Centurion University

Session 169:
Principle of Pyrosequencing

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PYROSEQUENCING:

- Genome Sequencing Utilizing Light-Emitting Luciferase and PCR-Reaction- Mixture-in-Oil Emulsion.
- A Swedish company known as Pyrosequencing marked this method for short fragments.
- pyros (Greek for “fire,” because light is produced)

Read lengths are around 200-300 bases.
400,000 reads of parallel sequencing
100mb of output per run
Run time 7.5 hours

- A luciferase is an enzyme which emits light in the presence of ATP. Several organisms, such as the American firefly and the poisonous Jack-o-lantern mushroom, produce luciferases.

Pyrosequencing Biochemistry:

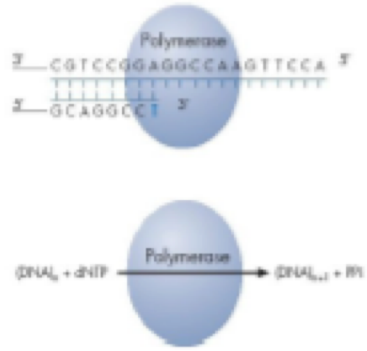
• In DNA synthesis, a dNTP is attached to the 3' end of the growing DNA strand. The two phosphates on the end are released as pyrophosphate (PPi).

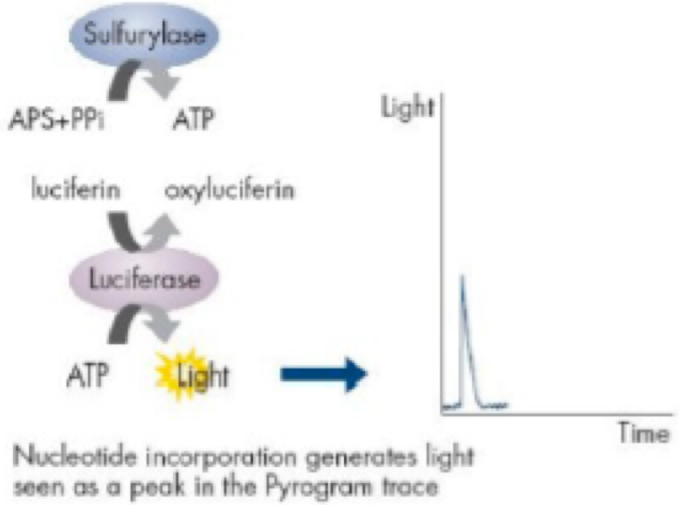
• ATP sulfurylase uses PPi and adenosine 5'-phosphosulfate to make ATP.

• Luciferase is the enzyme that causes fireflies to glow. It uses luciferin and ATP as substrates, converting luciferin to oxyluciferin and releasing visible light.

– The amount of light released is proportional to the number of nucleotides added to the new DNA strand.

• After the reaction has completed, apyrase is added to destroy any leftover dNTPs.





Nucleotide incorporation generates light seen as a peak in the Pyrogram trace

General working scheme:

• DNA fragment is amplified with PCR: one of the two primers used is labeled with biotin.

• Streptavidin-bound beads are taken.

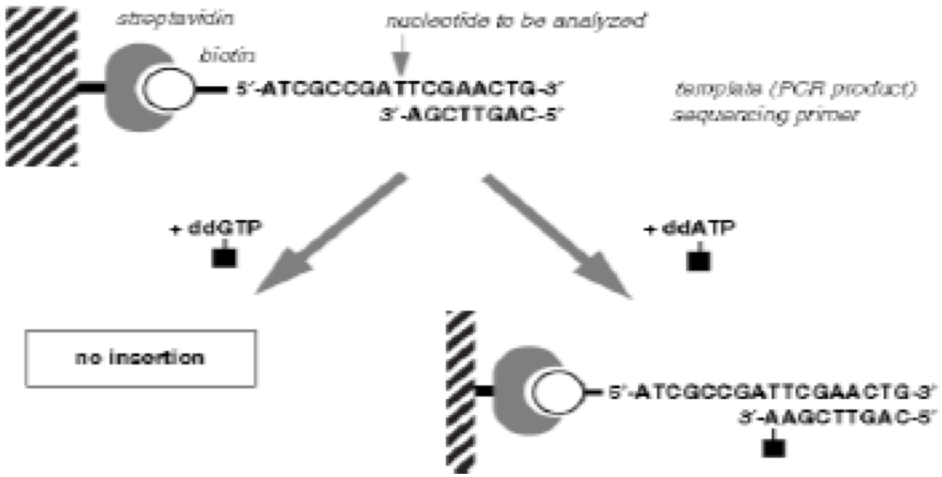
• Amplified DNA is coupled with Streptavidin-bound beads and denatured with NaoH.

• The biotin labeled chain remained bound to pellets.

• Washing step: complementary chain is washed, single strand left (biotin labeled).

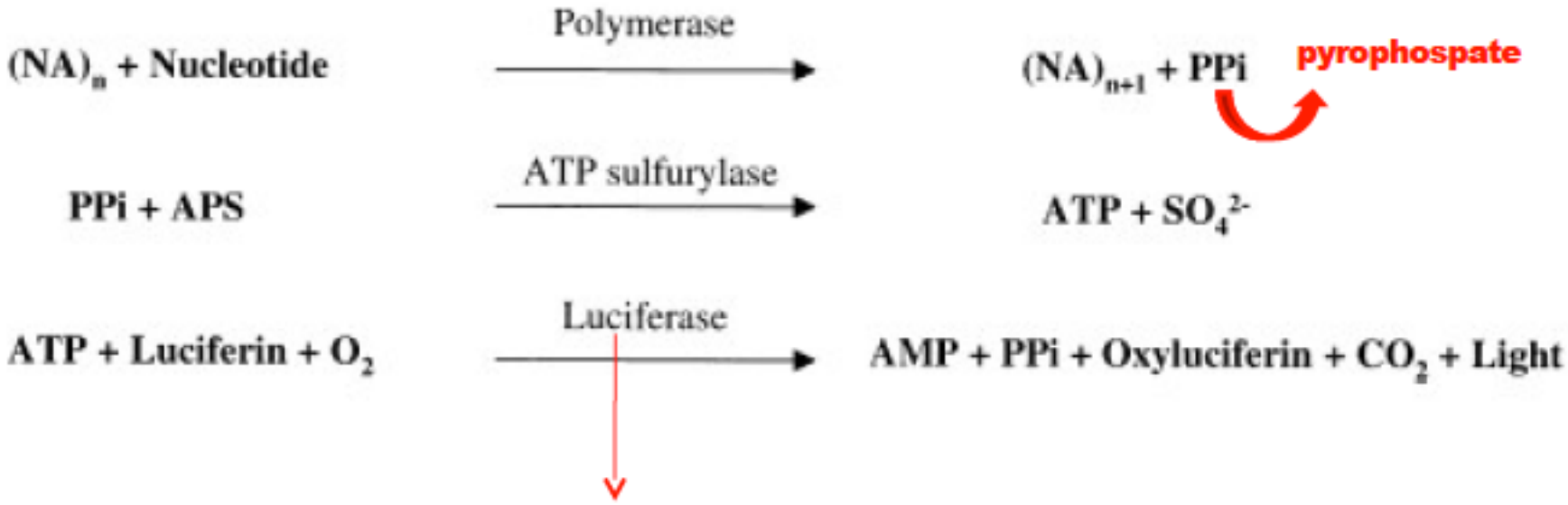
• Template is pipetted onto a microtiter plate.

- Sequencing primer, substrate mixture (i.e., adenosine-5' phosphosulfate [APS] and luciferin), enzymes (i.e., DNA polymerase, ATP sulfurylase, luciferase, and apyrase) are added.

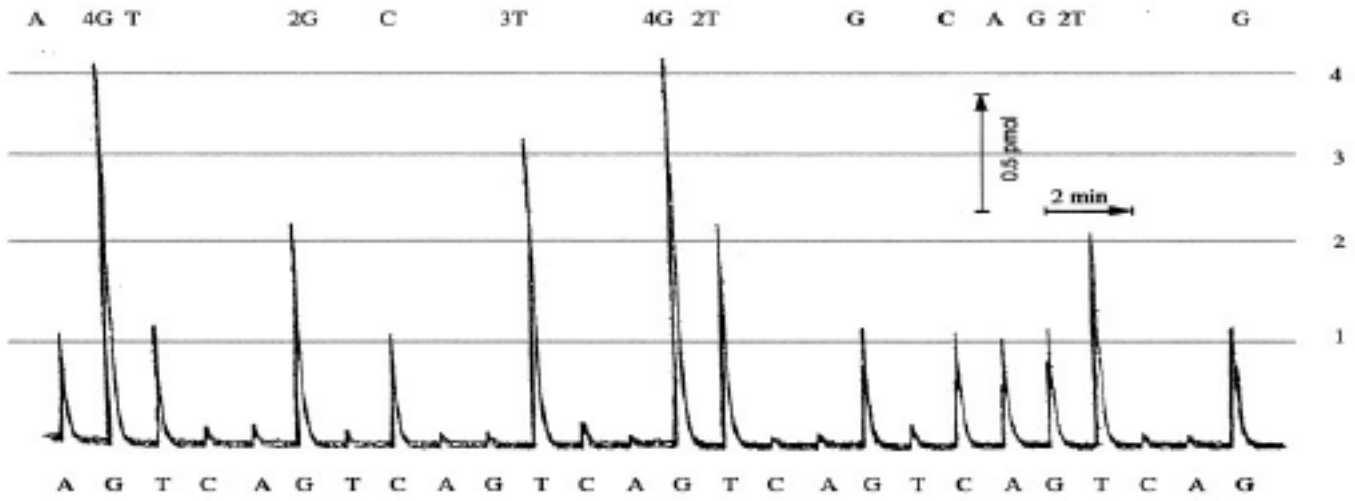


General working scheme Cont….

- The preparation is placed in the machine.
- The primer is first hybridized on the template, and this is followed by a gradual DNA synthesis.
- The machine adds one of the four necessary nucleotide triphosphates and lets the reaction take its course.
- If the dNTP added is complementary to the next base of the template, it is inserted by the DNA polymerase, and a pyrophosphate (PPi) molecule is released.



• The light production is recorded during the entire procedure by means of a camera;
• it is quantified, evaluated, and presented in the form of signal peaks.
• If two or three of the same nucleotides follow one another in the template, several nucleotides will be installed in a single round, and the light production will be twofold or threefold as high as normal;
• if no nucleotide is inserted, it remains pitch black in the well.
• To remove surplus dNTPs and to complete the reaction rapidly, the dNTPs, can be broken down into dNDP and phosphate by adding apyrase



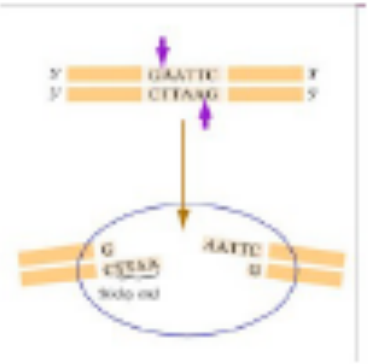
Step 1: Preparation of the DNA

• DNA is fragmented: To start, the DNA is sheared into 300-800 bp fragments, and the ends are “polished” by removing any unpaired bases at the ends.

• The DNA strand’s ends are made blunt with appropriate enzymes

• “A” and “B” adapters are ligated to the blunt ends using DNA ligase

• The strands are denatured using sodium hydroxide to release the ssDNA template library (sstDNA).

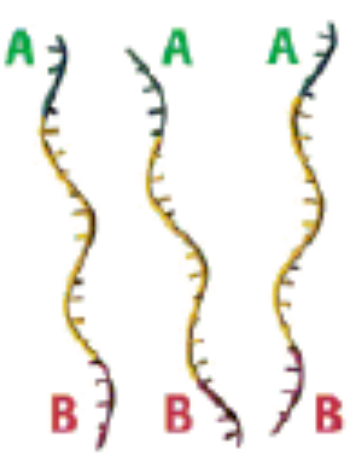


The Adaptors:

• The A and B adapters are used as priming sites for both amplification and sequencing since their composition is known.

• The B adapter contains a 5’ biotin tag used for mobilization.

• The beads are magnetized and attract the biotin in the B adaptors.



Step 2: Cloning of the DNA (emPCR)

• Using water-in-oil emulsion, each ssDNA in the library is hybridized onto a primer coated bead.

• By limiting dilution, an environment is created that allows each emulsion bead to have only one ssDNA.

• Each bead is then captured in a its own emulsion micro-reactor, containing in it all the ingredients needed for a PCR reaction.

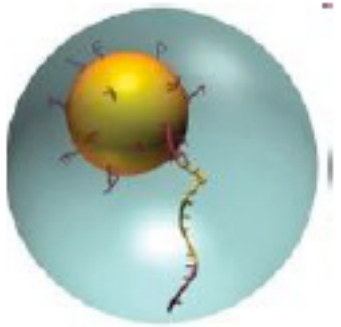
• PCR takes place in each of these beads individually, but all in parallel.

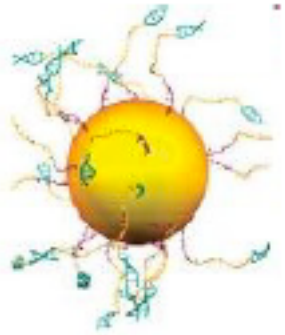
• This activity as a whole is emPCR.

✓ One adapter contains biotin, which binds to a streptavidin-coated bead. The ratio of beads to DNA molecules is controlled so that most beads get only a single DNA attached to them.

✓ Oil is added to the beads and an emulsion is created. PCR is then performed, with each aqueous droplet forming its own microreactor.

✓ Each bead ends up coated with about a million identical copies of the original DNA.





Step 3: Sequencing

• Utilizing the A adapter, a primer is added to the ssDNA.

• The beads are now loaded into individual wells created from finely packed and cut fiber-optics (PicoTiterPlate device).

• The size of the wells do not allow more than one ssDNA bead to be loaded into a well.

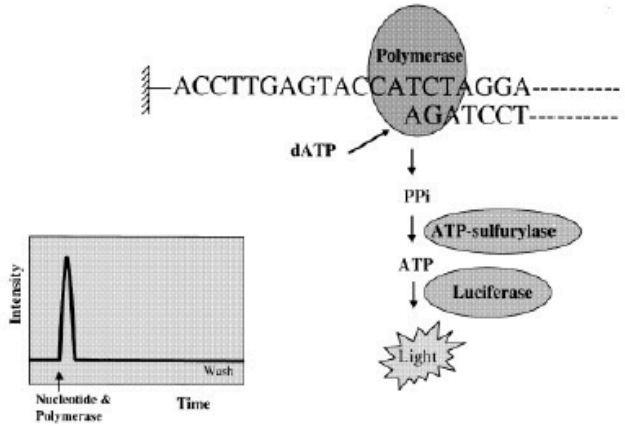
• Enzyme beads and packing beads are added. Enzyme beads containing sulfurase and luciferase, and packing beads used only to keep the DNA beads in place.

• Above the wells is a flow channel, passing nucleotides and apyrase in a timed schedule.

Pyrosequencing

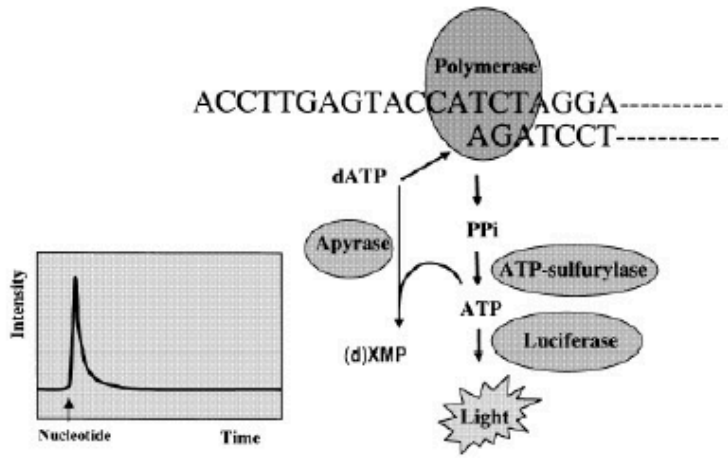
1st Method

- Solid Phase
  - Immobilized DNA
  - 3 enzymes
  - Wash step to remove nucleotides after each addition



2nd Method

- Liquid Phase
  - 3 enzymes + apyrase (nucleotide degradation enzyme)
    - Eliminates need for washing step



- In the well of a microtiter plate:
  - primed DNA template
  - 4 enzymes
- Nucleotides are added stepwise
- Nucleotide-degrading enzyme degrade previous nucleotides

• **Advantages:** a rapid method for the analysis of individual base polymorphisms with a high throughput–up to 4500 tests can be analyzed–and with a second, related system, you can sequence up to 48,000 probes per day.

✓ Accurate
✓ Parallel processing
✓ Easily automated
✓ No need for gel electrophoresis

- **Disadvantages:**

➢ This is not a procedure for simple research laboratories.
➢ The acquisition of such a system is profitable only for routine laboratories in which a large quantity of standardized analyses must be carried out, such as for the characterization of microorganisms, diagnosis of diseases, or analysis of allele frequencies.
➢ The work required is considerable, and special software is required for the instrument and for the analysis.
➢ A complete system costs $130 to $200,000. An attempt to analyze a SNP, for instance, costs about $1.25.

Refrences:

• Detailed overview of the system:
- http://www.454.com/products-solutions/multimedia-presentations.asp
• Pyrosequencing animation:
- http://www.youtube.com/watch?v=bFNjxKHP8Jc&feature=related
- http://www.pyrosequencing.com/DynPage.aspx?id=7454
• Sequencing step animation:
- http://www.youtube.com/watch?v=kYAGFrbGl6E