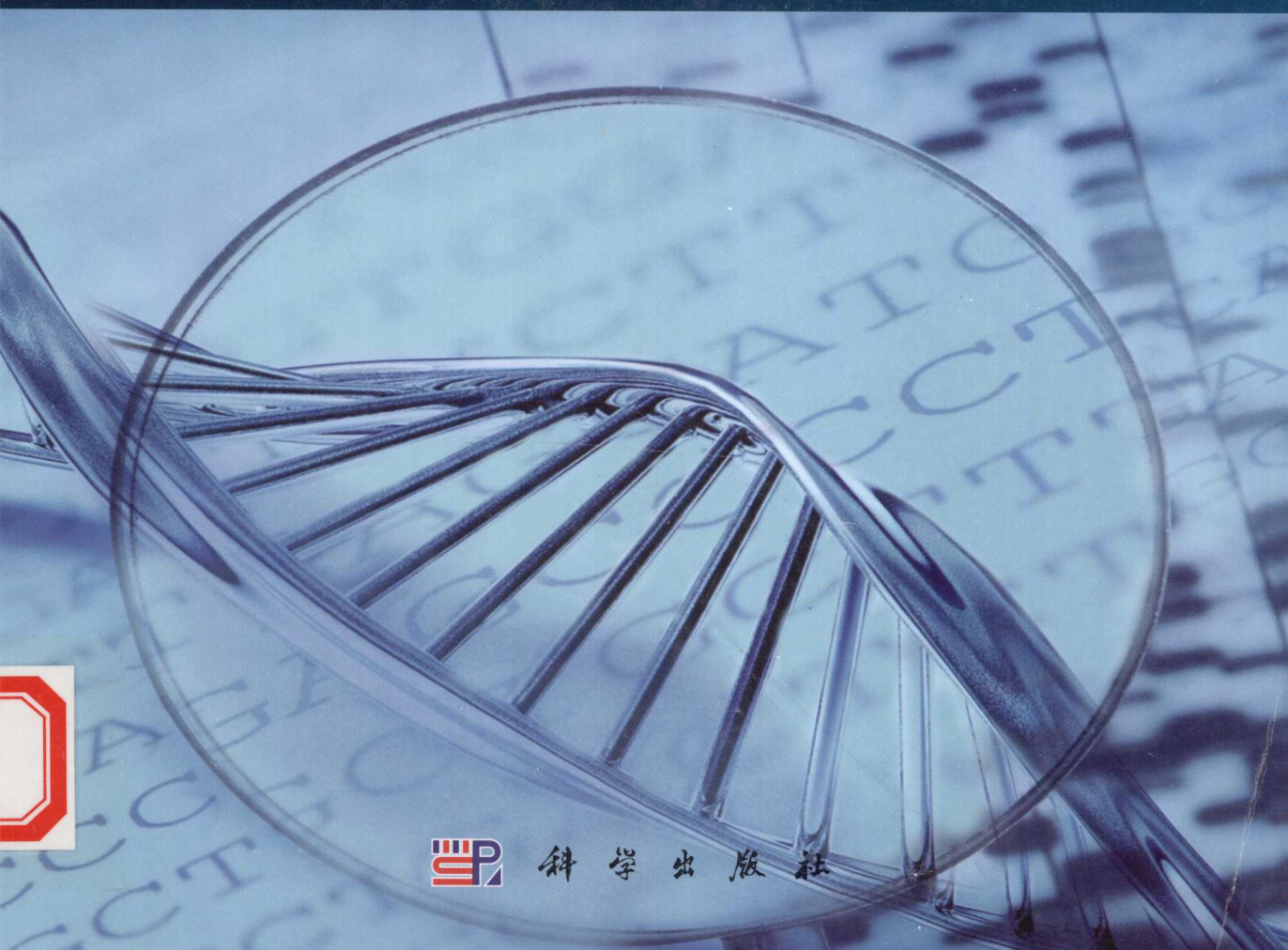
生命科学实验指南系列

Metagenomics
Methods and Protocols

元基因组学：方法和步骤
（影印版）

〔德〕斯特赖特 W.R.　丹尼尔 R.　著



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内 容 简 介

本书涵盖了元基因组文库的构建、元基因组文库的筛选技术和针对不同活性目标产物的筛选方法，并且提供了详细实用的实验操作方法，可以帮助希望从事元基因组学研究的科技人员尽快建立相关的实验平台。

本书适合于从事环境微生物学、微生物生态学等相关专业的高年级本科生、研究生，以及相关研究工作者等参考使用。

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METHODS IN MOLECULAR BIOLOGY™

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Preface

Metagenomics is a key technology to explore the DNAs from not-yet-cultivated microbes in their natural habitats. Theoretically, the microbial DNA isolated from an environmental sample represents the collective DNA of all the indigenous microorganisms and is named the metagenome. Metagenomes can be quite diverse, and, depending on the microbial community analyzed, several hundred up to several thousand different species and genomes can be present in a single metagenome. Typically, soil metagenomes are rather complex with several thousand species present, while microbial communities growing under extreme conditions (i.e., hot springs) are usually rather limited in their complexity and biodiversity. The primary goal of metagenomics is to explore this almost unlimited biodiversity. The last 10 years have already paved the way for the culture-independent assessment and exploitation of complex microbial populations for basic and applied research. Metagenomics has been defined as function-based or sequence-based cultivation-independent analysis of the collective microbial genomes present in an environment. The developed metagenomic technologies are used to complement or replace culture-based approaches and bypass some of their inherent and well-known limitations.

Besides identification of new biomolecules, metagenomics has proven to be a powerful tool for exploring the ecology, metabolic profiling, and comparison of complex microbial communities. Profiling the functions encoded by a microbial community rather than the types of organisms producing them provides a means to distinguish environmental samples on the basis of the functions selected for by the local environment and reveals insights into features of that environment. Another application of metagenomics is the genomic characterization of uncultivated microorganisms and complex communities. In addition, large-scale sequencing approaches of metagenomic DNA have been applied to reconstruct genome fragments and near-complete genomes from uncultivated species and natural consortia.

The main application area of metagenomics is the mining of metagenomes for genes encoding novel biocatalysts and drug molecules for bioindustries. Due to the complexity of most metagenomes, new sensitive and efficient high-throughput screening techniques that allow for fast and reliable identification of genes encoding suitable biocatalysts from complex metagenomes have been invented. Screens of metagenomic libraries have been based either on nucleotide sequence (sequence-driven approach) or on metabolic activity (function-driven approach).

This current book gives an overview and introduction to basic methods commonly used in laboratories that have a strong background in microbial metagenomics. All chapters are written by experts in the field, and our goal is that this book serves those who are interested in establishing metagenomics in their laboratories as a manual. Within the book, we have tried to address all working steps involved in this field: Starting from the DNA isolation from soils and marine samples to the construction and screening of the libraries, and finally we offer some advise with respect to the bioinformatic tools available to screen large sequences. An overview on strategies involved in the isolation of DNAs from environmental samples is given in the first four chapters together with the main strategies that

are currently used for the construction of metagenome libraries. Chapters 5–8 describe protocols linked to the expression of metagenome libraries in different host strains. Those include simple protocols for the construction of a library in broad host range vectors but also rather sophisticated protocols to handle *Sulfolobus* as a host strain. Furthermore, the book contains a significant number of chapters that describe a wide variety of screening technologies used for the identification of different enzymes or other biomolecules using function- and sequenced-based technologies. Altogether, the 15 chapters describe a diverse range of screening protocols for metagenome libraries. In our view, this is a very complete description of available screening protocols for all major biocatalysts and allows an easy setup of these screens in any microbiology lab.

Wolfgang R. Streit
Rolf Daniel

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Contents

Chapter 1

Molecular Methods to Study Complex Microbial Communities

José M. Vieites, María-Eugenia Guazzaroni, Ana Beloqui, Peter N. Golyshin, and Manuel Ferrer

Abstract

Microbes, which constitute a major fraction of the total biomass, are the main source of biodiversity on our Planet and play an essential role in maintaining global processes, which ultimately regulate the functioning of the Biosphere. Recent emergence of "metagenomics" allows for the analysis of microbial communities without tedious cultivation efforts. Metagenomics approach is analogous to the genomics with the difference that it does not deal with the single genome from a clone or microbe cultured or characterized in laboratory, but rather with that from the entire microbial community present in an environmental sample; it is the community genome. Global understanding by metagenomics depends essentially on the possibility of isolating the entire bulk DNA and identifying the genomes, genes, and proteins more relevant to each of the environmental sample under investigation. Following on this, in this chapter, we provide an analysis of methods available to isolate environmental DNA and to establish metagenomic libraries that can further be used for extensive activity screens.

Key words: Metagenomics, Cosmid, Fosmid, Phage library, Screening

1. Introduction

Microbes, the most abundant organisms on Earth, play a major role in maintaining global element cycling processes and facilitating the self-sustainable functioning of the Biosphere. From this point of view, it is crucial to generate a thorough understanding of these key microorganisms and processes they facilitate. However, at present, we simply do not know the extent of the functional diversity that microbes encompass: a classical theoretical analysis endeavors a population of prokaryotes on Earth of about 10^{30} bacteria, few order of magnitude higher than the

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number of stars in the known Universe (estimated 10^{22}–10^{24}) (1–3), with most microbes being members of complex communities. Invertebrate guts are certainly one of the most dense and diverse niches [10^{9}–10^{11} cells per mL of gut fluid (4)], followed by soil [10^{7}–10^{9} cells per gram (5)], and oligotrophic superficial sea- and freshwater [10^{5}–10^{6} bacteria per milliliter (6)]. Any individual survey to study such diversity is limited due to the relatively poor capacity of growth of most microorganisms that is offered even by rather sophisticated resources available for culturing (7). To circumvent this problem, a wide range of approaches collectively described as "metagenomics" have been developed to study communities through the analysis of their genetic material without culturing individual organisms (8). Metagenomics is analogous to genomics with the difference that it does not deal with the single genome from a clone or microbe cultured or characterized in laboratory, but rather with that from the entire microbial community present in an environmental sample; it is the community genome. Metagenomics represents a strategic concept that includes investigations at three major interconnected levels (sample processing, DNA sequencing, and functional analysis), with an ultimate goal of getting a holistic view of the functioning of microbial World. While many of the technical limitations to processing of samples have been overcome in the last decade (multiwell DNA extractions, single-cell isolation, sequence analysis by technologies such as 454 or Solexa platforms), we believe that the major hurdles still are (1) the adequate metagenome coverage, since genes of different organisms are be present in very different concentrations in the DNA used to construct the libraries or for sequencing, (2) the integrating and filtering gene sequences and experimental evidences to facilitate functional assignments of unknown genes, organisms, and communities and to recreate functional networks, and (3) the computational aspects of data archiving, analysis ,and visualization of vast numbers of DNA sequences which are released to databases. In this respect, lessons from 20 years of metagenomics and four of high-throughput DNA sequencing [first analyses of microbial communities through massive sequencing were published in 2004 (9, 10)] tell that giga-base amounts of environmental sequences can easily be generated to a large extent, but only a fraction of them can properly be annotated in terms of gene functions (~50% of the potential protein-coding genes lacked any functional assignment). More importantly, DNA sequences per se are not that helpful in linking genes to specific functions as we know that more than 60% of genes are ubiquitous and have similar housekeeping functions in different organisms. Therefore, in this chapter, we try to provide a broad view on current technical issues to illustrate the potential of getting appropriate metagenomic material to create representative gene libraries, as the first step for analysing community genomes.

Following the above observations, the principal measure of phylogenetic relatedness, and thus of biodiversity, is the sequence of the 16S ribosomal RNA gene in prokaryotes and its equivalent 18S rRNA gene in eukaryotes. Determination of very large numbers of such sequences has revealed that natural environments contain vast numbers of diverse microorganisms, but only a fraction of them can properly be analyzed (11). This "great plate count anomaly" (12), in fact, observed from early 1930s stimulated the development of new efficient tools to circumvent problems linked to the cultivation of microbes in artificial medium, the so-called metagenomics (13). These are often described as culture-independent approaches and, in terms of the organisms being accessed and mined, this is the case. However, the need for large amounts of cell biomass for gene and genomic analysis always requires cultivation of a producer microbe, except for DNA sequencing which requires direct separation of cells and bulk DNA. The difference here is that cultivation refers to that of a surrogate organism, the host exploited as a reservoir for archiving the harvested genetic resources. Considering these requirements, metagenomics is often based on a general strategy of producing large amount of environmental DNA to achieve two goals: (1) discovery of new gene sequences coding for enzymes and drugs and (2) random sampling and archiving of the genomes from a small subset of organisms present in an environment for subsequent in silico analysis (14, 15). Both research windows are essential as the microorganisms are known to be the "gatekeepers" of environmental processes. However, it is essential to note that the relative abundance of representatives of a certain group of microbes is not necessarily linked to the importance of that group in the community functioning: common organisms may not necessarily play a critical role in a community despite their numbers, and organisms that only muster 0.1% fraction (e.g., nitrogen fixers) can be of pivotal importance. What this means, in terms of microbial ecology, is that the structural and functional information based on more reductionist approach, that is, classical functional genomics based on single organisms, may not provide appropriate understanding of complex communities.

To date, much of the research has been focused on bulk DNA sequencing. The analysis of samples at sequence level somehow has lower resolution but can access much greater genomic information of untapped microbial biodiversity in many environments. In contrast, the second approach shows better options to link specific microbes to specific ecological functions. In one of the first examples, the Sargasso Sea genome sequencing project, the authors performed a size-selective filtration for enrichment of the microbes of a certain size (10). Actually, new developments involve the direct separation of cells or preferably the enrichment using ^{13}C-labeled compound directly related to primary ecological functions (16). A particular elegant strategy combines the extraction of almost

complete genomes from uncultured microbes in complex communities (with up to 5,000 species) by high-resolution stable isotope probing (SIP) to reconstruct their metabolisms and to link specific microbes, whose DNA is separated by ultracentrifugation, to specific ecological functions (17). Here, authors provided a good genome coverage of dominant organisms under dynamic utilization of different nutrients and were able to link environment-specific organisms and processes that are catalyzed by these microbes. However, despite their great potential, the main drawback of enrichment methodologies is the danger of a nonproportional accumulation of fast-growing microbes in the community, which is not necessarily relevant to the native ecosystem, followed by the reduction of the natural diversity in the sample.

We should also point out that metagenomics is not limited to prokaryotes: eukaryotic microbial diversity is also enormously diverse and are hence of a great interest for exploration of their functional diversity. Owing to the problem of introns in eukaryotes, considerable effort has been invested in the isolation of RNA and its conversion to cDNA, rather than dealing with genomic DNA. This requires isolation of full-length mRNAs, reverse transcribing them, and analysis of the cDNA libraries. Here, the RNA extraction technique is critical, since it needs to extract RNA from thick-walled cells of fungi and yeasts, and their spores. Further, as a complement to the long-standing trend towards reductionism, metagenomics seeks to treat the community as a whole. However, this is not an easy task, specially for sample processing, as we know that environmental samples also contain picoeukaryotes (size <2–3 μm) whose population composition varies dynamically in response to both seasonal and spatial gradients in environment (3, 18). Therefore, a general strategy for sample processing is recommended for metagenomics analysis in the future, in which parts of microbial communities are processed separately, using single microdroplets and cell-free translation systems together with cell sorting ("single-cell genomics"), accompanied by the integration these data with those obtained using mixed microbial communities (19–21). Finally, we should consider that genome coverage is an ephemeral term in complex communities, since individual community members are be present in varying numbers in a sample and their genomes are extracted with different efficiencies (see Note 1). Therefore, the genes of different organisms will be present at very different concentrations in the DNA used as material to construct the libraries or to sequencing. For this reason, attempts to obtain (or even calculate the size of) a sample providing good coverage of all genomes present in a sample are rare and limited to samples from extreme environments (22), known to contain microbial communities of very limited complexity/diversity. Further advances in this field are demanded to appropriately reconstruct the metabolism in more complex

microbial communities. Below, we list a series of protocols for extraction of environmental DNA and further production of metagenomic libraries.

2. Materials

2.1. Sampling

1. 500-kDa NMWL ultrafiltration disc (Biomax polyethersulfone, Millipore).
2. Filtration device Pellicon TFF 0.1 μm (Millipore™).
3. 500-kDa filtration disc Amicon® system (Millipore).
4. TE buffer pH 8.0.
5. Disruption buffer: 0.2 M NaCl, 50 mM Tris–HCl, pH 8.0.
6. Nycodenz (0.8–1.3 g/mL in distilled H_2O).
7. Phosphate-buffered saline (PBS) buffer: 137 mM NaCl, 2.5 mM KCl, 10 mM phosphate, pH 7.4.

2.2. DNA Extraction

1. Lysozyme solution (10% w/v).
2. RNase solution (1% w/v) free of DNase.
3. Proteinase K (1% w/v).
4. SDS solution (10% w/v).
5. Cetyltrimethylammonium bromide (CTAB) solution.
6. 10% w/v in 0.7 M NaCl.
7. $ChCl_3$:Isoamyl alcohol.
8. Isopropanol.
9. Ethanol (70% v/v).
10. TE buffer, pH 8.0.
11. DNA Clean & Concentrator from Zymo Research Corp.

2.3. DNA Isolation with Kits

1. UltraClean MegaPrep (MoBio Laboratories, Inc.).
2. G'NOME® DNA Extraction Kit (BIO101).

2.4. Nucleic Acid Quantification

1. Quant-iT™ PicoGreen® Kit.

2.5. 16S rRNA Gene Libraries Construction

1. pGEMT-Easy (Promega).
2. T4 DNA ligase buffer (10×).
3. T4 DNA ligase.
4. Primer 16F530 (5′-TTCGTGCCAGCAGCCGCGG-3′).
5. Primer 16R1492 (5′-TACGGYTACCTTGTTACGACTT-3′).
6. *Escherichia coli* DH5α.

2.6. Metagenomic Libraries Construction

1. CopyControl™ Fosmid Library Production kit (EPICENTRE).
2. GELase (EPICENTRE) kit.

2.6.1. In pCCFOS Vector 1

3. Microcon-100 (Millipore) concentrator membrane (100 kDa cut-off).
4. 3 M Sodium Acetate (pH 7.0).
5. TE buffer, pH 8.0.
6. 70% ethanol and 100% ethanol.
7. PD buffer: 10 mM Tris–HCl pH 8.3, 100 mM NaCl, 10 mM $MgCl_2$.
8. *E. coli* EPI300-T1^R.
9. Chloramphenicol 12.5 mg/mL.
10. LB (Luria–Bertani) medium.

2.6.2. In pLAFR3 Cosmid

1. Large-Construct Kit (Qiagen).
2. Tetracycline (Tc) 10 μg/mL.
3. ATP-dependent exonuclease.
4. *Hin*dIII, *Eco*RI, *Sau*3AI, and *Bam*HI enzymes.
5. Shrimp Alkaline Phosphatase (SAP; from Biotec ASA).
6. Microcon-100 (Millipore).
7. GeneClean Kit (BIO101).
8. Gigapack XL (Stratagene).
9. NEB3 and 1 (New England Biolabs Buffers 1 and 3).
10. 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.
11. GELase (EPICENTRE).
12. Bovine Serum Albumin (BSA) – nuclease free (10×).
13. Ligation Buffer NEB1 (10×).
14. T4 DNA Ligase (New England Biolabs).
15. SM buffer: 50 mM Tris–HCl pH 7.5, 0.1 M NaCl, 8.5 mM $MgSO_4$, and 0.01% (w/v) gelatin.
16. Chloroform.
17. *E. coli* DH5α or XL1Blue.
18. 1 M $MgSO_4$.
19. 2% (w/v) maltose.
20. LB medium.

2.6.3. In Lambda Zap® Express System

1. Zap Express pBK vector (Stratagene).
2. *Sau*3AI enzyme.
3. 10× BSA.
4. NEB1 (New England Biolabs Buffer 1).