ComінLabs 2022

dnarXiv: Biotechnology part

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Objectives, limitations and strategies

Synthetic DNA assemblies

- Objectives and strategies
- Proofs of concepts: 10 fragments assembly
- Compare methods: how ?
- Compare methods: results
- Ligation optimization
- Current work

Others approach: random assembly of large fragments

Objectives, limitations and strategies

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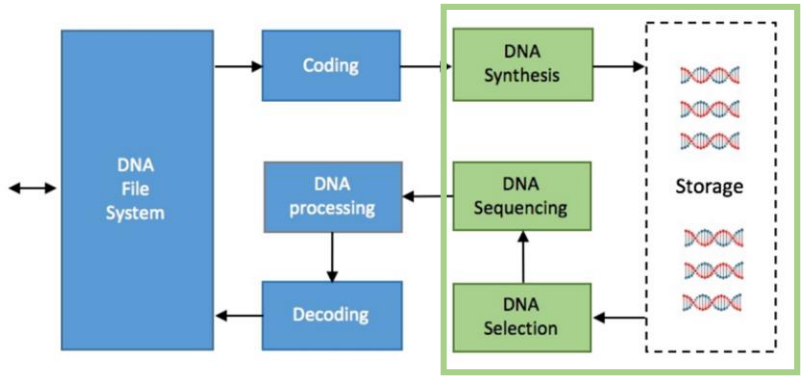
Objectives:

➔ **Test *in vitro* the solution developed *in silico* by the computer scientists**

- Need to develop the biotechnology part of the DNA storage pipeline
- 3 major blocks: DNA Synthesis, DNA Selection and DNA sequencing



Limitations:

➔ DNA synthesis is done externally and is the main bottleneck of the biotechnology part

- **Size limited:** dsDNA gene fragment 1.8 kb ; ssDNA oligo 300nt.
- **Expensive:** 1 Mb (10^6 bp) library cost 50,000€ to synthesize (with eBlock from Integrates DNA Technologies).

Strategies:

➔ **Assumption**: Thanks to the development of DNA synthesis by enzymes, the price will drop by the same order as with the advent of the New Generation Sequencing (NGS). In the near future (10 years) it will be possible to synthesize larger DNA molecules at lower cost.

➔ **Consequences**: Today we need to find a way to test our **DNA storage pipeline** with a **low-cost synthesis strategy**

Synthetic DNA assemblies

Objectives and strategies

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Objectives:

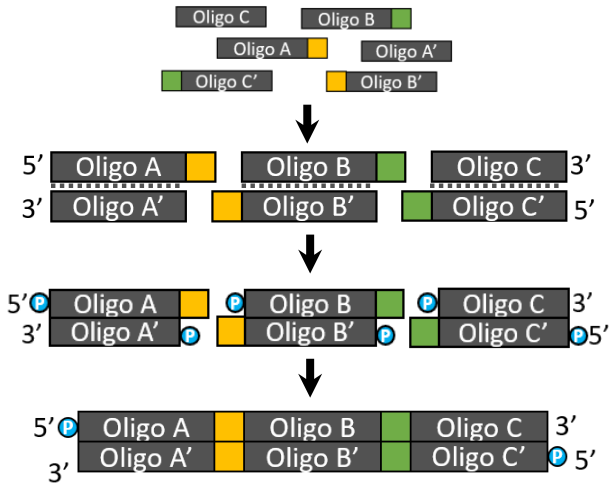
➔ **Construct a large DNA molecule from small ones**

- **How ?** Ordered assemblies of oligonucleotides or gene fragment.
- **Why "large DNA molecule"?** To take advantage of the Nanopore sequencing technology we need long read (up to 1 Mb)
- **Why "ordered assemblies" ?** To increase the information density encoding in DNA. Ordered assemblies help decode steps so we need less index regions or non-coding regions.

Strategies:

➔ **Based on pre-existing DNA assembly techniques**: Golden Gate and Gibson methods

1. **Design** oligos of 60nt and order the synthesis from an external supplier (IDT)
2. **Oligonucleotides annealing**: hybridization of complementary oligos to forms dsDNA.
3. **Phosphorylation** of 5' extremities through T4 PNK enzyme.
4. **Ordered** fragments **ligation** through the overhang and DNA ligase enzyme.

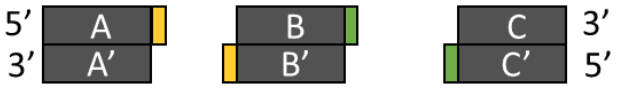


Synthetic DNA assemblies

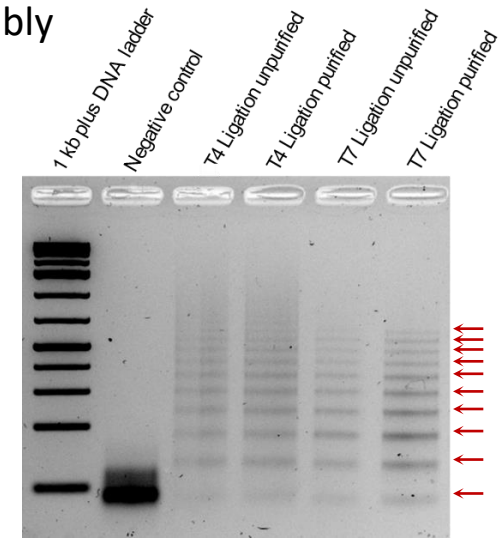
Proofs of concepts: 10 fragment assembly

➔ Try to **ordered assembly 10 fragments** (= 60 nt oligo) with **2 approaches** of DNA assembly techniques:

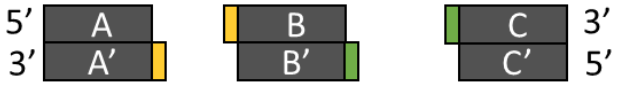
Gibson 4



- Golden Gate like method with **4nt** overhang
- **3’ overhang** / sticky end
- Overhang are designed with NEB tool (GETSET™)
- **Ordered** assembly



GoldenGate 4



- Golden Gate like method with **4nt** overhang
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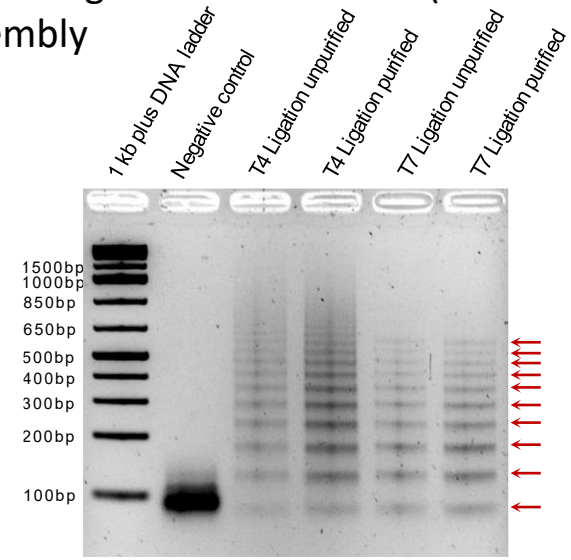


➔ Electrophoresis analysis on agarose gel shown 10 bands corresponds to all possibilities of fragments assemblies.

➔ Methods work with T4 and T7 DNA ligase. The frequency of assembly distribution depends on the final size (= the reaction isn’t total).

Synthetic DNA assemblies

Compare methods: how ?

Objectives:

➔ **Determine for each method:**

- Distribution of assembly size
- Correct or incorrect junction
- Assembly fidelity (yield)

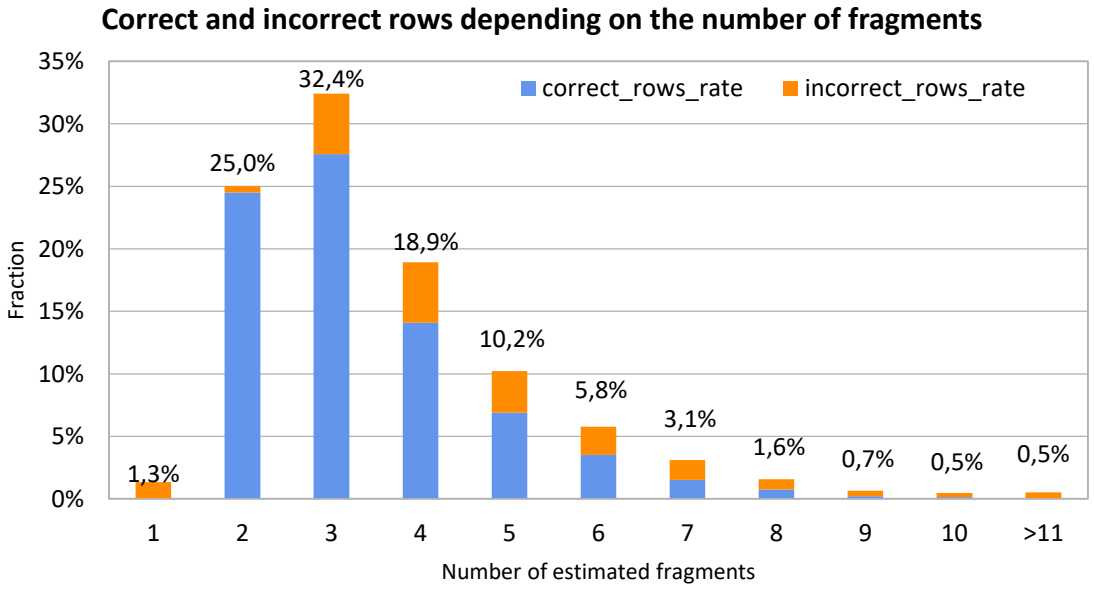
How:

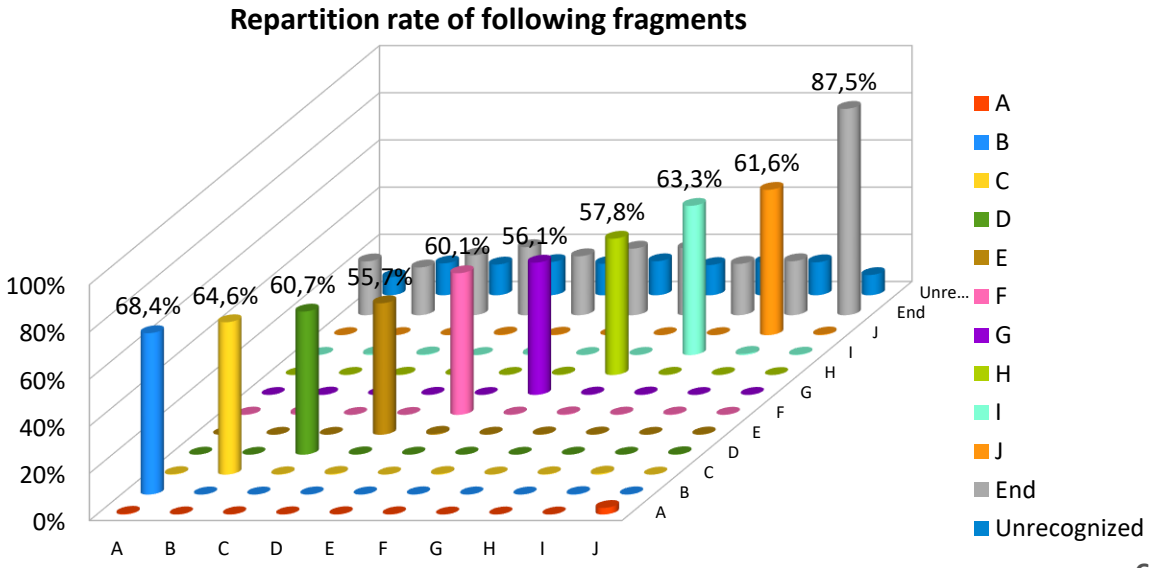
➔ Nanopore sequencing

➔ Internal software to:

- Decoding the information: did we find the encoding message?
- How many fragments do we have in each read?
- Are the fragments being in the right order?

➔ **Results example** for Gibson 4 method with T7 DNA ligase





Synthetic DNA assemblies

Compare methods: results

Summary of results:

➔ The results match with the electrophoresis analysis on agarose gel.

➔ **Gibson 4 and Golden Gate 4 shown similar results**: overhang position at 3' or 5' seems to have no impact.

➔ **T7 DNA ligase has better ligation "fidelity" than T4 DNA ligase**

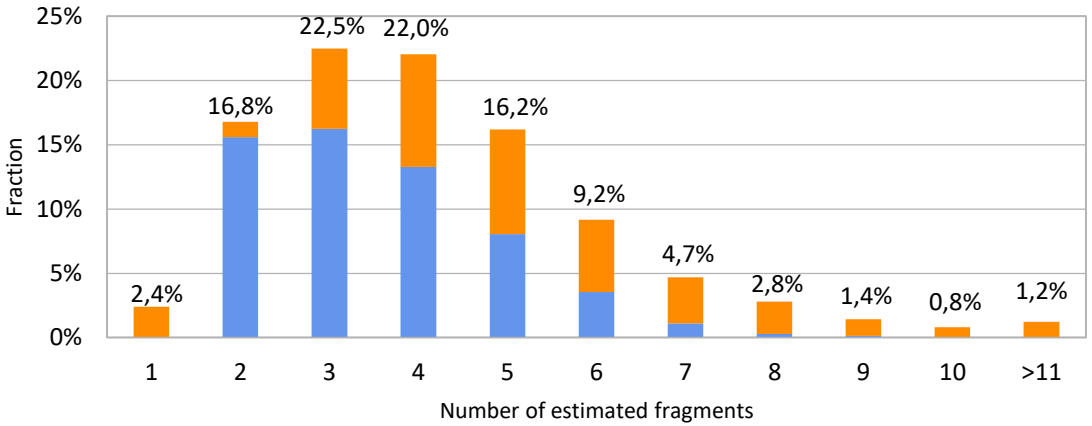
- T7 DNA ligase: average correct ligation = 97,7%
- T4 DNA ligase: average correct ligation = 89,5%
- It's due to enzyme capacity to ligate blunt end or not.

➔ In all cases we **success to decode the encoding message**.

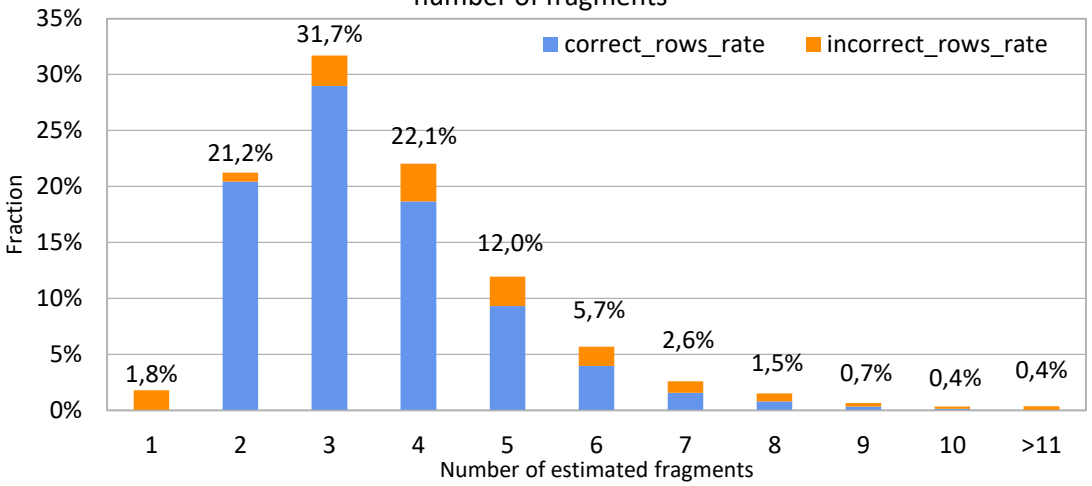
➔ **DNA assemblies are in majority composed of 3 fragments**

- Something limit the reaction
  - During the phosphorylation step
  - And/or during the ligation step

Gibson 4 T4 DNA ligase: correct and incorrect rows depending on the number of fragments



Gibson 4 T7 DNA ligase: correct and incorrect rows depending on the number of fragments



Synthetic DNA assemblies

Ligation optimization

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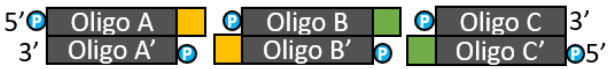
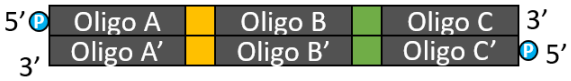
<u>Many things have been tested</u>:

➔ **During the phosphorylation step:**



- **Phosphorylation before or after dimerization** => significant improvement for phosphorylation before dimerization.
- **Two successive phosphorylation cycles** => small effect.

➔ **During the ligation step:**



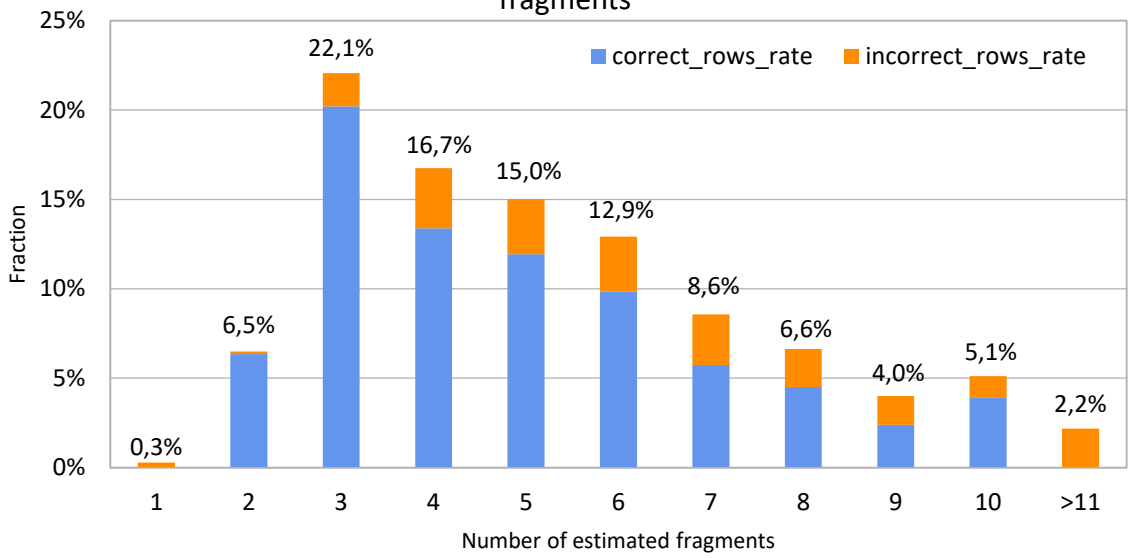
- **Pyrophosphatase concentration gradient** => no significant effect.
- **T7 DNA ligase concentration gradient** and **incubation time** => no significant effect.
- **Creatine kinase concentration gradient** with "**Energy-mix**"=> no significant effect.
- **Magnesium chloride concentration gradient** => no significant effect.
- **Temperature switching cycle** => no significant effect.
- **Ligation without blunt ends and sequential ligation** of blunt ends with T7 and T4 DNA ligase => no significant effect.

Synthetic DNA assemblies

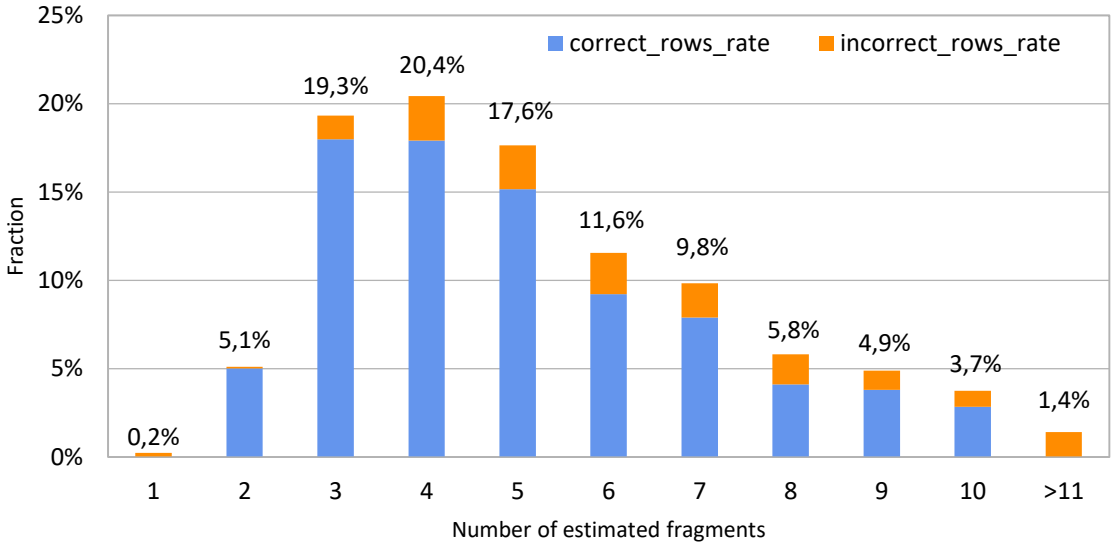
Ligation optimization

Results after optimization:

Gibson 4. Correct and incorrect rows depending on the number of fragments



Golden Gate 4 . Correct and incorrect rows depending on the number of fragments



➔ Much better but still something limit the assembly size.

➔ **Current work on a hypothesis**: is the limit caused by the presence of special fragments ?

- Synthesize oligos : 30% are at the wrong size (caused by coupling problems during synthesis).
- The synthesis is ordered so the truncated fraction is always on the 3' side, so only 1 overhang is affected.
- **Consequence**: during the oligo dimerization step, the resulting fragments can have one correct overhang instead of two. When these fragments are ligated to the assembly, they block elongation = “stop” fragment.

Synthetic DNA assemblies

Future work

New assembly tests with optimized conditions:

- 10 fragments of 60nt with 4nt overhang
- 20 fragments of 60nt with 4nt overhang
- 10 fragments of 60nt with 6nt overhang
- 10 fragments of 120nt with 4nt overhang
- 20 fragments of 60nt with 6nt overhang
- 10 fragments of 60nt with 4nt overhang with a step of purification of oligos before dimerization (chromato by IDT).

Objectives:

- **Compare the effect of the size of the overhang** : *10 frag 60 nt overhang 4nt vs 10 frag 60 nt overhang 6nt*
- **Compare the effect of the number of fragments**: *10 frag 60 nt overhang 4nt vs 20 frag 60 nt overhang 4nt*
- **Compare the effect of the size of the fragments**: *10 frag 60 nt overhang 4nt vs 10 frag 120 nt overhang 4nt*
- **Test the hypothesis about « stop » fragments**: *10 frag 60 nt overhang 4nt vs 10 frag 60 nt overhang 4nt* (purified oligos)

Others approach

Random assembly of large fragments

<u>Another approach</u> to test our DNA storage pipeline with a **low-cost synthesis strategy**

<u>New assembly method</u>:

1. Genomic DNA from BAC (Bacterial Artificial Chromosome 100 – 350 kb)
2. Sonication to have ~200 bp fragments (population of fragments)
3. Polishing of the DNA ends (DNA pol I + dNTPs) to have blunt ends.
4. Ligation with EcoRI adapters (= restriction site): blunt ends T4 DNA ligase Purification
5. Ligation DNA adapter with T7 DNA ligation: EcoRI cohesive ends ligation
6. Purification

<u>Objectives</u>:

➔ To define the maximum possible theoretical size in the assembly.
➔ To see how the assembly of a large DNA molecule is done. Identify potential limitations or problems.
➔ Produce larger reads during nanopore sequencing: identify critical points of the protocol to avoid damaging large DNA molecules.
➔ Bioinformatics: challenge to see if we can (re)found the information contained in the plasmid just with adapter info.