NEB EXPRESSIONS

A scientific update from New England Biolabs | Issue II, 2013

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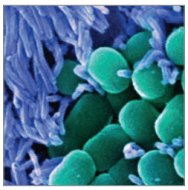
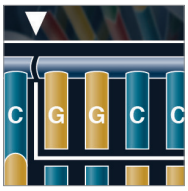
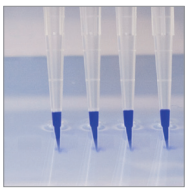
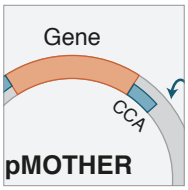
Contents

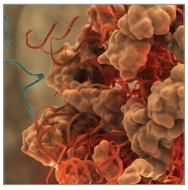
Issue II, 2013

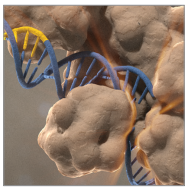












COVER PHOTO

Purple pansy (*Viola tricolor*) on the campus of New England Biolabs, photographed by Bree Hall.

FEATURE ARTICLE

Addressing Challenges in Microbiome DNA Analysis

Among the very many "-omes" now studied and discussed (1), microbiomes have received increasing attention in recent months, from both scientists and the general public. Used to describe the communities of microorganisms and their genes in a particular environment, including a body or part of a body, "microbiome" is becoming an increasingly common term in everyday language. One challenge in microbiome genome analysis is addressing the presence of host DNA in samples. As such, improved methods for solving this problem are needed.

Fiona Stewart, Ph.D. and Erbay Yigit, Ph.D.,
New England Biolabs, Inc.

Introduction

A wealth of information about the composition of, and interactions between, the constituent microbes of a microbiome can provide insight into both the function and dysfunction of the host organism, as well as the host-microbiome unit as a whole. In particular, the relationships amongst and between resident microbes (bacteria, archea and fungi) and their hosts have recently become the topic of fervent research; the number of microbiome research publications has been steadily increasing since 2003 (2). Such research has demonstrated that the microbiome communities of individuals are unique, as are the microbiome communities of specific sites within an individual (reviewed in 3). In humans, the number of microorganisms present is estimated to exceed the number of human cells by 10-fold (4). Studies of the human microbiome (including the Human Microbiome Project (HMP) [www.hmpdacc.org] (5), and MetaHIT, the metagenomics of the intestinal tract [www.metahit.eu] (6)) may be the best known, and have led to the understanding that the human microbiome may be critical to health and disease.

Until relatively recently, the role of the microbiome was unknown, and an organism's microbial load was considered to be potentially nothing more than cellular "hitchhikers", having little impact on the organism's functioning. Now, it is understood that an organism's microbiome can influence many processes within the host organism. Discoveries including the role of the microbiome in conditions and disease states, such as obesity, diabetes mellitus and cardiovascular disease (reviewed in 7), have led to the potential for development of microbiome-based diagnostic and therapeutic tools. Additionally, the unique nature of an individual's microbiome has enabled matching of skin-associated bacteria, on objects such as a keyboard, to specific individuals, leading to the potential for use in forensic applications (8). It should be noted that microbiome research is not limited to humans, and research into microbiomes of non-human organisms is also increasing rapidly in environmental and agricultural areas of research (9).

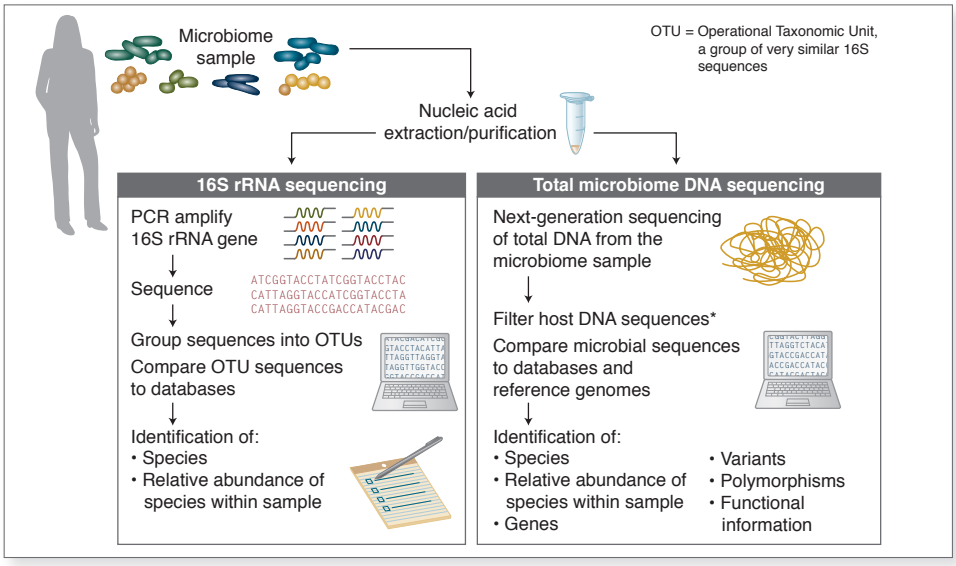
Although it is still not possible to isolate and culture the vast majority of microorganisms (estimated to be over 95%), analysis of total nucleic acid from microbiome samples has enabled significant advances in the field. Furthermore, advances in sequencing technologies have enabled significant progress in microbiome nucleic acid analysis.

Current Methods of Analysis

The majority of microbiome DNA studies to date have employed 16S analysis (Figure 1). This analysis method takes advantage of the 16S rRNA gene that is specific to prokaryotes and some of the archaea and is not found in eukaryotes. 16S rRNA genes from different species have significant homology, but the gene also includes hypervariable regions that are generally species-specific, and are determined by the microbial composition of the community. These characteristics enable the use of universal primer pairs to amplify 16S genes from many organisms in the same PCR reaction and then, through subsequent sequencing of the PCR products, the individual species represented can be identified.

While the 16S method is a fast and relatively inexpensive way to survey, at high throughput, the microbial organisms present within a sample, it provides very little information regarding function. Additionally, determining optimal PCR primers (for specific sample types and to distinguish between some species) can be challenging. In contrast, sequencing of the total DNA of a microbiome sample does not have these limitations and provides a more complex range of information. Through the identification of microbial sequences, genes, variants and polymorphisms, this method enables determination of information on microbiome species diversity and, also, putative functional information. Such sequencing-based studies have enabled the creation of many databases, including the Human Oral Microbiome Database (HOMD) [www.homd.org] (10). Approximately 700 prokaryotic species are present in the human oral cavity, and the stated goal of the HOMD database

Figure 1. Microbiome DNA Analysis Methods



While 16S analysis is fast and inexpensive, it provides little information regarding function. More detailed information can be obtained through microbiome sequencing, particularly once host DNA is removed.

** For many samples, host DNA constitutes a high percentage of sequence reads. Removal of host DNA, and enrichment of microbial DNA substantially increases the percentage of sequence reads from the microbial sequences of interest.*

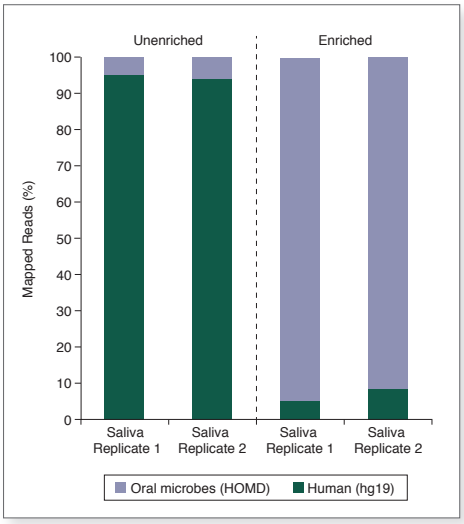
project is to provide taxonomic and genomic information on these species. Comparison of microbiome sample sequences to databases, such as HOMD, further enables discovery, including genes, pathways and their relative frequencies in the sample.

Overcoming Difficulties with Microbiome Samples

Many microbiome samples are overwhelmed with host DNA, and the HMP has reported especially high levels of human DNA in soft tissue samples, such as mid-vagina and throat samples. Saliva samples also contain high levels of human DNA (11). In contrast, although human DNA is generally all but absent from fecal samples, some infections can substantially increase the level of human DNA in such samples, likely due to widespread cell lysis during bacterial infection.

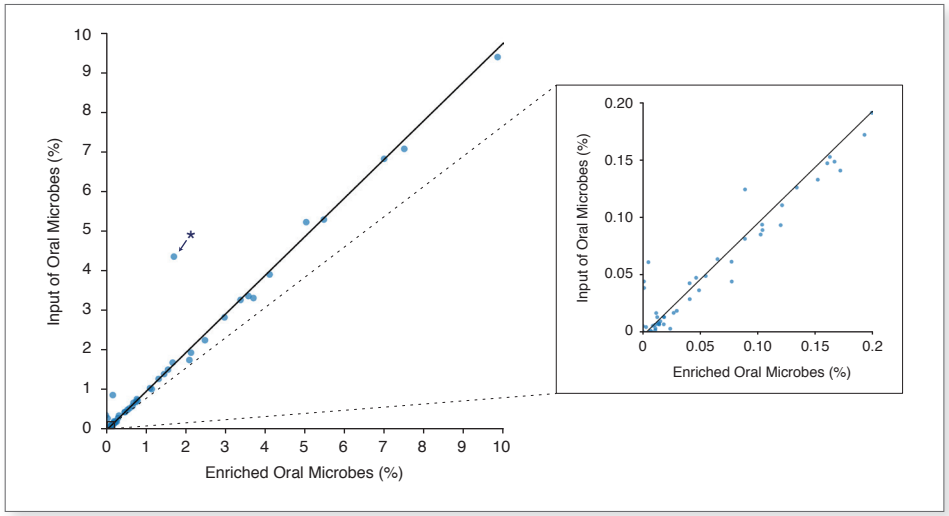
The presence of contaminating host genomic DNA in a microbiome sample complicates the genetic analysis of these samples. Since a single human cell contains approximately 1,000 times more DNA than a single bacterial cell (approximately 6 billion bp versus 4-5 million bp), even a low level of human cell contamination within a microbiome sample can substantially complicate the sample processing and sequencing. As a result, in the case of total microbiome DNA sequencing studies, only a small percentage of sequencing reads from such samples pertain to the microbes of interest, and therefore a large percentage of sequencing reads (host) have to be discarded. Consequently, obtaining sufficient sequence coverage of the microbiome DNA can become cost-prohibitive or even technically infeasible. Therefore, methods to enrich microbiome DNA are useful, and, in some cases, critical for sequencing of the microbiome. However, until now, options for such enrichment have been limited to selective cell lysis, with the disadvantages of a requirement for live cells, and low bacterial DNA recovery.

Figure 2. Salivary Microbiome DNA Enrichment



DNA was purified from pooled human saliva DNA (Innovative Research) and enriched using the NEBNext Microbiome DNA Enrichment Kit. Libraries were prepared from unenriched and enriched samples and sequenced on the SOLiD 4 platform. The graph shows percentages of 500M-537M SOLiD4 50 bp reads that mapped to either the Human reference sequence (hg19) or to a microbe listed in the Human Oral Microbiome Database (HOMD)[10]. (Because the HOMD collection is not comprehensive, ~80% of reads in the enriched samples do not map to either database.) Reads were mapped using Bowtie 0.12.7[13] with typical settings (2 mismatches in a 28 bp seed region, etc.).

Figure 3. Microbiome Diversity is Retained After Enrichment with the NEBNext Microbiome DNA Enrichment Kit



DNA was purified from pooled human saliva DNA (Innovative Research) and enriched using the NEBNext Microbiome DNA Enrichment Kit. Libraries were prepared from unenriched and enriched samples, followed by sequencing on the SOLiD4 platform. The graph shows a comparison between relative abundance of each bacterial species listed in HOMD[10] before and after enrichment with the NEBNext Microbiome DNA Enrichment Kit. Abundance is inferred from the number of reads mapping to each species as a percentage of all reads mapping to HOMD. High concordance continues even to very low abundance species (inset). We compared 501M 50 bp SOLiD4 reads in the enriched dataset to 537M 50 bp SOLiD4 reads in the unenriched dataset. Reads were mapped using Bowtie 0.12.7[13] with typical settings (2 mismatches in a 28 bp seed region, etc).

** Niesseria flavescens – This organism may have unusual methylation density, allowing it to bind the enriching beads at a low level. Other Niesseria species (N. mucosa, N. sicca and N. elognata) are represented, but do not exhibit this anomalous enrichment.*

The NEBNext® Solution

The NEBNext Microbiome DNA Enrichment Kit addresses this problem by providing a quick and effective way to remove contaminating host DNA, thereby enriching for microbiome DNA. The kit exploits the different prevalences of CpG methylation in the genomes of microbial and eukaryotic organisms. Eukaryotic DNA, including human DNA, is methylated at CpGs, while methylation at CpG sites in microbial species is rare.

The NEBNext Microbiome DNA Enrichment Kit uses a magnetic bead-based method to selectively bind and remove CpG-methylated host DNA. The kit contains the MBD2-Fc protein, which is composed of the methylated CpG-specific binding protein MBD2, fused to the Fc fragment of human IgG. The Fc fragment binds readily to Protein A, enabling effective attachment to Protein A-bound magnetic beads. The MBD2 domain of this protein binds specifically and tightly to CpG methylated DNA. Application of a magnetic field then pulls out the CpG-methylated (eukaryotic) DNA, leaving the non-CpG-methylated (microbial) DNA in the supernatant (see workflow figure on Page 5).

Microbiome Enrichment of Human Saliva

Human saliva samples can be especially challenging, due to high levels of human genomic DNA and the poor-quality of the DNA itself. Despite these sample challenges, the data shown in Figure 2 demonstrates that substantial enrichment of microbiome DNA from saliva was achieved using the NEBNext Microbiome DNA Enrichment Kit.

An important consideration when assessing the validity of microbiome enrichment is that the enrichment should not be biased, and the diversity of microbiome organisms in the sample should remain intact after enrichment. As shown in Figure 3, measurement of the relative abundance of

species represented in HOMD was equivalent between unenriched and enriched samples. Interestingly, *Neisseria flavescens*, highlighted with *, was a unique outlier in this comparison and may have unusual methylation density, which enables binding to the MBD-Fc beads at a low level. It is notable that other *Neisseria* species (*N. mucosa*, *N. sicca* and *N. elognata*) are also represented, but do not exhibit this anomalous enrichment.

Conclusion

From forensic microbial "fingerprints" to disease-causing pathogens, microbiomes comprise a vast and varied microcosm with a surprising degree of influence over the health and function of the host organism. The potential for significant and exciting discoveries to be achieved with microbiome analysis is enormous, but will require improved tools and methods to make this a reality. As a step towards this goal, the NEBNext Microbiome DNA Enrichment Kit now makes it possible to substantially enrich a variety of sample types for non-host, microbial DNA, while retaining microbial diversity, and thereby improving the quality and cost-effectiveness of downstream analyses and data generation.

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Scientific Contribution

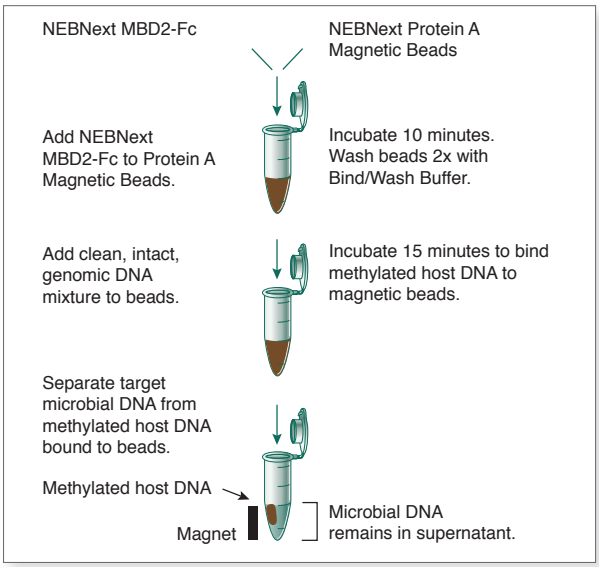
The scientific contributors to this article include: George R. Feehery, Erbay Yigit, Bradley W. Langhorst, Fiona J. Stewart, Eileen T. Dimalanta, Sriharsa Pradhan, James MacFarland, Christine Sumner and Theodore B. Davis.

NEW PRODUCT

NEBNext Microbiome DNA Enrichment Kit

Microbiome DNA analysis can be challenging due to the high percentage of host DNA present in samples. The NEBNext Microbiome DNA Enrichment Kit facilitates enrichment of microbial genomic DNA from samples containing methylated host DNA (including human), by selective binding and removal of CpG-methylated host DNA. Importantly, microbial diversity remains intact after enrichment.

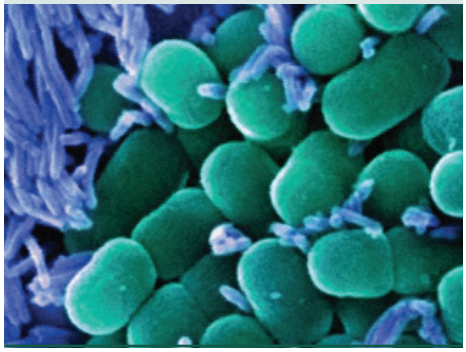
Microbiome DNA Enrichment Kit Workflow



The MBD2-Fc protein binds specifically to CpG methylated DNA. In the NEBNext Microbiome DNA Enrichment workflow, MBD2-Fc is attached to Protein A magnetic beads, enabling capture of methylated DNA, while the microbial DNA remains in the supernatant.

Advantages

- Effective enrichment of microbial genomic DNA from contaminating host DNA
- Fast, simple protocol
- Enables microbiome whole genome sequencing, even for samples with high levels of host DNA
- Compatible with downstream applications, including next generation sequencing on all platforms, qPCR and end point PCR
- Suitable for a wide range of sample types
- No requirement for live cells



Ordering Information

PRODUCT	NEB #	SIZE
NEBNext Microbiome DNA Enrichment Kit	E2612S	6 reactions

For information, visit
www.neb.com/microbiome

FAQ Spotlight

CutSmart Buffer for Restriction Enzymes

Q: How is NEB's new buffer system going to help me?

A: *Although the previous buffer system worked well, NEB is continuously looking for ways to enhance the convenience and performance of its products for our customers. Our new buffer system includes BSA in all reaction buffers, and no longer contains DTT. By adding BSA to the reaction buffer, we were able to offer even more enzymes that cut in a single buffer (> 200). This improves ease-of-use, especially when performing double digests. In addition, it eliminated the need to add BSA when setting up restriction enzyme digests.*

Q: If I have an old tube of Restriction Enzyme, what NEBuffer should I use?

A: *All NEB Restriction Enzymes have color coded labels for the appropriate NEBuffer; this system can either be used with the previously supplied NEBuffer or with the newly recommended buffer.*

Q: I currently have an old tube of Restriction Enzyme – is it still active in the new buffer?

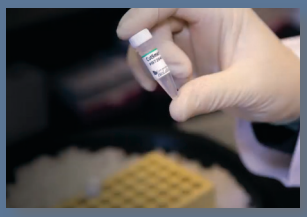
A: *Yes. The new buffers are mostly identical, except that BSA has been added directly to the buffer and DTT has been removed. BSA will not harm the reaction and may even enhance it in some cases. Extensive testing has shown that DTT is not required in the reaction.*

Q: I am still using your previous buffer system. Where can I find performance information for the previous buffers?

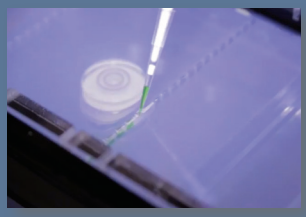
A: *Information for the previous buffer system is available on every restriction enzyme product page at www.neb.com, as well as from the Double Digest Finder and Activity Performance Chart, which are both available online.*

Visit **NEBCutSmart.com** for the full list of FAQs.

LEARN MORE WITH OUR VIDEO TECH TIPS AT WWW.NEB.COM/RESTRICTIONENZYMES



How will NEB's CutSmart Buffer help me?



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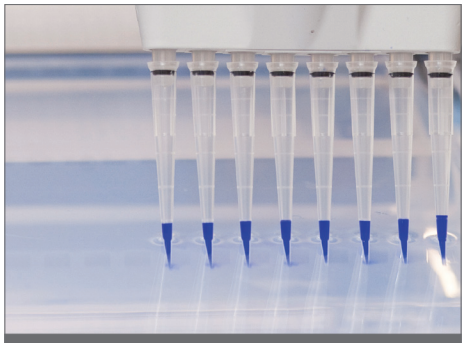

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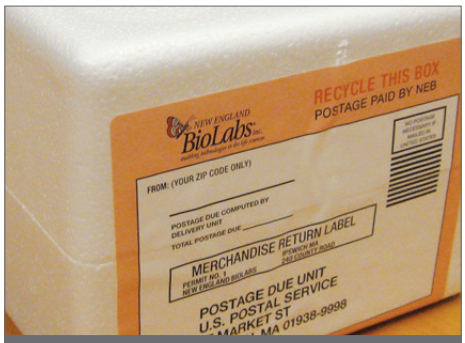

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APPLICATION NOTE

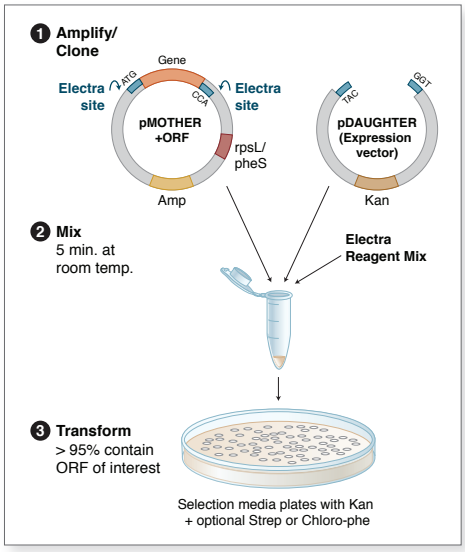
Rapid, Scarless Cloning of Gene Fragments Using SapI, T4 DNA Ligase and the DNA2.0 Electra Vector System

Laura Whitman, M.S., Medini Gore, M.S., Jon Ness, Ph.D., Elias Theodorou, Ph.D., and Jeremy Minshull, Ph.D., DNA2.0

Introduction

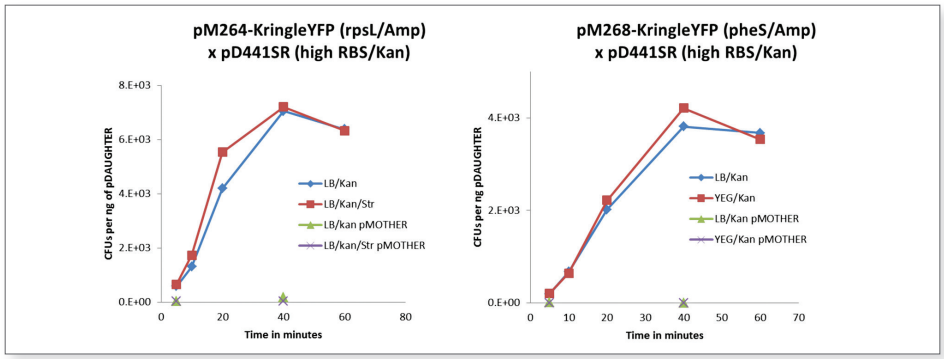
While the traditional cloning workflow has been used in laboratories for decades, new techniques have emerged that allow for rapid assembly of multiple genetic fragments. Examples of these technologies include Golden Gate Assembly and Gibson Assembly® (for more information, see review in (1)). Furthermore, systems that facilitate the easy movement of DNA elements from one context to another allow many genetic constructs to be rapidly built and tested. The Electra Vector System, developed by DNA2.0, is a universal cloning system similar to FX cloning (2), that utilizes the type IIS restriction enzyme SapI and T4 DNA Ligase in a single-tube reaction (Figure 1). In this reaction, SapI recognizes a 7 bp non-palindromic recognition sequence in the cloning vector, and leaves a 3 bp 5´ overhang after digestion for ordered assembly. This process leaves no cloning scars and does not require PCR or other mutation-inducing amplification. Any vector can be easily converted by DNA2.0 to function as an Electra vector. A collection of IP-free bacterial, mammalian and yeast DAUGHTER expression vectors have been constructed to function as Electra vectors. Any gene that is provided in a MOTHER vector can be quickly and efficiently moved into any DAUGHTER vector, allowing the gene to be tested under different conditions, including promoters, ribosome binding sites, C- and N-terminal tags and/or fusions. In this experiment, the gene encoding KringleYFP (yellow fluorescent protein) was cloned into a series of DAUGHTER vectors and then analyzed.

Figure 1: Single-Tube Digestion and Ligation Reaction Using the Electra System



The MOTHER vector, carrying the gene of interest, is mixed with a linearized DAUGHTER vector in the presence of SapI, T4 DNA Ligase, and ATP. The reaction is incubated for 5 minutes at room temperature, transformed into NEB 10-beta competent E. coli and then plated on LB/agar plates containing the appropriate antibiotic.

Figure 2. Cloning Time Course with *E. coli* DAUGHTER Expression Vectors



A gene encoding KringleYFP was cloned into an ampicillin-resistant MOTHER vector with an rpsL counter-selection gene (pMOTHER264, left panel) or a PheS counter-selection gene (pMOTHER268, right panel). The MOTHER vectors were mixed with a pre-linearized E. coli DAUGHTER expression vector with inducible T5 promoter (pDAUGHTER441-SR) in the presence of SapI, T4 DNA Ligase and ATP. Reaction mixtures were transformed into NEB 10-beta competent E. coli after various reaction times, and plated onto nutrient agar with kanamycin (blue lines) or kanamycin plus MOTHER counter-selection agent (red lines).

Results

A MOTHER vector carrying a gene encoding KringleYFP (yellow fluorescent protein) was mixed with an *E. coli* expression DAUGHTER vector in the presence of SapI (NEB #R0569), T4 DNA Ligase (NEB #M0202) and ATP (NEB #P0756) for 5–60 minutes. Reactions were transformed into NEB 10-beta competent *E. coli* (NEB #C3019) and plated onto LB agar plates supplemented with appropriate selective antibiotics. Transformants resulted from reactions that were incubated for as little as 5 minutes and maximum efficiency was obtained by 40 minutes. Almost all transformants showed an inducible fluorescent yellow phenotype, indicating accurate movement of the gene from the MOTHER into the expression DAUGHTER vectors. DAUGHTER constructs are selected because MOTHER and DAUGHTER vectors use different antibiotic resistance markers. Since MOTHER and DAUGHTER are present in the transformation mixture, a fraction of the cells transformed with a DAUGHTER construct will also take up and maintain the MOTHER construct. MOTHER vectors therefore also carry a counter-selection marker, either rpsL (streptomycin sensitivity) or PheS (phenylalanine analog p-chlorophenylalanine sensitivity). Plating transformants onto media that contains both DAUGHTER selection antibiotic and MOTHER counter-selection agent reduces (rpsL) or completely eliminates (PheS) transformants that carry the MOTHER (Figure 2). In a separate experiment, crude PCR product of the KringleYFP gene was successfully cloned into an *E. coli* DAUGHTER vector without any PCR reaction treatment or cleanup, using a similar single-tube protocol of 5 minutes at room temperature (data not shown).

Summary

The Electra Vector system from DNA2.0, combined with SapI and T4 DNA Ligase from New England Biolabs, enables quick and efficient transfers of genes into any DAUGHTER vector, either from a MOTHER vector or directly from a PCR reaction. Furthermore, transfer into multiple DAUGHTER vectors facilitates convenient testing of expression system parameters, and enables rapid optimization of genetic constructs. Unlike alternative systems, there are no intellectual property entanglements, no unwanted mutations from error-prone polymerases, and no sequence scars to affect expression and function.

The Electra system can also be used to combine multiple sequence elements simultaneously, facilitating the easy construction of combinatorial libraries, either in a single tube, or in individually enumerated combinations (data not shown).

For more information, see dna20.com/electra

Data & figures courtesy of DNA2.0. Figure 1 was adapted for style.

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FEATURED PRODUCT

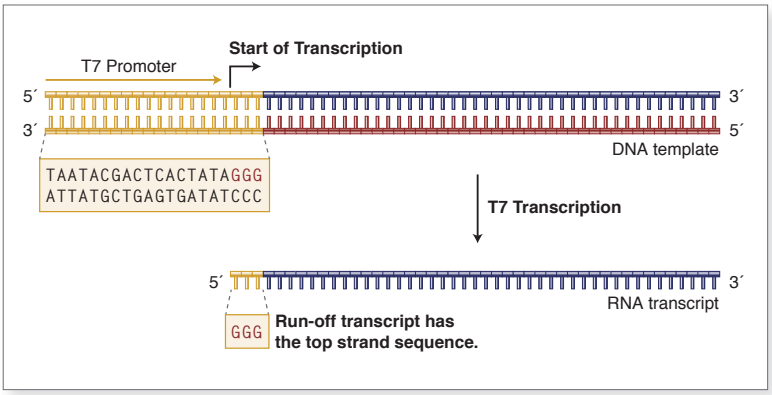
T7 Quick High Yield RNA Synthesis Kit

The T7 Quick High Yield RNA Synthesis Kit offers robust *in vitro* RNA transcription for a variety of applications. Yields of up to 180 µg of RNA can be obtained from a standard 20 µl reaction. The master mix format allows for quick reaction setup, requiring pipetting of two master mix reagents with template and water. This results in a reduced chance of errors due to fewer pipetting steps during reaction set up. DNase I and LiCl are included for DNA template removal and quick RNA purification.

Advantages

- **Streamlined format**
- **Flexibility** – enables incorporation of cap analogs, radiolabeled and modified nucleotides
- **High Yield** – up to 180 µg of RNA from a standard 20 µl reaction
- **High Quality Transcripts** – optimized formulation for increased transcript integrity over extended reaction times

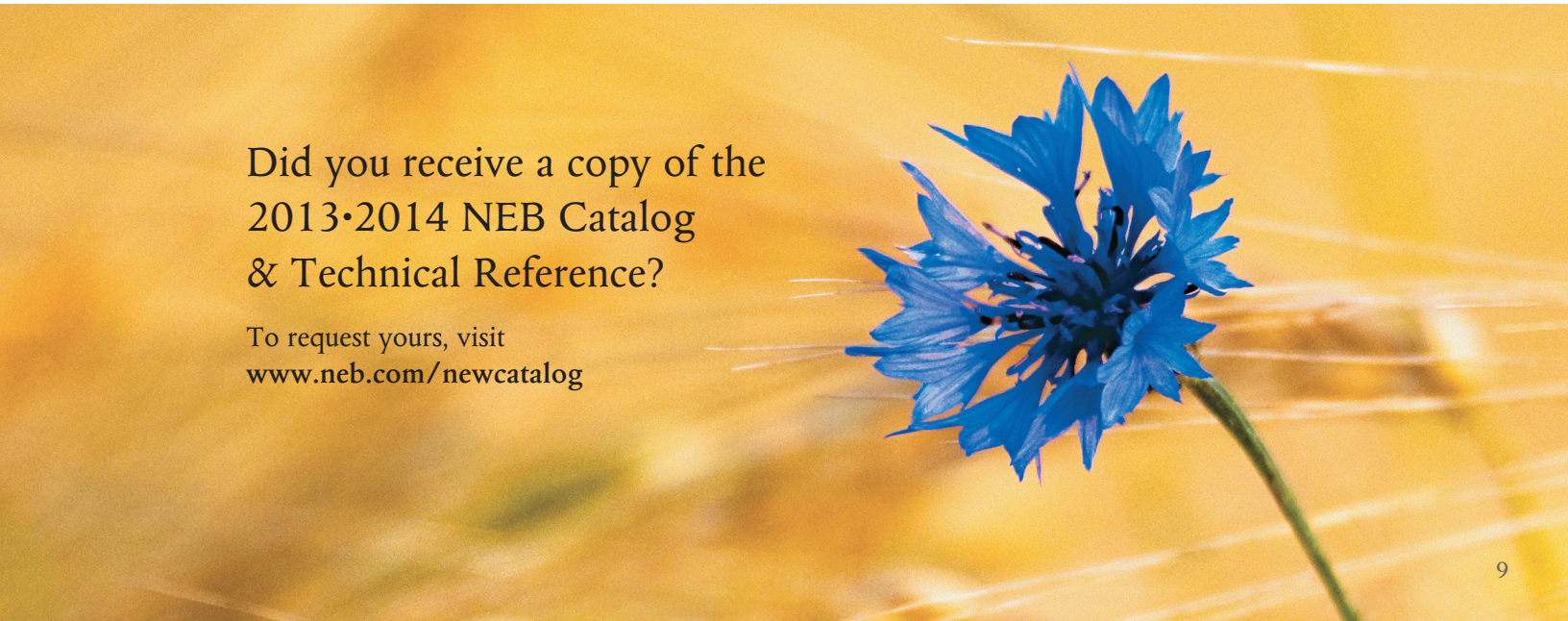
Transcription by T7 RNA Polymerase



T7 RNA Polymerase utilizes a minimal promoter sequence (shown in gold). Upon transcription, the run-off transcript has the top strand sequence.

Ordering Information

PRODUCT	NEB #	SIZE
T7 Quick High Yield RNA Synthesis Kit	E2050S	50 reactions

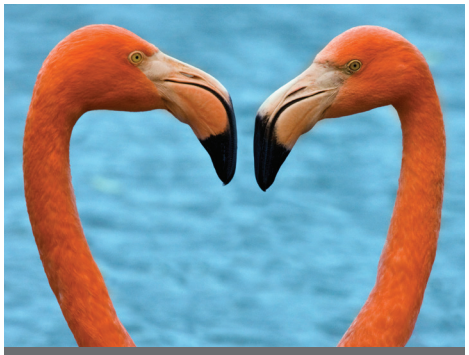


How many DNA Polymerases do you need in your freezer?

You only need TWO – One*Taq*® and Q5® DNA Polymerases, the complete solution for your PCR needs.

Whether you are performing routine PCR, or amplifying more challenging templates, New England Biolabs has the solution for you. Choose One*Taq* DNA Polymerase for routine amplification of standard, AT- and GC-rich templates, at a price-point that beats the competition. Choose Q5 High-Fidelity DNA Polymerase when you need high-fidelity amplification. With a fidelity > 100X *Taq* DNA Polymerase and 2X Thermo Scientific® Phusion® DNA Polymerase, Q5 delivers superior performance for complex templates with high-AT and -GC content.

Don't fill your freezer with DNA polymerases that can only amplify some of your templates. Choose One*Taq* and Q5 and clean out your freezer once and for all!



1 One*Taq* DNA Polymerases

2 Q5 High-Fidelity DNA Polymerases

Advantages of One*Taq*

- Exceptional performance across a wide range of templates
- Robust yields with minimal optimization
- Convenient product formats
- Hot start version allows room temperature reaction setup and does not require a separate activation step
- Compatible with standard *Taq* protocols

Advantages of Q5

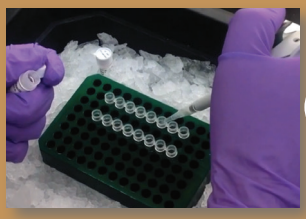
- Highest fidelity amplification available (> 100X higher than *Taq* and 2X higher than Thermo Scientific Phusion)
- High specificity and robust yields with minimal optimization
- Superior performance for a broad range of amplicons (from high AT to high GC)
- Fast with short extension times
- Amplifications up to 20 kb for simple templates, and 10 kb for complex ones

For more information, visit
www.neb.com/OneTaq and Q5PCR.com

LEARN MORE WITH OUR VIDEO TECH TIPS AT Q5PCR.COM

How to Amplify GC-rich DNA

Choose the Right DNA Polymerase for PCR



Why Choose Q5 High-Fidelity Polymerase?

WANT MORE?
NEB scientists have the expertise you need. Use the video carousel on **Q5PCR.com** to find additional video tech tips from NEB scientists.

TECHNICAL TIPS

PCR Troubleshooting Guide

The following guide can be used to troubleshoot PCR reactions. Additional tips for optimizing reactions can be found in the technical reference section of our website, **www.neb.com**.

PROBLEM	POSSIBLE CAUSE	SOLUTION
Sequence errors	Low fidelity polymerase	• Choose a higher-fidelity polymerase, such as Q5 (NEB #M0491) or Phusion (NEB #M0530)* High-Fidelity DNA Polymerases
	Suboptimal reaction conditions	• Reduce number of cycles • Decrease extension time
	Unbalanced nucleotide concentrations	• Prepare fresh deoxynucleotide mixes
	Template DNA has been damaged	• Start with a fresh template • Try repairing DNA template with the PreCR® Repair Mix (NEB #M0309) • Limit UV exposure time when analyzing or excising PCR product from the gel
	Desired sequence may be toxic to host	• Clone into a non-expression vector • Use a low-copy number cloning vector
Incorrect product size	Incorrect annealing temperature	• Recalculate primer Tm values using the NEB Tm calculator (www.neb.com/TmCalculator)
	Mispriming	• Verify that primers have no additional complementary regions within the template DNA
	Improper Mg^{++} concentration	• Adjust Mg^{++} concentration in 0.2–1 mM increments
	Nuclease contamination	• Repeat reactions using fresh solutions
No product	Incorrect annealing temperature	• Recalculate primer Tm values using the NEB Tm calculator (www.neb.com/TmCalculator) • Test an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor primer design	• Check specific product literature for recommended primer design • Verify that primers are non-complementary, both internally and to each other • Increase length of primer
	Poor primer specificity	• Verify that oligos are complementary to proper target sequence
	Insufficient primer concentration	• Primer concentration can range from 0.05–1 µM in the reaction. Please see specific product literature for ideal conditions
	Missing reaction component	• Repeat reaction setup
	Suboptimal reaction conditions	• Optimize Mg^{++} concentration by testing 0.2–1 mM increments • Thoroughly mix Mg^{++} solution and buffer prior to adding to the reaction • Optimize annealing temperature by testing an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor template quality	• Analyze DNA via gel electrophoresis before and after incubation with Mg^{++} • Check 260/280 ratio of DNA template
	Presence of inhibitor in reaction	• Further purify starting template by alcohol precipitation, drop dialysis or commercial clean-up kit • Decrease sample volume
	Insufficient number of cycles	• Rerun the reaction with more cycles
	Incorrect thermocycler programming	• Check program, verify times and temperatures
	Inconsistent thermocycler block temperature	• Test calibration of heating block
	Contamination of reaction tubes or solutions	• Autoclave empty reaction tubes prior to use to eliminate biological inhibitors • Prepare fresh solutions or use new reagents
	Complex template	• Use One*Taq* DNA Polymerases • For GC-rich templates, use One*Taq* DNA Polymerase (NEB #M0480) with One*Taq* GC Reaction Buffer (plus One*Taq* High GC Enhancer, if necessary) or Q5 High-Fidelity DNA Polymerase (NEB #M0491) with the High GC Enhancer • For longer templates, we recommend LongAmp® *Taq* DNA Polymerase (NEB #M0323)
Multiple or non-specific products	Premature replication	• Use a hot start polymerase, such as Q5 Hot Start High-Fidelity (NEB #M0493) or One*Taq* Hot Start (NEB #M0481) DNA Polymerases • Set up reactions on ice using chilled components and add samples to thermocycler preheated to the denaturation temperature
	Primer annealing temperature too low	• Recalculate primer Tm values using the NEB Tm Calculator (www.neb.com/TmCalculator) • Increase annealing temperature
	Incorrect Mg^{++} concentration	• Adjust Mg^{++} in 0.2–1 mM increments
	Poor primer design	• Check specific product literature for recommended primer design • Verify that primers are non-complementary, both internally and to each other • Increase length of primer • Avoid GC-rich 3′ ends
	Excess primer	• Primer concentration can range from 0.05–1 µM in the reaction. Please see specific product literature for ideal conditions
	Contamination with exogenous DNA	• Use positive displacement pipettes or non-aerosol tips • Set-up dedicated work area and pipettor for reaction setup • Wear gloves during reaction setup
	Incorrect template concentration	• For low complexity templates (i.e., plasmid, lambda, BAC DNA), use 1 pg–10 ng of DNA per 50 µl reaction • For higher complexity templates (i.e., genomic DNA), use 1 ng–1 µg of DNA per 50 µl reaction

*Phusion DNA Polymerase was developed by Finnzymes Oy, now a part of Thermo Fisher Scientific. This product is manufactured by New England Biolabs, Inc. under agreement with, and under the performance specifications of Thermo Fisher Scientific. Phusion® is a registered trademark and property of Thermo Fisher Scientific.

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