Pure & Appl. Chem., Vol. 63, No. 8, pp. 1089–1096, 1991.
Printed in Great Britain.

ADONIS 2069222091000965

Diagnosis of genetic diseases by DNA technology

Maurizio Ferrari, Laura Cremonesi, Paola Carrera and Pierangelo Bonini

Istituto Scientifico H.S.Raffaele, Lab. Centrale, Milano, Italy

Abstract - The development of recombinant DNA technology has allowed the study of the molecular pathology of inherited diseases in man. Two main molecular approaches are employed: direct study for detection of molecular defects and indirect detection by linkage analysis. A variety of new technologies and their applications have provided a powerful new tool in the diagnosis of inherited diseases.

INTRODUCTION

During the last twenty years the recombinant DNA technology has developed very powerful and sensitive techniques, which are listed in Table 1, useful in the study and for the identification of the molecular defects of human inherited diseases.

DNA technology has allowed an enormous increase of basic knowledge on inherited diseases and has had great effect on biodiagnostics; in general it has increased the knowledge on the human genome. The development of DNA based diagnostic tests for genetic disorders is still in progress. It will take time for experimental validation and for standardization of protocols. However their transferibility to the clinical field will depend on other factors, like the simplicity of procedures, the speed of execution and the cost of the analysis. Since 1973, 75 human genes have been mapped (ref. 1). Table 2 shows all the genes mapped in the 1st edition of "Human Gene Mapping". Every year, during the Human Gene Mapping conference, the DNA Committee has three primary responsibilities: a) summarizing information on cloned and mapped human genes, b) summarizing information on polymorphisms detected by using molecular techniques, and c) the development of a nomenclature appropriate for loci identified by anonymous DNA probes and assignment of symbols to those loci. Since 1983, these data have been collected also on Database to facilitate and to speed up the access (ref. 1). There are also others Database with the list of papers on cloned sequences both for genes and for anonimous sequences (ref. 2). The number and chromosome distribution of cloned sequences and polymorphisms are shown in Fig. 1 and Table 3 (10th edn of Human Gene Mapping).

The nature of the genetic lesion has now been established at the DNA level in many diseases: the majority , 58, are due to deletions, 52 are caused by point mutations and 34 by insertions, duplications and gene rearrangements (ref. 3). A variety of chromosomal alterations have also been analysed and well characterized (ref. 4).

The advent of molecular biology techniques has expanded our ability to diagnose inherited diseases. In fact , it is possible to carry out the analysis at the DNA level and it is not necessary to know the gene product, which gives three advantages: a) the analysis should be done for example on limphocytes and not only on the disease target cells, b) it allows carrier detection and, c) it allows the molecular defect characterization.

Two main molecular approaches are possible for genetic studies: a) indirect detection by linkage analysis, b) direct detection of the molecular alterations of genes.

TABLE 1. Recombinant DNA technology

- Restriction Endonucleases	- Vectors
- Cloning	- Oligonucleotides
- Libraries	- P C R (b)
- Electrophoresis	- Sequencing
- Blotting	- Probes
- Mapping	- DGGE (c)
- PFGE (a)	- SSCP (d)
- Chemical Cleavage	- RNAseA mismatch analysis

(a) Pulse Field Gel Electrophoresis
(b) Polymerase Chain Reaction
(c) Denaturing Gradient Gel Electrophoresis
(d) Single Strand Conformational Polymorphism

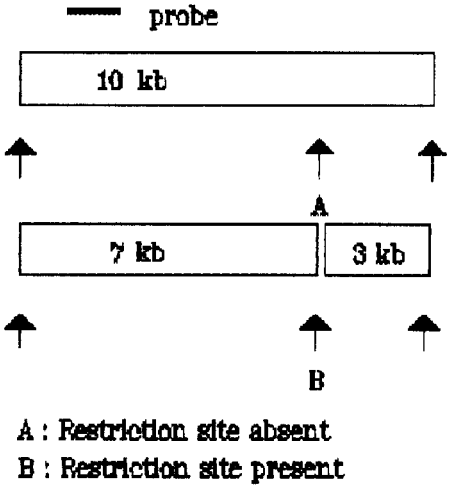


Fig. 2. RFLPs associated with base changes that alter restriction endonuclease sites. After the cleavage we will obtain DNA fragments of different molecular weight.

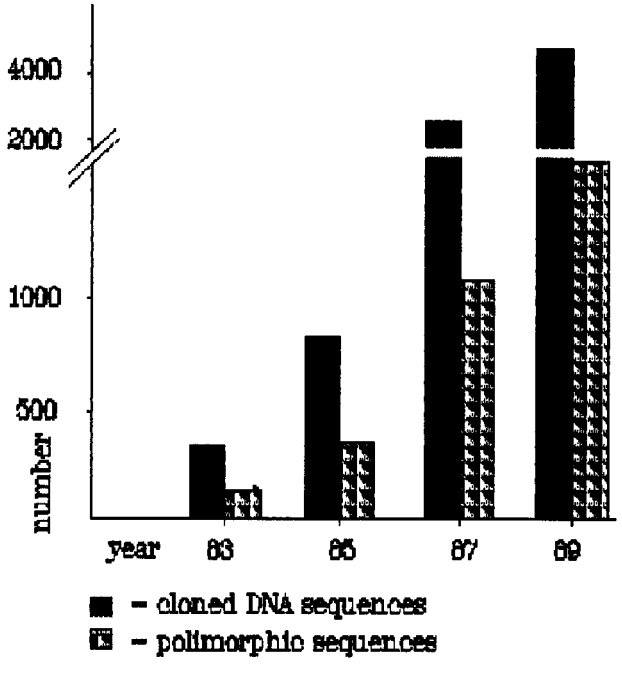


Fig. 1. Cumulative annual number of cloned sequences and polymorphisms

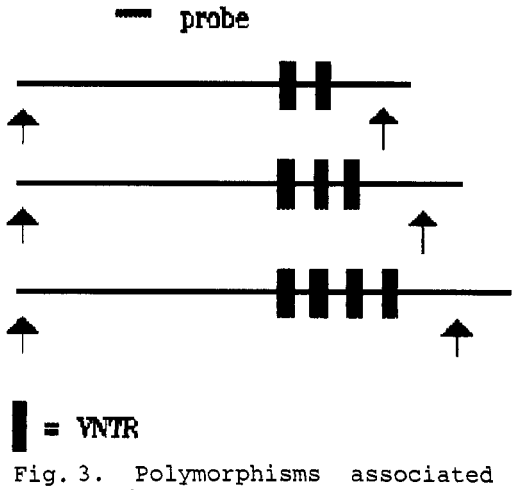


Fig. 3. Polymorphisms associated with variable tandem repeats.

INDIRECT DETECTION: POLYMORPHISMS AND LINKAGE ANALYSIS

The indirect analysis is very useful in the diagnosis of those diseases in which the affected structural gene is not known. The analysis can be done by Polymorphisms analysis.

DNA Polymorphisms: The human genome (3 billion bp) normally carries one variation in every 200-300 nucleotides localized in non-coding regions of DNA. Two or more allelic variations are known as Polymorphisms if their frequency in the general population is not less than 1%. Polymorphisms resulting after digestion of DNA with restriction enzymes are called Restriction Fragment Length Polymorphisms (RFLPs) (ref. 5). Another kind of polymorphism is due to a Variable Number of Tandem nucleic acid Repeat sequences (VNTRs) (ref.6), useful for example in the diagnosis of hemophilia A (ref. 7). In Fig. 2 and 3 the RFLP and VNTR mechanisms are respectively explained.

TABLE 2. Summary of Human Gene Map. New Haven HGM 1 - 1973

Chromosome	Mendelian Markers	In Vitro Markers
1	Iqh+, Cae, Fy, AOD Amy1, Amy2, EL1, Rh	PGM1, PGD, PPH, UGPP, FH, GuK, Pep-C,RN5S,AK-2
2	Acp1, MNSs	IDH-1, MDH-1, Gal1PT, If1, Hb
3		no assignment
4 or 5		Hb,ade+B,Es-Act
5		If2, Hex B
6		Me-1, IPO-B, PGM3, HLA
7		MPI,PKIII,HexA
8		no assignment
9		no assignment
10		GOT-1, HK
11		LDH-A,Es-A1,AL AcP2
12		LDH-B,Pep-B,TPI Gly+A, CS
13		RNr
14		RNr, NP
15		RNr
16	αHp	APRT
17		TK
18		Pep-A
19		GPI
20		ADA
21	Ag	RNr,IPO-A,AVP
22		RNr
X	mp,rp,rs,oa,Xg, ich,cbD,cbP,sp,md, mdc,HemA,HemB,Xm, MPS2	PGK,αGal,HPRT, G6PD,TATr

TABLE 3. Chromosome distribution of cloned human DNA (HGM 10)

Chrom	Genes			Anonimous DNA segmt		All cloned DNA	
	mapped	cloned	poly*	tot	poly*	tot	poly*
1	181	98	33	97	88	278	121
2	103	62	26	65	53	168	79
3	63	40	17	184	46	247	63
4	71	45	28	157	96	228	124
5	66	32	9	103	86	169	95
6	98	61	28	76	42	174	70
7	104	41	21	417	155	521	176
8	56	30	14	72	36	128	50
9	58	25	9	44	33	102	42
10	55	30	13	87	65	142	78
11	133	68	37	404	67	537	104
12	86	50	15	37	31	123	46
13	27	17	8	61	42	88	50
14	50	33	25	33	23	83	48
15	48	25	8	71	38	119	46
16	55	32	9	255	98	310	107
17	83	57	21	217	112	300	133
18	22	14	6	28	22	50	28
19	75	35	15	53	33	128	48
20	28	17	3	24	15	52	18
21	34	16	7	144	48	178	55
22	55	31	11	108	60	163	71
X	175	44	21	478	191	663	212
Y	14	8	2	192	9	216	11
XY				10	6		6

* polymorphism

A condition for the diagnostic use of RFLPs is a strong association of the polymorphism with the disease gene. The probability of recombinational events will depend on their distance. The genetic distance is expressed as recombination fraction and varies from 0 to 0.5 morgan (M). Also for close genes, recombination might occurr between the RFLPs and the structural gene; to prevent false negatives it is advisable to use more than one RFLP. The degree of linkage must be established by extensive studies on families.

RFLPs analysis allows to score the non random association between a disease allele and a polymorphic locus (linkage disequilibrium). To be informative, the family must show heterozigosity for the analysed polymorphism.

RFLPs analysis is usually carried out by Southern blotting technique (ref. 8): peripheral blood limphocytes DNA is extracted and digested by restriction endonucleases which cut the DNA at specific palindromic sequences. The resulting fragments are then separated by electrophoresis in an agarose gel, denatured and blotted onto nylon membranes. DNA fragments are then hybridized with isotopically labeled complementary probes.

In Fig.4 a prenatal diagnosis based on RFLP analysis is shown.

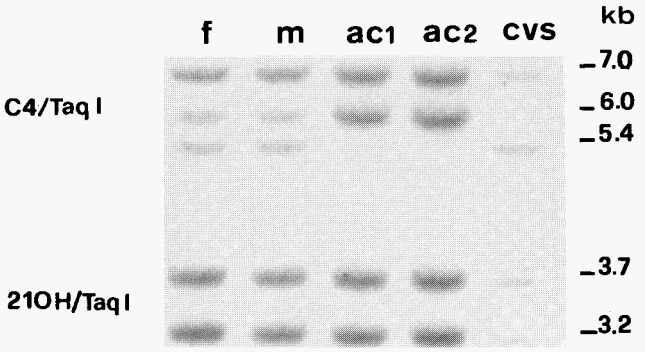


Fig. 4. Prenatal diagnosis in a family with 21-hydroxilase deficiency.
The probe/enzyme combination C4B550/TaqI evidentiates two polymorphic bands (6.0 and 5.4 kb) on the C4B gene, close to 21OHase-B gene (min 0.1% recombination) and a 7.0 kb constant band on the C4A gene. The probe/enzyme combination 21OH/TaqI recognizes the 21OHase-B active gene (3.7 kb) and the 21OHase-A pseudogene (3.2 kb).
In this family the RFLP analyzed is fully informative since both parents are heterozygotes for the polymorphism. The proband (CVS),inheriting both normal alleles from parents, results to be normal.
F.: father, M.: mother, AC1, AC2: affected children, CVS: chorionic villus sample.

Table 4 lists some of the most frequent inherited diseases for which this kind of analysis is carried out. Actually RFLPs analysis is used for the diagnosis of 74 diseases both autosomic and X-linked (ref. 3).

DIRECT DETECTION

The direct detection is possible when the affected gene is known and its structure is well defined. This approach is very sensitive and quick, in particular for those diseases in which the molecular defect shows little variability. In fact, the higher the heterogeneity of mutations, the more complex the design of diagnostic approach. The direct approach is feasible in the diagnostic field only after the definition of the different kinds of mutations in the various populations. Thus, when the complexity of genetic alterations is vast or when research is still at preliminary stages, the direct detection is used in combination with RFLPs analysis.

TABLE 4. Diagnosis of human inherited diseases by DNA technology.

	Direct	Indirect
Autosomal Dominant		
Adult Polycystic Kidney	-	+
Huntington Chorea	-	+
Osteogenesis Imperfecta (t IV)	-	+
Myotonic Dystrophy	-	+
Tuberous Sclerosis	-	+
Autosomal Recessive		
Thalassaemia beta and alfa	+	+
Phenylketonuria	+	+
Alpha-1-antitrypsin def.	+	+
21-hydroxylase deficic.	+	+
Cystic Fibrosis	+	+
Adenosine deaminase def.	+	+
Wilson disease	-	+
Retinoblastoma	+	+
Wilms	-	+
X-linked		
DMD/DMB	+	+
Haemophilia A and B	+	+
Ornithine transcarbamylase def.	+	+
Granulomatous disease chronic	+	-
Retinitis pigmentosa	-	+
Fragile X syndrome (Xq27-3)	-	+

TABLE 5. Diagnosis of human inherited diseases by PCR (1988-89) (ref.10)

Adenosine deaminase deficiency
ApoB deficiency
Becker and Duchenne Muscular Dystrophy
Cystic Fibrosis
Familial hypercholesterolemia
Glucose-6-phosphate dehydrogenase
Gaucher's disease
Haemophilia A
Haemophilia B
Hereditary persistence of fetal haemoglobin
Hypoxanthine phosphoribosyltransferase deficiency
Insulin resistance type A
Lesch-Nyhan syndrome
Leukocyte adhesion deficiency
Non insulin dependent diabetes mellitus
Osteogenesis imperfecta
Ornithine transcarbamylase deficiency
Phenylketonuria
Retinoblastoma
Sickle cell anaemia
Tay-Sachs disease
Thalassaemia beta
Thalassaemia delta
von Willebrand disease type IIA

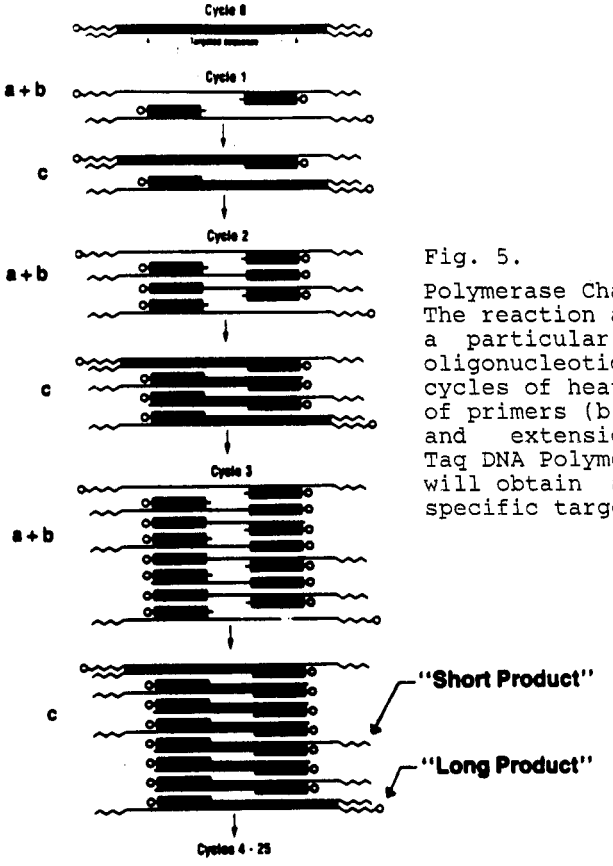


Fig. 5.

Polymerase Chain Reaction.
The reaction allows the specific amplification of a particular DNA segment flanked by two oligonucleotide primers. It involves repeated cycles of heat denaturation (a) of DNA, annealing of primers (b) to their complementary sequences and extension (c) of the annealed primers by Taq DNA Polymerase. At the end of the reaction we will obtain an exponential enrichment of the specific target fragment.

The rapid expansion of DNA technology and the development of new more sensitive techniques has remarkably increased the usefulness and potentiality of direct approach in prenatal diagnosis and carrier screening.

The most appropriate diagnostic methods will be chosen for each kind of disease even combining different techniques.

As we reported, at the DNA level there should be many different kinds of alterations: extensive deletions, insertions and rearrangements, can be scored on Southern blots as a band shift. On the other hand, if we consider smaller deletions/insertions or mutations, such as point mutations, the analysis by Southern blotting is not very sensitive because it is feasible only when the mutation alters a restriction enzyme site. In the last few years the introduction of the Polymerase Chain Reaction (PCR) technique (ref. 9) has advantaged the possibility to carry out genetic disease diagnoses that were impossible using conventional methods.

The PCR is a powerful method that allows to amplify over a millionfold specific DNA target single copy sequences. The reaction principles are shown in Fig. 5. The PCR has made possible to diagnose many inheritable diseases as is shown in Table 5.

When a polymorphism does not alter any restriction sequence or when we must screen for point mutations, the employment of Allele Specific Oligonucleotides (ASO) is useful. This tecnique, not very sensitive on single copy genes (ref. 11), is more efficient if utilized on amplified DNA spotted on dot blots (ref. 12). It allows to discriminate between different alleles by the use of synthetic oligonucleotides that must be hybridized under conditions that permit stability of only perfectly matched duplexes. The application of this technology is particularly feasible for the screening of populations and for carrier detection (example: beta-thalassemia , Cystic Fibrosis, alfa-1-Antitrypsin, Phenylketonuria). The use of PCR combined with ASO is also realible by non isotopic means of detection (ref. 13) with a potential widespread use in diagnostic laboratories.

PCR is also employed for detection of large deletions as for example on the dystrophin gene. By two sets of simultaneous amplifications of coding regions at the human dystrophin gene it is possible to detect about 98% deletions in Duchenne and Becker Muscular Dystrophy patients (ref. 14, 15). This strategy is very appropriate in these patients because of the large percentage of cases (about 60%) due to the presence of heterogeneous intragenic deletions.

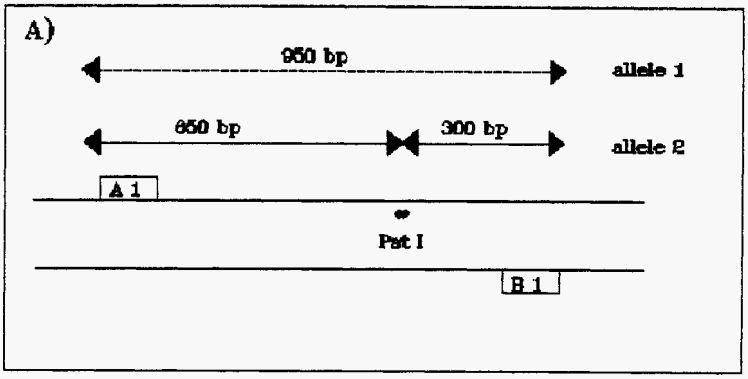
In those cases in which the mutation is unknown, RFLP analysis coupled to PCR can be used. By cutting specific amplified DNA with a restriction enzyme it is possible to directly analyse the RFLP on a agarose gel, without the use of radioisotopes. This strategy takes less than one day in comparison with longer Southern blotting and hybridization method. In Figure 6 is shown an amplification for detection of KM-19 polymorpfism for prenatal diagnosis in a family with Cystic Fibrosis.

NEW TECHNOLOGIES

We will briefly consider the most recent and significant technologies and we will outline the more advantageous aspects. In general these new technologies have the advantage of enhancing the resolution and the diagnostic potentiality and to increase the basic knowledge on research and characterization of new mutations and polymorphisms.

DNA sequencing

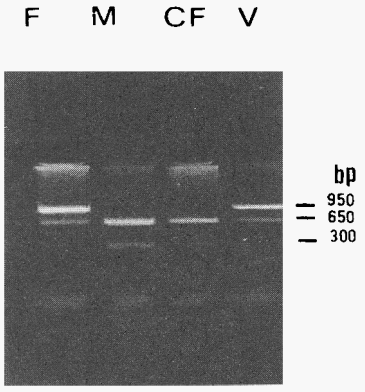
The two most used DNA sequencing methods are the chemical Maxam-Gilbert method (ref.16) and Sanger's enzymatic chain-terminating method (ref. 17). Such methods are subjected to cloning, mantainance and use of systems dependent on vectors and living host cells. More recently, the possibility to directly sequence PCR products (ref. 18), both from genomics or DNA-RNA templates, has greatly simplified sequencing because it does not need libraries construction, screening and subcloning. This method also facilitates automation for large-scale sequencing (ref. 19).



A) Amplification scheme, A1 and B1: primers flanking the Pst I polymorphic site.

B) Agarose gel analysis of amplified fragments after Pst I digestion.

B)



F: father, heterozygotes, alleles 1/2

M: mother, homozygotes, alleles 2/2

AC: affected child: homozygotes, alleles 2/2

V: proband: heterozygotes, alleles 1/2

Fig. 6. RFLPs analysis on PCR amplified DNA.

Chemical cleavage

This procedure allows detection, definition and localization of single base pair mutations. Labeled heteroduplex DNA molecules containing mismatched base pairs, are incubated with osmium tetroxide or hydroxylamine and then treated with piperidine to cleave the DNA at the modified mismatched base. DNA cleavage is detected by denaturing gel elecrophoresis (ref. 20).

Denaturing gradient gel electrophoresis (DGGE)

DGGE is very useful for the initial screening and detection of new polymorphisms or mutations, before precisely defining the nature of nucleotidic variation. This tecnique allows the resolution of DNA molecules differing by single base changes. Using a polyacrilamide gel containing a linear gradient of DNA denaturants (urea/formamide) (ref.21) it is possible to resolve mutant from normal homoduplexes because of the different melting properties of the DNA molecules. These different melting behaviors can be increased by adding a GC-rich clamp ending a primer (ref. 22, 23).

Single strand conformation polymorphisms (SSCP)

This technique, described by Orita et al in 1989, (ref. 24, 25) is a method to detect base changes on amplified or cloned DNA sequences. Specific DNA regions labeled and denatured are analysed in non-denaturing polyacrilamide gels. The electrophoretic mobility of single-stranded molecules will depend on their size and also on their sequence allowing detection of allelic variants or mutations.

RNAseA mismatch analysis

This strategy is based on the capability of the enzyme to digest only not hybridized RNA molecules. Thus it is possible to identify the site of a lesion in a gene by hybridizing a normal RNA molecule to the RNA or DNA to be analyzed. The mismatch region is identified by gel elecrophoresis after the RNAseA cleavage (ref. 26).

Pulse field gel electrophoresis (PFGE)

PFGE is a technique for resolving megabase restriction fragment length variations. By alternating the electric field, DNAs are able to reorient and differentially move through the gel (ref. 27). Conventional elecrophoresis does not resolve DNA fragments larger than 50 kb.

This technique has many applications: a) detection of polymorphisms (ref. 28), b) analysis of large rearrangments due to recombinational events as unequal crossovers (ref. 29), c) molecular mapping and construction of long-range genomic restriction maps (ref. 30).

CONCLUSIONS

DNA technology has led possible the diagnosis of many genetic diseases. It will play an increasing role in research and diagnosis of a growing number of inherited diseases, that will become amenable. The increase of knowledge and the technical development will be exploited and quickly transferred to the clinical laboratories. However, the widespread diffusion and the transferibility of these technologies will need to develop protocols and to take care of possible ethical problems.

REFERENCES

1. K.K. Kidd, A.M. Bowcock, J. Schmidtke, R.K. Track, F. Ricciuti, G. Hutchings, A. Bale, P. Pearson and H.F. Willard, Cytogenet. Cell. Genet., 51, 622-947 (1989).
2. J. Schmidtke and D.N. Cooper, Nucleic Acid Research, 17, r173-281 (1989).
3. D.N. Cooper, J. Schmidtke, Hum. Genet., 83, 307-334, 1989.
4. V.A. Mc Kusic, Mendelian Inheritance in man - 10th edn, J. Hopkins Univ. Press, Baltimore, (1990).
5. D. Botstein, R.L. White, M. Skolnick and R. Davis, Am. J. Hum. Genet., 32, 314-331 (1980).
6. A.J. Jeffreys, V. Wilson and S.L. Thein, Nature, 314, 67-73, (1985).
7. I. Oberle, G. Camerino, R.Heilig, L. Grunebaum, J.P. Cazenave, C. Crapanzano, P.M. Mannucci and J.L. Mandel, N. Engl. J. Med., 312, 682-686 (1985).
8. E. Southern, J. Mol. Biol., 98, 503 (1975).
9. R. K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich and N. Arnheim, Science, 230, 1350-1354 (1985).
10. J. Reiss and D.N. Cooper, Hum. Genet., 85, 1-8, 1990.
11. B.J. Conner, A.A. Reyes, C. Morin, K. Itakura, R.L. Teplitz and R.B. Wallace, Proc. Natl. Acad. Sci., USA 80, 278-282 (1983).
12. R.K. Saiki, T.L. Bugawan, G.T. Horn, K.B. Mullis and H.A. Erlich, Nature, 324, 163-166 (1986).
13. R.K. Saiki, C.A. Chang, C.H. Levenson, T.C. Warren, C.D. Boehm, H.H. Kazazian and H.A. Erlich, N. Engl. J. Med., 319, 537-541 (1988).
14. J.S. Chamberlain, R.A. Gibbs, J.E. Ranier, P.N. Nguyen and C.T. Caskey, Nucleic Acid Research, 16, 11141-11156 (1988).
15. A.H. Beggs and L.M. Kunkel, J. Clin. Inv., 85, 613-619 (1990).
16. A.M. Maxam, W. Gilbert, Proc. Natl. Acad. Sci., USA 74, 560-564, 1977.
17. F. Sanger, S. Nicklen and A. R. Coulson, Proc. Natl. Acad. Sci., USA 74, 5463-5467 (1977).
18. M.A.Innis, K.B. Myambo, D.H. Gelfand and M.A.D. Brow, Proc. Natl. Acad. Sci., USA 85, 9436-40, 1988.
19. Landegren, U.R. Kaiser, C.T. Caskey and L. Hood, Science, 242, 229-237, 1988.
20. R.G.H. Cotton, N.R. Rodriguez and R.D. Campbell, Proc. Natl. Acad. Sci., USA 85, 4397-4401 (1988).
21. S.G. Fischer and S. Lerman, Proc. Natl. Acad. Sci., USA 80, 1579-1583 (1983).
22. R.M. Myers, N. Lumelsky, L.S. Lerman and T. Maniatis, Nature, 313, 495-498 (1985).
23. R.M. Myers, S.G. Fischer, T. Maniatis and L.S. Lerman, Nucleic Acid Research, 13, 3111-3129 (1985).
24. M. Orita, H. Iwahana, H. Kanazawa, K. Hayashi and T. Sekiya,Proc. Natl. Acad. Sci., USA 86, 2766-2770 (1989).
25. M. Orita, Y Suzuki, T. Sekiya and K. Hayashi, Genomics, 5, 874-879 (1989)
26. J.T. Stout and C.T. Caskey, Trends Genet., 4, 175-178,1988.
27. D.C. Schwartz and C.R.Cantor, Cell, 37, 67-75, 1984.
28. S.K. Lawrance and C.L. Smith, Genomics, 8, 394-399, 1990.
29. S. Collier, P.J. Sinnot, P.A. Dyer, D.A. Price, R. Harris and T. Strachan, The Embo J., 8, 1393-1402, 1989.
30. I. Dunham, C.A. Sargent, J. Trowsdale and R.D. Campbell, Proc. Natl.Acad, Sci., USA 84, 7237- 7241, 1987.