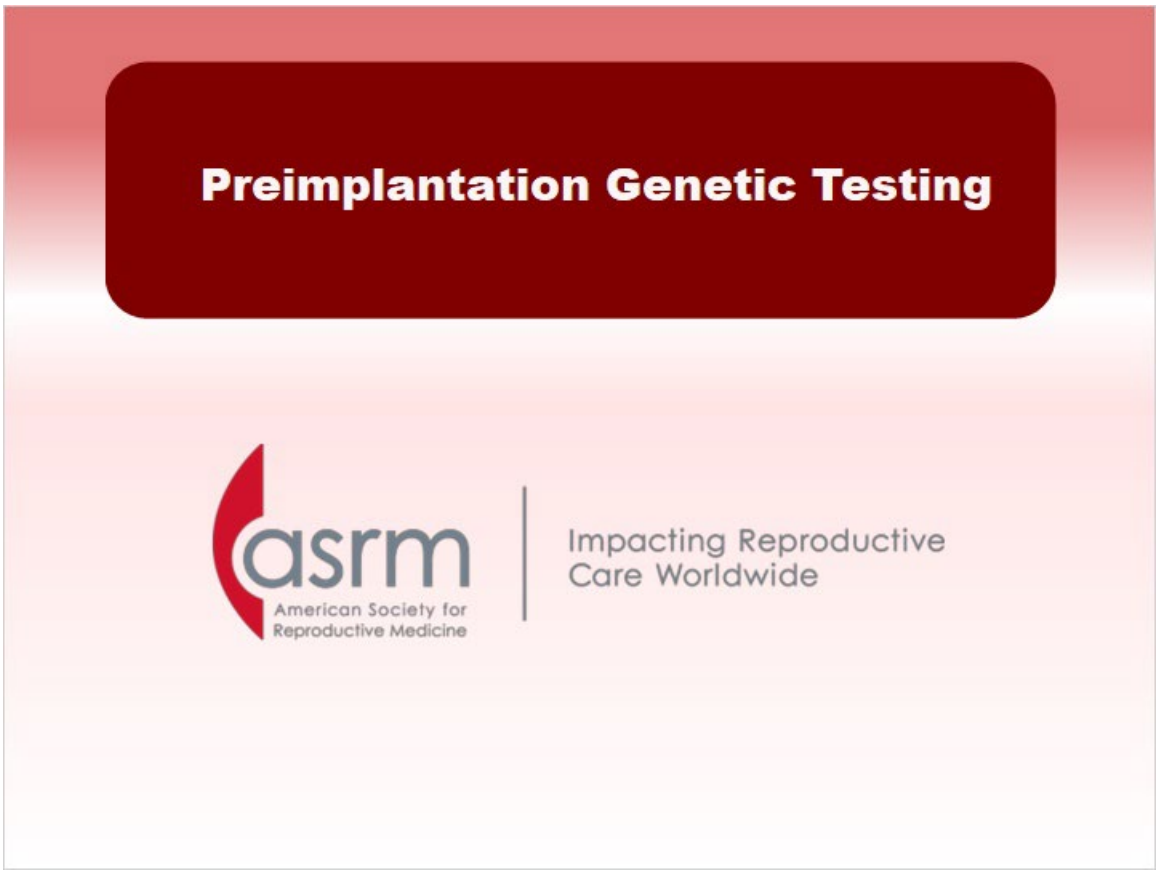
LABCC100 Lesson 8

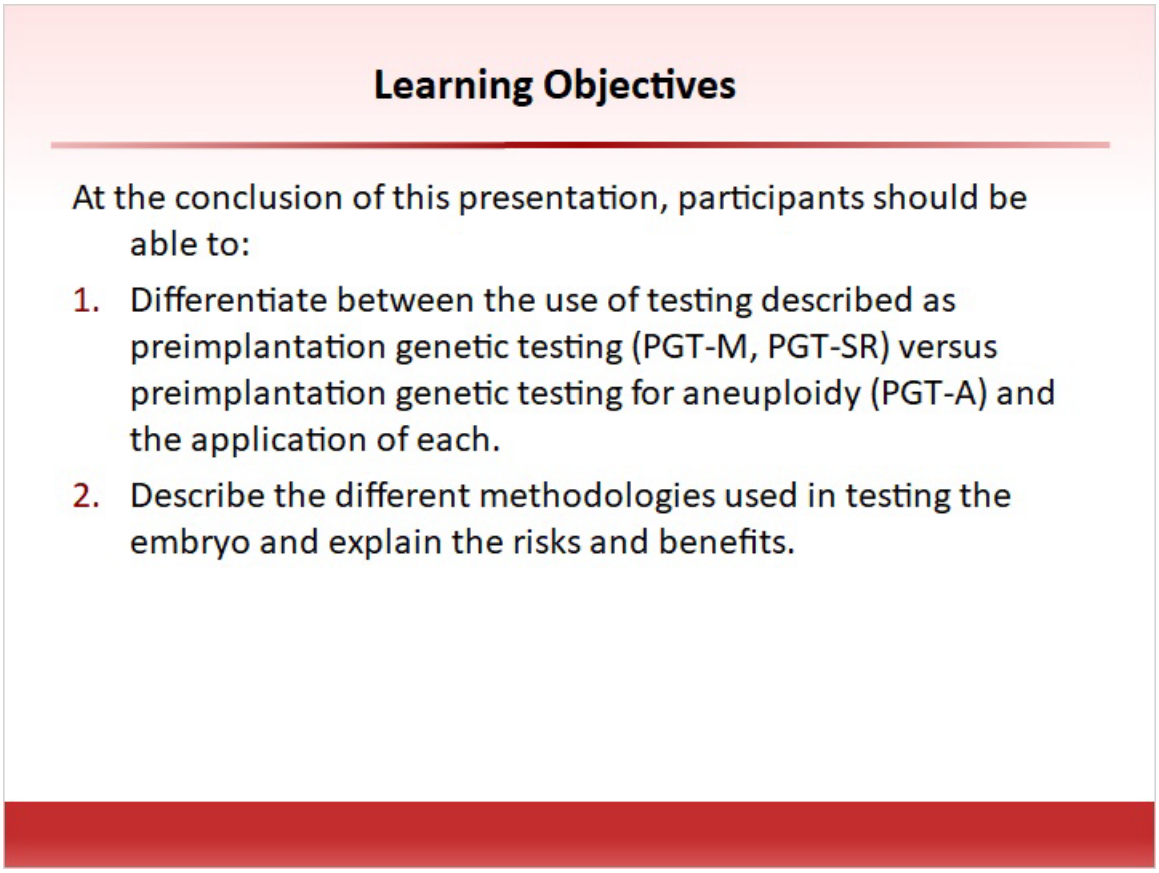
1.1 Preimplantation Genetic Testing



Notes:

Welcome to the American Society for Reproductive Medicine's eLearning modules. The subject of this presentation is Preimplantation Genetic Testing.

1.2 Learning Objectives

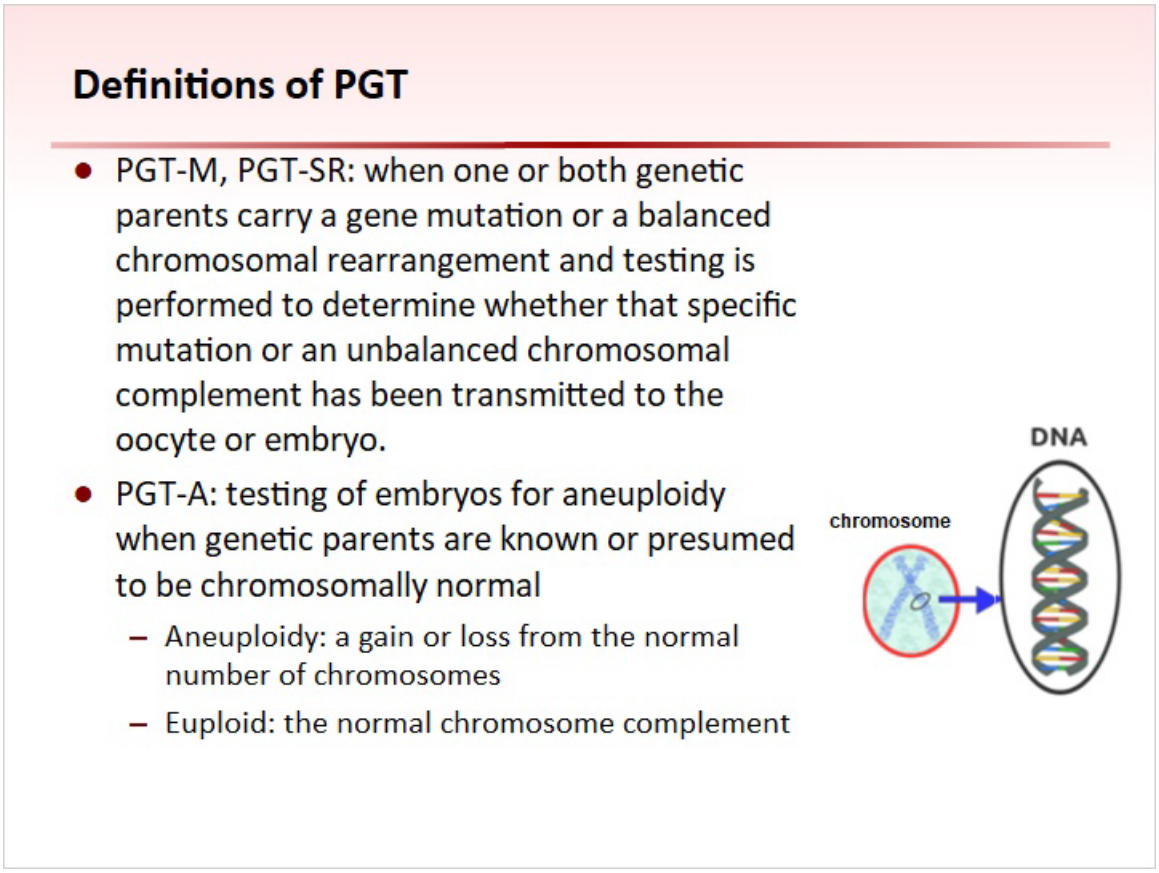


Notes:

At the conclusion of this presentation, participants should be able to:

1. Differentiate between the use of testing described as preimplantation genetic testing (PGT-M, PGT-SR) versus preimplantation genetic testing for aneuploidy (PGT-A) and the application of each.
2. Describe the different methodologies used in testing the embryo and explain the risks and benefits.

1.3 Definitions of PGT



Notes:

With the adoption of the International Glossary on Infertility Care in 2017, the terminology for preimplantation genetic testing (PGT) has been updated. PGT-M and PGT-SR refer to testing of an embryo when one or both genetic parents carry a gene mutation or a balanced chromosomal rearrangement, respectively, to determine whether that specific mutation or an unbalanced chromosomal complement has been transmitted to the oocyte or embryo. The term PGT-A, formerly known as PGS, refers to testing of embryos for aneuploidy when the genetic parents are known or presumed to be chromosomally normal. The definition for aneuploidy is a gain or loss from the normal number of chromosomes, while euploid is defined as the normal chromosome complement.

1.4 Who Might Benefit?

Notes:

For at-risk couples, PGT might be one of the options for them to have a healthy child. For PGT-M and PGT-SR, this would include couples who carry, or may they themselves have, genetic diseases or chromosomal rearrangements. For PGT-A, theoretically this might be an option for couples who may produce embryos with chromosomal errors due to advanced maternal age, repeated miscarriages, or previously failed IVF attempts. However, limited benefit has only been demonstrated in select randomized controlled trials.

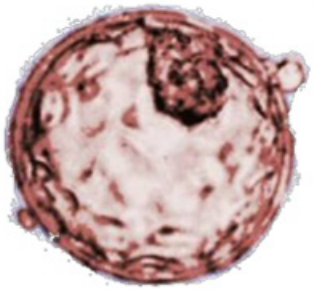
The use of PGT-A may allow a single euploid embryo to be transferred without an effect on the pregnancy rate while reducing the incidence of multiple pregnancy.

This module will provide discussion of the different aspects of PGT once the material is removed from the oocyte/embryo. Embryo biopsy procedures are discussed in a separate module.

1.5 General Overview of PGT Process

General Overview of PGT Process

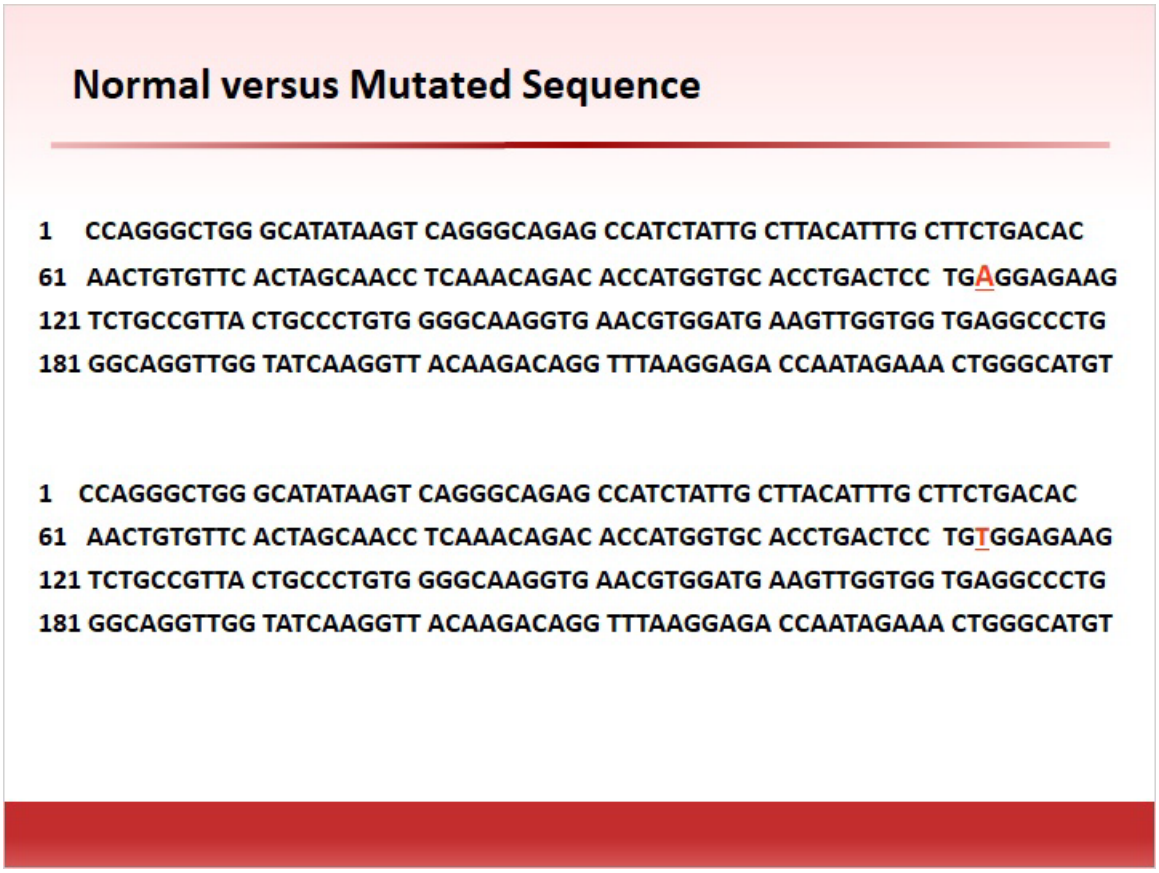
1. Cell(s) removed from oocyte (polar body), 6-8 cell embryo or from the trophectoderm of a blastocyst.
2. Cell(s) placed in a sterile microcentrifuge tube for molecular technologies (or fixed to a glass slide if fluorescence in situ hybridization [FISH] is being used)
3. Cell(s) lysed and subjected to molecular analysis
4. Embryo remains in incubator during diagnosis or may be cryopreserved while awaiting results.
5. After diagnosis, embryos selected for transfer to uterus, generally day 4-6 in a fresh cycle or for a frozen embryo transfer cycle, depending on the stage of development.



Notes:

During the embryo biopsy procedure, cellular material from the oocyte or developing embryo is removed and placed into a sterile microcentrifuge tube for most procedures. The exception is fluorescence in situ hybridization (FISH) where material is adhered to a slide with fixative. FISH is not commonly used today. With such small quantities of DNA for samples in a microcentrifuge tube, it is difficult to extract the DNA as in conventional molecular techniques. Rather, a lysis buffer is usually added to the tube to allow greater accessibility to the DNA. Whether the buffer is added by the biopsy specialist or at the molecular testing facility, the volume is strictly controlled since it may affect the reaction concentration during the actual molecular testing.

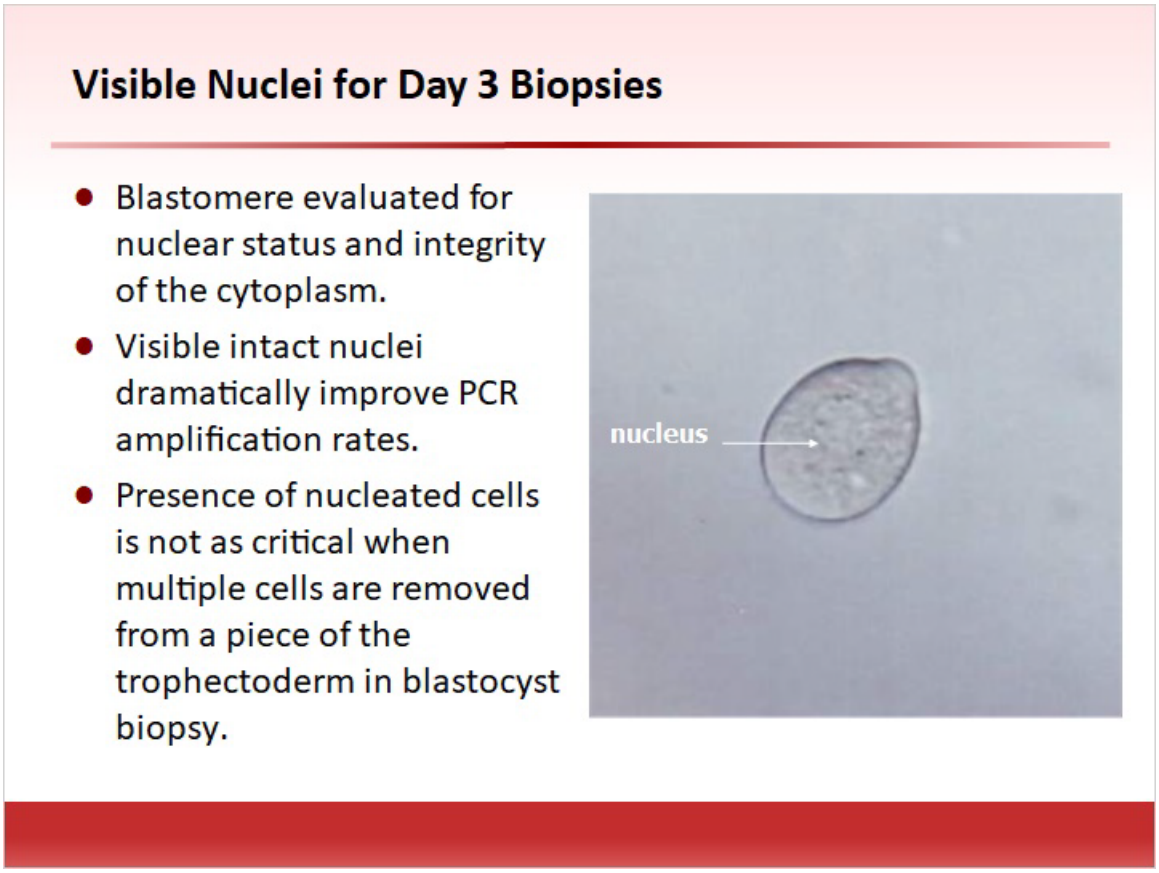
1.6 Normal versus Mutated Sequence



Notes:

As an example, shown here is the partial sequence for the hemoglobin gene. Part of the normal gene is shown in the first set while the lower set shows the single nucleotide point mutation that causes sickle cell anemia if both copies are inherited. At first glance, it is difficult to determine the difference.
The change of the base adenine (A) to thymine (T), emphasized in red, is enough insufficiency to cause sickle cell anemia when two copies of the mutation are inherited. In this example, only 240 bases are listed. Imagine if there was a single nucleotide mutated in the almost 4000 base pairs in the beta globin gene. Or, in the almost 3 billion base pairs in our genome. This is the task when PGT-M is performed for single gene defects: to find a mutation in a single polar body or in 1 to 5 or more cells in the embryo or blastocyst. That means that the diagnostic technique must have high sensitivity and specificity.

1.7 Visible Nuclei for Day 3 Biopsies

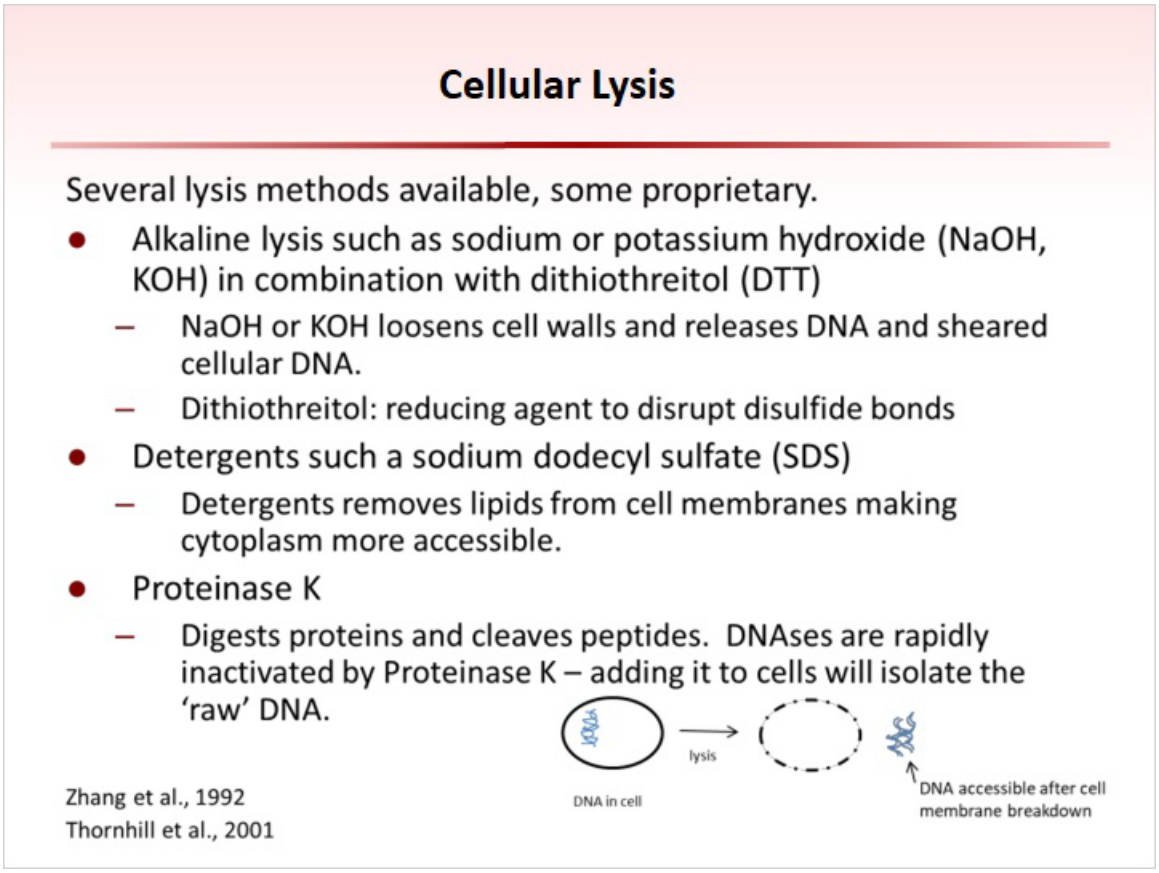


Notes:

In cleavage-stage biopsies, there is a dramatic improvement in amplification rates in cells that have a visible nucleus compared with those that do not. The appearance of nucleated cells seems not to be as critical when biopsying the trophectoderm since multiple cells are removed.

It is unclear why there is a difference in the amplification rates for single cells. There are several theories:
1) There was no DNA in the cell to begin with or the DNA is degenerating.
2) With a visible nucleus, the interphase DNA is in a 'friendlier' configuration. When the nuclear envelope breaks down, the DNA moves into the cytoplasm and is more tightly coiled to minimize loss before cytokinesis and may therefore not be as accessible for denaturing and PCR.

1.8 Cellular Lysis



Notes:

Several lysis methods are available, some of which are proprietary. Many PGT laboratories adopted the use of a strong alkaline lysis solution. Alkaline solutions loosen the cell walls and release the DNA or sheared cellular DNA. Dithiothreitol or DTT is a reducing agent that disrupts the disulfide bonds of the DNA, making it more accessible. Another lysis method uses sodium dodecyl sulfate (SDS), which is a detergent. Cell membranes are comprised of a lipid layer; detergents remove lipids from the cell membranes, making the cytoplasm more accessible. SDS may be used in conjunction with proteinase K which digests proteins and cleaves peptides. DNAses are rapid inactivated by proteinase K so adding it to cells will aid in isolating the raw DNA.

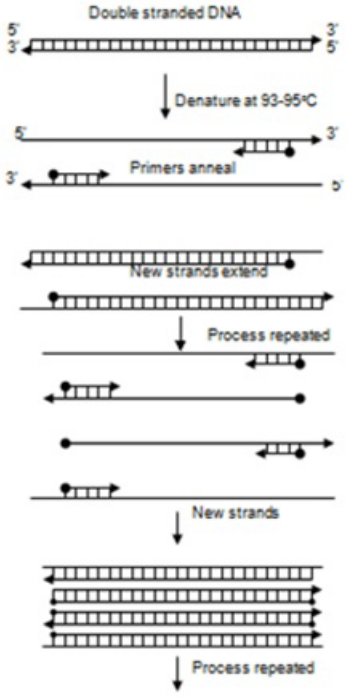
1.9 Molecular Techniques

Molecular Techniques

- Many initial PGT techniques for single gene defects use some form of the polymerase chain reaction (PCR) or real-time PCR (qPCR).

1. Denaturation of the template DNA
2. Annealing of the primers
3. Extension of the new strands aided by the polymerase and dNTPs

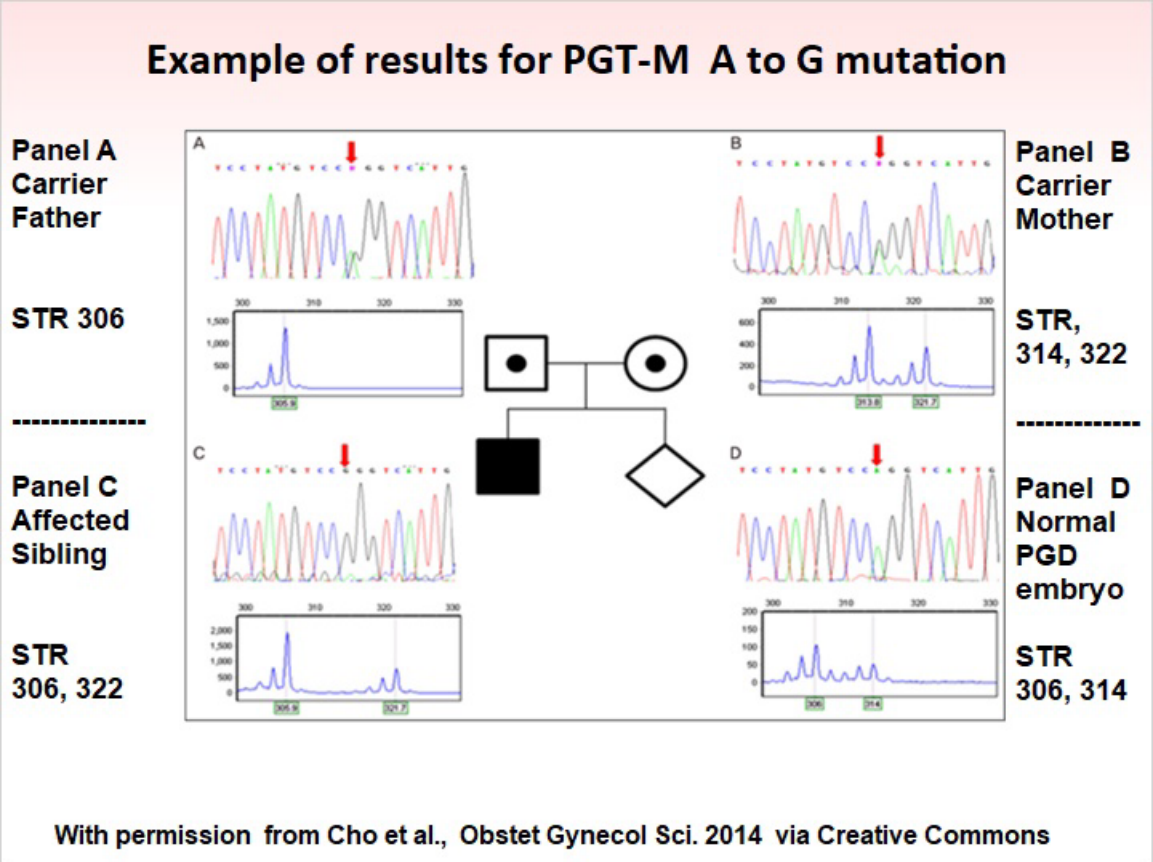
- 3 basic steps of PCR are repeated until sufficient DNA product can be detected via gel electrophoresis or more commonly through generated sequencing images.



Notes:

Many PGT techniques for single gene defects use some form of the polymerase chain reaction (PCR) or real-time PCR (qPCR). The three basic steps of PCR are:
1)Denaturation of the template DNA.
2)Annealing of the primers to their complementary sites, and
3)Extension of the new strands aided by the polymerase and dNTPs
The steps are repeated until sufficient DNA product can be detected via gel electrophoresis or more commonly through generated sequencing images as shown next.

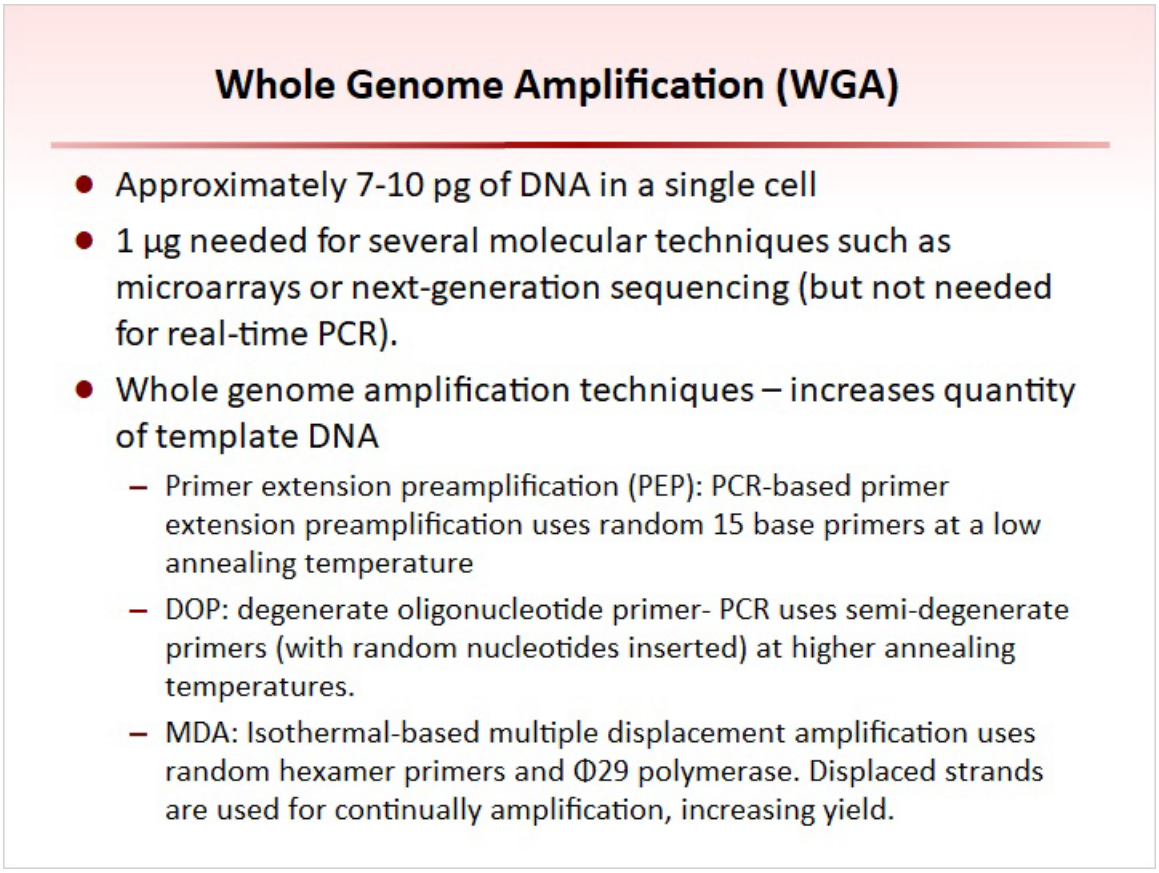
1.10 Example of results for PGT-M A to G mutation



Notes:

This figure illustrates results from PGT-M for a mutation for Type 1 citrullinemia, an autosomal recessive disorder that causes ammonia and other toxic substances to accumulate in the blood. The father is the square with the dot in panel A while the mother is the circle with the dot in panel B. Both are heterozygous carriers for the A to G mutation (marked by the red arrow) since they have both the A (shown in green) and the G (shown in black) on the electropherogram at the top of each panel. The lower figures in panels A and B also illustrate the capillary electrophoresis results of the short tandem repeat (STR) microsatellite markers that identify each parental allele: 306 base pairs for the father and 314 and 322 base pairs for the mother. Panel C illustrates the homozygous affected sibling with two affected alleles, shown as the black G and indicated by the red arrow. Panel D shows the PGT-M blastomere with the normal sequence, the Green A at the red arrow. The STR markers show both the 306 base pairs of the paternal allele and the 314 base pairs of the maternal allele, which rules out allele dropout.

1.11 Whole Genome Amplification (WGA)



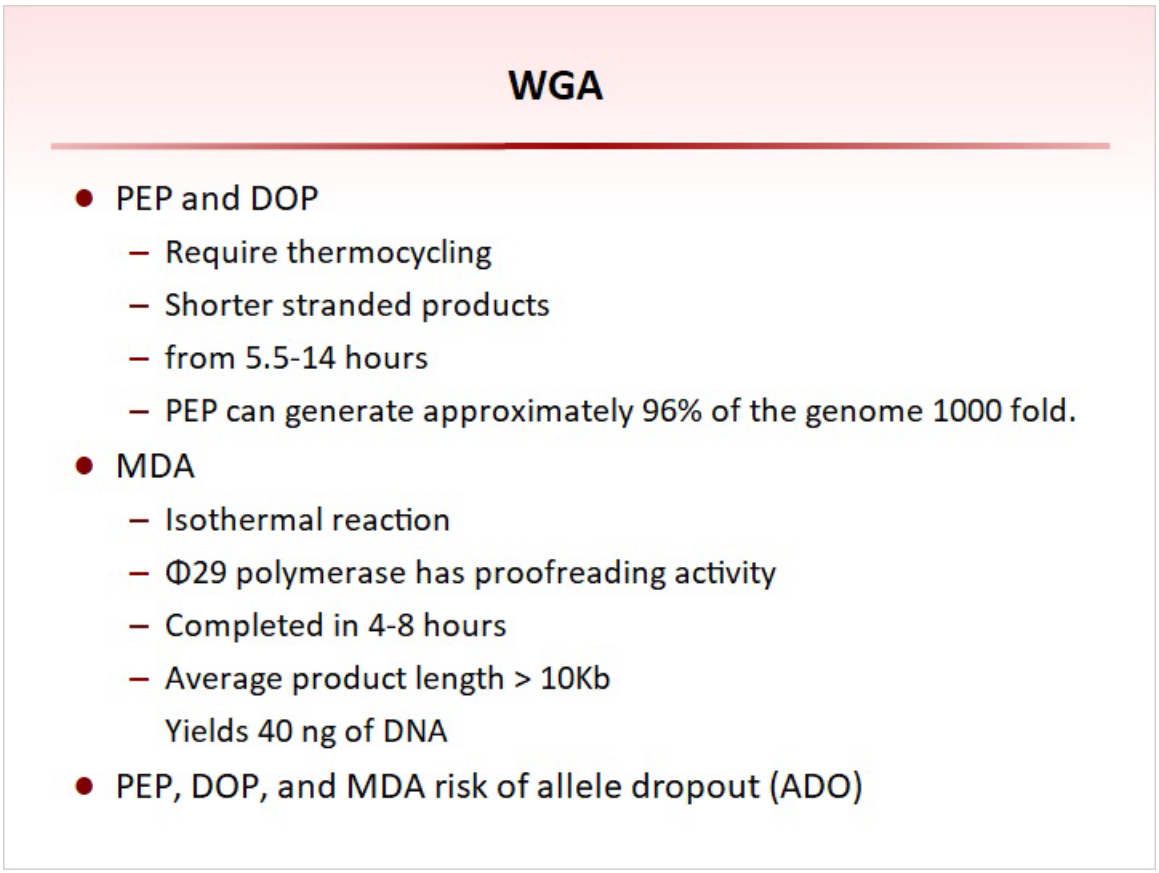
Notes:

A single cell contains approximately 7-10 picograms of DNA. That is insufficient when using several molecular techniques such as the use of microarray technology or next-generation sequencing. In order to increase the quantity of DNA, a whole genome amplification technique (or WGA) may be used. Initially used for forensic samples or other low copy templates, WGA may now routinely be used in several diagnostic techniques in PGT. An exception is that WGA is not needed when analyzing the samples via real-time PCR.

Several WGA techniques are available. PCR-based primer extension preamplification (PEP) uses random 15 base primers at a low annealing temperature while degenerate oligonucleotide primer (DOP) PCR uses semidegenerate primers (with random nucleotides inserted) at higher annealing temperatures.

Isothermal-based multiple displacement amplification (MDA) is currently more commonly used. Random hexamer primers and a specialized Φ29 polymerase are used at a constant temperature, 30 degrees Celsius, to replicate the majority of the genome. As the primers anneal and DNA strands are replicated, the new strands serve as templates for additional amplification, thus increasing the yield.

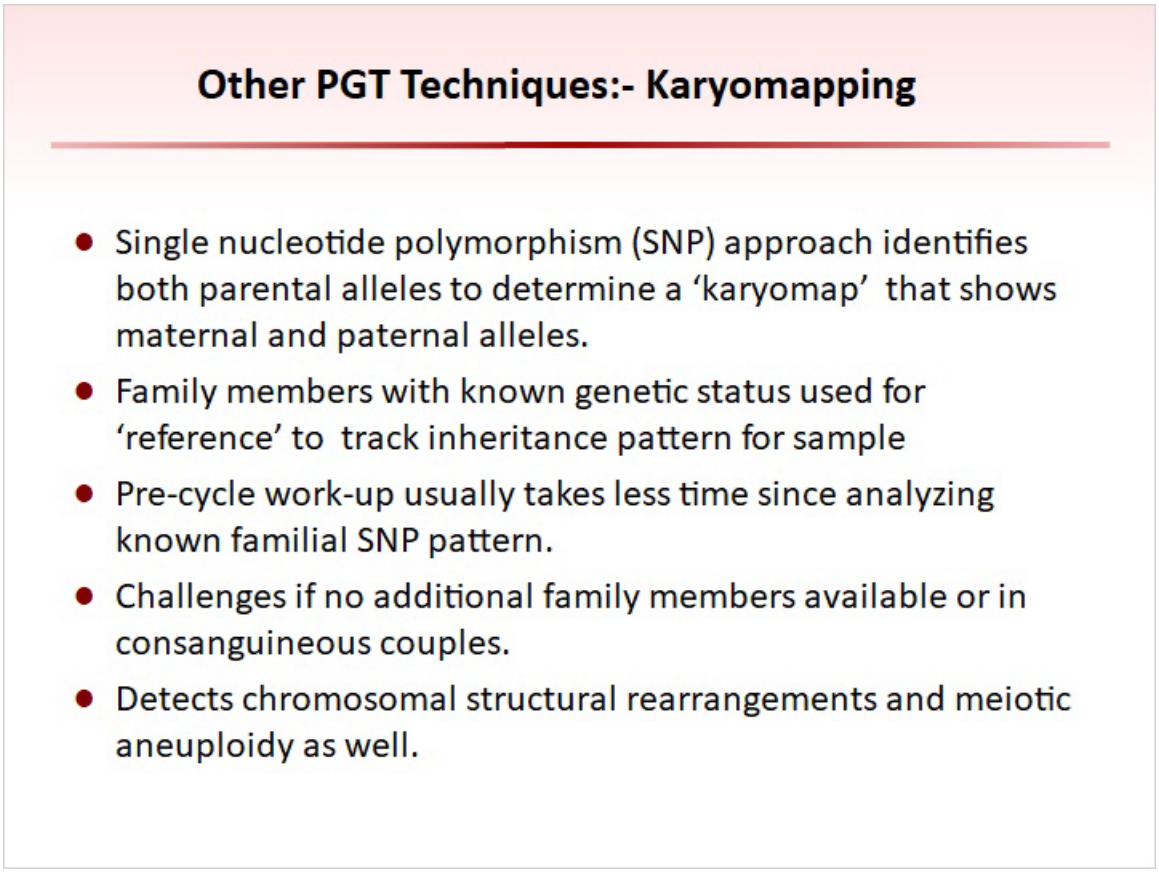
1.12 WGA



Notes:

The PEP and DOP techniques require the use of a thermocycler and may take from 5.5 to 14 hours to complete. PEP can generate a 1000-fold increase in approximately 96% of the genome.
MDA is performed at a constant temperature so no specialized equipment is needed. The Φ29 polymerase has proofreading capability, that is, 3-prime to 5-prime exonuclease activity for higher amplification fidelity. The average strand length is larger than 10 kilobases. MDA can yield as much as 40 nanograms of DNA.
One drawback of the whole genome amplification techniques is that ADO or allele dropout can occur. Allele dropout is the random preferential amplification of one of the two alleles present in the sample. Allele dropout and its implications will be further explained.

1.13 Other PGT Techniques:- Karyomapping



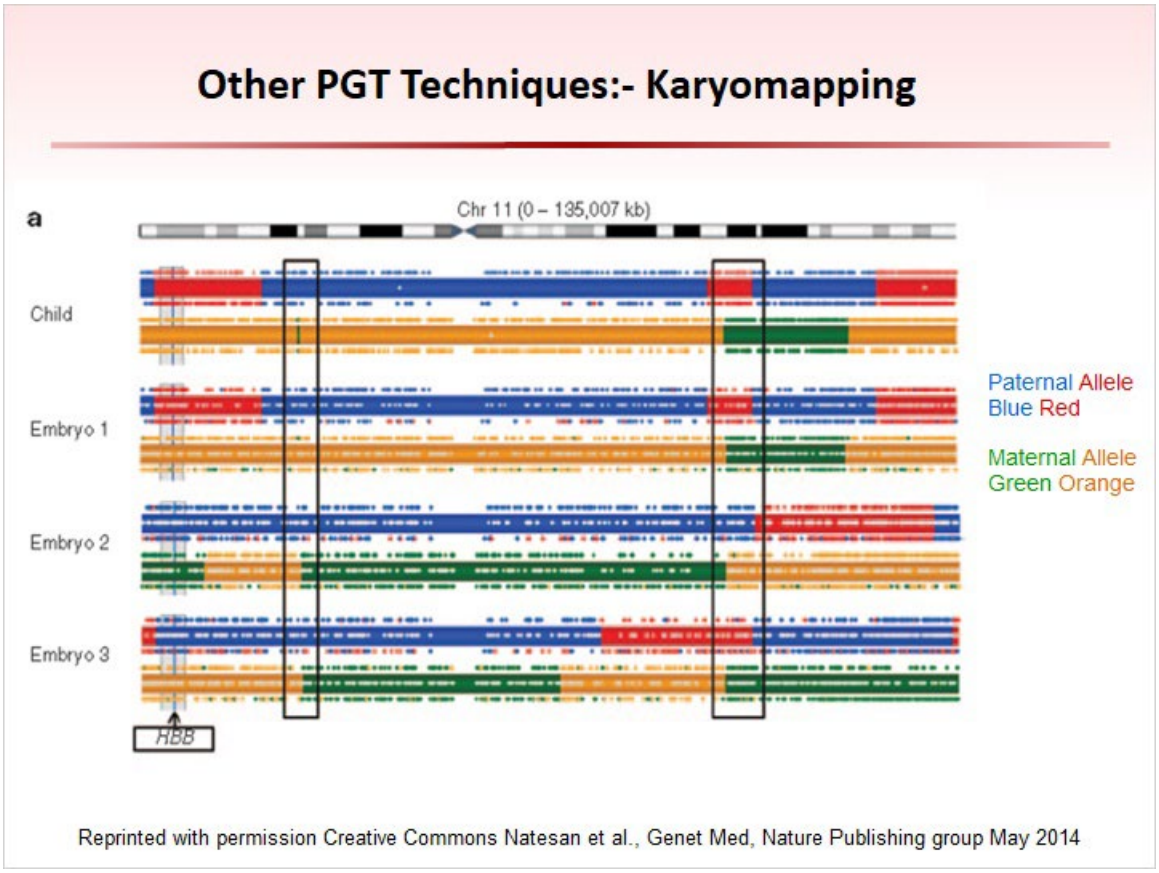
Notes:

Karyomapping is another technique that has been successfully used for single gene defect testing. After a whole genome amplification step, this technique uses single nucleotide polymorphisms or (SNPs) to identify both parental alleles in the sample. SNPs will be explained in further detail a little later in this module. Identifying parental alleles will aid in detection if recombination events have occurred. The use of thousands of SNP data is then constructed to design a 'map' to trace the origin of the genetic material inherited from each of the parents.

The pre-cycle work-up time is usually less than with other PGT-M preliminary work-ups because the analysis targets a known familial pattern. The technique is more difficult to perform when there are no additional affected family members or if the couple is consanguineous.

Karyomapping can be used to detect chromosomal structural rearrangements and detect aneuploidy as well.

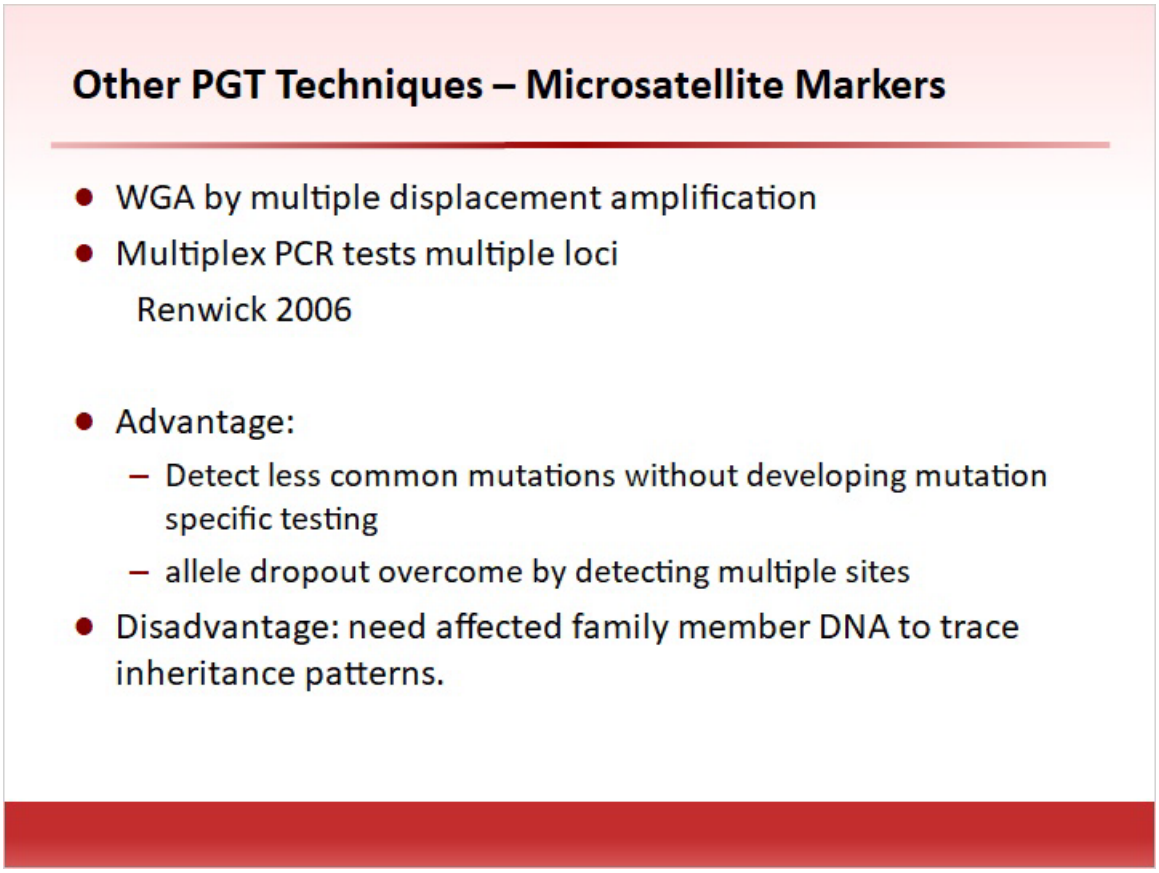
1.14 Other PGT Techniques:- Karyomapping



Notes:

In this figure, the paternal affected allele is mapped in blue and the normal allele in red. The maternal affected allele is mapped in green and the normal allele is orange. The HBB gene locus for beta thalassemia is recognized in the gray area on the left. An unaffected child was born after transfer of embryo #1, mapped with both parental normal alleles in red and orange. Embryo #2 inherited the affected paternal allele, in blue, and the affected maternal allele in green. Embryo number three is a carrier and inherited the affected paternal allele in blue and the normal maternal allele in orange. Karyomapping for chromosome copy number testing is undergoing validation.

1.15 Other PGT Techniques – Microsatellite Markers



Notes:

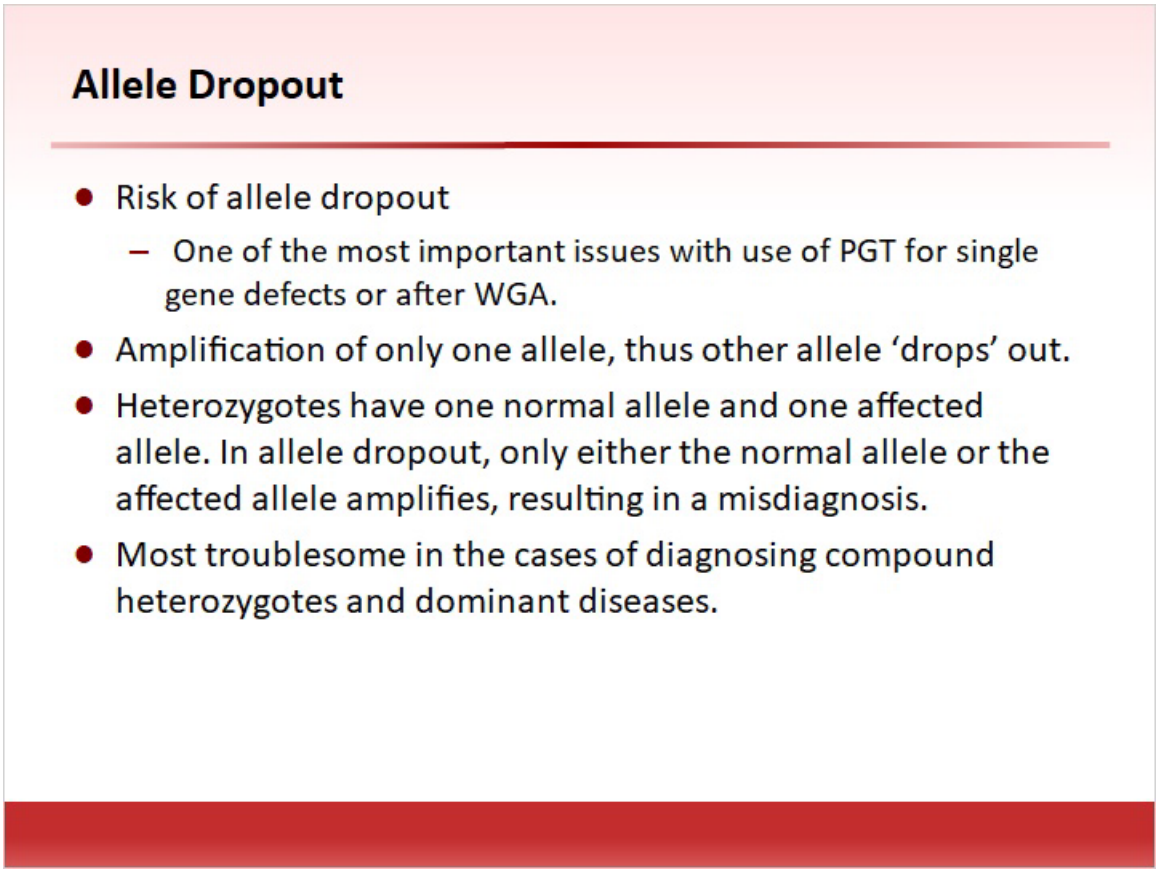
Renwick and colleagues reported on the use of microsatellite markers rather than direct mutation analysis to test the biopsied sample. The sample was subjected to PCR using multiple primers targeting multiple microsatellite markers after a whole genome amplification step increased the amount of DNA. These microsatellite markers are a method of identifying an individual, such as a DNA fingerprint, so a haplotype can be constructed. The haplotype is the combination or set of those markers on a chromosome that are generally inherited together as the set. This type of PGT-M is used by many laboratories.

One of the advantages of microsatellite markers is that it can be used to identify less common mutations without having to develop a specific sensitive test for direct mutation analysis. For example, if a couple presents with a less common mutation for cystic fibrosis, this methodology might allow a faster time frame in pre-cycle development, rather than direct mutation analysis since the only workup is determining which markers will be informative. Allele dropout, as explained next, may be overcome by the detection of multiple sites.

The disadvantage of the technology is that familial studies must be performed with an affected family member. The technique is essentially tracking the inheritance pattern in

the embryo from the maternal and paternal alleles.

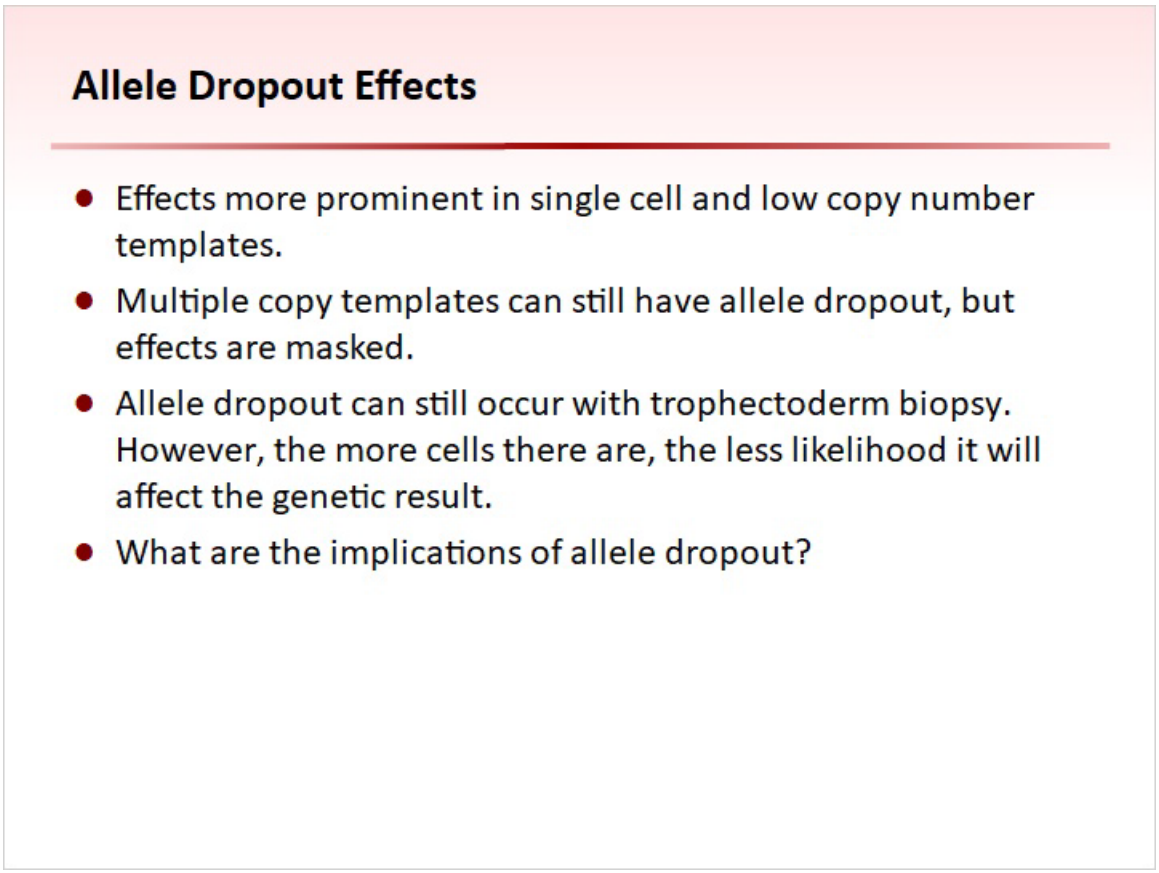
1.16 Allele Dropout



Notes:

One of the most important issues regarding the use of PGT for single gene defects or after WGA is the risk of allele dropout. Allele dropout is the preferential amplification of only one allele, thus the other allele 'drops' out. It is random which allele will be amplified.
The heterozygote has one normal allele and one affected allele. In allele dropout, either the normal allele or the affected allele amplifies, not both, resulting in a potential misdiagnosis. The most troublesome cases are when the gene of interest is autosomal dominant and thus the heterozygote is the affected condition, or in the case where the parents each carry a different mutation for the same gene and the compound heterozygote is the affected condition.
In the normal condition, as is in the homozygous affected, both alleles are the same. In those cases, if one allele drops out, the genetic answer is still the same since both alleles are the same.

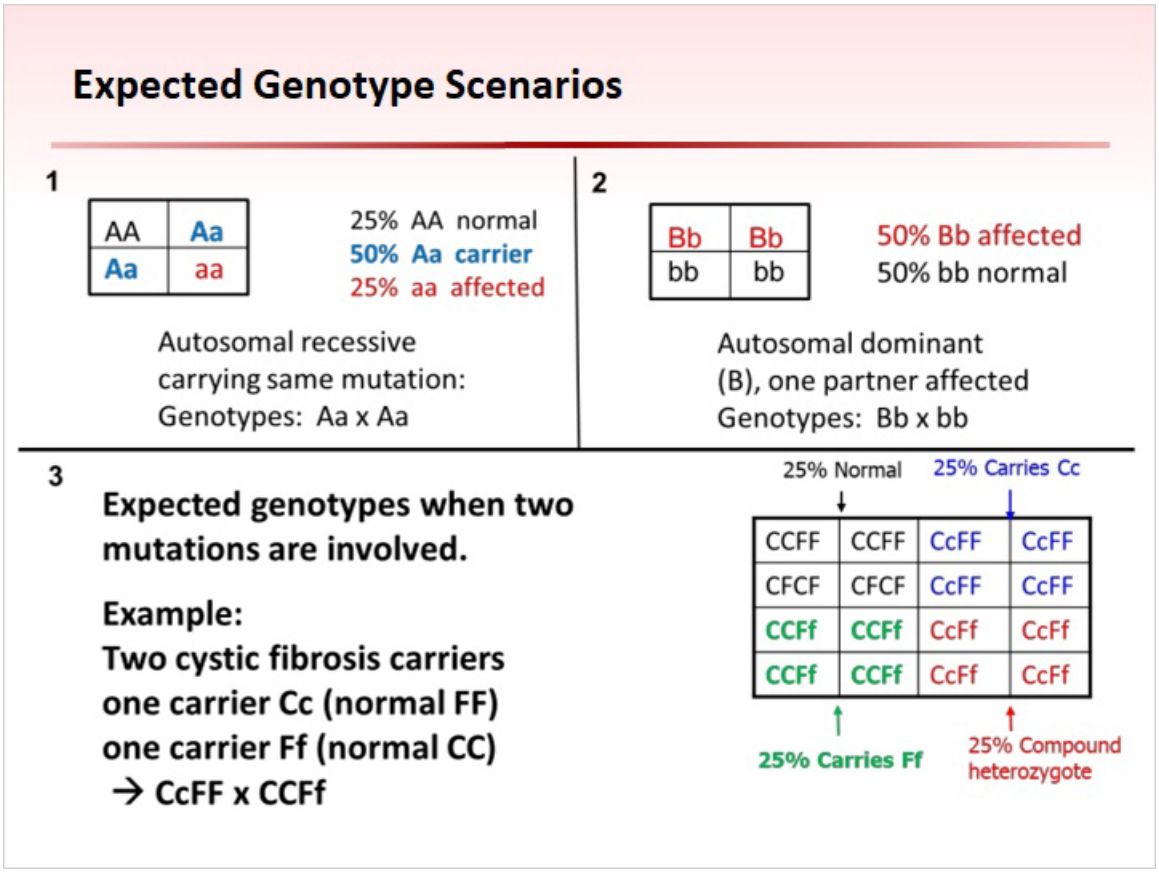
1.17 Allele Dropout Effects



Notes:

The effects are more prominent with the use of single cells (such as in PGT-M) or a few cells-multiple copy DNA templates do not have the same issues because there are so many cells that can compensate if allele dropout occurs.
It stands to reason that multiple copies of either allele will be produced when multiple cells are present so the effects of allele dropout will be masked even if it did occur in individual cells of a multi-cell DNA template.
Allele dropout can still happen with trophectoderm biopsy. However, the more cells there are, the less likelihood it will affect the genetic result.
The next section will demonstrate the implications of allele dropout and what might result.

1.18 Expected Genotype Scenarios



Notes:

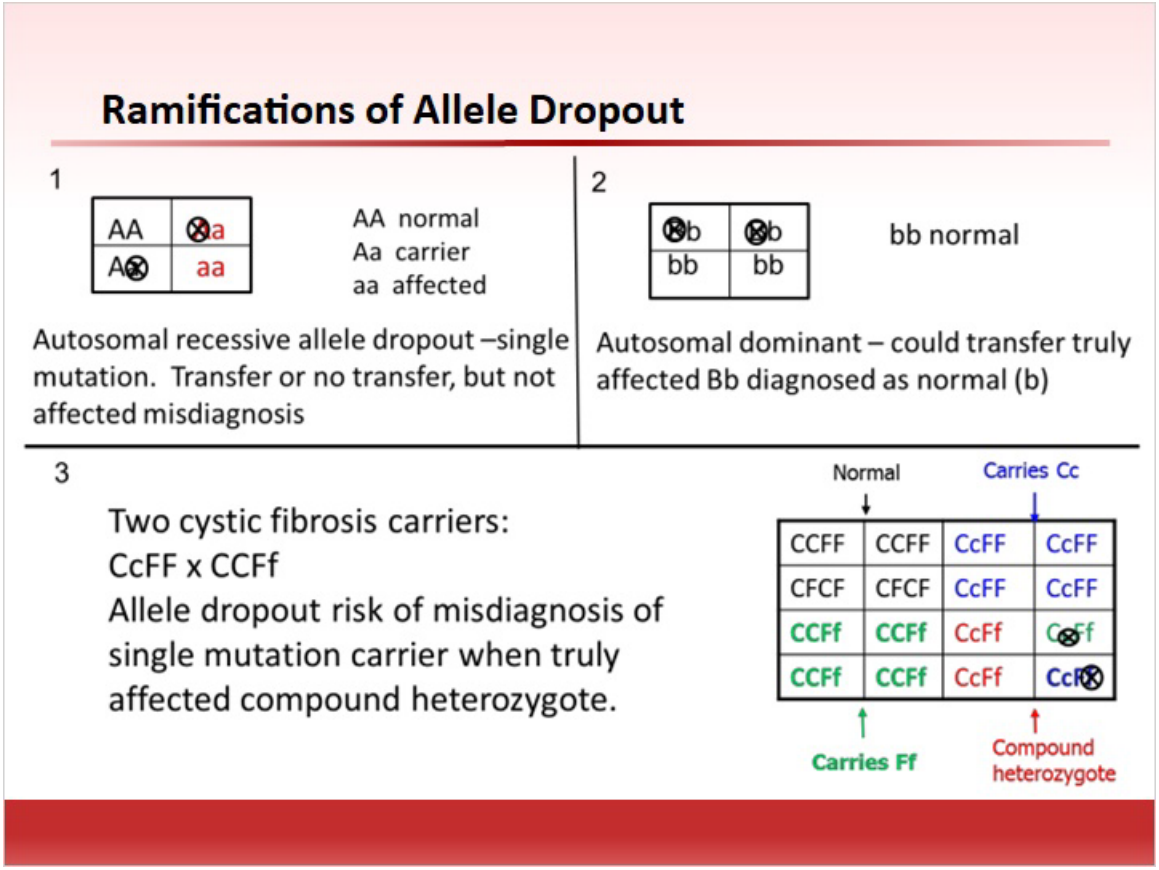
The easiest way to determine the implications of allele dropout is to go back to the Punnett square.

Panel 1 demonstrates what is the expected genotype in an autosomal recessive disease such as cystic fibrosis when there are two carrier parents. It is expected that 25% of the samples would be homozygous normal, AA; 50% of the samples to be heterozygotes or carriers, Aa; and 25% of the samples to be homozygous affected, aa for both alleles.

In Panel 2 in the upper right of the slide, PGD for an autosomal dominant disease like Marfan syndrome, one of the parents is affected, generally as the heterozygote. In this instance, this is illustrated by Bb. In making the diagnosis, the statistical probabilities are that 50% of the embryos will be affected as Bb and 50% of the embryos will be normal as bb.

Finally, the third panel in the lower half of the slide shows what occurs when the two parents carry different mutations of cystic fibrosis, identified here as either mutation C or F. The risks for normal, carrier, and affected are still the same at 25%, 50%, and 25% respectively. Here, the affected condition occurs when both mutations are inherited in the offspring. This is called a compound heterozygote.

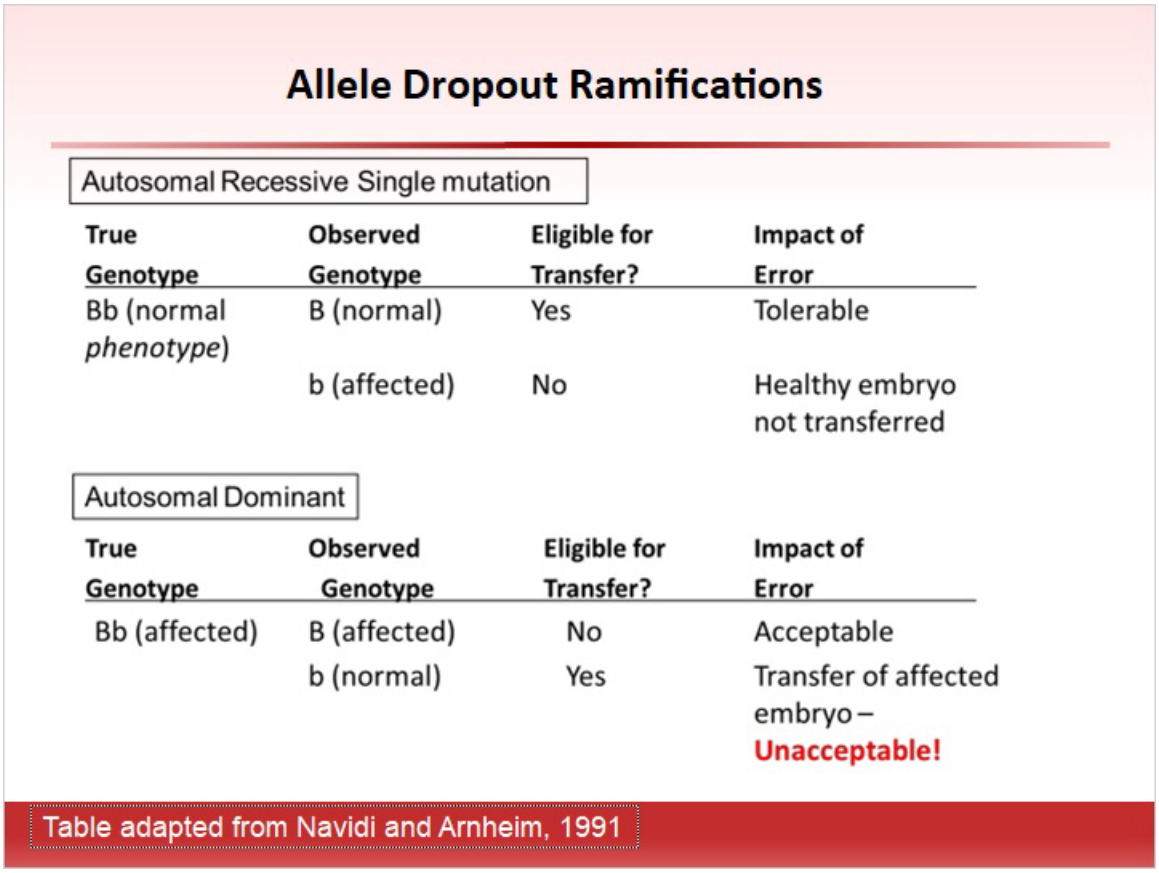
1.19 Ramifications of Allele Dropout



Notes:

What happens if allele dropout occurs? In the first panel, if the dominant allele (A) or the recessive allele (a) drops out in the heterozygote, as denoted by the X, it would either result in the transfer of a phenotypically normal embryo, or removal of an embryo from those eligible for transfer.
In panel 2, the effects of allele dropout are more pronounced in the dominant condition. For example, an embryo is diagnosed as normal (b), but in reality, allele dropout occurred and the affected allele (B) was not detected, a misdiagnosis would result if that embryo was transferred and implanted.
In panel 3, a similar scenario could result in allele dropout and a misdiagnosis for that compound heterozygote that was diagnosed as a single mutation carrier in error.

1.20 Allele Dropout Ramifications



Notes:

Another way to look at the risks was first characterized in 1991. In autosomal recessive conditions with a single gene mutation, the impact of allele dropout could be tolerable if the phenotypically normal embryo is transferred, even though the genotype was truly a heterozygote. If the embryo was misdiagnosed as affected due to allele dropout, that embryo would not be transferred. It takes away a potential embryo for transfer, but would not result in a potential misdiagnosed fetus/baby.
In autosomal dominant conditions, if the embryo is diagnosed as affected, it would not be transferred. However, the greatest ramification occurs when allele dropout results in a true heterozygous affected embryo being diagnosed as normal and eligible for embryo transfer. If implantation occurs, an affected fetus/baby would result.

1.21 Allele Dropout Ramifications: Two Mutations

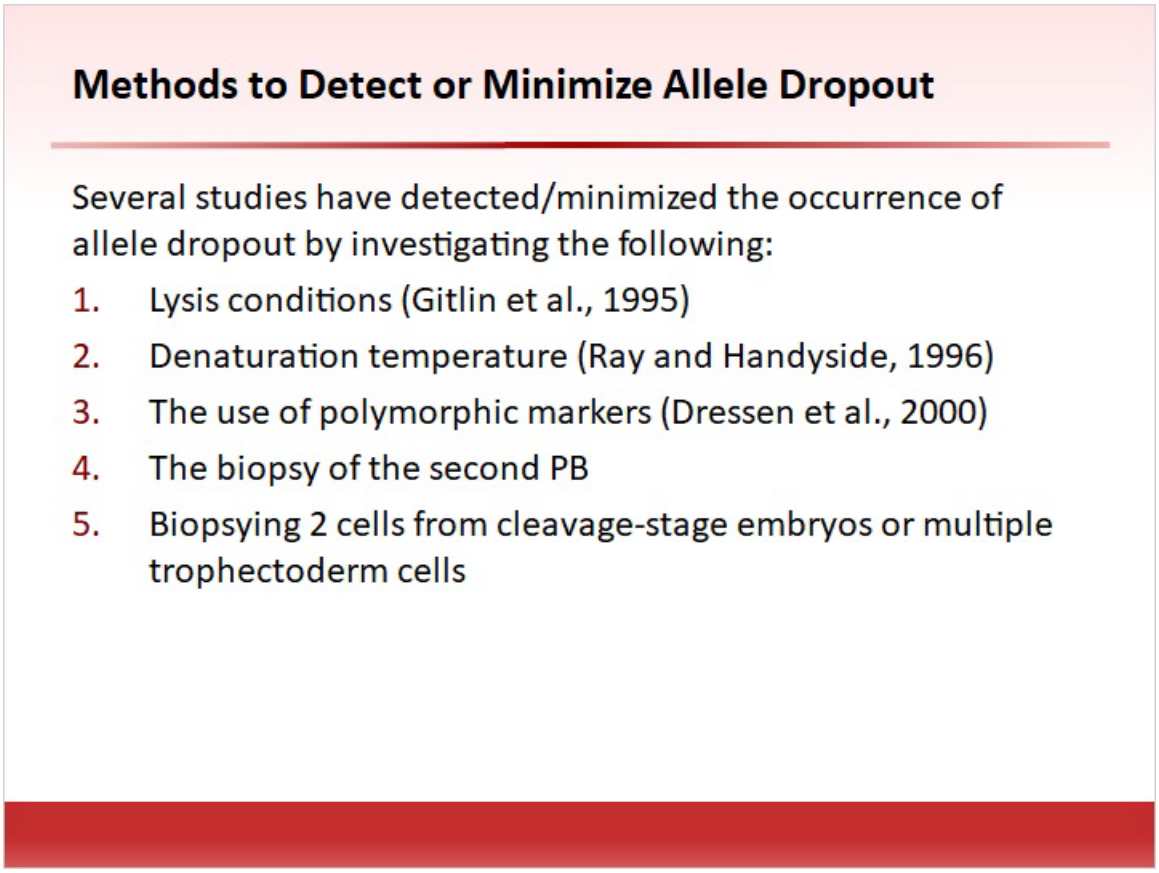
Allele Dropout Ramifications: Two Mutations

True Genotype	Observed Genotype	Eligible for Transfer?	Impact of Error
CcFf (affected)	CFf (normal)	Yes	Transfer of affected embryo
	cFf (affected)	No	Acceptable
	CcF (normal)	Yes	Transfer of affected embryo
	CF (normal)	Yes	Transfer of affected embryo
	cF (normal)	Yes	Transfer of affected embryo
	Cf (normal)	Yes	Transfer of affected embryo
	cf (affected)	No	Acceptable – no transfer

Notes:

One can see the ramifications of a misdiagnosis attributed to allele dropout in the case where parents carry two different mutations of the same autosomal recessive condition. At least two documented cases of misdiagnoses due to allele dropout have been reported in the literature in the early days of PGD. Strom and Rechitsky reported in 1991 that only one diagnosed mutation of cystic fibrosis (other mutation unknown) occurred when the embryo was diagnosed as normal, whereas it was truly a carrier for both the known and unknown mutation. The second case reported by Grifo and colleagues in 1998 was with two different diagnosed mutations of cystic fibrosis, when allele dropout occurred. The couple learned of the misdiagnosis at amniocentesis and terminated the pregnancy. Today, the use of polymorphic markers increases the detection of allele dropout and may have prevented the transfer of an embryo diagnosed in error.

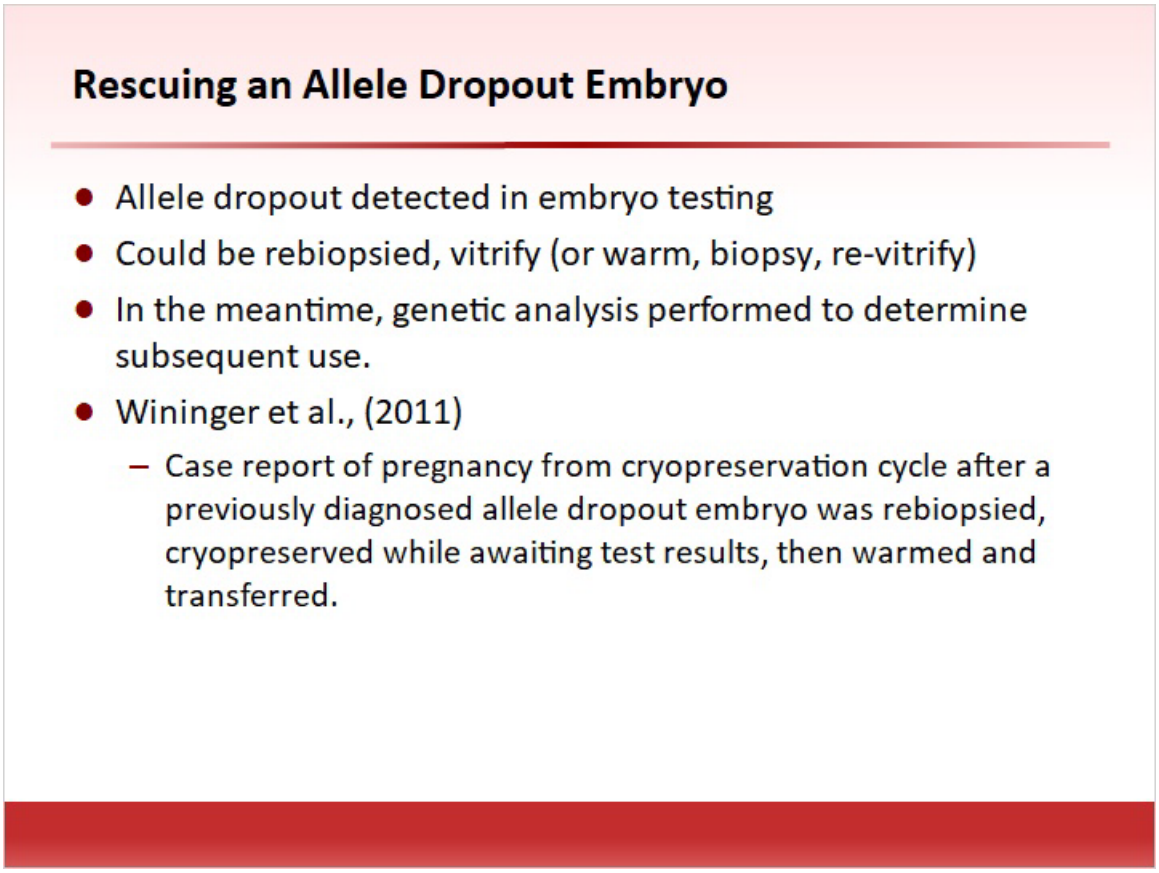
1.22 Methods to Detect or Minimize Allele Dropout



Notes:

Several studies have investigated the incidence of allele dropout and developed strategies to detect and minimize the incidence. Gitlin and colleagues in 1995 investigated several different DNA lysis methods in single heterozygous cells and determined amplification sensitivities and specificities. Ray and Handyside demonstrated the next year that an increased denaturation temperature for the first several rounds of PCR decreased the incidence of allele dropout by enhancing better DNA strand separation. Several years later, Dressen and colleagues advocated the use of informative polymorphic markers that can detect the allele from each parent, thus ensuring the detection of one or both alleles amplifying. In combination, these techniques have aided in either minimizing the occurrence of allele dropout or detecting its incidence to prevent an embryo transfer of the potentially misdiagnosed embryo.

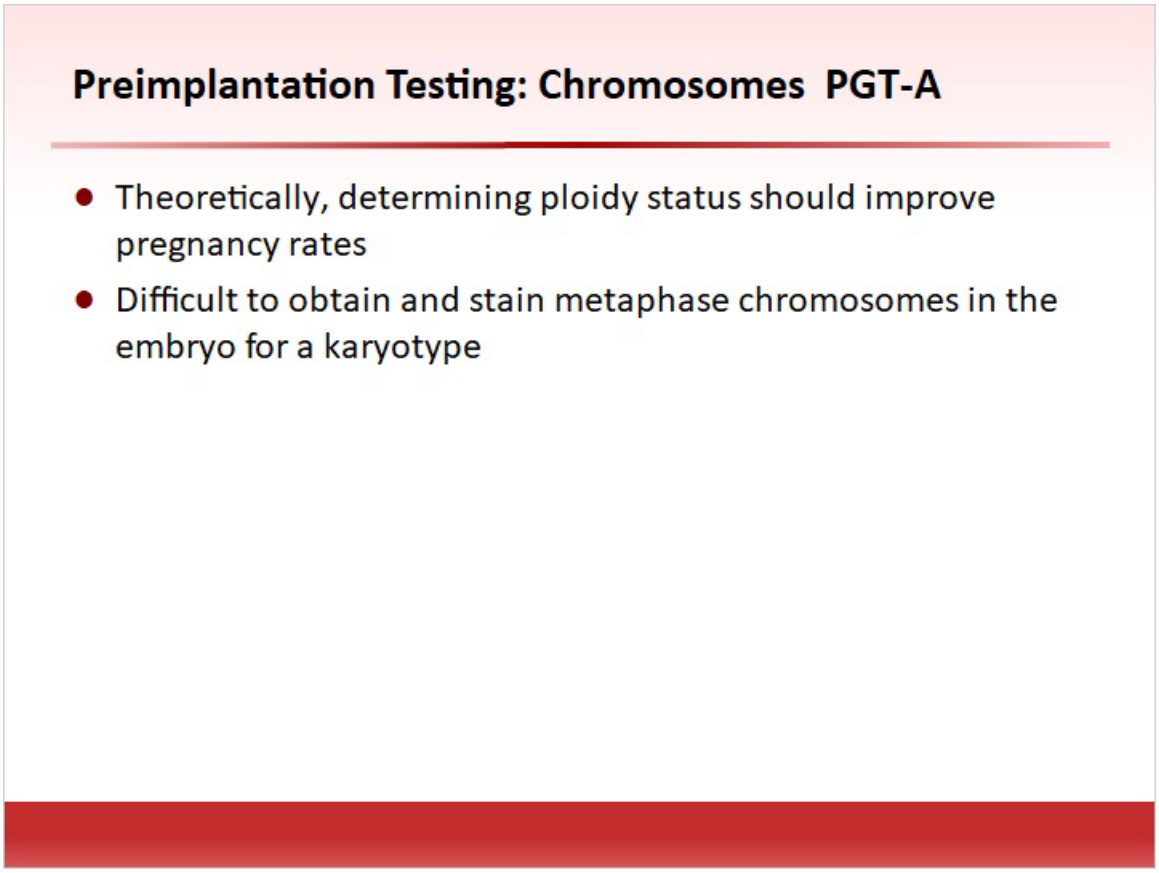
1.23 Rescuing an Allele Dropout Embryo



Notes:

What can be done with an embryo in which allele dropout has been detected? For this embryo or other embryos where there is no result or inconclusive results, there is the option that the embryo can be rebiopsied so material can be reanalyzed and then the embryo can be cryopreserved if this was for a planned fresh embryo transfer cycle. It is likely that the uterus is not synchronized for transferring the embryos in that same fresh cycle. If the embryo has already been cryopreserved, the embryo could be warmed or thawed, re-biopsied, then cryopreserved a second time while awaiting results. In a case report by Wininger and colleagues, a previously diagnosed allele dropout embryo was rebiopsied and cryopreserved. Upon reanalysis, the embryo was found to be normal. The embryo was warmed, transferred, and resulted in the live birth of a healthy infant.

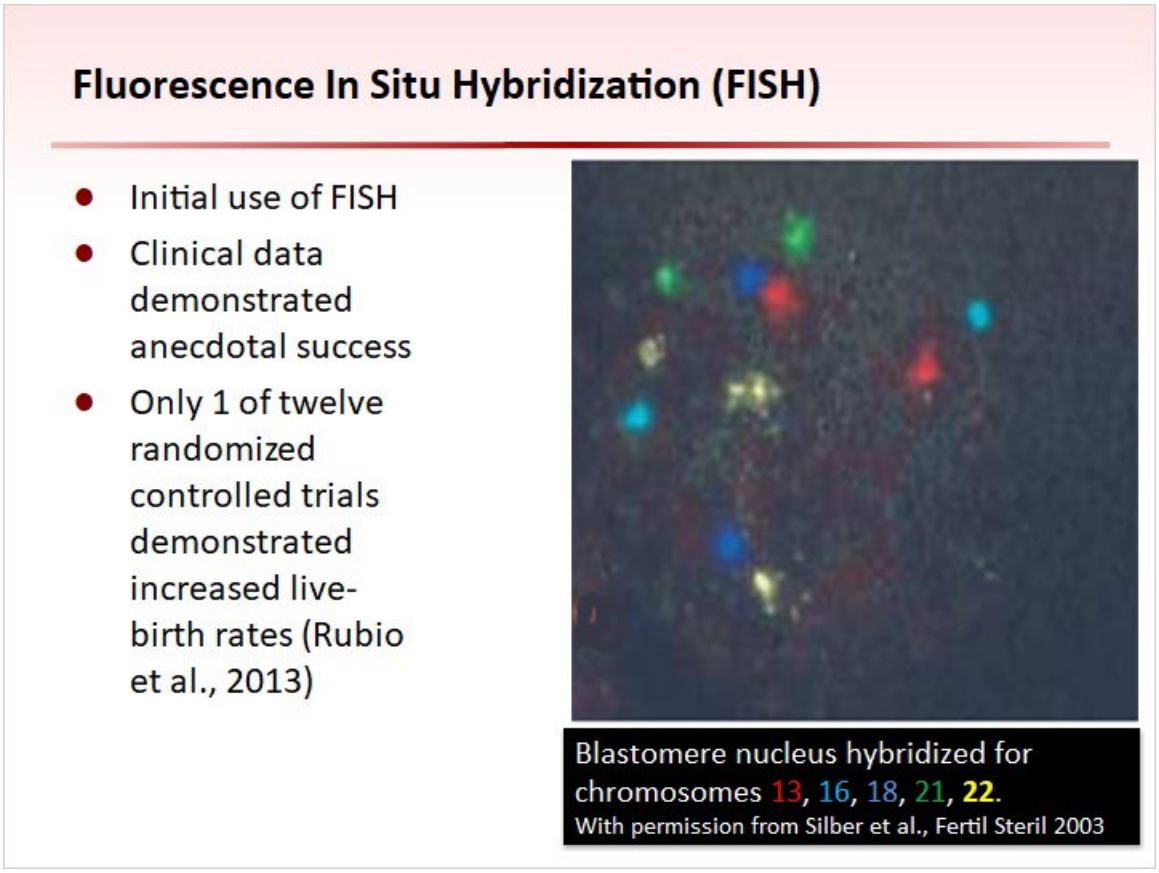
1.24 Preimplantation Testing: Chromosomes PGT-A



Notes:

PGT-M for single gene defects has been well established as an option to prevent transferring embryos with single gene defects. The use of PGT-A for chromosome testing has escalated in many IVF clinics even though the effects of its widespread use have been met with some uncertainty. Theoretically, if embryos could be diagnosed to determine the chromosome status, pregnancy and live-birth rates should improve since aneuploidy can cause failures of implantation and/or miscarriages.
It is difficult to obtain and stain metaphase chromosomes from the oocyte or embryo, thus methods other than conventional cytogenetics are used for determining chromosome copy number in the embryo.

1.25 Fluorescence In Situ Hybridization (FISH)

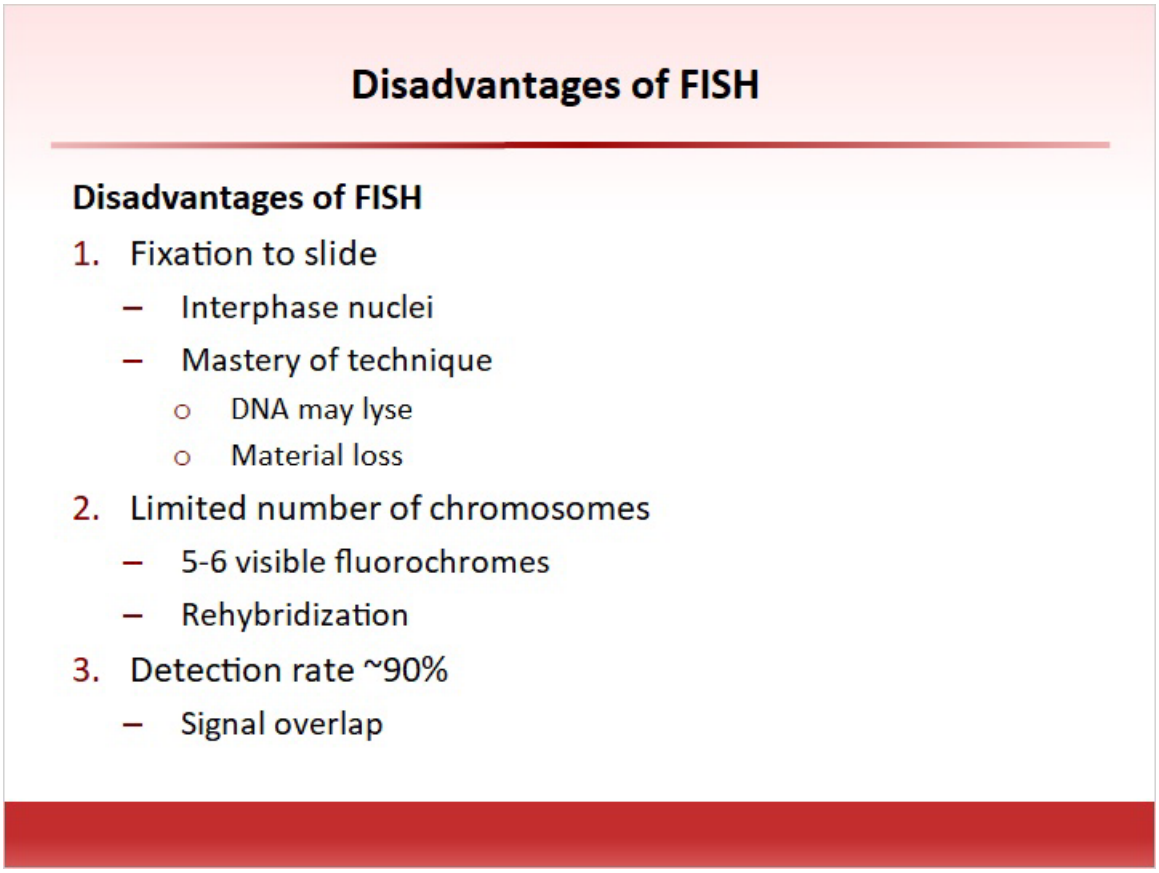


Notes:

Initially, fluorescence in situ hybridization or FISH was routinely used to determine the number of selected chromosomes. Although anecdotal reports suggested an increase in the pregnancy and live-birth rates, only one of 12 randomized, controlled trials demonstrated an increase in live-birth rates.

This image demonstrates fluorescent probes after hybridization of a blastomere nucleus fixed to a slide. Chromosome 13 is labeled in red, chromosome 16 in aqua, chromosome 18 in dark blue, chromosome 21 in green, and chromosome 22 is labeled in yellow. This demonstrates an embryo normal for each of these chromosomes (2 each of the selected chromosomes). At approximately the 10 o'clock position, the investigators deemed the golden color as debris. In this instance, it is easy to see one of the disadvantages of the methodology since it requires skilled personnel to make the correct analysis.

1.26 Disadvantages of FISH

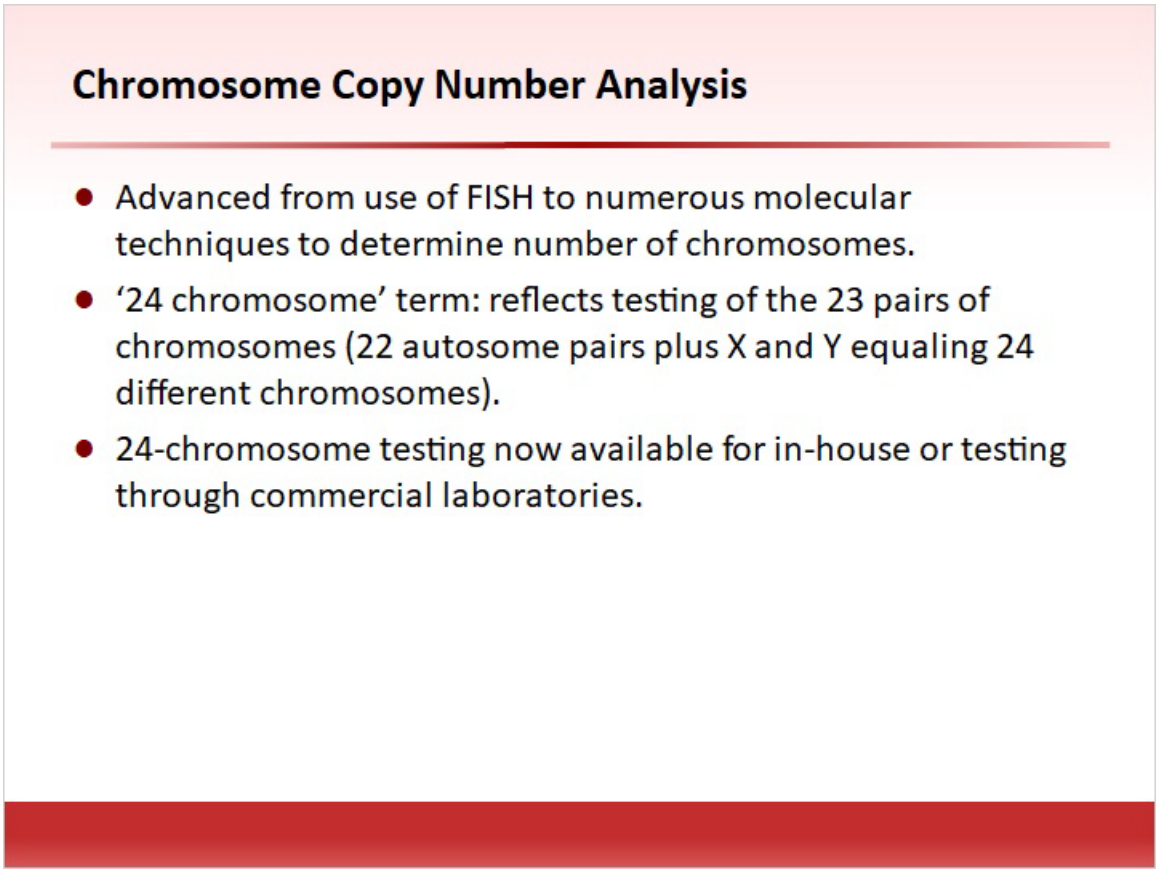


Notes:

There are several disadvantages of using FISH that has led to its diminished use in embryology:

1. FISH requires that the cell be in interphase and fixed to a slide. This fixation technique has proved difficult for some to accurately master. In other instances, parts of the DNA may lyse and material could be lost during the fixation process.
2. FISH is limited by the number of chromosomes that could be analyzed at one time due to the fact that only 5-6 fluorochromes are available. Generally, FISH is performed in two rounds; fluorochromes are analyzed in the first round, then washed off and rehybridization occurs with a second set (and potentially a third set) of probes.
3. The detection rate for FISH appears to be approximately 90%. Fluorescent signals can overlap, resulting in an error in detecting the accurate number of chromosomes.

1.27 Chromosome Copy Number Analysis



Notes:

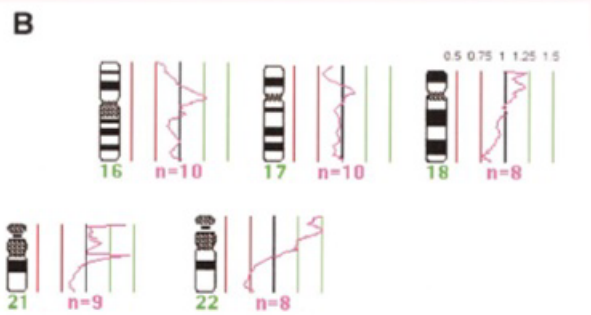
The field has advanced from the use of FISH to numerous molecular techniques to determine the number of chromosomes.
The term '24 chromosome' is used to reflect the testing of the 23 pairs of chromosomes (the 22 autosome pairs plus X and Y equaling 24 *different* chromosomes).
24-chromosome testing is now available for in-house or testing through commercial laboratories. Several of these techniques will be discussed.

1.28 Different Methodologies for PGT-A: CGH

Different Methodologies for PGT-A: CGH

Comparative genomic hybridization (CGH)

- WGA
- Sample versus control labeled with different fluorochromes, hybridized and compared to slide with known normal metaphase spread. Analyzed by filtered fluorescent microscope using specialized software.



With permission from Wilton. Complete karyotyping of embryos using CGH. Fertil Steril 2003

Figure illustrating partial results for CGH on an embryo for chromosomes labeled in green 16, 17, 18, 21, 22. The solid black line represents the test:reference ratio of one. Straight red line represents a chromosome excess and green line a deficiency.

Notes:

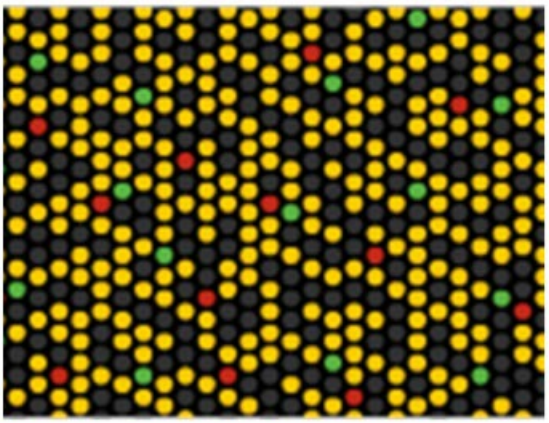
One of the first techniques used in 24-chromosome analysis was comparative genomic hybridization or CGH. CGH involves first using whole genome amplification to increase the template available.
The sample DNA is labeled in one fluorochrome while a known normal control is labeled with another. These two samples are combined and allowed to hybridize to a known normal metaphase spread. A fluorescent microscope and specialized software allow for the determination of the chromosome copy number.
This technique is rarely used for current PGT-A analysis. CGH is labor-intensive, requires several days before results are known, and the need for a metaphase spread was cumbersome.

1.29 Different Methodologies for PGT-A: Array CGH

Different Methodologies for PGT-A: Array CGH

Array CGH (aCGH)

- WGA
- Sample versus control labeled with different fluorochromes hybridized and compared to microarray: a slide spotted with segments of DNA or DNA libraries, or bacterial artificial chromosomes (BAC). Analyzed by microarray reader and specialized software.

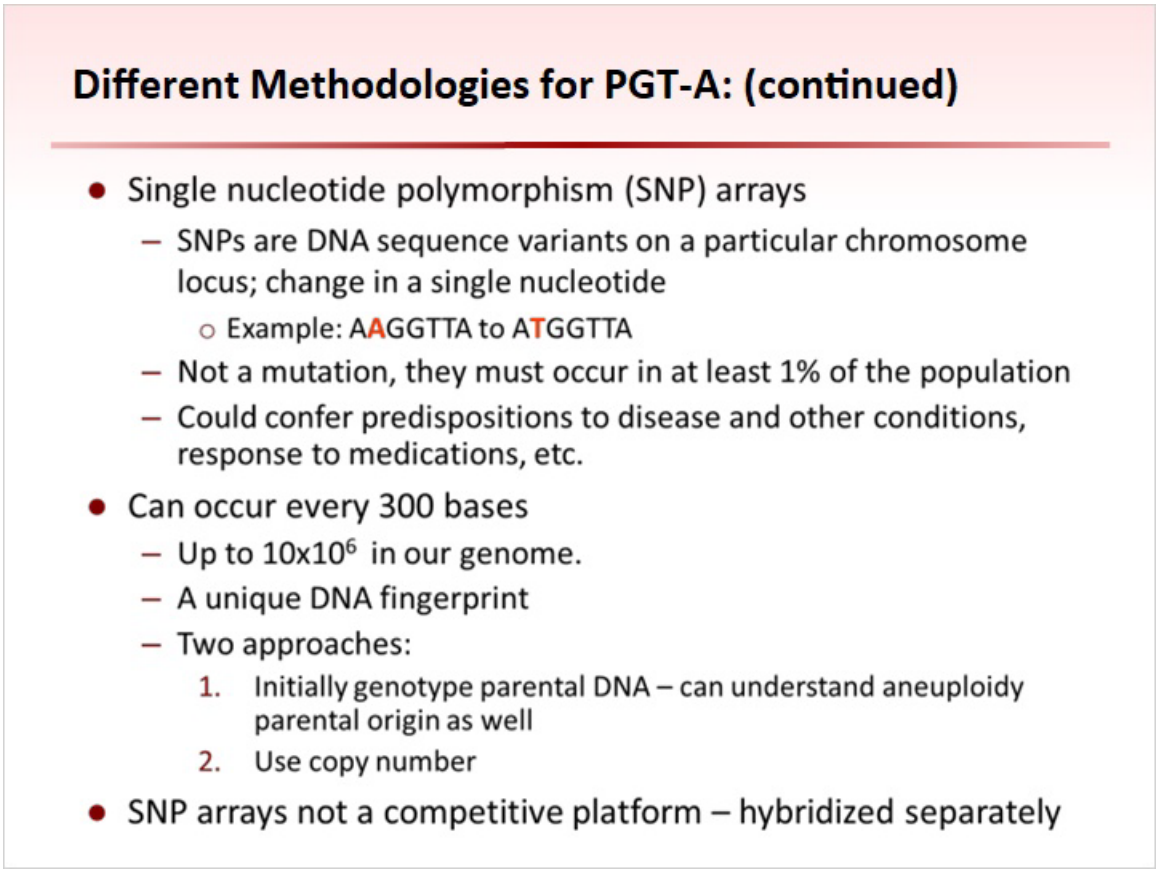


Here the control samples are green and the unknown is red. The resulting merged image demonstrating the three colors – yellow is normal since both red and green hybridize; green is excess control, red excess sample.

Notes:

Similarly, in array CGH, the unknown sample and the known control are labeled with different color fluorochromes after the use of a whole genome amplification technique. Array CGH differs from conventional CGH in that the sample and control DNA are instead hybridized to a DNA microarray so there is no need for the metaphase spread. The microarray slide is spotted with segments of DNA, DNA libraries, or bacterial artificial chromosomes (known as BACs). The samples are analyzed by a microarray reader. This is a raw image from a microarray reader. In this instance, the control samples are labeled with a green fluorochrome while the unknown sample is labeled in red. The merged image produces a yellow color (green plus red) when the unknown is present in normal amounts compared with a normal control. Excess control, as in the case of a monosomy for the unknown, would be represented by a green signal on the merged image. Excess sample, such as is the case with a trisomy, would then be represented by excess red. Specialized software is used to determine the actual intensities of the fluorochromes and actual chromosome copy number.

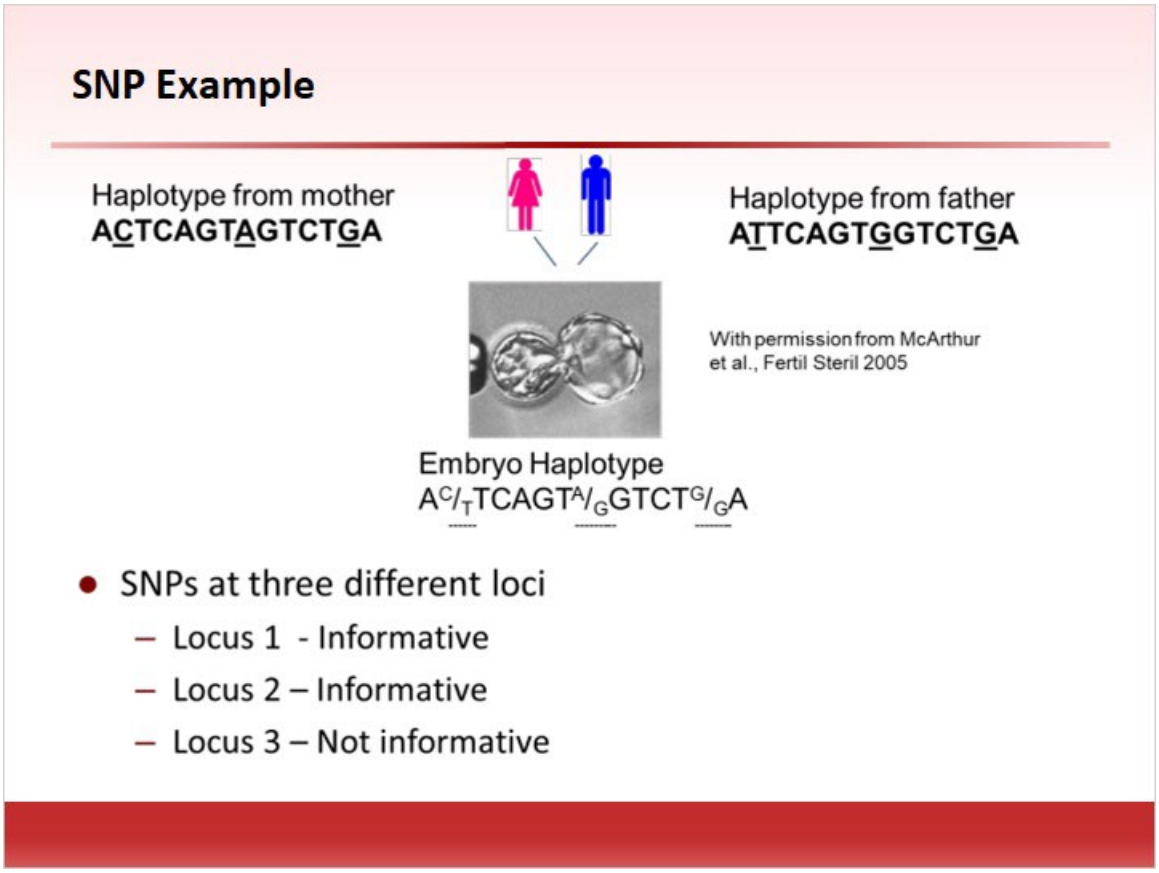
1.30 Different Methodologies for PGT-A: (continued)



Notes:

SNPs, or single nucleotide polymorphisms, are DNA sequence variants on a particular chromosome locus. A SNP represents a change in a single nucleotide, in this example , AAGGTTA to ATGGTTA. This is not a mutation, but rather a variant that must occur in at least 1% of the population. SNPs could confer predispositions to certain disease and other conditions, or may affect an individual's response to medications, etc.
SNPs can occur every 100-300 bases; there are up to 10 million in our genome (with 3 billion base pairs). A combination of SNPs creates a unique DNA fingerprint. There are two approaches used in PGT when using SNP arrays. One of the first methods used determined the genotype or several SNPs of the prospective parents. This will allow the determination of which SNPs are 'informative' or can be used in the diagnosis of the embryo. The SNP results also may be used to determine the origin of the aneuploidy; that is, whether the extra or missing chromosome came from the maternal or paternal partner. A second approach does not require the pre-cycle use of parental DNA, but rather copy number variation is used to determine if the normal numbers of each chromosome are present. Unlike array CGH, SNP arrays are not a competitive platform.

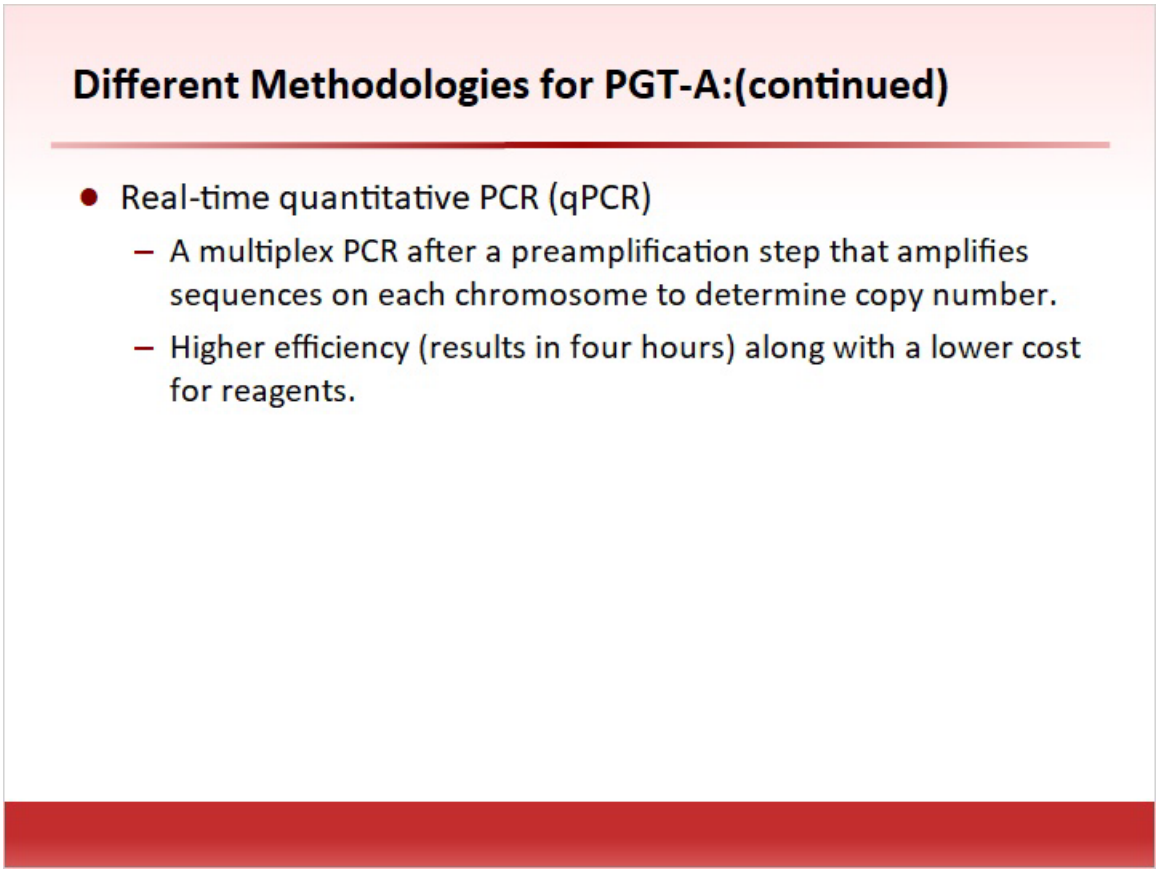
1.31 SNP Example



Notes:

In this example, there are 3 different loci underlined to determine the SNP. The mother has the nucleotide C at the first locus, followed by an A at the second and a G at the third loci of interest. The father has the nucleotide T at the first locus, followed by a G and another G at the second and third loci. In this example, one can differentiate if both alleles are present in the first and second locus since the nucleotides are different. This is termed 'informative.' In the third locus, both parents would contribute a guanine and therefore it cannot be determined if both parental alleles are present. Since there are thousands of SNPs, a limited number can be informative to accurately determine the presence of the allele from both the mother and father.

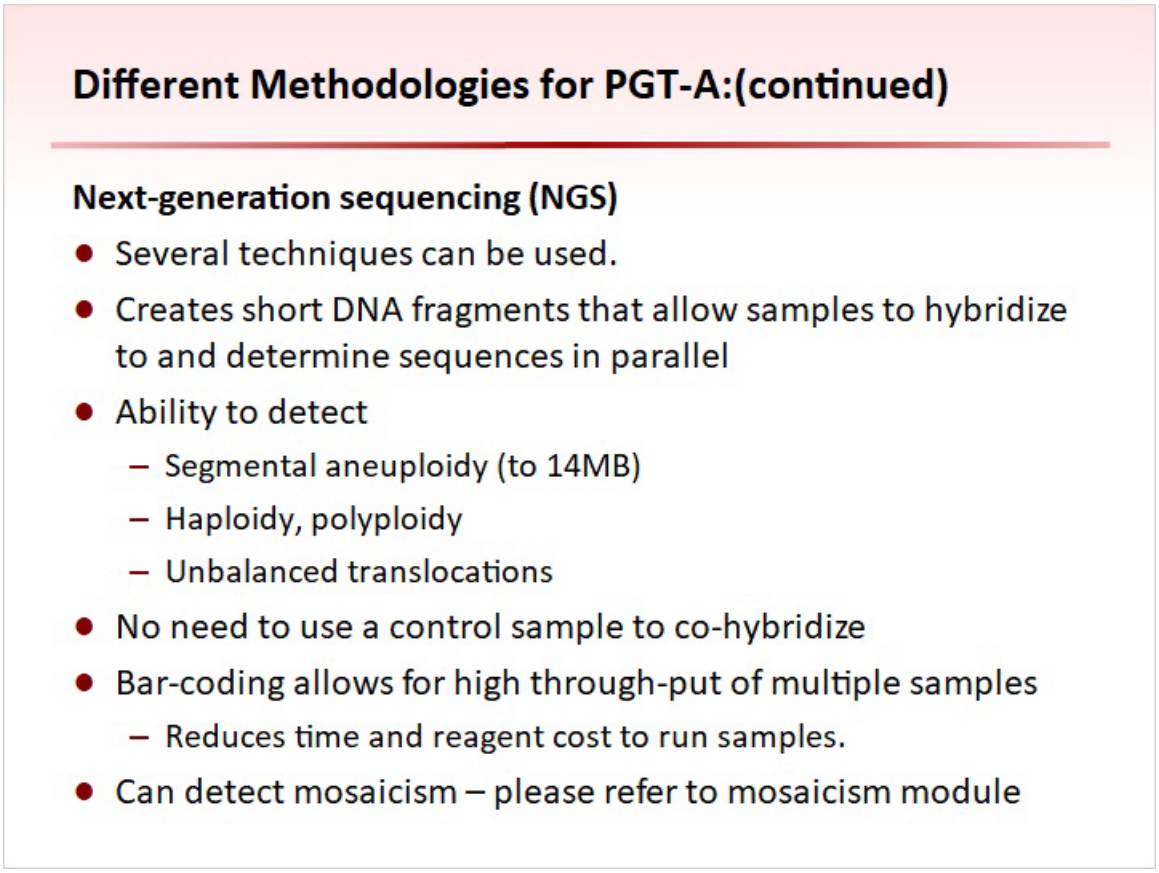
1.32 Different Methodologies for PGT-A:(continued)



Notes:

Real-time quantitative PCR, also known as q-PCR, has also been used successfully for determination of chromosome copy number. Unlike array CGH or SNP arrays, real-time PCR does not require the use of a whole-genome amplification step. Instead, a preamplification step is utilized that enables an increase in the quantity of specific targets, thus allowing a robust quantity of template that will then be subjected to real-time PCR. This preamplification step and the actual qPCR process can be performed in approximately four hours, as opposed to the need for several hours of a WGA and sample hybridization as in array techniques.

1.33 Different Methodologies for PGT-A:(continued)



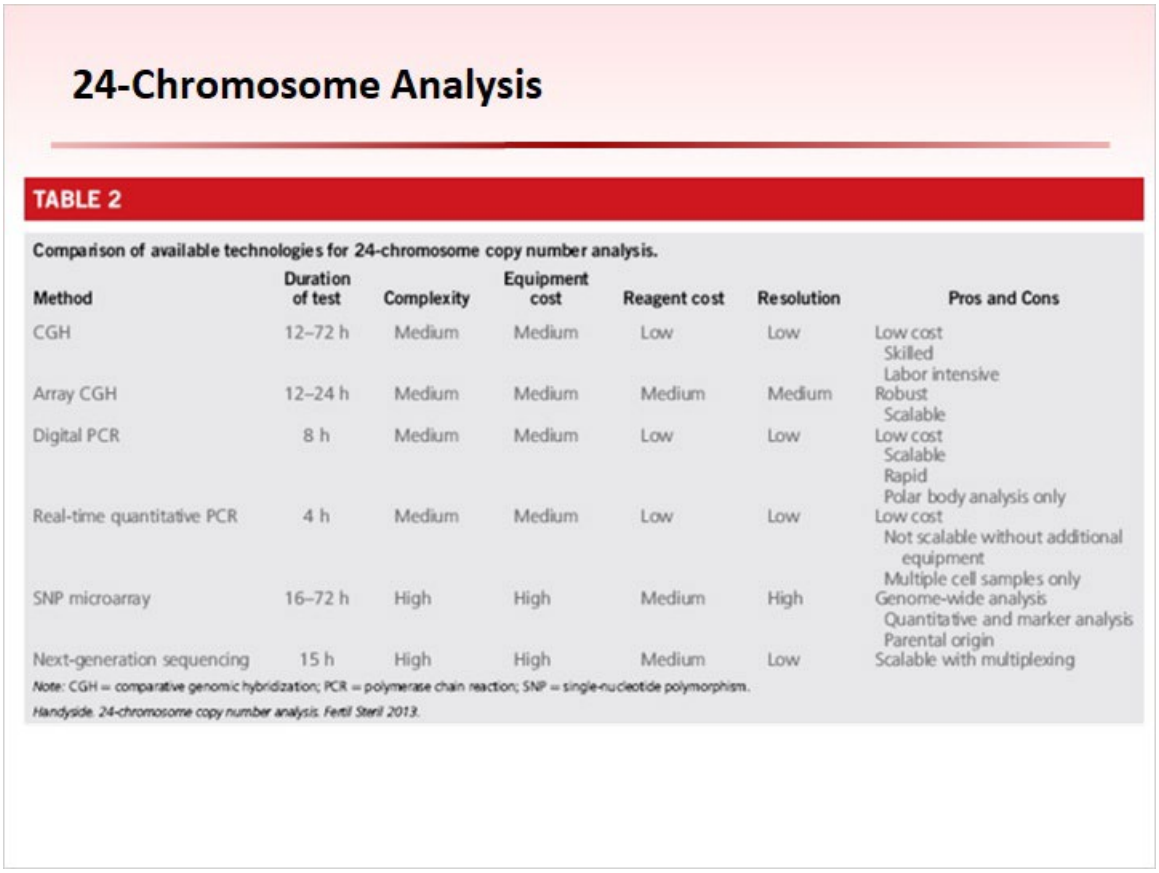
Notes:

Next-generation sequencing, or NGS, encompasses several protocols that can be used to create DNA sequences. This allows the ability to detect segmental aneuploidy, or small deleted or duplicated sequences down to 14 megabases. NGS can also detect haploidy and polyploidy that usually cannot be detected by conventional array CGH. NGS methodologies can also detect unbalanced translocations. One advantage of NGS technologies is that there is no need to co-hybridize each sample with a control, but rather one control sample can be used for all.

Bar-coding may be used to allow a higher throughput of multiple samples. Bar coding can be described as the insertion of short unique DNA sequences that are used to later isolate and identify the sample. With a unique tag, several samples can be analyzed in the same run, then sorted according to their tag.

Lastly, NGS can aid in the detection of mosaicism in a sample. For further learning regarding mosaicism, please refer to the separate course in the series.

1.34 24-Chromosome Analysis



24-Chromosome Analysis

TABLE 2

Comparison of available technologies for 24-chromosome copy number analysis.

Method	Duration of test	Complexity	Equipment cost	Reagent cost	Resolution	Pros and Cons
CGH	12–72 h	Medium	Medium	Low	Low	Low cost Skilled Labor intensive
Array CGH	12–24 h	Medium	Medium	Medium	Medium	Robust Scalable
Digital PCR	8 h	Medium	Medium	Low	Low	Low cost Scalable Rapid Polar body analysis only
Real-time quantitative PCR	4 h	Medium	Medium	Low	Low	Low cost Not scalable without additional equipment Multiple cell samples only
SNP microarray	16–72 h	High	High	Medium	High	Genome-wide analysis Quantitative and marker analysis Parental origin
Next-generation sequencing	15 h	High	High	Medium	Low	Scalable with multiplexing

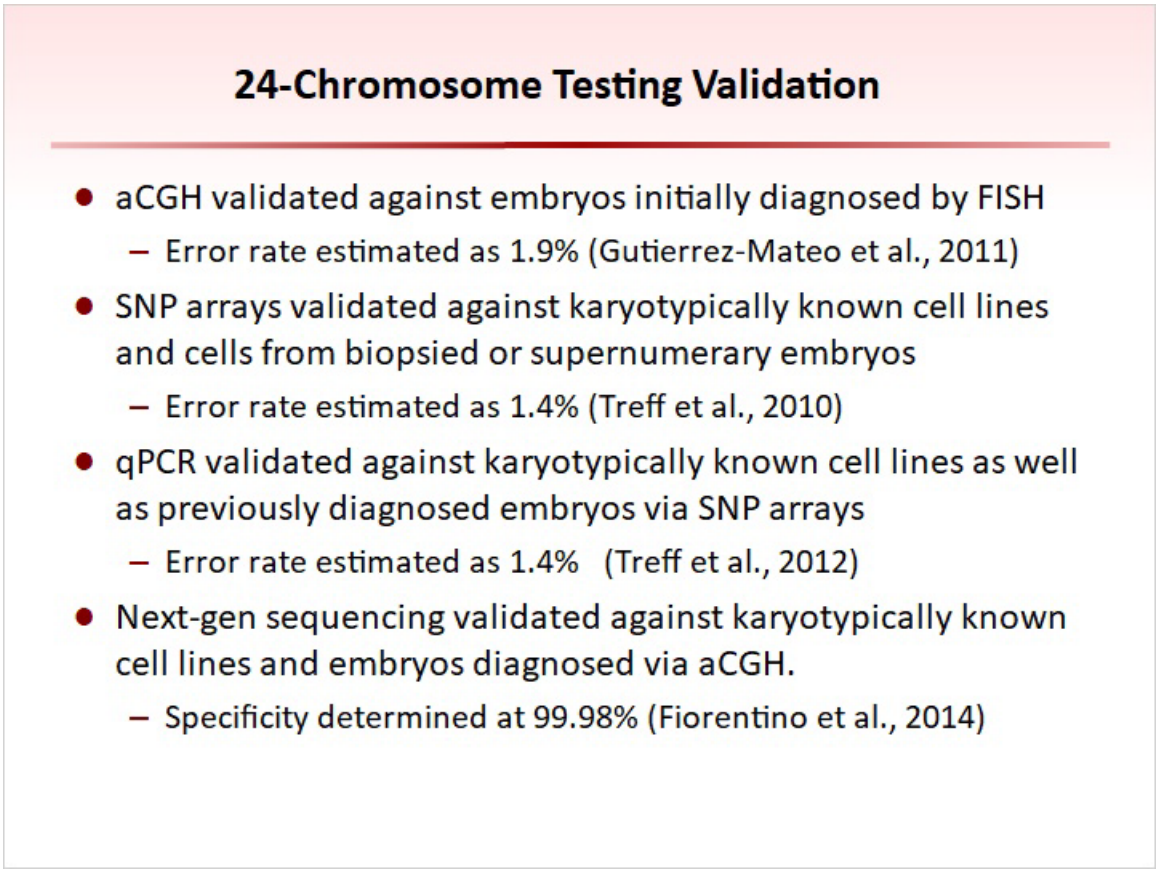
Note: CGH = comparative genomic hybridization; PCR = polymerase chain reaction; SNP = single-nucleotide polymorphism.

Handyside. 24-chromosome copy number analysis. Fertil Steril 2013.

Notes:

There are many factors to consider when deciding the methodologies used to test for 24 chromosomes. Is the test reliable and reproducible? Is it predictive? What is the duration of the test? How difficult is the test to perform? What is the cost, not only for the equipment, but for the reagents and other expendables. Although a patient or clinic may not need to know the actual raw material cost if they are using a reference lab that sets the fee, it is still important to understand. Lastly, it is important to recognize the resolution or the ability to detect small deletion or other changes on the chromosome.

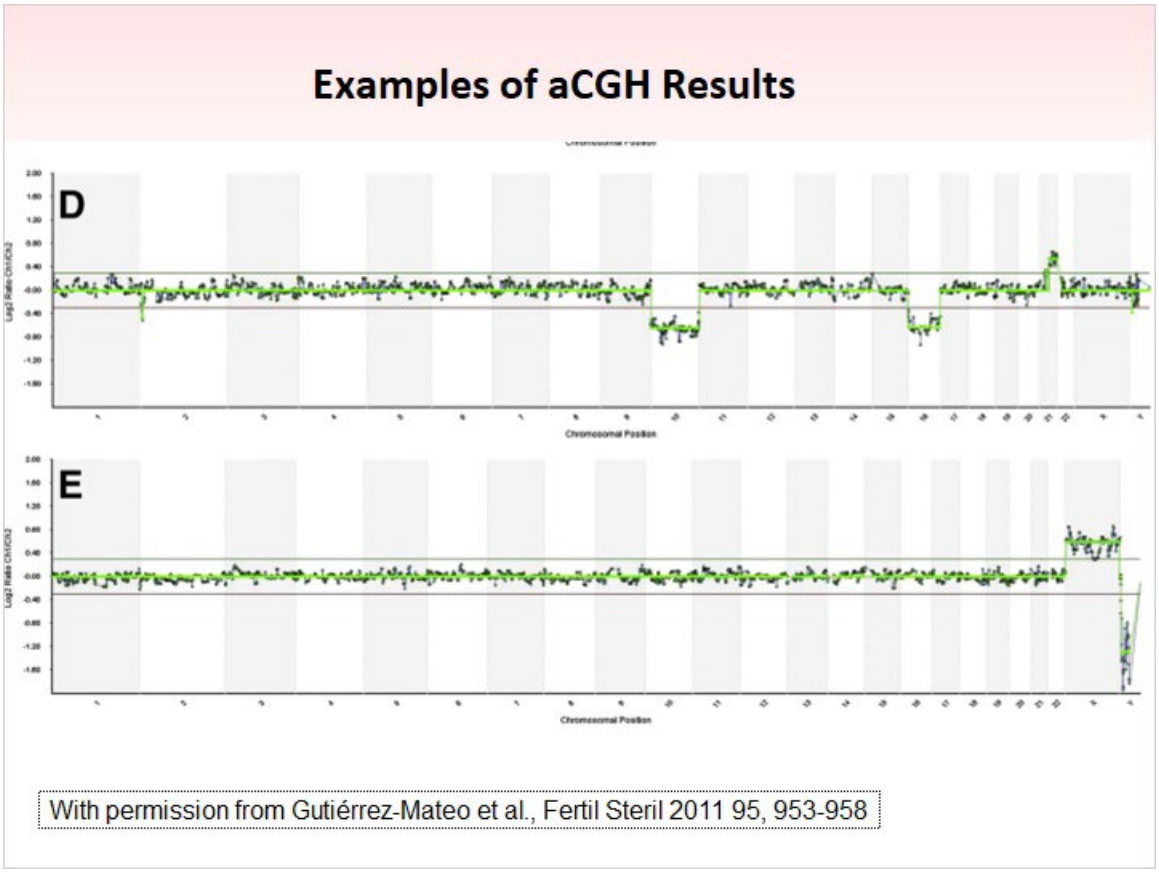
1.35 24-Chromosome Testing Validation



Notes:

The 24-chromosome testing methodologies have been validated by various means. Array CGH was validated by utilizing the remainder of embryos previously diagnosed by FISH. The researchers estimated the error rate to be approximately 1.9%. SNP arrays were validated using single cells from known fibroblast or lymphoblast cell lines. In addition, blastomeres biopsied from embryos or multiple cells from supernumerary embryos were also analyzed. The error rate for SNP arrays was estimated to be 1.4%. Real-time PCR analysis was validated in a similar manner as the SNP arrays using known cell lines and cells from embryos. Additionally, embryos that were previously diagnosed using SNP arrays were rebiopsied and tested for chromosome copy number via real-time PCR. The error rate was estimated to be 1.4%.
Similarly, a next-generation sequencing method was validated, first using known cell lines, followed by embryos that were previously diagnosed via array CGH.

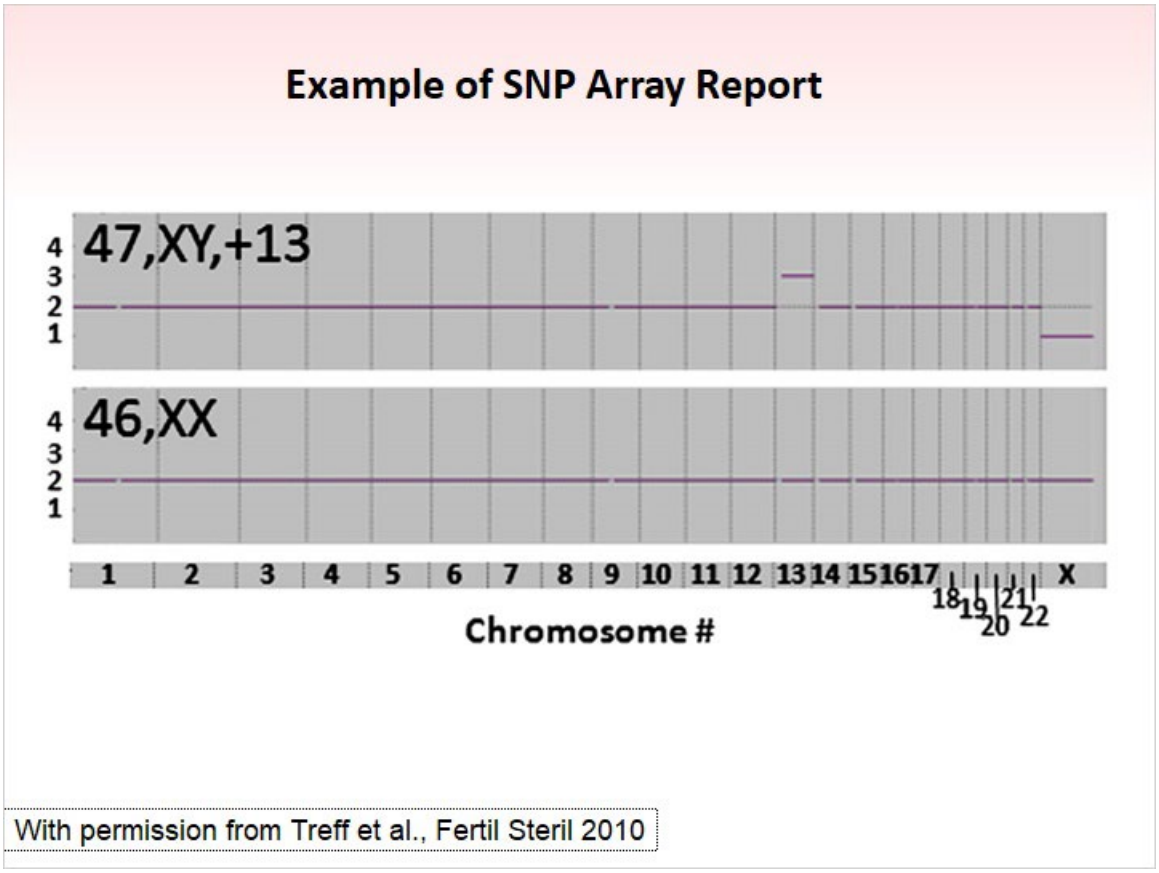
1.36 Examples of aCGH Results



Notes:

The two panels show array CGH results from two embryos. The X axis lists the specific chromosome number while the Y axis is the log scale. A normal result displays between the two horizontal lines. The panel labeled D then is diagnosed as 45,XY,-10,-16,+21 since the measurement for chromosomes 10 and 16 is below the horizontal line, indicating a monosomy while the measurement for chromosome 21 is above the horizontal line, indicating a trisomy. Panel E is a normal female 46,XX. The values all fall between the horizontal lines except the X (indicating there are two Xs when compared to a normal male) and missing a Y (indicating that there is no Y chromosome present and thus a female).

1.37 Example of SNP Array Report



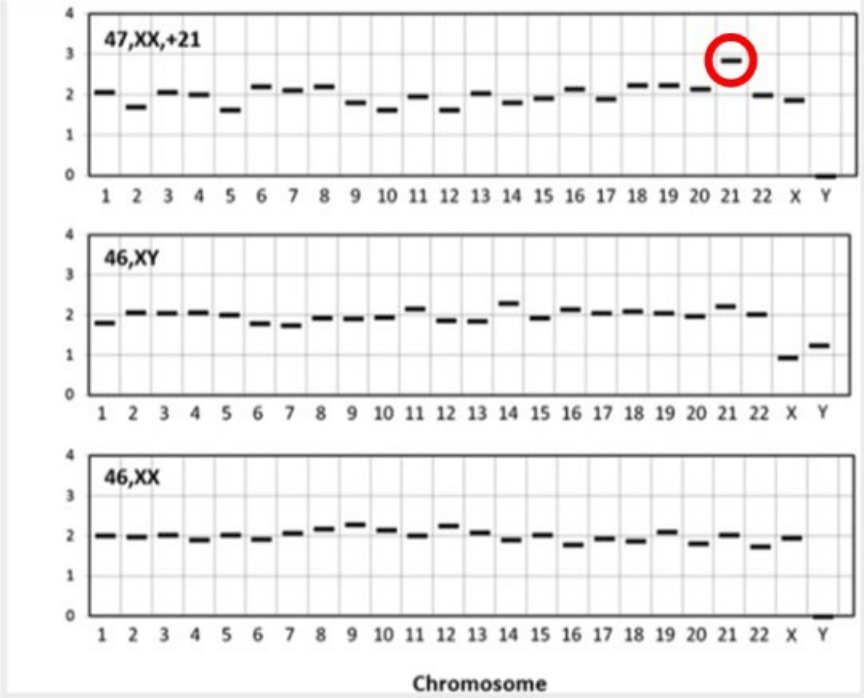
With permission from Treff et al., Fertil Steril 2010

Notes:

In these results from a SNP array, the specific chromosome is listed on the X axis while the chromosome copy number is listed on the Y axis. Two would indicate the normal value. In the upper panel, there are 3 for chromosome 13, indicating trisomy 13. Additionally, one can see that there is only one X chromosome, the presumption then is that the other is a Y chromosome.

1.38 Results for qPCR Testing

Results for qPCR Testing



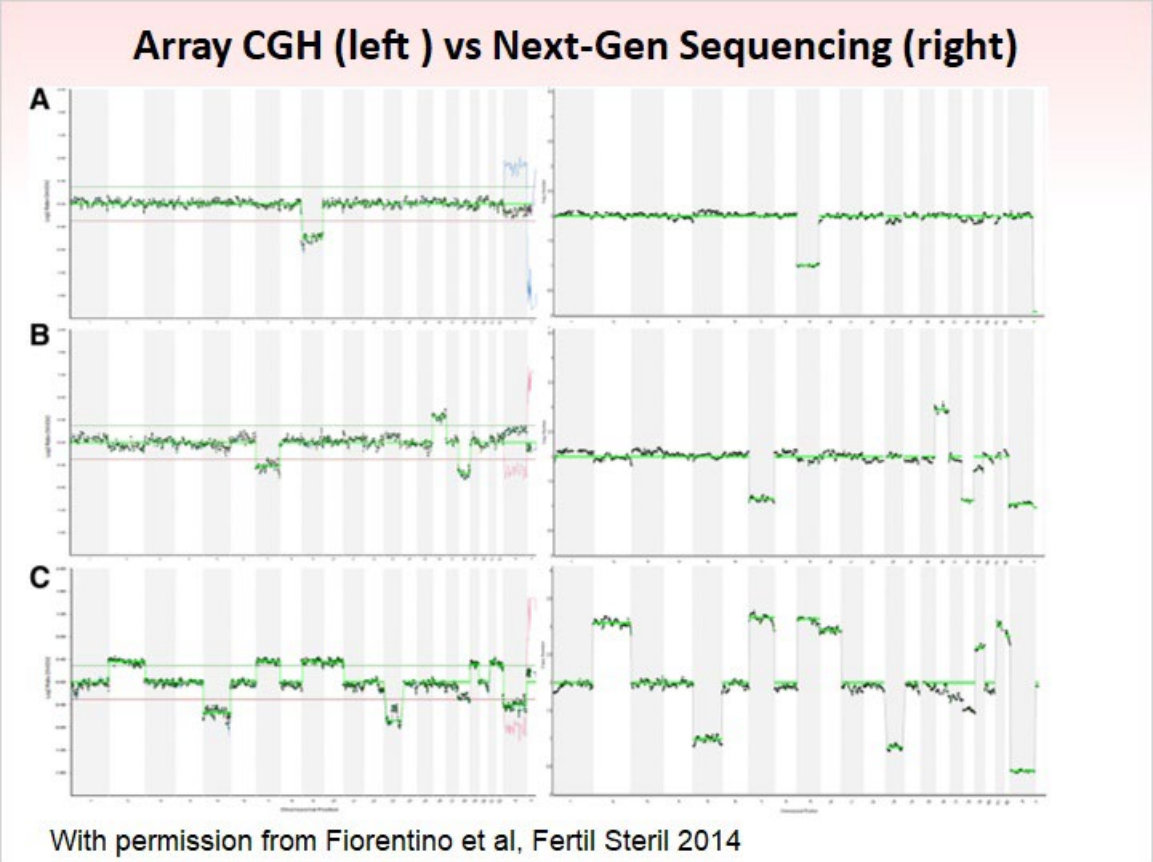
Examples of qPCR-based 24-chromosome copy number results from 5-cell samples derived from nine cell lines with previously well characterized karyotypes.

Treff. 4-hour qPCR-based 24-chromosome CCS. Fertil Steril 2012.

Notes:

These panels illustrate the results for qPCR testing. The specific chromosome is listed on the X axis while the number of chromosomes is listed on the Y axis. In the top panel, one can see an extra chromosome 21, circled in red. The diagnosis for this embryo is then 47,XX,+21. The middle and lower panels reflect the results for a normal male and normal female, respectively. One can see the how the results are displayed on the right to indicate where one X and one Y is present in the middle panel for a normal male or two X chromosome copies and no Y chromosome present in the lower panel for a normal female.

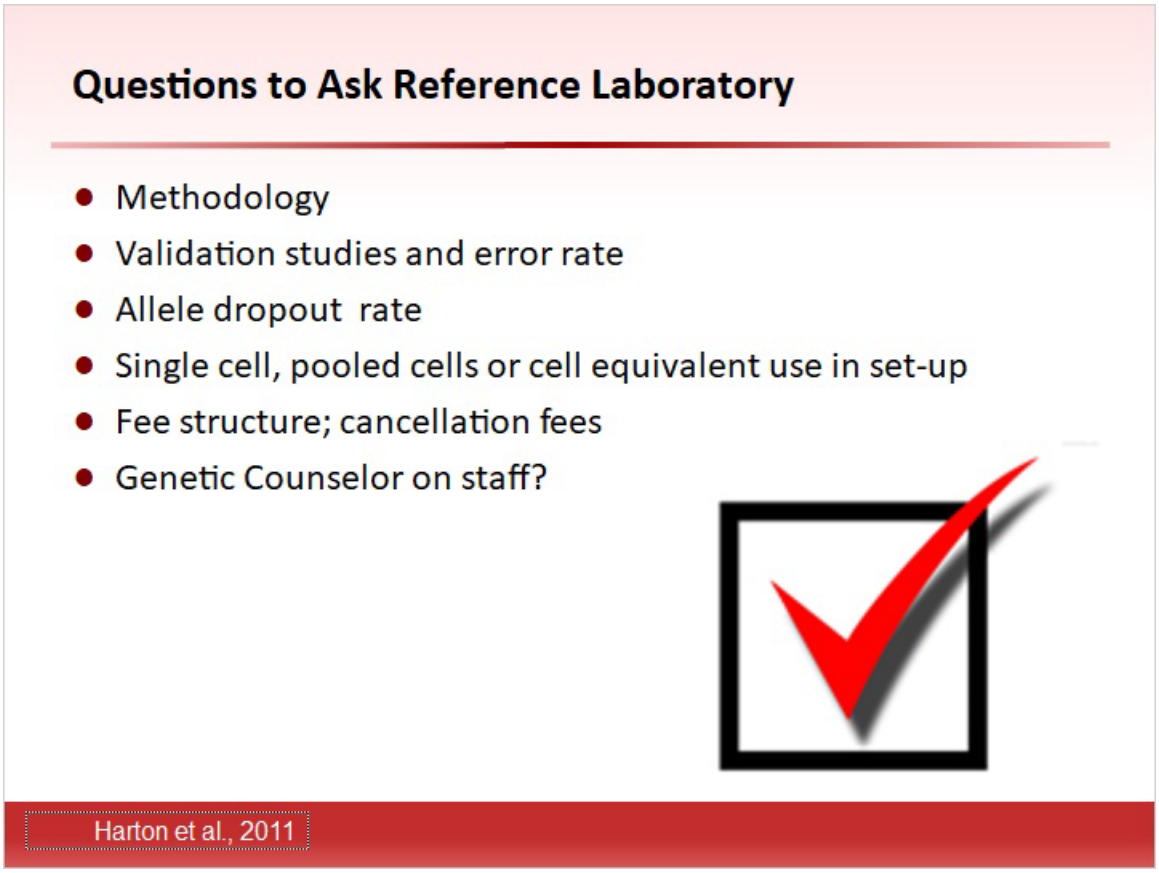
1.39 Array CGH (left) vs Next-Gen Sequencing (right)



Notes:

This figure illustrates aneuploidy results from cleavage-stage embryos detected by array CGH in the left panels versus the same WGA product subjected to next-gen sequencing on the right. The results themselves would appear similar regardless of cleavage- or blastocyst-stage testing. For array CGH on the left panels, the x-axis is the chromosome number while the y-axis is the log scale; above or below the red/green line, respectively, indicates extra or missing chromosomes. For next-gen sequencing on the right panels, the x axis is the chromosome number while the Y axis is the copy number of chromosomes 0,1,2,3,4 with the green line representing the normal two.
Panel A is from an embryo showing monosomy 9. Panel B is an embryo showing monosomy 7, monosomy 18, and trisomy 16. Panel C shows results from an embryo with trisomies of chromosomes 2, 7, 9, 10, 19, 21, and 22 in addition to monosomies of chromosomes 5, 13, and X.

1.40 Questions to Ask Reference Laboratory



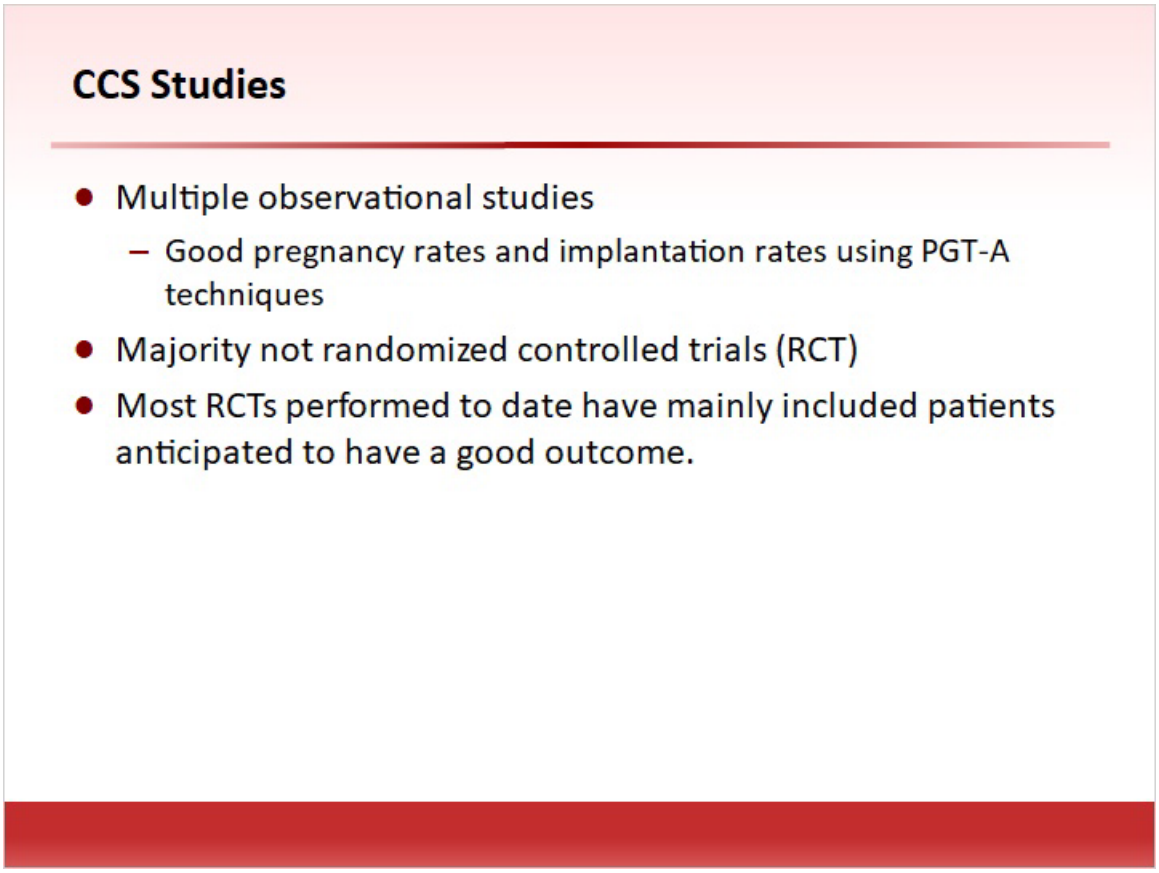
Notes:

Most PGT samples will be sent out to a specialized PGT reference laboratory since most IVF labs do not have their own in-house facilities. When using an outside lab, there are some important considerations. What is the methodology that they use for diagnosis? What error rate do they estimate and how were those validation studies performed? What is their allele dropout rate for single-gene testing? For single-gene tests, how long will it take to optimize a specific test for your patient? What type of sample will they need and from which other family members? Do they use single cells or pooled cells for the optimization testing or do they dilute the sample down to what they have calculated to be equivalent to a single or multiple cells. This is important since it might affect the sensitivity of the test.

It is also important to understand the reference lab's fee structure. If the patient does not proceed with the PGT, is there a refund or is there a cancellation fee?

Does the reference lab have genetic counselors on staff that can assist in obtaining informed consent prior to the procedure or serve as a resource after the test results are received?

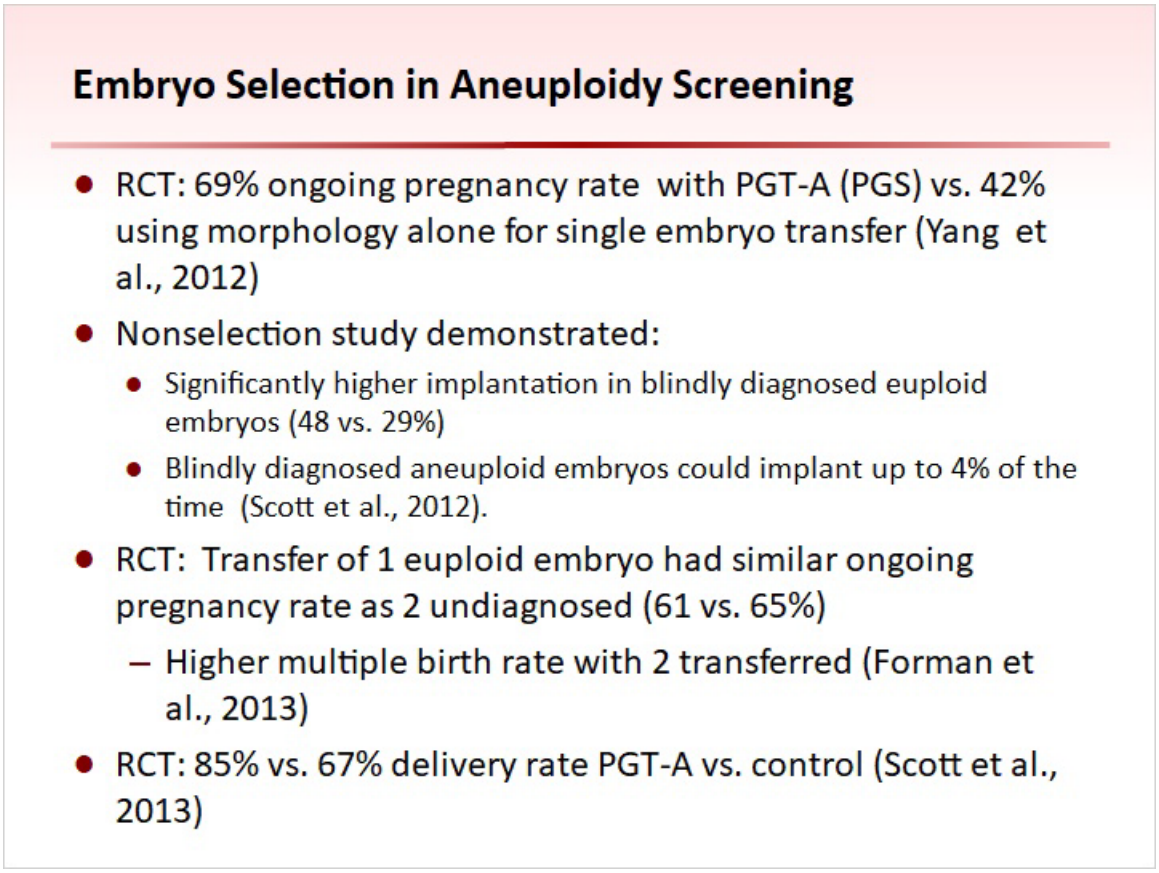
1.41 CCS Studies



Notes:

Multiple observational studies have demonstrated good pregnancy rates and implantation rates using CCS techniques such as array CGH, SNP arrays, real-time PCR, and next-generation sequencing. However, the majority of the studies were not randomized, controlled trials (RCTs). Note that many of the RCTs performed to date have mainly included patients anticipated to have a good outcome and one should carefully evaluate the data presented. The conclusions are presented on the next slide.

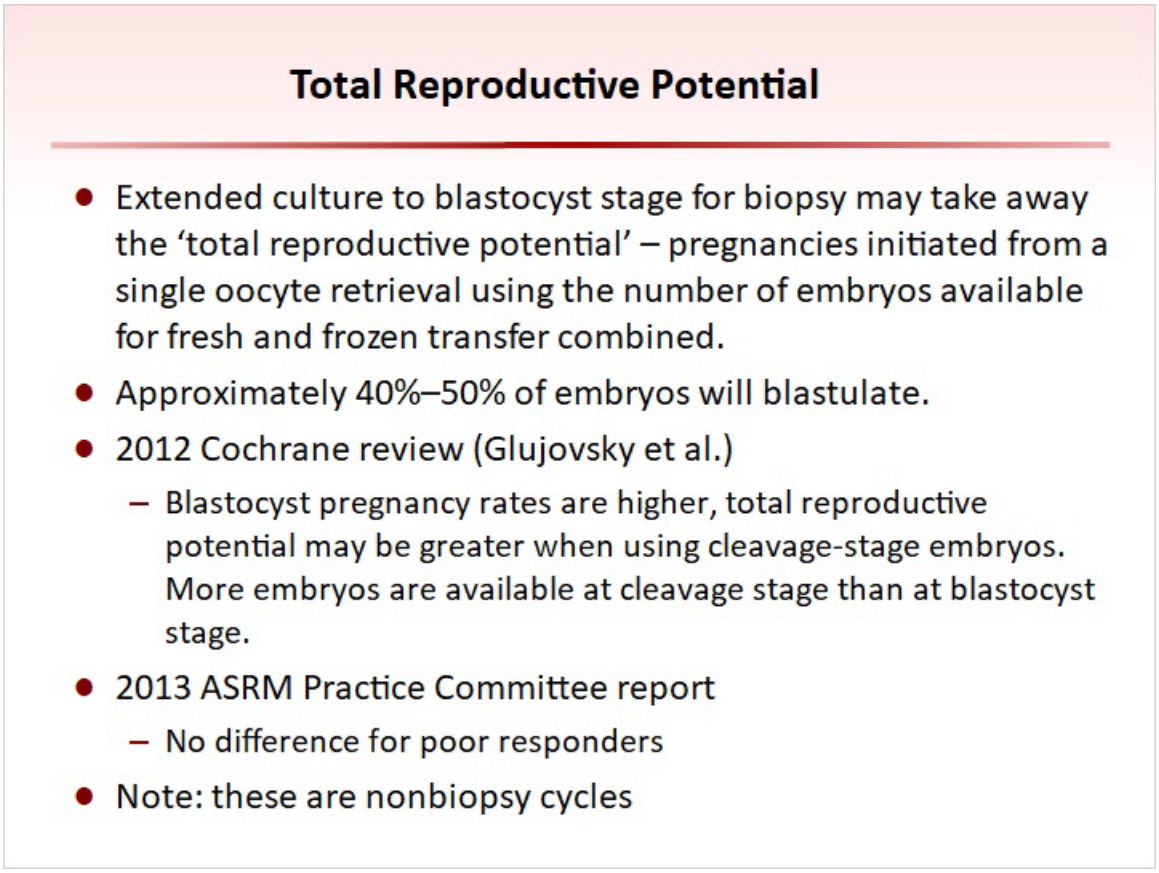
1.42 Embryo Selection in Aneuploidy Screening



Notes:

Research in the use of PGT-A for embryo selection and transfer is rapidly evolving. Conclusions for current studies are shown here. A randomized controlled trial involving younger women demonstrated a significantly higher ongoing pregnancy rate when selecting embryos based on ploidy, rather than on morphology alone.
Another study transferred embryos without knowing the ploidy status initially. When the PGT-A results were available, implantation rates were significantly higher for euploid embryos. It should be noted that of 99 embryos transferred that were subsequently diagnosed as aneuploid, 4 actually implanted and were healthy babies.
A randomized controlled trial demonstrated that when 1 euploid blastocyst was transferred, the pregnancy rate was similar to the transfer of 2 untested blastocysts. The 2-embryo transfer group had a significantly higher incidence of multiple gestation.
Lastly, a randomized controlled trial with both groups receiving 2 blastocysts demonstrated a significantly higher delivery rate in the PGT-A group.

1.43 Total Reproductive Potential



Notes:

The concept of total reproductive potential should be considered in this discussion. Extended culture to blastocyst stage for biopsy may take away the 'total reproductive potential,' that is, those pregnancies initiated from a single oocyte retrieval using the number of embryos available for fresh and frozen transfer combined. On average, approximately 40%-50% of embryos will blastulate.

A 2012 Cochrane review concluded that although blastocyst pregnancy rates are higher, the total reproductive potential may be greater when using cleavage-stage embryos since generally more embryos are available at the cleavage stage than at the blastocyst stage. Similarly, the ASRM Practice Committee report found that in good responder patients, pregnancy rates were higher with blastocyst culture, but there was no difference in poor responder patients.

Note that these studies were not conducted on patients undergoing preimplantation genetic testing. However, the information may be an important consideration in poor responder patients pursuing PGT-A whose embryos may not develop to blastocysts.

1.44 Results: Embryo Selection by Morphology vs Biopsy

Results: Embryo Selection by Morphology vs Biopsy

	Biopsy group (aCGH evaluation)	Control Group (Morphology only)
Number randomized	55	48
Female age	31.2 ±2.5 years *P*>0.05	31.5±2.7 years
Number Day5 blastocysts	8.3±2.1 *P*>0.05	8.1±2.4
Number blastocysts analyzed	425 (aCGH)	389 (morphology only)
Number aneuploid	191 (44.9%)	N/A
Blastocysts remaining after SET	171	341
Number blastocysts cryopreserved after SET	64 (1.2/pt)	157 (3.2/pt) *P*=.017
Clinical Pregnancy ≥20 weeks	38 (69.1%)	20 (41.7%) *P*=.0009

Data from Yang et al (2012)., except additional highlighted data from Liu et al (2012).

Notes:

Here is an example as to how to address the total reproductive potential. Recall that the RCT by Yang and colleagues demonstrated significantly higher ongoing pregnancy rates after aneuploidy screening as opposed to embryo selection by morphology only. In a subsequent publication investigating the same group of patients, it was revealed that significantly more blastocysts were cryopreserved in the control group.
The majority of data presented here are from the original study while the yellow highlighted data are from the subsequent study. Only excellent quality blastocysts were considered for cryopreservation. Although those in the control group have more embryos, only euploid embryos in the biopsy group were cryopreserved. In the initial study, 45% of the embryos were diagnosed as aneuploid. It is interesting to note that if one predicted a 55% euploid rate in the control group, one would have expected 18 pregnancies. That is calculated by multiplying the original 48 embryos by the known 55% euploidy rate and a 69% implantation rate as seen in the euploid group. Although this is purely hypothetical, it is possible that since the control group has more than twice as many available embryos and if all the embryos are eventually transferred, the two groups may eventually have similar outcomes based on the total reproductive potential.

1.45 Subsequent Frozen Embryo Transfer Cycles

Subsequent Frozen Embryo Transfer Cycles

- Patients initially not pregnant returned for cryopreserved embryos.
- Pregnancy rates similar to fresh cycles
- Biopsy group significantly higher implantation vs. control group and similar to fresh cycle (69%)

Table 4 Comparison of clinical outcomes of the subsequent FET cycles between the morphology assessment plus aCGH screening (Group A) and the morphology assessment alone (Group B)

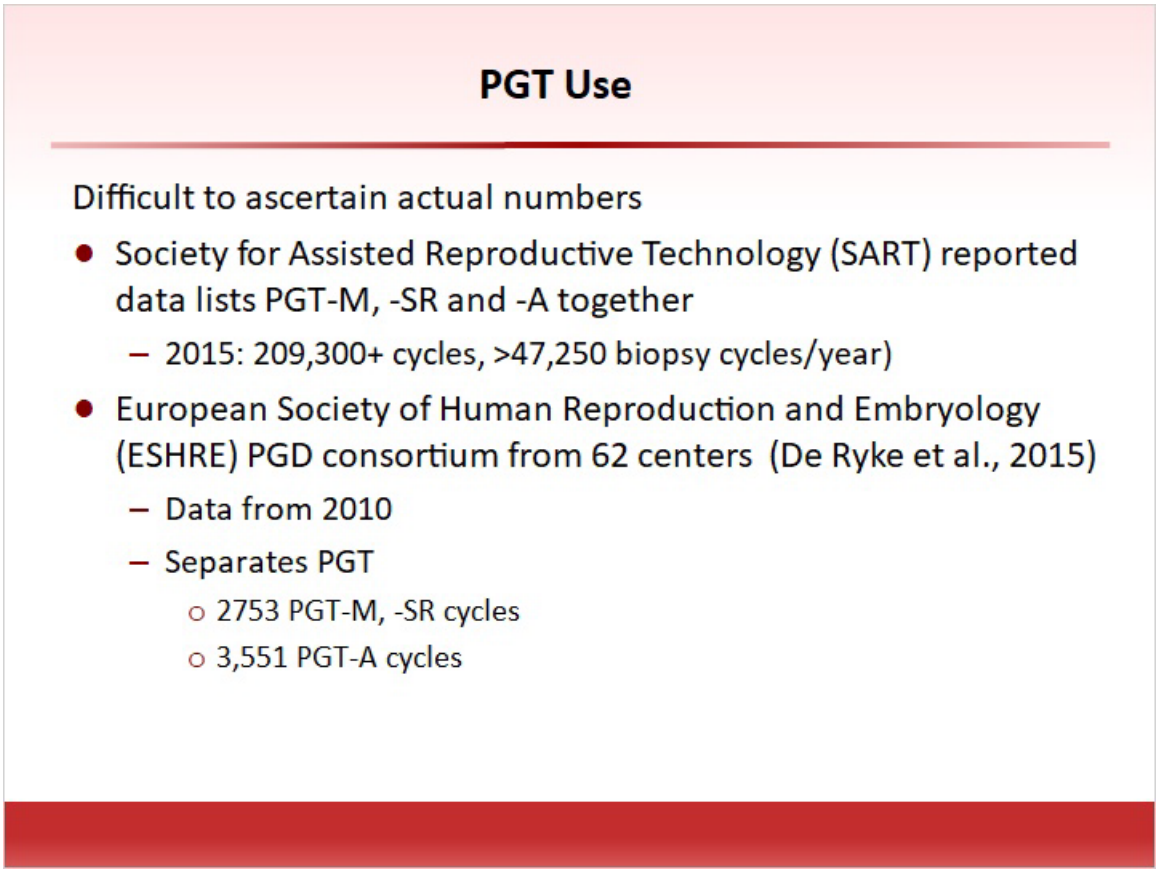
Parameters	Group A	Group B	*p* value
Patients completed FET cycles	15	23	
Patients with SET	10 (66.7%)	4 (17.4%)	
Patients with DET	5 (33.3%)	19 (82.6%)	0.006[a]
Clinical pregnancy	10 (66.7%)	12 (52.2%)	0.583[a]
Ongoing pregnancy (≥20 weeks GA)	10 (66.7%)	10 (43.5%)	0.286[a]
Implantation	13 (65.0%)	14 (33.3%)	0.038[a]
Twin pregnancy	3 (30.0%)	2 (16.7%)	0.624[b]
Miscarriage	0	2 (16.7%)	0.481[b]

SET = single embryo transfer, DET = double embryo transer, GA = gestational age; [a]by Chi-square analysis; [b]by Fisher's exact test.

Notes:

Patients from the 2012 Yang study who were not pregnant returned for transfer of their cryopreserved embryos. Rather than a single embryo transfer as in the original study, some of the patients elected to transfer two embryos; the overall survival rate for both groups was approximately 90%. Interestingly, the ongoing pregnancy rates were similar between the fresh and frozen cycles. In the biopsy group A, the fresh pregnancy rate was 69.1% compared with 66.7% in the frozen transfer cycle. In control group B, the fresh pregnancy rate was 41.7% compared with 43.5% in the frozen transfer cycle. The implantation rates were also similar in the biopsy group between fresh and frozen cycles. However, the implantation rate in the control group was lower in the frozen transfer cycle as compared with the fresh (33.3% and 41.7%, respectively) although this was not significant.

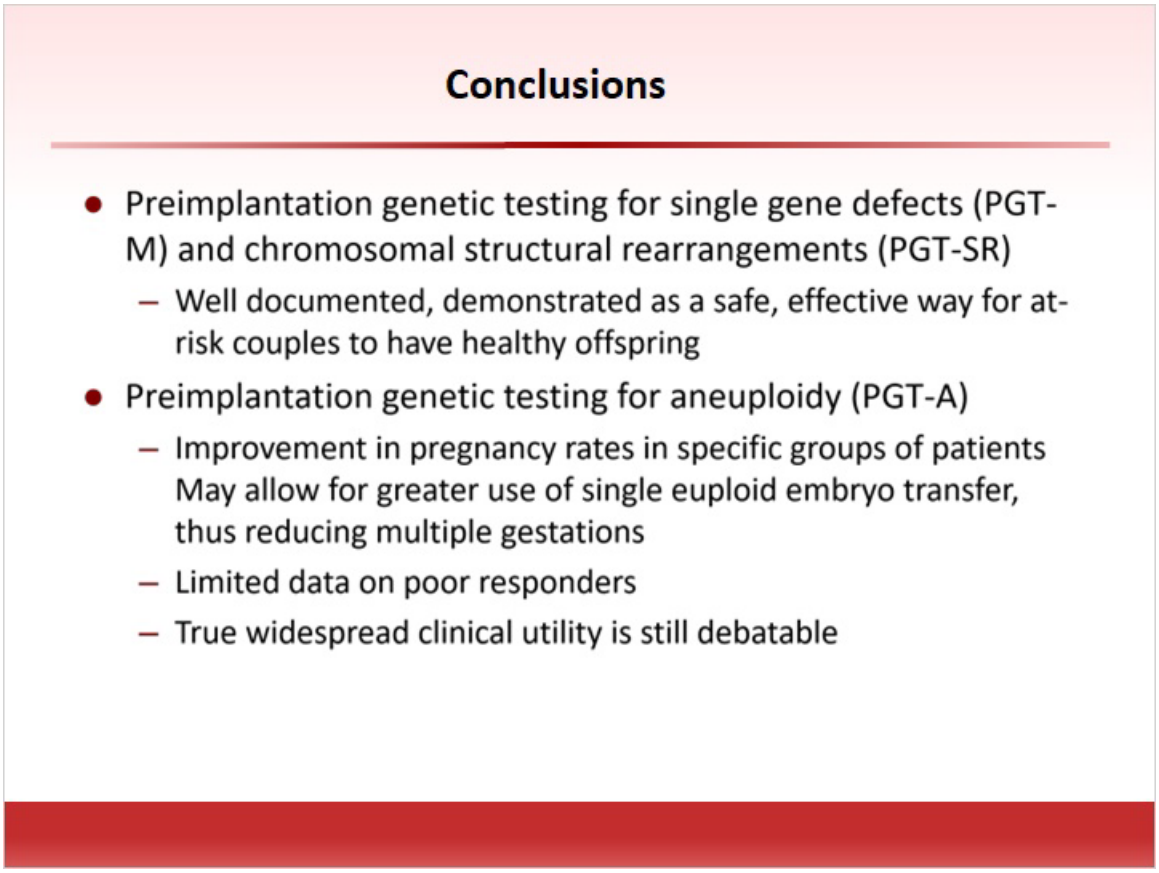
1.46 PGT Use



Notes:

It is difficult to accurately ascertain the number of PGT cycles performed across all laboratories. Currently, SART reports PGT cycles in aggregate. The European Society of Human Reproduction and Embryology (ESHRE) PGD consortium collects data from 62 centers worldwide, primarily in Europe and Asia. It is important to note that in 2010, FISH was the primary methodology for PGT-A cycles.

1.47 Conclusions



Notes:

The use of preimplantation genetic testing for single gene defects and for chromosomal structural rearrangements has been well documented and has been demonstrated as a safe, effective way for at-risk couples to have healthy offspring.
The use of preimplantation genetic testing for aneuploidy has demonstrated improvement in pregnancy rates in specific groups of patients. It may aid in the decision to transfer a single euploid embryo without a reduction in the pregnancy rate, while greatly reducing the multiple gestation rate. However, there are limited published data on poor responder patients and further studies are warranted, especially in this population. Because of this, the true widespread clinical utility is still debatable.

1.48 Thank you!

Notes:

Thank you for participating in this educational activity.