Failed DNA amplification

PGDIS
May 8-11 2016
Bologna, Italy

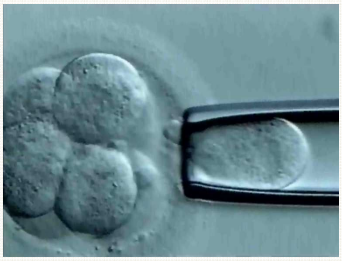
Anıl Biricik

GENOMA - Molecular Genetics Laboratory
Rome – Milan
Italy

biricik@laboratoriogenoma.it

What is Preimplantation Genetic Diagnosis (PGD)?

- PGD is a very early form of prenatal diagnosis.
- Its intended goal is to significantly reduce a couple's risk of transmitting a genetic disorder or chromosomal abnormality, by diagnosing a specific disorder in oocytes or early human embryos that have been cultured in vitro, before a clinical pregnancy has been established.



- Only the unaffected embryos would then be transferred to the uterus.
- The great advantage of PGD over prenatal diagnosis is that a potential termination of pregnancy is avoided.
- Opportunity for couples to prevent the physical and psychological trauma, and ethical-moral problems associated with possible termination.

PREIMPLANTATION GENETIC DIAGNOSIS

PGD to reduce recurrent genetic risk

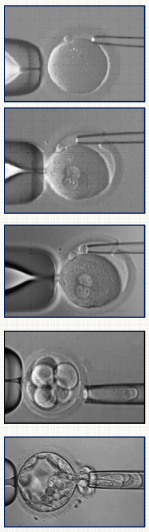
- **Single Gene Disorders**
  - Autosomal Recessive
  - Autosomal Dominant
  - X-Linked
- **Late onset disorders**
- **Inherited Predisposition to Cancer**
- **HLA Genotyping**
- **Structural chromosomal abnormalities**
  - Translocations (reciprocal, Robertsonian)
  - Deletions
  - Duplications
  - Inversions

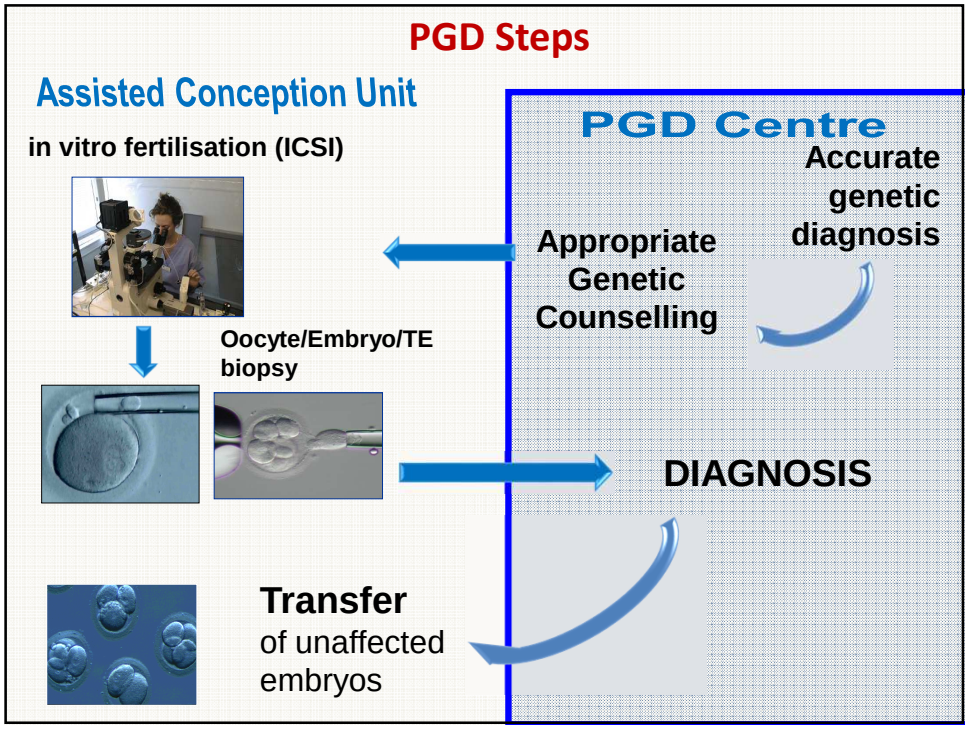
PGD to improve IVF outcome

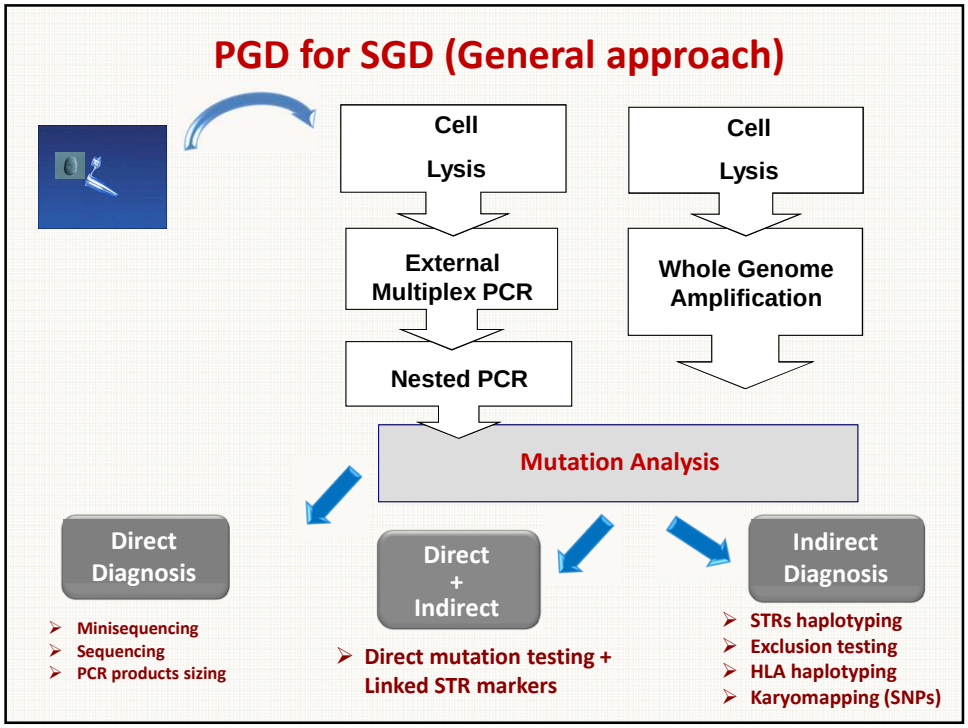
- **Aneuploidy Screening (PGS)**
  - Advanced Maternal Age
  - Repeated Implantation Failure
  - Recurrent Early Abortions
  - Severe Male Infertility

PGD classification according to the cell tested

- **1^ Polar Body (1PB)**
  - **Pre-conception Genetic Diagnosis (PCGD) (Before ICSI) for couples with ethical concerns (Fiorentino et al., 2008)**
  - **Pre-Zygotic Genetic Diagnosis (After ICSI, before syngamia) Switzerland – Austria – Germany**
- **1^ & 2^ Polar Body (1PB+2PB) RGI – Chicago**
- **Blastomeres from cleavage stage embryos (day-3)**
  - Single blastomere biopsy is advised (Goossens V. et al., Hum Reprod 2007)
- **Blastocyst (TE) biopsy**
- **Innovative alternative sources!**
  - **Blastocoele, cfDNA, exosomes**




PGD Steps
Assisted Conception Unit
in vitro fertilisation (ICSI)
PGD Centre
Accurate genetic diagnosis
Appropriate Genetic Counselling
Oocyte/Embryo/TE biopsy
DIAGNOSIS
Transfer
of unaffected embryos


PGD for SGD (General approach)
Cell Lysis
Cell Lysis
External Multiplex PCR
Whole Genome Amplification
Nested PCR
Mutation Analysis
Direct Diagnosis
Direct + Indirect
Indirect Diagnosis
Minisequencing
Sequencing
PCR products sizing
Direct mutation testing + Linked STR markers
STRs haplotyping
Exclusion testing
HLA haplotyping
Karyomapping (SNPs)

PGS (General approach)

Fluerescent In Situ Hybridisation (FISH)

- Cell fixation and probe hybridisation

Locus Specific Amplification (CCS)

- Quantitative Polymerase Chain Reaction (**qPCR**).

Whole Genome Amplification (CCS):

- Array comparative genomic hybridization (**Array-CGH**)
- Single-nucleotide polymorphism microarrays (**SNP-array)**
- Next-generation sequencing (**NGS**)

First Step:

An accurate, reliable effective amplification of limited DNA material

PB, Blastomeres:	6pg
Trophectoderm samples:	40-60pg

Loci specific multiplex PCR

Whole Genome Amplification

Loci specific multiplex PCR

PROS:

- Cost Effective
- PB, Blastomere, TE

CONS:

- Case specific optimisation
- Only the targeted region analysis
- Limited applications
- In-house protocols.
- Experienced staff.

Whole Genome Amplification

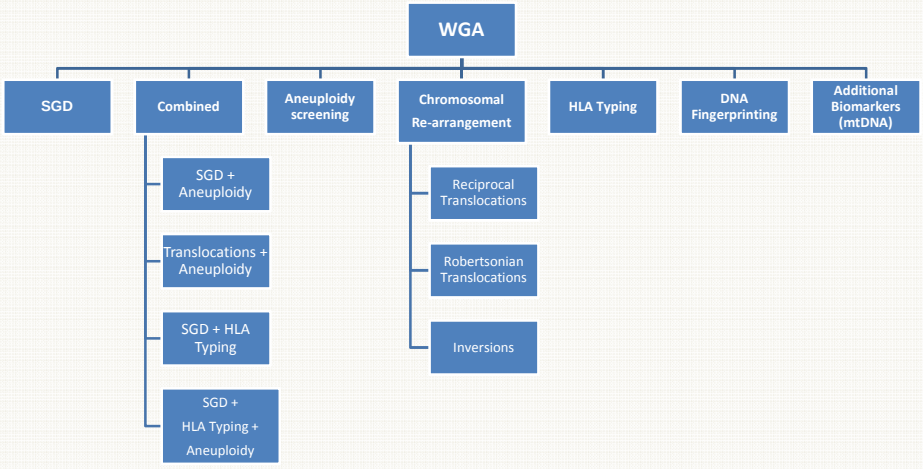
PROS:

- Standardized Universal protocol
- Applications for all genomic regions
- Ideal for CCS and combined indications

CONS:

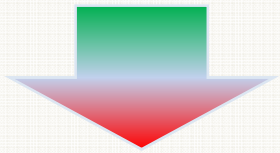
- Cost
- Better results for TE than PB, Blastomere,

Current vision: unique protocol for different PGD applications



MAIN DIFFICULTIES FOR AMPLIFICATION BASED PROTOCOLS:

I. CONTAMINATION
II. ALLELE DROP-OUT (ADO)
III. AMPLIFICATION FAILURE



MISDIAGNOSIS,
REDUCED IVF SUCCESS

I. CONTAMINATION

1. Internal Factors (Cumulus cells, sperm cells)

- Use of ICSI instead of IVF.
- Denuding the oocyte of cumulus cells

2. Extraneous Factors (Environment, cross contamination, etc.)

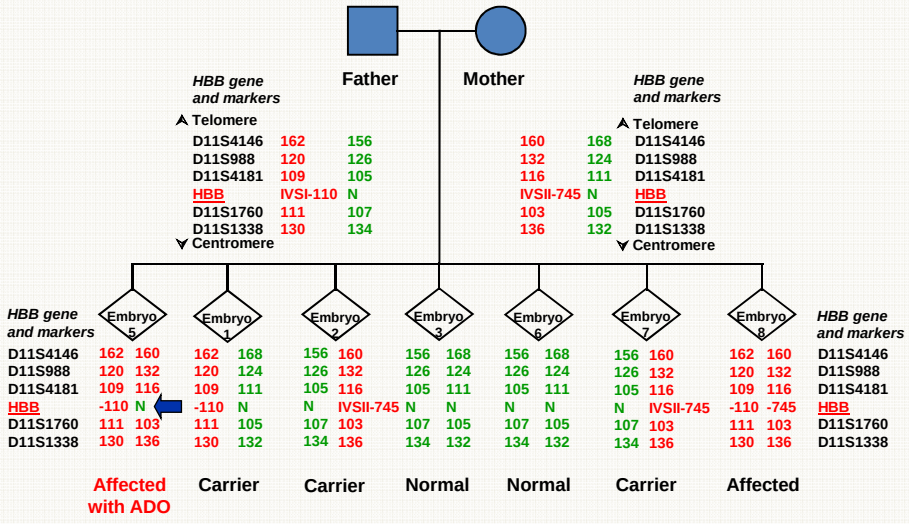
- Necessary precautions and sterile conditions (Mask, hair-cover, gloves…)
- Sterile regents for lysis and amplification process
- Blank control amplifications
- Internal STR markers detection (PCR based approaches)
- Organisation of PGD Lab: separare rooms and equipments for each applications

(ESHRE PGD consortium best practice guidelines…G. Harton et.al Hum Rep. 2010)

II. ALLELE DROP-OUT (ADO)

- 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 is common in single cell analysis (PB, blastomere), not expected at blastocyst analysis.
- An undetected ADO event leads to misdiagnosis

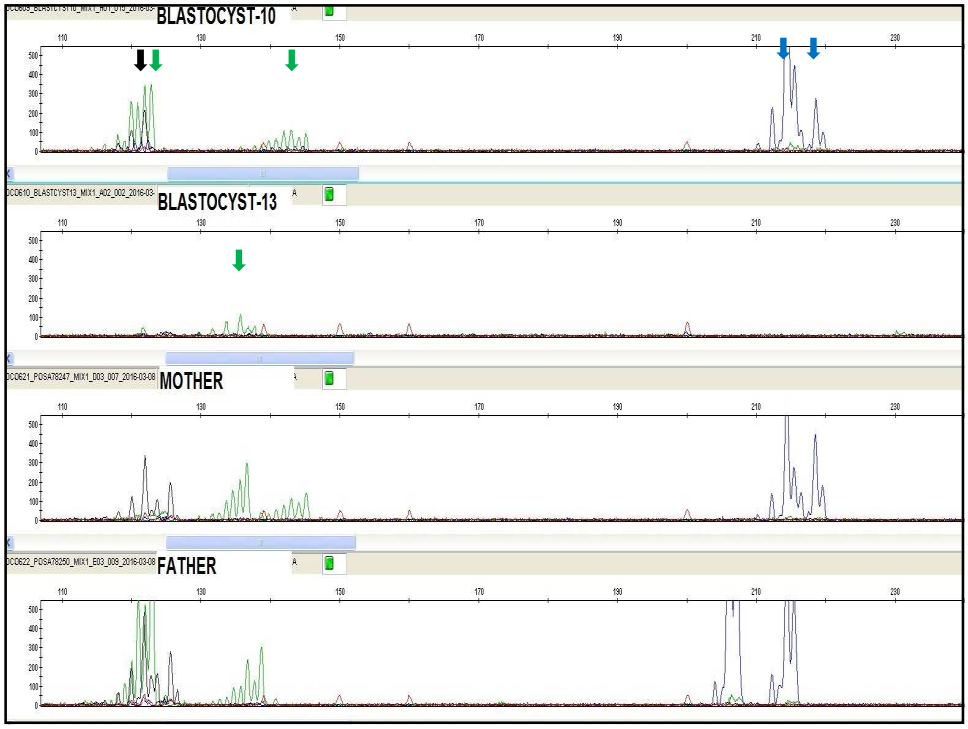
Avoidance of misdiagnosis due to ADO

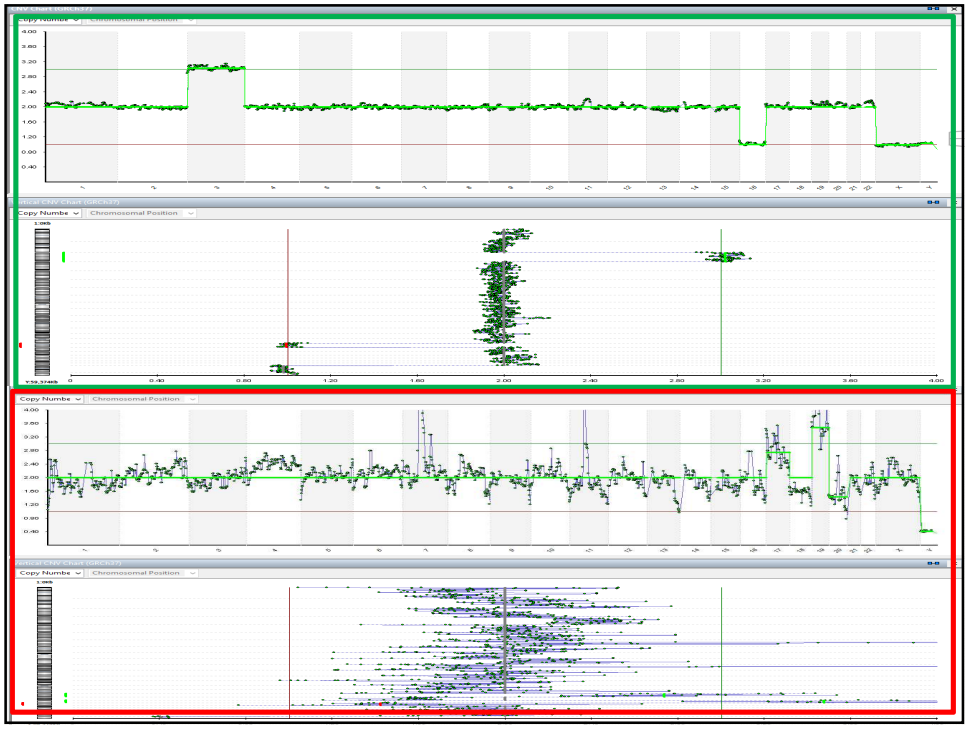


Fiorentino et. al., (2006) Hum Reprod 21: 670-684

III. AMPLIFICATION FAILURE

- No Amplification
  - Cells without nucleus
  - Lost of sample during tubing process
- Partial/Preferential Amplification (Not conclusive results)
  - Apoptotic cells with degredated DNA
  - Inappropriate amplification protocol





III. AMPLIFICATION FAILURE

1. Culture medium
2. Embryo Quality (Degredated or anucleated cells)
3. Embryo biopsy and tubing process
4. Cell lysis
5. Amplification Method (single cell PCR, WGA) and thermal cycling conditions(tubes, min oil, thermal cycler..)
6. Downstream applications efficiency (PCR, qfPCR, miniseq, aCGH, SNP analysis, NGS…)

1. Culture medium

- Calcium Magnesium-free culture medium is always RECOMMENDED
- Excess amount of medium should be avoided during tubing process (wash steps)
- Denaturated medium proteins may inhibit PCR reactions. They should be avoided by centrifugation or filtering (nested PCR).
- Medium Blank samples should be analysed for the

2. Embryo quality

Grade A (I)

Grade B (II)

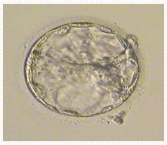
Grade C (III)

Grade D (IV)

Is embryo morphology correlated with the amplification success and diagnostic accuracy?

AMPLIFICATION → DIAGNOSIS ✓

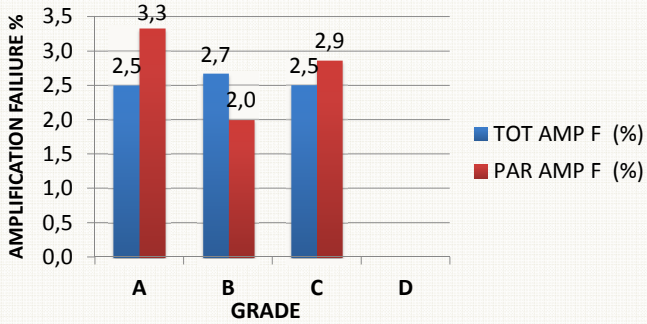
?



AMPLIFICATION → DIAGNOSIS ✗

N=350

GradeA: 85
GradeB: 120
GradeC: 145
GradeD: 0



2. Embryo quality

Jos Dreesen *et. al.; Eur J. Hum. Gen; (2014) 22. 1012-1018*

Sensitivity of PCR based PGD higher among Embryos with **good morphology** (100%) compared with those with **poor morphology** (94.9%)
N=940
P=0.032

No significant difference on **Specifity** (p=0.057) and **Diagnostic Accuracy** (p=0.999)

3. Embryo Biopsy and Tubing process

- **Embryo Biopsy**

- Laser, Mechanical, Acide Tyrode's
- Biopsy and tubing process should be done by trained operators

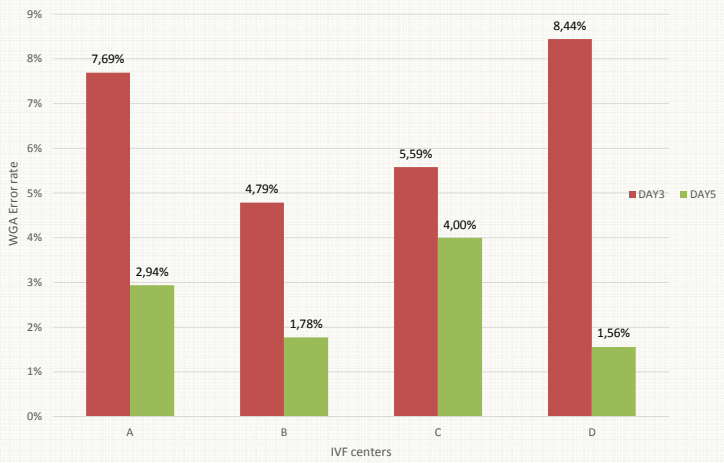
- **Post-biopsy washes**

- Biopsied samples should be rinsed at least twice in PBS droplets.
- One transfer pipette should be used for each sample.
- Last wash droplets should be used as blank control of embryo.

- **Correct volume of Tubing (1-2ul)**

- **Overlay with Mineral OIL (Transport PGD)**
- Molecular biology grade

- Amplification success between IVF centres



4. Cell Lysis

Single Cell PCR:

- Alkaline Lysis Buffer, Proteinase K (No strong evidences about the differences on lysis efficiency)
- PH neutralisation after lysis is essential !!!

Whole Genome Amplification:

- Cell extraction buffer, cell extraction enzyme.

Effect of freezing/thawing before lysis:

Freezing NOT RECOMMENDED (Storage at 4°C for long term)

- Significant differences for Accurate amplification and ADO levels (p=0.0091)

Piyamongkol *et. al.; Mol. Hum. Rep; (2003);V:9-N:7; 411-420*

5. Amplification Method (SCP, WGA)

Single Cell Multiplex Amplification:

- Until 20-plex amplification for different loci
- Correct PCR primer combinations is important (fragment size, annealing temperature)
- Intra-Assay control Samples (Negative Control, DNA control, Single Cell)

Whole Genome Amplification:

1. Multiple Displacement Amplification (MDA)
2. Degenerate – Oligonucleotide-Primed PCR (DOP-PCR)
3. Multiple annealing and looping based amplification cycles (MALBAC)

OVERALL AMPLIFICATION PERFORMANCE

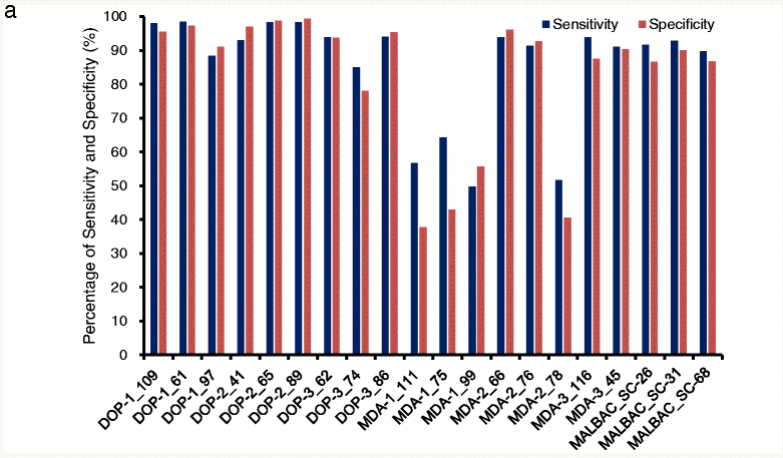
	Multiplex PCR (n=7621)		WGA* (n=7494)	
	D3	**D5**	**D3**	**D5**
Nr. Biopsied	5762	1859	2464	5030
Nr. Diagnosed (%)	5387 (93.5)	1789 (96.2)	2283 (92.7)	4828 (96.0)
Nr. Non-Diagnosed (%)	375 (6.5)	70 (3.9)	181 (7.9)	202 (4.2)
Total AF	200 (3.7)	27 (1.5)	90 (3.9)	53 (1.1)
Preferential/Partial Amplification	175 (3.2(	43 (2.4)	91 (4.0)	149 (3.1)

*PicoPlex, Rubicon Genomics

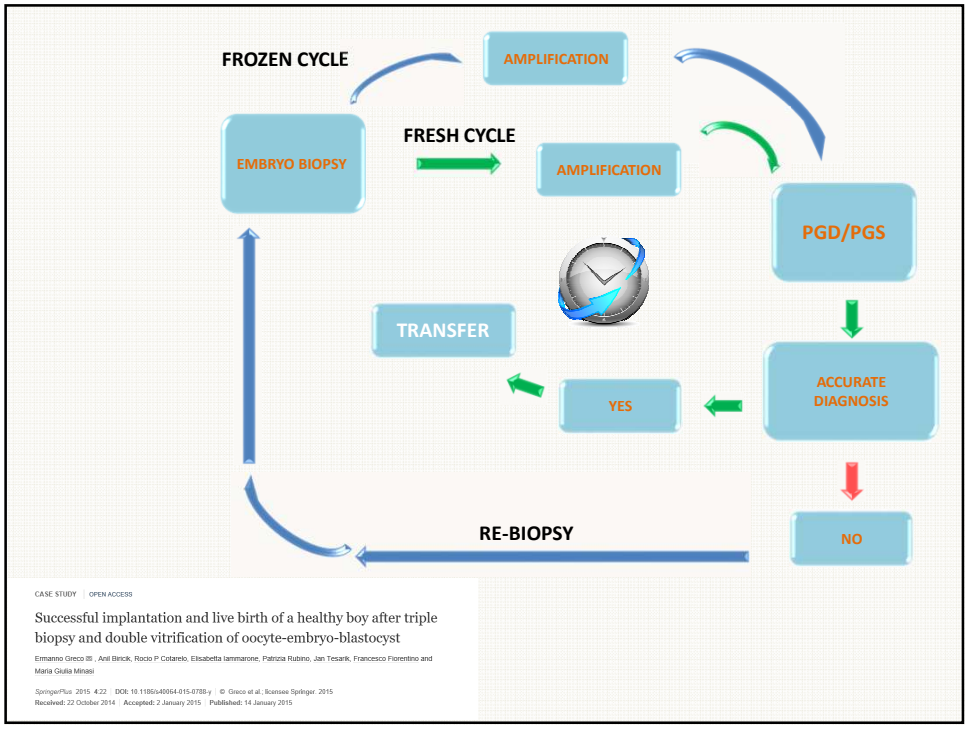
(Unpublished data)

"Comparison of variations detection between whole-genome amplification methods used in single-cell resequencing"

a



Yong Hou *et. al, GigaScience (2015), 4:37*



CONCLUSIONS

- Gold-standards should always be applied in each steps of IVF-PGD procedure for an accurate diagnosis.
- Several Best –Practice guidelines are pubblished and being updated to guide IVF and PGD laboratories.
- Correct PGD strategy should be choosen according to the indications. Diagnostic accuracy should be calculated before the application of new methods.
- Re-biopsies and appropriate freezing protocols does not affect embryo viability but help to conclude the diagnosis.

Thank you…

Anıl Biricik

biricik@laboratoriogenoma.it