Whole genome amplification from single cell and its optimization for better genomic coverage

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Whole genome amplification (WGA)

- WGA is an in vitro method to amplify DNA in a sample to generate amplified DNA for further molecular genetic analysis

- WGA is particularly useful for samples with limited DNA content

- **Preimplantation genetic diagnosis (PGD) relies on the genetic material taken from a single cell**
- **PGD utilizes direct PCR on the lysed single cells**
- **PCR has enough sensitivity for DNA analysis in a single cell**

- **Low quantity DNA in a single cell presents a number of challenges including**
  - **contamination**
  - **amplification failure**
  - **preferential amplification**
  - **allele dropout (ADO)**
- **Single cell can be analyzed only once**
- **Independent confirmation and additional analysis are impossible**

- **Will WGA be a solution?**

WGA methods used in PGD

- Primer extension preamplification PCR (PEP-PCR)
- Degenerate oligonucleotide primed PCR (DOP-PCR)
- OmniPlex WGA
- Multiple displacement amplification (MDA)

Primer extension preamplification-PCR (PEP-PCR)

- **First introduced in 1992 in the analysis of single sperm typing to study genetic recombination**
- **Total degenerate 15-mer PCR primers (hexamers were not successful)**
- **Generates a smear of DNA fragments that are visible on agarose gel**
- **Coverage at least 78%**

Zhang et al., 1992, PNAS

Protocol

- **Fifty amplification cycles**
  - **Denaturation 1-minute at 92°C**
  - **Annealing 2-minute step at 37°C**
  - **Ramping step of 0.1°C/sec to 55°C**
  - **Polymerase extension 4-minute step at 55°C by using Taq polymerase**

Zhang et al., 1992, PNAS

Improved PEP (IPEP)

- IPEP differs from original PEP
  - cell lysis in Expand Lysis buffer
  - use of a mix of *Taq* polymerase and proofreading *Pwo* polymerase in whole genome amplification
  - an additional cyclical elongation step at 68°C for 30 seconds before the denaturation step at 94°C

Dietmaier et al. 1999, AJP

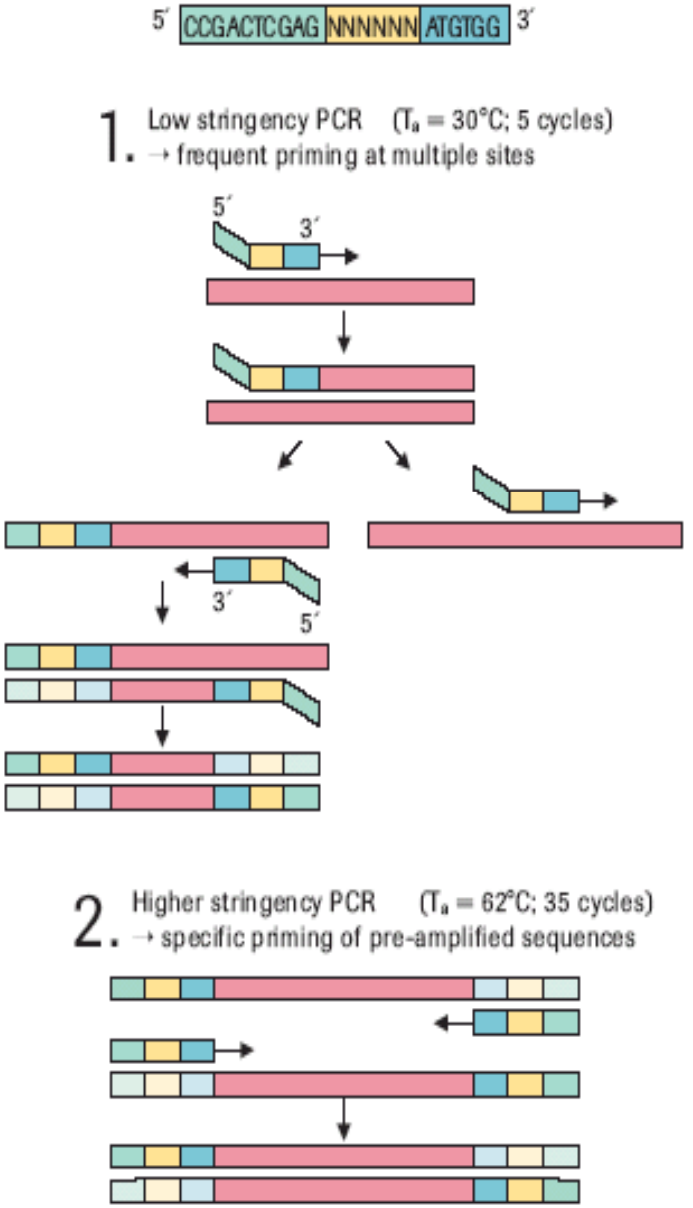
- **IPEP-PCR is more effective than the conventional PEP-PCR**
- **IPEP-PCR supports**
  - **microsatellite analysis**
  - **accurate sequencing**
  - **mutation detection methods**

Dietmaier et al. 1999, AJP

- **PEP in PGD**
  - Aspartylglucomaminuria
  - Infantile neuronal ceroid lipofuscinosis
  - Familial amyloidosis of the Finnish type
  - Beta-thalassemia
  - Familial adenomatous polyposis coli
  - Cystic fibrosis
  - Duchenne muscular dystrophy

Degenerate oligonucleotide-primed PCR (DOP-PCR)

- **Partially degenerate sequence oligonucleotides**
  - **5'-CCGACTCGAGNNNNNNATGTGG-3'**
  - **Primers randomly anneal to the genome**
- **Low initial annealing temperature, ensures priming from multiple sites**
- **Annealing temperature is increased to allow more specific priming**
- **Coverage is theoretically genome wide**



Telenius et al., 1992, Genomics

- **DOP-PCR allowed first CGH on single cell**
  - **Provided reliable detection of trisomies and sex in a blind study**
- **Hybridization of DOP-PCR products to normal metaphase chromosomes produced**
  - **strong even signals with no obvious sites of amplification deficiency or excess**

Wells et al., NAR 1999

Genomic coverage: proportion of successful amplifications at specific loci after various forms of WGA

Locus	WGA methods					
	PEP	T-PCR	DOP25	DOP50	alu	Total
D21S11	1.00	0.88	0.73	0.80	0.41	0.77
D21S1414	0.90	0.65	0.76	0.85	0.45	0.76
D18S535	0.90	0.69	1.00	0.95	0.80	0.86
CFTR	0.95	0.95	0.74	1.00	0.86	0.92
[beta]-globin	0.90	0.94	0.86	0.91	0.52	0.85
FACC	0.80	0.41	0.81	0.91	0.19	0.64
Amg X	0.91	0.82	0.95	0.80	0.47	0.80
Amg Y	0.90	0.50	0.80	0.80	0.67	0.73
APC	0.95	0.91	0.91	0.95	0.80	0.94
HMSH2	0.90	0.87	0.89	0.91	0.72	0.86
Total	0.91	0.76	0.85	0.89	0.59	0.81

Wells et al., NAR 1999

- **CGH with PEP amplified DNA technique failed to give any accurate results due to signal intensity**
- **DOP25 was particularly successful as incorporation of fluorescent nucleotides during the second amplification reaction**

Wells et al., NAR 1999

OmniPlex

- OmniPlex is a whole genome amplification method allows generating a representative amplification of genomic DNA
- Utilizes amplification technology based upon
  - random fragmentation of genomic DNA
  - conversion of the resulting fragments to PCR amplifiable OmniPlex Library molecules flanked by universal priming sites
  - OmniPlex library is then PCR amplified using universal oligonucleotide primers

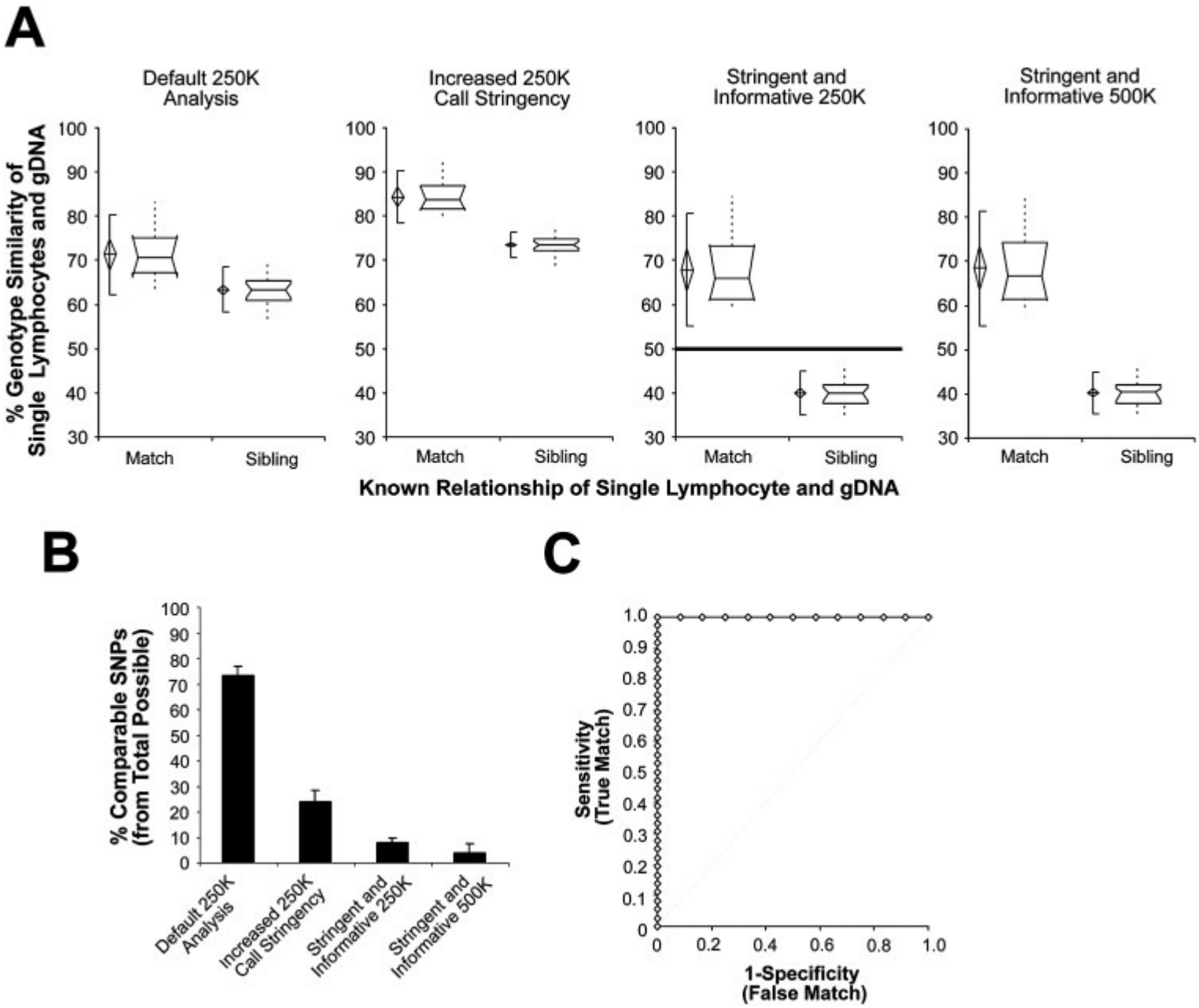
Protocol

- Proteinase K digestion 1 hr at 50° C
- 4 min at 99° C for fragmentation
- Sample cooled down to 4 °C in a thermocycler
- 3 µl library mix added to each proteinase K-digested cell
  - 2 min at 95° C
  - down to 4° C and kept on ice
  - 1 µl of library preparation enzyme
- Thermo cycling
  - 20 min at 16° C,
  - 20 min at 24° C,
  - 20 min at 37° C,
  - 5 min at 75° C
  - cool down to 4° C.
- 60 µl amplification mastermix added to the sample
- PCR
  - 3 min at 95° C
  - 25 cycles of 30 s at 94° C
  - 5 min at 65° C
  - cool down to 4° C

- **OmniPlex successfully amplified genomic material from sources**
  - **Saliva**
  - **whole blood**
  - **blood card**
  - **buccal swab**
  - **bacterial artificial chromosome**
  - **formalin-fixed, paraffin-embedded tissues**

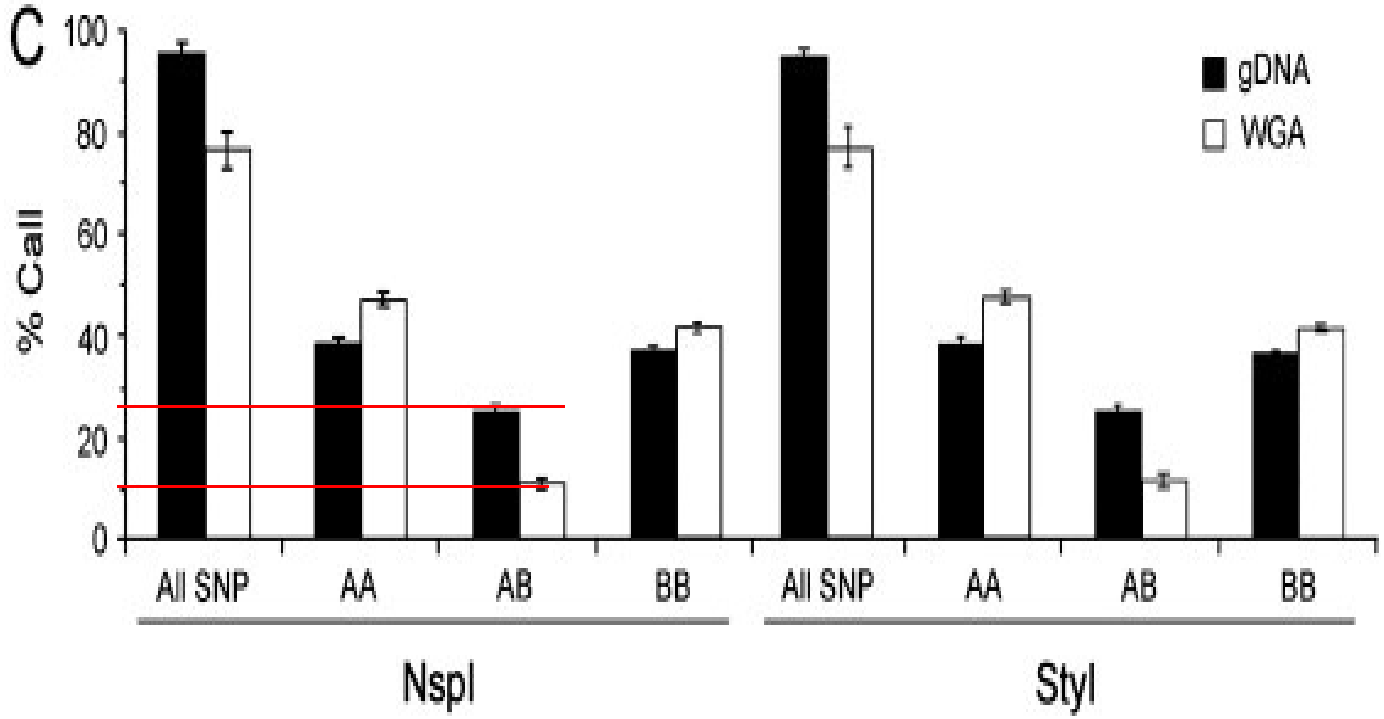
- **PGD for β-thalassaemia and HLA typing**
  - **Amplification efficiency 88-94%**
  - **ADO 6-19%**

Chen et al., 2008, RBM Online



Treff et al., 2009. Fertil Steril

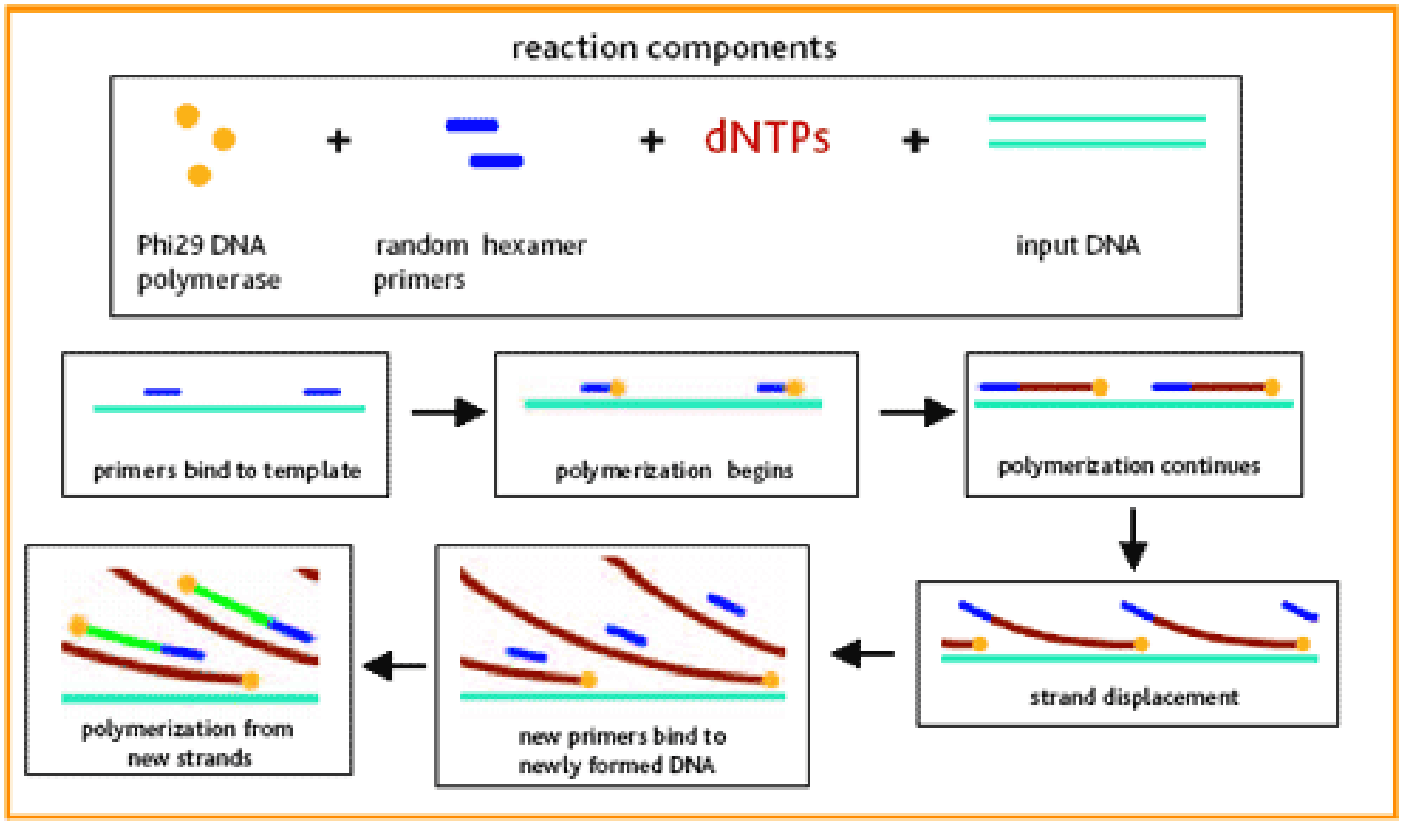
OmniPlex in Array



Treff et al., 2009. Fertil Steril

Multiple displacement amplification (MDA)

- **MDA is an isothermal method that utilizes**
  - **bacteriophage phi29 DNA polymerase**
  - **random hexamer (NNNNNN) for amplification**



- **MDA is capable of generating large fragments (>10 kb in size)**

- **Internal proofreading activity results in much lower misincorporation rates relative to *Taq* DNA polymerase**

Use in PGD

- CGH
- aCGH
- Beta-thalassemia
- Morquio disease
- X-linked retinoschisis
- Duchenne muscular dystrophy
- X-linked adrenoleukodystrophy
- Non-ketotic hyperglycinaemia
- Brachydactyly type B
- Diandric complete hydatidiform mole
- Marfan syndrome
- Fragile X syndrome
- Huntington disease
- Zellweger syndrome
- Cystic Fibrosis

Preimplantation Genetic Haplotyping (PGH)

- 127 PGH for 101 couples
  - Cystic fibrosis (50 cycles, 37 couples)
  - Huntington disease (29, 24)
  - Duchenne/Becker muscular dystrophy (17, 15)
  - Spinal muscular atrophy (SMA) (8, 6)
  - Epidermolysis bullosa (EB) (7, 4)
  - Sickle cell disease (SCD) (6, 6)
  - Alport syndrome (3, 2)
  - Fragile X syndrome (3, 3)
  - Prader-Willi syndrome (1, 1)
  - Familial partial lipodystrophy (1, 1)
  - Haemophilia A (1,1)
  - CF + Haemophilia A (1, 1)
- 26 babies born, 13 ongoing pregnancies
- MDA coupled with PGH provides a robust, efficient and successful alternative to single cell PCR for monogenic diseases

Renwick et al., RBM Online in press

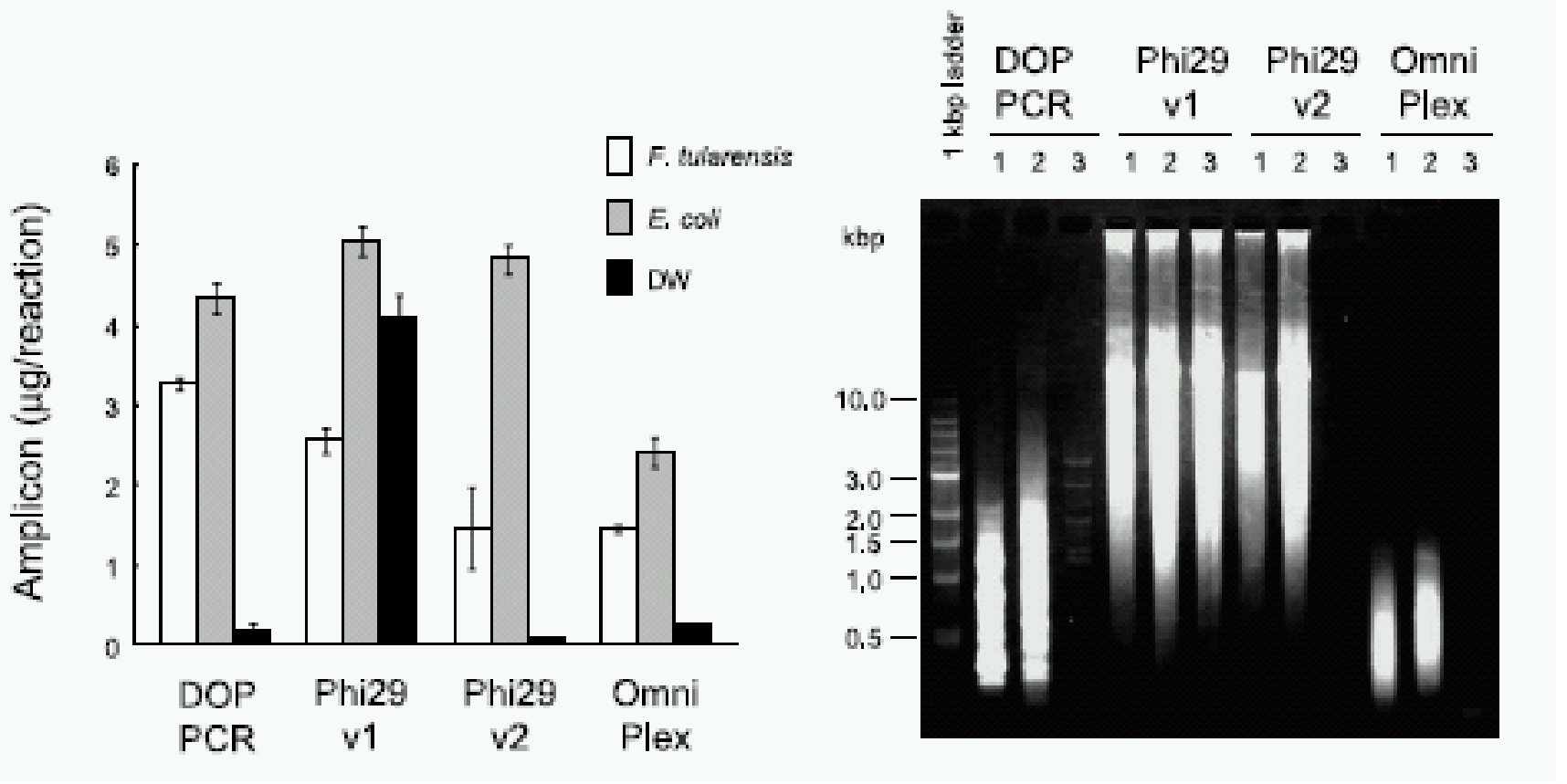
Achondroplasia	Maple syrup urine disease
Adreno Leukodystrophy (ADL)	Metachromatic Leukodystrophy
Albinism, oculocutaneous type IA	Microphthalmia/ anophthalmia
Alpha thalassemia/mental retardation syndrome	Mucopolysaccharidosis, type I (Hurler Syndrome)
Arginino Succinic Acidemia	mucopolysaccharidosis, type II (Hunter syndrome)
Ataxia Telangiectasia Mutated	Mucopolysaccharidosis, type IVA, Morquio syndrome A
Biotinidase deficiency	mucopolysaccharidosis, type VI (Maroteaux-Lamy syndrome)
Bosley-Salih-Alorainy syndrome	Niemann Pick disease (NPD)
B-Thalassemia	Non-ketotic hyperglaycemia
Canavan Disease	Osteogenesis Imperfecta Type IV
Carnitine Acylcarnitine translocase deficiency	Phenol Ketonuria (PKU)
Chronic Granulomatous disease (CGD)	Progressive Familial Intrahepatic Cholestasis 1
Citrullinemia	Propionic Acidaemia
Congenital Adrenal Hyperplasia (CAH)	Sanfilippo type B (MPS-IIIB)
Cystic Fibrosis	Sanjad Sakati Syndrome
Duchenne muscular dystrophy (DMD)	Severe combined immunodeficiency disease
Ehlers-Danlos Syndrome	Sickle cell
Fragile X	Smith Lemli Opitz Syndrome; SLOS
Galactosomia	Spinal muscular atrophy / Werdnig Hoffmann
Glucose-6-phosphate dehydrogenase deficiency	Sulfite Oxidase deficiency
Glutaric Acidemia type 1	Tyrosinemia type I
Glycogen storage disease type II, Pompe disease	Very long-chain acyl-CoA dehydrogenase deficiency
GM1-Gangliosidosis	Wiskott-Aldrich Syndrome
Hereditary non-syndromic sensorineural deafness	X-linked Hydrocephalus
Hyperinsulinemia	Zellweger Syndrome

DOP vs MDA in CGH

- 189 oocyte or PB using one of two WGA methods
  - DOP-PCR 159/183 (86.6%)
    - yielding 200–4,000 bp smear
  - MDA 5/6 (83.3%)
    - average fragment sizes of 10 000 bp
- No difference in fluorescence intensities between samples amplified by the DOP-PCR and with the MDA in CGH

Fragouli et al., 2006, Hum Reprod

DOP - MDA - OmniPlex



Uda et al., 2007, Jpn J Infect Dis

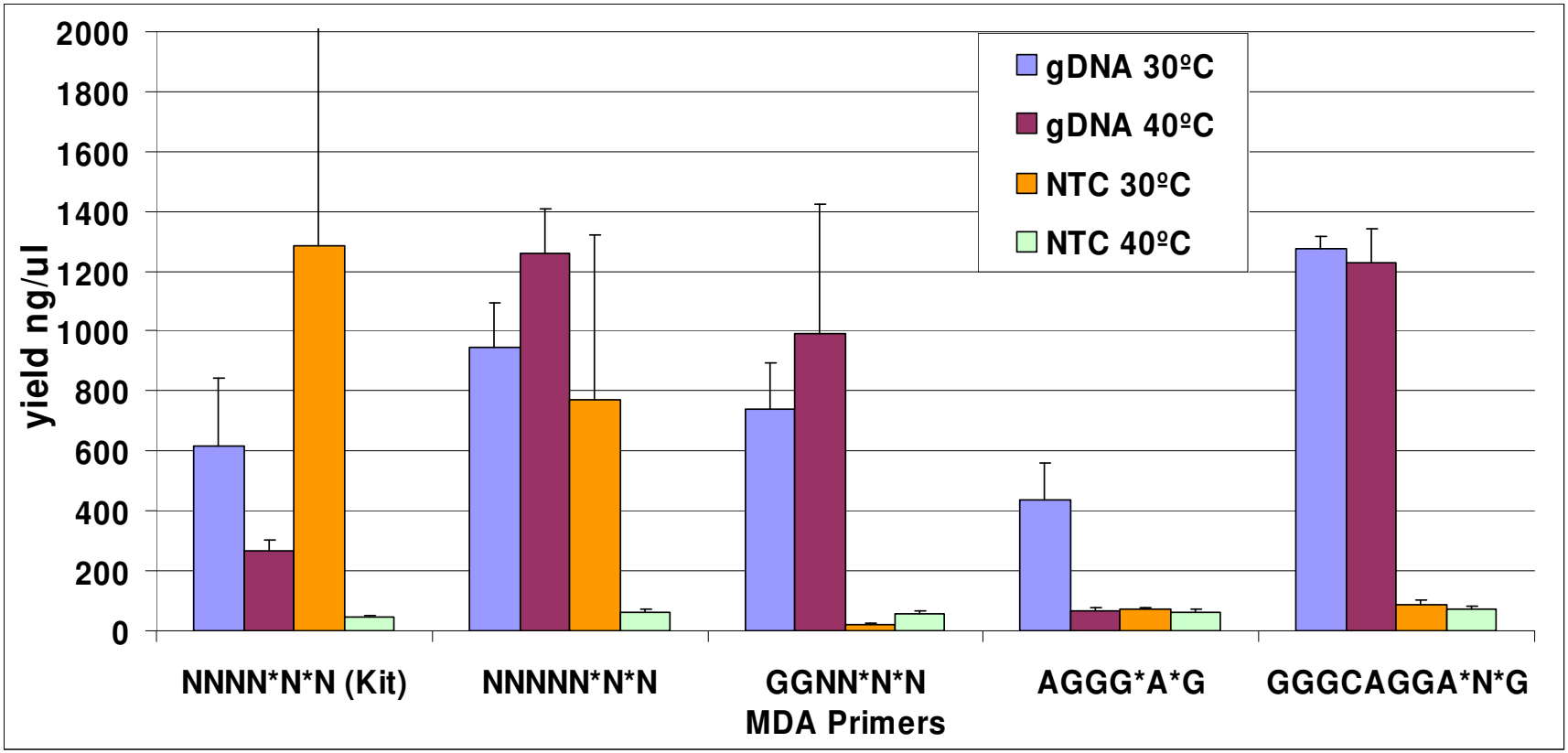
- DOP-PCR, OmniPlex and MDA have been successfully applied in PGD and arrays
- Among these WGA technologies
- MDA is more widely applied into PGD clinical application than other PCR-based WGA methods
    - Very simple to perform
    - Requiring only the addition of reagents (primers, polymerase, and nucleotides) in a single, isothermal step

- **MDA is still facing some challenges such as**
  - **Template independent DNA synthesis (TIDA)**
  - **Allele drop out (ADO)**
- **TIDA has been addressed by technical modifications on the MDA protocol**
  - **reducing the reaction volume resulted in suppression of TIDA (Kumar et al., 2008)**

MDA Modifications

- A random hexamer (NNNNNN) in kit constituted of a pool of 4096 (4^6) primers
- Four primers either non degenerate or partially to complete degenerate with their length ranging from 6 to 10 base pairs were used
  - NNNNNNN
  - GGNNNN
  - AGGGAG
  - GGGCAGGANG
- Primers were NNNNNN random hexamer primer utilized by the Repli-g kit was used as reference
- Temperature
  - 30°C
  - 40°C

MDA reactions in presence and absence of DNA template



Affymetrix SNP call analysis

Sample	% SNP Call
Genomic DNA	95.1
Kit 30	96.5
Kit 40	96.3
W30	96.9
W40	95.0

W30 and W40 -- MDA reactions using GGGCAGGANG primer at 30°C and 40°C

Single cell amplification efficiency

- NNNNNN 88%
- GGNNNN 72%
- AGGGAG <10%
- GGGCAGGANG <10%

MDA of single cell at 40°C had a very low amplification yield compared to 30°C

Conclusions

- Primer and temperature modifications resulted in increasing specific DNA yield and eliminating TIDA in gDNA
- They did not help to improve single cell MDA
- ADO still presents a challenge to PGD

Increasing number of cells

	1 blastomere removed	2 blastomeres removed	P value
# of embryos	229	399	
# of STRs	740	1334	
MDA failure	34 (15)	20 (5)	0.000
Allele dropout	91 (12.3)	101 (7.6)	0.000

Effects on Pregnancy and Implantation

	Transferred embryos generated from			
	1 blastomere removed	2 blastomeres removed	Mixture of 1 and 2	Overall
Cycles with embryo transfers	55	58	25	138
Implanted/transferred (%)	28/99 (28)	37/109 (34)	14/51 (31)	79/259
Total pregnant (%)	27 (50)	32 (55)	13 (52)	72

- **Removing two cells improves the efficiency of MDA**
- **ADO is still present**
- **ADO might be an inherent problem with single cell amplifications**

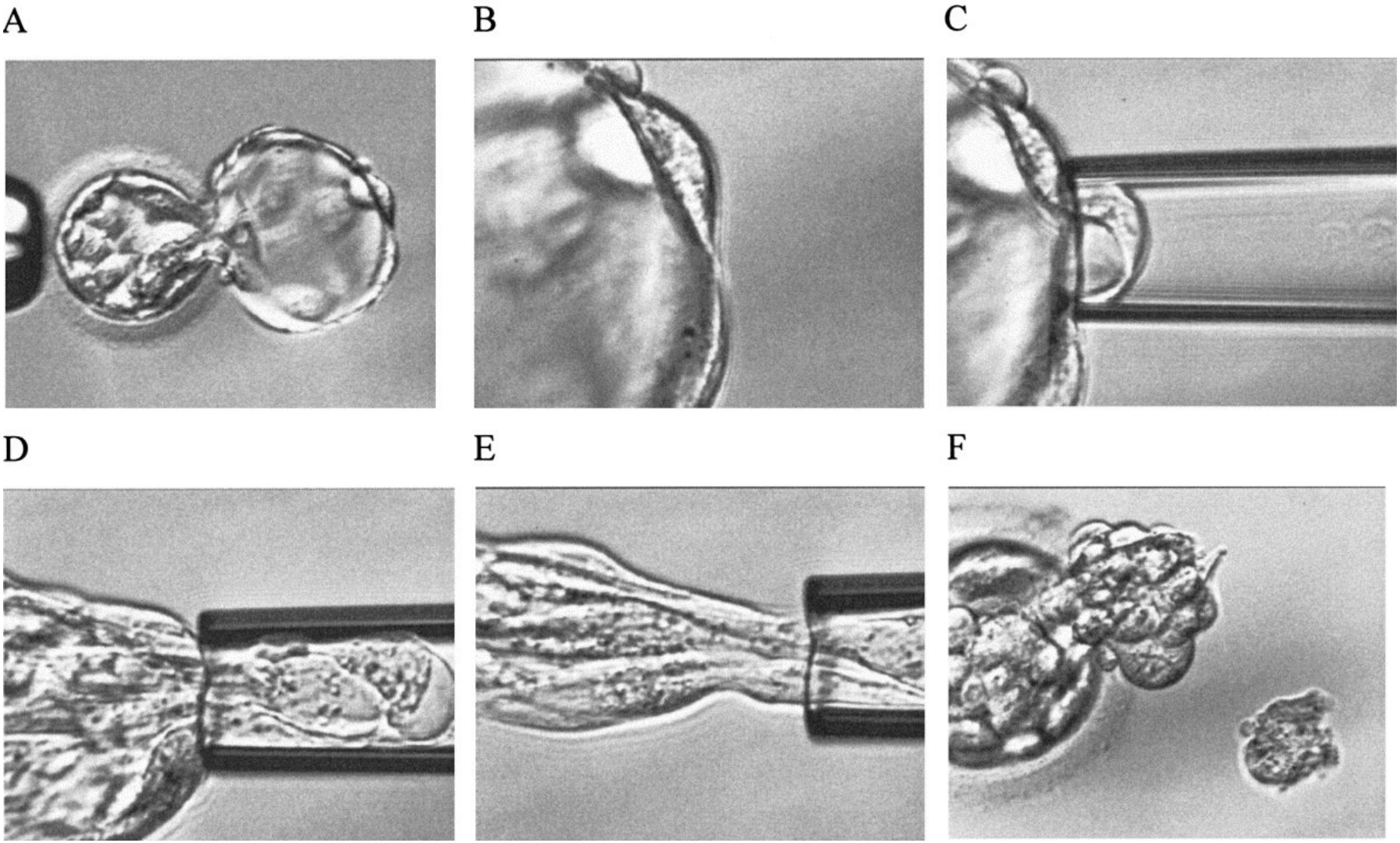
No. markers (N)	ADO (a) 0.12	0.28	0.35
2	0.899	0.607	0.474
3	0.968	0.754	0.618
4	0.990	0.846	0.723
5	0.997	0.903	0.799
6	0.999	0.939	0.854
7	1.000	0.962	0.894
8	1.000	0.976	0.923
9	1.000	0.985	0.944
10	1.000	0.991	0.960

$(N = 10,\ a = 0.35)$ for the detection of trisomy with 95% confidence.

Renwick et al., 2007, Prenatal Diagnosis

- **With the introduction of**
  - **Karyomapping**
  - **Parental support technology**
  - **DNA fingerprinting**

- **ADO could be overcame during the analysis**



McArthur et al., Fertil Steril 2005

Thank you