “Defining plate properties” window on Applied Biosystems 3500 Data Collection software.

3.1.8. Select “Assign Plate Contents”

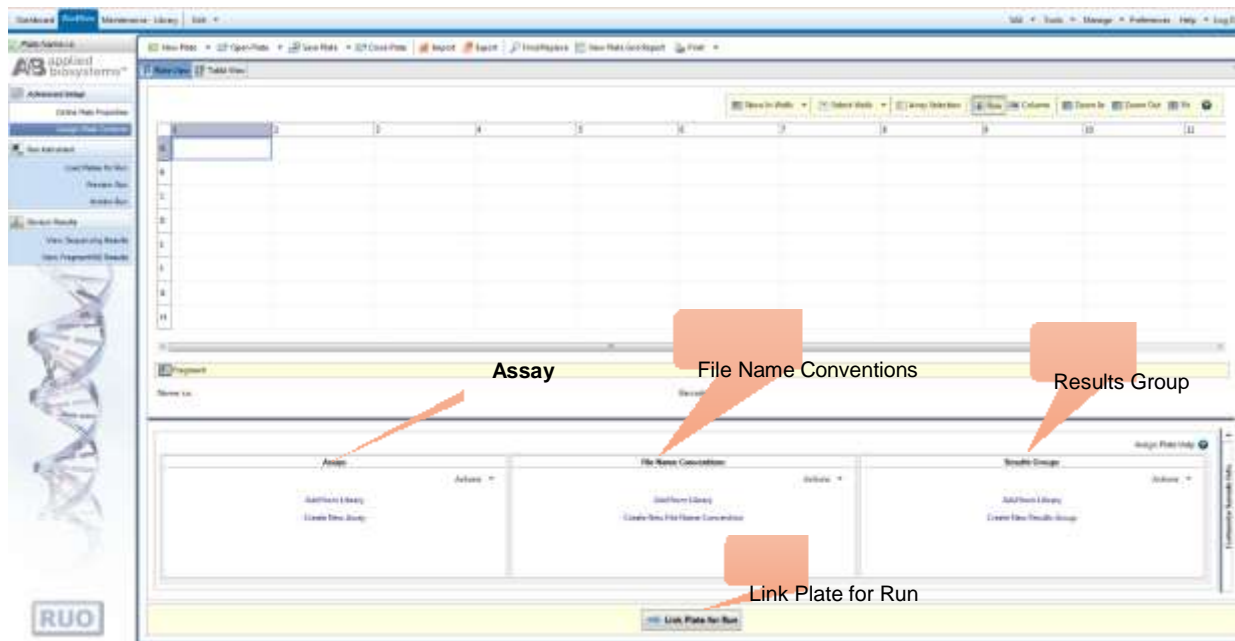


Figure 12. Screenshot for the “Assign Plate Contents” window on Applied Biosystems 3500 Data Collection software

- Define sample names to wells.
- In the *Assign Plate Window* (Figure 12), 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 are 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 13). Select “Start Run” after loading the plate.

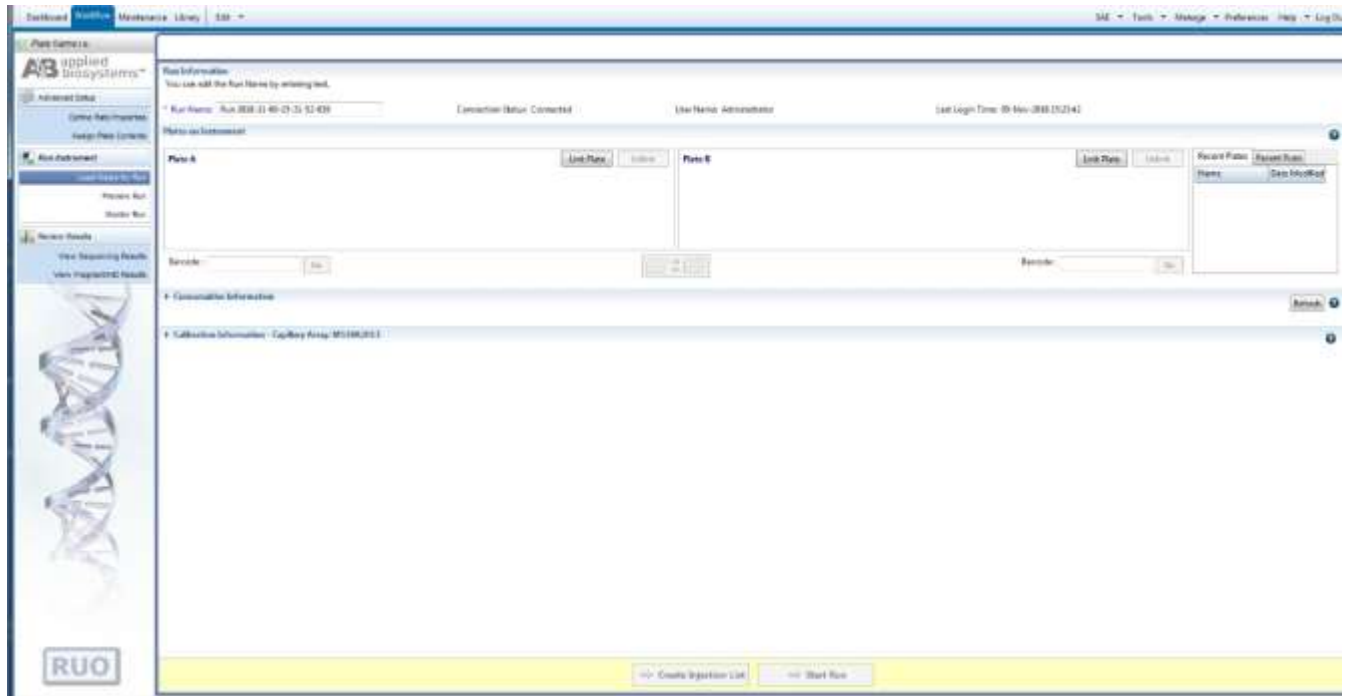


Figure 13. Screenshot for the “Run Information” window on Applied Biosystems 3500 Data Collection software

3.2. Sample preparation for capillary electrophoresis (3500 Series instruments)

Please note: The Size Standard used in the AneuSure® Max Plus v2 kit is GT600.

- Vortex and spin 9 µl (X number of samples) Hi-Di™ Formamide and 1 µl GT600 (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
- Pipette 10 µl of the prepared size standard mix to the required number of well and add 0.2-0.5 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 GT600 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).
- In a multi-capillary Analyzer, injections take place simultaneously on all the capillaries. Therefore, 1 entire column (8 samples) or 3 entire columns (24 samples) must be pipetted onto the plate. If fewer samples are to be analysed, the empty well positions must be filled with 10µl Hi-Di Formamide.
- To get reliable allele call, inject at least one allelic ladder for each set of 24 samples (one allelic ladder per injection for 24-capillary instrument or one allelic ladder per 3 injections for 8-capillary instrument).
- Actual room temperature may influence the performance of the multi-capillary instrument which could result into shoulder peaks or split peaks, so make sure that the ambient conditions are maintained as described by the instrument manufacturer.

4. Result analysis and Interpretation

4.1. Software for sample analysis

- For AneuSure® Max Plus v2 , the Applied Biosystems fragment analysis software compatible with your genetic Analyzer is recommended. This kit is compatible with GeneMapper® *ID-X* software. Analysis method depends on the software version.

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® Max Plus v2 results

AneuSure® Max Plus v2 PCR products are observed with 6-dye system on an electropherograms in the GeneMapper® *ID-X* software. For the analysis, import AneuSure® Max 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® *ID-X* 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 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 between 100 to 600 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.

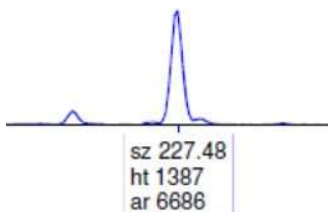


Figure 21. Allele plots generated with GeneMapper *ID-X* software for homozygous peak

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.

Note: we have to know that the larger fragment height is usually a small fraction smaller than the shorter fragment. The longer the second fragment, the more this difference would be. So when interpreting results one has to take this into account.

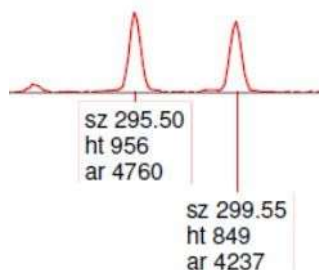
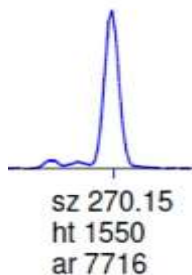


Figure 22. Allele plots generated with GeneMapper *ID-X* software for heterozygous peak. It is known that for the same locus, the peak height for the longer repeat size is proportionally smaller than the smaller size (299.55 vs 295.50). So, one has to be careful when trying to see if di-allelic trisomy 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.



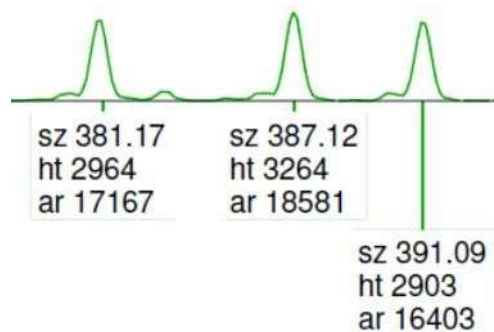
Figures 23. Allele plots generated with GeneMapper *ID-X* software, peak height and area are shown in the label

- Result for a sample with normal copy number for a chromosome will show homozygous or heterozygous pattern for all STR markers in the AneuSure® Max 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 *ID-X* software for tri-allelic trisomy, peak height and area are shown in the label. Usually alleles heights should be close to 1:1:1 ratio.

- **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.

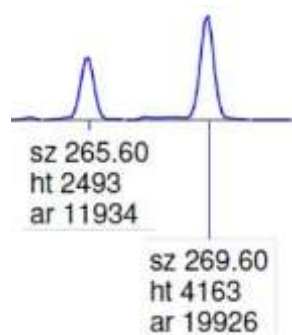


Figure 25. Allele plots generated with GeneMapper *ID-X* software for di-allelic trisomy, peak height and area are shown in the label

- 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 inserted more chromosome specific markers for each chromosome to circumvent re-testing with chromosome specific markers.

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 including 7/X and 18/X SD markers.
- 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[®] Max Plus v2 Kit is a segmental duplication marker which means it represents sequence from both 7 and X chromosomes. The same is true about 18/X marker. This is another SD marker. These markers are extremely useful in case of Turner syndrome and in case of Monosomy. See the example profile for Turner Syndrome generated using AneuSure[®] Max Plus v2 on page 33.

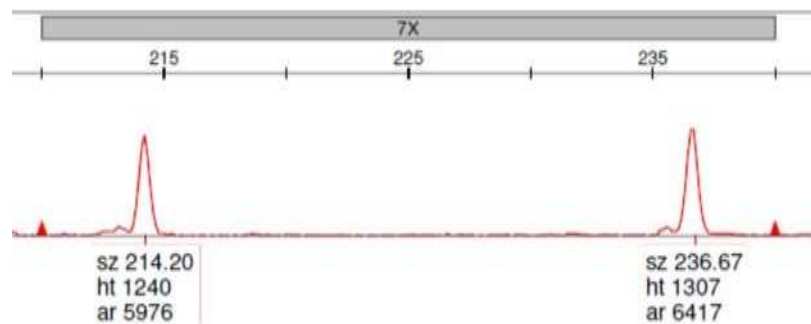


Figure 26. Normal male 7X or monosomy X – height ratio is ≥ 1.8

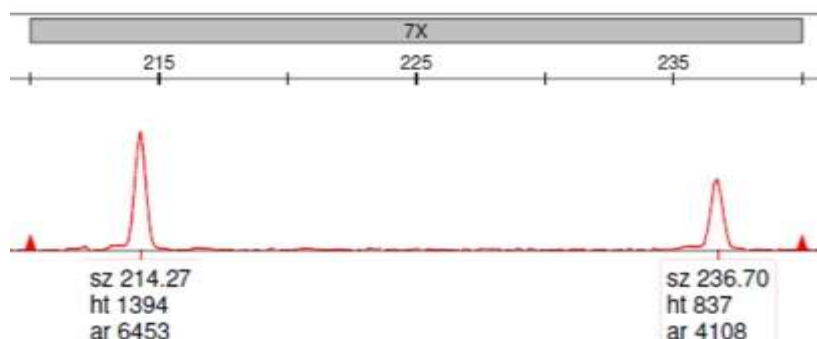


Figure 27. Normal female 7X – height ratio is 0.8 to 1.4 which are seen when the sample is from a male or a female affected with Turner Syndrome.

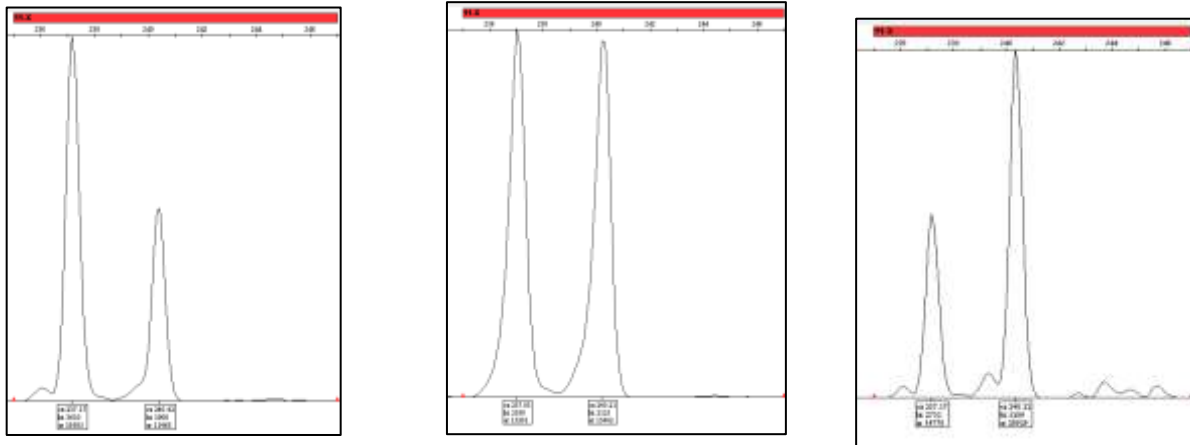


Figure 28. The figure on the left shows a normal male 11/X or monosomy X (Turner Syndrome if the sample is from a female) – height ratio should be $\geq 1.8:1$ since there are two chromosome 7 and one chromosome X. The figure in the middle shows a normal female sample (almost 1:1 ratio). The figure on 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 Normal Female profile (GT QCDF150 included in all AneuSure® Max Plus v2 Kit)



Figure 29. Example of normal female profile generated using AneuSure® Max Plus v2 kit. No aneuploidy is seen. The SMA markers are present, indicating that this sample is not affected with SMA. The data has been generated with GeneMapper *ID-X* software and 3500xL Genetic Analyzer.

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



Figure 30. An example of a fetal sample with no aneuploidy but his (since AMXY and other sex chromosome markers as well as 7X segmental duplication indicates so) DNA does not show any aneuploidy. However, we only see SMN2 fragment. Therefore, he is most probably affected with SMA. MLPA result indicated that the sample was indeed affected with SMA (data not shown). The profile has been generated with GeneMapper *ID-X* software and 3500xL Genetic Analyzer.

5.2. Trisomy 21 – ‘Down Syndrome’

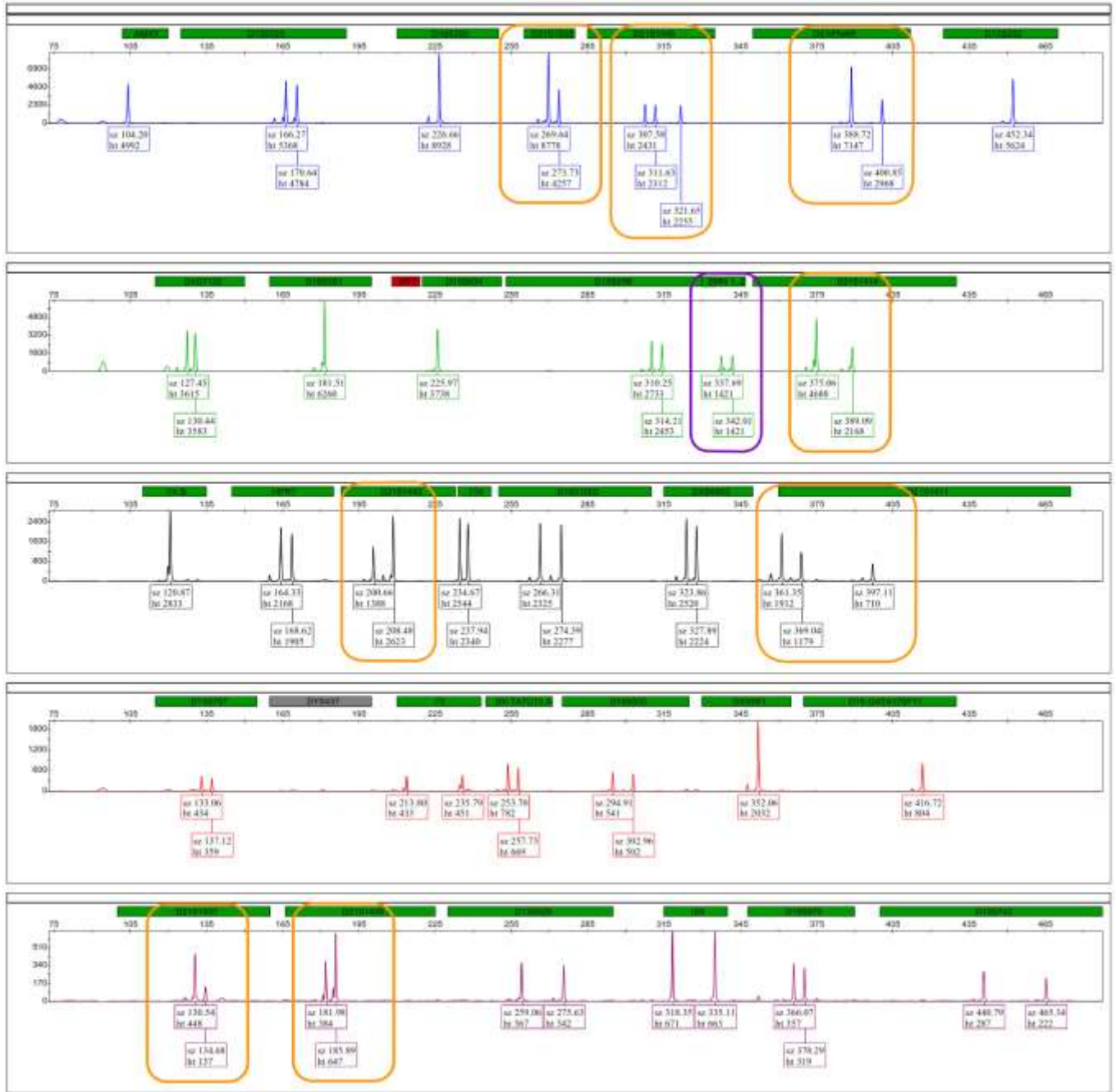


Figure 31. Example of a Down Syndrome profile generated using AneuSure® Max Plus v2 kit. Makers indicating trisomy of chromosome 21 are boxed. The data has been generated using GeneMapper ID-X software and 3500xL Genetic Analyzer.

5.3. Trisomy 18 – ‘Edward Syndrome’

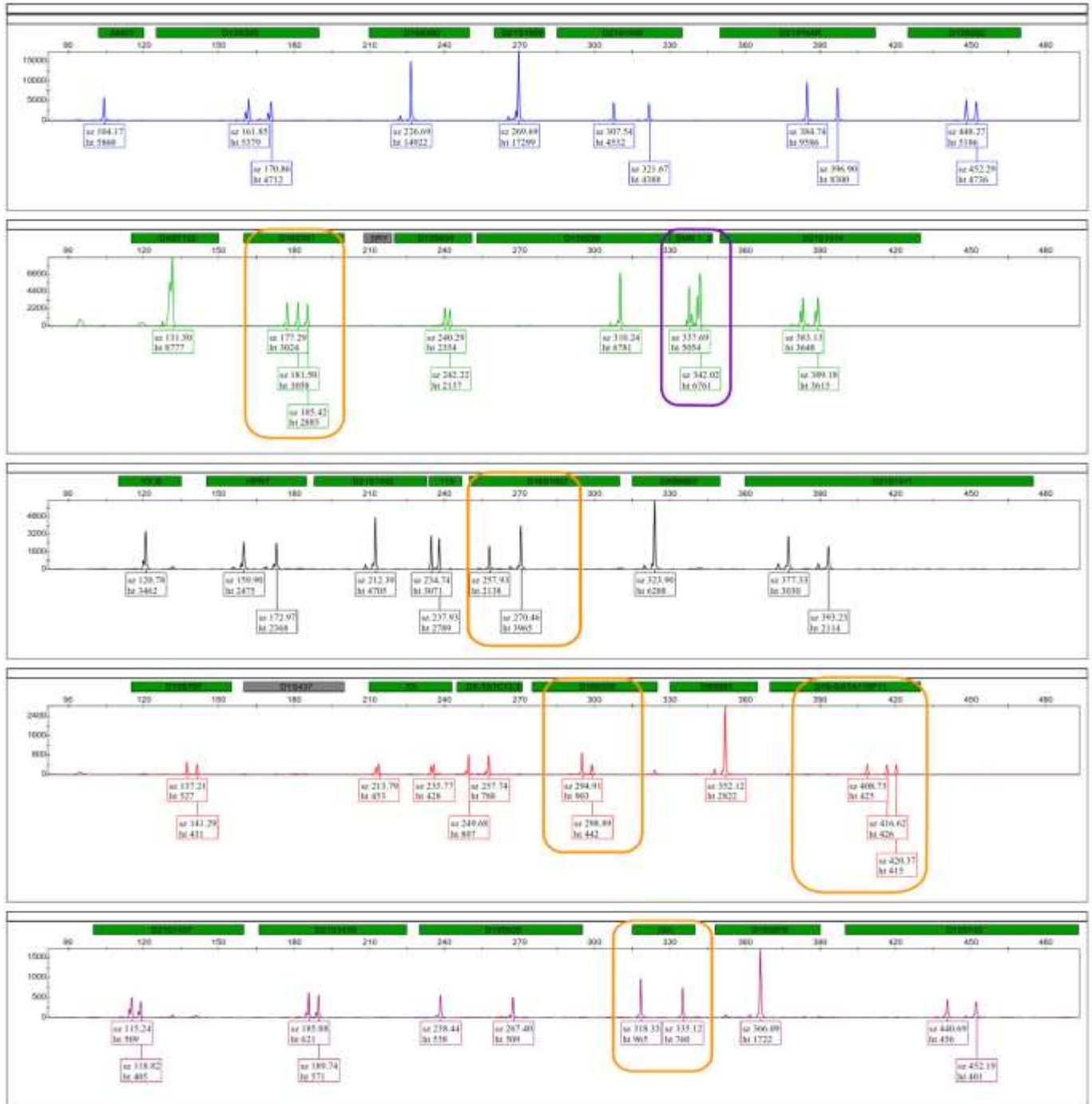


Figure 32. Example of Edward Syndrome profile generated using AneuSure® Max Plus v2 kit. Markers indicating trisomy of chromosome 18 are boxed. The data has been generated using GeneMapper *ID-X* software and 3500xL Genetic Analyzer.

5.4. Trisomy 13 – ‘Patau Syndrome’

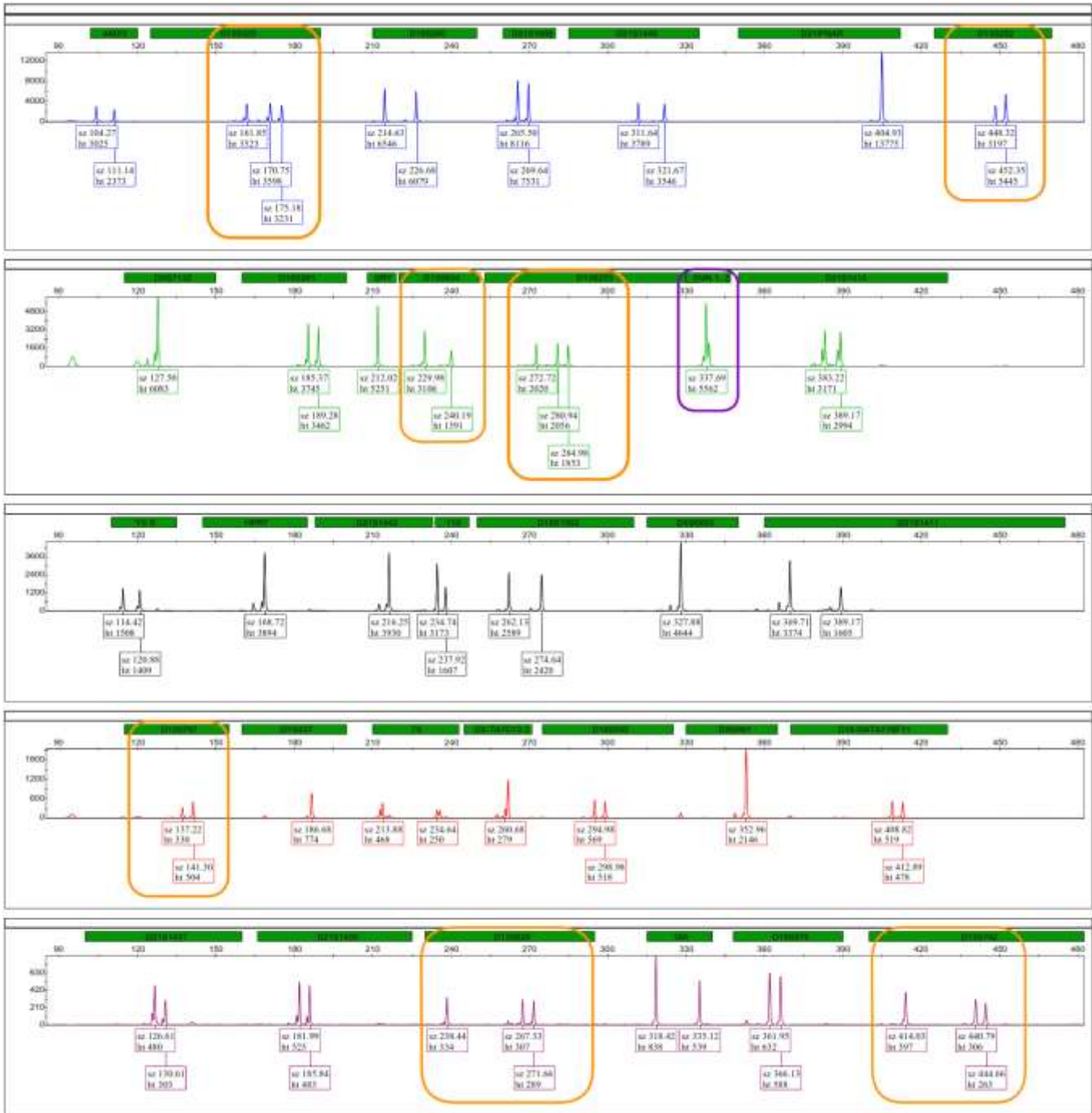


Figure 33. Example of Patau Syndrome profile generated using AneuSure® Max Plus v2 kit. Makers indicating trisomy of chromosome 13 are boxed. The data has been generated using with GeneMapper *ID-X* software and 3500xL Genetic Analyzer.

5.5. Klinefelter Syndrome (XXY)

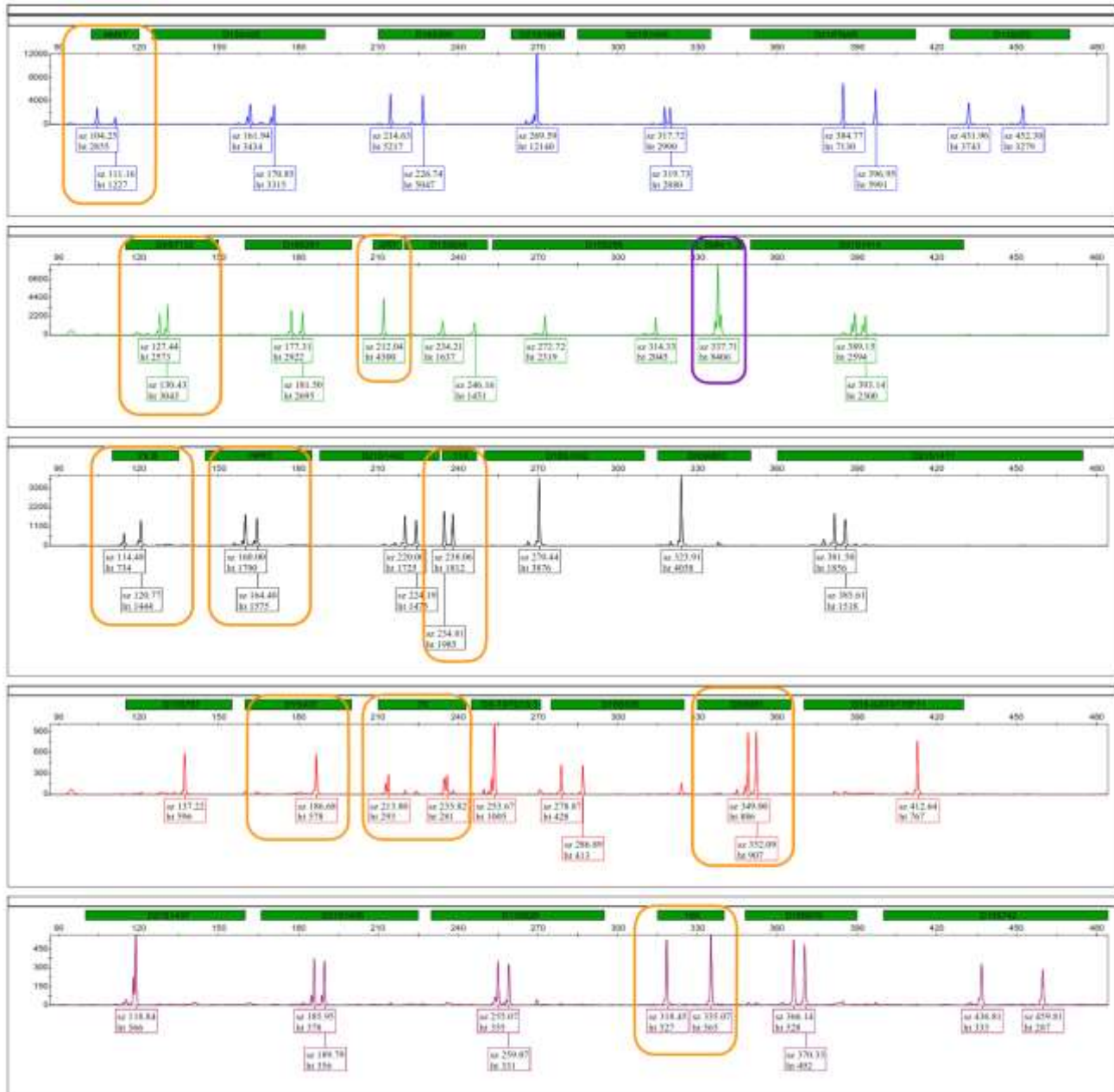


Figure 34. Example of Klinefelter Syndrome profile generated using AneuSure® Max Plus v2 kit. Markers indicating Klinefelter Syndrome are boxed. The segmental duplication makers (i.e., 7/X, 11/X and 18/X) also shows imbalanced results indicating two copies of chromosome X and one copy for chromosome Y. The AMXY, YX B and SRY markers indicate a male sample. The data has been generated using with the GeneMapper *ID-X* software and 3500xL Genetic Analyzer.

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.</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® Max 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 GT600 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® Max 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 seems 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 GTM6 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 GT600 Size Standard.
 - b) Incorrect size standard is used. We recommend using GT600 with AneuSure[®] Max Plus v2 Kit to obtain optimum results.
-

7. Limitations and Disclaimer

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

AneuSure[®] Max Plus v2 Kit is designed to detect only 21, 18 and 13 chromosomes specific trisomies and sex-chromosome aneuploidy. 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[®] Kit is for Research Use Only and user bears all the responsibility for its use in clinical practice.
Max Plus[®]
v2

Please consult best practice guidelines when using any QF-PCR kits including AneuSure[®] Kit.
Max Plus v2

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):285-288.