Issue I 2016

NEB expressions

a scientific update

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be **INSPIRED**
drive **DISCOVERY**
stay **GENUINE**

Five Steps to a Greener Lab:

A roadmap to environmental action

The laboratory is a place of inspiration, curiosity, ingenuity, and often altruism. Researchers are, by and large, a thoughtful and deeply invested group, but the environmental and ecological consequences of life science research are not typically at the forefront of researchers' minds when planning their investigations. Is it possible to minimize your environmental impact when working in the laboratory?

I. START THE DISCUSSION

The first step towards raising awareness about greener lab practices is to start the discussion with your coworkers. Share your concerns, brainstorm ideas together, and perhaps provide a list of green-labs-themed websites they can review. Several sites have sprung up in the past few years, including **www.mygreenlab.org**, the Labs21 Wiki, **www.green.harvard.edu/labs** and **www.labconscious.com**. Labconscious® began as a community of concerned life scientists, seeking to crowdsource solutions to the field's pervasive environmental challenges (see feature 1). "We've been really energized by the engagement we've seen with our blog and social network accounts," said Josh Resnikoff, contributing editor of Labconscious. "Life scientists are starting to take a critical look at the resources that their research demands, and they're starting to find smarter alternatives for a number of wasteful processes."

Next, speak to your environmental health and safety officer to ensure that your lab is taking full advantage of the green programs available. While some institutes and universities have realized the value of pro-environmental policies for the life sciences, others have been slower to adopt new processes. For instance, most universities offer basic recycling services, which collect paper products, beverage bottles and cans, but there are a number of specialized recycling methods that can address the various new streams of recyclables that are commonly used in life science research, such as polystyrene, polyethylene and polypropylene.

Be aware that laboratory safety policies frequently dictate how certain types of laboratory waste must be treated, whether it's incineration, sterilization or neutralization. Each of these methods is associated with its own environmental concerns; when possible, choose methods and chemicals that don't require regulated disposal. When regulated disposal is required, as for certain chemicals, consider secondary treatment methods that can minimize the amount or volatility of disposable materials, like distillation.

Once you've identified a few tactics for cutting your lab's environmental footprint, share your ideas with a larger group: present your findings at the next lab meeting, prepare a list for inclusion in your departmental newsletter, or hold a departmental seminar. You may be surprised by how receptive your coworkers are to your suggestions.

II. RECYCLE PLASTICS

Lab consumables are commonly made from plastics, as they are lightweight and extremely stable. Unfortunately, plastic decomposes slowly, if at all. Therefore, recycling is the most responsible way of disposing of the copious amounts of plastic generated in the lab. Small things, such as using refillable tip boxes or storage boxes, can help reduce the amount of plastic used. Items such as empty media bottles can also be recycled. Nucleic acid purification, in particular, is notorious for generating large amounts of plastic waste in the laboratory. To learn more about how NEB is working to minimize the impact of nucleic acid purification on the environment, see pages 6–7.

Labconscious is an open community for researchers to share ideas, protocols and best practices that help reduce the environmental footprint of bench science. Sponsored by New England Biolabs, it is our hope that Labconscious will become an educational platform and resource repository that will connect companies and brands with end users, and be used to identify greener processes and products. Together, we can try to make a better world with better labs.

Start the discussion at **labconscious.com**. Subscribe to the blog, follow the social channels and submit your ideas, stories and photos that relate to sustainability.

NEB'S SHIPPING BOX RECYCLING PROGRAM

NEB established the first shipping box recycling program over thirty-five years ago. Still in practice today in the U.S. and in some locations internationally, the program diverts polystyrene from landfills. Customers are provided with a return address label, and simply seal the box with the label and return via their local mail provider. It couldn't be more simple!

Many life science reagents require controlled temperature shipping, and expanded polystyrene (EPS) is the material of choice for protecting and insulating temperature-sensitive products. EPS is extremely stable, taking upwards of one million years to decompose in a landfill. EPS recycling is available in a few locations, but an EPS reuse program, like NEB's shipping box recycling program (see feature 2) and Andrew Markley's Styrofoam® box recycling program at the University of Wisconsin are even greener alternatives. Andrew received one of NEB's Passion in Science Awards™ in 2014, and has since worked to spread his box reuse program to other universities (see feature 3). The same material, polystyrene, is used in the fabrication of disposable serological pipettes, commonly used in sterile cell culture techniques. Recycling of serological pipettes may be available near you, but be sure to investigate BioSafety Level (BSL) restrictions for their applications.

If your institution's recycling program is limited, a good first step is to speak with your local environmental health and safety officer. Learning what provisions are available for new recycling programs, or what chemical safety rules must be adhered to, will help to guide your search for next steps.

III. CONSERVE WATER

Water consumption in life science research (and manufacturing) is extremely intensive. Things such as buffer production, cleaning of glassware, sterilization and incubation in water baths can all utilize large amounts of water.

There are several ways to conserve water in the lab. When making buffers or other aqueous solutions, follow an SOP; dumping out batches of incorrectly made buffers wastes both time and resources. Washing glassware by hand can be highly water efficient, but isn't a realistic option for certain labs; newer dishwashers can be connected to deionized and filtered water supplies, allowing for multiple rinses of important glassware. If your lab uses a water flow vacuum system, consider investing in a small vacuum pump; you can choose a size that fits your lab's particular needs and available space. Choose recirculating water baths, when possible. Consider installing aerators or water misers to minimize the amount of water that pours out of the faucet. While some of these improvements may be out of your hands, suggestions can always be passed on to your environmental health and safety officer or facilities manager.

AN NEB CUSTOMER WITH A PASSION FOR RECYCLING

Expanded polystyrene (EPS, also known as Styrofoam) has a low consumer-recycling rate due to its high transportation costs. In 2012, Andrew had the idea of collecting EPS boxes on campus and reusing them locally. Together, with the University of Wisconsin (UW) Office of Sustainability, they applied for and received over $100,000 in EPA funding for an undergraduate team to set up this system. In a year, UW went from no campus EPS recycling to collection sites in 26 buildings, reusing or recycling close to a semi-truck load monthly. Now, the university resells EPS boxes (as well as packing peanuts) through the campus surplus store, and provides boxes to local biotech companies for reshipment. The rest is delivered to a local EPS recycler. The hope is to use funds received by selling boxes to make campus EPS collection financially self-sustainable. Success with the UW program has led to Andrew replicating this program at two other universities.

AN INNOVATIVE METHOD FOR WASTEWATER TREATMENT

When NEB designed its facility in Ipswich, MA, one of the goals was to be more environmentally sound. As such, NEB chose to implement a Solar Aquatics® System on its campus to treat wastewater. Housed in a beautiful greenhouse filled with tropical plants, the system utilizes and accelerates the processes found in streams and wetlands to purify the water according to tertiary standards. While visitors may stop at the greenhouse to marvel at the beauty inside, many do not know it is treating the entire campus' wastewater for groundwater recharge.

To learn more about the water treatment process, visit **www.neb.com/aboutNEB**.

NEB's pledge to protect the environment extends to its local watershed; as such NEB has a unique solution for treating its wastewater and returning it to the ground (see feature 4).

IV. REDUCE ENERGY CONSUMPTION

Who hasn't unlocked the lab door, after a long weekend, to find that the lights had been on the whole time? Some laboratories are equipped with "smart" lights, activated by motion sensors; but if your lab is not, be mindful about turning the lights off. Furthermore, much of the climate control is determined by your facilities department. Still, there are several ways that every researcher can help save energy in the lab; it will just take some small adjustments to your established routines.

One of the major, researcher-related energy costs in the lab can be the easiest to avoid – skip the post-PCR hold. Plan your PCR to finish before you leave for the day or ask a labmate to move your tubes to the refrigerator, because your 4°C-hold step wastes significant energy.

Another simple, yet effective way to save energy is to shut the biosafety cabinet (tissue culture hood) sash. By shutting the sash, you stop the laminar-flow fan from running when it's not needed. Save even more energy by shutting the sash of the fume hood. In 2008, the Office for Sustainability at Harvard University initiated a shut the sash program, and has demonstrated significant energy savings as a result (For more information, visit **www.green.harvard.edu/programs/green-labs/shut-sash-program**).

continued on page 4...

To learn more about sustainability in the laboratory, view episode 6 of NEB TV

www.neb.com/NEBtv

You can also visit #shutthesash to see how other labs have adopted this philosophy.

Freezers are also known to consume large amounts of energy in the lab. Eliminate cold air loss by periodically scraping down the gasket that seals the freezer to remove any ice that has accumulated. Also, by keeping the coils on the rear of the freezer unit free from dust and debris, you can prevent the freezer from working harder to achieve and maintain temperature. Lastly, by simply organizing the freezer contents and creating a map to post on the freezer door, open times will be shorter and less frequent.

V. SHARE RESOURCES

Chemicals

Always review your experimental protocol; in some cases, greener chemicals can replace toxic chemicals. For example, one could consider replacing ethidium bromide, which is commonly used for staining DNA in agarose and acrylamide gels, with a safer stain such as GelRed™ or SYBR® safe. When safer options are not available, consider buying the smallest amount that will serve your purpose, or try to source your chemicals from a "shared" source. Find out if your institution has a shared chemical repository, and if not, work to set one up.

Equipment

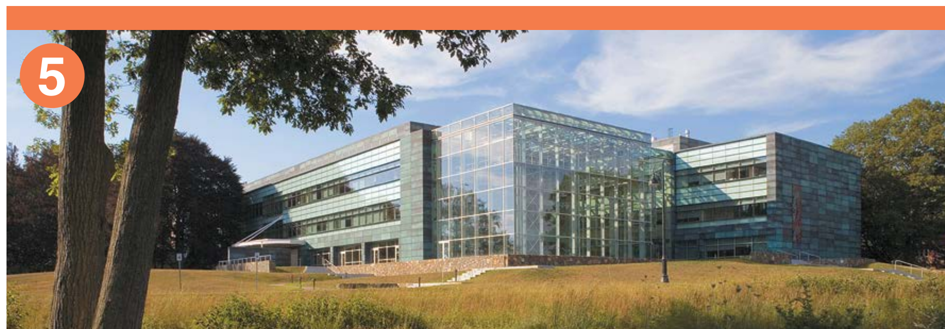
Perhaps you have changed your research focus and have equipment on your bench that you no longer need. Rather than disposing it, designate a place in the building for equipment reuse. There is probably another researcher in the building that can use it, and you may find something you were looking for as well! For example, in 2013, the Harvard University Office for Sustainability piloted a "Reuse Room" where researchers can deposit specific items for recycling and reuse, including equipment, glassware, NEB Styrofoam coolers and gel packs. This program has been very successful for them. To learn more, visit www.laboratoryequipment.com/articles/2014/04/collaborative-sustainability.

SUMMARY

You've already taken the first step; you've educated yourself about a number of greener actions you can get started with. The next step is up to you, but we'd like to suggest that you keep learning – learn what your institution and local governance can do for you. Learn what's recyclable, what can be reused, and how to reduce your lab's waste profile. Then, help to raise awareness amongst your coworkers. Work together to come up with a plan, tailor that plan specific to your department and then work to get leadership support.

At NEB, we're committed to reducing our own environmental footprint (see feature 5) and we are always open to suggestions as to how we can do better. We encourage you to share your ideas at labconscious.com or labconscious@neb.com, so that we can all benefit from each other's ideas, big or small.

Bringing your eco-mindedness into the laboratory can be easy; all it takes is a bit of research into your institution's policies, and a commitment to making life science research greener. Start small, question convention, and know that you can make a difference!



WHAT IS LEED® CERTIFICATION?

Leadership in Energy and Environmental Design (LEED) certification is a distinction awarded based on a suite of environmentally focused standards. These include site sustainability, water efficiency, energy conservation and atmospheric protection, choice of building materials and resources, indoor environmental quality, innovation and building design. LEED certification can be awarded at the laboratory level, or for entire buildings.

NEB commissioned the building of a LEED-certified laboratory facility in Ipswich, MA. Many choices were made to optimize energy usage, choose responsibly-sourced building materials and conserve resources through building design. For a list of examples, visit www.neb.com/about-neb/environmental-commitment.

References:
www.LabConscious.com
www.labs21century.gov

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Test your Sustainability I.Q.

Now that you have read about ways to start greening your laboratory, test your knowledge with our sustainability quiz.*

Questions kindly provided by Josh Resnikoff at Labconscious and Quentin Gilly at the Office for Sustainability at Harvard University.

1. Which of the following use the MOST energy in a typical laboratory?
   a) Biosafety Cabinets
   b) Fume Hoods
   c) -80°C Freezers
   d) Computers

2. Which of the following is recyclable?
   a) Pipette tip boxes
   b) Falcon® tubes
   c) Sterilized aluminum foil caps
   d) Cardboard packaging
   e) All of the above

3. Which one of these traditional reagents has a green alternative?
   a) Ethidium bromide
   b) Coomassie Stain
   c) Petri dishes
   d) Kimwipes®
   e) All of the above

4. How much energy does a lab use, compared to non-lab learning space?
   a) Less energy
   b) The same amount of energy
   c) Up to 4X more energy
   d) Up to 10X more energy

5. Which of the following practices DOES NOT improve the energy efficiency or longevity of a lab freezer?
   a) Cleaning filters regularly
   b) Defrosting freezers once ice build-up exceeds one quarter inch
   c) Keeping extra spaces in the freezer shelves so that air can circulate more efficiently
   d) Keeping a freezer map to minimize the amount of time the door is open

6. Which of the following substances render a plastic unrecyclable upon contact?
   a) LB broth
   b) Ethanol
   c) Agarose
   d) Sodium azide

7. Which of the following is NOT a recommended lab practice when preparing to leave for a long break?
   a) Closing all blinds/shades in the lab
   b) Shutting down and unplugging equipment that will not be in use
   c) Closing all sashes on hoods
   d) Stocking up on lab supplies that could increase in price next year

8. What is the most effective power management strategy for the office space?
   a) Unplugging items from outlets when not in use (like cell phone power cords)
   b) Using a central printer rather than individual printers for common office spaces
   c) Taking the stairs instead of the elevator in your building
   d) All of the above help save energy for office space

9. Boxes used for shipping samples with dry ice CANNOT be reused due to federal guidelines.
   True or False

10. Plastic media bottles CANNOT be recycled under any circumstances.
   True or False

For full questions and answers, including further explanation, visit **www.facebook.com/NEBiolabs**

Answers may vary depending on institutional policies.

Answer key: 1) b 2) e 3) e 4) c 5) c 6) d 7) d 8) d 9) False 9) False

NEW PRODUCT

Monarch™

Nucleic Acid Purification Kits

Migrate to Monarch.

NEW Monarch Nucleic Acid Purification Kits from NEB

It's time to transform your DNA purification experience. NEB's Monarch Nucleic Acid Purification Kits are optimized for maximum performance and minimal environmental impact. Our unique thin-walled column design uses less plastic, prevents buffer retention, eliminates the risk of carryover contamination, and enables elution in smaller volumes. The result: high performing DNA purification for your downstream applications.

Optimize your results with our unique column design

- Improved recovery of concentrated, pure DNA
- Low volume elutions, resulting in highly-concentrated DNA
- No buffer retention, eliminating the risk of carryover contamination

Enhance your DNA purification experience

- Fast, user-friendly protocols
- Columns designed for easy labeling and handling
- Improved buffer system for robust performance

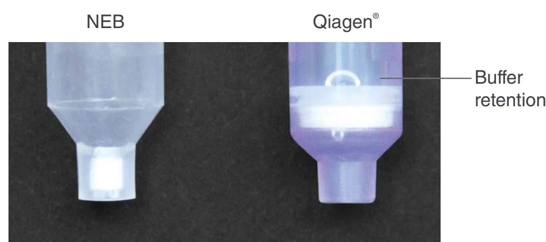
Feel good about choosing Monarch

- Significantly less plastic in every kit*
- Custom-designed, thin-walled columns and collection tubes
- Responsibly sourced and recyclable packaging
- Packaging and protocol cards printed with water and soy-based inks
- Reusable kit boxes

Choose Monarch kits for pure value

- Buffers and columns available separately
- No additional shipping or handling charges**
- Competitive pricing

Designed for performance



Designed for sustainability – Monarch kits*...

have up to 44% less plastic

could eliminate >140 tons of plastic each year

use recyclable packaging materials

use boxes made from 100% post-consumer paper

* Visit NEBMonarchPackaging.com for details.
** In the US and select subsidiary locations. Contact your local distributor for shipping policies.

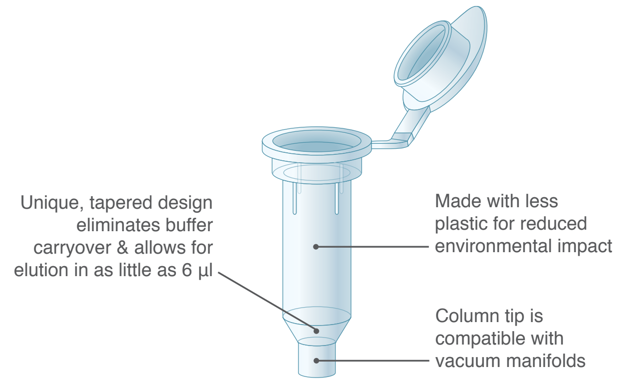
Monarch DNA Gel Extraction Kit

Rapidly purify up to 5 µg of concentrated, high-quality DNA from your agarose gels, with no need to adjust pH. Elute in as little as 6 µl for a more concentrated sample.

Monarch PCR & DNA Cleanup Kit (5 µg)

Purify DNA from a variety of enzymatic reactions, including PCR, restriction digestion, ligation and reverse transcription.

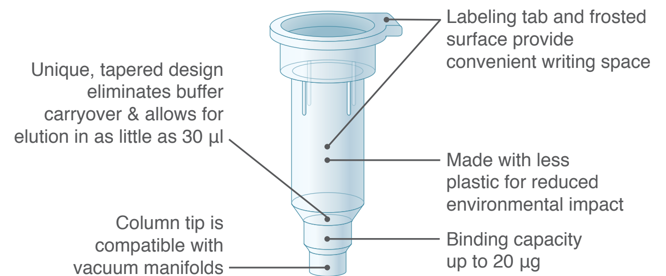
FIGURE 1: **Optimized design of the columns supplied with the Monarch DNA Gel Extraction and PCR & DNA Cleanup Kits**



Monarch Plasmid Miniprep Kit

This kit employs familiar cell resuspension, alkaline lysis and neutralization steps, with the additional benefit of color indicators in the buffers to monitor completion. Elute in lower volumes for more concentrated, highly pure DNA samples.

FIGURE 2: **Optimized design of columns supplied with Monarch Miniprep Kit**



Interested in learning more?

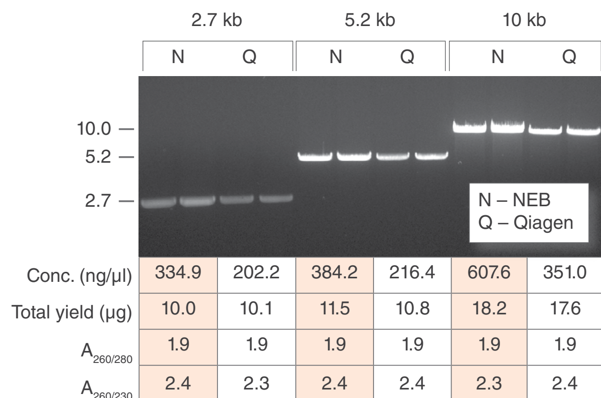
To learn more and request a sample, visit **NEBMonarch.com**

ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
Monarch DNA Gel Extraction Kit	T1020S/L	50/250 preps
Monarch PCR & DNA Cleanup Kit (5 µg)	T1030S/L	50/250 preps
Monarch Plasmid Miniprep Kit	T1010S/L	50/250 preps
COLUMNS SOLD SEPARATELY		
Monarch DNA Cleanup Columns (5 µg)	T1034L	100 columns
Monarch Plasmid Miniprep Columns	T1017L	100 columns
BUFFERS AVAILABLE SEPARATELY		
Monarch DNA Cleanup Binding Buffer	T1031L	235 ml
Monarch DNA Wash Buffer	T1032L	25 ml
Monarch DNA Elution Buffer	T1016L	25 ml
Monarch Gel Dissolving Buffer	T1021L	235 ml
Monarch Plasmid Lysis Buffer (B2)	T1012L	2 x 27 ml
Monarch Plasmid Neutralization Buffer (B3)	T1013L	110 ml
Monarch Plasmid Resuspension Buffer (B1)	T1011L	55 ml
Monarch Plasmid Wash Buffer 1	T1014L	2 x 27 ml
Monarch Plasmid Wash Buffer 2	T1015L	30 ml

FIGURE 3: **Monarch Plasmid Miniprep Kits consistently produce more concentrated plasmid DNA with equivalent yield, purity and functionality as compared to the leading supplier**

Preps were performed according to manufacturer recommended protocols using 1.5 ml aliquots of the same overnight culture. One microliter of each prep was digested with HindIII-HF® (NEB #R3104) to linearize the vector and the digests were resolved on a 1% w/v agarose gel.



	2.7 kb N	2.7 kb Q	5.2 kb N	5.2 kb Q	10 kb N	10 kb Q
Conc. (ng/µl)	334.9	202.2	384.2	216.4	607.6	351.0
Total yield (µg)	10.0	10.1	11.5	10.8	18.2	17.6
$A_{260/280}$	1.9	1.9	1.9	1.9	1.9	1.9
$A_{260/230}$	2.4	2.3	2.4	2.4	2.3	2.4

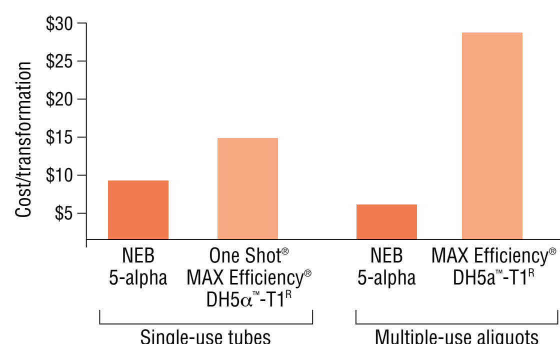
FEATURED PRODUCTS

Clone with confidence.

NEB 5-alpha Competent *E. coli* – new formats available

A versatile *E. coli* strain, NEB 5-alpha is a derivative of DH5α™ and has the same genetic features as this popular cloning strain. NEB 5-alpha offers high transformation efficiencies, convenient formats and value pricing. Whether you are doing routine cloning, subcloning or looking for a high efficiency (electrocompetent) format, NEB 5-alpha is the ideal strain for you.

FIGURE 1: **Take advantage of the low cost per transformation with NEB 5-alpha***

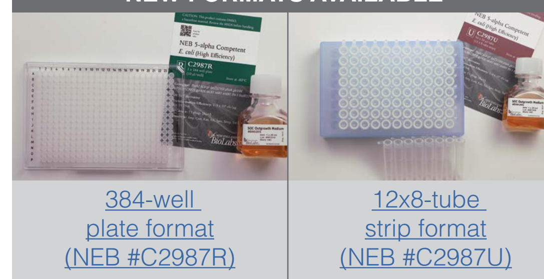


* *Calculations were based on list price and recommended transformation volumes.*

advantages

- A derivative of the popular DH5α strain
- Free of animal products
- No dry ice charges with any NEB competent cell shipments
- Choose from high efficiency, subcloning and electrocompetent formats

NEW FORMATS AVAILABLE



384-well plate format (NEB #C2987R)

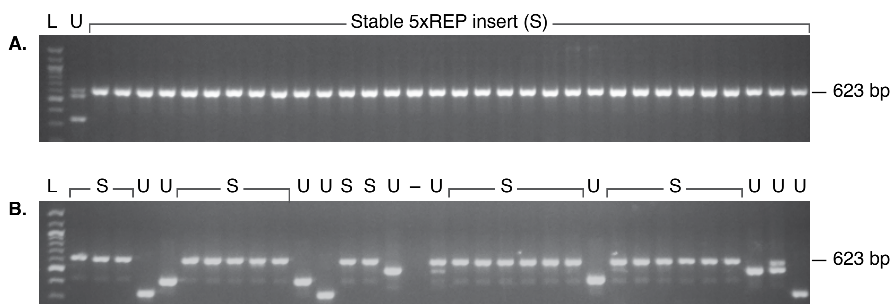
12x8-tube strip format (NEB #C2987U)

NEB Stable Competent *E. coli*

NEB Stable Competent *E. coli* is the ideal strain for cloning unstable inserts, such as direct or inverted repeats. It is the recommended host strain for cloning genes into retroviral/lentiviral vectors. NEB Stable Competent *E. coli* offers high efficiency and is competitively priced.

FIGURE 2: **NEB Stable enables the isolation of plasmid clones containing repetitive DNA elements**

Plasmid pUC-5xREP contains five 32-bp repeats, making it unstable in a recombination-proficient strain. A) NEB Stable competent cells or B) Stbl3™ competent cells were transformed with 2 µl of a pUC-5xREP Gibson Assembly® reaction containing 2.2 ng (0.00125 pmol) pUC19 vector and approximately 1 ng (0.0028 pmol) 5xREP insert. Transformed cultures were plated on LB plates containing 100 µg/ml ampicillin and incubated overnight at 30°C. The next day, colony PCR was performed using M13/pUC polylinker primers to analyze 5xREP insert stability.



L = TriDye™ 100 bp DNA Ladder (NEB #N3271)
U = Unstable insert with deletions
S = Stable 5xREP insert
– = No insert detected

advantages

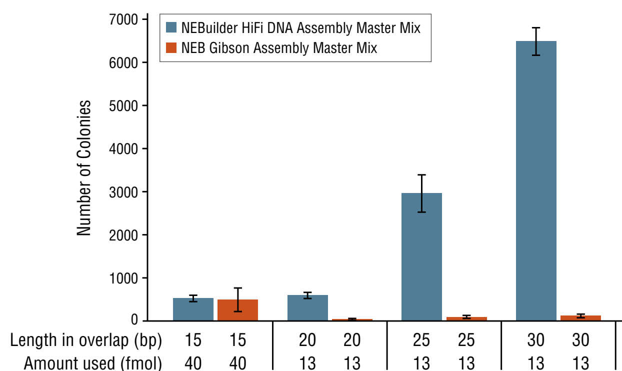
- Improved cloning of direct repeats and inverted repeats
- Recommended host strain for cloning genes into retroviral/lentiviral vectors
- Free of animal products
- No dry ice charges with any NEB competent cell shipments
- Value pricing

NEBuilder® HiFi DNA Assembly

NEBuilder HiFi DNA Assembly enables virtually error-free joining of DNA fragments, even those with 5´- and 3´-end mismatches. Available with and without competent *E. coli*, this flexible kit enables simple and fast seamless cloning utilizing a new proprietary high-fidelity polymerase. Make NEBuilder HiFi your first choice for DNA assembly and cloning.

FIGURE 3:
NEBuilder HiFi DNA Assembly offers improved efficiency and accuracy with lower amounts of DNA by increasing overlap length

Reactions were set up in a 4-fragment assembly reaction according to recommended reaction conditions. Amount of DNA and size of overlap is shown.



advantages

- Enjoy simple and fast seamless cloning in as little as 15 minutes
- Screen fewer constructs, with virtually error-free high-fidelity assembly
- Use for both "standard-size" cloning and larger gene assembly products, up to 12 fragments
- Use NEBuilder HiFi in successive rounds of assembly, because it removes 5´- and 3´-end mismatches
- Bridge two ds-fragments with a synthetic ssDNA oligo for simple and fast construction (e.g., linker insertion or gRNA library)
- Switch from other systems easily, as NEBuilder HiFi is compatible with Gibson Assembly- designed (and other) fragments
- No licensing fee requirements from NEB for NEBuilder products

For help with designing primers, try NEBuilder Assembly Tool at **NEBuilder.neb.com**

Bridge two dsDNA fragments with a synthetic ssDNA oligo

Visit **NEBuilderHiFi.com** and learn how NEBuilder HiFi DNA Assembly bridges dsDNA with a ssDNA oligo

ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987P	1 x 96 well plate (20 µl/well)
	C2987R	1 x 384 well plate (10 µl/well)
	C2987I	6 x 0.2 ml/tube
	C2987H	20 x 0.05 ml/tube
	C2987U	96 x 50 µl/tube (12 x 8-tube strips)
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S	10 rxns
NEBuilder HiFi DNA Assembly Master Mix	E2621S/L/X	10/50/250 rxns
NOW AVAILABLE:		
NEBuilder HiFi DNA Assembly Bundle for Large Fragments – Includes NEB 10-beta Competent *E. coli*, for assemblies larger than 15 kb	E2623S	20 rxns

Improved method for assembly of linear yeast expression cassettes using NEBuilder HiFi DNA Assembly Master Mix

Saulius Vainauskas, Ph.D. and Christopher H. Taron, Ph.D., New England Biolabs, Inc.

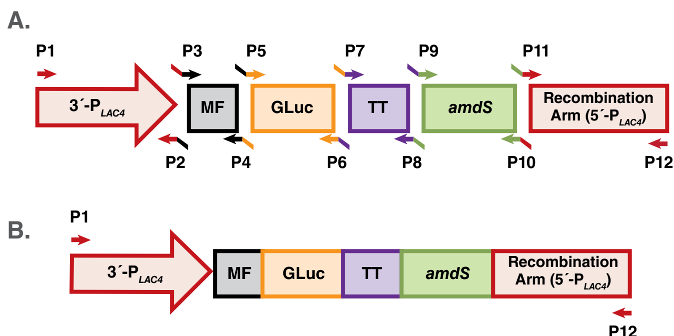
INTRODUCTION

Heterologous protein production in yeast expression systems (i.e., *Kluyveromyces lactis* and *Pichia pastoris*) normally involves insertion of a linear expression cassette into a target locus in the host genome (1–3). Typically, an expression cassette is assembled in *E. coli* by first cloning a gene of interest into a circular expression vector. The expression cassette comprises DNA encoding a strong yeast promoter upstream of a heterologous gene of interest, a downstream transcription terminator sequence and a selectable marker gene. The entire cassette is flanked by locus-specific targeting sequences on either end. The assembled vector is then amplified by propagation of the host *E. coli* cells, isolated by standard DNA preparation techniques, and subjected to restriction digestion to create a linear expression cassette. The linear fragment is then introduced into yeast whereby it integrates into a target locus on the host chromosome.

While this approach for expression strain construction has been accepted methodology for over two decades, it does have some limitations. For example, expression vector assembly using traditional cloning techniques is a multi-day process. Additionally, one is limited to the use of restriction sites that are present in the expression vector and the use of the expression machinery (i.e., promoter, terminator, locus targeting sequence, etc.) that is built into the vector. Finally, for cloning of a gene whose product is highly toxic to *E. coli*, serendipitous protein expression during vector assembly in bacteria can yield clones having deleterious mutations (1,4). The use of *in vitro* DNA assembly methods to construct linear expression cassettes suitable for direct introduction into yeast circumvents each of these limitations while shaving days off of expression strain construction time. In the presented method, we highlight the use of NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621) to rapidly assemble expression cassettes for use with NEB's *K. lactis* Protein Expression Kit (NEB #E1000).

FIGURE 1: *In vitro* assembly of a linear expression cassette.

(A) Primer pairs P1 through P12 were used in PCR to amplify individual functional domains of the expression cassette. (B) The domains were assembled using the NEBuilder HiFi DNA Assembly Master Mix according to the recommended protocols, and the assembled linear expression cassette was amplified by PCR using primers P1 and P12. In this experiment, the CYC1 transcription terminator (TT) was used.



Interested in learning more?

Visit www.neb.com/E2621 to **download the full application note**, which includes data and tables not shown.

PROTOCOL

Experimental Design – Liner expression cassette assembly

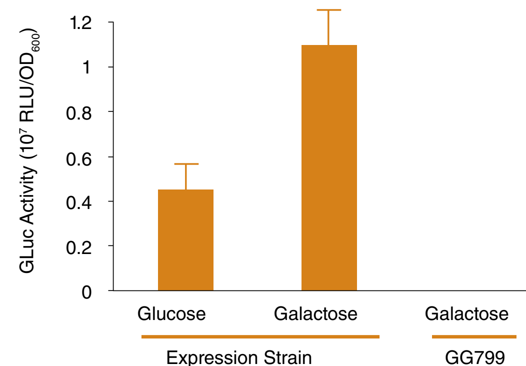
In this experiment, multiple pairs of primers were designed using the NEBuilder Assembly Tool (**NEBuilder.neb.com**) to permit amplification of individual functional domains of an expression cassette (table not shown). Each resulting amplicon contained 22 bp overlaps with adjacent domains (Figure 1A). Purified PCR products were combined and treated with NEBuilder HiFi DNA Assembly Master Mix and the fully assembled expression cassette was amplified using primers P1 and P12 (Figure 1B) to obtain sufficient quantities for introduction into *K. lactis* cells.

RESULTS:

A transformation efficiency of about 8 x 10^3 colonies/µg DNA was obtained with the *in vitro* assembled expression construct, a similar efficiency to that observed when using a cloned expression cassette liberated from a pKLAC2 expression construct. *K. lactis* cells harboring at least one integrated copy of the *in vitro*-assembled expression cassette successfully secreted *Gaussia princeps* luciferase (GLuc) into the growth medium (Figure 2).

FIGURE 2: Expression of *Gaussia princeps* luciferase (GLuc) in *K. lactis* GG799 cells.

The NEBuilder-assembled linear expression cassette (shown in Figure 1B) was used to transform *K. lactis* GG799 cells. A representative transformant was cultured in triplicate in rich medium (yeast extract/peptone) supplemented with glucose or galactose as a carbon source. Untransformed GG799 cells were grown in medium containing galactose as a negative control. GLuc enzyme activity secreted into the growth medium was measured using the BioLux® *Gaussia* Luciferase Assay Kit (NEB #E3300). GLuc expression from the LAC4 promoter is repressed in the presence of glucose, but is derepressed in galactose, as expected (1-3).



CONCLUSION:

In this application note, the use of NEBuilder HiFi DNA Assembly Master Mix to rapidly assemble a linear expression fragment for introduction into *K. lactis* was demonstrated. The method permits fast preparation of linear expression cassettes in 1-2 days whereas the classical *E. coli* cloning approach takes at least 4-5 days. The *in vitro* assembly method is also suitable for the cloning of toxic genes because it eliminates the potential for accumulation of deleterious mutations during expression vector construction steps in bacteria. Moreover, NEBuilder HiFi DNA Assembly permits more flexibility in the creation of constructs having different promoters, signal sequences, reporter genes and integration sites compared to using pKLAC2 in the *K. lactis* Protein Expression Kit. The general methodology outlined in this application note can also be applied more widely to assemble linear DNAs containing expression machinery for introduction into other yeasts (e.g. *Pichia pastoris*), fungi, bacteria, plant cells or animal cells.

ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
NEBuilder HiFi DNA Assembly Master Mix	E2621S/L/X	10/50/250 rxns
K. lactis Protein Expression Kit	E1000S	1 kit

References:
1. Colussi, P.A. and Taron, C.H (2005) *Appl. Environ. Microbiol.* 71,7092–7098.
2. van Ooyen, A.J.J., et al. (2006) *FEMS Yeast Res.* 6,381–392.
3. Read, J.D., et al. (2007) *Appl. Environ. Microbiol.* 73,5088–5096.
4. Gibbs, M., et al. (2004) *FEMS Yeast Res.* 4,573–577.

Joining of Difficult to Ligate dsDNA Fragments with Blunt/TA Ligase Master Mix

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INTRODUCTION

T4 DNA Ligase is routinely used to ligate breaks on one strand of a dsDNA molecule (nicks), or to ligate a variety of double-stranded breaks. This includes “cohesive ends”, with two or more bases of complementary single-stranded regions (such as those generated by BamHI-HF®), and fully base-paired fragments or “blunt-ends” (such as those generated by EcoRV). Certain ligation substrates are known to be difficult to ligate, with sluggish rates and poor yields even with prolonged incubation periods and high enzyme concentration. Commonly encountered ends that are difficult to ligate include single base overhangs, with 5´-single base overhangs (such as those generated by BstNI) showing almost no ligation (< 5%) by T4 DNA Ligase under typical conditions.

The Blunt/TA Ligase Master Mix (NEB #M0367) is a formulation of T4 DNA Ligase (NEB #M0202) pre-mixed with its reaction buffer and proprietary ligation enhancers in a convenient, single-tube 2X mixture. In most cloning applications, combining equal volumes of master mix and a solution of vector and insert will provide rapid ligation and high colony yield after transformation, equaling, and sometimes exceeding, the Quick Ligation™ Kit (NEB #M2200). Additionally, the Blunt/TA Ligase Master Mix has been found to give superior ligation yields for particularly difficult to ligate substrates, substantially exceeding that of any other ligase formulation.

Here, we demonstrate the use of Blunt/TA Ligase Master Mix for the ligation of difficult-to-ligate dsDNA fragments. The substrates tested were prepared from short, synthetic oligonucleotides purchased from IDT, and annealed using standard conditions (Figure 1). Each substrate contains one oligonucleotide 5´-phosphorylated and 3´-FAM labeled to allow ease of quantitation.

- **Nicked DNA Substrate:** a 50 bp dsDNA with a break on one strand between nucleotides 20 and 21. The 30-base strand was 5´-phosphorylated and 3´-FAM labeled.
- **Cohesive-end Substrate:** a 30-base strand 5´-phosphorylated and 3´-FAM labeled with a 26-base complement strand, resulting in a substrate with a 4-base, 5´-overhang modeling the DNA ends generated by treatment with BamHI-HF (NEB #R3136).
- **3´-A/T Single-base Overhang Substrate:** a 29-base strand 5´-phosphorylated and 3´-FAM labeled with a 30-base complement strand, and a second fragment with a 21-base oligonucleotide annealed to a 20-base complement strand modeling fragments generated by Hpy188I (NEB #R0617), a 3´ single-base dA overhang on one fragment and a dT on the other. This substrate also serves as a surrogate for a “TA cloning” substrate, such as insertion of a *Taq* DNA Polymerase amplicon with a 3´ adenosine into a “T-vector”, or for adapter ligation during an NGS library prep.
- **5´-A/T Single-base Overhang Substrate:** a 30-base strand 5´-phosphorylated and 3´-FAM labeled with a 29-base complement strand, and a second fragment with a 20-base oligonucleotide annealed to a 21-base complement strand modeling fragments generated by BstNI (NEB #R0168), a 5´ single-base dA overhang on one fragment and a dT on the other.

FIGURE 1: **Substrate sequences tested**

Nicked DNA substrate

```
  5'-TATAACTTTACTTCTATTGC pTGATGGGACCTACAATGTACCAGAAGCGTC-3'FAM
3'AM-ATATTGAAATGAAGATAACG—ACTACCCTGGATGTTACATGGTCTTCGCAG-5'
```

Cohesive end substrate (BamHI-HF)

```
5'-pGATCCGGACCTACAATGTACCAGAAGCGTC-3'FAM
    3'-GCCTGGATGTTACATGGTCTTCGCAG-5'
```

3′ A/T single base overhang substrate (Hpy188I)

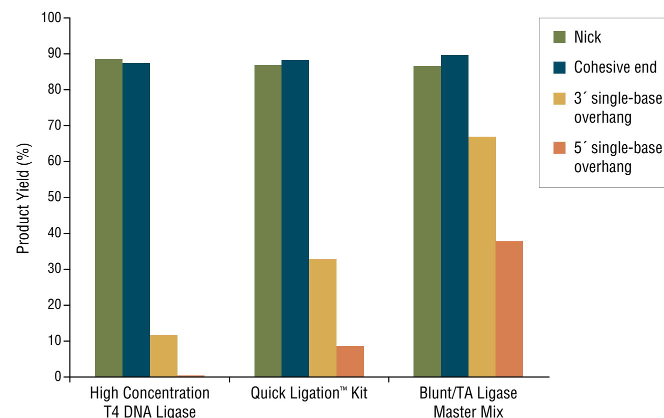
```
5'-TATAACTTTACTTCTATGTCA-3'   5'-pGATGGGACCTACAATGTACCAGAAGCGTC-3'FAM
3'-ATATTGAAATGAAGATACAG-5'    3'-TCTACCCTGGATGTTACATGGTCTTCGCAG-5'
```

5′ A/T single base overhang substrate (BstNI)

```
5'-TATAACTTTACTTCTCCTCC-3'    5'-pAGGAGGGACCTACAATGTACCAGAAGCGTC-3'FAM
3'-ATATTGAAATGAAGAGGAGGT-5'     3'-CCTCCCTGGATGTTACATGGTCTTCGCAG-5'
```

FIGURE 2: **Blunt/TA Ligase Master Mix improves yields for ends that typically react slowly**

Yields of final ligation product for all reaction conditions using high concentration T4 DNA Ligase, the Quick Ligation Kit and Blunt/TA Master Mix. Nick, cohesive end and 3´ single-base overhang substrates were incubated for 15 minutes; the 5´ single-base overhang was incubated for 1 hour.



RESULTS

The extent of ligation in each reaction was visualized by detection of the FAM-labeled strands by high throughput capillary electrophoresis, which allows for high accuracy in quantitation and product identification. For both nicked DNA and cohesive ends, all three ligase products perform similarly, with high ligation yields in a short reaction time. For the more difficult to ligate 3´-A/T single base overhang, ligation produces “AppDNA” in addition to ligated product. AppDNA is a reaction intermediate in ligation, where the ligase has transferred an enzyme-conjugated 5´phosphoryladenosine (Ap) group to the 5´-phosphate of the DNA substrate, producing an activated 5´-5´ AppN linkage at the 5´ terminus. In the course of the ligation of an easily ligatable substrate, this intermediate is not often observed. For very difficult ligations, the intermediate step can dissociate prematurely, accumulating in solution as a side product. Improved ligation yields are observed with the Quick Ligation Kit, but the Blunt/TA Master Mix produces even higher yields of ligated DNA. For the exceedingly difficult to ligate 5´A/T-single base overhang substrate, almost no reaction is observed when using high concentration T4 DNA Ligase. The Quick Ligation Kit produces predominantly AppDNA, but the Blunt/TA Master Mix again provides superior yields of ligated DNA (Fig. 2).

CONCLUSION

When attempting to ligate dsDNA ends that typically react slowly and with poor ligation yield, the Blunt/TA Ligase Master Mix will provide superior ligation yields over other T4 DNA Ligase formulations. When ligating more robust substrates (cohesive ends, nicks, blunt ends) the Blunt/TA Master Mix performs similarly to the Quick Ligation Kit in terms of yield and reaction time, and can be substituted for other T4 DNA Ligase preparations for convenience.

ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
Blunt/TA Ligase Master Mix	M0367S/L	50/250 rxns

Interested in learning more?

Visit **www.neb.com/M0367** to **download the full application note**, which includes figures not shown.

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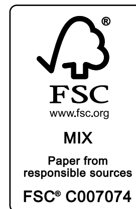

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