



GT AZFScreen Plus v2

User Manual

CAT# GT-11304

A kit for the
Detection of Y Chromosome Microdeletions and
Klinefelter Syndrome

Produced by

GENETEK BIOPHARMA GmbH

Research Use Only

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1. GT AZFScreen Plus v2 Overview

- Accurate detection of Y chromosome microdeletions (AZFa, AZFb and AZFc) and Klinefelter syndrome based on the latest recommendation by the **EAA/EMQN** Best Practice Guidelines for Molecular Diagnosis of Y-chromosomal Microdeletions: State of the art 2023, by Krausz et. al., 2023 ([DOI: 10.1111/andr.13514](https://doi.org/10.1111/andr.13514)).
- Can be used on extracted DNA from blood or blood on DNA banking card (DBC™) or diluted blood using GT Blood Diluting buffer.
- Accurate detection of Klinefelter syndrome in infertile men
- Multiplex amplification of 36 markers in a single reaction
- 5-dye fragment analysis using capillary electrophoresis
- Analyzed using 5-dyes capillary electrophoresis system. Compatible with the Compact Spectrum CE System from Promega and Applied Biosystems™ 3130/xl, 3500, 3500/xl, SeqStudio platforms.

1.1. Intended Use

The GT AZFScreen Plus v2 kit is an in vitro diagnostic product for the detection of Y chromosome microdeletions (AZFa, AZFb, and AZFc). It can also be used for the detection of Klinefelter syndrome.

1.2. GT AZFScreen Plus v2 Markers

The kit includes 26 STS (Sequenced Tagged Sites), 3 SD (Segmental Duplication), and 7 X-STRs (Short Tandem Repeat), in total 36 markers. GT AZFScreen Plus v2 kit is developed for the detection of Y chromosome microdeletions – a frequent genetic cause of infertility in men. It can also detect Klinefelter syndrome, another cause of infertility in men, though we have a specific kit named AZFScreen Plus for the detection of Klinefelter syndrome.

STS or a Sequence-tagged site is a unique 200-500 base pair DNA sequence site in the genome with a single occurrence. It's easy to amplify with PCR when specific primers are used and hence STS markers are used as markers to locate the specific positions (deletions, mutations etc.) in the genome.

SDs or Segmental Duplications are typically of 1 to 400 kb length DNA sequences recurring at multiple sites within the genome. Studies have shown correlation between segmental duplications and regions of chromosomal uncertainty, due to the recurrent rearrangement by nonallelic homologous recombination in these regions, SDs are landmarks for deletion, duplication or inversion of original genome sequences.

The GT AZFScreen Plus v2 kit consists of 36 markers amplifying 40 loci (includes 8 segmental duplication loci, two for each of AMXY, X/Y b, ZF Y/X and 11/X) (Table 1) which are designed and selected specifically according to EAA/EMQN 2023 [guideline](#) for AZF and others for sex typing and quantifying the X chromosome content for Klinefelter Syndrome. These markers include: sY82, sY84, sY86, sY88, sY625, M259, sY1064, sY1065, and sY1182 for the *AZFa* region; sY105, sY121, sY127, sY131, sY130, sY134, sY153, sY1192, sY1191 and sY1224 markers for the *AZFb* region; sY254, sY255 and sY157 markers are for the *AZFc* region and sY1191 for the gr/gr region. There are three internal control markers SY90, ZFX/Y, and sY14 (SRY). The sY160 marker is for the Heterochromatin region. The proximal breakpoint markers for the *AZFa* are sY82 and sY1064 and the distal breakpoint markers are sY1182 and sY88. The proximal breakpoint markers for the *AZFb* are SY105 and sY121/sY1224, and the distal breakpoint markers are sY1192 and sY153. The essential marker for the *AZFc* has been recommended to be sY160.

The AMXY, X/Y b, ZF Y/X and 11/X and X-chromosome STR markers (i.e., DXDMDSU1.6, DXS10101, DXS8377, DXS7132, DXDMDS13.3, DXS981, DXS6809) can aid the detection of Klinefelter syndrome by giving two peaks for each segmental duplication markers. Each of these markers' heights should be twice the other one since in Klinefelter males, there are two X chromosomes and one Y chromosome. Therefore, the AMXY, X/Y b, and ZF Y/X loci in a male sample should show twice the height for the X chromosome than for the Y chromosome. As we know, the XYB, AMXY, and ZF Y/X markers amplify locations that are common between the X and Y chromosomes. So, a normal male is expected to have a 1:1 ratio between the height of peaks for these markers, indicating the same number of X and Y chromosomes. But in a Klinefelter male, a 2:1 ratio between the heights of the peaks for these two markers indicates the existence of two X and one Y chromosomes. The same argument is valid for 11/X loci. The X chromosome-specific STR markers can aid sample authenticity, when most of these loci are informative in a Klinefelter male.

For a full understanding of Y-chromosome microdeletion, its interpretation, the science behind each marker, and how to interpret its presence or absence along with other markers, we strongly recommend reading and understanding the EAA/EMQN 2023 recommendation. It can be downloaded from [here](#).

Table 1: The Markers used in the GT AZFScreen Plus v2 kit.

No.	Markers/Dyes	Chromosome	Size Range	Chr. Location
1	sY153 (AZFb)	Y	90-110	Yq11.223
2	sY1191 (AZFb)	Y	115-135	Yq11.223
3	DXDMDSU1.6	X	148-190	Xp21.2
4	sY127 (AZFb)	Y	195-225	Yq11.223
5	sY157 (AZFc)	Y	250-265	Yq11.223
6	sY625 (AZFa)	Y	272-292	Yq11.221
7	sY1224 (AZFb)	Y	293-313	Yq11.222
8	DXS10101	X	315-375	Xq26.3
9	sY105 (AZFb)	Y	383-403	Yq11.222
10	sY1192 (AZFb)	Y	450-470	Yq11.223
11	Y/X b	Y, X	110-130	Yp11.2
				Xq21.31
12	sY90 (internal control)	Y	150-170	Yq11.221
13	sY121 (AZFb)	Y	197-217	Yq11.222
14	sY160 (Heterochromatin)	Y	260-280	Yq12
15	sY1064 (AZFa)	Y	295-310	Yq11.21
16	M259 (AZFa)	Y	350-375	Yq11.221
17	SRY(SY14)	Y	385-400	Yp11.2
18	sY130 (AZFb)	Y	80-100	Yq11.223
19	AMXY	X, Y	105-125	Xp22.2
				Yp11.2
20	sY86 (AZFa)	Y	170-190	Yq11.221
21	ZFY/X	X, Y	191-211	Yp11.2
				Xp22.11
22	sY84 (AZFa)	Y	220-240	Yq11.221
23	11/X	11, X	235/247	11q22.3
				Xp11.21
24	sY82 (AZFa)	Y	270-290	Yq11.21
25	sY1065 (AZFa)	Y	305-325	Yq11.221
26	sY254 (AZFc)	Y	330-350	Yq11.223
27	sY134 (AZFb)	Y	385-400	Yq11.223
28	DXS8377	X	430-490	Xq28
29	sY255 (AZFc)	Y	95-115	Yq11.223
30	DXS7132	X	150-200	Xq11.2
31	sY131 (AZFb)	Y	205-225	Yq11.223
32	DXDMDS13.3	X	235-280	Xp21.2
33	sY1182 (AZFa)	Y	295-310	Yq11.221
34	DXS981	X	345-380	Xq13.1
35	sY88 (AZFa)	Y	385-400	Yq11.221
36	DXS6809	X	438-485	q21.33

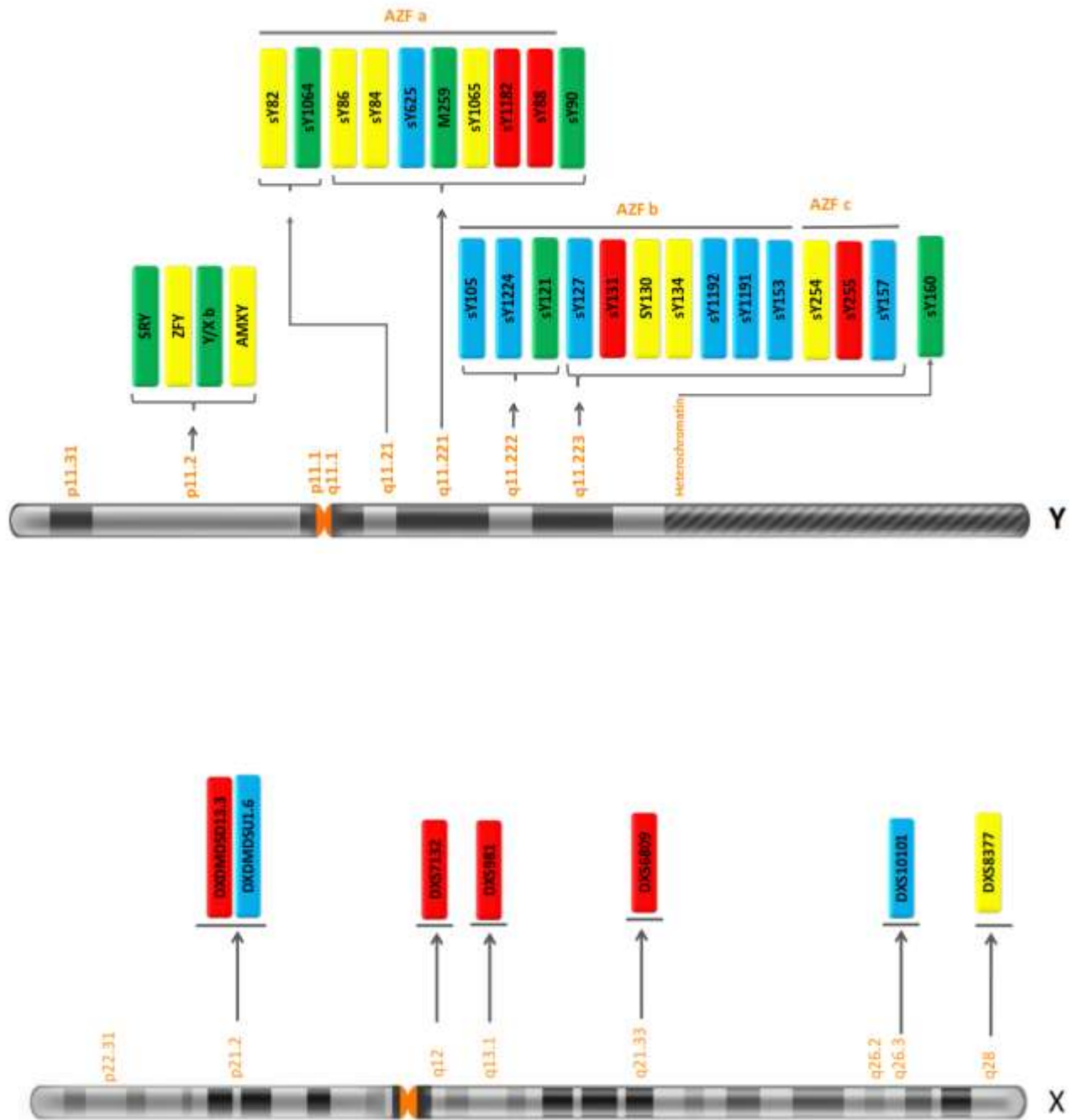


Figure 1. GT AZFScreen Plus v2 markers' distribution on human X and Y Chromosomes.

1.3. Five-dye fragment analysis

ABI 3130, 3130xl, and 3500 and 3500xL Genetic Analyzer (Applied Biosystems®) are recommended for the 5-dye capillary electrophoresis of amplified products.

Table 2: The fluorescent dyes used in GT AZFScreen Plus v2 kit

vv

Name	6-FAM	GT2907	GT2712	GT1803	GT500
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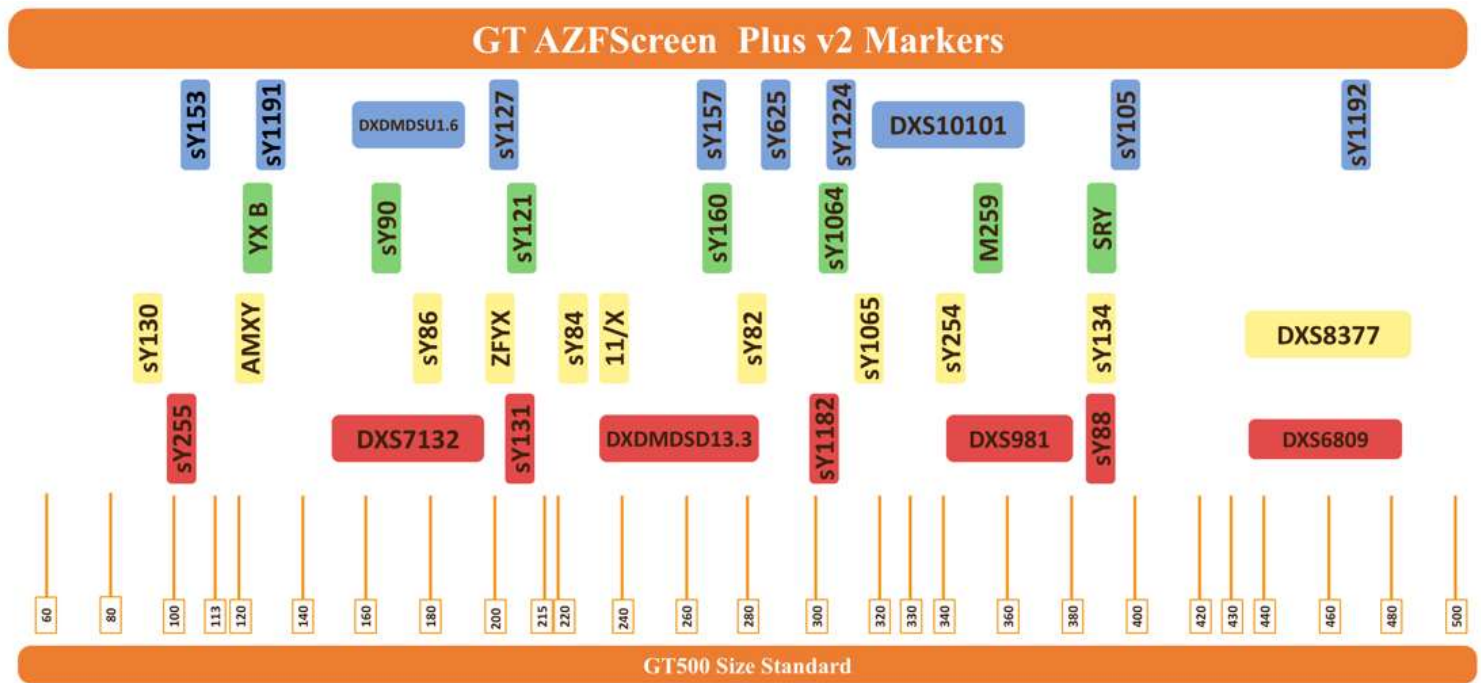


Figure 2. The diagram shows the distribution and placement of the GT AZFScreen Plus v2 Kit markers with GT500 Size Standard. There are 36 markers amplifiable in a single multiplex PCR.

2. PCR

2.1. Storage Condition

- Store at -20 °C
- Keep the primer mix in a dark place (because of fluorescently labeled 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 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 and the GTM5 v2 Matrix Standard are amplicons and should be stored in the post-PCR area. In each run, negative control should be added to determine the possible sources 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 3: Provided with the Kit in Box A and Box B. They should be kept separately. Box A in one freezer and Box B is in another freezer (PCR product)

BOX-A			BOX-B		
	Tube Label	Tube cap colour		Tube Label	Tube cap colour
1	PCR Mix		1	GT500 Size Standard	
2	Primer Mix		2	GTM5 v2 (Optional)	
3	GT HSTaq				
4	GT QCDM102 (Control DNA-50ng/μl)				
5	GT QCW (H2O)				

Not provided with the GT AZFScreen Plus v2 (but are needed)

- Reagents and equipment for DNA extraction
- Equipment and consumables for amplification (i.e., Thermal Cycler, Micropipette, Filter Tips, etc.)
- Applied Biosystems Genetic Analyzer (ABI 3130/xI 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. PCR amplification by GT AZFScreen Plus v2

- DNA can be extracted from blood. This kit also works for blood samples on filter paper such as DNA Banking Card (DBC™). 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. GT AZFScreen Plus v2 components

Table 4: 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. GT AZFScreen 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 5: 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	5-60 min
27-30 Cycles					

- After completion of PCR, store the PCR products at in a refrigerator 4-8°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 for running on the Genetic Analyzer at later days.
- 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 QC DM102 as a Quality 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).
- GT AZFScreen Plus v2 Kit is validated using 50 cm capillary array and POP7 as well as on 36 cm array and POP4 using 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 GT AZFScreen 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.

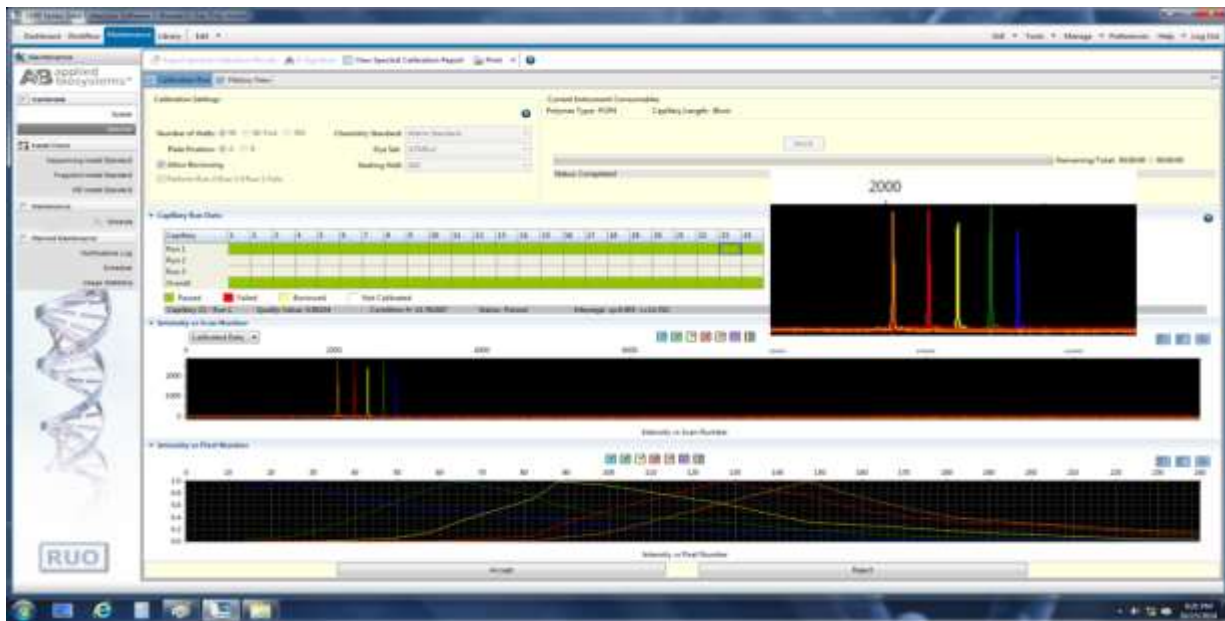


Figure 3. An example of a successful spectral calibration with GT 5-dye system on Applied Biosystems Genetic Analyzer 3500xL

- 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 the Oven Temperature and Detection Cell Temperature numbers turn green.



Figure 4. Dashboard of Applied Biosystems 3500 Data Collection software

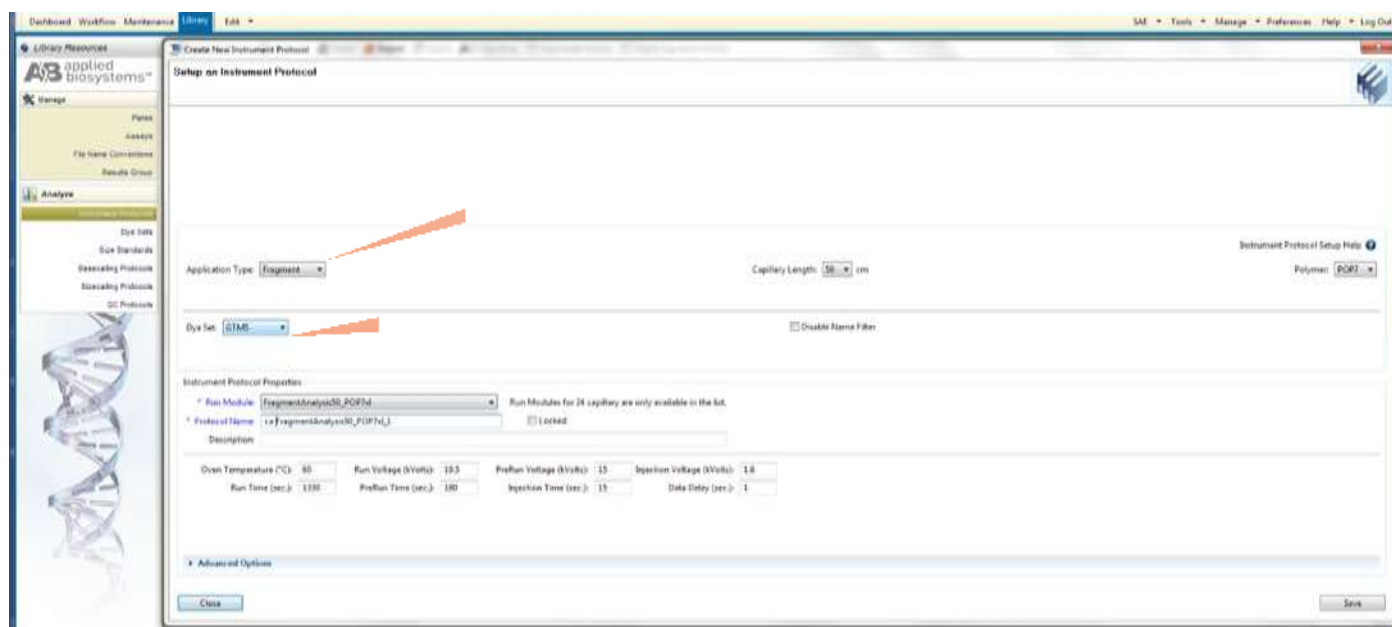


Figure 5. Screenshot for the “Create New Instrument Protocol” window on Applied Biosystems 3500 Data Collection Software.

- User can apply settings as shown in the Figure 5. Make sure that you select GTM5 v2 as a *Dye Set* (same name as was used to perform the GTM5v2 spectral calibration).

Onset of first analysis of GT AZFScreen 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 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 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 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 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.

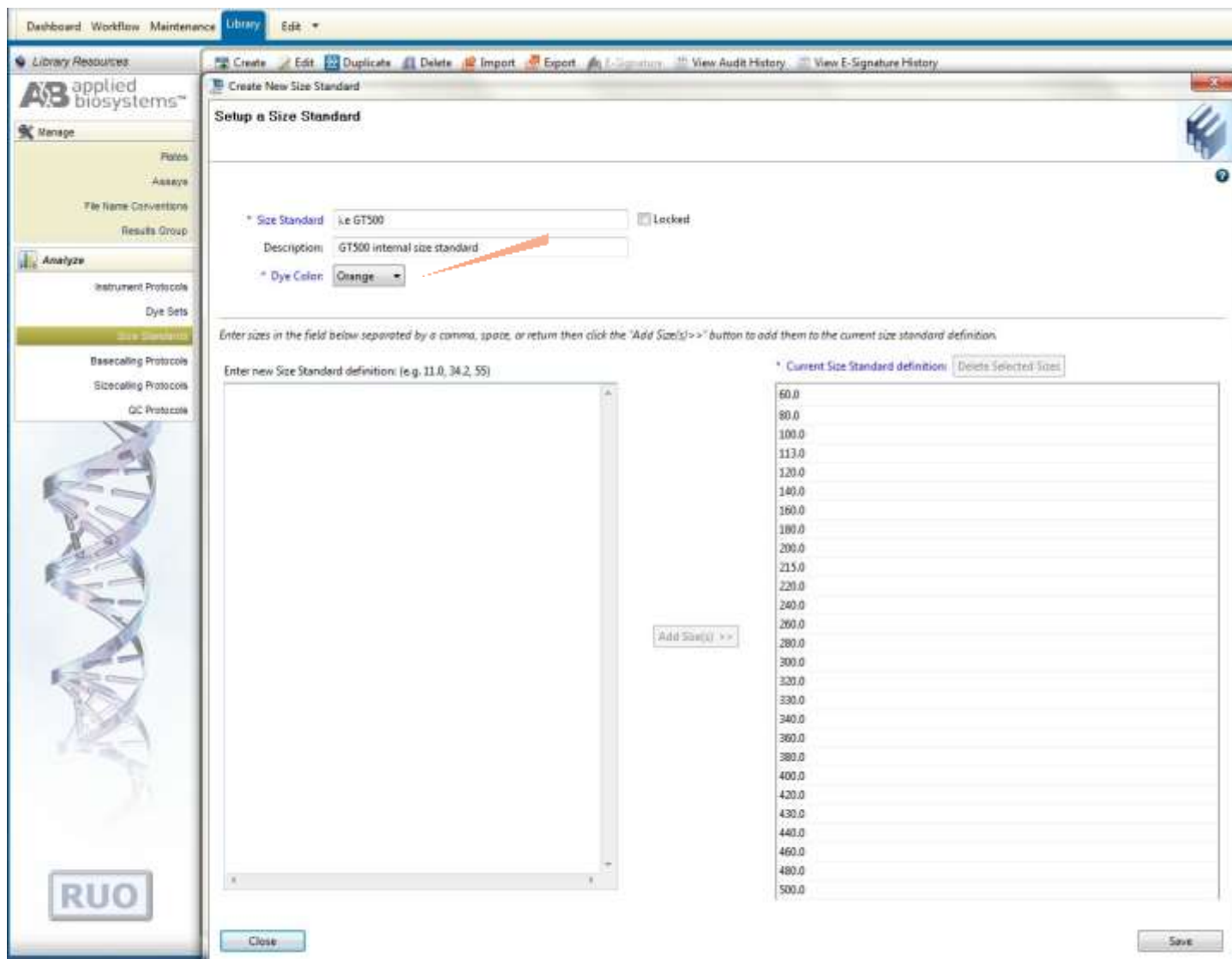


Figure 6. Screenshot for the “Create New Size Standard” window on Applied Biosystems 3500 Data Collection software

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. GT500” and select the *Size Standard* “GT500”

Users can select settings as shown in the Figure 7 or alternatively may define these settings based on internal validation condition for GT AZFScreen Plus v2 on the Applied Biosystems® 3500/3500xL Genetic Analyzer.

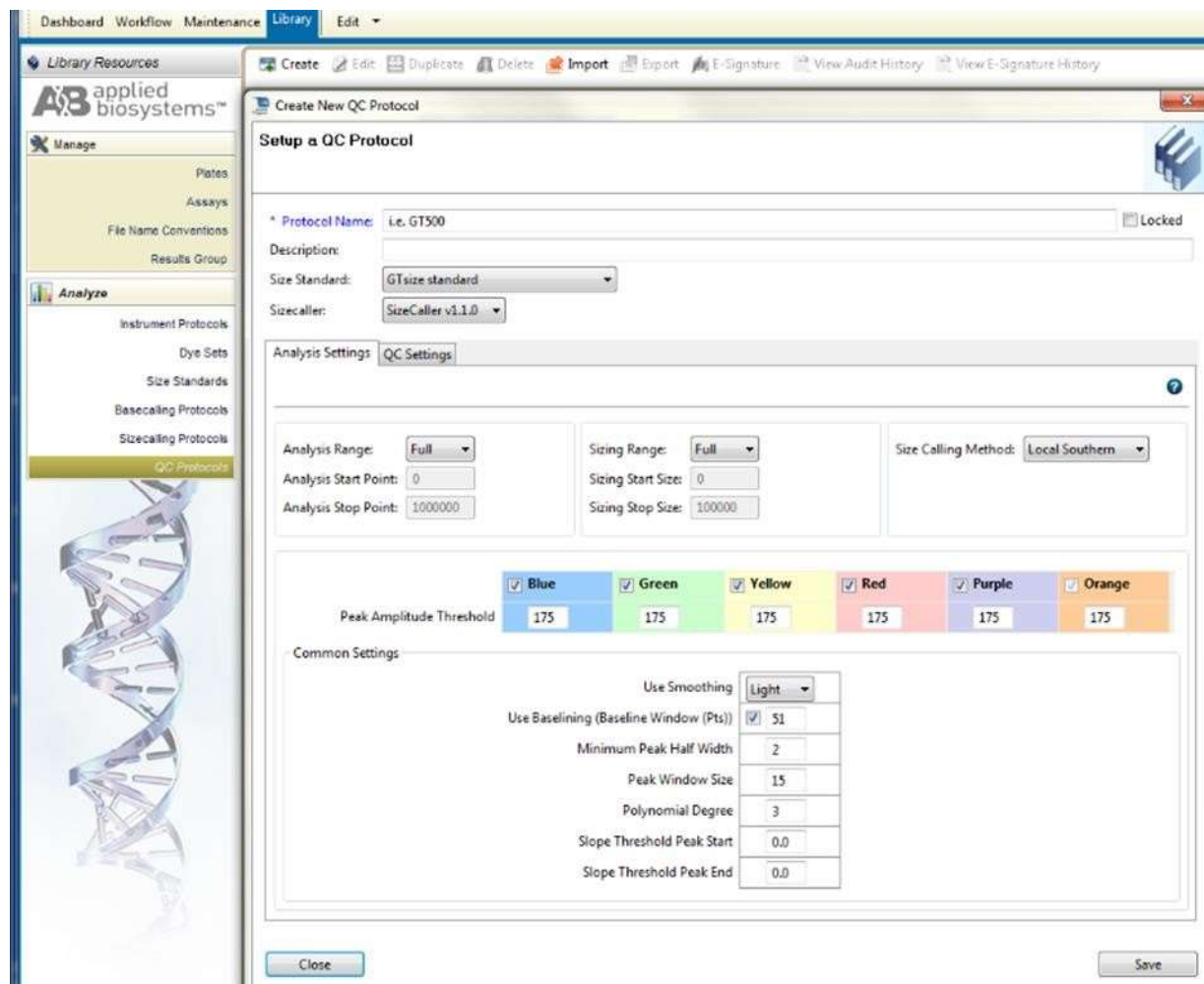


Figure 7. Screenshot for the “Create New QC Protocol” window on Applied Biosystems 3500 Data Collection software

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.

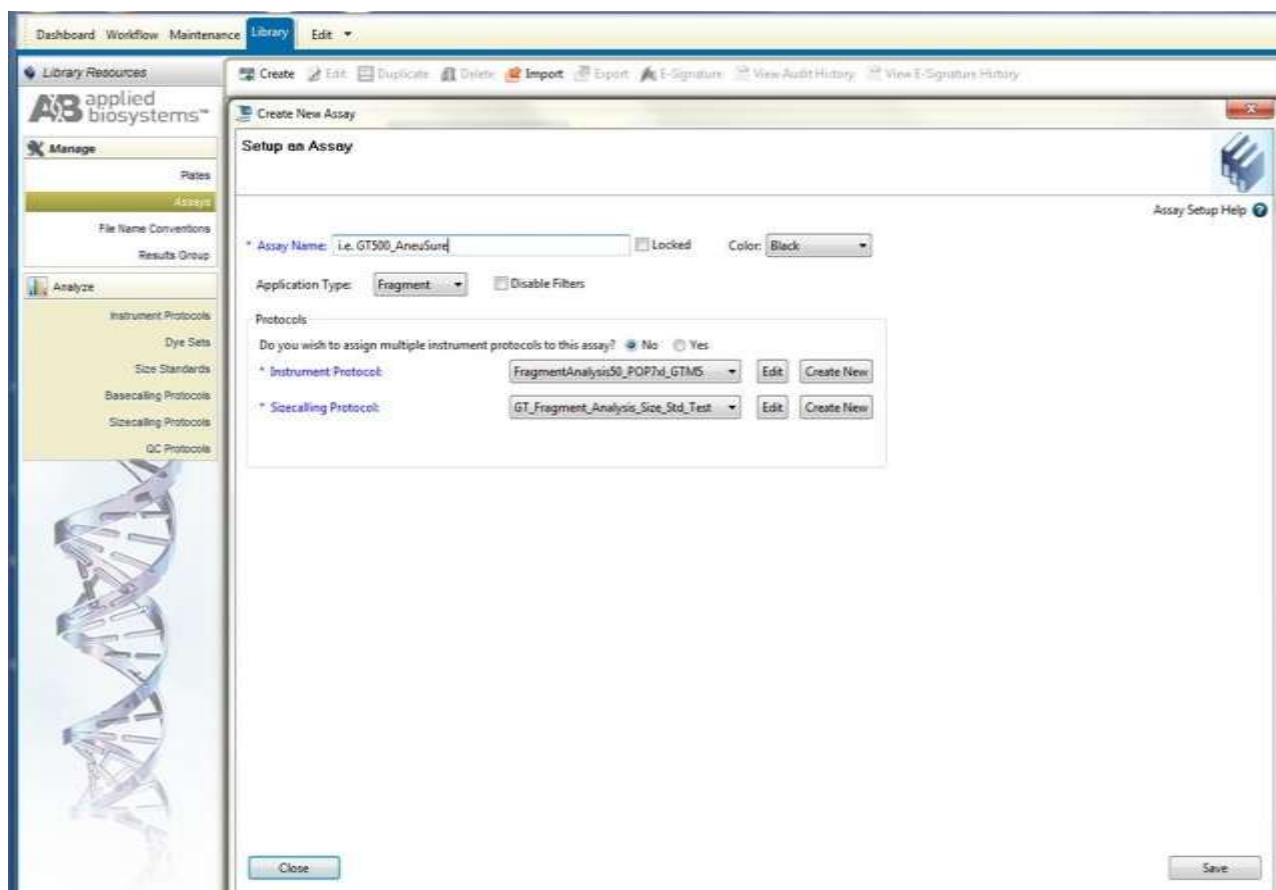


Figure 8. Screenshot for the “*Create New Assay*” window on Applied Biosystems 3500 Data Collection software

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

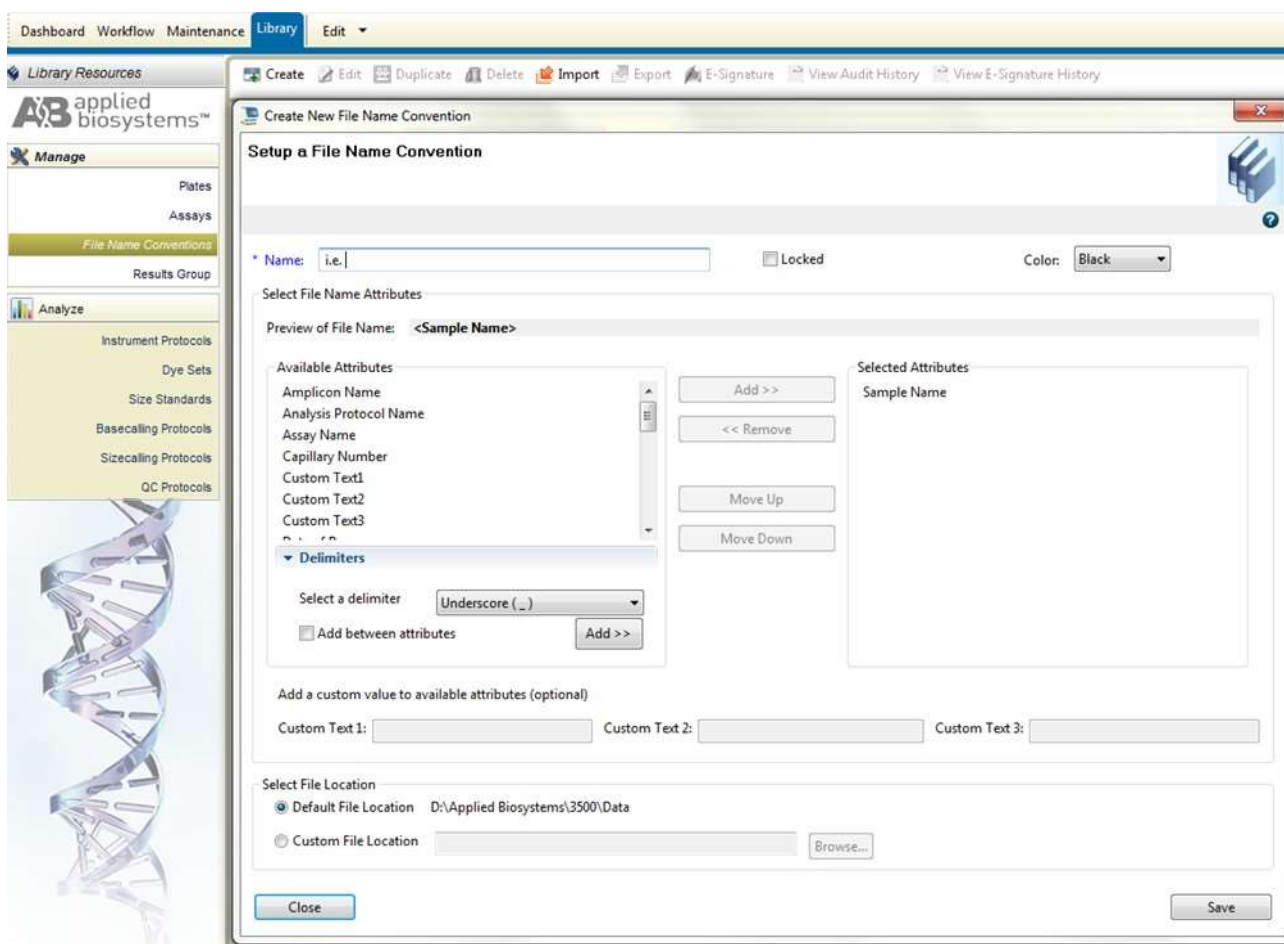


Figure 9. Screenshot for the “Create New File Name Convention” window on Applied Biosystems 3500 Data Collection software

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

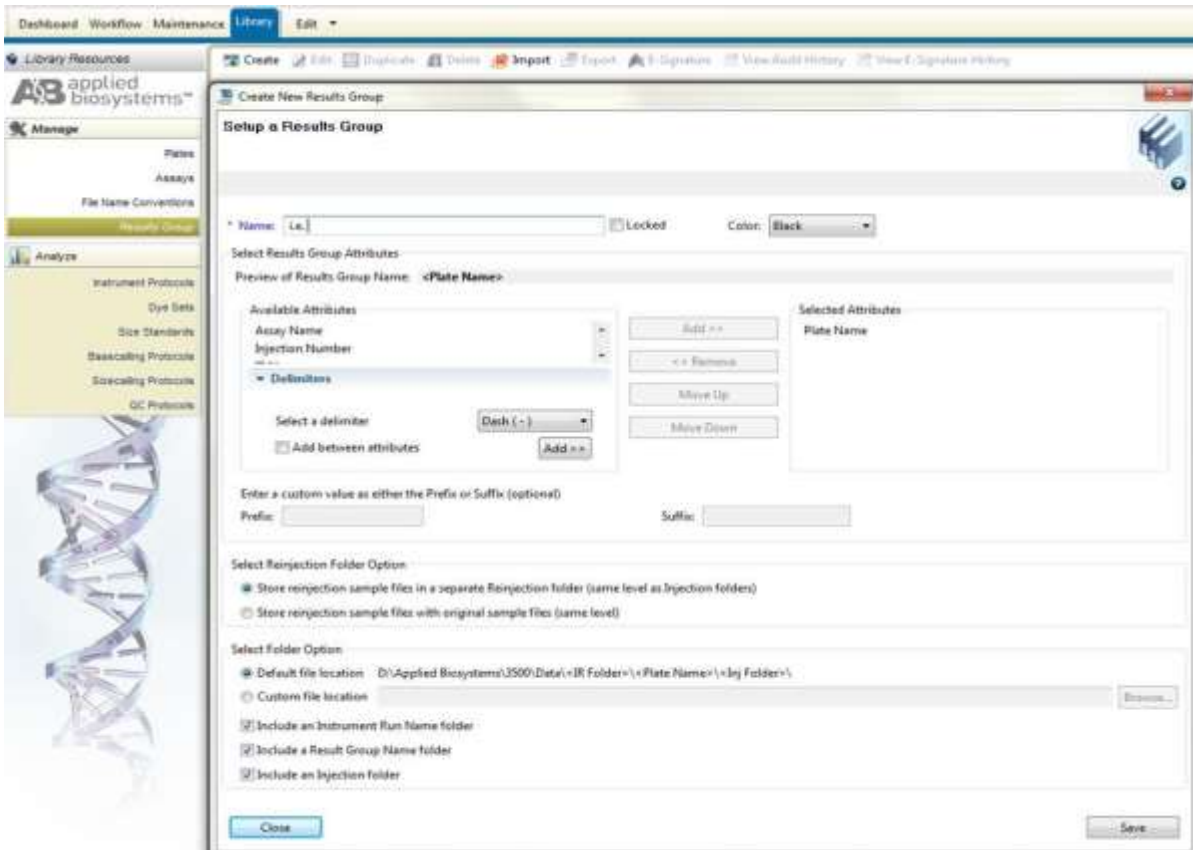


Figure 10. Screenshot for the “Create New Result Group” window on Applied Biosystems 3500 Data Collection software

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

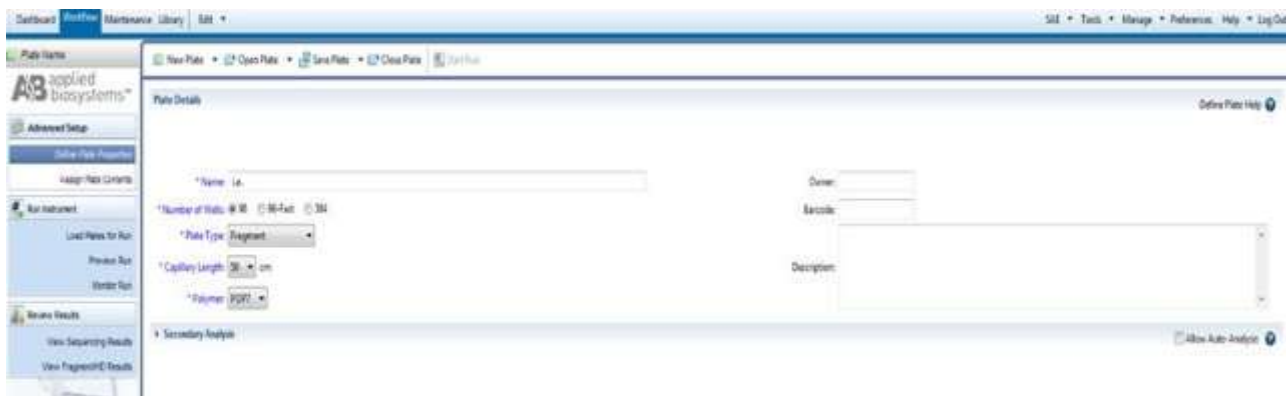


Figure 11. Screenshot for the “Defining plate properties” window on Applied Biosystems 3500 Data Collection software

3.1.8. Select “Assign Plate Contents”

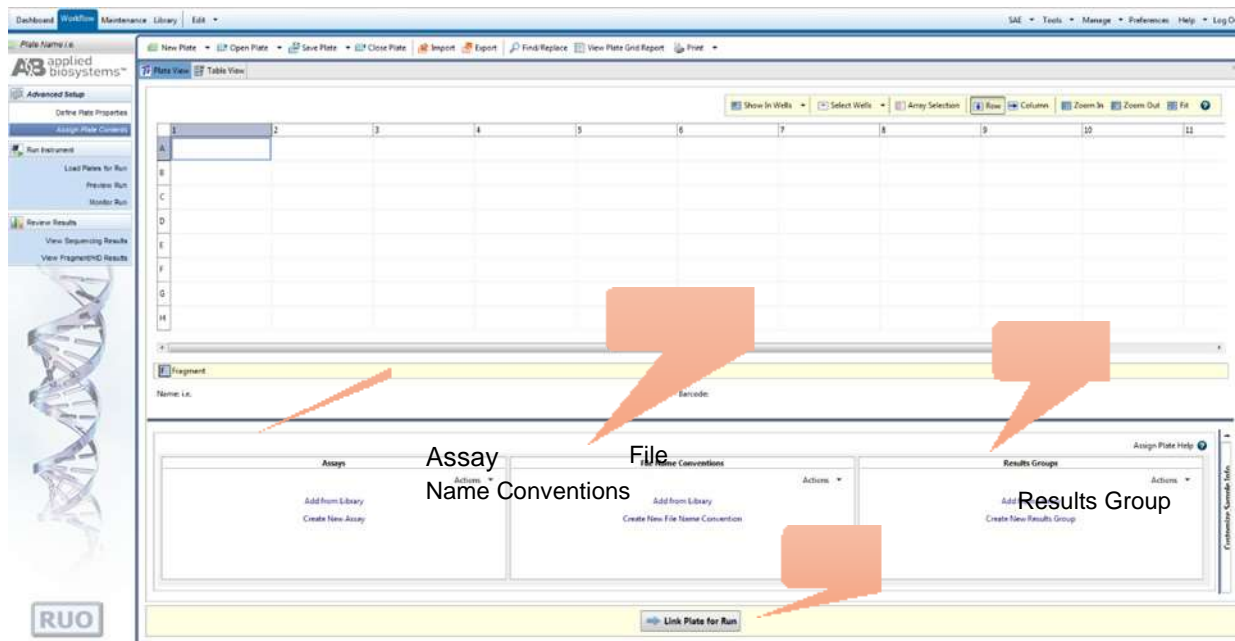


Figure 12. Screenshot for the “Assign Plate Contents” window on Applied Biosystems 3500 Data Collection Software

- 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 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.

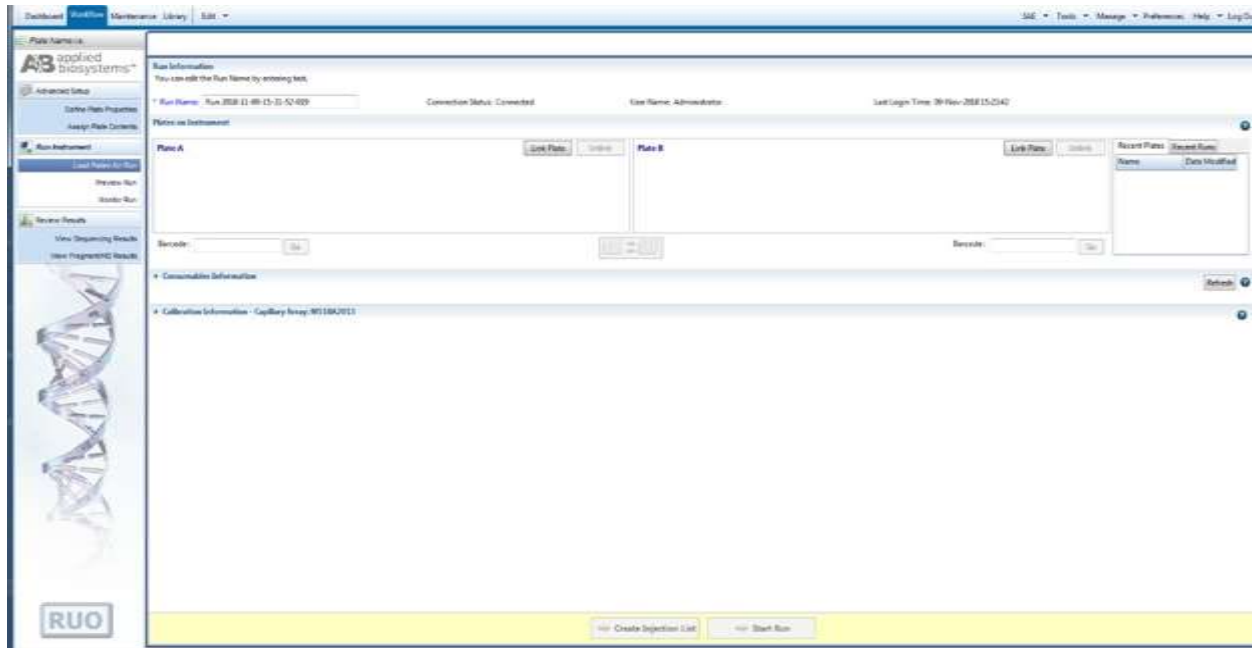


Figure 13. Screenshot for the “Run Information” window on Applied Biosystems 3500 Data Collection software

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

Make sure that maintenance and installation of the capillary array, buffers, and polymer are done according to the 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 the 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 GT AZFScreen 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.

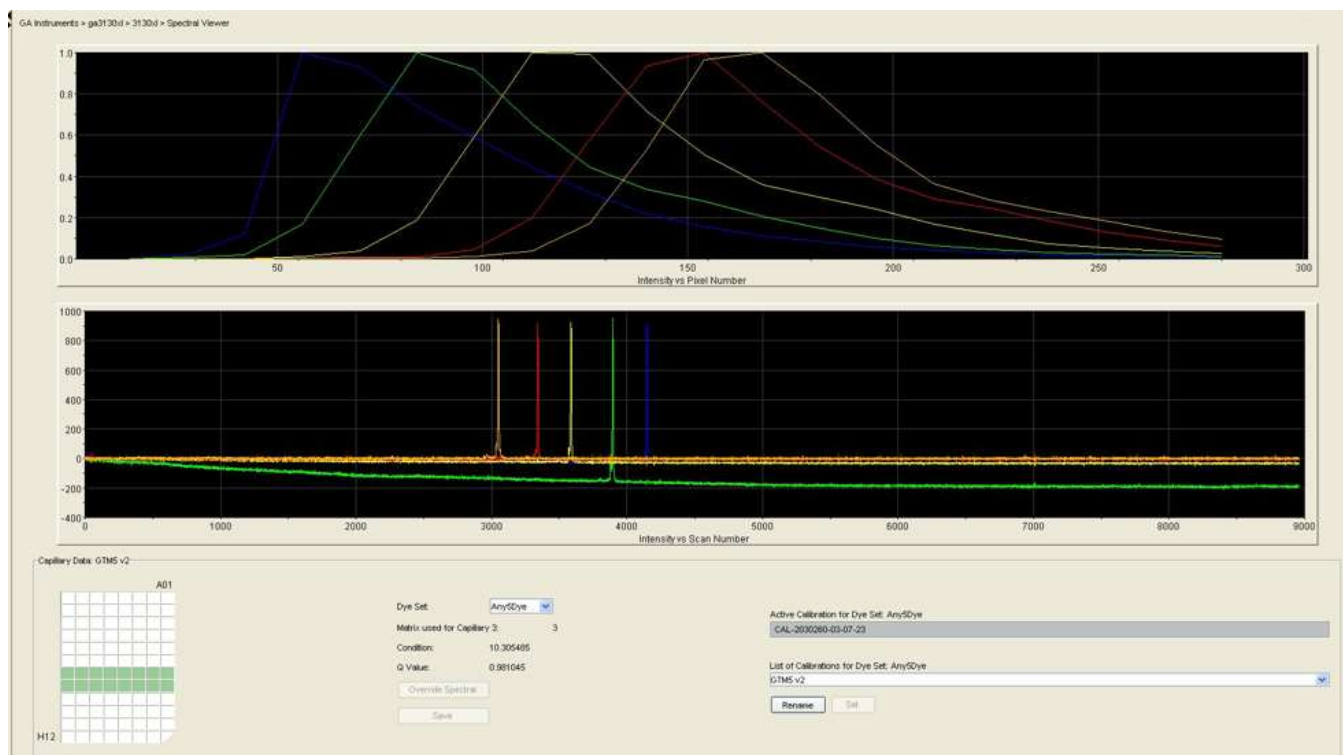


Figure 14. An example of a successful spectral calibration with GT 5-dye system on Applied Biosystems Genetic Analyzer 3130xl

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 (GT AZFScreen Plus v2)
- b) Type: Regular
- c) Template: FragmentAnalysis50_POP7 (default template for the capillary array and polymer used)
- d) Click OK

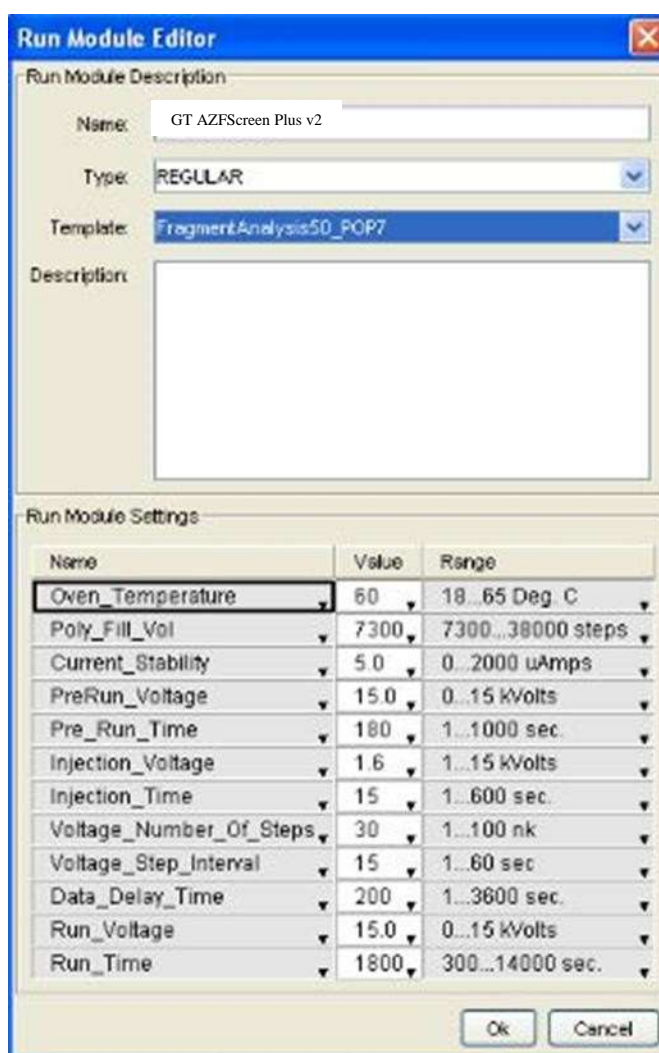


Figure 15. Screenshot for the “Module Manager” window on Applied Biosystems 3130 Data Collection software

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 (GT AZFScreen Plus v2)
- c) Type: Regular
- d) Run Module: Select the Run Module created (GT AZFScreen Plus v2)
- e) Dye Set: Any5Dye
- f) Click OK

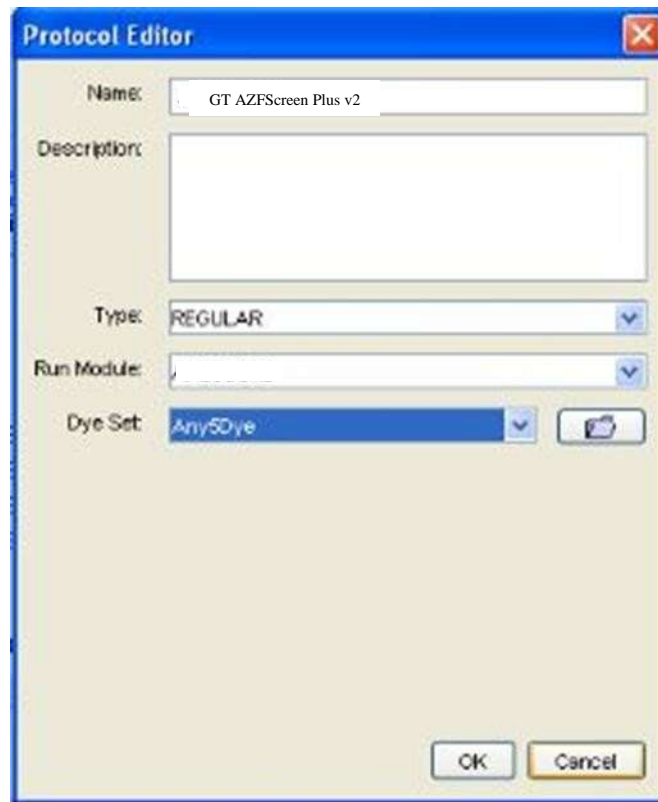


Figure 16. Screenshot for the “Create New Instrument Protocol” window on Applied Biosystems 3130 Data Collection software

3.2.3. Set up a Plate for run

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

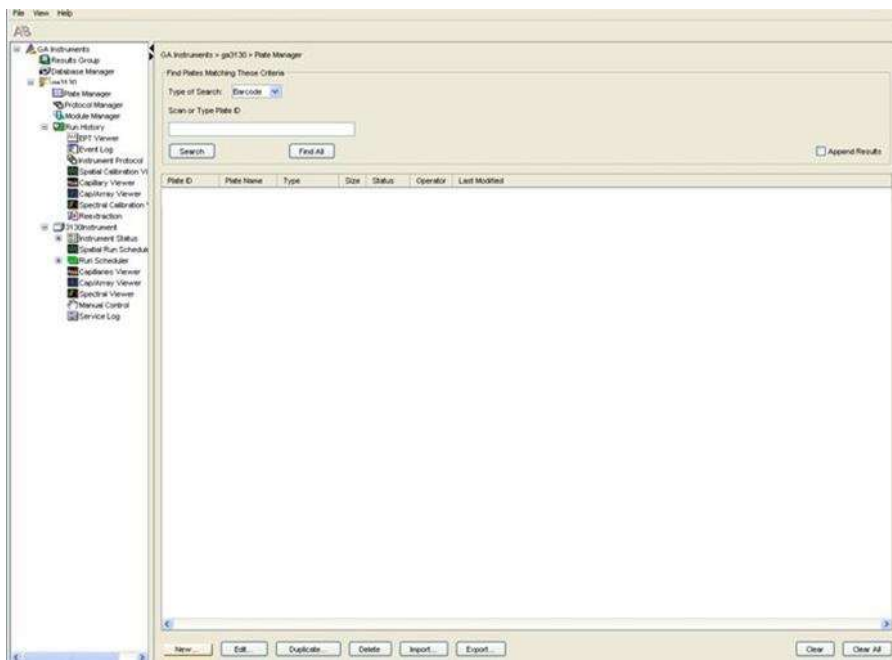


Figure 17. Screenshot for the “Plate Manager” window on Applied Biosystems 3130 Data Collection Software.

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

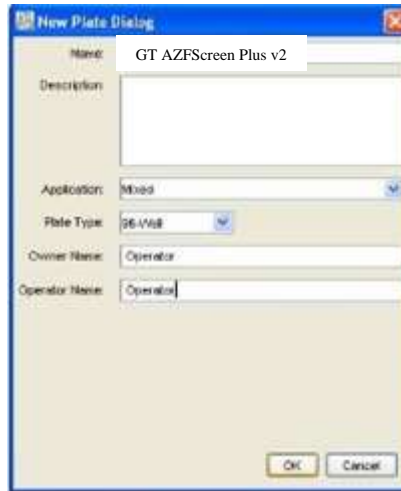


Figure 18. Screenshot for the “New Plate Dialog” window on Applied Biosystems 3130 Data Collection software

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

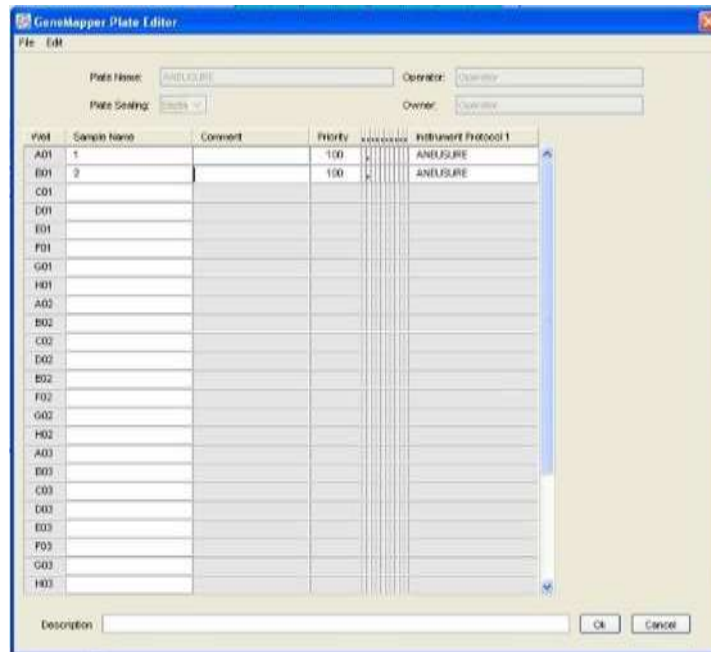


Figure 19. Screenshot for the “GeneMapper Plate Editor” window on Applied Biosystems 3130 Data Collection software

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

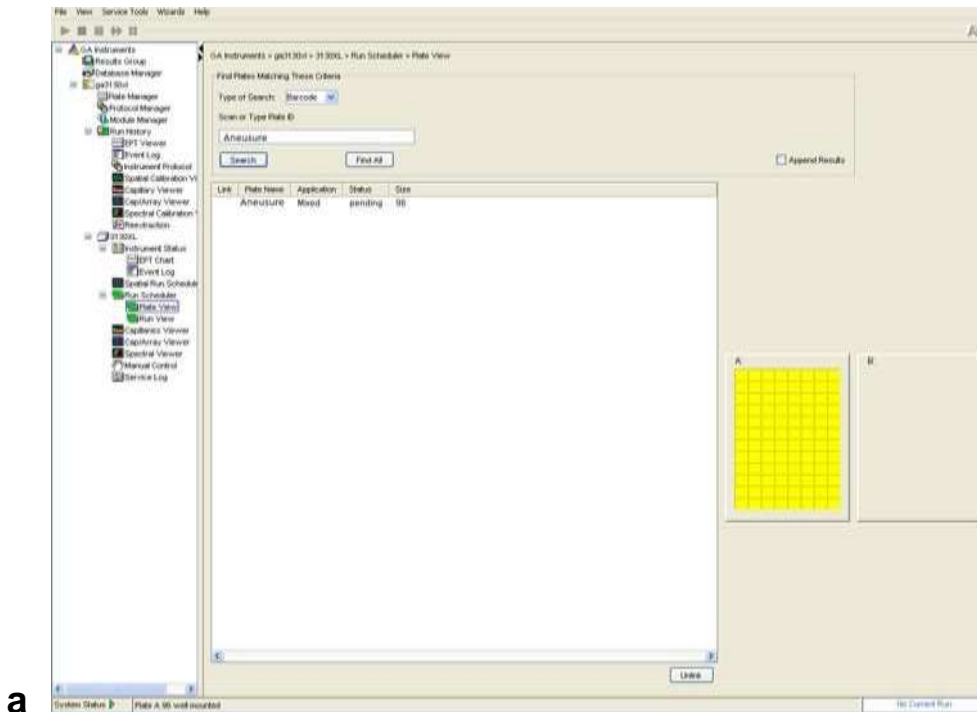


Figure 20 a. "Plate view" window on Applied Biosystems 3130 Data Collection software.

- 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.

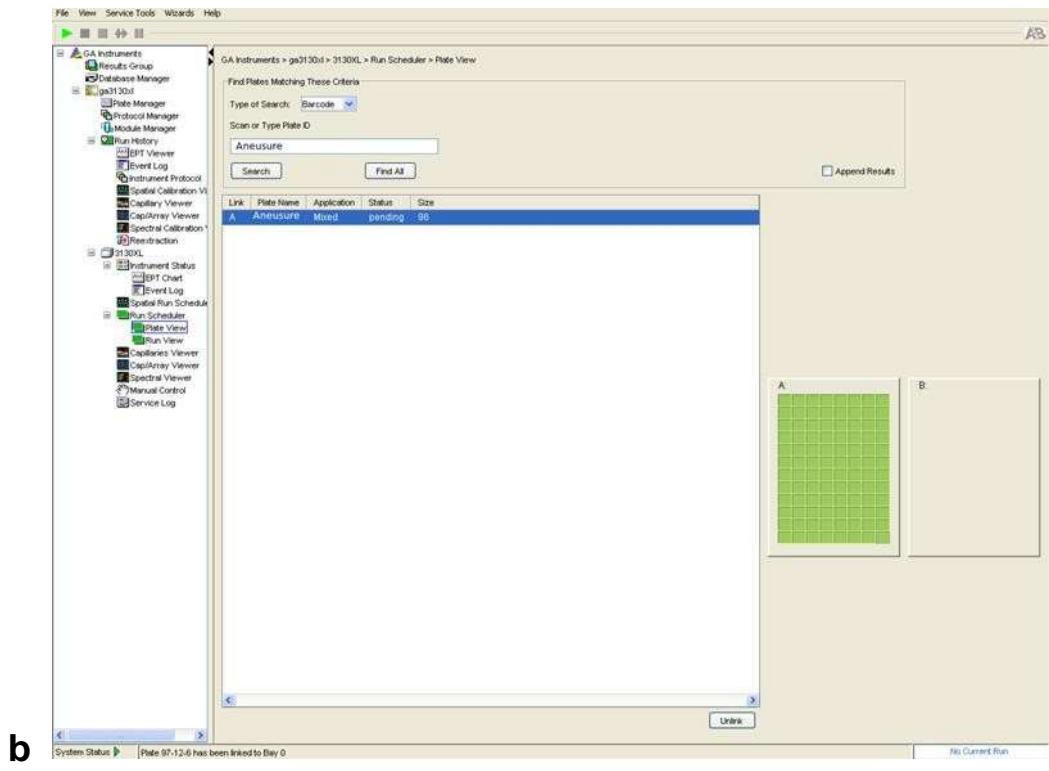


Figure 20 b. “Plate view” window on Applied Biosystems 3130 Data Collection Software.

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

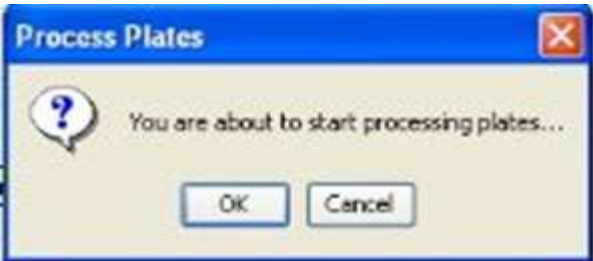


Figure 21. “Process Plates dialog” window on Applied Biosystems 3130 Data Collection software.

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

Please note: The Size Standard used in the GT AZFScreen 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.
- 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:
 - 94°C for 3 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 GT AZFScreen Plus v2, the Applied Biosystems fragment analysis software compatible with the Genetic Analyzer in use is recommended. This kit is compatible with GeneMapper Software. The analysis method depends on the software. Our customers have also been able to use the Compact Spectrum CE System from Promega to run and analyze the GT AZFScreen Plus v2 kit.

Each diagnostic lab should have individual interpretation and reporting procedures and criteria. To develop such a procedure, the use of “**EAA/EMQN best practice guidelines for molecular diagnosis of Y chromosomal microdeletions**” is recommended. You can download it from - <https://www.emqn.org/best-practice/>.

4.2. General guideline for the analysis of GT AZFScreen Plus v2 results

GT AZFScreen Plus v2 PCR products are observed on 5-dyes systems and analyzed using GeneMapper® or similar software. For the analysis, import GT AZFScreen Plus v2 panels from the Genetek website under the supporting document on the kit webpage. 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 sizes may differ slightly between individuals but are usually constant within a person and his/her parents on the same run. It has been observed that sizes may differ from 1-3 bp if the same sample if it is run on different days or with different instruments. However, this does not affect the results and their interpretations.
- 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. However, the height does not affect the result interpretation unless the heights are so low that interpretation can not be achieved (less than 50 RFC). However, for segmental duplication markers, the heights differ in non-Kleinfelter males.
- 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 below – see Example profile for the GT QCDDM control DNA.
- There should not be excessive bleed-through between dye colors or “Pull-up” effect in the electropherograms if spectral calibration has successfully been carried out.

- Successful amplification must result in at least one peak for each marker (except for Y chromosome markers which would be absent in a normal female sample if a female or a male sample with a female karyotype sample is being analyzed).

5. Examples of results

5.1. A male profile (GT QCDM102 included in GT AZFScreen Plus v2 Kit)

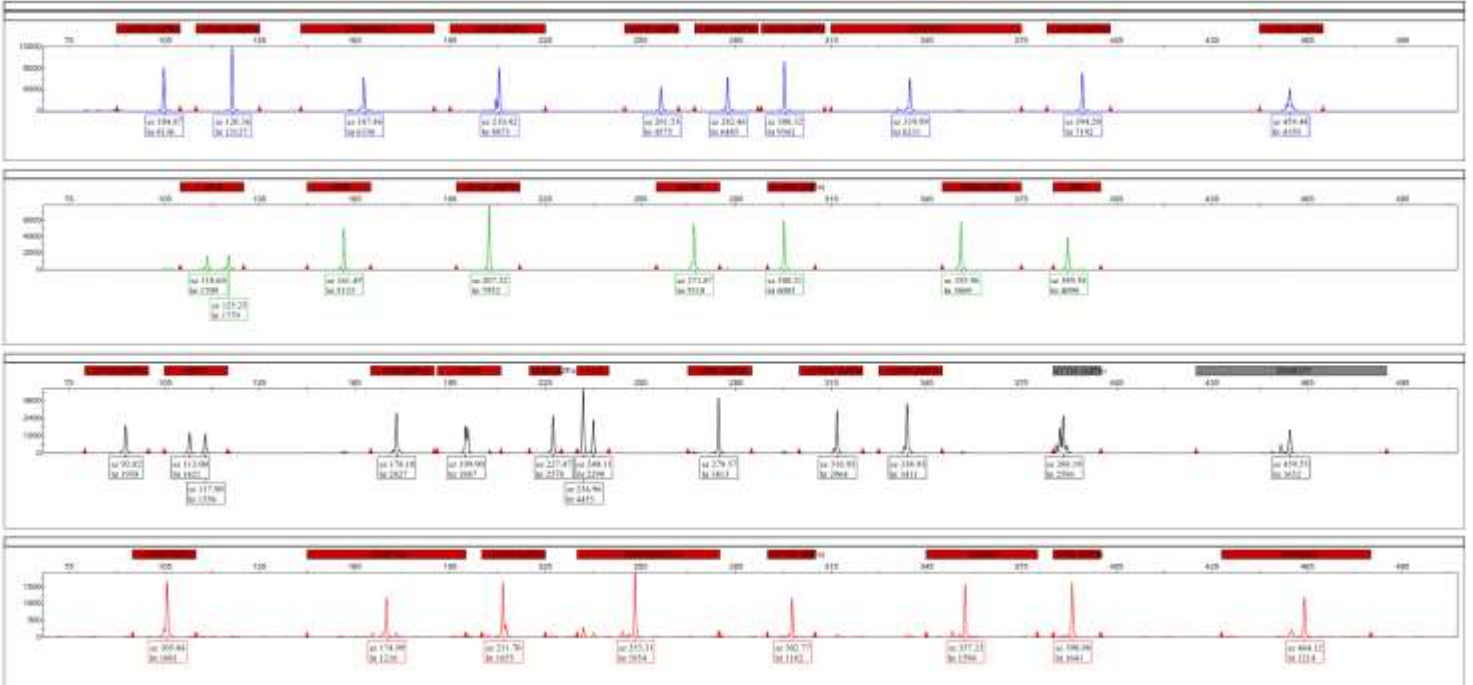


Figure 22. An example of a fertile male profile with no Y chromosome microdeletion. AMXY and XYB show two peaks indicating male. But both have almost the same height indicating a non-Klinefelter male sample is being analyzed. The 11/X marker shows a 2:1 ratio for chromosomes 11 and X. No Y-specific marker is absent indicating absence of microdeletion.

5.3. Male sample profile with Y chromosome microdeletion.

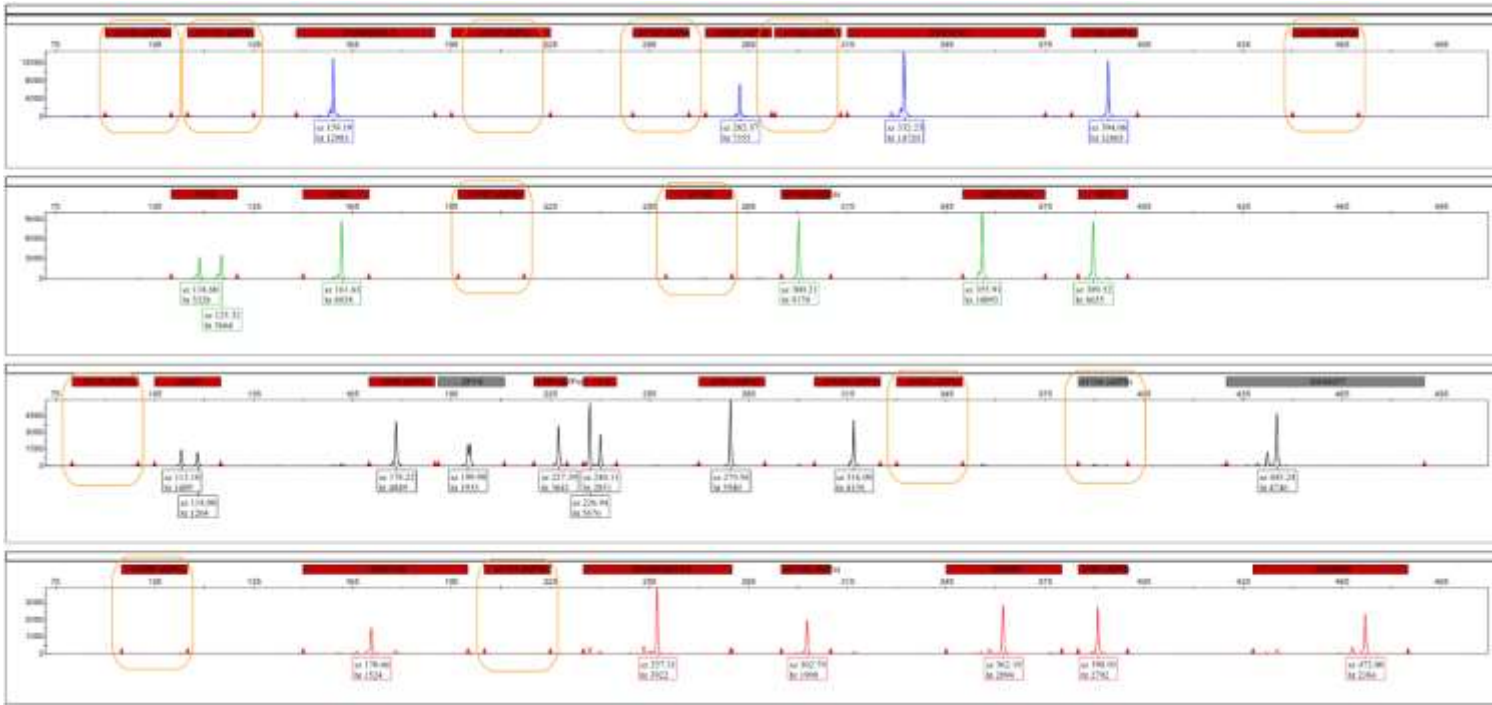
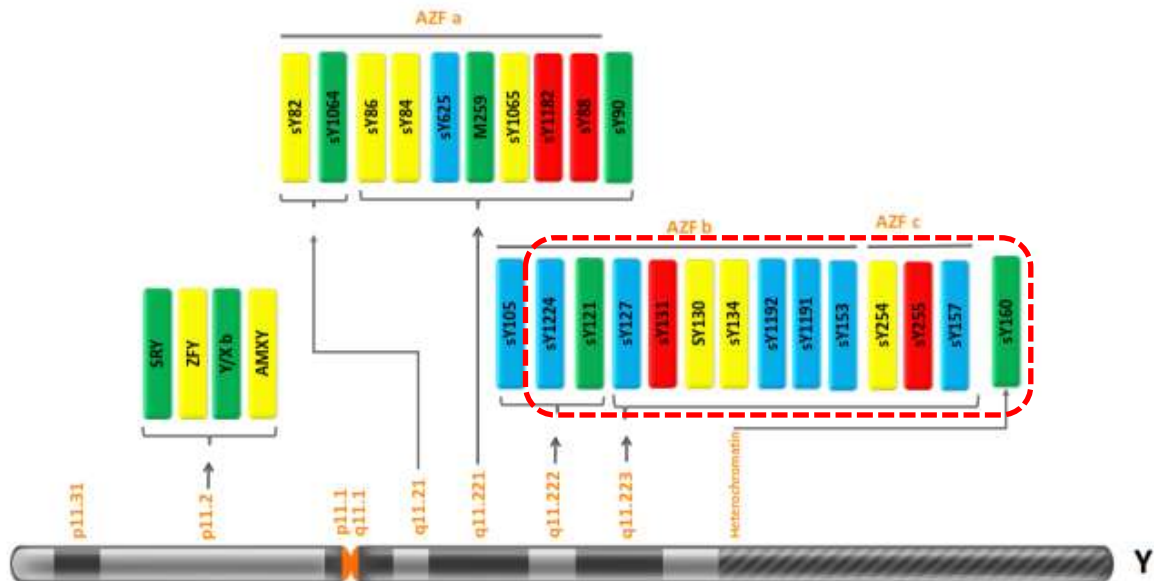


Figure 24. An example of male profile with Y chromosome microdeletion covering part of the AZFb, AZFc and heterochromatin regions. The boxed markers show the absence of PCR products indicating the deletion of these markers for the AZFb, AZFc and heterochromatin regions on the Y chromosome.



5.3. Male sample profile with Y chromosome microdeletion.

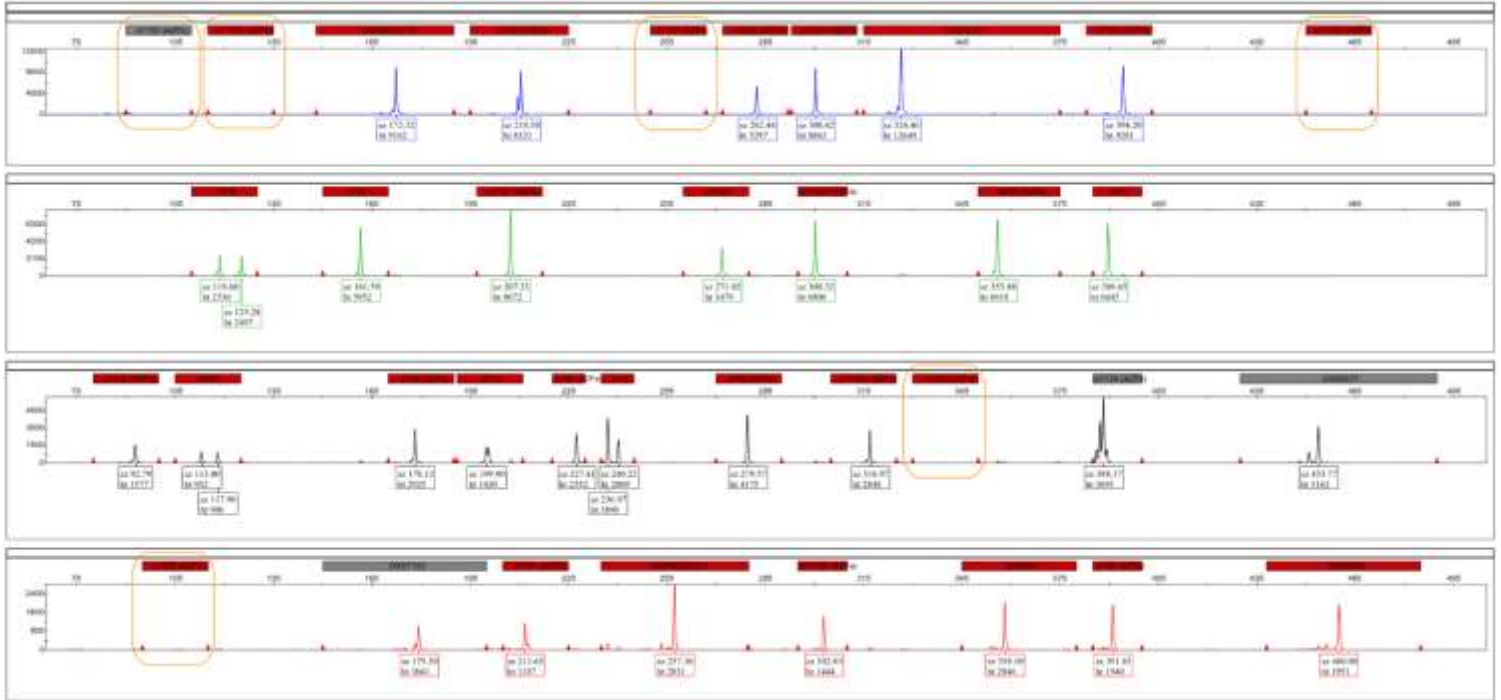
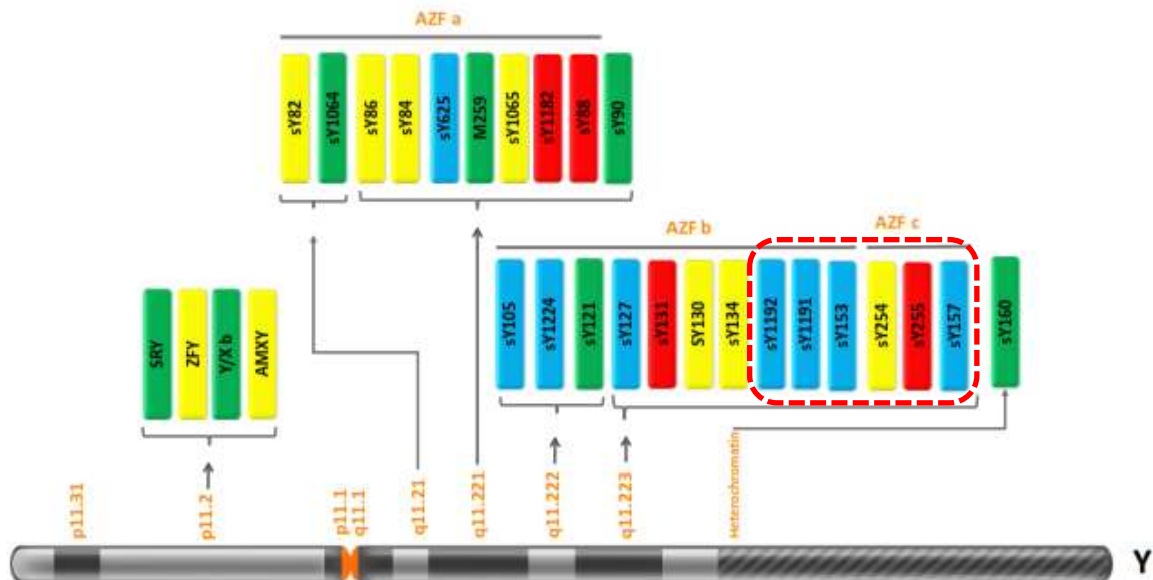


Figure 25. An example of a male profile with Y chromosome microdeletion around the AZFb region. Boxed markers show the absence of PCR products, indicating deletion of these markers for the AZFb region on the Y chromosome.



5.4. Male sample profile with klinefelter syndrome (XXY)

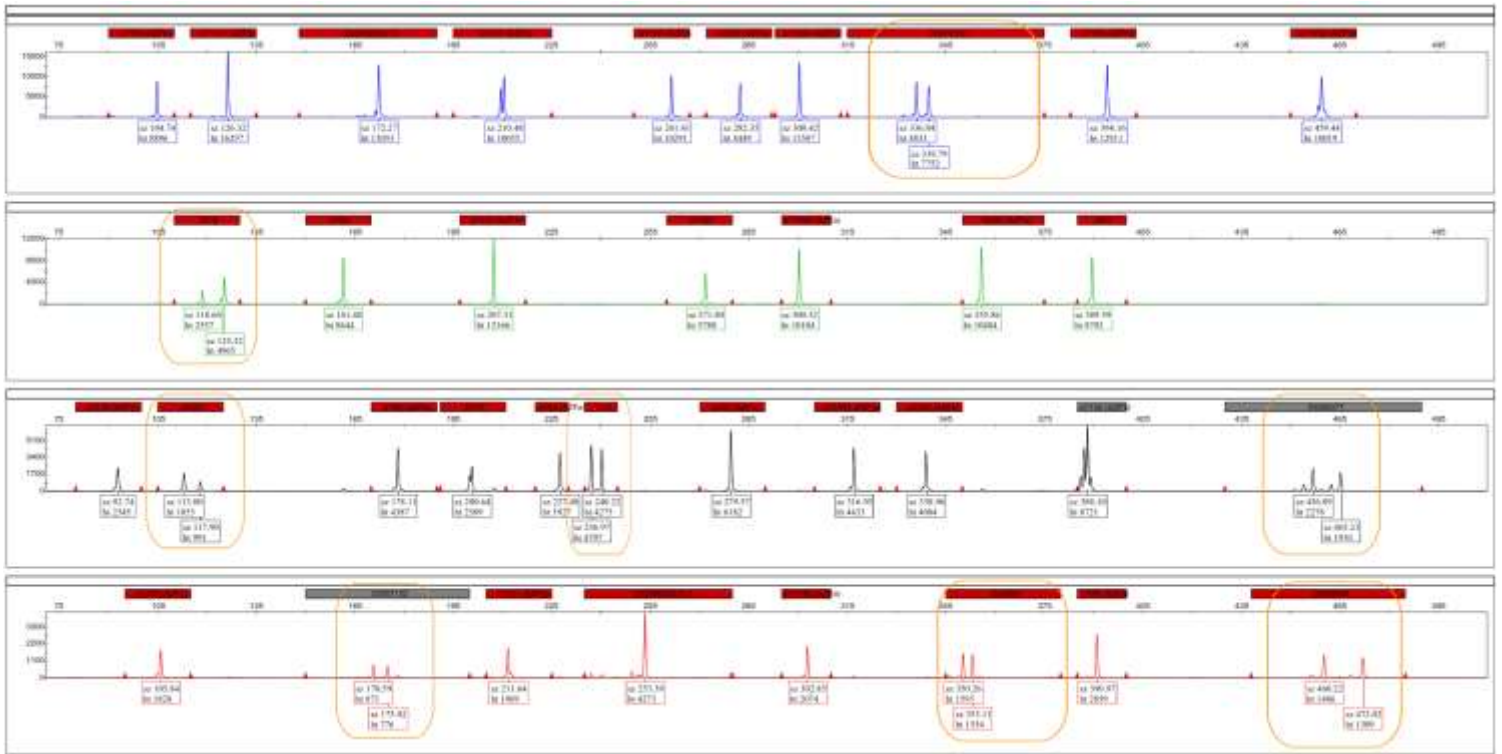


Figure 26. An example of a male profile with Klinefelter syndrome generated with GeneMapper® IDX v1.5 and 3500xL Genetic Analyzer. Since the person is affected with Klinefelter syndrome, then the X-related fragments heights are twice of the other on the Y chromosome (i.e. AMXY and Y/X B, indicating XXY). Also, the DXS10101, DXS8377, DXS7132, DXS981, and DXS6809 STR markers each have two peaks, supporting a Klinefelter sample is being analyzed (since Y markers have bands there, it is a XXY sample). The 11/X peaks are almost equal heights indicating two X chromosomes since a live person can not be monosome for both chromosomes 11 and X.

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. GT AZFScreen 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 QCDM provided with GT AZFScreen 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 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.

	<p>Polymer-caused artifacts, check Polymer expiration date and storage time as mentioned in the manufacture guide.</p>
<p>Off-scale peaks</p>	<p>If off-scale peaks after primer peaks are observed –</p> <ul style="list-style-type: none"> • 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.
<p>No sizing data or size quality fails</p>	<ul style="list-style-type: none"> • 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 GT AZFScreen Plus v2 Kit to obtain optimum results.

7. Limitations and Disclaimer

Any result obtained from GT AZFScreen 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 GT AZFScreen 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.

GT AZFScreen Plus v2 Kit is designed to detect Y chromosomes specific microdeletions (AZFa, AZFb, and AZFc) and Klinefelter syndrome. It will not detect other chromosome abnormalities or defect. User must carefully inspect any case of Maternal Cell Contamination and sample mix-up 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.

GT AZFScreen Plus v2 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 kit including GT AZFScreen Plus v2 Kit.

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. Krausz C., Navarro-Costa P., Wilke M and Tüttelmann F. (2023) EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: State of the art 2023. *Andrology* 12(1) 1-18, DOI: 10.1111/andr.13514.
2. DNA Fragment Analysis by Capillary Electrophoresis User Guide by Applied Biosystems® Publication Number 4474504.
3. Best Practice Guidelines for Internal Quality Control in Genetic Laboratories by Association for Clinical Genetic Science
4. 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.