Thema Gentechnologie

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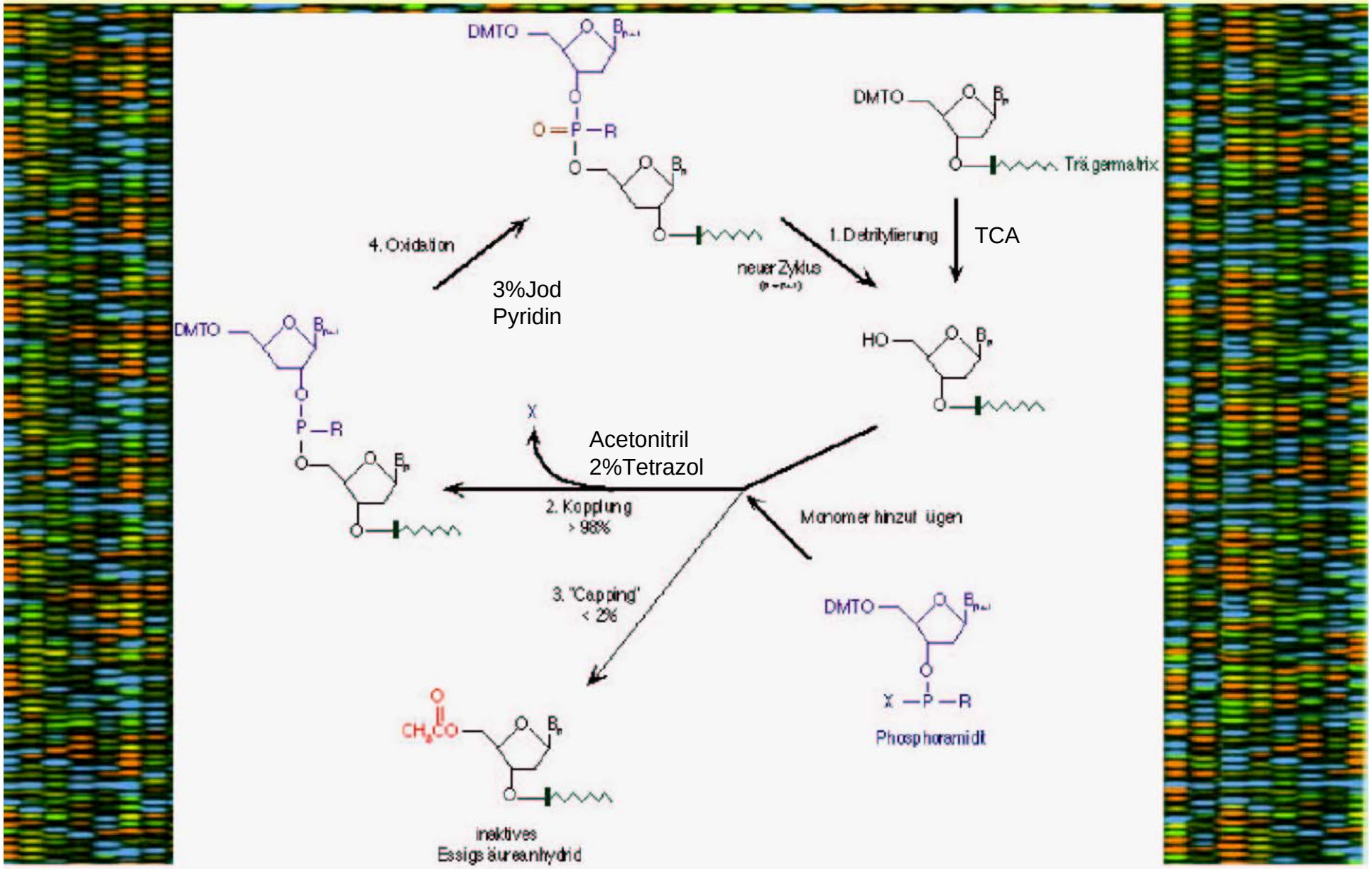
Institut für Molekulargenetik

10. VL

23. 06. 2009

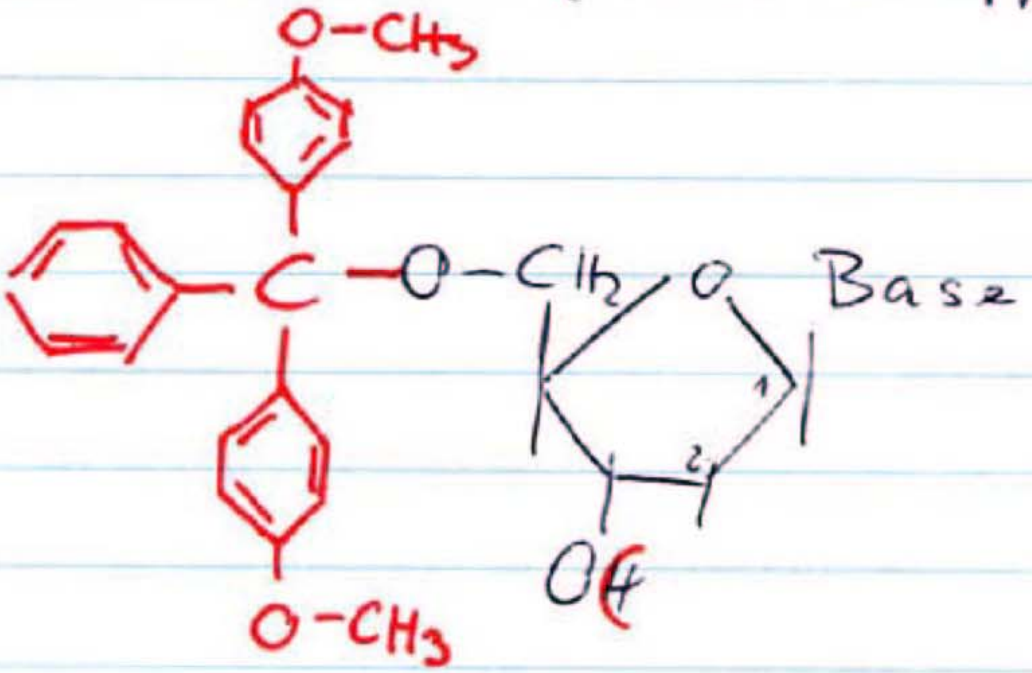
Künstliche Gene durch chemische DNA-Synthese



Künstliche Gene durch chemische DNA-Synthese

DMT-Zucker

geschützter Zucker (5'OH-Gruppe)

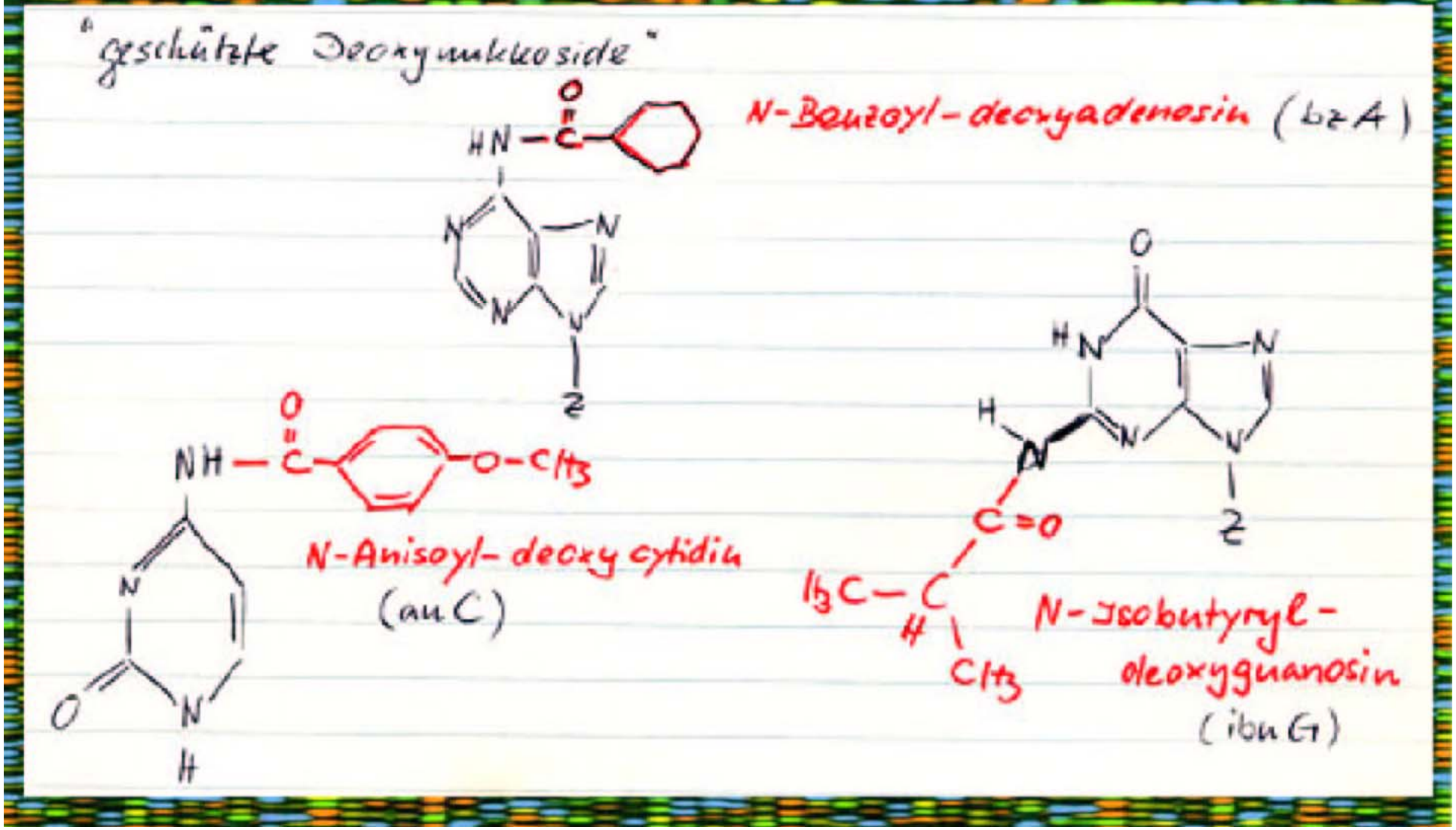


Abspaltung → mit Säure z.B. TCA

5'O-Dimethoxytrityl-deoxyribonucleosid

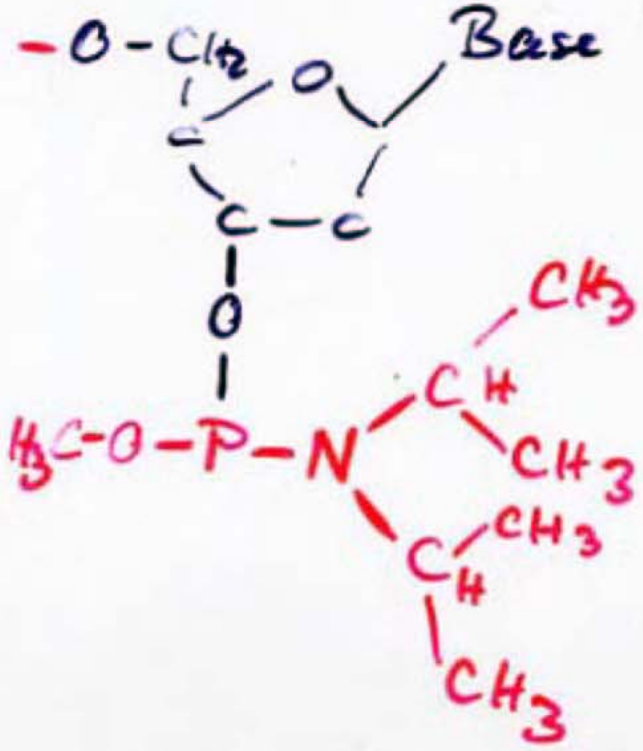
Künstliche Gene durch chemische DNA-Synthese

„geschützte“ Nukleotide



Künstliche Gene durch chemische DNA-Synthese
Phosphoamidit

3'OH-Gruppe: nicht geschützt, sondern reaktiv



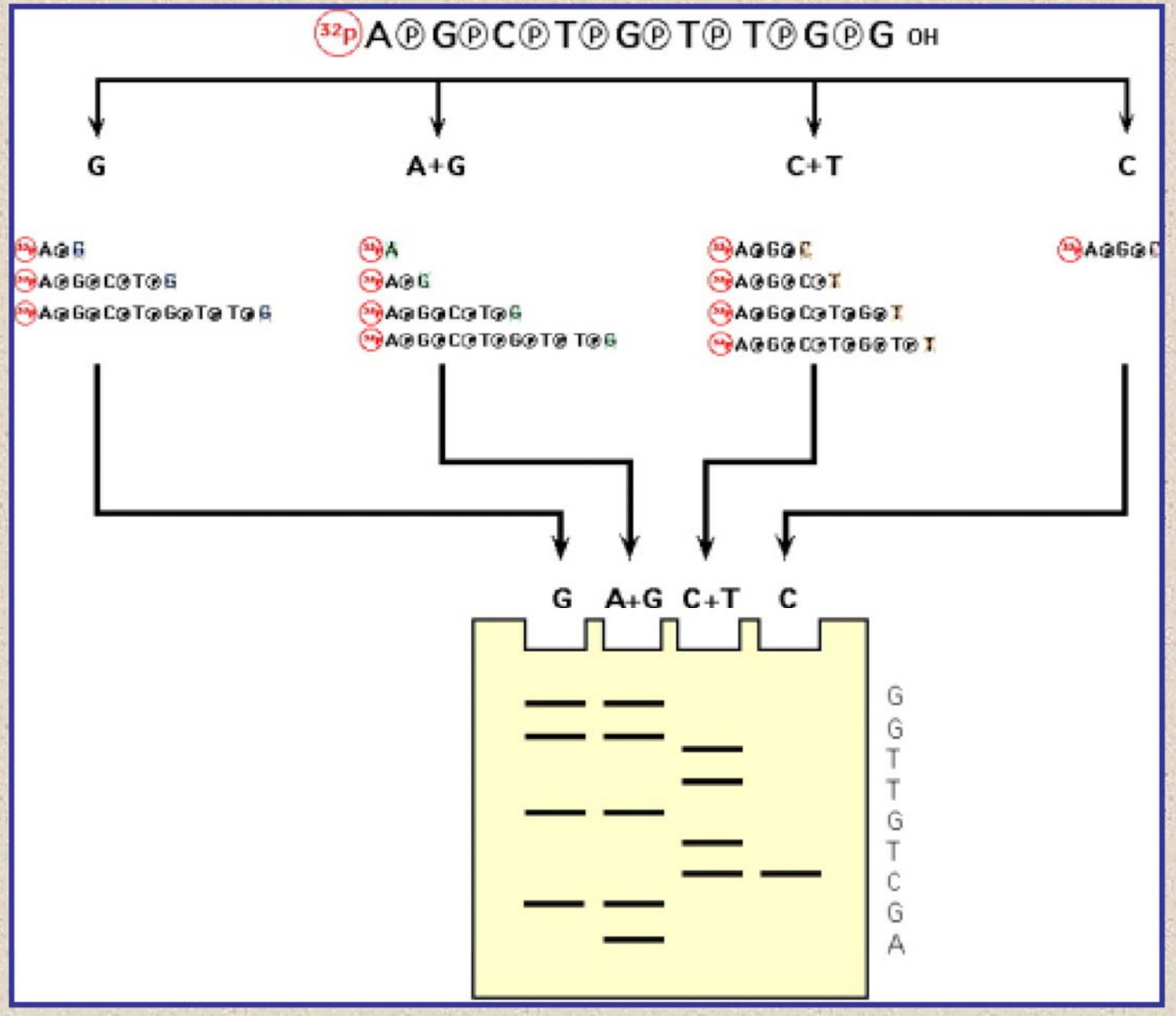
protonierles Phosphoamidit

DNA-Sequenzierung

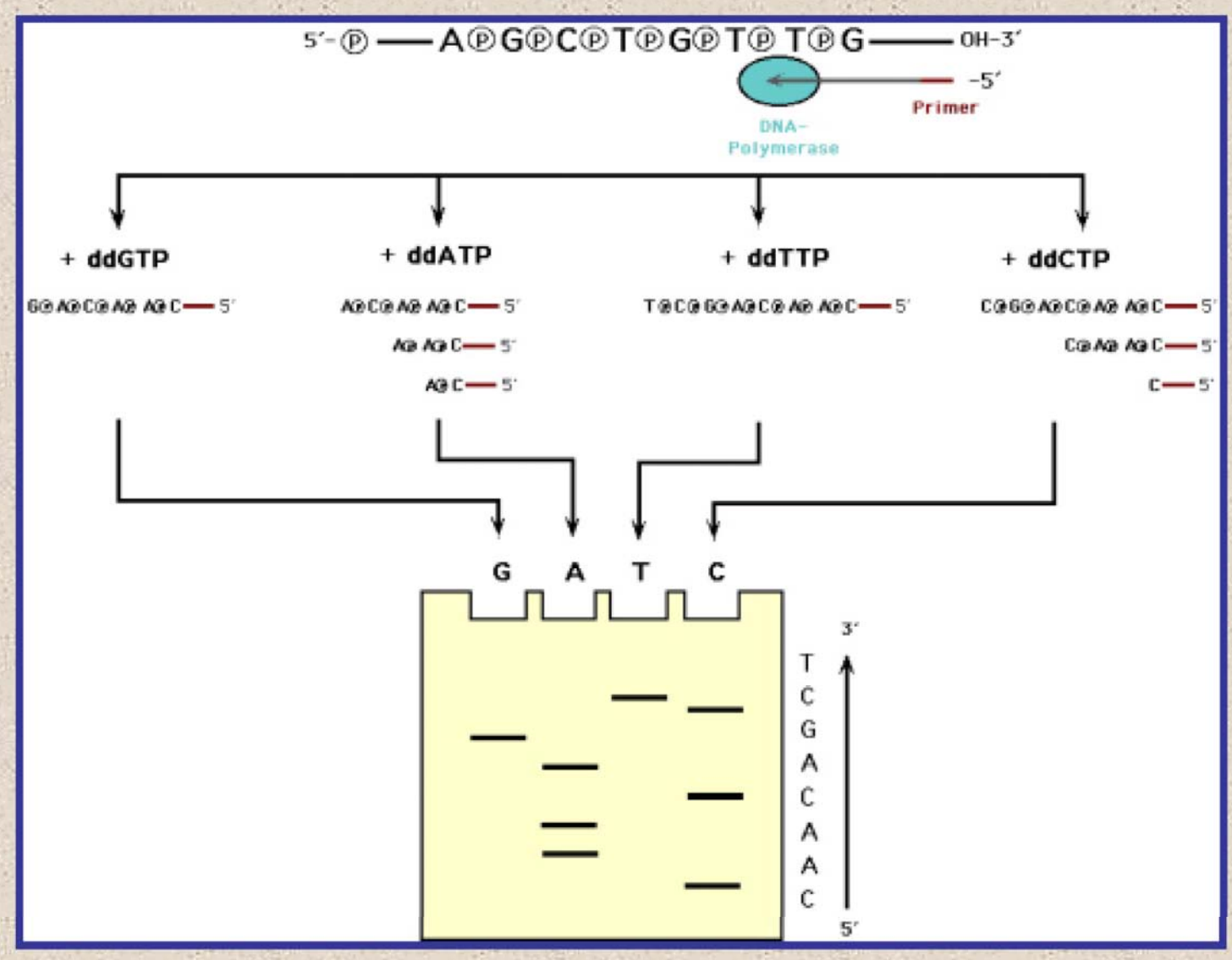
3 schnelle Methoden:

1. Maxam und Gilbert (= chemische Sequenzierung)
2. Sanger-Sequenzierung (=enzymatische Sequenzierung)
3. On-line-Sequenzierung (Fluoreszenz-basierte Sequenzierung)

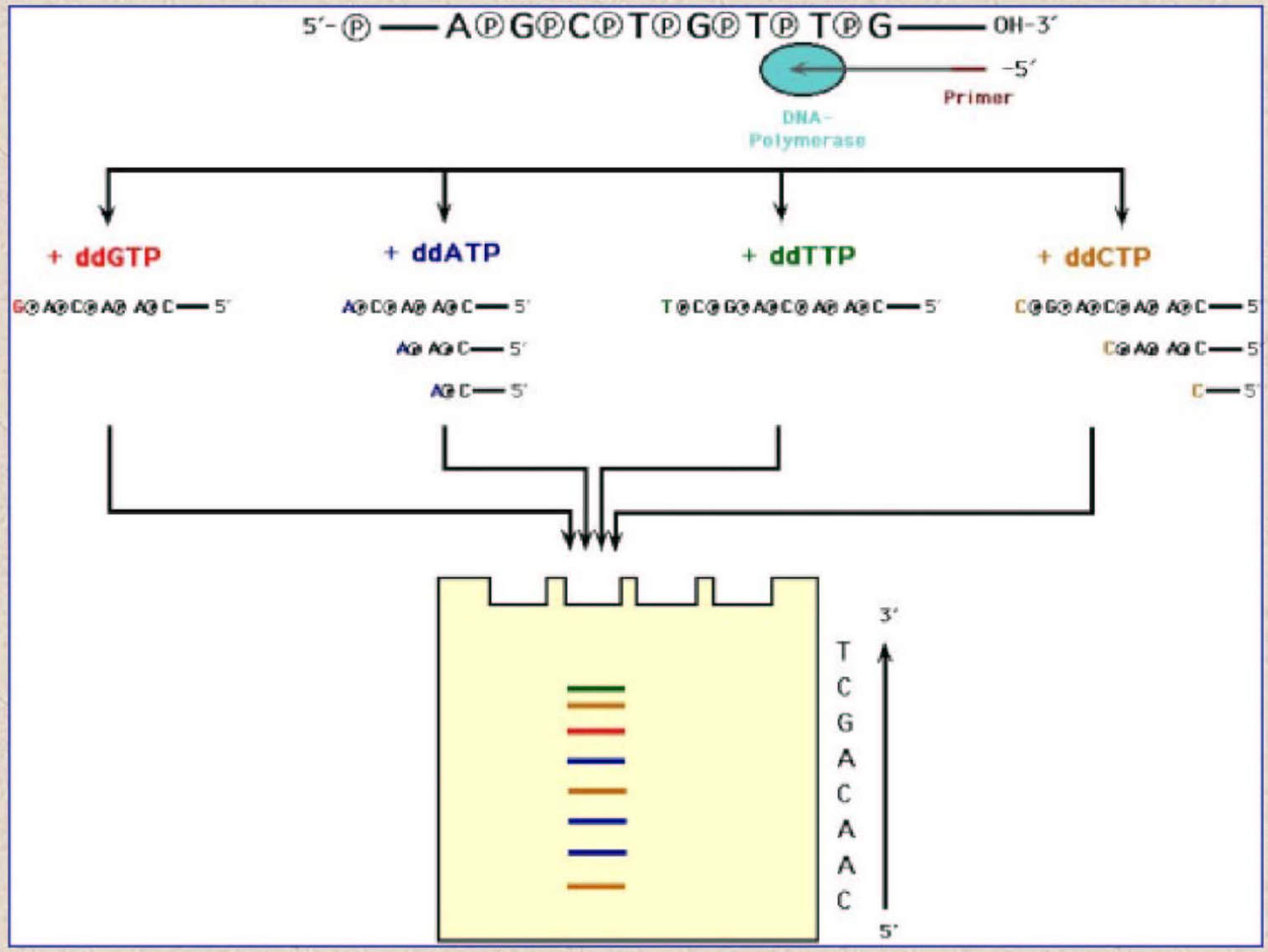
Maxam und Gilbert, 1978



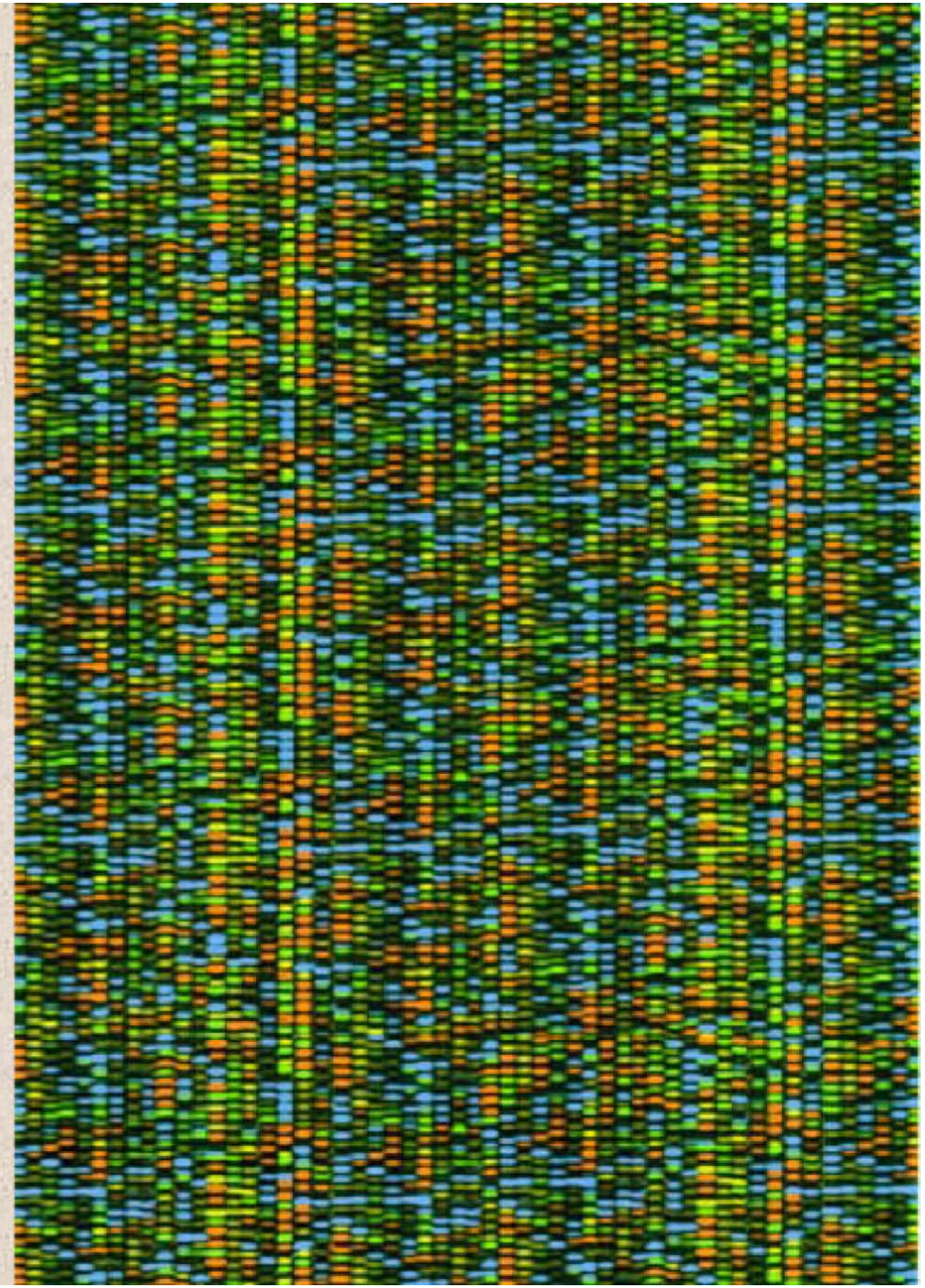
Kettenabbruch-Sequenzierung nach Sanger 1975-77



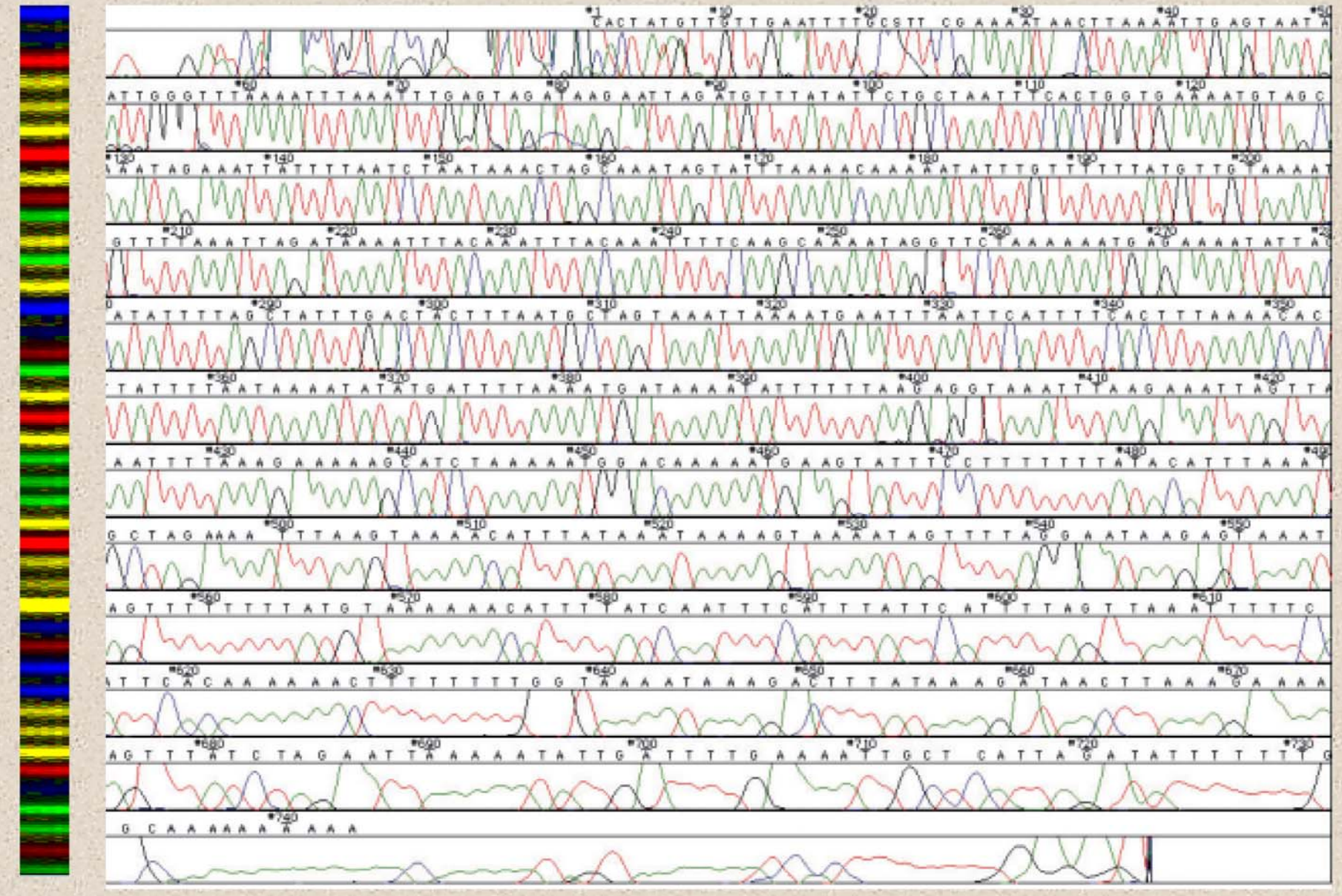
„Automatische“ DNA-Sequenzierung

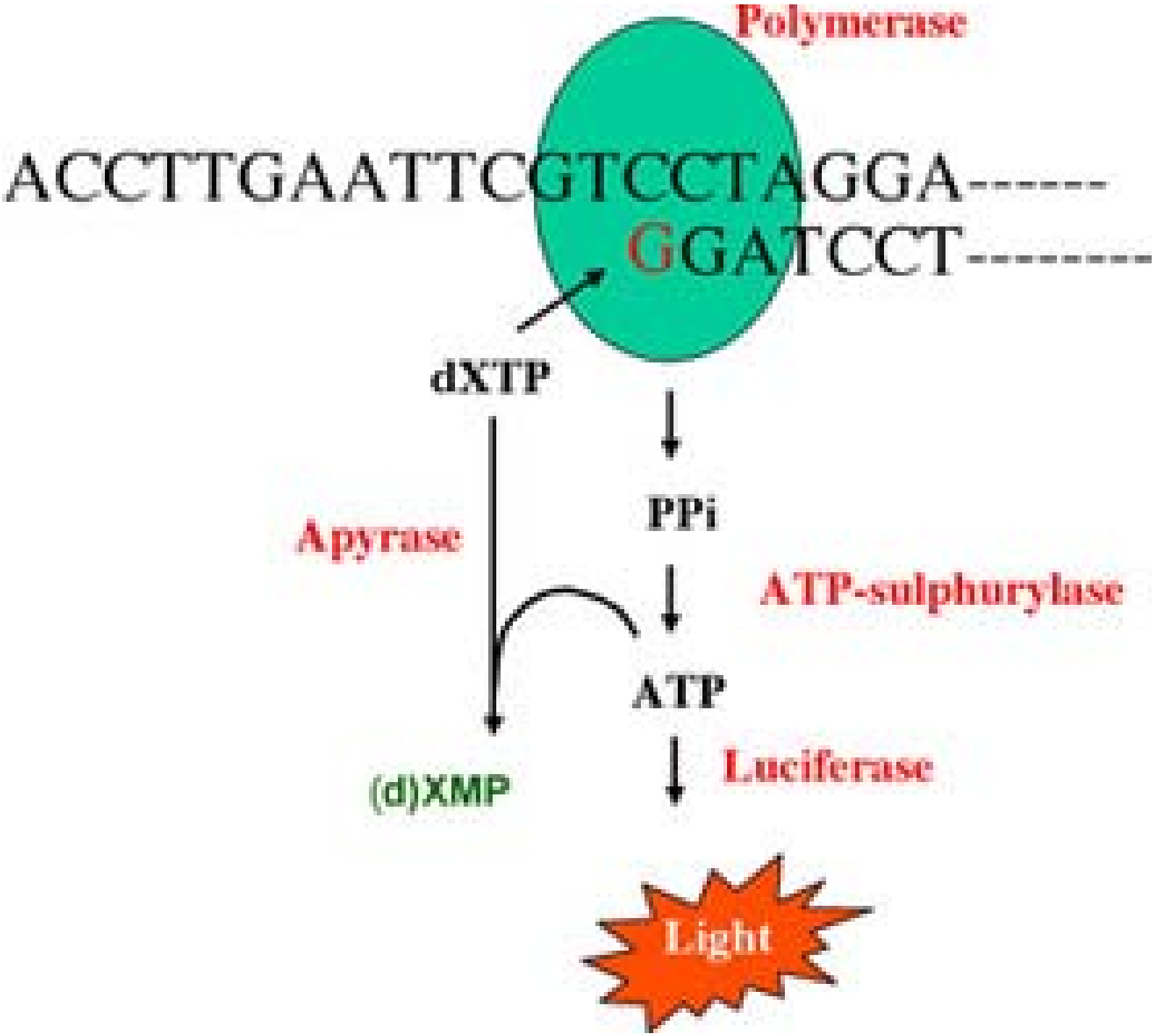


typisches Bild einer online Sequenzierung

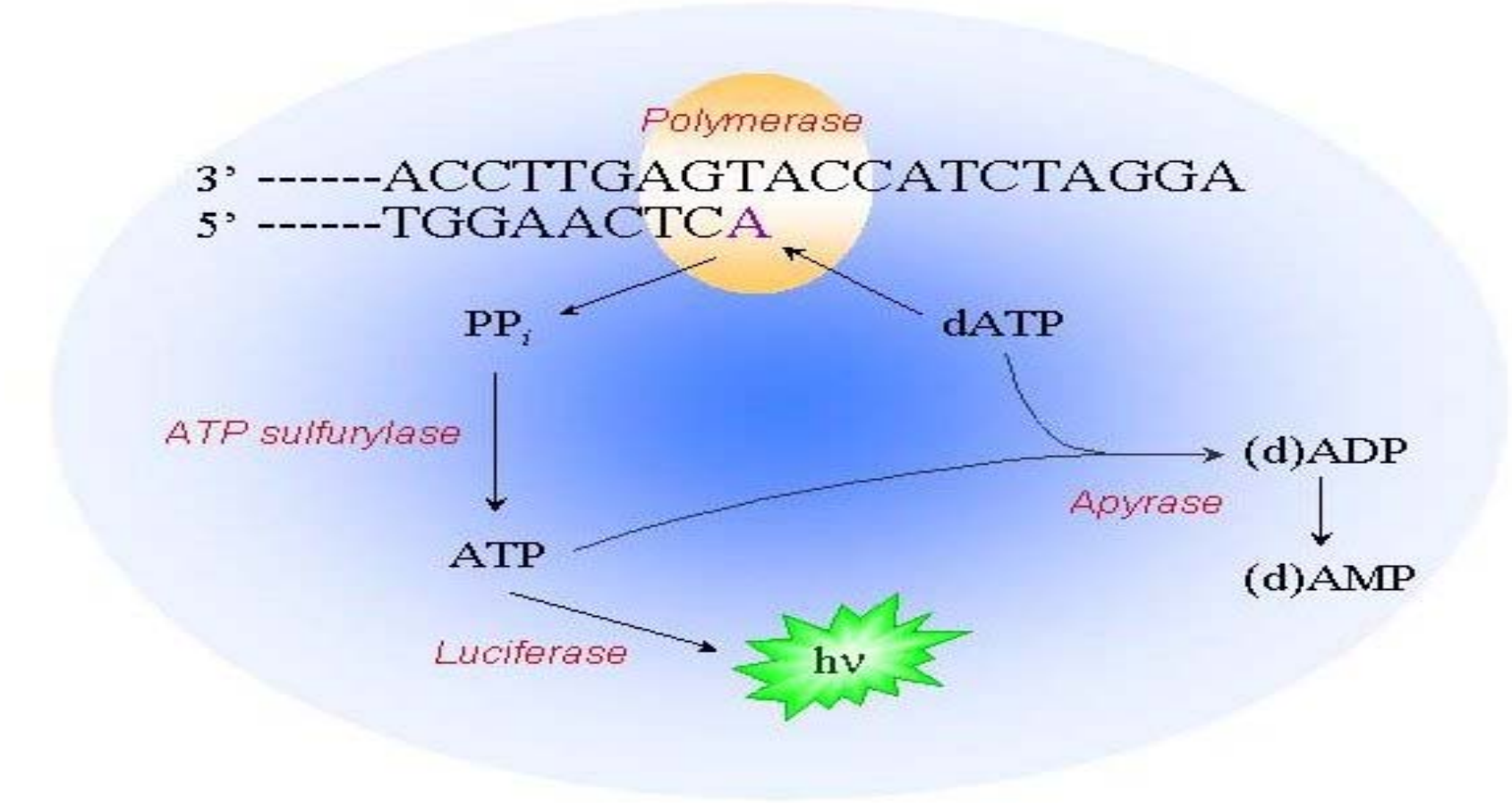


Sequenzdaten- Chromatogramm

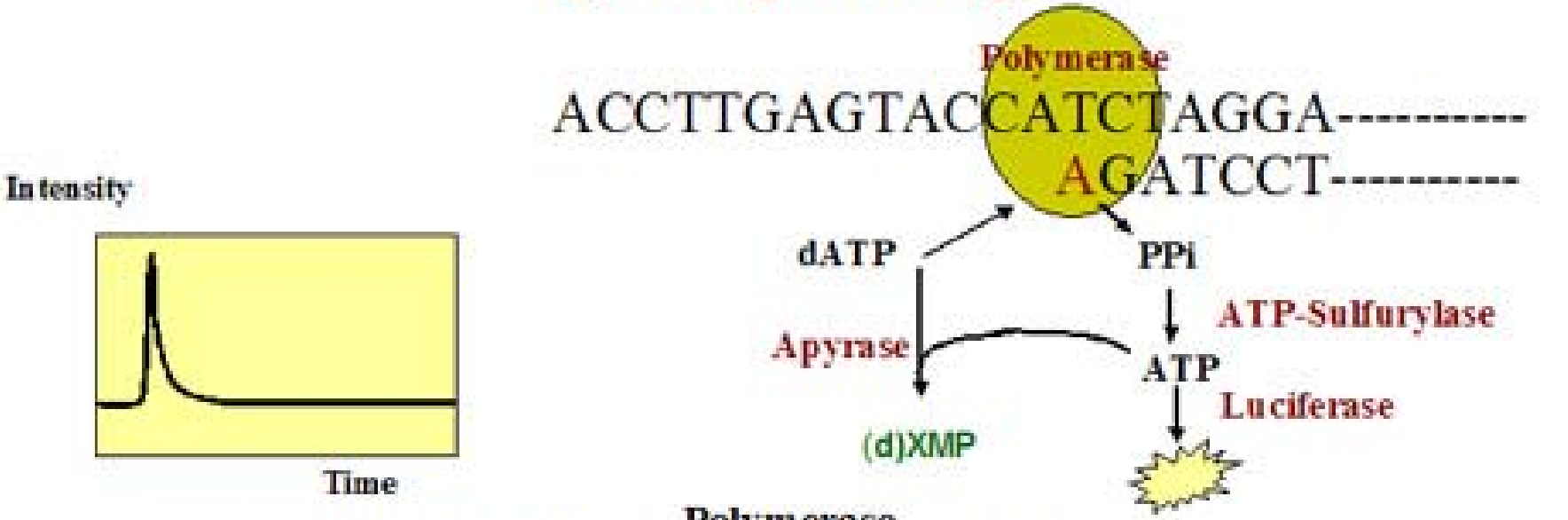



Polymeraase
ACCTTGAATTCGTCCTAGGA------
GGATCCT-------
dXTP
PPi
Apyrase
ATP-sulphurylase
ATP
Luciferase
(d)XMP
Light

Pyrosequenzierung



Pyrosequencing



$DNA_N + dNTP \xrightarrow{\text{Polymerase}} DNA_{N+1} + PPi$

Coupled Reactions

$PPi + APS \xrightarrow{\text{Sulfulylase}} ATP$

$ATP + O_2 + \text{Luciferin} \xrightarrow{\text{Luciferase}} AMP + PPi + CO_2 + \text{Oxyluciferase} + \text{Light}$

Degradation Reactions

$ATP + dNTP \xrightarrow{\text{Apyrase}} ADP + dNDP + Pi$

$ADP + dNDP \xrightarrow{\text{Apyrase}} AMP + dNMP + Pi$

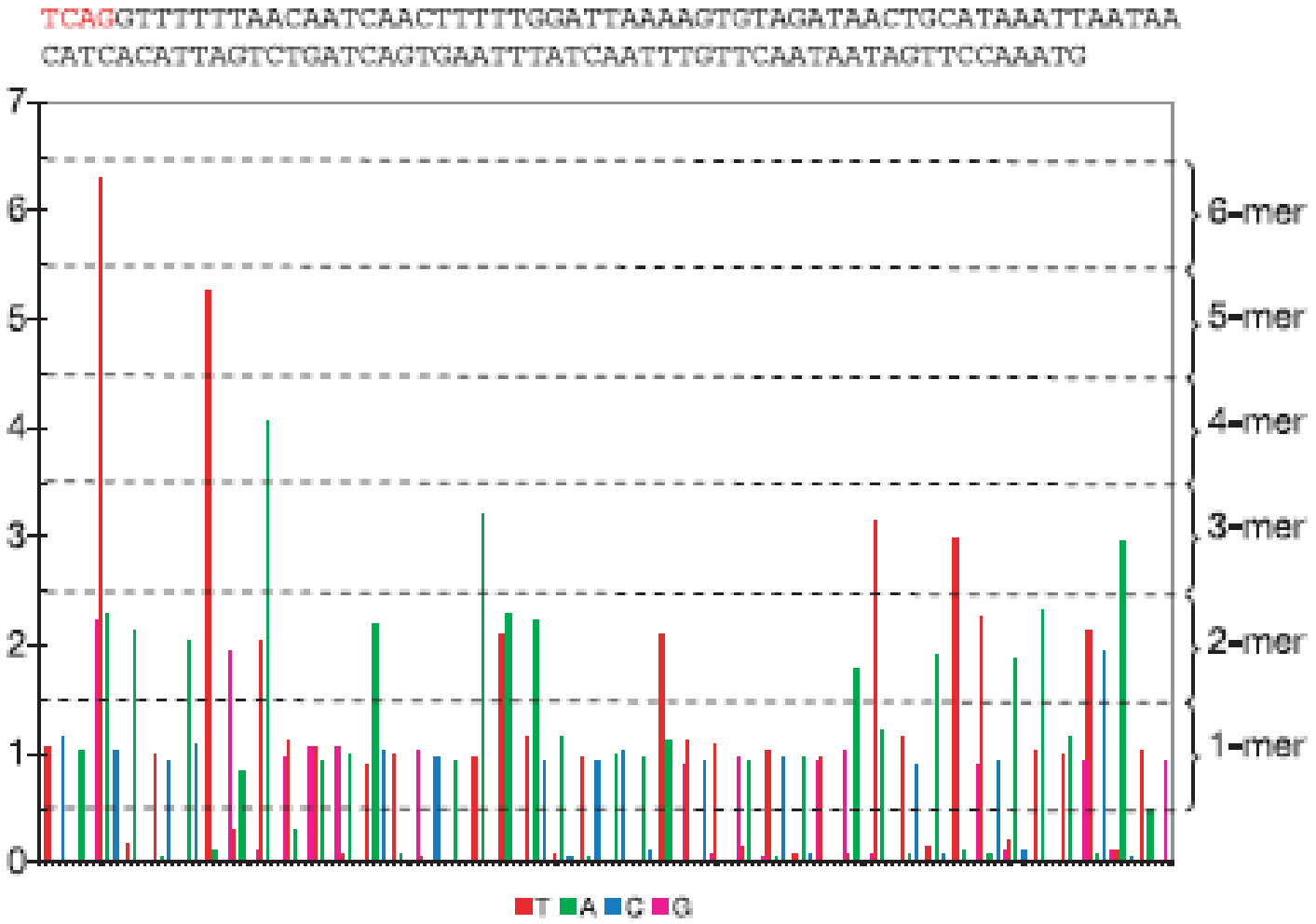


Figure 3 | Flowgram of a 113-bases read from an *M. genitalium* run. Nucleotides are flowed in the order T, A, C, G. The sequence is shown above the flowgram. The signal value intervals corresponding to the various homopolymers are indicated on the right. The first four bases (in red, above the flowgram) constitute the 'key' sequence, used to identify wells containing a DNA-carrying bead.

The Pyrosequencing™ technology is a relatively new DNA sequencing method originally developed here at KTH at the Department of Biotechnology. The technology has been commercialized and is today marketed by Biotage AB. The technique utilizes the cooperativity between four different enzymes and the phenomenon of bioluminescence to monitor the incorporation of nucleotides into the DNA. A short description of the steps in the Pyrosequencing process is given below.

Initial step

The reaction mixture consists of the four enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase), different substrates needed for the reactions and the single stranded DNA to be sequenced.

Step 1 - Polymerase

One of the four nucleotides dNTP (dATP, dCTP, dGTP, dTTP) is added to the reaction mixture. If the added nucleotide is complementary to the base in the DNA strand, it is incorporated and inorganic pyrophosphate (PP_i) is released.

Step 2 - ATP sulfurylase

The PP_i is converted into ATP by the enzyme ATP sulfurylase.

Step 3 - Luciferase

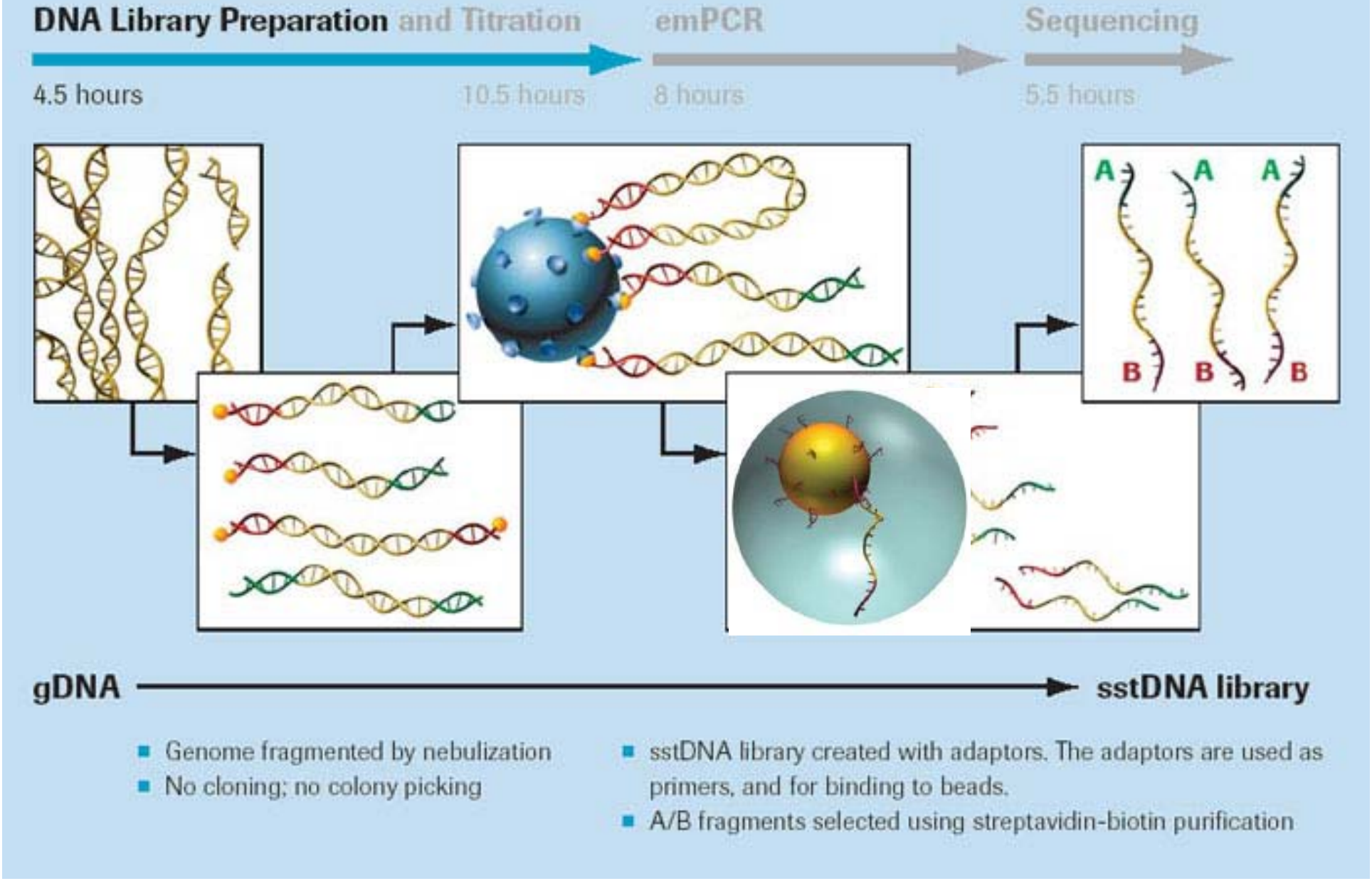
The luciferase catalyzes a reaction where ATP is used to generate light. The amount of light is proportional to the amount of ATP, and hence also proportional to the amount of incorporated nucleotides via the PP_i. The light is then detected by a CCD camera.

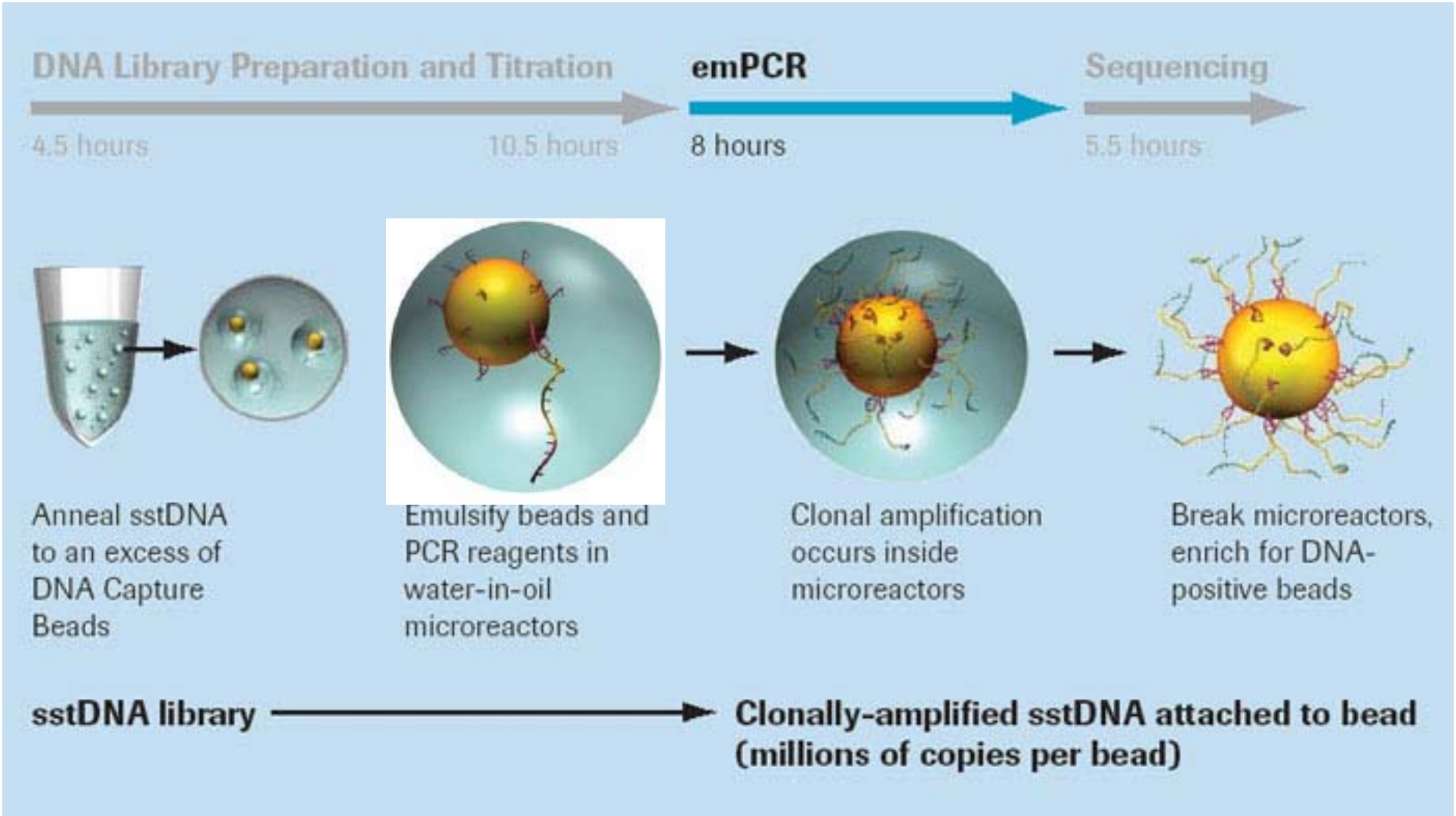
Step 4 - Apyrase

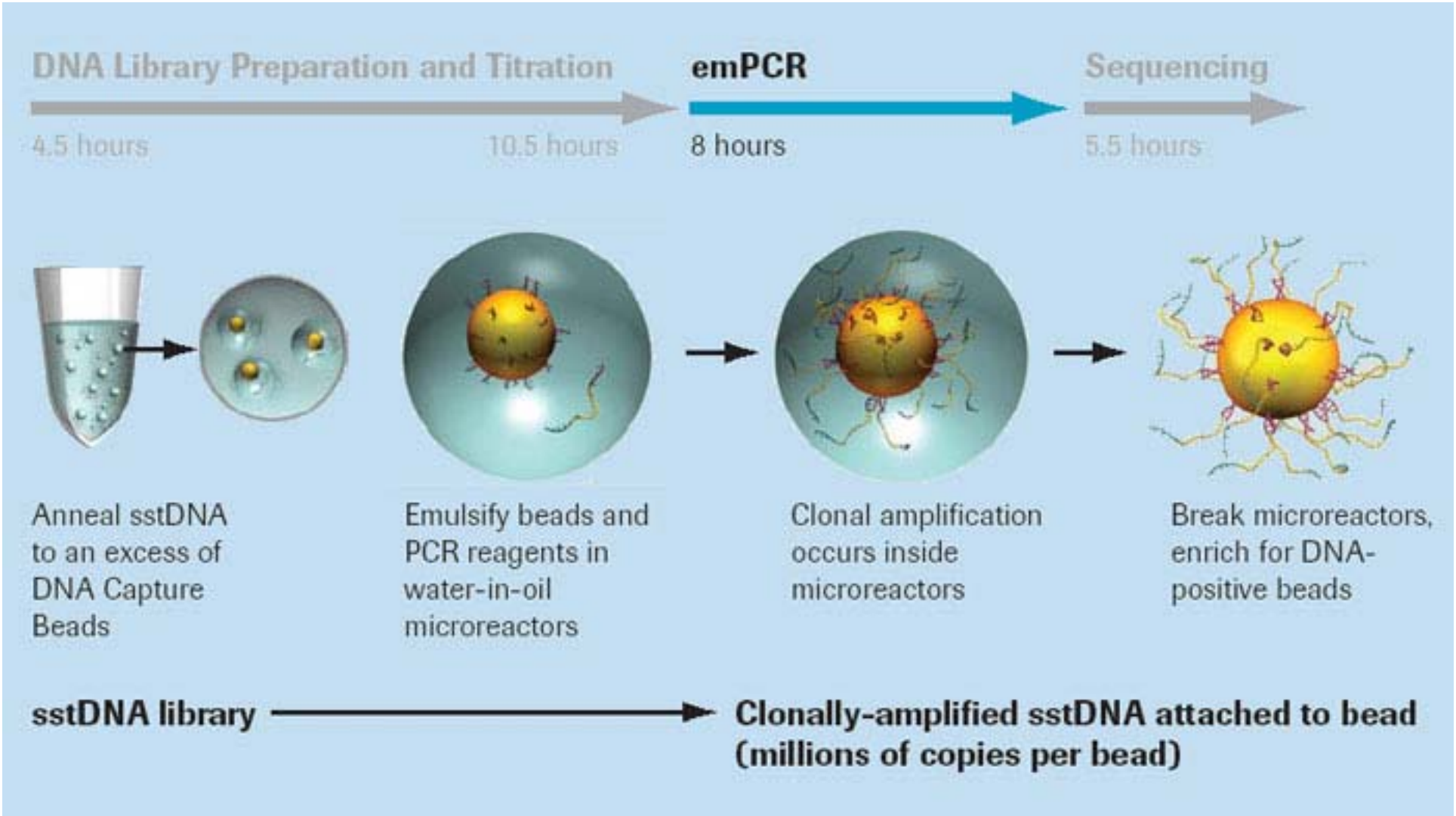
Remaining dNTP and ATP are degraded by the apyrase before the next nucleotide in the iterative cycle is added to the reaction mixture.

My research is devoted to developing a good mathematical model of the reaction system. This will help us to understand the mechanisms governing the system in detail. Once a satisfactory model has been developed, it can be used to optimize the method with respect to substrate and enzyme concentrations as well as the choice of enzymes (kinetic parameters). As the demand for even better DNA sequencing techniques is steadily increasing, as new applications arise, there is a lot to gain by optimization.

Massensequenzierung
mit Roche 454/FLX Sequencer




DNA Library Preparation and Titration
4.5 hours
10.5 hours
emPCR
8 hours
Sequencing
5.5 hours
Anneal sstDNA to an excess of DNA Capture Beads
Emulsify beads and PCR reagents in water-in-oil microreactors
Clonal amplification occurs inside microreactors
Break microreactors, enrich for DNA-positive beads
sstDNA library
Clonally-amplified sstDNA attached to bead (millions of copies per bead)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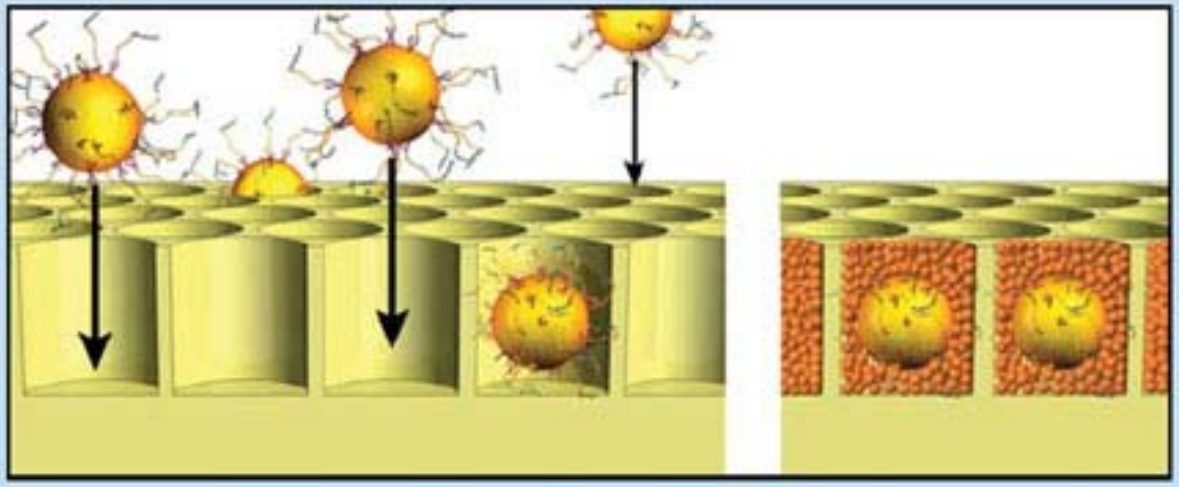
Clonal amplification occurs inside microreactors
Break microreactors, enrich for DNA-positive beads
sstDNA library
Clonally-amplified sstDNA attached to bead (millions of copies per bead)

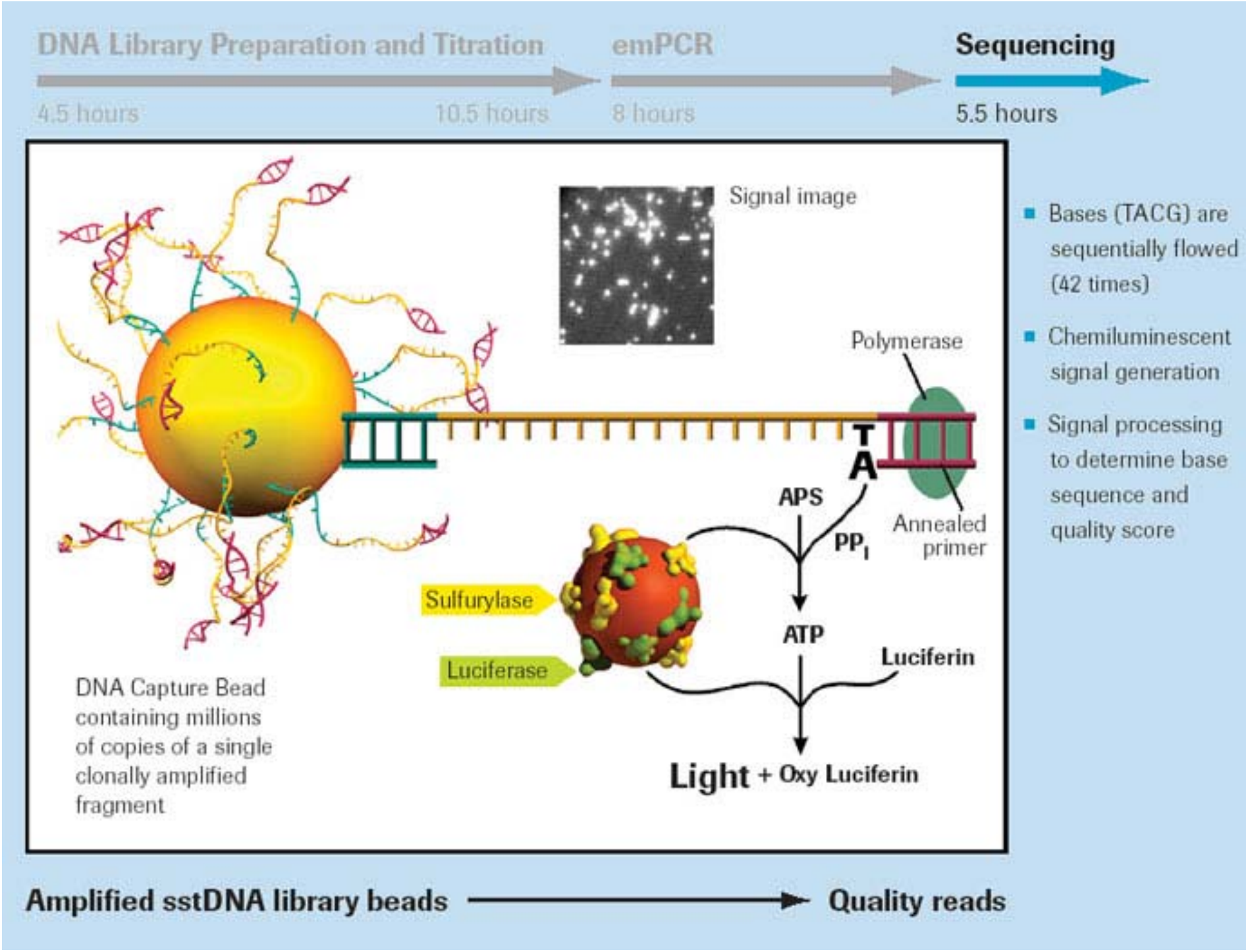
DNA Library Preparation and Titration → emPCR → **Sequencing**

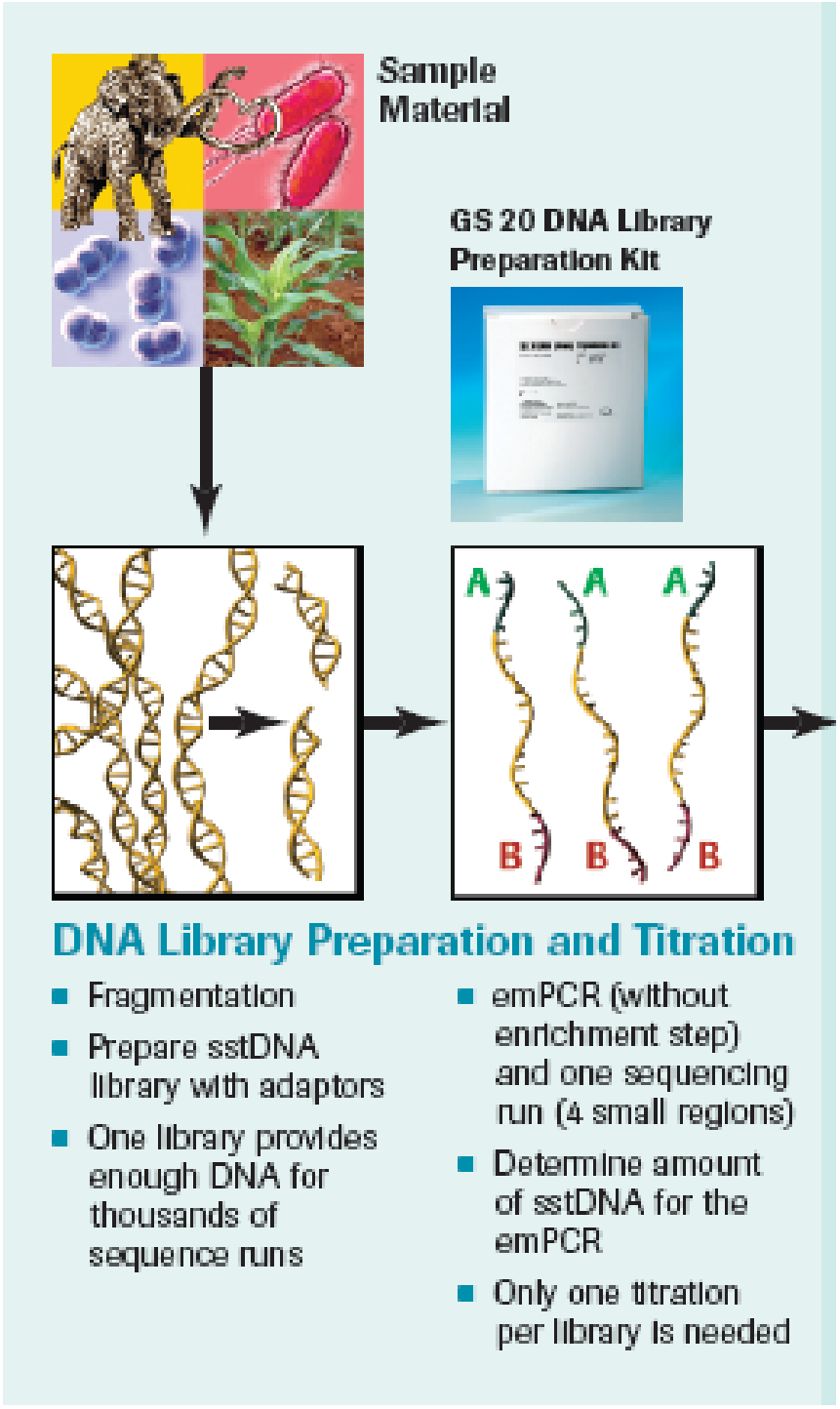
4.5 hours – 10.5 hours | 8 hours | 5.5 hours



- Well diameter: average of 44 µm
- A single clonally amplified sstDNA bead is deposited per well
- 200,000 reads obtained in parallel on large-format PicoTiterPlate device

Amplified sstDNA library beads → **Quality reads**

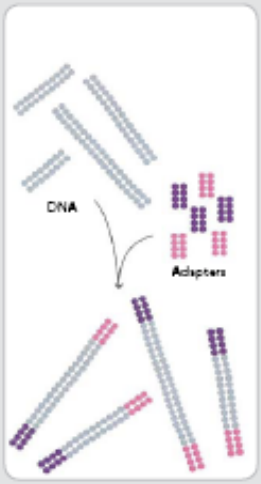

DNA Library Preparation and Titration
4.5 hours
10.5 hours
emPCR
8 hours
Sequencing
5.5 hours
Signal image
Polymerase
T
A
APS
PPi
Annealed primer
Sulfurylase
Luciferase
ATP
Luciferin
Light + Oxy Luciferin
DNA Capture Bead containing millions of copies of a single clonally amplified fragment
Bases (TACG) are sequentially flowed (42 times)
Chemiluminescent signal generation
Signal processing to determine base sequence and quality score
Amplified sstDNA library beads
Quality reads



20 Millionen Basenpaare
in 4,5 Std,
Ein Bakteriengenom
in einer Woche

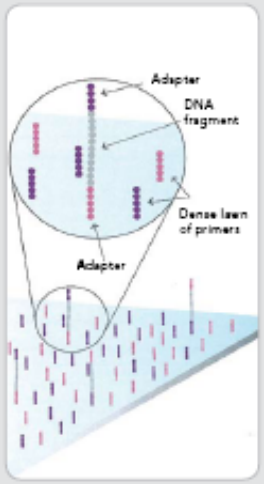
SEQUENCING TECHNOLOGY OVERVIEW

1. PREPARE GENOMIC DNA SAMPLE



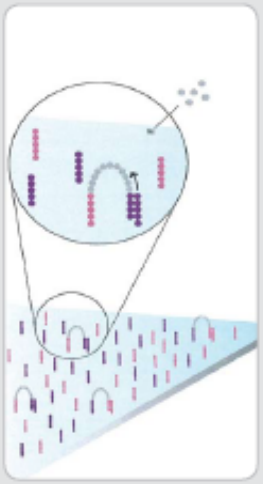
Randomly fragment genomic DNA and ligate adapters to both ends of the fragments.

2. ATTACH DNA TO SURFACE



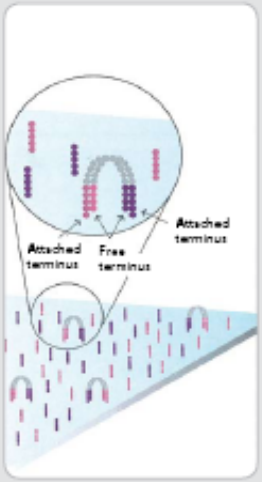
Bind single-stranded fragments randomly to the inside surface of the flow cell channels.

3. BRIDGE AMPLIFICATION



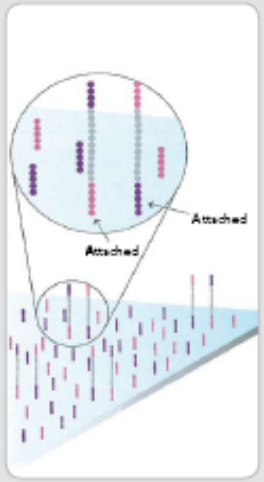
Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.

4. FRAGMENTS BECOME DOUBLE-STRANDED



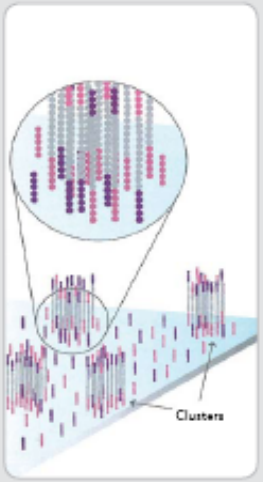
The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate.

5. DENATURE THE DOUBLE-STRANDED MOLECULES



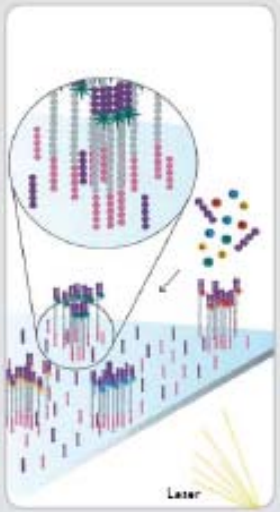
Denaturation leaves single-stranded templates anchored to the substrate.

6. COMPLETE AMPLIFICATION



Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell.

7. DETERMINE FIRST BASE

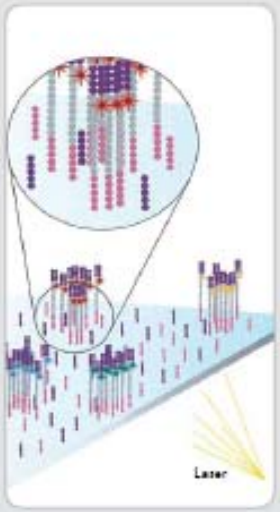


The first sequencing cycle begins by adding four labeled reversible terminators, primers, and DNA polymerase.

8. IMAGE FIRST BASE

After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified.

9. DETERMINE SECOND BASE

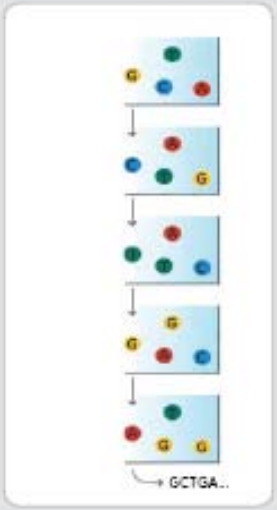


The next cycle repeats the incorporation of four labeled reversible terminators, primers, and DNA polymerase.

10. IMAGE SECOND CHEMISTRY CYCLE

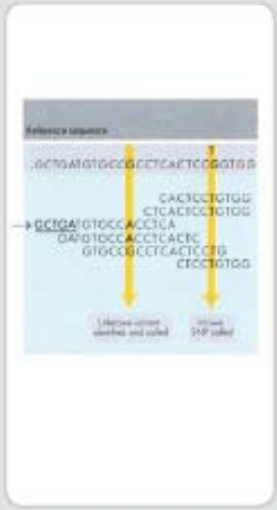
After laser excitation, the image is captured as before, and the identity of the second base is recorded.

11. SEQUENCING OVER MULTIPLE CHEMISTRY CYCLES



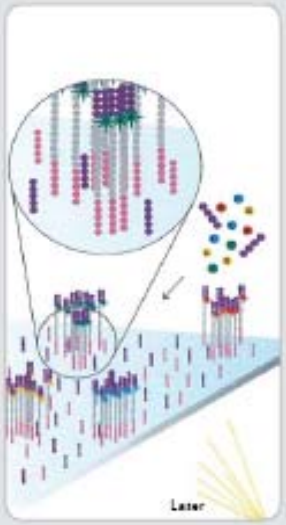
The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

12. ALIGN DATA



The data are aligned and compared to a reference, and sequencing differences are identified.

7. DETERMINE FIRST BASE



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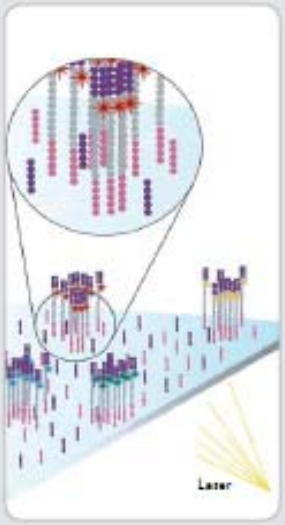

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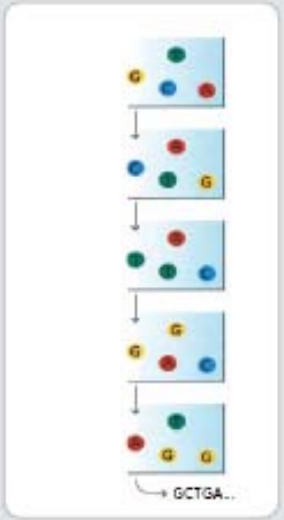

The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

12. ALIGN DATA

The data are aligned and compared to a reference, and sequencing differences are identified.

Next generation sequencing