BARKODING DNA BAKTERI

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- **DNA barcoding: towards an inventory of life**

- A DNA barcode is a short gene sequence taken from a standardized portion of the genome, used to identify species

What is DNA Barcoding

- A way of identifying samples based on a short standardized gene-region

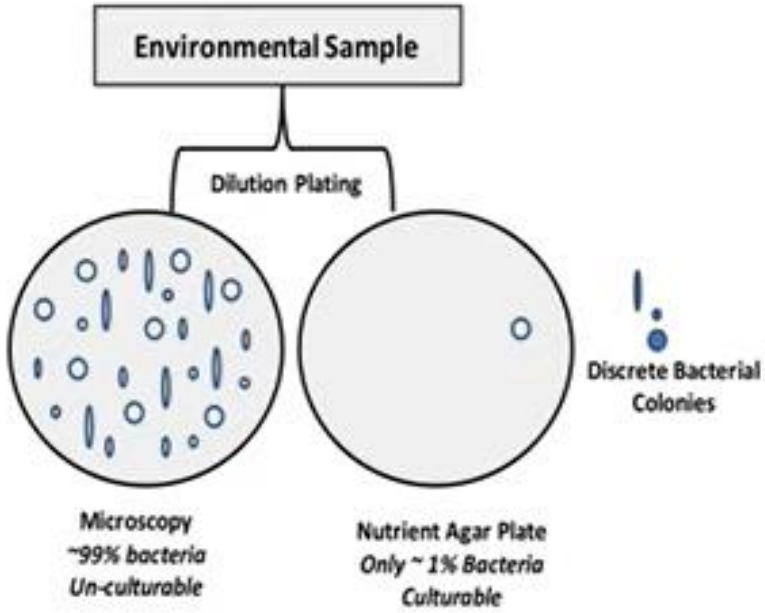
- Keywords : Identify , Samples , Species , Gene , Short , Standardized

DNA Bar-Code for Identification of Microbial Communities

- new molecular techniques have enabled scientist for robust identification of species.
- DNA barcode system provides authentic species identification in large number without delay and error.
- Several gene markers have been used for the identification of diverse microbial communities in DNA barcoding system.
- For the identification of bacteria, *16S rRNA* gene is an important marker.
- There is no specific marker for virus. → outer core protein and *K-mer* based barcode system for identification of blue tongue virus (BTV) and human enterovirus (HEVs), respectively

Great plate count anomaly

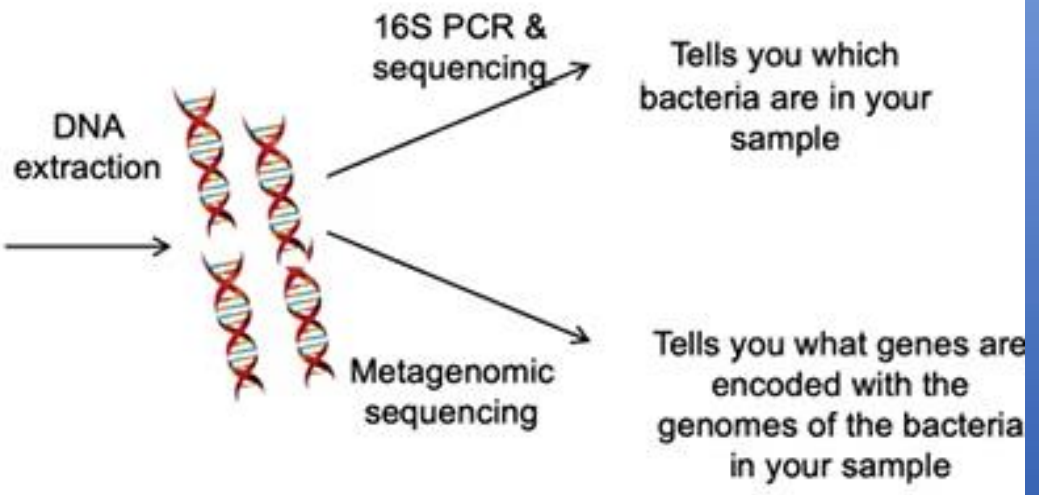
- Many more bacterial cells observed under the microscope than grown under standard laboratory conditions
- Need culture independent methods to examine the uncultivated majority



Harwani, 2013, Microbiology 2(9)

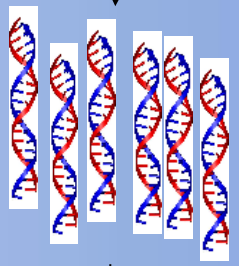
Molecular detection of microbiota

- Detection of microbial DNA directly from the environment
  - Sequencing of taxonomic marker genes (16S sequencing)
  - Sequencing of all DNA (metagenomics)

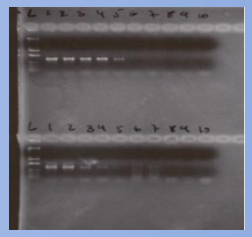


How do we estimate bacterial community composition ?

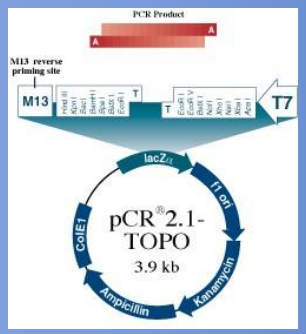
DNA extraction



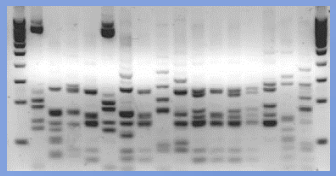
Primer design and PCR



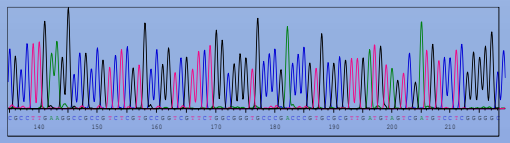
TA cloning



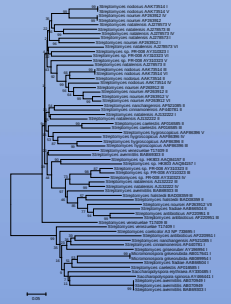
Library screening



Sequencing



Phylogenetic analysis

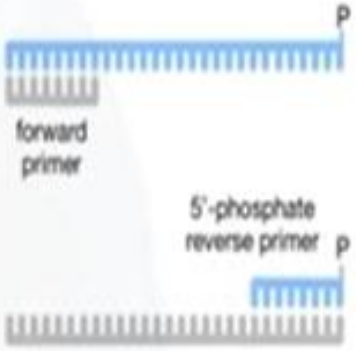


Comparison to other samples – hypothesis testing

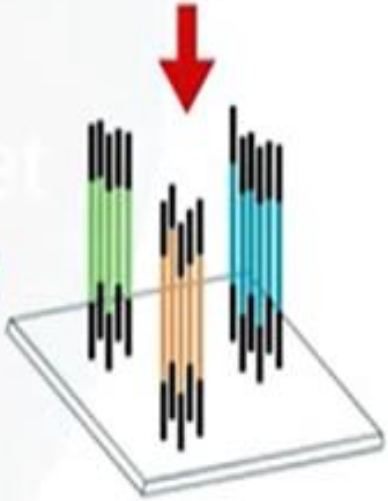
Principle of 16S rRNA Gene Sequencing

Sample

DNA Extraction



PCR



DNA Library Construction

DNA Sequencing

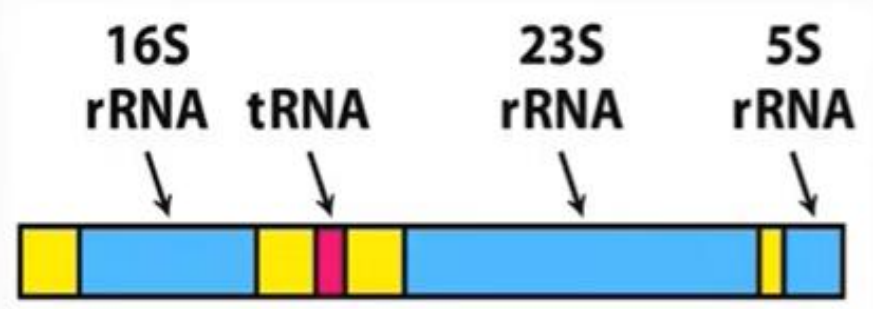
DNA Analysis

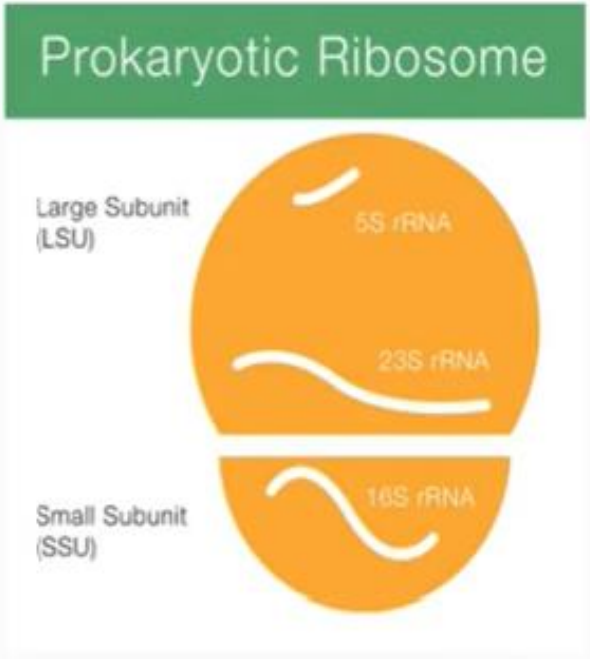
16S rRNA

CD Genomic
The Genomics Services Company

rRNA:

1. Universality
2. Activity in cellular functions
3. Extremely conserved structure and sequence

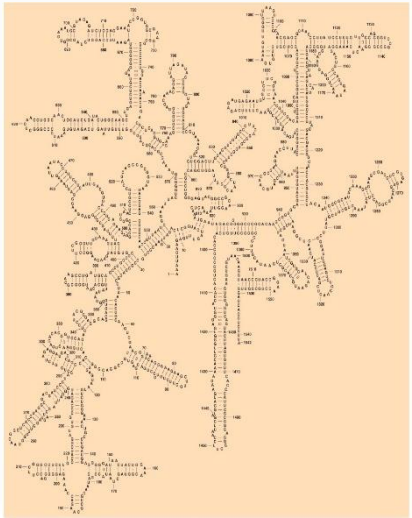


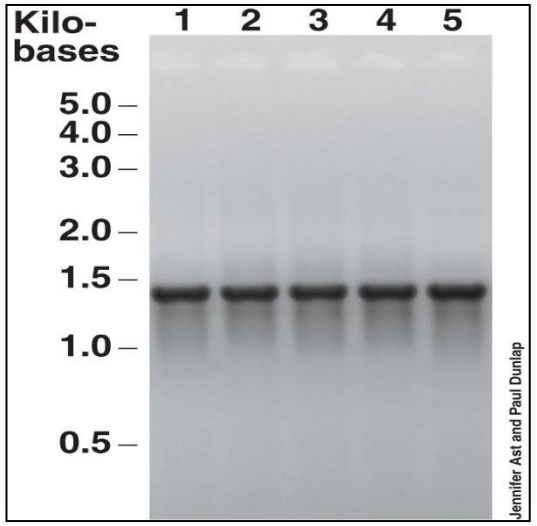


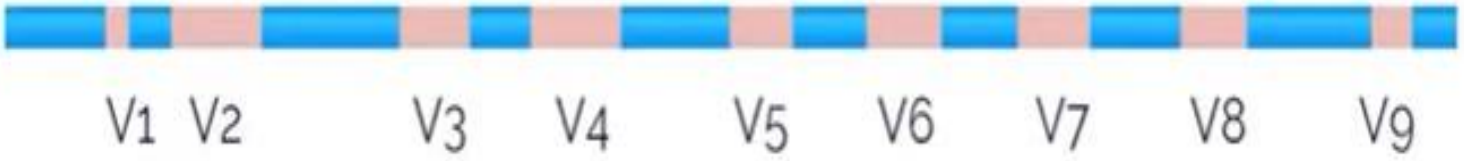
What Is 16s rRNA sequencing?

16S rRNA:

- ✓ Risosomal RNA
- ✓ Phylogenetic markers
- ✓ 1542 bp







Library Preparation

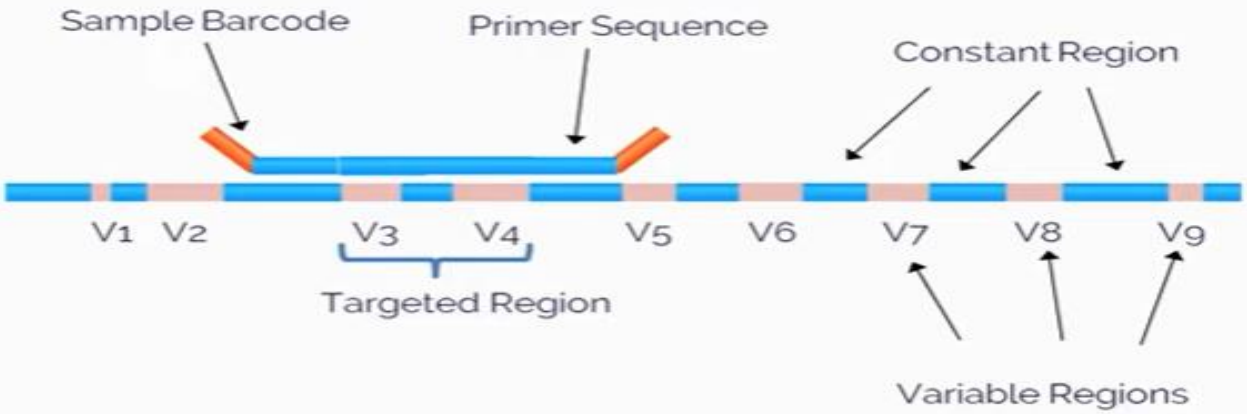


Table 1. Primer sets for the amplification of 16S rRNA. F=forward, R=reverse

Name of primer	Sequence	Name of primer	Sequence
8F	AGAGTTTGATCCTGGCTCAG	785F	GGATTAGATACCCTGGTA
27F	AGAGTTTGATCMTGGCTCAG	805R	GACTACHVGGGTATCTAATCC
336R	ACTGCTGCSYCCCGTAGGAGTCT	806RB	GGACTACNVGGGTWTCTAAT
337F	GACTCCTACGGGAGGCWGCAG	907R	CCGTCAATTCCTTTRAGTTT
337F	GACTCCTACGGGAGGCWGCAG	928F	TAAAACTYAAAKGAATTGACGGG
341F	CCTACGGGNGGCWGCAG	1100F	YAACGAGCGCAACCC
515FB	GTGYCAGCMGCCGCGGTAA	1100R	GGGTTGCGCTCGTTG
518R	GTATTACCGCGGCTGCTGG	1492R	CGGTTACCTTGTTACGACTT
533F	GTGCCAGCMGCCGCGGTAA		

CD Genomics
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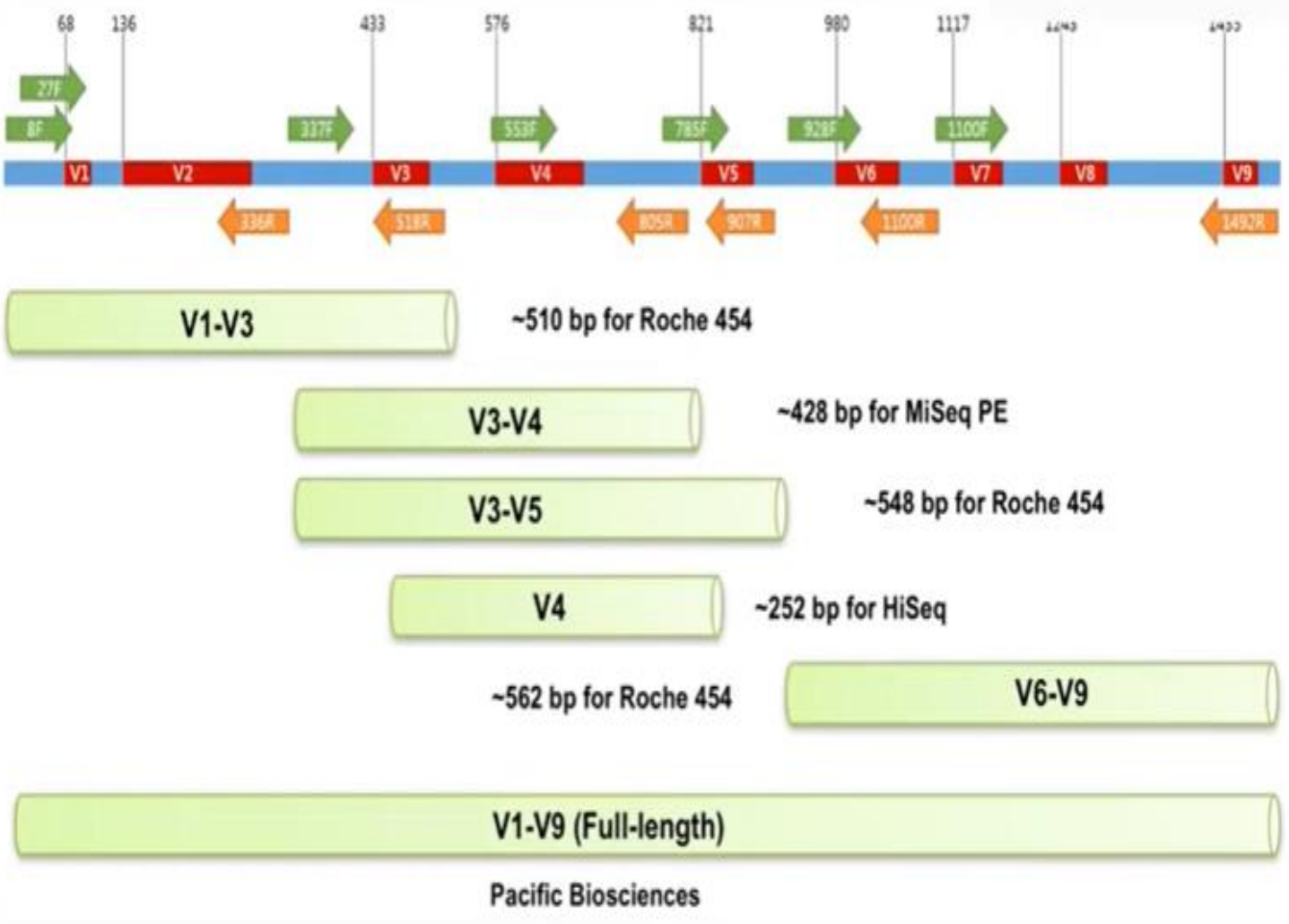
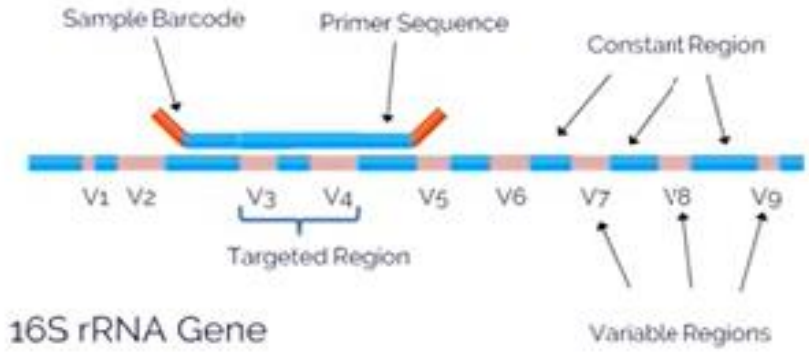
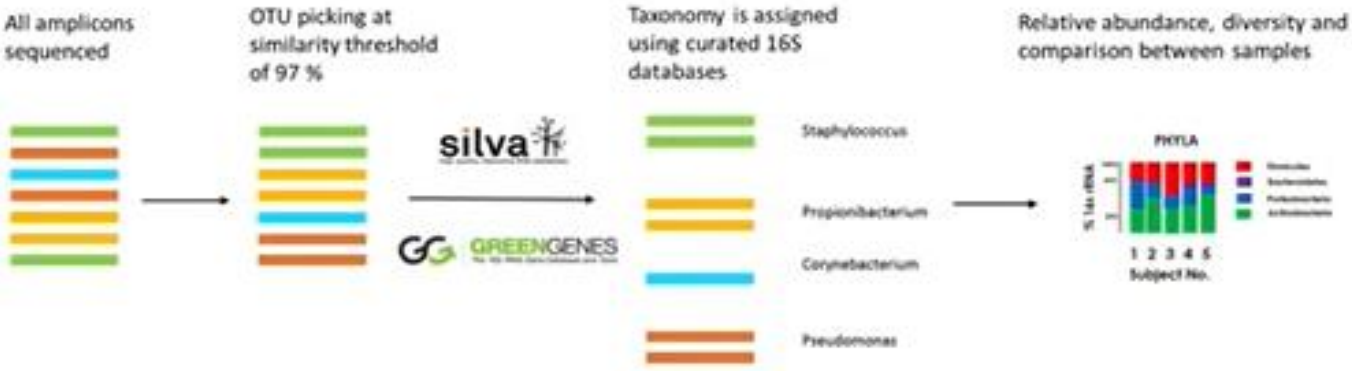


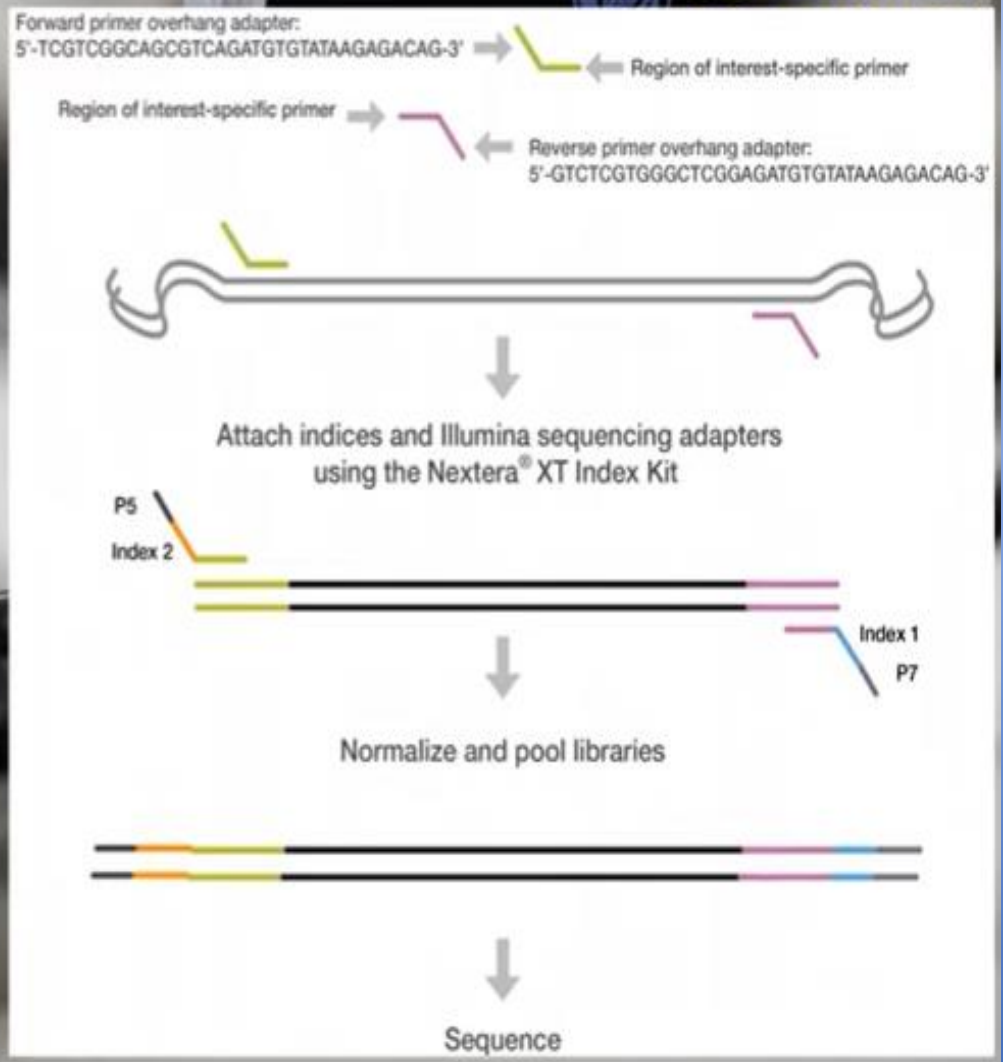
16S rRNA gene sequencing



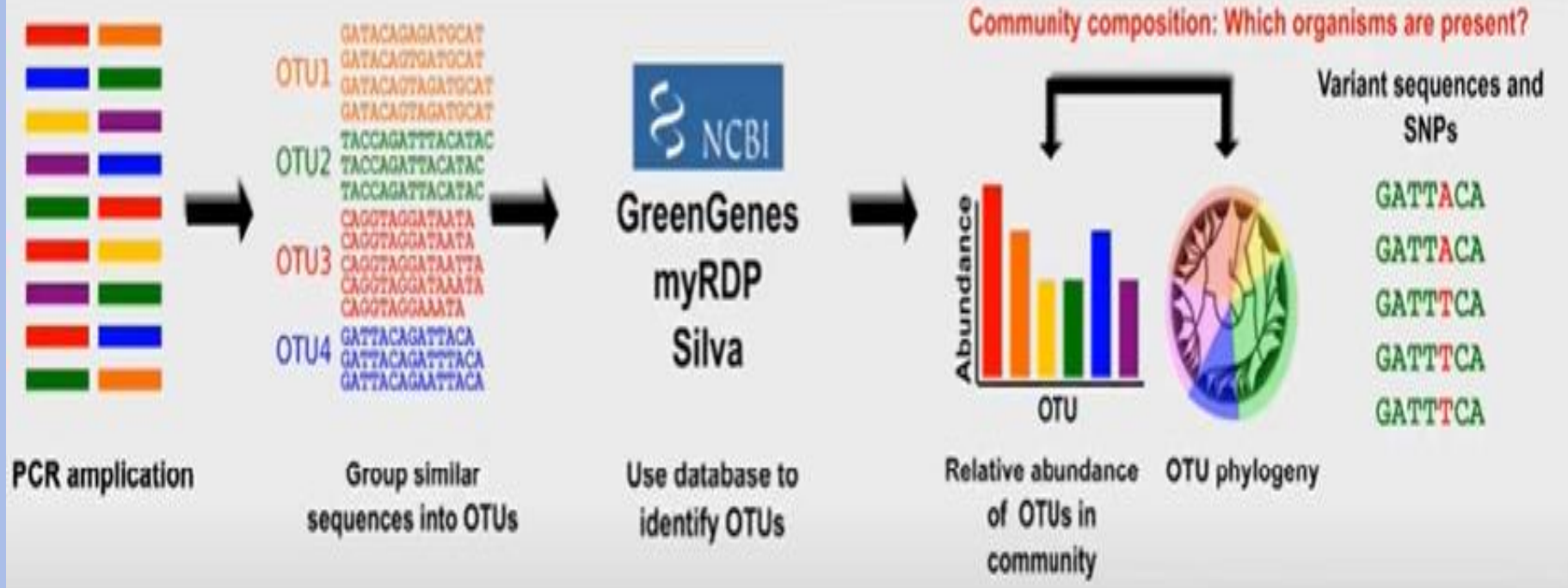


Sequencing

- For example: Illumina MiSeq
  - V3 and V4 region
  - Purification
  - Quantification
  - Pool



Bioinformatics

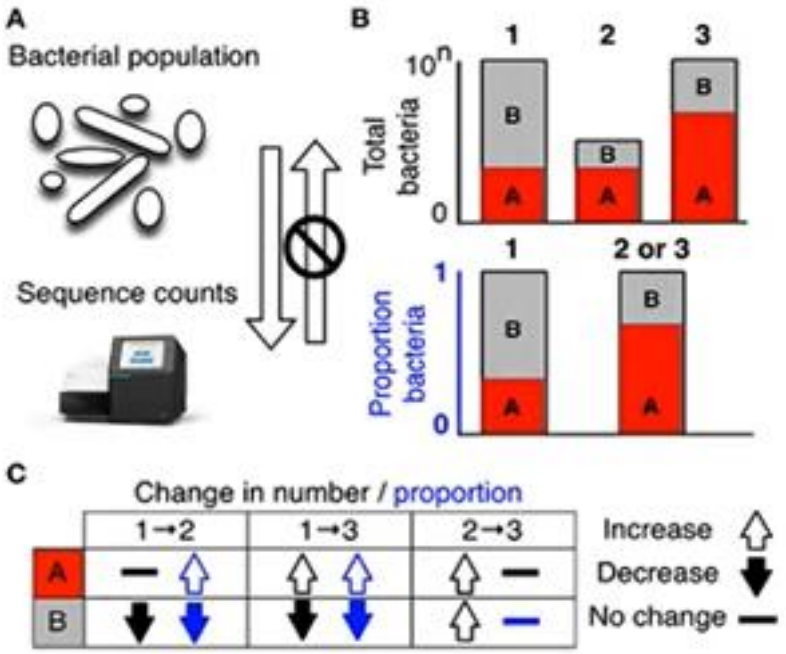


Advantages of 16S data

- Relatively cheap
- High throughput
  - Many samples, increase statistical power
- Well developed analysis tools and reference databases

Limitations of 16S data

- Taxonomy only, and at limited resolution
- Relative proportions of bacterial taxa
  - Not quantitative
  - Compositional



Gloor et al., 2017, Frontiers in Microbiology, 15

APPLICATIONS

- 16S rRNA gene sequencing is a powerful tool for determining the species composition and community structure of microbial populations within complex biological samples. This approach enables the study of microbial diversity and provides insights into how microbial communities interact within their ecosystems.
- In clinical microbiology, 16S rRNA gene sequencing is employed to investigate microbial communities associated with diseases. This aids in understanding the mechanisms of disease and in the development of targeted treatments.
- This technique is also valuable for identifying and classifying microorganisms in a variety of environmental samples, such as soil and water.
- In the food industry, 16S rRNA gene sequencing can be used to study microorganisms in fermented foods and to identify food-borne pathogens. This contributes to food safety by enabling the identification of microbial communities present in food products.

APPLICATIONS

- In agriculture, this method is utilized to enhance crop health by examining the microbial communities associated with soil and plants.
- In agriculture, this method is utilized to enhance crop health by examining the microbial The study of the human microbiome also benefits from 16S rRNA gene sequencing, as it allows researchers to explore microbial diversity and its impact on human health.
- Additionally, comparing bacterial 16S rRNA sequences can lead to the discovery of novel species.
- Examples of applications of 16S rRNA gene sequencing include the Human Microbiome Project, which characterizes human-associated microbial communities; the Global Ocean Sampling Expedition, which explores microbial diversity in the oceans; soil microbiome studies; research into antibiotic resistance in hospitals; and monitoring microbial communities in wastewater treatment facilities.

Summary

- Molecular methods overcome culturing bias
- 16S rRNA gene sequencing commonly used to characterise microbiome
- Cheap and accessible
- Data has limitations

Review Article

DNA Barcoding on Bacteria: A Review

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Bacteria are omnipotent and they can be found everywhere. The study of bacterial pathogens has been happening from olden days to prevent epidemics, food spoilage, losses in agricultural production, and loss of lives. Modern techniques in DNA based species identification are considered. So, there is a need to acquire simple and quick identification technique. Hence, this review article covers the efficacy of DNA barcoding of bacteria. Routine DNA barcoding involves the production of PCR amplicons from particular regions to sequence them and these sequence data are used to identify or "barcode" that organism to make a distinction from other species.

1. Introduction

Nowadays DNA barcoding has become a justifiable tool for the assessment of global biodiversity patterns and it can allow diagnosis of known species to nontaxonomists [1]. DNA barcoding is a fast, accurate, and standardized method for species level identification, by using short DNA sequences. In 2003, Hebert from the University of Guelph, Ontario, Canada, proposed a new technique called DNA barcoding. Hebert and his associates published a paper entitled "*Biological identifications through DNA barcodes*". They proposed a new system of species identification, that is, the discovery of species by using a short segment of DNA from a standardized region of the genome. That DNA sequence can be used to identify different species. DNA barcoding is the standardized research that facilitates biodiversity studies like species identification and discovery. This technique helps researchers to understand genetic and evolutionary relationships by assembling molecular, morphological, and distributional data [2]. Species-level identification by DNA barcoding is usually adapted by the recovery of a short DNA sequence from a standard part of the genome [3]. The sequence of barcode from each unknown specimen was then compared with a library of reference barcode sequences obtained from individuals of recognized identity [4]. DNA barcoding is an obligatory tool for species detection and specimen identification [5]. Using standardized identification method is very advantageous for mapping of all the species on Earth, especially when DNA sequencing technology is inexpensively obtainable. The term "DNA barcode" suggests that the standardized DNA sequences can identify taxa in the same way as the 11-digit Universal Product Code identifies retail products in market [6]. Lambert et al. (2005) scrutinized the opportunity of using DNA barcoding to measure the past diversity of the Earth's biota [7].

The barcode of life data system (BOLD) is an informatics workbench assisting the possession, analysis, storage, and publication of DNA barcode records. It links a traditional bioinformatics opening by collecting morphological, molecular, and distributional data. BOLD is freely accessible to any researcher with awareness in DNA barcoding. It helps the assembly of records that meet the standards required to gain barcode designation in the global sequence databases by affording specialized services [8]. BOLD could serve as the universal starting point for identification of species, which would convey users to refer to specialized databases, for example, pathogenic strains, endangered species, and disease vector species [9]. DNA barcoding study

ANALISIS SEKUENSING 16S rRNA DI BIDANG MIKROBIOLOGI

Tristia Rinanda

Abstrak. Gen 16S ribosomal RNA (16S rRNA) memiliki daerah yang *conserved* (lestari) sehingga tepat digunakan dalam *Polymerase Chain Reaction* (PCR) dan analisis sekuensing untuk menentukan taksonomi, filogeni dan keanekaragaman antar spesies. Gen ini juga memiliki *hypervariable region* yang merupakan ciri khas tiap mikroorganisme. Analisis sekuensing gen 16S rRNA sudah banyak digunakan di bidang mikrobiologi. Metode berbasis molekuler ini dinilai cepat dan akurat dalam mengidentifikasi bakteri patogen serta memiliki sejumlah keunggulan dibandingkan metode mikrobiologi konvensional. ***(JKS 2011; 3:172-177)***

Kata kunci : Analisis sekuensing, 16s rRNA, mikrobiologi

Abstract. The 16S rRNA gene is the most conserved DNA in all cells. For this reason, genes that encode the rRNA (rDNA) have been used extensively on PCR and sequencing analysis method to determine taxonomy, phylogeny (evolutionary relationships) and also to estimate rates of species divergence. This gene also consists of hypervariable region which is specifically characterized every organism Sequencing analysis has been widely use in microbiology. This molecular based method is becoming a powerful technology for identification of bacterial isolates in the human clinical diagnostic laboratory and also has several advantages compare to conventional method. ***(JKS 2011; 3:172-177)***

Key words: Sequencing analysis, 16S rRNA, microbiology

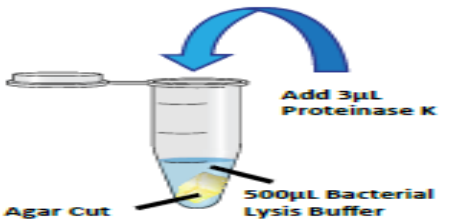
Pendahuluan

Di bidang mikrobiologi, identifikasi mikroorganisme penyebab infeksi memegang peranan yang sangat penting. Hal ini berkaitan dengan ketepatan terapi, pencegahan transmisi, serta pencegahan terjadinya resistensi antimikroba. Identifikasi mikroorganisme penyebab infeksi secara konvensional dilakukan melalui metode pembiakan dan dilanjutkan dengan pemeriksaan karakteristik fisiologis dan biokimia. Metode ini membutuhkan waktu yang lebih lama. Terlebih lagi pada beberapa mikroorganisme yang sulit untuk dibiakkan seperti *mycobacterium* dan virus tertentu. Saat ini dikembangkan metode identifikasi berbasis molekuler yang lebih cepat dengan tingkat sensitivitas dan spesifisitas yang tinggi, yaitu dengan analisis sekuensing gen 16S rRNA (16S *ribosomal Ribonucleic acid*/Asam ribonukleat pengkode ribosom 16S, S menyatakan *Svedberg*, yaitu satuan ukuran ribosom). Gen 16S rRNA juga sering disebut sebagai 16S rDNA (16S *ribosomal deoxyribose nucleatic acid*), namun menurut konsensus dari *American Society for Microbiology* (ASM), istilah 16S rRNA dinilai lebih tepat[1,2].

Gen pengkode RNA ribosomal (rRNA) adalah gen yang paling lestari *(conserved)*. Porsi sekuens rDNA dari tiap organisme yang secara genetik berkorelasi umumnya adalah sama. Dengan demikian setiap organisme yang memiliki jarak kekerabatan tertentu dapat disejajarkan sehingga lebih mudah untuk menentukan perbedaan dalam sekuens yang menjadi ciri khas organisme tersebut. Daerah yang lestari ini juga yang menyebabkan gen ini dapat digunakan sebagai primer universal yang digunakan dalam Polymerase Chain Reaction (PCR) serta dapat ditentukan urutan nukleotidanya melalui sekuensing[3,4]. Gen pengkode rRNA digunakan untuk menentukan taksonomi, filogeni (hubungan evolusi) serta memperkirakan jarak keragaman antar spesies *(rates of species divergence)* bakteri. Perbandingan

Tristia Rinanda adalah Dosen Bagian Mikrobiologi Fakultas Kedokteran Universitas Syiah Kuala

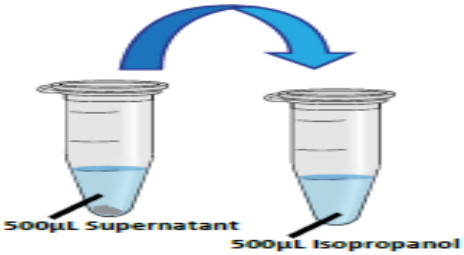
SHORT PROTOCOL- Bacterial DNA Barcoding



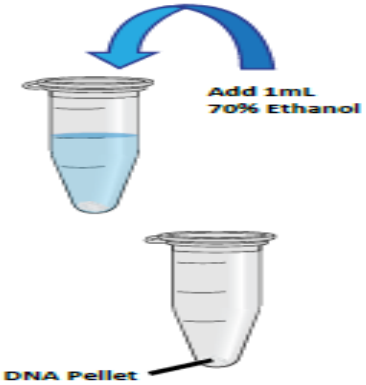
Prepare pure bacterial culture into ***1.5mL tube**
0.5cm x 0.5cm agar cut; or cell pellet from < 2mL liquid

Transfer **500 µL Bacterial Lysis buffer** into tube above. Add **3 µL of Proteinase K solution.** *Vortex to mix, spin down briefly.*

Incubate the mixture at 56°C for 30 min or overnight.
Centrifuge 14,000 -16,000 ×g for 10 min



Transfer **500 µL of supernatant** into
***new 1.5 mL Tube with 500 µL of Isopropanol**
Invert tube to mix gently
Centrifuge 14,000 -16,000 ×g for 10 min
Discard flow-through



Add **1mL *70% Ethanol.**
Centrifuge 14,000 -16,000 ×g for 5 min
Discard flow-through
Air dry the pellet for 3 min

Re-suspend the dried pellet in **50 µL of TE Buffer**
Incubate at 56°C for < 1hr.

Measure OD reading.
Dilute nucleic acid into the range of **15-25ng/uL.**
Use **2 µL of diluted** nucleic acid as DNA template for **PCR** using **Table 1 and Table 2.**

Highlight:
*** 1.5 mL tubes, Isopropanol and 70% ethanol are not supplied with the kit**

Table 1: Preparation of PCR Mix

Number of Reactions	16S Primer Mix (µL)	16S Enzyme (µL)	16S PCR Buffer (µL)	Total PCR Mix (µL)
2	20	1.0	25	46
3	30	1.5	37.5	69
4	40	2.0	50	92
5	50	2.5	62.5	115
6	60	3.0	75	138
7	70	3.5	87.5	161
8	80	4.0	100	184
9	90	4.5	112.5	207
10	100	5.0	125	230

ote 1:

The PCR Mix must be freshly prepared.

The recommended DNA template amount in each PCR is 30ng to 50ng.

Each PCR consists of 23µL of PCR Mix and 2uL of diluted DNA Template.

Both NTC (No Template Control) and positive control reactions are recommended to be included into each round of PCR preparation.

For each positive control reaction, use 1 µL of the provided plasmid positive control (5ng/µL) as DNA Template.

For each NTC reaction, use 1 µL of the provided TE Buffer as DNA Template.

Table 2: PCR Cycle Protocol

No.	Steps	Time	Temp. (°C)	No. of Cycles
1	Initial Denaturation	2 min	94	1
2	Denaturation	10 sec	98	25 cycles
3	Annealing	30 sec	53	
4	Extension	60 sec	68	

ote 2:

Always check the presence of PCR end products, which is ~**1. 5kb** size on agarose gel electrophoresis before submitting it for **1st BASE Sequencing+ PLUS Services.**

If you have alternative sequencing service provider, please purify the PCR products (PCR purification reagents not provided with this kit) before sequencing.

ANALISIS SEKUENSING 16S rRNA DI BIDANG MIKROBIOLOGI

- Gen 16S ribosomal RNA (16S rRNA) memiliki daerah yang conserved (lestari) → Polymerase Chain Reaction (PCR) dan analisis sekuensing →untuk taksonomi, filogeni dan keanekaragaman antar spesies.
- Gen ada hypervariable region yang merupakan ciri khas tiap mikroorganisme.
- Analisis sekuensing gen 16S rRNA → di bidang mikrobiologi.
- Metode molekuler ini → cepat dan akurat dalam mengidentifikasi bakteri patogen dan memiliki keunggulan dibandingkan metode mikrobiologi konvensional.→ untuk mikroorganisme yang tidak bisa dikultur

- Sebagian besar prokariot memiliki 3 jenis rRNA, yaitu 5S, 16S dan 23S.
- Gen 16S dan 23S rRNA memiliki ukuran yang cukup untuk dianalisis.
- Gen 16S rRNA sekitar 1550 pb dan sekitar 500 basa di bagian ujung sekuens adalah daerah yang disebut dengan hypervariable region.
- Gen 16S rRNA → salah satu gen yang telah dikarakterisasi → digunakan untuk identifikasiM.O
- Ribuan sekuens dari berbagai isolat klinis dan dari lingkungan telah terkumpul di satu database yaitu National Center for Biotechnology Information (NCBI) yang dapat diakses pada www.ncbi.nlm.nih.gov,
- Ribosomal Database Project yang dapat diakses di www.cme.msu.edu/RDP/html/index.html.

Aplikasi Analisis Sekuensing 16S rRNA di Bidang Mikrobiologi

- Langkah analisis sekuensing dimulai dengan mengisolasi DNA dari kultur bakteri
- DNA yang diperoleh akan dijadikan sebagai template dalam tahap amplifikasi dengan PCR
- Primer yang digunakan dalam PCR adalah primer 16S rRNA yang bersifat universal sekitar 1500 pb,
- Produk PCR yang telah dimurnikan ditentukan urutan nukleotidanya dengan metode sekuensing.
- Primer dalam sekuensing, forward saja atau reverse saja
- Sekuens DNA terbentuk dari hasil pensejajaran pembacaan primer reverse dan forward disebut sebagai sekuens konsensus (consensus sequence).
- Sekuens konsensus ini dibandingkan dengan data sekuens di database software tertentu.

- **Beberapa database yang dapat digunakan untuk membandingkan sekuens 16S rRNA antara lain:**

1. GenBank (http://www.ncbi.nlm.nih.gov/)
2. Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu/html/)
3. Ribosomal Database Project European Molecular Biology Laboratory (http://www.ebi.ac.uk/embl/)
4. Smart Gene IDNS (http://www.smartgene.ch)
5. Ribosomal Differentiation of Medical Microorganisms (RIDOM) (http://www.ridom.com/)