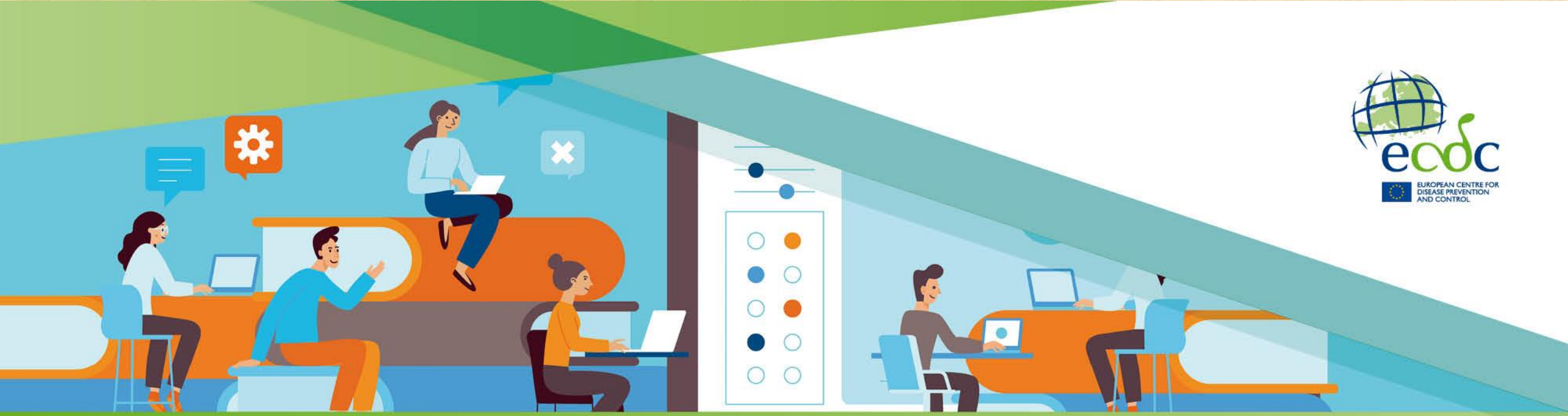


GenEpi-BioTrain Virtual Training 27

Clinical application of Nanopore for diagnostic 16S rDNA sequencing analysis

2026-05-15

Anna Norén, Elin Loo, Lili Andersson-Li, Samuel Lampa, Sofia Stamouli

Content overview

- Introduction
- Session 1: 16S sequencing background and analysis tools
  - Part 1: Background: 16S biology, Nanopore sequencing
  - Part 2: Open-source tools for ONT 16S sequencing data analysis
  - Intro to exercises
- Session 2: Interpretation of results
  - Part 1: Reporting, alignment quality metrics
  - Part 2: Clinical interpretation
  - Recap of exercises

Aim and learning objectives

Aim

The aim of this session is to give the learners a good understanding of why 16S sequencing is routinely applied in clinical settings, and why Nanopore sequencing is a good fit.

Specific learning objectives

At the end of this session, you will be able to:

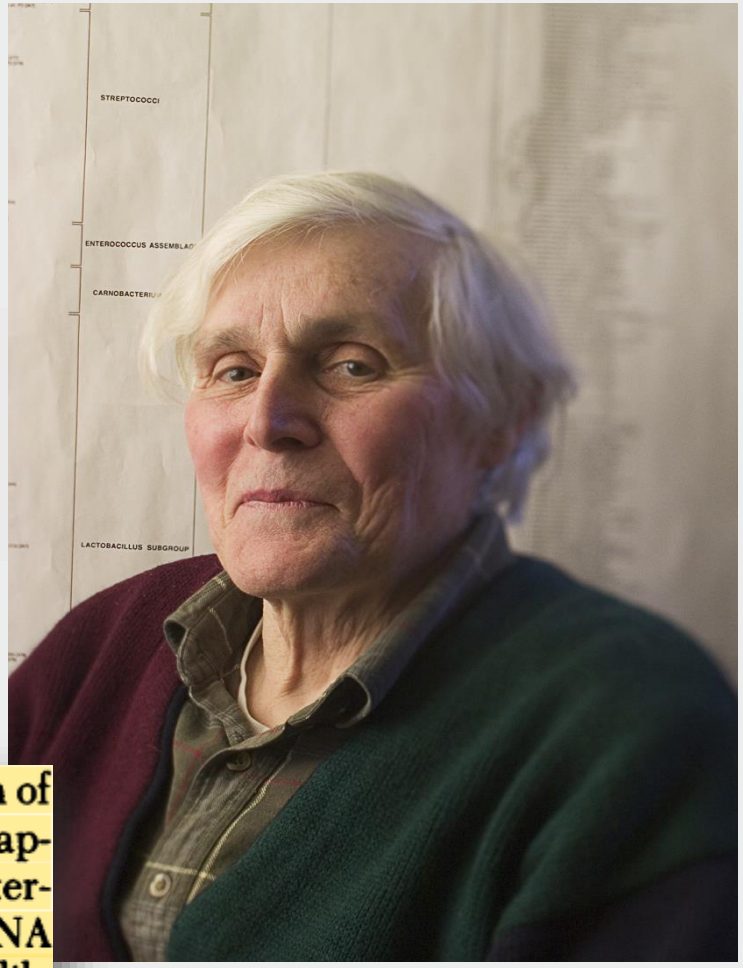
1. Explain why the 16S rRNA gene is used for bacterial species assignment
2. Explain why Nanopore sequencing is suitable for 16S sequencing
3. Explain in which situations 16S sequencing is regularly used

Session 1

16S sequencing background and analysis tools

The history of 16S rRNA

- Carl R. Woese suggested the use of 16S rRNA gene to classify bacteria already in 1977, in a paper with George E. Fox



Proc. Natl. Acad. Sci. USA
Vol. 74, No. 11, pp. 5088–5090, November 1977
Evolution

Phylogenetic structure of the prokaryotic domain: The primary kingdoms

(archaebacteria/eubacteria/urkaryote/16S ribosomal RNA/molecular phylogeny)

CARL R. WOESE AND GEORGE E. FOX*

Department of Genetics and Development, University of Illinois, Urbana, Illinois 61801

Communicated by T. M. Sonneborn, August 18, 1977

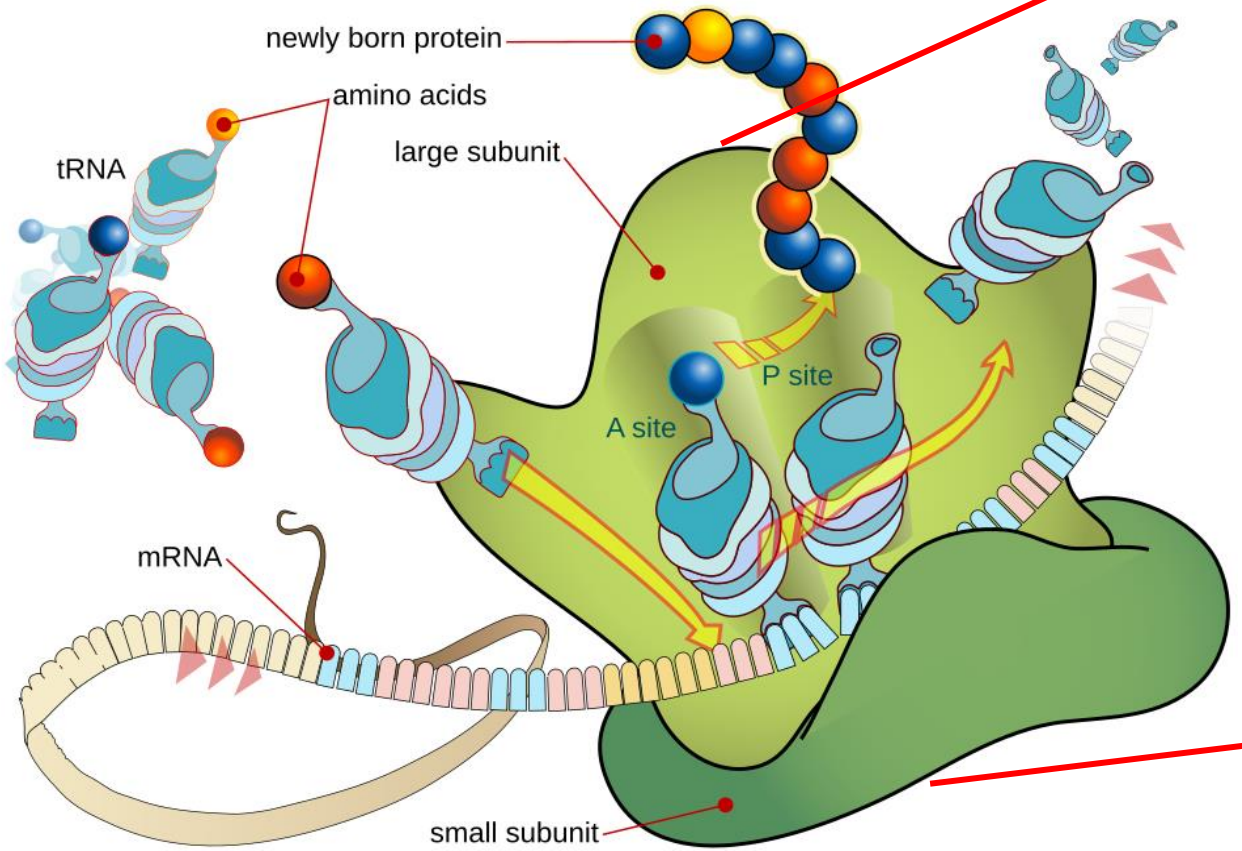
ABSTRACT A phylogenetic analysis b
RNA sequence characterization reveals
represent one of three aboriginal lines o
bacteria, comprising all typical bacteria; (*i*
containing methanogenic bacteria; and (*iii*
represented in the cytoplasmic compo
cells.

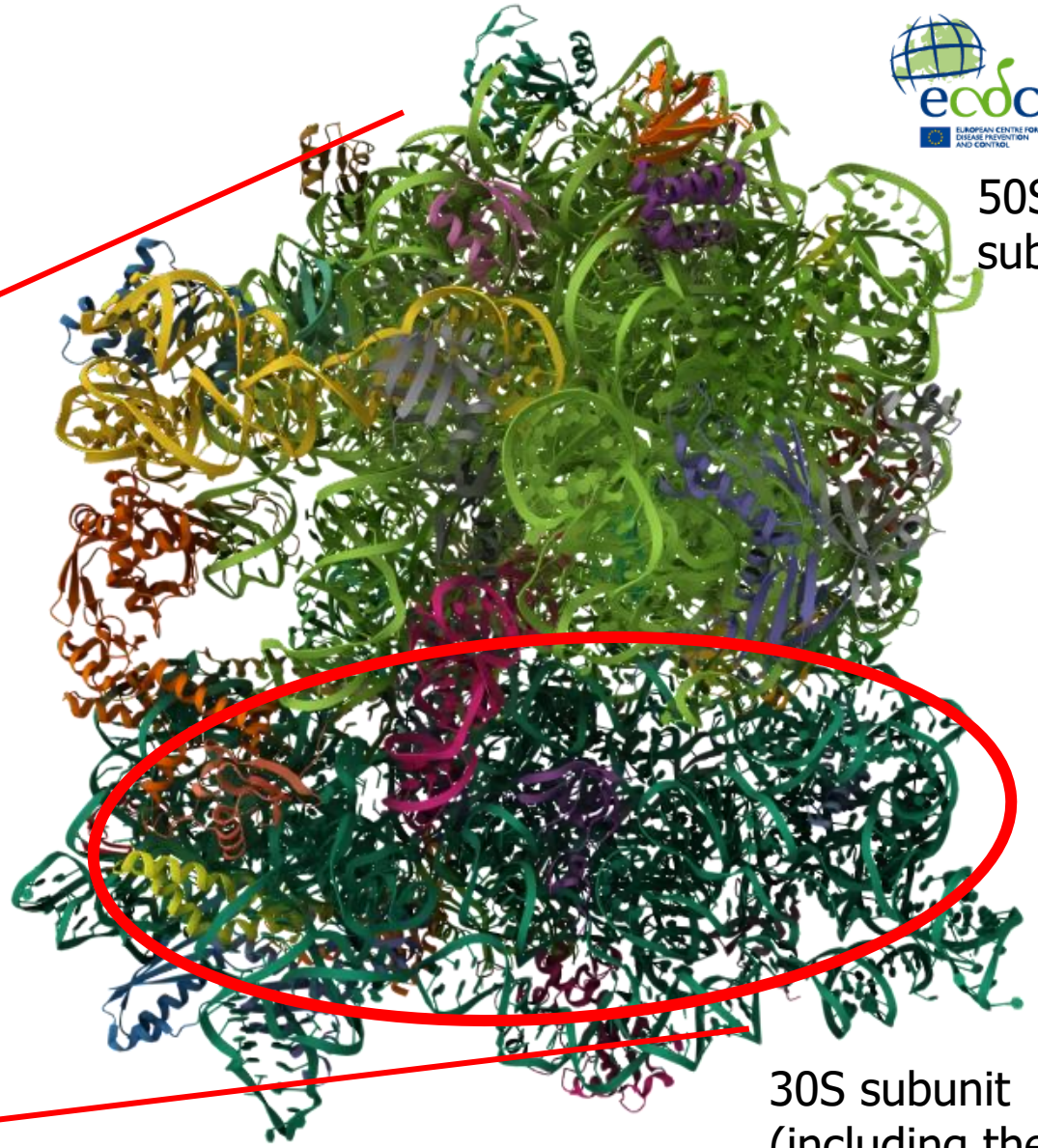
The biologist has customarily structured
certain basic dichotomies. Classically, wh
animal. The discovery that bacteria, whi
considered plants, resembled both plants
plants and animals resembled one anothe
tion of the issue in terms of a yet more bas
eukaryote versus prokaryote. The striking differences between
eukaryotic and prokaryotic cells have now been documented
in endless molecular detail. As a result, it is generally taken for

> To determine relationships covering the entire spectrum of extant living systems, one optimally needs a molecule of appropriately broad distribution. None of the readily characterized proteins fits this requirement. However, ribosomal RNA does. It is a component of all self-replicating systems; it is readily isolated; and its sequence changes but slowly with time—permitting the detection of relatedness among very distant species

domain has arisen but once; all extant eukaryotes stem from a
common ancestor, itself eukaryotic (2). A similar prejudice holds
for the prokaryotic domain (2). [We elsewhere argue (6) that
a hypothetical domain of lower complexity, that of "pro-

The bacterial ribosome

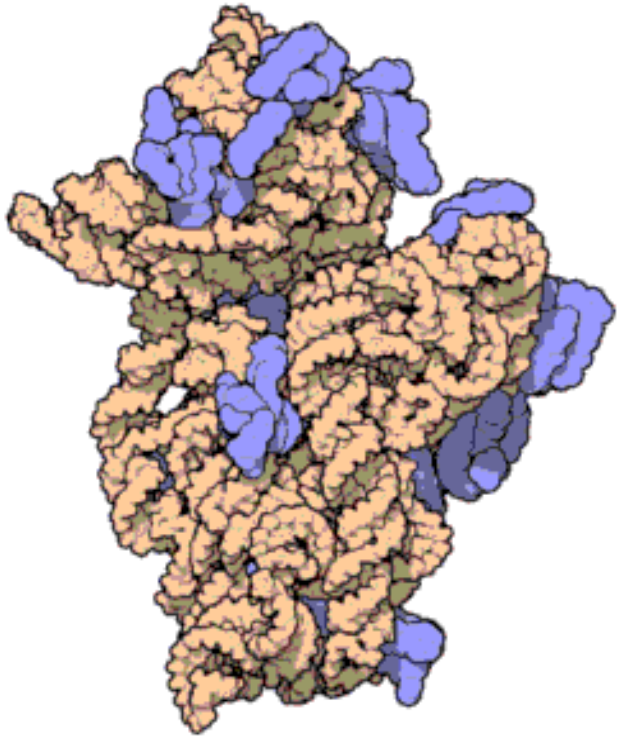




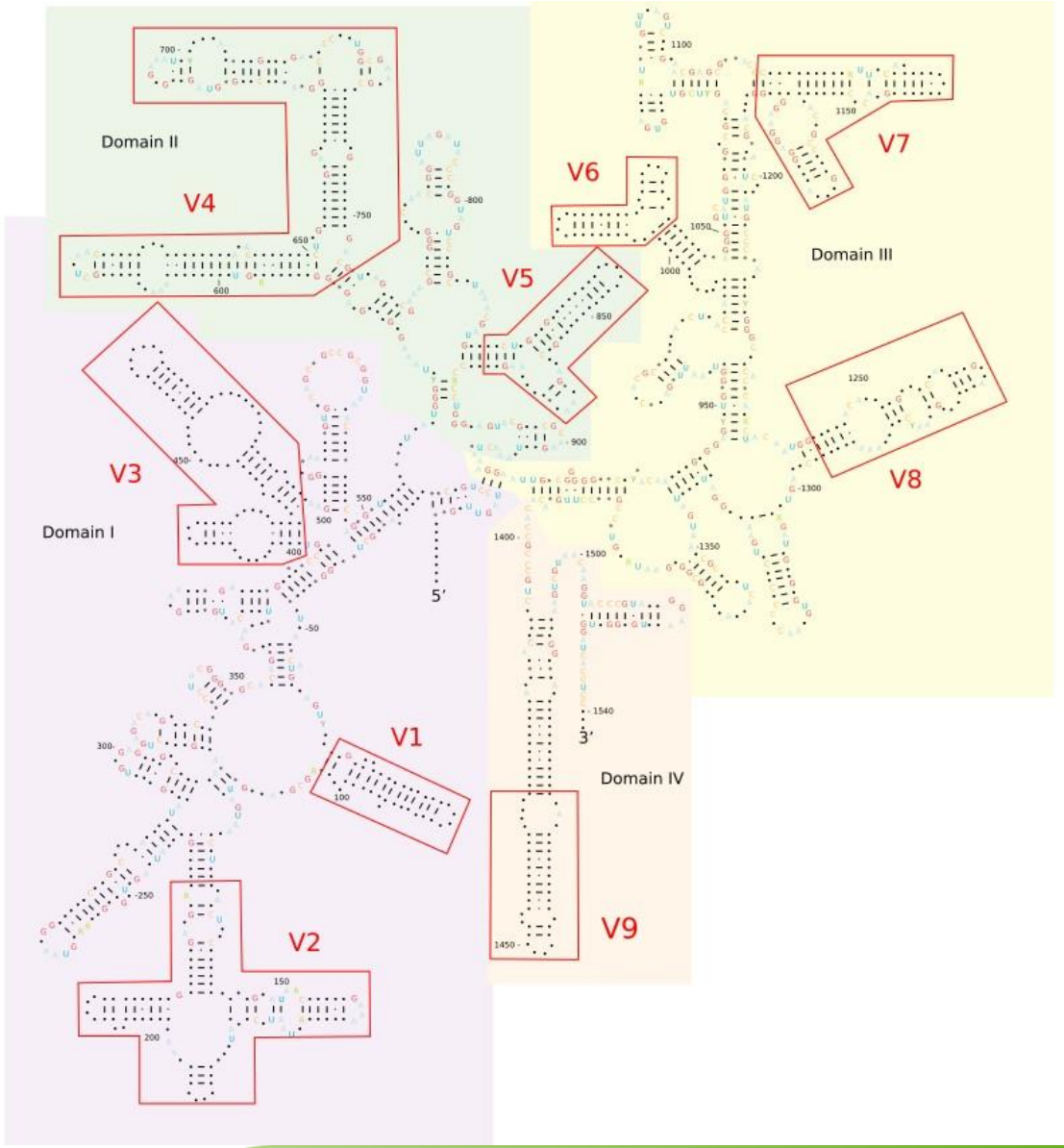
50S subunit

30S subunit (including the 16S rRNA)

16S rRNA

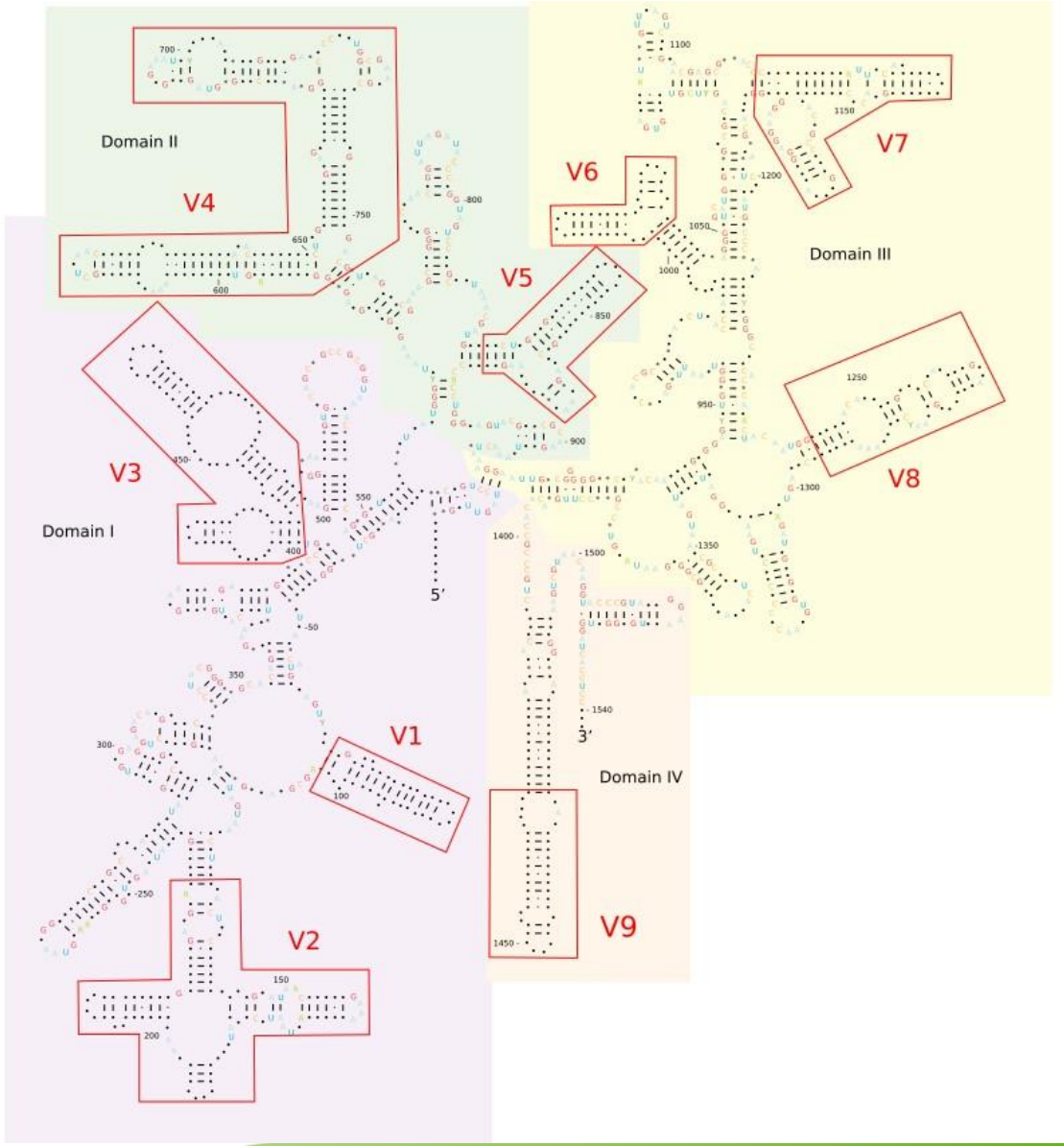


Animation by David S. Goodsell, RCSB Protein Data Bank - Molecule of the Month at the RCSB Protein Data Bank, Public Domain, https://commons.wikimedia.org/w/index.php?curid=2839678



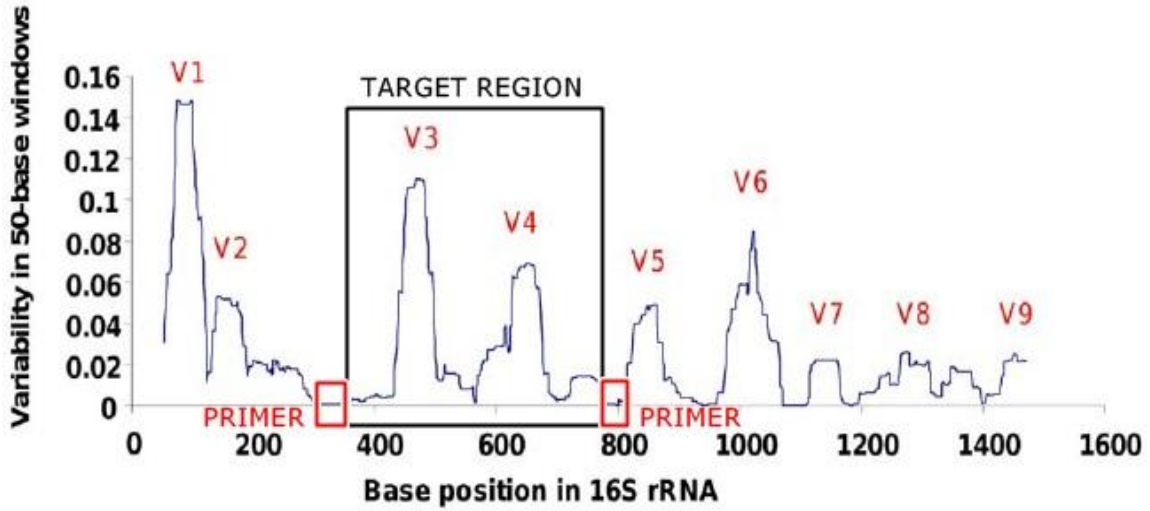
The 16S rRNA molecule and gene

- Uses the gene for the 16S RNA subunit of the prokaryotic ribosome
- Virtually all bacteria have at least one copy
- Bacteria can have 1-15 copies but most common with 2-7
- The full length is ca 1500 bp
- 9 hyper variable regions V1-V9 spread out across the gene.



The 16S rRNA molecule and gene

- Short-read NGS only covers selected variable regions, e.g: V1-V2 (300bp),V3-V4 (400bp)



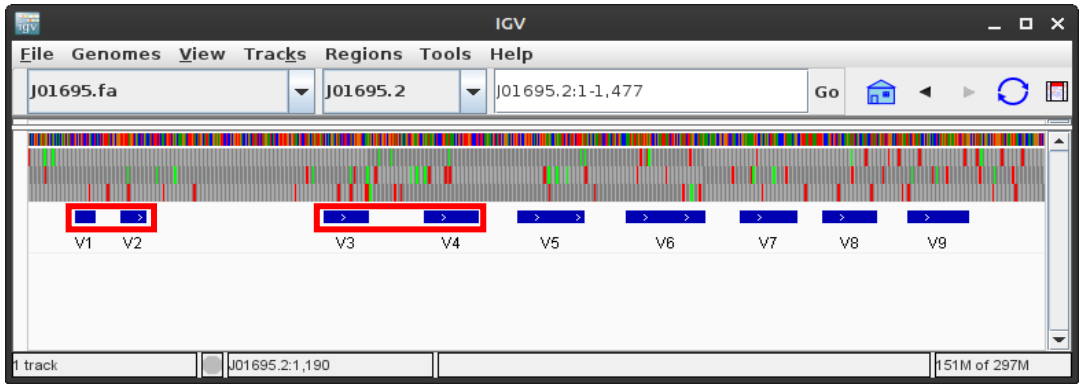


Image: Bodilis J, Nsigue-Meilo S, Besaury L, Quillet L. (2012). *Variable Copy Number, Intra-Genomic Heterogeneities and Lateral Transfers of the 16S rRNA Gene in Pseudomonas.* PLoS ONE 7(4). Via Wikimedia Commons. CC BY 4.0

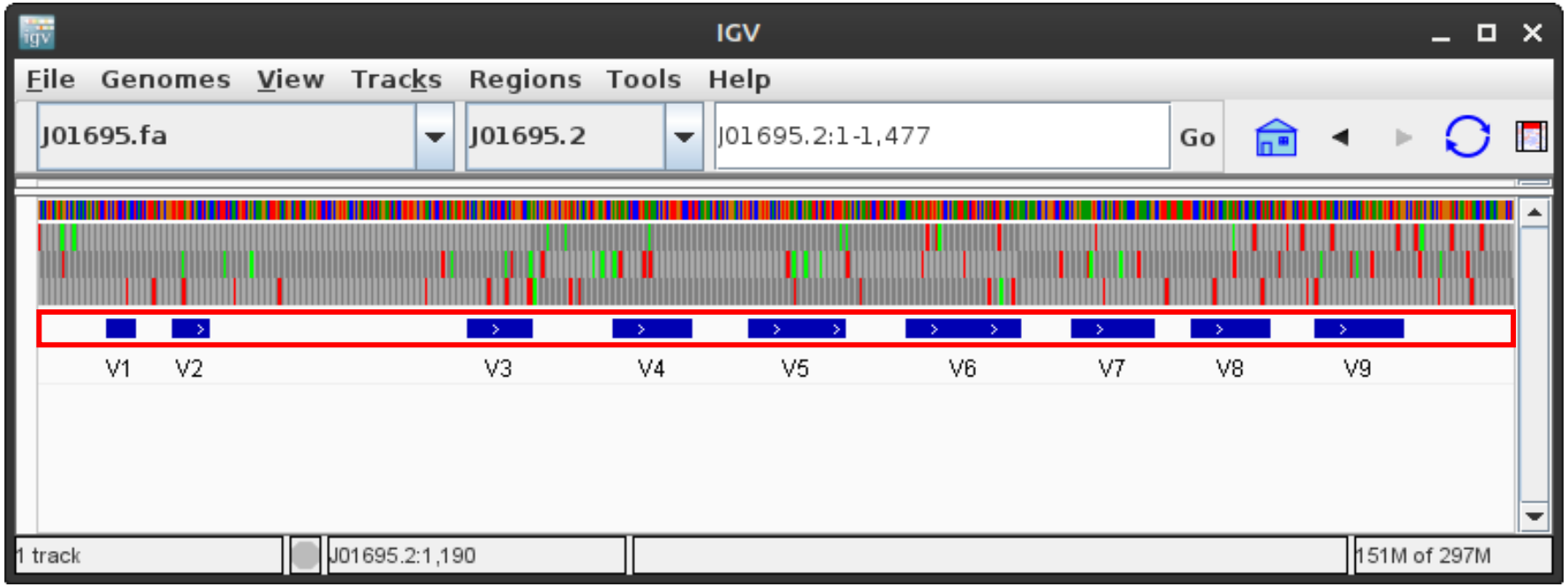
Poll slide

Why would you use the 16S gene for bacterial species typing?

Why would you use the 16S gene for bacterial species typing?

Why Oxford Nanopore (ONT) sequencing for 16S?

- Long read: can covers the full ~1500 bp gene, covering V1-V9 plus sequences in between in single reads
- Allows to reach species level rather than only genus level



Oxford Nanopore Sequencing

- Single DNA molecule strands passing through a protein based nanopore
- Bases passing through the pore creates a change in the Ion pore current passing through the pore, which can be detected.

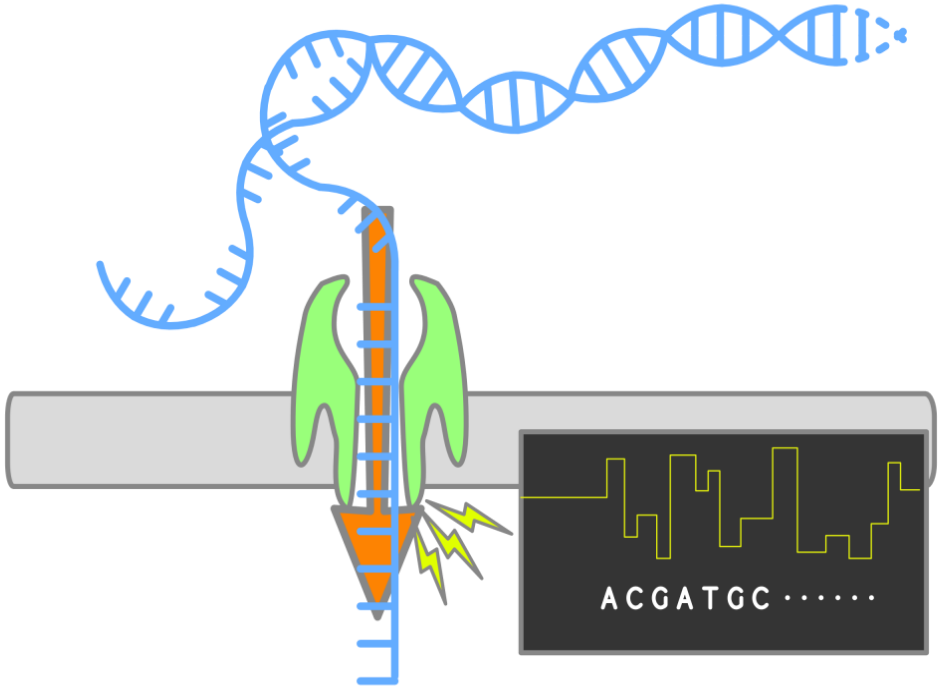


Figure By DataBase Center for Life Science (DBCLS) - https://doi.org/10.7875/togopic.2020.01, CC BY 4.0, https://commons.wikimedia.org/w/index.php?curid=86372818

Unique features of Oxford Nanopore Sequencing

- Native DNA sequencing
  - PCR not strictly needed (but still used for 16S sequencing)
- Real-time sequencing
  - Data can be analyzed while sequencing is on-going.
- Adaptive sequencing
  - Unwanted reads can be ejected to enhance yield of wanted regions

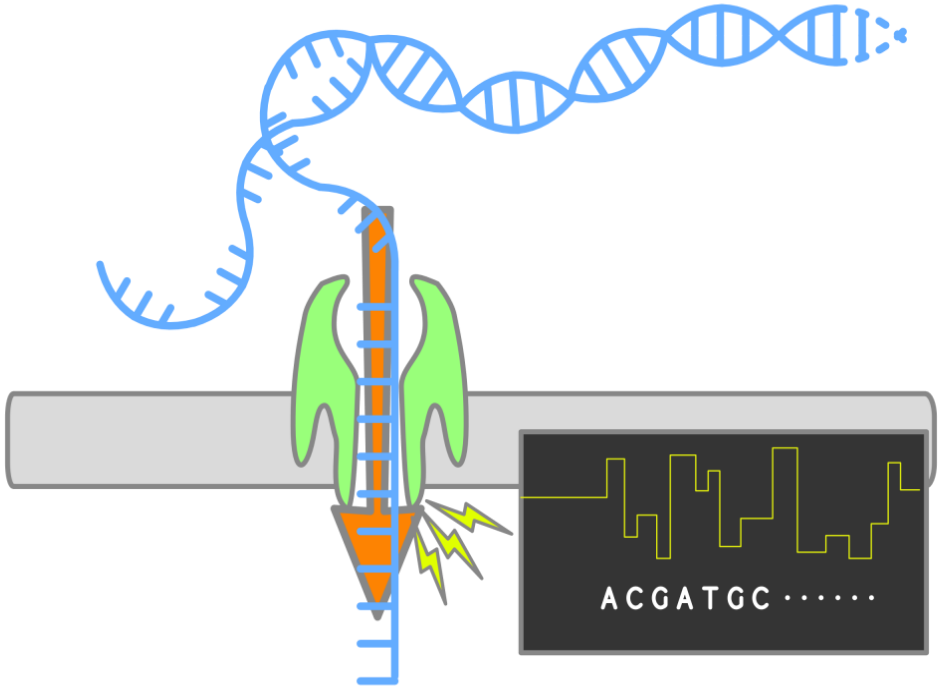


Figure By DataBase Center for Life Science (DBCLS) - https://doi.org/10.7875/togopic.2020.01, CC BY 4.0, https://commons.wikimedia.org/w/index.php?curid=86372818

Main challenge of: Oxford Nanopore Sequencing

- The electrical signal from the nanopore needs base calling
- Signal patterns don't correspond to individual bases, but to a window of bases "k-mers"
- This makes base calling computationally demanding.
- Is typically run on GPUs (Dorado)
- The quality is still not on par with Illumina.

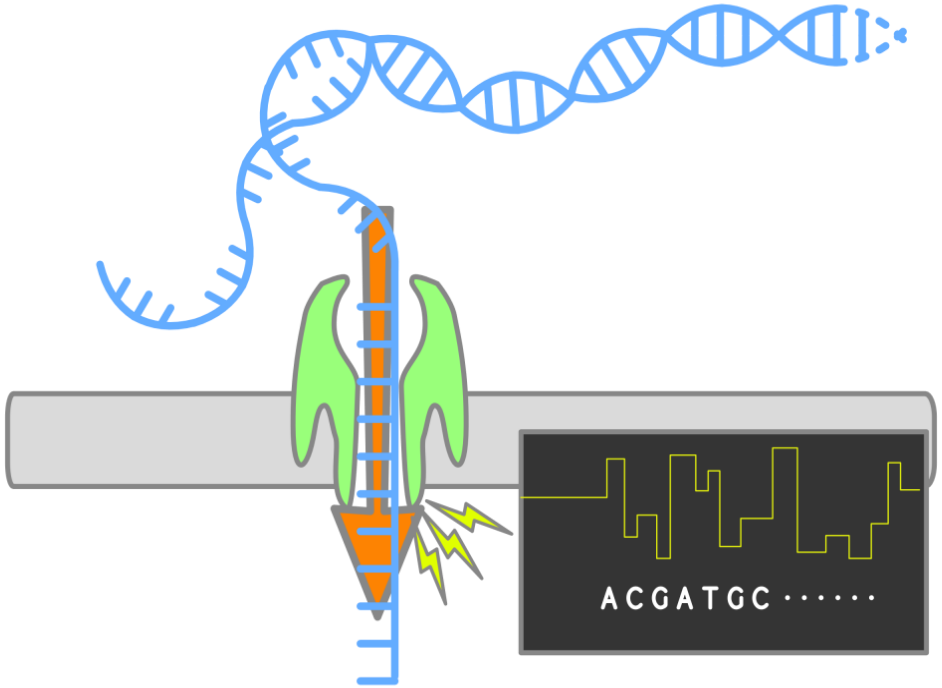
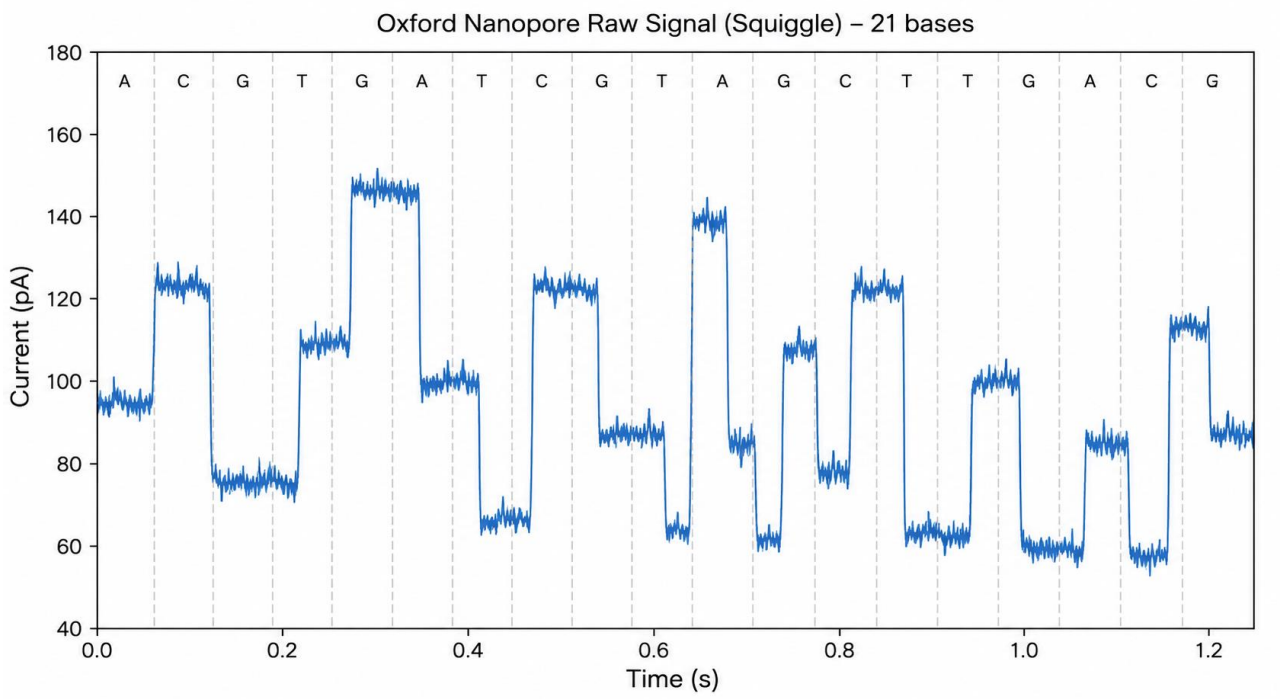


Figure By DataBase Center for Life Science (DBCLS) - https://doi.org/10.7875/togopic.2020.01, CC BY 4.0, https://commons.wikimedia.org/w/index.php?curid=86372818

Nanopore base call quality

- Dependent on base calling mode (high vs super accuracy)
- Q15-Q20
  (Q20 = 99% accuracy)
- Requires consideration to reach species level accuracy



What are some advantages of Oxford Nanopore sequencing for 16S analysis?

🤔

What are some advantages of Oxford Nanopore sequencing for 16S analysis?

Why is the basecalling of Nanopore so computationally demanding?

🤔

Why is the basecalling of Nanopore so computationally demanding?

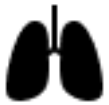
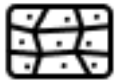
Clinical 16S sequencing: When and why?

- In most cases, culture-based methods are the primary test
- Cultivation is important because it can also detect antibiotic resistance
- 16S sequencing is useful when cultivation is insufficient or misses bacteria

Examples where 16S sequencing may help:

- Patients with prior antibiotic exposure
- Anaerobic bacteria that are difficult to culture
- Slow-growing bacteria
- Unknowns

Sample types

- Cerebrospinal fluid (CSF) 
- Synovial fluid 
- Pleural fluid
- Bronchoalveolar lavage (BAL) 
- Tissue 
- Other
  - Eye samples 
  - Abscesses 
  - Abdominal tap

Wet lab process for 16S Nanopore sequencing

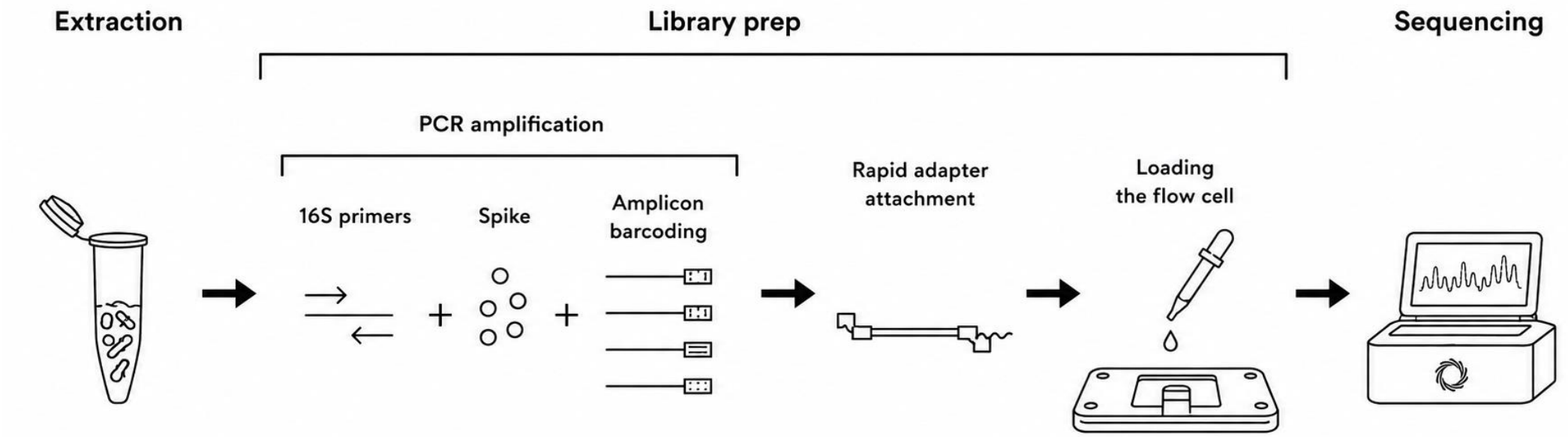


Image: Generated with ChatGPT / Dall-E based on a detailed description of the process. The image has been manually vetted for accuracy.

For which samples is 16S sequencing analysis beneficial? 🤔

For which samples is 16S sequencing analysis beneficial?

In summary

List of learning points in this session:

- The 16S rRNA gene is present in virtually all bacteria and contains 9 hypervariable regions (V1–V9) used for species identification
- Nanopore long reads cover the full ~1500 bp gene, enabling species-level identification
- Short-read NGS (e.g. Illumina) only covers selected variable regions, limiting resolution to genus level
- ONT base call quality (Q15–Q20) requires some special considerations to achieve reliable species-level accuracy
- 16S sequencing complements culture when cultivation fails - e.g. prior antibiotic exposure, slow-growing bacteria etc.

Recommended material to explore further

- Johnson, Jethro S., et al.
  "**Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis.**"
  Nature communications 10.1 (2019): 5029.
  https://doi.org/10.1038/s41467-019-13036-1
- Curry, Kristen D., et al.
  "**Emu: species-level microbial community profiling of full-length 16S rRNA Oxford Nanopore sequencing data.**"
  Nature methods 19.7 (2022): 845-853.
  - Paper: https://doi.org/10.1038/s41592-022-01520-4
  - BioRxiv: https://doi.org/10.1101/2021.05.02.442339

Acknowledgements

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The revision and update of this training material was commissioned by ECDC to Karolinska University Hospital with the direct involvement of Anna Norén, Elin Loo, Lili Andersson-Li, Samuel Lampa, Sofia Stamouli