Update on clinical use of PCR and the future

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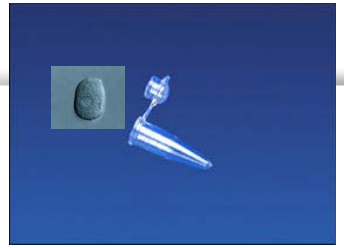
PGD Process



Cell Lysis

External Multiplex PCR

Nested PCR

Mutation Analysis

External primers for the amplification of:

- **the gene regions involved by mutations**
- **linked STR markers for ADO detection**
- **STR markers for detection of aneuploidies, in patients with advanced maternal age (>37 y.o.)**

2 μl of the primary PCR reaction product are used in separate second round PCR reactions for each locus

Electrophoresis of PCR products

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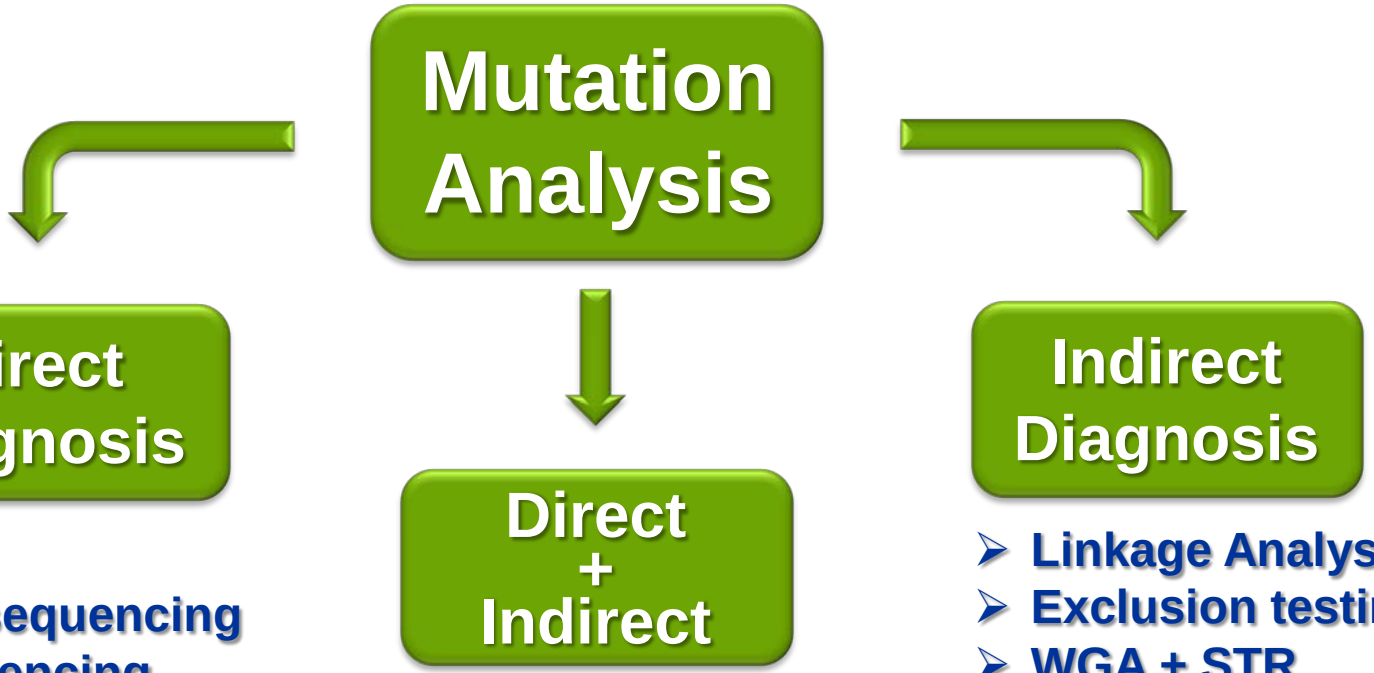
MUTATION ANALYSIS



- Minisequencing
- Sequencing
- Restriction enzime digestion
- PCR products sizing
- SSCP-DGGE
- ARMS / D-ARMS
- Molecular beacons

- Direct mutation testing + Linked STR markers

- Linkage Analysis
- Exclusion testing
- WGA + STR haplotyping

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MUTATION ANALYSIS

Direct Diagnosis

Minisequencing Single Base Extension

Template

Primer

CCATGACTGATTCC

NNNNNNAGCCTGGTACTGACTAAGGCNNNNNNN

Interrogation target

ddTTP + ddCTP + ddATP + ddGTP

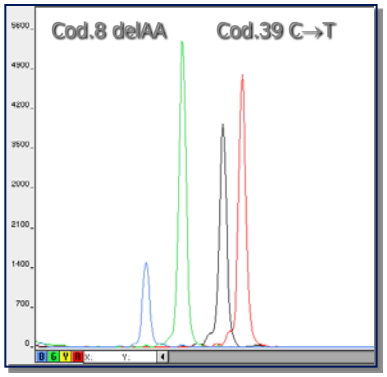
+

AmpliTaq®DNA Polymerase FS +Reaction Buffer

Extend and Terminate Primer

CCATGACTGATTCC ddGTP

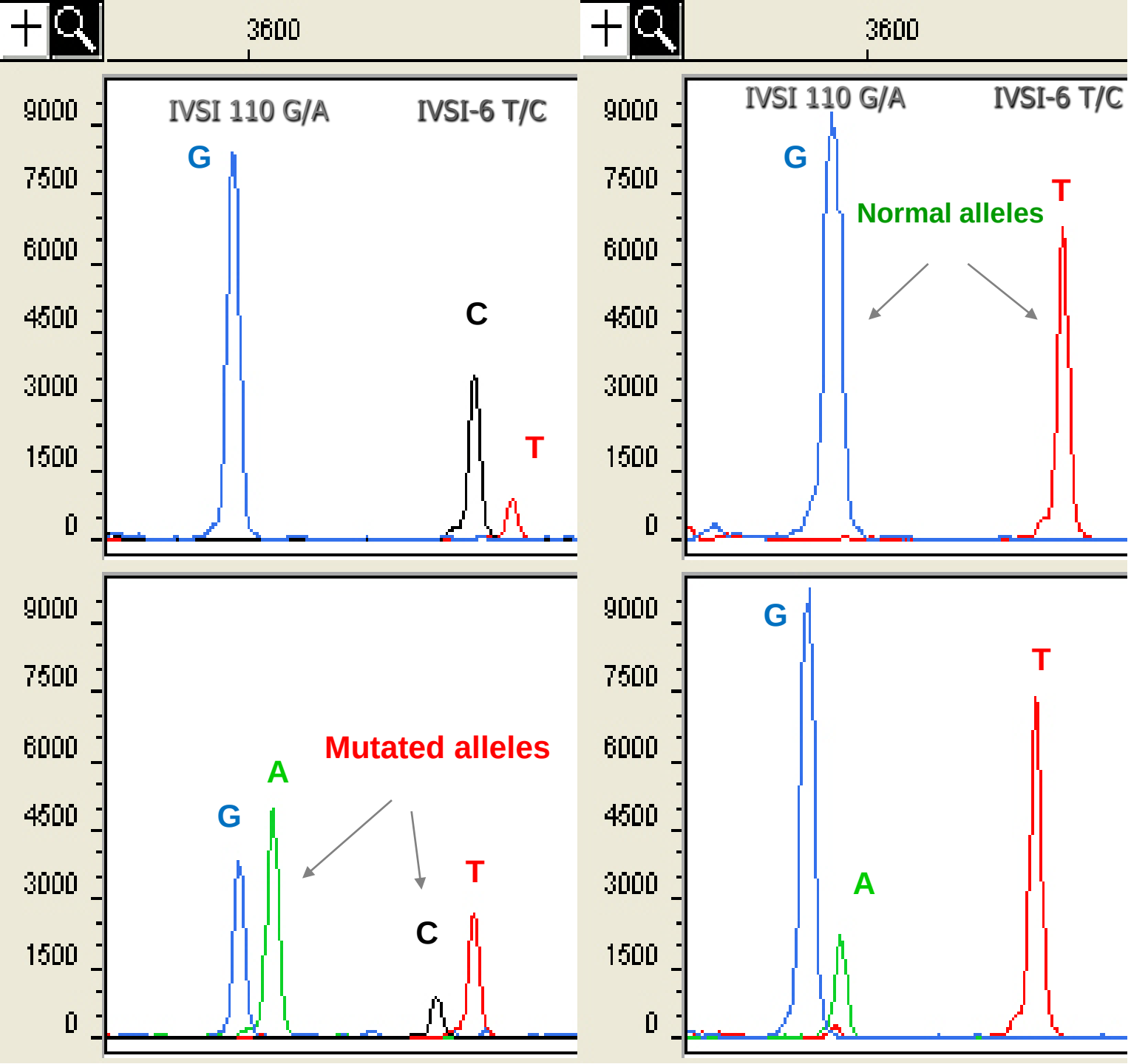
NNNNNNAGCCTGGTACTGACTAAGGCNNNNNNN



Repeat

Electrophoresis

Genotyping



Beta Thalassemia

MUTATION ANALYSIS

Direct + Indirect Diagnosis

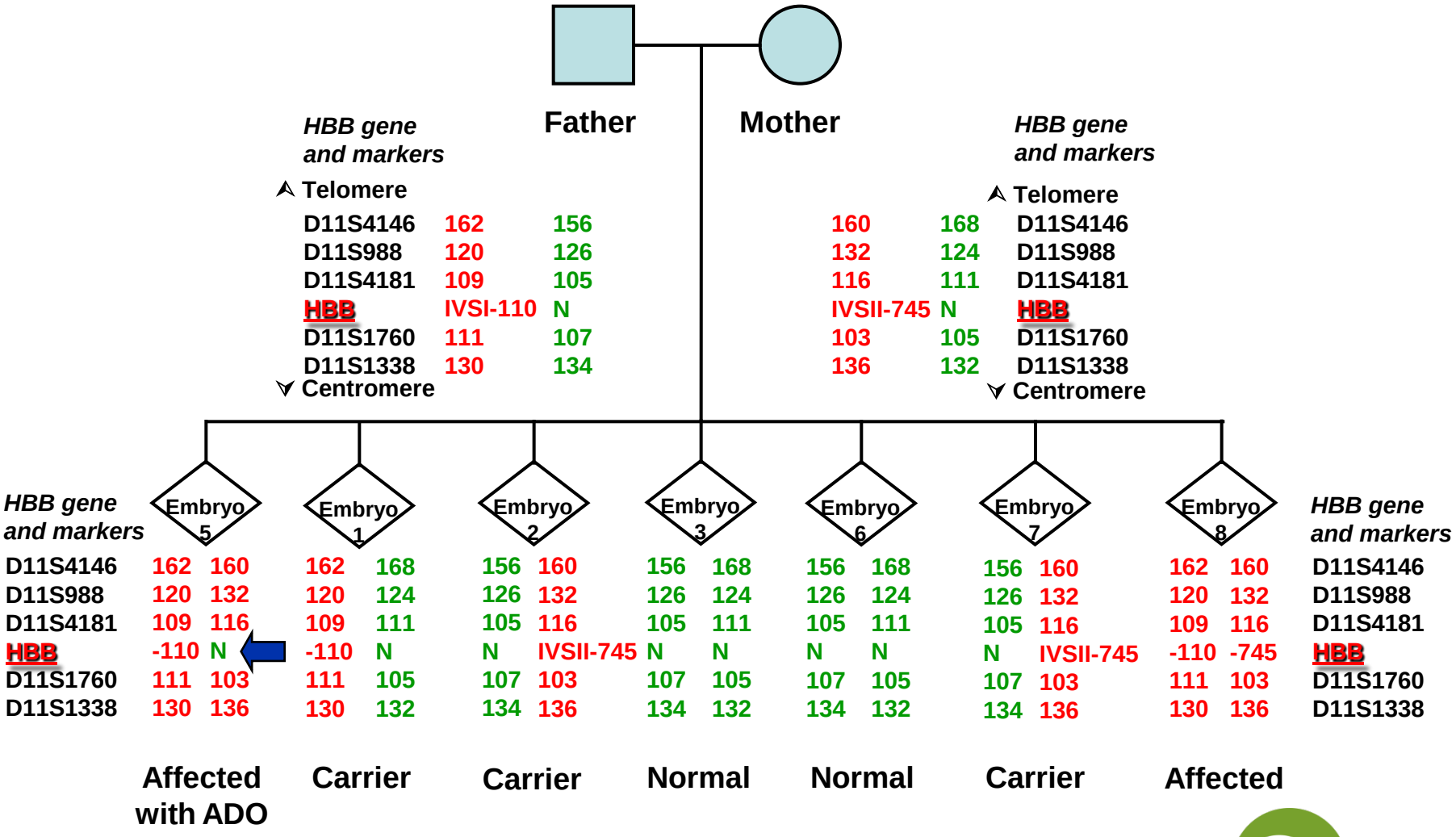
The use of STR markers in PGD procedure

- Represents a diagnostic tool for indirect mutation analysis, providing an additional confirmation of the results obtained with the direct genotyping procedure
- provides a control of misdiagnosis due to undetected ADO
- provides an additional control for contamination with exogenous DNA
- Provides information on embryo's chromosomes copy number
- PGD protocols for SGD are not appropriate for clinical practice without including a set of linked STR markers

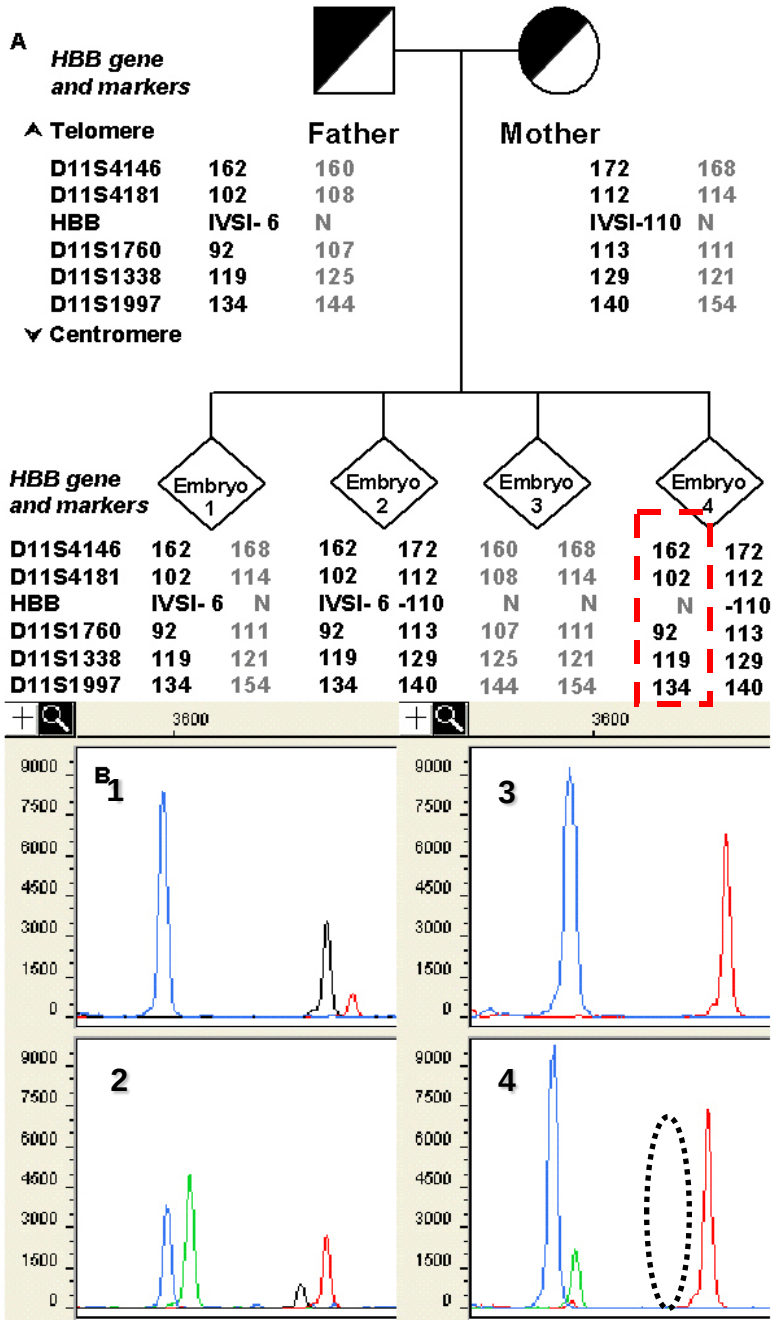
Allele drop-out

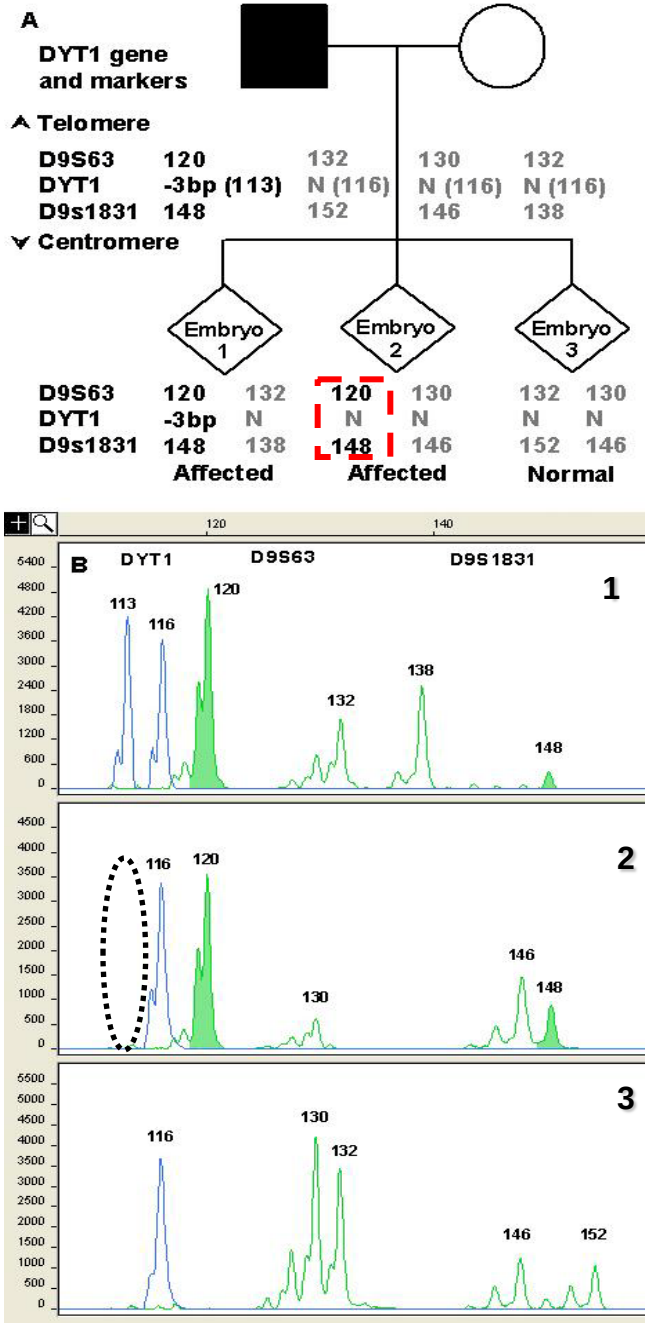
- Allele drop-out (ADO) is defined as the non-amplification of one allele when performing PCR at the single cell level.
- This phenomenon can only be demonstrated in heterozygote cells, which show a homozygous pattern when ADO has occurred
- ADO occurs in all cell types, e.g. blastomeres, lymphocytes, buccal cells and fibroblasts.
- An undetected ADO event leads to misdiagnosis

Avoidance of misdiagnosis due to ADO



Avoidance of misdiagnosis due to ADO





MUTATION ANALYSIS

Indirect Diagnosis

Indications for indirect diagnosis

- **Direct mutation testing is not possible**
  - **The mutation is unknown**
  - **The mutation is a large deletion/insertion with unknown breakpoints**
- **Direct mutation testing is not efficient**
  - **The gene region to be amplified is refractory to PCR (e.g. GC-rich)**
  - **Presence of a pseudogene**
- **Genes with a wide spectrum of mutations**
  - **indirect diagnosis as a general protocol for different couples**
- **Preimplantation HLA matching**
  - **flexible indirect HLA typing protocol applicable to a wide spectrum of possible HLA genotypes**
- **Exclusion testing**
  - **e.g. Huntington disease**

Indirect diagnosis: Pros / Cons

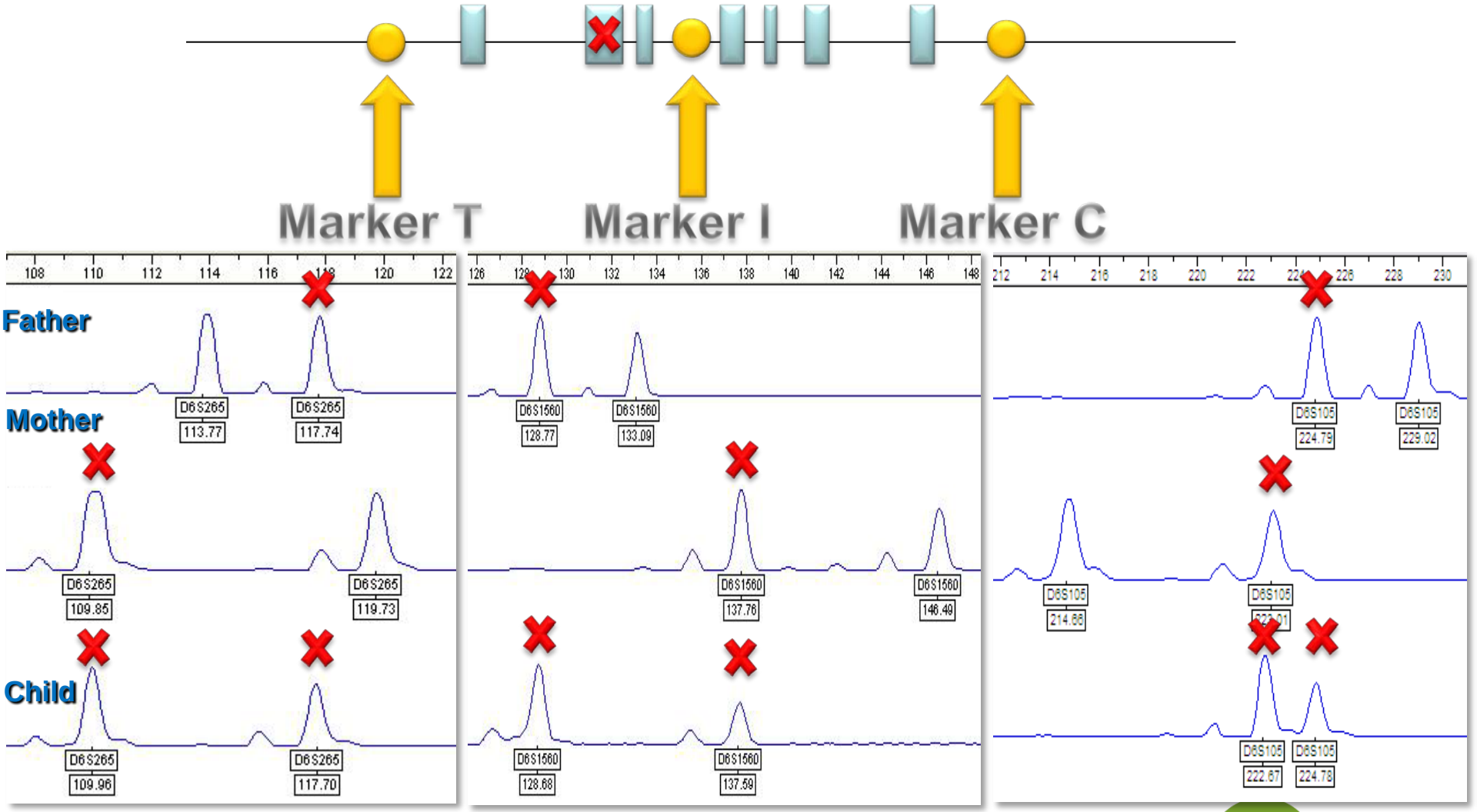
Advantages:

- No mutation analysis
  - same protocol useful for many couples
- Useful for rare disorders with private mutations

Disadvantages:

- Applicable to informative couples with family history
  - At least two affected family members needed
- Not applicable in cases of *de novo* mutation and no previous pregnancies

Principle of indirect diagnosis



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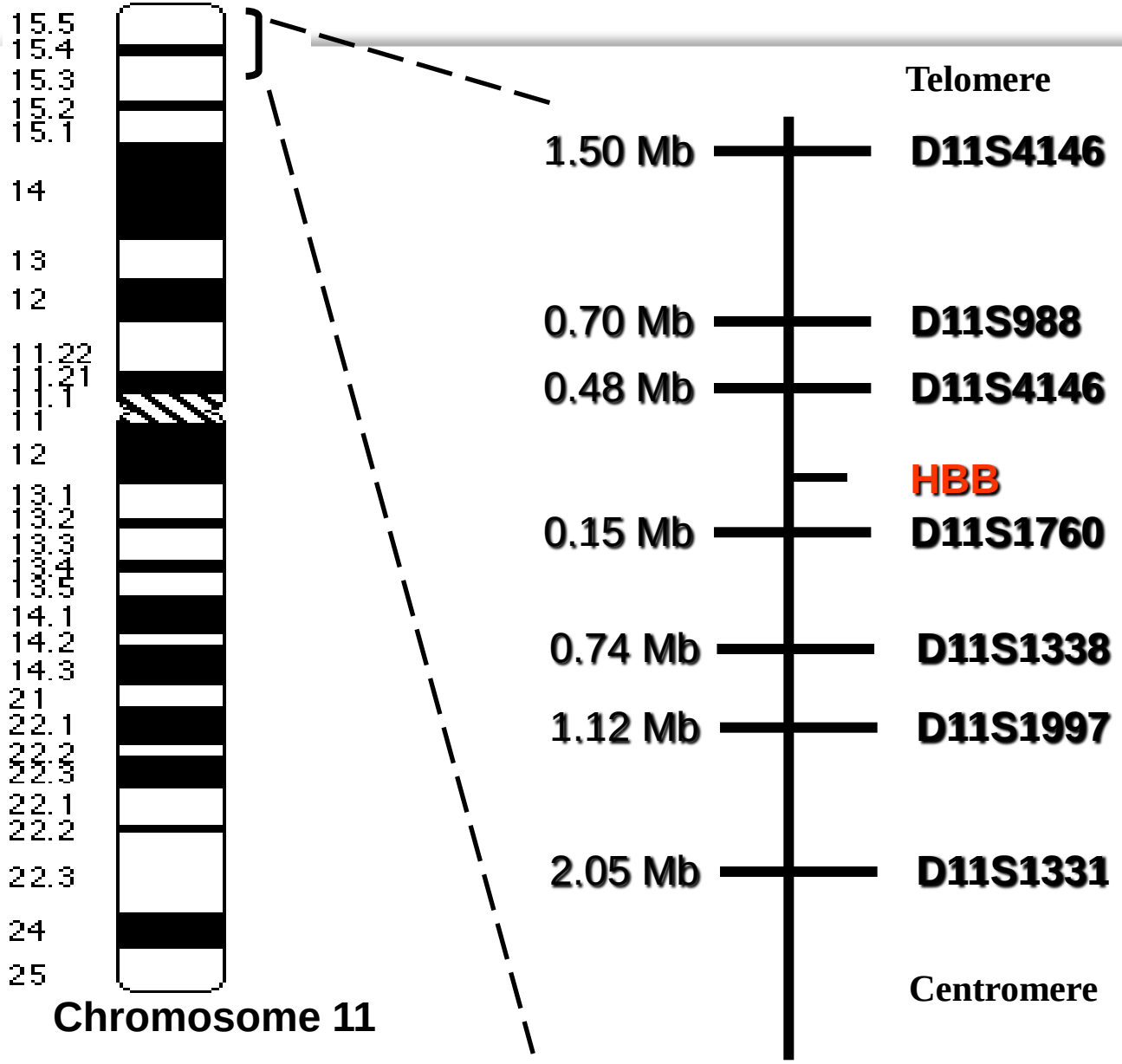
How to build the haplotypes?

- Selection of the STR markers linked to the disease causing gene

The choice of linked STR markers



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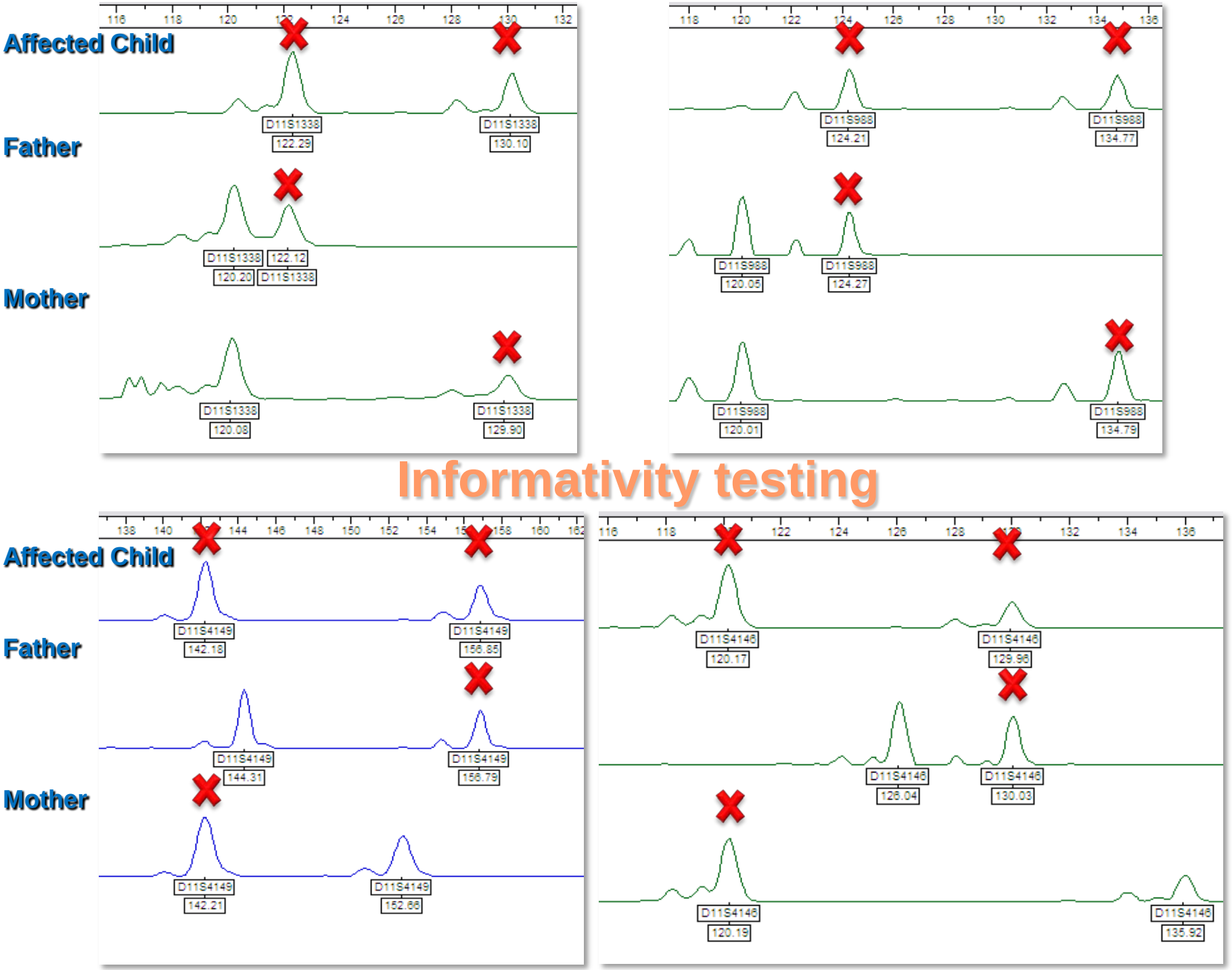
How to build the haplotypes?

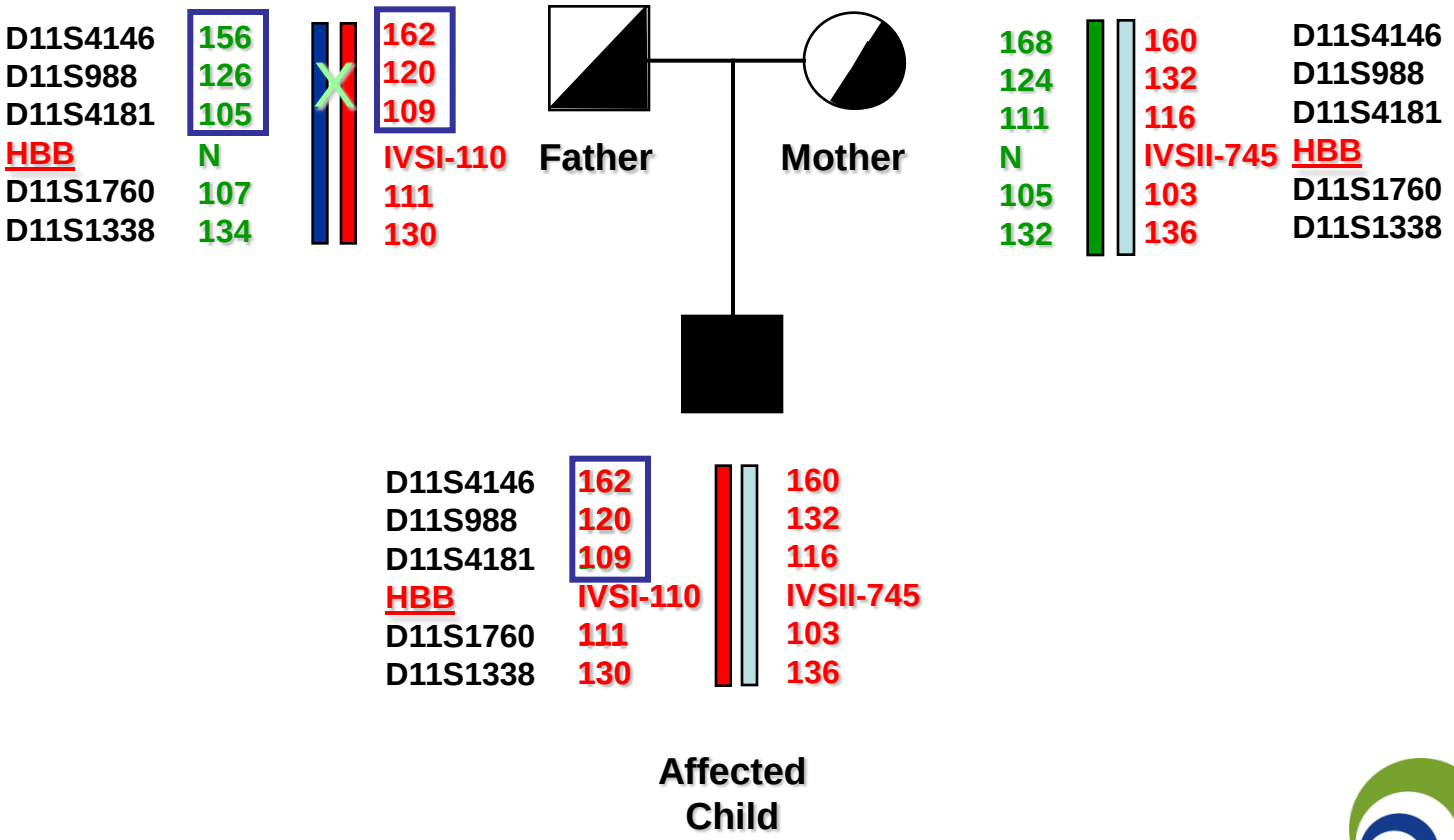
- Selection of the STR markers linked to the disease causing gene
- Evaluation of the informativity of the markers:
- Selection of the informative markers
  - Preferably fully informative (i.e., 4 different alleles, father a/b and mother c/d)
- Identification of the alleles associated with the mutation/disease


Affected Child
Father
Mother
D11S1338
122.29
D11S1338
130.10
D11S1338
122.12
120.20
D11S1338
D11S1338
120.08
D11S1338
129.90
D11S988
124.21
D11S988
134.77
D11S988
120.05
D11S988
124.27
D11S988
120.01
D11S988
134.79
Informativity testing
Affected Child
Father
Mother
D11S4149
142.18
D11S4149
156.85
D11S4149
144.31
D11S4149
156.79
D11S4149
142.21
D11S4149
152.66
D11S4146
120.17
D11S4146
129.96
D11S4146
126.04
D11S4146
130.03
D11S4146
120.19
D11S4146
135.92

How to build the haplotypes?

- Selection of the STR markers linked to the disease causing gene
- Evaluation of the informativity of the markers:
- Selection of the informative markers
  - Preferably fully informative (i.e., 4 different alleles, father a/b and mother c/d)
- Identification of the alleles associated with mutation/disease
- Determination of the haplotypes

DETERMINING HAPLOTYPES FOR LINKAGE ANALYSYS



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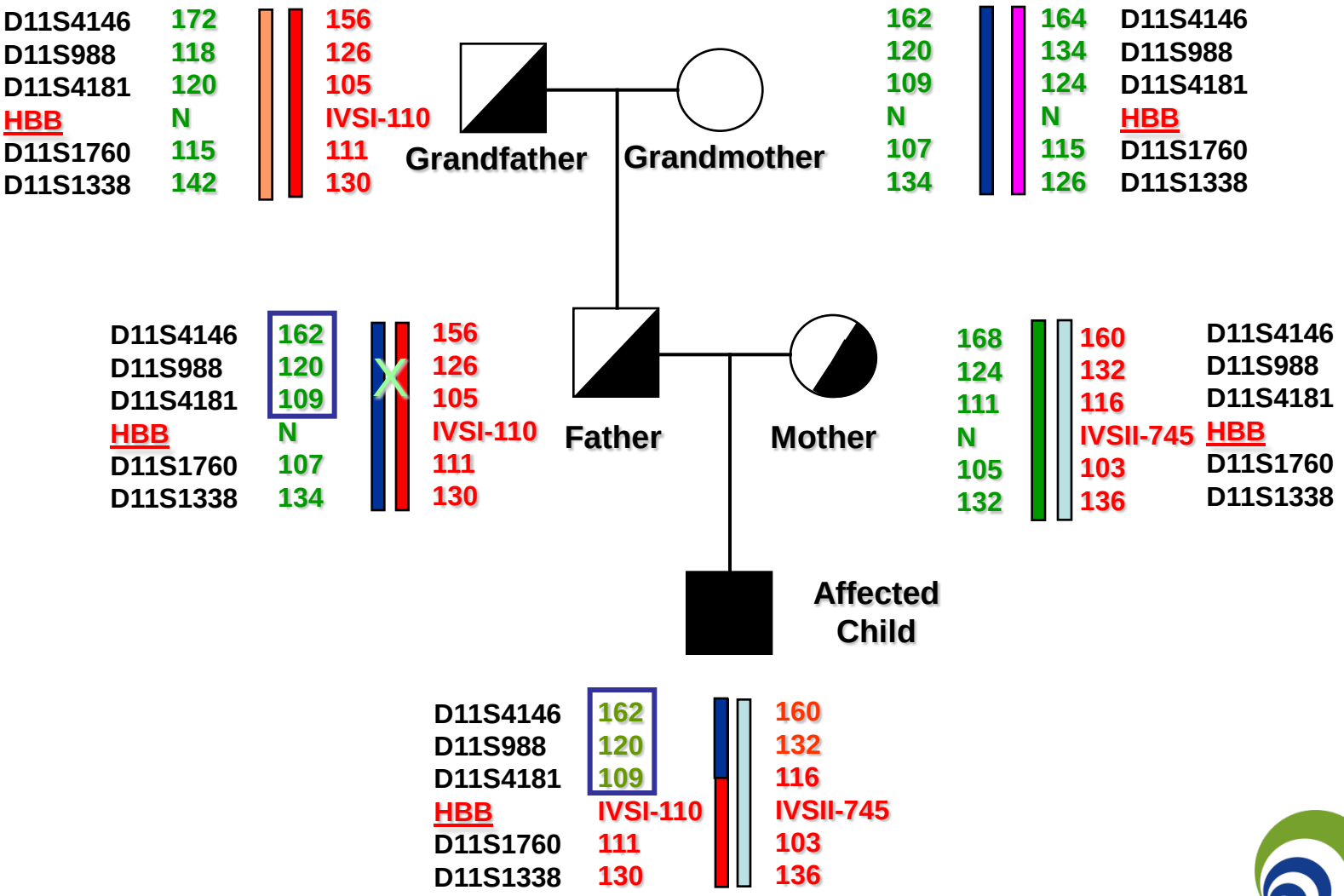
DETERMINING HAPLOTYPES FOR LINKAGE ANALYSYS



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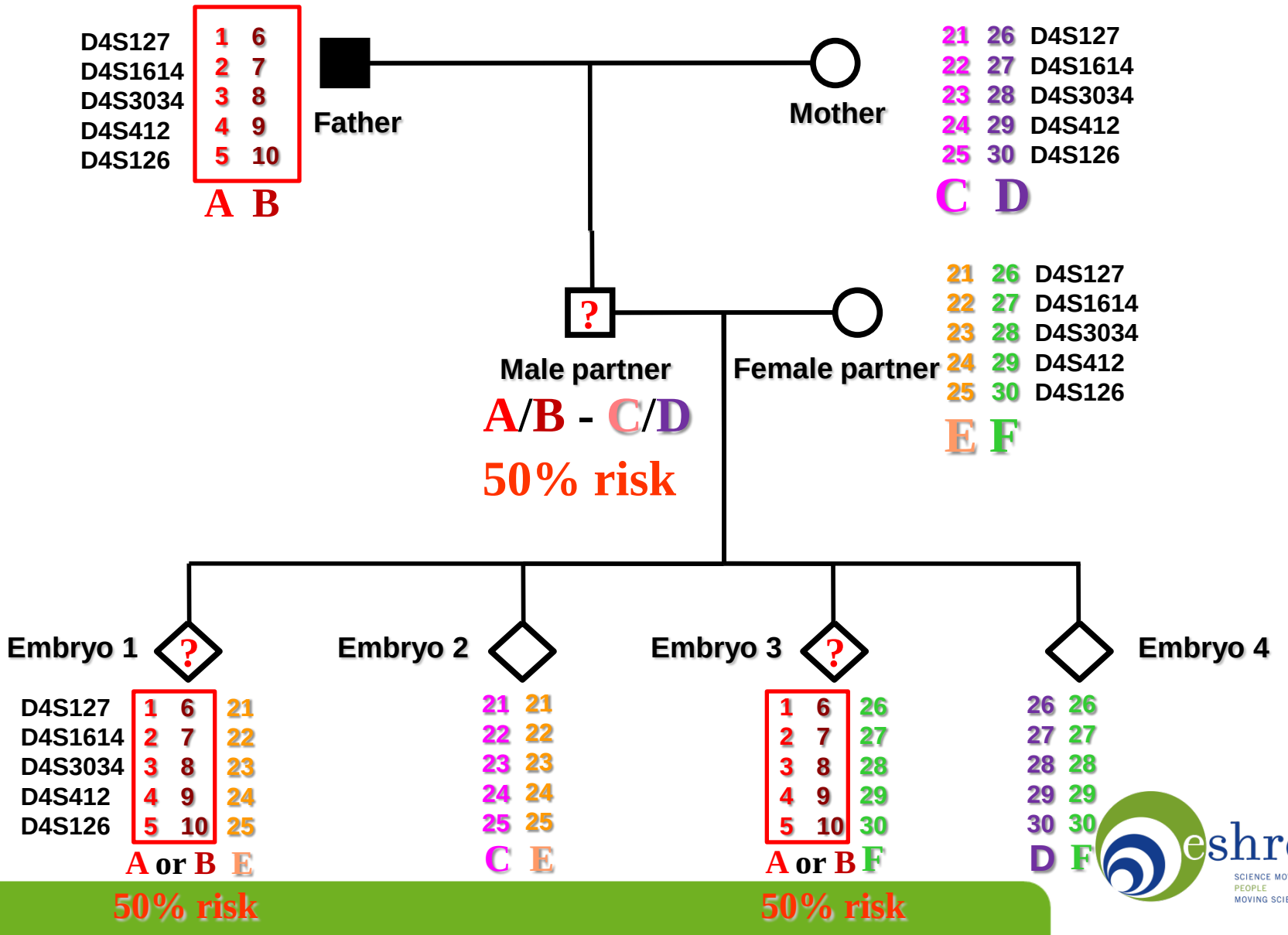
Linkage-based PGD protocols: general guidelines

- Type of markers:
  - STRs, preferably tetra-nucleotide repeat (di-nucleotide repeat are also acceptable)
- Location of STR markers:
  - preferentially intragenic or extragenic, very closed to the gene (max 1 Mb of distance) to reduce the risk of recombination events
- Heterozygosity of STR markers
  - High (>0.8) to improve informativity of the markers
- No. of STR markers
  - Preferably 4, 2 upstream and 2 downstream
- Size of the alleles
  - Small product size (preferably < 250 bp) to improve PCR efficiency
- Number of family members
  - At least two generations or affected family members

Indirect Diagnosis

Exclusion Testing

Exclusion of HD using linkage



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Indirect Diagnosis

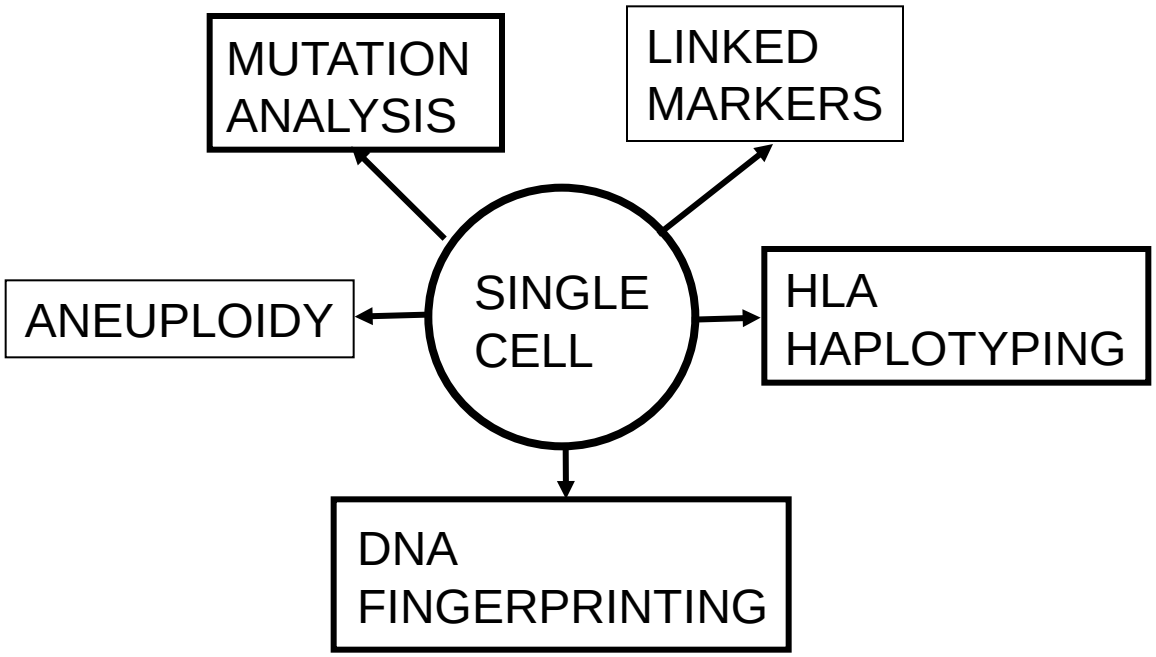
WGA

+

Haplotyping

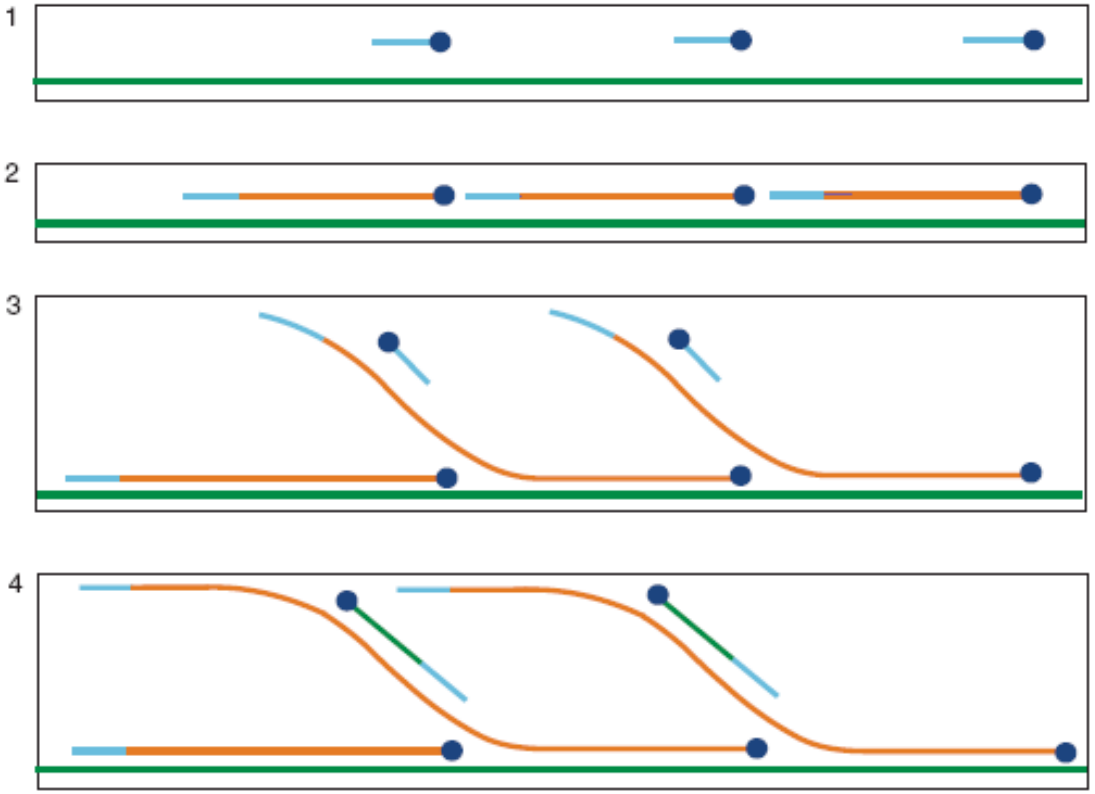
Whole Genome Amplification (WGA)

- Universal first amplification step
- WGA product analysis in conventional facilities
- No requirement for development of special single cell/mutation detection tests



Multiple Displacement Amplification

- Isothermal, no cycling involved (incubation at 30°C)
- Random priming using exonuclease resistant modified random hexamers
- Polymerase makes strand and displaces other strand, e.g. F29 polymerase
- 104-106-fold amplification
- Obtaining µgs of DNA



Spits et al., 2006, Nature Protocols, Vol 1(4): 1965-1970

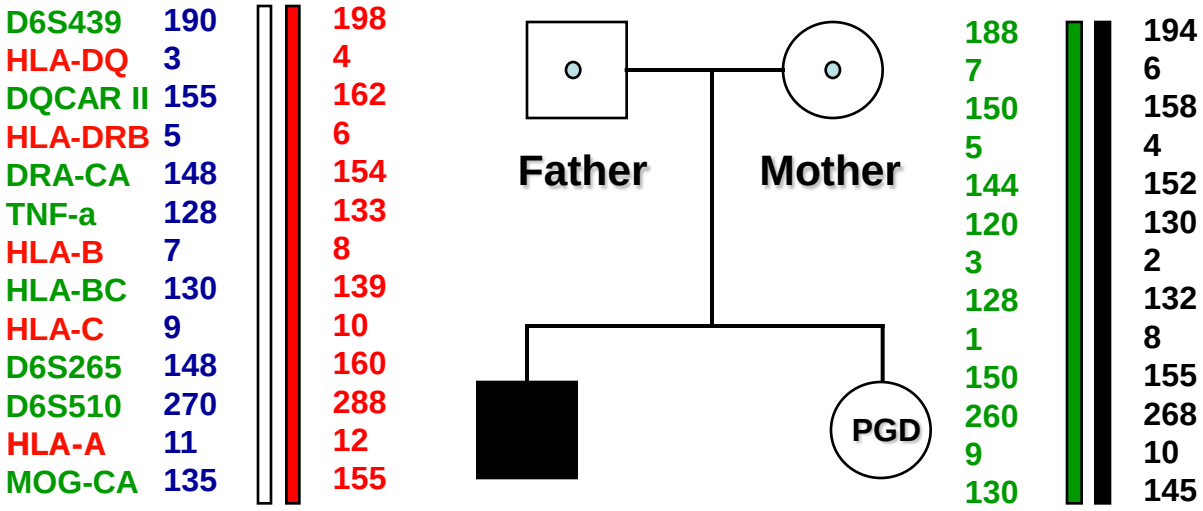
MDA and PGD

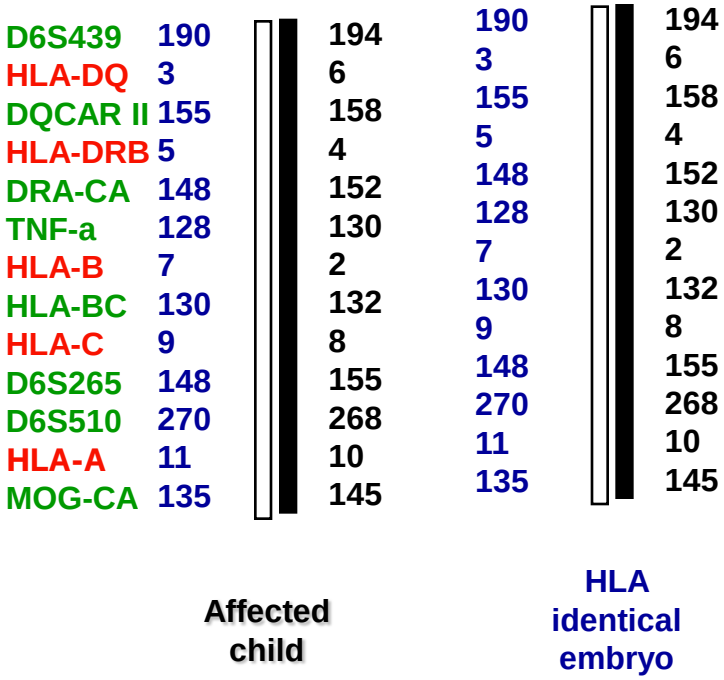
- Use for haplotyping in PGD for monogenic disease (PGH)
  - High ADO rate, many markers have to be included in the protocol
- Use for array-CGH in PGS
- A combination of both

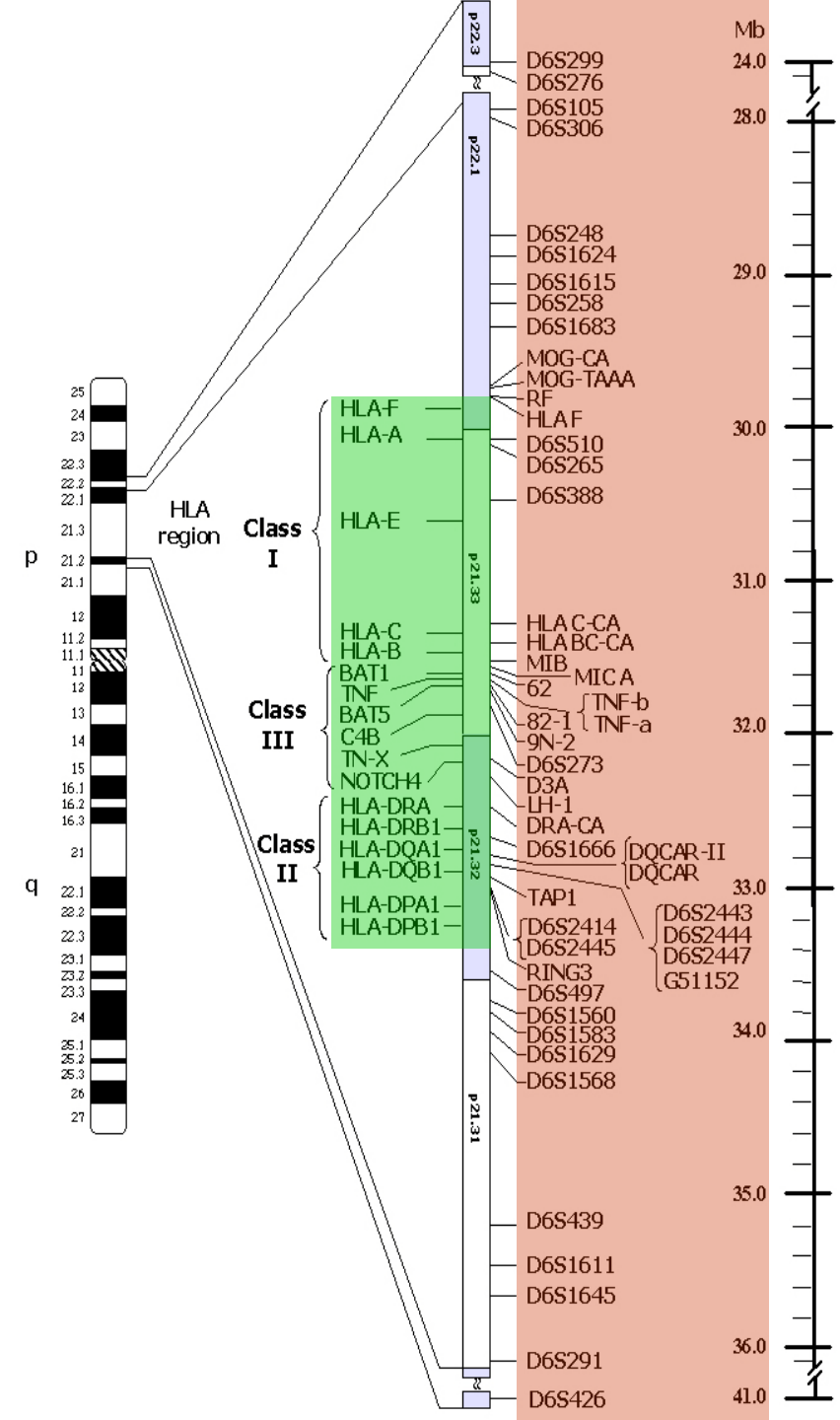
STR markers: Other application in PGD

Preimplantation HLA Matching

Preimplantation HLA Matching by STR haplotying







HLA STR haplotyping

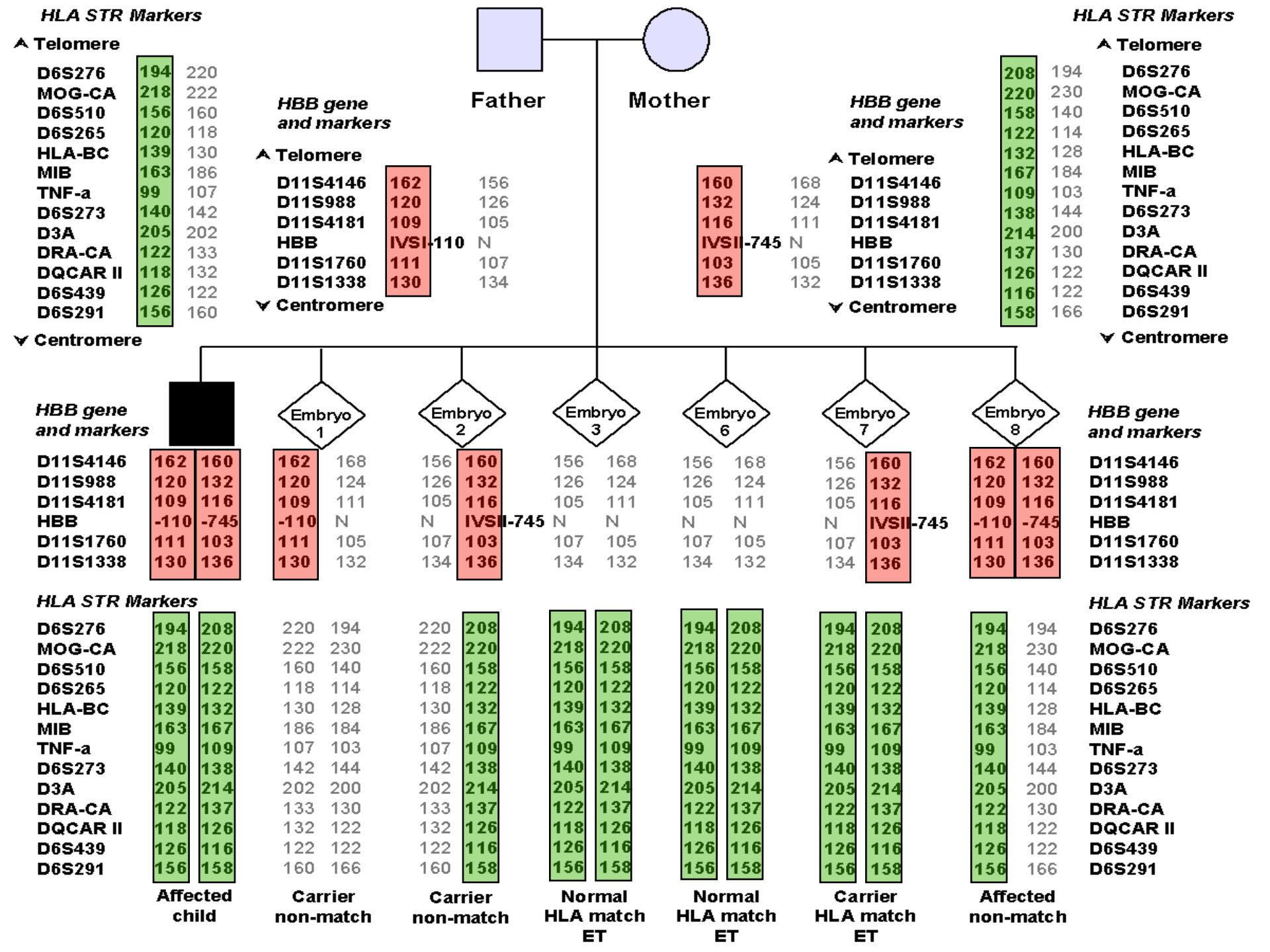
Indirect typing of the HLA region by segregation analysis of the STR alleles

The HLA identity of the embryos with the affected sibling is ascertained evaluating the inheritance of the matching haplotypes.

A panel of 50 different STRs is studied during the pre-clinical work-up

At least 8 informative markers, evenly spaced throughout the HLA region are selected to be used in clinical PGD

Achievement of an accurate mapping of the whole HLA region


HLA STR Markers
Telomere
D6S276 194 220
MOG-CA 218 222
D6S510 156 160
D6S265 120 118
HLA-BC 139 130
MIB 163 186
TNF-a 99 107
D6S273 140 142
D3A 205 202
DRA-CA 122 133
DQCAR II 118 132
D6S439 126 122
D6S291 156 160
Centromere
HBB gene and markers
Telomere
D11S4146 162 156
D11S988 120 126
D11S4181 109 105
HBB IVSI-110 N
D11S1760 111 107
D11S1338 130 134
Centromere
Father
Mother
HBB gene and markers
Telomere
160 168 D11S4146
132 124 D11S988
116 111 D11S4181
IVSII-745 N HBB
103 105 D11S1760
136 132 D11S1338
Centromere
HLA STR Markers
Telomere
208 194 D6S276
220 230 MOG-CA
158 140 D6S510
122 114 D6S265
132 128 HLA-BC
167 184 MIB
109 103 TNF-a
138 144 D6S273
214 200 D3A
137 130 DRA-CA
126 122 DQCAR II
116 122 D6S439
158 166 D6S291
Centromere
HBB gene and markers
D11S4146 162 160
D11S988 120 132
D11S4181 109 116
HBB -110 -745
D11S1760 111 103
D11S1338 130 136
Embryo 1
162 168
120 124
109 111
-110 N
111 105
130 132
Embryo 2
156 160
126 132
105 116
N IVSII-745
107 103
134 136
Embryo 3
156 168
126 124
105 111
N N
107 105
134 132
Embryo 6
156 168
126 124
105 111
N N
107 105
134 132
Embryo 7
156 160
126 132
105 116
N IVSII-745
107 103
134 136
Embryo 8
162 160
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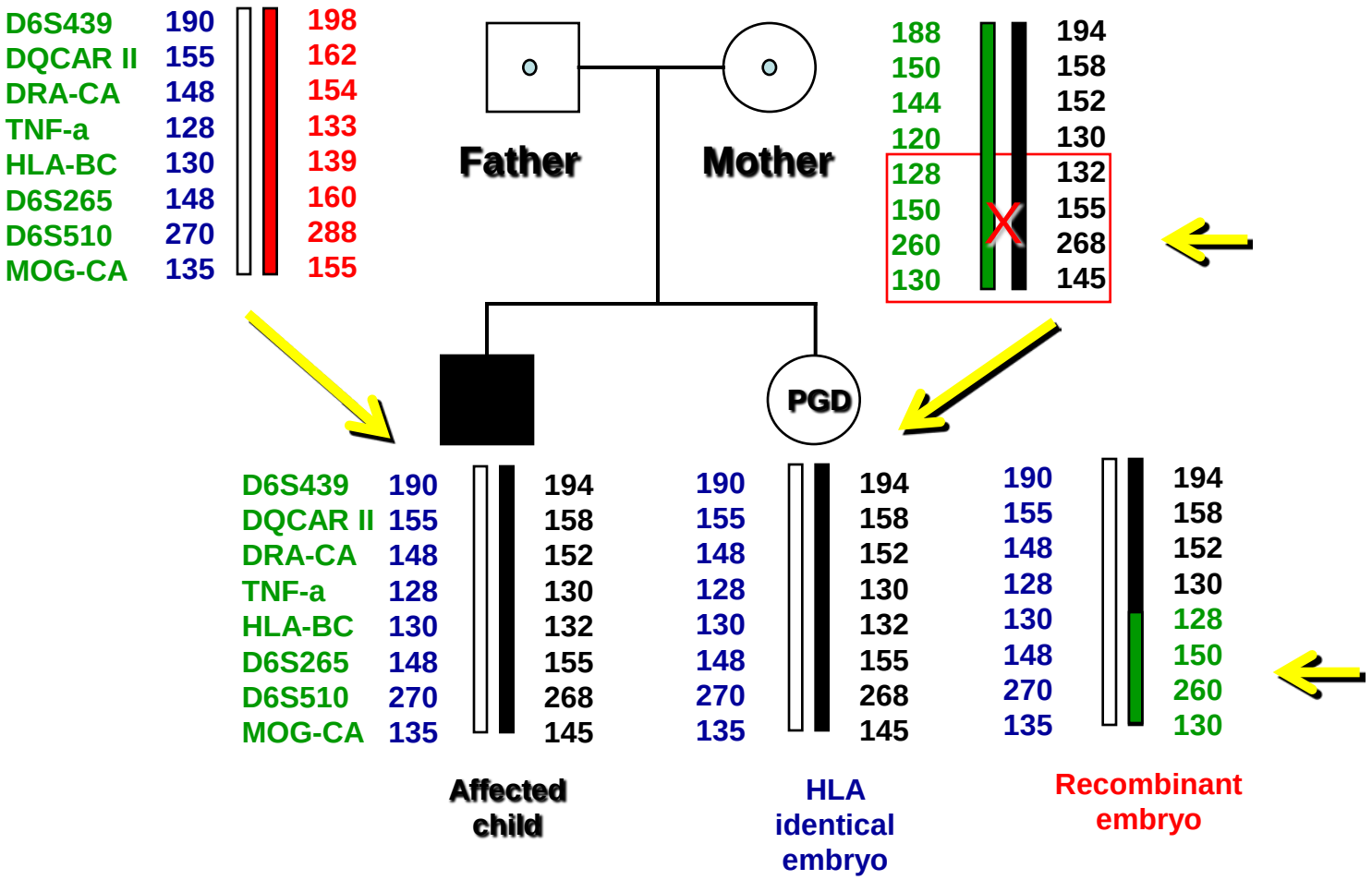
D6S439 126 116
D6S291 156 158
220 194
222 230
160 140
118 114
130 128
186 184
107 103
142 144
202 200
133 130
132 122
122 122
160 166
220 208
222 220
160 158
118 122
130 132
186 167
107 109
142 138
202 214
133 137
132 126
122 116
160 158
194 208
218 220
156 158
120 122
139 132
163 167
99 109
140 138
205 214
122 137
118 126
126 116
156 158
194 208
218 220
156 158
120 122
139 132
163 167
99 109
140 138
205 214
122 137
118 126
126 116
156 158
194 208
218 220
156 158
120 122
139 132
163 167
99 109
140 138
205 214
122 137
118 126
126 116
156 158
194 194
218 230
156 140
120 114
139 128
163 184
99 103
140 144
205 200
122 130
118 122
126 122
156 166
HLA STR Markers
D6S276
MOG-CA
D6S510
D6S265
HLA-BC
MIB
TNF-a
D6S273
D3A
DRA-CA
DQCAR II
D6S439
D6S291
Affected child
Carrier non-match
Carrier non-match
Normal HLA match ET
Normal HLA match ET
Carrier HLA match ET
Affected non-match

The use of STR markers in HLA matching procedure

- The same strategy can be used for different cases (and allele combinations)
- STRs provide an additional control for contamination with exogenous DNA
- The whole HLA complex can be covered, allowing the detection of recombination events between HLA genes.

Avoidance of misdiagnosis due to recombination



STR markers: Other application in PGD

Detection of chromosomal Aneuploidies

Aneuploidy Detection by using STR markers:

microsatellite

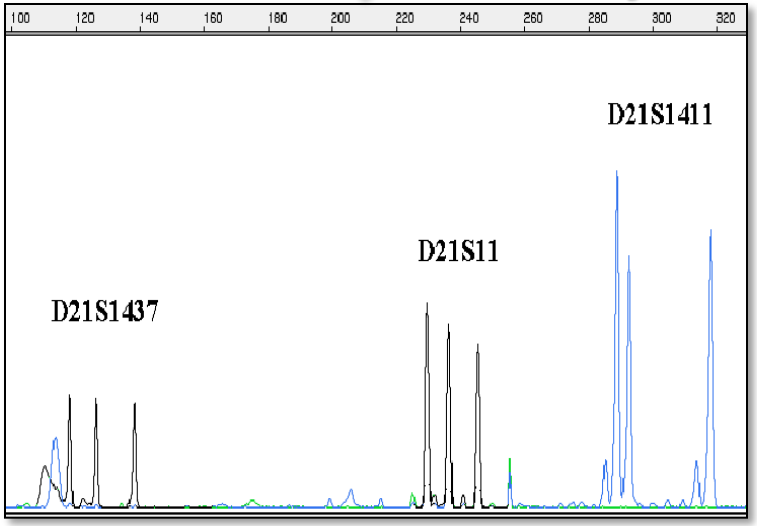
$(gata)_n$

gacctaatc**gata gata gata** taccgtta — **Allele 1**

gacctaatc**gata gata gata gata** taccgtta — **Allele 2**

gacctaatc**gata gata gata gata gata** taccgtta — **Allele 3**

Alleles are distinguishable by PCR product length



Embryo with trisomy 21

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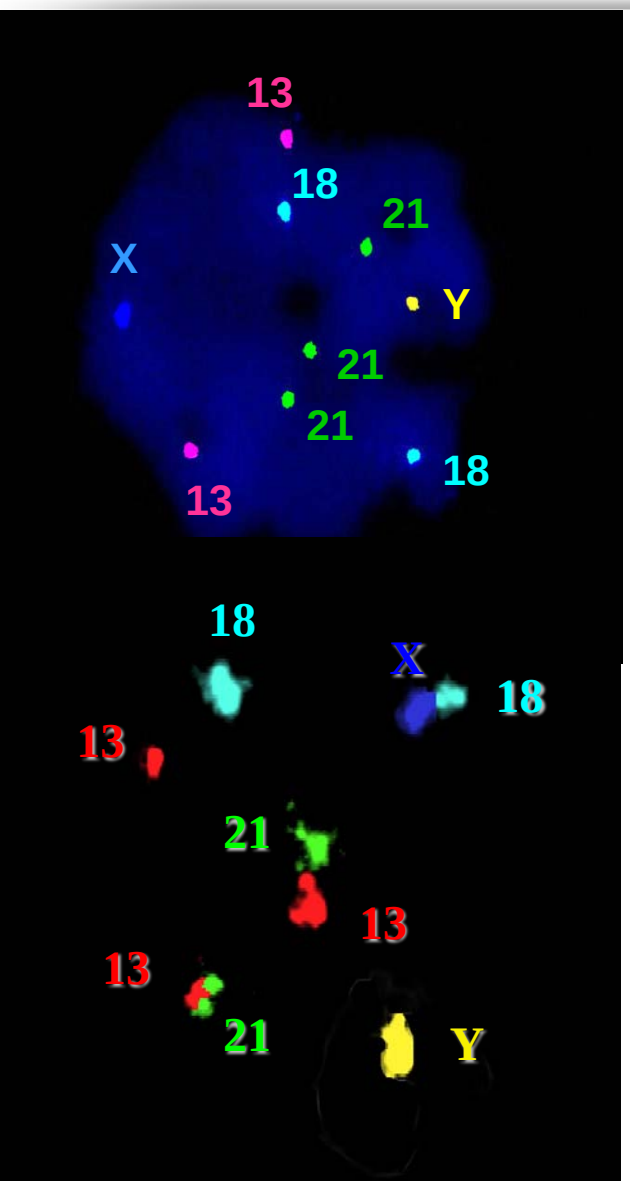
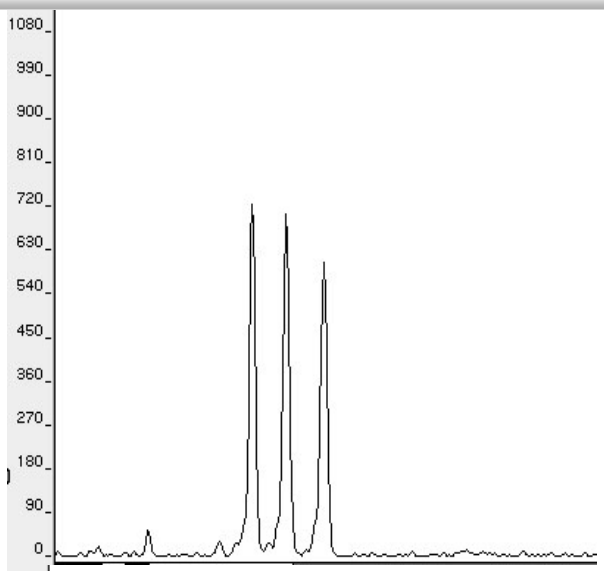
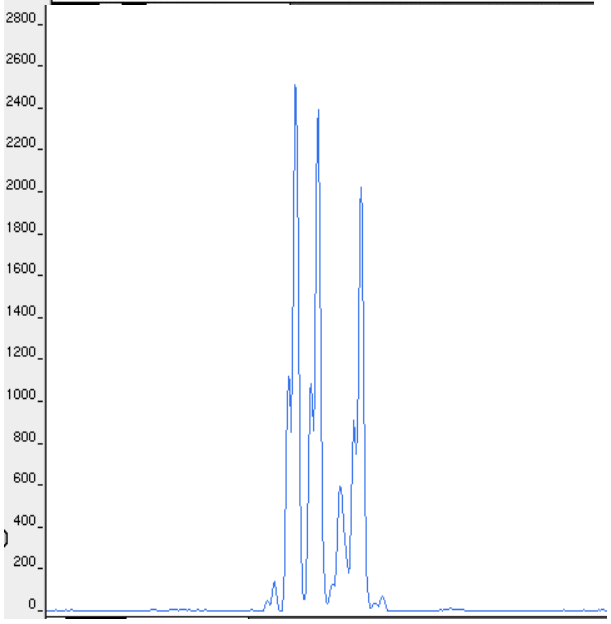
Aneuploidy Detection by using STR markers



Trisomy 21



Trisomy 13



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STR-based PGS: advantages

- Rapid procedure;
- amenable to automation.
- Cell fixation is not necessary
  - Solve suboptimal fixation problems, easier procedure for transport PGD
- Overcome to several technical limitation of FISH procedure:
  - Overlapping signals, split signals, lack of signals, cross-hybridization, polymorphisms, limited availability of the probes, combination of colours
- Possibility to perform combined testing
  - e.g. PGS + Translocation; PGS + SGD
- Tracking of parental origin allows:
  - UPD diagnosis, with the exception of isodisomy
  - Identification of the parental origin of aneuploidies
- A DNA fingerprint is achievable from each embryo
  - Identification of embryos that have implanted
- A potential lower error rate (<1%)
- Fairly inexpensive to run compared to purchasing commercial FISH probes for each translocation
- Unique expertise for PGD (unique lab equipments and staff)

The evolution of PGD for Chromosomal Translocation

Molecular-based PGD protocol for detection of unbalanced embryos

PGD for chromosomal translocation by FISH

- Fluorescent *in situ* hybridisation (**FISH**) is the method of choice for detecting unbalanced chromosome rearrangements on embryos.
- FISH is known to have several **limitations**, primarily deriving from errors inherent to the procedure (e.g., signal overlap, signal splitting, poor probe hybridization, etc.), which may lead to incorrect interpretation of the results and a potentially adverse outcome.
- Interpretation errors may lead to:
    - The loss of suitable (normal/balanced) embryos for transfer (which can impact pregnancy rates).
    - the errant transfer of unbalanced embryos (which can lead to pregnancy loss or the birth of children with congenital anomalies).
- Improvements have been established to diminish the error rate of the technique but certain shortcomings still remain.
- FISH error rates, including false negatives and false positives, have been estimated around **7-10%**.

PCR-based PGD approach for translocations

- **Development and clinical application of an alternative strategy for detection of chromosomal imbalances on embryos derived from both reciprocal and Robertsonian translocation carriers.**

- **Optimization of a molecular-based PGD approach in order to:**

  - **improve the reliability of the PGD procedure**

  - **overcome to the technical limitations of FISH technique**

  - **Use a unique expertise (lab equipments and staff) for PGD**

Methods

The procedure involves testing of single blastomeres by fluorescent multiplex PCR analysis of polymorphic short tandem repeat (STR) markers:

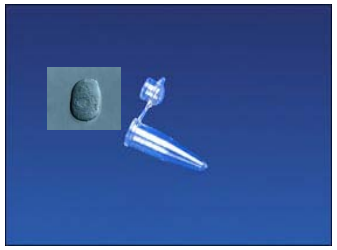
- **Reciprocal Translocations: STR markers flank translocation breakpoints**
- **Robertsonian Translocations: STR markers are located at any point along the chromosomes involved**
- **Patients with advanced maternal age (≥ 38 years old): STR markers were also included to determine the copy number of chromosomes 13, 14, 15, 16, 18, 21, 22, X, Y**

STRs characteristics

The selected STR markers were:

- **Tetranucleotide repeats**, in order to achieve reduced stuttering artefacts and to facilitate data interpretation;
- **Fully informative** heterozygous markers presenting non-shared alleles (i.e., 4 different alleles, male partner a/b and female partner c/d; or 3 different alleles, translocation carrier a/b, other partner c/c), so that segregation of each allele could be clearly determined;
- **At least 3** fully informative STR for each chromosome, in order to avoid misdiagnosis due to possible multiple ADO occurrences;
- **Located distant from the breakpoints**, because the limited resolution of the karyotype could lead to a wrong assignment of the breakpoints.

PGD Process



Cell Lysis

External Multiplex PCR

External primers for the amplification of:

- STR markers for translocation
- STR markers for detection of aneuploidies, in patients with advanced maternal age (>37 y.o.)

2 μl of the primary PCR products

Nested PCR (fluorescent)

Electrophoresis of PCR products

Data Interpretation

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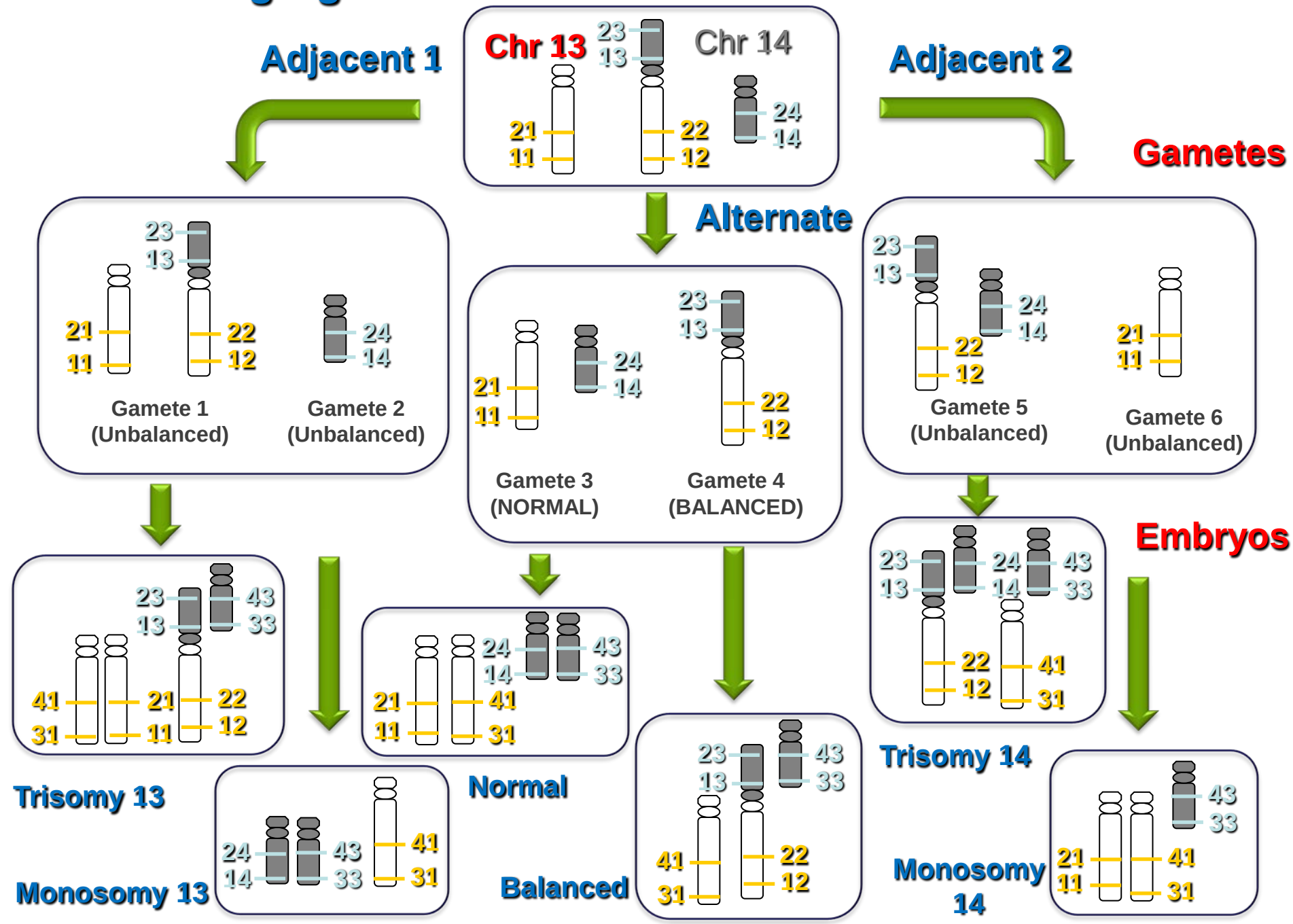
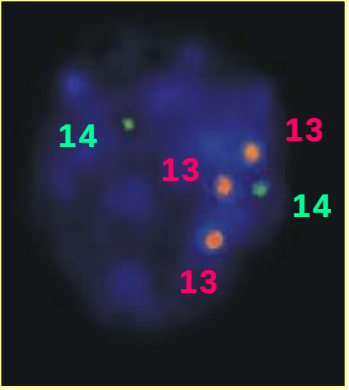
Classification of the results

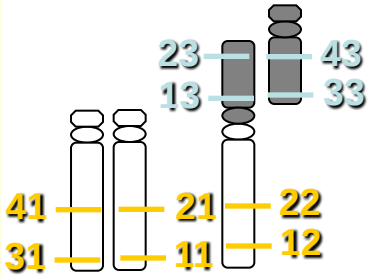
Embryos were diagnosed as:

- **Normal-Balanced**, if PCR results clearly indicated **2 signals** (peaks) for each chromosome tested (**disomic profile**);
- **Unbalanced**
  - **trisomies** (3 peaks – **trisomic profile**),
  - **monosomies** (1 peak – **monosomic profile**)
  - **nullisomies** (no PCR signals for all the markers tested)

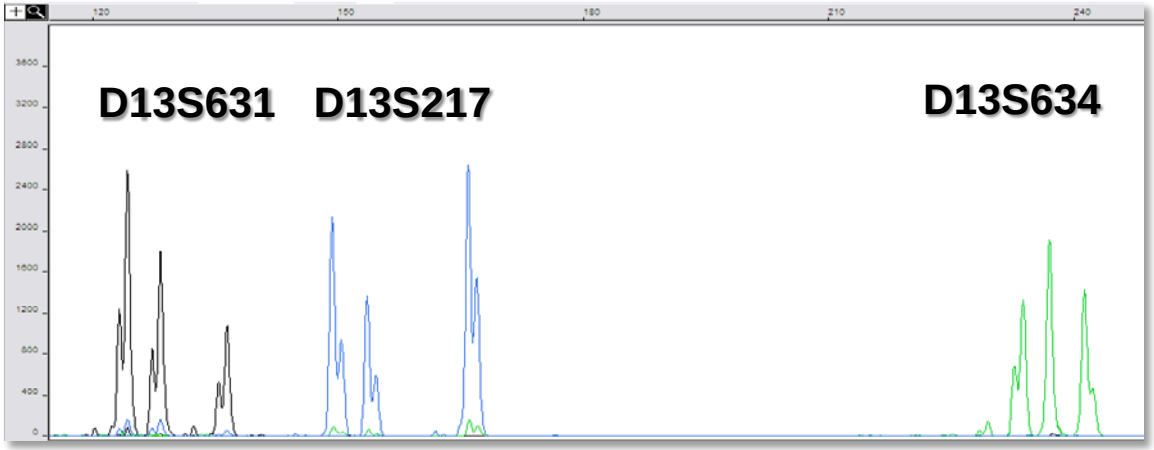
Segregation of Robertsonian Translocations

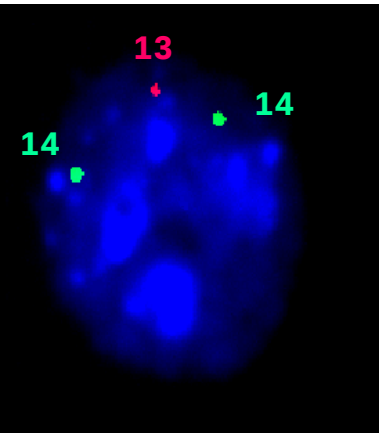


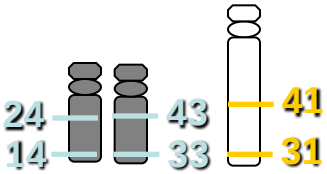




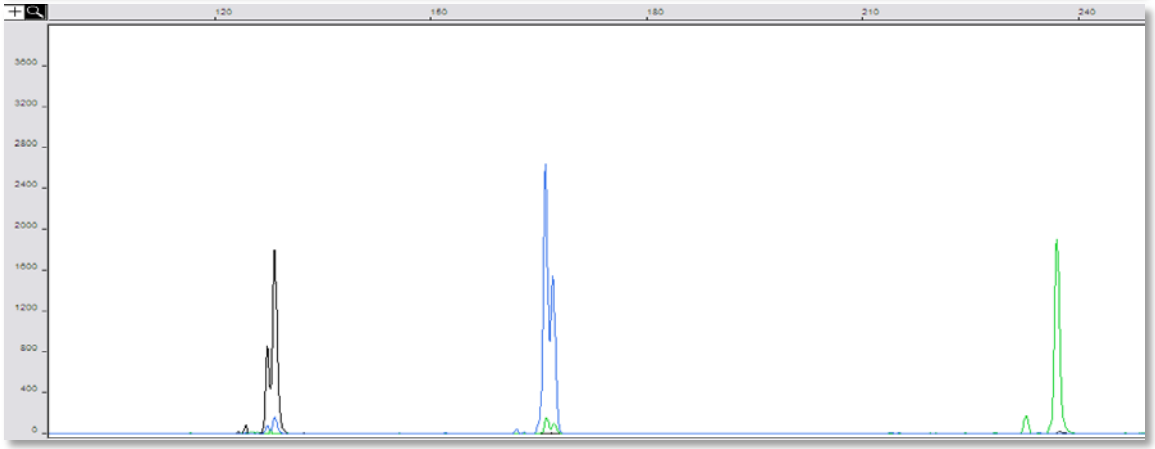
Trisomy 13

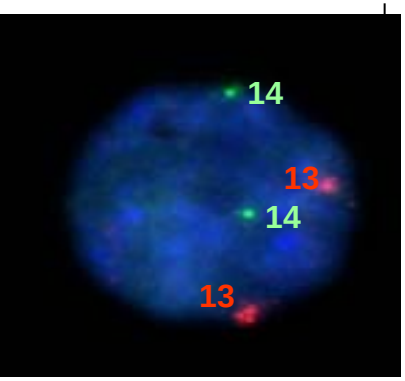


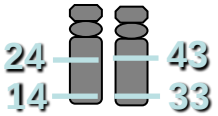


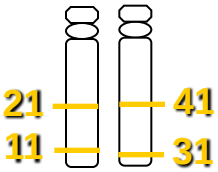


Monosomy 13

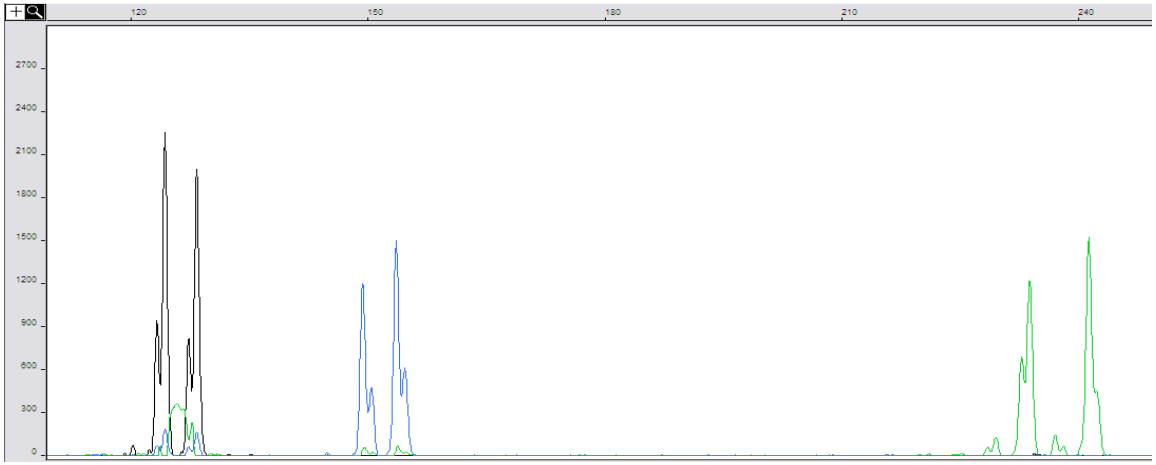


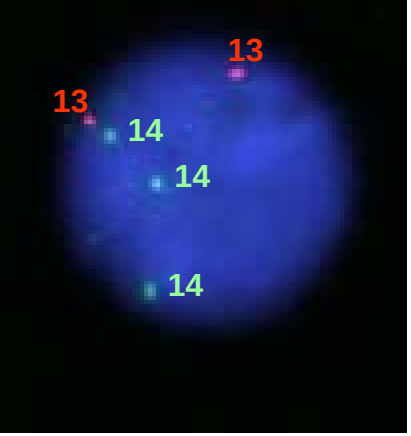


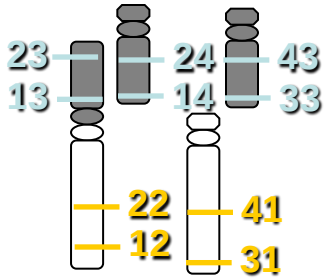




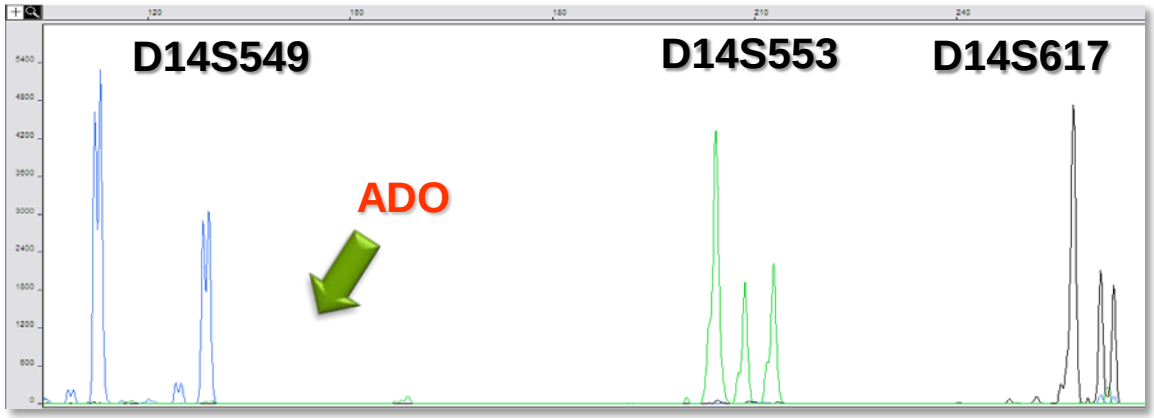
Normal

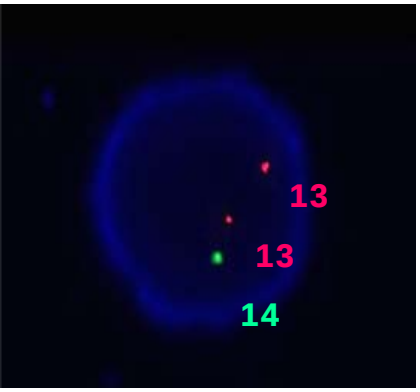


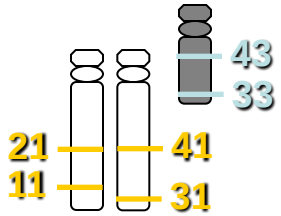




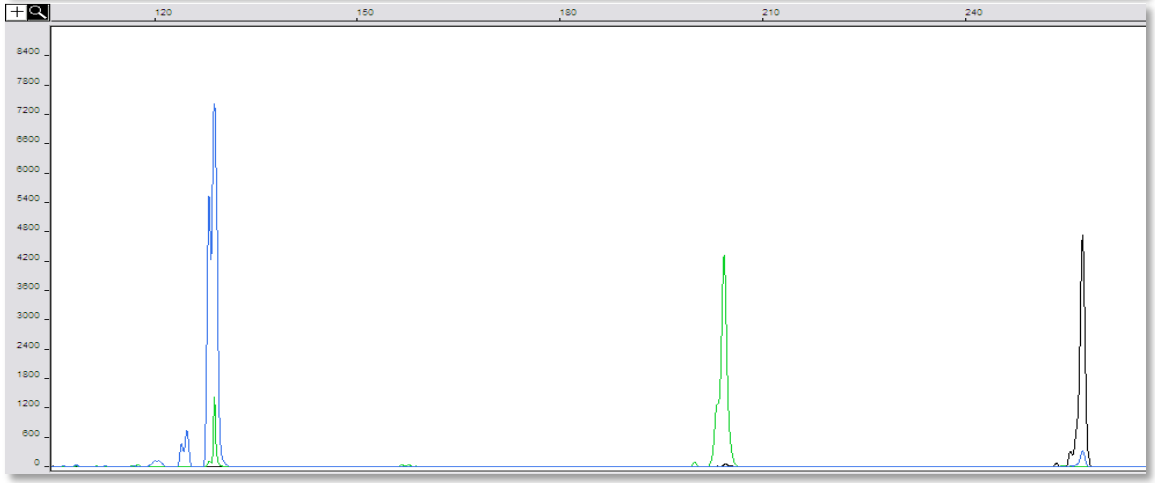
Trisomy 14

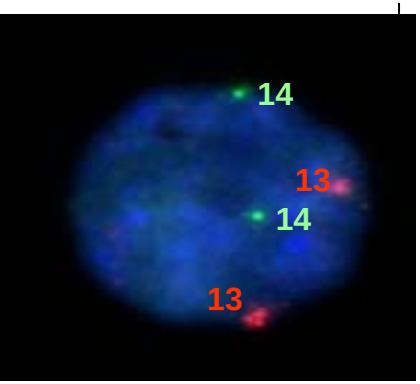


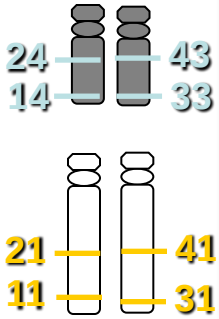




Monosomy 14







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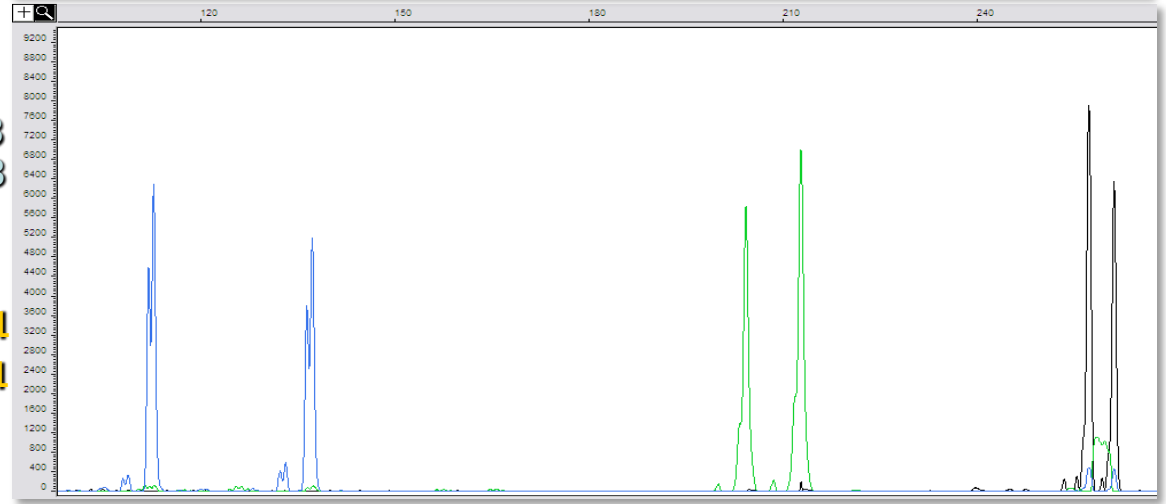
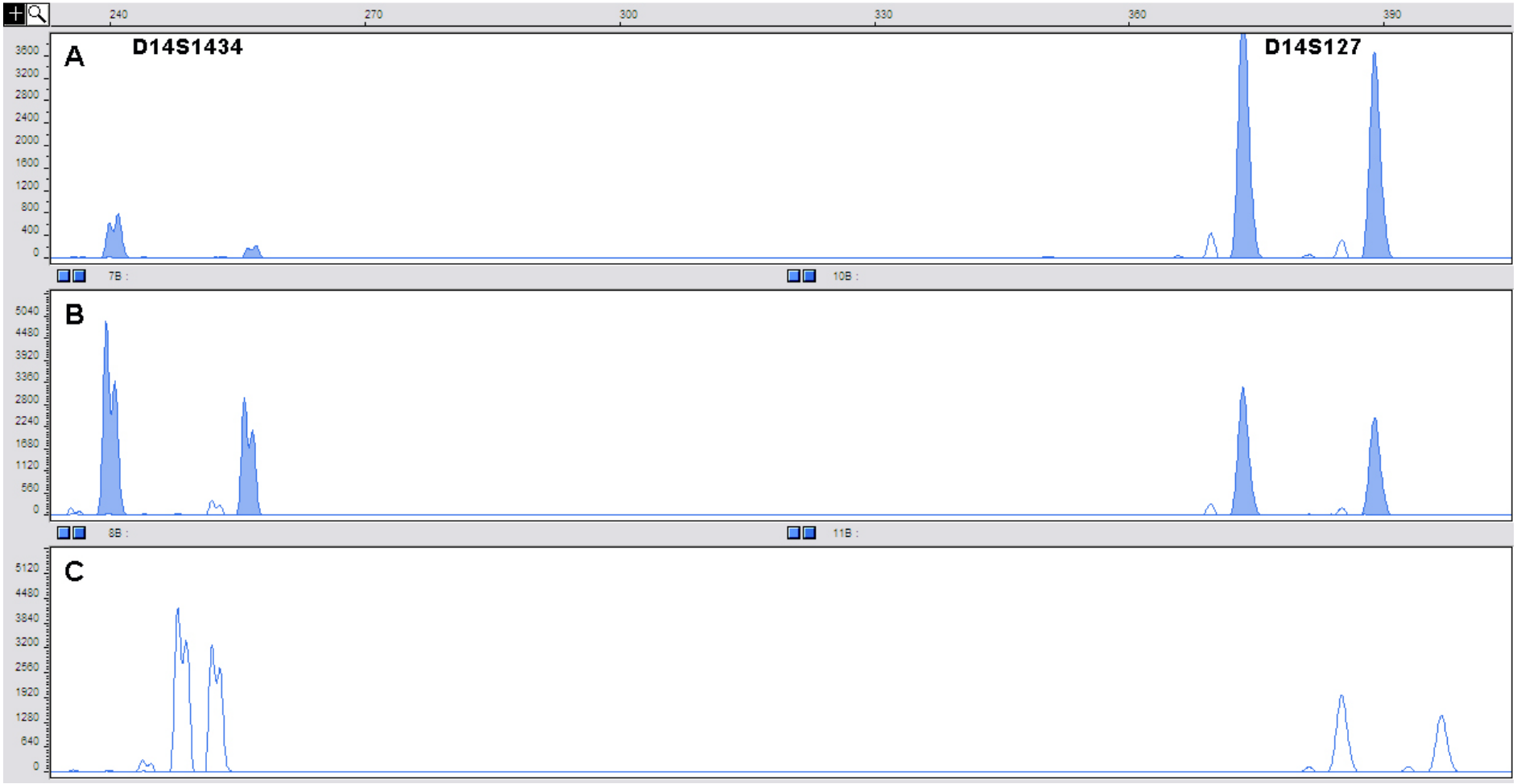


STR-based PGD for translocations: advantages

- Easy procedure and data interpretation
- Amenable to automation
- Rapid procedure (<12 h)(4-6 h for 1PB testing)
- Cell fixation (PBs or blastomeres) is not necessary
  - Solve suboptimal fixation problems, easier procedure for transport PGD
- Overcome to several technical limitation of FISH procedure:
  - Overlapping signals, split signals, lack of signals, cross-hybridization, polymorphisms, limited availability of the probes, combination of colours
- Possibility to perform combined testing
  - e.g. Translocation + PGS; Translocation + SGD with or w/o PGS
- Post-hybridization wash and re-probing are not necessary for combined testing
- UPD can be detected
- Lower error rate
- Low expensive
- A DNA fingerprint is achievable from each embryos
  - Identification of embryos that have implanted

STR-based PGD for translocations: UPD detection



Uniparental disomy (UPD) detection on embryos from a PGD case for Robertsonian translocation (13;14). The embryo (A) inherited alleles only from one parent (B) and failed to inherit an allele from the other (C).

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STR-based PGD for translocations: disadvantages

- Affected by contamination
- Affected by ADO – Preferential Amplification
- Recombination risk in cases of 1PB testing

Clinical application: pregnancies and babies

Clinical outcome	Total
No. of cycles	27
No. of couples	27
No. of embryo transfers	24
No. of transfers cancelled	3
No. of embryos transferred	52
Average embryos transferred	1.8±0.9
No. clinical pregnancies	18
No. of embryos implanted (gestation sacs)	31
No. of foetal heartbeats	29
No. foetuses after 12^ weeks of gestation	24
- Triplets	1
- Twins	4
- Singleton	13
Clinical pregnancy rate per OR	66.7%
Clinical pregnancy rate per ET	75.0%
Implantation rate	59.6%
No. of pregnancies delivered	10
No. of babies born	13

Fiorentino *et al*. Fert Steril, in press.

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Clinical outcome: comparison with FISH studies

Reference	Cycles/ Couples	Maternal age (Mean±SD)	No. clinical pregnancies	Clinical pregnancy rate/ET	Clinical pregnancy rate/OR	Implantation rate
Robertsonian translocation						
– Goossens et al. (34)	1009 / NA	33.5	213	29.0	21.1	16.0%
– Verpoest et al. (35)	94 / 54	32.2±5.0	24	38.1%	25.5%	NA
– Munnè et al. (36)	133 / 88	34.0	30	42.7%	37.6	NA
– Gianaroli et al. (37)	35 / 22	35.5±3.7	13	59.1%	37.1%	44.4%
– **This study**	**15 / 15**	**37.6±4.8**	**9**	**69.2%**	**60.0%**	**57.7%**
Reciprocal translocation						
– Goossens et al. (34)	1973 / NA	33.0	264	22.9%	13.4%	13.1%
– Verpoest et al. (35)	190 / 90	33.0±4.5	22	23.2%	11.6%	NA
– Lim et al. (38)	51 / 34	31.3±3.1	14	38.6%	33.3%	24%
– Otani et al. (7)	36 / 29	32.7±2.9	17	NA	47.2%	NA
– Munnè et al. (36)	338 / 239	36.1	79	34.1%	23.4%	NA
– Gianaroli et al. (37)	29 / 24	34.0±5.3	3	27.3%	10.3%	20.0%
– **This study**	**12 / 12**	**34.4±3.2**	**9**	**81.8%**	**75.0%**	**61.5%**
Cumulative translocations						
– Goossens et al. (34)	2982 / NA	33.2	477	25.3%	16.0%	14.2%
– Verlinsky et al. (39)	469 / NA	NA	123	34.6%	NA	NA
– McArthur et al. (40)[a]	21 / NA	NA	7	50%	NA	50%
– Verlinsky et al. (6)	183 / 130	33.2	45	35.7%	24.6%	24.7%
– **This study**	**27 / 27**	**36.1±4.4**	**18**	**75.0%**	**66.7%**	**59.6%**

Thank you……..

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