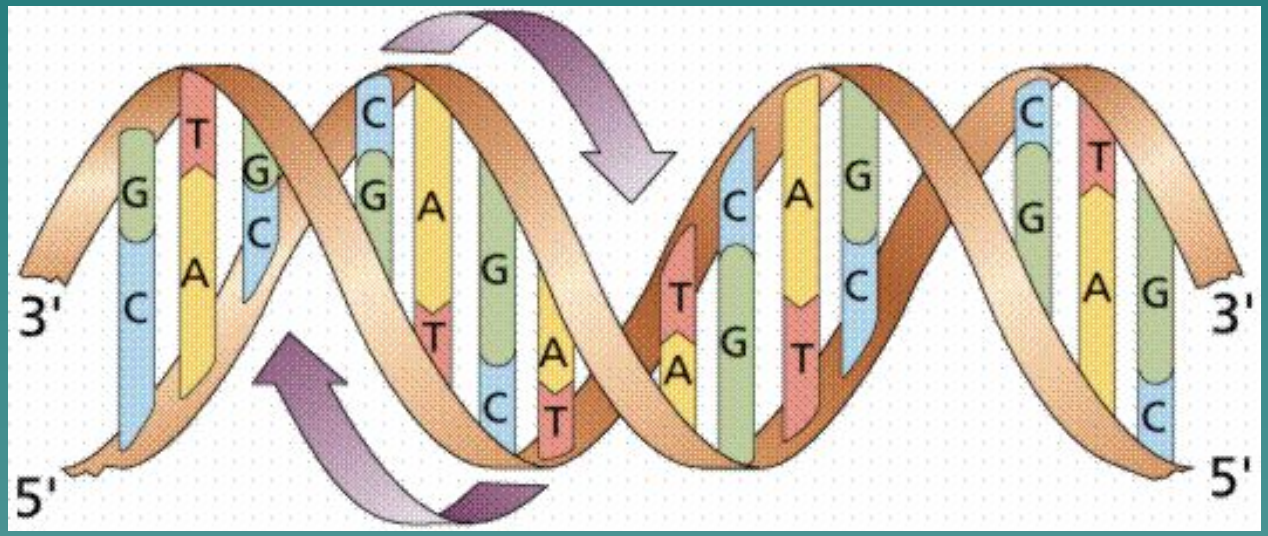
Полимеразная цепная реакция
Polymerase Chain Reaction



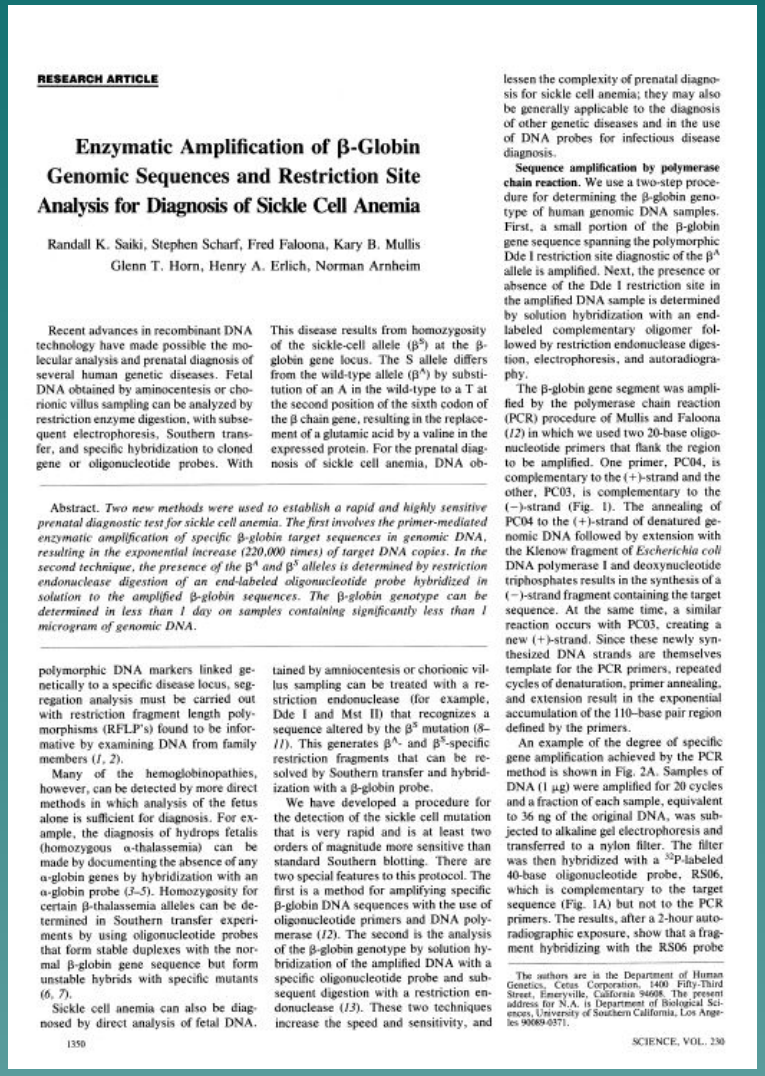
История метода

- В 1971 г. норвежский учёный Хьелль Клеппе предложил способ амплификации ДНК с помощью пары коротких одноцепочечных молекул ДНК - синтетических праймеров.

ПЦР была изобретена в 1983 году американским биохимиком Кери Муллисом.

История метода

Science. 1985. V. 230. P. 1350-1354



RESEARCH ARTICLE

Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia

Randall K. Saiki, Stephen Scharf, Fred Faloona, Kary B. Mullis
Glenn T. Horn, Henry A. Erlich, Norman Arnheim

Recent advances in recombinant DNA technology have made possible the molecular analysis and prenatal diagnosis of several human genetic diseases. Fetal DNA obtained by aminocentesis or chorionic villus sampling can be analyzed by restriction enzyme digestion, with subsequent electrophoresis, Southern transfer, and specific hybridization to cloned gene or oligonucleotide probes. With

Abstract. *Two new methods were used to establish a rapid and highly sensitive prenatal diagnostic test for sickle cell anemia. The first involves the primer-mediated enzymatic amplification of specific β-globin target sequences in genomic DNA, resulting in the exponential increase (220,000 times) of target DNA copies. In the second technique, the presence of the β^A and β^S alleles is determined by restriction endonuclease digestion of an end-labeled oligonucleotide probe hybridized in solution to the amplified β-globin sequences. The β-globin genotype can be determined in less than 1 day on samples containing significantly less than 1 microgram of genomic DNA.*

polymorphic DNA markers linked genetically to a specific disease locus, segregation analysis must be carried out with restriction fragment length polymorphisms (RFLP's) found to be informative by examining DNA from family members (*1*, *2*).

Many of the hemoglobinopathies, however, can be detected by more direct methods in which analysis of the fetus alone is sufficient for diagnosis. For example, the diagnosis of hydrops fetalis (homozygous α-thalassemia) can be made by documenting the absence of any α-globin genes by hybridization with an α-globin probe (*3*–*5*). Homozygosity for certain β-thalassemia alleles can be determined in Southern transfer experiments by using oligonucleotide probes that form stable duplexes with the normal β-globin gene sequence but form unstable hybrids with specific mutants (*6*, *7*).

Sickle cell anemia can also be diagnosed by direct analysis of fetal DNA. This disease results from homozygosity of the sickle-cell allele (β^S) at the β-globin gene locus. The S allele differs from the wild-type allele (β^A) by substitution of an A in the wild-type to a T at the second position of the sixth codon of the β chain gene, resulting in the replacement of a glutamic acid by a valine in the expressed protein. For the prenatal diagnosis of sickle cell anemia, DNA obtained by amniocentesis or chorionic villus sampling can be treated with a restriction endonuclease (for example, Dde I and Mst II) that recognizes a sequence altered by the β^S mutation (*8*–*11*). This generates β^A- and β^S-specific restriction fragments that can be resolved by Southern transfer and hybridization with a β-globin probe.

We have developed a procedure for the detection of the sickle cell mutation that is very rapid and is at least two orders of magnitude more sensitive than standard Southern blotting. There are two special features to this protocol. The first is a method for amplifying specific β-globin DNA sequences with the use of oligonucleotide primers and DNA polymerase (*12*). The second is the analysis of the β-globin genotype by solution hybridization of the amplified DNA with a specific oligonucleotide probe and subsequent digestion with a restriction endonuclease (*13*). These two techniques increase the speed and sensitivity, and lessen the complexity of prenatal diagnosis for sickle cell anemia; they may also be generally applicable to the diagnosis of other genetic diseases and in the use of DNA probes for infectious disease diagnosis.

Sequence amplification by polymerase chain reaction. We use a two-step procedure for determining the β-globin genotype of human genomic DNA samples. First, a small portion of the β-globin gene sequence spanning the polymorphic Dde I restriction site diagnostic of the β^A allele is amplified. Next, the presence or absence of the Dde I restriction site in the amplified DNA sample is determined by solution hybridization with an end-labeled complementary oligomer followed by restriction endonuclease digestion, electrophoresis, and autoradiography.

The β-globin gene segment was amplified by the polymerase chain reaction (PCR) procedure of Mullis and Faloona (*12*) in which we used two 20-base oligonucleotide primers that flank the region to be amplified. One primer, PC04, is complementary to the (+)-strand and the other, PC03, is complementary to the (−)-strand (Fig. 1). The annealing of PC04 to the (+)-strand of denatured genomic DNA followed by extension with the Klenow fragment of *Escherichia coli* DNA polymerase I and deoxynucleotide triphosphates results in the synthesis of a (−)-strand fragment containing the target sequence. At the same time, a similar reaction occurs with PC03, creating a new (+)-strand. Since these newly synthesized DNA strands are themselves template for the PCR primers, repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the 110–base pair region defined by the primers.

An example of the degree of specific gene amplification achieved by the PCR method is shown in Fig. 2A. Samples of DNA (1 μg) were amplified for 20 cycles and a fraction of each sample, equivalent to 36 ng of the original DNA, was subjected to alkaline gel electrophoresis and transferred to a nylon filter. The filter was then hybridized with a ^{32}P-labeled 40-base oligonucleotide probe, RS06, which is complementary to the target sequence (Fig. 1A) but not to the PCR primers. The results, after a 2-hour autoradiographic exposure, show that a fragment hybridizing with the RS06 probe

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Нобелевская премия 1993

Использование ПЦР

Год	Число публикаций
1985	1
1986	3
1987	8
1988	139
1989	698
1990	2 668
1995	11 890
2000	16 430
2004	20 075

Применение ПЦР

- Клонирование генов
- Генотипирование организмов
- Диагностика наследственных, инфекционных и онкологических заболеваний
- Идентификация личности и установление родства; криминалистика
- Анализ древних останков, эволюционная биология
- Детекция ГМО

Основные этапы ПЦР

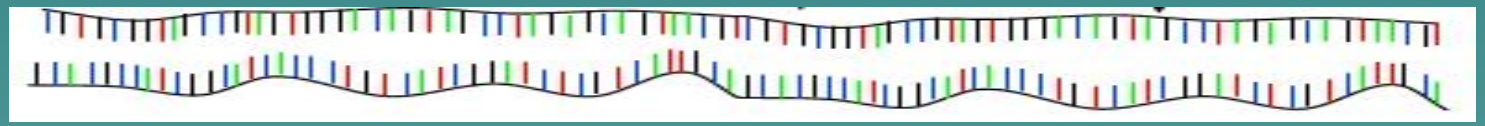
- ДЕНАТУРАЦИЯ
- ОТЖИГ ПРАЙМЕРА
- ЭЛОНГАЦИЯ

1) Денатурация ДНК

95°C

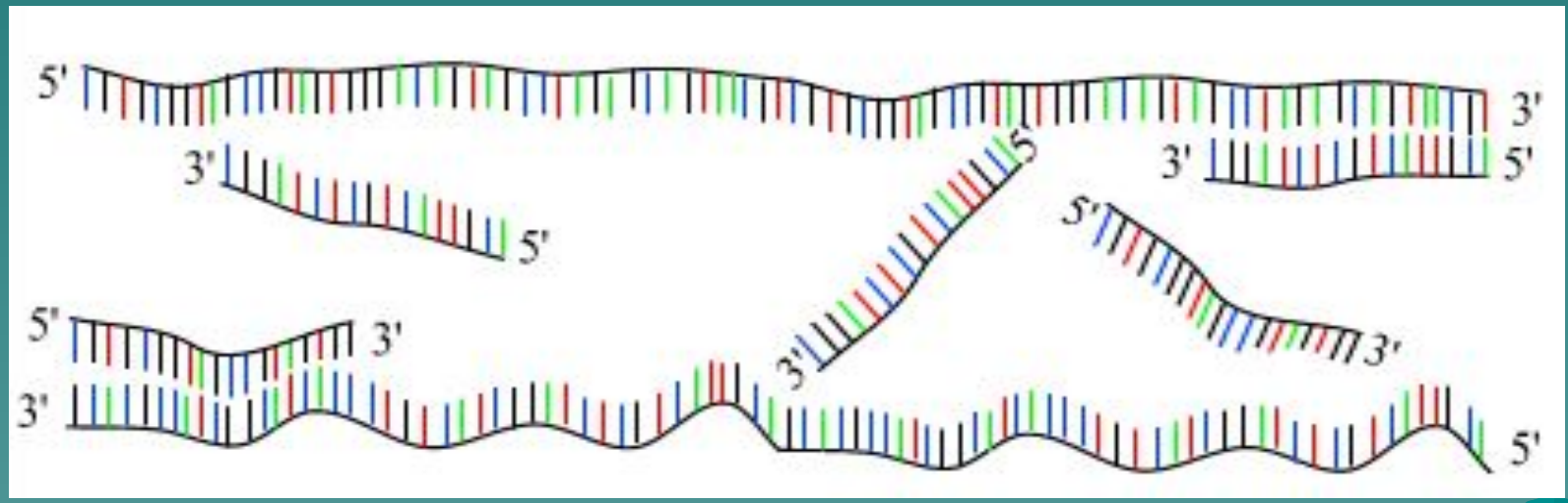
Нагревание

Охлаждение



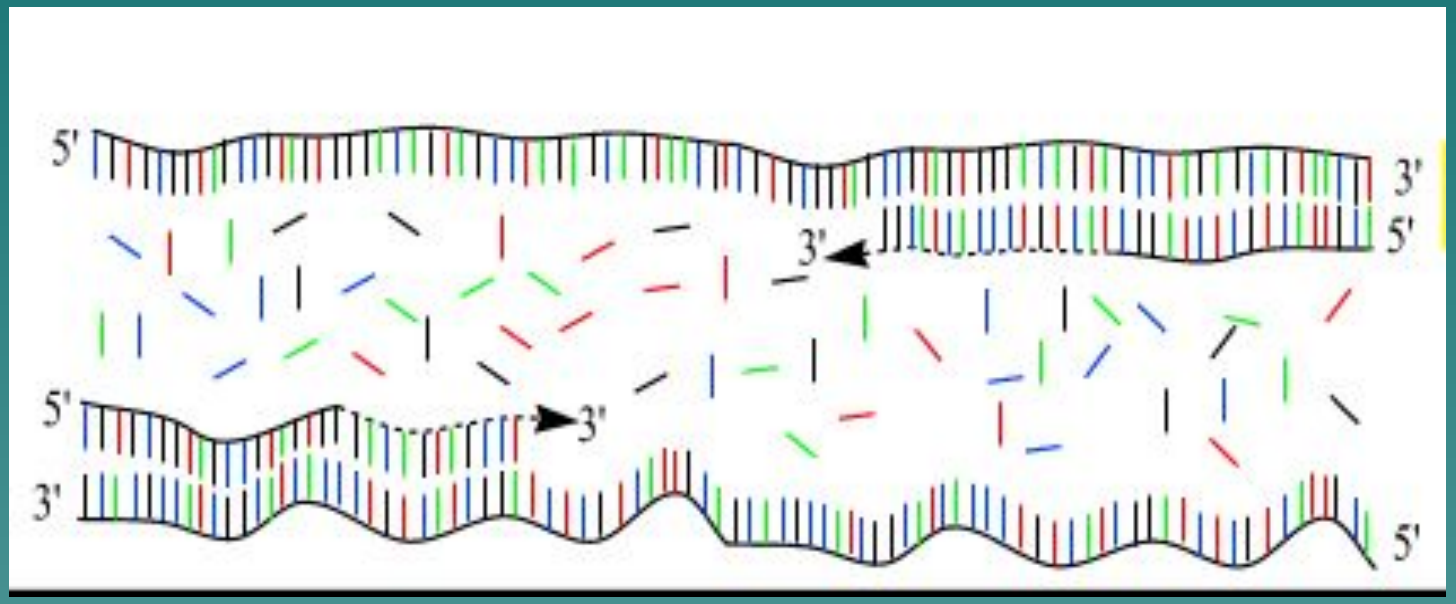
2) Отжиг (гибридизация) праймеров

Температура отжига зависит от длины и состава праймеров (50 – 70°С)



3) Элонгация

72°C



Дезоксирибонуклеозидтрифосфоты
(dATP, dGTP, dCTP, dTTP)

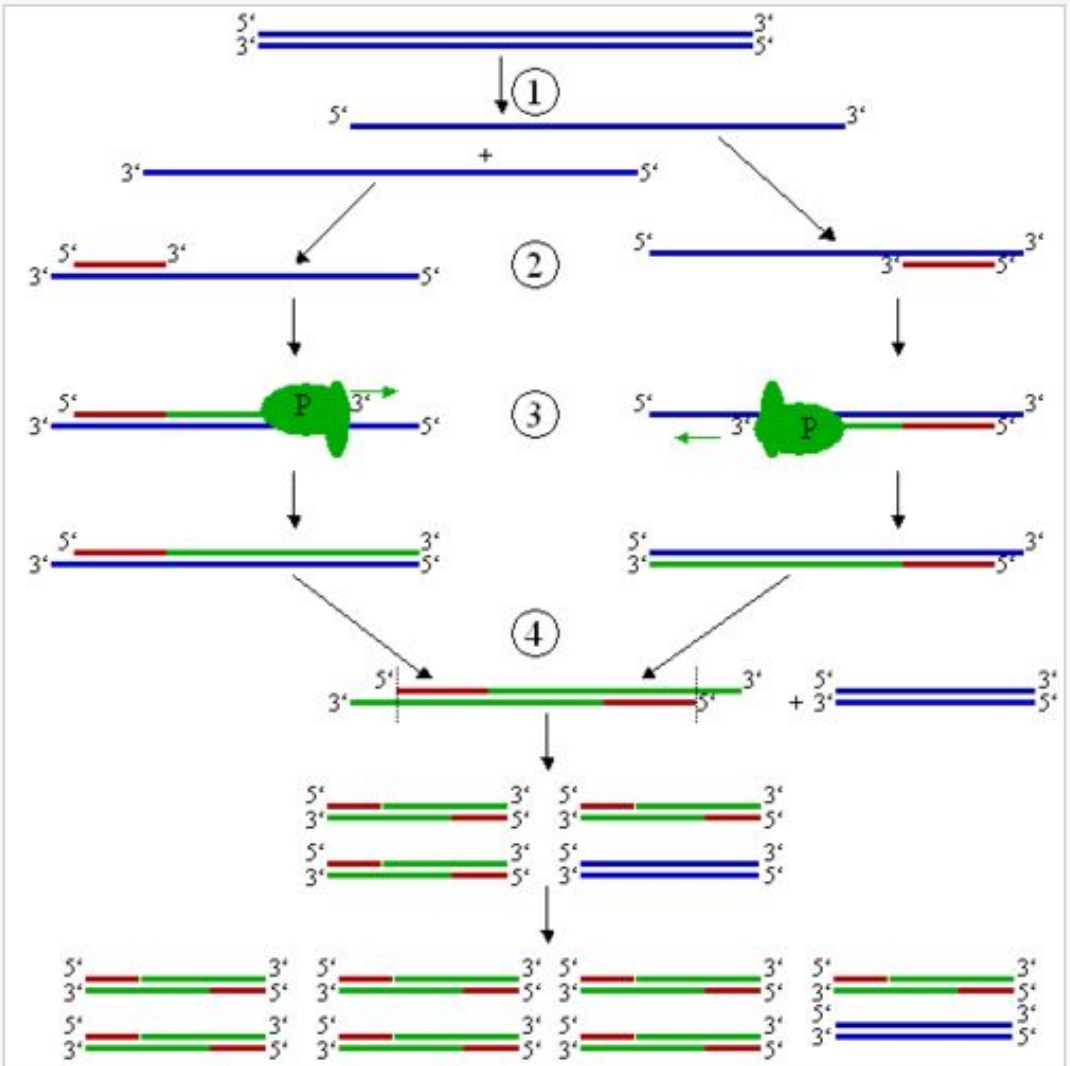
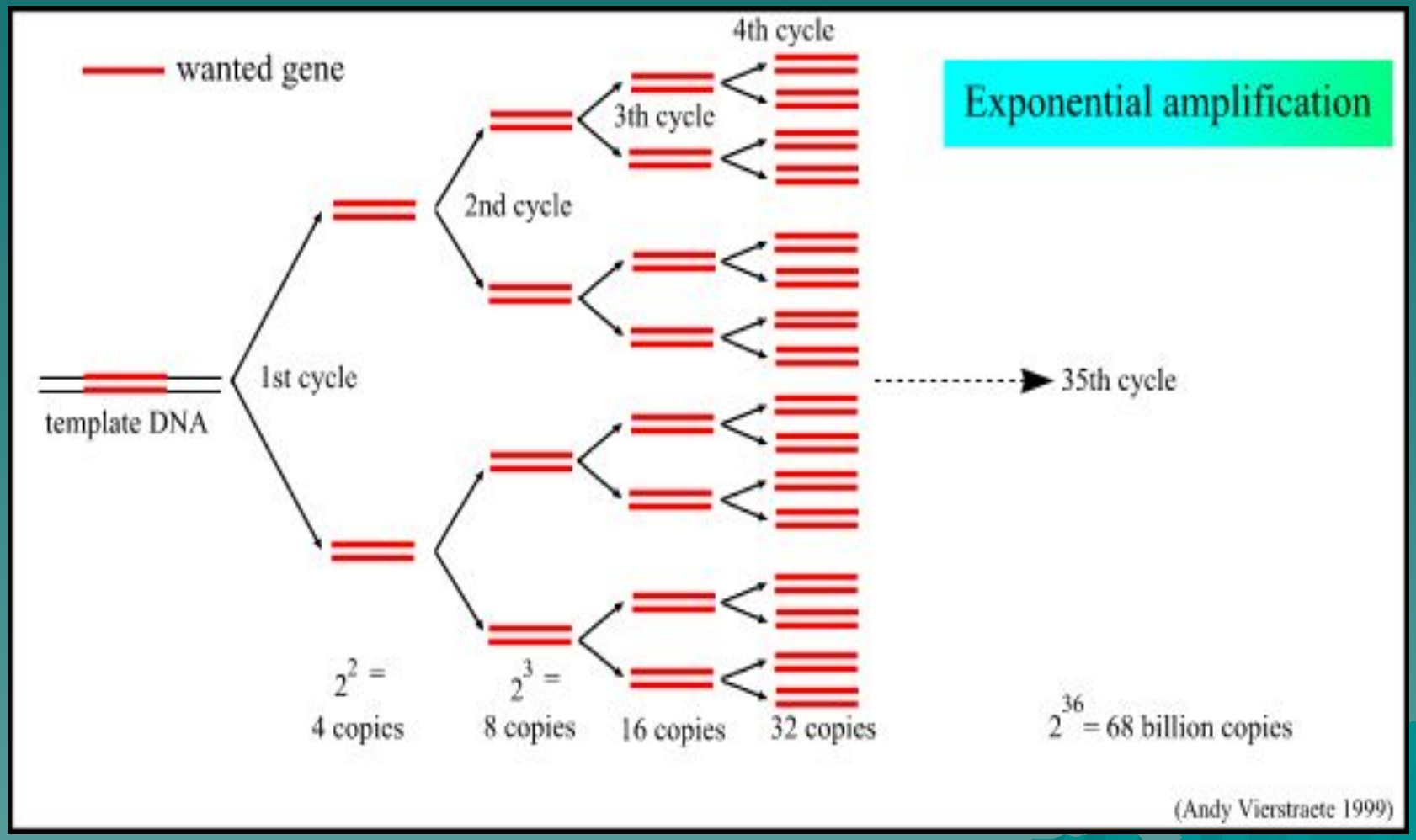


Рис. 2: Схематическое изображение первого цикла ПЦР. (1) Денатурация при 94—96 °C. (2) Отжиг при 68 °C (например). (3) Элонгация при 72 °C (P=полимераза). (4) Закончен первый цикл. Две получившиеся ДНК-цепи служат матрицей для следующего цикла, поэтому количество матричной ДНК в ходе каждого цикла удваивается

Увеличение ампликона происходит в геометрической прогрессии



ДНК-полимеразы, используемые при проведении ПЦР

Termus aquaticus – бактерия, живущая в горячих источниках Йеллоустонского национального парка США при температуре, близкой 85 °C

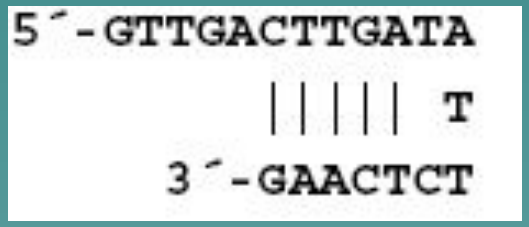
ДНК-полимеразы, используемые при проведении ПЦР

ДНК-полимераза	Время активности при 95°С (мин)	5'-3' экзонуклеазная активность (+/-)	3'-5' экзонуклеазная активность (+/-)
Taq	**40**	+	-
Tth	**20**	+	-
Pfu	**120**	-	+
Vent	**400**	-	+
Deep Vent	**1300**	-	+
UlTma	**50**	-	+
Pwo	**120**	-	+

Подбор праймеров

- Длина праймеров – 18-30 нуклеотидов
- Содержание GC должно составлять 45-55%
- Разница в температурах отжига между прямым и обратным праймером не более $5^{o}C$
- Не должны формировать димеры на 3-концах

- Не должны формировать шпилек на 3-конце



Основные компоненты для проведения ПЦР

- ДНК-матрица
- ДНК-полимераза
- Буфер:
  - 10 mM Tris-HCl, pH = 8,0-8,8;
  - 50 mM KCl;
  - 1,5-2.5 mM $MgCl_2$
- Праймеры
- dNTP

Дополнительные компоненты для проведения ПЦР

- ДМСО (5%) – улучшает амплификацию GC-богатых матриц и отжиг праймеров
- Формамид (1-5%) – улучшение специфичности реакции
- Глицерин (1-10%), БСА (0,01-0,1%), Triton X100 (0.05-0.1) – стабилизаторы фермента
- $(NH_4)_2SO_4$ (1-10%) – улучшение отжига праймеров

Ингибиторы ПЦР

Ингибитор	Концентрация ингибитора
SDS	>0,005%
фенол	>0,2%
этанол	>1%
изопропанол	>1%
ацетат натрия	>5 мМ
хлористый натрий	>25 мМ
EDTA	>0,05 мМ
гемоглобин	>1 мг/мл
мочевина	>20мМ

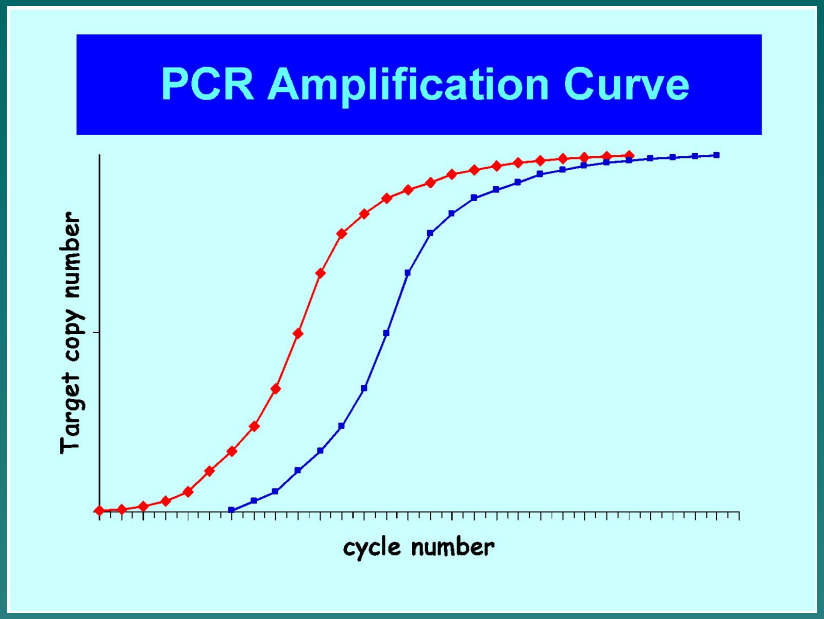
Программа для проведения ПЦР

95˚С- 2 мин (горячий старт - денатурация)

2) 94˚С -30 сек (денатурация)
3) 62˚С -45 сек (отжиг)
4) 72˚С -40 сек (элонгация)

25-40циклов

72˚С -3-5 мин (конечная элонгация)



Эффект «выхода на плато»

- Истощение субстратов (dNTP и праймеров)
- Падение активности dNTP и фермента
- Накопление ингибиторов, например, пирофосфатов и ДНК-дуплексов
- Конкуренция за реагентов неспецифическими продуктами или праймер-димерами
- Концентрация специфического продукта и неполная денатурация при высокой концентрации продуктов амплификации.