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# Quantitative fluorescent polymerase chain reaction (QF-PCR) for the rapid prenatal diagnosis of common fetal aneuploidies V.1

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ABSTRACT

Quantitative fluorescent polymerase chain reaction (QF-PCR) is reliable and efficient method for the rapid prenatal diagnosis of common fetal aneuploidies. In this protocol we first describe a procedure for isolation of DNA from amniocytes and chorionic villi with the use of commercial kit intended for the isolation of nucleic acids from viruses, which, taking into account the phenomenon of increased fragmentation of the DNA material of fetal origin, proved to be appropriate for prenatal diagnosis. (Section 1). As part of the isolation procedure, we also include a procedure for treating amniotic fluid with visible contamination (blood with possible origin from the mother).The presented procedure consists of simple physical separation of the maternal blood cells from the fetal material in order to reduce the failure rate due to maternal cell contamination.

In the second part of the protocol (Section 2) we are presenting procedure for performing the QF-PCR analysis. Here, we describe an in-house one-tube multiplex QF-PCR method including 20 PCR markers (15 STR markers and 5 fixed size) for rapid prenatal diagnosis of chromosome 13, 18, 21, X and Y aneuploidies, which targeted in total 26 genomic positions. Of them, three markers (D13S258, D13S305 and D13S1817) were for analysis of the aneuploidies for the chromosome 13, four (D18S386, D18S390, D18S391 and D18S535) for the chromosome 18, four (D21S1411, D21S1414, D21S1435 and D21S1446) for the chromosome 21 and six for the analysis of the sex chromosome aneuploidies. Two of the sex chromosome markers (DXS6803 and XHPRT) amplified sequences only on chromosome X (for counting chromosome X), two (AMELX/Y and DXYS218) co-amplified sequences of both X and Y chromosomes (for counting chromosomes X and Y in male samples and chromosome X in female samples), one (TAF9B) was for co-amplification of sequences on chromosomes X and 3 (for counting chromosome X) and one (SRY) located on chromosome Y was for male sex determination. Three remaining markers (MYPT2/Y, DYS448 and CDY1/2) were used simultaneously for detection of the Y chromosome aneuploidies and microaberrations in the azoospermia factor region “c” (AZFc), located on the Y chromosome.

For the cases with inconclusive results, we used additional QF-PCR reactions specific for given chromosome (Section 3). For chromosomes 13, 18 and 21 the primers were combined in one multiplex reaction each: four STR markers for chromosome 13 (D13S742, D13S628, D13S634 and D13S631), three STR markers for chromosome 18 (D18S51, D18S1367 and D18S978) and four STR markers for chromosome 21 (D21S1437, D21S11, D21S1412 and D21S1441). For the sex chromosomes we have used three markers as separate PCR reactions (for DXS6809, DXS996 and X22) and additionaly if needed, previously published multiplex reaction [File 1] for detection of sex chromosome aneuploidies in infertile patients.

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0221227

File 1.pdf

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| AmpliTaq Gold™ 360 DNA Polymerase | 4398823 | Thermo Fisher Scientific |

| NAME | CATALOG # | VENDOR |
|---|---|---|
| GeneScan™ 500 LIZ™ dye Size Standard | 4322682 | Thermo Fisher Scientific |
| Hi-Di™ Formamide | 4311320 | Thermo Fisher Scientific |
| High Pure Viral Nucleic Acid Kit | 11858874001 | Roche |
| 5' Labeled Primers | 450056 | Thermo Fisher Scientific |

DNA isolation from amniocytes/chorionic villi/blood

1 Perfom one-hour DNA isolation protocol using the commercial High Pure Viral Nucleic Acid kit (Roche Applied Science, Mannheim, Germany):

*Prepare the material for isolation:*

1. For amniotic fluid -> in a 1.5 ml tube collect amniotic fluid, centrifuge 13000 x g for 5 minutes and remove the supernatant; repeat the procedure several times (depends on the amount of amniocytes and/or volume of amniotic fluid); after the last spinning, apart from the precipitate, also retain 200 ul supernatant; vortex and proceede with isolation according to the manufacturer's protocol.
2. For chorionic villi -> in a 1.5 ml tube collect one or several chorionic villi and add up to 1.5 ml of 1x PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl; pH 7.4), centrifuge at 13,000 x g for 5 minutes and remove the supernatant; repeat the procedure one more time, discard the supernatant, add 200 µl 1x PBS and proceeded with isolation according to the manufacturer's protocol.
3. For blood -> proceeded with isolation according to the manufacturer's protocol.

*Perform isolation with High Pure Viral Nucleic Acid kit according to the folowing procedure:*

- Add 200 µl Binding buffer
- Add 50 µl Proteinase K solution (supplied as apart of the kit); mix immediately.
- Incubate for 10 min at +72°C.
- Add 100 µl Binding Buffer and mix well.
- To transfer the sample to a High Pure Filter Tube:

• Insert one High Pure Filter Tube into one Collection Tube.
• Pipet the entire sample into the upper reservoir of the Filter Tube.
• Insert the entire High Pure Filter Tube assembly into a standard table-top centrifuge.
• Centrifuge 1 min at 8,000 × g.

- After centrifugation:

• Remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
• Combine the Filter Tube with a new Collection Tube.

- After combining the Filter Tube with a new Collection Tube:

• Add 500 µl Inhibitor Removal Buffer (previosly prepared according to manufactutrer's description) to the upper reservoir of the Filter Tube.
• Centrifuge 1 min at 8,000 × g.

- After centrifugation:

• Remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
• Combine the Filter Tube with a new Collection Tube.

- After removal of inhibitors:

• Add 450 µl Wash Buffer (previosly prepared according to manufactutrer's description) to the upper reservoir of the Filter Tube.
• Centrifuge 1 min at 8,000 × g and discard the flowthrough.

- After the first wash and centrifugation:

• Remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
• Combine the Filter Tube with a new Collection Tube.
• Add 450 µl Wash Buffer to the upper reservoir of the Filter Tube.
• Centrifuge 1 min at 8,000 × g and discard the flowthrough. • Leave the Filter Tube-Collection Tube assembly in the centrifuge and spin it for 10 s at maximum speed (approx. 13,000 × g) to remove any residual Wash Buffer.
The extra centrifugation time ensures removal of residual Wash Buffer.

- Discard the Collection Tube and insert the Filter Tube into a nuclease-free, sterile 1.5 ml microcentrifuge tube.
- To elute the nucleic acid:

• Add 30 µl for amniocytes or 50 µl (chorionic villi or blood)of Elution Buffer to the upper reservoir of the Filter Tube.
• Centrifuge the tube assembly for 1 min at 8,000 × g.

- Dissolve the pelleted DNA with the pipetor and leave at 37 $^{o}$C for one hour.
- Measure the DNA concentration and proceed to the QF-PCR reaction step.

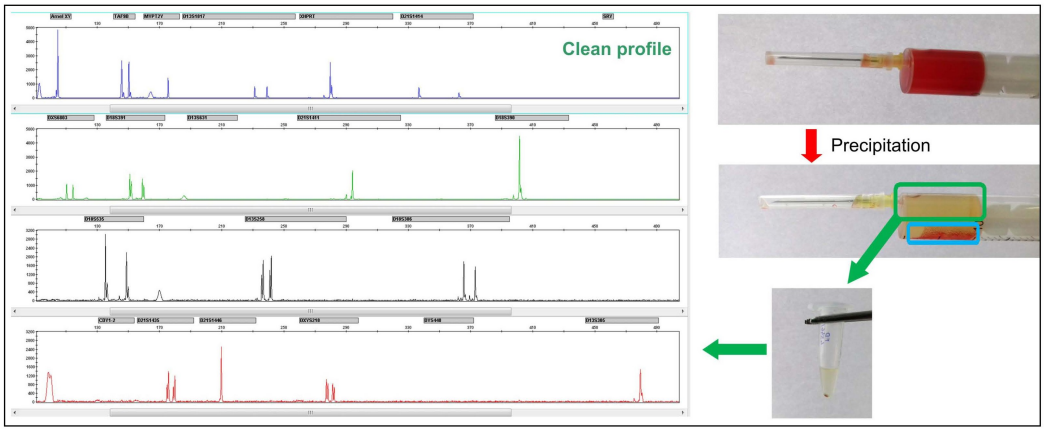
## 1.1 Treatment of maternal cell contamination in amniotic fluids

In order to improve the outcome of the analysis of amniotic fluids defined as samples with maternal cell contamination (MCC), perform physical separation of the potentially contaminating material:

1. Store the syringe with the amniotic fluid on +4$^{O}$C for 6-12 hours in order to precipitate (precipitation could also be performed over night);
2. After separation of the two clearly visible phases, firstly collect the upper clear phase in a 1.5 ml tube without dissolving the lower phase; centrifuge at 11,000 x g for 5 minutes and discard the supernatant; repeat until all of the upper clear phase is collected, and after last centrifugation retain 200 ul supernatant;
3. After that, collect the lower phase separately in a new 1.5 ml tube; centrifuge at 11,000 x g for 5 minutes and discard the supernatant, but retain 200 ul supernatant;
4. After centrifugation of the collected amniotic fluid, perform separate isolation (as previosly described) and QF-PCR analysis for the two colected pellets .

*Visualisation of the procedure for the treatment of maternal cell contamination in amniotic fluids is shown in the following figure:*

**A)**




**B)**

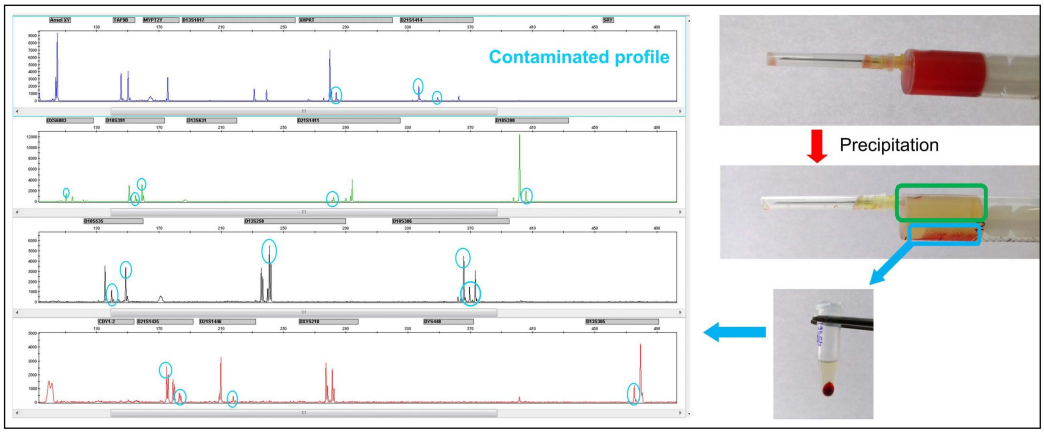



**Illustration of the treatment of the contaminated amniotic fluids.** Contaminated amniotic fluid is precipitated for 6-12 hours and then **A)** Upper clear phase (green color) was collected resulting in amplification of a pure uncontaminated profile of the fetus and **B)** Afterwards, lower contaminated phase (blue color) was collected separately wherein the amplification showed maternal cell contamination. Blue circles show the contaminating alleles from the mother.

Main QF-PCR reaction

## 2

This section describes the main QF-PCR reaction and consists of the following steps:

2.1) Prepare QF-PCR multiplex primer mix;
2.2) Perform PCR reaction;
2.3) Capillary electrophoresis of the PCR product and analysis of the results.

## 2.1

QF-PCR primer mix is prepared in a total volume of 200 µl.

Sequences of the primers, their GC percentages, melting points and amount of each primer used in preparation of the QF-PCR mix are shown in the folowing table:

| Name | STR or fixed size (fs) | Sequence 5 `-> 3` | GC % | Tm 0C | Amount (in µl) used in the primer mix from 50 pmol/µl of Forward/Reverse primer stocks | Concentration in the QF-PCR mix (nM) | Final concentration in the QF-PCR reaction (nM) |
|---|---|---|---|---|---|---|---|
| AMEL-X/Y-F | fs | 6-FAM-CCC TGG GCT CTG TAA AGA ATA GTG | 50 | 61 | 2 | 500 | 50 |
| AMEL-X/Y-R | | ATC AGA GCT TAA ACT GGG AAG CTG | 46 | 62 | | | |
| DXS6803-F | STR | VIC-GAA ATG TGC TTT GAC AGG AA | 40 | 55 | 15 | 3750 | 375 |
| DXS6803-R | | CAA AAA GGG ACA TAT GCT ACT T | 36 | 55 | | | |
| DXYS218-F | STR | PET-AAT CTG GGC CTG AAG CAT TT | 45 | 58 | 10 | 2500 | 250 |
| DXYS218-R | | AAG AGC GAA ACT CCG TCT CA | 50 | 60 | | | |
| TAF9B-F | fs | 6-FAM-TTT GAC AGG TAG TTT TGG GTC A | 41 | 58 | 3 | 750 | 75 |
| TAF9B-R1 | | TGG TTT TGC CTA GGT CCA GT | 50 | 60 | | | |
| XHPRT-F | STR | 6-FAM-ATG CCA CAG ATA ATA CAC ATC CCC | 46 | 61 | 7 | 1750 | 175 |
| XHPRT-R | | CTC TCC AGA ATA GTT AGA TGT AGG | 42 | 56 | | | |
| D13S258-F | STR | NED-ACC TGC CAA ATT TTA CCA GG | 45 | 57 | 8 | 2000 | 200 |
| D13S258-R | | GAC AGA GAG AGG GAA TAA ACC | 48 | 56 | | | |
| D13S305-F | STR | PET-AAC TAA TGC AAG GAA ATT TGT GG | 35 | 57 | 12 | 3000 | 300 |
| D13S305-R | | TGA GGA CCT GTC GTT ACG AAT | 48 | 59 | | | |
| D13S1817-F | STR | 6-FAM-ACC GGA CCT CAG ATC TGA AT | 50 | 58 | 20 | 5000 | 500 |
| D13S1817-R | | TGC CTG AGT AAA GGA AGT GG | 50 | 58 | | | |
| D18S386-F | STR | NED-TCA GGA GAA TCA CTT GGA AC | 45 | 55 | 10 | 2500 | 250 |
| D18S386-R | | TCC ATG AAG TAG CTA AGC AG | 45 | 55 | | | |
| D18S390-F | STR | VIC-GCC CAG GAT GAG GAG GTA AT | 55 | 59 | 6 | 1500 | 150 |
| D18S390-R | | TCC TGG TCT GAG GTG TCA TT | 50 | 59 | | | |
| D18S391-F | STR | VIC-TGG ACT TAC CAC AGG CAA TG | 50 | 58 | 5 | 1250 | 125 |
| D18S391-R | | TTC ACT ATT CCC ATC TGA GTC AC | 44 | 58 | | | |
| D18S535-F | STR | NED-AGA CAG AAA TAT AGA TGA GAA TGC A | 32 | 57 | 8 | 2000 | 200 |
| D18S535-R | | TCA TGT GAC AAA AGC CAC AC | 45 | 57 | | | |
| D21S1411-F | STR | VIC-ATG ATG AAT GCA TAG ATG GAT G | 36 | 55 | 10 | 2500 | 250 |
| D21S1411-R | | AAT GTG TGT CCT TCC AGG C | 53 | 58 | | | |
| D21S1414-F | STR | 6-FAM-AAA TTA GTG TCT GGC ACC CAG TA | 44 | 60 | 12 | 3000 | 300 |
| D21S1414-R | | CAA TTC CCC AAG TGA ATT GCC TTC | 46 | 61 | | | |
| D21S1435-F | STR | PET-CCC TCT CAA TTG TTT GTC TAC C | 45 | 57 | 13 | 3250 | 325 |
| D21S1435-R | | GCA AGA GAT TTC AGT GCC AT | 45 | 57 | | | |
| D21S1446-F | STR | PET-ATG TAC GAT ACG TAA TAC TTG ACA A | 32 | 57 | 12 | 3000 | 300 |
| D21S1446-R | | GTC CCA AAG GAC CTG CTC | 61 | 58 | | | |
| SRY-F | fs | 6-FAM-CGG AGA AGC TCT TCC TTC CT | 55 | 59 | 6,5 | 1625 | 162,5 |
| SRY-R | | TAA GTG GCC TAG CTG GTG CT | 55 | 62 | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| MYPT2/Y-F | fs | 6-FAM-CTC ACT ACA TGA CAT TCA GG | 45 | 54 | 5 | 1250 | 125 |
| MYPT2/Y-R | | GTT TCT TCC CAG TAT CTA GTA CAG TGC | 45 | 62 | | | |
| DYS448-F | STR | PET-CAA GGA TCC AAA TAA AGA ACA GAG A | 36 | 58 | 10 | 2500 | 250 |
| DYS448-R | | GGT TAT TTC TTG ATT CCC TGT G | 41 | 56 | | | |
| CDY1/2-F | fs | PET-TAT TGA GAC CCT TGC ACC TG | 50 | 58 | 2 | 500 | 50 |
| CDY1/2-R | | GTT TCT TGG AGT TTC CCT TCT GTC ACC | 48 | 65 | | | |
| | | | | | 176,5µl+23.5µl H2O | | |

2.2 Perform main QF-PCR reaction in volume of 20 µl consisting of 1X AmpliTaq Gold 360 Buffer (Applied Biosystems Inc), 1.875 mM MgCl2, 187.5 µM each of the four dNTPs (dATP, dCTP, dGTP, and dTTP), 1.5 µl 360 GC Enhancer (Applied Biosystems Inc), 50-667 nM primers (Table 1), 0.75 U AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems Inc) and 10-50 ng DNA (variable amount of DNA concentration with a minimum of 2 ng/µl for succsesful multiplex PCR).

- Mix for each sample:

| | |
|---|---|
| *H2O* | *6,30 µl* |
| *10x PCR Buffer* | *2 µl* |
| *25 mM MgCl2* | *1,5 µl* |
| *2.5mM dNTP* | *1,5 µl* |
| *GC rich enchancer* | *1,5 µl* |
| *PCR primer mix* | *2 µl* |
| *Taq Gold 360 DNA polymerase (5U/µL)* | *0,2 µl* |
| *DNA + H2O(if if required)* | *5 µl* |
| *Total* | *20 µl* |

- Thermal cycling conditions:

| | |
|---|---|
| *- Initial denaturation* | *95°C 10'* |
| *x 29 cycles* | |
| *- Denaturation* | *95°C 45"* |
| *- Annealing* | *58°C 1'* |
| *- Extension* | *72°C 1'30"* |
| *- Final Extension* | *60 °C 30'* |
| *- Final hold* | *4 °C* |

- Perform PCR reaction in Thermal Cycler (*"AB 2720"* or *"Veriti 96 well"* Thermal Cyclers in our case).

2.3 Capillary electrophoresis of the PCR products is performed on ABI Genetic Analyzers, in our case with ABI 3500 and ABI 3130. The procedure for preparation of the sample for capillary electrophoresis is same for both instruments:

- Combine 1 µl of the finished PCR reaction with 12 µl of Hi-Di Formamide and 0.15 µl of GeneScan 500 LIZ Size Standard;
- Denature on 95°C for 10' and place on ice;
- Run on ABI Genetic analyzer for automatic capillary electrophoresis and analyze with GeneMapper analysis software according to manufacturer's protocol.

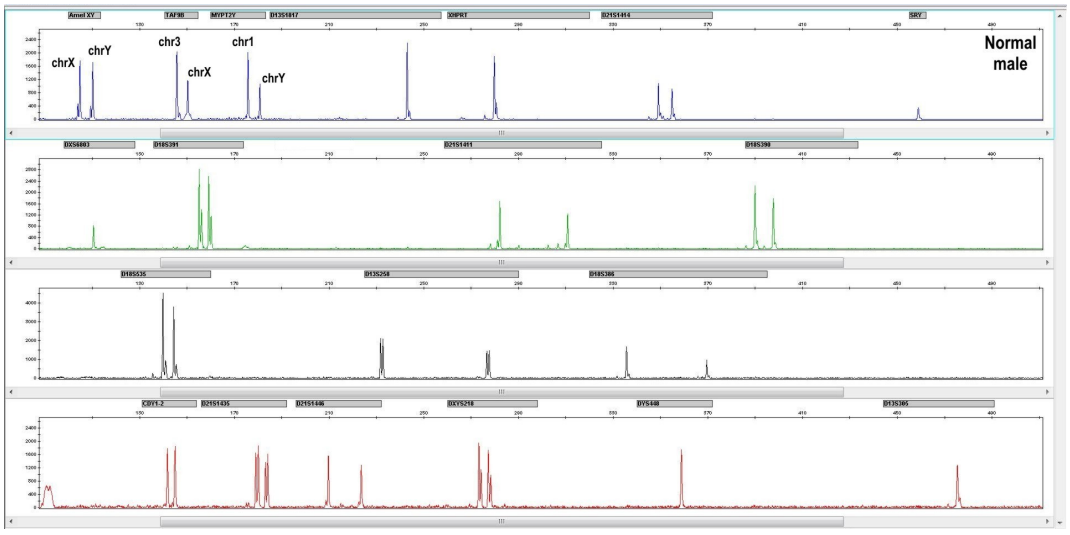
Technical specifications for running the automatic capillary electrophoresis on the ABI 3500 Genetic analyzer are:
- Polimer: POP-7;
- Capillary array: 50 cm, 8-Capillary;
- Dye Set: G5;
- Oven temperature: 60 °C;

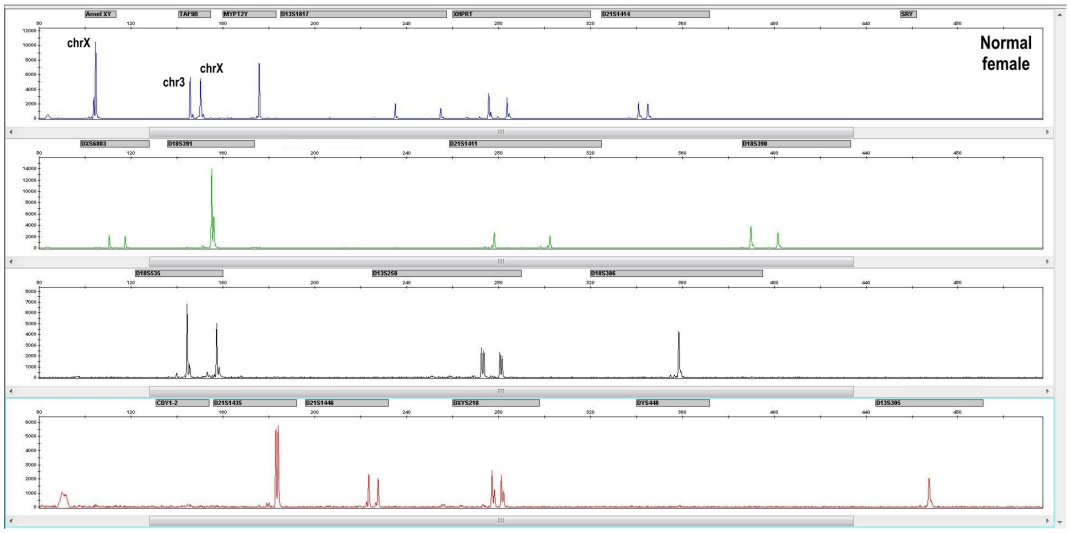
- Injection voltage: 1.6 kVolts;
- PreRun voltage: 15 kvolts;
- PreRun time: 180 sec.;
- Injection time: 3 sec;
- Run voltage: 19.5 kvolts;
- Run time: 1200 sec.

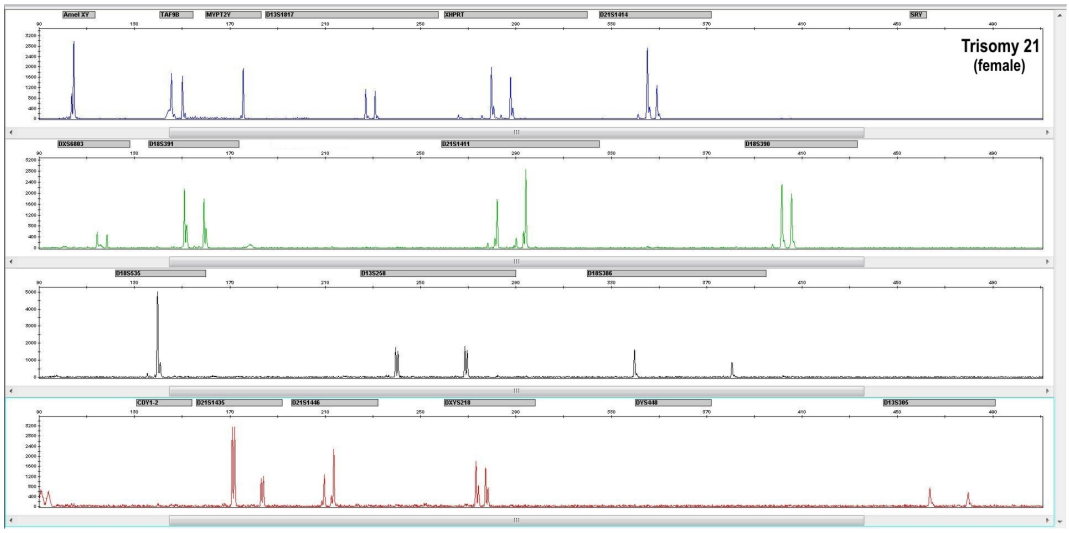
Technical specifications for running the automatic capillary electrophoresis on the ABI 3130 Genetic analyzer are:

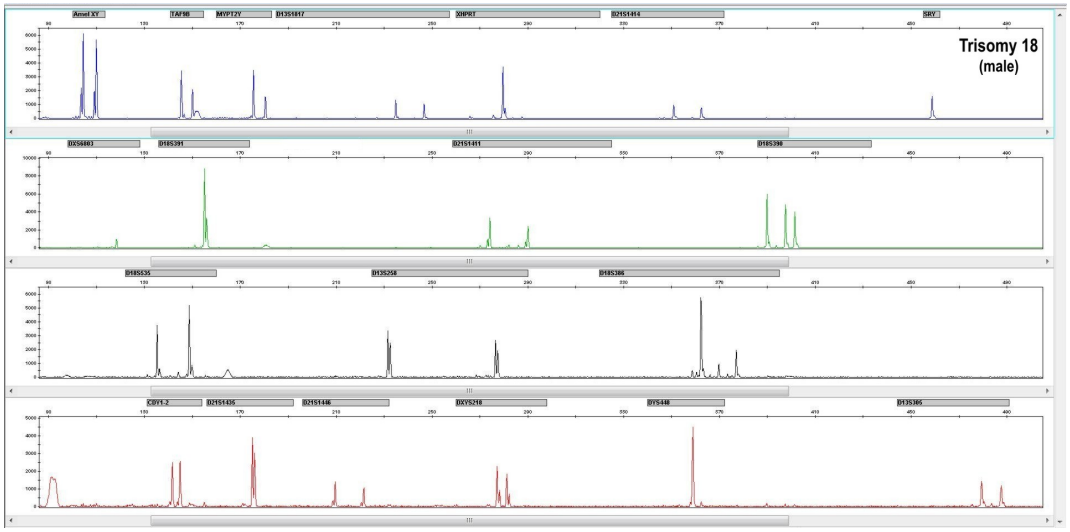
- Polimer: POP-4;
- Capillary array: 36 cm, 4-Capillary;
- Dye Set: G5;
- Oven temperature: 60 $^{O}$C;
- Injection voltage: 1.2 kVolts;
- PreRun voltage: 15 kvolts;
- PreRun time: 180 sec.;
- Injection time: 8 sec;
- Run voltage: 15 kvolts;
- Run time: 2500 sec.

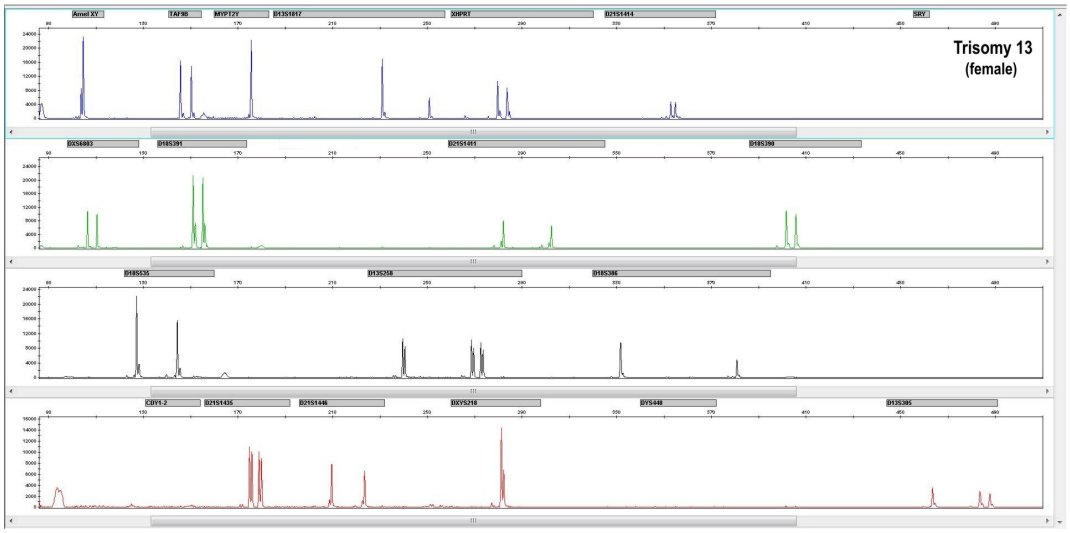
Examples for electropherograms from normal male, normal female, Trisomy 21, Trisomy 18, Trisomy 13, Triploidy, Klinefelter sy., Turner sy. and Trisomy X samples are shown below:

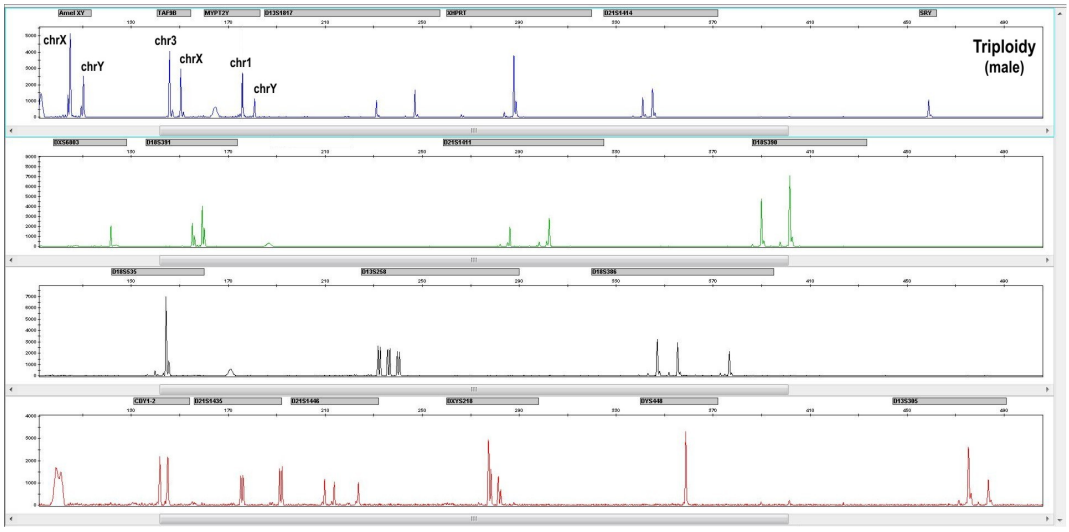


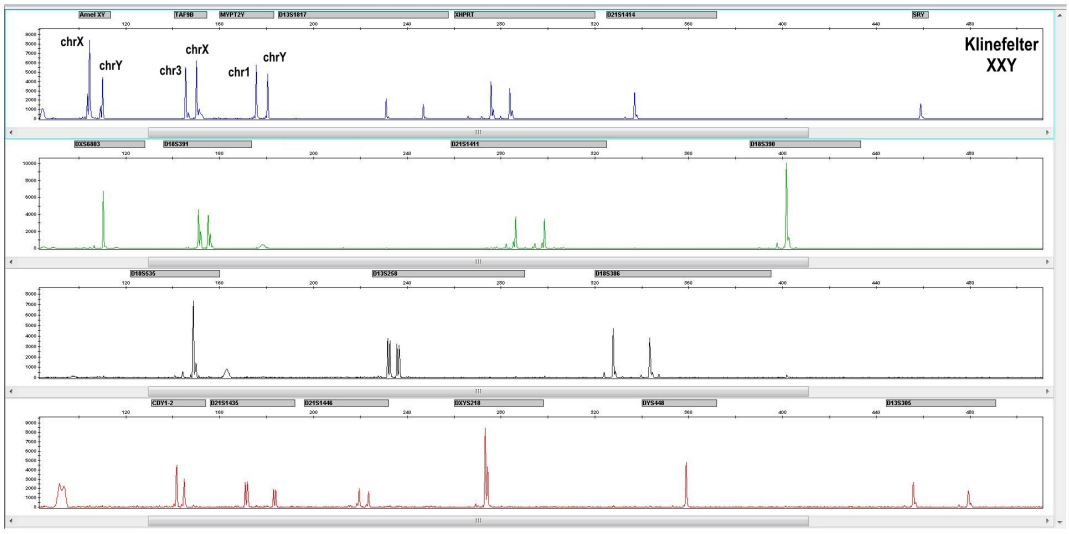



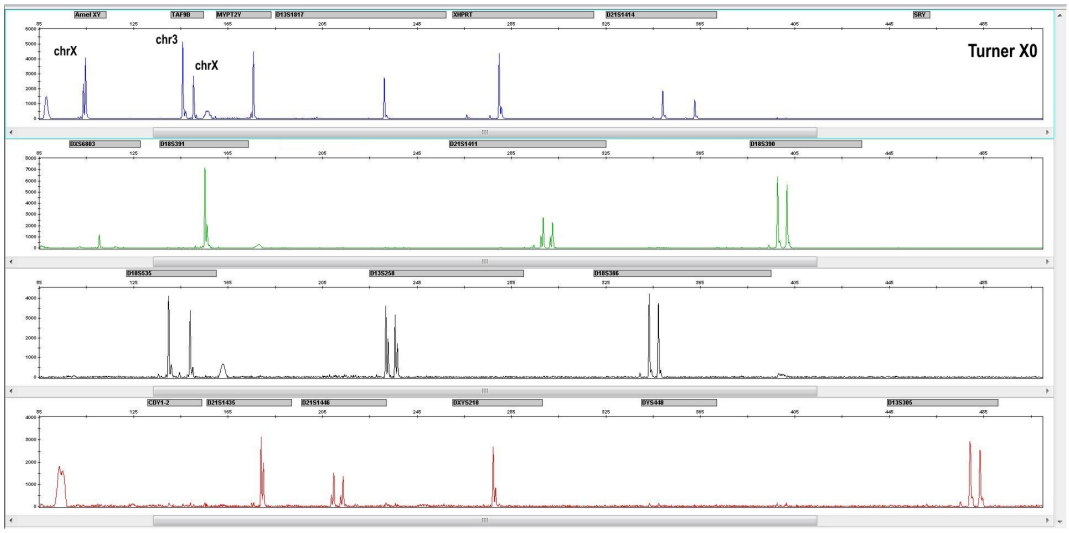


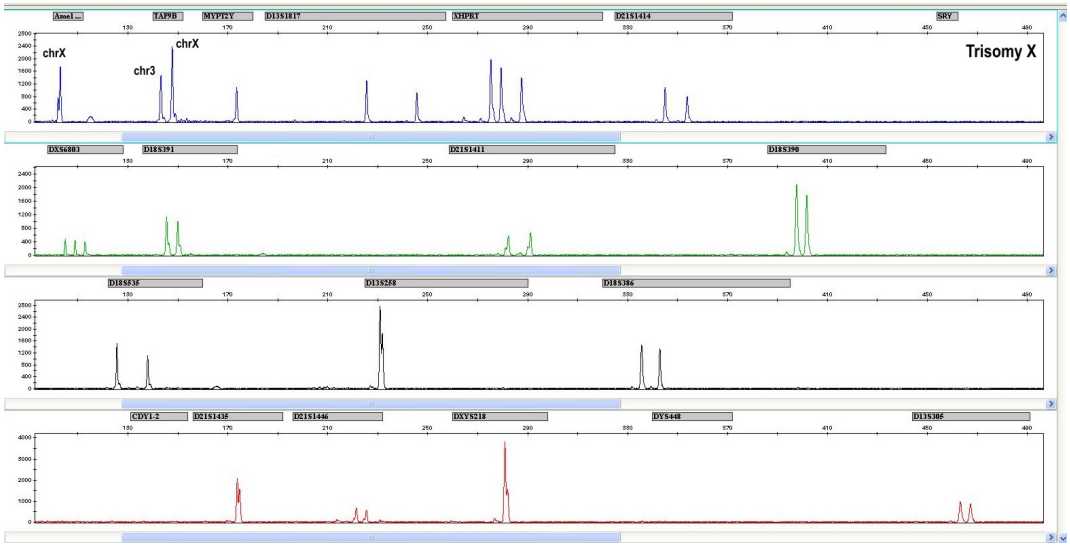








## Additional QF-PCR reactions specific for given chromosome

3

In this section, tecnical details are given about the chromosome specific backup markers. For chromosomes 13, 18 and 21, markers are combined in separate mixes, while for the sex chromosomes three markers are analyzed in single reactions.

Steps for performing QF-PCR reactions specific for given chromosome are essentialy the same as main QF-PCR reaction and consists of the following steps:

3.1) Prepare QF-PCR multiplex primer mix / singleton mix;
3.2) Perform PCR reaction;
3.3) Capillary electrophoresis of the PCR product and analysis of the results.

3.1 QF-PCR primer mixes for chromosomes 13, 18 and 21 are prepared in a total volume of 60 µl while primers for sex chromosomes are prepared as singletons with a concentration of 10 pmol/µl of the forward+reverse primer.

Sequences of the primers, their GC percentages, melting points and amount of each primer used in preparation of the QF-PCR mix are shown in the folowing table:

| Target chromosome | Name | Sequence 5 `-> 3` | GC % | Tm 0C | Amount (in µl) used in the primer mix from 50 pmol/µl of Forward/Reverse primer stocks | Concentration in the QF-PCR mix (nM) | Final concentration in the QF-PCR reaction (nM) |
|---|---|---|---|---|---|---|---|
| Chr 13 | D13S742-F | VIC-ATA ACT GGG CTA GGA ATG GAA ATA | 38 | 60 | 4 | 3333.3 | 166.7 |
| Chr 13 | D13S742-R | GAC TTC CCA ATT CAG GAG GAC T | 50 | 62.1 | | | |
| Chr 13 | D13S628-F | 6-FAM-TAA CAT TCA TTG TCC CTT ACA GAT | 33 | 58.3 | 12 | 10000.0 | 500.0 |
| Chr 13 | D13S628-R | GCA AGG CTA TCT AAC GAT AAT TCA | 38 | 60.3 | | | |
| Chr 13 | D13S634-F | VIC-TCC AGA TAG GCA GAT TCA AT | 40 | 54.3 | 16 | 13333.3 | 666.7 |
| Chr 13 | D13S634-R | CCT TCT TCT TCC CAT TGA TA | 40 | 54.3 | | | |
| Chr 13 | D13S631-F | VIC-GGC AAC AAG AGC AAA ACT CT | 56.84 | 45 | 4 | 3333.3 | 166.7 |
| Chr 13 | D13S631-R | TAG CCC TCA CCA TGA TTG G | 56.47 | 52.63 | | | |
| Chr 13 | D13S258-F | NED-ACC TGC CAA ATT TTA CCA GG | 45 | 56.8 | 2 | 1666.7 | 83.3 |
| Chr 13 | D13S258-R | GAC AGA GAG AGG GAA TAA ACC | 47.62 | 55.7 | | | |
| | | | | | = 38 µl + 22 µl H2O | | |
| Chr 18 | D18S51-F | NED-GAG CCA TGT TCA TGC CAC TG | 55 | 60.5 | 4 | 3333.3 | 166.7 |
| Chr 18 | D18S51-R | CAA ACC CGA CTA CCA GCA AC | 55 | 60.5 | | | |
| Chr 18 | D18S1367-F | 6-FAM-TTG GTT CAT CCA AAC ATG GT | 40 | 54.3 | 4 | 3333.3 | 166.7 |
| Chr 18 | D18S1367-R | ATA ACT GCA GAG AAC GTT GC | 45 | 56.4 | | | |
| Chr 18 | D18S978-F | VIC-TTC TTC AGT ATC ATC TTG TGC C | 41 | 58.4 | 6 | 5000.0 | 250.0 |
| Chr 18 | D18S978-R | GCC AAATGT AGA TCT TGG GA | 45 | 56.4 | | | |
| Chr 18 | D18S391-F | VIC-TGG ACT TAC CAC AGG CAA TG | 50 | 58.4 | 2 | 1666.7 | 83.3 |
| Chr 18 | D18S391-R | TTC ACT ATT CCC ATC TGA GTC AC | 43.5 | 58.2 | | | |
| | | | | | = 16 µl + 44 µl H2O | | |
| Chr 21 | D21S1437-F | PET-ATG TAC ATG TGT CTG GGA AGG | 48 | 59.5 | 4 | 3333.3 | 166.7 |
| Chr 21 | D21S1437-R | TTC TCT ACA TAT TTA CTG CCA ACA | 33 | 58.3 | | | |
| Chr 21 | D21S11-F | NED-TAT GTG AGT CAA TTC CCC AAG TGA | 42 | 62 | 4 | 3333.3 | 166.7 |
| Chr 21 | D21S11-R | GTT GTA TTA GTC AAT GTT CTC CAG | 38 | 60.3 | | | |
| Chr 21 | D21S1412-F | 6-FAM-CGG AGG TTG CAGTGA GTT G | 58 | 59.5 | 4 | 3333.3 | 166.7 |
| Chr 21 | D21S1412-R | GGG AAGGCT ATG GAG GAG A | 58 | 59.5 | | | |
| Chr 21 | D21S1441-F | 6-FAM-CAA AGC TGC AGT GAG CTG TA | 50 | 58.4 | 4 | 3333.3 | 166.7 |
| Chr 21 | D21S1441-R | CTC AGG GAA CTG ATG GTC AC | 55 | 60.5 | | | |
| Chr 21 | D21S1411-F | VIC-ATG ATG AAT GCA TAG ATG GAT G | 36.4 | 54.8 | 4 | 3333.3 | 166.7 |
| Chr 21 | D21S1411-R | AAT GTG TGT CCT TCC AGG C | 52.6 | 58.2 | | | |
| | | | | | = 20 µl + 40 µl H2O | | |
| Chr X+Y | X-22-F | 6-FAM-TAA TGA GAG TTG GAA AGA AA | 30 | 50.2 | / | / | 500.0 |
| Chr X+Y | X-22-R | CCC ATT GTT GCT ACT TGA GA | 45 | 56.4 | | | |
| Chr X | DXS6809-F | VIC-TGA ACC TTC CTA GCT CAG GA | 50 | 58.4 | / | / | 500.0 |
| Chr X | DXS6809-R | TCT GGA GAA TCC AAT TTT GC | 40 | 54.3 | | | |
| Chr X | DXS996-F | VIC -AAATTCTTGCTTAGGCCACTCTAGG | 44 | 61 | / | / | 500.0 |
| Chr X | DXS996-R | GTTGTTCTGGATCGTATGCTAGG | 48 | 59 | | | |

3.2 Perform main QF-PCR reaction in volume of 20 µl consisting of 1X AmpliTaq Gold 360 Buffer (Applied Biosystems Inc), 1.875 mM MgCl2, 187.5 µM each of the four dNTPs (dATP, dCTP, dGTP, and dTTP), 1.5 µl 360 GC Enhancer (Applied Biosystems Inc), 83-500 nM primers (Table 1), 0.75 U AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems Inc) and 10-50 ng DNA (variable amount of DNA concentration with a minimum of 2 ng/µl for succsesful multiplex PCR).

- Mix for each sample:

| | |
|---|---|
| *H2O* | *7,30 µl* |
| *10x PCR Buffer* | *2 µl* |
| *25 mM MgCl2* | *1,5 µl* |
| *2.5mM dNTP* | *1,5 µl* |
| *GC rich enchancer* | *1,5 µl* |
| *PCR primer mix* | *1 µl* |
| *Taq Gold 360 DNA polymerase (5U/µL)* | *0,2 µl* |
| *DNA + H2O(if required)* | *5 µl* |
| *Total* | *20 µl* |

- Thermal cycling conditions:

| | |
|---|---|
| *- Initial denaturation* | *95°C 10'* |
| *x 29 cycles* | |
| *- Denaturation* | *95°C 45"* |
| *- Annealing* | *58°C 1'* |
| *- Extension* | *72°C 1'30"* |
| *- Final Extension* | *60°C 30'* |
| *- Final hold* | *4°C* |

- Perform PCR reaction in Thermal Cycler (*"AB 2720"* or *"Veriti 96 well"* Thermal Cyclers in our case).

3.3 Capillary electrophoresis of the PCR products is performed on ABI Genetic Analyzers, in our case with ABI 3500 and ABI 3130. The procedure for preparation of the sample for capillary electrophoresis is same for both instruments:

- Combine 1 µl of the finished PCR reaction with 12 µl of Hi-Di Formamide and 0.15 µl of GeneScan 500 LIZ Size Standard;
- Denature on 95°C for 10' and place on ice;
- Run on ABI Genetic analyzer for automatic capillary electrophoresis and analyze with GeneMapper analysis software according to manufacturer's protocol.

Technical specifications for running the automatic capillary electrophoresis on the ABI 3500 and ABI 3130 Genetic analyzers are same as for main QF-PCR reaction.

Examples for electropherograms from samples with Trisomy 21, Trisomy 18 and Trisomy 13 are shown below: