Pre-examination Process (for labs)
Validation of test - II

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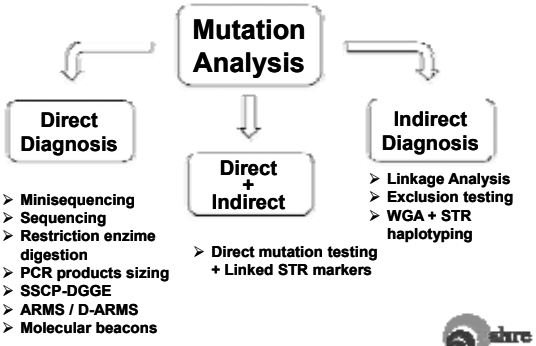
MUTATION ANALYSIS



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

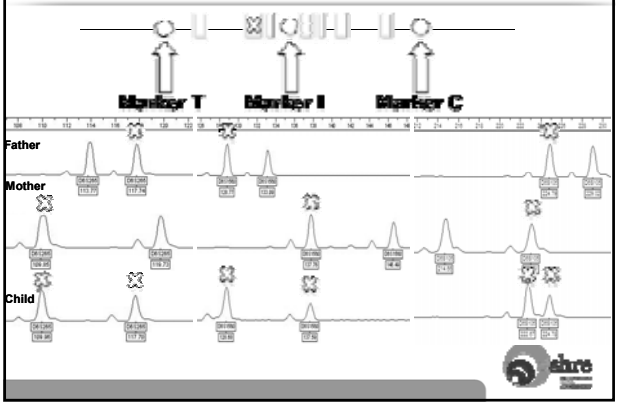
Advantages:

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

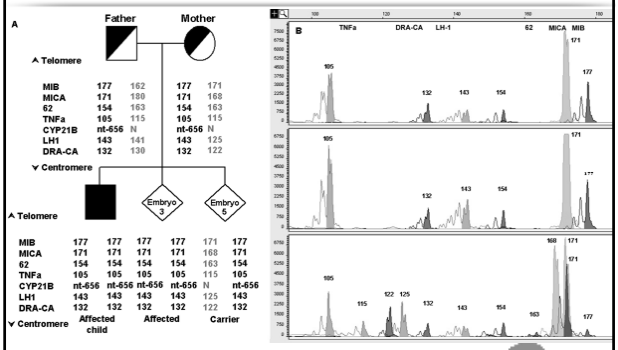
Disadvantages:

- 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



PGD for 21-OH deficiency by linkage analysis



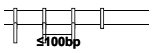
Genetic Markers

- **Single nucleotide polymorphisms (SNPs)**
  - single base change in genomic DNA
  - one per every 500 - 1000 base pairs
  - less informative than microsatellite markers
  - low mutation rate* (≈ 10^{-9})

TGCATTGCGTAGGC
TGCATTCCGTAGGC

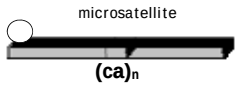
- **Short Tandem Repeats (STRs) or Microsatellites**
  - 1 every few kb, high degree of heterozygosity
  - mutation rate* ≤ 10^{-3}
  - Types:
    - di- (CA)n,
    - tri- (CAG)n,
    - tetra- (GATA)n.
    - Penta – (AAAAT)n nucleotide repeats
  - Minisatellites (VNTR - variable number tandem repeats)
  - 1 every few kb
  - mutation rate* ≤ 10^{-1}

TGCTCACACACACACAGC
TGCTCACACA------GC



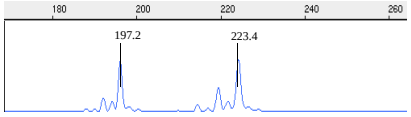
*per generation

Microsatellites Markers



the repeat region is variable between samples while the flanking regions where PCR primers bind are constant

gacctaatc **ca ca** taccgtta **Allele 2**
gacctaatc **ca ca ca ca ca** taccgtta **Allele 5**

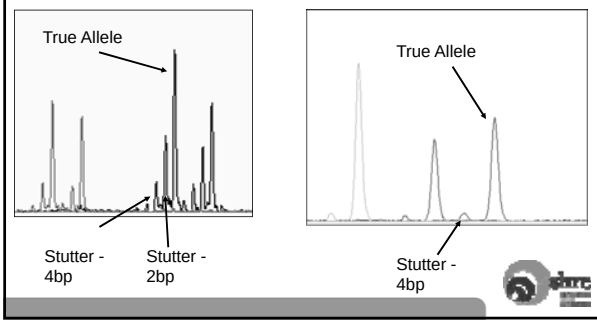


Alleles distinguishable by PCR product length

Homozygote = both alleles are the same length
Heterozygote = alleles differ and can be resolved from one another

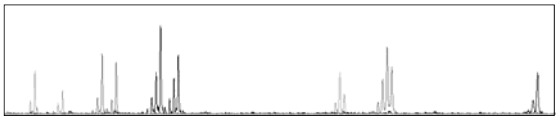
Microsatellite Characteristics

Stutter Peaks: Di-nucleotide vs Tetra-nucleotide Repeats



Microsatellite Marker Types
Dinucleotide Repeat Markers

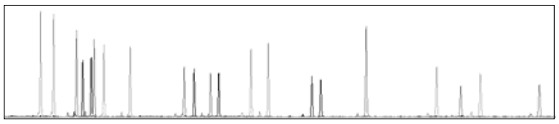
E.g.: (CA)(CA)(CA)…(CA)n



- Abundant coverage
- Characteristic stutter patterns
  - Interpretation can be complex

Microsatellite Marker Types
Tetranucleotide Repeat Markers

E.g. (TCTA)(TCTA)…(TCTA)n

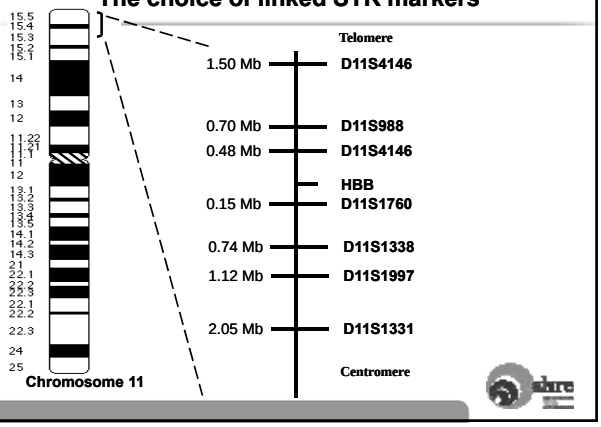


- Well characterised
- Discrete allele peaks
- Low, predictable and measurable stutter peaks
  - Easier interpretation

How to build the haplotypes?

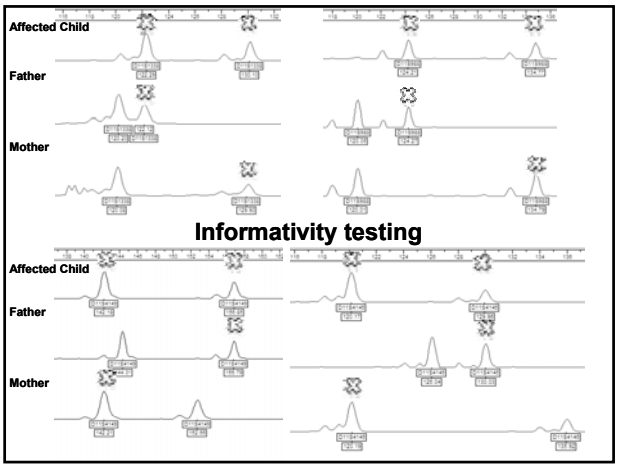
- Selection of the STR markers linked to the disease causing gene

The choice of linked STR markers



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



How to build the haplotypes?

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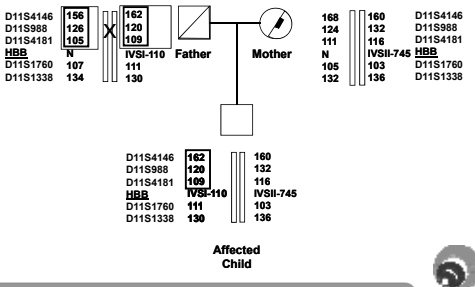
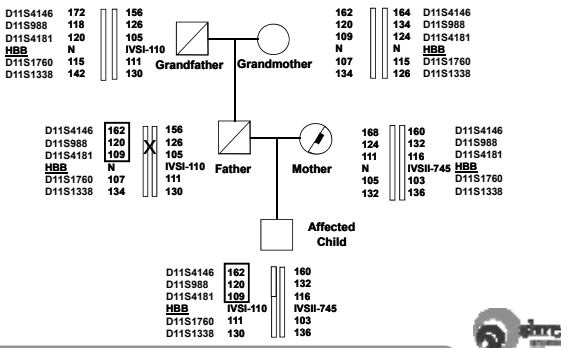
- Identification of the alleles associated with mutation/disease
- Determination of the haplotypes

DETERMINING HAPLOTYPES FOR LINKAGE ANALYSYS



DETERMINING HAPLOTYPES FOR LINKAGE ANALYSYS



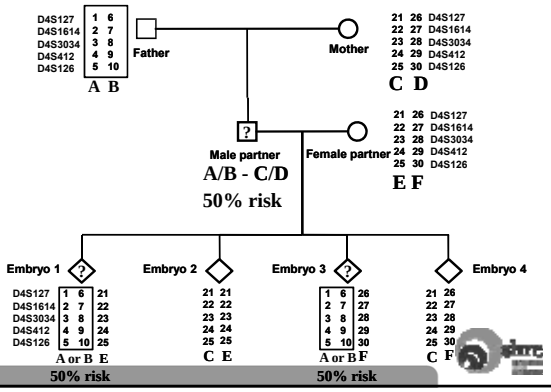
Linkage-based PGD protocols: general guidelines

- 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

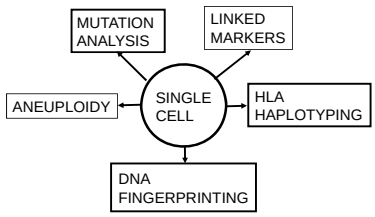


Indirect Diagnosis

WGA
+
Haplotyping

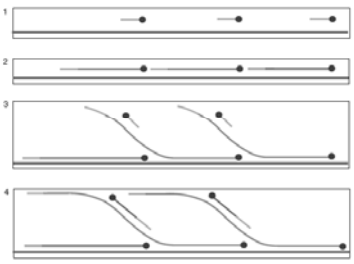
Whole Genome Amplification (WGA)

- 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

MUTATION ANALYSIS

Direct + Indirect Diagnosis

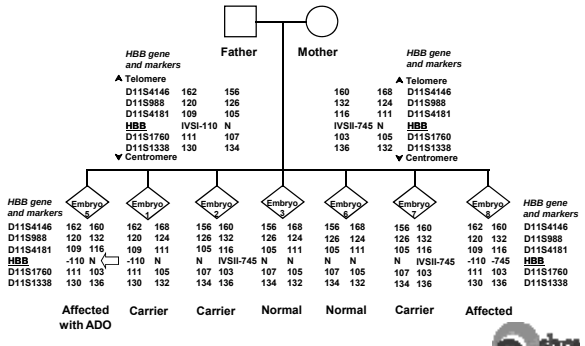
The use of STR markers in PGD procedure

- 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

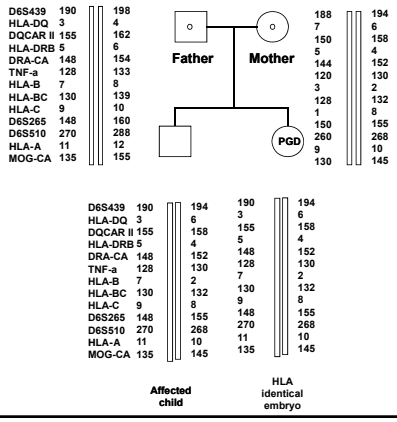
Avoidance of misdiagnosis due to ADO

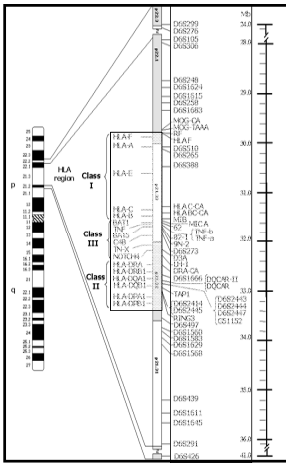


STR markers: Other application in PGD

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.

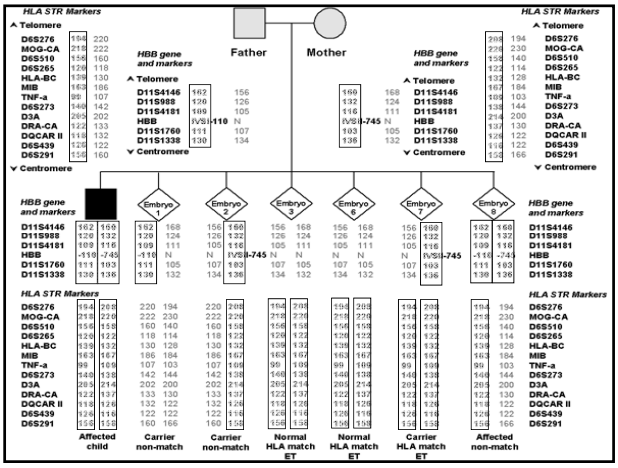
A panel of 50 different STRs is studied during the pre-clinical work-up

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

Informativity testing for preimplantation HLA matching

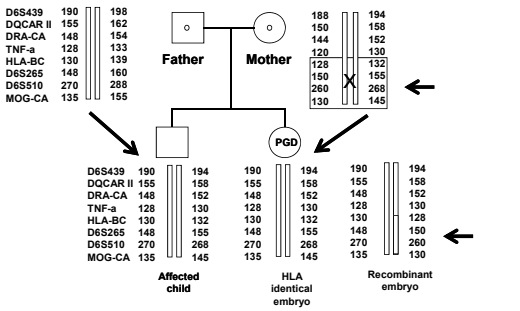
MARKER	MOTHER		FATHER		CHILD	
TNF1	107	114	105	114	105	114
D6S510	155	140	151	148	151	140
D6S426	120	128	120	124	120	128
MIC A	168	171	171	168	171	171
D6S273	142	144	136	142	136	144
D6S276	218	220	216	211	216	220
LH1	144	146	141	144	141	146
DQ CAR II	131	118	130	123	130	118
DRA CA	130	137	130	139	130	137
MOG CA	215	206	225	206	225	206
HLA BC CA	117	129	120	132	120	129
D6S265	110	114	120	116	120	114
D6S291	156	160	158	158	158	160
TNF2	111	111	113	111	113	111
82-1	111	111	104	111	104	111
G51152	145	145	147	147	147	145
D3A	200	202	202	202	202	202
RING3 CA	126	124	124	126	124	124
62	156	163	156	163	156	163
D6S439	120	122	120	122	120	122
MIB	177	177	172	177	172	177
D6S105	141	141	139	153	139	141



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

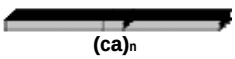


STR markers: Other application in PGD

Detection of chromosomal Aneuploidies

Aneuploidy Detection by using STR markers:

microsatellite



$(ca)_n$

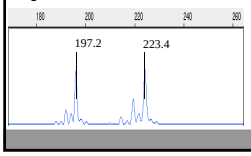
gacctaatc **ca ca** taccgtta
gacctaatc **ca ca ca ca ca** taccgtta
gacctaatc **ca ca ca ca ca ca ca** taccgtta

AMA
RIF
RM

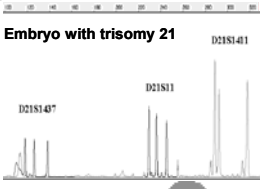
Chromosomes:
13, 14, 15, 16, 18, 21, 22, X ,Y

Allele 1
Allele 2
Allele 3

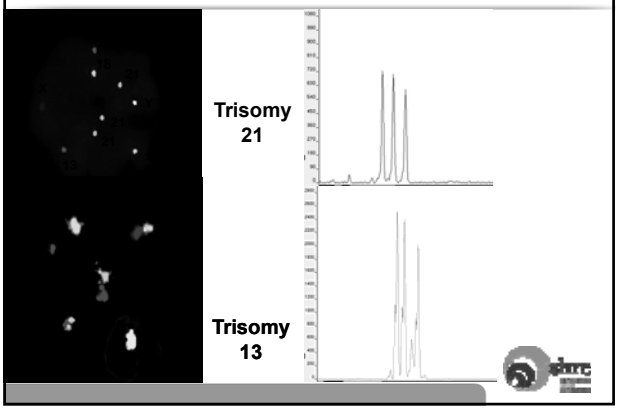
Alleles are distinguishable by PCR product length



Embryo with trisomy 21



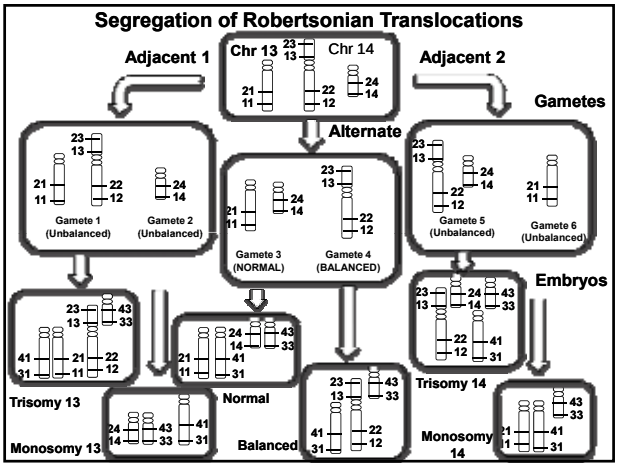
Aneuploidy Detection by using STR markers

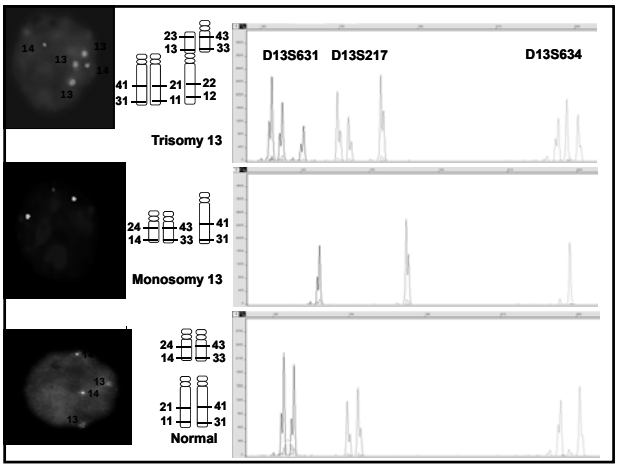


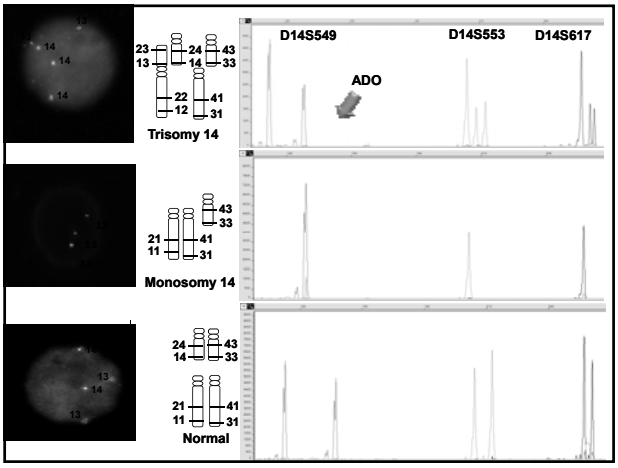
STR markers: Other application in PGD

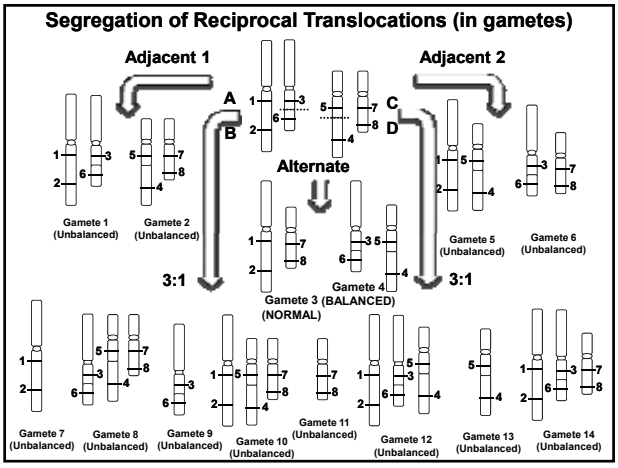
Detection of unbalanced chromosomal translocations

Segregation of Robertsonian Translocations

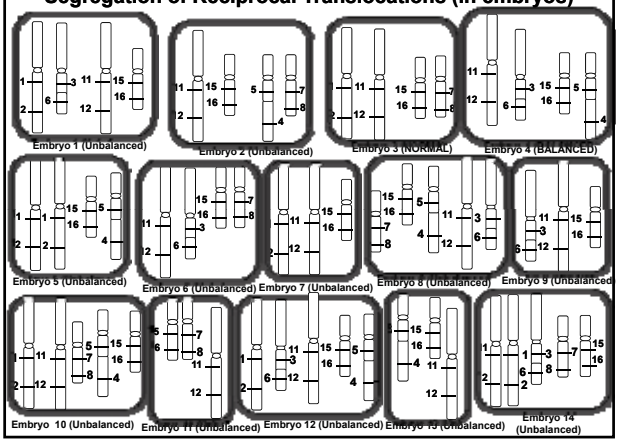



D13S631
D13S217
D13S634
Trisomy 13
Monosomy 13
Normal

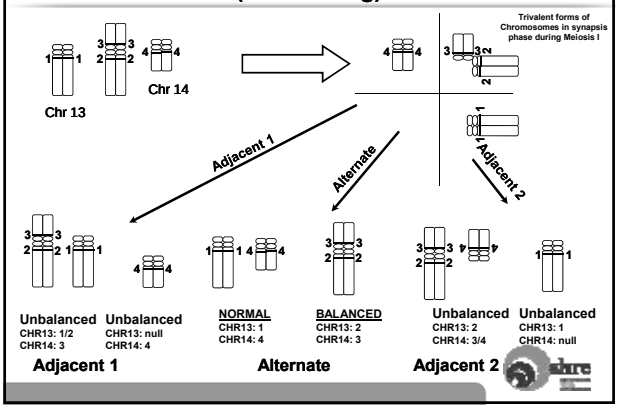

D14S549
D14S553
D14S617
ADO
Trisomy 14
Monosomy 14
Normal


Segregation of Reciprocal Translocations (in gametes)
Adjacent 1
Adjacent 2
A
B
C
D
Alternate
3:1
3:1
Gamete 1 (Unbalanced)
Gamete 2 (Unbalanced)
Gamete 3 (BALANCED) (NORMAL)
Gamete 4
Gamete 5 (Unbalanced)
Gamete 6 (Unbalanced)
Gamete 7 (Unbalanced)
Gamete 8 (Unbalanced)
Gamete 9 (Unbalanced)
Gamete 10 (Unbalanced)
Gamete 11 (Unbalanced)
Gamete 12 (Unbalanced)
Gamete 13 (Unbalanced)
Gamete 14 (Unbalanced)

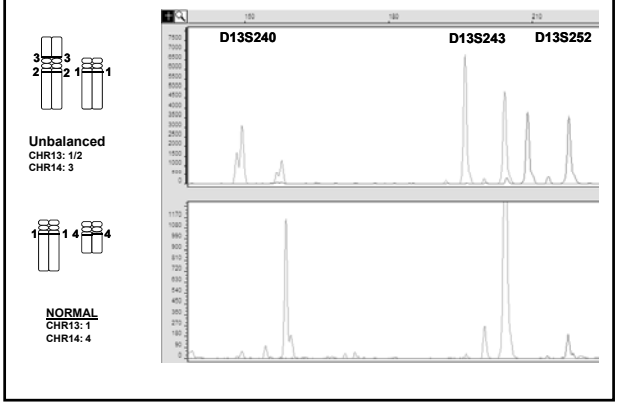
Segregation of Reciprocal Translocations (in embryos)



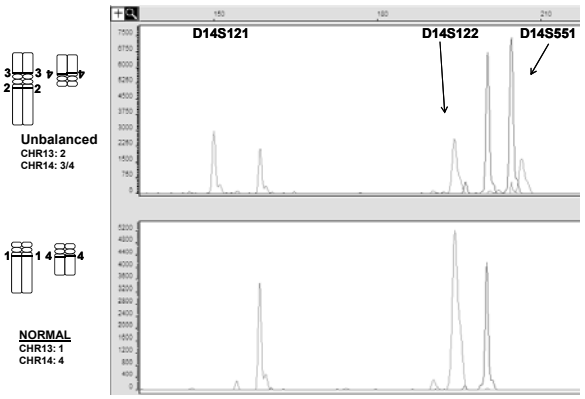
Segregation of Robertsonian Translocations (1PB testing)



PCGD for Robertsonian Translocations by 1PB testing



PCGD for Robertsonian Translocations by 1PB testing



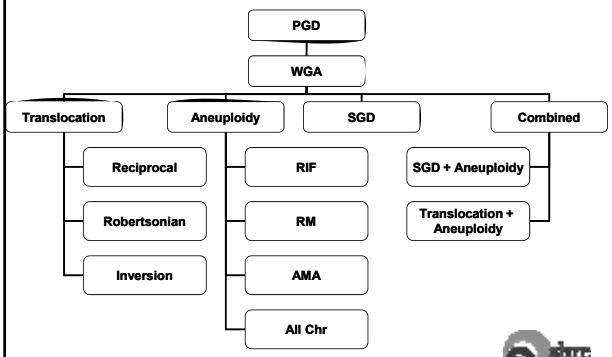
STR-based PGD for translocations: advantages

- 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: disadvantages

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

Future vision: unique protocol for PGD



Pre-examination Process (for labs)

Validation of the PGD Protocol

Developing a PGD protocol

- Confirmatory testing of the mutation(s)
- Choice of the PGD strategy (direct mutation diagnosis, linkage)
- Informativity testing for linked/un-linked STR markers
- Test multiplex PCR: combination of primers, etc.
- Application to the single cell level (i.e. single lymphocytes, fibroblast, buccal cells, etc.)
- Amplification efficiency, contamination and ADO rates in heterozygous samples.
- Test on spare blastomeres (depending on availability)
- Ready for PGD

Preliminary PGD work-up: parameters to be evaluated

- Pre-clinical tests on single cells (lymphocytes, fibroblasts, amniocytes, buccal cells, etc.)
  - At least 50 cells in total
- Amplification efficiency > 90 % should be aimed
  - Lower efficiency coincides with higher ADO
- ADO rates should be as low as possible (preferably <10%)
  - ADO is mostly influenced by PCR method (conventional vs. fluorescent)
- The contamination rate should be less than 5% (preferably zero)
  - At least 50 cell wash blanks

ESHRE PGD Consortium guidelines, Hum Reprod. 2005 Jan;20(1):35-48

One-cell versus two-cells biopsy

- Doubts and discussion about one vs two-cell biopsy
  - Risk of misdiagnosis (e.g. autosomal dominant disorders)
  - Claims that higher implantation rate is achieved after one-cell biopsy
- Randomized control trial (Goossens V. et al., Hum Reprod 2007)
  - Removal of two blastomeres significantly decreases the likelihood of blastocyst formation
  - one-cell biopsy results in a significantly lower diagnositic efficiency
  - live birth delivery rates were not statistically different for one- and two-cell biopsy
- RECOMMANDATION: diagnosis of only 1 cell requires a robust PGD protocol