What is in our toolbox for PGT?

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Universitair Ziekenhuis Brussel

PGT-M

- Haplotyping by MF-PCR
- Haplotyping by SNP array
- Direct mutation testing (+ haplotyping)
  - → SNVs (e.g. *HBB*)
  - → Small deletions and duplications (<20bp) (e.g. *CFTR*)
  - → Repeat disorders (e.g. *HTT*)
  - → Duplication (e.g. CMT1A *PMP22*)
  - → Junction PCR (e.g. large intragenic deletions)

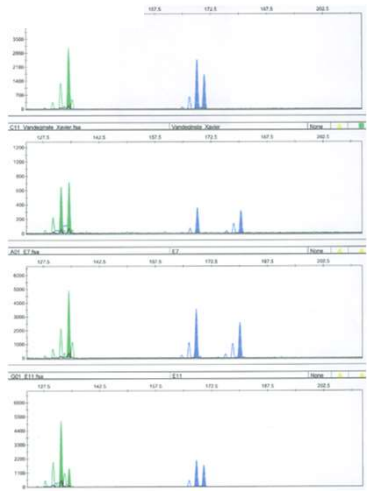
Multiplex Fluorescent PCR (MF-PCR)

- Targeted haplotyping
- +/- direct mutation testing
- D3 biopsy
- -> Fresh transfer D5 possible
- Analysis cannot be repeated on same biopsy

MF-PCR

- D3 biopsy -> PCR -> Fragment length analysis -> D5/6 fresh transfer or cryo

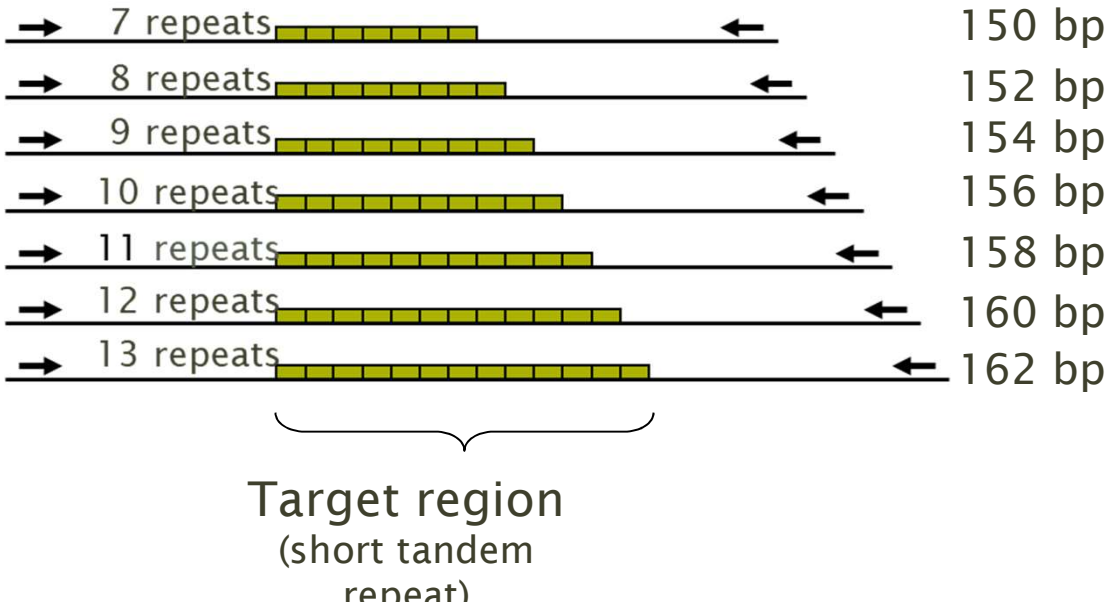
+polymerase
dNTP's,
fluorescent
primers,
buffer



MF-PCR using STRs

- STR: Short Tandem Repeat
- variation in number of 2, 3 or 4 bp repeats -> many alleles

```
ttactgccag ttggtccgct atctctgtca aaatggacgc tgcattccaa ctcctgggag
gagaaaaacc cctgtgctgg cacacacaca cacacacaca tggtgagtgt attaacaacc
agggttcgta cacctgtcag tgccgagctg gatatcagag cacactcacg cggacagaat
```



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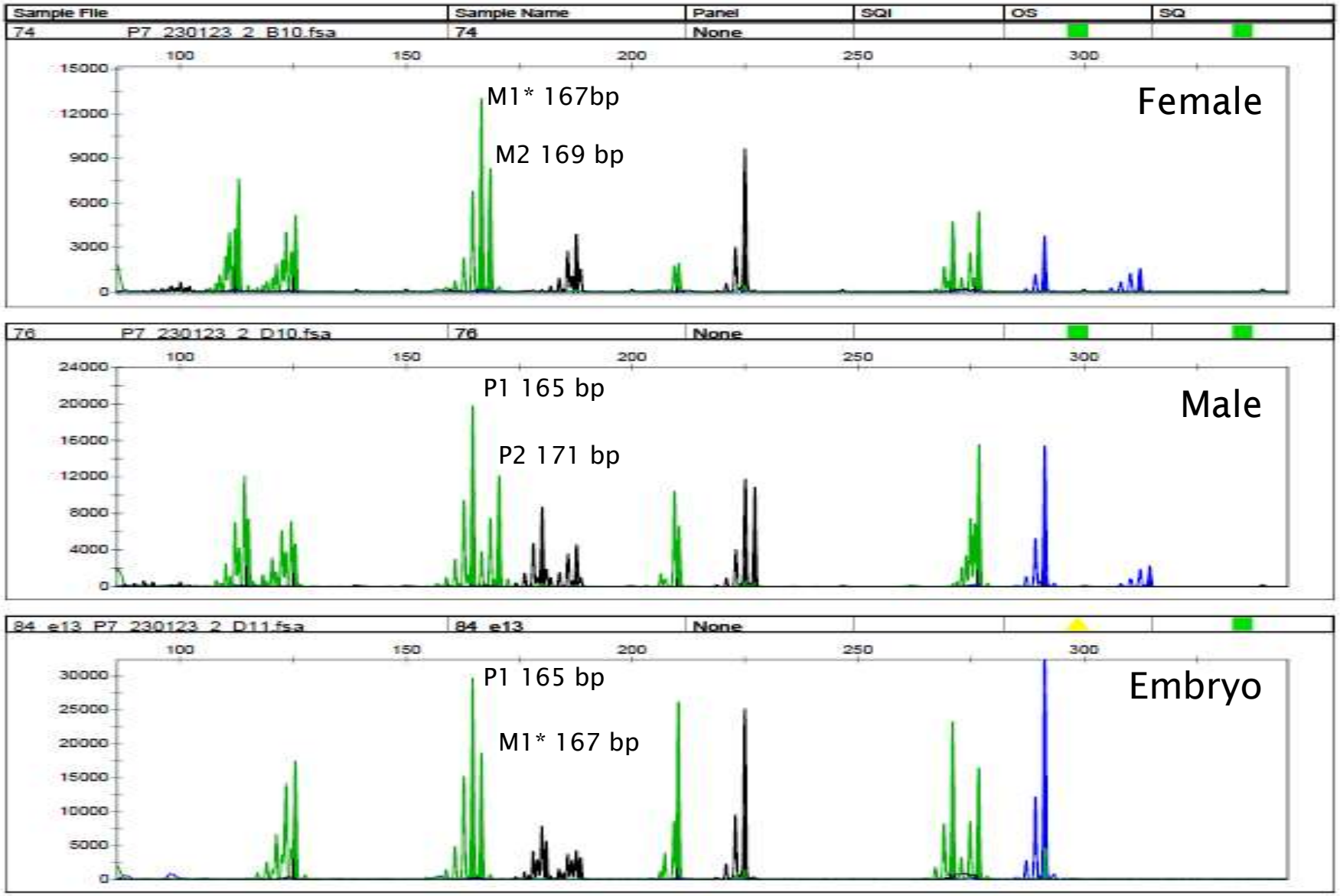
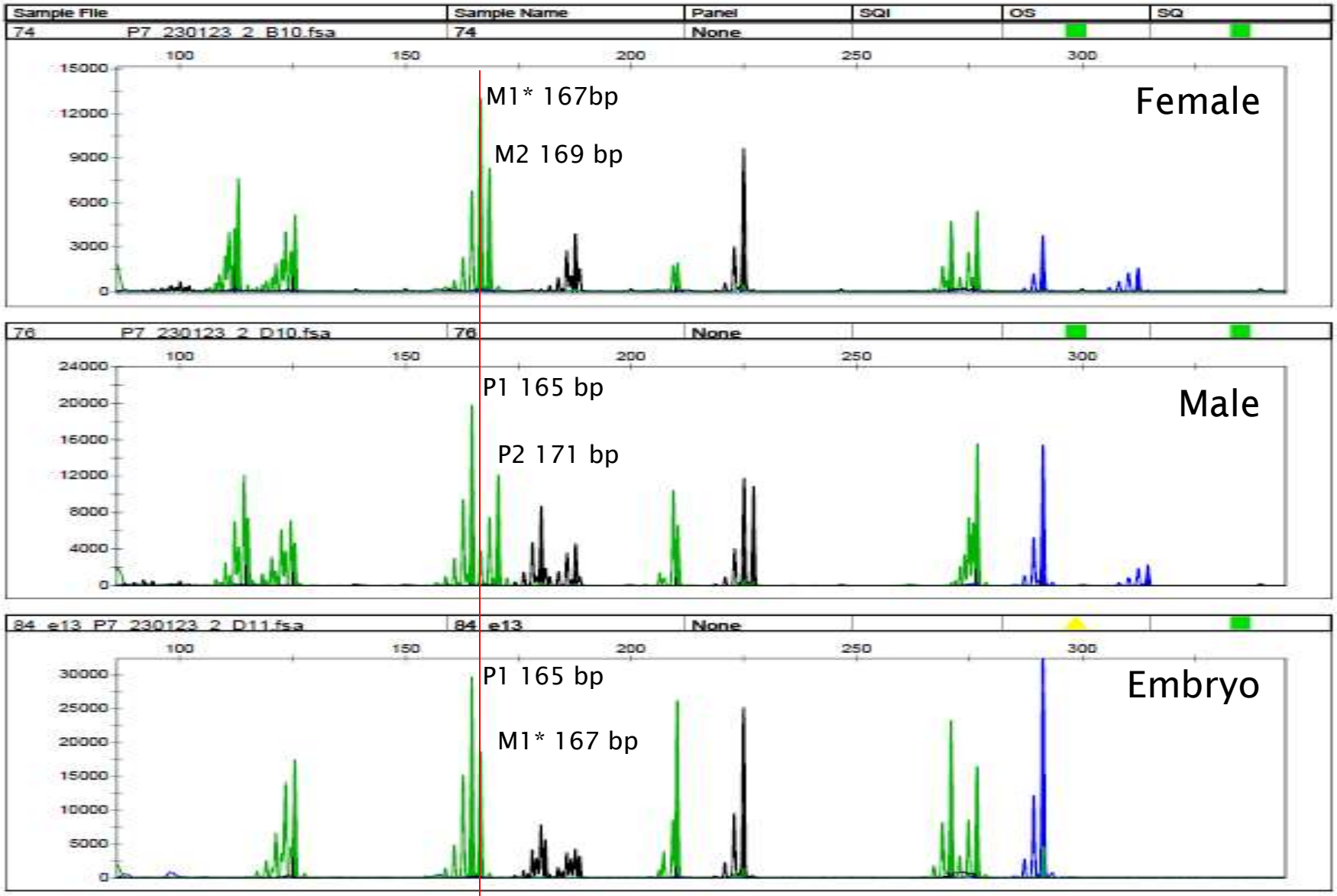
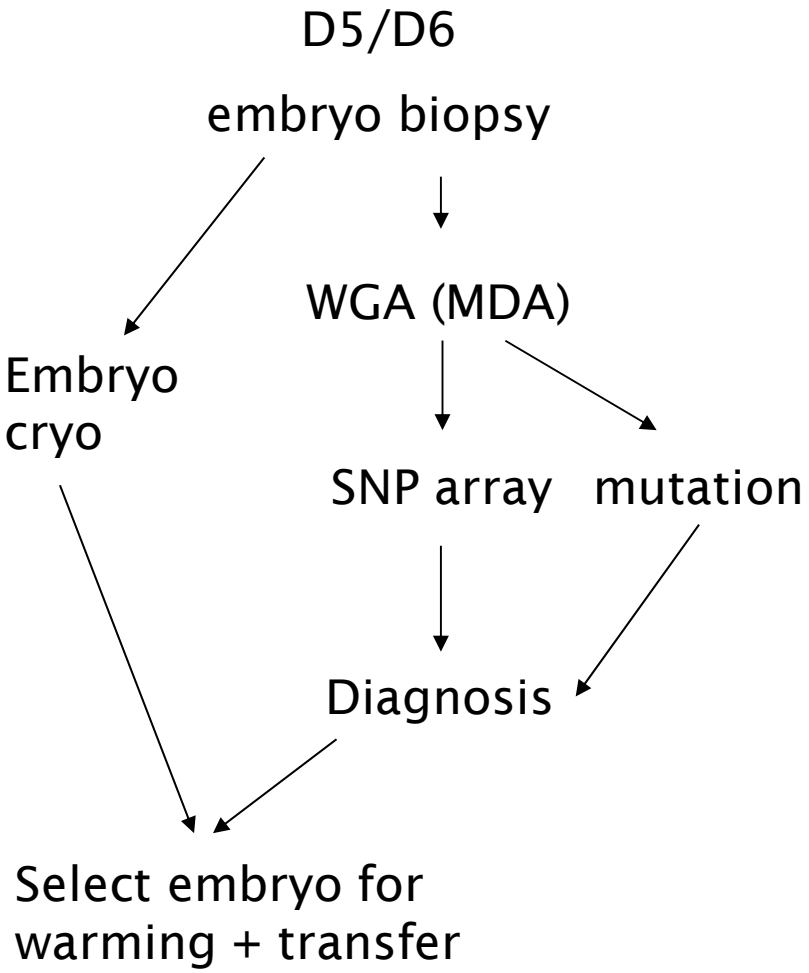
Multiplex Fluorescent PCR (MF-PCR)

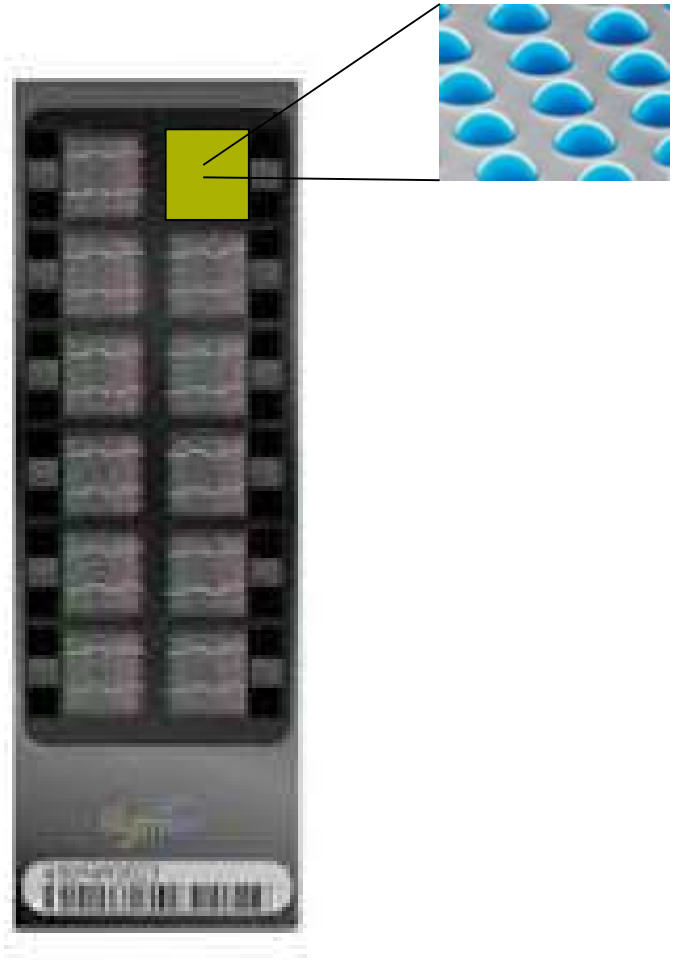


Multiplex Fluorescent PCR (MF-PCR)



SNP array – Vitrolife Karyomapping





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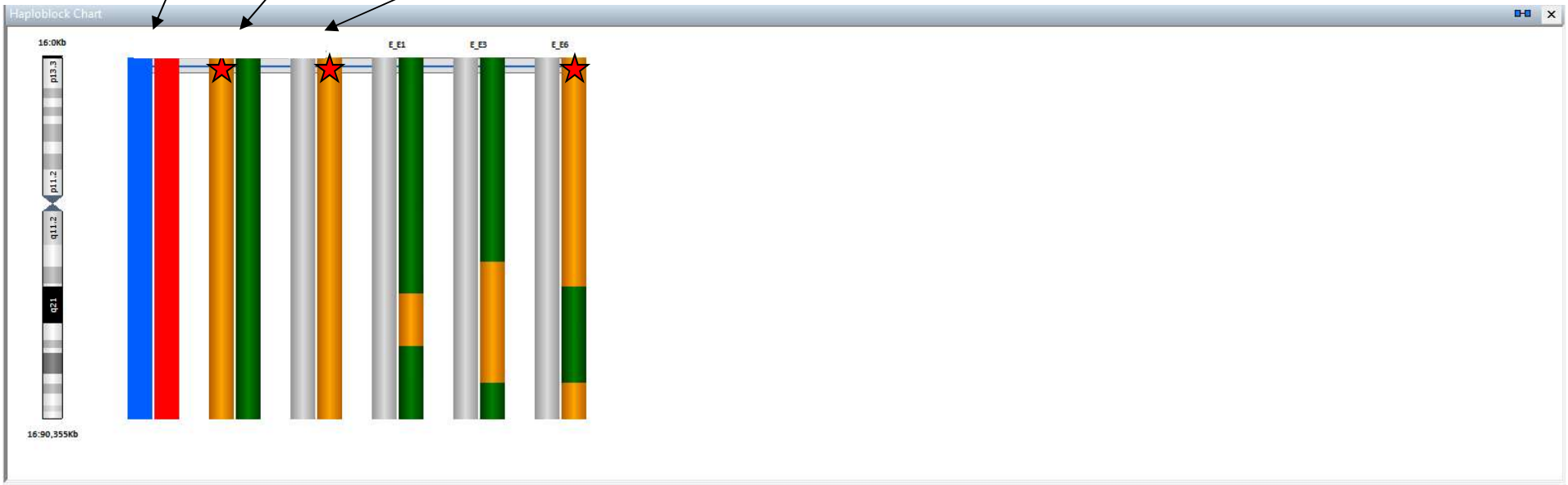
SNP vs STR

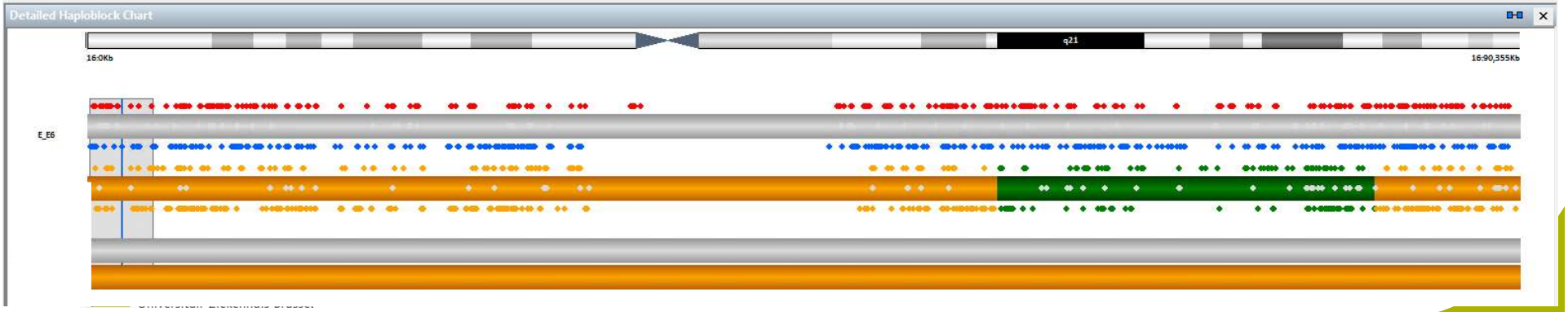
- SNP:
  - → AGTCATGGGCAGCCTGTT
  - → AGTCATGGACAGCCTGTT

- STR
  - → AGTCATGG<u>CACACACACACACACACACACACACACACACACA</u>GCCTGTT (17xCA)
  - → AGTCATGG<u>CACACACACACACACACACACACACACACACA</u>GCCTGTT (15xCA)

Example SNP array (PKD1)

Male Female* Mother of female*





SNP array

- Genome wide haplotyping
- Can be combined with direct mutation testing on WGA (MDA)
- D5/D6 biopsy (trophectoderm)
- PGT-M can be combined with PGT-SR and PGT-A

Direct mutation testing

- MF-PCR
- SNP array

} Haplotyping

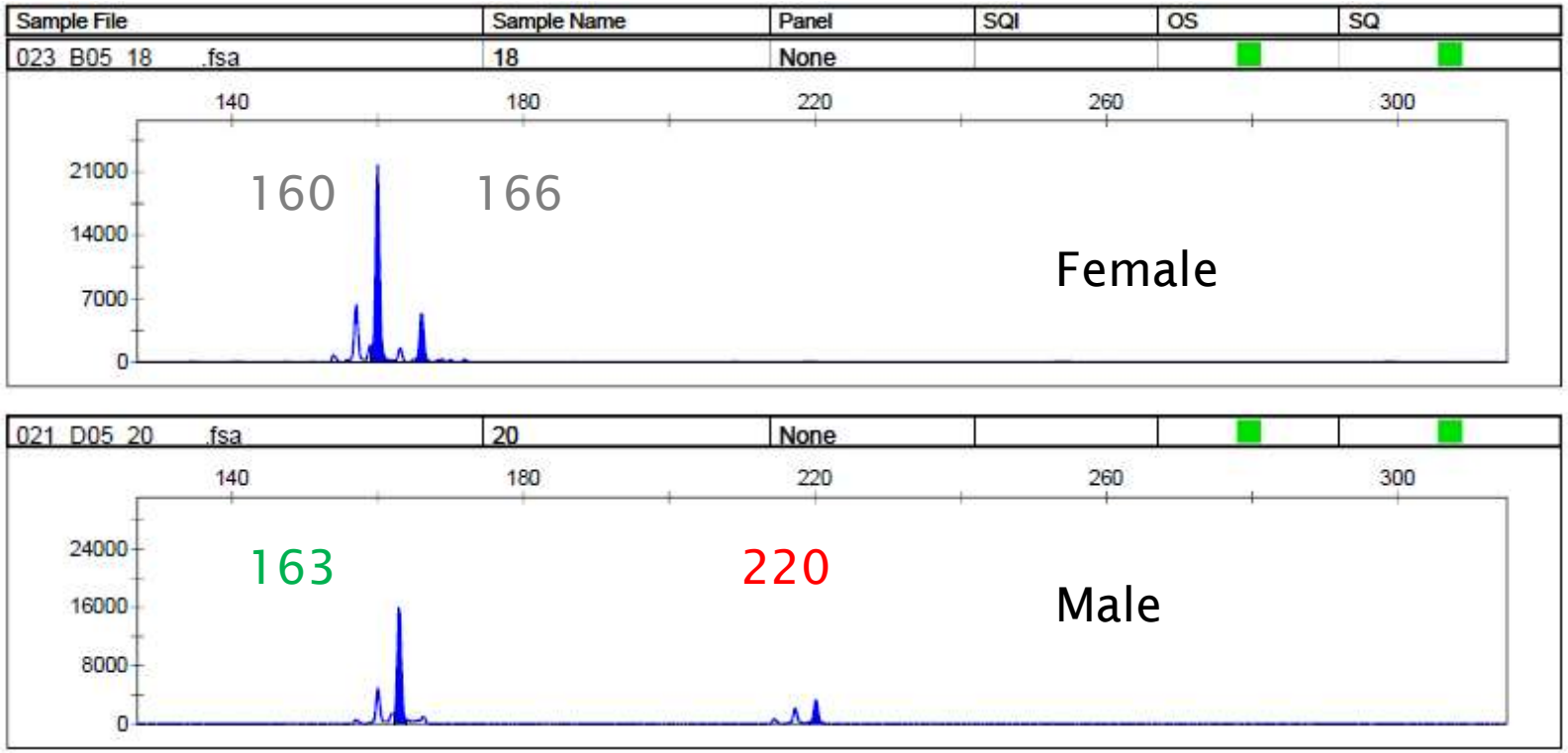
→ PGT cannot be applied for all couples with only haplotyping

→ Direct mutation testing can be required

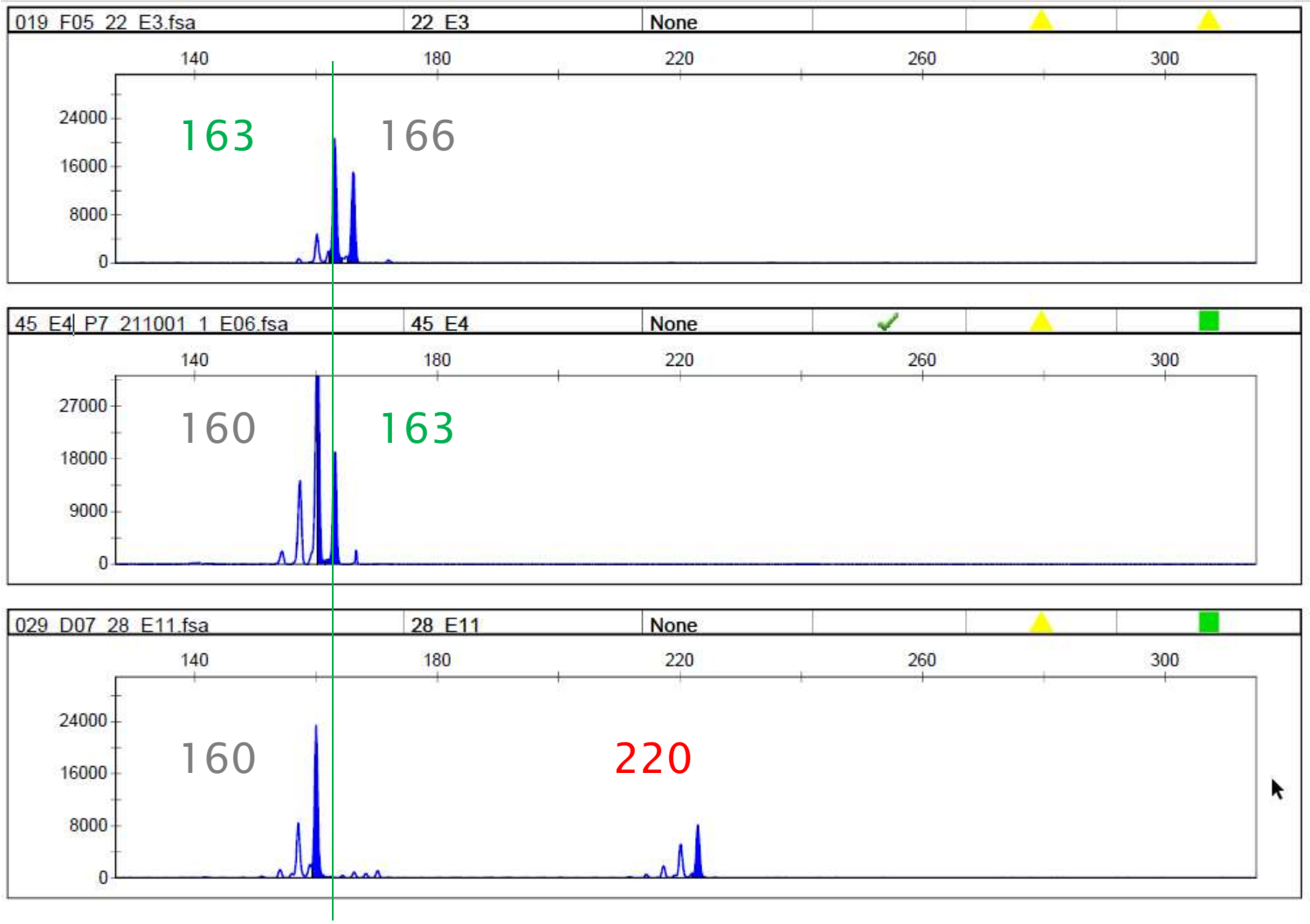
- MF-PCR: include mutation fragment in multiplex
- SNP array: PCR on WGA

Repeat expansion disorder

- Example *HTT*, *DMPK*, *FMR1*,…



Repeat expansion disorder



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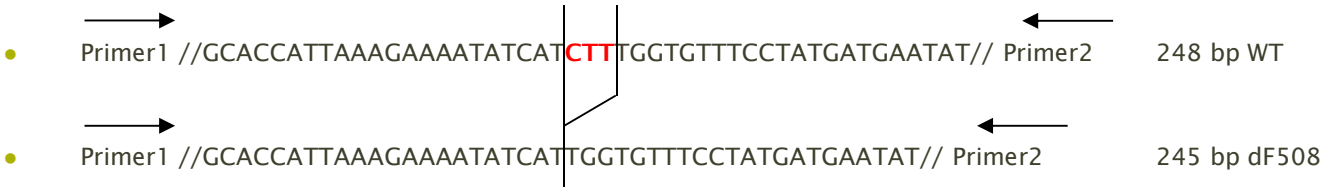
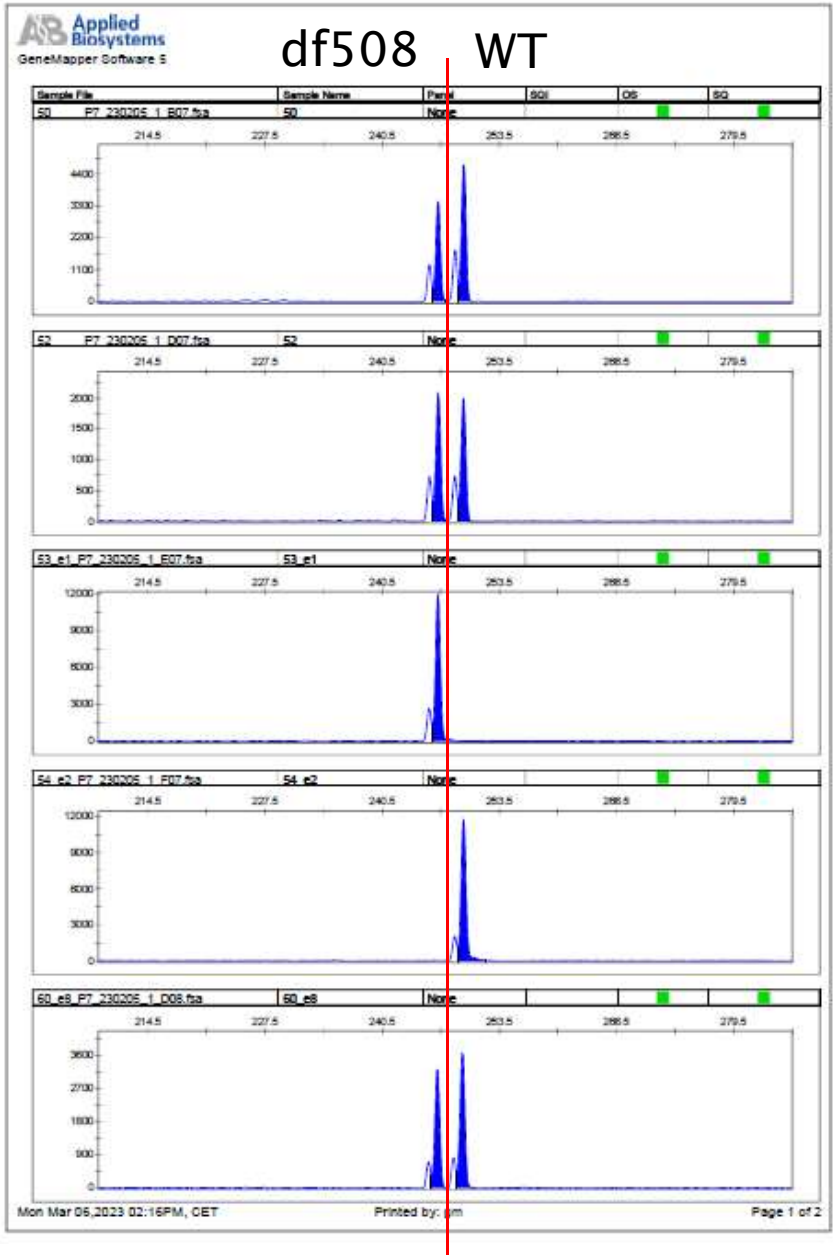
Direct mutation testing – small del/dup

- Example *CFTR* c.1521_1523delCTT (dF508)





Carrier (female)

Carrier (male)

Affected embryo

Unaffected embryo

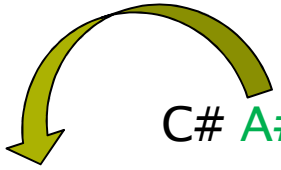
Carrier embryo

Direct mutation testing - SNV

5’-ATGTCTGACCGTCCAGTGTGCCGTC-3’ Fw-strand

3’-TACAGACTGGCAGGTCACACGGCAG-5’ Rv-strand

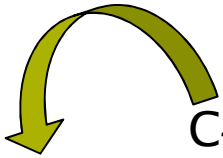
Mut A -> C



WT template C# A# T# G#

5’-ATGTCTGACCGT Fw-primer

3’-TACAGACTGGCATGTCACACGGCAG-5’ Rv-template



Template with mut C# A# T# G#

5’-ATGTCTGACCGT Fw-primer

3’-TACAGACTGGCAGGTCACACGGCAG-5’ Rv-template

Direct mutation testing - SNV

5’-ATGTCTGACCGTMCAGTGTGCCGTC-3’ Fw-strand

3’-TACAGACTGGCAKGTCACACGGCAG-5’ Rv-strand

Mut A -> C

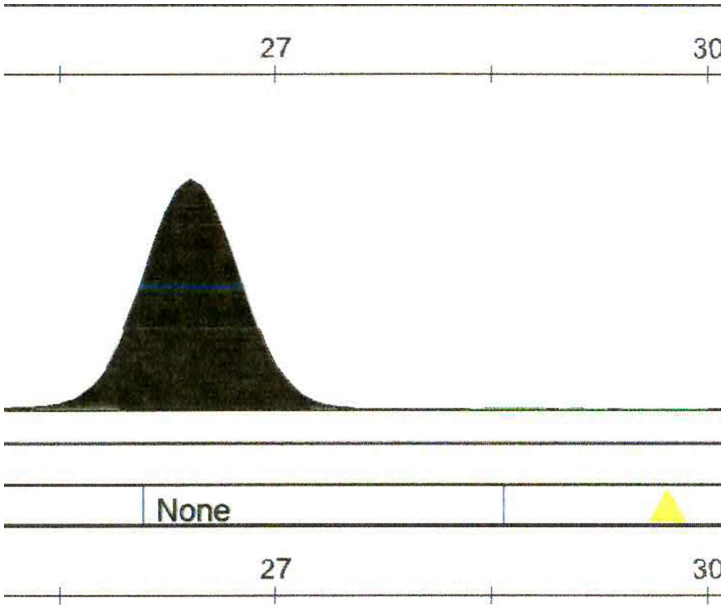
5’-ATGTCTGACCGTA# Fw-primer

3’-TACAGACTGGCATGTCACACGGCAG-5’ Rv-template

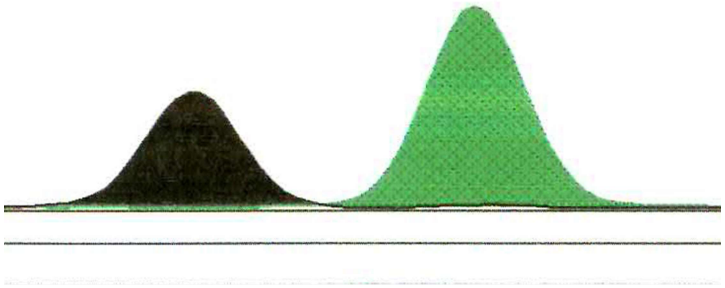
5’-ATGTCTGACCGTC# Fw-primer

3’-TACAGACTGGCAGGTCACACGGCAG-5’ Rv-template

Direct mutation testing - SNV



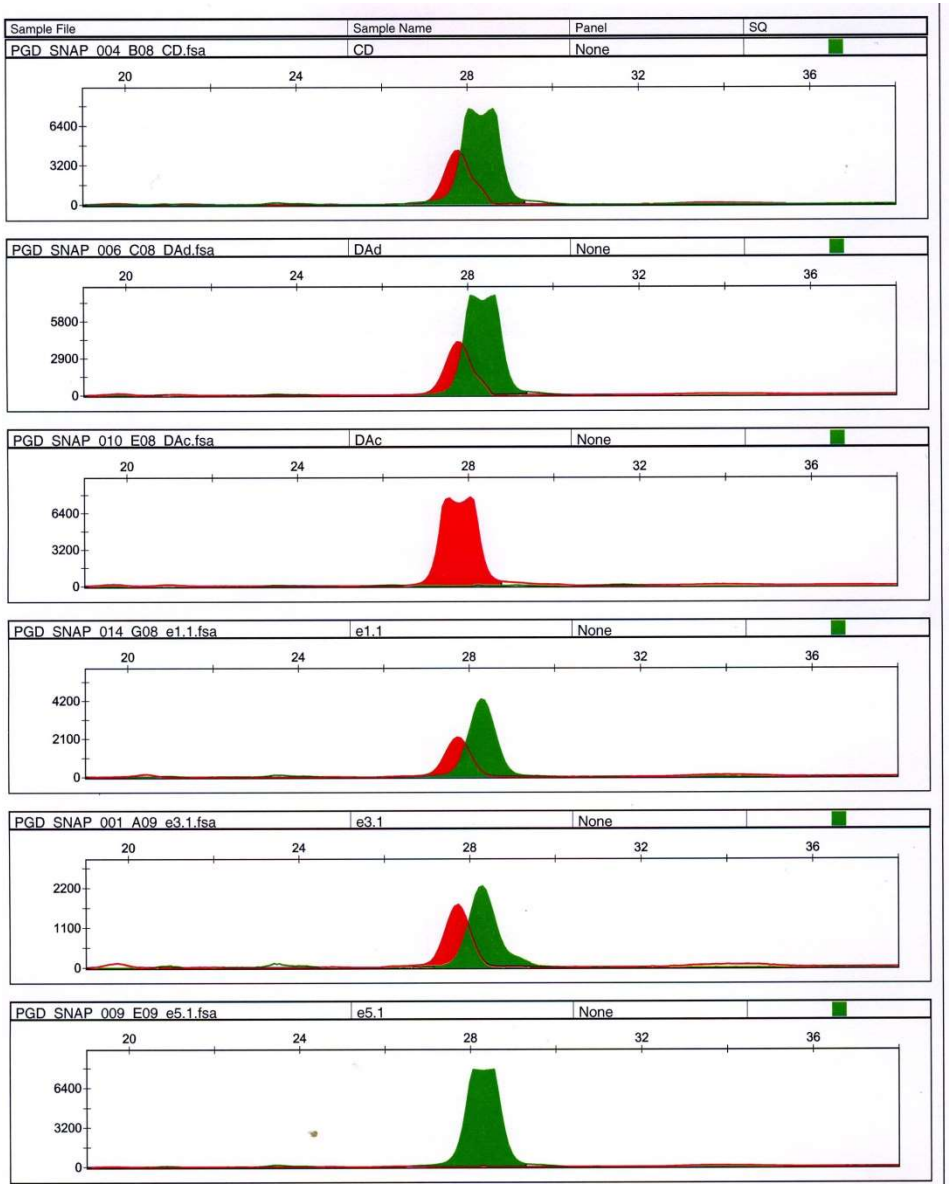
C or C/C



C/A

Direct mutation testing - SNV

c.20A>T
of sickle cell
mutation
in *HBB*



genomicDNA
of carrier
mother

genomic DNA
of carrier
father

genomic DNA
of affected
child

cell from a
carrier embryo (E1)

cell from a
carrier embryo (E3)

cell from
homozygous
normal embryo (E5)

PGT-SR

- Shallow genome sequencing
- SNP array
  - → Haplotyping / Copy number / BAF
  - → Workup required
  - → Distinguish normal from balanced SR
- Direct detection of SR junctions

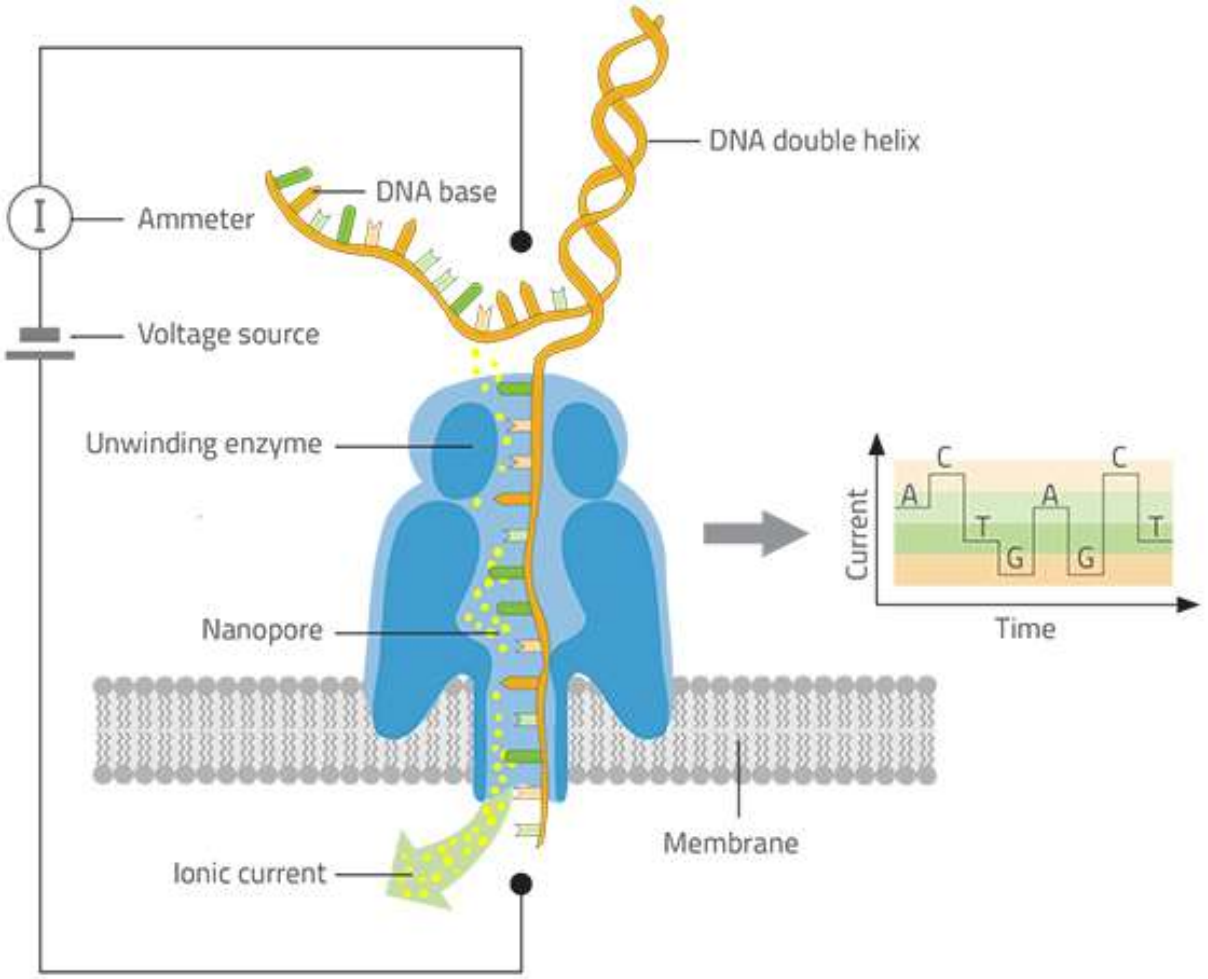
Direct detection of SR - problem

- Unbalanced SR: Shallow genome sequencing has a resolution of 0,5-5Mb.
  → Smaller deletions and duplications are not detected
- Direct detection of balanced structural rearrangements is difficult if not characterized to the molecular (bp) level
  → No simple targeted test can be developed

Direct detection of SR - solution

- Long read sequencing
  - → Oxford Nanopore Technologies platform
  - → Generate long reads across the genome
  - → Find 'Split reads' at the locus of interest
  - → Determine rearrangement to the molecular level.
- Develop tailored PCR to detect the rearrangement directly

Nanopore sequencing



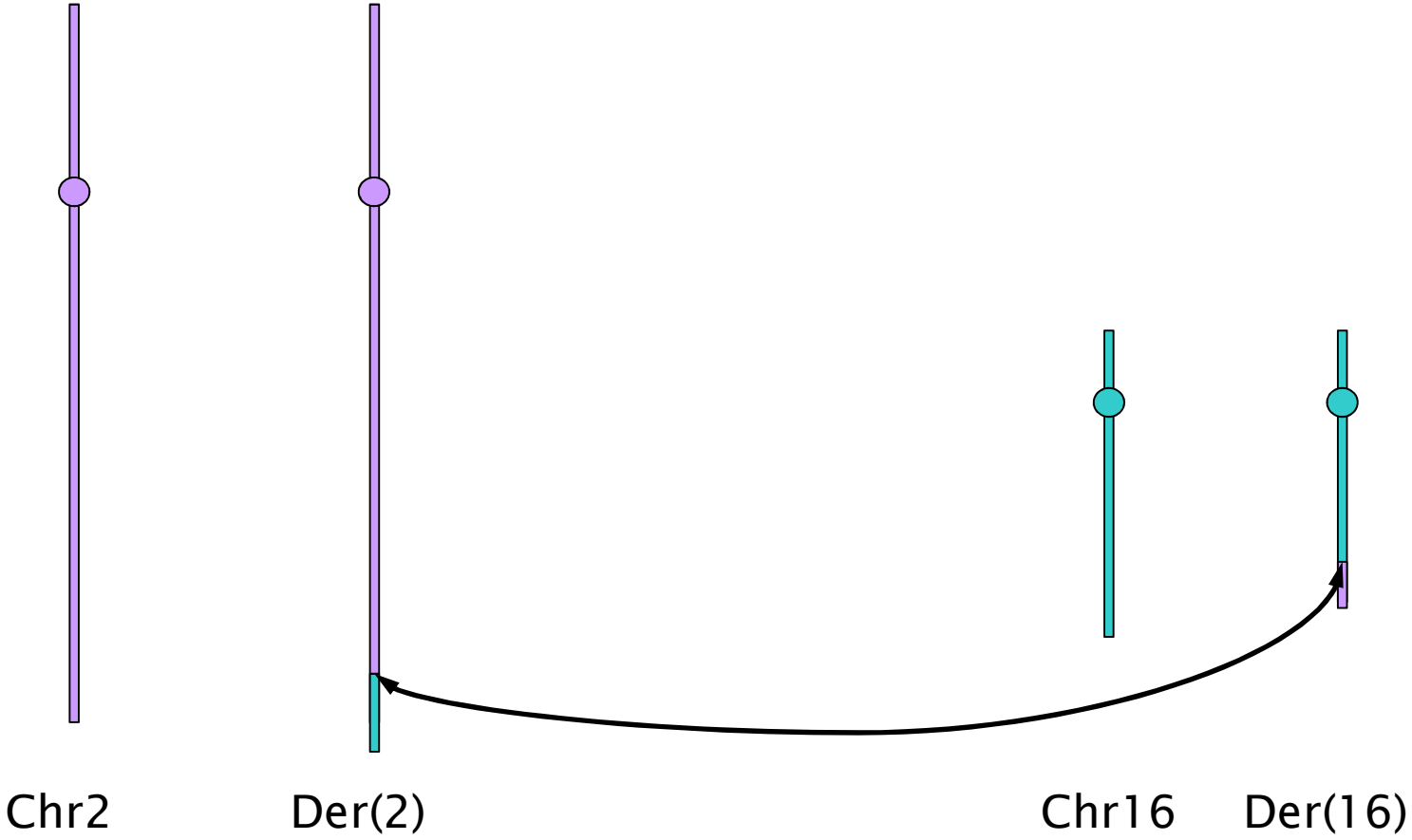
Nanopore sequencing

- Long reads (>10k)
  → allow sequencing over breakpoints
- High error rates
  → confident calling of single base changes difficult (requires high coverage)
  → do not hamper mapping
  → Suitable for detection of breakpoints of structural variants

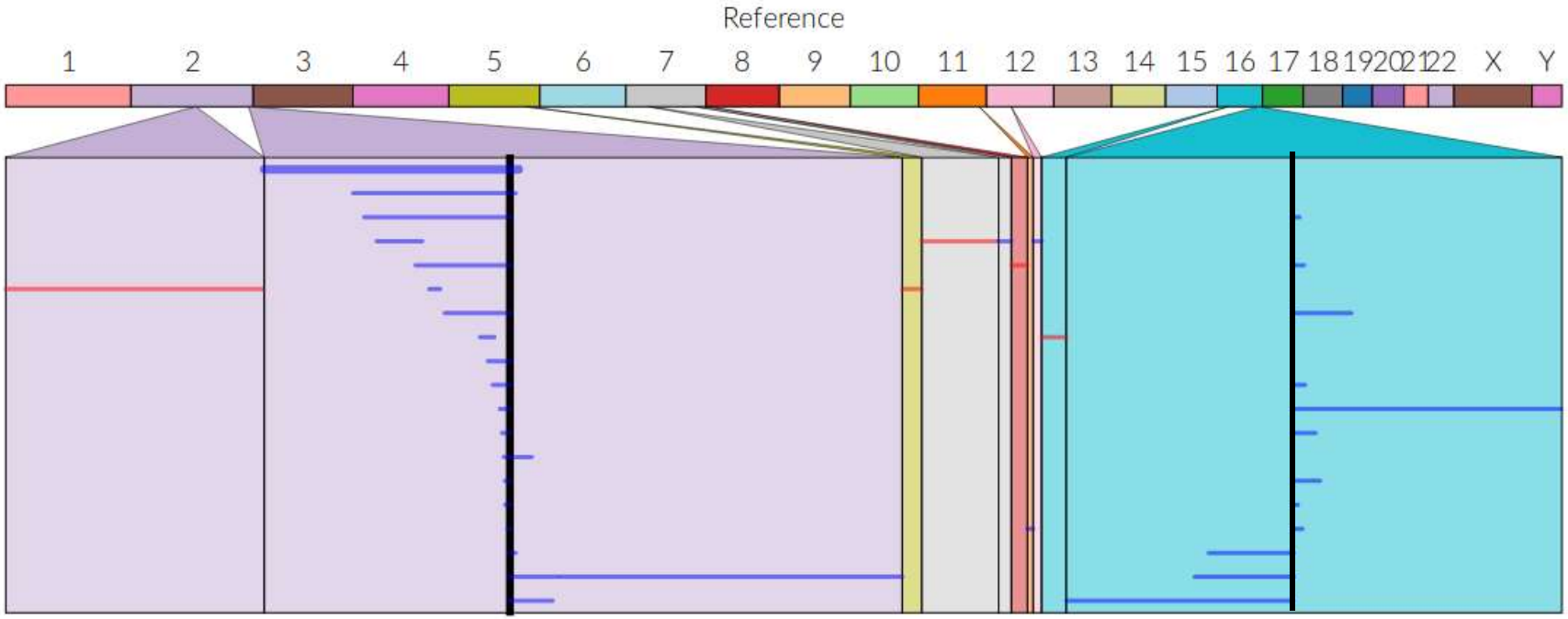
Nanopore sequencing

- GridION device in the lab since may 2019 (Oxford nanopore technologies).

t(2;16)(q37.1;q24.1)



Example t(2;16)(q37.1;q24.1)



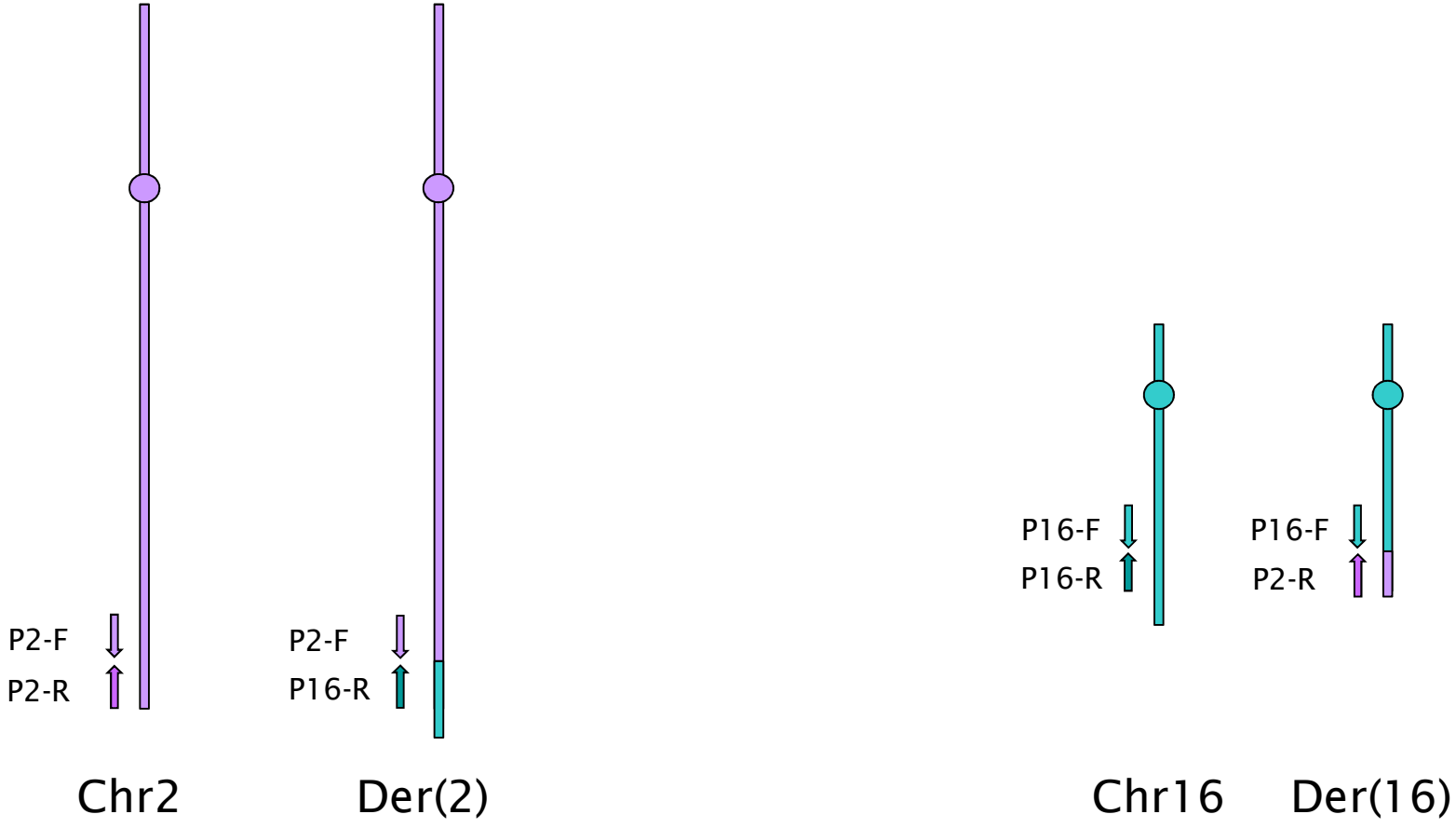
t(2;16)(q37.1;q24.1)

TAGGGGACGGTGGACGGCCAGAGCCCCCTCTCTCTTTCTCTCTCTCTCTTTGCCCGGTTTCTGTAATGAGGAAG
TTCTCCGCGGCTCAGTTTCCTTTCCCTCACTGAGCGCCTGAAACAGGAGATCAGTCAGTTAAGCTGGTGGCAGC
AGCCGAGGCCACCAGAGGCAACGGGCGGCAGGTTGCAGTGGAGGGGCCTCCGCTCCCCTCGGTGGTGTGTG
TGGGTCCTGGGGGGTGCCTGCCGGCCCAGCCGAGAGGCCCACGCCCACCATGGTCCCCTGCTGGAACCATGG
CAACATCACCCGCTCCAAGGCGGAGGAAGCACTTTCCAGGACAGGGCAAGGACAGGAGCTTCCTCGTGCGTG
CCAGCGAGTCCATCTCCCGGGCATACGCGCTCTGCGTGCTGTGAGTACAACCTGCTCCCTCCCCGGGCACAGAT
ATGACAGAGGGGGGCTTAGAGGGGGGCCCAGCTTTGAGATGGGTTGTTCTTAGGTCACAGGACAGAGTGATC
TGACATGCACACTTCCCCGCCACCCTGTCATGGACCTTGTCCTTGGAGTTCAGAGAGCTGGTCTCGTGGCTGTA
GCACTCTTGATTCAGGAAAATTGTGCTTGTCTGTCTCTCTATTATCTCTCTCTCTCTCTCTCTACCTACCTACCT
ATCTACCAACCAACCAACCTATCTAAAATCTATCTATAATCTATTAGTTTTCTTGCTTCACAAATGACTTAAGGCA
ACTCGCTTTCGGGTTGATGGAAGAAAAAAATACAAGTAGCAAACTTGGATGAAGTTGGAAGAAACTTGGCAC
CCACACTGTGTCTGGCATGTGCAGATATGAGTGATGGTCTATAAATTTAGGATCTTATTAACAGCCAAAGCAAA
GGTACAATTTTTCCATGTATCTGGTTTATAGTATCCACAGGATACAAGCATGCTAGTTGATCAAATGTTGCATCT
ATATCTTTGTGATTAACACCTGACAGATATTTCTATGATGCGTTCTGACAGGGTTACACATGACAGAGTTGGCT
CTGTCTATGTTGTACTTAATATTATTGTAGAAAGAGAAAAATTGAGTATATCTTGTGAAAAATAGTTCCATTTGA

Consensus sequence at breakpoint

```
read12 ·····ATGGGACC-TTGTCCTTGG-AGTT-CAG--AGAGCTGGTCTC-GTG-GCTGTAGCACTCTTTGA¶
read8 ·····GTGG-ACA-CCGTCCTTGGGAGTTTCAG--AG--CTGGTCTC-GTGAGCTGTAGCACTCT-TGA¶
read11 ·····ATGG-ACC--TGTCCTTGG-AGTT-CAGAGAGAGCTGGTCTC-GTG-GCTGTAGCACT--TGA¶
read10 ·····ATGG-ACC-TTGTCCTTGG-AGTT-CAG--AGAGCTGGTCTC-GTGGGCTGTAGTACTCT-TGA¶
read13 ·····ATGG-ACC-TTGTCCTTGG-AGTT-CAG--AGAGCTGGTCTC-GTG-GCTGTAGCACTCT-TGA¶
read2 ·····ATGG-ACCTTTGTCCTTGG-AGTT-CAG-GAGAGCTGGTCTCTGTG-GCTGTAGCACTCT-TGA¶
Read6 ·····ATGG-ACC-TTGTCCTTGG-AGTT-CAG--AGAGCTGGTCTC-GTG-GCTGTAGCACTCT-TGA¶
consensus ····ATGG-ACC-TTGTCCTTGG-AGTT-CAG--AGAGCTGGTCTC-GTG-GCTGTAGCACTCT-TGA¶
```

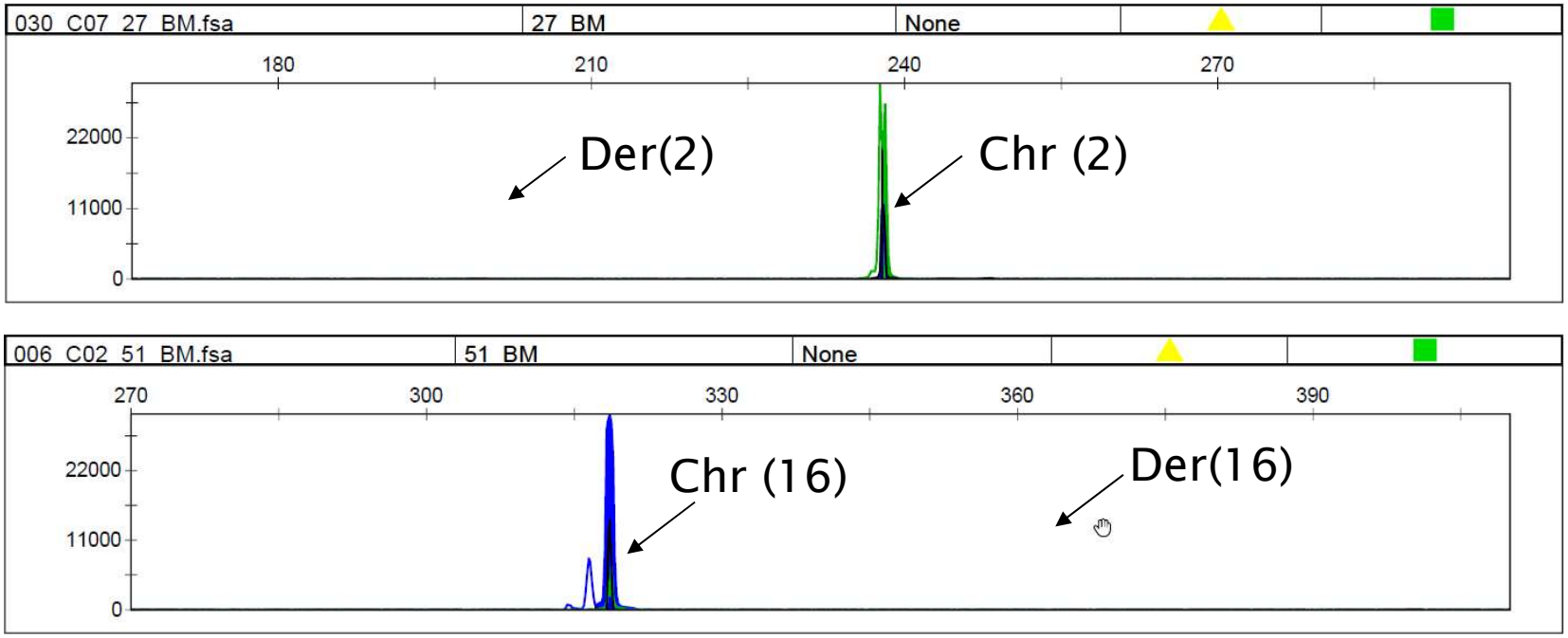
t(2;16)(q37.1;q24.1)



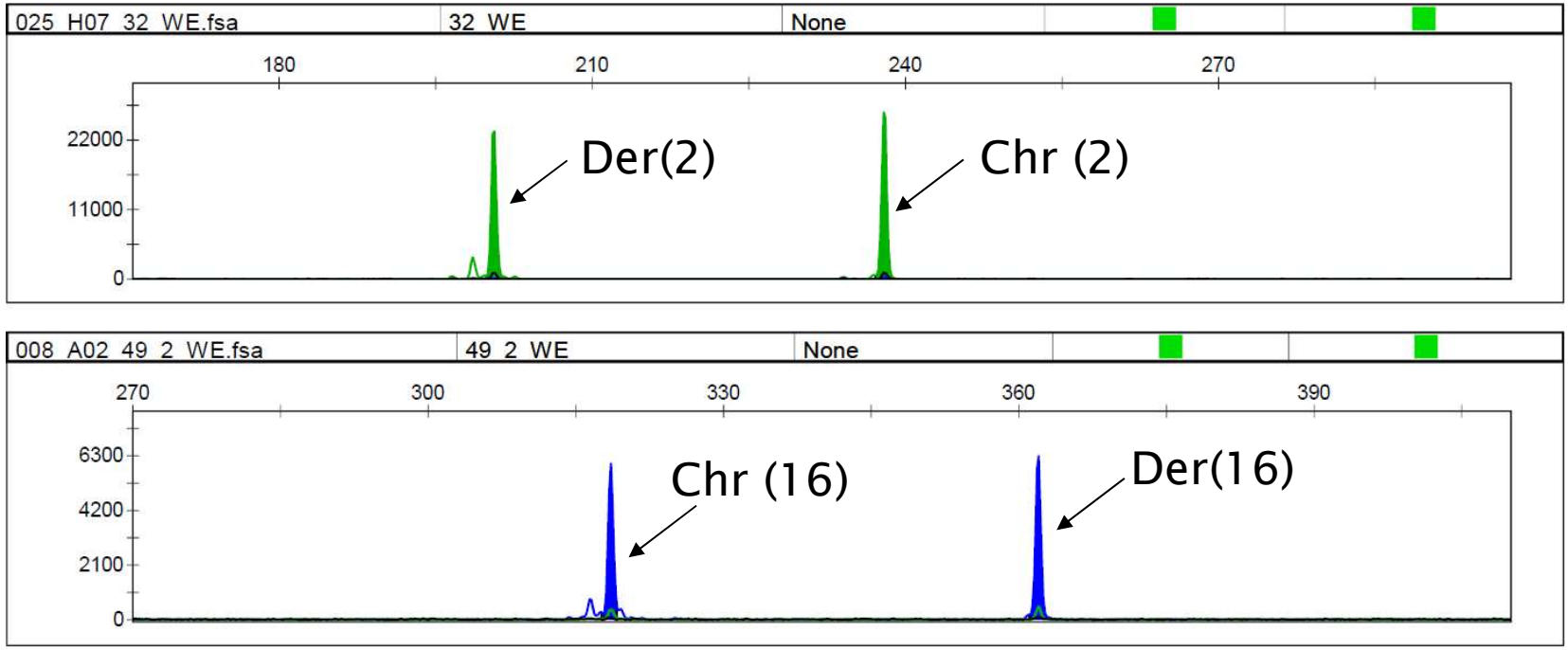
Analysis of familial samples

	primer	label	sequence
1	t(2;16)WE_2F	HEX	ATGGACCTTGTCCTTGGAGTT
2	t(2;16)WE_2R	/	CTGGGCATCTTTGAGCTGAT
3	t(2;16)WE_16F	FAM	CAAGTGGTTATTCCCCTTAACC
4	t(2;16)WE_16R	/	GCCTTAAGTCATTTGTGAAGCA
Mix for PCR:	amplicon	label	expected size
primers 1,2,4	chr2 (1 en 2)	HEX	237bp
	der2 (1 en 4)	HEX	200bp
primers 2,3,4	chr16 (3 en 4)	FAM	318bp
	der16 (3 en 2)	FAM	361bp

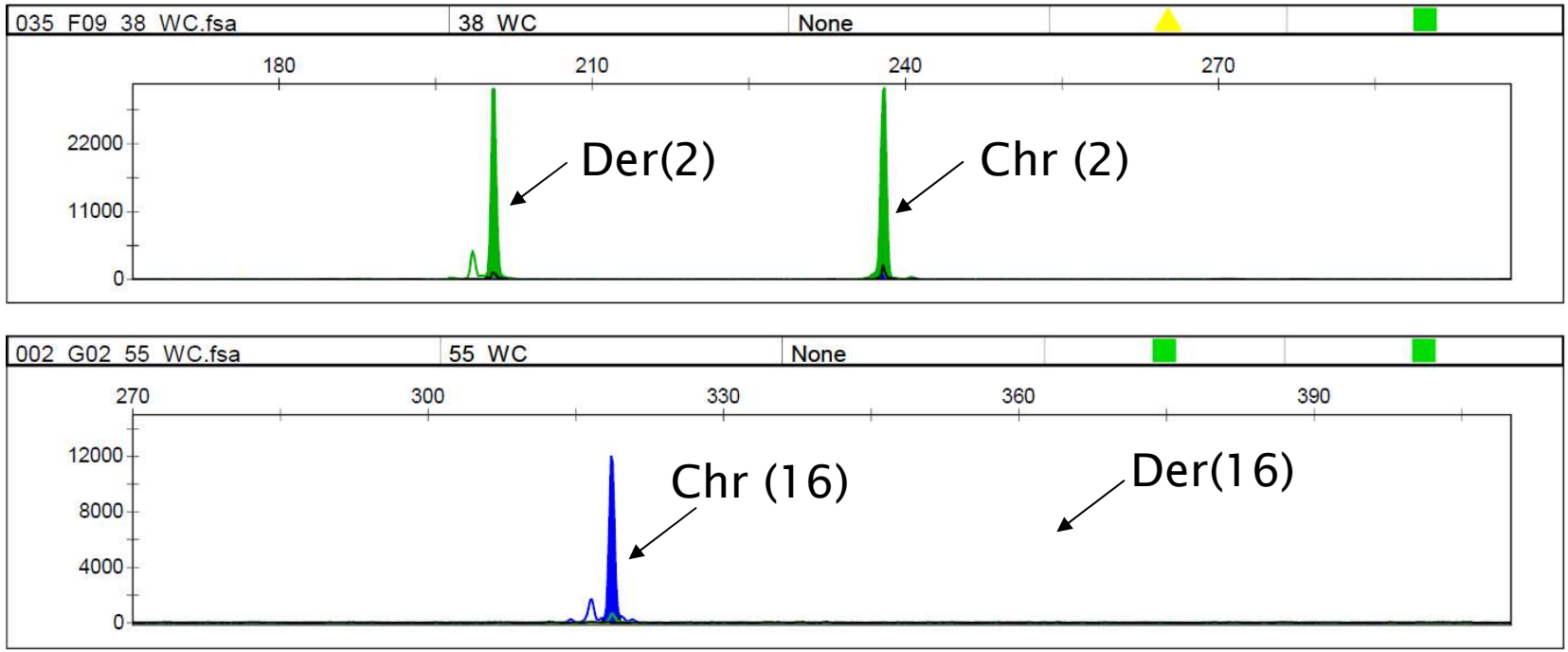
Individuals without transloc. (n=4)



Balanced transloc. carriers (n=2)



Unbalanced transloc. carrier (n=1)



- 46,XX,der(2)t(2;16)(q37.1;q24.1)

Reanalysis of MDA from embryos from previous PGT cycles

	chr2	der(2)	chr16	der(16)	Conclusion	Diagnosis SNP array
cycle 1						
E3	Y	Y	Y		unbalanced	unbalanced, Adj1 with der(2)
E4	Y		Y	Y	unbalanced	unbalanced, Adj1 with der(16)
E6	Y		Y		normal (or trisomy)	normal
E7	Y	Y	Y		unbalanced	unbalanced, Adj1 with der(2)
cycle 2						
E1	Y		Y		normal (or trisomy)	normal
E5	Y	Y	Y		unbalanced	unbalanced, Adj1 with der(2)
cycle 3						
E1	Y	Y	Y	Y	balanced (or trisomy or 3:1)	balanced
E2	Y		Y	Y	unbalanced	unbalanced, Adj1 with der(16)
E3	Y	Y	Y		unbalanced	unbalanced, Adj1 with der(2)
E7	Y		Y		normal (or trisomy)	normal
E9	Y	Y	Y		unbalanced	unbalanced, Adj1 with der(2)
E10	Y	Y	Y		unbalanced	unbalanced, Adj1 with der(2)
E11	Y		Y		normal (or trisomy)	normal

Breakpoint analysis and diagnosis

- Result from junction PCR itself is not a diagnosis
  → ADO or contamination is possible
  → PCR is not quantitative

- Eg. Der(2) and Der(16) observed.

	chr 2	der(2)	chr 16	der(16)
Embryo X	Present	Present	Present	Present
Balanced t.	1	1	1	1
3:1 segregation	2	1	1	1
3:1 segregation	1	1	2	1
MII non disjunction	1	2	1	1
MII non disjunction	1	1	1	2
4:0 segregation	2	1	2	1
…				

SNP array + breakpoint detection

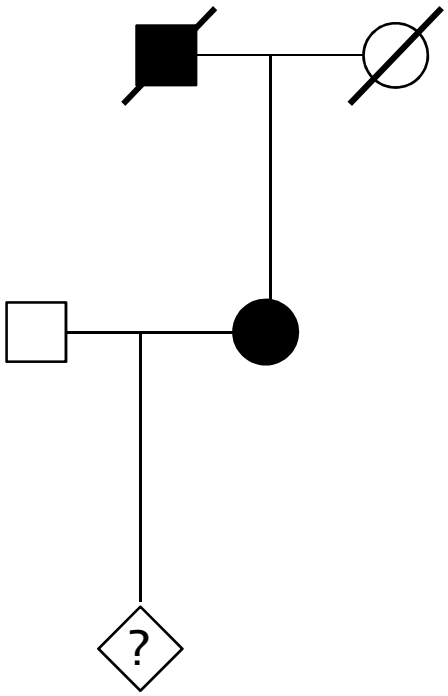
- Breakpoint detection will be used together with SNP array
- Diagnosis is reliable if based on
  - → Log2R + BAF (noisy if low call rate)
  - → Breakpoint detection
  - → Assigning an embryo (high call rate) as reference allows haplotyping -> extra confirmation

PGT-A

- NGS (shallow genome sequencing)
- SNP array
  → APCAD for detection of deletion/monosomy or duplication / trisomy

Case 1 – *LMNA*

- Cardiomyopathy
- c.1072G>T
- Parents both deceased
- No children
- -> How to proceed?

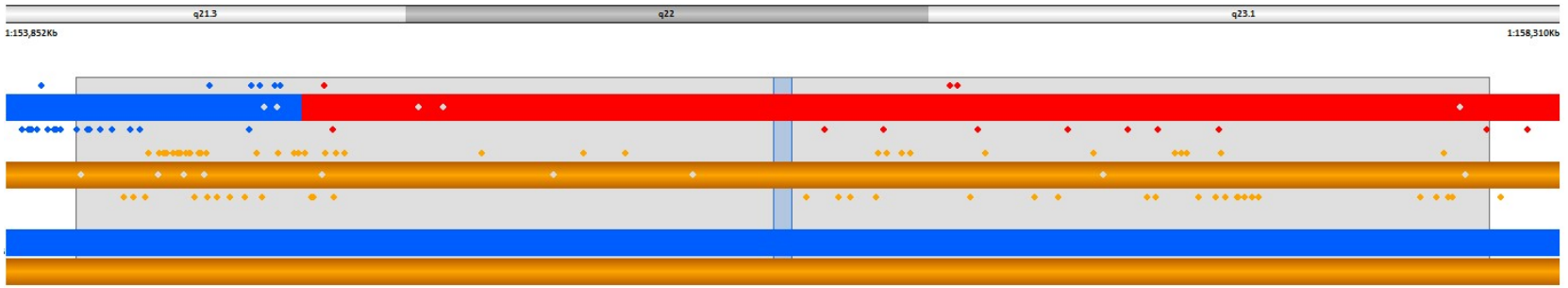


Case 1 – *LMNA*

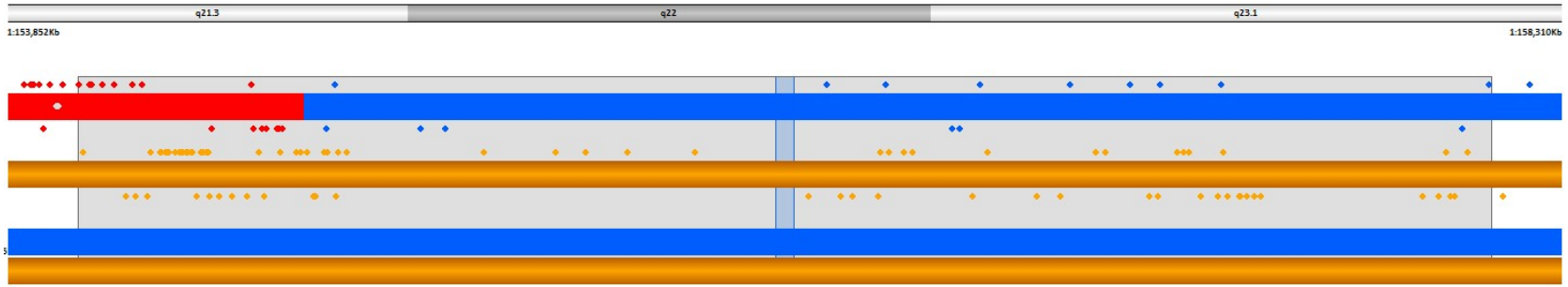
- Workup
  - → Check if sufficient informative SNPs ✓
  - → Check if the mutation can be detected ✓
- Cycle strategy
  - → Biopsy good quality embryos (TE biopsy)
  - → + collect poor quality embryos for analysis
  - → Put together haplotype and mutation detection to reach a diagnosis

Cycle 1 results

- E1 (seg) Mut: G/T*



- E2 Mut: G/T*



Case 1 cycle

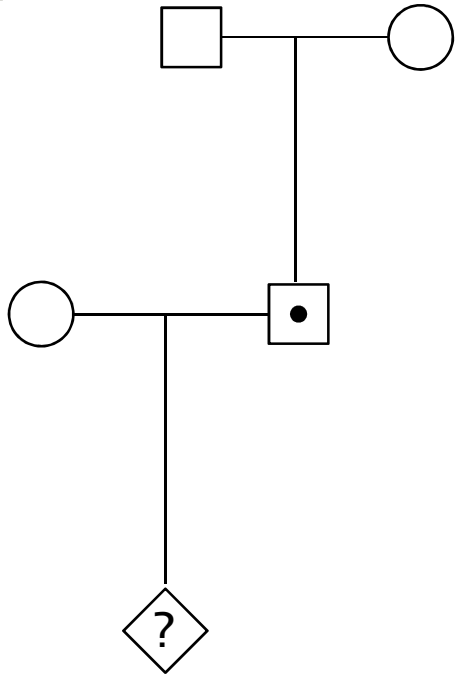
	Haplotype Maternal	Haplotype Paternal	G>T Mutation	PGT-A
cycle 1				
E1 (seg)	M1	P2	G/T*	disomy
E2	M1	P1	G/T*	disomy
E3 (seg)	M1	P1	G/T*	disomy
E4 (seg)	/	P1	G	monosomy
E5 (seg)	M1+M2	/	G/T*	UPD
E6 (seg)	?	?	G/T*	?
cycle 2				
E1 (seg)	M2	P2	G	disomy
E2	M2	P1	G	disomy
E3	M2	P2	G	disomy
E4	M2	P2	G	disomy
E5 (seg)	M1	P2	G/T*	disomy
E6	M1	P1	G/T*	disomy
E7	M2	P2	G	disomy
E8 (seg)	M1	P1	G/T*	disomy
E10 (seg)	M2	P2	G	disomy
E12	M2	P2	G	disomy

Case 1 segregation analysis

	Haplotype Maternal	Haplotype Paternal	G>T Mutation	PGT-A	Conclusion
cycle 1					
E1 (seg)	M1	P2	G/T*	disomy	affected
E2	M1	P1	G/T*	disomy	affected
E3 (seg)	M1	P1	G/T*	disomy	affected
E4 (seg)	/	P1	G	monosomy	monosomy
E5 (seg)	M1+M2	/	G/T*	UPD	UPD
E6 (seg)	?	?	G/T*	?	no diagnosis (QC)
cycle 2					
E1 (seg)	M2	P2	G	disomy	unaffected
E2	M2	P1	G	disomy	unaffected
E3	M2	P2	G	disomy	unaffected
E4	M2	P2	G	disomy	unaffected
E5 (seg)	M1	P2	G/T*	disomy	affected
E6	M1	P1	G/T*	disomy	affected
E7	M2	P2	G	disomy	unaffected
E8 (seg)	M1	P1	G/T*	disomy	affected
E10 (seg)	M2	P2	G	disomy	unaffected
E12	M2	P2	G	disomy	unaffected

Case 2

- Male with balanced insertion ins(2;6)(q31;21.1p23)
  - → Hoog risico op ongebalanceerd kind
  - → Grote segmenten

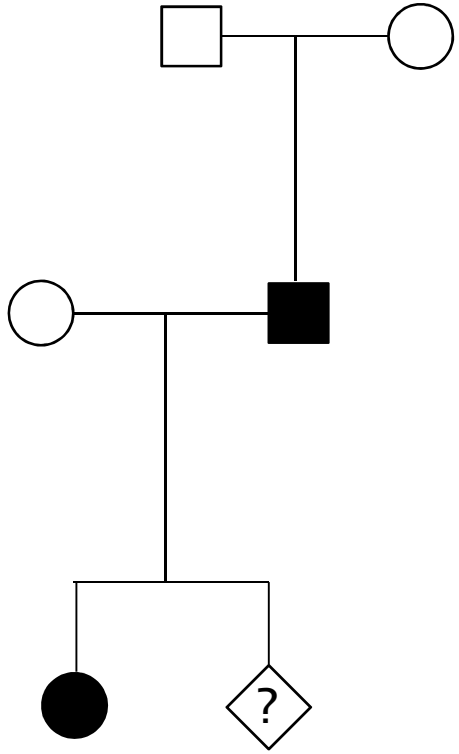


Case 2 PGT1 (shallow sequencing)

- 5 embryos
  - → 5/5 normal or balanced
  - → 4/5 transferable (1 aneuploid)
  - → 1 transfer -> pregnancy -> child
- Child showed cleidocranial dysplasia.
  - → Causative gene *RUNX2* is located on chr6 -> ?
  - → Insertion cause of CCD?
  - → Male examined and has mild CCD phenotype
  - → Mutation analysis of *RUNX2* showed no mutations

Case 2

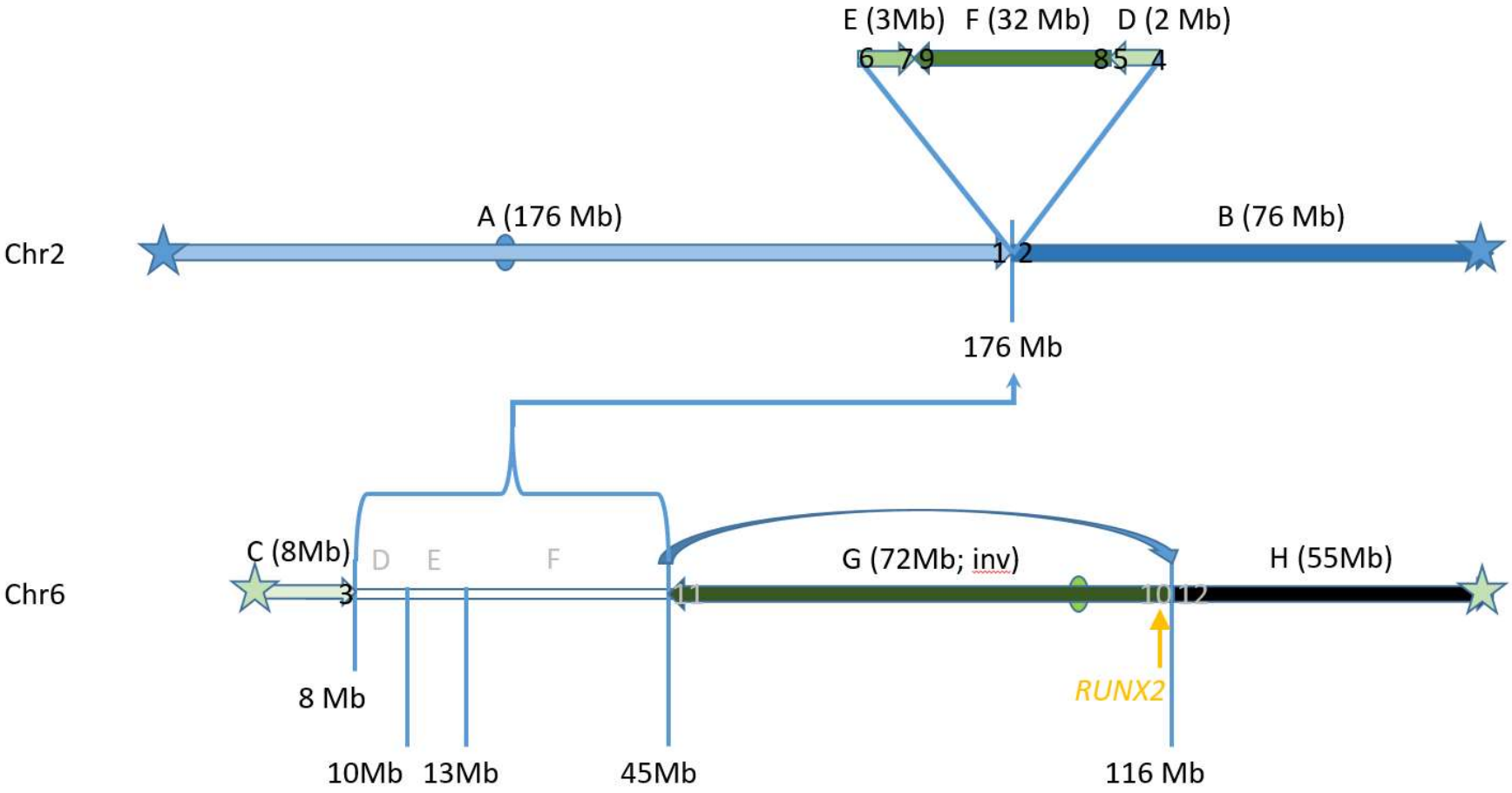
- Male and child with balanced insertion ins(2;6)(q31;21.1p23) with effect on *RUNX2* gene
- Both CCD
- -> Distinguish normal from balanced
- ->?



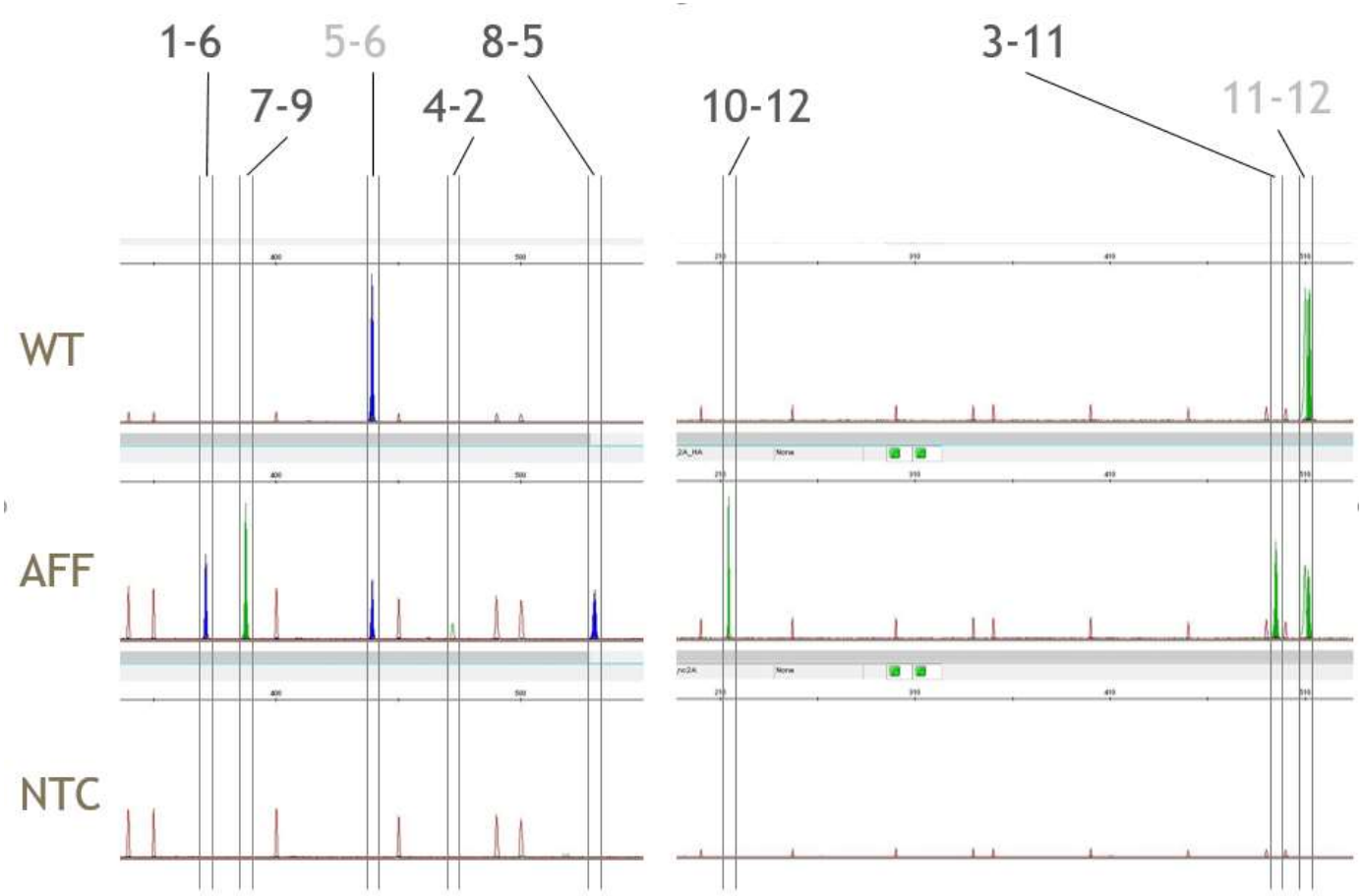
Long read sequencing

- 1 breakpoint at 380kb of *RUNX2* gene
  - → Likely cause of CCD
  - → Even if not cause (e.g. missed intronic pathogenic variant) -> also shared haplotype between father and daughter to be excluded
- In total 6 breakpoints (and new junctions) were detected!
  - → Much more complex rearrangement
  - → 6 junction PCRs were designed

Case 2


E (3Mb)
F (32 Mb)
D (2 Mb)
6
7
9
8
5
4
Chr2
A (176 Mb)
B (76 Mb)
1
2
176 Mb
Chr6
C (8Mb)
D
E
F
G (72Mb; inv)
H (55Mb)
3
11
10
12
RUNX2
8 Mb
10Mb
13Mb
45Mb
116 Mb

Case 2 – direct detection of junctions



Multiplex 1 Multiplex 2

Case 2 PGT2 results

cycle 2	chr6 5-6 (control)	chr 6 11-12 (control)	der(2) 1-6	der(2) 7-9	der(2) 8-5	der(2) 4-2	der(6) 3-11	der(6) 10-12	Haplotypes	copy number	Diagnosis
E4	V	V	/	/	/	/	/	/	normal	/	normal
E6	V	V	/	/	/	/	/	/	normal	/	normal
E9	V	V	/	/	/	/	/	/	normal	/	normal
E10	V	V	V	V	V	V	/	/	der (2), normal chr 6	CN gain 6p	unbalanced
E13	V	V	V	V	V	V	/	/	der (2), normal chr 6	CN gain 6p	unbalanced
E16	V	V	/	/	/	/	V	V	der (6), normal chr 2	CN loss 6p	unbalanced
E17	V	V	V	V	V	V	/	/	der (2), normal chr 6	CN gain 6p	unbalanced
E21	V	V	V	V	V	V	V	V	balanced	/	balanced
E23	V	V	/	/	/	/	/	/	normal	/	normal
E24	V	V	V	V	V	V	/	/	der (2), normal chr 6	CN gain 6p	unbalanced
E28	V	V	V	V	V	V	/	/	der (2), normal chr 6	CN gain 6p	unbalanced

Case 2 – PGT2

- 11 embryos
  - → 4 normal (no SR)
  - → 1 balanced SR
  - → 6 unbalanced
- Resulted in a second child for the couple