Issue II **2017**

NEB expressions

a scientific update

in this issue

Peony (*Paeoniaceae*) blooming on the New England Biolabs campus, Ipswich, MA. Photograph taken by Penny Devoe.

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Overcoming the challenges of applying target enrichment for translational research

by Andrew Barry, M.S., New England Biolabs, Inc.

Target enrichment is used to describe a variety of strategies to selectively isolate specific genomic regions of interest for sequencing analysis. The wide array of approaches presents challenges in selecting the appropriate technology for the growing number of research and clinical applications to which the sequencing data will ultimately be applied.

INTRODUCTION

In recent years, several techniques have emerged to enrich for specific genes of interest. When determining the appropriate target enrichment technology to use, one must first consider the primary goal of the study. For example, different approaches will be used if the aim is to identify known variants already shown to have clinical implications, versus discovering novel nucleic acid variants that may be associated with a given phenotype. Variant identification lends itself to more focused enrichment strategies, while variant discovery is driven by trade-offs between sequencing costs and target territory, as well as available sample cohort sizes for a given study.

As translational research seeks to bridge fundamental laboratory research and clinical treatment regimens for patients, there is an emerging need to balance discovery of novel nucleic acid variants, identification of known variants, and studies aimed at revealing associations with clinical phenotypes. Recent advances in sequencing technologies have revolutionized the field of genomic research, making tractable the application of whole genome and whole exome sequencing for broad discovery of germline genomic variants. However, despite these advances, the oncology field is fraught with the complexity of detangling the underpinnings of tumorigenesis, progression, and resistance mechanisms driven by somatic variants present at extremely low abundance in mixtures of malignant and stromal cells. These complexities necessitate increases in the depth of sequencing coverage to confidently call somatic variants, making broader scale approaches infeasible from an economic and practical standpoint.

To overcome these challenges, focused gene panels are being applied to patient samples. The size of the panel is highly variable, trending toward decreased genomic content as assays progress from pure research and discovery applications to clinical diagnostic assays. Furthermore, clinical applications raise the question of incidental findings and how to report them, introducing challenges for diagnostic assays based on sequencing entire genomes. This trend demonstrates the practical need for continued use of target enrichment strategies across the gamut of translational research activities.

TARGET ENRICHMENT APPROACHES

There are a number of different target enrichment approaches that can be grouped into three generalized categories: in-solution hybridization, multiplex PCR, and "alternative approaches", which span a wide variety of techniques.

In-solution hybridization-based approaches, originally developed for whole exome sequencing, use biotinylated oligonucleotides to capture genomic regions of interest (1). Commercially-available kits use DNA or RNA baits ranging from 50-150 nucleotides. Researchers have adapted this technique for more focused panels, ranging down to tens of kilobases in target territory, with limited success in maintaining specificity for target regions.

Multiplex PCR-based enrichment is most often employed for highly focused panels targeting a smaller territory than in-solution hybridization, and is typically limited to 150-200 amplicons (2). Using a pool of primers, enrichment is accomplished through PCR amplification of the targeted regions, which is followed by adaptor ligation or a second round of PCR using tailed primers to include sequencing adaptors. Scaling this technology has presented a challenge in maintaining target coverage uniformity.

A number of alternative approaches have been developed in an attempt to bridge the gap between hybridization and PCR-based approaches. Examples of these hybrid approaches include multiplex extension ligation (3), molecular inversion probes (MIPS)/padlock probes (4), nested patch PCR (5), and selector probes (6). These technologies can be broadly characterized as having more complex workflows, requiring splitting of samples into separate reactions, and creating challenges in target coverage uniformity.

NEBNext Direct® for target enrichment

NEBNext Direct is a novel target enrichment method that addresses several drawbacks that exist in alternative enrichment technologies (Table 1). Enrichment is achieved through direct hybridization of biotinylated DNA baits to denatured, fragmented molecules, which are subsequently captured using magnetic streptavidin beads (Figure 1, page 3). Unlike alternative in-solution hybridization protocols, the NEBNext Direct protocol does not require library preparation prior to hybridization of oligonucleotide probes. This feature reduces the overall amount of amplification that is required throughout the protocol and enables single-stranded DNA to be captured along with denatured, double-stranded DNA.

Conversion of captured fragments to sequence-ready libraries is achieved by the ligation of a loop adaptor to the proximal 3´ end of the captured molecule. During these steps, the bait / target molecules remain bound to the magnetic streptavidin beads and are processed in a single reaction tube. This eliminates sample loss and improves overall conversion efficiency.

TABLE 1:
Enrichment Challenges and Advantages of NEBNext Direct

Challenge	NEBNext Direct Advantage
Specificity across panel sizes	Enzymatic removal of off-target sequence
Uniformity of coverage	Individual synthesis of baits & empirical balancing
Sensitivity to detect variants	Unique Molecule Indexes for PCR duplicate marking & consensus variant calling
Degraded or low quality samples	Short baits that extend across molecules, targeting both DNA strands

Following ligation of the 3′ adaptor, the bait is extended across the entirety of the captured molecule, resulting in double stranded DNA that is ready for ligation of the 5′ unique molecular identifier (UMI) adaptor. This adaptor contains a 12 bp random sequence that is incorporated discretely into each molecule, indexing each molecule prior to amplification. This index can be used to identify duplicate molecules, thereby reducing artifacts that can lead to false positive variant calls.

Once the 5′ adaptor is ligated, the 3′ loop adaptor is cleaved, and the target molecule is PCR amplified off of the bait complex. It is important to note that the bait strand is not perpetuated through the PCR amplification and is not present in the final, sequencer-ready library.

The coverage plots of NEBNext Direct libraries are unique for a hybridization-based approach in that reads have a defined 3′ end, resulting in coverage plots that resemble PCR-based libraries, yet the approach allows for flexibility in tiling across longer targets. Disambiguation of PCR duplicates is accomplished by two features of the NEBNext Direct library: A variable 5′ end and a 12 bp randomized UMI that is incorporated into the 5′ adaptor.

CHALLENGES OF TARGET ENRICHMENT FOR TRANSLATIONAL RESEARCH

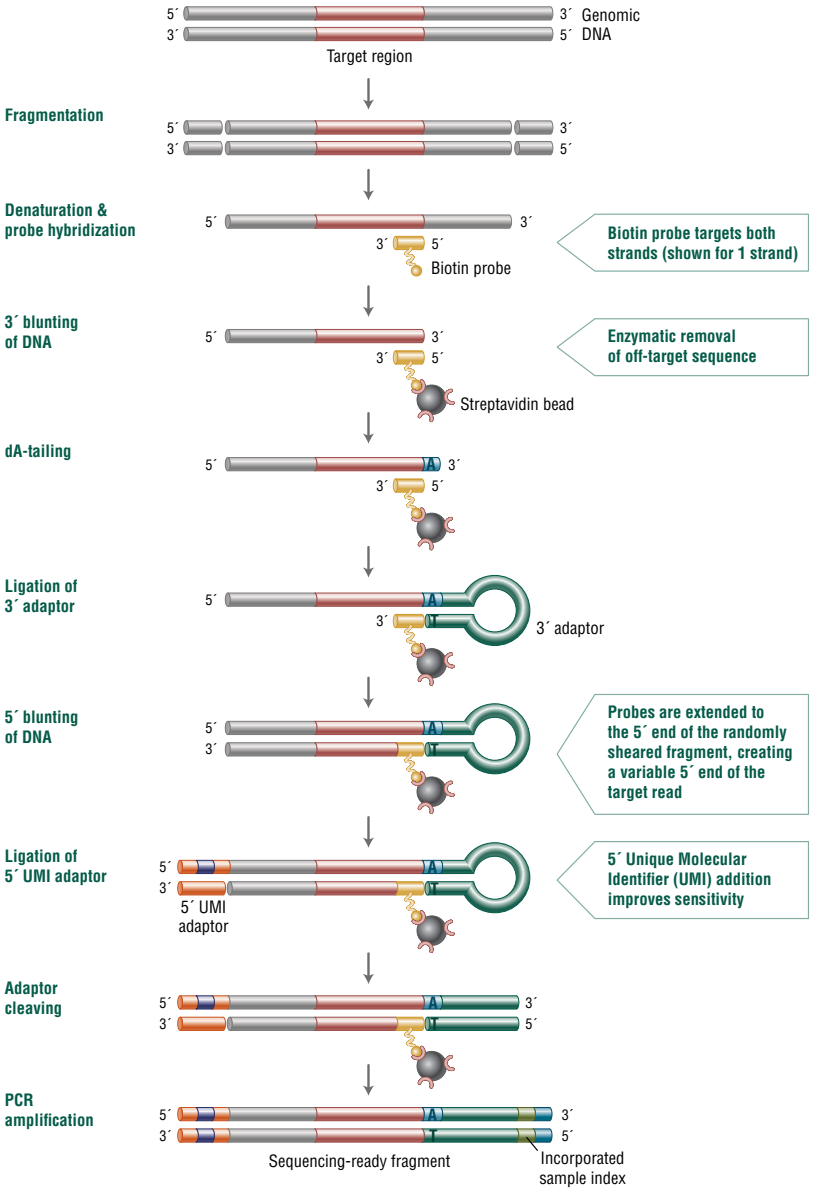
Specificity of target enrichment

For any study that necessitates enrichment of specific targets over more comprehensive sequencing approaches, specificity becomes more important as it directly translates to the amount of sequencing required to achieve the minimum coverage threshold to reliably detect variants of a given frequency. Specificity is typically measured by looking at the percentage of sequencing data that is derived from the targeted regions relative to the data that is aligned to other parts of the reference genome.

Enrichment of genomic regions is typically achieved by either amplifying the desired regions through PCR to generate enough copies of the targeted regions over the untargeted regions, or through hybridization of complementary biotinylated oligonucleotide probes to fragmented DNA molecules, where specificity is driven through careful control of melting temperatures and buffer composition to promote hybridization.

Specificity for target regions is enhanced using NEBNext Direct through both the hybridization of specific baits, as well as through enzymatic removal of off-target sequence. The enzymatic treatment removes both off-target sequence of molecules unbound to baits, as well as the regions of molecules upstream of where the baits are bound. This additional means of driving specificity enables the bait hybridization to be shorter, lasting only 90 minutes in duration. This differs from a typical hybridization-based approach,

FIGURE 1:
NEBNext Direct target enrichment workflow



in which randomly fragmented molecules are captured overnight, and without any removal of upstream off-target sequences, read coverage resembles a normal distribution.

While specificity for targeted regions using traditional hybridization approaches is typically quite high for larger panels up to whole exome, specificity typically decreases as the size of the targeted region decreases. Thus, smaller panels typically result in an increased proportion of sequencing lost to off-target regions. In contrast, the NEBNext Direct approach maintains high specificity across a broad range of target territory, from single genes or exons to hundreds of kilobases, eliminating the need to use different technologies for different panels (Table 2, page 4).

Uniformity of coverage across targets

One of the drawbacks to many available target enrichment methods is the inability to enrich different targets with equivalent efficiency. The result requires an increase in the overall coverage for all targets to achieve the minimum depth of coverage required to reliably call variants. One of the main factors influencing coverage unevenness is the sequence composition of the targeted regions themselves, with different efficiencies for sequences comprised of GC or AT rich regions.

Depending on the approach, the target enrichment strategy being employed may be more or less susceptible to the need for balancing melting temperatures across any complementary oligonucleotide baits or PCR primers that are employed in the enrichment process. Challenges

continued on page 4...

in uniformity can also arise from any downstream PCR that is used to generate sufficient material for the sequencing process, as various DNA polymerases demonstrate biases toward targets that may include secondary structure.

Using multiplex PCR-based workflows, primer design is limiting as melting temperatures must match within each panel and primer-primer interactions and primer cross-talk must be considered. These constraints can lead to variations in coverage uniformity between targets. Partitioning individual amplification reactions into emulsion droplets can alleviate some of these constraints and improve target uniformity (12), but this approach requires investment in instrumentation as well as additional workflow steps.

Oligonucleotides utilized during NEBNext Direct enrichment are individually synthesized, which enables bait pools to be carefully optimized based on empirical testing. Individual baits are balanced, allowing fine tuning of target coverage. Additionally, the bait design algorithm optimizes new bait design based on outcomes from prior results. Further, because the specificity is not solely driven through melting temperatures alone, NEBNext Direct allows increased flexibility in bait design.

The result is coverage across targets that can be optimized, demonstrating high degrees of uniformity and diminishing the overall amount of sequencing required to identify nucleic acid variants (Figure 2).

Sensitivity to detect nucleic acid variants

Perhaps the most critical aspect is the sensitivity of an approach to detect nucleic acid variants, as this is often the primary goal of studies in humans where target enrichment is employed. This is measured as the ability of an assay to detect nucleic acid variants that are present at a given frequency, referred to as variant allele frequency (VAF) or mutation allele frequency (MAF). Biologically, in the context of solid tumors, this is a function of the mixture of stromal and tumor cells, as well as the heterogeneity of tumor cells, and the existence of subclonal variants that are associated with tumorigenesis. Utilization of sequence data for the approximation of allele frequency is achieved through counting of sequence reads that possess a given variant. Quantitative assessment of sequence reads is challenged through the presence of duplicate molecules, or molecules that are identified through sequencing as having the same genomic coordinates. Depending on the target enrichment method that was employed to prepare the samples for sequencing, disambiguation of molecules that have arisen from discrete copies of genomic DNA versus those resulting from PCR amplification can be difficult or impossible to ascertain.

Disambiguation of PCR duplicates is accomplished by two features of the NEBNext Direct library: A variable 5´ end and a 12 bp randomized UMI that is incorporated into the 5´ adaptor. The amount of coverage one can expect from a given panel should be measured once duplicate molecules are removed in order to determine if the coverage is deep enough to reliably call a variant as a true-positive variant (Figure 3, page 5).

Difficult sample types

Whether for research or clinical applications, translational genomics often examines samples that are derived from patients. Patient tissue can be compromised by processes used to collect, preserve, store, extract nucleic acids from, and ultimately prepare for sequencing-based assays.

FIGURE 2:
NEBNext direct delivers higher coverage uniformity than alternative approaches.

Plot shows the uniformity across targets for each panel, measured as the percentage of bases above 25% of the mean target coverage. Samples were processed in duplicate according to the manufacturer's suggested protocol using the recommended amount of DNA input. DNA used was a blend of 24 HapMap samples. Samples were sequenced on an Illumina® MiSeq® per the manufacturer recommendation. Representative data across 2 replicates are shown.

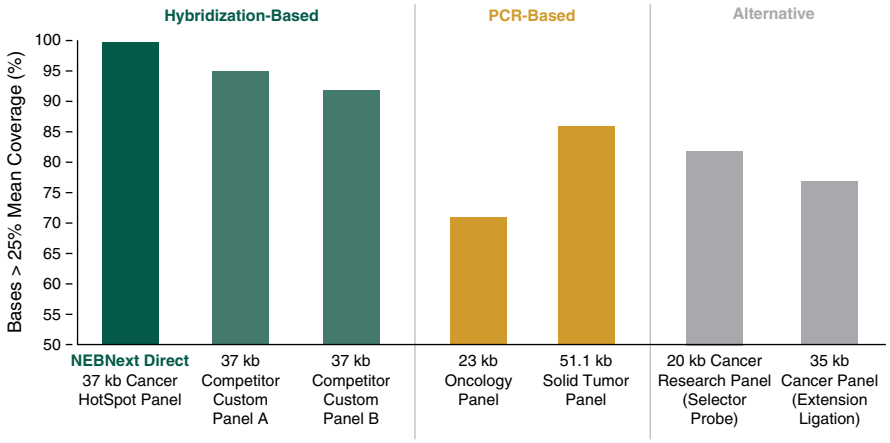


TABLE 2:
Specificity and uniformity of NEBNext Direct panels

Panel Size (kb)	Specificity (% Reads on Target)	Uniformity (% bp >20% MTC*)
15.2	99.4	99.3
15.9	96.1	100
20.4	99	99.5
36.8	92.5	98.7
76.4	91	98.5
93	95.9	99.35
217	90	99.23

** bp – base pairs MTC – Mean Target Coverage*

The most widely used technique for the storage and preservation of tissue derived from patient samples involves fixing the tissue in formalin, and embedding the fixed sample in paraffin. DNA derived from formalin-fixed, paraffin embedded (FFPE) samples has been shown to contain varying degrees of degradation, accumulation of base-specific errors, DNA breaks with damaged ends, and are often present in extremely low quantities (7-9). The recent application of target enrichment to circulating cell-free DNA molecules offers a less invasive means of monitoring cancer progression. Cell-free DNA derived from solid tumors is biologically present in relatively short fragments of 150-160 bp, which can present challenges using traditional enrichment approaches as both cell-free and FFPE tissue-derived nucleic acids contain high amounts of ssDNA (10, 11).

Using in-solution hybridization based enrichment presents challenges, as an upfront library must be prepared prior to hybridization to long (>100 bp) baits, and can result in sample loss. Moreover, degradation of FFPE derived nucleic acids can create shorter library inserts not optimal for hybridization to longer baits. Finally, the initial library generation step requires dsDNA; thus, the approach disregards ssDNA that may be present in the original sample due to DNA damage.

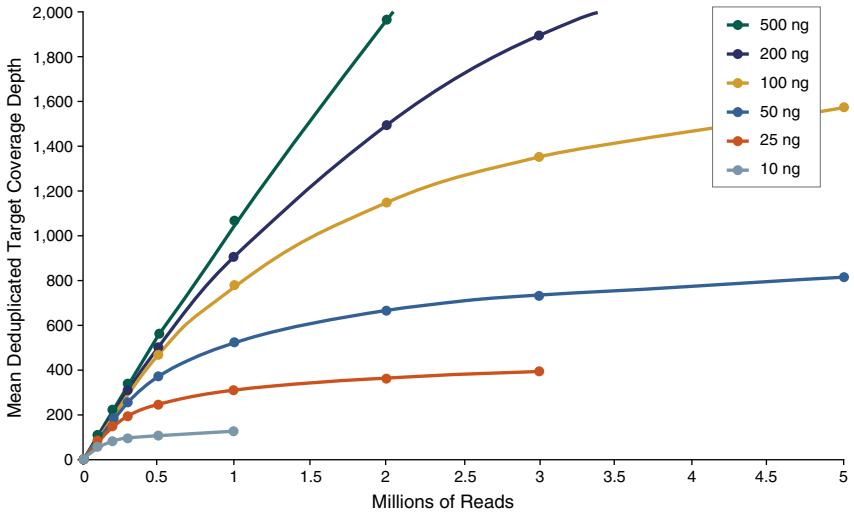
Multiplex PCR also presents challenges in targeting degraded samples, as the ability to successfully anneal both primers on a given molecule is difficult as DNA input molecule length is decreased due to degradation.

The short (~45-55 nucleotide) baits used in NEBNext Direct enrichment provide an increased probability of binding to shorter fragments, and the independent targeting of both strands of DNA offers improved opportunity to capture degraded fragments. The approach also contains an optional phosphorylation step to ensure the ends of target DNA are prepared for ligation of adaptors.

FIGURE 3:

NEBNext Direct is able to achieve high depths of sequence coverage across a broad range of inputs.

Mean depth of coverage relative to sequencing depth is shown across a range of DNA inputs. A blend of 24 HapMap samples were enriched using the 37 kb NEBNext Direct Cancer HotSpot Panel and sequenced on an Illumina MiSeq using 2 x 75 base pair sequencing. Coverage is shown after the removal of PCR duplicates using the information from the unique molecular identifier (UMI).



CONCLUSION

NEBNext Direct target enrichment overcomes several challenges translational researchers face in selectively enriching for certain genomic targets for clinical research. Providing the flexibility to use a single approach across a wide range of target content, NEBNext Direct allows enrichment of a single gene, up to panels comprised of hundreds of genes, without compromising performance as targets change. NEBNext Direct provides the specificity and coverage uniformity to maximize sequencing efficiency, in order to realize the benefits of target enrichment. Furthermore, intrinsic properties of the approach lend themselves to improved sensitivity, and have proven amenable to challenging sample types, typical of translational workflows. Combining the best aspects of hybridization-based enrichment and multiplex PCR enrichment, without the trade-offs, NEBNext Direct is a single-day, easy-to-use protocol that can be applied to advance translational research.

Learn more about NEBNext Direct at **NEBNextDirect.com**

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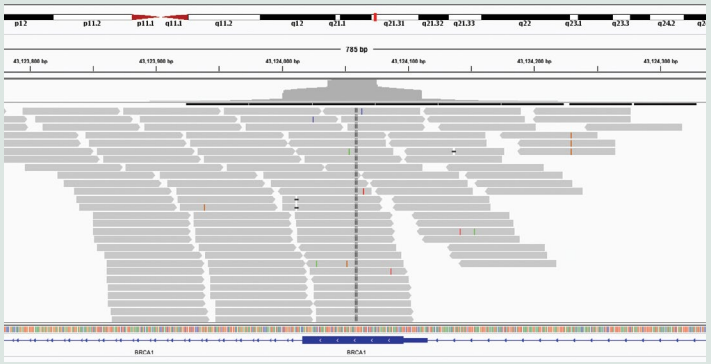
Now available: the NEBNext Direct BRCA1/BRCA2 Panel

The **NEBNext Direct BRCA1/BRCA2 Panel (NEB # E6627S/L/X)** enriches the complete exon content of BRCA1 and BRCA2 genes for NGS analysis. NEBNext Direct employs a unique enrichment workflow that hybridizes baits directly to genomic DNA, without the need for upfront library preparation. The BRCA1/BRCA2 panel demonstrates high specificity and unmatched coverage uniformity across a wide range of DNA inputs, allowing highly sensitive calling of germline and somatic variants, while maximizing sequencing efficiency.

IGV plot showing complete and even coverage across exon 1 of the BRCA1 gene

Integrative Genomics Viewer (IGV) Plot of exon 1 from BRCA1 gene showing read level coverage obtained using 50 ng DNA enriched using the NEBNext Direct BRCA1/BRCA2 Panel. Sequencing was performed on an Illumina MiSeq using 2 x 75 paired-end sequencing.



Benefits

- Generate full (100%) coverage of all protein coding regions in BRCA1 and BRCA2 genes
- Obtain highly uniform sequencing across exon target – 100% of base pairs in panel have coverage greater than 20X of the mean target coverage
- Save time with a 1-day workflow that combines enrichment with library preparation
- Distinguish molecular duplicates, reducing false-positive variants and improving assay sensitivity
- Produce high depths of target coverage across a wide range of DNA input amounts for germline and somatic variants

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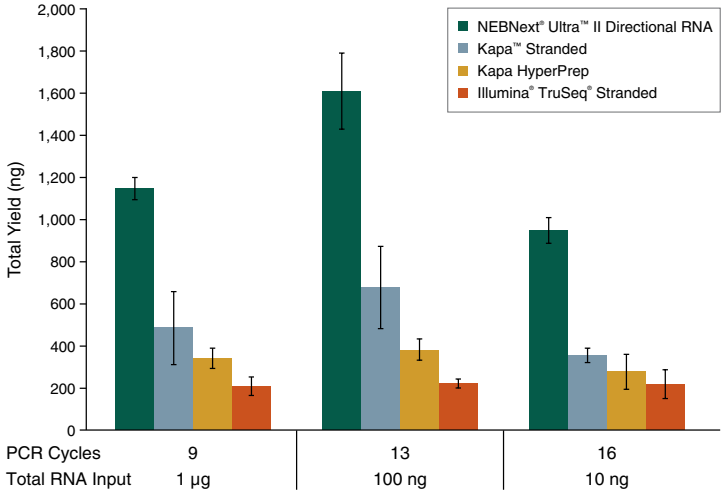
Do you need increased sensitivity and specificity from your RNA-seq experiments? Do you have ever-decreasing amounts of input RNA? To address these challenges, our next generation of RNA library prep kits have been reformulated at each step, resulting in several fold higher yields of high quality libraries, enabling use of lower input amounts and fewer PCR cycles. The kits have streamlined, automatable workflows and are available for directional (strand-specific, using the "dUTP method"(1,2)) and non-directional library prep, with the option of SPRISelect® beads for size selection and clean-up steps.

FIGURE 1:
NEBNext Ultra II Directional RNA produces the highest yields, from a range of input amounts

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent® #740000) and libraries were made using the NEBNext Ultra II Directional RNA Kit (plus the NEBNext poly(A) mRNA Magnetic Isolation Kit), Kapa Stranded mRNA-Seq Kit, Kapa mRNA HyperPrep Kit and Illumina TruSeq Stranded mRNA Kit. The input RNA amount and number of PCR cycles are indicated. Library yields from an average of three replicates are shown.



"The Ultra II RNA kit has allowed us to reduce the input for directional poly(A)+ RNA-seq libraries by a factor of 10 or more. We can now make a library with only 10 ng high quality total RNA and get the same gene expression profile as for 1 µg input. We've even pushed the input as low as 1 ng for very high quality total RNA. The new Ultra II RNA kit makes RNA-seq achievable for low yield samples, we actually need more RNA for quality control than for library prep! Furthermore, the library prep protocol is streamlined compared to the previous Ultra RNA kits, including a reduction in AMPure bead clean-ups and PCR cycles, resulting in better libraries for less time and resources."

– **Jen Grenier, Ph.D.**, Director of RNA Sequencing Core (RSC), Center for Reproductive Genomics, Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University

Advantages

- **Generate high yield**, high-quality libraries even with limited amounts of RNA:
  - 10 ng – 1 µg total RNA (poly(A) mRNA workflow)
  - 5 ng – 1 µg total RNA (rRNA depletion workflow)
- **Minimize bias**, with fewer PCR cycles required
- **Increase library complexity** and transcript coverage
- **Increase flexibility** by ordering reagents specific to your workflow needs
  - Directional and non-directional kits available
  - rRNA depletion and poly(A) mRNA isolation reagents supplied separately
  - Adaptors and primers (12-, 96-, and dual index) supplied separately
- Enjoy the **reliability of the gold standard SPRISelect size selection and clean-up beads**, supplied in just the amounts you need
- Save time with **streamlined workflows**, reduced hands-on time, and automation compatibility
- Rely on **robust performance**, even with low quality RNA, including FFPE

For more information and to request a sample, visit **UltraIIRNA.com**

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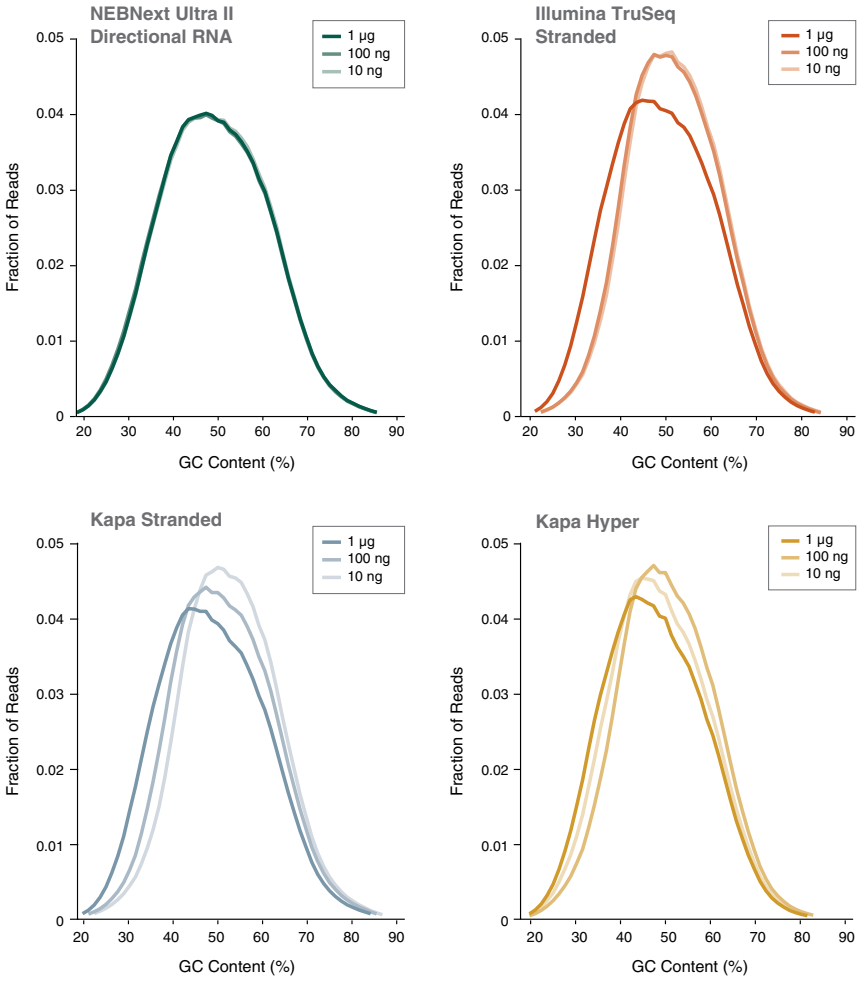
NEBNext® Ultra™ II RNA Library Prep Kits

FIGURE 2:
NEBNext Ultra II Directional RNA libraries provide uniform GC content distribution, for a broad range of input amounts

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent #740000), and libraries were made using the NEBNext Ultra II Directional RNA Kit (plus the NEBNext Poly(A) mRNA Magnetic Isolation Module), Illumina TruSeq Stranded mRNA Kit, Kapa Stranded mRNA-Seq Kit and Kapa mRNA HyperPrep Kit. Libraries were sequenced on an Illumina NextSeq® 500 using paired-end mode (2x76 bp). Reads were mapped to the hg19 reference genome. GC content distribution for each library was calculated using mapped reads. Ultra II Directional RNA libraries had uniform GC content distribution across a range of input amounts, as indicated by the curve overlap. For other kits the GC content distribution changed with different input amounts, indicating the introduction of input-dependent sequence bias.



OTHER PRODUCTS YOU MIGHT BE INTERESTED IN:

PRODUCT	NEB #	SIZE
NEBNext rRNA Depletion Kit (Human/Mouse/Rat)	E6310S/L/X	6/24/96 rxns
NEBNext rRNA Depletion Kit (Human/Mouse/Rat) with RNA Sample Purification Beads	E6350S/L/X	6/24/96 rxns
NEBNext Poly(A) mRNA Magnetic Isolation Module	E7490S/L	24/96 rxns
NEBNext Ultra II RNA First Strand Synthesis Module	E7771S/L	24/96 rxns
NEBNext Ultra II Directional RNA Second Strand Synthesis Module	E7550S/L	24/96 rxns
NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module	E6111S/L	20/100 rxns
NEBNext Multiplex Oligos for Illumina (Index Primers Set 1, 2, 3, 4)	E7335, E7500, E7710, E7730S/L	24/96 rxns
NEBNext Multiplex Oligos for Illumina (96 Index Primers)	E6609S/L	96/384 rxns
NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1)	E7600S	96 rxns

Available kits:

- **NEBNext Ultra II Directional RNA Library Prep Kit for Illumina**
  Includes optimized mixes for directional RNA library preparation (fragmentation, cDNA synthesis, end repair/dA-tailing, adaptor ligation and PCR enrichment steps) for sequencing on the Illumina platform.
  NEB # E7760S/L 24/96 rxns

- **NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads**
  Includes optimized mixes for directional RNA library preparation (fragmentation, cDNA synthesis, end repair/dA-tailing, adaptor ligation and PCR enrichment steps) plus SPRISelect® beads for size selection and clean-up.
  NEB # E7765S/L 24/96 rxns

- **NEBNext Ultra II RNA Library Prep Kit for Illumina**
  Includes optimized mixes for non-directional RNA library preparation (fragmentation, cDNA synthesis, end repair/dA-tailing, adaptor ligation and PCR enrichment steps) for sequencing on the Illumina platform.
  NEB # E7770S/L 24/96 rxns

- **NEBNext Ultra II RNA Library Prep with Sample Purification Beads**
  Includes optimized mixes for non-directional RNA library preparation (fragmentation, cDNA synthesis, end repair/dA-tailing, adaptor ligation and PCR enrichment steps) plus SPRISelect beads for size selection and clean-up.
  NEB # E7775S/L 24/96 rxns

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Versatile	•					•
Fast growth (< 8 hours)		•				
Toxic gene cloning		•	•			•
Large plasmid/BAC cloning				•		
Dam/Dcm-free plasmid growth					•	
Retroviral/lentiviral vector cloning						•
RecA	•		•	•		•
FORMATS						
Chemically competent	•	•	•	•	•	•
Electrocompetent	•	•		•		
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FIGURE 1:
Benefit from high transformation efficiencies

Transformation efficiencies were compared using manufacturers' recommended protocols. Values shown are the average of triplicate experiments.

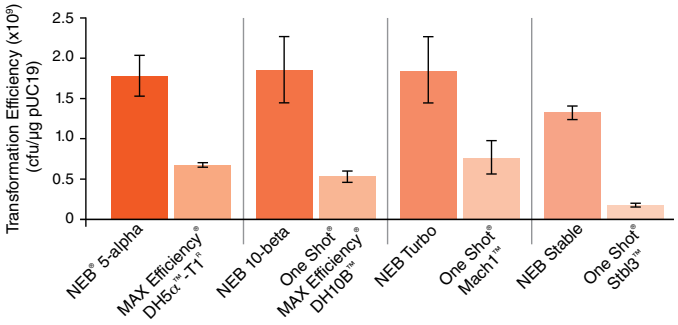
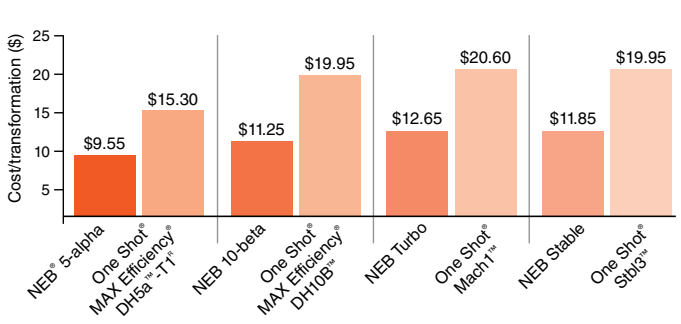


FIGURE 2:
Take advantage of the low cost per transformation

Calculations were based on list price and recommended transformation volumes.



**Offer valid in the U.S. only, limit one per customer, while supplies last.

Enhancing Transformation Efficiency

Transformation efficiency is defined as the number of colony forming units (cfu) that would be produced by transforming 1 µg of plasmid into a given volume of competent cells. However, 1 µg of plasmid is rarely transformed. Instead, efficiency is routinely calculated by transforming 100 pg–1 ng of highly purified supercoiled plasmid under ideal conditions. Transformation Efficiency (TE) is calculated as: TE = Colonies/µg/Dilution. Efficiency calculations can be used to compare cells or ligations. Our recommended protocols and tips to help you achieve maximum results are presented here:

TRANSFORMATION TIPS

Thawing

- Cells are best thawed on ice
- DNA should be added as soon as the last trace of ice in the tube disappears
- Cells can be thawed by hand, but warming above 0°C decreases efficiency

Incubation of DNA with Cells on Ice

- Incubate on ice for 30 minutes. Expect a 2-fold loss in TE for every 10 minutes this step is shortened.

Heat Shock

- Both temperature and time are specific to the transformation volume and vessel. Typically, 30 seconds at 42°C is recommended.

Outgrowth

- Outgrowth at 37°C for 1 hour is best for cell recovery and for expression of antibiotic resistance. Expect a 2-fold loss in TE for every 15 minutes this step is shortened.
- Use NEB 10-beta/Stable Outgrowth Medium for 10-beta and Stable Competent *E. coli.* Use SOC for all other strains.
- Outgrowth medium gives 2-fold higher TE than LB medium
- Incubation with shaking or rotation results in 2-fold higher TE

Plating

- Selection plates can be used warm or cold, wet or dry with no significant effects on TE
- Warm, dry plates are easier to spread and allow for the most rapid colony formation

DNA

- DNA should be purified and resuspended in water or Tris-EDTA Buffer
- Up to 10 µl of DNA from a ligation mix can be used with only a 2-fold loss of efficiency
- Purification by either a spin column or phenol/chloroform extraction and ethanol precipitation is ideal
- The optimal amount of DNA is lower than commonly recognized. Using clean, supercoiled pUC19, the efficiency of transformation is highest in the 100 pg–1 ng range. However, the total colonies which can be obtained from a single transformation reaction increase up to about 100 ng.

DNA CONTAMINANTS TO AVOID

CONTAMINANT	REMOVAL METHOD
Detergents	Ethanol precipitate
Phenol	Extract with chloroform and ethanol precipitate
Ethanol or Isopropanol	Dry pellet before resuspending
PEG	Column purify or phenol/chloroform extract and ethanol precipitate
DNA binding proteins (e.g., ligase)	Column purify or phenol/ chloroform extract and ethanol precipitate

Featured Videos

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How to perform a transformation with NEB competent cells

Tips for successful transformations with NEB competent cells

Online Tools

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Getting ready to perform a gel extraction?

Here are six tips to help you get the best results possible:

1 Melt your agarose completely

The number one reason that users see low yields with gel extraction procedures is because the agarose plug is not completely melted. When this happens, DNA remains trapped inside the agarose and cannot be extracted properly.

2 Minimize exposure to UV light

Excise the gel slice as quickly as possible, as exposure to UV light damages DNA. As long as the excision is done quickly, damage done to the DNA will be negligible.

3 Use the smallest agarose plug possible

Run your DNA in the narrowest well possible, and trim off any excess agarose. There are many reasons for this one! The less agarose in solution, the more efficient the extraction will be. The larger your agarose plug is, the longer it will take to melt. It will also require more dissolving buffer, which introduces more salts and other components to the purification. Also, if the plug is greater than 160 mg, the volume of agarose plus buffer will exceed the volume of the column reservoir (800 µl), and will require that your sample be loaded onto the column in two steps.

4 Ensure there is no ethanol in your eluate

Ethanol contamination can interfere with downstream applications. Frits, which are common in many manufacturers' columns, often retain small droplets of buffer, also known as buffer retention. This can contaminate your eluate. Monarch columns are designed without a frit, which eliminates buffer retention.

If you suspect that the tip of your Monarch column has come into contact with wash buffer after a spin, give the column another short spin to ensure that your column is ethanol-free.

5 Warm your Elution Buffer to 50°C for large fragments

Heating your Elution Buffer before applying to the column can increase efficiency, especially for large fragments (>10 kb).

6 Use the recommended amount of Dissolving Buffer

The Monarch protocol requires four volumes of Dissolving Buffer to be added to your gel slice. Other manufacturers' protocols differ, so when using our kit, be sure to use the recommended volume.

NEB's Monarch® DNA Gel Extraction Kit (NEB #T1020) rapidly and reliably purifies up to 5 µg of concentrated, high quality DNA from agarose gels. For a complete list of products, including buffers and columns sold separately, visit **NEBmonarch.com**.

It's time to unplug and pick up a good book.

For some "light" reading, we recommend the 2017-18 NEB Catalog & Technical Reference. Browse through to find the latest information on NEB's products, including recommendations for which product will work best for your application. Take a look at our popular technical reference section, which includes extensive information on maximizing product performance, technical tips, troubleshooting guides, and much more. And when you need a break from planning your research, read about the inspiring work of our 2016 Passion in Science Awards™ winners.

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