NEBNext® for Illumina®

NGS SAMPLE PREPARATION

NEW ENGLAND BioLabs®

be INSPIRED
drive DISCOVERY
stay GENUINE

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TOOLS & RESOURCES

Visit NEBNext.com to find:

- The full list of products available
- Video protocols
- Online tutorials to help with product selection, general handling tips and more
- Access to **NEBNext Selector Tool**, our online tool for help with selecting the right NEBNext product
- NEBNext citations
- Protocols & FAQs

12 QUICK TIPS FOR LIBRARY PREPARATION

Why Choose NEBNext?

The NEBNext suite of products supports Illumina sequencing with sample preparation tools that streamline workflows, minimize inputs, and improve library yields and quality. NEBNext sample preparation kits are available for genomic DNA, ChIP DNA, FFPE DNA, Microbiome DNA, RNA and small RNA samples. In addition to the extensive QCs performed on individual kit components, all NEBNext kits for Illumina are functionally validated by preparation of a library, followed by Illumina sequencing. NEBNext library preparation reagents are available in multiple kit and workflow formats, for maximum convenience and flexibility. Use of NEBNext products has been cited in over 1,000 publications.

WHATS NEW IN NEBNEXT?

- Novel DNA fragmentation integrated into library prep, with the **NEBNext Ultra II FS DNA Library Prep** kits
- **NEBNext Ultra II RNA** kits (directional and non-directional) – the next generation of RNA library construction
- **NEBNext Direct® BRCA1/BRCA2 Panel**
- **NEBNext rRNA Depletion** with optional Agencourt® RNAClean® XP beads

NEBNext Formats – Kits & Modules:

Looking for convenience?

Kits are the most convenient option, as they include reagents for the entire library preparation workflow. NEBNext kits include primarily master mix reagents that minimize the number of components and pipetting steps.

Looking for flexibility?

NEBNext modules provide the most flexible option, as modules contain reagents for individual steps in the library preparation workflow. A series of NEBNext modules can be combined to cover the entire library prep workflow, or a subset of NEBNext modules can be combined with other reagents to enable a customized workflow for your specific needs.

Additionally, adaptors and primers are supplied separately from the NEBNext kits (as NEBNext Oligos modules)*. This allows for increased flexibility in combining adaptors, primers and reagents.

**except in the case of the Small RNA Kit, which includes adaptors and primers.*

Visit **NEBNextSelector.neb.com** to access the **NEBNext Selector Tool**, our online tool for help with selecting the right NEBNext product

Bulk & custom formats:

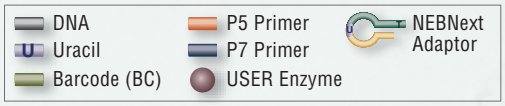
Choose NEBNext's bulk and custom formats when your reagent needs exceed standard volumes, or you require a specialized formulation. As enzyme manufacturers, we are able to easily provide customized reagents, kits and modules to meet your specific NGS sample prep workflow needs. For more information, please contact **NEBsolutions@neb.com.**

Not just library construction:

The NEBNext product line extends beyond library construction, with reagents for repair of FFPE DNA, depletion of rRNA, enrichment of microbiome DNA, and qPCR-based library quantitation.

DNA Library Preparation Overview

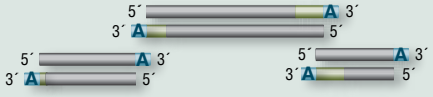
PRODUCT	INPUT AMOUNTS
NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB #E7805)	100 pg – 0.5 µg DNA
NEBNext Ultra II FS DNA Library Prep with Sample Purification Beads (NEB #E6177)	
NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB #E7645)	500 pg – 1 µg DNA
NEBNext Ultra II DNA Library Prep with Sample Purification Beads (NEB #E7103)	
NEBNext Oligos (including 12-plex, 96-plex and dual index primers) (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600	N/A



Ultra II FS DNA Workflow

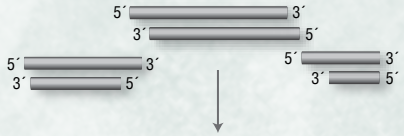
1 and 2 DNA Fragmentation, End Repair & dA-Tailing

- Enzymatic fragmentation
- Generation of blunt-ended fragments (filling in/ chewing back 3´ & 5´ overhangs)
- 5´ phosphorylation
- Creation of single A 3´ overhang enables ligation to adaptors with single T overhangs



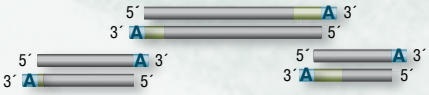
1 DNA Fragmentation (Not Required for ChIP)

- Fragmentation by acoustic shearing, nebulization or enzyme-based methods



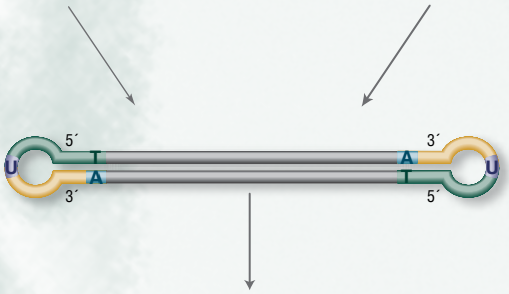
2 End Repair & dA-Tailing

- Generation of blunt-ended fragments (filling in/ chewing back 3´ & 5´ overhangs)
- 5´ phosphorylation
- Creation of single A 3´ overhang enables ligation to adaptors with single T overhangs



3 Adaptor Ligation

- Ligation of short adaptors (contain sequences required downstream)
- A novel hairpin loop structure increases ligation efficiency & minimizes adaptor-dimer formation

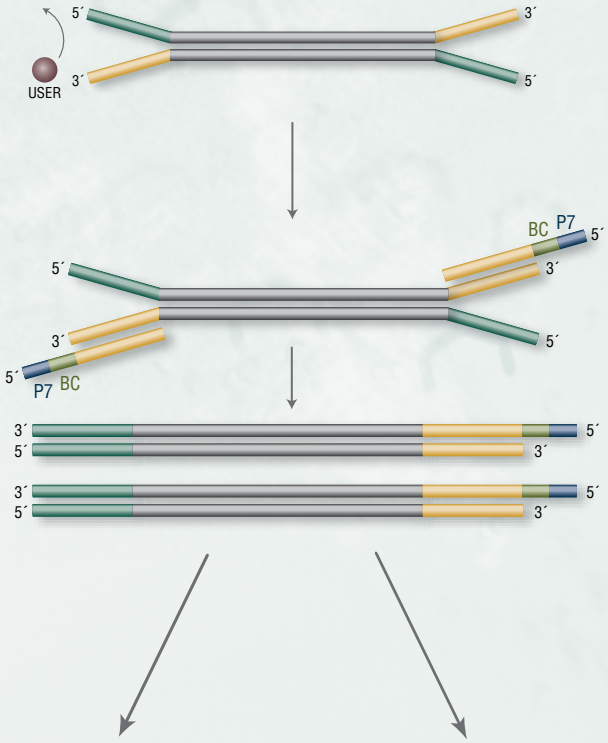


4 U Excision

- Removal of uracils in NEBNext Adaptor loop by USER® Enzyme (to make accessible for PCR)

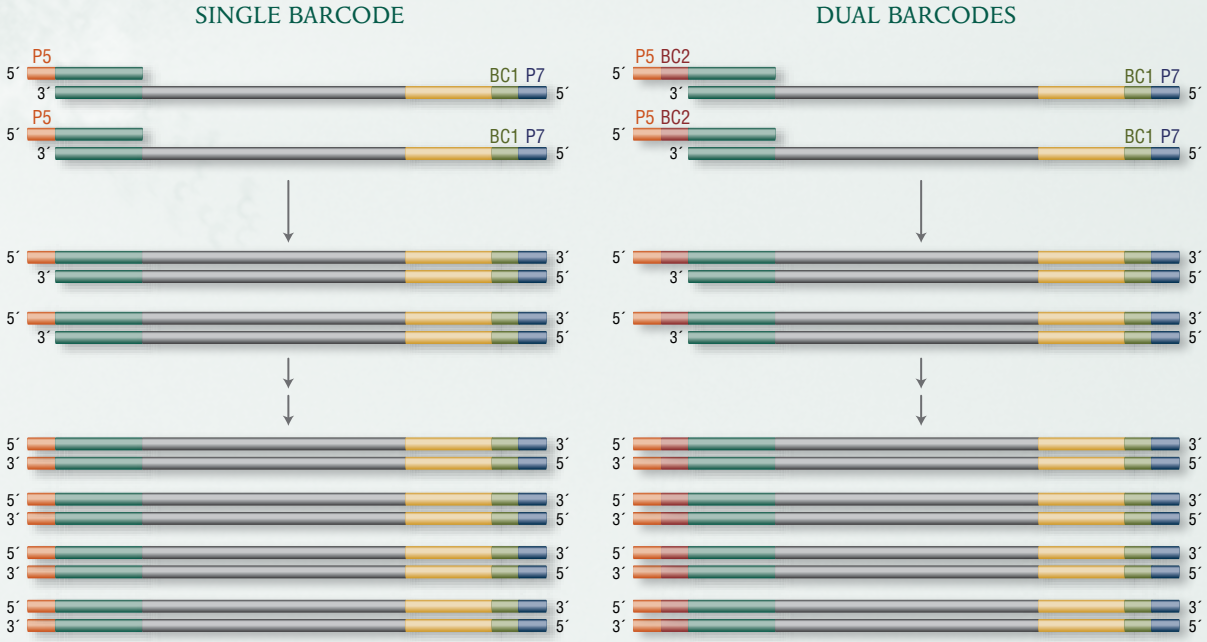
5 PCR Enrichment

- Amplification using a high-fidelity polymerase:
  - Selects for molecules with an adaptor at each end
  - Increases library yield
  - Incorporates barcodes/indices to enable multiplexing, and P5 & P7 sequences required downstream



NEBNext Oligos

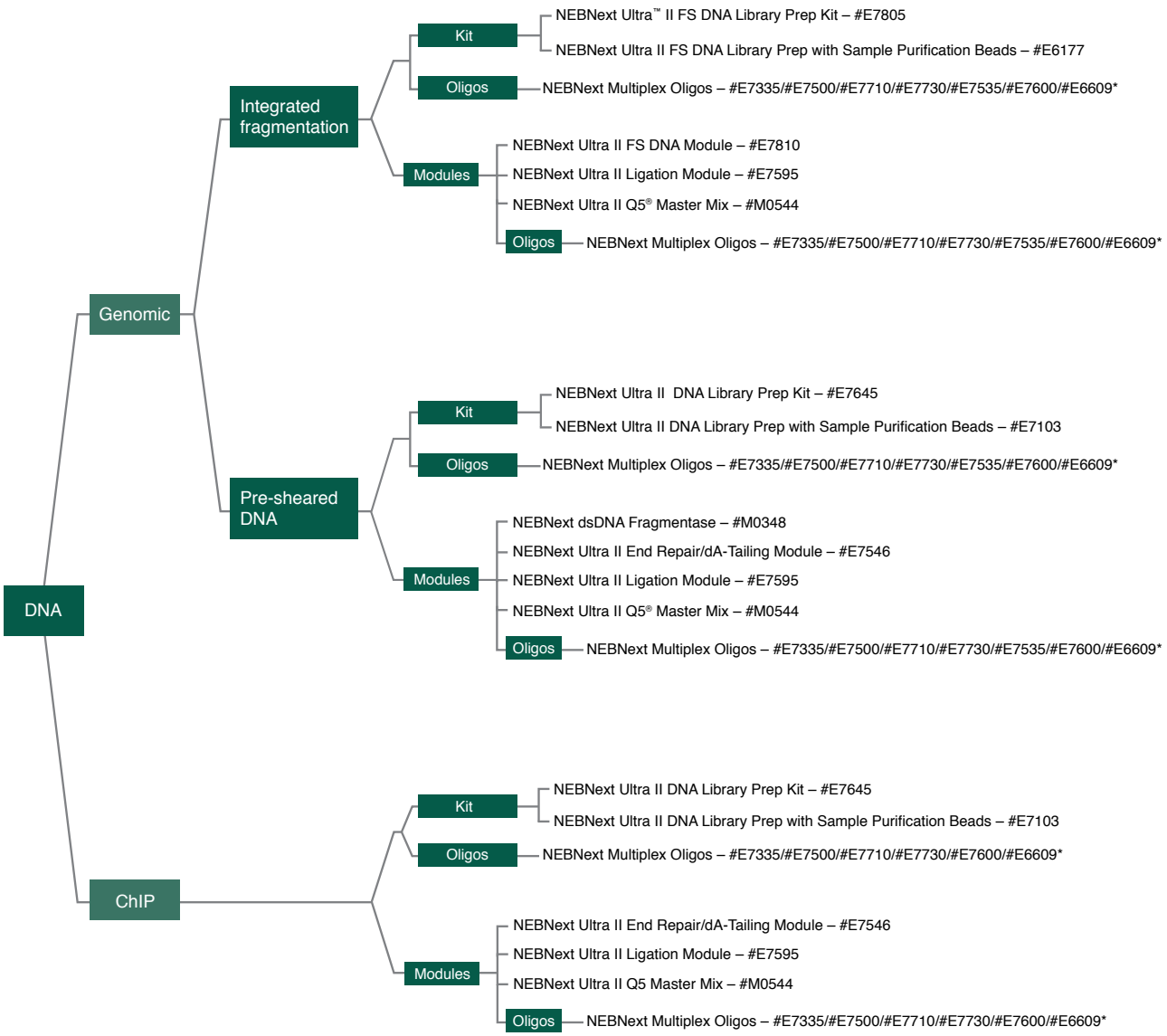
- Barcodes incorporated using NEBNext primers
- Single- or dual-barcode primer options available



DNA Product Selection

For DNA, NEBNext kits are available for sample preparation of genomic DNA, ChIP DNA, and FFPE DNA. Ultra II kits utilize fast, streamlined and automatable workflows with novel master mixes that have been designed for performance with a broad range of input amounts, from pg to µg of DNA. NEBNext DNA workflows are also available in module format, which provide the ability to easily customize sample preparation. Adaptors and primers (NEBNext Oligos) and FFPE DNA repair reagents are supplied separately.

This chart will help you to determine the best NEBNext product for your Illumina DNA library preparation. You can also use our online tool, **NEBNext Selector at nebnextselector.neb.com**, to choose the best products for your needs.



** Singleplex Oligos are also available.*
Reagents for the original Ultra workflow and standard workflow are also available.
See ordering information.

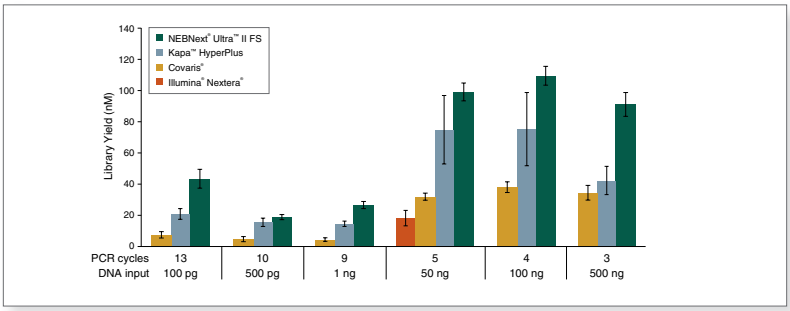
NEBNext Ultra II Kits for DNA Library Prep

The original Ultra II DNA Library Prep Kits for Illumina (NEB #E7645, E7103) enable construction of libraries from pre-sheared DNA, with an input range of 500 pg to 1 µg. With our latest kits, we have built upon our Ultra II DNA library prep workflow to create a fragmentation system.

NEBNext Ultra II FS DNA Library Prep Kit – *a fragmentation system with library construction*

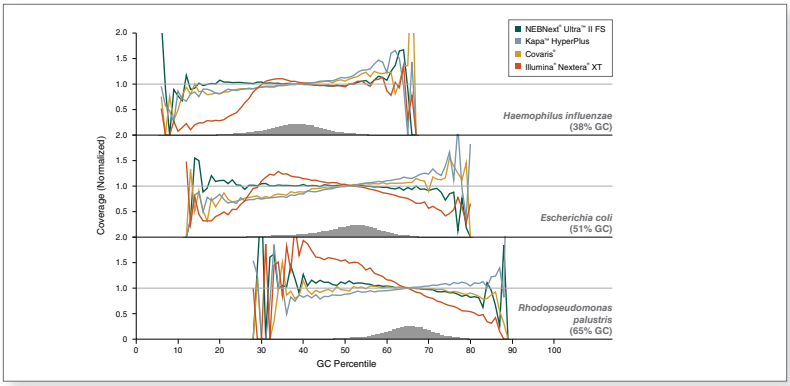
The Ultra II FS kit includes a new DNA fragmentation reagent, which is also combined with end repair and dA-tailing reagents, enabling these steps to be performed in the same tube, with no clean-ups or sample loss. The same fragmentation protocol is used for any input amount (100 pg–0.5 µg), or GC content.

NEBNext Ultra II FS DNA produces the highest yields, from a range of input amounts.



Libraries were prepared from Human NA19240 genomic DNA using the input amounts and numbers of PCR cycles shown. For NEBNext Ultra II FS, a 20-minute fragmentation time was used. For Kapa HyperPlus libraries, input DNA was cleaned up with 3X beads prior to library construction, as recommended, and a 20-minute fragmentation time was used. Illumina recommends 50 ng input for Nextera, and not an input range; therefore, only 50 ng was used in this experiment. "Covaris" libraries were prepared by shearing each input amount in 1X TE Buffer to an insert size of ~200 bp using a Covaris instrument, followed by library construction using the NEBNext Ultra II DNA Library Prep Kit (NEB #E7645). Error bars indicate standard deviation for an average of 3–6 replicates performed by 2 independent users.

NEBNext Ultra II FS DNA provides uniform GC coverage over a broad range of GC composition.



Libraries were prepared using 1 ng of a mix of genomic DNA samples from Haemophilus influenzae, Escherichia coli (K-12 MG1655), Rhodopseudomonas palustris and the library prep kits shown, with 9 PCR cycles for consistency across samples, and sequenced on an Illumina MiSeq®. NEBNext Ultra II FS libraries were prepared using a 20-minute fragmentation time. For Kapa HyperPlus libraries, input DNA was cleaned up with 3X beads prior to library construction, as recommended, followed by a 25-minute fragmentation time. "Covaris" libraries were prepared by shearing 1 ng of DNA in 1X TE Buffer to an insert size of ~200 bp using a Covaris instrument, followed by library construction using the NEBNext Ultra II DNA Library Prep Kit (NEB #E7645). Reads were mapped using Bowtie 2.2.4 and GC coverage information was calculated using Picard's CollectGCBiasMetrics (v1.117). Expected normalized coverage of 1.0 is indicated by the horizontal grey line, the number of 100 bp regions at each GC% is indicated by the vertical grey bars, and the colored lines represent the normalized coverage for each library.

ADVANTAGES OF NEBNEXT ULTRA II FS

- Perform fragmentation, end repair and dA-tailing with a **single enzyme mix**
- Experience **reliable fragmentation with a single protocol**, regardless of DNA input amount or GC content
- Prepare high quality libraries from a **wide range of input amounts:** 100 pg–0.5 µg
- Generate high yields with increased reaction efficiencies and minimized sample loss
- Use with input DNA in standard buffers (TE, Tris-HCl) and water
- Save time with a **streamlined workflow:** ~ 2.5 hours, with < 15 minutes hands-on time
- Vary incubation time to generate a wide **range of insert sizes**
- Available with optional SPRIselect® beads for **gold standard size selection** and clean-up

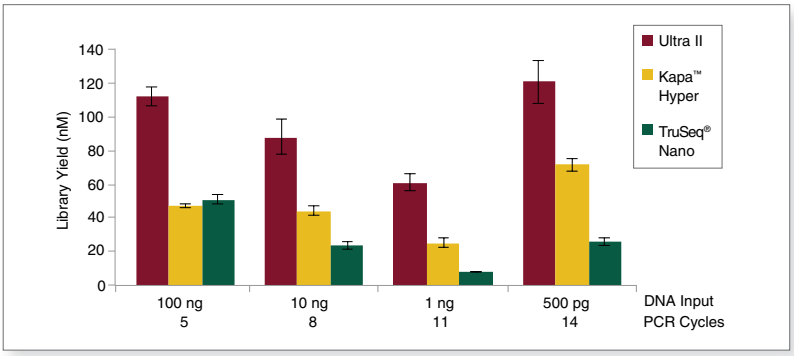
TOOLS & RESOURCES

View extensive performance data in our Technical Notes, which can be downloaded at **NEBNextUltraII.com**

View and download performance data generated by NEBNext Ultra II FS DNA users at **NEBNextUltraII.com**

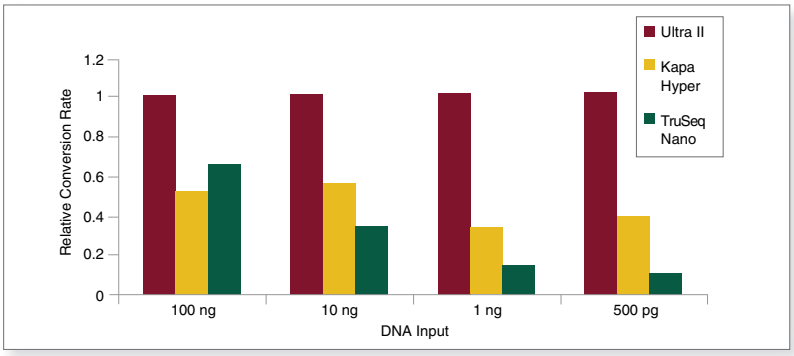
NEBNext Ultra II DNA Library Prep Kit – *for pre-sheared DNA*

The NEBNext Ultra II DNA Library Prep Kit produces high yield libraries from a broad range of input amounts, using pre-sheared input DNA.



Libraries were prepared from Human NA19240 genomic DNA using the input amounts and numbers of PCR cycles shown. Manufacturers' recommended protocols were followed, with the exception that size selection was omitted.

NEBNext Ultra II DNA produces high rates of conversion to adaptor-ligated molecules from a broad range of input amounts.



Libraries were prepared from Human NA19240 genomic DNA using the input amounts and library prep kits shown without an amplification step, and following manufacturers' recommendations. qPCR was used to quantitate adaptor-ligated molecules, and quantitation values were then normalized to the conversion rate for Ultra II. The Ultra II kit produces the highest rate of conversion to adaptor-ligated molecules, for a broad range of input amounts.

Ultra II DNA libraries provide high quality sequencing data.

DNA INPUT	LIBRARY KIT	TOTAL READS	% MAPPED	% DUPLICATION	% CHIMERAS
100 ng	Ultra II	419,093,838	96	1.87	0.48
	Kapa Hyper	419,097,926	96	2.00	0.60
	TruSeq Nano	419,086,546	97	1.91	0.53
1 ng	Ultra II	226,860,968	96	3.96	0.44
	Kapa Hyper	226,857,578	96	11.40	0.53
	TruSeq Nano	226,857,754	97	34.80	0.41

Libraries were prepared from Human NA19240 genomic DNA using the input amounts and library prep kits shown, following manufacturers' recommendations. Libraries were sequenced on the Illumina NextSeq 500. Reads were mapped to the GRCh37 reference using Bowtie 2.2.4. This data illustrates that the NEBNext Ultra II DNA Library Prep Kit enables high quality sequence data, even with very low input amounts.

% Mapped: *The percentage of reads mapped to Human GRCh37 reference.*
% Duplication: *The percentage of mapped sequence that is marked as duplicate.*
% Chimeras: *The percentage of reads that map outside of a maximum insert size or that have the two ends mapping to different chromosomes.*

ADVANTAGES OF NEBNEXT ULTRA II DNA, FOR PRE-SHEARED DNA

- Get more of what you need, with **high library yields**
- Use to generate high quality libraries with **inputs as low as 500 pg and as high as 1 µg**
- Improve library complexity with **fewer PCR cycles**
- Prepare libraries from ALL of your samples, including **GC-rich** and **FFPE** samples
- Compatible with bisulfite sequencing workflows
- Improve library complexity with **fewer PCR cycles**
- Save time with **streamlined workflows**, reduced hands-on time, and automation compatibility
- Enjoy the flexibility and reliability of the gold standard **SPRIselect size selection and clean-up beads**, supplied in just the amounts you need

TOOLS & RESOURCES

View additional performance data in our Technical Notes, which can be downloaded at **NEBNextUltraII.com**

View the NEBNext Ultra II DNA protocol video for protocol steps, and tips for optimization

NEBNext Ultra II FS DNA and Ultra II DNA Workflows and Product Details

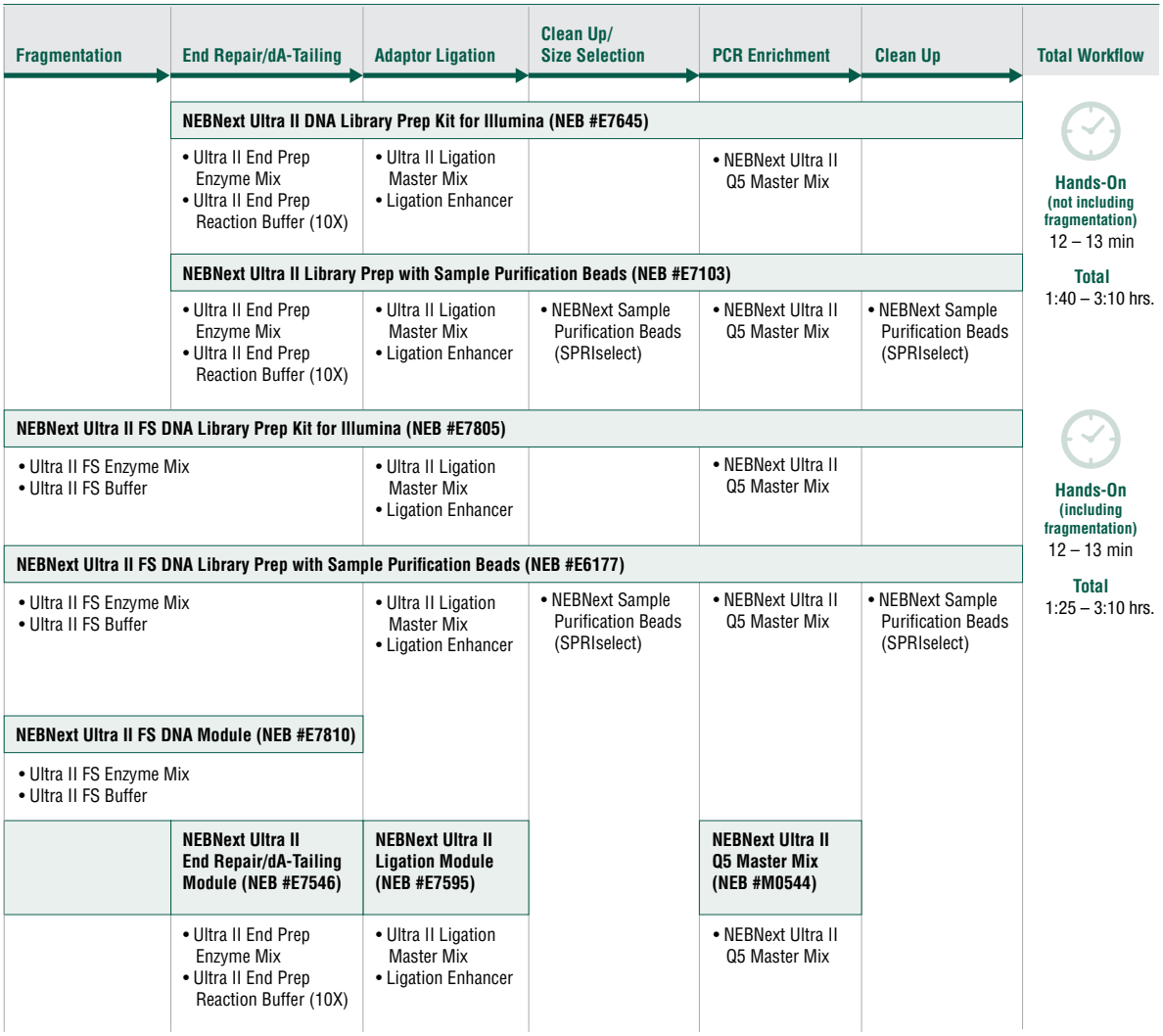
In addition to stringent QCs on individual components, the NEBNext DNA kits are also functionally validated by preparation of a library, followed by Illumina sequencing. Reagent lots are reserved specifically for inclusion in NEBNext kits. Note that adaptors and primers are supplied separately. For more information, see page 20.

Ultra II for DNA kit overview

	ENZYMATIC FRAGMENTATION INCLUDED	INPUT AMOUNTS	AVAILABLE WITH OPTIONAL SPRIselect® BEADS	COMPATIBLE WITH PCR-FREE WORKFLOWS	COMPATIBLE WITH BISULFITE SEQUENCING WORKFLOWS
NEBNext Ultra II FS Kits (NEB #E7805, #E6177)	Yes	100 pg – 0.5 µg	Yes (NEB #E6177)	Yes	No
NEBNext Ultra II DNA Kits (NEB #E7645, #E7103)	No	500 pg – 1 µg	Yes (NEB #E7103)	Yes	Yes

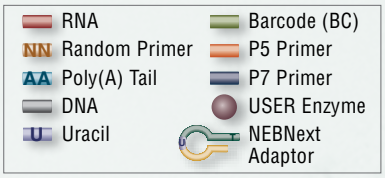
NEBNext Ultra II kit components

Input Ultra II DNA Workflow: 500 pg – 1 µg Ultra II FS DNA Workflow: 100 pg – 0.5 µg



Workflow for RNA Library Preparation

PRODUCT	INPUT AMOUNTS
NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760)	5 ng - 1 µg Total RNA (rRNA Depletion Workflow) 10 ng - 1 µg Total RNA (poly(A) mRNA workflow)
NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads (NEB #E7765)	
NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770)	
NEBNext Ultra II RNA Library Prep with Sample Purification Beads (NEB #E7775)	
NEBNext Oligos (including 12-plex, 96-plex and dual index primers) (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600)	



1 RNA Enrichment (rRNA Depletion or Poly(A) mRNA Isolation)

- Removal of rRNA (> 80% of total RNA) or enrichment for mRNA
- NEBNext Library Prep kits are compatible with either method

2 RNA Fragmentation & Random Priming

- Fragmentation by incubation with divalent cations (e.g., Mg^{++}) or enzymes (e.g., RNase III)
- Hybridization of random primers

3 First Strand cDNA Synthesis

- Reverse transcriptase lacking RNase H activity is optimal (does not degrade RNA in RNA:DNA complex)
- For directional RNA library preparation, Actinomycin D is added:
  – To inhibit DNA-dependent DNA Polymerase activity of RT & inhibit second strand synthesis/increase strand specificity

4 Second Strand cDNA Synthesis

- Generation of nicks & gaps in RNA by RNase H, enabling second strand synthesis by nick translation
- Sealing of breaks in second strand by *E. coli* DNA ligase
- For Directional RNA library preparation, second strand labeled with uracils by dUTP incorporation

DIRECTIONAL

NON-DIRECTIONAL

5 End Repair, dA-Tailing & Adaptor Ligation

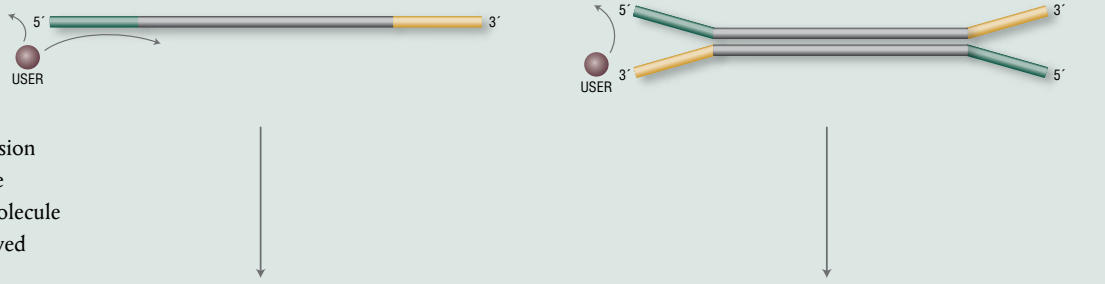
- Generation of blunt, phosphorylated ends
- Addition of single A 3´ overhang (enables ligation to adaptors with single T overhangs)
- Ligation of short adaptors (contain sequences required downstream)
- NEBNext adaptors increase ligation efficiency & minimize adaptor-dimer formation

6 U Excision

- Removal of uracils in NEBNext Adaptor loop by USER Enzyme (to make accessible for PCR)

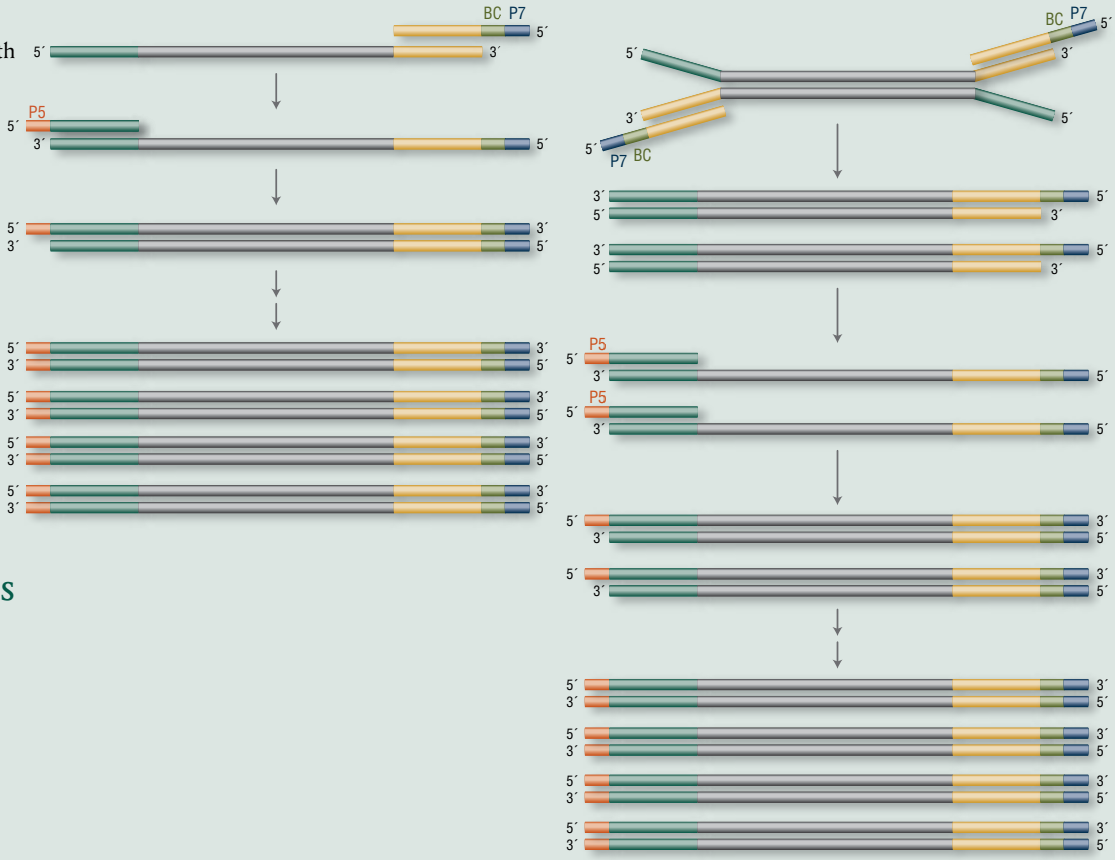
Directional Only

- Selective removal of second strand through excision of uracils by USER Enzyme
- Result is single-stranded molecule with different adaptor-derived sequences on each end



7 PCR Enrichment

- Amplification using a high-fidelity polymerase:
  - Selects for molecules with an adaptor at each end
  - Increases library yield
  - Incorporates barcodes/ indices to enable multiplexing, and P5 & P7 sequences required downstream



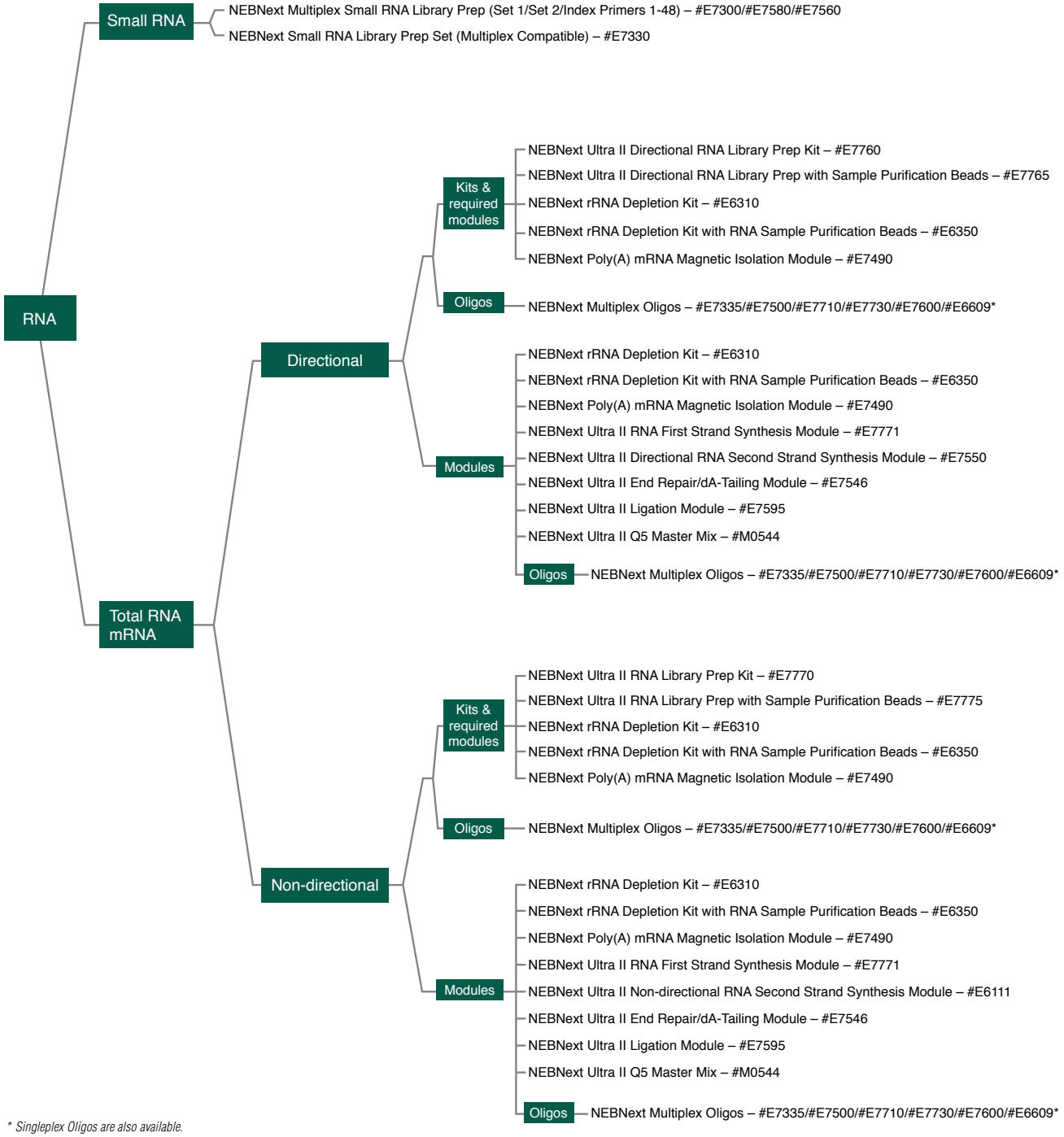
NEBNext Oligos

- Barcodes incorporated using NEBNext primers
- Single- or dual- barcode primer options available

RNA Product Selection

NEBNext Ultra II RNA kits have streamlined, automatable workflows and are available for directional (strand-specific, using the "dUTP method") and non-directional library construction. The kits are compatible with poly(A) mRNA enrichment or ribosomal RNA depletion, for low nanogram to microgram total RNA inputs. The kits are also available with the option of SPRISelect® beads for size-selection and clean-up steps. Our novel Small RNA workflow has been optimized to minimize adaptor-dimers, while producing high-yield, high-diversity libraries. See page 16 for more information. Modules offer the ability to customize sample preparation, and are available for directional and non-directional RNA library prep workflows. Adaptors and primers (NEBNext Oligos) are supplied separately.

Use this chart to determine the best NEBNext product for your Illumina RNA library preparation. You can also use our online tool, **NEBNext Selector at NEBNext Selector.com**, to choose the best products for your needs.



** Singleplex Oligos are also available.*

*** Set of individual reagents is also available. See ordering information.*

Ultra II RNA

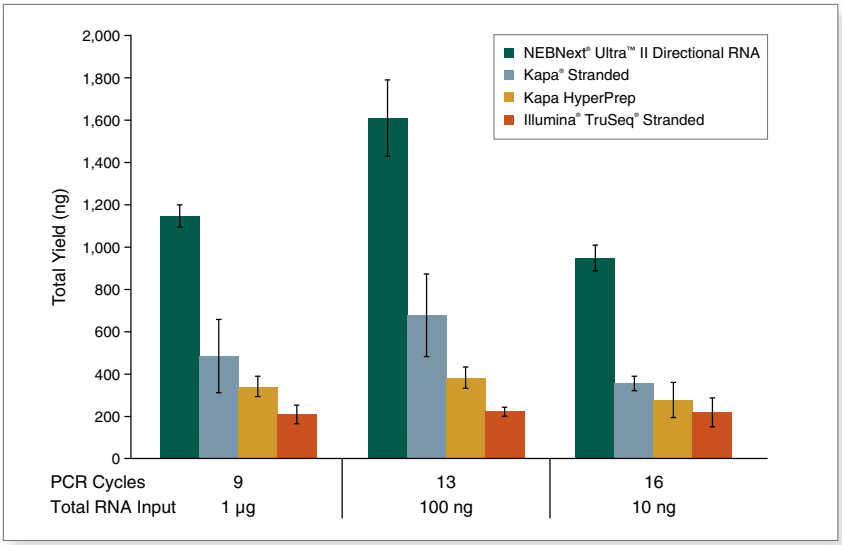
The latest generation of NEBNext kits for RNA enable directional (strand-specific) or non-directional library construction from low ng to µg input amounts, and are compatible with poly(A) mRNA enrichment or rRNA depletion. Workflows are streamlined with minimal hands-on time, and are automatable. Information on NEBNext rRNA Depletion and NEBNext Poly(A) mRNA enrichment is available on page 21.

Directional & Non-Directional RNA Library Preparation

Standard methods for RNA library preparation do not retain information on the DNA strand from which the RNA strand was transcribed. The ability to obtain information on the originating strand is useful for many reasons including the identification of antisense transcripts, determination of the transcribed strand of noncoding RNAs, and determination of expression levels of coding or non-coding overlapping transcripts. Overall, the ability to determine the originating strand can substantially enhance the value of a RNA-seq experiment.

Several methods have been developed for directional (strand-specific) RNA sequencing, involving different library preparation methods. The NEBNext Ultra Directional RNA Library Prep Kit uses the "dUTP method" (1,2) for strand-specificity, as shown in the overview on pages 10–11.

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



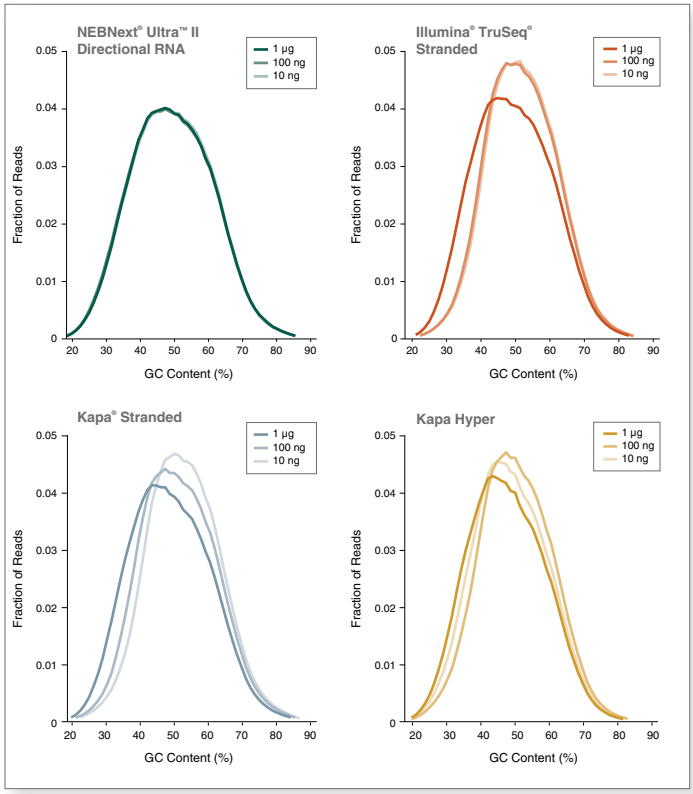
Poly(A)-containing mRNA was isolated from 10 ng, 100 ng and 1 µg of Universal Human Reference RNA (Agilent® #740000) and libraries were made using the NEBNext Ultra II Directional RNA Kit (plus the NEBNext poly(A) mRNA Magnetic Isolation Kit), Kapa Stranded mRNA-Seq Kit, Kapa mRNA HyperPrep Kit and Illumina 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.

ADVANTAGES OF NEBNEXT ULTRA II RNA

- **Generate high yield**, high-quality libraries, even with limited amounts of RNA:
  - 5 ng – 1 µg total RNA (rRNA depletion workflow)
  - 10 ng – 1 µg total RNA (poly(A) mRNA 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- and 96-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

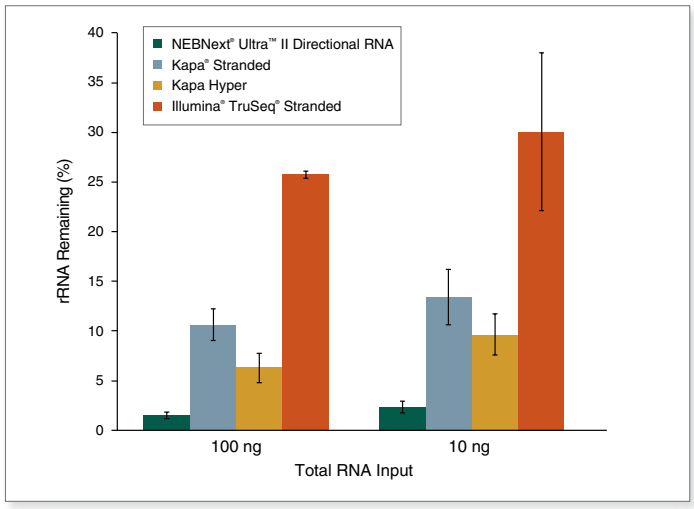
References
1. Parkhomchuk, D., et al. (2009) Nucleic Acids Res. 37, e123.
2. Levin, J.Z., et al. (2010) Nature Methods 7, 709–715.

NEBNext Ultra II Directional RNA libraries provide uniform GC content distribution, at 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 (2 x 76 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, whereas for other kits the GC content distribution changed with different input amounts, indicating the introduction of input-dependent sequence bias.

NEBNext Ultra II Directional RNA with NEBNext rRNA Depletion results in the lowest remaining ribosomal RNA levels with FFPE samples



Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149. RIN 2.5) and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2 x 76 bp). Read pairs were assessed to be rRNA if they contain 6 or more 32 base matches to 18S, 28S, 5S, 5.8S, 16S or 12S human rRNA sequences (mirabait 4.9). Percent rRNA remaining was calculated by dividing rRNA reads by the total number of reads passing instrument quality filtering. Average percent rRNA remaining is shown for three replicates. Error bars indicate standard deviation. The NEBNext rRNA Depletion Ultra II Directional RNA workflow is the most efficient in removing rRNA from total FFPE RNA.

Ultra II RNA Workflows and Product Details

In addition to stringent QCs on individual components, the NEBNext RNA kits are functionally validated by preparation of a library, followed by Illumina sequencing. Reagent lots are reserved specifically for inclusion in NEBNext kits. Adaptors and primers are supplied separately (NEBNext Oligos). For more information, see page 20.

NEBNext kit components

Input Poly(A) mRNA Workflow: 10 ng – 1 µg rRNA Depletion Workflow: 5 ng – 1 µg

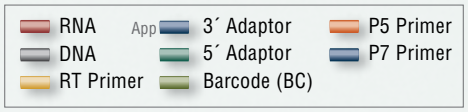
	mRNA Isolation/ rRNA Depletion	mRNA Fragmentation	First Strand cDNA Synthesis	Second Strand cDNA Synthesis	End Repair/ dA Tailing	Adaptor Ligation	Size Selection	PCR Enrichment	Clean Up	Total Workflow
Ultra II Directional Kits		NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760)								
		NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads (NEB #7765)								
		• First Strand Synthesis Reaction Buffer • First Strand Synthesis Enzyme Mix • Random Primers • Strand Specificity Reagent		• Second Strand Synthesis Reaction Buffer with dUTP Mix • Second Strand Synthesis Enzyme Mix • Nuclease-free Water	• End Prep Enzyme Mix • End Repair Reaction Buffer	• Ultra II Ligation Master Mix • Ligation Enhancer • Adaptor Dilution Buffer	• NEBNext Sample Purification Beads (SPRIselect) – NEB #E7765 only	• NEBNext Ultra II Q5 Master Mix	• NEBNext Sample Purification Beads (SPRIselect) – NEB #E7765 only	Hands-On 27 min Total 5:30 – 5:40 hrs. 6:40 – 6:50 hrs.
Ultra II Non-directional Kits		NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770)								
		NEBNext Ultra II RNA Library Prep with Sample Purification Beads (NEB #E7775)								
		• First Strand Synthesis Reaction Buffer • First Strand Synthesis Enzyme Mix • Random Primers • Strand Specificity Reagent		• Second Strand Synthesis Reaction Buffer • Second Strand Synthesis Enzyme Mix • Nuclease-free Water	• End Prep Enzyme Mix • End Repair Reaction Buffer	• Ultra II Ligation Master Mix • Ligation Enhancer • Adaptor Dilution Buffer	• NEBNext Sample Purification Beads (SPRIselect) – NEB #E7775 only	• NEBNext Ultra II Q5 Master Mix	• NEBNext Sample Purification Beads (SPRIselect) – NEB #E7775 only	Hands-On 27 min Total 5:30 – 5:40 hrs. 6:40 – 6:50 hrs.
Ultra II Modules	**rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310)** • RNase H/RNase H Reaction Buffer • rRNA Depletion Solution • Probe Hybridization Buffer • DNase I/DNase I Reaction Buffer • Nuclease-free Water	**Magnesium RNA Fragmentation Module (NEB #E6150)** • RNA Fragmentation Buffer • RNA Fragmentation Stop Solution	**Ultra II RNA First Strand Synthesis Module (NEB #E7771)** • First Strand Synthesis Reaction Buffer • First Strand Synthesis Enzyme Mix • Random Primers • Strand Specificity Reagent	**Ultra II Directional RNA Second Strand Synthesis Module (NEB #E7550)** • Second Strand Synthesis Enzyme Mix • Second Strand Synthesis Reaction Buffer with dUTP	**NEBNext Ultra II End Repair/ dA-Tailing Module (NEB #E7546)** • Ultra II End Prep Enzyme Mix • Ultra II End Prep Reaction Buffer	**NEBNext Ultra II Ligation Module (NEB #E7595)** • Ultra II Ligation Master Mix • Ligation Enhancer		**NEBNext Ultra II Q5 Master Mix (NEB #M0544)** • NEBNext Ultra II Q5 Master Mix		
	rRNA Depletion Kit (Human/Mouse/Rat) with RNA Sample Purification Beads (NEB #E6350) • RNase H/RNase H Reaction Buffer • rRNA Depletion Solution • Probe Hybridization Buffer • DNase I/DNase I Reaction Buffer • Nuclease-free Water • NEBNext RNA Sample Purification Beads			**Ultra II Non-directional RNA Second Strand Synthesis Module (NEB #E6111)** • Second Strand Synthesis Enzyme Mix • Second Strand Synthesis Reaction Buffer						
	Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) • Oligo d(T)25 beads • RNA Binding Buffer • Wash Buffer • Nuclease-free Water									

* Including poly(A) mRNA isolation
** Including rRNA depletion

Note that reagents for the original Ultra workflow and standard workflow are also available. See ordering information.

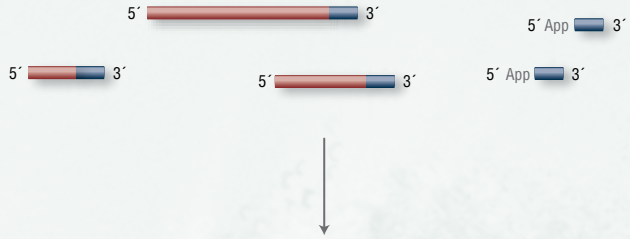
Workflow for Small RNA Library Preparation

PRODUCT	TOTAL RNA
NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 1) (NEB #E7300)	100 ng–1 µg
NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 2) (NEB #E7580)	100 ng–1 µg
NEBNext Multiplex Small RNA Library Prep Kit for Illumina (Index Primers 1–48) (NEB #E7560)	100 ng–1 µg
NEBNext Small RNA Library Prep Set for Illumina (Multiplex Compatible) (NEB #E7330)	100 ng–1 µg



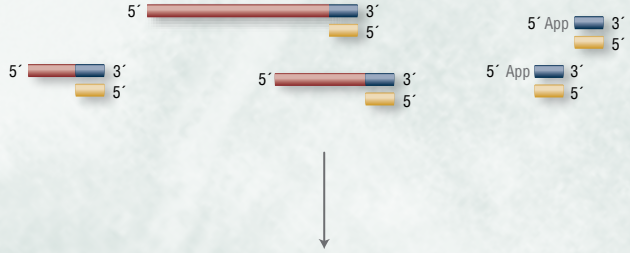
1 3´ Adaptor Ligation

- Input is purified total RNA
- Ligation of 5´-adenylated, 3´-blocked, single-stranded DNA adaptor to 3´ end of RNA



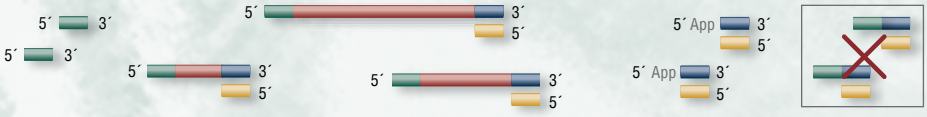
2 Primer Hybridization

- Hybridization of RT primer to 3´ adaptor-ligated molecules & any remaining 3´ adaptors



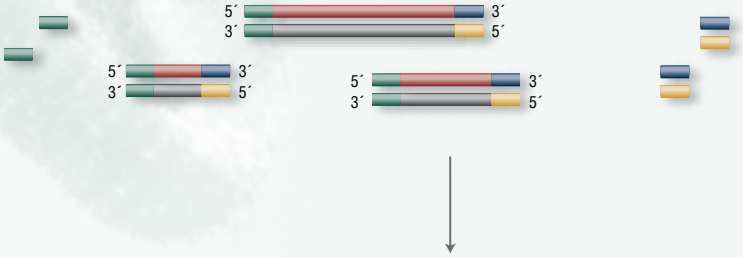
3 5´ Adaptor Ligation

- Preferential ligation of 5´ adaptor to single-stranded molecules (and therefore not to double-stranded 3´ adaptor:RT primer hybrid molecule)
- Result is minimized formation of adaptor-dimers



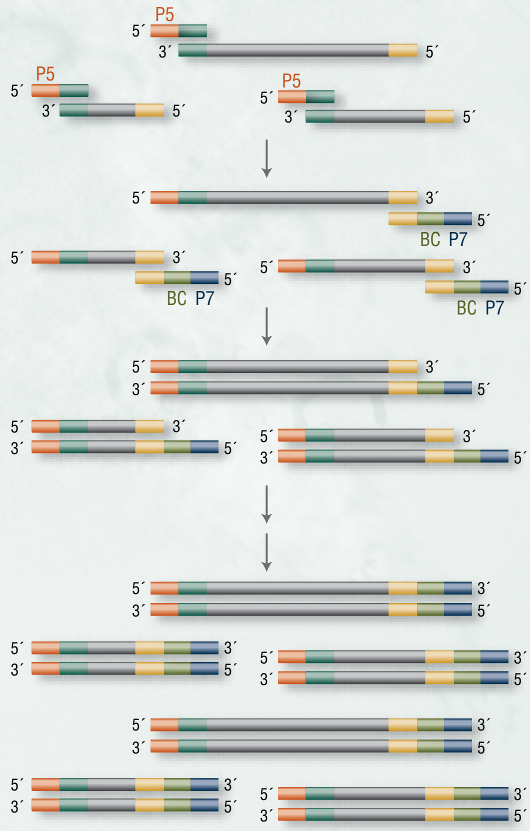
4 First Strand cDNA Synthesis

- Extension from RT primer synthesizes first strand cDNA
- Reverse transcriptase lacking RNase H activity is optimal (does not degrade RNA in RNA:DNA complex)



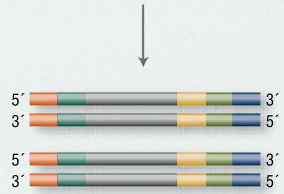
5 PCR Enrichment

- Amplification with a high-fidelity polymerase:
  - Selects for molecules with an adaptor at each end
  - Increases library yield
  - Incorporates barcodes/indices to enable multiplexing, and P5 & P7 sequences required downstream



6 Size Selection

- Ensures that only Small RNAs of interest are included in final library



Small RNA Workflow and Product Details

Our novel Small RNA workflow has been optimized to minimize adaptor-dimers while producing high-yield, high-quality libraries. Adaptors and primers are included in the Small RNA kits, and multiplexing options are available. The Multiplex kits contain index primers, and the Multiplex-Compatible kit enables use with your own barcode system.

In addition to stringent QCs on individual components, the NEBNext Small RNA kits are functionally validated by preparation of a library, followed by Illumina sequencing. Reagent lots are reserved specifically for inclusion in NEBNext kits.

NEBNext kit components

Input 100 ng – 1 µg

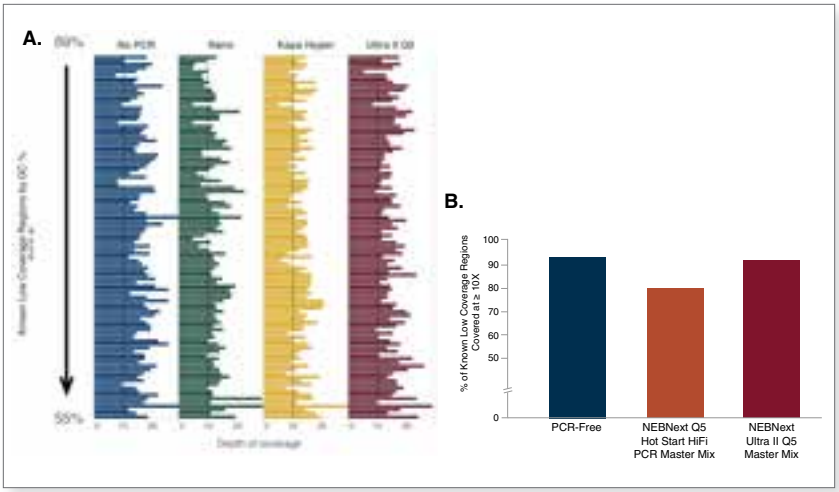
3´ Adaptor Ligation →	Primer Hybridization →	5´ Adaptor Ligation →	First Strand Synthesis →	PCR Enrichment →	Size Selection →	Total Workflow
NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 1 NEB #E7300, Set 2 NEB #E7580)						**Hands-On Time** 30 min **Total Time** 6 hrs
• 3´ Ligation Enzyme Mix • 3´ Ligation Reaction Buffer (2X) • 3´ SR Adaptor	• SR RT Primer	• 5´ Ligation Enzyme Mix • 5´ Ligation Reaction Buffer (10X) • 5´ SR Adaptor • Nuclease-Free Water	• RNase Inhibitor, Murine • M-MuLV Reverse Transcriptase (RNase H⁻) • First Strand Synthesis Reaction Buffer	• LongAmp® *Taq* 2X Master Mix • SR Primer • Index Primers 1–12 (Set 1) • Index Primers 13–24 (Set 2)	• Gel Loading Dye, Blue (6X) • Quick-Load® pBR322 DNA-MspI Digest • DNA Gel Elution Buffer (1X) • Linear Acrylamide • TE Buffer	
NEBNext Multiplex Small RNA Library Prep Kit for Illumina (Index Primers 1-48) (NEB #E7560)						**Hands-On Time** 30 min **Total Time** 6 hrs
• 3´ Ligation Enzyme Mix • 3´ Ligation Reaction Buffer (2X) • 3´ SR Adaptor	• SR RT Primer	• 5´ Ligation Enzyme Mix • 5´ Ligation Reaction Buffer (10X) • 5´ SR Adaptor • Nuclease-Free Water	• RNase Inhibitor, Murine • M-MuLV Reverse Transcriptase (RNase H⁻) • First Strand Synthesis Reaction Buffer	• LongAmp *Taq* 2X Master Mix • SR Primer • NEBNext Index 1-48 Primers for Illumina	• Gel Loading Dye, Blue (6X) • Quick-Load pBR322 DNA-MspI Digest • DNA Gel Elution Buffer (1X) • Linear Acrylamide • TE Buffer	
NEBNext Small RNA Library Prep Set for Illumina (Multiplex Compatible) (NEB #E7330)						**Hands-On Time** 30 min **Total Time** 6 hrs
• 3´ Ligation Enzyme Mix • 3´ Ligation Reaction Buffer (2X) • 3´ SR Adaptor	• SR RT Primer	• 5´ Ligation Enzyme Mix • 5´ Ligation Reaction Buffer (10X) • 5´ SR Adaptor • Nuclease-Free Water	• RNase Inhibitor, Murine • M-MuLV Reverse Transcriptase (RNase H⁻) • First Strand Synthesis Reaction Buffer	• LongAmp *Taq* 2X Master Mix • SR Primer • Index Primer 1	• Gel Loading Dye, Blue (6X) • Quick-Load pBR322 DNA-MspI Digest • DNA Gel Elution Buffer (1X) • Linear Acrylamide • TE Buffer	

High Yields and Minimized GC Bias with the NEBNext Ultra II Formulation of Q5® High-Fidelity DNA Polymerase

To ensure that sequence data reflects exactly the sequence of the original sample, it is essential that amplification of libraries be performed uniformly and with high fidelity. Historically, high-fidelity polymerases have been more susceptible to difficulties in PCR amplification of GC- rich and other challenging regions. If such bias occurs in library amplification, this can lead to uneven sequence coverage, challenges in sequence assembly and even "missing" sequence.

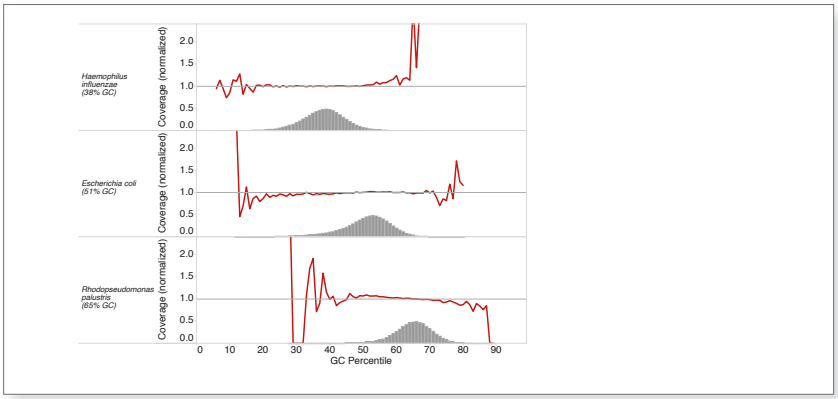
The NEBNext Ultra II Q5 Master Mix (NEB #M0544) is the latest formulation of Q5 DNA polymerase that has been optimized for robust, high-fidelity amplification of next-generation sequencing (NGS) libraries. This formulation further improves the uniformity of amplification of libraries, including superior performance with GC-rich regions.

NEBNext Ultra II Q5 Master Mix provides improved coverage of known low coverage regions of the human genome



Libraries were prepared from Human NA19240 genomic DNA. One library was not amplified. The other two libraries were amplified using 5 cycles of PCR with NEBNext Q5 Hot Start HiFi PCR Master Mix (NEB #M0543) or with NEBNext Ultra II Q5 Master Mix (NEB #M0544). Libraries were sequenced on an Illumina NextSeq® 500. 420 million 75 bp reads were randomly extracted from each dataset, representing an average coverage of 10X. Reads were mapped to the hg19 reference genome using Bowtie 2.2.4. Reads on each region were counted using bedtools v2.19.1. ***A:*** *The number of reads overlapping distinct low coverage regions of the human genome (1) are shown for each library.* ***B:*** *From the 420 million 75 bp reads randomly extracted from each dataset, 10X coverage was expected. The % of difficult regions covered at ≥ 10X are shown for each library. The NEBNext Ultra II Q5 Master Mix provides improved coverage of these known low coverage regions, without drop-outs, and shows similar coverage to the unamplified sample.*

NEBNext Ultra II Q5 Master Mix provides uniform GC coverage with a broad range of GC composition



Libraries were made using 100 ng of the genomic DNAs shown and the NEBNext Ultra II DNA Library Prep Kit. Libraries were amplified using the NEBNext Ultra II Q5 Master Mix, and sequenced on an Illumina MiSeq. GC coverage information was calculated using Picard's CollectGCBiasMetrics (v1.117). Expected normalized coverage of 1.0 is indicated by the horizontal grey line, the number of 100 bp regions at each GC% is indicated by the vertical grey bars, and the colored lines represent the normalized coverage for each library. NEBNext Ultra II Q5 Master Mix provides uniform GC coverage regardless of the GC content of the DNA.

ADVANTAGES OF NEBNEXT ULTRA Q5

- Optimized for high yields in NGS library amplification
- Minimizes GC bias, with superior performance across the GC spectrum
- Ultra-high-fidelity amplification with Q5, the highest-fidelity polymerase (2)
- Aptamer-based hot start without a separate activation step, for room-temperature reaction set-up

PRODUCT	SIZE
NEBNext Ultra II Q5 Master Mix (NEB #M0544S/L)	50/250 rxns

Reference:

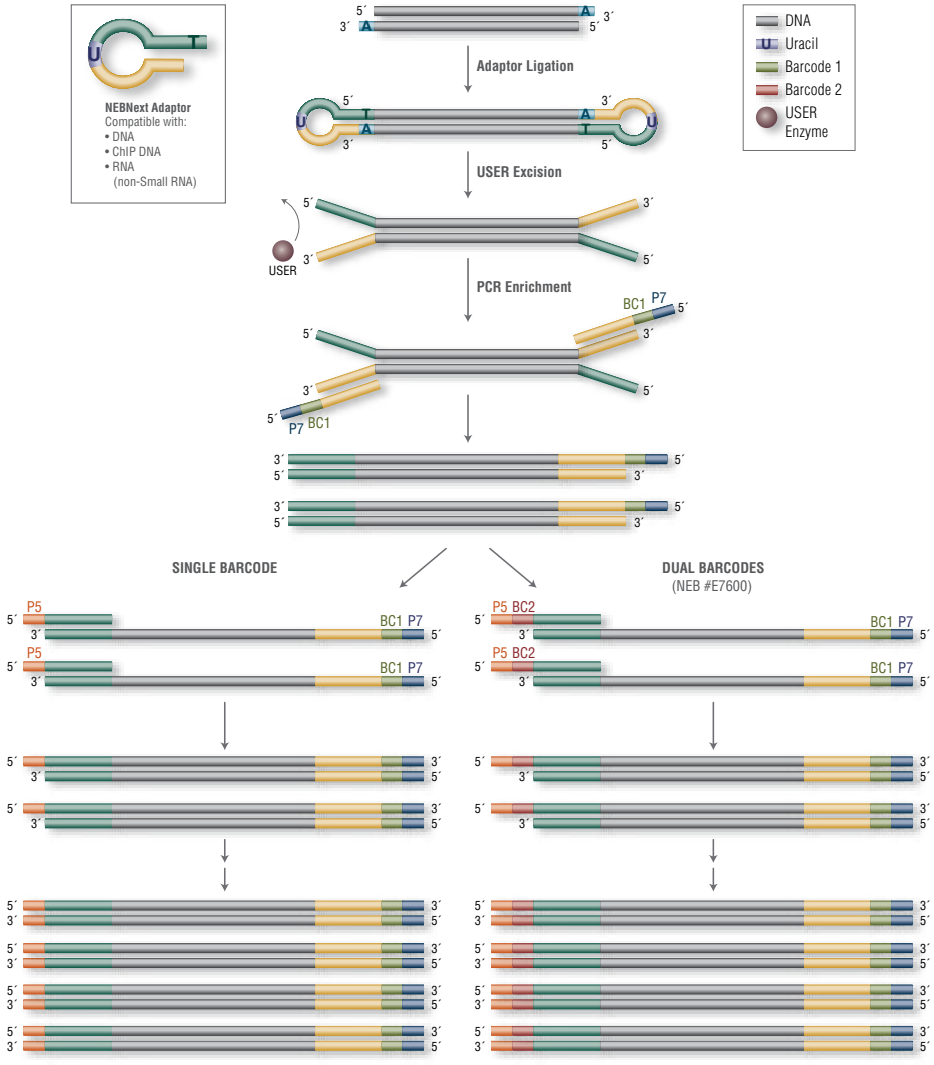
1. *Aird, D. et al. (2011). Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. Genome Biology 12(2), R18.*
2. *Popatov, V. and Ong, J.L. (2017). Examining Sources of Error in PCR by Single-Molecule Sequencing. PLoS ONE. 12(1):e0169774.*

NEBNext Adaptors and Primers

Designed for use in library prep for DNA, ChIP DNA and RNA (but not Small RNA), the NEBNext Adaptors enable high-efficiency adaptor ligation and high library yields, with minimized adaptor-dimer formation. Incorporating a novel hairpin loop structure, the NEBNext Adaptor ligates with increased efficiency to end-repaired, dA-tailed DNA. The loop contains a U, which is removed by treatment with USER Enzyme (a mix of UDG and Endo VIII), to open up the loop and make it available as a substrate for PCR. During PCR, barcodes can be incorporated by use of the NEBNext index primers, thereby enabling multiplexing. NEBNext Oligos can be used with NEBNext products, and with other standard Illumina-compatible library preparation protocols.

Single- or dual-barcode primer options are available. A methylated version of the NEBNext Adaptor is also available for use with bisulfite sequencing protocols.

Workflow demonstrating the use of NEBNext adaptors and index primers



ADVANTAGES

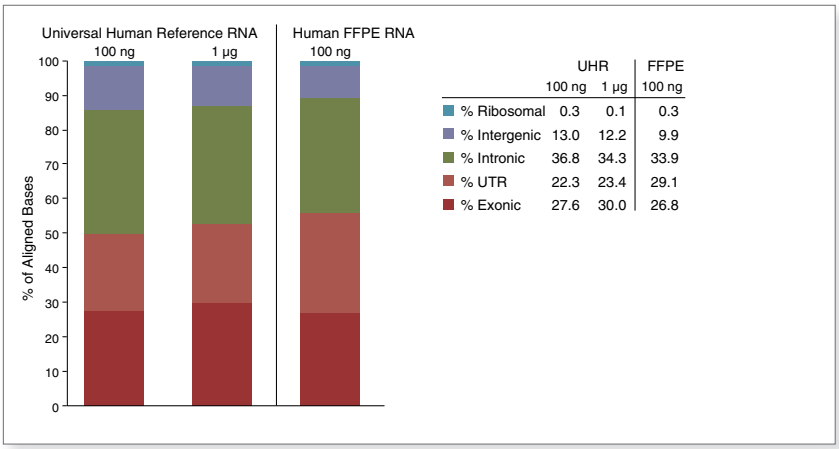
- Increased ligation efficiency
- Minimized adaptor-dimer formation
- Increased library yields
- Increased sample identification specificity (dual barcodes)
- Large number of single barcodes/ indices available
  - 4 sets of 12 indices
  - 1 set of 96 indices supplied in a 96-well plate with a pierceable foil seal
- Index pooling guidelines and sample sheets are provided

PRODUCT	# INDICES	SIZE
NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) (NEB #E7335S/L)	12	24/96 reactions
NEBNext Multiplex Oligos for Illumina (Index Primers Set 2) (NEB #E7500S/L)	12	24/96 reactions
NEBNext Multiplex Oligos for Illumina (Index Primers Set 3) (NEB #E7710S/L)	12	24/96 reactions
NEBNext Multiplex Oligos for Illumina (Index Primers Set 4) (NEB #E7730S/L)	12	24/96 reactions
NEBNext Multiplex Oligos for Illumina (96 Index Primers) (NEB #E6609S/L)	96	96/384 reactions
NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) (NEB #E7600S)	8 x 12	96 reactions
NEBNext Multiplex Oligos for Illumina (Methylated Adaptor, Index Primers Set 1) (NEB #E7535S/L)	12	24/96 reactions
NEBNext Singleplex Oligos for Illumina (NEB #E7350S/L)	12	12/60 reactions

NEBNext rRNA Depletion

The NEBNext rRNA Depletion Kit (Human/Mouse/Rat) enables efficient removal of ribosomal RNA (rRNA) from total RNA for human, mouse and rat RNA samples. This RNase H-dependent method uses DNA probes that tile completely across the target regions, with no gaps, so that even short RNA fragments (such as are present in low quality, degraded samples) are removed, as well as the longer, intact RNA molecules present in high quality samples. rRNA depletion with a broad range of input amounts (10 ng to 1 µg) is completed in under 2 hours, with less than 10 minutes of hands-on time.

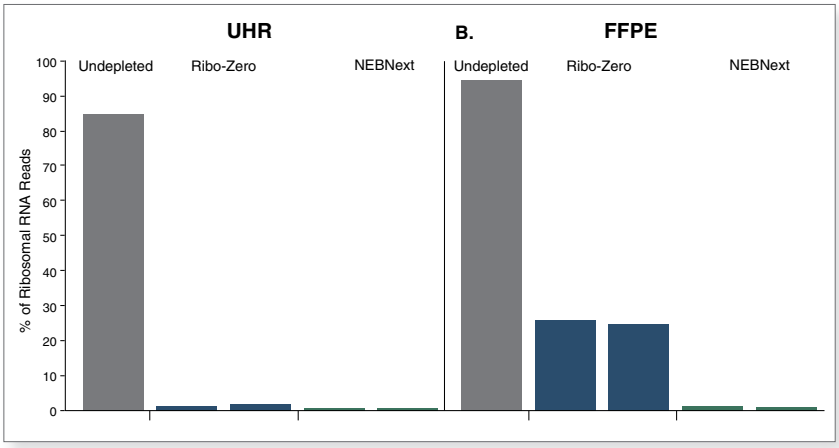
The NEBNext rRNA Depletion Kit efficiently removes rRNA from intact and degraded FFPE (RNA), while retaining coding and non-coding transcripts



	UHR 100 ng	UHR 1 µg	FFPE 100 ng
% Ribosomal	0.3	0.1	0.3
% Intergenic	13.0	12.2	9.9
% Intronic	36.8	34.3	33.9
% UTR	22.3	23.4	29.1
% Exonic	27.6	30.0	26.8

RNA-seq libraries were generated from Universal Human Reference Total RNA (UHR, Agilent) or FFPE-Human Normal Adult Tissue (Biochain, R8235XXX-PP/PM). RNA was treated with the NEBNext rRNA Depletion Kit, and the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420) was used to prepare libraries for sequencing on an Illumina NextSeq instrument. Reads were mapped to the GRCh38 human reference genome using Hisat, and base distribution was calculated using Picard's RNA-seq Metrics Tool using the Gencode v23 annotation.

Transcript expression correlation between depleted and non-depleted libraries



Universal Human Reference Total RNA (UHR, Agilent) was depleted using either the NEBNext rRNA Depletion Kit or the Ribo-Zero® Magnetic Gold Kit (Epicentre) or the NEBNext rRNA Depletion Kit. All libraries were prepared at 100 ng using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420) and sequenced on an Illumina NextSeq 500 instument. Reads were identified as ribosomal using Mirabait with human 18S, 28S, 5S, 5.8S, 12S and 16S ribosomal RNA sequences as baits.

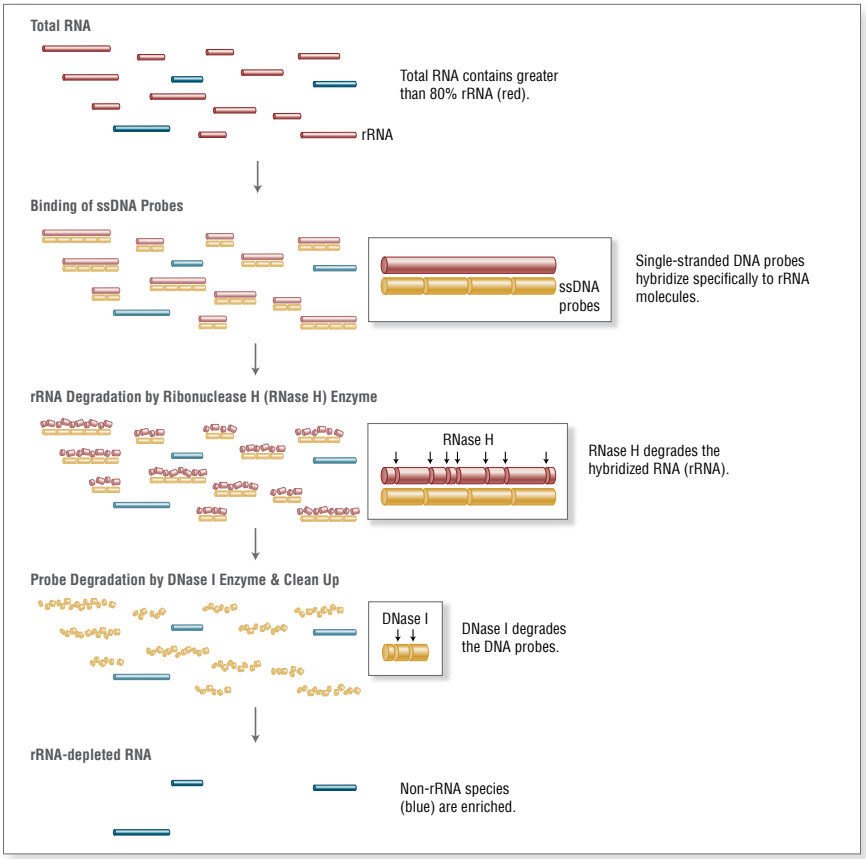
PRODUCT	SIZE
NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E6310S/L/X)	6/24/96 rxns
NEBNext rRNA Depletion Kit (Human/Mouse/Rat) with RNA Sample Purification Beads (NEB #E6350S/L/X)	6/24/96 rxns
ALSO AVAILABLE	SIZE
NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490S/L)	24/96 rxns

ADVANTAGES

- A single kit that **performs reliably well for all of your RNA samples**:
  – FFPE (degraded) or high-quality (intact) RNA
  – 10 ng to 1 µg input amounts
- Remove > 95% of rRNA, and **obtain more relevant sequence reads** from your sample
- **Obtain a more complete transcriptome picture** through retention of noncoding & incomplete RNAs that are lost with oligo d(T) poly(A) mRNA enrichment methods
- Suitable for use with human, mouse or rat samples
- Easily integrated upstream of any downstream random-primed cDNA synthesis protocol
- No more need to homebrew: A reliable & convenient NGS-validated kit for the "RNase H protocol" (1,2) for rRNA depletion
- Available with optional Agencourt® RNAClean® XP Beads, supplied in just the amounts you need

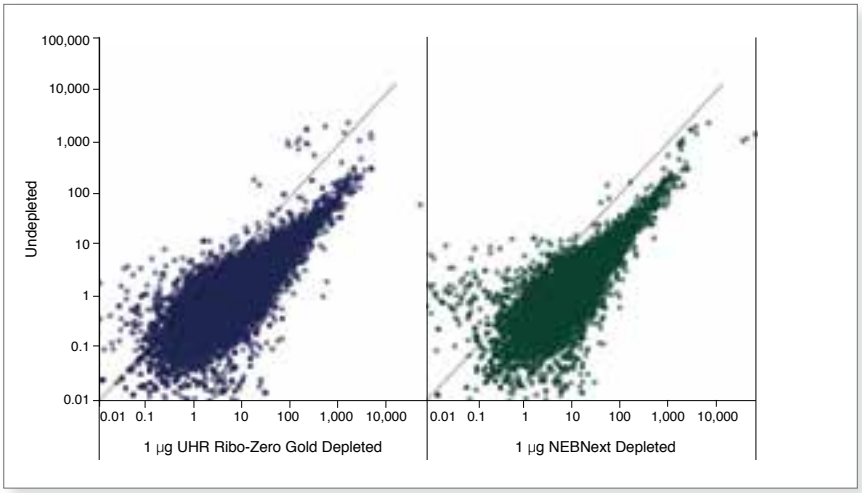
References
1. Adiconis, X. et al. (2013). Nature Methods 10; 623-629.
2. Morlan, J.D. et al. (2012). PLoS One 7, e42882.

NEBNext rRNA Depletion Kit workflow



Total RNA (0.1-1 µg) is hybridized with single stranded DNA probes targeting cytoplasmic (5S, 18S, 28S, 5.8S rRNAs) and mitochondrial (12S and 16S rRNAs) ribosomal RNA, followed by RNase H digestion to degrade targeted RNA. Finally, DNA probes are digested with DNase I. The ribosomal-depleted RNA is purified using Agencourt RNAClean XP beads. Ribosomal RNA depletion can be immediately followed by RNA-seq library preparation.

Transcription expression correlation with undepleted libraries



1 µg of Universal Human Reference Total RNA (Agilent) was depleted using either the NEBNext rRNA Depletion Kit or the Ribo-Zero Magnetic Gold Kit (Epicentre). Libraries were prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420) and sequenced on an Illumina NextSeq 500 instrument. Reads were mapped to all CDS entries from the GRCh38 Enzembl release 81 reference using Salmon (1). Transcripts per million values were correlated between an undepleted sample and depleted libraries. Transcript levels from the NEBNext-depleted library were better correlated than those from the Ribo-Zero depleted library.

NEBNext rRNA Depletion Kit workflow times

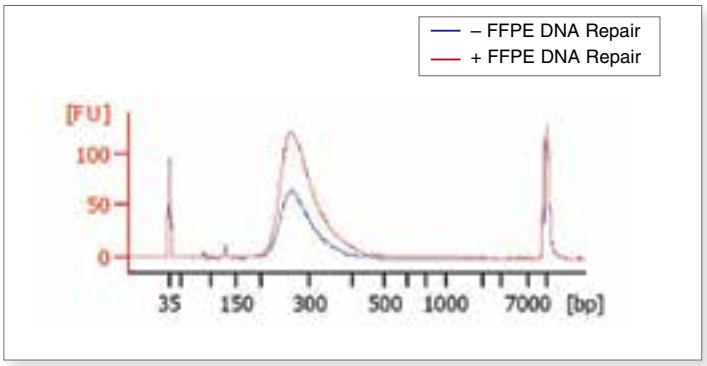
	Input Amount	Time				Workflow Time
		RNA/Probe Hybridization	RNase H Digestion	DNase I Digestion	Clean Up	
Kit	10 ng – 1 µg	Hands-On				Hands-On
		2 min.	2 min.	2 min.	2 min.	8 min.
		Total				Total
		22 min.	32 min.	32 min.	27 min.	1 hr., 53 min.

Reference
1. https://github.com/COMBINE-lab/salmon.

NEBNext FFPE DNA Repair Mix

Archiving of clinical materials as Formalin-Fixed, Paraffin-Embedded (FFPE) samples is a common practice. However, the methods used for fixation and storage significantly damage and compromise the quality of nucleic acids from these samples. As a result, it can be challenging to obtain useful information, including high-quality sequence data, especially when sample amounts are limited. The NEBNext FFPE DNA Repair Mix is a cocktail of enzymes formulated to repair DNA, and specifically optimized and validated for repair of FFPE DNA samples. Incorporation of the FFPE DNA Repair Mix into Next Generation Sequencing (NGS) workflows can increase yields and overall library success rates, while also improving sequence quality (1).

Effect of FFPE DNA Repair Mix on library yields

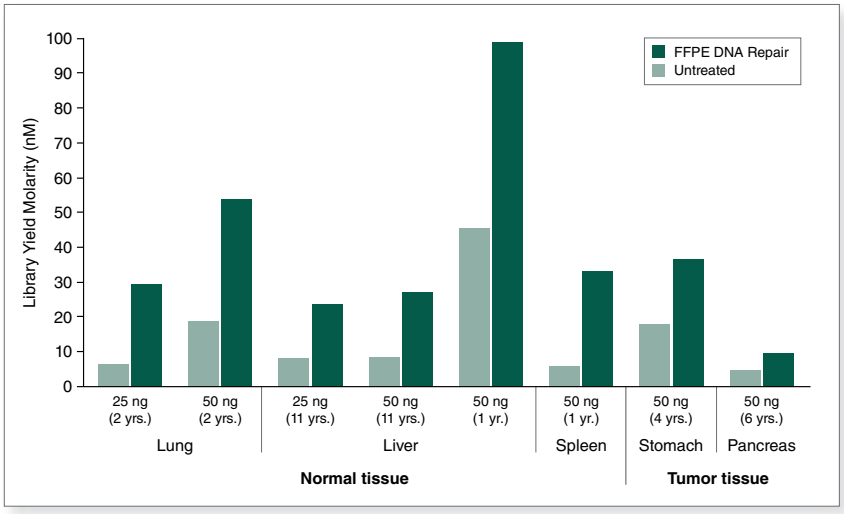


An example of Agilent Bioanalyzer® traces of libraries prepared from stomach tumor FFPE DNA that was treated with the FFPE DNA Repair Mix, or was untreated, before library construction. Yield improvements of 101% to 458% have been observed.

ADVANTAGES

- Increase library yield
- Increase library quality
- Use before library prep for any NGS platform
- No alteration of DNA sequence
- Rely on NEB's NGS validation process for FFPE DNA library prep

FFPE DNA repair increases NGS library yields



FFPE DNA samples (25 ng or 50 ng) from a variety of tissue types were treated with the NEBNext FFPE DNA Repair Mix, or were untreated, before library construction. Library yields were measured using the Agilent Bioanalyzer, and ratios of library yields with and without treatment with the NEBNext FFPE DNA Repair Mix were calculated. Yield improvements with these samples ranged from 101% to 458%.

Types of FFPE DNA damage and their ability to be repaired by the NEBNext FFPE DNA Repair Mix

FFPE DAMAGE TYPE	REPAIRED BY THE FFPE DNA ENZYME REPAIR MIX?
Deamination of cytosine to uracil	Yes
Nicks and gaps	Yes
Oxidized bases	Yes
Blocked 3´ ends	Yes
DNA fragmentation	No
DNA-protein crosslinks	No

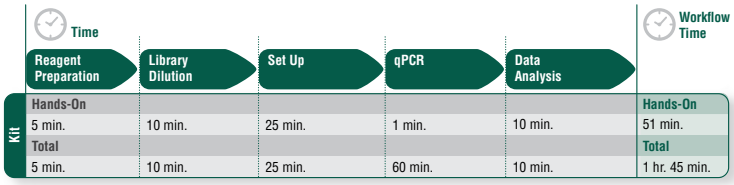
PRODUCT	SIZE
NEBNext FFPE DNA Repair Mix (NEB #M6630S/L)	24/96 rxns

Reference
1. Chen. L. et al. (2017) Science 355, 752-756.

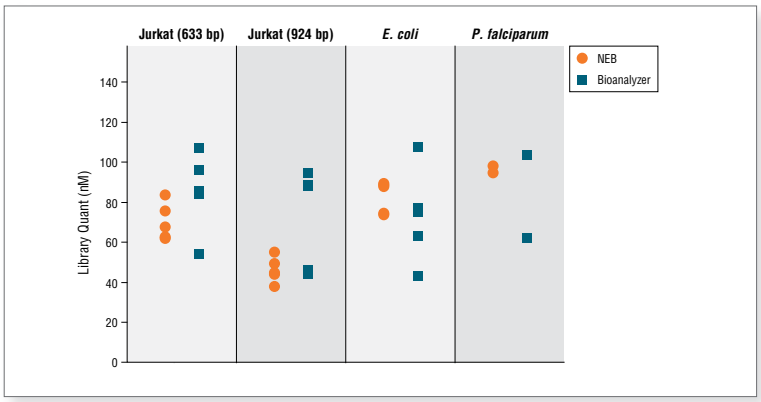
NEBNext Library Quant Kit for Illumina

Accurate quantitation of next-generation sequencing libraries is essential for maximizing data output and quality from each sequencing run. For Illumina sequencing specifically, accurate quantitation of libraries is critical to achieve optimal cluster densities, a requirement for optimal sequence output. qPCR is considered to be the most accurate and effective method of library quantitation, providing considerably higher consistency and reproducibility of quantitation. qPCR-based methods quantitate only those molecules that contain both adaptor sequences, thereby providing a more accurate estimate of the concentration of the library molecules that can be sequenced. The NEBNext Library Quant Kit delivers significant improvements to qPCR-based library quantitation for next-generation sequencing.

NEBNext Library Quant Kit for Illumina (NEB #E7630) workflow

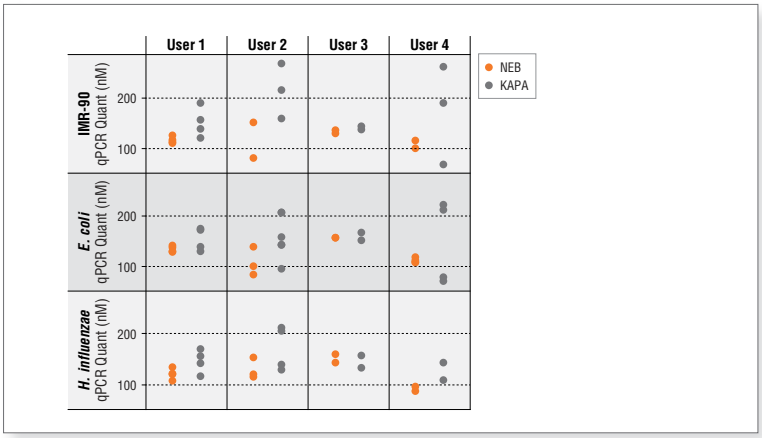


Comparison of quantitation by qPCR and electrophoretic methods



Concentrations of 4 libraries were determined by the NEBNext Library Quant Kit (orange) and compared to values measured using the Aglient Bioanalyzer (blue). Compared to NEBNext's qPCR-based method, the Bioanalyzer concentrations displayed a greater level of variation.

Greater reproducibility of library quantitation with the NEBNext Library Quant Kit



Three 340–400 bp libraries were quantitated by 4 different users 2–4 times using either the NEBNext or Kapa™ Library Quantification Kit (Universal). A notable improvement in quantitation consistency was observed for concentrations determined by the NEBNext Kit (orange) versus those from the Kapa kit (gray).

ADVANTAGES

- Be confident in your quant values, as our kit provides **more accurate and reproducible results** than other methods and kits
- Get up and running quickly with our **easy-to-use kit**, containing Library Dilution Buffer, optimized master mix, 4 standards and ROX dye
- Simplify your reaction setup with **fewer pipetting steps** and a **single extension time** for all libraries
- Quantitate more libraries per kit, **as only 4 standards are required**
- **Use with all your libraries**, regardless of insert size, GC content and preparation method
- Save money with our value pricing

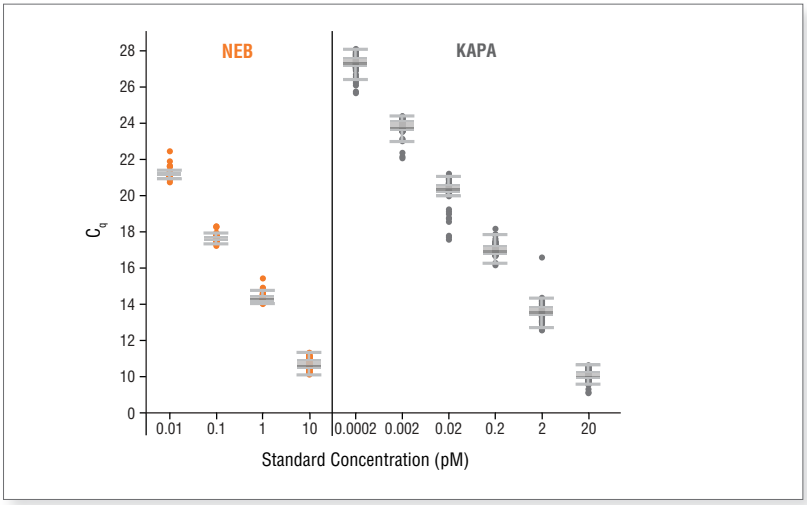
TOOLS & RESOURCES

Use NEBioCalculator at **NEBioCalculator.neb.com** to calculate your qPCR-based library quant values

Download our application note, "Improved library quantitation for a broad range of library types using the NEBNext Quant Kit for Illumina" at **www.neb.com/E7630**

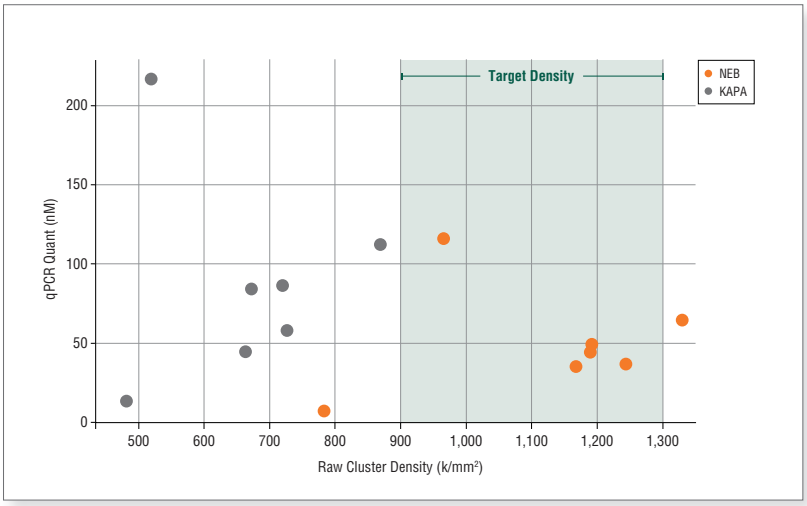
PRODUCT	SIZE
NEBNext Library Quant Kit for Illumina (NEB #E7630S/L)	100/500 rxns
NEBNext Library Dilution Buffer (NEB #B6118S)	7.5 ml

Greater lot-to-lot consistency of standards with the NEBNext Library Quant Kit



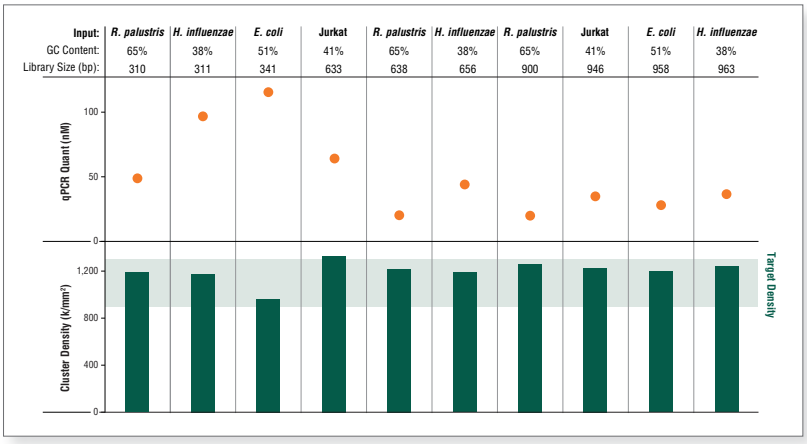
Accurate qPCR quantitation requires the use of high-quality DNA standards with known concentrations. The NEBNext Library Quant Kit contains 4 standards produced with a high level of both quantitation accuracy and consistency. This figure shows data from > 70 total runs from 4 lots of both NEBNext (orange) and Kapa (gray) standards, with all Cq values plotted. Box and whiskers indicate mean and quartiles. The NEBNext Library Standards displayed much lower variation in Cq, resulting in more consistent quantitation performance.

The NEBNext Library Quant Kit values enable optimal cluster densities



Seven different libraries were quantitated using either the NEBNext Library Quant Kit (orange) or the Kapa Library Quantification Kit (Universal) (gray). Undiluted library concentrations ranged from 2–200 nM. Libraries were diluted to 8 pM and loaded onto a MiSeq® instrument (v2 chemistry; MCS v2.4.1.3). Libraries quantitated with the NEBNext kit resulted in a raw cluster density average of 1160 k/mm², directly in the optimal range of 900–1300 k/mm². In contrast, libraries loaded based on the Kapa quantitation averaged only 660 k/mm².

With NEBNext, optimal cluster density is achieved from quantitated libraries with a broad range of library size and GC content

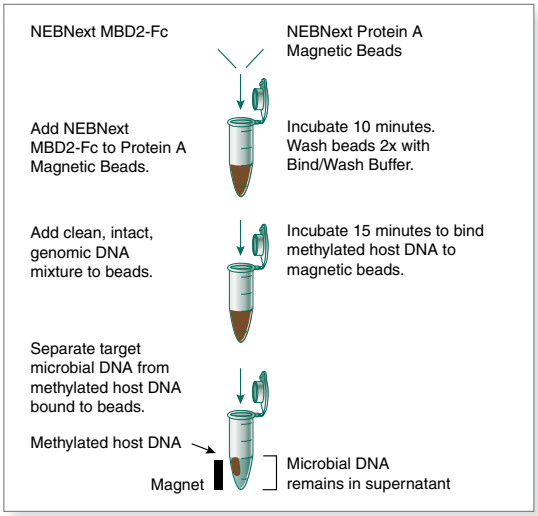


Libraries of 310–963 bp from the indicated sources were quantitated using the NEBNext Library Quant Kit, then diluted to 8 pM and loaded onto a MiSeq® (v2 chemistry; MCS v2.4.1.3). Library concentrations ranged from 7–120 nM, and resulting raw cluster density for all libraries was 965–1300 k/mm² (ave. =1199). Optimal cluster density was achieved using concentrations determined by the NEBNext Library Quant Kit for all library sizes.

NEBNext Microbiome DNA Enrichment Kit

Microbiome DNA analysis can be challenging due to the high percentage of host DNA present in many samples. The NEBNext Microbiome DNA Enrichment Kit facilitates enrichment of microbial DNA from samples containing methylated host DNA (including human), by selective binding and removal of the CpG-methylated host DNA. Importantly, microbial diversity remains intact after enrichment (1). If desired, the host DNA captured on the magnetic bead pellet can be eluted, and a protocol is provided for this.

Microbiome DNA Enrichment Kit workflow

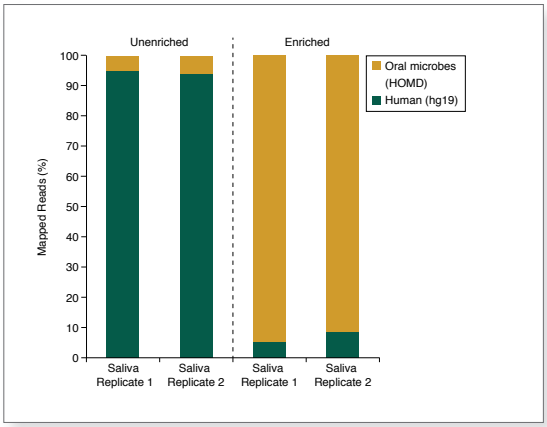


The MBD2-Fc protein binds specifically to CpG methylated DNA. In the NEBNext Microbiome DNA Enrichment workflow, MBD2-Fc is attached to Protein A magnetic beads, enabling capture of methylated DNA, while the microbial DNA remains in the supernatant.

ADVANTAGES

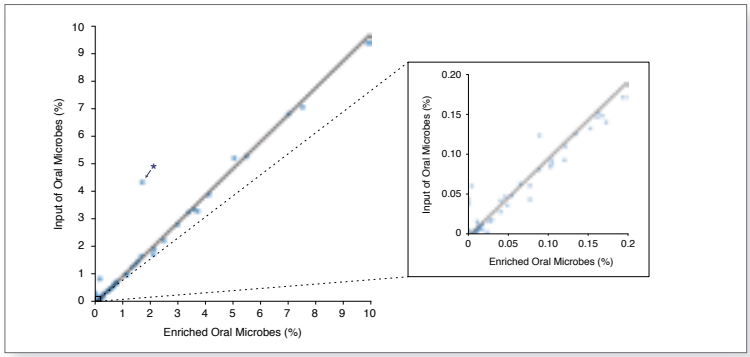
- Effective enrichment of microbial DNA from samples containing contaminating host DNA
- Fast, simple protocol
- Enables microbiome whole genome sequencing, even for samples with high levels of host DNA
- Compatible with downstream applications including next generation sequencing on all platforms, qPCR and end-point PCR
- Suitable for a wide range of sample types
- No requirement for live cells
- Optional protocol to retain separated host DNA
- Also effective for separation of organelle DNA (e.g. mitochondria, chloroplast) from eukaryote nuclear DNA (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 500 M-537 M SOLiD 4 50 bp reads that mapped to either the Human reference sequence (hg19) or to a microbe listed in Human Oral Microbiome Database (HOMD)[3]. (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[4] with typical settings (2 mismatches in a 28 bp seed region, etc.).

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 SOLiD 4 platform. The graph shows a comparison between relative abundance of each bacterial species listed in HOMD[3] before and after enrichment with the NEBNext Microbiome DNA Enrichment Kit. High concordance continues even to very low abundance species (inset). We compared 501 M 50 bp SOLiD 4 reads in the enriched dataset to 537 M 50 bp SOLiD 4 reads in the unenriched dataset. Reads were mapped using Bowtie 0.12.7[4] with typical settings (2 mismatches in a 28 bp seed region, etc).

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

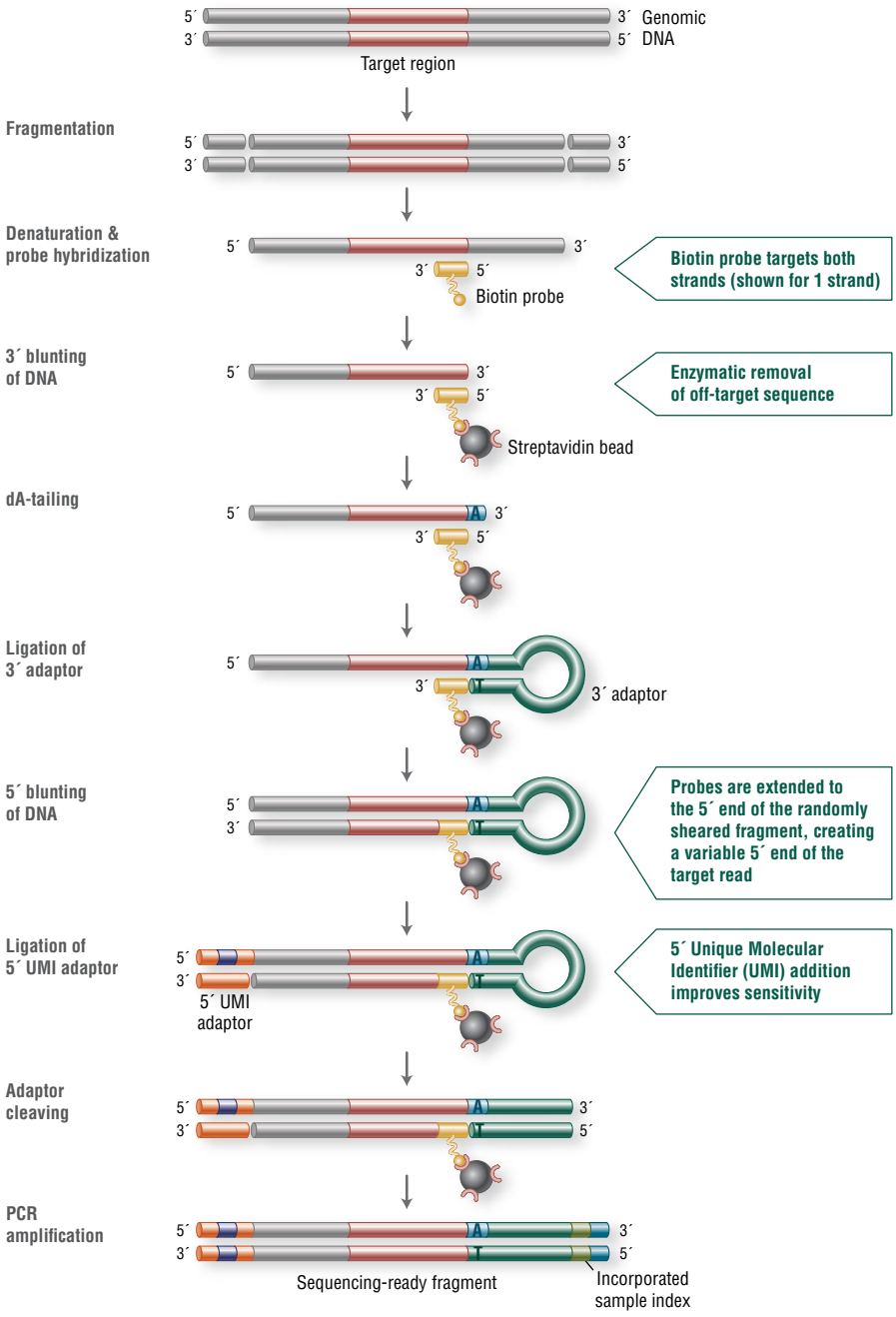
PRODUCT	SIZE
NEBNext Microbiome DNA Enrichment Kit (NEB #E2612S/L)	6/24 rxns

References
1. Feehery, G. R. et al. (2013). PLoS One 8, e76096.
2. Yigit, E. et al. (2014). Applications in Plant Sciences 2014 2 (11): 1400064
3. Chen, T., et al. (2010) Database, Vol. 2010, Article ID baq013, doi: 10.1093/database/baq013
4. Langmead B., et al. (2009) Genome Biol. 10:R25 doi:10.1186/gb-2009-10-3-r25

NEBNext Direct® for Target Enrichment

Using a novel approach to target enrichment, the NEBNext Direct Cancer Hotspot Panel enables highly specific hybridization-based capture of 190 common cancer targets from 50 genes. The NEBNext Direct technology offers significant advantages over both traditional in-solution hybridization and multiplex PCR protocols. Target enrichment is combined with library preparation, reducing processing time and minimizing sample loss. Ideal for automation, NEBNext Direct enables deep sequencing of genomic regions of interest for the discovery and identification of low frequency variants from challenging sample types.

NEBNext Direct employs a fast hybridization–based workflow that combines capture with library preparation



PRODUCT	SIZE
NEBNext Direct Cancer HotSpot Panel (NEB #E7000S/L/X)	8/24/96 rxns
NEBNext Direct BRCA1/BRCA2 Panel (NEB #E6627S/L/X)	8/24/96 rxns

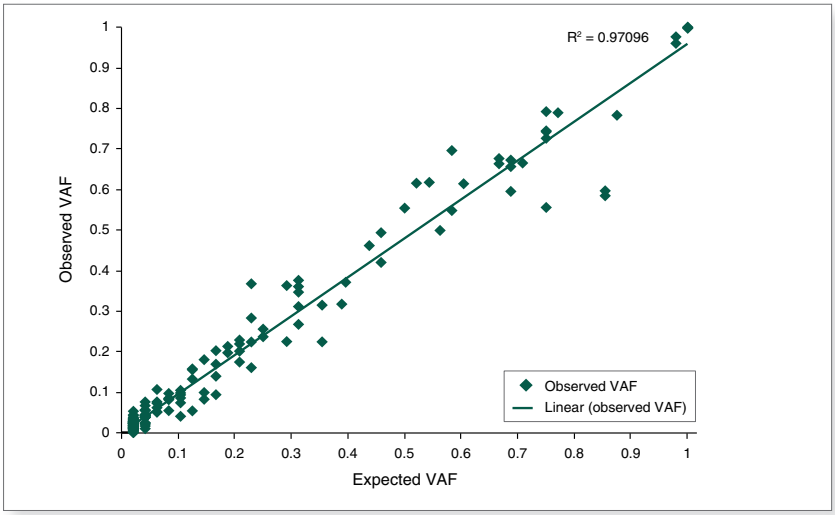
ADVANTAGES

- No upfront library prep: enriches targets and converts into a **sequence-ready library in 1 day**
- Generate a **higher percentage of your sequencing reads** aligning to your targets
- Eliminate the need to over-sequence, **reducing cost per sample**
- Obtain **uniform sequencing of all targets,** regardless of GC content
- Generate high quality libraries with **limited input amounts and degraded DNA samples,** including FFPE and ctDNA
- Distinguish molecular duplicates, reducing false positive variants and **improving sensitivity**

For research use only, not intended for diagnostic use.

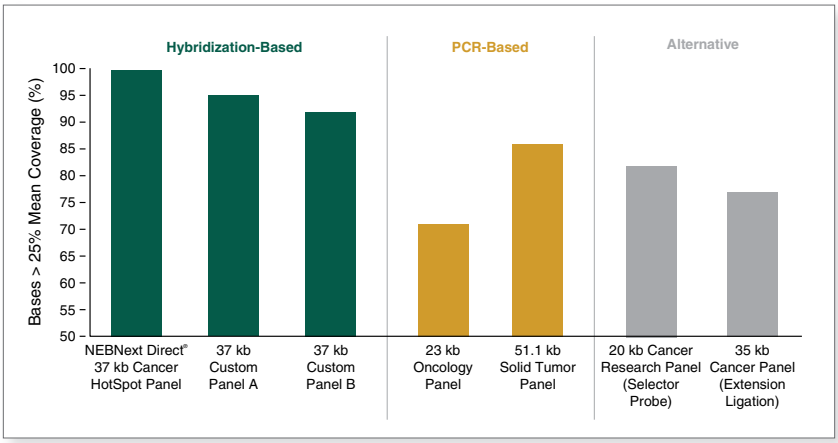
View additional information, including performance data and videos, at **NEBNextDirect.com**

The NEBNext Direct Cancer HotSpot Panel demonstrates the ability to accurately detect a range of nucleic acid variants.



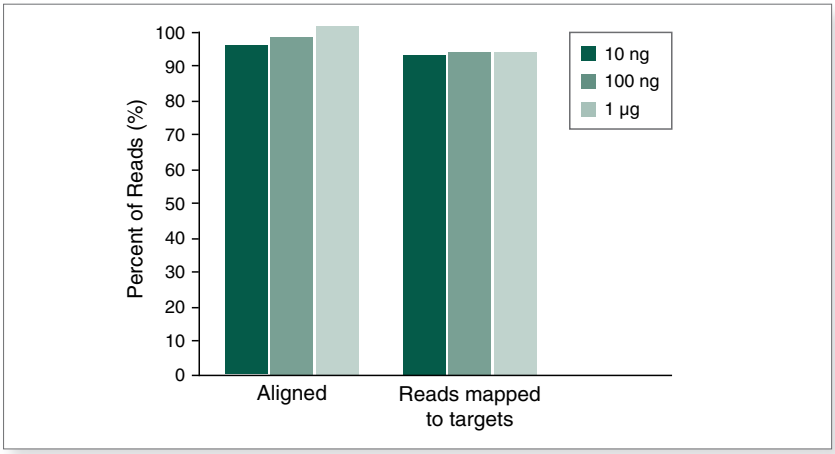
This figure shows the expected versus observed variant allele frequencies (VAF) across the range of well-characterized variants present in a pool of 24 HapMap samples screened against the NEBNext Direct Cancer HotSpot Panel. 100 ng of input DNA was used, samples were sequenced on the Illumina MiSeq® using 2 x 75 bp sequencing, and standard data analysis and variant calling algorithms were used. We were able to successfully detect 100% of the 168 truth variants present across a range of 2-100% VAF. The high degree of linearity across this broad dynamic range demonstrates the ability of the NEBNext Direct Cancer HotSpot Panel to accurately predict variant allele frequencies across a broad dynamic range.

NEBNext direct delivers higher coverage uniformity than alternative approaches.



Plot shows the uniformity across targets for each panel, measured as the percentage of bases below 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.

The NEBNext Direct BRCA1/BRCA2 Panel delivers highly efficient enrichment of BRCA1 and BRCA2 coding regions with a high percentage of reads mapping to targets



This histogram shows the % of reads aligned to the human genome, and the % of reads mapped to the targets included in the BRCA1/BRCA2 Panel across different input DNA amounts. 10 ng, 100 ng and 1 µg of purified genomic DNA was enriched using the NEBNext Direct BRCA1/BRCA2 Panel. Sequencing reads were generated on an Illumina MiSeq with 2 x 75 bp reads, 8 bp Sample ID, and 12 bp unique molecule ID. Sequencing read alignments were performed with BWA-MEM and PCR duplicates were filtered using the UMIs.

Technical Tips: Library Preparation

DNA Sample Input Guidelines

Integrity of DNA

- Start with as high quality DNA as possible. The quality of the input material directly affects the quality of the library. DNA quality can be assessed using gel electrophoresis. Impurities such as detergents or probes, or damaged DNA can usually be seen as a smear on the gel. RNA impurities are often seen at the bottom of the gel. Absorbance measurements can also be used as an indication of DNA purity. Ideally, the ratio of the absorbance at 260 nm to 280 nm should be between 1.8 – 2.0. However, measurements can be affected by the presence of RNA or small nucleic acid fragments.

Quantitation of DNA

- It is important to quantify accurately the DNA sample prior to library construction. Fluorescence based detection which utilizes dsDNA specific dyes, such as the Qubit® from Life Technologies, is more accurate than UV spectrometer based measurements, as the presence of RNA or other contaminants can result in overestimation of the amount of the DNA sample.

RNA Sample Input Guidelines

Integrity of RNA

- It is important to start with high quality RNA. The use of degraded RNA can result in low yield or failure to generate libraries. We recommend determining RNA quality using the RNA Integrity Number (RIN) estimated by the Agilent Bioanalyzer. The RNA sample should have a RIN value higher than 7.
    - :: Integrity and size distribution of total RNA can be checked by electrophoresis on a denaturing agarose gel and staining with ethidium bromide. The ribosomal RNA bands should appear as sharp bands on the stained gel. For eukaryotic samples, intact total RNA will have sharp, clear bands corresponding to 28S and 18S. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is completely intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit the 2:1 ratio of high quality RNA. Completely degraded RNA will appear as a very low molecular weight smear.
- RNA should be completely free of DNA. DNase digestion of the purified RNA with RNase-free DNase is recommended.

Quantitation of RNA

- It is important to quantify accurately the RNA sample prior to library construction. The concentration can be estimated with the Agilent Bioanalyzer on a pico or nano chip. Alternatively, RNA concentration can be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer such as a NanoDrop®. However, free nucleotides or other organic compounds routinely used to extract RNA will also absorb UV light near 260 nm and will result in an over-estimation of the RNA concentration.

Bead-based clean-ups and size selection

- Be careful when transferring material not to disturb the bead pellet
- Be sure to vortex the beads just before use – they should be a uniform suspension
- Do not over-dry the beads. This can make resuspension difficult and reduce yield.
- Bead-based clean-ups and size-selection are explained in the Ultra II video

Barcodes

- When you are using a subset of the barcodes supplied in a kit, or using barcodes from more than one kit, it is important to optimize the combination of barcodes used, to ensure balanced sequencing reads. We provide recommendations for NEBNext barcode combinations at NEBNext.com.
- Open only one index primer vial at a time, to minimize the risk of contamination

NEBNext Reagents for DNA Sample Preparation:

KITS FOR ILLUMINA DNA LIBRARY PREPARATION		NEB #	SIZE
DNA & ChIP	NEBNext Ultra II FS DNA Library Prep Kit for Illumina	E7805S/L	24/96 rxns
	NEBNext Ultra II FS DNA Library Prep with Sample Purification Beads	E6177S/L	24/96 rxns
	NEBNext Ultra II DNA Library Prep Kit for Illumina	E7645S/L	24/96 rxns
	NEBNext Ultra II DNA Library Prep with Sample Purification Beads	E7103S/L	24/96 rxns
	NEBNext Ultra DNA Library Prep Kit for Illumina	E7370S/L	24/96 rxns
	NEBNext DNA Library Prep Master Mix Set for Illumina	E6040S/L	12/60 rxns
	NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina	E6240S/L	12/60 rxns
MODULES & ENZYMES		**NEB #**	**SIZE**
DNA & ChIP	NEBNext FFPE DNA Repair Mix	M6630S/L	24/96 rxns
	NEBNext Microbiome DNA Enrichment Kit	E2612S/L	6/24 rxns
	NEBNext Ultra II FS DNA Module	E7810S/L	24/96 rxns
	NEBNext Ultra II End Repair/dA-Tailing Module	E7546S/L	24/96 rxns
	NEBNext Ultra II Ligation Module	E7595S/L	24/96 rxns
	NEBNext Ultra II Q5 Master Mix	M0544S/L	50/250 rxns
	NEBNext Ultra End Repair/dA-Tailing Module	E7442S/L	24/96 rxns
	NEBNext Ultra Ligation Module	E7445S/L	24/96 rxns
	NEBNext dsDNA Fragmentase	M0348S/L	50/250 rxns
	NEBNext End Repair Module	E6050S/L	20/100 rxns
	NEBNext dA-Tailing Module	E6053S/L	20/100 rxns
	NEBNext Quick Ligation Module	E6056S/L	20/100 rxns
	NEBNext Q5 Hot Start HiFi PCR Master Mix	M0543S/L	50/250 rxns
	NEBNext High-Fidelity 2X PCR Master Mix	M0541S/L	50/250 rxns
	NEBNext dsDNA Fragmentase Reaction Buffer v2	B0349S	6 ml
ADAPTORS & PRIMERS		**NEB #**	**SIZE**
	NEBNext Multiplex Oligos for Illumina (Index Primers Set 1)	E7335S/L	24/96 rxns
	NEBNext Multiplex Oligos for Illumina (Index Primers Set 2)	E7500S/L	24/96 rxns
	NEBNext Multiplex Oligos for Illumina (Index Primers Set 3)	E7710S/L	24/96 rxns
	NEBNext Multiplex Oligos for Illumina (Index Primers Set 4)	E7730S/L	24/96 rxns
	NEBNext Multiplex Oligos for Illumina (96 Index Primers)	E6609S/L	96/384 rxns
	NEBNext Multiplex Oligos for Illumina (Methylated Adaptor, Index Primers Set 1)	E7535S/L	24/96 rxns
	NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1)	E7600S	96 rxns
	NEBNext Singleplex Oligos for Illumina	E7350S/L	12/60 rxns
TARGET ENRICHMENT		**NEB #**	**SIZE**
	NEBNext Direct Cancer HotSpot Panel	E7000S/L/X	8/24/96 rxns
	NEBNext Direct BRCA1/BRCA2 Panel	E6627S/L/X	8/24/96 rxns
LIBRARY QUANTITATION		**NEB #**	**SIZE**
	NEBNext Library Quant Kit for Illumina	E7630S/L	100/500 rxns
	NEBNext Library Dilution Buffer	B6118S	7.5 ml
DNA ENRICHMENT		**NEB #**	**SIZE**
DNA	NEBNext Microbiome DNA Enrichment Kit	E2612S/L	6/24 rxns
DNA REPAIR		**NEB #**	**SIZE**
DNA	NEBNext FFPE DNA Repair Mix	M6630S/L	24/96 rxns

NEBNext Reagents for RNA Sample Preparation:

KITS FOR ILLUMINA RNA LIBRARY PREPARATION		NEB #	SIZE
Directional RNA	NEBNext Ultra II Directional RNA Library Prep Kit for Illumina	E7760S/L	24/96 rxns
	NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads	E7765S/L	24/96 rxns
	NEBNext Ultra Directional RNA Library Prep Kit for Illumina	E7420S/L	24/96 rxns
Non-directional RNA	NEBNext Ultra II RNA Library Prep Kit for Illumina	E7770S/L	24/96 rxns
	NEBNext Ultra II RNA Library Prep with Sample Purification Beads	E7775S/L	24/96 rxns
	NEBNext Ultra RNA Library Prep Kit for Illumina	E7530S/L	24/96 rxns
Small RNA	NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 1)	E7300S/L	24/96 rxns
	NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 2)	E7580S/L	24/96 rxns
	NEBNext Multiplex Small RNA Library Prep Kit for Illumina (Index Primers 1-48)	E7560S	96 rxns
	NEBNext Small RNA Library Prep Set for Illumina (Multiplex Compatible)	E7330S/L	24/96 rxns
MODULES & ENZYMES		**NEB #**	**SIZE**
RNA	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 Magnesium RNA Fragmentation Module	E6150S	200 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 RNA First Strand Synthesis Module	E7525S/L	24/96 rxns
DNA	NEBNext Ultra End Repair/dA-Tailing Module	E7442S/L	24/96 rxns
	NEBNext Ultra Ligation Module	E7445S/L	24/96 rxns
	NEBNext End Repair Module	E6050S/L	20/100 rxns
	NEBNext dA-Tailing Module	E6053S/L	20/100 rxns
	NEBNext Quick Ligation Module	E6056S/L	20/100 rxns
	NEBNext Ultra II Q5 Master Mix	M0544S/L	50/250 rxns
	NEBNext Q5 Hot Start HiFi PCR Master Mix	M0543S/L	50/250 rxns
	NEBNext High-Fidelity 2X PCR Master Mix	M0541S/L	50/250 rxns
ADAPTORS & PRIMERS		**NEB #**	**SIZE**
	NEBNext Multiplex Oligos for Illumina (Index Primers Set 1)	E7335S/L	24/96 rxns
	NEBNext Multiplex Oligos for Illumina (Index Primers Set 2)	E7500S/L	24/96 rxns
	NEBNext Multiplex Oligos for Illumina (Index Primers Set 3)	E7710S/L	24/96 rxns
	NEBNext Multiplex Oligos for Illumina (Index Primers Set 4)	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
	NEBNext Singleplex Oligos for Illumina	E7350S/L	12/60 rxns
LIBRARY QUANTITATION		**NEB #**	**SIZE**
	NEBNext Library Quant Kit for Illumina	E7630S/L	100/500 rxns
	NEBNext Library Dilution Buffer	B6118S	7.5 ml

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